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Low genetic diversity in the masked palm civet *Paguma larvata* (Viverridae)

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Abstract

The masked palm civet is distributed through south-east Asia, China and the Himalayas. Because of its potential role in the severe acute respiratory syndrome (SARS) epidemic, it has become important to gather information on this species, and notably to provide a tool to determine the origin of farm and market animals. For this purpose, we studied the genetic variability and the phylogeographic pattern of the masked palm civet *Paguma larvata*. First, two portions of mitochondrial genes, cytochrome *b* and the control region, were sequenced for a total of 76 individuals sampled from China, the Indochinese region and the Sundaic region. Results indicated a low genetic variability and suggested a lack of a phylogeographic structure in this species, which do not allow inferring the geographic origin of samples of unknown origin, although it is possible to distinguish individuals from China and the Sundaic region. This low variation is in contrast to the well-marked morphological differentiation between the populations in the Sundaic and Chinese–Indochinese regions. We also used five microsatellite loci to genotype 149 samples from two wild and four farmed populations in China, where the masked palm civet is farmed and where the SARS coronavirus was isolated. These analyses also showed a reduced variability in Chinese civets and showed that farmed populations did not exhibit a lower genetic diversity than wild populations, suggesting frequent introductions of wild individuals into farms.

Introduction

The masked palm civet *Paguma larvata* (Mammalia, Carnivora, Viverridae) is distributed in tropical and subtropical zones across the Himalayas, China, Indochina, the Malay Peninsula, Sumatra and Borneo (Jennings & Veron, 2009; Fig. 1). In China, it is mainly distributed in the southern regions, but it can also be found in Hubei, Shaanxi, Shanxi, Sichuan and Xizang provinces (Gao, 1987; Wang, 2003; Smith & Xie, 2008). The species has been introduced to Japan (Corbet & Hill, 1992).

There is no recent taxonomic revision of this species and no consensus on the validity of subspecies. Based mainly on facial pattern and body colour, up to 15 subspecies of masked palm civet have been recognized (Wozencraft, 2005), and up to nine in China (Jiang, Li & Zeng, 2003).

Although quite common in the original habitat, the masked palm civet may face a serious threat of local extinction due to habitat destruction and hunting (Sodhi *et al*., 2004; Steinmetz, Chutipong & Seuaturien, 2006). In China, the masked palm civet is considered a delicacy, and was sold in markets in Guandong, Guangxi and Hong Kong (Tan, 1989). People started eating masked palm civets in the 1950s and this increased to a larger scale after the 1990s (Jiang *et al*., 2003). Civet farming began to develop very quickly all over China and, by 2003, there were 660 farms in China, with a total of 40 000 civets (Jiang *et al*., 2003). In farms from the Hubei province, captive civets were reported as locally trapped, and newly trapped animals were regularly brought to the farms (pers. obs.). However, this may not be typical of other farms, where captive breeding may be the main source of animals.

Severe acute respiratory syndrome-associated coronavirus (SARS-like CoV) was first isolated from the masked palm civet from live market animals (Guan *et al*., 2003), but to our knowledge, it was never isolated from wild civets. Whether the infected market civets had been trapped in the wild or were from farms is not known. Their geographic origin was uncertain and, as wildlife trade from neighbouring countries to China is important (see Li & Li, 1997; Bell,

