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Cytogenetic Analysis of the Formosan Pangolin, *Manis pentadactyla pentadactyla* (Mammalia: Pholidota)

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Sheng-Hai Wu, Ming Chen, Shih-Chien Chin, Dong-Jay Lee, Pao-Yin Wen, Li-Wen Chen, Bao-Tyan Wang, and Hon-Tsen Yu (2007) Cytogenetic analysis of the Formosan pangolin, *Manis pentadactyla pentadactyla* (Mammalia: Pholidota) *Zoological Studies* **46**(4): 389-396. We analyzed the karyotypes of the Formosan pangolin, *Manis pentadactyla pentadactyla*, a subspecies of the Chinese pangolin, *Manis pentadactyla*. Pangolins belong to the mammalian order Pholidota and are primarily anteaters. Since eutherian *Sry* is highly conserved, we first conducted gene mapping of pangolin *Sry* by means of cross-species fluorescence *in situ* hybridization (FISH). The existence of a homologous *Sry* gene in the pangolin Y chromosome is demonstrated. We also report karyotyping by G-banding, C-banding, and Ag-NOR staining. The 2N and FN numbers of the Formosan pangolin are 40 and 70, respectively, which agree with the numbers reported for the subspecies *Manis pentadactyla aurita* from the Asian mainland. However, we found 2 or 4 nucleolar organizer regions (NORs) in the Formosan pangolin, whereas the Chinese pangolin has 3 or 4. An ideogram for this species is proposed. http://zoolstud.sincica.edu.tw/Journals/46.4/389.pdf

Key words: Cytogenetics, Heterochromatin, *Manis pentadactyla*, Pangolin, Silver-NOR.

Pangolins (or scaly anteaters), belonging to the order Pholidota, occupy a unique and interesting niche among mammals. The body is covered with overlapping scales, and their diets consist primarily of ants and termites (Nowak 1999). The phylogenetic position of the Pholidota has been a matter of debate. Some proposed that Pholidota is a sister group to Xenarthra, and both orders are situated at the basal position of the eutherian tree (Novacek 1992). However, a recent phylogenetic study comparing mitochondrial coding sequences among 60 mammalian species claimed that Pholidota is a sister group of the Carnivora (Arnason et al. 2002). The *Sry* gene is evolutionarily conserved in eutherian mammals except in some rodent species (Graves 2002). We therefore attempted to assign the *Sry* gene to the karyotype of the Pholidota represented by the Formosan pangolin with fluorescence *in situ* hybridization (FISH) to examine the conservative Y chromosome structure in mammals.

Currently, 7 species of pangolins are recognized, including 3 species distributed in Asia: the Chinese pangolin (*Manis pentadactyla*), the Indian pangolin (*Manis crassicaudata*), and the Malayan pangolin (*Manis javanica*) (Gaudin and Wible 1999). The original description of the Chinese pangolin was based on specimens from Taiwan,

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and the nominal subspecies, *M. pentadactyla pentadactyla*, is reserved for this population (hereafter referred to as the Formosan pangolin). There are 2 other subspecies, *M. p. aurita* from China (hereafter referred to as the Chinese pangolin), and *M. p. pusilla* from Hainan I., China (Heath 1992). Geographically, this species is distributed in North India, Bhutan, Nepal, possibly Bangladesh, across Myanmar to northern Indo-China, throughout most regions of southern China, and the islands of Hainan and Taiwan. *Manis pentadactyla* is threatened throughout its entire distributional range due to the alleged medicinal value of its scales in traditional Chinese medicine. The claimed therapeutic effects include enhancing microcirculation, antipyrexia, and anti-rheumatism (Li 1590). Fortunately, the Formosan subspecies (*M. pentadactyla pentadactyla*) has been protected under a wildlife conservation law for over 10 yr, and increases in wild populations have been witnessed in Taiwan.

