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INVASION NOTE

Wing shape changes: a morphological view of the *Diabrotica virgifera virgifera* European invasion

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Abstract An analysis of the hind wing morphology (size and shape) within and among western corn rootworm, *Diabrotica virgifera virgifera* LeConte, populations over a large geographic scale in Europe was conducted. The changes in hind wing shape and size detected were related to identifiable invasion processes (i.e. multiple introduction events into Europe), first characterised using genetic markers. Overall implications from this work suggest that geometric morphometric techniques can be used to detect population changes related to invasions and could therefore serve as a cheaper and more accessible alternative 'biomarker' to more expensive and specialised-use genetic markers, such as microsatellites or SNPs, when investigating biological invasions.

Keywords Western corn rootworm · Invasions · Wing shape · Geometric morphometrics

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Introduction

Biological invasions continue to have major negative impacts upon biodiversity, public health, food biosecurity and agriculture (Ehrenfeld 2010). Changes in global trade practices coupled with often relaxed quarantine and bio-security procedures has greatly contributed to the spread and movement of species beyond their endemic range. Indeed even in countries with strict quarantine and bio-security measures, many imported products go unchecked due to an over stretched workload (Stanaway et al. 2001), making it impossible to completely rule out the threat of a biological invasion. As is common in a biological invasion, the start of the invasion occurs many years before the invasion itself is detected. An example of a successful invasion that has followed this model is that of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, into Europe from the USA (Miller et al. 2005). As has been chronicled many times in the literature, it is suspected that the *D. v. virgifera* was introduced into Europe via Serbia in the early 1980s (Bača et al. 1994) and it was not until the 1990s that it began to cause economic levels of damage to corn production in Croatia and neighbouring countries (Igrc Barčić et al. 2003; Szalai et al. 2010). Since the 1990s research in Europe has focused on *D. v. virgifera* biology (Igrc Barčić et al. 2003; Szalai et al. 2010), ecology (Igrc Barčić et al. 2003; Szalai et al. 2010) and most recently population genetic tools (Miller et al. 2005; Ciosi et al. 2010) and geometric morphometrics

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(Benítez et al. 2014a) have been used to assist in understanding historical and contemporary aspects of the invasion process.

It is well known that the *D. v. virgifera* is one of the most important pests of corn production and in the USA it is referred to as the "billion dollar bug" (Sappington et al. 2006). *D. v. virgifera* is now present in all corn growing areas in the USA and Europe and chemical and rotation resistant forms are common in the USA (Gassmann et al. 2014), but not in the Europe (Ciosi et al. 2009). In Europe *D. v. virgifera* is established throughout much of continent but is still actively spreading. An in depth population genetics investigation of the invasion phases (introduction, establishment and spread) conducted by Lemic et al. (2015a) revealed that the three phases often overlap and are still occurring concurrently in various locations in Europe. It has been estimated that *D. v. virgifera* populations have an average dispersal rate of approximately 40 km/year and a growth rate that allows them to quadruple in abundance annually when inadequately controlled (Igrc Barčić et al. 2003; Szalai et al. 2010). *D. v. virgifera* is known to be moving in an east to west direction in Europe and its continuous spread makes it one of the most serious pests of corn production in continental Europe.

While population genetic studies of *D. v. virgifera* in Europe have provided baseline data and information on invasion processes (Ciosi et al. 2008; Ivkosic et al. 2014; Lemic et al. 2015a) there is still a lack of information on how its morphology (phenotype) has changed during the invasion process. Recent advances in geometric morphometric techniques have enabled its application to evaluate phenotypic changes that may have occurred during the invasion process. Wing morphology (size and shape) is a critical element of an insect's dispersal capacity (DeVries et al. 2010). Determining the dispersal capabilities of invasive species is vital to understanding how individuals and populations change in response to the new environments they invade (Bouyer et al. 2007; Benítez et al. 2014a). Li et al. (2010) recognized the need to understand the relationship between *D. v. virgifera* morphometric traits and its dispersal and invasiveness. Geometric morphometrics is a useful technique in quantifying the morphological variation within and among species, and this methods begin with the collection of two-dimensional coordinates of biologically definable landmarks (Bookstein 1991) such as

