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Evolutionary directional asymmetry and shape variation in *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae): an example using hind wings

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The western corn rootworm *Diabrotica virgifera virgifera* LeConte is a pest of maize in the USA and Europe and especially a problem in particular regions of Croatia. In the present study, patterns of variation in hind wing shape were examined. The first objective was to examine the influence of soil type on 10 populations of *D. v. virgifera* sampled from three regions in Croatia that differed according to edaphic factors and climate. The second objective was to investigate the potential evolutionary presence of directional asymmetry on hind wings. Geometric morphometrics was used to examine these objectives by quantifying the morphological variation within and among individuals and populations. Overall, *D. v. virgifera* hind wing shape changed according to major soil type classifications in Croatia. The three hind wing morphotypes found varied because of basal radial vein differences, related to landmarks 1, 3, 7, and 14. The findings of the present study show that hind wing shape in *D. v. virgifera* can be used to differentiate populations based on edaphic factors and may have application as a monitoring tool in the integrated management of *D. v. virgifera*. In an evolutionary context, the presence of directional asymmetry in the hind wings of *D. v. virgifera* adds to the ever growing data on the evolution of insect wings.

ADDITIONAL KEYWORDS: asymmetry – geometric morphometrics – rootworm – soil type – western corn – wing shape.

INTRODUCTION

For two decades, Croatian maize-growing areas have been the subject of invasion by the beetle pest *Diabrotica virgifera virgifera* LeConte. This particular pest was introduced into Europe in the early 1990s from the USA (Igrc Barčić, Bažok & Maceljski, 2003) and has subsequently spread through much of continental Europe (EPPO, 2012). In Croatia, *D. v. virgifera* is now established in the eastern and central parts of the country and there is evidence that its east to west spread is in part determined by soil type and hence suitability of soils for larval development (Toepfer & Kuhlmann, 2006; Kos, 2011). Within Croatia, *D. v. virgifera* infests maize fields from the eastern (chernozemic soils), central (ground water gley soils), and northern (alluvial soils) regions of the country (Bogunović *et al.*, 1996). As a consequence of the differing soil types in each of these regions (above), the prevailing microhabitat parameters vary,

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with the most important parameters being that of soil moisture and temperature; both of which are known to impact upon egg survival and larval development in Croatia (Kos, 2011), as well as elsewhere in Europe (Toepfer & Kuhlmann, 2006) and in the USA (Godfrey *et al*., 1995). At present, *D. v. virgifera* egg and larval abundance is greatest from central-northern Croatia (i.e. Gola) where maize grows in fields that are characterized by geologically young and agriculturally fertile alluvial soils (Kos, 2011).

Extensive population genetic surveys of *D. v. virgifera* in central and south-eastern Europe have failed to revel how individuals and populations have changed genetically as a result of the invasion process or indeed the habitats invaded (Ciosi *et al*., 2008, 2010; Lemic, Mikac & Bažok, 2013). Rather, these studies have revealed only that little genetic variation exists among populations spanning Italy, Austria, Hungary, Slovenia, Croatia, and Serbia. In particular, extensive population genetic surveys of *D. v. virgifera* spanning eastern, central, and northern Croatia from soon after they were first detected (i.e. approximately 1996) to the present time (2011) have revealed minimal genetic differences between populations and among regions (Lemic *et al*., 2013). Bouyer *et al*. (2007) suggested that the influence of environment on the genotype of an organism takes much longer to manifest than on the phenotype of an organism and, as such, the study of environmental influences on populations and individuals should be made on phenotypic, rather than genotypic, characters. Indeed, Bouyer *et al*. (2007) was able to demonstrate this for the tsetse fly *Glossina palpalis gambiensis* Vanderplank, when comparing wing shape and size differences with population genetic differences along an ecological cline, where geometric morphometrics analysis of wing shape and size demonstrated clinal differences, a result that was not mirrored by the microsatellite markers used. Mikac, Douglas & Spencer (2013) recently used wing shape and size as a population marker to demonstrate that differences in hind wing shape were detectable among *D. v. virgifera* sampled from maize-soybean rotation resistant and susceptible populations in the USA. Mikac *et al*. (2013) recommended the use of geometric morphometrics as a tool to understand how hind wing shape and size have changed during the invasion of maize-growing areas in the USA and Europe.