In recent years, relationships between some Taiwanese mammal species and their Asian mainland counterparts have been reevaluated by cytogenetic approaches, e.g., the Formosan and Chinese muntjacs (Chiang et al. 2004). Given the scarcity of genetic data for pangolins, we were interested in applying these cytogenetic approaches to the Formosan subspecies. Furthermore, the karyotype of *M. pentadactyla* is still a matter of uncertainty. The reported diploid number varies from 2N = 36 to 42, with some geographical differences. For example, 2N = 42 was reported from Taiwan (Makino and Tateishi 1951), 2N = 40 from China (Chen et al. 1991), and 2N = 36 and 40 from India (Ray-Chaudhuri et al. 1969, Satya-Prakash and Aswathanarayana 1972, Chakrabarti et al. 1982). Up to this point, the most comprehensive karyotypes of *M. pentadactyla* have been reported for the Chinese pangolin, including G-banded, C-banded, and silver-NOR-stained karyotypes (Chen et al. 1991). Yet no karyotypes obtained by modern methods have been reported for the Formosan pangolin, and no G-banded ideograms have ever been published for the species. In order to examine the phylogenetic relationship between Formosan and Chinese pangolins, we compared G- and C-banded and Ag-NOR-stained karyotypes of the Formosan form with those of the Chinese form and defined the subspecies *M. pentadactyla pentadactyla* with cytogenetic approaches.

MATERIALS AND METHODS

Sampling

Peripheral blood was collected from 7 Formosan pangolins (2 females and 5 males) housed in the Taipei Zoo, Taiwan. These animals were brought to the zoo as injured individuals under a wildlife conservation and rescue program in Taiwan. Venous blood (2 ml) was drawn, transported to the laboratory in heparin-containing tubes at room temperature, and prepared within 24 h after sampling.

Metaphase preparation

Lymphocytes were cultured in RPMI 1640 (Gibco, Grand I, NY, USA) with 10% fetal bovine serum, antibiotics (a 1% mixture of penicillin, streptomycin, and amphotericin B), and 0.2% phytohemagglutinin (PHA, Sigma, St Louis, MO, USA) at 37°C for 72 h in a CO_2 (5%) incubator. Colcemid (Gibco) was added (at a concentration of 0.1 μg/ml) 20 min before harvesting lymphocytes to induce metaphase arrest. KCl (0.075 M) was used as a hypotonic treatment for at least 20 min, followed by fixation with a freshly made batch of Carnoy's fixative (3: 1 methanol to glacial acetic acid), and the fixed cell suspension was stored at 4°C. The cell pellets were resuspended in Carnoy's fixative prior to slide preparation. Metaphase spreads were prepared according to standard protocols (Chiang et al. 2004, Wang et al. 2004). Slides were air-dried for at least 48 h or baked at 95°C in an oven for 30 min. Slides were then frozen at -20°C for future analysis.

Sry fluorescence *in situ* hybridization (FISH) (Ferguson-Smith 1997)

A commercial human locus-specific FISH probe aimed at *SRY* (Yp11.3, Spectrum Orange, (Vysis, Downer's Grove, IL, USA) was initially used as the hybridization probe, but it failed to give consistent results. A homemade homologous FISH probe was then aimed at the human *SRY* locus to cross-hybridize itself to the pangolin metaphase cells. Hybridization was performed according to a published protocol (including denaturation, hybridization, and post-hybridization washing), with minor modifications (Pinkel et al. 1986). Details of the procedure are described as follows.

Genomic DNA was isolated from the leuko-

cytes of a normal human male using the Genomic DNA Purification Kit (Gentra, Minneapolis, MN, USA). The human *SRY* region was amplified by a polymerase chain reaction (PCR) in 50 μl of a reaction mixture containing 0.2 mM dNTP, 2.5U Pro Taq DNA Polymerase (Protech, Taipei, Taiwan), and 0.4 μM of each specific set of primers. PCR primers (Canto et al. 2004) were chosen to produce 6 partially overlapping fragments (each with a length of about 1 kb, thus giving a total length of about 3 kb in the human genome), encompassing sequences from the *SRY* coding and flanking regions (5' and 3'). PCR conditions were followed as published (Canto et al. 2004), with minor modifications. PCR products were purified using Gene-Spin™ (Protech) and labeled by nick translation using Biotin-Nick Translation Mix (Roche, Basel, Switzerland) to produce biotin-labeled probes for FISH. For hybridization, slides were treated with 2x SSC at 37°C for 60 min and with 0.002% pepsin in 0.01 N HCl at 37°C for 3 min. After re-fixing for 10 min in 1x PBS containing 50 mM $MgCl_2$ and 1% formaldehyde, the slides were dehydrated in an ethanol series. Slides were denatured in 2x SSC (pH 7.0 containing 70% formamide) for 5 min at 73°C and again dehydrated using an ethanol series. For hybridization of the denatured slides, 5 μl of biotinylated probe DNA was co-precipitated with or without a 100-fold excess of human C_0t-1 DNA (Invitrogen, Carlsbad, CA, USA) and re-dissolved in 2x SSC containing 50% formamide and 10% dextran sulfate. The hybridization mixture was then denatured for 10 min at 80°C. Pre-annealing of repetitive DNA was carried out for 30 min at 37°C. The slides were hybridized overnight in a moist chamber at 37°C, then washed for 3 min in 0.4x SSC containing 0.3% NP-40 at 70°C (for human metaphases) or 3 min in 0.5x SSC at 68°C (for pangolin metaphases) and for 3 min in 4x SSC containing 0.1% Tween 20 at room temperature. Slides were then blocked with 4x SSC containing 3% BSA and 0.1% Tween 20 at 37°C for 30 min. The hybridization signal was detected with Cy5-conjugated streptavidin (Cambio, Cambridge, UK). Chromosomes were counterstained with 0.125 μg/ml DAPI added to Antifade (Vysis).

