

Whence the Red Panda?

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The evolutionary history of the red panda (*Ailurus fulgens*) plays a pivotal role in the higher-level phylogeny of the “bear-like” arctoid carnivoran mammals. Characters from morphology and molecules have provided inconsistent evidence for placement of the red panda. Whereas it certainly is an arctoid, there has been major controversy about whether it should be placed with the bears (ursids), ursids plus pinnipeds (seals, sea lions, walrus), raccoons (procyonids), musteloids (raccoons plus weasels, skunks, otters, and badgers [mustelids]), or as a monotypic lineage of uncertain phylogenetic affinities. Nucleotide sequence data from three mitochondrial genes and one nuclear intron were analyzed, with more complete taxonomic sampling of relevant taxa (arctoids) than previously available in analyses of primary molecular data, to clarify the phylogenetic relationships of the red panda to other arctoid carnivorans. This study provides detailed phylogenetic analyses (both parsimony and maximum-likelihood) of primary character data for arctoid carnivorans, including bootstrap and decay indices for all arctoid nodes, and three statistical tests of alternative phylogenetic hypotheses for the placement of the red panda. Combined phylogenetic analyses reject the hypotheses that the red panda is most closely related to the bears (ursids) or to the raccoons (procyonids). Rather, evidence from nucleotide sequences strongly support placement of the red panda within a broad Musteloidea (*sensu lato*) clade, including three major lineages (the red panda, the skunks [mephitids], and a clearly monophyletic clade of procyonids plus mustelids [*sensu stricto*, excluding skunks]). Within the Musteloidea, interrelationships of the three major lineages are unclear and probably are best considered an unresolved trichotomy. These data provide compelling evidence for the relationships of the red panda and demonstrate that small taxonomic sample sizes can result in misleading or possibly erroneous (based on prior modeling, as well as conflict between the results of our analyses of less and more complete data

sets) conclusions about phylogenetic relationships and taxonomy. © 2000 Academic Press

INTRODUCTION

Relationships among arctoid carnivorans have been difficult to resolve. In particular, the pandas have taken center stage because of their peculiar specializations and controversy regarding their evolutionary relationships. The highly endangered, high-profile giant panda (*Ailuropoda melanoleuca*) has dominated many prior phylogenetic discussions and analyses, but in fact its position (as a bear, representing the sister lineage to all other living ursids) has been well documented for some time (Davis, 1964; see Flynn *et al.*, 1988 and Wyss and Flynn, 1993). The most puzzling question in panda relations, the evolutionary position of the red or lesser panda (*Ailurus fulgens*), has yet to be resolved. This lack of resolution is due primarily to its unique morphology and peculiar specializations to herbivory (e.g., complexly elaborated cheek teeth, especially the premolars, with P2-3 morphology unique among carnivorans; small extra “thumb,” a radial sesamoid, on the hand; distinctive red fur; cranial modifications, including very large zygomatic arch and temporal fossa widths; powerful jaw apparatus, including very robust jaw cross-sectional area, tall coronoid process, and highest moment arm of the temporalis muscle among living carnivorans [Radinsky, 1981]). In addition, the red panda retains some primitive characteristics of the basicranium and ear region, features that have been modified in potential close relatives. Unfortunately, the red panda has not been included in many molecular studies.

Geoffroy-Saint-Hilaire and Cuvier (1825) first described the red panda as closely resembling a raccoon (procyonid), although they gave it the name *Ailurus* based on its superficial likeness to that of the domestic cat. Subsequent morphological and molecular evidence has supported several phylogenetic hypotheses, including (1) related to the ursids (or ursids plus pinnipeds; Sarich, 1973; Wozencraft, 1989; Wyss and Flynn, 1993; Vrana *et al.*, 1994); (2) related to the procyonids (Greg-

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ory, 1936; Thenius, 1979; O'Brien *et al.*, 1985; Goldman *et al.*, 1989; Wayne *et al.*, 1989; Slattery and O'Brien, 1995; questionably, Flynn *et al.*, 1988; Wang, 1997); (3) related to the musteloids, including procyonids plus some or all "mustelids" (see below; Schmidt-Kittler, 1981; Flynn and Nedbal, 1998; Bininda-Emonds *et al.*, 1999); (4) sister taxon to the giant panda either with uncertainty about their broader relationships (Segall, 1943) or with the panda clade as sister taxon to ursids (Ginsburg, 1982); and (5) an unresolved monotypic lineage within the arctoids (Ledje and Árnason, 1996a,b; Wolsan, 1993).

Bininda-Emonds *et al.* (1999) included analysis of all carnivoran species, but developed their phylogeny by compositing tree topologies of prior studies (via "super-tree" consensus methods of "matrix representation using parsimony analysis"), rather than from primary character data. In a study focused on higher-level carnivoran phylogeny rather than the placement of *Ailurus*, Flynn and Nedbal (1998) determined that the red panda is sister taxon to Musteloidea (*sensu stricto*, s.s.), based on analysis of a less complete taxonomic (no skunks) and molecular character (no 16S rRNA) data set.

