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Genetic diversity estimates in *Trichogramma* populations from sugarcane cropping systems in India

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Abstract: Determining the genetic diversity among the populations of natural enemies can help in identifying the strain best suited to a particular geographical location. A combination of molecular methods like ITS 2 sequence analysis and RAPD markers were employed to study the genetic diversity in the extensively used biocontrol agent, *Trichogramma.* The BLASTN of the diagnostic ITS 2 region classified all the populations as *Trichogramma chilonis* Ishii barring the population from Andhra Pradesh ecosystem. The respective ITS 2 sequences have been submitted in nucleic acid database. The dominant markers like RAPD gave 19% polymorphic bands with population specific markers for eight populations. The *Trichogramma* populations in the Indian subcontinent are genetically related as revealed from the analysis and can provide some clues for effective biological control. Hence, the present study provides novel data critical to the successful biological control using *Trichogramma* species. The diversity estimates will help in selecting the populations best suited for biological control in specific agro-climatic zones.

Key words : Axygen gel extraction kit, Biological control, Sugarcane ecosystem, *Trichogramma*

Introduction

India is one of the largest sugarcane producers in the world with an average of approximately 300 million tonnes and the cane production is expected to rise steadily. Sugarcane is infested by about 288 insects, of which nearly two dozen cause heavy losses to the quality as well as quantity of the crop. Biological control of crop pests is yet to be explored in India. Practically every crop pest has its natural enemies in the form of parasites, predators and pathogens. Characterization of these natural enemies will help in improving the efficiency of biological control against crop pests. *Trichogramma* are group of minute parasitic wasps, having a wingspread of about 1/50th of an inch. Despite its size, it is an efficient destroyer of eggs of many agricultural pests in their early stage. These parasitic wasps disperse readily in search for over 200 species of eggs and parasitize, but do not feed on or harm vegetation. The most common mode of reproduction in *Trichogramma* is haplodiploidy in which females (diploids) arise from fertilized eggs and males (haploids) arise from unfertilized eggs. *Trichogramma* has been a potential biocontrol agent in a variety of crops such as cotton, corn, tomatoes, avocados, walnuts, pecans, apples, alfalfa, etc (Hassan, 1993).

There is a need for molecular markers for natural enemies to establish phylogenetic relatedness for identification of various biotypes and to assess heritable variation for population genetics and ecological investigations. Identification of *Trichogramma* wasps is particularly difficult due to their minute size and complexity (Pinto and Stouthamer, 1994). The ISSR (Inter Simple Sequence Repeats) technique has been used to differentiate close individuals, due to its high polymorphism level, reproducibility and low cost. Borba *et al.* (2005) measured the level of genetic differentiation among five populations of *Trichogramma*, three belonging to the species *T. pretiosum*, one to *T. atopovirilia* and one to *T. bruni* using 26 ISSR markers, of which 11 were highly polymorphic. Li (2007) molecularly characterized and differentiated four most commonly occurring *Trichogramma* species, using molecular markers like RAPD along with direct amplification of the Internal Transcribed Spacer 2 (ITS 2) region of ribosomal DNA. At the species and intraspecies levels, the ITS1 and ITS 2 regions have been often used as taxonomic tools for insect identification especially to identify the closely related groups. Stouthamer and Kazmer (1994) used ITS 2 DNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the *Trichogramma.* The intra species variations in *Trichogramma* are minimal vis-a-vis interspecies variation.

Our study was to determine the intra species variation in the *Trichogramma* populations collected from sugarcane cropping systems in Indian subcontinent. This study can provide very important clues as the population of parasitoids from distinct geographical regions may differ in relevant biological characteristics of importance to biological control.

Material and methods

Tirchogramma populations were collected by National Bureau of Agriculturally Important Insects (NBAII) Bengaluru from different geographical agro-climatic zones of Uttar Pradesh, Punjab, Haryana, Maharashtra, Karnataka, Andhra Pradesh, Orissa and Tamil Nadu in India.

From individual *Trichogramma* populations, DNA was extracted using Chelex 100 method (Ciociola *et al*., 2001), quantified spectrophotometrically and by gel electrophoresis. The PCR amplification was carried out using the ITS-2 primers designed using the sequence information of ribosomal DNA of the wasps and custom synthesized from Sigma-Aldrich chemical company. Each PCR reaction volume of 25 µl contained 5 µl of 50 ng of DNA template and 20 µl of the PCR mix [2.5 ìl 10X buffer, 2.5 mM each of dNTPs; 10 pM of each ITS 2 forward and reverse primers (5'-TGTGAACTGCAGGACACATG-3' and

