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Genetic and phenotypic effects of hybridization in independently introduced populations of the invasive maize pest *Diabrotica virgifera virgifera* in Europe

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Abstract

The North American western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) was introduced into Europe several times during the end of the 20th century. Outbreaks in north-western Italy (NW Italian) and central and south-eastern Europe (CSE European) have merged in 2008 and insects interbreed since then. This study compared the genetic diversity (multi-locus genotype analyses at 13 microsatellites markers) and ten phenotypic traits among the CSE European and NW Italian populations as well as their hybrid offspring. All insects were reared under standardised laboratory conditions. Neutral genetic polymorphism appeared moderate in parental and hybrid populations, compared to North American populations. Some increase in neutral genetic variability was detected in the hybrids` expected heterozygosity and allelic richness compared to parental populations when family structures were considered. In 70% of the assessed phenotypic traits, the population type (CSE European, NW Italian, hybrids) influenced a trait, but averages in hybrids never exceeded those in their parents. Population type did not influence fecundity or adult lifespan (reflecting fitness) and not the proportion of adults flying (reflecting dispersal capabilities). There was no evidence yet that hybridization influences variability of phenotypic traits. In conclusion, there are only few indications that hybrids between the two overlapping invading European populations may in the longer term, take advantage through higher neutral genetic diversity and subsequent phenotypic adaptability.

Introduction

Biological invasions are threats to biodiversity, human health, and agricultural production (Pimentel, *et al.*, 2001). The acceleration of trade and transport during the last 200 years has led to a growing importance of invasions (Wittenberg & Cock, 2001). In this context, insects are of particular importance because they may vector diseases or become agricultural pests. Although an abundant literature exists on this topic, few biological invasions of insects are entirely understood (Facon *et al.*, 2008). This is largely because of experimental limitations. As a consequence,

hypotheses on the key factors determining the probability of success or failure of insect invasions such as the role of genetic diversity or hybridization are mostly theoretical.

Among introduced species, only a small fraction becomes established, and an even smaller fraction of these becomes invasive, which is known as the rule of the 1/10th (Williamson & Fitter, 1996). To successfully establish, spread, and become invasive, the species must adapt to new environments. Broad tolerance and plasticity as well as a large phenotypic variability are commonly associated with adaptability, *i.e.* the ability to respond to selection (Baker, 1965; Facon *et al.*, 2008). Large phenotypic variability may also be associated with large genetic diversity (Mueller-Schaerer *et al.*, 2004; Roush, 1990). However, genetic diversity is hypothesised to be lower within introduced populations than within source populations due to genetic bottlenecks during introduction events (Allendorf & Lundquist, 2003; Barrett & Kohn, 1991; Sakai *et al.*, 2001). Nevertheless, some introduced species become highly invasive, which is known as the genetic paradox in invasive species (Frankham, 2005).

Several introductions of the same organism in neighbouring locations and subsequent admixture of the invading populations may increase genetic diversity (genetic variation theory) (Fagan *et al.*, 2002). Crossing may also generate novel genotypes (evolutionary novelty theory). Increased genetic diversity and new gene combinations may then lead to further evolution of invasiveness (Ellstrand & Schierenbeck, 2000), because they (a) may contribute to an increase of phenotypic variability and thus a better adaptability (Ellstrand & Schierenbeck, 2000), and (b) may increase the fitness of some individuals due to their transgressive phenotypes, *i.e.* extreme phenotypes in crossed populations that exceed those of either parental pure population (Rieseberg *et al.*, 1999).

The western corn rootworm, *Diabrotica virgifera virgifera* leconte, (Coleoptera: Chrysomelidae), is one well-studied example of a successful invader that became a major pest of cultivated maize, *Zea mays* L. in North America and Europe. *Diabrotica v. Virgifera* is hypothesised to have evolved with maize or related grasses in Mexico or Central America several thousand years ago (Branson and Krysan. 1981; Krysan & Smith, 1987). It likely reached the southern USA with the introduction of its host plant maize (Krysan & Smith, 1987; Smith, 1966). Since the 1950s, *D. v. virgifera* rapidly expanded its range from the southwestern region of the US Corn Belt north-eastwards, reaching the east coast of the USA and Canada during the 1980s (Gray *et al.*, 2009; Spencer *et al.*, 2005). In the late 20th and early 21st century it was introduced on several occasions to Europe (Miller *et al.*, 2005) probably through a bridgehead effect via the USA (Guillemaud *et al.*, 2011). Consequently, independent populations of *D. v. virgifera* exist in Europe (Ciosi *et al.*, 2008; Kim *et al.*, 2007). Neutral genetic diversity in introduced European populations appeared slightly smaller than in North American populations, likely as a result of bottlenecks. For example, European samples displayed less than half the number of alleles per microsatellite locus (mean N_{al}) than North American populations; and a heterozygosity *H* of less than 70 % (Ciosi *et al.*, 2008). Whether this reduction in genetic diversity had any effect on the phenotype of *D. v. virgifera* and its fitness as an invader, was unknown.

Two of the largest spreading *D. v. virgifera* populations in Europe are the central and south-eastern European (CSE European) and the north-western Italian (NW Italian) population. Both populations display a strong neutral genetic differentiation with a mean pairwise fixation index estimates *Fst* of 26% (Bermond *et al.*, 2012). From 2008 onwards, crossings naturally occurred between individuals belonging to both differentiated populations forming a hybrid (or admixture) zone in the provinces of Treviso, Venetia, or Eastern Veneto in northern Italy (Bermond *et al.*, 2012). A slight increase of the neutral genetic variability was shown in this hybrid population (Bermond *et al.*, 2012)., Then Bermond *et al.*, (2014) collected hybrid and parental individuals from the field and measured two phenotypic parameters of fitness (survival under starvation and mating probability). However, results did not reveal any impact of the hybrid status. It became apparent that a larger variety of phenotypic traits would need to be assessed under standardized laboratory conditions that would exclude parental as well as age- and nutrition-related effects from the field (Li *et al.*, 2014). This may allow to conclude whether hybridization events can affect the invasion of *D. v. virgifera* in Europe.

Therefore, we aimed to analyse and compare the neutral genetic as well as phenotypic variability in *D. v. virgifera* from the two main European outbreaks as well as in their hybrids, through rearing and crossing them under strictly standardised laboratory conditions. The results were hoped to help in estimating whether the hybridization of the invading *D. v. virgifera* populations may increase their fitness and/or adaptability, and therefore render the invasion of this alien maize pest in Europe even more problematic.

Materials and methods

Origin and crossing of *Diabrotica* v. *virgifera*

Experiments were conducted on six laboratory-reared *D. v. virgifera* populations, *i.e.* two parental and four hybrid populations. The parental populations were the central and south-eastern European population (CSE European) and the north-western Italian population (NW Italian), as defined by Miller *et al.* (2005). Hybrid populations were the four possible crossings of both (Table 1).

Table 1. Origin, rearing, and sample size of invasive parental north-western Italian (NWIT) and central and south-eastern European (CSEE) populations and hybrid populations of *Diabrotica virgifera virgifera*.

