Heredity (2017) 118, 330–339

www.nature.com/hdy

ORIGINAL ARTICLE

Genetic hitchhiking and resistance evolution to transgenic *Bt* toxins: insights from the African stalk borer *Busseola fusca* (Noctuidae)

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Since transgenic crops expressing *Bacillus thuringiensis* (*Bt*) toxins were first released, resistance evolution leading to failure in control of pests populations has been observed in a number of species. Field resistance of the moth *Busseola fusca* was acknowledged 8 years after *Bt* maize was introduced in South Africa. Since then, field resistance of this corn borer has been observed at several locations, raising questions about the nature, distribution and dynamics of the resistance trait. Using genetic markers, our study identified four outlier loci clearly associated with resistance. In addition, genetic structure at neutral loci reflected extensive gene flow among populations. A realistically parameterised model suggests that resistance could travel in space at speed of several kilometres a year. Markers at outlier loci delineated a geographic region associated with resistance spread. This was an area of approximately 100 km radius, including the location where resistance was first reported. Controlled crosses corroborated these findings and showed significant differences of progeny survival on *Bt* plants depending on the origin of the resistant parent. Last, our study suggests diverse resistance mutations, which would explain the widespread occurrence of resistant larvae in *Bt* fields across the main area of maize production in South Africa.
Heredity (2017) **118,** 330–339; doi:10.1038/hdy.2016.104; published online 26 October 2016

INTRODUCTION

Adaptation to human driven environmental pressures present insightful illustrations of the various facets of contemporary evolution and their implications. The evolution of resistance, the process by which a population of organisms acquires a genetically based decrease of susceptibility to a toxic compound (Tabashnik *et al.*, 2009), may result in spectacularly fast responses to strong selective pressures (e.g., Mallet, 1989). Genetically modified crops, expressing *Bacillus thuringiensis* (*Bt*) toxins, are expected to result in elevated and continuous selection whenever *Bt* crops dominate the landscape (Tabashnik *et al.*, 2013).

Evolution of resistance to *Bt* toxins expressed by crops is a direct threat to sustained control of pest populations, which may seriously impact agricultural yields. Even if a history of 10 – 20 years has revealed the first empirical trends (Tabashnik *et al.*, 2013), the questions of how fast resistance to *Bt* crops toxins is likely to evolve and how quickly it may spread remain challenging. While *Bt* crops have been deployed for almost two decades, loss of control in pest populations, due to field-evolved resistance, arose in a few pest species only. However, decrease of susceptibility to transgenic *Bt* toxins (field-evolved resistance), which has not led to control failure, has been observed in many species (Tabashnik *et al.*, 2013). Empirical knowledge about the spatial dynamics of resistance spread is scarce. Extensive gene flow is commonly observed in pest populations (e.g., Daly and Gregg, 1985; Korman *et al.* 1993; Bourguet *et al.*, 2000; Endersby *et al.*, 2007; Kim *et al.*, 2009) and may promote rapid diffusion of resistance alleles across space (e.g., Peck *et al.*, 1999; Bourguet *et al.*, 2000).

The main option for delaying resistance evolution is the refuge strategy, which amounts to planting non-*Bt* plants within or surrounding Bt fields, allowing the survival of some susceptible larvae in a *Bt*-dominated environment. The proportion of refuge crop required to effectively delay resistance evolution is strongly dependent on the recessiveness of the resistance trait and, more generally, on the survival of heterozygote-resistant individuals at the dose of toxin expressed in plant tissues (e.g., see Vacher *et al.*, 2003; Vacher *et al.*, 2004).

The nature and uniformity of the genetic bases of resistance to *Bt* toxins are thus crucial aspects influencing resistance evolution and management (e.g., Tabashnik *et al.*, 1998). Multiple recessive mutations that cause resistance to *Cry*1A toxins in Lepidoptera have been reported (see Heckel *et al.*, 2007) in genes encoding Cadherin-like proteins (e.g., Morin *et al.*, 2003) and ABC transporters (e.g., Baxter *et al.*, 2011). In the field, diverse alleles conferring resistance were identified within Chinese populations of *Helicoverpa armigera* (Zhang *et al.*, 2012), including recessive resistance alleles at the Cadherin locus

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Received 29 January 2016; revised 29 July 2016; accepted 8 September 2016; published online 26 October 2016

as well as two non-recessive mutations, one of which was not linked to this locus.

Strong selection acting at a particular resistance locus is expected to affect not only the frequency of the selected mutation but also flanking polymorphisms. Alleles present on the ancestral chromosome on which a favourable mutation arose will tend to increase in frequency as the mutation invades the population, a consequence, which is known as the genetic hitchhiking effect (Kojima and Schaffer, 1967; Maynard-Smith and Haigh, 1974). Selective shifts may therefore give rise to strong linkage disequilibrium, i.e., some non-random allelic associations at the population level, between loci of the genomic region under selection.

Various population genetics approaches have aimed at taking advantage of such phenomena to capture signatures from positive selection (Jensen, 2014), notably in non-model organisms (e.g., de Villemereuil and Gaggiotti, 2015). The size of a hitchhiking effect depends on several factors, including: age and frequency of the mutation under selection; the intensity of selection; and recombination distance to the locus under selection. In particular, the initial frequency of the selected allele in the population before a selective shift, as well as the age of a mutation, has a crucial role (Maynard-Smith and Haigh, 1974; Jensen, 2014). As a consequence, the effectiveness of genome scans is lowered when e.g.: populations have undergone a particular demographic history (e.g., bottleneck); a recessive allele is involved; or selection acts on an ancient and previously neutral allele (Teshima *et al.*, 2006; Jensen, 2014).

In South Africa, the pest *Busseola fusca* (Lepidoptera, Noctuidae) evolved resistance to the *Bt* toxin *Cry*1Ab expressed by transgenic maize within 8 years of the technology being released (Van Rensburg, 2007). Although this pest species is seldom hosted by wild grasses (Le Rü et al., 2006), it is responsible for substantial yield losses in maize, sorghum (Kfir *et al.*, 2002) and more marginally sugarcane crops (Assefa *et al.*, 2015). Being endemic to Sub-Saharan Africa and widely distributed throughout the region (Dupas *et al.*, 2014), *B. fusca* is considered a pest of economic importance. To date, field resistance in *B. fusca* remains one of the few cases in the world where *Bt* crop failure has clearly been documented (see Tabashnik *et al.*, 2013). Resistance evolution in this species was initially ascribed to an insufficient implementation of the refuge strategy (Kruger *et al.*, 2012; Van den Berg *et al.*, 2013). Nonetheless, the existence of a dominant resistance trait, such as found in this pest (Campagne *et al.*, 2013), is expected to have greatly accelerated the spread of resistance and to have drastically reduced refuge efficiency. Furthermore, the presumed low survival of resistant individuals on non-*Bt* plants is unlikely to have mitigated the fitness benefits conferred by the resistance trait since no fitness cost associated with resistance was found in *B. fusca* (Kruger *et al.*, 2014).

