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Preliminary assessment of the genetic population structure of the enigmatic species *Laonastes aenigmamus* (Rodentia: Diatomyidae)

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Described in 2005, *Laonastes aenigmamus* is the only species of Diatomyidae. The known distribution of this rodent encompasses only the rugged mountains of the Khammouan karst in central Lao People's Democratic Republic. We used a sample of 52 specimens to survey population structure by sequencing 887 base pairs of the cytochrome-*b* gene. The overall haplotype diversity was low (0.789 ± 0.039 *SD*), with 14 haplotypes identified, whereas the nucleotide diversity was high (0.015 ± 0.008 *SD*). Phylogenetic and haplotypic network reconstructions revealed 3 well-supported and rather divergent lineages with mutational steps ranging from 28 to 32. Identified haplotype groups correspond to localities, suggesting that populations of *L. aenigmamus* are geographically structured. Mismatch distributions suggest population stability. An exact test for population differentiation confirms a significant level of differentiation. Taking into account human pressure increasingly threatening this ecosystem, we provide preliminary insights on the genetically discrete population structure of this enigmatic mammal species.

Key words: cytochrome *b*, Diatomyidae, *Laonastes aenigmamus*, population fragmentation, wildlife conservation

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In 1996 scientists announced the discovery of a new rodent species, called ''kha-nyou,'' in a rural market of Khammouan Province in Lao People's Democratic Republic where animals were being sold for food. This species was described formally in 2005 and named *Laonastes aenigmamus*, and it never has been observed outside of this restricted area (Jenkins et al. 2005). Its phylogenetic relationships are under debate. Initially, its anatomy looked remarkable enough to define a new genus, *Laonastes*, and a new family, Laonastidae (Jenkins et al. 2005), classified within Hystricognathi, a group of rodents now mostly represented in Africa and South America. Later, based on refutation of the hystricognath diagnosis and using comparison with Asiatic rodent fossils, Dawson et al. (2006) proposed *L. aenigmamus* to be a survivor of an extinct Asian family, Diatomyidae. According to the fossil record members of Diatomyidae occurred from 33.9 million years ago (mya; early Eocene) to 11.6 mya (late Miocene) and became extinct 11 mya. When classifying *L. aenigmamus* as a diatomyid, the authors considered it as the sole known remaining representative of this family; they proposed *L. aenigmamus* as a living fossil and a ''Lazarus'' taxon (Dawson et al. 2006; Huchon et al. 2007). The only other comparable example in mammals is *Dromiciops gliroides*, a living South American marsupial of the Microbiotheriidae, a family otherwise known only from Miocene deposits (Palma and Spotorno 1999). Finally, relying on various gene markers, Huchon et al. (2007) unambiguously placed *L. aenigmamus* as a sister group of the gundies (Ctenodactylidae). Ctenodactylid rodents originated in Asia, where they became extinct, and subsequently dispersed to Africa. Four genera including 5

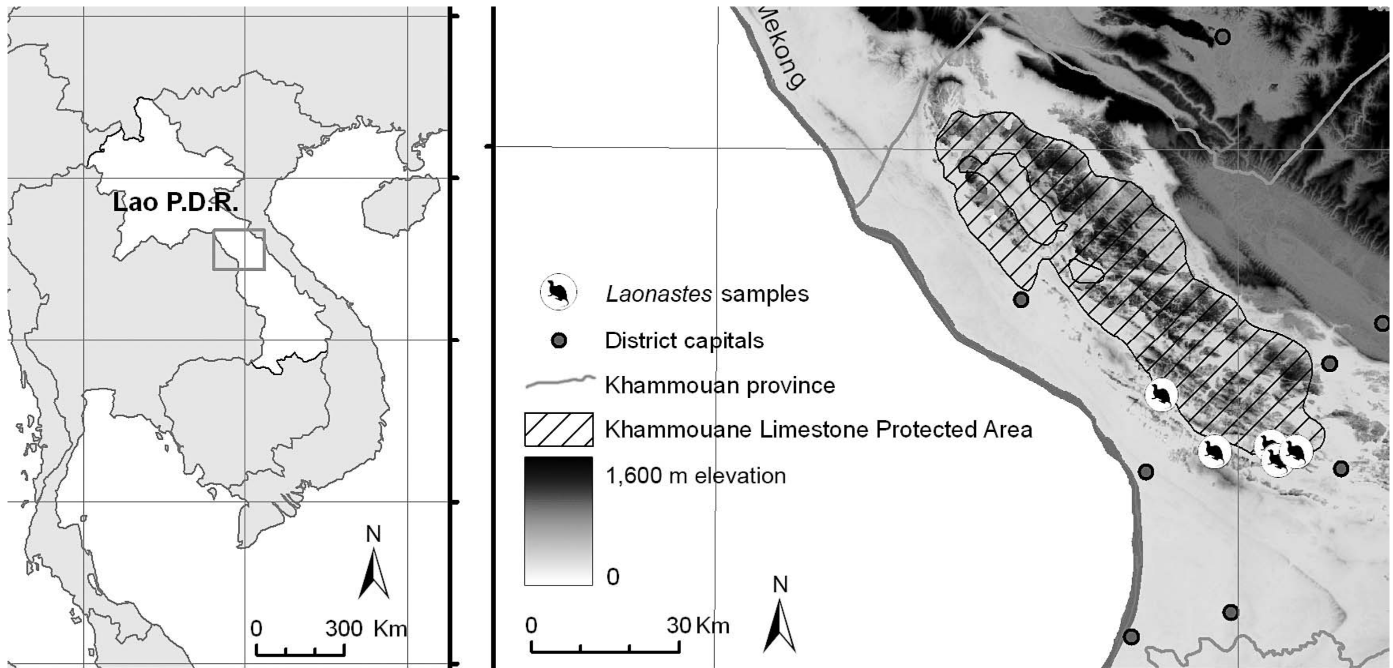

FIG. 1.—Location of Phou Hin Poun National Biodiversity Conservation Area and sampling localities for this study in Lao People's Democratic Republic.

species are currently living in desert rocky ecosystems on the periphery of the Sahara (Wilson and Reeder 2005).