5'-GTCTTGCCTGCTCTGAG-3') located in the 5.8S and 28S region of rDNA respectively; 1 µl of *Taq* polymerase (1 unit/µl) and 12.5 µl of autoclaved Milli Q water]. The above reaction mixture was amplified in a BIO-RAD thermocycler with a cycling program of 3 minutes. of initial denaturation at 94ºC, followed by 35 cycles with of denaturation at 94ºC for 40 seconds, annealing at 54ºC for 45 seconds; extension at 72ºC for 45 seconds and a final extension for 5 minutes at 72ºC (Gonçalves *et al*., 2006). Amplicons were analyzed by 1.2% agarose gel electrophoresis run in 1X TAE buffer (100 mM Tris-HCl, pH 8.0; 83 mM acetic acid; 1 mM EDTA) at 60 V. The gels were stained with 0.5 mg/ml ethidium bromide solution and visualized under UV light. The size of the amplified products was compared to 100 bp marker (Axygen, California, USA) and the bands of interest were then eluted using Axygen Gel Extraction kit and sequenced in automated DNA sequencer (Bangalore Genei, Bengaluru, India).

Forty RAPD primers (Sigma-Aldrich chemical company, Bengaluru, India) were used (Table 1) for the diversity analysis. Amplification was done in a 25 ìl reaction volume containing 35 ng of template DNA; 2.5 ìl 10X buffer, 2.5 mM of dNTPs; 10 pM of primer and 0.3 ì l *Taq* polymerase (3U/ì l conc.). PCR conditions were maintained at 94°C for 4 minutes and 45 cycles of 94°C for 1 minutes (denaturation), 40°C for 1 minutes (annealing) and 72°C for 2 minutes (extension) each, followed by 10 minutes of final elongation at 72°C. The random amplification was performed thrice for all the samples and reproducible primers were selected for data analysis. ISSR primers based on the sequences based on *Trichogramma* Westwood populations (Borba *et. al*., 2005) were also taken up for initial screening. The ISSR PCR was carried out using the same PCR program with an exception of an annealing temperature of 55°C.

Amplicons were analyzed on 1.5% agarose gel run in 1X TAE buffer at 60 V. The gels were stained with 0.5 mg/ml ethidium bromide solution and visualized under UV light. The size of the amplified products was compared to 100 bp marker. The bands were scored with the help of UVI BANDMAP software (Uvipro Platinum, Cambridge, UK).

Table 1. Sequence of the RAPD primers used for the characterization of *Trichogramma* populations

Primer name	Primer Sequence	Primer name	Primer Sequence
A01	CAGGCCCTTC	RA6	TCTTCGAGGA
A06	CGTCCCTGAC	RA7	AGCACTTCGG
B02	TGATCCCTGG	RA8	CACCGTTCTG
B08	GTCCACACGG	RA21	AGGCCGTATC
C04	CCGCATCTAC	RA31	AACCGACGGG
C14	TGCGTGCTTG	RA32	TGCCCTGCCT
D06	ACCTGAACGG	RA35	AAGCTCCCCG
D12	CACCGTATCC	RA36	GGGGGTCGTT
E02	GGTGCGGGAA	RA45	TACCACCCCG
E09	CTTCACCCGA	RA46	CCAGACCCTG
F05	CCGAATTCCC	RA47	CGGGAACCGA
F07	CCGATATCCC	RA48	GAAGGCGCGT
G01	CTACGGAGGA	RA49	GTCACTCCCC
G14	GGATGAGACC	RA50	GCTGTGCAGC
H05	AGTCGTCCCC	RA51	TGTCCCGGGTG
RA1	GTCTGACGGT	RA52	GGCACCACCA
RA2	CAGCTCAAGT	RA53	TCCGTCGTGG
RA3	CGATCGAGGA	RA54	CGTAGCGCGA
RA4	GCAGAGCATC	RA58	GCCCCATCAC
RA5	AAGCAGCAAG	RA59	CGGGCAACGT

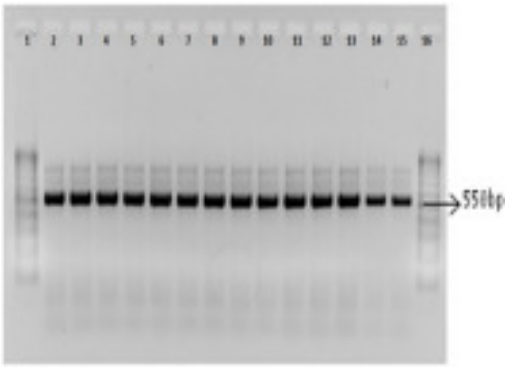

Fig 1. PCR amplification of ITS 2 region in *Trichogramma* populations from different regions

