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Deciphering and dating the red panda's ancestry and early adaptive radiation of Musteloidea

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ABSTRACT

Few species have been of more disputed affinities than the red or lesser panda (*Ailurus fulgens*), an endangered endemic Southeast Asian vegetarian member of the placental mammalian order Carnivora. This peculiar carnivoran has mostly been classified with raccoons (Procyonidae) or bears (Ursidae), grouped with the giant panda (*Ailuropoda melanoleuca*) in their own family, or considered a separate lineage of equivocal ancestry. Recent molecular studies have indicated a close affinity of the red panda to a clade of procyonids and mustelids (weasels, otters, martens, badgers, and allies), but have failed to unambiguously resolve the position of this species relative to mephitids (skunks and stink badgers). We examined the relationship of the red panda to other extant species of the carnivoran suborder Caniformia using a set of concatenated ~5.5-kb sequences from protein-coding exons of five nuclear genes. Bayesian, maximum likelihood, and parsimony phylogenetic analyses strongly supported the red panda as the closest living relative of a clade containing Procyonidae and Mustelidae to the exclusion of Mephitidae. These three families together with the red panda (which is classified here as a single extant species of a distinct family, Ailuridae) compose the superfamily Musteloidea, a clade strongly supported by all our phylogenetic analyses as sister to the monophyletic Pinnipedia (seals, sea lions, walruses). The approximately unbiased, Kishino–Hasegawa, and Templeton topology tests rejected ($P < 0.05$) each of all possible alternative hypotheses about the relationships among the red panda and mephitids, procyonids, and mustelids. We also estimated divergence times for the red panda's lineage and ones of other caniform taxa, as well as the ages of the first appearance datums for the crown and total clades of musteloids and the total clades of the red panda, mephitids, procyonids, and mustelids. Bayesian relaxed molecular-clock analysis using combined information from all sampled genes yielded a ~42-Myr timescale to caniform evolution and provided evidence of five periods of increased diversification. The red panda's lineage and those of other extant musteloid families are estimated to have diverged during a 3-Myr interval from the mid-Early Oligocene to near the Early/Late Oligocene boundary. We present fossil evidence that extends the early adaptive radiation of the total clade of musteloids to the Eocene–Oligocene transition and also suggests Asia as a center of this radiation.

1. Introduction

The red or lesser panda was introduced to Western naturalists in 1821 (Hardwicke, 1826) and a few years later (Geoffroy Saint-Hillaire and Cuvier, 1825) was assigned its current scientific name, *Ailurus fulgens*, which means "shining cat." It was the only panda known to Western science until 1869, when the giant panda (*Ailuropoda melanoleuca*) was first reported, albeit originally as a bear, not a panda (David, 1869). Both species are endemic to an area encompassing south-central China and the Himalayas, occur in high-altitude bamboo forests, subsist primarily on bamboo, and resemble each other in a number of anatomic and behavioral features related to their peculiar vegetarian specialization, so unusual for members of Carnivora. Mainly for these reasons, it was common in the past to regard both species as close relatives. As the red panda is in some respects similar to raccoons, whereas the giant panda exhibits many characteristics of bears, there had

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long been disagreement over whether the pandas should be classified in the raccoon family Procyonidae or the bear family Ursidae. Alternatively, they have been united in their own family (variously referred to as Ailuridae or Ailuropodidae) or recognized as single extant representatives of Ailuridae and Ailuropodidae, respectively (for a recent review of literature, see Bininda-Emonds, 2004).

Even though the bear nature of the giant panda was convincingly demonstrated almost half a century ago (Davis, 1964) and the perception of this species as part of a sister lineage to all other living ursids has become ultimately widespread (O'Brien et al., 1985; Bininda-Emonds et al., 1999; Bininda-Emonds, 2004), the relationships of the red panda have remained puzzling and highly controversial despite a variety of data classes examined. Only over the last two decades, attempts to decipher the enigma of the red panda's affinities, whether based on morphologic or genetic grounds or both, have resulted in an impressively broad range of hypothesized relationships. These have included placements in close affinity with ursids alone (e.g., Wozencraft, 1989) or ursids and pinnipeds (seals, sea lions, walruses; e.g., Vrana et al., 1994); among procyonids (e.g., Dragoo and Honeycutt, 1997); basally to a clade containing both ursids and procyonids as well as some other caniforms, including basal ones such as canids (dogs; e.g., Schreiber et al., 1998); or, at last, at or near the base of a clade comprising procyonids and mustelids (weasels, otters, martens, badgers, and their relatives). The last placement, initially postulated on the basis of chiefly fossil evidence (Schmidt-Kittler, 1981; Wolsan, 1993a), over recent years has repeatedly been recovered by phylogenetic analyses on combined molecular sequences from multiple loci, delivering unprecedented amounts of character data (Flynn and Nedbal, 1998; Flynn et al., 2000, 2005; Yu et al., 2004a, 2008; Delisle and Strobeck, 2005; Fulton and Strobeck, 2006, 2007; Sato et al., 2006; Yu and Zhang, 2006; Árnason et al., 2007; Peng et al., 2007; Yonezawa et al., 2007). Although confidence in a basal position of the red panda to the procyonid–mustelid clade has grown with increase in the genomic and taxonomic coverage of the sequence data, the persisting uncertainty about the relationship of the red panda to another living musteloid clade, the mephitids (skunks and stink badgers), has not been resolved decisively. The mephitids themselves had long been viewed as mustelids based on fossil and other morphologic features (e.g., Schmidt-Kittler, 1981; Wozencraft, 1989; Wolsan, 1993a, 1999; Wyss and Flynn, 1993; Bininda-Emonds et al., 1999; Sato et al., 2004), but the overwhelming contradictory evidence that has accumulated from various genetic sources (Ledje and Árnason, 1996; Dragoo and Honeycutt, 1997; Flynn et al., 2000, 2005; Sato et al., 2004, 2006; Delisle and Strobeck, 2005; Fulton and Strobeck, 2006, 2007; Árnason et al., 2007; Peng et al., 2007; Yonezawa et al., 2007; Yu et al., 2008; and references therein) persuasively argue for a position outside a clade containing mustelids and procyonids.

