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Rapid molecular identification of *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitizing the eggs of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae) in Serbia

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Abstract

Background: The European corn borer (ECB), *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae), is the major pest of maize (*Zea mays* Linnaeus, 1753) in Serbia. One potential method for managing this pest is the augmentative release of naturally occurring egg parasitoids of the genus *Trichogramma*. The first step in this process is accurately identifying the naturally occurring species and estimating their natural distribution and abundance. Molecular identification, based on differences in DNA sequences, has commonly been employed for the identification of *Trichogramma* species. A simple, quick, and accurate molecular assay is urgently required for the identification of two common *Trichogramma* species, associated with ECB in Serbia: *T. brassicae* Bezdenko, 1968 and *Trichogramma evanescens* Westwood, 1833. Such an assay will facilitate an expansive survey of resident populations of *Trichogramma* associated with ECB across agricultural growing regions of Vojvodina province.

Results: A species-specific multiplex PCR assay for the 2 species was developed and validated that assay using a sample of 79 parasitoid wasps reared from ECB egg masses collected from sample sites across Vojvodina province. *Trichogramma brassicae* was confirmed as the dominant egg parasitoid of ECB in this region, accounting for 77 of the 79 wasps (97.47%). The remaining 2 were confirmed as *T. evanescens*. *Trichogramma brassicae* was detected at all 12 sample sites, while *T. evanescens* was detected at only 2 plots, Mokrin and Nakovo.

Conclusions: The species-specific multiplex PCR assay presented herein can provide the basis of a quick, cheap, and reliable means for identifying the species of *Trichogramma* that parasitize ECB egg masses in Serbia. Two currently documented species, *T. brassicae* and *T. evanescens*, are readily diagnosed by the size of the PCR product they produce in the assay. Any additional species are expected to not produce a band of a diagnostic size. Such species would subsequently be identified by sequencing, which may also allow them to be promptly incorporated into a revised assay.

Keywords: Maize, Species-specific multiplex PCR, *Trichogramma brassicae*, *Trichogramma evanescens*, ITS2

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Background

Maize (*Zea mays* Linnaeus, 1753) is the number one crop in Serbia. The largest areas of maize production are located in the northern province of Vojvodina, where it is grown on about 620,000 ha, with a total production of over 4 million tons (Latković et al., 2018). Although the production of maize is affected by several harmful insects, the most important maize pest in Serbia is the European corn borer (ECB), *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera, Crambidae) (Čamprag et al., 1983). Management techniques commonly used to reduce ECB infestations include changes to cultural practices, the planting of resistant cultivars, and the application of chemical insecticides (Maissle et al., 2010), the last being the most commonly employed approach. Chemical insecticides are not entirely effective against ECB because of the relatively short period the insect spends outside the plant prior to boring into it. Furthermore, there are general concerns about the effects of insecticides on beneficial insects and the wider environment, and specific concerns among the general public about insecticide residues on agricultural products (Phoofolo, 1997). Due to such growing awareness, biological control methods are becoming more favored and increasingly important (Ivezić et al., 2020).

One particular biological control method that has attracted a lot of attention is the augmentative release of naturally occurring species of the genus *Trichogramma* Westwood, 1833 (Hymenoptera: Trichogrammatidae) (Grushevaya, 2020). *Trichogramma* spp. are tiny (<1mm) wasps that parasitize the eggs of many insect species and are widely used in inundative and inoculative biological control programs against several pests in the EU (Golbaghi et al., 2020). Several species of *Trichogramma* have been identified as promising biological control agents of ECB, including *T. brassicae* and *T. evanescens* (Van Schelt and Ravensberg, 1991). Both species appear to be widespread across Europe and are reared in commercial facilities for release as biological control agents.

