A molecular solution to the riddle of the giant panda's phylogeny

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Although it is generally agreed that the giant panda (Ailuropoda melanoleuca) *is a member of the order Carnivora, there has long been disagreement over whether it should be classified with bears, raccoons or as a single member of its own family. Four independent molecular and genetic measures lead to a consensus phylogeny for the giant and lesser pandas. The lesser panda diverged from New World procyonids at approximately the same time as their departure from ursids, while ancestors of the giant panda split from the ursid lineage much later, just before the radiation which led to modern bears. The giant panda's divergence was accompanied by a chromosomal reorganization which can be partially reconstructed from the ursid karyotype, but not from that of procyonids or the lesser panda. The apparently dramatic, but actually limited, distinctions between the giant panda and the bears in chromosomal and anatomical morphology provide a graphic mammalian example of the discordance of molecular and morphological (and chromosomal) evolutionary change.*

THE taxonomic status of the giant panda and its diminutive Chinese relative, the lesser panda, has been a biological puzzle since their description by western naturalists a century ago[1,2]. Although there is some agreement that the lesser panda was a member of the raccoon family (Procyonidae), a survey of over 40 published phylogenetic treatises[3-5] shows that the giant panda has been classified with almost equal frequency in Ursidae (bear family), in Procyonidae and as the single member of a separate family, Ailuropodidae. The panda does look like a bear but has some characteristics and habits that are unusual for bears: (1) Despite its classification in the order Carnivora, it is largely herbivorous, subsisting primarily on bamboo. (2) The giant panda has extremely large forequarters and reduced hindquarters, which account for its ambling gait. The enlargement of the anterior end is associated with a huge specialized masticatory apparatus which has some parallel in the lesser panda. (3) The male genitalia in the giant panda are tiny, cylindrical, S-shaped and posteriorly directed, very similar to the lesser panda. (4) The giant panda has six digits on its forepaw, resulting from the evolutionary extension of the radial sesamoid to an awkward, but functional, opposable thumb[3,6]. (5) Finally, the giant panda does not behave like a bear[7,8]. Most bears hibernate, the giant panda does not; bears roar, whereas the giant panda bleats[7,8].

The proponents of grouping of the giant and lesser panda in the Procyonidae have emphasized the similarity of the two in tooth structure, skull architecture, penis morphology and colour patterning[4,9]. Davis[3], using anatomical data, and Leone and Wiens[10] and Sarich[11], using serological data, classified the panda as a bear. Davis's conclusions were disputed as being uncritical[12,13] and the immunological data were often ignored[14]. Moreover, recent fossil evidence described by Chinese palaeontologists[15-17] and the widely divergent karyotype of bears versus pandas[18,19] lent support to the separate family interpretation.

Here we report the use of various molecular methods to estimate evolutionary distances between the giant panda, the lesser panda and their supposed closest relatives. We considered three molecular techniques: (1) DNA hybridization between species using unique-sequence cellular DNA; (2) genetic distance based on electrophoretic mobility of >50 homologous isozyme loci; and (3) immunological distance of serum proteins. Analysis and interpretation of quantitative differences involving these techniques in extant species is based on the 'molecular clock' hypothesis[20-22]. As a control for the procedures and as a strategy for calibrating evolutionary time in these species, we performed the same estimates on tissues of the Hominoidea (apes and man), an extensively studied group[23-29]. Finally, a comparative cytological analysis of highly extended G-banded chromosomes from pandas and bears is presented. Our results converge on a consensus phylogeny of the pandas.
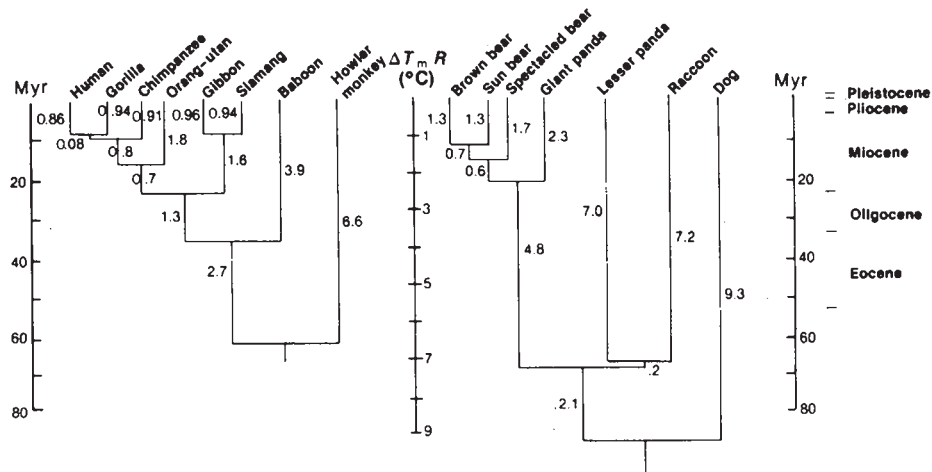
DNA hybridization

