Hemoglobin of Pandas: Phylogenetic Relationships of Carnivores as Ascertained with Protein Sequence Data

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Dedicated to Prof. Dr. Dr. Dr. h.c. Heinz A. Staab for his 60th birthday

The generally accepted relationships of pandas [1–3], pinnipeds [4, 5], and other carnivores [5–7], as determined with traditional and molecular data, are evaluated with the protein sequences of six different polypeptide types combined in an extended tandem aligment. Phylogenetic trees constructed from the protein sequences by the maximum parsimony method [8, 9] do not refute the relationships of terrestrial carnivore families, as developed from other evidence [5]. However, they do disagree with the commonly accepted categorization of pandas [1–3] and pinnipeds [4, 5]. The most parsimonious trees revealed by the parsimony analysis suggest instead that the pandas (either separately or together) are closely related to the families Mustelidae and Procyonidae, and that the pinnipeds are a monophyletic (rather than a diphyletic) group.

Extant families in the mammalian order Carnivora are traditionally [6] divided into the superfamilies Feloidea (cats and allies) and Canoidea [Canidae (dogs and foxes), Mustelidae (badgers and minks), Odobenidae (walruses), Otariidae (sea lions), Phocidae (true seals), Procyonidae (racoons), and Ursidae (bears)]. Within the Canoidea, the family Canidae is usually regarded as the sister group of the other members (which collectively form the superfamily Arctoidea of Tedford [5]. Traditional [6] and paleontological data [5] furthermore suggest that the arctoids may be further subdivided into two different clades, as represented by: the families Mustelidae, Phocidae, and Procyonidae; and the families Odobenidae, Otariidae, and Ursidae. Pinnipeds (the families Odobenidae, Otariidae, and Phocidae) are therefore diphyletic according to traditional evidence [4, 5].

Molecular approaches have also been used to investigate the phylogenetic relationships of carnivores. In a recent analysis of different molecular and cytological data sets, O'Brien et al. [2] concluded that *Ailuropoda melanoleuca* (greater panda) is closely related to the family Ursidae, whereas *Ailurus fulgens* (lesser panda) may be allied to the family Procyonidae. However, their phylogenies were based on few representatives of the order (two to three families) and on the assumption of a molecular clock (constant rate of evolution). In an unrelated study of pinnipeds, Sarich [10], using immunological distances, suggested that the families Otariidae and Phocidae are closely related, rather than distantly allied as proposed from traditional evidence [4, 5]. However, in both cases, molecular sequences were not considered in the analyses.

In this study, a molecular phylogeny of carnivores is constructed from an enlarged body of protein sequence data by the maximum parsimony algorithm [8, 9, 11]. In the enlarged matrix, the order Carnivora is best represented by α- and β-hemoglobins and then by lens α-crystallin A, fibrinopeptides A and/or B, myoglobin, and cytochrome c in that order. The α- and β-hemoglobins

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                  NA        A_______________________________________________________________AB  B_____________________
Greater Panda a                                            10     Ala                 Ile           20
Lesser  Panda a  Val-   -Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val-Lys-Ser-Thr-Trp-Asp-Lys-Leu-Gly-Gly-His-Ala-Gly-Glu-Tyr-
Lesser  Panda ß  Val-His-Leu-Thr-Gly-Glu-Glu-Lys-Ala-Ala-Val-Thr-Gly-Leu-Trp-Ser-Lys-Val-       -Asn-Val-Asp-Glu-Val-
Greater Panda ß                                        10                                              20
                 ___________________________________________C___________________________CD

Greater Panda a                         30                                      40
Lesser  Panda a  -Gly-Gly-Glu-Ala-Leu-Glu-Arg-Thr-Phe-Ala-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe-   -Asp-Leu-
Lesser  Panda ß  -Gly-Gly-Glu-Ala-Leu-Gly-Arg-Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe-Asp-Ser-Phe-Gly-Asp-Leu-
Greater Panda ß                             30                                      40
                     D_____________________________    E______________________________________________________________

Greater Panda a         50                                                      60                              Thr
Lesser  Panda a  -Ser-Pro-                   -Gly-Ser-Ala-Gln-Val-Lys-Ala-His-Gly-Lys-Lys-Val-Ala-Asp-Ala-Leu-Thr-Leu-
Lesser  Panda ß  -Ser-Ser-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-Lys-Val-Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Asn-Ser-Phe-Ser-Glu-
Greater Panda ß       Thr  51                 Asn              60                                       70
                  ___________EF                              F_______________________________  ___FG

