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MOLECULAR ANALYSIS OF SOME FORENSICALLY SIGNIFICANT DIPTERANS ASSOCIATED WITH DECOMPOSING PIG (SUS SCROFA) CARCASS IN IWO SOUTH-WESTERN NIGERIA USING THE COI GENE

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Abstract: Identification of many forensically important insects has been achieved using morphological keys with only a few relying on molecular techniques. Yet, information regarding the molecular identification of Dipteran flies from sub-Saharan Africa is scarce. Therefore, this study used the COI gene to analyze fly specimens collected from decomposing pig (*Sus scrofa*) carcasses in Iwo South-Western Nigeria. Several batches of eggs from carcasses were reared to adults and preserved in 75% ethanol for subsequent identification using molecular techniques in triplicate. A total of 450 bp sequences was obtained from BLAST analysis of the samples from the population, which has led to the identification of four families consisting of five species with the following breakdown: *Sarcophaga africa* (95.4%), *Chrysomya putoria* (99.1%), *Chrysomya inclinata* (98.3%), *Tricogena rubricosa* (89.6%), and *Chirosia flavipennis* (88.8%). The phylogenetic analysis identified *Chrysomya chloropyga* (AY139694) as the same as *C. putoria* and *S. africa* as the same as *Sarcophaga cruentata,* suggesting them as sister species. This study concluded that *T. rubricosa* and *C. flavipennis* are implicated in carrion decomposition, which provides crucial insights for forensic investigations.

Keywords: *Carrion flies, COI gene, decomposition, forensic entomology, identification.*

1. Introduction

Fly communities are crucial biological indicators of time elapsed since the death of an organism (Sharma & Gaur, 2015). Calliphorids (blowflies), Sarcophagids (fleshflies), and Muscids (houseflies) are among the first insects to colonize corpses (Oliveira-Costa, 2003; Reibe & Madea, 2010) as they feed on soft tissues of fresh carcasses. Thus, these species have been included in most forensic investigations. However, accessibility to identification tools has been a significant obstacle to the progression of forensic entomology in most African countries, particularly Nigeria, where the incidence of kidnap, banditry, rape, and other societal vices continues to rise (Ngwama, 2014). It is disheartening that medico-legal investigations, particularly involving the adoption of a forensic entomology approach, remain at its rudimentary stage despite the recent events in the country. Therefore, it is imperative to take steps to produce evidence conducive to swift and accurate prosecution to curb these social vices. The first step in this undertaking involves the accurate identification of these flies, whether they are in the egg, larval, pupal, or adult stage, to the species level. Morphological keys remain the main component in most methods for the identification of insects, whose effectiveness extends only to the family level. Improvements have been seen in these morphological approaches with further identification of key features of adult fly species by Carvalho & Mello-Patiu (2008) in South America, and later certain blowflies by Lutz *et al*. (2018) in Africa. However, similarities between species remain a crucial challenge to morphological identification. Recent advances in research have explored the use of molecular methods in identifying forensically important flies. These methods offer a precise, rapid, and reliable identification of different developmental stages of flies (Sperling, Anderson & Hickey, 1994; Marlgorn & Coquoz, 1999; Oliveira *et al*., 2011). Research has identified the COI gene (Cytochrome Oxidase subunit I) as a suitable marker for the identification of insect species (Harvey, Dadour, & Gaudieri 2003; Nelson, Wallman, and Dowton, 2007; Tuccia *et al.,* 2016). Recently, Sontigun *et al*. (2018) identified 16 species of blowflies in Thailand using the COI and COII genes. Similarly, several other studies have adopted molecular techniques to identify numerous fly species, particularly those with medico-legal significance (Sperling, Anderson & Hickey,

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1994; Marlgorn & Coquoz, 1999; Ames, Turner & Daniel, 2006; Oliveira *et al*., 2011). However, this identification has been limited to temperate regions, prompting the need to identify forensically important fly species native to the tropical region, particularly sub-Saharan Africa. Therefore, this study sought to identify some forensically significant fly species from decomposing carcasses in Iwo South-Western Nigeria using molecular techniques.