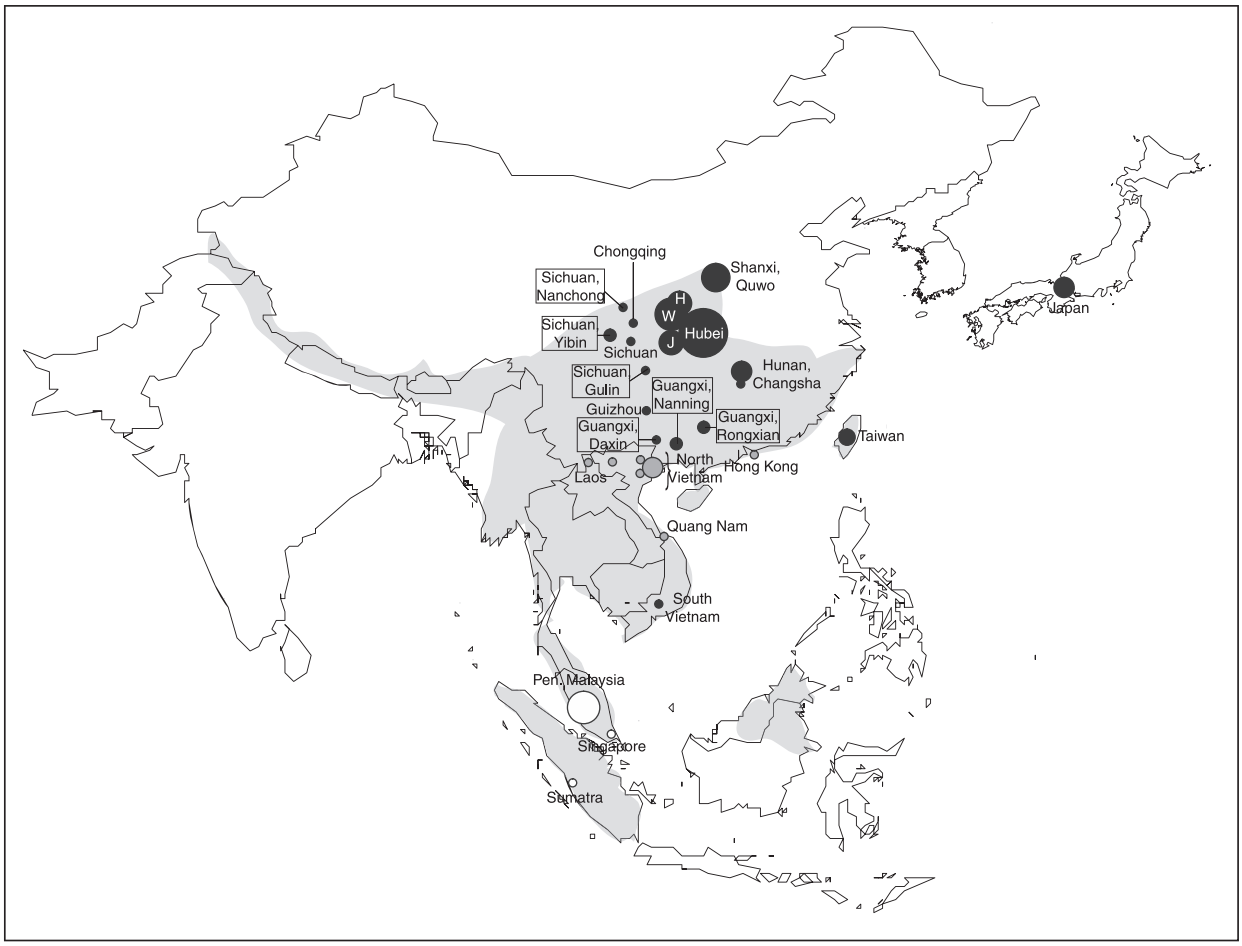

Figure 1 Natural distribution of the masked palm civet *Paguma larvata,* in grey. Molecular sampling is denoted on this map by the circles (whose size is proportional to the sampling's size). The colour of circles indicates the different genetic groups retrieved.

Robertson & Hunter, 2004), it is vital to develop a tool to identify their geographic origin. Although the SARS-like CoV has now been discovered in horseshoe bats as well (Li *et al.*, 2005*a*,*b*), Song *et al.* (2005) have described the role played by masked palm civets as intermediate hosts during the 2003/2004 outbreak in Guangdong. However, the origin of this virus is still debated (see Li *et al.*, 2005*a*,*b*; Janies *et al.*, 2008).

The purpose of the present study was to evaluate the genetic diversity in the masked palm civet and to provide a phylogeographic pattern in order to determine the geographic origin of farm and market civets. This may allow for future comparisons of the species' population structure with the occurrence of the virus in its population. For this purpose, we sequenced a portion of two mitochondrial genes, the cytochrome *b* (Cyt*b*) and the control region (CR), both shown to be suitable markers for carnivore species phylogeography (e.g. Li *et al.*, 2005*a*,*b*; Marmi *et al.*, 2006; Cosson *et al.*, 2007; Veron *et al.*, 2007), and analysed five polymorphic microsatellite markers in Chinese wild and farmed populations.

Materials and methods

Sample collection

For the mitochondrial study, 76 hair and tissue samples (see Table 1) were obtained from different parts of the range of the masked palm civet, from various sources (see Acknowledgments) or collected by the authors. Two individuals of common palm civet *Paradoxurus hermaphroditus* were used as an outgroup according to Patou *et al.* (2008). For the microsatellite study, the sampling was expanded to 149 individuals from China only. They were sampled from two wild and four farm populations, as follows: one wild population from the south-east of Sichuan province north-west of the Liangshan Mountain range (from Xihua University Museum) ($n = 17$); one wild population from Houhe National Nature Reserve in Hubei ($n = 49$); four farm populations: Nibin farm in Sichuan province ($n = 20$); Wufeng farm in Hubei province ($n = 23$); Quwo farm in Shanxi province ($n = 12$); Rongxian farm in Guangxi province ($n = 28$).

Table 1 List of the samples used in this study

Species	Locality	*n*=	Samples numbers	GenBank accession numbers Cyt*b*/Dloop	Origin
Paguma larvata	China, Sichuan, Yibin Farm	2	L-122; L-129	393/470; 394/471	Farm
P. larvata	China, Hunan, Changsha Farm	3	L-70; L-71; L-121	424/501; 425/502; 392/469	Farm
P. larvata	China, Hubei, Wufeng Farm	1	L-142	395/472	Farm
P. larvata	China, Shanxi, Quwo Farm	6	L-84; L-85; L-86; L-87; L-88; L-89	436/513; 437/514; 438/515; 439/516; 440/517; 441/518	Farm
P. larvata	China, Hubei, Wufeng N.R	6	L-76; L-115; L-116; L-167; L-168; L-169	428/505; 387/464; 388/465; 397/474; 398/475; 399/476	Field
P. larvata	China, Hubei, Jishou N.R	5	L-93; L-187; L-188; L-189; L-190	444/521; 405/482; 406/483; 407/484; 408/485	Field
P. larvata	China, Hubei, Houhe N.R.	5	L-75; L-79; L-114; L-119; L-182	427/504; 431/508; 386/463; 391/468; 402/479	Field
P. larvata	China, Hubei	11	L-77; L-78; L-110; L-111; L-112; L-117; L-118; L-170; L-171; L-184; L-185	429/506; 430/507; 382/459; 383/460; 384/461; 389/466; 390/467; 400/477; 401/478; 403/480; 404/481	Field
P. larvata	China, Chongqing	1	L-72	426/503	Field
P. larvata	China, Hunan, Changsha	1	L-94	445/522	Field
P. larvata	China, Hunan	1	L-81	433/510	Field
P. larvata	China, Guangxi, Rongxian	2	L-92; L-113	443/520; 385/462	Field
P. larvata	China, Guangxi, Daxin	1	L-90	442/519	Field
P. larvata	China, Guangxi, Nanning	2	L-82; L-83	434/511; 435/512	Field
P. larvata	China, Guizhou	1	L-80	432/509	Field
P. larvata	China, Sichuan, Gulin	1	L-97*	446/523	Field (Xihua University Museum)
P. larvata	China, Sichuan, Nanchong	1	L-108*	381/458	Field (Xihua University Museum)
P. larvata	China, Sichuan	1	L-99*	447/524	Field (Xihua University. Museum)
P. larvata	Taiwan	3	L-46; L-47; L-48	411/488; 412/489; 413/490	Field
P. larvata	South Vietnam	1	L-44	409/486	Captive
P. larvata	North Vietnam	3	L-49; C-292; C-317	414/491; 378/455; 379/456	Captive
P. larvata	North Vietnam, Cuc Phong National Park	1	L-51	416/493	Field
P. larvata	North Vietnam, Sa Phin	1	L-59	419/496	Field
P. larvata	North Vietnam	1	C-137	377/454	Field
P. larvata	Vietnam, Quang Nam Province	1	TC-113	452/529	Field
P. larvata	North Laos, Phou Dendin Cons. Area	1	L-16	396/473	Market
P. larvata	Peninsular Malaysia	2	L-45; L-66	410/487; 423/500	Zoo
P. larvata	Malaysia	4	L-55; L-57; L-60; L-61	417/494; 418/495; 420/497; 421/498	Zoo
P. larvata	Singapore	1	L-50	415/492	Zoo
P. larvata	Indonesia, Sumatra, Kerinci National Park	1	L-64	422/499	Field
P. larvata	Japan, Otsu, Shiga prefecture	1	C-399	380/457	Field

Table 1. Continued.