Serbia is one of the countries, at the southeast border of Europe, that does not actively use *Trichogramma* species in its agricultural practices, despite evidence suggesting high activity of native populations of these insects (Ivezić et al., 2020). Not all *Trichogramma* species (or populations) perform equally well in terms of mass rearing or field dispersal and parasitism rates (Borba et al., 2005). Morphological identification of *Trichogramma* requires expert taxonomic knowledge, due to their minute size (<1 mm long) and a general lack of reliable characters (Stouthamer et al., 1999). Consequently, alternative identification methods have been developed. Sequences of the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal DNA are one accepted means of identifying *Trichogramma* species (Fahriye et al., 2009). An initial

survey of the Kikinda region identified two species of *Trichogramma* (*T. brassicae* and *T. evanescens*) parasitizing ECB eggs, based on sequences of the ITS2 gene (Ivezić et al., 2018). A second, more geographically expansive study identified the same two species, again based on DNA sequences, but this time of the mitochondrial cytochrome oxidase subunit 1 (COI) gene (Ivezić et al., 2020). In both studies, *T. brassicae* was found to be far more abundant in maize than was *T. evanescens*.

The utility of ITS2 and COI sequences as diagnostic tools for *Trichogramma* (and many other taxa) is widely accepted, but obtaining a DNA sequence incurs significant costs (time and money). One way that can be achieved is through the development of species-specific PCR primers that can be run alone, or better still, in a multiplex reaction. Species-specific multiplex PCR incorporates several PCR primers that bind to the same DNA strand, each of which is specific to only one “target” species (Rugman-Jones et al., 2020). When combined in a single reaction with a single complementary PCR primer that is “universal” to all target species, the multiplex PCR results in the production of a PCR product of a unique size for each of the target species, allowing specimens to be identified directly following gel electrophoresis of the PCR product (Rugman-Jones et al., 2020).

The aims of this study were (1) to develop a diagnostic species-specific multiplex PCR assay for *T. brassicae* and *T. evanescens* in Serbia and (2) to use that assay to survey resident populations of *Trichogramma* associated with ECB across agricultural growing regions of Vojvodina province.

Methods

Multiplex PCR design

Previous studies of the egg parasitoids of ECB in Serbian maize resulted in the identification of just two species, *T. brassicae* and *T. evanescens* (Ivezić et al., 2020). Identification in these studies was based on DNA sequences of the ITS2 and/or COI genes. An assay based on the former gene that was chosen to develop ribosomal sequences typically show lower levels of intraspecific variation than mitochondrial sequences. Ivezić et al. (2018) resulted in the acquisition of ITS2 sequences from 26 specimens, 25 of which were *T. brassicae* and the other *T. evanescens* (GenBank accessions MW377755-MW377780). These were combined with a further 31 sequences of *T. brassicae* and 50 sequences of *T. evanescens* retrieved from GenBank and aligned in MAFFT version 7.050 (<http://mafft.cbrc.jp/alignment/software/>) using the Q-INS-I iterative strategy (Katoh and Standley, 2013). This alignment (available in the [supplementary material](#)) was used as the basis for designing species-specific PCR primers using the Primer3 (v.0.4.0) software (Rozen and Skaletsky, 2000). Criteria for the design of species-specific primers were as follows: (1) they should