Population type	Origin	Year of field collection or year of crossing	Tested generations in laboratory	*N .a*dults tested in genetic/phenotypic analyses	*N* families within experimental series 1	2
Parental	CSEE (Serbia)	2006	G_2	144 / 132	23	20
	NWIT	2006	G_2	37/37	7	NA
Hybrid	CSEE females (Serbia) X NWIT males	2007	F_{x1}*	148 /116	28	17
	NWIT females X CSEE males (Serbia)	2007	F_{x1}*	146/142	21	13
	CSEE females (Hungary) X NWIT males	2007	F_{x1}*	141/140	23	26
	NWIT females X CSEE males (Hungary)	2007	F_{x1}*	149/142	21	25

Genetic *D. v. virgifera* population definitions as per Miller *et al.*, (2005). *N* families within experimental series = number of genetic families reconstructed for each experimental series using the microsatellite markers. * Fx1 is the first generation after crossing, but beetles being the second generation reared under standardised laboratory conditions to avoid parental effects originating from the field.

Adults of the CSE European population were collected from maize fields near Crvenka in Severnobacki County in northern Serbia and near Kondoros in Bekes County in southern Hungary (only 200 km distant from each other). Adults from the NW Italian population were collected from a maize field near Como in Lombardy County in northern Italy. Adults were shaken from the maize plants into a funnel with an attached gauze bag. In a climate chamber, adults were reared in gauzed cages (45 × 45 × 60 cm) for egg laying according to Krysan & Miller (1986) and Singh & Moore (1999) under standardized conditions similar to conditions during hybridisation and for trait assessments as described in detail below.

Adults of the first laboratory generation (G_1) of the CSE European and NW Italian populations were crossed in the laboratory. Newly emerged adults from both populations were sexed according to antenna length (Kuhar & Youngman, 1995; Staetz *et al.*, 1976) and tarsus characteristics (Hammack & French, 2007). For crossing, single CSE European females were kept with single NW Italian males (or one NW Italian female with one CSE European male) in small gauze-covered plastic cylinders (dia.: 120 mm, h: 140 mm) for five days to allow mating. Two soft, unripe kernels of organically-produced maize, one 13 × 13 × 13 mm piece of zucchini flesh, one 13 × 13 × 13 mm piece of pumpkin flesh (Li *et al.*, 2014), and a 5 × 5 × 5 mm piece of artificial pollen diet (Branson & Jackson, 1988; Singh & Moore, 1999) were provided as food, as well as a 10 × 5 × 5 mm cube of 15% watery agar. After 5 days, female-male pairs were double-checked for correct sex identification to avoid mistakes in crossings. Then, about 100-200 crossed pairs were pooled and transferred into a larger gauze cage for oviposition. Importantly, the CSE European female X NW Italian male population was reared separately from the NW Italian female X CSE European male population.

The parental populations were mass-reared until G_2 and the hybrid populations until F_{x1} for experimentation. Parental effects - the fact that the phenotype of an individual is affected by the environment of its parents (Johnston *et al.*, 2004) - were reduced because each *D. v. virgifera* was reared under similar standardised laboratory conditions as per Li *et al.*, (2014) for more than one generation. Those conditions were the same as for the trait assessments described below.

Assessment of neutral genetic traits and their variability

DNA extraction and genotyping

All D. v. *virgifera* adults assessed for phenotypic traits, had been stored in 90-96% ethanol until DNA extraction. Individuals were then washed in 0.065% NaCl at least three times to remove ethanol from the tissues (Bermond *et al*., 2012). DNA was extracted from half of each body cut lengthwise, using the DNeasy tissue kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) with an elution volume of 100 ml (AE buffer, Qiagen, Hilden, Germany). Thirteen microsatellite loci *of D. v. virgifera* as described by Bermond *et al.*, (2012), were amplified using three separate multiplex pcrs performed in a PTC-225 MJ Research thermocycler (GMI, Minnesota, USA).

Family reconstruction

In the rearing, mated females had been grouped per population for oviposition, so that the offspring from different females were mixed. This resulted in cages in which several families of various sizes were present. This family structure is expected to affect genetic variability estimates. Therefore, the microsatellite markers were used to reconstruct the kinship between individuals, which allowed reconstructing siblings and thus considering the family structure in the genetic analyses. Families were reconstructed using the COLONY v. 2.0.1.1 software of R (Wang, 2004) (R Development coreteam, 2012). COLONY implements a maximum likelihood method to group individuals with siblings on the basis of their multi-locus microsatellite marker - defined genotypes. The connection between most likely siblings was obtained through a simulated annealing algorithm (Wang 2004). This method takes different types of genotyping errors into account when working with markers (dropout, mutations, contamination, false alleles) and can be used with or without knowledge of the parental genotypes. In our case, we did not have access to the genotypes of each sibling's parents. Therefore, we performed four independent runs for each population to reconstruct the families. Siblings were formed after observing consensus groups of individuals in all the four different runs (Table 1). Only the individuals assigned to a genetic family were used for those genetic and phenotypic analyses that considered the family structure.

Assessment of neutral genetic variation

Neutral genetic variation was calculated after pooling individuals from the two experimental series per population (see below), because the pairwise fixation index (F_{ST}) between both series was not higher than 1%. The four hybrid populations were not pooled for genetic analyses because of a slight genetic differentiation detected with the microsatellite markers (see below). Neutral genetic variation within populations was evaluated by determining the mean number of alleles per locus (*A*) and the mean expected heterozygosity (*He*) (Nei, 1987) using GENECLASS version 2.0.h software (Piry, *et al.*, 2004). We compared the mean number of alleles between populations by estimating allelic richness (*AR*) based on the smallest sample size using the rarefaction method of Petit *et al.*, (1998) in Fstat version 2.9.3 software (Goudet, 2001). We calculated individual inbreeding coefficients (F_{IS}) in each population and tested for deviations from the Hardy-Weinberg (*HW*) equilibrium using probability-test of "Genepop on the Web" -software (Raymond and Rousset, 1995a, b) similar to the exact Hardy-Weinberg tests of Weir & Cockerham (1984).

Then, neutral genetic variation within populations was analysed a second time, this time accounting for family structures of the studied individuals, thus allowing the extraction of inter-family neutral genetic variation. From each population, 100 samples were randomly selected by sampling one individual per reconstructed family using the COLONY v. 2.0.1.1 software of R (R Development coreteam, 2012). From these, we generated one hundred values of *DC*, *AR*, *He*, F_{IS} and probabilities associated with the Hardy Weinberg equilibrium test using Genepop 4.0 software (Raymond & Rousset 1995a, b). To calculate *AR*, seven families (the minimum number of families per population) were randomly drawn per population. This resampling procedure was used to correctly estimate mean values of *DC*, *AR*, *He*, F_{IS} and their standard deviations when considering family structure. The 100 samples are not independent samples that could be used as independent observations to perform parametric tests.

When analysing statistics of genetic diversity such as *He* and *AR*, the unit of observation was the locus (loci are independent in a coalescence framework, not individuals). Thus, the homogeneity of *He* and *AR* among populations was tested using non-parametric Friedman test (with the locus as repeat unit) taking and not taking the family structure into account. In case of detecting an overall heterogeneity of *He* and *AR* among samples, the hypothesis of an equal genetic variation between each hybrid and parental population was tested using the two-sided Wilcoxon signed rank tests. Due to multiple comparisons, the level of significance of each of

the Wilcoxon test results was corrected with the false-discovery rate procedure of Benjamini & Hochberg (1995).

