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The taxonomic status of *Lepus melainus* (Lagomorpha: Leporidae) based on nuclear DNA and morphological analyses

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Abstract

The taxonomic status of the species *Lepus melainus*, the Manchurian black hare, is intensely debated. It is considered either as a valid species or a black color morph of *L. mandshuricus*, the Manchurian hare. Herein, we evaluate the validity of *L. melainus* using 24 morphological traits and two nuclear DNA loci (TG=466bp; MGF=592bp) from newly collected specimens. Except for winter pelage, we fail to discover significant morphological differences between *L. melainus* and *L. mandshuricus*. Analysis of the nuclear DNA sequences reveals lack of reciprocal monophyly between *L. mandshuricus* and *L. melainus*, as they form one single clade with high bootstrap support; in addition, morphometric and morphological analyses found no specific differentiation between forms corresponding to *L. mandshuricus* or *L. melainus*. Together with the fact that the range of *L. melainus* is completely within that of *L. mandshuricus*, our study supports the recognition of *L. melainus* as a melanistic morph and junior synonym of *L. mandshuricus*.

Key words: *Lepus mandshuricus*, pelage, skull, TG gene, MGF gene, hares, taxonomy

Introduction

The Chinese hares (*Lepus*) are widely distributed throughout China from the Qinghai-Tibetan plateau to near sea level, and from mainland to the islands of Taiwan and Hainan. Northeastern China is characterized by the presence of several endemic species (Hoffmann & Smith 2005; Pan *et al*. 2007), but the validity of one of these species, the Manchurian black hare, *L. melainus* Li and Luo, is intensely debated (Flux & Angermann 1990; Hoffmann & Smith 2005; Wu *et al*. 2005).

The occurrence of a melanistic form of hare in northeastern China was first reported in 1870 from Ussuriland by Przewalsky (Loukashkin 1943), and later noted again by Sowerby (1923), who reported it as a black form of *L. mandshuricus* Radde. Two subspecies were described for these black hares: *L. brachyurus niger* from the Lower Amur River (Noack 1891), and *L. mandshuricus melanonotus* from Ussuri (Ognev 1922). Both taxa were relegated to the synonymy of *L. mandshuricus* by subsequent authors (Loukashkin 1943; Ellerman & Morrison-Scott 1951). Subsequently, Li & Luo (1979) described a new species, *L. melainus*, based on two blackish brown specimens in which the anterior upper premolar had a deep median re-entrant angle. The species was recognized by Flux & Angermann (1990). Hoffmann (1993) and Hoffman & Smith (2005), however, considered *L. melainus* as a synonym of *L. mandshuricus* because it occurred entirely within the range of *L. mandshuricus*, as well as *L. timidus* Linnaeus and *L. tolai* Pallas—the ecological sympatry of four species of hares was unprecedented. An extremely small mitochondrial (mt) DNA sequence divergence between *L. melainus* and *L. mandshuricus* was later reported (Wu *et al*. 2005), but the taxonomic conundrum remained unanswered.

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Although mtDNA can be used to infer phylogenetic relationships, the introgression of mtDNA among species caused by interspecific hybridization can result in biased mtDNA genealogies compared with the actual species relationships (Ballard & Whitlock 2004). This observation is critical for hares because unidirectional introgression of mtDNA from *L. timidus* into *L. europaeus* Pallas was shown to have taken place in Sweden during the 19th century (Thulin *et al*. 1997). Ancient introgression of mtDNA was also reported from *L. timidus* into *L. granatensis* Rosenhauer and *L. europaeus* in the Iberian Peninsula (Alves *et al*. 2003; Melo-Ferreira *et al*. 2005, 2007). Moreover, introgression may also exist among Chinese hares (Alves *et al*. 2006; Alves *et al*. 2008), as the mtDNA of *L. mandshuricus* seems to be from either *L. timidus* or *L. sinensis* Gray (Liu *et al.* unpublished data). Therefore, unlinked nuclear gene loci should be used to trace the evolutionary history of *Lepus* and clarify controversial taxonomical issues.

Herein, we test the null hypothesis of conspecificity between *L. melainus* and *L. mandshuricus*. We use two nuclear DNA loci (Thyroglobulin—TG, and Stem cell factor—MGF) and comparisons of skull morphology to assess the taxonomic validity of *L. melainus*. In order to reject the null hypothesis of conspecificity, and accept both *L. mandshuricus* and *L. melainus* as valid species, we must demonstrate the presence of distinct morphological differentiation or reciprocal monophyly.

Materials and methods

Sampling

Specimens and tissue samples examined in this study were collected from the focal species' type localities and surrounding areas (Fig. 1; Table 1); voucher and tissue samples are stored in the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences. Based on the species diagnoses of Li & Luo (1979), five specimens plus five additional tissue samples (n=10) were assigned to the Manchurian hare (*L. mandshuricus*), and two specimens plus four more tissue samples (n=6) to the Manchurian black hare (*L. melainus*). For comparison, nine specimens of the mountain hare (*L. timidus*) were used in the morphological analyses and ten additional tissue samples representing five well recognized species of Chinese hares were included in the nuclear DNA analysis (Table 1).

