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Molecular identification of *Zeugodacus cucurbitae* (Coquillett, 1899) (Diptera: Tephritidae) using PCR-RFLP

Молекулярно-генетическая идентификация вида *Zeugodacus cucurbitae* (Coquillett, 1899) (Diptera: Tephritidae) методом ПЦР-ПДРФ

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Key words: Fruit fly, molecular identification, COI gene, plant quarantine.

Ключевые слова: мухи-пестрокрылки, молекулярная идентификация, ген COI, карантин растений.

Abstract. This paper discusses the method of molecular identification of the melon fly *Zeugodacus cucurbitae* (Coquillett) based on restriction fragment length polymorphism-polymerase chain reaction (PCR-RFLP). This approach, which involves the analysis of nucleotide composition of the 5'-end of the mitochondrial COI gene fragment, allows for the detection of differences in the nucleotide sequences of this region and serves as a rapid method for identification and differentiation *Z. cucurbitae* from other fruit fly species, which larvae can also be found in the fruits of cucurbit plants.

Резюме. В данной работе представлен метод молекулярно-генетической идентификации африканской дынной мухи *Zeugodacus cucurbitae* (Coquillett) с использованием метода полимеразной цепной реакции — полиморфизма длин рестрикционных фрагментов (ПЦР-ПДРФ). Данный подход, основанный на анализе нуклеотидного состава 5'-конца фрагмента митохондриального гена COI, позволяет выявлять различия в нуклеотидных последовательностях этого участка и служит методом быстрой идентификации и дифференциации *Z. cucurbitae* от некоторых видов мух-пестрокрылок, личинки которых также могут быть обнаружены в плодах тыквенных растений.

Introduction

The melon fly *Zeugodacus cucurbitae* (Coquillett, 1899) is an economically important pest on fruits of mostly Cucurbitaceae family plants [White, Elson-Harris, 1992; De Meyer et al., 2015]. The species originated from Asia and had spread to Africa, Australia, and Oceania [Virgilio et al., 2010; De Meyer et al., 2015]. To prevent further spread of this pest to other territories, a quick and reliable method to identify the melon fly is required.

The importance of developing molecular genetic methods arises from the fact that morphological identification is labor-intensive and requires highly qualified specialists. In addition, the preimaginal stages of fruit flies, which are the life stages most often found in fruits, are the most difficult to identify because the diagnostic protocols for them are not fully developed or completely absent [Kandybina, 1977]. It is also worth noting that some of the morphological features used to identify the larvae of the Tephritidae family members show considerable variability [Kamayev et al., 2020].

DNA barcoding is widely used to identify pest fruit flies including the melon fly [Barr et al., 2012; Barr et al., 2018; Doorenweerd et al., 2020; EPPO, 2021]. It involves the amplification of mitochondrial cytochrome oxidase I (COI) gene region, followed by sequencing and comparing the obtained nucleotide sequences with the reference sequences from the dedicated databases (BOLD, NCBI GenBank) [Ratnasingham, Hebert, 2007; Benson et al., 2018]. However, the analysis using this method is rather time-consuming, and is also not always reliable, since some sequences in the databases may be assigned to incorrect species. This is a consequence of

errors made by the researchers during the preliminary morphological identification of insect samples [Doorenweerd et al., 2020].

Some studies have suggested to use polymerase chain reaction by restriction fragment length polymorphism (PCR-RFLP) analysis for tephritid pest differentiation [Armstrong et al., 1997; Muraji, Nakahara, 2002; Chua et al., 2010; Harbi et al., 2022]. To identify *Z. cucurbitae* by this method, the COI gene region [Chua et al., 2010; Harbi et al., 2022], as well as the 18S and ITS regions [Armstrong et al., 1997], and 12S and 16S were used [Muraji, Nakahara, 2002]. It is worth noting that the samples studied in some of these works were represented by a small number of species, which were often obtained from a single geographic region.