Little is known about the ecology of kha-nyou because it rarely has been observed alive by scientists (Herbreteau et al. 2006). To date, this species has been recorded only from the southeastern edge of the Khammouan Limestone within the Phou Hin Poun National Biodiversity Conservation Area, Lao People's Democratic Republic (Jenkins et al. 2005; Fig. 1). Created in 1993, this area covers 1,580 km^2 and includes unique biotopes over spectacular karst topography (Robichaud et al. 2001). This geologic formation belongs to the Quy Dat limestone massif, which was derived from sedimentation in late Carboniferous seas in central Laos and north-central Vietnam (Duckworth et al. 1999; Fontaine and Workman 1997). This ecosystem is composed primarily of tower karst topography, with a dry, open xerophytic flora on the limestone and small pockets of mixed tropical, moist deciduous and evergreen rain forests in lowlands (Musser et al. 2005). Local residents inhabit lowland areas because steep tower karsts are unsuitable for human habitation. Large plain areas have been cleared for paddy cultivation, shifting agriculture, and roads.

In such a fragmented habitat hunters report that kha-nyou are caught exclusively in limestone karsts and never have been observed or captured in plains. This mostly inaccessible habitat seems to have provided *L. aenigmamus* with a rare niche, theoretically exempt from human pressure, where this docile animal has survived. In such a context we present here the 1st preliminary survey on the population structure of *L. aenigmamus*. To achieve this goal we sequenced 887 base pairs (bp) of the cytochrome-*b* gene (*Cytb*) from 52 specimens of *L. aenigmamus*. An understanding of its population genetics is a prerequisite to determining the status of this probably endangered species.

MATERIALS AND METHODS

Animal sampling and DNA extraction, amplification, and sequencing.—In February and March 2006, along the road from Thakek to Mahaxai in Khammouan Province (Lao People's Democratic Republic), 52 specimens of *L. aenigmamus* were processed. The specimens came only from village markets. Animal sampling was performed under the approval of the National Agriculture and Forestry Research Institute, Vientiane, Lao People's Democratic Republic. This research benefits from an official agreement between the French National Center for Scientific Research and the Lao National Agriculture and Forestry Research Institute to promote the conservation of *L. aenigmamus* (agreement 0963/PAF, ''Regulation on the Conservation of Kha-nyou (Nou Heen)'') following guidelines approved by the American Society of Mammalogists (Gannon et al. 2007). Specimens were obtained from different village markets where they were sold dead for food, as is done routinely in this area. Hunters indicated during interviews the karst where they trapped each animal. To minimize the impact of our sampling on local hunting, only toes were collected for this study. Samples were preserved in 100% ethanol at the Center for Biology and Management of Populations laboratory for subsequent DNA extractions. These animals came from 4 localities named according to the closest village (Appendix I; Fig. 2): 3 specimens were trapped by local hunters in Thamel (17°26′50.776″N, 104°57′19.959″E), 46 animals were from Na Dee (17°25′54.546″N, 105°04′14.484″E), 1 was from Phonlai (17°28′09.454″N, 105°03′31.401″E), and 2 were from Na Tung (17°26′48.472″N, 105°06′36.812″E). Tissue samples were preserved in 100% ethanol. DNA was extracted

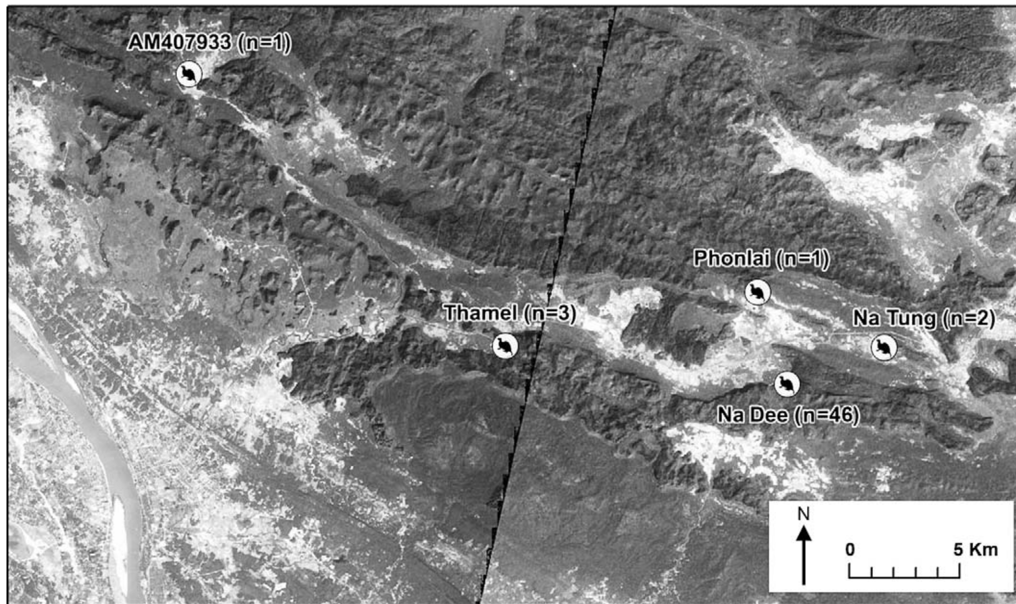

FIG. 2.—Aerial photograph showing the sampling localities of specimens of *Laonastes aenigmamus* in this study; Khammouan Limestone, Lao People's Democratic Republic. Constructed from a mosaic of Aster images (17 February 2004, 26 December 2004, and 7 March 2005) acquired from the United States Geological Survey.

using the DNeasy Tissue Kit (Qiagen, Germantown, Maryland). A portion of *Cytb* was amplified following standard procedures and using 2 newly designed primers: LaoFW (5′-ACCAATGACATGAAAAATCATCGTT-3′) and LaoREV (5′-ACATGAATYGGAGGYCAACCWG-3′). We used mitochondrial *Cytb* because it has been used widely as a molecular marker for investigating phylogenetic or phylogeographic relationships in rodents (Bradley and Baker 2001; Hewitt 2004). Polymerase chain reaction mixtures (25 μl) contained 1X buffer (10 mM of Tris-HCl, pH 8.3, 50 mM of KCl, and 2 mM of $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate, 1 μM of each primer, 0.5 unit of Taq polymerase (Promega, Madison, Wisconsin), and 20 ng of DNA. Polymerase chain reaction parameters were set as follows: 4 min at 94°C, 25 cycles of 30 s at 94°C, 1 min 30 s at 63°C for annealing, 1 min 30 s at 72°C for extension, and a final extension step of 10 min at 72°C. Polymerase chain reaction products were sent to Macrogen Inc. (Seoul, South Korea) for sequencing. Both strands were sequenced and edited in BioEdit version 5.09 (www.mbio.ncsu.edu/bioedit/bioedit.html); alignment was performed using CLUSTAL W (Thompson et al. 1994) and optimized manually.

