



Short Note

Genetics reveals that free-ranging chipmunks introduced to Italy have multiple origins

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Abstract

The Siberian chipmunk *Eutamias sibiricus* is native to north-eastern Asia, but alien populations, mostly composed by a low number of individuals, have been established in many western countries through the pet trade. Large native ranges, such as that of Siberian chipmunks, and number of introduction events may explain the establishment success of alien mammals. In Italy, the largest population of Siberian chipmunks occurs in an urban park of Veneto (NE Italy), whereas other 3 nuclei are currently composed by less than 10 individuals. In this work we aimed at assessing the origin of Siberian chipmunks introduced to Italy. We analyzed a region of the mtDNA gene cytochrome-b to infer the native geographic range of chipmunks introduced to Italy. Our genetic analysis (N = 8 samples) revealed a multiple origin of alien chipmunks in Italy, with individuals from Siberia (collected in Rome) and from Korea (collected both in Rome and northeastern Italy), with Korean chipmunks (i.e. those also present in central Europe) showing the highest invasive success. The ongoing taxonomic splitting of his species, if accepted by the scientific community, will require a revision of the European Regulation 1143/2014, which currently bans the trade of Siberian chipmunk only, with no explicit consequences on Korean and Chinese taxa.

Introduction

Biological invasions represent one of the leading causes of the ongoing sixth global biodiversity crisis (Pimentel, 2014; Bellard et al., 2016; Boudouresque et al., 2017; Ceballos et al., 2017). Currently, near 12,500 alien species occur in Europe (DAISIE, 2008) and over 1,000 of them are invasive (Vilà et al., 2010; Bacher et al., 2018), i.e. they dramatically affect native ecosystems, requiring high costs for their management each year (Kettunen et al., 2008). In the last 10 years, a number of strategies and policies have been set up to disentangle and classify the impacts of established species, and to limit further damages by biological invaders (Blackburn et al., 2014; Crowley et al., 2016; Rabitsch, 2016; Turbè et al., 2017). This allowed researchers to identify factors and main traits determining severity and typologies of impacts (Evans, 2018). So-called "black lists" include invasive species exerting the strongest impacts on native biodiversity. Black lists may be based on locally addressed studies or inferred by evidences from other areas (e.g. Lowe et al., 2000; Pergl et al., 2016; Nentwig et al., 2018). These lists have been mainly developed to increase the awareness of the problems for local stakeholders and policy makers (Crowley et al., 2016; Nentwig et al., 2018). The most important European policy on alien species is the EU Regulation 1143/2014, which currently lists 49 species banned for trade, possession, breeding, transport and release in the wild (Genovesi et al., 2014). This list includes invasive alien species in

Europe, prioritized following evidence-based risk assessments carried out through horizon scanning (Carboneras et al., 2018; Nentwig et al., 2018). Accordingly, the first step to design "black list" regards a critical and accurate identification of the introduced taxon and its original extent of occurrence, to better ascertain its invasiveness (Boykin et al., 2011; Comtet et al., 2015; Mazzamuto et al., 2016).

The Siberian chipmunk *Eutamias sibiricus* is the only Palearctic chipmunk, showing a wide extent of occurrence (Mori et al., 2018a). Being introduced in high numbers (being very popular as a pet), it also represents a successful invasive species (D'Ovidio and Pirrone, 2018; Mori et al., 2018a). This squirrel is currently facing a taxonomic re-definition, which may also result in legislative and management repercussions (Lee et al., 2008; Obolenskaya et al., 2009; Patterson and Norris, 2016; Mori et al., 2018c). Skull morphology, allopatric distribution, bioacoustics patterns and genetic analyses on mitochondrial DNA suggest that the Korean lineage of this species may represent a divergent species—called *Eutamias barberi*—with respect to northern groups (i.e. *E. s. sibiricus*) and Chinese (i.e. *E. s. senescens*) clades (Lee et al., 2008; Pisanu et al., 2013). Chipmunks introduced to France belong to the Korean lineage (Pisanu et al., 2013), but data on other European populations are still lacking. In our work, we aimed at identifying the origins of Siberian chipmunks who established populations in Italy (Mori et al., 2018a). We expected that the most of individuals belongs to the Korean lineage, being the most traded one (see Dal Farra et al., 1996) and we discussed potential management and legislative consequences.