G-banding, C-banding, and silver-NOR staining

Staining of metaphase chromosomes was performed according to published protocols (Chiang et al. 2004), which are briefly described as follows. At least 20 metaphase cells were analyzed after the following treatments.

For G-banding (Seabright 1971), slides were treated with 0.05% trypsin/EDTA for 10-15 s at room temperature and stained with Wright's dye for 60-80 s.

For C-banding (Sumner 1972), slides were treated with 0.2 N HCl at room temperature for 1 h, rinsed with double-distilled water (ddH_2O), followed by treatment in an alkali solution containing 5% $Ba(OH)_2$ at 50°C for 10 min, then rinsing with ddH_2O. Slides were incubated in 2x SSC at 60°C for 1 h before staining with Wright's dye for 60-80 s.

For silver-NOR staining (Goodpasture and Bloom 1975), slides were treated with gelatin and 50% silver nitrate solution (3: 4 v/v) at 65°C for 2-4 min, followed by washing with 3% acetic acid and ddH_2O, then were stained with Wright's dye after air-drying.

Image capture and analysis

Fluorescence microscopy integrated with a cooled CCD camera system and Smart Capture software for chromosome arrangement (Cytovision Chromophour System, Applied Imaging, Carlsbad, CA, USA) was used to investigate and analyze the FISH results.

RESULTS

Sry FISH

A human *SRY* homologue was revealed in the pangolin genome by the successful hybridization of the human *SRY* locus-specific probe to the pangolin Y chromosomes, and this produced a FISH signal. Of the 14 metaphase spreads analyzed, 11 produced doublet signals (Fig. 1a), and 3 produced a single signal. All interphase nuclei revealed hybridization signals. Figure 1b reveals the results of the external control experiments using the human homemade *SRY* probe hybridized to human metaphase cells, which yielded doublet signals corresponding to the *SRY* region of the human Y chromosome, as expected.

Diploid number (2N) and fundamental number (FN)

In total, 187 metaphase spreads were obtained with the preparations from the 7 individu-

als. The diploid number of the Formosan pangolin was 40 (Fig. 2), which agrees with previous studies (summarized in Table 1). For a convenient comparison, we followed the chromosomal arrangement in the Chinese pangolin (see Chen et al. 1991). The male chromosome complement includes 10 metacentrics (chromosomes 1-5), 18 submetacentrics (chromosomes 6-13, X, and Y), 6 acrocentrics (chromosomes 14-16), and 6 telocentrics (chromosomes 17-19) according to the classification by Levan et al. (1964). The fundamental number (FN) was therefore 70 (the number of arms excluding the sex chromosomes). We classified the 40 chromosomes of the Formosan pangolin (Table 2, Fig. 2b) by counting at least 10 metaphase spreads.

G-banding

Among the 20 metaphase spreads were counted in each animal, 5 were analyzed and 2 karyotyped (Fig. 2a). In total, 112 bands were identified through our preparation and stained with the Giemsa-Trypsin-Wright method. Here we present the 1st ideogram (Fig. 2b) for *M. p. pentadactyla*.

C-banding

Most centromeres could be stained with the standard C-banding technique; however, prominent constitutive heterochromatin was noted in most parts of chromosome no. 2 and the Y chromosome, the pericentromeric region of chromosome nos. 3 and 15, and the terminal regions of chromosome no. 4, no. 7, and the X chromosome.

