Molecular Genotypic Diversity of Populations of Brinjal Shoot and Fruit Borer, *Leucinodes Orbonalis* and Development of SCAR Marker for Pesticide Resistance

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Abstract

The brinjal shoot and fruit borer, *Leucinodes orbonalis* is a destructive pest of *Solanum melongena*. The control of *L. orbonalis* with extensive application of synthetic chemical insecticides resulted in the development of resistance with known genetic heterogeneity among populations. Understanding the genetic diversity of their populations is important in developing strategies for their management. The present investigation was performed to characterize populations of *L. orbonalis* for their genetic diversity in the entire region of Tamil Nadu, South India using random amplified polymorphic DNA (RAPD) primers as a tool of the molecular marker. Among sixty random 10-mer primers, only ten primers generated reproducible and scorable banding profile. Among the ten different random primers, the primers namely OPG 7, OPG 8, OPS 2 and OPS 7 generated the highest genetic variation with over 80% genetic polymorphism. Phylogram analysis produced 18 clusters with 8 major and 10 minor clusters. Cluster analysis, statistical fitness, population structure and analysis of molecular variance confirmed the significant genetic variation among different populations. A trait specific marker obtained through RAPD was cloned, sequenced and used to develop a stable diagnostic SCAR marker for DNA fingerprinting to distinguish the populations. Amplification of this locus in the samples of 20 different populations indicated recognition of the trait for pesticide resistance in 12 populations. The results suggest that the biochemical nature of host plant varieties of this insect pest and variation in the application of different insecticides are essential contributing factors for the genotypic variations observed among populations of *L. orbonalis*.

Introduction

Brinjal, popularly known as eggplant (*Solanum melongena* L.) is a native vegetable crop of India and has been cultivated in the country for over 4000 years. It is a commercially cultivable vegetable crop among the members of family Solanaceae. India produces about 13.44 m MT of brinjal from an area of 0.722 M ha with average productivity of 18.6 MT/ha (Yadav et al. 2015). This crop has been infested by a major insect pest, *Leucinodes orbonalis* throughout the year with a yield loss of 40–80% (Alam et al. 2003). Pest control by synthetic chemical insecticides in eventual manner promotes resistance, appearance of secondary pests, harm to non-target organisms, accumulation of chemical residues in soil and thereby leading to contaminated environment

(Alam et al. 2003; Eswara and Srinivasa 2004). The stress that is caused in this context leaves no choice to the populations of *L. orbonalis* but to adapt with the altered measures in their behavioural pattern, reproduction etc. Apparently, the regular insecticide usage in a way helped *L. orbonalis* not only to be uncontrolled but also having developed with genetic resistance via genotypic variations in their genome (Alyokhin et al. 2008). This absolutely necessitates the molecular marker-based analysis to uncover the genotypic variations happening in the species population and to frame an effective plan under insect pest management programmes (Smith and Wayne 1996; Symondson and Liddell 1996). There are barely limited numbers of works available in India on the study of genetic variation in the population of *L. orbonalis* using molecular markers (Karthikeyan et al. 2005; Geetharajalakshmi et al. 2006; Marimuthu et al. 2009; Ghante et al. 2013). Therefore, the present investigation was aimed to characterize populations of brinjal shoot and fruit borer, *L. orbonalis* for their genetic diversity throughout the entire region of Tamil Nadu (South India) using RAPD random primers as a tool of the molecular marker and further developing the suitable Sequence Characterized Amplified Region (SCAR) marker to identify the pesticide resistance population.

Materials And Methods

Insect collection

Fifth instar larvae of *L. orbonalis* (Lepidoptera: Crambidae) were collected from the infested fruit and shoot of brinjal from twenty different locations (brinjal cultivation fields) of Tamil Nadu as stated in Table 1 and Fig. 1 (Population-1: Madurai; Population-2: Dindigul; Population-3: Theni; Population-4: Namakkal; Population-5: Erode; Population-6: Karur; Population-7: Salem; Population-8: Virudhunagar; Population-9: Tirunelveli; Population-10: Dharmapuri; Population-11: Kanyakumari; Population-12: Villupuram; Population-13: Trichy; Population-14: Pudukottai; Population-15: Tanjore; Population-16: Ariyalur; Population-17: Perambalur; Population-18: Coimbatore; Population-19: Tirupur; Population-20: Ooty). The collected fifth instar larvae were stored in 70% ethanol and brought to the laboratory.

Isolation of genomic DNA

Larvae were dissected out to remove the entire gut and the remaining larval tissue was washed with distilled water. The genomic DNA was purified from fifth instar larvae using HiPurA™ (HiMedia Laboratories, Mumbai, India) genomic DNA purification kit. Purified genomic DNA was quantified using a spectrophotometer (Eppendorf).

