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Mitochondrial DNA sequence evolution in the Arctoidea

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ABSTRACT **Some taxa in the superfamily Arctoidea, such as the giant panda and the lesser panda, have presented puzzles to taxonomists. In the present study, ≈397 bases of the cytochrome *b* gene, 364 bases of the 12S rRNA gene, and 74 bases of the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes from the giant panda, lesser panda, kinkajou, raccoon, coatimundi, and all species of the Ursidae were sequenced. The high transition/transversion ratios in cytochrome *b* and RNA genes prior to saturation suggest that the presumed transition bias may represent a trend for some mammalian lineages rather than strictly a primate phenomenon. Transversions in the 12S rRNA gene accumulate in arctoids at about half the rate reported for artiodactyls. Different arctoid lineages evolve at different rates: the kinkajou, a procyonid, evolves the fastest, 1.7–1.9 times faster than the slowest lineage that comprises the spectacled and polar bears. Generation-time effect can only partially explain the different rates of nucleotide substitution in arctoids. Our results based on parsimony analysis show that the giant panda is more closely related to bears than to the lesser panda; the lesser panda is neither closely related to bears nor to the New World procyonids. The kinkajou, raccoon, and coatimundi diverged from each other very early, even though they group together. The polar bear is closely related to the spectacled bear, and they began to diverge from a common mitochondrial ancestor ≈2 million years ago. Relationships of the remaining five bear species are derived.**

The Arctoidea (Canoidea) is an extant superfamily of Carnivora that contains four families: Canidae, Ursidae, Procyonidae, and Mustelidae (1). The relationships of some arctoid carnivores, such as the giant panda and lesser panda, are a continuing controversy to taxonomists.

The giant panda is a specialist bamboo feeder and might well be the most popular wild animal worldwide. Is the giant panda a bear; is it, like the lesser panda, a member of the Procyonidae (raccoon family); or is it in its own family? On the basis of comparative anatomical studies, immunological distances, DNA hybridization, isozyme electrophoresis, karyological evidence, and palaeontological information, the giant panda has been classified into the Ursidae, the bear family (2–6). However, the giant panda shows differences from bears in its genital structure, behavior, hemoglobin sequences, and restriction fragment length polymorphisms of mitochondrial DNA (mtDNA) that indicate that it is not closely related to bears (1, 7–9).

The lesser panda has been variously placed in the Procyonidae, in its own family (Ailuridae), or in the Ursidae or allied with the giant panda (1, 10–12).

Classification of ursids at the generic and species level remains controversial. For example, seven species are usually recognized and organized into from two to six genera (1, 12, 13).

In the present study, we sequenced segments of mitochondrial cytochrome *b*, 12S rRNA, $tRNA^{Pro}$, and $tRNA^{Thr}$ genes (a total of 835 bp) and examined the relationship among all species of the Ursidae, the giant panda, the lesser panda, and three representatives of procyonids (coatimundi, kinkajou, and raccoon).†

MATERIAL AND METHODS

Samples. The giant panda (*Ailuropoda melanoleuca*) and American black bear (*Euarctos americanus*) samples were provided by R. Montali (National Zoological Park, Washington, DC) and F. Allendorf (University of Montana, Missoula, MT), respectively. The lesser panda (*Ailurus fulgens*), kinkajou (*Potos flavus*), coatimundi (*Nasua narica*), raccoon (*Procyon lotor*), spectacled bear (*Tremarctos ornatus*), Asiatic black bear (*Selenarctos thibetanus*), brown bear (*Ursus arctos*), polar bear (*Thalarctos maritimus*), sun bear (*Helarctos malayanus*), and sloth bear (*Melursus ursinus*) samples were collected at the San Diego Zoo.