Here we explore interspecific variation in ~5.5 kb of nuclear DNA to estimate the phylogenetic relationship of the red panda to other extant caniforms. By using diverse analytic methods, both probabilistic and parsimony ones, we demonstrate consistently strong support for a close relationship of the red panda to a clade of mustelids and procyonids to the exclusion of mephitids. This is the first compelling resolution of the red panda's phylogenetic placement, a convincing solution to an almost 200-year-old evolutionary riddle of this species. This finding deciphers higher-level hierarchical relationships within Musteloidea, which in combination with the recent identification of the first appearance datums for major musteloid and extinct closely related lineages (Wolsan, 2005) allows, for the first time, an efficacious estimation of the timing, tempo, and mode of early musteloid diversification. To address this issue, we estimate multigene divergence times for the red panda's lineage and ones of other caniform taxa under a relaxed molecular clock, as well as the ages of the first appearance datums for the principal recognized musteloid and extinct closely related lineages. In addition, we address the issue of an early center for musteloid evolution and also present the climatic context of the early adaptive radiation of musteloids. Part of the phylogenetic results obtained in this study have been reported at two meetings (Wolsan and Sato, 2007a; Sato and Wolsan, 2008).

2. Materials and methods

2.1. Data collection

2.1.1. Gene and taxon sampling

Nucleotide sequences were obtained from five protein-coding exons of five nuclear genes (Table 1). The sequence data were either newly generated or compiled from previously published DDBJ, EMBL, and GenBank accessions (Table 2). A total of 51 species were sampled, including the red panda and 42 other species of all relevant clades of the carnivoran suborder Caniformia (dog-like carnivorans, constituting the ingroup) and eight species of the carnivoran suborder Feliformia (cat-like carnivorans, used as a collective outgroup; Table 2). A sister relationship of Feliformia to Caniformia is well established on the basis of evidence from both genetics (e.g., Flynn et al., 2005) and morphology (e.g., Flynn et al., 1988; Wyss and Flynn, 1993; Wesley-Hunt and Flynn, 2005).

2.1.2. DNA isolation, amplification, and sequencing

Total genomic DNA was first extracted from tissues preserved in ethanol using the phenol–chloroform method (Sambrook and Russell, 2001), followed by amplification via two nested polymerase chain reactions (PCRs) carried out in an automated thermal cycler (model PC 808, Astec, Fukuoka, Japan) with the following conditions (same for both nested PCRs): a cycle of denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s; and a cycle of extension at 72 °C for 10 min. In the first PCR, each 50-μl reaction mixture contained 10× Ex Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1.25 U of Ex Taq (Hot Start version) polymerase (Takara, Shiga, Japan), 0.8 μM of each primer, and 0.1–0.5 μg of genomic DNA. The primer pairs applied were as follows (Table 3): APOB-F8487 and APOB-R9826, BRCA1-F997 and BRCA1-R2047, RAG1-F1842 and RAG1-R2951, R+IRBP335 and −IRBP1531, and vWF-F241-dog and vWF-R1507-dog. A 1-μl aliquot of each reaction mixture after the first nested PCR was used as a template for the second nested PCR in a 50-μl reaction mixture with the same reagents except that the following sets of primer pairs were applied (Table 3): APOB-F8487 and APOB-R9324, and APOB-F9287 and APOB-R9826; BRCA1-F997 and BRCA1-R1509, and BRCA1-F1428 and BRCA1-R2047; RAG1-F1851 and RAG1-R2486, and RAG1-F2357 and RAG1-R2951; R+IRBP335 and U−IRBP734, R+IRBP724-short and U−IRBP1145-short, and R+IRBP1085 and −IRBP1531; and vWF-F241-dog and vWF-R816, vWF-F611 and vWF-R1076, and also vWF-F1072 and vWF-R1507-dog. The products of the second PCR were then sequenced using the original second-PCR primers and the Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol, followed by runs on an ABI 310, ABI3100 Avant, or ABI3130 automated sequencer.

2.2. Phylogeny estimation

2.2.1. Sequence alignment

All sequences were aligned manually (Kjer et al., 2007) through multiple alignment in DNASIS Pro version 2.6 (Hitachi Software