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Molecular identification of *Trichogramma* species parasitizing *Ostrinia nubilalis* in corn and pepper in south–east border of Europe

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ABSTRACT

The current study extends the faunistic survey of *Trichogramma* species parasitizing ECB to all agricultural growing regions in Serbia. Specimens of *Trichogramma* were reared from parasitized egg masses of ECB collected from field-grown corn and pepper crops. The number of egg masses parasitized varied by location. Using sequences of mitochondrial cytochrome oxidase I (COI) gene, we examined intra and interspecific variation in *Trichogramma* parasitizing the ECB egg masses. Seventy specimens were successfully sequenced of which, 65 were identified as *T. brassicae*. The remaining five were identified as *T. evanescens*, and this second species was only collected from pepper.

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Biological control; COI; PCR; Wolbachia

1. Introduction

Corn (*Zea mays L*) is one of the most important grain crops in the world. In Serbia, it is grown on 1-1,2 million hectares. More than half of it (52%) is on the territory of the province of Vojvodina and the rest is in the areas below the rivers Sava and Danube. Although, the production of corn is affected by several harmful insects, the most important pest of corn in Serbia is the European corn borer (hereinafter: ECB) *Ostrinia nubilalis* (Hübner 1796) (Lepidoptera, Pyralidae) (Čamprag et al. 1983). ECB is a highly polyphagous pest and can reproduce on many host plants. However, its preferred host is maize, and the large scale cultivation of maize has contributed to its rapid spread over large areas. In addition to the damage it causes in maize, ECB has been gaining increasing importance as a pest of pepper fruits in Serbia (Sekulić et al. 2005).

Various control methods of ECB have been promoted worldwide, with the application of chemical insecticides being the most commonly employed tactic. However, because ECB larvae spend most of their life inside the plant, chemical control is not easy, and there has always been interest in alternative control methods (Suverkropp et al. 2001). Due to the growing awareness of the impact of various chemical pesticides on the environment, biological control methods are becoming more favored and increasingly important. One such method is the augmentative release of commercially available species of the genus *Trichogramma* Westwood, 1833 (Hymentoptera: Trichogrammatidae).

Several species of *Trichogramma* have been identified as promising biological control agents of ECB (Beglyarov et al. 1977; Bigler 1986; van Schelt and Ravensberg 1991; Hassan 1993). *Trichogramma* are egg parasitoids that attack more than 200 lepidopteran host species, including the pest groups of borers, webworms, loopers, leafworms, fruitworms, cutworms, bollworms and armyworms (Knutson 1998). As a result, they have been widely used in inundative and inoculative biological control programs in more than 30 countries, in agricultural crops (e.g. corn, cotton, sugarcane, fruit trees, vegetables) and natural forests (Hase 1925; Sweetman 1958; Stinner 1977; King et al. 1985; Li 1994; Smith 1996; van Lenteren 2000; van Lenteren et al. 2003). Indeed, *Trichogramma* have been successfully used for inundative biological control of lepidopteran pests for more than 120 years (Smith 1994; van Lenteren 2000). Furthermore, it has been estimated that over 16 million ha globally receive parasitoid inundative releases (Hassan 1997; van Lenteren et al. 2003). This history includes releasing *Trichogramma* wasps to control ECB populations. In Europe, *Trichogramma* spp. are released against ECB in French (Meissle et al. 2010) and German (Hassan 1981) cornfields

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Hassan (1997) and van Lenteren et al. (2003) reported that over 16 million ha receive parasitoid inundative releases.

Trichogramma are common natural enemies in agricultural areas of the Mediterranean (Pintureau 1987; Silva et al. 1999; Oztemiz 2007) and several species have previously been identified as promising biological control agents of ECB, including *T. maidis* (Bigler 1986; van Schelt and Ravensberg 1991), *T. evanescens* (Hassan 1993) and *T. brassicae*, (Burgio and Maini 1995). Seven species of *Trichogramma* were previously recorded from the former Yugoslavia, *T. evanescens* (Boucek 1977; Injac and Krnjajic 1990), *T. minutum* (Boucek 1977), *T. embryophagum* (Voegelé et al. 1978), *T. pretiosum* (Danon 1989), *T. brassicae*, *T. cacoeciae* and *T. oleae* (Pintureau 1987, 2008), but little is known about their distribution in current day Serbia. Resident populations have been recorded in Serbia (Krnjajić 2002; Tancik 2017; Ivezić et al. 2018) but, identification to species level has only been done in isolated areas (Krnjajić 2002; Tancik 2017; Ivezić et al. 2018). Furthermore, little is known about intraspecific variation in these species (Ivezić et al. 2018). In *Trichogramma*, traits such as fecundity, egg maturation rate and offspring sex-ratio, have been shown to be influenced by intraspecific genetic variation (Wajnberg 2010; Wajnberg et al. 2012; Coelho et al. 2016). All such traits could affect the performance of a particular strain (or genotype) as a biological control agent. Thus, before we can truly assess the potential for large scale use of these parasitoids in biological control in Serbia, it is essential to first identify and characterize the native populations of *Trichogramma* countrywide. It is expected that such autochthonous/native species are likely to be the best adapted to environmental conditions in a specific ecosystem (Whitman and Nordlund 1994).

