Int J Legal Med (2007) 121: 1–8
DOI 10.1007/s00414-005-0056-8

ORIGINAL ARTICLE

Jeffrey D. Wells · Diana W. Williams

Validation of a DNA-based method for identifying Chrysomyinae (Diptera: Calliphoridae) used in a death investigation

Received: 30 June 2005 / Accepted: 27 September 2005 / Published online: 19 November 2005

Abstract Many authors have proposed DNA-based methods for identifying an insect specimen associated with human remains. However, almost no attempt has been made to validate these methods using additional observations. We tested a protocol for identifying insects in the blow fly subfamily Chrysomyinae (Diptera: Calliphoridae) often found to be associated with a human corpse in Canada or the USA. This method uses phylogenetic analysis of DNA sequence from a short segment of the mitochondrial gene for cytochrome oxidase one (COI). Test chrysomyine COI sequences were obtained from 245 newly sequenced specimens and 51 specimens from the published literature. Published sequences from representatives of nonchrysomyine genera were also included to check for the possibility of a false positive identification. All of the chrysomyine test haplotypes were correctly identified with strong statistical support, and there were no false positives. This method appears to be an accurate and robust technique for identifying chrysomyine species from a death investigation in this geographic region. The far northern species *Protophormia atriceps* was not evaluated; therefore, caution is required in applying this method at very high latitudes in North America.

Keywords Forensic entomology · Mitochondrial DNA · Cytochrome oxidase · Phylogeny · Species determination

Introduction

Correct identification of an insect specimen is usually a crucial early step in a forensic entomological analysis [28]. Although traditionally accomplished using anatomical characters, this can be difficult or impossible for the immature stages of many species [3]. Sperling et al. [29] first proposed that such specimens should be identified using mitochondrial DNA (mtDNA). The majority of published insect mtDNA sequences cover some portion of the genes for cytochrome oxidase subunits one and two (COI + COII) [7]. Consequently, most authors have advocated the use of a region within COI + COII for forensic insect identification (e.g., [8, 14, 20, 40]). However, some closely related species could not be distinguished using the COI + COII sequence [36, 42]. Protocols are available for characterizing other mtDNA [37, 43] or nuclear [26, 30] loci in forensically important Diptera, and some of these may prove to be more useful than COI + COII for some taxa.

A locus that works well for diagnosing species of one taxonomic group may not work for a different group. The use of mtDNA sequence data for species determination requires an assumption of reciprocal mtDNA monophyly when comparing a particular species with its closest relative in the data set [22, 24]. In other words, unless each morphological species under consideration corresponds to a single mtDNA lineage, it will not be possible to assign at least some sample haplotypes to a single species (Fig. 1).

Not surprisingly, carrion flies show intraspecific variation in mtDNA haplotypes [35], and geographic variation is also possible [31]. Therefore, we believe that validation of any molecular genetic species test for these insects requires a population and geographic survey of the target locus. Wells and Sperling [40] proposed a method for identifying all forensically important species in the blow fly subfamily Chrysomyinae (Diptera: Calliphoridae) likely to be found in a human corpse in Canada or the USA. This test relies on a short region of the COI sequence from an evidence specimen and maximum parsimony phylogenetic analysis [33] to potentially associate a haplotype from an unidentified specimen with a haplotype from an identified specimen. The current study aims to validate this analytical method using newly generated and published but

J. D. Wells (✉)
Department of Biology, West Virginia University,
Morgantown, WV 26506, USA
e-mail: jdwells@mail.wvu.edu
Fax: +1-304-2936363