When considering the family structure, the tests of Hardy-Weinberg equilibrium and homogeneity of *He* and *AR* among populations were performed 100 times, *i.e.* once for each of the 100 random draws (see above). The aim was to get a point estimate of the *p*-values via the resampling procedure and the averaging over the 100 values. We thus considered the means of the 100 *p*-values obtained from the random draw of 100 samples and their standard deviation.

Assessment of phenotypic traits and their variability

Ten phenotypic traits were assessed following Li *et al.*, (2009; 2010) for each population (Table 1). These included six fitness traits (adult lifespan, fecundity, overwintering survival of eggs, survival from eggs to adulthood, overall fitness), two activity traits (proportion of adults flying, flight take-off response) and three morphometric traits (fresh body weight, elytra length, elytra width) (Table 5).

Fitness traits

To assess adult lifespan and fecundity, newly emerged male-female pairs were transferred into small bioassay containers (climate chamber: L: D, 24: 18°C, light regime L: D, 14: 10, r.h. 40 to 60%). The containers consisted of two plastic urinalysis cups (diameter: 48 mm, height: 80 mm), stacked one inside the other providing 175 cm^3 of space as per Toepfer *et al.*, (2012). The upper cup had a 10 - mm hole in the bottom to give the female access to the lower, soil-filled cup for egg-laying. Abundant - for the insects unlimited - food was provided in each container. This was, two soft, unripe kernels of organically-produced maize, one 13 × 13 × 13 mm piece of zucchini flesh, one 13 × 13 × 13 mm piece of pumpkin flesh (Li *et al.*, 2009), and a 5 × 5 × 5 mm piece of artificial pollen diet (Branson & Jackson, 1988). A 10 × 5 × 5 mm cube of 15% watery agar served as a water source for the adults. Food and agar were changed every 5 to 7 days. All pairs of *D. v. virgifera* were provided with the same amount of food, as *D. v. virgifera* fitness and activity is influenced by diet experience (Mabry *et al.*, 2004).

To assess adult lifespan, bioassay containers were daily checked for live and dead adults. The date of death was recorded, lifespan calculated, and dead adults removed. The experiment was stopped after 70 days because it was thought that this period was long enough to reliably reflect the total adult lifespan (Li *et al.*, 2009). The proportion of females and males surviving until day 70 was calculated for each tested population.

To assess fecundity, two teaspoons of moist, sterile black field soil (sieved at 0.15 mm mesh size; 25-35 w% moisture) were placed into the lower cup of the bioassay containers after 7 days maturation period (Branson & Johnson, 1973; Hill, 1975). Then, every 14 days after adult emergence, the lower cup of the bioassay container (containing soil and eggs) was removed and replaced with a new one. The soil with eggs was washed with tap water through a 0.25 mm sieve, and recovered eggs were counted. The experiment was stopped after 70 days (Li *et al.*, 2009). The realised fecundity was calculated for each individual female. Eggs were then stored in sterile moist soil for pre-diapause at 24°C during two weeks (Branson, 1976; Krysan, 1972), and then for diapause at 6 to 8°C during another five months (Krysan, 1982).

To determine the overwintering survival of eggs, the soil with eggs was washed through a sieve after five months diapause. Egg survival per individual parental female of each tested population was assessed under stereomicroscope according to Modic *et al.*, (2005). To determine survival from eggs to adulthood, 200 successfully overwintered viable eggs were incubated at a temperature of L: D, 25: 21°C for 14 to 21 days to initiate hatching. As not each female had laid enough eggs to obtain 200 eggs after overwintering, this experimental part was not conducted on an individual female base, but with pooled egg batches.

About 2,000 to 3,000 organic, untreated maize seeds (var. Gavott UFA Semences, Bussigny, Switzerland) were soaked in water for 24h and germinated in a plastic tray (330 × 190 × 110 mm) with a gauze lid. Three days after germination, ready-to-hatch eggs were transferred onto the seeds in the plastic trays and maintained at a temperature of L: D, 25: 21°C, and light regime of L: D, 14: 10. Emerging larvae found unlimited maize roots. After 14 to 20 days, third instar larvae were transferred along with the maize seedlings into gauze-covered cylinders (diameter: 120 mm, h: 140 mm) containing sterilised field soil for pupation (sieved at <5 mm mesh size, 25-35 w% moisture). The transferred maize still provided food for the larvae until entering the soil for pupation. Adults started to emerge about one week later. Adult emergence was recorded daily until no emergence was anymore recorded during four consecutive days. The total number of emerged adults was divided by the initial number of 200 incubated viable eggs. Survival from eggs via all developmental stages until adulthood was calculated by combining the data from the overwintering survival of eggs with the survival from larvae and pupae until adulthood.

The overall fitness of each individual *D. v. virgifera* female equals the net reproductive rate *R* of a population (Begon *et al.*, 1990) and was calculated according to Lombaert and al. (2008) assuming a 50 : 50 sex ratio (Spencer *et al.*, 2009; Toepfer & Kuhlmann, 2006):

*Fitness = Larvae to adult survival * Adult sex ratio * Realised fecundity * Egg overwintering survival*

Activity traits

Flight activity was assessed as a measure of dispersal capability using seven-day old adults (Naranjo, 1991). Activity measures on young adults better reflect activity differences between individuals and between populations than measures on mature adults which are influenced by nutritional status and egg load (Li *et al.*, 2010). Flight activity was assessed by measuring the proportion of adults flying and the flight take-off response as described by Li *et al.*(2010) between 14:00 and 16:00 under laboratory conditions of 24°C and 60% relative humidity. Flight stands consisted of a wooden pin (h: 40 mm, diameter 10 mm) fixed onto the end of an inverted white plastic funnel (h: 160 mm, diameter 135 mm at base). The base of the funnel was surrounded with water to prevent the adults from walking off the stand. An individual adult was gently transferred onto the base of the funnel using an aspirator device. Following adult release, the incidence of take-off and the time from release to take off were recorded. The trial ended when the adults flew off the stand or when 300 seconds had elapsed (Li *et al.*, 2010). The proportion of adults flying and mean time until take-off was calculated for female and male adults (non-fliers were recorded as taking more than 300 seconds). Tested adults were returned to the bioassay containers.

Morphometric traits

Fresh body weight, elytra length and elytra width were measured on each individual *D. v. virgifera* within 24 h following adult emergence (= initial morphometric traits without feeding). This is, because measures on young adults are less influenced by nutritional status and egg load than older adults (Li *et al.*, 2014). The tested young adults were assumed to no longer be teneral, as they were fully coloured and did not have the light grey soft body typical of adults just after emergence.

Fresh body weight was measured by transferring adults into a small plastic container and weighting them on a 0.1 mg to 160 g precision scale (Fox & Scheibly, 2006). Individuals were then returned to the bioassay containers.