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Materials and methods

Study sites

We collected hairs of Siberian chipmunks through hair-tube transects located where this species occurs in Italy, i.e. three urban parks (Sigurtà Garden Park in Valeggio sul Mincio, Villa Ada and Villa Doria-Pamphili in Rome) and the riverbanks of the Piave river at Cesa di Limana in the province of Belluno (Mori et al., 2018a; Zozzoli et al., 2018).

We used plastic hair-tubes (length=25 cm; diameter=4.5 cm), baited with nutcream and nuts, and horizontally secured on tree trunks (1 m above the ground: Zozzoli et al., 2018). Hair-tubes contained two plastic plates, one per entrance, with adhesive tapes placed on the upper side to capture squirrel hairs (for hair-tubes survey details, see Zozzoli et al., 2018). Collected hairs were identified at the microscope through the comparison with a reference collection (Department of Life Sciences, University of Siena) and a specific atlas (Teerink, 1991). Chipmunk hairs may be distinguished from those of syntopic *Sciurus vulgaris* (Teerink, 1991), as showing a roundish section (kidney-shaped in *S. vulgaris*), a mosaic-scaled cuticular pattern in the central part of guard hair (regular-waved in *S. vulgaris*), multicellular medullar composition (thicker in *S. vulgaris*: Zozzoli et al., 2018).

Molecular analyses

We extracted the DNA from 8 hair samples of Siberian chipmunk, i.e. 1–3 per sampling site. Chipmunks are known to be territorial and they show aggressive displays towards potential conspecifics (Getty, 1981). Siberian chipmunks show different personalities: bold chipmunks have larger home ranges and take more risks, thus being more likely to be trapped, with respect to shy ones (Boyer et al., 2010; Montiglio et al., 2012). We collected samples from tubes located over 250 m one-another (i.e. the max straight line crossed by chipmunks, across their territories: (Marmet et al., 2009), to avoid a double genetic analysis on the same individual (see Zozzoli et al., 2018, for sampling design). DNA was extracted through liquid nitrogen and phenol-chloroform purification protocol: about 100 mg hair samples were grinded using liquid nitrogen, mortar and pestle, following Nerva et al. (2016). DNA pellets were re-suspended in 150 µL of elution solution and then cleaned using the DNA Clean and Concentration 25 kit (Zymoresearch, CA, USA), to further separate DNA pellets from PCR inhibiting molecules. We amplified a 628 bp fragment of the cytochrome b (cyt-b) mitochondrial gene. Despite morphological identifications may allow researchers to distinguish among different (sub)species of Siberian chipmunks according to the coat colour (1% error: Obolenskaya et al., 2009), genetic analysis of mitochondrial cyt-b sequences is reported to be the best way to assess their geographical origin (Lee et al., 2008; Pisanu et al., 2013; Lisovsky et al., 2017). The gene fragment was amplified by PCR using primers developed for Siberian chipmunks (Pisanu et al., 2013). PCR reactions were run through a 2720 Thermal Cycler (Applied Biosystems), following Pisanu et al. (2013). The electrophoresis was run for 35' on a 1% agarose gel, and then sequenced using the dideoxy chain termination method (Sanger, 1977) at BioFab Research (Rome, Italy), with the specific forward primer (cyt-b_For: Pisanu et al., 2013). The obtained sequences were visually cleaned at the 5' and 3' ends by looking at the chromatogram. At the end, to confirm the correct origin of the obtained sequences, we compared our sequences to the ones deposited in the NCBI database using BLASTx and BLASTn ((Altschul et al., 1990).