Phylogenetic analyses.—Nucleotide sequences of 52 *Cytb* specimens were included with a total length of 887 bp. Three sequences from GenBank were added to our original data set (National Center for Biotechnology Information accession numbers DQ139932, 292 bp; DQ139933, 292 bp; and AM407933, 642 bp). For each specimen, the date of collection, locality, phylogenetic lineage (A–C), haplotype designation (Hd), and GenBank accession number are given (Appendix I). No geographic location was given for 2 specimens (DQ139932 and DQ139933—Jenkins et al. 2005). A 3rd (AM407933) was obtained from a specimen collected near Ban Mauang village, on the southeastern edge of Khammouan Limestone National Biodiversity Conservation Area (Huchon et al. 2007). *Ctenodactylus vali* (AJ389532) and *Massoutiera mzabi* (AJ389533) were used as outgroups following Huchon et al. (2007). Phylogenetic reconstructions were performed with various approaches, including neighbor-joining (Kimura 2-parameter [K2P] distances—Saitou and Nei 1987), maximum-parsimony (heuristic search and tree-bisection-reconnection branch-swapping algorithm—Fitch 1971), and maximum-likelihood approaches. All analyses were performed in PAUP* version 4.01 (Swofford 2000). The model of molecular evolution that best fits our data set was selected on the basis of the Akaike information criterion as implemented in Modeltest version 3.7 (Posada and Crandall 2001). Node support was assessed through bootstrap percentage (Felsenstein 1985). Finally, a Bayesian approach (MrBayes—Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2001; Yang and Rannala 1997) using the Metropolis-coupled Markov chain Monte Carlo algorithm (4 chains, burn-in value: 420,000 generations, convergence ~0.5%; 2,000,000 iterations; all other parameters set to default values) was used. Bayesian posterior probabilities were obtained from the 50% majority rule consensus of trees sampled every 20 generations after removing trees obtained before the chains reached an apparent plateau (burn-in determined by empirical checking of likelihood values). A minimum spanning network was constructed using the median-joining method implemented in Network version 4.2.0.1 (www.fluxus-engineering.com; Bandelt et al. 1999). Two sequences, DQ139932 and DQ139933 (Huchon et al. 2007), were excluded from the network data set due to the lack of global positioning system positions.

Population differentiation.—To further investigate population structure we conducted 2 sets of analyses: all sequences of *Laonastes* were included (52 specimens plus 3 GenBank

