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RAPD-PCR characterization of *Bacillus thuringiensis* soil isolates from Mizoram with insecticidal activity against *Culex tritaeniorhynchus*

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Abstract

Background & Aim: Vector-borne diseases are one of the major public health problems in developing countries. *Culex tritaeniorhynchus* (Diptera: Culicidae) could be a vector of filariasis and West Nile encephalitis. The use of entomopathogenic bacteria like *Bacillus thuringiensis* (Bt) as larvicides is a viable alternative for the biological control mosquitoes with a low environmental impact. Hence, an attempt was made to explore the abundance of Bt in Mizoram soils from different habitats, testing their toxicity against *C. tritaeniorhynchus* and characterization by following acetate selection, mortality, dissimilarity analysis and RAPD-PCR assay methods. **Method:** In this present study, nine soil isolates (Mzubt 2,4,5,6,11,23,25,26 and 29) were identified, characterized and evaluated against *C. tritaeniorhynchus.* The new isolates were compared with two reference strains - Bt *israelensis* 4Q1 and Bt *aizawai* 4J3 obtained from Bacillus Genetic Stock Center, USA. **Results:** The mzubt 2 and mzubt 6 isolates showed higher larvicidal activity than other isolates, against *C. tritaeniorhynchus*. RAPD-PCR assay has been optimized that discriminate *Bacillus thuringiensis* (Bt) isolates from nine different habitats soil covering five districts of Mizoram. All the studied isolates showed a diverse RAPD patterns and were different from each other in relation to habitats, toxicity and type of *cry* gene present. High polymorphism was observed between Bt isolates which was authenticated through high PIC, RP, EMR and MI values. Further, three major clusters were identified through dissimilarity analysis. **Conclusion:** No relationship found between the type of cry gene in Bt and their toxicity against mosquitoes. The importance of the isolation of native strains of Bt in the mosquito management program in Mizoram is highlighted based on the findings.

Keywords: Bt isolates, toxicity, RAPD-PCR, dissimilarity analysis

1. Introduction

Bacillus thuringiensis Berliner (Bt) is a spore-forming bacterium producing crystal proteins which is toxic to insects[1]. The crystals (Cry) are composed of proteins, or δ-endotoxins, varying in quantity and type depending on the Bt strain. Each type of crystal protein is characterized by a specific host range, and based upon differences in sequence and specificity, insecticidal crystal δ-endotoxins have been classified into more than 300 groups of Cry proteins[2].

Random amplification of polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR) is one of the marker techniques widely used in genome characterization using a single primer which is able to anneal and prime at multiple location throughout the genome producing a spectrum of polymorphic amplification products. The advantage of RAPD is that no prior knowledge of the genome is necessary[3-5]. RAPD analysis has been applied to differentiate isolates of Bt strains[6].

The distribution of this bacterium in Mizoram soil is abundant and identified the cry gene content of the Bt isolates using PCR[7]. In continuation of the earlier study, Bt strains were isolated from soils of different habitats to know the geographical diversity with the prediction of their insecticidal activity through polymerase chain reaction (PCR).

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2. Materials and Methods

2.1 Isolation of Bt from soil samples

A total of 100 soil samples from nine different habitats [Jhum, Shrub, River bed, Fish pond, Grass, Flower garden, Wayside (Road), Barren land] covering five districts (Champhai, Lunglei, Aizawl, Lawngtlai, Mamit) in Mizoram, India were used for isolation of *Bt*. All the soil samples were collected aseptically from top to a depth of 10 cm after scrapping off the surface material with a sterile spatula. Samples were stored in labeled sterile plastic bags at room temperature until processed.

Bt strains were isolated employing the technique developed by Travers et al.[8] from the soil samples of different locations of Mizoram. Soil samples (1 g) were incubated in a shaker for 4 hours at 37°C in 10 ml of Luria Broth (LB) buffered with 0.25 M sodium acetate (pH 6.8). 1 ml of the sample was serially diluted to 103 times, heat shocked at 80°C for 3 – 5 minutes. Then the samples were spread on LB agar plates. Two standard strains – *Bt israeliensis, Bt aizawai* were provided by Dr.Zeigler (BGSC, USA) were used. The selected strains are streaked on LB agar and incubated overnight at 37°C and are then subsequently then inoculated in LB broth overnight in a shaker at the same conditions. A total of 20 - 30 isolates were selected after identification and biochemical characterization[6].

