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Phylogenetic relationships of the Asian palm civets (Hemigalinae & Paradoxurinae, Viverridae, Carnivora)

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ABSTRACT

The Viverridae (Mammalia, Carnivora), one of the least studied groups of carnivorans, include two subfamilies of Asian palm civets: Hemigalinae and Paradoxurinae. The relationships between and within these two subfamilies have never been thoroughly tested using an extensive molecular sample set. In this study, we gathered sequences of four genes (two mitochondrial: Cytochrome *b* and ND2 and two nuclear: β -fibrinogen intron 7 and IRBP exon 1) for eight of the eleven extant species representing these two subfamilies. The results showed that: (1) the Asian palm civets (Hemigalinae and Paradoxurinae) have a single origin and form the sister-group of the (Genettinae + Viverrinae) clade, (2) the Hemigalinae (including the otter civet *Cynogale bennettii*) are monophyletic, (3) the Paradoxurinae are monophyletic and (4) the small-toothed palm civet (*Arctogalidia trivirgata*) is an early offshoot within the Paradoxurinae. Using a relaxed molecular clock analysis, the differentiation of the (Hemigalinae + Paradoxurinae) was inferred to occur in the Late Oligocene/Early Miocene.

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1. Introduction

The Viverridae are medium-sized, nocturnal, solitary carnivorans (Nowak, 1999). They are elusive and poorly known, and until recently, were long overlooked by researchers. Wozencraft (2005) recognized four subfamilies: Hemigalinae, Paradoxurinae, Prionodontinae and Viverrinae. However, recent studies have shown that the Prionodontinae (Asian linsangs—*Prionodon*) are a sister-group of the Felidae and should now be erected as a family, the Prionodontidae (Gaubert and Veron, 2003; Gaubert et al., 2005). Gaubert and Cordeiro-Estrela (2006) have argued that the Viverrinae should be split into two subfamilies: the Viverrinae (terrestrial civets) and the Genettinae (*Genetta* and *Poiana*).

The Hemigalinae and Paradoxurinae—the Asian palm civets—are two subfamilies confined to South and South-East Asia,

whereas the Viverrinae are distributed across Asia and Africa, and the Genettinae across Africa and part of Europe. Although little is known about the ecology of most of the palm civet species, they are generally nocturnal, some are arboreal and frugivorous (Grassman, 1997; Nowak, 1999; Veron, 1999; Mudappa, 2001), and others are omnivorous or invertebrate eaters (Kowalczyk, 1989; Nowak, 1999). Some palm civets may play an important role in seed dispersal (Corlett, 1998). These taxa are of important conservation concern, with five species listed in the IUCN (2007) Red List of Threatened Species as endangered or vulnerable (<http://www.iucnredlist.org>), although this situation could be underestimated given the scarcity of data about their current biological status (Schreiber et al., 1989). The destruction of habitat by intensive deforestation is the major threat to this group (Brooks et al., 1999; Laurance, 1999; Brook et al., 2003; Sodhi et al., 2004).

To date, the systematics of the Hemigalinae and Paradoxurinae has been mainly assessed by morphological data (Pocock, 1933; Gregory and Hellman, 1939; Wozencraft, 1989; Veron, 1994, 1995). Molecular studies of feliform carnivorans have included very few representatives of these subfamilies due to the difficulties in obtaining biological material (see Veron and Heard, 2000; Gaubert

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and Veron, 2003; Flynn et al., 2005; Gaubert and Cordeiro-Estrela, 2006). These two subfamilies were suggested to be sister-groups in molecular studies, but with poor support (Veron and Heard, 2000) or few representatives (Flynn et al., 2005; Gaubert and Cordeiro-Estrela, 2006).

The phylogenetic relationships within each subfamily have been little studied and much debated. Notably, the affinities of the otter civet (*Cynogale bennettii* Gray, 1837) (Hemigalinae) have been questioned. This rare, semi-aquatic civet (see Veron et al., 2006) has a peculiar and specialized morphology that led some authors to place it in a separate subfamily, the Cynogalinae Pocock, 1933 (followed by Gregory and Hellman, 1939). However, its basi-cranial region displays features typical of the Hemigalinae (Veron, 1994, 1995). The small-toothed palm civet *A. trivirgata* (Gray, 1832) exhibits some peculiar morpho-anatomical characters, notably the absence of a perineal gland in males (Pocock, 1933), which is present in both genders of viverrids that are known to show this structure (see Gaubert et al., 2005). This characteristic, together with its distinctive dental features and its strict arboreality, led some authors to put it in a monotypic subfamily, the Arctogalidiinae Pocock, 1933. *A. trivirgata* was also deemed a primitive offshoot of the viverrid stock by Gregory and Hellman (1939).

All the genera of these two subfamilies are monotypic, with the exception of *Paradoxurus*. However, only the common palm civet *P. hermaphroditus* has previously been included in molecular studies, while the golden palm civet (*Paradoxurus zeylonensis*), the brown palm civet (*Paradoxurus jerdoni*) and the *incertae cedis* Mentawai palm civet (*Paradoxurus hermaphroditus lignicolor*) have not been included. Thus, the relevance of the genus *Paradoxurus* and the validity of these different species have never been assayed.

We provide here a phylogeny of the Hemigalinae and Paradoxurinae, with a special emphasis on never before studied species, the otter civet and the brown palm civet, and on the problematic small-toothed palm civet. Four genes were sequenced, two mitochondrial (Cytochrome *b* and ND2) and two nuclear (β -fibrinogen intron 7 and IRBP exon 1), in order to reach different resolution scales (as has been achieved in other carnivore studies; e.g. Gaubert and Veron, 2003; Yoder et al., 2003). We also estimated dates of origin of these two subfamilies based on molecular data, calibrated with viverrid fossils.

2. Materials and methods

2.1. Taxonomic sample and DNA extraction

Given the elusive way of life of Hemigalinae and Paradoxurinae species, it was very difficult to obtain fresh samples. However, for our molecular analyses we were able to obtain biological samples for almost all the species (Table 1), from various sources (see Acknowledgments). Some sequences from GenBank were also included in the dataset. One Felidae (*Panthera leo*) and one Herpestidae (*Herpestes javanicus*) were chosen as outgroups to the Viverridae, according to previous large-scale molecular phylogenies (Flynn and Nedbal, 1998; Yu et al., 2004; Flynn et al., 2005). Total genomic DNA was isolated from fresh biological samples (hairs or tissues) following a CTAB-based protocol (Winnepenninckx et al., 1993). For the museum collection samples (teeth), we performed extractions in a separate “Ancient DNA” room to avoid contamination and followed a specific protocol proposed by Hassanin et al. (1998).

