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# The spatial genetic differentiation of the legume pod borer, *Maruca vitrata* F. (Lepidoptera: Crambidae) populations in West Africa

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## Abstract

The legume pod borer, *Maruca vitrata*, is an endemic insect pest that causes significant yield loss to the cowpea crop in West Africa. The application of population genetic tools is important in the management of insect pests but such data on *M. vitrata* is lacking. We applied a set of six microsatellite markers to assess the population structure of *M. vitrata* collected at five sites from Burkina Faso, Niger and Nigeria. Observed polymorphisms ranged from one (marker 3393) to eight (marker 32008) alleles per locus. Observed and expected heterozygosities ranged from 0.0 to 0.8 and 0.0 to 0.6, respectively. Three of the loci in samples from Nigeria and Burkina Faso deviated significantly from Hardy-Weinberg Equilibrium (HWE), whereas no loci deviated significantly in samples from Niger. Analysis of molecular variance (AMOVA) indicated that 67.3% level of the genetic variation was within individuals compared to 17.3% among populations. A global estimate of  $F_{ST}=0.1$  (ENA corrected  $F_{ST}=0.1$ ) was significant ( $P\leq 0.05$ ) and corroborated by pairwise  $F_{ST}$  values that were significant among all possible comparisons. A significant correlation was predicted between genetic divergence and geographic distance between subpopulations ( $R^2=0.6$ ,  $P=0.04$ ), and cluster analysis by the program STRUCTURE predicted that co-ancestry of genotypes were indicative of three distinct populations. The spatial

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genetic variance among *M. vitrata* in West Africa may be due to limited gene flow, south-north seasonal movement pattern or other reproductive barriers. This information is important for the cultural, chemical and biological control strategies for managing *M. vitrata*.

**Keywords:** microsatellites, cowpea pests, West Africa

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## Introduction

The legume pod borer, *Maruca vitrata* Fabricius (Lepidoptera: Crambidae), is one of the major pest of grain legumes in the tropics and subtropics. Its emergence as a serious pest is attributed to an extensive host plant range, distribution and persistence. The geographic range of *M. vitrata* extends from northern Australia and East Asia through sub-Saharan Africa (Taylor, 1967; Raheja, 1974; Katayama & Suzuki, 1984; Ke *et al.*, 1985; Sharma, 1998) to the Americas (Wolcott, 1933; Taylor, 1967; Munroe, 1995). The larval stages of *M. vitrata* are destructive within agricultural and forest eco-systems as they feed on the tender parts of the plant stems, peduncles, flower buds, flowers and pods (Singh & Jackai, 1988) of more than 39 host plants belonging mostly to the family Fabaceae (Singh & van Emden, 1979; Sharma *et al.*, 1999; Arodokoun *et al.*, 2006).

In West Africa, *M. vitrata* is one of the major pests of cowpea, especially in the countries of Nigeria, Niger and Burkina Faso, which are the major cowpea producing areas. Cowpea production in West Africa accounts for more than 80% of the world's production (Ortiz, 1998; FAOSTAT, 2000; Sawadogo, 2009), but typical infestations by *M. vitrata* can cause yield reductions of 20 to 80% (Taylor, 1967; Raheja, 1974; Katayama & Suzuki, 1984; Ke *et al.*, 1985; Singh *et al.*, 1990; Sharma, 1998). The year-to-year crop losses and challenges faced in the effective field control have led to the identification of this pest as a major threat to economic and humanitarian well-being in developing and under-developed nations. Efforts to enhance the effectiveness of biological control agents that attack *M. vitrata* were partially successful following the importation of the parasitoid wasp, *Apanteles taragamae*, into Benin from Taiwan by the International Institute of Tropical Agriculture (IITA) (Dannon *et al.*, 2010). However, this has been hindered by the lack of population genetic data and information regarding the structure of *M. vitrata* populations in West Africa. Although significant advances have been made to understand the life-history and distribution patterns of *M. vitrata* using light trap and field studies (Ba *et al.*, 2009; Baoua *et al.*, 2011), extensive population-level data are still needed for deployment of biocontrol agents to be effective (Margam *et al.*, 2011). The application of population genetic data to biological control of *M. vitrata* will provide better information on how many distinct genotypes exist and the effect this can have on the parasitoid population over time. If different distinct genotypes exist, then this could result in the formation of specialized parasitoid populations on the different host genotypes in a process termed sequential speciation. So, over time, parasitoids on a particular genotype or host population may be isolated from other parasitoids on other populations. As herbivorous insects and their parasitoids interact with their environment on a fine spatial and temporal scale, sequential radiation may be quite

common (Feder & Forbes, 2010). More importantly, detailed population genetics data can be used to better target biological control interventions. First, the origin of a given pest can be traced by comparing the population genetics of the same organisms across continents where it occurs, thus allowing to focus the search for efficient natural enemies in areas where both the pest and its antagonistic organisms have co-evolved (Roderick, 1996). Once the most efficient natural enemies have been identified, their deployment in the field can be guided by the genetic structure of the target pest population in the likely area of introduction (Roderick & Navajas, 2003).

