Arch Biol Sci. 2018;70(3):425-432 https://doi.org/10.2298/ABS171103002I

Molecular identification of *Trichogramma* egg parasitoids of *Ostrinia nubilalis* in northeastern Serbia

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Received: November 13, 2017; **Revised**: January 12, 2018; **Accepted**: January 18, 2018; **Published online**: January 23, 2018

Abstract: The European corn borer *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Pyralidae) is a major pest of corn (*Zea mays* Linnaeus, 1753) in Serbia. Regular monitoring of *O. nubilalis* in the region of Kikinda (province of Vojvodina, Serbia) demonstrated a high percentage of parasitized eggs of this pest. Preliminary identification of the egg parasitoids based on morphological traits revealed that they belong to the genus *Trichogramma* Westwood, 1833 (Hymenoptera, Trichogrammatidae). The objective of this study was to identify the autochthonous *Trichogramma* species present in the region of Kikinda by using sequences of ribosomal DNA. Specimens of *Trichogramma* were reared from parasitized eggs of *O. nubilalis* collected at different localities in the region of Kikinda. The ITS2 gene was sequenced and subjected to BLAST searches of GenBank NCBI (National Center for Biotechnology Information). The majority of collected specimens were identified as *Trichogramma brassicae* Bezdenko, 1968, and a single specimen as *T. evanescens* Westwood, 1833. Intraspecific variation of *T. brassicae* was assessed by amplifying the mitochondrial cytochrome oxidase I (COI) gene. Wasps were also tested for the presence of the endosymbiotic bacteria *Wolbachia* Hertig, 1936 using *Wolbachia*-specific 16S and *wsp* primers as a preliminary test for evidence of thelytoky. Understanding of the diversity and biology of autochthonous *Trichogramma* species is a key step in the potential commercial implementation of these egg parasitoid wasps in the protection of corn fields in Serbia.

Key words: ITS2, *Ostrinia nubilalis,* PCR, *Trichogramma spp., Wolbachia*

INTRODUCTION

Corn is the most important cash crop in Serbia, with the country being one of the top ten world exporters [1]. Although the production of corn is affected by several harmful insects, the most important pest of corn in Serbia is the European corn borer (ECB) *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera, Pyralidae) [2]. The caterpillars of this species damage the ears, stalks and leaves of corn, significantly reducing the yield quality and quantity. The damage caused by *O. nubilalis* in Serbia is fostered by the practice of constant growth of maize in the same fields, without crop rotation, and by the inadequate use of pesticides [2]. Various control methods of *O. nubilalis* have been promoted, but due to the growth of environmental awareness, biological control methods are becoming favored and increasingly important.

Egg parasitoids of the genus *Trichogramma* Westwood, 1833 (Hymenoptera: Trichogrammatidae) have been successfully used for inundative biological control of many lepidopterous pests for more than 120 years [3,4]. Indeed, releasing *Trichogramma* to control ECB populations at the egg stage is one of the successful methods worldwide [5]. At least five *Trichogramma* species *(T. evanescens* Westwood, 1833*; T. brassicae* Bezdenko, 1968; *T ostriniae* Pang and Chen, 1974; *T. dendrolimi* Matsumura, 1926*, T. nubilale* Ertle and Davis, 1975) are known to be effective and have been released in the field to control *O. nubilalis* and its sibling species, the Asian corn borer *Ostrinia furnacalis* (Guenée, 1854) [6]. Among them, *T. evanescens*

was released in the former USSR [7] and Germany [8], while *T. brassicae* (syn. *T. maidis* Pintureau and Voegelé, 1980) was used to control *O. nubilalis* in other European countries such as Switzerland [9], France, Italy, Austria and the Netherlands [10]. These parasitic wasps are common natural enemies in agricultural areas of the Mediterranean [11-13], and during this study, their presence was registered in Serbia as well. Monitoring of ECB in the region of Kikinda in the province of Vojvodina in northeastern Serbia, has also revealed high rates of ECB egg parasitization by native *Trichogramma*. Identification of resident populations of *Trichogramma* wasps to species level has not been done in Serbia so far, thus limiting the potential for large scale use of these parasitoids in biological control. Therefore, it is essential to both identify the native populations of *Trichogramma* wasps and to understand their biology, since autochthonous species are likely to be the best adapted to environmental conditions in a specific ecosystem [14].

