DNA BASED TECHNIQUES FOR THE IDENTIFICATION OF FORENSICALLY IMPORTANT FLIES (DIPTERA): A REVIEW

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ABSTRACT: DNA based methods become important in the identification of insects when we don't have complete sample for identification and the available sample is either broken or fragmented and unable to provide complete information necessary for the identification. We have number of DNA based techniques which can be used for the identification of insect's samples. We are therefore presenting here the review of various DNA based techniques commonly used in the species identification.

KEYWORDS: Diptera, Forensic entomology, mtDNA, PMI, RAPD

INTRODUCTION:

Benecke and Wells (2001) suggested that at a time when many aspects of forensic science are dominated by recent advances in the field of molecular biology, it is no surprise that DNA technology should also become a tool of the forensic entomologists. At present, efforts to develop these tools are still mostly at the research stage. However, they have the potential to move very quickly into widespread use by those who analyze insect evidence in forensic investigations (Byrd and Castner, 2001).

DNA typing of biological material has become one of the most powerful tools for potential identification in forensic medicine and in criminal investigation since 1985 (Benecke, 1997). Benecke and Wells (2001) emphasized that advantages of using DNA provides a huge amount of diagnostic information compared to some older techniques (such as blood group typing), it is present in all biological tissues, and it is much more resistant to environmental degradation than most other biological molecules (e.g. proteins). The entire DNA content of an organism is termed its genome and techniques that extract all regions of DNA from a biological sample are said to yield genomic DNA (Byrd and Castner, 2001). Newton and Graham (1997) suggested that a locus that is less than about 1000 bp in length can be easily amplified using a method known as PCR. Primer is the key ingredient in PCR analysis. It is a short piece of DNA that anneals to the sample DNA in the specific location, where the replication can begin. There are specific primers to anneal at known locations on the sample DNA (Benecke and Wells, 2001). The resulting amplified PCR product will be from a known locus. The nonspecific primers anneal somewhere on the sample DNA which is not known to the investigator. Despite this fact, the resulting PCR product can still be used for some analysis even through one does not know what has been amplified (Benecke and Wells, 2001; Byrd and Castner, 2001). The major advantages of PCR based techniques are that they are faster (requiring hours or days rather than weeks) and require much less sample DNA than methods without PCR (Hillis *et al*., 1996). Another benefit is that loci for PCR analysis have been developed that are less than about 350 bp in length, allowing the use of sample DNA that is very degraded and broken into small pieces (Byrd and Castner, 2001).

DNA BASED MOLECULAR TECHNIQUES

i. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP analysis has been used to identify closely related species of forensic importance from different life stages (Sperling *et al*., 1994; Malgorn and Coquoz, 1999; Thyssen *et al*., 2005). It is a fast, easy, low cost technique for routine diagnostic purposes (Litjens *et al*., 2001; Thyssen *et al*., 2005). In PCR-RFLP analysis, the detection of polymorphic patterns between individuals is based on differences in the sizes of the restriction fragments obtained from the amplified DNA region generated by a specific endonuclease or a multiple set of restriction enzymes (Thyssen *et al*., 2005). Hall and Smith (1991) used restriction enzyme analysis of PCR products (PCR-RFLP) to differentiate African and European honey bee races, and Simon *et al*. (1993) used PCR-RFLP to characterize periodic cicada broods. The latter authors indicated that the PCR-RFLP is less expensive and has higher resolution than standard RFLP analysis (Simon, 1991). Sperling *et al*. (1994) modified the PCR-RFLP technique to differentiate 3 species of blow flies. They used restriction fragment length differences in a 348 bp sequence from the mitochondrial COI gene. They were able to identify larval specimens who had been stored in 75% ethanol at room temperature for up to 1 month and adults which had been dried and stored at room temperature for 5 days.

ii. Random Amplified Polymorphic DNA (RAPD)

