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MOLECULAR IDENTIFICATION OF FIVE SPECIES OF FAMILY MUSCIDAE (INSECT:DIPTERA) FROM BASRAH, SOUTH IRAQ

[1]Zainab. I. Al-frhany and [2]Dhia. K. Kareem
[1,2]Dipartment of Biology, Collage of education for pure science, University of Basrah, Basrah, Iraq, [1]Zainabdaykh@gmail.com, [2]dhia.Kareem@uobasrah.edu.iq

Abstract

Muscidae are a very large and diverse family, so it is difficult to identify them morphologically. Therefore, this study used PCR for five Muscidae species: *Musca domestica*, *Musca sorbens*, *Muscina stabulans*, *Stomoxys calcitrans*, and *Lispe orientalis*, The results of the study showed that the morphological definition matches the molecular definition whose genetic sequences matched globally equivalent species recorded in GenBank at rates ranging from (99.84% - 99.15%) . For the first time, the genetic sequence of each species was documented and preserved on the National Center for Informatics of Life Technology website NCBI under special accession numbers with information indicating the names of researchers and the place of collection. The branches of the phylogenetic tree showed the values of genetic distances for matching and affinity of species with their equivalent species from different geographical environments, and showed high genetic affinity with some species with a genetic distance equal to zero.

Keywords: Muscidae, Musca domestica, PCR, NCBI, COXI, Phylogenetic.

Introduction

The family Muscidae belongs to the Phylum of arthropods, of the insect class, Pterygota subclass, and within the Division Endopterygota, the largest family in the order Diptera and the Musciode superfamily (Pape *et al*., 2011), It is one of the well-studied families and it is called the house fly family due to the spread of the house fly *Musca domestica* all over the world (Pape and Thompson, 2013). Members of the Muscidae family are of medical and veterinary importance due to their ability to transmit diseases to humans and animals, and their developing larvae on carcasses are used as an indicator for estimating the post-mortem period in forensic medicine (Achint and Singh, 2021). There are more than 5000 species of house flies within 170 genera and the Muscidae is highly represented all over the world (Kutty *et al*., 2014). Although many modern and different methods of phylogenetic analysis have been used, the classification of this family remains unstable, variable and controversial (Grzywacz *et al*., 2017). Phenotypic identification of flies of medical and veterinary importance is complex due to their morphological similarity (Sing and Achtinm, 2017). In order to diagnose any species on the basis of phenotypic features, we need a taxonomic key and researchers skilled in the classification of insects, morphological identification requires a long time, and there is no taxonomic key that covers all stages of flies, so entomologists resort to rapid and alternative diagnosis of taxonomic keys, and molecular diagnosis is valuable in diagnosing species that are difficult to distinguish based on phenotypic features, and also aiding in the diagnosis of undiagnosed species, cytochrome oxidase 1 (COXI) is preferred for accurate diagnosis and strain distinction (Achtin and Singh, 2021). The first subunit of cytochrome oxidase

is one of the genes in the mitochondrial genome that is widely used in animal molecular diagnostics (Mokosuli, 2013). The COXI gene encodes the enzyme Cytochrome Oxidase, a protein complex that is found on the inner membrane of mitochondria and is composed of 13 subunits whose function is to catalyze the transfer of electrons and protons and to produce up to 95% of the energy of living eukaryotic cells (Johnson and Rolff, 2013). It has been successfully used to diagnose many species of flies of criminal importance in different parts of the world (Aly and Mahmoud, 2016). Since it is possible to successfully differentiate species of house flies using the COXI gene, it is a good marker for phylogenetic analysis and verification of species geographical distribution patterns due to variation in this gene (Achtin and Singh, 2021). DNA-based methods are widely used to determine any stage of the insect life cycle, whether the samples are alive, dead, or preserved (Aly and Wen, 2013). One of the advantages of mitochondrial DNA is that it has a small and simple structure, a high number of copies, and is easy to isolate These advantages have made it an important tool in the study of genetic and geographical evolution of breeds, population genetics, and lineage tracking (Yuan *et al*., 2015). The use of mitochondrial DNA is advantageous compared to genomic DNA because of the high mutation rates and multiple copies in the cell, and the mtDNA encoding of the cytochrome oxidase gene is particularly useful for studying population genetics and evolutionary relationships of species (Dogac, 2016). This gene is 650 base pairs long and is effectively used to identify various insects of medical and veterinary importance (Onder *et al*., 2019). The COXI genetic sequence has been successfully used to diagnose many flies of medical and veterinary importance (Kavitha and Nazni, 2013)

Materials & Methods

Insect sample collection: The current study was conducted from August 2021 to July 2022, samples were collected from a different area of Basrah province, killed by freezing and kept in plastic containers.