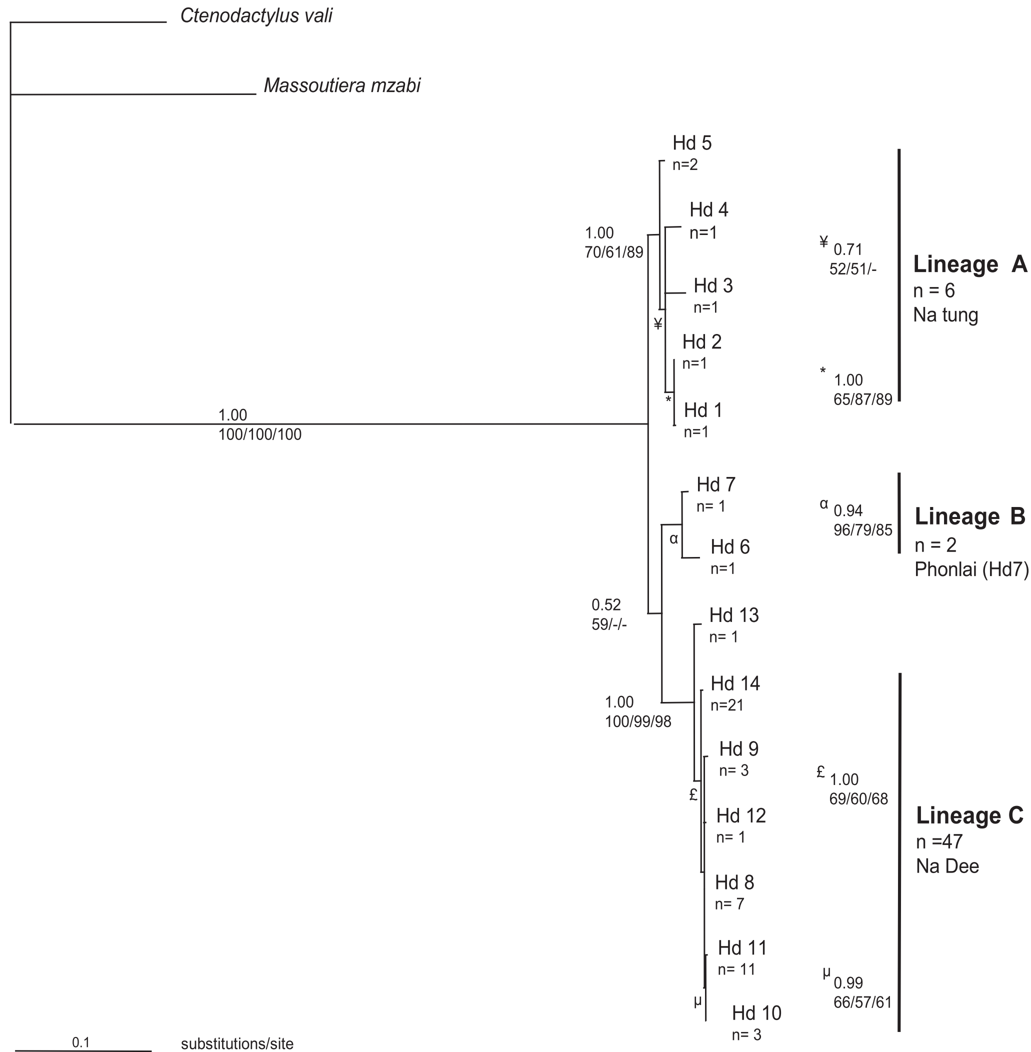

FIG. 3.—Maximum-likelihood tree of the 14 mitochondrial DNA haplotypes under study. Haplotypes are identified by GenBank accession numbers (Appendix I). Numbers of specimens (*n*) within each haplotype (Hd) and lineages (A, B, and C) are indicated. The sequences of *Ctenodactylus vali* and *Massoutiera mzabi* were used as outgroups. Support values for major nodes are indicated, with Bayesian posterior probabilities above and bootstrap values (neighbor-joining/maximum-parsimony/maximum-likelihood) below.

sequences); and we focused on the 3 haplotype groups that were recovered by phylogenetic methods (lineages A, B, and C; respectively 6, 2, and 47 specimens; Fig. 3), which appear to correspond to 3 localities. For each data set genetic characteristics were examined through haplotype (Hdi) and nucleotide (π) diversities (Nei 1987) and their standard deviations ($\pm$ *SD*—Tajima 1993). Fu's F_S statistic (Fu 1997), Tajima's *D* (Tajima 1989), and R_2 values (Ramos-Onsins and Rozas 2002) were calculated using both DnaSP version 4.50.3 (Rozas et al. 2003; http://www.ub.es/dnasp) and Arlequin 3.1 (Excoffier et al. 2005). R_2 was estimated to infer the long-term demographic history of the lineages. R_2 is based on the difference between the number of singleton mutations and average number of nucleotide differences among sequences within a population sample and represents a better test than other measures when sample sizes are small (Ramos-Onsins and Rozas 2002). Significance of R_2 was evaluated by comparing the observed value with a null distribution simulated under the neutral coalescent process with 10,000 replicates, using the empirical population sample size and observed number of segregating sites. Furthermore, mean distances between lineages were obtained using a K2P model of nucleotide substitution (500 bootstrap replicates; seed = 94,129) available in the MEGA 4.1 package (Tamura et al. 2007). The significance of the differences between the haplotype groups was investigated using a hierarchical analysis of molecular variance (AMOVA) and the exact statistic for population differentiation based on haplotype frequencies, both using Arlequin software. This statistic tests the hypothesis that the distribution of haplotypes between each pairwise comparison of populations is random and uses a 100,000-step Markov chain procedure (Raymond and Rousset 1995). Finally, to detect potential population expansion patterns we performed mismatch distribution analyses using all 52 sequences and on cluster C ($n = 47$) using DnaSP. We used the same program to calculate Harpending's (1994)

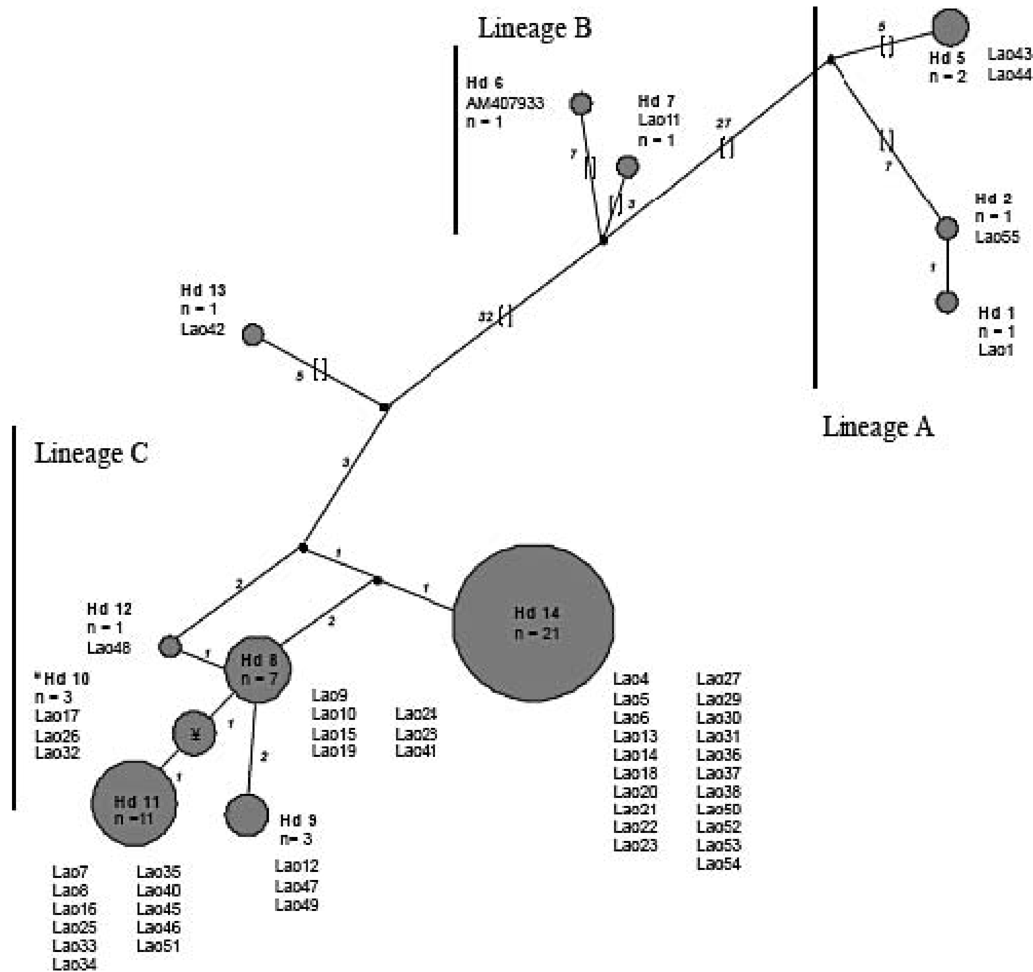

FIG. 4.—Minimum spanning network constructed using the 53 mitochondrial cytochrome-*b* sequences of *Laonastes aenigmamus*. Haplotypes (Hd) are clustered into 3 lineages: lineages A, B, and C. Branch lengths are proportional to the number of mutational steps (indicated above each branch). Numbers of haplotypes (*n*) within each haplogroup are indicated within the circles, and haplotypes are listed near the circles, whose areas are proportional to the number of individuals sharing the same haplotype.

raggedness index (Hri) to measure the smoothness of the observed distributions.