Four years after being first recorded in the field in 2006, resistance in *B. fusca* was observed at a distance of ~40 km from the location reported initially (Kruger *et al.*, 2011). By 2011, *Bt* maize fields infested by *B. fusca* were observed in various places up to ~400 km distance from the initial location (see van den Berg *et al.*, 2013). The latter sequence of observations raises a number of puzzling questions regarding both the initial distribution and spatial dynamics of resistance traits to *Bt*-maize in this species. Although gene flow may either swamp local resistance evolution or contribute to the spread of resistance alleles (Bourguet *et al.*, 2000), little is known about the levels of migration and gene flow taking place in populations of *B. fusca* at sub-regional scales.

On the basis of empirical genetic data collected in the field, controlled crosses, and theoretical considerations, the purpose of this study was to investigate dynamics and diversity of resistance evolution in field populations of *B. fusca*. Our aims were to: (i) to evaluate the extent of gene flow in this pest within and between sites distributed across the major region of maize cultivation in South Africa; (ii) to identify markers associated with resistance and further investigate spatial distribution of resistance traits; and (iii) to explore the geographic variability of resistance inheritance in *B. fusca* within the study region.

MATERIALS AND METHODS

Study context and sampling

The first official observation of field resistance in *B. fusca* was recorded in the Christiana area (Van Rensburg, 2007; Kruger *et al.*, 2011) which is located at the western extremity of the main maize production area (Van den Berg *et al.*, 2013). Available data indicate, between 1998/1999 and 2010/2011, an average proportion of 60% *Bt* maize in the area where resistance was first reported (Kruger *et al.*, 2012). The landscape is composed of 20% crops (the main one being maize) while most of the matrix consists of semi-natural or natural habitats (source: FAO GeoNetwork, www.fao.org). In this area, populations of *B. fusca* are bivoltine with a diapause stage between cropping seasons. Sampling was performed during the growing season 2010/2011, i.e., $\sim k \approx 26$ generations after *Bt* maize was released, across the main region of maize production in South Africa (stretching across more than 600 km East-West).

To carry out population genetics analyses, 20–25 larvae per field were sampled in a total of 28 fields (14 non-*Bt* fields and 14 *Bt* fields, Table 1). Sampling was performed within maize fields at advanced stage of development (late vegetative stage or higher), and consisted in five collection points distributed along a line, separated by ~50 m. At each point, we collected five larvae originating from five different plants separated by a few metres from each other. Care was taken to sample larvae in their fourth instar at least, in order to minimise the number of susceptible larvae collected in *Bt* fields. Larvae originating from different plants were conserved separately in 90% ethanol. Finally, a crude assessment of field damage level was performed in most sampled fields by recording the number of plants with leaf damage in three separate rows chosen at random (at a distance > 20 m from each other, depending on an arbitrary number of steps) of 100 plants each (see Table 1). In order to perform controlled crosses, an additional number of larvae was collected in *Bt* fields at sites where resistance had been reported.

Genetic markers

Total DNA isolation of 20 mg of larvae thoraces was done using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was determined using a Nanodrop (Thermo Scientific, Wilmington, DE, USA). The amplified fragment length polymorphism (AFLP) reaction was performed as described by Vos *et al.* (1995) with slight modifications. A quantity of 300 ng of DNA was digested using the following restriction enzymes: EcoRI and PstI. Digestion was made overnight at 37 °C with five units of EcoRI (New England Biolabs, Ipswich, MA, USA), and five units of PstI (New England Biolabs) in a total volume of 25 µl. Ligation of double-stranded EcoRI and PstI adaptors to the ends of the restriction fragments was performed (for 8 h at 15 °C) by adding 10 pmol of EcoRI and PstI adaptors, five units T4 DNA ligase (Promega, Madison, WI, USA). A 5 µl volume of 10× diluted ligation products were used as a template for pre-amplification, using 10 pmol of EcoRI and MseI primers, 0.4 mM dNTPs, 1.5 mM $MgCl_2$ and 1 unit of GoTaq Flexi DNA Polymerase (Promega) in a final volume of 25 µl. The pre-amplification thermocycle profile was: 94 °C for 5 min, followed by 20 cycles at 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min and 72 °C for 5 min. Selective amplification steps were performed using 5 pmol EcoRI (+NN) and PstI (+NN) primers with 2.5 µl of 20× diluted preamplification product in a final volume of 12.5 µl. Each selective amplification reaction mixture contained 0.4 mM dNTPs, 1.5 mM $MgCl^2$ and 0.5 unit of GoTaq Flexi DNA Polymerase (Promega). The selective amplification thermocycler profile was: 94 °C for 2 min, followed by 12 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, followed by 23 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and 72 °C for 5 min. The different primers combinations used were: EcoRI+AA/PstI+AG, EcoRI+AG/PstI+AG,

EcoRI+GA/PstI+AT, EcoRI+AG/PstI+AT, EcoRI+GA/PstI+AG, EcoRI+CA/PstI+AA, EcoRI+CT/PstI+AT, EcoRI+TG/PstI+GA and EcoRI+GG/PstI+AC. AFLP markers were scored according to the absence/presence of peaks using Genemapper V4.1 (Invitrogen/ThermoScientific, Wilmington, DE, USA). To optimise the reliability of the markers, 65 individuals were processed twice. Loci characterised by more than three discrepancies (5%) were considered prone to genotyping errors and were discarded. A total of 629 polymorphic markers were retained for further statistical analysis. The overall genotyping error rate per locus (see Bonin *et al.* 2004), calculated as the ratio between observed number of differences and total number of comparisons, was 1.3%.

Data analysis

The inbreeding coefficient F_{IS} was estimated at both population and individual levels, for each sampled field using the software I4A, which is designed to deal with dominant genetic markers (Chybicki *et al.*, 2011). After testing the convergence of different run lengths, data were analysed using 10 000 burn-in and 50 000 sampling iterations considering flat priors for the inbreeding coefficients. Estimates were provided with 95%-credibility intervals.

Rapid resistance evolution is expected to have left specific signatures in the genome of *B. fusca* as directional selection imposed by *Bt* toxins may result in increased differentiation between populations at hitchhiking loci. Using Bayescan (see Foll and Gaggiotti, 2008), we scanned the genome of this pest for loci at which genetic differentiation between populations (F_{ST}) is significantly higher than the genome baseline (outlier loci). Two sets of markers were defined and analysed separately: non-outlier loci, expected to reflect neutral processes such as genetic drift and gene flow between populations; and outlier loci, further tested for being associated with resistance.