Deoxyribonucleic Acid (DNA) extraction: The thoracic region and legs were isolated from whole and frozen insects collected from study areas previously, and sometimes from the whole body. The study included five species of the Muscidae, namely Musca domestica, Lipse orientalis, Muscina stabulans, Musca sorbens and Stomoxys calcitrans The isolated sections were crushed in a ceramic slurry to obtain a homogeneous powder of 0.020 g, using the gSYNCTM DNA extraction kit, which was provided by Geneaid and the DNA was extracted according to the steps mentioned in the of the producing company's protocol. To separate DNA from tissues with some changes except that 400 µl of GST Buffer buffer solution was added to Eppendorf containing the sample powder.

Primers: Polymerase chain reaction (PCR) was performed to obtain the mitochondrial cytochrome oxidase gene mtCOX1 using general primers. The primers were designed by Bioneer Company (Table 1) and the primers were dissolved by adding 90 µl of Nuclease Free Water.

Table (1): General primer sequences for five species of house flies selected in the current study.

species	Gene name	Primer sequences	Primer lenght
Musca domestica	F COX I	GGTCAACAAATCATAAAGATATTG	24
Musca sorbens			
Muscina stabulans	R	TAAACTTCAGGGTGACCAAAAAATCA	26
Stomoxys calcitrans			
Lispe orientalis			

polymerase chain reaction (PCR)program:

The components mentioned in Table (2) were mixed inside an Eppendrof tube using a shaking device and placed in the thermocycler. The device worked according to a program shown in Table (3), and after the amplification process was completed, the electrophoresis analysis of the products was carried out.

Table (2): Components of a polymerase chain reaction mixture

N	Components	Volum
1	Master Mix	5 µL
2	Forward Primer	2 µL
3	Resever Primer	2 µL
4	DNA	5 µL
5	Nuclease Free Water	11 µL
6	Total	25 µL

Table (3): Polymerase device work program to amplify the studied gene segments**.**

STEPS	Temperature	Time	Number of cycle
Initial denaturation	**94 C**	**1 M**	**1**
Denaturation	**94 C**	**1 M**	**35X**
Annealing	**50 C**	**30 S**	
Etension	**72 C**	**30 S**	
Final Etension	**72 C**	**60 S**	**1**

Electrophoresis analysis: After completing the work program of the polymerase device, the electrophoresis was performed, the weight of the agar used was 0.50 mg, 5 µL of DNA ladder (100-5000) was added in one hole, and 5 µL of PCR product samples in the other slot. Voltage at 85 V for 1 hour, after completion of the electrophoresis, the agarose template was transferred and a UV scan was performed to see the magnified PCR product bundles and imaged directly with the camera.

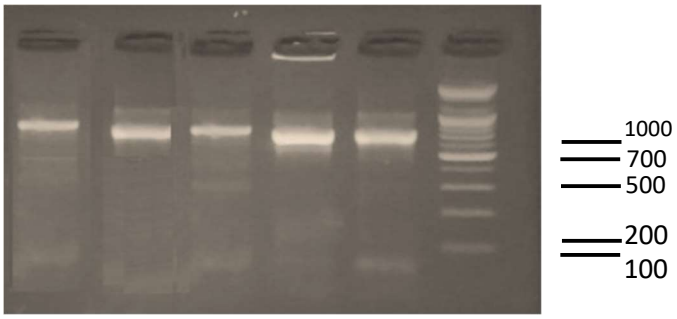
Sequences analysis: Send 15 microliters of PCR results samples to Yang Ling Biotechnology CO. (China) to perform DNA sequence analysis of the mitochondrial COX1 gene using a Genetic analyzer, and the sequence segments were recorded in the Genebank for all five species of house flies studied.

genetic identification: The results were analyzed in practice and And make alignment and filtering of the studied pieces by using Bio Edit Bioinformati programs (SoftwareV. 7.2.6, Network V.5, Mega 7.0.26,) as the pieces became smaller, the sequence of matrices was performed to identification species by applying the BLAST program, and to compare species With identical species in other geographical regions, the evolutionary tree was drawn and the genetic distance between them was calculated.