Elytra length and width (*i.e.*, single measurement of both elytrons together across the dorsum) were measured according to Li *et al.*, (2009;) and Mabry *et al.*, (2004). Elytra length and width were chosen among other morphometric traits, such as hind tibia length or head capsule width because measures of elytra characters seem to well-reflect fecundity, life span and activity of *D. v. virgifera* (Li *et al.*, 2009; Li *et al.*, 2010). Adults were placed on a cool but not frozen pad (Icepack Migros, Delémont, Switzerland) to limit their activity during the measurements with a micrometre scale to the nearest 0.06 mm under a stereomicroscope (16 × magnification).

Analyses of phenotypic trait differences among populations

The "Family structure" was considered as a random factor in the applied linear mixed models (LMM) for normal distributed errors, generalized linear mixed models (GLMM) for other error distributions, or mixed Cox models for time-series data. The environmental variance was kept minimal because all animals were reared in the same laboratory conditions. Under these conditions, the inter-family variance of a trait reflects its genetic variance. This allowed us to compare the mean values and genetic variances of each phenotypic trait between populations. Our models allowed testing whether hybridization had an impact on the means and variances of phenotypic traits. The same models were used for each trait with the independent variables "Population type" (with the modalities NW Italian, CSE European (Serbia), each of four hybrids), "Sex" (female and male) and "Experimental series" (series 1 and 2) as fixed effects. We determined for each trait, whether the inter-family variance (the genetic variation proxy) varied or not between the "Population types". For each trait, we compared a model with a nested random structure "Population type | Family" with a model with a simple random structure "1 | Family" (Table 2). The best model was chosen on the basis of the smallest value of the Akaike information criterion (AIC) (Burnham and Anderson, 2004). For some traits, only one or both sexes together were concerned in the phenotypic measures (fecundity, overwintering survival of eggs, survival of larvae and pupae until adulthood). Consequently, the two sub-types of generic models used to cover all the studied traits and factors were as follows:

*Phenotypic trait ~ Population type * Sex + Experimental series + Random factor*

Phenotypic trait ~ Population type + Experimental series + Random factor

From these pre-established models we (i) determined the best random structure and its significance in the model; (ii) estimated the significance of the fixed effects; and (iii) tested the equality of traits for all comparisons among parental and hybrid populations. For that, multiple comparisons of the means of the phenotypic traits were conducted using the R package "multcomp" (R Development coreteam, 2012) and through defining a matrix of contrasts. The obtained *p*-values allowed us to determine which comparisons of mean phenotypic traits were significantly different between population types (see parental and hybrid populations in Table 2).

All ten phenotypic traits (fecundity, egg overwintering survival, survival from eggs to adulthood, fitness index, adult lifespan, proportion of adults flying, flight take-off response, fresh body weight, elytra length, elytra width,) were analysed. Most traits were analysed by using LMM (with a normal error distribution). For that, data have been transformed to improve normality with a square-root transformation for fecundity and survival from eggs to adulthood, and with a logarithmic transformation for the fitness index. The proportion of adults flying (a binomial distribution of the error) was analysed by using a GLMM. The adult lifespan and flight take-off response were

Table 2. Comparison of averages and variability of phenotypic traits among invasive north-western Italian (NWIT) and central and south-eastern European (CSEE, here Serbia) populations and their hybrid populations of *Diabrotica virgifera virgifera*; using linear generalized mixed model for each phenotypic trait. See p-values for differences between averages of traits. See AIC for differences in variability, *e.g.* the random factor "population type | family" was never statistically selected for any of the assessed phenotypic traits reflected in consistently higher AIC values of this random structure than for "1 | Family".

Explanatory fixed factors		Overall fitness	Fecundity	Over-wintering egg survival	Survival from eggs to adulthood	Adult lifespan	Proportion of adults flying	Flight take-off response	Fresh body weight	Elytra length	Elytra width
Population type	*p**	*0.022*	0.31	*<10⁻³*	*<10⁻³*	*0.18*	*0.27*	*0.009*	*<10⁻³*	*<10⁻³*	*0.0048*
	Diff.	NWIT > CSEE; NWIT > Hybrid; CSEE ~ Hybrid	NWIT ~ CSEE ~ Hybrid	NWIT > CSEE > Hybrid	NWIT > CSEE; NWIT > Hybrid; CSEE ~ Hybrid	NWIT ~ CSEE ~ Hybrid	NWIT ~ CSEE ~ Hybrid	NWIT < Hybrid < CSEE	CSEE > NWIT ~ Hybrid	CSEE > NWIT ~ Hybrid	CSEE ~ NWIT; NWIT ~ Hybrid; CSEE > Hybrid
Sex	*p**	-	-	-	-	*<10⁻³*	*<10⁻⁵*	*<10⁻⁵*	*<10⁻³*	*<10⁻³*	*<10⁻³*
	Diff.					F>M	M>F	M>F	F>M	F>M	F>M
Population type : sex	*p**	-	-	-	-	0.19	0.71	0.25	0.26	0.76	0.94
Experimental series	*p**	*<10⁻³*	0.65	*<10⁻³*	*<10⁻³*	*<10⁻⁵*	*<10⁻³*	*<10⁻⁵*	*<10⁻³*	*<0.05*	*<0.05*
	Diff.	2>1	1~2	1>2	2>1	1>2	2>1	2>1	2>1	2>1	2>1
Explanatory random factors											
1 | Family	AIC **	527.4 (ns)	2617 (ns)	2670 (ns)	633 (ns)	36.7 (ns)	381.5 (s)	175.8 (s)	3034 (s)	34 (s)	-653 (s)
Population type | Family	AIC **	536.5	2625	2678	638	-	391.3	176.1	3044	43	-644
Over-dispersion (>1)	Φ	-	-	-	-	-	0.88	-	-	-	-

**p*-values associated with the generic statistical model used for each of the phenotypic measured traits, **AIC values of the model, obtained for the different tested random structures (1|Family or Population type | Family) and for each of the measured trait. Hierarchical significant relations (< or >) of averages of traits between pure parental populations NWIT, CSEE (Serbia) and hybrid populations with « ~ » representing no significant differences (see details in Materials and Methods); Sex: M(ales) and F(emales); Experimental series: 1 and 2; Population type: sex: interaction between population and sex; Population type | Family: estimates « inter-families » variance for each modality of « Population type» ; 1 | Family estimates only one « inter-families » variance for the dataset; (s) indicates a significant random effect at $p = 0.05$; (ns) indicates no significant random effect (-) indicates that data are not available, for example no « sex » effect because measures are collected only on females.

analysed by using a mixed Cox models (lifespan time-series censored to 70 days, flight take of response to 300 sec). Normality of data had been visually confirmed for untransformed data as well as after-transformation- data before applying any model.

The over-dispersion of binomial data has been tested for the proportion of adults flying by computing the over-dispersion parameter Φ, the ratio of the residual deviance of the model over the residual degrees of freedom.

