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Development and Validation of a Real-Time PCR Assay for Identification of a Common *Agrotis* Species: *Agrotis segetum* (Lepidoptera: Noctuidae)

P. Matlabdokht[1], L. Fekrat[1*] M. Zakiaghl[1], G. H. Moravej[1], and A. Shirvani[2]

ABSTRACT

The common cutworm, *Agrotis segetum*, is a serious soil pest of many vegetable and field crops all over the world. Morphological identification of *Agrotis* species is predominantly performed on adults due to the deficiency of adequate identification keys for immature stages. In international trade, the immature life stages are frequently being intercepted at point of inspection, challenging the possibilities of morphological identification. To realize a rapid and reliable identification for all stages of *A. segetum*, a TaqMan real-time Polymerase Chain Reaction (PCR) was developed based on the mitochondrial *Cytochrome Oxidase I (COI)* gene. All specimens of *A. segetum* (including various life stages) were detected and no cross-reactivity was observed with 5 non-target *Agrotis* species in the specificity tests. The tests showed to be repeatable, reproducible, and robust. The assay performed equally well with crushed insects and purified DNA, so, the efficiency was added by removing DNA extraction step. The method has proven to be suitable tools for routine identification of all life stages of *A. segetum* considering the speed, specificity, as well as sensitivity of the assay.

Keywords: Mitochondrial cytochrome oxidase I, Qualitative real-time PCR.

INTRODUCTION

The genus *Agrotis* Ochsenheimer, 1816 (Noctuidae: Noctuinae), consists of approximately 300 species throughout the world, with the exception of Polar Regions (San Blas, 2014). The members of the genus, commonly known as cutworms, feed on roots and foliage of their host plants and many species are considered as significant pests for a number of crops in agriculture, horticulture, and forestry all around the world (Zethner, 1980). The common cutworm (turnip moth), *Agrotis segetum* Denis & Schiffermüller, is nearly a cosmopolitan insect pest that frequently lives on the ground feeding on seedlings of almost all vegetable and field crops as well as many other plants (El-Salamouny *et al*., 2003).

Morphological identification of *Agrotis* adult species is undemanding (e.g., Feizpoor *et al*., 2014), although meticulous examination of genital structures is generally requisite (Blas *et al*., 2013). Pheromone/light traps are tremendously straightforward and well-liked methods for collecting and monitoring adult male noctuid moths particularly in regions where it is prudent to monitor for destructive insect pests (Lewter and Szalanski, 2007). As these traps may attract more than one species and samples often degrade as adult moths typically remove a large portion of their scales while fluttering around inside the traps, morphological diagnosis of collected species

[1]Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.
[2]Department of Plant Protection, Faculty of Agriculture, Shahid Bahonar University of Kerman, 76169-133 Kerman, Islamic Republic of Iran.
*Corresponding author; e-mail: fekrat@ferdowsi.um.ac.ir

