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MOLECULAR IDENTIFICATION AND DESIGN OF SPECIFIC PRIMERS FOR QUARANTINE AND NON-QUARANTINE FRUIT FLY SPECIES (*DROSOPHILA SUZUKII, DROSOPHILA SIMULANS* AND *DROSOPHILA MELONOGASTER*)

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ФЕДЕРАЛЬНОЕ ГОСУДАРСТВЕННОЕ АВТОНОМНОЕ ОБРАЗОВАТЕЛЬНОЕ УЧРЕЖДЕНИЕ ВЫСШЕГО ОБРАЗОВАНИЯ

На правах рукописи

НАСЕРЗАДЕ ЮСЕФ

МОЛЕКУЛЯРНАЯ ИДЕНТИФИКАЦИЯ И ДИЗАЙН СПЕЦИФИЧЕСКИХ ПРАЙМЕРОВ ДЛЯ КАРАНТИННЫХ И НЕКАРАНТИННЫХ ВИДОВ ПЛОДОВЫХ МУШЕК (*DROSOPHILA SUZUKII, DROSOPHILA SIMULANS* И *DROSOPHILA MELONOGASTER)*

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INTRODUCTION

The relevance of research. The fly species *Drosophila* (Diptera: *Drosophilidae*) has served as a basic model system in animal genetics for more than a century [1], becoming a reliable source of the most important information about many biological processes. In addition to an extensive *Drosophila* scientific heritage, *D. melanogaster* is associated with a high-quality annotated reference sequence [2–4] and a large set of experimental possibilities. However, most of the knowledge gained from *Drosophila* research has not been used to solve applied entomological problems, since *Drosophila* is not recognized as a species that is seriously dangerous for human economic activity.

Drosophila suzukii is a dangerous pest of many small fruits, particularly those of the genera Vaccinium, Rubus, Rubus fruits (raspberry, blackberry, red raspberry, strawberry, gooseberry, boysenberry, etc.), Prunus, Fragaria, Vitis, Ficus, Actinidia, Rhamnus, Lonicera, Sambucus, and others. As host plants, plant species from 15 families are used [5]. *Drosophila suzukii* is also recognised as the vinegar or wine fly. Even though they infect overripe, fallen, or rotting fruit, most vinegar flies are not pests. *D. suzukii* females, on the other hand, lay their eggs in ripening fruits, and the larvae develop in the fruits, making them soft and unfit for consumption.

This species originated and is widespread in the zoogeographic region of the Eastern Palearctic belt with a temperate climate. However, relatively recently, it was introduced to North America and Europe [5, 6]. Hansen [7] provides the most recent and thorough analysis of this pest's distribution, host range, economic impact, and treatment in Europe. Many areas of Europe are currently experiencing a rapid spread of this pest. From the shore to the higher areas, it can be found at various altitudes. It is advised to mention the usage of PCR-based identification systems [7] due to the rapid expansion.

For the most recent details on the geographic range of this species. Fly species *Drosophila simulans*, which is a member of the same subgroup as *D. melanogaster* species, is closely related to *D. melanogaster* [8].

This pest (*Drosophila simulans*) is not yet on the Russian quarantine list, but it has been studied as part of the project because it is very similar to *Drosophila suzukii*.

The degree of development of the topic. The aims of this research was to create and evaluate a fast and affordable PCR assay that would enable the detection of various fruit flies, including *Drosophila suzukii, Drosophila simulans*, and *Drosophila melanogaster*, for use by quarantine organizations where precise control and identification are crucial.

Research goal and tasks: Since today in Russia, with a large volume of imports and exports of fruits and vegetables, there is no full-fledged analysis of *Drosophila suzukii*, including accurate diagnostic methods, the purpose of this study was to find a way to quickly identify this type of dangerous fruit flies, as well as improve methods for identifying closely related species of *Drosophila simulans* and *Drosophila melanogaster*. to achieve this goal, the following tasks were set:

Development of accurate and sensitive primers for real-time PCR and classical PCR for the detection of non-quarantine pests *Drosophila melanogaster*.

2. Development of an accurate and sensitive primer for real-time PCR and classical PCR for the detection of non-quarantine pests *Drosophila simulans*.

3. Development of accurate and sensitive primers for real-time PCR and classical PCR for the detection of quarantine pests *Drosophila suzukii*.

4. Optimization of the developed primers on different types of fruit flies that are genetically closely related to each other.

5. Checking the selectivity of primers with different types as a marker.

6. Study of the developed primers for evaluation and analysis of efficiency in agricultural laboratories in Iran and Russia.

In this paper, theoretical methods were used for the initial study and review of the scientific literature. Most of the project is hands-on and done in the lab and then carefully studied by various software methods.

Scientific novelty of the research. As part of dissertation research for the first time:

• A phylogenetic analysis of *Drosophila* species was carried out together with other closely related species as a marker.26

• Primers have been developed that accurately identify the desired genetic regions in *Drosophila suzukii*, *Drosophila simulans*, *Drosophila melanogaster* species, as well as primers using markers to confirm their accuracy for real-time PCR and classical PCR

• Created primers are used to evaluate the effectiveness of existing diagnostic methods in agricultural laboratories in Iran.

• Conducted large-scale molecular identification with precise differentiation of species to identify quarantine and non-quarantine objects *D. suzukii*. *D.simulans*, *D. melanogaster* at the AllRussian Center for Plant Quarantine (VNIIKR).

• Conducted molecular identification with precise delineation of species to identify fruit flies *D. suzukii*. *D.simulans*, *D. melanogaster* in Iran.

Theoretical and practical significance. The theoretical and practical significance lies in the development of new specific primers for *Drosophila* sp. Thus, in this dissertation, for the identification of *Drosophila* species, nine pairs of primers were developed. For the molecular identification of *D. suzukii* five pairs of primers were developed (12. dsuz. F/R, 12. dsuz. F/R and 3. dsuz. F/R for classical PCR) and (1. dsuz. F/R. Probe, 3. dsuz. F/R for real-time PCR). For molecular identification of *D. melanogaster*, two pairs of primers were developed. (4.DM F/R for classical PCR) and (3.DM.F/R.Probe for real-time PCR). For molecular identification of *D. simulans*, two pairs of primers were developed. (6.ds F/R for classical PCR) and (ds.F/R.Probe for real-time PCR). According to the test results of primers developed in Iran, they can be used in laboratories in other countries. The proposed methods can be used for express diagnostics of *Drosophila* Sp. Designing specific primers for populations of interest can be a useful tool to help biologists expand and continue their research.

Basic provisions for defense:

- Study of the molecular variability of *Drosophila* sp. population.
- Validate and improve classical and real-time PCR methods for *D. suzukii*, *D.*

simulans and *D. melanogaster* in Russian federation and Iran.

- Development of new specific real-time and classical primers for molecular diagnostics of *Drosophila* sp.
- Assess the accuracy of the results obtained with the developed primers.
- Study of the selectivity of the designed primers among related species.
- Compare the classical and real-time PCR.

The results obtained in the framework of the dissertation were reported with title: (SPECIFIC IDENTIFICATION METHOD FOR DROSOPHILA MELANOGASTER), and discussed at НАУКА И ИННОВАЦИИ-СОВРЕМЕННЫЕ КОНЦЕПЦИИ (Moscow, May 22, 2020) / resp. ed. D.R. Khismatullin. – Moscow: Infiniti Publishing House.

Publication of research results. Publications retrived from the outcomes of the dissertation research, 8 papers were published, including 2 in scientific journals listed in the International Journal database and 2 in the Scopus databases, 2 in peer-reviewed journals listed on the BAK list, and 1 original scientific articles presented at conferences.

Contribution by the author personally. The applicant took part in establishing the study's goals and objectives, collected and analyzed the data, processed and evaluated the information, and generated publications as a co-author.

Structure and volume of thesis. An introduction, three chapters, conclusions, and a bibliography make up the dissertation work. On 127 pages, the information is presented along with 79 tables, 52 figures, and diagrams. There are 84 sources in the list of references.

Acknowledgment. The author is grateful to his supervisor (Dr. Elena Pakina) from RUDN university of Moscow. Also Special thanks to Ms. Galina Bondarenko of the All-Russian Plant Quarantine Center (VNIIKR) for all the advice and training she gave me during the investigation. Finally, I must thank my wonderful wife Niloufar, as without her support (mental, motivational, and financial) I may never have completed this thesis.

CHAPTER 1. LITERATURE REVIEW

1.1. Disadvantages and problems of *Drosophila* sp

1.1.1. *Drosophila* sp

Approximately 2000 of the over 3850 species that make up the family *Drosophilidae* are *Drosophila* species (Table.1). *Drosophila* species are well known due to the widespread use of *Drosophila melanogaster* in genetic research and as the common vinegar flies connected to rotting and overripe fruit [9]. *Drosophila* species are well-known annoyance pests in homes, grocery stores, fruit markets, and restaurants [10]. During the fermentation of fruit and the production of wine, *Drosophila* species are also well recognized to be annoyance pests [11].

Table 1. – Classification of *Drosophila suzukii*

Scientific name	*Drosophila suzukii* Matsumura, 1931 [12, 13]
Synonyms	*Drosophila indicus* Parshad & Paika, 1965[14] *Leucophenga suzukii Matsumura*, 1931[14] *Drosophila suzukii* subsp. *indicus* Pashan & Paika, 1964[14]
Conventional name	Spotted wing *Drosophila*, cherry *Drosophila*
Known hosts	Includes *Fragaria* spp., *Prunus* spp., *Rubus* spp., *Vaccinium* spp., *Vitis* spp., and *Morus* spp.
Distribution	Asia, North America and Europe.

Hauser [15] has provided an excellent comparative diagnostic report of *D. suzukii*. Additionally, Vlach [16] has offered a useful key for the identification.

1.1.2. Identity

Name: *Drosophila suzukii* (Matsumura 1931) [14].

Synonym*: Leucophenga suzukii* Matsumura 1931.

Taxonomic position: Diptera, Brachycera: *Drosophilidae*; *Drosophila*, subgenus: *Sophophora*.

EPPO code: DROSSU.

Recently, the *Drosophila* gene has become the subject of special scientific discussions [17, 18]. According to most scientists, it is likely that the next generation of the *Drosophila* gene will raise the Sophophora gene to the level of an independent one [19]. A group of melanogaster species, including *Drosophila suzukii*, is included in the subgenus Sophophora [7]. However, a proposal to the International Committee of Zoological Nomenclature to retain the melanogaster group within the genus *Drosophila* was rejected due to the importance of *Drosophila melanogaster* for genetic research [20]. It is expected that the name *Drosophila suzukii* will eventually be changed to *Sophophora suzukii*.

1.1.3. Main host plants and all species affected

Fruits may contain larvae or pupae of *Drosophila suzukii*. *Drosophila* eggs can also be discovered on fruits, albeit it is very difficult to find them because of how little they are. Adults are also susceptible to traps [21]. *Drosophila suzukii* has a wide range of hosts, including:

1. *Actinidia*
2. *Diospyros kaki* (*persimmon*)
3. *Ficus carica (common fig)*
4. *Fragaria (strawberry)*
5. *Fragaria ananassa (strawberry)*
6. *Prunus (stone fruit)*
7. *Prunus avium (sweet cherry)*

8. *Prunus domestica (plum)*
9. *Prunus persica (peach)*
10. *Ribes (currants)*
11. *Rubus (blackberry, raspberry)*
12. *Rubus armeniacus (Himalayan blackberry)*
13. *Rubus fruticosus (blackberry)*
14. *Rubus idaeus (raspberry)*
15. *Rubus laciniatus (cutleaf blackberry)*
16. *Rubus loganobaccus (loganberry)*
17. *Rubus ursinus (boysenberry)*
18. *Vaccinium (blueberries)*
19. *Vaccinium angustifolium (Lowbush blueberry)*
20. *Vaccinium corymbosum (blueberry)*
21. *Vitis (grape)*
22. *Vitis vinifera (grapevine)*

1.1.4. Distribution of *Drosophila suzukii*

D. suzukii is thought to be native of eastern and southeastern Asia, including China, Japan and Korea [22], although little is known about its geographical origin. According to references reported by Hauser (2011) [23], The potential exists that the species was brought to Japan at the turn of the century and is not indigenous to the nation.

1.1.5. Introduced Distribution

D. suzukii has been introduced to the several Hawaiian Islands, including Oahu [24]. It has also been, together with organic cargo, this species was introduced to North America and Europe. *D. suzukii* has recently been recorded in Iran as well, which indicates the expansion of its territory into the Middle East and Central Asia. [25]. Réunion has also reported it. (Figure 1), (EPPO, 2018).

Figure 1. – Distribution map of *Drosophila suzukii* (https://www.cabi.org/isc/datasheet/109283#toDistributionMaps) [27]

In 1980, the Spotted Wing *Drosophila* was identified for the first time in Hawaii at an elevation of more than 1000 m [28]. Later, it was also detected at altitudes that were more moderate. This species was found in California in 2008, according to records [29]. (The species' identification was established in 2009) [30]. On the other hand, there is no current evidence of this species' distribution in these regions. Recent data indicate that there are no *Drosophila suzukii* colonies in Central or South America, and there is currently no evidence to support this hypothesis [31]. *Drosophila suzukii* was first discovered in Europe in 2009 in the Italian province of Trento [32]. Later, in southern Italy's Tuscany and Calabria, the discovery's accuracy was confirmed (IASMA and Maria). Recent works have established the existence of the species in a number of Mediterranean-area countries, including Spain, where the spotted *Drosophila* was found in 2008, and France in 2009 [33].

Since then, *Drosophila suzukii* has spread to other countries, including Belgium, Switzerland, Slovenia, and Germany [34]. The media also reported that *Drosophila suzukii* was recorded on extensive vineyards in the Azores in Portugal [35]. These reports also need further development. It is also known that the *spotted Drosophila* is included in the list of dangerous quarantine pests in Russia.

1.2. *Drosophila simulans*

1.2.1. Scientific classification for *Drosophila simulans*

One of the fruit fly species that shares a genetic ancestor with *D. melanogaster* is *Drosophila simulans*. According to studies, Alfred Sturtevant made the discovery of fly genetics in 1919 [36], when he noticed that the *D. melanogaster* and *D. simulans* flies used in Thomas Hunt Morgan's Columbia University laboratory were actually two different species. Males have different external genitalia, and viewers with laboratory training may tell females apart based on their color and outward appearance (Table 2).

Table 2. – Scientific classification for *Drosophila simulans*

Kingdom	*Animalia*
Subgenus	*Sophophora*
Class	*Insecta*
Order	*Diptera*
Generation	*Arthropoda*
Genus	*Drosophila*
Species subgroup	*melanogaster subgroup*
Species group	*melanogaster group*
Species	*D. simulans*
Species complex	*simulans complex*
Binomial name	*Drosophila simulans*

Currently, the most widely used strategy for the control of *D. simulans* in agriculture is the use of specialized chemicals [37, 38]. Because insecticides are aimed mainly at adults, *D. simulans*, which have a high reproductive capacity and numerous offspring, their repeated application is necessary (Figure 2).

Figure 2. – Map of *Drosophila Simulans* Distribution

(https:// DistributionMaps www.cabi.org/isc/datasheet/109283#to) [22, 39]

The California Department of Nutrition and Agriculture later discovered and identified adults of this species of fruit fly in the United States in 2009. In just 3 years, *Drosophila simulans* has spread (or been introduced) across North America from California to Oregon, Washington, British Columbia, Florida, Utah, Louisiana, North Carolina, South Carolina, Wisconsin, Michigan, Alberta, Manitoba, Ontario, and Kebek [40]. By 2012, the presence of this species was recorded all over the world, for the most part: in Hawaii, Mexico, Spain, Italy, France, Hungary, the Netherlands, Portugal, Great Britain, Slovenia, Croatia, Switzerland, Austria, Germany and Belgium [41, 42]. In 2012 and 2013, *Drosophila simulans* was found in South America, namely in the southern states of Brazil (in five locations). Then the researchers predicted the spread of *Drosophila* sp data to neighboring countries (Figure 3).

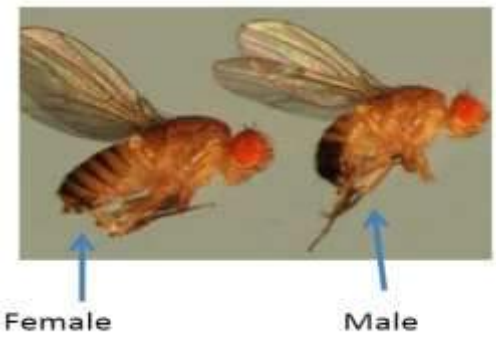

Figure 3. – *Drosophila simulans*, female and male [43]

At present, there are almost no countries left in Europe in which representatives of *Drosophila simulans* have not been found.

1.3. *Drosophila melanogaster*

1.3.1. Main Host plants and all species affected

Over 2000 of the over 4200 species that make up the *Drosophilidae* family are *Drosophila* species (Figure 4).(table 3) [44].

1.3.2. Scientific classification

Table 3. – Scientific classification for *Drosophila melanogaster* [43].

Kingdom	*Animalia*
Subgenus	*Sophophora*
Genus	*Drosophila*
Order	*Diptera*
Class	*Insecta*
Family	*Drosophilidae*
Phylum	*Arthropoda*
Species classification	Subgroup of *Drosophila melanogaster*
species class	Group of *Drosophila melanogaster*
Species	*D. melanogaster*
Species complex	*Drosophila melanogaster complex*
Binomial name	*Drosophila melanogaster*

Figure 4. – Picture for *Drosophila sp.* [45]

Table 4. – Main Host plants and all species affected

1	*Citrus*
2	*Citrus sinensis* (navel orange)
3	*Ficus*
4	*Malus domestica* (apple)
5	*Mangifera indica* (mango)
6	*Opuntia* (Pricklypear)
7	*Opuntia ficus-indica* (prickly pear)
8	*Prunus* (stone fruit)
9	*Prunus persica* (peach)
10	*Quercus alba* (white oak)
11	*Rubus* (blackberry, raspberry)
12	*Vitis vinifera* (grapevine)

Insects such as the fruit *Drosophila sp* pose a major problem and risk to the fruit industry, as they cannot be completely eradicated or contained. Targeted integrated pest management (IPM) has been implemented in the Russian Federation in order to lessen the financial risks of effects on the fruit and vegetable business. Farmers should also ascertain the pest as soon as possible, namely whether *Drosophila melanogaster* larvae are present in the harvested berries [46]. Currently, it is impossible to identify between

D. Melanogaster and other species without the use of sophisticated instruments. (Figure 5).

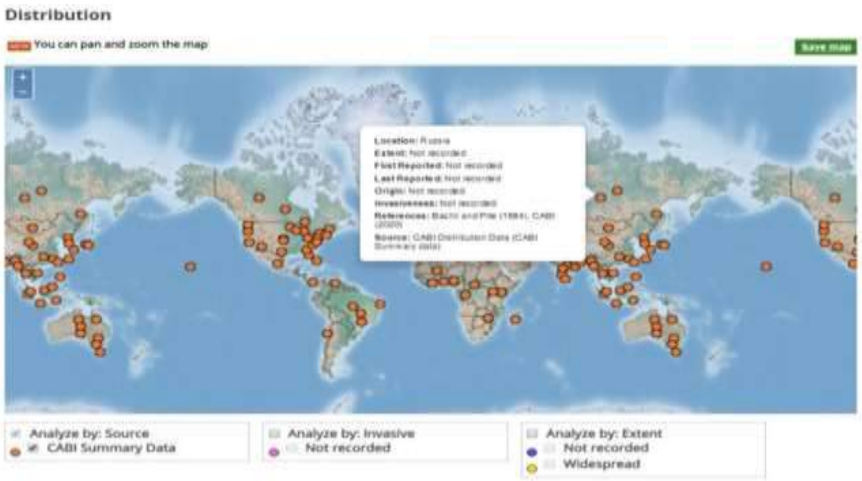

Figure 5.- *Drosophila melanogaster's* distribution is shown on a map. (https://www.cabi.org/isc/datasheet Maps)[46]

1.3.3. Life cycle of *D. Melanogaster*

The *D. melanogaster* life cycle lasts approximately 10 days at 25°C (Figure 6.7). A single fertile female can lay hundreds of eggs and *Drosophila* embryogenesis lasts approximately 24 h.

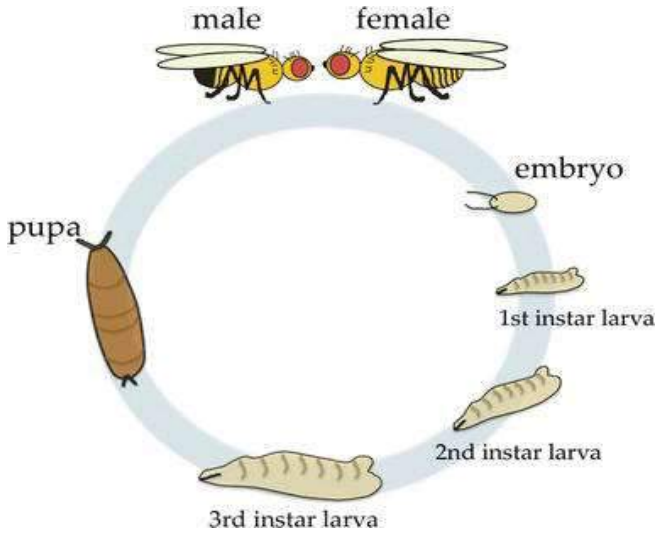

Figure 6. – Life cycle of *Drosophila melanogaster* [41]

Figure 7. – Female fruit fly (*Drosophila melanogaster*) (left) and male (right)[11]

1.3.4. Distribution and harmfulness of *Drosophila* sp. in Russia and Iran

For seven years, two species of exotic pests, fruit flies, have been introduced to Iran. This was first recorded in 2008 when an African species of *Zaprionus indianus* Gupta was found on an orange farm in southern Iran. The introduction of this species was blamed on state authorities, who mistakenly issued a quarantine permit for the import of tons of orange fruits from Egypt. Previously, the exporting country, Egypt, was declared infested with *Drosophila sp* pests [8, 16].

Since 2008, Z.indianus has been effectively expanding its range throughout the country, seriously threatening Iranian fruit production, and affecting many fruits and horticultural crops, especially figs. The second introduction of the pest *D. suzukii* is thought to be the result of poor border control with Pakistan, the only country bordering Iran known to be home to spot winged fruit flies [1]. There is no information about the species *D. suzukii* in other countries neighboring Iran. Iran and Pakistan have a complex long border (about 1000 km), which is often crossed by smugglers.

The area suffers from poverty and high unemployment due to prolonged drought and a lack of infrastructure. Thus, in these conditions, smugglers trade in various goods, including agricultural products. Thus, pest-infested smuggled fruits from Pakistan are most likely the reason for the introduction of *D. suzukii* into Iran. (Figure 8).

Figure 8. – Distribution map of *Drosophila* sp. [43]

1.4. Molecular Studies

1.4.1. Using PCR for molecular identification of *Drosophila* sp.

A DNA polymerase, magnesium, nucleotides, primers, the DNA template to be amplified, and a thermocycler are all that are required for normal PCR. The PCR mechanism is as straightforward as its goal: Precursors align to the single DNA strands, double-stranded DNA (dsDNA) is heat denatured, primers are expanded by DNA polymerase, and the outcome is two copies of the original DNA strand. One cycle of amplification is the process of denaturation, annealing, and elongation over a range of temperatures and periods. Every cycle step needs to be tailored to the particular template and primer set being utilized. After this cycle has been completed roughly 20–40 times, the amplified result can be examined.