In this study, we expanded sampling of both taxa and characters beyond all previous analyses of primary molecular phylogenetic data by including sequences (3450 nucleotides; all confirmed by double-stranded sequencing) from three mitochondrial genes (cytochrome *b*, 12S rRNA, and 16S rRNA) and one nuclear intron (transthyretin intron I) for 17 carnivoran species representing all caniform families, including a wide diversity of arctoids, and several feliform outgroups. We also performed extensive analyses of phylogeny robustness (parsimony and likelihood bootstrap, parsimony decay indices, likelihood ratio tests of branch lengths) and tests of statistical support for the proposed *Ailurus* phylogeny or alternatives (Wilcoxon signed rank, Kishino-Hasegawa, compare-2 permutation).

METHODS

Data Collection

Nucleotide sequence data used in this study were compiled from previously published studies and GenBank accessions or sequenced for 16S rRNA (spectacled bear, giant panda, red panda) and transthyretin intron I (spotted skunk, striped skunk). Sequencing methods were similar to those previously described in Dragoo and Honeycutt (1997) and Flynn and Nedbal (1998). When sequence data from the same species were not available for all genes, sequences from very closely related species (e.g., same genus or subfamily) were used as proxies, assuming that variation within closely related taxa was insignificant compared to the variation among the higher-level study taxa. Sources of

published data and GenBank accession numbers are shown in Table 1. The composite (concatenated; after removal of ambiguously aligned nucleotides) nucleotide sequence data comprised a total of 3450 bp (851 bp of nuclear transthyretin intron I, 1140 bp of mt cytochrome *b*, 964 bp of mt 12S rRNA, 495 bp of mt 16S rRNA) for 17 carnivoran taxa (15 caniforms, 13 of which were arctoids, and 2 feliform outgroups).

Phylogenetic Analyses

New sequences were joined to existing alignments (Árnason *et al.*, 1995; Dragoo and Honeycutt, 1997) by eye. Ambiguous regions were excluded from the analyses. Phylogenetic analyses were conducted with PAUP 4.0d59 (Swofford, 1998). For maximum-parsimony analyses, the following character weightings were used: (1) transversion substitutions only for 12S and 16S rRNA genes, (2) the conservative weighting approach (tranversions only at 3rd positions, all substitutions at 2nd positions, all substitutions at 1st positions with changes involving leucine recoded as a "Y") of Irwin *et al.* (1991) for cytochrome *b* sequences, and (3) equally weighted substitutions within the transthyretin intron, following Flynn and Nedbal (1998). In the case of transthyretin, transition/transversion and nucleotide position biases are not applicable to this putatively noncoding region, and prior analyses of a broader suite of carnivorans showed no tree topology differences between equal-weighting parsimony and rate-heterogeneous likelihood analyses of transthyretin data. Incorporating among-site rate heterogeneity in likelihood analyses of the entire molecular data set of the current study yielded no conflict (only less resolution, in the likelihood tree) in the comparative tree topologies. Maximum-likelihood analyses were performed using a general time-reversible model of sequence evolution incorporating rate heterogeneity among sites using a "discrete gamma" model. Substitution model parameters were estimated via maximum-likelihood and the most-parsimonious tree. Bootstrap (BS) values for maximum-parsimony analyses of the concatenated molecular data set were obtained using 1000 replicates, each consisting of 10 heuristic (100 heuristic for the individual gene/intron analyses only; tree bisection-reconnection) searches in which the input order of the taxa was randomized. Bootstrap values for maximum-likelihood analyses of the concatenated molecular data set were obtained using 100 replicates and the "asis" addition sequence of taxa. In addition, the decay index (DI; Bremer, 1988), representing the number of extra steps required for a clade not to be unequivocally supported, was used in maximum-parsimony analyses. Three statistical tests [Wilcoxon signed rank (Templeton, 1983), Kishino-Hasegawa (Kishino and Hasegawa, 1989), and compare-2 permutation (Swofford, 1998)] were applied to determine whether

TABLE 1
Taxon and Gene Sampling, with GenBank Accession Nos.