Species	Locality	*n*=	Samples numbers	GenBank accession numbers Cyt*b*/Dloop	Origin
P. larvata	Japan	3	ML-372; ML-373; ML-374	448/525; 449/526; 450/527	Field
P. larvata	Hong-Kong	1	ML-375	451/528	Field
Paradoxurus hermaphroditus	China, Guangxi, Daxin	1	L-91	453/531	Field
P. hermaphroditus	Philippines	1	FMNH, LRH 3167/C-199	-/530	Field

*Museums specimens. GenBank accessions numbers range from *EU910377* to *EU910531*, only the three last numbers are reported in the table.

DNA extraction and sequencing

The extraction and PCR procedures are the same as those described in Cosson *et al.* (2007). The 3′ end of Cyt*b* (*c.* 150 bp) and the CR from the 5′ end down to the second variable domain (*c.* 850 bp) were amplified using the following primers for amplification and sequencing: L126: 5′-CGA AGCATAATATTCCGAC-3′, H526: 5′-ACATACTGGG CAAGCACAG-3′ (this study), LCR1 and HCR2 (Cosson *et al.*, 2007); L400: 5′-TAATCGCTAGTCCCCATGAA-3′, H810: 5′-CTGCGTCGAGACCTTTACG-3′, LCR2: 5′-GT ACCTCTTCTCGCTCCGGG-3′, HSC2: 5′-TTGTTTGTG GGGTTTGGCAAGA-3′, L840: 5′-CATGTAGCTGGACT TATTCT-3′ and H1340: 5′-GTTCATATTTCACCATGGG GTTAAC-3′ (this study). The fragments amplified using these primers pairs overlap for about 100 bp, allowing for appropriate assembling of the CR sequence. PCR products were visualized in a 1.5% agarose gel to check for expected size of fragments and specific amplification. Positive PCR products were purified using the 'QiaQuick PCR Purification Kit' (Qiagen, Holden, Germany). Both strands (light and heavy) were sequenced in all cases using an automated DNA sequencer (ABI3100, Applied Biosystems, Foster city, CA, USA). Sequences were treated using Sequencher 4.14 (Gene Codes Corporation, Ann Harbor, MI, USA) and then the BLASTN 2.2.14 program (Altschul *et al.*, 1997) to check for contamination. Sequences were aligned in BioEdit (Hall, 1999).

Isolation of microsatellite markers and genotyping

Five polymorphic microsatellite markers were isolated and characterized (see Chen *et al.*, 2008), and were used for screening the two wild populations and four farm populations in China. For genotyping of the samples, PCR products were electrophoresed using an ABI 3100 DNA sequencer. The fragment length of the PCR products was determined with GeneScan (version 2.1, Applied Biosystems), and marker genotypes were assigned to the animals using Genotyper (version 2.5, Applied Biosystems).

Phylogenetic and phylogeographic analyses

Phylogenetic analyses were performed using Bayesian inference (BI). Parameters were set according to MrModeltest 2.0 (Nylander, 2004). BI analyses were performed with MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003) using 5 000 000 generations and five Metropolis-coupled Markov chains Monte Carlo (MCMCMC) with nodal support assessed by Bayesian posterior probabilities (BPP). The burn-in period was chosen after having explored output parameters with Tracer v 1.3 (Rambaut & Drummond, 2004). Analyses were run twice to ensure convergence of the results.

We used the Arlequin 2.0 software (Excoffier, Laval & Schneider, 2005) to carry out several analyses: (1) analysis of molecular variance (AMOVA, Excoffier, Smouse & Quattro, 1992) to test for genetic differentiation between putative geographical regions and (2) computation of haplotypic as well as nucleotidic diversity (π) for each of the groups.

In the absence of clear partitions retrieved by the phylogenetic analyses, AMOVA were performed for different putative geographical partitions to see which could better describe our data that is find the partition that minimizes the intra-population variability and maximizes the inter-population variability. Several geographical partitions were tested: (1) Group 1: 'China' versus Group 2: 'Indochinese region and allies' + 'Sundaic region'; (2) Group 1: 'China' + 'Sundaic region' versus Group 2: 'Indochinese region and allies'; (3) Group 1: 'China' + 'Indochinese region and allies' versus Group 2: 'Sundaic region'. As two Chinese haplogroups were suggested, partitions were also attempted with these two distinct groups but this did not improve the description of our data significantly. Specimens from Japan (C-399; ML-372; ML-373; ML-374), Hong Kong (ML-375) and Taiwan (L-46; L-47; L-48) were included in the same group as Vietnamese samples (C-317; L-51; C-292; L-59) as they share their haplotype.

A mismatch distribution of the pairwise differences among haplotypes was produced to test for the hypothesis of a sudden population expansion. Genetic distances among samples were computed using Mega v 3.1 (Kumar, Tamura & Nei, 2004) under the Kimura two parameters (K2P) model. The software TCS (Clement, Posada & Crandall, 2000) was used to reconstruct a haplotype network using the statistical parsimony method with a 95% confidence interval and gaps were treated as missing data. Some ambiguities (loops) were observed in the network. They were resolved using an empirical criterion derived from the coalescent theory that is the frequency, topological and geographical criteria identified by Pfenninger & Posada (2002).