Preservation of the products of molecular sequences in the genebank: The products of the molecular sequencing of the mtCOX1 gene of the five types of house flies from Basrah Province were documented for the first time in the DJB Gene Database (DNA Data Bank of Japan). For each of those species in Basrah Province, a serial number was obtained for each DNA segment, with the name of the researcher, the species name and the information of the studied gene fixed. The data was published on the site of the gene bank, and it was applied with the species registered from different regions of the world through the genetic sequence of the species deposited in the gene bank, and the percentage was determined percentage of match.

Results

DNA extraction and amplification: This study used the polymerase chain reaction (PCR) method for the molecular identification of five species of the house fly family for the first time in Iraq and Basrah Province by detecting the cytochrome oxidase mtCOXI gene, The results of the molecular identification PCR showed the appearance of the gene for *Musca domestica* at a molecular weight of 710pb, the gene for *Musca sorbens* at a molecular weight of 800pb, for *Muscina stabulans* at a molecular weight of 690pb and *Stomoxys calcitrans* at a molecular weight of 750pb, while the gene for *Lispe orientalis* appeared at a molecular weight of 650pb, and the molecular weights were calculated by comparison with the Ladder (DNA Marker) **(picture 1).**



Picture1: Amplification product for mtCOXI primer on agarose gel.

Genetic code analysis and documentation of genetic sequences: The nucleotide sequences of the DNA fragments of the mitochondrial gene of selected species of the house fly family were analyzed and, after alignment and filtering, it was found that the sequences of the selected species **(Figure 1)** matched the genomic sequences of their counterparts conserved as references in the gene bank with prices ranging from 99.15 to 99.84. All species were subjected to similarity analysis using the Blast genetic sequencing software. mtCOXI of selected species of the fly family was included in the NCBI Gene Bank for the first time as future sources for the house fly family in Basrah and Iraq, in which fragments and their lengths were recorded and given an independent accession number for each fragment **(Table 4).**

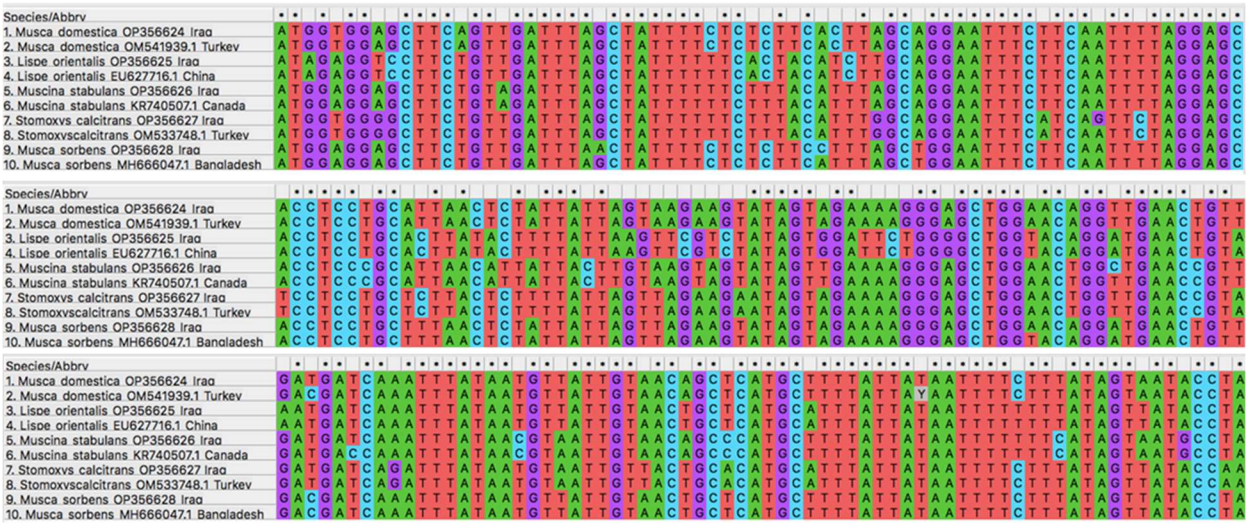


Figure 1: Multiple sequence alignment highlighted with four colours to indicate the similarity and differences within the translated amino acids encoded by COX1 gene of the identified species (analysed by Mega X).