RESULTS

The 52 sequences of *L. aenigmamus* obtained could be aligned unambiguously with a total of 887 base positions; none of the sequences had stop codons indicative of nonfunctional nuclear copies. Considering the 52 sequences, 11 unique haplotypes were detected in the data set, and 5 of them were unique to individuals. The 3 reference sequences downloaded from GenBank were unique (Huchon et al. 2007), thus increasing the total number of sequences to 55 and unique haplotypes to 14 (Appendix I), with a total of 39 polymorphic sites. The highest-frequency haplotype (38%) was shared by 21 specimens (Fig. 4). The sequences have been deposited in GenBank (accession numbers FJ492865–FJ492875; Appendix I). Using Modeltest, the model of nucleotide substitution that best fits the data was K2P. Phylogenetic reconstructions performed with various approaches (neighbor-joining, maximum-parsimony, maximum-likelihood, and MrBayes) revealed 3 haplotype groups, named lineages A, B, and C. The maximum-likelihood tree is presented in Fig. 3. The minimum spanning network constructed using the median-joining method confirms that the haplotypes split into 3 lineages. They are separated by high genetic distances, ranging from 28 to 32 mutational steps (Fig. 4). A relationship was found between the haplotype groups identified and the geographic origins of the specimens, coming from Na Tung (lineage A), Na Dee (lineage C), Phonlai (lineage B), and Thamel (lineage A) sites. Two individuals have haplotypes (Hd5 and Hd13) with no matches on lineages identified (Fig. 4). Those 2 specimens came from Thamel. Across the 4 localities sampled the number of unique haplotypes ranged from 2 (Na Tung and Phonlai sites) to 6 (Na Dee site). When considering the 55 sequences, the number of segregating sites ranged from 11 to 39 among lineages; nucleotide diversities varied from 0.3319 to 1.7182; haplotype diversities varied from 0.7234 to 0.933 (Table 1). Fu's F_S statistic and Tajima's D values (Table 1) were not significant, supporting a nonexpanding population signal (Tajima 1989). None of the mismatch distribution tests (for the entire data matrix or

Table 1.—Genetic diversity statistics for the total data set and the 3 lineages A, B, and C with N_H: number of unique haplotypes; N_{site}: number of polymorphic sites; Hdi: haplotype (gene) diversity; π: nucleotide diversity; sim_F_S: simulated F_S; obs_F_S: observed F_S.

	n	N_H	N_{site}	$\pi \times 100 \pm SD$	Hdi $\pm$ *SD*	Fu's F_S Probability (sim_$F_S \leq$ obs_F_S)	Tajima's D[a]	R_2[b]
Total	55	14	39	1.523 ± 0.008	0.789 ± 0.039	3.972	−0.998	0.103
						$P = 0.895$	$P > 0.10$	$P = 0$
Lineage A	6	5	12	1.718 ± 0.011	0.933 ± 0.122	2.397	−0.295	0.205
						$P = 0.536$	$P > 0.10$	$P = 0$
Lineage B	2	2	11	1.713 ± 0.018	1.000 ± 0.500	—[c]	—	—
Lineage C	47	7	16	0.332 ± 0.002	0.723 ± 0.045	2.587	−0.551	0.108
						$P = 0.851$	$P > 0.10$	$P = 0$

[a] Significance ($P < 0.05$) assessed by assuming that D follows the beta distribution.
[b] Significance ($P < 0.05$) assessed by comparing observed R_2 with null distribution. Distribution of test statistic under null hypothesis of constant growth generated by 10,000 coalescent replicates simulated using the observed number of segregating sites and sample sizes for each population.
[c] — = not available.

lineage C) showed indications of population expansion (Figs. 5a and 5b). In addition, the observed mismatch distribution did not fit the predicted unimodal distribution of a population in expansion (Hri = 0.1478, $n = 55$, $P = 0.06$). Lineages A and B were not tested using this method because of their low sample numbers ($n = 6$ and 2, respectively).

Across the 55 sequences overall nucleotide diversity was high (0.0153), but haplotype diversity was low (0.789). The mean K2P distances (*Md*) between the 3 lineages were $Md_{CB} = 0.084 \pm 0.016$ *SD*, $Md_{AB} = 0.064 \pm 0.014$ *SD*, and $Md_{AC} = 0.053 \pm 0.012$ *SD*. In addition, AMOVA revealed that lineages were significantly ($F_{ST} = 0.921$; $P < 10^{-5}$) different, with 92% of the total variance being explained by interpopulation variability, whereas intrapopulation variability represents only 8% (1,023 permutations; 20,000 bootstrap). An exact test for population differentiation performed in Arlequin revealed that lineages A and B ($P = 0.00$) and lineages B and C ($P = 0.00267$) are significantly differentiated.

Discussion

Because *L. aenigmamus* rarely is observed alive in the wild, mainly due to the inaccessibility and ruggedness of its habitats, the 52 specimens were obtained only from dead animals sold for food in local markets. Therefore, it was not possible to use an intensive sampling scheme. Although our sampling is insufficient to fully comprehend the population structure of *L. aenigmamus*, we present here preliminary insights on the genetic diversity found.

