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

HUGO A. BENÍTEZ1,2*, DARIJA LEMIC3, RENATA BAŽOK3, CLAUDIO M. GALLARDO-ARAYA4 and KATARINA M. MIKAC5

1 Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK
2 Instituto de Alta Investigación, Universidad de Tarapacá, Casilla, 7-D Arica, Chile
3 Department for Agricultural Zoology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, Zagreb 10000, Croatia
4 Departamento de Química Orgánica I, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Madrid, Spain
5 Institute for Conservation Biology and Environmental Management, University of Wollongong, Wollongong, NSW 2522, Australia

Received 3 September 2013; revised 11 September 2013; accepted for publication 11 September 2013

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. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, 1–. 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 Barcˇic´, 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,
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 reveal 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 & Kalezic, 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 Mikanović, 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...
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 megapixel) 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

---

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

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

---

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

© 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, ••, ••••••
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).

**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).
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, Diabrotica virgifera virgifera. The 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

**DISCUSSION**

![Figure 3](image-url)
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 (soil 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, Stupovacˇa, 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 3. Procrustes analysis of variance for both centroid size and shape of Diabrotica virgifera virgifera, characterized by matching symmetry

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

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

<table>
<thead>
<tr>
<th>Locations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.008**</td>
<td>0.005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.007*</td>
<td>0.008*</td>
<td>0.009*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.008*</td>
<td>0.006</td>
<td>0.006</td>
<td>0.009*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.013**</td>
<td>0.013**</td>
<td>0.012**</td>
<td>0.015**</td>
<td>0.011**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.012**</td>
<td>0.011**</td>
<td>0.011**</td>
<td>0.016*</td>
<td>0.01*</td>
<td>0.007*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.008*</td>
<td>0.009*</td>
<td>0.01**</td>
<td>0.009*</td>
<td>0.01**</td>
<td>0.014**</td>
<td>0.013**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.009*</td>
<td>0.008*</td>
<td>0.007*</td>
<td>0.006</td>
<td>0.009*</td>
<td>0.015**</td>
<td>0.016**</td>
<td>0.009**</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.011**</td>
<td>0.011**</td>
<td>0.01**</td>
<td>0.01**</td>
<td>0.011**</td>
<td>0.016**</td>
<td>0.016**</td>
<td>0.007*</td>
<td>0.008*</td>
</tr>
</tbody>
</table>

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.
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 chernozem 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.

ACKNOWLEDGEMENTS

This research was financially supported by the Unity through Knowledge Fund through the Ministry of Science, Education and Sports of the Republic of Croatia; project: 178-1782066-2064 (risk estimation systems: basis for the integrated control of corn pests) and a University of Wollongong, Australia URC Small Grant 2012. We thank the three anonymous reviewers for their helpful comments.

REFERENCES


© 2013 The Linnean Society of London, Biological Journal of the Linnean Society, 2013, ••, ••–••


