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CYTOLOGICAL AND CYTOCHROME C OXIDASE II (COII) GENE CHARACTERIZATION OF THE BEAN PODBORER, *Maruca vitrata* (Fabricius) (LEPIDOPTERA: CRAMBIDAE)

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ABSTRACT

The bean podborer, *Maruca vitrata* (Fabricius) was characterized through its karyotype and mitochondrial *cytochrome c oxidase II* (*COII*) gene. The chromosomes are irregular in shape, with number ranging from 23 to 66, relative lengths of 0.010 to 0.073 and with no definite centromeric sites. The partial nucleotide sequence of the *COII* gene is 662 base pairs that encodes 199 amino acids. This was submitted to GenBank as *Maruca testulalis* (Geyer), synonym of *M. vitrata*, and was assigned Accession No. AY899212.1. The putative initiation codon was ATG at position 64 to 66 and encodes the amino acid methionine. The gene has high A-T content of 81% with A-T bias in codon usage that preferred the third position. The alignment of the *COII* gene with those of other members of family Crambidae that are available in GenBank, revealed that it is most related to *Heliothis virescens*.

Key words: *cytochrome c oxidase II, COII,* karyotype, *Maruca vitrata*

INTRODUCTION

Grain and vegetable legumes such as mungbean, string beans, cowpea and snapbeans are very important crops among Asians. They have comparatively high protein contents, making them cheap substitutes for meat as protein source. Much effort has been exerted to improve the yield of both grain and vegetable legumes to meet the demand of our increasing population. However, problems on insect pests have always been a threat to the stability of legume production. One of the major insect pests attacking legumes is the polyphagous bean podborer, *Maruca vitrata* (Fabricius) [=*M. testulalis* (Geyer)], which causes severe damage to flower buds and flowers as well as green pods and peduncles.

It was reported that 40-50% loss of flowers and 40% damaged pods could be caused by *M. vitrata* (Taylor 1967). According to Valdez (1989), this pest can cause higher yield loss than pre-flowering pests like leafhopper, aphids, and beanfly and requires a more expensive insecticidal control. Heavy spraying is practised by farmers to control this pest and it is being done at 2-3 days interval starting at pod stage, resulting to 11-13 sprayings for the entire growing period (Aganon et al. 1997). Considering the extent of damage that this pest inflicted, there is not much priority given in the country. Moreover, genetic studies on our local bean podborer populations are also scarce. Such studies are necessary in the development of a sound pest management strategy. The goal of this study was to evaluate the cytological and

molecular structure of the bean podborer, which will serve as benchmark information for future studies on genetic variations of this pest. Moreover, this can also be utilized to reconcile species complex issues in *Maruca*, previously reported by other authors (Taylor 1967; Margam et al. 2010).

MATERIALS AND METHODS

Damaged pods and flowers with podborer larvae were collected from the field and reared in the Entomology Laboratory, Institute of Plant Breeding. Newly emerged male adults were collected, preserved and fixed in Farmer's solution (3 parts absolute ethyl alcohol: 1 part glacial acetic acid) for cytological studies. The remaining adults were placed in glass vials and stored at -20°C for molecular studies.

The lacto-aceto-orcein squash technique described by Barrion and Manalo (2002) was used in this study. Each male adult was placed on a glass slide, the head and thorax were removed and discarded. The abdomen was macerated using bent forceps to expose the male reproductive organ, adding 1-2 drops of 1:3 acetic acid-methanol solution. The preparation was stained with lacto-aceto-orcein, then covered with glass cover slip. The prepared slide was covered with a piece of blotting paper and the cells were flattened using pencil eraser. Slides were temporarily sealed with paraffin-Canada balsam and examined under light microscope. Photomicrographs of the chromosomes during diakinesis stage were taken under oil immersion objective. The number, sizes and shapes of the chromosomes were recorded/noted. The relative length of each chromosome was computed using the formula:

Relative length = length of the chromosome/total length of all chromosomes

The protocol used in the isolation of total genomic DNA was that of Sujin et al. (2002) and Cho (2003) with a slight modification. Thoraces were pulverized in liquid nitrogen using mortar and pestle, and homogenized in buffer (sterile 10 mM NaCl, 10 mM Tris, 25 mM EDTA, and 0.5% SDS with proteinase K). The homogenate was incubated for 12-18 hours at 50°C with continuous shaking. One volume of chloroform: phenol: isoamyl alcohol (25:25:1) was added, mixed, and centrifuged for 10 min at 15,000 x g. The aqueous phase was transferred to a new centrifuge tube and mixed with 0.5 volume of sterile 7.5 M ammonium acetate. Genomic DNA was precipitated with 2 volumes of 100% cold ethanol, spooled, washed twice with 70% ethanol, dried, suspended in 0.1 ml of 1.0 mM Tris, 0.1 M EDTA and stored frozen until used.

