

# Morphometrics and Pelage Characterization of Longtailed Macaques (*Macaca fascicularis*) from Pulau Bintan, Indonesia; Singapore; and Southern Vietnam

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Cynomolgus (or longtailed) macaques (*Macaca fascicularis*) are used extensively as laboratory animals in biomedical research. Their use in Singapore, an emerging biomedical hub in Southeast Asia, is now increasing widely, with research subjects currently originating from Singapore, Vietnam, and Pulau Bintan, Indonesia. Limited data exist on the genetic and phenotypic polymorphisms and phylogenetic relationships of these groups, and the animals are used as research subjects without regard to potential differences or homogeneity. Here we characterize their phenotypes by using established primatology tools to detail morphometrics and pelage erythrism and saturation. Pelage analyses supported the Gloger rule, in which heavily pigmented forms predominate near the equator, with Singaporean and Bintan macaques having darker pelage than Vietnamese macaques. Morphometric variation patterns suggest a tendency toward insular dwarfism and correlate generally with the Bergmann rule, in which body mass increases with latitude and colder climate. Although the 3 populations all belong to the nominotypical subspecies *M. f. fascicularis*, phenotypic differences are evident and are valuable tools to analyze their phylogeographic history and phylogenetic relationships.

Singapore, the city-state at the southern tip of peninsular Malaysia, is an emerging biomedical research hub in Southeast Asia in response to government initiatives making biomedical research the country's fourth economic pillar.<sup>1</sup> Singapore has increased translational research projects by using different animal species including the cynomolgus (or longtailed) macaques (*Macaca fascicularis*), the most common Southeast Asian nonhuman primate species and the most commonly used primate model, after the rhesus macaque (*M. mulatta*; a closely related species), for the study of human diseases. Local institutions currently use macaques originating from Singapore and Long Thanh in southern Vietnam and have recently started using animals from Pulau Bintan, Indonesia.

The genus *Macaca* represents successful primate adaptive radiations with respect to geographic distribution, ranking second only to human beings.<sup>3</sup> For example, *M. fascicularis* occupies a vast geographic range in mainland and insular Southeast Asia, extending from approximately 21°N to 10°S latitude and from 92°E to 126°E longitude.<sup>6</sup> In addition, aberrant *M. fascicularis* groups were introduced by man into areas including Mauritius and Pulau Tinjil, Indonesia. *M. fascicularis* is 1 of the most genetically diversified primates, with an estimated proposed 50 specific (including *Macaca cynomolgus* and *M. irus*<sup>18</sup>) or subspecific names.<sup>6</sup> However, only 10 subspecies have been recognized recently based on character-state discontinuity, the critical requisite for subspecific recognition.<sup>6</sup> This situation

means that these geographic populations vary with at least 1 character being nearly completely or completely discontinuous with the same corresponding character in *M. f. fascicularis*, the nominotypical subspecies.<sup>6</sup> Currently, macaque populations in Bintan, Singapore, and southern Vietnam are considered nominotypical with the populations found in the Malay Peninsula, Java, and Borneo.<sup>6</sup> Although this assumption may be true, phenotype differences are evident based on this study's findings. Evaluating the data used to characterize genetic and phenotypic polymorphisms and phylogenetic relationships among these groups of macaques are essential, because they are used as research subjects without regard to potential differences or homogeneity among populations.

Minimizing genetic and phenotypic variations have proven to be important factors in research success. Animal model suitability is influenced by the level of diversity and genetic subdivision (or substructure) of the population from which research subjects are selected.<sup>20</sup> Subjects originating in regions separated by biogeographic barriers (for example, rivers, mountain ranges) have long precluded or restricted gene flow. Their use as research subjects in the same experiment or in studies of the same disease as animals from a population with increased gene flow, for example, increases the proportion of phenotypic differences due solely to genetic differences (that is, genetic subdivision) among the subjects.<sup>19</sup>

Phenotypic relationship data also can be used to benefit colony and breeding management. In addition, biologic characters, including those described here, are helpful in species identification. For example, tail length and pelage color are the 2 most important morphologic characters used to classify rhesus and cynomolgus macaques.<sup>11</sup>

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The current report is part of a larger project establishing phylogenetic relationships among *M. fascicularis* used in Singapore's biomedical research industry. To date, data on their morphologic and genetic distinctions and associated genetic relationships are limited compared with other geographic populations of the same species. Here we focus on using standard primatology tools to quantitatively investigate and establish various phenotypes and patterns of morphologic variations.