DNA hybridization of unique-sequence cellular DNA between heterologous species is a common technique in the measurement of phylogenetic affinity[28-32]. Primary fibroblast cells from four primate index species (*Homo sapiens*, human; *Pan troglodytes*, chimpanzee; *Gorilla gorilla*, gorilla; and *Hylobates concolor*, black gibbon) were cultured in the presence of radiolabelled ³H-thymidine and their high C_0t (≥ 200) DNA was purified using hydroxyapatite. This presumed 'single-copy' DNA was hybridized, using an S_1 nuclease assay[29], to excess cold DNA extracted from the same species and from three additional primates (Fig. 1). Similarly, reciprocal DNA hybridization curves were derived between each of four index carnivore species (*Procyon lotor*, raccoon; *Ailuropoda melanoleuca*, giant panda; *Ailurus fulgens*, lesser panda; and *Ursus arctos*, American brown bear). Changes in the average thermal stability (see Fig. 1 legend) were also measured between these species and two additional ursids: *Tremarctos ornatus*, spectacled bear; and *Helarctos malayanus*, Malayan sun bear (Fig. 1).

Data matrices (Fig. 1) were analysed using five distinct phylogenetic algorithms: the distance Wagner procedure[33-35], the UPGM algorithm[36], the neighbourliness method[37], the MATTOP program[38] and the Fitch–Margoliash[39] algorithm. All the algorithms produced phylogenetic trees that support the presented topologies, although the actual distances varied depending on the method used. The hominid tree shows an excellent agreement both in topology and in arm lengths with trees developed elsewhere[25-28], the only confusion being the trichotomy between African apes and man which has been under debate for some time[26-29,31,40-43]. The derived carnivore topology places the giant panda as an early leg of the ursid lineage occurring before the divergence of the modern bears but after the ursid–procyonid split. The first bear in the ursid proper radiation was the ancestor of the spectacled bear (*T. ornatus*) which conforms to its morphological assignment to generic status in the family Ursidae[44]. The lesser panda, the ursids and the procyonids seemed to split from each other at approximately the same time. Although the tree (Fig. 1) favours an association between the raccoon and the lesser panda, the errors associated with the large distances between lesser panda, raccoon and the bears do not allow unequivocal resolution of the trichotomy.

Isozyme genetic distance

Isozyme genetic distance is a statistical calculation, developed

Fig. 1 Average thermal stabilities ($\Delta T_m R$, °C; 2–9 experiments) derived from DNA hybridization of genomic DNAs of indicated species. Primary fibroblast cultures were established for each species from skin or organ biopsies of primate and carnivore species. Cultured cells from four primates (human, chimpanzee, gorilla and gibbon) and four carnivores (brown bear, giant panda, lesser panda and raccoon) were grown in the presence of ^{3}H-thymidine and the non-repetitive DNA was isolated and hybridized to each of the species listed in the left-hand column of each table[29]. ΔT_m is the difference in T_m between the other DNA-DNA hybrids and the T_m of the homologous hybrids. $\Delta T_m R$ is the thermal stability difference for all non-repeated DNA, and is the ΔT_m value corrected for the normalized percentage of hybridization to the labelled index species[28–30]. Distance matrices for phylogenetic inference were judged to be adequate, based on four different measurements: first, the standard deviations of the estimates (which were based on 2–9 hybridizations per pair) were low (±0.2 °C for $\Delta T_m R$ <5 °C, and ±0.5 °C for $\Delta T_m R$ values ⩾5 °C). Second, the reciprocal estimates were in close agreement, as evaluated by calculating the mean % deviation from the mean[60]: 2.1% for the primates and 1.8% for carnivores. Third, the metricity of the distance matrix is evidenced by conformance of all the three-way combinations to