Results

Neutral genetic traits and their variability

When not taking family structures into account, hybrid and parental populations appeared moderately polymorph with 6.08 alleles per locus (±4.01 SD) over all samples. The mean number of alleles ranged from 3.23 (±1.83) alleles for the parental NW Italian population up to 5.23 (±3.68) for the hybrid population of NW Italian females X CSE European males (Hungary) (Table 3). The mean expected heterozygosity was moderate and ranged from 0.43 for the parental NW Italian population up to 0.52 for the hybrid population NW Italian females X CSE European males (Hungary). An excess of heterozygosity (negative F_{IS} in Table 3) and significant deviations from the Hardy-Weinberg equilibrium (Table 3) were found for all populations.

No differences in expected heterozygosity (*He*) were observed between hybrid and parental populations (Friedman test, c^2=6.1, df=5, p=0.3) (Table 4). However, allelic richness (*AR*) differed between these populations (Friedman test, c^2=23.96, df=5, p=0.0002). The number of alleles in hybrid populations usually appeared slightly higher than in the parental populations (Tables 3 and 4), except for the comparison between hybrids of CSE European females (Serbia) X NW Italian males and the NW Italian parents.

Table 3. Neutral genetic variation of invasive north-western Italian (NWIT) and central and south-eastern European (CSEE) populations and hybrid populations of *Diabrotica virgifera virgifera* without considering family structures (all reared and studied individuals) and with considering family structures (100 random re-samples).

	N	*A (DC)*	*AR*	*He*	*p-HW*	F_{IS}
Populations without family structure						
CSEE (Serbia)	144	3.46 (±1.90)	3.01 (±1.42)	0.45	*<10⁻³*	-0.05
NWIT	37	3.23 (±1.83)	3.23 (±1.83)	0.43	*0.00*	-0.06
CSEE female (Serbia) X NWIT male	148	4.31 (±3.09)	4.04 (±2.62)	0.50	*<10⁻³*	-0.14
NWIT female X CSEE male (Serbia)	146	4.69 (±2.87)	4.08 (±2.37)	0.50	*<10⁻³*	-0.10
CSEE female (Hungary) X NWIT male	141	4.54 (±3.18)	4.16 (±2.67)	0.51	*<10⁻³*	-0.16
NWIT female X CSEE male (Hungary)	149	5.23 (±3.68)	4.57 (±2.68)	0.52	*<10⁻³*	-0.08
Populations with family structure						
CSEE (Serbia)	43	3.29 (±0.06)	3.99 (±2.30)	0.46 (±0.01)	0.42 [±0.22]	-0.02 [±0.02]
NWIT	7	2.89 (±0.07)	2.85 (±1.54)	0.45 (±0.03)	0.86 [±0.15]	0.02 [±0.07]
CSEE female (Serbia) X NWIT male	45	4.17 (±0.06)	3.73 (±2.27)	0.54 (±0.01)	0.15 [±0.15]	-0.07 [±0.01]
NWIT female X CSEE male (Serbia)	34	4.40 (±0.09)	3.56 (±1.89)	0.54 (±0.01)	0.26 [±0.22]	-0.09 [±0.02]
CSEE female (Hungary) X NWIT male	49	4.31 (±0.10)	4.08 (±2.24)	0.59 (±0.01)	*<10⁻³* [±<10⁻³]	-0.15 [±0.01]
NWIT female X CSEE male (Hungary)	46	4.94 (±0.12)	3.91 (±2.33)	0.56 (±0.01)	*0.02* [±0.03]	-0.08 [±0.02]

A = mean number of alleles per locus determined by direct counts (*DC*) and allelic richness (*AR*). *AR* is based on the smallest sample size (N = 37 and N = 7 for several loci in the Italian population). *He*: mean expected heterozygosity (Nei. 1987). The standard deviations of *DC*, *AR* and *He*, shown here in ( ), are calculated between loci. *FIS*: genes frequency correlation between individuals per population (Weir & Cockerham, 1984). *p-HW*: p-values associated with the exact tests of deviation from Hardy Weinberg (*HW*) equilibrium with italics values if significant at p<0.05. When considering family structure, the average over the 100 random samples of *DC*, *AR*, *He*, *FIS* and *p-HW* is shown. The average inter-locus standard deviation over the 100 random samples is shown for *DC*, *AR*, *He* in ( ). The standard deviations of *p-HW* and *FIS* computed among the 100 re-samples are shown in [ ].

Table 4. Comparison of neutral genetic variation among invasive parental north-western Italian (NWIT) and central and south-eastern European (CSEE) populations and hybrid populations of *Diabrotica virgifera virgifera* without considering family structures (all reared and studied individuals) and with considering family structures (100 random re-samples).

	Parental populations, P of He		Parental populations, P of AR	
	NWIT	CSEE (Serbia)	NWIT	CSEE (Serbia)
Populations without family structure, hybrid populations				
CSEE female (Serbia) X NWIT male	0.37	0.56	*0.04*	*0.01*
NWIT female X CSEE male (Serbia)	0.29	0.05	*0.01*	*0.02*
CSEE female (Hungary) X NWIT male	0.22	0.08	*0.03*	*0.01*
NWIT female X CSEE male (Hungary)	0.11	0.05	*0.01*	*0.00*
Populations with family structure, hybrid populations				
CSEE female (Serbia) X NWIT male	*<10⁻⁵*	*<10⁻⁵*	*<10⁻⁵*	*<10⁻⁵*
NWIT female X CSEE male (Serbia)	*<10⁻⁵*	*<10⁻⁵*	*<10⁻⁵*	*<10⁻⁵*
CSEE female (Hungary) X NWIT male	*<10⁻⁵*	*<10⁻⁵*	*<10⁻³*	0.9
NWIT female X CSEE male (Hungary)	*<10⁻⁵*	*<10⁻⁵*	*<10⁻³*	*<10⁻³*

Without family structure, *p*-values of the Wilcoxon signed ranks tests for an equal mean expected heterozygosity (*He*) and allelic richness (*AR*) for each pair of sample, in italics if significant at p<0.05 after false discovery rate correction according to (Benjamini and Hochberg, 1995). When considering family structure, the average over the 100 random samples of *p*-values of the Wilcoxon signed ranks tests for an equal mean expected heterozygosity (*He*) and allelic richness (*AR*) for each pair of sample, in italic if significant at p<0.05 after false discovery rate correction.

Based on multi-locus genotype analyses at 13 microsatellites, 7 to 28 families were reconstructed in the parental and hybrid populations (mean 20.4±6.1; Table 1).

When family structures were considered, this is, when sampling multiple times only one individual per family, allele number was low in each population and ranged from 2.89 (±0.07) for NW Italian to 4.94 (±0.12) for NW Italian females X CSE European males (Hungary) (Table 3). The mean expected heterozygosity was moderate ranging from 0.45 (±0.03) for NW Italian to 0.59 (±0.01) for CSE European females (Hungary) X NW Italian males (Table 3). The largest mean allelic richnesses and heterozygosities were observed in the same hybrid population, with an *AR* of 4.08 (±2.24) and a *He* of 0.59 (±0.01) for the hybrid CSE European females (Hungary) X NW Italian males (Table 3). In most cases, the populations did not deviate from the Hardy-Weinberg equilibrium (Table 3).

The heterozygosity of hybrid populations appeared higher than of the parental populations in all possible comparisons (Tables 3 and 4). The same was true for allelic richness in 87.5% of comparisons between hybrid and parental populations. Only one comparison (CSE European female (Hungary) X NW Italian male hybrid *versus* CSE European (Serbia)) did not reveal differences in allelic richnesses (Table 5).