The quality and quantity of the DNA isolated were determined using a 1% Low Electroendosmosis (LE) agarose in 1X Tris-borate-EDTA (TBE). A 50 ml agarose gel containing 45 ml nanopure water, 5 ml sterile 10X TBE, and 0.5 g agarose was prepared in a mupid gel moulder. For each DNA sample, 3 µL of aliquot was dispensed in a tube containing 17 µL of 1X loading buffer to make 20 µL volume on the prepared gel starting on the fifth well. Ten µL of prepared lambda standards (Sigma): 5, 10, 20, and 40 ng/µL, were loaded in the first four wells of the gel. The gel was run at 100 V for 15 min, then viewed in the UV trans-illuminator. A photograph of the gel was taken using Polaroid Land Camera and the concentrations (in ng/µL) of the DNA samples were estimated by comparing the intensity of the fluorescence with those of the standards used.

The amplification of the mitochondrial *cytochrome c oxidase II* (*COII*) gene was done through polymerase chain reaction using the primer pair: forward 5'-ATGGCAGATTAGTG-3' and reverse 5'-GTTTAAGAGACCAGTACTTG-3' (Choong-on et al. 1999). PCR amplifications were carried out in 20 µL volume containing 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer and 1 U Taq polymerase using the PTC Thermo Cycler (MJ Research). The following amplification conditions were used: denaturation at 94°C for 30 sec, annealing at 47°C for 1 min, and extension at 70°C for 2 min for 35 cycles. A final single cycle was performed following the same conditions, except that the extension step was done for 7 min.

The amplicon was ligated into plasmid p-GEM-T Easy using the p-GEM-T Easy vector system (Promega) and transformed into competent *Escherichia coli* cells. The clone was submitted for sequencing using PRISM DNA sequencer (Applied Biosystems, Inc.) at Michigan State University DNA Sequence and Synthesis facility. The *COII* gene amino acid sequence was compared with those o other *M. vitrata* and *M. testulalis* accessions reported in the GenBank using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and aligned with othe crambid species using NCBI COBALT (http://www.st-va.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi?).

RESULTS AND DISCUSSION

The cytological characteristics of *M. vitrata* adult males are shown in Figure 1. The chromosomes are irregular in shape and vary from rod, oblong, pear-shaped, ovoid, droplet-like, to circular. The chromosome count ranged from 23 to 66 with modal chromosome number of 26. The relative lengths varied from 0.010 to 0.073 for the shortest and longest chromosomes, respectively. The chromosomes observed from all populations did not show or possess definite centromeric sites.

The results obtained are typical of most insect groups, including Lepidoptera, that possess extreme variability in the chromosome number. Demayo et al. (1994) reported that rice leaffolders *Cnaphalocrosis patnalis* (Bradley) (=*Marasmia patnalis* Bradley) and *Cnaphalocrosis medinalis* (Guenée) had chromosome numbers of 7-75 and 10-75, respectively. The chromosomes are holokinetic with modes of 28 for *C. patnalis* and 30 for *C. medinalis*. In carob moth, *Ectomyelois ceratoniae* (Zeller), the mitotic spermatogonia chromosomes observed by Mediouni et al. (2004) were 62, of holokinetic type, mostly rod-shaped and lacking primary constrictions. Meanwhile, codling moth, *Cydia pomonella* (L.), karyotype showed 56 rod-shaped chromosomes, also of holokinetic type, and likewise devoid of centromeres (Fukova et al. 2005).

The procedure developed by Sujin et al. (2002) and Cho (personal communication, 2003) was successfully used in the isolation of total genomic DNA from *M. vitrata*. A slight modification was employed due to the small size of the specimens. Thus, instead of isolating DNA from a single individual, five insects were used. This was originally developed for *H. armigera* but also worked well on *M. vitrata* and yielded positive results (data not shown). The protocol was suitable for use even for dried or improperly preserved podborer.