Thermal stabilities ($\Delta T_m R$, °C)

	Human	Chimpanzee	Gorilla	Gibbon
Human	—	1.9	1.8	5.2
Chimpanzee	1.8	—	1.9	4.9
Gorilla	1.8	2.0	—	5.0
Orang-utan	3.5	NT	3.6	NT
Gibbon	5.0	5.0	5.1	—
Siamang	5.1	4.9	5.0	1.9
Baboon	7.7	7.6	7.8	7.7
Howler	13.0	13.3	NT	13.1

Thermal stabilities ($\Delta T_m R$, °C)

	Brown bear	Giant panda	Lesser panda	Raccoon
Brown bear	—	4.3	14.0	14.4
Sun bear	2.5	NT	NT	NT
Spectacled bear	3.3	NT	NT	NT
Giant panda	4.8	—	14.4	14.2
Lesser panda	13.9	14.1	—	14.3
Raccoon	14.4	14.7	13.9	—
Dog	18.7	18.3	18.9	18.5

the triangle inequality[33,35]. Metricity or conformance to the triangle inequality is not an absolute indicator of clock-like behaviour because a distance may be metric but still exhibit variation in evolutionary rates[35]. Thus, metricity is a necessary, but not sufficient, condition for an evolutionary clock. Fourth, the apparent constancy of the DNA clock was evaluated by demonstrating that internal species were equidistant from an outside group (howler monkey and dog, respectively), the relative rate test[21,40]. The phylogenetic trees shown here were derived from the Fitch-Margoliash algorithm[39] based on maximum parsimony. The actual trees were drawn to scale using the KITSCH of the PHYLIP program, given by J. Felsenstein (University of Washington). This program computes a rooted topology based on the assumption of an evolutionary molecular clock rendering all terminal species as contemporaneous. The numbers are the leg lengths of the unrooted tree generated by the Fitch-Margoliash algorithm in the absence of the above assumptions. This tree is rooted by the midpoint of the two most distal species in the network. Placing a timescale with some degree of confidence on the three derived molecular topologies is difficult. Although the carnivore fossil record is unusually good and has been studied intensively, even the commonly accepted dates for divergence of these families can be incorrect by up to 25–50%. For example, the best geological dates for the time of procyonid-ursid divergence vary from 30 to 50 Myr ago. A strategy we have used for setting our molecular clock was to take advantage of the demonstration that the primate and carnivore clocks seem to run at the same rate[31,48]. We use a timescale based on Pilbeam's date of the human-orang-utan divergence occurring at ~16 Myr BP[28,31,53]. Because the human-orang-utan split occurred near the middle of the timescale, the errors are not amplified at the stem or the root of the trees. The derived topology places the hominid-Old World monkey split at 35 Myr BP, a date which is not at variance with fossil or molecular conclusions previously presented[28,29,40,53,61]. NT, not tested.

and improved by Nei[45,46], for measuring the degree of allelic substitutions at a group of loci between populations (or species) based on the electrophoretic mobility of soluble proteins. The distance estimate, D, is defined as the average number of gene differences per locus between individuals from two test populations. Within the limits of certain specific assumptions relating to electrophoretic resolution and relative rates of nucleotide substitution, the genetic distance estimates increase proportionately with the amount of time the compared populations have been reproductively isolated[43,45–47]. The derived ape topology (Fig. 2) agrees with the DNA topology (Fig. 1) and with previous studies in placing the orang-utan as an early branch before the unresolved trichotomy between man and African great apes.

The same five phylogenetic algorithms applied to the DNA data were used to derive a topology of the giant panda and its relatives using isozyme data (Fig. 2). In general, the most parsimonious and most consistently derived relationship was the same and differed only in branch length. As illustrated by the Fitch-Margoliash tree (Fig. 2), the giant panda diverged from the ursid line just before the radiation of modern bears, but long after the ursid-procyonid split. After the giant panda divergence, the next split involved the spectacled bear, followed by a three-way split (another unresolved trichotomy) between the brown, black and sun bears. The lesser panda and the raccoon seem to have shared a common ancestor for a short time after procyonid divergence.