PCR-RAPD analysis

PCR-RAPD analysis was carried out according to Lima et al. (2000). The amplification was performed in 20µl of the reaction mixture with PCR buffer (2X Master Mix-Red-Gene Technologies, Chennai, India) containing Tris-HCl pH 8.5, 3mM MgCl₂, 0.4mM dNTP, 25 pM primer, 0.2 units *Taq* DNA polymerase, sterile water, 25 ng genomic DNA, inert red dye and stabilizer. Initially, a total of 60 RAPD 10-mer random primers (Eurofins Genomics, Bangalore, India) were screened for selection of suitable primers for further analysis. Among the 60 primers, 10 primers were further chosen based on the production of a consistent and scorable amplification pattern in RAPD profile of *L. orbonalis*. The amplification reactions were performed in a thermal cycler (MyGene™ Series Peltier Thermal Cycler, LongGene MG25+, Version 3.20) programmed for an initial denaturation (94°C for 5 min) followed by 35 cycles (each cycle comprised denaturation for 1 min at 94°C, annealing for 1 min at 37°C and extension for 2 min at 72°C) with a final extension of 72°C for 7 min and stored at 4°C. The amplified PCR products were loaded on 1.5% (w/v) agarose gel and the gel was electrophoresed at 100V for 3h using 1X tris-borate-EDTA (TBE) buffer. The gel was stained with ethidium bromide, observed under UV trans-illuminator and photographed.

Genetic analysis

The percentage of polymorphism was calculated using amplified allelic fragments showing unique multi-locus RAPD fingerprints. Polymorphic information content (PIC) was calculated with the help of PIC Calc program (Nagy et al. 2012). Most distinct bands from RAPD profile were chosen for genetic statistical analysis. The amplified fragments were scored as 1 (presence of a band) and 0 (absence of a band). The RAPD data were analyzed for similarity coefficients to find out the genotypic variation among populations using PAST version-2 software (Hammer et al. 2001). Dendrograms were constructed with bootstrap values based on the Jaccard's similarity coefficient (Real 1999).

AMOVA analysis

The genetic variance observed in RAPD data was assessed using analysis of molecular variance (AMOVA) with GenAlEx software version-6.5 (Peakall and Smouse 2012). It was used to estimate the total molecular variance observed among and within populations of *L. orbonalis*.

Statistical fitness analysis

Genetic structure, cluster analysis and cophenetic correlation coefficient analysis (CCC) were performed using UPGMA (Un-weighted Pair Group Method with Arithmetic Mean). Population distribution was assessed by principal component analysis (PCA). Principle coordinate analysis (PCO) was generated using PAST version-2 software (Hammer et al. 2001). The major substantial components to infer from PCA were determined by both Jolliffe cut-off value and broken stick model (Jolliffe 2002; Legendre and Legendre 2012). The value of accuracy of a principal component over 0.60 or less than 0.60 was considered as significant (McGarigal et al. 2013).

Population structure analysis

Population structure and gene flow analysis was carried out using 20 different populations of *L. orbonalis* with a model based on clustering to subdivide genotypes into genetic subpopulations (SPs) using the software STRUCTURE v.2.3.4 (Pritchard et al. 2000). The number of SPs that existed in the samples were analyzed purely based on PCR-RAPD profiles. Each *L. orbonalis* population was considered as a member of a different subgroup (Admixture model; ALPHAPROPSD = 0.20). The number of subgroups (k) in the population was determined by running the programme with k values varying from 1 to 10 with five independent runs for each k value. To determine the most appropriate k value, Markov Chain Monte Carlo (MCMC) (Karandikar 2006) simulations were set to 100,000 and data were collected over 500,000 MCMC replications in each run. The k value was detected by employing an *ad hoc* statistic ΔK based on the rate of change in the log probability of data between successive *K* values using Structure Harvester (Evanno et al. 2005).

Cloning of trait specific gene fragment (SCAR marker)

A FavorPrep™ Gel/PCR purification kit (Favorgen, Europe) was used to excise the polymorphic band from the agarose gel. The extraction method was followed according to the manufacturer's protocol. The purified product was sequenced using the Sanger sequencing method on an ABI 3730 XL (Applied Biosystems, USA). The pGEM®T vector system I was purchased from the Promega and the ligation of extracted polymorphic band were performed according to the manufacturer's protocol. High-Efficiency JM109 cells (non-competent) were purchased from Promega. These cells were quadrant streaked in prepared M9 + B1 plates (minimal media) to obtain a single colony. The colony is propagated for competent cell preparation using CaCl₂ and stored in aliquots at -80°C. Luria Bertani medium (LB) plates with ampicillin, IPTG and X-gal were prepared. The transformation was performed by a heat shock of JM109 High-Efficiency competent cells mixed with ligation reaction. Transformation culture was plated onto the LB/ampicillin/IPTG/X-gal plates and screened (blue/white) for recombinant (white) colonies.

Primer designing and amplification of SCAR marker in *L. orbonalis* population

The plasmid DNA was isolated from the recombinant colonies. The M13 primers of transcription initiation site in pGEM®T vector system I were used to amplify the vector sequence followed with trait specific gene sequence residing in isolated plasmid to identify the orientation of the gene fragment. The product was sequenced by Sanger's method (Applied Biosystems). The nucleotide sequence of the SCAR marker was then used to design a set of specific primers for amplification of SCAR marker for the detection of insecticide resistance populations of *L. orbonalis*.

