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Geographic distribution of phylogenetically-distinct legume pod borer, *Maruca vitrata* (Lepidoptera: Pyraloidea: Crambidae)

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Abstract *Maruca vitrata* Fabricius is a pantropical lepidopteran pest of legumes. Phylogenetic analysis of a mitochondrial cytochrome *c* oxidase-I gene (*cox1*) fragment indicates that three *Maruca* sp. mitochondrial lineages have unique geographic distributions [lineages 1 and 2: Australia, Taiwan, and West Africa (Niger, Nigeria, and Burkina Faso), and lineage 3: Puerto Rico]. The haplotype (T30, T114) is specific to lineages 1&2 and was assayed by *Nsi*I and *Sac*I polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) within population samples; it was not observed in the Puerto Rican samples, but was nearly fixed among samples from West Africa, Australia and Taiwan (85.5–100%). Re-sequencing and phylogenetic analyses of PCR-RFLP defined *cox1* haplotypes indicate that nucleotide diversity is highest among samples from West Africa. Phylogenetic reconstruction based upon ribosomal DNA (rDNA) internal transcribed spacer-2 (ITS-2) sequences provided additional evidence for three *Maruca* sp. clades. These data suggest that multiple unique *Maruca* species or subspecies are present worldwide, which has implications for the management of this pest species-complex.

Keywords Legume pod borer · *cox1* · Barcoding · Population genetics · *Maruca*

Introduction

The legume pod borer (LPB), *Maruca vitrata* Fabricius (Lepidoptera: Pyraloidea: Crambidae; Syn: *Maruca testulalis*), is distributed through the tropical and subtropical regions of the world. Although the Indo-Malaysian

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region is considered to be the most probable region of origin for the genus *Maruca*, including *M. vitrata* [1], the geographic range of this insect extends from northern Australia and East Asia through sub-Saharan Africa [2–6] to the Caribbean, Central America, and Hawaii [2, 7, 8]. The larval stages of *M. vitrata* are destructive within agricultural and forest eco-systems as they feed on flowers and pods of more than 39 host plants, including two non-leguminous hosts [9, 10]. Host plants are mainly species from the Family Fabaceae (leguminous plants) [11–13]. *M. vitrata* attacks *Vigna unguiculata* subsp. *unguiculata* (cowpea), *Vigna unguiculata* subsp. *sesquipedalis* (yard-long bean), *V. radiata* (mung bean), *Glycine max* (soybean), *Pueraria phaseoloids* (puero), *Phaseolus lunatus* (lima bean), and *Cajanus cajan* (pigeonpea) and often causes significant yield losses in sub-Saharan Africa [2, 6], Southeast Asia [14–16], South Asia [17–19] and central and South America. The cultivation of these legumes for food has increased due to the advent of double cropping and inter-cropping systems in subtropical regions [20], and has resulted in *M. vitrata* emerging as major threat to legume production in developing and underdeveloped nations.

Control of *M. vitrata* damage to field crops depends mainly on chemical insecticide applications [6], but success is variable due in part to (i) the web structures larvae construct which shields them from insecticide sprays [2], (ii) the evolution of resistance to insecticides [21, 22], and (iii) the cost of chemical sprays in developing nations [23, 24]. Much of sub-Saharan Africa is inhabited by subsistence farmers [25], and the continent as a whole shows low per capita growth in crop production compared to worldwide averages [26]. Pest control measures have improved the quality and quantity of crop output in most parts of the world [27], but limited access to chemical technology by farmers in Africa continues to hinder increases in food production. Other efforts for enhancing cowpea production include breeding for native resistance to insect pests [28, 29], biological control, and use of biopesticides [24] including *Bacillus thuringiensis* (Bt) toxins [30]. *Bt*-Cowpea designed to resist *M. vitrata* is being developed [31].

Insect resistance management (IRM) practices are crucial for effective and sustainable control of insect pests. Development and application of IRM requires a thorough understanding of the biology and ecology of the target pest species. Lack of population genetic data including DNA sequences, molecular markers, and information on population structure hinders the development of IRM protocols for *M. vitrata*. Furthermore, species within the genus *Maruca* are morphologically hard to distinguish, and *M. vitrata* has historically been surmised to be a species complex [2]. Past and more recent light-trap studies in sub-Saharan Africa suggest south to north migration of *M. vitrata* [32, 33]. If true, this confounds any estimates of population variability, genetic structure, and gene flow. DNA sequence divergence in the mitochondrial cytochrome *c* oxidase I (*cox1*) gene has recently been established for species-level identification (DNA barcoding), and has been applied to differentiate species within a species-complex [34–37].

