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Reticulate evolution: frequent introgressive hybridization among chinese hares (genus *Lepus*) revealed by analyses of multiple mitochondrial and nuclear DNA loci

Jiang Liu¹, Li Yu^{1*}, Michael L Arnold³, Chun-Hua Wu^{2,5}, Shi-Fang Wu², Xin Lu⁴ and Ya-Ping Zhang^{1,2*}

Abstract

Background: Interspecific hybridization may lead to the introgression of genes and genomes across species barriers and contribute to a reticulate evolutionary pattern and thus taxonomic uncertainties. Since several previous studies have demonstrated that introgressive hybridization has occurred among some species within *Lepus*, therefore it is possible that introgressive hybridization events also occur among Chinese *Lepus* species and contribute to the current taxonomic confusion.

Results: Data from four mtDNA genes, from 116 individuals, and one nuclear gene, from 119 individuals, provides the first evidence of frequent introgression events via historical and recent interspecific hybridizations among six Chinese *Lepus* species. Remarkably, the mtDNA of *L. mandshuricus* was completely replaced by mtDNA from *L. timidus* and *L. sinensis*. Analysis of the nuclear DNA sequence revealed a high proportion of heterozygous genotypes containing alleles from two divergent clades and that several haplotypes were shared among species, suggesting repeated and recent introgression. Furthermore, results from the present analyses suggest that Chinese hares belong to eight species.

Conclusion: This study provides a framework for understanding the patterns of speciation and the taxonomy of this clade. The existence of morphological intermediates and atypical mitochondrial gene genealogies resulting from frequent hybridization events likely contribute to the current taxonomic confusion of Chinese hares. The present study also demonstrated that nuclear gene sequence could offer a powerful complementary data set with mtDNA in tracing a complete evolutionary history of recently diverged species.

Background

The genus *Lepus* (the jackrabbit and hares; order Lagomorpha) is widespread in Africa, Europe, Asia, and North and Central America. The number of *Lepus* species currently recognized ranges from 24 to 32 [1-4]. In China, hares (*Lepus*) are distributed from the Qinghai-Tibetan Plateau to near sea level, and from the mainland to the island of Hainan. To date, the evolutionary history of Chinese hares (Lagomorpha: *Lepus*) is poorly defined, mainly due to uncertain taxonomic classification [2,3,5-7].

Many taxonomic schemes based on morphology have been proposed for Chinese hares and classifying them into various numbers of species. The earliest studies divided extant Chinese hares into seven species: *L. capensis* (Cape hare), *L. sinensis* (Chinese hare), *L. oiostolus* (Woolly hare), *L. mandshuricus* (Manchurian hare), *L. timidus* (Mountain hare), *L. yarkandensis* (Yarkand hare) and *L. hainanus* (Hainan hare) [8-12]. Subsequent analyses, incorporating a large number of morphometric features (e.g., body measurements and skull characters) conducted by Luo [13], assigned Chinese hares into nine species by adding 2 species: *L. melainus* (Manchurian black hare) and *L. comus* (Yunnan hare). Several recent classifications, however, have reignited the debate. Hoffmann & Smith [3] divided *L. capensis* into two species: *L. tibetanus* and *L. tolai*, and

* Correspondence: yuli1220@yahoo.com.cn; zhangyp@mail.kiz.ac.cn

¹Laboratory for Conservation and Utilization of Bio-resource & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, 650091, PR, China

Full list of author information is available at the end of the article

placed *L. melainus* into *L. mandshuricus*. Pan *et al.* [7] also did not support the specific status of *L. melainus*, and assigned *L. tibetanus* within *L. tolai* (corresponding to *L. capensis*). Wu *et al.* [14], based on four mitochondrial genes, also suggested that *L. mandshuricus* and *L. melainus* should be a single species, but rejected the specific status of *L. capensis*. Liu *et al.* [15], using skull characters and nuclear gene sequences, supported the synonymy of *L. melainus* and *L. mandshuricus*. Hence, taxonomic classifications within Chinese hares remain uncertain and necessitate further analyses that include additional taxa and discrete sequence data.

Hybridization among wild mammalian species may lead to the introgression of genes and genomes across the species barrier yielding a reticulate evolutionary pattern (evolution characterized by genetic exchange among different evolutionary lineages) among these species and thus taxonomic uncertainties. As reviewed by Nosil [16], speciation with gene flow could be common; however, the impact of hybridization on the speciation process in animals remains poorly defined. Studies have revealed that introgressive hybridization often leads to the complete replacement of mtDNA on a regional scale [17-22] or even throughout a species' distribution [23]. Such replacements appear to be more common than previously appreciated and can affect the origin and adaptation of organisms [24].

If hybridization occurs between recently diverged species, then morphological intermediates between these species may be produced thus contributing to the taxonomic confusion. Such a phenomenon seems to occur within the genus *Lepus*. Several previous studies have demonstrated that introgressive hybridization has occurred among some species within *Lepus*. Thulin *et al.* [25] and Thulin & Tegelström [26,27] demonstrated the unidirectional introgression of *L. timidus* mtDNA into *L. europaeus* that was introduced into Sweden during the 19th century. Morphological intermediates between the two species, believed to be hybrids, have also been observed since the introduction of *L. europaeus* [28]. In addition, hybrids between *L. timidus* females and *L. europaeus* males are easily acquired in captivity [29] and the F₁ hybrids show intermediate characters [30]. Furthermore, Alves *et al.* [31] and Melo-Ferreira *et al.* [17,32] described an ancient mtDNA introgression of *L. timidus*, which is now extinct from Iberia, into *L. granatensis* and *L. europaeus* in the Iberian Peninsula. A reverse direction of mtDNA introgression from *L. europaeus* into *L. timidus* was also reported in the Alps by Zachos *et al.* [33] and in Russia by Thulin *et al.* [34]. Near gene sequence-ization and evolution.

In view of these findings, it is possible that introgressive hybridization events may also occur between Chinese *Lepus* species, most of which overlap geographically with other species of this genus [3,7,13]. If introgression

among *Lepus* species does indeed occur it may contribute to the current taxonomic confusion of Chinese hares [21,35].

