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Comparison of mitochondrial genome sequences of pangolins (Mammalia, Pholidota)

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ABSTRACT

The complete mitochondrial genome was sequenced for three species of pangolins, *Manis javanica*, *Phataginus tricuspis*, and *Smutsia temminckii*, and comparisons were made with two other species, *Manis pentadactyla* and *Phataginus tetradactyla*. The genome of Manidae contains the 37 genes found in a typical mammalian genome, and the structure of the control region is highly conserved among species. In *Manis*, the overall base composition differs from that found in African genera. Phylogenetic analyses support the monophyly of the genera *Manis*, *Phataginus*, and *Smutsia*, as well as the basal division between Maninae and Smutsiinae. Comparisons with GenBank sequences reveal that the reference genomes of *M. pentadactyla* and *P. tetradactyla* (accession numbers NC_016008 and NC_004027) were sequenced from misidentified taxa, and that a new species of tree pangolin should be described in Gabon.

1. Introduction

Pangolins (Manidae) are scaly mammals, sole representatives of the order Pholidota. According to the morphological analysis of Gaudin et al. [1], which was based on 395 osteological characters, the eight extant species of pangolins (Pholidota, Manidae) can be classified into three genera: (1) *Manis* representing the four Asian species (*M. pentadactyla* – Chinese pangolin, *M. javanica* – Sunda pangolin, *M. culionensis* – Philippine pangolin, and *M. crassicaudata* – Indian pangolin); (2) *Phataginus* for the two species of African tree pangolins (*P. tricuspis* – White-bellied pangolin, and *P. tetradactyla* – Black-bellied pangolin); and (3) *Smutsia* representing the two species of African ground pangolins (*S. gigantea* – Giant pangolin, and *S. temminckii* – Temminck's pangolin). In addition, Gaudin et al. [1] suggested that the family Manidae can be divided into two subfamilies, the Asian subfamily Maninae containing only the single genus *Manis*, and the African subfamily Smutsiinae uniting the genera *Smutsia* and *Phataginus*. Although the two species of African tree pangolins are sometimes considered as monotypic genera, i.e., *Phataginus tricuspis* and *Uromanis tetradactyla* [2], we consider here only three genera of pangolins (*Manis*, *Phataginus* and *Smutsia*), as proposed by Gaudin et al. [1].

In this study, the complete mitochondrial genome was sequenced for the three genera of Pholidota, as represented by the species *M. javanica*, *P. tricuspis* and *S. temminckii*, and comparisons were performed with all mitochondrial data available in GenBank, including the genomes of *M. pentadactyla* [3] and *P. tetradactyla* [4], as well as the sequences from five markers, i.e., two protein-coding genes,

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subunit I of the cytochrome *c* oxidase (*COI*) and cytochrome *b* (*Cytb*), two rRNA genes (*12S* and *16S*), and the control region (*CR*). Our three main objectives were (1) to better understand the molecular evolution of the mitochondrial genome in the order Pholidota, (2) to examine phylogenetic relationships among pangolin species, and (3) to identify potential taxonomic inconsistencies at the species level.

2. Materials and methods

2.1. DNA extraction, amplification, and sequencing

Total DNA was extracted from tissue culture cells of *M. javanica* (Thailand, tissue code: 1999.508) and muscle samples of *P. tricuspis* (Gabon, tissue code: 2005.GLC14) and *S. temminckii* (Tanzania, tissue code: SUN106) using the QIAGEN DNeasy Tissue Kit (Qiagen, Hilden, Germany).

