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The utility of chemical signals as phylogenetic characters: an example from the Felidae

OLAF R. P. BININDA-EMONDS[1]

Section of Evolution and Ecology, One Shields Avenue, University of California at Davis, Davis, CA 95616, USA.

DENISE M. DECKER-FLUM[2]

Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996, USA

JOHN L. GITTLEMAN[3]

Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22904-4328, USA

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Chemical secretions that are explicitly tied to species recognition may potentially be informative for phylogenetic reconstruction, especially when traditional morphological or molecular characters lack resolution. Anal sac secretions from 16 species within the family Felidae (order Carnivora) were chemically analysed and their utility as phylogenetic characters was assessed. Results were generally consistent across the different chemical data types (e.g. glycolipids, neutral lipids, or phospholipids). Two major clades were indicated, falling out according to body size: one for species greater than 30 kg (*Panthera*, *Uncia*, and *Puma*) and another for those less than 12 kg (remaining species). The primary solutions agreed with respect to the species pairs *Prionailurus*+*Leptailurus*, *Caracal*+*Lynx*, *Oncifelis*+*Leopardus*, *Otocolobus*+*Felis*, *Panthera leo*+*P. pardus*, and *P. tigris*+*Uncia*. The only area of disagreement between chemical types was the positioning of the mountain lion (*Puma concolor*); however, this species appears to cluster with the cheetah (*Acinonyx jubatus*) in the 'big cat' clade. Although our solutions differ from six previously proposed hypotheses of felid phylogeny (morphological and molecular), the previous estimates all differ strongly amongst themselves reflecting the historical uncertainty regarding felid systematics. Phylogenies derived from the lipid data were very robust and decisive. Few equally most parsimonious trees were obtained, consistency indices were much higher than their expected values, and bootstrap and Bremer support values were also high. Thus, our findings illustrate the species-specific nature of chemical signals and their usefulness as phylogenetic characters.

ADDITIONAL KEYWORDS: phylogeny – systematics – Felidae – chemical signals – lipids – evolution.

INTRODUCTION

Phylogeny reconstruction requires measurable, homologous characters that provide an accurate record of evolutionary history. Typically, morphological and molecular characters meet these requirements and thus are most often used in systematics. Recently, however, there has been a resurgence in the use of 'non-traditional' characters (e.g. Brooks & McLennan, 1991; de Queiroz & Wimberger, 1993; Winkler & Sheldon, 1993; McCracken & Sheldon, 1997). At least five reasons underlie this trend. (1) Material used in morphological or molecular studies, such as cranial/postcranial skeletons or tissue/blood, are often unavailable due to rarity of specimens and/or conservation restrictions for endangered taxa. (2) Detailed natural history information is increasingly abundant,

[1] Present address: Institute of Evolutionary and Ecological Sciences, Leiden University, Kaiserstraat 63, PO Box 9516, 2300 RA Leiden, The Netherlands.
[2] Present address: Biology Department, Southern State Community College, 100 Hobart Drive, Hillsboro, OH 45133, USA.
[3] Corresponding author: E-mail: JLGittleman@virginia.edu

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providing more opportunities to investigate phylogenetic patterns in other kinds of traits. (3) Accumulating evidence suggests that non-traditional characters such as behavioral or ecological traits yield useful information about evolutionary pattern in addition to historical reconstruction (see below). (4) When phylogenies based on molecular and morphological data disagree, other characters often resolve incompatibilities. (5) Last, but perhaps most important, a widespread consensus exists that the best phylogenetic hypothesis is the one supported by the most independent lines of evidence (Mickevich, 1978; Farris, 1983; Penny & Hendy, 1986; Kluge, 1989; Novacek, 1992; de Jong, 1998).

Studies across diverse taxa indeed show that behavioral and ecological characters may be as informative as morphological characters (e.g. de Queiroz & Wimberger, 1993; Gittleman & Decker, 1994) though this will depend to some extent on the taxa and specific characters involved (see Gittleman *et al.*, 1996). Unfortunately, discussions of character selection have been either/or in kind: a character is useful for phylogeny reconstruction because it can be shown to be a derived feature uniquely shared by some species or it should be discarded because it is homoplastic (e.g. convergent or has been secondarily lost in some species). Characters with an obvious functional component have often been purposely ignored in phylogenetics because of the dual assumption that the limited number of solutions to a similar selection pressure obscures or even provides a false estimate of evolutionary history (for a discussion, see Wyss, 1989; Proctor, 1996). However, what is a homoplasy at one level may serve as a useful homology at another, more inclusive one. Comparative morphology is rich with examples: long legs within different groups of carnivorous mammals reflect increased running capacity and systematic placement; enlarged lung capacity is associated with both flying and being a bird; flippers are common to aquatic mammals for locomotion, but are sufficiently different morphologically to define the major groups. Early ethologists recognized the phylogenetic utility of some functional characters. Lorenz (1941, 1971) and Tinbergen (1951) focused on display behaviours because such characters were informative for both systematic and functional study. The advent of molecular systematics now raises a new but parallel question: are there phenotypic characters derived from genetic homologues that reflect *both* functional and phylogenetic information?

The present paper is a phylogenetic analysis of biochemical (lipid) compounds in the scent glands of 16 species within the cat family (Felidae). We find chemical signals to be useful phylogenetic characters. We suggest that the biochemistry of scent in felids contains both functional and phylogenetic information, an idea first proposed for scent gland lipids by Kluge (1989). Also, because they are distinct from traditional morphological and molecular characters, chemical signals serve as useful *independent* characters in verifying phylogenies.

FELID PHYLOGENY AND CHEMICAL SIGNALS

Despite intensive systematic study, there remains considerable debate about relationships within the Felidae. The crown group containing all extant species evolved rapidly in the last 16 million years (Bininda-Emonds, Gittleman & Purvis, 1999), resulting in relative morphological uniformity (e.g. see Werdelin, 1983; Van Valkenburgh, 1989) compared to other carnivore families (Radinsky, 1981); most phenotypic variation among felids relates to differences in body size (Ewer, 1973; Gittleman, 1985). Uncertainty regarding felid phylogeny is reflected in the contentious taxonomy of the family, where as few as two genera (the cheetah, *Acinonyx jubatus*, versus all other species lumped under *Felis*) to as many as 19 have been recognized historically (Kitchener, 1991). To facilitate comparisons, we standardized the generic nomenclature according to Wozencraft (1993) and that of higher level groups according to O'Brien *et al.* (1996).