2.2. DNA amplification and sequencing

Four genes were sequenced for this study: two mitochondrial (Cytochrome *b* and ND2) and two nuclear (a non-coding fragment:

intron 7 of the β -fibrinogen and a coding one: exon 1 of the IRBP). Primers used were: Cytochrome *b*: L14724, L15146, H15149, L15408, L15513, Cytb17 (see Gaubert et al., 2004a, for details) and L14841 (Veron and Heard, 2000); ND2: ND2-Met, ND2-Trp, ND2-IntF and ND2-IntR (see Perez et al., 2006); β -fibrinogen intron 7: BFG-F and BFG-R (Yu and Zhang, 2005) and IRBP: U29 and L559 (Gaubert and Cordeiro-Estrela, 2006). PCR cycles used for DNA amplification were: a first denaturation step at 94 °C for 4 min and the following cycle repeated 35 \times : 94 °C for 30 s/55 °C (mitochondrial) and 59–60 °C (nuclear) for 40 s/72 °C for 40 s; and a last step at 72 °C for 7 min. For the museum samples, genes were sequenced in smaller overlapping fragments because of the poor quality and fragmentation of the DNA and we performed a two-step PCR, as proposed by Debruyne et al. (2003): with a first step at 94 °C for 3 min, 10–15 cycles as follows: 94 °C for 1 min/50 °C for 90 s/72 °C for 90 s and a second step repeated 25 \times : 94 °C for 30 s/54 °C for 40 s/72 °C for 40 s, ended by 7 min at 72 °C. PCR products were visualized in a 1.5% agarose gel and positive PCR products were directly purified by MinElute PCR Kit (Qiagen). Both strands (Light and Heavy) were sequenced in all cases with an automated DNA sequencer (Beckman CEQ 2000 DNA Analysis System or Applied Biosystems 3730XL). We used the BLASTN 2.2.13 program (Altschul et al., 1997) to check for contamination. Sequences were aligned manually in Bioedit (Hall, 1999).

2.3. Phylogenetic analysis

The four datasets were first treated separately to evaluate their individual signal and to detect any incongruence. Each dataset was then explored by three reconstruction methods: Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI), given their different optimality criterion and their respective reconstruction artefacts (Felsenstein, 1978; Siddall, 1998; Pol and Siddall, 2001). The datasets were then combined into a single matrix. Specimens of *P. jerdoni* (MNHN 1998) and *C. bennettii* were not represented for nuclear genes and were coded as missing data for these genes.

The PAUP* 4b10 program (Swofford, 2001) was used to perform the parsimony analysis with heuristic search using 100 random addition sequence and performing Tree-Bisection-Reconnection (TBR) branch-swapping (Swofford et al., 1996), with 1000 bootstrap replicates (Felsenstein, 1985). We used PHYML (Guindon and Gascuel, 2003) to run the ML analyses under the best-fitting model chosen by MODELTEST version 3.7 (Posada and Crandall, 1998) according to the hLRT criterion. We ran 100 bootstrap (BP) replicates. For BI, we used MRBAYES 3b4 (Huelsenbeck and Ronquist, 2001). The best-fitting models were estimated with MRMODELTEST 2.0 (Nylander, 2004). Four cold (heating parameter = 0.2) Metropolis-Coupled Markov Chains Monte Carlo (MCMCMC) were run for 5×10^6 generations and one tree was sampled every 100 generations. Output parameters were analysed using TRACER (Rambaut and Drummond, 2004) to make sure that a sufficient number of generations were run and to decide the length of the “burn-in” period using Effective Sample Size (ESS) and Auto-Correlation Time (ACT) values. Analyses were run twice independently to check for convergence of the results. Bayesian Posterior Probabilities (BPP) were used to assess statistical support. The combined matrix was also partitioned into the four genes and into codon positions for the coding genes to improve both estimation and accuracy of the phylogeny (Nylander et al., 2004; Brandley et al., 2005) in allowing parameters (base frequencies, rate matrix, shape parameter, proportion of invariable sites) to vary for each partition. Pairwise genetic distances were computed under a Kimura-two-parameters (K2P) model in MEGA 3.1 (Kumar et al., 2004). We also used RRTREE (Robinson-Rechavi and Huchon, 2000) to perform statistical tests for comparison of evolutionary rates. This