DNA Isolation and Nucleotide Sequencing. Total genomic DNA was isolated from cultured fibroblasts, muscle, liver, and kidney tissues by methods adapted from Lansman *et al.* (14). Conserved primer pairs L14724/H15149 (15), L1091/H1478, and L15926/H00651 (16) were used to amplify segments of cytochrome *b*, 12S rRNA genes, and the D-loop region (containing segments of $tRNA^{Pro}$ and $tRNA^{Thr}$ genes) by PCR. Double-stranded PCR amplifications were performed for 30–40 cycles by using cloned *Pyrococcus furiosus* DNA polymerase (Stratagene) according to the manufacturer's instructions, with denaturation for 45 s at 95°C, annealing for 1 min at 40–50°C, and extension for 1–4 min at 73°C. PCR products were purified in 1.5–2.0% SeaPlaque agarose (FMC). The amplification product to be sequenced was excised from the gel, melted in STE (0.1 M NaCl/10 mM Tris·HCl/1 mM EDTA, pH 8.0) at 70°C, and then extracted once with phenol/chloroform. DNA was precipitated with 2 vol of ethanol at −20°C and suspended in water. Double-stranded DNA was directly sequenced with Sequenase Version 2.0 (United States Biochemical) using heat denaturation. Both the original PCR primers and internal primers could be used as sequencing primers. Our procedure was based on the observation that adding the Sequenase buffer after the heat denaturation ensured a good sequencing reaction. Briefly, 7 μl of gel-purified DNA was annealed with 1 μl of sequencing primer (0.5 pmol/μl) by heating at 98°C for 5 min, followed by rapid cooling on ice; 5× Sequenase buffer (2 μl) was added to the mixture, followed by labeling, termination, and stopping reactions performed according to the protocol provided by the manufacturer. Products of these reactions were electrophoresed on a 7% polyacrylamide/50% urea gel, dried, and subjected to autoradiography.

All sequences were aligned using PC/GENE program Version 6.6 (IntelliGenetics) and checked by eye. Because of the difficulty of aligning segments caused by too many length variations, D-loop regions were deleted from the data set, and

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L21859–L21891, L22070, L22163, and L22164).

only cytochrome *b*, 12S rRNA, and tRNA gene sequences were used in the phylogenetic analysis.

Phylogenetic Analysis. The variable nucleotide positions and insertions/deletions were analyzed using PAUP Version 3.0 (17). All nucleotide-substitution characters were specified as unordered. Insertions/deletions were coded as single characters, regardless of length. Two pairs of outgroups (bovine and zebra sequences and bovine and horse sequences) were used for rooting the phylogenetic trees (refs. 15 and 18 and U. Arnason, personal communication), since suitable sequences from carnivore species had not been found.

Branch-and-bound searches were performed to ensure that all minimum-length trees were identified (17).

Bootstrap analyses consisted of 100 heuristic replications. As many as 100 trees were held for each bootstrap replication (17).

Different weighting approaches were employed for cytochrome *b* and RNA genes, as described in the *Results* and *Discussion*.

Relative-Rate Test. The number of nucleotide substitutions per site (K) was calculated with Kimura's two-parameter method employing DNADIST program in PHYLIP Version 3.4 (19). The bovine sequence (18) was used as outgroup (C) to compute the differences in the number of substitutions per site between taxa A and B ($K_{AC} - K_{BC}$), using the formulas in Li (20), where K_{AC} and K_{BC} are the numbers of substitutions per site between taxa A and C and between taxa B and C, respectively.

RESULTS AND DISCUSSION

Approximately 397 bases of cytochrome *b* gene (nt 14,514–14,910), 364 bases of 12S rRNA gene (nt 884–1233), and 74 bases of tRNA genes (nt 15,721–15,791) were sequenced for each sample. Unless otherwise stated, position numbers in parentheses indicate sequences corresponding to the bovine mtDNA sequences (18). Our sequences have been aligned with a limited amount of length variation.

Sequence Variation. There are 166, 91, and 31 sites in cytochrome *b*, 12S rRNA, and tRNA gene regions characterized with substitution, respectively. There are 27 and 2 sites in 12S rRNA and tRNA gene regions characterized with insertion/deletion, respectively, and no insertion/deletion was observed in cytochrome *b* gene region.

The pattern of variation at the different codon positions of cytochrome *b* gene is generally similar to that previously reported (15). However, the ratio of substitution rates at the first and second positions of codons shows greater variation, ranging from 2 to 13 times, with an average of 5.

