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Identification of a Death-scene Maggot using Standardized Molecular Methods: *Sarcophagabullata* Parker 1916 (Sarcophagidae) Out-numbers Blowflies (Calliphoridae) on an Urban Cadaver in Southeastern Texas

Rekha Raghavendra[1], Christopher P. Randle[2] and Sibyl Rae Bucheli[2]*

[1]*School of Medicine Case Western Reserve University, Cleveland OH, USA*
[2]*Department of Biological Sciences Sam Houston State University, Huntsville, TX, USA*

Abstract

In forensic entomology,fly data including maggot age are frequently used to help estimate the time since death.Accurate identification of the maggot to species is critical for time since death estimations. However, within a family, maggots are notoriously difficult to identify to species.In this study, we employ phylogenetic datafrom the mtDNAgenes COI and COII to identify an unknown maggot to species (member of the family Sarcophagidae) harvested from a cadaver in June 2009 in Harrison County, Texas. The most closely related species to our unknown maggot was SarcophagabullataParker 1916, a somewhat common carrion-feeding species in southeastern United States that is now gaininggreater recognition as a forensically significant species.

Keywords: Forensic entomology; case study; Sarcophagidae; Sarcophagabullata Parker 1916

Introduction

Decomposition of a large mammalian carcass is greatly accelerated through the action of insects belonging to the order Diptera (flies) [1]. In southeastern Texas, initial colonizers include members ofthe families Calliphoridae (blow flies), Sarcophagidae (flesh flies), and Muscidae (house flies), with blow flies and flesh flies often arriving and laying eggs or giving birth tomaggots (rather than laying eggs) within minutes of death (unpublishedrecords from cadavers at the Southeastern Texas Applied Forensic Science Facility at the Center for Biological Field Studies at Sam Houston State University). Maggots acquirebiomass as a function of physiological time rather than calendar time and therefore develop at a predictable rate. Since flies arrive and lay eggs or maggots immediately, they are considered useful tools for estimation of the time that has elapsed since death, or the postmortem interval (PMI), by estimating the time since maggot colonization [1-7]. By recreating the conditions of the death scene in the laboratory and working backwards through time to determine the age of the oldest maggot, the forensic entomologist can correlate the age of the maggot to the PMI [1-7].

Identification of maggots to species remains a challenging aspect to forensic science even though maggots are frequently collected evidence during a death scene investigation. Identification keys are not currently available for all life stages are not currently available andmaggots are difficult to identify particularly at early life stages because morphological features among maggots are similar, rendering them virtually undistinguishable beyond the family level [8-12]. Molecular data can aid in the identification of larvae where morphology is limited in utility [8-12]. In this study, we employ an established phylogenetic protocol by Wells et al. [10] using the mitochondrial DNA genes of COI and COIIto identify an unknown maggot of the family Sarcophagidae harvested from a cadaver in Harrison County, Texas.

Materials and Methods

Specimens: The unknown maggot was recovered from a body discovered in June 2009 in Harris County, TX, and was the largest observed maggot and most abundant larval type; in fact, no other species were collected despite law enforcement agents reporting the remains to be in a state of fresh/bloated decomposition.The unknown maggots were identified as members of the family Sarcophagidae using standard morphological features of the spiracular complex but could not be further identified (Peterson 1960). Common species of Sarcophagidae which frequent cadavers in this area include *Sarcophaga (Neobellieria)bullata* Parker, 1916 and *Sarcophaga (Bercaea) africa* (Wiedemann 1824: 49) (=*cruentata* Meigen 1826;=*haemorrhoidalis* auct.) [14-16]. Proper species identification is critical to generate proper growth curves for age estimation; Wells et al. [10] demonstrate that these two species grow at rates disparate enough to create as much as a 24 hour discrepancy.

DNA extraction: Genomic DNA was extracted from the unknown maggotstarting with tissue homogenization using a Disruptor Genie TM and followed by a standardChelex DNA extraction method [17].

Amplificationand Sequencing: PCR protocols were modified from Wells et al. [10] using their published primers for COI and COII in various combinations (Table 1) and carried out in 50 µl volumes including 1X PCR buffer (Promega, Madison WI), 0.4 µM forward and reverse primers, 0.2mMdNTPs, 2.5UGoTaq polymerase (Promega, Madison WI), with 3 µl of template DNA. PCR reaction conditions were as follows: 94°C for 2 min(initial denaturation), continued with 35 cycles of 94°C for 1 min (denaturation), 50°C for 1 min (primer annealing), 72°C for 2 min (extension), and 72°C for 10 min (final extension). PCR products were visualized on 1% agaroseand purified

***Corresponding author:** Sibyl Rae Bucheli, Department of Biological Sciences Sam Houston State University, Huntsville, TX, USA, Tel: 936-294-1550; Fax: 396-294-3940; E-mail: bucheli@shsu.edu

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using a QIAquick PCR Purification Kit (QIAGEN INC., Valencia, CA). The COI and COII regions were sequenced on a Bechman-Coulter 8000 CEQ Genetic Analyzer using the GenomeLab DTCS Quick Start Kit method.Contig assembly was performed using Geneious [18].