hind wing vein intersections in *D. v. virgifera* (Mikac et al. 2013; Benítez et al. 2014a; Lemic et al. 2014). In application of the technique to understand invasive *D. v. virgifera* Mikac et al. (2013) investigated wing size and shape differences between resistant and non-resistant populations in the USA, and Lemic et al. (2014) and Benítez et al. (2014a) investigated wing shape variation in *D. v. virgifera* populations on micro-geographic scale. Despite recent use of geometric morphometrics in understanding *D. v. virgifera* there is no data on how hind wing size and shape have changed during their invasion of corn growing areas in Europe. In this paper we examined the hind wing morphology (size and shape) within and among *D. v. virgifera* populations over a larger geographic scale than previously conducted and further quantify whether morphometric techniques (e.g. Lemic et al. 2015a), can be used in lieu of population genetic techniques to describe patterns related to their biological invasion.

Materials and methods

Sample sites and specimen collection

Adults were collected by hand from maize plants in July 2012 from two locations in Croatia, two locations in Italy, one location in Hungary, Serbia and Austria each (Fig. 1). The specimens collected (n = 560) were preserved in 70 % ethanol and sex was determined through the examination of the abdominal apex prior to hind wing dissection. 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 and Mantel 2010).

Data analysis

Slide mounted wings were photographed using a Leica DFC295 digital camera (3 M Pixel) on a trinocular mount of a Leica MZ16a stereo-microscope and saved in JPEG format using the Leica Application Suite v3.8.0 (Leica Microsystems Limited, Switzerland). 14 landmarks were digitized (see Fig. 2 in Benítez et al. 2014a) defined by vein junctions or vein terminations using the software program tpsDIG v 2.17 (Rohlf 2013). Cartesian coordinates were extracted for all landmarks and the shape information was extracted

Fig. 1 The 2012 collection sites of adult *D. v. virgifera* in Croatia, Italy, Serbia, Hungary and Austria

Fig. 2 PCA of *D. v. virgifera* by Country indicating two distinct morphotypes based on geographic location and time of introduction
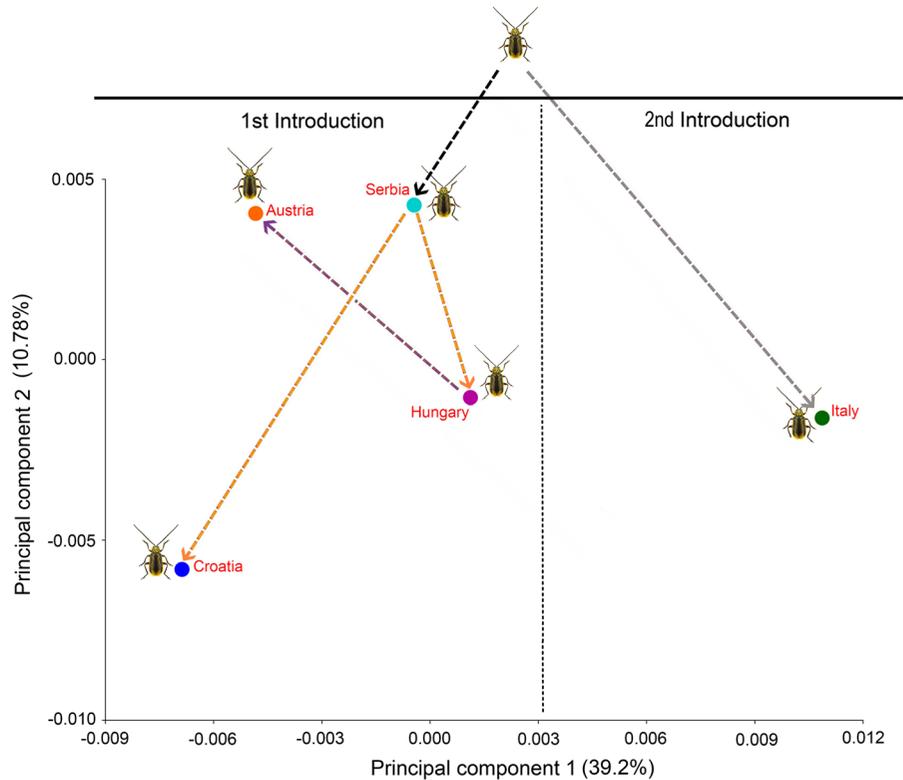
using a full Procrustes fit (Rohlf and Slice 1990). Measurement error is always undesirable but more or less inevitable in morphometric studies, it therefore requires to be minimized so that it does not interfere with the most subtle effects of interest under study (Arnqvist and Martensson 1998). To avoid possible effect on the morphometric data, the left and right wings were digitized twice and a Procustes ANOVA was calculated (Klingenberg and McIntyre 1998). The MS relate to the individual effect and were used as an estimator of an individual's variation, while the MS related to the interaction (individual* side) left or right side were used as an estimator of FA and compared with the digitized Error 1 in order to avoid this possible problem, that ended up being negligible (Klingenberg and McIntyre 1998, Klingenberg 2002).

