

Phylogeny of the bears (Ursidae) based on nuclear and mitochondrial genes

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Received 31 May 2003; revised 12 January 2004

Available online 1 April 2004

Abstract

The taxonomic classification and phylogenetic relationships within the bear family remain argumentative subjects in recent years. Prior investigation has been concentrated on the application of different mitochondrial (mt) sequence data, herein we employ two nuclear single-copy gene segments, the partial exon 1 from gene encoding interphotoreceptor retinoid binding protein (IRBP) and the complete intron 1 from transthyretin (TTR) gene, in conjunction with previously published mt data, to clarify these enigmatic problems. The combined analyses of nuclear IRBP and TTR datasets not only corroborated prior hypotheses, positioning the spectacled bear most basally and grouping the brown and polar bear together but also provided new insights into the bear phylogeny, suggesting the sister-taxa association of sloth bear and sun bear with strong support. Analyses based on combination of nuclear and mt genes differed from nuclear analysis in recognizing the sloth bears as the earliest diverging species among the subfamily ursine representatives while the exact placement of the sun bear did not resolved. Asiatic and American black bears clustered as sister group in all analyses with moderate levels of bootstrap support and high posterior probabilities. Comparisons between the nuclear and mtDNA findings suggested that our combined nuclear dataset have the resolving power comparable to mtDNA dataset for the phylogenetic interpretation of the bear family. As can be seen from present study, the unanimous phylogeny for this recently derived family was still not produced and additional independent genetic markers were in need.

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Keywords: Interphotoreceptor retinoid binding protein; Transthyretin; Evolution; Phylogenetic analysis

1. Introduction

The bear family Ursidae includes seven species and has been suggested to consist of two to seven genera (Eisenberg, 1981; Ewer, 1973; Hall, 1981; Nowak, 1991). Even though the closest relative of the giant panda is the bears, it is still controversial if the giant panda is a bear (Davis, 1964; Goldman et al., 1989; Hashimoto et al., 1993; Nash and O'Brien, 1987; Nash et al., 1998; O'Brien et al., 1985; Sarich, 1973; Van Valen, 1986; Wayne et al., 1989; Zhang and Ryder, 1993).

Up to now, the taxonomic classifications and phylogenetic relationships within the *Ursidae* remain subjects of controversies. The main problem is that the family *Ursidae* represents a typical example of rapid evolutionary radiation and recent speciation events, dating back to mid-Miocene about 20 million years ago (Goldman et al., 1989; Kurten, 1968; Waits et al., 1999). For this reason, attempts to clarify relationships among the seven bear species based on a variety of molecular studies have encountered challenge. In particular, attention of these studies has been restricted to mitochondrial DNA (mtDNA) because of its relatively small effective population size and rapid rate of sequence evolution as attractive advantages for the reconstruction of phylogeny in this case. However, though conclusive for the early

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divergence of the spectacled bear relative to other bear species, yet all analyses of mtDNA sequences failed to portray a congruent phylogenetic scenario for species that subsequently evolved within the Ursidae (Talbot and Shields, 1996a,b; Waits et al., 1999; Zhang and Ryder, 1994; see Figs. 1A–F). In addition, the fact that all genes comprising mt genome are inherited as a single, haploid linkage unit has been a well-known limitation on phylogenetic reconstruction because the resulting mt gene trees are unlikely to reflect one independent estimate of the species tree (Giannasi et al., 2001; Johnson

and Clayton, 2000; Moore, 1995; Page, 2000). Hence, future effort should be put into the exploitation of independent sources of phylogenetic characters (Giannasi et al., 2001; Wu, 1991). However, no additional information from nuclear DNA or Y chromosome markers is available so far to expound the taxonomic and phylogenetic issues within the Ursidae family.

In this paper, we are the first to employ nuclear DNA data, that is, exon 1 sequence from the gene encoding interphotoreceptor retinoid binding protein (IRBP) and the intron 1 from the transthyretin (TTR) gene in phylogenetic study of bear family. Both genes are single-copy nuclear protein-coding loci presented in all mammalian genomes and include four exons and three introns (Borst et al., 1988; Duan et al., 1991; Fong et al., 1990; Liou et al., 1989; Tsuzuki et al., 1985; Wakasugi et al., 1985) and have proved to be useful in reconstructing phylogenetic relationships among Carnivoran lineages (Flynn and Nedbal, 1998; Yoder et al., 2003). DNA sequences from these two nuclear genes for all extant bear species, together with previously published mt data, were used here, in both separate and combined analyses, with a view to: (1) gain new insight into the resolution of the evolutionary history of this group, (2) compare evolutionary dynamics between nuclear and mt genes as well as their phylogenetic performance for the estimation of bear phylogeny, and (3) examine the congruence among gene trees based on these two unlinked loci and combined data set.

2. Materials and methods

2.1. DNA samples and PCR amplifications

Sequence data from the first part of exon 1 of the IRBP gene and the first intron of the TTR gene were considered for all the seven species of bears including the spectacled bear (*Tremarctos ornatus*), the sloth bear (*Melursus ursinus*), the American black bear (*Ursus americanus*), the Asiatic black bear (*Ursus thibetanus*), the sun bear (*Helarctos malayanus*), the brown bear (*Ursus arctos*) and the polar bear (*Ursus maritimus*), plus the giant panda (*Ailuropoda melandeuca*) (see Table 1), of which 13 out of 16 were produced for this study. The published TTR intron 1 sequences of spectacled bear, brown bear, and the giant panda were extracted from GenBank (Flynn and Nedbal, 1998). Taxonomic classification of bear species followed Wozencraft (1993). For each species, total genomic DNA was isolated from whole blood or frozen tissues following standard protocols (Sambrook et al., 1989).

Primers were designed to amplify segments corresponding to nucleotides 217–1531 of IRBP gene of human sequence (Stanhope et al., 1992; see Fig. 2) and nucleotides 635–1628 of TTR gene of human sequence

