

# DNA-based identification of forensically important Chrysomyinae (Diptera: Calliphoridae)

Jeffrey D. Wells<sup>a,\*</sup>, Felix A.H. Sperling<sup>b</sup>

<sup>a</sup>Department of Justice Sciences, University of Alabama at Birmingham, Birmingham, AL 35294, USA

<sup>b</sup>Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

## Abstract

Identifying an insect specimen is an important first step in a forensic–entomological analysis. However, diagnostic morphological criteria are lacking for many species and life stages. We demonstrate a method for using mitochondrial DNA sequence data and phylogenetic analysis to identify any specimen of the blow fly subfamily Chrysomyinae likely to be collected from a human corpse within Canada or the USA. The reliability of the method was illustrated by analyzing specimens designed to mimic the information likely to be obtained from highly degraded specimens as well as specimens collected from widely separated geographic locations. Our sequence database may be suitable for another country provided the investigator knows the local fly fauna well enough to narrow the choice of chrysomyine species to those used in this study. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Forensic entomology; Mitochondrial DNA; Molecular systematics; *Phormia*; *Protophormia*; *Chrysomya*; *Comptosomyiops*; *Cochliomyia*

## 1. Introduction

A carrion-fly maggot is by far the most common type of insect evidence collected during a death investigation. Determining the species of such a sample is an important first step in a forensic entomological analysis [1,2]. Blow fly species in the subfamily Chrysomyinae that are likely to be collected from a human corpse in America north of Mexico can be identified using keys for the adult stage [3,4] and third larval instar [5]. Although, species-diagnostic anatomical characters have been reported for the egg and early larval instars of some chrysomyine species (e.g. [6,7]), we are not aware of a comprehensive key covering all immature stages of all forensically important species for any geographic region.

Mitochondrial DNA (mtDNA) can be used to identify all life stages of a carrion fly [8]. This is the second in a series of papers, the first covering the Sarcophagidae [9], in which we present a means for using mtDNA sequence to identify all forensically important flies within Canada and the USA

from a particular taxonomic group. This study was designed to be a comprehensive treatment of carrion-feeding Chrysomyinae for only this geographic region. However, it may be possible for a forensic scientist in another country to rely on our data for identification purposes provided he or she knows the local fauna well enough to narrow the choice to the species presented herein.

Our approach relies on the principle of including sequence data from an unidentified specimen in a phylogenetic analysis of homologous data from identified specimens. By pairing the unknown specimen with its closest relative it may be possible to avoid difficulties arising from our current lack of knowledge concerning intraspecific variation in these insects [9].

## 2. Materials and methods

### 2.1. Specimens

The specimens used for DNA extraction are shown in Table 1. Species listed in the Chrysomyine genera *Phormia*, *Protophormia*, *Cochliomyia*, and *Chrysomya*, with the exception of the obligate parasite *C. bezziana*, are all carrion

\* Corresponding author. Tel.: +1-205-934-2069;  
fax: +1-205-934-2067.  
E-mail address: jwells@uab.edu (J.D. Wells).

Table 1  
Specimens newly sequenced for this study<sup>a</sup>

Species	Genbank accession no.	Location	Method <sup>b</sup> and duration of preservation (year)
<i>Phormia regina</i> (Meigen)	AF295550	Hopland, California	FF, <0.1
<i>Phormia regina</i> (Meigen)	Not submitted	Birmingham, Alabama	FF, 0.5
<i>Protophormia terraenovae</i> (Rob.-Des.)	AF295553	White Mts., California	ETOH, <0.1
<i>Protophormia atriceps</i> (Zetterstedt)	AF295560	Sognefjell, Norway	P, 18
<i>Protocalliphora sialia</i> Shannon & Dobroscky	AF295559	Rigaud, Quebec	ETOH, 2.0
<i>Cochliomyia macellaria</i> (F.)	AF295555	Gainesville, Florida	ETOH, <0.1
<i>Cochliomyia macellaria</i> (F.)	Not submitted	Birmingham, Alabama	FF, 0.75
<i>Comptosomyia callipes</i> (Bigot)	AF295549	Berkeley, California	FF, <0.1
Unidentified second instar larva	Not submitted	Martinez, California	ETOH, <0.1
<i>Chrysomya megacephala</i> (F.)	AF295551	between Lae and Bulolo, Papua New Guinea	ETOH, 1.0
<i>Chrysomya megacephala</i> (F.)	Not submitted	Birmingham, Alabama	FF, 1.0
<i>Chrysomya chloropyga</i> (Wiedemann)	AF295554	Near Chilbre, Panama	ETOH, 4.5
<i>Chrysomya varipes</i> (Macquart)	AF295556	Adelaide, Australia	ETOH, 0.3
<i>Chrysomya norrisi</i> James	AF295552	Wau, Papua New Guinea	ETOH, 1.0
<i>Chrysomya semimetallica</i> (Malloch)	AF295562	Near Hoskins, Papua New Guinea	ETOH, 1.0
<i>Chrysomya bezziana</i> Villeneuve	AF295548	Bogor, Indonesia	FF, <0.1
<i>Eucalliphora latifrons</i> Hough	AF295557	Burnaby, British Columbia	FF, 1.0
<i>Hypoderma lineatum</i> (De Villiers)	AF295558	Lethbridge, Alberta	FF, <0.1