DNA is frequently amplified using PCR for later use in experiments. Additionally, PCR can be used for genetic testing or the detection of pathogenic DNA [17].

Preparing a master mix for several reactions is advised because PCR is a highly sensitive technology and very tiny amounts are needed for each reactions. In order to ensure that each reaction will have the same quantity of enzyme, dNTPs, and primers, the master mix must first be well mixed before being divided by the number of reactions. Numerous vendors also provide PCR mixes that already include everything but the primers and the DNA template, such as Enzo Life Sciences.

Guanine/Cytosine-rich (GC-rich) areas provide a problem for conventional PCR methods. Sequences having a higher GC content are more stable than those with a lower GC level. Furthermore, secondary structures like hairpin loops frequently arise in GC-rich sequences. As a result, during the denaturation step, it is challenging to entirely separate GC-rich double strands. As a result, DNA polymerase encounters obstacles when attempting to create the new strand.

This can be enhanced with a higher denaturation temperature, and modifications to a higher annealing temperature and shorter annealing duration can stop the unintended binding of GC-rich primers [19]. The amplification of GC-rich sequences can be improved with additional reagents. The secondary structures produced by GC interactions are disrupted by DMSO, glycerol, and betaine, which makes it easier to separate the double strands.

CHAPTER 2. MATERIAL AND METHODS

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effective by molecular approaches [9]. To detect *Drosophila* spp. DNA, a number of primer-based assays have been created, some of which can be used to monitor predation [18, 19, 25]. But these tests need to be done in real time.

Name: *Drosophila suzukii* [14].

Synonym: *Leucophenga suzukii* [14].

Taxonomic position: Diptera

Brachycera: *Drosophilidae; Drosophila*, [14].

Subgen: *Sophophora*. EPPO code: DROSS˘Ă. [14].

2.1.Extraction larva from fruits and Preparation samples

- The study used material collected at the All-Russian Center for Plant Quarantine (VNIIKR), Moscow, Russia. This material contains fruits, larvae, insects, pupae, DNA that we have selected from different countries, and different fruits. (Figure 9).
- Some specimens of other fruit fly species have been studied in the laboratory of Varamina Agricultural University in Tehran, Iran.

Figure 9. – Extraction of the larva from the sugar apple (*Annona squamosa)*

Figure 10. – Selection of larvae and pupae of the sugar apple (*Annona squamosa*)

Besides, to study the species composition of the genus *Drosophila* spp, we selected material from Egypt, Turkey, Canada, Mexico, Iran, and in the Russian Federation territory and were selected from a variety of hosts, including: sugar apple, citrus, berries, raspberries (larvae), blueberries (larvae and pupae), Mellon, Grapes, apples, pomegranates, persimmon, tangerines, mango. (Figure 10). We studied samples of larva, pupae and isolated DNA. All material (plant tissue and isolated DNA) was stored at a temperature of -20°C.

Figure. 11. – Checking the morphology of fruit fly larvae and pupae under a microscope

After separating the larvae from the fruits shown in the sample, we examine them morphologically according to the microscopic identification instructions. However, as the project aims to identify molecular, do not investigate further.

2.2. Preparation of *Drosophila spp*. and another species of *Drosophila* for DNA extraction

The DNA Extran-2 Kit, kit No. NG-511-100 (Synthol, Russian Federation), was used to extract DNA from the investigated material (insects and larvae) by treating the preparations with proteinase K, followed by the removal of proteins without the use of organic solvents. Since *Drosophila* sp are extremely small, tissues were physically destroyed using homogenizing pestles. For urgent diagnostic needs, our quick DNA extraction approach offers a time benefit. With the use of a NanoDrop 2000 spectrophotometer, DNA extracts were measured [22]. (Thermo Fisher Scientific Inc., USA).

Figure 12. – Preparation of *Drosophila* spp. and another species for DNA extraction

2.1.1. The protocol of DNA extraction. DNA Extran-2 Kit (“Synthol”, Russian Federation)

DNA was extracted from the study material by following the manufacturer's instructions and processing the specimens by Proteinase K, eliminating the proteins without extracting them with organic solvents, and using the «DNA Extran-2 Kit», set No. NG-511-100 ("Synthol," Russian Federation) (larvae and insect) [4]. Due to the small size of *Drosophilae* species, physical tissue disruption was accomplished with

homogenization pestles. This quick DNA extraction technique saves time, which is useful for urgent diagnostic needs. DNA extracts were evaluated using a NanoDrop 2000 spectrophotometer (manufactured by Thermo Fisher Scientific Inc., USA). [22, 44].

Table 5. – Chemicals used in the extraction of DNA [10].

	Name	Description
1	Proteinase K	Reagent for cell lysis, 1.0 µl
2	Lysis solution 2	Reagent for cell lysis, 31 µl
3	Precipitating solution 1	Reagent for wasp ›protein ctenia, 11µl
4	Precipitation solution 2	DNA Precipitation Reagent, 31 µl
5	Wash Solution	DNA washing reagent, 41 µl
6	DNA dissolution	DNA Dissolution Reagent, 11 µl
7	2-mercaptanol	Reagent for cell lysis, 0.2 µl
8	Glycogen	DNA Co-Precipitation Reagent, 0.2 µl

2.3. Extraction and purification of DNA

First day:

1. At first, the number of test tubes with a volume of 1.5 or 2 µl following the number of analyzed samples was written and put an additional test tube for negative control. In all tubes (except negative), added 2-3 pupae or insects.

2.1. 20 µl of Lysing Solution 2 (No. 2) added to each tube and grind the pupae or insects as much as possible in the microtube with a Teflon pestle. Then 280 µl of Lysing Solution 2 (No.2) and 1 µl of 2-mercaptoethanol (No.7) added.

2.2. 10 µl of Proteinase K (No.1) added to the tubes and mix on the vortex.

2.3. At this stage, the samples left overnight at 56 °C in the thermometer.

Second day

3.1. 100 µl Solution (No.3) added to the samples. The contents of the tubes mixed on the vortex for 20 seconds.

3.2. The mixture was centrifuged at 13,000 rpm for 5 minutes. At this scale, a dense cause rapid established at the tube's bottom.

4.1. In clean 1.5-2 ml, tubes added 2 µl DNA co-precipitator (No.8).

4.2. The supernatant containing the DNA transferred in test tubes.

4.3. 300 µl of Precipitation Solution 2 (No. 4) added and mixed by inversion (10-12 times) until a visible DNA precipitate appears.

4.4. The mixture centrifuged at 13,000 rpm. 5 min. Then the supernatant drained.

5.1. Added 400 µl Wash Solution (No. 5) and mixed several times by turning to wash the DNA.

5.2. After that tubes centrifuged for 2 min at 13,000 rpm. Then carefully removed supernatant.

5.3. The tubes lid was opened and put in the thermometer at 37 °C for 10-15 min until the alcohol has completely evaporated.

5.4. Then 50 µl of Elution Solution (No.6) added. Stirred and warmed at 65 °C for 5 minutes until the DNA is dissolved.

5.5. At the end DNA solution stored at -20 °C for long storage.

2.4. Primers used in this study are listed below

Table 6. – Designed primers for classical and real-time PCR

NO	Primer name	Primer sequence (5' to 3')	Method	Target genes	Reference
1	12.dsuz. F 12.dsuz.R	5'- CCTTCGTGAAGCCTTCTACCG -3' 5'- GCACTCTTGATGGGAAGATC -3'	PCR	*Drosophila suzukii*	This study
2	*2. dsuz. F* *2.dsuz.R*	5'- TCCTGCAGAAGGGATACGGA -3' 5'- AACCACAGCGAACACCAGAA -3'	PCR	*Drosophila suzukii*	This study
3	1.dsuz F 1. dsuz R 1. dsuz Probe	5'- CCTTCGTGAAGCCTTCTACCG -3' 5'- GCACTCTTGATGGGAAGATC -3' 5'- CAACCGTTCTGGTGTTCGCTG -3'	Real-Time PCR	*Drosophila suzukii*	This study
4	7.dsuz. F 7.dsuz.R	5'- CCTTCGTGAAGCCTTCTACCG -3' 5'- GCACTCTTGATGGGAAGATC -3'	PCR	*Drosophila suzukii*	This study
5	3.dsuz F 3. dsuz R 3. dsuz Probe	5'- GGCGCCGGTGTCTGCCTGC -3' 5'- CTGGTTTGATTGTGCTGCTGC -3' 5'- GGCAATGGAACAGGGAAATTCC -3'	Real-Time PCR	*Drosophila suzukii*	This study
6	3.DM F 3.DM R 3.DM Probe	5'- GGCGCCGGTGTCTGCCTGC -3' 5'- CTGGTTTGATTGTGCTGCTGC -3' 5'- GGCAATGGAACAGGGAAATTCC -3'	Real-Time PCR	*Drosophila melanogaster*	This study
7	4.DM F 4.DM R	5'- AAGCTCTTCGGCATGGTGAT -3' 5'- CCAGTCCATAGCCCTTCTGC -3'	PCR	*Drosophila melanogaster*	This study
8	6.ds F 6.ds R	5'- CCCAAGGATCGTGCTCTGTT -3' 5'- TCCACACAATCGTCTCGCAA -3'	PCR	*Drosophila Simulans*	This study
9	5.ds F 5.ds R 5.ds Probe	5'- GCAACTTCTTCATTAACCTCG -3' 5'- CTGGGGTGTGTGGGCTGATGT -3' 5'- GATAGTAGCACAGACCACCG -3'	Real-Time PCR	*Drosophila Simulans*	This study

2.5. Polymerase chain reaction

Primer Dro-Suz A390 (5′- TTGAACTGTTTACCCACCTCTT -3′) as a forward and Dro-Suz S390 (5′- GGTATTCGGTCTAATGTAATACCC -3′) as a reverse were used as a universal primers for sequencing. (Table 7).

Table 7. – *Drosophila suzukii* identification using a qualitative time PCR assay [4]

Target genes	Primer	Primer sequence (5´-3´)	Refrences
Drosophila suzukii (COI)	Dro-Suz A390	TTGAACTGTTTACCCACCTCTT	Bogdanowicz.2000 [14]
Drosophila suzukii (COI)	Dro-Suz S390	GGTATTCGGTCTAATGTAATACCC	Bogdanowicz.2000 [14]

Forward primer is indicated by A, and reverse primer by S. The group primer sequences' lowercase letters denote changes to the original primers. [22, 47]

To make the PCR mixture, I used 0.5 l (10 pmol) of each primer, 5 l of screen-mix (HS-5x), 17 l of water, and 1 l of DNA (Table2). There should be a total of 25 l. The tubes should then be placed in a VertiTM thermocycler or PCR equipment (Applied Biosystems, USA). (Table 9). The reaction mixture appeared to be as follows: Screen Mix-HS [4], a pre-made PCR mixture (Evrogen, Russia). PCR conditions: 30 seconds of primer annealing at 58 degrees Celsius, 30 seconds of elongation at 72 degrees Celsius, and 5 minutes of completed elongation at 72 degrees Celsius. [22]. (Table10-16).

Table 8. – Composition of the PCR reaction mixture

Ingredients	For a sample
Forward Primer : Dro-Suz A390	0.5µl (10 pmol)
Reverse Primer : Dro-Suz S390	0.5µl (10 pmol)
Master mix 5x screen-mix (HS-5x)	5µl
H_2O	17µl
Template DNA	2µl
Total	25µl

Table 9. – PCR reaction mixture composition

Ingredients	For a sample
Forward Primer: Doro-suz S390	0.5µl
Reverse Primer: Doro-suz A390	0.5µl
Master mix 10x (Green)	2.5µl
H_2O	19.5µl
Template DNA	2µl
Total	25µl

Table 10. – PCR amplification and sequencing

Step	Temperature (◦C)	Duration
Initial denaturation	95	90 s
Cycle (35×)	95	15 s
Annealing	58	30 s
Extension	72	30 s
Final extension	72	5 min
Hold	8	Forever

Table 11. – *Drosophila suzukii* identification using a qualitative time PCR assay [4].

Object genes	Primer	Primer sequence (5´-3´)	Reference
Drosophila suzukii (COI)	Dsuz 6	TGGAACTGTTTACCCACCTCGT	Murphy., et al.2015 [14]
Drosophila suzukii (COI)	Dsuz 1	TGTATTCGGTCTAATGTAATACT	Murphy., et al.2015 [14]

Table 12. – The PCR preparation plan and solution

Ingredients	For a sample	×4
Forward Primer: Dsuz 6	1.5µl	6.5 µl
Reverse Primer: Dsuz 1	1.5µl	6.5 µl
Master(Screen mix) mix 5x	5µl	20µl
H_2O	16µl	64 µl
Template DNA	1µl	1µl
Total	25µl	97µl

Table 13. – Conditions for temperature cycling and reaction composition.

Step	Temperature (◦C)	Duration
Initial denaturation	95	2min
Cycle (35×)	95	10 s
Annealing	61	40 s
Extension	72	20 s
Final extension	72	10 min
Hold	8	Forever

Table 14. – A qualitative time PCR assay for determining the different *Drosophila species* [4].

Target genes	Primer	Цепочка праймера (5′-3′)	Reference
Drosophila spp.	1248 F	TGGAACTGTTTACCCACCTCGT	Bogdanowicz.2000[21]
Drosophila spp.	1248 R	TGTATTCGGTCTAATGTAATACT	Bogdanowicz.2000[21]

Table 15. – The components of the PCR reaction mixture

Reagents	For a sample	×5
Forward Primer: 1248 F	0.5µl	2.5 µl
Reverse Primer: 1248 R	0.5µl	2.5 µl
Master mix 5x (Green)	5µl	25µl
H_2O	18µl	90 µl
Template DNA	1µl	1µl
Total	25µl	125µl

Table 16. – Conditions for temperature cycling and reaction composition

Step	Temperature (◦C)	Duration
Initial denaturation	95	5 min
Cycle (40×)	95	30 s
Annealing	64	30 s
Extension	72	1:30 s
Final extension	72	10 min
Hold	8	Forever

Table 17. – A qualitative time PCR assay for identifying different *Drosophila* species [4]

Target genes	Primer	a primer sequence (5´-3´)	Reference
Drosophila spp.	S1859	5′-GGAACAGGATGAACAGTTTAACCGCC-3′	Bogdanowicz.2000[21]
Drosophila spp.	A2191	5’-CCCGGTAAAATTAAAATATAAACTTC-3’	Bogdanowicz.2000[21]

S stands for the forward primer and A for the reverse primer. The group primer sequences' lowercase letters denote changes to the original primers [21].

In a VertiTM thermocycler, the primers were used to amplify mitochondrial COI gene fragments. S1859 (5′-GGAACAGGATGAACAGTTTAACCGCC-3′) and A2191 (5’-CCCGGTAAAATTAAAATATAAACTTC-3’) suggested by Bogdanowicz et al. [22]. (Applied Biosystems, United States) (Table 13); the reaction mixture is described as follows: Screen Mix-HS is a pre-made PCR mixture (Evrogen, Russia).

35 cycles of denaturation at 95 °C for 15 seconds each, primer annealing at 55 °C for 30 seconds, elongation at 72 °C for 90 seconds, and final elongation at 72 °C.

Table 18. – *Drosophila melanogaster* identification using a qualitative time PCR assay: reaction composition and temperature cycling requirements

Target genes	Primer	Primer sequence (5´-3´)	Reference
	Droso-S391	AAATAACAATACAGGACTCATATcc	(Bogdanowicz et al., 2000) [15]
	Droso-A381	gTAATACGCTTACATACATaAAGGTATA	(Bogdanowicz et al., 2000) [15]

A denotes the forward and S the reverse primer. Lower-case letters in the group primer sequences indicate modifications of the original primers [21, 22].

Primer Droso-S391 (5′- AAATAACAATACAGGACTCATATcc -3′) as a forward and Droso-A381 (5′- gTAATACGCTTACATACATaAAGGTATA -3′) as a reverse were used for sequencing. In order to make PCR mix I used 0.5µl (10 pmol) µl of each primers, 5 µl of screen-mix (HS-5x), 17 µl H_2O and 1 µl DNA (Table 15).

There should be a combination of 25 ml [4]. The tubes should then be positioned in a VertiTM thermocycler or PCR equipment (Applied Biosystems, USA). The reaction

mixture appeared to be as follows: Screen Mix-HS is a pre-made PCR mixture (Evrogen, Russia). After 90 seconds of denaturation at 95 °C, 40 cycles of PCR are performed, including 15 seconds at 90 °C, 30 seconds at 63 °C for primer annealing, 30 seconds at 72 °C for elongation, and a final 5 minutes at 72 °C [4]. (Table 15). The Droso-S391 and Droso-A381 primers [4]. target several Drosophila species and generate a 220 bp amplicon. 2 l of DNA extract (10 pmol), 5x PCR Master Mix, Screen-mix (HS-5x), 0.5 M of each primer [4], and 17 l of water have been used in each 25 ml reaction. For both primer pairs, the PCR conditions were the same. (Tables 20, 21)

Table 19. – Composition of the PCR reaction mixture

Ingredients	For a sample	×12
Forward Primer : Droso-S391	0.5µl (10 pmol)	6 µl
Reverse Primer : Droso-A381	0.5µl (10 pmol)	6 µl
Master mix 5x screen-mix (HS-5x)	5µl	60 µl
H_2O	17µl	204 µl
DNA	2µl	24 µl
Total	25µl	300 µl

Table 20. – PCR amplification, DNA extraction, and sequencing

Step	Temperature (◦C)	Duration
Initial denaturation	95	10 min
Cycle (35×)	95	15 s
Annealing	55	30 s
Extension	72	1:30 s
Final extension	72	90 s
Hold	8	Forever

Table 21. – PCR reaction mixture composition

Reagents	For a sample	×6
Forward Primer : 2.dsuz.F	0.5µl (10 pmol)	3 µl
Reverse Primer : 2.dsuz.R	0.5µl (10 pmol)	3 µl
Master mix 5x.RED	5µl	30 µl
H_2O	18µl	108 µl
Template DNA	1µl	1 µl
Total	25µl	145 µl

By 35 cycles of 20 seconds each at 94 degrees, 50 degrees, and 68 degrees. At 68 °C, the final elongation ran for 3 minutes.

2.6. Gel Electrophoresis

Following PCR, the samples underwent the following agarose gel electrophoresis analysis: By utilizing a mini oven to melt 500 mg of agarose in 0.5 l of 1X TBE buffer, the molten gel was created. Ethidium bromide was added to the molten gel at a final concentration of 0.5 g/ml once it had cooled to about 60°C. By gently swirling, the solution was thoroughly combined [22].

An appropriate comb was picked out and placed in the electrophoresis apparatus while the agarose solution cooled in order to create the sample slots in the gel. The mold, which had a comb on it, was filled with the warm agarose solution [22].

A tiny amount of electrophoresis buffer was poured on top of the gel when it had completely set (30–45 minutes at room temperature), and a comb was carefully removed. It was necessary to add enough electrophoresis buffer to fill the gel to a depth of around 1 mm. The necessary 6x gel-loading buffer was combined with 2 l quantities of the PCR product (5 l). Using a disposable micropipette, the sample mixture was gradually fed into

the slots of the submerged gel. Depending on the anticipated molecular size of the amplicon, a voltage of 150 V was applied to the solution to cause it to migrate towards the positive anode for 30 to 60 minutes at room temperature.

The electric current was shut off once the samples or dyes had moved through the gel far enough. The gel was initially seen by a UV detector, and then a picture of it was taken with a computer-assisted NTAS® gel imager equipment using the GDS Version 3.32 software and saved on disks as TIFF files.

2.7. PCR product purification

Utilizing Thermo Scientific GeneJET, the PCR product was extracted and purified. PCR products were seen using a UV lamp after electrophoresis, and the gel band was removed using a razor blade or scalpel that was at least 2 mm broader than the band on either side.

Binding Buffer was added at a ratio of 1:1 to the finished PCR mixture (i.e. 100 mL of Binding Buffer for every 100 mL of the reaction mixture). [47–50] Thoroughly blended.

I increased the concentration of the solution and placed it in the GeneJET purification column. Flow-through was discarded after centrifugation for 30-60 seconds. The GeneJET purification column received 700 ml s of Wash Buffer. After centrifuging for 30-60 seconds, return the purification column to the collecting tube, discarding the flow-through. After centrifuging the empty GeneJET purification column for an additional minute, transfer the GeneJET purification column to a clean 1.5 mL micro centrifuged tube. After adding 50 mL of removal buffer [22] to the GeneJET purification column membrane in the center, the top solution was discarded and the bottom solution was used. They were placed in new tubes and numbered as before.

Each sample was added to the machine in a volume of 2 l for measurement, which was then processed by a computer program. The purified PCR product was kept at - 20° C once the Gene JET purification column was discarded.

2.8. Nano Drop™ 2000/2000c Spectrophotometers

The NanoDrop Spectrophotometer from NanoDrop Technologies is made for determining the quantities of nucleic acids in samples with a volume of 1 l. The process that was used was as follows: The lower optical surface was filled with one microml of double-distilled water. To clean the upper optical surface, the lever arm was briefly closed. Then, the lever arm was raised, and a soft cloth was used to clean both optical surfaces. The nucleic acid molecule (DNA 50) setting in the Nano-Drop program was chosen. By selecting "blank" and loading a 1 l solution buffer from the KIT, the blank measurement was carried out. Both optical surfaces were cleaned after the blank had been created. By loading 1 l and choosing "measure," the nucleic acid sample was measured. Both of the optical surfaces were cleaned after the measurement was finished.

2.9. Phylogenetic and sequencing analyses

Thermo Scientific Gene JET Gel Extraction Kit was used to purify the amplified PCR product, which was then sequenced by Bioneer in Iran and Genetic Analyzer AB-3500 (Applied Biosystems, USA) in Russia using the Sanger's Dideoxy cycle. The NCBI BLAST website (http://www.ncbi.nlm.nih.gov/BLAST) compared sequencing results to the GeneBank genetic sequence database [10, 51, 52]. The sequence alignment editor BioEdit v.7.0.5.3 [53] was used to check, change, and align the sequence [22].

The sequences genetic distances between the sequences were calculated using Kimora's two-parameter model. The tree diagrams were created using the maximum likelihood methodology (ML method), which is a feature of the Mega 10 software. [54]. Bootstrap The correctness of the tree diagrams was examined and confirmed using a test that involved building 1000 different trees. The results are shown as percentage values, and both the freshly sequenced DNA and the DNA sequences that are homologous to those under examination but are already in the GeneBank were investigated. The following three steps are involved in PCR: Extension, annealing, and denaturation. To create single-stranded DNA molecules from double-stranded ones, the genetic material

must first be denatured [10, 22]. The complementary portions of the single stranded molecules are then annealed with the primers. They are lengthened in the third stage by the DNA polymerase. These processes are all temperature-sensitive, and the usual choices are 94°C, 60°C, and 70°C, respectively. Successful reactions depend on effective primer design.

2.10. Primer Design

2.10.1. Molecular diagnostic

The identification of a pest accurately and quickly is the first step in its effective treatment. Quick identification methods for developing pests are not always available. *Drosophila suzukii*, or spotted-wing *Drosophila*, is an example of this (Diptera: *Drosophilidae*). *Drosophila suzukii* is a serious pest in both countries where the fly is already well established and countries where it is not yet present [55] [56, 57]. Additional GenBank sequences are included as well.

Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA, USA) [10, 22] was used to manually create and certify a primer combination that targets *D. suzukii* [58]. The two main chemistries upon which real-time PCR is based are fluorescent probe-based chemistry and DNA-binding dye-based chemistry. Non-fluorescent oligos that are easily produced are also desired.

