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Genetic characterization of *Callosciurus* (Rodentia: Sciuridae) Asiatic squirrels introduced in Argentina

M. GABRIELLI[1], Y. P. CARDOSO[1], V. BENITEZ[2], A. C. GOZZI[2], M. L. GUICHÓN[2], & M. S. LIZARRALDE[1]*

[1]*Centro Regional de Estudios Genómicos (CREG), Universidad Nacional de La Plata, La Plata, Pcia. de Bs. As, Argentina, and* [2]*Departamento de Ciencias Básicas, Universidad Nacional de Luján, Luján, Pcia. de Bs As, Argentina*

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Abstract
Squirrels have been traded in the pet market for several decades, and numerous species have established in the wild. The Asiatic species *Callosciurus erythraeus* and *Callosciurus finlaysonii* have been introduced into other parts of Asia, in Europe and South America. In this study, (1) we conducted a genetic characterization of *C. erythraeus* introduced into Argentina and compared them with native and introduced populations in Asia, and (2) we analyzed genetic variation among the four invasion foci in Argentina in order to corroborate that the pathway of invasion was a single introduction event in the country and subsequent translocations. We analyzed mitochondrial (cytochrome b, Cyt b; cytochrome oxidase c subunit I, COI and D-loop) and nuclear (recombination activating gene I, RAGI) DNA markers using the classical method (DNA barcoding gap analysis) and also the Automatic Barcode Gap Discovery method (ABGD). The markers D-loop, COI, and RAG1 indicated that the introduced squirrels from the different invasion foci formed a monophyletic group that, together with only one haplotype for the D-loop and COI markers, supported the hypothesis of one introduction event into Argentina followed by subsequent translocations. Unexpectedly, sequences from squirrels captured in Argentina were more related to *C. finlaysonii* than to *C. erythraeus* for D-loop and Cyt b markers. However, intraspecific variation among sequences of *C. erythraeus* belonging to different subspecies or collected in different regions was large and comparable with the distance to the sequences from Argentina. The ABGD method also indicated large genetic variability within *C. erythraeus* and close proximity between squirrels from Argentina and *C. finlaysonii*. The complex taxonomy of *Callosciurus*, as occurs with the sister species *C. erythraeus* and *C. finlaysonii*, requires a thorough systematic revision. A simultaneous analysis of diagnostic morphological characters and genetic markers is needed and will provide new insight regarding the worldwide invasion of Asiatic squirrels.

Keywords: *Invasive species, Argentina, Asiatic squirrels, mitochondrial DNA, nuclear DNA*

Introduction

The pet trade is a common pathway of introduction of several species worldwide and it is a constant source of individuals that may establish wild populations as a consequence of either accidental escapes or deliberate releases (Hulme et al. 2008; Keller et al. 2011). Squirrels have been traded in both the legal and illegal pet markets for several decades, and numerous species have now been established in the wild, some of which are considered invasive species (Palmer et al. 2008; Bertolino 2009). The most frequent vectors of squirrel introductions are the pet market, private citizens and zoos (Bertolino 2009). Several mammalian species are intensively hunted and traded in Asia within and between nations, where squirrels are sold in fresh food and pet markets (Timmins & Duckworth 2008). The tropics, particularly the forests of South and Southeast Asia, are hotspots of squirrel diversity; however, most studies have focused on temperate squirrel species, raising the need for further research to understand the ecological and taxonomical complexity of tropical squirrels (Kropowski & Nandini 2008).

Squirrels of the subfamily Callosciurinae are native to the Indomalayan Region. The genus *Callosciurus* (Sciuridae: Rodentia) occurs over a large portion of the Indochinese and Malaysian subregions and it is classified into 15 species (Corbet &

*Correspondence: M. S. Lizarralde, Centro Regional de Estudios Genómicos (CREG) Universidad Nacional de La Plata. Boulevard 120 y 62, 1900 La Plata, Bs. As. Argentina. Tel/Fax: +54 11 42758100. Email: mlizarralde@fibertel.com.ar: mslizarralde@gmail.com

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Hill 1992). The species *Callosciurus erythraeus* (Pallas, 1779) and *Callosciurus finlaysonii* (Horsfield, 1824) have been introduced into other parts of Asia, Europe and South America as a result of the pet trade (Bertolino 2009). Wild populations of *C. finlaysonii* have only been reported in Italy (Bertolino et al. 1999), while *C. erythraeus* has been recorded in Argentina, Belgium, France, Japan and the Netherlands (Lurz et al. 2013). However, a recent study conducted in Japan described some individuals that were morphologically identified as *C. erythraeus* but genetically closer to *C. finlaysonii* (Oshida et al. 2007). The authors assumed that both species have been introduced into Japan (Oshida et al. 2007), where a new haplotype of *C. finlaysonii* was described in a subsequent study (Kuramoto et al. 2012). Recently, Oshida et al. (2013) found that *C. erythraeus griseimanus* was more closely related to *C. finlaysonii* than to *C. erythraeus cf. hendeei*, and suggested that *C. e. griseimanus* could be considered a distinct species; all of which reinforces the need for further genetic studies.

In 1970, 10 squirrels were introduced close to Luján city, in the province of Buenos Aires (Argentina), and founded the first population of exotic squirrels in South America (Guichón & Doncaster 2008). These arboreal squirrels were morphologically identified as *C. erythraeus thai* (Aprile & Chicco 1999); however, the subspecies could also correspond to *C. erythraeus atrodorsalis* (Cassini & Guichón 2009). Variation in pelage colour has been described in the original site of introduction, in Luján, where individuals had the typical olive brown agouti dorsal pelage with a black stripe on the back and reddish ventral coloration. However, squirrels with yellow-creamy underparts and no black stripe on their backs were also found (Cassini & Guichón 2009). Other populations of *C. erythraeus* have been introduced in three Argentinian provinces due to its charismatic appearance (Benitez et al. 2013). Interviews with local residents indicated that squirrels were subsequently translocated from the invasion focus in Luján to other locations within Argentina, where they were established as a consequence of intentional releases (Guichón et al. 2005; Benitez et al. 2013).

In this study, we aimed to analyze genetic variation among the invasion foci of *C. erythraeus* established in Argentina and their similarity with native and introduced populations in Asia. Therefore, the objectives of this study were (1) to conduct a genetic characterization of the squirrels introduced in Argentina using both mitochondrial and nuclear DNA markers, and (2) to analyze genetic variation among the invasion foci in Argentina in order to corroborate the pathway of invasion as a single introduction event and subsequent translocations into new areas.

