






Защита растений Plant protection

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Phylogenetic analysis and designing new primers for molecular identification of *Drosophila suzukii*

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Abstract. The family Drosophilidae includes over 3750 species worldwide and over 2000 of these are species of *Drosophila*. Spotted wing drosophila (SWD), *Drosophila suzukii* is one of the most dangerous species in this family. The insects live on undamaged ripening fruits, using its peculiar serrated ovipositor to break the skin of fresh ripening fruits and lay eggs in it. *Drosophila* species are very difficult and practically impossible to detect at larval stages. The present investigation was conducted at the All-Russian Plant Quarantine Center and Agrarian and Technological Institute of RUDN University, Moscow, Russia in 2018—2020. The aim of this study was to investigate the method of accurate and rapid identification of *D. suzukii*, and to design specific primer pairs for pest identification by Real-Time PCR method. The real-time quantitative PCR is a fast, sensitive, repeatable and accurate method for quantifying gene transcript levels. In this study, we designed specific primers (4.Dsuz.FRP) for Real-Time PCR to identify *D. suzukii* from other relative species. Although *D. suzukii* is absent in the Russian Federation and has not been reported so far, the project could be a precautionary measure.

Keywords: identification, original sequences, phylogenetic tree, *Drosophila suzukii*, PCR, plant quarantine

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Introduction

The spotted wing *Drosophila* (SWD), *Drosophila suzukii*, was originally described by Matsumura in Japan in 1931 [1]. Members of the *Drosophila* genus are not generally considered pests since their larvae are primarily developed on damaged or rotting fruits. Nevertheless, *D. suzukii* infests healthy ripening fruits while still on the plant. *Drosophila suzukii* larvae devour the fruit pulp within the fruits rendering them unmarketable and reducing the processed fruit exceptional [2, 3]. Furthermore, the wounds created at the infested fruits throughout ovipositing provide access to secondary fungal or bacterial infections leading to additional fruit tissue disintegrate [4–8]. *Drosophila suzukii* includes exceptional kinds of hosts; for this reason, this type of agricultural pest has come to be a world major pest (Fig. 1) of a large variety of commercial fruit crops [9, 10]. The aim of the research was to molecularly become aware of and draw a phylogenetic tree for *Drosophila suzukii* and distinguish it from other species.



Fig. 1. Worldwide-confirmed distribution of *D. suzukii* (August 2020) [11].
(<https://www.cabi.org/isc/datasheet/109283#toDistributionMaps>)

The principal purpose of this study was to categorize the molecular species of *D. suzukii*. Our purpose was to create an accurate and unique identity technique based on primers designed for *D. suzukii*, this approach is rapid and more efficient than techniques based totally on morphological identity [5, 12, 13]. The polymerase chain reaction turned into created to discover insects as a reliable and price-effective approach. In addition, in various molecular identification methods, one of the most important parts is the primer design. In this project, we design a specific pair of primers based on Real-time PCR.

Materials and Methods

DNA was extracted from the material under project (insect and larvae, from laboratory collection) by treating the specimens with Proteinase K accompanied with removal

of proteins with no extraction with natural solvents and using «DNA Extran-2 Kit», set № NG-511-100 («Synthol», Russian Federation) according to manufacturer’s instructions.

PCR-products purification. A 1:1 volume of Binding Buffer was added to the completed PCR mixture (e.g. 100 µL of Binding Buffer per every 100 µL of the reaction mixture) and mixed thoroughly. Then the solution was transferred up to the GeneJET purification column, centrifuged for 30...60 s. The flow-through solution was discarded. 700 µL of Wash Buffer was added to the GeneJET purification column and centrifuged for 30...60 s. The flow-through was discarded. The purification column was placed back into the collection tube. Then the empty GeneJET purification column was centrifuged for 1 min. After that the GeneJET purification column was transferred to a clean 1.5 mL microcentrifuged tube. Then 50 µL of Elution Buffer was added to the center of the GeneJET purification column membrane and centrifuged for 1 min.

Sequencing. DNA extracts were quantified on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). Sequencing was done according to the generally accepted protocol with the use of Genetic Analyzer AB-3500 (Applied Biosystems, USA).

The *Drosophila spp.* primers, S1859, and A2191 are targeting several *Drosophila* species and generate an amplicon of 220-bp length. PCR conditions had been identified for each primer pairs: each 25 µL response protected 2 µl of DNA extract (10 pmol), 5x PCR grasp mix, and display-blend (HS-5x), zero.5 µM each primer, 17 µl water.