The taxonomy and identification of *Trichogramma* has historically been problematic (Stouthamer et al. 1999). Diagnostic morphological features are limited and those that are useful require specialized knowledge for interpretation (Nagaratti and Nagaraja 1968, 1971; Pinto and Stouthamer 1994). Morphological identification remains difficult mostly because of their minute size (<1 mm long) and the lack of reliable characters (Stouthamer et al. 1999). Consequently, alternative identification methods such as the use of reproductive compatibility studies (Pinto et al. 1991) and the incorporation of molecular methods (Stouthamer et al. 1999) 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 (Stouthamer et al. 1999). In addition, sequences of the mitochondrial cytochrome oxidase subunit I (COI) are commonly used in 'DNA barcoding' studies to identify biological material to the species level (Hebert et al. 2003). Barcoding has been shown to be particularly useful for insects (Zaldívar-Riverón et al. 2010; Malausa et al. 2011), including trichogrammatids (Thiruvengadam et al. 2016). Currently, around 210 species of *Trichogramma* are formally recognized worldwide (Pinto 2006), but this figure may change considerably with increasing use of molecular methods as tools for identification (Del Pino et al. 2013). Sequences of the COI gene also provide a very powerful tool with which to characterize within-species molecular diversity (Mardulyn and Whitfield 1999; Correa et al. 2016).

A further factor that may influence the success of *Trichogramma* wasps in release programs is their mode of reproduction (Stouthamer et al. 1999). The most common mode of reproduction in *Trichogramma* is arrhenotoky, in which females develop from fertilized eggs and males from unfertilized eggs (Nazeri et al. 2015). However, thelytokous reproduction also occurs in some populations/species, in which unfertilized eggs also develop as females. In *Trichogramma*, thelytoky is typically induced by the endosymbiotic bacterium *Wolbachia* (Louis et al. 1993; Stouthamer et al. 1993). All-female populations may be advantageous in the field of biological control since only females parasitize eggs, and, assuming similar levels of fecundity, all-female populations are expected to have higher growth rates, making them easier and cheaper to mass-rear (Stouthamer 1993). Considering the influence that *Wolbachia* may have on host reproduction and fitness, the presence of this bacterium in Serbian populations of *Trichogramma* needs to be investigated to assure the success of potential biological control programs.

The aim of this study was to identify the resident *Trichogramma* species associated with ECB across all agricultural growing regions of Serbia. *Trichogramma* species were identified by sequencing the COI gene and those sequences were further used to investigate the genetic structure of populations collected in different parts of Serbia. Finally, we also tested these Serbian *Trichogramma* for the presence of *Wolbachia*.

2. Material and methods

Trichogramma populations were sampled at 21 different sites/localities in Serbia (Figure 1 and Table 1). These sites covered four natural geographic regions separated from each other by natural barriers and/or relatively large distances: (I) a cluster of sites in the Autonomous Province of Vojvodina, an area north of the River Danube and close to the borders with Croatia, Hungary and Romania; (II) a central region comprising three sites 50–100 km south of Belgrade; (III) an isolated eastern site close to the confluence of the borders with

Romania and Bulgaria; and (IV) an isolated area in the south of the country, surrounded on three sides by mountains (Figure 1).

Sampling took place between late July and mid-September 2018; dates correspond with the activity of II and III generation of ECB (Čamprag et al. 1983). Seventeen of the sites were sampled on a single occasion, but two samples were taken at both Vrbas (corn and pepper) and Nakovo (different dates), three samples were taken at Leskovac (different corn fields) and four samples were taken at Vršac (two different pepper fields, on two different dates; Table 1). Thus, a total of 28 samples were taken. For each sample, 100 randomly selected plants of corn or pepper were inspected for the presence of ECB egg clusters. The total number of ECB egg clusters, and of parasitized egg clusters, was recorded. A single parasitized egg cluster from each sample was transported to the laboratory and maintained to enable the rearing of the parasitoids (Table 1). Following emergence, the wasps from each parasitized egg cluster were identified as male or female and preserved in labeled vials with 95% ethanol and stored at –20 °C.

2.1. DNA extraction

Molecular studies were conducted at the National Institute of Agricultural Research (INRA), Sophia Antipolis, France. Genomic DNA was isolated from ethanol-preserved females using the QuickExtract DNA Extraction Solution 1.0 kit (LU-QE09050 LUCIGEN) following the manufacturer's protocol. Where possible, three female *Trichogramma* were randomly selected from those that emerged from each of the 28 laboratory-maintained ECB egg clusters and individually placed in 20 µL of extraction buffer and incubated for 15 min at 65 °C and then for 2 min at 98 °C. There were three exceptions:

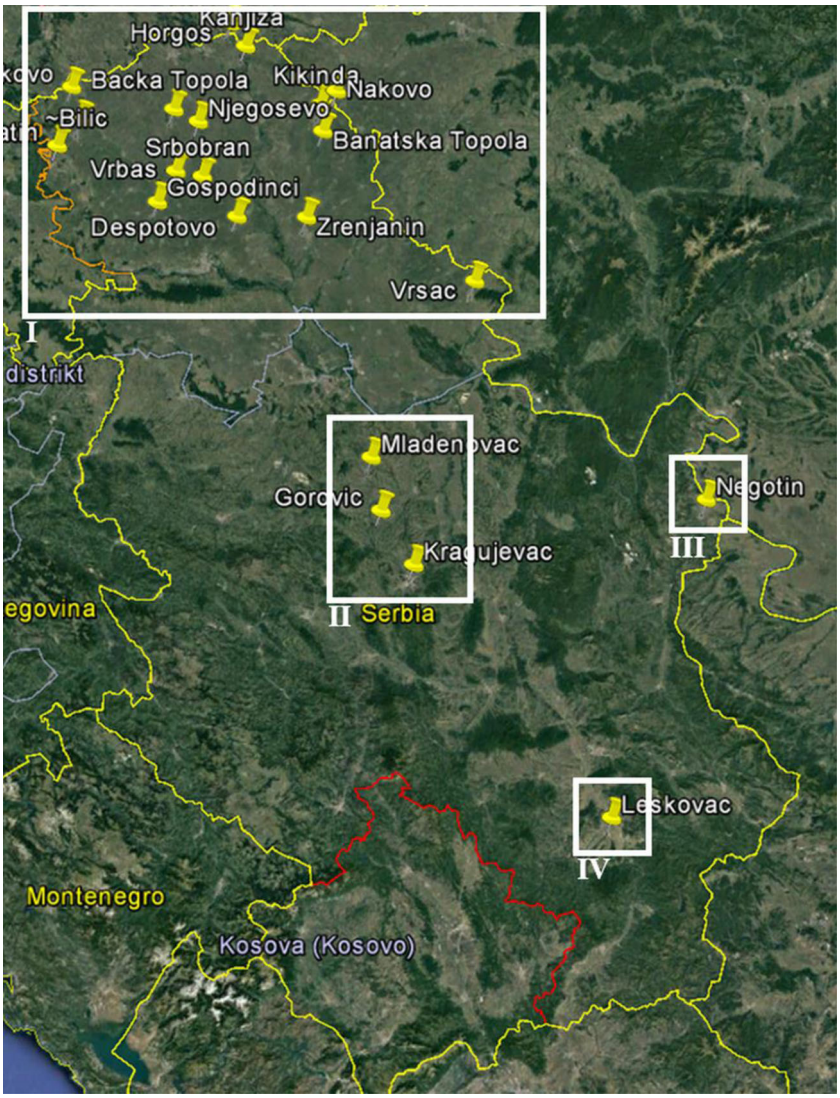

Figure 1. Locations in Serbia where corn and pepper crops were sampled in search of parasitized ECB egg masses. Sampling locations lay within one of four natural geographic regions: (I) an area north of the River Danube and close to the borders with Croatia, Hungary and Romania; (II) a central region 50–100 km south of Belgrade; (III) an isolated eastern site close to the confluence of the border with Romania and Bulgaria; and (IV) an isolated area in the south of the country, surrounded on three sides by mountains. Samples were collected in 2018. Map from Google Earth (earth.google.com/web/).

Table 1. Details of twenty eight samples of Serbian corn or pepper crops taken in search of parasitized ECB egg masses.

Region	Site	Sample number	Crop	Date of collection	Total egg clusters	Parasitized egg clusters	
						No	%
I	Despotovo	IA001	Corn	8 August 2018	5	1	20.0
I	Bačka Topola	IA004	Corn	16 September 2018	12	5	41.7
I	Njegoševo	IA008	Corn	4 August 2018	8	3	37.5
I	Vrbas	IA010	Corn	27 August 2018	3	1	33.3
I	″	IA011	Pepper	9 August 2018	4	2	50.0
I	Vršac	IA015	Pepper	8 August 2018	13	6	46.1
I	″	IA016	Pepper	8 August 2018	9	5	55.5
I	″	IA017	Pepper	10 August 2018	3	1	33.3
I	″	IA018	Pepper	10 August 2018	3	1	33.3
I	Horgoš	IA023	Corn	15 August 2018	11	4	36.4
I	Kanjiža	IA024	Corn	15 August 2018	13	7	53.8
I	Srbobran	IA027	Corn	22 August 2018	10	3	30.0
I	Gospođinci	IA028	Pepper	8 August 2018	5	1	20.0
I	Bilić	IA031	Corn	13 September 2018	14	5	35.7
I	Apatin	IA033	Corn	13 September 2018	6	3	50.0
I	Gakovo	IA034	Corn	13 September 2018	5	4	80.0
I	Zrenjanin	IA035	Corn	10 August 2018	14	6	42.8
I	Kikinda	IA038	Corn	11 August 2018	5	1	20.0
I	Nakovo	IA042	Corn	14 August 2018	3	1	33.3
I	″	IA043	Corn	13 September 2018	5	3	60.0
I	Banatska Topola	IA046	Corn	11 September 2018	8	5	62.5
II	Kragujevac	IA048	Corn	13 September 2018	5	1	20.0
II	Mladenovac	IA029	Corn	15 August 2018	9	1	11.1
II	Gorović	IA053	Corn	16 September 2018	7	1	14.3
III	Negotin	IA030	Corn	08 August 2018	8	1	12.5
IV	Leskovac	IA012	Corn	15 August 2018	6	1	16.7
IV	″	IA013	Corn	15 August 2018	4	1	25.0
IV	″	IA014	Corn	15 August 2018	5	1	20.0

For each sample, 100 randomly selected plants of corn or pepper were inspected. Also see Figure 1 for geographic location of the sites.

only two females emerged from samples IA013 and IA033; and, only one from IA023. In total, 80 individual females were extracted.

