Invasion of Europe by the western corn rootworm, *Diabrotica virgifera virgifera*: multiple transatlantic introductions with various reductions of genetic diversity

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Key words. Multiple introductions, microsatellites, invasion success, redistribution of genetic variance, founder effects, loss of genetic variation

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Abstract

The early stages of invasion involve demographic bottlenecks that may result in lower genetic variation in introduced populations as compared to source population/s. Low genetic variability may decrease the adaptive potential of such populations in their new environments. Previous population genetic studies of invasive species have reported varying levels of losses of genetic variability in comparisons of source and invasive populations. However, intraspecific comparisons are required to assess more thoroughly the repeatability of genetic consequences of colonization events. Descriptions of invasive species for which multiple introductions from a single source population have been demonstrated may be particularly informative. The western corn rootworm (WCR), *Diabrotica virgifera virgifera*, native to North America and invasive in Europe, offers us an opportunity to analyze multiple introduction events within a single species. We investigated within- and between-population variation, at eight microsatellite markers, in WCR in North America and Europe, to investigate the routes by which WCR was introduced into Europe and to assess the effect of introduction events on genetic variation. We detected five independent introduction events from the northern US into Europe. The diversity loss following these introductions differed considerably between events, suggesting substantial variation in introduction, foundation and/or establishment conditions. Genetic variability at evolutionarily neutral loci does not seem to underlie the invasive success of WCR in Europe. We also showed that the introduction of WCR into Europe resulted in the redistribution of genetic variance from the intra- to the interpopulational level contrary to most examples of multiple introductions.

INTRODUCTION

Invasive species may present a major threat to biodiversity, ecosystem integrity (reviewed in McKinney & Lockwood, 1999; Olden *et al.*, 2004), agriculture and fisheries (Pimentel *et al.*, 2001). They may also present public health risks (e.g. Ruiz *et al.*, 2000). We therefore need to improve our understanding of the processes underlying their success or failure. Another reason that motivates the study of biological invasions is that recently introduced species may be seen as natural experiments, providing opportunities to investigate the genetic consequences of the early stages of colonization (e.g. Cadotte *et al.*, 2006; Sax *et al.*, 2005). The repeated introductions of a given species, in different geographic locations, provides spatial replicates of colonization (reviewed in Bossdorf *et al.*, 2005; Roman & Darling, 2007). In such cases, it is possible to evaluate the repeatability of genetic consequences of colonization events (Ayala *et al.*, 1989) by comparing different introduced populations.

It is difficult to detect biological invasions in their early stages (small number of founder individuals, long period with low population densities) and such invasions may also be unpredictable (the location and time of introduction are generally unknown), making them difficult to study directly (e.g. Grevstad, 1999). There are therefore few detailed descriptions of population dynamics and structure during early phases of invasion and founder events remain largely unstudied. Analysis of the genetic variation of recently introduced and source populations can be used to provide indirect information about the first steps of the invasion process. The initial phases of invasion (introduction and establishment) are often associated with a founder effect — a loss of genetic variability with respect to the source population, due to the small number of founder individuals and small population size during the first few generations (e.g. Dlugosch & Parker, 2008). By contrast, multiple introductions may increase the genetic variability of the invasive population especially when several genetically differentiated source populations contribute to the invasion (e.g. Facon *et al.*, 2003; Kang *et al.*, 2007; Kolbe *et al.*, 2004). Analyses of the genetic variability of invading populations hence provide insight into the historical demography of the introduction and establishment phases of invasion.

Ecological conditions in the new environment may vary greatly from those in the area of origin, representing an adaptational challenge for newly introduced populations (reviewed in Reznick & Ghalambor, 2001; Schierenbeck & Aïnouche, 2006). Within population genetic variability, thought to determine the capacity of populations to adapt to new environments, may therefore be crucial to successful invasion although some examples of successful invaders display very low genetic variability (reviewed in Novak & Mack, 2005; Wares *et al.*, 2005). This hypothesis, although intuitive, has rarely been tested with actual introduced populations, due to the lack of reports of failed invasions and of genetic patterns of repeated independent introductions of a single species (Lockwood *et al.*, 2005; but see Kelly *et al.*, 2006; Roman, 2006; Stockwell *et al.*, 1996; Voisin *et al.*, 2005).

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a major pest of cultivated corn, *Zea mays* L. Most of the damage to this crop is caused by larvae feeding on the root system of maize (Levine *et al.*, 2002). This pest species probably originated in Central America (Branson & Krysan, 1981; Smith, 1966), but the current southernmost limit of its modern distribution is northern Mexico (Krysan & Smith, 1987). It is likely that WCR evolved with corn in Mexico and reached what is now the southwestern USA about 3000 years ago with the introduction of its host plant (Krysan & Smith, 1987). More recently, WCR rapidly expanded its range from the south-western region of the US Corn Belt in the 1950s,

reaching the east coast of North America during the 1980s (Metcalf, 1983; Spencer *et al.*, 2005). It was recently introduced into Europe, where it was first observed near Belgrade, Serbia, in 1992. An international network has since monitored its spread throughout Europe (Kiss *et al.*, 2005a), and has provided an annually updated, detailed description of the distribution and spread of WCR in Europe. This monitoring is mandatory within the European Union and serve as a powerful tool to detect new introductions of WCR into Europe, making it unlikely that a large and persistent outbreak remains undetected. Two types of infested area have been identified: 1) areas of continuous spread (in Central and South-Eastern (CSE) Europe and north-western (NW) Italy) that correspond to "successful invasions" and 2) several disconnected outbreaks that did not persist over time and/or did not spread. These outbreaks correspond to "unsuccessful invasions". The CSE Europe outbreak now extends over eleven countries, from Austria to the Ukraine and from Southern Poland to Southern Serbia. The first disconnected outbreak was discovered near Venice in 1998. Since then, new disconnected outbreaks have been detected, in NW Italy and Switzerland (canton Ticino) in 2000, north-eastern (NE) Italy in 2002 (Pordenone) and 2003 (Udine), Northern Italy (Trentino), Eastern France, Switzerland, Belgium, the United Kingdom and the Netherlands in 2003, and the Parisian region, France in 2002, 2004 and 2005. Unsuccessful invasive outbreaks can be classified in two categories. Outbreaks detected in North Switzerland, Belgium, Netherlands and the Parisian region did not persist over time and are currently extinct. We refer to these as "extinct outbreaks". Outbreaks detected in NE Italy, Eastern France and the United Kingdom have persisted over time but did not undergo geographic expansion. We refer to these as "established but non spreading outbreaks". A recent population genetics study by Miller *et al*. (2005) showed that the different WCR introduction foci in Europe probably resulted from both the intracontinental movement of insects and repeated transatlantic introductions from North America. Miller *et al.* (2005) suggested that independent introductions were probably responsible for at least the CSE Europe, NW Italy and Paris-2002 outbreaks. WCR thus provides us with an opportunity to analyze introduced populations in the early phases of invasion, and represents an ideal biological model for assessing the details and repeatability of genetic consequences of colonization events, through the comparison of different introduced populations. Miller *et al*. (2005) focused on the statistical inference of WCR introduction routes and did not describe genetic variation within and between the populations they investigated. Moreover, they did not genetically study several European foci as well as American populations of WCR. There is thus so far no precise description of the worldwide geographic distribution of the genetic variability of WCR.

We reanalyzed the data of Miller *et al*. (2005), investigated additional American and European WCR samples, so as to cover most of the geographic distribution of *D. virgifera virgifera*, and addressed the following issues: 1) we inferred the most probable source population and introduction route of each European outbreak; 2) we documented the effect of multiple introductions on the overall genetic variance of WCR in its introduction range in Europe (more specifically, we analyzed the balance between intra- and interpopulation genetic variance in the introduced range compared to the source geographic area); 3) finally, we evaluated the intraspecific repeatability of losses of genetic variation between independent introductions by comparing different outbreaks originating from the same source population. Based on this analysis, we evaluated the relationship between the invasion success and genetic variation of introduced populations of WCR.

MATERIALS AND METHODS

Sample collection

Samples of WCR from European outbreaks were collected at ten sites in five countries (see details in Table 1 and Figure 1). In CSE Europe, the sample studied was collected close to the site at which this species was first observed in Europe — Belgrade Airport in Serbia (only one sample from CSE Europe was used because unpublished results have shown little or no genetic differentiation between sites in this outbreak). The European samples from CSE Europe, Friuli, Piedmont, Paris-2, and Alsace (Eastern France) studied here were those investigated by Miller *et al*. (2005). We also sampled a site (Trentino) corresponding to a small disconnected outbreak observed in 2003 in northern Italy and two sites corresponding to the large outbreak in NW Italy: Lentate in Italy (Lombardy) and Balerna in southern Switzerland (SW). In this area, WCR was first detected in 2000, the year in which this outbreak was first detected in Piedmont, from which we also collected a sample (Oleggio). The sample collected close to Roissy Airport near Paris (Paris-1 sample) studied by Miller et *al.* (2005) was small. We therefore obtained and genotyped additional individuals from this site. We reprocessed the individuals collected by Miller *et al*. (2005) from Alsace, France, for which microsatellite data were missing, to try to fill in the gaps where possible. Finally, we included a sample from the outbreak near Heathrow Airport (London, UK) first detected in 2003 in the analysis. These European sampling sites correspond to all the outbreaks detected in Western Europe between the first observation of WCR in Europe and 2006, with the exception of three outbreaks for which no beetles were detected after 2003: the outbreaks discovered in Belgium and the Netherlands in 2003, and the outbreak detected near Venice in North-Eastern Italy in 1998. In three of the outbreaks (Alsace, Paris-2, and Friuli), sampling was performed before any eradication attempts. In the four other outbreaks (CSE Europe, NW Italy, UK and Pairs-1) eradication attempts occurred before the sampling. In these latter outbreaks, eradication activities principally consist of aerial application of pyrethroid insecticides and the establishment of crop rotation in subsequent years.

