Original Article

Molecular Species Identification of Six Forensically Important Iranian Flesh Flies (Diptera)

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Abstract

Background: Flesh flies (Diptera: Sarcophagidae) are considered as myiasis agents and important evidences in forensic investigations. However, their use has been restricted because, at all larval stages and female adults, morphological species identification is difficult or very challenging. This study investigated to test utility of mitochondrial cytochrome oxidase subunit I (mt-COI) sequences for differentiation of six forensically important Iranian flesh flies namely, *Sarcophaga crassipalpis*, *S. flagellifera*, *S. hirtipes*, *S. aegyptica*, *S. africa* and *S. argyrostoma*.

Methods: Male specimens were morphologically identified to species level and then the genomic DNA of the flies were extracted and subjected to polymerase chain reaction (PCR) against mt-COI gene. The PCR products were sequenced and the obtained sequences were analyzed for the species specific restriction fragment length polymorphisms (RFLPs).

Results: Rate of genetic variation between species was 6–10% which was enough to find restriction enzymes (RE) that were able to produce species-specific RFLP profiles. Combinations of three REs: BsrFI, RsaI and HinfI, provided diagnostic bands for identification of the six *Sarcophaga* species.

Conclusion: The results of this study showed that molecular markers such as RFLPs enhancing the use of evidence from flesh flies in forensic investigation. However, lack proper restriction sites in the COI region inhibited introduction of a single restriction enzyme for easy species identification. It is recommended to apply larger part of DNA such as combination of COI and COII genes to provide better RFLP markers for species identification of flesh flies.

Keywords: Sarcophagidae; Forensic entomology; Flesh fly, Mitochondrial cytochrome oxidase subunit I (mt-COI)

Introduction

A range of insects present on a corpse can be used as evidence in forensic investigations to estimate the post-mortem interval (PMI). Since rate of development in insects is species-specific, commonly, estimation of PMI using insect evidence requires accurate species identification, with subsequent examination of thermos biological profiles to determine insect age (1-4). When larvae or pupae in various stages of development are collected from the site of investigation and the growth rates of samples are known, an approximate time of oviposition or larviposition can be estimated (5). For accuracy, forensic entomologists preferentially use evidence from initial corpse colonizers, such as flesh flies (Diptera: Sarcophagidae) and blow flies (Diptera: Calliphoridae) (2). It can be argued that sarcophagids have the potential to provide a more accurate PMI estimate than calliphorids, based on differences in their life cycles (6). Sarcophagids lay live larvae (viviparous), providing developed immatures ready to start feeding immediately. However, most calliphorids lay eggs (oviparous), which will only hatch into larvae when the correct environmental condi-

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tions are met (3). Despite the prospective use of sarcophagids in forensic investigations, their use to date has been overshadowed by calliphorids. This is due to the difficulties of morphological species-level identification at any life stage of flesh flies, and a lack of documented thermos-biological profiles of these insects.

Sarcophagid flies comprise more than 2,500 species in more than 100 genera, of which approximately 800 species belong to the genus *Sarcophaga* Meigen (7), widespread, and often among the first insects to colonize a corpse (6). The genus is notorious for having many species with a highly similar morphological appearance, females are difficult to identify, and immature stages are often unknown or lack species-specific diagnostic characters (6, 8). Adult sarcophagids can be easily identified at the family level, as most species share the characteristic features of longitudinal stripes on the thorax and a tessellated/chequered abdominal pattern. Identification of the species of the genus *Sarcophaga* is traditionally accomplished on males using characters of the phallus (9, 10). However, species-level identification is difficult and requires examination of subtle morphological variation of bristle placement and length, hair coloration, body pigmentation and genitalic structure of adults (7). This is unfortunate because the PMI is sometimes estimated using larvae and pupae. Rearing larvae and pupae to the adult level may allow identification but is time consuming, often not possible for all species, and may result in a number of unidentifiable females. Considering this, molecular-based approaches for species identifications have been proposed to eliminate issues with identifications based exclusively on taxonomy (11, 12).

Species identification is essential for determining growth rates, as these rates are species-specific (2). Therefore, species identification is a key step in estimating the PMI from entomological evidence. The traditional species identification method is dependent on the morphological features of insects and is not easily applicable to immature samples such as eggs, larvae, and pupae (4-9). Moreover, only a few expert taxonomists specialize in forensically important insect species.

