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SHORT REPORTS

Diversity analysis reveals genetic homogeneity among Indian populations of legume pod borer, *Maruca vitrata* (F.)

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Abstract
Legume pod borer, *Maruca vitrata,* has lately emerged as one of the major insect pests of pigeonpea causing considerable crop losses. Thus, efficient management of *M. vitrata* is an important component for sustained pigeonpea productivity for which information on insect diversity could be useful. Present study was undertaken to evaluate the diversity in *M. vitrata* populations collected from major pigeonpea growing areas of India using molecular markers, *Cytochrome C Oxidase subunit 1* (*cox1*) and *Translational Elongation Factor-1α* (*tef-1α*). Genomic DNA from larvae of different populations was extracted; 709 bp and 550 bp fragments of *cox1* and *tef-1α* were PCR-amplified, cloned and sequenced. Comparison of sequences of different populations using multiple sequence alignment did not show any differences in *cox1* and *tef-1α* sequences within the Indian populations. However, further analysis based upon *cox1* sequences has revealed moderate nucleotide diversity ($\pi = 0.26174$) among Indian and global *M. vitrata* populations, whereas nucleotide diversity within Indian populations is nonsignificant ($\pi = 0.00226$). Additionally, phylogenetic analysis of *cox1* sequences grouped all the Indian populations into one cluster while that of global were completely separate indicating a different ancestral background. This is a maiden attempt for diversity assessment of Indian *M. vitrata* populations that established them to be genetic homologs with different ancestral background.

Keywords Pigeonpea · *Maruca vitrata* · *Cytochrome c Oxidase subunit 1* (*cox1*) · *Translational Elongation Factor-1 alpha* (*tef-1α*) · Genetic diversity

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.], one of the most important legume crops in India, suffers stagnated productivity due to various biotic and abiotic stresses (Rana et al. 2016). Biotic stress caused by insect pests is considered as one of the major constraints in pigeonpea cultivation. Among ~200 insect pests that ravage pigeonpea, *Helicoverpa armigera* (gram pod borer), *Maruca vitrata* (spotted pod borer), *Melanagromyza obtusa* (pod fly), *Exelastis atamosa* (plume moth), *Lampides boeticus* (blue butterfly), *Mylabris* spp. (blister beetles), are considered to be prominent (Sujithra and Subhash 2014).

The spotted pod borer, *M. vitrata* (Fabricius) (Lepidoptera: Crambidae), is one of the major constraints hampering productivity in pulses globally. This pest can be found on 45 different hosts including legumes and non-legumes (Sharma 1998; Arodokoun et al. 2003). In India, green gram, soybean, beans, black gram, cowpea and pigeonpea are known to be preferred hosts of *M. vitrata* (Mahalakshmi et al. 2016). The genus *Maruca* comprises of eight species, viz. *M. amboinalis*, *M. aquitilis*, *M. bifenestralis*, *M. fuscalis*, *M. nigropicalis*, *M. simialis*, *M. testualis* and *M. vitrata*. However, *M. testualis* was found to be similar to *M. vitrata* (Fabricius) which is the only species causing

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economic damage on food legumes (Periasamy et al. 2015). Up to 80% yield, loss is reported in various vegetables and crops due to damage by *M. vitrata* (Singh et al. 1990; Afun et al. 1991; Dreyer et al. 1994; Ulrichs and Mewis 2004). In India, extreme losses of pigeonpea yield has been recorded which is about $US30 million annually (Sharma et al. 1999). Infestation of *M. vitrata* occurs mostly in legume-growing areas of India, viz. Karnataka (Krishnamurthy 1936), Uttar Pradesh (Patel and Singh 1977), Bihar (Saxena 1978), Madhya Pradesh (Saxena 1978), Delhi (Saxena 1978), Tamil Nadu (Sundara Babu and Rajasekaran 1984), Gujarat (Venkaria and Vyas1985), Andhra Pradesh (Rao et al. 1986), Orissa (Prasad et al. 1989), Haryana (Srivastava et al. 1992; Mahalakshmi et al. 2016). Hence, mitigation of this pest is pertinent toward crop improvement in pigeonpea.