This real-time PCR method is easily compatible with high-resolution melt (HRM) analysis [10], which can distinguish between target and non-target DNA by a single base pair [59, 60]. We chose the COI gene as the target because it has been well described for a number of *Drosophila melanogaster* species, including sequences species in within *D. suzukii* species subpopulation.

Adult females of this species differ from other *Drosophila spp* in that they oviposit on marketable, ripening fruit rather than rotting fruit, which is accomplished by using a serrated ovipositor to pierce the fruit's surface. Most *Drosophila* spp are not horticultural

pests. *D.Suzukii* has a diverse range of hosts in its native habitats in Asia and the United States, with tiny fruits and cherries becoming the primary economic problems. [61].

However, since their feeding causes visible damage to the produce, the majority of suspected *Drosophilidae* in transit or discovered during orchard surveys are larval. [10]. Since previously stated, it is nearly impossible to correctly identify them unless they are raised by adults. Many facilities around the world may lack the quarantine protection required to raise pests like *D. suzukii*, and egg raising failure rates may be quite high, making this a high-risk operation [10, 22].

Furthermore, the long-term aspect of identification rearing, which involves fresh product worth hundreds of thousands to millions of dollars [10, 22]. , can be frustrating. In these cases, molecular recognition techniques may be useful. Polymerase chain reaction (PCR) techniques have been used for many years to identify pests and diseases all over the world [62]. The results showed that these primers accurately identify the gene's location as well as the unique for *Drosophila suzukii*.

CHAPTER 3. RESULTS AND DISCUSSION

More than 1500 of the more than 3,750 species that make up the *Drosophilidae* family are *Drosophila* species [62]. Different species of *Drosophila* are well-known in both the scientific community as genetic study subjects and in everyday life and commercial pursuits including the production and storage of fruits and vegetables.After all, they cause serious harm to products on the shelves of markets and shops, in restaurants and canteens, as well as in ordinary homes and wineries.

Since its discovery in California in 2008, the spotted winged *Drosophila suzukii* (Matsumura) (Diptera: *Drosophilidae*) has become a significant problem in the United States. Following that, her existence was documented in 35 states across the American continent [63]. Their ability to puncture fruit peels and deposit eggs even on developing fruits makes them dangerous and harmful.

Currently, the *Drosophila suzukii* has a significant host plant population in both its native Asia and other distribution areas, particularly in the United States. Berries and little fruits are particularly vulnerable. The spotted *Drosophila* may damage complete fruits from the inside only when they are in the maturation stage, which is the problem.

It is challenging to distinguish between species in zones of sympatry since the ovipositor's serrations and the black dots on the wings are present in 150 other *Drosophila* species. Male *D. subpulchrella Takamori* and Watabe black spots resemble those of *D. suzukii* extremely closely in terms of position and shape [64].

The occasional absence of black wing spots in common male *D. suzukii* can lead to misidentification with other closely related *Drosophila* species whose males do not have wing spots, including: *D. ashburneri Tsacas*, *D. immacularis Okada, D. lucipennis Lin, D. mimetica Bock* and *Wheeler, D. oshimai* Choo and *Nakamura*, and *D. unipectinata Duda*. Instead, other characteristics can be used for identification, such as genital ridges on the forelegs.

D. suzukii has one row of ridges on the first and one row on the second tarsal segment, whereas *D. biarmipes* [10] has two ridges on the first jaw.

The identification of females has issues. It is simple to distinguish *D. suzukii* from closely related species like *D. biarmipes* based on the length and shape of the ovipositor, but more challenging to do so from other species with extremely similar ovipositors, such *D. immigrans* Sturtevant and *D. subpulchrella* [65]. In these situations, the taxonomist may make the ultimate determination based on the ratio of the spermatheca's size to that of the ovipositor.

Drosophila melanogaster, a common vinegar fly (Diptera: *Drosophilidae*), can only lay eggs on rotting or otherwise harmed fruit (such as broken grapes after rain or being eaten by birds).

Drosophila suzukii Matsumara, on the other hand, uses a serrated ovipositor to colonize undamaged fruits and is an invasive Asian pest that was presented to Europe and north America in 2008 [66].

The primary pest of cherries, raspberries, and blueberries is the Asian berry *Drosophila*, although it can also infect different grape kinds, particularly those with delicate skins [67]. It is conceivable that *D. melanogaster* can lay eggs in berries that have already suffered *D. suzukii* fruit destruction.

Thus, oviposition of *D. suzukii* facilitates access to the fruit for *D. melanogaster*, resulting in more damage due to a mixture of the two species than would be expected from exposure to a single species. However, the tests were carried out with sterilized berries, which were then dipped in acid rot extract and placed in tanks in the laboratory. Such observations cannot be transferred to field conditions [68].

New and established *Drosophila suzukii* and other quarantine pests such as Drosophila simulans and *Drosophila melanogaster* have been recognized as a major threat to fruit and vegetable production. For growers, gardeners, researchers and farmers around the world, *Drosophila suzukii* is a serious quarantine pest.

As previously, stated, it is and almost difficult to pinpoint these specific fruit flies until they are fully grown. Such research is risky because not all research facilities can maintain the quarantine security required to produce pests like *D. suzukii*.

After all, the probability of their spread to other objects is high [68]. In view of the potential danger of such studies, the method of molecular identification is quite effective and safe.

At present, there is no method to distinguish *Drosophila* sp. Larvae from two or three other garden species. Therefore, in a laboratory study, infected fruits were left for 1-2 weeks to allow the larvae to develop and turn into adult flies that are easier to identify [69].

To date, only DNA sequencing [70] has been used to identify *D. suzukii* larvae. The PCR amplification [22] was created as a dependable molecular technique for identifying insects that are harmful to commercial operations. These studies sought to offer a number of pairs of targeted primers for molecular identification utilizing traditional PCR and real-time PCR techniques.

To then compare them. In addition, these identification methods do not require morphological identification due to their high accuracy.

To more accurately assess the effectiveness of the primers, we tested them on other fruit fly species in the laboratory of Varamin Agrarian University, Tehran Province, Iran. The results obtained are discussed in the “results” section.

3.1. Molecular Diagnostic *Drosophila* sp.

Throughout the course of the research, the primers 1248 F (5'-TGGAACTGTTTACCCACCTCGT-3') and 1248 R (5'-TGTATTCGGTCTAATGTAATACT-3') were employed as forward and reverse, respectively (Table 22). A universal primer is Primer 1248. Additionally, after sequencing

the DNA with this set of primers, we altered them in BioEdit proG using new GenBank *Drosophila* genetic data.

No	Sample ID	Name of primer	No. Electrophoreses	Host	Country	Date	Results
1	1.174-1_A2191.u3fn.ds.	1248F 1248R	1	Melon	Turkey	2019.04.09	*Drosophila suzukii*
2	2.174-1_A2191.u3fn. ds.	1248F 1248R	2	Sugar apple	Mexico	2019.04.09	*Drosophila suzukii*
3	3.174-1_A2191.u3fn. ds.	1248F 1248R	3	Melon	Turkey	2019.04.09	*Drosophila simulans*
4	4.174-1_A2191.u3fn. ds.	1248F 1248R	4	Melon	Turkey	2019.04.09	*Zaprionus indianus*
5	5.174-7_A2191.u3fn. ds.	1248F 1248R	5	GRAPE WINE	Mexico	2019.04.09	*Drosophila simulans*
6	6.174-7_A2191.u3fnds.	1248F 1248R	6	Grip wine	Egypt	2019.04.09	*Drosophila melanogaster*
7	7.174-7_A2191.u3fn. ds.	1248F 1248R	7	Melon	Turkey	2019.04.09	*Drosophila .melanogaster*

3.2. Identification of *Drosophila suzukii* (Dro-Suz A390, S390 и Droso-S391, Droso-A381)

Accurate and quick pest identification is the first step in effective pest management. There aren't always quick identification techniques available to identify newly developing pests. Likewise, *Drosophila suzukii*, the spotted fruit fly, exhibits this behavior (Diptera: *Drosophilidae*). *Drosophila suzukii* is a serious pest in both countries where the fly has established itself and countries where it is currently absent. [71]. Two pairs of primers were created to monitor the activity of *D. suzukii* at the species and life level of

Drosophila spp. at the genus-specific level. The mitochondrial cytochrome c oxidase (COI) subunit 1 gene was the target region for DNA amplification using species-specific *D. suzukii* primers [72].

3.2.1. Design (12. dsuz. F/R) for classical PCR for *Drosophila suzukii*

Fly and predator DNA was amplified in PCR Thermal Cyclers (Applied Biosystems, Hamburg, Germany) with 1.5 µl of DNA extract, 0.5 µM of each primer (12.dsuz.F/R). The sequences were aligned in BioEdit [72], along with additional sequences obtained from GenBank. Using Primer Premier 5 (Palo Alto, USA. PREMIER Biosoft International), a primer pair targeting *D. suzukii* was manually created and confirmed (Figure 13).

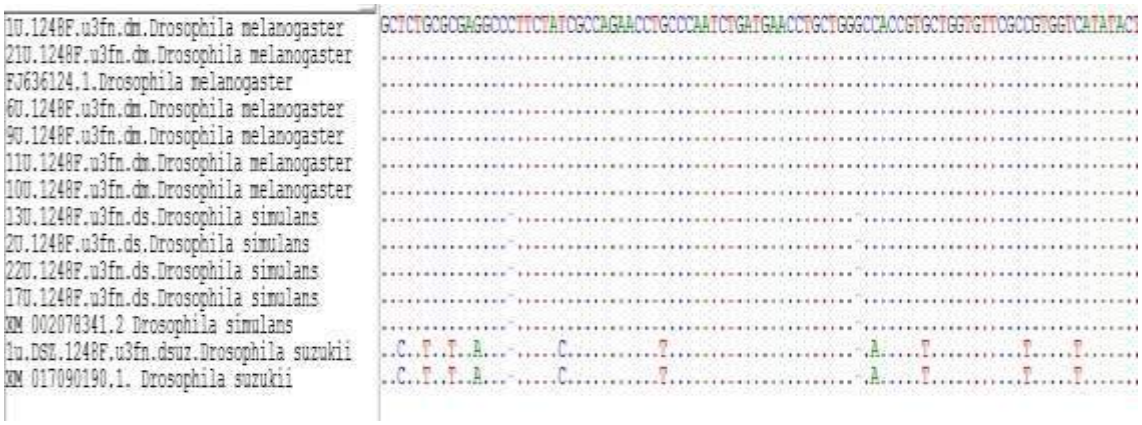


Figure 13. – COI gene fragment sequences and primer constructs - different codes

Table 23. – Identification of the genetic codes of *Drosophila suzukii*

NO	Spices	Number of letter										
		69	70	78	81	84	104	116	131	137	149	155
1	*Drosophila melanogaster*	T	T	G	C	G	T	G	C	G	C	C
2	*Drosophila simulans*	T	T	G	C	G	T	G	C	G	C	C
3	*Drosophila suzukii*	C	C	T	T	A	C	T	A	T	T	T

Table 24. – Primer sequences and sizes of resulting amplicons

Primer name	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC %
12.dsuz.F	CCTTCGTGAAGCCTTCTACCG	Plus	21	475	496	57.14	58
12.dsuz.R	GCACTCTTGATGGGAAGATC	Minus	20	530	550	50.00	60
Длина	191						

F stands for the forward primer, and R for the reverse primer.

Based on the matching of two sets of primers, DrosoATAT-S391 (5′-AAGATA-3′) as direct and Droso-A381 (5′-gTAATACGCTTACATACATaAAGGTATA-3′) as reverse [22], Dro-Suz A390 (5′-TTGAACTGTTTACCCACCTCTT -3′) as direct, and Dro-Suz S390 (5′-GGTATTCGGTCTAATGTA Following DNA sequencing, we edited this set of primers in ProG e BioEdit using additional Drosophila gene sequences from GenBank. Then, two exclusive primers were created. Primer 12. dsuz. R (5' GCACTCTTGATGGGAAGATC -3') was employed as the reverse and 12. dsuz. F (5′-CCTTCGTGAAGCCTTCTACCG -3′) as a straight line (Tables 23 and 24).

3.2.2. Test of selectivity of designed primer pairs

Electrophoresis (Figure 14) 1. *Drosophila suzukii* (Mexico), (Turkey), (Egypt) 2. *Drosophila simulans*. M - Molecular weight marker 3. *Drosophila melanogaster*. Electrophoresis picture (Figure 13) 1. *Drosophila suzukii* (Mexico), (Turkey), (Egypt) 2. *Drosophila simulans*. M-Molecular weight marker 3. *Drosophila melanogaster*.Negative control: DNA extraction control and amplification control. In the electrophoresis photograph (Figure 14), all three species of *D. suzukii*, *D. simulans*, and *D. melanogaster* were tested using a primer that shows that the designed primer (12.dsuz.F /R) detects only *D. suzukii*. Moreover, there are no positive results for other species in the picture.

Figure 14. – Electrophoregram of traditional PCR amplification products with different primer contamination of *D. suzukii*: (12.dsuz.F/R). 1. *Drosophila suzukii*. Copies from Mexico, Turkey, Egypt. 2. *Drosophila simulans*. M - Marker (10[illegible] **END *DROSOPHILA MELONOGASTER*)** [illegible]ntrol (dH_2O)

3.2.3. Test of designed primer pairs (12. Dsuz F/R) with different regions of *D. suzukii*

Susceptibility test 12. Dsuz F/R with varying amounts of *D.suzukii* DNA, K: negative control (dH_2O); K^+: *Drosophila suzukii*. M: DNA, marker (100–1000) b.p. (Figure 15). A value of 1.5 µl shows the best electrophoresis images among the samples. These primers are only able to identify the species *Drosophila suzukii*.

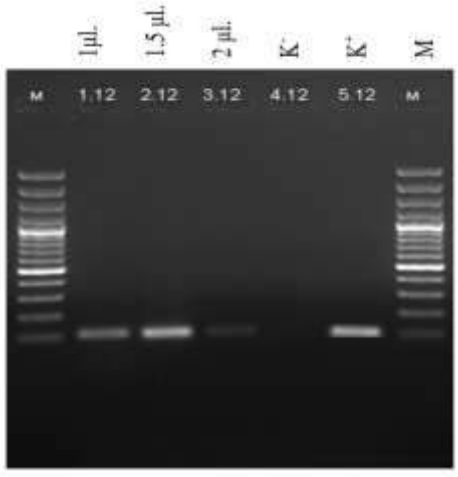


Figure 15. – Electropherogram of conventional PCR amplification products with primer: (12.dsuz.F/R). 1.12. *Drosophila suzukii*. 2.12. *Drosophila suzukii*. M - Molecular weight marker 3.12. *Drosophila suzukii*. 4.12. K-: negative control (dH_2O): DNA extraction control and an amplification control. 5.12. Positive control: DNA isolation from *Drosophila suzukii*

PCR is advised. Molecular diagnostics can be used as a quick and effective identification method when specimens are not in good enough condition for accurate morphological identification or when only unfinished samples are available. This study and others suggest that not all investigations may benefit from the use of standard primers. Various writers [72]. Confirm our findings as well. Due to the close proximity of the genetic codes of several *Drosophila* species, some primers are unable to distinguish between multiple subspecies. Therefore, the World Quarantine Organization may benefit greatly from the creation of more sensitive primers. The suggested strategy for detecting this economically significant invasive species paves the way for more precise surveillance and identification while simultaneously reducing the likelihood of incorrect identification. The findings demonstrated that these primers (12.dsuz.F/R) accurately identify the gene area and are additionally particular to *Drosophila suzukii*.

3.2.4. Primer (7. dsuz. F / R) for classical PCR for *Drosophila suzukii*

In a PCR Thermal Cycler (Applied Biosystems, Hamburg, Germany) using 1.5 L of DNA extract and 0.5 M of each primer (7.dsuz.F/R), the DNA of predators and flies was amplified. Sequences were aligned in BioEdit [73]. In addition, additional sequences from GenBank and control samples *Zaprionus indianus* and *Megaselia scalaris* were employed (markers). Primer Premier 5 was used to manually construct and certify a pair of primers that target *D. suzukii* (Palo Alto, USA, International PREMIER Biosoft). (Figures16).

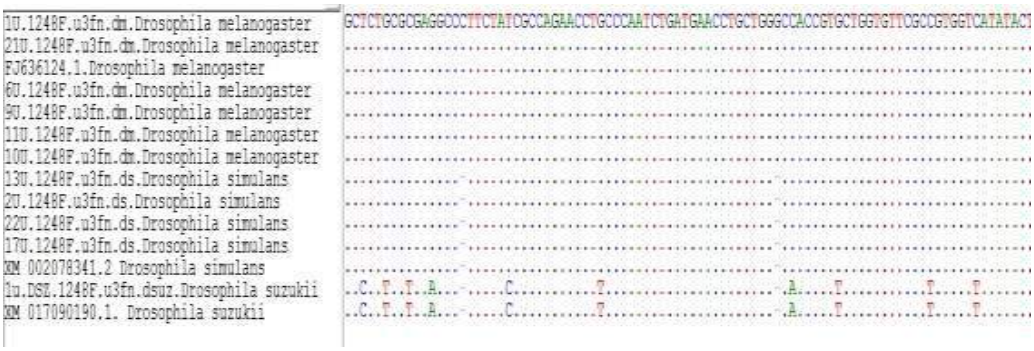


Figure 16. – Different primer designs and COI gene fragment sequences s

Table 25. – Identification of various genetic codes of *Drosophila* sp. with markers *Zaprionus indianus* and *Megaselia scalaris*

№	Spices	Number of letter										
		69	70	78	81	84	104	116	131	137	149	155
1	*Drosophila melanogaster*	T	T	G	C	G	T	G	C	G	C	C
2	*Drosophila simulans*	T	T	G	C	G	T	G	C	G	C	C
3	*Zaprionus indianus*	T	T	G	T	G	T	C	G	G	C	C
4	*Megaselia scalaris*	T	T	C	C	G	T	G	C	G	C	C
5	*Drosophila suzukii*	C	C	T	T	A	C	T	A	T	T	T

Table 26. – Primer sequences and sizes of resulting amplicons

Primer name	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
7.dsuz.F	CCTTCGTGAAGCCTTCTACCG	Plus	21	475	496	57.14	58
7.dsuz.R	GCACTCTTGATGGGAAGATC	Minus	20	530	550	50.00	60
Product length	191						

F stands for the forward primer, and R for the reverse primer.

Based on the alignment of two pairs of Primers Dro-Suz A390 (5′-TTGAACTGTTTACCCACCTCTT -3′) as a forward and Dro-Suz S390 (5′-GGTATTCGGTCTAATGTAATACCC -3′) as a reverse, and Droso-S391 (5′-AAATAACAATACAGGACTCATATcc-3′) as a forward and, Droso-A381 (5′-gTAATACGCTTACATACATaAAGGTATA-3′) as a reverse [22, 25].

We used this pair of primers for sequencing, then after sequencing the DNAs, and edited them in BioEdit Software and additional *Drosophila* sequences from GenBank and *Zaprionus indianus* and *Megaselia scalaris* were used as control samples (markers). Therefore, we design a pair of proprietary primers. Primer 7. dsuz. F (5′-

CCTTCGTGAAGCCTTCTACCG -3′) as a forward and 7. dsuz. R (5′ GCACTCTTGATGGGAAGATC -3′) as a reverse were used (table 25 and 26).

3.2.5. Primer selectivity (7.dsuz.F/R) with other quarantine fruit fly species

Electrophoresis image (Figure 6) 1, 2 and 3 *Drosophila suzukii* from: (Mexico), (Turkey), (Egypt) 4. *Drosophila simulans*. 5. *Drosophila melanogaster*. 6. *Zaprionus indianus*, 7. *Megaselia scalaris* as a marker. M - Marker (100–1000) b.p. Negative control: DNA extraction control and amplification control.

All three species of *D. suzukii*, *D. simulans*, and *D. melanogaster* were tested using the primer in the electrophoresis image (Figure 17), displaying the effectiveness of the specially developed primer (7.dsuz.F/R), which only identifies *D. suzukii*. These primers can only identify species of *Drosophila suzukii*. Recommended PCR. When specimens are too damaged for accurate morphological identification or when ready specimens are not available, molecular diagnostics can be employed as a rapid and effective identification method.

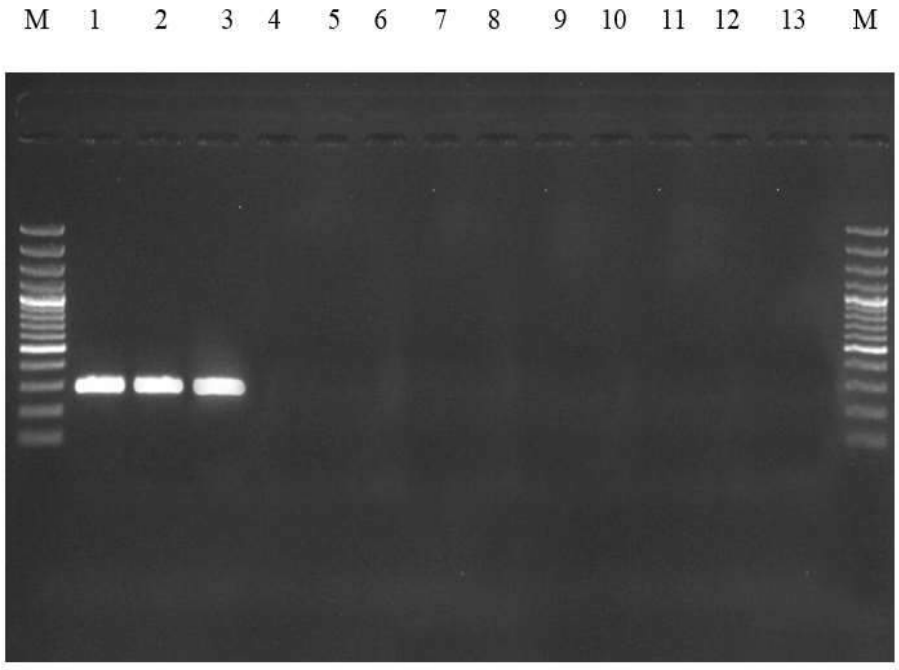


Figure 17. – Electrophoresis of traditional PCR amplification products with different *D. suzukii* contaminant primer: (7.dsuz.F/R)

3.3. Primer designing for *D. suzukii* specific real-time PCR

Primers 3. Dsuz. F (5-GGCGCCGGTGTCTGCCTGC-3), 3.Dsuz.R (5-CTGGTTTGATTGTGCTGCTGC-3), and 3. Dsuz. P (5-GGCAATGGAACAGGGAAATTCC -3), are adopted as forward, reverse, and probe, respectively. The PCR mix was created using 1 ml (10 pmol) 5 ml s of master-mix5dd (HS-5x), 16 ml [10] of water, and 1 ml of DNA The Real-Time PCR unit was a CFX 96 (Bio Rad, USA) with a cumulative volume of 25 ml. Screen-Mix, a ready-to-use PCR mixture, was used in the reaction (Evrogen, Russia) [10]. 94 degrees for five minutes, then 40 cycles of 95 degrees for thirty seconds, 70 degrees for twenty seconds, and 72 degrees for thirty seconds. After the amplification, the findings of a melting curve analysis subsequently combined. (Software by CFX Maestro).

The designed primers (3. Dsuz F and R) were used to diagnose of 13 *Drosophila* species samples under 60 ^{0}C temperature. In figure, two upward peaks were shown that indicated our positive control with *D. suzukii* (from Egypt and Turkey). There were no other melt peaks noted. Various writers [74]. Also, support our judgment. Due to the close proximity of the genetic codes of different *Drosophila species*, some primers are unable to distinguish between numerous subspecies. The World Quarantine Organization can therefore benefit much from creating primers that are more sensitive. (Figures. 18).