The transfer of genes and genomes across the species barrier can provide novel genetic material for natural selection to work upon and, thus, may change the evolutionary direction of one or both of the intermixing taxa [36-46]. Previous studies, however, have revealed that there was a lack of nuclear DNA introgression in hares from Iberia and Sweden. Alves [47] used 14 autosomal protein loci to assay the genetic variability in *L. granatensis* populations from Iberia and detected no significant differentiation between the populations with introgressed *L. timidus* mtDNA and non-introgressed populations. Similar results were also obtained from a microsatellite analysis [48,49]. Based on SNP analyses of 10 autosomal, two X-linked and one Y-linked loci, Melo-Ferreira *et al.* [50] suggested that autosomal introgression appeared to be mostly sporadic or undetectable and that sex-chromosome introgression was completely absent in hare species of Iberia. Alves *et al.* [51] and Roca *et al.* [52] attributed the lack of nuclear introgression to recurrent crossing of hybrid females with males of the invading species, coupled with male hybrid sterility or inviability (Haldane's rule). This pattern of crossing could eventually lead to the replacement of one nuclear genome within a few generations. This inference was also supported by the results of Thulin *et al.* [53]. By analyzing microsatellites, Thulin *et al.* [53] detected a small number of specimens with genotypes consistent with those of F₁ hybrids in Sweden. From this observation it was hypothesized that the transferred nuclear genes from *L. timidus* had disappeared from the *L. europaeus* populations during the 50 generations since the introduction and initial hybridization. In view of these reports, if introgression among Chinese *Lepus* species does indeed occur, what then is the genomic legacy of the introgressed nuclear genes and genomes? At one extreme, they may disappear over subsequent generations. At the other extreme, some hybrid genotypes in the Chinese populations may actually possess elevated fitnesses thus leading to continued introgression and the possibility of the fixation of introgressed nuclear DNA, and eventually to the formation of novel hybrid lineages (i.e. hybrid species).

In the present study, four mtDNA genes and one nuclear DNA locus from 124 Chinese hare individuals collected at 54 localities in China were analyzed in order to test for the possibility of introgressive hybridization and to help clarify the taxonomy of Chinese hares.

Results

MtDNA and Nuclear sequence characterizations

Sequences for four mtDNA genes, cytochrome b (Cyt b; 1140 bp), cytochrome c oxidase subunit I (COX I; 1450 bp), NADH dehydrogenase subunit 4 (ND4; 1378 bp)

and control region (D-loop; 589 bp), were obtained successfully from 116 Chinese hare individuals, with eight museum skin samples failing to yield full-length sequences due to the poor quality of the isolated DNA. The mtDNA genes appear to be of mitochondrial origin, rather than nuclear copies of mtDNA-like pseudogenes, as indicated by the fact that the amino acid sequences of the Cyt b, COX I and ND4 genes did not possess premature stop codons or frameshifting insertions/deletions. In addition, the phylogenetic analyses based on separate mtDNA fragments were generally concordant (data not shown).

The sequence characteristics of the four mtDNA fragments and one nuclear gene, Stem cell factor (MGF), are summarized in Table 1. As seen from Table 1,

a total of 201 sequences from 119 samples with a length of 593 base pairs were aligned at the MGF locus. Full-length sequences could not be obtained from five museum skin samples. A total of 72 haplotypes were defined from the 58 polymorphic sites, 44 of which were potentially phylogenetically informative.

Phylogenetic inference from MtDNA

The best fitting model for the Bayesian analysis using the combined mtDNA data was “GTR+I+G” with the following parameter settings: Base = (0.2968, 0.2771, 0.1192, 0.3069), Nst = 6, Rmat = (3.2812, 75.0100, 6.1253, 2.5585, 57.7249, 1.0000), Rates = gamma Shape = 1.0115, and Pinvar = 0.5638. Neighbour-joining (NJ) and Bayesian analyses of the combined mtDNA dataset yielded identical tree topologies (Figure. 1). The Chinese *Lepus* species included eight lineages and were grouped into five major clades. Sequence divergence (uncorrected pairwise distance; p-distance) among the eight mtDNA lineages based on the separate mtDNA fragments are presented in Additional file 1 (2.2-8.5% in Cyt b, 2.6-8.6% in COX I, 3.2-11.1% in ND4 and 5.9-15.3% in the control region). As seen from Figure 1, *L. hainanus* branched first, followed by the sister-grouping of *L. comus* and *L. oiostolus*. In another portion of the phylogeny, *L. sinensis* was most basal, *L. timidus* and *L. capensis* are closely related, and

L. yarkandensis and a portion of the *L. capensis* individuals, which are defined here as *L. capensis-2*, grouped together. The support for the monophyly of these eight lineages was 1.00 for Bayesian posterior probability (PP) and 93%-100% for NJ bootstrap sampling (BS). Support for relationships among the five clades was 0.98-1.00 for PP and 59%-93% for BS.

As illustrated in Figure 1, sequences from *L. hainanus*, *L. comus*, *L. oiostolus*, *L. sinensis* and *L. yarkandensis* showed species-specific monophyly; however, this was not the case for *L. capensis*, *L. timidus* and *L. mandshuricus*. The sequences of *L. capensis* were mainly divided into two lineages. Besides the lineage that included the majority of *L. capensis* sequences, five sequences of *L. capensis* from Xinjiang Province of China formed a separate mtDNA clade (*L. capensis-2*) (1.00 for PP, 100% for BS), which was a sister clade to *L. yarkandensis*. In addition, another eight sequences of *L. capensis* were placed within two other lineages: three (CA14, CA15 and CA35) in a *L. timidus* lineage (1.00 for PP, 93% for BS), and five (CA9, CA11-CA13 and CA36) in a *L. sinensis* lineage (1.00 for PP, 100% for BS). Similarly, the sequences of *L. timidus* were also divided into two lineages: one included the majority of the *L. timidus* sequences, and the other included two sequences of *L. timidus* (T10 and T11) from Inner Mongolia Province of China that grouped within the *L. sinensis* lineage. Remarkably, we found that among the fourteen *L. mandshuricus* sequences, nine were placed within the *L. timidus* lineage and the remaining five grouped within the *L. sinensis* lineage.

Phylogenetic inference from nuclear gene

The recombination test with the nuclear gene MGF gene data failed to detect any signal for recombination. The NJ tree for the nuclear gene MGF, based on the analyses of 72 haplotypes from the 119 individuals, is presented in Figure 2. The Bayesian inference (BI) analysis yielded a result similar to that of the NJ analysis.

The MGF gene tree in Figure 2 divided the Chinese *Lepus* species into six lineages (*L. hainanus*, *L. comus*,

Table 1 Characteristics of the gene segments used in this study

Gene	Total number of samples	Total number of sequences	Sequence length	Informative sites	Variable sites	Nucleotide Composition				Ti/Tv	Heterozygotes (%)
						A	T	G	C		
Cyt b	116	116	1140	262	297	28.2	30.3	12.7	28.8	6.6	
COI	116	116	1450	283	304	26.4	32.3	17.4	23.8	6.1	
ND4	116	116	1378	346	385	30.8	31.4	10.5	27.3	6.6	
D-loop	116	116	590	204	220	28.7	28.7	11.7	30.9	2.9	
Total mtDNA	116	116	4558	1095	1206	28.5	31.1	13.4	27.0	5.4	
MGF	119	201	593	44	58	29.7	32.9	17.2	20.1	2.5	70.59