Twenty-five overlapping fragments of the mitochondrial genome were amplified by PCR using primers published in previous studies [5–8], as well as four new primers specially designed for this study: DLU400 or DLU400M1/12SL41 or 12SL200; 12SU1230/12SL2226M1LA; 12SU829/16SL518; 16SU365/16SL1056; 16SU946/N1L64; Uleu or U16S1421/LMet or LMet2; N1U840/N2L492; N2U354M2 or IleU/AsnL; TrpU/C1L705; UTyr or C1U246/C1L1017; C1U897M1/C2L15M1; SerU or SerUM1/A8L1; C2U603M4/C3L168; A6U654M1 or A6U654M2/GlyLM1; C3MANIU (5′- CAA TAT ATC AAT GAT GAC GTG A-3′)/LARGMANI (5′- GTT GAY TTG TTT GTG ATG CTC A-3′); C3U780 or C3U780M3/N4L366 or N4L366M2; UArg or UArgM1/N4L918M1 or

Table 1
Characteristics of the mitochondrial genome in three species of the family Manidae.

Name of gene	Location (Size in bp)			Strand	Start codon	Stop codon
	M. javanica[a]	*P. tricuspis*[b]	*S. temminckii*[c]			
tRNA-Phe	1–68 (68)	1–68 (68)	1–68 (68)	H		
12S ribosomal RNA (12S)	69–1028 (960)	69–1026 (958)	69–1027 (959)	H		
tRNA-Val	1029–1094 (66)	1027–1093 (67)	1028–1093 (66)	H		
16S ribosomal RNA (16S)	1095–2665 (1571)	1094–2648 (1555)	1094–2647 (1554)	H		
tRNA-Leu (UUR)	2666–2739 (74)	2649–2723 (75)	2648–2722 (75)	H		
NADH dehydrogenase subunit 1 (ND1)	2743–3699 (957)	2727–3683 (957)	2727–3683 (957)	H	ATG	TAA
tRNA-Ile	3699–3767 (69)	3683–3751 (69)	3683–3751 (69)	H		
tRNA-Gln	3765–3837 (73)	3749–3820 (72)	3749–3820 (72)	L		
tRNA-Met	3839–3907 (69)	3822–3890 (69)	3822–3890 (69)	H		
NADH dehydrogenase subunit 2 (ND2)	3908–4948 (1041)	3891–4934 (1044)	3891–4934 (1044)	H	ATA	TAG
tRNA-Trp	4947–5013 (67)	4933–4998 (66)	4933–5000 (68)	H		
tRNA-Ala	5017–5085 (69)	5002–5070 (69)	5003–5071 (69)	L		
tRNA-Asn	5087–5159 (73)	5072–5144 (73)	5073–5145 (73)	L		
Origin of L-strand replication (OL)	5159–5196 (38)	5144–5180 (37)	5145–5181 (37)			
tRNA-Cys	5193–5257 (65)	5177–5241 (65)	5178–5242 (65)	L		
tRNA-Tyr	5258–5324 (67)	5242–5308 (67)	5243–5309 (67)	L		
Cytochrome *c* oxidase subunit I (COI)	5326–6876 (1551)	5310–6863 (1554)	5311–6864 (1554)	H	ATG	AGA[a] AGG[b,c]
tRNA-Ser (UCN)	6872–6940 (69)	6855–6923 (69)	6856–6924 (69)	L		
tRNA-Asp	6948–7014 (67)	6931–6997 (67)	6932–6998 (67)	H		
Cytochrome *c* oxidase subunit II (COII)	7015–7698 (684)	6998–7681 (684)	6999–7682 (684)	H	ATG	TAA
tRNA-Lys	7701–7764 (64)	7685–7750 (66)	7685–7751 (67)	H		
ATP synthase F0 subunit 8 (ATP8)	7766–7969 (204)	7752–7958 (207)	7754–7954 (201)	H	ATG	TAA[a,c] TAG[b]
ATP synthase F0 subunit 6 (ATP6)	7927–8606 (681)	7913–8592 (681)	7915–8594 (681)	H	ATG	TAA
Cytochrome *c* oxidase subunit III (COIII)	8607–9391 (785)	8593–9377 (785)	8595–9379 (785)	H	ATG	TAN*
tRNA-Gly	9391–9459 (69)	9377–9444 (68)	9379–9447 (69)	H		
NADH dehydrogenase subunit 3 (ND3)	9460–9806 (347)	9445–9791 (347)	9448–9794 (347)	H	ATA	TAN*
tRNA-Arg	9807–9873 (67)	9792–9858 (67)	9795–9861 (67)	H		
NADH dehydrogenase subunit 4L (ND4L)	9874–10,170 (297)	9859–10,155 (297)	9862–10,158 (297)	H	ATG	TAA
NADH dehydrogenase subunit 4 (ND4)	10,164–11,541 (1378)	10,149–11,526 (1378)	10,152–11,529 (1378)	H	ATG	TNN*
tRNA-His	11,542–11,609 (68)	11527–11594 (68)	11530–11598 (69)	H		
tRNA-Ser (AGY)	11610–11668 (59)	11595–11653 (59)	11599–11657 (59)	H		
tRNA-Leu (CUN)	11670–11740 (71)	11654–11723 (70)	11657–11725 (69)	H		
NADH dehydrogenase subunit 5 (ND5)	11741–13561 (1821)	11724–13544 (1821)	11726–13546 (1821)	H	ATT	TAA
NADH dehydrogenase subunit 6 (ND6)	13545–14069 (525)	13528–14055 (528)	13530–14057 (528)	L	ATG	AGA[a] TAG[b,c]
tRNA-Glu	14070–14138 (69)	14056–14124 (69)	14058–14126 (69)	L		
Cytochrome *b* (Cytb)	14142–15281 (1140)	14128–15267 (1140)	14130–15269 (1140)	H	ATG	AGA
tRNA-Thr	15282–15348 (67)	15268–15336 (69)	15270–15338 (69)	H		
tRNA-Pro	15348–15413 (66)	15336–15401 (66)	15338–15403 (66)	L		
D-loop	15414–16576 (1163)	15402–16570 (1169)	15404–16559 (1156)			

