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DNA-Based Identification and Molecular Systematics of Forensically Important Sarcophagidae (Diptera)*

ABSTRACT: Sarcophagid flies have many characteristics that make them ideal forensic indicators. However, their utility is severely limited because it is difficult or impossible to determine the species of a sarcophagid larva, and in many instances an adult specimen, based on anatomy. We developed a database of mitochondrial DNA sequence data that makes it possible to identify all sarcophagid species likely to be found feeding on a human corpse at an urban location in Canada or the USA. Analyses were based on a 783 base pair region of the gene for cytochrome oxidase subunit one (COI). The species analyzed, including some of no forensic importance that were included for purposes of phylogenetic comparisons, were members of the genera *Sarcophaga, Peckia, Blaesoxipha, Ravinia, Wohlfahrtia, Brachicoma* (all Sarcophagidae), and *Musca* (Muscidae).

KEYWORDS: forensic science, entomology, mtDNA, cytochrome oxidase, species identification, maggot, death investigation, postmortem interval estimation, molecular systematics

The larvae of sarcophagid flies (commonly called "flesh flies") potentially are among the most useful insects for investigation of suspicious human death. These flies are widespread and common. They are often the first insects to colonize a corpse, and we suspect that the large size of many species makes them particularly likely to be noticed and collected by a forensic investigator. Furthermore, in the experience of the senior author, the Sarcophagidae may be the only carrion-fly larvae found on a body that is physically isolated, e.g., covered by garbage. This may, in part, reflect the fact that, in contrast to other forensically important flies, sarcophagids deposit live larvae rather than eggs, and these may be dropped in the vicinity of the corpse when an adult female cannot physically reach the body (1). Although sarcophagid larvae and adults are easily distinguished from other dipteran families such as the blow flies

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(*Calliphoridae*) (2,3), the forensic utility of the Sarcophagidae has been greatly hampered by our inability to recognize the individual species. Identification of specimens is an essential first step during a forensic entomological analysis, the aim of which is usually estimation of the postmortem interval (PMI) (4). The species of sarcophagid larvae likely to be found feeding on a human corpse cannot be separated based on anatomy, even to the level of genus, and for many species it is only the adult males that can be identified with certainty.

Although variation in rearing methods makes it difficult to compare many studies of sarcophagid development, it appears that widespread and common species with overlapping ranges can grow at very different rates. For example, the most rapidly developing individuals of *Sarcophaga* (=*Neobelliera*) *bullata* required 16 days to reach the adult stage at 26.7°C (5), while the most rapid *S. africa* (=*S. haemorrhoidalis*) required 10.5 days at the same temperature (6). Thus misidentification of a sarcophagid specimen could produce an error of greater than one day when estimating PMI.

The use of mitochondrial DNA (mtDNA) to identify the species of forensic specimens has been demonstrated for vertebrate (7,8) and insect tissues (9–11). In this study we developed an mtDNA sequence database that permits the identification of all sarcophagid species likely to be found feeding on a corpse in a habitat other than pristine forest within Canada or the USA. We refer to urban areas as well as nonpristine "nature" such as gardens, open parks, and agricultural land. The sarcophagid faunas of natural forests in these countries and of various kinds of habitats in other regions of the world include carrion-feeding species that we have not examined, and there is a huge number of sarcophagid species that do not inhabit vertebrate carrion. We also included species representing genera that normally feed on either feces or live mammalian tissue. Because such larvae may inhabit a body prior to death, a failure to distinguish them from a carrion-breeding species could lead to an overestimate of the time since death (12). If properly identified, such species may provide evidence of antemortem abuse or neglect.

