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Resolving Phylogeny at the Family Level by Mitochondrial Cytochrome Oxidase Sequences: Phylogeny of Carrion Beetles (Coleoptera, Silphidae)

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We investigated the phylogenetic relationships of carrion beetles (Coleoptera, Silphidae) using 2094 bp of their mitochondrial cytochrome oxidase subunit I and II and tRNA leucine gene sequences. Shorter fragments of this gene region previously have been used to establish generic relationships in insects. In this study, they provided more than sufficient resolution, although the third positions of the protein-coding sequences reached saturation for the deeper divergences. This first published phylogeny for the Silphidae comprises 23 species from 13 genera sampled across the geographic range of the family. In addition, we included species from three related families as outgroups. One of these families, the Agyrtidae, was, until recently, included in the Silphidae, but its resolution here justifies its current position as a separate family. The silphid subfamilies Nicrophorinae and Silphinae are monophyletic in all analyses. All genera for which several species were sampled are supported as monophyletic groups, with the exception of the genus *Silpha*. European and North American representatives of two *Nicrophorus* species described from both continents are supported as each others' closest relatives. The lineage that colonized Gondwanaland and that most likely originated in the Palearctic is the most basal within the Silphinae. © 2000 Academic Press

INTRODUCTION

The Silphidae constitute a relatively small family of beetles encompassing about 172 species. Despite their small number, silphids are distributed worldwide, with the center of their distribution located in the Palearctic. Of the 22 genera currently recognized, 7 occur in the Palearctic only, 7 spread from the Palearctic into other geographic regions, and the remaining 8 genera are small in terms of species numbers and are endemic to the Australian, Ethiopian, Nearctic, Neotropical, or Oriental regions (Table 1; data from Hatch, 1928; Schawaller, 1979, 1981, 1996a,b; Peck and Anderson, 1985; Peck and Miller, 1993). The worldwide distribution of siphids, including Holarctic and Gondwanaland elements (Peck and Anderson, 1985), clearly reflects the antiquity of the family. Silphid fossils are rare and are known primarily from the Tertiary (see Madge, 1980), but one of the oldest known coleopteran fossils from the upper Triassic, *Pseudosilphites triassicus,* has been judged morphologically similar to silphids and a possible ancestor of the Staphylinoidea, the superfamily containing the Silphidae (Zeuner, 1930).

The taxonomy and systematic position of the Silphidae have been vastly rearranged since the exhaustive treatment by Hatch (1928). The family is currently placed within the Staphylinoidea in close relationship to the Staphylinidae. Many groups still ranked as subfamilies or tribes within the Silphidae in Hatch's (1928) compilation, e.g., the subfamilies Leptininae, Catopinae, and Coloninae and the tribes Agyrtini and Lyrosomini, have since been raised to family rank (Lawrence and Newton, 1982; Newton and Thayer, 1992; Newton, 1998). Cladistic analyses based on morphology have shown that these groups are well separated from the Silphidae, and they confirm the close relationship between the Silphidae and the Staphylinidae (Beutel and Molenda, 1997; Hansen, 1997). Within the Silphidae, two subfamilies are currently recognized, the Silphinae and the Nicrophorinae (Lohse and Lucht, 1989; Newton and Thayer, 1992; Peck and Miller, 1993), although Hansen (1997) suggests that the Silphinae may in fact be paraphyletic. Within the Silphidae, few attempts have been made to clarify the phylogenetic relationships within or between genera. The notable exceptions include sister group relationships that have been established for *Oxelytrum* and *Ptomaphila* (genera native to South America and Australia, respectively) and *Nicophorus* and *Ptomascopus* (the two genera in the Nicrophorinae) and a cladistic analysis of the New World *Nicrophorus* species based on morphology (Peck, 1982; Peck and Anderson, 1985). However, no phylogeny for the family has been published thus far, and the relationships among the gen-

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TABLE 1

Biogeography of Silphid Genera

Genus	Holarctic	Palearctic	Nearctic	Neotropical	Ethiopian	Oriental	Australian
Silphinae							
Ablattaria		4					
Aclypea	1	9	1				
Calosilpha[a]		4				3	
Chrysosilpha[a]						5	
Dendroxena		2					
Deutosilpha[a]						1	
Diamesus						2	
Eusilpha[a]		7					
Heterosilpha			2				
Heterotemna		2					
Isosilpha[a]		1					
Necrodes		3	1			1	
Necrophila[a]			1				
Oiceoptoma		3	3				
Oxelytrum				8			
Phosphuga		1					
Ptomaphila							3
Silpha		17			3	1	
Silphosoma				1	1		
Thanatophilus	1	11	5		4		
Nicrophorinae							
Nicrophorus[b]	2	35	13	5		3	
Ptomascopus		3					

Note. The number of species in each major region is given, counting each species only once, according to the center of its distribution.
[a] A pending revision is going to join these species in the genus *Necrophila* (S. B. Peck, pers. commun.).
[b] Revision with description of several new Palearctic species pending (D. S. Sikes, pers. commun.).

era, as well as the biogeography of the family, still await a comprehensive analysis.

The biology of silphid beetles offers at least two interesting aspects for behavioral and ecological research. First, the genus *Nicrophorus* exhibits a highly developed breeding biology that is unique among beetles and involves parental care of larvae by both sexes (Eggert and Müller, 1997; Scott, 1998). Second, silphids exhibit an array of different feeding specializations. As suggested by their common name, the majority of carrion beetles feed on carcasses of either vertebrates or invertebrates, although the genera *Ablattaria, Dendroxena,* and *Phosphuga* are highly specialized predators of snails or caterpillars (Heymons *et al.,* 1927, 1928; Heymons and von Lengerken, 1932) and one genus, *Aclypea,* has been a serious agricultural pest of beets in the past (Chittenden, 1903; Blunck and Janisch, 1925; Heymons and von Lengerken, 1929, 1930; Anderson and Peck, 1984).

Our long-term goal is to reconstruct the evolution of feeding specializations in the family. This phylogenetic analysis is a first step toward this objective, although we still need more comparative ecological data for a comprehensive analysis of the evolution of feeding habits. In this paper, we establish a phylogenetic hypothesis for the family Silphidae and examine the monophyly of the subfamilies and several genera. The study is based on DNA sequences of 2094 bp of the mitochondrial cytochrome oxidase subunit I (COI) and II (COII) genes and the tRNA leucine (UUR) gene between the two. We sampled 13 genera of the family involving a total of 23 species, including European and North American representatives of the genera *Necrodes, Nicrophorus, Oiceoptoma,* and *Thanatophilus.* We also included populations from both continents of two species of *Nicrophorus* that have been described as native to both regions.