Diversity analysis of insects is a much conserved way to assess phenotypic and genetic changes they adapt to overcome various hurdles for survival. Inter-population genetic differences arising due to DNA polymorphism can be detected by employing molecular markers. Apart from RAPD-PCR, RFLP or SSR markers, mitochondrial gene *Cytochrome c Oxidase subunit I* (*cox1*) has been widely used in several studies to decipher genetic diversity among individuals of different populations. Extra-chromosomal mitochondrial DNA is smaller in size (~ 16 kb) and plays a major role in cell metabolism and regulatory mechanism (Taanman 1999). The gene arrangements of animal mitochondrial genomes are stable for longer period of evolution (Boore 1999). Further, *Translational Elongation Factor-1 alpha* (*tef-1α*) is mostly used as an internal control gene during expression analysis in any organism. However in arthropods, the well-characterized *tef-1α* used for many systemic studies in various taxonomic groups (Djernæs and Damgaard 2006) has revealed genetic diversity in a much conserved way. In spite of *M. vitrata* being an important pest in Indian agriculture, to the best of our knowledge there has been hardly any attempt to assess its genetic diversity. Thus, the present study was undertaken to characterize the diversity among Indian populations of *M. vitrata* using *cox1* and *tef-1α*. This is the first ever report to use *tef-1α* as a molecular marker to determine genetic variation in Indian *M. vitrata* populations.

Materials and methods

Ethics statement

All the larvae were collected from agricultural fields of the places mentioned below. Hence, no special permission was needed to collect these insects.

Insect collection

Larvae of *M. vitrata* were identified by characterizing their morphology as cited at http://idtools.org/id/leps/lepintercept/pdfs/vitrata.pdf and collected from pigeonpea growing fields of different agro-climatic zones of northern and southern parts of India, viz. Delhi (28.7041°N, 77.1025°E), Kanpur—Uttar Pradesh (26.4499°N, 80.3319°E), Almora—Uttaranchal (29.8150°N, 79.2902°E), Banjar—Himachal Pradesh (31.6377°N, 77.3441°E), Adilabad—Telangana (19.0809°N, 79.5603°E), Guntur—Andhra Pradesh (16.3067°N, 80.4365°E), Gulbarga—Karnataka (17.9689°N, 79.5941°E), Raichur—Karnataka (16.2120°N, 77.3439°E), Bengaluru—Karnataka (12.9716°N, 77.5946°E), Dharwad—Karnataka (15.4589°N, 75.0078°E), Kasaragod—Kerala (12.5102°N, 74.9852°E) during 2017–2018 and preserved in 95% ethanol. For each of the populations, genomic DNA was extracted from five individual larvae separately except for the population from Kasaragod, Kerala, where only three larvae were used.

Extraction of DNA

DNA was extracted separately from each larva of late third and fourth instar of different populations following the extraction procedure as described by Doyle and Doyle (1990) using 2X hexadecyltrimethylammonium bromide (CTAB) with minor modifications. DNA samples were quantified by Nanodrop® 2000, and quality was checked on 0.8% (w/v) agarose gel.

PCR amplification and sequencing of *cox1* and *tef-1α*

About 100 ng genomic DNA was used for PCR analysis using universal *cox1* primer pair (LCO: 5′-GGTCAACAA ATCATAAAGATATTGG-3′and HCO: 5′-TAAACTTCA GGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) and *tef-1α* gene-specific primer pair (Forward Primer: 5′-GCC AACATCACCACTGAA-3′ and Reverse Primer: 5′-CTA GCTACTTCTTGCCCTTG-3′) (Margam et al. 2011). PCRs were performed in 25 µl volume containing 0.5 µl of DyNAzyme II DNA polymerase (2 U/µl) (Thermo Scientific), 10X PCR buffer (2.5 µl) (Thermo Scientific), dNTPs (0.2 mM), forward and reverse primers (0.2 µM) and 19.5 µl of nuclease free water (Ambion, USA). The program of PCR cycles for both *cox1* and *tef-1α* was performed as follows: 95 °C for initial denaturation for 5 min followed by 35 cycles of 95 °C for 1 min, annealing at 48 °C and 60 °C for 1 min for *cox1* and *tef-1α,* respectively, extension at 72 °C for 1 min followed by a final extension of 72 °C for 10 min using Eppendorf thermal cycler (Eppendorf, Germany). Amplified

products (10 µl) were resolved on 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized by alpha image analyzer (USA). After electrophoresis, *cox1* and *tef-1α* gene fragments of each of the populations were cloned into pGEM-T easy vector (Promega, USA) and sequenced by Sanger sequencing method using ABI 3730XL DNA sequencer (Applied Biosystems) at Agri Genome Labs Pvt. Ltd. (Kochi, Kerala, India).