Materials and Methods

Specimens

DNA was extracted from adult male sarcophagids (Table 1). Single specimens of each species were used with the exception of *Sarcophaga argyrostoma* and *S. crassipalpis* for which two specimens of each species were analyzed. The samples included the 9 sarcophagid species likely to be found feeding on dead human tissue in

TABLE 1—*Specimens used in this study.*

Species	GenBank Accession No.	Location	Method‡ and Duration of Preservation
Sarcophaga bullata Parker	AF259506	Hopland, CA	FF, 0.75 yr
S. cooleyi Parker	AF259507	White Mts., CA	ETOH, 0.75 yr
S. africa Wiedemann	AF259508	Berkeley, CA	FF, 0.5 yr
S. crassipalpis Macquart	AF259510	Berkeley, CA	FF, <0.1 yr
...	....	Birmingham, AL	FF, 1.25 yr
S. ruficornis Macquart	AF259511	Oahu, HI	P, 15.2 yr
S. argyrostoma Rob.-Desvoidy	AF259512	Alexandria, Egypt	P, 1.0 yr
...	....	Ohio S. U. colony	ETOH, <0.1 yr
S. peregrina Rob.-Desvoidy	AF259509	U. Hawaii colony	ETOH, <0.1 yr
Peckia chrysostoma Wiedemann	AF259515	Moorea, Fr. Polynesia	ETOH, 0.75 yr
Blaesoxipha plinthopyga Wied.	AF259514	Hopland, CA	FF, 0.75 yr
Ravinia lherminieri Rob.-Desvoidy	AF259513	Hopland, CA	FF, 0.5 yr
Wohlfahrtia vigil (Walker)	AF259516	Nome, AK	P, 13.25 yr
Brachicoma devia Fallén	AF259517	Lejre, Denmark	ETOH, <0.1 yr
Musca domestica L.	AF259518	U. C. Berkeley colony	FF, <0.1 yr

* Nomenclature follows (13).
† FF = fresh frozen, ETOH = 95% ethanol, P = pinned and dried.

urban areas of Canada and the USA, although several of these are found in other countries as well (13). Representatives of the widespread fecal-feeding genus *Ravinia,* and of a genus that parasitizes warm-blooded vertebrates, *Wohlfahrtia,* were included for reasons described in the introduction. All of the carrion-breeding species we examined are classified as members of the subfamily Sarcophaginae, while *Wohlfahrtia* is in the Paramacronychiinae (13). In an effort to reduce the taxonomic bias in our phylogenetic analysis (see Results and Discussion) we added a second paramacronychiine species that is of no forensic relevance. *Brachicoma devia* is a predator of immature bumblebees. Although much of our sample material had been preserved fresh at −70°C, we had little difficulty processing dried and pinned museum specimens, including a *Sarcophaga ruficornis* that was 15 years old. Vouchers have been deposited in the insect collection of the U.S. National Museum.

DNA Extraction

Genomic DNA from thoracic muscle, or in the case of pinned specimens the entire thorax, was extracted using QIAamp tissue columns (Qiagen Inc., Valencia, CA) following the manufacturer's instructions.

Amplification and Sequencing

The PCR amplification primers and conditions used are described in (14). Automated sequencing of PCR product was conducted using Applied Biosystems (Foster City, CA) technology; either a 377 DNA sequencer using the Dye Terminator Cycle Sequencing Ready Reaction Kit or with a 310 genetic analyzer using the BigDye™ Terminator Cycle Sequencing Kit. The sequence was determined for both forward and reverse DNA strands.

Computer Analyses and Software

Sequences were confirmed and aligned manually using Sequence Navigator (Applied Biosystems, Foster City, CA). All other analyses were performed using PAUP 4.0b2 (15). *Chrysomya albiceps* (Wiedemann) (Calliphoridae, GenBank accession AF083657) and *Musca domestica* L. (Muscidae) were used as outgroups for the phylogenetic analyses.