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might be tremendously arduous (Lewter and Szalanski, 2007). Moreover, the early detection of any pest, including *A. segetum*, in different agro-ecosystems is of prime importance for implementing any control strategies. Nonetheless, the immature stages are usually being intercepted instead of adults, and in most cases, for the continuation of trade, identification cannot be interrupted until a specimen is reared to the adult stage. Furthermore, the descriptions of immature stages are often restricted to the last instars, and since young larvae differ considerably from fully-grown ones, these descriptions cannot be applicable for the earlier instars. Besides, it is noteworthy to say that, for many *Agrotis* species, the larvae are not described at all, so, the possibilities for morphological identification of these stages are limited. Hence, morphological identification of *Agrotis* larvae requires expertise and experience or may not be possible at all. The pupal morphology is also inadequately known, with dissimilarities between species only described for a few species. Based on the foregoings, for efficient diagnosis of adults and immature stages, either captured during domestic surveys or intercepted at ports of entry, means of identification other than morphology are indispensable. In such situations, using a molecular diagnostic protocol might be very useful, especially when dealing with specimens that are in poor condition. Various molecular methods have been successfully used for identification of insect pests, each of which has their pros and cons. Although most of the currently used molecular methods such as DNA barcoding allow identification, they require post-PCR analysis steps that increase the processing time for samples. Some methods such as single or multiplex PCR need less labor, cost, and time than the DNA barcode assay, however, they still require a post-amplification step to visualize the PCR products, which in its turn, increases the time and risk of contamination. Moreover, since in multiplex PCR designing primers should be based on the regions where sequences of various target species differed, in some cases when gene sequences are very similar, particularly in closely related species, it may not be possible to design specific primers to differentiate them. Real-time PCR is a molecular method that has hitherto been utilized for detection and diagnosis of various insects (Huang *et al*., 2010; Dhami and Kumarasinghe, 2014; Tembrock *et al*., 2017; Koohkanzadeh *et al*., 2018). Assay time reduction, post-PCR electrophoresis elimination, potential of scaling for high throughput testing as well as higher level of sensitivity and specificity are the merits of real-time PCR compared with conventional PCR (Logan and Edwards, 2004; Tembrock *et al*., 2017). Moreover, real-time PCR eliminates the necessity to process and sequence the final PCR product, the lengthiest step in DNA barcoding. Highly species-specific PCR primers or probes designed in Real-time TaqMan PCR give positive reactions with DNA of the target species. A positive diagnosis is determined by the measurably lower number of PCR cycles taken for the amplicon to reach a given concentration for fluorescence to be detected (Li *et al*., 2019). In terms of sensitivity, specificity, susceptibility to inhibition and handling effort, the method has better performance compared to some other conventional PCR-based methods (Baric *et al*., 2006; Zhang *et al*., 2016).

In the current study, we aimed to develop a real-time PCR assay based on TaqMan for fast, quick, and reliable identification of *A. segetum*. To demonstrate assay specificity and sensitivity, some other morphologically similar or closely related non-target *Agrotis* species that might be found in vegetable fields were to be evaluated to ensure that the probe region is diagnostic for *A. segetum*.

MATERIALS AND METHODS

Sample Collection

Agrotis adult specimens were collected by using pheromone-light traps fitted at different potato fields in Khorasan-e-Razavi Province during the 2017 growing season.

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DNA Extraction

Total genomic DNA was extracted from middle and hind legs on one side of the body of adult specimens, a 2–3 mm piece of the larval body or pupae using a Qiagen DNeasy Blood and Tissue Kit according to manufacturer's instructions.

Primer and Probe Design

Mitochondrial *COI* gene sequence of *A. segetum* (MH924167) was aligned with homologous sequences of *A. segetum* and also several other *Agrotis* species downloaded from GenBank (Table 1) by ClustalW implemented in MEGA6 software (Tamura *et al*., 2013) and the resulting alignment was curated manually. The software AlleleID 7.84 was used to design the primers and the probes. The designed primers and probes were then compared with the locations in a COI alignment in order to choose combinations that would maximize discrimination between *A. segetum* and other examined noctuid species. The COI primers produced an amplicon of 91bp. The resulting primers and probe information is included in Table 2.

Real-time PCR Protocol

All real-time PCR experiments were carried out on an ABI7300 real-time PCR instrument. Gradients of temperatures (60–65°C) and primer concentrations (300 and 400 nM) were utilized for optimizing the PCR conditions. The PCR components are shown in Table 3. The real-time cycling parameters were as follows: an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 94°C for 5 seconds, and annealing and extension at 63°C for 30 seconds.

Specificity Analyses

A total of 5 non-target species were tested for specificity evaluation (Table 1). The identity of non-target specimens was confirmed by the fifth author. Each of these control species were either sympatric, closely related, or have shared host plants with *A. segetum*. The samples were also trialed using a TaqMan 18S internal control real-time PCR (Applied Biosystems, USA)

Table 1. List of species utilized in primer and probe design alignment.