Prior studies in sub-Saharan Africa, including Burkina Faso, have suggested that seasonal flowering patterns of the different host plants on a south-north gradient may influence the migration of *M. vitrata* (Ba *et al.*, 2009). This seasonal movement occurs from temperate conditions along the coast into the Savannas of West Africa as the rainy season progresses (Bottenberg *et al.*, 1997). Migrating *M. vitrata* find favorable feeding and reproductive conditions on a succession of different host plants and, thereby, increase the population size and density with each successive generation. Despite the results of previous studies, many questions remain regarding the timing and spatial scale of *M. vitrata* migration patterns. A preliminary survey that genotyped *M. vitrata* from West Africa using 11 single nucleotide polymorphism (SNP) markers indicated that the population may show genetic subdivision (Margam *et al.*, 2011). Corroborating evidence is still necessary in order to validate the conclusions drawn from prior SNP analyses, and it is yet to be seen whether alternate molecular genetic markers, such as highly polymorphic microsatellites, have a similar potential to differentiate populations.

We developed and applied a set of microsatellite markers to estimate the genetic variability, population structure and gene flow among *M. vitrata* in the West African countries of Niger, Nigeria and Burkina Faso. The main objective of this study was to assess the genetic variability in the *M. vitrata* populations across West Africa, where these data will be useful for (i) determining effective areas for the release of biocontrol agents and (ii) the recommendation of insect resistance management (IRM) protocols aimed at minimizing the threat of selection for insecticide resistance alleles in the major cowpea producing areas in West Africa.

## Material and methods

### DNA sequence libraries

A combined assembly from Roche 454 reads of a premolt 4th to 5th instar *M. vitrata* larval EST library and Sanger-based EST reads from whole *M. vitrata* adults (referred to as the

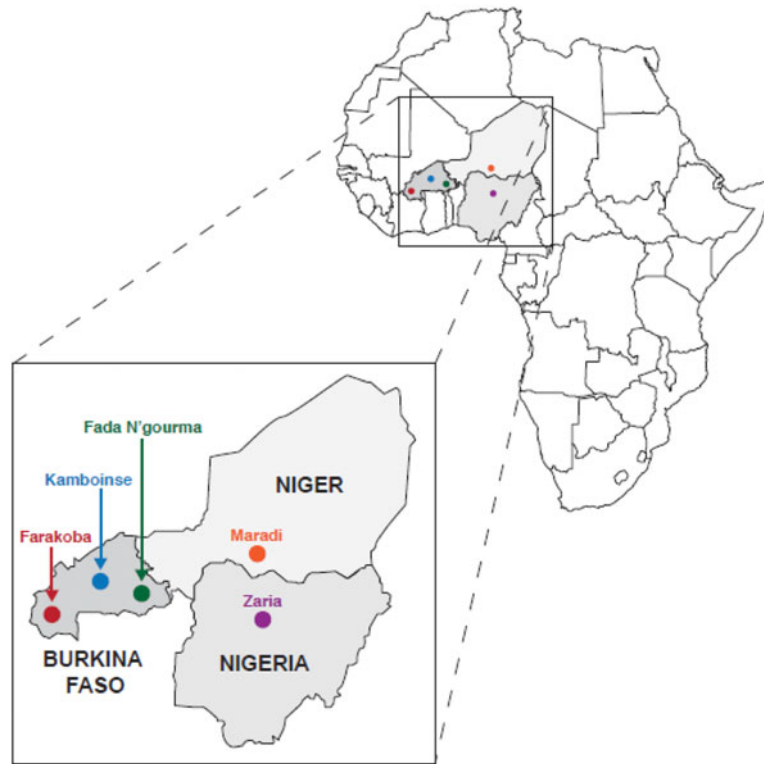


Fig. 1. Map showing locations in West Africa (Niger, Nigeria and Burkina Faso) where the *M. vitrata* samples were collected.

'reference assembly') was generated from *M. vitrata* collected from Maradi in Niger; Zaria in Nigeria; and Fada, Farakoba and Kamboinse in Burkina Faso between 2005 and 2007 (see Margam *et al.*, 2011: fig. 1 provides a map of the locations). Specifically, adult and larval samples from Nigeria were collected from a cowpea field around Zaria. The adults were collected using light traps. Samples from Niger were also collected from a light trap at Maradi field station while samples from Burkina Faso were collected in cowpea plots at the Institut de l'Environnement et de Recherches Agricoles (INERA) Fada, Farakoba and Kamboinse stations. This microsatellite study was conducted using DNA samples from 72 individuals from Niger, 53 individuals from Nigeria and 175 individuals from Burkina Faso (Fada: 40, Farakoba: 86 and Kamboinse: 49). Additionally, a microsatellite repeat-enriched partial genomic library was constructed using biotinylated (CA)<sub>15</sub> and (GA)<sub>15</sub> probes. Biotin probe-selected fragments were ligated into the pBluescript SK+ vector, which was used to transform the *Escherichia coli* strain XL1 Blue (Stratagene) by electroporation. Transformants were plated on LB agar containing 20 µg ml<sup>-1</sup> ampicillin and clones were picked, cultured and plasmid DNA purified at the Purdue University Genomics Core Facility (PUGCF), West Lafayette, Indiana, USA. Additionally, PUGCF performed sequencing of plasmid inserts on an ABI 3730XL sequencer, as well as vector sequence trimming, and PHRED quality parameter assessment and trimming at q < 30 (99% base call accuracy). Sanger sequence reads from the microsatellite-enriched library were assembled with CAP3 (Huang & Madan, 1999) using default parameters, and contig and singleton sequences pooled into a single FASTA-formatted file.

