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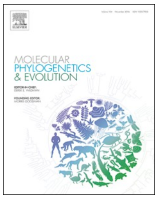

Contrasting phylogeographic histories between the continent and islands of East Asia: Massive mitochondrial introgression and long-term isolation of hares (Lagomorpha: *Lepus*)

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ABSTRACT

Hares of the genus *Lepus* are distributed worldwide, and introgressive hybridization is thought to be pervasive among species, leading to reticulate evolution and taxonomic confusion. Here, we performed phylogeographic analyses of the following species of hare across East Asia: *L. timidus*, *L. mandshuricus*, *L. coreanus*, and *L. brachyurus* collected from far-eastern Russia, South Korea, and Japan. Nucleotide sequences of one mitochondrial DNA and eight nuclear gene loci were examined, adding sequences of hares in China from databases. All nuclear DNA analyses supported the clear separation of three phylogroups: *L. timidus*, *L. brachyurus*, and the *L. mandshuricus* complex containing *L. coreanus*. On the other hand, massive mitochondrial introgression from two *L. timidus* lineages to the *L. mandshuricus* complex was suggested in continental East Asia. The northern population of the *L. mandshuricus* complex was mainly associated with introgression from the continental lineage of *L. timidus*, possibly since the last glacial period, whereas the southern population of the *L. mandshuricus* complex experienced introgression from another *L. timidus* lineage related to the Hokkaido population, possibly before the last glacial period. In contrast to continental hares, no evidence of introgression was found in *L. brachyurus* in the Japanese Archipelago, which showed the oldest divergence amongst East Asian hare lineages. Our findings suggest that glacial–interglacial climate changes in the circum-Japan Sea region promoted distribution shifts and introgressive hybridization among continental hare species, while the geographic structure of the region contributed to long-term isolation of hares on the islands, preventing inter-species gene flow.

1. Introduction

It is now widely accepted that evolutionary processes in animals can present opportunities for inter-species gene flow after speciation without complete fusion of populations of the two species (Mayr et al., 1996). A number of molecular studies have suggested introgressive hybridization in hundreds of species pairs (Mallet, 2005; Toews and Brelsford, 2012). Introgressive hybridization is thought to play important roles during the speciation and adaptation processes (Abbott et al., 2013; Mallet, 2005; Seehausen, 2013), and pervasive introgression among closely related species leads to reticulate evolution. In particular, complete replacement of the original mitochondrial genome via introgression is an extreme result of hybridization events, causing nuclear-mitochondrial discordance (Good et al., 2015; Irwin et al., 2009; Kutschera et al., 2014; Pons et al., 2014; Seehausen, 2013; Toews et al., 2014; Willis et al., 2014; Zieliński et al., 2013). Extensive mitochondrial introgression may be caused by demographic events such as distribution shifts (Currat et al., 2008; Seixas et al., 2018) or driven by selective advantages (Boratyński et al., 2014; Doiron et al., 2002; Toews et al., 2014), but these hypotheses remain controversial.

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The genus *Lepus* represents a remarkable animal group, as it has undergone reticulate evolutionary processes due to frequent introgressive hybridization. Approximately 30 species have been identified from various habitats across North America, Asia, Europe, and Africa (Smith et al., 2018). All modern species in this genus have been inferred to be evolutionarily young lineages that arose via rapid expansion during the last ~5 million years (Matthee et al., 2004; Robinson and Matthee, 2005), and more than 10 species in the Holarctic region have been associated with introgressive hybridization (Alves et al., 2008; Jones et al., 2018; Liu et al., 2011b; Melo-Ferreira et al., 2012, 2014a; Wu et al., 2011). Specifically, introgression between the arctic/boreal species of *L. timidus* occupying northern Eurasia and other temperate species has been reported. For example, ongoing introgressive hybridization between *L. timidus* and *L. europaeus* (temperate species in Europe) has been detected in the Alps, Fennoscandia, and southwestern Russia (Levänen et al., 2018a, b; Thulin et al., 1997, 2006; Zachos et al., 2010). Moreover, past massive mitochondrial introgressions from *L. timidus* have been suggested in three temperate species in the Iberian Peninsula (*L. europaeus*, *L. castroviejoi*, and *L. granatensis*), where *L. timidus* is thought to have expanded its range during the last glacial period but is currently absent (Melo-Ferreira et al., 2005, 2007). In particular, *L. granatensis* exhibits geographical structuring of its mitochondrial lineages; the original haplotypes of *L. granatensis* have remained in the southern Iberian Peninsula, and haplotypes with introgression from *L. timidus* increase in frequency toward the north (Melo-Ferreira et al., 2005). This introgression has been attributed to distribution shifts between arctic/boreal and temperate species due to climate changes since the end of the Late Pleistocene (Acevedo et al., 2015; Melo-Ferreira et al., 2011; Seixas et al., 2018).

Because of the wide distribution range of *L. timidus*, from northern Europe to Northeast Asia, introgression between *L. timidus* and other temperate species may be relatively common across the Eurasian continent (Alves et al., 2008; Liu et al., 2011b; Melo-Ferreira et al., 2007, 2012). East Asia—including China, far-eastern Russia, Korea, and Japan—represents one of the most species-rich regions for hares, including *L. timidus* (see species distribution ranges in Fig. 1). However, as these species were historically classified based on morphology and biogeography, their taxonomic status and relationships remain controversial. Moreover, only one molecular phylogenetic study has examined most *Lepus* species in East Asia, and that study relied solely on mitochondrial DNA (mtDNA) (Wu et al., 2005). Subsequently, Liu et al. (2011b) focused on phylogenetic relationships among nine species of hares only from China using four mtDNA loci and one nuclear DNA (nDNA) locus, and concluded that the hares they examined, including *L. timidus*, had undergone multidirectional introgression events and reticulate evolution. Furthermore, they suggested that the original mitochondrial lineage of *L. mandshuricus* in northeastern China was completely replaced by lineages with introgression from *L. timidus* and *L. sinensis* in southeastern China (Liu et al., 2011b), while other populations outside of China have not yet been surveyed. In our previous study, based on one mtDNA locus (Kinoshita et al., 2012), we suggested that past massive mitochondrial introgression from *L. timidus* to *L. coreanus* might have occurred in the Korean Peninsula. These studies suggest that frequent and massive introgressions of mtDNA among East Asian hares have led to confusion regarding their taxonomic and phylogenetic relationships. On the other hand, *L. brachyurus*, the hare species endemic to the Japanese Archipelago, is suggested to have a long independent history, beginning before the Middle Pleistocene (Nunome et al., 2010, 2014; Yamada et al., 2002), despite the possibility of secondary contact and introgressive hybridization with *L. timidus* and other continental species across land bridges during glacial periods. Comparative genetic studies on continents and insular isolates are currently of fundamental importance to understand the geographical factors in evolutionary divergence and shaping biodiversity of a focal area (Patiño et al., 2017), while only a handful of phylogeographic studies have been conducted in East Asia including both sides of the archipelago and the continent (e.g., Aoki et al., 2018; Kinoshita et al., 2015; Sakka et al., 2010). The circum-Japan Sea region,