In North America, we choose a sampling scheme that allows the description of the genetic structure of WCR in its native continent. Kim and Sappington (2005a) showed that there is little to no genetic differentiation between US populations of WCR form Texas to the East Coast of the USA; Krysan & Smith (1987) showed that the state of Durango, in northern Mexico, is the southernmost limit of the geographic distribution of WCR in America. For our analysis we choose samples from locations that represent the genetic variability of WCR from Texas to the East Coast of the USA and that were previously analyzed by Kim & Sappington (2005a), namely Pennsylvania, Illinois, Texas. To those three samples, we added samples collected at the southernmost limit of WCR distribution in North America and at an intermediate locality in Arizona near the border with Mexico.

In invasive outbreaks (CSE Europe and NW Italy), where population densities were high, adult beetles were sampled with aspirator devices or butterfly nets. In the other outbreaks (UK, the three French outbreaks and Friuli), because of the very low population densities, WCR adults were trapped with sexual pheromone-based sticky traps used for WCR monitoring in Europe. When beetles were collected with aspirator devices or butterfly nets, the insects were sampled within one day in a unique maize field. For each site sampled using the trap method, the collection of individual beetles could be separated by a few days and a few kilometers. The number of individuals in each sample is given in Table 1.

DNA extraction and microsatellite analysis

Template material for polymerase chain reaction (PCR) amplification of microsatellites was obtained using three different protocols. DNA was prepared from a single leg per individual in 25

µl 15% Chelex (Bio-Rad, Hercules, CA) supplemented with 2 µg/µl proteinase K (Euromedex, Mundolsheim, France), as described by Estoup *et al*. (1996) for two individuals from the Paris-1 sample. For the other insects of the Paris-1 sample and all individuals from Alsace, DNA was extracted from the thorax of each specimen, using the DNeasy tissue kit (Qiagen, Hilden, Germany). For the other insects, the "salting out" rapid extraction protocol (Sunnucks & Hales, 1996) was used to extract DNA from the head of each individual. Prior to using the latter two extraction protocols, individuals were washed at least three times in 0.065% NaCl, to remove ethanol from the tissues. Subsequently, each head or thorax was cut and placed in a 1.5 ml microcentrifuge tube, frozen in liquid nitrogen and pulverized with a micropestle. DNA was extracted from the pulverized material.

Six dinucleotide (DVV-D2, DVV-D4, DVV-D11, DVV-D5, DVV-D8, DVV-D9) and two trinucleotide (DVV-T2 and DVV-ET1) microsatellite loci (Kim & Sappington, 2005b; Miller *et al.*, 2005) were amplified in two separate multiplex PCR reactions, and analyzed as described by Miller *et al*. (2007). Allele scoring was standardized between this study and that of Kim & Sappington (2005a), using a panel of common reference DNA samples (not shown), as reported by Kim *et al*. (2008).

Summary statistics of genetic variation

Genetic variation within populations was quantified by determining the mean number of alleles per locus, *A,* and mean expected heterozygosity, *H* (Nei, 1987). *A* is highly dependent on sample size (e.g. Leberg, 2002), rendering comparisons between populations potentially problematic. We therefore used GenClone 1.0 (Arnaud-Haond & Belkhir, 2007) to estimate *A* for a sample size between one and the actual size of the sample considered, using the multiple subsampling method (Leberg, 2002). Exact tests for population differentiation (Raymond & Rousset, 1995a) were carried out for all pairs of populations, with GENEPOP (Raymond & Rousset, 1995b). As this test involves non orthogonal and multiple comparisons, a sequential Bonferroni correction was applied (Sokal & Rolf, 1995 p.236). GENEPOP was also used to calculate pairwise F_{ST} estimates (Weir & Cockerham, 1984) as statistics summarizing genetic variation between populations, and to test for Hardy-Weinberg equilibrium, with the probability test approach.

Identification of source populations

The most probable source population for each European outbreak was identified by calculating the mean multilocus individual assignment likelihood of each introduced outbreak sample *i* to each sample of possible source populations *s* (hereafter denoted $L_{i\to s}$ (see Pascual *et al.*, 2007; and Rannala & Mountain, 1997)). Pascual *et al.* (2007) showed, by computer simulation, that $L_{i\to s}$ efficiently identifies the actual source population of a recently introduced population, even if the candidate source populations display only weak differentiation (i.e. display low F_{ST}) and if the introduced population endured a strong founder event. More specifically, $L_{i\to s}$ values remain similar in expectation for a large range of founder event intensities, though its variance increases, as high-frequency alleles tend to be retained after a founder event. Individuals in introduced populations subject to bottlenecks therefore tend to bear alleles present at high frequency in the source population, resulting in high individual assignment likelihoods in the actual source population. $L_{i\to s}$ values were calculated with GENECLASS 2 (Piry *et al.*, 2004). No ad hoc statistical test has yet been described for formally comparing mean individual assignment likelihoods (as well as F_{ST}). Moreover, non-parametric tests, such as the Friedman analysis of variance by rank or pairwise

Wilcoxon signed rank test, using the locus as the repetition unit, are not sufficiently powerful (due to limited number of loci) for such comparisons in the context of the present study.

Therefore, for each European outbreak, the most probable source population was simply identified as that with both the highest $L_{i \to s}$ value and the lowest F_{ST}-value with this outbreak. However, as only a small fraction of the large geographic range of WCR in North America has been sampled, the selected populations may not be the "true" source population per se, corresponding instead simply to the most probable of the source populations studied.

Multiple introductions in a single location are expected to leave a genetic signature for migrants originating from sources genetically differentiated from the outbreak considered. Because of the number of loci we used, only migrants of first generation would be detectable (see Rannala & Mountain (1997) for a discussion on the power of statistical tests of assignment). To detect multiple introductions, two methods were therefore applied: 1) the detection method of first generation migrants of Paetkau *et al*. (2004) implemented in GeneClass2 (ver. 2.0, Piry *et al*. (2004)) was used. 10000 individuals were simulated per population and the likelihood calculation of Rannala & Mountain (1997) was used. The statistics used was the individual assignment likelihood to the population where the individual was sampled. 2) A multimodal distribution of the individual assignment likelihood value of an outbreak into each putative source population can be observed when first generation migrants introduced from different sources are frequent in the outbreak (unpublished results). We thus tested the unimodality of the distribution of assignment likelihood value of individuals belonging to each European population into each possible source population (normality test of the data using a Kolmogorov-Smirnov test).

RESULTS

The Lombardy and SW samples were considered as a single population sample, as they displayed no significant genetic differentiation (see below). The Pennsylvania and Illinois samples are referred to as the "northern US sample" below. Microsatellite allele frequencies for each locus and population are listed in the Appendix. The mean number of alleles per locus and expected heterozygosity are given for each population in Table 1.

Genetic variation within populations

The complete dataset of WCR samples showed substantial polymorphism, with a mean of 12.375 alleles per locus over all samples. The number of alleles varied from 6 for the DVV-D5 and DVV-ET1 loci to 23 for the DVV-D8 locus. All 99 observed alleles were present in North America and 58 of these alleles were detected in Europe. In North America, all loci were polymorphic in all samples, whereas, in Europe, some loci were monomorphic in some samples (e.g. the DVV-D5 locus, which was monomorphic in CSE Europe and all Italian samples; see Appendix). Significantly fewer alleles were found in Europe than in North America (mean A when pooling all populations within each continent = 7.250 and 12.375, respectively; Wilcoxon's signed rank test, $p = 0.008$), and expected heterozygosity (mean among populations) was lower in Europe than in America (0.457 and 0.681, respectively; Wilcoxon's signed rank test, $p = 0.008$).

The standardization of A as a function of smallest sample size (i.e. *MSS* in Table 1) made it possible to compare samples. In North America, the samples from Mexico, Texas and Arizona were

genetically more diverse than those from the northern US (Illinois and Pennsylvania) (Wilcoxon's signed rank tests, $p \leq 0.024$). Expected heterozygosities (H in Table 1) in North America range from 0.644 (Pennsylvania) to 0.753 (Mexico). H was significantly higher in Mexico than in Texas and in the northern US samples (Wilcoxon's signed rank tests, $p \leq 0.04$).