Immunological distance

The third method is that of immunological distance[21,22] and we summarize here the results of Sarich[11]. The procedure measures the displacement of immunological titration curves between

species based on amino-acid substitutions that have occurred in homologous proteins. The extensive and comprehensive molecular evolutionary studies of Sarich, Wilson and collaborators have demonstrated the constancy of the albumin clock in several vertebrate taxa by using microcomplement fixation as a measure of immunological distance[11,21,22,40,48,49]. Sarich prepared both albumin and transferrin (from two species, brown bear and raccoon) to generate a carnivore tree which is reproduced in Fig. 3. These results also placed the giant panda on the ursid line before the divergence of modern bears, but after ursid-procyonid split. The position of the lesser panda gave conflicting results with albumin (which favoured the ursid lineage) versus transferrin (which placed the lesser panda equidistant from both bear and raccoon), probably because of the error associated with the large distances involved. Although he shows caution in interpreting the lesser panda placement, Sarich's main conclusion was clearly supported by both albumin and transferrin immunological distances.

Karyological evidence

The karyological relationship between the bears, panda and raccoon seems to emphasize the differences between bears and giant pandas, but, on closer analysis, has become particularly informative. The giant panda has 42 metacentric chromosomes whereas members of the genus Ursus (black, Malayan sun, and brown bears) have 74 mainly acrocentric chromosomes[18,19]. Wurster-Hill and Bush[19] prepared G-trypsin-banded karyotypes of the giant panda and were able to recognize only three homologies between giant panda and bear chromosomes. To extend these observations to a higher level of resolution, we transformed brown bear and giant panda primary fibroblasts

Fig. 2 Isozyme genetic distance (*D*) computed from 44 loci in primates and 50 loci in carnivores. The enzymes studied are homologous with enzymes examined previously in the domestic cat and man[62-65]. Electrophoretic procedures were predominantly on starch gels using standardized protocols. For the primates, 44 enzyme systems were resolved: ACP1, -2, ADA, APRT, AK, ALB, ACY, CAT, CPKB, DIA4, ENO1, ES1, ESD, GALA, GDH1, GAPD, GLO1, GOT1,2, G6PD, GPI, GSR, GUS, HBB, HK1, IDH1, -2, LDHA, -B, MDH1, MPI, PEPA, -B, PGM1, -2, -3, 6-PGD, PGAM, NP, PP, PK, SOD1, -2 and TPI (see ref. 63 for nomenclature). The same systems, with the exception of GAPD, were scored in the carnivores. In addition, the following enzymes were resolved in the carnivore study: FUCA, GPT, HEXA, PEPC, ES2 and ES3[63]. Each system

	Human	Chimpanzee	Gorilla
Chimpanzee	0.31		
Gorilla	0.25	0.21	
Orang-utan	0.40	0.35	0.38

	Raccoon	Lesser panda	Giant panda	Spectacled bear	Malayan sun bear	Brown bear
Lesser panda	0.81					
Giant panda	0.97	0.93				
Spectacled bear	0.89	0.95	0.50			
Malayan sun bear	0.94	0.94	0.68	0.39		
Brown bear	0.98	1.19	0.61	0.45	0.21	
Black bear	1.02	1.33	0.99	0.57	0.36	0.45