Data analysis, sequence alignment and phylogenetic analysis

The sequences of *cox1* and *tef-1α* generated for 11 Indian *M. vitrata* populations in this study were compared with *cox1* sequences from 11 countries of Asia, Africa and Oceania (Periasamy et al. 2015), downloaded from National Centre for Biotechnology Information (NCBI) database for comparative genetic study. All the sequences were imported into FASTA format for alignment by setting default parameters in ClustalW (Jeanmougin et al. 1998) algorithm into MEGAX (Kumar et al. 2018) software application package. To search for homologs of both the genes, sequences from each population were subjected to Nucleotide Basic Local Algorithm Search Tool (nBLAST) of NCBI (https://www.ncbi.nlm.nih.gov/). The sequences were analyzed and submitted to NCBI for obtaining GenBank accessions (Table 1). Phylogenetic trees for *cox1* and *tef-1α* were constructed in MEGAX using maximum likelihood method considering 1000 bootstrap replications under distance models Tamura 3-parameter (T92) (Tamura 1992) and Kimura 2-parameter (Kimura 1980). Some measures of DNA sequence variation between the populations including haplotype diversity (hd) and nucleotide diversity (π) were evaluated using DnaSP 5.10.1 (Librado and Rozas 2009). To understand the population demography and to ensure whether the sequences of both the genes are conventional to neutrality or not, neutrality tests like Tajima's *D* (1989), Fu's and Li's *D**, *F** (1993) and Fu's *F* (1997) were also performed using DnaSP 5.10.1 (Librado and Rozas 2009). Pairwise F_{ST} values were calculated employing Arlequin 3.5 software (Excoffier and Lischer 2010) to know the actual genetic variation among and within populations.

Table 1 Details of samples used for genetic diversity analysis of *M. vitrata* from different populations

Location	States/continents	Accession no.		Latitude	Longitude
		cox1	*tef-1α*		
India					
New Delhi	Delhi	MK681906	MK681895	28.7041°N	77.1025°E
Kanpur	Uttar Pradesh	MK681907	MK681896	26.4499°N	80.3319°E
Almora	Uttarakhand	MK681908	MK681897	29.8150°N	79.2902°E
Banjar	Himachal Pradesh	MK681909	MK681898	31.6377°N	77.3441°E
Adilabad	Telangana	MK681910	MK681899	19.0809°N	79.5603°E
Guntur	Andhra Pradesh	MK681911	MK681900	16.3067°N	80.4365°E
Gulbarga	Karnataka	MK681912	MK681901	17.9689°N	79.5941°E
Bengaluru		MK681913	MK681902	12.9716°N	77.5946°E
Raichur		MK681914	MK681903	16.2120°N	77.3439°E
Dharwad		MK681915	MK681904	15.4589°N	75.0078°E
Kasaragod	Kerala	MK681916	MK681905	12.5102°N	74.9852°E
Global					
Lao PDR	Asia	KM987734	_	_	_
Malaysia		KM987735	_	_	_
Vietnam		KM987736	_	_	_
Thailand		KM987739	_	_	_
Bangladesh		KM987713	_	_	_
Indonesia		KM987747	_	_	_
Taiwan		KM987759	_	_	_
Kenya	Africa	KM987732	_	_	_
Nigeria		GU288704	_	_	_
Benin		KM987733	_	_	_
Australia	Oceania	GU288707	_	_	_

Results and discussion

Maruca vitrata is one of the major pests affecting various legumes resulting in 80–90% crop losses in India as well as other countries worldwide (Singh et al. 2014). Diversity analysis to understand population genetics of such a devastating pest is a prerequisite for designing efficient management strategies. In this direction, information on genetic diversity of Indian populations of *M. vitrata* is quite important. Mitochondrial DNA-based marker is a very useful tool for identification of genetic variation, speciation of insects and to know their ancestral history (Kruse and Sperling 2001; Armstrong and Ball 2005; Valade et al. 2009). *M. vitrata* diversity analysis based on *cox1* has revealed the presence of different putative subspecies among the populations of Asia and sub-Saharan Africa (Periasamy et al. 2015). Since the Indian populations have not yet been analyzed to assess the diversity, present study is undertaken to quantify genetic variability in *M. vitrata* within Indian populations and comparing with the rest of the world using two versatile molecular markers, *cox1* and *tef-1α*.