Our data also provide an opportunity to examine the rate of evolution and amount of phylogenetic information contained in the sequences across various taxonomic levels. Fragments of the mitochondrial gene region analyzed here have been frequently used to address phylogenetic relationships in insects, but only at the generic level or below have relationships been successfully resolved (e.g., Brower, 1994; Brown *et al.,* 1994b; Emerson and Wallis, 1995; Funk *et al.,* 1995; Kelley and Farrell, 1998; Köpf *et al.,* 1998; Caterino and Sperling, 1999). At the family level, this gene region rarely gives satisfactory resolution (Brown *et al.,* 1994a; Miura *et al.,* 1998) and often proves to be unreliable (Dowton and Austin, 1997; Mardulyn and Whitfield, 1999; Maus *et al.,* unpublished), while at still higher levels, cytochrome oxidase sequences are not suitable for resolving relationships (Liu and Beckenbach, 1992; Howland and Hewitt, 1995; Frati *et al.,* 1997). We use our data set in comparison to these

TABLE 2

Beetles Examined in This Study and Their Origin

Family	Genus	Species	Origin
Agyrtidae	*Agyrtes*	*bicolor* Castelnau	Achkarren, Baden-Württemberg, Germany
	Necrophilus	*hydrophiloides* Guérin-Meneville	Vancouver Island, Canada
Leiodidae	*Catops*	sp.	Achkarren, Baden-Württemberg, Germany
	Liodes	sp.	Achkarren, Baden-Württemberg, Germany
Staphylinidae	*Aleochara*	*curtula* (Gravenhorst)	Freiburg, Baden-Württemberg, Germany
Silphidae			
Silphinae	*Ablattaria*	*laevigata* (Fabricius)	Illmitz, Burgenland, Austria
	Aclypea	*opaca* (Linnée)	Gorleben, Niedersachsen, Germany
	Dendroxena	*quadrimaculata* (Scopoli)	Freiburg, Baden-Württemberg, Germany
	Heterosilpha	*ramosa* (Say)	Boulder, Colorado, USA
	Necrodes	*littoralis* (Linnée)	Illmitz, Burgenland, Austria
		surinamensis (Fabricius)	Wolf Lake, Ontario, Canada
	Necrophila	*americana* (Linnée)	Wolf Lake, Ontario, Canada
	Oiceoptoma	*inequale* (Fabricius)	Normal, Illinois, USA
		thoracica (Linnée)	Freiburg, Baden-Württemberg, Germany
		novaboracense (Förster)	Wolf Lake, Ontario, Canada
	Phosphuga	*atrata* (Linnée)	Freiburg, Baden-Württemberg, Germany
	Ptomaphila	*lacrymosa* Schreibers	Kangaroo Island, Australia
	Silpha	*obscura* Linnée	Stubaital, Tirol, Austria
		tristis Illiger	Bielefeld, Nordrhein-Westfalen, Germany
		tyrolensis Laicharting	Arlberg, Tirol, Austria
	Thanatophilus	*lapponicus* (Herbst)	Wolf Lake, Ontario, Canada
		rugosus (Linnée)	Bielefeld, Nordrhein-Westfalen, Germany
		sinuatus (Fabricius)	Freiburg, Baden-Württemberg, Germany
		truncatus (Say)	Carrizozo, New Mexico, USA
Nicrophorinae	*Nicrophorus*	*defodiens* Mannerheim	Wolf Lake, Ontario, Canada
		investigator Zetterstedt	Wolf Lake, Ontario, Canada
		investigator Zetterstedt	Freiburg, Baden-Württemberg, Germany
		vespilloides Herbst	Wolf Lake, Ontario, Canada
		vespilloides Herbst	Freiburg, Baden-Württemberg, Germany
	Ptomascopus	*morio* Kraatz	Iwakura, Kyoto, Japan

studies to investigate under which conditions resolution can be satisfactorily achieved.

MATERIALS AND METHODS

Insects

Silphid beetles were sampled from 13 genera across different geographic regions to gain as balanced a representation as possible (Table 2). Outgroup species were chosen from the families Agyrtidae, Leiodidae, and Staphylinidae, according to current morphological hypotheses. All three families are considered to be close relatives of the Silphidae, but the actual sister group remains a matter of some debate (Madge, 1980; Newton and Thayer, 1992; Beutel and Molenda, 1997; Hansen, 1997). Most insects were kept frozen at −20°C until DNA extraction. Other specimens were obtained in 100% ethanol, and one specimen (*Heterosilpha ramosa*) collected in 1969 was obtained pinned and dried from the University of Colorado Museum collection. A dried specimen of the Oriental genus *Chrysosilpha, C. viridis* from the Philippines, yielded insufficient DNA amplification products for sequencing.

DNA Extraction and PCR

To extract DNA, only clean legs and antennae of individual carrion beetles were used to avoid contamination with gut content or phoretic mites. Only for the much smaller outgroup species (*Agyrtes bicolor, Aleochara curtula, Catops* sp., *Leiodes* sp.) were whole individuals used. After grinding the tissue in liquid nitrogen, we followed the protocol recommended by the QIAgen tissue kit (QIAgen, Hilden, Germany). Total genomic DNA was eluted in 400 μl 10 mmol Tris–HCl buffer, pH 8.4. Amplification of the 2094-bp gene region was accomplished by amplifying two smaller overlapping fragments using C1-J-1562 5′-TGAKCYGGAATASTAGGAICATC-3′ (B. D. Farrell, pers. commun.) in combination with TL2-N-3020 5′-GGAGCTTAAATCCAATACACTATTCTGCC-3′ as a PCR primer and C1-J-2441 5′- CCAACAGGAATTAAAATTTTTAGATGATTAGC-3′ in combination with C2-N-3661 5′-CCACAAATTTCTGAACATTGACCA-3′ as a primer; the nomenclature for primers and sequences of C1-J-2441 and C2-N-3661 follow Simon *et al.* (1994). Typical reactions were prepared in 50-μl volumes using 2 units of *Taq*-Polymerase and 2 μl of genomic DNA at 2.5 mmol $MgCl_2$ and 0.8 mmol dNTP

concentration. The amplification involved 2-min denaturing at 95°C, followed by 35 cycles of 45-s denaturing at 95°C, 1-min primer annealing at 47°C, and 1-min extension at 72°C, after which a final 5-min extension at 72°C was used. Reactions were then cooled to 4°C until removal. To amplify difficult templates, smaller fragments were generated using the same internal primers as for sequencing (see below). PCR products were cleaned of enzyme and remaining primer using a PCR purification kit (Nucleospin; Machery and Nagel, Düren, Germany) and recovered in 50 μl H_2O.

Sequencing

Both strands of the PCR products were sequenced by the Sanger dideoxy method in a cycle sequencing reaction using the Thermosequenase kit (Amersham, Braunschweig, Germany). The reactions were electrophoresed and analyzed on a Licor 4000 L automatic sequencer (MWG Biotech, Ebersberg, Germany). In the sequencing reactions, infrared-labeled primers C1-J-1562, TL2-N-3020, and C2-N-3661 (see PCR protocol) as well as internal primers C1-J-2166 5′- GGAGGTGGAGACCCAATTTTATACCAAC-3′, C1-N-2188 5′- CCYGGTAAAATTAAAATATAAACTTCRGG-3′, and TL2-J-3045 5′-CAGATTAGTGCATTGGATTTAAAC-3′ were used. Sequences were read with the Licor Image-Analysis software (Ver. 2.3 and 4.0) and then exported to Sequencher V3.0 for alignment (Gene Codes Corp., Ann Arbor, MI). Sense and antisense strands were checked against each other. Overall, 85% of each sequence was generated from several primers independently, with the exception of two species for which the reproducibility of one of the sequencing primers failed, so that a larger part of the sequence could be read only from one strand. In addition, the congruence of sequences in the 600-bp region of overlap between the two PCR products permitted us to ascertain the identity of the samples.

Phylogenetic Analyses

The aligned sequences were analyzed for their phylogenetic information with PAUP* (Vers. 4.0 d64 and b2; Swofford, 1999) on a Macintosh G3 computer. The homogeneity of base frequencies across taxa was assessed using the χ^2 test implemented in PAUP*. Maximum-parsimony trees were generated in 100 random-addition replicates of heuristic searches. Bootstrap support of individual nodes was assessed by 500 bootstrap replicates, each comprising 10 heuristic random-addition searches. As an additional means of assessing the robustness of the most-parsimonious tree, branch support values (Bremer, 1988, 1994) were calculated using AutoDecay (Vers. 4.0.1; Erikson, 1998) in 100 random-addition searches evaluating individual nodes. In two additional parsimony analyses, transversions were weighted 2 times and 10 times more heavily than transitions, respectively. The information contained in third codon positions was assessed by running parsimony analyses with all data except third position and with third positions only.