lie in stretches of the ITS2 that display minimal levels of intraspecific variation particularly at the 3' end of the primer, (2) they should work in tandem with the "universal" forward primer ITS2 for (Stouthamer et al. 1999) which is located in the highly conserved 5.8S adjacent to ITS2, (3) at least one nucleotide at the 3' end of the primer must be unique to its target, (4) complementarity between primers should be minimal to avoid the production of primer dimers, (5) they should all work at similar annealing temperatures, and (6) they should produce species-specific amplicons that differ sufficiently in size to allow their diagnosis using standard agarose gel electrophoresis. The specificity of several primers for each species was further assessed by conducting BLAST searches of the primer sequences against the ITS2 sequences of eight further *Trichogramma* species that are common in the Mediterranean region: *T. bourarachae*, *T. cacoeciae*, *T. cordubensis*, *T. dendrolimi*, *T. euproctidis*, *T. nerudai*, *T. oleae*, and *T. pinto* (GenBank accessions listed in Sumer et al. 2009 (Table 2)). Any primers with significant similarity to these additional species were immediately rejected. Several primers were subsequently tested individually and in multiplex PCR against DNA from the original 26 specimens (Ivezić et al. 2018), resulting in the identification of a set of primers, which gave the best combination of the desired characteristics (Table 1 and Fig. 1). The resulting assay was performed in individual 25 µl reactions containing 1X DreamTaq PCR mastermix (ThermoScientific), 0.2 µM of each primer (Table 1), and 2.0 µl template DNA. Cycling was done on a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA) programmed for an initial 3-min denaturation step at 95 °C, followed by 38 cycles of 20 s at 95°C, 20 s at 55°C, 1 min at 68°C, and a final extension of 3 min at 68°C. Amplification was visualized by electrophoresis of 5 µl of each PCR product on a 1.5% agarose gel stained with ethidium bromide. To estimate the size of the PCR products, a 100 bp molecular weight marker was used (Fermentas). Verified *Trichogramma evanescens* DNA was used as a positive control and the PCR reagent mix without DNA as a negative control.

Identification of native populations of *Trichogramma*

Trichogramma wasps were reared from parasitized egg clusters of ECB collected from the leaves of maize sampled

at 12 different corn fields/plots in Vojvodina (Fig. 2). Sampling took place between late July and late September 2017 in correspondence with the activity of II and III generations of ECB (Čamprag et al., 1983). At each site, 100 randomly selected plants of corn were inspected for the presence of ECB egg clusters using protocols detailed in Reid et al. (1996). Four of the sites were sampled on a single occasion (Čoka, Senta, Vrbas, and Gakovo), three sites were sampled on 2 different dates (Bašaid, Banatska Topola, and Stankić), 4 sites were sampled on 3 different dates (Mokrin, Idoš, Nakovo, and Despotovo), and only one site was sampled on 4 different dates (Njegoševo) (Table 2). Across all these sampling efforts, a total of 40 parasitized egg clusters were collected and transported to the laboratory, where they were subsequently stored in individual glass tubes (25±1 °C, 65±10% RH, 16:8 h [L:D] photoperiod), until the emergence of the adult parasitoids. Following emergence, the wasps from each parasitized egg cluster were preserved in labeled vials with 95% ethanol and stored at -20 °C.

Genomic DNA was isolated from the ethanol-preserved females, using a HotSHOT method (Truett et al. 2000), following the protocol described by Ivezić et al. (2018). Where possible, 2 female *Trichogramma* were randomly selected from those that emerged from each of the 40 laboratory-reared ECB egg clusters. Specimens were then identified to species, using the multiplex-PCR assay detailed above.

Results

The species-specific multiplex PCR assay yielded amplicons (bands) of a diagnostic size for each of the target species, *T. brassicae* (199bp) and *T. evanescens* (480bp; Fig. 1). Validation of the assay was sought by aiming to identify 2 female parasitoids emerging from each of 40 collected ECB egg clusters. This was possible for all but one egg cluster (JL40; Table 2), which only yielded a single female wasp. Thus, a total of 79 females were identified, using the species-specific multiplex PCR assay (e.g., Fig. 1). Seventy-seven out of 79 wasps (97.47%) were identified as *T. brassicae*, and the remaining 2 were confirmed as *T. evanescens*. No specimens failed to amplify. *Trichogramma brassicae* was detected at all 12 sample sites, while *T. evanescens* was detected at only 2 plots, Mokrin and Nakovo (Table 3). In both instances, the *T. evanescens* individual emerged from an egg cluster that also yielded a *T. brassicae* individual, indicating multi-parasitism of the cluster