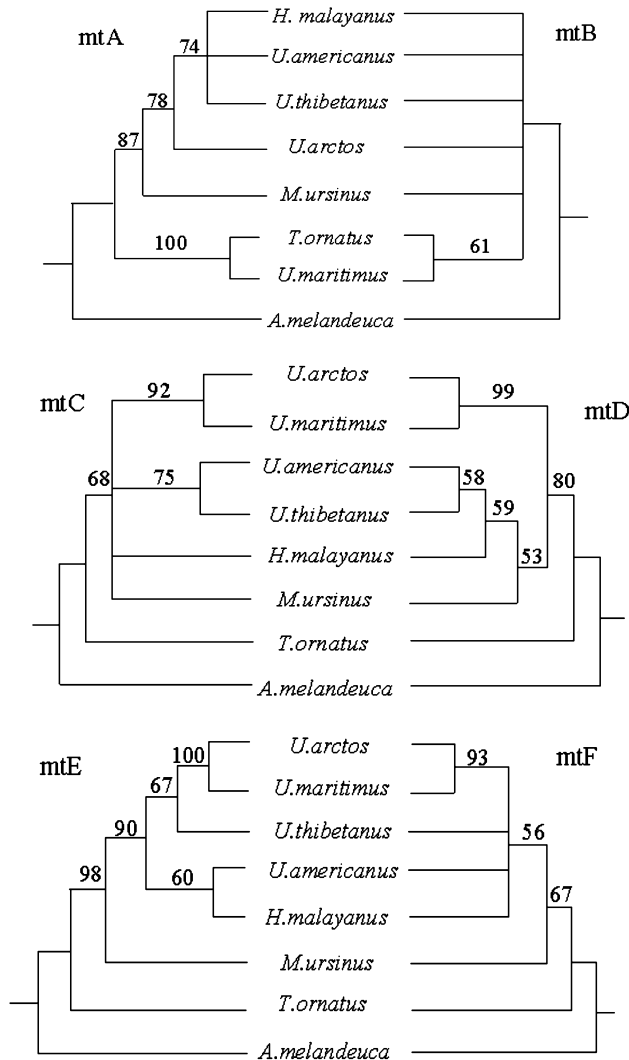


Fig. 1. Competing hypotheses of phylogenetic relationships within family Ursidae deduced from mitochondrial sequences (mtA–F). The topologies above were constructed based on (mtA) partial cytochrome *b* gene (Zhang and Ryder, 1993), (mtB) partial 12SrRNA, tRNA^{Pro}, and tRNA^{Thr} gene (Zhang and Ryder, 1993), (mtC) MP analysis of complete cytochrome *b*, tRNA^{Pro}, and tRNA^{Thr} genes (Talbot and Shields, 1996a,b), (mtD) NJ analysis of complete cytochrome *b*, tRNA^{Pro}, and tRNA^{Thr} genes (Talbot and Shields, 1996a,b), (mtE) partial D-loop region, cytochrome *b*, 12SrRNA, tRNA^{Pro}, and tRNA^{Thr} genes (Zhang and Ryder, 1994), (mtF) combined analysis of six gene segments of mtDNA sequence data (Waits et al., 1999). Corresponding bootstrap support were indicated above each branch.

Table 1
Samples and sequences used in this study

Taxa		Subfamily	Sample source	Nuclear dataset		Mitochondrial datasets*		
Scientific name	Common name			IRBP exon 1	TTR intron 1	Cytb	tRNA ^{Thr} / tRNA ^{Pro}	12SRNA
<i>Ailuropoda melandeuca</i>	The giant panda	<i>Ailuropodinae</i>	Sichuan Province, China	AY303836 ^a	AF039738 ^b	U23552 ^c	U23553 ^c	L21887 ^g
<i>Tremarctos ornatus</i>	Spectacled bear	<i>Tremarctinae</i>	San Diego Zoo, USA	AY303840 ^a	AF039740 ^b	U23554 ^c	U23555 ^c	L21883 ^g
<i>Ursus thibetanus</i>	Asiatic black bear	<i>Ursinae</i>	Yunnan Province, China	AY303841 ^a	AY303847 ^a	AB020910 ^d	U23559 ^c	L21890 ^g
<i>Ursus americanus</i>	American black bear	<i>Ursinae</i>	University of Montana	AY303837 ^a	AY303844 ^a	AF303109 ^e	AF303109 ^e	AF303109 ^e
<i>Ursus maritimus</i>	Polar bear	<i>Ursinae</i>	San Diego Zoo, USA	AY303843 ^a	AY303848 ^a	AF303111 ^e	AF303111 ^e	AF303111 ^e
<i>Ursus arctos</i>	Brown bear	<i>Ursinae</i>	Heilongjiang Province, China	AY303842 ^a	AF039741 ^b	AF303110 ^e	AF303110 ^e	AF303110 ^e
<i>Melursus ursinus</i>	Sloth bear	<i>Ursinae</i>	San Diego Zoo, USA	AY303838 ^a	AY303845 ^a	U23562 ^c	U23563 ^c	L21884 ^g
<i>Helarctos malayanus</i>	Sun bear	<i>Ursinae</i>	Yunnan Province, China	AY303839 ^a	AY303846 ^a	U18899 ^f	U18900 ^f	L21882 ^g

Note. taxonomic denomination followed classification of Wozenkraft (1993). (a) Sequences new to this study. (b–g) Sequences taken from the database. Data sources are (b) Flynn and Nedbal (1998), (c) Talbot and Shields (1996a), (d) Matsuhashi et al. (1999), (e) Delisle and Strobeck (2002), (f) Talbot and Shields (1996b), and (g) Zhang and Ryder (1993).

* Accession numbers of D-loop sequences in mt gene dataset are unavailable and the sequences were obtained from publication (Zhang and Ryder, 1994).

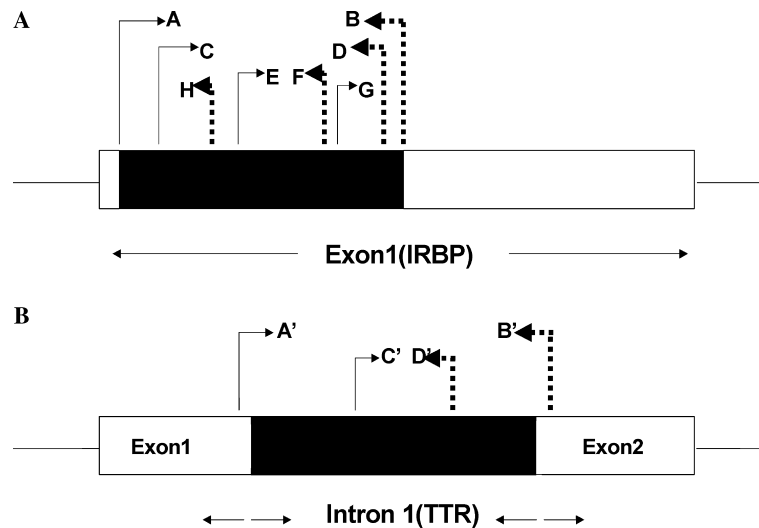


Fig. 2. Location of primers along and around sequenced fragments of IRBP (A) and TTR (B) genes. (A) A217 5'-ATGGCCAAGGTC CTCTTGGATAACTACTGCTT-3'; B1531 5'-CGCAGGTCCATATAGGTGTCTCCGTGTCCTG-3'; C4 94 5'-ACGAGGTTCTGGAGGGCAA TGTGG-3'; D1275 5'-ACGGCCCGCACCAGGAGCCTG-3'; E835 5'-GGCGTGGCTGAGGACATCACTTAC-3'; F954 5'-GGACACGGGC ACGGTGAGGAAG-3'; G1160 5'-ACCGTGTGCCACCCCTGCTGC-3'; H546 5'-CCAGCTTGCTCACCACCTCCTG-3' and (B) A'625 5'-TG CCTCG CTGGACTGGTATT-3'; B'1628 5'-GACAGCATCTAGAACTTTGACCAT-3'; C' 5'-TACAAGTAGTAAGTGGGAATGAC-3'; D' 5'-TT CTGCCTCCGGACATGCTGCG-3'. Numbers in the primer names refer to the position of the 5' end of the primer in the published human sequence.

(with some flanking exon 1 and exon 2 sequences; Tsuzuki et al., 1985; see Fig. 2). Additional internal primers were derived from consensus sequences among species used in this study with a view to sequence the remaining portion of the exon and the intron (see Fig. 2). Double-

stranded polymerase chain reaction (PCR) amplification was carried out using the following parameters: 95 °C initial hot start (5 min), 35 cycles of 94 °C denaturation (1 min), 50–63 °C annealing (1 min), and 72 °C extension (1 min).

