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Fahd M Abd Algalil
A) Department of Zoology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, India
B) Department of Zoology, Faculty of Applied Sciences, Dhamar University, Dhamar, Yemen

Sureshchandra P Zambare
Department of Zoology, Faculty of Applied Sciences, Dhamar University, Dhamar, Yemen

Correspondence
Fahd M. Abd Algalil
Ph.D. Research Student
Department of Zoology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra State-India

Molecular identification of forensically important flesh flies (Diptera: Sarcophagidae) using COI Gene

Fahd M Abd Algalil and Sureshchandra P Zambare

Abstract
The traditional identification and taxonomy among the flesh flies of the *Sarcophagidae* family can be done based on the morphology of the male genitalia. Nonetheless, Morphological identification of male genitalia sometimes not up to the standard which means that sometimes misidentifications can occur. Barcoding of mitochondrial DNA (mtDNA) Cytochrome C Oxidase subunit I gene (COI) for identification of three species of *Sarcophagidae* which are forensically important was done. *Sarcophaga ruficornis*, *Sarcophaga dux* and *Sarcophaga peregrina* were collected from Maharashtra State, India and used for mtDNA identification. The mean of nucleotide composition frequently distribution within *Sarcophaga* were A= 29.99%, T=37.84%, C= 16.31% and G= 15.85%. *S. ruficornis* and *S. dux* are common ancestor species they are shearing most common ancestor characters and *S. peregrina* is out of a group of *S. ruficornis* and *S. dux*. The results showed that the value of COI gene as an identification marker, since its nucleotide variability endows reliable distinction to be drawn between species. These sequences can be used for correct species identification for future workers.

Keywords: Forensic entomology, sarcophagidae, mitochondrial DNA, cytochrome C oxidase subunit I gene

1. Introduction

The collected entomological evidences from the crime scenes can provide many of the useful information of murders and suspicious deaths by to approximate the minimum postmortem interval (PMI), time of the year, manner and place of death [1-3].

The identification of insects based on DNA using immature insects or fragments of puparium and adult insects can be performed and provide a much faster way for identification and thus facilitate the successful conclusion of a case [4-7]. However, molecular identification techniques will probably tend to be used more for confirmation of traditional morphological methods rather than on their own in forensic cases [8-9].

It was suggested that the method of molecular identification of DNA region is the best choice for the identification of all animals on the earth, this method is known as barcoding DNA and it should be standardized. This approach utilizes a region of 658-bp of the gene encoding the Mitochondrial C Oxidase subunit I (COI) gene [10-12]. This region has been shown to be appropriate region for the identification of a range of taxa, for gastropod [13], butterflies [14], birds [15], mayflies [16], fish [17], blowflies [18] flesh flies [19-21] and cephalopods [22]. The supporters of barcoding (COI) of mtDNA visualized the construction of a universally accessible, accurate database comprising all animal COI sequences; this database will serve as the source for a global bio-identification system for animals [11]. There are many benefits for using of DNA barcoding for species identification and discovery [12], although Wheeler, [23] discussed the concept to be hotly debated. It was assured that an identical approach to species identification would consolidate global research efforts and be useful for the identification of those species with medical, economic or environmental importance [24]. The construction of a barcode database could expose new DNA barcodes that may indicate provisional new species [14].

In this study, COI gen subunit I was used for molecular identification of three species of forensically important flesh flies *Sarcophaga ruficornis*, *Sarcophaga dux* and *Sarcophaga peregrina* (Diptera: Sarcophagidae) Which are collected from Maharashtra State, India.

2. Materials and Methods

2.1 Specimens

Three samples of *Sarcophagidae* flies were collected from different localities. Males were identified using morphological characteristics of the male genitalia with various identification keys [25- 29]. For molecular identification, Legs were removed from male adult specimens and kept in Eppendorf tube containing 3ml of absolute alcohol. Removing of the legs tissues was done in the sterile condition and sterilized in 70% Ethanol and sent for DNA extraction.

2.2 Extraction of DNA

DNA was extracted from the specimen separately by using QIAamp-DNA Mini kit following the manufacturer's instruction with the tissue protocol, Tissue samples were cut into small pieces, and placed in a 1.5ml micro-centrifuge tube, and 180µl of Buffer ATL.QIAGEN proteinase K was added and incubated at 56 °C overnight until the tissue completely lyses.

2.3 Amplification

Gene COI fragment was amplified using Universal primers LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3'25 base pair and HCO2198 5'-TAAACTTCAGGCTGACCAAAAAATCA-3' 26 base pair [30]. 1µL of template DNA was added in 20µL of PCR reaction solution. The PCR reaction was performed with Initial denaturation at 94 °C for 5 min and then 35 amplification cycles at 94 °C for 45 sec, 55 °C for 60 sec, and 72 °C for 60 sec with a final Extension at 72 °C for 10 min.