Species	Accession number
Agrotis segetum	MH924167 (this study) KM572518, HQ955316, HQ955317, JF415462, HM393504, KT988755, JN988482, JN988485, JN988481, JN988483, KR183254, JX411853
A. clavis	KM573319, KM573076, JF860001, JF415459
A. ipsilon	GU686965, GU438724, GU090283, KM573001, KM572047, HM436275, HM415317, HQ951105, HQ951104, HQ951103, HQ961652, HQ961653, KJ393618, KJ388585, KJ383690, KJ388780, KJ388550, KJ388115
A. exclamationis	KM573623, KM573507, JF859961, HM914211
A. cinerea	JF415457, JF860102

Table 2. TaqMan probe and primers used in the current study.

Name	Sequence	Tm (°C)
AsegF	ACAGGATGAACAGTTTATC	68.9
AsegR	CAGCTAAATGAAGGGAAA	58.3
AsegProb	3′-FAM-TCTACTGATCTACCTCCATGAGCAAT-TAMARA-'5	58.3

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Table 3. Reaction components and cycling conditions of real-time PCR assay.

Reaction composition			Cycling conditions			
Component	Final concentration		Step	Temperature (°C)	Time (s)	
	Simple	Duplex				
Primer F	400 nM	400 nM	Initial denaturation	95	30	
Primer R	400 nM	400 nM	Denaturation	94	5	×40 cycles
Probe (FAM)	200 nM	200 nM	Annealing/extension	63	30	
18SF		50 nM				
18SR		50 nM				
18S Probe (VIC)		50 nM				
PCR Buffer	2.5µl	2.5 µl				
Taq polymerase	0.5	0.5				
dNTPs	0.5 µl	0.5 µl				
DNA template	1 µl	1 µl				
ddH_2O	Adjust volume to 25 µl					

as stated by manufacturer's instructions in the duplex format i.e. *A. segetum* primers and probe, along with 18S internal control primers and probe, to make sure that non-amplification was an outcome of non-target samples instead of insufficient quality or quantity of the used DNA or the existence of inhibitory compounds

Sensitivity Analyses, Amplification Efficiency, Repeatability and Reproducibility of the Assay

Sensitivity of the real-time PCR assay was evaluated using a series of serial dilutions of *A. segetum* DNA with the same primer concentration. The primary DNA concentration was measured using a spectrophotometer (Thermo) and, afterwards, a 10-fold dilution series was created using the previous solution and sterile water in a 1:9 ratios at each step. Real-time PCR conditions were the same as those previously mentioned. The Cq values for each dilution were plotted against logarithmic values of target copy numbers. The efficiency was computed and converted to percentage efficiency by the formulas $E= 10^{(-1/slope)}$ (Ritz and Spiess, 2008) and $E\%= (E-1)\times100$ (Dhami and Kumarasinghe, 2014), respectively. Furthermore, r^2 was recorded as the fit of the slope.

The repeatability (intra-run variation) and reproducibility (inter-run variation) of the assay were measured as percentage coefficient of variation (%CV). To compute %CV for each sample, four samples were examined in 3 replicates in four identical but separate runs and the resulting data were compared to make an estimation of reproducibility. To decrease the time and improve the convenience of the assay, the sensitivity of the method on *A. segetum* samples was evaluated by eliminating DNA extraction step and using crushed individuals as template. For this test, one individual was crushed in 20 µL of sterile distilled water and the resulting homogenized solution was employed as DNA template for the real-time PCR reaction.

