Syst. Biol. 52(5):571–593, 2003

ISSN: 1063-5157 print / 1076-836X online
DOI: 10.1080/10635150390235368

Type I STS Markers Are More Informative than Cytochrome *b* in Phylogenetic Reconstruction of the Mustelidae (Mammalia: Carnivora)

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Abstract.— We compared the utility of five nuclear gene segments amplified with type I sequence-tagged site (STS) primers versus the complete mitochondrial cytochrome *b* (cyt *b*) gene in resolving phylogenetic relationships within the Mustelidae, a large and ecomorphologically diverse family of mammalian carnivores. Maximum parsimony and likelihood analyses of separate and combined data sets were used to address questions regarding the levels of homoplasy, incongruence, and information content within and among loci. All loci showed limited resolution in the separate analyses because of either a low amount of informative variation (nuclear genes) or high levels of homoplasy (cyt *b*). Individually or combined, the nuclear gene sequences had less homoplasy, retained more signal, and were more decisive, even though cyt *b* contained more potentially informative variation than all the nuclear sequences combined. We obtained a well-resolved and supported phylogeny when the nuclear sequences were combined. Maximum likelihood and Bayesian phylogenetic analyses of the total combined data (nuclear and mitochondrial DNA sequences) were able to better accommodate the high levels of homoplasy in the cyt *b* data than was an equally weighted maximum parsimony analysis. Furthermore, partition Bremer support analyses of the total combined tree showed that the relative support of the nuclear and mitochondrial genes differed according to whether or not the homoplasy in the cyt *b* gene was downweighted. Although the cyt *b* gene contributed phylogenetic signal for most major groupings, the nuclear gene sequences were more effective in reconstructing the deeper nodes of the combined tree in the equally weighted parsimony analysis, as judged by the variable-length bootstrap method. The total combined data supported the monophyly of the Lutrinae (otters), whereas the Melinae (badgers) and Mustelinae (weasels, martens) were both paraphyletic. The American badger, *Taxidea taxus* (Taxidiinae), was the most basal taxon. Because hundreds of type I STS primer sets spanning the complete genomes of the human and mouse have been published and thus represent many independently segregating loci, the potential utility of these markers for molecular systematics of mammals and other groups is enormous. [Cytochrome *b*; intron; Mammalia; Mustelidae; nuclear DNA; phylogeny; sequence-tagged sites.]

During the last two decades, DNA sequences have become the dominant tool in assessing the phylogenetic relationships among a wide variety of organisms. The overwhelming majority of molecular systematic studies on animals have used mitochondrial genes for which the availability of universal primers (Kocher et al., 1989, for three loci; Sorenson et al., 1999, for all mitochondrial loci in vertebrates) has made the collection of DNA sequence data relatively simple. Nevertheless, mitochondrial genes represent only a very small handful of the tens of thousands of loci that comprise the genomes of animals. Despite the recent completion of several genome projects (e.g., *C. elegans* Sequencing Consortium, 1998; International Human Genome Sequencing Consortium, 2001), the nuclear genomes of most species remain unexplored, yet they represent a large untapped resource of molecular phylogenetic data.

One of the ways in which the nuclear genomes of organisms are being made available is through a research approach known as comparative genomics. This approach uses homologous sequence information from one organism to assign putative function to an unknown gene and/or serve as a guide to map the position of genes in another organism (Brown, 1999). In recent years, efforts to construct comparative genomic maps in mammals have led to the development of thousands of markers that define specific landmarks in the nuclear genomes of model species (e.g., mouse and human), which are known as sequence-tagged sites (STSs). STSs are short regions of DNA about 200–1,000 base pairs (bp) in length and are specifically detected by polymerase chain reaction (PCR) in the presence of all other genomic sequences (Olson et al., 1989). In other words, a PCR primer that amplifies a unique sequence and maps to a single position in the genome defines an STS. Three classes of STS loci are recognized: type I, type II, and type III (O'Brien et al., 1999). Type I loci are based on single-copy coding genes (exons and introns) and help define homologous landmarks that are conserved across a wide range of taxa. Type II loci are based on repetitive DNA markers, such as microsatellites, and are highly polymorphic, making them useful for pedigree and population analyses. Type III loci are biallelic single-nucleotide polymorphisms within coding and noncoding regions and are also informative for pedigree and population studies, although they are less polymorphic than type II loci.

Of the three classes of STS loci, type I markers are likely to be the most useful for studies in molecular systematics for several reasons. First, primers derived from well-mapped genomes (e.g., human and mouse) can be used to amplify homologous loci in map-poor species. This is possible because the primers are located in sequence regions (usually exons) that are highly conserved among distantly related taxa. Second, by definition, primers are expected to amplify a single sequence, reducing the possibility of amplifying paralogous sequences, which are a potential hazard because many nuclear genes belong to gene families. Third, type I loci are abundant; in mammals, hundreds of loci have been characterized (Venta et al., 1996; Lyons et al., 1997; Jiang et al., 1998). Finally, type I loci are usually targeted at introns and are thus likely to be informative across both shallow and deep levels of evolutionary divergence.

Given the availability of a variety of different genes for use in molecular systematics, two critical problems concern the sequences that are best (data quality) for phylogeny reconstruction and the amount of sequence data necessary (data quantity) to resolve relationships for a particular group. Numerous papers in the systematics literature have been devoted to determining the answers to these questions, especially in the context of comparing the relative merits of mitochondrial and nuclear genes (e.g., Graybeal, 1994; Baker and DeSalle, 1997; Prychitko and Moore, 2000; Springer et al., 2001). In general, it is difficult to predict beforehand whether a specific gene will be informative for a particular group until phylogenetic analysis is actually attempted (Graybeal, 1994; Mitchell et al., 2000). Nonetheless, the attributes that distinguish mitochondrial and nuclear genes are well known and provide guidance as to how to best employ these different markers for phylogeny reconstruction.

The popularity of mitochondrial DNA (mtDNA) stems in part from the distinctive characteristics that make it useful for inferring relationships among closely related taxa, including a high rate of substitution relative to single-copy nuclear genes (Brown et al., 1979). However, a significant shortcoming of mitochondrial genes is that they are all inherited as a single linkage group and thus represent only one of the many thousands of gene trees that comprise a species phylogeny (Hey, 2000). Ultimately, the corroboration or refutation of any phylogenetic hypothesis based on a single gene can only come from sequencing genes that belong to different linkage groups (Brower et al., 1996). For phylogenetic studies on animals, this means turning to loci from the nuclear genome.

Nuclear gene sequences are being increasingly incorporated into systematic studies (e.g., Hey and Kliman, 1993; Slade et al., 1994; DeBry and Seshadri, 2001). Exon sequences have proven useful in some instances (e.g., Serizawa et al., 2000). However, intron sequences are favored because of their presumed lack of functional constraint, leading to higher rates of substitution and a greater number of sites free to vary relative to exons. Most studies utilizing nuclear genes are still restricted to a small number of single loci, but theoretical and simulation studies suggest that sequences from multiple nuclear loci will often be required to resolve a species phylogeny for several reasons. First, based on the difference in coalescence times between mitochondrial and nuclear loci, Moore (1995) showed that many nuclear genes are needed to achieve the same resolving power as a single mtDNA gene, particularly among closely related taxa. Second, patterns and processes of substitution vary from locus to locus and perhaps even regionally within genomes (Matassi et al., 1999). Sampling sequence from a single gene may introduce biases caused by "location-dependent processes" (Cummings et al., 1995). Sampling short segments of multiple loci from different regions of the genome can mitigate such biases (Cummings et al., 1995). Third, it is likely that species trees represent composites of gene trees of varying topologies (Maddison, 1996, 1997). That is, different genes may possess phylogenetic signal for different groupings within a phylogeny because of the variation in patterns of substitution across loci and lineages as well as the stochastic nature of lineage sorting. Sequences from multiple regions of the genome are therefore required to understand how phylogenetic signal (its mode and variance of distribution; Maddison, 1996) is distributed across a phylogeny.

Previously, we used complete sequences of the mitochondrial cytochrome *b* (cyt *b*) gene to examine the phylogenetic relationships among otters (Lutrinae) and their position within the Mustelidae, a large and ecomorphologically diverse family of mammalian carnivores that also includes badgers, martens, and weasels (Koepfli and Wayne, 1998). Although some nodes were well supported by bootstrap values, deeper nodes encompassing relationships among taxa from different mustelid subfamilies were poorly supported. Moreover, we were unable to find strong support for the monophyly of otters. These results were most likely due to a combination of a high level of homoplasy in the cyt *b* sequences and the presence of short internodes at deeper levels of the mustelid tree, which suggested that a rapid radiation had occurred (Koepfli and Wayne, 1998). However, it would be useful to compare these results with those based on nuclear genes in order to test the validity of our earlier findings. The availability of type I STS primers now makes such a comparison possible.

In this study, we assessed the relative performance of five nuclear gene segments amplified with type I STS primers versus the complete mitochondrial cyt *b* gene in resolving phylogenetic relationships within the Mustelidae. We used separate and combined phylogenetic analyses to address questions regarding the levels of homoplasy, incongruence, and information content within and among loci. Furthermore, we compared the efficiency of the cyt *b* sequences and the concatenated nuclear sequences in reconstructing different regions of the mustelid phylogeny. Finally, we discuss the potential utility of genomic resources such as type I STS markers to the field molecular systematics.

Overview of Mustelid Systematics

The Mustelidae is the largest family of the caniform carnivores, consisting of 24 extant genera and 65 species, which have been divided into six subfamilies (Wozencraft, 1993): Lutrinae (otters), Melinae (badgers), Mellivorinae (honey badger), Mephitinae (skunks), Mustelinae (weasels and martens), and Taxiidinae (American badger, *Taxidea taxus*). Mustelids exhibit a tremendous range of ecomorphological diversity, from species that are fossorial (badgers) to those that are semi- or completely aquatic (otters). Traditional subfamilial boundaries have been drawn along these broad ecomorphological lines, raising the possibility that groupings represent phenetic grades rather than phyletic clades.

Bryant et al. (1993) assessed the relationships among the extant genera and the monophyly of the subfamilies in the Mustelidae defined by Simpson (1945) using cladistic analysis of 46 morphological characters.

They found that the Lutrinae and Mephitinae were monophyletic, the Mustelinae was paraphyletic, and the Melinae was polyphyletic. However, bootstrap support for most nodes was low, and only three clades were strongly supported: (1) a monophyletic Lutrinae, (2) *Arctonyx* + *Meles*, and (3) a monophyletic Mephitinae + *Mydaus* (Bryant et al., 1993).

Studies utilizing biochemical and molecular approaches, however, have cast doubt on the monophyly of the family itself. Evidence from karyology (Wurster and Benirschke, 1968), immunological techniques (Ledoux and Kenyon, 1975), DNA hybridization (Arnason and Widegren, 1986; Wayne et al., 1989), mtDNA sequences (Vrana et al., 1994; Ledje and Arnason, 1996a, 1996b; Dragoo and Honeycutt, 1997) and nuclear DNA sequences (Flynn et al., 2000) generally indicate that skunks diverged long before the evolution of the other mustelid subfamilies. Moreover, the study by Dragoo and Honeycutt (1997) strongly links *Mydaus* (stink badgers) with skunks in a monophyletic group (Mephitidae) distinct from the Mustelidae.

Beyond these issues, there have been few molecular studies that have examined relationships exclusively within the Mustelidae (Masuda and Yoshida, 1994; Koepfli and Wayne, 1998). These studies have been limited in scope in terms of taxonomic sampling, however, either because they involved relationships of mustelids found in one country (Masuda and Yoshida, 1994) or they primarily focused on resolving relationships within one subfamily (Koepfli and Wayne, 1998). Furthermore, both studies were based on mitochondrial cyt *b* sequences and lacked robust support for deeper level relationships within the Mustelidae. Therefore, the additional data provided by the multiple nuclear gene sequences we examined might help to begin to resolve some of the long-standing controversies that have plagued mustelid systematics in the past.

Materials and Methods

Sampling Design

We obtained tissue samples from single individuals of the species listed in Table 1. Samples included 22 species of Mustelidae representing 16 genera and four subfamilies. We did not include skunks (Mephitinae) in our study because molecular evidence has shown that skunks and the related stink badgers (*Mydaus*, traditionally placed in the Melinae) diverged prior to the evolution of the Mustelidae. Two species of Procyonidae, the raccoon (*Procyon lotor*) and the ringtail (*Bassariscus astutus*), were

TABLE 1. Species, common name, and source of taxa used in this study. Classification is based on Wozencraft (1993).