Materials and methods

Between February 2007 and June 2010, we obtained tissue samples for DNA analysis of 30 individuals captured in all known invasion foci of *C. erythraeus* in Argentina: (1) 13 from Luján, Buenos Aires province (34°33'S, 59°07'W), (2) seven from Escobar (34°21'S, 58°48'W), Buenos Aires province, 35 km from Luján, (3) five from Cañada de Gómez (32° 48'S, 61°23'W), Santa Fe province, 290 km from Luján, and (4) five from La Cumbrecita (31°53'S, 64°46'W), Córdoba province, 610 km from Luján (Benitez et al. 2013) (Figure 1). Squirrels inhabit rural and urbanized areas where they use highly fragmented woodland patches mainly composed of introduced tree species. In each study site, 30–50

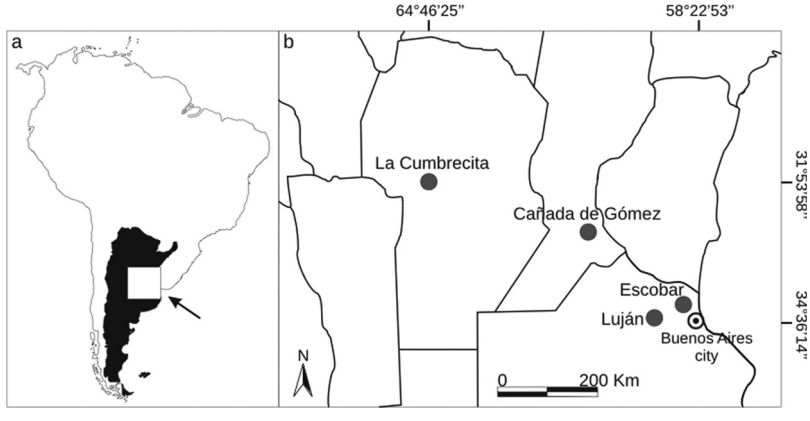

Figure 1. (**a**) Location of the area of interest in Argentina (white square) and (**b**) study areas in the four invasion foci located next to the cities (black dots) of Luján and Escobar in Buenos Aires province, Cañada de Gómez in Santa Fé province, and La Cumbrecita in Córdoba province (lines divide provinces).

cage traps were set at fixed points 40 m apart on tree branches during four consecutive days. Captured squirrels were anaesthetized subcutaneously using a ketamine-acepromazine mixture, pelage coloration was described, tissue samples from one ear were preserved in 96% ethanol, and individuals were released at the same site of capture.

We used the mitochondrial gene *cytochrome oxidase c subunit I* (COI, 650 bp) and a 500-bp fragment of the control region (D-loop) to compare among individuals from the different invasion foci in Argentina and with *Callosciurus* sequences available in GenBank, from both their native and introduced ranges. These two molecular markers were chosen for their high intraspecific variability, which allows evaluation of the genetic variation of the squirrels in Argentina. To complement the genetic characterization of these squirrels within the genus *Callosciurus*, some samples were sequenced using the mitochondrial gene *cytochrome b (*Cyt b) and the nuclear autosomic locus of the recombination activating gene 1 (RAG1). These markers are less variable than COI and D-loop but useful for phylogenetic approaches, and have been previously used for *Callosciurus*, allowing the comparison with sequences available in GenBank. DNA was extracted using the salt-extraction protocol (Aljanabi & Martínez 1997). D-loop sequences were amplified using the universal primers Thr-L15926: 5′-CAATTCCCCGGTCT TGTAAACC-3′ located in the neighbouring tRNA-pro gene and DL-H16340: 5′-CCTGAAG TAGGAACCAGATG-3′ (Vila et al. 1999). The Cyt b gene was amplified using a combination of the primers LMVZ055′-CGAAGCTTGATATGAAAAA CCATCGTTG-3′ (Smith & Patton 1993) and H15910 5′-GATTTTTGGTTTACAAGACCGAG-3′ (Oshida et al. 2000). The COI gene was amplified and sequenced in the Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario (University of Guelph, Guelph, Ontario) using the cocktail primers described in Ivanova et al. (2007) as part of a collaboration between Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET) and International Barcode of Life (IBOL). Finally, the RAG1 gene was amplified using primers designed by Steppan et al. (2004). Thermal profile for all markers are detailed in the Appendix; polymerase chain reaction (PCR) products were examined on 1% agarose gels and both chains (forward and reverse) were automatically sequenced in an ABI3100 sequencer (MACROGEN, Inc. Korea). Chromatograms were edited using the program BIOEDIT 7.0 (Hall 1999). The sequences were aligned using Clustal X (Thompson et al. 1997).

A molecular phylogenetic approach was used to describe the genetic similarity of the squirrels captured in Argentina to available sequences of the four markers of *C. erythraeus*, its sister species *C. finlaysonii* and the congenerics *C. caniceps*, *C. prevostii*, *C. orestes*, *C. notatus*, *C. nigrovittatus* and *C. inornatus* (see Appendix). We also used sequences of other squirrel genera as outgroup species. For the D-loop marker, we sequenced 23 samples obtained in the four invasion foci of Argentina (Luján, Escobar, Cañada de Gómez and La Cumbrecita) and used 74 available sequences of the genus *Callosciurus*, including sequences of *C. erythraeus* from native (China and Taiwan) and introduced (Japan) ranges, *C. finlaysonii* from native (Laos and Thailand) and introduced (Japan) ranges, *C. prevostii* and three species of the genus *Petaurista*. For the Cyt b marker, we sequenced samples obtained in one invasion focus of Argentina (Cañada de Gómez) and used 30 sequences of *Callosciurus* that included *C. erythraeus* from native (Vietnam and Taiwan) and introduced (Japan) ranges, *C. finlaysonii* from its native range, *C. prevostii*, *C. nigrovittatus*, *C. inornatus*, *C. notatus* and *C. caniceps*. We also included two sequences of the species *Lariscus insignis* and *Sciurus lis*. For the COI marker, we sequenced samples from 23 squirrels captured in the four invasion foci of Argentina, and used 11 sequences available of *C. erythraeus* from its native range (Vietnam and China), *C. prevostii*, *C. orestes*, *C. notatus* and the outgroup *Dremomys rufigenis*. For RAG1, we sequenced four samples obtained in three invasion foci of Argentina, and used four sequences of *C. erythraeus* from native ranges (China and Taiwan), one of *C. prevostii* and the outgroup *Sundasciurus philippinensis*. We found no available sequences of *C. finlaysonii* for the markers COI and RAG1.