RESULTS

Real-Time PCR

By using the TaqMan probe and primer pairs, *A. segetum* specimens were successfully diagnosed, disregarding their life stages (Figure 1). Moreover, when specimens belonging to different populations of *A. segetum* were utilized, they were all identified successfully (Figure 2). All of *A. segetum* specimens evaluated with the multiplex real- time PCR created Cq values for both COI (FAM) and 18S

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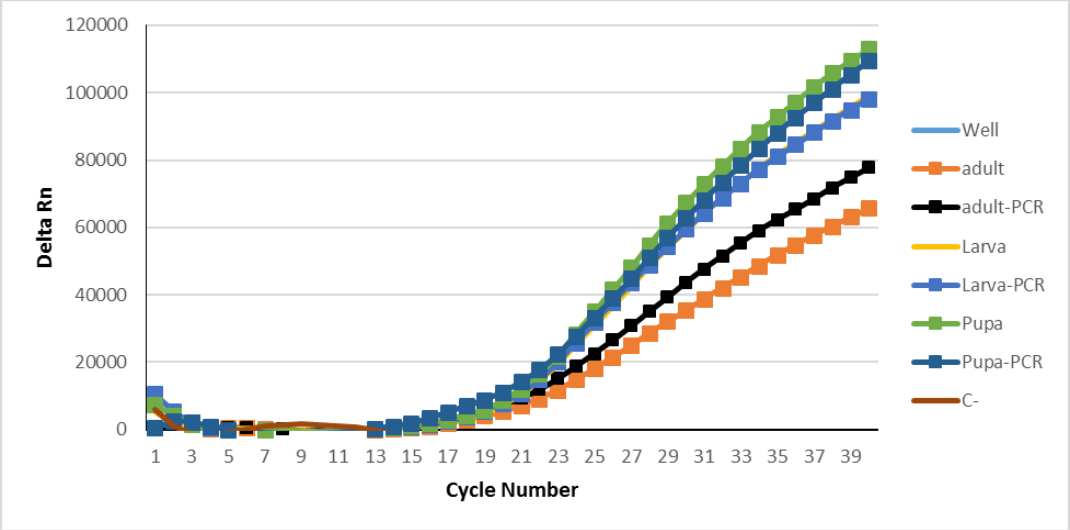

Figure 1. Amplification plot of various developmental stages of *A. segetum* with optimized primer (400 nM) and probe concentrations (200 nM).

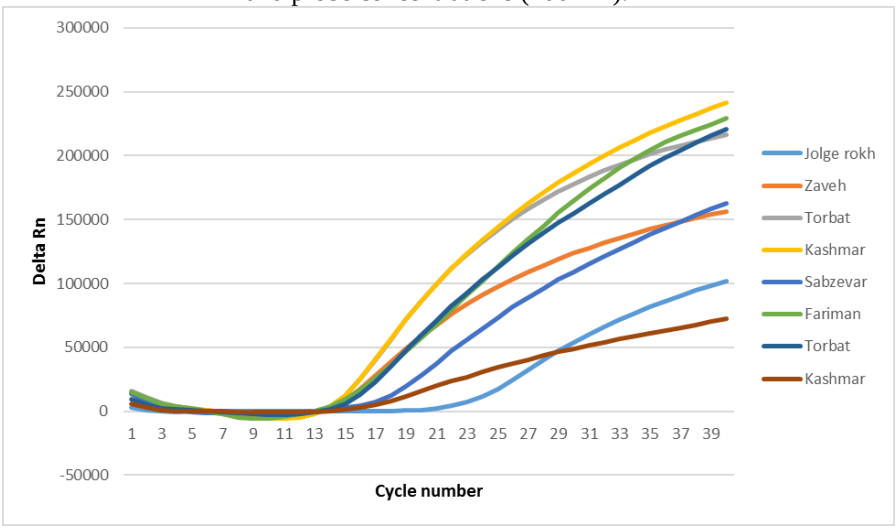

Figure 2. Amplification plot of specimens belonging to different *populations* of *A. segetum*.

markers (Figure 3; Table 4). Similar ranges of Cq values for the *A. segetum* specimens using the COI probe system (FAM), either multiplexed (11.74–12.65) or not multiplexed (11.29–12.68) with 18S in the assay, indicated that the assay was not negatively impacted by multiplexing. The cut-off of the assay was considered 30 cycles to reduce the probability of non-specific binding. Nevertheless, nonspecific amplification did not occur.