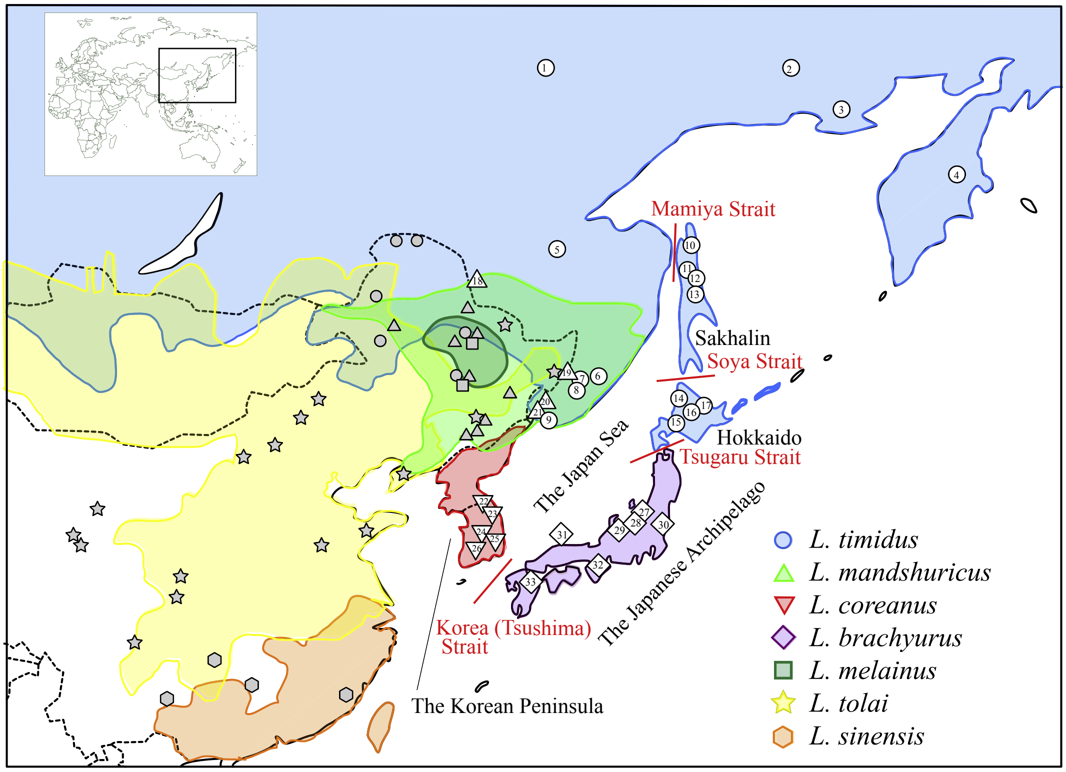

Fig. 1. Sample locations and distribution areas of *Lepus* species investigated in this study. Locations with numbers indicate sampling points (Supplementary Table 1), and those without numbers are database sequences (Supplementary Table 3).

surrounded by the Japanese Archipelago and the Asian continent, has experienced drastic climate and geological changes, leading to the intermittent formation of land bridges during glacial periods. These characteristics of this region may have played an important role in the demographic and evolutionary histories of many organisms in East Asia (Aoki et al., 2018; Dobson, 1994; Hanazaki et al., 2017; Kawamura, 2007; Kinoshita et al., 2015; Millien-Parra and Jaeger, 1999). Therefore, phylogeographic study of all *Lepus* species in this region will provide insights into the evolutionary mechanisms that have shaped the current fauna of eastern Asia.

In this study, we examined the evolutionary history of all hare species in the circum-Japan Sea region based on multiple nDNA loci and the mitochondrial cytochrome *b* (*Cytb*) gene. We hypothesized that introgression from *L. timidus* to temperate hare species has caused unclear phylogenetic relationships among East Asian hares, and that the evolutionary impacts of introgression would differ between the continental and archipelagic sides of the circum-Japan Sea region. Our results reveal the spatial and temporal aspects of the recurrent introgression events associated with distribution shifts between *L. timidus* and temperate species since the Middle Pleistocene.

2. Materials and methods

2.1. Sampling and sequencing analysis

In total, 97 tissue samples from four *Lepus* species—*L. timidus* ($n = 42$), *L. mandshuricus* ($n = 14$), *L. coreanus* ($n = 10$), and *L. brachyurus* ($n = 31$)—were obtained from road-killed or hunted animals that were collected for the present study and previous studies (Kinoshita et al., 2012; Nunome et al., 2010, 2014). Sampling locations are shown in Fig. 1 and Supplementary Table 1. All samples were preliminarily identified based on collection location and morphological traits such as coat color patterns and skull characteristics, and these classifications were consistent with the results of subsequent analyses based on nDNA loci reported below. Total genomic DNA was extracted using standard phenol-chloroform extraction. We amplified 7,088-bp fragments, representing the maximum total length of six autosomal gene loci (*Mgf*, *Tg*, *Tshb*, *Sptbn1*, *Mc1r*, and *Asip*), one X chromosomal locus (*Phka2*), one Y chromosomal locus (*Sry*), and one mitochondrial gene locus (*Cytb*). Polymerase chain reaction (PCR) analysis was performed following previously described methods (Kinoshita et al., 2012; Nunome et al., 2014). Sequences of the two nDNA loci (*Mgf* and *Phka2*) represented intron regions; the *Mc1r* sequence was from an exon region; and *Tg*, *Tshb*, *Sptbn1*, *Asip*, and *Sry* sequences included both intron and exon regions. The amplified region for each locus and the primers used are shown in Supplementary Table 2. Sequences of the *Cytb* and *Sry* loci for some samples derived from our previous studies (Kinoshita et al., 2012; Nunome et al., 2010, 2014) were downloaded from international DNA databases. Novel sequences used in the present study were registered in databases under the accession numbers LC131889–LC132693. Sequences were aligned using ProSeq ver. 2.91 (Filatov, 2001). The haplotypes of each nDNA fragment were determined using the coalescent-based Bayesian algorithm available in PHASE software (Stephens et al., 2001), including all sequences from our sample set in the same run for each locus. Separate sequences of the *Asip* locus (region including exon 2, intron 2, exon 3, and exon 4) were concatenated for PHASE and subsequent analyses. A small number of sequences could not be phased, mainly due to the presence of two or more singletons, and all phased sequences were subjected to downstream analysis. Furthermore, the *Mgf* and *Cytb* gene sequences of five species from China (*L. timidus*, *L. mandshuricus*, *L. melainus*, *L. tolai*, and *L. sinensis*) were obtained from databases (Liu et al., 2011a, b). Location information for sequences collected from databases and species distribution areas are shown in Fig. 1 and Supplementary Table 3.