Early phylogenetic studies used a wide range of characters, including skin pattern and colour (Pocock, 1917; Weigel, 1956), tongue morphology (Sonntag, 1923), structure of the anterior portion of the zygomatic arch (Lonnberg, 1926), and overall morphological structure (Haltenorth, 1936). More recent studies have used chromosomal data (Roubin, De Gouchy & Klein, 1973; Wurster-Hill & Gray, 1973, 1975; Benirschke, Edwards & Low, 1974; Wurster-Hill, 1974; Soderlund *et al.*, 1980), dental features (Glass & Martin, 1978), physiology (Hemmer, 1976), and various types of DNA sequence data (Wayne *et al.*, 1989; Janczewski *et al.*, 1992, 1995; Masuda *et al.*, 1994; Pecon Slattery *et al.*, 1994). On the whole, these characters have been mainly useful at generic levels and have not provided sufficient resolution at more inclusive levels.

Six competing hypotheses (see Fig. 1; trees include only taxa examined in this study) exist for phylogenetic relationships across all Felidae: Hemmer (1978), Herrington (1986), Salles (1992), two from O'Brien *et al.* (1996; includes the important studies of Collier & O'Brien (1985) and Janczewski *et al.* (1995)), and Bininda-Emonds *et al.* (1999). The six hypotheses, hereafter referred to as the 'rivals' (following Mason-Gamer & Kellogg, 1996), differ in character selection, whether (and with what taxa) outgroup comparisons were made, and methods of phylogenetic analysis. The phylogenies of Hemmer (1978), Herrington (1986), and Salles (1992) are based on morphological features,

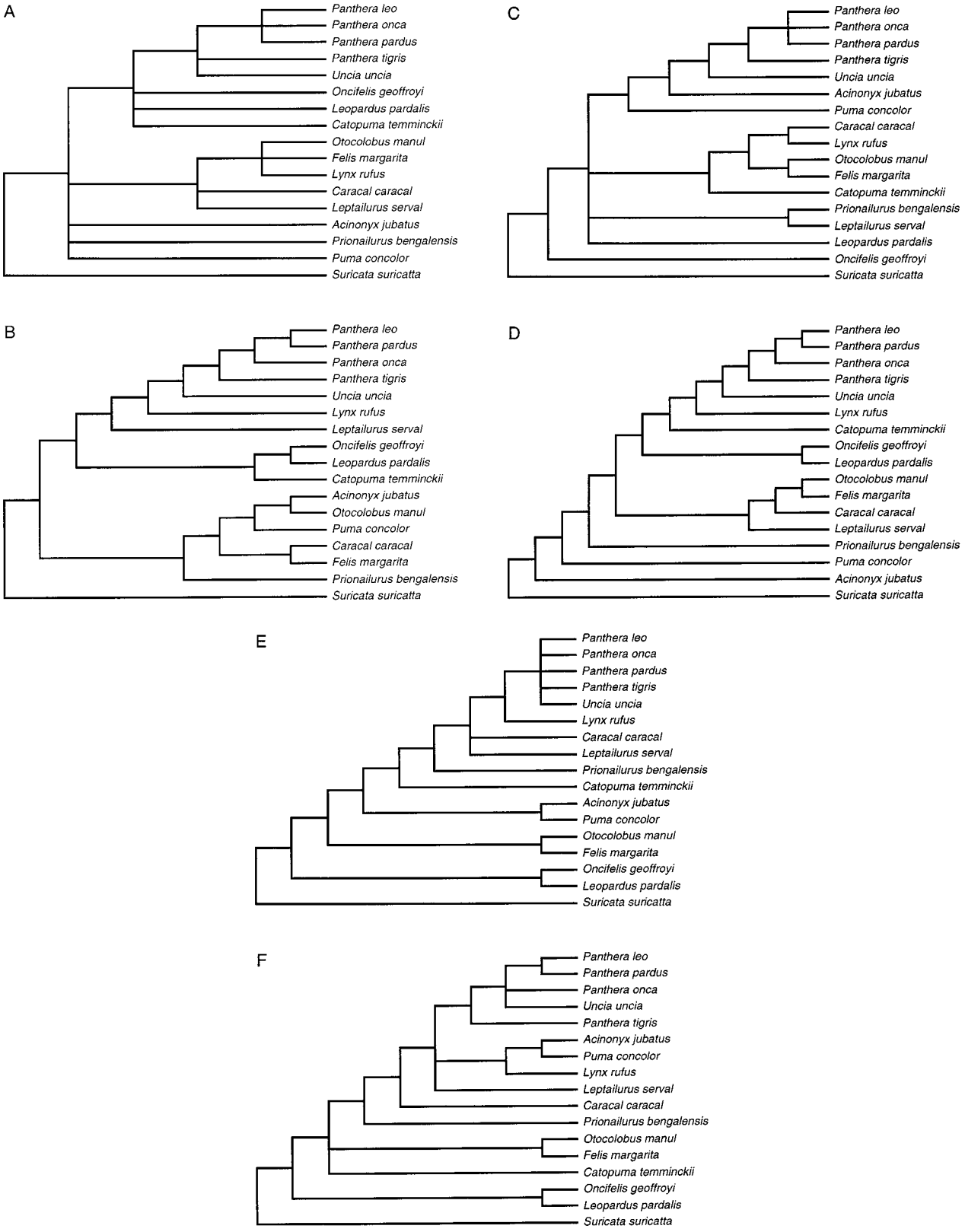

Figure 1. Major hypotheses of felid phylogeny: (A) Hemmer (1978), (B) Herrington (1986), (C) Salles (1992), (D) Bininda-Emonds *et al.* (1999), (E) O'Brien *et al.* (1996: figure 3.1), and (F) O'Brien *et al.* (1996: figure 3.2). The trees have been pruned to include only those taxa examined in this study.

primarily skull and dental characters. Hemmer (1978) also used subjective views of biogeography and, unlike the other two studies, did not use an explicit methodology to produce a tree. The remaining three hypotheses represent consensus phylogenies. The two trees from O'Brien *et al.* (1996) are based on a combination of immunological distances, isozyme electrophoresis, karyology, endogenous retroviruses, and a partial sequence analysis of the mitochondrial genes 12S rDNA and cytochrome *b*; they differ mostly in resolution. Bininda-Emonds *et al.* (1999) present a MRP supertree (*sensu* Baum, 1992; Ragan, 1992; Sanderson, Purvis & Henze, 1998) that combined 38 previously published hypotheses of felid phylogeny derived from morphological, molecular, and behavioural data.

