Research Article

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Monitoring techniques of the western corn rootworm are the precursor to effective IPM strategies

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Abstract

BACKGROUND: The western corn rootworm (WCR) is economically the most important pest of maize in Croatia. To predict WCR adult population abundance and variability, traditional, genetic and morphometric monitoring of populations was conducted over time through each phase of the WCR invasion process in Croatia.

RESULTS: Through traditional monitoring it was shown that WCR established their current population and reached economic densities after 14 years persisting in the study area. Regression-tree-based modelling showed that the best predictor of WCR adult abundance was the total amount of rainfall. Genetic monitoring indicated that genetic differentiation increased over time at the intrapopulation level, and morphometric monitoring indicated that wing morphotypes varied according to edaphic landscape changes.

CONCLUSION: Traditional population metric surveys are important in WCR integrated pest management (IPM), as such surveys can be effectively used to predict population abundances. Novel-use monitoring techniques such as genetics and geometric morphometrics can be used to provide valuable information on variation within and among populations. The monitoring techniques presented herein provide sound data to assist in the understanding of both WCR ecology and population genetics and may provide more information than that currently available using traditional techniques (e.g. sticky traps), and as such these additional techniques should be written into IPM for WCR.

Keywords: population abundance; regression tree; population genetics; wing shape analyses

1 INTRODUCTION

The western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) is an invasive beetle species accidentally introduced from North America into Europe. The pest was first recorded in Europe in 1992 near Belgrade, Serbia,[1] and since then has spread throughout much of the continent.[2] WCR adults were first detected in eastern Croatia in 1995[3] and have since spread west, now infesting over 28 000 km^2 of arable land. It has been estimated that WCR populations have an average dispersal rate of approximately 40 km year^{-1} and a growth rate that allows them to quadruple in abundance annually when inadequately controlled.[4,5] While other insect species are known to have an adverse effect on maize production (e.g. the European corn borer,[6] wireworms[7,8]), WCR is now recognised as the most serious pest of maize production in Europe. The life history of the WCR is such that eggs laid during summer overwinter in soil only to emerge the following spring as larvae that immediately commence feeding upon the roots of recently sown maize.[9] The resulting damage leads to stalk lodging and yield losses, culminating in economic levels of damage to crops. The first serious damage to maize crops from WCR was observed in the Baranja region of eastern Croatia in 2002 where yield was reduced by 85%.[4] Economic losses are found in other parts of Europe, and to date WCR has been recorded in 22 European countries.[2]

Traditional monitoring of WCR populations in Croatia started in 1996,[10] using pheromone and yellow sticky traps, and this initially allowed for the rapid detection and assessment of its spread.[11] Most recently, however, genetic and morphometric techniques have been used to monitor WCR in an attempt to provide targeted strategies for management of the species.

1.1 Traditional pest monitoring and prediction using yellow sticky traps and pheromone traps

Using pheromone trapping, it was possible to monitor the occurrence and abundance of WCR adults and predict the damage inflicted upon maize crops in the following year. It was suggested

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that a weekly catch of 22 WCR per Pherocon AM® yellow sticky trap from week 29 to week 31 of the year would result in economic levels of root damage the following year.[12]

Ongoing WCR-ecology-based research in the United States and Europe[13–16] and in Croatia[7,12,17] has yielded important information on distribution and abundance and habitat parameters (i.e. climate, soil characteristics, vegetation, relief, etc.). Having identified key habitat parameters, it has been possible to predict infestation levels annually and thus inform farmers about appropriate control strategies required for that and the following year's maize crop. For example, larval emergence can be predicted by the abundance of adults and eggs in the year preceding the repeated sowing of maize.[12] The methods used to assess WCR abundance are basic and rely on the visual inspection of both individual plants and yellow sticky traps or pheromone traps, such as Csalomon PAL traps.

According to good agricultural practice (e.g. Ministry of Agriculture)[18] measures, WCR control must be based on population level predictions that adhere to the principles of integrated pest management (IPM). Determining the factors that positively or negatively affect or limit the growth of WCR populations will facilitate the development of IPM strategies aimed at slowing the spread of individuals and thus mitigating damage to maize crops on a national and potentially international scale.

1.2 Genetic monitoring

For over a decade, population genetic theory and techniques have been used in the effective control and ongoing management of invasive species.[19–21] An understanding of a pest's population genetic structure, gene flow and dispersal patterns has helped to control the impact invasive species have had on global agriculture and food resources, as seen for species such as Colorado potato beetle *Leptinotarsa decemlineata* Say,[21] multicoloured Asian lady beetle *Harmonia axyridis* Pallas,[22] boll weevil *Anthonomus grandis* Boh.[23] and WCR.[24,25]

The *Diabrotica* genetics consortium recognised the importance that molecular markers could play in assisting pest management strategies, and consortium members were encouraged to use a core set of markers to investigate WCR population genetic structure, gene flow and dispersal patterns and share their data globally with members.[26] To assist in and facilitate the sharing of genetic data on WCR, Kim *et al.*[27] developed a core set of microsatellite markers for the WCR, from which it was possible to obtain key population genetic metrics.[24,28,29] There are now numerous studies on the invasion genetics of WCR both in the United States[30] and in Europe[24,25,28,29,31] that have used the core set of markers developed by consortium members. However, the genetic data generated using these markers is yet to be used to assist in the monitoring of existing and new populations or to inform or evaluate management practices by country or region. Evidently, the scope is to use the WCR core set in the monitoring of WCR populations to assist in the management of the species.

1.3 Geometric monitoring

The expense and inaccessibility (i.e. required expertise) of genetic techniques to survey WCR were the impetus to search for novel-use existing techniques to monitor WCR. The novel-use existing method would need to be easy to use, inexpensive and able to yield a lot of information quickly; these criteria were satisfied by geometric morphometrics. After almost two decades of traditional (distribution and abundance) and genetic monitoring of WCR in Croatia, geometric morphometric monitoring was tested with the aim of assessing whether WCR wing shape and size was influenced by specific habitat parameters that could pave the way for the discovery of a population marker. Bouyer *et al.*[32] first demonstrated this for the tsetse fly *Glossina palpalis gambiensis* Vanderplank when they compared wing shape and size differences with population genetic differences found along an ecological cline. The authors found that geometric morphometric differences in wing shape and size were clinal, a result not mirrored by the microsatellite markers they used. Bouyer *et al.*[32] explained this result by stating that the influence of the surrounding environment on an organism's genotype takes much longer to manifest than on its phenotype, thus making geometric morphometrics a much more useful tool than genetics to detect changes in populations in the short term.

Wing morphology (size and shape) is a critical element of an insect's dispersal capacity. Determining the dispersal capabilities of invasive species is vital to understanding how they adapt to new environments,[33] as well as for strategic planning ahead of the invasion front.[34] Recent advances in geometric morphometric techniques (i.e. shape analysis) renders the quantification of wing morphology (size and shape) a readily accessible tool for investigating population or geographic differences and for possibly inferring dispersal ability.[35,36] Geometric morphometric is useful in quantifying the morphological variation within and among species, and geometric morphometric methods begin with the collection of two- or three-dimensional coordinates of biologically definable landmarks,[37] such as hind wing vein intersections in WCR.[38] However, morphometric data are yet to be used to assist in the monitoring of existing or new populations or to inform or evaluate management practices by country or region.