Microsatellite analyses

Allele frequencies, the mean number of alleles per locus, observed heterozygosity (H_o) and expected heterozygosity (H_e) were computed using the Genetix software package (Belkhir *et al.*, 1996). Comparison of alleles' number between different sample sizes and measurement of the allelic richness (i.e. number of alleles independent of sample size) were performed using the program FSTAT v 2.9.3 (Goudet, 2001). A Hardy–Weinberg (HW) test for each locus in each population and global tests for all populations were performed with Genepop (Raymond & Rousset, 1995). The program FSTAT was also used to test for linkage disequilibrium (LD) between the polymorphic loci within each species and calculate two different measures of the genetic differentiation over subpopulations (F_{st}, Kimura & Crow, 1964; R_{st}, Kimura & Ohta, 1978) as well as inbreeding coefficients (F_{is}). The sequential Bonferroni correction was applied to derive significance levels for the analysis involving multiple comparisons (Rice & Gaines, 1989). The genetic divergence between each pair of six palm civet populations based on allele frequencies was calculated according to Nei's D_A genetic distance (Nei, Tajima & Tateno, 1983) using Population v 1.2.28 (http://www.cnrs-gif.fr). Genetic distances; trees were also constructed from these genetic distances values using the neighbour-joining clustering (Saitou & Nei, 1987). Bootstrap re-sampling ($n = 1000$) was performed to assess the robustness of dendrogram topologies. We also used a clustering method that does not use a spatial prior, as implemented in Structure 2.0 (Pritchard, Stephens & Donnelly, 2000), to detect the maximum number of populations (K), five independent runs of $K = 1$–6 were performed with 1×10^6 MCMC repeats and a burn-in of 1×10^6. K was identified using the maximal values of Ln $P_{(D)}$ (the posterior probability of the data for a given K). Moreover, the program Bottleneck v1.2.02 was used to test for heterozygosity excess and to determine whether allele distributions within populations had been affected by recent changes in the size of the two wild populations (Cornuet & Luikart, 1996; Luikart & Cornuet, 1998).

Results

Sequencing

We gathered portions of Cyt*b* and CR sequences for 76 specimens of *Pag. larvata* (GenBank accession numbers *EU910377–EU910531*; Table 1). For a few samples, we were unable to obtain the complete sequence owing to the DNA's quality. The 3′ end of the CR was composed of highly repeated dinucleotidic motifs (CA), whose number differed for each individual and was excluded from the analyses. We thus used a composite fragment located at [15164–15491] and [15713–16088] positions (plus a short fragment between these two boundaries) of the Javan mongoose complete mitochondrial genome (*Herpestes javanicus* AY873843, Penny & McLenachan, 2005) corresponding to the 3′ end region of the Cyt*b* (*c.* 150 bp), the first hyper-variable region (HVR1) and the central conserved domain of the CR. These parts of the CR did not give any alignment problems and were thus aligned by eye. The dataset included 811 characters and 26 parsimony-informative sites.

Phylogenetic analysis

The consensus tree provided in BI (model: HKY + I + G including two substitutions' categories and a burn-in period fixed at 3000 generations) yielded a polytomy with few supported clades (Fig. 2). The monophyly of *Pag. larvata* was strongly supported (BPP = 1.00), and a few clades were well supported: (1) wild civets from Hubei and Guangxi (L-77; L-111; L-171; L-90); (2) two wild civets from Hubei, (L-93; L-190); (3) three civets from Malaysia (L-55; L-61; L-66); (4) two others from Hunan Changsa Farm and from Chongqing (L-121; L-72); (5) two wild civets from Hubei Province (L-118; L-184); (6) two farmed civets, one from Hunan and one from Shanxi (L-86; L-142). Two civets (L-16; L-50) exhibited long branches in the phylogenetic tree and comparison of their genetic distances (K2P) confirmed this assertion.

Genetic variability

Among the three partitions tested for AMOVAs, partition (1) (China vs. the remaining regions) was the one that maximized the percentage of variation (*c.* 19.6%), explained by differences among groups while within-population variations still explained *c.* 70% of the total variation. These results suggested that the Chinese group was the most differentiated group among the three. This was also reflected by the pairwise F_{st} (F_{st} China/Sundaic = 0.035; F_{st} China/Indochina = 0.025; F_{st} Sundaic/Indochina = 0.014). Each of the three groups exhibited almost the same genetic characteristics (Table 2). Genetic distances ranged from 0 to 2.5% within the species, with a mean pairwise genetic distance of 0.57%. The mismatch distribution, including all specimens (Fig. 3), yielded a unimodal distribution corresponding to the expected distribution in case of a rapid population expansion.

Haplotype network

The 76 individuals corresponded to 50 haplotypes differing from 1 to 15 mutations (see Fig. 1 for the geographical distribution of these haplotypes). The haplotype network (Fig. 4) was rooted by haplotype L-97 (Sichuan province). Individuals from the Sundaic region were all connected to this haplotype, exhibiting a star-like structure. One of the Sundaic individuals (L-50, Singapore) was connected by a higher number of mutations ($n = 5$) to the root haplotype. Indochinese region and allies' (Japan, Hong Kong and Taiwan) individuals were nested within the network and were poorly differentiated from the Chinese individuals. We noticed one distinct Indochinese individual (L-16) from Northern Laos, differing from the root haplotype by four mutations. For the Chinese group, no geographical structure (either between the different Chinese provinces or