<sup>a</sup> Most specimens were used to produce a reference database of 2.3 kb mtDNA sequence per species. The Alabama specimens of *P. regina*, *C. macellaria*, and *C. megacephala*, as well as the unidentified larva, were used to test our method for species identification based on a 304 bp amplicon. Previously published sequences that were also used are listed in Section 2.

<sup>b</sup> FF = fresh frozen or immediately extracted; ETOH = 95% ethanol; P = pinned and dried.

feeders. *C. varipes*, *C. norrisi*, *C. semimetallica*, and *C. bezziana* are not known to occur in the Western Hemisphere. They were included in order to produce a more reliable phylogenetic analysis (see below). Similarly the chrysomyine *Protocalliphora sialia*, an ectoparasite of nestling birds [10], was also included to assist in clarifying relationships among species. The calliphorine blow fly *Eucalliphora latifrons* and the oestrid *Hypoderma lineatum* were newly sequenced as outgroups for this study. *C. chloropyga* has not been reported north of Panama, but this introduced species may eventually reach the USA [5]. We realize that many scientists consider the *C. chloropyga* found in the New World to be *Chrysomya putoria*, however, we choose to be taxonomically conservative, because Dear [4] did not distinguish between the two species in his monograph.

*C. bezziana* DNA was kindly supplied by an Australian CSIRO laboratory (see Acknowledgements) and was derived from multiple larvae. However, our analytical approach is designed to accommodate intraspecific variation (see Section 3) and even obvious heteroplasmy does not cause problems with interpretation such as may occur with identification of individual humans [11].

We were not able to obtain specimens of the tropical carrion-feeding species *Cochliomyia aldrichi*, *Cochliomyia minima*, or *Chloroprocta idiodea* (= *C. fuscipennis*), which could be found in the USA. However, the two *Cochliomyia* species have only been reported from the Florida Keys and other islands, and *C. idiodea* is rare in

the USA and not likely to be found north of the most southern part of Texas [3].

Initial laboratory work determined the sequence for the mtDNA region including the genes for cytochrome oxidase subunits one and two (COI + II) along with the gene for mitochondrial transfer RNA leucine (tRNA-leu) for one individual of each species. We designated these data as our “reference” sequences and they, along with similar published sequences, constitute our database for specimen identification. In order to evaluate the utility of this database for the identification of insects from a variety of crime scenes, we mimicked the analysis likely to be performed during a forensic investigation. That is, we added additional “test” sequences from chrysomyine flies that were obtained from individuals collected and preserved as a small second instar larva during an actual homicide investigation or as adult flies collected far from the location of the reference specimen (Table 1). These test sequences were very short (304 bp), because forensic specimens are often poorly preserved and only a small amount of sequence data can be obtained. In addition to our own test specimens, we analyzed a 166 bp sequence of a *P. terraenovae* from France (GenBank accession number AF017426 [12]).