We conducted separate analyses for each molecular marker and estimated appropriate substitution models using the Akaike Information Criterion as implemented in MrAIC (Nylander 2004). The model of sequence evolution that best fitted our sequences data for D-loop and COI markers was HKY+G+I. The best model of sequence evolution for Cyt b marker was TN+G and for RAG1 marker the best evolution model was HKY+G+I. We analysed three phylogenetic reconstruction methods. First, we performed maximum likelihood (ML) phylogenetic reconstruction using TreeFinder (Jobb et al. 2004) and calculated confidence intervals for the edges of the ML tree using bootstrapping (Felsenstein 1985), based on 1000 repetitions. Second, we conducted Bayesian inference (BI) analysis using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Four chains were run simultaneously (three heated, one cold) for 20,000,000 generations, with tree space sampled every 100th

generation. After a graphical analysis of the evolution of the likelihood scores, we discarded the first 300,000 generations as burn-in. We used the remaining trees to calculate the consensus tree. Third, we performed a maximum parsimony (MP) analysis using TNT 1.1 (Goloboff et al. 2008) and a traditional search using tree bisection reconnection (TBR) with 1000 random sequence additions, and jacknife support values based on 1000 replicates. For each marker, we computed a distance matrix of the Kimura two-parameter (K2P) model (Kimura 1980). In addition to the classical method (DNA barcoding gaps analysis, based on K2P distances) we explored species limits using the Automatic Barcode Gap Discovery method (ABGD) (Puillandre et al. 2012a). This method of DNA taxonomy automatically finds the distance at which a barcode gap occurs and sorts the sequences into putative species based on this distance (Puillandre et al. 2012a). Therefore, it is applicable as an independent tool without an *a priori* species hypothesis, and it provides insight into whether the taxonomic identification based on morphological features has any genetic support. This method is also advantageous in terms of computation time and does not require an ultrametric tree that relies heavily on the correctness of the speciation model (Puillandre et al. 2012a). We used several mitochondrial markers (D-loop, Cyt b and COI) to avoid problems such as the presence of pseudogenes, incomplete lineage sorting or introgression (Puillandre et al. 2012b). The alignments were uploaded to http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html and ABGD was run with the default initial settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, X relative gap width = 1.5, Nb bins = 20) and both distance models (Jukes-Cantor and Kimura).

Results

Squirrels analyzed in this study showed high variability in pelage colour and included individuals with a reddish ventral pelage and a black stripe on the back, others with yellow-creamy underparts and no black stripe on their backs, and intermediate variations such as reddish belly with yellow-creamy or orange chest, groin and/or genital area, and with or without a black stripe on the back (Appendix).

We found only one haplotype by sequence alignment of the D-loop marker in squirrels captured in Argentina. The ML, BI and MP phylogenetic trees obtained had similar topologies and we therefore only show the BI tree with the support values of the three analyses (Figure 2). The haplotype found in Argentina has not been previously sampled in other populations and was phylogenetically closer to sequences of *C. finlaysonii*, particularly to the two haplotypes sampled in Japan that had been morphologically identified as *C. erythraeus* (Oshida et al. 2007).

Sequence alignment of the Cyt b marker comprised 700 bp positions. The ML, BI and MP phylogenetic trees obtained had similar topologies (Figure 3). The haplotype found in Cañada de Gómez, Argentina, has not been described in other populations though it was highly related to *C. finlaysonii*. *C. erythraeus* appeared as sister group. However, one sequence described as *C. finlaysonii* from Thailand was also included in this group. This sequence was strangely identical to one *C. erythraeus cf. hendeei* from Vietnam. A similar misclassification seemed to occur with a sequence of *C. caniceps* from Malaysia that was grouped together with *C. nigrovittatus* instead of its conspecifics (Figure 3). It must be noted that sequences of *C. erythraeus griseimanus* separated from the other sequences of *C. erythraeus* that grouped with *C. finlaysonii*.

For the COI marker we found only one haplotype in the four invasion foci of Argentina, as we expected given the lower evolution rate of the COI marker compared with D-loop analysis. The ML, BI and MP phylogenetic trees obtained had similar topologies (Figure 4). We obtained a non-resolved polytomy among the individuals captured in Argentina in the monophyletic group with *C. erythraeus* from China, and the sequence of *C. erythraeus flavimanus* from Vietnam.

For the RAG1 marker, the sequence alignment comprised 887 bp and we found eight variable sites in the squirrels captured in Argentina, resulting in four haplotypes. The ML, BI and MP phylogenetic trees obtained had similar topologies (Figure 5). Squirrels captured in Argentina formed a monophyletic group; however, *C. erythraeus* did not form a monophyletic group and the sequences obtained in Taiwan were closer to the sequences from Argentina than those from China (Figure 5).

We estimated the percent sequence divergence amongst taxa for each marker separately and calculated the sequence divergence within and between each species of *Callosciurus*. Pairwise comparisons of *Callosciurus* species for D-loop (Table I) and Cyt b markers (Table II) indicated that the smallest percent sequence divergence amongst species occurred between the specimens identified as *C. finlaysonii* and squirrels captured in Argentina (5.88% for D-loop; 5.39% for Cyt b). These values were two times smaller than the divergence between *C. erythraeus* and