#### Microsatellite prediction, repeat filtering and marker development

The EST 'reference assembly' and DNA sequence files were merged and used as input for the program SciRoKo (Kofler *et al.*, 2007), where a search for arrays ≥ 10 units at di-, tri- and tetranucleotide repeat loci was specified. The *M. vitrata* sequence from +250 to -250 of predicted microsatellite repeats was parsed from FASTA files using 'SciRoKo's Little Helper' application (Chunk size 50,000,000; overhead 0) and corresponding positional information exported in tab-delimited format. Repetitive DNA has been described in proximity to microsatellite loci in Lepidopteran genomes and proven problematic for the development of locus-specific molecular genetic markers (please see Discussion). In order to identify repetitive DNA adjacent to *M. vitrata* microsatellites, we employed a bioinformatic pipeline to predict loci with homologies to known Lepidopteran transposable elements and showing sequence similarity to other unrelated *M. vitrata* microsatellites. To do this, the *Bombyx mori* transposon database was downloaded (file silkworm\_glean\_transposons.fa.tar.gz at <http://sgp.dna.affrc.go.jp/pubdata/index.html>) and was queried with *M. vitrata* microsatellite sequences using the blastn algorithm and results filtered for *E*-values ≤ 1 × 10<sup>-15</sup> (low-complexity filter was not used). NCBI nr database accessions identified as microsatellite loci from Lepidoptera were downloaded and queried with *M. vitrata* microsatellite sequences as described previously. *Maruca vitrata* sequences that produced a 'hit' with an *E*-value ≤ 1 × 10<sup>-15</sup> to a *B. mori* transposon or Lepidopteran microsatellite sequence were removed from the merged *M. vitrata* dataset.

Table 1. *Maruca vitrata* primer sequences used for microsatellite amplification reactions.

Locus	Primer (dye label) and sequence (5'-3')	Repeat	Size (bp)
C32008 <sup>E</sup>	F-(MAX)AAAAAGCGTTATATGTTTATTAGT R-GAAATTTTTAACGGAGATACAATCA	(CATA) <sub>3</sub>	163
7_02K06 <sup>A</sup>	F-(FAM)ATTTGTCAGAATGGTATCTTACGT R-CCTCTGGGTCATAATTATATTGTTC	(GAT) <sub>6</sub>	151
C3393 <sup>E, 1</sup>	F-(ROX)AGACCCCAAAGTGGAGAA R-ACGTTACGAACCTCTGTT	(GAA) <sub>5</sub>	91
C0444 <sup>E, 1</sup>	F-(FAM)AAAGGAACTACGCCGTCAGG R-GTTGAGCGATCTTGGCACAG	(CAA) <sub>8</sub>	102
C0241 <sup>E</sup>	F-(TAM)GACGAAACAAGGCCTACCAG R-GGTACTTCYGACGTTGTTCC	(GAT) <sub>9</sub>	165
C0325 <sup>E</sup>	F-(ROX)CGAAAAGAAACACCGCTCTG R-CAGTCTGTTCAGWCTCTTCAGTGG	(GAA) <sub>7</sub>	173

E, EST-derived primer pair; A, anonymous genomic sequence-derived primer pair; 1, PCR multiplexed primers.

The remaining *M. vitrata* sequences were used as input for BatchPrimer3 (<http://probes.pw.usda.gov/batchprimer3/index.html>; You *et al.*, 2008), and primer pairs were picked with the SSR screening and primers module [pattern type = di-, tri- or tetranucleotide; product size = 90 to 300 (150 opt); primer size = 18 min. 21 opt. 26 max.; primer T<sub>m</sub> = 58 min. 60 opt. 65 max). Primer and predicted PCR amplified genome sequence information was used as input for the program MultiPLX 2.0 (<http://bioinfo.ut.ee/multiplx/>; Kaplinski *et al.*, 2005), where potential primer-primer interactions were predicted using a monovalent salt concentration of 50 mM and a Mg concentration of 1.5 mM. Alignment energies for all primer pairs and products, and locus groupings used the normal stringency conditions according to author instructions. Oligonucleotides were synthesized and 5'-dye labels were added to forward primers by Integrated DNA technologies (Coralville, Iowa, USA).

#### *Maruca vitrata* sampling and genotyping

*Maruca vitrata* samples were collected from West African sites and genomic DNA extracted as described by Margam *et al.* (2011), and DNA concentrations were adjusted to ~10 ng μl<sup>-1</sup> with nuclease free water. PCR reactions of 25 μl were set up with 2.0 mM MgCl<sub>2</sub>, 150 μM dNTPs, ~15 ng DNA, 1.75 pmol each primer (multiplex reactions indicated in table 1), 2 μl 5X PCR buffer and 0.3125 U GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA), then amplified using the touchdown thermocycler program TD2 (Coates *et al.*, 2009). A total of 2 μl of each of the five PCR products for individual samples was pooled, diluted to a total volume of 128 μl, and a 5 μl aliquot submitted to the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign for separation on an ABI Prism 3730 × 1 Analyzer with the LIZ500 internal standard. Fragment analysis was performed using Peak Scanner software 1.0 (Applied Biosystems, Foster City, CA, USA) for data scoring and GeneMapper software for the identification and classification of alleles present per locus and per individual.