In Europe, A was highly heterogeneous between samples, varying from 1.75 (MSS = 1.711) in Friuli to 5.75 (MSS = 4.374) in the UK (Table 1). The UK and Alsace samples had significantly higher allelic diversities than any other European sample (Wilcoxon's signed rank tests on MSS, $p \leq 0.024$ for each test) except for comparisons of the UK sample to both the Parisian samples. Mean expected heterozygosity ranged from low to medium values in Europe (about 0.3 in Friuli to 0.6 in Alsace and the UK). No significant differences of genetic variability could be detected between extinct (Paris-1 and 2), established but not spreading (UK, Alsace and Friuli) and invasive (NW Italy and CSE Europe) outbreaks (global test: Friedman's test by rank performed over loci, $p > 0.5$ for both A and H; invasive vs others: Wilcoxon's test over loci $p \geq 0.164$ for both A and H; and extinct vs others: Wilcoxon's test over loci, $p \geq 0.194$ for both A and H).

Genetic variation between populations

Most pairwise comparisons showed significant genetic differentiation ($p < 0.05$; Table 2), with large to very large F_{ST} estimates (mean = 0.16, SD = 0.11). In North America, pairwise genetic differentiation ranged from weak in the northern US (F_{ST} = 0.01) to considerable between northern US and Mexico (mean F_{ST} = 0.11, SD = 0.01). Most sample pairs in Europe displayed significant differentiation, with high F_{ST} values (mean = 0.19, SD = 0.12), with the exception of SW-Trentino, SW-Lombardy and Trentino-Lombardy pairs, for which F_{ST} estimates were below 0.01 (mean = 0.002, SD = 0.003). SW and Lombardy were not significantly differentiated (Fisher's exact test, p = 0.86), with an F_{ST} value of zero, and were hence pooled together for subsequent analysis.

A high level of genetic differentiation was observed for most intercontinental comparisons (mean pairwise F_{ST} values = 0.15, SD = 0.09), with the exception of comparisons between the UK sample and samples from the northern US, for which an F_{ST} value of only about 0.01 was obtained. Intercontinental pairwise F_{ST} decreased from the South-West to the North-East for American samples (mean F_{ST} (SD) of 0.25 (0.09), 0.17 (0.07), 0.11 (0.06), 0.12 (0.07), 0.10 (0.06), for comparisons of the European samples with Mexico, Arizona, Texas, Illinois and Pennsylvania sample, respectively).

Identification of the most representative source populations

The hypothesis of a single source population for each European outbreak was never rejected. All 77 normality tests performed suggest that assignment likelihood values of European individuals into the eleven potential source populations are approximately normally distributed (Kolmogorov-Smirnov tests, $p > 0.05$ for all tests), so that the unimodality of the individual assignment likelihood distributions was never rejected. Using the method of Paetkau *et al.* (2004), we found that two European individuals were classified as first generation migrants ($p < 0.05$ for both individuals), one in the UK, statistically assigned into Texas or Pennsylvania (-10Log(L) = 4.55 and 4.56, respectively), and one in Paris-1 assigned into UK. These migrants probably correspond to multiple introductions from the most representative source population identified for each of these outbreaks. Overall, we found no evidence for multiple introductions from various differentiated source populations into the European outbreaks.

The most probable source population of each European sample *i* was identified by analyzing the F_{ST} values of all sample pairs including sample *i* and all mean individual assignment likelihoods of sample *i* into sample *s* ($L_{i\to s}$ values expressed on a –log scale). The deduced most probable source population for each outbreak was identified as the sample with both the highest $L_{i\to s}$ and the lowest F_{ST} value (Table 2). These criteria identified the northern US population as the most representative source population for CSE Europe, the UK, Paris-2 and Alsace. For all the NW Italian and Swiss samples, minimum F_{ST} estimates and maximum $L_{i\to s}$ identified a sample from the same region as the most probable source. If these NW Italian and Swiss samples were considered to correspond to a single outbreak, then their most probable source population was Pennsylvania in the northern US. Both F_{ST} and $L_{i\to s}$ values suggested that the Paris-1 population originated in the UK, and that the Friuli population originated in CSE Europe.

A detailed investigation of allelic frequency distributions (see Appendix) supported our identification of the most probable source population for each outbreak. A sample from the source population should contain all the alleles present in samples corresponding to introductions from that population. All the alleles of the Friuli population were found in CSE Europe, and all the alleles of the CSE Europe, UK, Paris-2 and NW Italy samples were found in the northern US sample. A single rare allele of the Paris-1 population (allele 207 of DVV-D2) was not present in the sample of its most probable source, the UK. Allelic distributions also made it possible to reject alternative hypotheses. For instance, the UK is unlikely to be the source of the Piedmont population, given the presence of allele 198 at locus DVV-D11 and alleles 208 and 234 at locus DVV-D8 in the Piedmont population, and the absence of these alleles in the UK. The UK is also unlikely to be the source of the Paris-2 population, as alleles 198 at locus DVV-D11, 152 at DVV-D9 and 214 at DVV-D8 were present in the Paris-2 population but absent from the UK sample.

Comparison between introduced populations and their most representative source populations

The mean number of alleles was smaller for all European outbreak samples than for their inferred source populations (Table 1 and Figure 2). *MSS* was, on average, 38.2% (SD = 20.5%) lower and *H* was 25.1% (SD = 15.1%) lower in European populations than in their inferred sources (Figure 2). The decrease in the number of alleles was significant in all cases (Wilcoxon's signed rank tests, p = 0.016 for all tests) other than for comparisons of the samples from Alsace and the UK with the sample from Illinois (Wilcoxon's signed rank tests, p = 0.25 and 0.156 respectively) and for the comparison of the Paris-1 and UK populations (Wilcoxon's signed rank test, p = 0.062). A significant decrease in expected heterozygosity was observed only for comparisons of the Piedmont and Pennsylvania populations and the Friuli and CSE Europe populations (Wilcoxon's signed rank tests, p = 0.008 and 0.016 respectively).

The loss of variability differed markedly between outbreaks (Figure 2). Genetic bottlenecks were weakest for the UK and Alsace populations, with a loss of less than 16% *MSS,* whereas the other outbreak populations showed *MSS* losses exceeding 28% (Figure 2). The loss of expected heterozygosity was also highly heterogeneous, with a loss of less than 18% for Parisian samples and samples from the UK and Alsace and a loss of more than 29% for Italian samples and CSE Europe.

When considered individually, European outbreak populations were generally significantly less variable than northern US sample (see above). However, overall, the global European gene pool contained almost as much genetic variation as that of the northern US sample. The number of alleles was similar in the northern US sample and the global European gene pool (A = 8.25 and

7.25, respectively, and *MSS* = 8.25 and 6.23, respectively; Wilcoxon's signed rank test, $p = 0.218$ and 0.032 for *A* and *MSS*) (Figure 2). The 11 alleles (concerning all eight loci) present in the northern US sample but not in Europe were all rare (frequency ≤ 2%). Expected heterozygosity was nevertheless significantly lower in the global European gene pool (0.457) than in the northern US sample (0.647) (Wilcoxon's signed rank test, $p = 0.008$).

The UK and Alsace populations were genetically very variable (Table 1) and had a variability similar to that of the northern US sample. However, they were far from being solely responsible for the high allelic diversity found within the global European gene pool. Removing the UK and Alsace populations from the global European gene pool decreased the number of alleles by only 12 % (from 58 to 51 alleles). The global European gene pool was rapidly increased by successive introductions (Figure 3): 46.5 % of the 58 European alleles arrived with the first introduction of WCR into Serbia in 1992, and 33 % of the total allelic diversity (19 additional alleles) was added during the second recorded introduction (in NW Italy in 2000). Subsequent introductions added 15.5 % (9 additional alleles in the UK and Paris-1 introductions), 2% (1 allele in Alsace) and 3% (2 alleles in the Paris-2 population) to the overall allelic diversity of European populations. Hence allelic variability doubled in a very short period, between 1992 — the year in which WCR was first detected (27 alleles) — and 2004 (58 alleles). On average, the genetic diversity loss was not significantly different between outbreaks that had been subjected to eradication activity (Paris-1, UK, NW Italy and CSE Europe) and those that had not (Alsace, Paris-2 and Friuli), with a mean loss of *MSS* of nearly 33% and a mean loss of *H* of nearly 21% in both outbreak categories (Wilcoxon's test performed over loci, $p > 0.204$ for both tests).

DISCUSSION

In this study, we analyzed the worldwide genetic variation of the invasive western corn rootworm *Diabrotica virgifera virgifera*. We considered almost all known European outbreaks (CSE Europe, NE Italy, NW Italy, the Parisian region and Alsace in France, and the UK), with the exclusion of those whose low density or rapid disappearance, subsequent to eradication attempts made sampling impossible. Moreover, samples collected in the USA and Mexico, cover much of the American geographic distribution of WCR. We detected five independent introduction events from the northern US into Europe (see Figure 4 for an illustration of the suggested routes of introduction. The diversity loss following these introductions differed considerably between events, suggesting substantial variation in introduction, foundation and/or establishment conditions. Finally, our results indicate that the introduction of WCR into Europe resulted in the redistribution of genetic variance from the intra- to the interpopulational level.

Routes of introduction of WCR

Our results show a decrease in genetic variability from Mexico to the north-eastern USA. This observation is consistent with the hypothesis that WCR originated in the neotropics (Branson & Krysan, 1981; Smith, 1966), subsequently colonizing North America following the expansion of corn cultivation (Krysan *et al.*, 1977).