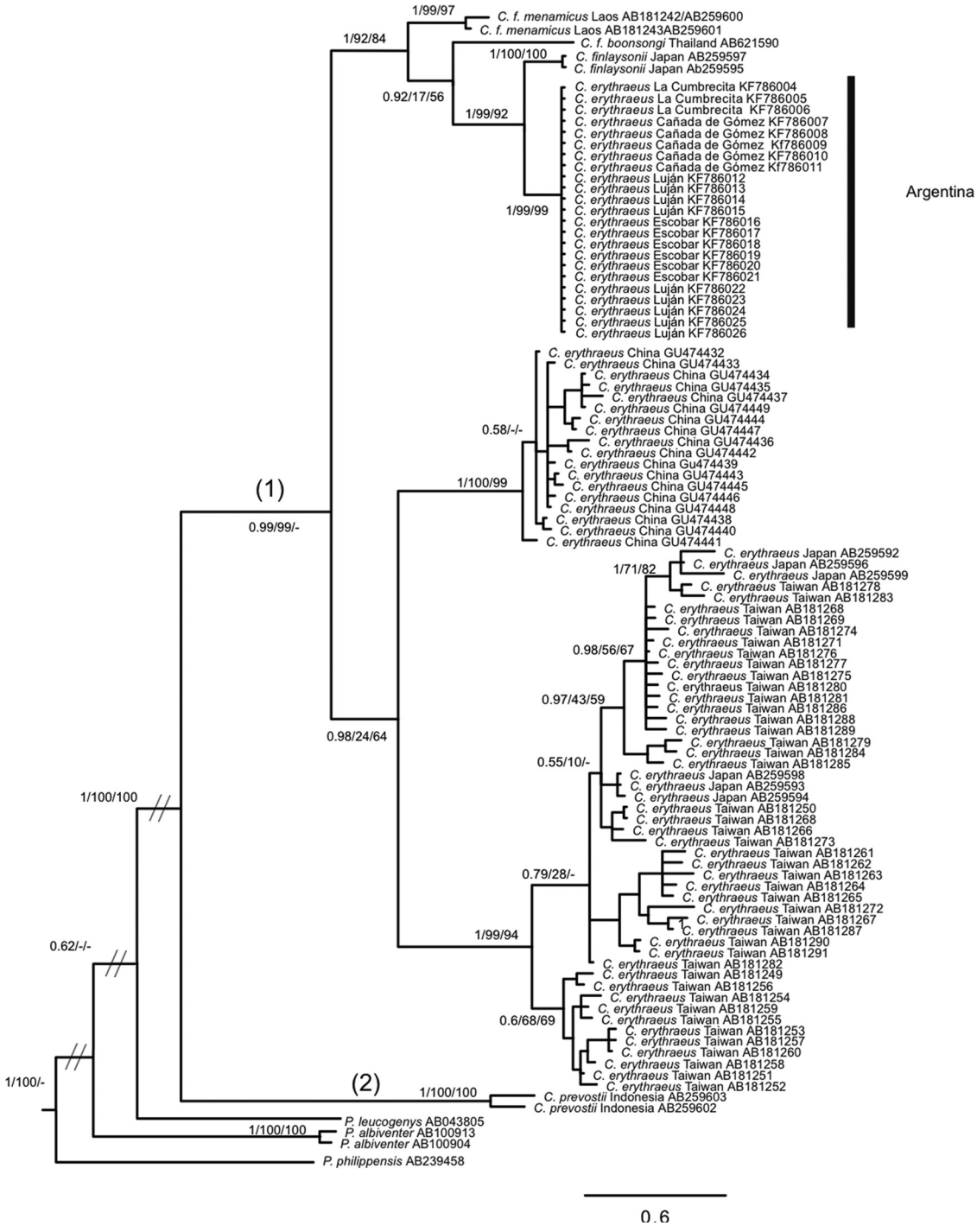

Figure 2. Bayesian inference trees of *Callosciurus* genus. Tree derived from D-loop. Numbers next to branches are Bayesian posterior probabilities, jackknife support values followed by bootstrap values, respectively. Numbers between brackets on a branch that defines a clade correspond to the groups obtained with the Automatic Barcode Gap Discovery (ABGD) method for the marker.

C. finlaysonii; however, divergence between Cyt b sequences from Argentina and *C. erythraeus* (10.96%) and *C. erythraeus griseimanus* (10.89%) was similar to the divergence between *C. erythraeus* and *C. erythraeus griseimanus* (10.24%) (Table II).

For the COI gene (Table III), the smaller divergence (7.34%) was between the squirrels captured in Argentina and *C. erythraeus* from China, which diverged by 8.8% from *C. erythraeus flavimanus* from Vietnam. For RAG1 (Table IV), we found

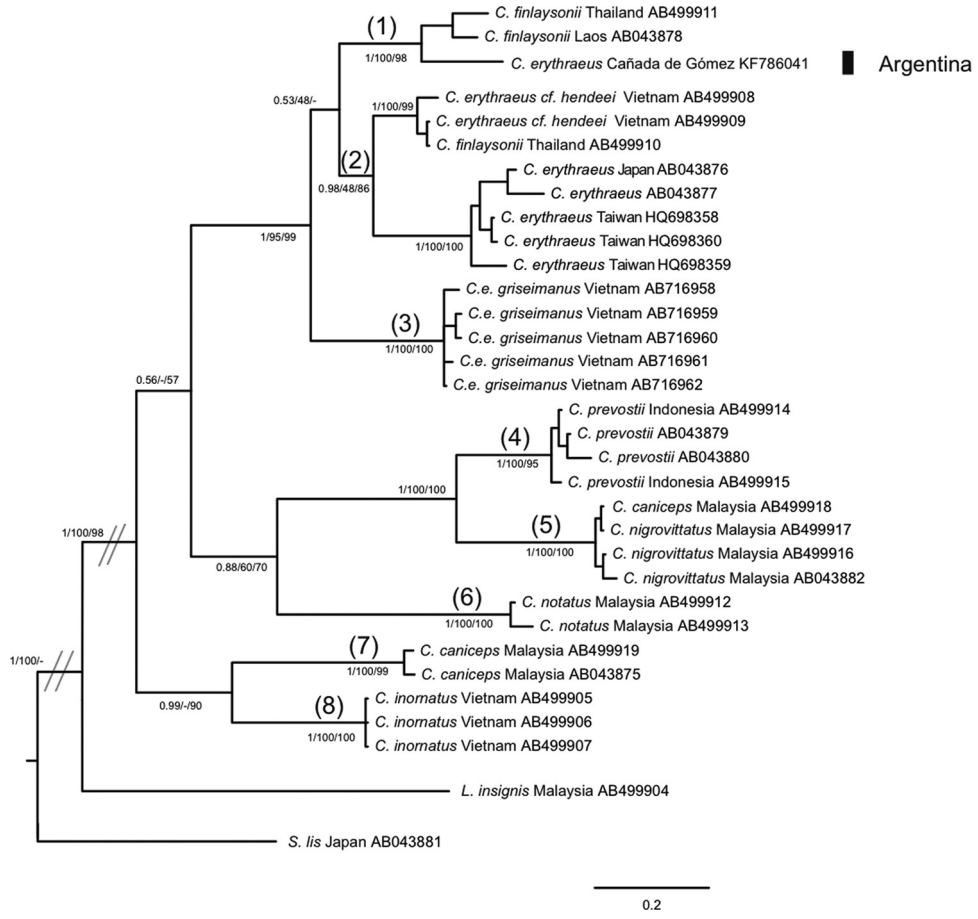

Figure 3. Bayesian inference trees of *Callosciurus* genus. Tree derived from cytochrome b (Cyt b). Numbers next to branches are Bayesian posterior probabilities, jackknife support values followed by bootstrap values, respectively. Numbers between brackets on a branch that defines a clade correspond to the groups obtained with the Automatic Barcode Gap Discovery (ABGD) method for the marker.

that the smaller sequence divergence (1.53%) was between the squirrels captured in Argentina and *C. erythraeus*, which diverged by 2.01% from *C. prevostii*.