In order to visualize hind-wing shape variation related to invasions processes, in multidimensional space, the entire dataset was analysed using principal component analysis (PCA) (Jolliffe 2002) based on the covariance matrix of the individual wing shape and average by Country shape (Jolliffe 2002; Lemic et al. 2014). The PCA as an ordination multivariate analysis was used as it better reflects the shape morphospace (Klingenberg 2013). To further quantify the magnitude of shape variation between countries and invasion phases Procrustes Distances and Mahalanobis values, that represent multivariate shape differences, were computed (Klingenberg and Monteiro 2005). In order to evaluate the effect of size on the shape a multivariate regression of the wing shape on centroid size was performed. All the morphometrics analysis were performed using the software MorphoJ 1.06e (Klingenberg 2011).

Results

The Procrustes ANOVA for assessing the measurement error showed that the mean square for individual variation exceeded (MS = 0.00006) the error (MS = 0.000007). Allometry effects on the dataset were taken into account, however, due to considerably small values a correction was not necessary for the data (predicted: 0.92 %, $P < 0.001$). A PCA of the covariance matrix of the average shape showed that wing shape followed a clear patterns associated with the introduction of *D. v. virgifera*, namely PC1 explained the first introduction of the species into Europe from eastern Europe to western

and then southern Europe, while the second introduction was separated from the first and occured into Italy (Fig. 2). The level of hind wing shape variation showed that the first three PCs accounted for 60.13 % (PC1 = 39.2 %; PC2 = 10.78 %; PC3 = 10.1 %) of the total shape variation and provided a sound estimate of the total amount of wing shape variation. The Procrustes and Mahalanobis distances, and associated permutation tests (corrected for multiple comparisons), showed significant relationships among groups (Table 1). Mahalanobis distances were greatest for pairwise comparisons between Italy and all other Countries suggesting that *D. v. virgifera* in Italy had more pronounced hind-wing shape differences overall (Table 1).

Discussion

In this paper we examined the hind wing morphology (size and shape) within and among *D. v. virgifera* populations over a larger geographic scale than previously conducted and further quantified whether morphometric techniques can be used in lieu of population genetic techniques to describe patterns related to their invasion (i.e. geographic introduction pathways and entry points). Both PCA and the two distance measures demonstrated that hind wing shape and size could reliably be used to patterns related to the invasion process of *D. v. virgifera* that correspond to the findings from population genetics studies of the same populations collected in early years (1995–2011).

For the purposes of this study *D. v. virgifera* were collected over 160,000 km^2 of agricultural corn production area and with over 500 specimen analysed, the data presented here represents a major morphological investigation of one invasive species. The dataset describes phenotypic changes across a major geographic corridor of invasion by this pest into corn growing areas in Europe. The data also demonstrates that population differences found using microsatellite markers (Lemic et al. 2015a) can also be detected at the same geographic locations using morphometric techniques.