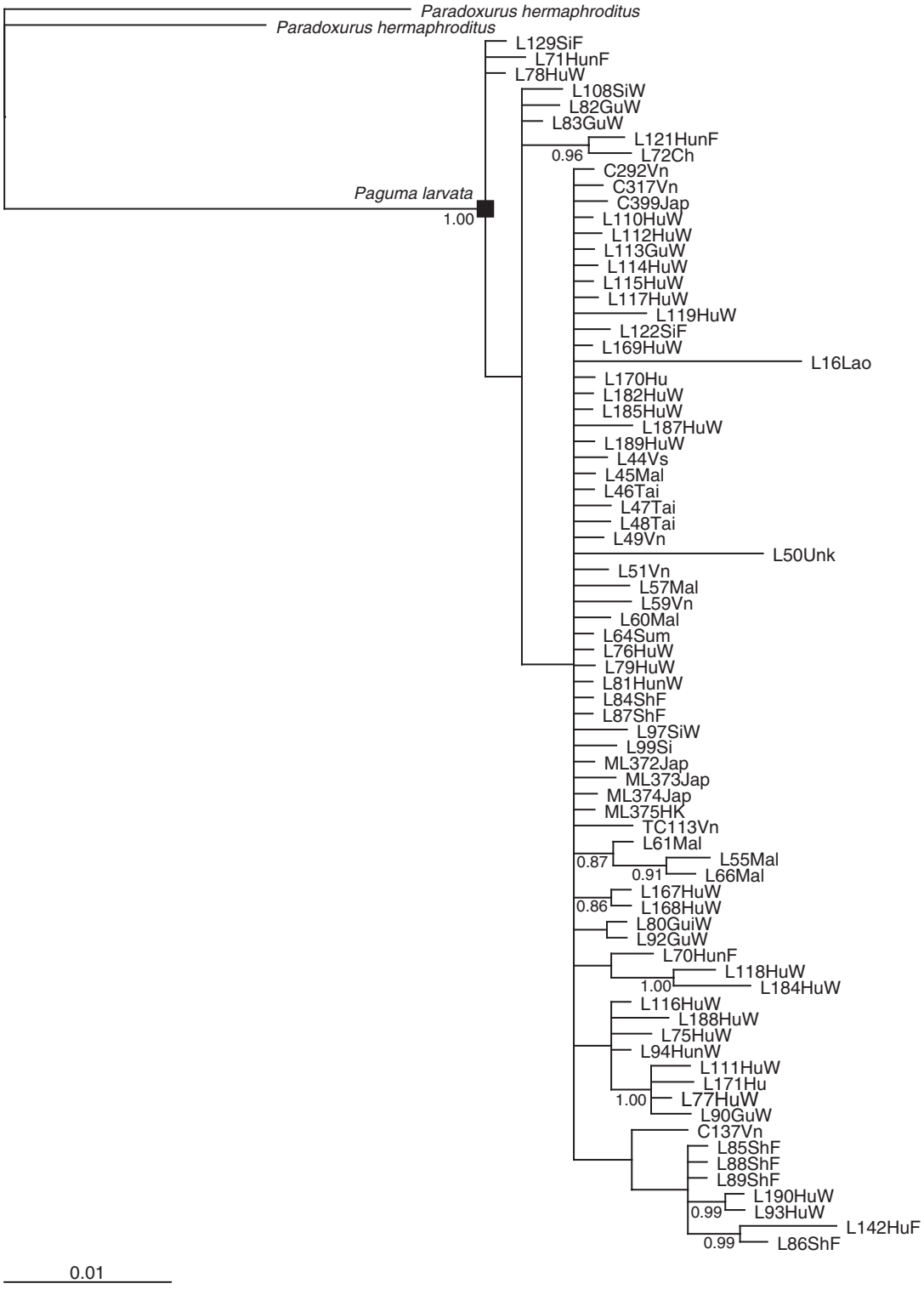

Figure 2 Phylogenetic tree obtained in Bayesian inference from the analysis of the Cyt*b*+CR dataset. Only BPP>0.8 are reported at the nodes. Individual's identification is followed by two or three letters referring to the locality of this specimen: Ch, Chongquing; Gu, Guangxi; Gui, Guizhou; Hu, Hubei; Hun, Hunan; Sh, Shanxi; Si, Sichuan; Tai, Taiwan; Jap, Japan; Vn, North Vietnam; Vs, South Vietnam; Mal, Malaysia; Lao, Laos; Sum, Sumatra. Letters W or F means 'wild' or 'farmed'. Cyt*b*, cytochrome *b*; CR, control region; BPP, Bayesian posterior probabilities.

Table 2 Genetic diversity indices of mtDNA for the two groups of masked palm civets

	China	Indochina and allies (Japan, Hong-Kong, Taiwan)	Sundaic region
n	52	16	8
Haplotypic diversity (*H*)	0.00 (±0.0056)	0.00 (±0.0018)	0.00 (±0.052)
Nucleotidic diversity (π)	0.0046 (±0.003)	0.0042 (±0.002)	0.0036 (±0.002)
Average number of nucleotide differences (*K*)	3.76	3.38	2.93
Genetic distances range (%)	0–1.3	0–1.9	0–1.4

In parentheses are reported the associated standard deviations. The genetic distances range refers to the minimum and maximum pairwise genetic distances (MEGA, K2P) computed within each group.
K2P, Kimura two parameters.

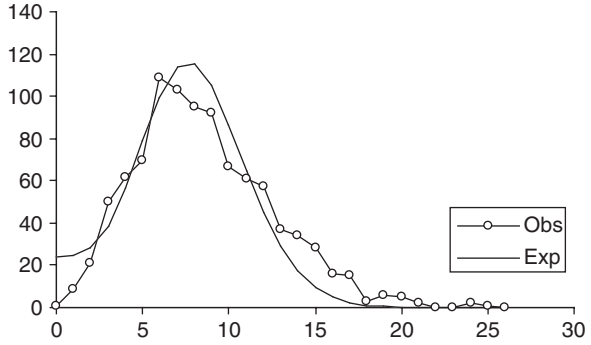

Figure 3 Mismatch distribution describing the frequency of pairwise differences among all mitochondrial haplotypes of masked palm civet. The expected distribution in case of a sudden population expansion is shown as a solid line and the observed distribution as a dotted line and empty circles.

between the different farms) was retrieved. An important result was the fact that individuals from the Sundaic region were not directly connected to the Chinese haplogroups, while individuals from the Indochinese region and allies were connected to both Chinese and Sundaic individuals.

Microsatellites

Microsatellite characterization and overall genetic variability

The five loci were highly polymorphic in *Pag. larvata* with 43 alleles observed and the allele number for each locus ranging from 3 (PC119) to 14 (PC58) (Table 3). Allele frequency distributions showed 9 rare (<5%) of a total of 43 alleles summed over loci, for a mean of 20.9%. The overall F_{is} values per locus ranged from −0.028 (PC119) to 0.204 (PC14), showing an overall F_{is} of 0.111 (Table 3). Pairwise F_{st} values for different civet populations ranged from −0.001 to 0.132, and no pairwise F_{st} value differed significantly from zero.