TABLE 1 Specimens and tissue samples of Chinese hares, genus *Lepus*, used in the morphometric and phylogenetic analyses. For heterozygous individuals, the GenBank numbers of the two chromosomes copies are provided. Acronyms: KIZ—Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences; MA—*L. mandshuricus*; ME—*L. melainus.*

Species	Collection locality and province	Specimen numbers	Tissue sample codes	GenBank numbers	
				TG	MGF
L. mandshuricus	Mudanjiang, Heilongjiang		MA1		HM233340
L. mandshuricus	Sunwu, Heilongjiang		MA2	HM233390	HM233326 HM233339
L. mandshuricus	Zhalantun, Neimenggu		MA4	HM233391	HM233325 HM233338
L. mandshuricus	Harbin, Heilongjiang		MA5		HM233342
L. mandshuricus	Harbin, Heilongjiang		MA6	JF750699	HM233337
L. mandshuricus	Zhanhe, Heilongjiang	KIZ019692	MA7	JF750706	HM233334 HM233349
L. mandshuricus	Zhanhe, Heilongjiang	KIZ 019689	MA8	JF750701	HM233328 HM233348
L. mandshuricus	Zhanhe, Heilongjiang	KIZ 019690	MA9	JF750703	HM233329 HM233335
L. mandshuricus	Beian, Heilongjiang	KIZ 019691	MA10	HM233388	HM233350
L. mandshuricus	Zhanhe, Heilongjiang	KIZ 019695	MA11	JF750707	HM233327 HM233346

..... *continued on the next page*

TABLE 1 (continued)

Species	Collection locality and province	Specimen numbers	Tissue sample codes	GenBank numbers	
				TG	MGF
L. melainus	Harbin, Heilongjiang		ME1	JF750704 JF750708	HM233344
L. melainus	Harbin, Heilongjiang		ME2	JF750705	HM233324 HM233336
L. melainus	Harbin, Heilongjiang		ME3		HM233330 HM233343
L. melainus	Harbin, Heilongjiang		ME4		HM233331 HM233341
L. melainus	Zhanhe, Heilongjiang	KIZ 019696	ME5	JF750700	HM233332 HM233347
L. melainus	Zhanhe, Heilongjiang	KIZ 019694	ME6	JF750702	HM233333 HM233345
L. timidus	Caoer, Neimenggu	KIZ 019681			
L. timidus	Caoer, Neimenggu	KIZ 019697			
L. timidus	Caoer, Neimenggu	KIZ 019693			
L. timidus	Mohe, Heilongjiang	KIZ 019682			
L. timidus	Wuerqihan, Neimenggu	KIZ 019683			
L. timidus	Zhanhe, Heilongjiang	KIZ 019684			
L. timidus	Mohe, Heilongjiang	KIZ 019685			
L. timidus	Xinlin, Heilongjiang	KIZ 019686			
L. timidus	Wuerqihan, Neimenggu	KIZ 019688			
L. timidus	Harbin, Heilongjiang		L. timidus-1	JF750696 JF750698	JF750675 JF750677
L. timidus	Harbin, Heilongjiang		L. timidus-2	JF750697	JF750676 JF750678
L. comus	Zhaotong, Yunnan		L. comus-1	JF750688	JF750680
L. comus	Guangnan, Yunnan		L. comus-2	JF750687 JF750689	JF750679 JF750681
L. oiostolus	Daocheng, Sichuan		L. oiostolus-1	JF750690 JF750693	JF750682
L. oiostolus	Saka, Tibet		L. oiostolus-2	JF750691	JF750683
L. hainanus	Datian, Hainan		L. hainanus-1	JF750695	JF750684 JF750686
L. hainanus	Danzhou, Hainan		L. hainanus-2	JF750692	JF750685
L. yarkandensis	Yuli, Xinjiang		L. yarkandensis-1	JF750709	JF750672
L. yarkandensis	Yuli, Xinjiang		L. yarkandensis-2	JF750694 JF750710	JF750673 JF750674

Morphological comparison

In addition to pelage color variation, the following external measurements were taken from 5 *L. mandshuricus* (KIZ019689-019692, KIZ019695) and 2 *L. melainus* (KIZ019694, KIZ019696) specimens: length of head and body (HB), tail length (TL), hind foot length (HF), and ear length (EL). These data were combined with the external measurements of 43 *L. mandshuricus* and 5 *L. melainus* specimens from Luo (1988) and used in T- tests as implemented in SPSS 13.0 (2004). In addition, the following 24 cranial measurements were taken from five *L. mandshuricus* (KIZ019689-019692, KIZ019695), two *L. melainus* (KIZ019694, KIZ019696) and nine *L. timidus*

(KIZ019681-019686, KIZ019688, KIZ019693, KIZ019697) specimens with a digital caliper graduated to 0.1 mm following Pan *et al*. (2007): greatest length of skull (GLS), condylobasal length (CBL), basal length (BL), palatal length (PL), post-palatal length (PPL), rostrum length (RSL), length of upper tooth row (LUTR), length of upper molars (LUM), length of diastema (LD), length of incisive foramina (LIF), breadth of incisive foramina (BIF), breadth of mesopterygoid fossa (BMF), cranial height (CH), cranial breadth (CB), zygomatic breadth (ZB), interorbital breadth (IOB), breadth of palatal bridge (BPB), length of palatal bridge (LPB), length of auditory bulla (LAB), nasal length (NL), mandible length (ML), length of lower tooth row (LLTR), length of lower molars (LLM), and length of mandible joint (LMJ). All 24 variables were used in principal component analysis (PCA) and one-way analysis of variance (ANOVA) as implemented in SPSS 13.0 (Inc 2004). Finally, we also checked anterior upper premolar of these specimens, presumably a diagnostic character for the Manchurian and Manchurian black hares (Li & Luo 1979).