Phenotypic traits and their variability

Variability of phenotypic traits

The random factor "population type | family" was never statistically selected for any of the assessed phenotypic traits (see consistently higher AIC values of this random structure in Table 2). This indicates that inter-family intra-population variances, or genetic variances, were not statistically different between parental and hybrid populations.

Table 5. Averages and variability (confidence intervals) of phenotypic traits of invasive parental north-western Italian (NWIT) and central and south-eastern European (CSEE, here Serbia) populations and their hybrid populations of *Diabrotica virgifera virgifera*.

Phenotypic trait	Averages (Confidence intervals)																	
	Both sexes						Females						Males					
	Parental		Hybrid population				Parental		Hybrid population				Parental		Hybrid population			
	CSEE (Serbia)	NWIT	CSEE females (Serbia) X NWIT males	NWIT females X CSEE males (Serbia)	CSEE females (Hungary) X NWIT males	NWIT females X CSEE males (Hungary)	CSEE (Serbia)	NWIT	CSEE (Serbia) females X NWIT males	NWIT females X CSEE males (Serbia)	CSEE females (Hungary) X NWIT males	NWIT females X CSEE males (Hungary)	CSEE (Serbia)	NWIT	CSEE females (Serbia) X NWIT males	NWIT females X CSEE males (Serbia)	CSEE females (Hungary) X NWIT males	NWIT females X CSEE males (Hungary)
N	129-132	35-37	111-116	135-142	135-140	138-142	31-33	16-19	54-58	67-73	62-69	63-70	65-67	16-18	54-58	65-69	68-71	69-72
Fitness traits																		
Overall fitness	11.6 (4.2-30)	15.1 (5.4-39.2)	12.4 (7.3-20.9)	10.2 (3.7-26.3)	8.9 (3.1-23.3)	12.3 (4.5-31.4)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Fecundity (egg counts)	489 (220-865)	603 (277-1053)	466 (318-644)	439 (189-794)	392 (159-732)	524 (246-909)	489 (220-865)	603 (277-1053)	466 (318-644)	439 (189-794)	392 (159-732)	524 (246-909)	NA	NA	NA	NA	NA	NA
Overwintering egg survival (%)	41.6 (30.0-53.2)	65.4 (52.5-78.3)	35.4 (29.4-41.4)	36.2 (24.7-47.7)	30.9 (14.3-42.5)	35.1 (23.6-46.7)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Survival from eggs to adulthood (%)	6.5 (2.7-13.6)	6.5 (4.1-9.5)	8.7 (6.7-12.0)	6.0 (2.5-12.6)	6.1 (2.5-12.9)	5.0 (2.0-10.9)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Adult lifespan (days)+	61 (52-70)	62 (53-71)	62 (54-70)	60 (51-69)	58 (48-68)	58 (48-68)	59 (50-68)	68 (63-73)	64 (58-70)	59 (51-67)	58 (48-68)	57 (47-67)	62 (53-71)	57 (35-69)	61 (51-71)	61 (51-71)	58 (48-68)	59 (49-68)
Activity traits																		
Proportion of adults flying (%)	91.9 (53.5-99.2)	96.5 (64.0-99.9)	96.3 (89.5-98.9)	94.9 (63.6-99.6)	92.1 (54.4-99.2)	91.5 (52.4-99.2)	84.3 (35.7-97.9)	93.0 (43.9-99.6)	92.9 (80.2-97.5)	90.1 (46.9-98.9)	84.8 (36.6-98.0)	82.8 (33.3-97.6)	96.7 (60.2-99.8)	98.7 (68.3-100)	98.6 (91.7-99.8)	98.1 (70.6-99.9)	96.8 (61-99.8)	96.3 (57.6-99.8)
Flight take-off response (sec) +	41 (11-71)	17 (4-30)	22 (0-44)	34 (1-67)	23 (1-45)	24 (0-48)	51 (20-82)	28 (13-43)	32 (2-62)	55 (15-95)	36 (8-64)	29 (1-57)	30 (1-59)	5 (2-8)	12 (0-24)	15 (0-30)	10 (1-19)	20 (0-40)
Morphometrics																		
Fresh bodyweight (mg)	11.7 (10.5-12.95)	9.0 (7.5-10.4)	9.9 (9.3-10.6)	10.8 (9.6-12.1)	10.3 (9.1-11.6)	11.1 (9.9-12.3)	12.5 (10.8-14.15)	9.8 (7.9-11.8)	10.4 (9.6-11.3)	10.9 (9.3-12.7)	11.1 (9.4-12.8)	11.7 (10.1-13.5)	11.1 (9.5-12.6)	8.1 (6.3-9.8)	9.4 (8.6-10.2)	10.8 (9.3-12.4)	9.6 (8.1-11.1)	10.5 (8.9-12.0)
Elytra length (mm)	4.4 (4.2-4.5)	4.2 (4.0-4.4)	4.2 (4.1-4.3)	4.3 (4.1-4.5)	4.2 (4.1-4.4)	4.3 (4.2-4.5)	4.5 (4.3-4.6)	4.3 (4.1-4.4)	4.3 (4.2-4.4)	4.4 (4.2-4.5)	4.3 (4.2-4.5)	4.4 (4.3-4.6)	4.3 (4.1-4.5)	4.1 (3.9-4.3)	4.1 (4.0-4.3)	4.2 (4.0-4.4)	4.1 (3.9-4.3)	4.2 (4.0-4.4)
Elytra width (mm)	2.5 (2.4-2.6)	2.4 (2.3-2.5)	2.4 (2.3-2.5)	2.5 (2.4-2.6)	2.4 (2.3-2.5)	2.5 (2.4-2.6)	2.5 (2.4-2.6)	2.5 (2.4-2.6)	2.4 (2.4-2.5)	2.5 (2.4-2.6)	2.5 (2.4-2.6)	2.5 (2.4-2.6)	2.4 (2.3-2.6)	2.4 (2.2-2.5)	2.3 (2.3-2.4)	2.4 (2.3-2.5)	2.4 (2.3-2.5)	2.4 (2.3-2.5)

All *D. v. virgifera* were reared as male-female pairs and tested under standardised laboratory conditions. Data of experimental series 1 and 2 have been pooled here as obtained from the LMMs, GLMMs and mixed Cox models+. NA= not applicable.

The random factor "1 | family" however significantly affected about half of the traits; this is, fresh bodyweight, elytra length and width, the proportion of adults flying as well as the flight take-off response, all indicating that an inter-family, intra-population variance, *i.e.* an intra-population genetic variance, exists for these traits.

No signs of over-dispersion of data was detected for the proportion of adults flying (Φ<1) (Table 2).

Averages of phenotypic traits

The population type affected average levels of about 70% of the assessed phenotypic traits of *D. v. virgifera*: overall fitness, overwintering survival and survival from eggs to adulthood, as well as the flight take-off response, fresh body weight, elytra length and width (LMMs and mixed Cox model tests, p<0.05) (Table 2). On the other hand, population type did not affect average fecundities (LMM test, p=0.31), adult lifespans (mixed Cox model test, p=0.18) and proportions of adults flying (GLMM test, p=0.27) (Table 4).