2.2. nDNA sequence analyses

First, we assessed the phylogenetic relationships among East Asian hares by constructing individual-based networks using eight nDNA loci (*Mgf*, *Tg*, *Tshb*, *Sptbn1*, *Mc1r*, *Asip*, *Phka2*, and *Sry*). Genetic distance matrices were calculated for each nDNA locus based on the p-distances of all phased sequences using MEGA ver. 6.0 (Tamura et al., 2013). The matrices for each locus were converted to a single genetic distance matrix using the standardized method in POFAD v1.07 (Joly and Bruneau, 2006). A multilocus network based on the converted matrix was then constructed using the NeighborNet algorithm (Bryant and Moulton, 2004) in SplitsTree v.4.14.2 (Huson and Bryand, 2006). Median-joining networks were also constructed for each nDNA locus using the program Network ver. 4.6.1.3 (Bandelt et al., 1999), and indels were treated as single mutations. Sequences of Chinese *Lepus* species obtained from databases (Supplementary Table 3) were also used in constructing the network of the *Mgf* locus.

Four autosomal gene loci (*Mgf*, *Tg*, *Tshb*, and *Sptbn1*) were subjected to multilocus species tree construction, and their divergence times were estimated using the *BEAST package of BEAST 2 ver. 2.4.7 (Bouckaert et al., 2014). Database sequences of *Oryctolagus cuniculus* and *L. americanus* deposited by Matthee et al. (2004) were used as outgroup taxa. In this Bayesian coalescent approach, incomplete lineage sorting during speciation is efficiently accommodated, but subsequent gene flow is not modeled (Leaché et al., 2013). Therefore, we did not use outlier sequences that were not grouped into species-specific phylogroups in the network analysis. The input file for the *BEAST program was generated by the program BEAUti 2 ver. 2.4.7 using a Yule process model and a strict clock with a normal prior value of 0.0022 ± 0.001 (mean ± standard deviation) for the substitution rate of the *Mgf* locus. This substitution rate was calculated using the previously estimated calibration point of *O. cuniculus* and *Lepus* of 11.8 million years ago (MYA) and standard deviation of 1.24 MYA (Matthee et al., 2004). The best-fit nucleotide substitution model was selected for each gene locus using Bayesian information criterion (BIC) scores that were calculated with MEGA 6. Three independent MCMC analyses were performed with *BEAST for 5×10^7 generations, with trees sampled every 5,000 generations. Each log file was checked to confirm convergence to the stationary posterior distribution and sufficient ESS (effective sample size) for each parameter greater than 200 using the program Tracer ver. 1.6 (Rambaut and Drummond, 2013). The log files of three independent runs were combined using the program LogCombiner (provided in the BEAST 2 package), with the first 10% of the sampled parameters discarded as burn-in. A maximum clade credibility tree with median node heights was then obtained using TreeAnnotater ver. 2.4.7 and visualized using FigTree ver. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Phylogenetic and dating inference with mtDNA

Phylogenetic trees based on complete Cyt *b* gene sequences were constructed using the Bayesian inference (BI) and maximum likelihood (ML) methods. In these analyses, we added sequences of Chinese *Lepus* species (Supplementary Table 3), *L. americanus* (AY292733), and *O. cuniculus* (AY292717) as outgroup taxa. The best-fit substitution model (HKY + G) was selected based on the BIC score calculated in MEGA 6. Reconstruction of the phylogenetic tree and estimation of divergence times through BI were conducted using BEAST 2. The dataset was analyzed using a Bayesian uncorrelated log-normal relaxed-clock model, using a coalescent model with constant population size as the tree prior. A calibration point was set based on the divergence time between *O. cuniculus* and *Lepus*, following a normal distribution with a mean of 11.8 MYA and standard deviation of 1.24 MYA (Matthee et al., 2004). Bayesian searches and convergence verification were carried out as described above. A maximum clade credibility tree was visualized in FigTree ver. 1.4.2, and this tree was annotated with median node heights using TreeAnnotater (ver. 2.3.0). Reconstruction of ML phylogenetic trees was conducted using the program MEGA 6. Trees were obtained from heuristic searches using as-is sequence addition and NNI (nearest-neighbor interchange) branch swapping. Topology support was assessed using non-parametric bootstrap analysis (Felsenstein, 1985), with 1,000 replicates using the same settings implemented in the ML heuristic search. The median-joining network for the *L. timidus* clade in the phylogenetic trees was constructed using the program Network. In this analysis, partial 617-bp sequences of Cyt *b* were used, adding database sequences of introgressed mtDNA from *L. timidus* to three temperate hares in the Iberian Peninsula [(*L. europaeus* DQ883027-DQ88303, *L. granatensis* DQ882960-DQ883026, and *L. castroviejoi* DQ883038 examined in Melo-Ferreira et al. (2007)].

2.4. Inference of the introgressive hybridization

Because incongruence was detected between the species tree estimated from nDNA loci and the mitochondrial gene tree, we used JML ver. 1.3.0 (Joly, 2012) to distinguish evidence of introgression from incomplete lineage sorting. The JML method (Joly et al., 2009) uses posterior predictive checking to determine whether the observed distance between the sequences of two species is smaller than the expected minimum distance under a model assuming no hybridization (the incomplete lineage sorting scenario). To estimate the expected minimum distances of *Cytb* sequences, we simulated 9,001 gene trees of *Cytb* using the posterior species trees from the *BEAST analysis described above, discarding the first 10% as burn-in, and using the locus-specific parameters of the *Cytb* gene from the previous BEAST analysis for the locus. If the observed genetic distance of a particular pair of *Cytb* sequences is lower than the significance level ($P = 0.01$) based on the posterior predictive distribution of the expected minimum distance, the incomplete lineage sorting scenario is rejected, and we can conclude that the observed nuclear-mitochondrial discordance is a result of introgressive hybridization.

3. Results

3.1. Network and clustering analyses based on eight nDNA loci

Partial sequences of six autosomal gene loci (*Mgf*, *Tg*, *Tshb*, *Sptbn1*, *Mc1r*, and *Asip*) and two sex chromosomal gene loci (X-linked *Phka2* and Y-linked *Sry*) were successfully obtained from all 97 samples of the four *Lepus* species from East Asia; namely, *L. timidus*, *L. mandshuricus*, *L. coreanus*, and *L. brachyurus*. The lengths of each locus are shown in Supplementary Table 2. Diploid sequences of the *Sptbn1*, *Mc1r*, and *Asip* loci from all individuals, *Mgf* and *Tg* loci from 90 individuals, and *Tshb* and *Phka2* loci from 95 individuals were successfully separated into haplotypes using PHASE software (Supplementary Table 1). The sum of the maximum consensus length of all nDNA loci was 5,948 bp including the *Sry* locus, and 7,088 bp when including the mitochondrial *Cytb* gene.

