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Molecular evidence revealed *Lepus hainanus* and *L. peguensis* have a conspecific relationship

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Abstract

Accurate species delimitation in *Lepus* was often hindered by highly conserved morphology and frequent introgression. In this study, we used rigorous molecular species delimitation methods to evaluate the taxonomic status of Hainan hare (*Lepus hainanus*) which has been traditionally identified as a distinct species, or a subspecies of Burmese hare (*L. peguensis*). The genetic distance and phylogenetic network support *L. hainanus* and *L. peguensis* are conspecific. However, the phylogenetic species concept and Bayesian species delimitation analysis based on combined mtDNA supported they are different species. The discordance between different methods can be explained by different species criterion. By taking into account our conflict results, we hold the opinion that adoption of the phylogenetic species concept and Bayesian species delimitation analysis would increase the risk of taxonomic inflation of island biota or otherwise spatially isolated population. Conservatively, we suggest that *L. hainanus* and *L. peguensis* are conspecific based on the results of our genetic divergence and phylogenetic network exclusively.

Keywords

Lepus hainanus, *Lepus peguensis*, mitochondrial genes, MGF gene, taxonomy

History

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Introduction

Hainan Island is the largest tropical island of China and is separated from the Leizhou Peninsula by approximately 30 km of Qiongzhou Strait. It is known to have a high level of species diversity including 76 mammal species (subspecies) (Xu et al., 1983). Five of them have been recognized as endemic species to the island mainly based on morphological characters, including Hainan gymnure (*Neohylomys hainanensis*), Hainan gibbon (*Nomascus hainanus*), Hainan giant flying squirrel (*Petaurista hainana*), Hainan small flying squirrel (*Petinomys electilis*) and Hainan hare (*Lepus hainanus*) (Huang et al., 1995; Xu et al., 1983). Except for *N. hainanensis*, which was regarded as a distinct species due to its significant morphological variation with other living Hylomyinae, the other four species were regarded as subspecies level based on morphological traits (Corbett & Hill, 1992). Recently, molecular genetic studies have been conducted on *N. hainanensis*, *N. hainanus and P. hainana*, and revealed clear species-level differences between them and the congeneric species of China mainland (He et al., 2012; Roos et al., 2007; Yu et al., 2006). However, up to now, the molecular taxonomic status of *L. hainanus* has still been unclear.

L. hainanus is only found in the Hainan Island and was firstly described as a distinct species by Swinhoe (1870). Later, it was regarded as a subspecies of Burmese hare *L. peguensis* (Allen, 1940; Ellerman & Morrison-Scott, 1951). Gureev (1964) and Flux & Angermann (1990) regarded the taxonomic status of *L. hainanus* as provisional specific status. Huang et al. (1995) described *L. hainanus* as a distinct species, and reported that the body size, cranial length and tympanic bulla length of *L. hainanus* were all smaller than that of *L. peguensis*. Besides, unlike *L. hainanus,* the back of *L. peguensis* is cervinus mixed with black. Previously, the most adopted characteristics for taxonomic determination of *Lepus* were morphological traits such as body size, tail length, cranial morphology and the pelage coloration (Corbett & Hill, 1992). However, the use of morphological traits for species designations is easy to be confounded by plastic responses to the external environment. For example, previous studies have shown that the hares on islands tend to be dwarfed (Lomolino, 1985; Thulin et al., 2012) and some studies demonstrated that the change of food types could contribute to the variation in cranial morphology in rodents (Kiliaridis et al., 1985). Besides, variations in coat coloration were found to match environmental background color of the habitats in which they were distributed (Stoner et al., 2003). So the taxonomic determination of species is challenging when molecular data is absent. For example, *L. melainus* was originally diagnosed as a distinct species for having black pelage with a white speckle on the forehead and a reentrant angle on the anterior upper premolars (Luo, 1988). However, Liu et al. (2011a) suggested that *L. melainus* represented a synonym of Manchurian hare (*L. mandshuricus*) based on molecular evidence. Recent years, molecular studies have revealed complex reticulate evolutionary scenarios, such as introgressive hybridization between 'good species' in genus *Lepus*, e.g. between *L. capensis* and *L. yarkandensis* (Wu et al., 2011), and between *L. timidus* and *L. mandshuricus* (Liu et al., 2011b). Continual introgressive hybridization confounded species identification within this genus. In this study, three mtDNA genes (cytochrome *b*, Cyt *b*; cytochrome c oxidase subunit I, COX I; NADH dehydrogenase subunit 4, ND4) and intron variation of one nuclear gene (Stem cell factor, MGF) were used to test whether these taxa merit species status by different species delimitation methods.