Species	Common name	Source and voucher/reference number[a]
Mustelidae		
Lutrinae		
Aonyx capensis[b]	Cape clawless otter	J. Dallas, University of Aberdeen, Aberdeen, U.K.
Amblonyx cinereus	Asian small-clawed otter	IZL 1056
Enhydra lutris[b]	sea otter	J. Bodkin, U.S. Fish and Wildlife Service
Lontra canadensis	North American river otter	LSU-MNS 2132
Lontra felina	marine otter	C. Vila, University of California, Los Angeles
Lontra longicaudis	Neotropical otter	MVZ 4913
Lutra lutra[b]	Eurasian otter	J. Ruiz-Olmo, Dir. General del Medi Natural, Spain
Lutra maculicollis	spotted-necked otter	Brookfield Zoo, Brookfield, IL; ISIS 24033
Pteronura brasiliensis	giant otter	Hagenbeck Zoo, Hamburg, Germany; no. 1384
Mustelinae		
Eira barbara	tayra	MSB 58756
Galictis vittata	greater grison	MVZ 155226
Gulo gulo[b]	wolverine	UAM 24083
Ictonyx striatus	zorilla	Brookfield Zoo, Brookfield, IL; ISIS 880085
Martes americana	American marten	H. Henry, Rocky Mountain Research Station
Martes pennanti	fisher	E. York, University of Massachusetts
Mustela erminea[b]	ermine	UAM 32764
Mustela frenata	long-tailed weasel	TK 23477
Mustela vison[b]	American mink	TK 29694
Melinae		
Arctonyx collaris	hog badger	MVZ 186562
Meles meles[b]	Eurasian badger	E. Geffen, Tel Aviv University, Tel Aviv, Israel
Melogale moschata	Chinese ferret badger	MVZ 186565
Taxidiinae		
Taxidea taxus[b]	American badger	MSB 64932
Procyonidae		
Procyoninae		
Bassariscus astutus	ringtail	M. Gompper, Columbia University, New York
Procyon lotor[a]	raccoon	UAM 33819

[a]IZL = Institute of Zoology, London; LSU-MNS = Louisiana State University, Museum of Natural Sciences, Baton Rouge; MSB = Museum of Southwestern Biology, University of New Mexico, Albuquerque; MVZ = Museum of Vertebrate Zoology, University of California, Berkeley; TK = Museum of Texas Tech University, Lubbock; UAM = University of Alaska Museum, Fairbanks.

[b]Two different individuals were typed for mitochondrial and nuclear loci.

used as outgroups based on the sister-group relationship between the Mustelidae and Procyonidae. This relationship is well supported by multiple lines of evidence (morphology: Flynn et al., 1988; DNA sequences: Ledje and Arnason, 1996a, 1996b; Dragoo and Honeycutt, 1997; Flynn and Nedbal, 1998; Flynn et al., 2000).

For cyt *b*, sequences were either derived from our previous study (GenBank accessions AF057118–AF057132; Koepfli and Wayne, 1998) or newly generated. For 9 of the 24 taxa (Table 1), a different individual was used for the nuclear loci and the cyt *b* gene, and for 3 of these (*Gulo gulo*, EMBL X94921; *Meles meles*, EMBL X94922; and *Procyon lotor*, EMBL X94930), the cyt *b* sequence was from the study by Ledje and Arnason (1996a). These differences should not have a strong affect on the phylogenetic analyses because we found intraspecific polymorphism in the cyt *b* gene to be generally low (Koepfli and Wayne, 1998). In addition, our earlier study included *Mustela putorius*, which has been replaced here with *M. frenata*.

DNA Isolation, Amplification, and Sequencing

Total genomic DNA was extracted from tissues with standard phenol/chloroform methods and recovered by ethanol precipitation (Sambrook et al., 1989). Segments of five nuclear genes were amplified by PCR with the type I STS primers listed in Table 2. Primers for nuclear loci include the amplification of complete introns as well as 5′ and 3′ flanking exon sequences, except in the case of APOB, which amplify a partial fragment from exon 29 of this gene. New cyt *b* sequences were amplified with primers L14609, L14974, H15149, L15408, and H15915, as described by Koepfli and Wayne (1998). We found that all loci amplified equally well with a standard set of PCR conditions. Thirty cycles of amplification were performed in a programmable thermal cycler (Perkin Elmer Cetus model 9600 or MWG-Biotech Primus 96 Plus) at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 45 sec, followed by one cycle at 72°C for 5 min. Negative (no DNA) controls were included with all amplifications. Reaction products were gel purified with an Ultra Clean Kit (Mo Bio Laboratories). PCR products were then cycle sequenced directly using the same PCR primers and the ABI PRISM BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems). Sequencing reactions were precipitated following the manufacturer's protocol and run on an ABI PRISM 377 DNA Sequencer. For most species and loci, both strands were sequenced to ensure accuracy.

We were unable to amplify APOB from *Aonyx capensis* and *Galictis vittata*, despite modifying the PCR reagent concentrations and cycling conditions. A possible explanation for the lack of amplification of APOB in these two taxa is primer-template mismatch, which can affect the PCR product yield, especially if the mismatches are concentrated at the 3′ end of the primer (Kwok et al., 1990). Consequently, for these two taxa, characters for the APOB gene were coded as missing (with a question mark) in the phylogenetic analyses of the combined data sets.

Phylogenetic Analysis

We aligned all nuclear DNA sequences with Clustal W (Thompson et al., 1994) and then adjusted them by eye. Alignment of cyt *b* sequences was easily accomplished by eye alone. The five nuclear gene data sets contained insertions and deletions (indels) of variable length, which were treated accordingly depending on the optimality criterion used to reconstruct trees. Thirty-one heterozygous sites (equal peak heights in electropherograms) were scored as polymorphisms (e.g., A and G = R; T and A = W). All of our nuclear DNA sequences were judged to be orthologous based on the nucleotide and amino acid sequence similarity of the flanking exons (or total exon in the case of APOB) between the 24 taxa and the human sequences derived from GenBank. Furthermore, for the four loci that included an intron, the sequences at the exon–intron splice junctions all conformed to the GT–AG rule (Breathnach et al., 1978).

We performed phylogenetic analyses on the following data partitions: (1) individual loci, (2) combined nuclear data, and (3) total combined data (nuclear plus mitochondrial). Maximum parsimony (MP) and maximum likelihood (ML) trees were generated for all partitions using PAUP* (Swofford, 2002). For parsimony analyses, all characters were equally weighted. Differentially weighted analyses using implied weights (Goloboff, 1993) were also conducted in the analysis of

TABLE 2. Gene symbol and name, fragment of gene amplified, and primers used in this study.

Gene symbol	Gene name	Fragment amplified[a]	Primers	Reference
APOB	apolipoprotein B	exon 29 (portion of)	F: GGCTGGACAGTGAAATATTATGAAC R: AATCAGAGAGTTGGTCTGAAAAAT	Jiang et al., 1998
FES	feline sarcoma protooncogene	intron 14	F: GGGGAACTTTGGCGAAGTGTT R: TCCATGACGATGTAGATGGG	Venta et al., 1996
CHRNA1	cholinergic receptor, nicotinic, alpha polypeptide 1 precursor	intron 8	F: GACCATGAAGTCAGACCAGGAG R: GGAGTATGTGGTCCATCACCAT	Lyons et al., 1997
GHR	growth hormone receptor	intron 9	F: CCAGTTCCAGTTCCAAAGAT R: TGATTCTTCTGGTCAAGGCA	Venta et al., 1996
RHO1	rhodopsin	intron 3	F: TACATGTTCGTGGTCCACTT R: TGGTGGGTGAAGATGTAGAA	Venta et al., 1996

[a]For STS loci, exon and intron numbers correspond to homologous locations in human genes. GenBank accession numbers and references for human sequences are as follows: APOB: M19828, Ludwig et al., 1987; FES: X06292, Roebroek et al., 1985; CHRNA1: NM000079, Noda et al., 1983; GHR: Z11802, Godowski et al., 1989; RHO1: U49742, Nathans and Hogness, 1984.

the total combined data to downweight noisy (homoplastic) characters, especially in the cyt *b* data set. Homoplastic characters are downweighted in proportion to their extra number of steps according to a concavity function constant (k), which varies from mild (e.g., $k = 6$) to strong ($k = 1$). Because it is not possible to know *a priori* how much homoplastic characters should be downweighted, we conducted searches with $k = 2, 4, 6, 8$, and 10 to explore the effects of different concavities on tree topology. We employed heuristic searches with 50 replicates of random stepwise addition and tree bisection-reconnection branch swapping. To explore the effect of indels on the parsimony analyses, we created two versions of each of the nuclear DNA data sets in which indels were either coded as missing or recoded according to the strategy of Barriel (1994). This strategy expresses the phylogenetic information contained in indels by treating them as single events regardless of length or complexity. Question marks are used for the coding of indels associated with subsequent substitutions and for minimizing the number of steps on the tree. Coding indels as distinct presence/absence characters has been justified on theoretical grounds (Simmons and Ochoterena, 2000). Both treatments (indels missing versus recoded) resulted in identical topologies, but because the recoded matrices contained more information (Table 3), we only present and discuss results based on these analyses.

Nodal support in parsimony analyses was evaluated by bootstrapping (BP; Felsenstein, 1985), jackknifing (JK; Farris et al., 1996), and Bremer support (BS; Bremer, 1988, 1994). For BP, we used 1,000 pseudoreplicates employing the same search conditions as described above. Only 200 pseudoreplicates were used in the implied weighting searches with concavity functions (k) of 4, 6, 8, and 10 (1,000 pseudoreplicates were used with $k = 2$). Trees were jackknifed using PAUP* (Swofford, 2002) with 1,000 replicates and the same search conditions as described above but with all uninformative characters excluded (Cunningham, 1997) and 50% of the characters deleted in each replicate. BS analyses were facilitated using TreeRot 2 (Sorenson, 1998). To assess the relative contribution of each data set to the total BS value at each node in the two combined analyses, we conducted partitioned BS (PBS) analyses (Baker and DeSalle, 1997; Gatesy et al., 1999), also with the program TreeRot 2. However, raw BS values are of limited use in judging nodal support because variation in tree shape and branch lengths have a large impact on the statistical interpretation of these support values (DeBry, 2001). This makes it difficult to even interpret support values on different nodes of a tree derived from a single data set (e.g., a BS value of 8 for one node and 6 for another node on the same tree does not necessarily mean that the first node is better supported than the second). Therefore, the BS and PBS values we report should be treated with caution. We primarily use these measures as a way to evaluate the relative contribution of informative characters from different data sets to the nodal support on the total combined data tree.

We also measured the index of data decisiveness (DD; Goloboff, 1991) to assess how each partition differed in the information content related to the support for the most parsimonious tree(s). Trees that are much shorter than the average length of all possible trees for a given data set are considered to be highly decisive, regardless of the amount of homoplasy present (Goloboff, 1991). DD is calculated as $(\bar{S} - S)/(\bar{S} - M)$, where $\bar{S}$ is the average length of all trees, S is the length of the most-parsimonious tree(s), and M is the sum of the minimum possible number of steps. The average length of all trees ($\bar{S}$) was estimated by generating 100,000 random trees in PAUP* (Swofford, 2002).

Using equally weighted MP, we compared the performance of the concatenated nuclear data versus the cyt *b* data to recover specific nodes in the total combined tree with the variable-length bootstrap method, where bootstrap support is tested as a function of the number of resampled characters (Springer et al., 1999; Teeling et al., 2000). This analysis provides a graphical measure of the relative efficiency of the two data sets in reconstructing the phylogeny on a per-nucleotide basis.

For ML analyses, indels were coded as missing. We used ModelTest 3.06 (Posada and Crandall, 1998) to determine the model and parameters of DNA substitution that best fit each of the partitions. Models were chosen among a set of 56 models according to the hierarchical likelihood ratio test. Models and parameters were then

TABLE 3. Sequence characteristics of the separate and combined data partitions used in the study.

Partition	No. characters[a]	No. observed variable sites[a]	No. PI sites[a]	No. indels	No. PI indels	Empirical base frequencies (%)[b]			
						A	C	G	T
APOB	301/299	62/63	34/35	1	1	31.6/26.3	21.8/28.8	20.3/17.1	26.3/27.9
FES	436/413	145/157	78/81	12	5	19.0/13.9	32.0/29.9	27.7/33.3	21.3/22.9
CHRNA1	385/359	112/121	58/61	10	3	26.0/22.6	24.5/33.5	26.5/25.1	23.0/18.8
GHR	634/627	150/162	71/75	11	4	30.1/33.0	19.1/17.5	18.7/19.3	32.1/30.1
RHO1	284/284	67/68	39/38	2	0	19.1/17.6	30.1/40.2	28.3/23.9	22.5/18.3
CYT *b*	1,140	524	454	na[c]	na	29.2/29.8	29.7/41.7	13.7/7.4	27.4/21.2
Nuclear	2,040/1,982	536/571	280/290	36	13	25.6/22.5	24.8/28.8	23.6/24.9	26.0/23.8
All data	3,180/3,122	1,060/1,095	734/744	36	13	26.9/27.1	26.7/36.7	19.9/14.0	26.5/22.2

[a]Number of characters or sites with gaps coded as missing/gaps coded according to Barriel (1994). PI = parsimony informative.
[b]All sites/informative sites only.
[c]na = not applicable.

specified in heuristic searches employing the same search conditions as described for the parsimony searches, except that only one replicate of random stepwise addition was used. Based on the results from ModelTest 3.06, a discrete approximation of the gamma distribution (NCAT = 4) was employed for all data partitions. Nodal support in ML trees was evaluated using 300 pseudoreplicates of BP with MaxTrees = 1 (DeBry and Olmstead, 2000) for the individual nuclear loci. Only 100 pseudoreplicates were used for cyt *b*, the combined nuclear data, and the total combined data.