Results

PCR-RAPD analysis

PCR was carried out using sixty random RAPD primers on genomic DNA samples isolated from the larvae of *L. orbonalis* collected at twenty different locations in Tamil Nadu, South India. Each genomic DNA material was a pooled population sample from four individual larvae of *L. orbonalis*. Primers that produced consistent and scorable amplified DNA fragments were then submitted to the second round of amplification. The selected ten various random primers were applied to larval DNA samples obtained from twenty different populations of *L. orbonalis* for their diagnostic ability to distinguish genetic variability among samples of populations (Table 2). The ten random primers produced 1375

scorable bands for the pooled twenty different populations (Fig. 2). The total scorable bands were equal to 68.75 bands / population for 10 primers or 137.5 bands/primer for 20 populations. Otherwise, it was calculated as an average of 6.88 bands/population/primer. Among the ten selected random primers that produced consistent RAPD profiles, seven different primers namely, OPA 7, OPG 7, OPG 8, OPK 6, OPS 2, OPS 7 and OPS 8 produced over 70% polymorphic bands (Table 3, Fig. 3) for the 20 different populations of *L. orbonalis*. Within these seven random primers, OPG 7, OPG 8, OPS 2 and OPS 7 were responsible for the production of over 80% polymorphic bands. It was interesting to observe that the primer, OPS 8 revealed 100% polymorphic bands. The size of the amplified DNA fragments was ranged from 2750 bp to 200 bp.

Genetic relationship among the amplified RAPD profiles of various populations of *L. orbonalis* was revealed by the Jaccard similarity coefficient values as shown in Table 4. A UPGMA based Jaccard coefficient similarity values were ranged from 0.39 to 0.72. Of the pair-wise relationships observed among 20 different populations of *L. orbonalis*, the population collected at Karur and Dindigul districts showed a minimum similarity index of 0.39 and a maximum similarity index value of 0.72 between the populations of Karur and Salem. The dendrogram constructed based on the RAPD profiles for the 20 different populations resulted in 6 major clusters and 14 minor clusters. Most of the populations of *L. orbonalis* viz., Dindigul, Theni, Ooty, Perambalur, Ariyalur, Kanyakumari, Karur, Tirupur, Villupuram, Dharmapuri, Salem, Tirunelveli, Namakkal and Tanjore occurred in minor clusters while the other remaining populations of Erode, Pudukottai, Trichy, Madurai, Virudhunagar and Coimbatore were observed in the major clusters (Fig. 3). The total number of bootstrap values observed in cluster node was 19 and the values ranged from 0 to 100%. Out of 19 cluster nodes, node 7 (36.8%) showed the higher bootstrap value of 30-100% and node 12 (63.2%) produced a lower bootstrap value of 0-16%. The average bootstrap value was 20.1%. However, the bootstrap value was observed as 100% in the final node of the cluster. This value showed that nodes of clusters were well supported; only internode was found to be poorly supported. Karur and Salem districts showed the highest bootstrap value of 43% followed by Theni-Dindigul and Kanyakumari-Ariyalur each with 36% and Dharmapuri-Tanjore and Villupuram-Tirupur showed each with 35%. Remaining populations revealed lower bootstrap values (Fig. 3). It was thus confirmed based on the cluster grouping and bootstrap values that all the *L. orbonalis* samples (i.e., among populations) collected at 20 different locations in Tamil Nadu, India were genetically distinct populations.

AMOVA analysis

Hierarchical analysis of *L. orbonalis* diversity was carried out using AMOVA to know the variation that existed among and within populations. This analysis showed the degrees of freedom (df) value within the populations and among populations as 2 and 17, respectively. Sum of squared deviations (SS) value within the populations and among populations were 0.218 and 2.532, respectively. The mean of squared deviation (MS) values within the populations and among populations were 0.109 and 0.149, respectively. The percentage of molecular variance within the populations was less than 1% and among populations, it was above 99%. The PhiPT, PhiPT max, Phi'PT values were -0.043, 0.851 and -0.050, respectively. The AMOVA analysis using RAPD data revealed genetic variation among the populations rather than within populations of *L. orbonalis*.

Statistical fitness analysis

PCA and PCO analyses were applied to determine the genetic diversity among populations of *L. orbonalis* collected at different locations. In PCA, the first and third component axes exhibited a total variance of 11.8% and 8.4%, respectively, and eigenvalue was 2.2 and 1.5, respectively. Hence, based on the Euclidean similarity index, PCA was plotted with the first and third components. This showed that several populations were closely dispersed in first component axes. Significant overlap arisen mostly at third component among different populations of *L. orbonalis* which suggested that different sites in the PCA plot were straightforward to estimate the genetic diversity. To proof read the PCA, the loading analysis was implemented. In this, based on the latitude and longitude sites, the species were correlated at the third component level, which reinforced the accuracy of PCA. PCA scatter diagram showed the Jolliffe cut-off value of 0.10267 and the first 8 principal components (PCs) with eigen values greater than (2.22296 to 1.04826) the cut-off value (Fig. 4). PCs related to the covariance matrix had eigen values bigger than the average (all the eigen values) and confirmed those were significant. PCO was produced according to the Euclidean similarity index; the first and third coordinate axes accounted for 11.8% and 8.4% total variance with eigen value of 356.6 (Fig. 5). PCO plot was made using the first and third coordinate and the Tirupur, Ariyalur, Villupuram and Kanyakumari districts were in third coordinate.