Taxon ^a /Gene	Cytochrome <i>b</i> ^b	12S rRNA ^c	16S rRNA ^d	Transthyretin ^e
Herpestidae				
Mongoose or Cusimanse: <i>Crossarchus obscurus</i>				AF039726
(Java mongoose): <i>Herpestes auropunctatus</i>	X94926	Y08506		
(Cape gray mongoose): <i>H. (=Galerella) pulverulentus</i>			U78332	
Felidae				
Cat: <i>Felis sylvestris</i>	X82296	Y08503		AF039724
(Ocelot): <i>Felis pardalis</i>			U78331	
Canidae				
Red fox: <i>Vulpes vulpes</i>	X94929	Y08508		AF039733
(Grey fox): <i>Vulpes cinereoargenteus</i>			U78348	
Wolf: <i>Canis lupus</i>		Y08507	U78329	AF039732
(<i>Canis familiaris</i>)	X94920			
Ursidae				
Brown bear: <i>Ursus arctos</i>	X82038	Y08519	U78349	AF039741
Spectacled bear: <i>Tremarctos ornatus</i>	U23554		AF306947 ^f	AF039740
(Black bear): <i>Ursus americanus</i>		Y08520		
Giant panda: <i>Ailuropoda melanoleuca</i>	X94918	Y08521	AF306945 ^f	AF039738
Pinnipedia				
Sea lion: <i>Zalophus californianus</i>	X82310	Y08525	U78350	AF039745
Walrus: <i>Odobenus rosmarus</i>	X82299	L&A 1996a	U78343	AF039743
Bearded seal: <i>Erignathus barbatus</i>	X82295			AF039742
(Grey seal): <i>Halichoerus grypus</i>		X72004		
(Harbour seal): <i>Phoca vitulina</i>			X63726	
Procyonidae				
Kinkajou: <i>Potos flavus</i>			U78344	AF039737
(Olingo): <i>Bassaricyon gabbii</i>	X94931	Y08509		
Raccoon: <i>Procyon lotor</i>	X94930	Y08510	U78345	AF039736
Mustelidae (s.s.)				
River otter: <i>Lontra longicaudus</i>	X94923		U78335	AF039734
(Sea otter): <i>Enhydra lutris</i>		Y08512		
Long-tailed weasel: <i>Mustela frenata</i>			U78339	AF039735
(European polecat): <i>Mustela putorius</i>	X94925	Y08516		
Mephitidae				
Spotted skunk: <i>Spilogale putorius</i>	X94928	Y08518	U78346	AF306949 ^f
Striped skunk: <i>Mephitis mephitis</i>	X94927	Y08517	U78338	AF306948 ^f
<i>Ailurus</i>				
Red panda: <i>Ailurus fulgens</i>	X94919	Y08511	AF306946 ^f	AF039739

^a In some cases the same species had not been sequenced for all genes. The most closely related taxa, listed in the table, that had been sequenced for those genes were incorporated into the analyses.

^b Cytochrome *b*: Ledje and Árnason, 1996a; Árnason *et al.*, 1995; Talbot and Shields, 1995.

^c 12S rRNA: Ledje and Árnason, 1996b; Árnason *et al.*, 1993.

^d 16S rRNA: Árnason and Widegren, 1986; Árnason and Johnsson, 1992; Dragoo and Honeycutt, 1997.

^e Transthyretin: Flynn and Nedbal, 1998.

^f New GenBank accessions, this paper.

the level of support for the placement of the red panda in the maximum-parsimony analyses is significantly higher than that for alternative hypotheses of phylogenetic relationships.

Prior to combining all genes into a single phylogenetic analysis, we tested for significant conflict in phylogenetic signal among the genes (Bull *et al.*, 1993). Specifically, we performed maximum-parsimony reconstruction on each gene separately (including estimation of nodal bootstrap proportions) and then compared the most-parsimonious trees from each analysis. Genes that yielded topological differences, supported by nodal bootstrap propor-

tions >70%, were not combined (de Quiroz, 1993; Flynn and Nedbal, 1998). This approach is similar to the conditional data combination (CDC) approach of Bull *et al.* (1993). Results from these individual gene analyses were twofold. First, the bootstrap criterion did not reject the null hypothesis of homogeneity in phylogenetic signal among the gene sequences, enabling them to be combined in a single analysis. Second, separate analyses of individual genes alone were unable to completely resolve phylogenetic relationships involving the red panda, highlighting the value of combining multiple data sets in reconstructing resolved and robustly supported phylogenies.

TABLE 2
Statistical Tests of Phylogenetic Hypotheses

Hypotheses tested	Wilcoxon signed rank	Kishino–Hasegawa		Compare-2 permutation
		Parsimony	Likelihood	
Hypotheses in Fig. 1A versus				
Red panda + bear clade	$Z = 2.737^{**}$	$T = 3.149^{**}$	$T = 3.323^{***}$	T-PTP = 0.001, 22 steps
Red panda + raccoon clade	$Z = 1.651$	$T = 1.900$	$T = 1.814$	T-PTP = 0.001, 13 steps
Hypotheses in Fig. 1B versus				
Red panda + bear clade	$Z = 1.988^*$	$T = 2.287^*$	$T = 2.873^{**}$	T-PTP = 0.001, 18 steps
Hypotheses in Fig. 1C versus:				
Red panda + bear clade	$Z = 2.230^*$	$T = 2.568^*$	$T = 3.421^{***}$	T-PTP = 0.001, 21 steps
Red panda + raccoon clade	$Z = 2.737^{**}$	$T = 3.132^{**}$	$T = 2.452^*$	T-PTP = 0.001, 15 steps

Note. The constraint trees used to test the hypotheses were the most parsimonious or most likely topology having the appropriate relationships constrained. For example, in the first test in which all taxa were included, the unconstrained most-parsimonious tree (Fig. 1A) was tested against the most-parsimonious tree having a monophyly constraint of the red panda and bears. Significance indicates that the hypothesis tested (e.g., red panda + bear clade) was rejected.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