The size of the total combined data set, in terms of taxa and nucleotides, and the model of evolution used for this data set (GTR+I+G; general time reversible with some invariable sites and with variable sites assumed to follow a gamma distribution) prevented a thorough ML search with BP because of the computational effort involved. Bayesian analysis offers an alternative model-based phylogenetic inference method that is not only more computationally efficient (Larget and Simon, 1999) but also permits tree topology and nodal support to be estimated simultaneously (Lewis, 2001). We performed Bayesian phylogenetic analysis on the total combined data partition with the program MrBayes (Huelsenbeck, 2000), which employs a Markov Chain Monte Carlo (MCMC) approach for sampling the joint posterior probability distributions. We ran the Markov chain for 500,000 generations, sampling trees every 100 generations, with four independent chains running simultaneously (allowing a greater amount of tree space to be searched). Two runs were performed to ensure consistent results. Likelihood values for trees were estimated using the GTR+I+G model of evolution. Following each run, 5,000 trees were imported into PAUP*, and a 50% majority-rule consensus tree was constructed after deleting the first 1,000 trees (=burn-in value), thereby excluding any trees that had not yet converged on a stable log likelihood. In addition, plots of generation number versus log-likelihood score showed that the MCMC had reached a stable log likelihood score within 50,000 generations in both runs.

Finally, we assessed the heterogeneity in phylogenetic signal (i.e., incongruence) among the individual data sets and partitions. We used the level of bootstrap support in MP and ML trees to measure the degree of conflict among topologies recovered in the separate analyses (de Queiroz et al., 1993; Flynn and Nedbal, 1998). In light of the findings of Hillis and Bull (1993), we selected a bootstrap value of 70% as an indicator of strong support and as the threshold value between conflicting (≥70%) and nonconflicting (<70%) nodes.

RESULTS

Sequence Characteristics of the Gene Segments

Our data set comprised a total of 3,180 characters (including gaps) for the 24 taxa included in our study (except for *A. capensis* and *G. vittata*, which each had 2,879 bp). We inferred a total of 36 indels among the five nuclear genes, which ranged in size between 1 and 19 bp, with most indels being 1 bp long. Thirteen of these indels were parsimony-informative; the rest were restricted to a single taxon (Table 3). All loci except RHO1 contained informative indels. The single indel in the APOB gene segment was in-frame, whereas for the four gene segments that contained exon and intron sequences, all indels were found in the intron regions. When gaps were recoded (Barriel, 1994), the total combined data set contained 3,122 characters. Sequence characteristics of the eight data partitions are summarized in Table 3. All new sequences were deposited in GenBank, and the alignments for each data set are available from the Entrez PopSet database (accessions AF498107–AF498231).

All nuclear gene segments contained observed variable sites and parsimony-informative sites, but the proportion of each of these differed among genes, ranging between 21%–38% and 12%–21%, respectively (Table 3). These differences can be attributed to the amount of intron sequence contained by each gene relative to the total sequence length. Accordingly, CHRNA1 and FES each contained a high number of observed variable sites (88% and 72% intron sequence, respectively), followed by RHO1 and GHR (35% and 33% intron sequence, respectively) and then APOB (0% intron sequence).

Sequences from the cyt *b* gene contained more observed variable and parsimony-informative sites when compared with either individual or combined data sets of nuclear loci (Table 3). Specifically, the cyt *b* gene contained 6–13 times more parsimony-informative sites than did the individual nuclear loci. Furthermore, although the cyt *b* gene and combined nuclear data partition (with gaps uncoded) had roughly similar numbers of observed variable sites (524 vs. 536, Table 3), the cyt *b* sequences contained nearly twice as many parsimony-informative sites (454 vs. 280, Table 3) despite being almost 50% shorter in length. The majority of observed variable and parsimony-informative sites in the cyt *b* gene were found in the third position of codons, as we found previously (Koepfli and Wayne, 1998).

The ranges of uncorrected pairwise sequence divergence (minimum = comparison within ingroup; maximum = comparison between ingroup and outgroup) for the six genes were as follows: APOB, 0–9.8%; FES, 0.5–16.4%; CHRNA1, 0.8–13.8%; GHR, 0.3–8.3%; RHO1, 0.4–9.9%; and CYT *b*, 5.5–20.6%. Evaluation of mean nucleotide frequencies (all sites and informative sites only) among the five nuclear genes showed that some loci exhibited compositional bias, but the direction of this bias differed among genes (Table 3). Only for APOB and GHR did the best-fit models of substitution suggest the influence of base composition bias (Table 4), possibly because these two loci contained the greatest proportion of exon sites (100% and 67%, respectively). Furthermore, the χ^2 test of homogeneity of nucleotide frequencies of the individual and combined nuclear loci showed no significant heterogeneity among the 24 taxa (results not shown). When the nuclear DNA sequences were combined into a single matrix, frequencies for the four nucleotides were nearly equivalent (Table 3). Conversely, the cyt *b* gene

TABLE 4. Best-fitting substitution models and parameter values for the separate and combined partitions determined using ModelTest (Posada and Crandall, 1998). A dash indicates the parameter value was not estimated according to best-fit model. The model parameters estimated from the Bayesian phylogenetic analyses of the total combined data are also presented.

	Partition									
									All data, Bayesian	
Model/parameter	APOB	FES	CHRNA1	GHR	RHO1	CYT *b*	Nuclear	All data	Run 1	Run 2
Best-fit model[a]	TrN+G	K2P+G	K2P+G	HKY+G	K2P+G	GTR+I+G	K3Puf+G	GTR+I+G	GTR+I+G	GTR+I+G
Nucleotide frequency										
π_A	0.3060	0.2500	0.2500	0.2975	0.2500	0.3398	0.2623	0.2692	0.2684	0.2684
π_C	0.2051	0.2500	0.2500	0.1995	0.2500	0.3458	0.2430	0.2787	0.2797	0.2796
π_G	0.2055	0.2500	0.2500	0.2043	0.2500	0.0863	0.2292	0.1985	0.1989	0.1992
π_T	0.2834	0.2500	0.2500	0.2986	0.2500	0.2281	0.2655	0.2536	0.2531	0.2527
Ts/Tv ratio[b]	—	3.2719	3.2260	2.5090	3.7429	—	—	—	—	—
Gamma shape parameter	0.4146	0.7784	0.4725	0.4806	0.2937	1.2996	0.4764	0.3965	0.3996	0.4003
Proportion of invariable sites	—	—	—	—	—	0.5133	—	0.3673	0.3674	0.3646
No. substitution types	6	2	2	2	2	6	6	6	6	6
R-matrix										
A ↔ C	1.0000	—	—	—	—	3.3230	1.0000	1.2451	1.4064	1.4383
A ↔ G	3.3367	—	—	—	—	99.8254	5.0179	8.3713	9.3890	9.6423
A ↔ T	1.0000	—	—	—	—	3.3496	0.6587	0.8805	0.9997	1.0366
C ↔ G	1.0000	—	—	—	—	0.0001	0.6587	0.3956	0.4635	0.4866
C ↔ T	9.2140	—	—	—	—	74.6752	5.0179	14.3850	16.2594	16.8057
G ↔ T	1.0000	—	—	—	—	1.0000	1.0000	1.0000	1.0000	1.0000

[a]G = gamma distribution of variable sites; TrN = Tamura-Nei (Tamura and Nei, 1993); K2P = Kimura two parameter (Kimura, 1980); HKY = Hasegawa–Kishino–Yano (Hasegawa et al., 1985); K3Puf = Kimura three parameter (Kimura, 1981).

[b]Ts/Tv = transition/transversion.

showed a conspicuous deficit in the frequency of G (Table 3), which is characteristic of the vertebrate mitochondrial genome (Kocher et al., 1989). This deficit is still apparent when the cyt *b* sequences are combined with the nuclear sequences in the total combined data set (Table 3). The χ^2 test of homogeneity revealed a significant difference in base composition among taxa in the cyt *b* gene when only informative sites were examined ($P \ll 0.00001$).

Phylogenetic Analyses of Individual Loci

Although none of the gene trees are identical, several clades were consistently recovered with the six loci in both MP and ML analyses (Fig. 1). First, the monophyly of the Mustelidae was well supported (BS = 10+; BP = 100%). Seven of the 13 informative indels found in the nuclear loci provide support for this clade. Second, the two Old World badgers, *Meles meles* and *Arctonyx collaris*, were placed as sisters with strong support (BS = 2–22; >80% BP in MP and ML trees), and both taxa shared an indel at the FES locus (Fig. 1). Third, the three species of *Lontra* (New World river otters) received strong support (BS = 1–8; BP = 83%–100%) in analyses of FES, CHRNA1, GHR, and cyt *b* but were weakly supported by APOB and RHO1. Fourth, a group containing the three species of *Mustela* had weak to strong support (BS = 1–4; BP = 58%–96%), and within this clade, *M. frenata* and *M. vison* were strongly united by five of the six genes and shared an informative indel at the GHR locus (Fig. 1). Fifth, the two species of *Martes*, *Gulo*, and *Eira* were monophyletic (except for CHRNA1) with variable support (BS = 1–5; BP = <50%–97%). These taxa shared an indel at the FES locus (Fig. 1). The last informative indel was found at the CHRNA1 locus and was shared by *Aonyx capensis* and *Amblonyx cinerea*. It is noteworthy that all informative indels were associated with internodes that have ≥70% BP values, and these values are maintained even when the indels are coded as missing in MP analyses (not shown).

Deeper nodes were much less congruent among trees, but these generally had low support and/or little resolution (e.g., lutrine monophyly, Fig. 1). Nuclear gene trees based on more informative sites (i.e., FES and CHRNA1, Table 3) were more resolved than were gene trees with fewer informative sites (i.e., GHR and RHO1). Even though ML trees were more resolved than MP trees, the resolved branches generally had low bootstrap support (<50%). The only exception to this was the cyt *b* gene tree, where the ML tree had more nodes with >50% bootstrap support than did the MP tree (15 vs. 9 nodes, Fig. 1).

There was a sharp contrast between the nuclear genes and the cyt *b* gene in the amount of homoplasy, character congruence, and decisiveness, as judged by the consistency index (CI), retention index (RI), and DD, respectively (Table 5). Although the cyt *b* gene contained a greater amount of phylogenetic signal overall, it had more than twice the amount of homoplasy (CI = 0.307, RI = 0.326) and was the least decisive (DD = 0.210)

when compared with the nuclear genes. Furthermore, the lower RI for the cyt *b* tree suggests that multiple substitutions along terminal branches were more likely to erase synapomorphies at internal nodes.

Base composition was significantly heterogeneous for the informative sites of the cyt *b* gene, suggesting that base composition may be evolving in a nonstationary manner for this marker. This finding raises the possibility