D. W. Williams
US Army Investigation Laboratory,
Forest Park, GA 30297, USA

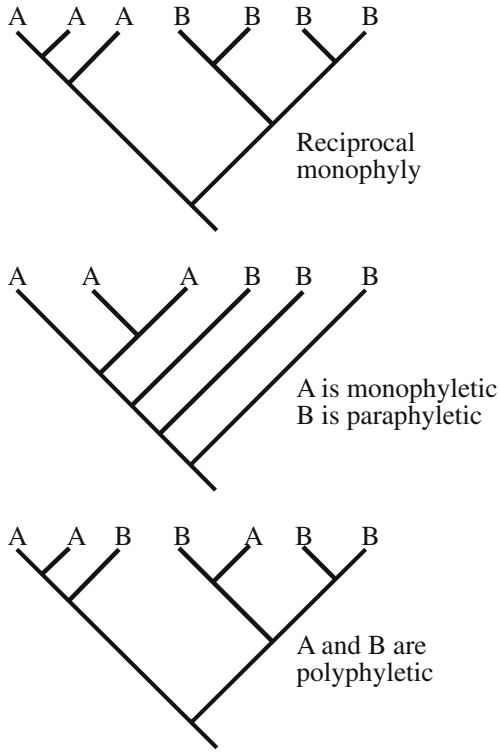

Fig. 1 Hypothetical results of a phylogenetic analysis of mitochondrial DNA haplotypes for species A and B, showing some possible relationships and their implications for a reliable species-diagnostic test. If reciprocal monophyly exists, then a haplotype obtained from an additional member of one of these two species will be grouped with one lineage or the other, and thereby identified. If a monophyletic + paraphyletic arrangement exists, then an additional haplotype that fell within the A lineage would be identified, but one falling between the two species could either be one of them. A polyphyletic arrangement does not support a species diagnosis

previously untested chrysomyine haplotypes from a wide range of geographic locations.

Materials and methods

Specimens and published COI haplotypes

Table 1 lists the 245 adult flies sequenced for this study as well as previously published COI haplotypes representing 59 specimens, not including those of Wells and Sperling [40], that were tested. These represent all but one of the Chrysomyinae species likely to be found in a human corpse in Canada or the USA [40]. We were unable to obtain additional specimens or published sequence data for *Protophormia atriceps*, which was included in the 2001 paper. However, this fly is only found at very high latitudes in North America (Alaska to Greenland; [13]). Therefore, we believe that our results are valid for death investigations in the USA, southern Canadian, and farther north if an investigator has good reason to exclude *P. atriceps* as a candidate species.

An effort was made to obtain specimens or genetic data covering as wide a geographic range as possible to determine if this species-diagnostic procedure could similarly be widely applied. *Compsomyiops callipes* (=*Paralucilia wheeleri*), which was only sampled from California (Table 1), has been reported north of Mexico only in the western part of the USA [13]. *Chrysomya albiceps* and *Chrysomya putoria*, which spread from Africa to Brazil in the 1970s [11], have not yet been collected as far north as the USA. We suspect, however, that their ranges in the Western hemisphere are likely to expand north as has recently occurred with *C. albiceps* in Europe [10, 25].

In addition to the chrysomyine haplotypes, we also considered homologous sequence data from a representative selection of other forensically important Diptera (Table 1). The purpose was to test for the possibility of our analytical method yielding a false positive, i.e., a nonchrysomyine specimen mistakenly identified as one of the chrysomyine species. Although not all of these data are from species that occur in North America, they represent genera that are found here.

The new fly specimens in Table 1 were all captured with a hand net at a decayed meat bait, with the exception of the *Protophormia terraenovae* specimens from France, which were purchased as fishing bait. Their true geographic origin is therefore uncertain, although we think that it is probably somewhere in Europe. They were also almost certainly from a colony and therefore were potentially inbred. However, the six specimens we processed produced five different COI haplotypes based on about 1 kb of sequence (data not shown). Therefore, it appears that these specimens can conservatively be considered a sample size of five for purposes of an mtDNA survey.

The time and manner of specimen preservation prior to laboratory processing varied with regard to good DNA preservation conditions, including some that were fresh-frozen at −70°C and some that were held for several years at room temperature in ethanol with a range of 70–90% concentration.