Pairwise alignments of cyt-b DNA sequences were performed using ClustalW (Higgins and Sharp, 1988) (Appendix A). The best evolutionary model used for our phylogenetic analyses was selected using the algorithm implemented in MEGA7 (Kumar et al., 2016), which gives BIC (Bayesian Information Criterion) score (Tab. 1). Tamura-Nei model with Invariant sites (TN93+I) resulted the best model for the considered data. Origins of our samples were then inferred through the Neighbour-Joining and the Maximum Likelihood methods (1000 bootstrap replicates), based on the Tamura-Nei model (Tamura, 1993). Accession numbers of the reference sequences used in this work are reported in Tab. 2. The number of base substitutions per site (aver-

Table 1 – Maximum Likelihood (a) and Neighbour-Joining (b) trees on mtDNA sequence of the cyt-b of Siberian chipmunks including sequences from the native range (black) and those from Italian sampling areas (red); other chipmunk species are also included as outgroup (blue).

Model	BIC	Invariant	Gamma
TN93+I	5882.162388	0.567608938	n/a
TN93+G	5883.520258	n/a	0.277472063
HKY+I	5885.449082	0.577473228	n/a
HKY+G	5885.503662	n/a	0.269074595
TN93+G+I	5886.725559	0.524016072	2.463778087
HKY+G+I	5891.146146	0.534753106	2.546245569
GTR+G+I	5913.068268	0.528820109	3.186141524
GTR+I	5916.948568	0.567635038	n/a
GTR+G	5920.44041	n/a	0.292840802
T92+I	5978.464725	0.570301976	n/a
T92+G	5980.293395	n/a	0.294073149
T92+G+I	5984.002694	0.515509628	2.314092349
K2+I	6010.553529	0.574066771	n/a
K2+G	6015.832754	n/a	0.304673391
K2+G+I	6018.345273	0.536354445	3.569508737
TN93	6095.087847	n/a	n/a
HKY	6103.10025	n/a	n/a
GTR	6120.246802	n/a	n/a
T92	6177.092585	n/a	n/a
K2	6201.741278	n/a	n/a
JC+I	6259.622256	0.556010399	n/a
JC+G+I	6269.330988	0.555201283	200
JC+G	6270.838104	n/a	0.371651943
JC	6425.002012	n/a	n/a

aging over all sequence pairs) was calculated involving 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. A total of 628 positions occurred in the final dataset. Pairwise distances and differences in base composition bias per site were calculated using the same parameters. A haplotype network has been created to inspect the phylogeographic relationships among specimens of *Eutamias sibiricus sensu lato* using TCS 1.21 (Clement et al., 2000). A connection limit of 78 steps was used as the minimum step number to link the two species, *E. sibiricus* and *E. barberi*, into one single haplotype network, following the instructions of TCS 1.21 (Clement et al., 2000). The output of TCS has been graphically improved with tcsBU (Mùrias et al., 2016) and Adobe Illustrator CC 2015.

Results

We obtained a total of 8 sequences of Siberian chipmunk from 4 Italian populations (Accession numbers: Tab. 2; Fig. 1). All individuals from Italy were clustered by the phylogeographic analysis with the samples from southern Korea (Fig. 1), but for one individual from Rome (Villa Ada) which clustered within the Siberian clade (Figs. 12). The number of base substitutions per site from averaging over all sequence pairs was 0.116. Composition and pairwise distances are reported in Appendices 2 and 3, respectively.

Discussion

Most of the introduced chipmunks to Italy can be reasonably ascribed to the *E. barberi* clade, thus providing support to our expectation. Our findings fit with the morphological identification by Dal Farra et al. (1996), as well as with the intense pet-trade routes between Europe and Korea in the 1980s (Pisanu et al., 2013). A single individual belonging to the Siberian lineage was detected in Villa Ada, together with Korean ones, thus supporting the hypothesis of multiple release events due to the local abandonment of undesired pets (Benassi and Bertolino, 2011; Mori et al., 2018a). Also chipmunk populations introduced to France belonged to the Korean taxon (i.e. to the *E. barberi* clade: Chapuis,