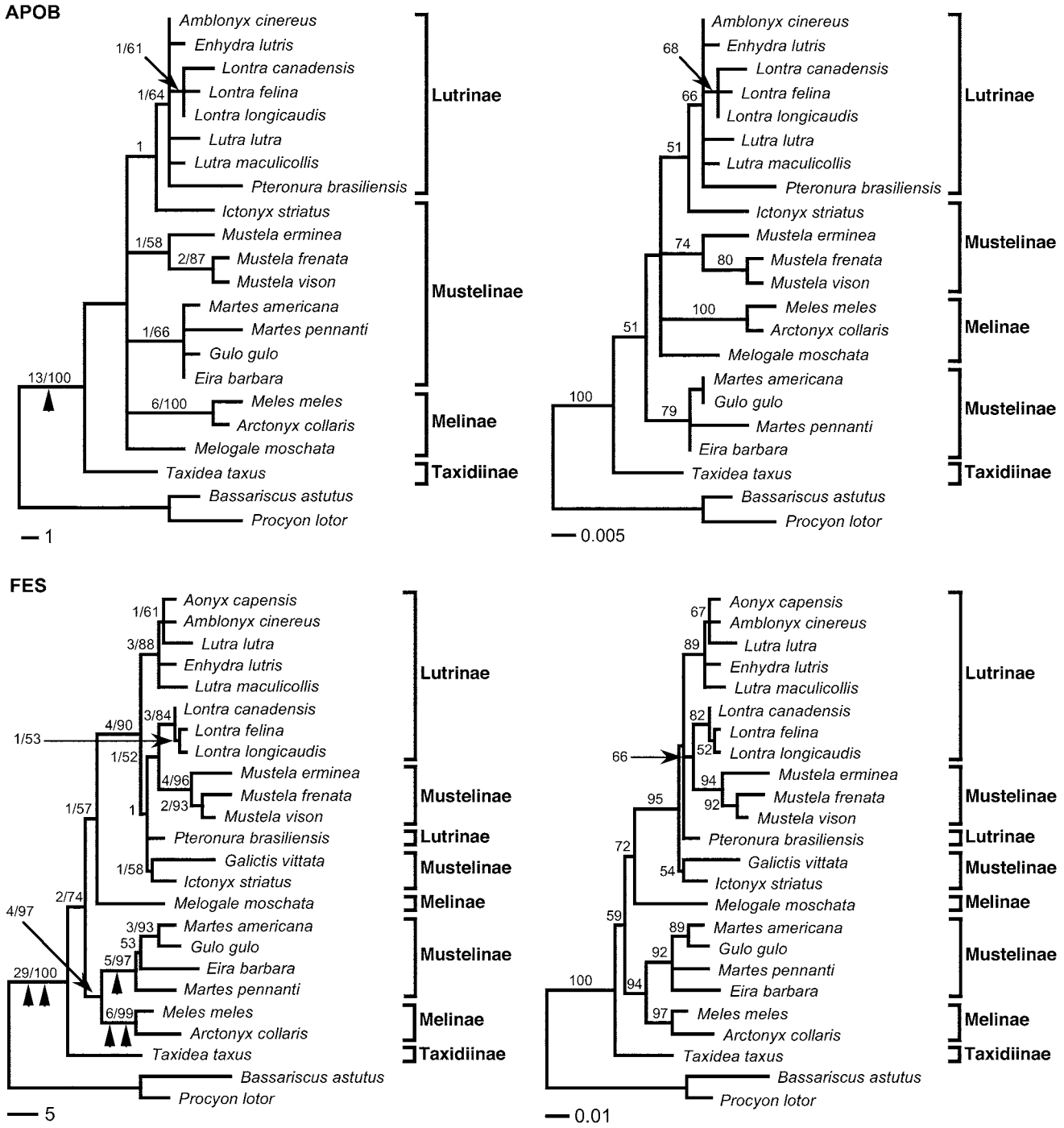

FIGURE 1. Strict consensus MP (left) and ML (right) trees inferred from phylogenetic analysis of the six individual loci. For MP trees, BS/BP values (of 1,000 pseudoreplicates and ≥50%) are shown above internodes. Arrowheads below internodes indicate location of parsimony-informative indels. Branch lengths are proportional to the number of substitutions per site (see scale bars). Tree statistics for MP trees are presented in Table 5. For likelihood trees, BP values (of 300 pseudoreplicates and ≥50%) are shown above internodes. The substitution model and parameters obtained using ModelTest 3.06 (Posada and Crandall, 1998) for each ML tree are presented in Table 4. The negative log likelihood (−lnL) for each ML tree is as follows: APOB, 895.096; FES, 1790.471; CHRNA1, 1467.116; GHR, 2115.685; RHO1, 980.852; CYT *b*, 9583.799. *(Continued)*

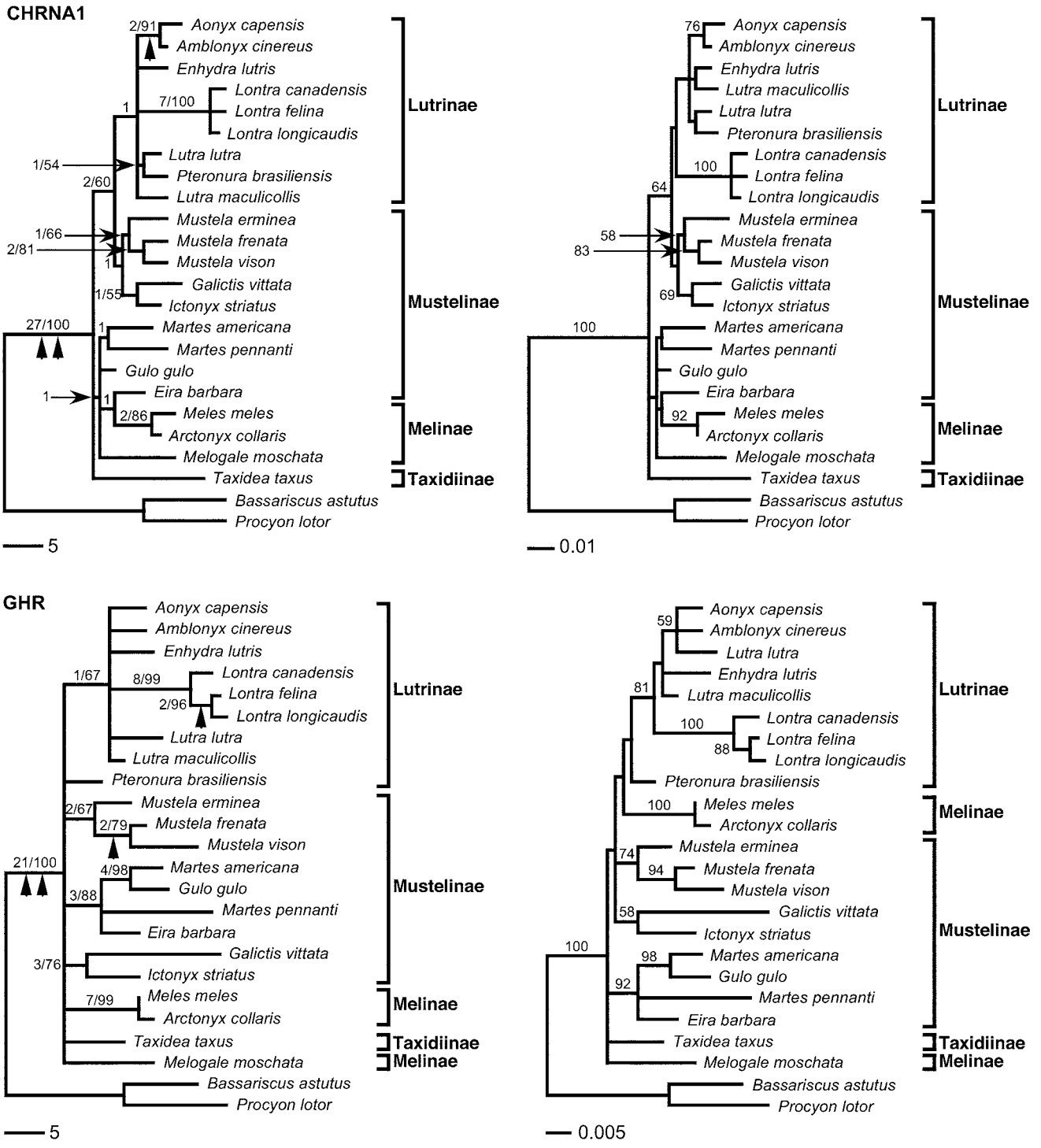

FIGURE 1. (Continued)

that convergence in base composition between unrelated taxa could lead to the estimation of well-supported, but incorrect, relationships (e.g., Lockhart et al., 1994). To determine whether this indeed was the case, we analyzed the cyt *b* data using the minimum evolution method (Rzhetsky and Nei, 1992) with the LogDet/paralinear transformation (Lake, 1994; Lockhart et al., 1994), which corrects for nonstationary base composition. We used ML analysis of the two equally parsimonious MP trees derived from the equally weighted cyt *b* data (Fig. 1) to fit the data to the GTR+I model to estimate the proportion of invariable sites. This parameter was incorporated

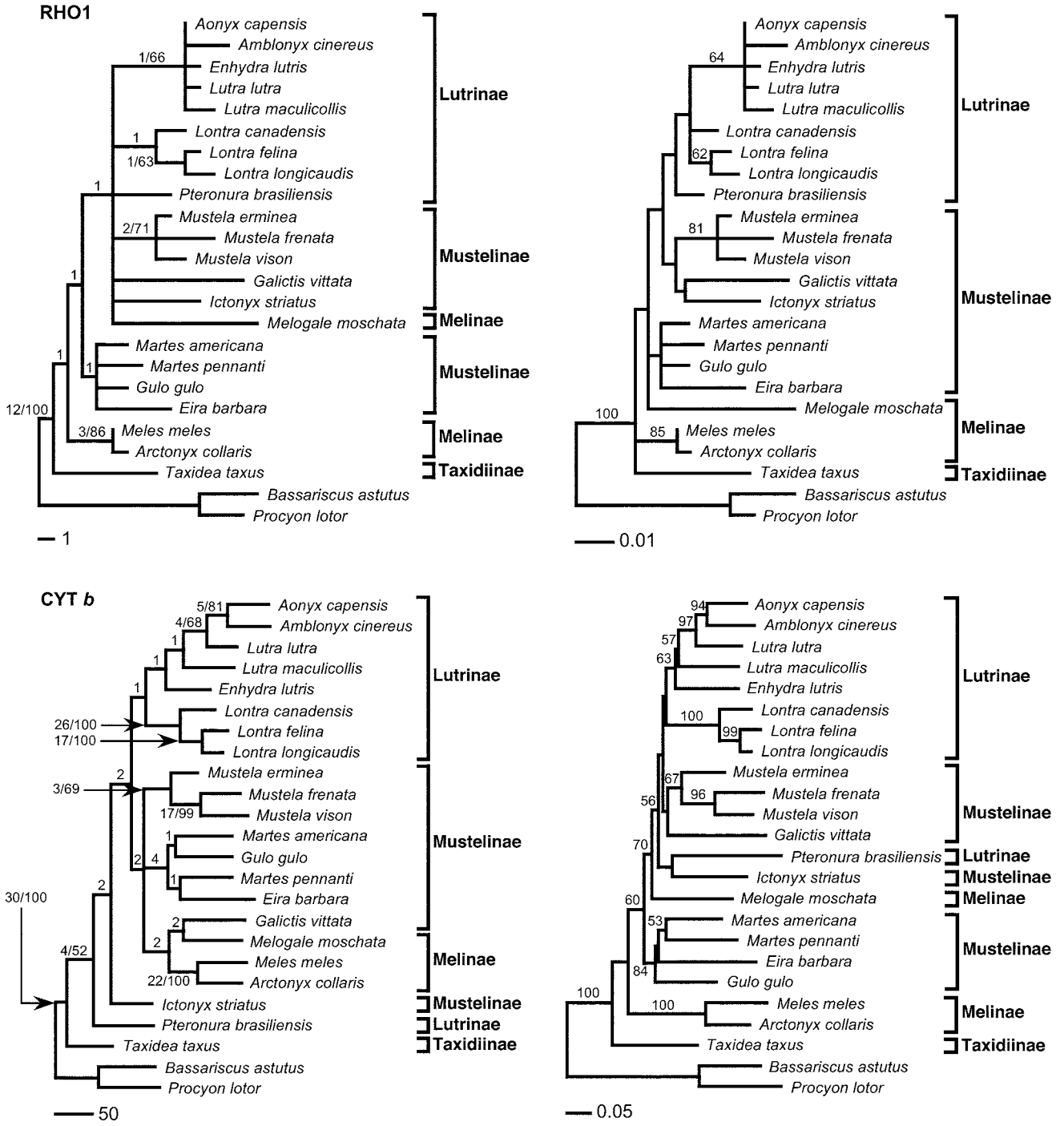

FIGURE 1. (Continued)

into the transformed distances to account for rate heterogeneity (i.e., LogDet+I). The proportions of invariable sites for the two cyt *b* MP trees were estimated to be 0.5362 and 0.5363. The minimum evolution tree with LogDet+I transformed distances was topologically similar to the strict consensus of the equally weighted MP tree (Fig. 1) but differed in the relationships among the deeper nodes (results not shown). Importantly, however, bootstrap analysis of the minimum evolution tree (using 1,000 psedoreplicates) showed the same well supported nodes as found in the MP and ML trees (Fig. 1), suggesting that convergence in base composition is not biasing the MP and ML analyses of the cyt *b* data.

ModelTest selected different models and parameters of substitution for different genes and partitions using hierarchical likelihood ratio tests (Table 4; Posada and

TABLE 5. Tree statistics from MP analyses of separate and combined partitions.

Partition	No. equally parsimonious trees	Tree length	CI[a]	RI	DD
APOB	26	82	0.731	0.841	0.821
FES	1	218	0.717	0.838	0.816
CHRNA1	5	175	0.658	0.748	0.719
GHR	1,303	216	0.680	0.772	0.750
RHO1	42	102	0.625	0.738	0.700
CYT *b*	2	2169	0.307	0.326	0.210
Nuclear	3	805	0.667	0.776	0.749
All data	3	3002	0.373	0.433	0.343

[a]Excluding uninformative characters.

Crandall, 1998). Simpler models with fewer parameters were selected for the individual nuclear genes compared with the cyt *b* gene.

Tests of Incongruence

We did not detect significant differences in the distribution of phylogenetic signal among the individual partitions. None of the gene trees had nodes that conflicted with ≥70% bootstrap support (Fig. 1). Clades that were well-supported in one gene tree were also supported in others, while clades that were discordant among the individual partitions were always weakly supported (<70% bootstrap support) in both MP and ML trees. The lack of significant incongruence as judged by BP may reflect the low variability of the nuclear genes and the high amount of homoplasy in the cyt *b* gene, both of which result in gene trees that were incompletely resolved and/or weakly supported.