We constructed an individual-based multilocus network using all eight nDNA loci (Fig. 2A). In the network, three phylogroups were clearly delineated for East Asian hares, two of which emerged as species-specific clusters corresponding to *L. timidus* and *L. brachyurus*. The other phylogroup comprised *L. coreanus* and *L. mandshuricus* individuals, and this phylogroup was referred to as the *L. mandshuricus* complex. Haplotype networks were also constructed for each nuclear gene locus (Fig. 2B and Supplementary Fig. 1). Although network topologies differed among loci, the three phylogroups of East Asian hares were apparent in all networks. In the *Mgf* network, which includes database sequences of Chinese hares, individuals of *L. melainus* were integrated into the *L. mandshuricus* complex, while sequences of *L. sinensis* and *L. tolai* formed a single cluster (Fig. 2B). However, some *L. timidus* and *L. mandshuricus* individuals possessed outlier haplotypes that were not included in any of the species-specific phylogroups (Fig. 2B, Supplementary Fig. 1, and Supplementary Table 1). Some of those haplotypes clustered into phylogroups of other species for each locus. For example, haplotypes of *L. mandshuricus* GK247 and GK286 were included in the *L. timidus* clade in the *Mgf* network (Fig. 2B), and that of *L. timidus* GK189 was included in the *L. mandshuricus* complex clade in the *Tg* network (Supplementary Fig. 1A). In addition, the two outlier haplotypes found in three *L. timidus* individuals (GK199, GK201, and GK203) at the *Tg* locus corresponded to sequences (HM233456–HM233457, HM233460, HM233462, HM233464–HM233465, and HM233351–HM233353) deposited in databases as *L. capensis* (*L. tolai* in China).

3.2. Phylogenetic and chronological inferences based on nDNA loci

Based on phylogroups and local populations, a species tree for East Asian hares was reconstructed using four nDNA loci (*Mgf*, *Tg*, *Tshb*, and *Sptbn1*; Fig. 3). The divergence time between East Asian hares and *L. americanus* was estimated as 2.94 MYA with a 95% credibility interval (HPD) of 1.67–4.36 MYA. Among eastern hares, *L. brachyurus* was inferred to have diverged at 1.88 MYA (95% HPD: 1.16–2.68 MYA), and the subsequent divergence of the *L. timidus* and *L. mandshuricus* complex clades was 1.57 MYA (95% HPD: 0.85–2.18 MYA). However, the order of divergence among the three phylogroups was unclear due to low nodal support for the latter bifurcation (0.78 for BI). The divergence time for the node of the *L. timidus* clade (including the continental, Sakhalin, and Hokkaido populations) was estimated to be 0.05 MYA (95% HPD 0.01–0.08 MYA), and that of the *L. mandshuricus* complex clade (between *L. mandshuricus* and *L. coreanus*) was estimated to be 0.11 MYA (95% HPD: 0.03–0.20 MYA), although the accuracy of these estimates of intra-phylogroup divergence timing was likely limited due to the low mutation rates of nDNA loci.

3.3. Phylogenetic inferences and tests for mtDNA introgression

A Bayesian phylogenetic tree based on the mitochondrial *Cytb* region was divided into four main clades (*L. timidus*, *L. tolai*, *L. sinensis*, and *L. brachyurus* clades; Fig. 4). The *L. tolai* and *L. sinensis* clades were named following a previous study (Liu et al., 2011b). No species-specific clade corresponding to the *L. mandshuricus* complex was found. All sequences of *L. mandshuricus* from far-eastern Russia were integrated into the *L. timidus* clade, while those from northeastern China and of *L. melainus* (currently considered as synonym of *L. mandshuricus*) belonged to either the *L. timidus* or *L. sinensis* clades (described in Liu et al. (2011a,b). Meanwhile, two *L. timidus* and three *L. tolai* sequences from China were included in the *L. sinensis* clade (reported by Liu et al. (2011b). Some structure was present within the *L. timidus* clade, with continental and Sakhalin *L. timidus* populations forming a clade (designated the Tim1 lineage; 0.95 BI) in which the aforementioned haplotypes of *L. mandshuricus* were included. All sequences of *L. timidus* from Sakhalin formed a subclade (the Sakhalin clade; 0.99 BI) within the Tim1 lineage, including one sequence from an individual of *L. timidus* collected in far-eastern Russia. By contrast, all haplotypes of *L. timidus* from Hokkaido were resolved as monophyletic (the Hokkaido clade; 0.98 BI), with the haplotypes of *L. coreanus* and one *L. mandshuricus* individual forming a sister clade (0.98 BI) to the Hokkaido lineage; the clade comprising the Hokkaido and *L. coreanus* clades was designated the Tim2 lineage (0.98 BI). The topology of the ML phylogenetic tree was generally consistent with that of the Bayesian phylogenetic tree (only bootstrap values are shown in Fig. 4). Consequently, the three mitochondrial types recovered from the *L. mandshuricus* complex ("*L. sinensis*," "*L. coreanus* (Tim2)," and "Tim1" types) were observed in northeastern China, the Korean Peninsula, and northeastern China and southern far-eastern Russia, respectively (see map in Fig. 4). Furthermore, several haplotypes of the Tim1 lineage were shared between *L. timidus* and *L. mandshuricus*, and introgressed haplotypes from *L. timidus* to three temperate species of the Iberian Peninsula (Melo-Ferreira et al., 2007) were also integrated into the Tim1 lineage (Supplementary Fig. 2).

The divergence time of East Asian hares from *L. americanus* was estimated to be 5.00 MYA (95% HPD: 3.24–7.57 MYA), earlier than the time estimated from the species tree analysis using nDNA loci (2.94