was examined in extracts of erythrocytes, leukocytes and cultured fibroblasts for each species. The single exception was the American black bear, for which only cultured cells were available. Hence, only 36 of the 50 test loci could be evaluated. This difference dramatically affected the data, as the measured distances to other species were consistently greater than expected from the phylogenetic relationship. Because of the inequivalence of *D* values for the black bear, these data were not considered in the computation of outside-group distances. Genetic distances were calculated using the methods of Nei[45,46]. Phylogenetic trees are derived using the Fitch–Margoliash algorithm, as described in Fig. 1 legend. The other algorithms[33-39] produced similar trees with these data. Fitch's unique neighbourliness algorithm[37] yielded nearest-neighbour values which showed that the lesser panda and raccoon had the highest scores, that is, they are the nearest neighbours most often in four-way comparisons, followed closely by the three ursids sun, brown and black bears.
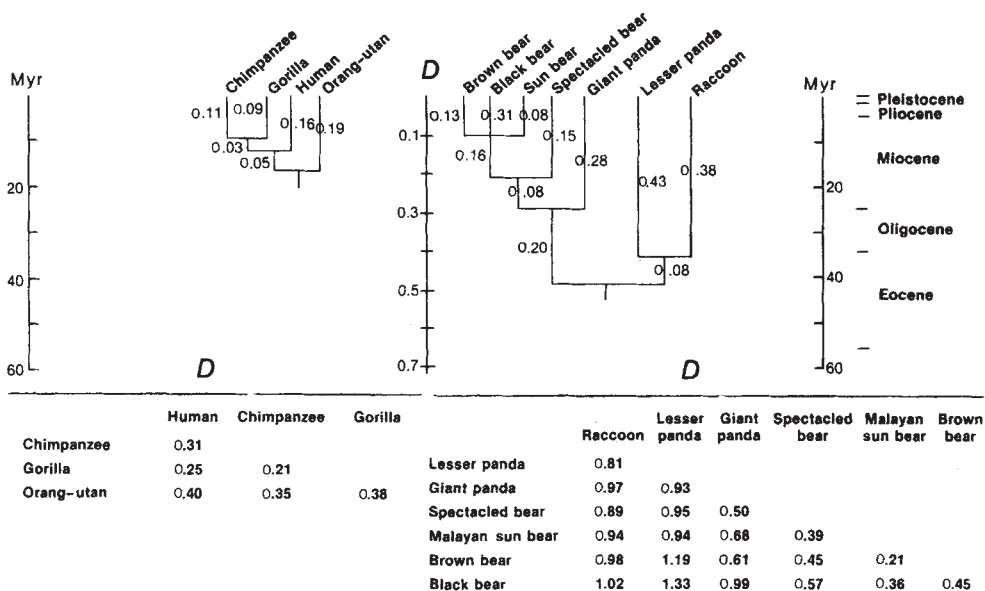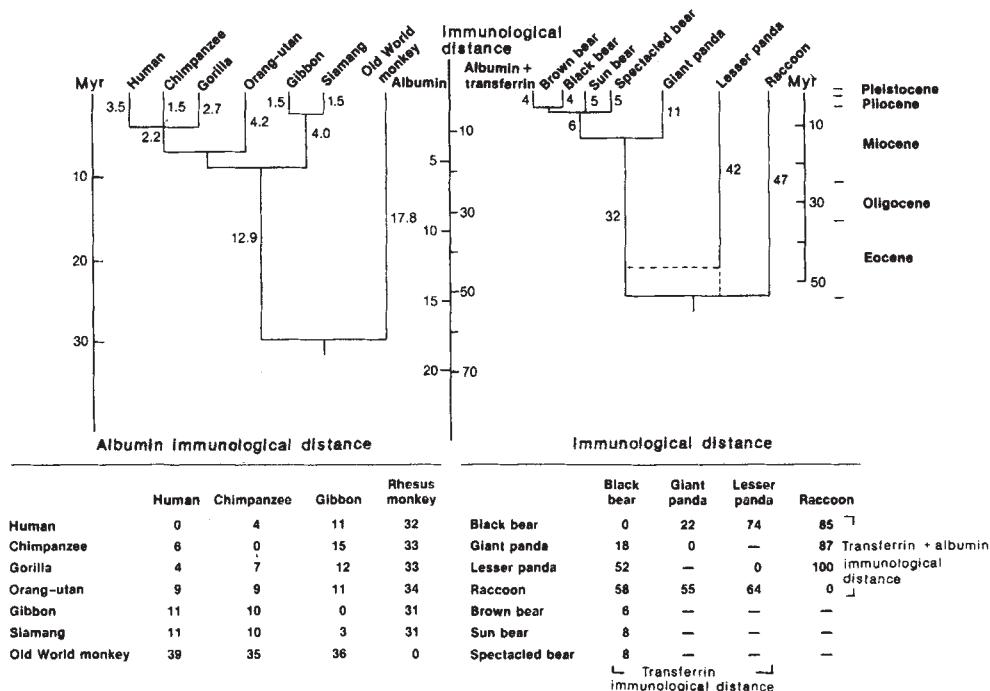
Fig. 3 Phylogenetic relationships of primates and carnivores based on immunological distance. Immunological distance was defined as 100 × the logarithm of the index of dissimilarity, the factor by which the dilution of a heterologous serum is increased to fix the amount of complement fixed in the homologous reaction. Primate distances are derived from the albumin index of dissimilarity presented by Sarich and Wilson[40]. Carnivore distances were based on transferrin plus albumin immunological distance data (upper right of table)[11]. The topology was constructed using the Fitch–Margoliash procedure[39] outlined in Fig. 1. The additional ursid species were added to the tree using the transferrin immunological distances presented in the lower left of the table. Because of the inequivalence of evolutionary rates of the three immunological distance metrics, the alignment of the two trees was determined on the basis of the timescale presented by Sarich and Wilson[40] and by Sarich[11]; this timescale is shown on the left of the figure, while the Pilbeam[53] timescale is presented on the right.