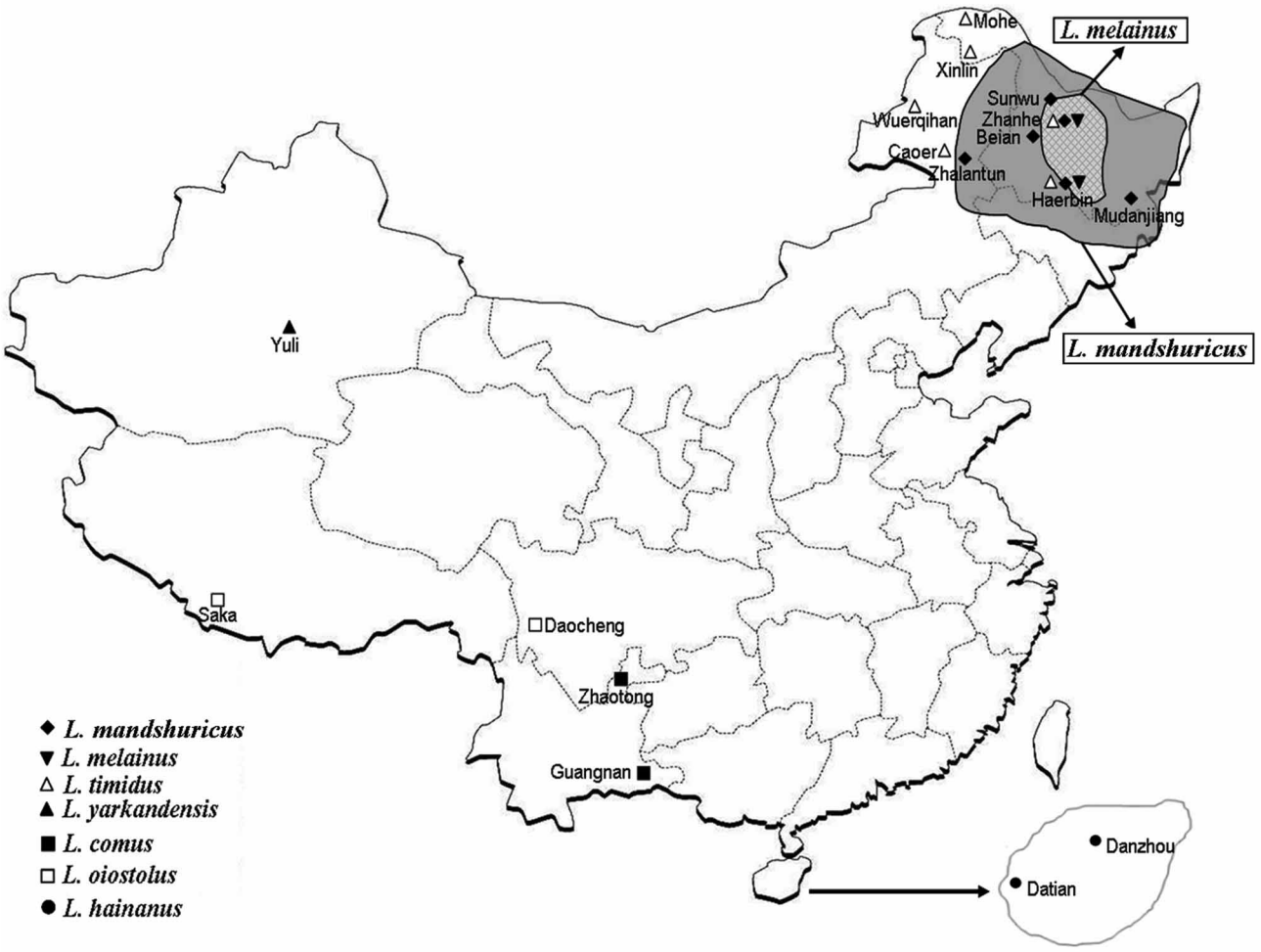

FIGURE 1. Geographical distribution of the samples examined in this study and the species ranges of *Lepus melainus* and *L. mandshuricus* in China.

DNA sequence analyses

Total genomic DNA was extracted using a method modified from the standard phenol/chloroform extraction process (Sambrook *et al*. 1989; Wu *et al*. 2000). Using the primers from Matthee *et al*. (2001, 2004), two nuclear DNA fragments (Thyroglobulin or TG, and Stem cell factor or MGF) were amplified from 26 tissue samples (Table 1). The primers were situated in exon regions, and faster evolving intron sequences were amplified. Amplified PCR products were purified and sequenced in both directions with an ABI PRISM 3730 DNA sequencer. Fragments with heterozygous sites were cloned into PMD18-T vector (Takara, China) and transformed into ultracompetent *E. coli* cells (Takara, China). Six clones per ligation reaction were sequenced in both directions to separate the alleles. Acquired sequences were submitted to GenBank for BLAST searching (Altschul *et al*. 1997) to assess homology.