Specificity of the Assay

All 5 non-target *Agrotis* species evaluated in the multiplex assay produced Cq values for the 18S (VIC) probe ranging between 6.14 and 8.38. None of the non-target species produced a Cq value with the *A. segetum* (FAM) probe (Table 4).

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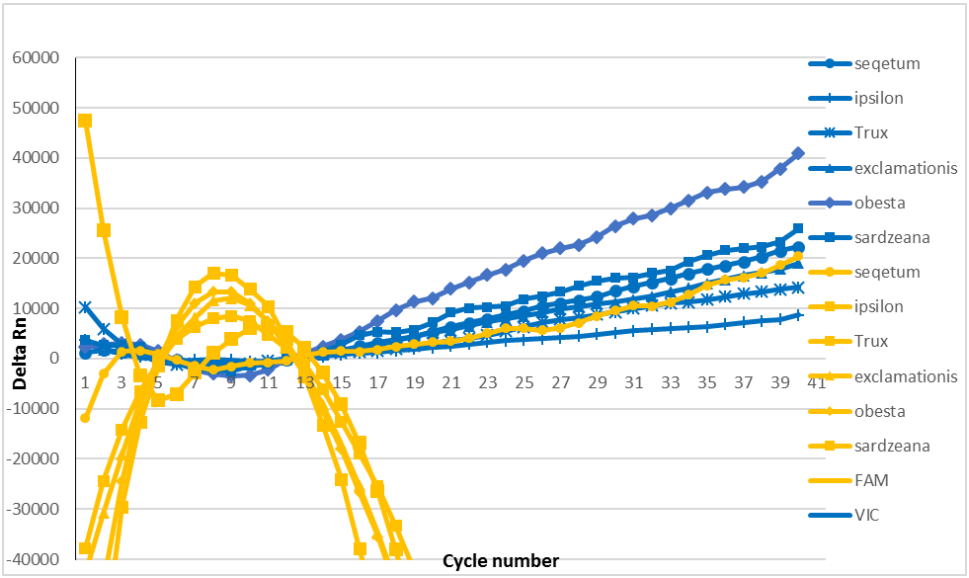

Figure 3. Amplification plot of different *Agrotis* species with *A. segetum* primers and probe (yellow) and 18S internal control (blue).

Table 4. List of fruit fly species used in the real-time PCR assays.

Species	Life stage	Origin	COI		18S Internal control	
			Result	Mean Cq	Result	Mean Cq
A. segetum	Adult	Torbat-e-Heidarieh	+	12.21	+	8.77
A. segetum	Adult	Zaveh	+	12.28		
A. segetum	Adult	Fariman	+	12.17	+	8.15
A. segetum	Adult	Golgeh-Rokh	+	12.44		
A. segetum	Adult	Feiz abad	+	11.77	+	9.15
A. segetum	Adult	Kashmar	+	12.20		
A. segetum	Larva	Golgeh-Rokh	+	15.10		
A. segetum	Pupa	Golgeh-Rokh	+	12.55		
A. ipsillom	Adult	Golgeh-Rokh	-	NA	+	8.38
A. trux	Adult	Kerman	-	NA	+	7.27
A. exclamationi	Adult	Kerman	-	NA	+	6.42
A. obests	Adult	Kerman	-	NA	+	6.14
A. sardzeana	Adult	Kerman	-	NA	+	6.44

Sensitivity and Performance of the Assay

As expected, the Cq values of the reactions increased with decreasing DNA template concentrations (Figure 4). The PCR efficiency (97%) was within the generally accepted range for an efficient PCR reaction (95–105%) (Figure 4). The correlation coefficient (r) of the calibration curve was 0.970.