Maximum-likelihood (ML) analyses were carried out with PAUP*4d64 using three different models of rate distribution among sites that mirror the properties of our data set to different degrees. We used the HKY85 model of base substitution (Hasegawa *et al.*, 1985), which allows for unequal base frequencies, as actually observed in our data. Empirical base frequencies were used and the ratio of transitions to transversions was estimated from the data. In all three analyses, 10 heuristic searches with random input order were conducted. In the first analysis, all sites were assumed to be variable and to have equal substitution rates. In a second analysis, we allowed for invariable sites but assumed equal substitution rates at the variable sites. The third analysis refined the model further in recognizing four different site classes with different substitution rates: first, second, and third codon positions and sites belonging to the tRNA or noncoding bases. In all three analyses, the rate parameters and the transition to transversion ratio of the desired model of sequence evolution were estimated from the most-parsimonious tree. This strategy is recommended (Swofford *et al.*, 1996; Swofford, 1998) because ML analyses that provide parameter estimates for each tree evaluated are prohibitively time-consuming.

Differences between the most-parsimonious tree of the unweighted analysis, the most-parsimonious tree identified by maximum-likelihood analyses, and the most-parsimonious tree in which the genus *Silpha* was constrained to be monophyletic were assessed by the likelihood ratio test (Kishino and Hasegawa, 1989). MacClade (Vers. 3.04; Maddison and Maddison, 1992) was used to partition data, to calculate steps and transition to transversion ratios for clades in the most-parsimonious trees, and to draw trees.

RESULTS AND DISCUSSION

Alignment and Translation of COI and COII

Alignment of the mostly protein-coding sequences was unambiguous, including the placement of a few gaps in, and immediately after, the tRNA leucine gene. The length of the tRNA varied between 64 and 66 bases, all length differences being caused by indels in the TΨC arm.

As reported for several mitochondrial genes in insects (Crozier and Crozier, 1993; Flook *et al.*, 1995; Dobler and Farrell, 1999) the cytochrome oxidase I gene seems to be terminated by a single T rather than by a complete stop codon. The position and identity of the start codon for the COII gene, however, varies between the sequences. All silphids, the agyrtid *Agyrtes bicolor,* and the staphylinid *Aleochara curtula* have an ATG or ATA start codon one to four bases downstream from the tRNA leu (Fig. 1). In three spe-

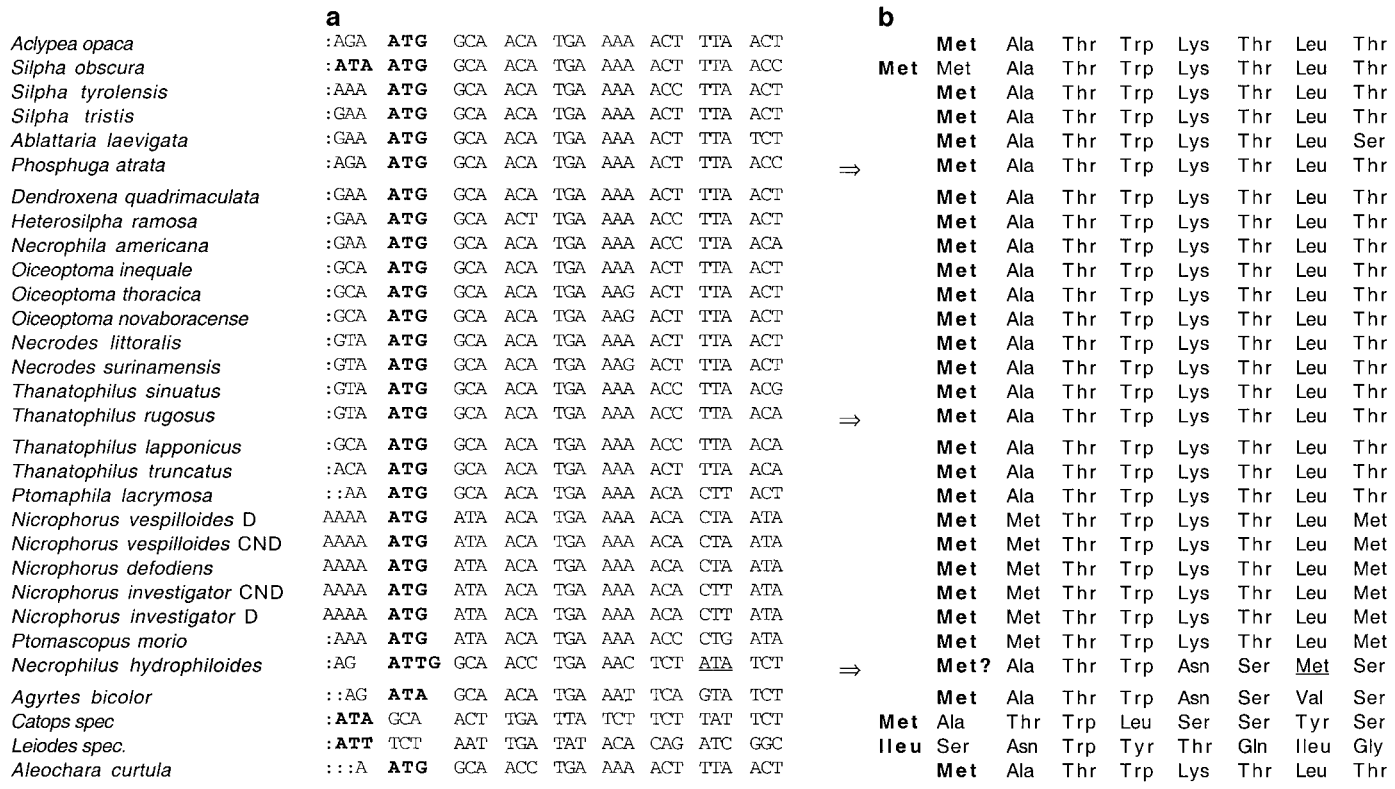

FIG. 1. Inferred start codons for cytochrome oxidase II. (a) DNA sequence with potential start codons in boldface; (b) amino acid sequence. Presumptive start codons are in boldface; an alternative start codon in *Necrophilus hydrophiloides* is underlined. Country codes for the conspecific *Nicrophorus* sequences: CND, Canada; D, Germany.

cies, COII is longer by one amino acid at the 5′ end and is initiated either by ATT (*Leiodes* sp.) or by ATA (*Catops* sp., *Silpha obscura*). In the agyrtid *Necrophilus hydrophiloides,* the first start codon in the same reading frame as that of other beetles lies seven triplets further downstream (underlined in Fig. 1). There is, however, a frame-shifted ATT codon one base upstream of the start codon present in the majority of the species. Neither of these two known start codons is likely to represent the actual start to translation of COII in *N. hydrophiloides.* With respect to the downstream start codon, it would be unclear why translation was initiated only by the second start codon and why the sequence upstream from this start codon is highly conserved between the two agyrtid species. If instead translation was initiated by the frame-shifted ATT codon, the translation product would be only 11 amino acids in length. Therefore, we suggest that *N. hydrophiloides* may have an anomalous start codon of four bases in length, ATTG, which would initiate translation in the same reading frame and at the same site as in the other species. Since the sequence around these positions was unambiguous among several fragments generated from independently amplified PCR products, no sequencing error can be involved. An anomalous ATAA four-base start codon for COI has been reported in *Drosophila melanogaster* and *D. yakuba* (de Bruin, 1983; Clary and Wolstenholme, 1985). Translational frameshifts that lead to a violation of the triplet-based reading frame have been reported in various virus, bacteria, and yeast genes, mammalian antizyme genes, and, most recently, the bird mitochondrial NADH dehydrogenase subunit III gene (Farabaugh, 1996; Mindell *et al.,* 1998). Four-base start codons in insect mtDNA genes may represent yet another instance of such alternative translation mechanisms (de Bruin, 1983).