Table 1

List of the sequences used in this study

(Sub)Family	Species	GenBank	References	Samples, information
<i>Cytochrome b</i>				
Hemigalinae	<i>Chrotogale owstoni</i>	AF125144	Veron and Heard (2000), Gaubert et al. (2004a)	Zoo
Hemigalinae	<i>Cynogale bennettii</i>	DQ683992	This study	Tissue/TC-417/C-515/Malaysia
Hemigalinae	<i>Hemigalus derbyanus</i>	AF125143	Veron and Heard (2000), Gaubert et al. (2004a)	Zoo
Paradoxurinae	<i>Arctictis binturong</i>	AY048793	Albert, 2001	Unknown
Paradoxurinae	<i>Arctogalidia trivirgata</i>	AF125140	Veron and Heard (2000), Gaubert et al. (2004a)	Zoo
Paradoxurinae	<i>Paguma larvata</i>	AF125151	Veron and Heard (2000), Gaubert et al. (2004a)	Unknown
Paradoxurinae	<i>Paradoxurus hermaphroditus</i>	AF511056	Gaubert et al. (2004a)	Vietnam
Paradoxurinae	<i>Paradoxurus jerdoni</i>	DQ683993 (700 bp)	This study	Teeth/MNHN 1998/ML-JD/India
Paradoxurinae	<i>Paradoxurus jerdoni</i>	DQ683994	This study	Skin/TC-470/L-29/India
Viverrinae	<i>Genetta genetta</i>	AY241922	Gaubert et al. (2004b)	Algeria
Viverrinae	<i>Genetta servalina</i>	AF511053	Gaubert et al. (2004b)	Gabon
Viverrinae	<i>Viverra tangalunga</i>	AF511045	Gaubert et al. (2004a)	Philippines
Herpestidae	<i>Herpestes javanicus</i>	NC006835	Penny & McLenachan, 2005 (direct subm.)	Unknown
Felidae	<i>Panthera leo</i>	AF053052	Cracraft et al., 1998	Unknown
<i>ND2</i>				
Hemigalinae	<i>Chrotogale owstoni</i>	DQ683984	This study	Tissue/TC-2092/C-138/Vietnam
Hemigalinae	<i>Cynogale bennettii</i>	DQ683983	This study	Tissue/TC-417/C-515/Malaysia
Hemigalinae	<i>Hemigalus derbyanus</i>	DQ683987	This study	Tissue/TC-22/C-209/Malaysia
Paradoxurinae	<i>Arctictis binturong</i>	DQ683980	This study	Tissue/TC-94/C-151/Unknown
Paradoxurinae	<i>Arctogalidia trivirgata</i>	DQ683981	This study	Tissue/TC-1/C-35/Unknown
Paradoxurinae	<i>Arctogalidia trivirgata</i>	DQ683982	This study	Tissue/TC-27/C-124/Unknown
Paradoxurinae	<i>Paguma larvata</i>	DQ683990	This study	None/C-72/Unknown
Paradoxurinae	<i>Paradoxurus hermaphroditus</i>	AY170056	Yoder et al. (2003)	Philippines
Paradoxurinae	<i>Paradoxurus jerdoni</i>	DQ683988 (250 bp)	This study	Teeth/MNHN 1998/ML-JD/India
Paradoxurinae	<i>Paradoxurus jerdoni</i>	DQ683989	This study	Skin/TC-470/L-29/India
Viverrinae	<i>Genetta genetta</i>	DQ683985	This study	None/C-9/France
Viverrinae	<i>Genetta servalina</i>	AY170058	Yoder et al. (2003)	Unknown
Viverrinae	<i>Viverra tangalunga</i>	AY170055	Yoder et al. (2003)	Philippines
Herpestidae	<i>Herpestes javanicus</i>	NC006835	Penny & McLenachan, 2005 (direct subm.)	Unknown
Felidae	<i>Panthera leo</i>	AY170043	Yoder et al. (2003)	Unknown
<i>β-fibrinogen intron 7</i>				
Hemigalinae	<i>Chrotogale owstoni</i>	EF680505	This study	Tissue/TC-2092/C-138/Vietnam
Hemigalinae	<i>Hemigalus derbyanus</i>	EF680508	This study	Tissue/TC-23/C-215/Malaysia
Paradoxurinae	<i>Arctictis binturong</i>	EF680503	This study	Tissue/TC-94/C-151/Unknown
Paradoxurinae	<i>Arctogalidia trivirgata</i>	EF680504	This study	Tissue/TC-27/C-124/Unknown
Paradoxurinae	<i>Paguma larvata</i>	AY634380	Yu and Zhang (2005)	China
Paradoxurinae	<i>Paradoxurus hermaphroditus</i>	EF680510	This study	Tissue/LRH3167/C-199/Philippines
Paradoxurinae	<i>Paradoxurus jerdoni</i>	EF680511	This study	Skin/TC-470/L-29/India
Viverrinae	<i>Genetta genetta</i>	EF680506	This study	(DNA) C-9/France
Viverrinae	<i>Genetta servalina</i>	EF680507	This study	(DNA) C-20/Gabon
Viverrinae	<i>Viverra tangalunga</i>	EF680512	This study	Hairs/TC-534/ML-86/Malaysia
Herpestidae	<i>Herpestes javanicus</i>	EF680509	This study	Tissue/TC-297/C-374/Bangladesh
Felidae	<i>Panthera leo</i>	AY634374	Yu and Zhang (2005)	Zoo
<i>IRBP intron 1</i>				
Hemigalinae	<i>Chrotogale owstoni</i>	DQ683127	This study	Tissue/TC-2092/C-138/Vietnam
Hemigalinae	<i>Hemigalus derbyanus</i>	AY170082	Yoder et al. (2003)	Zoo
Paradoxurinae	<i>Arctictis binturong</i>	DQ683125	This study	Tissue/TC-94/C-151/Unknown
Paradoxurinae	<i>Arctogalidia trivirgata</i>	DQ683126	This study	Tissue/TC-1/C-35/Unknown
Paradoxurinae	<i>Paguma larvata</i>	AY525040	Yu et al. (2004)	China
Paradoxurinae	<i>Paradoxurus hermaphroditus</i>	AY170086	Yoder et al. (2003)	Philippines
Paradoxurinae	<i>Paradoxurus jerdoni</i>	DQ683128	This study	Skin/TC-470/L-29/India
Viverrinae	<i>Genetta genetta</i>	DQ267565	Gaubert and Cordeiro-Estrela (2006)	Unknown
Viverrinae	<i>Genetta servalina</i>	AY170088	Yoder et al. (2003)	Unknown
Viverrinae	<i>Viverra tangalunga</i>	AY170085	Yoder et al. (2003)	Philippines
Herpestidae	<i>Herpestes javanicus</i>	AY170081	Yoder et al. (2003)	Unknown
Felidae	<i>Panthera leo</i>	AY170073	Yoder et al. (2003)	Unknown

The length of incomplete sequences is reported after their GenBank Accession number. Sample information includes: Type of biological material/Tissue number/DNA number/Geographic origin (MNHN: Museum National d'Histoire Naturelle, Paris, France; FMNH: Field Museum of Natural History, Chicago, USA; NMS: National Museums of Scotland, Edinburgh, UK).

program compares substitution rates between DNA sequences grouped (or not) in phylogenetically defined lineages: a species (*Arctogalidia trivirgata* for example) or a group of species (e.g. Paradoxurinae) is chosen and its mutation rate is compared to other species (e.g. remaining Viverridae species) and to an outgroup (e.g. Feliformia species).

2.4. Dating of divergence events

The fossil record is quite fragmentary concerning the Viverridae since fossils are almost absent for the Asian subfamilies. The cali-

bration points had to be taken outside these two subfamilies, within the Viverrinae and Genettinae. The earliest fossil attributed to the Viverridae is *Herpestides* (Hunt, 1991, 1996) and is dated 23 millions years (MY) old, while the fossil *Semigenetta*, described by Helbing (1927) (see also Ginsburg, 1999), is also dated ca. 23 MY old. The taxonomic attribution of *Herpestides* is debated and Gaubert and Cordeiro-Estrela (2006) attribute this fossil to the Viverrinae or Genettinae. *Semigenetta* is seen as an early viverrine-like fossil (Qiu and Gu, 1986; Montoya et al., 2001). According to these latter reinterpretations, the (Viverrinae + Genettinae) clade was calibrated at 23 MY old. The genus *Genetta* is acknowl-

edged in Africa around 8 MY (McDougall and Feibel, 2003; Werdelin, 2003) and we thus fixed the origin of the *Genetta* clade at 8 MY.