High assay reproducibility was indicated by low variation in CV% values for each of the samples tested within individual and between different runs (Table 5).

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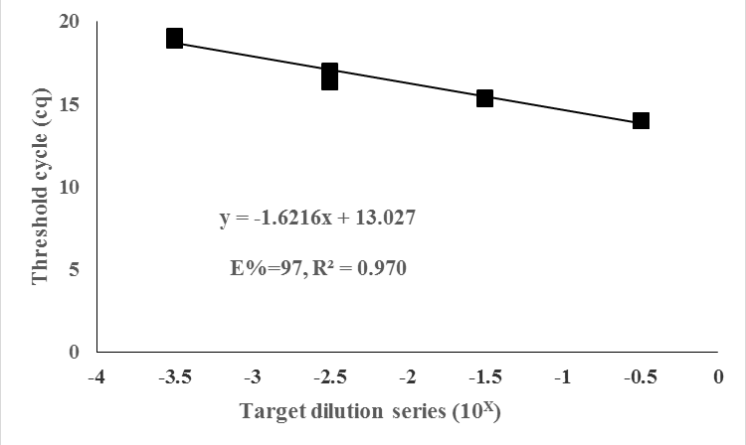

Figure 4. Efficiency of the real-time PCR assay for *A. segetum* diagnosis. Dilution series were used to create calibration curves for efficiency calculations. The standard curve was built from threshold cycle values against the log of target dilution series. Based on curve statistics, the assay efficiency is 97.0% and the r^2 (fit)= 0.970.

Table 5. Repeatability (the intra-run variation of a sample) and reproducibility (inter-run variation of a sample) of the assay, measured as percentage Coefficient of Variation (%CV).

sample	Repeatability (Run1, %CV)	Repeatability (Run2, %CV)	Repeatability (Run3, %CV)	Repeatability (Run4, %CV)	Reproducibility (%CV)
1	0.315997	0.066423	0.571416	0.424938	0.478643427
2	0.157762	0.180777	0.01967	0.358708	0.495793512
3	0.491761	0.339157	0.265039	1.336366	0.896001952
4	0.87233	0.403763	0.711157	0.483693	0.649481906

By excluding DNA extraction step and utilizing crushed individuals as template, the results did not change and the target *A. segetum* samples were amplified successfully within the Cq cut-off 30 cycles (Figure 5).

DISCUSSION

Biosecurity of borders vastly relates to the power of expeditious and reliable detection and identification of pests threatening the global trade. Morphology-based methods for determining the identity of intercepted pests are generally untrustworthy, time consuming, and often incapable of identification of immature stages. There are numerous molecular methods that can be applied to support early detection of insect or mite pests (Fekrat *et al*. 2015; Kouhkanzadeh *et al*., 2017; Sabahi *et al*., 2017a, 2017b; Feizpour *et al*., 2018, Sinaie *et al*., 2018). The cost and time required to process a sample are considered paramount factors in selecting the method. Real-time PCR-based techniques have recently revolutionized the diagnosis process and are growingly being used to identify various pests all around the world (Dhami *et al*., 2016a, 2016b; Zhang *et al*., 2016; Kouhkanzadeh *et al*., 2018).

In the current study, the identity of *A. segetum* specimens was determined explicitly using a real-time PCR assay notwithstanding their developmental stages. The method decreased the overall identification time by removing post-PCR processing. Furthermore, consistent results were acquired by omitting the DNA isolation step and using crushed specimens as template. The approximate time required