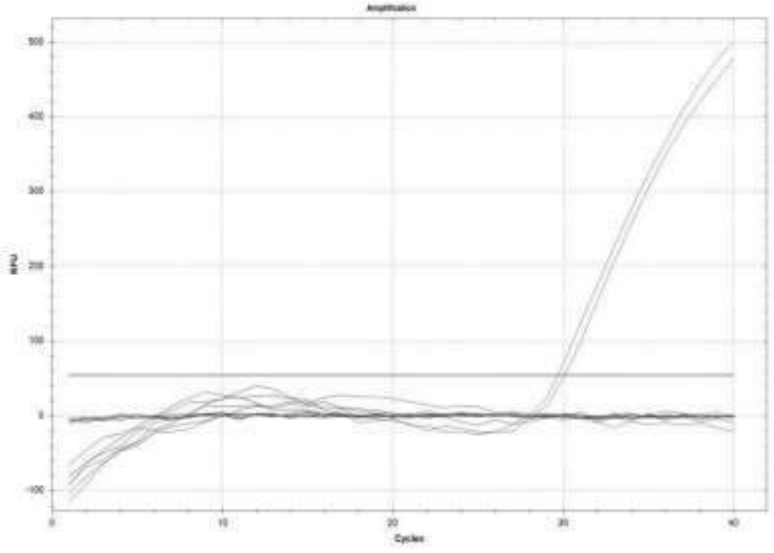


Figure 18. – Efficiency of real-time PCR for *D. suzukii* (primers3. Dsuz F and R)

Table 27. – Quantification Data for *D. suzukii.* With primers (3. Dsuz F and R)

Well	Fluor	Target	Content	Cq Mean
A01	FAM	Pos Ctrl	25,19	25.19
A03	FAM	unknown	N/A	0.00
C01	FAM	unknown	26,17	26.17
C03	FAM	unknown	N/A	0.01
D01	FAM	unknown	N/A	0.14
D03	FAM	unknown	N/A	1.00
E01	FAM	unknown	N/A	3.02
E03	FAM	unknown	N/A	0.02
F01	FAM	unknown	N/A	2.00
F03	FAM	unknown	N/A	2.09
G01	FAM	unknown	3,83	3.83
G03	FAM	unknown	N/A	0.00
A05	FAM	Neg Ctrl	N/A	0.00

Only 2 of the 13 samples with an unclear identity (Table 27.) were successfully amplified and had the proper melt peak temperature to be correctly classified as *D.suzukii* (positive control) (figure.23). The amplification of any more samples was negative. The number of target copies in your sample correlates with the Cq values, which are inversely proportional to the number of target nucleic acid in your specimens. Areas 25.19 and 26.17 have therefore seen the appearance of Cq values, and there are peaks there. Lower

Cq values (typically below 29 cycles) indicate high amounts of a target sequence. Higher Cq values (above 38 cycles) mean lower amounts of the target nucleic acid.

Table 28. – The list of *Drosophila* species used in this work for the detection of *D. suzukii* by real-time PCR (primers 3. Dsuz. F/R PROBE-FAM**)**

NO	Species Identification	Country: (laboratory Data , Russian quarantine)	Consequence of real-time PCR
1	*Drosophila melanogaster*	Egypt	
2	*Drosophila suzukii*	Turkey	+
3	*Drosophila simulans*	Russia	
4	*Drosophila suzukii*	Turkey	+
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	*Sarcophagi similis*	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*K*$^{-}$	Water	-

Due to successful amplification and the correct melt peak temperature, only two of the 13 samples with unknown identities (1, 2) were correctly identified as *Drosophila suzukii* (positive control) (Table 28). The sample number was the best of them (1). Other samples did not exhibit positive amplification. (Figures. 19).

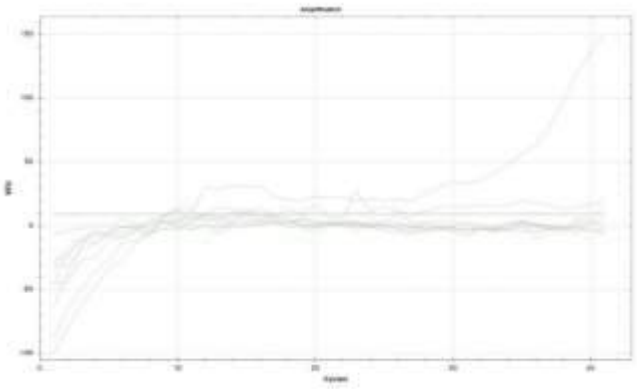


Figure 19. – Efficiency of real-time PCR for the identification of *Drosophila suzukii* (primers 3.Dsuz. F/R PROBE-FAM) (Repetition number 2)

Table 29. – Quantification Data for *D. suzukii.* With *primers* (3. Dsuz F and R)

Well	Fluor	Target	Cq Mean
A01	FAM	Neg Ctrl	0.00
A02	FAM	unknown	27.05
B01	FAM	unknown	25.89
B02	FAM	unknown	0.00
C01	FAM	unknown	40.29
C02	FAM	unknown	11.86
D01	FAM	unknown	9.19
D02	FAM	Pos Ctrl	14.55
E01	FAM	unknown	2.49
E02	FAM	unknown	10.84
F01	FAM	unknown	0.00
F02	FAM	unknown	1.67
G01	FAM	unknown	11.87
G02	FAM	unknown	3.17

Table 30. – List of *Drosophila* species used in this paper, which were used to determine *D. suzukii* By real-time PCR (primers 3. Dsuz. F/R PROBE-FAM**)**

NO	Species Identification	Country: (laboratory Data from Russian quarantine)	Result of real-time PCR
1	*Drosophila melanogaster*	Turkey	
2	*Drosophila suzukii*	Egypt	+
3	*Drosophila simulans*	Russia	
4	*Zaprionus indianus*	Turkey	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Turkey	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	unknown	
9	Sarcophagi similis	Turkey	
10	*Zaprionus tuberculatus*	China	
11	*Zaprionus indianus Malloch*	unknown	
12	*Drosophila funebris*	Turkey	
13	*K*$^{-}$	Water	-

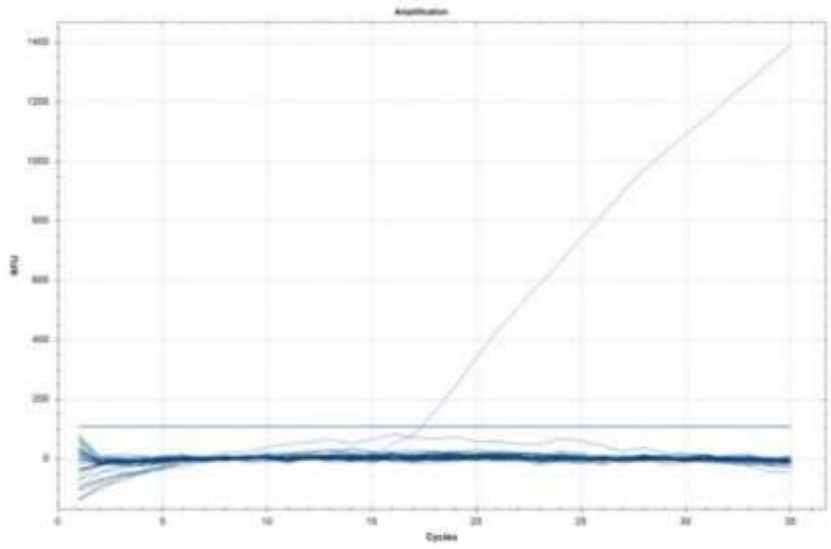


Figure 20. – Efficiency of real-time PCR for the identification of *Drosophila suzukii* (primers 3. Dsuz. F/R PROBE-FAM). (Repetition number 3)

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 31. – Quantification Data for *D. suzukii.* with *primers* (3. Dsuz F and R)

Well	Fluor	Target	Cq Mean
A01	FAM	Neg Ctrl	0.00
A02	FAM	unknown	1.09
B01	FAM	Pos Ctrl	28.11
B02	FAM	unknown	1.02
C01	FAM	unknown	3.29
C02	FAM	unknown	1.24
D01	FAM	unknown	2.11
D02	FAM	unknown	0.74
E01	FAM	unknown	1.97
E02	FAM	unknown	3.3
F01	FAM	unknown	0.01
F02	FAM	unknown	2.65
G01	FAM	unknown	1.76
G02	FAM	unknown	3.17

Table 32. – List of *Drosophila* species used in this paper, which were used to determine *D. suzukii* by real-time PCR (primers 3. Dsuz. F/R PROBE-FAM**)**

NO	Species Identification	Country: (laboratory Data from Russian quarantine)[22]	Result of real-time PCR
1	*Drosophila melanogaster*	Turkey	
2	*Drosophila suzukii*	Egypt	+
3	*Drosophila simulans*	Russia	
4	*Zaprionus indianus*	Turkey	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Turkey	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	unknown	
9	Sarcophagi similis	Turkey	
10	*Zaprionus tuberculatus*	China	
11	*Zaprionus indianus Malloch*	unknown	
12	*Drosophila funebris*	Turkey	
13	K^-	Water	-

Through successful amplification and the correct melt peak temperature, only one of the 13 samples of unknown identity (3) was correctly identified as *Drosophila suzukii* (positive control) (Table 32). The sample number was the best of them (1). Other samples did not exhibit positive amplification.

3.4. Real-time PCR design (1. dsuz. F/R) for *Drosophila suzukii*

To design specific primer to identify for *Drosophila suzukii*. I used basic primers 1248 F (5′- TGGAACTGTTTACCCACCTCGT -3′) as a forward and 1248 R (5′- TGTATTCGGTCTAATGTAATACT -3′) as a reverse, we used this pair of primers for sequencing, then after sequencing the DNAs, and edited them in BioEdit Software and additional *Drosophila* sequences from GenBank. In addition, design a pair of Primers (1. dsuz. F/R. Probe FAM) where designed for Real Time PCR. (Table.33).

All primers designed were evaluated qualitatively in two or three replications:

Table 33. – Primer sequences and resulting amplicon sizes

Name of primer (u3fn1.dsuz)	Sequence (5'->3')	Length	GC%	T^0m
1. dsuz F	CCTTCGTGAAGCCTTCTACCG	21	57.14	58
1. dsuz R	GCACTCTTGATGGGAAGATC	20	50.00	60
1. dsuz Probe FAM	CAACCGTTCTGGTGTTCGCTG	21	52	62

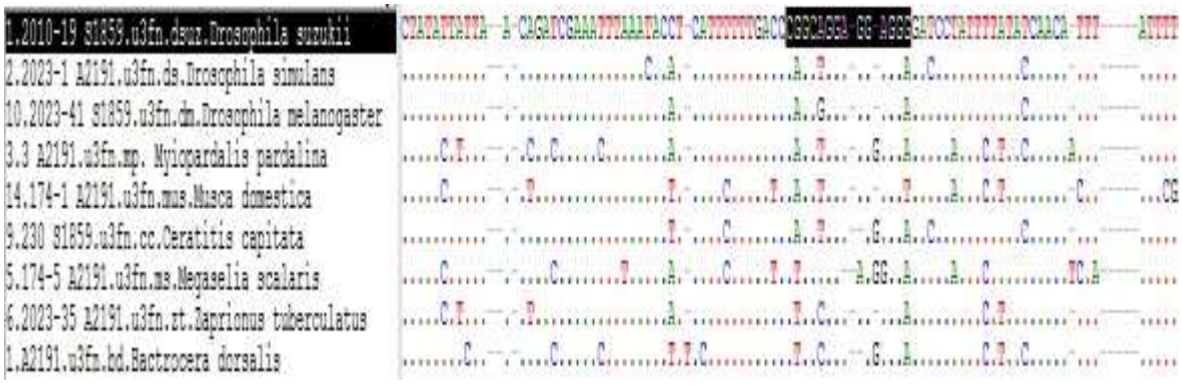


Figure 21. – COI gene fragment sequences and primer designs for *Drosophila suzukii* with various codes

Table 34. – Identification of different *Drosophila suzukii* genetic codes with primer 1. dsuz. For Real Time. *Drosophila suzukii*

NO	*Species*	*Number of letter with primer 1 .dsuz.*														
		295	315	318	321	323	325	337	345	348	352	375	384	385	386	387
1	*mp. Myiopardalis Pardalina*	T	A	T	A	-	A	A	G	A	A	T	G	G	T	A
2	*mus. Musca Domestica*	T	A	T	T	-	A	A	G	A	T	T	C	G	A	T
3	*cc.Ceratitis Capitata*	T	A	T	T	-	A	A	G	A	A	T	T	A	G	A
4	*ms. Megaselia Scalaris*	T	T	T	A	-	A	T	A	G	A	A	T	A	G	A
5	*zt.Zaprionus Tuberculatus*	T	A	T	A	-	A	T	G	A	A	T	T	A	G	A
6	*dm. Drosophila melanogaster*	T	A	T	A	-	A	A	G	A	A	T	T	A	G	A
7	*ds. Drosophila simulans*	T	A	C	A	-	A	A	G	A	A	T	C	G	A	T
8	*bd. Bactrocera Dorsalis*	C	A	T	T	T	C	T	G	A	A	T	C	G	A	T
9	*dsuz.Drosophila suzukii*	T	A	T	C	-	A	G	G	A	G	T	T	T	G	A

3.5. Optimization with Primer Real Time PCR (1. dsuz. F/R PROBE-FAM)

It is necessary to set the optimal primer temperature. Samples should be tested by PCR at different temperatures. This graph (Figure 22) presents 12 samples for identification with a primer (1. dsuz. F/R PROBE-FAM) and a temperature gradient from T: 60°C to T: 640° C, six of *D. suzukii*, 6 samples as a negative unit (Table 34), resulting in a temperature T: 60 °C. (Figure 22) shows that the best peak is the top peak.

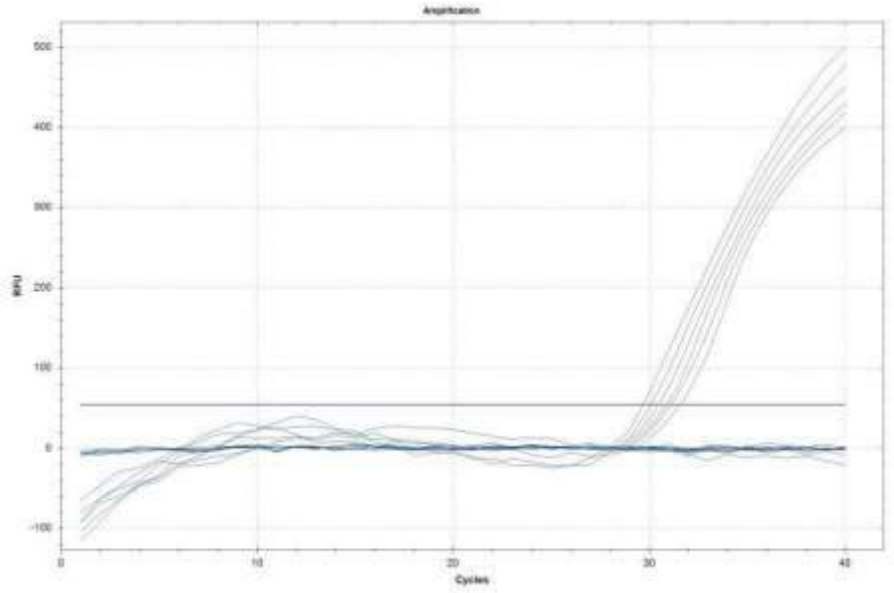


Figure 22. – Efficiency of real-time PCR for the identification of *D. suzukii* (primers1. dsuz)

Tip: Protocol

1: 95, 0°C for 10:00

2: 95, 0°C for 0:40

3: Gradient 60, 0°C / 64, 0°C for 0:40

4: GOTO 2, 39 more times

Table 35. - Quantification Data for *D. suzukii*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	FAM	Pos Ctrl (*D.suzukii)*	27,12	27,12	60.00
A03	FAM	Neg Ctrl	N/A	0,00	60.00
C01	FAM	Pos Ctrl (*D.suzukii)*	26,11	26,11	61.00
C03	FAM	Neg Ctrl	N/A	0,00	61.00
D01	FAM	Pos Ctrl (*D.suzukii)*	29,68	29,68	62.00
D03	FAM	Neg Ctrl	N/A	0,00	62.00
E01	FAM	Pos Ctrl (*D.suzukii)*	30,09	30,09	63.00
E03	FAM	Neg Ctrl	N/A	0,00	63.00
F01	FAM	Pos Ctrl (*D.suzukii)*	29,46	29,46	64.00
F03	FAM	Neg Ctrl	N/A	0,00	64.00
G01	FAM	Pos Ctrl (*D.suzukii)*	29,58	29,58	64.01
G03	FAM	Neg Ctrl	N/A	0,00	64.01

Six of the twelve *D.suzukii* identifying samples (samples 1, 3,5, 7, 9, and 11 in Table.35) were successfully amplified and had the proper melt peak temperature, resulting in an accurate identification of *D.suzukii* (positive control) (figure.22). As a result, Cq values have appeared and peaked in the range of 27.12 to 29.58.

The amplification of any more samples was negative. The number of target copies in your sample correlates with the Cq values, which are proportional to the concentration of target nucleic acid in your specimens. A target sequence is present in large concentrations when the Cq value is lower (usually below 29 cycles). Lower concentrations of the target nucleic acid are indicated by higher Cq values (above 38 cycles).

3.5.1. Result (1.dsuz. F/R-FAM) for *Drosophila suzukii* with all fruit fly species

To determine the specificity of the designed primer; also, make sure that the primer can detect the species *Drosophila suzukii*. We compare the *D.suzukii* specimen with other species of the same family and other fruit flies.

Tip: Protocol

1: 95, 0°C for 1:00

2: 95, 0°C for 0:40

3: 60, 0°C for 0:40

4: GOTO 2, 40 more times

Table 36. – Comparison of *Drosophila suzukii* with all fruit fly species (1. dsuz. F/R PROBE.FAM)

№	List of Species
1	*mp. Myiopardalis Pardalina*
2	*mus. Musca Domestica*
3	*cc.Ceratitis Capitata*
4	*mc. Megacelia Scalaris*
5	*zt.Zaprionus Tuberculatus*
6	*dm. Drosophila melanogaster*
7	*ds. Drosophila simulans*
8	*bd. Bactrocera Dorsalis*
9	*dsuz.Drosophila suzukii*

Additionally, DNA was added to each tube (Figure 22).

Figure 23. – The process of adding DNA with all species of fruit flies

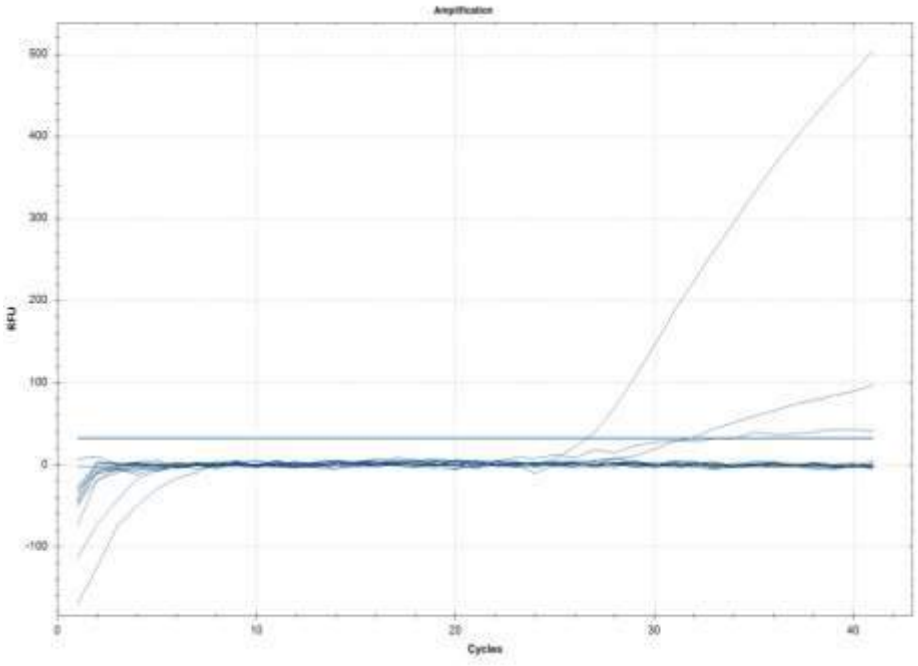


Figure 24. – Efficiency of real-time PCR for the identification of *D. suzukii* (primers1. dsuz.F /R PROBEFAM)

15 samples were submitted for identification in this study (Figure 24) using primer (1. dsuz.F /R PROBEFAM) at 600 °C. 11 were unknown, while 3 from *Drosophila suzukii* were negative.

Two of these have been conclusively identified as being *Drosophila suzukii*; samples (1 and 2) had melting peaks that were within the acceptable range of 60.08 °C and stood out from other species. There were no further melting peaks noted. [10]

Table 37. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila suzukii* by Real-time PCR (primers 1. dsuz. F/R PROBEFAM).

NO	Subspecies' name	Country:(lab Data from Russian quarantine)	Result of real-time PCR [10, 22]
1	*Drosophila melanogaster*	Russia	
2	*Drosophila suzukii*	Egypt	+
3	*Drosophila simulans*	Canada	
4	*Drosophila suzukii*	Turkey	+
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	Sarcophagi similis	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianusMalloch*	Turkey	
13	*Drosophila funebris*	Iran	
14	*K*$^{-}$	Water	-

Table 38. – Quantification Data for *D. suzukii*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	FAM	Pos Ctrl (*D.suzukii)*	25,14	27,12	60.08
A03	FAM	Pos Ctrl (*D.suzukii)*	21,31	21,31	60.08
C01	FAM	unknown	N/A	3,12	60.08
C03	FAM	unknown	N/A	4,22	60.08
D01	FAM	unknown	N/A	1,68	60.08
D03	FAM	unknown	N/A	0,00	60.08
E01	FAM	unknown	N/A	1,04	60.08
E03	FAM	unknown	N/A	1,19	60.08
F01	FAM	unknown	N/A	0,00	60.08
F03	FAM	unknown	N/A	5,72	60.08
G01	FAM	unknown	N/A	6,00	60.08
G03	FAM	unknown	N/A	2,00	60.08
B03	FAM	unknown	N/A	1,19	60.08
A01	FAM	Neg Ctrl	N/A	0,00	60.08

Due to successful amplification and the appropriate melting peak temperature, 2 of the 13 accessions of *Drosophila* sp. (Table 38) were positively identified as spotted *Drosophila* (positive) (Figure 23. 24). Cq values therefore appeared in regions 21, 31, up to 25, and 14, where the peaks peaked.