Test sequence and laboratory techniques

The region of COI we used to mimic data from an evidence specimen is a 304 base sequence corresponding to positions 1029–1332 of GenBank accession number L14946 [29]. We refer to this in the context of a validation study as a test sequence or haplotype, and an individual insect from which it was derived as a test specimen. This region can be amplified using primers C1-J-2495(5′ CAGCTACTTTAT GAGCTTTAGG; [29]) and C1-N-2800 (5′ CATTTCAA GYTGTGTAAGCRTC; [40]). Although a larger region can easily be amplified from fresh tissue using a single polymerase chain reaction (PCR) reaction, we generated either this segment or considered only this portion of a longer sequence, because this short region can often be characterized from a very degraded specimen. DNA was extracted from the thoracic muscle or legs using a DNeasy tissue kit (QIAGEN, Valencia, CA). PCR reactions were carried out

Table 1 Sources of the test COI haplotypes for this study

Chrysomyinae newly sequenced for this analysis			GenBank record or otherwise previously published COI haplotype			
Species	Collection site	Number of specimens	Accession number	Collection site	Number of specimens	Reference
Protophormia terraenovae	Paris, France[a]	5	L14946	British Columbia, Canada	1	Sperling et al. [29]
	Morgantown, WV	34	AF295553[b]	White Mountains, CA	1	Wells and Sperling [40]
Phormia regina	White Mountains, CA	10	AF295550[b]	Hopland, CA	1	Wells and Sperling [40]
	Berkeley, CA	10				
	Santa Barbara, CA	6				
	Lyle Grove, WA	7				
	Tucannon R., WA	9				
	Orr, MN	2				
	Madison County, IA	8				
	Morgantown, WV	10				
	Quantico, VA	10				
	Birmingham, AL	10				
Cochliomyia macellaria	Cabo Rojo, Puerto Rico	4	AF022370	French Guiana	1	Vincent et al. [34]
	Key Largo, FL	5	AF295555[b]	Gainesville, FL	1	Wells and Sperling [40]
	Birmingham, AL	2				
	Sarita, TX	5				
	Baja California, Mexico	5				
	Rsva.Sanguaré, Colombia	3				
	Plata Had, Colombia	2				
Compsomyiops callipes	Martinez, CA	20				
	Berkeley, CA	14				
	White Mountains, CA	1				
Chrysomya megacephala	Mayaguez, Puerto Rico	1	AY092761	Taipai, Taiwan	1	Chen et al. [8]
	Cabo Rojo, Puerto Rico	2	AB080252	Western Australia	1	Harvey et al. [14]
	Miami, FL	10	AB112830	KwaZula-Natal, South Africa	1	
	Birmingham, AL	15	AB112841	Brisbane, Australia	1	Harvey et al. [15]
	Sulawesi, Indonesia	1	AB112846	Perth, Australia	1	Harvey et al. [15]
	Guam	1	AB112847	Perth, Australia	1	Harvey et al. [15]
	Moorea, Fr. Polynesia	1	AB112848	Pretoria, South Africa	1	Harvey et al. [15]
			AB112856	Kitwe, Zambia	1	Harvey et al. [15]
			AB112861	Kitwe, Zambia	1	Harvey et al. [15]
			AF295551[b]	Nr. Lae, Papua New Guinea	1	Wells and Sperling [40]

Table 1 (continued)