The routes of WCR introduction in Europe were studied by Miller *et al.* (2005), using model-based Bayesian approaches to the analysis of genetic variability. Miller *et al.* (2005) demonstrated that there have been at least three independent introductions of WCR from North America to Europe

over the past two decades, leading to the CSE Europe, NW Italy and Paris-1 outbreaks. They also showed that the NE Italian Friuli population corresponded to a secondary introduction from CSE Europe. However, they were unable to draw firm conclusions about the origins of the Paris-2 and Alsace populations. Our analysis supports the conclusions of Miller *et al.* (2005) concerning the CSE Europe, NE and NW Italy populations, but additional data for the Paris-1 and Alsace populations and analysis of the UK population have provided new information.

The UK outbreak appears to have resulted from a direct introduction of WCR from North America, with the Paris-1 population probably corresponding to a secondary introduction from the UK. The UK population being the source population of the Paris-1 outbreak may initially appear illogical, as WCR was first detected in the Parisian region in 2002 but was not detected in the UK until one year later (Kiss *et al.*, 2005a). However, observation dates strongly reflect the effort devoted to WCR monitoring. The first report of WCR in France in 2002 prompted the monitoring of English corn fields, beginning in the summer of 2003 (Cheek *et al.*, 2004; Ostoja-Starzewski, 2005) and resulting in the first detection of WCR. In addition, large trap counts at one English site in 2003 indicated that the pest had likely been present for at least one year prior to its detection (Cheek *et al.*, 2004). This information strongly suggests that WCR was present in the UK before 2003 and thus have possibly served as the source of the Paris-1 outbreak. Our data also indicate that the Alsace outbreak, rather than corresponding to a secondary introduction from other European populations, likely originated from a direct introduction from the northern US. We also found that the Paris-2 population was probably founded by individuals originating from the northern US. Finally, the weak genetic structure of populations from NW Italy and Switzerland suggested that these populations probably correspond to a single outbreak.

Our results hence indicate that there have been five independent introductions from the northern US into Europe (Figure 4) that led to the CSE Europe, NW Italy, the UK, Paris-2 and Alsace populations. Secondary introductions of WCR within Europe were probably responsible for two additional outbreaks: the UK may have been the source of the Paris-1 population and CSE Europe is the most probable source of the Friuli population in NE Italy. The occurrence of multiple introductions of WCR in Europe is consistent with a growing number of analyses of invasive species (e.g. Chen *et al.*, 2006; Facon *et al.*, 2003; Fonseca *et al.*, 2000; Kang *et al.*, 2007; Kolbe *et al.*, 2004), suggesting that multiple introductions of invasive species may be a common phenomenon (reviewed in Bossdorf *et al.*, 2005; Roman & Darling, 2007).

Uncertainty relating to inferences on routes of introduction

Due to the considerable genetic similarity between UK and Northern US, it was difficult to firmly exclude UK as the putative source population of the European outbreaks. However, the low but significant level of genetic differentiation between the UK and northern US populations appears to be sufficient to distinguish between populations assigned to the northern US and the UK. A careful examination of allelic frequency distributions also revealed the presence of alleles absent from the UK in some European outbreaks. Based on an approximate Bayesian computation (ABC) approach, Miller *et al.* (2005) rejected the possibility that an unstudied population already established in Europe (such as that the UK outbreak, which was not studied by Miller *et al.* (2005)) was the source of the CSE Europe, Paris-1 and NW Italy outbreaks. Therefore our analysis as well as that of Miller *et al.* (2005) suggest that the UK was not the source of most European outbreaks.

Our analysis of the data set presented in this study show that UK, Paris-2, Alsace, CSE Europe and NW Italy outbreaks were not successive introductions, i.e. they did not originate from each

other. They thus correspond to independent introductions from their own source population. Strictly, we cannot exclude the possibility that an unstudied population already established in Europe (a "ghost population") was the origin of these outbreaks. Several lines of evidence refute this latter hypothesis. To be a viable source of new outbreaks, a population would probably need to be persistent over time and reasonably large. Detected but unsampled introduced populations (the Netherlands, Belgium and Venice area in Italy) were geographically very limited and did not persist over time (Kiss *et al.*, 2005a). Populations that were not detected by the European monitoring network may have existed. But precisely because they were not detected, these undiscovered outbreaks were probably too small and not sufficiently persistent to be the origin of the studied outbreaks. Moreover, as mentioned previously, Miller *et al*. (2005) rejected the "ghost scenario" hypothesis for Paris-1 and 2, Alsace, CSE Europe, and NW Italy. We therefore conclude that five independent introductions of WCR have occurred form Northern US into Europe (Figure 4).

Heterogeneity in loss of diversity

Most European outbreaks of WCR (the UK, Alsace, Paris-2, NW Italy and CSE Europe populations) had the same source population (northern US). This circumstance has provided us with a rare opportunity to analyze multiple instances of the same type of demographical event (i.e. the foundation of new population) within a single species. The history of WCR introduction into Europe thus provides an opportunity to directly compare the effects of independent introductions from the same original gene pool. Our findings show considerable heterogeneity in genetic differentiation between outbreaks and between outbreak and source populations, leading us to reject the hypothesis of homogeneity or repeatability in loss of genetic variability between introductions. The differences in diversity loss were not accounted for by differences in time between the introduction and sampling of populations. The French and Friuli populations were sampled the year they were first detected, but nonetheless differed considerably in terms of loss of diversity compared to their respective sources. Thus, we conclude that the observed variation in the loss of genetic variability may reflect differences in conditions for the introduction, foundation or establishment of populations (e.g. number of founder individuals, number of introductions involved in each outbreak and population dynamics after introduction). Stochastic or deterministic processes, such as eradication attempts, may account for the observed heterogeneity. However, in the particular case of WCR, eradication activity does not seem to be an explanatory factor of the observed heterogeneity in loss of diversity.

Previous population genetic studies of invasive species have reported a wide range of genetic variability loss during introductions (Facon *et al.*, 2003; Holland, 2001; Johnson & Starks, 2004; Kolbe *et al.*, 2004; Lindholm *et al.*, 2005; Ross *et al.*, 1996; Tsutsui *et al.*, 2000; Zayed *et al.*, 2007). However this heterogeneity corresponds to differences in diversity loss between studies focusing on different species (see Cox (2004), Wares *et al*. (2005), Bossdorf *et al.* (2005) and Roman & Darling (Roman & Darling, 2007) for reviews). In that respect, WCR allowed heterogeneity of diversity loss to be investigated at the intraspecific level (see also Kelly *et al.*, 2006; Roman, 2006; Stockwell *et al.*, 1996; Voisin *et al.*, 2005).

Recent reviews have suggested that many successful invasive species suffer no major loss of diversity, suggesting a link between the genetic variation of introduced populations and invasion success. In 29 studies of invasive animals reviewed by Wares *et al*. (2005), introduced populations were found to contain about 80% of the native genetic diversity. Similarly, more than 65% of the invasive species reviewed by Bossdorf *et al.* (2005) and Roman & Darling (Roman & Darling, 2007) showed no significant loss of diversity with respect to native populations. For WCR, repeated

introductions from the same genetic pool have occurred, making it possible to analyze the link between genetic variation and invasion success within this species. We found that genetic variability within the introduced WCR populations was heterogeneous and that their establishment or invasive success was apparently not related to the level of the genetic variability of the various introduced outbreaks. The extinct Parisian outbreaks and the non spreading Alsace and UK outbreaks were as diverse as or more diverse than the successfully invasive CSE European and NW Italian outbreaks. This suggests that, at least for invasive pest species subject to human control and eradication, such as WCR, high levels of genetic diversity may not be the key determinant of a successful invasion. However, we measured only evolutionarily neutral genetic variation, through microsatellite markers, and such variation is often weakly correlated with that involved in the adaptive potential of introduced populations in a novel environment (for reviews see McKay & Latta, 2002; Merila & Crnokrak, 2001; Reed & Frankham, 2001). Alternative explanations for the success or failure of WCR invasion may include differences in pest management efforts, such as monitoring and pesticide treatments. The success of the initial European introduction (CSE Europe, first detected in 1992 (Kiss *et al.*, 2005a)) may in part be due to the absence of monitoring of this species during its early phase of establishment, allowing it to reach high densities before control attempts were implemented.

Redistribution of genetic variance in relation to multiple introductions

If all the European outbreaks are combined, the genetic variation observed in the invaded area is similar to that found in the northern US. Thus, recurrent introductions from the same original gene pool resulted in an increase in overall European genetic variability over time, with at least a doubling of allelic diversity within a span of 12 years.

Demonstrations of multiple introductions based on previous population genetics analyses, such as those of Kolbe *et al.* (2004), Facon *et al.* (2003) or Genton *et al.* (2005), have mostly shown a redistribution of interpopulation genetic variance into intrapopulation variance (but see Kelly *et al.*, 2006; Stockwell *et al.*, 1996; Voisin *et al.*, 2005). This is of evolutionary importance in terms of adaptation, as natural selection acts on intrapopulation variance (e.g. Falconer & Mackay, 1996). This shift may be accounted for by a single invaded area experiencing multiple introductions from genetically differentiated source populations. The case of WCR is different in that its invasion of Europe has resulted in the redistribution of genetic variance from intrapopulation level to the interpopulation level. Interpopulation variance accounted for 1% of total variance in the northern US and 19% in Europe. The genetic variation contained in a single non structured gene pool (northern US) has been distributed among several introduced, unconnected and genetically differentiated populations over a large area (the European continent).