Divergence ages were estimated using a relaxed molecular clock, as implemented in the PAML/MULTIDIVTIME package (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002). Baseml (PAML 3.14b, Yang, 1997) was used to estimate model parameters, and ESTBRANCHES was then used to estimate branch lengths of the rooted tree and to generate a variance–covariance matrix from the dataset. Finally, we performed a Bayesian estimation of divergence times via Markov chain Monte Carlo using MULTIDIVTIME. The Markov chain was run for 1×10^6 generations with a tree sampled every 100 generations and a burn-in period of 1×10^4 generations. We used different submatrices due to the absence of *Cynogale bennettii* in the nuclear dataset (see below). Analyses were performed with (i) the combined mitochondrial dataset (Cytochrome *b* and ND2) including *C. bennettii*, (ii) the combined nuclear dataset (β -fibrinogen intron 7 and IRBP exon 1) lacking *C. bennettii* and (iii) the complete dataset (four genes) without *C. bennettii*.

3. Results

3.1. Sequencing results

We obtained 27 new sequences: three sequences of Cytochrome *b* (1140 bp), 10 of ND2 (1044 bp) 10 of β -fibrinogen intron 7 (645 bp) and four of IRBP (502 bp) (GenBank accession numbers are listed in Table 1). Due to the quality of the tooth sample of *P. jerdoni*, only 700 bp of Cytochrome *b* and 250 bp of ND2 were usable, but the skin sample provided whole sequences for the analyses. Sequences obtained from these two samples were nearly identical. We did not obtain nuclear sequences (β -fibrinogen intron 7 and IRBP exon 1) for *C. bennettii*. This tissue sample was not stored in appropriate conditions (inappropriate buffer and temperature), which likely damaged the DNA and made the nuDNA amplifications unsuccessful (given its reduced copy number compared to mtDNA). Extractions were also attempted on museum specimens of *Diplogale hosei* (MNHN CG 1892–2288, mounted skin), *Macrogalidia musschenbroekii* (MNHN CG 1868–1327, mounted skin) and *Paradoxurus zeylonensis* (MNHN CG 1876–2018, tooth), but these proved unfruitful.

3.2. Phylogenetic results

The parameters and the results of single-gene analyses are summarised in Appendixes A and B, respectively. The genes showed different levels of resolution depending on taxonomic levels. The mitochondrial genes did not resolve deep phylogenetic relationships, namely the (Hemigalinae + Paradoxurinae), (Viverrinae + Genettinae) and Paradoxurinae clades. The nuclear exon (IRBP) proved not variable enough to resolve the phylogeny among the Asian palm civets. However, the nuclear intron 7 of the β -fibrinogen brought strong support, whatever the taxonomic scale. The combined analysis (3331 characters) provided robust phylogenetic results as shown in Fig. 1. Analyses of the combined matrix strongly supported the monophyly of the Viverridae (node 9; BPP = 1.00; BP_{ML} = 100; BP_{MP} = 100), of the (Viverrinae + Genettinae) clade (node 8; BPP = 1.00; BP_{ML} = 99; except a BP_{MP} = 60) and a single origin for the (Hemigalinae + Paradoxurinae) clade (node 1; BPP = 1.00; BP_{ML} = 100; BP_{MP} = 83).

The monophyly of Hemigalinae was also well supported (node 2; BPP = 1.00; BP_{ML} = 100; BP_{MP} = 98), and the relationship (*Cynogale* (*Chrotogale*, *Hemigalus*)) was favoured by most of the analyses. However, the Bayesian trees of the ND2 and the parsimony analy-

sis of the complete dataset showed a polytomy within Hemigalinae.

In the single-gene analyses, a substantial difference was observed between the nodal support assessing the monophyly of the Paradoxurinae (including *A. trivirgata*) and those assessing the monophyly of the remaining Paradoxurinae species (*Arctictis*, *Paguma* and *Paradoxurus*). All markers, except the β -fibrinogen intron 7, failed to recover the monophyly of the Paradoxurinae (including *A. trivirgata*), while each of them strongly supported the monophyly of the (*Arctictis*, *Paguma* and *Paradoxurus*) clade (see Appendix B). Sequences of a second individual of *A. trivirgata* were then included in the analyses to exclude any possibility of species misidentification or errors in the sequences.

In the combined analyses (*Arctictis* (*Paguma*, *Paradoxurus*)) (node 4; BPP = 1.00; BP_{ML} = 100; BP_{MP} = 99) as well as the Paradoxurinae including *A. trivirgata* (node 3; BPP = 1.00; BP_{ML} = 100; BP_{MP} = 81) constituted strongly supported monophyletic groups.

The two species of the genus *Paradoxurus* clustered together (node 6) and presented a genetic distance (K2P) of 10.7% for Cytochrome *b* (within Viverridae pairwise distances ranged from 7.4% to 21.9%), 9.8% for ND2 [9.8–23%], 1.4% for intron 7 of the β -fibrinogen [1.4–9.5%] and 1.6% for the IRBP exon 1 [1.0–5.5%].

A relative-rate test, performed using RRTREE, showed that *C. bennettii* had a significantly higher evolutionary rate in the Cytochrome *b* for non-synonymous substitutions ($p = 0.048$ at a 95% threshold) and for synonymous substitutions in the ND2 gene, compared to the other Viverridae ($p = 0.00066$) and Hemigalinae ($p = 0.014$). Conversely, *A. trivirgata* did not exhibit a significantly higher evolutionary rate compared to the other Paradoxurinae ($p = 0.023$) and Viverridae ($p = 0.013$), except for synonymous substitutions in ND2.

3.3. Estimation of divergence times

All the divergence time estimates, from each of the three dating analyses, are reported in Table 2. The most striking result was the difference between the mitochondrial and nuclear estimates, which did not overlap. Divergence times estimated from the mitochondrial dataset were far more ancient, and had higher standard deviations, compared to those estimated from the nuclear dataset. Combining the four genes yielded divergence dates estimates whose standard deviations were reduced and which overlapped those of the nuclear and not those of the mitochondrial data. As a consequence, only the results of the combined analyses are further discussed.