DNA barcoding is also useful for understanding the ecology, distribution, and environmental adaptation of a species. In the current study, DNA barcoding of a 500 bp *M. vitrata* mitochondrial *cox1* gene fragment barcode was used to determine the global distribution of haplotypes within the genus. We used sequence of rDNA internal transcribed spacer (ITS) region to corroborate conclusions drawn from DNA barcodes. Sub-species distribution also was estimated from a larger population sample by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays, and provided a method for molecular differentiation within the species in lieu of direct sequencing.

Materials and methods

Sample collection

Maruca vitrata samples from Australia were from a colony at CSIRO, Entomology, Canberra, Australia (source populations for the colony were collected from two different geographical sites during 2008). Samples from Nigeria were collected from a cowpea field at Maigana (near Zaria) in 2006. Samples from Niger were collected from a light trap at Maradi field station during 2006. Samples from Burkina Faso were collected in cowpea plots at the Institut de l'Environnement et de Recherches Agricoles (INERA) Kamboinse station during 2006. Puerto Rican samples were collected from white bean (*Phaseolus vulgaris*) near Lares, Puerto Rico, during 2008 and 2009. Samples from Taiwan were obtained from AVRDC—The World Vegetable Center in Tainan, Taiwan.

DNA sequencing and phylogenetics

Genomic DNA was isolated from whole adult or larval tissue using a DNeasy animal tissue kit following manufacturer instructions (Qiagen, Valencia, CA). Oligonucleotide primers HC02198 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ and LCO1490: 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ [38] were used for PCR amplification of mitochondrial cytochrome *c* oxidase I (*cox1*) DNA barcode region. PCR reactions were performed in 50 µl reaction volumes that contained 1 µl template DNA (15–25 ng), 5 pmol of each primer, 5 µl

10× PCR buffer, 0.4 µl *Taq* polymerase (New England Biolabs, Ipswhich, MA), 1.2 µl 10 mM dNTP and 41.4 µl deionized H_2O. Thermal cycling was carried out on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany), and performed using a 95°C denaturation step for 2 min, followed by 5 cycles of 95°C for 30 s, 45°C for 45 s, 72°C for 1.5 min, followed by 31 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 45 s and a final cycle of 72°C for 8 min. A 10 µl aliquot of PCR product was separated on a 1% agarose gel that contained 0.5 µg/ml ethidium bromide at 90 V for 30 min, and data acquired on a ChemiDoc XRS gel imaging system (BioRad, Hercules, CA). Remaining 40 µl of PCR product was purified using Qiaquick PCR purification kits (Qiagen, Valencia, CA) following the manufacturer protocols.

Cycle sequencing of purified PCR products was performed using 1 µl of PCR-purified template, 1 µl of 10× BigDye™ (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit) (Applied Biosystems, Foster City, CA), 1 µl of 10 pm forward or reverse primer (HCO2198 or LCO1490), 6 µl of ddH_2O using the conditions: 95°C for 2 min, followed by 98 cycles of 95°C for 10 s, 50°C for 5 s, 60°C for 4 min. Cycle sequencing products were precipitated using an ethanol–sodium acetate procedure followed by resuspension in 35 µl of ddH_20 of which 15 µl was separated on an ABI sequencer (Applied Biosystems, Foster City, CA) at the Purdue University Genomics Facility.

FASTA formatted data from *M. vitrata* sequence reaction were imported into the MEGA 4.0 software package sequence alignment application [39], and multiple sequence alignments performed with the ClustalW algorithm using default parameters (gap opening penalty 15, gap extension penalty 6.66, weight matrix IUB, and transition weight of 0.5). The Basic local alignment search tool (BLAST) [40] was used to query the national center for biotechnology information (NCBI) non-redundant nucleotide database and protein database with *M. vitrata cox1* sequence data in blastn and blastx searches. The coding frame of the *cox1* fragment was defined within the MEGA 4.0 alignment file, and substitution mutations were identified manually. The nucleotide diversity (π), base composition (θ), transition to transversion bias (R; [41]), disparity in substitution among nucleotide sites (Disparity Index), and test substitution homogeneity [42] of the aligned sequence was analyzed by MEGA 4.0 [39].

The phylogenetic relationships among *M. vitrata* mitochondrial *cox1* sequences were inferred using the Neighbor-Joining method of Saitou and Nei [43] from estimated genetic distances [44] computed using the Kimura 2-parameter (K2P) method of sequence evolution [45]. Similarly close-neighbor-interchange (CNI) search was used to examine the neighborhood of the NJ tree to find the potential ME tree under the K2P model of sequence evolution [46]. Both NJ and ME reconstruction methods incorporated 1,000 bootstrap pseudoreplicates to determine the number of possible trees supporting each node [47], and each reported on a strict consensus tree [48].