RESULTS

Our related earlier study (Flynn and Nedbal, 1998) indicated strong support for placement of the red panda as a member of the clearly monophyletic Musteloidea (*sensu lato*, s.l.), although both molecular sampling and taxonomic coverage of arctoids were less complete (no mt 16S rRNA sequences, no skunks). However, as in this study, separate analyses of individual nucleotide sequences alone (transthyretin intron I, mt cytochrome *b*, mt 12S rRNA; Flynn and Nedbal, 1998) were unable to completely or robustly resolve phylogenetic relationships of the red panda within the well-supported clade of Musteloidea (s.l.). For example, analyses of the transthyretin intron sequences effectively yielded a trichotomy of procyonids/mustelids/red panda, as none of the three taxa were linked to one another with strong support (>70 BS; procyonids and mustelids were linked only weakly [BS/DI = 53/1] to the exclusion of the red panda). To further test hypotheses concerning phylogenetic relationships of the red panda, we applied the statistical test of Kishino and Hasegawa (1989), using maximum-likelihood (implemented using the PHYLIP software package of Felsenstein, 1993) and the Flynn and Nedbal (1998) data set. This test compares the mean and variance of log-likelihood differences between constraint trees to determine whether the means are significantly different. If one assumes the robustly supported relationships in Flynn and Nedbal (1998) (1) monophyly of all caniform families (note that the data from Flynn and Nedbal do not include any skunks, thus mustelid monophyly is not at issue), (2) monophyly of pinnipeds, and (3) basal placement of the Canidae within the caniforms, then there are five possible sister clades (otters/weasels, raccoons, otters/weasels + raccoons,

bears, and pinnipeds) to the red panda. Pinnipeds were included because their phylogenetic position within Arctoidea may influence the statistical tests for support of the position of the red panda. In Flynn and Nedbal (1998), the pinnipeds were of uncertain position within the Arctoidea (depending on the data sets analyzed) and could be the sister group to any of three larger clades (bears, raccoons + otters/weasels [with or without the red panda], and all the remaining Arctoidea). In combination, 15 different constraint trees (3 alternative phylogenetic placements for pinnipeds, multiplied by 5 alternatives for the red panda) were compared against one another to determine the relative character support for alternative phylogenetic hypotheses. The 12S rRNA gene lacked sufficient resolving power to differentiate among any of the alternative hypotheses of relationships for the red panda. The transthyretin intron always demonstrated significant character support for placement of the red panda within a clade containing mustelids and procyonids (Musteloidea, s.l.), but interrelationships of the taxa within that musteloid clade were equivocal (Table 2). The cytochrome *b* gene demonstrated significant character support for a monophyletic mustelid/procyonid clade, excluding the red panda. It was only when combined, however, that the transthyretin intron and cytochrome *b* sequences provided significant resolving power to definitively place the red panda as the sister group to the other Musteloidea (mustelids and procyonids). The phylogenetic position of pinnipeds, however, remained equivocal.

Phylogenetic parsimony analyses of the three individual mitochondrial genes (cytochrome *b*, 12S rRNA, 16S rRNA) and the nuclear transthyretin intron I compiled for the current analysis were tested for signifi-

cant conflict in phylogenetic signal (Bull *et al.*, 1993). Topological conflicts with nodal bootstrap support >70% were considered indicative of significant conflict in the phylogenetic signal, therefore excluding them from a combined analysis (Bull *et al.*, 1993; de Quiroz *et al.*, 1995; Flynn and Nedbal, 1998). There were no cases of significant conflict or heterogeneity in phylogenetic signal, indicating that all data could be combined in a single "total evidence" analysis. None of the individual mitochondrial gene analyses alone resulted in a well-resolved topology among the major arctoid carnivoran clades. However, all of them supported monophyly of the Caniformia (BS = 91–100), canids (BS = 100 in all), and skunks (BS = 100 in all). Cytochrome *b* and 16S rRNA both showed robust bootstrap support for pinniped monophyly and relationships within that clade, whereas 12S rRNA did not. Cytochrome *b* and 12S rRNA showed moderate to strong support for mustelid (s.s., excluding skunks) monophyly, ursid monophyly, and relationships within the ursid clade, whereas 16S rRNA did not. The transthyretin intron data yielded a topology and nodal support that were almost identical to those in Flynn and Nedbal (1998), even though the previous study included many more feliforms but no skunks. Although the topologies for both transthyretin analyses were identical, the addition of skunks to the current study lowered bootstrap support below 50% for the associations between procyonids and mustelids (s.s.) and between pinnipeds and Musteloidea (s.l.), yielding unresolved polytomies of ursids/pinnipeds/musteloids (and procyonids/red panda/skunks/mustelids [s.s.], within the musteloids [s.l.]). In Flynn and Nedbal (1998) each of those clades had a decay index of 1 and bootstrap support of 53 and 66, respectively.

Phylogenetic parsimony analyses of the four concatenated nucleotide sequences for all 17 taxa provided strong support for monophyly of previously well-corroborated clades of carnivorans (e.g., Arctoidea, Ursidae [including *Ailuropoda*], and Pinnipedia; see Wyss and Flynn, 1993 and Flynn and Nedbal, 1998). These analyses also indicated monophyly of the skunks, as well as their distant relationship to the mustelids (hence, these analyses could support recognition of a distinct mephitid clade and taxon). In contrast, Wolsan (1999) suggested that skunks ("mephitines") are close to otters (lutrines), within a traditionally conceived Mustelidae. We note that Wolsan's conclusion was based on comparisons of skunk basicrania to otters only, with no other carnivoran taxa included explicitly in the comparison and no character matrices or cladistic analyses provided.