Greater Panda a         70                                     80                                       90
Lesser  Panda a  -Ala-Val-Gly-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu-Ser-Ala-Leu-Ser-Asp-Leu-His-Ala-His-Lys-Leu-Arg-Val-
Lesser  Panda ß  -Gly-Leu-Lys-Asn-Leu-Asp-Asn-Leu-Lys-Gly-Thr-Phe-Ala-Lys-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val-
Greater Panda ß                             80                                      90
                 G___________________________________________________________________________ GH                  H___

Greater Panda a                             100                                  110 Ser
Lesser  Panda a  -Asp-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-Cys-Leu-Leu-Val-Thr-Leu-Ala-Cys-His-His-Pro-Ala-Glu-Phe-Thr-
Lesser  Panda ß  -Asp-Pro-Glu-Asn-Phe-Lys-Leu-Leu-Gly-Asn-Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-Gly-Lys-Glu-Phe-Thr-
Greater Panda ß         100                                    110                                      120
                  _______________________________________________________________________________HC

Greater Panda a         120                                    130                                      140
Lesser  Panda a  -Pro-Ala-Val-His-Ala-Ser-Leu-Asp-Lys-Phe-Phe-Ser-Ala-Val-Ser-Thr-Val-Leu-Thr-Ser-Lys-Tyr-Arg
Lesser  Panda ß  -Pro-Gln-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His
Greater Panda ß                             130                                     140
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Fig. 1. Protein sequences of the α- and β-hemoglobins from the greater panda (*Ailuropoda melanoleuca*) and the lesser panda (*Ailurus fulgens*). Only the variable positions of *Ailuropoda* relative to *Ailurus* are shown. *A, B, C* ... helical regions. *AB, BC, CD* ... interhelical regions [18]