2.4 Purification

Unincorporated PCR primers and dNTPs were removed from the PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the LCO1490/HCO2198 primers. Sequencing reactions were performed using an ABI PRISM Big DyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems).

2.5 DNA Sequencing

Sequencing forward and reverse was performed on each template using universal primers LCO1490 and HCO2198. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. Sequencing was performed by using ABI 3730xl sequencer (Applied Biosystems).

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of the sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences [31]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and removes alignment noise [32]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as the other existing phylogeny programs using simulated data while being one of the order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering [33].

The alignment of Diptera sequences was carried out using the ClustalX v2.0.12, and the alignment was prepared with BioEdit Sequence Alignment Editor V7.0.5.3. To avoid interferences in the analyses due to the presence of stop codon, the sequences ends were cut. The analysis was, therefore, made with 658 bp from COI barcode fragment, each sequence was submitted to Barcoding of Life Database (BOLD; available online at www.boldsystems.org). All sequences were entered into BOLD under the project name DNA barcoding of forensically important *Calliphoridae* and *Sarcophagidae* flies (DBCS). All sequences of the samples were storage and preliminary barcoding analyses were done. Sequences were submitted to NCBI and the GenBank Accession Numbers of the investigated samples with worldwide known sequences of similar species are as shown in (Table 1).

3. Result and discussion

The collected samples of flesh fly morphologically identified as *Sarcophaga ruficornis*, *Sarcophaga dux* and *Sarcophaga peregrine*, these species belong to *Sarcophaga* genus and subgenus of (*Liopygia, Liosarcophag* and *Bottcherisca*) respectively. The mean of nucleotide composition frequency distribution within the genus of *Sarcophaga* were A=29.99%, T=37.84%, C=16.31%, G=15.85% and the SE was 0.28, 0.36, 0.53 and 0.09 respectively. Sharma *et al*, [16] did the sequencing of 30 specimens belonging to 10 species of Indian *Sarcophagidae* and observed that the average nucleotide composition for 450 bp region of the ten species was A=30.10%, T=40.58.6%, C=16.08%, G=13.24%, and the data showed that maximum A+T bias was observed in *S. hirtipes* (72.2%) and lowest observed in *S. macroauriculata* (69.3%). But our data showed that maximum A+T bias was observed in *S. dux* (68.69%) and the lowest was observed in *S. peregrine* (66.72 %). The content of A+T biases found during the current study was observed to be in agreement with the characteristics of the bases composition of the mDNA of other dipteran insects which are ranging from 72.6% to 82.2% [16, 34].

Sharma *et al*, [17] shown the average nucleotide composition of the 465 bp region of the ten species was A=29.19%, T=37.20%, C=16.61% and G=17.00% and they reported that the maximum A+T bias was observed in *P. ruficornis* (67.50%) and lowest in *P. hirtipes* (64.50%) and our result has shown that the A+T bias in *S. ruficornis* was (68.09%).

Meiklejohn *et al.,* [35] reported that A=29.66%, T=37.02%, C=17.43% and G=15.89%, While Bajpai and Tewari, [36] eported the A=3%, T=40%, C=15% and G=14% as the average nucleotide composition frequency distribution within the genus of *Sarcophaga* for five species (*S. ruficornis, S. argyrostoma*, *S. dux, S. albiceps* and *S. knabi*). These results are similar to our record with a slight difference which may be due to the variation in the subgenus of the barcode species.

According to Kimura`s 2 parameter model (K2P), the mean distance of sequence divergence at the COI barcode region among these sarcophagid flies at species and the family level was 0.0%. While the minimum, maximum, mean and standard error of the distance of sequence divergence at the genus level were 7.87%, 8.58%, 8.22% and 0.1% respectively. Likewise, the minimum, maximum, mean and standard error of the distance of sequence divergence according to the Pairwise Distance model were 7.45%, 8.05%, 7.75% and 0.08% respectively.

In the Kimura`s 2 parameter model, the barcode Gap analysis shown the minimum, maximum and mean distribution of the intra-specific distance to the nearest neighbor species were 7.87%, 8.23% and 7.99% respectively and the SE is 0.06%, while according to Pairwise Distance model the minimum, maximum, mean and SE distribution of the intra-specific

distance to the nearest neighbor species are 7.45%, 7.75%, 7.55 and 0.05 respectively.

The current result has been shown that the *S. dux* is the nearest neighbor species of *S. ruficornis* with 7.87% distance and *S. peregrina* is the nearest neighbor species of *S. ruficornis* with 7.75% distance [36]. Bajpai and Tewari, [36] reported that the *S. dux* is the nearest neighbor species of *S. ruficornis* with 9.4% distance and *S. ruficornis* is nearest neighbor species of *S. peregrine* with 8.23% distance.

The BOLD taxon ID tree shown that *S. ruficornis* and *S. dux* are common ancestor species; they are sharing the most ancestor characters, while *S. peregrina* is out group of *S. ruficornis* and *S. dux* (Fig. 1).