Comparison of analyses of individual data sets versus the "total evidence" analysis indicated no significant conflicts among them, supporting the assumption that incorporating sequence data from taxon proxies

would not affect the topology of the higher-level phylogeny.

Our analyses (Fig. 1A) strongly rejected the phylogenetic hypotheses that the red panda (*Ailurus*) was most closely related either to the ursids (22 more steps than the most-parsimonious tree to support an ursid/red panda clade) or to the procyonids (13 more steps than the most-parsimonious tree to support a procyonid/red panda clade). Instead, there is very strong support (BS/DI = 100/19) for a Musteloidea clade (s.l.; see Flynn and Nedbal, 1998) associating the red panda, skunks, and a clade comprising the mustelids (s.s., otter and weasel) and procyonids (raccoon and kinkajou). Within that clade of Musteloidea (s.l.), the Procyonidae and Mustelidae (s.s.) are robustly supported as closest relatives (maximum-likelihood BS = 100; parsimony BS = 85/DI = 6), but there is only weak evidence of a sister group relationship between the red panda and the skunks (maximum-likelihood BS <50; parsimony BS = 54/DI = 3). Conservative assessment of the results would indicate an unresolved trichotomy of the three main lineages within the Musteloidea (s.l.: red panda/skunks/procyonids + mustelids [s.s.]). In addition to bootstrap and decay indices, the three statistical tests [Wilcoxon signed rank (Templeton, 1983), Kishino–Hasegawa (Kishino and Hasegawa, 1989), and compare-2 permutation (Swofford, 1998); Table 2] also confirmed the significance of these phylogenetic results and rejected (at $P < 0.05$ to $P < 0.001$ probability levels) alternative hypotheses of phylogenetic relationships traditionally proposed for the red panda. However, when the skunks were included in the analyses (Table 2 [top]; Fig. 1A), the significance was borderline for two of the three tests (Wilcoxon signed rank, Kishino–Hasegawa) in rejecting a sister group relationship between the raccoon clade and the red panda. We presume that this is due to the increased homoplasy, lower nodal synapomorphy number, and reduced nodal support (compare bootstrap/decay index in Figs. 1A–1C), with the associated reduction in probability of rejecting the null hypothesis for alternative geometries of relationship that typically accompanies addition of taxa when number of characters is constant.

DISCUSSION

Phylogeny

Our analyses of the molecular data from four genes/introns show very strong support for a broad musteloid (s.l.) clade, distinct from all other arctoids. Both *Ailurus* and skunks are closely related to a monophyletic crown clade of Musteloidea (s.s., procyonids + mustelids [excluding skunks]), and there is weak support for a skunk/*Ailurus* clade. Conservative interpretation of the data would consider the three musteloid (s.l.) lin-