*TAN and *TNN indicates the incomplete stop codon, where N or NN is the 5′ terminus of the adjacent tRNA gene, which presumably formed a stop codon by posttranscriptional polyadenylation.

[a] *Manis javanica*.

[b] *Phataginus tricuspis*.

[c] *Smutsia temminckii*.

N4L1071R; N4U681/Leu2L or Leu2LM1; Ser2U/N5L652 or N5L652M1; N5U501/N5L1214; N5U1146M5/N5L154M5; N6UT298 (5′- GGC TCA ATC AAA CTA TAC TTC CT-3′)/ CBLT298 (5′-AGT TTC ATC ATG CTG AGA TG-3′); N6RU102 or N6RU102M1/CBL402 or CBL402M1; GluMA/ProMA; CBU162 or U844/L482; and UThr13/L482. Amplifications were done in 20 μl using 3 μl of Buffer 10× with $MgCl_2$, 2 μl of dNTP (6.6 mM), 0.12 μl of Taq DNA polymerase (2.5 U, Qiagen, Hilden, Germany) and 0.75 μl of the two primers at 10 μM. The standard PCR conditions were as follows: 4 min at 94 °C; 5 cycles of denaturation/annealing/extension with 45 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C, followed by 10 min at 72 °C.

Both strands of PCR products were sequenced using Sanger sequencing on an ABI 3730 automated sequencer at the Centre national de séquençage (Genoscope) in Évry (France). The sequences were edited and assembled using Sequencher 5.1 (Gene Codes, Corp., Ann Arbor, MI, USA).

2.2. Phylogenetic analyses

The analyses based on complete mitochondrial genomes included five species of the order Pholidota: *M. javanica*, *M. pentadactyla*, *P. tetradactyla*, *P. tricuspis*, and *S. temminckii*. Four genera, representing different orders of Laurasiatheria, were used to root the Pholidota tree: *Boselaphus* (Cetartiodactyla), *Canis* (Carnivora), *Ceratotherium* (Perissodactyla), and *Rhinolophus* (Chiroptera). GenBank accession numbers of the DNA sequences are provided on the tree of Fig. 2. Complete mitochondrial genomes were aligned using MUSCLE [9] and then further adjusted by eye with Se-Al v2.0a11 [10]. All ambiguous regions, i.e., involving ambiguity in the position of gaps, were excluded from the analyses to avoid erroneous hypotheses of primary homology. For this reason, the control region was not included in the final alignment. The length of the reduced alignment is 14,926 nt (available upon request to AH). The phylogenetic tree was constructed using PAUP* version 4 [11] based on the Maximum Likelihood (ML) method and the GTR + I + G model selected by the Akaike information criterion under jModelTest 2 [12]. Bootstrap percentages (BP) were computed using 1000 replicates.