Silver-NOR staining

We found 4 nucleolar organizer regions (NORs) in the satellite region on both chromosome 18s and 19s (Fig. 3b) in preparations from 2 male individuals, whereas 2 NORs were found in the satellite region on both chromosome 19s in the preparations from the remaining 5 individuals we examined (3 males and 2 females, Fig. 3c). The numbers of NORs in the Formosan pangolins were therefore 2 or 4 in our analysis.

DISCUSSION

Sry FISH

Mammalian sex chromosomes are believed to have evolved from autosomal pairs. The testis-determining *SRY* gene is involved in the formation of the male phenotype in humans, and its homologues are found in other eutherian mammals (Grüzner et al. 2004). Successful hybridization of the human *SRY* locus-specific probe to the pangolin Y chromosome suggests that the *SRY* locus has been highly conserved during mammalian evolution. The homemade probe, which consists of 6 overlapping amplicons encompassing the entire human *SRY* locus including the 5' and 3' flanking regions with a total length of 3 kb in the human genome, produced a strong signal in both the human and pangolin Y chromosome after suppression with repetitive C_ot-1 DNA. The quality of

Table 1. Summary of reports on the chromosome numbers of *Manis pentadactyla*. Chromosomes are categorized as either bi-armed (B) or telocentric (T)

	Autosomes			Sex chromosome			
Locality	2N	B	T	X	Y	FN[h]	Sample size (sex included)
Taiwan	42	n/a	n/a	B[i]	n/a	-	1 (♂)[a,g]
India	36	16	1	B	T	66	1 (♂)[b]
India	36	16	1	B	n/a	66	1 (♀)[c]
India	40	16	3	B	B	70	4 (♀ + ♂)[d]
China	40	16	3	B	B	70	5 (♀ + ♂)[e]
Taiwan	40	16	3	B	B	70	7 (♀ + ♂)[f]

[a]Makino and Tateishi 1951; [b]Ray-Chaudhuri et al. 1969; [c]Satya-Prakash and Aswathanarayana 1972; [d]Chakrabarti et al. 1982; [e]Chen et al. 1991; [f]this study. [g]Based on serial paraffin sections of testes n/a, non-available. [h]Excluding sex chromosomes. [i]Reported as subterminal J-shaped and therefore bi-armed.

the cross-hybridization FISH experiments we obtained indicates that our method is suitable for overcoming difficulties during hybridization of the human probe to homologous pangolin sequences. The *SRY* and SOX family genes (*SRY*-related family genes) share a highly conserved sequence similarity in their DNA-binding domains, the high mobility group (HMG) domain (at least > 50% identity in nucleotide sequences), with each other and among different mammalian species. The highly conserved HMG sequence was thus the genetic basis for cross-hybridization in our study, using the human *SRY* as a probe to prove the existence of homologous gene sequences in the pangolin genome. The pangolin Y chromosome and the human Y chromosome are both mainly composed of heterochromatin. Cloning of the pangolin *Sry* locus may further demonstrate this and awaits future study.