Geometric morphometrics is useful when quantifying the morphological variation within and among populations and species, and begins with the collection of two- or three-dimensional coordinates of biologically definable landmarks (Bookstein, 1991) (e.g. hind wing vein intersections in *D. v. virgifera*) (Mikac *et al*., 2013). Among the parameters possible to investigate with morphometrics is asymmetry, the study of which has flourished in the past two decades (Møller & Swaddle, 1997; Polak, 2003; Graham *et al*., 2010). Van Valen (1962) defined directional asymmetry (DA) as occurring when 'there is normally a greater development of a character on one side of the plane or planes of symmetry than on the other'. The basic study of asymmetry started with Van Valen (1962) and, in more recent times, the focus has been on linear distances, with many studies using the framework of analysis developed by Leamy (1984) and Palmer & Strobeck (1986). Directional asymmetry is common in animals and humans, particularly in internal organs (Mittwoch, 1988; Ligoxygakis, Strigini & Averof, 2001; Boorman & Shimeld, 2002; Toga & Thompson, 2003; Palmer, 2004). However, conspicuous external asymmetries are less widespread but have evolved in many lineages, including fishes, birds, and mice (Palmer, 2004). Subtle DA is commonly found using geometric morphometric methods and DA is widespread (Auffray *et al*., 1996; Smith, Crespi & Bookstein, 1997; Klingenberg, McIntyre & Zaklan, 1998; Debat *et al*., 2000; Ivanović & Kalezić, 2010; Klingenberg, Debat & Roff, 2010). In the present study, a geometric morphometric approach was used to address objectives with applied and evolutionary importance. The first objective was applied and aimed to assess whether *D. v. virgifera* hind wing shape changed according to major soil type classification in Croatia. The second objective was evolutionary based and aimed to examine whether DA could be extracted from the hind wing shape of *D. v. virgifera* in a bid to improve the general understanding of the presence of this type of asymmetry in Coleoptera.

MATERIAL AND METHODS

SAMPLE SITES AND SPECIMEN COLLECTION

Adult *D. v. virgifera* were collected by hand from corn, *Zea mays* L., in July 2011 from 10 locations in Croatia (Fig. 1). All specimens were processed in accordance with methods outlined by Mikac *et al*., 2013 (Table 1). For subsequent analyses, locations were grouped by soil type. Group 1 consisted of locations: Rugvica, Stupovača, Požega, and Slavonski Brod, which were characterized by ground water gley soils. Group 2 consisted of locations: Stari Mikanovci, Nuštar, Otok, Vrbanja, and Gunja, which had alluvial soils. Group 3 consisted of the location Gola, which had chernozemic soil (Bogunović *et al*., 1996). All specimens collected were preserved in 70% ethanol and sex was determined through the examination of the abdominal apex prior to hind wing dissection (White, 1977). Left and right hind wings were removed from each individual and slide-mounted using the fixing agent