The ABGD method for the D-loop data set resulted in seven groups for the recursive partition with prior of 0.001, 0.002, 0.003, 0.005, 0.008, 0.013, 0.022; six groups with 0.036, and two with 0.6 and 0.1. The primary partition was stable on the range of prior values with two groups (Figure 2). When lowering the relative width of the barcoding gap (X-value) and increasing the prior intraspecific limit, we obtained more groups than the number of species defined by taxonomy. We were not able to identify a barcode gap for the D-loop data set. Using the Cyt b dataset, we obtained one partition (i.e., no barcoding gap) using the standard settings, though eight groups separated when lowering the X-value to 1.1 (see Figure 3), with a prior of intraspecific divergence up to 0.0215, that were similar to the number of species defined by taxonomy. For the COI dataset, the number of groups for the recursive partition was six with the entire prior (0.1–0.001). The primary partition was stable on the whole range of prior values, and in all cases we obtained the same six groups indicated in Figure 4, that were similar to the number of species defined by the taxonomy.

Discussion

The markers, D-loop, COI and RAG1, used in this study indicated that the introduced squirrels from the different invasion foci in Argentina formed a monophyletic group. Together with the description of only one haplotype for D-loop and COI markers obtained in the four foci, our results support the information collected in interviews with local residents that indicated a single introduction event into Argentina followed by translocations into new areas within the country. Management decisions should focus on these invasion pathways to prevent further expansion (Borgnia et al. 2013). Variation in pelage

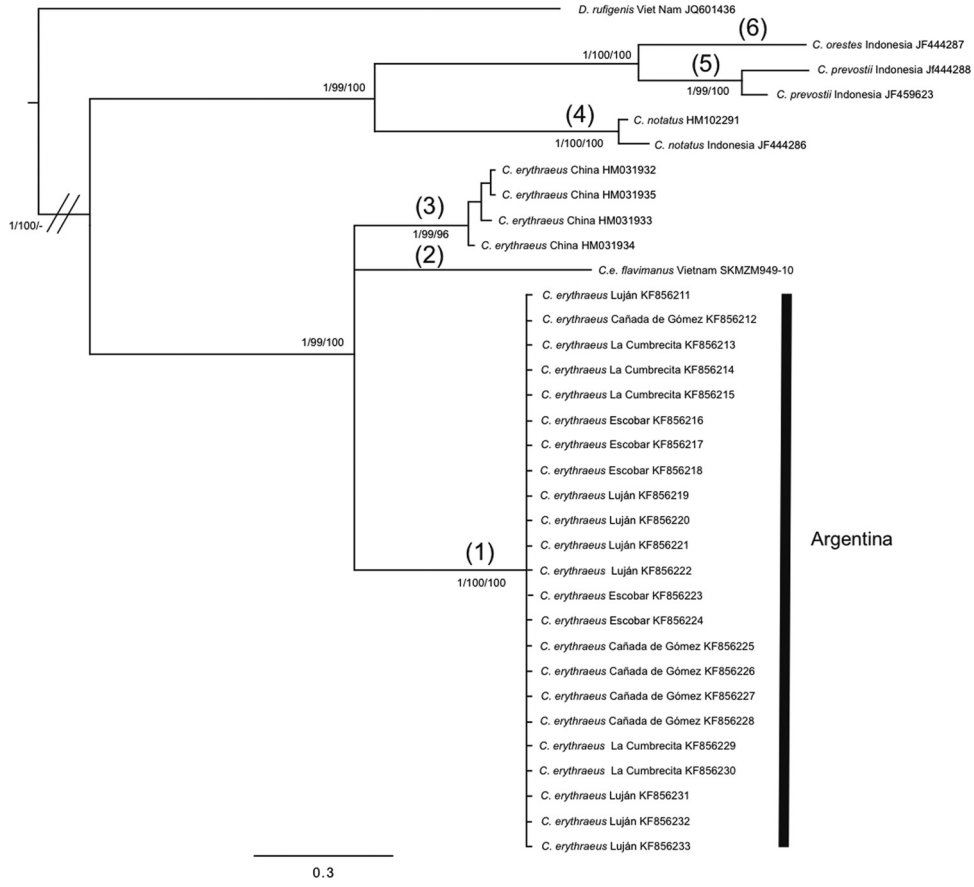

Figure 4. Bayesian inference trees of *Callosciurus* genus. Tree derived from citochrome oxidase c subunit I (COI). Numbers next to branches are Bayesian posterior probabilities, jackknife support values followed by bootstrap values, respectively. Numbers between brackets on a branch that defines a clade correspond to the groups obtained with the Automatic Barcode Gap Discovery (ABGD) method for the marker.

colour among squirrels captured in Argentina showed no genetic support. Unexpectedly, squirrels captured in Argentina were more related to *C. finlaysonii* than to *C. erythraeus* for D-loop and Cyt b markers. However, intraspecific variation among sequences of *C. erythraeus* belonging to different subspecies or collected in different regions was large and comparable with the distance to the sequences from Argentina. We could not resolve the relationship between the sequences from Argentina and *C. erythraeus* using the COI and RAG1 markers.

The classification of the 15 species of the genus *Callosciurus* is based on morphological features, and also several subspecies have been described on the basis of pelage variations (Moore 1961; Moore & Tate 1965; Chakraborty 1985; Corbet & Hill 1992). However, identification of individuals may be difficult given the large number of species and subspecies, many of which closely resemble each other and often show considerable colour variation (Moore & Tate 1965; Chakraborty 1985; Corbet & Hill 1992). The species *C. erythraeus* and *C. finlaysonii* have been referred to by Timmins and Duckworth (2008) as the *C. erythraeus-C. finlaysonii* complex. High sequence divergence within *C. erythraeus* supports the idea that at least this species could represent a species complex (Clare et al. 2007). Regional variation in *C. finlaysonii* is so extreme that it is doubtful that all these forms constitute discrete species (Corbet & Hill 1992), while hybrids may occur in certain regions where both species occur (Moore & Tate 1965; Timmins & Duckworth 2008). Genetic introgression between subspecies, and even between species, has been reported in other squirrels given the hybridization that frequently occurs in pet markets and stores (Wettstein et al. 1995; Good et al. 2008; Hird & Sullivan 2009; Chang et al. 2011). Oshida et al. (2007) and Kuramoto et al. (2012) concluded that the squirrels introduced into Japan that had been identified as *C. erythraeus* but were genetically closer to *C. finlaysonii* could be regarded either as *C. finlaysonii* or as hybrids between both species. However, no studies have yet evaluated whether

