THE USE OF MICROSATELLITE MARKERS AS A TOOL FOR DISTINCTION BETWEEN WESTERN CORN ROOTWORM (*Diabrotica virgifera virgifera* [Coleoptera: Chrysomelidae]) POPULATIONS

Melita ŠTRUKELJ[1], Špela MODIC[2], Aleš SEDLAR[3], Stephan WINTER[4], Jaka RAZINGER[5]

[1, 2, 5]Plant Protection Department, Agricultural Institute of Slovenia, Slovenia
[3]Crop Science Department, Agricultural Institute of Slovenia, Slovenia
[4]Plant Virus Department, Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany

ABSTRACT

The western corn rootworm (WCR) is an economically important pest of maize in North America and Europe. It was first discovered in Europe in Serbia in 1992, and in 1998 in NW Italy. Presently there is still some controversy whether these two population outbreaks originate from a single or multiple introductions. Genetic monitoring of WCR utilising molecular markers was reported both on a multinational and micro geographic scale. The aim of our study was to implement two widely used molecular approaches for typing the WCR individuals, microsatellite and mtDNA markers and test them on a limited available number of Slovenian WCR samples from the location and period when admixture was occurring.

Keywords: *Diabrotica v. virgifera*, microsatellite markers, population genetics, Western corn rootworm

IZVLEČEK

UPORABA MIKROSATELITNIH MARKERJEV ZA RAZLIKOVANJE MED POPULACIJAMI KORUZNEGA HROŠČA (*Diabrotica virgifera virgifera* [Coleoptera: Chrysomelidae])

Koruzni hrošč, *Diabrotica v. virgifera* LeConte (Coleoptera: Chrysomelidae), je gospodarsko pomemben škodljivec koruze v Severni Ameriki in Evropi. V Evropi je bil prvič potrjen 1992 v Srbiji in leta 1998 v SZ Italiji. Trenutno še ni jasno ali gre za enkratni vnos iz ZDA, ali za dva ločena vnosa. O nadzoru koruznega hrošča na genetskem nivoju z uporabo molekularnih markerjev poročajo raziskave tako na mednarodnem nivoju kot tudi na ožjih geografskih območjih. Naš namen je bil vpeljati dve uveljavljeni molekularni metodi za tipizacijo koruznega hrošča in jih testirati na

[1] PhD, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
[2] MSc, ibid.
[3] PhD, ibid.
[4] PhD, Inhoffenstraße 7B, 38124 Braunschweig, Germany
[5] PhD, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia

omejenem vzorcu koruznega hrošča iz lokalitete in obdobja mešanja populacij.

Ključne besede: koruzni hrošč, *Diabrotica v. virgifera*, mikrosatelitni markerji, populacijska genetika

1 INTRODUCTION

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a maize pest which is hypothesised to have originated in Mexico. It has expanded into North America (Krysan and Smith, 1987) in the last century, and from there to Europe (Miller *et al*., 2005). Its invasion of Europe was not officially recorded until 1992, this is, from a damaged maize field near Belgrade airport Surčin in Serbia (Baca, 1994). In 1998, WCR was first found in Italy, at Tessari airport near Venice (Furlan *et al*., 1999). In 2003, it reached Udine (Furlan *et al*., 2005), near the Slovenian-Italian border. In the same year (2003) the first beetles were caught in the west of Slovenia, near Nova Gorica, and in the east of the country, in Prekmurje (Urek and Modic, 2004). In 2009 the entire territory of Slovenia was officially declared an infested area (Modic and Knapič, 2010).

WCR causes considerable economic damage to maize *Zea mays* (L.) (Levine and Oloumi-Sadeghi, 1991). The control and management of an alien invasive species, like WCR, is often hindered by a lack of understanding about their geographic source, a number of introduction events, genetic structure, and gene flow (Mack *et al*., 2000). The molecular markers such as microsatellites could play in informing pest management strategies through accurate estimates of population genetic structure, gene flow and dispersal (Sappington *et al*., 2006).

Populations of WCR in Europe have been extensively analysed in comprehensive studies covering multiple countries (Ciosi *et al*., 2011), localized studies in proposed centres of origin, such as Italy and Serbia, as well as neighbouring countries, such as Slovenia, Croatia and Hungary (Bermond *et al*., 2012; 2014). European populations have been compared to American populations (Ciosi *et al*., 2008; Ivkosic *et al*., 2014). NE Italy has been proposed as a secondary contact site between the invasive outbreaks in NW Italy and Serbia resulting in admixture (Bermond *et al*., 2012). However, with the exception of Prekmurje region (Bermond *et al*., 2012) little sampling and subsequent microsatellite analysis has been performed in Slovenia that is in the direct vicinity of the admixture site. For the limited number of samples analysed in this study, we assumed that the individuals caught in eastern Slovenia (Prekmurje) belonged to the population originating from Serbia (eastern population), whereas the ones caught near Nova Gorica (Primorska) belonged to the population originating from north Italy (western population).