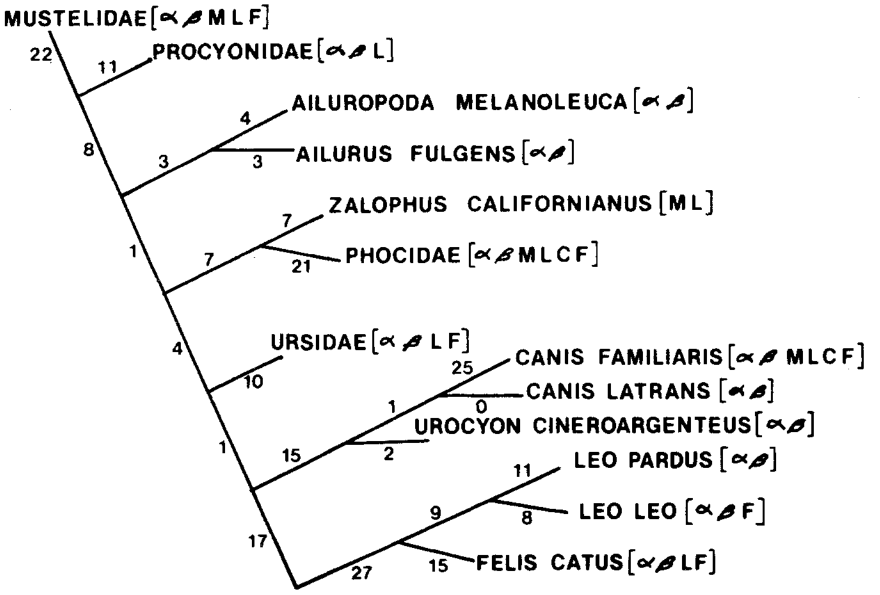

Fig. 2. One of the most parsimonious trees [nucleotide replacement (NR) score = 3553] supported by the amino acid sequences of the study group (carnivores) and outgroups (other gnathostomes) [8]. The abbreviations in parentheses correspond to amino acid sequences available for each OTU [11], as follows: *α* α-hemoglobin; *β* β-hemoglobin; *M* myoglobin; *L* lens α-crystallin A; *C* cytochrome c; and *F* fibrinopeptides A and/or B. Values next to branches represent link lengths which summarize the total number of nucleotide replacements along individual internodes [8, 9]. Families of carnivores in the tree reflect hybrid OTU's which were formed from the protein sequences of confamilial members [11], as follows: (1) family Mustelidae [*Meles meles* (European badger, αβMF) + *Mustela vison* (eastern mink, LF)]; (2) family Phocidae [*Halichoerus grypus* (gray seal, LF) + *Mirounga leonina* (elephant seal, C) + *Phoca vitulina* (harbor seal, αβM)]; (3) family Procyonidae [*Bassariscus* sp. (ring-tailed cat, L) + *Procyon lotor* (racoon, αβ)]; and (4) family Ursidae [*Melursus ursinus* (sloth bear, L) + *Ursus arctos* (grizzly bear, F) + *U. maritimus* (polar bear, αβ)]. This tree is rooted by outside reference to the outgroups (not shown) [19, 20]. Methods. The protein sequences of the 90 eutherian and other gnathostome OTU's used by Miyamoto and Goodman [11] were employed in the present analysis with the following exceptions: The ribonuclease sequences used by them were not included in the present study, since such data are still not available for carnivores [21]. However, their data matrix was updated with the α- and β-hemoglobins of seven carnivores [13, 22, 23]: [*Ailuropoda melanoleuca* (giant panda); *Ailurus fulgens* (lesser panda); *Canis latrans* (coyote); *Felis catus* (domestic cat); *Leo pardus* (amur-leopard); *Urocyon cineroargenteus* (gray fox); and *Ursus maritimus* (polar bear)] and by a supraspecific hybrid representing the order Psittaciformes [24]: [*Ara ararauna* (blue-and-yellow macaw, αβ) + *Melopsittacus undulatus* (budgerigar, L)]. Phylogenetic trees based on the modified sequence matrix were constructed from initial input cladograms by the maximum parsimony algorithm [8, 9]. In the analysis, the branching patterns of the outgroups were fixed in all input trees [except for the UPGMA and the distance Wagner dendrograms (see below)] according to the most parsimonious phylogeny reported by Miyamoto and Goodman [11]. The initial branching arrangements for the study group (carnivores) were obtained from several different traditional and molecular studies available in the literature [1–7], as well as from UPGMA and the distance Wagner clustering of pairwise minimum mutation distances [8, 9, 14–16, 20, 22, 25]. The point at which the outgroups joined the network of the study group (carnivores) was chosen as the root of each cladogram under consideration [19, 20]

of *Ailuropoda* and *Ailurus* are now included, as are several sequences of other carnivores not previously considered (Figs. 1 and 2). More importantly, all living families in the order are now represented by at least one OTU (operational taxonomic unit), except for the canoid family Odobenidae and feloid taxa Hyaenidae and Viverridae. Each carnivore OTU in the matrix is represented by an average of nearly 3.5 different polypeptide chains.

The α- and β-hemoglobins of *Ailuropoda* and *Ailurus* are considerably more similar to each other than to any other carnivore including *Procyon lotor* (racoon) [12] and *Ursus maritimus* (polar bear) [13]. The hemoglobins of the two pandas differ at six amino acid positions, which reflects a minimum mutation distance of only seven [14] (Fig. 1). In contrast, the sequences of *Ailuropoda* and *Ailurus* differ from those of *U. maritimus* and *P. lotor* by minimum mutation distances of 11 and 18, respectively. *Ailuropoda* and *Ailurus* would be grouped together by phenetic methods using overall similarity (i.e., UPGMA) [15].

Several most parsimonious trees, each with a nucleotide replacement (NR) score of 3553 are supported by the tandemly aligned data [9, 16]. The alternatives revealed by the maximum parsimony method vary from the one shown in Fig. 2 by their arrangements of pandas and pinnipeds [Phocidae and *Zalophus* (sea lion)], and by their positions of Canidae [*Canis familiaris* (domestic dog), *C. latrans* (coyote), and *Urocyon cineroargenteus* (gray fox)] and Ursidae relative to each other. *Ailurus* is more closely related to the families Mustelidae and Procyonidae than *Ailuropoda*, according to one other parsimonious solution. The position of pinnipeds varies such that they are represented in another reconstruction as the sister group of all other canoids. One additional parsimonious solution reverses the positions of the families Canidae and Ursidae. In all cases, the congeners *Canis familiaris* and *C. latrans* and *Leo leo* (lion) and *L. pardus* (amur-leopard) unite to form separate monophyletic genera.

The most parsimonious trees constructed from the protein sequence data do not support the relationships of pandas and pinnipeds relative to other carnivores, as hypothesized from either traditional evidence or DNA hybridizations [1–5] (Fig. 2). *Ailuropoda* and *Ailurus* are not closely related to the families Ursidae and Procyonidae, respectively, as generally accepted by other investigators. Instead, the most parsimonious solutions based on amino acid sequences suggest that *Ailuropoda*, *Ailurus*, and the clade of Mustelidae/Procyonidae are best treated as separate lineages of a trichotomy [17]. With respect to pinnipeds, the most parsimonious trees suggest that the families Otariidae (*Zalophus*) and Phocidae are closely related, as hypothesized by Sarich [10] from immunological distances. Therefore they do not support the more widely accepted hypothesis that the pinnipeds are diphyletic [4, 5], rather than monophyletic. Clearly, the relationships of both pandas and pinnipeds warrant further cladistic analyses with additional protein sequences.