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ATTTAATCGCAACAATGGTTATTCTCTACTAATCATAAAGATATTGGAACTTTATATTTTATTTTCGGAGCTTGAGCAGGTATAGTAGGA    1557

ACTTCATTAAGAATTCTTATTCGAGCAGAACTAGGCCATCCGGGTGCATTAATTGGAGATGACCAAATTTATAATGTAATTGTTACAGCC    1647

CATGCTTTTATTATAATTTTTTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAATTGATTAGTACCAATTATACTAGGAGCCCCA    1737

GACATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGACTTTTACCCCCAGCATTAACATTACTTCTAGTAAGTAGTATAGTAGAAAAC    1827

GGAGCTGGAACAGGATGAACTGTTTACCCTCCTTTATCTTCTAACATCGCCCATGGAGGAGCTTCTGTTGATTTAGCCATTTTTTCCCTA    1917

CATTTAGCCGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTATTACTACAGTTATTAATATACGATCTACAGGTATTACATTTGATCGA    2007

ATACCTTTATTTGTTTGATCTGTAGTAATTACAGCTTTACTTTTACTTCTTTCCCTACCTGTACTTGCTGGAGCAATTACTATACTATTA    2097

ACTGATCGAAATATTAATACTTCATTCTTTGACCCTGCAGGAGGGGGAGATCCAATTCTTTATCAACATTTATTTTGATTCTTTGGACAT    2187

CCTGAAGTTTATATTTTAATTTTACCAGGATTTGGAATAATTTCCCATATTATTAGTCAAGAA    2250
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FIG. 1—*Sequence of a portion of the gene for cytochrome oxidase subunit one from* Sarcophaga bullata. *Base position numbers correspond to homologous positions in* Drosophila yakuba *(16).*

Results and Discussion

Sequence data from a 783 bp region of COI corresponding to positions 1468–2250 of *Drosophila yakuba* (GenBank accession number NC_001322, 16) were obtained for all specimens (Figs. 1 and 2).

DNA-Based Identification of Specimens

Once sequence data have been obtained from an unidentified specimen they can be quickly compared to the huge collection of identified, or "type," sequences accessible using the online BLAST search engine of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). An exact match with one and only one species represented by a COI sequence in the database will reveal the species of the unknown specimen.

However, in many cases there will not be an exact match because of intraspecific haplotype variation or because the species is not represented in the database. Preliminary observations support a difference in the magnitude of intra- versus interspecific sequence divergence in these insects. A world-wide survey of *Chysomya albiceps* and *C. rufifacies* (Calliphoridae) found that these flies, which

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          7215847780367893582403492352480369256845702581736281570369258171369025681406 9
S. coo.   .............................GTCT......TC...T......G.....G............T...
S. afr.   ......................T.TCT....T..T......C................G.......TTC.
S. per.   .....C................T.TCT.....C...TC.....G.........CG.....T..T.TT
S. cra    ........C.......A....C......T.T.T.......T....T........G....C..T.......TTCT
S. ruf.   ........C.......A....C......T.TCT.........T............CC.T....T..TT.T
S. arg.   ................A....C.C.A...T.T.......T....T...........CC.T.......TT..
R. lhe.   G..G.....TT...T.A...T.......T.A.AA........T..T...C....TC.......GT.....TTTT..
B. pli.   .....CC..T....TCA...G......TT.A.A..CTT.CT..C.T...CC...T.........T.....T.AT..
P. chr.   ....A.C..T.........ATC.....CT.T.AATC....T...CT...C...G....T...C......T...T..
W. vig.   GG.T...CAT....T.A.......TA..T.A.TAT..TA.TC......G.C...T..C.TC...TTTAGT.G.TA.
B. dev.   .G......C..AT.T.AAT.T.TCTAATT.A.AA.....T...CT.C.C.AT...........TTA.T..T.AT
M. dom.   ..TT....C....ATT...A....TA.TT.ACT.TC...CT.T.CT...C....T.....G....TTTA.T..TTA.