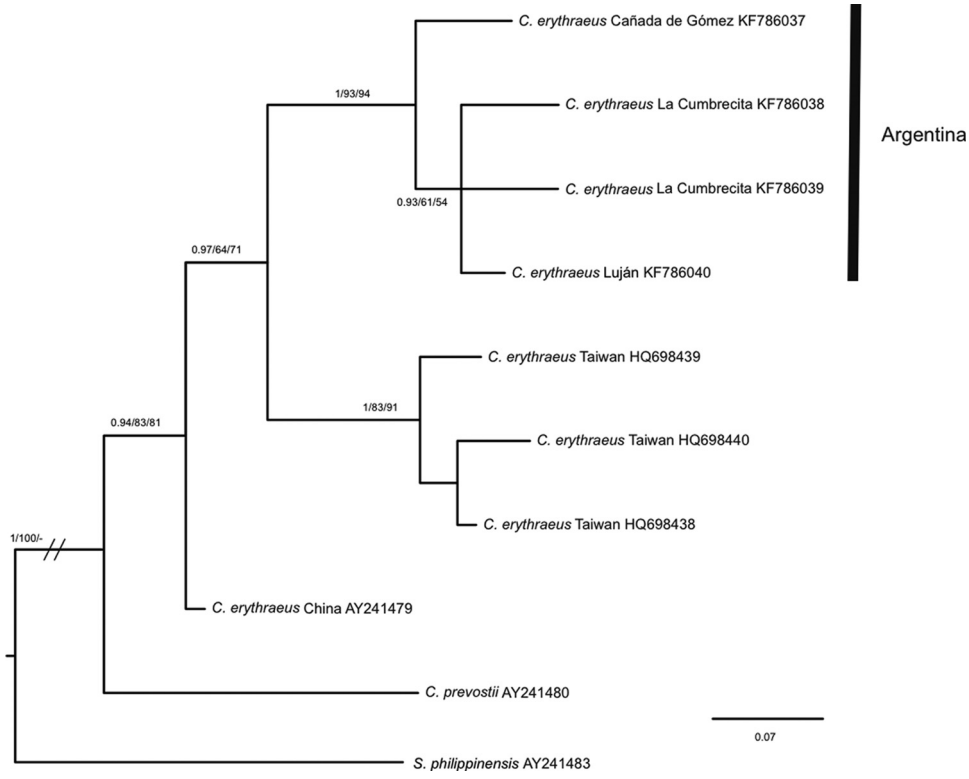

Figure 5. Bayesian inference trees of *Callosciurus* genus. Tree derived from recombination activating gene I (RAGI). Numbers next to branches are Bayesian posterior probabilities, jackknife support values followed by bootstrap values, respectively.

Table I. Percentage of sequence divergence (Kimura 1980) of *Callosciurus* species for the D-loop marker.

	C. erythraeus Arg.	*C. Finlaysonii*	*C. erythraeus*	*C. prevostii*
C. erythraeus Arg.	0			
C. finlaysonii	5.88	6.80		
C. erythraeus	13.59	13.13	6.87	
C. prevostii	21.54	21.2	22.48	3.19

the present classification of *Callosciurus* has any genetic support.

Phylogenetic relationships among *Callosciurus* species indicate three major lineages, one of which is characterized by mainland species (Oshida et al. 2011). The Indochina Peninsula lineage contains two sublineages: *caniceps-inornatus* and *erythraeus-finlaysonii*, which is composed by *C. e. griseimanus*, *C. erythraeus cf. hendeei* and *C. finlaysonii* (Oshida et al. 2013). Interestingly, *C. e. griseimanus* proved to be

Table II. Percentage of sequence divergence (Kimura 1980) of *Callosciurus* species for the cytochrome b (Cyt b) marker.

	C. erythraeus Arg.	*C. erythraeus*	*C.e. griseimanus*	*C. finlaysonii*	*C. prevostii*	*C. caniceps*	*C. inornatus*	*C. nigrovittatus*	*C. notatus*
C. erythraeus Arg.	0								
C. erythraeus	10.96	4.74							
C.e. griseimanus	10.89	10.24	0.72						
C. finlaysonii	5.39	9.85	9.7	2.46					
C. prevostii	20.14	17.9	16.93	17.66	0.93				
C. caniceps	18.29	17.13	16.87	16.22	18.72	0.72			
C. inornatus	15.83	14.36	14.56	14.66	18.53	11.51	0		
C. nigrovittatus	20.96	17.8	18.51	18.61	9.46	18.58	18.41	0.57	
C. notatus	17.76	16.52	17.33	16.73	17.02	17.8	18.85	16.54	1

Table III. Percentage of sequence divergence (Kimura 1980) of *Callosciurus* species for the cytochrome oxidase c subunit I (COI) marker.

	C. erythraeus Arg.	*C. erythraeus*	*C.e. flavimanus*	*C. prevostii*	*C. orestes*	*C. notatus*
C. erythraeus Arg.	0					
C. erythraeus	7.34	0.33				
C.e. flavimanus	9.63	8.8	0			
C. prevostii	19.06	18.18	18.87	2.38		
C. orestes	19.21	18.23	19.58	7.67	0	
C. notatus	18.6	17.53	18.07	13.41	13.51	0.94

Table IV. Percentage of sequence divergence (Kimura 1980) of *Callosciurus* species for the recombination activating gene I (RAGI) marker.

	C. erythraeus Arg.	*C. erythraeus*	*C. prevostii*
C. erythraeus Arg.	0.64		
C. erythraeus	1.53	0.51	
C. prevostii	2.75	2.01	0

more closely related to *C. finlaysonii*; however, genetic distances among the three forms were very similar, suggesting a polytomic phylogenetic relationship which remained unresolved (Oshida et al. 2013). Using the same Cyt b marker, we could not resolve the relationship between these species, as in the previous study by Oshida et al. (2013). However, the monophyletic group formed by *C. e. grisemaneus* from Vietnam was supported by our analyses (molecular phylogenetic approach and ABGD method) and was used as an independent group to estimate genetic distances. These results indicate that certain subspecies of *C. erythraeus* could be more closely related to *C. finlaysonii* than to other conspecifics.

Sequence divergence indicated that the squirrels captured in Argentina were more closely related to *C. finlaysonii*, when this comparison was possible (D-loop and Cyt b markers), than to *C. erythraeus*. However, divergence between squirrels captured in Argentina and *C. erythraeus* was similar to the divergence between subspecies of *Callosciurus* (e.g. between *C. erythraeus* and *C. erythraeus griseimanus* using the Cyt b marker). Sequence divergence using the COI marker also resulted in a similar genetic distance between the squirrels captured in Argentina and *C. erythraeus* (7%) and between *C. erythraeus* from China and *C. erythraeus flavimanus* from Vietnam (9%).

The ABGD method was efficient when intraspecific diversity for the molecular marker was lower than the interspecific diversity, i.e. when sequences sampled within the same species were more similar than sequences sampled from different species. That was the case for COI and Cyt b markers, although with a shorter barcode gap for the latter. Squirrels from Argentina were grouped with *C. finlaysonii* using the Cyt b marker and were separated from *C. erythraeus* using the COI marker. *C. erythraeus* was separated into several groups with both markers, which is consistent with the other analyses. For the D-loop marker, the ABGD method indicated multiple species hypotheses (e.g. one species split into two, or several species merged into a single one). Therefore, the D-loop marker was not entirely consistent in defining species limits using this method due to intra- and interspecific distance overlap. This is a consequence of the high mutation rate for the D-loop marker and a theoretical limitation of the ABGD method and, more generally, of the methods that identify species limits based on the barcode gap, because these methods use the population mutation rate, estimated from the data set, as the main parameter (Puillandre et al. 2012a).