The results presented here clearly separate out populations from Italy and those from the first known introduced population in central and southeastern Europe (see Miller et al. 2005; Ciosi et al. 2008), a result mirrored by Lemic et al. (2015a) who

Table 1 Pairwise Procrustes and Mahalanobis distances between countries

	Procrustes distances				Mahalanobis distances			
	Austria	Croatia	Hungary	Italy	Austria	Croatia	Hungary	Italy
Croatia	0.01*				1.64***			
Hungary	0.01**	0.01***			2.02***	1.19***		
Italy	0.01**	0.01***	0.01***		2.61***	2.56***	2.47***	
Serbia	0.008[ns]	0.01*	0.006[ns]	0.013***	1.77***	1.42**	1.34*	2.95***

* $P < 0.05$; ** $P < 0.001$; $P < 0.0001$; *ns* not significant

demonstrated this using genetic markers. Also, the hind-wing shape and size differences presented here that follow an east to west direction of spread, are corroborated by Igrc Barčić et al. 2003 (see Fig. 2), where it was shown that Serbia was the geographic source of *D. v. virgifera* in Croatia and Hungary, while Austrian populations arose from Hungary. Based on Lemic et al. (2015a) and the results of this study it is further possible to conclude that the Italian *D. v. virgifera* population have no link to the aforementioned populations and originate from a separate and most likely more recent introduction from the USA. It is important to note that while this conclusion using genetic markers required the analysis of *D. v. virgifera* over a period of two decades (Lemic et al. 2015a), through the use of geometric morphometrics a clear picture of the invasion process was possible from the sampling of *D. v. virgifera* in a single time period (i.e. here in 2012 only vs. Lemic et al. (2015a) in which samples from 1996 to 2011 were genotyped).

Lemic et al. (2015a) found unusually high genetic differentiation between Italy and southern Europe comparisons, including high differentiation between Italian populations separated by a short geographic distances during a key phase of their invasion of the area. The results shown here are further confirmation that phenotypic differences are likely to manifest in a much shorter periods of time than genotypic differences (after Bouyer et al. 2007). Future surveys are planned that include hind-wing shape and size comparisons of *D. v. virgifera* from the USA and Europe that will provide baseline hind-wing shape and size data on endemic populations and also information on the effect of environmental and management practises (i.e. *Bt* corn, rotation resistant etc.), as conducted by Mikac et al. (2013). Having such information would facilitate the implementation of appropriate control methods (wing shape in insects can indicate changes in environmental conditions, see: Hoffmann et al. 2005). Given that *D. v. virgifera* in the USA have developed

resistance to a number of control measures (Ciosi et al. 2009; Gassmann et al. 2014) and that intercontinental introductions continue (Lemic et al. 2015a), the possibility of resistant alleles entering Europe remains a concern. Therefore, future integrated control strategies should consider incorporating geometric morphometrics into monitoring and management plans.

Our population genetics studies (Lemic et al. 2013; Ivkosic et al. 2014; Lemic et al. 2015a) have shown that alleles within the invaded areas have not had enough time to become fixed within populations to serve as a bio-marker for monitoring purposes (Lemic et al. 2015b). There are a number of possible explanations for this including drift versus selection etc. In contrast phenotypic changes have been detected in the same populations for which genetic markers showed no difference (Benítez et al. 2014a, b; Lemic et al. 2014). While we acknowledge that each marker can and does address its own aim, if the aim is to discover a population marker that can be used to conduct biomonitoring then a marker based on phenotypic differences is more suitable than a population genetic marker (Bouyer et al. 2007; Mikac et al. 2013). To further test whether phenotypic differences can be used as a biomarker over genetic markers the technique must be applied to other insect pests for which population genetic information exists, such as the Colorado potato beetle (Grapputo et al. 2005). Finally, phenotypic differences should be discernible in populations subjected to control practises and further studies should focus on whether a uniform phenotypic pattern of change is noted according to control practice or whether a single set change occurs only. Again this should be investigated for invasive insects for which robust population genetic studies already exist (e.g. Colorado potato beetle: Grapputo et al. 2005; Codling moth: Franck et al. 2007; Franck and Timm 2010).

Finally the results of this study have important considerations for invasive species monitoring and

management, particularly where genetic capabilities and monetary investment in such techniques maybe not be feasible. In addition to Mikac et al. (2013), Benítez et al. (2014a), Lemic et al. (2014), this study has demonstrated an affordable and accessible technique that reliably demonstrates hind-wing shape and size differences can be used to reveal populations differences and thus can be used as a biomarker.

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