As a part of the present study aiming at the development of a method for *Z. cucurbitae* molecular identification, the region of the COI gene, which is considered the most universal molecular marker for identifying tephritid flies, was chosen for the analysis [Lunt et al., 1996; Doorenweerd et al., 2020]. As a result, a method for identification of the melon fly *Z. cucurbitae* using the PCR-RFLP was developed using the COI gene.

Material and methods

The samples for the study were adults and 3rd instar larvae of tephritid flies: *Z. cucurbitae*, *Zeugodacus tau* (Walker, 1849), *Bactrocera dorsalis* (Hendel, 1912), *Bactrocera latifrons* (Hendel, 1912), *Dacus* spp., *Ceratitis capitata* (Wiedemann, 1824) and *Anastrepha grandis* (Macquart, 1846) (Table 1). The specimens were preliminarily identified based on their morphological characteristics.

DNA was isolated from a single leg of an adult insect or from a 3 mm long fragment of larval body. To extract DNA, the previously described method was used [Galinskaya et al., 2016].

To search for the suitable primers in the COI gene, an approximately 849 bp region was chosen, which included the positions 2120–2968 in the complete mitochondrial genome of *Z. cucurbitae* (GenBank number: JN635562.1). This COI gene region is sufficiently variable and suitable for differentiating closely related species [Lunt et al., 1996]. Specific forward and reverse primers Z-F (5'-TGATTCTTTGGACACCCTGAAG-3') и Z-R (5'-CAGGTGGTGTATTTTGAAGTCCT-3') were designed using the Primer-BLAST [Ye et al., 2012] software for genetic identification of *Z. cucurbitae* (Fig.1). Hairpin and dimer formation tests and melting point calculations were performed using the OligoCalc online calculator [Kibbe, 2007]. Primers were synthesized by Evrogen (Russia).

To amplify the fragment under study, the ScreenMix-HS ready-made PCR mix (Evrogen) was used. The reaction was carried out according to the manufacturer's instructions in the volume of 25 µl. PCR conditions: 94 °C — 5 min; 35 cycles: 94 °C — 1 min, 60 °C — 30 s, 72 °C — 1 min; 72 °C — 5 min. Amplification

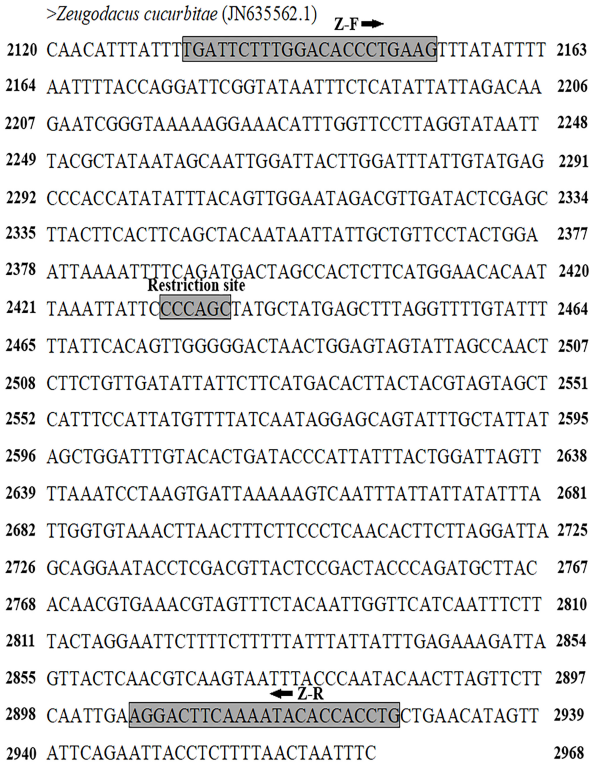

Fig. 1. Primers annealing regions and a restriction site in the COI gene sequence of *Z. cucurbitae*.

Рис. 1. Области гибридизации праймеров и сайт рестрикции в последовательности гена COI вида *Z. cucurbitae*.

products were separated by electrophoresis in the 1.5 % agarose gel with EtBr. To estimate the size of the fragments, the DNA Ladder 100+ bp (Evrogen) was used. Purification of PCR products and Sanger sequencing were performed by Evrogen. The PCR products were sequenced on an Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific, USA). The resulting chromatograms of the forward and reverse sequences for each sample were combined and edited in the SeqMan NGen program (DNAStar, Madison, Wisconsin, USA).