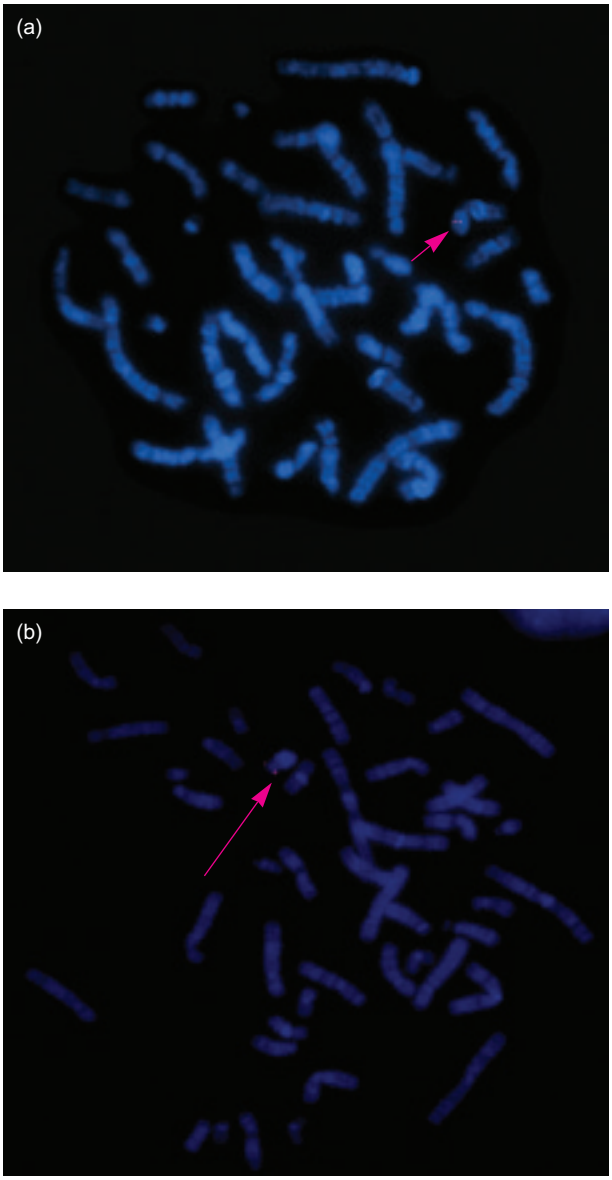


Fig. 1. (a) Human *Sry* locus-specific probe showing a hybridization signal over the pangolin Y chromosome (red arrow, DAPI-counterstained). (b) Human *Sry* locus-specific probe showing a hybridization signal over the human Y chromosome (red arrow, DAPI-counterstained).

Comparison between Formosan and Chinese pangolins

The 2N (= 40) and FN (= 70) of the Formosan pangolin are compatible with those of the Chinese pangolin (Chen et al. 1991). These results are not unexpected given that these are subspecies of the same species, and there is little reason to suppose that they exhibit dramatic intraspecific chromosomal variations like that observed in house mice (e.g., Nachman et al. 1994) or in the common shrew (e.g., Searle and Wojcik 1998), since the stasis of karyotypes remains the norm in most mammalian species.

Makino and Tateishi (1951) reported 2N = 42 for the Formosan pangolin. However, they used serial paraffin sections to examine the testes of a pangolin obtained from Taiwan, and their results

Table 2. Measurements of pangolin chromosomes (*Manis p. pentadactyla*)

Chromosome no.	Arm ratio (mean ± SD; *n*)	Attribute
1	1.5 ± 0.01; 13	Metacentric
2	1.33 ± 0.01; 11	Metacentric
3	1.6 ± 0.01; 19	Metacentric
4	1.2 ± 0.01; 19	Metacentric
5	1.67 ± 0.01; 19	Metacentric
6	2.5 ± 0.03; 18	Submetacentric
7	1.89 ± 0.02; 17	Submetacentric
8	1.75 ± 0.01; 18	Submetacentric
9	2.33 ± 0.02; 19	Submetacentric
10	2.33 ± 0.03; 17	Submetacentric
11	3.0 ± 0.03; 12	Submetacentric
12	2.67 ± 0.01; 14	Submetacentric
13	2.0 ± 0.01; 16	Submetacentric
14	10.0 ± 0.11; 18	Acrocentric
15	9.0 ± 0.14; 18	Acrocentric
16	5.5 ± 0.07; 19	Acrocentric
17	∞; 18	Telocentric
18	∞; 17	Telocentric
19	∞; 17	Telocentric
Y	1.75 ± 0.01; 16	Submetacentric
X	1.80 ± 0.01; 15	Submetacentric

were most likely prone to errors inherent in that method. The more-precise techniques used in this study and that of Chen et al. (1991) should result in greater accuracy. The discrepancy between our results and the 2N numbers (36 and 40) reported for the Chinese pangolins from India seems to have originated from the incorrect identification of the animals, because 2 species of pangolins (*M. pentadactylus* and *M. crassicaudata*) sympatrically occur there (Chakrabarti et al. 1982, Aswathanarayana 2000). Therefore, the 2N and FN numbers for *M. pentadactyla* can be confidently concluded as being 40 and 70. The 2N is 36 and FN is 66 for *M. crassicaudata* (Aswathanarayana 2000). No genetic data are available for *M. javanica* (the Malayan pangolin).

Chromosomes 18 and 19 are the smallest telocentric chromosomes, and it is difficult to differentiate between the two in both the Formosan pangolin (Fig. 1b) and the Chinese pangolin (see Chen et al. 1991). The G-banded karyotype and ideogram obtained in this study provide a better resolution than that previously reported for the Chinese pangolin (see Chen et al. 1991). All together, we are able to differentiate 112 bands in the Formosan pangolin, offering the first G-banded ideogram for the species and a basis for future comparisons with other species in the genus *Manis*.