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Materials and methods

Sample collection

One *L. hainanus* was collected from Datian, Dongfang, Hainan province of China and six *L. peguensis* were collected from Dak Lak province and Dong Nai province of Vietnam. The sequence data for Cyt *b*, COX I, ND4 and MGF from another six species of genus *Lepus* published by Liu et al. (2011b) were extracted from GenBank and added to our dataset (Heterozygotes were exclusive of our analyses).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from liver with a DNA isolation kit (Omega). Primers used in this study were summarized in Table 1. DNA samples were amplified in 50 μL reactions containing 50 ng of genomic DNA, 0.5 μM of each primer, 5 μL of 10×PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP and 2 Units of Taq polymerase. PCR was performed with 95 °C for 4 min, followed by 30 cycles with 30 s at 95 °C, 30 s at 50–56 °C, 60 s at 72 °C, and a final extension cycle at 72 °C for 7 min. PCR products were cloned into the pEasy-T1 vector with an Easy-T1 cloning kit (TransGen Biotech Co., Ltd, Beijing, China), and the recombinant plasmids were transformed into the *Escherichia coli* strain TOP10 for sequencing analysis.

Genetic analysis

Multiple alignment of these sequences was performed using the Clustal X2 (Larkin et al., 2007, Dublin, Ireland) and haplotypes were identified by DnaSP v5 (Librado & Rozas, 2009, de Barcelona, Spain). The distance values by Kimura's two-parameter model (K2P) for inferring evolutionary distance (Kimura, 1980, Tokyo, Japan) were estimated by MEGA v5 (Tamura et al., 2011).

Phylogenetic analysis

The combined mtDNA sequences were analyzed using Bayesian inference (BI) as implemented in BEAST (Drummond et al., 2012, Auckland, New Zealand). The best-fit model of DNA substitution for the BI analysis was assessed by MrModelTest2.2 (Nylander, 2004, Uppsala, Sweden) based on the Akaike Information Criterion (AIC). The nucleotide substitution models were the combined mtDNA evolution of GTR+I+G with the following parameters setting: *nst* = 6; rates = invgamma. BEAST was run for10 million generations and trees were sampled by every 100 generations. The resulting log files were examined using Tracer 1.4 (Rambaut & Drummond, 2007a, Auckland, New Zealand) to confirm that the likelihood was stationary and adequate mixing of the MCMC chains and to ascertain whether the two separate runs had converged on the same results. Then, TreeAnnotator v1.4.8 (Rambaut & Drummond, 2007b, Auckland, New Zealand), which is part of the BEAST software package, was used to determine adequate burn-in (20%) and to compute the maximum clade credibility tree. The consensus tree is used as a guide topology for the subsequent BP&P analysis (Yang & Rannala, 2010, London, England).

A minimum-spanning network (MSN) was calculated under the program TCS v1.21 (Clement et al., 2000, Salt Lake City, UT) with a 95% connection limit. The program calculates the probability of a parsimonious connection between haplotypes by determining the probability of unobserved substitutions.

Divergence times were also performed by BEAST using combined mtDNA dataset, and employing an uncorrelated lognormal relaxed molecular clock, GTR+I+G models, and application of two previously published calibration points calibrated a phylogenetic tree (Liu et al., 2011b).

Bayesian species delimitation

We used a Bayesian approach for species delimitation as implemented by the program BP&P, v2.2 (Yang & Rannala, 2010, London, England) based on the three mtDNA-encoded genes. Because prior distributions can affect the results of MCMC analyses, we followed suggestions presented in previous work (Leaché & Fujita, 2010; Yang & Rannala, 2010) by using three combinations of prior settings: (1) a relatively large ancestral population with shallow divergences ($\theta = 1, 10$; $\tau_0 = 2, 2000$), (2) a relatively large ancestral population with deep divergences ($\theta = 1, 10$; $\tau_0 = 1, 10$), and (3) a relatively small ancestral population and shallow divergences ($\theta = 2, 2000$; $\tau_0 = 2, 2000$). We used the topology from the BEAST species tree as the BP&P guide tree and performed multiple runs using algorithm 0 with fine-tune parameters 15, 20 for ε and algorithm 1 with fine-tune parameters 1, 1.5, 2 for ε and 0.5, 1, 2 for *m* to confirm the stability of the rjMCMC. MCMC analyses were run for 500,000 generations, sampled every 50 generations with a burn-in of 10,000. We also broke up the large guide trees into subtrees to test them separately using above prior settings to ensure stability among runs.