Primer S1859 (5'-GGAACAGGATGAACAGTTTAACCGCC-3') as a forward and A2191 (5'-CCCGGTAAAATTTAAAATATAAACTTC-3') as a reverse were used (Table 1) and, in a Veriti™ thermocycler (Applied Biosystems, USA). The reaction mixture was as follows: ready-to-use PCR mixture Screen Mix-HS (Evrogen, Russia). PCR conditions: denaturation at 95 °C for 90 Sec was followed by 39 cycles, including 30 sec at 90 °C; primer annealing at 61 °C for 15 sec; elongation at 72 °C for 30 sec; final elongation at 72 °C for 5 min (Table 2).

Table 1

Primers for the identification of *Drosophila spp.*

Target genes	Primer	Primer sequence (5'-3')		
<i>Drosophila spp.</i>	S1859	5'-GGAACAGGATGAACAGTTTAACCGCC-3'	Bogdanowicz et al. [14]	2000
<i>Drosophila spp.</i>	A2191	5'-CCCGGTAAAATTTAAAATATAAACTTC-3'	Bogdanowicz et al. [14]	2000

Table 2

A list of sequences for identification of *Drosophila suzukii* with Real-time PCR

No	Species	Country: (Data from laboratory of Russian Plant Quarantine Center)	Result of real-time PCR
1	<i>Drosophila suzukii</i>	Egypt	+
2	<i>Drosophila suzukii</i>	Turkey	+
3	<i>Drosophila melanogaster</i>	Turkey	
4	<i>Drosophila simulans</i>	Canada	
5	<i>Drosophila persimilis</i>	USA	
6	<i>Drosophila rhopaloa</i>	Japan	
7	<i>Drosophila kikkawai</i>	China	

The end of the table 2

No	Species	Country: (Data from laboratory of Russian Plant Quarantine Center)	Result of real-time PCR
8	<i>Drosophila ficusphila</i>	Japan	
9	<i>Drosophila erecta</i>	Brazil	
10	<i>Drosophila obscura</i>	Japan	
11	<i>Drosophila yakuba</i>	USA	
12	K- extraction		
13	K- amplification	Water	

Primer design for Real-time PCR, for the identification of Drosophila suzukii. Primer 4.Dsuz.F (5'- CCTTCGTGAAGCCTTCTACCG -3') as a forward and 4.Dsuz.R (5'- GCA*****AGATC -3') as a reverse and 4.Dsuz.Probe (5'- CAA*****TTCGCTG -3') as a probe were used. 1 µl (10 pmol) of each primer, 5 µl of master-mix 5dd (HS-5x), 16 µl H₂O and 1 µl DNA were used to make PCR mixture. The total volume of 25 µl, in a CFX 96 (Bio Rad, USA), was maintained and run-in Real-Time PCR unit. The reaction mixture was ready-to-use PCR mixture Screen-Mix (Evrogen, Russia) with the following procedure: 94 °C for 5 min, followed by 39 cycles of 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 30 s. After the amplification, a melting curve analysis was performed, and the results had been averaged.

Results and Discussion

D. suzukii has a wide range of hosts both in its native habitats in Asia and the United States, with small fruits and cherries being the main economic concerns [1, 15–17]. Significant damage has been observed in several commercial soft fruits, such as blackberries, blueberries, cherries, raspberries, strawberries, tomatoes, grapes, cherries, figs, kiwis [18–20].

As shown in the phylogenetic tree (fig. 2), *D. suzukii* family members, such as *D. simulans* and *D. melanogaster*, are very close in genetic code, making it more difficult to identify. Moreover, this further highlights the need to design proprietary primers. Finally, we recommend this primer to other researchers (Table 3).

In this study, we have developed a Real-time PCR assay for the detection and identification of *D. suzukii* (Fig. 3).

In these results, we designed the primers 4.Dsuz.F (5'- CCTTCGTGAAGCCTTCTACCG -3') as a forward and 4.Dsuz.R (5'- GCA*****AGATC -3') as a reverse and 4.Dsuz.Probe (5'- CAA*****TTCGCTG -3') as a probe. In addition, we had nine samples for identification with originally designed primer (4.Dsuz) and we had only two positives of *Drosophila suzukii* as the positive control, 9 unknown cases, their names are listed in Table 1, respectively.

In addition, there is a case of the negative control (sample 3 up to 11) for checking PCR and extraction DNA processes. Two were accurately identified as *Drosophila suzukii*; two samples (number: 1, from Egypt and number: 2 from Turkey) showed melt peaks in the acceptable range of T: 58 °C results for and were clearly distinguished from another species. No other melt peaks were reported.