The genetic distance matrix was also studied using nMDS approach based on three dimensions. The stress values for the three axes were 0.2431 (axes 1= 0.4989, axes 2= 0.06648, axes 3= 0.02801). The nMDS three-dimensional scatter plot result was figured using the second and third coordinate based on the Euclidean similarity index (Fig. 6). The position of the *L. orbonalis* population was found to be consistent with the grouping pattern of the UPGMA clustering, and the *L. orbonalis* population adaptations on the three-dimensional nMDS plot were linked with the latitude and longitude site of the species collection on the world map. The nMDS Shephard's plot produced similar value in both obtained rank and target rank with a range from 20 to 180 which confirmed that the nMDS plot represented an excellent fit to the evolutionary distance matrix between collection sites. In histogram investigation, the frequency of similarity value was also 0.1 to 1.0 which was corresponded to Jaccard's similarity coefficients value. The CCC value was 0.7 which identified that the clustering result was very acceptable for the genetic similarity matrix calculation.

Population structure analysis

The structure analysis produced that 20 populations were broadly divided into three subpopulations (SPs) as SP1, SP2 and SP3. The molecular variance among the SPs showed different confirmation for gene flow between SPs. An analysis of the precise population structure (K), (K value 1 to 10 with 5 runs for each K value independently) was analyzed and the LnP (D) value was used for all the 20 samples. Structure analysis showed maximum DK value, K=3. These three SPs also confirmed that Jaccard's similarity coefficient analysis. Theni, Karur, Dindigul, Salem, Madurai, Tirunelveli, Namakkal and Erode district species

were placed in SP1. Pudukottai, Dharmapuri, Tanjore, Virudhunagar and Villupuram district species were placed in SP2. Similarly, Ariyalur, Kanyakumari, Ooty, Coimbatore, Perambalur and Tirupur district species were placed in SP3. The results of the structure produced that all the three SPs had an admixture of alleles and only one pure species (Theni) was observed. Karur, Dindigul, Salem, Madurai, Tirunelveli, Namakkal, Erode, Dharmapuri, Ariyalur, Kanyakumari, Ooty and Coimbatore populations contained 85-98% pure alleles. The AMOVA analysis also produced a consistent relationship, representing high intra-population variation, which confirmed that the SPs had a strong genetic structure (Fig. 7).

Development of SCAR marker based on trait specific locus

Based on the higher level of polymorphic profile generated by the random primer OPS 08, a trait specific locus representing insecticide resistant gene fragment was identified. This trait specific polymorphic band with a size of approximately 0.5 kb was purified from the agarose gel and subjected to the electrophoresis to confirm its purity (Fig. 8) for its development in to a SCAR marker. It was then used as insert DNA for cloning into the pGEM[®]T vector system for nucleotide sequencing and trait specific designing of primers. The trait specific locus representing insecticide resistant gene (SCAR marker) was ligated in the lacZ region of pGEM[®]T vector system-I (with a vector size of 3 kb). The ligation reactions were transformed into the competent cells of JM109 strain. Both recombinant and non-recombinant colonies were observed on the LB plates in presence of ampicillin incubated overnight at 37°C (Fig. 9). The isolated plasmid was then quantified using NanoDrop UV-Vis Spectrophotometer (Eppendorf, USA). The quantity of plasmid from the recombinant colony was 893 µg/ml with the purity of 1.06 at the absorbance of 260/280 nm. The identified SCAR marker as a diagnostic tool for insecticide resistance subjected to sequencing resulted in a product size of 402 bp (Fig. 10). Specific primers (18 bp - sense primer sequence: 5' TAATGTTGCTGTGATACG 3' and a 20 bp - antisense primer sequence:

5' GAATATGTCTTCTGTGAT 3') were subjected for amplification of SCAR marker-based detection of insecticide resistance using various genomic DNA isolated from twenty different populations of *L. orbonalis*. The PCR results indicated that an amplified product with an expected size of approximately 400 bp in

L. orbonalis populations collected at Dindigul, Theni, Namakkal, Karur, Salem, Tirunelveli, Dharmapuri, Kanyakumari, Villupuram, Tanjore, Coimbatore and Tirupur by the diagnostic SCAR marker (Fig. 11). It was found that all these 12 populations were resistant to insecticides through the trait diagnosed by the development of SCAR marker with successful employment of this tool among populations.