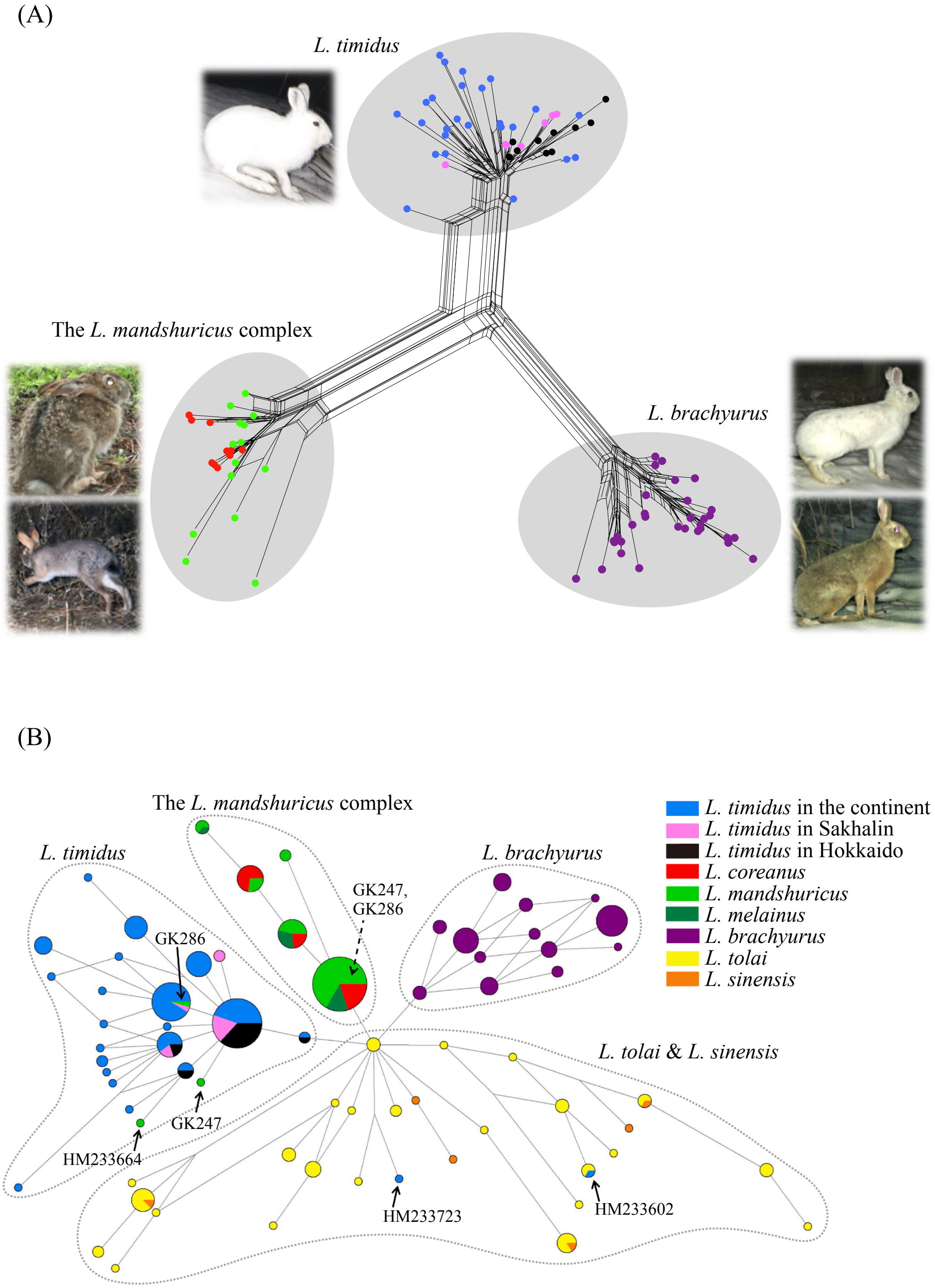

Fig. 2. Networks based on eight nuclear DNA (nDNA) loci. (A) Multilocus network of East Asian hares constructed using the NeighborNet algorithm in SplitsTree. For this network, the genetic distances among eight nDNA loci were standardized using POFAD. Phylogroups are shaded in gray. (B) Median-joining network of *Mgf* locus haplotypes of East Asian hares and database sequences of Chinese hares. Each gap in the sequences was treated as a single substitution. Phylogroups are denoted with dotted lines. Outlier haplotypes that did not cluster into a species-specific phylogroup are marked with arrows and labeled with sample codes of individuals or accession numbers of sequences. Alternative species-specific alleles of these individuals are shown with dotted arrows. Two *Lepus mandshuricus* individuals (GK247 and GK286) possessed outlier and species-specific haplotypes in heterozygous state.

MYA, 95% HPD: 1.67–4.36 MYA), although the same calibration point for the divergence time of *Lepus* and *Oryctolagus* was used in both estimations. One reason for this discrepancy is likely a saturation problem in the mtDNA, which would reduce the distance between the time to the most recent common ancestor (tMRCA) estimates of *Lepus* species and *Oryctolagus*. Considering the overlap in the estimated divergence times for *L. brachyurus* (95% HPD: 1.64–4.36 MYA from nDNA phylogeny and 2.35–5.41 MYA from mtDNA phylogeny), the gap between the two phylogenies decreases in younger branches. The estimated divergence times for mitochondrial lineages of other East Asian hares were later than that of *L. brachyurus*, ranging from the Pliocene to the Early Pleistocene (*L. sinensis*, 2.94 MYA with 95% HPD of 1.76–4.24 MYA,

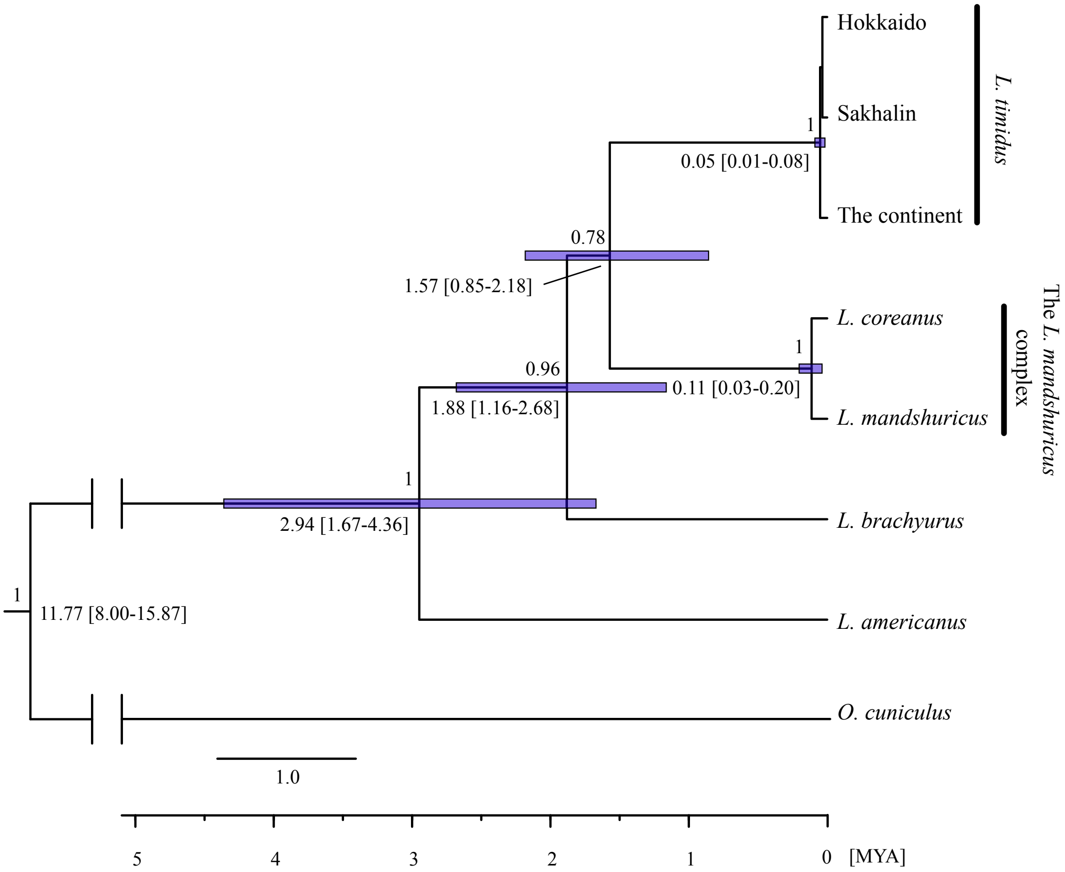

Fig. 3. Species tree constructed using four nDNA loci (*Mgf*, *Tg*, *Tshb*, and *Sptbn1* sequences) of four *Lepus* species in East Asia and two outgroups, *L. americanus* and *Oryctolagus cuniculus*, using *BEAST. The tree is a maximum clade credibility tree from our species tree analysis; numbers along the branches of the tree are the posterior probabilities of nodes and estimated divergence times (means), with their 95% confidence intervals noted in parentheses.