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          5810379025812478013425776921470136791258247036057145703658270358173467925681
S. coo.   .........T.......................A...TTT.......T.....C.T...................
S. afr.   G.C......TT.....G......T..TA.........TTT.......T..T.C..T..C.........A..AC.TG
S. per.   .........T.....C....CC.T.............TT...T....TCC..T..TA.............TT.....
S. cra    ................G......T.GG..........TTT.......T..T....T........A......A..C.
S. ruf.   .........T...........C.........A...TTT.......T..T.T..........A......A..C.
S. arg.   ...A.....TT.......A....T.........A...TTT......CT..T.TC.T.......A.........T.
R. lhe.   .....TACTT.TCC...TA......T...AA......TTT...C..CTCCT....AA..TT.......A..A..TC
B. pli.   .......CTT.T.....TA....T..T..........TT...A..A.CT.TC.TAT.....A.........T.
P. chr.   ....C....T.......T...T....TAA......TTT..G....A..T.T..A......A.A...G..A..C.
W. vig.   .....TACCTT......TAT.....TT...ACTAG..TTTA....G.ACCT.T.GT.T.GT.AT....A..A....
B. dev.   .....TAC..T...T..TAT....G.....CC.....TTT...C.A.T.CTT...ACT.GTTA.A.TGA..A.T..
M. dom.   .G...TA..TT...TC.TATA.CG..T.TA...A.ATTTT.T..A..T.CT.TC.A......AT.C..A..A...C


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          0111122333344444555555666677777888990011112223333444455566677888999912222222 333
          4034628124703479023568124701369258240302581470369258157067281470699403479256
S. coo.   C.................T........................A.C.TAC.....GC.............
S. afr.   .....A....C.CC....TA..TT......C.T..T........T..G..A..T.A.......G...C.......TC.
S. per.   .G...AA...........TA..TT.CT..C....AT........T.T...A.C..AC.C.T..C.AC.T.....T..
S. cra    ......A.....CC......TATT.G...A.T..T..C...CTTC.A..A..TTACCC.TCTC.....C....TC.
S. ruf.   ......A......C......TATT.....A.T..T..C..C.C....A..A.CTTACC..T.TC.....CG...T..
S. arg.   ..CCT.A....C....CC...A...C..A....T.C......T.TA.A....A.C..T.T...........T..
R. lhe.   ......A.A.CT..TACT..TAAT.AT....T..T..C.....T..A..A.C.TAC......C.....C...CT..
B. pli.   ...........T..TA......TT......T...T........A.....ACC.C........T.......
P. chr.   .........T..CCTA......TT.A...A...AT.A....A...TAT.A..T.....CTC.C....G.....T..
W. vig.   ...C..C..G.T..TACT...A.T...TA...C..TA.C...CT..C.TA..T......T.GCA..CT.T...AC.
B. dev.   ..CC..A.......TA....TAT.TATTAA...AT.A.C....T.T...AG....C...T.T.A......TG.T.G
M. dom.   ......AT...TA.TA..TATAT.T.T....T......TA......AT.AT....CC.C....A...T.....T..
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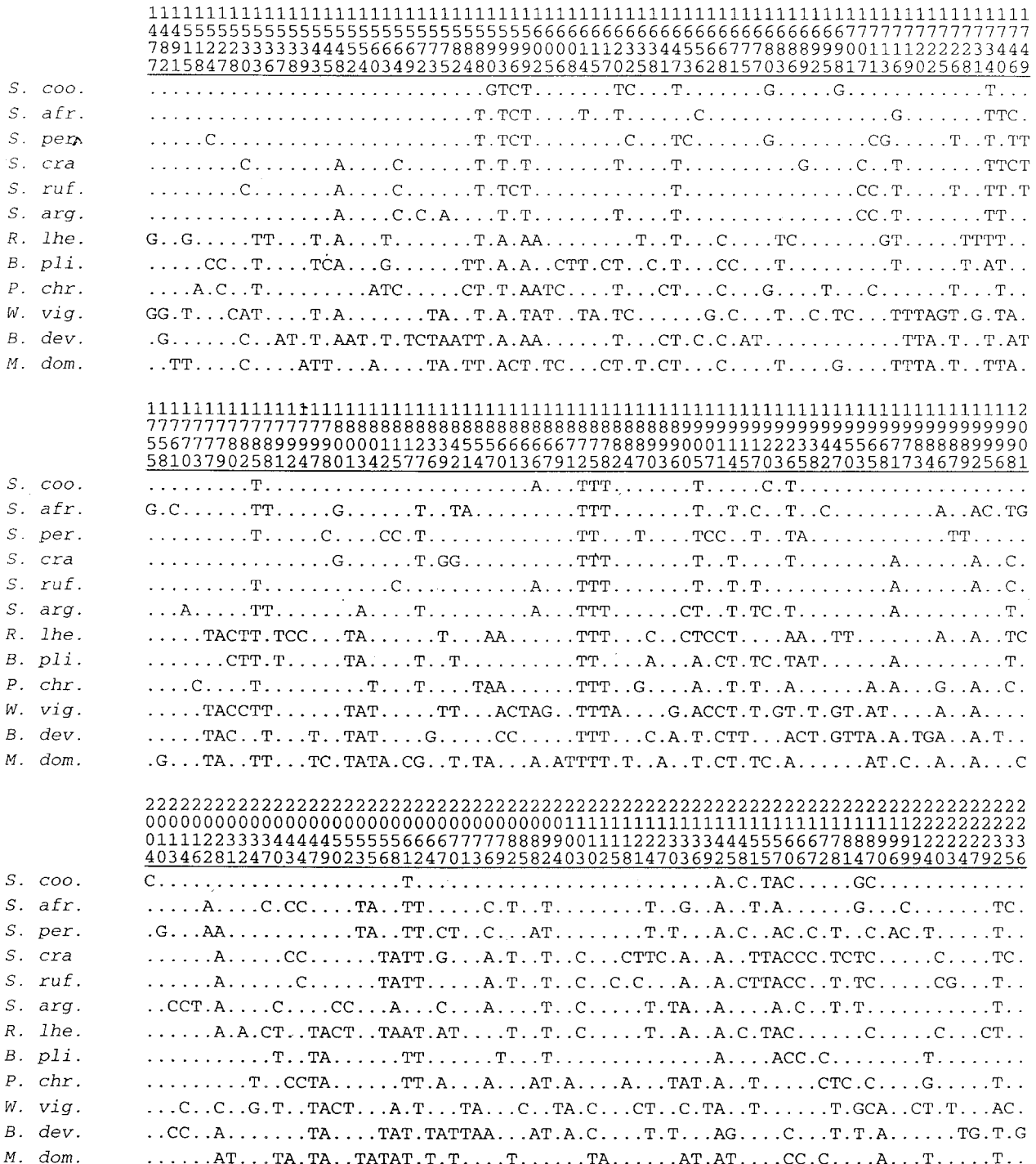
FIG. 2—*All mitochondrial DNA sites that varied between the species examined in this study. Base position numbers are those used in Fig. 1. A dot indicates the same nucleotide found in* Sarcophaga bullata. *See Table 1 for a full description of species.*