The taxonomy and identification of *Trichogramma* has historically been problematic because of their small size (<1 mm) and lack of reliable characters [15]. Diagnostic morphological features are very limited and those that are useful require specialized knowledge for interpretation [16-18]. The problem was solved by the establishment of molecular alternatives for reliable identification. Stouthamer et al. 15] first showed the usefulness of the DNA sequence of a ribosomal gene, the internal transcribed spacer 2 (ITS2), as a general identification method for *Trichogramma*, and this method has been successfully used to characterize and describe species of *Trichogramma* [19]. Following this lead, interspecific variation in ITS2 has been exploited by several researchers, resulting in the development of diagnostic molecular keys for the identification of *Trichogramma* species occurring in particular areas [11,20,21], in particular crops [17,19], and simply as a method to distinguish between closely related species [15,22-28].

The aim of this study was to identify the *Trichogramma* spp. parasitizing eggs of *O. nubilalis* in the corn fields of Serbia. Since different genotypes may perform differently (in terms of rearing and parasitization), we were also interested in quantifying genetic variations within species and determining the presence of the endosymbiotic bacterium *Wolbachia* Hertig, 1936. *Wolbachia* is known to induce parthenogenesis in several species of *Trichogramma* [18], resulting in heavily female-biased populations, which in turn are beneficial for biological control (since only females parasitize eggs).

MATERIALS AND METHODS

While monitoring the ECB in different corn fields in the region of Kikinda, 150 egg clusters of this pest have been registered. Among them, 97 egg clusters of ECB were parasitized. Twenty-eight of these parasitized egg clusters were collected and used for rearing parasitoids (Supplementary Table S1). Host eggs were identified by the specific structure of the egg cluster and the morphological traits of emerged larvae from non-parasitized eggs. Parasitized egg clusters were sampled from leaves of corn between late July and mid-August 2016, which corresponded to the early oviposition period of females of the second generation of ECB. Parasitized egg clusters were stored in vials at room temperature, and after a few days the first adult *Trichogramma* wasps started to emerge. Emerged wasps from each parasitized egg cluster were preserved in labeled vials with 95% ethanol. Vials were stored in a refrigerator at 4°C for 4 months prior to molecular analyses.

DNA extraction and amplification

Molecular studies were conducted at the Department of Entomology, University of California, Riverside, USA. Genomic DNA was extracted from a single female wasp of each sample (in total 28 samples) using a HotSHOT method in a total volume of 48 μL [29]. The HotSHOT method is rapid and nondestructive. As such, morphological traits of the wasps are preserved and can be accessed later should the need arise. Extracted rDNA was stored in a freezer at -20°C.

The ITS2 region was amplified in a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA) using the following primers: forward, 5′-TGTGAACTGCAGGACACATG-3′ (ITS2-forward) located in the 5.8S ribosomal DNA (rDNA); and reverse 5′-GTCTTGCCTGCTCTGAG-3′ (ITS2rev-Trich) located in the 28S rDNA [15]. PCR was performed in 25-μL reaction

volumes containing 5 μL of DNA template, 1X Thermopol PCR buffer (New England Biolabs, Ipswich, MA, USA), an additional 1 mM of $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, 400 μM of dUTP, 0.2 μM each of the forward and reverse primers and 1.5 U Taq polymerase (NEB). The PCR cycling program was 3 min at 95°C, followed by 37 cycles of 45 s at 92°C, 45 s at 53°C and 1 min and 15 s at 72°C, with 3 min at 72°C after the last cycle. Amplification was confirmed by electrophoresis of 5 μL of the PCR product on a 1.5% agarose gel stained with ethidium bromide. To determine the size of the PCR product, a 100-bp molecular weight marker was used (Fermentas). *Trichogramma pretiosum* DNA was used as a positive control and the PCR reagent mix without DNA was a negative control.