## Materials and Methods

**Animals.** Wild-caught macaques from Singapore (1°18'N, 103°51'E) and Bintan (1°4' N, 104°30' E), Indonesia (Table 1) were sampled from outdoor colonies maintained by the SingHealth Experimental Medicine Centre (SEMC) and PT MacFauna (of Maccine Pte, Singapore), respectively (Figure 1); both facilities are AAALAC-accredited. The colony at SEMC has been maintained for at least 3 y, whereas PT MacFauna had kept its colony for at least 1 y before the study period. Meanwhile, captive-bred male macaques from southern Vietnam were purchased initially by SEMC from Nafovanny in Long Thanh (10°46' N, 106°57' E), Dong Nai Province, Southern Vietnam, for select studies. All macaques were adult, as judged by their permanent dentition and fully erupted third molars and canines. They were seronegative for simian immunodeficiency virus. With few exceptions they were also seronegative for B virus (*Cercopithecine herpesvirus 1*), simian T-cell leukemia virus, and simian retrovirus. In addition, Vietnamese and Singaporean macaques were seronegative for measles (rubeola). Routine health monitoring and care also included tuberculosis testing, deworming, and physical examination. Overtly pregnant, infantile, and juvenile macaques were excluded from the study, because phenotypes can vary with the stage of physical or physiologic development. All macaques were fed twice daily with commercially available primate diets (Vietnamese and Singaporean macaques: Diet 5049, PMI Nutrition International, Brentwood, MO; Bintan macaques: Primate Diet, Perfect Companion, Bangkok, Thailand). Protocols conformed to the guidelines set by Singapore's National Advisory Committee for Laboratory Animal Research and were approved by both the SingHealth and Maccine Institutional Animal Care and Use Committees. All animals were sedated with ketamine (5 to 10 mg/kg IM) for initial data collection.

**Pelage characterization.** Previous characterization data of the pelage of *M. fascicularis* have been limited to subjective saturation and erythrim interpretations. Here, we used a color reflectometer (Chroma Meter CR300, Minolta, Osaka, Japan)<sup>12</sup> that emits a standardized light (D65); measures the light reflected through an 8-mm aperture; and provides values for L\* [lightness, ranging from darkest (0) to lightest (100)], a\* [green (-60) to red (+60)], and b\* [blue (-60) to yellow (+60)]. Pelage readings were taken at the vertex of the animal's head (crown), interscapular dorsum, and lateral left thigh and data were used to calculate color attributes based on the Munsell color system.<sup>2</sup> These attributes include chroma (also called saturation or intensity), the degree of color strength and the amount of gray perceived by the human eye and calculated as  $\sqrt{(a^*2 + b^*2)}$  value (also called brilliance, or lightness), which is designated as L\* and is related to a given color's light strength; and hue (H°), characterizing the degree to which red can be distinguished from green or blue from yellow and calculated as  $\tan^{-1}(b^*/a^*)$ .<sup>16</sup>

**Body weight and morphometrics.** Personnel taking morphometric measurements as described previously<sup>8</sup> were blinded to each other's results, for purposes of accuracy and comparison.

Measurements were repeated in the cases of large discrepancies (5 mm for long parameters, like total length, and 2 mm for shorter ones, like ear length) between the 2 readings; otherwise, the average value was calculated and recorded. Body weight (measured to the nearest 0.1 kg) and 5 linear morphometric parameters (defined in following sections), were determined by using a recently calibrated weight scale and standard metric tape measure, respectively. Total length was measured on dorsally recumbent and fully extended animals as the greatest length (to the nearest millimeter) in straight line from the tip of the rostrum to tip of the tail (excluding terminal tail hairs). Tail length was measured as the combined length (to the nearest millimeter) of the caudal vertebral series from the tail base to the tail tip (excluding terminal tail hairs). Head and body length was calculated as total length minus tail length. Hindfoot length was measured as the straight-line distance (in millimeters) from the calcaneus to the tip of the longest toe of a fully extended right foot. Ear length was measured as the straight-line distance (in 0.5 mm) from the notch below ear to the tip of the upper edge of the pinna of the right ear.