The availability of such DNA database full or partially fragment will facilitate forensic cases by allowing immature stages to be identified [37- 40]. The results obtained in the present study can prove that the sequencing of COI gene has the potential for identification of *sarcophagid* flies.

Table 1: Showing voucher codes, collection locality and accession numbers for Sequences available worldwide for the species under current investigation

S. No.	Species	Voucher ID	GenBank	Country	Collection locality	GPS (Longitude and Latitude)	Reference
1	Sarcophaga dux	FZ1	KT353006	India	Near Paithan dam M.S.	75.3785 N and 19.4824 E	**This study**
		S69	EF405937	Malaysia	Selangor (KS)	3˚20’33.03”N and 101˚14’48.48”E	Tan *et al.* (2010)
		S132	EF405938	Malaysia	Terengganu	5˚12’19.38”N and 103˚12’5.75”E	Tan *et al.* (2010)
		SY9	EF405939	Malaysia	Kelantan	5˚58’20.65”N and 102˚14’43.96”E	Tan *et al.* (2010)
			HM016685	China	Datong, Shanxi	40:07N and113:13E	Yadong *et al.* (2010)
			HM016686	China	Yongzhou, Hunan	26:42N and 111:61E	Yadong *et al.* (2010)
			HM016687	China	Urumqi, Xinjiang	43:46N and 87:36E	Yadong *et al.* (2010)
			HM016688	China	Xining, Qinghai	36:37N and 101:49E	Yadong *et al.* (2010)
			HM016689	China	Changsha, Hunan	28:12N and 112:59E	Yadong *et al.* (2010)
			GU269982	China	Yongzhou, Hunan	26:42N and 111:61E	Yadong *et al.* (2010)
			GU269984	China	Wanning, Hainan	18:80N and 110:39E	Yadong *et al.* (2010)
			HM016690	China	Wanning, Hainan	18:80N and 110:39E	Yadong *et al.*(2010)
2	Sarcophaga ruficornis	FZ4	KT353008	India	Padegaon M.S.	75.2864N and 19.888E	**This study**
		S21	EF405940	Malaysia	Kuala Lumpur	03°07'51"N and 101°39'22"E	Tan *et al.* (2010)
		SY5	EF405941	Malaysia	Kelantan	05°58'21"N and 102°14'44"E	Tan *et al.* (2010)
		A365	JX495081	India	Punjab, Patiala	30˚22’45.94”N and 76˚17’22.89”E	Sharma *et al.* (2014)
		A366	JX495082	India	Punjab, Patiala	30˚22’45.94”N and 76˚17’22.89”E	Sharma *et al.* (2014)
			AF259511	America	Oahu		Wells *et al.* (2001)
3	Sarcophaga peregrina	FZ3	KT353007	India	Near Paithan dam M.S.	75.3747N and 19.485	**This study**
			AF259509	America	U. Hawaii Colony		Wells *et al.* (2001)
			HM016674	China	Urumqi, Xinjiang	43:46N and 87:36E	Yadong *et al.* (2010)
			HM016676	China	Changsha, Hunan	28:12N and 112:59E	Yadong *et al.* (2010)
			HM016677	China	Urumqi, Xinjiang	43:46N and 87:36E	Yadong *et al.* (2010)
			HM016678	China	Lanzhou, Gansu	36:06N and 103:83E	Yadong *et al.* (2010)
			HM016679	China	Yongzhou, Hunan	26:42N and 111:61E	Yadong *et al.* (2010)
			HM016680	China	Changsha, Hunan	28:12N and 112:59E	Yadong *et al.* (2010)
			GU269979	China	Xining, Qinghai	36:37N and 101:49E	Yadong *et al.* (2010)
			HM016681	China	Nanning, Guangxi	22:47N and 108:21E	Yadong *et al.* (2010)
		SCH2	EF405927	Malaysia	Cameron Highlands	04°30'50"N and 101°2523E	Tan *et al.* (2010)
		SCH9	EF405928	Malaysia	Cameron Highlands	04°30'50"N and 101°2523E	Tan *et al.* (2010)
		D407	JX507335	India	Bilaspur, HP	31˚20’48.42”N and 76˚44’36.70”E	Sharma *et al.* (2015)
		D409	JX507334	India	Bilaspur, HP	31˚20’48.42”N and 76˚44’36.70”E	Sharma *et al.* (2015)

Fig 1: BOLD taxon ID tree of barcode records of three Sarcophagidae species collected from Maharashtra, India

4. Conclusion

The barcoding of mtDNA using COI gene is perfect and quick method which is capable of identification and phylogenetic analysis of *sarcophagid* flies. The present study has been done to identify three forensically important flesh fly species of Indian origin using mtDNA- COI gene. The results can be rather helpful for the future workers in their investigations and enhance the data for the advancement of specific markers for the identification purposes.

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