Genetic variability and HW equilibrium in Chinese populations

The Guangxi and Hubei farms and Hubei wild populations showed a high degree of genetic diversity (Table 4). In contrast, the lowest genetic diversity was observed in the Shanxi farm population and the Sichuan wild population in all measures of genetic diversity. The results of the HW equilibrium test (Table 5), computed at each locus showed that 26 of 30 locus–population combinations deviated from the HW equilibrium ($P<0.05$) after correction for multiple tests across populations and loci. Almost all the deviated cases are related to the positive F_{is}, indicating an HW equilibrium deviation in the direction of the heterozygote deficit. Significant LD was only found between loci PC14 and PC58 in the wild Hubei population.

Genetic distances

The genetic distances ranged from 0.042 (Hubei wild and farm populations) to 0.238 (Sichuan and Shanxi farm populations) (Table 6). The pairwise F_{st} ranged from −0.001 (Hubei wild and farm populations) to 0.132 (Shanxi and Sichuan farm populations) (Table 6). A genetic distance tree of the six civet populations (Fig. 5) was reconstructed and yielded three highly supported distinct lineages (bootstrap values of 100): (1) Hubei wild and farm populations were grouped together; (2) the Sichuan wild population and the Shanxi farm population clustered with the Guangxi farm population; (3) the Sichuan farm population was separate from these two branches. However, no structure was identified in the Bayesian clustering analysis (the maximal values of $\mathrm{Ln}\,P_{(D)} = -2034.9$ for a given $K = 1$) implemented in Structure.

Test for the genetic signature of population decline in two wild civet populations

According to the Wilcoxon sign-rank test, under the infinite allele model, stepwise mutation model and two-phase model, for the two wild populations neither of the *P*-values approached significance of heterozygote excess at the 5% level. The two wild populations exhibited a normal allele frequency distribution shape. Genetic tests for a population bottleneck performed on each of the two wild populations provided no significant genetic signature of populations' decline.

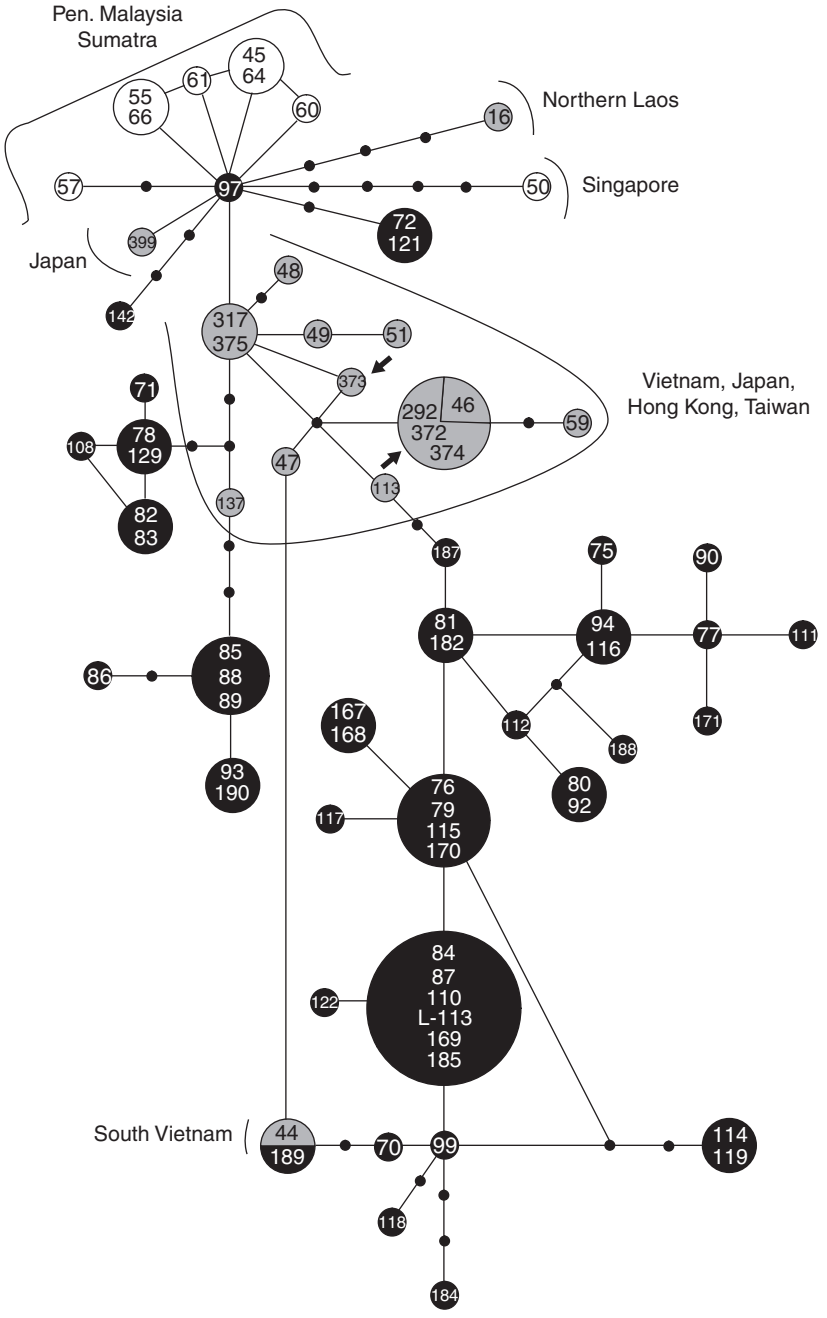

Figure 4 Mitochondrial haplotype network produced by TCS. In black, samples from China; in grey, samples from the Indochinese region (Vietnam, Laos) and allies (Japan, Taiwan, Hong Kong); in white, samples from the Sundaic region (Malaysia, Indonesia). Small circles: median vectors inferred by TCS. The size of the circles is proportional to the number of individuals sharing a haplotype. The arrows indicate the introduced populations (Japan).