Phylogenetic Analyses of Combined Nuclear Data

The Kimura three parameter model (Kimura, 1981) with a shape parameter of the gamma distribution best described the concatenated nuclear data set (Table 4). Figure 2 shows the ML tree based on combined analysis of the five nuclear loci. In contrast to the analyses of the individual loci, all nodes were well resolved except for *Eira barbara* and *Martes pennanti*. MP resulted in three equally parsimonious trees because of alternative placements of *E. barbara* and *M. pennanti* relative to the clade that joined *Gulo gulo* and *Martes americana*, but otherwise the topology was the same as shown in Figure 2. As expected, the level of homoplasy (CI = 0.667, RI = 0.776) and decisiveness (DD = 0.749) were comparable to the values obtained in the individual analyses of the nuclear genes (Table 5). The majority of clades were strongly supported by BS, BP (MP and ML), and JK, with only nodes 4 and 13 receiving low support (Fig. 2). The tree supported the monophyly of the Lutrinae, whereas the Melinae and Mustelinae were both polyphyletic. The American badger, *Taxidea taxus*, was the most basal taxon and sister to a clade that contained all other mustelid taxa. As in the separate analyses, *Arctonyx* and *Meles* were sister taxa. These taxa were joined with low to moderate support to the clade composed of *Eira*, *Gulo*, and the two species of *Martes*. Furthermore, *Gulo* clustered strongly with *M. americana*, suggesting that *Martes* is paraphyletic with respect to *Gulo*. However, further sampling of other species of *Martes* is required to confirm this result. The Chinese ferret-badger (*Melogale moschata*) formed a distinct lineage in the middle of the tree, but its placement was weakly supported (MP and ML BP and MP JK values of <70%). The remaining mustelines formed two separate clades, one containing the three species of *Mustela* and the other containing *Galictis vittata* and *Ictonyx striatis*. The latter clade formed the sister group to the otters with moderate support (Fig. 2). Otters were subdivided into three primary lineages with moderate to strong support (range of MP BS = 71%–100%; Fig. 2).

Although the tree in Figure 2 is well resolved and supported, it is unlikely that the five nuclear genes contributed equally to the structure of this combined tree because the individual analyses (Fig. 1) showed different degrees of resolution among the genes. Therefore, we used PBS analysis to assess the relative contribution of each of the five nuclear genes to the total BS value at each node in the combined tree (Table 6). Several aspects of the PBS analysis are noteworthy. First, the number of negative contributions was low; only 5 of the 98 values were negative. This suggests that there is little conflicting information among the five nuclear genes in the combined analysis. Second, the genes differ considerably in their contribution to the overall support of the combined tree. For example, FES contributes the most support (30.1%), while RHO1 contributes the least (9.6%). Even though APOB contained the fewest number of observed variable and parsimony informative sites (Table 3), it contributed slightly more support to the combined tree than did RHO1 (13.7% vs. 9.6%, respectively). Third, the partitioned BS of each gene is highly variable across nodes. In most cases, nodes receive positive support from one to four genes, with four nodes (1, 4, 5, and 18) receiving support from only a single gene. Only three nodes (12, 19, and 20) receive positive support from all five genes, and these are among the nodes that were consistently recovered in the individual analyses (Fig. 1).

Phylogenetic Analyses of the Total Combined Data

There were no conflicting nodes with ≥70% bootstrap support between the concatenated nuclear gene tree and the cyt *b* gene tree (Figs. 1, 2). MP searches under implied weights using five different concavity function constants ($k = 2$, 4, 6, 8, and 10) all resulted in the same topology. Bootstrap values were broadly similar across these different analyses. Therefore, we only report results from the differentially weighted analyses where $k = 2$.

The results of our MP analyses differed according to the two primary weighting schemes we employed (Fig. 3). The equally weighted analysis resulted in three equally parsimonious trees, the strict consensus of which is shown in Figure 3A. This tree was incongruent in two respects with the tree in Figure 2. First, otters were no longer monophyletic because *Pteronura brasiliensis* grouped with *Galictis vittata* + *Ictonyx striatis*, and

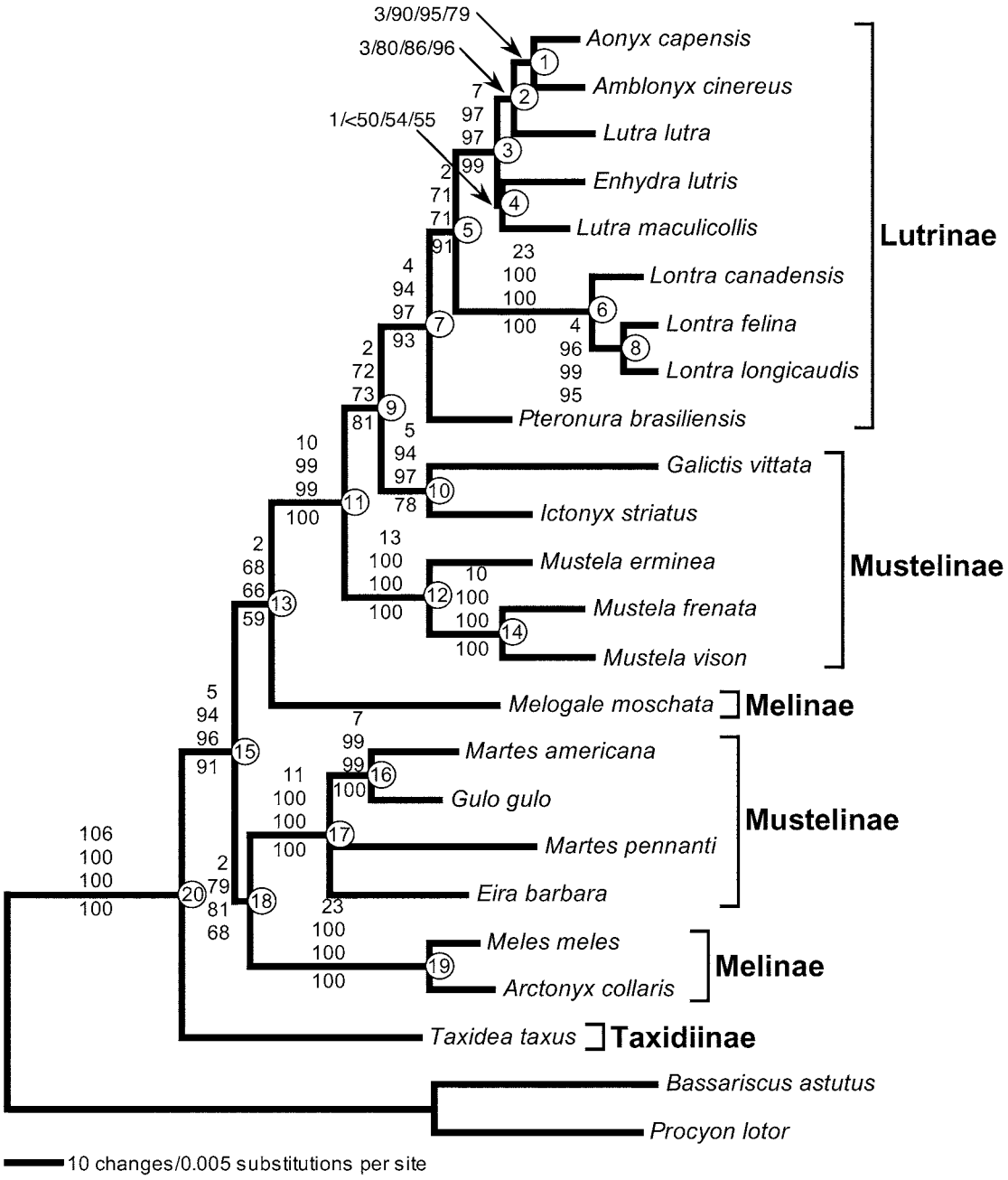

FIGURE 2. ML tree based on combined analysis of the five nuclear DNA sequence data sets (−lnL = 7415.122). Searches using parsimony resulted in three equally parsimonious trees. A strict consensus of these three trees was identical in topology to the likelihood tree. Tree statistics for the parsimony trees are presented in Table 5. The substitution model and parameters used in the ML search are presented in Table 4. BS, MP BP (out of 1,000 pseudoreplicates), MP JK (out of 1,000 pseudoreplicates), and ML BP (out of 1,000 pseudoreplicates) are shown from top to bottom at the internodes. Resolved nodes are indicated by circled numbers and correspond to those in Table 6. Branch lengths are proportional to the number of substitutions per site, as shown by the scale bar.

second, *Melogale moschata* was joined as the sister taxon to *Arctonyx collaris* + *Meles meles* (Fig. 3A). Neither of these relationships, however, was well supported (BS = 1, <50% BP and JK support). The level of homoplasy increased (CI = 0.373, RI = 0.433) and decisiveness decreased (DD = 0.343) relative to the tree based on the combined nuclear data alone (Table 5). In contrast, the tree found under implied weights (Fig. 3B) differed from the one in Figure 2 only in the placement of the clade containing *A. collaris* + *M. meles*.

Although the BS was increased at many nodes in both trees, there was a striking difference between the two analyses in the relative contribution of the nuclear and cyt *b* data sets to the BS at each node (Table 7). In the

TABLE 6. Partitioned BS values for the 20 nodes of the MP tree based on the concatenated nuclear data. Node numbers and total BS values correspond to those shown in Figure 2 only.

Node	Partition					Total BS for node
	APOB	FES	CHRNA1	GHR	RHO1	
1	—	0	3	0	0	3
2	0	1	0	2	0	3
3	0	3	1	1	2	7
4	0	0	1	0	0	1
5	0	0	−1	3	0	2
6	1	4	8	10	0	23
7	1	0	1	1	1	4
8	0	0	0	3	1	4
9	1	1	0	0	0	2
10	—	1	1	2	1	5
11	0	6	3	0	1	10
12	2	5	1	2	3	13
13	0	1	0	0	1	2
14	3	2	2	3	0	10
15	2	2	1	−1	1	5
16	1	3	−1	4	0	7
17	3	6	−1	3	0	11
18	0	3	0	0	−1	2
19	6	7	5	9	2	29
20	14	30	29	21	12	106
Total	34	75	53	63	24	249
% of total	13.7	30.1	21.3	25.3	9.6	

equally weighted analysis, much of the nodal support provided by the nuclear data was negative, in marked contrast to the PBS analysis of the combined nuclear data alone (Table 6). Moreover, cyt *b* provided more overall support than did the combined nuclear data (62.8% vs. 37.2%, respectively). In the implied weighting analysis, however, this result was reversed and the PBS of the nuclear data was the same or similar to that of the total BS at each node for the combined nuclear tree (i.e., compare last column in Table 6 with fifth column in Table 7). The actual nodal support provided by the cyt *b* gene also emerges and shows that this support is both overestimated at some nodes (e.g., node 3, Table 7) and underestimated at others (e.g., node 8, Table 7) with respect to the equally weighted analysis. Importantly, the results show that cyt *b* contributes negative or no information with regards to the placement of *P. brasiliensis* and *M. moschata* (nodes 7 and 13 in Fig. 3B), respectively.

The topologies of the ML and Bayesian analysis trees were identical, and these in turn were mostly identical to the differentially weighted MP tree (Figs. 3B and 4, respectively), except for the placement of *Enhydra lutris* with respect to *Lutra maculicollis*. The differentially weighted MP phylogeny unites these as sister species, but the BP and JK support for this group is very low (54% and 53%, respectively). However, the PBS analyses (Tables 6, 7) indicate that one of the nuclear loci (CHRNA1) and the cyt *b* gene contribute support to this clade. In contrast, the ML/Bayesian phylogeny places *E. lutris* as the sister taxon to the four species of Old World river otters, including *L. maculicollis*. The ML BP support for this relationship is low (69%). We believe that the low BP values from the MP and ML analyses, as well as the low JK support in the differentially weighted MP tree, suggest that the current data are insufficient and that additional data are therefore required to decide between these two hypotheses.

Nodal support as measured by BP (ML) and posterior probabilities (Pr, Bayesian) was generally high across the tree (BP > 70%, Pr ≥ 0.95; Fig. 4). Posterior probabilities were higher in most cases than BP support, most likely because of the limited number (100) of BP replicates performed in the ML analysis and the conservative nature of the BP in general (Hillis and Bull, 1993).

Efficiency of Nuclear Versus Mitochondrial DNA Sequences

We contrasted the efficiency of the concatenated nuclear data versus the cyt *b* data in recovering specific nodes with the variable-length BP method, using the trees of the differentially weighted MP analysis (Fig. 3B) of the total combined data as a reference. We found that the nuclear DNA sequences recovered nodes with high BP support as or more efficiently (i.e., with less data) than did the cyt *b* sequences (Fig. 5). For example, both data sets showed equivalent performance in recovering nodes that defined more recent clades (e.g., node 6 in Fig. 3B). For deeper nodes, however, the nuclear data always outperformed the cyt *b* data (Fig. 5). Consistent with the results of the PBS analyses, cyt *b* showed a near-complete absence of support for several key clades (nodes 7, 9, 11, and 13), including the monophyly of otters (node 7). For the last three nodes illustrated (12, 17, and 27; Fig. 5), the results suggest that two to three times more mitochondrial sequence would be required to reach the high support provided by the concatenated nuclear data. However, any conclusions derived from the variable-length BP analyses apply only with respect to the equally weighted parsimony analyses.