The *Maruca cox1* sequence was also verified using the BOLD (www.boldsystems.org; [49]) by inputting the nucleotide sequence representing each of the 14 haplotypes in this study in the specimen identification tool using the option "species level barcode records" on BOLD. BOLD uses a hidden Markov model [50] for finding the best match for the input sequence in the data set. Two reports, one with similarity match and the other with phylogenetic tree, were generated from BOLD's specimen identification tool.

The internal transcribed spacer (ITS) regions 1 (ITS1) and 2 (ITS2), and 5.8S rRNA gene were sequenced from the same *Maruca* sp. samples from which *cox1* data were generated. Both ITS regions are size and sequence variable between species, but since the regions evolve by concerted evolution within a species they are a target for the molecular discrimination of species. Oligonucleotide primers ITS4 5′-TCC TCC GCT TAT TGA TAT GC-3′ and ITS5: 5′-GGA AGT AAA AGT CGT AAC AAG-3′ [51] were used for PCR amplification of the ITS regions. PCR and DNA sequencing reactions and phylogenetic analyses were conducted as described above for the amplification of *cox1* regions.

Restriction assays and population sample screens

PCR reactions and thermocycler conditions were run as described above for the *M. vitrata* mitochondrial *cox1* gene fragment, except volumes were scaled to 10 µl. Entire PCR products were added to a 20 µl reaction that contained 1× of restriction enzyme buffer and 0.1 units of *Sac*I (Promega, Madison, WI) or *Nsi*I (New England Biolabs) restriction endonuclease. Reactions were incubated overnight at 37°C, and entire reaction volumes separated by 1.5% agarose gel electrophoresis as described previously.

Results

DNA sequencing and phylogenetics

Universal barcoding primers for *cox1* amplified a ~650 bp product from all *M. vitrata* samples and sequences from the amplified region were obtained from 48 individuals. The resulting sequence reads were trimmed based on base call Phred quality parameter scores ≥20 [52], and used to generate a 500 bp multiple sequence alignment (GenBank accessions in Table 1). Analysis of the *cox1* gene fragments showed a high A+T content (39.1% A and 31.4% T

averaged across the multiple sequence alignment), and a low mean difference in base composition per site (mean composition distance = 0.016). A total of 41 substitution mutations were predicted (Table 1; nucleotide diversity = $\pi = 0.0266$ = mean number of substitutions per site between two sequences) with 27 being transition mutations (ratio of transitions to transversions = ts/tv = $k = 2.90$) that gave a transition to transversion bias (R) = 1.99. The homogeneity of the observed substitution pattern across all sequences was assessed by calculating whether the difference in base composition bias among sequences was more than what would have occurred by chance alone. This substitution pattern homogeneity test indicated that significant departures occurred in 71 pairwise sequence comparisons ($P \leq 0.05$ following 1,000 Monte Carlo simulations).

Analysis of the *cox1* sequences after exclusion of all 3rd codon positions generated a mean nucleotide composition of 23.8% A and 30.4% T. This did not deviate from the 25% expected by random chance ($\chi^2 = 1.22$, d.f. = 1, $P \geq 0.32$), and resulted in a mean composition distance of 0.002. Furthermore, the parameters of nucleotide diversity $\pi = 0.008$ and transversion bias $R = 1.29$, respectively, suggested that sequences within 1st and 2nd codon positions showed a lower chance for incurring >1 substitution mutation over evolutionary time, and a bias for transversion bias was no greater than that observed for all nucleotide sites. Results from a test for homogeneity of substitution patterns

Table 1 Forty-one variable nucleotide positions present among 14 *Maruca vitrata* mitochondrial haplotypes (MvHT01–MvHT13) that were defined by alignment of 23 *cox*I sequences 500 bp in length, with 24 substitution mutations (6 haplotypes) being unique to the Puerto Rican samples

Genbank accession number	18	30	54	57	72	87	114	129	153	165	210	213	243	249	258	261	264	267	309	327	333	Haplotype
GU288705	T	C	T	G	T	T	C	T	C	C	A	A	T	T	T	T	A	A	A	G	C	MvHT01
GU288698																				A		MvHT02
GU288699																						MvHT03
GU288691										T							G			A		MvHT04
GU288682																				A		MvHT05
GU288695	C																G					MvHT06
GU288703		T	A	A	C	C	T	C	C				C	A	C	C		T	T	A	T	MvHT07
GU288660		T	A	A		C	T	C	C				C	A	C	C		T	T	A	T	MvHT08
GU288672	C	T	A	A		C	T	C	C				C	A	C	C		T	T	A	T	MvHT09
GU288661		T	A	A		C	T	C	C				C	A	C	C		T	T	A	T	MvHT10
GU288673		T	A	A		C	T	C	C				C	A	C	C		T	T	A	T	MvHT11
GU288670		T	A	A		C	T	C	C				C	A	C	C		T	T	A	T	MvHT12
GU288669		T	A	A		C	T	C	C				C	A	C	C		T	T	A	T	MvHT13
GU288666		T	A	A		C	T	C	C		G	C	C	A	C	C		T	T	A	T	MvHT14