To analyse the set of non-outlier loci, Euclidean genetic distances between individuals were computed (Peakall and Smouse, 2006) and genetic differentiation (Φ_{ST}) among fields was estimated with an analysis of molecular variance (Excoffier *et al.*, 1992; Peakall, Smouse 2006). Statistical significance was tested using 999 permutations. A between-field analysis was performed on Principal Coordinate Analysis (PCO) axes (ade4 package, R Core Team, 2012). In addition, pairwise kinship coefficients (F_{ij}) were estimated using the software SPAGeDi (Hardy and Vekemans, 2002). Estimation of kinship coefficients based on dominant markers requires an externally supplied estimate of the inbreeding coefficient. As the vast majority of individuals did not exhibit evidence of inbreeding (see Results section) we used $F=0$. Average kinship was then calculated within and between fields. The genetic neighbourhood size (N_b*), a measure of the local extent of breeding, was estimated following Hardy's (2003) procedure: $N_b^* \approx (F_0-1)/b$, where F_0 is the average kinship for adjacent individuals (within a field) and b is the slope of the linear decay between kinship and the natural logarithm of distance. Pairs of geographic distances higher than 100 km were excluded (see Hardy, 2003) and a confidence interval was calculated using 999 bootstrap iterations. N_b^* is an empirical estimate of the theoretical demographic parameter $N_b=4\pi\sigma^2\rho_e$, where ρ_e denotes the effective population density (e.g., see Rousset 1997; Hardy, 2003) and σ^2 (km^2 per generation) is the axial variance of an isotropic Gaussian dispersal distribution ($2\sigma^2$ being the mean squared parent–offspring-displacement).

Association between field resistance and the genetic structure at outlier loci was then investigated. Analyses of population clustering were performed exclusively on individuals which were sampled in *Bt* fields. Ward's method for hierarchical clustering based on pairwise genetic distances (Peakall and Smouse, 2006) and the software STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) were

Table 1 General characteristics of the 28 fields sampled: IL is the infestation level (%); *n*, the sample size; *F* the inbreeding coefficient in a given field and its 95% estimation interval ($IC_{95\%}$)

Field ID	*Longitude*	*Latitude*	*Altitude (m)*	*Crop*	*IL (%)*	*n*	*F*	$IC_{95\%}$
3	24.76	−27.75	1096	Corn[a]	15	25	0.011	(0.000–0.053)
5	24.70	−29.10	1130	Corn[a]	5	25	0.007	(0.000–0.037)
7	24.72	−29.29	1100	Corn[a]	5	25	0.010	(0.000–0.047)
43	25.96	−28.04	1136	Corn[a]	10	25	0.011	(0.000–0.049)
65	24.67	−28.05	1149	Corn[a]	>30	25	0.026	(0.002–0.081)
2	24.85	−27.93	1150	Corn	15	25	0.011	(0.001–0.082)
4	24.79	−27.71	1130	Corn	5	25	0.009	(0.000–0.042)
6	24.74	−29.17	1100	Corn	na	25	0.009	(0.000–0.041)
11	26.79	−27.41	1136	Corn	5	25	0.013	(0.005–0.111)
89	28.82	−25.72	1473	Corn	5	25	0.009	(0.000–0.043)
1	25.47	−27.68	1213	Sorghum	>30	24	0.031	(0.002–0.098)
8	26.23	−28.11	1290	Corn[a]	5	20	0.010	(0.000–0.047)
10	27.09	−26.69	1356	Corn	na	25	0.044	(0.007–0.115)
12	28.55	−27.94	1754	Corn[a]	>30	25	0.058	(0.012–0.136)
13	28.24	−27.79	1615	Corn[a]	15	25	0.028	(0.001–0.093)
14	28.60	−27.75	1670	Corn[a]	5	25	0.030	(0.001–0.093)
15	28.08	−27.46	1638	Corn[a]	5	25	0.024	(0.001–0.081)
17	27.70	−26.70	1461	Corn	5	25	0.008	(0.000–0.038)
24	29.66	−26.79	1660	Corn	5	25	0.019	(0.000–0.073)
40	26.12	−26.22	1454	Corn	10	23	0.023	(0.001–0.087)
41	25.51	−27.23	1363	Corn[a]	>30	25	0.008	(0.000–0.038)
42	26.54	−27.77	1288	Corn	15	24	0.009	(0.000–0.043)
51	28.08	−28.10	1623	Corn[a]	>30	25	0.037	(0.002–0.106)
54	26.77	−26.22	1520	Corn[a]	5	25	0.021	(0.001–0.070)
57	26.59	−25.96	1551	Corn	25	25	0.011	(0.000–0.052)
64	26.94	−26.14	1529	Corn	25	24	0.012	(0.001–0.057)
71	26.01	−26.39	1427	Corn	5	24	0.034	(0.003–0.099)
75	25.63	−26.80	1387	Corn[a]	15	24	0.010	(0.000–0.049)

na, fields for which information is not available.
[a]*Bt* field.

used to identify genetic clusters. The latter analysis was executed under the admixture model and correlated allele frequencies, with 10 000 burn-in iterations and 50 000 main iterations. The optimal number of genetic clusters (K) was determined following the procedure described in Evanno *et al.* (2005), with five replicates for each K-value. For the sake of comparison, the same analysis was performed using the set of non-outlier loci.

The difference in marker frequency at outlier loci between *Bt* and non-*Bt* fields was tested using generalised linear mixed models (Venables and Ripley, 2002) with spatially correlated random effects, assuming a binomial distribution. Crop type (i.e., *Bt* vs. non-*Bt*) was treated as a fixed effect and the geographic coordinates of the fields were considered as random components of the analysis. Correlations between marker frequencies at outlier loci were evaluated with Pearson's correlation coefficient (R Core Team, 2012). Linkage disequilibrium between outlier loci was tested following Hill's procedure (Hill, 1974), which provides a parametrically explicit maximum-likelihood computation method for dominant markers. Both a standardised estimate of linkage disequilibrium (r) and its associated $\log_{10}$-likelihood ratio (LR) were calculated.

Controlled crosses

Heritability of resistance traits provides insights into the genetic bases of resistance. To explore the geographic variations in larvae survival on *Bt* maize, we performed single-pair crosses between resistant parents and a strain of *a priori* susceptible larvae originating from Tanzania (Tz), where no *Bt* crops are grown. Three sites, where resistance had been reported, were selected: Valhaarts (Va), an irrigation scheme close (~50 km) to the location, where resistance was first reported and in which resistance has been previously studied (Kruger *et al.*, 2011); Lichtenburg (Li) at 180–200 km distance from the initial location; and Bethleem (Be), at ~300 km from the initial location (see also Figure 1). Controlled crosses were performed between adults originating from *Bt* fields and susceptible individuals: Va × Tz; Li × Tz; and Be × Tz. Progeny survival was assessed in a greenhouse on *Bt* and non-*Bt* maize planted in pots. Maize plants were inoculated with 20–30 neonates, which were gently transferred with a paintbrush within the whorl of young maize plants (i.e., from the 5th leaf collar stage). Plants were contained in fine-meshed nets to avoid larvae escaping. The number of individuals surviving on a plant was recorded after a period of 21 days. Preliminary observations indicate that survival after 2 weeks on *Bt* maize of individuals originating from Tanzania, is typically $\leqslant 2\%$. A fraction of the offspring were reared on non-*Bt* maize as a control. About 1700 larvae were used in the experiment. Survival of the progeny on maize was compared using Fisher's exact tests (R Core Team, 2012). Contingent upon emergence of F1 adults and various experimental constraints, sib-crosses were undertaken in order to test whether homozygosity could reinforce resistance in F2 offspring of selected parents. One sib-cross was successfully performed among individuals originating from the Li × Tz cross and which survived on *Bt* maize.