Determination of genetic variability of different Indian populations

Molecular marker *cox1* has been found to be useful in evolutionary studies across the animal phyla (Pentinsaari et al. 2016; Tay et al. 2017). For example, South American tomato pinworm (*Tuta absoluta*) populations from India and Nepal have shown genetic homogeneity with populations from other countries of Europe, South America, Africa and Middle-East Asia (Shashank et al. 2018). The gene arrangements in mitochondrial genome are highly conserved in animal phyla. But exceptionally, insects are having highly variable gene orders mostly in tRNA regions but not in rRNA and protein coding regions (De Mandal et al. 2014). Among 13 protein coding genes in insect mitochondria, ND4, ND5, ND2, cytb and *cox1* come under good group of phylogenetic performers in revealing the evolutionary relationships among distant relatives (Zardoya and Meyer 1996). However, among all the mentioned genes, *cox1* is considered as the best molecular marker for evolutionary studies as well as phylogenetic analysis. In 2003, *cox1* was for the first time reported as an important tool for DNA barcoding, taxonomic identification and speciation (Hebert et al. 2003). Due to large size and high rate of nucleotide substitution, *cox1* helps in differentiating cryptic species (De Mandal et al. 2014).

In the present study, *cox1* (709 bp) and *tef-1α* (550 bp) were successfully amplified from single larval DNA isolated from different populations (Figs. 1, 2). Sequencing

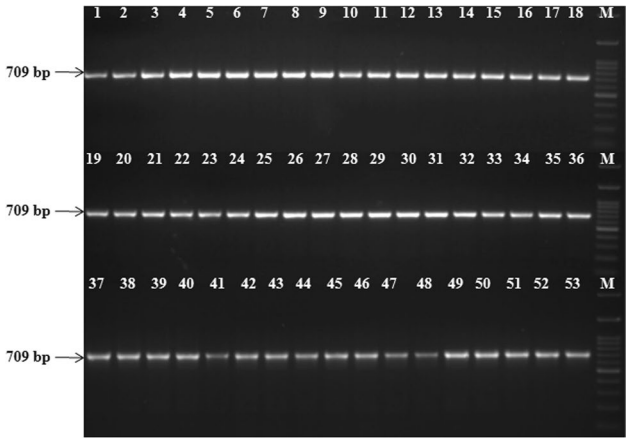

Fig. 1 PCR amplification of *cox1* (709 bp): Lanes: 1–5: Delhi; 6–10: Kanpur; 11–15: Almora; 16–20: Banjar; 21–25: Adilabad; 26–30: Guntur; 31–35: Gulbarga; 36–40: Raichur; 41–45: Bengaluru; 46–50: Dharwad; 51–53: Kasaragod

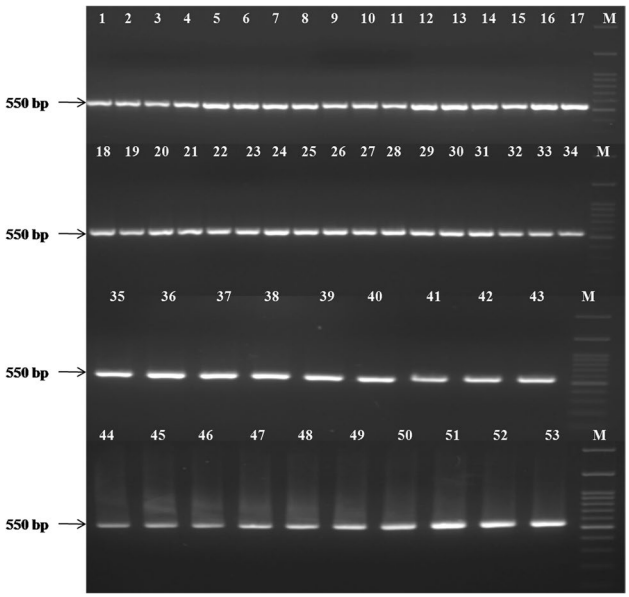

Fig. 2 PCR amplification of *tef-1α* (550 bp): Lanes: 1–5: Delhi; 6–10: Kanpur; 11–15: Almora; 16–20: Banjar; 21–25: Adilabad; 26–30: Guntur; 31–35: Gulbarga; 36–40: Raichur; 41–45: Bengaluru; 46–50: Dharwad; 51–53: Kasaragod

of the amplified products cloned into pGEM-T vector followed by analysis using NCBI blast confirmed the sequence authenticity in all the eleven populations. Since the present study used *tef-1α* for the first time in diversity analysis of *M. vitrata*, no prior sequences were found in the NCBI database for comparative study as well as statistical tests. Alignment of sequences for both the genes from different populations did not show any significant variation among them indicating homogeneous genetic background of the Indian populations analyzed.

