

Improving Maggot Therapy: Mass Rearing and Molecular Identification of *Lucilia Sericata* (Meigen, 1826) Larvae in Maggotarium, School of Health, Shiraz

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Received date: September 16, 2019; Accepted date: September 30, 2019; Published date: October 07, 2019

Citation: Bagheri M, et al. (2021) Improving Maggot Therapy: Mass Rearing and Molecular Identification of *Lucilia Sericata* (Meigen, 1826) Larvae in Maggotarium, School of Health, Shiraz. Cell Mol Biol, Vol.67 Iss.6 No:201.

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Abstract

Introduction: *Lucilia sericata* is a member of the *Calliphoridae* family of insect class. This species has a complete metamorphosis and feeds on dead and decaying tissues as necrophage species. Sterile larvae of this species are used in maggot therapy for wound healing.

Objectives: The aim of this study was to breed and identify the morphological and molecular identification of the *Lucilia sericata* species in Maggotarium of Shiraz School of Health.

Methods: *Lucilia sericata* species were bred under standard conditions in the maggotarium of Shiraz School of Health. They were identified using morphological methods and then, molecular identification and phylogenetic analysis were performed using PCR and CO1 diagnostic marker.

Discussion: In this study, due to the importance of *Lucilia sericata* species in the method of maggot therapy, accurate and correct knowledge of this species can lead to the proper larval therapy. Increasing the resistance of wounds to antibiotics has led to the use of maggot therapy in the past.

Key words: *Lucilia*; Rearing; CO1; Maggot therapy

Introduction

The *Calliphoridae* family is one of the largest and most diverse families of flies. Up to 1500 species in 97 genera of this family have been identified so far, which are distributed in neotropical regions and a large number of them in Africa and southern Europe. From Iran, 20 species of three subspecies, *Calliphorinae*, *Chrysomyinae*, and *Luciliinae* have been reported so far.

This family have been considered by many researchers in the field of medical sciences, forensic medicine, veterinary medicine, pharmaceutical and biomedicine due to the possibility of easy and fast breeding, ability and growth of this insect on cheap materials, the rapid output of the target product, the non-toxicity of the target product, and the stability of the physiological parameters of the insect during the biotechnology process. Also, many studies are still being done on this family.

Lucilia sericata belongs to the *Calliphoridae* family. This species is mostly distributed in hot and humid regions of the world, although they are also present in areas with dry climates. It is a domesticated species and has a high adaptability. *Lucilia sericata* has a complete metamorphosis (Holometaboluse). Life cycle of this species includes egg stages, three larval stages (1, 2, and 3), pupa, and adult. The eggs are placed in clusters of about 2000-3000 on animal carcasses and excrement. About 18 to 24 hours later, they will be opened and entered

the larval stage. The larvae are milky, conical in shape with anterior and posterior spirals, and after 4 to 5 days, they turn into pupae [1]. Pupae usually have a hard, brown and black coating, and finally, after 6 to 14 days, they turn into adult flies. These flies are capable of mass production in the standard environment and conditions of the insectarium. Adults are mostly metallic green. The antenna is tripoded and aristate, and the RS rifle is branched out twice. Frontal sutures are well visible and calypters have been grown well in them. The average lifespan of an adult is usually 7 days. One of the nutritional habits is that it feeds on corpse and dead tissues; therefore, it has a special place in medical sciences, veterinary medicine, and forensic medicine. One of the useful and practical uses of the larvae of *L. sericata* is in the field of Maggot therapy. Maggot therapy is a useful, effective and controlled method using sterile larvae of *L. sericata* to treat a variety of acute and chronic wounds. This method was approved by the US FAD in 2004 with the approval of K033391. This method is also used to treat wounds, which are caused by bed sores, traumatic skin injuries, and burns [2].