DNA-based approaches have been developed in an effort to improve accessibility to methods of species identification. The mitochondrial encoded cytochrome c oxidase subunit I (COI) gene has been shown to be a major candidate gene to identify forensically important insects (12-14). Numerous studies have evaluated the effectiveness of COI gene, with the approach shown to be unreliable for some Diptera (15, 16) but also proven successful for many groups of invertebrates, such as mosquitoes, cockroaches, and black flies (17-22). Although the COI gene has been used widely for species identification of forensic insects (23-29), however, only a few previously reported studies have used the COI gene for a few forensically important species in Iran (30). Therefore, there has been little effort to characterize the COI haplotypes of Iranian Sarcophagidae fly species. This study examined about 711 nucleotide sequences of the 5’ end of COI gene of six Sarcophagidae fly species from the genus *Sarcophaga* collected in Iran. It is hoped that the results from this study will assist with the implementation of Iranian Sarcophagidae in forensic investigations.

Materials and Methods

Specimen collection

Trapping at decayed meat baits (sheep’s or chicken’s liver and fish carcasses) in plastic bottle fly traps, and hand netting were employed to collect adult sarcophagid specimens in Tehran provinces at the Laleh Park in center of Tehran as well as the livestock shopping center and close vicinity of slaughter houses in the east and south of Tehran, Iran. The captured flies in entomological nets immediately transferred into glass jars. The jars or baits harboring flies were then transported in a polystyrene icebox to the laboratory of Medical

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Entomology, School of Public Health, Tehran University of Medical Sciences (SPH-TUMS). Live adult were transferred individually into a bottle trap including sand and meat to lay eggs at 28 °C±1, 40%±5 relative humidity and 12h photoperiodicity, protected with an external net curtain to avoid the entry of other insect species. After laying eggs, the dead specimens were identified morphologically by using the taxonomic keys (7, 30, 31). To identify the specimens to species level, the male specimens were dissected and the genitalia of each male specimen were examined. All specimens then were kept in 70% ethanol and stored at -20 °C for further molecular investigation in the laboratory of SPH-TUMS.

DNA Extraction, Amplification and Sequencing

One to three legs from each known adult male sarcophagid specimen were used as tissue for total genomic DNA extractions using the Qiagen DNA extraction kit, following the protocols recommended by the manufacturer (Qiagen, Hilden, Germany). The DNA was resuspended in 50–100µl of fresh TE solution (1 mM Tris–HCl (pH 8), 0.1mM EDTA) and subsequently stored at 4° C. The COI region of approximately 711bp was amplified and sequenced using the primers of COI-F 5'-GG TCAACAAATCATAAAGATATTGG-3' and COI-R 5'-TAAACTTCAGGGTGACCAAA AAATCA-3' and thermal cycling conditions explained previously (18, 19, 33). The PCR products were visualized on a UV transilluminator following electrophoresis on a 1.2% agarose gel containing ethidium bromide. The PCR products were purified from seized gels according to the supplier's guidelines (Amersham Pharmacia Biotech, USA) and used directly for DNA sequencing in an automatic sequencer (Seqlab, Goä ttingen, Germany). The sequences were generated in both directions, and the resultant chromatograms were edited using ChromasPro Version 1.33 (available online at www.technelysium.com.au/ChromasPro.html) and aligned using ClustalW2 (34). Homology of the sequences with GenBank sequences was assessed using FASTA search (https://www.ebi.ac.uk/Tools/sss/). Consensus sequences for the COI region was deposited in the GenBank database. Multiple alignments of the nucleotide sequences were performed using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2).

Following sequencing, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was used to distinguish the six species. Based on sequence variation among the six species, three restriction enzymes were selected by manufacturer's NEBcutter V2.0 program (http://nc2.neb.com/NEBcutter2/) and a physical map provided (Fig. 1). For restriction fragment assays, the 711bp COI PCR products were digested separately with restriction endonuclease BsrFI, RsaI, and HinfI (Vivantis) according to the manufacturer's recommendation. Then, the digested sample was resolved by electrophoresis in 2.5% TBE agarose gel, stained with ethidium bromide and viewed under UV-illumination. The restriction patterns were photographed and analyzed.

Results

Morphological Species Identifications

In the present study, 273 specimens were collected from Tehran district and 86 specimens comprising six species of Sarcophagidae were identified using the taxonomic literature. These specimens collectively represent six identifiable taxa: *S. argyrostoma* Robineau-Desvoidy 1830, *S. africa* Wiedemann 1824, *S. aegyptica*/*dux* Salem 1935/Thomson 1869, *S. crassipalpis* Macquart 1839, *S. flagellifera* Grunin 1964, and *S. hirtipes* Wiedemann 1830. These identifications were based on male morphological characters, as these are the only specimens that can be reliably identified. Difficulties were encountered in accurately identifying some of the 273 specimens using the available taxonomic keys. To further assist with identifications, each 'unknown' sequence was com-

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pared with the available data in Genbank, however no conclusive matches were obtained. We cannot confidently associate the unknown *Sarcophaga* species with a particular subgenus, and it is possible that these could represent new species, given that no broad work on the Iranian *Sarcophaga* fauna has been carried out in the past. It is notable that there are 68 reported Sarcophagidae species in Iran (35).