Results

DNA sequences data

Three mtDNA genes (Cyt *b*, 1140 bp; COX I, 1449 bp; ND4, 1377 bp) and one *L. peguensis* nuclear gene intron (Stem cell factor, MGF, 589 bp) were obtained successfully from *L. hainanus* and specimens. Gene sequences generated by this study have been deposited in GenBank under Accession Nos. KF723323–KF723342.

Genetic analysis

We compared the pairwise sequences divergence among 7 species (Table 2), and the genetic distance (K2P) between *L. hainanus* and *L. peguensis* were ranged from 2.60% to 3.35% in Cyt *b*, 1.61% to 2.32% in COX I, 1.85% to 5.09% in ND4, while other interspecific variations were at least greater than 6.69% in Cyt *b*, 6.57% in COX I, 8.78% in ND4.

There was no haplotype shared between *L. hainanus* and *L. peguensis*. The Network based on analysis of MGF haplotypes in

Table 1. Gene name and primer sequences used in the study.

Gene name	Primers	Annealing temperature	Reference
COX I	F: ATCAACTGGCTTCAATCTACTTCT R: GGCTTGAAACCAGTCTTAGGG	53 °C	This study
ND4	F: ATCTTCCTAGCGTTTATCTTTGCC R: AGGATAATGATTGAGACGGCTATT	53 °C	This study
Cyt *b*	F: ACCAATGACATGAAAAATCATCGTT R: TCTCCATTTCTGGTTTACAAGAC	50 °C	Irwin et al., 1991
MGF	F: AAATATCAGTCTTGAATCTTAC R: TTTTAGATGAATTACAGTGTCC	50 °C	Matthee et al., 2004

Table 2. The genetic distance (K2P) of 6 species of genus *Lepus*, based on three mtDNA genes (%).

Taxa	1	2	3	4	5	6	7	8	9	10
L. peguensis hap1	0.000 (COX I)									
	0.000 (ND4)									
	0.000 (Cyt *b*)									
L. peguensis hap2	1.97									
	1.40									
	2.05									
L. peguensis hap4	1.90	0.07								
	1.40	0.00								
	2.15	0.26								
L. peguensis hap5	2.18	0.35	0.28							
	1.47	0.07	0.07							
	2.42	0.35	0.44							
L. hainanus DT	1.61	2.11	2.04	2.32						
	1.92	1.85	1.85	1.92						
	3.35	2.79	2.88	3.15						
L. hainanus H1	1.61	2.11	2.04	2.32	0.00					
	4.93	5.01	5.01	5.09	3.67					
	3.25	2.69	2.79	3.06	0.44					
L. hainanus H11	1.61	2.11	2.04	2.32	0.00	0.00				
	2.07	2.00	2.00	2.07	0.29	3.67				
	3.16	2.60	2.70	2.97	0.53	0.44				
L. comus C2	7.12	7.35	7.28	7.59	7.04	7.04	7.04			
	9.87	9.09	9.09	9.17	9.59	10.81	9.59			
	10.26	9.95	10.26	10.35	10.14	10.46	10.14			
L. sinensis S2	8.80	8.96	8.88	9.21	8.56	8.56	8.56	7.44		
	9.89	9.98	9.98	10.07	9.46	11.12	9.28	9.81		
	8.79	8.80	8.91	9.19	9.01	9.11	9.01	9.51		
L. yarkandensis Y2	8.38	8.46	8.38	8.70	8.06	8.06	8.06	7.58	7.66	
	11.54	11.45	11.45	11.54	11.18	11.09	11.18	10.29	10.25	
	9.40	9.60	9.72	10.00	9.60	9.92	9.60	7.98	9.01	
L. capensis CA20	7.75	7.99	7.91	8.23	7.75	7.75	7.75	7.13	7.52	6.57
	10.16	9.89	9.89	9.98	9.46	10.86	9.28	8.78	9.41	9.89
	10.49	10.48	10.60	10.88	10.27	10.37	10.27	7.69	8.74	6.69

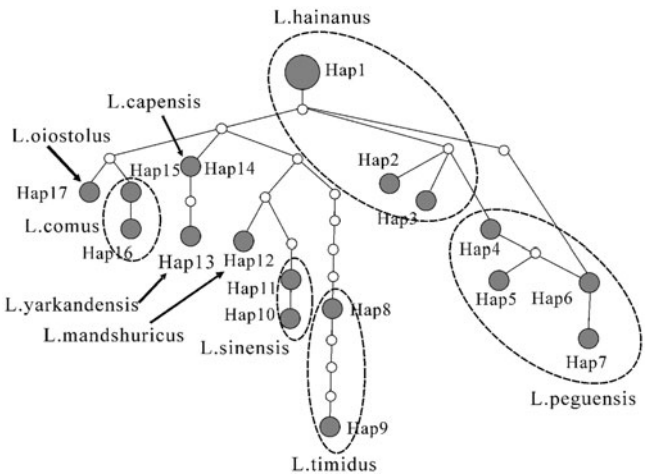

Figure 1. Statistical parsimony network showing genetic relationships of *Lepus* based on MGF gene. The sizes of filled black circles representing haplotypes reflect the number of sequences that share a haplotype. Each haplotype is numbered. Inferred intermediate haplotypes, either not sampled, or extinct, are represented by small non-colored circles.