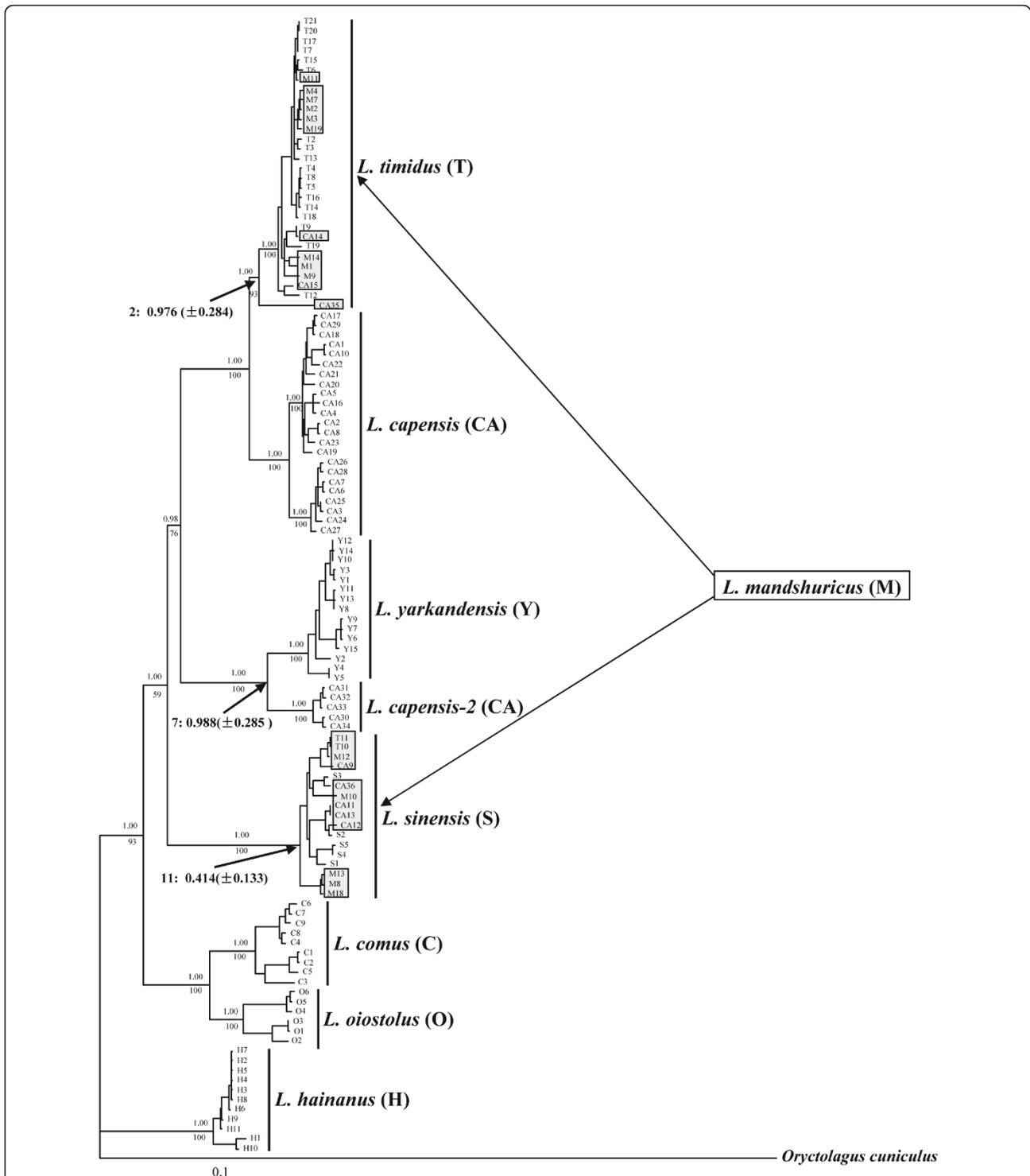
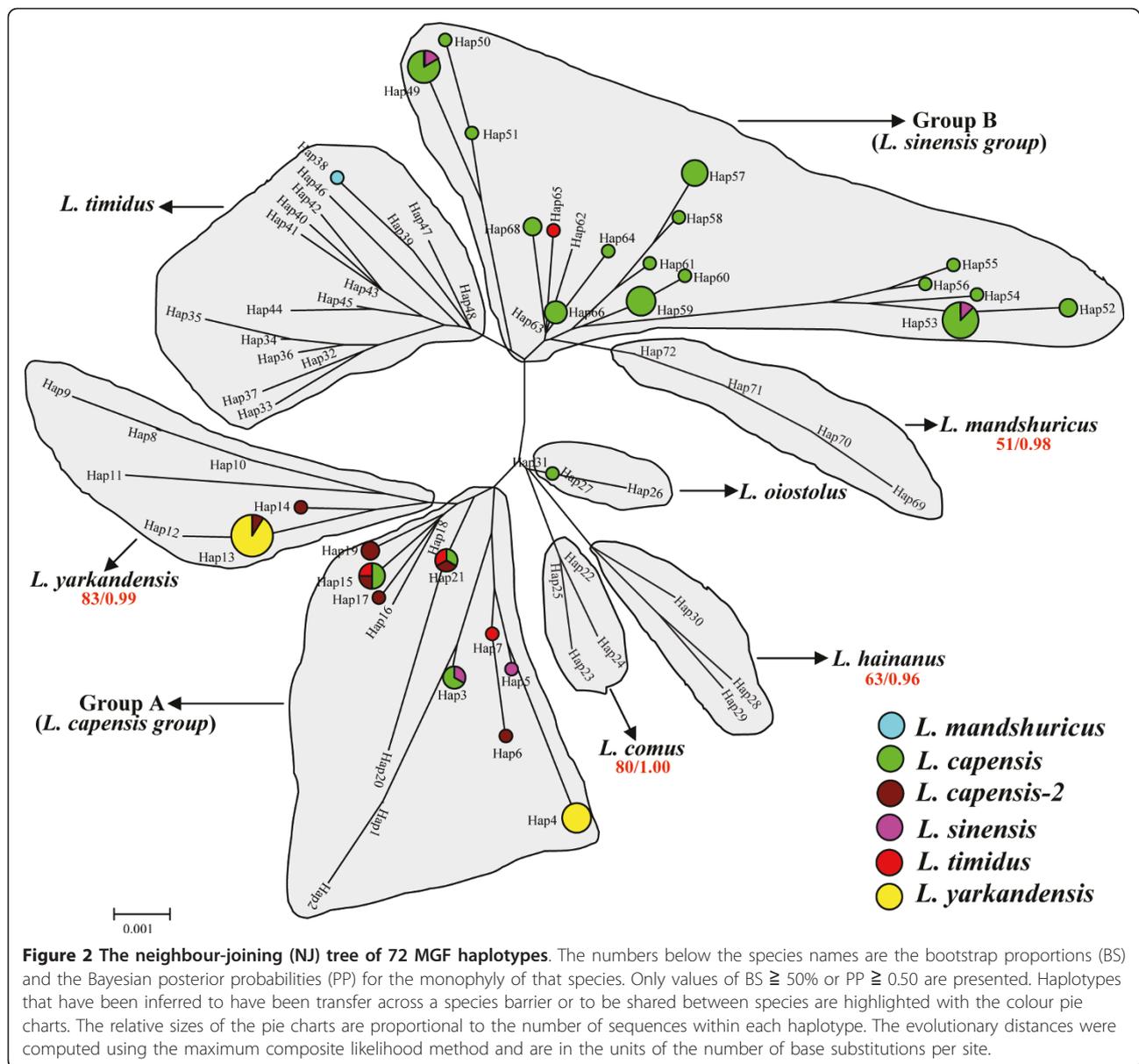


Figure 1 The neighbour-joining (NJ) tree derived from the combined data of four mtDNA fragments. *Oryctolagus cuniculus* was used as the outgroup. The numbers above the branches are the Bayesian posterior probabilities (PP) and the numbers below the branches are the bootstrap proportions (BS). Abbreviations of each species name are presented in the parentheses following the species name, and this abbreviation was used in the sequence names. Sequence names correspond to the sample codes listed in Additional file 4 and Additional file 2. The boxed and highlighted sequences indicated the samples with an introgressed mitochondrial genome. MtDNA of *L. mandshuricus* are completely replaced by mtDNA from *L. timidus* or *L. sinensis*, suggesting the absence of a “true” *L. mandshuricus* mtDNA lineage.