#### Genetic structure of *M. vitrata*

The mean number of alleles per locus, observed heterozygosity and expected heterozygosity were used to estimate within population genetic variability using Arlequin version 3.5.1.2. (Excoffier & Lischer, 2010). Analysis of molecular

variance (AMOVA) was also performed using Arlequin 3.5.1.2. Variance components were used to compute fixation indices, and their significance was tested at 1000 permutations as described by Weir & Cockerham (1984) in Arlequin. The program Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004) was used to estimate the frequency of null alleles and other genotyping errors such as stuttering and allele drop out. Null alleles are suspected for a given locus when the Micro-Checker 2.2.3 program rejects Hardy-Weinberg Equilibrium (HWE) among genotypes and if the excess homozygote genotypes are evenly distributed among allele size classes. Because some of the alleles harbored potential null alleles, corrected pairwise  $F_{ST}$  estimates were calculated for all populations by applying the ENA correction in the FREENA package (Chapuis & Estoup, 2007; Chapuis *et al.*, 2008).  $F_{ST}$  values were estimated following Weir (1996) while the null allele frequencies for each locus and population was analyzed following the expectation maximization (EM) algorithm (Dempster *et al.*, 1977). Exact tests using a Markov chain were used to test the deviation from HWE at each locus and population (Guo & Thompson, 1992) as implemented in Arlequin 3.5.1.2. Locus-by-locus  $F$ -statistics and pairwise  $F_{ST}$  estimates were also calculated using Arlequin 3.5.1.2. Due to the fact that pairwise  $F_{ST}$  estimates among sample sites represent multiple comparisons that are considered simultaneously, application of a significance threshold that treats these comparisons as a single comparison (that is  $\alpha = 0.05$ ) fails to recognize that as the number of comparisons increases the probability that any comparison will differ by random chance also increases (Miller, 1981). Specifically, when multiple hypothesis tests are performed, the experiment-wise (EW) Type I error is quickly increased at the rate of  $1 - (1 - \alpha)^k$ , where  $k$  is the number of hypothesis tests performed. To account for the presence of multiple dependent tests within our pairwise  $F_{ST}$  estimates, we implicated a correction to the significance thresholds used and determined the critical value according to B-Y method by Benjamini & Yekutieli (2001). Isolation by distance (Slaktin, 1993) was tested by analyzing the independence between geographical and genetic distances (Bohonak, 2002). The relationship was assessed by the Mantel test after 1000 permutations using a program IBD version 1.52 (<http://www.bio.sdsu.edu/pub/andy/IBD.html>).

The program STRUCTURE 2.3.3. uses a model-based clustering to predict population structure using genotypic

Table 2. Microsatellite motifs predicted from *M. vitrata* DNA sequence sources (combined library), that was partitioned into (CA) and (GA) repeat enriched library (microsatellite library), and expressed sequence tag (EST) library (EST library) sources. The mean length of repeat arrays (L) and frequency of nucleotide mismatch within repeat arrays (MisMch).

Motif	Combined library			Microsatellite library			EST library		
	Count	L	MisMch	Count	L	MisMch	Count	L	MisMch
AC	107	57.1	1.6	107	57.1	1.6	0		
AG	11	56.6	1.3	11	56.6	1.3	0		
ATC	14	25.8	0.4	0			14	25.8	0.4
AAT	12	18.4	0.3	0			12	18.4	0.3
AAAT	11	17.8	0.2	0			11	17.8	0.2
	155								

marker data from individual samples, where the model assigns proportions of individual genotypes to one of  $K$  populations (Pritchard *et al.*, 2000). To accomplish this, we used an admixture model to define individual ancestry and included data of sampling location as *a priori* informative descriptors of potential shared co-ancestry among the genotypes (LOCPRIOR command) according to the STRUCTURE 2.3.3 modification described by Hubisz *et al.* (2009). STRUCTURE 2.3.3 was run using an initial burn-in of 100,000 iterations followed by 100,000 iterations. We ran ten replicates with each value of  $K$  ranging from 1 to 10. The 'real' value of  $K$  (number of potential unique populations represented by the *M. vitrata* genotypes within our sample) was estimated from the  $\ln \Pr(X|K)$  values output for each replicate of  $K=1$  to  $K=10$  using the  $m|L''(K)/sL(K)$  statistic described by Evanno *et al.* (2005). In brief, the 'real' value of  $K$  within our dataset was determined by where the  $\ln \Pr(X|K)$  maximized the value of  $m|L''(K)/sL(K)$ . A graphical display of individual coancestry ( $Q$ -matrix) data generated within STRUCTURE 2.3.3 output was performed using the program Distruct (Rosenberg, 2004).