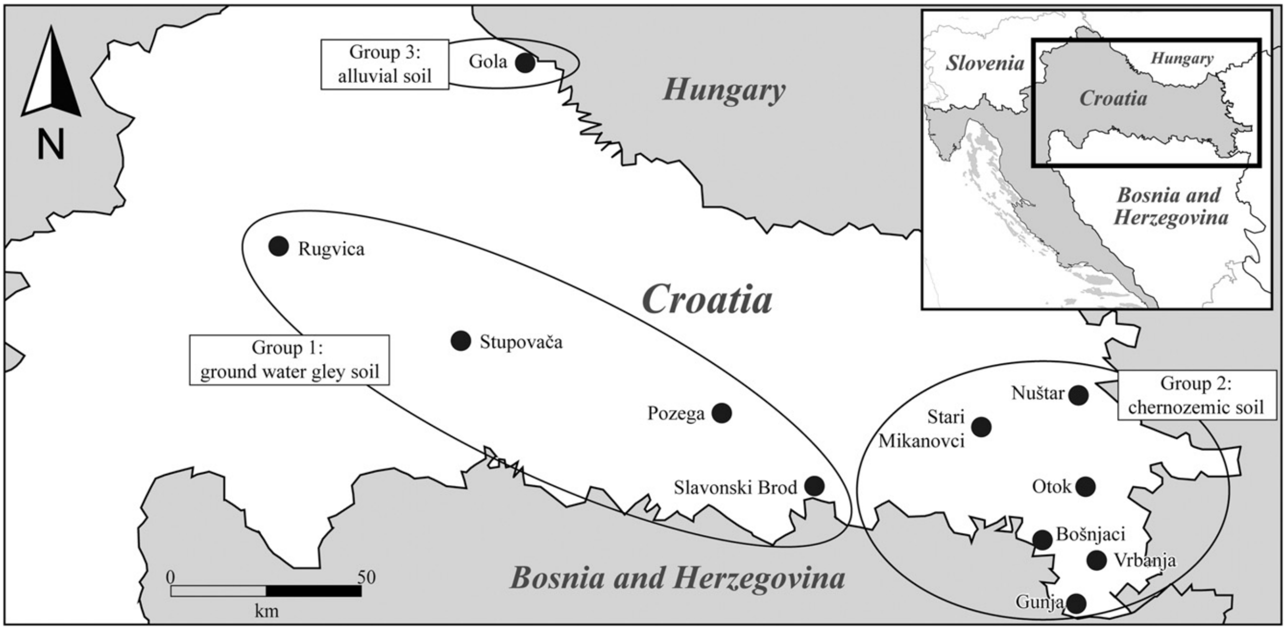

Figure 1. Geographical locations sampled in Croatia, grouped according to major prevailing soil type and climate.

Table 1. Geographical locations and coordinates of the 10 populations of *Diabrotica virgifera virgifera* sampled per sex and left and right wings

	Male		Female	
Location/coordinates	Left wing (*N*)	Light wing (*N*)	Left wing (*N*)	Light wing (*N*)
Gola: 46°11′41.69′N; 17°2′52.21′E	15	17	15	15
Gunja: 44°53′26.70′N; 18°49′23.82′E	15	15	15	15
Mikanovci: 45°17′13.73′N; 18°32′50.61′E	18	15	15	15
Nuštar: 45°19′50.75′N; 18°49′56.48′E	15	15	15	16
Otok: 45°8′38.15′N; 18°52′3.19′E	15	15	15	15
Požega: 45°20′41.43′N; 17°39′44.00′E	9	9	20	21
Rugvica: 45°44′24.11′N; 16°13′43.06′E	7	7	24	24
Slavonski Brod: 45°10′17.07′N; 17°58′9.75′E	13	13	17	16
Stupovača: 45°32′15.89′N; 16°50′18.06′E	8	8	21	23
Vrbanja: 44°59′7.43′N; 18°54′54.39′E	15	15	15	15

Euparal (Australian Entomological Supplies, Melbourne, Australia) based on standard methods (Upton & Mantel, 2010).

Wing landmark acquisition

Slide-mounted wings were photographed using a Leica DFC295 digital camera (3 meagpixel) on a trinocular mount of a Leica MZ16a stereo-microscope and saved in JPEG format using the LEICA APPLICATION SUITE, version 3.8.0 (Leica Microsystems Ltd). Fourteen type 1 landmarks (Fig. 2) defined by vein junctions or vein terminations (Bookstein, 1991) were used in morphometric analyses. Specimens for which wing veins were damaged or where the wing tissue had folded during the slide mounting process were discarded and excluded from further analysis.