Recently, genetic monitoring of WCR on a micro-geographic scale was reported using both microsatellite and mtDNA markers (Ivkosic *et al*., 2014). The aim of our study was to implement these two molecular approaches for typing the WCR individuals and test them on a limited available number of Slovenian WCR samples from the period when admixture was occurring.

2 MATERIALS AND METHODS

2.1 WCR sampling and DNA purification

Adults were sampled within the monitoring of WCR in maize fields in Slovenia carried out by Agricultural Institute of Slovenia (Figure 1). Samples of WCR from Austria were sent from colleagues from AGES. Genomic DNA was extracted from adult WCR individuals using the InviMag Plant DNA Mini Kit/ KF96 (Stratec Biomedical AG, Birkenfeld, DE) following the user's manual and checked for integrity on 1% agarose gel.

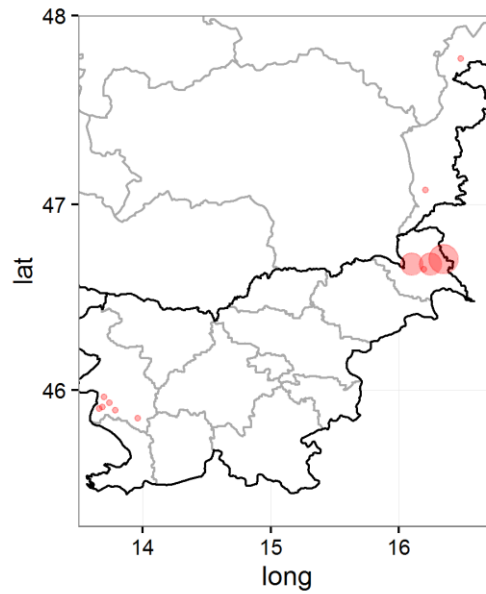

Figure 1: Distribution of sampled WCR, *D. v. virgifera*, in Slovenia and Austria.

2.2 DNA barcoding

Partial mitochondrial cytochrome oxygenase subunit I (mtCOI) was amplified using primers C1-J-2195 (5' – TTGATTTTTTGGTCATCCAGAAGT – 3') and L2-N-3014 (5' – TCCAATGCACTAATCTGCCATATTA – 3') according to Frohlich et al., 1999. PCR assay was conducted using 2 µl of template DNA in a total reaction volume of 25 µl. The PCR reaction mix contained 2.5 µL of 10×PCR buffer, 0.5 µL of 40 mM dinucleotide triphosphates (dNTPs), 1.25 µL of 50 mM $MgCl_2$, 0.5 µL of each primer, 0.25 µL *Taq* DNA polymerase and 17.5 µL dH_2O. All PCR reagents were purchased from Invitrogen. Thermal cycling conditions were an initial 3 min at 95°C, followed by 1 min at 95°C, 1 min at 52°C and 1 min at 72°C for 35 cycles, and 5 min at 72°C. The resulting PCR amplicons were checked on a 3% agarose gel, excised from the gel and sequenced from both directions using the amplification primers.

2.3 Genotyping and data analysis

Genotyping was conducted using a core set of six WCR microsatellite markers: *DVV-D2*, *DVV-D4*, *DVV-D8*, *DVV-T2* (Kim and Sappington, 2005), *Dba05* and *Dba07* (Waits and Stolz, 2008) (Table 1). Thermal cycling conditions were 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min (55°C in case of *DVV-D2*) and 72°C for 1 min followed by a final incubation at 72°C for 10 min. PCR products were separated using QIAxcel Advanced System (Qiagen) and scored for polymorphisms. The allele frequencies were estimated using GenAlex v 6.5. Locus Dba07 was excluded from analysis due to unsuccessful amplification.

Table 1: Core set WCR markers recommended for population genetics studies (Kim et al., 2008). The number of alleles and size range for samples tested in our study is specified.

Locus name	Primer sequences (5'-3')	No. of alleles	Size range (bp)	GenBank accession no.
DVV-D2	F: CACGCAGCACTTAATTGGTTT R: CTATGCCTCCCAATTCGTGT	5	187-212	AY738532
DVV-D4	F: TGTGTGCAGTGTCCCGTTAT R: GTGGCCAGTATTCACGACCT	7	192-244	AY738534
DVV-D8	F: AAGGCAGGTAGTAATGTTGGTGA R: TCATCACTAATGGGGAAACGA	4	206-248	AY738538
DVV-T2	F: ATCGGTTTTGGCTGGATATG R: GTTCAACAACTCGCAAACCA	5	209-275	AY738546
Dba05	F: GCTGAGGAGGCTTATGTC R: CAATGGAGGTTGGCTATT	2	223-226	EF524280
Dba07	F: ATCGGTGTAACTTTTCCACA R: CACATCGGCATAGGATAGAC	-	-	EF524282