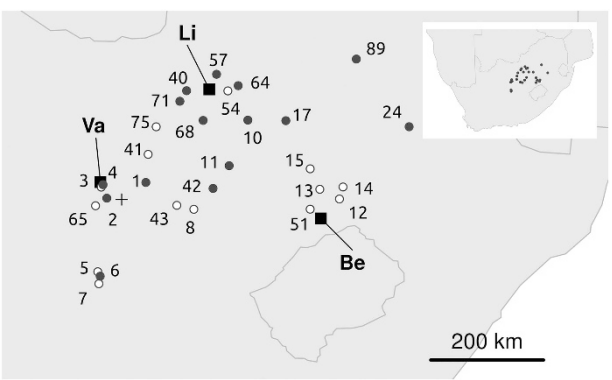

Figure 1 Map of the South African localities where larvae of *Busseola fusca* were collected. Empty dots represent *Bt* fields; dark-grey dots, non-*Bt* field; square dots, locations sampled for the controlled crosses (Va, Be and Li). Location where resistance was initially reported is represented by a cross. Numbers are the field IDs as displayed in Table 1.

Theoretical resistance spread

One approach for understanding the geographic distribution of resistance is to model the spatial spread of resistance alleles. In our survey, larvae were found to survive on *Bt* maize at various locations which also correspond to sites where resistance was reported (Van den Berg *et al.,* 2014), translating into a vast expanse of approximately 200 km × 300 km. Assuming relatively simple genetic bases of resistance, the most elementary scenarios consistent with these observations could include: (i) a rapid propagation of a unique mutation conferring resistance from an initial location; or (ii) ancient and widespread resistance mutation(s), before the selective shift. We explored the feasibility of a dominant mutation spreading across space, from an initially confined distribution, under the selective pressure of *Bt* maize. Resistance was considered to involve a single locus, with a resistance allele (R) of frequency q, and a susceptible allele (S) of frequency $1-q$. For modelling purposes, the spatial distribution of *Bt* and non-*Bt* plants is considered continuous and random. The proportion of *Bt* crop in the landscape (ω) is assumed to determine the survival probability (e.g., see Tabashnik *et al.*, 2008; Jin *et al.*, 2015) of the three genotypes (RR, RS and SS): $W_{SS}=1-\omega$, $W_{RS}=(1-\omega)+h\times\omega$ and $W_{RR}=1$, where h denotes the dominance of the resistance allele. Spatial spread of a resistance allele under selection was considered under a demogenetic system of deterministic reaction-diffusion equations proposed by Tyutyunov *et al.* (2008) and slightly modified to our purpose (see Supplementary Appendix S1). The reaction term of the model accounts for local increase of resistance allele frequency, in relation to ω, h, W_{SS}, W_{RR}, q and the birth rate β per individual and per generation. The diffusion term captures the effect of individual dispersal under diffusive Brownian motion (diffusion constant D), scaling to $2\sigma^2=4D$ after one generation.

RESULTS

Genetic structure at non-outlier loci

The five primer combinations yielded 629 polymorphic and reproducible fragments in *B. fusca*, for the entire set of 693 individuals. All individuals had different AFLP profiles, differentiated by at least eight markers. Estimates of population inbreeding coefficient were low, ranging between 0.007 and 0.058 in the 28 sampled fields (Table 1), indicating no strong heterozygote deficiency. Similarly, estimates of median individual inbreeding were <0.01 in ~97% of individuals. The outlier analysis carried out with all 28 fields detected 26 loci characterised by $F_{ST}>0.07$ (logarithm of the posterior odds, $\log_{10} PO>1$) while the non-outliers were characterised by a lower differentiation $F_{ST}=0.027$ (s.d. $=0.008$). On this basis, loci were split into two groups: 'outlier' and 'non-outlier' loci and were further analyzed separately.

Evidence for extensive gene flow between populations was found when investigating the genetic structure of non-outlier loci. Overall genetic differentiation among fields was low but non-null ($\Phi_{ST}=0.03$, $P<0.001$). Even at long distances (>400 km), average pairwise genetic differentiation between sampled fields was weak ($\Phi_{ST}\approx 0.04$). In spite of a lack of genetic differentiation at broad spatial scales, a smooth pattern of isolation by distance was found. Firstly, pairwise Φ_{ST} were associated with geographic distances ($R=0.47$; $P<0.001$). Similarly, kinship coefficients were significantly correlated with log-transformed distances ($R=0.52$; $P<0.001$; Figure 2a). Parameters of the latter regression yielded the estimate for the neighbourhood size, $N_b{}^*\approx(F_0-1)/b=706$ with a 95% bootstrap interval (526, 1010), which translates into $\rho_e.\sigma^2\approx 55$ (i.e., with $N_b{}^*\approx 4\pi\sigma^2\rho_e$), which is well above the theoretical threshold of $\rho_e.\sigma^2>1$ at which a population distributed in a continuous space behaves nearly as a panmictic population (see Maruyama, 1972). Secondly, a between-class (i.e., fields) PCO (Figure 2b) revealed congruent mapping between B-PCO

axes and the geographical origin of the field-collected populations (see Figure 1 for the locations); e.g., clusters of fields (5, 6, 7), (2, 3, 4) and (54, 64, 68, 71, 75). The small amount of variation captured by the analysis (6.7% of the total inertia; 1.7% onto axes 1 and 2) consistently reflected low levels of genetic structure.

Outlier loci and resistance

The genetic structure of *Bt* fields at outlier loci revealed two spatially segregated groups of *Bt* fields, henceforth A_1 and A_2 (Figure 3). The optimal number of clusters obtained using a Bayesian clustering method (STRUCTURE) was $K=2$ in both sets of loci; higher values of K did not result in better fits of the model (see Supplementary Appendix S2). Although essentially no genetic structure appeared in non-outliers, a clustering based on outlier loci brought to light a clearer pattern which was further supported by the Ward's clustering method (Figure 3). One cluster was composed of five *Bt* fields (3, 5, 7, 43 and 65) which were spatially aggregated (area A_1, Figure 1) in the surroundings of the location where resistance was first reported. While the extent of A_1 could be sketched as an area of ~100 km radius, A_2 was defined as an outgroup which included the remaining nine *Bt* fields.

A second round of outlier analysis (using Bayescan) was performed within each group A_1 and A_2, including the four closest non-*Bt* fields (1, 2, 4 and 6) in A_1 and the remaining ones in A_2. These additional analyses did not yield outliers which were not detected previously