TABLE 2—*Pair-wise percent sequence divergence for a 783 bp region of COI. See Table 1 for a description of specimens.*

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14
1. *M. domestica*	…													
2. *C. albiceps*	9.7	…												
3. *B. devia*	12.3	12.4	…											
4. *W. vigil*	12.9	13.2	12.4	…										
5. *R. lherminieri*	12.1	11.4	12.1	12.1	…									
6. *B. plinthopyga*	11.0	10.4	12.9	12.4	8.7	…								
7. *P. chrysostoma*	12.0	9.8	12.5	13.5	11.2	9.1	…							
8. *S. bullata*	12.2	10.7	12.2	12.8	10.1	7.5	8.4	…						
9. *S. cooleyi*	11.2	10.6	12.6	12.8	9.7	7.7	8.7	3.5	…					
10. *S. africa*	12.0	10.5	13.8	13.2	10.7	9.1	9.2	6.6	6.3	…				
11. *S. peregrina*	12.8	11.0	13.7	14.7	10.9	9.3	9.2	7.0	6.1	7.7	…			
12. *S. crassipalpis**	12.8	11.1	12.5	13.8	9.6	9.5	8.4	7.5	6.8	6.3	8.4	…		
13. *S. ruficornis*	11.8	9.8	12.3	13.0	8.8	8.9	7.9	6.6	5.6	7.2	7.2	3.3	…	
14. *S. argyrostoma**	12.0	10.3	11.9	12.9	10.0	8.6	9.2	6.8	6.0	7.4	8.0	6.1	5.4	…