between *L. timidus* and *L. tolai*, 0.87 MYA with 95% HPD of 0.53–1.43 MYA). The tMRCA for *L. brachyurus* was estimated to be 0.95 MYA (95% HPD: 0.52–1.55 MYA), and these lineages are more than twice as old as other East Asian *Lepus* species (*L. sinensis*, *L. tolai*, and *L. timidus*). Within the *L. timidus* clade, the Tim2 lineage branched from the Tim1 lineage around 0.40 MYA (95% HPD: 0.23–0.65 MYA), before the diversification of the Tim1 lineage at 0.30 MYA (95% HPD: 0.17–0.48 MYA). The divergence time of the two clades in the Tim2 lineage (the Hokkaido and *L. coreanus* clades) was estimated to be 0.30 MYA (95% HPD: 0.17–0.50 MYA), and the tMRCAs of these clades were estimated at 0.15 MYA (95% HPD: 0.07–0.26 MYA) and 0.12 MYA (95% HPD: 0.04–0.24 MYA), respectively. The tMRCA for the Sakhalin clade was estimated at 0.05 MYA (95% HPD: 0.01–0.11 MYA).

In the JML test, all observed *Cytb* sequence distances of all individual pairs between individuals of *L. timidus* in Hokkaido and *L. coreanus* or between individuals of *L. timidus* on the continent and *L. mandshuricus* were below the significance level based on posterior predictive distributions ($P = 0.0001–0.0014$ and $0.0001–0.0039$, respectively), strongly suggesting that introgressive hybridization events occurred between these species pairs. On the other hand, the minimum observed distances and probability between *L. brachyurus* and *L. timidus* could be explained by a scenario without introgression ($P = 0.9997$).

4. Discussion

4.1. Molecular phylogenetic implications for taxonomy of East Asian hares

Hares in East Asia (*L. timidus*, *L. mandshuricus*, *L. coreanus*, and *L. brachyurus*) are thought to be closely related, but their taxonomic classifications have long been debated (Chapman and Flux, 1990; Flux and Angermann, 1990; Smith et al., 2018; Wu et al., 2005). In the present study, three clearly distinctive phylogroups, representing *L. timidus*, *L. brachyurus*, and the *L. mandshuricus* complex (consisting of *L. mandshuricus* and *L. coreanus*) were delineated across all examined nDNA loci including X- and Y-chromosomal genes (Fig. 2 and Supplementary Fig. 1). In addition to *L. coreanus*, *L. melainus* in northeastern China showed genetic synonymy with *L. mandshuricus* at the *Mgf* loci (Fig. 2B; Ge et al., 2012; Liu et al., 2011a). Ge et al. (2012) suggested that *L. melainus* was a local population of the black coat color variant of *L. mandshuricus*. In our sample set, a similar variant was also observed in far-eastern Russia (*L. mandshuricus* GK249 and GK277). Based on the present molecular analyses, we suggest that the *L. mandshuricus* complex (including *L. mandshuricus*, *L. coreanus*, and *L. melainus*) should be treated as a single species, *L. mandshuricus* (Radde, 1861).

4.2. Introgression in continental East Asia

The results of phylogenetic analysis using the mitochondrial *Cytb* gene were discordant with the nDNA analyses. Although deeply divergent mitochondrial clades were recognized for *L. timidus*, *L. tolai*, *L. sinensis*, and *L. brachyurus*, we could not identify a species-specific clade representing the *L. mandshuricus* complex, and all sequences from that group were assigned to clades containing *L. sinensis* or *L. timidus* (Fig. 4). It has been suggested that *L. mandshuricus* (as well as *L. melainus*) in northeastern China lost its original mitochondrial lineage through complete replacement by introgressed mitochondrial sequences from *L. timidus* and *L. sinensis* (Liu et al., 2011b). The present study further suggested that mitochondrial introgression from *L. timidus* of the Tim1 lineage on the continent has spread in northern populations