L. oiostolus, *L. yarkandensis*, *L. timidus* and *L. mandshuricus*) and two complexes (Group A and Group B). The haplotypes from *L. hainanus*, *L. comus*, *L. oiostolus*, *L. yarkandensis*, *L. timidus* and *L. mandshuricus* formed their own species-specific clades. Exceptions to this phylogenetic signal were one *L. capensis* haplotype (Hap31) that was included within the *L. oiostolus* clade, one *L. mandshuricus* haplotype (Hap38) that was included within the *L. timidus* clade, one *L. capensis-2* haplotype (Hap14) that fell within the *L. yarkandensis* clade and one *L. yarkandensis* haplotype (Hap13) that was shared with *L. capensis-2*.

The majority of the *L. sinensis* and *L. capensis* haplotypes were admixed and fell within either Group A or

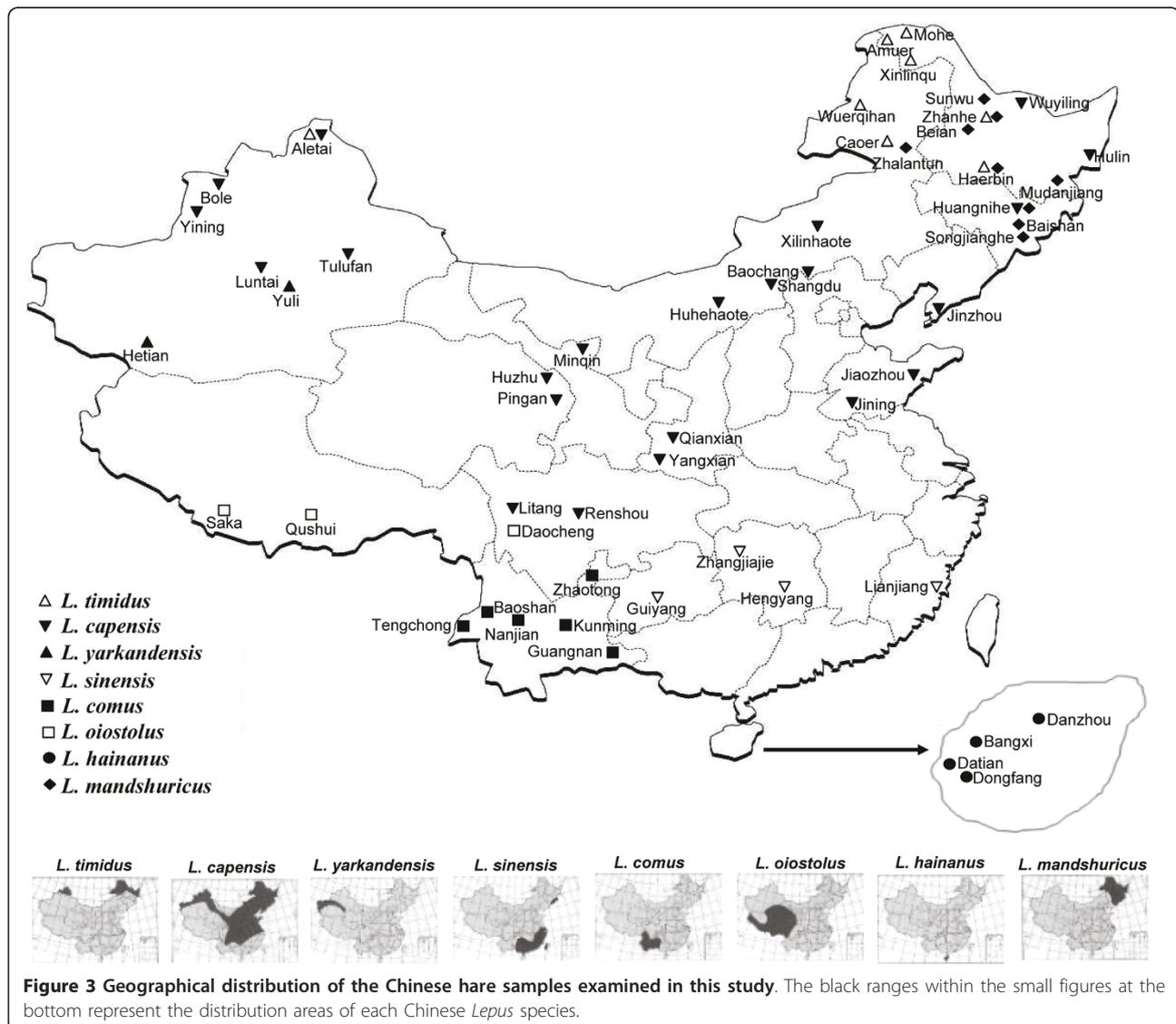
Group B (Figure 2). Group A includes 14 haplotypes, of which five were from *L. capensis*, three (Hap6, 17, 19) were from *L. capensis-2*, and Hap 4, 5, 7 were from *L. yarkandensis*, *L. sinensis* and *L. timidus*, respectively. For the three remaining haplotypes, Hap15 and Hap21 were shared by *L. capensis*, *L. capensis-2* and *L. timidus*, and Hap3 was shared by *L. capensis* and *L. sinensis*. Fourteen of the 19 haplotypes in Group B were from *L. capensis*. In addition, one *L. timidus* haplotype (Hap65), two *L. sinensis* haplotypes (Hap62, 63) and two haplotypes (Hap49, 53) shared between *L. sinensis* and *L. capensis* were also grouped into Group B. One *L. yarkandensis* haplotype (Hap 4) was grouped into Group A and Hap13 was shared between *L. capensis-2* and *L. yarkandensis*,

suggesting a recent hybridization event between *L. yarkandensis* and a lineage in Group A. The disjunct geographical distributions of *L. yarkandensis* and *L. sinensis* (Figure 3) led to the definition of Group A as the “*L. capensis* group” and Group B as the “*L. sinensis* group” (Figure 2).