About 60% of average levels of phenotypic traits differed between the parental NW Italian and CSE European populations (Table 2). The CSE European population (Serbia) was slightly heavier and had longer elytra than the NW Italian population. Both populations were comparably often flying, but the NW Italian adults took-off faster for flying than the CSE European adults. In addition, NW Italian population had a better egg overwintering survival, a better survival from eggs to adulthood and therefore a slightly better overall fitness than the CSE European population. In all such cases, absolute differences were tiny.

Hybrid populations usually appeared either intermediate between the parental populations; or similar to the less performing parental population (Table 2). Only three of the assessed ten phenotypic traits were, in their averages, comparable between hybrids and their parental populations (adult lifespan, fecundity, proportion adults flying). In many cases hybrids were close to the least performing parental population, *i.e.* in body weight, elytra length and width, survival from eggs to adulthood as well as overall fitness. Only the flight take-off response of hybrids was truly intermediate between the parental populations. And only the eggs of hybrid populations overwintered less successful than those of both parental populations.

Average levels of activity and morphometric traits always depended on sex (LMMs, GLMM and mixed Cox models' tests, p<0.0001). Young female beetles were heavier and larger (longer and wider) than young males, but performed flights less often and less quick (Table 4). Females lived longer than males.

Interaction between "population type" and "sex" was never significant (LMMs, GLMM and mixed Cox models' tests, p>0.05) (Table 2) indicating that the average levels of phenotypic traits in parental and hybrid populations did not differently vary between females and males.

The experimental series slightly affected phenotypic traits (LMMs, GLMM and mixed Cox model tests, p<0.05) except fecundity (LMM test, p=0.65) (Table 2). Often, the averages of traits in the experimental series 2 were slightly superior to those of the series 1 (Table 2).

Discussion

Our findings complement works on the European invasion history of the maize pest *D. v. virgifera* but also add to our understanding of the role of hybridization events in invasion processes in general (Bermond *et al.*, 2012; Ciosi *et al.*, 2008; Ciosi *et al.*, 2011; Gray *et al.*, 2009; Miller *et al.*, 2005). We compared the neutral genetic variability (multi-locus genotype analyses at 13 microsatellite-markers) as well as average levels and variabilities of ten phenotypic traits between parents and hybrids of two invasive *D. v. virgifera* populations. These were the distinct north-western Italian and central and south-eastern European populations (Miller *et al.*, 2005). Despite hybridization occurring *in natura* in northern Italy since 2008 (Bermond *et al.*, 2012), we decided to investigate hybridisation in the laboratory. This was because (a) *D. v. virgifera* were difficult to mass-collect in the hybridization zone due to containment measures (Furlan *et al.*, 2002), (b) several generations of hybrids were likely to be present rendering it difficult to classify or replicate genotypic classes in a meaningful way (Johnston *et al.*, 2004), and (c) standard conditions were needed during rearing to avoid varying parental effects originating from the field (Campbell & Meinke, 2010); as well as insect age- and nutrition-related effects. We are aware that tiny variations of external conditions may already change the environmentally determined phenotype, potentially masking the genetic background of a phenotypic variation. We tried to avoid such environmental influences by (a) conducting all experiments in climate chambers with similar conditions, (b) assessing all traits only on young adults of exactly the same age (Li *et al.*, 2009), (c) providing always the same amount of abundant (=unlimited) food to larvae and adults, (d) investigating populations in two experimental rearing series, and (e) investigating populations that had been reared for at least one generation in the laboratory (Li *et al.*, 2014). Still, we cannot entirely exclude that tiny changes in external factors may have influenced the phenotype of *D. v. virgifera*. For example, analyses detected slightly higher average levels of some phenotypic traits in experimental series 2 than in experimental series 1; and few slightly lower average levels (Table 2). As those differences between both experimental series appeared consistently across populations, they probably reflect slight changes in external factors. They have therefore been considered as random effects in our statistical analyses, meaning they may explain some data variance but are irrelevant to the biological invasiveness questions. We moreover believe that the trait data of the studied *D.v.virgifera* populations are statistically sound as multiple comparison tests were applied with fdr-correction due to the large number of tested traits and populations that reduces the power of p -values, and family effects were considered in the variance analyses.

Impact of hybridization on genetic traits

The here-reported moderate to low polymorphism of alleles per locus and the moderate heterozygosity in the independently invading populations of *D. v. virgifera* in CSE Europe and NW Italy underline their only moderate neutral genetic variability compared to that of North American populations. This phenomenon had already been documented for *D. v. virgifera* (Kim & Sappington, 2005; Kim *et al.*, 2007; Ciosi *et al.*, 2008; Miller *et al.*, 2005), and is typical for invaders having experienced genetic bottlenecks during introduction events (Allendorf & Lundquist, 2003; Barrett & Kohn, 1991; Sakai *et al.*, 2001).

Same principles apply when individuals of the same species are introduced geographically separated, as it was the case for *D. v. virgifera* in Europe (Miller *et al.*, 2005). It is therefore not surprising that both studied parental populations of *D. v. virgifera* genetically differ, such as in allele frequencies (Ciosi *et al.*, 2008) as shown by a fixation index (F_{ST}) as large as 26% (Bremond *et al.*, 2012).

And since both parental populations genetically differ, their crossings were expected to generate variations at many genes and to increase the neutral genetic variability of hybrid populations (Facon *et al.*, 2008). In a first analyses which did not consider family structures, there was little evidence on effects of hybridization on genetic variation. No differences in expected heterozygosity

were observed between hybrid and parental populations. Moreover, allelic richness was found only slightly higher in hybrid populations than in parental populations. In contrast, when accounting for family structures (re-sampling of only one individual per family) (Wang 2004), hybrid populations exhibited a higher expected heterozygosity and allelic richness than their parental populations (Table 4). This confirms indications from analyses of some *in natura* samples of *D. v. virgifera* from the hybrid zone in the Veneto area in Italy by Bermond *et al.*, (2012). Both, the higher expected heterozygosity and the higher allelic richness are indications that a crossing of independently invading *D. v. virgifera* populations can indeed increase their genetic variability or even reconstruct their variability as compared to the situation in the area of origin (Ciosi *et al.*, 2011). We had then hypothesised that such increases in genetic variability as well as potential new gene combinations may contribute to phenotypic variability and thus adaptability of *D. v. virgifera* (Ellstrand & Schierenbeck, 2000).

Impact of hybridization on phenotypic traits

The studied phenotypic traits had been chosen because they reflect the capacity of *D. v. virgifera* to disperse and invade new environments (flight activity), or because they may be related to species' fitness, either directly (fecundity, survival, lifespan) or indirectly (morphometrics) (Li *et al*., 2010). Indeed, we found that the population type (CSE European, NW Italian, hybrids) influences averages of 70% of the assessed phenotypic traits, but hybrids did not exceed their parents` performances. In contrast, no evidence has been found that the population type (parental or hybrid) influences the variance of any of the phenotypic traits.