Sequence Variability

With 31% A, 37% T, 15% G, and 17% C, the sequenced genes show the typical AT bias of insect mitochondrial DNA and cytochrome oxidase genes in particular (Clary and Wolstenholme, 1985; Crozier and Crozier, 1993; Frati *et al.,* 1997). Although there was some variation in base frequencies between species, with the outgroup *Aleochara curtula* showing the strongest AT bias (34% A, 40% T, 13% G, 13% C), no significant heterogeneity between taxa was detected ($\chi^2 = 96.31$, $df = 87$, $P = 0.232$).

Of the 2094 nucleotide sites examined, 47% were variable and 39% were parsimony informative (Table 3). The majority of the variable sites were third codon positions (65%), although first (24%) and second (9%) positions also contained a considerable fraction of the

TABLE 3

Summary Statistics Based on the Most-Parsimonious (MP) Tree (Fig. 2)

	All sites	First positions	Second positions	Third positions	tRNA
No. of characters	2094	675	674	674	71
No. variable	975	230	86	632	27
No. parsimony informative	821	181	54	563	23
No. of autapomorphies	154	49	32	69	4
Steps in MP tree	4716	832	151	3646	87
Consistency index[a]	0.30	0.34	0.59	0.29	0.41
Retention index	0.44	0.56	0.79	0.38	0.67
No. of steps/character	2.3	1.2	0.2	5.4	1.2
Transitions/transversions	1.4	4.1	0.9	1.1	4.2

[a] Excluding parsimony uninformative characters.

variable sites, while the tRNA gene contributed only a small fraction (3%) of the variability. The greatest sequence divergence observed within the ingroup species lay just below 20%. When the outgroup species were included, maximal divergence amounted to 24%. The lowest divergence was found in the intraspecific transatlantic comparisons of the two *Nicrophorus investigator* samples (2.6%) and of the two *N. vespilloides* samples (3.6%).

To compare our data with those of previous studies employing subsets of the same gene region, we reanalyzed a representative sample of them. The analysis reveals that uncorrected sequence divergences between genera in the Silphidae are at the higher end of those observed in other families (Brower, 1994; Brown *et al.,* 1994a; Emerson and Wallis, 1995; Howland and Hewitt, 1995; Köpf *et al.,* 1998; Miura *et al.,* 1998; Caterino and Sperling, 1999; Chippindale *et al.,* 1999). Greater distances within a family were recorded in a study on Collembola, where the families are assumed to be older than those of other insects (Frati *et al.,* 1997) and in studies where the comparisons were across families that are especially large (Funk *et al.,* 1995; Mardulyn and Whitfield, 1999). The divergence among families in our data set (Table 4) is markedly greater than that between genera, but not unusually so (Brown *et al.,* 1994a; Howland and Hewitt, 1995; Dowton and Austin, 1997; Frati *et al.,* 1997; Miura *et al.,* 1998; Mardulyn and Whitfield, 1999). Compared to studies conducted at a similar or a higher taxonomic level, the percentage of variability in our data set is somewhat lower, with first and second positions being more conserved than those in both studies that have failed to resolve the phylogeny of interest (Howland and Hewitt, 1995; Dowton and Austin, 1997; Frati *et al.,* 1997; Mardulyn and Whitfield, 1999) and those that have succeeded in doing so (Brown *et al.,* 1994a; Miura *et al.,* 1998). Thus, these sequence statistics appear to be weak predictors of the phylogenetic information content of a data set.

Phylogenetic Analysis

Unweighted maximum-parsimony analysis resulted in a single most-parsimonious tree of a length of 4716 steps that had a consistency index of 0.33 and a retention index of 0.44 (Fig. 2). Because we did not want to make assumptions about the still contentious relationships among the outgroups, we rooted the tree on an internal branch within the outgroup. The resulting outgroup topology coincides closely with the families as they are currently defined and groups the Agyrtidae with the Leiodidae in concurrence with recent views (Newton and Thayer, 1992; Beutel and Molenda, 1997; Hansen, 1997; Newton, 1998). The subfamilies Nicrophorinae and Silphinae and all genera represented by several species, with the exception of the genus *Silpha*, are well supported as monophyletic groups by both bootstrap and branch support values. The genus *Silpha* appears to be paraphyletic, with the representatives of three other genera, *Aclypea*, *Dendroxena*, and *Phosphuga*, being nested within *Silpha*. The resolution of the ingroup was very robust to the choice of outgroup. The addition of two chrysomelid sequences (*Aphthona lutescens* and *Longitarsus tabidus*, S. Dobler, unpublished) or the inclusion of fewer outgroup species produced an identical, or in a few cases, a nearly identical tree, in which only *P. lacrymosa* was shifted to the base of the genus *Thanatophilus*.

Phylogenetic Information of Third Positions

Because 77.3% of the steps in the most-parsimonious tree occur at third positions, we examined whether the phylogenetic information contained in them is compromised by saturation. A pairwise comparison of third position sequence divergence versus total divergence shows that third position distances reach up to 50% in the comparisons between ingroup and outgroup species at an overall sequence divergence of just above 20% (Fig. 3). Under the simplest model of sequence evolution, one that assumes equal base frequencies (Jukes and Cantor, 1969), complete saturation is reached only at 75% divergence. However, since insect mtDNA is AT

TABLE 4

p-Distances (above Diagonal) and Transition/Transversion Ratio (below Diagonal) between All Species Studied