RAPD is a useful technique designed for the detection of sequence polymorphism in organisms where previous nucleotide sequence data is not available. RAPDs are based on random priming using short primers of arbitrary sequence that act as both forward and reverse primers in the PCR reaction. The primers produce a series of bands that may be visualized using agarose gel electrophoresis and each of the products represents a single genetic locus (Hill and Crampton, 1994). The first application of RAPD to differentiate forensically relevant insects was done by Benecke (1998b). He found the RAPD technique unsuitable for

species identification due to many disadvantages like high information density; variable patterns between most (if not all) known species and different peaks, heights and shapes. Few more limitations of RAPDs are, non genetic variation in the analysis of the progeny of controlled mating, indicating some artifacts in priming, the co-migration of products of equal size and homologous loci that are difficult to identify (Palumbi, 1996). So, the applicability and utility of RAPDs in forensic casework is not that much convincing.

iii. PCR-Amplified Fragment Length Polymorphism (PCR-AFLP)

AFLP is a powerful method that combines techniques from classical hybridization based (Jeffrey *et al*., 1985) and PCR-based (Weber and May, 1989) genotyping strategies. Therefore, analysis of AFLPs has the potential to become a powerful DNA fingerprinting technique for studying genetic relationships and genetic diversity in arthropods (Vos *et al*., 1995; Reineke *et al*., 1999; Bensch and Kesson, 2005). The AFLP technique has several advantages: (1) reproducibility over a wide range of template concentrations due to the high stringency annealing conditions; (2) production of a large number of observable molecular markers due to the fact that the entire genome is subject to restriction digestion (Blears *et al*., 1998; Savelkoul *et al*., 1999); (3) a high discriminative capacity since multiple combinations of selective nucleotides can be used to observe additional markers (Sevelkoul *et al*., 1999). AFLP has been used to construct linkage maps (Hawthorne, 2001; Parsons and Shaw, 2002) and to study genetic relationships (Katiyar *et al*., 2000; Ravel *et al*., 2001; Salvato *et al*., 2002) and diversity (Kakouli-Duarte *et al*., 2001) among insects. The strength of the technique was demonstrated in a study conducted by Parsons and Shaw (2001) in which AFLP was used to differentiate morphologically cryptic species in the genus *Laupala* (Beckert *et al*., 2010).

iv. DNA Sequencing

Sanger introduced his 'plus and minus' method for DNA sequencing in 1975 (Sanger, 1975; Sanger and Coulson, 1975). This was a critical transition technique leading to the modern generation of methods that have completely dominated sequencing over the past 30 years. The key to this advance was the use of polyacrylamide gels to separate the products of primed synthesis by DNA polymerase in order of increasing chain length. The method analyzed the products of DNA polymerase reactions that extended a primer annealed to a single-stranded DNA template, as Wu and Kaiser (1967) had done in sequencing the lambda cohesive ends (Hutchison, 2007). Maxam and Gilbert (1977) developed a DNA sequencing method that was similar to the Sanger and Coulson method (1975) in using polyacrylamide gels to resolve bands that terminated at each base throughout the target sequence, but very different in the way that products ending in a specific base were generated. Their method started with a double stranded DNA restriction fragment radio labeled at one end with 32^{P}. The fragment was then cleaved by basespecific chemical reactions. One reaction cleaves at both purines (the A+G reaction), one preferentially at A (A>G), one at pyrimidines (C+T) and one at cytosine's only (C). Unlike the plus and minus method, the chemical method produced bands for every sequence position, including those within homopolymer runs. This advantage led to early widespread adoption of the chemical method following its publication (Hutchison, 2007). The problems with the plus and minus method were solved when Sanger developed 'the dideoxy method (Sanger *et al*., 1977). The underlying concept was to use chain terminating nucleotide analogs rather than subsets of the four natural dNTPs to cause base-specific termination of primed DNA synthesis. In the original implementation, both arabinoside triphosphates and 20,30-dideoxy nucleoside triphosphates were tried. These analogs are incorporated in a sequence-specific manner by *E. coli* Pol I, but the enzyme is unable to further extend the growing DNA strand (in the case of the ddNTPs simply because of the lack of a 30 hydroxyl group). Synthesis was carried out in the presence of all four dNTPs, one of which was 32^{P} labeled (Hutchison, 2007).