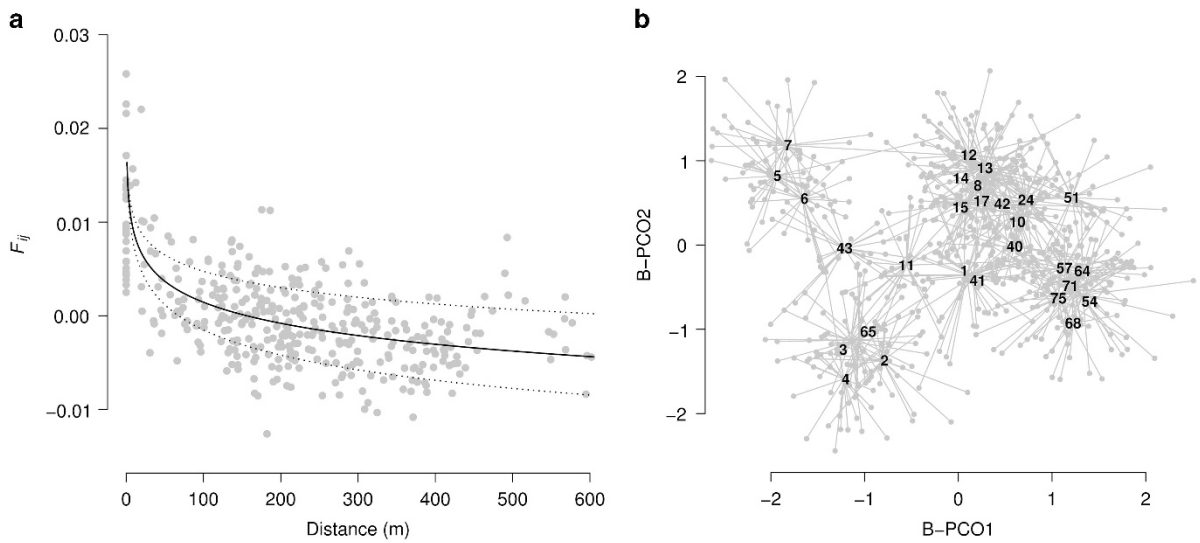

Figure 2 Isolation by distance in *B. fusca* populations (non-outlier loci). (**a**) Pairwise kinship coefficients (F_{ij}) as a function of distance (km). The curve corresponds to the regression of kinship as a function of log-transformed distance; dotted lines represent the 95% confidence interval of the regression slope. (**b**) Between-field analysis performed on Principal Coordinate (PCO) axes. Barycentre of each field sampled is denoted by its ID (number); individuals collected in the different fields correspond to grey dots; lines relate each individual to its field of origin.

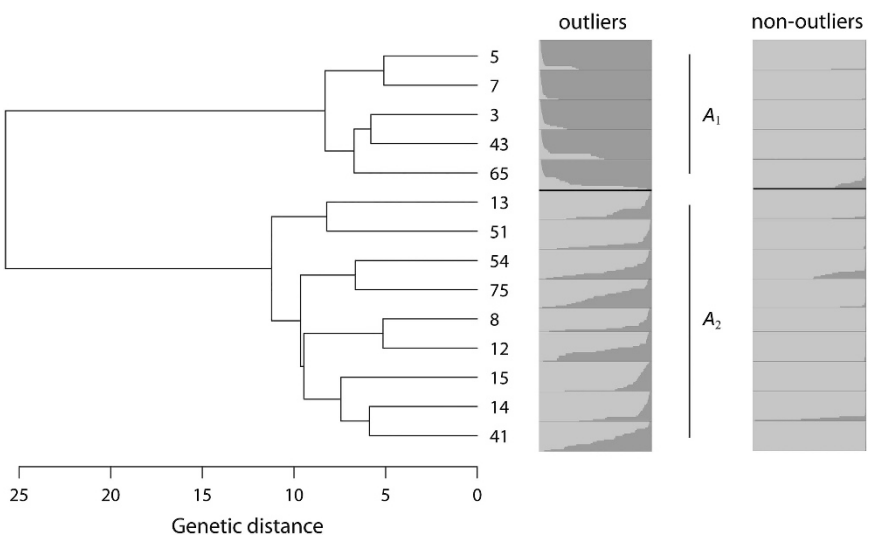

Figure 3 Comparison of population structures between both the outliers and non-outlier loci, for individuals collected in Bt fields. The dendrogram (left) was obtained based on Euclidean genetic distances obtained with the data set of outlier loci. Two genetic groups are distinguished: A_1 and A_2. Comparative STRUCTURE plots (right) of the 14 *Bt* fields using outlier loci and non-outlier loci. The number of clusters in each analysis was set at $K=2$. The length of individual bars corresponds to the probability of membership to a cluster. Numbers identify the *Bt* field sampled.

Table 2 Overview of the outlier scan.

Area			A_1			A_2		
Locus	F_{ST}	*log(PO)*	f_{Bt}	f_{nBt}	P	f_{Bt}	f_{nBt}	P
73	0.10	>1000	—	—	—	—	—	—
81	0.14	>1000	0.91	0.66	0.254	0.72	0.75	0.974
110	0.20	>1000	0.56	0.29	0.597	0.11	0.18	0.147
184	0.08	1.6	—	—	—	—	—	—
187	0.07	1	0.98	0.87	0.231	—	—	—
192	0.12	>1000	0.71	0.99	0.005**	—	—	—
204	0.14	>1000	0.67	0.97	0.002**	—	—	—
208	0.07	1	—	—	—	—	—	—
231	0.08	1.3	—	—	—	—	—	—
299	0.08	1.3	0.66	0.93	0.004**	—	—	—
306	0.08	1.2	—	—	—	—	—	—
331	0.14	>1000	—	—	—	0.80	0.83	0.639
369	0.15	>1000	0.98	0.68	0.002**	0.81	0.82	0.677
390	0.10	2.5	—	—	—	0.63	0.69	0.602
392	0.08	1.4	—	—	—	—	—	—
492	0.13	>1000	0.01	0.02	0.675	0.09	0.19	0.057
572	0.10	>1000	—	—	—	—	—	—
596	0.12	>1000	—	—	—	0.96	0.92	0.110
615	0.15	>1000	—	—	—	0.78	0.80	0.844
617	0.16	>1000	0.75	0.48	0.319	—	—	—
631	0.08	1.6	—	—	—	—	—	—
738	0.16	>10	0.97	0.81	0.700	0.88	0.95	0.176
759	0.11	2.4	—	—	—	—	—	—
782	0.12	>1000	—	—	—	0.90	0.82	0.421
818	0.11	>1000	—	—	—	—	—	—
821	0.09	1.3	—	—	—	0.77	0.82	0.534

The three first columns display outlier loci that were detected while performing an overall analysis. In each geographical region, A_1 and A_2, comparisons of marker frequency between *Bt* fields (f_{Bt}) and non-*Bt* fields (f_{nBt}) were carried at various outlier loci. The *P*-values refers to a mixed generalised linear model (binomial family) with spatially correlated random effects. —, Locus detected as an outlier in the overall analysis but no longer significant when analysing A_1 and A_2 separately. Note that the absence of contrasts between *Bt* and non-*Bt* fields was verified at all outlier loci within each zone but are not displayed here for the sake of simplicity. Significance level: $^{**}P<0.01$.

(Table 2). However, some outliers returned in the first round were not detected as significant ($\log_{10} PO<1$) in the second round, presumably due to a reduction in statistical power.

The association of marker frequency at outlier loci with *Bt* and non-*Bt* fields was then examined. Mixed generalised linear model with spatially correlated random effects showed significant differences in frequency at four outlier markers (L192, L204, L299 and L369) in A_1 ($P<0.005$, Table 2) whereas no significant association was found in A_2.