Genbank accession number	345	348	372	373	375	376	378	386	394	405	422	429	430	432	442	447	456	483	490	492	Haplotype
GU288705	T	G	A	T	A	A	T	A	T	A	C	A	C	A	C	A	A	C	C	T	MvHT01
GU288698																					MvHT02
GU288699					G																MvHT03
GU288691																					MvHT04
GU288682																G					MvHT05
GU288695					G			G													MvHT06
GU288703	C	A		C		T	C		C				C	T	T			T	T	A	MvHT07
GU288660	C	A		A		T	C		C				C	T	T			T	T	A	MvHT08
GU288672	C	A		C		T	C		C				C	T	T			T	T	A	MvHT09
GU288661	C	A		A		T	C		C	G			C	T	T			T	T	A	MvHT10
GU288673	C	A		C		T	C		C	G			C	T	T			T	T	A	MvHT11
GU288670	C	A	G	C		T	C		C	G		T	C	T	T			T	T	A	MvHT12
GU288669	C	A		C		T	C		C	G	T		C	T	T			T	T	A	MvHT13
GU288666	C	A		C		T	C		C	G			C	T	T		G	T	T	A	MvHT14

among 1st and 2nd codon positions indicated (1) no significant departures from a random distribution, (2) a correction with the gamma parameter was not required, and (3) which sites were likely most appropriate for subsequent phylogenetic analyses as described below.

A 166 residue *cox1* amino acid (aa) sequence was derived from *M. vitrata* sequence data (Supplemental Fig. S1), from which multiple sequence alignments predicted three non-synonymous aa changes, with the serine^{126} to threonine^{126} mutation present only in the ten Puerto Rican samples. Similarly, a change from leucine to methionine at aa position 125 appeared only in five African samples from Nigeria and Burkina Faso. Analysis of the predicted aa sequence suggested an overall ratio of synonymous to non-synonymous substitution mutations ($d_S/d_N = 31/3 = 10.3$), wherein >90% of observed substitutions were predicted to be silent. A codon-based *Z*-statistic supported the rejection of the null hypothesis that assumed a strictly-neutral mode of evolution within 68% of pairwise *cox1* sequence comparisons ($P \geq 0.05$). A Tajima's D statistic of −0.611 was estimated from *cox1* sequence data (number of segregating sites = $S = 147$; [53]), wherein results suggest an excess of low frequency polymorphism that may be influenced by population size expansion or selection.

Base composition disparity, transition to transversion bias, and an unequal substitution distribution among sites can affect phylogenetic inference [54]. The preceding *Maruca* sp. *cox1* sequence analysis suggested that the 1st and 2nd codon positions evolved similarly to the Kimur-2-Parameter (K2P) model of sequence evolution. A Neighbor Joining (NJ) phylogeny using a matrix of genetic distance estimates (*D*) that were constructed from 1st and 2nd codon position changes ($D^{\text{Total}} = 0.0104$) was built. The resulting strict consensus NJ tree shows a topology with three clades (total branch length = 0.061; Fig. 1a), one consisting of 100% Puerto Rican samples ($D^{\text{Lineage 3}} = 0.0004$) and the remaining two clades comprised of sequences from African, Asian, and Australian samples ($D^{\text{Lineage 1}} = 0.0158$, $D^{\text{Lineage 2}} = 0.0176$; Fig. 1a). Consistency of the predicted NJ tree topography across phylogenetic methods was inferred by congruency with a tree constructed by the minimum evolution (ME) method (Fig. 1b).

A search of the GenBank non-restricted (nr) database resulted in no accessions that correspond to *Maruca* sp. *cox1* sequence data (current 11-20-2009). Results from a specimen identification search of the Barcode of Life Database (BOLD) on November 21, 2009 showed that the sample from Nigeria (NGA1; lineage 2 in Fig. 1) has 100% nucleotide sequence similarity to a *M. vitrata* sequence sample and 95.78% similarity to a Crambid moth specimen (genus: crambiolep01 species: biolep01). The sequence from NGA1 had 92.37% nucleotide sequence similarity to 5 *Maruca* sp. samples. In contrast, the Puerto Rican sample PR1 from clade 3 showed 98.72% similarity to the BOLD specimen genus: crambiolep01 species: biolep01, and returned 31 *M. vitrata* specimens with nucleotide sequence similarities between 95.55 and 95.08% to the query sequence (Table 2).