## 2.2. Additional published sequences

A number of published cytochrome oxidase sequences obtained from GenBank were included in our reference

database. These were the carrion-feeding chrysomyine species *Chrysomya rufifacies* (GenBank accession no. AF083658) and *C. albiceps* (AF083657) [14]. The primary screwworm *Cochliomyia hominivorax* (AF260826 [20]) is, like *C. bezziana*, an obligate parasite of warm-blooded vertebrates [3]. It was included primarily for systematic purposes, although we can imagine a situation in which larvae of screwworm species would be found in a human killed by an infestation [13]. In such a case, it would be essential, for forensic purposes, to recognize a species in which development begins prior to the host's death. *C. hominivorax* has been eradicated from North America [13], but reintroduction of the species is possible.

The Calliphoridae *Lucilia* (= *Phaenicia*) *sericata* (L14947) and *L. illustris* (L14945) [8] were included as outgroups.

### 2.3. DNA extraction

Genomic DNA from thoracic muscle, or in the case of the pinned specimen the entire thorax, was extracted using QIAamp tissue columns (QIAGEN INC., Valencia, CA) following the manufacturer's instructions. For most tissue samples 300 µl of elution buffer was used. Because the *P. atriceps* specimen had been preserved in a dry state for 18 years, we suspected that it contained relatively degraded DNA. Therefore the *P. atriceps* extraction was eluted in only 100 µl in order to increase DNA concentration. DNA from *E. latifrons* was extracted using a phenol/chloroform method [8].

### 2.4. Amplification and sequencing

PCR primers were purchased from Operon Technologies (Alameda, CA). The primer sequences are listed in Table 2. Amplification of *P. atriceps* template was successful only for primer pairs TY-J-1460/C1-N-1687, C1-J-2495/C1-N-2800, and C1-J-2319/C1-N-2514. Other PCR reagents were purchased from Promega Corp. (Madison, WI). Each 50 µl PCR reaction mix was prepared using 5 µl 10X buffer, 10 pmol each dNTP, 10 pmol each primer, 200 µmol MgCl<sub>2</sub>, 1–15 µl DNA extract, and enough H<sub>2</sub>O to complete the total 50 µl volume. Taq polymerase (0.5–1.0 µl) was added to each tube during the initial denaturation phase.

The thermal cycler program consisted of an initial denaturation step of 95°C for 3 min, followed by an annealing step of 45°C and an extension step of 72°C for 1.5 min. This was followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min. The final cycle was the same as the previous one except for an extension step of 5 min duration.

PCR product was cleaned using a QIAquick PCR Purification Kit (QIAGEN INC., Valencia, CA), and cycle sequencing product was cleaned using spin columns filled with Sephadex<sup>®</sup> G-50 beads (Sigma-Aldrich, Milwaukee, WI). Automated sequencing was conducted with a PE-Biosystems (Foster City, CA) 373 or 377 DNA sequencer using the Dye Terminator Cycle Sequencing Ready Reaction Kit, or with a 310 genetic analyzer using the BigDye<sup>™</sup> Terminator Cycle Sequencing Kit. The sequence was determined for both forward and reverse DNA strands.

Table 2  
PCR primers used in this study<sup>a</sup>

	Location <sup>b</sup>	Sequence	Other primer(s) with which it was paired
1	TY-J-1460	TACAATTATCGCCTAAACTTCAGCC	2, 4
2	C1-N-1687	CAATTTCCAAATCCTCCAATTAT	1
3	C1-J-1751	GGATCACCTGATATAGCATTCCC	6, 8
4	C1-N-1840	AGGAGATAAACAGTTCAC/TCC	1
5	C1-J-2183	CAACATTTATTTTGATTTTTTGG	11
6	C1-N-2191	CCCGGTAAAATATAAACTTC	3
7	C1-J-2319	TAGCTATTGGAC/TTATTAGG	10, 13
8	C1-N-2293	AGTAAACCAATTGCTAGTATAGC	3
9	C1-J-2495	CAGTACTTTATGAGCTTTAGG	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

<sup>a</sup> C1-J-2319 and C1-N-2514 were newly designed for this investigation. The original references for the other primer sequences are given in [14].

<sup>b</sup> Nomenclature of [21]. The numbers indicate the 5' position of the synthesized fragment.

DNA from test specimens was amplified using only the primers C1-J-2495 and C1-N-2800.

### 2.5. Computer analyzes and software

Sequences were confirmed and aligned manually using Sequence Navigator (PE-Biosystems, Foster City, CA). All other analyzes were performed using PAUP 4.0b2 [15].