Cranial measurements were performed on macaques euthanized for study endpoints. Animals were euthanized by overdose of pentobarbitone sodium (100 mg/kg IV; Valabarb, Jurox, New South Wales, Australia) and decapitated, and skin and muscle tissues were removed from the cranium. Specimens were soaked in warm water overnight to facilitate further tissue removal for cranial measurement determination by using a sliding caliper. Greatest length of skull was measured from the most anterior point on the rostrum to the most posterior point on skull. Postrostral length was measured from the most inferior point on either orbital margin to the most posterior point on skull. Rostral length was measured as the most anterior point on the rostrum to the most inferior point on either orbital margin. Zygomatic breadth was measured as the distance between the most lateral points on the zygomatic arches.

Relative morphometrics<sup>6</sup> based on measurements above were calculated as follows:

Relative tail length = tail length / head and body length × 100

Relative hind foot length = hindfoot length / head and body length × 100

Relative ear length = ear length / head and body length × 100

Relative zygomatic breadth = zygomatic breadth / greatest length of skull × 100

Rostral-postrostral ratio = rostral length / postrostral length × 100

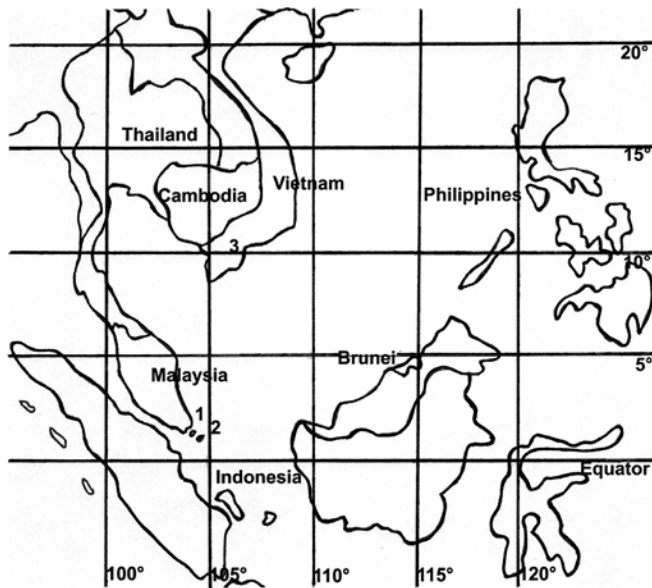
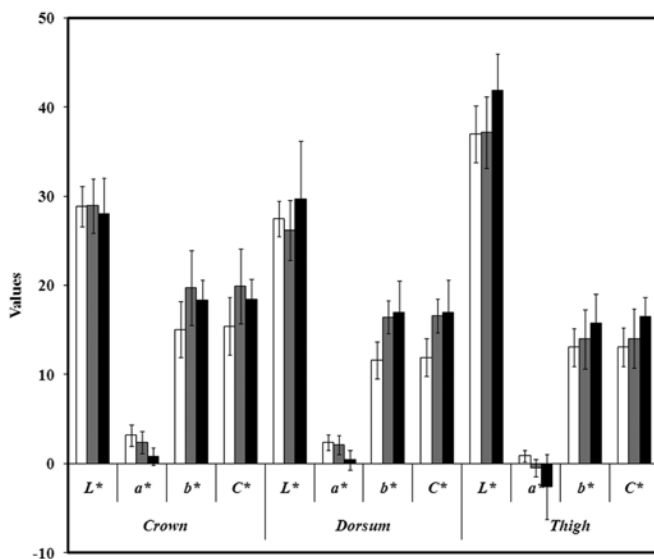
**Statistical analyses.** Data analyses were performed by using Minitab 15.1.20.0 (State College, PA). Means and standard deviations were calculated for male and female *M. fascicularis* of each geographic population. For pelage characterization, we applied ANOVA and principal components analysis to identify data patterns showing similarities and differences, by using established guidelines for interpreting differences.<sup>13</sup> Cluster analysis was applied to population means of pelage variables to acquire dendrograms based on Euclidean distance measures.<sup>12</sup> Finally, we used *t* tests to determine significant differences in morphometrics between groups according to sexual dimorphism and origin. Significance was defined as a *P* value of less than 0.05 for both ANOVA and *t* tests.