Together, the six hypotheses highlight the major phylogenetic problems we examine here: (1) persistent unresolved nodes within the *Panthera* genus of O'Brien *et al.* (1996); (2) morphological data placing the putative pantherine cat *Catopuma temmincki* outside of the pantherine lineage in contrast to the molecular data; (3) putative monophyly between the cheetah (*Acinonyx jubatus*) and mountain lion (*Puma concolor*); and (4) well-supported major lineages of uncertain relationship to one another.

To try to resolve these relationships, we use a new source of characters. Chemical signals have been used in taxonomic studies of insects (e.g. Buschinger, 1975; Vane-Wright, Schultz & Boppre, 1992) and snakes (e.g. Tolson, 1987; Kluge, 1989), but have been little used in mammalian systematics. This is surprising given the large literature on the evolution and function of mammalian scent-marking. Chemical signals are secreted from the scent sacs of mammals and used to communicate among individuals within a species. How mammals encode chemical signals, either intra- or interspecifically, is unclear (see Natynczuk & Macdonald, 1994). However, relative to other communicatory mechanisms (e.g. visual, auditory, physical), it seems likely that chemoreception of scent requires quite sophisticated (and temporally constant; Schaal & Porter, 1991) perceptual and cognitive structures that would enhance species-specific rather than interspecific qualities (Albone, 1984).

Among carnivores, felids exude a lot of chemical signals from numerous glands (e.g. anal, facial, interdigital, supracaudal) as well as in their urine and faeces (Macdonald, 1985). Chemical signals of lipids show considerable variation across the Felidae and may possess both functional and systematic value. Descriptive studies have shown that, in general, scent-marking in felids is species-specific with regard to the exudate source, target of scent, and contextual function such as individual or sexual recognition (Asa, 1993; Leyhausen, 1979; Mellen, 1993; Wemmer & Scow, 1977). In this paper, we apply phylogenetic methodology to chemical signals among species within the Felidae to examine the phylogenetic utility of chemical signals and whether they may serve as independent characters for testing rival morphological and molecular phylogenies.

METHODS

CHEMICAL SECRETIONS

Collection of samples

Details of the following methodology for collection and chemical analysis of scent secretions are described in Decker, Ringelberg & White (1992). Anal sac secretions were collected from individuals of 16 felid species housed in zoos primarily in the United States (see Table 1). Samples were taken by curators or veterinarians when individual animals were anaesthetized for medical purposes. Because of the opportunistic nature of data collection, sample sizes are uneven across species; sample size, however, did not effect quantity or quality of chemical constituents represented in each species (Decker, 1996). Secretions were gathered with a sterile cotton swab by either swabbing the internal surface or, after squeezing, the external surface of the anal sac. Based on studies of another carnivore species, the domestic ferret (*Mustela putorius*: Crump, 1980), we assume that samples of scent secretions collected from captive individuals resemble those in natural populations.

Collected secretions were immediately inserted into a Teflon-lined screw cap test tube and placed on dry ice. A control swab was collected at the same time by waving a sterile cotton swab in the air. Samples were transported to the Center for Environmental Biotechnology, The University of Tennessee, for chemical analyses. The procedure for transporting samples is effective for retaining the heavier lipids (C12–C30), which are of principle interest in this study (Decker *et al.*, 1992).

Chemical extraction

All chemical analyses follow the methods of White *et al.* (1979); further details are outlined in Decker *et al.* (1992) for applying these methods to detect specific and numerous compounds in the anal sacs of various carnivore species. Each secretion sample was extracted for a minimum of 4 h with a modified Bligh and Dyer solvent system, consisting of chloroform, methanol, and a phosphate buffer in a ratio of 1:2:0.8. Equal amounts of water and chloroform were then added to the samples, separating them into aqueous and organic phases. The upper aqueous layer was discarded and

Table 1. List of species studied, sample sizes of collected secretions, and source of sample

Species	Common name	Sample size	Zoo
Acinonyx jubatus	Cheetah	9	Binder Park Zoo, Louisville Zoo, Sacramento Zoo, San Antonio Zoo, Toledo Zoo
Caracal caracal	Caracal	4	Central Florida Zoo, Dallas Zoo, San Antonio Zoo
Catopuma temminckii	Asiatic golden cat	1	San Antonio Zoo
Felis margarita	Sand cat	1	Granby Zoo
Leopardus pardalis	Ocelot	3	Cheyenne Mt. Zoo
Leptailurus serval	Serval	2	Sacramento Zoo, Santa Ana Zoo
Lynx rufus	Bobcat	1	San Francisco Zoo
Oncifelis geoffroyi	Geoffroy's cat	2	Bronx Zoo, Sacramento Zoo
Otocologus manul	Pallas' cat	1	Bronx Zoo
Panthera leo	Lion	32	Dallas Zoo, Detroit Zoo, John Ball Zoo, Kings Dominion, Louisville Zoo, Oakland Zoo, Riverbanks Zoo, San Francisco Zoo, Seneca Park Zoo, Topeka Zoo, Zoo Atlanta
Panthera onca	Jaguar	3	Sacramento Zoo
Panthera pardus	Leopard	5	Knoxville Zoo, Lowry Park Zoo, Miller Park Zoo, Toledo Zoo
Panthera tigris	Tiger	22	Bronx Zoo, Central Florida Zoo, Cheyenne Mt. Zoo, Dallas Zoo, Detroit Zoo, Knoxville Zoo, Lowry Park Zoo, Miller Park Zoo, Pittsburgh Zoo, San Francisco Zoo, Seneca Park Zoo, Sunset Zoo, Toledo Zoo, Topeka Zoo, Zoo Atlanta
Prionailurus bengalensis	Bengal cat	2	Bronx Zoo
Puma concolor	Mountain lion	3	Central Florida Zoo, Lowry Park Zoo, Topeka Zoo
Uncia uncia	Snow leopard	12	Dallas Zoo, Lake Superior Zoo, Miller Park Zoo, Sacramento Zoo, San Francisco Zoo, Toledo Zoo, Tulsa Zoo

the lower organic (lipid containing) phase was transferred to Teflon-lined screw cap test tubes and dried under a stream of nitrogen at room temperature.