In this paper we use several survey techniques to examine (i) how traditional monitoring (using pheromones and yellow sticky traps) can predict population abundance and be used in WCR management and control, (ii) how genetic monitoring (using microsatellites) can detect differences in genetic population structure during all phases of the WCR invasion process and (iii) how geometric monitoring (using hind wing shape and size) can be used to define WCR wing differences based on specific soil types, and how wing differences influence WCR dispersal capabilities and invasion dynamics.

2 MATERIALS AND METHODS

2.1 Traditional pest monitoring and prediction using yellow sticky and pheromone traps

In the eastern Croatian region of Vukovar-Sirmium, WCR adult abundance was monitored annually from 1996 to 2009 (Fig. 1). Pheromone traps were used to monitor WCR from 1996 to 2004, while yellow sticky traps were used from 1996 to 2009. Although Multiguard® traps were initially used in 2000, they were replaced with Pherocon AM® yellow sticky traps. The switch was made to facilitate the comparison of data from surveys conducted in the United States that used Pherocon AM®.[10] Each year, sets of one pheromone and one yellow sticky trap were placed in 3–38 maize fields. Traps were placed in the fields at distances of 30–50 m apart. Each set of traps was examined weekly from mid-June until the end of September. The raw number of WCR caught was recorded, and individuals were then removed and stored in ethanol. Pheromone capsules and yellow sticky traps were replaced once a month during the annual trapping period.

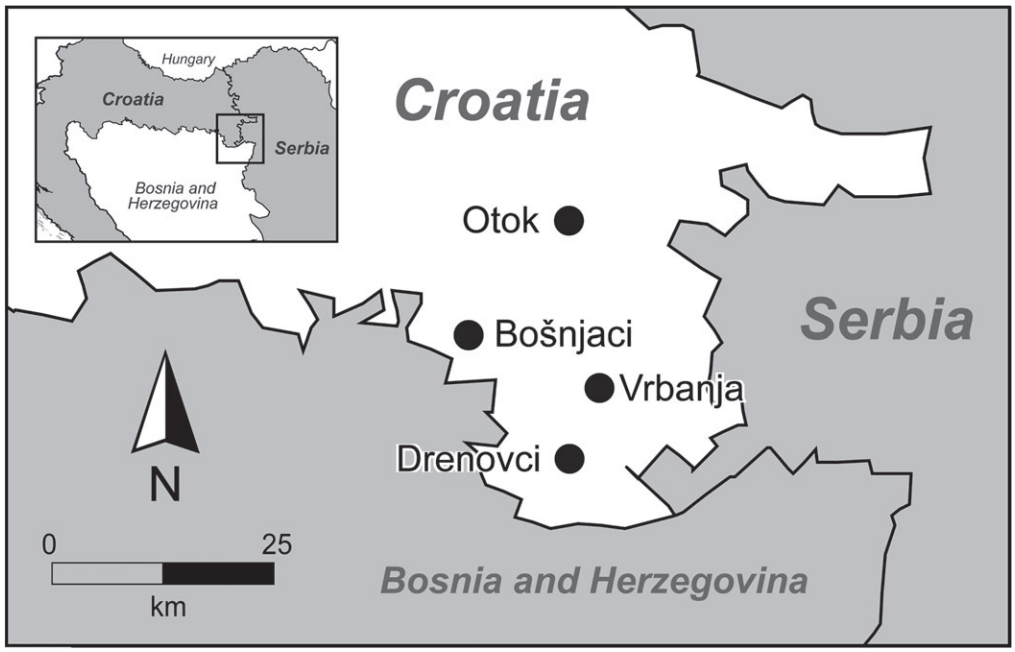

Figure 1. Sampling locations in the Vukovar-Sirmiun region, where monitoring based on trap captures was conducted and where adult WCR individuals were collected from maize fields in 1996, 2009 and 2011 for genetic and morphometric analyses.

From 2007 to 2009, 9–10 fields were included in the survey per year. Three Pherocon AM® yellow sticky traps were placed in each field, at least 30 m apart. Traps were placed in fields every year in mid-June. Trapping occurred from week 25 to week 35 of the year. Climatic conditions (mean weekly temperatures and weekly amount of rainfall from week 25 to week 35 of the year; mean annual temperature and total amount of rainfall) were obtained from the Croatian Meteorological and Hydrological Service from 2007 to 2009 and analysed per site.

During the same time period, approximately 1.5 kg of soil was sampled from each field. Samples were taken by a pedological probe from five locations in each field site at the depth of the plough layer (approximately 30 cm). Soil testing and analyses were conducted by the pedology laboratory of the Department of Soil Science at the University of Zagreb. Specific analyses included soil sediment grain size (contents of coarse and fine sand, coarse and fine silt and clay), humic content and pH.

Soil texture was determined by sieving and sedimentation techniques.[39] Based on the particle size of soil samples, soils were classified as coarse sand (2–0.2 mm), fine sand (0.2–0.063 mm), coarse silt (0.063–0.02 mm), fine silt (0.02–0.002 mm) and clay (<0.002 mm).[40] Humic content was analysed using Tjurin's method.[41] According to international standards and Croatian norms,[42] soil pH in H_2O and KCl was determined using Beckman's electrometrical pH meter.

Crop rotation is randomly practised in the region surveyed[4,11,43,44] and involves the annual rotation of maize with a WCR non-host crop (e.g. soybean *Glycine max* L.); it provides a simple control and management solution, as WCR larvae are unable to survive on crops other than maize. Owing to numerous WCR infestations of maize in Europe, the European Union (EU) in 2003 implemented mandatory pest management strategies, such as crop rotation, to prevent its further spread.[45] In this study, maize was grown either in monoculture (continuous maize) or in rotation with wheat *Triticum aestivum* L., soybean and sugar beet *Beta vulgaris* L. (first-year maize).

2.1.1 *Data analysis*

A Pearson's correlation was conducted to examine the relationship between WCR collected (in fields of first-year maize and continuous maize) and the mean annual temperature, total amount of rainfall, content of coarse and fine sand, content of coarse and fine silt, content of clay, humic content and soil pH in H_2O and KCl. Analyses were performed using SAS/STAT v.9.1.[46]

Following the basic correlation, a regression tree analysis was performed in R v.2.30,[47,48] using the package 'tree'. All variables (number of collected WCR beetles in fields of first-year maize and in fields of continuous maize, average air temperature, total amount of rainfall, content of coarse and fine sand, content of coarse and fine silt, content of clay, humic content and soil pH in H_2O and KCl) were included in a regression tree analysis model. Regression trees are a form of exploratory data analysis that consider which variables contribute to the greatest level of variability explaining the response variables,[49] here the abundance of WCR. The most parsimonious model selected was the model that explained the greatest level of variation within the first split of the regression tree output. Because not all the variables were included in each run of the model (as it is assumed that at least ten data points are required to complete a statistically valid regression analysis),[49] a number of model iterations were used, where different combinations were employed and where variables were either added or subtracted. If a variable was not included in the model (i.e. it did not significantly contribute to explaining as much variability as other variables), it was substituted with another variable. This process continued until the most parsimonious model remained.