Dating results (Table 2) placed the origin of the two Asian subfamilies at 24.8 MY ago (95% confidence interval (CI): 20.7–29.1, Late Oligocene–Early Miocene) and the divergence of the (Viverrinae + Genettinae) at 23 MY ago (95% CI: 20.7–24.0 MY, Late Oligocene–Early Miocene). Concerning the two strictly Asian subfamilies, the divergence time of the Paradoxurinae was inferred at the same period of time (21.9 MY ago, 95% CI: 17.6–26.5 MY) and that of the Hemigalinae (here defined as *Hemigalus* + *Chrotogale*) in the Late-Middle Miocene (11.6 MY ago, 95% CI: 8.4–15.4 MY). These results are reported on a chronogram (Fig. 2).

4. Discussion

4.1. Phylogenetic relationships of the Asian palm civets

Until the 1990s, the Viverridae were considered as the first group to have diverged within the Feliformia and thus to have retained some ancestral traits of the earliest feliforms. Some taxa were placed among the Viverridae on the basis of shared morphological traits, but some of these characters are now deemed plesio-

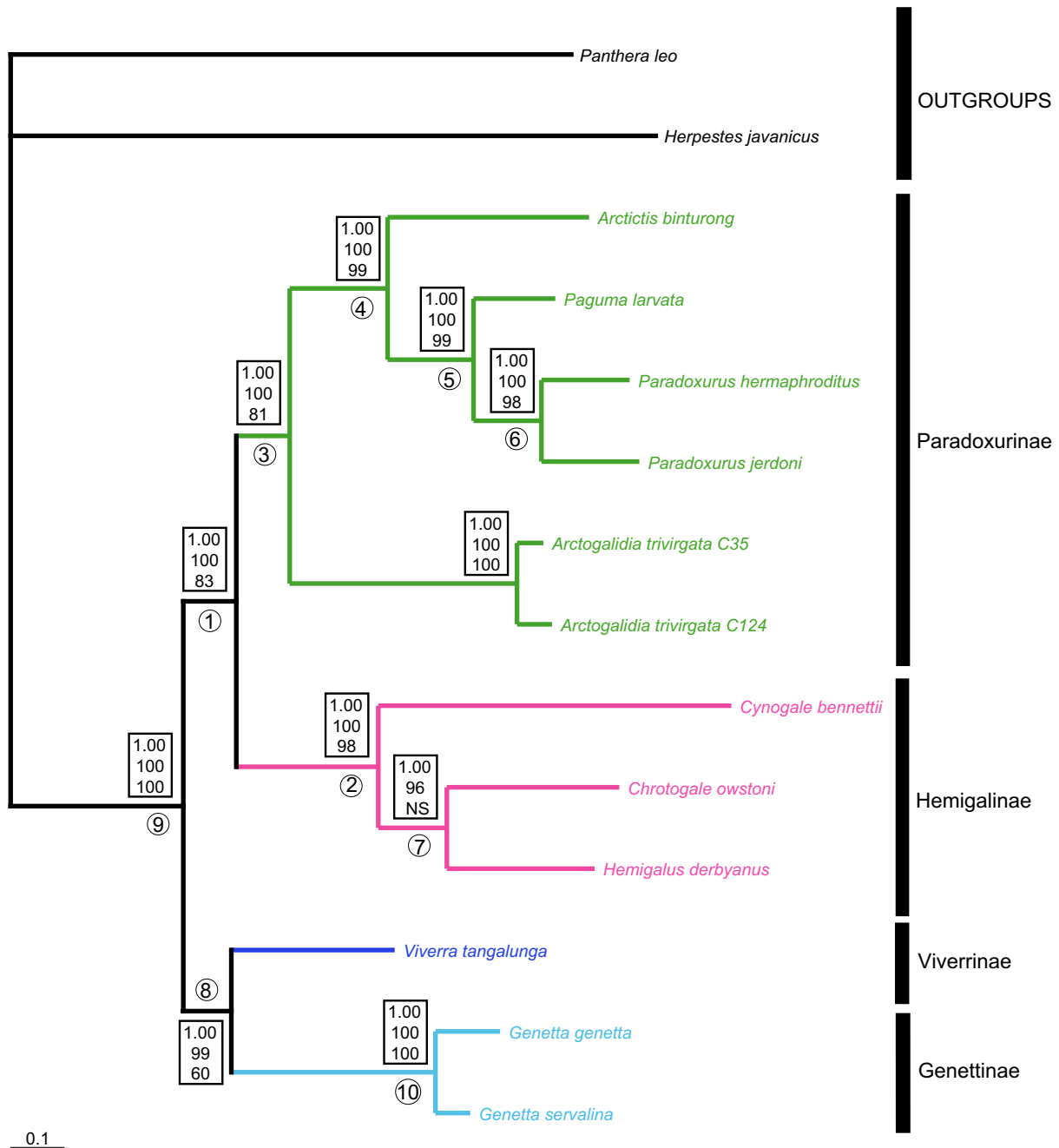


Fig. 1. Phylogenetic tree obtained for the combined matrix Cytochrome *b* (1140 bp) + ND2 (1044 bp) + β -fibrinogen intron 7 (645 bp) + IRBP exon 1 (502 bp) in Bayesian inference (MRBAYES). The dataset was partitioned into gene and codon positions for coding genes. Analyses were run for 5×10^6 generations with a tree sampled every 100 generations and a “burn-in” period of 5×10^5 generations (5000 trees discarded). The BPP (on top)/BP_{ML}>50/BP_M>50 are reported vertically for each node of the phylogenetic tree. (NS) means that this node is not supported (i.e. BP < 75 or BPP < 0.95) by a given analysis.

omorphous (Gaubert et al., 2005). Several studies led to the exclusion of some ‘viverrid-like’ taxa and helped redefine the Viverridae *sensu stricto* as a monophyletic group (Veron and Catzeflis, 1993; Veron, 1995; Veron and Heard, 2000; Gaubert and Veron, 2003; Yoder et al., 2003; Gaubert et al., 2004a, 2005), but little attention was paid to the Hemigalinae and Paradoxurinae. This study confirms the definition of the Viverridae as containing the Genettinae, Hemigalinae, Paradoxurinae, and Viverrinae. Molecular dating inference provided a divergence date estimate for the Viverridae in the Late Oligocene at 26.9 MY (95% CI: 23.4–30.5 MY). This is in agreement with the results yielded by Koepfli et al. (2006), who estimated the divergence of the Viverridae at 25.2 MY (95%

CI: 18.9–33.2 MY), while it is more recent than the estimation of Gaubert and Cordeiro-Estrela (2006) at 34.3 MY (no confidence interval).