Table (4) Percentages of genotypic sequences of mtCOXI fragment gene segments matching in selected fly species with those conserved in the genebank and accession numbers in the genebank:

N	Species	Accession numbers in the gene bank	Accession numbers of identical species in the gene bank	Match ratio
1	*Musca domestica*	OP356624	OM541939	99.48%
2	*Musca sorbens*	OP356628	MH666047	99.15%
3	*Muscina stabulans*	OP356626	KR740507	99.68%
4	*Stomoxys calcitrans*	OP356627	OM533748	99.77
5	*Lispe orientalis*	OP356625	EU627716	99.84%

Phylogenetic tree: The results of the phylogenetic tree analysis of the selected species in the current study targeting the mtCOXI gene showed that the rootstock of this tree is *Cephslopina*

titllator, and the genetic tree analysis confirmed that the domestic *Musca domestica* was genetically close to isolate number OM541939. 1 from Turkey, that local type II *Musca sorbens* was genetically related to isolate MH666047.1 from Bangladesh, local type III *Muscina stabulans* was genetically related to isolate number KR740507.1 from Canada, while local type IV *Stomoxys calcitrans* was genetically close to isolate OM533748.1 Turkey, and the fifth local species *Lispe orientalis* was genetically close to isolate EU627716.From China, the interspecific genetic distance in tree branches was recorded between 0.0173-0.000 **(Fig. 2).**

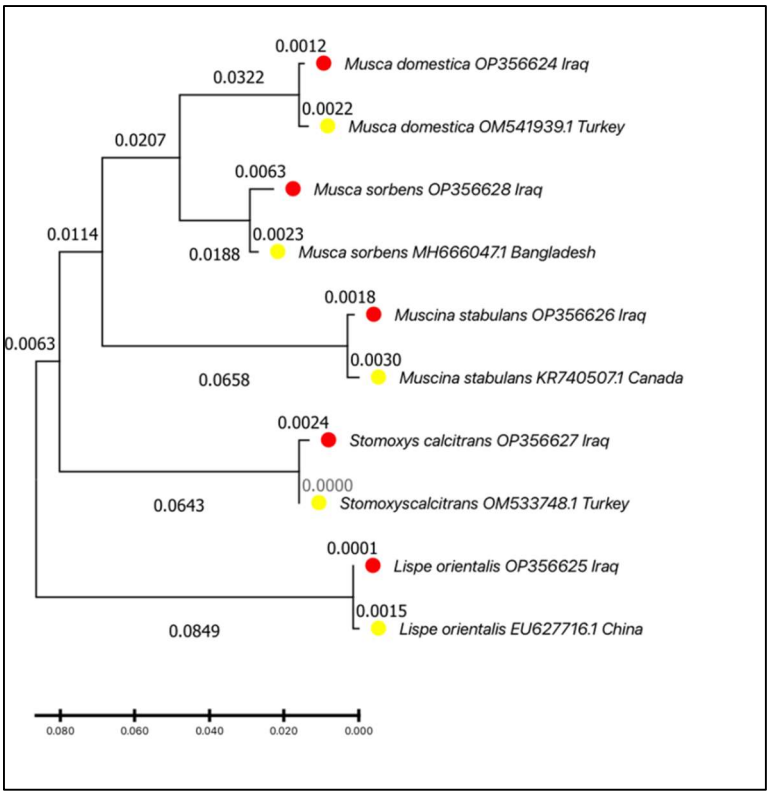


Figure (2): Phylogenetic tree analysis of the identified species targeting the translated amino acids of (COX1) gene. These were deposited in the global gene bank as can be seen as in yellow circles with accession numbers followed by the identified species. analysed using neighbor-joining method in Mega X.