As Miller *et al.*, (2005), Ciosi *et al.*, (2008) and our above-presented results showed, independently invading populations of *D. v. virgifera* in Europe differ in their neutral genetic variability. Consequently, there had been indications that also the phenotype of these populations might differ, such as higher fecundity of NW Italian adults than CSE European adults (S. Vidal pers. comm. 2010) or higher mobility of NW Italian than CSE European adults (K. Gloyna pers. comm.). These reports, however, were based on laboratory observations of field-collected adults. This is problematic because field collected adults may differ in age, nutritional status, or egg laying status (Li *et al.*, 2014). They may have moreover experienced different environmental influences during their larval stage, which are known to influence the morphometrics and fecundity of adults (Branson *et al.*, 1988; Li *et al.*, 2014). Therefore, we used laboratory-reared second-generation insects from the NW Italian and CSE European population and investigated them under standardized conditions, as discussed above. As a result, a number of assessed phenotypic traits appeared, in their averages, comparable between the two pure parental European populations. Nevertheless, about 60% of the averages of phenotypic traits indeed differed between both populations. For example, eggs of the NW Italian population survived slightly better than of the CSE European population leading to an overall better fitness index for NW Italian than for CSE European *D. v. virgifera*, despite similar fecundity and adult lifespan (Table 2). Moreover, NW Italian adults were usually a bit lighter and flew off faster than CSE European adults (confirming K. Gloyna, pers. comm.). Although absolute differences were tiny, they may lay in the genetic differentiation of both populations and the selection on them (see below).

As for crossings, the expectation was that the average of phenotypic traits of hybrids will be intermediate compared with their parental populations, which has been supported by many studies (*e.g.* Baker, 1965; Barrett and Kohn, 1991; Facon *et al.*, 2008). This is also reflected in our study, where hybrid populations usually appeared either intermediate between the parental populations (flight take-off response); or similar to the less performing parental population (body weight, elytra length and width, survival from eggs to adulthood, overall fitness, Table 2). Only three of the ten assessed phenotypic traits were, in their averages, comparable between hybrids and their parental populations (adult lifespan, fecundity, proportion adults flying). None of the studied phenotypic traits showed a superior mean performance relative to the parental population, however, eggs of hybrid populations overwintered slightly less successful than those of both parental populations. In general, this confirms Bermond *et al.*, (2012) who did not find any impact of hybrid status on two phenotypic traits (survival and mating success) among field –collected beetles from Northern Italy. In other words, no heterosis effect was observed, although heterosis is invoked to explain the advantage of hybrids in invasions (*e.g*. Drake 2006). The absence of observable heterosis in the present study may be due to the fact that deleterious mutations were not frequent enough in the parental populations, so that no differences in homozygosity of deleterious mutations can be found between hybrid and parental populations. The fate of deleterious mutations in recently invading populations is a new area of research in biological invasions (*e.g*. Peischl *et al.*, 2013) and may be considered in more biological models on *D. v. virgifera* in the future.

Whilst a number of phenotypic traits differed in their average levels between parental populations, no differences in variability have been detected between both parental populations and between hybrid and parental populations. The provided efforts to reconstruct the genetic families inside each population type to better estimate a proxy of the genetic variability did not meet our expectation in better detecting variability changes in phenotypic traits. Indeed, the family structure only allowed us to detect inter-family variance for some phenotypic traits (proportion of flying adults, flight take-off response, fresh bodyweight, elytra sizes), but not between populations (Table 2). This is difficult to explain, as the found phenotypic differentiation in some traits between the parental populations should have led to a larger variability of those traits in hybrid populations. It might be that the studied populations sizes were not large enough to detect difference in variation, it might be that there is just no difference between variability of these traits between population, or there might be genetic reason (see below).

Link between genetic or phenotypic variability and invasiveness

In general, it is difficult to translate genetic variability into variability of certain phenotypic (selected genetic) traits, because genetic analyses only target a limited number of loci, and only the neutral genetic variability. Moreover, relationships of certain phenotypic traits to certain gene patterns are unknown for *D. v. virgifera*. The chance to link neutral genetic traits to selected genetic traits is therefore low.

Large genetic variability is often considered an asset for invading populations since it may represent a large spectrum on which selection processes could play, thus allowing a better adaptation of the alien species to the new environment (Estoup *et al.*, 2016). This theoretical case seems, *in natura*, rare. Invading populations often experience losses in genetic variation due to genetic bottlenecks and founder effects. Still, some bottlenecked populations are able to successfully colonize and disperse through new environments. They sometimes reach such proliferation that they become highly destructive pests in the newly invaded area. Recent prominent examples are the American origin lepidopterans *Spodoptera frugiperda* in Africa and Asia or *Tuta absoluta* in Africa, Asia and Europe (Anonymous, 2020). Also, the here-presented study on *D. v. virgifera* perfectly illustrates such a situation. As hypothesised, the invading *D. v. virgifera* in Europe have indeed a relatively low

to moderate neutral genetic variability compared to their native populations in North America, and are genetically slightly different to those (see above, and Ciosi *et al.*, 2008; Miller *et al.*, 2009). Interestingly, Li *et al*. (2014) found some evidence that European *D. v. virgifera* may also slightly differ phenotypically from US American *D. v. virgifera*, and this is in the averages as well as in the variability of some, but not all assessed traits. For example, US American *D. v. virgifera* were more variable in their egg overwintering survival, proportions of adults flying and flight-take off response than NW Italian and CSE European populations; but variability in morphometric traits were found similar across populations. Moreover, the overall phenotypic variability (average coefficient of variation of traits) did not differ among those populations (Li *et al*., 2014). We hypothesise that bottlenecks may not have been intense enough to lead to a significant decrease in the variability of the considered traits in the invading populations compared to source populations.

Despite a slightly reduced genetic, and (in few traits) phenotypic variability, the separately invading and genetically distinct NW Italian and CSE European populations became two successful outbreaks, providing another example of the genetic paradox in invasions (Facon *et al.*, 2008). Reasons behind such discrepancies might be that the genetic variation proxy may have been inadequate and the used neutral genetic markers may have not well-reflect the genetic variation of ecologically relevant traits. Second, a relatively low genetic and phenotypic variability may be even advantageous in cases where single evolutionary shifts in a small introduced population favours invasion success, something that is hypothesised in the frequently observed bridgehead pattern of invasion routes of species (Lombaert *et al.*, 2010). Or third, the genetic and phenotypic variability play a minor role in biological invasion (Facon *et al.*, 2008), and other factors, such as the natural enemy release (leaving specific and effective natural enemies behind in the area of origin), are the key factors behind invasion success (Wittenberg & Cock, 2001).

Conclusions

Hybridization events, as simulated in laboratory between separately invading *D. v. virgifera* populations in Europe, seem to slightly augment neutral genetic variability; but do not lead to an immediate augmentation of average levels or variability of phenotypic traits. This underlines first findings from studying *in natura* samples by Bermond *et al.* (2014), overall suggesting that no major effect of hybridization between the main invading outbreaks of *D. v. virgifera* in Europe is, at least in short term, expected on their invasion dynamics. However, evolutionary selection processes may act on the hybrid populations and may select for certain traits in the mid- and long term, something worth to be studied in the next decade.

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