2.2 Genomic DNA Isolation and Quantification

DNA was extracted and was used as a template for PCR[9]. The cultures were incubated overnight at 30°C in LB agar at 37˚C. After 16-20 hrs one loop full of culture was transferred to 300µl of milliQ water and vortexed. It was then kept in - 80˚C for 15 minutes. The frozen DNA was immediately transferred to boiling water and kept for 10 minutes. The resulting cell lysate was briefly spun at 6000rpm for 3-4 seconds. The supernatant was used as the DNA template. The genomic DNA was quantified and diluted to 50ng/µl using Biophotometer Plus (Eppendorf).

2.3 RAPD-PCR profiling

Twenty six random primers (Bangalore's genei) were screened to differentiate the Bt isolates (Table I). Amplification reactions were carried out in 10µl volumes containing 2mM Tris - Hcl taq buffer, 1.5 mM of MgCl2, dNTP 2mM, BSA 0.8 %, primer 0.4µM, taq polymerase

TableI: Primer used for RAPD-PCR analysis

Primer name	Primer Sequence (5'—3')	Primer name	Primer Sequence (5'—3')
BT-1	CAGGCCCTTC	BT-14	CCGGCGGCGC
BT-2	CAATCGCCGT	BT-15	TGCCGAGCTG
BT-3	TCATCGCGCT	BT-16	CAAACGTCGG
BT-4	GCGATCCCCA	BT-17	GAGAGCCAAC
BT-5	CAGCACCCAC	BT-18	ACGGCCGACC
BT-6	GTGAGGCGTC	BT-19	CGCCCCCATT
BT-7	GAACGGACTC	BT-20	TGCAGTCGAA
BT-8	GGTGCGGGAA	BT-21	AGGCCGCTTA
BT-9	GTTTCGCTCC	BT-22	CCGGGCAAGC
BT-10	AAGAGCCCGT	BT-23	AGGATCAAGC
BT-11	AACGCGCAAC	BT-24	CAGGCGCACA
BT-12	CCCGTCAGCA	BT-25	AAACAGCCCG
BT-13	ACGCGCCCTA	BT-26	TGTCAGCGGT

1 unit, and 50 ng of template DNA. The PCR program was as follows - 4 min at 94°C, 35 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 2 min followed by a final extension for 5 min at 72°C. Amplified DNA fragments were analyzed in 1.5% agarose gel at 50 volt in 1x TAE buffer[10]. Agarose gels are visualize using UVP gel documentation system and analyzed by Doc-ITLS image analysis software (UVP, Cambridge, UK).

2.4 Data analysis

Amplified products were scored as either present (1) or absent (0). A data matrix was prepared to determine the genotypes. The data matrix was used to calculate dissimilarity using the Jaccard function supported by Darwin 5 software.

$$d_{ij} = \frac{b+c}{a+(b+c)}$$

Where, d_{ij}: dissimilarity between units *i* and *j*

a: number or variables where X_i = presence and X_j = presence; *b*: number or variables where X_i = presence and X_j = absence; *c*: or variables where X_i = absence and X_j = absence.

Cluster analysis and factorial and co-ordinates analysis of the cluster was done by the same software. Based upon the above method, phylogenetic tree is being created. The reliability and robustness of the phenograms were tested by bootstrap analysis for 5,000 bootstraps for computing probabilities in terms of percentage for each node of the tree using the DARwin software[11].

The genotyping data from RAPD PCR was further used for assessing the discriminatory power of the primers by evaluating six parameters of the following: - polymorphism percentage, frequency, polymorphism information content (PIC), resolving power (RP), effective multiplex ratio (EMR) and marker index (MI). The PIC of each RAPD marker was computed as $PIC_i = 2f_i\ (1 - f_i)$; where PIC_i is the polymorphic information content of the marker *i*, f_i is the frequency of the amplified allele (band present), and $(1-fi)$ is the frequency of the null allele[12]. PIC was averaged over the fragments for each primer combination. The MI was calculated using formula, $MI = PIC - EMR$, where, effective multiplex ratio (EMR) is the total number of polymorphic loci/fragments per primer[11]. Resolving Power, this is based on the distribution of alleles within the sampled genotypes. Resolving power of each primer combination was calculated using formula, $RP=\Sigma I_b$; where, I_b represents band informativeness expressed as $I_b = 1 - (2 X I0.5 - pI)$, where, *p* is the fraction of the total accessions in which the band is present[13].