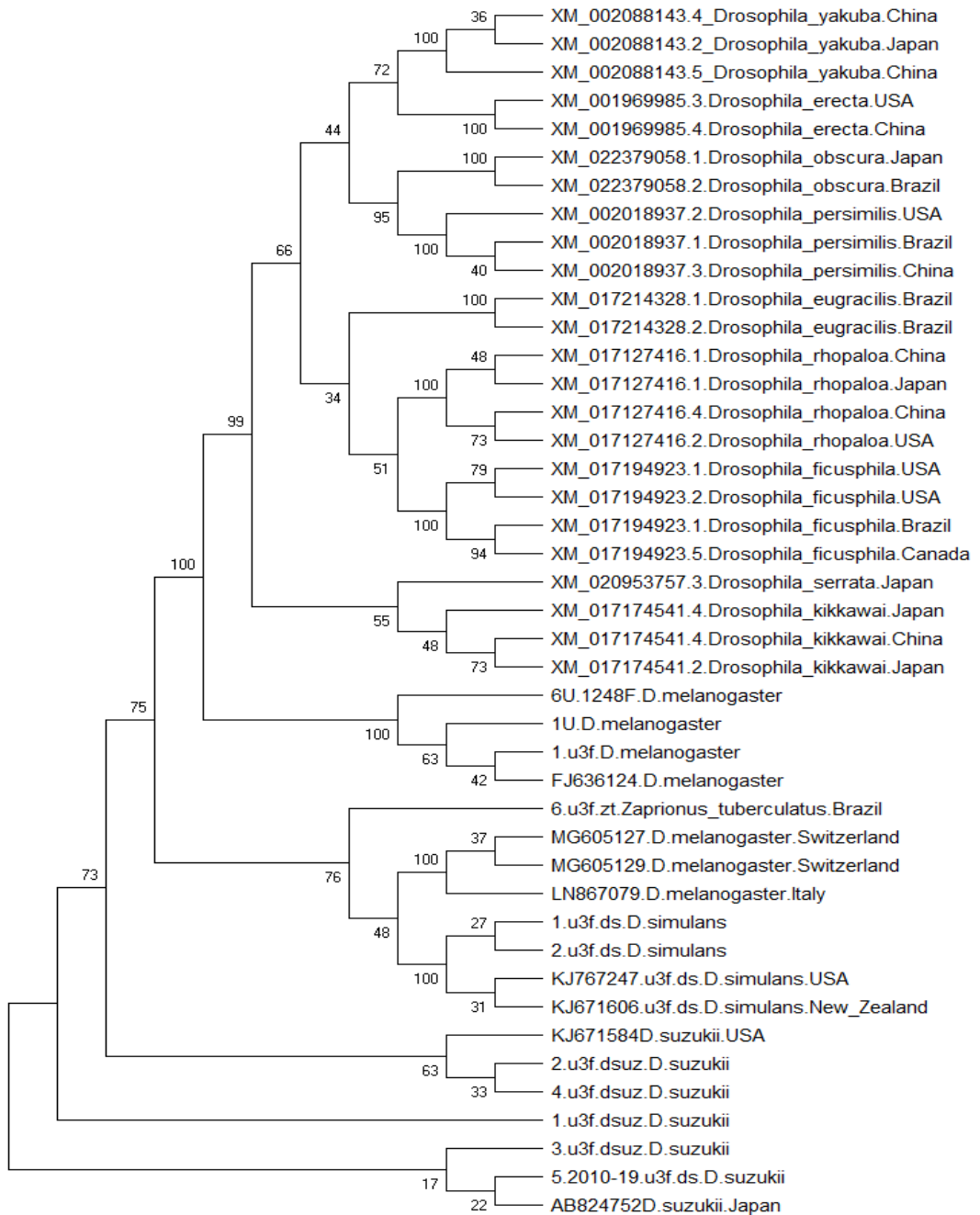


Fig. 2. Phylogenetic tree for *Drosophila* spp.

Identification of different *Drosophila suzukii* genetic codes

No	Species	Number of letters											
		99	101	114	119	123	140	141	143	160	165	171	181
1	<i>Drosophila suzukii</i>	T	C	C	T	C	T	C	C	C	C	T	A
2	<i>Drosophila simulans</i>	C	T	T	C	T	G	T	G	C	T	C	T
3	<i>Drosophila melanogaster</i>	A	T	C	T	C	T	T	A	T	T	C	T
4	<i>Drosophila persimilis</i>	C	T	T	C	T	G	T	G	C	T	C	T
5	<i>Drosophila rhopaloea</i>	C	T	T	C	T	G	T	G	C	T	C	T
6	<i>Drosophila ficusphila</i>	A	T	C	T	G	T	T	C	T	T	C	T
7	<i>Drosophila kikkawai</i>	G	T	C	T	G	T	T	A	T	T	C	T
8	<i>Drosophila erecta</i>	C	T	T	C	T	G	T	G	C	T	C	T
9	<i>Drosophila yakuba</i>	C	T	T	C	T	G	T	G	C	T	C	T
9	<i>Drosophila obscura</i>	G	T	C	T	T	T	T	C	T	T	C	T