2. Materials and Methods

Specimen Collection and Preparation

Egg deposits and larvae present on different parts (eyes, nostril, mouth, ears, anus, and body folds) of six (6) pig carcasses were collected into 50-mL universal bottles. The collected samples were transported to the laboratory and transferred into rearing cages. The eggs were placed in 300-mL plastic bowls with a lid. A ventilation panel with a dimension of 20 mm x 25 mm was made on the lids, which were then covered using woven clothes with a dimension of 24 mm x 29 mm to allow air into the bowl. The pork was served as a main diet. The bowls were placed in the rearing cages in which sawdust was added to serve as bedding for the larvae to pupate before emerged. Upon the emergence of adult flies, they were collected using universal bottles and freeze-killed at -20 ℃, according to a method of Lonsdale, Dixon & Gennard (2004). This step is crucial to prevent the rapid degradation of DNA before preserving the specimens in 75% ethanol for 96 h. These adult flies were then mounted and identified using the established morphological keys in Carvalho & Mello-Patiu, 2008, Szpila et al., 2015, and Lutz et al., 2018. These identified flies were preserved again in 75% ethanol for further molecular identification. Four of each of the fly samples were put into 20-mL universal bottles and added with 2 mL of 75% ethanol. Each bottle was labelled and sent to the Biochemistry and Microbiology Department of the University of Fort-Hare, South Africa for molecular analysis. Ethical clearance was sought from the Health Research Ethic Committee (HREC) Institute of Public Health Obafemi Awolowo University (HREC No: IPHOAU/12/1611).

DNA Extraction

Upon the collection, ethanol was completely removed from the flies. The removal was achieved by rinsing the flies in Petri dishes containing phosphate buffer saline (PBS) three to four times. The flies were subsequently chopped using a sterile blade, removed into a 2-mL centrifuge tube, and vortexed. Then, 20 µL of Proteinase K (PK) (for the digestion of contaminating proteins) and 200 µl of cell lysis buffer (CLD) were added to the homogenized samples. The suspensions were incubated at 56 ℃ for 2 h, followed by centrifugation at 15,000 rpm for 1 minute, from which the supernatants were aliquoted into sterilized 2-mL centrifuge tubes. Subsequently, DNA extraction was performed using the commercial kit, Promega ReliaPrep® gDNA Tissue Miniprep System (Madison, USA) (lot number: 0000318464). The protocol provided by the manufacturer was strictly followed and the eluted DNA was preserved at -20 ℃ until further use.

DNA Amplification and Sequencing

A portion (450 bp) of the COI gene of the DNA was amplified using a set of oligonucleotides: forward primer of 5'-CAGCTACTTTATGAGCTTTAGG-3'; reverse primer of 5'-CATTTCAAGCTGTGTAAGCATC-3'. The protocol by Sharma et al. (2015) was followed with slight modifications.

A reaction mixture of 25 µL consisting of 14 µl of master mix, 1 µL each of 10 pmol/L of the forward and reverse primers, 4 µL of RNase nuclease-free water, and 5 µL of DNA template was prepared to perform the polymerase chain reaction (PCR). The amplification was carried out under these cycling conditions; initial denaturation at 94 °C for 3 min, followed by denaturation at 93 °C for 30 sec, annealing at 50 °C for 30 sec, elongation at 72 °C for 60 sec, and a final elongation at 72 °C for 5 min. A negative control was used for the PCR to spot false positives or any likelihood of recontamination.

The transillumination method using 1.5 % agarose gel stained with ethidium bromide (EtBr) was applied to visualize the PCR products. The positive amplicons were subsequently sequenced bi-directionally using an ABI3500xl automated DNA sequencer with a 50-cm Capillary array and POP7 (all supplied by Applied Biosystems).

Editing of Sequences, Blast Search, and Accession Numbers Allocation

The Geneious program version 11.1.5 was used to produce consensus sequences for each positive PCR product using the collected and corrected forward and reverse nucleotide sequence strands. The data produced after the editing were submitted to the BLAST program in GenBank for homology search against other curated sequences in GenBank (http://blast.ncbi.nlm.nih.gov) (Table 1). All the curated sequences were placed on the "highly similar sequences" parameter on the blast tool to reveal their identity. Assembled sequences were subsequently deposited to the NCBI GenBank database, and the following accession numbers OM492409-OM492417 were assigned.

Sequence Analysis

The sequences generated were submitted to a Clustal W program in MEGA software version 11 for sequence alignment (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were subsequently performed using nine nucleotide sequences, and a neighbor-joining tree was drawn with branch lengths of the same unit as those used for evolutionary distances (Tamura et al., 2011). Evolutionary distances were calculated using the Maximum Composite Likelihood method, according to Tamura et al. (2004). The variation in the gene within and between species was also determined using the Kimura two-parameter model.

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3. Results

A total of 52 individuals of over 450 bp of the COI gene were sequenced. The BLAST search revealed that the analyzed flies belonged to four families (*Sarcophagidae*, *Calliphoridae*, *Anthonomylidae*, and *Rhinophoridae*) (Table 1). A comparison between the sequences to their reference strains in the GenBank revealed a sequence similarity ranging from 99.19% to 88.85% (Table 2).