## Results

### DNA sequence libraries

The combined assembly of *M. vitrata* EST read data produced a total of 3499 contigs of  $452.9 \pm 279.9$  bp (see Margam *et al.*, 2011, for details). Sequencing of 480 clones from the  $(CT)_n$  and  $(GT)_n$  microsatellite repeat resulted in a total of 461 high quality sequences of  $285.8 \pm 200.8$  bp (131.8 kb total; GenBank accession numbers JN685509–JN685580). Assembly of the  $(CT)_n$  and  $(GT)_n$  microsatellite repeat sequences using CAP3 resulted in 46 contigs ( $5.9 \pm 7.2$  sequences per contig) and 69 singletons that were merged into a single dataset with a mean length of  $442.1 \pm 300.3$  bp.

### Microsatellite prediction, repeat filtering and marker development

A search for microsatellite-like repeats within 115 sequences in file 'MvMsatCAGA.fasta' predicted a total of 118, 26 and 11 di-, tri- and tetranucleotide repeat motifs, respectively. All of the 118 putative dinucleotides were predicted from the (AC) and (AG) repeat enriched libraries, and respectively showed a mean length of 57.1 nt (28.5 repeats) and 56.6 nt (28.3 repeats; table 2). In contrast, all putative tri- and tetranucleotide repeats were characterized from EST library sequences. The mean mismatch of nucleotides within the predicted array was  $\geq 3.7$ -fold higher among

dinucleotides compared to either the tri- or tetranucleotide repeat groups. Filtering of sequences  $\pm 250$  bp of putative *M. vitrata* microsatellite repeats against *B. mori* transposon-like sequences resulted in identification of 12 putative repetitive elements ( $E$ -values  $\leq 2.0 \times 10^{-16}$ ; similarity  $\geq 81.5\%$ ;  $L = 164.7 \pm 111.0$  bp; remaining data not shown). An analogous blastn search of the NCBI nr nucleotide database accessions from Lepidoptera that contained microsatellite sequences indicated that sequence similarities existed with 28 *M. vitrata* microsatellite flanking sequences ( $E$ -values  $\leq 2.0 \times 10^{-16}$ ; similarity  $\geq 86.7\%$ ;  $L = 245.5 \pm 77.8$  bp; remaining data not shown). In total, 40 sequences were identified within *M. vitrata* microsatellite flanking sequences (34.8%) and were removed from the file prior to PCR primer design. All of the filtered sequences were derived from the anonymous (AC) and (AG) microsatellite-enriched library.

The design of oligonucleotide primers from the remaining 75 sequences using Batch Primer3 resulted in 24 pairs (11 from anonymous microsatellite enriched library and 13 from EST sequence). Preliminary analysis by PCR resulted in the successful amplification for one of 11 anonymous microsatellite markers (9.1%) and five of 13 EST-derived microsatellite markers (38.5%; results not shown). PCR primer multiplex pair design using MultiPLX resulted in the prediction of two loci being suitable for co-amplification (markers C3393 and C0444), where primer alignment energies were at a maximum of  $-5.1$  kcal mol $^{-1}$  compared to  $-6.0 \pm 0.9$  kcal mol $^{-1}$  for all remaining possible primer pairs (remaining data not shown).

### Genetic structure of *M. vitrata*

The six microsatellite loci used to screen *M. vitrata* samples collected at five African sites showed significant deviation from HWE at 13 instances following 30 locus-by-site calculations (supplementary data), thus subsequent estimations of population subdivision used ENA corrected values to account for potential influence of null alleles. The mean number of alleles per locus was similar across all sample sites, and the overall observed heterozygosity was less than expected ( $F_{IS} \geq 0.1$ ) for all populations except at Fada, Burkina Faso ( $F_{IS} = -0.0$ ; table 3). Also the  $F_{IS}$  estimates were negative for only two loci (CO241 and CO325) across all populations (table 4). The locus-by-locus  $F_{ST}$  estimates derived from the five populations ranged from  $-0.0$  (ENA corrected  $F_{ST} = 0.0$ ; marker 32008) to 0.3 (ENA corrected  $F_{ST} = 0.0$ ; marker 7\_02K06; remaining data not shown). The subsequent global estimates of  $F_{ST}$  across all loci and all populations were moderate (uncorrected  $F_{ST} = 0.1$ , ENA corrected  $F_{ST} = 0.1$ ) and significant (95% CI = 0.0–0.3, 95% CI with ENA

Table 3. Characteristics of the *M. vitrata* individuals across West Africa showing the number of alleles ( $N_a$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_E$ ) and fixation index ( $F_{IS}$ ) per sample site (values rounded up to 1 decimal place).

Location	$N_a$ (mean per locus)	$H_o$	$H_E$	$F_{IS}$	Loci not in HWE	Number of Null alleles
Niger	25 (4.2)	0.2	0.2	0.1	0	2
Nigeria	27 (4.5)	0.2	0.3	0.2	4	2
Fada	18 (3.0)	0.3	0.3	-0.0	3	2
Farakoba	23 (3.8)	0.2	0.3	0.2	3	3
Kamboinse	18 (3.0)	0.2	0.3	0.4	3	1

Table 4. Estimates of total number of alleles ( $N_a$ ), mean estimated frequency of null alleles, fixation index ( $F_{IS}$ ) and  $F_{ST}$  across microsatellite loci (values rounded up to 1 decimal place).