The search for the species-specific restriction sites for *Z. cucurbitae* in the fragment under study was carried out using the REBASE software [Roberts et al., 2015]. As a result, the 5'-CCCAGC-3' site was identified, corresponding to the restriction endonuclease Gsa I (SibEnzyme, Russia). After amplification of the fragments using the Z-F and Z-R primers a restriction reaction was carried out in the volume of 30 µl (3 µl SE-Buffer W, 3 µl BSA, 3 µl restriction enzyme Gsa I, 5 µl PCR product, and 16 µl ddH_2O) at 70 °C for 1 hour. Restriction products were separated in the 1.5 % agarose gel with EtBr by electrophoresis. DNA Ladder 100+ bp (Evrogen) was used as the DNA size marker.

The present work is registered in ZooBank (www.zoobank.org) under LSID urn:lsid:zoobank.org:pub:8B48DFB3-82F2-4E59-8217-880286CAB2EA

Table 1. List of the studied species
Таблица 1. Список изученных видов

Species	Place and date of collection	Number and sex of specimens	GenBank accession number
Zeugodacus cucurbitae (Coquillett, 1899)	Vietnam, Hanoi, 25.VI.2019	1♂, 1♀	OR989967, OR989968
	Vietnam, Hanoi, 23.VIII.2019	5♀♀, 1 larva	OR989970–OR989974, OR989969
	Indonesia, Java, Sumur, 19.XI.2016	1♂, 1♀	OR989975, OR989976
	Tanzania, VII.2019	2♂♂	OR989977 OR989978
Zeugodacus tau (Walker, 1849)	Vietnam, Hanoi, 25.VI.2019	1♂, 1♀	OR989979, OR989980
Bactrocera dorsalis (Hendel, 1912)	Thailand, XI.2018	4♂♂, 4♀♀	–
Bactrocera latifrons (Hendel, 1912)	Vietnam, Hanoi, 23.VII.2019	2♂♂	–
	Vietnam, 24.VIII.2019	1♀	–
	Tanzania, Morogoro, 25–29.XII.2019	1♂, 1♀, 2 larvae	–
Dacus sp.	Tanzania, Morogoro, 12.I.2020	1♂	–
Dacus sp.	Kenya, Kitale, Mount Elgon National Park, 14.V.2019	1♂	–
Ceratitis capitata (Wiedemann, 1824)	Türkiye, XII.2018	3♂♂, 5♀♀, 2 larvae	–
Anastrepha grandis (Macquart, 1846)	Peru, Quillabamba, 04.IV.2016	2♂♂, 1♀	–

Results

As a result, a fragment of the COI gene was amplified in 3 replicates for 44 samples of the Tephritidae family representatives (Table 1), which larvae can be found in the fruits of Cucurbitaceae (Fig. 2). A fragment of 796 bp was amplified in all specimens of *Z. cucurbitae* and *Z. tau* (Fig. 3). The amplicons were sequenced and deposited in NCBI GenBank (accession numbers: OR989967–OR989980) (Table 1).

Based on the results of the analysis of the amplified sequences, a restriction site (5'-CCCAGC-3') specific for *Z. cucurbitae* was detected (Fig. 1). Gsa I endonuclease (5'-CCCAG↑C-3'/3'-G↓GGTCG-5') cuts the 796 bp PCR fragment into the fragments of 304 bp and 492 bp (Fig. 4). It was shown that *Z. tau* does not have this site in the amplified sequence, and its restriction digestion is thus impossible.

In addition, *in silico* analysis was performed. In the course of studying 375 *Z. cucurbitae* and 79 *Z. tau* sequences, both obtained by us and downloaded from NCBI GenBank, the presence of the restriction site (5'-CCCAGC-3') was confirmed only in the target species. Thus, it appears that it is possible to differentiate *Z. cucurbitae* from *Z. tau* by PCR-RFLP.