2.2. Molecular characterization of *Trichogramma* spp.

A fragment (about 700 bp) of the mitochondrial COI gene was amplified using the primers: LCO 1490 (5-GGTCAACAAATCATAAAGATATTGG-3) and HCO 2198 (5-TAAACTTCAGGGTGAC CAAAAAATCA-3) (Folmer et al. 1994). PCR amplifications were carried out in a total volume of 25 µL with 1 µL of DNA. PCR was performed with the Multiplex PCR Master Mix (QIAGEN, # 206145) with a final concentration of 3 mM $MgCl_2$ and 0.125 μL of each of the two primer solutions (100 μM). The PCR conditions were as follows: 95 °C for 15 min; 40 cycles at 94 °C for 30 s, 50 °C for 90 s, 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. The presence and size of PCR products was confirmed using a QIAxcel DNA Fast Analysis Kit (QIAGEN S.A.S) on a QIAxcel Advanced System (QIAGEN S.A.S). Financial constraints allowed the PCR products to be Sanger-sequenced in only one direction (with primer HCO2198), by GENEWIZ (Essex, UK). The COI sequences obtained in the current study were aligned using MAFFT version 7.050 (Katoh and Standley 2013) and the Q-INS-i algorithm, employing default settings. The aligned COI sequences were trimmed to a uniform length (542 bp) and a haplotype network was constructed using the statistical parsimony method of Templeton et al. (1992) in the software program TCS, version 1.21 (Clement et al. 2000). All haplotypes were translated and checked for stop codons and indels, to reduce the chances of including sequencing errors or NUMTs in subsequent analyses (Song et al. 2008), and subject to BLAST searches of the GenBank database (NCBI-National Center for Biotechnology Information) to identify matches (>99% similarity) with existing GenBank accessions. All sequences generated in this study were deposited in GenBank (accession nos. MT049959-MT050028).

Two species were identified in our samples, one common, one rare (see Results). Genetic variation was only examined in the common species. *Trichogramma* are not strong fliers and are thought to disperse on a local scale by hopping from plant to plant, walking, riding the wind and hitch-hiking on other insects (phoresy). Little is known about the dispersal abilities of natural *Trichogramma* populations but several studies have looked at dispersal of laboratory reared *Trichogramma* from a mass release site, and recorded localized spread of as much as 400 m, typically downwind (e.g. Bigler et al. 1990; Smith 1996; Kuske et al. 2003; Chapman et al. 2009; Fatouros and Huigens 2012; Sharma and Aggarwal 2015). This may facilitate passive dispersal of *Trichogramma* into neighboring crop fields and hence, localized genetic mixing across intensively farmed areas. In contrast, the ability of *Trichogramma* to actively migrate longer distances has not been rigorously investigated, but two separate studies that looked at dispersal of mass-released wasps into adjacent 'non-target' habitats both concluded that

any migration was limited to very short distances and likely dependent on the nature of that habitat, and the habitat preferences of the parasitoid (Orr et al. 2000; Kuske et al. 2003). The food plant species of its hosts, may also directly or indirectly affect *Trichogramma*. For example, simple differences in plant structural complexity may favor different parasitoid species or genotypes (reviewed by Romeis et al. 2005). In light of these factors, and since each of our 28 samples yielded a maximum of only three data points (DNA sequences), to facilitate a more meaningful analysis, the samples were divided into five population groups, based on geographic region (I–IV) and crop type (corn was sampled in all regions; pepper was only sampled in region I). This created five arbitrarily defined population groups, and genetic variation within these groups was examined in terms of haplotype number, haplotype diversity and nucleotide diversity (π), calculated using DnaSP Version 5.10.01 (Librado and Rozas 2009). Divergence between the population groups was estimated as the number of amino acid differences per site from averaging over all sequence pairs within and between groups (p-distance), calculated in MEGA version 6 (Tamura et al. 2013). The geographic distribution of the mitochondrial haplotypes was also mapped using PhyloGeoViz Version 2.4.5 (Tsai 2011) and visualized using GPS Visualizer (https://www.gpsvisualizer.com/map_input).

2.3. Wolbachia infection status

The DNA of each of the 80 extracted wasps was tested for the presence of *Wolbachia* using an established diagnostic marker (Dedeine et al. 2001). We attempted to amplify a 400 bp fragment of the *Wolbachia ftsZ* gene using 1.2 μL of DNA in 15 μL of final reaction volume, composed of 0.12 μL Taq (5 U/μL) (QIAGEN, # 201203), 1x PCR Buffer (QIAGEN), 1.5 mM $MgCl_2$, 0.5 μM forward FtsZ-F (TTGCAGAGCTTG GACTTGAA) and reverse FtsZ-R (CATATCTCCGCC ACCAGTAA) primers (Dedeine et al. 2001), and 200 μM dNTP. The PCR conditions were as follows: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min, with final elongation at 72 °C for 10 min. The presence of amplicons was determined with the QIAxcel DNA Fast Analysis kit (QIAGEN SAS) on the QIAxcel analyzer. *Wolbachia* infected *Psyttalia lounsburyi* Silvestri, 1913 (Hymenoptera: Braconidae) and uninfected *P. lounsburyi* were used as positive and negative controls, respectively.