Morphometric analysis

Each landmark was digitized using TPSDIG, version 2.16 (Rohlf, 2008) for which *x* and *y* co-ordinates were generated to investigate hind wing shape. Initially, the symmetric component of hind wing shape was calculated from the means of original and reflected copies. Then, an asymmetric component was

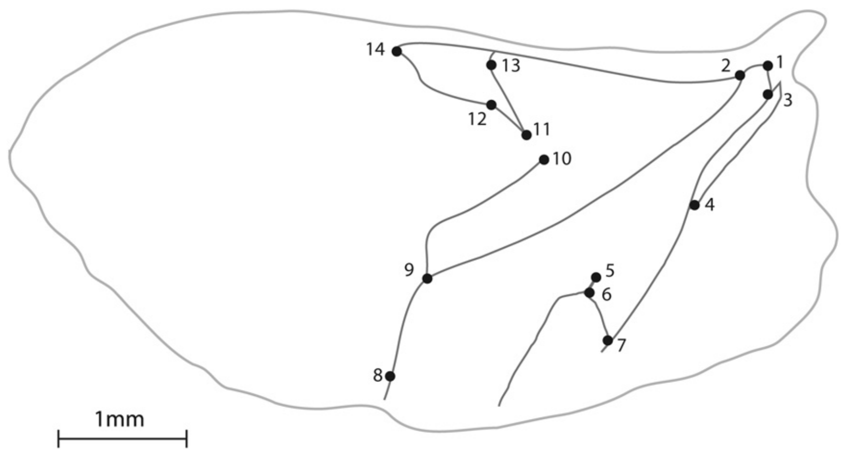

Figure 2. Representation of the 14 morphological landmarks identified on the hind wings of *Diabrotica virgifera virgifera*.

computed from the differences between the original and reflected copies (Klingenberg & McIntyre, 1998; Klingenberg, Barluenga & Meyer, 2002). Measurement error (ME) is of critical importance when analyzing symmetries (Palmer, 1994). To assess the significance of DA relative to ME, the left and right wings of 30 individual beetles were digitized twice (Klingenberg & McIntyre, 1998). The interlocation differences were assessed using Procrustes distances, which were the product of a canonical variates analysis (CVA). The results were reported as Procrustes distances and the respective significance (*P*) values for these distances, after permutation tests (10 000 runs), were reported. A general discussion of how Procrustes distances are used in geometric morphometrics is provided by Mikac *et al*. (2013).

Directional asymmetry and multivariate analysis

Directional asymmetry was tested using an analysis of variance (ANOVA) of Procrustes transformed hind wing shape data that considered individual and side effects, and the interaction between both. In the ANOVA, the mean squares (MS) was related to the individual effect and was used as an estimator of an individual's variation, whereas the MS related to the interaction (individual × side) for the left or right side was used as an estimator of DA. The existence of DA can be tested statistically through the main effect of 'side'. The shape variation in the entire dataset was analyzed using principal component analysis (PCA) based on the covariance matrix of symmetric and asymmetric components of hind wing shape variation. The shape variation component is the mean of the left and right sides of the hind wings, whereas the asymmetric component represents an individual's left–right differences (Klingenberg *et al*., 2002).

All morphometric and statistical analyses were performed using MORPHO J, version 1.04a (Klingenberg, 2011).

RESULTS

Morphometric analysis

The Procrustes ANOVA for assessing the measurement error showed that the MS for individual variation exceeded the measurement error (Table 2). The three location/soil type groups were differentiated according to the movement of landmarks 1, 3, 7, 8, and 14. At the individual group level, for group 1, the mean hind wing shape showed expansion of landmarks 3 and 7, resulting in an elongated hind wing morphotype (Fig. 3). For group 2, the mean hind wing shape showed the expansion of landmarks 2, 3, and 14, resulting in a narrowed hind wing morphotype (Fig. 3). Individuals from group 3 were characterized by narrow hind wings, with the movement of hind wing landmarks being for positions 3, 7, and 14 (Fig. 3).

A Procrustes ANOVA for hind wing shape showed highly significant differences among localities ($P < 0.0001$) and between sex ($P < 0.0001$) (Table 3). The PCA of hind wing shape variation showed that the first three PCs accounted for 47.6% (PC1 = 21.03%; PC2 = 15.05%; PC3 = 11.57%) of the total shape variation and provided a reasonable approximation of the total amount of wing shape variation. The CVA showed significant differences among locations based on Procrustes distances (Table 4).