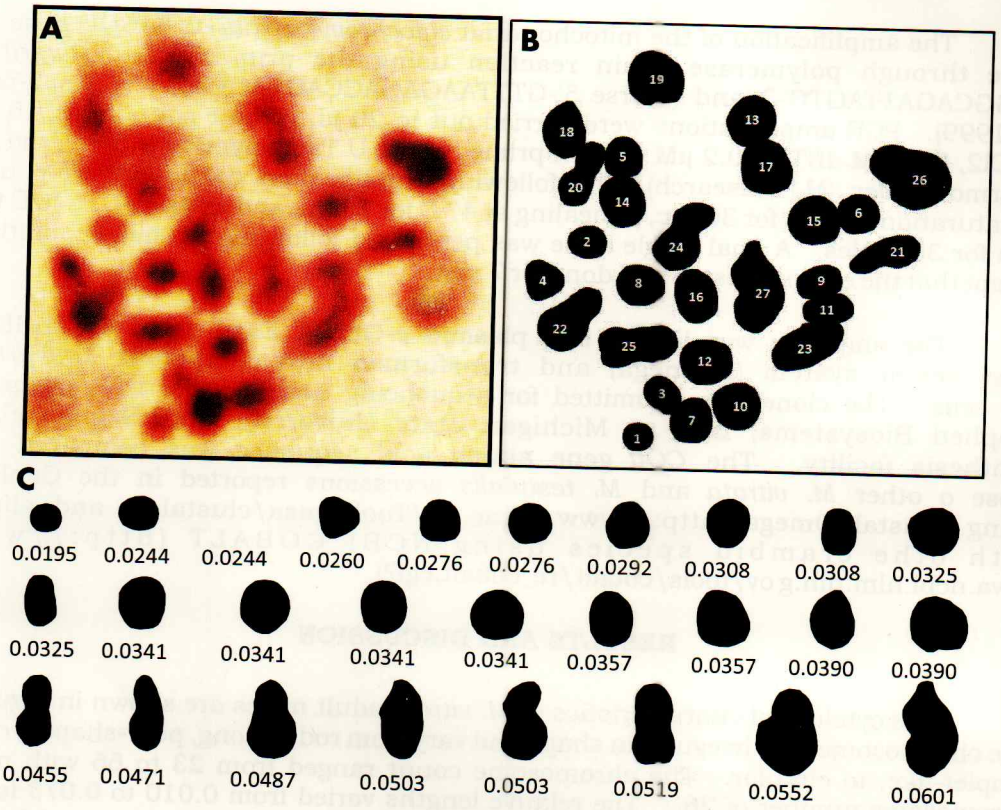

Figure 1. Representative karyogram of *Maruca vitrata* (Fabricius) showing photomicrograph (A) interpretative drawings (B) of 27 chromosomes with respective relative length (C).

The partial mitochondrial DNA sequence of the *COII* gene of *M. vitrata* is presented in Figure 2. The nucleotide sequence was submitted to GenBank and was assigned the accession number AY899212.1. The partial *COII* gene obtained is composed of 662 base pairs and encodes 199 amino acids. The putative initiation codon was ATG at position 64-66 and encodes for the amino acid methionine. The initiation codon is similar to that obtained by Margam et al. (2011), Agunbiade et al. (2014), and Zou et al. (2014) with GenBank Accession # HM751150.1, KJ466365.1, and KJ623250.1 respectively (www.ncbi.nlm.nih.gov/genbank). On the other hand, Coates et al. (2005) reported that the initiation codon in crambid species like *Ostrinia furnacalis* (Guenee) and *O. nubilalis* (Hübner), was ATA, which also codes for methionine. However, the *COII* gene sequences obtained by these authors were 682 base pairs. Since the gene sequence obtained in this study was shorter at 662 base pairs, the termination codon cannot be distinctively determined but reports on *M. vitrata* and *Ostrinia* spp. were set at single nucleotide T (Margam et al. 2011; Coates et al. 2005). Morlais and Severson (2002) reported that TCG (Ser) is used as the initiation codon in dipterans and further suggested that the start codon may differ among insects.