Sequences obtained in the present study were deposited in GenBank under accession numbers HM233388, HM233390, HM233391 and JF750687–JF750710 for TG and HM233324–HM233350 and JF750672–JF750686 for MGF.

Sequences were aligned using Clustal X1.81 (Thompson *et al*. 1997) and refined by visual inspection. Given that recombination events within a fragment may influence the phylogenetic tree topology, recombination tests were conducted by using Sawyer's (1989) method implemented in the program GENECONV (Sawyer 2000) prior to tree construction. The default parameters were used except that the mismatch penalties varied from small (gscale = 1) to infinite (gscale = 0).

Phylogenetic analyses of the individual nuclear genes were performed using PAUP* 4.0b10 (Swofford 2002) for maximum likelihood (ML) analyses. In ML analysis, the best-fit models of sequence evolution were selected using the Akaike Information Criterion (AIC; Akaike 1974; Posada & Buckley 2004) with Modeltest version 3.7 (Posada & Crandall 1998). The chosen models and their parameters were used to infer the ML trees with the heuristic algorithm, 10 random-addition sequence replicates, and TBR branch swapping. Nodal reliability under ML analysis was assessed using a bootstrap resampling of 100 replicates (BP; Felsenstein 1985). The TG and MGF sequences of *Sylvilagus obscurus* obtained from Matthee *et al*. (2004) were used to root the tree.

Results

Morphological evidence

Color variation. All specimens used for the morphological comparisons were collected in winter so that pelage-color variation was not influenced by seasonal effects. Among 16 specimens, five (KIZ019689, KIZ019690, KIZ019691, KIZ019694, KIZ019695) were identified as Manchurian hares (*L. mandshuricus*) according to the following morphological characters: (1) dorsal pelage generally blackish brown and flank hairs blackish brown for about 2/3 of the base length with white or buffy brown on the terminal tips (1/3 hair length); (2) ventral pelage generally pale blackish brown; owl hairs blackish brown with buffy-brown tips (1/3–1/2 of hair length); throat hairs with short buffy-brown tips (1/5 of hair length); chest hairs with white tips (1/4–1/3 hair length) and blackish roots; and belly hairs with 1/2 buffy brown tips and 1/2 blackish brown base. In addition, two specimens (KIZ019690, KIZ019692) had a white speckle on the forehead comprised of 115–120 white hairs.

Two specimens (KIZ019694, KIZ019696) were identified as the Manchurian black hare (*L. melainus*) according to the diagnosis of Li & Luo (1979): blackish brown color on the entire body. Sparse, short white hairs mixed in long blackish brown hairs occurred on the throat of both specimens. A few shorter, pure white hairs (about 2/3 the length of the blackish brown hairs) mixed in blackish brown chest hairs.

Morphometric variation

T-test analyses found no significant differences ($p > 0.05$) between the external measurements (HB, TL, HF, and EL; Table 2) of *L. mandshuricus* and *L. melainus*. ANOVA revealed significant differences in all of the 24 cranial measurements ($p < 0.001$) among *L. timidus*, *L. mandshuricus*, and *L. melainus*; nevertheless, while *L. timidus* differed significantly from *L. mandshuricus* and *L. melainus* (Least-Significant Difference, post hoc multiple comparisons), there were no significant differences between *L. mandshuricus* and *L. melainus*.

The PCA analysis of all 16 specimens based on 24 skull variables was performed to assess overall similarity (Fig. 2). The factor loadings are shown in Table 3. The first principal component (PC1), a size factor, accounted for 59.36% of the total variance. Most variables were strongly correlated with it (e.g. GLS, LUTR, CBL, BL, LLTR, and PPL). The second principal component (PC2) accounted for 11.88% of the total variance. It was strongly correlated with ZB and BIF. The third principal component (PC3) accounted for 7.43% of the total variance and it was highly correlated with LPB. As shown in Fig. 2, *L. timidus*, *L. mandshuricus*, and *L. melainus* could be divided into two groups by the first principal component, with *L. melainus* greatly overlapped with *L. mandshuricus* to the extent of forming one group apart from *L. timidus*.

Anterior upper premolar variation. Inspection of anterior upper premolars (P^3) of *L. melainus* and *L. mandshuricus* showed that all specimens had a median re-entrant angle, except for one specimen of *L. melainus* (KIZ019696), which had a deeper re-entrant angle on only one side (left P^3).

TABLE 2. External and cranial measurements of Chinese hares, genus *Lepus*. Summary statistics are mean, standard deviation, sample size, and observed range for each species. The asterisk (*) means that the sample size is unknown. All measurements in millimeters.