Conflicts between the mitochondrial and nuclear gene trees

Three major differences were demonstrated between the mtDNA and nuclear gene trees: (i) no species-specific mtDNA lineage was identified for *L. mandshuricus*. All the mtDNA sequences of *L. mandshuricus* were identified as belonging to either *L. timidus* or *L. sinensis*; however, almost all of the MGF sequences from *L. mandshuricus* clustered together within a single clade; (ii) a new monophyletic lineage, *L. capensis-2*, was identified in the

mtDNA analysis, but the MGF haplotypes of *L. capensis-2* were divided into two portions (the *L. yarkandensis* clade and the *L. capensis* group); and (iii) the mtDNA analysis suggested that the introgression among Chinese *Lepus* species was likely unidirectional, while, in contrast, the MGF data indicated the occurrence of bidirectional introgression among these species. For example, from the mtDNA analysis, four *L. capensis* sequences were grouped with *L. sinensis*, suggesting the unidirectional introgression of mtDNA from *L. sinensis* into *L. capensis*; however, the MGF tree identified one haplotype (Hap3) which was nested in the *L. capensis* group and two haplotypes (Hap49, 53) which were nested in the *L. sinensis* group as sharing haplotypes between *L. capensis* and *L. sinensis*, consistent with the predicted pattern for bidirectional introgression. In addition, the fact that nine *L. capensis* and two *L. sinensis* individuals were identified as



heterozygotes at the MGF locus, with alleles belonging to the *L. capensis* and *L. sinensis* groups, further supports the hypothesis of bidirectional introgression (Additional file 2). Details about the discrepancy between the mitochondrial and nuclear genes are summarized in Additional file 2.

Dating the Divergence Times

The Bayesian tree used for divergence time estimation is presented in Additional file 3. In contrast to the mtDNA tree shown in Figure 1, the mtDNA tree in Additional file 3 included only representative sequences from each major lineage, and excluded sequences that demonstrated only a few base pair differences. The speciation time of Chinese hares was estimated at 3.116 MYA (± 0.731 MYA, Additional file 3). The estimated times of introgressive hybridization events are presented in Figure 1.

Discussion

Introgressive hybridization among Chinese *Lepus* species

As seen from the analyses of mtDNA and nuclear DNA sequences of Chinese hares, most of the phylogenetic conflicts between the mtDNA and nuclear DNA gene trees suggest multiple episodes of introgression. Alternatively, incomplete lineage sorting of the nuclear gene may also result in the observed “mixing” of the nuclear gene haplotypes; however, most of the samples with the discrepancy between the mitochondrial and nuclear genotypes have congruence between their morphology and nuclear genotype except for some *L. capensis* and *L. sinensis* individuals (Additional file 2). Although some of the *L. capensis* and *L. sinensis* individuals have incongruence between their morphology and nuclear genotype, the “*L. capensis* group” and the “*L. sinensis* group” is divergent enough to distinguish each other (Figure 2), therefore, the nuclear MGF gene is informative enough to distinguish Chinese hare species and the observed “mixing” of nuclear gene haplotypes likely is due to introgressive hybridization rather than incomplete lineage sorting. In addition, the observation that a large number of the heterozygous genotypes have alleles from divergent clades (Additional file 2) is also inconsistent with the alternative hypothesis of incomplete lineage sorting. The evidence suggests that introgression has occurred between seven pairs of the hare species examined here.

L. sinensis \times *L. capensis*, *L. sinensis* (♀) \times *L. mandshuricus* (♂), *L. sinensis* \times *L. timidus*

Five *L. capensis* individuals (CA9, CA11- CA13 and CA36) were placed within the *L. sinensis* lineage on the mtDNA tree (Figure 1). These results likely reflect recent introgression of *L. sinensis* mtDNA into *L. capensis*; however, results from the nuclear gene analysis suggested bidirectional and recent introgression between

these species, as evidenced by the observation that the MGF haplotypes from *L. capensis* and *L. sinensis* were admixed and fell within two distantly related groups. Furthermore, one haplotype (Hap3) that nested in the *L. capensis* group and two haplotypes (Hap49, 53) that nested in the *L. sinensis* group were shared between *L. capensis* and *L. sinensis* (Figure 2). In addition, ten *L. capensis* individuals (CA1, CA2, CA10, CA17-CA19, CA21, CA22, CA29 and CA31) and two *L. sinensis* individuals (S2 and S3) were identified as heterozygotes at the MGF locus for two alleles from *L. capensis* and *L. sinensis* (Additional file 2). The available data, however, cannot eliminate the possibility that some of the heterozygotes were produced by ancient hybridization events.

Five individuals (M8, M10, M12, M13 and M18) of *L. mandshuricus* were grouped into the *L. sinensis* lineage on the mtDNA tree (Figure 1), while their MGF haplotypes fell within the *L. mandshuricus* clade (Figure 2). This can be explained by a unidirectional mtDNA introgression from *L. sinensis* into *L. mandshuricus*. In addition, two individuals of *L. mandshuricus* (M10 and M12) have low sequence divergence relative to the *L. sinensis* individuals, thus supporting a recent age for the hybridization events. The divergence time estimation indicated that the remaining three *L. mandshuricus* individuals (M8, M13 and MA18) shared a most recent ancestor with the *L. sinensis* samples at ca. 0.414 MYA (± 0.133 MYA) (Figure 1). This would suggest that the mitochondrial genome of *L. sinensis* introgressed into *L. mandshuricus* no later than 0.414 MYA. Our estimate of the time of introgression may be biased somewhat if additional extant lineages were not sampled, or if there have been extinction events. Indeed, such a bias has been inferred in the case of *Lepidiolamprologus* [54]. However, in the present study three ancient hybridization events, involving five species (Figure 1; also see below), were detected. This strongly supports the hypothesis of ancient hybridization and not solely recent introgression events.

Two individuals of *L. timidus*, T10 and T11, nested within the *L. sinensis* clade on the mtDNA tree (Figure 1), while their MGF haplotypes grouped within the *L. timidus* clade (Figure 2), once again suggesting mtDNA introgression (in this case, from *L. sinensis* into *L. timidus*). In addition, the detection of one *L. timidus* haplotype (Hap65) within the *L. sinensis* group on the MGF gene tree (Figure 2) was consistent with the occurrence of nuclear DNA introgression from *L. timidus* into *L. sinensis*. Taken together, the mtDNA and nuclear DNA analyses supported reciprocal introgression between *L. timidus* and *L. sinensis*. An alternative explanation would be that the introgressive hybridization of *L. sinensis* \times *L. capensis*, *L. sinensis* (♀) \times *L. mandshuricus* (♂), and *L. sinensis* (♀) \times *L. timidus* (♂) resulted from an initial hybridization between *L. sinensis*

and *L. capensis*, *L. mandshuricus* or *L. timidus*, followed by a second hybridization among *L. capensis*, *L. mandshuricus* and *L. timidus*. Supporting this latter hypothesis was the inference of hybridization between *L. timidus* and *L. mandshuricus* as well as *L. timidus* and *L. capensis* (see below).