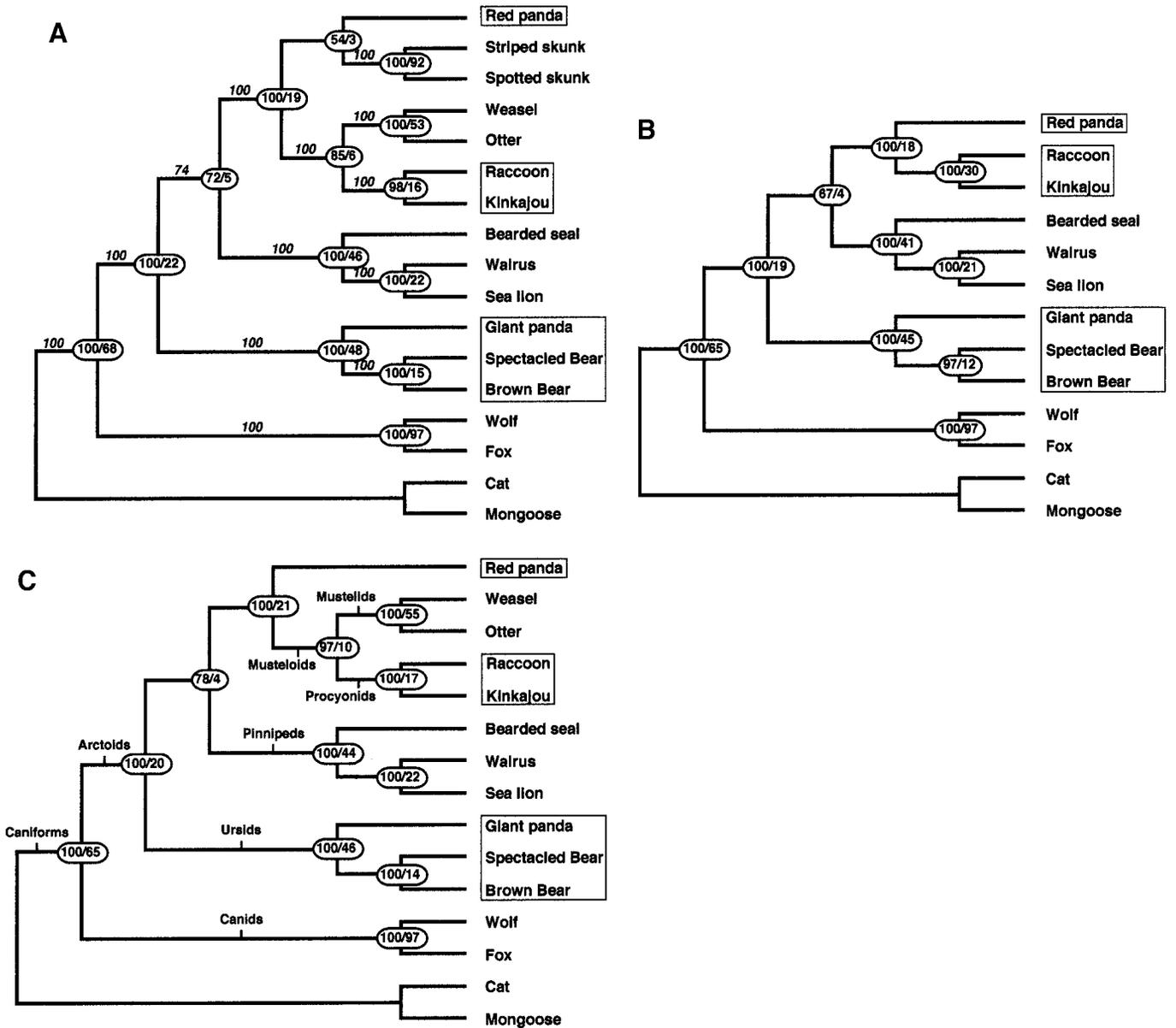


FIG. 1. Phylogenetic position of the red panda, inferred from phylogenetic analysis of concatenated nucleotide sequences from three mitochondrial genes (12S rRNA, 16S rRNA, and cytochrome *b*) and one nuclear intron (transthyretin intron I). Maximum-parsimony searches were conducted using 100 heuristic (tree bisection–reconnection) searches in which the input order of the taxa was randomized. Bootstrap proportions are to the left of the slash, and decay indices are to the right. Maximum-likelihood bootstrap proportions are shown in italics above the branches. Three separate analyses were conducted to assess the effects of taxonomic sampling and phylogenetic position on determination of the closest sister group and precise phylogenetic relationships of the red panda, including (A) a “total evidence” analysis of the complete taxonomic sampling of this study (including taxa constant for all three analyses [feliform outgroups, canids, ursids (including giant panda), and pinnipeds] plus procyonids, skunks, and Mustelidae *sensu stricto* [s.s.]), (B) procyonids only (no mustelids/skunks), and (C) both procyonids and mustelids (s.s.) but no skunks. When all taxa were included (A) a single most-parsimonious tree (length, 1731; CI, 0.508; RI, 0.597) resulted. Maximum-likelihood analyses resulted in a similar topology ($-\log$ likelihood, 23080.407; estimated gamma shape parameter, 0.4662), except that the relatively weak linkage between skunks/red panda (BS 54/DI 3) was not replicated, and instead skunks were the sister group to the musteloid clade of mustelids (weasel, otter) plus procyonids (raccoon, kinkajou), with the red panda more basal to that clade. When the skunks and mustelids (s.s.) were excluded (B) a single most-parsimonious tree (length, 1264; CI, 0.567; RI, 0.612) that was topologically identical to that inferred from maximum-likelihood ($-\log$ likelihood, 19823.489; gamma, 0.4983) resulted. When the skunks alone were excluded (C) a single most-parsimonious tree (length, 1467; CI, 0.534; RI, 0.605) that was topologically identical to that inferred from maximum-likelihood ($-\log$ likelihood, 21862.159; gamma, 0.4637) resulted. Relative topologies of relationships (for the overlapping taxic subsets) among the three analyses are identical; they differ in robustness of support for various nodes/clades and in resolution of the interrelationships of the red panda, skunks, mustelids (s.s.), and procyonids (including different, each weakly supported, placements of skunks relative to red panda and other musteloids in the maximum-parsimony versus the maximum-likelihood results for the complete taxonomic sample (A)).

eages (skunks/*Ailurus*/procyonids + mustelids [s.s.]) as an unresolved trichotomy.

Bininda-Emonds *et al.* (1999) proposed a similar relationship of *Ailurus* to a Procyonidae + Mustelidae clade (they enforced monophyly of Mustelidae, including skunks), although their result was derived through a very different method of tree-consensus analysis of phylogenies published between 1981 and 1995 (with varying taxon and character sampling regimes, often nonindependent among source analyses), rather than primary character data. They further emphasized that this relationship was exceptionally weakly supported (decay index of 1) and was explicitly present in only one source tree—the placement arises largely through parsimony optimization of matrix data from three classes of potentially conflicting trees (4 source trees relating *Ailurus* to, or including it within, procyonids; 10 source trees relating procyonids to mustelids, without explicitly addressing *Ailurus*; 11 source trees uniting *Ailurus* with ursids, which was not globally parsimonious [and presumably helped pull *Ailurus* basally, because of “character” homoplasy of tree structure “resemblance” to ursids]). This conflict would have been further emphasized by studies published prior to Bininda-Emonds (1999; but after their 1995 inclusion date), such as Dragoo and Honeycutt (1997; mephitid monophyly, outside other mustelids plus procyonids) or Flynn and Nedbal (1998; *Ailurus* sister taxon to Mustelidae [s.s.] plus Procyonidae). We have greater confidence in the largely congruent and more robust phylogenetic results of our analyses of multiple primary nucleotide data sets (applying multiple tests of node support and tree topology) from a taxonomically diverse subset of arctoid species. Sampling large numbers of species, alone, does not ensure greater tree resolution or accuracy—that is dependent on the “true” phylogenetic position and distribution of the species sampled, the information content of the genes (or other character systems) sampled, and the hierarchical level of relationships being assessed. For example, adding taxa that break long branches or sampling more slowly evolving gene systems should be more informative about higher-level phylogeny than adding numerous closely related species or sampling rapidly evolving genes.