It is also effective in chronic diabetic ulcers, osteomyelitis, and ulcers created after cancer surgery. Chronic wounds, which are primarily associated with severe and prolonged inflammation, stop the proliferation of cells and cause the extracellular matrix to be regenerated incompletely. Excessive ECM overexpression temporarily and its ineffective removal from the wound surface usually stop the

healing of chronic wounds. Necrotic tissue is also a good place for the accumulation of various pathogens and the production of biofilms, which in turn cause the infection of dead cells and wounds. Traditional treatments for chronic wounds include surgery, enzymatic debridement, and rinsing. The most important drawbacks of these methods are pain, mechanical damage to healthy tissues, and human error [3]. Maggot therapy can be used as an alternative, classical and controlled method instead of the mentioned methods to treat wounds. Important advantages of this method include cleaning the wound surface, disinfecting and accelerating wound healing. The mechanical movement of larvae on the wound and the production and secretion of various digestive enzymes by them are two very important factors in accelerating wound healing. Maggot excretions / secretions contain a variety of digestive enzymes, including carboxypeptase A, B (carboxypeptidase A, B), collagenase-amino peptidase (aminopeptidases collagenases), aspirin and serine protease (aspart), trypsin- and chymotrypsin-like ones., and metalloproteases, which are active in a wide range of pH. In larval secretions, by breaking down fibronectin and converting it into biological active components, fibroblasts are multiplied and migrated. Today, with the advancement of biotechnology, new research is being done on the use of flies products in the pharmaceutical industry, and the production of recombinant proteins from some *Lucilia* members is being developed and evaluated [4]. As briefly mentioned above, due to the importance of members of this subfamily requires the necessary accuracy and precision. Blow flies have a wide variety of species and their morphological diagnosis is confusing and difficult, and due to the similarity of the species, errors in morphological diagnosis may occur. However, the accurate identification of this species of insect can be done by experts and experienced people. Due to the importance of accurate identification of species, the use of molecular methods to accurately identify and confirm morphological methods is recommended. In general, for molecular identification, a variety of nucleotide and mitochondrial loci and different gene markers are used to accurately identify species. The CO1 Cytochrome c oxidase has a high degree of nucleotide diversity and it is considered by many specialists to identify species. It was first proposed by Harbor and it is well established that this barcode can be used as a suitable marker for identification. Different species of humans, birds, and insects has been considered by many biotechnologists and molecular entomologists as a marker that has been successful in differentiating two species. We also decided to use this method to identify the species of *Lucilia sericata* in the maggotarium of Shiraz School of Health due to the specificity of this marker.

Material and Methods

The fly species

The first *L. sericata* was bought from Shiraz Wound healing and Biotherapy center.

Expected size	(5' _3') Primer sequence	Primer name	Gene
300bp	ACTATTAGTAAGAAGAATAG	Forward	CO1
	AAGTTGCAGGAGAGTAGTTG	Revers	

Table 1: The sequence of designed forward and revers primers.

Breeding

The larvae were transferred to cages (45×45×45) in the Shiraz health school Maggotarium. There were chicken liver and water-sugar inside the cages for flies feeding. The larvae were placed on chicken liver as well as a special container for laying eggs. The eggs were transferred to a new cage. They were exposed to a standard condition; 12-h light/dark cycle with humidity of 40–60% at 16–25°C.

Collection and identification

The larvae and adults of species were collected from cages (Figure 1).



Figure 1: Shiraz Health School Maggotarium.

Identified using the morphological identification key and then, they were confirmed by molecular methods.

Molecular methods

DNA extraction: The genomic DNA of samples was extracted using the instructions of the AccuPrep® Genomic DNA Extraction Kit – Bioneer.

Primer design

The mRNA sequences of cytochrome oxidase subunit 1 (COI) gene, partial cds, and mitochondrial genes of flies were obtained from the NCBI base gene bank. Then, obtained sequences were entered into the Gene Runner software separately and copied in the MEGA 6.0 software. In this application, they were aligned and based on the conserve points between the compared genes, forward and reverse primers were designed using Oligo7.0 software program and Gene Runner version 4.0 [2].