Sperling *et al.* (1994) sequenced 2300 bp of mtDNA genes encoding COI, COII and tRNA-leucine from each of the three forensically important blow flies; *Phormia regina, Phaenicia sericata* and *Lucilia illustris.* They found 118 nucleotide differences between the *L. illustris* and *P. sericata*, 186 between *L. illustris* and *P. regina* and 192 between *P. sericata* and *P. regina*. They identified immature larvae within one day of the receipt of specimens and suggested that this molecular approach can also be possible for identification of dead insect material. Gleeson and Sarre (1997) sequenced a 472 bp region of the mitochondrial COI of *Lucilia cuprina dorsalis* and *Lucilia cuprina cuprina* from six Australian regions and also from South Africa and Malaysia. Malgorn and Coquoz (1999) sequenced two fragments 297 bp and 304 bp of length from the 5 species of forensically important blow flies.

Wallman (2001) studied the morphological characteristics of third instar larvae of common carrion-breeding *Calliphora* blow flies. Wallman and Donnellan (2001) studied a partial sequence of mtDNA COI and COII genes for forensically important Australian blow flies. Results revealed abundant phylogenetically informative nucleotide substitutions for identification of calliphorid species. A total of 639 aligned sites for the COI sequences were included containing 524 invariant sites and 80 parsimony informative sites. Wells and Sperling (2001) identified fourteen American species of Calliphoridae on the basis of COI, COII and tRNA-leucine genes. Approximately 2.3 kb of sequence was obtained for each of the sample except *P. atriceps*, for which a total of 678 bp was recovered. They found that COI+COII sequence is ≤1% divergent within carrion fly species and ≥3% divergent between species. They further confirmed that there is no overlapping between intraspecific and interspecific percent sequence variation in forensically important carrion species. Wells *et al.* (2001b) published sequence data of the COI gene at nucleotide positions 1477–2250 for the species *Bercaea africa* (geographic origin Berkeley, California), *Liopygia argyrostoma* (geographic origin Alexandria, Egypt) and *Liopygia crassipalpis* (geographic origin Berkeley, California).

Stevens *et al.* (2002) sequenced 28S rRNA, COI and COII gene markers from *L. sericata* and *L. cuprina* collected from Europe, Africa, North America and Hawaii. The degree of variation observed across the 2329 nucleotides of the mitochondrial COI + COII gene varied widely across all specimens of *L. cuprina* and *L. sericata.* All specimens were also well separated from the two outgroup species, *L. caesar* and *C. vicina*. *L. cuprina* sequences, excluding those from Hawaii, showed between 2 and 11 intraspecific nucleotide differences. *L. cuprina* from Hawaii showed 53–59 nucleotide differences from other specimens of *L.*

cuprina, but only 16–20 nucleotide differences from global *L. sericata* sequences; global, non-Hawaiian specimens of *L. cuprina* showed between 45 and 55 nucleotide differences with global *L. sericata* sequences. The global sample of *L. sericata* specimens showed 0–6 intraspecific nucleotide differences, of which sequences from only two specimens were identical.

Harvey *et al*. (2003a) sequenced forensically significant calliphorids from South Africa, Swaziland, Botswana and Zimbabwe and Australia over an 1167 base pair region of the COI gene. The lowest interspecific variation was between the two sister species pairings of L. *sericata* and *L. cuprina* at 3%, and *C. rufifacies* and *C. albiceps* at 3.5%. At a higher level, the subfamilies Chrysomyinae and Luciliinae displayed greater than 8% variation across all species. Harvey *et al*. (2003b) used 278 bp region of COI for 3 species of calliphorids commonly associated with the corpses in Western Australia, *Calliphora dubia*, *Chrysomya rufifacies* and *L. sericata*, in addition to specimens of *Calliphora augur* and *Chrysomya megacephala*. Results provided high support for separation of congeneric species.