Although the traditional notion of the red panda as a relative of the procyonids is correct at some broad hierarchical level, there is no support for placement of *Ailurus* within the Procyonidae, or as a closest sister taxon, in our parsimony analyses, the bootstrap and decay index assessments of phylogeny robustness, or the three statistical tests. Many prior conclusions of phylogenetic relationships of the red panda within the Arctoidea were biased by the taxonomic sampling used. For example, some studies did not include bears and/or skunks (or even any mustelids [s.s.]) in the analysis, yielding a de facto relationship between the red panda

and the procyonids. Because those two taxa were the only members of the clearly monophyletic Musteloidea (s.l.) sampled, they necessarily would be more closely related to each other than to any other arctoids or caniforms. A similar result is seen in our limited taxon sampling analysis (Fig. 1B).

Results of an earlier investigation of hemoglobin protein amino acid sequences (Tagle *et al.*, 1986) resemble those of our analysis of multiple genes in their support of a sister group relationship between the pandas (either separately or together) and a clade of Musteloidea (s.s.—the mustelids and procyonids; no skunks were included). However, those results must be interpreted with great caution, as they proposed a relationship (linkage of red and giant pandas) in strong conflict with almost all later studies, derived from less rigorous analysis of more restricted taxonomic and character data sets.

The possible sister group relationship between the skunks and the red panda presented herein (Fig. 1A), although very weakly supported, represents a hypothesis that has never been proposed before. Adding new and more extensive character data (e.g., multiple genes or molecular data sets, or molecular plus morphological data) can greatly benefit phylogenetic analyses. Our results also document that increased taxon sampling (especially representatives of all possible in-group taxa) can enhance phylogenetic analyses by yielding a better-resolved topology through addition of new character combinations (new synapomorphy) or breaking up of long branches (changing optimization/homology presumptions). This can result in support for previously unsuspected hypotheses of relationships.

It has long been recognized that methods of estimating evolutionary relationships are sensitive to the number and taxonomic distribution of the species included (Lanyon, 1985; Lecointre *et al.*, 1993). Notwithstanding this fact, much previous research in carnivoran phylogeny has downplayed the importance of broad taxonomic sampling. In fact, the majority of studies that have provided evidence suggesting that the red panda is closely related to the procyonids ignored the pinnipeds, the mustelids (s.s.—otters, badgers, weasels), and the skunks. Pinnipeds are an ancient and diverse group of arctoid carnivorans, mustelids have been hypothesized to be closely related to the procyonids, and skunks now appear to be an early diverging arctoid lineage (separate from the mustelids with which they had traditionally been classified; e.g., Arnason and Widegren, 1986 and Wayne *et al.*, 1989 [molecular hybridization data]; Vrana *et al.*, 1994 and Dragoo and Honeycutt, 1997 [sequence data]). Because many prior studies did not include these diverse taxa (of significance because of their positions within arctoid phylogeny), they may have drawn incomplete or inaccurate conclusions about the evolutionary history of the red panda. This could lead to unfortunate conse-

quences for related evolutionary studies or conservation policies.

To demonstrate the effects of taxonomic sampling, we reanalyzed our data after excluding all “mustelid” (s.l.) species. As in antecedent studies by others, our results provide strong support (BS/DI = 100/18; Table 2) for a close relationship between the red panda and the procyonids (Fig. 1B), which clearly is only a partial (and ultimately misleading) “snapshot” of the red panda evolutionary picture. A reanalysis of the data after inclusion of a weasel and otter (mustelids, s.s.) provided strong support (BS/DI = 97/10; Table 2) for placement of the red panda not as the closest sister group to the procyonids alone, but rather to a broader clade consisting of both the procyonids and the mustelids (Fig. 1C). This provides a more complete phylogenetic picture, but with some pieces still missing. The final piece of the puzzle was brought to light by recent molecular evidence (e.g., DNA hybridization data of Arnason and Widegren, 1986 and Wayne *et al.*, 1989; nucleotide sequence data of Dragoo and Honeycutt, 1997) which placed skunks not in their traditional position as closely related to other mustelids, but rather outside of a larger clade of the remaining mustelids (s.s.) plus procyonids. Our data place the red panda in a position—as a basal musteloid—similar to that suggested for the monophyletic clade of skunks (mephitids). Clearly, the addition of not only mustelids generally, but also skunks in particular, has a major impact on inferring the evolutionary affinities of the red panda. Our combined gene results provide robust evidence for placing both the skunks (with strong support for skunk monophyly) and the red panda basal to the procyonid/mustelid (s.s.) clade, but the relationship between the skunks and the red panda remains poorly resolved (Fig. 2 and Table 2). Parsimony bootstrap and decay analyses weakly supported (BS 54/DI 3) a link between the skunks and the red panda, whereas a maximum-likelihood analysis (-log likelihood, 23080.407; gamma, 0.4662) did not support that relationship (even though the overall tree topology is similar), instead linking skunks as closest sister group to the clade of procyonids plus mustelids (s.s.).