2.2. Sequencing and data analyses

Purified PCR products were directly sequenced with an ABI automated DNA sequencer and sequences were then determined in both directions for each of the eight species and submitted for BLAST searching (Altschul et al., 1997) in GenBank to ensure that required sequences had been amplified.

Alignments of nuclear IRBP and TTR data were first conducted separately using program CLUSTAL X (Thompson et al., 1997) with default parameters and verified by eye. The aligned sequences representing eight species are available as Supplementary Material online. IRBP exon 1 was sufficiently conserved as expected with few insertions or deletions, while TTR intron 1 displayed apparent length variation with a region of large indels spanning about 60 bps among short TAAA repeats at the 3' end, characteristic of Caniformians (Flynn and Nedbal, 1998). This ambiguous region made alignment difficult and was omitted from further phylogenetic analyses.

Pairwise divergence values were estimated by the method of Tamura and Nei (1993) (TN93) for IRBP data and TTR data with the computer software programs MEGA (Kumar et al., 2001). The resultant values were then used for comparisons of substitution rate between both gene segments. The hypothesis of molecular clock was examined for our both data sets using the method of relative-rate test (Takezaki et al., 1995) with the aid of the software program PHYLTEST (Kumar, 1996). The *g₁* statistic for the skewness of tree length distributions (Hillis and Huelsenbeck, 1992) performed in PAUP*4.0 (Swofford, 1998) was used as a sensitive measure to examine if our two nuclear data sets possess valuable phylogenetic information. Before reconstructing phylogenetic relationships, we also took a plot of the number of transitions and transversions versus TN93 distance as a measure of detecting substitution saturation using DAMBE program (Xia, 2000). Because transitions and transversions in the case of nuclear IRBP and TTR genes were accumulating linearly and gave no indication of saturation effect (data not shown), so all substitutions in both genes were used for phylogenetic inference. With the aim of maximizing the explanatory power of phylogenetic estimates, we used conditional data combination (CDC) approach (Bull et al., 1993; de Queiroz et al., 1995) to analyze multiple datasets in this study. Prior to phylogenetic reconstruction, partition homogeneity test (PHT or ILD test, Farris et al., 1994, 1995; PAUP*4.0, Swofford, 1998) was conducted to assess compatibility of phylogenetic signal in these two data sets.

Traditional maximum parsimony (MP) and maximum likelihood (ML) analyses of aligned sequences were performed using PAUP*4.0 (Swofford, 1998) for both separate and concatenated datasets. We designated

the giant panda for outgroup rooting on the basis of the belief that the giant panda branched off earlier than the seven bear species on the evolutionary tree (Nash and O'Brien, 1987; Nash et al., 1998; Talbot and Shields, 1996a,b; Waits et al., 1999; Wayne et al., 1991; Zhang and Ryder, 1993, 1994). In the MP analysis, we adopted the exhaustive search algorithm with TBR branch swapping, random addition sequence for taxa, MULPARS, and 1000 replicate per search. Branches were collapsed if their maximum length equaled zero. All characters were treated as unordered and nucleotide substitutions in each gene segment as equal weight. Gaps were treated as missing data. For model-based ML analyses, we initially introduced hierarchical likelihood ratio tests (hLRT) to compare the goodness of fit of 56 nucleotide substitution models using program ModelTest version 3.06 (Posada and Crandall, 1998) for all data sets. Once an appropriate model was established, a ML tree was constructed using this explicit model of evolution. Reliabilities of phylogenetic relationships were evaluated using nonparametric bootstrap analysis (Felsenstein, 1985) for MP and ML trees (1000 replicates for MP and 100 replicates for ML), with bootstrap values exceeding 70 interpreted as well supported (Hillis and Bull, 1993). Partitioned Bremer support analysis (PBS; Baker and DeSalle, 1997; Bremer, 1988, 1994) was also conducted with the program TreRot.v2 (Sorenson, 1999) to measure the respective contribution of each gene partition made toward the total Bremer support for nodes of multigene-based tree topology.

In addition, a recently developed Bayesian approach (Larget and Simon, 1999) was likewise performed for inference of bear phylogeny using MrBayes2.01 (Huelsenbeck and Ronquist, 2001). The nucleotide evolution model, being the very one as determined in ML analyses by Modeltest, was incorporated in Bayesian method. For combined dataset, different substitution rates for IRBP and TTR gene partitions were allowed using site-specific model. Posterior probabilities were estimated and used to assess support for each branch in inferred phylogeny, with probabilities $\geq 95\%$ being indicative of significantly supported (Reeder, 2003).

2.3. Comparison with mitochondrial data set

The mitochondrial DNA genes have long been considered to be a rich reservoir of information and the availability of mt studies in bear family would provide an opportunity for comparative assessment of phylogenetic utilities between our nuclear and mtDNA genes. Mitochondrial sequence data for all extant bear species used here were right obtained from GenBank database (see Table 1). In this study, five mt genes including partial D-loop and 12SrRNA as well as

complete cytochrome *b* (cytb), tRNA^{Thr}, and tRNA^{Pro} were gathered to constitute mt gene partition in this study. Alignments of these mt gene segments by use of program CLUSTAL X (Thompson et al., 1997) were much straightforward, except for that of D-loop region requiring several regions of single- and multiple-base pair indels and an ambiguously aligned region about 50 bp was removed. TN93 values for cytb, tRNA^{Thr}, tRNA^{Pro}, 12SrDNA, and D-loop were calculated to compare substitution rates between them and also nuclear genes. Considering all mt gene sequences were virtually inherited as one linkage group, so these five mt gene segments were concatenated into a single partition at the beginning and analyzed simultaneously under MP, ML, and Bayesian optimization criteria as described above. Though evaluation of the third positions of the cytb and the other four regions using DAMBE program revealed no signal for saturation effect, several weighting schemes were still attempted in MP analysis for concatenated mt data to examine the influence of weighting on phylogeny estimation and also compensate for substitution patterns heterogeneity among various gene regions.

Combinability between mt and nuclear datasets was also examined by PHT test. In sum, five different data sets were generated and analyzed in this study: (1) nuclear IRBP exon, (2) nuclear TTR intron, (3) combined nuclear IRBP and TTR, (4) combined five mt gene regions, and (5) combined nuclear and mt data sets. Among the resulting tree topologies derived from them, combined data phylogeny were compared using the Wilcoxon signed-ranks test (Templeton, 1983), as implemented in PAUP*. In addition, several other competing hypotheses with regard to evolutionary relationships among extant bear species based on prior studies were also constructed with the use of MacClade version 3.07 (Maddison and Maddison, 1992) and brought into comparison.