Discussion

Determining the genetic variability is one of the important tools used in designing pest control practices of an integrated pest management programme. Variation at the genetic level seen largely in insect pest species are through their interaction with environmental factors by the natural selection process and this offers the basis for evolutionary change (Marimuthu et al. 2009). Gene flow, genetic drift and natural selection interact in equilibrium to produce genetic variations among populations (Futuyma and Peterson 1985). Though natural selection does not reflect directly on phenotype, it is a major factor causing a genetic differentiation at the molecular level. Random amplified polymorphic DNA (RAPD) is an efficient assay system useful for the study of genetic variability (Beeman and Brown 1999; Infante-Malachias et al. 1999; Bas et al. 2000; Gill et al. 2006). The polymorphic genomic DNA provided by RAPD markers allows rapid identification and isolation of chromosome-specific DNA fragments, its deletion or addition in the genetic pool of large chromosomal segment (Jain et al. 2010). In the present study, genetic diversity among the populations of brinjal shoot and fruit borer, *L. orbonalis* in Tamil Nadu, India was demonstrated using RAPD random primers as molecular markers. This work was formed because of the inadequate research works reported previously on the genetic diversity of brinjal pest, *L. orbonalis*. Genetic variations based on RAPD analysis was reported only in five different populations of *L. orbonalis* with a restricted area of study (Karthikeyan et al. 2005). Based on the intensity of brinjal growing farms such as high, moderate and low-intensity cultivations, populations of *L. orbonalis* were collected at 10 different regions, revealed intraspecific variations based on random primers (Geetharajalakshmi et al. 2006). Similar such work was carried out by Marimuthu et al. (2009), reporting only two major clusters with no variations among populations using RAPD assays. Detection of genetic variations in the populations of *L. orbonalis* in north Karnataka was screened in ten locations with varying degrees of genetic similarity (Ghante et al. 2013). This prompted to attempt to reveal the variation among the populations of *L. orbonalis* from twenty different locations on the entire region of the state of Tamil Nadu.

Populations of the brinjal shoot and fruit borer, *L. orbonalis* collected from different brinjal growing areas in our study throughout Tamil Nadu revealed genetic variations. Of the ten different random primers used, the primers namely OPG 7, OPG 8, OPS 2 and OPS 7 generated highest genetic variation with over 80% polymorphism among the populations of *L. orbonalis* in the present investigation. It was also interesting to observe that the primer, OPS 8, revealed 100% polymorphism. A UPGMA based similarity coefficient values were ranged from 0.39 to 0.72. The dendrogram constructed based on the RAPD profiles for the 20 different populations resulted in 6 major clusters and 14 minor clusters. Based on the cluster grouping and bootstrap values, it was confirmed that all *L. orbonalis* samples collected at 20 different places of Tamil Nadu, India were genetically distinct populations. This could be due to different cropping pattern, varieties of brinjal cultivation, application of insecticides and their types to manage this insect pest. During collection, it was observed that at least a dozen varieties of brinjal cultivated at different locations. Studies on the biochemical basis of resistance to *L. orbonalis* and their correlation with the shoot and fruit borer infestation in selected brinjal genotypes showed that less susceptible genotypes had the higher amount of polyphenol oxidase, phenylalanine ammonium lyase and lignin and a lower amount of reducing sugar (Khorsheduzzaman et al. 2010). This biochemical basis of resistance in plants could definitely been a contributing factor for the genetic variations observed among populations of *L. orbonalis*. A field study conducted for screening the 52 cultivable brinjal genotypes from the 14 states of India concluded with the resistance towards *L. orbonalis* only by four genotypes where rest were categorized as tolerant and highly susceptible (Rameash et al. 2015). An extensive report on socio-economic assessment of brinjal cultivation in Tamil Nadu with context to insect resistance stated 32% percent (higher) of major constraints in production was by insect damage (Ashok et al. 2019).

Brinjal growers mostly depend on insecticides to battle this huge damage-causing insect pest. Reports reveal that synthetic chemical insecticides dominate in the control of *L. orbonalis* (Latif et al. 2010). In India, over 95% of farmers apply insecticides more than 40 sprays per season with two or three times a week (Alam et al. 2006). A survey also revealed that farmers exclusively rely on the application of insecticides in managing this pest. The insecticides used

mostly are organophosphates, carbamates and synthetic pyrethroids (Alam et al. 2003). Brief information of insecticides used in the collected sample sites against *L. orbonalis* infesting brinjal crops is given in Table. 5. There is also variation in the application of these insecticides from field to field and the efficacy of the type of insecticides used against *L. orbonalis* in a particular location might have different levels of toxic action (Latif et al. 2010). In our present study, among 20 populations of *L. orbonalis* assayed for genetic characterization, the population collected at Karur and Dindigul districts showed a minimum similarity index of 0.39. But at the same time, a maximum similarity index value of 0.72 was recorded in the populations of Karur and Salem. The distance between Karur to Dindigul and Karur to Salem is only around 90 kilometers and with this same distance, we could able to observe both minimum and maximum similarity index. Under such circumstances, it could be reasonable to believe that the variation in the application of nature of insecticides brought about the enormous gap in similarity indices.