1	ATT	ATG	GCA	GAT	TAG	TGC	AAT	GGA	TTT	AAA	30
31	CCC	CAT	TTA	TAA	AGG	ATT	ATC	CTT	TTT	TTA	60
61	GAA	ATG	GCA	ACA	TGA	TCA	AAT	CTT	AAT	TTA	90
		M	A	T	W	S	N	L	N	L	
91	CAA	AAT	GGA	GCA	TCT	CCT	TTA	ATA	GAA	CAA	120
	Q	N	G	A	S	P	L	M	E	Q	
121	ATT	ATT	TTT	TTT	CAC	GAT	CAC	ACT	TTA	ATT	150
	I	I	F	F	H	D	H	T	L	I	
151	ATT	TTA	ATT	ATA	ATT	ACA	ATT	TTA	GTT	GGT	180
	I	L	I	M	I	T	I	L	V	G	
181	TAT	TTA	ATA	ATA	AGA	TTA	ATA	TTT	AAT	AAA	210
	Y	L	M	M	S	L	M	F	N	K	
211	TAT	ATT	AAT	CGA	TTT	TTA	TTA	GAA	GGT	CAA	240
	Y	I	N	R	F	L	L	E	G	Q	
241	ATA	ATT	GAA	TTA	ATT	TGA	ACT	ATT	TTA	CCA	270
	M	I	E	L	I	W	T	I	L	P	
271	GCA	ATT	ACT	TTA	ATT	TTT	ATT	GCC	CTA	CCA	300
	A	I	T	L	I	F	I	A	L	P	
301	TCT	TTA	CGA	TTA	TTA	TAT	TTA	TTA	GAT	GGA	330
	S	L	R	L	L	Y	L	L	D	G	
331	TTA	AAT	AAT	CCC	TTA	ATT	ACC	CTA	AAA	TCA	360
	L	N	N	P	L	I	T	L	K	S	
361	ATT	GGA	CAT	CAA	TGA	TAT	TGA	AGA	TAT	GAA	390
	I	G	H	Q	W	Y	W	S	Y	E	
391	TAT	TCA	GAC	TTT	CAC	AAT	ATT	GAA	TTT	GAT	420
	Y	S	D	F	H	N	I	E	F	D	
421	TCT	TAT	ATA	ATT	CCA	TCA	AAT	GAA	ATA	ACT	450
	S	Y	M	I	P	S	N	E	M	T	
451	CCT	AAT	AGA	TTT	CGT	TTA	CTA	GAT	GTA	GAT	480
	P	N	S	F	R	L	L	D	V	D	
481	AAT	CGA	ATT	GTT	CTA	CCA	ATA	AAT	AAT	CAA	510
	N	R	I	V	L	P	M	N	N	Q	
511	ATT	CGA	ATT	ATA	GTT	ACA	GCA	ACA	GAT	GTA	540
	I	R	I	M	V	T	A	T	D	V	
541	ATT	CAC	TCT	TGA	ACT	ATC	CCA	TCA	TTA	GGG	570
	I	H	S	W	T	I	P	S	L	G	
571	GTA	AAA	GTA	GAT	GCT	AAC	CCA	GGA	CGT	CTT	600
	V	K	V	D	A	N	P	G	R	L	
601	AAT	CAA	ACT	AAT	TTT	TAT	ATT	AAT	CGC	CCT	630
	N	Q	T	N	F	Y	I	N	R	P	
631	GGA	ATT	TTT	TAT	GGT	CAA	TGC	TCT	GAA	ATT	660
	G	I	F	Y	G	Q	C	S	E	I	
661	TG										

Figure 2. Nucleotide sequence of *cytochrome c oxidase* (*COII*) gene (Genbank Acc. No. AY899212.1) of *Maruca vitrata* (syn. *M. testulalis*), collected from the Philippines, following the invertebrate mitochondrial translation table. The predicted amino acid sequence is shown below the nucleotide sequence.

Table 1. Frequency of codons in *cytochrome c oxidase II* (*COII*) of *Maruca vitrata* (Genbank Acc. No. AY899212.1) from the Philippines following the invertebrate mitochondrial code.

Codon	Amino Acid	Freq	Codon	Amino Acid	Freq	Codon	Amino Acid	Freq	Codon	Amino Acid	Freq
ATT	I	25	TAT	Y	9	CTT	L	2	GTT	V	3
ATC	I	1	TAC	Y	0	CTC	L	0	GTC	V	0
ATA	M	10	TAA	Ter	0	CTA	L	4	GTA	V	4
ATG	M	1	TAG	Ter	0	CTG	L	0	GTG	V	0
ACT	T	6	TCT	S	5	CCT	P	3	GCT	A	1
ACC	T	1	TCC	S	0	CCC	P	1	GCC	A	1
ACA	T	4	TCA	S	5	CCA	P	6	GCA	A	4
ACG	T	0	TCG	S	0	CCG	P	0	GCG	A	0
AGT	S	0	TGT	C	0	CGT	R	2	GGT	G	3
AGC	S	0	TGC	C	1	CGC	R	1	GGC	G	0
AGA	S	3	TGA	W	5	CGA	R	4	GGA	G	5
AGG	S	0	TGG	W	0	CGG	R	0	GGG	G	1
AAT	N	16	TTT	F	10	CAT	H	1	GAT	D	7
AAC	N	1	TTC	F	0	CAC	H	4	GAC	D	1
AAA	K	3	TTA	L	21	CAA	Q	7	GAA	E	7
AAG	K	0	TTG	L	0	CAG	Q	0	GAG	E	0

Table 2. Base composition of *cytochrome c oxidase II* (*COII*) gene of *Maruca vitrata* (Genbank Acc. No. AY899212.1) from the Philippines at the three codon positions.