Wells *et al*. (2004) sequenced 593 bp of COI from the 24 specimens of *Chrysomya chloropyga* (Wiedemann) and *C. putoria* (Wiedemann) (Diptera: Calliphoridae) which are closely related Afrotropical blow flies that breed in carrion and latrines. The results showed 5 haplotypes (i.e. mitochondrial genotypes) designated A–E. The two haplotypes (C and D) recovered from *C. putoria* were also recovered from some *C. chloropyga*, so neither of these haplotypes was found to be unique to *C. putoria*. The deep divergence of mtDNA COI between specimens of *C. chloropyga* was more than twice that found in a worldwide survey of *C. rufifacies* (Wells and Sperling, 1999). Both the high frequency and basal position of haplotype E suggest that that is the oldest in their sample (Crandall and Templeton, 1996). All of the polymorphic sites represent silent substitutions, i.e. all haplotypes code for the same amino acid sequence. Zehner *et al*. (2004) described DNA sequence data of twelve species from subfamily Sarcophaginae, on the basis of a segment of COI and ND5 genes. The two specimens of most species showed identical sequences. Within the genus *Sarcophaga* the genetic distances are relatively low, i.e. 2.7%–4.7% in the case of COI and 2.3%–4.1% in the case of ND5. The distances among the COI and ND5 sequences within the other Sarcophaginae investigated range from 6.1% to 10.5% and 6.5% to 13.2%, respectively.

Saigusa *et al*. (2005) reared dipteran nymphs that were early colonizers on the corpse to adult and identified them morphologically. Meanwhile they were sequenced over a 304 base pair region of the mitochondrial COI. Six species belonging three genera of Calliphoridae (*Calliphora lata, C. vicina, Lucilia cuprina, L. illustris, L. sericata, Chrysomya pinguis*) and two species of Sarcophagidae (*P. crassipalpis, P. similis*) were collected and identified. Each fly had somewhat different ecological features such as seasonal dominance and habitat. The COI sequences of each species were unique and distinguishable from each other, although they showed high homology. Wallman *et al*. (2005) analyzed four genes COI, COII, ND4 and ND4L of mitochondrial DNA of 33 species of Australian Calliphoridae. The results showed that the combination of these four genes should identify most species reliably, although some very closely related taxa could still be misdiagnosed.