The AMOVA analysis using RAPD data showed that the genetic variation was observed among the populations rather than within populations of *L. orbonalis*. The population structure analysis revealed the formation of three subpopulations as SP1, SP2 and SP3. The molecular variance among the SPs showed different confirmation for gene flow between SPs. A population may be considered as a single unit. However, in many species and situations, populations are separated into smaller units. Such separation might be either due to ecological factors where habitats are not continuous or behavioural features with the relocation of populations consciously or unconsciously. In such a situation, the genetic links among its parts may vary, depending on the actual amount of gene flow taking place. There are at least three reasons with which a population is considered structured. They are (a) occurrence of genetic drift in several of its subpopulations, (b) no uniform migration throughout the population or (c) the process of mating is not random throughout the population

(De Vicente et al. 2004). Consequently, the structure of a population influences the extent of genetic variation and its distribution patterns. Our study with twenty different populations subdivided or separated into three major subpopulations could definitely due to one or more of the reasons explained here. Our previous study has verified the phylogeographical relationships among the insect pest, *L. orbonalis* to validate the population genetic structure based on mitochondrial COI gene sequences (Palraju et al. 2018). Exploring further with RAPD markers could essentially set a base for deriving the sequence characterized amplified region (SCAR) marker to identify the resistance traits. In that context, generation of molecular or trait specific characters using different populations of a species or at various stages of taxonomy could be used as diagnostic tools after screening with synthetic short oligonucleotide primers. In order to establish genetic relationships, it is either identified as polymorphic amplified fragments between species within the genus or constant amplified fragments diagnostic for a genus. Fragments that are amplified at polymorphic loci at the species level could be used to recognize members of a given species if that fragment is constant among all members of that species in order to ascertain trait-specific character (Hadrys et al. 1992). Use of molecular markers in mapping experiments has identified quantitative trait loci that determine the insecticide resistance phenotypes in insects (Ranson et al. 2000). Likewise, using random amplified DNA with RAPD markers, genetic loci have been mapped in lesser grain borer, *Rhyzoperthadominica* that determines high-level resistance to phosphine (Schlipalius et al. 2002). Also, conversion of RAPD markers into SCAR markers has been reported and applied to improve the low reproducibility of RAPD (Paran and Micheltmore 1993). SCAR is a genomic fragment localized in a single genetically defined locus that can be amplified by PCR using a pair of specific primers. It is low in sensitivity to PCR reaction conditions, allowing for an excellent reliability among various laboratory conditions (Fernandez et al. 2003). This study dealing with various populations of *L. orbonalis* suggests the possibility of exploring co-dominant markers through transfer of RAPD amplified fragments into SCAR marker. A trait specific marker obtained through RAPD in *L. orbonalis* was developed as a stable diagnostic SCAR marker for DNA fingerprinting to distinguish the populations of brinjal shoot and fruit borer collected at different geographical locations in Tamil Nadu, South India for insecticide resistance. The results have shown that the developed SCAR marker could be highly useful as a trait specific identification tool, particularly for distinguishing insecticide resistant populations in *L. orbonalis*.

Declarations

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All authors have contributed equally

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CRedit authorship contribution statement

Palraju Murali: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Funding acquisition.

Karuppiiah Hilda: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization.

Muthusamy Ramakrishnan: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing - review & editing.

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Sreeramulu Bhuvargavan: Investigation, Data curation, Writing - review & editing.

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Tables

Table 1

Collection sites of the larval populations of *L. orbonalis*

S. No.	Districts	Abbreviation
1.	Madurai	MDU
2.	Dindigul	DGL
3.	Theni	TNI
4.	Namakkal	NKL
5.	Erode	ERO
6.	Karur	KRR
7.	Salem	SLM
8.	Virudhunagar	VHN
9.	Tirunelveli	TVL
10.	Dharmapuri	DMI
11.	Kanyakumari	KKR
12.	Villupuram	VPM
13.	Trichy	TRY
14.	Pudukottai	PKI
15.	Tanjore	TNJ
16.	Ariyalur	ALR
17.	Perambalur	PMR
18.	Coimbatore	CBE
19.	Tirupur	TRR
20.	Ooty	OTY

Table 2

List of RAPD primers used for initial screening of various populations of brinjal shoot and fruit borer, *L. orbonalis*

S. No	OPA	OPG	OPJ	OPK	OPS	
1.	OPA-01	OPG-01	OPJ-01	OPK-01	OPK-11	OPS-01
2.	OPA-02	OPG-02	OPJ-02	OPK-02	OPK-12	OPS-02
3.	OPA-03	OPG-03	OPJ-03	OPK-03	OPK-13	OPS-03
4.	OPA-04	OPG-04	OPJ-04	OPK-04	OPK-14	OPS-04
5.	OPA-05	OPG-05	OPJ-05	OPK-05	OPK-15	OPS-05
6.	OPA-06	OPG-06	OPJ-06	OPK-06	OPK-16	OPS-06
7.	OPA-07	OPG-07	OPJ-07	OPK-07	OPK-17	OPS-07
8.	OPA-08	OPG-08	OPJ-08	OPK-08	OPK-18	OPS-08
9.	OPA-09	OPG-09	OPJ-09	OPK-09	OPK-19	OPS-09
10	OPA-10	OPG-10	OPJ-10	OPK-10	OPK-20	OPS-10
Primers marked with bold letters produced consistent and scorable banding profile in PCR-RAPD						