Chrysomyinae newly sequenced for this analysis			GenBank record or otherwise previously published COI haplotype			
Species	Collection site	Number of specimens	Accession number	Collection site	Number of specimens	Reference
Chrysomya rufifacies	Cabo Rojo, Puerto Rico	3	None	Adelaide, Australia	1	Wells and Sperling [39]
	Miami, FL	10	None	Ceram, Indonesia	1	Wells and Sperling [39]
	Honolulu, HI	6	None	Moorea, French Polynesia	1	Wells and Sperling [39]
	Birmingham, AL	13	None	Kerrville, TX	1	Wells and Sperling [39]
			AB080253	Western Australia	1	Harvey et al. [14]
			AB080254	Western Australia	1	Harvey et al. [14]
			AB080255	Western Australia	1	Harvey et al. [14]
			AB112828	Perth, Australia	1	Harvey et al. [15]
			AB112845	Perth, Australia	1	Harvey et al. [15]
			AY092760	Taipai, Taiwan	1	Chen et al. unpublished
Chrysomya albiceps			AB112836	KwaZula-Natal, South Africa	1	Harvey et al. [15]
			AB112839	Pretoria, South Africa	1	Harvey et al. [15]
			AB112840	Pretoria, South Africa	1	Harvey et al. [15]
			AB112842	KwaZula-Natal, South Africa	1	Harvey et al. [15]
			AB112849	Deka, Zimbabwe	1	Harvey et al. [15]
			AB112851	Manzini, Swaziland	1	Harvey et al. [15]
			AB112854	Manzini, Swaziland	1	Harvey et al. [15]
			AB112858	Deka, Zimbabwe	1	Harvey et al. [15]
			AB112865	Manzini, Swaziland	1	Harvey et al. [15]
			None	Campinas, Brazil	1	Wells and Sperling [39]
			None	Bloemfontein, South Africa	2	Wells and Sperling [39]
			AF083657[b]	Alexandria, Egypt	1	Wells and Sperling [39]
Chrysomya putoria			AY139693	Grahamstown, South Africa	3	Wells et al. [42]
			AY139694	Grahamstown, South Africa	9	Wells et al. [42]
			NC_002697	Brazil	1	Junqueira et al. [17]
			AB112831	Kitwe, Zambia	1	Harvey et al. [15]
			AB112835	Snake Island, Botswana	1	Harvey et al. [15]
			AB112855	Snake Island, Botswana	1	Harvey et al. [15]
			AB112860	Kitwe, Zambia	1	Harvey et al. [15]
			AF295554[b]	Panama	1	Wells and Sperling [40]
Ophyra capensis			AY818106			Cai et al. unpublished
Musca domestica			AY818108			Cai et al. unpublished

Table 1 (continued)

Non-Chrysomyinae haplotypes from forensically important genera used to test for a false positive		
Species	Accession number	Reference
Fannia armata	AF104623	Bernasconi et al. [4]
Cynomya cadaverina	AF259505	Wells et al. [41]
Calliphora vicina	AY842603	Wallman et al. [37]
Aldrichina grahami	AY818124	Cai et al. unpublished
Sarcophaga africa	AY315642	Zehner et al. unpublished
Megaselia scalaris	AF217464	Morehead et al. unpublished

Locations are within the USA unless otherwise stated
[a]Purchased from a fishing supply shop. See "Materials and methods"
[b]Reference specimen from the phylogenetic data file used for comparative analysis (see "Materials and methods"). Only those reference individuals from an otherwise unlisted location are shown here in order to give the full geographic range of samples. See Wells and Sperling [40] for a description of the reference data set

using a commercial master mix (Promega, Madison, WI, Catalog #M7505) following the manufacturer's instructions. The thermal cycler program consisted of an initial denaturation of 95°C for 3 min, 45°C for 1 min, 72°C for 1.5 min, followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min, and a 4°C hold. PCR products were cleaned using a QIAquick PCR purification kit (QIAGEN) and then cycle-sequenced using BigDye reagents (Applied Biosystems, Foster City, CA). The sequence was detected using several Applied Biosystems 310, 3100-Avant, or 3130 capillary instruments. In most cases, only one of the DNA strands was sequenced.

Computer analysis

Sequence files were edited and aligned using Sequence Navigator (Applied Biosystems), phylogenetic analysis was performed using phylogenetic analysis using parsimony (PAUP) 4.0b2 [32]. The PAUP data set was that used by Wells and Sperling [40] and consisted of 2.3 kb COI + COII reference sequences for the outgroup species *Hypoderma lineatum* (Oestridae), *Eucalliphora latifrons* (Calliphoridae: Calliphorinae), *Lucilia sericata* (Calliphorinae), and *Lucilia illustris*. Reference ingroup taxa were complete COI + COII haplotypes for a variety of Chrysomyinae (see Fig. 2) plus a 678 base sequence from *P. atriceps*, which included the test COI region.