DISCUSSION

Properties and Relative Utility of the Nuclear and Mitochondrial DNA Sequences

By themselves, the five nuclear DNA fragments were of limited utility in resolving the relationships within the Mustelidae. Because of their short length and slow rate of evolution, the nuclear sequences contained too few informative characters to resolve adequately all the nodes of the gene trees, although some trees were more resolved than others (e.g., FES; Fig. 1). However, the low resolution of the individual nuclear gene analyses was overcome when we concatenated the nuclear sequences (Fig. 2). The signal of the individual and combined nuclear partitions had little homoplasy, as shown by their high CIs across the trees (Table 5). Furthermore, the RI and DD values of the nuclear data were similarly high (Table 5).

We ascribe the low amount of homoplasy and high decisiveness in the nuclear data to the combination of low among-site rate variation (Yang, 1996; Davis et al., 1998), relatively low base composition bias, and the slow rate of evolution of these markers. Because four of the nuclear

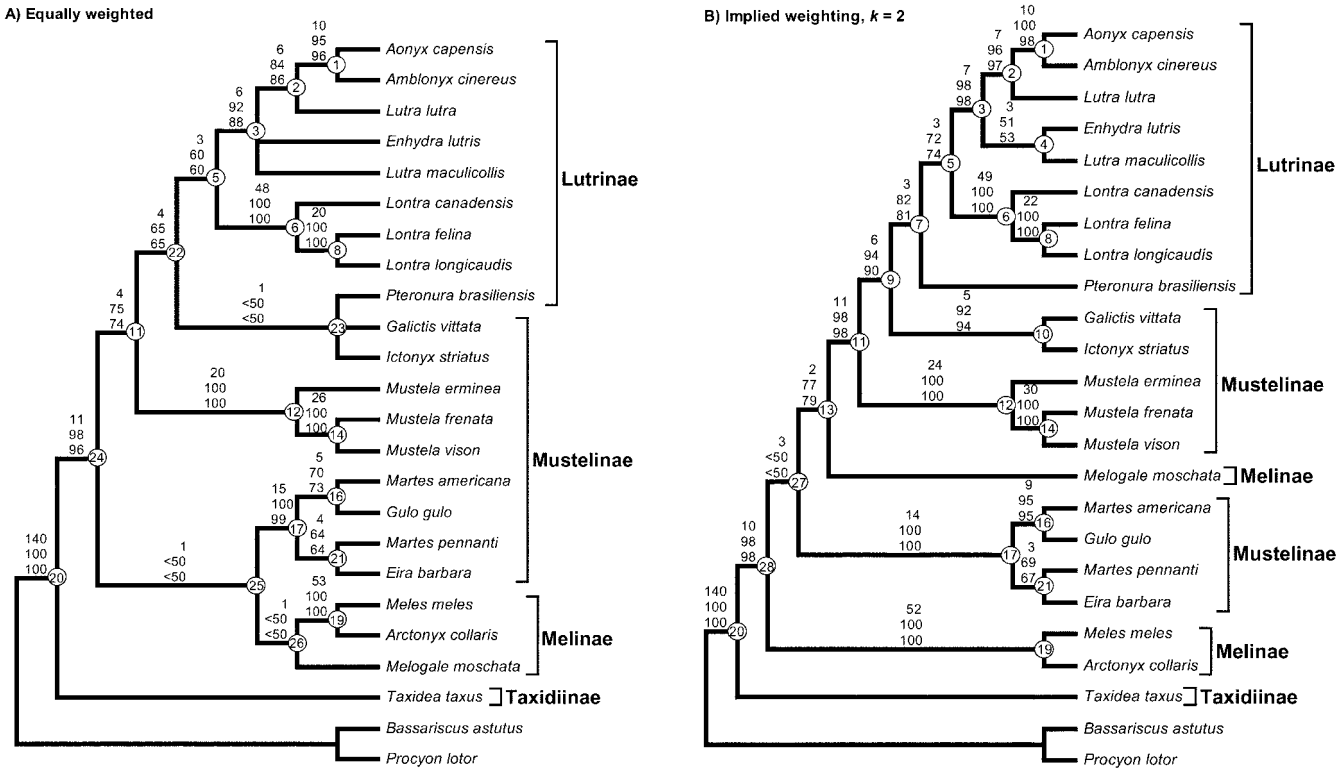

FIGURE 3. Most-parsimonious trees based on equal weighting (A) and implied weighting (B) analyses of the total combined data. The equally weighted tree is a strict consensus of three equally parsimonious trees, whereas the tree based on implied weighting is the single most-parsimonious tree. BS, BP (out of 1,000 pseudoreplicates), and JK values are shown from top to bottom above the nodes. Resolved nodes are indicated by circled numbers and correspond to those in Table 7. Tree statistics for the equally weighted tree are presented in Table 5. The tree derived under implied weights had the same CI and RI values as the equally weighted tree but had a tree length (L) of 3,003 and a Goloboff fit of −497.92.

gene segments contained noncoding intron sequences, the potential for multiple substitution is reduced because more sites are free to vary (Palumbi, 1989). However, all of our sequences also contained coding exon sequences, which are subject to the same differences in functional constraint across codon positions as the cyt *b* gene (i.e., third positions change more often than first and second positions). As a result, among-site rate variation is expected to be higher in these sequences (lower gamma shape parameter; Yang, 1996). The fact that four of the five nuclear gene segments contained both exon and intron sequences explains the low to moderate estimates of the gamma shape parameter we observed (Table 4). Even so, the potential for multiple substitutions (and thus homoplasy) is mitigated by the slow rate of evolution of the nuclear sequences. Indeed, Graham and Olmstead (2000), following the reasoning of Felsenstein (1981, 1983), have suggested that slowly evolving sequences with a low gamma shape parameter value basically approximate a model where all sites are evolving at the same rate. This is because even if sites change at different rates, the slow rate of evolution ensures that most sites will experience few changes (Graham and Olmstead, 2000).

In contrast, the cyt *b* data contained more informative variation than the individual or combined nuclear data (Table 3). Furthermore, the minimum and maximum pairwise divergence values for the cyt *b* data were higher than those for the individual nuclear DNA fragments (see Results). These observations are consistent with the higher rate of substitution of mitochondrial versus single copy nuclear genes (Brown et al., 1979). However, cyt *b* was characterized by a high level of homoplasy, which compromised much of its phylogenetic signal in the equally weighted MP analysis. CIs and RIs across trees, as well as DD values, were all uniformly low for the cyt *b* data (Table 5). Although the cyt *b* gene tree was more resolved than the nuclear gene trees, many nodes were poorly supported in both MP and ML analyses (Fig. 1). The cyt *b* gene was characterized by a high amount of among-site rate variation ($\alpha = 0.16$, excluding the proportion of invariable sites) and base compositional bias (Table 3), which is typical of a mitochondrial protein-coding gene (Brown, 1985; Irwin et al., 1991). Most of the nucleotide changes are synonymous substitutions that occur at third codon positions (Koepfli and Wayne, 1998). Substitutions at the other two codon positions are limited by constraints that effectively prevent amino acid replacements (nonsynonymous changes). The high base composition bias, particularly at third codon positions (Koepfli and Wayne, 1998), further limits the amount of change that a site can undergo, leading to substitution

TABLE 7. Partitioned BS values for the nodes of the trees based on equally weighted parsimony (unweighted) and parsimony with implied weighting ($k = 2$) of the total concatenated data. Node numbers and total BS values for the equally weighted parsimony analysis correspond to those shown in Figure 3A, whereas those for the parsimony analysis with implied weighting correspond to the tree shown in Figure 3B. PBS values for the equally weighted MP analyses are derived from three equally parsimonious trees, hence the decimal numbers. We standardized node numbers according to those in Figure 2. Therefore, nodes numbered 1–20 correspond to the same nodes (and monophyletic groups) found in Figure 2. Nodes without values indicate a monophyletic group not found in Figure 2. Nodes 21–28 are monophyletic groups *unique* to the two MP analyses of the total combined data. Here again, nodes without values correspond to monophyletic groups not found in either Figures 3A or 3B. For example, the monophyletic group denoted by node 28 was recovered in Figure 3B but not in Figure 3A.

	Equally weighted			Goloboff fit, $k = 2$		
Node	Combined nuclear genes	Cyt *b*	Total BS	Combined nuclear genes	Cyt *b*	Total BS
1	2.2	7.6	10	3	7	10
2	−2.3	8.3	6	3	4	7
3	−4.3	10.3	6	7	0	7
4				1	2	3
5	−5.3	8.3	3	2	1	3
6	24.2	23.8	48	23	26	49
7				4	−1	3
8	5.7	14.3	20	4	18	22
9				2	4	6
10				5	0	5
11	12.2	−8.2	4	8	3	11
12	14.2	5.8	20	13	11	24
13				2	0	2
14	8.2	17.8	26	10	20	30
15						
16	−4.3	9.3	5	7	2	9
17	9.9	5.1	15	12	2	14
18						
19	20.7	32.3	53	31	21	52
20	105.7	34.3	140	105	35	140
21	−12.3	16.3	4	−2	5	3
22	5.3	−1.3	4			
23	−11.3	12.3	1			
24	−5.3	16.3	11			
25	−11.3	12.3	1			
26	−11.3	12.3	1			
27				−2	5	3
28				6	4	10
Total	140.6	237.4	378	244	169	413
% of total	37.2	62.8		59.1	40.9	

bias (Collins et al., 1994). This limitation is supported by the fact that the best-fitting model for the cyt *b* gene was the GTR model, which incorporates the inequality of substitution rates among the six classes of substitution. Therefore, even though the higher rate of substitution in the cyt *b* gene results in more variation per unit time, this variation is concentrated at sites where multiple substitutions are prevalent and whose character space is restricted (Naylor et al., 1995; Hassanin et al., 1998).

The difference in the patterns of substitution between the nuclear and cyt *b* sequences in the equally weighted MP analyses also showed that the concatenated nuclear data were more efficient than the cyt *b* data for recovering the deeper nodes of the mustelid phylogeny (Fig. 5). Although the cyt *b* gene contained more informative variation, the high level of homoplasy diminished its efficiency. Indeed, the results of the variable-length BP indicate that a substantial amount of mitochondrial sequence data (with properties similar to those of the cyt *b* gene) is required to resolve some nodes before it can match the efficiency provided by the nuclear DNA sequence data alone (e.g., nodes 12, 13, 17, and 27 in Fig. 5). Furthermore, the concatenated nuclear sequences were as efficient as the cyt *b* sequences in recovering more recent clades (node 6 in Fig. 5). It should be remembered, however, that the individual nuclear loci varied with respect to the various nodes supported, and that the combination of the five loci was required in order to find support throughout the tree. Furthermore, we reiterate that the contrast in the efficiency between the combined nuclear data versus the cyt *b* data was only evaluated under equally weighted MP. The cyt *b* sequences might demonstrate improved efficiency at recovering deeper nodes using a model-based approach such as ML, which can better accommodate base composition bias and high among-site rate heterogeneity. Springer et al. (2001), however, found that even under ML, four concatenated mitochondrial genes were less efficient than a similar number of concatenated nuclear genes at recovering certain deep-level clades within eutherian mammals. Despite these caveats, our results are in agreement with those of Cummings et al. (1995), who found that