Variability of *cox1* Sequences across Indian and global populations

Sequences of *cox1* in Indian populations were compared with that of available global populations retrieved from NCBI revealed total mean nucleotide composition to be 34%, 37%, 14% and 15% for A, T, G, C, respectively. The mean AT and GC was 71% and 29%, respectively. High AT content is a common feature of *cox1* stretch in arthropods (Shashank et al. 2018). Statistical tests were performed for 22 sequences of *cox1* that included both Indian as well as global populations. A low nucleotide diversity (π) of 0.00226 was observed in case of Indian populations and 0.00582 among world populations (Table 2). Further, demographic population history analyzed independently through neutrality tests for the two sets of populations gave negative Tajima's *D* value which is statistically insignificant indicating low-frequency polymorphism among these populations. But, comparison of Indian populations with that of global gave a statistically significant positive ($P<0.001$) Tajima's *D* value. Hence, it can be inferred that moderate amount of polymorphism exists between Indian and global populations. Additionally, AMOVA revealed 1.54 and 101.54% genetic variation among and within populations, respectively (Table 3). Fixation index (F_{ST}) refers to a common measure to evaluate the degree of genetic variation (Willing et al. 2012). In the present study, the calculated F_{ST} was −0.015 that revealed absence of considerable variation among the populations analyzed. Negative F_{ST} values can be inferred as no genetic differences between the populations compared. F_{ST} values play a significant role in the assessment of genetic diversity based on extensive knowledge accruing from literature (Shashank et al. 2018; Jaramillo et al. 2001).

Homology search, multiple sequence alignment and construction of phylogenetic tree

Homology search result of *cox1* sequence of each population of *M. vitrata* using blastn depicted 99.43% sequence identity with complete mitochondrial genome of *M. vitrata* (accession no. KP327715.1). Likewise, *tef-1α* sequence of *M. vitrata* was 91.82% identical to *Spodoptera frugiperda* (Accession no. KT218669.1). Additionally, *cox1* and *tef-1α* sequences of *Bombyx mori* (Accession Nos. NC_002355, NM_001044045) were considered as out groups for sequence alignment and tree construction. Based on sequence alignment (Figs. 3, 4), it could be inferred that there was no major difference among the populations. All the populations were genetically similar, and their sequences were same except for variation in one or two nucleotides. However, maximum likelihood phylogenetic tree of all the 22 populations (Fig. 5) established that populations from India and other countries did not share a common ancestor. Since this is the first report of using *tef-1α* as a molecular marker for genetic diversity analysis of *M. vitrata* in Indian populations, other neutrality tests could not be performed.

Table 2 Genetic variability of *cox1* sequences of different populations of *M. vitrata*

Types of analysis	Population from Asian and African countries	Indian population	Total
Sample size	11	11	22
No. of haplotypes (h)	11	6	17
Haplotype diversity (*hd*)	1.000	0.727	0.935
Nucleotide diversity (π)	0.00582	0.00226	0.26174
No. of segregating sites (S)	11	7	255
Fu and Li's *F**	− 1.13446 ($P>0.10$)	− 1.85949 ($P>0.10$)	2.55173 (**$P<0.02$)
Fu and Li's *D**	− 1.01417 ($P>0.10$)	− 1.74973 ($P>0.10$)	1.62510 (**$P<0.02$)
Fu's *F*	− 9.959	− 2.164	5.693
Tajima's *D*	− 0.96987 ($P>0.10$)	− 1.34124 ($P>0.10$)	3.39807 (***$P<0.001$)

Fu and Li's *F**, *D** and Tajima's *D* value of India-Asia-Africa-Oceania combined population set are significant, i.e., $P<0.02$ and $P<0.001$, respectively. While others have $P>0.10$ which is insignificant