3. Results

3.1. Sequences characteristics

For IRBP the resulting data is about a 1.3 kb region of coding sequence from exon 1. Few indels are found except that the spectacled bear (*Tremarctos ornatus*) presents a 6 bp autapomorphic insertion at site 1082–1087 compared to other bear species. On average the sequences has a substantially high G+C-rich bias (mean = 64.6%), especially in the third codon positions (82.1%). In contrast, the base composition in TTR intron is slightly A–T biased (mean = 54.1%) and the sequence data demonstrates length variation ranging from 989 to 993 nucleotides after the exclusion of the ambiguous region. The observation of AT rich in our TTR data set is in accord with the distinctive feature of noncoding sequences, which suffer less functional constraints (Prychitko and Moore, 1997). The estimated ratios of transition to transversion were 5.23 for IRBP and 2.16 for TTR gene, suggesting the apparent tendency against transversions among the bear species in our nuclear genes. Sequence divergence (TN93 distances) between the *Ursidae* species ranges from 0.16 to 1.27% for IRBP and from 0.3 to 1.5% for TTR (see Table 2). If the outgroup was taken into account, then the average number of nucleotide differences per site between giant panda and bear ingroup is 1.67% for IRBP and 3.20% for TTR, respectively. Table 3 shows these sequence characteristics not only in nuclear genes but also those in five mt genes for comparison. D-loop had the largest percentage of variable (31.01%) and parsimony-informative sites (17.72%) while the nuclear IRBP had the least of both (2.89 and 0.31%, respectively; Table 3). It can be seen that significant differences in modes and rates of sequence evolution exist among different regions, especially between nuclear and mt genes. Calculations of pairwise divergences revealed that

Table 2

Percentage sequence divergences for nuclear (IRBP/TTR) and combined five mt gene regions of bear species based on Tamura and Nei (1993) method

	1	2	3	4	5	6	7	8
1. <i>Ursus arctos</i> (brown bear)	—	2.60	8.90	10.45	9.27	8.20	15.78	15.27
2. <i>Ursus maritimus</i> (polar bear)	0.16/0.40	—	8.29	10.17	8.34	8.38	15.93	15.77
3. <i>Ursus thibetanus</i> (Asiatic black bear)	0.32/0.51	0.16/0.51	—	8.60	9.85	9.43	15.63	17.00
4. <i>Ursus americanus</i> (American black bear)	0.39/0.41	0.24/0.61	0.24/0.30	—	10.01	8.71	15.42	17.76
5. <i>Ursus ursinus</i> (sloth bear)	0.39/1.43	0.24/1.22	0.24/1.12	0.32/1.43	—	8.66	14.53	16.43
6. <i>Ursus malayanus</i> (sun bear)	0.39/1.23	0.24/1.02	0.24/0.92	0.32/1.23	0.32/0.40	—	15.12	16.84
7. <i>Tremarctos ornatus</i> (spectacled bear)	1.27/1.54	1.11/1.54	1.11/1.43	1.19/1.54	1.19/1.54	1.03/1.33	—	19.37
8. <i>Ailuropoda melandeuca</i> (giant panda)	1.67/3.21	1.51/3.20	1.51/3.10	1.59/3.21	1.59/3.42	1.43/3.21	2.00/3.01	—

Note. The numbers above the diagonal are for the combined five mt genes, and those below the diagonal are for nuclear (IRBP/TTR) genes.

Table 3
Summary statistics for nuclear and mitochondrial gene segments used in this study

	Nuclear dataset		Mitochondrial dataset				
	IRBP exon 1	TTR intron 1	Cytochrome <i>b</i>	tRNA ^{Thr}	tRNA ^{Pro}	12SRNA	D-loop
Aligned sites (bp)	1280	1007	1140	72	65	349	316
A%	18.4	25.6	29.6	30.2	34.6	37.0	25.3
C%	32.8	24.3	30.5	23.1	29.8	21.9	25.3
G%	31.8	21.6	13.6	16.2	14.6	15.5	18.5
T%	17.0	28.5	26.3	30.4	21.0	25.6	30.8
Variable sites	37 (2.89%)	48 (4.77%)	349 (30.61%)	14 (19.44%)	17 (26.15%)	52 (14.90%)	98 (31.01%)
Parsimony-informative sites	4 (0.31%)	13 (1.29%)	178 (15.61%)	7 (9.72%)	4 (6.15%)	24 (6.88%)	56 (17.72%)
Ti:Tv ratio	5.23	2.16	21.52	3.5	∞ ^a	11.1	16.13
Mean TN distance (%)	0.53	1.03	12.10	6.73	7.64	5.49	13.17
within ingroup	(0.16–1.27)	(0.3–1.54)	(2.33–18.10)	(1.47–14.09)	(0–16.9)	(1.47–10.28)	(4.69–21.31)
Mean TN distance (%)	1.67	3.20	18.66	15.99	15.03	9.20	21.3
between outgroup and ingroup							

^a 5 Tis and zero Tvs.

mt genes evolved about 10 times faster than nuclear intron and 20 times than nuclear exon in general. This rate heterogeneity noted in bear family agree with the result from mammalian mt and single-copy nuclear DNA comparison, in which larger than 10 times of rate difference has been well-documented (Brown et al., 1982). The substitution rate of TTR intron 1 was about two times higher than that of IRBP exon 1 in nuclear gene comparisons while D-loop the fastest, followed by cytb, tRNA, and 12SrRNA in mt gene comparisons.

3.2. Phylogenetic inference from nuclear genes

Figs. 3A and B show trees based on separate analyses of IRBP exon and TTR intron, respectively. For IRBP gene, the MP and ML analyses recovered identical tree topology with similar nodal support (Fig. 3A). At the

base of the family Ursidae, the lineage to the spectacled bear first branched off from the subfamily ursine representatives, and then within the latter, the sun bear first separated from the remaining bear species but this was not significantly supported (bootstrap values <70%). The brown and polar bears were grouped together, an expected association having been well-confirmed by fossil record and most molecular evidences, albeit with weak supports (bootstrap values <70%) in the IRBP analyses. As can be seen, TTR intron is less conserved and evolves at a more rapid rate compared to IRBP exon, so it affords a relatively significant amount of phylogenetic signal. The MP and ML tree based on TTR intron were the same topology and showed similar bootstrap values (Fig. 3B). Obviously, the striking feature of TTR intron gene tree in contrast to that of IRBP exon was the well-supported sister group relationship between sun bear and sloth bear (bootstrap support >90%). Undeniably, the clustering of Asiatic and American black bears was also an indelible aspect in this analysis, however, it was not robust (bootstrap support <70%).

Because partition homogeneity test presented no evidence for phylogenetic conflict between nuclear IRBP and TTR gene partitions ($P = 0.34$), then an alternative dataset comprising both nuclear sequences (≈ 2 kb) was constructed for phylogenetic inferences. The resultant MP and ML tree topologies based on combined data with improved nodal support (Fig. 4) compared to those based on separate IRBP and TTR data was not only identical with each other, but with the individual TTR intron gene tree as well, clearly signifying that the TTR sequence data contributed greatly to the topology of combined nuclear DNA tree. As an alternative to traditional tree-making methods, Bayesian analysis of pooled data was also performed and produced identical topology, with high posterior probabilities for all resolved nodes (Fig. 4). The ancestor of spectacled bear

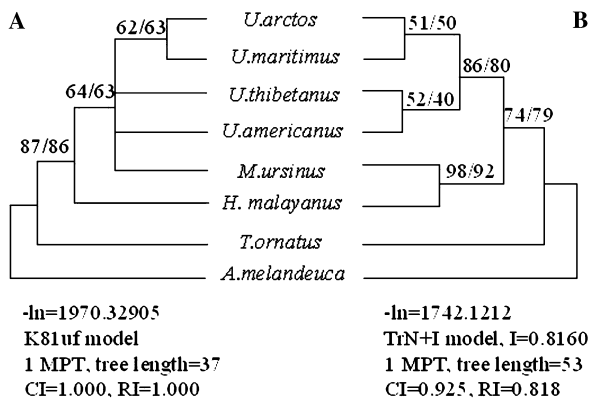


Fig. 3. Phylogenetic hypotheses based on maximum parsimony (MP) and maximum likelihood (ML) analyses of IRBP exon sequences (A) and TTR intron sequences (B). Numbers above branches are bootstrap values (MP/ML). MP tree statistics including the number of most parsimonious trees (MPTs), tree length, consistency index (CI), and retention index (RI) are shown. Best-fitting model used in ML analysis and proportion of invariable sites (I) are also indicated.