Ames *et al*. (2006) studied 523 bp region of COI in two blow fly species *Calliphora vicina* (Robineau-Desvoidy) and *Calliphora vomitoria* (Linnaeus) of UK. The differences consist of 17 base substitutions resulting in a variation level of 4% between species. Nelson *et al*. (2007) sequenced 658 bp fragment of the COI gene from 56 specimens representing all nine *Chrysomya* species and three calliphorid species were taken as outgroups. All species were resolved as reciprocally monophyletic on the NJ tree. Mean intraspecific and interspecific sequence divergences were 0.097% (range 0–0.612%, standard error = 0.119%) and 6.499% (range 0.458–9.254%, standard error=1.864%), respectively. In one case, a specimen that was identified morphologically was recovered with its sister species on the NJ tree. They found COI barcode to be suitable for the identification of *Chrysomya* species from the east coast of Australia. Wells and Williams (2007) tested Chrysomyinae COI sequences obtained from 245 newly sequenced and 51 specimens from the published literature. Alessandrini *et al*. (2008) bred immature specimens found from human corpses recovered in Italy to obtain adult flies. DNA was extracted and analyzed by a 900 bp fragment of COI and COII genes, using four degenerate primers. MtDNA analysis identified seven Diptera species: *C. vicina, L. sericata, Sarcophaga crassipalpis, Sarcophaga argyrostoma, Chrysomya albiceps, Phormia regina, Musca domestica* with sequence identity varying between 97% and 100%, because of intraspecific and geographic variations. They confirmed genetic identification by morphological analysis. Harvey *et al*. (2008) utilized 1167 bp of the COI gene from 119 specimens from 22 countries, and confirmed the utility of the COI gene in identifying most species. The species *L. cuprina*, *Ch. megacephala*, *Ch. saffranea*, *Ch. albifrontalis* and *Calliphora stygia* were unable to be monophyletically resolved based on these data. At the species level, all of the species has bootstrap support of over 94% except *Ch. megacephala* (paraphyletic, but with a large internal clade with 83% support), *L. sericata* (81%), and *Ch. putoria* (69%). Most specimens were accurately assigned to their respective species. The exceptions were *C. stygia* and *C. albifrontalis*, which were intermingled; *Ch. megacephala*, which formed a paraphyletic grade with respect to a monophyletic (*C. rufifacies* + *C. albiceps*) and a monophyletic *Ch. saffranea* because of two Malaysian specimens; and *L. cuprina*, which formed two distinct clades that were collectively paraphyletic with respect to *L. sericata*. One clade of *L. cuprina* consisted of individuals from Australia, Senegal and Uganda, while the other represented Taiwan, Thailand and Hawaii. Smith and Baker (2008) sequenced 524 bp region of COI gene of four blow fly species *P. regina, C. vomitoria, L. caesar and L. illustris* in UK. Pairwise analysis of published COI sequences revealed that across a 524 bp region there was a mean sequence identity of 91.8% across eight blow fly species (ranging from 87.9% to 97.5%). Honda (2008) also observed 99% similarity of *Phoenicia sericata* collected by the his students and earlier published by Wallman *et al.* (2005) and Stevens *et al*. (2002). Song *et al*. (2008c) demonstrated phylogenetic relationship among 15 sarcophagid flies representing six subgenera of genus *Sarcophaga* on the basis of partial DNA sequences of two mitochondrial genes, cytochrome b and COI. Results revealed monophyly of three subgenera i.e. *Parasarcophaga*, *Boettcherisca* and *Liopygia*. Their analysis showed that the subgenus *Parasarcophaga* and *Pandelleisca* were not monophyletic. This study showed no resolution for the phylogenetic positions of *S. (Pandelleisca) similis* and *S. (robineauella) coei*. Furthermore, the MP analysis and the likelihood function analysis revealed incongruent phylogenetic relationship between the *Scopariiformis iwuensis* clade and the *Polystylata hui* clade.

Park *et al*. (2009a) amplified and sequenced the full length of the COI gene of the Luciliinae fly species collected in Korea. The results showed that the COI sequences are instrumental in identifying Luciliinae fly species. The maximum intraspecific distances were 0.2%, 0.0%, 0.8%, and 0.4% for *P. sericata*, *H. ligurriens*, *L. caesar*, and *L. illustris*, respectively. Because only one individual of *L. ampullacea* was available, intraspecific variation in this species was not estimated. Interspecific distance was the lowest between *L. caesar* and *L. illustris* (1.4%-1.9%) and the highest between *L. ampullacea* and *H. ligurriens* (8.1%). A phylogenetic tree generated from COI sequences of Korean Luciliinae flies showed no paraphyly within the genera or species levels. Park *et al*. (2009b) again reported the full length sequences of the COI gene of four Calliphorinae fly species collected in Korea (five individuals of *Calliphora vicina*, five *Calliphora lata*, four *Triceratopyga calliphoroides* and three *Aldrichina grahami*). The intraspecific sequence distances ranged from 0.0% to 0.4% whereas interspecific distances from 3.8% to 6.4%, which indicated that species level identification is possible with these sequences. Reibe *et al*. (2009) analyzed a 229 bp fragment of the mitochondrial COI gene from all 53 Calliphoridae specimens collected in Bonn, Germany. All specimens were identified correctly in the Blast searches except for *L. caesar* and *L. illustris*. When comparing the sequences of both species, it became obvious how very similar they were. They diverged by only 2 bp (0.87%). In comparison, the average intraspecific variation in *L. caesar* was 1.57%. The divergence between the subfamilies Calliphorinae and Luciliinae was 8%–9%, between Luciliinae and Chrysomyinae was 9%–11%, and between Calliphorinae and Chrysomyinae was 11%–12%.Saigusa *et al*. (2009) compared 304 bp sequences of COI of 8 species under 4 genera of order Diptera. Eggs and larvae were recovered from the corpse during forensic autopsies and then reared in a growth chamber under controlled conditions. The emerging adults were identified based on the shape of the genitalia. The most homologous sequences with *L. ampullacea* were those of *L. illustris*, with 0.9145 or 26 differences, and the analyzed sequences of *L. ampullacea* were different from all other 8 species. Therefore, this species could also be identified using the molecular method.