These four loci were therefore considered strong candidates associated with resistance to *Bt* toxins. Spatial distribution of these putative hitchhikers within A_1 was highly consistent (Figure 4): marker frequency in the *Bt* fields was either systematically lower (L192, L204 and 299) or systematically higher (L369) than in the non-*Bt* fields. It is worth noticing that these contrasts were stronger in the two most Southern *Bt* fields of A_1 (5 and 7), suggesting a higher resistance allele frequency in these fields. Overall, allelic frequencies at the four outlier markers (in the 28 fields) were significantly inter-correlated (see also Supplementary Appendix S3). Pearson's correlation coefficients were: $0.6<R<0.7$ ($P<0.001$) in the pairs (L192-L204), (L192-299) and (L204-299); $R=-0.51$ ($P=0.005$) in the pair (L204-L369); $R=-0.44$ ($P=0.017$) in the pair (L192-L369); and not significant in one pair of loci only, $R=-0.30$ ($P=0.115$).

In addition, linkage disequilibrium was detected among putative hitchhikers within the smaller and more homogeneous area A_1. Highly significant pairwise linkage disequilibrium values $0.42<|r|<0.47$ ($\log_{10}$-likelihood ratios $LR\geqslant 3$) were obtained for the pairs of loci (L192-L369), (L204-L369) and (L204-L299), while the remaining three pairs were characterised by *r*-values>0.25: $r=0.38$ for the pair (L192-L369) ($LR=2.4$), $0.25<|r|<0.32$ ($LR>1.13$) for (L299-L369) and (L192-L299). Consistently, genetic differentiation at these four outlier loci was elevated between *Bt* and non-*Bt* fields of A_1 ($\Phi_{ST}=0.24$; $P<0.001$) as well as between *Bt* fields of A_1 and A_2 ($\Phi_{ST}=0.21$; $P<0.001$). By contrast, it was low ($\Phi_{ST}<0.05$) within either type of field (*Bt* or non-*Bt*) and within each area (A_1 and A_2).

Controlled crosses

The site (Va) was located in the area A_1 in which genetic contrasts were observed, while (Li) and (Be) were located farther apart in A_2 (see Figure 1). Overall survival was 0.67 when offspring was reared on non-*Bt* maize, significantly higher ($P<0.001$) than in offspring reared on *Bt* maize (Figure 5). When reared on *Bt* maize, survival of Va × Tz offspring (0.32) was clearly higher than both Li × Tz and Be × Tz offspring ($P<0.001$). Given substantial heterogeneity within the two latter crosses, the highest survival in each type of offspring (0.11, 0.13) was compared with that of Va × Tz offspring reared on *Bt*; it was unambiguously confirmed that progeny obtained with individuals from Valhaarts outperformed progeny of the other crosses on *Bt* maize ($P<0.001$; Figure 5). Although survival in a Li × Tz progeny reared on *Bt* maize was particularly low (6/383), a F2 sib-cross between two out of the six survivors resulted in significantly increased survival on *Bt* maize (51/251, $P<0.001$), suggesting the existence of a recessive resistance trait in (Li).

Theoretical resistance spread

On the basis of a demogenetic system of deterministic reaction-diffusion equations, we show that the lower propagation speed (V) of resistance is a constant which takes the form (Supplementary Appendix S1, see also Tyutyunov *et al.*, 2008):

$$V=\sqrt{2\sigma^2\cdot\beta\cdot h\cdot\omega} \qquad [1]$$

The time required for a concentric wave of resistance to travel a distance d (km) may simply be approximated by $T\approx d/V$ generations.

From controlled crosses performed in the laboratory we estimated that a female of *B. fusca* produces on average ~250 viable neonates (see also Khadioli *et al.*, 2014). In laboratory experiments, survival (egg to adult stage) of *B. fusca* has been estimated to be, respectively, of order 40% and 15–20% in optimal and in sub-optimal temperature conditions (Khadioli *et al.*, 2014). In a different species of Noctuidae (*Helicoverpa zea*) the estimate of survival to adulthood in the field was close to 5% (Vargas and Nishida, 1980). Assuming a 1:1 sex ratio, we determined a conservative estimate of $\beta=0.05\times 250/2\approx 6$.

A resistance allele being propagated under constant selective pressure typically results in fast rapid spread. On the basis of equation (1), we explored the scale at which a dominant resistance allele may spread within ~26 generations (i.e., 13 years, and assuming $h=1$, $\omega=0.6$, $\beta=6$) from an initially local distribution. Selection due to a moderate fraction of *Bt* crop in the landscape ($\omega\approx 0.6$) and operating on a fully dominant resistance allele ($h=1$) yields speeds of several kilometres per year, e.g., 4.6 km per year $<V<$ 17 km per year for $1<\sigma^2<10$. Over 26 generations, the spread of resistance would attain a radius of several tens of kilometres (i.e., ~60–220 km) (Figure 6). Under the same parameter values, a high axial dispersal variance ($\sigma^2\approx 25\,\text{km}^2$ per generation) would be required for the