Directional asymmetry

The Procrustes ANOVA confirmed a pattern of DA with significant differences in the side factor detected for size ($F = 4.31$; $P < 0.001$) and shape ($F = 6.98$; $P < 0.0001$) (Table 5).

Table 2. Measurement error procrustes analysis of variance for both centroid size and hind wing shape of *Diabrotica virgifera virgifera* characterized by matching symmetry

	SS	MS	d.f.	*F*	*P*	Pillai's Trace	*P* (parameter)
Centroid size							
Effect							
Individual	0.003	0.0001	29	0.1	1		
Side	0.0007	0.0007	1	0.71	0.407		
Individual × Side	0.02	0.001	29	661.8	< 0.0001		
Error 1	**0.00009**	**0.000002**	**60**				
Shape							
Effect							
Individual	0.05	0.00007	696	1.15	0.03	14.91	< 0.0001
Side	0.0006	0.00002	24	0.39	0.99	0.86	0.3
Individual × Side	0.04	0.00006	696	9.17	< 0.0001	11.81	< 0.0001
Error 1	**0.01**	**0.00007**	**1440**				

Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances (i.e. dimensionless). Values shown in bold represent the values of the measurement error.

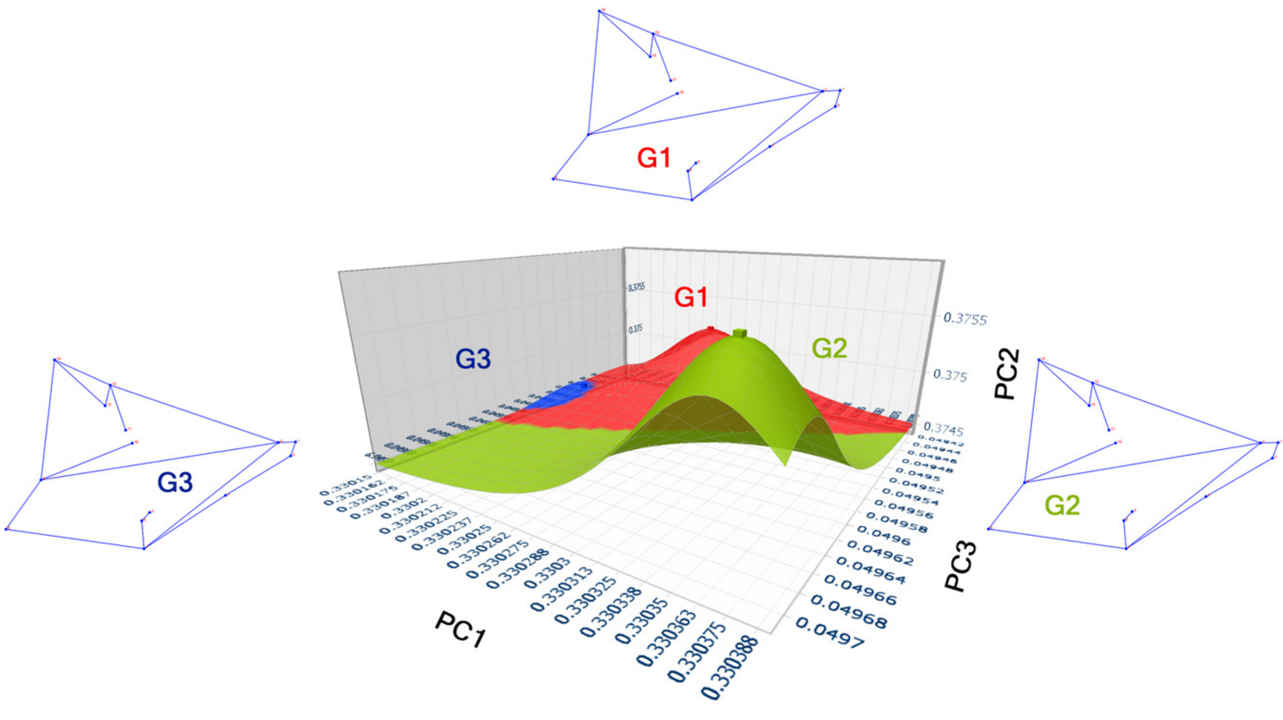

Figure 3. Three-dimensional volume dispersion graph of the average shape for the three location groups. The volumes simulate the shape space of variation founds. Group (G) 1, red (Rugvica, Stupovača, Požega and Slavonski Brod). Group 2, green (Stari Mikanovci, Nuštar, Otok, Vrbanja and Gunja). Group 3, blue (Gola). PC, principal component.

DISCUSSION

A geometric morphometric approach was used to investigate the variation in hind wing shape and size between both sexes of multiple populations of the beetle pest, *D. v. virgifera*. *Diabrotica v. virgifera* hind wing shape changed according to major soil type classification in Croatia. Three main shape differences were found (Fig. 3) and could be differentiated based on the three major soil types found in each broad area. The volume graph indicated that overall hind wing shape variation for *D. v. virgifera* was most important in landmarks 1, 3, 7, and 14. These landmarks relate to the basal radial vein and are the key

Table 3. Procrustes analysis of variance for both centroid size and shape of *Diabrotica virgifera virgifera*, characterized by matching symmetry

	SS	MS	d.f.	*F*	*P*	Pillai's Trace	*P* (parameter)
Centroid size							
Effect							
Location	10.99	1.09	10	6.53	**< 0.0001**		
Sex	0.25	0.25	1	1.51	0.22		
Individual	51.13	0.16	304	90.72	< 0.0001		