Table 3 Characterization of the five microsatellites analysed in six palm civet populations

Locus	T_a	No. of alleles	Size r ange (bp)	F_{is}	F_{it}	F_{st}	R_{st}
PC14	50	11	184–212	0.204**	0.250**	0.058**	0.019
PC55	50	6	180–198	0.074	0.111	0.040	−0.015
PC119	55	3	111–115	−0.028**	0.010**	0.037	0.045
PC29	54	9	190–226	0.087	0.099	0.013**	−0.006
PC58	52	14	137–165	0.111	0.133	0.024	0.064
All loci	–	43	–	0.111	0.142	0.035	0.039

*The statistical significance of the test. F_{is} and F_{it} computed within different civet populations and in the total population, respectively.
*$P<0.05$. **$P<0.01$.

Discussion

Mitochondrial diversity in masked palm civets

The genetic divergence among the studied *Pag. larvata* populations was quite low (mean pairwise genetic distance *c.* 0.6%) and did not express any clear phylogeographic signal. The sequenced markers (Cyt*b* and CR) are usually variable in other carnivores (see studies by Davison *et al.*, 2001; Cosson *et al.*, 2007; Tchaicka *et al.*, 2007; but see Pérez-Haro *et al.*, 2005). Comparatively, for a similar CR fragment (HVRI, 346 bp), the variability was lower in the masked palm civet than in closely related species: the binturong *Arctictis binturong* (Cosson *et al.*, 2007) and the common palm civet *Par. hermaphroditus* (Patou *et al.*, unpubl. data). The genetic variability in masked palm civets was also explored in a nuclear gene (nuclear intron 7 of the β-fibrinogen; M.-L. Patou, unpubl. data), which also showed low variability (mean pairwise genetic distance *c.* 0.3%). Comparable results were obtained for the complete Cyt*b* gene by J. Chen (unpubl. data) and Masuda *et al.* (2008). Moreover, the microsatellite analyses did not contradict these findings.

These molecular results were in contrast with the marked morphological differences observed among the masked palm civet populations (Pocock, 1934*a*,*b*; G. Veron, pers. obs.), suggesting unlinked evolutionary mechanisms at the molecular and morphological levels in this species. In fact, the relationships between morphological and molecular evolution have long been debated (e.g. Omland, 1997; Bromham *et al.*, 2002). An empirical study on rodents (Renaud, Chevret & Michaux, 2007) concluded that correlations between morphological and molecular evolution can be lost when organisms had to answer to an ecological change. In this case, morphological changes may occur rapidly (as observed in specialized taxa; e.g. semi-aquatic genet *Genetta piscivora*, which exhibits very distinctive morphological features but small genetic distance values to other genets, Gaubert *et al.*, 2004). In the masked palm civet, two main morphotypes can be distinguished and their distribution limit appears to be located in southern Thailand by the study of a high number of museum specimens ($n = 225$; from AMNH, New York; BMNH, London; MCZ, Cambridge; MNHN, Paris; USNM, Washington; Institute of Zoology, Beijing; and observation of wild and captive individuals, G. Veron, pers. obs.). Specimens from Malaysia and Indonesia do not have the black and white head pattern, but have a yellowish face, and their body colour is dark brown. Specimens from the other parts of the range display the typical black and white head pattern with a conspicuous white longitudinal band on the nose, and the

Table 4 Number of samples, mean number of alleles per locus (MNA), mean allelic richness (A_R), expected heterozygosity (H_e) at Hardy–Weinberg equilibrium, observed heterozygosity (H_o), and inbreeding coefficient (F_{IS})

Population	Sample no.	MNA	A_R	H_e	H_o	F_{IS}
Sichuan wild	17	4.60	4.40	0.586	0.354	0.406*
Sichuan farm	20	5.20	4.83	0.601	0.544	0.097
Hubei farm	23	5.60	4.90	0.649	0.563	0.134
Hubei wild	48	6.60	4.93	0.638	0.586	0.083
Shanxi farm	12	3.60	3.58	0.468	0.409	0.131
Guangxi farm	28	6.00	5.22	0.662	0.656	0.008

Probability from multi-loci test that there is heterozygote excess.
*$P<0.05$ after correction for multiple tests.

Table 5 Expected heterozygosity and results of the exact tests for HW disequilibrium

Locus	Sichuan wild	Sichuan farm	Hubei farm	Hubei wild	Shanxi farm	Guangxi farm	Mean	SD	Total H_e
PC14	0.768	0.810*	0.788**	0.780**	0.540**	0.830*	0.753	0.097	0.815
PC55	0.602*	0.581*	0.702**	0.715**	0.757	0.602**	0.660	0.068	0.688
PC119	0.063**	0.142**	0.294**	0.307**	0.000**	0.366**	0.195	0.135	0.251
PC29	0.660**	0.612**	0.585**	0.533**	0.359**	0.647**	0.566	0.102	0.580
PC58	0.837**	0.859	0.874	0.858**	0.684*	0.863*	0.829	0.066	0.864
Mean	0.586	0.601	0.649	0.638	0.468	0.662	0.601	0.065	0.640
SD	0.274	0.253	0.202	0.198	0.271	0.179	0.230	0.038	0.218

*$P<0.05$. **$P<0.01$.
HW, Hardy–Weinberg.

Table 6 Nei's D_A genetic distance (below the diagonal) and mean F_{ST} estimates (above the diagonal) between each pair of six civet populations

Population	Sichuan wild	Sichuan farm	Hubei farm	Hubei wild	Shanxi farm	Shanxi wild
Sichuan wild	+	0.020	0.017	0.018	0.072	0.023
Sichuan farm	0.099	+	0.007	0.022	0.132	0.036
Hubei farm	0.116	0.076	+	−0.001	0.107	0.030
Hubei wild	0.109	0.070	0.042	+	0.064	0.035
Shanxi farm	0.196	0.238	0.219	0.160	+	0.119
Guangxi wild	0.146	0.125	0.127	0.108	0.220	+

Nei's D_A and pairwise F_{ST} are measures of genetic distance and genetic differentiation between populations, respectively. $P<0.05$ from a multilocus test that there is genetic differentiation in population pair.