## 3. Results and discussion

### 3.1. Sequence data

Approximately 2.3 kb of sequence corresponding to base positions 1467–3771 of *Drosophila yakuba* (GenBank accession NC-001322 [16]) was obtained for each of the reference samples with the exception of the 18-year-old pinned *P. atriceps*, for which a total of 678 bp was recovered from the two regions flanked by primer pairs TY-J-1460/C1-N-1687 and C1-J-2319/C1-N-2800. No insertions or deletions greater than two base pairs in length were observed with the exception of an 18 bp insertion relative to the others in the *P. sialia* sequence between the tRNA-leu and COII genes. All new reference sequences have been deposited in the GenBank database (see Table 1 for accession numbers). The single PCR amplicon from each test specimen produced a 304 bp sequence.

The fact that our *C. bezziana* DNA was from more than one individual was probably the reason we observed apparent heteroplasmy in the form of large underlying electropherogram peaks at three positions. These all represented silent substitutions, point mutations that do not alter the amino acid sequence, and this is the type of intraspecific variation in COI + II sequence we have observed in other carrion flies [14,17].

### 3.2. Phylogenetic analysis

There are a variety of phylogenetic tree-building algorithms to choose from depending on an investigator's philosophy and research goals [18]. For the method outlined in this paper, we prefer the commonly used criterion of maximum parsimony (MP) with equal weight for all substitutions [9]. MP work well for purposes of identification (see below), the analysis requires relatively little time to perform on the average personal computer, and we have found it to be relatively easy to explain the analysis to a lay person. However, with this data set as well as our earlier study of Sarcophagidae [9], maximum likelihood (ML) allowing the program to estimate transition and transversion rates appeared to perform equally well for pairing test and reference sequences with >95% bootstrap support (not shown). We say "appeared to perform equally well" because ML is so computationally intensive that with these data an iMac G3 computer with a machine speed of

500 MHz was able to complete only 11 bootstrap replications within a 24 h period. As a result we stopped the analysis after 60 replications. In contrast 1000 MP bootstrap replications on the same computer required 2.5 min. Neighbor joining based on log-determinant/paralinear distance, although fast and easy to implement, performed poorly compared to MP or ML in that the sister-group relationship between most test and reference sequences had 91–92% bootstrap values (not shown).

The single most parsimonious phylogeny of the haplotypes used in this study is shown in Fig. 1. All previously identified test specimens were paired with the correct reference haplotype with  $\geq 97\%$  bootstrap support. Based on this method the larva collected from a human corpse is identified as *C. callipes*, a conclusion that is supported by the large number of adults of that species that were collected at the scene.

The *C. macellaria* test haplotype differed from the *C. macellaria* reference haplotype by two silent substitutions and the larval haplotype differed from the *C. callipes*

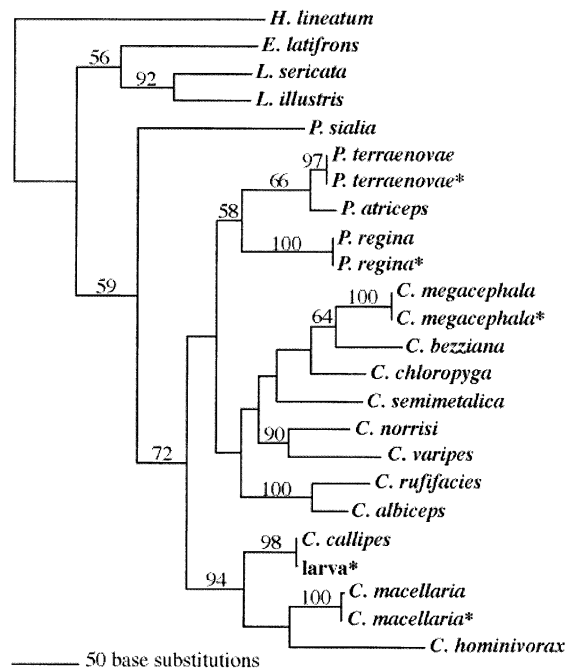


Fig. 1. Single most parsimonious phylogeny of blow flies in the subfamily Chrysomyinae calculated using 10 random stepwise additions [15]. The phylogeny is presented in the form of a phylogram in which branch length indicates the number of base substitutions separating haplotypes [15]. Analysis was based on a 2.3 kb sequence from the genes for COI, COII, and tRNA-leu for most specimens. Numbers indicate percent bootstrap support for a particular branch (1000 replications). Specimens marked by an asterisk were represented by 304, or 166 bp in the case of *P. terraenovae*, in order to mimic very degraded forensic evidence. See Table 1 and Section 2 for a more complete description of the specimens.