## Results

**Pelage characterization.** Figure 2 provides means and standard deviations of examined pelage variables. Vietnamese macaques had the lightest and most vivid pelage, except for a dark crown, which was similar to that of Bintan and Singapo-

**Table 1.** Cynomolgus macaques (*M. fascicularis*) evaluated in the present study

Geographic distribution	Pelage characterization		Body weight and morphometrics	
	Male	Female	Male	Female
Singapore	14	17	38	34
Bintan, Indonesia	5	45	0	37
Long Thahn, Vietnam	10	0	15	0

**Figure 1.** Map of Southeast Asia showing the areas sampled in the study. Cynomolgus macaques from Singapore (1); Bintan, Indonesia (2); and Long Thanh, Vietnam (3) all belong to the nominotypical species *M. f. fascicularis*.**Figure 2.** Means and standard variations of pelage variables of cynomolgus macaques (*M. fascicularis*) from Bintan (white bars,  $n = 50$ ), Singapore (gray bars,  $n = 31$ ) and Vietnam (black bars,  $n = 10$ ), where  $n =$  number of samples. Readings were taken at the vertex of the head, interscapular dorsum and lateral left thigh  $L^*$  = lightness, ranging from darkest (0) to lightest (100);  $a^*$  = green (-60) to red (+60);  $b^*$  = blue (-60) to yellow (+60) and chroma calculated as  $\sqrt{a^{*2} + b^{*2}}$ .

rean macaques but with a prominent crown crest (Figure 3). In contrast to Vietnamese macaques, Bintan and Singaporean *M. fascicularis* had darker dorsal and thigh coats.

Principal components analysis resulted in 12 variables, with the first 5 principal components accounting for 88% of the total variability of pelage phenotypes (Table 2). Therefore, most of the data structure could be captured in 5 underlying dimensions, with the remaining dimensions being less important.  $b^*$  and chroma of crown and dorsal pelage were the marker variables because they accounted for much of the total variance of the first component, with  $L^*$  and chroma of the thigh being less reliable. The second component was a contrasting level of all 3  $a^*$  values with the rest of the variables except for  $b^*$  and chroma of the thigh.

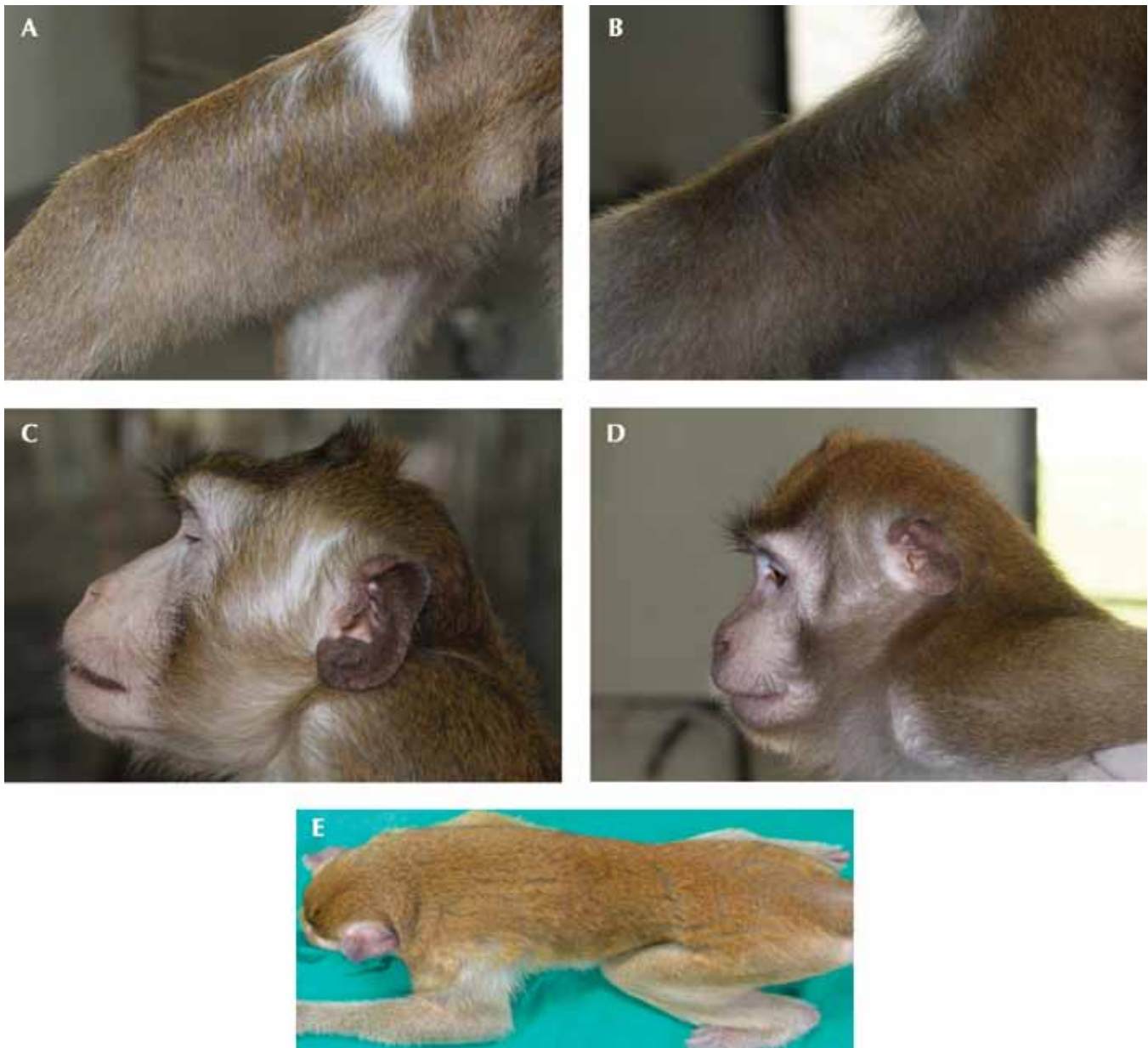
Dendograms based on the Euclidean distances of pelage variables mean values were generated (Figure 4). Overall pelage characterization differences grouped Bintan and Singaporean *M. fascicularis* together but close to the Vietnamese population. We attributed this assignment to the high distance level of the Vietnamese macaques' coat color lightness. Pelage color saturation grouped Singapore and Vietnam macaques together, because Bintan macaques were at a distant level of 6.56, thus lowering the overall distance of Vietnamese cynomolgus macaques based on pelage phenotypes.