A second PCR was used to amplify a section of the mitochondrial gene (mtDNA), cytochrome oxidase c subunit 1 (COI), commonly used for DNA barcoding studies [30]. PCR was again performed in 25-μL reactions containing 2 μL of DNA template (concentration not determined), 1X ThermoPol PCR Buffer (NEB), an additional 1 mM of $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, 400 μM dUTP, 4% (v/v) BSA (NEB), 1 U Taq polymerase (NEB) and 0.2 μM each of the primers LCO1490 and HCO2198 [31]. The thermocycling protocol was an initial denaturing step of 2 min at 94°C, followed by five 30 s cycles at 94°C, 1 min and 30 s at 45°C, and 1 min at 72°C, followed by a further 35 30-s cycles at 94°C, 1 min and 30 s at 51°C, 1 min at 72°C, and a final extension of 5 min at 72°C. Amplification was confirmed by gel electrophoresis as described above.

Sequencing and identification

PCR products were purified with the Illustra ExoPro-Star kit (GE Healthcare Life Sciences). Ten μL of PCR product was mixed with 1 μL of alkaline phosphatase and 1 μL of exonuclease I and incubated at 37°C for 30 min. The reaction was terminated with an 80°C step for 20 min. Each purified PCR product was sent for direct sequencing in both directions at the Institute for Integrative Genome Biology (University of California, Riverside). Forward and reverse reads were manually aligned and checked for agreement in the BioEdit Sequence Alignment Editor ver. 7.2.5. BLAST searches of the GenBank NCBI database were conducted to determine the similarity of each sequence to those already deposited in the database. Sequence matches >99% were used to diagnose the specific identity of each specimen. All sequences were deposited in GenBank.

Identification of the presence of Wolbachia

The *Wolbachia* infection status of each molecularly identified specimen of *Trichogramma* (1-28) was initially assessed by amplifying the 16S gene of the bacterium using primers and protocols detailed in Werren and Windsor [32]. For specimens testing positive in the initial assessment, the more informative *wsp* gene was subsequently amplified in 25-μL PCR reactions containing 1 X Thermopol PCR buffer (NEB), an additional 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, 400 μM dUTP, 2% (v/v) BSA (NEB), 0.8 μM each of the forward (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and reverse (5’-AAA AAT TAA ACG CTA CTC CA-3′) primers [33] and 1 U Taq polymerase (NEB). PCR cycling conditions were a 2-min pre-dwell at 94°C, followed by 38 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a post-dwell period of 10 min at 72°C. PCR products were run on a 1% agarose gel stained with ethidium bromide to determine if the PCR had been successful, and the amplified products of the *wsp* gene were purified and sequenced as described above. Sequences were deposited in GenBank.

RESULTS

Initially, the research was encouraged by findings of a high percentage of parasitized eggs of *O. nubilalis* (Table 1). Examination of egg clusters of *O. nubilalis* collected in the Kikinda region between late July and mid-August 2016 revealed a high portion of parasitization that ranged between 50 % and 100 %, depending on the locality and date of collection. For the entire period of collection, the parasitization rate was between 48.18% (at the locality Stankić) and 78.35% (at the locality Nakovo), while for the entire region, 67.38% of the collected egg clusters were parasitized (Table 1).

Table 2. Data on findings of parasitized egg clusters of *Ostrinia nubilalis* in the region of Kikinda (July-August 2016).