2.2 Genetic monitoring

Methods used to collect and process WCR for microsatellite genotyping are outlined in Lemic *et al.*[24] A subset of the datasets of Lemic *et al.*[24] and Ivkosic *et al.*[25] were used in this study and provided the genotypes for WCR sampled ($n = 294$) in 1996, 2009 and 2011 in the Vukovar-Sirmium region (Fig. 1).

2.2.1 Data analysis

The number of alleles, Weir and Cockerham's[50] inbreeding coefficient (F_{IS}) and Weir and Cockerham's[50] θ (F_{ST}) for each population and locus were estimated using FSTAT v.2.9.3.2.[51] Observed (H_o) and expected (H_e) heterozygosity and deviations from Hardy–Weinberg equilibrium (HWE) for each population were estimated using GENEPOP on the web v.4.0.10.[52] Exact tests, to calculate significant differences between populations, were estimated using the Markov chain method with 10 000 dememorisation steps, 10 000 batches and 10 000 iterations, also using GENEPOP on the web. Exact tests are considered to be accurate even for small sample sizes and low-frequency alleles.[52] Bayesian model-based clustering was used to investigate genetic clusters in STRUCTURE v.2.3.3.[53] A series of ten independent runs for each value of K between 1 and 13 were conducted. In each run, an admixture model and a burn-in period of 100 000 iterations were used. Sampling locations were not used as informative priors in an effort not to force STRUCTURE to consider sampling locations as putative populations. Probability estimates were obtained after 1 000 000 Markov chain Monte Carlo iterations. Evanno's method,[54] as implemented in STRUCTURE HARVESTER v.0.6.92,[55] was used to estimate the most likely number of genetic clusters (ΔK). The presence of recent population bottlenecks within the 1996, 2009 and 2011 populations was assessed using the program BOTTLENECK v.1.2[56] incorporating two models [i.e. a stepwise mutation model (SMM) and a two-phase mutation model (TPM)]. The TPM incorporates both the stepwise and the multiple-step model. The TPM was run for 100 000 simulations using a proportion of 95% SMM in TPM, with 5% variance. The Wilcoxon signed rank test was applied to determine significant deviations of heterozygosity (H_e) across all loci relative to the expected drift mutation equilibrium.[57] Analyses of allele frequency distribution, to detect a mode shift, were used as an additional indicator of population bottlenecks.[57]

2.3 Geometric monitoring

2.3.1 Specimen collection and wing preparation

Adult WCR were collected by hand from maize plants in July 2011 from four locations in the Vukovar-Sirmium region. These locations were characterised by drier weather conditions and chernozemic soil types (Fig. 1). All specimens were processed as per methods outlined by Mikac *et al.*[38]

2.3.2 Wing landmark acquisition

Slide-mounted wings were photographed using a Leica DFC295 digital camera (3 M Pixel) on a trinocular mount of a Leica MZ16a stereomicroscope and saved in JPEG format using the Leica Application Suite v.3.8.0 (Leica Microsystems Limited, Heerbrugg, Switzerland). Fourteen type 1 landmarks (Fig. 2) defined by vein junctions or vein terminations were identified.[37]

2.3.3 Morphometric analysis

Each landmark was digitised using the software program tpsDIG v.2.16,[58] for which x, y coordinates were generated to investigate hind wing shape. The total wing shape variation was analysed using principal components analysis (PCA). Interlocation differences were assessed using Procrustes distances, which were the product of a canonical variate analysis (CVA). In order to avoid confounded products, the covariance matrix used to analyse the interlocation differences was pooled by sex. The results were reported as Procrustes distances, and the respective P-values for these distances, after permutation tests (10 000 runs), were reported.

3 RESULTS

3.1 Traditional pest monitoring and prediction using yellow sticky and pheromone traps

During the 14 years of WCR monitoring in the Vukovar-Sirmium region, 62 821 beetles were captured, of which 48 001 adults were captured on pheromone traps and 14 820 adults on yellow sticky traps (Table 1). Pheromone traps were found to be very sensitive for early detection purposes. The highest population density of WCR was recorded in 2003, when the average number of adult WCR on pheromone traps was $n = 1275$, and on yellow sticky traps $n = 177$.

The relationship between the average number of WCR captured per trap and climatic conditions (mean weekly temperature and rainfall) from week 25 to week 35 of the year was established for the 2007–2009 data (Figs 3a to c). The average number of WCR per field was higher in years with higher amounts of precipitation and during which lower summer temperatures prevailed.

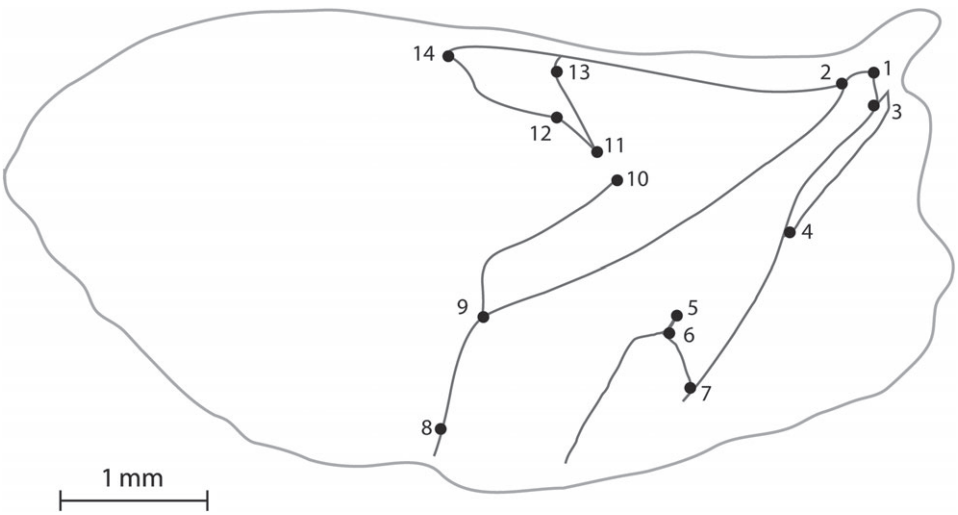

Figure 2. Representation of the 14 morphological landmarks identified on the hind wings of WCR.