**Body weight and morphometrics.** Figure 5 provides the means and standard deviations of the morphometrics. Size phenotypes were analyzed differently than were pelage phenotypes primarily because of macaque sexual dimorphism, which was most marked for Singaporean macaques at the greatest length of the cranium (male:female = 1.27) and body weight (male:female = 1.35; data not shown). Although the postrostral length of the male craniums was shorter than that for female, relative postrostral length was still higher for male than female macaques.

The total body length of Singaporean female macaques was 5% ( $P < 0.05$ ) longer than that of Bintan females because of longer tails (10% longer) and not because of head and body length (13% shorter). Hindfoot length, ear length, and body weight were not significantly different between these 2 populations of female macaques. Meanwhile, Vietnamese adult male macaques were significantly ( $P < 0.05$ ) heavier (by as much as 44%) and larger than their Singapore counterparts in all parameters, especially ear length (22% longer), hindfoot length (20%), and head and body length (21%). Postrostral length was not significantly longer for Singaporean than Vietnamese male macaques. Overall body proportions indicated relative total length was significantly ( $P < 0.05$ ) longer for Singaporean than Vietnamese male macaques.

## Discussion

Present-day phenotypes of mammals evolved due to adaptations to environmental settings and constraints, phylogenetic inertia (influence of an ancestor on a descendent), and drift.<sup>4</sup> Their study, including that of pelage characterization<sup>12</sup> and morphometrics, entails geomorphologic considerations, especially for mammals with wide inter- and intraspecific ranges, such as macaques. Singapore and Bintan are shallow-water fringed islands (within 120-m bathymetric line),<sup>6</sup> less than 40 km from each other. Where Bintan is almost 400 m above sea



**Figure 3.** (A) Vietnamese male macaque of lighter lateral left thigh pelage. (B) Singaporean male macaque of darker lateral left thigh pelage. (C) Vietnamese male macaque with a prominent crest to the head crown. (D) Singaporean male macaque with a less prominent crest to the head crown. (E) Vietnamese male macaque with the lightest dorsal pelage examined.