	L. mandshuricus			*L. melainus*		
	Luo(1988)	Li & Luo (1979)	This study	Luo(1988)	Li & Luo (1979)	This study
HB	435±29.0, 11 (394–500)	–	429.2±9.7, 5 (415–440)	425±14.0, 5 (410–430)	–	419±1.4, 2 (418–420)
TL	67±4.0, 12 (60–70)	–	65.6±8.7, 5 (57–78)	72±5.0, 5 (65–80)	–	64.5±14.8, 2 (54–75)
HF	121±29.0, 12 (120–138)	–	124±8.7, 5 (115–134)	116±5.6, 5 (111–125)	–	122±1.4, 2 (121–123)
EL	74±4.0, 12 (69–80)	–	69.8±5.7, 5 (62–78)	77± 1.8, 5 (75–80)	–	71.5±12.0, 2 (63–80)
GL	–	86.9, * (82.7–90.7)	86.8, 5 (84.17–89.45)	–	86.2, * (84.4–88.5)	86.3, 2 (86.3–86.3)
CBL	–	77.2, * (73.2–82.5)	80.4, 5 (78.33–83.04)	–	76.6, * (75.0–78.7)	79.6, 2 (79.0–86.3)
BL	–	68.8, * (66.0–72.4)	70.1, 5 (68.13–73.13)	–	70.0, * (67.0–73.0)	70.3, 2 (70.2–70.4)
LD	–	22.8, * (21.1–24.4)	23.0, 5 (22.2–24.5)	–	22.5, * (20.9–23.6)	22.2, 2 (22.0–22.4)
ZB	–	44.0, * (42.5–46.0)	43.0, 5 (42.2–43.9)	–	43.3, * (41.3–44.6)	42.7, 2 (42.1–43.3)
CB	–	34.2, * (33.0–35.5)	34.1, 5 (33.5–35.1)	–	34.3, * (33.8–34.9)	34.3, 2 (33.9–34.8)
LAB	–	10.4, * (9.6–11.7)	10.9, 5 (9.7–11.8)	–	10.7, * (10.0–11.2)	11.1, 2 (10.7–11.4)
BAB	–	7.6, * (7.2–8.2)	7.5, 5 (6.1–9.5)	–	7.5, * (7.0–8.0)	7.9, 2 (7.7–8.0)
BMF	–	7.9, * (7.0–8.7)	7.7, 5 (7.5–8.0)	–	7.7, * (6.3–8.6)	7.9, 2 (7.6–8.2)
LPB	–	7.9, * (7.0–9.0)	7.9, 5 (7.7–8.2)	–	8.2, * (7.2–8.9)	7.7, 2 (7.1–8.3)
LIF	–	20.4, * (19.0–22.0)	20.5, 5 (19.8–20.9)	–	20.6, * (19.5–21.3)	20.2, 2 (19.9–20.5)
BIF	–	8.5, * (7.5–9.1)	9.2, 5 (8.0–9.8)	–	8.7, * (7.9–9.5)	8.7, 2 (8.3–9.1)
NB	–	18.8, * (17.3–20.0)	17.9, 5 (16.8–18.8)	–	17.7, * (15.7–18.7)	17.5, 2 (17.3–17.8)
ML	–	66.3, * (62.0–71.1)	62.5, 5 (59.3–64.7)	–	68.1, * (66.1–70.3)	62.3, 2 (62.1–62.5)

TABLE 3. Factor loadings, eigenvalues and percentage of variance in principal component analysis for morphological characteristics of *Lepus melainus*, *L. mandshuricus* and *L. timidus*.

Variables	PC 1	PC 2	PC 3
GLS	0.974	0.045	0.069
LUTR	0.969	-0.082	-0.025
CBL	0.960	-0.036	0.143
BL	0.954	-0.042	0.118
LLTR	0.921	-0.183	-0.114
PPL	0.919	0.018	-0.062
LD	0.898	-0.058	0.024

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TABLE 3 (continued)

Variables	PC 1	PC 2	PC 3
ML	0.896	0.083	0.087
RSL	0.870	-0.111	0.111
PL	0.859	-0.237	0.333
CH	0.825	-0.134	-0.216
LIF	0.821	-0.123	0.058
BMF	0.810	0.039	-0.489
LLM	0.782	-0.154	0.062
IOB	0.736	-0.374	-0.391
BPB	0.731	0.504	0.121
LMJ	0.701	-0.199	0.426
LAB	0.641	0.587	0.117
CB	0.601	0.082	-0.388
LUM	0.506	-0.382	0.306
ZB	0.152	0.840	-0.154
BIF	0.528	0.645	0.235
NL	-0.071	0.600	0.569
LPB	-0.451	-0.507	0.646
Eigenvalues	14.264	2.852	1.907
Variance (%)	59.357	11.884	7.947