	Human	Chimpanzee	Gibbon	Rhesus monkey
Human	0	4	11	32
Chimpanzee	6	0	15	33
Gorilla	4	7	12	33
Orang-utan	9	9	11	34
Gibbon	11	10	0	31
Siamang	11	10	3	31
Old World monkey	39	35	36	0

	Black bear	Giant panda	Lesser panda	Raccoon
Black bear	0	22	74	85
Giant panda	18	0	—	87
Lesser panda	52	—	0	100
Raccoon	58	55	64	0
Brown bear	6	—	—	—
Sun bear	8	—	—	—
Spectacled bear	8	—	—	—

Transferrin + albumin immunological distance

Transferrin immunological distance

with an oncogenic retrovirus (feline sarcoma virus) and examined the preparations of extended early-metaphase chromosomes by G-trypsin banding (Fig. 4). Remarkably, nearly every large chromosome of the brown bear could be aligned with a giant panda chromosome arm. Sixteen such homologous comparisons are presented in Fig. 4. Alignments of smaller bear chromosomes (for example, UAR 19-24 in Fig. 4) were not attempted because homologies of 1-3 bands are likely to be coincidental. Nonetheless, the giant panda chromosomes seemed to be composed largely of bear chromosomes fused together as robertsonian translocations.

Only two of the banded giant panda or bear chromosomes had recognizable counterparts in the lesser panda or raccoon, thereby emphasizing the chromosomal consanguinity between bears and the giant panda[19]. On the contrary, 14 chromosomes of the lesser panda were strikingly homologous to chromosomes found in several procyonids and 10 of these were recognized as conservative ancestral 'carnivore chromosomes' found in several carnivore families other than Ursidae[50-52]. The simplest explanation of these data seems to affirm the molecular topologies; that is, the lesser panda and procyonids share a common ancestor at or subsequent to ursid divergence. The ursid line apparently