Locality	Date	Number of examined egg clusters	Parasitized egg clusters		Parasitization rate for the locality (%)
			No	%	
Kinđa	27.07.2016	8	6	75	63.59
	3.8.2016	7	4	57.14	
	9.08.2016	29	17	58.62	
Stankić	3.08.2016	6	3	50.00	48.18
	9.08.2016	22	12	54.55	
	17.08.2016	5	2	40.00	
Banatska Topola	3.08.2016	6	3	50.00	68.18
	9.08.2016	11	6	54.54	
	17.08.2016	6	6	100	
Nakovo	3.08.2016	8	4	80.00	78.35
	9.08.2016	9	6	66.67	
	12.08.2016	12	11	91,67	
	17.08.2016	8	6	75.00	
Banatsko Veliko Selo	3.08.2016	4	3	75.00	75.00
	9.08.2016	2	1	50.00	
	17.08.2016	7	7	100.00	
TOTAL		150	97	67.38	

Table 2. Haplotypes of *Trichogramma spp.* detected in the region of Kikinda in 2016.

Haplotype mark	Species	Specimens sharing the same haplotype
TbSERB-1	*T. brassicae*	ALEX-8, ALEX-13, ALEX-14, ALEX-19, ALEX-20, ALEX-1, ALEX-10, ALEX-28, ALEX-5, ALEX-25, ALEX-2, ALEX-11, ALEX-12, ALEX-23
TbSERB-2	*T. brassicae*	ALEX-3, ALEX-4, ALEX-17, ALEX-18, ALEX-27
TbSERB-3	*T. brassicae*	ALEX-26, ALEX-9
TbSERB-4	*T. brassicae*	ALEX-21
TeSERB-5	*T. evanescens*	ALEX-6

Twenty-six of the 28 tested samples yielded amplifiable ribosomal DNA. Two unique ITS2 sequences were identified among the samples. Samples that failed to amplify were one sample from Nakovo and one from Banatsko Veliko Selo (ALEX-22, ALEX -24). BLAST searches of GenBank revealed that by far the most abundant species (in 25 of 26 samples) was *T. brassicae*; 39 out of 40 GenBank accessions (deposited from multiple studies) that bore >99% similarity to our sequence were positively identified as *T. brassicae* by the authors (accession number KM279941), with the remaining accession being identified only to the genus level. The remaining sample (ALEX-6 from the

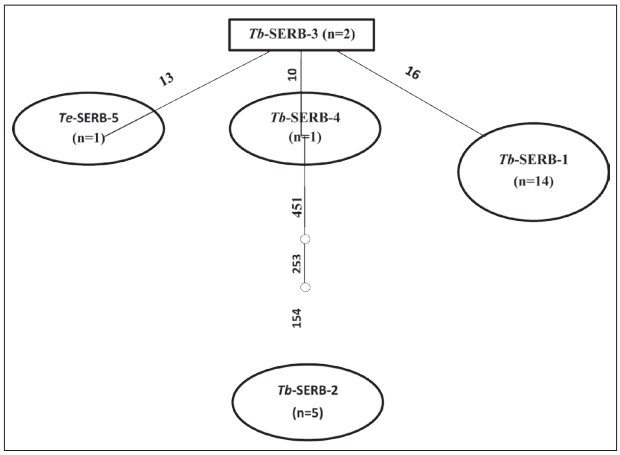

Fig. 1. COI haplotype network of *Trichogramma spp.* detected in the region of Kikinda, Serbia, 2016. Each connecting line between circles in the haplotype network represents a single nucleotide change, the number next to the line indicates the position in our sequences where that change occurred, and the circles represent hypothetical haplotypes that were not detected in our sample.

locality Stankić) was identified as *T. evanescens*; 37 out of 40 GenBank accessions (deposited from multiple studies) that bore >99% similarity to our sequence were positively identified as *T. evanescens* by the authors (accession number JF920459).

Amplification of the COI region of mitochondrial DNA was successful in only 23 samples (Table 2). In addition to the two samples for which ITS2 failed to amplify, sample numbers ALEX-7, ALEX-15 and ALEX-16 also failed. Among the 23 sequences, five haplotypes were detected (Table 2). All changes were synonymous. Two haplotypes (TbSERB-1 and TbSERB-2) were relatively abundant, being present in 14 and 5 individuals, respectively. The sequence positively detected in TbSERB-1 (at localities Kinđa, Stankić, Banatska Topola, Nakovo and Banatsko Veliko Selo) were previously detected in Iran (accession number KC488658), while the sequence positively detected in TbSERB-2 (at localities Kinđa, Nakovo and Banatsko Veliko Selo) was previously reported from material reared by Andermatt Biocontrol, Switzerland (accession number: FM210198). Surprisingly, the COI sequence of the one *T. evanescens* sample (identified by ITS2), TeSERB-5, was very similar to those of others (Fig. 1), and about 5% different from the nearest sequences attributed to *T. evanescens* in GenBank (e.g. KC488677).