2.5 Larvicidal bioassay

Culex tritaeniorhynchus larvae were collected from a fish pond in Lengpui, Aizawl, Mizoram and reared at the Department of Biotechnology, Mizoram University, Aizawl, Mizoram[14]. The third instar larvae used in bioassay belonged to the 2nd generation and were maintained at 27 ± 2°C with 65 ± 5% relative humidity and 12 h photoperiod.

One ml of the Bt broth containing of 8 x 10^5 cells/ml were added to 250 ml deionized water previously put in a 500 ml glass beaker (10 x 5 cm), where larvae of third instar of *C. tritaeniorhynchus* were individualized (50 insects per isolate). In controls, the broth was replaced by 100 µl of sterile deionized water[14]. The mortality was evaluated up to seven days after treatment. The data were corrected according to Abbot formula[15] and submitted to Tukey's multiple range test (P = 0.05)[16].

3. Results and Discussion

The survey work of Bt distribution in different habitats was done during 2009 - 2012. Bt was found to be ubiquitous in different habitats like jhum, aquatic (river bed and fish pond), grass, shrub, garden, barren land and

wayside (Road) (Table II). The Bt index ranged from 0.010 to 0.015. The Bt was abundant in aquatic habitat followed by forest and agricultural habitats. The Bt index was least in wayside and barren land (Table II). The toxicity of Bt against *C. tritaeniorhynchus* ranged between 60 and 95%. The Bt isolates Mzubt 2,4 and 6 exhibited 95% mortality against *C. tritaeniorhynchus*, whereas Mzubt 5,11,23 and 25 exhibited 70 - 75% mortality (Table II). The toxicity varied with different Bt isolates. From this study, it was observed that Mizoram soil is rich in Bt isolates showing toxicity towards dipteran insects. This is in accordance with our previous study on identification of dipteran specific cry gene 2, 4, 9 and 11 in Mizoram soil[7]. The study carried out by González et al[18] with Bt isolates from Cuba showed higher larvicidal activity than reference strain against both *A. aegypti* and *C. quinquefasciatus*. The variation within the type of cry genes was observed in the chosen Bt isolates. There is no relationship between the number of cry genes present and insect toxicity (Table II).

Of the 26 random primers screened (Table I), only 15 primers produced clear and highly reproducible amplicons and were considered for further analysis (Table III). The total number of polymorphic bands

Table II: *Bacillus thutingiensis* isolates from soil with site of collection, habitat and *cry* genes detected.

No.	Bt Strain ID	Site	Vegetation	Bt index[@]	Mortality (%)	Cry gene(s) present[6]
1	*Mzubt-2*	Chhippui	Jhum	0.012	95	Cry 2,9
2	*Mzubt-4*	Campus	Shrub	0.014	95	Cry 2,4
3	*Mzubt-5*	Chhimtuipui	River bed	0.015	75	Cry 2,3,4,9
4	*Mzubt-6*	Ramrikawn	Fish pond	0.014	95	Cry 2,3,9
5	*Mzubt-11*	Serkawr	Grass	0.014	70	Cry 1,2,4
6	*Mzubt-23*	Champhai	Grass	0.013	75	Cry 1,2,9
7	*Mzubt-25*	Lunglei	Flower garden	0.012	75	Cry 1,4,9
8	*Mzubt-26*	Chhippui	Wayside (Road)	0.011	80	Cry 1,9
9	*Mzubt-29*	Serkawr	Barren land	0.010	90	Cry 4,9
10	*Bt israelensis 4Q1*	Standard strains obtained from Bacillus Genetic Stock Center, The Ohio State University, Co-lumbus, USA			90	Cry 4, 11
11	*Bt aizawai 4J3*				60	Cry 1, 2, 7, 8, 9

[@]The *Bt* index was calculated for each sample as the number of isolates of *Bt*/number of isolates of sporulated bacilli