Discussion

We propose an approach for identification of *Z. cucurbitae* using the PCR-RFLP method. The designed Z-F

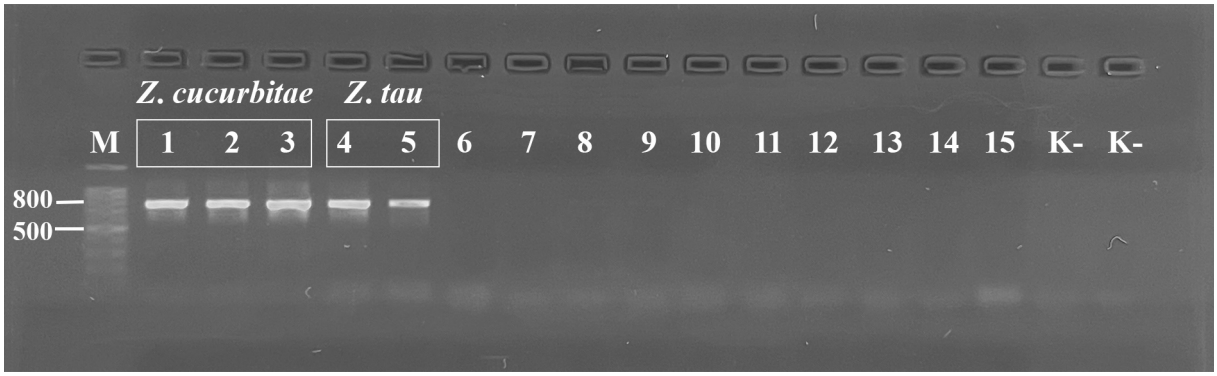

Fig. 2. Electrophoretic separation of the amplification products obtained with the Z-F and Z-R primers. Designations: 1, 2, 3 — *Z. cucurbitae* samples; 4, 5 — *Z. tau*; 6, 7 — *B. dorsalis*; 8, 9 — *B. latifrons*; 10, 11 — *Dacus* spp.; 12, 13 — *C. capitata*; 14, 15 — *A. grandis*; K- — negative control; M — DNA size marker.

Рис. 2. Электрофорез продуктов амплификации с праймерами Z-F и Z-R. Обозначения: 1, 2, 3 — образцы *Z. cucurbitae*; 4, 5 — *Z. tau*; 6, 7 — *B. dorsalis*; 8, 9 — *B. latifrons*; 10, 11 — *Dacus* spp.; 12, 13 — *C. capitata*; 14, 15 — *A. grandis*; K- — отрицательный контроль; M — маркер длины ДНК-фрагментов.

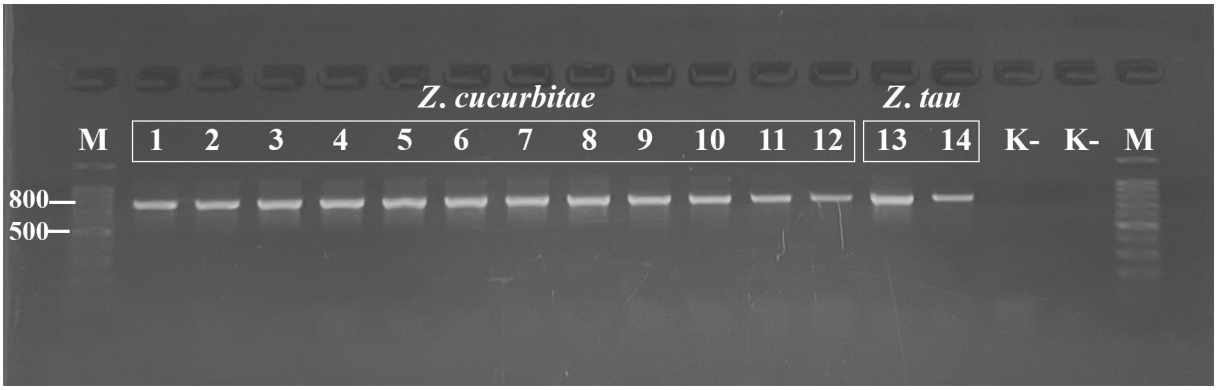

Fig. 3. The result of amplification with the Z-F and Z-R primers. Designations: 1–12 — *Z. cucurbitae* samples; 13, 14 — *Z. tau* samples; K- — negative control; M — DNA size marker.