Position	Base Composition							
	A		T		C		G	
	Freq	%	Freq	%	Freq	%	Freq	%
1st	71	35.7	56	28.1	35	17.6	37	18.6
2nd	56	28.1	81	40.7	37	18.6	25	12.6
3rd	92	46.2	93	46.7	12	6.0	2	1.0

Table 3. Percent homology of *cytochrome c oxidase II* (*COII*) amino acid sequences among the *Maruca vitrata* (syn. *M. testulalis*) reported in the GenBank.

Species GenBank Acc. No.	*M. testulalis** AAW81695.1	*M. vitrata* ADO13503.1	*M. testulalis* YP_009040742.1	*M. testulalis* AIA77352.1	*M. vitrata* AHY24045.1
M. testulalis* **AAW81695.1**	100.00	93.47	93.97	93.97	93.47
M. vitrata **ADO13503.1**		100.00	99.12	99.12	97.80
M. testulalis **YP_009040742.1**			100.00	100.00	97.80
M. testulalis **AIA77352.1**				100.00	97.80
M. vitrata **AHY24045.1**					100.00

*Philippine pod borer population.

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M. testulalis AAW81695.1     MATWSNLNLQNGASPLMEQIIFFHDHTLIILIMITILVGYLMMSLMFNKYINRFLLEGQM
M. vitrata ADO13503.1       MATWSNFNLQNGASPLMEQIIFFHDHTLIILMITVLVGYLMLSLFFNKYINRFLLEGQM
M. testulalis YP_009040742.1 MATWSNFNLQNGASPLMEQIIFFHDHTLIILIMITVLVGYLMLSLFFNKYINRFLLEGQM
M. testulalis AIA77352.1    MATWSNFNLQNGASPLMEQIIFFHDHTLIILIMITVLVGYLMLSLFFNKYINRFLLEGQM
M. vitrata AHY24045.1      MATWSNFNLQNGASPLMEQIIFFHDHTLIILIMTTILVGYLMLSLFFNKYINRFLLEGQM
              *******.************************** *.*******.**.***************

M. testulalis AAW81695.1     IELIWTILPAITLIFIALPSLRLLYLLDGLNNPLITLKSIGHQWYWSYEYSDFHNIEFDS
M. vitrata ADO13503.1       IELIWTILPAITLIFIALPSLRLLYLLDELNNPLITLSSIGHQWYWSYEYSDFNNIEFDS
M. testulalis YP_009040742.1 IELIWTILPAITLIFIALPSLRLLYLLDELNNPLITLKSIGHQWYWSYEYSDFNNIEFDS
M. testulalis AIA77352.1    IELIWTILPAITLIFIALPSLRLLYLLDELNNPLITLKSIGHQWYWSYEYSDFNNIEFDS
M. vitrata AHY24045.1      IELIWTILPAITLIFIALPSLRLLYLLDELNNPLITLKSIGHQWYWSYEYSDFNNIEFDS
              ************************** ********.************** ******

M. testulalis AAW81695.1     YMIPSNEMTPNSFRLLDVDNRIVLPMNNQIRIMVTATDVIHSWTIPSLGVKVDANPGRLN
M. vitrata ADO13503.1       YMTPVNEMNNNSFRLLDVDNRIVLPMGNQIRIMVTATDVIHSWTIPSLGVKVDANPGRLN
M. testulalis YP_009040742.1
YMTPVNEMNSNSFRLLDVDNRIVLPMGNQIRIMVTATDVIHSWTIPSLGVKVDANPGRLN
M. testulalis AIA77352.1    YMTPVNEMNSNSFRLLDVDNRIVLPMGNQIRIMVTATDVIHSWTIPSLGVKVDANPGRLN
M. vitrata AHY24045.1      YMTPMNEMNNNNFRLLDVDNRIVLPMGNQIRIMVTATDVIHSWTIPSLGVKVDANPGRLN
              ** * ***. *.************** ********************************