Positive amplification was not seen in any of the other samples. The quantity of destination nucleic acid in the samples is proportional to the Cq values, which are also proportional to the number of target copies in the sample. [10]

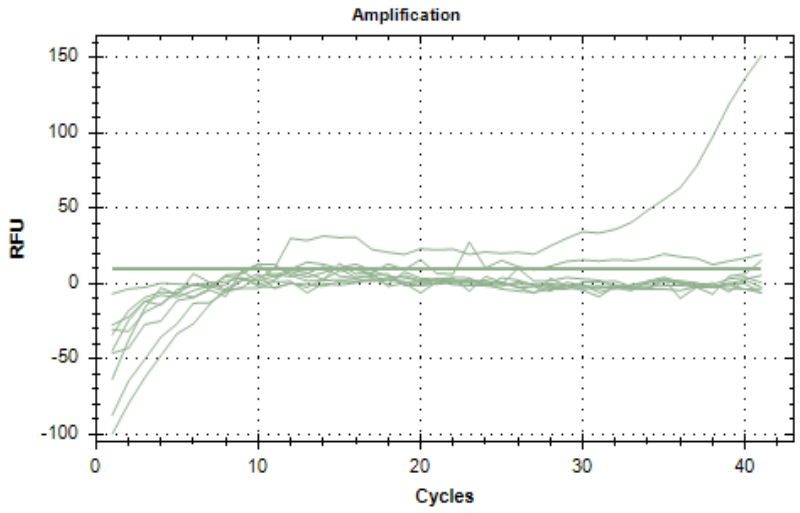


Figure 25. – Efficiency of the real-time PCR method in the identification of the *D. suzukii* species (primers1. dsuz. F/R PROBEFAM). (Repetition number 2)

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 39. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila suzukii* by real-time PCR (primers 1. dsuz. F/R PROBEFAM)

NO	Name of Species	Country: (Data from laboratory of Russian quarantine)	Result of real-time PCR
1	*Drosophila funebris*	Unknown	
2	*Drosophila suzukii*	Egypt	+
3	*Drosophila simulans*	Canada	
4	*Drosophila melanogaster*	Russia	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	*Sarcophagi similis*	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*Drosophila funebris*	Iran	
14	*K-*	Water	-

Table 40.- Quantification Data for *D. suzukii*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	FAM	Pos Ctrl (*D.suzukii)*	25,14	27,12	60.08
A03	FAM	Pos Ctrl (*D.suzukii)*	21,31	21,31	60.08
C01	FAM	unknown	N/A	3,12	60.08
C03	FAM	unknown	N/A	4,22	60.08
D01	FAM	unknown	N/A	1,68	60.08
D03	FAM	unknown	N/A	0,00	60.08
E01	FAM	unknown	N/A	1,04	60.08
E03	FAM	unknown	N/A	1,19	60.08
F01	FAM	unknown	N/A	0,00	60.08
F03	FAM	unknown	N/A	5,72	60.08
G01	FAM	unknown	N/A	6,00	60.08
G03	FAM	unknown	N/A	2,00	60.08
B03	FAM	unknown	N/A	1,19	60.08
A01	FAM	Neg Ctrl	N/A	0,00	60.08

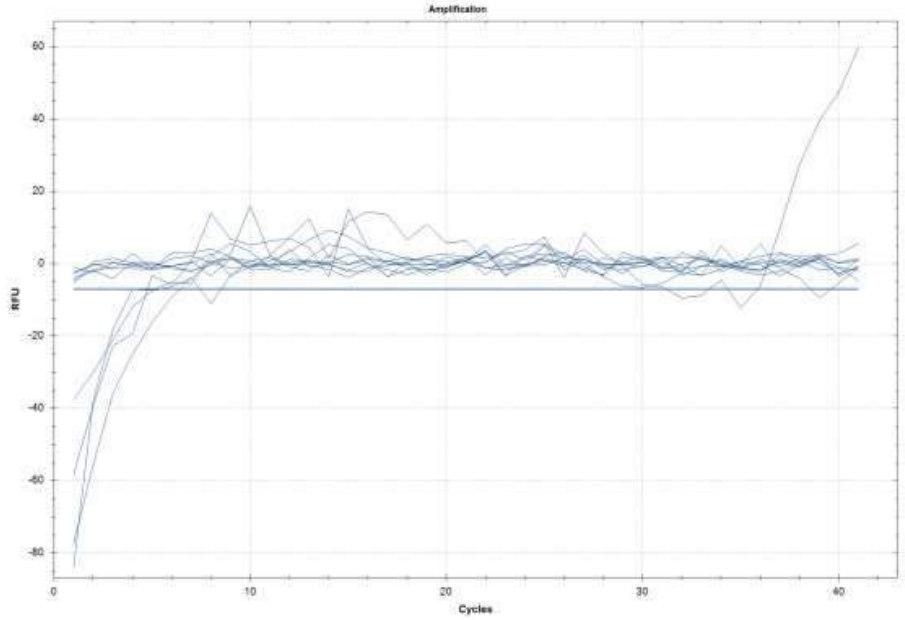


Figure 26. – Efficiency of the real-time PCR method in the identification of the *D. suzukii* species (primers 1. dsuz. F/R PROBEFAM) (Repeat three times)

Only one peak of the several peaks created in the graph has risen, signifying the identification of the target sample.

Table 41. – List of *Drosophila* species used in this paper, which were used to determine Drosophila *suzukii* by real-time PCR (primers 1. dsuz. F/R PROBEFAM)

NO	Name of Species	Country: (Data from laboratory of Russian quarantine)	Result of real-time PCR
1	*Drosophila funebris*	Unknown	
2	*Drosophila suzukii*	Egypt	+
3	*Drosophila simulans*	Canada	
4	*Drosophila melanogaster*	Russia	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	*Sarcophagi similis*	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*Drosophila funebris*	Iran	
14	*K-*	Water	-

Table 42. – Quantification Data for *D. suzukii*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	FAM	unknown	N/A	1,07	60.08
A03	FAM	unknown	N/A	3,33	60.08
C01	FAM	Pos Ctrl (*D.suzukii)*	N/A	14,68	60.08
C03	FAM	unknown	N/A	5,25	60.08
D01	FAM	unknown	N/A	1,12	60.08
D03	FAM	unknown	N/A	2,19	60.08
E01	FAM	unknown	N/A	0,81	60.08
E03	FAM	unknown	N/A	1,15	60.08
F01	FAM	unknown	N/A	2,01	60.08
F03	FAM	unknown	N/A	-01,33	60.08
G01	FAM	unknown	N/A	3,05	60.08
G03	FAM	unknown	N/A	1,42	60.08
B03	FAM	unknown	14,19	01,19	60.08
A01	FAM	Neg Ctrl	N/A	0,11	60.08

3.5.2. Result (1.dsuz. F/R-FAM) for *D.suzukii* with all fruit fly species in Iran

Samples (1) stood out substantially from other species and displayed melting peaks within the permitted range of T: 60.08°C. There were no further melting peaks identified.

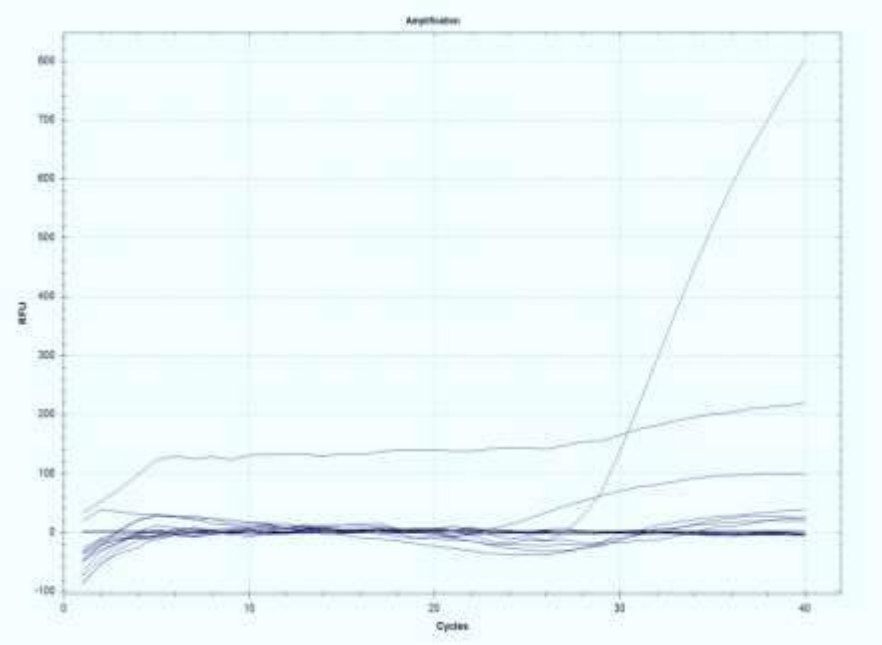


Figure 27. – Efficiency of the real-time PCR method in the identification of the *D. Suzukii* species (primers1. dsuz. F/R PROBE.FAM). (Repetition number No. 4)

Table 43. – The list of *Drosophila* species used in this work for the detection of *Drosophila suzukii* by real-time PCR (primers1. dsuz. F/R PROBE.FAM)

NO	Name of Species	Country: (Data from laboratory of Russian quarantine)	Result of real-time PCR
1	*Drosophila funebris*	Unknown	
2	*Drosophila suzukii*	Egypt	+
3	*Drosophila simulans*	Canada	
4	*Drosophila melanogaster*	Russia	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	*Sarcophagi similis*	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*Drosophila funebris*	Iran	
14	*K*-	Water	-

One growing peak was depicted in (figure 27), indicating a successful identification of *D. suzukii* (from Iran). There is no information on further melting peaks, likely as a result of the lower DNA content. Other writers [70, 74] also support our findings.

Table 44. – Quantification Data for *D. suzukii*.

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	FAM	unknown	N/A	1,07	60.08
A03	FAM	unknown	N/A	3,33	60.08
C01	FAM	Pos Ctrl (*D.suzukii)*	N/A	14,68	60.08
C03	FAM	unknown	N/A	5,25	60.08
D01	FAM	unknown	N/A	1,12	60.08
D03	FAM	unknown	N/A	2,19	60.08
E01	FAM	unknown	N/A	0,81	60.08
E03	FAM	unknown	N/A	1,15	60.08
F01	FAM	unknown	N/A	2,01	60.08
F03	FAM	unknown	N/A	-01,33	60.08
G01	FAM	unknown	N/A	3,05	60.08
G03	FAM	unknown	N/A	1,42	60.08
B03	FAM	unknown	14,19	01,19	60.08

1. Figure (27) displayed a single climbing peak, indicating a favorable outcome with *D.simulans* (from Iran). No further melting peaks were seen, most likely because there was less DNA material present. Other authors [21] also support our findings. Real Time PCR displays the data of amplification during the run after each cycle, whereas conventional PCR only provides results at the conclusion of the process.
2. Classical PCR results are in the form of bands in the gel whereas in Real Time PCR Ct or threshold value gives the measure of quality and quantity of the product. By examining the performance of both Real Time PCR and PCR results, it can be concluded that the best performance is Real Time.

3.6. Molecular Diagnostic of *Drosophila melanogaster*

3.6.1. Molecular identification of *Drosophila melanogaster* with (Droso-S391, A381)

I used Droso-A381 (5′-gTAATACGCTTACATACATaAAGGTATA-3′) as the reverse and the base primer Droso-S391 (5′- AAATAACAATACAGGACTCATATcc -3′) as the reverse [15, 22]. (Table 45).

We used this primer pair for sequencing, then after DNA sequencing and edited them in the BioEdit software and additional *Drosophila* sequences from GenBank. In addition, a couple of drivers have been developed (Table 45).

Table 45. – List of samples where used with different countries, hosts plant**. (**With Dro-Suz A390, S390 primer).

No	Sample ID	Name of primer	No. E.phoreses	Host	Country	Date	Results
1	1. A2191.u3fn.dm.	*Droso-S391, Droso-A381*	1	Melon	Turkey	2019.04.09	*Drosophila. melanogaster*
2	2. A2191.u3fn.dm.	*Droso-S391, Droso-A381*	2	Sugar apple	Mexico	2019.04.09	*Drosophila. melanogaster*
3	3. A2191.u3fn.dm.	*Droso-S391, Droso-A381*	3	Melon	Turkey	2019.04.09	*Drosophila. melanogaster*
4	K^-	*Droso-S391, Droso-A381*	4			2019.04.09	-

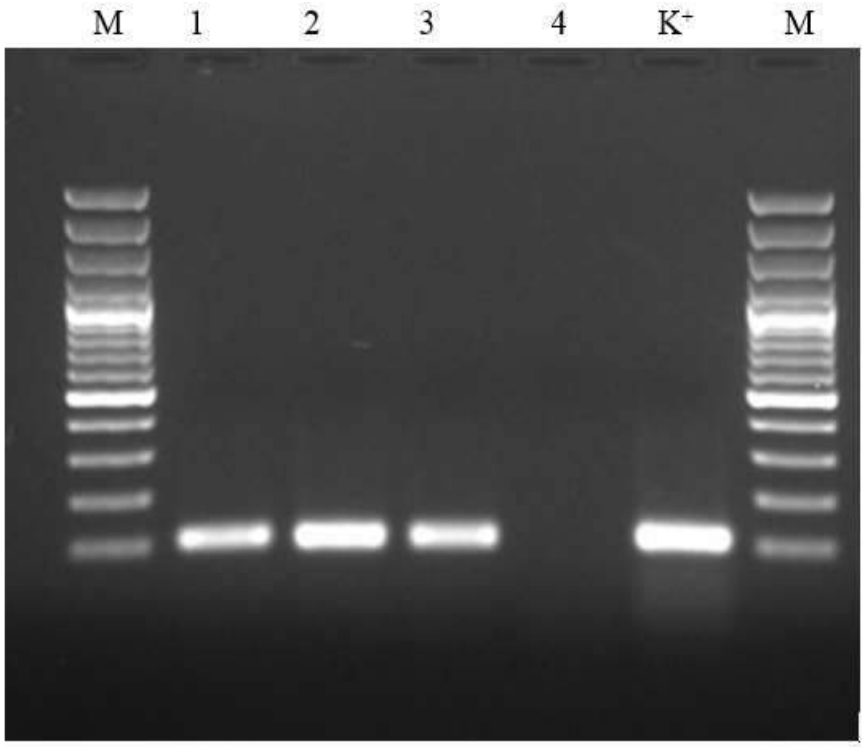


Figure 28. – Picture of Electrophoreses (Droso-S391, A381.)

Three samples are identified as *Drosophila melanogaster* (1, 2, and 3) in the electrophoresis image (Fig. 28), whereas the remaining samples are Positive and Negative Controls. The results demonstrated that these primers accurately identify the gene's area and even the specific for *Drosophila melanogaster* [10, 22].

Figure 29. – Different codes are found in the COI gene fragment and primer sequences.

Table 46. – Identification of several genomic codes from *Drosophila melanogaster* [42]

№	Spices	amount of letters										
		208	215	217	254	271	292	295	307	311	549	555
1	*Drosophila melanogaster*	C	C	T	T	G	C	T	G	A	G	C
2	*Drosophila suzukii*	A	T	G	C	C	A	C	T	T	C	G
3	*Drosophila Simulans*	A	T	G	C	C	T	C	C	T	C	G
4	*Drosophila subobscura*	A	T	G	G	T	A	A	C	G	A	G
5	*Drosophila immigrans*	A	T	G	G	C	A	C	T	T	C	G
6	*Zaprionus indianus*	A	T	G	C	C	A	C	T	T	C	G

Table 47. – Primer (4.dm.F/R) sequences and resulting amplicon sizes for *Drosophila Melanogaster* [43]

Drosophila Melanogaster	Sequence (5'->3')	Length	Start	Quit	Tm	GC %
4.Forward primer	AAGCTCTTCGGCATGGTGAT	20	122	141	59.75	50.00
4.Reverse primer	CCAGTCCATAGCCCTTCTGC	20	299	280	60.18	60.00
4.Product length	198					

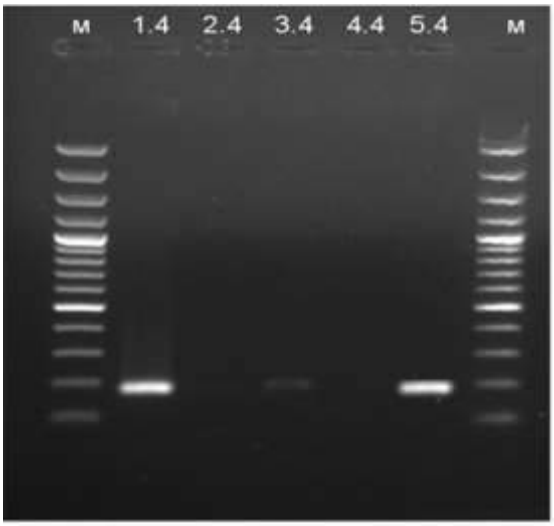


Figure 30. – ElectrophoreG and products of traditional PCR amplification: 1.4. *Drosophila melanogaster*. Samples from Turkey. 2.4. *Drosophila suzukii*. 3.4. *Drosophila simulans*. 4.4. k-5.4. To +. *Drosophila melanogaster*. Negative control: Control over DNA extraction and amplification. M: Marker of particle size

The electrophoresis photo (Fig.30), shows that one samples are *Drosophila Melanogaster* (1.4), one *Drosophila suzukii* (2.4) and one *Drosophila simulans* (3.4), the rest belonging to Negative and Positive Controls (5.4. *Drosophila melanogaster*). Only *Drosophila. Melanogaster* was observed, suggesting that the primer is only able to identify *Drosophila Melanogaster*.

Even before samples are too damaged for accurate morphological identification or only immature specimens are available, the suggested PCR molecular evaluation can be used as a rapid and convenient identification tool. In the case of this economically important invasive species [22], an alternative method of identification will allow for more precise surveillance and identification and may deter misidentification.

3.6.2. Primer design for identification of *D. melanogaster* with (LCO1490, HCO 2198)

In addition, I used base primers LCO1490 (5' GGTCAACAAATCATAAAGATATTGG-3') HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') [7] to design the primer (13.DM.F/R), (Figure 31) for classical PCR (Tables 46,47, 48 and 49).

Figure 31. – Different primer designs and COI gene fragment sequences s

Table 48. – Identification of several genomic codes from *Drosophila melanogaster*

№	Species	Number of letter										
		208	**215**	**217**	**254**	**271**	**292**	**295**	**307**	**311**	**549**	**555**
1	*Drosophila suzukii*	A	T	G	C	C	A	C	T	T	C	G
2	*Drosophila melanogaster*	C	C	T	T	G	C	T	G	A	G	C
3	*Drosophila simulans*	A	T	G	C	C	T	C	C	T	C	G
4	*Drosophila subobscura*	A	T	G	G	T	A	A	C	G	A	G
5	*Drosophila immigrans*	A	T	G	G	C	A	C	T	T	C	G
6	*Zaprionus indianus*	A	T	G	C	C	A	C	T	T	C	G

In (Table 48), the *Drosophila Melanogaster* is placed next to the other species to be examined in terms of the difference in genetic codes in the BioEdit software.

Table 49. – Primer (13.dm.F/R) sequences and resulting amplicon sizes for *Drosophila Melanogaster*

Drosophila Melanogaster	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
13.Forward primer	TGCTCCTGATATAGCATTCCCAC	Plus	23	187	209	59.99	47.83
13.Reverse primer	TTCCAGCGGATAGAGGTGGA	Minus	20	329	310	60.03	55.00

The electrophoresis image shows that one sample is *Drosophila melanogaster* (1.13), one *Drosophila simulans* (2.13) and one *Drosophila suzukii* (3.13), the rest are negative and positive control (5.13) *Drosophila melanogaster*. *Drosophila melanogaster*

was observed, suggesting that the primer is not only capable of identifying *D. suzukii*. (Figure 32).

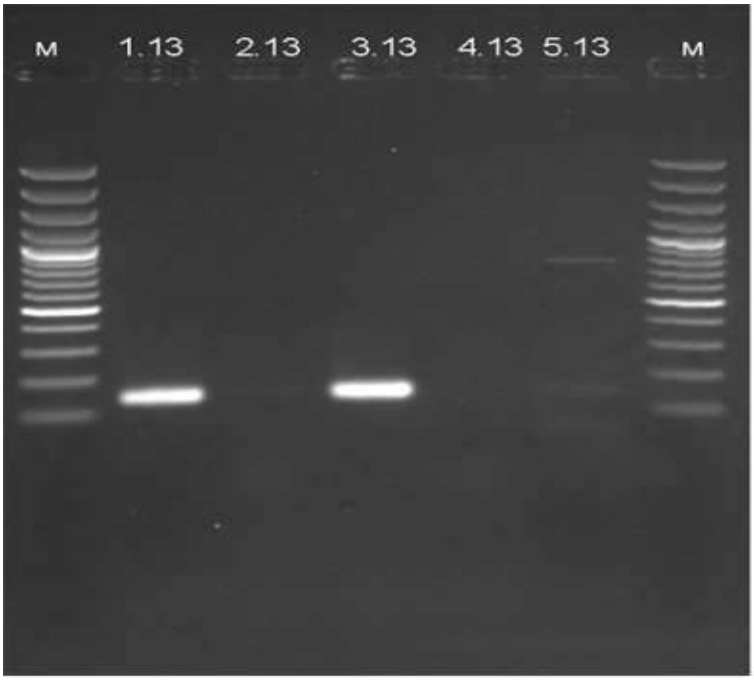


Figure 32. – Checking the selectivity of the developed primer pairs (13 dm. F/R) for conventional PCR. 1.13. *Drosophila melanogaster*. Samples from Turkey. 2.13. *Drosophila simulans* samples from Russia. 3.13. *Specimens of Drosophila suzukii from Egypt. 4.13. K-. 5.13. K+. Specimens of Drosophila melanogaster from Brazil. Negative controls include DNA extraction and amplification. M: Marker of molecular weight*

Despite the design accuracy of this primer, after several tests at different temperatures and conditions, it simultaneously identified both *Drosophila melanogaster* and *Drosophila suzukii*. This primer is not a completely specific primer for *Drosophila melanogaster*, but it is suitable for various *Drosophila species*.

3.6.3. Selectivity (13 dm.F/R) with other quarantine fruit flies

The electrophoresis photo shows that one samples are *Drosophila melanogaster* (1), one *Drosophila simulans* (2) and one *Drosophila suzukii* (3), With *Zaprionus*

indianus (4) and *Megaselia scalaris (5)* as a marker. Positive Controls (10) *Drosophila melanogaster*.

Drosophila melanogaster was observed, suggesting that the primer is only capable of *Drosophila melanogaster* (Figure 33, 37).

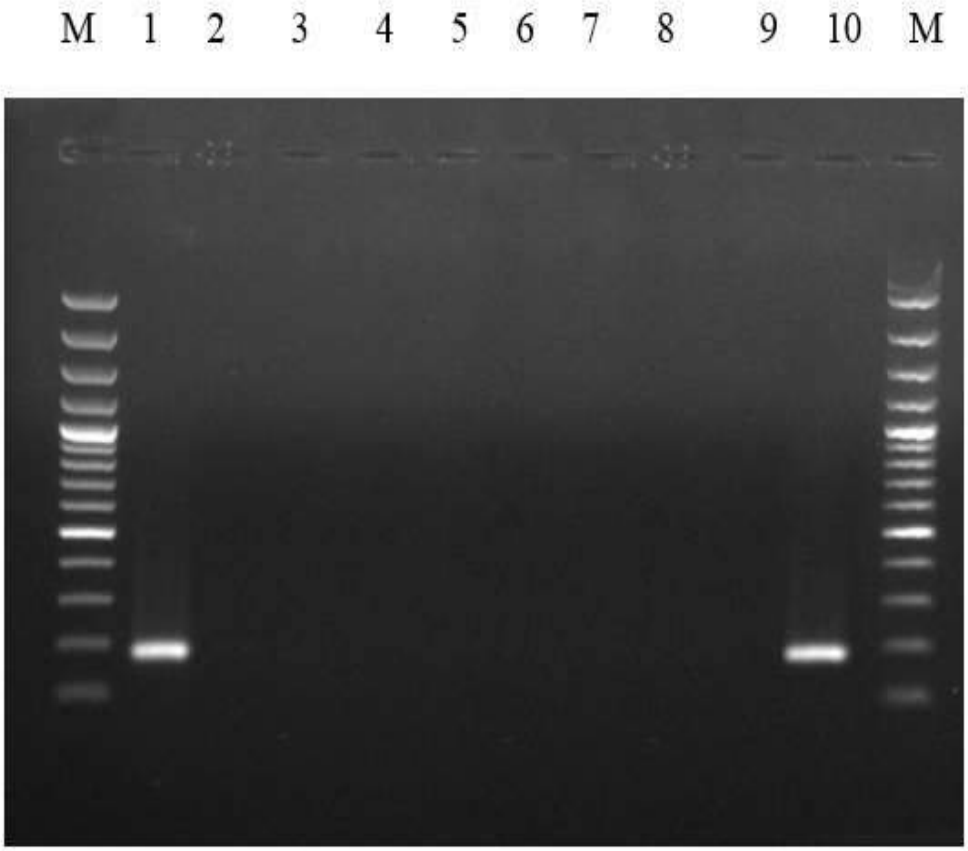


Figure 33. – Test of selectivity of designed primer pairs (13.dm.F/R) for convential PCR. With *Zaprionus indianus* and *Megaselia scalaris* as a marker

Despite the design accuracy of this primer, after several tests at different temperatures and conditions, it simultaneously identified both *Drosophila melanogaster* and *Drosophila suzukii*.