Locus	$N_a$	Mean null alleles frequency	$F_{IS}$	$F_{ST}$ uncorrected (ENA corrected)
C0444	7	0.0	0.1	0.0 (0.0)
C32008	10	0.1	0.1	-0.0 (0.0)
C3393	3	0.1	0.9	0.0 (0.0)
7_02K06	6	0.2	0.7	0.4 (0.3)
CO241	7	0.0	-0.1	0.0 (0.0)
CO325	5	0.0	-0.1	0.1 (0.1)

correction=0.0–0.2, with the lower bounds rounded to zero in one decimal place). The partitioning of population genetic variance from AMOVA results indicated that  $\geq 67.3\%$  resides within individuals, and correspondingly 17.3% and 15.4% of the total genetic variation, was among populations and among individuals (table 5). Pairwise comparison of  $F_{ST}$  estimations showed that a significant level of differentiation exists for all the possible comparisons after the sequential B-Y adjustment for the ENA corrected  $F_{ST}$  estimates across all loci (table 6). Specifically, a critical significance level was achieved at  $0.05/2.929=0.017$ . Results indicated that the  $P$ -values obtained from pairwise  $F_{ST}$  estimates ( $\leq 0.005$ ) were all statistically significant at the B-Y adjusted thresholds (table 6). Regression of uncorrected  $F_{ST}$  estimates and geographic distance (km) among West African sample sites showed a significant dependence of genetic variation on geographic distance ( $R^2=0.6$ , Mantel  $P=0.04$ ), and showed the relative genetic similarity ( $F_{ST}$  estimates) of genotypes at Niger and Nigeria, and among Burkina Faso samples (fig. 2). The ‘real’ number of populations ( $K$ ) estimated from the microsatellite-defined *M. vitrata* genotypes from the  $m|L''(K)|sL(K)$  statistic calculated from STRUCTURE 2.3.3 output achieved a maximum value of 14.5 at  $K=3$  and suggested that three genetically-distinct *M. vitrata* ancestries exist in West Africa. The three genetically distinct ancestries across collection sites were represented in fig. 3 as vertical bars with Niger, Nigeria and Fada, Burkina Faso primarily red; Farakoba, Burkina Faso primarily green; Kamboinse, Burkina Faso primarily green and red and a minor cluster represented by yellow across all sites.

## Discussion

### Microsatellite markers in Lepidoptera

The isolation and subsequent application of microsatellite loci as molecular genetic markers in Lepidopteran insects has

Table 5. Analysis of Molecular Variance (AMOVA) for Maradi, Niger, Zaria, Nigeria and Fada, Farakoba, and Kamboinse locations in Burkina Faso.

Source of Variation	df	SS	% of Variation
Among population	4	56.1	17.3
Among individuals	295	190.5	15.4
Within individuals	300	133.0	67.3
Total	599	379.6	100.0

often been difficult (Nève & Megléc, 2000; Ji & Zhang, 2004; Zhang, 2004), where studies have developed five or fewer loci for most species studied so far (reviewed in Ji *et al.*, 2004). These difficulties have been attributed to the high degrees of nucleotide sequence similarity between regions that flank different microsatellite loci (Megléc *et al.*, 2004) or the low frequency of microsatellites in Lepidopteran genomes (Ji *et al.*, 2003; Prasad *et al.*, 2005). Additionally, evidence suggests that Lepidopteran microsatellites may be derived from and mobilized by transposable elements (TEs) (Coates *et al.*, 2009, 2010, 2011, 2012; Tay *et al.*, 2010). Despite these associations with repetitive DNA, microsatellite markers have been developed for more than 40 Lepidopteran species in 35 genera (GenBank, 15 August 2006) and have proven to be useful for population genetic analyses. Within this study, we initially identified 155 unique *M. vitrata* microsatellite loci from which six were eventually considered informative genetic markers. Forty of 155 loci (25.8%) contained known repetitive element-like sequences previously identified within the *B. mori* genome assembly, which suggests that *M. vitrata* microsatellites may be associated with repetitive DNA as observed in other Lepidopteran species (Megléc *et al.*, 2004). High failure rates observed during microsatellite marker development are rarely reported, and the extent to which repetitive DNA can cause these failures has not been thoroughly investigated (Tay *et al.*, 2010). In the current study, we aimed to identify *M. vitrata* microsatellite loci which may contain repetitive sequences and remove them from consideration for molecular marker development. Despite these measures, an approximate 25% success rate for *M. vitrata* microsatellite markers suggest that screening for repetitive DNA from distantly related species may not be sufficient to identify all affected loci.

Microsatellite loci that deviate significantly from HWE show evidence of null alleles according to the distribution of homozygote-size classes. Microsatellite null alleles are commonly found among a wide range of taxa but have a particularly high incidence among species of Lepidoptera (Megléc *et al.*, 2004), Diptera (Lehmann *et al.*, 1997) and Orthoptera (Chapius *et al.*, 2005). The extent to which null alleles tend to overestimate the true population differentiation has not been investigated (Chapius & Estoup, 2007) but can lead to overestimates of population differentiation due to effects on subsequent calculations of  $F_{ST}$  and genetic distances (Slatkin, 1995; Paetkau *et al.*, 1997). Although null alleles were present at all microsatellite loci, correction with the ENA algorithm nonetheless allowed effective population genetic analysis. Specifically, analysis of these microsatellite markers resulted in conclusions analogous to those obtained from *M. vitrata* single nucleotide polymorphism (SNP) markers applied to the same populations (see next section).