3. Results

3.1. Field parasitism rates

Monitoring of ECB in 2018, conducted on 21 different sites on the territory of Serbia, revealed 203 egg clusters of ECB. Of those 203 egg clusters, 75 were parasitized. On a regional scale, the highest percentage of parasitized ECB egg clusters was registered in region I, the Autonomous Province of Vojvodina, with parasitization rate of 41.7%. This was followed by region IV with parasitization of 20.6%, while the lowest levels of parasitization were registered in regions II and III, at 15.3% and 12.5%, respectively. Comparing individual sample sites, parasitization rates (the percentage of egg masses parasitized) ranged between 11.1% and 80.0%, depending on the locality, date of collection and examined crop. The highest percentages of parasitized egg clusters were registered in corn fields at sites in region I (Gakovo [80.0%], Banatska Topola [62.5%] and Nakovo [60.0%]). The lowest percentages of parasitized egg clusters were also registered in corn fields, but in regions II (Mladenovac [11.1%] and Gorović [14.3%]) and III (Negotin [12.5%]) (Table 1).

Monitoring of corn and pepper fields was performed during the period of oviposition of females of II and III generation of ECB. Our data showed significantly higher activity of *Trichogramma* wasps registered in the first half of September, corresponding with the activity of the III generation ECB. Monitoring of oviposition of II generation of ECB revealed 31.5% of parasitized egg clusters, while during the activity of III generation of ECB parasitization rate was 45.5%.

3.2. Genetic characterization of *Trichogramma* spp. associated with ECB

Twenty-eight parasitized egg clusters were sampled and individual females were used for molecular identification of the *Trichogramma* species present on the territory of Serbia in the south east border of Europe. A total of 80 emerged wasps were examined and presence of both male and female adults were registered in all sampled egg cluster. Seventy out of eighty tested samples yielded amplifiable DNA. Among those samples, a 542 bp fragment of the COI gene revealed the existence of 18 haplotypes, 15 of which (65 specimens) matched or clustered tightly with GenBank accessions (e.g. KC488658 and FM210198) identified as *T. brassicae* (Figure 2, Supporting Information Table S1). The remaining three haplotypes (5 specimens) were identified as *T. evanescens*, closely matching GenBank accessions MG932147 and MG932180 (Figure 2, Supporting Information Table S1). All five individuals identified as *T. evanescens* were sampled in pepper fields in Vojvodina (i.e. region I). Across both species, only two haplotypes, both *T. brassicae*, were identical (100% match) to those previously deposited in GenBank. The haplotype Tbr-H1 has previously

been recorded in Iran (e.g. JX131627) and Tbr-H5 matched a commercial strain reared by Andermatt BioControl, Switzerland (FM210198) (Pasquer et al. 2009).

Genetic variation was examined further only for the most common species, *T. brassicae*. Across the five arbitrary population groups, the 542 bp sequence of COI contained 46 polymorphic sites, which resulted in the 15 haplotypes. In terms of the actual number of haplotypes detected, corn fields in region I appeared to be the most polymorphic population group, with 9 haplotypes, followed by pepper fields in the same region, with 6. However, since this region was the most intensely sampled, this could be a reflection of sampling effort (Table 2). Despite the differences in sample sizes, haplotype diversity and nucleotide diversity were similar across the five population groups (Table 2). Only three haplotypes (Tbr-H1, Tbr-H2 and Tbr-H5) were numerically abundant, being present in 32, 9 and 7 individuals, respectively (Figure 2; Supporting Information Table S1). These haplotypes were also three of only four that were detected in more than a single population group, the fourth being Tbr-H6 (Figure 3). The remaining 11 haplotypes were restricted to a single population group, and indeed, 9 of those haplotypes were only detected once (i.e. in a single specimen). Perhaps the most surprising finding was that in the only region in which we collected from both corn and pepper (region I), among the 9 haplotypes detected in corn and 6 detected in pepper, only one of these (the numerically dominant Tbr-1) was detected in both crops (Supporting Information Table S1). Indeed there was very little overlap between the haplotypes detected in pepper in region I and