Side	0.007	0.007	1	3.98	0.04		
Individual × Side	0.58	0.001	315	0.01	1		
Residual	0.22	0.22	1				
Shape							
Effect							
Location	0.03	0.0001	240	2.75	**< 0.0001**	1.7	**< 0.0001**
Sex	0.01	0.0006	24	12.8	**< 0.0001**	0.39	**< 0.0001**
Individual	0.37	0.00005	7296	2.67	< 0.0001	16.37	< 0.0001
Side	0.002	0.0001	24	5.66	< 0.0001	0.4	< 0.0001
Individual × Side	0.14	0.00002	7560	0.51	0.99		
Residual	0.0009	0.00003	24				

Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances (i.e. dimensionless). Values shown in bold represent location and sex effect.

Table 4. Canonical variate analysis of Procrustes distances at the 10 locations sampled in Croatia and associated significance values

	Procustes distances								
Locations	1	2	3	4	5	6	7	8	9
2	0.006								
3	0.008**	0.005							
4	0.007*	0.008*	0.009*						
5	0.008*	0.006	0.006	0.009*					
6	0.013**	0.013**	0.012**	0.015**	0.011**				
7	0.012**	0.011**	0.011**	0.016*	0.01*	0.007*			
8	0.008*	0.009**	0.01**	0.009**	0.01**	0.014**	0.013**		
9	0.009*	0.008*	0.007*	0.006	0.009*	0.015**	0.016**	0.009**	
10	0.011**	0.011**	0.01**	0.01**	0.011**	0.016**	0.016**	0.007*	0.008*

1, Gola; 2, Gunja; 3, Stari Mikanovci; 4, Nuštar; 5, Otok; 6, Požega; 7, Rugvica; 8, Slavonski Brod; 9, Stupovača; 10, Vrbanja. $^*P < 0.05$; $^{**}P < 0.001$.

anatomical characters used to distinguish among hind wing shapes in Croatia based on soil type. There was a mean of 10 hind wing morphotypes associated with edaphic factors (soli type and climate) in Croatia. In the eastern populations (Stari Mikanovci, Gunja, Vrbanja, Otok, and Nuštar) where the weather is drier, individuals had narrow hind wings, whereas, in central Croatia (Rugvica, Stupovača, Požega, Slavonski Brod), a mixture of individuals with elongated and narrow hind wings were found, most likely resulting from a mix of soil types and greater variation in climate. Finally, *D. v. virgifera* from north-western Croatia (Gola), where daily mean temperatures are lower than in the east, had narrow hind winds. This is the first study of its kind to show that patterns in hind wing morphology change according to local edaphic factors. Similar studies have shown shape variation in plants under field and common garden conditions (Téllez & Møller, 2006) or other examples of Arthropod adaptations to edaphic conditions (Villani *et al*., 1999).