Table 1. WCR capture numbers on different types of trap in monitoring conducted in the region of Vukovar-Sirmium in the period 1996–2009

Year of monitoring	Number of traps set		Number of beetles captured		Average number of beetles captured	
	Multigard® pheromone trap	Pherocon AM® yellow sticky trap	Multigard® pheromone trap	Pherocon AM® yellow sticky trap	Multigard® pheromone trap	Pherocon AM® yellow sticky trap
1996	38	27	699	10	18	0
1997	22	8	2398	74	109	9
1998	24	24	4416	198	184	8
1999	20	20	8565	1122	428	56
2000	14	14	8342	263	596	19
2001	11	11	5716	617	520	56
2002	9	9	5623	346	625	38
2003	8	8	10202	1414	1275	177
2004	4	4	2040	285	510	71
2005	0	5	–	442	–	88
2006	0	3	–	316	–	105
2007	0	30	–	5637	–	188
2008	0	27	–	1314	–	49
2009	0	27	–	2782	–	103

There was a significant correlation between adult WCR abundance in fields of first-year maize and temperature ($P < 0.01$), precipitation ($P < 0.01$) and soil clay content ($P < 0.05$). In fields of continuous maize, WCR adult abundance was associated only with precipitation ($P < 0.01$) (Table 2).

Regression tree analyses showed that the total amount of rainfall was the best predictor of WCR adult abundance in the present study. Higher abundances were predicted in years when the total amount of rainfall was greater than 680 mm (Fig. 4). The second most important predictor of adult WCR abundance was mean annual temperature (Fig. 4). When the temperature was lower than 11.55 °C, abundance was best predicted by soil pH in KCl. Higher abundances were expected in soils with a pH of less than 6.93 (Fig. 4). Only when the above predictors prevailed did repeated sowing have an influence on population abundance (Fig. 4).

3.2 Genetic monitoring

The number of alleles was low in all sampled periods (1996, 2009 and 2011). Significant deviations from HWE were observed in 1996 for loci DVV-T2, Dba05 and Dba07 in three of the four populations sampled (Table 3). In 2009, significant deviations from HWE were observed mostly for a single locus (DVV-D2) in three of the four populations sampled (Table 3). In 2011, significant deviations from HWE were observed only for one population (location Vrbanja) for three loci (Dba05, DVV-D8 and Dba07) (Table 3). The number of observed alleles ranged from two to six per locus, with an average of 3.1 in 1996, 3.3 in 2009 and 3.4 in 2011 across the six loci (Table 3). Heterozygosity estimates were low across most populations in 1996 and again in 2009, while values were highest in 2011 (Table 3). In general, H_e among populations was lower in 1996 and 2009 than in populations sampled in 2011. The H_e per population in 1996 ranged from 0.09 (Bošnjaci) to 0.18 (Vrbanja), in 2009 from 0.05 (Otok) to 0.19 (Bošnjaci) (Table 3) and in 2011 from 0.18 (Vrbanja) to 0.75 (Otok). The H_o per population in 1996 ranged from 0.08 (Drenovci) to 0.29 (Otok), in 2009 from 0.06 (Otok) to 0.29 (Otok, Vrbanja) and in 2011 from 0.20 (Vrbanja) to 0.87 (Vrbanja). Within populations, F_{IS} values per locus varied considerably (Table 2). The mean F_{IS} among populations ranged from −0.28 (Otok) to −0.13 (Bošnjaci) in 1996, from −0.19 (Bošnjaci) to 0.03 (Vrbanja) in 2009 and from −0.17 (Vrbanja) to 0.08 (Bošnjaci) in 2011.

3.2.1 *Genetic structure*

Pairwise population comparisons in 1996, 2009 and 2011 revealed low levels of genetic differentiation ($P < 0.05$) (Table 4). In 1996, pairwise genetic differentiation ranged from 0.003 to 0.037 (mean $F_{ST} = 0.021$). Pairwise genetic differentiation in 2009 ranged from −0.002 to 0.015 (mean $F_{ST} = 0.005$). In 2011, pairwise genetic differentiation ranged from −0.002 to 0.008 (mean $F_{ST} = -0.002$). Temporal population pairwise comparisons of populations from 1996 versus 2009 (mean $F_{ST} = 0.05$), 1996 versus 2011 (mean $F_{ST} = 0.04$) and 2009 versus 2011 (mean $F_{ST} = 0.03$) were significant for most populations after correction for multiple comparisons (Table 4).

3.2.2 *Genetic clustering*

The highest likelihood run ($n = 10$) and highest ΔK statistics were consistent for both models tested and yielded $K = 6$ for the combined 1996, 2009, 2011 dataset.

3.2.3 *Population bottleneck*

The Wilcoxon signed rank test showed a significant ($P < 0.05$) heterozygosity excess across all loci relative to drift mutation equilibrium, indicative of a bottleneck event having occurred in three of the four populations sampled in the Vukovar-Sirmium region in 1996. For the locations sampled in 2009 the opposite pattern was found, where three of the four populations sampled did not display evidence of a bottleneck (i.e. no mode shift). In contrast, all populations sampled in 2011 displayed evidence of bottlenecks, with each population having both significant heterozygosity excess and a mode shift.

3.3 Geometric monitoring

The PCA showed that the first three PCs accounted for 50% (PC1 = 23%, PC2 = 16%, PC3 = 12%) of the total hind wing shape