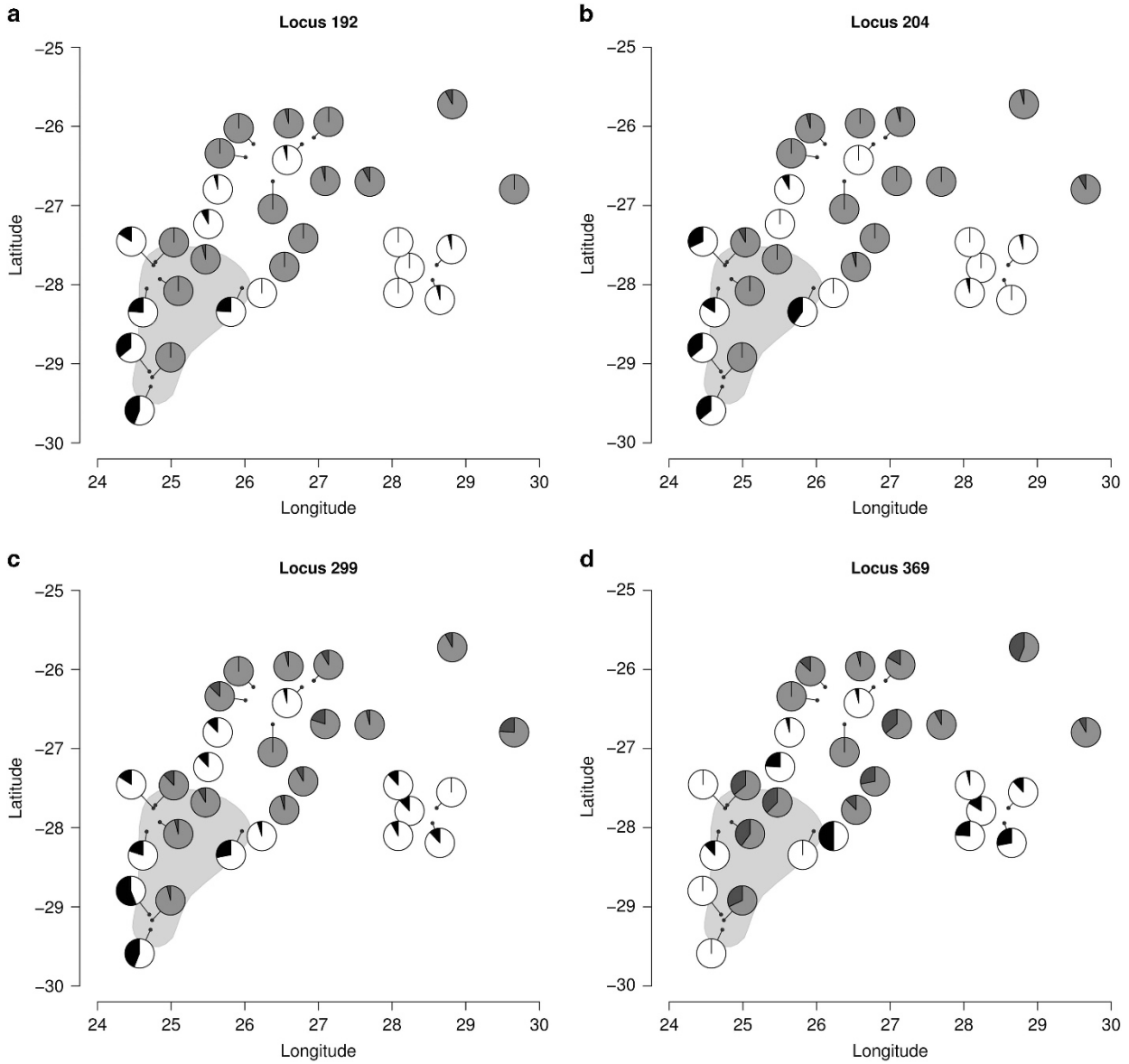

Figure 4 Marker frequency at four outliers loci for the different locations sampled. Black-and-white pies represent *Bt* fields; shaded-grey pies, non-*Bt* fields. The darker fraction of the pies corresponds to the absence of marker (i.e., homozygote for the null allele). The shaded area delineates the region A_1, while by default A_2 is composed of any fields outside this region.

resistance wave to travel from the location where resistance was first reported to the farthest Bt fields in which individuals were found to survive (Figure 6).

DISCUSSION

Being one of the few instances of field-evolved resistance leading to *Bt* crop failure (Tabashnik *et al.*, 2009; Tabashnik *et al.*, 2013), *Bt*-resistance in *B. fusca* may be considered an insightful study case in the context of *Bt* crop deployment worldwide. Our results confirmed that a sharp selective shift, as indicated by the extensive uptake of *Bt* maize (Kruger *et al.*, 2012), resulted in rapid evolution of resistance. Beyond the detection of outlier loci, our results provide further understanding of both gene flow and field resistance in this pest, notably in terms of spatial distribution, dynamics and diversity of resistance in the field.

Gene flow and resistance spread

In line with what was shown in several other moth species (e.g., Korman *et al.*, 1993; Nibouche *et al.*, 1998; Bourguet *et al.*, 2000; Han and Caprio, 2002), our empirical results reflect extensive gene flow and low levels of population inbreeding in *B. fusca*. To a first approximation, the whole population seems close to panmixia at coarse scales (700 km × 400 km). It has been suggested that *B. fusca* could be characterised by limited dispersal capacities (Sezonlin *et al.*, 2006). However, consistent with Dupas *et al.* (2014), our study did not reveal strong barriers to gene flow. Estimate of neighbourhood size in *B. fusca* ($N_b^* \approx 700$ individuals) had the same order of magnitude as in the European corn borer *Ostrinia nubilalis* ($N_b^* \approx 500$ on maize, see Martel *et al.*, 2003). Classical methods for estimating gene flow (see Rousset, 1997; Hardy, 2003) cannot yield a separate estimation of the parameters σ^2 and ρ_e, such that the product of these has to be considered, i.e., $\rho_e.\sigma^2 \approx 55$ in our study. Assuming an effective density of

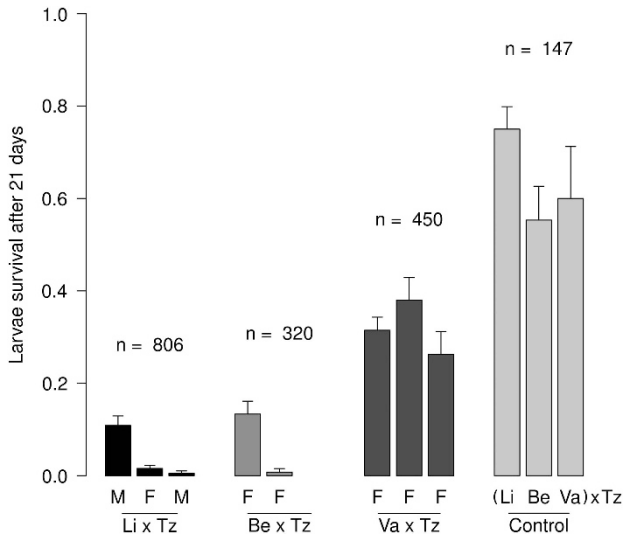

Figure 5 Survival of progeny on *Bt* maize after 21 days. Crosses were performed between individuals originating from a susceptible Tanzanian strain (Tz) and individuals originating from *Bt* fields in three different locations: Lichtenburg (Li×Tz), Bethleem (Be×Tz) and Valhaarts (Va×Tz), the latter being in the area A_1. The control consisted of the three same types of cross for which progeny was reared on non-*Bt* maize. M/F indicate whether the resistant parent was a male (M) or a female (F); error bars represent standard errors of the different proportions.

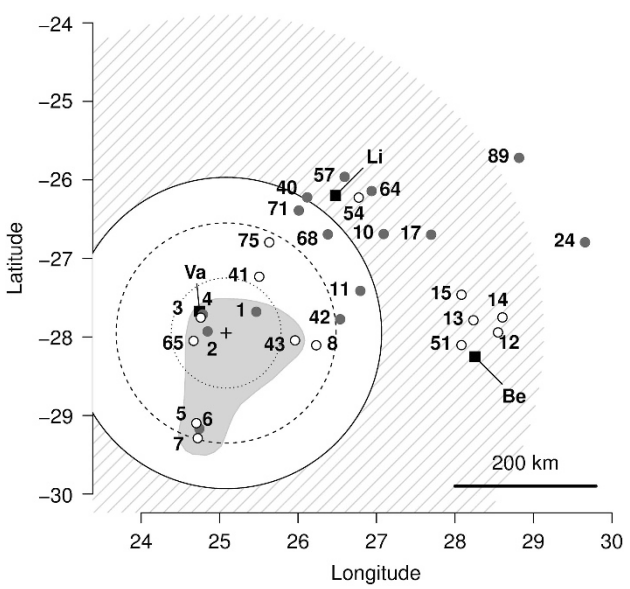

Figure 6 Expected resistance spread due to selection operated by *Bt* toxins ($\omega=0.6$) after $k=26$ generations. Resistance was assumed initially confined at the location where it was first reported (centre of the circles). Dotted, dashed and solid circles correspond to distances obtained with increased axial dispersal variance ($\sigma^2=0.75$, 3 and 6 km^2 per generation). External border of the hatched ring represents the dispersal parameter required $\sigma^2\approx 25$ km^2 to encompass all resistant populations collected in this study. Empty dots correspond to sampled *Bt* fields; dark-grey dots, non-*Bt* field; square dots, locations sampled for the controlled crosses (Va, Be and Li). Numbers are the field IDs as displayed in Table 1.