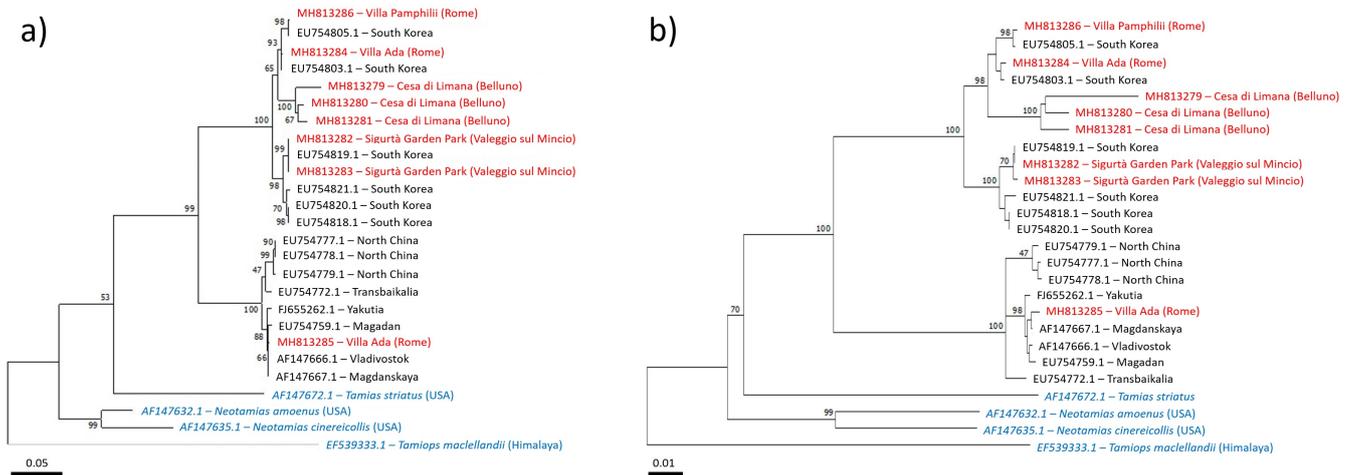


Figure 1 – Maximum Likelihood (a) and Neighbour-Joining (b) trees on mtDNA sequence of the cyt-b of Siberian chipmunks including sequences from the native range (black) and those from Italian sampling areas (red); other chipmunk species are also included as outgroup (blue).

Table 2 – List of reference sequences used to infer the origin of Italian specimens.

GeneBank Accession Number	Species	Isolation country
EU754777.1	<i>Eutamias sibiricus</i>	China
EU754778.1	<i>Eutamias sibiricus</i>	China
EU754779.1	<i>Eutamias sibiricus</i>	China
EU754818.1	<i>Eutamias sibiricus</i>	South Korea
EU754819.1	<i>Eutamias sibiricus</i>	South Korea
EU754805.1	<i>Eutamias sibiricus</i>	South Korea
EU754818.1	<i>Eutamias sibiricus</i>	South Korea
EU754821.1	<i>Eutamias sibiricus</i>	South Korea
EU754803.1	<i>Eutamias sibiricus</i>	South Korea
AF147666.1	<i>Eutamias sibiricus</i>	East Russia
FJ655262.1	<i>Eutamias sibiricus</i>	Russia-Yakutia-Sakha
EU754759.1	<i>Eutamias sibiricus</i>	Russia
AF147667.1	<i>Eutamias sibiricus</i>	Russia-Magdanskaya Oblast
EU754772.1	<i>Eutamias sibiricus</i>	Russia
MH813279	<i>Eutamias sibiricus</i>	Italy (Belluno 1)
MH813280	<i>Eutamias sibiricus</i>	Italy (Belluno 2)
MH813281	<i>Eutamias sibiricus</i>	Italy (Belluno 3)
MH813282	<i>Eutamias sibiricus</i>	Italy (Sigurtà 1)
MH813283	<i>Eutamias sibiricus</i>	Italy (Sigurtà 2)
MH813284	<i>Eutamias sibiricus</i>	Italy (Villa Ada 1)
MH813285	<i>Eutamias sibiricus</i>	Italy (Villa Ada 2)
MH813286	<i>Eutamias sibiricus</i>	Italy (Villa Pamphili)