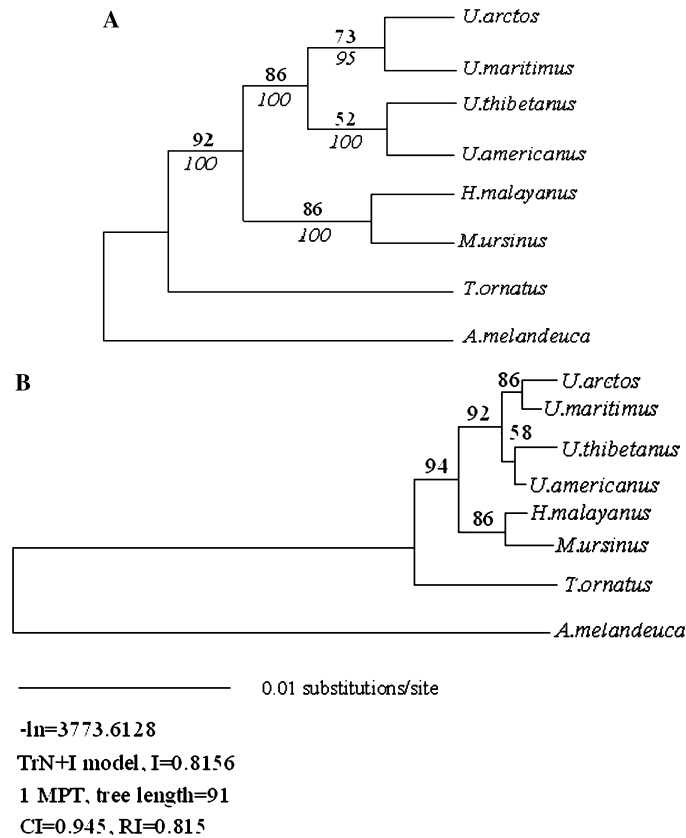


Fig. 4. Phylogenetic hypotheses based on analyses of concatenated IRBP exon and TTR intron sequences. Cladogram A (A) shows maximum parsimony (MP) tree, with bootstrap values indicated above branches. Bayesian analysis produced the same tree topology and the posterior probabilities were below branches. Phylogram B (B) shows maximum likelihood (ML) tree, with bootstrap values indicated above branches. Branch lengths are proportional to the number of nucleotide substitutions per sites. MP tree statistics including the number of most parsimonious trees (MPTs), tree length, consistency index (CI) and retention index (RI) are shown. Best-fitting model used in ML analysis and proportion of invariable sites (I) are also indicated.

was positioned most basally, followed by two distinct clusters, one corresponding to the sloth and sun bears (bootstrap support >85%, posterior probability = 100%), and the other including the remaining ursine species. Among the remaining ursine species, brown bear and polar bear was identified as sister taxa with robust bootstrap value and high posterior probability (bootstrap support >70%, posterior probability = 100%). The clustering of American black bear and Asiatic black bear (bootstrap support <70%, posterior probability = 95%) was placed as a clade closest to the lineage leading to the brown and polar bears. Taken total evidence together, we favored the results of concatenated nuclear analyses as the best compared to either IRBP or TTR gene analysis. Unless otherwise stated, only combined nuclear tree was referred for discussion.

3.3. Phylogenetic inference from concatenated mt data

Optimal trees based on MP, ML and Bayesian analyses for concatenated five mt data set (≈ 2 kb) were constructed as described above. Besides equally weighted

MP search, two additional weighting schemes were also applied in our mt analyses: (i) 4:7:7:10:3.5 weighting of gene regions cytb:tRNA^{Thr}:tRNA^{Pro}:12SRNA:D-loop according to substitution rate differences in each mt segment, (ii) 2, 4, 6, 8, and 10 times upweighting of transversions over transitions. All weighted parsimony analyses recovered a single identical most parsimonious tree, except for the case with characters equally weighted. In the former, the spectacled bear was positioned most basally with high bootstrap support, followed by the sloth bear (bootstrap values = 59–84%). Other ursine representatives were divided into two major clades. One was composed of the brown and polar bears with robust support (bootstrap values >95%), while in the second clade the sun bear was sister to the cluster comprising the Asiatic and American black bears. The close association of the sun bear with two black bears was supported by bootstrapping analysis above 50% in 4, 6, and 10 times transversion parsimony searches (Fig. 5A). Equally weighted MP analysis produced two most parsimonious trees, one of which being identical to that found by weighted analyses while the other differed in positioning the sun bear as sister to the brown and polar

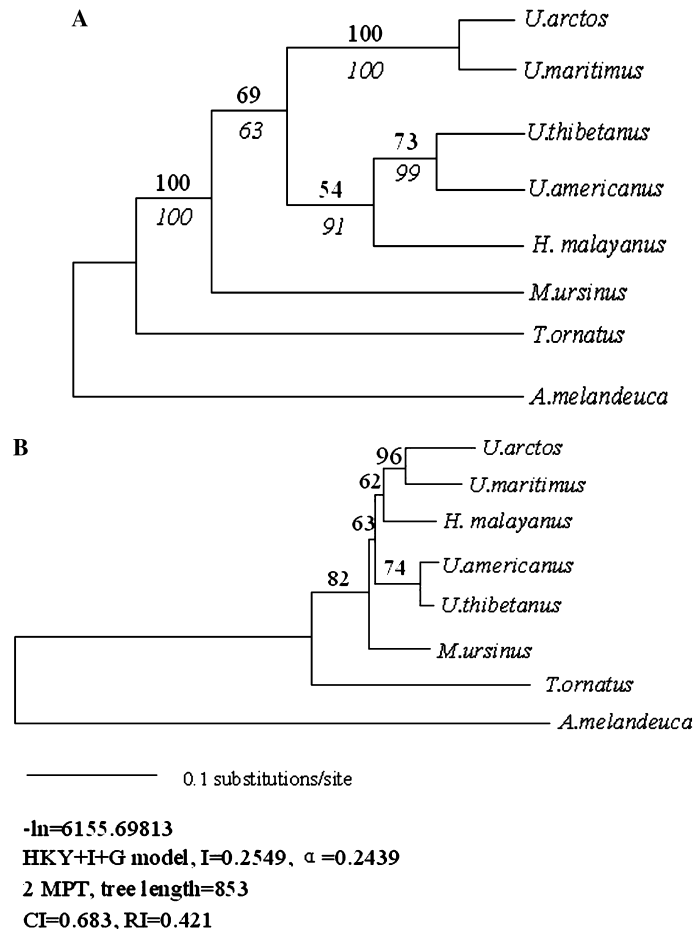


Fig. 5. Phylogenetic hypotheses based on analyses of concatenated five mtDNA genes. Cladogram A (A) shows weighted maximum parsimony (MP) tree, with bootstrap values indicated above branches. Bayesian analysis produced the same tree topology and the posterior probabilities were below branches. Phylogram B (B) shows maximum likelihood (ML) tree, with bootstrap values indicated above branches. Branch lengths are proportional to the number of nucleotide substitutions per sites. MP tree statistics including the number of most parsimonious trees (MPTs), tree length, consistency index (CI) and retention index (RI) are shown. Best-fitting model used in ML analysis and parameters including proportion of invariable sites (I) and gamma-shape parameter (α) are also indicated.

bears. The ML and Bayesian analyses based on the same data set yield identical tree topology to weighted MP analysis with similar levels of confidence (Fig. 5).