PAUP analysis involved 1,000 bootstrap replicates using the maximum parsimony optimality criterion, in which a tree implying the fewest number of point mutations is preferred [33], and the software default settings. Each test sequence was individually queried by running the PAUP

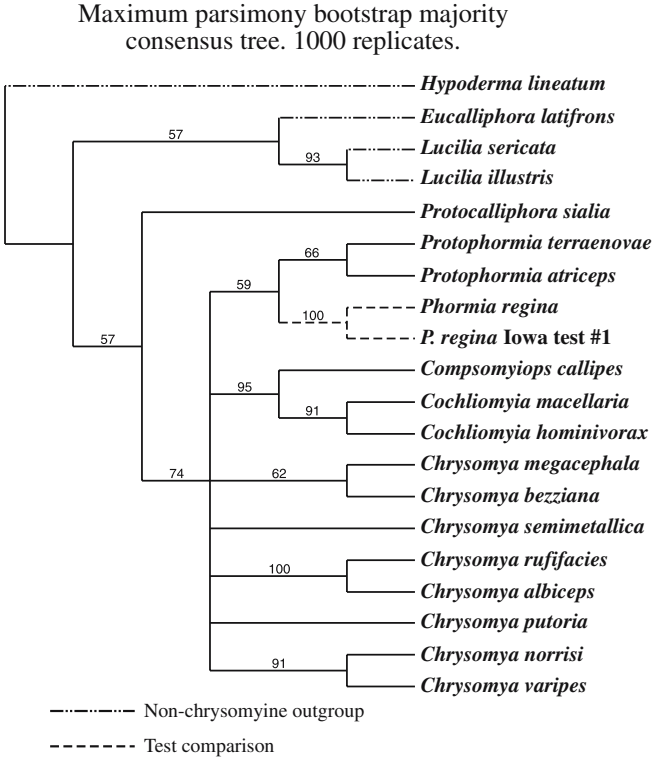

Fig. 2 An example of the results of our analyses. Each test COI haplotype listed in Table 1 was individually subjected to a maximum parsimony phylogenetic analysis following addition to the data set of Wells and Sperling [40]. A number on a branch is percent bootstrap support for that lineage

analysis using the reference sequences plus the test sequence (Fig. 2).

Results and discussion

The chrysomyine test sequences were paired with the correct reference haplotype with ≥92% bootstrap support for the *C. albiceps* test sequences and ≥97% for the other species (Table 2). None of the test sequences from non-chrysomyine genera paired with a chrysomyine reference haplotype in a majority consensus bootstrap tree. The PAUP data files are available by request. We conclude that the analytical procedure of Wells and Sperling [40] is a reliable and robust method for identifying insects in this subfamily associated with a human corpse in the USA outside of Alaska and in southern Canada. Because we did not evaluate *P. atriceps*, which is found at very high latitudes, a similar study should be performed with this species. If a death investigator can exclude *P. atriceps* as a candidate species based on other knowledge, such as season or geographic location, then this is not a concern. We suspect that identifying *P. atriceps* in this manner will be found to be valid given that the available COI haplotype of that species [40] is quite distinct from those of its closest relative *P. terraenovae*. Some of the taxonomic literature treats it as a member of the separate genus *Boreelus* [12].

Strong statistical support for correct identification was obtained, although some of the sequences we used were shorter than 304 bases (Table 2), either because only that portion of a published sequence overlapped the test region or because a poorly preserved specimen did not yield clean sequence data for the entire PCR product.

Table 2 Statistical support for the correct identification of the chrysomyine test specimens in this study based on maximum parsimony analysis of a short region of COI

Species	Number of test individuals included in analysis	Mean and range of percent bootstrap support for correct identification	Length of shortest sequence and bootstrap value
Protophormia terraenovae	40	99.7 (99–100)	304 bp (99)
Phormia regina	82	100	267 (100)
Cochliomyia macellaria	27	99.8 (98–100)	230 (98)
Compsomyiops callipes	35	98.9 (97–100)	215 (99)
Chrysomya megacephala	40	100	227 (100)
Chrysomya rufifacies	42	99.9 (99–100)	228 (100)
Chrysomya albiceps	13	93.9 (92–95)	304 (92)
Chrysomya putoria	17	99.9 (99–100)	281 (100)

The importance of defining a method like this in terms of geographic region is illustrated by the fact that COI data did not distinguish *C. putoria* from its sister species *Chrysomya chloropyga* [42]. These two flies are extremely closely related, resulting in a history of confusion concerning the taxonomic separation; however, they are clearly separate species with distinct bionomic traits [27]. Although they coexist in Africa, *C. chloropyga* has never been reported from the western hemisphere, permitting COI-based identification of *C. putoria* in the Americas. However, all four Chrysomya test species in this study are originally Old World flies that were introduced to the New World, where they became common and widespread within a few decades [2, 11, 16, 38]. When analyzing forensic entomological evidence, close attention must be paid to the scientific literature for any report of such range expansions.