$14<\rho_e<55$ individuals per km^2 therefore amounts to assuming a dispersal parameter $1<\sigma^2<4$ km^2. Similar effective population densities, i.e., $5<\rho_e<50$ individuals per km^2, have been proposed in other species of wild moths (Saccheri *et al.*, 2008) and moth pests (Martel *et al.*, 2003). Mean flight distance of several kilometres was observed in males of the moth pest *Spodoptera litura*, using a release-recapture experiment. In *B. fusca*, observations of dispersing females crossing an inter-field of 1.6 km have been reported (see Harris and Nwanze, 1992). Although dispersal distances and timing may differ between males and females, these observations are roughly compatible with a dispersal parameter σ^2 scaling to a few km^2 per generation.

Extensive gene flow in pest populations may affect the pace of resistance evolution by mitigating local increase of resistance with susceptible alleles originating from non-*Bt* fields, or by contributing to an accelerated spatial spread of resistance pockets. Besides gene dispersal (σ), the speed of resistance propagation crucially depends on the inheritance of resistance (i.e., h, the dominance parameter in our model) and the population ecology of a pest, notably its birth rate (β). These parameters would clearly deserve further consideration in order to better comprehend the spatial dynamics of resistance. Nonetheless, realistic numerical applications suggested a travelling speed of a few kilometres per generations. It seems plausible that a resistance pocket, initially small, may have quickly expanded into a bigger area of radius several tens of kilometres or more. Within 13 years ($k=26$ generations), one might expect the radius of such a pocket to increase by ~ 100 km (provided $\omega\approx 0.6$, $h=1$, $\beta=6$, $\sigma^2\approx 2.1$ km^2, $\rho_e\approx 26.8$), an order of distance which is commensurate with the size of A_1. However, our model suggested that only high dispersal variance could result in a propagation radius that would encompass all apparently resistant populations, which might lie at the margin of what can be reasonably assumed.

Outlier loci and resistance

While larvae surviving in *Bt* fields were observed across a vast area, a genetic signature associated with field-evolved resistance could be attributed to a genetic hitchhiking process within a smaller region only. The existence of such a pattern is also suggestive of a recent geographical spread of resistance. Indeed, due to genetic recombination, associations between resistance allele and alleles at linked loci are not expected to persist in time, unless recombination distance is very low (see Maynard-Smith and Haigh, 1974). Correlated allele frequencies and linkage disequilibrium are not necessarily indicative of physical linkage as they may also result from various stochastic processes at population level. Nevertheless, given an observed lack of neutral genetic structure, even at coarse spatial scale, we expect neither population differentiation, demographic history nor genetic drift to strongly affect linkage disequilibrium patterns in the wild.

In line with genetic hitchhiking arising due to resistance evolution, consistent differences in outlier marker frequencies were observed between *Bt* and non-*Bt* fields. The size of these differences could reflect an intermediate frequency of resistance alleles in A_1 (see also Supplementary Appendix S4). On the one hand, a review study (Tabashnik *et al.*, 2013) evaluated the proportion of resistant *B. fusca* individuals to be $\approx q^2+2hq(1-q)\geqslant 50\%$, in the core resistance region, which translates into a resistance allele frequency $q>0.25$ assuming a strictly dominant ($h=1$) monogenic trait. On the other hand further assuming a resistance allele exclusively associated with null alleles (a_0) at either outlier loci L192, L204 or L299, one would expect the frequency of homozygotes a_0/a_0 (corresponding to an absence of marker) to be close to $P(a_0/a_0)=q^2/(q^2+2hpq)$ in *Bt* fields, i.e., leading to $q=2\times P(a_0/a_0)/[1+P(a_0/a_0)]$. Given $P(a_0/a_0)\approx 0.3$ at these loci in *Bt* fields (see Table 2), we attain a crude estimate $q\approx 0.46$. Finally, relaxing the stringent assumptions of full linkage and strict dominance, this exercise suggests a resistance allele frequency $0.25<q<0.5$ (see also Supplementary Appendix S4).

Note that some outlier loci could not be associated with resistance, which suggests that these loci may not be related to resistance at all, or reflect a lack of power in some analyses, notably due to the use of dominant genetic markers. However, it cannot be excluded that a fraction of these outliers, loosely linked to the resistance trait, underwent quick hitchhiking but did not shift significantly during the time for which associations persisted, contributing to some levels of genetic differentiation between areas (see also Supplementary Appendix S5).

Uniformity of resistance

Since the first observations of field resistance in *B. fusca* were reported, in the Western part of the maize cutivation region, most conspicuous failures to control populations of *B. fusca* in *Bt* fields (e.g., see Van Rensburg, 2007; Kruger *et al.*, 2011) have been observed in the surroundings of this location. Moreover, the resistance trait associated with these failures appeared to be dominant (Campagne *et al.*, 2013). Our results showing differential progeny survival on *Bt* plants and genetic contrasts (between A_1 and A_2) support the idea of a dominant resistance trait being confined within A_1. Furthermore, it is coherent with the chronology of resistance development in South Africa (Van den Berg *et al.*, 2013) and with the levels of infestation in *Bt* fields perceived by farmers at different locations: perceived field infestations $>10\%$ mostly occurred within $100-150$ km from the site where resistance was initially reported (Kruger *et al.*, 2012; Van den Berg and Campagne, 2014).

By contrast, information about resistance found within A_2 is rather scarce (Van den Berg *et al.*, 2013). Even if survival of some susceptible individuals on plant tissues expressing a low dose of toxins might partly explain the development of larvae on *Bt* fields within A_2, our results would advocate for independent evolution of resistance. Indeed, increased survival on *Bt* maize obtained in a F2 sib-cross clearly suggested the existence of at least one recessive resistance trait in A_2. Similarly to what has already been reported in another pest (e.g., Zhang *et al.*, 2012; Jin *et al.*, 2013), diverse resistance mutations characterised by different levels of recessiveness might therefore be responsible for the observed pattern of widespread field resistance to *Bt* maize in South Africa.

Finally, our results draw a valuable picture of resistance distribution and dynamics for one of the few instances of resistance evolution leading to control failure in the field. Evidence suggests resistance being a nonuniform trait across the extended region of maize production. The distribution of the main dominant resistance trait seems confined to a smaller region (100 km radius). The scale at which the distribution of this trait takes place could correspond to a recent geographical spread as confirmed by theoretical considerations showing a fast propagation of a dominant resistance trait across space. This study illustrates both potential challenges and insights of a strong evolutionary response scaling, in space and time, with the selective pressure of drastic environmental change.

DATA ARCHIVING

AFLP data are available from Dryad doi:10.5061/dryad.n3r35.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank four anonymous reviewers for their constructive comments about earlier versions of the mansucript as well as Peter E Smouse and Stéphane Dupas for helpful discussions. PC was supported by IRD and Natural Environment Research Council grant NE/J022993/1; RP, J-FS and BLR were funded by IRD; and JVdB was supported by Biosafety South Africa (Grant 08-001).

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