The lack of examples of a redistribution of genetic variance from the intra- to the interpopulation level during multiple invasions probably results from the technical difficulties associated with the detection of multiple introductions from a single source. The genetic signatures of multiple and single introductions from a single source population are unlikely to be distinguished with commonly used genetic markers (most often mitochondrial markers) and statistical techniques (haplotypic networks or distance-based trees). Moreover, because of the rapid spatial spreading of most invasive populations, a late sampling of the invaded area is likely to result in the detection of a single homogenized and genetically diverse population irrespective of the number of introductions from a unique source population. WCR European outbreaks were detected and sampled at an early stage of the invasion process and hence probably before any secondary contact between outbreaks.

This allowed a redistribution of genetic variance from the intra to the inter population levels to be detected, which may actually correspond to a transitory state in the invasion process.

Natural selection acts on intrapopulation variance (e.g. Falconer & Mackay, 1996). The redistribution of genetic variance from the intra- to the interpopulation level in WCR may therefore jeopardize the adaptation of this species to new environmental conditions in Europe. However, geographically close invasive outbreaks, such as those corresponding to the CSE Europe and NW Italy populations, will probably overlap in the future, restoring much of the original intrapopulation genetic variance. It is worth pointing that northern US populations are polymorphic for adaptive traits, such as insecticide resistance (e.g. Meinke *et al.*, 1998; Parimi *et al.*, 2006) and resistance to crop rotation (Levine *et al.*, 2002). Chemical insecticide treatments and crop rotation strategies are also used in Europe against WCR (Kiss *et al.*, 2005b; Van Rozen & Ester, 2007). Therefore recurrent and independent introductions of WCR into Europe are likely to increase the probability of adaptations to management strategies being introduced, potentially increasing the invasiveness and economic impact of this pest.

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725 ***ACKNOWLEDGMENTS***

726 We thank Stefan Toepfer, Lorenzo Furlan, Sylvie Derridj, Gino Angeli, Mario Bertossa, Sharon
727 Cheek and Joe Ostoja-Starzewski for their assistance with sample acquisition and Benoît Facon for
728 critical reading of an earlier version of this manuscript. This work was funded by the French ANR
729 Biodiversité #ANR-06-BDIV-008-01.

730

731 **Figure legends**
732
733 **Figure 1:** Geographic distribution of WCR in 2006 and sampling sites. Distribution area, with sites
734 at which WCR was observed for at least one year is shown in gray.
735
736 **Figure 2:** Loss of genetic diversity in European invasive populations of WCR with respect to their
737 most representative source populations. White bars correspond to the % allelic diversity loss,
738 corrected for sample size, and gray bars correspond to the % mean expected heterozygosity (gene
739 diversity (Nei, 1987)) loss. Significant diversity losses are indicated by asterisks (based on
740 Wilcoxon's signed rank tests). For the two European outbreaks probably originating from a
741 secondary introduction from Europe (Friuli and Paris-1), diversity loss with respect to northern US
742 populations is also shown to illustrate the effect of successive introductions. For comparisons of the
743 entire area of invasion in Europe with the most probable source of the invasion, we pooled all
744 outbreaks originating from the northern US into a single sample referred to as global Europe (with
745 only the Piedmont sample included to represent the NW Italian outbreak).
746
747 **Figure 3:** Cumulated allelic richness (mean allele number per locus) in Europe during the invasion
748 by the western corn rootworm. The dotted line shows the allelic richness of the most representative
749 native source population (northern US).
750
751 **Figure 4:** Suggested routes of introductions of WCR in Europe. The dotted line encircles the NW
752 Italian outbreak.
753

Table 1: Western corn rootworm population samples used in this study, with statistics summarizing genetic variation within populations

Geographic area	Sample name	Location	1st obs.	N	Collection year	*A*		
						DC	*MSS*	*H*
North America	Mexico	Registrillo, Durango, Mexico	<1940	14	2001	7.250 (3.694)	6.154 (2.716)	0.753
	Arizona	Willcox, Arizona, USA	<1974	40	1998	9.000 (4.928)	5.524 (2.311)	0.681
	Texas	New Deal, Texas, USA	<1980	51	2004	8.125 (4.673)	5.493 (2.650)	0.675
	Illinois	Champaign, Illinois	<1974	60	2003	7.250 (5.120)	4.806 (2.189)	0.649
	Pennsylvania	Bellefonte, Pennsylvania	<1985	62	2003	7.500 (5.043)	4.798 (2.366)	0.644
Central South Eastern Europe area of spread	CSE Europe	Belgrade Airport, Serbia	1992	38	2003	3.375 (1.685)	2.912 (1.257)	0.453
Western European disconnected outbreaks	Friuli	Buttrio, Italy	2003	27	2003	1.750 (0.707)	1.711 (0.634)	0.293
	Trentino	Storo, Italy	2003	44	2004	2.875 (1.959)	2.430 (1.449)	0.361
	Piedmont	Oleggio, Italy	2000	40	2003	4.250 (3.151)	3.252 (2.060)	0.420
	Lombardy	Lentate, Italy	2001	44	2003	3.250 (2.816)	2.322 (1.499)	0.347
	SW	Balerna, Switzerland	2000	45	2003			
	Paris-1	Roissy Airport, France	2002	19	2003	3.750 (1.753)	3.160 (1.162)	0.510
	Paris-2	Pierrelaye, France	2004	74	2004	3.750 (1.581)	2.931 (0.722)	0.534
	Alsace	Schwindratzheim, France	2003	9	2003	4.625 (1.996)	4.625 (1.996)	0.581
	UK	Slough, United Kingdom	2003	36	2005	5.750 (3.770)	4.374 (2.212)	0.612

Note: 1st obs.: year of first observation of the outbreak. N: number of individuals analyzed per sample. ***A*:** average number of alleles per locus; standard deviations across loci are shown in brackets. *A* is given by direct counts (DC) and based on multiple subsampling (*MSS*), accounting for sample size variation. *MSS* is given for the smallest sample size (n = 9). *H*: mean expected heterozygosity (Nei, 1987). Significant deviation from Hardy Weinberg Equilibrium was observed for the Paris-2 sample only ($p<0.0001$).

Table 2: Pairwise estimate of F_{ST} (Weir & Cockerham, 1984) and mean individual assignment likelihood ($L_{i\rightarrow s}$) of each sample to each potential source population (Pascual *et al.*, 2007).

	Potential source populations														**Most likely source population**
	North America					**Europe**									
	Mexico	**Arizona**	**Texas**	**Illinois**	**Penn-sylvania**	**CSE Europe**	**Friuli**	**Trentino**	**Lombardy-SW**	**Piedmont**	**Paris-1**	**Paris-2**	**Alsace**	**UK**	
Arizona	0.0590	-	-	-	-	-	-	-	-	-	-	-	-	-	
Texas	0.0870	0.0295	-	-	-	-	-	-	-	-	-	-	-	-	
Illinois	0.1002	0.0501	0.0164	-	-	-	-	-	-	-	-	-	-	-	
Pennsylvania	0.1177	0.0638	0.0169	0.0094	-	-	-	-	-	-	-	-	-	-	
CSE Europe	0.224 (16.410)	0.167 (16.770)	0.103 (8.974)	0.109 (8.259)	**0.095** (**7.627**)	-	0.116 (11.143)	0.264 (17.760)	0.276 (19.266)	0.197 (13.196)	0.257 (11.080)	0.148 (12.130)	0.118 (8.960)	0.126 (8.581)	Pennsylvania
Friuli	0.319 (16.649)	0.276 (18.605)	0.226 (10.048)	0.229 (9.175)	0.218 (8.152)	**0.116** (**4.863**)	-	0.43 (19.620)	0.429 (20.986)	0.357 (15.354)	0.439 (14.479)	0.278 (12.777)	0.267 (9.425)	0.285 (11.301)	CSE Europe
Trentino	0.331 (16.010)	0.222 (13.740)	0.151 (8.674)	0.17 (8.498)	0.13 (7.708)	0.264 (11.534)	0.43 (18.465)	-	**0.005** (**3.812**)	0.028 (4.359)	0.299 (12.051)	0.17 (9.634)	0.256 (12.248)	0.149 (7.851)	Pennsylvania*
Lombardy-SW	0.37 (15.885)	0.257 (14.143)	0.178 (8.895)	0.202 (9.030)	0.152 (7.922)	0.276 (12.026)	0.429 (18.463)	**0.005** (**3.784**)	-	0.023 (4.400)	0.324 (12.297)	0.203 (10.321)	0.27 (12.280)	0.173 (7.984)	Pennsylvania*
Piedmont	0.285 (16.214)	0.177 (13.497)	0.103 (8.660)	0.116 (8.396)	0.082 (7.672)	0.197 (12.059)	0.357 (18.382)	0.028 (**6.957**)	**0.023** (7.354)	-	0.224 (11.380)	0.133 (10.482)	0.161 (11.241)	0.09 (7.814)	Pennsylvania*
Paris-1	0.223 (15.938)	0.136 (11.390)	0.105 (9.045)	0.068 (7.553)	0.087 (7.706)	0.257 (12.784)	0.439 (23.903)	0.299 (18.588)	0.324 (21.812)	0.224 (11.179)	-	0.154 (11.061)	0.095 (7.952)	**0.066** (**7.148**)	UK
Paris-2	0.207 (16.384)	0.143 (13.127)	0.074 (9.342)	0.069 (9.140)	**0.052** (**8.189**)	0.148 (11.703)	0.278 (18.206)	0.17 (14.494)	0.203 (16.341)	0.133 (11.507)	0.154 (11.224)	-	0.141 (11.201)	0.086 (9.912)	Pennsylvania
Alsace	0.100 (14.648)	0.06 (12.215)	0.042 (9.660)	**0.021** (**8.301**)	0.046 (8.940)	0.118 (13.178)	0.267 (19.268)	0.256 (19.240)	0.27 (21.460)	0.161 (12.901)	0.095 (10.165)	0.141 (15.418)	-	0.032 (8.928)	Illinois
UK	0.128 (16.125)	0.066 (13.203)	0.022 (9.077)	**0.008** (**8.097**)	0.013 (8.436)	0.126 (13.840)	0.285 (22.653)	0.149 (17.122)	0.173 (19.244)	0.09 (11.848)	0.066 (10.887)	0.086 (14.242)	0.032 (9.768)	-	Illinois