The distribution of constitutive heterochromatin revealed by C-banding showed a slightly different pattern in Formosan pangolins when compared with those of Chinese pangolins. Constitutive heterochromatin constituted the majority of chromosome no. 2 and the Y chromosome in both subspecies, and they appeared dark in the C-banding. However, a terminal heterochromatin block was noted in chromosome nos. 3, 4, 7, and 15 in Chinese pangolins, but was only found in chromosome nos. 3, 4, and 7 in Formosan pangolins. A large pericentromeric heterochromatin block was noted in chromosome no. 15 in both subspecies. In addition, the possibility of polymorphism in heterochromatin distribution was suggested in the X chromosome of Chinese pangolins. Some reports claimed that the X chromosome was mostly C-positive, while another claimed that it was only C-positive in both termini (Chen et al., 1991). We found that constitutive heterochromatin was only distributed in the terminal regions of the X chromosome in Formosan pangolins.

A slight difference between the karyotypes of the Formosan and Chinese pangolins was revealed by Ag-NOR staining, which indicates the location of the nucleolus. The number of NORs in the Chinese pangolin is 3 or 4, and the sites are near the satellites of chromosomes nos. 18 and 19 (Chen et al. 1991). In all of the metaphase cells we examined in the 5 Formosan pangolins (2 females and 3 males), the number of NORs was consistently 2, and they were restricted to chromosome no. 19. However, we observed NORs in both chromosome nos. 18 and 19 in another 2 individuals, which made the number of NORs 4.

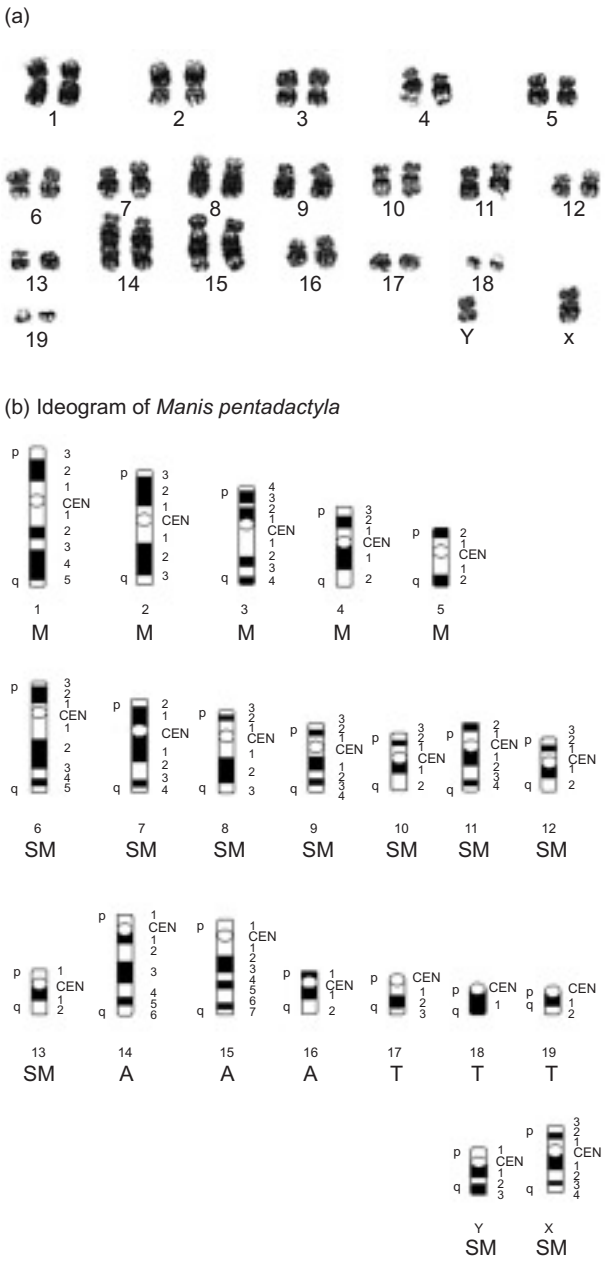
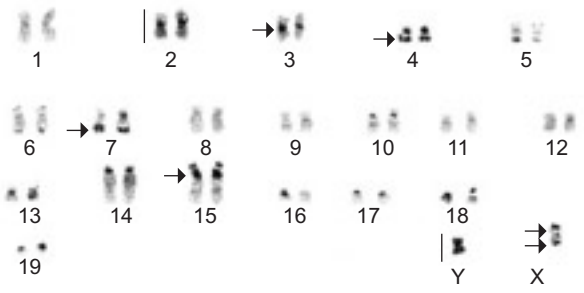
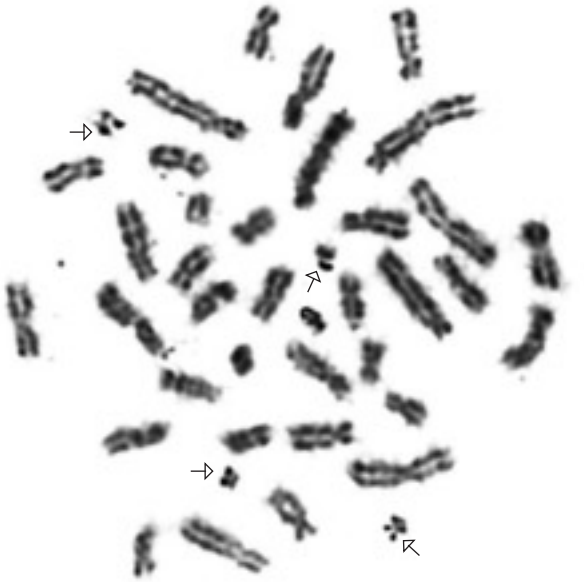