A ~1,450 bp fragment that comprised the internal transcribed spacer region 1 (ITS1), 5.8S ribosomal RNA, and ITS2 was PCR amplified using oligonucleotides ITS4 and ITS5 from all *Maruca* sp. samples, and the ITS2 region successfully sequenced from 32 samples from African (13), Asian (15), and Puerto Rican populations (8 individuals; results not shown). Sequence alignment showed an average nucleotide frequency (25.7% A and 29.2% T), which did not significantly deviate from the expected 1/4 ratios ($\lambda^2 = 0.73$, d.f. = 1, $P \geq 0.81$), and the mean difference in base composition per site when averaged across all sequences was 0.331. The mean number of substitutions per site (nucleotide diversity = $\pi = 0.175$), transition to transversion ratio = 21/47 = 0.447, and transition to transversion bias ($R = 3.96$) was higher among the aligned ITS2 regions compared to the *cox1* sequence alignment. Homogeneity of substitution pattern across all ITS2 sequences shows a substitution pattern that significantly departs from random chance in 79 of 666 pairwise sequence comparisons (11.9%, $P \leq 0.05$ following 1,000 Monte Carlo simulations). These sequence results likely indicate that the ITS2 region is not appropriate for phylogenetic inferences due to high rate of mutation. Regardless, the observation that the genetic distances within lineage 3 ($D^{\text{Lineage 3}} = 0.236$) and lineages 1 and 2 ($D^{\text{Lineage 1 and 2}} = 0.089$) based on ITS2 region sequence data are lower compared to the genetic distance between ($D^{\text{Lineage 3 vs. Lineage 1 and 2}} = 0.318$) supports the differentiation of clades based on the prior *cox1* data.

Restriction assay and population genetics

The ~650 bp PCR-amplified mitochondrial *cox1* gene fragment was predicted to contain substitution mutations within *Sac*I or *Nsi*I restriction endonuclease cleavage sites. The *Sac*I or *Nsi*I site mutations were tested by PCR-RFLP assays of 243 individual *Maruca* sp. DNA samples, and corresponding mitochondrial haplotypes determined (Table 3). Within the dataset, PCR-RFLP assays differentiated the T30, T114 mitochondrial *cox1* haplotype (cleave ++) from the C30, C114 haplotype (cleavage −) with 100% fidelity based on corresponding sequences. Results indicate that the mitochondrial *cox1* C30, C114 (cleave −) haplotype is present within populations from Burkina Faso (9.1%), Nigeria (14.5%), Niger (2.9%), and Puerto Rico (100%). The C30, C114 haplotype was not observed in Australian ($n = 22$) or Taiwanese population samples ($n = 11$). The corresponding haplotype diversity