L. timidus (♀) × L. mandshuricus (♂), L. timidus (♀) × L. capensis (♂)

Alves et al. [51] suggested that mtDNA introgression from *L. timidus* might occur in several other regions in addition to Iberia and our results confirm this hypothesis. In the *L. timidus* mtDNA lineage, nine *L. mandshuricus* individuals (M1-M4, M7, M9, M11, M14 and M19) and three *L. capensis* individuals (CA14, CA15 and CA35) were included (Figure 1). The MGF haplotypes of the nine *L. mandshuricus* individuals were grouped with the other *L. mandshuricus* haplotypes except for Hap38, which is nested within the *L. timidus* clade. The nuclear sequences of the three *L. capensis* individuals were placed in either the *L. capensis* group (CA15) or the *L. sinensis* group (CA35) (Additional file 2). In addition, one *L. timidus* haplotype (Hap7) was placed into the *L. capensis* group and two haplotypes (Hap15 and Hap21) were shared with *L. capensis* on the MGF gene tree (Figure 2). Our results thus suggest that the *L. mandshuricus* and *L. capensis* individuals likely reflect the capture of *L. timidus* mitochondrial genomes via several hybridization events between females of *L. timidus* and males of *L. mandshuricus* or *L. capensis*. Furthermore, the nuclear DNA analysis indicated that the hybridization events between *L. timidus* and *L. mandshuricus* or *L. capensis* were recent and a second hybridization event likely occurred between *L. capensis* and *L. sinensis* as well. An additional nine Cyt b sequences of *L. mandshuricus* accessed from Genbank (accession numbers: AY650894-AY650899, AJ279423, DQ793162, DQ793163) were also grouped into the *L. timidus* lineage on our Cyt b tree (data not show), further supporting the inference of mtDNA introgression from *L. timidus* into *L. mandshuricus*. Finally, one *L. timidus* Cyt b sequence (accession number: AY745108) collected from GenBank was grouped within the *L. capensis* lineage in the Cyt b phylogeny (data not show), suggesting the introgression of mtDNA from *L. capensis* into *L. timidus*. The *L. capensis* individual, CA35, shared a most recent common ancestor with *L. timidus* samples at 0.976 MYA (\pm 0.284 MYA) (Figure 1), indicating that the hybridization between *L. timidus* and *L. capensis* took place at about this point in time.

L. yarkandensis (♀) × L. capensis(♂), L. capensis × L. oiostolus

Several observations suggest the role of recent hybridization between *L. capensis* and *L. yarkandensis*. These

observations include: one MGF haplotype (Hap4) of *L. yarkandensis* nested within the *L. capensis* group; one MGF haplotype (Hap13) of *L. yarkandensis* shared with *L. capensis-2* (Figure 2); and two MGF alleles of four *L. yarkandensis* individuals (Y7, Y10, Y14, Y15) were grouped into either the *L. yarkandensis* and *L. capensis* groups (Additional file 2).

The newly identified *L. capensis-2* lineage might have originated through ancient hybridization between female *L. yarkandensis* and male *L. capensis* at ca 0.988 MYA (\pm 0.285 MYA) (Figure 1; also see below). Interestingly, one *L. capensis* haplotype (Hap31) was grouped into the *L. oiostolus* clade (Figure 2). This may reflect a recent hybridization event between *L. capensis* and *L. oiostolus*. In addition, hybridization between *L. oiostolus* and *L. capensis* was also evidenced by the fact that one *L. oiostolus* Cyt b sequence (accession number: AJ279427) from GenBank was nested within the *L. capensis* lineage (data not shown).

The possible forces driving the introgressive hybridization among Chinese Lepus species

Compared with introgression reported previously for other species complexes [18,21-23,54,55], especially within other *Lepus* species [17,25-27,31,32,50], Chinese hares demonstrated a previously unreported level of complexity. First, most of the previous studies within the genus *Lepus* reported only the presence of mtDNA introgression of *L. timidus*, in which unidirectional mtDNA introgression events from *L. timidus* into populations of *L. europaeus* in Sweden [25-27] and into *L. granatensis* and *L. europaeus* in Iberian Peninsula [17,31,32] were inferred, with the identification of nuclear introgression lacking or infrequent [48,50,53]. Similar scenarios have also been posited for several other species clades [18,22,23,54-56]. Second, although contemporaneous introgression has been reported for several non-hare species [21,57,58], most of these cases of introgression involve at the most two or three species. In comparison, the present study demonstrated that introgression among Chinese *Lepus* species has most likely been a continuous and recent process involving multiple waves of hybridization among six species.

Our results also suggest that introgression has occurred in multiple directions, involving both mitochondrial and nuclear DNA. The mtDNA of *L. mandshuricus* was replaced entirely by that of *L. timidus* and *L. sinensis*. Although several previous studies have provided convincing evidence for complete local or even range-wide replacement of mtDNA following hybridization in insects, fishes and reptilia [18,23,55,59], such observations are infrequent for mammals. The hybridization events among Chinese hares are possibly driven by species abundance asymmetries, mating preferences,

range expansion and low divergence between *Lepus* species.

In the studies of Thulin & Tegelström [27], Hubbs [60] and Wirtz [61], they suggested that the females of a rare species (e.g. *L. timidus*) would generally hybridize with the males of a common species (e.g. *L. europaeus*). In the present study, the hybridization between females of *L. timidus* (rare species) and males of *L. capensis* (common species), and between females of *L. timidus* (rare species) and males of *L. mandshuricus* (common species) are congruent with their conclusion. However, the hybridization between females of *L. sinensis* (common species) and males of *L. capensis* (common species) is inconsistent with this hypothesis. In addition, Thulin & Tegelström [27] and Grant & Grant [62] indicated that unidirectional hybridization would usually occur between the females of smaller species (e.g. *L. timidus*) and the males of larger-bodied species (e.g. *L. europaeus*). Among Chinese hares, hybridization between females of *L. sinensis* (smaller species) and males of *L. capensis* (larger species), *L. timidus* (larger species) or *L. mandshuricus* (larger species) support this hypothesis. In contrast, the hybridizations between female *L. timidus* (larger species) and male *L. capensis* (smaller species) or *L. mandshuricus* (smaller species) are inconsistent with this hypothesis. Species abundance and body size, though, might have been different in ancestral populations. The present study indicates that at least some of the hybridization events are recent, therefore, the forces driving the hybridization among Chinese *Lepus* species may include species abundance asymmetries and mating preferences.

The hybridizations among Chinese *Lepus* species might be a consequence of range expansion caused by the Pleistocene glaciations. Melo-Ferreira *et al.* [32] estimated the timing of mtDNA introgression from *L. timidus* into *L. granatensis* in Iberia at 33 000–35 000 years and suggested that it might have been favored by the climatic fluctuation during the Pleistocene glacial phenomena. Our latest study also strongly indicates that one of the Chinese *Lepus* species (*Lepus yarkandensis*) has undergone repeated population reductions and expansions during the Pleistocene glacial and interglacial periods, respectively [63]. These population size fluctuations may have contributed to the extensive gene flow among *Lepus yarkandensis* populations. Similarly, the alternation of glacial and interglacial periods during the Pleistocene likely contributed to the conditions necessary for of the overlapping ranges of different Chinese hare species, resulting in the observed introgressive hybridization. Morgan *et al.* [59] and Melo-Ferreira *et al.* [32] drew similar inferences for both mosquitoes in Southeast Asia and European hares, respectively. Further support for the effect of glaciation comes from the estimation of some of the introgression events

among the Chinese hare lineages as occurring during the Pleistocene (i.e. 0.414–0.988 MYA). Simulation studies have also suggested that range expansions could lead to asymmetric introgression from the resident species toward the invading species [64–66].