our study identified several specific sub-clades (Figure 1), moreover, *L. peguensis* fell into the *L. hainanus* sub-clade, inferring that *L. peguensis* derived from *L. hainanus.*

Phylogenetic analysis and divergence times

Bayesian phylogenetic tree based on combined mtDNA dataset was split into two deeply divergent and well-supported clades (Figure 2). The first included *L. hainanus*, *L. peguensis* and *L. europaeus*, and the second was sub-divided further into 6 branches, with all species formed their own species-specific clades. Within the first clade, *L. hainanus* was reciprocally monophyletic with *L. peguensis*.

Our phylogenetic dating analysis placed the deepest divergence within *Lepus* at approximately 2.52 million years ago (Mya), and speciation within *Lepus* took place during 2.0–1.0 Mya during the Pleistocene, producing 9 extant species (Figure 2). The divergence between *L. hainanus* and *L. peguensis* took place about 1.14 Mya, in the early Pleistocene ice ages. Our result was mainly in concordance with previous studies (Liu et al., 2011b; Wu et al., 2005).

Bayesian species delimitation

Here, we present a contrived example illustrating the Bayesian species delimitation (BSD) in *Lepus* by BP&P. In this example, heterozygotes were excluded from our phylogenetic and BSD analyses. We took gene tree inference in BEAST as our user-specified guide tree for BP&P analysis. BSD produced robust results that the posterior probabilities of all analyses performed on nine species were 1 under a variety of demographic scenarios. The BSD results for *Lepus* were shown in Figure 3. In BP&P analysis, posterior probabilities (*pp*) $\geq$0.95 were interpreted as strong support in favor of a speciation event (Leaché & Fujita, 2010). So our BSD results for genus *Lepus* supported nine species with high speciation probabilities on the guide tree.

Discussion

Phylogenetic analysis and divergence times

Our Bayesian inference (BI) results were mainly in concordance with previous studies (Liu et al., 2011b; Wu et al., 2005).

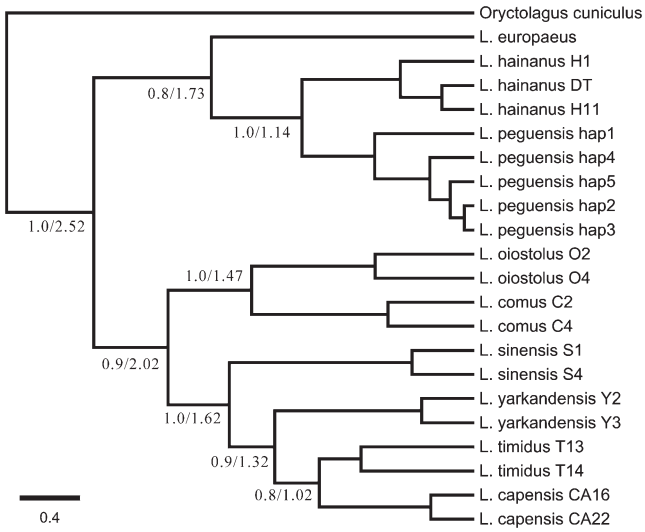

Figure 2. The Bayesian inference (BI) tree under the GTR+I+G evolution model based on the combined data of three mtDNA genes. Bayesian posterior supports of nodes and the divergence times for each clade are shown (shown on left and right of each clade-defining node, respectively). The core material was based from the study of Liu et al. (2011b), and here we sequenced an additional sample *L. hainanus* DT from Datian, Dongfang, Hainan, and six *L. peguensis* collected from Dak Lak province and Dong Nai province of Vietnam.