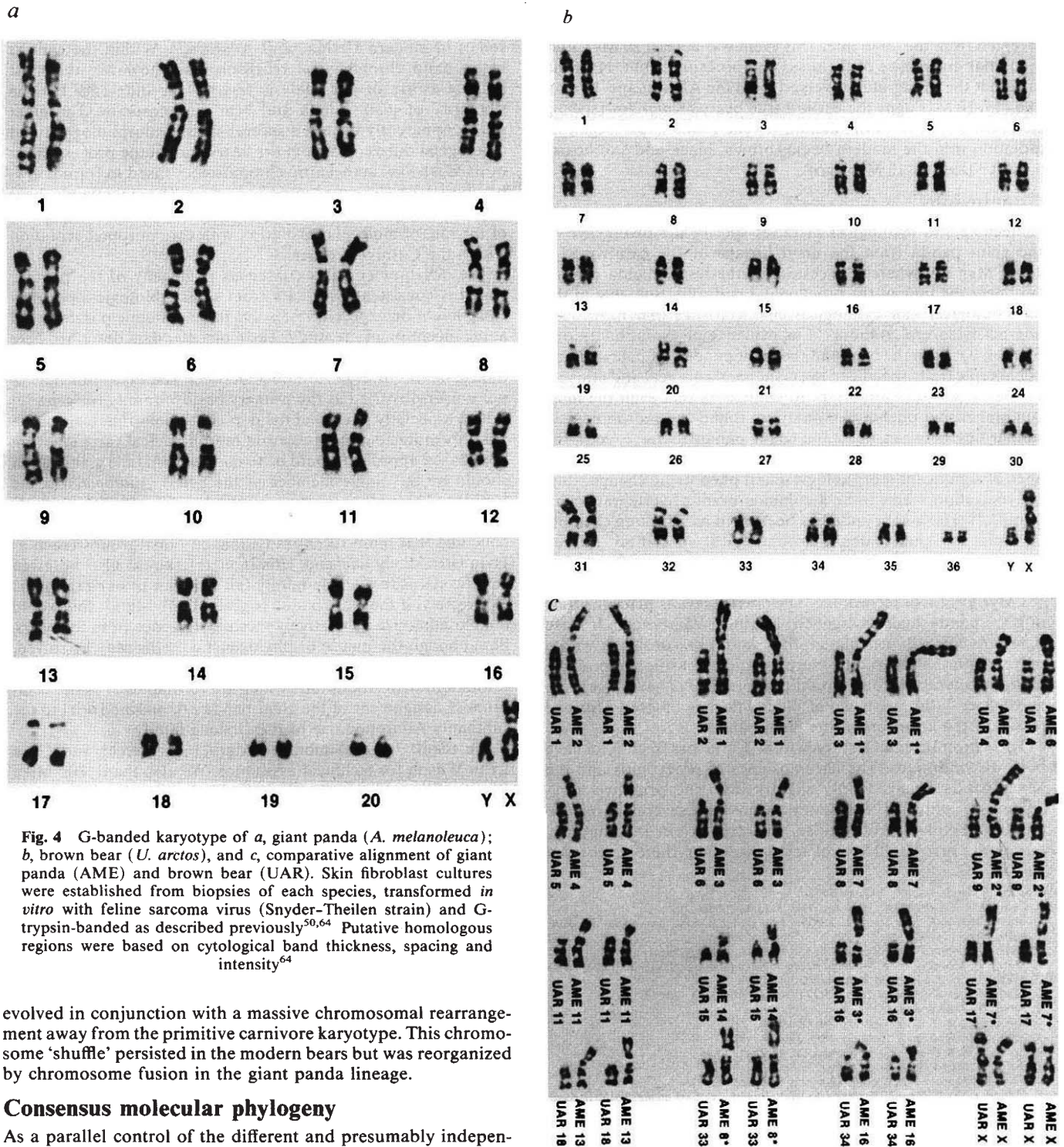
Fig. 4 G-banded karyotype of *a*, giant panda (*A. melanoleuca*); *b*, brown bear (*U. arctos*), and *c*, comparative alignment of giant panda (AME) and brown bear (UAR). Skin fibroblast cultures were established from biopsies of each species, transformed *in vitro* with feline sarcoma virus (Snyder-Theilen strain) and G-trypsin-banded as described previously[50,64] Putative homologous regions were based on cytological band thickness, spacing and intensity[64]

evolved in conjunction with a massive chromosomal rearrangement away from the primitive carnivore karyotype. This chromosome 'shuffle' persisted in the modern bears but was reorganized by chromosome fusion in the giant panda lineage.

Consensus molecular phylogeny

As a parallel control of the different and presumably independent techniques used here, we have applied the same methods to the construction of evolutionary trees of Hominoidae and representative simian species (Figs 1-3). Based on the 16-Myr calibration of the man–orang-utan split[53], the three primate phylogenies are topologically equivalent and in good agreement with the prevailing consensus of relationships between this group of primates[23-28,40-42]. Basically, the apes diverged from their common ancestor with the Old World monkeys 30–40 Myr BP. The gibbons split next between 20 and 25 Myr ago. The great apes began their radiation ~13–16 Myr ago with the orang-utan lineage followed by the ape–gorilla–human split 8–10 Myr ago. The agreement of our DNA and isozyme data with the immunological distance data and other topologies emphasizes the reliability of application of these same procedures to the carnivores.