Lipid separation and methylation

Silicic acid columns were prepared using 0.5 g Unisil (100–200 mesh, Clarkson Chemical Co., Inc., Williamsport, PA), activated at 100°C for 60 minutes and pre-extracted with chloroform. The total lipid (i.e. the entire organic layer of the neutral, glyco-, and phospholipids) was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 5 mL of chloroform, acetone, and methanol eluted the neutral, glyco-, and phospholipids. Each fraction was then dried under a stream of nitrogen. A mild alkaline methanolysis procedure was utilized to prepare methyl esters of the ester-linked fatty acids of each lipid fraction.

Gas chromatography (GC)

Dry methyl esters of neutral, glyco- and phospholipids were dissolved in iso-octane containing the internal standard of methyl nonadecanoate. Samples of 1 μL were injected onto a 50 m nonpolar, cross-linked methyl silicone fused silica capillary column (0.2 mm i.d., Hewlett Packard) in a Shimadzu GC-9A GC. A 30 s splitless injection at 270°C was used. Hydrogen, at a linear velocity of 35 cm/s, was the carrier gas with a temperature program starting at 100°C. The temperature was then increased at a rate of 10°C/min to 150°C. At 150°C, the temperature was increased 3°C every 5 minutes until the temperature peaked at 282°C. An equal detector response was assumed for all components. Peak areas were quantified with a programmable laboratory data system (Nelson Analytical 3000 Series Chromatography Data System, Revision 3.6). Tentative component identification prior to GC/MS was based on

comparison of the retention time data or co-elution with authentic standards.

Mass spectrometry (MS)

GC/MS analysis was performed on a Hewlett Packard 5996A GC/MS fitted with a direct capillary inlet utilizing the same chromatographic system as above except for the use of a helium carrier gas and the temperature program, which started at 100°C and increased to 280°C at 3°C/min for a total analysis time of 60 min. The electron multiplier voltage was between 1800 and 2000 V, the transfer line was maintained at 300°C, the source at 280°C and analyzer at 250°C, and the GC/MS was autotuned with DFTPP (decafluorotriphenylphosphine) at m/z 502 with an ionization energy of 70 eV. The data were acquired using the Hewlett Packard 6/VM data system. Using the Mass Spectrometry database, we had an 80% or greater success rate in specifically identifying a compound's structure (Decker, 1996).

PHYLOGENETIC ANALYSIS

Chemical compounds were coded as discrete presence/absence characters for each of glycolipids, neutral lipids, and phospholipids. The complete data matrix for each chemical includes over 400 compounds for each of 16 species (plus an outgroup species). Complete matrices are available in either Decker (1996) or online on TreeBASE (study accession number S513; matrix accession numbers M743-M745; http://www.herbaria.harvard.edu/treebase/).

Polarization of the characters (i.e. determining the primitive condition) was problematic. Phylogenetic relationships among families of the Feliformia (Felidae, Hyaenidae, Herpestidae, and Viverridae) are controversial (Flynn, Neff & Tedford, 1988; Wozencraft, 1989; Flynn, 1996; Bininda-Emonds *et al.*, 1999), making selection of an outgroup difficult (Maddison, Donoghue & Maddison, 1986). Indeed, as reflected in existing felid phylogenies, no consistent outgroups have been employed to date. Despite these potential problems, we used the meerkat, *Suricata suricatta* (Herpestidae), as an outgroup to root the tree. If high weight lipid compounds are species or taxon specific (Decker *et al.*, 1992; Decker, 1996), then it seems reasonable that those lipid compounds should be absent ancestrally for that taxon. Thus, it is encouraging that *Suricata* lacked all the putative felid-specific compounds. Also, outgroup analysis merely provides an *estimate* of the ancestral state. In any outgroup analysis, derived characters possessed by the outgroup will decrease the accuracy of this estimate, an error minimized by selecting an outgroup as closely related to the ingroup as possible. Admittedly, *Suricata* is reasonably distantly related to felids, but given the taxon-specific nature of the characters, any lipid compounds it shares with felids are likely to be universal among carnivores and therefore uninformative within felids (barring secondary losses which would be informative).

Hierarchically structured data sets possess two characteristics: (1) the optimal solution is shorter than those produced by random permutations of the character states, and (2) the distribution of all tree lengths is negatively skewed. Given the novel nature of our data source, it was desirable to test for both properties. However, processes other than evolutionary descent with modification can produce hierarchical structure (e.g. character non-independence). Therefore, the lack of either property is more informative than its presence (see Alroy, 1994; Bininda-Emonds & Russell, 1996). We tested our data for both characteristics using the permutation tail probability test (PTP; Faith & Cranston, 1991) and examining skewness statistics (with critical values from Hillis & Huelsenbeck, 1992), respectively, using PAUP* 4.0b2 (Swofford, 1999).

Phylogenetic analysis used PAUP*. We determined optimal solutions for each primary data matrix using branch-and-bound searching (thereby guaranteeing an optimal solution) under a parsimony criterion. Signal strength for each solution was summarized using the goodness-of-fit statistics CI, RC, and RI (see Farris, 1989), bootstrap frequencies (Felsenstein, 1985), and Bremer decay indices (Bremer, 1988; Källersjö *et al.*, 1992). The latter measure reveals how much less parsimonious a solution must be before a group of interest (e.g. a monophyletic *Panthera*) is contradicted. The longer a group persists uncontradicted in trees of increasing length, the more confident we can be in it. Bootstrap frequencies were determined from 1000 replicates using a branch-and-bound search algorithm.

Comparisons among phylogenetic hypotheses

Incongruence between competing phylogenetic hypotheses can be assessed with and without taking the underlying data into account. The simplest procedure involves comparing tree topologies only using a tree comparison metric. Of the metrics available, we used the partition metric (d_S; Penny & Hendy, 1985), which reveals the number of clades found in one tree or the other, but not both. Therefore, it treats polytomies as being real ('hard'; Maddison, 1989): for two trees to be identical, even the polytomies must be the same. Like other simple tree comparison metrics, the partition metric reveals only the absolute difference between two solutions; it does not statistically test the null hypothesis that the solutions are different. To assess the latter, we employed two different tests, each of slightly different properties.

First, we tested the significance of incongruence

between the primary chemical data matrices used in this study using the incongruence length difference test (ILD; Farris *et al.*, 1994), implemented in PAUP* as the partition homogeneity test. We did not use the ILD test with the rival hypotheses because, except for Salles (1992) and Bininda-Emonds *et al.* (1999), the underlying data matrices were unavailable. ILD tests were based on 1000 replicates using a branch-and-bound search algorithm. Based on the results of these tests, we pooled congruent primary data matrices (glycolipids + phospholipids: 'glycophospholipids'; see Results) and conducted analogous phylogenetic analyses as for each of the primary data sets.