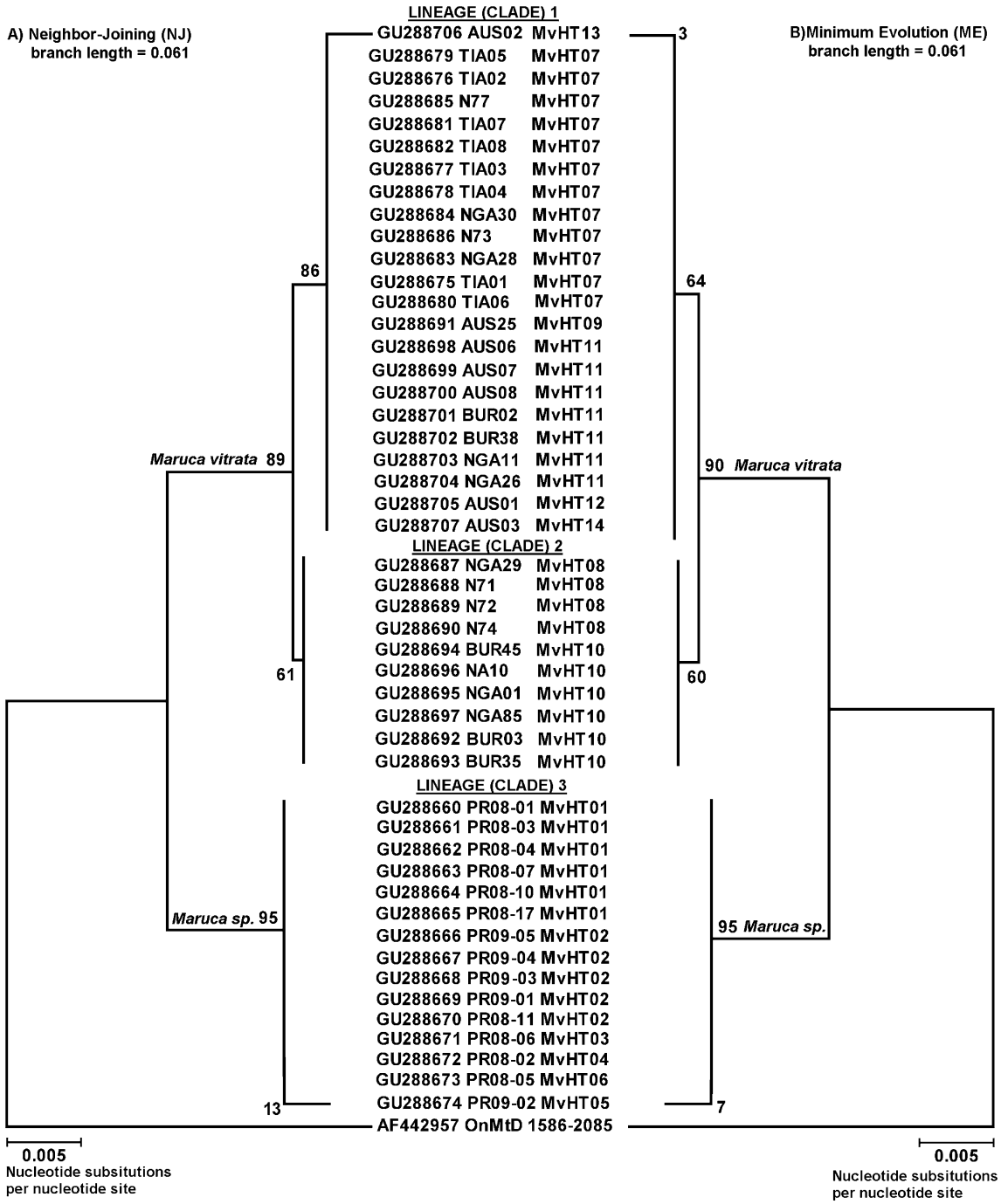

Fig. 1 Phylogenetic relationship among *Maruca* sp. based upon a 500 bp mitochondrial *cox*I gene fragments using (*A*) neighbor-joining (NJ), and (*B*) minimum evolution (ME) algorithms

(equal to the fractional proportion of individuals with a differing haplotype) ranged from 0.000 to 0.145, with samples from Australia and Taiwan being fixed for the T30, T114 haplotype and the Puerto Rican sample fixed for the C30, C114 haplotype. Samples from three African locations showed haplotype variation based on PCR-RFLP estimates (mean haplotype diversity = 0.088 ± 0.058).

Discussion

DNA sequencing and phylogenetics

The present study demonstrates that DNA barcoding of the mitochondrial *cox1* gene is a viable method of determining molecular diversity and global distribution of *M. vitrata* mitochondrial haplotypes. Molecular differentiation of

Table 2 Results from specimen identity search on Barcode of Life Database (BOLD) [49] against species level barcode records

Clade	Haplotype	BOLD species	BOLD specimen ID	% similarity
1	MvHT07	*M. vitrata*	IMLR042-08	100
1	MvHT09	*M. vitrata*	IMLR042-08	99.8
1	MvHT11	*M. vitrata*	LOQ162-04	100
1	MvHT12	*M. vitrata*	LOQ162-04	99.6
1	MvHT13	*M. vitrata*	LOQ162-04	99.8
1	MvHT14	*M. vitrata*	LOQ162-04	99.4
2	MvHT8	*M. vitrata*	PMANK371-06	99.8
2	MvHT10	*M. vitrata*	PMANK371-06	100
3	MvHT1	*biolep253*	BLPDB507-09	98.39
3	MvHT2	*biolep253*	BLPDB507-09	98.59
3	MvHT3	*biolep253*	BLPDB507-09	97.99
3	MvHT4	*biolep253*	BLPDB507-09	98.39
3	MvHT5	*biolep253*	BLPDB507-09	98.19
3	MvHT6	*biolep253*	BLPDB507-10	97.79

Sequences representing the haplotypes were used to confirm the identity

M. vitrata cox1 sequence data also was shown by both NJ- and ME-based phylogenies (Fig. 1), which suggests the presence of three distinct mitochondrial lineages or clades. A monophyletic group of all 15 *M. vitrata* sequence samples from Puerto Rico is suggested by ≥95% of bootstrap values, and correspondingly low genetic distance ($D^{\text{Lineage 3}} = 0.003$) and nucleotide diversity ($\pi^{\text{Lineage 3}} = 0.0197$) within the clade. A polyphyletic group of *M. vitrata* is predicted among *cox1* sequences obtained from Asia, Australia, and Africa, such that individuals with mitochondrial lineages 2 and 3 may be randomly distributed among the continents. Both the level of genetic differentiation within each lineage ($D^{\text{Lineage 1}} = 0.002$, $\pi^{\text{Lineage 1}} = 0.0024$; $D^{\text{Lineage 2}} = 0.002$, $\pi^{\text{Lineage 2}} = 0.0023$) and between clades 1 and 2 are low ($D^{\text{Lineage 1 vs. 2}} = 0.003$, $\pi^{\text{Lineage 1 vs. 2}} = 0.0017$), which further suggest that there is a recent common ancestry despite a broad geographic distribution among samples. By contrast, clade 3 shows a higher genetic distance compared to clade 1 ($D^{\text{Lineage 1 vs. 3}} = 0.058$) or clade 2 ($D^{\text{Lineage 2 vs. 3}} = 0.058$) than previously shown from other inter-clade comparisons. The unique genetic composition of clade 3 was further suggested by a higher total nucleotide diversity among all *M. vitrata* sequences ($\pi^{\text{Total}} = 0.0259$) compared to the estimates when partitioned by phyletic group.