There are a variety of forensic applications for a molecular species-diagnostic test, including the violation of hunting or conservation laws [9, 23], and the fraudulent labeling of food [6, 19, 21]. Although DNA-based species determination has been widely successful, certain conditions must be fulfilled before such a test can be considered reliable. One condition is the availability of known reference genetic data for all species relevant to the evidence in hand. In other words, the investigator must be confident that the evidence specimen, although unidentified, is a species for which correctly identified genetic data are available for comparison. Narrowing the set of candidate species can be accomplished using biological information that is independent of the evidence genotype, such as morphology, seasonality, or geographic distribution. It may also be that the genotypes of a given taxonomic group have been so well surveyed that association of an evidence specimen with a species in that group can be made based on genetic data alone.

In most situations, the species that are difficult or impossible to distinguish based on anatomical characters are close relatives. It is also these close relatives that may not be distinguishable with a genetic test, although that test may unambiguously separate more distantly related lineages (e.g., Birstein et al. [5]). Some closely related carrion flies differ greatly in forensically important aspects of their natural history, such as larval growth rate [18] or diapause response [1]. Therefore, the utility of a species-diagnostic data set depends on whether it allows one to separate such close relatives.

The authors of several forensic fly DNA papers have apparently not considered this problem in their experimental design. For example Ratcliffe et al. [26] claimed that PCR-restriction fragment length polymorphism (PCR-RFLP) of the internal transcribed spacer regions provides "accurate identification of forensically important [fly] species." This conclusion was based on their demonstration that each of the ten forensically important species yielded a unique PCR-RFLP pattern. However, Ratcliffe et al. [26] did not evaluate common and forensically important close relatives of their study species, close relatives that often co-

occur with the flies they investigated. It is not known if their procedure will distinguish between a species they examined, e.g., *Calliphora vicina*, and one they did not examine that might easily be found in the same corpse, e.g., *Calliphora vomitoria*. Therefore, their method cannot be considered accurate until it is evaluated using a more complete sample of taxa.

The genotyping and phylogenetic methods used in this paper are widely and commonly practiced, and we see no reason why an experienced and competent molecular geneticist or forensic mtDNA analyst with no entomological training cannot use them correctly. In our experience, crime laboratory analysts in the USA are reluctant to genotype nonhuman tissue; hence, the involvement of academic scientists is probably needed if this procedure is to aid death investigations to the greatest possible extent. In most circumstances, the best arrangement is a collaboration between an experienced forensic entomologist and a DNA expert, along with close consultation with other professionals concerned with the case. If possible, a duplicate tissue sample(s) must be preserved for potential independent testing. A DNA extraction negative control as well as the typical PCR negative control should be prepared, and, of course, sample security and chain of evidence documentation must be maintained.

Acknowledgements Many friends and colleagues either provided fly specimens or hosted JDW during a collecting trip. These included Whitney Banks (West Virginia U.), Michael Caterino (Santa Barbara Museum of Natural History), members of the Contra Costa, CA, Sheriff's Department, Patrick Erwin (U. Alabama at Birmingham), Emmanuel Gaudry (French Gendarmerie), M. Lee Goff (Chaminade U.), Jack Hayes (U. Texas at San Antonio), Hiromu Kurahashi (Japanese National Institute of Infectious Disease), Wayne Lord (Federal Bureau of Investigation), Paul Richards (U. Miami), Daniel Rubinoff (U. Hawaii), Nikolaos Schizas (U. Puerto Rico Mayaguez), and Marta Wollf (U. Antioquia). This work was supported in part by the US National Institute of Justice award 99-IJ-0034. The views expressed here are not necessarily those of the US Department of Justice.