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	*A. opaca*	—	0.111	0.115	0.100	0.109	0.121	0.112	0.138	0.153	0.126	0.136	0.129	0.132	0.141	0.139	0.147	0.158	0.145	0.169	0.189	0.195	0.197	0.176	0.186	0.185	0.194	0.199	0.204	0.242	0.169
2	*S. obscura*	1.511	—	0.088	0.106	0.112	0.118	0.123	0.147	0.152	0.135	0.141	0.140	0.134	0.142	0.136	0.146	0.158	0.143	0.156	0.188	0.188	0.186	0.169	0.174	0.171	0.187	0.192	0.195	0.222	0.155
3	*S. tyrolensis*	1.516	2.119	—	0.113	0.110	0.125	0.128	0.146	0.145	0.137	0.143	0.139	0.129	0.139	0.139	0.152	0.156	0.149	0.147	0.176	0.176	0.176	0.162	0.165	0.168	0.181	0.185	0.193	0.226	0.161
4	*S. tristis*	1.633	1.433	1.484	—	0.100	0.107	0.106	0.136	0.151	0.117	0.126	0.124	0.128	0.131	0.137	0.153	0.156	0.139	0.167	0.185	0.192	0.187	0.177	0.177	0.176	0.183	0.195	0.202	0.237	0.173
5	*A. laevigata*	1.522	1.330	1.290	1.080	—	0.104	0.110	0.130	0.135	0.117	0.136	0.128	0.126	0.136	0.138	0.147	0.145	0.142	0.151	0.176	0.176	0.180	0.167	0.165	0.165	0.187	0.184	0.193	0.224	0.163
6	*P. atrata*	1.739	1.490	1.534	1.372	1.455	—	0.112	0.149	0.153	0.132	0.152	0.143	0.131	0.144	0.141	0.150	0.154	0.147	0.163	0.180	0.182	0.186	0.181	0.177	0.170	0.191	0.193	0.195	0.227	0.168
7	*D. quadrimaculata*	1.786	1.909	1.840	1.763	1.614	1.560	—	0.137	0.142	0.127	0.139	0.127	0.124	0.144	0.130	0.142	0.163	0.142	0.158	0.180	0.187	0.190	0.180	0.182	0.184	0.194	0.199	0.207	0.230	0.174
8	*H. ramosa*	1.323	1.194	1.203	1.238	1.151	1.403	1.298	—	0.116	0.131	0.141	0.135	0.144	0.143	0.150	0.155	0.169	0.158	0.162	0.191	0.194	0.196	0.180	0.183	0.184	0.189	0.207	0.211	0.238	0.183
9	*N. americana*	1.170	1.273	1.321	1.143	1.213	1.312	1.331	1.241	—	0.136	0.141	0.137	0.145	0.150	0.156	0.163	0.168	0.164	0.164	0.193	0.196	0.197	0.184	0.184	0.185	0.198	0.202	0.205	0.234	0.180
10	*O. inequale*	1.157	1.385	1.367	1.042	1.077	1.273	1.500	1.076	1.220	—	0.071	0.058	0.114	0.110	0.131	0.133	0.149	0.128	0.159	0.184	0.184	0.183	0.174	0.175	0.169	0.190	0.188	0.195	0.227	0.168
11	*O. thoracica*	1.220	1.471	1.423	1.259	1.282	1.496	1.437	1.139	1.325	2.128	—	0.064	0.136	0.129	0.146	0.146	0.159	0.143	0.164	0.193	0.190	0.191	0.185	0.189	0.187	0.195	0.202	0.201	0.236	0.180
12	*O. novaboracense*	1.205	1.366	1.350	1.197	1.119	1.285	1.431	1.081	1.159	2.000	3.323	—	0.123	0.121	0.134	0.137	0.148	0.128	0.159	0.184	0.180	0.179	0.178	0.184	0.176	0.195	0.197	0.201	0.231	0.178
13	*N. littoralis*	1.099	1.114	0.971	0.956	0.948	1.167	1.106	1.120	1.112	0.992	1.236	1.116	—	0.109	0.124	0.137	0.157	0.138	0.152	0.166	0.169	0.169	0.165	0.166	0.167	0.190	0.185	0.199	0.228	0.162
14	*N. surinamensis*	1.240	1.056	1.126	1.102	1.154	1.295	1.407	1.085	1.110	1.238	1.304	1.306	1.253	—	0.127	0.134	0.150	0.132	0.155	0.180	0.179	0.179	0.169	0.171	0.160	0.187	0.193	0.198	0.232	0.161
15	*T. sinuatus*	1.157	1.029	0.979	0.979	1.065	1.093	1.308	1.060	1.000	1.195	1.349	1.336	0.985	1.261	—	0.113	0.132	0.127	0.158	0.175	0.181	0.177	0.176	0.178	0.167	0.194	0.192	0.195	0.236	0.165
16	*T. rugosus*	1.250	1.194	1.154	1.154	1.193	1.341	1.595	1.292	1.089	1.351	1.360	1.173	1.339	1.293	1.591	—	0.131	0.131	0.163	0.187	0.191	0.189	0.184	0.186	0.183	0.190	0.195	0.203	0.231	0.169
17	*T. lapponicus*	1.440	1.397	1.259	1.307	1.459	1.454	1.654	1.465	1.320	1.580	1.661	1.476	1.380	1.380	1.395	1.533	—	0.138	0.168	0.185	0.182	0.187	0.180	0.178	0.179	0.200	0.200	0.203	0.239	0.173
18	*T. truncatus*	1.221	1.184	1.175	1.117	1.193	1.117	1.339	1.012	1.091	1.054	1.267	1.153	1.242	1.069	1.229	1.284	1.298	—	0.155	0.179	0.176	0.178	0.174	0.176	0.177	0.193	0.193	0.198	0.233	0.170
19	*P. lacrymosa*	1.338	1.338	1.047	1.253	1.194	1.338	1.317	1.209	1.178	1.283	1.408	1.299	1.264	1.279	1.096	1.230	1.367	1.146	—	0.174	0.174	0.173	0.166	0.167	0.182	0.189	0.191	0.196	0.216	0.173
20	*N. vespilloides* D	0.931	0.940	0.825	0.905	0.957	0.864	1.005	0.975	0.900	1.071	1.140	1.088	0.859	1.000	1.112	1.083	1.060	0.979	0.946	—	0.036	0.069	0.115	0.115	0.147	0.197	0.198	0.197	0.219	0.172
21	*N. vespilloides* CND	0.906	0.912	0.795	0.937	0.902	0.849	1.015	0.995	0.948	1.104	1.146	1.066	0.892	0.989	1.112	1.075	1.000	0.978	0.926	3.353	—	0.069	0.111	0.111	0.147	0.206	0.198	0.199	0.219	0.175
22	*N. defodiens*	0.847	0.894	0.767	0.835	0.767	0.821	0.974	0.855	0.864	0.979	1.048	1.017	0.805	0.925	1.006	1.077	1.060	0.978	0.923	1.745	1.593	—	0.112	0.113	0.145	0.202	0.201	0.200	0.230	0.177
23	*N. investigator* CND	0.852	0.770	0.749	0.802	0.741	0.847	0.883	0.823	0.938	0.865	0.915	0.887	0.730	0.922	0.870	0.979	0.887	0.841	0.994	0.975	0.949	0.916	—	0.026	0.148	0.192	0.195	0.199	0.231	0.171
24	*N. investigator* D	0.901	0.772	0.721	0.742	0.704	0.779	0.879	0.804	0.919	0.795	0.907	0.862	0.785	0.928	0.891	0.974	0.887	0.852	0.971	0.950	0.924	0.966	7.833	—	0.148	0.196	0.194	0.205	0.234	0.170
25	*P. morio*	1.111	0.994	0.928	1.063	0.921	1.065	1.093	1.000	1.000	1.006	1.200	1.092	0.901	1.056	0.870	1.074	1.127	1.062	0.974	0.955	0.912	0.959	0.824	0.808	—	0.199	0.191	0.201	0.219	0.170
26	*N. hydrophiloides*	0.897	0.897	0.857	0.831	0.835	0.805	0.832	0.980	0.864	0.941	0.971	0.906	0.931	0.915	0.933	0.882	0.990	0.870	1.015	0.875	0.837	0.838	0.816	0.815	0.826	—	0.178	0.188	0.208	0.179
27	*A. bicolor*	0.820	0.901	0.741	0.930	0.830	0.850	0.909	0.916	0.879	1.000	1.072	1.005	0.817	0.900	0.868	0.989	0.922	0.859	1.021	0.776	0.824	0.870	0.777	0.735	0.838	0.956	—	0.179	0.195	0.171
28	*Catops* sp.	0.805	0.789	0.760	0.784	0.763	0.758	0.870	0.849	0.829	0.880	0.896	0.905	0.777	0.825	0.764	0.822	0.875	0.836	0.902	0.722	0.684	0.676	0.649	0.671	0.701	0.741	0.767	—	0.187	0.172
29	*Liodes* sp.	1.013	0.917	0.910	1.009	0.987	0.939	1.004	0.996	0.996	1.018	1.095	1.045	0.940	0.974	0.966	0.964	0.987	1.009	1.099	0.849	0.827	0.947	0.844	0.861	0.853	0.995	0.954	0.907	—	0.212
30	*A. curtula*	0.971	0.749	0.758	0.862	0.829	0.795	0.945	0.903	0.867	0.847	0.984	0.890	0.858	0.911	0.844	0.881	1.017	0.821	0.934	0.730	0.728	0.728	0.676	0.644	0.719	0.847	0.647	0.639	0.796	—