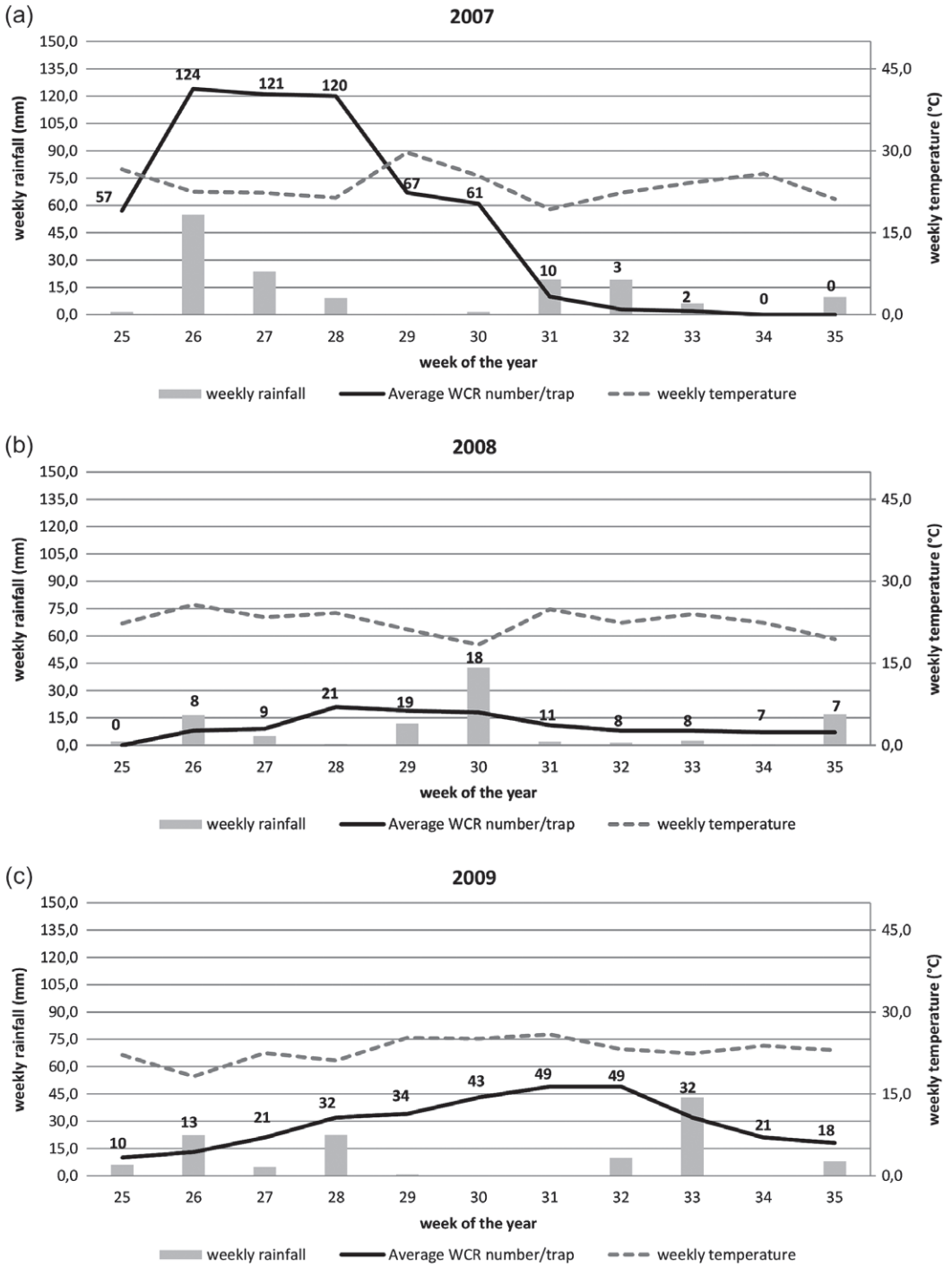

Figure 3. Average adult WCR captures per yellow sticky trap per week and climatic conditions in the Vukovar-Sirmium region in (a) 2007, (b) 2008 and (c) 2009.

variation and provided a reasonable approximation of the total amount of hind wing shape variation. The remaining 21 PCs each accounted for no more than 10.45% of the total variation. Average hind wing shape showed expansion of the proximal landmarks 2 and 3 and distal landmarks 8 and 14, resulting in a narrowed hind wing morphotype; moreover, landmarks 5 and 6 showed clear differences in all four populations studied (Fig. 5).

The Procrustes ANOVA indicated that shape variation among populations was highly significant. Conversely, centroid size differences were not significant (Table 5). The CVA showed significant differences for both Mahalanobis and Procrustes distances after permutation tests (10 000 permutation runs) (Table 6 and Fig. 5).

4 DISCUSSION

4.1 Traditional pest monitoring and prediction using yellow sticky and pheromone traps

Arable land in the Vukovar-Sirmium region spans 150 856 ha, with maize grown on 20 000–40 000 ha.[10] After the first appearance of

Table 2. Correlation values of climatic factors and physical and chemical properties of the soil with the WCR abundance in fields of first-year corn and in fields of continuous corn from 2007 to 2009, expressed by Pearson's correlation coefficient (*r*) and the statistical probability of error (*P*)[a]

	First-year corn		Continuous corn	
	r	*P*	*r*	*P*
Temperature (mean annual)	−0.36982*	0.0124	−0.20671	0.1890
Rainfall (total per year)	0.37883*	0.0103	0.41230**	0.0067
Coarse sand	−0.06692	0.6623	−0.12260	0.4392
Fine sand	0.28946	0.0538	0.26573	0.0890
Coarse silt	−0.12920	0.3976	−0.15182	0.3371
Fine silt	0.21390	0.1583	0.05620	0.7237
Clay	−0.30073*	0.0447	−0.11274	0.4772
pH in H_2O	−0.07080	0.6440	0.12389	0.4344
pH in KCl	−0.10231	0.5037	0.13659	0.3884
Humic (%)	0.16507	0.2785	0.00643	0.9678

[a] not significant;
*$P < 0.05$;
**$P < 0.01$.

WCR in 1995, Croatia started using traditional monitoring techniques to estimate adult WCR population levels and for the rapid detection of new populations in WCR-free locations. WCR captures during the first year of monitoring (after initial detection) were low. Early monitoring results showed that a population was established only 3–4 years after the introduction of WCR in this area. Since its introduction, permanent monitoring has occurred at specific sites in the study area, and this has allowed for the measurement of populations and how they fluctuate over time. According to 14 years of monitoring, pheromone traps proved to be more attractive to WCR than yellow sticky traps; specifically, Multiguard or PheroconAM® traps were most useful under high population abundance conditions. Bažok and Igrc Barčić[10] recommend the use of pheromone traps for early detection purposes and to measure population fluctuations over time, and only if economic adult population levels are present should yellow sticky traps (over the use of pheromone traps) be employed. Our results showed significant fluctuations in the average number of beetles per trap per period. According to Kiss *et al.*,[11] unfavourable weather conditions (low precipitation and high temperatures), crop rotation and to some extent control measures have an impact on reducing population numbers. The higher abundances of WCR found in 2000, 2003 and 2007 could also be attributed to the climatic conditions of the previous summers, which were characterised as hot and accompanied with precipitation (in July and August),[10] a situation favourable for oviposition. Soil moisture is an important factor for oviposition, and if the soil is sufficiently moist during August females will lay a larger number of eggs.[13,15,16,59,60] Extremely warm summers (especially July) are known to influence adult survival rates because of WCR's sensitivity to high temperatures (>30 °C).[60] Such temperatures can lead to weak oviposition and lower damage to maize in the following growing season. However, during hot summers, the effect of larval feeding on plant growth is much higher because larvae feed more within the root owing to a lack of moisture in the soil. Similar results were found by Ivezić *et al.*,[61] who in 2003 recorded a warm summer with a low amount of precipitation resulting in damage to maize that amounted to 80% yield losses. These results were confirmed in this study, where a correlation between WCR adult abundance in fields of first-year maize and temperature, precipitation and clay content in the soil was also found. In fields of continuous maize, adult WCR abundance was correlated only with precipitation. Based on these results, regression tree analyses were conducted on WCR adult populations to identify the factors that can predict population abundance in the following year. Regression modelling has provided a better understanding of how agroecological factors influence WCR adult population density. Our results confirm the findings of numerous