One of the interesting properties of vertebrate mtDNA is the mutational transition bias. This transition bias is known to decrease over time, with increasing sequence divergence, until an asymptotic value is reached. Allard and Honeycutt (21) suggested that at least for the 12S rRNA gene, the presumed transition bias observed for mammalian mtDNA divergence (22, 23) might represent a primate phenomenon rather than a general trend for all mammalian lineages. In the present study, however, the transition/transversion ratios for most pairwise comparisons of bear taxa at the third positions of codons in cytochrome *b* gene are very high, ranging from 6.0 to 28.0, and the ratios of $\approx$1.0 are reached between carnivore and perissodactyl taxa, presumably as a result of saturation. A similar pattern was observed in the 12S rRNA gene: prior to saturation, the transition/transversion ratios at low levels of divergence ($d < 10$) range from 3.1 to 16, much higher than those of artiodactyl and rodent comparisons at similar levels of divergence. Similar to primates, arctoids show greater variation in their transition/transversion ratio than do either artiodactyls or rodents. Our results suggest that the presumed transition bias may represent a trend for some mammalian lineages rather than just a primate phenomenon.

Stem regions and loop regions of mitochondrial rRNA and tRNA genes are subject to different selective and structural constraints (18, 24). Stem regions and loop regions of the 12S rRNA gene and the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes of our Arctoidea sequences were assigned according to the secondary-structure models of bovine 12S mitochondrial rRNA and mitochondrial tRNA (18, 25). The loop regions are found to be $\approx$1.4 times more variable than the stem regions. In addition, the majority of insertions/deletions occur in loop regions. Anderson *et al.* (18) noticed that transition/transversion bias was especially marked in the stem regions of tRNA genes. They suggested that the constraints of certain structural requirements favored transitions, since these allowed either Watson–Crick base pairs to mutate to the other via a G-U or A-C intermediate. Since functional RNA genes, from the small tRNAs to much larger 18S and 26S rRNAs, exhibit analogous secondary structures that rely on base pairing between nucleotide positions (26), Wheeler and Honeycutt (24) suggested that those RNA molecules would also show a similar mode of evolution. They proposed that to maintain secondary Watson–Crick base-pairing structure, if one substitution was fixed, then another complementary substitution was positively selected to ameliorate the negative effects of the first. These proposals are consistent with some of our data from arctoid RNA genes. In arctoid tRNA genes, there are 6 sites characterized by transversions out of 15 variable sites in the loop regions, whereas there is only one site characterized by transversion out of 20 variable sites in stem regions. In addition, several compensatory changes were observed in the stem regions (e.g., positions 15,751 and 15,768). In the 12S rRNA gene, many compensatory changes can also be inferred from stem regions (e.g., positions 1179–1182 and 1193–1196). However, some sites do not show structural constraint according to the proposed secondary structures of bovine RNAs (e.g., positions 889 and 904). This may be a result of either inaccurate secondary-structure models for divergent mitochondrial RNA molecules or limited functional importance of some stem nucleotide pairings.

Parsimony Trees. Because all the functional RNA gene sequences may show a similar model of evolution, we have combined 12S rRNA and tRNA gene sequences into one data set.

Previous work has shown that transversions accumulate linearly with time in the rRNA gene of artiodactyls (27). Transversions at third codon positions and both transitions and transversions at first and second codon positions accumulate in an approximately linear manner with time in the cytochrome *b* gene of mammals (15). Thus, parsimony analysis was performed by using transversions at the third codon positions and both transversions and transitions at the first and second codon positions for cytochrome *b* sequences and only transversions for RNA gene sequences.

The strict consensus tree (plus other groups supported by >70% bootstrap replications and topologically consistent with this tree) of the 4 shortest trees found using cytochrome *b* sequences is shown in Fig. 1*a*. The total steps for each of the shortest trees are 187. The strict consensus tree of the 13 shortest trees found using RNA gene sequences is shown in Fig. 1*b*. The length for each of the shortest trees is 67. These two trees are similar. It is interesting that the kinkajou–coatimundi branch in the strict consensus tree of parsimony data from RNA genes is weakly supported by bootstrap replications (Fig. 1*b*). Thus, the phylogenetic results of cytochrome *b* and RNA genes are generally consistent.