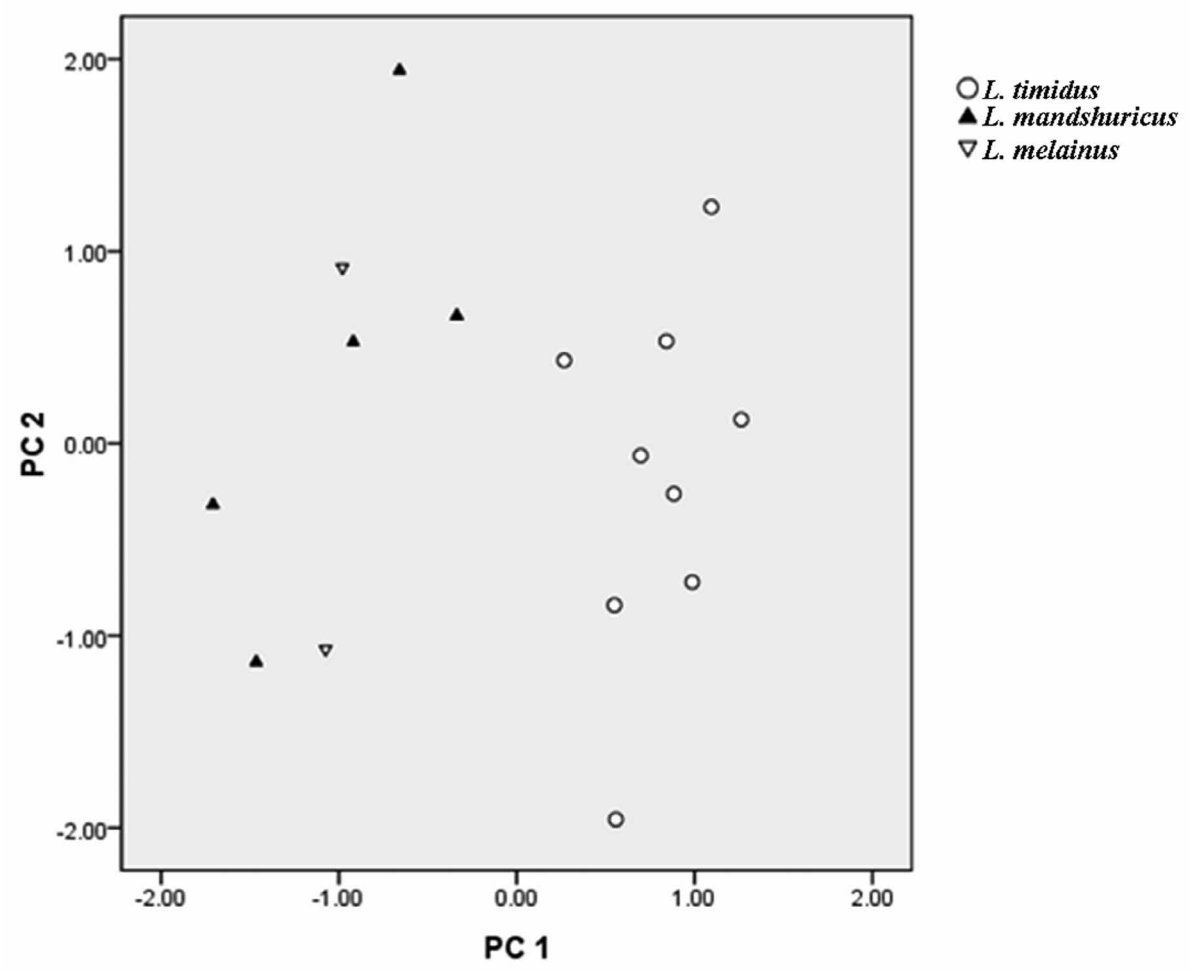

FIGURE 2. Plot of scores for principal components 1 and 2 of *Lepus melainus*, *L. mandshuricus*, and *L. timidus*. See able 3 for factor loadings, eigenvalues and percentage of variance of principal components.

Nuclear Gene Analyses

Sequence variation. Sequences of the TG fragment (466bp) were obtained successfully from 22 individual Chinese hares. Four museum skin samples (MA1, MA5, ME3 and ME4) failed to yield full-length sequences due to their poor quality. Five heterozygotes were detected in total, and there were 27 sequences in the final alignment. The average base frequencies were A=23.2%, G=25.2%, C=24.4%, and T=27.1%. There were 22 variable and 15 potentially parsimony-informative sites. Five transitions and one transversion were present. In comparison, we detected 16 heterozygotes from the 26 samples of the MGF locus. A total of 42 sequences had an aligned length of 592 bp. The average base frequencies for MGF were A=29.7%, G=17.2%, C =20.0% and T=33.0%. There were 21 variable and 16 potentially parsimony-informative sites. Three transitions were identified and no transversion was found.

Phylogenetic inference. Recombination tests for the two nuclear genes failed to detect signals of recombination. The best fitting model for maximum likelihood analysis in the TG gene data set was "K80+I" with the following parameter settings: TRatio=2.6659 and Pinvar=0.6619. The analysis based upon the TG gene, using sequences from 22 individuals, clustered *L. timidus* and *L. oiostolus* Hodgson together (Fig. 3). Furthermore, the TG gene clustered the sequences from *L. mandshuricus* and *L. melainus* into one group with high bootstrap support (88%). However, sequences of the TG gene failed to separate *L. hainanus* Swinhoe, *L. comus* Allen and *L. yarkandensis* Günther.