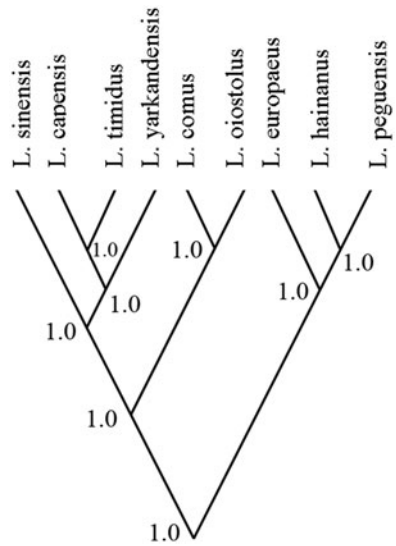

Figure 3. Bayesian species delimitation results for *Lepus* based on the three mtDNA genes. Speciation probabilities for each node are shown. BSD produced robust results that the posterior probabilities of all analyses performed on nine species were 1 by using all combinations of prior settings presented in previous work (Leaché & Fujita, 2010; Yang & Rannala, 2010).

Similarly to a previous study (Wu et al., 2005), we also observed the unexpected result that *L. europaeus* grouped with *L. hainanus* and *L. peguensis* as early diverging clade of the *Lepus.* This result inferred that *L. hainanus* had different evolutionary history with respect to the China mainland hares.

From our analysis of combined mtDNA dataset, the divergence time between *L. hainanus* and *L. peguensis* was estimated to be 1.14 Mya. Then, we took geological data and sea-level changes into consideration to find patterns that concurred with the molecular results, assuming that the dispersal events of *L. hainanus* to the Indo-Chinese peninsula were affected by the appearance of land-bridge between Hainan Island and Indo-Chinese peninsula. The area between Hainan Island and Indochinese peninsula was a shallow sea basin less than 100 meters in depth (Tougard, 2001), however, during the glacial periods of Pleistocene, the sea level dropped significantly up to 200 meters lower than at present (Lekagul & McNeely, 1988), so it is reasonable to assume that the rapid drops in sea levels would have created land routes that connected Hainan Island and Indo-Chinese peninsula enabling the dispersal of *L. hainanus* to Indo-Chinese peninsula. Similar scenarios that Hainan Island had opportunities for faunal exchange with Indo-Chinese peninsula have been given for other species, such as *Nomascus concolor* (Chatterjee, 2006) and *Geoemyda spengleri* (Gong et al., 2009), *Leiolepis reevesii* (Lin et al., 2010). The proposed scenario of a western expansion of *L. hainanus* into Indo-Chinese peninsula must be confirmed by new sampling efforts and more nuclear genes (e.g. microsatellite DNA).

Species delimitation and species concept

Species delimitation is often based on distance methods (DNA barcoding) in which a differentiation of samples or populations above a certain threshold of genetic divergence is considered as species criterion. Different authors of molecular taxonomic surveys have used different thresholds for the same gene (Bradley & Baker, 2001; Chang et al., 2011; Nicolas et al., 2012). Tobe et al. (2010) compared the relative values of Cyt *b* and COX I for identification of mammalian species. They suggested that a K2P<1.5% indicated the samples come from the same species and a K2P>2.5% indicated a different mammalian species for COX I and Cyt *b* using K2P model. Our results showed that K2P>2.5% with Cyt *b* between *L. hainanus* and *L. peguensis*, but K2P with COX I fell in the gray area of 1.5%≤K2P≤2.5%. The divergence in the mtDNA markers studied was marginal, so we would be reluctant to raise *L. hainanus* to species level on this data alone. The MGF network gave a discordant topology with phylogenetic analysis based on combined mtDNA dataset that *L. hainanus* was not monophyletic. Because of the recent origin of the group and geographic distribution separation, incomplete lineage sorting might explain this discordance. Our MGF network result provided additional evidence for conspecific status between *L. hainanus* and *L. peguensis.*

However, phylogenetic and BP&P results based on three mt genes gave an opposite pattern that *L. hainanus* and *L. peguensis* should be different species. Phylogenetic analysis supported that *L. hainanus* and *L. peguensis* were separately evolving lineages as defined by the phylogenetic species concept (PSC). In fact, adoption of the PSC would reclassify nearly all island subspecies as species (Hazevoet, 1996). PSC may result in taxonomic inflation of island biota or otherwise spatially isolated population (Zachos et al., 2013). Using BP&P, Mckay et al. (2013) found that geographic clusters within *Pycnonotus sinensis* and *P. hainanus* had higher speciation probability than the speciation probability of the split between the two subspecies (*Pycnonotus sinensis formosae* and *Pycnonotus sinensis orii*). So they suggested that BP&P may not be a conservative method for delimiting independently evolving population lineages. Whether BP&P overstated the speciation probabilities of island biota remains ambiguous. We therefore question the accuracy of our BP&P results and suggest that *L. hainanus* and *L. peguensis* have a conspecific relationship.

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Declaration of interest

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