reference haplotype by a single silent substitution. Haplotypes of the *P. terraenovae*, *P. regina*, and *C. megacephala* test specimens exactly matched those of the reference specimens. Although, we feel there is little reason to include identical sequences in a PAUP data file for most purposes of phylogenetic analysis, it is convenient to analyze all test data in the same manner. Furthermore, even if a test and a reference sequence match exactly, a low bootstrap value (<90%) might indicate that the test sequence is too short to permit definitive identification of the specimen.

Conversely, a high bootstrap number supporting a sister-group relationship between test and reference sequence is not by itself sufficient evidence that they are from the same species. If the correct reference species is missing from the data set, a strongly supported relationship may be found with the most closely related species (see *L. sericata* and *L. illustris* in Fig. 1). For that reason, it is essential that an investigator knows the local fly fauna well enough to be confident that the species of an unidentified specimen is represented in the reference database if exact identification is to be accomplished. However, even in situations where phylogenetic analysis does not reveal the exact species, it may still narrow the number of choices to the point, where useful forensic inferences can be made.

A potentially useful criterion for specimen identification is percent intraspecific sequence variation. That is, a specimen would be identified as the same species as a reference specimen if the two were joined by parsimony analysis and if they differed by less than the maximum value of intraspecific sequence variation. Although, the data are preliminary, we have found that COI + II sequence is  $\leq 1\%$  divergent within carrion fly species and  $\geq 3\%$  divergent between species [14,17]. Further, observations are needed before we can be confident that there is no overlap between intraspecific and interspecific percent sequence variation in forensically important carrion flies.

The traditional system of chrysomyine taxonomy groups the genera *Protocalliphora* + *Phormia* + *Protophormia* in the tribe Phormiini, and *Chrysomya* + *Compsoomyia* + *Cochliomyia* in the tribe Chrysomyini [3,4]. Fig. 1 represents our best estimate of the relationships among chrysomyine haplotypes, and this does not agree with the traditional taxonomic system. Because bootstrap numbers associated with the deeper branches are low, our results are not a convincing reason to abandon the tribal categories. However, as Rognes [19] points out, more recent analyzes of morphological characters also do not agree with the traditional classification system.

In conclusion, we believe that phylogenetic analysis using these reference data can determine the species of a chrysomyine specimen collected from a human corpse anywhere in Canada or the USA, with the possible exception of rare species at locations in the Florida Keys and near the Texas–Mexico border. This approach appears to be reliable for identifying highly degraded tissue as well as specimens collected from widely separated geographic locations.

## Acknowledgements

Fly specimens were kindly contributed by G.S. Anderson (Simon Fraser University, Canada), J. Byrd (Virginia Commonwealth University, USA), D. Colwell (Agriculture and Agri-Food Canada), J. Mendez (Universidad de Panama), K. Rognes (Høgskolen i Stavanger, Norway), R. Tellman (CSIRO Tropical Agriculture, Australia), J. Wallman (University of Adelaide, Australia), and M. Wood (Canadian National Insect Collection). V. Aswani (Smithsonian Tropical Research Institute, Panama), shared unpublished sequence data that, while not included in this paper, was very useful in helping us to evaluate our methods. This research was supported by US National Institute of Justice grants 97-IJ-CX-0035 to FAHS and 99-IJ-CX-0034 to JDW, and by US National Science Foundation fellowship INT-9311789 to JDW. The views expressed here are not necessarily those of the US Department of Justice.