Discussion

The Muscidae family is one of the most famous types of flies in the world, and despite extensive taxonomic research for more than a century, the classification of these flies is still weak (Haseyama et al., 2015), The lineage of flies is one of the most diverse lineages of insects, and this complicates systematic research in evolutionary biology, which includes reconstructing the tree of life of Diptera insects and integrating all available information to compare existing and extinct flies (Trautwein et al., 2012). The taxonomic keys were adopted to identify the members of the Muscidae, which depend on the phenotypic characteristics, including the characteristics of the

middle chest, leg and wing veins (Grzywacz et al., 2017), For example, members of the genus *Stomoxys* and members of *Musca* are similar in morphology and are approximately the same size, and both are spread and exist in the same habitats (Patra et al., 2018), As it is difficult to separate them by untrained people and needs a tool that facilitates their identification, such as PCR reaction, to confirm the phenotypic diagnosis (Sevidzem, 2020), The method of identification based on phenotypic traits gives wrong results, especially for non-expert researchers (Bosley, 2020), Therefore, in the current study, the selected species of the studied flies were identified by using molecular identification by PCR technique to amplify the mtCOXI gene in order to confirm the known species and to establish a database for them in the gene bank, and genetically identical rates ranged from 99.84% - 99.15 to the types of flies registered in Iraq in general and in Basrah in particular with Those species Recorded in the countries of Asia, North America and Europe, and this indicates that these species preserve their genetic makeup and are able to spread between continents and adapt to the environment and climatic changes, through which the relationship of climate to biodiversity can be studied. Avise (2000) explained that one of the most promising methods for determining biodiversity is genetic analysis of a small piece of the genome, since the genetic code enables the diagnosis of many species using one or more genes, thus helping the unskilled researcher in classification to diagnose the species of unknown specimen , the discovery of new species, the abundance of information about mtDNA, in particular the COXI gene, and it has been used to differentiate closely related species (Simon et al., 1994), It is characterized by its great influence on the inheritance of insects (Cameron, 2014), and it is characterized by preserving its genetic coding, as it is rarely exposed to deletion and addition mutations that cause a modification in its pattern, and it is one of the genes used in evolutionary studies because it is one of the well-preserved genes (Bybee et al., 2016). In the current study, a general primer was used for all studied species to obtain the mitochondrial COXI gene sequence, and this contributed to finding a match between genotype and phenotypic classificationThe current study is the first of its kind in Iraq and is consistent with the results of many studies, including the Bosly study (2020), the sequences of these species were included under the accession numbers in the gene bank representing their ownership and to be a genetic source for local flies species used by future studies in various parts of the world to compare with them, In the current study, we encountered difficulties in extracting the genetic material of the insects. The reason is due to the structure of the insect's exoskeleton, which consists of chitin, complex proteins and peptides. These compounds reduce the efficiency of solutions and proteinse enzyme and thus affect the purity and quantity of the extracted DNA (Timah and Semuel, 2017) for this The method of work included a change in the weight of the tissue used from 0.050 to 0.020, as well as the volume of GST, using 400 µl instead of 200 µl. The results of the alignment of the multiple sequences of the five studied species showed that they have a common origin, and they are closely related to each other, and this was confirmed by the high matching ratio of their genetic sequences and the matching of most of their amino acids, the species Lispe orientalis in Iraq is identical to the species recorded in China, noting that this type is recorded for the first time in Iraq and we were not able through the phenotypic definition to know its type. The phylogenetic tree of the five studied species of the house fly family

indicates a close relationship between the species, where five branches appeared and each branch represents a species and the species that corresponds to it globally and the values of the genetic distance between species and the furthest difference recorded in the branch carrying *Musca sorbens* and this indicates that It contains more mismatched nucleotide units than other species. Wiegmann and Yeastes (2017) indicated that the phylogenetic tree serves as an organizational framework for classifying and naming flies, understanding the pattern and timing of evolution, tracking phenotypic and environmental change, documenting species, understanding geographical distributions, and genetic differences recorded between the studied species in the branches of the tree, ranging from few to medium (0.0077-0.0797). Whereas for one species, the difference was significant (0.0064).

Conclusions

The results of the current study indicated that molecular identification could be an effective method for identifying flies and could be an alternative method to the traditional methods of identification.

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