The phylogenetic and network analyses showed that the population of *L. aenigmamus* is split into 3 well-supported lineages separated by high genetic distances. Moreover, lineages display average sequence (K2P) divergence values ranging from 6.4% (between haplotype groups A and B), to 5.3% (between B and C), to 8.4% (between A and C). In rodents, such levels of genetic differentiation are usually observed between subspecies and congeneric species (*Cryptomys* spp. [Faulkes et al. 2004], *Apodemus sylaticus* and *A. flavicollis* [Michaux et al. 2003, 2004], and *Mastomys* spp. [Mouline et al. 2008]). Bradley and Baker (2001) argued that generally in rodents intrapopulational, intrasubspecific, and intraspecific genetic K2P distances are 0–0.53%, 0–1.87%, and 0–6.29%, respectively, whereas interspecific distances range between 2.7% and 19.23%. In our study the K2P distances between lineages suggest intraspecific or interspecific values (Bradley and Baker 2001). Although it is premature to draw conclusions, our results call into question

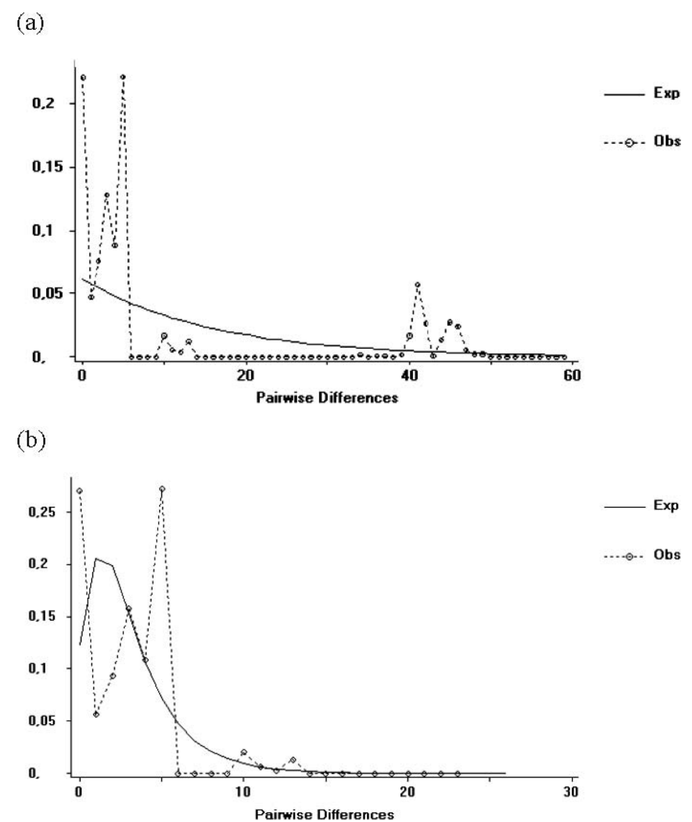

Fig. 5.—Mismatch distribution for mitochondrial DNA from a) all specimens ($n = 52$; expansion parameters: θ initial [θ before the population growth or decline] = 15.2, θ final [θ after the population growth or decline] = 1,000, and τ [the past demographic parameter] = 0), and b) lineage C ($n = 47$; θ initial = 2.0, θ final = 1,000, and τ = 1.0). Exp = expected frequency based on a growth–decline model of population size. Obs = observed frequency. Plots were generated with DnaSP version 4.50.3 (Rozas et al. 2003; http://www.ub.es/dnasp).

the taxonomic status of the kha-nyou. Further investigations should address this question using other molecular markers.

Beyond taxonomic aspects, genetic divergences in *L. aenigmamus* are of a magnitude usually associated with phylogeographic entities (Mouline et al. 2008; Riddle and Hafner 1999). According to hypotheses proposed in previous rodent studies, the genetic differentiation observed here suggests population fragmentation. The specimens were captured by hunters in 4 different areas, and the molecular lineages are mainly congruent with these locations. This could result from barriers to genetic exchange between populations, leading to the pronounced gaps between lineages. One hypothesis is that the tower karsts are isolated from each other such that kha-nyou individuals cannot disperse from one massif to another. Although little is known about migration abilities or ecologic plasticity of *L. aenigmamus*, it seems plausible that this animal is endemic to karstic mountains, being highly specialized ecologically. This hypothesis is reinforced by ecological observations and interviews with hunters that indicate specimens are caught exclusively in karsts and never observed in plains where anthropogenic pressure is high (Herbreteau et al. 2006). In such a context a preliminary hypothesis would be that populations are restricted to 1 karst or 1 group of connected karsts. Even if karsts are very close to each other, lowlands separating them could constitute real barriers for animals specialized for mountain habitat. Specimens from lineages B and C came from Phonlai and Na Dee sites, which are 2 distinct karsts separated by a plain about 9 km wide. Such habitat fragmentation has been highlighted as a primary factor in genetic structuring of wild populations (Harris 1984; Wilcove et al. 1986). The hypothesis of population fragmentation needs to be investigated rigorously by including nuclear markers and examining potential barriers to gene flow. Low haplotypic diversity and high nucleotide diversity also could be due to population isolation and subdivision in microhabitats. Such genetic differentiation is rare for a mammal. To our knowledge, very few other mammal species present such characteristics. Presently, the majority of mammal species having a restricted distribution are those that have undergone bottlenecks or reduction of their distribution, or both. These events generally have decreased their genetic diversity (orangutans [*Pongo abelii* and *P. pygmaeus*—Goosens et al. 2006], saiga antelope [*Saiga tatarica*—Kholodova et al. 2006], and fur seals [*Arctocephalus townsendi*—Weber et al. 2004]). Some other relict species or populations, well established and in restricted regions, also are characterized by a low level of genetic diversity (e.g., the endemic French Basque country population of the bank vole [*Myodes glareolus*], $\pi = 0.0024$—Deffontaine et al. 2009). However, some exceptions exist, such as the wood mouse (*A. sylvaticus*) population from Sicily (Michaux et al. 2003), which is characterized by surprisingly high levels of genetic diversity ($\pi = 0.013$). Nevertheless, these values do not exceed those observed for *L. aenigmamus.*

Another explanation for the observed levels of genetic differentiation might be biased sampling that favored the capture of closely related specimens. Given the difficulties of climbing karsts, hunters act in restricted areas and are prone to catch entire families of animals. However, the individuals that were used in the present study include specimens from 4 distinct sites and 2 independent studies (Huchon et al. 2007).