Table 1 PCR primers used in the species-specific multiplex PCR. Primers were designed in the Primer3 (v.0.4.0) software (Rozen and Skaletsky, 2000) from complete sequences of ITS2 rRNA

Species	Primer name	Primer sequence	Amplicon size (bp)
All	ITS2for	5'-TGTGAACTGCAGGACACATG-3'	-
<i>T. brassicae</i>	<i>T.bras-R</i>	5'-CGACGTATCACACTGCTCCT-3'	199
<i>T. evanescens</i>	<i>T.evan-R</i>	5'-GCCGTCGTTACCTACTTTCG-3'	480

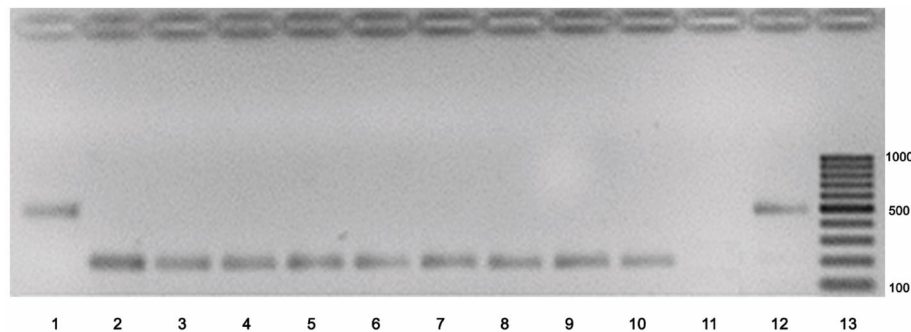


Fig. 1 Visualization of the species-specific multiplex PCR products identifying two species of *Trichogramma* that parasitize the eggs of the European corn borer, in Serbia. Lane legend, from right to left by ordered numbers: 1 *T. evanescens*; lanes 2–10 *T. brassicae*; lane 11 no template control; lane 12 positive control *T. evanescens*; lane 13 100bp DNA size standard (Fermentas Inc, USA). Products were run on a 1.5% agarose gel, stained with ethidium bromide

by both species. One of those egg clusters (JL14) yielded further 5 females (7 total); therefore, the molecular assay was used to identify each of them. Including the original 2 wasps, 5 of the females emerging from that cluster were *T. evanescens*, and 2 were *T. brassicae*.

Discussion

The species-specific multiplex PCR assay described herein relies on inter- and intraspecific variations in the DNA sequences of the locus that were targeted, ITS2. It

worked well in the particular agricultural system that was chosen, Serbian maize production. As such, it provided an excellent diagnostic tool for monitoring the *Trichogramma* species present in that specific system. Whether the assay, in its current format, would be applicable to further agricultural and natural ecosystems, in Serbia or further afield, or for identifying *Trichogramma* from the eggs of different insect pests, is unknown. The ITS2 region was targeted because it is typically well-conserved within species. However, it is