3.4. Phylogenetic inference from combined nuclear and mt data

The result of PHT test indicated that there was no significant incongruence between the nuclear and mitochondrial gene partitions ($P = 0.065$), although marginal, so simultaneous analyses of these two datasets were justified. The combined data set (≈ 4 kb) was analyzed using MP, weighted MP, ML, and Bayesian approaches in like manner. Bootstrap 50% majority-rule consensus MP tree under equally weighted schemes was given in Fig. 6A. This tree is congruent with the individual nuclear (Fig. 4) and mt (Fig. 5) trees in the placement of the spectacled bear as most basal to the other ursids, as well as the sister-group affinities between brown and polar bears (bootstrap support = 100%) and

between two black bears (bootstrap support = 73%). On the other hand, combined analysis of all available evidence revealed that the sloth bear emerged first from the subfamily ursine representatives (bootstrap values = 80%), a result equally found in mt gene trees but with improved support. The position of the sun bear appear unresolvable here instead of as sister group to the clade comprising two black bears in mt analyses (Fig. 5) nor as the closest species to the sloth bear in nuclear analyses (Fig. 4). Weighted parsimony analyses gave identical topology, except that the close relatedness of the sloth and sun bears was recovered in rate-based parsimony search, i.e., the first weighting scheme described in individual mt analyses, but the support for this hypothesis was low (bootstrap values < 70%). ML and Bayesian analyses (Fig. 6) produced the same groupings as in parsimony analysis. It should be noted that though total characters numbers in our nuclear and mt data sets were similar, they have great disparities in proportions of variable and parsimony informative site

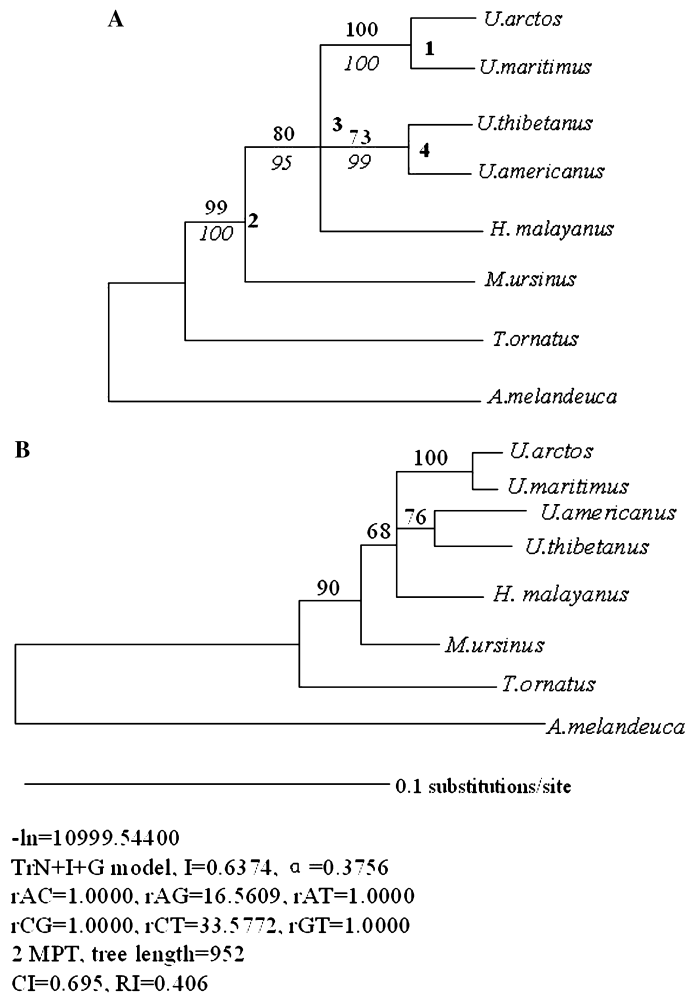


Fig. 6. Phylogenetic hypotheses based on analyses of concatenated nuclear and mt sequences. Cladogram A (A) shows maximum parsimony (MP) tree, with bootstrap values indicated above branches. Bayesian analysis produced the same tree topology and the posterior probabilities were below branches. Phylogram B (B) shows maximum likelihood (ML) tree, with bootstrap values indicated above branches. Branch lengths are proportional to the number of nucleotide substitutions per sites. MP tree statistics including the number of most parsimonious trees (MPTs), tree length, consistency index (CI) and retention index (RI) are shown. Best-fitting model used in ML analysis and parameters including proportion of invariable sites (I) and gamma-shape parameter (α) are also indicated.

(Table 3). Combination of these two datasets might face the unfavorable result that the large one would “swamp” the phylogenetic signal of the smaller one (Goto and Kimura, 2001; Hillis, 1987; Miyamoto and Fitch, 1995). Indeed, we can find that combined data showed more similarity to the mt gene tree in topology than the nuclear one. This would come in no surprise when we examined the partitioned Bremer support, which indicated that larger than 90% of the total PBS values was provided by mt genes (Table 4). However, despite this, analyses of combined nuclear + mt dataset under all methods did not support identical tree topologies from separate mt analyses, and moreover, except that the higher support for the close relatedness of the brown and polar bears, all recovered nodes in total evidence tree presented similar or even lower levels of confidence than nuclear trees, thus also providing evi-

dence of interactions between nuclear and mt genes in our combined analyses.