This primer is a fully specific primer for *Drosophila melanogaster* (Table 50).

3.6.4. Design (3.DM.F/R) for real-time PCR and identification of *Drosophila melanogaster*

I used basic primers LCO1490 (5’ GGTCAACAAATCATAAAGATATTGG-3') HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') [15, 75]. For sequencing, and add another sequences from Gen bank and edit in BioEdit (7) software. [54].

Table 50. – Primer (3.dm. F/R. ROX) for Real-time PCR, sequences and resulting amplicon sizes for *Drosophila Melanogaster*

Drosophila Melanogaster	Sequence (5'->3')	Length	Tm	GC%
3.F.u3fn.dm	GGCGCCGGTGTCTGCCTGC	19	69.16	78.95
3.Prob.u3fn.dm	CTGGTTTGATTGTGCTGCTGC	21	62.93	52.38
3.R.u3fn.dm	GGCAATGGAACAGGGAAATTCC	22	62.09	50.00

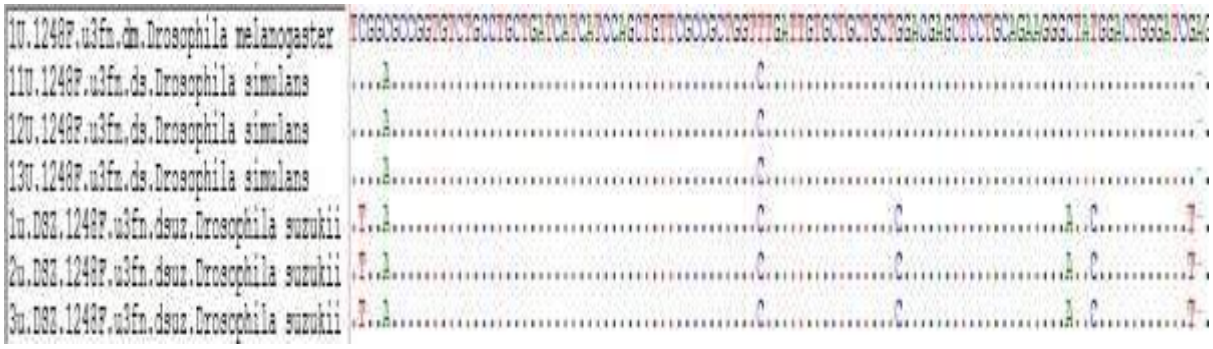


Figure 34. – Different primer designs and COI gene fragment sequences s

It is clear from (Figure 32) that *Drosophila* can be identified in certain codes in different regions of the sequence in the indicated area Melanogaster is distinct from the other species.

Tip: Protocol

1: 95, 0°C for 1:00

2: 95, 0°C for 0:40

3: 59, 4°C for 0:40

4: GOTO 2, 39 more times

Table 51. – The list of *Drosophila* species used in this work for the detection of *Drosophila melanogaster* by real-time PCR (primers 3.dm. F/R)

NO	Name of Species	Country: (Data from laboratory of Russian quarantine)	Result of
1	*Drosophila melanogaster*	Turkey	+
2	*Drosophila suzukii*	Egypt	
3	*Drosophila simulans*	Russia	
4	*Zaprionus indianus*	Canada	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Turkey	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	unknown	
9	*Drosophila melanogaster(from* Egypt)	Egypt	+
10	Sarcophagi similis	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Drosophila funebris*	Turkey	
13	*Drosophila melanogaster (54/89861)*	Iran	+
14	K^-	Water	-

Only three of the 13 samples with unclear identities (1, 9, 13) were successfully amplified and had the proper melt peak temperature to be positively identified as *Drosophila melanogaster* (positive control) (Table 51). The most effective were sample number (1 and 9). No other samples displayed successful amplification.

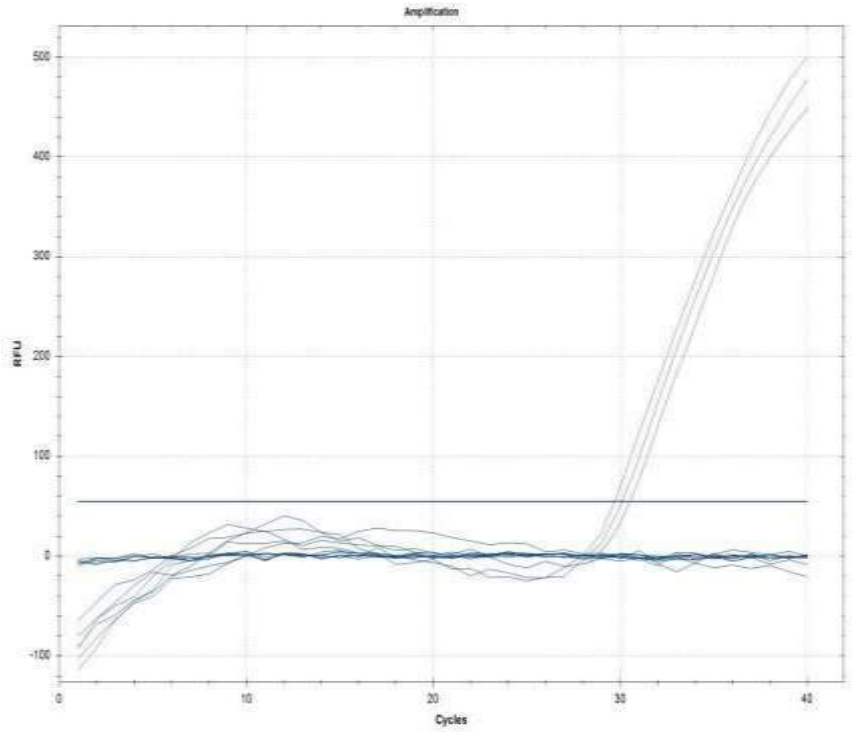


Figure 35. – Efficiency of real-time PCR for the identification of *Drosophila melanogaster* species (primers 3.dm. F/R. ROX)

In these findings, we have 13 samples for primer (primer 3.dm.F/R. ROX and T: 59.4) identification, 3 *Drosophila melanogaster* samples as passive controls, and 10 unidentified instances, each of whose names are presented in Table.52. (Figure 35). Additionally, it involves negative control (sample 13). Two of these were correctly recognized as *Drosophila melanogaster*; samples 1 and 9 had melt peaks that were within the permissible range of 59.04^{o}C and could be easily discriminated from those of other species. There were no further reported melt peaks.

Table 52. – Quantification Data for *Drosophila melanogaster*

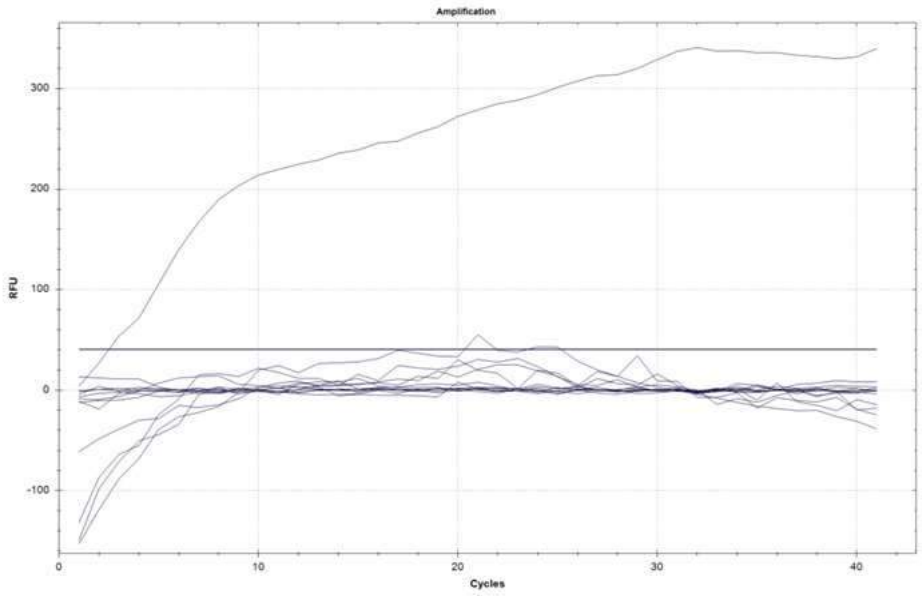
Well	Fluor	Target	Content	Cq Mean
A01	ROX	Pos Ctrl	K^+	29,29
A02	ROX	Unkn	2	3,00
A03	ROX	Unkn	3	0,00
A04	ROX	Unkn	4	0,00
Well	Fluor	Target	Content	Cq Mean
A05	ROX	Unkn	5	2,00
A06	ROX	Unkn	6	1,00
A07	ROX	Unkn	7	6,00
A08	ROX	Unkn	8	0,01
A09	ROX	Pos Ctrl	K+	24,88
A10	ROX	Unkn	10	1,00
A11	ROX	Unkn	11	0,00
A12	ROX	Unkn	12	0,01
A13	ROX	Pos Ctrl	K+	25,18
A14	ROX	Neg Ctrl	K^-	0,00

Only three of the fourteen samples (Table 52) with unclear identities were correctly identified as *D. melanogaster* (positive control) thanks to successful amplification and

the proper melt peak temperature (figure 36). No other samples displayed successful amplification.

The number of target copies in your sample correlates with the Cq values, which are proportional to the concentration of objective nucleic acid in your samples. A target sequence is present in large concentrations when the Cq value is lower (usually below 29 cycles). Lower concentrations of the target nucleic acid are indicated by higher Cq values (across 38 cycles).

Tip: Cq (Ct): The PCR cycle number at which your sample's response curve meets the threshold line is known as the Cq value or cycle quantification value. This number



indicates the number of cycles required to identify a genuine signal in your samples. Each sample in a real-time PCR run will have a reaction curve, and hence numerous Cq values. The Cq value for each sample is calculated and plotted by the cycler's software.

Figure 36. – Efficiency of real-time PCR for the identification of *Drosophila melanogaster* species (primers 3.dm. F/R. ROX) (Repetition number 2)

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 53. – Data for *Drosophila melanogaster* Quantification

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	ROX	Pos Ctrl (*D. melanogaster)*	Pos Ctrl	10,96	59.4
A02	ROX	unknown	N/A	2,50	59.4
B01	ROX	unknown	N/A	3.55	59.4
B02	ROX	unknown	N/A	3,3	59.4
C01	ROX	unknown	N/A	3,75	59.4
C02	ROX	unknown	N/A	1,67	59.4
D01	ROX	unknown	N/A	0,32	59.4
D02	ROX	unknown	N/A	0,11	59.4
E01	ROX	unknown	N/A	4,02	59.4
E02	ROX	unknown	N/A	3,74	59.4
F01	ROX	unknown	N/A	1,63	59.4
G01	ROX	unknown	N/A	0,87	59.4
H01	ROX	unknown	N/A	-00,22	59.4
A03	ROX	Neg Ctrl	N/A	0,00	59.4

Only one of the 14 samples with an unclear identity (Table. 54) was successfully amplified and had the proper melt peak temperature to be correctly identified as *D. melanogaster* (positive control) (figure.37). The amplification of any more samples was negative.

Table 54. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila melanogaster* by real-time PCR (primers 3.dm. F/R)

NO	Name of Species	Country: (Data from laboratory of Russian quarantine)	Result of real-time PCR
1	*Drosophila melanogaster*	Turkey	+
2	*Drosophila suzukii*	Turkey	
3	*Drosophila melanogaster*	Russia	
4	*Drosophila simulans*	Canada	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	Sarcophagi similis	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*Drosophila funebris*	unknown	
14	*K*$^{-}$	Water	-

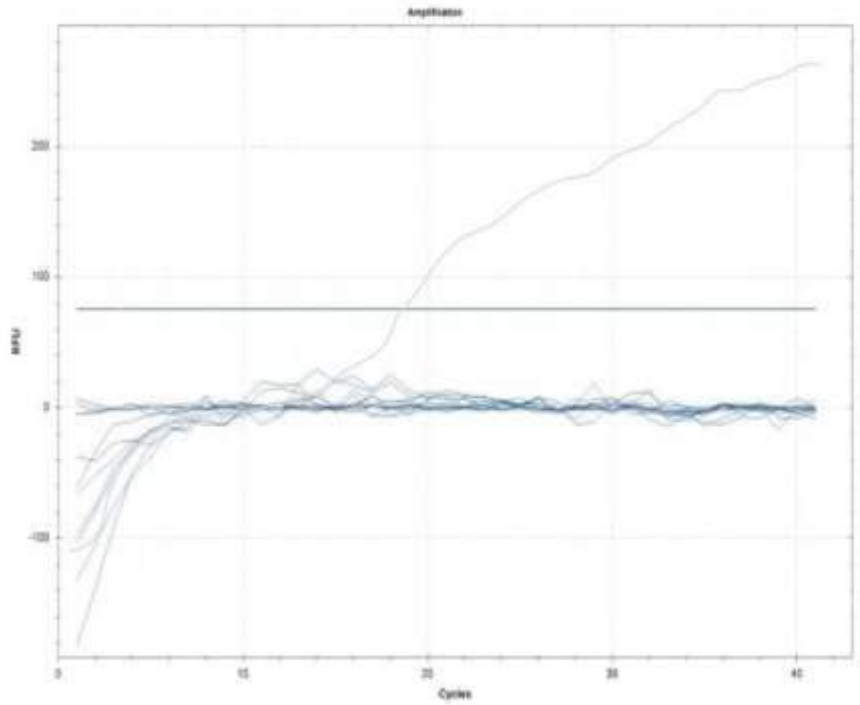


Figure 37. – Efficiency of real-time PCR for the identification of *Drosophila melanogaster* (primers 3.dm. F/R. ROX) (Repetition number 3)

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 55. – Data for *Drosophila melanogaster* Quantification [22]

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	ROX	Pos Ctrl (*D. melanogaster)*	Pos Ctrl	10,96	59.4
A02	ROX	unknown	N/A	39,16	59.4
B01	ROX	unknown	N/A	1.55	59.4
B02	ROX	unknown	N/A	1,12	59.4
C01	ROX	unknown	N/A	3,35	59.4

Well	Fluor	Target	Content	Cq Mean	Set Point
C02	ROX	unknown	N/A	1,67	59.4
D01	ROX	unknown	N/A	5,32	59.4
D02	ROX	unknown	N/A	0,11	59.4
E01	ROX	unknown	N/A	4,02	59.4
E02	ROX	unknown	N/A	3,74	59.4
F01	ROX	unknown	N/A	1,62	59.4
G01	ROX	unknown	N/A	33,71	59.4
H01	ROX	unknown	N/A	01,95	59.4
A03	ROX	Neg Ctrl	K^-	0,00	59.4

Table 56. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila melanogaster* by Real-time PCR (primers 3.dm. F/R) [22]

NO	**Name of Species**	**Country: (Data from laboratory of Russian quarantine)**	**Result of real-time PCR**
1	*Drosophila melanogaster*	Turkey	+
2	*Drosophila suzukii*	Turkey	
3	*Drosophila funebris*	Russia	
4	*Drosophila simulans*	Canada	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	Sarcophagi similis	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*Drosophila funebris*	unknown	
14	*K^-*	Water	

Only one of the 14 samples with an unclear identity (Table.56) was successfully amplified and had the proper melt peak temperature to be correctly identified as *D. melanogaster* (positive control) (figure 38). No other samples displayed successful amplification.

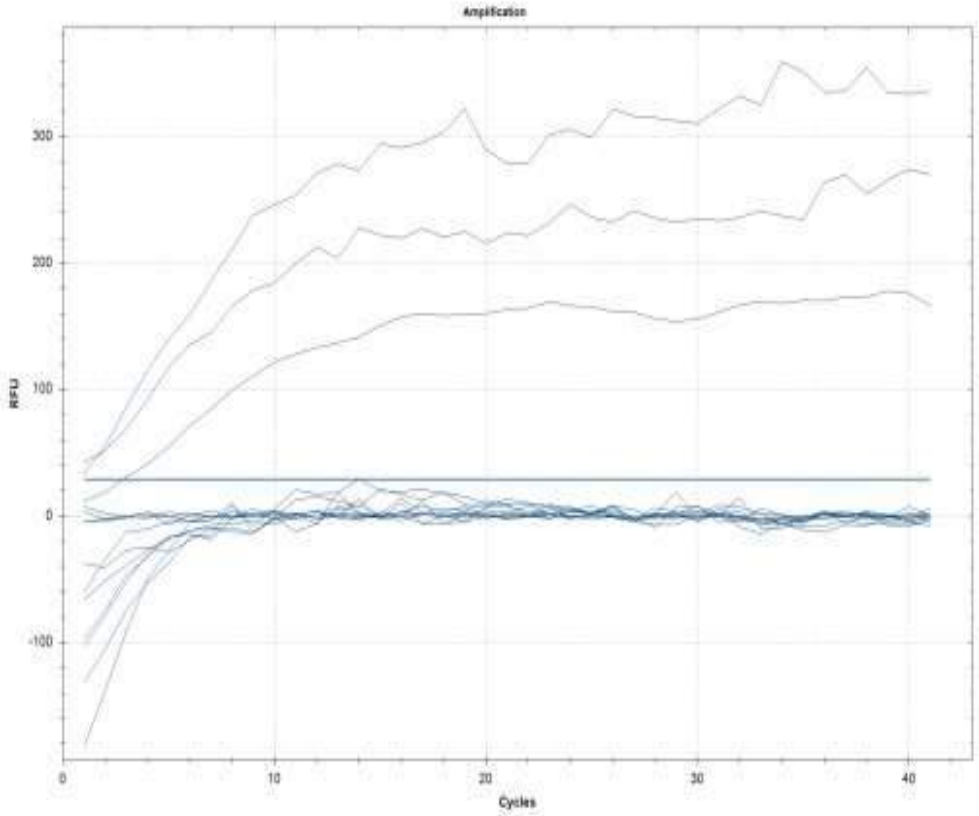


Figure 38. – Efficiency of real-time PCR for the identification of *Drosophila melanogaster* (primers 3.dm. F/R. ROX) (Repetition number 3)

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 57. – Quantification Data for *Drosophila melanogaster*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	ROX	Pos Ctrl (*D. melanogaster)*	Pos Ctrl	8,93	59.4
A02	ROX	unknown	N/A	2,50	59.4
B01	ROX	Pos Ctrl (*D. melanogaster)*	N/A	11.28	59.4
B02	ROX	unknown	N/A	3,3	59.4
C01	ROX	unknown	N/A	3,75	59.4
C02	ROX	Pos Ctrl (*D. melanogaster)*	N/A	19,89	59.4
D01	ROX	unknown	N/A	0,32	59.4
D02	ROX	unknown	N/A	0,11	59.4
E01	ROX	unknown	N/A	4,02	59.4
E02	ROX	unknown	N/A	3,74	59.4
F01	ROX	unknown	N/A	1,63	59.4
G01	ROX	unknown	N/A	0,87	59.4
H01	ROX	unknown	N/A	-00,22	59.4
A03	ROX	Neg Ctrl	N/A	0,39	59.4

Only three of the fourteen samples (Table 56) with unknown identities were correctly identified as *D. melanogaster* (positive control) thanks to effective amplification and the proper melt peak temperature (figure 38). No other samples displayed successful amplification.

Table 58. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila melanogaster* by real-time PCR (primers 3.dm. F/R)

NO	Name of Species	Country: (Data from laboratory of Russian quarantine)	Result of real-time PCR
1	*Drosophila melanogaster*	Turkey	+
2	*Drosophila suzukii*	Turkey	
3	*Drosophila melanogaster*	Russia	+
4	*Drosophila simulans*	Canada	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	unknown	
9	*Myiopardalis pardalina*	Turkey	
10	Sarcophagi similis	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Turkey	
13	*Drosophila melanogaster*	unknown	
14	*K*$^{-}$	Water	+

3.6.5. Result for *Drosophila melanogaster* with all species in Iran (Primer 3.dm.F/R.ROX). (Repetition number 4)

Samples (1 and 3) showed melting peaks in the moderate range of T: 59.4 C and differed from other species. There are no data on other melting peaks.

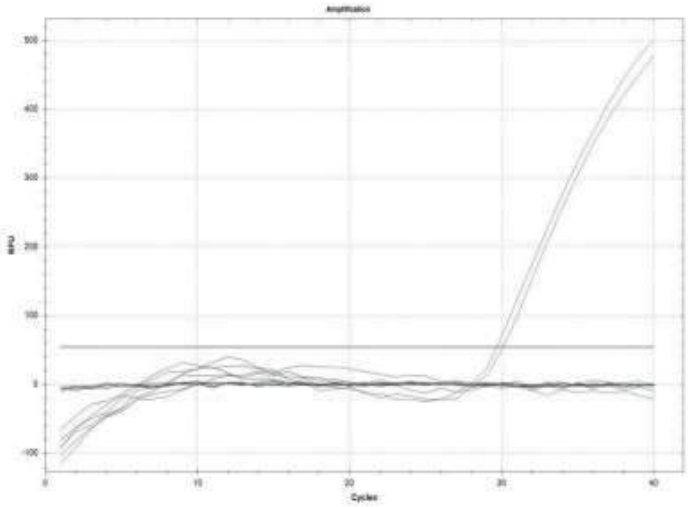


Figure 39. – Efficiency of real-time PCR for the identification of *D. melanogaster* (primers 3.dm.F/R.ROX (Repetition number 4)

Samples (1 and 3) showed melting peaks in the moderate range of T: 59.4 C and differed from other species. There are no data on other melting peaks.

Table 59. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila melanogaster* by real-time PCR with sample in Iran (primers 1. dsuz. F/R PROBEFAM)

№	Name samples	Country (data from VNIIKR)	Real-time PCR results
1	***Drosophila melanogaster***	Iran	+
2	***Drosophila melanogaster***	Iran	+
3	*Drosophila suzukii*	Turkey	
4	*Drosophila funebris*	Iran	
5	*Drosophila melanogaster*	Turkey	
6	*Drosophila simulans*	Russia	
7	*Zaprionus indianus*	Jordan	
8	*Ceratitis capitata*	Turkey	
9	*Megaselia scalaris*	Iran	-
10	*Bactrocera dorsalis*	Iran	
11	*Myiopardalis pardalina*	Turkey	
12	*Zaprionus tuberculatus*	Turkey	
13	*Zaprionus indianus Malloch*	Turkey	
14	K^-	Water	

In (Figure 39) shows two ascending peaks that indicate our positive result with *D. melanogaster* (from Iran and other regions). Probably due to the lower concentration of DNA substances, there are no data on other melting peaks. Other authors [76] also confirm our results.