Note: The only non significant pairwise differentiation exact test before and after correction for multiple comparisons was that between the Alsace and Illinois samples. The Lombardy and SW samples were considered as a single population sample (denoted Lombardy-SW), as they displayed no significant genetic differentiation. $L_{i\rightarrow s}$ values expressed on a –log scale are indicated in parentheses for the European outbreaks only. For each European outbreak, maximum $L_{i\rightarrow s}$ and minimum F_{ST} are indicated in bold typeface. For the Piedmont, Lombardy-SW and Trentino populations, maximum $L_{i\rightarrow s}$ and minimum F_{ST} with respect to all other samples are underlined. The most representative source population for each European outbreak is indicated in the last column. * indicates the most likely source of the single outbreak corresponding to the Piedmont, Lombardy-SW and Trentino samples.

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776 **Figure 1.**
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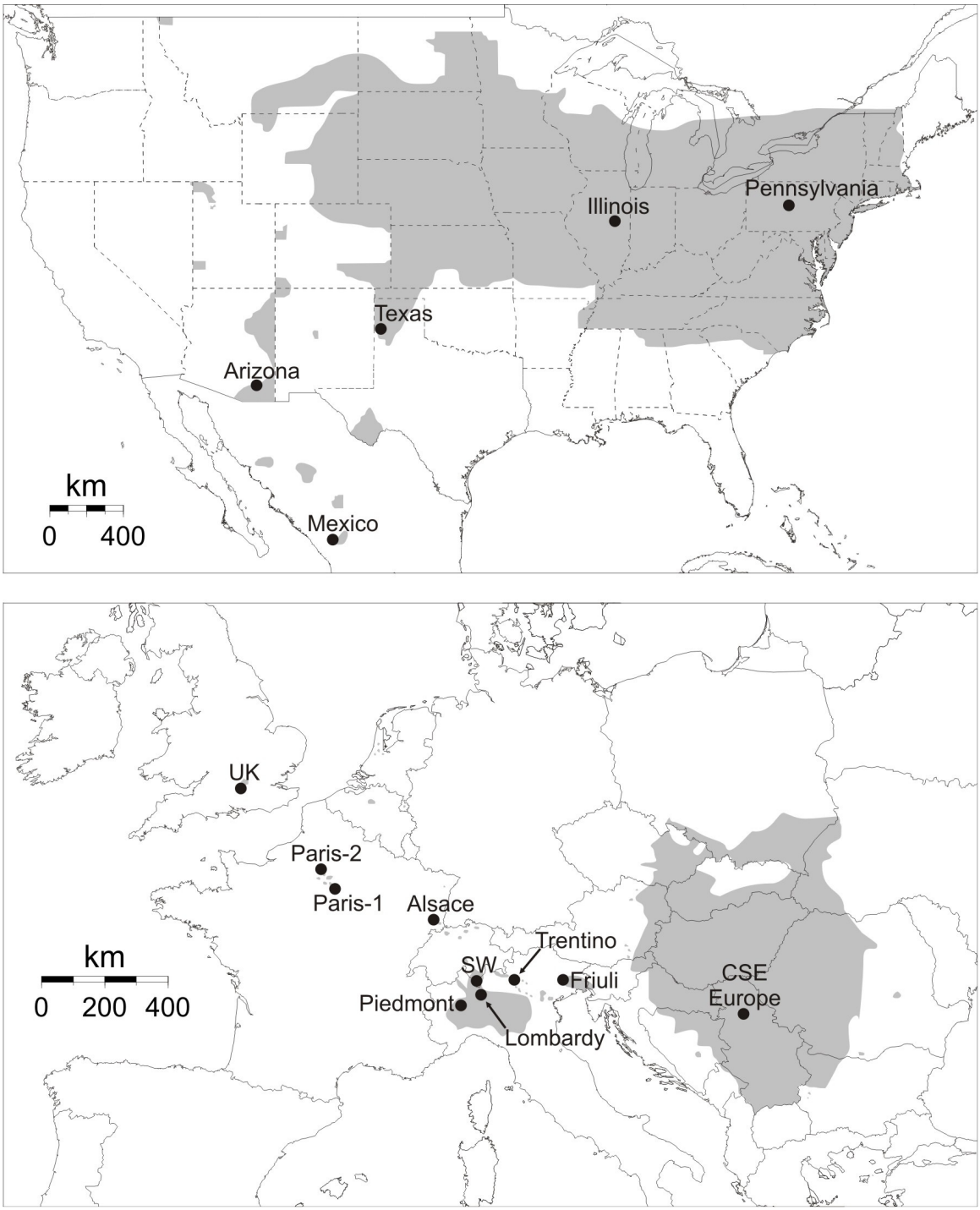

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782 **Figure 2.**
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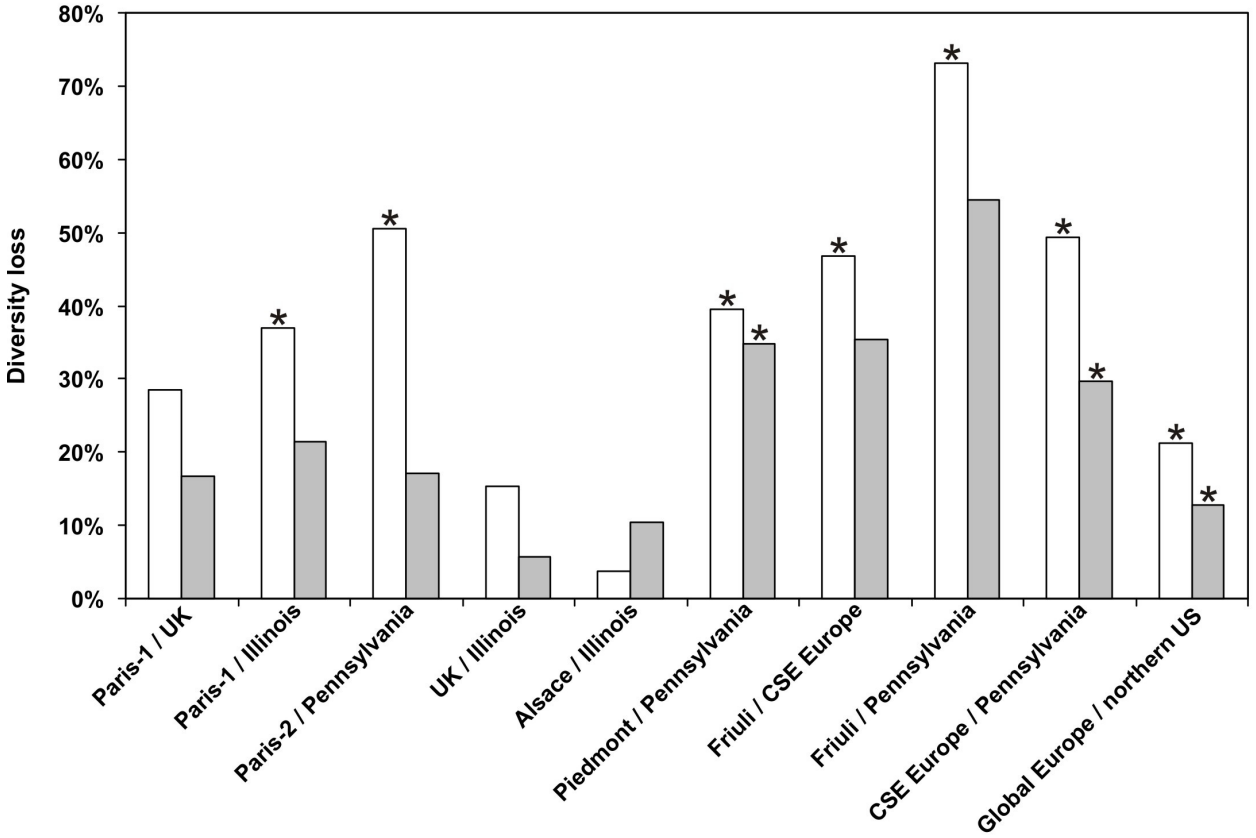

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Figure 3.