BLASTN searches were performed on NCBI [13] using the mtDNA genome of *P. tricuspis* as a query to extract all available mitochondrial sequences of Manidae. The sequences were filtered by mitochondrial markers (*COI*, *Cytb*, *12S*, *16S*, and *CR*) and aligned as explained above. Uncorrected nucleotide distances (D) were calculated in PAUP* for each datasets and the Neighbour-Joining (NJ) method was applied to reconstruct clusters of similar sequences, with BP computed after 1000 replicates.

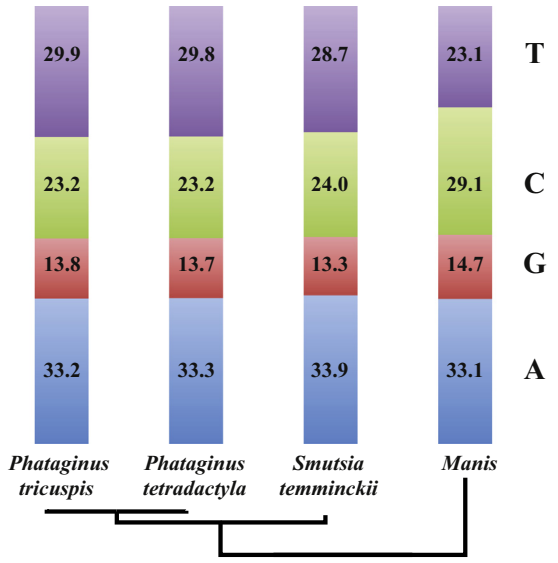

Fig. 1. (Colour online.) Overall base composition on the L-strand of the mitochondrial genome of five species of Manidae. The overall base composition of *M. javanica* was found to be identical to that of *M. pentadactyla*. The tree below was reconstructed using the NJ clustering method and a matrix of pairwise distances calculated by summing the percentage differences obtained for each of the four nucleotides A, G, C and T.

3. Results and discussion

3.1. Characteristics of the mitochondrial genome

Protein-coding, ribosomal and transfer RNA genes were identified by comparison with the two published genomes of Pholidota, *M. pentadactyla* [3] and *P. tetradactyla* [4]. The annotated sequences of *M. javanica*, *P. tricuspis*, and *S. temminckii* were deposited in GenBank under accession numbers KP306514-KP306516. The three new mitochondrial genomes of Manidae represent circular double stranded DNA molecules that range between 16,559 and 16,576 bp in length, and that contains the 37 genes described in Table 1. Among these genes, only *ND6* and eight tRNAs are encoded by the L-strand, whereas all the other genes are encoded by the H-strand. The gene

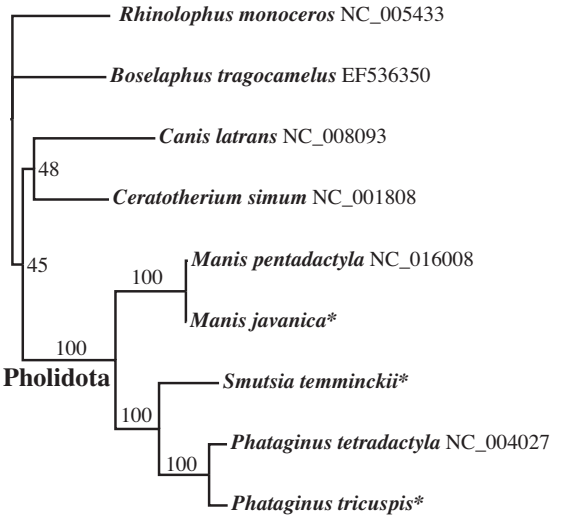

Fig. 2. Interspecific relationships within the order Pholidota as inferred from a DNA alignment of complete mitochondrial genomes. Species labelled with an asterisk (*) were sequenced for this study. Values on the branches are bootstrap percentages B.