Because the rate of nucleotide substitution at second positions is $\approx$20% the rate of first-position replacements or third-position transversions in the arctoid cytochrome *b* gene

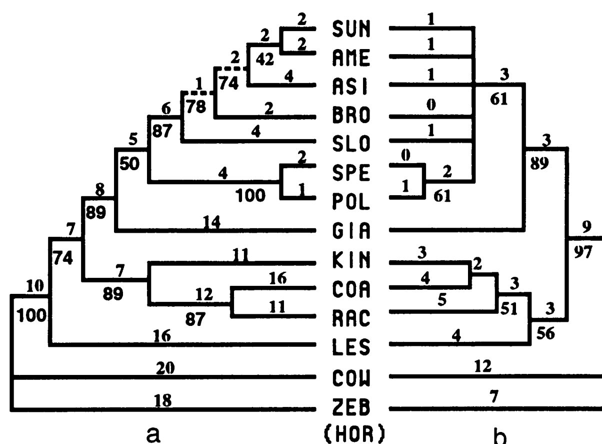

FIG. 1. Strict consensus trees of the shortest trees found using the branch-and-bound search in PAUP based on conservative changes (17). (*a*) Cytochrome *b* gene tree using replacements at the first and second positions of codons and transversions at the third position of codons and bovine and zebra sequences as outgroups. (*b*) 12S rRNA, $tRNA^{Pro}$, and $tRNA^{Thr}$ gene trees using transversions and bovine and horse sequences as outgroups. Groups compatible with the strict consensus tree and supported by the majority of bootstrap replications are indicated by dashed lines. Branches supported by ≥40% of bootstrap replications are indicated below the branches. The lengths of each branch are indicated above the branch. The consistency indexes of trees in *a* and *b* are 0.647 and 0.761, respectively. SUN, sun bear; AME, American black bear; ASI, Asiatic black bear; BRO, brown bear; SLO, Sloth bear; SPE, spectacled bear; POL, polar bear; GIA, giant panda; KIN, kinkajou; COA, coatimundi; RAC, raccoon; LES, lesser panda; ZEB, zebra; HOR, horse.

region, we have given a weight of 5 for second positions and weights of 1 for both first and third positions according to the suggestion of Irwin *et al.* (15). This weighting results in a strict consensus tree identical to the tree in Fig. 1*a*.

Insertions/deletions combined with transversions in RNA genes were also used for parsimony analysis. The resulting strict consensus tree of most parsimonious trees is identical to that in Fig. 1*b*. To test the influence of tRNA gene sequences, only transversions in 12S rRNA gene regions were used, but the identical strict consensus tree again resulted.

Wheeler and Honeycutt (24) suggested that, because of the character covariance, nucleotide substitutions in stems could not be interpreted as independent evolutionary events and should be either down-weighted by 50% or disregarded altogether. Compensatory changes in stem regions can be inferred from our RNA gene sequences, even though many taxa do not show perfect Watson–Crick base pairing. When stem regions were down-weighted by 50% to determine the influence of compensatory changes, the strict consensus tree is again identical to that in Fig. 1*b*.

A 12S rRNA gene fragment (28) from the tiger, a felid carnivore, was tested to root the phylogenetic tree in an attempt to resolve the uncertain placements in Fig. 1*b*. However, an identical strict consensus tree resulted.

Reanalysis of a portion of our data, including only bears and rooting with the giant panda, resulted in the identical strict consensus trees for both cytochrome *b* gene and RNA genes.

Evolutionary Rates of Cytochrome *b* Gene and 12S rRNA Gene. Placing a clock on molecular data is still a difficult problem. Sequence comparison of mtDNA genes suggests that transversions within artiodactyl rRNA genes accumulate in a linear manner with a rate of 0.2% per million years (27). Transversions at the third positions of mammal cytochrome *b* gene also accumulate nearly linearly, at a rate of 0.5% per million years (15). When we use these rates in the Arctoidea, however, divergence times based on cytochrome *b* gene are about twice as high as those based on 12S rRNA gene.

According to the fossil record, the oldest procyonids are believed to date to the early Oligocene, and the common ancestral forms of raccoons and kinkajou date back to the earliest Miocene (29). The fossil precursor of the giant panda *Agriarctos* occurred in the mid-Miocene (29, 30). The first tremarctine bear appears in the late Pliocene, and the divergence of this lineage from ursine bears probably dates to the mid- to late Miocene. The common ancestor of ursine bears appears in the Old World during the early Pliocene, 4–5 million years ago (31). The divergence times estimated on the cytochrome *b* gene clock agree with those of the fossil record. Our results suggest that artiodactyl rRNA genes evolve at about twice the rate of arctoid rRNA genes.