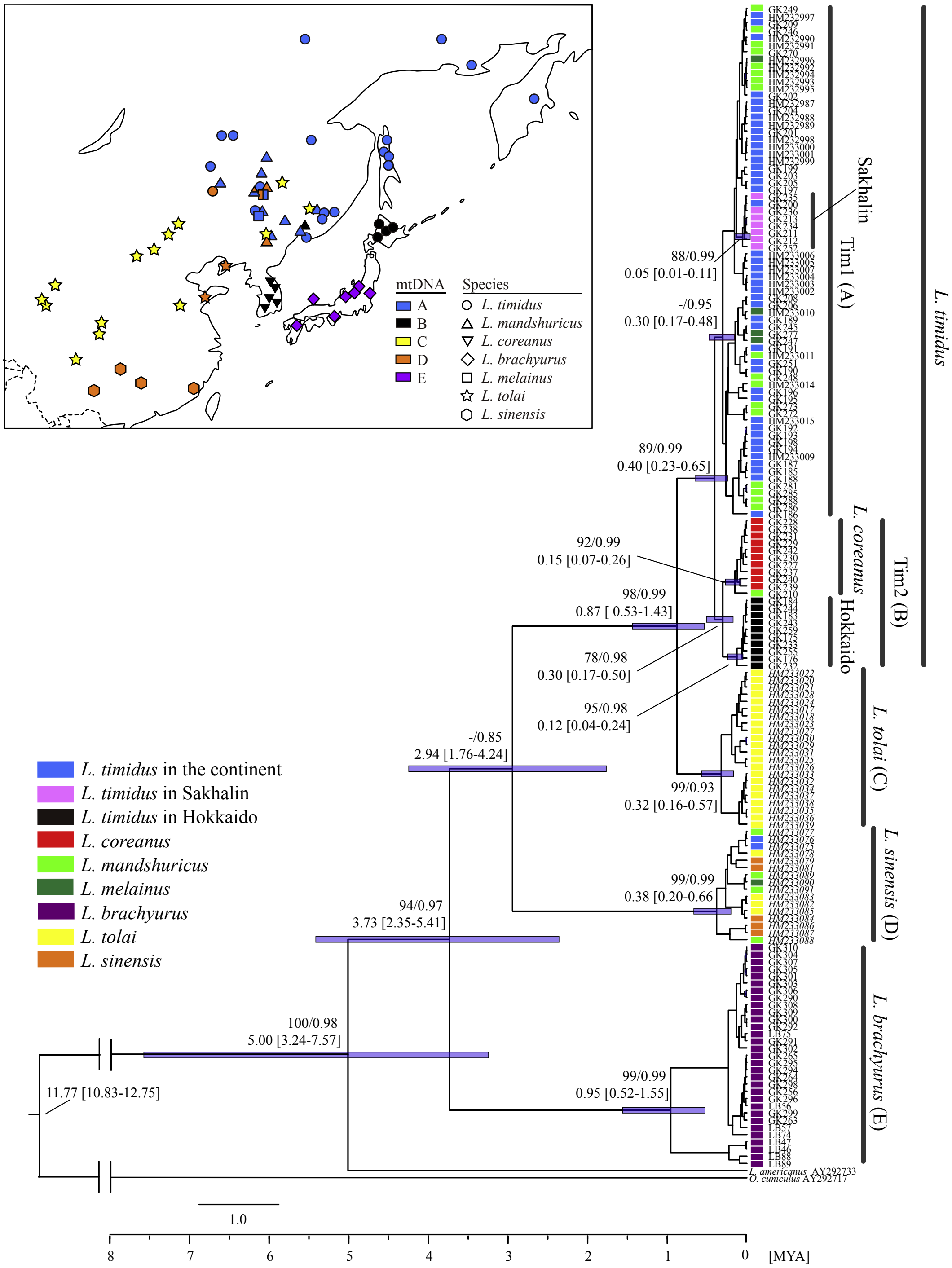

Fig. 4. Bayesian Inference (BI) tree constructed using *Cytb* (1,140 bp) sequences of seven East Asian hares and two outgroups, *Lepus americanus* and *Oryctolagus cuniculus*. Numbers along branches are posterior probabilities in the BI analysis (left), and bootstrap proportions in the tree using the maximum likelihood method (right) for the major nodes. Estimated divergence times (means) are also shown for the major nodes, with their 95% confidence intervals noted in parentheses. The map at the top left shows the geographic distribution of mitochondrial lineages found in East Asian hares. Sampling points for each species were colored according to the five mtDNA clades (A–E) in the phylogenetic tree. Clades C and D were of *L. tolai* and *L. sinensis*, respectively, based on Liu et al. (2011b), but clade C may represent an introgressed lineage from *L. timidus* to *L. tolai/L. sinensis* (D), or clade D may be the original lineage of the *L. mandshuricus* complex that introgressed to *L. tolai/L. sinensis* (C) and *L. timidus* (A).

of the *L. mandshuricus* complex as well as temperate species in Europe, while the Tim2 lineage (related to *L. timidus* in Hokkaido) has been introgressed into southern populations of the *L. mandshuricus* complex (Fig. 4 and Supplementary Fig. 2). In addition, a few haplotypes of nDNA loci were shared among the continental species (*L. timidus*, *L. mandshuricus*, and *L.tolai*), possibly indicating low levels of introgression at these nDNA loci (Fig. 2B and Supplementary Fig. 1).

In northeastern China, the occurrence of mitochondrial introgressions from *L. sinensis* to *L. timidus* and *L. tolai*, as well as *L. mandshuricus*, has also been suggested (Liu et al., 2011b), although the donor species (*L. sinensis*) is currently only found in the southeastern part of China. We note that *L. sinensis* is phylogenetically problematic with regard to *L. tolai,* as they are indistinctive in the *Mgf* network (Fig. 2B) despite clear separation of their mtDNA clades (clades C and D in Fig. 4). Two hypotheses could be offered to explain this complicated pattern of phylogenetic relationships among *L. sinensis*, *L. tolai*, and other species. One hypothesis is that clade D in the mtDNA phylogenetic tree (Fig. 4) comprised *L. tolai/L. sinensis*, and clade C represents an ancient introgressed lineage from *L. timidus* to *L. tolai* (around 0.87 MYA based on the estimated divergence time between clade C and the *L. timidus* clade). Another possibility is that clade D was the original mitochondrial lineage of the *L. mandshuricus* complex, which has subsequently been introgressed into *L. tolai/L. sinensis* and *L. timidus*. Due to the lack of nDNA data of hares in China, more rigorous analyses using multi-locus or genome-wide datasets must be carried out to clarify the cause of haplotype sharing among *L. sinensis*, *L. tolai*, and other species. Nevertheless, these results indicate that the continental side of the circum-Japan Sea region is one of the main regions of reticulate evolution, involving two lineages of *L. timidus* (Tim1 and Tim2), two populations of the *L. mandshuricus* complex (*L. mandshuricus* and *L. coreanus*), *L. tolai*, and *L. sinensis*. Because the circum-Japan Sea region is currently a boundary between the cold and warm temperate climatic zones (Beck et al., 2005; Garcia et al., 2014), demographic events associated with climatic changes during glacial-interglacial periods may have promoted the reticulate evolution of *Lepus* species, as reported in European species (Acevedo et al., 2015; Lado et al., 2018).

4.3. Process of massive mtDNA introgression from L. timidus

Based on genome-wide investigations, past massive mitochondrial introgression from the arctic/boreal species *L. timidus* to *L. granatensis*, a temperate species in the Iberian Peninsula, has been well studied. These studies have suggested that northward expansion of *L. granatensis* into the distribution range of *L. timidus* after the last glacial period induced frequent hybridization between males of the invading species (*L. granatensis*) and females of the resident species (*L. timidus*) at the invasive front (Seixas et al., 2018). In this scenario, signatures of introgression on the nuclear genome of the invader species would have been diluted by repeated backcross within their own populations, while the frequency of introgressed mtDNA received from the resident species might increase as populations at the invasive front grew, assuming that the population size and migration rate from the southern source populations were restricted (Melo-Ferreira et al., 2014b; Marques et al., 2017; Seixas et al., 2018). A similar pattern was observed along ongoing invasive fronts where *L. europaeus* is replacing *L. timidus* in northern Europe (Levänen et al., 2018a, b). Further study is required using genome-wide analysis and denser sampling to test this “invasive front” scenario for massive mitochondrial introgression from *L. timidus* into the *L. mandshuricus* complex, assuming the former as the resident donor of mtDNA, and the latter as the invasive recipient during a post-glacial period. Nonetheless, the present case of the *L. mandshuricus* complex provided novel insights, especially into the southern expansion of *L. timidus* during glacial periods, as a step preceding post-glacial distribution shifts and introgressive hybridization between these species.