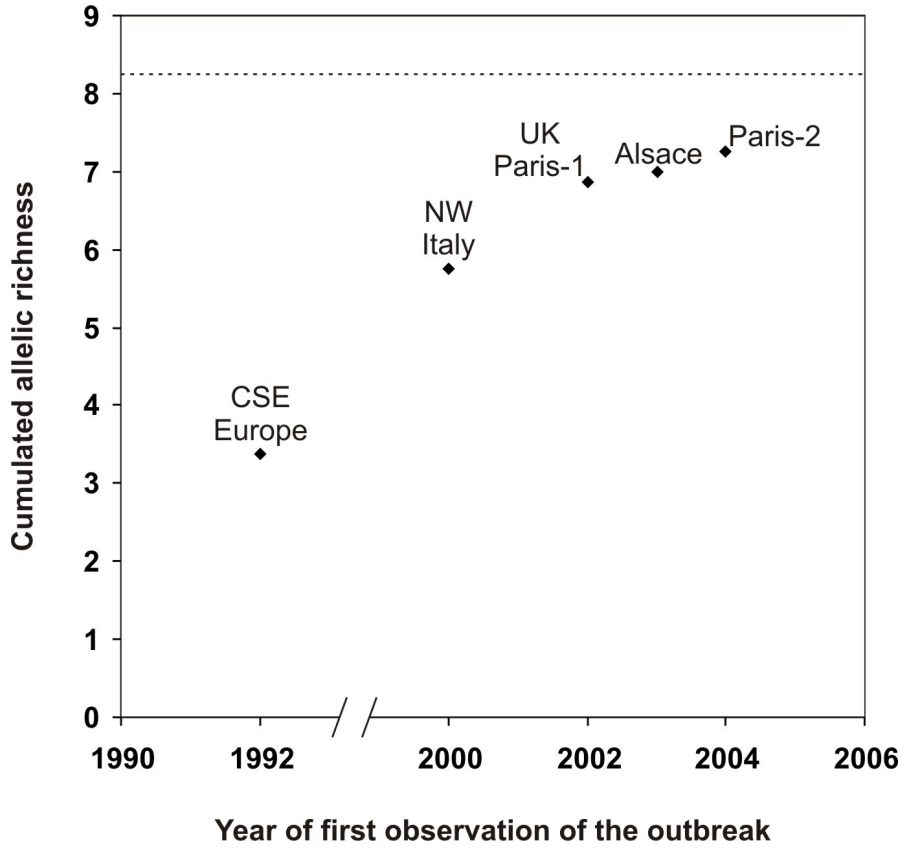
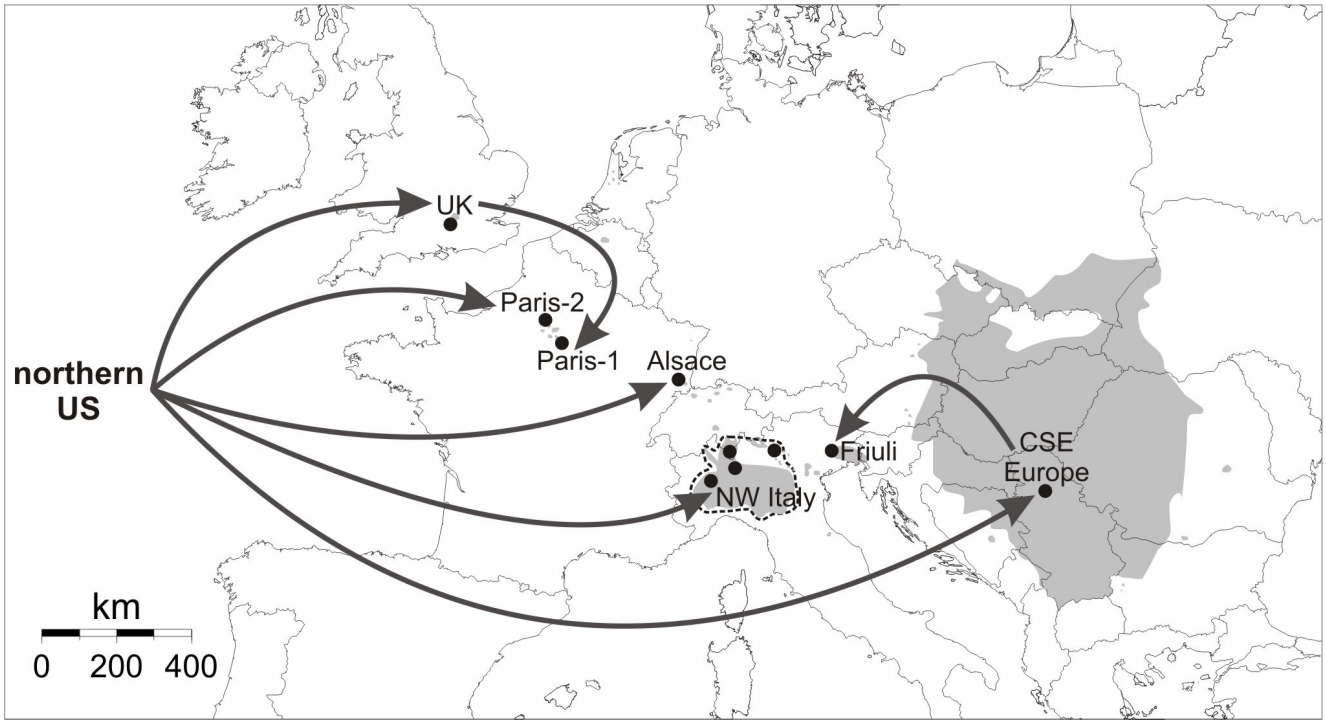

Cumulated allelic richness
9
8
7
6
5
4
3
2
1
0
UK
Paris-1
Alsace
Paris-2
NW
Italy
CSE
Europe
1990
1992
2000
2002
2004
2006
Year of first observation of the outbreak

Figure 4.


UK
Paris-2
Paris-1
Alsace
northern
US
Friuli
CSE
Europe
NW Italy
km
0 200 400

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Supplementary material

Table S: Allele frequency distributions of the WCR samples collected in North America and in Europe. The Lombardy and SW samples were considered as a single population sample (denoted Lombardy-SW), as they displayed no significant genetic differentiation.

		North America					Europe								
	Allele	Mexico	Arizona	Texas	Illinois	Pennsylvania	CSE Europe	Friuli	Trentino	Lombardy-SW	Piedmont	Paris-1	Paris-2	Alsace	UK
Locus DVV-D2															
Gene number		28	76	102	120	124	70	52	86	176	80	38	144	18	68
Allele number		7	11	8	8	9	4	3	2	3	4	6	4	6	9
	177	0.214	0.053	0	0	0	0	0	0	0	0	0.026	0	0.056	0.015
	179	0.143	0.039	0.118	0	0	0	0	0	0	0	0	0	0	0
	181	0.357	0.395	0.225	0.350	0.218	0.214	0.558	0	0	0.100	0.421	0.083	0.611	0.206
	183	0.071	0.382	0.392	0.367	0.548	0.329	0.058	0.802	0.750	0.663	0.316	0.681	0.111	0.412
	185	0.143	0.026	0	0	0	0	0	0	0	0	0	0	0	0
	187	0	0	0.029	0.050	0.024	0.100	0	0	0	0	0	0	0.111	0.147
	189	0.036	0	0	0.017	0.008	0	0	0	0	0	0	0	0.056	0.015
	191	0	0.013	0	0	0	0	0	0	0	0	0	0	0	0
	193	0	0.026	0	0	0	0	0	0	0	0	0	0	0	0
	197	0	0.026	0	0	0	0	0	0	0.006	0	0	0	0	0
	199	0	0.013	0.029	0.033	0.024	0	0	0.198	0.244	0.188	0	0.056	0	0.044
	201	0	0	0.137	0.067	0.097	0.357	0.385	0	0	0	0.026	0.181	0.056	0.029
	203	0	0.013	0.010	0.025	0.048	0	0	0	0	0.050	0.158	0	0	0.088
	205	0	0.013	0.059	0.092	0.024	0	0	0	0	0	0	0	0	0.044
	207	0	0	0	0	0.008	0	0	0	0	0	0.053	0	0	0
	217	0.036	0	0	0	0	0	0	0	0	0	0	0	0	0
Locus DVV-D4															
Gene number		28	76	102	120	124	70	52	84	176	80	38	134	18	66
Allele number		6	8	6	8	7	3	2	3	2	4	4	4	5	7
	219	0.536	0.132	0.098	0.175	0.210	0	0	0.012	0	0.013	0	0.030	0.111	0.152
	223	0	0	0.118	0.125	0.169	0.286	0.288	0	0	0	0.105	0.157	0.056	0.167
	225	0.036	0.197	0.510	0.442	0.452	0.643	0.712	0.750	0.739	0.775	0.342	0.761	0.333	0.394
	227	0.107	0.026	0.059	0.075	0.048	0	0	0	0	0.013	0.289	0	0.111	0.152
	229	0.071	0.026	0	0	0	0	0	0	0	0	0	0	0	0