References

1. Ash N, Greenberg B (1975) Developmental temperature responses of the sibling species *Phaenicia sericata* and *Phaenicia pallescens*. Ann Entomol Soc Am 68:197–200
2. Baumgartner DL, Greenberg B (1984) The genus *Chrysomya* (Diptera: Calliphoridae) in the New World. J Med Entomol 21: 105–113
3. Benecke M, Wells JD (2001) DNA techniques for forensic entomology. In: Byrd JH, Castner JL (eds) Forensic entomology: utility of arthropods in legal investigations. CRC Press, Boca Raton, pp 341–352
4. Bernasconi MV, Valsangiacomo C, Piffaretti JC, Ward PI (2000) Phylogenetic relationships among muscoidea (Diptera: calyptratae) based on mitochondrial DNA sequences. Insect Mol Biol 9:67–74
5. Birstein VJ, Doukakis P, DeSalle R (2000) Polyphyly of mtDNA lineages in the Russian sturgeon, *Acipenser gueldenstaedtii*: forensic and evolutionary implications. Conserv Genet 1:81–88
6. Bottero MT, Civera T, Anastasio A, Turi RM, Rosati S (2002) Identification of cow's milk in "buffalo" cheese by duplex polymerase chain reaction. J Food Prot 65:362–366
7. Caterino MS, Cho S, Sperling FAH (2000) The current state of insect molecular systematics: a thriving Tower of Babel. Annu Rev Entomol 45:1–54
8. Chen WY, Hung TH, Shiao SF (2004) Molecular identification of forensically important blow fly species (Diptera: Calliphoridae) in Taiwan. J Med Entomol 41:47–57
9. Cronin ME, Palmisciano DA, Vyse ER, Cameron DG (1991) Mitochondrial DNA in wildlife forensic science: species identification of tissues. Wildl Soc Bull 19:94–105
10. Grassberger M, Friedrich E, Reiter C (2003) The blowfly *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) as a new forensic indicator in central Europe. Int J Legal Med 117: 75–81
11. Guimaraes J, do Prado A, Linhares AX (1978) Three newly introduced blowfly species in southern Brazil (Diptera: Calliphoridae). Rev Bras Entomol 22:53–60
12. Hall DG (1948) Blowflies of North America. Thomas Say Foundation, Lafayette
13. Hall DG (1965) Calliphoridae. In: Stone A, Sabrosky CW, Wirth WW, Foote RH, Coulson JR (eds) A catalog of the Diptera of America north of Mexico. Smithsonian Institution Press, Washington, pp 922–932
14. Harvey ML, Dadour IR, Gaudieri S (2003a) Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in western Australia. Forensic Sci Int 131:134–139
15. Harvey ML, Mansell MW, Villet MH, Dadour IR (2003b) Molecular identification of some forensically important blowflies of southern Africa and Australia. Med Vet Entomol 17: 363–369
16. Jiron LF (1979) Sobre moscas califoridas de Costa Rica (Diptera: Cyclorrhapha). Brenesia 16:221–222
17. Junqueira AC, Lessinger AC, Torres TT, da Silva FR, Vettore AL, Arruda P, Azeredo Espin AM (2004) The mitochondrial genome of the blowfly *Chrysomya chloropyga* (Diptera: Calliphoridae). Gene 339:7–15
18. Kamal AS (1958) Comparative study of thirteen species of sarcosaprophagous Calliphoridae and Sarcophagidae (Diptera). 1. Bionomics. Ann Entomol Soc Am 51:261–270
19. Lenstra JA (2003) DNA methods for identifying plant and animals species in foods. In: Lees M (ed) Food authenticity and traceability. CRC Press, Boca Raton, pp 34–53
20. Malgorn Y, Coquoz R (1999) DNA typing for identification of some species of Calliphoridae. An interest in forensic entomology. Forensic Sci Int 102:111–119
21. Marko PB, Lee SC, Rice AM, Gramling JM, Fitxhenry TM, McAllister JS, Harper GR, Moran A (2004) Mislabelling of a depleted reef fish. Nature 430:309–310
22. Moritz C (1996) The uses of molecular phylogenies for conservation. In: Harvey PH, Leigh Brown AJ, Maynard Smith J, Nee S (eds) New uses for new phylogenies. Oxford University Press, Oxford, pp 203–214
23. Palumbi SR, Cipriano F (1998) Species identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. J Hered 89:459–464
24. Palumbi SR, Cipriano F, Hare MP (2001) Predicting nuclear gene coalescence from mitochondrial data: the three-times rule. Evolution 55:859–868
25. Povolny D (2002) *Chrysomya albiceps* (Wiedemann, 1918): the first forensic case in central Europe involving this blowfly (Diptera: Calliphoridae). Acta Univ Agric Silvic Mendel Brun 50:105–112
26. Ratcliffe ST, Webb DW, Weinzievr RA, Robertson HM (2003) PCR-RFLP identification of Diptera (Calliphoridae, Muscidae and Sarcophagidae)—a generally applicable method. J Forensic Sci 48:783–785
27. Rognes K, Paterson HEH (2005) *Chrysomya chloropyga* (Wiedemann, 1818) and *C. putoria* (Wiedemann, 1830) (Diptera: Calliphoridae) are two different species. Afr Entomol 13:49–70