Table III: Polymorphism in Bt isolates using RAPD analysis

Sl. No.	Primer used	Total no. of bands	Poly morphic bands	Mono morphic band(s)	Poly mor phism %	Frequency	PIC	RP	EMR	MI
1	BT-1	112	101	1	90.17	0.299	0.419	0.598	0.901	0.378
2	BT-3	44	33	1	75	0.181	0.296	0.362	0.750	0.222
3	BT-5	119	108	1	90.75	0.373	0.467	0.746	0.907	0.424
4	BT-6	96	85	1	88.54	0.241	0.365	0.482	0.885	0.323
5	BT-7	45	45	0	100	0.240	0.364	0.480	1.000	0.364
6	BT-8	94	72	2	76.59	0.294	0.415	0.588	0.765	0.317
7	BT-10	93	82	1	88.17	0.242	0.366	0.484	0.881	0.323
8	BT-11	109	98	1	89.9	0.267	0.391	0.534	0.899	0.351
9	BT-12	66	55	1	83.34	0.272	0.396	0.544	0.833	0.330
10	BT-15	156	134	2	85.89	0.429	0.489	0.858	0.858	0.420
11	BT-16	86	75	1	87.2	0.289	0.410	0.578	0.872	0.358
12	BT-17	108	97	1	89.81	0.350	0.455	0.700	0.898	0.408
13	BT-18	123	112	1	91.05	0.338	0.447	0.676	0.910	0.407
14	BT-23	100	89	1	89	0.303	0.422	0.606	0.890	0.375
15	BT-24	117	106	1	90.59	0.397	0.478	0.794	0.905	0.433
Total		1468	1292	16	88.01	4.515	6.188	9.030	0.880	0.363
						Average	0.412	0.602	0.877	0.362

PIC - Polymorphism information content; RP - resolving power; EMR - effective multiplex ratio; MI - marker index

observed was 1292, with BT 15 primer producing the maximum of 134 bands whereas BT7 primer produced a minimum of 45 bands. Polymorphism was found between Bt isolates from different habitats. The percent polymorphism ranged from 75 to 100%. The average PIC - 0.412 (0.296 – 0.478); RP - 0.602 (0.36 – 0.858); EMR - 0.877 (0.750 – 1.00) and MI - 0.362 (0.222 – 0.430) values were high showing better discriminatory power and marker efficiency. The polymorphism between isolates was high and the 15 primers, used in the present study, were efficient in differentiating the Bt isolates (Table III). The chosen primers produced a range of 3 to 14 bands, allowing confirming at least eight different groups per primer. RAPD reactions with the 15 primers yielded characteristic products ranging from 150 to 2500 bp. High number of amplification products was obtained with the primers BT1,10,11,15,18 and 24 followed by moderate (BT 5,6,8,12,16,17 and 23) and low by BT3 and 7 (Figure I). RAPD analysis of Bt isolates in Jordan revealed high polymorphism between