arrangement is the same as that found in other mammalian genomes. All protein-coding genes of the mtDNA have a methionine start codon (ATR), except *ND5* (ATT). Most protein-coding genes appear to be terminated by TAR, although this stop codon is incomplete in the *COIII*, *ND3* and *ND4* genes. In *M. javanica*, the *COI*, *Cytb*, and *ND6* genes are terminated by AGA or AGG.

The control region is highly conserved among the five species of Manidae, both in structure and length (sequence length varies between 1156 and 1169 bp), with three tandem RS2 repeats of 78/79 bp in the L domain, which are located just after a partial RS2 motif, i.e., "YATGTA-TAATCGTGCAT" (pos. 15,453 in *M. javanica*).

The overall base composition of the mt genome showed marked differences between the two Asian species (*Manis*) and the three African species (*Phataginus* and *Smutsia*), with higher percentages of C (29.1 *vs.* 23.2–24.0%) and G nucleotides (14.7 *vs.* 13.3–13.8%), and lower percentages of A (33.1 *vs.* 33.2–33.9%) and T nucleotides (23.1 *vs.* 28.7–29.9%) (Fig. 1).

3.2. Phylogeny and taxonomy

Maximum Likelihood analyses based on an alignment of 14,926 bp resulted in a robust tree (Fig. 2) with maximal BP (100%) for interspecific relationships within the family Manidae. The results are in full agreement with the morphological study of Gaudin et al. [1]: the genera *Manis* and *Phataginus* are found to be monophyletic and the family Manidae is divided into two geographic groups corresponding to the Asian subfamily Maninae (*Manis*) and the African subfamily Smutsiinae (*Smutsia* and *Phataginus*). However, the terminal branch lengths of the two species of *Manis* are unexpectedly short in the ML tree (Fig. 2). Nucleotide distances indicate that the mitochondrial genome of *M. javanica* is 99% identical to that of