Рис. 3. Результат амплификации с праймерами Z-F и Z-R. Обозначения: 1–12 — образцы *Z. cucurbitae*; 13, 14 — образцы *Z. tau*; K- — отрицательный контроль; M — маркер длины ДНК-фрагментов.

and Z-R primers allow us to differentiate *Z. cucurbitae* and *Z. tau* from *B. latifrons*, *B. dorsalis*, *C. capitata* and *A. grandis*, which larvae can be found in the fruits of cucurbit plants. Enzymatic treatment of the PCR product with Gsa I endonuclease (5'-CCCAG↑C-3'/3'-G↓GGTCG-5') allows accurate differentiation of the target species *Z. cucurbitae* from *Z. tau*.

The previously suggested PCR-RFLP identification methods [Armstrong et al., 1997; Muraji, Nakahara, 2002; Chua et al., 2010] are only partially applicable to *Z. cucurbitae* species and are not specifically aimed at identifying the melon fly. Moreover, in some papers it describes processing the gene fragments with several restriction endonucleases, which implies certain challenges in the interpretation of the obtained results.

The method we propose allows for rapid, inexpensive and targeted identification of *Z. cucurbitae*. This approach is useful for diagnosing not only adults, but also larvae, which is especially important in the work of plant quarantine and phytosanitary safety specialists. This is due to the fact that *Z. cucurbitae* is most often found in fruits at the larval stage, which is difficult to identify by morphological methods.

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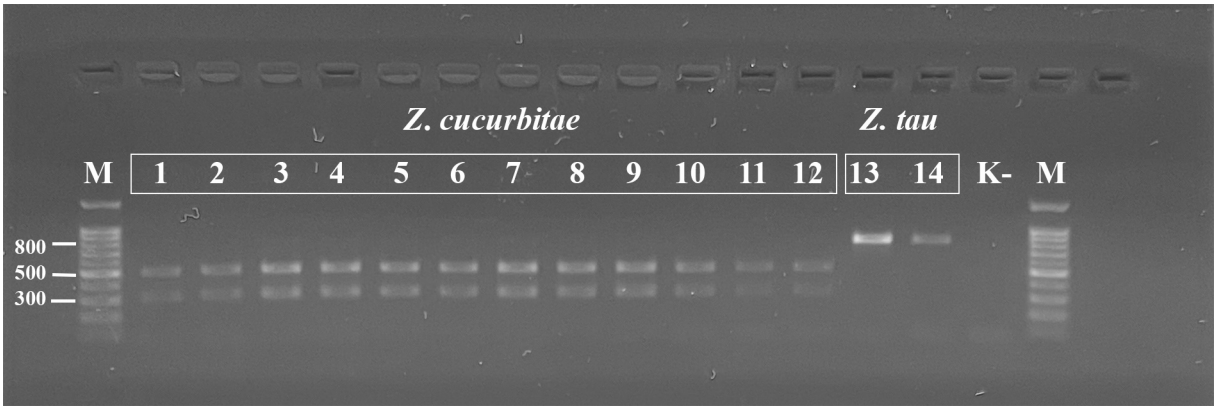

Fig. 4. The result of enzymatic treatment of the PCR products with Gsa I endonuclease. Designations: 1–12 — *Z. cucurbitae* samples; 13, 14 — *Z. tau* samples; K– — negative control; M — DNA size marker.

Рис. 4. Результат обработки ПЦР продуктов эндонуклеазой Gsa I. Обозначения: 1–12 — образцы *Z. cucurbitae*; 13, 14 — образцы *Z. tau*; K– — отрицательный контроль; M — маркер длины ДНК-фрагментов.

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