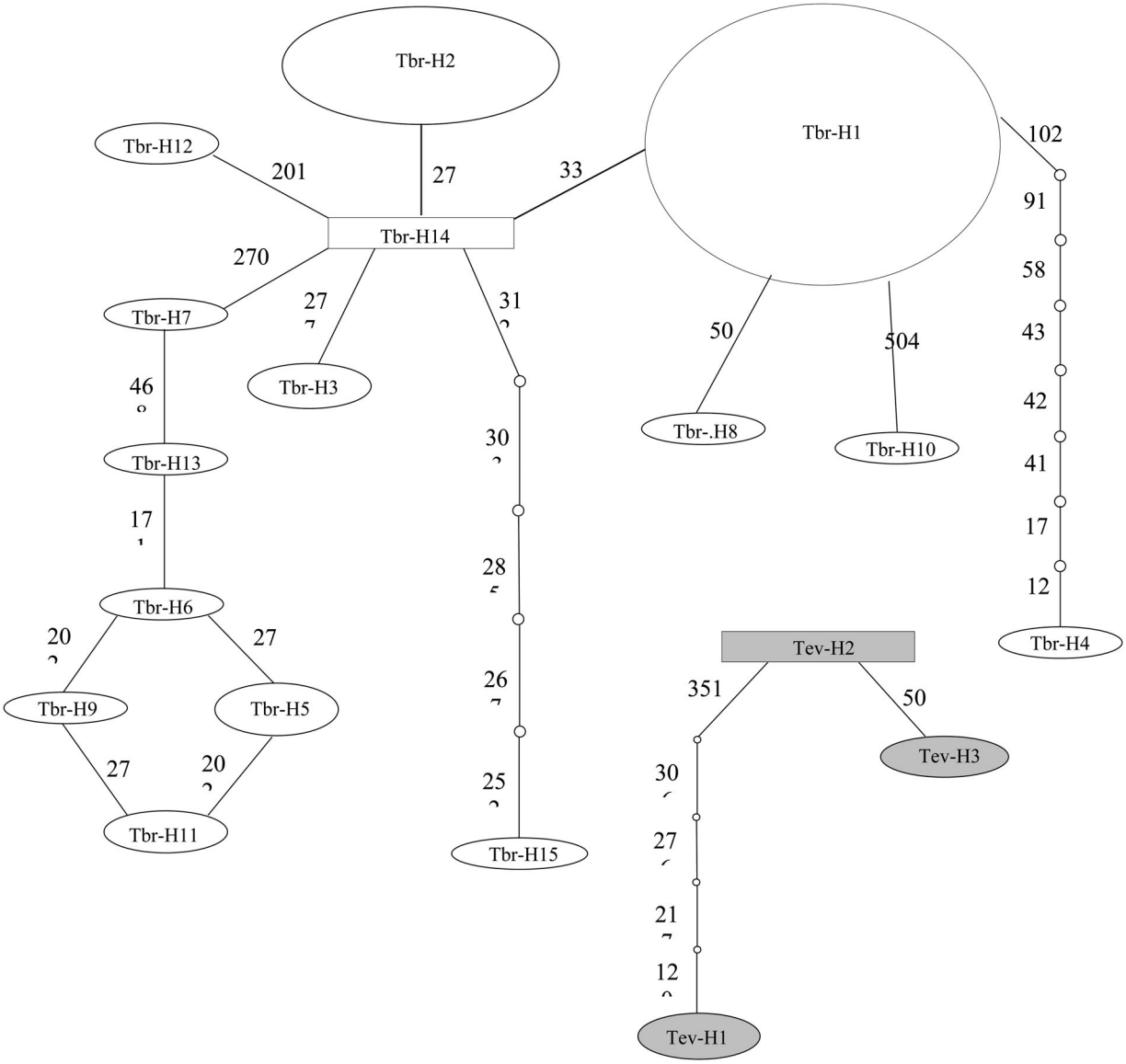

Figure 2. Network of mitochondrial haplotypes present in Serbian populations of two *Trichogramma* species found parasitizing eggs of the European corn borer in corn and pepper fields; *T. brassicae* (white) and *T. evanescens* (grey). Haplotype size is proportional to the number of specimens sharing a haplotype. Small circles represent unobserved inferred haplotypes and lines connecting haplotypes represent a single nucleotide mutational change. (number alongside the line denotes the position of the mutation in the 542 bp fragment of COI).

Table 2. Genetic variation present in *Trichogramma brassicae* parasitizing ECB egg masses in different regions/crops in Serbia.

Region/crop	*N*	*h*	Hd	л
I/corn	32	9	0.7258	0.0040
I/pepper	16	6	0.6167	0.0050
II/corn	8	3	0.7500	0.0051
III/corn	3	2	0.6667	0.0012
IV/corn	6	3	0.7333	0.0052

Variation was estimated from a 542 bp sequences of COI and is expressed in terms of haplotype number (*h*), haplotype diversity (Hd) and nucleotide diversity (л), calculated using DnaSP Version 5.10.01.

those found in corn anywhere in Serbia. Despite this disparity, estimates of p-distance between all population groups, regardless of crop type, were similar (0.004–0.008; Table 3). Finally, none of the *Trichogramma* samples tested positive for *Wolbachia*.

4. Discussion

The use of *Trichogramma* wasps in biological control is common worldwide but its potential has not been explored for many pests and in many geographic