In addition to the above factors, the widespread and complex patterns of reticulate evolutionary events among the Chinese *Lepus* lineages may also reflect the generally low levels of interspecific genetic differentiation. Previous studies have suggested that *Lepus* experienced rapid radiation [67,68]. The lack of chromosomal structural changes supports such a model of relatively recent, and rapid, diversification in this clade [69,70]. Low levels of genetic diversification between species could result in relatively fertile F₁ hybrids providing a bridge for further hybridization and thus introgression. Alternatively, low levels of genetic diversification between species may indicate that speciation processes of some Chinese *Lepus* species are not complete and there have been continuous gene flow among them. For example, the admixture of MGF gene between *L. sinensis* and *L. capensis* could be the consequence of an incomplete reproductive isolation between the two species. Further definition of the introgressive system characterizing Chinese *Lepus* species should provide a clearer resolution of the evolutionary factors that have resulted in this reticulate complex.

Taxonomic classification of Chinese hares

Based on the mtDNA and nuclear gene sequence analyses, the present study not only suggests that introgressive hybridization among Chinese *Lepus* species contributed to the current taxonomic confusion, but also provides insights into the previously obscure taxonomic classification of Chinese hares. Our mtDNA and nuclear DNA analyses supported the specific status of eight hare species, including *L. hainanus*, *L. comus*, *L. oiostolus*, *L. yarkandensis*, *L. timidus*, *L. mandshuricus*, *L. capensis* and *L. sinensis*. This finding is consistent with the morphological taxonomy of Pan *et al.* [7]. The division of *L. capensis* into two species (*L. tibetanus* and *L. tolai*), as suggested by Hoffmann & Smith [3], was not favored in the present study.

In the mtDNA analysis of *L. mandshuricus*, nine specimens were found to harbor *L. timidus* mtDNA haplotypes and three specimens contained *L. sinensis* mtDNA haplotypes, suggesting the absence of a diagnostic *L. mandshuricus* mtDNA lineage (Figure 1); however, the nuclear gene (MGF) analysis supported the monophyly of *L. mandshuricus*, which is consistent with its specific status (Figure 2). Furthermore, Liu *et al.* [15], using skull characters and nuclear gene sequences, also supported the specific status of *L. mandshuricus*, therefore, the discrepancy between the mtDNA and nuclear gene trees is most likely due to mtDNA introgression from *L. timidus* and *L. sinensis* into

L. mandshuricus. This finding reflects the importance of including nuclear markers for systematic studies of hares [71-73].

On the mtDNA tree, *L. capensis* and *L. timidus* formed sister taxa and have the lowest Cyt b sequence divergence (2.2%) among the species analyzed in this study (Figure 1, Additional file 1). The Cyt b sequence divergence of 7.6% between *L. capensis* and *L. sinensis* (Additional file 1), and the presence of *L. capensis* group and *L. sinensis* group on nuclear gene tree (Figure 2) supported the specific status of *L. capensis* and *L. sinensis*. As we discussed above, our analyses led to the inference that *L. timidus* and *L. sinensis* mitochondrial genomes had introgressed into *L. capensis* and that frequent nuclear DNA introgression had occurred between *L. capensis* and *L. sinensis*. These observations are inconsistent with those of Wu et al. [14], who concluded that *L. capensis* either does not exist in China as a unique taxon or has been replaced by *L. timidus*. In contrast, our data suggest the occurrence of continuous introgression from *L. timidus* into *L. capensis*.

In the present study, our mtDNA tree identified a new mtDNA lineage that might represent a previously unidentified hare species from Xinjiang Province. This is evidenced by the fact that the newly identified mtDNA lineage (*L. capensis*-2) was closely related to *L. yarkandensis* on the mtDNA tree (Figure 1) and the Cyt b sequence divergence (2.5%) between *L. capensis*-2 and *L. yarkandensis* is higher than that (2.2%) found between *L. capensis* and *L. timidus* (Additional file 1). This finding is consistent with the inference of Wu et al. [14] that there might be two or more new hare species in this area of China; however, the MGF haplotypes of the *L. capensis*-2 samples were divided into two different groups: (i) two (Hap13 and Hap 14) were nested in the *L. yarkandensis* clade, with one (Hap13) shared with *L. yarkandensis*; (ii) five (Hap6, 15, 17, 19, 21) fell into the *L. capensis* group, with two (Hap15 and Hap21) shared with *L. capensis* (Figure 2). In addition, the individuals of *L. capensis*-2 and *L. capensis* have no significant morphological differences, therefore, the alternative hypothesis, that this newly identified mtDNA lineage may reflect ancient mtDNA introgression from *L. yarkandensis* into *L. capensis*, seems more likely.

Conclusions

This study has revealed the first evidence of complex, ancient and recent introgressive hybridization among Chinese *Lepus* species thus providing a framework for understanding the patterns of speciation and the taxonomy of this clade. Frequent introgressive hybridization involving *L. timidus*, *L. oiostolus*, *L. sinensis*, *L. yarkandensis*, *L. capensis* and *L. mandshuricus* were detected. Both unidirectional mtDNA introgression and bidirectional nuclear DNA introgression were inferred. In contrast to the

previously reported introgression events within the genus *Lepus*, some of the mtDNA introgression events among Chinese *Lepus* species were estimated to be relatively ancient. We hypothesized that asymmetries in species abundance, mating preferences, range expansion and low levels of genetic divergence between *Lepus* species may have been causal for this reticulate evolutionary process. The existence of morphological intermediates and atypical mtDNA genealogies resulting from this reticulation might have contributed to the current taxonomic confusion among Chinese *Lepus* species. Our analyses supported the classification of Chinese *Lepus* into eight species. The present study also demonstrated that nuclear DNA could offer a powerful complement to the use of mtDNA genes for tracing the evolutionary history of recently diverged species. Future studies incorporating broader taxonomic sampling and unlinked genetic markers should provide additional resolution of the reticulate evolutionary patterns and systematic relationships among Chinese hares.