Relative Rate of Different Lineages in the Arctoidea. When the bovine sequence was used as an outgroup in the relative-rate test (18), the results show that the different arctoid lineages evolve at differing rates (Table 1). The kinkajou evolves the fastest, 1.7–1.9 times faster than the slowest lineage (spectacled and polar bears); the ursine bear lineage (sun, Asiatic black, American black, brown, and sloth bears) evolves ≈1.4 times faster than the spectacled bear lineage. To verify that the rate differences observed in our study represent a common phenomenon in the Arctoidea, more sequences should be investigated, and a more closely related outgroup should be employed.

Generation-time effect has been used to explain the higher substitution rates in monkeys than in humans and the higher rates in rodents than in primates (32). Our results may be explained only partly by this effect. The differences in substitution rates could be partly due to differences in the efficiency of the DNA repair system (33). As far as we know, there is no evidence indicating that the efficiency of DNA repair system varies in different arctoid lineages.

Our results suggest that caution should be exercised in using genetic distance data to infer phylogenetic trees in arctoid taxa.

Phylogenetic Relationships. Many questions about the phylogeny of the Arctoidea are still open. One of the interesting

Table 1. Differences in the number of nucleotide substitutions per site (K_{AC}–K_{BC}) between different taxa of Arctoidea

Sequence	Species A	Species B	K_{AB}	K_{AC}–K_{BC}
Cyt *b* gene	Sun bear	Kinkajou	0.216	−0.041
	Spectacled bear	Kinkajou	0.243	−0.056
	Giant panda	Kinkajou	0.251	−0.018
	Lesser panda	Kinkajou	0.218	−0.045
	Raccoon	Kinkajou	0.210	−0.021
	Coatimundi	Kinkajou	0.216	−0.016
	Sun bear	Spectacled bear	0.157	0.015
12S rRNA gene	Sun bear	Kinkajou	0.128	−0.030
	Spectacled bear	Kinkajou	0.145	−0.044
	Giant panda	Kinkajou	0.151	−0.017
	Lesser panda	Kinkajou	0.132	−0.012
	Raccoon	Kinkajou	0.122	−0.024
	Coatimundi	Kinkajou	0.108	−0.045
	Sun bear	Spectacled bear	0.085	0.015

The bovine sequence (18) was used as an outgroup (C). K_{AB} is the number of substitutions per site between taxa A and B. K_{AC} and K_{BC} are as defined in *Material and Methods*.

questions is the systematic position of the giant panda. Based on a detailed analysis involving a large number of anatomical characters, Davis (2) showed that the giant panda is a bear and suggested that convergent selection pressures must have been responsible for the seeming similarities between the two pandas. The fossil record shows that fossil bears fall into two groups, one giving rise to the large bears and the other branch giving rise to a number of genera and species that have since become extinct, leaving the giant panda as the last survivor. The lesser panda, through the Miocene genus *Sivanasua*, is more closely related to the New World procyonids than it is to the bears (including the giant panda) (for review, see ref. 34). The earlier (3) and recent (4, 35) studies involving chromosomal analysis, DNA hybridization, isozyme genetic distance, immunological distance, and two-dimensional protein electrophoresis support the viewpoint of Davis (2). However, on the basis of behavioral similarities between the giant panda and the lesser panda, some authorities have favored the two-panda viewpoint (1, 7). Support for the close evolutionary affinity of the two pandas has also been derived from hemoglobin amino acid sequence data and restriction fragment length polymorphisms of mtDNA (8, 9).

Our parsimony analysis indicates that the giant panda is more closely related to bears than to the lesser panda and the procyonids. It should be noted, however, that if we translate cytochrome *b* gene sequences into amino acid sequences by using mitochondrial genetic codes, the single most parsimonious tree groups the giant panda with the lesser panda and kinkajou, which are joined with spectacled and polar bear. Because of the limitation of our sequence data, it is not clear whether this is caused by convergence or other factors. Considering such long divergence time between the giant panda and bears compared with humans to chimpanzees, we favor placing the giant panda in a separate family, the Ailuropodidae (10, 29).

The systematic positions of the lesser panda, kinkajou, raccoon, and coatimundi are still unclear. Current consensus seems to classify the lesser panda into the Ailurinae, a subfamily of the Procyonidae, and the other three species into the Procyoninae, another subfamily of the Procyonidae (1, 12, 36, 37). However, some authorities place the lesser panda in its own family, the Ailuridae, or in the Ursidae (11, 29).