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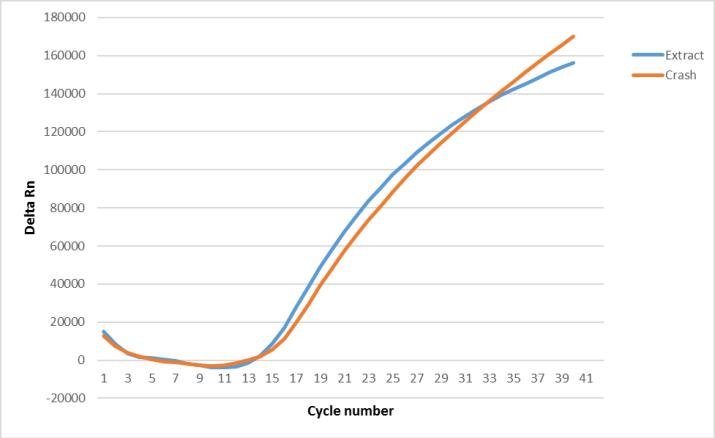

Figure 5. Amplification plot obtained during real time PCR on DNA of *A. segetum*: (a) With and (b) Without DNA extraction.

for executing the TaqMan real-time PCR was notably shorter in comparison with that needed for some other diagnosis methods like conventional PCR or DNA barcoding. Not only was the technique less labor-intensive and time-consuming, it had also high specificity and sensitivity with the capability of application in moderately equipped laboratories.

Lacking cross-reaction of the COI probe to any of five non-target *Agrotis* species incorporated in the study demonstrated that the assay was specific enough to differentiate *A. segetum* from other sympatric, closely related species or the species with shared host plants.

Quick outcomes as well as high specificity and sensitivity are the prime principles that should be considered during application of any diagnostic technique. These criteria were totally fulfilled with real-time PCR assay used here; however, saving time is the most outstanding merit of the technique compared to the traditional morphological or conventional PCR-based methods of diagnosis.

The high efficiency and sensitivity confirm the consistency of the results. Moreover, low intra-assay and inter-assay variance confirms the feasible application of the assay throughout various laboratories. This method of identification is also convenient when a huge number of insect specimens should be processed. However, this does not denote that the real-time PCR is appropriate in every situation. The method targets just one species and treats all other moths as non-targets in the assay. If identification of those non-targets to species level is required, using DNA sequences might be helpful.

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ایجاد و اعتبارسنجی روش Real-time PCR برای شناسایی گونه‌ای رایج از کرم‌های طوقه‌بر: *Agrotis segetum* (Lepidoptera: Noctuidae)

پ. مطلب دخت، ل. فکرت، م. زکی‌عقل، غ. ح. مروج و ا. شیروانی

چکیده

کرم طوقه‌بر، *Agrotis segetum*، یکی از آفات مهم بسیاری از محصولات زراعی و سبزیجات در سراسر دنیا است. شناسایی مورفولوژیکی گونه‌های *Agrotis* اغلب براساس خصوصیات افراد بالغ بوده و کلیدهای شناسایی برای مراحل نابالغ غالبا موجود نیست. در تجارت بین‌المللی، در ایستگاه‌های قرنطینه غالبا مراحل نابالغ آفات یافت شده که همین امر شناسایی مورفولوژیک آنها را دچار چالش می‌کند. برای شناسایی سریع و دقیق همه مراحل زیستی *A. segetum*، روش TaqMan real-time PCR بر اساس ژن میتوکندریایی COI مورد استفاده قرار گرفت. تمامی نمونه‌های *A. segetum* (شامل مراحل زیستی مختلف) شناسایی شدند و در آزمون‌های اختصاصیت، در هیچ یک از ۵ گونه دیگر *Agrotis* واکنش متقابلی مشاهده نشد. آزمایشات کاملا تکرارپذیر و قابل اعتماد بودند. آزمایشات با نمونه‌های له شده و استفاده از آنها به عنوان DNA الگو، به خوبی انجام شد که نشان‌دهنده این موضوع بود که سهولت آزمایش با حذف مرحله استخراج DNA قابل افزایش است. این روش، با در نظر گرفتن سرعت، کارایی و همچنین حساسیت، روشی مناسب برای شناسایی تمام مراحل زیستی *A. segetum* می‌باشد.

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