28. Smith KGV (1986) A manual of forensic entomology. Cornell University Press, Ithaca
29. Sperling FAH, Anderson GS, Hickey DA (1994) A DNA-based approach to the identification of insect species used for postmortem interval estimation. J Forensic Sci 39:418–427 (published erratum appears in letter from Wells JD, Sperling FAH (2000) J Forensic Sci 45:1358–1359)
30. Stevens J, Wall R (2001) Genetic relationships between blowflies (Calliphoridae) of forensic importance. Forensic Sci Int 120:116–123
31. Stevens JR, Wall R, Wells JD (2002) Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. Insect Mol Biol 11:141–148
32. Swofford DL (1998) PAUP, phylogenetic analysis using parsimony (and other methods), Version 4. Sinauer, Sunderland
33. Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics. Sinauer, Sunderland, pp 407–514
34. Vincent S, Vian JM, Carlotti, MP (2000) Partial sequencing of the cytochrome oxydase b subunit gene I: a tool for the identification of European species of blow flies for postmortem interval estimation. J Forensic Sci 45:820–823 (published erratum appears in letter from Wells JD, Sperling FAH (2000) J Forensic Sci 45:1358–1359)
35. Wagner DH (2000) Investigation of molecular population genetic substructure in forensically important flies. MS thesis, University of Alabama, Birmingham
36. Wallman JF, Donnellan SC (2001) The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in southeastern Australia. Forensic Sci Int 120:60–67
37. Wallman JF, Leys R, Hogendoorn K (2005) Molecular systematics of Australian carrion-breeding blowflies based on mitochondrial DNA. Invertebr Syst 19:1–15
38. Wells JD (2000) Introduced *Chrysomya* Robineau-Desvoidy (Diptera: Calliphoridae) flies in northcentral Alabama. J Entomol Sci 35:91–92
39. Wells JD, Sperling FAH (1999) Molecular phylogeny of *Chrysomya albiceps* and *C. rufifacies* (Diptera: Calliphoridae). J Med Entomol 36:222–226
40. Wells JD, Sperling FAH (2001) DNA-based identification of forensically important Chrysomyinae (Diptera: Calliphoridae). Forensic Sci Int 120:109–114
41. Wells JD, Introna FG, DiVella G, Campobasso CP, Hayes J, Sperling FAH (2001) Human and insect mitochondrial DNA analysis from maggots. J Forensic Sci 46:685–687
42. Wells JD, Lunt N, Villet MH (2004) Recent African derivation of *Chrysomya putoria* from *C. chloropyga* and mitochondrial DNA paraphyly of cytochrome oxidase subunit one in blowflies of forensic importance. Med Vet Entomol 18:445–448
43. Zehner R, Amendt J, Schutt S, Sauer J, Krettek R, Povolny D (2004) Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). Int J Legal Med 118:245–247