The mitochondrial DNA region encompassing the COI and COII genes of two Malaysian blow fly species, *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) were studied by Tan *et al.* (2009). This region, which spans 2303 bp and includes the COI, tRNA leucine and partial COII was sequenced from adult fly and larval specimens, and compared. Intraspecific variations were observed at 0.26% for *Ch. megacephala* and 0.17% for *Ch. rufifacies*, while sequence divergence between the two species was recorded at a minimum of 141 out of 2303 sites (6.12%). Tourle *et al*. (2009) studied COI and 28s rRNA gene in two phenotypically resembled blow fly species *Lucilia cuprina* (Weidemann) and *Lucilia sericata* (Meigen) collected from Bothasig, South Africa. The alignments were 439 bp for COI and 678 bp for 28S. Bajpai and Tewari (2010) studied the phylogenetic relationship between five species (*Sarcophaga ruficornis*, *Sarcophaga albiceps*, *Sarcophaga argyrostoma*, *Sarcophaga dux* and *Sarcophaga knabi*) of Sarcophagidae on the basis of COI and ND5. The COI gene revealed 71 variable sites in 296 bp long sequences and 26 sites were fund to be parsimony informative. Boehme *et al.* (2010) analyzed 111 specimens belonging to 13 species of families Calliphoridae, Piophidae and Muscidae originating from Frankfurt am Main, Germany on the basis of COI gene of mtDNA. Intraspecific variation ranged from 0% to 1.17% and interspecific variation occurred between 1.17% and 15.21%. DeBry *et al*. (2010) generated DNA data for *L. cuprina* from North America, including 1200 bp portions of both the mitochondrial COI gene and the nuclear 28S rRNA gene. With the new data, *L. cuprina* remains monophyletic for 28S but paraphyletic with respect to *L. sericata* for COI gene. However, they found that all flies that were identified as *L. cuprina* by morphology and had *L. sericata* like mtDNA form a distinctly monophyletic mtDNA clade.

Preativatanyou *et al*. (2010) demonstrated the application of partial mitochondrial COI and COII sequences for differentiation of forensically important blowflies in Thailand; *Ch. megacephala*, *Ch. rufifacies* and *L. cuprina* by PCR-RFLP. The PCR yielded a single 1324 bp sized amplicon in all blow fly specimens, followed by direct DNA sequencing. TaqaI and VspI predicted from the sequencing data provide different RFLP profiles among these three species. Sequence analysis revealed no significant intraspecific divergence in blow fly specimens captured from different geographical regions in Thailand. Tan *et al.* (2010) reported *I. martellata* (Senior-White) from peninsular Malaysia identified on morphological and molecular basis that included the COI, COII, tRNA leucine and a spacer region. This mtDNA region was observed to have a strong AT bias (average 69%), which is typical of insect mitochondrial DNA (Crozier and Crozier, 1992, 1993). distinct congeneric clusters were formed based on the sequence data.