level, Singapore is predominantly low, with more than 60% of the land surface below 30 m, and only 10% above 30 m.<sup>21</sup> Dong Nai province is a part of a zoogeographic core-area, composed of mainland Southeast Asia, Sumatra, Borneo, and Java, all interconnected by dry land approximately 18,000 y ago.<sup>6</sup> It is topographically a plain and flat land with rare scattered 200- to 800-m mountains and gradual declivity in the southward direction.<sup>26</sup> The 3 geographic areas sampled all belong to a stable block termed Sundaland, which refers to the Sunda shelf geologic area, that is, the Thai–Malay Peninsula south of the Isthmus of Kra, the larger islands of Sumatra, Borneo, Palawan and Java, and many smaller islands. Sundaland emerged during periods of low sea level, particularly during the 2.4-million-year Quaternary period, when sea levels were at least 120 m below present-day levels. The taxonomically diverse nonhuman primates on Sundaland comprise 27 species that exhibit relatively high levels of provinciality and endemism.<sup>14</sup>

Here, we disregarded unnatural constraints that typically affect the expression of phenotypes. In particular, rearing conditions (laboratory versus free-ranging), including the ‘nutritionally balanced’ commercial diet given to animals maintained in animal facilities, were not considered because of 2 reasons. One, the Singaporean and Bintan macaques studied, although wild-caught, have been maintained in their respective colonies for years and received commercial diets since capture. Two, all 3 groups were housed in outdoor colonies. Although there were differences in housing dimensions, it is important to note that caged animals—even those given commercial diets—tend to be smaller and have shorter morphometrics compared with free-ranging animals, as in the case of rhesus macaques.<sup>23</sup> This association is especially true for primates, given that rearing conditions affect the age at and sequence in which the epiphyses of bones fuse.<sup>15,23</sup> Another factor we disregarded was the macaques’ pathogen status, because even though some might

**Table 2.** Principal component analysis (eigenanalysis of the correlation matrix)

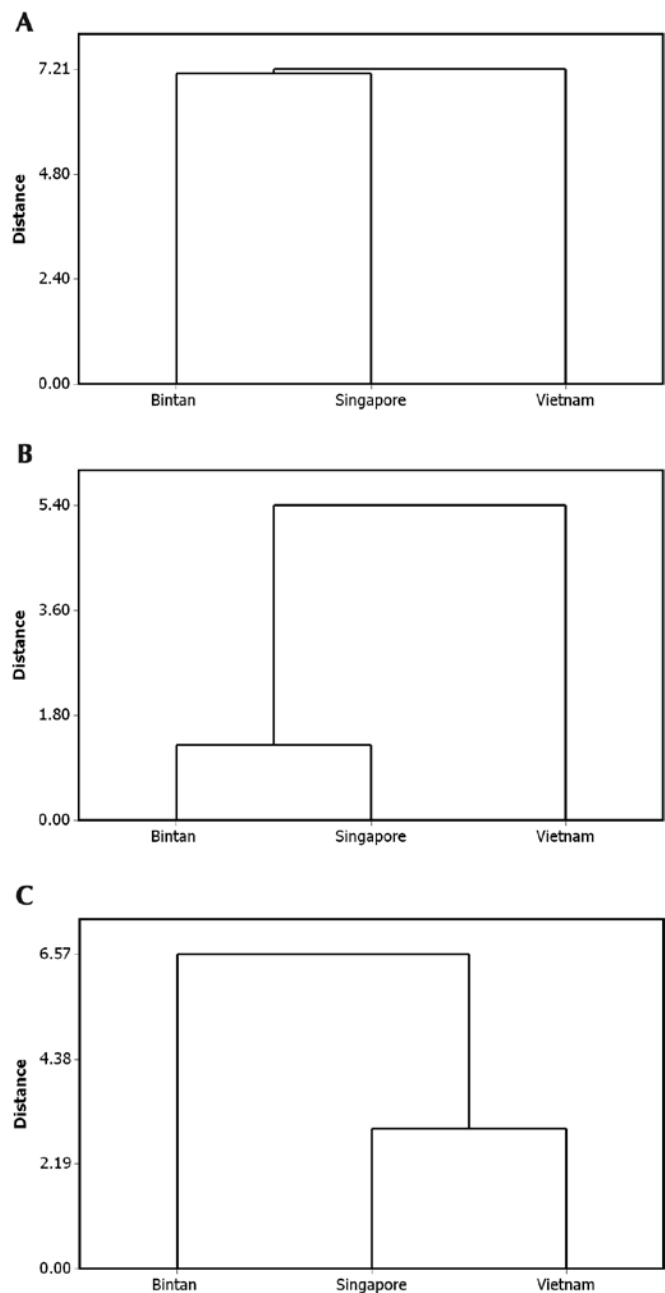
	PC1	PC2	PC3	PC4	PC5
Variance	3.94	2.55	1.87	1.14	0.98
Proportion	0.33	0.21	0.16	0.10	0.08
Cumulative	0.33	0.54	0.70	0.80	0.88
<b>Crown</b>					
L*	0.20	-0.15	-0.27	-0.43	0.50
a*	-0.05	0.31	-0.48	-0.34	-0.33
b*	0.43	0.05	-0.23	0.02	-0.35
Chroma	0.43	0.07	-0.25	0.00	-0.36
<b>Dorsal</b>					
L*	0.11	-0.37	0.07	-0.57	-0.04
a*	-0.06	0.42	-0.38	0.07	0.30
b*	0.45	0.07	0.06	0.28	0.22
Chroma	0.45	0.08	0.05	0.28	0.23
<b>Thigh</b>					
L*	0.21	-0.25	-0.19	-0.10	0.34
a*	-0.22	0.45	-0.13	-0.09	0.28
b*	0.19	0.41	0.41	-0.28	0.04
Chroma	0.20	0.33	0.46	-0.34	-0.04