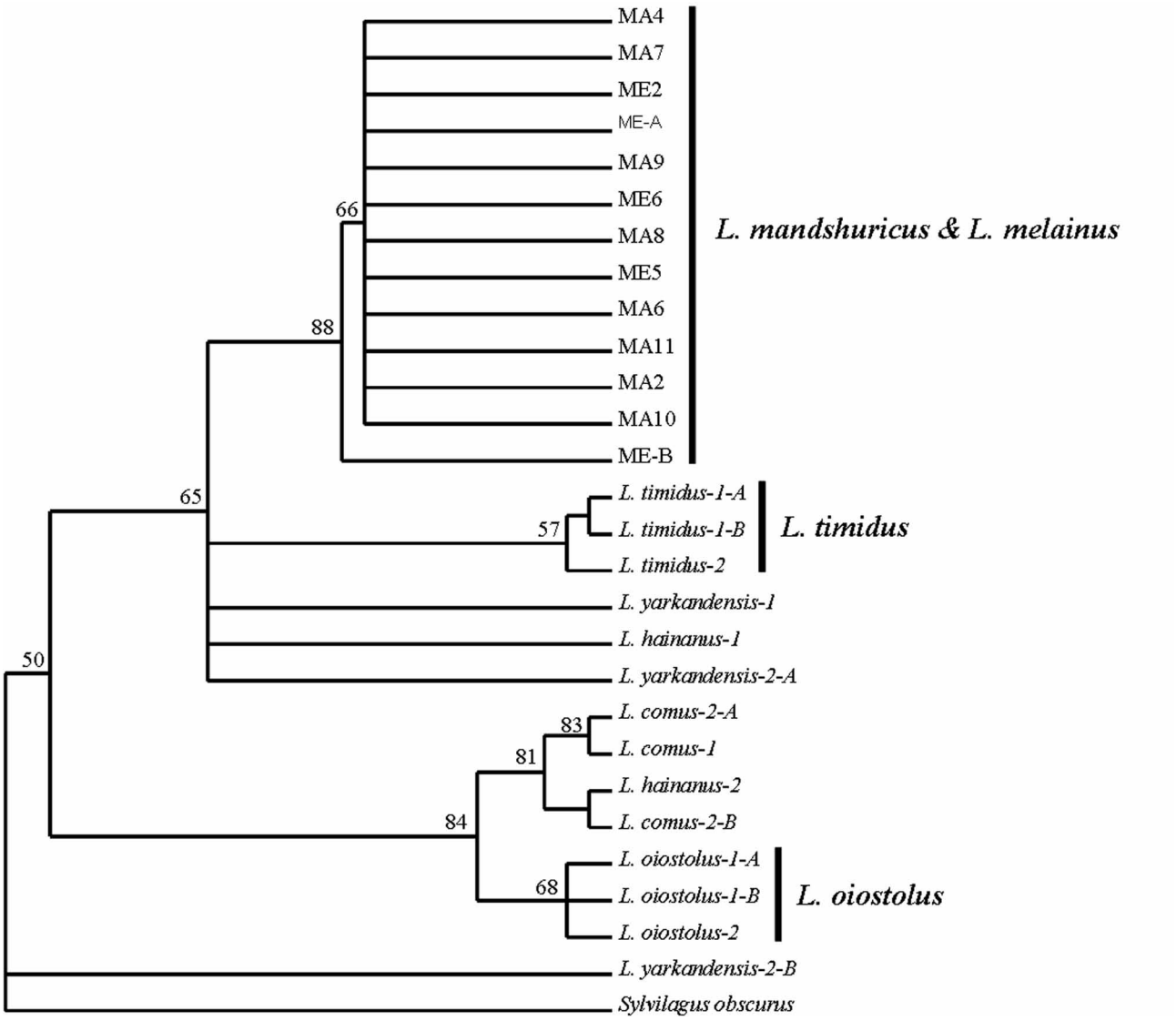

FIGURE 3. Maximum likelihood (ML) tree of the nuclear TG gene. Numbers above nodes represent bootstrap values. The sequence names correspond to the sample codes listed in Table 1. The letters A and B in the terminal names represent the two alleles of heterozygotic individuals.

The best fitting model for maximum likelihood analysis in the MGF gene data set was "K81uf+I" with the following parameter settings: Base frequencies = (0.2968, 0.1951 0.1765, 0.3316), Rate matrix = (1.0000, 2.5318, 0.0137), Pinvar = 0.7276. The MGF gene tree, based upon sequences from 26 individuals, served to separate *L. hainanus*, *L. comus*, *L. oiostolus*, *L. yarkandensis* and *L. timidus* from each other (Fig. 4). In addition, the MGF gene united *L. mandshuricus* and *L. melainus* into one group with high bootstrap support (86%).