Table 1. Reference sequences NCBI GenBank used in this Study

Family	Species	Accession Number	COI length	Region	Author	Reference
Anthomyiidae	*Chirosia flavipennis*	DQ657040	1-743	Singapore	Kutty *et al.*	Kutty *et al.* (2007)
	Chirosia cinerosa	MZ625164	1 to 658	Finland	Roslin *et al.*	Roslin *et al.* (2021)
	Palesisa nudioculata	NC_041657	1-16476	China	Han *et al.*	Unpublished
Calliphoridae	*Melinda viridicyanea*	GQ409335	1-725	Singapore	Kutty *et al*.	Kutty *et al*. (2010)
	Chrysomya putoria	FJ195384	1-1256	USA	Singh *et al.*	Singh *et al.* (2011)
	Chrysomya putoria	MH034007	1-313	South America	Mat *et al.*	Unpublished
	Chrysomya putoria	MH034006	1-313	South America	Mat *et al.*	Unpublished
	Chrysomya putoria	EU418542	1-1167	United Kingdom	Harvey *et al*.	Harvey *et al*. (2008)
	Chrysomya putoria	AF295554	1-2304	USA	Wells and Sperling	Wells and Sperling (2001)
	Chrysomya putoria	AB112835	1-1167	Australia	Harvey *et al.*	Harvey *et al.* (2003)
	Chrysomya putoria	AB112831	1-1167	Australia	Harvey *et al.*	Harvey *et al.* (2003)
	Chrysomya putoria	AB112860	1-1167	Australi	Harvey *et al.*	Harvey *et al.* (2003)
	Chrysomya inclinata	AB112857	1-1167	Australia	Harvey *et al.*	Harvey *et al.* (2003)
Sarcophagidae	*Sarcophaga africa*	JQ582120	1-1535	Belgium	Jordaens *et al.*	Jordaens *et al.* (2013)
	Sarcophaga africa	KF038000	1-2305	China	Guo *et al.*	Unpublished
	Sarcophaga cruentata	JN604570	1-1539	China	Ling *et al.*	Unpublished

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Rhinophoridae	*Tricogena rubricosa*	KP004882	1-634	Czech Republic	Ziegler and Tothova	Unpublished
	Tricogena caucasica	KP004881	1-736	Czech Republic	Ziegler and Tothova	Unpublished

Table 2. Percentage similarity index between the sequences of the collected carrion flies and the respective reference sequences from the GenBank

S/N	Specimen	Blast molecular identity	Percentage similarity
1	IWO1901.1	*Sarcophaga africa*	95.44%
2	IWO1902.1	*Chrysomya putoria*	99.19%
3	IWO1903.1	*Chrysomya putoria*	98.86%
4	IWO1904.1	*Chrysomya putoria*	94.47%
5	IWO1905.1	*Crysomya inclinata*	98.25%
6	IWO1906.1	*Chrysomya putoria*	98.91%
7	IWO1909.1	*Chirosia flavipennis*	88.85%
8	IWO1910.1	*Tricogen rubricosa*	89.60%
9	IWO1913.1	*Chrysomya putoria*	91.61%

Phylogenetic Analysis

C. putoria, C. inclinata, S. africa, C. flavipennis, and *T. rubricosa* were clustered with reference sequences to construct a phylogenetic tree according to Vogel *et al.* (2014) (Figure 1). The neighbor-joining trees of the sequences of COI genes from this study showed three distinct groups with a clear monophyletic cluster among them. The genus *Chrysomya* was divided into two clades: one clade consists of *C. putoria* while the other consists of *C. inclinata*. The genus *Chrysomya* formed a monophyletic group with *S. africa*. Similarly, *C. flavipennis* and *T. rubricosa* formed a monophyletic group with *Musca domestica.*