Principal components (PC) or loadings of  $\geq |0.30|$  are marker variables; loadings of  $< |0.30|$  are less important and probably can be discarded. Negative and high loadings indicate variables that are inversely related to an underlying component. b\* and chroma of crown and dorsal pelage were assigned as marker variables because they accounted for much of the total variance of PC1, with L\* and chroma of the thigh being less reliable.

have been seropositive for the infectious diseases listed earlier, all macaques were clinically healthy during study period. In addition, the animals were regularly dewormed to prevent parasitism.

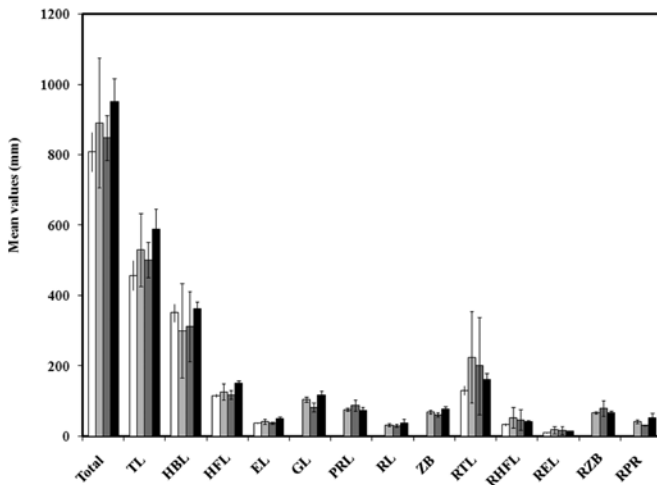
Pelage color is generally similar among adult, subadult, and juvenile male and female *M. fascicularis*.<sup>6,12,14</sup> The infant black natal coat is lost by 2 to 3 mo of age, and the progression to adult pelage is complete by 1 y.<sup>6,18</sup> Adults have gray to red-brown fur and light underparts,<sup>17</sup> with females having beards and males having cheek whiskers.<sup>18</sup> *M. f. fascicularis* has been described as having buff (light gray) to dark brown dorsal and thigh pelage and a golden-brown crown.<sup>6</sup> The results of the current study support this earlier description in regard to a nonerythristic specimen (erythristm defined as bright burnt-orange dorsal fur coloration) with dark crown from southern Vietnam and an intensely erythristic specimen from near Singapore and Bintan. Vietnamese macaques are pale yellowish-brown with a clearly defined dark crown resulting from a reduction to absence of pale crown hair bands, in contrast to most macaques, which possess a more brightly colored crown.<sup>6</sup> The dark color of Singaporean and Bintan macaques might correspond to the coat color evolution of terrestrial (or forest-floor-living) macaques, with a darker environment yielding a darker body color,<sup>12</sup> especially considering that Singapore was a closed tropical rainforest prior to the 1800s.<sup>21</sup>

Taken collectively, our pelage characterization data supports the Gloger rule, in which warm-blooded animals living in warmer, humid climates develop more melanin pigment,<sup>10</sup> resulting in a tendency for darker, more heavily pigmented skin and coat near the equator and lighter skin and coat pigmentation farther from it. Therefore, the original function of coat color

**Figure 4.** Dendrograms calculated for (A) all pelage variables, (B) value, and (C) chroma.

as camouflage against predation (mainly by felids) is replaced by thermoregulation with the coat color of equatorial island populations turning dark,<sup>25</sup> indicating melanism's importance for temperature regulation in humid habitats, such as tropical forests. The pelage characterization differences can also be explained by the phylogeographic history. It is important to note that pelage color may not remain static throughout an animal's life, with pigmentation loss occurring from stress, age, or illness.