2011; Pisanu et al., 2013). The morphology of chipmunks occurring in urban and periurban parks of Belgium, the Netherlands, Germany and Switzerland seem to show the same morphological phenotype of the Korean taxon: deep brown dark stripes (sharp black in Russian specimens), rufous brown head (greyish-brown in Russian specimens) and rufous-fiery red central pair of light dorsal stripes (sandy in Russian specimens: Obolenskaya et al., 2009; Mori et al., 2018c). Despite these morphological characteristics, genetic analyses are necessary to confirm this hypothesis. Accordingly, the importance of genetic analyses to assess origin of alien species populations even in taxa easily identifiable through morphological features, e.g. the Siberian chipmunk, increases where low densities limit the collection of biological samples (i.e. <10 individuals per study area in Belluno and in Rome, Italy: Mori et al., 2018a; Zozzoli et al., 2018). Furthermore, very few naturalized individuals of Siberian chipmunks have been photographed in Italy (with the exception of Sigurtà Garden Park: Mori et al., 2018b), as they are mainly present at low densities and difficult to detect, with the only

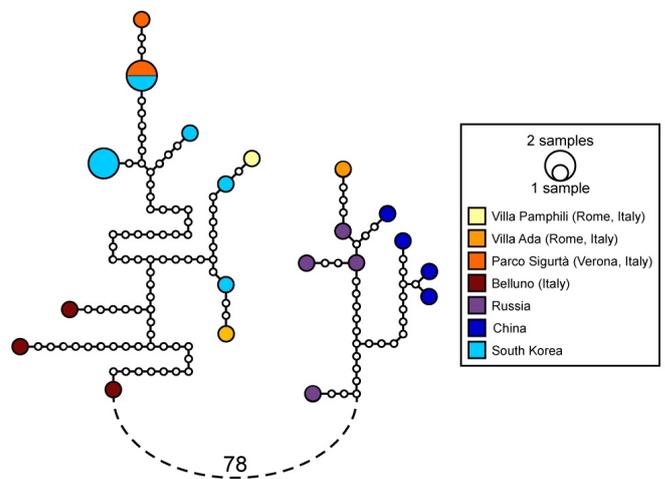


Figure 2 – Haplotype network on all the available sequences of Siberian chipmunk.

self-sustaining population occurring in Sigurtà Garden Park (Mori et al., 2018b). Nuclei of Siberian chipmunks in Rome could have been maintained by several releases, with very few evidences of reproduction (Benassi and Bertolino, 2011; Mori et al., 2018a).

So far, evidence of impacts by introduced chipmunks are mainly related to the spread of Lyme disease and human borreliosis, but they also include a potential parasite-mediated competition with small rodents (Marsot et al., 2013; Vourc’h et al., 2016; Mori et al., 2018d) and debarking of native trees (EM and RZ, unpublished). Therefore, established localized populations should be rapidly controlled or eradicated, according to the recommendations of the EU Regulation 1143/2014 (Carboneras et al., 2018; Nentwig et al., 2018). The taxon “*Eutamias sibiricus*” is currently included within the list of invasive alien species of Union concern (Nentwig et al., 2018). The Korean clade shows a very high genetic divergence (i.e. > 10%) from the main taxon, comparable to the genetic distance between North American chipmunk species, potentially representing a distinct species: *Eutamias barberi* (Lee et al., 2008). However, before the confirmation of this taxonomic redefinition, Lee et al. (2008) suggested to increase knowledge on distribution, morphology and diversity at nuclear genes of this clade before proposing it as a new species. If this species splitting will be confirmed and accepted by the scientific community, a new risk assessment should be rapidly conducted to avoid that the Korean chipmunks became widely traded again. ☞

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Sequence alignment**Table S2** Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 628 positions in the final dataset. Evolutionary analyses were conducted in MEGA7**Table S3** Estimates of Base Composition Bias differences between Sequences. Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 628 positions in the final dataset. Evolutionary analyses were conducted in MEGA7