The three molecular methods were also highly consistent in

the derived relationship within the carnivore radiations. Furthermore, a comparison of the primate and carnivore topologies using each procedure allows the correlation of the bear radiations with primate events regardless of the calibration date(s) used. Briefly, between 30 and 50 Myr ago, the progenitors of modern ursids and procyonids split into two lineages. Within 10 Myr of that event (possibly at its inception), the procyonid group split into Old World procyonids (represented today by the genus *Ailurus*, the lesser panda) and the New World procyonids (for example, raccoon, coatis, olingo and kinkajou). The lesser panda and giant panda clearly do not share a common ancestor after the ursid–procyonid split, emphasizing that the morphological similarities of the pandas are probably the result of parallel retention of ancestral characters that may have been lost (for example, in the bear) after their divergence from the

main line. At about the same time as the gibbons split from great apes (18-25 Myr ago), the ancestor of the giant panda diverged from the ursid line. This event was at least 20 Myr after the initial divergence of the ursid and procyonid split. Near the time that the orang-utan diverged from the African ape-human line (13-16 Myr ago) the earliest true bear, *Tremarctos* (spectacled bear), split from the ursid line. The genus *Ursus* began its radiation into the modern bears (brown, black and sun bears) 6-8 Myr later (8-12 Myr ago).

Conclusions

Molecular and cytological methods specify the divergence of the giant panda from the ursid lineage of the carnivores at 15-25 Myr BP, whereas ancestors of the lesser panda emerge very near the time of the procyonid-ursid split. But what of the morphological and cytological characteristics that have been offered by several authors[2,4,9] as evidence of evolutionary distinction between the bear and the giant panda? Of course, these phenotypic traits specific to the giant panda are real and unusual for bears, but it is important to emphasize that even the most comprehensive phenotypic analysis[3] found the morphological similarities between bear and giant panda to far exceed the number (and extent) of the differences. Furthermore, the appearance of significant morphological and phenotypic changes during speciation seems to be a common event of vertebrate evolution[54-56]. The giant panda has been cited as a striking example of rapid (or 'punctuated') morphological speciation[56], as its apparently dramatic anatomical divergence from bears (or raccoons) is accompanied by only a very recent (Pleistocene, <2 Myr BP) fossil record[15-17]. The present results place the time of giant panda-bear divergence back to 15-25 Myr ago. If these dates are correctly calculated, they would be consistent with a more traditional or gradual morphological transition to produce modern *Ailuropoda*, and would also predict, as have Eisenberg and Setzer[13], the existence of older Pliocene or even Miocene fossils of the ancestors of the giant panda.

The chromosomal history within Carnivora seems to have been discontinuous. The chromosomes of procyonids and the lesser panda changed only slightly from the primitive karyotype[50-52], which is today represented in several carnivore families (Felidae, Procyonidae, Viverridae). On the contrary, during the first 10-20 Myr of ursid evolution, there occurred a quantum chromosomal reorganization that is reflected today by the almost complete absence of 'carnivore-specific' chromosomes in modern Ursidae and *Ailuropoda*. Within the context of an ursid chromosomal reorganization, however, the most striking aspect of our analysis was not the difference but the similarity of giant panda and bear chromosomes. The giant panda apparently acquired several specific morphological and ethological differences from the bears and, in the process, most of its 'bear-like' acrocentric chromosomes fused to form a low-numbered, largely metacentric karyotype. The giant panda-bear phylogeny seems to provide a specific example of the uncoupling of the rate of molecular evolution with chromosomal evolution within the Carnivore order[57-59].

The development of a consensus phylogeny of the pandas, which is consistent with data from several biological perspectives, might be expected to resolve the taxonomic puzzle posed at the inception of the study. The molecular data described here provide a clear relationship between the species and narrow the timing of the divergence nodes between the taxa. But time is not universally used as the primary basis of family (or generic) status, especially in view of the non-continuous timescale of the possibly more adaptively relevant morphological variation. One time-based approach would be to conclude that the giant panda should be the single member of the genus *Ailuropoda* in the Ursidae. The phylogenies presented here would not preclude this assignment; however, if we accept this, should we not also conclude that more recent radiations in other groups (such as Hylobates, Pongidae and Hominoidae) should also be given generic status in a single family (all the apes in our example)? Conversely, if the giant panda is given family status, this would tend to minimize its relatively recent divergence from the bears. So, although the puzzle of phylogenetic timing may be solved, classification of the pandas may continue to be argued. One resolution of this question (which we recommend) is the compromise assignment of the giant panda (*A. melanoleuca*) to the subfamily Ailuropodinae of the Ursidae family.

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