Finally, a short-term genetic bottleneck, which might have occurred in a large ancestral population of *L. aenigmamus*, could have induced a decrease in haplotype diversity. Such an event might not have impacted nucleotide diversity if populations were large enough to maintain genetic variation. However, the impact of ancient or recent bottlenecks on extant population structure is still controversial, depending on the species under study. For example, a bottleneck had little impact on genetic diversity in the fur seals *Arctocephalus gazella* and *A. tropicalis* (Wynen et al. 2000) or box turtle *Terrapene ornata* (Kuo and Janzen 2004) but had a significant impact on another fur seal species, *A. townsendi* (Weber et al. 2004), and golden monkeys (*Rhinopithecus roxellana*—Haipeng et al. 2003).

In conclusion, an ancient microdifferentiation of *L. aenigmamus*, which appeared after its isolation in the karst of the Khammouan region, seems to be the most-parsimonious hypothesis explaining the particular genetic and phylogeographic pattern of this species. Survey studies such as those based on capture–mark–recapture would be useful in evaluating such a hypothesis because they would indicate dispersal distances. In addition, from a genetic perspective, nuclear and rapidly evolving markers such as microsatellite loci should provide a more-precise picture of the situation at population levels, allowing estimates of gene flow, dispersal, and movement patterns of this species. The resulting data will be pivotal for a better understanding of the evolutionary biology and population structure of *L. aenigmamus* and are needed for an accurate conservation policy of this enigmatic mammal species.

Acknowledgments

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Literature Cited

Bandelt, H. J., P. Forster, and A. Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16:37–48.

Bradley, R. D., and R. J. Baker. 2001. A test of the genetic species concept: cytochrome-*b* sequences and mammals. Journal of Mammalogy 82:960–973.

Dawson, M. R., L. Marivaux, C. K. Li, K. C. Beard, and G. Métais. 2006. *Laonastes* and the ''Lazarus effect'' in recent mammals. Science 311:1456–1458.

Deffontaine, V., et al. 2009. A relic bank vole lineage in the French Basque country highlights the biogeographic history of the Pyrenean region in Europe. Molecular Ecology 19:2489–2502.

Duckworth, J. W., R. E. Salter, and K. Khounboline. 1999. Wildlife in Lao PDR: 1999 status report. IUCN—The World Conservation Union/Wildlife Conservation Society/Centre for Protected Areas and Watershed Management, Vientiane, Laos.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin version 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47–50.

Faulkes, C. G., E. Verheyen, W. Verheyen, J. U. M. Jarvis, and N. C. Bennett. 2004. Phylogeographical patterns of genetic divergence and speciation in African molerats (family: Bathyergidae). Molecular Ecology 13:613–629.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Fitch, W. M. 1971. Towards defining the course of evolution: minimum change for a specific tree topology. Systematic Zoology 20:406–416.

Fontaine, H., and D. R. Workman. 1997. Vietnam. Pp. 774–782 in Encyclopedia of European and Asian regional geology (E. M. Moores and R. W. Fairbridge, eds.). Chapman & Hall, London, United Kingdom.

Fu, Y. X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925.

Gannon, W. L., R. S. Sikes, and the Animal Care and Use Committee of the American Society of Mammalogists. 2007. Guidelines of the American Society of Mammalogists for the use of wild mammals in research. Journal of Mammalogy 88:809–823.

Goosens, B., L. Chikhi, M. Ancrenaz, I. Lackman-Ancrenaz, P. Andau, and M. W. Bruford. 2006. Genetic signature of anthropogenic collapse in orang-utans. PLoS Biology 4:25.

Haipeng, L., S. J. Meng, Z. M. Men, Y. X. Fu, and Y. P. Zhang. 2003. Genetic diversity and population history of golden monkeys (*Rhinopithecus roxellana*). Genetics 164:269–275.

Harpending, H. C. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Human Biology 66:591–600.

Harris, L. 1984. The fragmented forest. University of Chicago Press, Chicago, Illinois.

Herbreteau, V., V. Nicolas, R. Cornette, and J. P. Hugot. 2006. Une récente découverte au Laos: le Kha-nyou rongeur énigmatique. Le Courrier de la Nature 213:28–33.

Hewitt, G. M. 2004. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London, B. Biological Sciences 359:183–195.

Huchon, D., et al. 2007. Multiple molecular evidences for a living mammalian fossil. Proceedings of the National Academy of Sciences 18:7495–7499.

Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755.

Huelsenbeck, J. P., F. Ronquist, R. Nielsen, and J. P. Bollback. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294:2310–2314.

Jenkins, P. D., C. W. Kilpatrick, M. F. Robinson, and R. J. Timmins. 2005. Morphological and molecular investigations of a new family, genus and species of rodent (Mammalia: Rodentia: Hystricognatha) from Lao PDR. Systematics Biodiversity 4:419–454.

Kholodova, M. V., et al. 2006. Mitochondrial DNA variation and population structure of the critically endangered saiga antelope. Oryx 40:103–107.

Kuo, C. H., and F. J. Janzen. 2004. Genetic effects of a persistent bottleneck on a natural population of ornate box turtles (*Terrapene ornata*). Conservation Genetics 5:425–437.

Michaux, J. R., M. G. Filippucci, and R. Libois. 2004. Phylogeographic history of the yellow-necked fieldmouse (*Apodemus flavicollis*) in Europe and in the Near and Middle East. Molecular Phylogeny and Evolution 32:188–198.

Michaux, J. R., E. Magnanou, E. Paradis, C. Nieberding, and R. M. Libois. 2003. Mitochondrial phylogeography of the woodmouse (*Apodemus sylvaticus*) in the western Palearctic region. Molecular Ecology 12:685–697.

Mouline, K., et al. 2008. Phylogeography of a Sahelian rodents species *Mastomys huberti*: a Plio-Pleistocene story of emergence and colonization of humid habitats. Molecular Ecology 17:1036–1053.