Table 60. – Quantification Data for *Drosophila melanogaster*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	ROX	Pos Ctrl (*D. melanogaster)*	Pos Ctrl	8,93	59.4
A02	ROX	unknown	N/A	2,50	59.4
B01	ROX	Pos Ctrl (*D. melanogaster)*	N/A	11.28	59.4
B02	ROX	unknown	N/A	3,3	59.4
C01	ROX	unknown	N/A	3,75	59.4
C02	ROX	Pos Ctrl (*D. melanogaster)*	N/A	19,89	59.4
D01	ROX	unknown	N/A	0,32	59.4
D02	ROX	unknown	N/A	0,11	59.4
E01	ROX	unknown	N/A	4,02	59.4
E02	ROX	unknown	N/A	3,74	59.4
F01	ROX	unknown	N/A	1,63	59.4

3.6.6. Comparison between Real Time PCR and classical PCR

In Real Time PCR, the amplified DNA is labelled – usually with fluorescent dyes enabling amplification of DNA visually by reading the fluorescence of each well. Fluorescence is measure after each cycling enabling testing to be read/analyzed in real-time.

Conventional PCR can only be visualized by running amplified DNA samples thru electrophoresis [23]. This is done at the conclusion of cycling run. Conventional PCR can also be referred to end-point PCR [3, 10, 22].

The amount of fluorescence emitted during amplification in Real Time PCR is exactly proportional to the volume of DNA amplified. There is no electrophoresis step needed to visualize DNA for Real Time PCR, but is required for conventional PCR. The steps for annealing and extension during replication of DNA is usually combined during Real Time PCR testing

3.7. Design (6.ds.F/R) for PCR and real-time PCR, identification *Drosophila simulans*

To design two specific primers to identify for *Drosophila Simulans*. I used basic primers 1248 F (5′- TGGAACTGTTTACCCACCTCGT -3′) as a forward and 1248 R (5′- TGTATTCGGTCTAATGTAATACT -3′) as a reverse, [15]. For DNA sequencing and design one, pair of Primers (6.ds.F/R) for Classic PCR. (Table.58.) In addition, (5.ds), (Table 60) For Real Time.

In (Figure 40), you can see that the sequence of *Drosophila simulans* differs from the rest of the species in some codes in several areas [10].

Figure 40.- Different COI gene fragment sequences and primer designs for *Drosophila simulans*

Table 61. - Different genetic codes from *Drosophila simulans* identified

NO	Subspecies' name	The letter's number																				
		92	101	108	111	121	587	599	629	752	754	755	756	760	787	794	803	809	812	826	827	828
2	*Drosophila simulans*	C	G	A	T	G	A	C	T	T	C	T	G	T	G	C	T	C	T	T	T	T
1	*Drosophila melanogaster*	T	T	C	G	T	T	T	T	C	T	C	T	T	C	T	T	C	T	A	C	C
3	*Drosophila suzukii*	T	T	C	G	C	T	T	C	C	T	C	T	C	C	C	C	T	A	A	C	C

Table 62. – Primer (6.ds.F/R) for PCR, sequences and resulting amplicon sizes for *Drosophila Simulans*

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
6.Ds.F	CCCAAGGATCGTGCTCTGTT	Plus	20	92	111	60.04	55.00
6. Ds.R	TCCACACAATCGTCTCGCAA	Minus	20	356	337	59.97	50.00
Product length	265						

The PCR products for the CO1 gene were subjected to electrophoresis in 1% agarose gel. A specific product size of 300 bp was observed. This is shown in (Figure 41). These primers are only able to identify *Drosophila simulans* species.

Figure 41. – Test of sensitivity of dsn.1 for and dsn.1 Rev with different DNA amount of *D. simulans*, K^-: negative control (dH_2O); K^+: *Drosophila simulans*. M: DNA, marker (100–1000) bp.

Figure 42. – Gel showing selectivity of designed primer pairs of 6.ds.F/R. 1, 2, 3: *Drosophila simulans.* 4-11: *Drosophila suzukii.* 12-19: *Drosophila melanogaster*, K^-: negative control (dH_2O); K^+: *Drosophila simulans*. M: DNA, marker (100–1000) bp.

3.7.1. Optimization with (5.ds.F/R.FAM) for *Drosophila simulans* with real-time PCR

Choose the best temperature for the primer (5.ds.F/R. FAM). Real Time PCR should test samples at different temperatures.

Table 63. – Primer (5.ds.F/R) for Real-time PCR, sequences and resulting amplicon sizes for *Drosophila Simulans* [22].

Drosophila Simulans	Sequence (5'->3')	Length	Tm	GC%
5. F.u3fn.ds	GCAACTTCTTCATTAACCTCG	21	55.26	42.86
5. Probe. u3fn.ds	CTGGGGTGTGTGGGCTGATGT	21	61.69	61.90
5. R. u3fn.ds. FAM	GATAGTAGCACAGACCACCG	20	57.51	55.00

Protocol

1: 95, 0°C for 10:00

2: 95,0°C for 0:40

3: Gradient 57,0°C / 61,0°C for 0:40

4: GOTO 2, 39 more times

3.7.2. Optimization of real-time PCR analysis for *D. simulans* (5.ds.F/R)

In these results, we have 12 samples for identification with primer (5.ds.F/R. FAM) and gradient of temperature T: 57 ^{0}C up to T: 61^{0}C, six of *D. simulans* as passive control, 6 of samples as negative control, of which their names are listed in Table.63 (Figure. 43), as result best temperature was T: 58, 6°C.

In (figure. 43), five upward peaks were shown that indicated our positive control with *D.simulans*. There were no further reported melt peaks. In the diagram of the peaks of the two samples of *D.simulans* are different, because both types of DNA extracted from simulans differed in quality, region, and amount. [22].

Figure 43. – Efficiency of real-time PCR for the identification of *D. simulans* (primers5.ds.F/R.FAM**)**

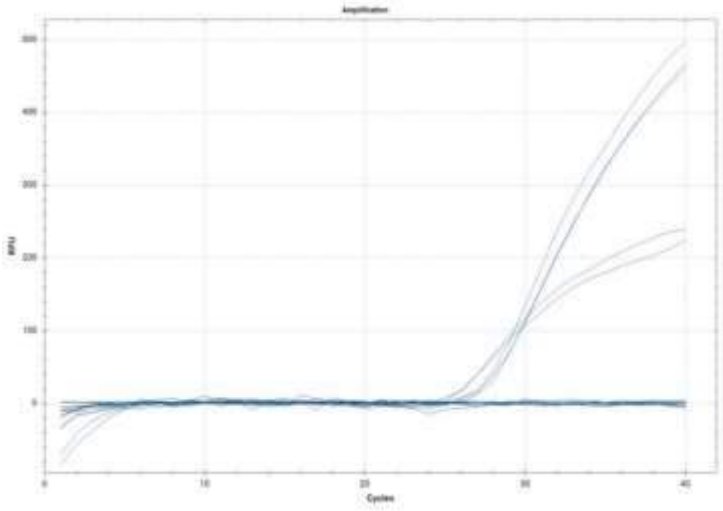


Table 64. – Quantification Data for *D. simulans*

Well	Fluor	Target	Content	Sample	Cq
A01	FAM	Pos Ctrl	*D.simulans*	28,32	28,32
A02	FAM	Neg Ctrl	37,59	37,59	0,000
B01	FAM	Pos Ctrl	*D.simulans*	12,72	12,72
B02	FAM	Neg Ctrl	25,60	25,60	0,000
D01	FAM	Pos Ctrl	*D.simulans*	26,01	26,01
D02	FAM	Neg Ctrl	35,81	35,81	0,000
E01	FAM	Pos Ctrl	*D.simulans*	25,46	25,46
E02	FAM	Neg Ctrl	22,27	22,27	0,000
G01	FAM	Pos Ctrl	*D.simulans*	24,18	24,18
G02	FAM	Neg Ctrl	29,42	29,42	0,000

Of the 10 samples of identity (Table.64), five samples were *D.simulans* (positive control) and the rest were negative control. We have Cq ≤29 in all *D. simulans* samples.

Figure 44. – The process of optimization temperature for *D. simulans*

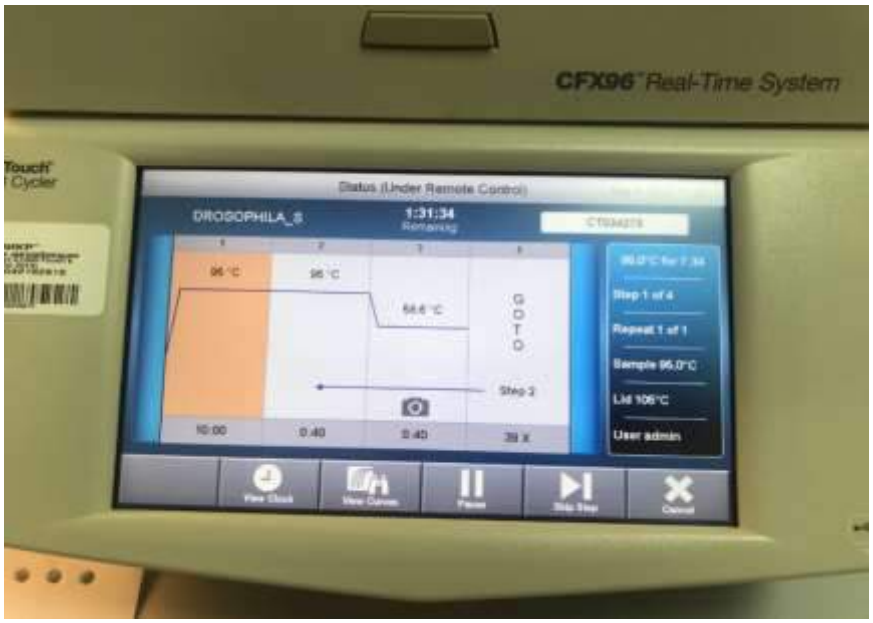


Figure 45. – Temperature for Real-Time PCR of *D. simulans*

3.7.3. Real time result for *D.simulans* with (5.ds.F/R.FAM) for all fruit flies (Repetition2)

To determine the specificity of the designed primer; also, make sure that the primer can detect the species *Drosophila simulans*. We compare the *D.simulans* specimen with other species of the same family and other fruit flies.

Protocol

1: 95, 0°C for 10:00

2: 95, 0°C for 0:40

3: 58, 6°C for 0:40

4: GOTO 2, 39 more times

Table 65. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila simulans* by real-time PCR (primers 5.ds.F/R. FAM)

NO	Name of the species	Country: (Data from Plant Quarantine Laboratory)	Result
1	***Drosophila simulans***	Turkey	+
2	*Drosophila suzukii*	Egypt	
3	*Drosophila melanogaster*	Russia	
4	*Zaprionus indianus*	Canada	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Turkey	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	Mexico	
9	Sarcophagi similis	Turkey	
10	*Zaprionus tuberculatus*	China	
11	*Drosophila funebris*	unknown	
12	***Drosophila simulans*** (ds/2***)***	Russia	+
13	***Drosophila simulans***	Russia	+
14	K^-	Water	-

In (Table 65) Of 13 samples of unknown identity, only 3 of them (1, 12 and 13) were positively identified as *Drosophila simulans* (positive control) due to successful amplification and correct melting peak temperature. Sample number. 1 was the best of them. The amplification of any more samples was negative. [35].

3.7.4. Specificity of high resolution melt peaks

We have 13 samples in these results for identification using primers (5.ds) and T: 58.6,3 *D. simulans* as passive controls, and 12 unknown cases, each of whose names are reported in Table 63.

Additionally, it involves negative control (sample 13). One was correctly recognized as *D. simulans*, and sample (1) displayed melt peaks that could be separated from those of other species and fell within the permissible range of 58.06 °C. There were no other melt peaks noted (Figure 46).

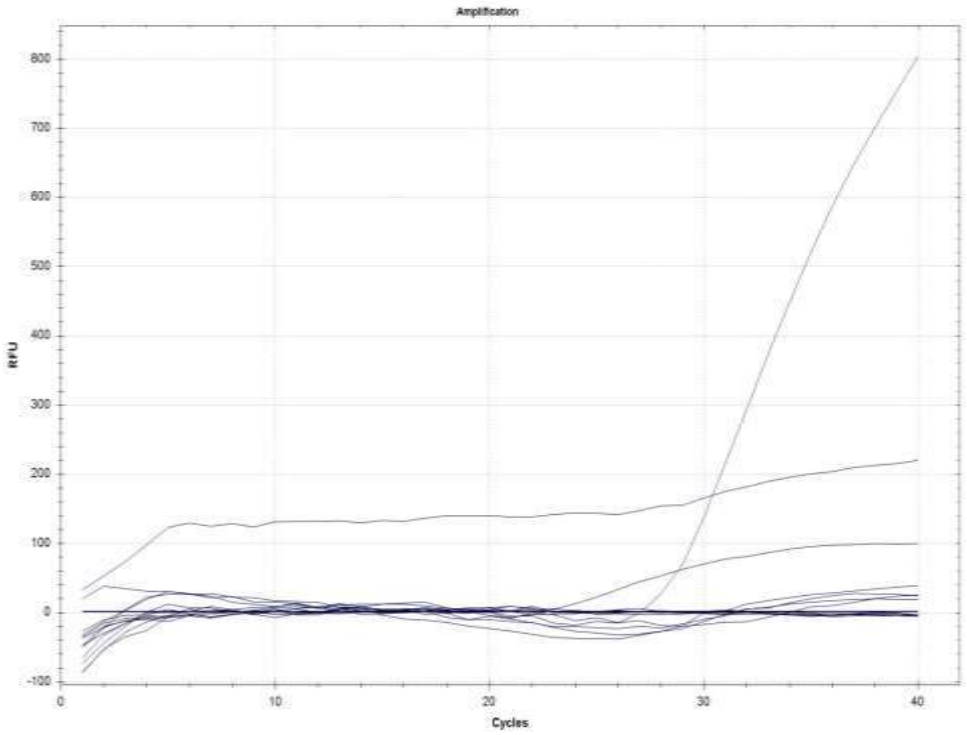


Figure 46. – Efficiency of real-time PCR for the identification of *Drosophila simulans* (primers 5.ds.F/R. FAM)

Table 66. – Quantification Data for *Drosophila simulans*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	8,93	59.4
A02	HEX	unknown	N/A	2,50	59.4
B01	HEX	unknown	N/A	39,44	59.4
B02	HEX	unknown	N/A	3,35	59.4
C01	HEX	unknown	N/A	3,75	59.4
C02	HEX	unknown	N/A	19,89	59.4
D01	HEX	unknown	N/A	0,32	59.4
D02	HEX	unknown	N/A	0,11	59.4
E01	HEX	unknown	N/A	4,02	59.4
E02	HEX	unknown	N/A	3,74	59.4
F01	HEX	unknown	N/A	1,63	59.4
G01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	2,87	59.4
H01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	11.28	59.4
A03	HEX	Neg Ctrl	N/A	0,39	59.4

Table 67. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila simulans* by real-time PCR (primers 5.ds.F/R. FAM)

NO	Name of the species	Country: (Data from Plant Quarantine Laboratory)	Result
1	***Drosophila simulans***	Turkey	+
2	*Drosophila suzukii*	Egypt	
3	*Drosophila melanogaster*	Russia	
4	*Zaprionus indianus*	Canada	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Turkey	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	Mexico	
9	Sarcophagi similis	Turkey	
10	*Zaprionus tuberculatus*	China	
11	*Drosophila funebris*	unknown	
12	***Drosophila simulans*** (ds/2***)***	Russia	+
13	***Drosophila simulans***	Russia	+
14	K^-	Water	-

In (figure 47), one upward peaks were shown that indicated our positive control with *D. simulans* (from Turkey). The second peak, which is very low, because it was selected from two *D.simulans*. Probably due to the lower concentration of DNA, There were no other melt peaks noted. Other authors [19, 76] also confirm our conclusion.

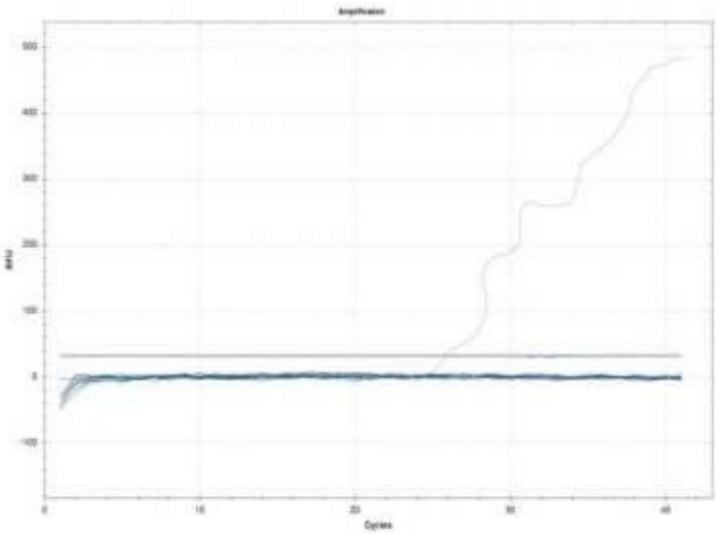


Figure 47. – Efficiency of the real-time PCR method in the identification of the *Drosophila simulans* species (primers 5.ds.F/R. FAM) (Repetition number 3)

Only one peak has shifted higher among the several peaks created in the graph, indicating the identification of the target sample.

Table 68. – Quantification Data for *Drosophila simulans.*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	HEX	Pos Ctrl (*D. simulans*)	Pos Ctrl	26,67	59.4
A02	HEX	unknown	N/A	3,14	59.4
B01	HEX	unknown	N/A	11.47	59.4
B02	HEX	unknown	N/A	1,65	59.4
C01	HEX	unknown	N/A	2,14	59.4
C02	HEX	unknown	N/A	1,69	59.4
D01	HEX	unknown	N/A	0,32	59.4
D02	HEX	unknown	N/A	0,11	59.4
E01	HEX	unknown	N/A	4,01	59.4
E02	HEX	unknown	N/A	3,22	59.4
F01	HEX	unknown	N/A	1,24	59.4
G01	HEX	unknown	N/A	0,87	59.4
H01	HEX	unknown	N/A	1,65	59.4
A03	HEX	Neg Ctrl	N/A	0,67	59.4

Only 1 of the 14 samples with undetermined identities (Table 68) was successfully amplified and had the proper melt peak temperature to be correctly identified as *D. simulans* (positive control) (figure 47). The amplification of any more samples was negative.

Table 69. – List of *Drosophila* species used in this paper, which were used to determine *Drosophila simulans* by real-time PCR (primers 5.ds.F/R. FAM)

NO	Subspecies' name	Country (Data from laboratory of Russian quarantine)	Result
1	*Drosophila simulans*	Turkey	+
2	*Drosophila suzukii*	Egypt	
3	*Drosophila melanogaster*	Russia	
4	*Drosophila simulans*	Canada	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	Mexico	
9	*Myiopardalis pardalina*	Turkey	
10	*Sarcophagi similis*	China	
11	*Zaprionus tuberculatus*	unknown	
12	*Zaprionus indianus Malloch*	Russia	
13	*Drosophila funebris*	Russia	
14	*K*-	Water	-

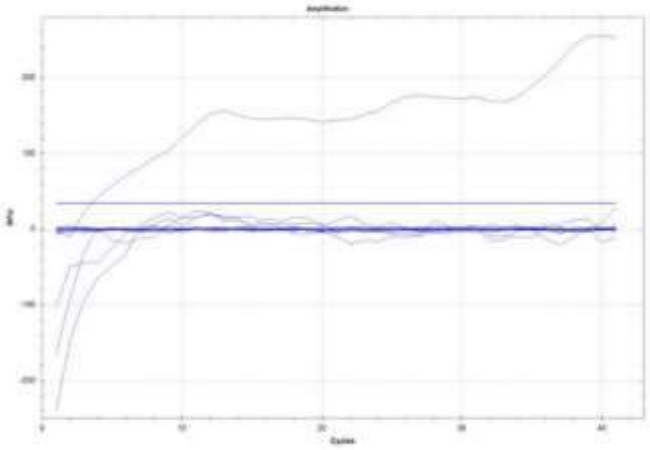


Figure 48. – Efficiency of the real-time PCR method in the identification of the all species of fruit flies (primers 5.ds.F/R. FAM) (Repetition number 4)

Table 70. – Quantification Data for *Drosophila simulans*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	12,09	59.4
A02	HEX	unknown	N/A	3,27	59.4
B01	HEX	unknown	N/A	1.27	59.4
B02	HEX	unknown	N/A	39,64	59.4
C01	HEX	unknown	N/A	2,98	59.4
C02	HEX	unknown	N/A	00,69	59.4
D01	HEX	unknown	N/A	39,32	59.4
D02	HEX	unknown	N/A	38,11	59.4
E01	HEX	unknown	N/A	00,00	59.4
E02	HEX	unknown	N/A	1,22	59.4
F01	HEX	unknown	N/A	-06,24	59.4
G01	HEX	unknown	N/A	1,87	59.4
H01	HEX	unknown	N/A	1,65	59.4
A03	HEX	Neg Ctrl	N/A	0,67	59.4

Only one of the 14 samples with unknown identities (Table 69) was successfully amplified and had the proper melt peak temperature to be correctly identified as *D. simulans* (positive control) (figure 49). No other samples displayed successful amplification.

Table 71. – The list of *Drosophila* species used in this work for the detection of *Drosophila simulans* by real-time PCR (primers 5.ds.F/R. FAM)

NO	Subspecies' name	Country: (Data from laboratory of Russian quarantine)	Result
1	*Sarcophagi similis*	unknown	
2	*Drosophila suzukii*	Egypt	
3	*Drosophila melanogaster*	Russia	
4	*Drosophila simulans*	Canada	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	+
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	Mexico	
9	*Myiopardalis pardalina*	Turkey	-
10	*Drosophila simulans*	Turkey	
11	*Zaprionus tuberculatus*	China	
12	*Zaprionus indianus Malloch*	Russia	
13	*Drosophila funebris*	Russia	
14	*K*$^{-}$	Water	

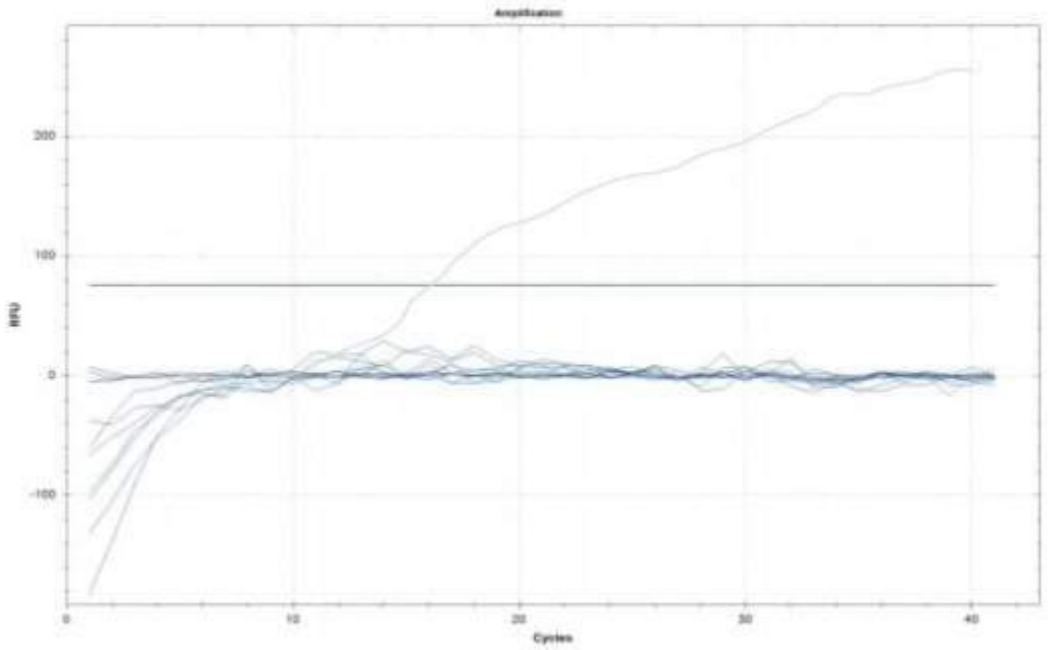


Figure 49. – Efficiency of the real-time PCR method in the identification of the *Drosophila simulans* species (primers 5.ds.F/R. FAM) (Repetition number 5)

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 72. – Quantification Data for *Drosophila simulans*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	12,09	59.4
A02	HEX	unknown	N/A	3,27	59.4
B01	HEX	unknown	N/A	1.27	59.4
B02	HEX	unknown	N/A	39,64	59.4
C01	HEX	unknown	N/A	2,98	59.4
C02	HEX	unknown	N/A	00,69	59.4
D01	HEX	unknown	N/A	39,32	59.4

Well	Fluor	Target	Content	Cq Mean	Set Point
D02	HEX	unknown	N/A	38,11	59.4
E01	HEX	unknown	N/A	00,00	59.4
E02	HEX	unknown	N/A	1,22	59.4
F01	HEX	unknown	N/A	-06,24	59.4
G01	HEX	unknown	N/A	1,87	59.4
H01	HEX	unknown	N/A	1,65	59.4
A03	HEX	Neg Ctrl	N/A	0,67	59.4

Only one of the 14 samples with an unclear identity (Table 72) was successfully amplified and had the proper melt peak temperature to be correctly identified as *D. simulans* (positive control) (figure 50). No other samples displayed successful amplification.