Table 3 Analysis of molecular variance (AMOVA) for the *cox1* sequences among and within Indian and global population

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation
Among populations	1	0.000	0.00842 Va	1.54
Within populations	180	29.888	0.22564 Vb	101.54
Total	181	29.888	0.22222	

Fixation index, F_{ST}: −0.01588

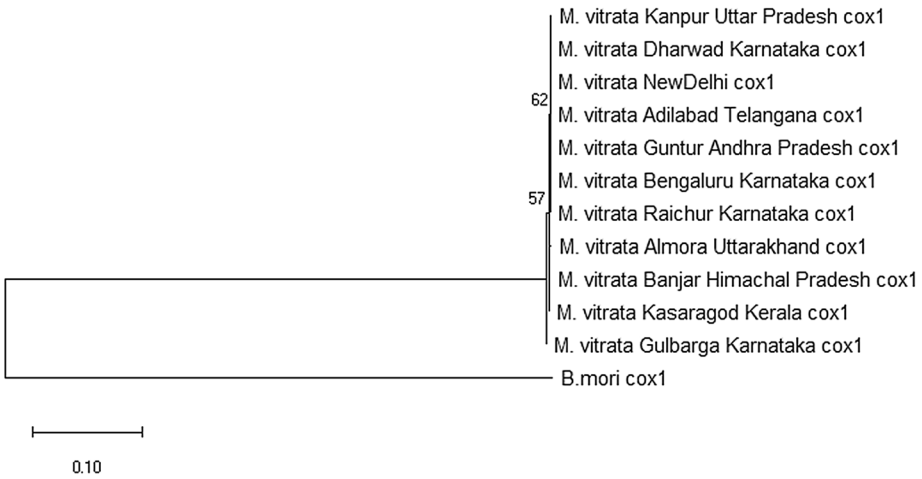

Fig. 3 Maximum likelihood phylogenetic tree of *cox1* sequences of *M. vitrata* from eleven populations. The tree was generated using Tamura 3-prameter model (1992) and 1000 bootstrap replicates

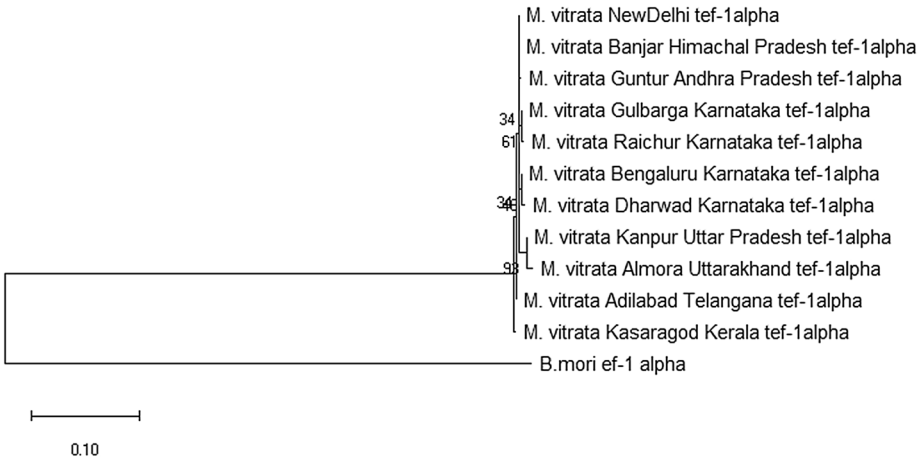

Fig. 4 Maximum likelihood phylogenetic tree of *tef-1α* sequences of *M. vitrata* from eleven populations. The tree was generated using Kimura 2-parameter model (1980) and 1000 bootstrap replicates

However, phylogenetic analysis of *tef-1α* of Indian populations revealed absence of genetic variation.

Results from the present study have confirmed that *M. vitrata* populations across different countries are genetically homogeneous. However, based on *cox1* analysis, emergence of a completely different clade for Asian-African-Australian population has clearly depicted ancestral difference. It has been reported that during expansion of the population, many insect species exhibit less genetic variation as a result of bottleneck and founder effects (Lindholm et al. 2005; Hawley et al. 2006). Less genetically variable populations can be considered as only a subset of the genetic diversity present in the native population, and furthermore, if populations are small, there must be a decreased genetic diversity (Roderick 2004). However, from the present inter- and intra-population study and data analysis of *M. vitrata*, it can be concluded that no genetic divergence exists among Indian populations. Further, *M. vitrata* from other countries belong to a different ancestral history and are homologous to Indian populations. The homogeneity observed across the populations of *M. vitrata* indicates its poor ability to overcome the management hurdles and also shows its recent spread across the country. This simplifies the designing of both traditional and biotechnological management strategies. This is the first attempt to generate molecular data for this emerging pest from different parts of India.