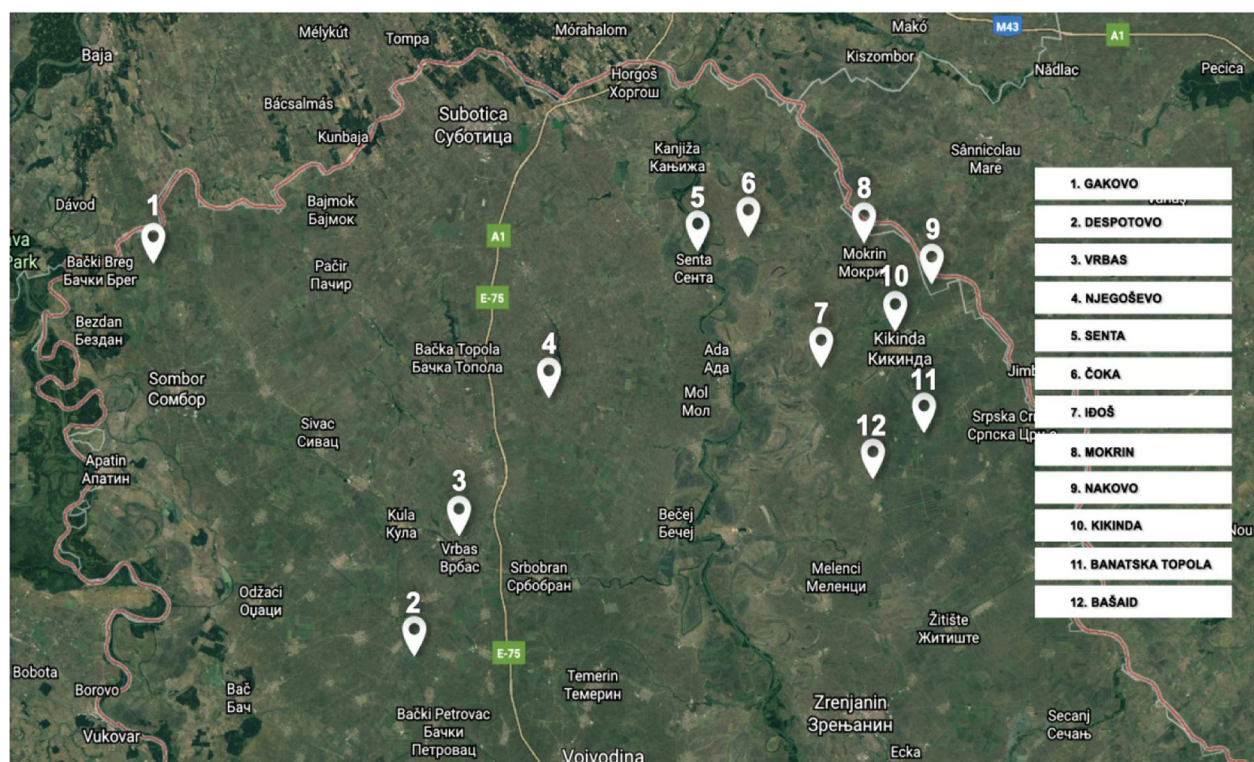


Fig. 2 Locations in Vojvodina where corn crops were sampled in search of parasitized ECB egg masses. Samples were collected in 2017. Map was extracted from Google Maps (earth.google.com/web/) with modifications in Adobe photoshop CC

Table 2 Data for forty samples of Serbian corn crops taken in search of parasitized ECB egg masses. For each sample, 100 randomly selected plants of corn were inspected

Plot	GPS coordinates	Collection date	Parasitized <i>O. nubilalis</i> egg clusters	
			Number	Label
Mokrin	45°53'0.39" N 20°28'15.41" E	20/07/2017.	1	JL1
		28/07/2017	2	JL2, JL3
		14/09/2017.	2	JL4, JL5
Iđoš	45°49'12.38" N 20°22'2.26" E	21/07/2017.	1	JL6
		07/08/2017.	1	JL7
		15/09/2017.	1	JL8
Bašaid	46°01'08.66" N 20°05'27.98" E	28/07/2017.	1	JL9
		13/09/2017.	2	JL10, JL11
Nakovo	45°51'51.54" N 20°35'18.37" E	04/08/2017.	1	JL12
		10/08/2017.	2	JL13, JL14
		14/09/2017.	1	JL15
Banatska Topola	45°40'41,71" N 20°29'13.02" E	28/07/2017.	1	JL16
		10/09/2017.	2	JL17, JL18
Stankić	45°51'08.11" N 20°25'03.22" E	08/09/2017.	2	JL19, JL20
		11/09/2017	1	JL21
Njegoševo	45°47'53.61" N 19°39'19.10" E	20/07/2017	1	JL22
		18/07/2017	1	JL23
		11/09/2017	1	JL24
		15/09/2017.	1	JL25
Despotovo	45°27'06.22" N 19°32'15.57" E	29/07/2017	2	JL26, JL27
		10/08/2017.	3	JL28, JL29, JL30
		17/9/2017.	3	JL31, JL32, JL33
Čoka	45°57'27.80" N 20°08'31.25" E	22/09/2017	1	JL34
Senta	45°53'53.92" N 20°05'14.01" E	22/09/2017	1	JL35
Vrbas	45°35'36.81" N 19°39'4.86" E	11/09/2017	2	JL36, JL37
Gakovo	45°54'41.07" N 19°04'42.11" E	18/09/2017	3	JL38, JL39, JL40
Total numbers for Vojvodina province			40	JL1 to JL40