Our lack of Bintan male and Vietnamese female macaques precluded detailed statistical comparisons of morphometrics, especially given that macaques exhibit a pronounced sexual dimorphism<sup>4,6</sup> most evident in body size and weight and resulting from different durations of growth between sexes.<sup>25</sup> On average, male *M. fascicularis* weigh 4.7 to 8.3 kg, whereas female *M. fascicularis* weigh 2.5 to 5.7 kg.<sup>5</sup>



**Figure 5.** Means and standard deviations of morphometric variables of cynomolgus macaques (*M. fascicularis*) from Bintan (females: white bars,  $n = 37$ ), Singapore (males: light gray bars,  $n = 38$ ; females: dark gray bars,  $n = 34$ ), and Vietnam (males: black bars,  $n = 15$ ). Total, total length; TL, tail length; HBL, head and body length; HFL, hindfoot length; EL, ear length; GL, greatest length of skull; PRL, postrostral length; RL, rostral length; ZB, zygomatic breadth; RTL, relative tail length; RHFL, relative hindfoot length; REL, relative ear length; RZB, relative zygomatic breadth; and RPR, rostral–postrostral ratio. Note: Bintan samples lack cranial measurements.

Our results suggest geographic differences in morphometrics follow the Bergmann rule, in which warm-blooded animals from warm regions are smaller than those from cold regions.<sup>10</sup> Rhesus macaques inhabiting the Indochinese peninsula have been reported to be *M. fascicularis* hybrids,<sup>7</sup> with molecular studies indicating a recent introgression between the 2 species.<sup>22</sup> Vietnamese longtailed macaques may have acquired morphometric phenotypes similar to those of rhesus macaques, that is, a larger body size. Interestingly, this assumption might not be true for the regions of the peninsular core-area north of about 13°N, where skull length in both sexes decreases northward contrary to the Bergmann rule.<sup>9</sup> This retrograde tendency was explained as resulting from evolutionary interactions between *M. fascicularis* and other unspecified element(s) of the peninsula's rich nonhuman primate diversity and might explain our results of a shorter postrostral length for Vietnamese macaques, given that postrostral length is being directly proportional to the greatest length of the skull. Still, the rostral:postrostral length ratio of Vietnamese male macaques was the highest among the populations we evaluated, indicating the variable's sexual and geographic differences, the former probably resulting from both a higher rate and greater duration of allantoic growth of the rostrum in males<sup>9</sup> and the latter because of the Bergmann rule and interspecies introgression. It would be interesting to obtain cranial measurements from female Vietnamese and Bintan *M. fascicularis*. Bintan cynomolgus macaques apparently have significantly smaller cranial sizes than do those from the rest of the Riau Archipelago<sup>9</sup> and perhaps including nearby Singapore.

Variation patterns generally indicated positive allometry (larger individuals of a species have proportionally larger traits), with Vietnamese macaques having longer morphometric variables (including cranial measurements) than did Singaporean (and likely Bintan) macaques. These include the length of the nonprehensile tail, which in *M. fascicularis* is equal to or longer than the head and body length.<sup>17</sup> Tail length, although contributing much to the animal's total length, was initially found to follow a geographic cline, in which northern populations had

shorter tails than did southern populations.<sup>11</sup> In addition, our data show that *M. fascicularis* possibly has a tendency toward insular dwarfism, the process and condition of size reduction among large animals, especially mammals, when their gene pool is limited to a very small environment, like islands. Size differences in *M. fascicularis* populations on islands presumably indicate the effect of postglacial isolation, whereas the differences in the core-area represent quasi-continental variation.<sup>9</sup>

In conclusion, we have identified phenotypic differences of *M. fascicularis* used in Singapore's biomedical research industry by using standard primatology tools and in relation to their geographic origins. Although these macaques belong to the same subspecies, variation in the pattern of their pelage color and morphometrics are evident and can be explained by established biologic principles.

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