To circumvent the lack of data matrices for the rival hypotheses, we employed the Kishino–Hasegawa test in PAUP* (see Kishino & Hasegawa, 1989). The Kishino–Hasegawa test allows the comparison of competing tree topologies in the context of an underlying data matrix (the primary matrices here). Methodologically, the test operates by estimating the variance across characters of the difference in length among topologies. The variance then forms the basis for a paired *t*-test. In essence, the Kishino–Hasegawa test examines whether a data matrix is able to accommodate a competing topology, either because the competing topology is very similar (small length difference) to the optimal one or because the signal possessed by the underlying data are not very decisive (high variances). Ideally, one would perform reciprocal tests using each competing data matrix; however, the unavailability of most of the rival data matrices did not allow this.

RESULTS

Characteristics of the data

The specific higher molecular weight (C12–C30) lipids examined in this study were restricted to the felid species only; all compounds were absent in *Suricata*. Chemical signals were also constant within a species (i.e., a chemical was not absent in one individual but present in another) despite large qualitative differences across species (Decker *et al.*, 1992; Decker, 1996). Possible effects of diet, time of sampling, seasonality or other factors were not controlled for; however, given that no intraspecific variation was observed in qualitative differences for each compound (among individuals, not compounds), it is unlikely that these factors would obscure phylogenetic patterns.

All three primary matrices had over 400 characters, of which roughly 70% were phylogenetically informative (Table 2). A similar proportion of informative characters occurred in the combined matrices as well (see below). All matrices in this study contained significant hierarchical clustering information as revealed by highly significant PTP values and skewness statistics (Table 3).

Phylogenies of chemical signals

Analyses of glycolipids, neutral lipids, phospholipids, and two combined data sets each produced at most only three equally most parsimonious solutions (Table 2, Fig. 2). Decisiveness in the data is further substantiated by the high values of the goodness-of-fit statistics, indicating relatively low levels of homoplasy. In particular, CIs were noticeably higher than the value expected for studies with the same number of taxa (derived from Sanderson & Donoghue, 1989). Support for the different solutions was strong (Fig. 2). Bootstrap values for most nodes are in excess of 80%, particularly for the tree obtained when all three primary matrices were combined. Bremer decay indices are likewise high and reflect bootstrap values quite closely.

All solutions indicate two major clades within felids. Interestingly, despite the lack of any obvious allometric correlations in the lipid data (see Decker, 1996), membership in the clades is associated with body size. Species weighing more than 30 kg comprise one clade (*Acinonyx*, *Panthera*, *Uncia*, and usually *Puma*), while those weighing less than 12 kg comprise the other (remaining species). We loosely refer to these hereafter as the 'big cat' and 'small cat' clades, respectively. Beyond this, the primary solutions agree only with respect to several species pairs: *Prionailurus* + *Leptailurus*, *Caracal* + *Lynx*, *Oncifelis* + *Leopardus*, *Otocolobus* + *Felis*, *Panthera leo* + *P. pardus*, and *P. tigris* + *Uncia*. However, a majority rule consensus tree reveals more structure deeper in the tree (Fig. 3).

The main source of disagreement among the primary data sets is the unstable placement of the mountain lion, *Puma concolor*. For glycolipids (Fig. 2A), *Puma* clustered equally parsimoniously at three positions at or near the base of the 'small cat' clade. The remaining data sets placed *Puma* with the remaining 'big cats', either with the cheetah, *Acinonyx jubatus*, to form the sister group to the remaining species (phospholipids; Fig. 2C) or within *Panthera* + *Uncia*, possibly including *Acinonyx* with it in one of the two equally most parsimonious solutions (neutral lipids; Fig. 2B).

The ILD test revealed that only the glyco- and phospholipid data matrices were not significantly incongruent ($P = 0.135$; all other combinations had $P = 0.001$) despite the similar topologies all three primary matrices produced. In fact, the topologies produced by the glyco- and neutral lipid data sets are absolutely more similar than either are to the phospholipids as measured by the partition metric (Table 5). Thus, although all primary matrices possess strong, unequivocal signal, this appears especially true of the neutral lipids and less so of the phospholipids, as

Table 2. Summary statistics for the primary and combined data sets and resultant trees. MPT = number of equally most parsimonious solutions. Expected CI values were derived from Sanderson and Donoghue (1989)

Data set	Total characters	Informative characters	Taxa	MPT	Length	CI	Expected CI	RI	RC
Glycolipids	407	289 (71.0%)	17	3	519	0.775	0.588	0.780	0.604
Neutral lipids	477	349 (73.2%)	17	2	619	0.753	0.588	0.814	0.613
Phospholipids	432	290 (67.1%)	16	1	563	0.758	0.603	0.773	0.587
'Glycophospholipids'	839	579 (69.0%)	17	1	1092	0.759	0.588	0.768	0.583
All three	1316	928 (70.5%)	17	1	1741	0.744	0.588	0.772	0.574

Table 3. Examining for hierarchical structure within the lipid data sets. PTP shows the number of replicates out of 1000 producing a solution as short or shorter than the original data matrix and is equivalent to a *P* value. Skewness statistics were derived from a random sample of 1 million trees with critical values obtained from Hillis and Huelsenbeck (1992) for 25 taxa and 500 binary characters

Data set	PTP	Skewness statistics g_1	Critical value
Glycolipids	0.001	−1.059	−0.08
Neutral lipids	0.001	−0.764	−0.08
Phospholipids	0.001	−0.826	−0.08
'Glycophospholipids'	0.001	−0.896	−0.08
All three	0.001	−0.823	−0.08

witnessed by their respectively slightly higher and lower values for RI and RC (Table 2). The Kishino–Hasegawa test supports these observations (Table 4). Only the phospholipid data matrix is able to accommodate an alternative topology, that of the glycolipids. For the neutral lipids, *P* values were less than 0.0001 in every case.

Combining the glyco- and phospholipid matrices yielded a tree with an identical topology to the glycolipid tree except that *Puma* groups with *Acinonyx* (Fig. 2D). When all three matrices were combined, the resultant tree was identical with that for glycolipids for the small cat clade and with the neutral lipids for the big cat clade except that *Puma* and *Acinonyx* again formed sister species (Fig. 2E). In both cases, combining the data had little or no effect on support or goodness-of-fit values (Table 2). The topologies for the combined matrices were generally absolutely closer to those of the primary matrices than the latter were amongst themselves (Table 5). Predictably, the Kishino–Hasegawa test revealed that the primary data matrices were able to accommodate only those combined topologies into which they had some input (Table 4). The same was generally true in reverse: the combined matrices produced trees that were not significantly different from the trees of their constituent primary matrices.