Selection of Sequences for comparison: NCBI nucleotide MEGABLAST was used to identify 100 closest Genbanksequence matches to the unidentified maggot sequence.These were downloaded, and aligned with the unknown sequence using the MUSCLE [19] algorithm in Geneious [18]. Of these, 81 sequences were chosen that had sufficient overlap with each other and the unknown sequence to allow unambiguous alignment. Multiple accessions for species were retained when available, including two sequences each from *Sarcophaga africa* and *S. bullata*. Additionally, a sequence obtained from *Eucalliphora latifrons* (Calliphoridae) was selected as an outgroup. The reduced matrix was realigned using the MUSCLE [19] algorithmfor phylogenetic analysis.

Phylogenetic Analysis: Maximum parsimony searches were conducted using WinClada [20] as a shell program. The parsimony ratchet [21] was implemented with 200 iterations (10% of the matrix sampled; one tree held per iteration). The tree generated via the ratchet search was the starting tree for a more thorough analysis conducted in NONA ver. 2.0 [22] using the commands "rs 0; hold 1000; mult* 50." Parsimony jackknife percentages (23) were calculated in NONA ver. 2.0 [22] with 1000 replications (200 search steps; one starting tree per replication; rs 0). For maximum likelihood (ML) analysis, the most appropriate stationary model of evolution was inferred using the Akaike Information Criterion [24] in jMODELTEST [25,26]. ML searches were performed using GARLI 1.0 (27) using the default configuration. One thousand non-parametric bootstrap replicates were analyzed with two search replicates each to obtain clade support. Phyutility [28] was used to generate the majority rule consensus of 1,000 bootstrap trees.

Results

NCBI nucleotide MEGABLAST returned a COI sequence from *Sarcophaga bullataas* the best sequence match with 97% sequence identity, while *S. africa* obtained 92% identity and the outgroup, *Eucalliphora latifrons* obtained an identity of 86%. Sequence alignment resulted in a matrix of 2,305 characters. Parsimony searches resulted in a single most parsimonious trees (L=3921;CI=0.30; RI=0.65). AIC identified GTR+Γ as the best fitting model of evolution. The maximum likelihood tree obtained anln likelihood score = -21,146.499. MP and ML trees were largely congruent, differing only in the resolution of clades that were poorly supported and inconsequential in the identification of the known sequence. While overall clade support was low, the two most probable species matches, *Sarcophaga africa* and *S. bullata* were separated by several strongly supported nodes (jackknife and bootstrap >80%; Figure 1 (included as supplementary data)). Both parsimony and likelihood identified the unknown sequence as sister to the two sequences of *Sarcophaga bullata* (jackknife =100%; bootstrap = 67%). *Sarcophaga polistensis* (jackknife =85%; bootstrap = 89%) is sister to this clade, and *S. cooleyi* is sister to the clade including *S. polistensis-S. bullata* (jackknife =95%; bootstrap = 97%). *Sarcophaga polistensis* occurs in Texas, but is not known to feed on carrion [14]. *Sarcophaga cooleyi* is not known to occur in Texas. Therefore, evidence best supports the hypothesis that the unknown maggot is *S. bullata*.

Discussion

Many modern forensic techniques that employ DNA profiling to make associations between individuals and individuals, individuals and locations, and/or individuals and events (such RFLP analysis, PCR analysis, STR analysis, AmpFLP, DNA family relationship analysis, Y-chromosome analysis, mitochondrial analysis) [29] are sound due to the process of evolution acting on marker loci. Marker similarityis interpreted as evidence for shared ancestry [30]. Overall, the process leads to situations where more closely related organisms share in common more regions of their DNA. In most situations, DNA profiling analyses are based in principles of phylogenetics (the study of evolutionary relatedness among groups of organisms) and population genetics (the study of the effects of evolutionary processes on allele frequenciesin populations) [30,31]. In a growing number of situations, it has been useful to extend methods commonly employed in human DNA analyses to non-human organisms (for a discussion see 29). For species identification of unknown organisms, modern methods of