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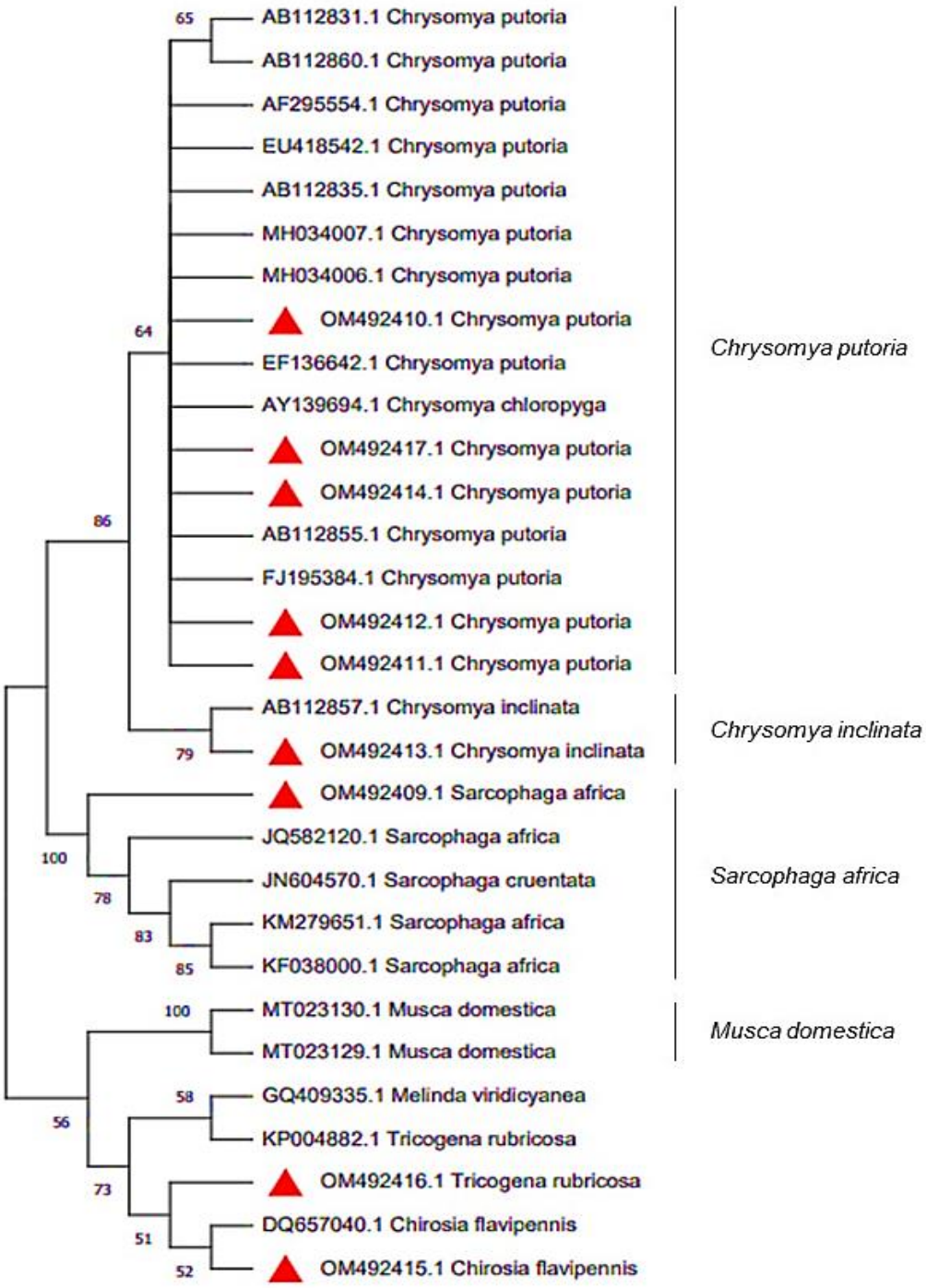

Figure 1. Neighbor-joining tree of COI sequences (450 bp) demonstrates evolutionary relationships among species.
Genetic Distance

Based on the 450 bp of COI sequences, the mean percentage of the interspecific variation between the carrion flies collected from this study varied from 0.01% to 0.25% (Table 2). *C. putoria* and *C. flavipenis* showed the highest variation (0.25%) while the least was observed between *C. putoria* and *C. putoria* (0.01%).

Species pairs of *C. putoria/C. inclinata*, *C. flavipenis/T. rubricosa*, and *T. rubricosa/S. Africa was* separated by 0.09%, 0.17%, and 0.22%, respectively. Similarly, the intraspecific distance values of the sequenced data varied from 0.03% to 7.33% (Table 3) with the highest value recorded in *T. rubricosa.* The calculated pairwise distance indicates that *C. Putoria* differs only by 0.01%, whereas *C. putoria* and *C. inclinata* differ only by 0.03%.