The complex taxonomy of certain *Callosciurus* species and subspecies, such as described for *C. erythraeus* and *C. finlaysonii*, and the genetic and morphological data reported in these last decades require a thorough systematic revision. Misidentification in the field cannot be discarded in certain cases. However, identification based on genetic data cannot be fully applied until a simultaneous analysis of diagnostic morphological characters and genetic markers is conducted. These studies will provide new insight regarding *Callosciurus*' status in its native area and will aid the study of worldwide invasion pathways of introduction.

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Appendix

Polymerase chain reaction thermal cycling amplification profiles.[a]

Locus[b]	Number of cycles	Denaturation		Annealing		Extension	
		Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)
RAG1	40	0,75	94	1	55	1.5	72
D-loop	40	0.75	94	0.5	56	0.75	72
COI	40	0.75	94	0.5	54	1	72
cyt-*b*	40	0,75	94	1.5	55	2	72

[a]Each thermal cycling profile includes a beginning denaturation of 94°C for 5 min, 30–40 cycles of amplifications and a final extension of 72°C for 5 or 10 min.

[b]Locus: *RAG1*, recombination activating gene 1; *D-loop*, the control region of DNA mitochondrial; *COI*, mitochondrial *cytochrome oxidase c subunit I* gene; cyt-*b*, mitochondrial cytochrome *b* gene.

Species name; locality of collection; *GenBank* accession numbers of markers; pelage colouration: of the belly (red, orange or creamy), other underparts such as chest, groins or genital area (red, orange or creamy), and the back (black stripe, faint black stripe or no stripe) of the squirrels captured in Argentina, and references of samples used in this study.

Species name	Locality	GenBank accession number				Pelage colour (belly/other underparts/back)	References
		Cyt b	D-loop	RAG1	COI		
C. erythraeus	Argentina: Luján, Buenos Aires		KF786012	KF786040	KF856233	red/red/black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786013		KF856231	red/red/black stripe	present study
C. erythraeus.	Argentina: Luján, Buenos Aires		KF786014		KF856219	red/red/black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786015		KF856232	red/creamy/no stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires				KF856211	orange/red/no stripe	present study
C. erythraeus	Argentina: Cañada de Gómez, Santa Fe.		KF786011	KF786037	KF856212	red/creamy/black stripe	present study
C. erythraeus	Argentina: Cañada de Gómez, Santa Fe.		KF786010		KF856228	red/red/light black stripe	present study
C. erythraeus	Argentina: Cañada de Gómez, Santa Fe.	KF786041	KF786009		KF856227	red/creamy/black stripe	present study
C. erythraeus	Argentina: Cañada de Gómez, Santa Fe.		KF786008		KF856226	red/creamy/black stripe	present study
C. erythraeus	Argentina: Cañada de Gómez, Santa Fe.		KF786007		KF856225	red/creamy/black stripe	present study
C. erythraeus.	Argentina: La Cumbrecita, Córdoba.				KF856213	red/creamy/black stripe	present study
C. erythraeus	Argentina: La Cumbrecita, Córdoba.		KF786006		KF856214	creamy/creamy/no stripe	present study
C. erythraeus	Argentina: La Cumbrecita, Córdoba.		KF786005	KF786038	KF856229	red/creamy/black stripe	present study
C. erythraeus	Argentina: La Cumbrecita, Córdoba.				KF856230	red/orange/black stripe	present study
C. erythraeus	Argentina: La Cumbrecita, Córdoba.		KF786004	KF786039	KF856215	red/creamy/faint black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires		KF786016		KF856216	red/creamy/faint black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires		KF786017			red/creamy/faint black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires		KF786018		KF856217	red/creamy/black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires		KF786019			red/orange/black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires		KF786020		KF856224	red/orange/black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires		KF786021		KF856223	red/red/black stripe	present study
C. erythraeus	Argentina: Escobar, Buenos Aires				KF856218	red/red/black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires.				KF856220	creamy/creamy/no stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires.				KF856221	red/creamy/no stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires				KF856222	creamy/red/faint black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786022			red/creamy/black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786023			red/creamy/black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786024			creamy/orange/faint black stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786025			creamy/creamy/no stripe	present study
C. erythraeus	Argentina: Luján, Buenos Aires		KF786026			red/creamy/faint black stripe	present study
C. erythraeus	Taiwan: Kaohsiung: Tianchi	HQ698358		HQ698438			Chang et al. (2011)
C. erythraeus	Taiwan: Nantou: Zhongxingxincun	HQ698359		HQ698439			Chang et al. (2011)
C. erythraeus	Taiwan: Nantou: Shishan	HQ698360		HQ698440			Chang et al. (2011)
C. erythraeus	China,Yunnan, Jingdong, Wuliangshan, Raomalu			AY241479			Steppan et al. (2004)

(*Continued*)

(Continued).

Species name	Locality	GenBank accession number				Pelage colour (belly/other underparts/back)	References
		Cyt b	D-loop	RAG1	COI		
C. erythraeus	China		GU474432				Guo et al. (2011)
C. erythraeus	China		GU474433				Guo et al. (2011)
C. erythraeus	China		GU474434				Guo et al. (2011)
C. erythraeus	China		GU474435				Guo et al. (2011)
C. erythraeus	China		GU474436				Guo et al. (2011)
C. erythraeus	China		GU474437				Guo et al. (2011)
C. erythraeus	China		GU474438				Guo et al. (2011)
C. erythraeus	China		GU474439				Guo et al. (2011)
C. erythraeus	China		GU474440				Guo et al. (2011)
C. erythraeus	China		GU474441				Guo et al. (2011)
C. erythraeus	China		GU474442				Guo et al. (2011)
C. erythraeus	China		GU474443				Guo et al. (2011)
C. erythraeus	China		GU474444				Guo et al. (2011)
C. erythraeus	China		GU474445				Guo et al. (2011)
C. erythraeus	China		GU474446				Guo et al. (2011)
C. erythraeus	China		GU474447				Guo et al. (2011)
C. erythraeus	China		GU474448				Guo et al. (2011)
C. erythraeus	China		GU474449				Guo et al. (2011)
C. erythraeus	Taiwan		AB181249				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181250				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181251				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181252				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181253				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181254				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181255				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181256				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181257				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181258				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181259				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181260				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181261				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181262				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181263				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181264				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181265				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181266				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181267				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181268				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181269				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181270				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181271				Oshida et al. (2006)

(*Continued*)

(Continued).