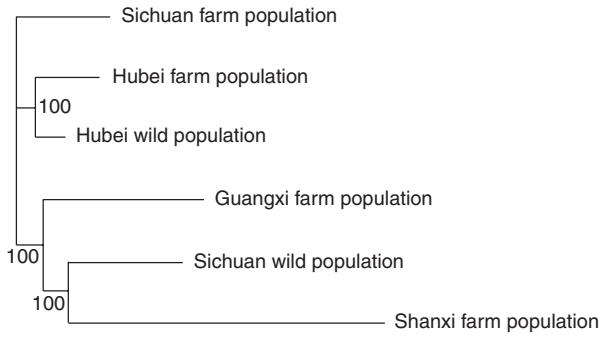

Figure 5 Neighbour-joining (NJ) analysis of six civet populations based on Nei's D_A genetic distance. The numbers at the nodes indicate bootstrap percentages for 1000 bootstraps.

body colour is lighter, varying from beige, yellowish and grey to light brown. The northern morphotype seems to display much more variability in the colour and head pattern than the southern one. The head and body length of the northern and southern morphotypes does overlap, while Hainan and Taiwan specimens are smaller (head and body length not exceeding 540 and 600 cm, respectively) than specimens from other populations (head and body length up to 790 cm).

However, despite the low variability and lack of structure, in our molecular results, none of the Sundaic individuals was found in the China–Indochina clade, suggesting a putative population subdivision. Three haplogroups of masked palm civets were suggested through our analyses: (1) Sundaland, (2) the Indochinese region, Japan, Hong Kong and Taiwan, poorly differentiated from (3) China. The Indochinese region and allies haplogroup was connected to the other two. In general, haplotypes were not highly differentiated from each other, with few missing intermediates inferred. The Sundaic haplogroup exhibited a high number of haplotypes relative to the low number of samples. Besides, one haplotype (L-50 from Singapore) appeared to be quite divergent. This suggested a putative variability in this region. Diverging haplotypes were also found in Vietnam and Northern Laos (L-16). Individuals from Taiwan, Hong Kong and Japan shared haplotypes with Vietnamese samples.

Taiwan and Hong Kong individuals were closely related to those from Vietnam, rather than to Chinese civets, as could have been expected. However, Taiwan and Hong Kong masked palm civets were not believed to have been introduced. The subspecies from Taiwan, whose most striking morphological difference is the smaller size, cannot be validated from our results.

In Japan, although several authors suggest that the masked palm civet may be native (Sasaki, 1991; Masuda *et al.*, 2008), there are clear mentions of introductions (e.g. Kuroda, 1955; Dobson, 1994). They took place in the 1940s, but the exact date is unclear (Kuroda, 1955; Nawa, 1965). Civets were believed to come from Taiwan or South China and brought as cage animals (Kuroda, 1955). In our study, except for one individual (C-399), which was only connected to the root haplotype, other samples from Japan shared haplotypes with Vietnam and Taiwan individuals or were closely linked to them. This result could support an Indochinese origin for the Japanese civets. Our results did not support a Sundaic or a Chinese origin.

The poor variability exhibited by the Chinese individuals as well as the shape of the mismatch distribution suggested a recent population expansion. On the other hand, Wilcoxon's test did not suggest the occurrence of a bottleneck. The expansion could be a natural northern expansion during Pleistocene as suggested by Tong (2006), or a more recent expansion due to human activity and notably by moving these animals for the farming and consumption purposes. Civet trade is important (Bell *et al.*, 2004) and farming in China was conducted on a large scale. We found no museum specimens ($n = 225$) having localities more northerly than 30°9'N. Corbet & Hill (1992) mapped the distribution up to 35°0'N in China; nowadays, the species is recorded as far north as 39°9'N (Zhang, 1997). The lack of genetic variability and of structure within the Chinese samples does not agree with the division of the Chinese populations into nine subspecies as suggested by Gao (1987) and Jiang *et al.* (2003), and, in fact, the morphological evidence does not support this subdivision either.

Comparison of the genetic diversity of wild and farmed masked palm civets in China

Our microsatellite analysis results suggested a reduced population subdivision across the six Chinese populations studied. The mean F_{st} value of 0.035 from all loci indicated that 96.5% of the genetic variation was explained by the differences among individuals and 3.5% by the differentiation among populations. The greatest genetic differentiation occurred between Shanxi and Sichuan farm populations ($F_{st} = 0.132$), which indicated that perhaps they originated from two distinct populations. The lowest genetic differentiation occurred between Hubei wild and farm populations ($F_{st} = -0.001$), which was consistent with the fact that all individuals in Hubei farm were cultured many years ago from the Hubei wild population. There was also reduced differentiation between the Hubei and the Sichuan wild populations ($F_{st} = 0.018$). We are uncertain whether this value was an accurate measure of the level of genetic differentiation between different civet subspecies. To fully address this issue, however, more rigorous investigation with a greater number of samples from different wild populations will be needed.

The results of this study showed that the farm civet populations did not exhibit a lower genetic diversity than the wild populations in China. Wild civet populations were an important genetic resource for farm populations. Many local farms introduced different wild populations and crossbred these with local populations, which resulted in a genetic exchange between different populations. Whether captive breeding was beneficial or harmful for native civet populations is unknown, but this practice may

accelerate the crossbreeding and genetic exchange between different populations.

Conclusions

The low genetic diversity of mitochondrial DNA and the lack of a structure among the masked palm civet populations did not allow inferring the geographic origin of an animal with unknown origin. Indeed, we found no molecular evidence supporting the monophyly of any particular clade matching with a peculiar farm, Chinese province or geographical region, and our results did not support any subspecies subdivision. However, three main haplogroups were put forward while the individuals from Malaysia and Indonesia exhibited a putatively higher genetic diversity. The Chinese farmed populations did not exhibit a lower genetic diversity than the Chinese wild populations, and this probably resulted from bringing in new wild individuals regularly. By these captures, the wild population may be at risk. The discovery of the SARS-like CoV in masked palm civets in the Southern China markets has led to the interdiction of the farming of this species. Whether this has not been replaced by increased local trapping and wildlife traffic is unknown, but should be considered.

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