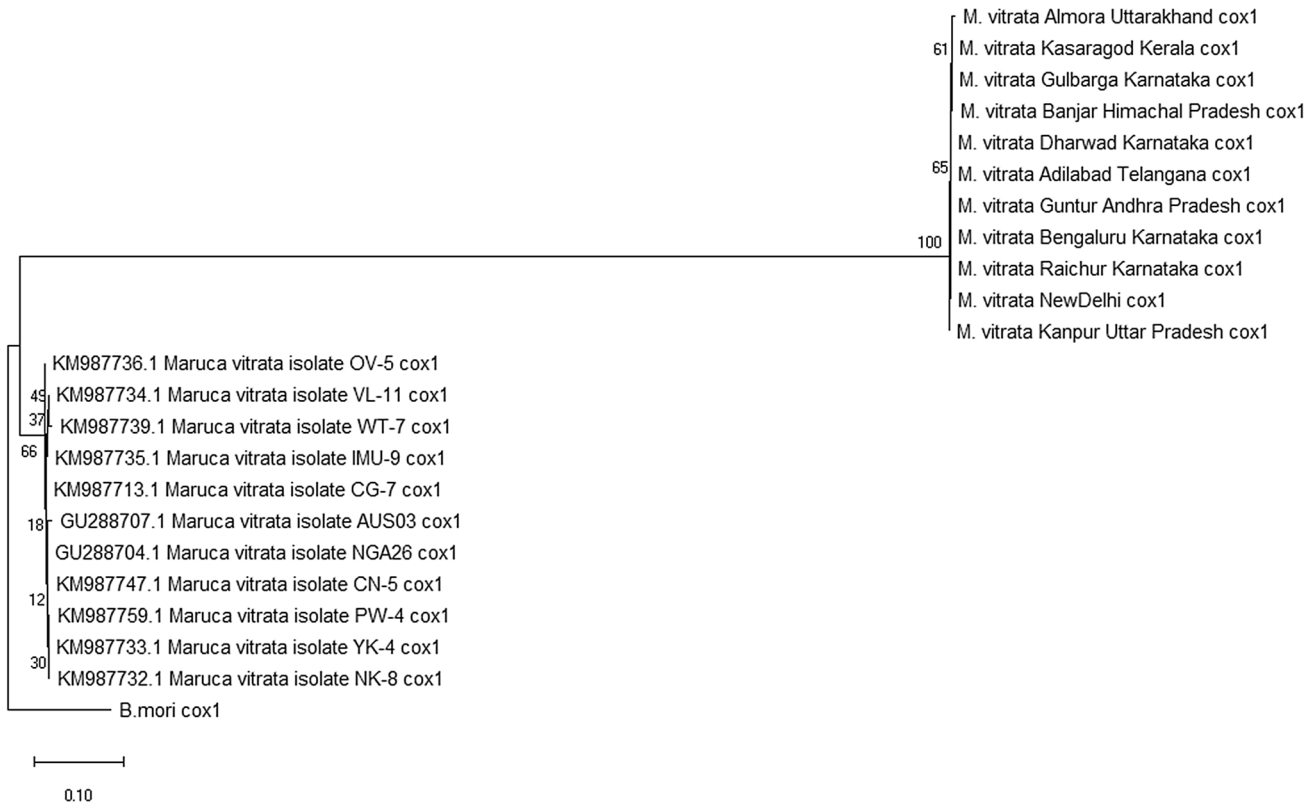

Fig. 5 Maximum likelihood phylogenetic tree of *cox1* sequences of *M. vitrata* populations from India and other countries of Asia and Africa. The tree was generated using Tamura-3 parameter model (1992) and 1000 bootstrap replicates

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Author contributions MC and JY performed all the molecular analysis. SV and SPR collected the larval samples. UR helped in conceptualization and manuscript preparation. MC and NS contributed to data analysis. RS helped in data Analysis and manuscript preparation. Supervision: UR.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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