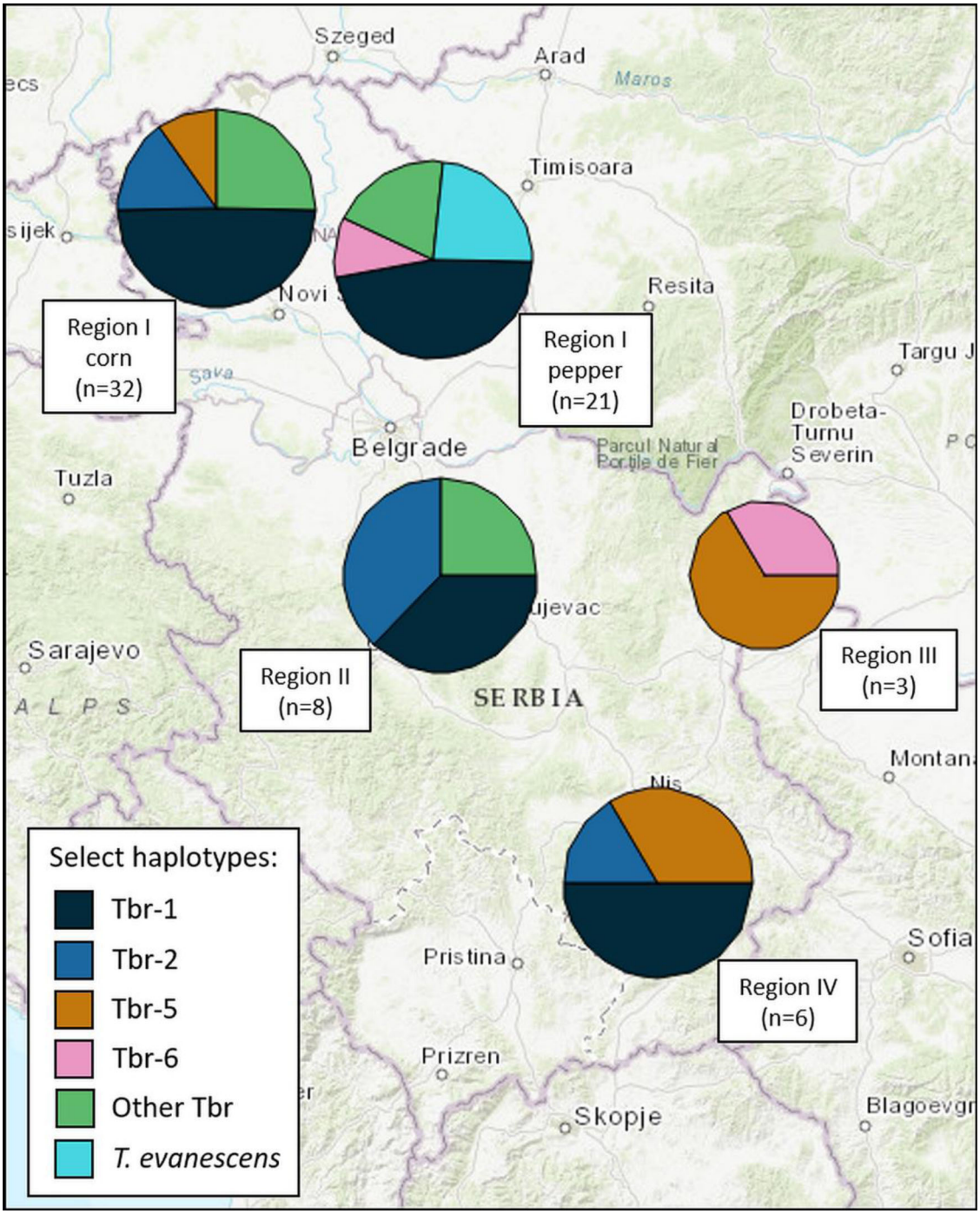

Figure 3. Geographic distribution of *Trichogramma* COI haplotypes parasitizing ECB egg masses in corn and pepper fields across Serbia. Sixty-five of 70 sequenced specimens were identified as *T. brassicae*, which in turn harbored 15 haplotypes. Only four haplotypes (singled out) were recorded in multiple regions and/or crops. A second species, *T. evanescens*, was found only in pepper fields in region I. Map was produced using PhyloGeoViz Version 2.4.5 and visualized using GPS Visualizer.

Table 3. Genetic divergence between the populations of *Trichogramma brassicae* parasitizing ECB egg masses in different regions/crops, in Serbia.

Region/crop	I/corn	I/pepper	II/corn	III/corn	IV/corn
I/corn	0.004				
I/pepper	0.005	0.005			
II/corn	0.005	0.006	0.005		
III/corn	0.008	0.008	0.006	0.001	
IV/corn	0.004	0.005	0.005	0.006	0.005

Estimates of p-distance were calculated from a 542 bp sequence of the COI gene in MEGA version 6. Diagonal element represents within region/crop divergence.

regions. Serbia is one of the countries at the south east border of Europe that does not currently use *Trichogramma* in its agricultural systems. However, monitoring of ECB on the territory of Serbia since 2010 has shown the presence of *Trichogramma*-parasitized egg clusters and significant rates of parasitism (Krnjajić 2002; Tancik 2017; Ivezić et al. 2018). In the current study, we identified two different *Trichogramma* species parasitizing the eggs of ECB in two different crops in Serbia, corn and pepper. Across different agricultural regions, parasitism rates ranged between 12.5% and 80.0%. Comparable parasitism rates have been reported previously. At two sites in the region of Kikinda (Vojvodina), natural parasitism of ECB in corn during July and August 2016 was reported as 48.2% and 78.3% (Ivezić et al. 2018). Similarly, a multi-year study of ECB in sweet corn fields in north-west Serbia at the locality of Ruski Krstur (Vojvodina) also documented natural parasitism of ECB by *Trichogramma* spp. (Tancik 2017). In the study of Tancik, the rate of egg parasitism varied from 9.3% to 73.6% during the period from 2004 until 2007 suggesting that, although, *Trichogramma* occurred constantly, their number greatly fluctuated from year to year. Clearly, *Trichogramma* have the potential to achieve high levels of parasitization in both corn and pepper in Serbia and their use as potential biological control agents warrants further investigation.