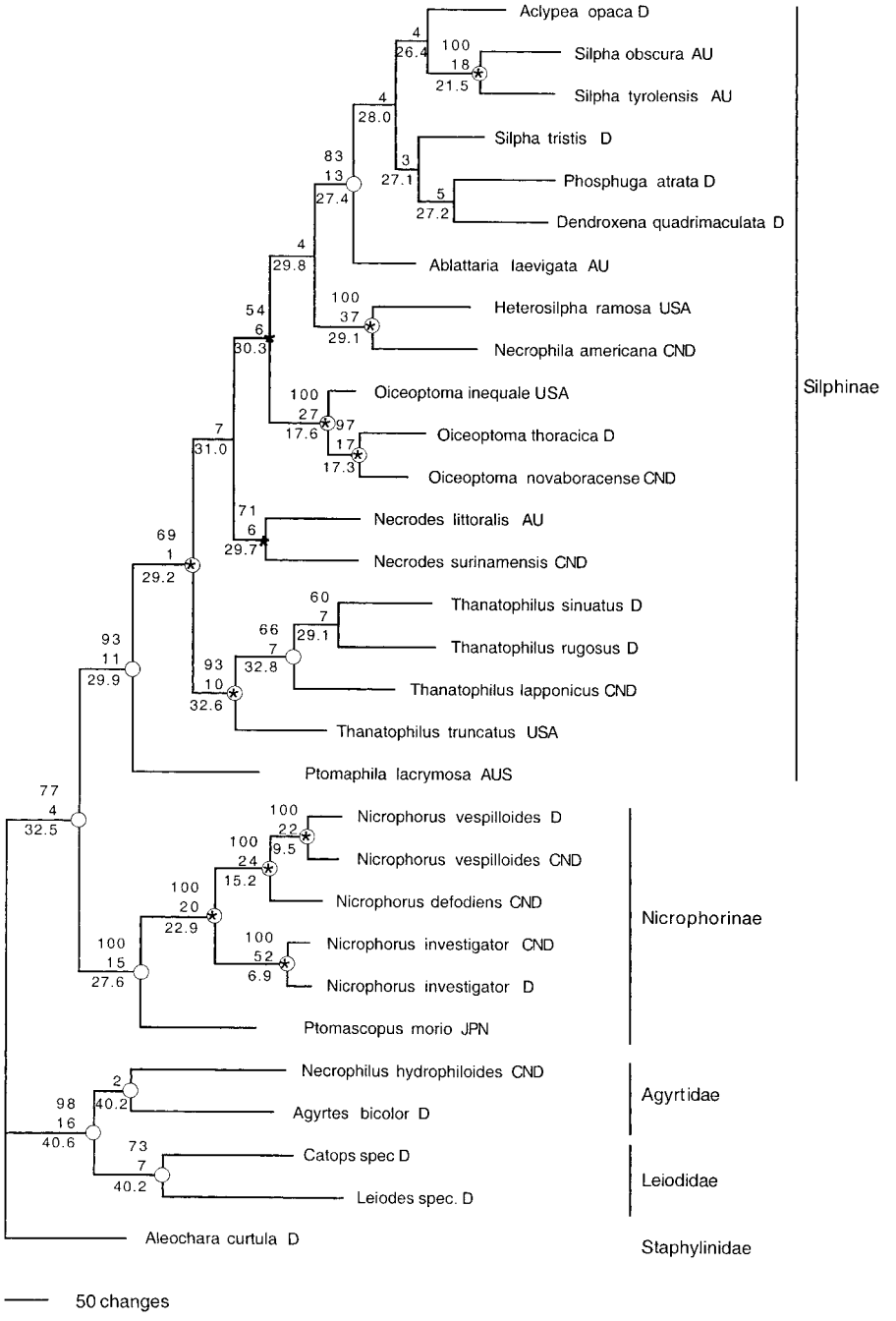

FIG. 2. Single unweighted maximum parsimony tree. The length of the tree was 4716 (consistency index 0.31). Numbers above branches give bootstrap values >50% calculated in 500 replicates of 10 heuristic searches each and Bremer support values for the adjacent node. Numbers below branches correspond to mean third position divergences between the taxa descended from that node. * Marks nodes supported by bootstrap values >50% in an analysis based on third codon positions only, o marks those supported in an analysis excluding third codon positions. The tree was rooted at an internal branch within the outgroup. The codes after the species names give the country of origin: AU, Austria; AUS, Australia; CND, Canada; D, Germany; JPN, Japan; USA, United States of America.

biased, saturation will be reached at lower levels. The general pattern in this plot (Fig. 3) shows that at low overall sequence divergence, i.e., among closely related species, third position distances increase linearly. The fact that more distantly related species may have lower third position divergences than closer relatives illustrates the preferential accumulation of further substitutions at first and second positions and suggests that saturation at the third position starts at around 30% overall divergence.

We also investigated third position saturation by calculating the mean third position divergence of taxa descended from individual nodes (Mitchell *et al.*, 1997). This yielded consistently increasing values of up to

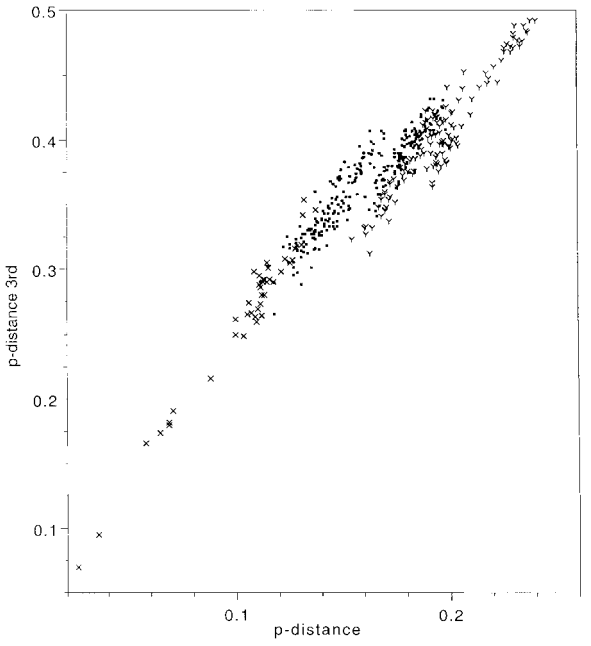

FIG. 3. Correlation of third codon position sequence divergence between all species pairs relative to overall sequence divergence (given as p-distances). Comparisons between closely related groups (sister species, species within genera, subfamily Nicrophorinae) are indicated by x, comparisons between ingroup and outgroup species by y, and all remaining comparisons by squares.

about 30% when proceeding from the tips toward the root of the tree (Fig. 2). Beyond 30%, the average third position divergence stagnates or even decreases slightly with successively deeper nodes. Among the outgroup species, third position divergence levels out at around around 40% (Fig. 2), suggesting that sequence evolution is under different constraints in these species. Nevertheless, third positions do not contain enough phylogenetic information (i.e., they are too homoplastic) to resolve deeper nodes. Evaluating the data excluding third codon positions versus evaluating third positions alone reveals that the phylogenetic signal is well distributed between these two subsets. Using only third positions in a maximum-parsimony analysis resolves the closely related groups with good bootstrap support, while a parsimony analysis excluding third codon positions recovers the deeper nodes and some of the intrageneric relationships with high bootstrap support. In both analyses the relationships within *Nicrophorus* are resolved with high support (minimum bootstrap value is 77%). Obviously, the two partitions complement each other in their ability to resolve the phylogeny across the whole data set.