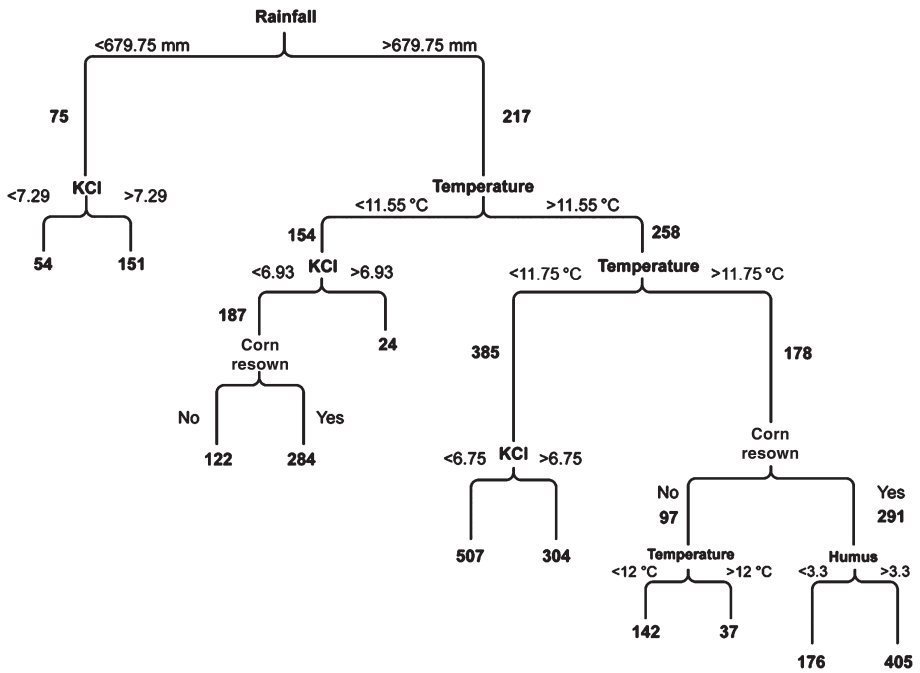

Figure 4. Environmental variables most influential in predicting WCR abundance using the regression tree procedure.

Table 3. Allelic diversity of WCR sampled in 1996, 2009 and 2011 in the Vukovar-Sirmium region[a]

Population	Microsatellite loci						
	DVV-D2	DVV-T2	Dba05	DVV-D4	DVV-D8	Dba07	Mean
Bošnjaci, 1996	$n = 30$[b]	$n = 30$	$n = 27$	$n = 30$	$n = 30$	$n = 30$	$n = 29.50$
Number of alleles[c]	4	2	2	4	4	3	3.17
F_{IS}[d]	−0.10	−0.23	−0.21	0.10	−0.17	−0.16	−0.13
H_o[e]	0.25	0.12	0.16	0.16	0.24	0.21	0.18
H_e[f]	0.22	0.09	0.13	0.17	0.20	0.18	0.17
Otok, 1996	$n = 26$	$n = 30$	$n = 27$	$n = 29$	$n = 25$	$n = 27$	$n = 27.33$
Number of alleles	3	2	2	3	5	3	3.00
F_{IS}	0.28	−0.93	−0.70	−0.13	−0.17	−0.03	−0.28
H_o	0.11	0.29	0.23	0.16	0.21	0.17	0.20
H_e	0.15	0.15*	0.13*	0.14	0.18	0.16	0.15
Drenovci, 1996	$n = 25$	$n = 27$	$n = 27$	$n = 22$	$n = 26$	$n = 25$	$n = 25.33$
Number of alleles	4	2	2	3	4	3	3.00
F_{IS}	−0.11	−0.93	−0.41	0.30	−0.01	−0.41	−0.26
H_o	0.21	0.26	0.11	0.08	0.18	0.20	0.17
H_e	0.19	0.13*	0.11	0.11	0.17	0.14*	0.14
Vrbanja, 1996	$n = 24$	$n = 30$	$n = 27$	$n = 30$	$n = 28$	$n = 28$	$n = 27.83$
Number of alleles	4	2	2	4	4	3	3.17
F_{IS}	0.12	−0.42	−0.70	−0.27	−0.01	−0.20	−0.25
H_o	0.16	0.20	0.23	0.24	0.18	0.21	0.20
H_e	0.18	0.14*	0.13*	0.18	0.17	0.17*	0.16
Bošnjaci, 2009	$n = 30$	$n = 28$	$n = 30$	$n = 27$	$n = 30$	$n = 30$	$n = 29.17$
Number of alleles	4	2	2	3	5	3	3.17
F_{IS}	−0.46	−0.01	−0.40	−0.23	0.01	−0.02	−0.19
H_o	0.22	0.12	0.21	0.19	0.18	0.20	0.19
H_e	0.15*	0.11	0.15	0.15	0.18	0.19	0.16
Otok, 2009	$n = 30$	$n = 28$	$n = 30$	$n = 29$	$n = 30$	$n = 30$	$n = 29.5$
Number of alleles	4	2	2	4	4	3	3.17
F_{IS}	−0.46	−0.10	0.07	−0.08	−0.05	0.00	−0.10
H_o	0.24	0.06	0.14	0.19	0.21	0.18	0.17
H_e	0.16*	0.05	0.14	0.17	0.19	0.18	0.15
Drenovci, 2009	$n = 26$	$n = 25$	$n = 26$	$n = 25$	$n = 26$	$n = 26$	$n = 25.67$
Number of alleles	5	3	2	3	4	5	3.67
F_{IS}	−0.11	−0.10	−0.67	0.15	0.11	−0.16	−0.13
H_o	0.18	0.06	0.21	0.12	0.16	0.18	0.15
H_e	0.16	0.05	0.12*	0.14	0.17	0.15	0.13
Vrbanja, 2009	$n = 30$	$n = 30$	$n = 30$	$n = 30$	$n = 30$	$n = 30$	$n = 30$
Number of alleles	4	2	2	3	6	4	3.5
F_{IS}	0.20	0.06	0.02	0.09	−0.19	0.01	0.03
H_o	0.14	0.08	0.15	0.17	0.24	0.19	0.16
H_e	0.17*	0.08	0.15	0.18	0.20	0.19	0.16
Bošnjaci, 2011	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$
Number of alleles	6	4	3	3	4	3	3.8
F_{IS}	0.154	−0.018	0.26	−0.222	0.26	0.031	0.0788
H_o	0.60	0.53	0.40	0.73	0.47	0.67	0.57
H_e	0.68	0.51	0.52	0.58	0.60	0.66	0.59
Otok, 2011	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$
Number of alleles	4	3	2	3	4	3	3.2
F_{IS}	0.194	−0.207	0.153	−0.115	−0.111	−0.044	−0.022
H_o	0.63	0.44	0.44	0.75	0.81	0.69	0.63
H_e	0.75	0.35	0.50	0.65	0.71	0.64	0.6
Drenovci, 2011	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$
Number of alleles	5	2	2	3	4	3	3.2
F_{IS}	0.111	−0.175	0.084	0.067	−0.077	−0.278	−0.0455
H_o	0.67	0.33	0.47	0.53	0.80	0.73	0.59
H_e	0.72	0.28	0.49	0.55	0.72	0.56	0.55
Vrbanja, 2011	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$	$n = 15$
Number of alleles	4	2	2	3	6	4	3.5
F_{IS}	−0.124	−0.077	−0.59*	−0.422	−0.22*	0.42*	−0.4227
H_o	0.80	0.20	0.80	0.87	0.87	0.40	0.63
H_e	0.69	0.18	0.50	0.60	0.69	0.66	0.55

[a] *, significant deviation from Hardy–Weinberg equilibrium.
[b] *n*, number of individuals scored per locus.
[c] Total number of alleles.
[d] F_{IS}, Weir and Cockerham's[52] inbreeding coefficient.
[e] H_o, observed heterozygosity.
[f] H_e, expected heterozygosity.