The *Ostrinia* species complex is a model for speciation of Lepidoptera and provides a basis for comparison with our observations from *M. vitrata*. An estimated overall nucleotide diversity (π^{Total}) of 0.0259 among *M. vitrata cox1* sequences is similar to the range of 0.0015 to 0.0723 estimated among *Ostrinia* sp. using *cox1*I sequence data [55, 56], but higher than the 0.0130 shown between *O. nubilalis* and *O. furnacalis* mitochondrial genomes [57]. The level of mitochondrial variation among *M. vitrata* sequences also is higher than that previously observed within the species *O. furnacalis* [58] or *O. nubilalis* (0.005–0.008) [59], such that the molecular variation within *M. vitrata cox1* samples may suggest a species complex exists.

A second set of molecular evidence was collected by sequencing the internal transcribed spacer 2 (ITS2) region of a subsample of *M. vitrata* within *cox1* clades 1, 2, and 3. Subsequent ITS2 sequence analysis suggests a similar conclusion to that derived from the *cox1* analyses, namely, that *M. vitrata* from lineage 3 show a higher degree of molecular differentiation when compared to lineages 1 and 2 ($D = 0.236$) than between lineages 1 and 2 ($D = 0.089$).

The Barcode of Life Database (BOLD) is a repository of *cox1* sequence data from animal specimens and serves as a standardized framework for identification of species [49]. BOLD describes 45,097 species of Lepidoptera, for which 36,432 have associated *cox1* barcode sequence information as of November 20, 2009. A search of BOLD indicates that *cox1* sequences within clades 2 and 3 show ≥99.40% similarity to previous *M. vitrata* barcode submissions, and 92.37% similarity to *Maruca* sp. submissions. These results suggest that sequences within clades 2 and 3 likely

Table 3 The distribution of *Maruca* sp. mitochondrial *cox*I haplotype determined by *Sac*I and *Nsi*I PCR-RFLP assays (T30,T114 or C30,C114 haplotypes)

Population	Continent	Host plant	T30,T114	C30,C114	*n*	*Ht*
Australia	Australia	*Vigna mungo*	22	0	22	0.000
Burkina Faso	Africa	*Vigna unguiculata*	50	5	55	0.091
Nigeria	Africa	*Vigna unguiculata*	53	9	62	0.145
Niger	Africa	*Vigna unguiculata*	67	2	69	0.029
Taiwan	Asia	*Sesbania cannabina*	11	0	11	0.000
Puerto Rico	North America	*Phaseolus vulgaris*	0	24	24	0.000
			203	40	243	

n Number in the population sample, *Ht* estimated haplotype diversity within population

correspond to *M. vitrata sensu stricto*, and represent specimens belonging to the species *vitrata*. BOLD submissions for *Maruca* sp. show lower similarity to clades 2 and 3 sequences and provide evidence that sequence diversities $\leq$0.0763 are present within the genus. An analogous level of 0.0723 was estimated among *Ostrinia* sp. [55]. Sequence variation with *Maruca* sp. was further indicated by observation of 95.55 and 95.08% similarity of clade 1 *cox1* barcode sequence with *M. vitrata* specimens within BOLD, and that unspecified BOLD specimens from the family Crambidae that show >95.55% similarity to clade 1 samples may belong to a *Maruca* species complex.

As is the case with most of the phylogenetic analyses, the point at which sequence divergence indicates speciation is ambiguous [60, 61], but analogies with known studies of other Lepidoptera genera suggest the levels observed between clade 3 and clades 1 and 2 may indicate different species. The observation of sequence differences between *Maruca* sp. is bolstered by companion evidence showing unique non-overlapping geographic distributions. Field specimens identified as *Maruca* have been described as a collection of morphologically indistinguishable but closely-related species, which were proposed to comprise a collection of different species [2]. Despite prior field observations and taxonomic assumptions, the molecular data presented here provides phylogenetic and phylogeographic evidences for species differentiation within the genus *Maruca*.