clear from the alignment of ITS2 sequences retrieved from GenBank that the locus was not 100% homogenous across all specimens/populations from different studies ([supplementary file](#)). Indeed, the species-specific primer designed for *T. evanescens* actually spanned a variable stretch which was subject to a 2–4 bp insertion in 10 of the 50 (20%) existing GenBank accessions. Without access to that DNA, there was no way to certain that the assay would result in the amplification of DNA from those specimens, although there was some hope in the fact that the first 9 bases from the 3' end of the primer were identical across all 50 accessions. It cannot be certain exactly how the introduction of further species into the Serbian maize system might affect the assay, but non-significant similarity was found between the species-specific primers and the ITS2 sequences of 8 further *Trichogramma* species that are common in the

Mediterranean region (Sumer et al. 2009). It was more likely that specimens of additional species would simply fail to produce any PCR product. As such, the integrity of the extracted DNA of any specimen that did not yield an amplicon should be confirmed and followed by the amplification and sequencing of the complete ITS2 gene. If a “new” species was detected, it was then likely that it could be included in a redesigned assay.

The identification of arthropod pests and natural enemies using traditional morphological keys and/or DNA sequences of appropriate genes is critical, if expensive and time-consuming part of surveying any new area/ecosystem. Compared with traditional morphological identification, which requires lengthy processing and slide mounting of specimens, coupled with expert knowledge of the genus, the multiplex PCR assay allowed the accurate diagnosis of a large number of *Trichogramma* specimens in just a few

Table 3 Results of the molecular identification of sampled *Trichogramma* individuals on the territory of Vojvodina in 2017

Plot	Labels of sampled <i>O. nubilalis</i> egg cluster	Number of tested <i>Trichogramma</i> specimens	Identified species
Mokrin	JL1	2	<i>T.brassicae</i> <i>T. evanescens</i>
	JL2	2	<i>T.brassicae</i>
	JL3	2	<i>T.brassicae</i>
	JL4	2	<i>T.brassicae</i>
	JL5	2	<i>T.brassicae</i>
Iđoš	JL6	2	<i>T.brassicae</i>
	JL7	2	<i>T.brassicae</i>
	JL8	2	<i>T.brassicae</i>
Bašaid	JL9	2	<i>T.brassicae</i>
	JL10	2	<i>T.brassicae</i>
	JL11	2	<i>T.brassicae</i>
Nakovo	JL12	2	<i>T.brassicae</i>
	JL13	2	<i>T.brassicae</i>
	JL14	2	<i>T. evanescens</i> <i>T.brassicae</i>
Banatska Topola	JL15	2	<i>T.brassicae</i>
	JL16	2	<i>T.brassicae</i>
	JL17	2	<i>T.brassicae</i>
Stankić	JL18	2	<i>T.brassicae</i>
	JL19	2	<i>T.brassicae</i>
	JL20	2	<i>T.brassicae</i>
Njegoševo	JL21	2	<i>T.brassicae</i>
	JL22	2	<i>T.brassicae</i>
	JL23	2	<i>T.brassicae</i>
Despotovo	JL24	2	<i>T.brassicae</i>
	JL25	2	<i>T.brassicae</i>
	JL26	2	<i>T.brassicae</i>
Čoka	JL27	2	<i>T.brassicae</i>
	JL28	2	<i>T.brassicae</i>
	JL29	2	<i>T.brassicae</i>
Senta	JL30	2	<i>T.brassicae</i>
	JL31	2	<i>T.brassicae</i>
	JL32	2	<i>T.brassicae</i>
Vrbas	JL33	2	<i>T.brassicae</i>
	JL34	2	<i>T.brassicae</i>
	JL35	2	<i>T.brassicae</i>
Gakovo	JL36	2	<i>T.brassicae</i>
	JL37	2	<i>T.brassicae</i>
	JL38	2	<i>T.brassicae</i>
	JL39	2	<i>T.brassicae</i>
	JL40	1	<i>T.brassicae</i>