Musser, G. G., A. L. Smith, M. Robinson, and D. P. Lunde. 2005. Description of a new genus and species of rodent (Murinae, Muridae, Rodentia) from the Khammouan Limestone National Biodiversity Conservation Area in Lao PDR. American Museum Novitates 3497:1–31.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Palma, R. E., and A. E. Spotorno. 1999. Molecular systematics of marsupials based on the rRNA 12S mitochondrial gene: the phylogeny of Didelphimorphia and of the living fossil microbiotheriid *Dromiciops gliroides* Thomas. Molecular Phylogenetics and Evolution 13:525–535.

Posada, D., and K. A. Crandall. 2001. Selecting the best-fit model of nucleotide substitution. Systematic Biology 50:580–601.

Ramos-Onsins, S., and J. Rozas. 2002. Statistical properties of new neutrality tests against population growth. Molecular Biology and Evolution 19:2092–2100.

Raymond, M., and F. Rousset. 1995. An exact test for population differentiation. Evolution 49:1280–1283.

Riddle, B. R., and D. J. Hafner. 1999. Species as units of analysis in ecology and biogeography: time to take the blinders off. Global Ecology and Biogeography 8:433–441.

Robichaud, W., C. W. Marsh, S. Southammakoth, and S. Khounthikoummane. 2001. Review of the national protected area system of Lao PDR. Lao–Swedish Forestry Programme and Department of Forestry, International Union for Conservation of Nature, Vientiane, Lao PDR.

Rozas, J., J. C. Sánchez-Delbarrio, X. Messegyer, and R. Rozas. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Swofford, D. L. 2000. PAUP* 4.0: phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tajima, F. 1993. Measurement of DNA polymorphism. Pp. 37–59 in Mechanisms of molecular evolution (N. Takahata and A. G. Clark, eds.). Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evoution 24:1596–1599.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22(22):4673–4680.

Weber, D. S., B. S. Stewart, and N. Lehman. 2004. Genetic consequences of a severe population bottleneck in the Guadalupe fur seal (*Arctocephalus townsendi*). Journal of Heredity 95:144–153.

Wilcove, D. S., C. H. McLellan, and A. P. Dobson. 1986. Habitat fragmentation in the temperate zone. Pp. 237–256 in Conservation biology: the science of scarcity and diversity (M. E. Soulé, ed.). Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.

Wilson, D. E., and D. M. Reeder (eds.). 2005. Mammal species of the world: a taxonomic and geographic reference. 3rd ed. Johns Hopkins University Press, Baltimore, Maryland.

Wynen, L. P., et al. 2000. Postsealing genetic variation and population structure of two species of fur seals (*Arctocephalus gazella* and *A. tropicalis*). Molecular Ecology 9:299–314.

Yang, Z., and B. Rannala. 1997. Bayesian phylogenetic inference using DNA sequences: a Markov chain Monte Carlo method. Molecular Biology and Evolution 14:717–724.

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Appendix I

List of specimens of *Laonastes aenigmamus* and associated data. For each specimen the date of collection (day/month/year), locality, phylogenetic lineage (A–C), haplotype designation (Hd) for cytochrome *b*, and GenBank accession number are given in parentheses (na = not available).

Lao1 (02/02/2006, Na Tung, A, Hd1, FJ492865); Lao4, Lao5, Lao6 (02/02/2006, Na Dee, C, Hd14, FJ492875); Lao7, Lao8 (02/02/2006, Na Dee, C, Hd11, FJ492872); Lao9, Lao10 (02/02/2006, Na Dee, C, Hd8, FJ492869); Lao11 (03/02/2006, Phonlai, B, Hd7, FJ492868); Lao12 (03/02/2006, Na Dee, C, Hd9, FJ492870); Lao13, Lao14 (03/02/2006, Na Dee, C, Hd14, FJ492875); Lao15 (03/02/2006, Na Dee, C, Hd8, FJ492869); Lao16 (03/02/2006, Na Dee, C, Hd11, FJ492872); Lao17 (03/02/2006, Na Dee, C, Hd10, FJ492871); Lao18 (03/02/2006, Na Dee, C, Hd14, FJ492875); Lao19 (03/02/2006, Na Dee, C, Hd8, FJ492869); Lao20, Lao21, Lao22, Lao23 (03/02/2006, Na Dee, C, Hd14, FJ492875); Lao24 (03/02/2006, Na Dee, C, Hd8, FJ492869); Lao25 (03/02/2006, Na Dee, C, Hd11, FJ492872); Lao26 (03/02/2006, Na Dee, C, Hd10, FJ492871); Lao27 (03/02/2006, Na Dee, C, Hd14, FJ492875); Lao28 (03/02/2006, Na Dee, C, Hd8, FJ492869); Lao29, Lao30, Lao31 (03/02/2006, Na Dee, C, Hd14, FJ492875); Lao32 (03/02/2006, Na Dee, C, Hd10, FJ492871); Lao33, Lao34, Lao35 (03/02/2006, Na Dee, C, Hd11, FJ492872); Lao36, Lao37, Lao38 (03/02/2006, Na Dee, C, Hd14, FJ492875); Lao40 (03/02/2006, Na Dee, C, Hd11, FJ492872); Lao41 (03/02/2006, Na Dee, C, Hd8, FJ492869); Lao42 (04/02/2006, Thamel, C, Hd13, FJ492874); Lao43, Lao44 (04/02/2006, Thamel, A, Hd5, FJ492867); Lao45 (04/02/2006, Na Dee, C, Hd11, FJ492872); Lao46 (04/02/2006, Na Dee, C, Hd11, FJ492872); Lao47 (04/02/2006, Na Dee, C, Hd9, FJ492870); Lao48 (04/02/2006, Na Dee, C, Hd12, FJ492873); Lao49 (04/02/2006, Na Dee, C, Hd9, FJ492870); Lao50 (04/02/2006, Na Dee, C, Hd14, FJ492875); Lao51(04/02/2006, Na Dee, C, Hd14, FJ492872); Lao52, Lao53, Lao54 (04/02/2006, Na Dee, C, Hd14, FJ492875); Lao55 (08/02/2006, Na Tung, A, Hd2, FJ492866); AM407933 (na, na, B, Hd6, AM407933); DQ139932 (na, na, A, Hd3, DQ139932); DQ139933 (na, na, A, Hd4, DQ139933).