A fundamental step in the development of any biological control program utilizing *Trichogramma* is the identification and choice of species and/or strains to use: not all *Trichogramma* species, populations or genotypes (strains) perform equally well, be it in terms of mass rearing or field dispersal and parasitism rates (Van Driesche and Bellows 1996). For example, key traits such as fecundity, egg maturation rate, and offspring sex-ratio, have been shown to be influenced by intraspecific genetic variation (Wajnberg 2010; Wajnberg et al. 2012; Coelho et al. 2016). Therefore, the first step for the implementation of these beneficial insects in the program of biological control of pests is the accurate identification and genetic characterization of those native species that might be considered for use as biological control agents. Across agricultural growing regions in Serbia, the current study found that *T. brassicae* was the predominant species parasitizing ECB eggs in both corn and pepper fields. A second species, *T. evanescens*, was also identified, but in much lower numbers, and only from pepper fields. This is very much in line with an earlier study that focused only on corn, and only on a single growing region, Kikinda (Ivezić et al. 2018). That earlier study did recover a single specimen of *T. evanescens* from corn, but considered alongside the current study, it suggests that *T. evanescens* is naturally rare in corn. However, while *T. brassicae* was also the predominant species in pepper, *T. evanescens* was more likely to be encountered in this crop.

The findings of our study can be used to both justify and direct the rearing and augmentative (or inundative) release of *Trichogramma* in Serbia, perhaps most importantly in the regions with lower levels of natural parasitism. Outside of Serbia, in western regions of Europe, both *T. brassicae* and *T. evanescens* are used for biological control of ECB. *Trichogramma brassicae* is already commercially produced for biological control of ECB in maize in Switzerland, Germany and France (Suverkropp et al. 2001). Indeed, one of the haplotypes identified in our study (Tbr-5) matches that of a strain that is already mass-reared for commercial sale and release in Europe (Pasquer et al. 2009). Similarly, *T. evanescens* has also been advocated as a biological control agent in many countries of Western Europe (Hassan 1981; Bigler 1986; Hluchý et al. 2004; Tancik and Cagáň 2004). The fact that these two species can be cheaply mass-produced on eggs of stored product pests like *Sitotroga cerealella* Olivier 1789 (Lepidoptera: Gelechiidea) or *Ephestia kuehniella*. Zeller 1879 (Lepidoptera: Pyralidae) strongly facilitate their commercial utilization in Serbia. If we must choose between these two species, then *T. brassicae* is probably the most parsimonious option, based simply on its natural abundance relative to *T. evanescens* in both corn and pepper. However, it would be interesting to continue the monitoring of both species in case that changes.

With 15 haplotypes, there was a high degree of genetic diversity in Serbian *T. brassicae* populations. Each of these haplotypes may be potentially considered for use in a biological control program. However, almost half of the *T. brassicae* specimens characterized were of just one haplotype, Tbr-H1. This haplotype occurred in four of the five population groups sampled, and in each of those, accounted for around 50% of the population. One interpretation of this finding is that this haplotype is particularly successful under natural (field) conditions, and may therefore be a good candidate for

use in a biological control program. Future research should investigate the suitability of this haplotype for mass-rearing. This haplotype may be a particularly useful introduction in the east of the country (Region III) where it was not actually detected in our study. A mountain range separates the eastern region from the rest of the country, which may have acted as a natural barrier to the spread of this otherwise successful haplotype. That said, we recommend that sampling effort in the eastern (and central and southern) region should be increased to gain a more reliable picture of genetic diversity in those areas. Furthermore, a more rigorous approach would be to compare the suitability of several of the available haplotypes/strains (Coelho et al. 2016).

An alternative approach to initiating a mass-rearing effort in Serbia could be to import *T. brassicae* from a mass-rearing facility outside of the country. It is interesting to note that one of the haplotypes detected in this study (Tbr-5) is shared with a strain of *T. brassicae* that is already in mass-rearing in Switzerland (or at least was a decade ago; Pasquer et al. 2009). This similarity is currently based on just a relatively short fragment of one mitochondrial gene. Future work might seek to extend the genetic comparison of the two forms. With this in mind, it is also worth noting that 13 of the 15 Serbian *T. brassicae* haplotypes were new, in the sense that this study is the first time they have been deposited in GenBank. Any one of these haplotypes could potentially prove to be a better biological control agent than what is currently available, attracting foreign commercial interest.

One further factor that can influence the choice of *Trichogramma* strains is infection with the endosymbiotic bacterium *Wolbachia* (Huigens et al. 2004). *Wolbachia* can induce thelytokous parthenogenesis in *Trichogramma* creating heavily female-biased populations that can be beneficial to biocontrol (since only females parasitize eggs). For example, with no males produced, populations grow faster in mass-rearing, and since females are the ones that control the pest in the field, greater parasitism rates may be achieved (Almeida 2004). As it happens, none of the Serbian wasps showed any presence of *Wolbachia*.

The results of this study are particularly interesting because they reveal new information about the biodiversity of *Trichogramma* spp. in an area without previous deliberate introduction of these insects. The obtained data will certainly benefit the potential commercial use of these insects and the implementation of conservation biocontrol strategies promoting the naturally high ECB parasitism rates achieved by resident Serbian *Trichogramma* populations. Molecular characterization of additional *Trichogramma* species from Serbia will help to clarify the *Trichogramma* fauna native to Serbia and it will help in a rational choice for testing and identifying the best species to be used for biological control.

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