hours. Importantly, such a diagnosis can be completed by a person that has training only in relatively straightforward laboratory techniques (DNA extraction, PCR, and gel electrophoresis), thus helping to overcome the taxonomic impediment that may be associated with identifying large numbers of very small parasitoid wasps. A further advantage of the assay was that it allows the identification of females, which, in *Trichogramma*, can often only be identified by their association with males (Pinto, 1997). This is particularly important since *Trichogramma* populations often display heavily female-biased sex-ratios (Stouthamer et al., 1999).

ECB control in maize, in Serbia, is a very complex task, primarily due to the difficulties associated with the application of insecticides in advanced stages of corn development and because of the long oviposition period of ECB females. Given the negative side effects of chemical insecticides, the use of alternative strategies for ECB control should be considered by relevant decision-making and biological control stakeholders. The use of the multiplex assay in this study again demonstrated the widespread occurrence of 2 resident species of *Trichogramma* parasitizing the eggs of ECB. Coupled with the findings of surveys conducted in 2016 and 2018 (Ivezić et al. 2018, 2020), it is clear that *Trichogramma* species, particularly *T. brassicae*, are important, naturally occurring egg parasitoids of ECB in Kikinda and other areas of Serbia. Indeed, natural rates of parasitism by *Trichogramma* spp. have been reported to be as high as 70% in north-west Serbia, although that rate fluctuates year to year (Tancik, 2017). Collectively, these studies strongly suggest the potential for using *Trichogramma* in a biological control program. While the multiplex PCR assay described herein provided a useful tool for surveying naturally occurring *Trichogramma* populations, should future management plans for ECB in Serbia adopt an approach that does incorporate biological control, this assay (or something similar) may also provide an efficient means of monitoring the immediate success of field-released wasps and/or longer term genetic trends within parasitoid populations (Roltsch et al. 2021). Furthermore, this assay has a practical application for simply assisting with quality control in any future mass-rearing facilities, ensuring that rearing stocks are not invaded by another strain or species.

Conclusions

It is clear that the naturally occurring egg parasitoid *T. brassicae* and to a much lesser extent *T. evanescens* parasitize a significant number of ECB egg masses in Serbian maize. As such, Serbian agriculture should seriously consider developing a biological

control plan against ECB that focuses on the augmentative release of mass-reared *T. brassicae*. Species-specific multiplex PCR can provide a quick, cheap, and reliable tool to assist the planning, implementation, and future monitoring of such augmentative releases.

Abbreviations

ECB: European corn borer; ITS2: Internal transcribed spacer 2; COI: Mitochondrial cytochrome oxidase subunit 1 gene

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41938-021-00414-5>.

Additional file 1.. All ITS2 for alignment and multiplex primer design

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Authors' contributions

AI conducted field and laboratory work and participated in the interpretation of the obtained results as well as in writing of the manuscript. PRJ designed species-specific primers for multiplex PCR and was an important contributor in data analysis and writing of the manuscript. BT revised the manuscript and made a significant contribution to the final version of submitted paper. All authors contributed equally to this work. All authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published manuscript and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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