Conservatively, we consider the musteloid interrelationships as an unresolved trichotomy among the red panda, the skunks, and a procyonid/mustelid (s.s.) clade. Our study yields strong evidence for rejecting both of the previous hypotheses of a closest sister group relationship between the red panda and either (1) the ursids or (2) the procyonids. It also provides very strong support for *Ailurus* being a basal member of the Musteloidea (s.l.), but new questions arise as to the precise phylogenetic affinities among the red panda, the skunks (mephitids, to the exclusion of other Mustelidae, s.s.), and the strongly supported musteloid (s.s.) clade of procyonids plus mustelids (s.s., excluding skunks). Inclusion of a broad set of species, represent-

ing all the major clades (*Ailurus*, pinnipeds, mephitids, mustelids [s.s.], procyonids, ursids [including *Ailuropoda*]) relevant to the phylogenetic hypothesis being tested, enabled us to provide a more accurate and rigorously tested depiction of the evolutionary history of the red panda. It is not a bear, nor closely related to the giant panda, nor a raccoon, nor a lineage of uncertain affinities. Rather it is a basal lineage of musteloid, with a long history of independence from its closest relatives (skunks, raccoons, and otters/weasels/badgers). That long separation from other musteloids could argue for recognition of the red panda lineage as a separate higher-level taxonomic entity (e.g., Ailuridae, within the Musteloidea), although erection of a taxon Ailuridae would not (by itself) be informative as to its precise phylogenetic position relative to other musteloids and arctoids.

Temporal History

One can use temporal ranges of the oldest fossil taxa (first stratigraphic appearance) in each of the three main musteloid lineages to directly, or phylogenetically, calibrate the minimum clade divergence ages (Norell, 1992; Flynn, 1996). Even though the red panda lineage has a very poor fossil record, its phylogenetic relationships (as a basal musteloid) suggest that the lineage has a minimum age at least as old as the Early Miocene and possibly as old as the Late Eocene or the Early Oligocene (using the “restrictive/younger” and “permissive/older” clade membership criteria of Flynn, 1996 for various early fossil musteloid species). In fact, using any of the three possible resolutions of the trichotomy of musteloids, and the known oldest fossils of lineages or their closest sister taxa (McKenna and Bell, 1997; but see Wolsan, 1999 for a contrasting view of the age of the oldest known skunk), all three lineages must have been present by the Early Miocene (~20–24 Ma) and may have minimum divergence ages as old as the Late Eocene or the Early Oligocene (~31–37 Ma). Bininda-Emonds *et al.* (1999) presented a different approach to estimating node divergence ages for their consensus “supertree,” averaging literature estimates derived from both the fossil record (using the approach of Wayne *et al.*, 1991) and the fossil-calibrated molecular branch-length data (applying a “local molecular clock”). As these averaged ages incorporate putative clocks, they appear to represent estimated actual (maximum) divergence ages, rather than minimum divergence ages, and are dependent upon the particular, small number of fossil-calibrated nodes used to constrain the “local clock”. Bininda-Emonds *et al.*'s (1999) averaging of nine literature estimates for the Musteloidea (s.l., common ancestor of *Ailurus*, and a clade of mustelids [enforced monophyletic] plus procyonids) indicated a node divergence age of 29.3 Ma (median = 29.3, mean = 27.9, SE = 2.5), and six literature estimates for Musteloidea (s.s.) indicated a divergence age

of 28.1 Ma (median = 28.1, mean = 29.4, SE = 2.1). As they constrained Mustelidae to be monophyletic, this cannot be compared directly to our skunk/*Ailurus*/musteloid (s.s.) analysis, but their estimate of the actual divergence age of the *Ailurus* lineage from the rest of the Musteloidea is intermediate between our entirely fossil/phylogeny-calibrated younger ("restrictive" clade membership) and our older ("permissive" clade membership) minimum divergence age estimates.

Relevant to the deep roots of the musteloid (s.l.) lineages, when the skunks were included in the analyses, the significance was borderline for two of the three statistical tests (Wilcoxon signed rank, Kishino-Hasegawa) in rejecting a potential sister group relationship between the raccoon clade and the red panda (Table 2 [top]; Fig. 1A). This indicates that phylogenetic results (both topology and robustness of support), and tests of their significance, can be very sensitive to taxonomic sampling and the effects of long-branch attraction among lineages that have been separated for a very long time. Future phylogenetic studies should strive to maximize both character data and taxonomic sampling (including representative taxa from as many potential subclades as is possible, for both fossil and living taxa [at least for morphology]) and explore the phylogenetic ramifications of applying various models of molecular evolution in the data analyses.

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