. The majority of northeastern Eurasia was not covered by glacial ice (Grosswald 1980, 1998; Svendsen et al., 2004), and land bridges appeared from the continent to Hokkaido via Sakhalin during most glacial periods. *Lepus timidus* of the Tim2 lineage likely expanded onto Hokkaido from the continent during a glacial period around 0.15–0.30 MYA, based on the tMRCA of the Hokkaido clade and its divergence time from sister lineages (Fig. 4). On the other hand, *L. timidus* of the Tim1 lineage subsequently expanded to Sakhalin Island around 0.05 MYA, likely during the last glacial period (Fig. 4). Southward expansion of *L. timidus* may have occurred at least twice in the continent during the last glacial period and earlier one, synchronously with the migration waves to the islands of the Tim2 and Tim1 lineages. In this case, southern edge populations of *L. timidus*, established during each glacial period, would have suffered from post-glacial northward invasions by the *L. mandshuricus* complex, inducing introgressive hybridization.

The northern range of the *L. mandshuricus* complex currently overlaps with the distribution range of *L. timidus*. In addition, multiple mitochondrial haplotypes of Tim1 are shared between these species, and possible signatures of nDNA introgression with *L. timidus* were found only in the northern population of the *L. mandshuricus* complex. These patterns suggest that massive introgression of the Tim1 lineage occurred recently, likely since the last glacial period, or is still ongoing. On the other hand, the time of introgression of the Tim2 lineage into the *L. mandshuricus* complex would be older than that of the Tim1 lineage, at a maximum of 0.30 MYA, according to the divergence time between the Tim2 lineages of *L. timidus* in Hokkaido and *L. coreanus* (Fig. 4). These assumptions are consistent with the scenario of two temporally distinct expansion waves of *L. timidus* during glacial periods and the subsequent northward withdrawals of the species contributing to recurrent introgressions to the *L. mandshuricus* complex. Considering regional cooling in east Siberia due to Eurasian ice sheet extension covered a broader area during the penultimate glacial maximum (≈0.14 MYA) than during the last glacial maximum 18,000 years ago (Colleoni et al., 2009), the range of *L. timidus* of the Tim2 lineage could have expanded farther into southern areas, such as the Korean Peninsula, prior to the expansion of the Tim1 lineage.

In the case of *L. granatensis*, several mitochondrial sublineages of Tim1, introgressed from *L. timidus*, showed a geographic structure within the distribution range of *L. granatensis*, which is likely the trace of a preexisting population structure of the donor species during the last glacial period (Melo-Ferreira et al., 2011). However, our results suggested that the current geographic structure of the introgressed mitochondrial lineages (the Tim2 and Tim1) within the range of the *L. mandshuricus* complex was caused by the recurrent expansion waves of *L. timidus* since the late Middle Pleistocene (Fig. 4).

4.4. Long independent histories of insular hares

The extent of introgression among and between islands and continents is one of the most important factors shaping the genetic diversity and speciation processes of insular organisms (Warren et al., 2015; Patiño et al., 2017). In contrast to the frequent introgression observed on the continental side of the circum-Japan Sea region, no evidence was found for introgression to *L. brachyurus* in the Japanese Archipelago at any locus. The divergence time between *L. brachyurus* and the continental hares (*L. timidus* and the *L. mandshuricus* complex) was 1.88 MYA based on nDNA sequences (Fig. 3), roughly corresponding to the main emergence of the Korea (Tsushima) Strait separating the continent from the Japanese Archipelago (Kitamura and Kimoto, 2006; Osozawa et al., 2012). Although land bridges are thought to have formed several times from the late Early Pleistocene to the Middle Pleistocene (Ohshima, 1990; Osozawa et al., 2012), interspecies gene flows across the strait have likely been prevented after their speciation. In addition, the Tsugaru Strait between Hokkaido Island and the southern islands of Japan is the current boundary between *L. timidus* and *L. brachyurus*, and is regarded as an important biogeographic boundary (Blakiston’s line) dividing multiple species pairs of continental boreal species and Japanese endemic species (Dobson, 1994; Kawamura, 2007; Millien-Parra and Jaeger, 1999). While phylogenetic studies have suggested that some boreal terrestrial mammals, including *L. timidus*, have colonized Hokkaido via a northern route during the Middle Pleistocene or earlier, few species could have extended farther south, crossing the Tsugaru Strait (reviewed in Sato et al., 2016). There is no fossil evidence that *L. timidus* crossed the strait, and *L. brachyurus* was distributed immediately to the south of the strait during the Late Pleistocene (Sawaura et al., 2018). Our results indicated that the Tsugaru and Korea (Tsushima) Straits acted as strong barriers preventing genetic interaction between *L. brachyurus* and continental hare species.

The oldest divergence time and tMRCA were calculated for *L. brachyurus* from East Asian hares using mitochondrial phylogenetic analysis (Fig. 4), which suggested ancient colonization and a long independent demographic history in the Japanese Archipelago, as described previously (Nunome et al., 2010, 2014; Wu et al., 2005; Yamada et al., 2002). Three Japanese islands (Honshu, Shikoku, and Kyushu) isolated by the Tsugaru and Korea (Tsushima) Straits are home to more than 30 non-volant terrestrial mammalian species, more than two thirds of which, including *L. brachyurus*, are endemic to Japan (Ohdachi et al., 2015). In addition, *L. brachyurus* and other Japanese mammals exhibit apparent genetic population structures (Kirihara et al., 2013; Nagata et al., 1999; Nunome et al., 2010, 2014; Suzuki et al., 2004; Tomozawa and Suzuki, 2008; Tsuchiya et al., 2000) that suggest existence of multiple refugia within the main islands of Japan (Honshu, Shikoku, and Kyushu) concurrently with climate changes since the Pleistocene. The long independent history of *L. brachyurus* may have promoted its adaptation to the local insular environment, which may have allowed this insular species to become competitively dominant over later migrants from the continent, as hypothesized for other endemic Japanese species (Sato et al., 2016), which may have contributed to the maintenance of this lineage without introgression. Owing to the contrasting evolutionary trends with regard to inter-species gene flows, further genome-wide investigations comparing the continental and insular hares in East Asia would provide deeper insights into impacts of introgression on the local adaptation and speciation processes.

5. Conclusions

Our multilocus approach revealed that massive mitochondrial introgression from *L. timidus* to the *L. mandshuricus* complex (*L. mandshuricus* and *L. coreanus*) has occurred in continental East Asia. Although mitochondrial introgression in European temperate species was contributed by the *L. timidus* Tim1 lineage, possibly related to the last glacial period, introgression in the *L. mandshuricus* complex is likely related to the two expansion events of *L. timidus* (via the Tim1 and Tim2 lineages) that have occurred since the Middle Pleistocene. On the other hand, *L. brachyurus* in the Japanese Archipelago has experienced a long independent history, likely beginning before the Middle Pleistocene, without involvement in the reticulate evolution of continental species. These results suggest that glacial-interglacial climate changes and the geographic structure of the circum-Japan Sea region greatly impacted the contrasting evolutionary histories of hares between the continent and islands.

7. Declarations of interest

None.

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Declarations of interest

none

Appendix A. Supplementary material

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