4. Discussion

4.1. Utilities of molecular markers

The partial exon 1 region of nuclear IRBP gene has been widely used to infer phylogenies of various groups in previous studies and demonstrated to be informative at different taxonomic ranks from mammalian orders (Debry and Sagel, 2001; Smith et al., 1996; Springer et al., 1997, 2001; Stanhope et al., 1992, 1996) to rodent species (Serizawa et al., 2000; Suzuki et al., 2000) whereas the TTR intron 1 fragment was believed to be a useful genetic marker for settling interfamilial and

Table 4
Results of partitioned bremer support (PBS) analyses to each node on the total evidence MP tree based on combined nuclear and mt dataset

Nodes ^a	Nuclear dataset			Mitochondrial dataset					Combined nuclear and mt gene tree (Fig. 6A)	
	IRBP exon 1	TTR intron 1	Combined nuclear genes	Cytb	tRNA ^{Thr}	tRNA ^{Pro}	12SRNA	D-loop		Combined mt genes
1	1	1	2	14	-4	0	1	13	24	26
2	2	1	3	10	0	-1	8	7	24	27
3	-1	0	-1	8	-4	-1	0	5	8	7
4	0	1	1	14	0	0	-1	-9	4	5
Total	2 (3.08%)	3 (4.61%)	5 (7.69%)	46 (70.77%)	-8 (-12.31%)	-2 (-3.08%)	8 (12.31%)	16 (24.62%)	60 (92.31%)	65

^aNodes are numbered as in Fig. 6A.

intergeneric relationships, especially within Carnivores (Flynn and Nedbal, 1998; Flynn et al., 2000; Walton et al., 2000; Yoder et al., 2003). We are among the first to use both nuclear genes to phylogenetic studies of bears. In our case, the IRBP gene trees showed little resolution due to lowest sequence divergence in the event of tracking the evolutionary history for such a young lineage while the functionally unconstrained TTR intron 1 data retained more signal in clarifying relationships among closely related bears, though some recovered nodes were less robust. Combinations of these two nuclear gene loci revealed much improved support for most nodes. The mt genes examined here were shown to evolve at a much faster rate and held more informative characters than either nuclear gene but they contained a higher level of homoplasy, as evidenced by lower CI and RI values, resulting in a less ideal phylogeny also with some weak-supported nodes. In summary, we found that for bears, the nuclear TTR gene served better than IRBP gene, and combined nuclear sequences were capable of resolving relationships of recently diverged species comparable to the mt genes. It was interesting to note that concatenated analyses of nuclear and mt genes in this study did not show remarkably improved resolution and confidence in phylogenetic estimates as expected, possibly due to the extremely heterogeneous rates of evolution and levels of homoplasy between these two gene partitions.

4.2. Phylogeny of family Ursidae

In our study, spectacled bear was clearly shown to depart furthest from the other six bear species in every analysis. However, there was no agreement on the branching order within the subfamily *ursine* based on both separate and simultaneous analyses of nuclear (IRBP and TTR genes) and mitochondrial (combined cytb, tRNA^{Thr}, tRNA^{Pro}, 12SRNA, and D-loop genes) datasets. Various elements may bear the responsibility for these inconsistent relationships, but the fact that the diversification of six bear species took place within a short period of evolutionary time should not at any rate be overlooked. The topological concordance of these data sets supported a sister relationship between the brown and polar bears, especially receiving both high bootstrap supports and posterior probabilities in combined IRBP + TTR, mt, and nuclear + mt trees (bootstrap supports = 73–100%, posterior probabilities = 96–100%). All analyses except for that based on independent IRBP gene region suggested that the two black bears were closely related and formed sister taxa with weak to moderate levels of support (bootstrap values = 52–76%) but with high posterior probabilities (= 95–100%). This result was in accordance with that of Talbot and Shields (1996a,b) based on complete mtDNA cytb, tRNA^{Thr}, and tRNA^{Pro} sequences

(bootstrap values = 58–75%), as well as that of Waits et al. (1999) derived from six different mtDNA gene segments involving all the ursid species (bootstrap values = 38–58%). In addition, this connection of the American and Asiatic black bears presented here has also been formerly suggested by some authorities according to known fossil information (Kurten and Anderson, 1980), in which these two black bears were depicted to resemble in habits and both derived from *Ursus abstrusus*. The positionings of the sloth and sun bears, however, were enigmatic based on our analyses in this paper. Different conclusions in this regard were reached depending on the gene regions analyzed. IRBP data analysis placed the sun bear as sister to all remaining ursine bears but this received weak support (bootstrap value <70%). TTR and combined nuclear dataset clearly indicated sister-taxa association of sun bear and sloth bear with high robustness (bootstrap support >85%, posterior probability = 100%). This opinion was not unreasonable from the morphological standpoint, in which sloth bear and sun bear distinguished markedly from other bears by their morphological and behavioral specialization due to adaptive change (Goldman et al., 1989; Hall, 1981; Nowak and Paradiso, 1983). Moreover, as in Goldman et al. (1989), this relationship uniting the sun and sloth bears has also been revealed either in phylogenetic tree derived from 122 allozyme character states or phenetic tree based on genetic distances of 44 allozyme loci. Bininda-Emonds et al. (1999) deduced the same view from their supertree construction for Ursidae, in which variously sourced relevant information reported since 1970, not only that of molecular data, was integrated and analyzed. In contrast, both mt and concatenated nuclear + mt trees were in agreement about the placement of the sloth bear as the earliest diverging species among the ursine bears with moderate bootstrap support (values = 63–80%) and varying posterior probabilities (63% in mt Bayesian tree and 95% in nuclear + mt Bayesian tree). This resolution has also been suggested in previous studies based on the analyses of other mt gene combination (Waits et al., 1999; Zhang and Ryder, 1994). On the other hand, they differed in the placement of the sun bear, either as the sister species to the clade composed of the two black bears in the mt trees (bootstrap support = 54–62%, posterior probabilities = 91%) or as an unresolved species sharing polytomy with the brown/polar bears and the two black bears clade in concatenated nuclear + mt tree. Therefore, the areas of most topological incongruence centered on the sloth and sun bears. However, branch reliability tests derived from these analyses showed that the mt phylogeny had weak support for their resolution of these two bears while the nuclear phylogeny provided strongest signal in grouping them. The nuclear + mt one did not conclusively resolved the position of the sun bear but placed more confidence in

the early diverging status of the sloth bear compared to the mt analysis. To conclude, we considered that based on our present available DNA sequence data, some relationships among the family Ursidae seemingly were resolved, such as the earliest divergence of the spectacled bear and sister-taxa status of brown/polar bears and of Asiatic/American black bears, but some still required confirmation by analyzing additional character information, such as the precise positions of the sun and sloth bears in bear phylogeny.

4.3. Comparison among gene trees

In our study, the tree topologies based on the analyses of novel nuclear, mt, and combined nuclear + mt genes differed not only from each other but also from any prior mtDNA based phylogenetic findings. Various hypotheses about branching patterns of ursine bears have been advocated depending on mt gene segments and tree-building methods used (Fig. 1). For example, Zhang and Ryder (1994), on the basis of combined analyses of five mt gene regions (Fig. 1E), positioned the two black bears distantly with poor support, that is, the Asiatic black bear formed a cluster with the brown/polar bears and the American black bear with the sun bear. Talbot and Shields (1996a,b), while resolving sister-taxa relationship between the brown and polar bears as well as between the two black bears with similar bootstrap support as that in this study, has varying placement of the sun and sloth bears under different analytical methods constructed from three complete mt genes (Figs. 1C–D). Another recent study of Waits et al. (1999) based on six partial mt gene segments from all bear species (Fig. 1F) recognized even a less resolved tree, in which not only the phylogenetic status of the two black bears and sun bear were ambiguous as a result of extremely low bootstrap values but also the support for the early diverging sloth bear was not very robust. Corresponding tests (Templeton's test) were carried out to examine the degree of significant difference not only between trees produced in present study but also between them and those previous competing phylogenies. The results were summarized in Table 5. On the one hand, test indicated significant topological incongruence between nuclear and mt trees described in this paper. When combined nuclear + mt gene analyses were likewise under consideration, our mt tree was the best tree for the combined dataset. However, nuclear and two of previous alternative hypotheses (Figs. 1D–E) also cannot be rejected by that dataset. On the other hand, the same two (Figs. 1D–E) were judged not significantly different from our mt estimates of bear phylogeny whereas in sharp contrast none of prior mt trees was supported by nuclear data under the Templeton's test. Phylogenetic incongruence between nuclear and mitochondrial genes has also been reported in *Drosophila*