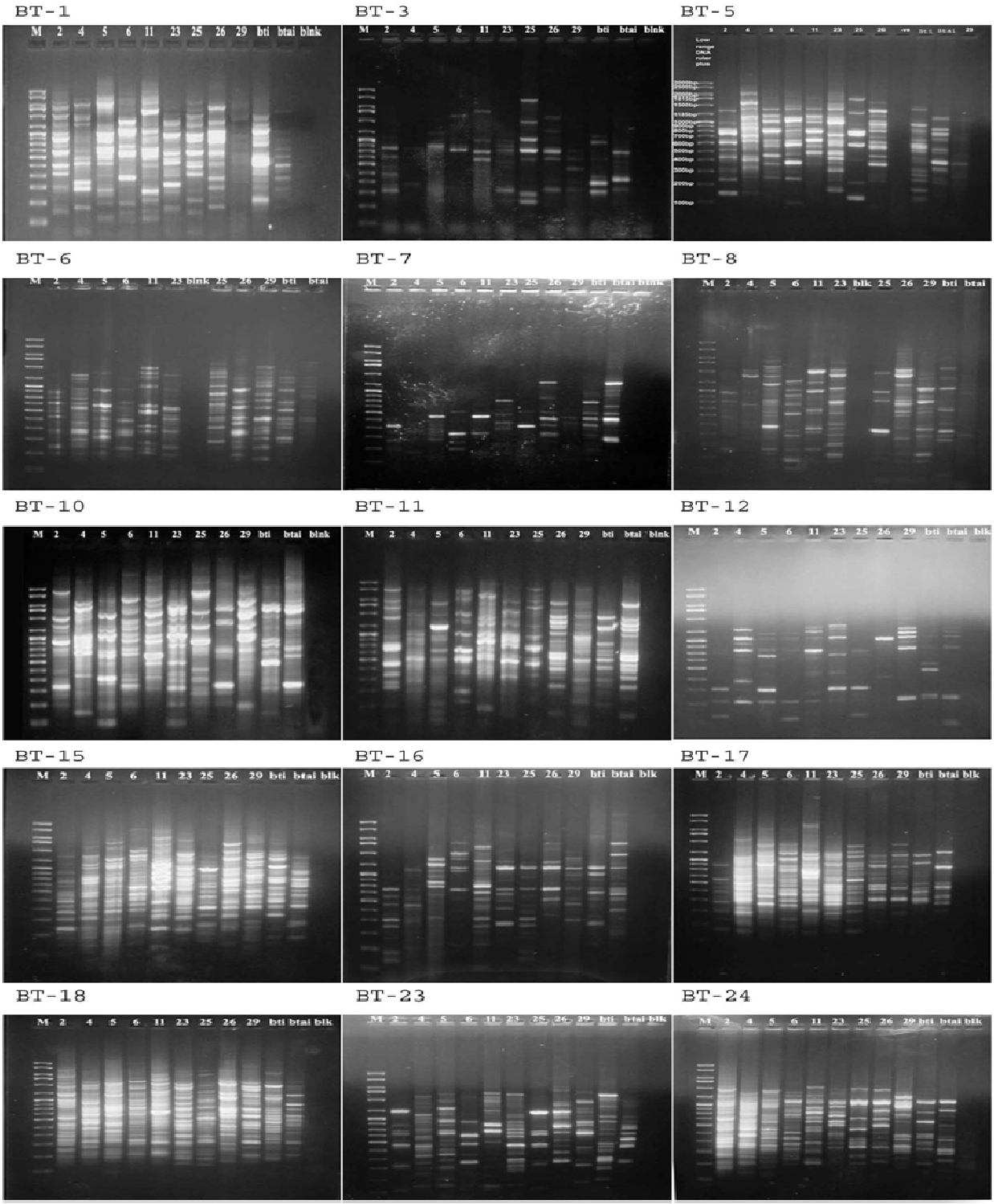

Figure I: Gel electrophoresis photo of the 15 primers selected for RAPD analysis.

M = Low range DNA ruler plus (Marker sizes in base pairs – 3000, 2500, 1815, 1500,1185, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100); Strain no *Mzubt* = 2, 4, 5, 6, 11, 23,25,26,29; *Bti*= *Bacillus thuringiensis israelensis*; *Btai* = *Bacillus thuringiensis aizawai;* Blnk or –ve = blank

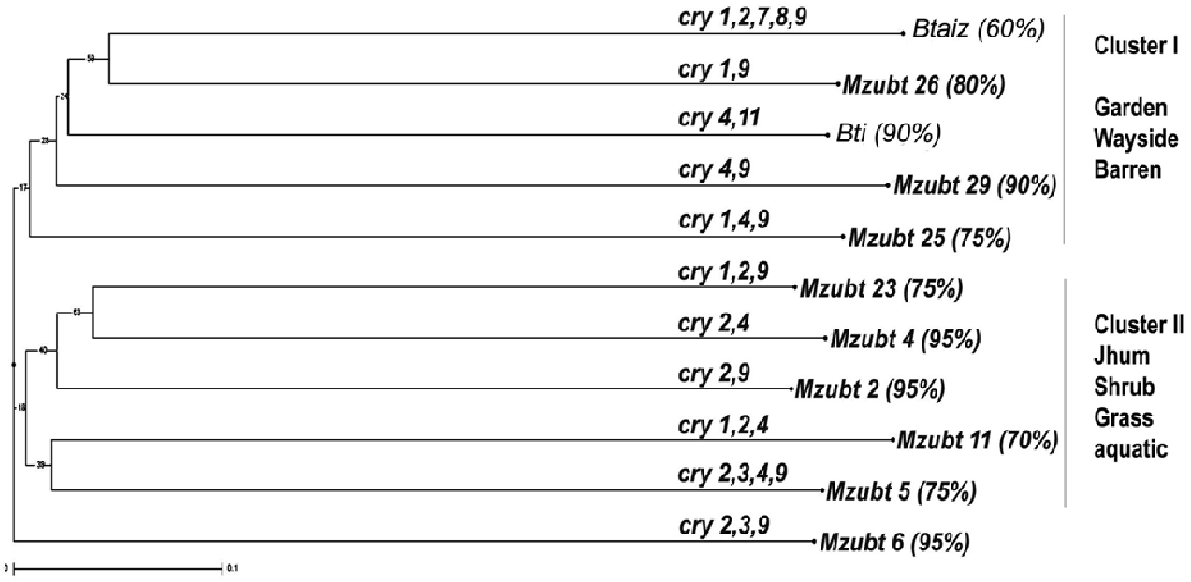

Figure II: Dendrogram constructed using RAPD-PCR data of *Bacillus thuringiensis* isolates with two standard strains *Bt. israeliensis and Bt. aizawai*