Fossil evidence demonstrates that procyonids already showed considerable heterogeneity in the Miocene, foreshadowing the great diversity of the living survivors. The raccoon, coatimundi, kinkajou, and lesser panda are quite different from each other and could well be divided into several different families or subfamilies (for review, see ref. 34).

The strict consensus tree for our cytochrome *b* sequences indicates that the lesser panda may be a sister group of ursids and procyonids (Fig. 1*a*). In the strict consensus tree for RNA gene sequences, however, the lesser panda groups with procyonids. However, this is supported only by 56% of bootstrap replications (Fig. 1*b*). It also should be pointed out that in the RNA gene regions the average number of transversions between the lesser panda and bears and between the lesser panda and procyonids is very close (refs. 12 and 13, respectively). These results suggest that the lesser panda is closely related neither to bears nor to procyonids.

Our sequence data suggest that the raccoon, coatimundi, and kinkajou may represent an early radiation within the Arctoidea.

In considering the combined evidence, it seems reasonable to place the lesser panda in its own family and place the kinkajou, raccoon, and coatimundi in different subfamilies or even in different families. Given the lesser panda is the only member of the family, it is particularly worthy of conservation.

The systematics of the seven true bear species have caused considerable confusion (Table 2). The spectacled bear has been considered a very primitive bear and has been alternatively placed in its own genus or subfamily, distant from other bear species. Mayr (34) discussed two possibilities for the relationships of arctoid hemoglobin sequences. The first explanation has been confirmed by spectacled bear hemoglobin sequence data; the spectacled bear is somewhat intermediate between pandas and other bears (38). As mentioned above, the cytochrome *b* amino acid sequence data resulting from translation of the gene sequences group the spectacled bear lineage with the giant panda rather than with other bears, which may also indicate certain affinity between the spectacled bear lineage and the giant panda.

The relationships among six ursine species have not been resolved in the previous studies. According to the fossil record, the brown, American black, and polar bears arose in the Old World in the early Pliocene. The Asiatic black bear might be the closest living relative of the American black bear, and the polar bear might have evolved from a mid-Pleistocene brown bear (31). To our knowledge, the relationships have not been resolved with any molecular methods reported before (4, 34, 39).

Our mtDNA sequences provide evidence that the polar bear is very closely related to the spectacled bear. Their mtDNA sequences diverge from a common ancestral mtDNA dating ≈2 million years ago. The fossil record and nuclear genomic data separate these two species and place the polar bear close to the other bears. The reason for the difference between data derived from the mitochondrial genome and the nuclear genome is not clear. One possible explanation is that both polar bear and spectacled bear are less diverged from the ancestral mtDNA sequence, and they share most of the primitive features. Indeed, our results show that mtDNAs from these two species evolve more slowly than those from other bears.

The phylogenetic relationships among five ursine bear species can be inferred from our cytochrome *b* gene sequence data (Fig. 1*a*). The ancestor of the sloth bear diverged first,

Table 2. Systematics of the Ursidae

	Genus and species			
Common name	Hall (13) and Nowak (12)	Wozencraft (11) and Thenius (29)	Ewer (1), Morris (36), and Corbet and Hill (37)	Eisenberg (10)
Spectacled bear	*Tremarctos ornatus*	*Tr. ornatus*	*Tr. ornatus*	*Tr. ornatus*
Brown bear	*Ursus arctos*	*U. arctos*	*U. arctos*	*U. arctos*
American black bear	*Ursus americanus*	*U. americanus*	*U. americanus*	*Euarctos americanus*
Polar bear	*Ursus maritimus*	*U. maritimus*	*Thalarctos maritimus*	*Th. maritimus*
Asiatic black bear	*Ursus thibetanus*	*U. thibetanus*	*Selenarctos thibetanus*	*S. thibetanus*
Sun bear	*Ursus malayanus*	*Helarctos malayanus*	*H. malayanus*	*H. malayanus*
Sloth bear	*Ursus ursinus*	*Melursus ursinus*	*M. ursinus*	*M. ursinus*

followed by the brown bear. The sun bear and the American black bear are sister taxa relative to the Asiatic black bear.

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