Fig. 2. Chromosomes of *Manis p. pentadactyla*. (a) G-banded karyotype; (b) proposed ideogram. M, metacentric; SM, submetacentric; A, acrocentric; T, telocentric.

(a) C-banding



(b) Silver-NOR staining: four signals



(c) Silver-NOR staining: two signals

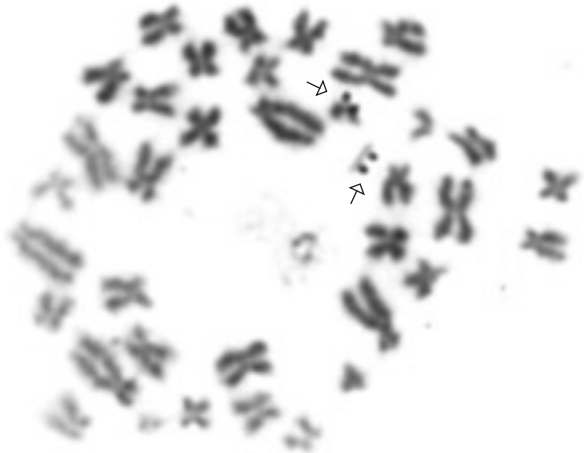


Fig. 3. (a) C-banding. Black arrows indicate the heterochromatic blocks, including prominent pericentromeric blocks in chromosome nos. 3 and 15, and prominent terminal blocks in chromosome nos. 4 and 7, and the X chromosome; vertical bars indicate that chromosome no. 2 and the Y chromosome largely consist of heterochromatin. (b) and (c) Silver-NOR staining (black arrows) of chromosomes of *Manis p. pentadactyla*, indicating that the numbers of silver-NOR were 4 and 2, respectively.

NORs may be seen as a secondary constriction in conventional karyotyping with a distally associated satellite (Miller and Therman 2001). Active NORs can be strongly stained with a silver nitrate solution because a specific nucleolar protein quickly reduces the ionic silver to native silver (Gebrane-Younes et al. 1997). Notably, the repetitive genes encoding the rRNA, which we call the rDNA, may participate in speciation. One line of evidence that NORs are involved in the speciation process is nuclear dominance, an epigenetic phenomenon in which a selective silencing of the rDNA of a parent species occurs when speciation involves interspecific hybridization. This phenomenon is commonly found in plants, but is also found in a diverse group of animals, including insects, amphibians, and a few mammals (Labhart and Reeder 1985, Chen and Pikarrd 1997, Chen et al. 1998). Numbers of NORs are influenced by transcriptional activity of rDNA and may vary because of chromosomal rearrangements such as translocation and deletion. Nonetheless, the number of NORs may differ among different cell types of a single individual, among different cells of even the same slide preparation, and among different individuals. Whether this slight difference (3 or 4 of the Chinese pangolin vs. 2-4 of the Formosan pangolin) has an evolutionary significance with respect to population differentiation in Chinese versus Formosan pangolins warrants further study by utilizing the rDNA as the cross-species FISH probe to distinguish if there is a differential distribution of rDNA in these subspecies.

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