Transition/Transversion Ratio and Weighted Parsimony

The native ratio of transitions to transversions is most likely to be manifest when comparing the most recently diverged lineages, because transitions should be more frequent and accumulate more rapidly but be erased by later transversions at the same site (Brown *et al.,* 1982; DeSalle *et al.,* 1987). In comparisons across older divergences, however, this ratio should be shifted toward transversions. Hence, the maximum ratio of transitions to transversions is normally observed in comparisons within species: between the two *N. investigator* samples, the ratio of transitions to transversions was 7.8 for the 52 unambiguous substitutions, and for the two *N. vespilloides* samples, the ratio was 3.4 for the 74 substitutions. In the more divergent comparisons both among the ingroup and between the ingroup and the outgroup species, the ratio levels out at around 0.75 (Table 4). Calculated over all species in the most-parsimonious tree, the transitions account for 58% of all substitutions. The theoretical expectation for transition saturation lies at 28.6% (Liu and Beckenbach, 1992). As in other studies on lower taxonomic levels in insects (Funk *et al.,* 1995; Funk, 1999), the cytochrome oxidase genes are not yet saturated in the group investigated here despite the observed onset of third position saturation (Fig. 3).

To explore the sensitivity of the parsimony analysis to differential weighting of transitions versus transversions, we reanalyzed the data giving transversions 2 times and 10 times more weight than transitions. Both analyses resulted in single trees almost identical to the one from the unweighted analysis. The 2-fold tree (CI = 0.32) differs from the equal weight tree only in the positions of *Phosphuga atrata, Dendroxena quadrimaculata,* and *Silpha tristis,* which are resolved in a pectinate manner in the order given. Bootstrap support for individual nodes is very similar to that of the equal weight tree. Weighting transversions 10-fold over transitions produces a similar but less well-supported tree (CI = 0.29; overall lower bootstrap support). Rearrangements in the ingroup concern the branching order of the same species as before (. . .(*D. quadrimaculata* (*A. laevigata* (*P. atrata* ((*S. tyrolensis, S. obscura*) (*S. tristis, A. opaca*)))))). In addition one of the outgroup species, *Aleochara curtula,* is placed inside the Silphidae at the base of the Nicrophorinae in the shortest tree. The overall lower level of support for this tree, along with the unlikely arrangement of the outgroup species, suggests that this weighting scheme is too extreme. The general robustness of the tree topology to differential weighting of transversions over transitions lends additional support to the phylogenetic hypothesis depicted in the equal weight tree (Swofford, 1991), with some caution about the exact resolution of the species discussed above.

Maximum-Likelihood Analysis

Three maximum-likelihood analyses, based on the HKY85 model of sequence evolution (Hasegawa *et al.,* 1985) but assuming different patterns of rate heterogeneity among sites, were conducted. The first assumed that all sites evolve at equal rates and clearly oversimplifies the properties of our data, the second

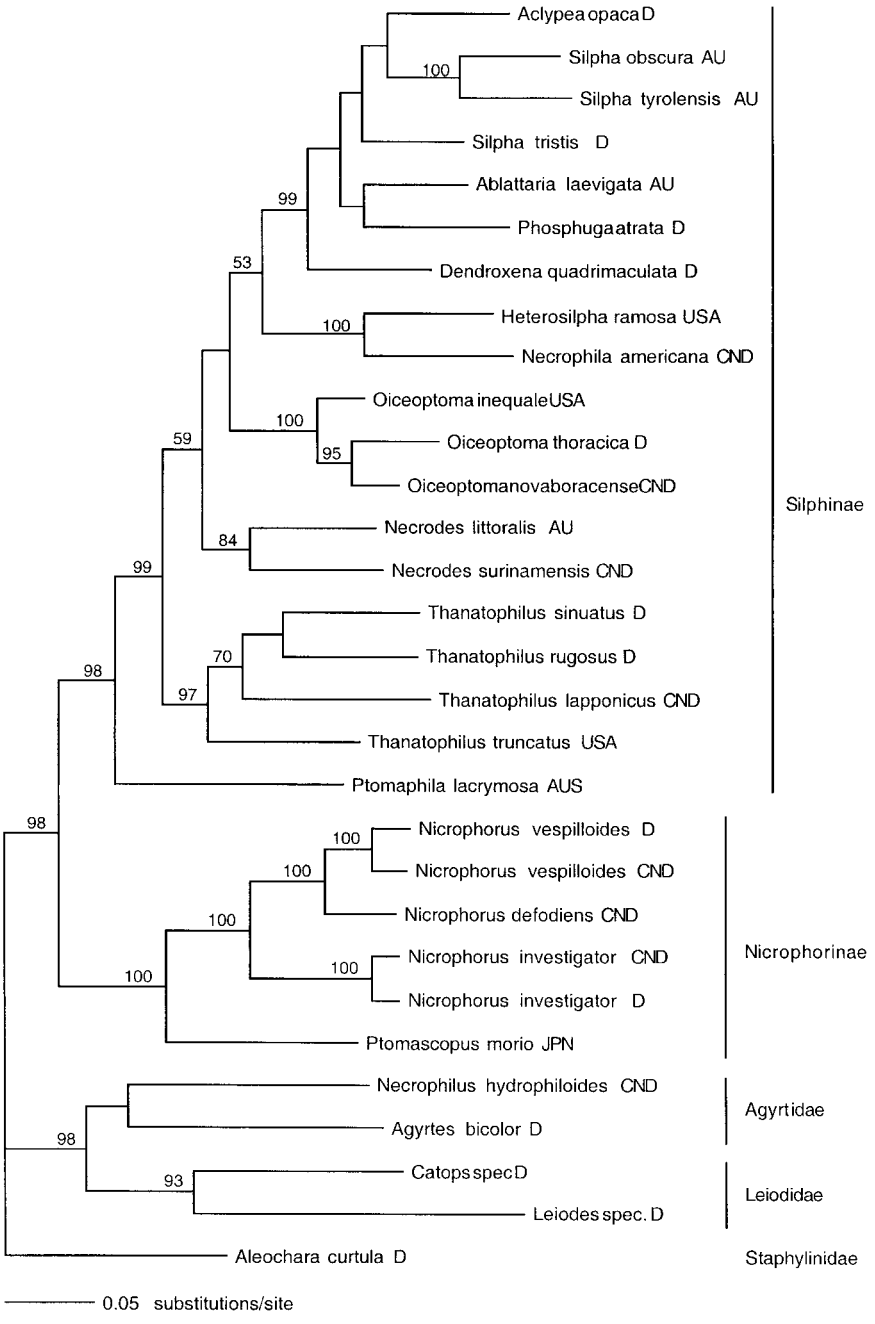

FIG. 4. Maximum-likelihood tree with branch lengths assuming the HKY85 model with four site-specific substitution rate classes (1st, 2nd, and 3rd codon position and tRNA including noncoding bases). Numbers above the branches give bootstrap values in 500 replicates of neighbor-joining analysis based on maximum-likelihood distance under that model. Codes for countries of origin as in Fig. 2.