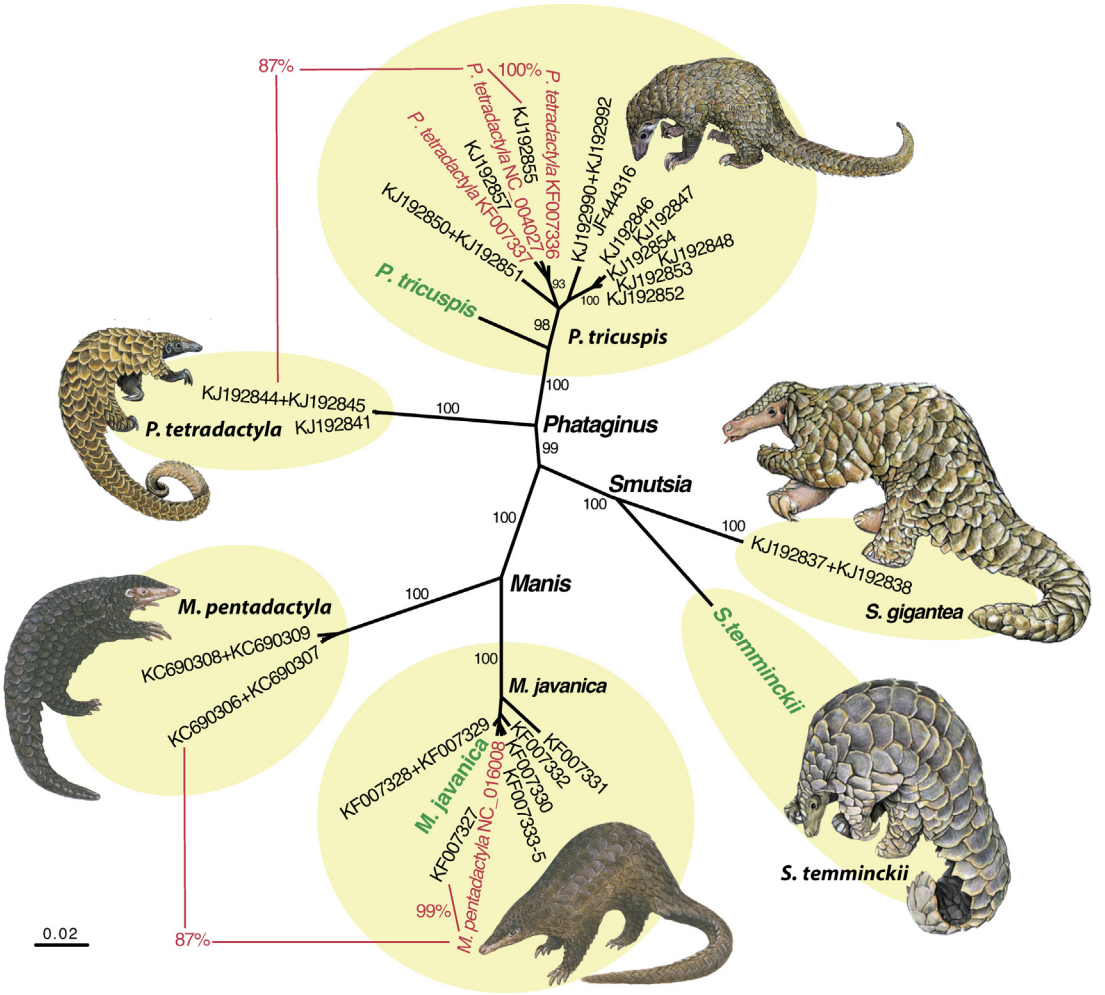

Fig. 3. (Colour online.) Neighbour-joining tree reconstructed from COI sequences. Genomes sequenced for this study are indicated in green. Sequences from GenBank written in red are suspected to have been misidentified. The percentages in red represent nucleotide distances. Values on the branches are bootstrap percentages more than 90%. The six clusters highlighted in yellow correspond to the six following species of pangolin: *Manis javanica*, *M. pentadactyla*, *Phataginus tricuspis*, *P. tetradactyla*, *Smutsia gigantea* and *S. temminckii*. Illustrations are modified from Francis [18] for *Manis* species, and from Kingdon and Hoffmann [15] for *Phataginus* and *Smutsia* species.

M. pentadactyla published by Qin et al. [3] (NC_016008), suggesting species misidentification for one of them. To solve this issue, the mitochondrial genomes were compared to all homologous fragments available for Pholidota in GenBank. Five mtDNA alignments were analysed using the NJ clustering method, i.e., *COI* (38 taxa and 658 nt), *Cytb* (35 taxa and 402 nt), *12S* (38 taxa and 395 nt), *16S* (25 taxa and 521 nt), and *CR* (56 taxa and 1264 nt). In Fig. 3, the *COI* tree shows the existence of six robust clusters (BP = 100) corresponding to the following species: *M. javanica*, *M. pentadactyla*, *P. tricuspis*, *P. tetradactyla*, *S. gigantea* and *S. temminckii*. On the one hand, the NC_016008 genome of *M. pentadactyla* fell into the *M. javanica* cluster ($0.2 < D < 3.5$ %), which is composed of representatives from China, Malaysia and Thailand. On the other hand, the NC_016008 genome appears to be highly divergent from *COI* sequences of *M. pentadactyla* from Taiwan and China (provinces of GuangDong, Hainan, and Hunan) ($13.2 < D < 13.7$ %). Similar results were found for three other markers (*Cytb*, *12S* and *CR*) using mitochondrial sequences produced by independent teams (Appendices 1, 2 and 4). The analyses suggest, therefore, that the NC_016008 genome published in Qin et al. [3] belongs to *M. javanica* rather than *M. pentadactyla*. However, the specimen was collected from Guangxi, a Chinese province where *M. javanica* is not currently recorded (Fig. 4). Accordingly, two hypotheses can be proposed to explain the presence of *M. javanica* in Guangxi: (1) the specimen was not native to Guangxi, but was imported in China from a Southeast Asian country (Vietnam, Lao PDR, Myanmar, Cambodia, Malaysia, Thailand or Indonesia) to satisfy the persistent demand for meat and scales used in traditional medicines [14]; (2) alternatively, the species *M. javanica* may also occur in sympatry with *M. pentadactyla* in the Guangxi province, implying that the geographic range currently provided by the IUCN [2] for *M. javanica* is incomplete. To resolve this and to ensure that optimal conservation strategies are in place, the possible occurrence of *M. javanica* in Guangxi should be reassessed urgently.