Table 3

Total number of bands, number of monomorphic and polymorphic PCR-RAPD fragments and their percentages generated by selected random primers among various populations of

L. orbonalis collected at different locations in Tamil Nadu, South India

S. no.	Selected primers	Total no. of bands	Monomorphic		Polymorphic	
			Bands	%	Bands	%
1	OPA 1	161	100	62.11	61	37.88
2	OPA 7*	143	40	27.97	103	72.02
3	OPA 8	125	40	32.00	85	68.00
4	OPG 7*	151	20	13.24	131	86.75
5	OPG 8*	151	40	13.24	131	86.75
6	OPK 6*	91	20	21.97	71	78.02
7	OPS 2*	115	20	17.39	95	82.60
8	OPS 3	181	100	55.24	81	44.75
9	OPS 7*	140	20	14.28	120	85.71
10	OPS 8*	117	00	00.00	117	100
* Random primers significantly produced > 70 % polymorphism among populations of <i>L. orbonalis</i>						

Table 4

Jaccard's similarity coefficient showing genetic relationship among the populations of *L. orbonalis* from different locations in Tamil Nadu

Districts	MDU	DGL	TNI	NKL	ERO	KRR	SLM	VHN	TVL	DMI	KKR	VPM	TRY	PKI	TNJ	ALR	PMR	CBE	TRR	OTY
MDU	1.00																			
DGL	0.51	1.00																		
TNI	0.58	0.56	1.00																	
NKL	0.65	0.54	0.58	1.00																
ERO	0.57	0.51	0.56	0.55	1.00															
KRR	0.65	0.51	0.54	0.65	0.65	1.00														
SLM	0.66	0.49	0.54	0.68	0.57	0.72	1.00													
VHN	0.55	0.47	0.56	0.58	0.51	0.57	0.54	1.00												
TVL	0.57	0.52	0.63	0.65	0.60	0.61	0.64	0.63	1.00											
DMI	0.58	0.47	0.50	0.60	0.53	0.62	0.59	0.66	0.57	1.00										
KKR	0.55	0.46	0.42	0.57	0.52	0.54	0.55	0.61	0.59	0.54	1.00									
VPM	0.61	0.45	0.44	0.61	0.54	0.61	0.60	0.59	0.58	0.58	0.60	1.00								
TRY	0.56	0.52	0.58	0.62	0.67	0.70	0.67	0.61	0.66	0.59	0.60	0.64	1.00							
PKI	0.59	0.50	0.53	0.63	0.60	0.61	0.68	0.63	0.64	0.59	0.62	0.65	0.66	1.00						
TNJ	0.55	0.45	0.52	0.66	0.59	0.62	0.63	0.58	0.57	0.68	0.55	0.60	0.65	0.66	1.00					
ALR	0.62	0.47	0.45	0.56	0.56	0.55	0.54	0.54	0.57	0.57	0.65	0.60	0.67	0.57	0.56	1.00				
PMR	0.49	0.50	0.48	0.54	0.58	0.53	0.55	0.57	0.57	0.57	0.56	0.56	0.63	0.57	0.63	0.62	1.00			
CBE	0.53	0.44	0.50	0.53	0.55	0.59	0.58	0.55	0.57	0.53	0.55	0.54	0.64	0.54	0.56	0.56	0.56	1.00		
TRR	0.53	0.39	0.40	0.52	0.52	0.56	0.57	0.53	0.49	0.57	0.51	0.63	0.60	0.53	0.60	0.55	0.53	0.53	1.00	
OTY	0.59	0.47	0.46	0.59	0.51	0.57	0.55	0.57	0.52	0.53	0.55	0.52	0.59	0.56	0.59	0.60	0.60	0.59	0.59	1.00

Table 5

Details of insecticides used in the fields of brinjal against the larval infestation of shoot and fruit borer, *Leucinodes orbonalis*