Table 4. Pairwise estimates of F_{ST} (θ) (below diagonal) and geographic distances (km) (above diagonal, indicated once) of WCR sampled in Vukovar-Sirmium region in 1996, 2009 and 2011. Values in bold were significant after corrections for multiple comparisons for within individual years spatial differences ($n = 4$, $P < 0.05$) and for between years temporal and spatial differences ($n = 66$, $P < 0.05$)

	Population	Bošnjaci	Drenovci	Otok	Vrbanja	Bošnjaci	Drenovci	Otok	Vrbanja	Bošnjaci	Drenovci	Otok
		1996				2009				2011		
1996	Bošnjaci		24	30	20							
	Drenovci	0.022		30	7							
	Otok	0.037	0.018		22							
	Vrbanja	0.003	0.025	0.022								
2009	Bošnjaci	**0.047**	**0.076**	**0.048**	**0.048**							
	Drenovci	0.031	**0.083**	**0.065**	0.045	0.015						
	Otok	**0.027**	**0.070**	**0.056**	**0.055**	0.006	0.012					
	Vrbanja	**0.015**	**0.056**	0.034	**0.027**	0.004	−0.002	−0.004				
2011	Bošnjaci	0.022	0.053	**0.089**	0.035	**0.065**	**0.066**	**0.075**	**0.063**			
	Drenovci	0.001	0.055	0.068	0.014	**0.050**	0.016	**0.043**	**0.030**	0.008		
	Otok	**0.003**	0.049	0.058	0.011	**0.019**	0.012	**0.019**	0.010	−0.002	−0.008	
	Vrbanja	0.010	0.071	**0.080**	0.030	**0.032**	0.007	**0.029**	**0.013**	0.005	−0.005	−0.011

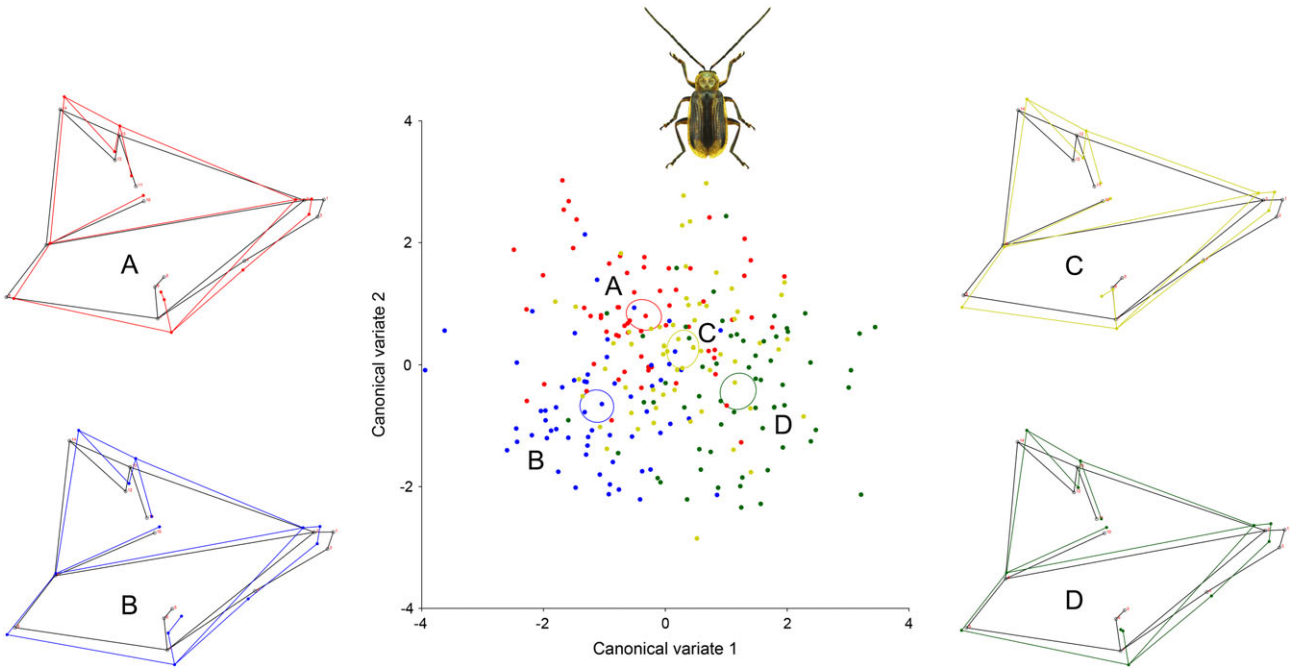

Figure 5. Canonical variate analysis (CVA) of four WCR populations. The first two CV axis points represent individuals of every population with corresponding 95% confidence intervals (coloured circles) and the wireframe visualisation of the average shape for all populations (black line average shape, coloured line target shape). Colours represent populations: A – red: Drenovci; B – blue: Otok; C – yellow: Bošnjaci; D – green: Vrbanja.

others who have shown that lower average yearly temperatures, higher amounts of rainfall and neutral or slightly acidic soils are important predictors for higher population abundances of adult WCR.[16,17,59,62,63]

It is important to point out that larval population levels, and how these translate into adult densities, has not been investigated. Larval population size and its relationship with continuous maize have been investigated, although Igrc Barčić *et al.*[64] showed that an edge effect for WCR egg laying can reach approximately 20 m into neighbouring maize fields. It is possible to see larval damage in rotated fields caused by non-resistant WCR variants (until now only present in US maize–soybean rotation). Generally, the adult population represents the colonisation of new fields and is not only a result of the larval development in a single field. If the fields are small in size and narrow (as is often the case in Croatia), WCR adult populations in first-year maize may be the result of larval development as well as climatic conditions.

4.2 Genetic monitoring

Microsatellite-based estimates of WCR genetic differentiation showed that genetic variation was low to moderate during the 15 years of monitoring in eastern Croatia. Overall results from the population genetic analyses indicate that the populations investigated are genetically similar and exist as a single large population in the Vukovar-Sirumium region. Similar patterns of consistently low F_{ST} estimates among populations sampled at small scales are common in Europe.[24,25,65] Low F_{ST} values may indicate gene flow, but they could also mean that WCR populations have not

Table 5. Procrustes ANOVA for both centroid size and shape of WCR hind wings, (dimensionless), characterised by matching symmetry (left and right wings). Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances

Centroid size							
Effect	SS	MS	df	*F*	*P*	Pillai trace	*P* (param)
Location	0.787	0.197	4	1.05	0.384		
Individual	25.663	0.187	137	73.9	<0.0001		
Shape							
Effect	SS	MS	df	*F*	*P*	Pillai trace	*P* (param)
Location	0.009	9.364E-05	96	2.02	<0.0001	1.24	<0.0001
Individual	0.153	4.638E-05	3288	2.83	<0.0001	16.67	<0.0001