Table 6. Pairwise comparisons of *M. vitrata* samples showing the corrected  $F_{ST}$  estimates (below diagonal) and significance of corresponding comparisons ( $P$ -values) as indicated above the diagonal. Significance thresholds were evaluated using a Benjamini and Yekutieli (B-Y) adjusted  $\alpha=0.017$ .

	Niger	Nigeria	Fada	Farakoba	Kamboinse
<b>Niger</b>	–	0.005*	<0.001*	<0.001*	0.001*
<b>Nigeria</b>	0.2	–	<0.001*	<0.001*	0.004*
<b>Fada</b>	0.2	0.2	–	<0.001*	0.002*
<b>Farakoba</b>	0.2	0.2	0.1	–	0.001*
<b>Kamboinse</b>	0.2	0.2	0.2	0.2	–

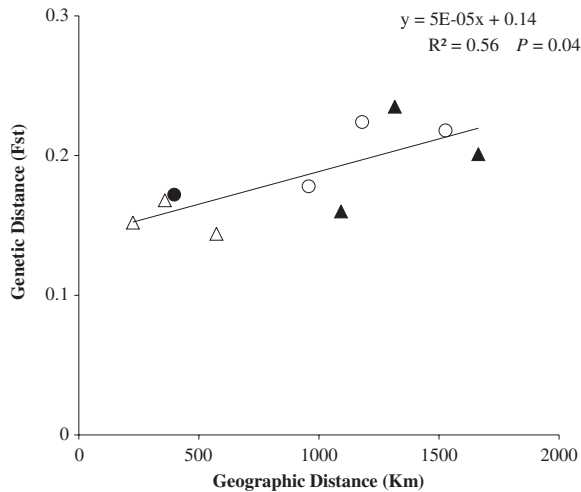


Fig. 2. Geographic distance versus genetic distance among populations of *M. vitrata* using  $F_{ST}$ . Correlations and probabilities were estimated from a Mantel test with 10,000 repeats of bootstrapping.  $\Delta$ , Comparisons among Burkina Faso (Fada, Farkoba and Kamboinse);  $\bullet$ , Niger and Nigeria;  $\blacktriangle$ , Niger and locations in Burkina Faso;  $\circ$ , Nigeria and locations in Burkina Faso are indicated.

#### Genetic structure of *M. vitrata* in West Africa

At the population level, our ENA corrected microsatellite genotype data suggested that a moderate level of genetic differentiation may be present among *M. vitrata* sample sites in West Africa. Consistent with previous studies in other insects, a majority of the total genetic variation is within individuals compared to between sample sites (Coates & Hellmich, 2003; Juan *et al.*, 2004; Timmermans *et al.*, 2005). Furthermore, our microsatellite results agree with an analogous study using *M. vitrata* SNP markers in West Africa (Margam *et al.*, 2011), where genetic structure was detected between Niger and Nigeria, and Burkina Faso sample sites and an overall  $K=3$  obtained from STRUCTURE results. The current study also indicated three distinct populations of *M. vitrata* in West Africa and suggests that analysis of microsatellite and SNP markers can provide equivalent results and conclusions. The findings from that study, however, showed significant pairwise  $F_{ST}$  estimates between eastern (Niger and Nigeria) and western sample sites from Burkina Faso, and all other comparisons indicated a lack of genetic divergence while findings from our study indicated a significant divergence in all pairwise populations using a critical  $P$ -value determined using B-Y adjustment method (Benjamini & Yekutieli, 2001).

Our population differentiation results derived from microsatellite data may be expected since SNPs typically have two alleles per locus due to their low mutational rate (Hancock, 1999; Zhang & Hewitt, 2003) compared to multi-allelic microsatellites that have higher power per locus for estimating genetic divergence or gene flow when using  $F$ -statistics or assignment tests (Vignal *et al.*, 2002; Brumfield *et al.*, 2003; Morin *et al.*, 2004). Studies further suggest that measures of pairwise genetic relationships using SNPs would require analysis of more than five times more loci as compared to microsatellites (Blouin *et al.*, 1996; Glaubitz *et al.*, 2003) and suggest that the current set of microsatellite markers will be more efficient at detecting population subdivision compared to SNP markers within future population genetic analyses.