Districts	Common name ^a	Trade name	Manufacturer	Dose
1. Madurai	Carbosulfan	Marshal 25	FMC Corporation	50ml/acre
2. Dindigul	Deltamethrin	Deniel EC	Greenland Crop Sciences	50ml/acre
3. Theni ^b	Phyrethroids	Flame	Tatva Crop Science	70ml/acre
4. Namakkal	Fenvalerate	Fantom 20C	Chemet Chemicals Ltd.	60ml/acre
5. Erode	Fenvalerate	Fantom 20C	Chemet Chemicals Ltd.	60ml/acre
6. Karur ^b	Spinosad	Tracer 240SC	Dow Agrosiences	60ml/acre
7. Salem	Chlorantraniliprole	Coragen 20SC	Dupont	50ml/acre
8. Virudhunagar	Deltamethrin	Deniel EC	Greenland Crop Sciences	50ml/acre
9. Tirunelveli ^b	Chlorantraniliprole	Coragen 20SC	Dow Agro Sciences	60ml/acre
10. Dharmapuri	Flubendiamide	Belt 48SC	Bayer Crop Science	50ml/acre
11. Kanyakumari	Triazophos	Parashute	Paramount Pesticide Ltd.	50ml/acre
12. Villupuram	Cybermethrin	Ammo	FMC Corporation	50ml/acre
13. Trichy	Spinosad	Tracer 240SC	Dow Agrosiences	60ml/acre
14. Pudukottai	Triazophos	Parashute	Paramount Pesticide Ltd.	50ml/acre
15. Tanjore	Chlorantraniliprole	Coragen 20SC	Dupont	50ml/acre
16. Ariyalur	Chlorantraniliprole	Coragen 20SC	Dupont	50ml/acre
17. Perambalur	Enamectin benzoate	Timer 1.9EC	HebbeVian	200ml/acre
18. Coimbatore	Malathion	Shrithion	Sri Ram Agro Chemicals	140ml/acre
19. Tirupur	Fenvalerate	Fantom 20C	Chemet Chemicals Ltd.	60ml/acre
20. Ooty	Deltamethrin	Deniel EC	Greenland Crop Sciences	50ml/acre

^aMaximum use of insecticide type among various synthetic chemicals used against the infestation of *L. orbonalis* ^bBrinjal fields observed with higher incidence of larval infestation despite application of synthetic insecticides

Figures

Fig. 1

Figure 1

Image of Tamil Nadu geographical map showing collection sites of brinjal shoot and fruit borer, *L. orbonalis* in Tamil Nadu, South India

Fig. 2

Figure 2

PCR-RAPD profile generated by ten selected random decamer primers with genomic DNA isolated from various populations of *L. orbonalis*. Lanes 1: Madurai-MDU; 2: Dindigul-DGL; 3: Theni-TNI; 4: Namakkal-NKL; 5: Erode-ERO; 6: Karur-KRR; 7: Salem-SLM; 8: Virudhunagar-VHN; 9: Tirunelveli-TVL; 10: Dharmapuri-DMI; 11: Kanyakumari-KKR; 12: Villupuram-VPM; 13: Trichy-TRY; 14: Pudukottai-PKI; 15: Tanjore-TNJ; 16: Ariyalur-ALR; 17: Perambalur-PMR; 18: Coimbatore-CBE; 19: Tirupur-TRR; 20: Ooty-OTY. M - 100 bp DNA ladder (GeneDireX, Bio-Helix Co Ltd, Taiwan) run as standard molecular weight marker

Fig. 3

Figure 3

The dendrogram based UPGMA cluster analysis from the RAPD binary data showing the genetic variability among the populations of *L. orbonalis*

Fig. 4

Figure 4

PCA scatter diagram analysis showing the distributions of *L. orbonalis* populations

Fig. 5

Figure 5

PCO scatter diagram analysis showing the distributions of *L. orbonalis* populations

Fig. 6

Figure 6

The nMDS Shepard's plot showing similar values in obtained rank and target rank

Fig. 7

Figure 7

The subpopulations (SPs) showing admixture of alleles in genotypes of *L. orbonalis* populations based on structure analysis

Fig. 8

Figure 8

Electrophoretic profile (right) of trait specific locus extracted by gel purification from RAPD analysis. Lanes: I – 3 kb marker; II – 0.5 kb insert DNA (trait specific locus)

Fig. 9

Figure 9

LB plates supplemented with IPTG/X-Gal/Ampicillin (White colonies – recombinant; Blue colonies – non-recombinant) (A) Positive control plate showing both recombinant and non-recombinant colonies (B) Sample plate showing non-recombinant colonies (C) Sample plate showing recombinant colonies

Fig. 10

>Insert DNA sequence (402 bp)

```
ATTGTAATGTTGCTGTGATACGGCAGAGGCCAGTGGTTGTCCGGGAGCCAAATAT
GCGTTGTCCCTAGTTGCTTTTCAGCTGATGGAACCCGTCATGAGAGTGGTGGAGG
GTTTAAATTGTCTTGCTGACCTTTTAATTACTGGAATGTCCAGCCTCTAATTCCCT
GATTCTTGTTTGGAACGCATGGCTTCTGTTTCATAAGCTTCTTTAATATTGGTATTT
TGCCTGACCTGCATGAGTAGTCCTTCATATGGCACGCGAGAGGATATGGCTCTCT
GCGTAGGTGTGCGTGGCTCGTTGAGGTGCCTCTCTCTTCTCCTGTATAGCTGCTTC
CCATTTCCCTAACTCGTTCTGGAAGTTGTTGTGGCATATCAACAGGAAGACATAT
TCTTGGGTTCTGCT
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Figure 10

Nucleotide sequence of cloned RAPD fragment identified as SCAR marker

Fig. 11

Figure 11

PCR analysis with genomic DNA of different populations of *L. orbonalis* using designed SCAR primers (Locations indicated in red colour & marked with arrow at the top panel indicates populations positive for amplification with SCAR primers)