Location on the mtDNA			
Primer Sequence	Paired combination of primers used in this study		
1	TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	2, 4
2	C1-N-1687	CAATTTCCAAATCCTCCAATTAT	1
3	C1-J-1751	GGATCACCTGATATAGCATTCCC	6, 8
4	C1-N-1840	AGGAGGATAAACAGTTCAC/TCC	1
5	C1-J-2183	CAACATTTATTTTGATTTTTTGG	11
6	C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	3
7	C1-J-2319	TAGCTATTGGAC/TTATTAGG	10, 13
8	C1-N-2293	AGTAAACCAATTGCTAGTATAGC	3
9	C1-J-2495	CAGCTACTTTATGAGCTTTAGG	13, 14
10	C1-N-2514	AACTCCAGTTAATCCTCCTAC	7
11	C1-N-2659	GCTAATCCAGTGAATAATGG	5
12	C1-J-2792	ATACCTCGACGTTATTCAGA	16
13	C1-N-2800	CATTTCAAGT/CTGTGTAAGCATC	7, 9
14	TL2-N-3014	TCCAATGCACTAATCTGCCATATTA	9
15	C2-J-3138	AGAGCCTCTCCTTTAATAGAACA	18
16	C2-N-3389	TCATAAGTTCA[R]TATCATTG	12
17	C2-J-3408	CAATGATAT/CTGAAGT/ATATGA	18
18	TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT	15, 17

Table 1: PCR primers* used in this study.* Primers were taken from Wells et al. [10]. N-forward primer; J-reverse primer.

phylogenic analyses are the preferred method. In a now famous paper, Scadutoet al. [32] demonstrate the source of transmission of HIV strains by standard and rigorous phylogentic analysis (using maximum likelihood and Bayesian estimators). Such methods are frequently employed in insect identification (for a forensic focus on Calliphoridae and Sarcophagidae only see: [8-12,33-36]).

The intent of this study was identification to species of the largest larval flies harvested from a cadaver by using established phylogenetic protocols. These protocols have only been worked out for controlled situations and have not been used "in the field." Larvae were identified initially by standard morphological methods as members of the family Sarcophagidae. Both parsimony and likelihood trees generated from COI and COII mtDNA data matrices of GenBank data-based sequences and the largest unknown specimen strongly allied the unknown sequence as sister to *Sarcophaga bullata*. (Figure 1 (included as supplementary data)) shows all species included in the analysis and their GenBank accession numbers. In the analysis, Sarcophagidae forms a monophyletic group. Analysis of reference sequences downloaded from GenBank database shows little variation between species with different accession numbers. This suggests that the protocol developed by Wells et al. [10] for the use of reference sequences available in the GenBank database is a sensible tool to reveal identity ofan unknown specimen. Phylogenetic analysis using these reference sequences was able to determine the species of the flesh fly collected from a cadaver and hence and may be used to provide supporting information to aid in the estimation of the time sinceinsect colonization.

The occurrence of *Sarcophaga bullata* as the largest and most abundant species of larval fly recovered from the corpse is note-worthy. Despite the remains being reported by law enforcement as fresh/bloated, this species outnumbered members of the family Calliphoridae (no larvaeof Calliphoridae were recovered). While many published accounts of necrophagous species biodiversity of a corpse note the presence of *S. bullata*, no published accounts rely primarily on data provided by this species as the largest and most abundant member of the community for applied aspects of the science. Anecdotal accounts of the utility of this species in forensic applications exist; an entry made on the open-access on-line Encyclopedia Wikipedia discusses their forensic importance. Their abundance in this situation may be explained by the location of the corpse and time of death in terms of season. In June, southeastern Texas (Houston and surrounding cities) experiences average daytime high temperatures above 90˚F/32˚C, nighttime lows around 70˚F/21˚C, and relative humidity levels that fluctuate widely between 50% at noon and 90% at midnight (average minimum and maximum when not raining) [37]. This generally results in dehydration of tissues of the corpse at an accelerated rate (personal observations made of human decomposition at STAFS at CBFS at SHSU, Bucheli and Lindgren) when compared to published descriptions of cadavers at other forensic anthropology stations through out the United States (2; 3; 5; 6; 38; 39). Furthermore, extensive areas of Montgomery County are urbanized. Unpublished photos of crime scenes from variousHouston, TX, urban and rural locations reveal corpses with fewto no observed species of Calliphoridae and much greater numbers of Sarcophagidae (personal observations, Bucheli). Reasons for the absence of Calliphoridae may include the lack of a constant supply of large, fresh mammalian corpses due to urbanization in certain areas to sustain populations of significant size. The authors recognize this discussion as largely speculative but do so to draw attention to the fact that very little is known regarding the utility of *Sarcophaga bullata* in forensic situations.

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