Very few trends were apparent in the individual chemical data. With respect to the total evidence tree (Fig. 2E), there was no significant difference between the primary compounds in terms of the number of changes each contributed to any branch (for both ACCTRAN and DELTRAN optimizations). There was also no difference for each compound in the number of changes along a branch as a function of the depth of that branch. That is, all three compounds were changing homogeneously across the entire tree. Furthermore, changes along a given branch or in different parts of the tree were apparently random with respect to the molecular weight of the compound. While a significantly positive relationship exists between the fit of a compound to the tree (as measured by CI or RI) and its molecular weight ($P<0.0001$), the relationship is quite weak with a high amount of scatter ($r^2<0.100$). Finally, the big cat clade possesses slightly longer branch lengths on average than the small cat clade, although there is no apparent biological explanation for this observation. Altogether, we interpret these findings to mean that lipid compounds provide a reasonably unbiased source of information for phylogenetic inference.

CONGRUENCE WITH OTHER PHYLOGENIES

The rival phylogenies display little common structure (Figs 1 and 4). Most pairs of phylogenies share fewer than 50% of their clades in common as measured by the partition metric (Table 5). At best, only the clades of *Panthera* + *Uncia* (and *P. leo* + *P. onca* + *P. pardus* within that), *Oncifelis* + *Leopardus*, and *Otocolobus* + *Felis* are supported by more than half of the six phylogenies. These same clades were also found using our data, which strengthens our confidence in their existence.

The rival solutions were very different from those produced by both the primary and combined chemical data sets. Again, any pair of trees that we compared had 50% or fewer of their clades in common; differences

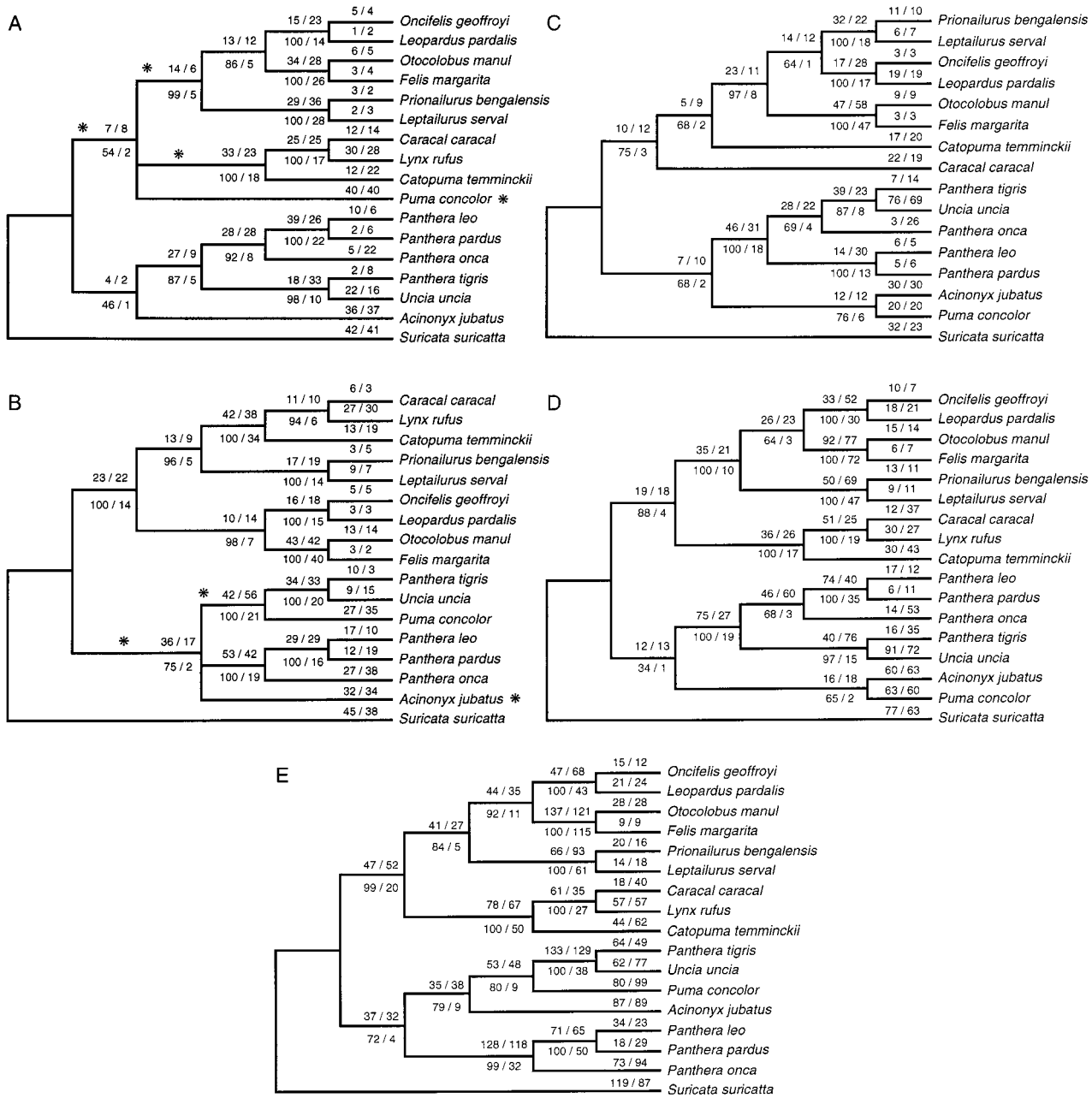

Figure 2. Phylogenetic trees derived from the analysis of (A) glycolipids, (B) neutral lipids, (C) phospholipids, (D) 'glycophospholipids', and (E) all three primary data sets simultaneously. Bootstrap frequencies (1000 replicates) and Bremer decay indices are found above and below each node respectively. (A) and (B) are strict consensus trees with asterisks marking the alternative placements for *Puma* and *Acinonyx*, respectively, among the equally most parsimonious solutions.