isolates which is in accordance with the present study[17]. The dendrogram obtained using DARwin is shown in Figure II. The overall topology of majority of *Bt* strains was dissimilar. The dendrogram represents three major clades wherein polymorphism was observed between cluster 1 (garden, wayside, barren land) and cluster 2 (jhum, shrub, grass, aquatic habitats). The first cluster was again divided into four sub clusters, namely Btaiz and Mzubt26; Bti; Mzubt29; and Mzubt25. The second sub cluster was also again divided into three subclusters namely Mzubt23 and Mzubt4; Mzubt 2; and Mzubt 11 and Mzubt5. The third cluster contained the isolate Mzubt6 (Figure II).

In conclusion, the new Bt isolates from Mizoram soil showed a strong activity against *C. tritaeniorhynchus*. Due to their distinctive characteristics, Mzubt2 and Mzubt6 isolates are great candidates for the development of new larvicidal formulations. The isolation of native strains with activity against dipteran pests, gives us new tools to be introduced into the mosquito management program.

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References

1. Feitelson J S, Payne J & Kim L, *Bacillus thuringiensis*: insects and beyond. *Biotechnol,* **10** (1992) 271–275.
2. Crickmore N, Zeigler D R, Schnepf E, Van Rie J, Lereclus D, Baum J, Bravo A & Dean D H, *Bacillus thuringiensis* toxin nomenclature, http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/ (2012).
3. Pradhan S, Basistha BC & Handique PJ, Determination of genetic fidelity of Micropropagated plants of *Zingiber officinale* cv-*Majuley* of Sikkim Himalaya using RAPD markers. *Int J Fundam Applied Sci,* **1** (2012) 20-23.
4. Williams J G K, Kubelik A R, Livak K J, Rafalski J A & Tingey S V, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *NAR,* **18** (1990) 6531-6535.
5. Welsh J & McClelland M, Fingerprint genomes using PCR with arbitrary primers. *NAR,* **18** (1990) 7213-7218.
6. Hansen B, Damgaard P H, Eilenberg J & Pedersen J C, Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated from leaves and insects. *J Invert Pathol,* **71** (1998) 106–114.
7. Zothansanga, Senthil Kumar N & Gurusubramanian G, PCR pathotyping of native *Bacillus thuringiensis* from Mizoram. *Sci Vision,* **11** (2011) 165-168.
8. Travers R S, Martin P A & Reichelderfer C F, Selective process for efficiency isolates of soils *Bacillus* spp. Appl Environ *Microbiol,* **53** (1987) 1263-1266.
9. Sambrook J & Russell D W, Molecular cloning – A laboratory manual. 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001).
10. Rai A R, Sudhir U, Meshram & Dongre A B, Optimization of RAPD-PCR for discrimination of different strains of *Bacillus thuringiensis. Romanian Biotechnol Lett,* **14** (2009) 4307-4312.

11. Perrier X & Jacquemoud-Collet J P, DARwin software, Version5.0.158, http://darwin.cirad.fr/darwin (2006).
12. Roldan-Ruiz I, Dendauw J, VanBockstaele E, Depicker A & Loose M D, AFLP markers reveal high polymorphic rates in ryegrasses (Lolium spp.). *Mol Breed,* **6** (2000) 125–134.
13. Prevost A & Wilkinson M J, A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor Appl Genet,* **98** (1999) 107–112.
14. World Health Organization, Report of the WHO informal consultation on the evaluation on the testing of insecticides. CTD/WHO PES/IC/96.1 (1996).
15. Abbott W S, A method of computing the effectiveness of an insecticide. *J Econ Entomol,* **18** (1925) 265–267.
16. Middha SK, Bhattacharjee B, Saini D, Baliga MS, Nagaveni MB & Usha T, Protective role of *Trigonella foenum graceum* extract against oxidative stress in hyperglycemic rats. *Eur Rev Med Pharmacol Sci*, **15** (2011) 427–435. PMID:21608438
17. Sadder M T, Hala K H & Luma AB, Application of RAPD technique to study polymorphism among *Bacillus thuringiensis* isolates from Jordan, *World J Microbiol Biotechnol,* **22** (2006) 1307–1312.
18. González A, Díaz R, Díaz M, Borrero Y, Bruzón R Y, Carreras B & Gato R, Characterization of *Bacillus thuringiensis* soil isolates from Cuba, with insecticidal activity against mosquitoes. *Rev Biol Trop,* **59** (2011) 1007-1016.