Our study advocates for a single origin of the Asian palm civets (Hemigalinae + Paradoxurinae). They appear as the sister-group to the (Viverrinae + Genettinae) clade (see also Gaubert and Cordeiro-Estrela, 2006), which are both Asian (terrestrial civets) and African (genets, African oiyans *Poiana* and African civet *Civettictis civetta*) in distribution. The monophyly of the Asian palm civets is strongly supported in the analyses of the combined dataset, and the intron 7 of the β -fibrinogen supports this node. The monophyly of the (Hemigalinae + Paradoxurinae) group is also supported by a single

Table 2
Results of dating analyses

Nodes	Fossils (MY)	mt DNA		nu DNA		all DNA	
		Divergence times (SD) (MY)	95% confidence intervals	Divergence times (SD) (MY)	95% confidence intervals	Divergence times (SD) (MY)	95% confidence intervals
Hemigalinae + Paradoxurinae (node 1)	–	39.4 (±5.5)	[27.8–48.3]	21.6 (±3.2)	[15.9–28.7]	24.8 (±2.1)	[20.7–29.1]
Hemigalinae (node 2)	–	27.6 (±4.8)	[18.4–37.2]	9.4 (±2.7)	[4.7–15.3]	11.6 (±1.8)	[8.4–15.4]
Paradoxurinae (node 3)	–	35.5 (±5.4)	[24.6–44.8]	17.6 (±3.2)	[11.8–24.5]	21.9 (±2.2)	[17.6–26.5]
<i>Arctictis</i> + <i>Paguma</i> + <i>Paradoxurus</i> (node 4)	–	24.5 (±4.4)	[16.1–33.2]	14.0 (±3.0)	[8.8–20.5]	15.9 (±2.0)	[12.0–20.0]
<i>Paguma</i> + <i>Paradoxurus</i> (node 5)	–	18.2 (±3.6)	[11.6–25.5]	6.5 (±2.2)	[2.9–11.6]	10.7 (±1.7)	[7.7–14.2]
<i>Paradoxurus</i> genus (node 6)	–	13.1 (±3.0)	[7.8–19.6]	3.1 (±1.5)	[0.8–6.7]	7.1 (±1.4)	[4.6–10.2]
<i>Hemigalus</i> + <i>Chrotogale</i> (node 7)	–	20.1 (±3.9)	[13.0–28.1]	9.4 (±2.7)	[4.7–15.3]	11.6 (±1.8)	[8.4–15.4]
Viverrinae + Genettinae (node 8)	23	34.4 (±5.0)	[24.4–43.4]	22.2 (±1.4)	[18.8–23.9]	23.0 (±0.8)	[20.7–24.0]
Viverridae (node 9)	–	40.9 (±5.7)	[29.1–49.5]	26.3 (±2.9)	[21.2–32.6]	26.9 (±1.8)	[23.4–30.5]
<i>Genetta</i> genus (node 10)	8	9.9 (±1.6)	[8.1–14.1]	8.9 (±0.8)	[8.0–11.0]	8.4 (±0.4)	[8.0–9.5]

The fixed calibration points are, according to the fossils, assigned to the Viverridae: *Herpestides* and *Semigenetta* (23 MY old) for the (Viverrinae + Genettinae) clade (node 8), and 8 MY old for the oldest known representative of the *Genetta* genus (node 10). The divergence times for each node (estimated by a relaxed molecular clock analysis) are presented with their associated standard deviation (SD) and 95% confidence interval. Analyses were performed for different datasets: (i) mitochondrial DNA: mtDNA (ii) nuclear DNA: nuDNA and (iii) all sequences: allDNA. Node numbers refer to Fig. 1.

non-ambiguous morpho-anatomical synapomorphy (symmetry of the M₁ buccal lobes, Gaubert et al., 2005, character 185).

The molecular dating analysis gave an estimated divergence date for the (Hemigalinae + Paradoxurinae) clade at 24.8 MY (95% CI: 20.7–29.1, Late Oligocene–Early Miocene). This estimation is similar to that of Gaubert and Cordeiro-Estrela (2006) who proposed a divergence time at 23.8 MY. The presence of *Semigenetta* in China, dated from the Middle Miocene (Qiu and Gu, 1986), proves the presence of the Viverridae in Asia at this time. The divergences within the (Hemigalinae + Paradoxurinae) clade took place between 24.8 and 7.1 MY. Many other Asian taxonomic groups underwent diversifications in this timeframe: Muridae (Suzuki et al., 2003); Sciuridae (Mercer and Roth, 2003); Dicuridae (Pasquet et al., 2006); Cervidae (Gilbert et al., 2006); and Felidae (Johnson et al., 2006). Climatic, vegetational and geological changes (Hall, 2001) may have been involved in these diversifications. Songtham et al. (2003) suggested that a drastic climatic change (from temperate to tropical) occurred in Asia in the Oligocene–Miocene. Morley (2000), Meijaard (2004) showed that before 20–21 MY ago, the Sunda shelf was covered by dry and seasonal vegetation, but that after 20 MY, this region became rainforest; an environmental change that may have allowed the palm civets, and probably other forest species, to diversify. During the Late Miocene to the Last Glacial Maximum, changes in sea levels (Haq et al., 1987) allowed for faunal dispersions between the Indo-Malayan region and the Sunda shelf, which resulted in further diversifications (e.g. Mercer and Roth, 2003).

4.2. The Hemigalinae

Strong molecular evidence argues for the monophyly of the Hemigalinae (including the otter civet). Morpho-anatomical data also supports this monophyly, with a dental synapomorphy (presence of a hypocone on the M₂, Gaubert et al., 2005, character 197) and a similar structure of the perineal glands (at least in *Cynogale* and *Hemigalus*). However, the inclusion of Hose's palm civet in the sample set is required to definitively assess the monophyly of this subfamily.