The relationships of carnivores, as hypothesized from traditional evidence, are not otherwise refuted by the most parsimonious trees supported by protein sequences (Fig. 2). Mustelidae and Procyonidae represent sister groups, according to the sequence data, which are allied to the families Canidae and Ursidae. The superfamily Feloidea (Felidae) constitutes the distantly derived lineage of Carnivora according to the molecular results. These higher-level relationships among terrestrial families are consistent with a cladogram of Tedford [5], proposed from traditional anatomical and paleontological evidence. The traditional arrangement of these five families into the two superfamilies Canoidea (Canidae, Mustelidae, Procyonidae, and Ursidae) and Feloidea (Felidae) [6] is therefore consistent with available amino acid sequence data.

Phylogenetic analysis of the sequence data was supported by National Institutes of Health (NIH grant R01 HL33940), National Science Foundation (NSF grant BSR 83-07336), and Alfred P. Sloan Foundation.

Received March 19, 1986

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1. Davis, D.: Fieldiana Zool. Mem. *3*, 1 (1964); Leone, C.A., Wiens, A.L.: J. Mammal. *37*, 11 (1956); Sarich, V.M.: Nature *245*, 218 (1973)
2. O'Brien, S.J., et al.: Nature *317*, 140 (1985)
3. Morris, D., Morris, R., in: The Giant Panda, p. 160. New York: Penguin, 1981; Thenius, E.Z.: Säugetierk. Mitt. *44*, 286 (1979)
4. McLaren, I.A.: Syst. Zool. *9*, 18 (1960); Mitchell, E.D.: ibid *16*, 350 (1967); Mitchell, E.D., in: Biology of the Seal, p. 12 (eds. K. Ronald, A.W. Mansfield). Rapp. P. -v. Reun. Conserv. int. Explor. Mer 1975
5. Tedford, R.H.: Syst. Zool. *25*, 363 (1976)
6. Stains, H.J., in: Orders and Families of Recent Mammals of the World, p. 491 (eds. S. Anderson, J. Knox Jones, Jr.). New York: Wiley 1984
7. Sarich, V.M.: Syst. Zool. *18*, 286 (1969); Simpson, G.G.: Bull. Am. Mus. Nat. Hist. *85*, 105 (1945)
8. Goodman, M., et al.: Syst. Zool. *28*, 132 (1979)
9. Goodman, M.: Prog. Biophys. Mol. Biol. *37*, 105 (1981)
10. Sarich, V.M.: Am. Zool. *15*, 826 (1975)
11. Miyamoto, M.M., Goodman, M.: Syst. Zool. (submitted)
12. Brimhall, B., et al.: Hemoglobin *2*, 351 (1978)
13. Hofman, O., Schreitmuller, T., Braunitzer, G.: Biol. Chem. Hoppe-Seyler *367*, 53 (1986)
14. Fitch, W.M., Margoliash, E.: Science *155*, 279 (1967)
15. Sneath, P.H.A., Sokal, R.R., in: Numerical Taxonomy, p. 230. San Francisco: Freeman 1973
16. Goodman, M., et al.: Acta Zool. Fennica *169*, 19 (1982)
17. Wurster-Hill, D.H., Bush, M.: Cytogen. Cell Genet. *27*, 147 (1980); Newnham, R.E., Davidson, W.M.: Cytogenetics *5*, 152 (1966); Collins, L.R., Page, J.K.: Ling-Ling and Hsing-Hsing, Year of the Panda. Garden City: Anchor Press/Doubleday 1973
18. Hofmann, O.: Thesis Univ. Munich 1986
19. Farris, J.S.: Syst. Zool. *31*, 328 (1982)
20. Farris, J.S.: Am. Natur. *106*, 645 (1972)
21. Beintema, J.J., Lenstra, J.A., in: Macromolecular Sequences in Systematic and Evolutionary Biology, p. 43 (ed. M. Goodman). New York: Plenum Press 1982
22. Goodman, M., et al., in: ibid., p. 115
23. Abbasi, A., Braunitzer, G.: Biol. Chem. Hoppe-Seyler *366*, 699 (1985); J. Prot. Chem. *4*, 57 (1985)
24. Godovac-Zimmerman, J., Braunitzer, G.: Biol. Chem. Hoppe-Seyler *366*, 503 (1985); Stapel, S.O., et al.: Nature (submitted)
25. Goodman, M., Czelusniak, J., Beeber, J.E.: Cladistics *1*, 171 (1985); Goodman, M., Weiss, M.L., Czelusniak, J.: Syst. Zool. *31*, 376 (1982)

Naturwissenschaften Buchbesprechungen

Verdunstung. Von H. Schrödter. Berlin-Heidelberg-New York-Tokyo: Springer 1985. 186 S., 17 Tab., DM 36,–.

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