* Identical sequence was found for two specimens (see Table 1).

are closest relatives and almost indistinguishable based on adult or larval anatomy, showed less than 1% sequence divergence within species and about 3% divergence between species (14). A USA-wide survey of *Phormia regina* (Calliphoridae) found ≤0.4% divergence in COI sequence (17). In this study we found no variation between specimens of *S. argyrostoma* or of *S. crassipalpis,* and greater than 3% sequence divergence between sarcophagid species (Table 2). If we knew that all carrion fly species displayed distinctly less divergence in COI sequence within compared with between species, then it would be possible to identify an unknown specimen based on some standard of percent similarity to a type sequence. Our limited knowledge of haplotype diversity within carrion-fly species makes this unwise at the present time.

Instead, identification is best accomplished by including the unidentified haplotype in a phylogenetic analysis including all available members of the same taxonomic group. The result will reveal the closest relative(s) of the unknown specimen to be found in the database. This will provide exact identification of the unknown if it is paired with a type sequence and if one is confident that no potentially better candidate species was excluded from the analysis. The results may fail to provide exact identification because of an incomplete set of type sequences or because the relationship of the unknown specimen is unresolved, i.e., it is grouped with >1 species with no indication of which is the closest relative. Even if this were to occur, such analysis will narrow the choice of species and an investigator may learn enough about the specimen to make useful forensic inferences. For example, if phylogenetic analysis paired a haplotype from an unidentified specimen with the *Ravinia lherminieri* haplotype we present here, this would strongly suggest that the unknown specimen was a member of the same genus. With this knowledge an investigator would be alerted to the possibility that the larva had been present in feces prior to the victim's death.

There is a wide selection of methods for constructing phylogenetic trees from DNA sequence data (18). In order to evaluate the effect choice of method may have on using phylogenetic reconstruction to identify a sarcophagid specimen we applied three common and easily implemented analyses that rely on very different algorithms. These were: 1) maximum parsimony with equal weights for all substitutions, 2) maximum likelihood allowing the program to estimate transition and transversion rates, and 3) neighbor joining based on log-determinant/paralinear distance (18).

The maximum parsimony tree, also called a cladogram, is shown in Fig. 3. This is a reasonable hypothesis of sarcophagid relationships based on our current knowledge (see below). The maximum likelihood tree and neighbor-joining trees were slightly different,

e.g., likelihood placed *Peckia* within the genus *Sarcophaga* and neighbor joining paired *Blaesoxipha* with *Ravinia,* but no difference between the topologies of these three trees had majority bootstrap support. Furthermore the most forensically important lineages with bootstrap support in the parsimony tree ((*bullata* + *cooleyi*) and (*argyrostoma* + (*ruficornis* + *crassipalpis*)) had bootstrap support in the other phylogenies. We believe that these similar results produced using different methods supports the robustness of the phylogenetic signal in our data set.

Although we find PAUP to be a particularly user-friendly software package, several other programs appear frequently in the systematic literature and there is little reason to think that they will not find the same optimal solution produced by PAUP. For example, maximum parsimony analyses using the default settings for the phylogenetic computer programs Hennig86, NONA, and Pee-Wee (for a comprehensive list of such software packages, many of which can be downloaded for free, see (18)) each produced the same single most parsimonious tree shown in Fig. 3. We are confident that investigators can employ phylogenetic analysis to identify fly specimens using the software that best fits his or her operating system, budget, and other analysis needs.

We emphasize that this data set was designed for identification of sarcophagid species found in locations in Canada and the USA that have been disturbed by human activity (see the introduction). It may be, however, that an experienced entomologist working in another situation can use our data to identify a specimen if he or she uses detailed knowledge of the local fauna to narrow the choice of possible species to those presented here.