The otter civet is one of the least known carnivore species with very few specimens in museum collections and scarce data on its ecology (Veron et al., 2006). Pocock (1933) suggested that the otter civet should be placed in a separate monotypic subfamily, the Cynogalinae, based on its derived morphology for a semi-aquatic lifestyle. This is the first time it has been included in a molecular phylogeny and the results showed it to be the sister-group to

(*Chrotogale owstoni* + *Hemigalus derbyanus*). *Cynogale* exhibits a long branch that might have affected the topology within the Hemigalinae (long branch attraction artefact; Felsenstein, 1978), but unfortunately no nuclear sequences were obtained to counteract this possible artefact. However, these relationships are congruent with traditional taxonomy and morphology. Due to its peculiar morphology, the position of *Cynogale* as sister-taxon to the other hemigalines was expected, although this could have been a similar situation to that of the aquatic genet (*Genetta piscivora*), which was previously placed in a separate genus but then shown to be a specialized genet (Gaubert et al., 2004a, 2004b). These two species exhibit specialized morpho-anatomical structures (Veron et al., 2006; Van Rompaey and Colyn, in press) that are related to their way of life (semi-aquatic and piscivores) and which have confused their systematics. Interestingly, the genetic and morphological divergence rates do not appear unbalanced in the otter civet, whereas the aquatic genet had a fast morphological evolution compared to its relatively low genetic distance to other genets (Gaubert et al., 2004a, 2004b).

4.3. The Paradoxurinae

The monophyly of the Paradoxurinae was strongly supported in the combined analyses. The contribution of intron 7 of the β -fibrinogen in the phylogenetic signal was valuable, while the other markers failed to resolve the position of the small-toothed palm civet (with regard to the other Paradoxurinae). All the species within this subfamily exhibit a peculiar dental morphology: reduction of the shearing blades of the carnassials and small premolars and molars (which bear low rounded cusps). This is likely related to their omnivorous to frugivorous diet, and according to our phylogenetic results, these dental features were present in the common ancestor of the Paradoxurinae. The monophyly of the Paradoxurinae is also supported by the similar structure of the perineal glands (Gaubert et al., 2005).

The small-toothed palm civet was found as the sister-group to the other Paradoxurinae species in the combined analyses. This species did not exhibit a significantly higher evolutionary rate compared to other Paradoxurinae (except for synonymous substitutions in ND2), so its positioning as sister-species to all other Paradoxurinae species should not have resulted from long branch attraction. The small-toothed palm civet was first described as *Paradoxurus trivirgatus* Gray, 1832, then placed in a separate genus (*Arctogalidia*) and monotypic subfamily Arctogalidiinae by Pocock (1933), Gregory and Hellman (1939), and then finally placed with-

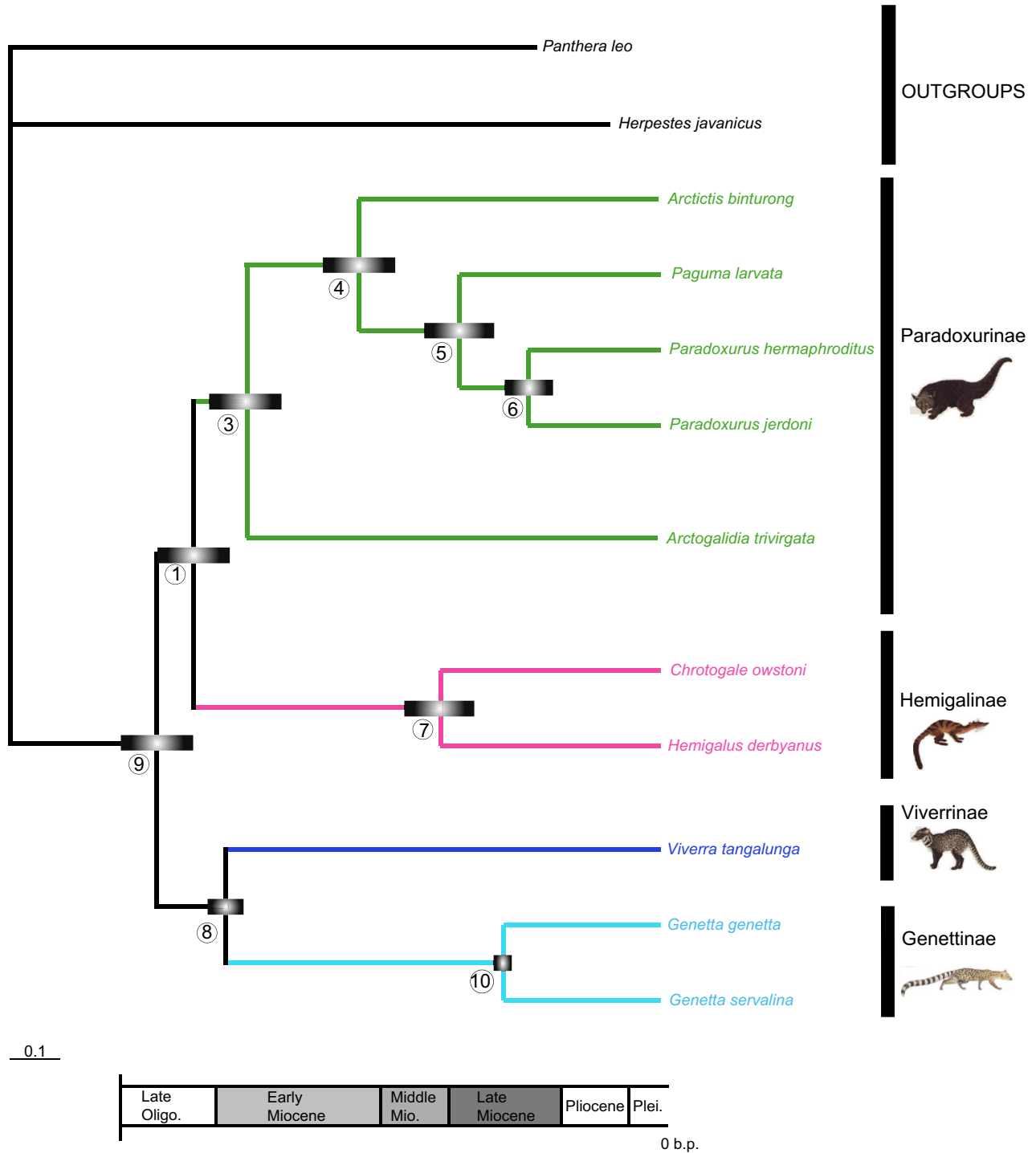


Fig. 2. Chronogram presenting the results of the dating analyses. For each node, the divergence times (estimated from the whole dataset) and their standard deviation (filled stripe) are reported. The geological time scale is presented along the bottom. Drawings are modified from Payne et al., 1985; Dorst and Dandelot, 1972.