Methods

Specimen collection

To avoid the impact of seasonal effect on the pelage color variations, a total of 124 Chinese hares were collected in winter from 54 localities in China (Additional file 4 and Figure 3). The taxonomic status of some Chinese hare species reflects a longstanding debate [2,3,5-7]. We adopted mainly the classification scheme of Luo [13] for this study, but assigned *L. melainus* into *L. mandshuricus*, therefore, there are eight putative Chinese *Lepus* species in our samples. As described by Luo [13], *L. capensis* and *L. sinensis* have significant differentiation in tail length; *L. capensis* has the longest tail length (80-125 mm) among Chinese *Lepus* species while *L. sinensis* has the shortest (30-60 mm). The winter pelage of *L. mandshuricus* samples examined here was entirely black, except for the ventral surfaces; this is a unique character for *L. mandshuricus*. In contrast, only *L. timidus* has a white winter pelage making the identification of animals belonging to this species similarly straightforward. *L. yarkandensis* has a smaller body size than *L. capensis* as evidenced by their average body weight (*L. yarkandensis*: 1635 g; *L. capensis*: 1987 g). In addition, a large, black speckle on the dorsal surface of the tail allows the identification of *L. capensis* individuals. All of the samples examined in the present analysis were collected from their typical distributional areas (Figure 3), further supporting the assignment of samples to various species. Samples with unavailable or uncertain morphological information were excluded from the present study. All collection was performed following animal use protocols approved by the Kunming Institute of Zoology Animal Care Committee.

DNA extraction and PCR amplification

Total genomic DNA was extracted using a modified phenol/chloroform method [74,75]. For hair and skin specimens, total genomic DNA was extracted with the Chelex-100 method [75-77]. We designed hare-specific primers from the published sequences of lagomorph species, and those that we sequenced to amplify four mtDNA fragments (Cyt b, COX I, ND4 and D-loop) from 124 individuals (Additional file 5). Due to its better phylogenetic performance demonstrated in the Leporidae study of Matthee *et al.* [78], one nuclear DNA fragment (MGF) was also amplified from 124 individuals using primers from Matthee *et al.* [78,79]. The primers are situated in the MGF exon regions, and the faster evolving intron sequence was amplified. Interestingly, high proportion of heterozygotes was found at the MGF locus.

Amplified PCR products were purified and sequenced in both directions with an ABI PRISM 3730 DNA sequencer. Homology of the acquired sequences was assessed by BLAST searching [80] of GenBank. Direct sequencing of the PCR products from the nuclear gene allowed the identification of heterozygous genotypes. Products from heterozygous individuals were cloned into the PMD18-T vector (Takara, China) and transformed into an ultracompetent *E.coli* cell line (Takara, China). Six clones per ligation reaction were sequenced in both directions to identify the heterozygous positions. Since the same DNA samples were used to amplify both the mtDNA and nuclear gene fragments, and the sequencing of mtDNA fragments did have any signal of admixture, thus we could rule out contamination as a cause of the heterozygosity at the nuclear locus. The mtDNA and nuclear gene sequences obtained in the present study have been deposited into GenBank with the following accession numbers: ND4 (HM232860 - HM232975); Cyt b (HM232976 - HM233091); COX I (HM233092 - HM233207); D-loop (HM233208 - HM233323) and MGF (HM233559 - HM233732).

Sequence data analysis

Sequences were aligned using Clustal X1.81 [81] and refined by visual inspection. Prior to phylogenetic analysis of nuclear sequence recombination tests were conducted by using Sawyer's method [82] with the program GENECONV [83]. The default parameters were used except that the mismatch penalties varied from small (gscale = 1) to infinite (gscale = 0).

Phylogenetic analyses were conducted using the NJ method in MEGA 4 [84] and BI method as implemented in MrBayes v3.0b4 [85] for the separate and combined mtDNA datasets and the nuclear DNA dataset. In the NJ analysis, gaps or missing data were excluded. Nodal supports were assessed using BS with 1000 replicates. The evolutionary distances were computed using the maximum composite likelihood method. Prior to BI analysis,

the program Modeltest 3.7 was used to identify the optimal model of DNA substitution [86]. The best-fit model was selected according to the Akaike information criterion (AIC) [87]. Bayesian analysis began with random starting trees and ran for 5×10^6 generations, with the Markov chains sampled every 100 generations. We checked the average standard deviation of split frequencies for parameter convergence in the BI analysis. In our BI analysis, the average standard deviation of split frequencies was close to 0.007 when the run ended. The first 1.25×10^6 generations were excluded as burn-in. The analysis was conducted twice to ensure that the Bayesian analyses were not trapped in local optima [88,89]. The remaining trees from both analyses were used to create a majority rule consensus tree where the percent of samples recovering the same clade represented the PP value of that clade.

Molecular clock test and divergence time estimation

The hypothesis of a molecular clock was examined for the combined mtDNA data set using the relative-rate test [90] with the program PHYLTEST [91]. Rate constancy was rejected at the 5% level, so we employed the relaxed Bayesian methods to estimate divergence times [92,93]. First, in the PAMLv3.14 package [94], we used BASEML to create the maximum likelihood output files needed for the MULTIDIVERGENCE package developed by Thorne *et al.* [92] and Kishino *et al.* [95]. Next, we used PAML2MODELINF to write "model files" needed to estimate the variance-covariance matrix for the four mtDNA fragments independently. The variance-covariance matrix estimation was performed using ESTBRANCHES. Finally, the output files from ESTBRANCHES were employed in MULTIDIVTIME to estimate the prior and posterior distributions of the divergence dates. Markov chain Monte Carlo analyses involved an initial burn-in (250,000 cycles), after which the Markov chain was sampled every 100th cycle a total of 20,000 times. Multiple independent runs were performed for the same data and prior distributions, but with different starting points to ensure stationarity. Two calibration points were used for estimating divergence time: (i) a literature-based divergence time between *L. comus* and *L. oiostolus* (1.83 ± 0.669 MYA) [14]; (ii) the earliest fossil record of *L. timidus* collected from the Middle Pleistocene (0.75MYA) [96,97].

Additional material

Additional file 1: Uncorrected pairwise distances (P-distance) among eight mtDNA lineages of Chinese hares based on the separate analyses of four mtDNA fragments.

Additional file 2: Details about the discrepancy between the mitochondrial and nuclear gene trees. Sample code corresponds to sequence name shown in Figure 1 and sample code in Additional file 4.

Additional file 3: Bayesian tree used for divergence time estimates and the divergence time for each major clades. Phylogenetic analyses are rooted with *Oryctolagus cuniculus*. The best substitute model GTR+I+G was used for Bayesian inference. The numbers above the branches are the Bayesian posterior probabilities (PP).

Additional file 4: Samples collected in this study. Sample code corresponds to sequence name shown in Figure 1 and sample code in Additional file 2.

Additional file 5: Primers used for mitochondrial fragments amplification and sequencing.

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Author details

¹Laboratory for Conservation and Utilization of Bio-resource & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, 650091, PR, China. ²State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Kunming 650223, China. ³Department of Genetics, University of Georgia, Athens, Georgia 30602, USA. ⁴Department of Zoology, College of Life Sciences, Wuhan University, Wuhan 430072, China. ⁵Utah State University Department of Animal, Dairy & Veterinary Sciences Old Main Hill 4700 Center for Integrated Biosystems Rm315 Logan, UT 84322-4700, USA.

Authors' contributions

JL, LY, CHW and YPZ designed the study. JL carried out the experiment work. JL and LY performed the sequence analyses. JL, LY and YPZ wrote the manuscript. MLA contributed to the manuscript revision. SFW and XL helped collect and identify samples. All authors read and approved the final manuscript.

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