## References

- [1] E.P. Catts, Analyzing entomological data, in: E.P. Catts, N.H. Haskell (Eds.), *Entomology and Death: A Procedural Guide*, Joyce's Print Shop, Clemson, SC, 1990, pp. 124–137.
- [2] J.D. Wells, L.R. LaMotte, Estimating the post-mortem interval, in: J.H. Byrd, J.L. Castner (Eds.), *Forensic Entomology: Utility of Arthropods in Legal Investigations*, CRC Press, Boca Raton, FL, 2001, pp. 259–281.
- [3] D.G. Hall, *Blowflies of North America*, Thomas Say Foundation, Lafayette, IN, 1948.
- [4] J.P. Dear, A revision of the New World Chrysomyini (Diptera: Calliphoridae), *Rev. Bras. Zool.* 3 (1985) 109–169.
- [5] J.D. Wells, J.H. Byrd, T.I. Tantawi, Key to third-instar Chrysomyinae (Diptera: Calliphoridae) from carrion in the continental United States, *J. Med. Entomol.* 36 (1999) 638–641.
- [6] D. Liu, B. Greenberg, Immature stages of some flies of forensic importance, *Ann. Entomol. Soc. Am.* 82 (1989) 80–93.
- [7] J.P. Spradbery, *A Manual for the Diagnosis of Screw-Worm Fly*, CSIRO Division of Entomology, Canberra, 1991.
- [8] F.A.H. Sperling, G.S. Anderson, D.A. Hickey, A DNA-based approach to the identification of insect species used for post-mortem interval estimation, *J. Foren. Sci.* 39 (1994) 418–427.
- [9] J.D. Wells, T. Pape, F.A.H. Sperling, DNA-based identification and molecular systematics of forensically important Sarcophagidae, *J. Foren. Sci.* (in press).
- [10] C.W. Sabrosky, G.F. Bennet, T.L. Whitworth, *Bird Blow Flies (Protocalliphora) in North America with notes on the Palaearctic Species*, Smithsonian Institution, Washington, 1989.
- [11] M.M. Holland, T.J. Parsons, Mitochondrial DNA sequence analysis — validation and use for forensic casework, *Foren. Sci. Rev.* 11 (1999) 21–50.
- [12] S. Vincent, J.M. Vian, M.P. Carlotti, Partial sequencing of the cytochrome oxidase-b subunit gene. Part I. A tool for the identification of European species of blow flies for post-mortem interval estimation, *J. Foren. Sci.* 45 (2000) 820–823.
- [13] J.P. Spradbery, Screw-worm fly: a tale of two species, *Agric. Zool. Rev.* 6 (1993) 1–62.

- [14] J.D. Wells, F.A.H. Sperling, Molecular phylogeny of *Chrysomya albiceps* and *C. rufffacies* (Diptera: Calliphoridae), *J. Med. Entomol.* 36 (1999) 222–226.
- [15] D.L. Swofford, PAUP, Phylogenetic Analysis Using Parsimony (and other methods), Version 4, Sinauer, Sunderland, MA, 1998.
- [16] D.O. Clary, D.R. Wolstenholme, The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization and genetic code, *J. Mol. Evol.* 22 (1985) 252–271.
- [17] D.H. Wagner, J.D. Wells, An investigation of reported regional genetic variation in the forensically important blow fly, *Phormia regina*, using mitochondrial DNA (abstract), *Proc. Am. Acad. Foren. Sci.* 6 (2000) 181.
- [18] D.L. Swofford, G.J. Olsen, P.J. Waddell, D.M. Hillis, Phylogenetic inference, in: D.M. Hillis, C. Moritz, B.K. Mable (Eds.), *Molecular Systematics*, Sinauer, Sunderland, MA, 1996, pp. 407–515.
- [19] K. Rognes, Blowflies (Diptera: Calliphoridae) of Fennoscandia and Denmark, E.J. Brill/Scandinavian Science Press, Leiden, 1991.
- [20] A.C. Lessinger, A.C.M. Junqueira, T.A. Lemos, E.L. Kemper, F.R. da Silva, A.L. Vettore, P. Arruda, A.M.L. de Azeredo-Espin, The mitochondrial genome of the primary screwworm fly *Cochliomyia hominivorax* (Diptera: Calliphoridae), *Insect Mol. Biol.* 9 (2000) 521–529.
- [21] C.F. Simon, F. Frati, A. Beckenbach, B. Crespi, H. Liu, P. Flook, Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers, *Ann. Entomol. Soc. Am.* 87 (1994) 651–701.