permitted invariable sites but assumed that all variable sites evolve at equal rates, which comes closer to the properties of the data, and the third allowed the three codon positions and the tRNA plus the few noncoding bases at its end to evolve at separate rates. This last model probably best represents the properties of our mostly protein-coding sequences. All three analyses resulted in the same tree (Fig. 4; tree description: (1) ti/tv ratio = 1.273, -ln likelihood = 25502.97; (2) ti/tv ratio = 1.612, proportion of invariable sites = 0.532, -ln likelihood = 22625.85; (3) ti/tv ratio = 1.709, relative rates 0.395, 0.070, 2.596 , 0.435, -ln likelihood = 22448.24). Except for the branching order of *A. laevigata*, *P. atrata*, *S. tristis*, and *D. quadrimaculata*, the single maximum-likelihood tree is identical to the results of the unweighted and the transversions-weighted-twofold parsimony analyses (Fig. 4). The absence of very short branches in both the maximum-likelihood tree and the most-parsimonious tree (Fig. 2) suggests that the phylogenetic information in our data

set is well distributed and provides sufficient information to trace all speciation events in this group. An assessment of the branch support by 500 bootstrap replicates of the neighbor-joining analysis, based on maximum-likelihood distances under the third model used here, shows a pattern very similar to that of the bootstrap values in the maximum-parsimony analyses. Branches with significant bootstrap support (>70%, Hillis and Bull, 1993) are identical in both analyses, with the support in general being higher in the ML tree. However, the support for the branching order of *Silpha* and its allies is low in all analyses.

Phylogeny of Silphidae

Our data provide a well-supported phylogenetic hypothesis for most of the species examined, because very few discrepancies between the different methods of analysis exist and support for the majority of nodes is high. The Agyrtidae, which were originally included in the Silphidae (Hatch, 1928; Madge, 1980; Peck and Miller, 1993), are diagnosed as more closely related to the family Leiodidae than to the Silphidae. This agrees with recent taxonomic restructuring based on cladistic analyses of adult and larval morphology (Lawrence and Newton, 1982; Beutel and Molenda, 1997; Hansen, 1997; Newton, 1998). Within the Silphidae, the separation of two monophyletic subfamilies, the Nicrophorinae and Silphinae, is well supported, as is the monophyly of the genera *Necrodes*, *Nicrophorus*, *Oiceoptoma*, and *Thanatophilus*. The Holarctic Nicrophorinae are resolved with strong support for all nodes and with no discrepancies between the methods of analysis used. The sister group relationship between *Nicrophorus defodiens* and *N. vespilloides* agrees with the cladistic analyses of Peck and Anderson (1985). The genus *Ptomascopus* has been judged to be the sister group to *Nicrophorus* based on morphology and breeding biology (Peck, 1982). This hypothesis is corroborated by our results. Parental brood care accordingly evolved only once in the Silphidae at the base of the genus *Nicrophorus* and has since been refined to considerable complexity (Eggert and Müller, 1997; Scott, 1998).

The subfamily Silphinae is, by far, more heterogeneous with respect to the number of genera, their geographic distribution, and the sequence divergence between them. Few discrepancies exist between the methods of analysis used, although the bootstrap support for the branching order of the genera is rarely strong. The Australian genus *Ptomaphila*, however, appears with good support as the most basal lineage within the Silphinae. The biogeographic implications of this finding will be discussed below.

The relationships within the well-supported group comprising the genera *Ablattaria*, *Aclypea*, *Phosphuga*, *Silpha*, and *Dendroxena* remain ambiguous. Possibly, the inclusion of more *Silpha* species (Table 1) could improve the resolution in this part of the tree. In addition to the data presented here, we attempted to gain a better resolution of this group using a more slowly evolving gene, the small subunit nuclear rDNA. This gene was too conserved, however, to provide sufficient information to resolve this group. The data presented here suggest that the genus *Silpha* is paraphyletic and that at least *Aclypea* is nested within it. A pending revision is going to join at least *Ablattaria* and *Phosphuga* into the genus *Silpha* (A. F. Newton Jr., pers. commun.). The support for a paraphyletic *Silpha* in our data is, however, not strong, because constraining the genus to be monophyletic in the maximum-parsimony analysis resulted in a tree that is 10 steps longer but, except for the position of *S. tristis*, congruent with the unweighted maximum-parsimony tree presented here. The difference between these two trees was not significant in a test comparing tree length (Templeton, 1983; $Z = -1141$, $P = 0.157$). There was also no significant difference between the constrained tree and the maximum-likelihood tree in a likelihood ratio test (Kishino and Hasegawa, 1989) that compares the likelihood scores of both trees under the simplest maximum-likelihood model used here (HKY85, ti/tv ratio estimated from the data, all sites evolving at equal rates: Δ ln likelihood = 8.494, $T = 0.077$, $P = 0.939$). As for the remaining taxa in the group, additional evidence resolving *Ablattaria laevigata* and *Phosphuga atrata* as sister groups supports the phylogenetic hypothesis suggested by our maximum-likelihood tree. Both species share a derived feeding biology within the Silphidae. They are specialized snail hunters and display similar morphological changes, namely a very slender and protruding head, an adaptation that allows them to enter the shells of their prey.

Biogeography

The family Silphidae apparently originated in the Palearctic, because this is where most genera and the highest number of species occur (Peck and Anderson, 1985; cf. Table 1). Our phylogeny agrees with this hypothesis and an original splitting of the two subfamilies in the Palearctic. Since the Australian genus *Ptomaphila* appears as the most basal lineage within the Silphinae, it is most likely that the colonization of Gondwanaland by the ancestor of *Ptomaphila* and its undoubted South American sister genus *Oxelytrum* (Peck and Anderson, 1985) occurred before the speciation events giving rise to the other genera of the Silphinae included in our analysis.

Several genera within the Silphidae have a Holarctic or even wider distribution (Table 1). In some cases, such as *Necrodes*, the species belonging to these genera are separated by large gaps. For all Holarctic genera, i.e., *Necrodes*, *Nicrophorus*, *Oiceoptoma*, and *Thanatophilus*, our analyses support the current classification and the monophyly of the genera. The 2 species of *Nicrophorus*, *N. investigator* and *N. vespilloides*, which have been described as native to both North America

and Europe, are supported as each others' closest relatives by our data. Peck and Anderson (1985) hypothesized that the 20 *Nicrophorus* species distributed across North and South America belong to four well-defined groups, *N. vespilloides* and *N. defodiens* representing one group and *N. investigator* another group, that may be descended from different ancestors that independently colonized North America. According to their hypothesis, both *N. investigator* and *N. vespilloides* originated in the Palearctic, and owing to their northerly distribution and concomitant cold tolerance, would have been able to migrate across the Bering land bridge into the Nearctic up until the end of the Pleistocene. This hypothesis could be assessed only, however, by a study sampling a much larger fraction of the species and the geographic range covered by *Nicrophorus*.

CONCLUSIONS

Our sequence analysis provides the first phylogeny of the biologically interesting family Silphidae that will permit reconstruction of the evolutionary events leading to their feeding specializations. Our study further pushes the limits of the taxonomic level at which resolution can still be satisfactorily achieved using mitochondrial gene sequences of the cytochrome oxidase genes. In comparison to other data sets at the same or higher taxonomic level, sampling in this study was more intensive, both in terms of species numbers and sequence length. Although the data set displays some evidence for third position saturation, the phylogeny can be resolved with confidence because the phylogenetic information is well distributed among codon positions and across the historic splitting events.

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