As a first test, five samples tested positive for *Wolbachia* based on the 16 S gene: samples ALEX-2 (from

Kinđa), ALEX-5, ALEX-11, ALEX-12 (from Stankić), and ALEX-23 (from Banatsko Veliko Selo). These five samples were all COI haplotype TbSERB-1, but not all specimens with this haplotype were infected (35.71% of the samples were positive for *Wolbachia*). Sequences of the 16 S gene are unlikely to contain enough resolution to determine conclusively the likely source of the bacterium *Trichogramma* itself, or its lepidopteran host (potentially present in the gut of the *Trichogramma*). PCR products were subsequently generated for the *wsp* gene and sequenced. Sequences of the *wsp* gene were identical to several previously deposited sequences in GenBank (accession number FJ441291), also attributed to a *Wolbachia* endosymbiont of *T. brassicae*. The same *wsp* gene region from the *Wolbachia* endosymbiont of *Ostrinia* (e.g. GU166588) was very different.

DISCUSSION

Mass release of *Trichogramma* spp. is a promising alternative for ECB control in the EU, avoiding or reducing the need for broad-spectrum insecticide applications [34]. According to the same authors, the parasitoid wasps are released against ECB on about 150000 ha of maize per year, mainly in France, Germany and Switzerland, with the widest implementation in France. It is accepted that there are three general approaches to biological control of pests: importation, augmentation (inoculative or inundative activities) and conservation of natural enemies [35,36]. Augmentation and conservation of autochthonous species are good choices because the natural enemy is already present and therefore likely to be well adapted to environmental conditions in a specific ecosystem [14]. For development of a successful inundative biological control program, it is necessary to reduce costs, facilitate mass production processes and establish efficient methods for providing a large number of beneficial insects at the appropriate time [37]. A large number of traits have been studied to improve biological control strategies, such as host searching behavior, dispersal, emergence rate of the parasitized eggs, percentage of non-deformed females, walking speed and walking activity [38-44]. Since only female wasps have the potential for pest control, the sex ratio is of particular importance for the mass rearing of parasitoids for release [45]. The use of thelytokous strains has been proposed to provide advantages for biological control because they produce a higher proportion of females [46]. However, the evidence for this is somewhat conflicting. The parasitism rate of *T. brassicae* on *Ephestia kuehniella Zeller*, 1879 eggs has previously been shown not to be affected by infection with *Wolbachia*, but infection was found to negatively affect individual fecundity [46]. Similar reduced levels of fecundity in *Wolbachia*-infected lines have also been reported for other *Trichogramma* species in both laboratory and greenhouse conditions [47-50]. Thus, when viewed at the individual level, it appears that *Wolbachia* infection is not good for biological control. However, if viewed at the population level, it is argued that any reduction in individual fecundity is more than compensated for by the much higher proportion of females present in a mass-reared *Wolbachia*-infected population. Relatively low levels of *Wolbachia* infection were detected in the Serbian *T. brassicae* populations sampled in this study. Among four haplotypes, only one haplotype was associated with infection, and then only at an apparent rate of about 36%. Such "mixed" infections appear to be quite common in *Trichogramma* species [51]. Any effects of this particular *Wolbachia* strain on its host's reproduction were beyond the scope of this initial study, but the DNA sequence of its *wsp* gene revealed it to be closely related to a strain associated with parthenogenesis in *T. pretiosum*. Therefore, in light of its potential incorporation into a mass-rearing program, this warrants further investigation.