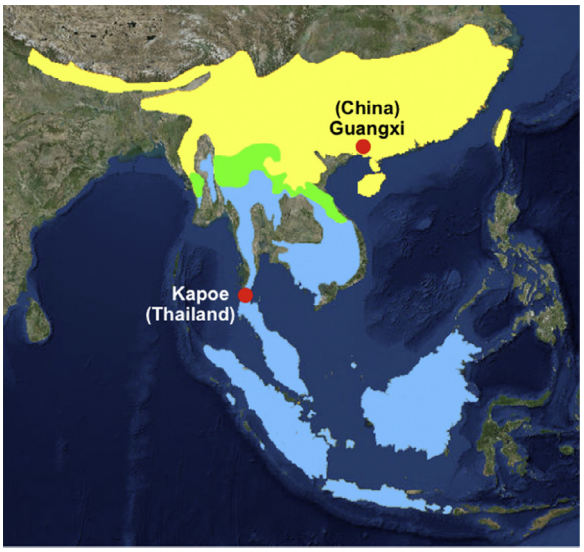

Fig. 4. (Colour online.) Geographic distributions of *Manis pentadactyla* (in yellow) and *M. javanica* (in blue) (modified from the IUCN [2]). The overlapping ranges are represented in green. The complete mitochondrial genome of *M. javanica* was sequenced from an individual collected in Kapoe district (Thailand), whereas the NC_016008 genome of *M. pentadactyla* published by Qin et al. [3] came from the Guangxi province of China.

A similar problem was found for the mitochondrial genome of *P. tetradactyla* published by Arnason et al. [4]. Indeed, the NC_004027 genome is highly divergent from mitochondrial sequences of *P. tetradactyla* produced in different labs (*COI*: 13%; *Cytb*: 15%; *12S* and *16S*: 6%), whereas it shares 99–100% of identity with *COI*, *Cytb*, *12S*, and *16S* sequences of *P. tricuspis* obtained by independent teams (Fig. 3 and Appendices 1–3). Here again, the most likely hypothesis involves species misidentification of the specimen sequenced by Arnason et al. [4].

Within *P. tricuspis*, the analyses of mitochondrial data revealed high nucleotide divergence between the Gabonese pangolin sequenced for this study and the specimens collected in Ghana, Nigeria and Cameroon: 5.9–6.7% for *COI*, 8.0–9.5% for *Cytb*, 1.5–2.6% for *12S*, 2.4–3.3% for *16S* (Fig. 3 and Appendices 1–3). Given that mitochondrial markers are available for all species of pangolin currently recognized in Africa, and that the holotype of *P. tricuspis* was described from Guinea in West Africa [15], these comparisons suggest the existence of a new species of tree pangolin in Gabon. This finding could be consistent with recent studies supporting the existence of a strong biogeographic barrier (named *charnière climatique*) between Cameroon and Gabon for both animals and plants endemic to tropical rainforests [16,17]. Further studies are needed to determine the morphology, geographic distribution and conservation status of this new taxon.

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Appendices 1–4. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2015.02.003.

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