Utility of COI for Phylogenetic Analysis of Sarcophagid Flies and Their Relatives

Although our primary purpose was to develop a forensic tool, these data provide an opportunity to evaluate the potential value of COI for basic biological studies of these insects. Many aspects of sarcophagid systematics are poorly understood, and the family is a part of a larger taxonomic group, the Oestroidea, for which a basic understanding of evolutionary relationships remains to be achieved (19). In such a situation a new source of phylogenetic information would be useful.

Figure 3 agrees with some hypotheses of sarcophagid relationships for which there is morphological support, and is not in conflict with previous hypotheses. These aspects of the cladogram are: 1) monophyly of the genus *Sarcophaga,* the subfamilies Paramachronychiinae and Sarcophaginae, and the family Sarcophagi-
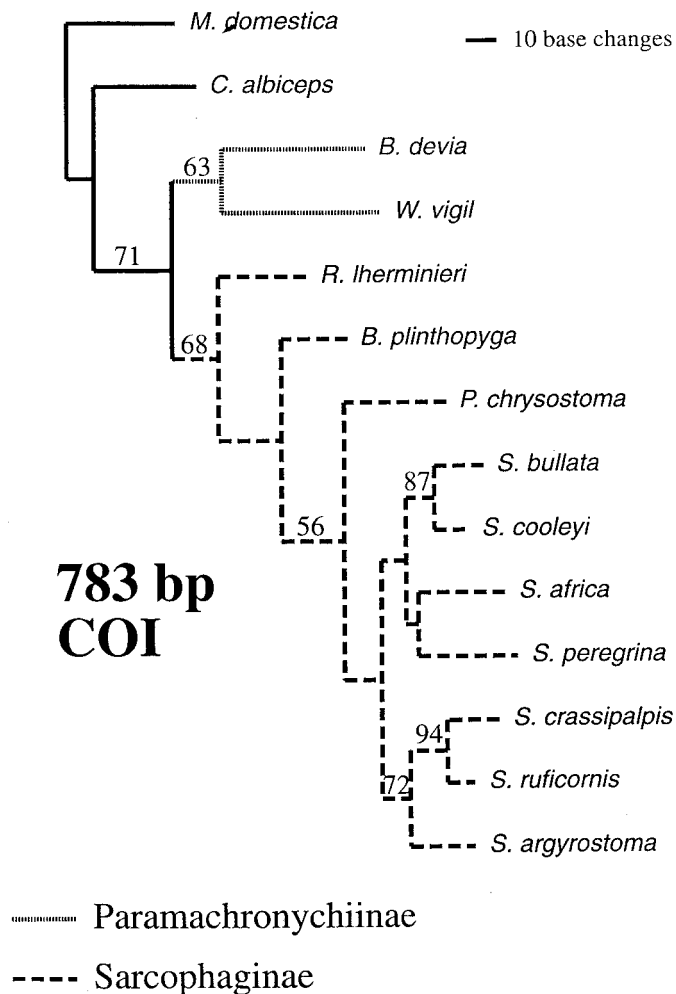
FIG. 3—*Single most parsimonious cladogram (heuristic search with 2000 random step-wise additions) based on a 783 bp region of mitochondrial DNA. Numbers on branches indicate percent bootstrap support if greater than 50% (5000 reps.). See Table 1 for a full description of species.*

dae, 2) the inclusion of *S. bullata* and *S. cooleyi* together in the subgenus *Neobellieria,* and of *S. argyrostoma, S. ruficornis,* and *S. crassipalpis* together in the subgenus *Liopygia* (13), and 3) the basal position of *Ravinia* within the Sarcophaginae (20). The topology corroborates an emerging pattern of early sarcophagine diversification in the New World (20, unpublished). The nested position of the New World *S. bullata* and *S. cooleyi* within the now widespread but originally Old World *Sarcophaga* spp. may seem at odds with a possible New World origin of the genus *Sarcophaga,* but on our cladogram this aspect of the topology is without bootstrap support and would as such be considered tentative at best. Moreover, sequences from many more exemplar taxa need to be incorporated into the analysis to make proper deductions on this issue. Because analysis based on COI is in agreement with those based on anatomy, we predict that it will be a useful genetic marker for further systematic studies of this and other oestroid families.

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