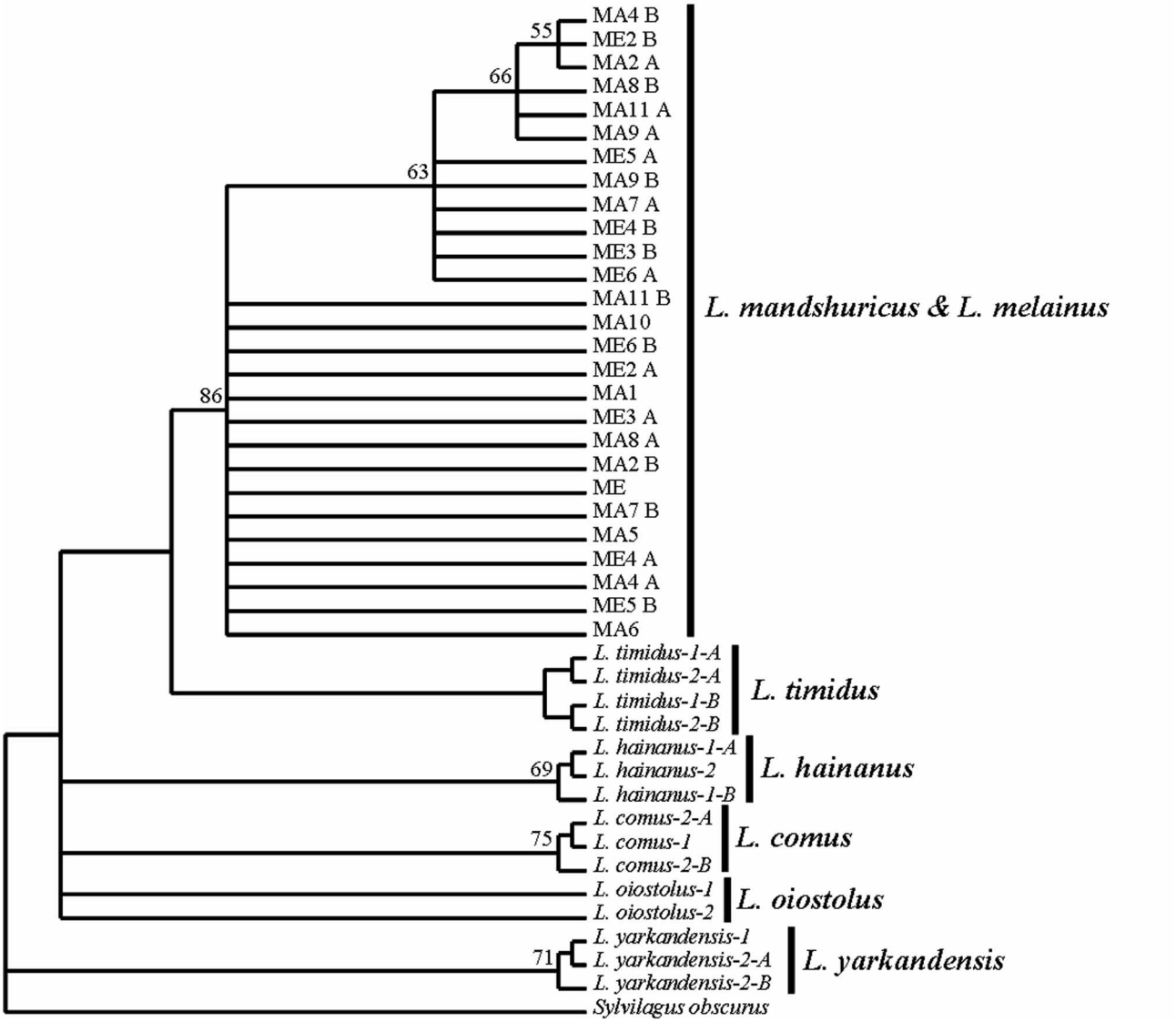

FIGURE 4. Maximum likelihood (ML) tree of the nuclear MGF gene. Numbers above nodes represent bootstrap values. The sequence names correspond to the sample codes listed in Table 1. The letters A and B in the terminal names represent the two alleles of heterozygotic individuals.

Discussion

Our study provided insights into the taxonomic status of *L. melainus*. The results strongly indicated that *L. melainus* is a junior synonym of *L. mandshuricus*; the former appears to be a melanistic morph of the latter species. This conclusion is inconsistent with the morphological analyses of Li & Luo (1979) and Flux & Angermann (1990), but supports the ecological analysis of Hoffmann (1993) and Hoffman & Smith (2005).

The original description of *L. melainus* diagnosed the species as having black pelage with a white speckle on the forehead and a re-entrant angle on the anterior upper premolars (Li & Luo 1979; Luo 1988). Two of our specimens (KIZ019694, KIZ019696) are typical *L. "melainus"* based on the color pattern. The dorsal pelage color in *L.*

mandshuricus varies from buffy brown to pure black on the head and body, with white venter, and buffy legs and inner ear surfaces. In comparison, the melanistic morphs have sparse and short white hairs scattered among the long blackish brown hairs, and some shorter, pure-white hairs mixed in the blackish brown hairs on the chest, which Li & Luo (1979) considered to represent individual variation. None of our specimens of *L. "melainus"* have a white speckle on the forehead; this condition, however, was observed in two specimens of *L. mandshuricus*.

Nevertheless, no other morphological characteristic separates the melanistic morphs from typical *L. mandshuricus*. All examined specimens of *L. "melainus"* and *L. mandshuricus* have a median re-entrant angle in the anterior upper premolars, except for one specimen of *L. "melainus"* that has a deep re-entrant angle on one side only. As Li & Luo (1979) reported, no other differences in external and cranial measurements separate *L. "melanius"* and *L. mandshuricus*, and this is reinforced by our statistical analyses.

We used two nuclear DNA fragments, TG and MGF, in order to avoid the influence of mtDNA introgression that is caused by interspecific hybridization, a common occurrence in hares and usually unaccompanied by nuclear introgression (Alves *et al*. 2003; Estonba *et al*. 2006; Freitas 2006; Melo-Ferreira *et al*. 2009). Although some sequence variation occurred among specimens of *L. mandshuricus* and *L. "melainus"*, both the TG and MGF gene trees found the two taxa in a single clade without reciprocal monophyly (Fig. 3 and Fig. 4). Given that no morphological or molecular differentiation corresponding to *L. mandshuricus* or *L. melainus* is found within this group, our results strongly corroborated the hypothesis that *L. melainus* should be best considered as a junior synonym of *L. mandshuricus*.

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