Directional asymmetry in wing size is widespread among insects, with left–right biased asymmetries being commonly observed (Pelabon & Hansen, 2008).

Table 5. Procrustes analysis of variance for both centroid size (CS) and shape (SH) of *Diabrotica virgifera virgifera*, characterized by matching symmetry

	Source of variation	SS	MS	d.f.	F	P	Pillai's Trace	P (parameter)
CS	Individual	62.26	0.19	314	107.4	< 0.0001		
	Side	**0.007**	**0.007**	**1**	**3.93**	**0.048**		
	Individual × Side	0.58	0.001	314	0.01	1		
SH	Individual	0.39	0.00005	7536	3.31	< 0.0001	17.11	< 0.0001
	Side	**0.002**	**0.0001**	**24**	**7**	**< 0.0001**	**0.41**	**< 0.0001**
	Individual × Side	0.11	0.00001	7536	0.42	1		

Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances (dimensionless). The effect of side (in bold) shows the presence of directional asymmetry in hind wings.

Attempts to estimate or reveal genetic variation in DA have repeatedly failed (Maynard Smith & Sondhi, 1960; Coyne, 1987; Tuinstra, De Jong & Scharloo, 1990; Monedero, Chavarrias & Lopez Fanjul, 1997), casting doubt on the evolutionary potential of DA (Maynard Smith *et al*., 1985; Lewontin, 2000). If true, this absence of genetic variation could possibly lead to evolutionary stasis in DA. Directional asymmetry corresponds to a fitness optimum resulting from some selective pressures acting on asymmetry (Pelabon & Hansen, 2008). Pelabon & Hansen (2008) reviewed 49 studies that showed DA in wing size for 47 insect species from seven orders (Diptera: 21 species; Hymenoptera: six species; Lepidoptera nine species; Mecoptera: one species; Orthoptera: two species; Thysanoptera: one species; Odonata: seven species). Currently, there are no data for DA in Coleoptera or Chrysomelidae. Directional asymmetry appears particularly sensitive to genetic perturbations such as those resulting from hybridization (Pelabon & Hansen, 2008). Hidden genetic variation remains and can be revealed by sudden changes in the system (Hermisson & Wagner, 2004). This may explain the results of studies by Leamy (1984), Klingenberg *et al*. (1998), Schneider *et al*. (2003) and Rego, Matos & Santos (2006) in terms of a release of hidden genetic variation in DA. These results are similar to those reported by Pelabon & Hansen (2008) in a recent review of DA in wing size in insects, where it was stated that more than one-quarter of the statistical tests and one-third of the species show significant DA. Klingenberg *et al*. (1998), Santos (2002), Santos, Iriarte & Cespedes (2005) and Carter, Osborne & Houle (2009) all found small but consistent DA in *Drosophila* species, showing the existence of left–right wing variation that has a genetic basis compared to other body parts or characters that do not.

Conclusions

Based on varying patterns in hind wing shape, it was possible to differentiate populations based on edaphic factors by region. These results are novel for *D. v. virgifera* and, as such, the use of morphometrics in the integrated management of *D. v. virgifera* warrants further investigation. *Diabrotica v. virgifera* hind wing shape changed according to major soil type classifications in Croatia. The three hind wing shapes found varied because of basal radial vein differences, relating to landmarks 1, 3, 7, and 14. Where a ground water gley soil type dominated, an elongated hind wing morphotype was found. By contrast, where a chernozemic or alluvial soil type dominated, a narrow hind wing morphotype was found. In addition to the applied results, the presence of DA in *D. v. virgifera* is a novel finding for the order Coleoptera and adds to ever growing pool of data on the general evolution of insect wings.

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