Table 5
Results of Templeton's test for hypotheses derived from our datasets and mitochondrial topologies defined in Fig. 1 (mtA–F)

Data set	Tree (Tree length and P values*)									
	Present nuclear (Fig. 4)	Present mt (Fig. 5)	nuclear+mt (Fig. 6)	mtA (Fig. 1)	mtB (Fig. 1)	mtC (Fig. 1)	mtD (Fig. 1)	mtE (Fig. 1)	mtF (Fig. 1)	
Combined mt	872 Z = -2.9673 P < 0.05	853 (best) —	863 Z = -3.1623 P < 0.05	980 Z = -10.2666 P < 0.05	1026 Z = -11.1283 P < 0.05	883 Z = -5.4772 P < 0.05	863 Z = -1.9612 NS	858 Z = -0.6299 NS	887 Z = -5.8310 P < 0.05	
Combined nuclear	91 (best)	99	99	110	113	99	98	100	100	
All available characters	—	Z = -2.8284 P < 0.05	Z = -2.8284 P < 0.05	Z = -3.7547 P < 0.05	Z = -3.7547 P < 0.05	Z = -2.8284 P < 0.05	Z = -2.6458 P < 0.05	Z = -3.000 P < 0.05	Z = -3.000 P < 0.05	
	963	952 (best)	962	1090	1139	982	961	958	987	
	Z = -0.1601 NS	—	Z = -3.1623 P < 0.05	Z = -9.7032 P < 0.05	Z = -11.4115 P < 0.05	Z = -4.4721 P < 0.05	Z = -0.1644 NS	Z = -0.5443 NS	Z = -5.000 P < 0.05	

* P < 0.05 indicated significantly less likely, NS indicated not significantly different from the best tree.

and Aves (Durando et al., 2000; Giannasi et al., 2001; Goto and Kimura, 2001). Conflicting signal between gene trees may be attributed to various factors and the probability of its occurrence increased especially when separation time between different species is short (Moore, 1995; Nei, 1987; Pamilo and Nei, 1988; Wu, 1991), as it is in present study. Incongruence may be due partly to dissimilar evolutionary histories in heterogeneous gene regions, or sampling error (Harris and Distotell, 1998). A variety of potentially relevant elements, including gene duplication, incomplete lineage sorting, and introgressive hybridization may account for it (de Queiroz, 1993; Giannasi et al., 2001; Page, 2000; Slowinski and Page, 1999). In our case, the choice of nuclear single-copy genes as sources of phylogenetic information has, first of all, removed paralogy problem, thus, it seemed that lineage sorting and introgressive hybridization are likely to be main candidates for explaining the conflicting results from mtDNA and nuclear genes (Moore, 1995). However, these speculations were always unable to be verified in practical phylogenetic examples. In addition, potential problems introduced by dramatic rate difference among and within gene regions maybe also in part gave rise to the discordance of mtDNA with the nuclear genes. Here and now we would not figure out which one hypothesis overtops another, but recommend that addition of independent gene loci is none the less essential to attain an unequivocal resolution of intricate issues within family Ursidae.

4.4. Implications for Ursidae radiation

The relative-rate test in our analyses suggested that the IRBP gene in the extant bear species seems to be evolving at an approximately constant rate, so does the TTR gene. Smith et al. (1996) tentatively estimated that the rate of IRBP sequence change was about 0.21% Myr when they applied the same IRBP region to the phylogenetic analyses of Cetacean and of their association with Artiodactyls. However, in the same principle if we chose the minimum date of split revealed by the fossil record between the giant panda and the rest of the bear species (12 Mya, Wayne et al., 1991; Thenius, 1979) as reference time in our case, then a remarkably low value of 0.139% Myr was produced. Coincidentally, it was also the case just in consideration of the third codon of the cytb gene in the bear species, which had been previously reported by Talbot and Shields (1996a,b) [6% Myr of ursids vs 10% Myr of other mammalian species (Irwin et al., 1991)]. As a result, on the one hand, certain interrelation in evolutionary process between nuclear and mt genomes was displayed, on the other hand, they also supplement evidence in support of an inclination of evolutionary rate slowdown by a big margin during the progressive radiation of family Ursidae relative to that for other certain mammalian species.

Molecular dating of the bear radiation has been attempted in several studies previously. Talbot and Shields (1996a,b), using *cytb* sequences, made a detailed estimation of divergence time for most extant bears. However, Waits et al. (1999) questioned their results for the reason that *cytb* gene of the family Ursidae seemed not to be evolving in a clock-like manner. Earlier dating information from Goldman et al. (1989) based on protein electrophoresis appear to give older dates than that of Talbot and Shields (1996a,b). Now we address the same question from the perspective of nuclear sequence data, given abundant fossil documents of living carnivores (Wayne et al., 1991) allowing us to draw a comparison of the dating results estimated from prior and present studies. Although contention regarding when the bears diversified persisted, all existing evidence from paleontological and molecular studies manifested that it invariably fell under the category between 6 and 15 Mya. Goldman et al. (1989) had proposed that South American spectacled bear split from the ursine line 10–15 Mya. In our instance, we argue for the same episode to be Late Miocene date of 6–8 Mya (95% confidence intervals = 3.5–12.5 Mya) based on the evolutionary rates of nuclear IRBP and TTR gene outlined above, and our mentioned date for the bear radiation was more in agreement with those revealed by fossil record (5–7 Mya, Kurten and Anderson, 1980; Wayne et al., 1991) than that measured by mitochondrial clock (12–13 Mya; Talbot and Shields, 1996a,b). Applying the same train of thought, we reasoned the remaining six closely related ursine bears characterized by a rapid radiation began their divergence from a common ancestor during the Pliocene epoch at 2–5 million years ago (95% confidence intervals = 0.7–6.9 Mya), a slightly earlier date than the paleontological date of 4–6 Mya (Kurten, 1968; Wayne et al., 1991) and previously thought of 4–8 Mya proposed by Goldman et al. (1989) as well as of 5–7 Mya by Talbot and Shields (1996a,b). The divergence time suggested by us at 1–1.5 Mya (95% confidence intervals = 0.01–3.08) for the split of the brown bear and the polar bear was consistent with *cytb* sequence (1–2 Mya, Talbot and Shields, 1996a,b) and protein electrophoresis (2–3 Mya, Goldman et al., 1989) but approximately 10 times older than the fossil records (0.07–0.1 Mya, Kurten, 1968; Wayne et al., 1991). Because of the unstable clustering patterns detected among other ursine bears in our phylogenetic analyses, we discontinued the further investigation of *ursine* radiation in detail for fear that unreliable conclusions would be reached.

Supplementary materials

The sequences reported in this paper have been deposited in the GenBank database. Accession Nos.: AY303836–AY303848.

Acknowledgments

We thank Dr. F. Allendorf for providing samples. This work was supported by National Natural Science Foundation of China (NSFC) and Chinese Academy of Sciences.

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