231	0.143	0.118	0.049	0.100	0.065	0.071	0	0.238	0.261	0.200	0.263	0.052	0.389	0.061
233	0	0.382	0.167	0.042	0.032	0	0	0	0	0	0	0	0	0.061
235	0.107	0.105	0	0.017	0	0	0	0	0	0	0	0	0	0
237	0	0	0	0.025	0.024	0	0	0	0	0	0	0	0	0.015
239	0	0.013	0	0	0	0	0	0	0	0	0	0	0	0

Locus DVV-D5

Gene number	26	80	102	120	124	74	34	82	172	80	38	128	18	72
Allele number	5	4	4	3	2	1	1	1	1	1	2	2	2	2
162	0.038	0	0	0	0	0	0	0	0	0	0	0	0	0
168	0	0	0.039	0	0	0	0	0	0	0	0	0	0	0
170	0.077	0.013	0	0	0	0	0	0	0	0	0	0	0	0
172	0.692	0.863	0.843	0.867	0.790	1.000	1.000	1.000	1.000	1.000	0.632	0.852	0.944	0.889
174	0.154	0.100	0.029	0.025	0	0	0	0	0	0	0	0	0	0
176	0.038	0.025	0.088	0.108	0.210	0	0	0	0	0	0.368	0.148	0.056	0.111

Locus DVV-D8

Gene number	24	80	102	120	124	74	34	84	172	80	38	126	18	72
Allele number	14	18	18	17	17	5	2	7	9	10	5	6	8	12
208	0	0.013	0	0	0	0	0	0	0	0.013	0	0	0	0
212	0.042	0.063	0.029	0.092	0.040	0	0	0	0	0	0	0	0.111	0.056
214	0.125	0.150	0	0.008	0	0	0	0	0	0	0	0.056	0	0
216	0.083	0.088	0.127	0.033	0.145	0	0	0.071	0.128	0.138	0.079	0.302	0.111	0.042
218	0.042	0.050	0.098	0.383	0.234	0.135	0	0.071	0.006	0.150	0.763	0.516	0.278	0.375
220	0	0.150	0.069	0.058	0.065	0	0	0.012	0	0.013	0.053	0	0.167	0.028
222	0.125	0.013	0.088	0.033	0.040	0	0	0.036	0.070	0.013	0	0.016	0.056	0.125
224	0.042	0.038	0.059	0.033	0.024	0	0	0.298	0.297	0.225	0	0.056	0	0.069
226	0	0.038	0.039	0.025	0.024	0	0	0	0	0	0	0	0	0.014
228	0.042	0.100	0.010	0	0	0	0	0	0	0	0	0	0	0
230	0.042	0.113	0.127	0.008	0	0	0	0	0	0	0	0	0	0
232	0.083	0.038	0.039	0.025	0.016	0	0	0	0	0	0	0	0	0
234	0.125	0.025	0.029	0.008	0.016	0	0	0	0	0.013	0	0	0	0
236	0.042	0.025	0.049	0	0.016	0	0	0	0	0	0	0	0	0
238	0.042	0.050	0.020	0.008	0	0	0	0	0	0	0	0	0	0
240	0.125	0	0.069	0.050	0.081	0	0	0.155	0.233	0.175	0	0	0	0.083
242	0.042	0	0.029	0.083	0.056	0.108	0	0	0.006	0	0.053	0	0.056	0.111
244	0	0	0.039	0.083	0.121	0.595	0.706	0	0	0.050	0.053	0	0.167	0.056

246	0	0	0.049	0.017	0.065	0.149	0.294	0	0.006	0	0	0	0.056	0.028
248	0	0	0.029	0.050	0.032	0.014	0	0.357	0.250	0.213	0	0.056	0	0.014
250	0	0.013	0	0	0.016	0	0	0	0.006	0	0	0	0	0
252	0	0.025	0	0	0.008	0	0	0	0	0	0	0	0	0
256	0	0.013	0	0	0	0	0	0	0	0	0	0	0	0
Locus DVV-D9														
Gene number	24	80	102	120	124	70	52	84	172	80	38	144	18	72
Allele number	5	5	6	3	6	2	1	3	2	2	2	3	3	2
128	0	0	0.020	0	0.008	0	0	0	0	0	0	0	0	0
130	0.208	0.063	0	0	0	0	0	0	0	0	0	0	0	0
136	0	0	0.010	0	0	0	0	0	0	0	0	0	0	0
138	0.292	0.313	0.294	0.292	0.250	0.100	0	0.464	0.378	0.250	0.421	0.299	0.111	0.347
140	0.292	0.450	0.569	0.567	0.597	0.900	1.000	0.524	0.622	0.750	0.579	0.438	0.778	0.653
142	0.125	0.138	0.088	0	0.024	0	0	0	0	0	0	0	0	0
150	0.083	0.038	0.020	0.142	0.105	0	0	0.012	0	0	0	0	0.111	0
152	0	0	0	0	0.016	0	0	0	0	0	0	0.264	0	0
Locus DVV-D11														
Gene number	28	76	102	120	124	56	50	84	176	78	38	82	18	66
Allele number	12	14	12	12	12	6	2	4	6	8	6	6	6	8
174	0.107	0.026	0	0	0	0	0	0	0	0	0	0	0	0
176	0.179	0.487	0.353	0.383	0.298	0.339	0	0	0	0.077	0.737	0.280	0.389	0.348
178	0.036	0	0.029	0.017	0.105	0.268	0	0.274	0.381	0.218	0.026	0	0	0.106
180	0.071	0.053	0	0	0	0	0	0	0	0	0	0	0	0
182	0.107	0.092	0.108	0.050	0.065	0	0	0	0	0.064	0.026	0.012	0.056	0.076
184	0.036	0.026	0	0	0	0	0	0	0	0	0	0	0	0
188	0.071	0	0	0	0	0	0	0	0	0	0	0	0	0
190	0	0.013	0	0	0	0	0	0	0	0	0	0	0	0
192	0.071	0	0	0	0	0	0	0	0	0	0	0	0	0
196	0.036	0	0.059	0.083	0.169	0.214	0.560	0.310	0.307	0.333	0.026	0.159	0.056	0.045
198	0.143	0.079	0.118	0.025	0.008	0	0	0	0.006	0.013	0	0.244	0	0
200	0.107	0.053	0.147	0.117	0.073	0	0	0.226	0.148	0.115	0.132	0	0.222	0.136
202	0	0.026	0.078	0.108	0.048	0	0	0	0.011	0.064	0.053	0	0.222	0.167
204	0.036	0	0.010	0.008	0.008	0	0	0	0	0	0	0	0.056	0
206	0	0.013	0.029	0.158	0.194	0.125	0.440	0.190	0.148	0.115	0	0.280	0	0.091
208	0	0	0	0.017	0.008	0.018	0	0	0	0	0	0	0	0

	210	0	0.039	0	0	0.008	0	0	0	0	0	0	0	0
	212	0	0.039	0.039	0.017	0	0.036	0	0	0	0	0	0.024	0	0.030
	214	0	0	0.020	0.017	0.016	0	0	0	0	0	0	0	0	0
	216	0	0	0.010	0	0	0	0	0	0	0	0	0	0	0
	228	0	0.039	0	0	0	0	0	0	0	0	0	0	0	0
	232	0	0.013	0	0	0	0	0	0	0	0	0	0	0	0
Locus DVV-T2															
Gene number		28	76	102	120	124	70	52	86	176	80	38	144	18	68
Allele number		5	6	6	3	3	2	1	1	1	3	2	2	3	3
	204	0.214	0.053	0.088	0	0	0	0	0	0	0	0	0	0	0
	210	0	0.132	0.245	0.317	0.298	0.100	0	0	0	0.075	0.447	0.313	0.167	0.206
	213	0.036	0	0	0	0	0	0	0	0	0	0	0	0	0
	216	0.036	0.013	0.010	0	0	0	0	0	0	0	0	0	0	0
	219	0.143	0.145	0.078	0.150	0.089	0	0	0	0	0.100	0	0	0.111	0.176
	222	0.571	0.592	0.569	0.533	0.613	0.900	1.000	1.000	1.000	0.825	0.553	0.688	0.722	0.618
	225	0	0.066	0	0	0	0	0	0	0	0	0	0	0	0
	240	0	0	0.010	0	0	0	0	0	0	0	0	0	0	0
Locus DVV-ET1															
Gene number		22	80	102	120	124	64	32	82	172	80	38	128	18	72
Allele number		4	6	5	4	4	4	2	2	2	2	3	3	4	3
	160	0	0.300	0.422	0.450	0.540	0.234	0	0.915	0.983	0.925	0.842	0.422	0.556	0.653
	163	0.364	0.250	0.284	0.283	0.202	0.234	0	0.085	0.017	0.075	0.132	0.164	0.111	0.250
	166	0.318	0.075	0.147	0.192	0.194	0.484	0.688	0	0	0	0.026	0.414	0.278	0.097
	169	0.273	0.300	0.127	0.075	0.065	0.047	0.313	0	0	0	0	0	0.056	0
	172	0	0.050	0.020	0	0	0	0	0	0	0	0	0	0	0
	178	0.045	0.025	0	0	0	0	0	0	0	0	0	0	0	0

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