often reached as high as 70% or more (Table 5). Results from the Kishino–Hasegawa test, which accounts for the signal within the chemical data sets, were similar. All the chemical matrices produced solutions that were significantly different from the rival topologies ($P<0.0001$ in every case; see Table 4). From both the partition metric and examining the *t*-values from the Kishino–Hasegawa test, the topology of Salles (1992) was usually the closest to those from our chemical data (although still a very poor fit), while that of Herrington (1986) was always the furthest. Much of the congruence with Salles (1992) could be because of the same recognized pattern of a 'big cat' clade (the 'small cat' clade is paraphyletic, however). Otherwise,

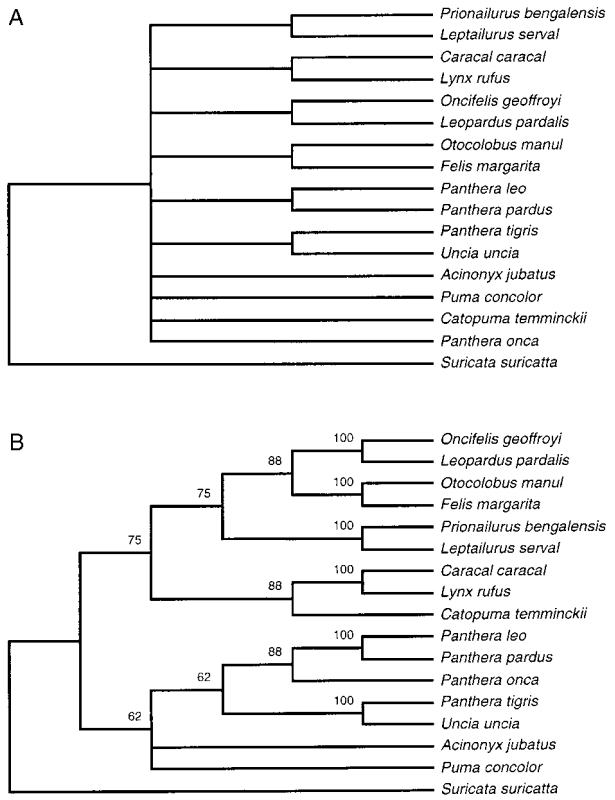

Figure 3. Strict (A) and 50% majority rule (B) consensus solutions for the trees in Figure 2. For the phospholipids, we assumed that *Lynx*, which we did not have data for, would cluster with *Caracal* as in all the other trees.

none of the rival topologies present analogous big or small cat clades as were found here.

DISCUSSION

Systematic characters

Our analysis demonstrates the conservative, species-specific nature of chemical signals, that they are possibly not as malleable to environmental conditions as traditionally regarded, and that these signals may be informative phylogenetically. Chemical compounds appear to contain both qualitative differences (presence/absence) among species that reflect phylogenetic history along with quantitative variation (amount of each chemical) that may be subject to environmental effects. In terms of what types of data are useful for phylogenetic analysis, we agree with the growing trend that a wide variety of characters will best illuminate phylogenetic relationships (Brooks & McLennan, 1991).

However, with more studies using 'non-traditional' characters for analysis, some may be more useful than others (e.g. Lee, Clayton & Griffiths, 1996). Among behavioral characters, we suggest that those relating to communicatory function, especially in reproductive contexts linked to species recognition, are likely to have greater phylogenetic signal than those that show more individual variability and less species-specific qualities (McCracken & Sheldon, 1997). Recent studies on behavioral phylogeny, both intra- (McLennan, 1991) and interspecific (McCracken & Sheldon, 1997), have emphasized that communicatory behaviours are often critical for species recognition and therefore frequently mirror phylogenetic history. Clearly, this is not always the case. Bird calls often function for interspecific communication rather than species-specificity. Nor is it exclusively true either. Many examples of grooming behavior in insects support phylogenetic relationships (Wenzel, 1992). But, communicatory signals may be regularly more phylogenetically informative than other behavioral/ecological characters. A general scan of studies cited in reviews of behavioral homology show that 9 of 20 (Wenzel, 1992) were related to modes of communication.

Biochemical communicatory characters may in fact usefully bisect a continuum between characters that are completely consistent versus completely malleable. One reason that communication may serve as an 'intermediate character' is that it is influenced by many factors including physical properties of the environment (for mammalian scent marks, the deposited substrate), perceptual systems (olfactory sensitivity), and the information signaled (e.g. reproductive status, territorial boundary, etc.). Yet, these are precisely the various cues that serve the evolution of communication within species and lead to species divergences (Marchetti, 1993). It would be interesting to test whether biochemical characters are indeed intermediate between the two character extremes. Further comparative analysis should also assess whether communication is consistently more phylogenetically informative than other behavioural/ecological characters.

The phylogeny of the Felidae

Significant advances have been made in our understanding of felid phylogeny (O'Brien *et al.*, 1996). However, there remain considerable unresolved relationships within the family, particularly at more inclusive phylogenetic levels (Johnson & O'Brien, 1997; Bininda-Emonds *et al.*, 1999). Indeed, in contrast to other carnivore families, multiple estimates of phylogenetic relationships in felids are generally not congruent with respect to the molecular marker used, samples sizes, or the age of the study (Bininda-Emonds, 2000). Our analysis therefore emphasizes the use of different types of data rather than one 'correct' data set toward resolving this difficult phylogenetic problem; concurrently, O'Brien and colleagues have promoted this approach for molecular data (e.g. O'Brien *et al.*, 1996) and Bininda-Emonds *et al.* (1999) for all types of data.

Table 4. Comparing rival solutions using a Kishino–Hasegawa test. Only those *P* values in bold face are significant at the 0.05 level corrected for multiple comparisons using a sequential Bonferroni technique (Rice, 1989)

	Data matrix								
	Glycolipids			Neutral lipids			Phospholipids		
Rival tree	Length	*t*	*P*	Length	*t*	*P*	Length	*t*	*P*
Glycolipids	—	—	—	674	4.53	**<0.0001**	579	1.73	0.0845
Neutral lipids	540	4.48	**<0.0001**	—	—	—	599	3.65	**<0.0003**
Phospholipids	555	3.86	**<0.0001**	723	9.07	**<0.0001**	—	—	—
'Glycophospholipids'	521	0.50	0.6177	660	5.22	**<0.0001**	571	0.98	0.3253
All three	530	2.21	0.0276	624	0.90	0.3697	587	2.58	0.0104
Hemmer	815	16.02	**<0.0001**	1052	19.31	**<0.0001**	788	11.13	**<0.0001**
Herrington	730	12.98	**<0.0001**	962	16.96	**<0.0001**	753	10.44	**<0.0001**
Salles	655	10.59	**<0.0001**	842	13.96	**<0.0001**	692	8.05	**<0.0001**
Bininda-Emonds *et al.*	671	11.74	**<0.0001**	892	14.32	**<0.0001**	662	7.02	**<0.0001**
O'Brien (3.1)	761	15.22	**<0.0001**	960	18.89	**<0.0001**	744	11.74	**<0.0001**
O'Brien (3.2)	702	12.71	**<0.0001**	917	16.49	**<0.0001**	692	10.05	**<0.0001**