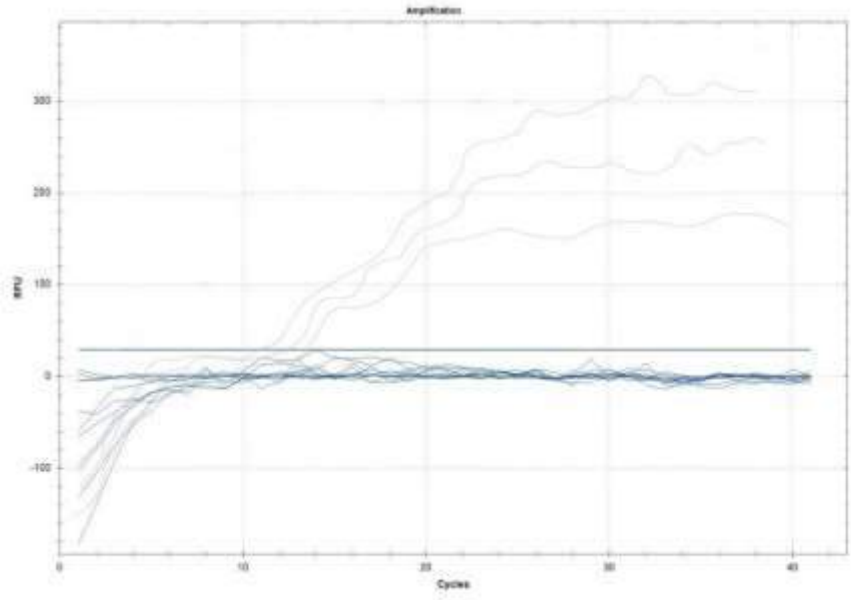


Figure 50. – Efficiency of the real-time PCR method in the identification of the *Drosophila simulans* species (primers 5.ds.F/R. FAM) (Repetition number 6)

Table 73. – The list of *Drosophila* species used in this work for the detection of *Drosophila simulans* by real-time PCR (primers 5.ds.F/R. FAM)

NO	Subspecies' name	Country: (Data from laboratory of Russian quarantine)	Result
1	*Sarcophagi similis*	unknown	
2	*Drosophila suzukii*	Egypt	
3	*Drosophila melanogaster*	Russia	
4	*Drosophila simulans*	Canada	
5	*Zaprionus indianus*	Turkey	
6	*Ceratitis capitata*	Turkey	+
7	*Megaselia scalaris*	unknown	
8	*Bactrocera dorsalis*	Mexico	
9	*Myiopardalis pardalina*	Turkey	-
10	*Drosophila simulans*	Turkey	
11	*Zaprionus tuberculatus*	China	
12	*Zaprionus indianus Malloch*	Russia	
13	*Drosophila funebris*	Russia	
14	*K*$^{-}$	Water	

Of the various peaks formed in the graph, only one peak has moved upwards, indicating the identification of the target sample.

Table 74. – Quantification Data for *Drosophila simulans*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	HEX	unknown	N/A	1,09	59.4
A02	HEX	unknown	N/A	2,51	59.4
B01	HEX	unknown	N/A	39,22	59.4
B02	HEX	unknown	N/A	3,35	59.4
C01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	14,54	59.4
C02	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	12.74	59.4
D01	HEX	Pos Ctrl (*D. simulans)*	Pos Ctrl	13,24	59.4
D02	HEX	unknown	N/A	0,12	59.4
E01	HEX	unknown	N/A	2,47	59.4
E02	HEX	unknown	N/A	1,65	59.4
F01	HEX	unknown	N/A	1,69	59.4
H01	HEX	unknown	N/A	1,11	59.4
A03	HEX	Neg Ctrl	N/A	0,63	59.4

Table 75. – The list of *Drosophila* species used in this work for the detection of *Drosophila simulans* by Real-time PCR (primer 5.ds.F/R. FAM)

NO	Subspecies' name	Country: (Data from laboratory of Russian quarantine)	Result
1	*Drosophila simulans*	Turkey	+
2	*Drosophila suzukii*	Egypt	
3	*Drosophila melanogaster*	Russia	
4	*Zaprionus indianus*	Canada	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Turkey	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	Mexico	
9	Sarcophagi similis	Turkey	
10	*Zaprionus tuberculatus*	China	
11	*Drosophila funebris*	unknown	
12	*Drosophila simulans (ds/3)*	Russia	+
13	*Drosophila simulans*	Russia	+
14	*K*$^{-}$	Water	

In (figure 51), 3 upward peaks were shown that indicated our positive control with *D. simulans* (from Turkey). The second peak, which is very low, because it was selected from 3, *D.simulans*. Probably due to the lower concentration of DNA, there were no other melt peaks noted. Other authors [13, 19, 77] also confirm our conclusion

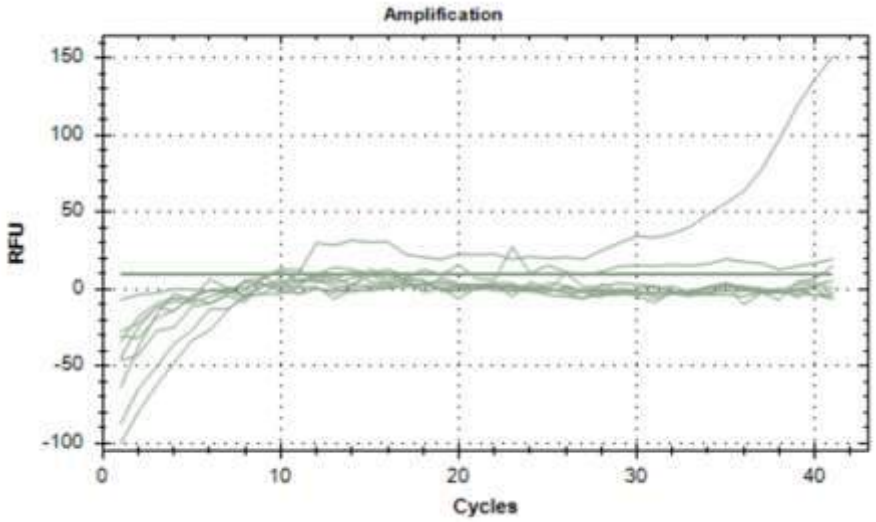


Figure 51. –Real time result for *Drosophila simulans* with all fruit fly species in Iran (primer 5.ds.F /R. FAM) (Repetition 7)

Of the various peaks formed on the graph, only one peak moved up, indicating identification of the target sample.

Table 76. – Quantitative data for *Drosophila simulans*

Well	Fluor	Target	Content	Cq Mean	Set Point
A01	HEX	Pos Ctrl (*D. simulans*)	Pos Ctrl (*D. simulans*)	12.04	59.4
A02	HEX	unknown	N/A	1.39	59.4
B01	HEX	unknown	N/A	28.28	59.4
B02	HEX	unknown	N/A	4.21	59.4
D02	HEX	unknown	N/A	0.14	59.4
E01	HEX	unknown	N/A	2.47	59.4
E02	HEX	unknown	N/A	2.63	59.4
F01	HEX	unknown	N/A	1.65	59.4
G01	HEX	unknown	N/A	2.24	59.4
H01	HEX	unknown	N/A	1.12	59.4
A03	HEX	Negative control	N/A	0.51	59.4

Table 77. - The list of *Drosophila* species used in this work for the detection of *Drosophila simulans* by real-time PCR (primer 5.ds.F/R. FAM).

NO	Subspecies' name	Country: (Data from laboratory of Russian quarantine)	Result
1	*Drosophila simulans*	Iran	+
2	*Drosophila suzukii*	Turkey	
3	*Drosophila melanogaster*	Russia	
4	*Zaprionus indianus*	Jordan	
5	*Ceratitis capitata*	Turkey	
6	*Megaselia scalaris*	Iran	
7	*Bactrocera dorsalis*	unknown	
8	*Myiopardalis pardalina*	Iran	
9	Sarcophagi similis	Turkey	
10	*Zaprionus tuberculatus*	China	
11	*Drosophila funebris*	Turkey	
12	K^-	Water	

In (Figure 51) a single ascending peak was shown which indicates our positive result for *D. simulans* (from Iran). Probably due to the lower concentration of DNA material, no other melting peaks are reported. Other authors [3, 77] also confirm our result.

3.8. Analysis of evolution using the Maximum Likelihood approach

The Neighbor-Joining approach was used to infer the evolutionary history [78]. The ideal tree is displayed, with a branch length sum of 5.00128004. In the bootstrap test (1000 repetitions), the percentage of duplicate trees in which the connected taxa clustered together are displayed next to the branches [22, 35, 78].

With branch lengths in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is rendered to scale. The Maximum Composite Likelihood technique [78] was used to calculate the evolutionary distances.

Furthermore, are expressed in proportion to the number of base substitutions per site. This study included 57 nucleotide sequences. Codon positions 1st+2nd+3rd+Noncoding were included. For each sequence pair, all unclear places were eliminated (pairwise deletion option). The final dataset contained 460 locations altogether. MEGA X was used to undertake evolutionary analyses [78-81].

We chose the COI gene as the target because it is well defined for a wide range of *Drosophila suzukii* species, including sequences available for specific species within the *D. suzukii* species subgroup (Table 78).

Drosophilid species one among the most popular widely studied organisms in biological science. However, the phylogenetic relationship of the subfamily *Drosophilidae*, which should serve as the basis for various studies using *Drosophilidae*, has not been established. To address this issue, we performed phylogenetic analyzes using nuclear DNA sequences for several species that had not been analyzed in previous studies.

In general, the topology of our tree of 21 species exactly matches the topology presented by van der (Figure 52) [80, 82].

Table 78. – Species and Accession number list used for the current study [3, 10, 22, 77]

NO	Species and Accession number	Country	Data from laboratory of Russian quarantine	Data from GenBank
1	*1U.D. melanogaster*	Turkey	✓	
2	*6U.1248F.D. melanogaster*	Egypt	✓	
3	*1.u3f.D. melanogaster*	Turkey	✓	
4	*2. u3f.D. melanogaster*	Egypt	✓	
5	*3. u3f.D. melanogaster*	Turkey	✓	
6	*4. u3f.D. melanogaster*	Turkey	✓	
7	*5. u3f.D. melanogaster*	Turkey	✓	
8	*6. u3f.D. melanogaster*	Mexico	✓	
9	*MG605127. D. melanogaster*	Switzerland		✓
10	*LN867079. D. melanogaster*	Italy		✓
11	*MG605129. D. melanogaster*	Switzerland		✓
12	*FJ636124. D. melanogaster*	Switzerland		✓
13	*1.u3f.ds.D. Simulans*	Turkey		
14	*2.u3f.ds.D. Simulans*	Iran	✓	
15	*3.u3f.ds.D. Simulans*	Turkey	✓	
16	*4.u3f.ds.D. Simulans*	Mexico	✓	
17	*MG605144.u3f.ds.D. Simulans*	Mexico	✓	
18	*KJ767247.u3f.ds.D. Simulans*	USA		✓
19	*KJ671606.u3f.ds.D. Simulans*	NewZealand		✓
20	*1.u3f.dsuz. D. Suzukii*	Canada		✓
21	*2.u3f.dsuz. D. Suzukii*	Turkey		
22	*1.2010-19.u3f.ds.D. Suzukii*	Mexico	✓	
23	*AB824772D.Suzukii*	Japan	✓	
24	*KJ671597D.Suzukii*	Italy	✓	✓
25	*AB824766D.Suzukii*	Japan		✓
26	*AB824752D.Suzukii*	Japan		✓
27	*KF312626D.Suzukii*	China		✓
28	*MG816102D.Suzukii*	unknown		✓
29	*MG816086D.Suzukii*	unknown		✓

30	*KJ671584D.Suzukii.*	USA		✓
31	*6.u3f.zt. ZaprionusTuberculatus*	Mexico		✓
32	*002088143.5 Drosophilayakuba.*	*China*		
33	017127416.1. *Drosophilarhopaloa*.	China		✓
34	002088143.2 *Drosophila yakuba.*	Japan	✓	✓
35	017127416.1. *Drosophila rhopaloa*	Japan		✓
36	017194923.1. *Drosophila ficusphila.*	USA		✓
37	017174541.4. *Drosophila kikkawai.*	China		✓
38	001969985.3. *Drosophila erecta.*	USA		✓
39	017214328.1. *Drosophila eugracilis.*	Brazil		✓
40	022379058.1. *Drosophila obscura.*	Japan		✓
41	002018937.2. *Drosophila persimilis*.	USA		✓
42	017194923.1. *Drosophila ficusphila.*	Brazil		✓
43	017127416.1. *Drosophila rhopaloa.*	China		✓
44	017194923.2. *Drosophila ficusphila*.	USA		✓
45	017174541.2. *Drosophila kikkawai*.	Japan		✓
46	001969985.4. *Drosophila erecta.*	China		✓
47	017214328.2. *Drosophila eugracilis*.	Brazil		✓
48	022379058.2. *Drosophila obscura*.	Brazil		✓
49	002018937.1. *Drosophila persimilis.*	Brazil		✓
50	017194923.2. *Drosophila ficusphila*.	USA		✓
51	017127416.2. *Drosophila rhopaloa*.	USA		✓
52	002018937.3. *Drosophila persimilis*.	China		✓
53	017174541.4. *Drosophila kikkawai*.	Japan		✓
54	020953757.3. *Drosophila serrata*.	Japan		✓

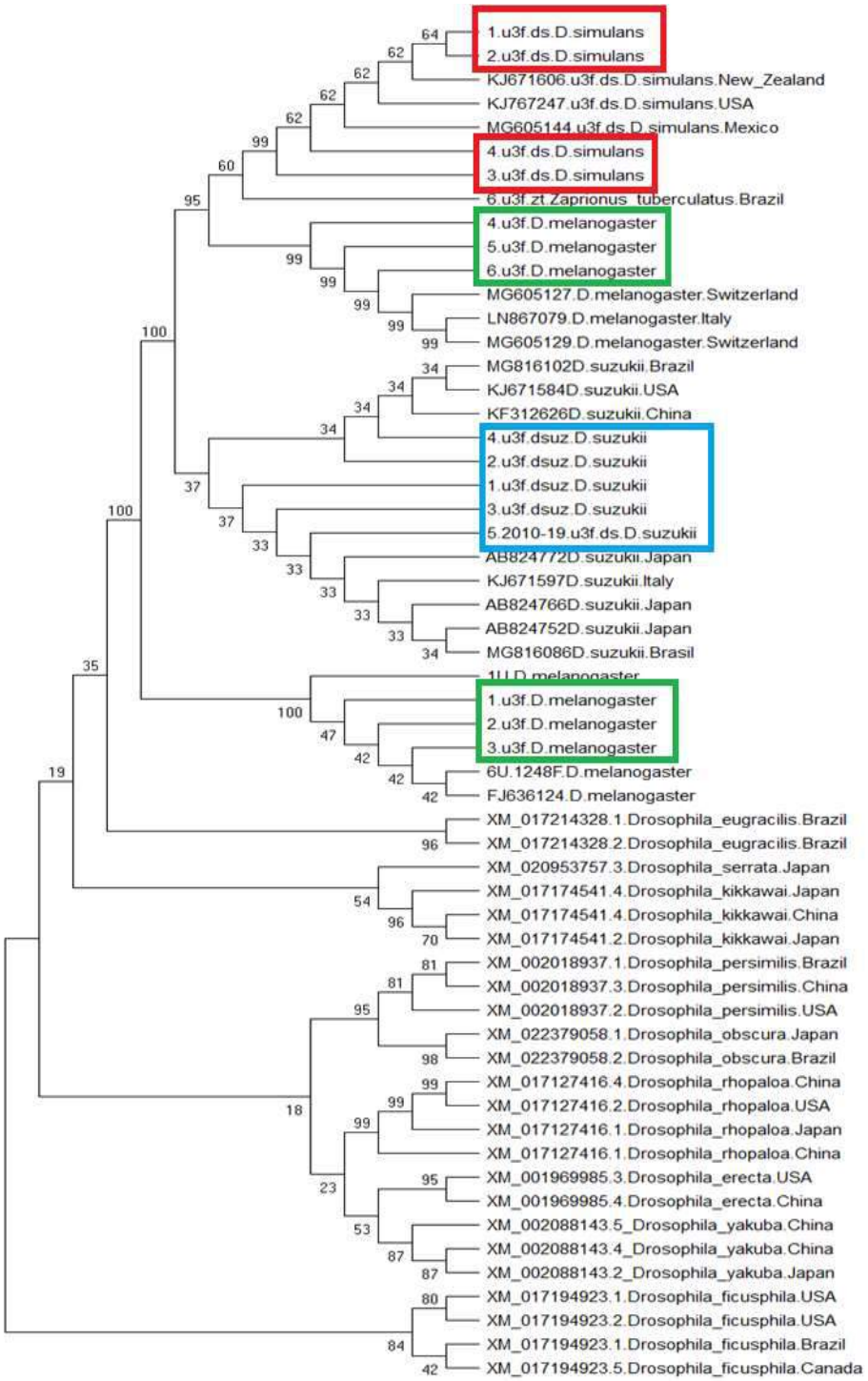


Figure 52. – Molecular phylogeny of the genus *Drosophila* and closely related genera (Diptera Drosophilidae) (Red colors *D. simulans*, Green colors *D. melanogaster*, Blue colors *D. suzuki*

3.9. The Importance of Primers

Accordingly, validated primers are critical for determining the specificity, sensitivity, and robustness of a PCR reaction. While it is usually possible to get a result using a PCR assay, it is not the same as getting the correct result, whether it is the presence/absence of a call to detect a pathogen or a mutation using an endpoint assay or precise RNA quantification. Copy numbers in real time. In fact, PCR is not as reliable as many people think, and the science behind DNA folding and match-and-mismatch hybridization needs to be taken into account. However, it is not always obvious why some primer combinations work or really do not work [23, 32, 83].

The critical parameter for the performance of a primer is its annealing temperature (Ta) and not it's melting temperature (Tm), since Ta determines the temperature at which the maximum amount of primer binds to its target. The optimal primer Ta must be established experimentally because primer design programs typically calculate Tms and, in any case, many use incorrect predictive parameters (Table 78).

Moreover, since the optimal annealing temperatures differ depending on the buffers, the results obtained with one master mix cannot necessarily be extrapolated to the second one. Even at optimal Ta, nonspecific amplification can occur, especially with "corrector" enzymes, caused not only by primer dimers, but also by the physical proximity of primer pairs at mismatched sites [3, 23, 36, 84].

Moreover, using a BLAST search alone does not guarantee primer specificity, because while the BLAST algorithm returns fast results, it may miss thermodynamically important hybridization events because it misjudged the gaps that duplex bulges generate. In addition, the effect of mismatches on duplex stability depends on the context of the sequence and is incorrectly caused by a sequence-independent approximation.

Table 79. – Comparison between conventional PCR and Real time

Conventional PCR	Real time PCR
Conventional PCR is more time consuming as it uses gel electrophoresis to analyze the amplified PCR products	In contrast, real-time PCR is less time consuming as it can detect amplifications during the early phases of the reaction
Conventional PCR collects data at End-point of the reaction	Real-time PCR collects data at the exponential growth phase of PCR
The end point results of the conventional PCR may not be very precise	the results of the real-time PCR are very precise
Real-time PCR is more labor-intensive and highly advanced than conventional PCR	Real-time PCR has a higher sensitivity level than traditional PCR
Conventional PCR has very poor resolution	Real-time PCR can detect very little changes due to the high resolution
End point detection of conventional PCR has short dynamic range	real-time PCR detection has wide dynamic range
Unlike real-time PCR, conventional PCR cannot discriminate between dead and live bacteria	Unlike conventional PCR, automated detection techniques are found in real-time PCR
conventional PCR uses ethidium bromide and UV light to visualize bands in the agarose gel medium	Real-time PCR uses fluorescent dye system to detect the products
Conventional PCR produces results only at the conclusion of the reaction.	Real Time PCR displays the data of amplification during the run after each cycle
Conventional PCR results are in the form of bands in the gel whereas in Real Time PCR Ct or threshold value gives the measure of quality and quantity of the product	Real Time PCR uses a special dye, which helps in production of signal with every cycle and the signal strength increases as the number of copies of the gene increase

CONCLUSION

Based on the study, the following conclusions can be drawn:

• For the identification of *Drosophila suzukii*, *Drosophila simulans* and *Drosophila melanogaster*, nine primers were designed: 5 primers for conventional PCR and 4 primers for real-time PCR with extremely high identification accuracy. Primers can accurately define the gene regions of the studied species by isolating them from several closely related *Drosophila* sp. some of which are on the list of quarantine objects.

• According to the phylogenetic tree, 3 species of *Drosophila suzukii*, *Drosophila simulans* and *Drosophila melanogaster*, each fell into a separate clade, the constructed phylogenetic tree also shows that primers designed with maximum accuracy identified each *Drosophila* species among is subspecies.

• The design of the primers included temperature optimization, and after three iterations of testing each sample, the optimal temperature for their use was selected.

• Almost all developed primers were also tested in the Quarantine Organization of Iran. The results of their sensitivity and accuracy were similar to those of Russia and approved for use by the Iranian quarantine service. However, the primer pair 12. primers dsuz F/R for classical PCR and 1.dsuz.F/R., developed for real-time PCR for the identification of *Drosophila suzukii*, did not show high accuracy and quality of identification in Iran.

• According to research and a phylogenetic tree created, *Drosophila simulans* is one of the related species of *Drosophila suzukii* and has a genetic code very close to *Drosophila melanogaster*. It is also included in the list of quarantine objects in many countries (including Canada, Poland, etc.). It is recommended that the fruit fly species *Drosophila simulans* be included in the list of quarantine objects in Russia.

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