Restriction assay and population genetics

Despite recent advances in DNA sequencing technologies, acquisition of data for the description of species differentiation at the population-level remains time-consuming and currently beyond the financial resources of most laboratories. Protocols that use molecular genetic markers to detect mutations within the DNA of target populations are available [62], of which PCR-RFLP assays allow for screening of insect populations at relatively low cost and technological requirements [63, 64]. PCR-RFLP assays are accessible to lesser-equipped laboratory facilities, which may promote the acquisition of population data from a broader range of samples. Although the genotype frequencies as determined by PCR-RFLP are prone to ascertainment bias, similar drawbacks are present for most single nucleotide polymorphism (SNP) and microsatellite markers [65, 66]. Ascertainment bias can be overcome partially by thorough sampling of specimens from all populations that are to be analyzed using downstream molecular markers [67] and determination of variation at >1 nucleotide position of each haplotype [68]. Specific steps were taken to minimize bias in our *Maruca* sp. PCR-RFLP assay development, including random coverage of individuals from all six sampled populations for *cox1* sequencing, and screening for population-level variation using two restriction enzymes (*Nsi*I and *Sac*I) diagnostic for the (C30, C114) or the (T30, T114) *cox1* haplotype (two SNP loci).

Results from the population screening confirmed conclusions that were drawn from phylogenetic analysis of *M. vitrata* DNA sequences, in that the mitochondrial haplotypes from Puerto Rican samples form a unique clade compared to other *Maruca* samples. More specifically, the (C30, C114) *cox1* haplotype defined by PCR-RFLP analysis is fixed within samples from Puerto Rico (cleave −), and provides additional evidence that clade 3 is a valid phylogenetic division within *Maruca* sp. Sequence diversity estimates and subsequent differentiation of clade 3 within NJ- and ME-base phylogenies suggested that all Puerto Rican specimens are derived from a species within the genus *Maruca* and further suggest the *Nsi*I and *Sac*I PCR-RFLP assays are diagnostic at the genus level. The *Nsi*I and *Sac*I PCR-RFLP assay also indicates that all specimens from Australia and Taiwan are the (T30, T114; cleavage ++) haplotype and suggest that *M. vitrata* haplotypes within clade 2 and 3 may be dominant in these geographic regions. It should be noted that sample numbers were low from Australia ($n = 22$) and Taiwan ($n = 11$), and more thorough sampling in the future will be required to refine estimates of species distribution.

Samples from the African continent, Burkina Faso ($n = 55$), Nigeria ($n = 62$), and Niger ($n = 69$), show a greater number of observed PCR-RFLP haplotypes compared to all other sample locations, in that both (T30, T114) and (C30, C114) types are present. Observed diversity across African sub-populations (mean = 0.088 ± 0.058) suggest that both *M. vitrata* (T30, T114; cleavage ++) haplotypes from clades 1 and 2, and the allied *Maruca* sp. (C30, C114; cleavage −) from clade 3 are present (Table 3; Fig. 1). Re-sequencing of 8 of the 16 PCR-RFLP-defined (T30, T114) haplotypes from Africa validated the fidelity of *Nsi*I and *Sac*I restriction assays in detecting the substitution mutations that resulted in elimination of enzyme recognition sites. The PCR-RFLP assays appear to be a relatively effective method for differentiation of *Maruca* sp. within lineage 3 from *M. vitrata* within clades 1 and 2. Additional field samples with associated morphological data will be required to determine if the PCR-RFLP haplotypes show fidelity in the differentiation of *M. vitrata* from other members within the genus.

Conclusions

As was observed in this study, the results from the DNA barcoding analyses lead to ambiguous species identification

when reference samples are lacking within current database versions, or may require flexible interpretation when an undescribed species is encountered [60, 61]. The diversity indices obtained, and phylogenetic analyses in the current study suggest that two *Maruca* sp. mitochondrial haplotypes are present and may represent distinct geographic distributions. Qualitative analysis of corresponding ITS2 region sequences corroborate conclusions drawn from the mitochondrial *cox1* barcode sequences. Defining species within the genus *Maruca* will require additional sequence evidence, traditional taxonomical approaches, and mating studies between the suspected species to determine mating success and the viability of any F_1 hybrids. Although several lepidopteran mitochondrial genomes have been recently sequenced [57, 69–72], the complete mitogenome for *Maruca vitrata* has not yet been sequenced. If available in the near future, a genome wide comparison of these haplotypes would shed more light on the origin of this genus. Further, previous evidence led to the hypothesis that the genus *Maruca* might have originated in Asia and spread to Africa only more recently [73, 74]. Results from our study add strength to this hypothesis and has possible implications for biocontrol programs. These findings are important to defining the genetics and ecology of this insect pest (i) for the development of effective control measures and IRM strategies prior to the release of transgenic *Bt*-toxin-expressing crops in developing nations and (ii) for the development of effective biological control programs.

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