Table 6. Results of CVA analysis with Procrustes and Mahalanobis distances and the respective *P*-values (in parentheses) for wing shape variation

	Drenovci	Otok	Vrbanja	Drenovci	Otok	Vrbanja
	Procustes *P*-value			Mahalanobis *P*-value		
Otok	0.008			1.713		
	(0.007)			(<0.0001)		
Vrbanja	0.011	0.011		1.989	2.348	
	(0.0001)	(0.0002)		(<0.0001)	(<0.0001)	
Bošnjaci	0.008	0.008	0.008	1.343	1.829	1.495
	(0.014)	(0.027)	(0.011)	(<0.0001)	(<0.0001)	(<0.0001)

yet attained genetic equilibrium. As shown in this study and confirmed by Lemic *et al.*[24] and Ivkosic *et al.*,[25] F_{ST} increased over time at the intrapopulation level. This result is supported by the findings of the STRUCTURE analysis, which revealed six different clusters, each potentially representative of populations sampled from 1996 to 2011. Previous work by Lemic *et al.*[24] showed only one cluster within Croatia, contrary to the findings from this study which was conducted on a microgeographic scale (i.e. 600 km^2). We suspect that the results found in this study have arisen because of a lack of consistency in crop rotation practices, which are known to differ greatly among primary producers in this area. This confirms the results from the temporal bottleneck analyses conducted on all populations during the three time periods investigated. The majority of populations sampled in 1996 underwent a bottleneck and a significant loss of genetic diversity (indicated by low H_e estimates), probably associated with the initial introduction of a small number of individuals into the region investigated. However, in 2009 there was limited evidence of bottlenecks occurring, and it was not until 3 years after mandatory crop rotation practices were implemented in Croatia that populations were perhaps forcibly fragmented and artificial bottlenecks created, as seen for all Vukovar-Sirmium populations sampled in 2011. We suspect that mandatory crop rotation practices are now working to fragment populations, making them easier to control and providing less opportunity for gene flow. Although genetic structure estimates indicated that a single population exists in the Vukovar-Sirmium region, Bayesian genetic clustering revealed the possibility of restricted gene flow, and the presence of genetic bottlenecks indicates the negative effect that crop management may have on the genetic diversity of WCR in this region.

Although traditional monitoring techniques provide valuable observational data of a population density in one specific area, genetic monitoring is necessary to evaluate the efficacy of the pest control techniques used. If mandatory pest management in this area (i.e. crop rotation) is effective, future analyses of genetic variation (underpinning the principles of genetic monitoring) should reveal moderately fragmented populations and a situation of increasingly restricted gene flow between populations.

It is not necessary to conduct annual genetic monitoring in all infested areas. Rather, what is recommended is the annual sampling of adults within the expansion front of the invasion and in newly invaded areas. In areas where WCR are established, sampling once every 2–3 years is sufficient to determine current levels of genetic diversity and associated population genetic measures. Once samples have been collected and genotyped at the core-set loci,[27] genotypes can be compared with an established database to inform ongoing management practices.[66] Comparing genetic results is possible to detect new introductions, possible admixture events, increase or decrease in genetic diversity, as a result of an establishment event in some area, and bottlenecks that occur or vanish during the invasion process, all of which can lead to certain conclusions concerning the effectiveness of control measures and provide insight into population genetics. New uncontrolled introductions and population admixture events should obviously be a sign of ineffective quarantine measures and poor customs control. Genetically diverse populations, as evidenced by increased diversity, may be due either to genetic drift or to admixture facilitated by human-assisted movement of WCR via international flights in the trade of commodities.[11] Previous findings[11] reveal patterns of mixed ancestry as a result of relaxed quarantine conditions in Europe and the ever-increasing international trade. Evidently, intercontinental WCR introductions are still occurring, and this finding highlights the need for the mitigation of new introductions of WCR from the United States and possibly from the EU to the United States (i.e. bidirectional gene flow) by implementing strict quarantine practices.[25] Knowledge of a pest's invasion history (source population) in a specific area, as well as the direction of its spread, is important for planning future strategies for its

control. Therefore, genetics together with other novel-use techniques can be used to detect the effect that various factors (e.g. resistance, changes in climatic conditions, diet) have on WCR.[67] Nevertheless, the use of genetic monitoring techniques are important in WCR management and can be effectively used to detect the possible entry points into Europe of control resistant variants currently only found in the United States. The future development of biomarkers to track the movement of rotation-resistant variants will depend on the implementation of genetic monitoring and its effectiveness. As always, there is the possibility that WCR established in Europe will adapt to selective pressure of crop rotation naturally and develop resistance *in situ*. For this reason, routine genetic monitoring should be written into integrated management practices for this species.[24,25,66]

4.3 Geometric monitoring

WCR hind wing shape changed according to major soil type classifications in Croatia. Previous studies differentiated three wing types based on soil characteristics such as soil temperature and moisture.[67] In the Vukovar-Sirmium region, only one soil type dominates (i.e. chernozemic) and dry weather conditions are characteristic.[68] Our results indicated that overall hind wing shape variation for WCR in the Vukovar-Sirmium region was most important in proximal landmarks 2 and 3 and distal landmarks 8 and 14. The expansion of these specific landmarks resulted in narrowed hind wing morphotypes. This confirms results from a previous study by Benitez *et al.*,[67] who also detected narrowed wing type for WCR from eastern Croatia. When we analysed populations by location, the results showed differentiation of all four populations based on expansion of landmarks 5 and 6. This means that populations from the small geographic area of Vukovar-Sirmium are significantly different, based on wing shape, and therefore there are four different morphotypes in the one relatively small geographic area (600 km^2). This result is a confirmation of the statement by Bouyer *et al.*[32] that the 'influence of environment on an organism's genotype takes much longer to manifest than on the phenotype of an organism', and contrary to our genetic findings where there were no differences found within a single time period or year. In addition, WCR hind wing shape changed not only by major soil type, as per Benitez *et al.*,[67] but also according to environmental conditions on a microgeographic scale, possibly influenced by different pest management practices.[67] Similar results have been shown where various management practices covary with wireworm capsule head shape.[69]

Wing shape morphotypes are known to influence the dispersal capabilities of flying insects.[70,71] According to previous studies on butterflies, a narrowed wing type is more efficient for flapping low-level flights.[72,73] In beetles, only studies regarding the flight morphology and evolution of wings have been performed.[74–78] Experimental studies on migration that is specifically connected with wing shape adaptations are needed in order to gain a better understanding of flight performance and its influence on invasiveness.

This is the first study to use a combination of different techniques (some novel use) to monitor WCR, and in doing so to detect interpopulation variation caused by environmental factors (e.g. climate, soil characteristics and pest management practices). The outcomes from this work suggest that the combined use of traditional monitoring (sticky traps, etc.), which can be effectively used to predict population abundance, and novel-use monitoring techniques, such as population genetics and geometric morphometrics, can be effectively used to estimate variation within and among populations. Through the combined application of traditional and novel-use monitoring techniques it will be possible to achieve better control and management of WCR in Europe.

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