Mitochondrial cytochrome oxidase subunit I (COI) Analysis

The Qiagen DNeasy Blood and Tissue kit (Qiagen) was used to extract genomic DNA from one to three legs of each specimen in each extraction to retain the integrity of the specimens. Known specific mitochondrion COI primers were used to minimize the possibility of amplifying nuclear pseudogenes. About 711 bp products of 5' end of COI gene were able to be amplified using the primer combination of COI-F and COI-R from the DNA extracted from the specimen legs. Direct PCR-sequencing were used and only strongly amplified products were sequenced. Further evidence that the obtained sequences were of mitochondrial origin came from BLAST search followed by the observation that they did not contain base ambiguities or premature stop codons upon translation. This mtDNA region was observed to have a strong AT bias (average 67%), which is typical of insect mitochondrial DNA (36). The ambiguous parts from the beginning and end of sequences for the six species were removed and finally 621bp reliable data were analyzed. Sequence comparison among species showed a maximum of 0.2% intra-specific variation for the species whereas the interspecific variation ranged 6.23–10%. The most divergence rate (10%) was observed between *S. argyrostoma* and *S. flagellifera* and the least rate (6.23%) was seen between *S. hirtipes* and *S. aegyptica*.

Based on nucleotide sequence analysis of the mtDNA-COI gene, a PCR-RFLP assay was developed for the diagnosis and differentiation of the six Iranian *Sarcophaga* species. It was accomplished at first stage by digesting the PCR products (711bp) with *Bsr*FI restriction enzyme. This enzyme cuts the PCR–amplified mtDNA-COI fragment of *S. aegyptica* into two bands of 511 and 200bp whereas it does not cut the amplicons of other five species and the amplicons remain intact (Fig. 2). At next step, RsaI enzyme was used for the five remaining species. This enzyme produces three profiles, 1) a unique profile for *S. hirtipes* with 485 and 226bp, 2) a specific profile for *S. felagellifera* by producing 643 and 68bp bands, and 3) a profile with two bands of 580 and 130bp for three species of *S. africa*, *S. crassipalpis*, and *S. argyrostoma* (Fig. 2). At third stage, HinfI enzyme was used to separate 1) *S. africa* with three bands of 514, 172, and 25bp, 2) *S. argyrostoma* with two bands of 550 and 80bp, and 3) *S. crassipalpis* with an intact 711bp band (Fig. 2).

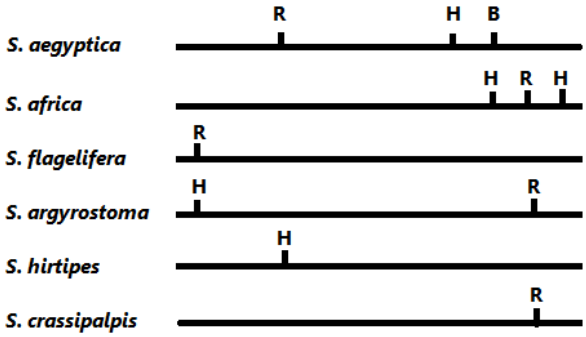

Fig. 1. Physical map of 711bp of 3' end of mtDNA-COI for six Iranian *Sarcophaga* species. B: BsrFI, R: RsaI, H: HinfI restriction enzymes

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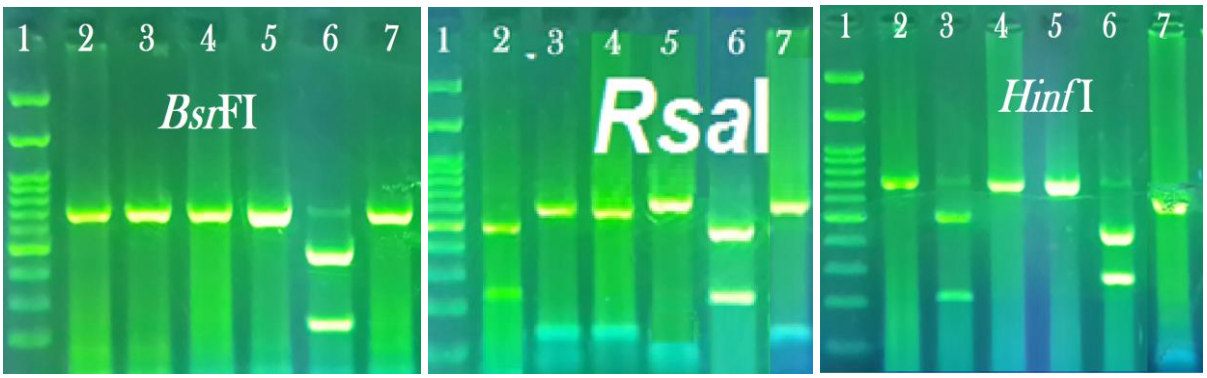