M. testulalis AAW81695.1     QTNFYINRPGIFYGQCSEI----------------------------
M. vitrata ADO13503.1       QTNFFINRPGIFYGQCSEICGANHSFMPIVIESISIKNFINWINNYS
M. testulalis YP_009040742.1 QTNFFINRPGIFYGQCSEICGANHSFMPIVIESISIKNFINWINNYS
M. testulalis AIA77352.1    QTNFFINRPGIFYGQCSEICGANHSFMPIVIESISIKNFINWINNYS
M. vitrata AHY24045.1      QTNFFINRPGIFYGQCSEICGANHSFMPIVIESISIKNFINWINNYS
              ****.***************
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Figure 3. Amino acid sequence alignment of cytochrome c oxidase II (COII) of *Maruca vitrata* (syn. *M. testulalis*) collected from the Philppines (GenBank. Protein ID # AAW81695.1) with other *M. vitrata* and *M. testulalis* COII protein sequences in the GenBank.

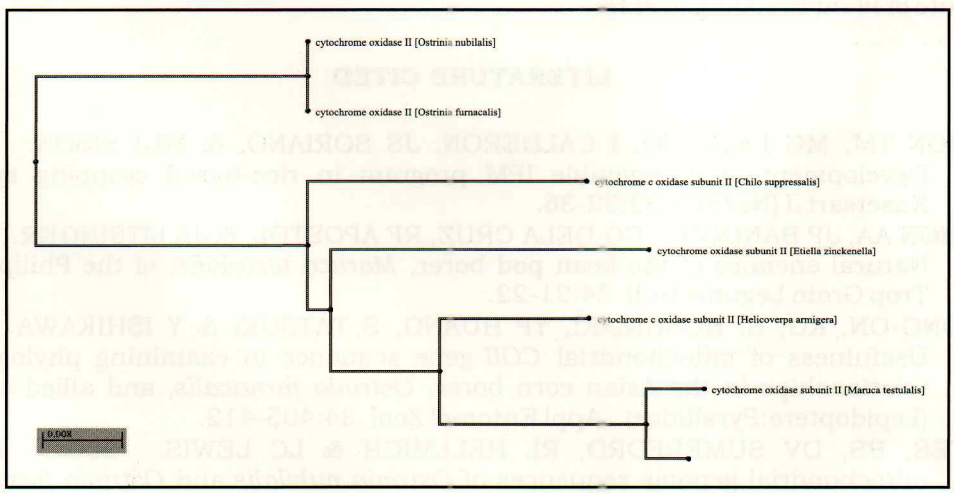

Figure 4. Cluster analysis (gene tree) of *COII* gene sequences created from the protein multiple sequence alignments among different members of the family Crambidae (*Maruca testulalis, Heliothis virescens, Helicoverpa armigera, Etiella zinckenella, Chilo suppressalis, Ostrinia furnacalis,* and *O. nubilalis* with their protein ID # AAW81695.1, AEF33917.1, BAH23861.1, BAH23057.1, AAY54543.1, ABX64445.1, AEV54861.1, respectively).

The *M. vitrata COII* gene has high A-T content, which is 81% of the entire sequence. The high A-T content is comparable to those in other species as expected for an insect mitochondrial *COII* gene (Frati et al. 1996; Choong-on et al. 1999). This was reflected by the abundance of A-T containing amino acids such as isoleucine (I), leucine (L), glutamine (N), methionine (M), and phenylalanine (F), which accounted for 41.2% of the amino acids encoded (Table 1). It was also observed that this A-T bias in codon usage preferred the third position with 92.9% as compared to the 1st and 2nd positions with only 63.8% and 68.8%, respectively (Table 2).

The deduced amino acid sequence of obtained *COII* gene was aligned with the complete genome of *M. vitrata* and *M. testulalis* accessions available in the NCBI GenBank. The Philippine population of *M. vitrata* (GenBank Acc. No. AAW81695.1) showed 93-94% homologies with the submitted *COII* genes and is most similar to those reported by Zou et al. (2014; GenBank Acc. No. YP_009040742.1).

Also, alignment with the other members of family Crambidae showed that, among those with sequences available at GenBank, the bean podborer is most closely related to *Heliothis virescens* (Fabricius) followed by *Helicoverpa armigera* (Hübner), *Etiella zinckenella* (Treitschke) and *Chilo suppressalis* (Walker). The two species of *Ostrinia* formed a separate cluster and seemed to be the most distant relatives in this particular analysis.

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