3 RESULTS AND DISCUSSION

We used MtCOI sequence analysis and microsatellite markers to study the diversity of WCR insect collected between 2007 and 2014. Almost identical sequences were obtained from the MtCOI gene fragments amplified from insects collected from different localities in Slovenia (data not shown). Similarly, only a single WCR haplotype was observed in studies on the diversity of WCR in Croatia and Serbia in years 2009 to 2011 (Ivkosic *et al*., 2014). This is in sharp contrast to the diversity recorded for WCR in 1996, where several haplotypes of the insect were observed. Using microsatellite genotyping we have observed allele frequencies shown in Table 2. Differences in the number of observed alleles were observed between Eastern and Western populations sampled in the period 2007-2008 as well as both Western populations sampled at different time periods however these may also be due to small sample number available. The most frequent allele for markers DVV_D4, DVV_D8 and DVV_T2 was the same in all three populations.

Table 2: Allele frequencies of WCR, *D. v. virgifera*, sampled in Slovenia, the number of tested samples (n) and the number of alleles (N) per individual loci.

Locus	**Allele/N**	**East 2007-08 (n = 7)**	**West 2007 (n = 7)**	**West 2014 (n = 5)**
DVV_D2	N	7	7	5
	187	0.43	0.21	0.1
	189	0.36	0.14	0.5
	193		0.36	
	210	0.21	0.29	0.3
	212			0.1
DVV_D4	N	5	6	4
	192			0.13
	208		0.08	
	226	0.2		
	228	0.4	0.58	0.5
	230	0.2	0.08	
	236	0.1	0.25	0.38
	244	0.1		
DVV_D8	N	2	5	3
	206		0.2	
	221		0.2	0.33
	246		0.2	
	248	1	0.4	0.67
DVV_T2	N	4	5	4
	209		0.1	
	211		0.2	0.13
	221		0.1	
	223	1	0.5	0.75
	275		0.1	0.13
Dba05	N	7	5	4
	223	0.86	0.5	
	226	0.14	0.5	1

Miller *et al*. (2005) proposed at least two distinct introductions of WCR from North America into Central and South-Eastern (CSE) Europe, one in North-Western Italy

and one in Serbia. These findings were corroborated by Lemic *et al*. (2015) and Bermond *et al*. (2012). The latter also reported that secondary contact between the two populations probably occurred in North Italy between in 2008. This secondary contact, or admixture, between the two outbreak populations, led to a partial restoration of genetic variation (Bermond *et al*., 2012). However, this partial restoration of the genetic heterogeneity did not have a measured impact on invasion success (Bermond *et al*. 2014). Ciosi *et al*. (2011) on the other hand found evidence that the invaded area probably corresponds to a single expanding population resulting from a single introduction. They also discovered that subsequent outbreaks in southern Germany and north-eastern Italy most likely originated from CSE Europe. So, apparently, the origin of the CSE European WCR population remains unclear. This study showed some differences in allele frequency between samples supposedly originating from the eastern (Serbia) or western (NW Italy) population (Table 2), but due to the limited number of samples analysed these data are inconclusive. For further comparison purposes, standardisation of allele calls should be performed from a standardised set of individuals since apparent allele sizes can differ based on the equipment and software used (Kim *et al*., 2008). Additionally, significantly larger sample size at each time-location should be analysed.

Population genetic principles and theory have been applied to *D. v. virgifera* to monitor the genetic variation present and the success of control measures used in Europe (Ciosi *et al*., 2011). Using microsatellite markers, it was possible to demonstrate the changes in genetic variation in central and south-east Europe where crop rotation was documented to effectively control the western corn rootworm. A likely consequence of crop rotation was to force once-connected populations of *D. v. virgifera* to fragment and thus restricting gene flow (Ciosi *et al*., 2011). Additionally, the introduction of WCR from North America itself resulted in a significant genetic bottleneck, as the number of alleles was low during the introduction phase (45% or the supposed North American population of origin) in southern Europe. However, repeated introductions and admixture events in southern Europe may have resulted in genetically diverse WCR populations that have attained 83% of all known alleles worldwide 14 years after their first introduction (Lemic *et al.*, 2015). As genetic diversity in southern Europe is approaching equality with populations of origin in the USA, there is a real possibility of resistant alleles entering Europe, potentially resulting in resistance to crop rotation as experienced in the USA (Meissle et al. 2011; Lemic *et al*. 2015). Additionally, a chance exists that already established European WCR populations will adapt to the selective pressure of crop rotation naturally and develop resistance in situ (Ivkosic *et al.* 2014).

4 CONCLUSIONS

Genetic monitoring can be used to enhance integrated pest management strategies for WCR control, for example by developing biomarkers to find and track rotation-resistant populations. Such data can be used to improve WCR pest management strategies in Slovenia and elsewhere.

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