in the Paradoxurinae by Simpson (1945). The peculiar morpho-anatomical traits of the small-toothed palm civet (the absence of a perineal gland in males, distinctive dental morphology, the presence of a long postorbital process and the early fusion of the ectotympanic and entotympanic, Pocock, 1933 and Veron, pers. obs.), its strict arboreal habits and divergence time estimates, suggest that this species was an early offshoot of the paradoxurine lineage. The estimation of divergence dates shows a long period of time between the divergence of the Paradoxurinae (including *A. trivirgata*) (21.9 MY; 95% CI: 17.6–26.5 MY) and that of the (*Arctictis*, (*Paguma*, *Paradoxurus*)) clade (15.9 MY, 95% CI: 12.0–20.0 MY). Nevertheless,

this assertion might have resulted from taxonomic sampling bias since the Sulawesi palm civet (*Macrogalidia musschenbroekii*) was not included in our study and might fill this “temporal gap”. This species needs to be included in a molecular phylogeny to confirm the monophyly of the Paradoxurinae.

The Viverridae are characterized by the presence of perineal glands (although its presence in *Poiana* and Sulawesi palm civet males is uncertain, see Pocock, 1933; Wemmer et al., 1983; Wozencraft, 1984; Gaubert et al., 2005). This is a feature absent in other feliformian families, and may have been present in the common ancestor of the Viverridae (Hunt, 2001). In the palm ci-

vets, the perineal gland is simple and less developed than in the terrestrial civets (Viverrinae) and this may be related to their arboreal habits. A perineal gland is absent in small-toothed palm civet males (Pocock, 1915) and according to our phylogenetic results, this absence in *Arctogalidia* should be considered as a secondary loss rather than a primitive feature.

The monophyly of the (*Arctictis*, (*Paguma*, *Paradoxurus*)) clade was unambiguously supported by every marker (both mitochondrial and nuclear) and the combined analyses. This clade is supported by a morphological synapomorphy, the union of the 3rd and 4th digit pads of the hind-foot (Veron, 1994). The phylogenetic relationships supported by our study are: (*A. binturong*, (*P. larvata* (*P. hermaphroditus*, *P. jerdoni*))). Our molecular analyses advocate that *Arctogalidia* and *Arctictis* were the first to have diverged within the Paradoxurinae, and that *Paguma* is the sister-taxon of *Paradoxurus*. The morphological characters that grouped *Paguma* and *Arctictis* in the morphological tree of Gaubert et al. (2005) were mainly related to the robustness and larger size of the skull and the teeth, and thus appeared by convergence.

Paradoxurus is the only polytypic genus within the Paradoxurinae. The number of species is still uncertain as it includes the Mentawai palm civet (considered as *incertae cedis* by Wozencraft, 2005). *P. hermaphroditus* has a large distribution throughout Asia and varies in size, coat pattern and colour throughout its range (Veron & Patou, pers. obs.), and more than 20 subspecies have been proposed (see Corbet and Hill, 1992). The other *Paradoxurus* species have been described mainly on the basis of coat colour and pattern (Pocock, 1934, 1939) and could either be different morphotypes of *P. hermaphroditus* or valid species. Our study showed significant genetic distance values between *P. hermaphroditus* and *P. jerdoni*, with the two sequenced specimens of this latter species clustering together in the analysis of the Cytochrome *b* dataset. These results may suggest that the brown palm civet is a valid taxon, which is supported by its distinctive morphology (brown uniform colour, two-third posterior parts lighter, no spots, no facial pattern, hairs on the neck reversed, large parastyle on the upper carnassial; Veron, pers. obs.). The taxonomic status of *Paradoxurus* species and subspecies remains to be investigated (Patou et al., in prep.).

In Pakistan, a new fossil species of *Paradoxurus* has been found and dated to the late Miocene (Flynn and Morgan, 2005), whose age (11.1 MY) is quite congruent with the divergence date for *Paradoxurus* obtained by our analyses. However, to our knowledge, this fossil has still not been described and its attribution to the Paradoxurinae remains to be checked. While the Paradoxurinae are mainly distributed in the Indochinese and Sundaic biogeographic regions (extending to the Himalayas for *Paguma*), some of the extant species of *Paradoxurus* do occur in the Indian region. If this fossil found in Pakistan is confirmed to be a *Paradoxurus*, then it shows that the presence of this genus outside South-East Asia is ancient.

4.4. Contribution of molecular data

The addition of nuclear data, especially of β -fibrinogen intron 7, significantly improved the phylogenetic resolution of deep nodes within the Hemigalinae and Paradoxurinae phylogeny. Although used previously in carnivorans by Yu and Zhang (2005), this was the first time it was used in the Viverridae and it proved to be a relevant phylogenetic marker for the systematic questions we addressed here. Another interesting aspect of this study was the discrepancies observed between the divergence dates estimates yielded by mitochondrial and nuclear datasets. Mitochondrial data provided far more ancient divergence estimates and wider 95% confidence intervals than the nuclear and combined datasets. A similar observation was made by Gilbert et al. (2006), who noticed a decrease in the confidence interval range when markers were com-

bined. However, contrary to our study, their nuclear datasets provided the widest confidence intervals. Our results, therefore, support the hypothesis of Yang and Yoder (2003), who argued in favour of combining markers in dating analyses, and who also warned of the putative weakness of dating procedures under certain conditions.

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Appendix A

Parameters of phylogenetic analyses provided by MODELTEST and MRMODELTEST and implemented for the phylogenetic analyses. Details of models and parameters include: selected models and estimates of the parameters' model for each partition. Nb cat., number of substitution categories; r(A–C) is the substitution rate between A and C; Ti/Tv: ratio between transitions and transversions; I: proportion of invariant sites; G: gamma distribution shape parameter. NA: means "Non Applicable".

Appendix B

Nodal support found in single-gene analyses for Cytochrome *b*, ND2, β -fibrinogen intron 7 and IRBP exon 1. Gene length/Informative sites number/Number of constant sites are reported under each gene. BP, bootstrap, BPP, Bayesian posterior probabilities. (<) means that this node is, by default, present in the phylogenetic tree but that it was not supported (i.e. BP < 70 or BPP < 0.95). Node numbers refer to Fig. 1.

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2008.03.026](https://doi.org/10.1016/j.ympev.2008.03.026).

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