Fig. 2. PCR-RFLP assay of 711bp of COI region digested with BsrFI, RsaI, and HinfI. Lane 1 is a 100bp plus ladder (Sinaclon, Iran), 2: *Sarcophaga hirtipes*, 3: *S. africa*, 4: *S. crassipalpis*, 5: *S. flagellifera*, 6: *S. aegyptica*, 7: *S. argyrostoma*

Discussion

This study was aimed at carefully evaluating the COI gene for species identification of six forensically important Iranian Sarcophagidae. Calculation of the percentage divergence between sequences is used to quantitatively evaluate the success of COI DNA. For successful species-level resolution using the COI approach, genetic variation between species (interspecific) exceeds that of within species (intraspecific) in which intraspecific variation should be less than 3% and interspecific variation should be more than 3% (37, 38). This was the case for all of the six Iranian Sarcophagidae examined in this study, where the percentage divergences lie between 6.23 to 10 percent through the 621bp compared. Similar result to this was obtained when different part of COI gene were previously evaluated for identification of Sarcophagidae (23, 24, 26, 27). Indeed, various fragments of the COI gene (for example: mini-barcode 127bp, 272–278bp, standard barcode 658bp, 700bp, and entire gene 1,535bp) that show low sequence divergence within species but high divergences among species. Hence, this gene can be employed as taxon "barcodes" and unknown samples can be placed accurately into species groups simply by calculating their pairwise genetic distances with reference sequences of a "barcode library" (37, 39). As such, an online COI identification system will provide a cost-effective and widely accessible identification tool for flies of forensic interest, even for immature or damaged specimens.

At present, such practice are being increased for species of the genus *Sarcophaga*, since COI sequence database has been developed for 12 species from the south of the Czech Republic (12), for seven species within Canada and the USA (6), for 17 Malaysian, two Indonesian, one Japanese species (40), for 16 Australian species (26), for 56 species in West Europe (25), for several Chinese species (23, 24, 28), for six Korean species (27), ten Iranian species (41) and recently for ten Indian species (29). However, a database for other regions in the world is lacking. Since the above mentioned studies have shown that intraspecific sequence divergence in *Sarcophaga* is usually well below 1.5%, whereas interspecific divergence is more than 2–2.5%, it can be indicated that the COI gene has a large potential as an identification tool in this forensically interesting taxon.

In this study, we introduce PCR-RFLP markers using 711bp of 3' end of COI gene to identify six species of the genus *Sarcophaga* that occur in Iran. This is the first mtDNA COI PCR-RFLP assay for species identification of Sarcophagidae in the literature. From the PCR-RFLP analysis using combination of three restriction enzymes, each species showed

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a distinct and unique restriction pattern compared to their closely related species. These six species are synanthropic and commonly present in the same collection site, and show high similarities in morphology and behavior.

These results suggest that PCR-RFLP assay are useful to distinguish these species. PCR-RFLP technique is a very cheap and rapid method for species identification of many organisms. This molecular assay was used to identify many species of different group of organisms such as rodents (42), pathogens (43-46), *Anopheles* spp (17, 18, 42) and to determine blood type within insect guts (47, 48). This will provide a cost-effective solution that so many specimens can be studied with no need to provide DNA sequencing. In this method, using a set of conserved primers, a tiny amount of DNA can be amplified and then be identified by restriction enzyme that overcomes lack of ample amount of flesh fly body parts.

Conclusion

The COI sequences of each species were unique and distinguishable from each other, although they showed high homology. Species identification from immature dipteran by the DNA sequences was simple and time-saving because there was no need to wait for adult emergence or knowledge of morphological keys. Nevertheless, given that sarcophagid larvae are likely to be found on a human corpse and cannot be identified based on anatomy, and that the identification of females is very difficult and requires strong taxonomic expertise, the use of the COI gene and or PCR-RFLP as a species identifier will greatly enhance rapid and correct species identification.

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The authors declare that there is no conflict of interests.

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