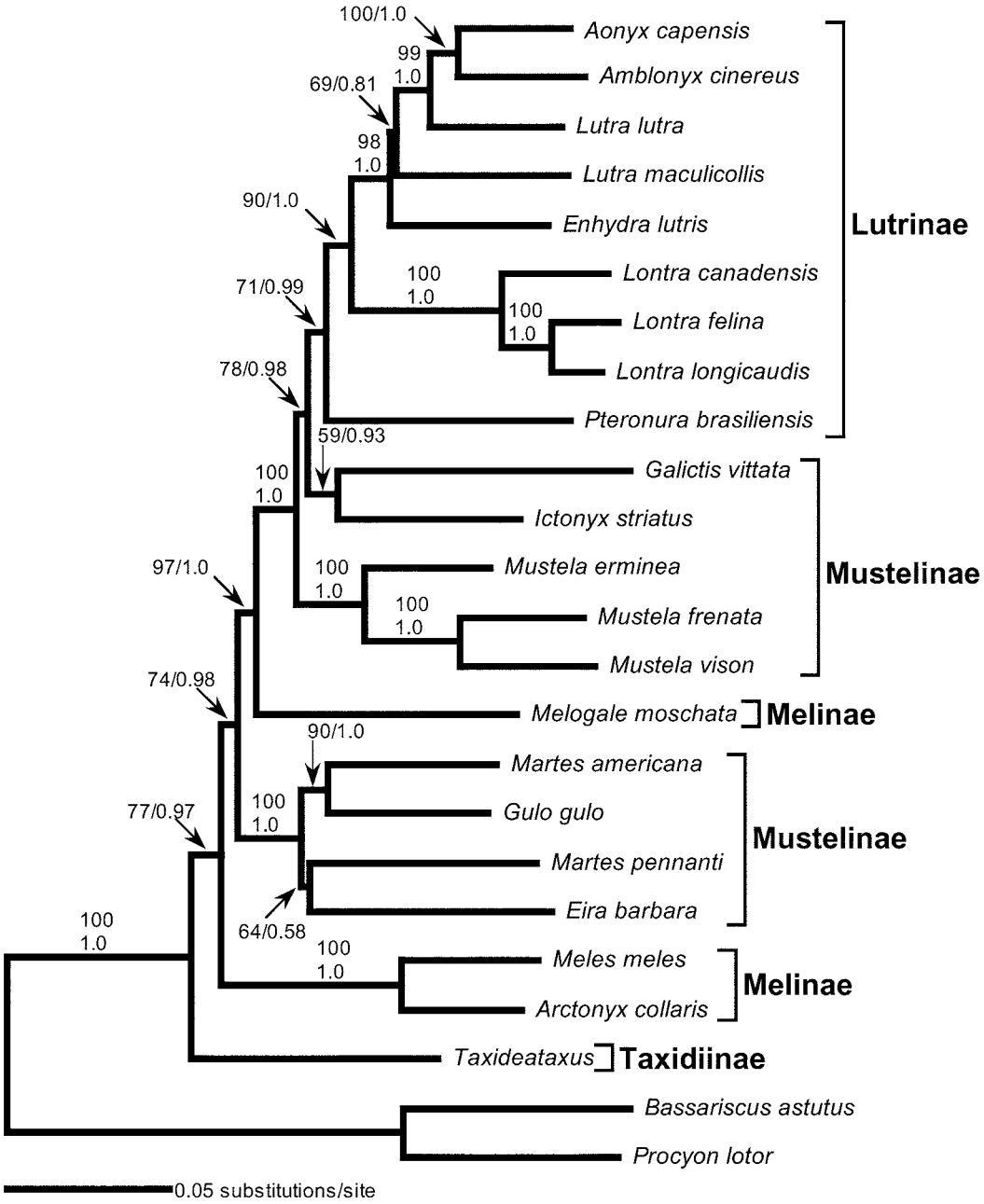

FIGURE 4. ML tree estimated from the total combined data (−lnL = 17642.031). Branch lengths are proportional to the number of substitutions per site (see scale bar). ML bootstrap (out of 100 pseudoreplicates)/Bayesian posterior probability values are shown at the nodes. The substitution model and parameters used in the ML search and estimated from the Bayesian analysis are presented in Table 4. The mean −lnL for the trees derived from the two runs using Bayesian phylogenetic analysis were 17671.426 (95% confidence interval = 17683.120–17661.660) for run 1 and 17671.660 (95% confidence interval = 17684.130–17662.320) for run 2.

nucleotides sampled from multiple loci from different regions of the genome may be more representative of the species phylogeny than nucleotides sampled from a single gene (see also Cao et al., 1994; Meyer, 1994). In fact, Cummings et al. (1995) specifically suggested that STSs were one among a class of markers that could be used for improving the recovery of the species phylogeny compared to contiguous blocks of sites drawn from a single gene.

Overall, our results are consistent with those of other recent studies that have compared the utility and efficacy of mitochondrial and nuclear DNA sequences in phylogeny reconstruction (e.g., Slade et al., 1994; Prychitko and Moore, 2000; Baker et al., 2001; Matthee et al., 2001; Springer et al., 2001). All of these studies reached the same conclusion that nuclear genes (exons and/or introns), especially when combined from different loci, were more informative and showed superior

performance for resolving relationships compared with mitochondrial genes. In all cases, the low amount of homoplasy exhibited by the nuclear genes was the reason given for the greater utility of the nuclear genes compared with the mitochondrial genes. These studies spanned a wide range of taxa and evolutionary divergences, suggesting that these observations are not restricted to certain genes or taxa.

Our incongruence analyses did not detect any significant incongruence among the five nuclear gene fragments (Fig. 1). This result was somewhat unexpected because it has been suggested that nuclear genes are more

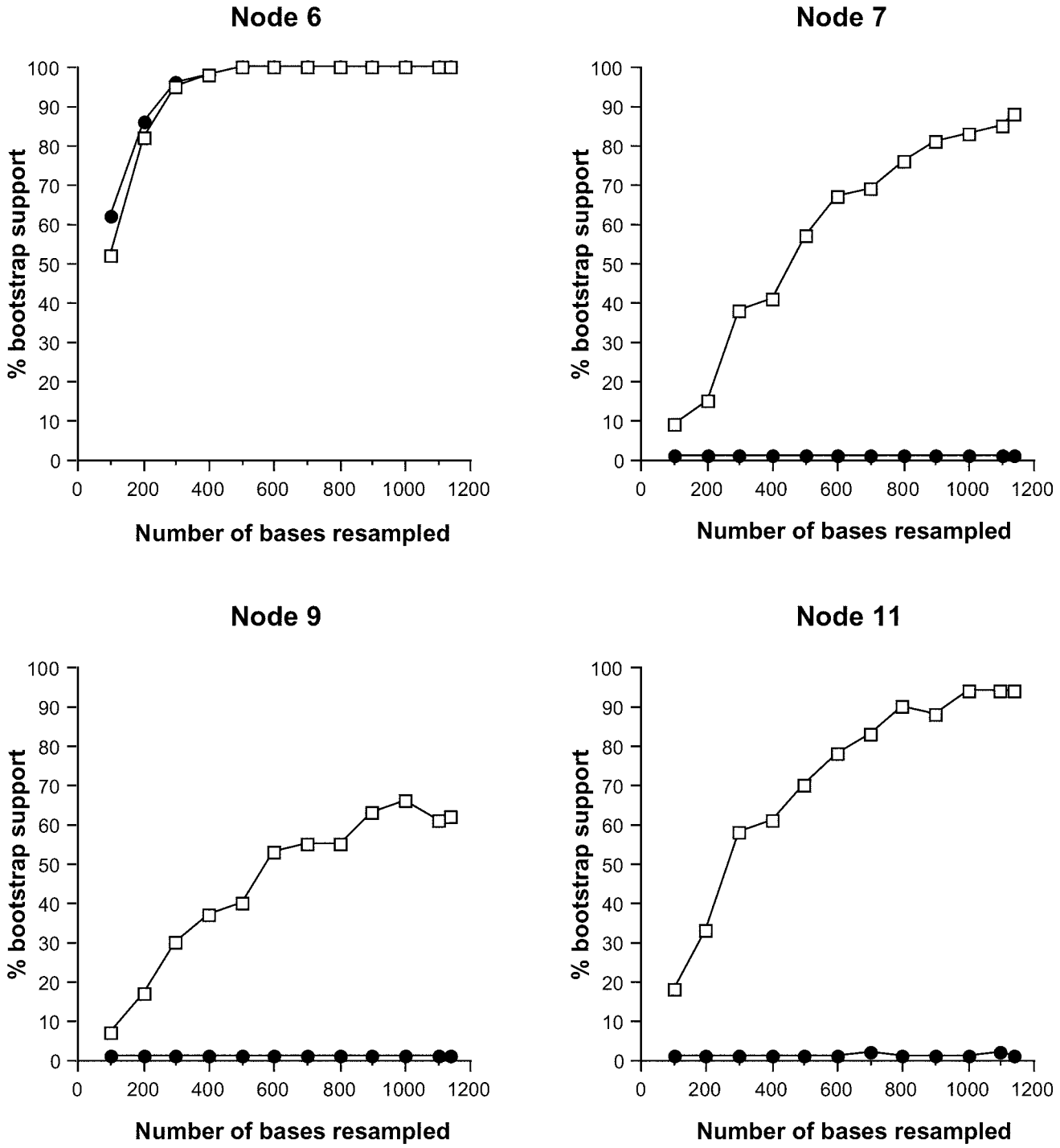

FIGURE 5. Plots of the variable-length BP results for the concatenated nuclear data (open squares) and cyt *b* data (filled circles) for eight nodes found in the differentially weighted MP analyses of the total combined data (Fig. 3B). The *x*-axis is the number of bases resampled, and the *y*-axis is the BP percentage (out of 200 pseudoreplicates). Bases were resampled in 100-bp increments up to 1,140-bp, which corresponded to the total size of the smaller cyt *b* data set. *(Continued)*

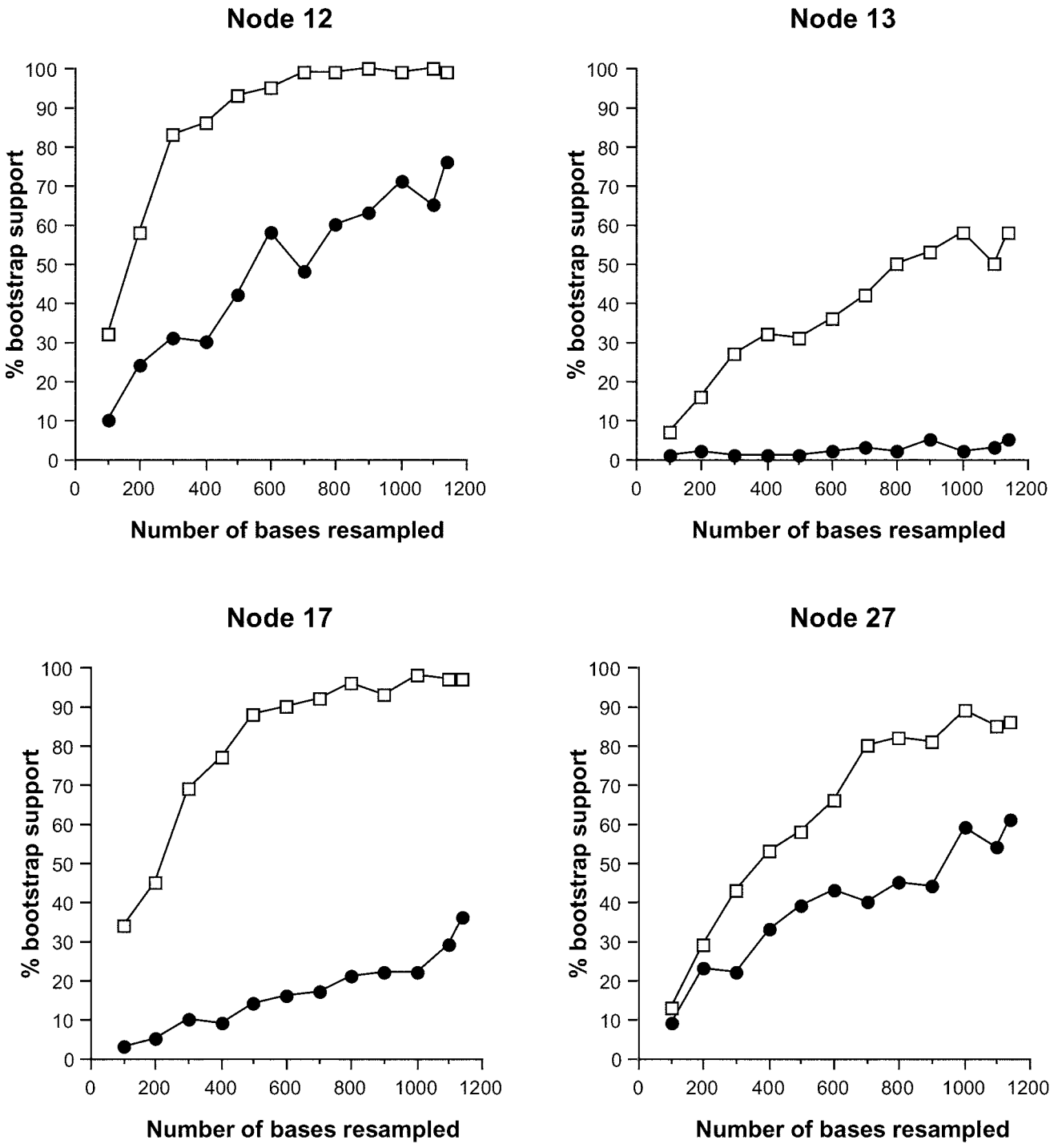

FIGURE 5. (Continued)

likely to be incongruent with one another because of their increased probability of lineage sorting (Moore, 1995). However, our small sample of five nuclear gene fragments does not exclude the possibility that lineage sorting will be found as additional loci are sequenced. We predict that some loci as yet unsequenced will be incongruent with the present trees because the deeper nodes of the combined data trees are short (Figs. 2, 4) and because there is an increased probability of lineage sorting along short nodes (Pamilo and Nei, 1988; Moore, 1995). In fact, the gene trees did differ widely in topology, but none of these differences were supported by high BS or BP values (Fig. 1). In the case of the nuclear genes, this incongruence reflects insufficient informative variation contained by these sequences (Wendel and Doyle, 1998). Support is always strong, however, where the gene trees

show congruence (Fig. 1), suggesting they are all tracking the same phylogenetic history.

Most often, studies that use multiple data partitions to reconstruct phylogenies will test for incongruence and, if none is found, combine their data sets and evaluate the support for nodes with bootstrapping. Because bootstrapping is a cumulative measure of support across the entire data matrix, however, it is not possible to evaluate how different data partitions contribute to the support of a particular node (Baker and DeSalle, 1997; Remsen and DeSalle, 1998). This issue is not trivial because relationships not found in the separate analyses often emerge in the combined analysis, and it is of interest to know how different data partitions interact to produce the structure of the combined tree (Chippendale and Wiens, 1994). The topology of the tree based on the concatenated nuclear data (Fig. 2) was most similar to the topology of the FES tree (Fig. 1). This is not surprising because the PBS analyses indicated that FES contributed the most information to the structure of the combined nuclear tree (Table 6). Furthermore, the PBS analysis of the combined nuclear tree (Fig. 2) clearly indicated that the nuclear loci are not contributing equally to the support of nodes or of the overall tree. While some nodes are supported by all loci, support for others, particularly deeper nodes, is patchy at best (e.g., nodes 12 and 19 versus nodes 2 and 9 in Fig. 2 and Table 6). These differences may reflect the true nature of the branch lengths at the different levels of the phylogeny. When a branch is long, there is a greater chance that more of the genome will reflect the ancestry of two taxa (Maddison, 1995). When a branch is short, however, the signal for shared ancestry in the genome is much more patchily distributed (Pamilo and Nei, 1988; Maddison, 1995). These results are also helpful in deciding which phylogenetic hypotheses require additional data and thus where future sequencing efforts should be directed. For example, it would be helpful to test the current position of *Melogale* (Fig. 2) with additional data from other loci.