Gene specific forward and reverse primers (GSP) were blasted using NCBI. The sequence of designed primers is shown in [Table 1].

Polymerase chain reaction

Using Gene runner and Mega 6 software, forward and reverse primers were designed for CO1 barcode. Primer sequences can be seen in Table 1. Co1 fragment was amplified using pair primer. The PCR reaction volume was 20 μ l and contained 1 μ l cDNA, 1 μ l of pair primer, 10 μ l master mixes and 7 μ l water. The amplification program included initial denaturation at 95°C for 5 min followed by 35 cycles, including denaturation at 95°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 80s, and an additional final extension at 72°C for 10 min [2].

Sequencing

The products of PCR were purified using Thermo Scientific GeneJET Gel Extraction Kit. The samples were sent to Phishgam Biotech Company in Tehran for sequencing.

Phylogenetic tree

Phylogenetic tree based on COI nucleotide sequences of flies from several worldwide geographical areas, including available data from Gene Bank was constructed by neighbor joining (NJ) method with the Kimura's 2-parameter model implemented in the MEGA_ version 6.1, and the trees were tested by 1000 bootstrap replicates [3].

Results

Morphological identification

In the present study, samples were collected from Maggotarium and identified as *Lucilia sericata* using morphological identification key.

Molecular identification

The expected band of 300 bp appeared on the gel (Figure 2).

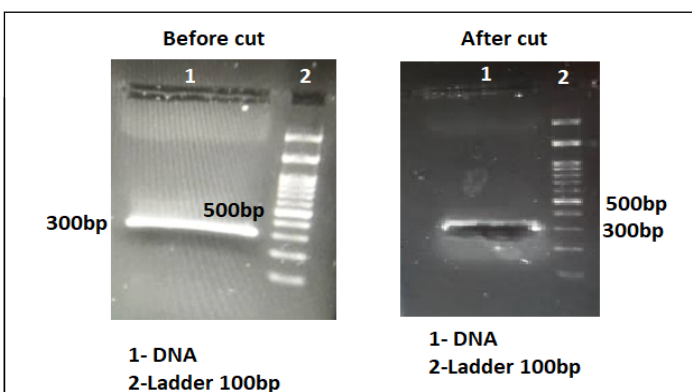


Figure 2: Number 1, PCR product of *Lucilia sericata* (CO1 marker), 300bp band; Number 2: ladder 100.

The results of the sequencing (Gel extraction) were blasted on NCBI site, and all samples were identified as *Lucilia Sericata*.

Phylogenetic analysis

In order to analyze and compare, the phylogenetic tree based on COI nucleotide sequences of different species of flies, their information was registered in the Gene Bank, which was constructed

by neighbor joining (NJ) method with the Kimura's 2-parameter model implemented in the MEGA_ version 6.1, and they were tested by 1000 bootstrap replicates (Figure 3) [4,5].

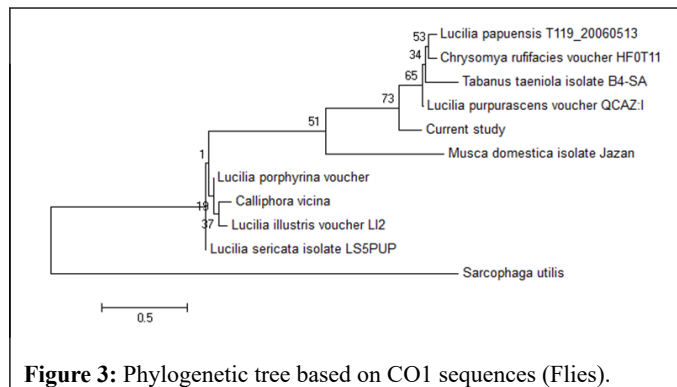


Figure 3: Phylogenetic tree based on CO1 sequences (Flies).