#### Effect of migration on the population structure in *M. vitrata*

Migration is a fundamental population process and is crucial to understanding the dynamics and persistence of populations of insects (Dingle, 1996). Despite the field observations that *M. vitrata* has high rates of migration, significant differences in  $F_{ST}$  estimates and partitioning of 17.3% of population variance between sample sites within AMOVA analysis suggested that *M. vitrata* showed reduced gene flow and genetic structuring in West Africa. This is evidenced in the  $F_{IS}$  estimates obtained for almost all the study sites except Fada, Burkina Faso where there was some evidence of outbreeding suggesting high gene flow in this population. Specifically, an isolation by distance (IBD) model suggested that subpopulation differentiation ( $F_{ST}$ ) or structured gene flow is a function of the geographical distance between them (Slaktin, 1993). Our analyses indicate that although *M. vitrata* has a seasonal south-north migration, there appears to be evidence of reduced gene flow. In agreement with this, Peterson & Denno (1998) reported that insect species with high- and low-dispersal rates tend to show less IBD compared to species with moderate dispersal capabilities. High levels of gene flow tend to homogenize the observed genetic variation across a geographic range, whereas low gene flow can result in the effective genetic isolation of subpopulations and genetic drift. We observed a positive correlation between geographic and genetic distances among the *M. vitrata* sample sites within this study ( $R^2=0.56$ ), suggesting that *M. vitrata* may have a structured migratory pattern. Although isolation by distance is not typical for species with high mobility (Arguedas & Parker, 2000), it has been reported for other Lepidoptera, such as *Chazara briseis* Linnaeus (Lepidoptera: Satyridae) (Johannesen *et al.*, 1997) and *Hesperia dacotae* Skinner (Lepidoptera: Hesperidae) (Britten & Glasford, 2002). Light trap data and sampling of *M. vitrata* in West Africa showed that *M. vitrata* does not infest



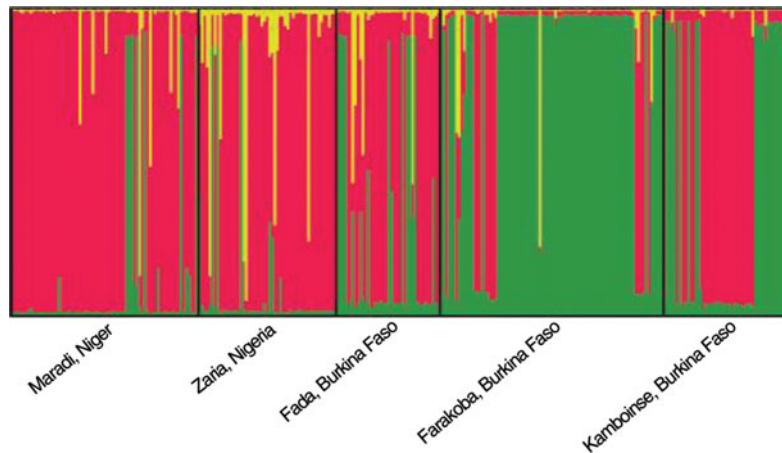


Fig. 3. Partitioning of the co-ancestry among microsatellite-defined *M. vitrata* genotypes generated from STURUTURE using the LOCPRIOR command for Maradi, Niger, Zaria, Nigeria and Fada, Farakoba and Kamboinse locations in Burkina Faso. For each, the estimated co-ancestry was derived from the Q-matrix for each individual and represented as vertical lines showing the proportion of the  $K=3$  segments that made up the individual genotype.

cowpea during the dry season in the northern extremes of its geographic range, even if cowpea is present (Bottenberg *et al.*, 1997; Ba *et al.*, 2009). Climatic factors such as lack of rain, and temperature or relative humidity may influence this observed spatial restriction. Research has also shown that *M. vitrata* survive on alternate host plants within the more humid southern regions and migrate to the northern regions over a period of several months (Bottenberg *et al.*, 1997). During the seasonal migration, the population of *M. vitrata* finds favorable conditions for multiplying on a succession of different host plants, which may result in temporal mating barriers due to differential larval maturation rates on the alternate host plants. This serves to emphasize the importance of ecological migration (movement of individuals) on population genetic migration (transfer of alleles). Gene flow cannot occur without ecological migration, but the effects of ecological migration on the population genetic structure can be tempered by factors such as temporal mating barriers between dispersing and resident individuals.

#### Implications for pest control

The implementation of effective cultural, chemical and biological control strategies to limit *M. vitrata* feeding damage to cowpea crops in West Africa is dependent upon a basic understanding of population structure and migration. Our results and previous ecological studies have implications for IRM strategies involving Bt-cowpea in West Africa. The implication is that in the north, resistance may spread only slowly among *M. vitrata* populations because the populations eventually die out during the dry season; in other words, the southern populations in endemic zones act as a source population. Also, the long-distance migration from the south to the north might be a source of susceptible populations into the northern part, which can slow down the evolution of resistance (if there are pockets of *M. vitrata* populations that survive in the north throughout the year). In the more humid south, *M. vitrata* can be found on different host plants throughout the year. Further studies should be conducted on population characterization on the different host plants,

especially in the more humid south, to determine if there are differences in populations on the different host plants or if geographic barriers restrict gene flow between them. Migration results in changes in allele frequency that are greater in the short term and smaller in the long term, leading to under- and overestimation of effective population size, respectively, if it is ignored (Wang & Whitlock, 2003). Using these polymorphic markers, studies should also be conducted to test how migration rate affects the effective population size of the *M. vitrata* populations. The results of this study also have implications for the implementation of control strategies involving the release of biocontrol agents against *M. vitrata*. In keeping with an endemic zone to migratory zone hypothesis, the conclusions of this study agree with the conclusions by Margam *et al.* (2011), which suggested that the deployment of biocontrol agents (for classical biological control) would be most logical in the endemic zone directly south of migratory regions where *M. vitrata* is a significant pest during the cowpea growing season.

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