The reasonably high incidence of parasitism of *O. nubilalis* eggs by autochthonous populations of *Trichogramma* wasps in the studied region of Serbia represents a great potential opportunity for biological control of *O. nubilalis.* However, a fundamental first step prior to mass rearing and commercial utilization of these beneficial insects is their correct identification. Consequently, understanding the biology and behavior of native populations of *Trichogramma* wasps should help us to choose the most suitable species for mass rearing and field release. The current study used DNA sequences of the ITS2 gene to identify the most prevalent species in Vojvodina as *T. brassicae*, with only a single specimen identified as a different species, *T. evanescens*. While public repositories such

as GenBank are prone to the inclusion of sequences that have been attributed to misidentified specimens (as erroneous data), we believe our diagnoses based on ITS2 are correct given that they aligned with multiple GenBank accessions deposited by multiple studies and multiple authors, and were designated as *T. brassicae* or *T. evanescens*, respectively. However, it is surprising that the COI sequence of our single *T. evanescens* specimen was not more divergent from those obtained for our *T. brassicae* specimens. The simplest explanation for this is overlapping levels of interspecific variation within this mitochondrial gene. Given our small sample size, it is difficult to expand on this hypothesis, except to say that in contrast to the nuclear ribosomal ITS2, the mitochondrial COI gene has not been well characterized in *Trichogramma*. Indeed, for the species *T. brassicae* and *T. evanescens*, the majority of accessions currently relate to unpublished work on specimens from a single country (Iran; KC488651-684). This is unlikely to capture the full "global" extent of variation within and between these species.

This species has previously been used to target *O. nubilalis* in several other European countries. Because of the high potential of these beneficial insects in Serbia it is necessary to implement *Trichogramma* wasps in the program of biological control of economically important pests, such as the ECB. As such, mass-rearing and release protocols of *T. brassicae* are strongly recommended for implementation in programs of biological control against *O. nubilalis* in Serbia. The biological control program targeting *O. nubilalis* in Serbia should focus on augmentative and conservation activities for upgrading and preserving resident *Trichogramma* populations. Augmentation includes activities in which populations of natural enemies are increased through mass production, periodic release (either inoculative or inundative) and colonization for suppression of native or introduced pests [52]. Beside inundative and inoculative activities for the commercial use of *Trichogramma* in Serbia, it is also necessary to preserve the autochthonous/native resident populations. Conservation of habitats and preservation of biodiversity, as the main prerequisites for successful implementation of biological control strategies, can be defined as the study and modification of human influences that allow natural enemies to realize their potential to suppress pests [52]. Further work is required to investigate potential differences in performance (e.g. host-seeking behavior, dispersal capacity, emergence rate) of different *T. brassicae* genotypes. This may be particularly relevant in the case of populations that are infected with *Wolbachia,* since this bacterium renders some *Trichogramma* species parthenogenetic and therefore more efficient in terms of biological control.

Acknowledgments: The study was supported by the Forecasting and Warning Service of Plant Protection in Serbia, the Ministry of Education, Science and Technological Development, Republic of Serbia (Projects TR31084 and III43007), "Adama SRB" DOO, and by the Department of Entomology, University of California Riverside. The authors are grateful to Branislav Trudić, MSc, Melita Dejanović, Dragan Kerkez, Dragana Dragomirov and Mladen Đuran from the Forecasting and Warning Service in Plant Protection of Serbia for the help in field work.

Author contributions: Aleksandar Ivezić was responsible for collecting the data and samples, the entire molecular analysis and coordination of article writing, Paul Rougman-Jones and Richard Stouthamer were equally responsible for hosting and supervising the molecular analysis, result interpretation and review of the draft version of the manuscript, Aleksandra Ignjatović Ćupina was responsible for the hypothesis design, proofreading the manuscript, choice of journal and technical preparation of the manuscript.

Conflict of interest disclosure: The authors declare that there are no conflicts of interest.

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Supplementary data

Supplementary data can be accessed via the following link: http://serbiosoc.org.rs/sup/Sup2365.pdf