In the present study, different species such as *Lucilia purpurascens*, *L. papuensis*, *L. porphyrina*, *L. illustris*, *L. sericata*, *chrysomya ruffifacies*, *Tabanus taeniola*, *Musca domestica*, and *Calliphora vicina* were compared with our study. *Sarcophaga utilis* was used as the outgroup of the phylogenetic tree [6]. According to the tree, current study and *Lucilia purpurascens* the same group and there was a closer relationship between the two species (73 nucleotide). *L. papuensis* and *chrysomya ruffifacies* are closely related and *Tabanus taeniola* will be placed in the next close relationship. *L. illustris*, *L. porphyrina*, and *Calliphora vicina* are also similar and they were placed in a separate subgroup [7].

L. sericata was clearly separated from them.

Discussion and Conclusion

In the present study, *Lucilia sericata* was identified using morphological and molecular methods.

The aim of this study was to identify the morphological and molecular method for identifying *Lucilia sericata*, and this species was reared on a mass scale in maggotarium at Shiraz School of Health. This species is considered as a suitable model for medical and pharmaceutical research, including the discussion of Maggot therapy. The proteins in larval secretions are now being used both chemically and also in the production of recombinant proteins. Due to the importance of this issue, it is important to accurately identify this species in medical discussions and maggot therapy, which uses sterile larvae of this species to heal wounds.

Temperature and humidity are two important factors in the breeding and growth of insects, including *Lucilia*, which were raised in the present study with the optimum temperature and humidity of larvae. The larvae were fed using fresh chicken liver and sugar water. In a study, Tachibana et al. tested artificial diet primarily composed of whole milk powder, dried yeast, and wheat germ in order to compare the *Lucilia sericata* larval diet and its effect on growth and development. They did not observe a significant difference between them on beef liver, although there was a small difference between artificial feeding and longer growth period. Otherwise, there was no difference between the mortality rate and the pupal weight between the artificial meal and the beef liver. The results of the present study are consistent with the study of Firoozfar et al.

Using the CO1 marker, we could identify the species *Lucilia sericata*, and Reibe et al. were able to identify different species with

this marker. Six different species was sampled in their study; *Calliphora vicina*, *Calliphora vomitoria*, *Lucilia caesar*, *Lucilia sericata*, *Lucilia illustris*, and *Protophormia terraenovae*.

In a study, which conducted by CHEN et al. in Taiwan, the molecular identification of important species in forensic medicine based on the CO1 marker was investigated. In this study, species in Taiwan were surveyed from early 2000 and identified using molecular data. Currently, eight species have been identified; *Chrysomya megacephala* (Fabricius), *Chrysomya pinguis* (Walker), *Chrysomya rufifacies* (Macquart), *Hemipyrellia ligurriens* (Wiedemann), *Lucilia bazini* Se'guy, *Lucilia cuprina* (Wiedemann), *Lucilia hainanensis* Fan, and *Lucilia prophyrina* (Walker).

Today, due to the development of societies and the increase in accidents and burns, as well as not following a proper diet among people and suffering from diabetes, the wounds, which are caused by these diseases and accidents, are spreading. Also, due to the resistance of various bacteria on the wounds to antibiotics, larval therapy is used as a classic and modern method and a supplement to surgical methods. Therefore, accurate knowledge of *Lucilia sericata* and its use in larval therapy is very important. Sometimes, due to the similarity of some diagnostic characters, this species may be misidentified and the larval therapy may be failed.

Acknowledgments

In this study, all those who helped in the implementation of this project should be thanked and appreciated. The respected staffs of

Shiraz Larval Therapy Center, the respected experts of the Insectarium and the laboratory of Shiraz School of Health are thanked. This article is extracted from the first part of the thesis of Ms. Masoumeh Bagheri, PhD student in Vector Biology and Control of Diseases, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran.

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