	Data matrix					
	'Glycophospholipids'			All three		
Rival tree	Length	*t*	*P*	Length	*t*	*P*
Glycolipids	1098	0.93	0.3548	1772	2.25	0.0248
Neutral lipids	1139	6.75	**<0.0001**	1769	4.25	**<0.0001**
Phospholipids	1118	2.20	0.0279	1841	5.81	**<0.0001**
'Glycophospholipids'	—	—	—	1752	1.18	0.2384
All three	1117	4.40	**<0.0001**	—	—	—
Hemmer	1603	20.45	**<0.0001**	2655	25.89	**<0.0001**
Herrington	1483	17.03	**<0.0001**	2445	22.00	**<0.0001**
Salles	1347	14.00	**<0.0001**	2189	17.88	**<0.0001**
Bininda-Emonds *et al.*	1333	13.68	**<0.0001**	2225	17.44	**<0.0001**
O'Brien (3.1)	1505	20.10	**<0.0001**	2465	24.71	**<0.0001**
O'Brien (3.2)	1394	16.84	**<0.0001**	2311	21.10	**<0.0001**

Table 5. Absolute differences between competing tree topologies as measured by the partition metric (standardized). Higher values indicate increasingly different topologies

Tree	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1) Glycolipids										
(2) Neutral lipids	0.286									
(3) Phospholipids	0.423	0.423								
(4) 'Glycophospholipids'	0.179	0.179	0.231							
(5) All three	0.250	0.107	0.385	0.143						
(6) Hemmer	0.500	0.571	0.538	0.536	0.607					
(7) Herrington	0.679	0.750	0.769	0.714	0.786	0.536				
(8) Salles	0.429	0.500	0.577	0.464	0.536	0.429	0.679			
(9) Bininda-Emonds *et al.*	0.607	0.679	0.692	0.643	0.714	0.536	0.571	0.607		
(10) O'Brien *et al.* (3.1)	0.607	0.679	0.538	0.571	0.714	0.464	0.643	0.607	0.571	
(11) O'Brien *et al.* (3.2)	0.571	0.643	0.500	0.536	0.679	0.500	0.679	0.643	0.607	0.393

Our analysis of felid chemical data is valuable because it provides another new and independent line of evidence. We have demonstrated a strong, decisive phylogenetic signal present in this underutilized data source. Phylogenetic analysis of three different lipid compounds obtained well-resolved, largely congruent,

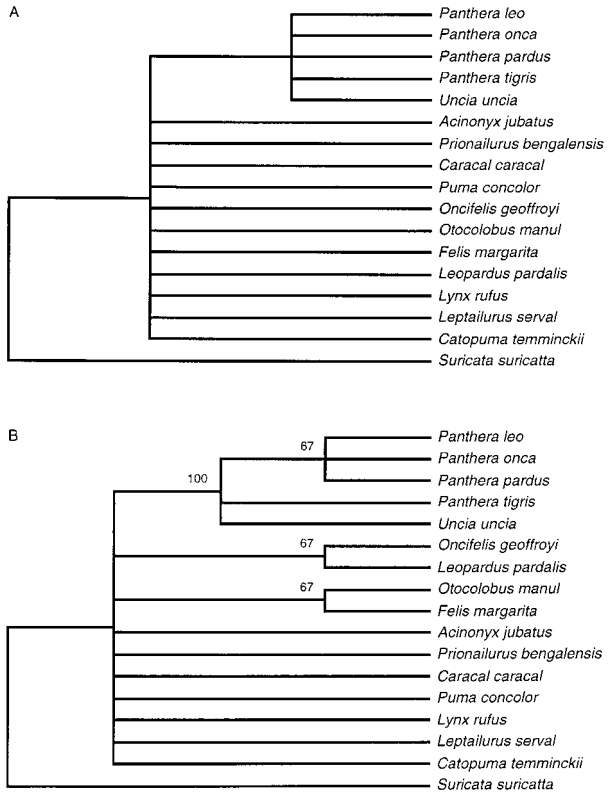

Figure 4. Strict (A) and 50% majority rule (B) consensus solutions for the trees in Figure 1.

and strongly supported findings. The strength of support is remarkable given the 'non-traditional' nature of our data source.

In general, phylogenetic relationships among felid species based on chemical signals reinforce areas of agreement among previous morphological, genetic, and molecular analyses. This is particularly striking with respect to the species pairs *Otocolobus manul*+*Felis margarita*, *Oncifelis geoffroyi*+*Leopardus pardalis*, and *Panthera pardus*+*Panthera leo*, which are consistent across all trees. Likewise, the separation of cats into two clades of 'big cats' versus 'small cats' that we found is generally supported by most studies. A clear size dichotomy does seem to exist in felids. Except for the European lynx, *Lynx lynx*, at about 20 kg, all cat species either weigh more than 30 kg or less than 12 kg. An expanded phylogeny based on chemical data would be useful to see if this size dichotomy does reflect evolutionary history.

Based on our analyses, two problem areas should receive attention in felid systematics, the positions of the puma and the cheetah. The position of the puma differs between the three primary lipid compounds in this study. Likewise, other authorities either cannot resolve the puma's affinity with other taxa (Hemmer, 1978), or place it in numerous different positions such as with the *Panthera* clade (Salles, 1992), with the cheetah (Herrington, 1986), or as a sister group to most species of *Felis* (Collier & O'Brien, 1985). With the cheetah, our results support the view that this species diverged early in the history of modern felids (Kral & Zima, 1980; Neff, 1983), though there is uncertainty about whether the cheetah is the sister taxon to the lynxes and *Panthera* group (Collier & O'Brien, 1985), to Pallas's cat (*Otocolobus manul*; Herrington, 1986), or to the *Panthera* group (Salles, 1992). Collection of further systematic characters, including non-traditional ones, will greatly aid in further resolving these phylogenetic and evolutionary problems.

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