Guo *et al*. (2011) identified 7 Chinese sarcophagid species *B. peregrina* (Robineau-Desvoidy), *P. similis* (Meade), *P. albiceps* (Meigen), *P. dux* (Thompson), *Bercaea africa* (Wiedemann), *Sarcophaga haemorrhoidalis* (Fallen), *Sarcophaga javanica* (Lopes) and *Phallosphaera gravelyi* (Senior-White) on the basis of sequencing of a 272 bp 'barcode' fragment of the mitochondrial COI gene. Phylogenetic analysis of the sequenced segments showed that all sarcophagid specimens were properly assigned into seven species, which indicated the possibility of separation congeneric species with the short fragments. Liu *et al.* (2011) studied nine species under three subfamilies of Calliphoridae on the basis of 278 bp region of COI gene. The maximum intraspecific divergences were less than 1.2%, except for *Ch. megacephala* at 4.3% and *L. sericata* at 2.1%. Levels of interspecific divergences varied from 4.5% to 15.4%. The lowest interspecific divergence (4.5%) was observed between the congeneric species *L. sericata* and *L. porphyrina*. Raghavendra *et al*. (2011) employed phylogenetic data from the mtDNA COI and COII genes for the identification of an unknown maggot to species level which was harvested from cadaver in June 2009 in Harrison County, Taxas. They successfully identified the species as *Sarcophaga bullata* Parker which is a common carrion species in southeastern United States. Singh and Wells (2011) reconstcurted phylogenies of the Chrysominae based on 2285 bp region of combined data from mitochondrial COI and nuclear carbamoylphosphate synthetase (CPS) genes. A sister group relationship between *Trypocalliphora* and *Protocalliphora* indicates that obligate bird parasitism evolved once within the Calliphoridae. For the first time all Neotropical genera (*Cochliomyia* Townsend, *Compsomyiops* Townsend, *Paralucilia* Brauer and Bergenstamm, *Hemilucilia* Brauer and *Chloroprocta* Wulp) were found to comprise a single lineage and *Chrysomya* Robineau-Desvoidy, traditionally a member of Chrysomyini was found to be closer to the Phormiini.

Similarly, *Hemilucilia* + *Chloroprocta* were a monophyletic group. Every genus for which they examined more than one species was found to be monophyletic.

Kavitha *et al.* (2012) identified 10 samples of blow fly larvae collected from different crime scenes in Malaysia on the basis of entire COI, COII and t-RNA leucine genes. They showed that 80% of the identified samples were in concordance with the morphological results. Xiong *et al*. (2012) studied 31 forensically important muscid flies collected from 15 locations in 11 provinces of China, and a 272 base pair region of the mitochondrial COI gene was sequenced. The monophyletic branches of the phylogenetic tree revealed that this marker is suitable for discrimination between these five species of four genera. Stamper *et al.* (2013) analyzed data from three mitochondrial gene fragments i.e. COI, COII and ND4 obtained from 43 species of Sarcophagidae representing 15 genera. The final alignment consisted of 2924 bp of DNA sequence data per taxon: COI 1539 bp; COII 683 bp; ND4 702 bp. They found no evidence of compositional differences between taxa.

Sharma *et al.* (2014) sequenced 450 bp region of COI from three species of flesh flies and concluded that COI gene has a lot of potential for species identification. Sharma *et al.* (2015a) phylogenetically analyzed ten species and sequenced 465 bp regions of COI gene. They concluded that COI gene is very good for interspecific species discrimination and as intraspecific variations was 0.0% in all the samples they suggested not to use it for itraspecific studies or to sequence whole gene instead of partial fragment. Sharma *et al.* (2015b) again analyzed 465 bp regions of two forensically important flesh flies by using different primer pair and showed that COI can easily distinguish between species.

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