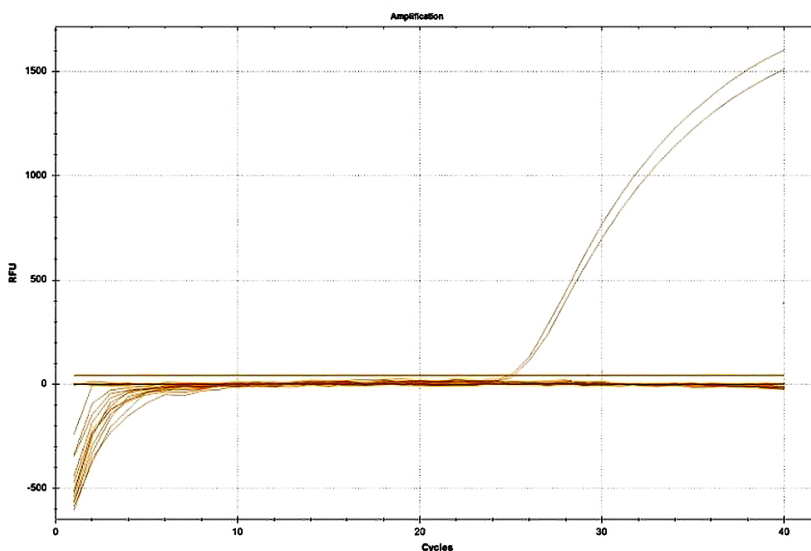


Fig. 3. The efficiency of the real-time PCR assay for the identification of *D. suzukii*

The research has proven that particular host fruits were preferred for ovipositing and development of larvae, at the same time as temperature performed an essential position in *D. suzukii* development, survival, and fecundity. We have used unique sequences in all stages and the primers were designed for the first time as well.

The primary comparison for the results of the sequence with the GeneBank genetic sequence database was performed by the NCBI BLAST web site (<http://www.ncbi.nlm.nih.gov/BLAST>). BioEdit v.7.0.5.3 [18] sequence alignment editor was used for sequence checking, alignment, and editing.

Conclusion

Molecular identification for all samples used within the improvement of the technique changed into done by way of PCR amplification. For a long time, polymerase chain reaction (PCR) methods had been used to classify pests, organisms and sicknesses around the sector. The outcomes showed that those primers appropriately diagnosed the gene region in addition to the specific region of *D. suzukii*. When samples are not sufficient for morphological identification or handiest immature samples are available, real-time PCR molecular detection may be used as a fast and efficient detection approach. For such an economically vital attacker, a one-of-a-kind identification method can also permit extra accurate tracking and detection and save you misidentification. Real-time PCR detection can be used as a fast and efficient detection device. Finally, a phylogenetic tree turned into identified for numerous specimens close to *D. suzukii*. As an end result, it is far located near the subspecies in the tree.

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Филогенетический анализ и разработка новых праймеров для молекулярной идентификации *Drosophila suzukii*

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Аннотация. Семейство Drosophilidae насчитывает более 3750 видов по всему миру, из них к роду *Drosophila* относится более 2000 видов, причем, *Drosophila suzukii* считается одним из самых опасных

представителей этого семейства. Личинок вредителя крайне трудно или практически невозможно обнаружить, так как азиатская ягодная дрозифила большую часть жизненного цикла проводит внутри плода. Вылупляющиеся из яиц личинки *Drosophila suzukii* питаются здоровыми плодами, вызывая размягчение тканей и загнивание плодов. Эксперименты проводились на базе Всероссийского центра карантина растений и в Аграрно-технологическом институте Российского университета дружбы народов в 2018—2020 гг. Цель исследования — подобрать метод точной и быстрой диагностики вредителя, разработать специфические пары праймеров для ПЦР в реальном времени. На сегодняшний день количественная ПЦР — это быстрый, высокочувствительный, воспроизводимый и наиболее точный метод диагностики. По результатам исследования были разработаны специфические праймеры (4.Dsuz.FRP), позволяющие с высокой точностью отличать *D. suzukii* от других родственных видов при использовании метода ПЦР в реальном времени. Хотя появление *D. suzukii* не было зафиксировано на территории Российской Федерации, исследование дает возможность быстро и с высокой точностью идентифицировать вредителя в случае его возможного завоза, а также поможет предотвратить его последующее распространение.

Ключевые слова: идентификация, исходная последовательность, филогенетическое дерево, *Drosophila suzukii*, ПЦР, карантин растений

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