Table 3. Percentage mean of interspecific distances of carrion flies with their respective standard error based on 450-bp COI sequences

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S/N	SPECIES	INTERSPECIFIC DISTANCE								
		1	2	3	4	5	6	7	8	9
1	*Tricogena rubricosa*		0.03	0.04	0.03	0.03	0.04	0.04	0.03	0.03
2	*Sarcophaga africa*	0.22		0.03	0.03	0.03	0.03	0.03	0.03	0.03
3	*Chrysomya putoria*	0.25	0.18		0.02	0.03	0.02	0.02	0.02	0.03
4	*Chrysomya putoria*	0.19	0.14	0.07		0.02	0.01	0.01	0.01	0.03
5	*Chrysomya putoria*	0.21	0.23	0.16	0.08		0.02	0.03	0.02	0.03
6	*Chrysomya putoria*	0.19	0.14	0.07	0.01	0.09		0.01	0.02	0.03
7	*Chrysomya putoria*	0.17	0.13	0.07	0.02	0.10	0.02		0.01	0.03
8	*Chrysomya inclinata*	0.18	0.14	0.09	0.04	0.12	0.05	0.03		0.03
9	*Chirosia_flavipennis*	0.17	0.22	0.23	0.19	0.25	0.18	0.17	0.19	

Table 4. Maximum percentage of intraspecific variation in COI sequences of Dipteran species

S/N	Species	Maximum variation within species
1	*Sarcophaga africa*	0.03±0.02
2	*Chrysomya putoria*	0.09±0.03
3	*Chrysomya inclinata*	0.05±0.01
4	*Chirosia flavipennis*	2.24±1.48
5	*Tricogena_rubricosa*	7.33±2.08

4. Discussion

Cytochrome c oxidase subunit 1 (COI) sequences were used to identify fly species from the Order Diptera among which are the genus: *Chrysomya, Sarcophaga, Chirosia*, and *Tricogena*. The results are comparable with those of Sontigun *et al*. (2018), who identified 16 species of forensically significant blowflies in Thailand, the majority from the genus *Chrysomya*. In addition, Khoso *et al*. (2015) successfully used COI gene sequences and phylogenetic analysis to identify Cyclorrhaphan flies. Carvalho & Mello-Patiu (2008) used advanced morphological keys to identify 12 families of carrion flies, of which *Calliphoridae, Muscidae Fannidae*, and *Sarcophagidae* were the most abundant genera found on carrion and cadaver in South America. *S. africa* identified in this study is comparable to that of Guo *et al*. (2013), who used a large fragment of COI and period gene to identify Sarcophagid flies in India. Harvey *et al*. (2003) conducted molecular identification of some Southern African and Australian blowflies, which led to the discovery of *C. inclinata*, the same species found in this current research. These similar findings demonstrate the vital role of the species in carrion decomposition irrespective of regional differences.

This current study was able to identify the forensically significant fly species using short fragments of 450 bp nucleotides similar to the previous studies using large fragments of ~710 bp (Mashaly *et al*., 2017) and1247 bp (Sotingun *et al*., 2018) nucleotides, and complete genome. The results are also consistent with the findings of Bosly (2020) who used a partial mitochondrial cytochrome oxidase gene of 272 bp to identify *Musca domestica* from Jazan, Saudi Arabia.

The molecular approach for the identification of forensically important flies is the key to a successful species identification method alternative to the exhaustive, time-consuming, and potentially error-prone morphological method. Despite the development of sophisticated and high-quality key features for morphological identification (Lutz *et al*., 2018) based on recent findings, the procedures remain cumbersome and unable to correctly identify down to species level. Although expensive, molecular identification can misidentify sister species; however, the method proves excellent for the accurate identification of organisms when used with other supportive analyses. Based on the tree analysis conducted in this study, *C. putoria* and *C. chloropyga* were identified as sister species. These two species had long been treated as synonymous despite being classified as distinct species (Rognes & Paterson, 2005). This finding demonstrates a case of misidentification. Similarly, *S. africa* and *S. curentata* appeared similar with just 78% bootstrap value. Additionally, *C. flavipennis* and *T. rubricosa* were implicated in carrion decomposition for the first time although with low bootstrap values. These findings provide an indication and further study is necessary to affirm their role in decomposition.

The morphological method also faces limitations when identifying the larval stages of flies that are forensically significant. In contrast, the DNA-based identification method has successfully identified different stages (first, second, third, pupa, and adult) in the life cycle of the carrion flies (Kavitha *et al*., 2013). The previous study further used an empty puparium sampled from ten different crime scenes, which revealed a 100% similarity, demonstrating the accuracy of the molecular method in species identification.

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5. Conclusion

Over the years, molecular techniques using the COI gene have demonstrated consistent success. Out of over two million animal sequences in the GeneBank, 75% are insects, the majority of which were identified using the COI sequences, boasting up to 100% identity similarity. This current study demonstrated a successful identification of forensically important fly species using a short fragment of the MT-CO1 gene (~450 bp). Given the effectiveness of molecular techniques, future forensic investigations may preferentially opt for the use of insects and their larvae for accurately determining the actual time, nature, and circumstances of death.

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