The PBS analyses were especially informative with regard to the total combined analyses. In the equally weighted analysis, addition of the cyt *b* sequences causes much of the positive support of the concatenated nuclear sequences to become negative. Furthermore, the resolution and BP/JK support of the equally weighted total combined tree was decreased relative to that of the tree based on the nuclear sequences alone (compare Figs. 2 and 3A). These results stem from the fact that equally weighted MP does not correct for multiple substitutions (Felsenstein, 1978). Because the cyt *b* data contained two to three times more homoplasy than the nuclear data (Table 5), combination of the two data sets biases the total combined analysis towards the cyt *b* data. Reed and Sperling (1999) reported a similar interaction when they combined mitochondrial and nuclear DNA data in their phylogenetic analysis of swallowtail butterflies of the genus *Papilio* (see Johnson and Clayton, 2000, for another example). The larger number of homoplastic characters in the cyt *b* data set overwhelm the few unambiguous synapomorphies at the deeper nodes that are provided by the concatenated nuclear data. This not only causes the average BP/JK percentage to decrease in the equally weighted tree but also results in some anomalous groupings (e.g., the joining of *Pteronura brasiliensis* with *Galictis vittata* + *Ictonyx striatus*). Under such conditions, downweighting more homoplastic characters can improve phylogenetic resolution (Chippendale and Wiens, 1994). Indeed, the PBS of the nuclear gene partition was restored and the true support of the cyt *b* gene was revealed when the characters were downweighted in proportion to the amount of homoplasy they contained (Goloboff, 1993). The weighting is based on a concavity function of the homoplasy of a character and its fit to a tree (i.e., its reliability, Goloboff, 1993) and is implemented during the tree search. Although the implied weights method suffers from being starting-point dependent, we believe that this approach is less drastic than downweighting or excluding an entire class of characters *a priori* (e.g., all third position transitions) that may contribute phylogenetic information to the hierarchy of the tree. Not surprisingly, the ML and Bayesian trees of the total concatenated data were nearly identical to the differentially weighted MP tree. Because both ML and Bayesian methods incorporated the same model of substitution (GTR+I+G in the case of the total concatenated data set; see Table 4) during their searches, they were more resistant to the effects of homoplasy in the cyt *b* data.

The claim is often made that mitochondrial sequences are more useful for resolving recent nodes while nuclear sequences are more appropriate for resolving older nodes (e.g., Johnson and Clayton, 2000; Quattro et al., 2001). Because nuclear genes evolve more slowly, they are postulated to have greater utility at levels of divergence where the signal from mitochondrial sequences has become saturated. The PBS analysis of the total combined data with implied weighting allows us to assess the veracity of this claim in the Mustelidae. Examination of the PBS values in Table 7 indicates that although the amount of support provided by the cyt *b* gene is lower relative to the combined nuclear data, the cyt *b* gene nevertheless contributes some signal at nearly every level of the phylogeny. At three nodes (3, 10, and 13), cyt *b* contributes no support, whereas at one node (7) it contributes negative support. However, in partial agreement with expectations, cyt *b* does seem to provide more support among recent nodes (e.g., nodes 1, 6, 8, and 14). Conversely, the nuclear DNA sequences provide more support at the deeper levels of the phylogeny, but they also contribute to the support at recent nodes (see also Prychitko and Moore, 2000).

Mustelid Systematics

The phylogenies based on the differentially weighted MP and ML/Bayesian analyses (Figs. 3B, 4) provide a new phylogenetic hypothesis for the Mustelidae that can be compared with those derived from earlier studies. Except for the alternative placements of *Enhydra lutris* and *Lutra maculicollis* (see Results), the topologies from the

two analyses are the same. The combined molecular data strongly support the monophyly of the Lutrinae (otters), which agrees with the results of the morphological study by Bryant et al. (1993). In our previous analysis based on cyt *b* alone, we were unable to find strong support for a monophyletic Lutrinae, and spectral analysis suggested the presence of conflicting signal in these data (Koepfli and Wayne, 1998). The main reason for this conflict was the problematic placement of the giant otter, *Pteronura brasiliensis*. These earlier observations are corroborated by our current results. Despite the higher taxonomic sampling, the individual MP and ML trees of the cyt *b* gene join all otter taxa together (but with <50% BP and JK support and a BS value of 1) except *P. brasiliensis* (Fig. 1). *Pteronura* is placed anomalously either basally in the tree (MP) or joined with *Ictonyx* (ML). Furthermore, the PBS analysis confirms that the cyt *b* data contribute negative, and therefore conflicting, support for otter monophyly (Table 7). Indeed, the strong support for otter monophyly is entirely derived from the nuclear DNA sequences and more specifically from four of the five nuclear partitions (Table 6). Our results also support our previous finding (Koepfli and Wayne, 1998) that otters are divided into three primary clades, with *Pteronura* being the most basal lineage within the Lutrinae and *Enhydra* joined to a clade that also includes *Amblonyx*, *Aonyx*, and *Lutra*. Furthermore, our nuclear DNA data corroborates van Zyll de Jong's (1972, 1987) decision based on morphology to separate *Lutra* and *Lontra* into separate clades (Old World and New World river otters, respectively).

The combined molecular data clearly suggest that the Mustelinae is not monophyletic (Figs. 3B, 4), which is consistent with both previous morphological (Bryant et al., 1993) and molecular (Masuda and Yoshida, 1994; Koepfli and Wayne, 1998) analyses. This subfamily contains many genera, several of which are monotypic, and whose relationships have been difficult to decipher. Indeed, Anderson (1989) referred to the Mustelinae as a "catchall" for both fossil and recent taxa. Although we have only sampled six of the nine genera currently classified in the Mustelinae (Wozencraft, 1993), our results separate these six genera into three clades. First, we find a well-supported clade composed of *Eira–Gulo–Martes*, which is also supported by an informative indel from the FES gene segment. Furthermore, *Martes* appears paraphyletic in this clade. Both the nuclear and cyt *b* data strongly support an association between *Martes americana* and *Gulo*, but only the cyt *b* data support the sister relationship between *Martes pennanti* and *Eira* (Figs. 3B, 4; Table 7). The second and third clades consist of the three species of *Mustela* and the sister genera *Galictis* and *Ictonyx*, respectively. The association of *Galictis vittata* and *Ictonyx striatis* is surprising, given that the former taxon is distributed in Central and South America while the latter is restricted to Africa. Nevertheless, this relationship consistently received strong support across all the analyses except with the ML BP (Figs. 3B, 4; Table 7). Our previous study based on cyt *b* did not include *Galictis* or *Ictonyx*, and we therefore inferred that *Mustela* formed the sister group to the lutrines. However, the present study indicates that the *Galictis–Ictonyx* clade is the sister to the lutrines.

As for the badgers, our results imply that the Melinae is not monophyletic. Both individual and combined analyses, as well as the presence of two informative indels at the FES gene, strongly support the sister relationship of *Arctonyx* with *Meles*, which has been recognized by previous researchers based on morphology (Pocock, 1921; Petter, 1971; Long and Killingley, 1983; Wozencraft, 1989; Bryant et al., 1993). Our results place *Arctonyx–Meles* near the base of the mustelid phylogeny, between *Taxidea* and all other mustelids (Figs. 3B, 4). In contrast, Bryant et al. (1993) placed these two taxa in a more derived position as the sister group to the Lutrinae, but with low BP support. The Chinese ferret-badger, *Melogale moschata*, formed a distinct lineage that was sister to the monophyletic group that includes the *Mustela* clade, the *Galictis–Ictonyx* clade, and the lutrine clade. Although support for this placement in the combined analyses was strong, the PBS analyses indicate that this support is derived from only single contributions by two of the nuclear gene segments and none from the cyt *b* gene (Tables 6, 7). Nonetheless, our results are consistent with the observations of Pocock (1921) and Petter (1971), who classified *Melogale* apart from the other badger taxa. However, our combined molecular data contradict the morphological data of Bryant et al. (1993), who placed *Melogale* as the most basal taxon in the Mustelidae. Instead, we find strong support for the placement of *Taxidea* as the outgroup to the remaining mustelids, a position that has never before been suggested. Wozencraft (1989, 1993) placed *Taxidea* into its own subfamily, Taxidiinae, because this badger possesses basicranial features not found in Old World badgers. Our previous study using cyt *b* sequences (Koepfli and Wayne, 1998) included *Meles* and *Taxidea* but not *Arctonyx* or *Melogale*. In that study, *Meles* was placed as the most basal taxon, followed by *Taxidea*, but this topology may have been due to long-branch attraction to the two distantly related outgroups used in the original study (*Bassaricyon* and *Procyon*). In any case, these relationships were associated with low BP values (<60%). The addition of *Arctonyx* in the present study breaks the long branch of *Meles* in the cyt *b* gene trees (Fig. 1) and thereby results in *Taxidea* as the outgroup to the rest of the mustelids. Although we have only sampled 16 of the 20 genera presumed to belong to the Mustelidae (with the three genera of the Mephitinae and *Mydaus* excluded), our combined molecular data provide an initial framework of relationships that can be further tested with sampling from both additional sequence data and additional taxa.

Prospect of Type I STSs for Future Studies

We have shown that nuclear DNA sequences amplified and sequenced using type I STS primers are useful for reconstructing the phylogenetic relationships within the Mustelidae. The combined nuclear sequences had less homoplasy and were more decisive and efficient than were sequences of the cyt *b* gene from the same taxa. Our

study is similar in approach to the study by Slade et al. (1994), which demonstrated the applicability of short nuclear gene sequences amplified with conserved primers to resolve relationships among a small sample of pinniped taxa. Those authors suggested that three conditions must be satisfied for such an approach to be useful: (1) PCR primers must amplify orthologous sequences, (2) the resulting sequences should be easy to align, and (3) the sequences should contain sufficient variation to resolve the phylogenies of the taxa under study. The sequences we obtained with type I STS primers readily satisfy criteria 1 and 2. As for the third criterion, concatenation of the nuclear sequences was able to overcome the low amount of variation we found in the individual nuclear gene partitions (Fig. 2).

Compilations of nuclear genes (<20) applicable to molecular systematic studies of animals have been previously published (Friedlander et al., 1992, 1994; Graybeal, 1994). However, their application has been limited because the level at which the loci were most informative was not intensively investigated and/or only studied in certain taxa. In other cases, primers for single nuclear genes have been published for use in specific taxonomic groups (e.g., birds; Groth and Barrowclough, 1999; Prychitko and Moore, 1997). The development of abundant markers such as type I STSs offers exciting prospects for studies of both molecular evolution and systematics because researchers will gain unprecedented accessibility to the nuclear genomes of a wide array of species. The fact that hundreds of primer pair sequences that span the complete genomes of such model species as human, dog, and mouse have already been published (Venta et al., 1996; Lyons et al., 1997; Jiang et al., 1998) implies that hundreds of independently segregating loci can be used to estimate species trees and provide a better understanding of the temporal spacing between branching events. Although these markers have been recently applied to understanding the higher level relationships among placental mammals (Murphy et al., 2001), they have also been used to support the recognition of two species of African elephants (*Loxodonta*; Roca et al., 2001). Therefore, these sequences can potentially be used to resolve both ancient and recent relationships. Although the primers that have been published thus far were developed from mammalian taxa, they have been shown to amplify in other vertebrate groups as well (Lyons et al., 1997). STS primers provide a way to unlock the rich phylogenetic signal contained in the genomes of many organisms and thereby complement other systematic data.

ACKNOWLEDGMENTS

We thank the individuals and institutions listed in Table 1 for providing the tissue and DNA samples used in this study. In addition, we thank the following individuals who assisted in the provision of samples: Carla Cicero (Museum of Vertebrate Zoology), Joseph Cook and Gordon Jarrell (University of Alaska Museum), Bill Gannon and Terry Yates (Museum of Southwestern Biology), and Nicky Ladkin and Robert J. Baker (Museum of Texas Tech University). We are grateful to Kate Griffiths for providing excellent laboratory assistance in the initial stages of this research. We thank Don Buth, Chris Simon, Jack Sullivan, and two anonymous reviewers for comments and suggestions that improved the manuscript. This work was supported in part by research funds from the Department of Organismic Biology, Ecology and Evolution awarded to K.-P. Koepfli and by the U.S. National Science Foundation.

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First submitted 4 April 2002; reviews returned 9 August 2002; final acceptance 26 April 2003
Associate Editor: Jack Sullivan