Species name	Locality	GenBank accession number				Pelage colour (belly/other underparts/back)	References
		Cyt b	D-loop	RAG1	COI		
C. erythraeus	Taiwan		AB181272				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181273				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181274				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181275				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181276				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181277				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181278				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181279				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181280				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181281				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181282				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181283				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181284				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181285				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181286				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181287				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181288				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181289				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181290				Oshida et al. (2006)
C. erythraeus	Taiwan		AB181291				Oshida et al. (2006)
C. erythraeus	Izu-ohshima Island, Japan		AB259592				Oshida et al. (2007)
C. erythraeus	Izu-ohshima Island / Fukue Island, Japan		AB259593				Oshida et al. (2007)
C. erythraeus	Izu-ohshima Island, Japan		AB259594				Oshida et al. (2007)
C. erythraeus	Hamamatsu, Japan		AB259596				Oshida et al. (2007)
C. erythraeus	Izu Peninsular, Japan		AB259598				Oshida et al. (2007)
C. erythraeus	Miyazaki, Japan		AB259599				Oshida et al. (2007)
C.e.cf. hendeei	Tam Dao, Vietnam	AB499908					Oshida et al. (2011)
C.e.cf. hendeei	Tam Dao, Vietnam	AB499909					Oshida et al. (2011)
C. erythraeus	Ohshima, Tokyo, Japan	AB043876					Oshida et al. (2001b)
C. erythraeus	?	AB043877					Oshida et al. (2001b)
C. erythraeus	Qiongzhong, China				HM031932		Lu et al. (2012)
C. erythraeus	Qiongzhong, China				HM031933		Lu et al. (2012)
C. erythraeus	Qiongzhong, China				HM031934		Lu et al. (2012)
C. erythraeus	Qiongzhong, China				HM031935		Lu et al. (2012)
C.e. flavimanus	Vietnam				SKMZM949-10*		BOLD
C.e. griseimanus	Cat Tien National Park, Vietnam	AB716958					Oshida et al. (2013)
C.e. griseimanus	Cat Tien National Park, Vietnam	AB716959					Oshida et al. (2013)
C.e. griseimanus	Cat Tien National Park, Vietnam	AB716960					Oshida et al. (2013)

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Species name	Locality	GenBank accession number				Pelage colour (belly/other underparts/back)	References
		Cyt b	D-loop	RAG1	COI		
C.e. griseimanus	Vinh Cuu Nature Reserve, Vinh Cuu, Vietnam	AB716961					Oshida et al. (2013)
C.e. griseimanus	Vinh Cuu Nature Reserve, Vinh Cuu, Vietnam	AB716962					Oshida et al. (2013)
C. finlaysonii	Hamamatsu, Japan		AB259595				Oshida et al. (2007)
C. finlaysonii	Hamamatsu, Japan		AB259597				Oshida et al. (2007)
C. f. menamicus	nearby Vientianne, Laos		AB181242/ AB259600				Oshida et al. (2007)
C. f. menamicus	nearby Vientianne, Laos		AB181243/ AB259601				Oshida et al. (2007)
C. f. boonsongi	Thailand		AB621590				Kuramoto et al. (2012)
C. finlaysonii	Thailand	AB499910					Oshida et al. (2011)
C. finlaysonii	Thailand	AB499911					Oshida et al. (2011)
C. finlaysonii	near Vientianne, Laos	AB043878					Oshida et al. (2001b)
C. prevostii	Sumatra Island, Indonesia		AB259602				Oshida et al. (2007)
C. prevostii	Sumatra Island, Indonesia		AB259603				Oshida et al. (2007)
C. prevostii	Palembang, Indonesia	AB499914					Oshida et al. (2011)
C. prevostii	Palembang, Indonesia	AB499915					Oshida et al. (2011)
C. prevostii	?	AB043879					Oshida et al. (2001b)
C. prevostii	?	AB043880					Oshida et al. (2001b)
C. prevostii	Indonesia, Kayan Mentarang Nature Reserve				JF459623		BARCODE 2011
C. prevostii	Indonesia, Kayan Mentarang Nature Reserve				JF444288		BARCODE 2011
C. prevostii	Zoo captive			AY241480			Steppan et al. (2004)
C. caniceps	Negeri Sembilan, Malaysia	AB499918					Oshida et al. (2011)
C. caniceps	Negeri Sembilan, Malaysia	AB499919					Oshida et al. (2011)
C. caniceps	Negeri Sembilan, Malaysia	AB043875					Oshida et al. (2001b)
C. inornatus	Co Ma, Thuan, Chau, Son La, Vietnam	AB499905					Oshida et al. (2011)
C. inornatus	Hon, Phu Yen, Son La, Vietnam	AB499906					Oshida et al. (2011)
C. inornatus	Hon, Phu Yen, Son La, Vietnam	AB499907					Oshida et al. (2011)
C. nigrovittatus	Negeri Sembilan, Malaysia	AB499916					Oshida et al. (2011)
C. nigrovittatus	Negeri Sembilan, Malaysia	AB499917					Oshida et al. (2011)
C. nigrovittatus	Negeri Sembilan, Malaysia	AB043882					Oshida et al. (2011)
C. notatus	Negeri Sembilan, Malaysia	AB499912					Oshida et al. (2001b)
C. notatus	Negeri Sembilan, Malaysia	AB499913					Oshida et al. (2011)
C. notatus	USA				HM102291		Cooper et al. (2007)
C. notatus	Indonesia, Kayan Mentarang Nature Reserve				JF444286		BARCODE 2011

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(Continued).

Species name	Locality	GenBank accession number				Pelage colour (belly/other underparts/back)	References
		Cyt b	D-loop	RAG1	COI		
C. orestes	Indonesia, Kayan Mentarang Nature Reserve				JF444287		BARCODE 2011
Dremomys rufigenis	Viet Nam: Quang Nam, Tra Giac				JQ601436		BARCODE 2012
Lariscus insignis	Negeri Sembilan, Malaysia	AB499904					Oshida et al. (2011)
Sciurus lis	Fukui Pref., Japan	AB043881					Oshida et al. (2001b)
Petaurista albiventer			AB100913				Oshida et al. (2004)
P. albiventer			AB100904				Oshida et al. (2004)
P. philippensis			AB239458				Oshida et al. (2011)
P. leucogenys			AB043805				Oshida et al. (2001a)
Sundasciurus philippinensis				AY241483			Steppan et al. (2004)

*Sequence retrieved from Barcode of Life Data Systems.