Legend	
Lane 1: Marker	Lane 2: AP1-Andhra Pradesh
Lane 3: PN1-Maharashtra	Lane 4: SH1-Shanjahanpur (Uttar Pradesh)
Lane 5: UP2-Uttar Pradesh	Lane 6: LK4-Lucknow (Uttar Pradesh)
Lane 7: PJ1-Punjab	Lane 8: PJ2-Punjab
Lane 9: KA1 Karnataka	Lane 10: OR1-Orissa
Lane 11: TN1-Tamil Nadu	Lane 12: HR1-Haryana
Lane 13: TN2-Tamil Nadu	Lane 14: UP1-Uttar Pradesh
Lane 15: LK3-Lucknow (Uttar Pradesh)	Lane 16: Marker

Amplified bands were scored as present (1) or absent (0) for each DNA sample. Ambiguous bands that could not be clearly distinguished were not scored. Data from all RAPD results were used to compute the measures of genetic distances for all populations. In addition genetic diversity was measured by the percentage of polymorphic bands at population, region or species level, from the total number of bands surveyed.

Data was analyzed from the amplifications that were reproducible after three repetitions. Bands observed in each lane were compared with all the other lanes of the same gel and were scored as present (1) and absent (0). Fragment sizes were estimated based on the 1Kb DNA ladder (Axygen) using BANDMAP software (Uvipro Platinum). The dendrograms were developed based on genetic distance by UPGMA method (Sneath and Sokal, 1973).

Results and discussion

The PCR using ITS 2 primers with all the 14 populations of *Trichogramma* produced an amplicon of about 525-550 bp (Fig 1). The ITS 2 sequences of populations revealed that 13 populations match to *T. chilonis* and one population from Andhra Pradesh belong to *T. pretiosum*. All monomorphic bands in the ITS 2 amplification were detected and the diversity estimates were made after three rounds of sequencing. All the ITS 2 sequences were deposited in GenBank and the accession numbers were obtained (Table 2).

Multiple sequence analysis of the ITS 2 fragments was done using CLUSTAL W and NJ Tree was plotted using the ITS 2 sequence of the populations. The NJ tree (Fig 2) constitutes three

Table 2. The genebank accession numbers of ITS 2 regions of the fourteen *Trichogramma* populations collected from the sugarcane cropping systems in India

Population /Isolates	Location	Identification based on ITS 2 sequences	Gen Bank Accession Number
PJ 1	Punjab (Jalandhar)	*Trichogramma chilonis*	FN568064
PJ 2	Punjab (Nawanshahar)	*Trichogramma chilonis*	FN568065
UP 1	Uttar Pradesh (Lucknow)	*Trichogramma chilonis*	FN568066
UP 2	Uttar Pradesh (Lucknow)	*Trichogramma chilonis*	FN599802
KA1	Karnataka (Mandya)	*Trichogramma chilonis*	FN566844
HR 1	Haryana (Ambala)	*Trichogramma chilonis*	FN568056
TN 1	Tamil Nadu (Coimbatore)	*Trichogramma chilonis*	FN665798
TN 2	Tamil Nadu (Cuddalore)	*Trichogramma chilonis*	FN566843
AP 1	Andhra Pradesh (Anakapalli)	*Trichogramma pretiosum*	FN568059
PN 1	Maharashtra (Pune)	*Trichogramma chilonis*	FN568057
OR 1	Orissa (Bhuvaneshwar)	*Trichogramma chilonis*	FN568058
LK 3	Uttar Pradesh (Lucknow)	*Trichogramma chilonis*	FN599803
LK 4	Uttar Pradesh (Lucknow)	*Trichogramma chilonis*	FN665797
SH 1	Uttar Pradesh (Shahjahanpur)	*Trichogramma chilonis*	FN665799

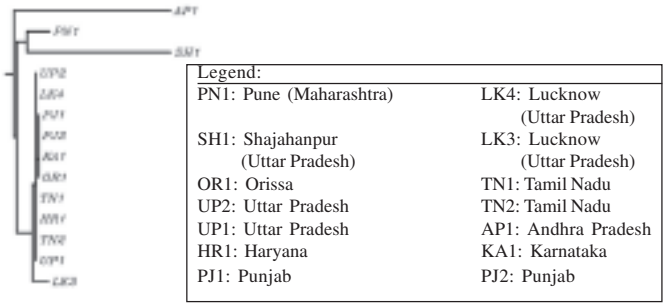

Fig 2. N-J tree showing the Intra species variation in the ITS 2 region of *Trichogramma*

distinct clusters from fourteen populations. Population from Andhra Pradesh (AP1) forms a unique cluster suggesting that it is different from other 13 populations, while two populations PN1 (Maharashtra) and SH1 (Uttar Pradesh) formed a separate sub-cluster.

The sequence similarity analysis revealed 98-100% similarity between 10 populations and approximately 80% in four populations (Table 3). Four populations had a variation of more than 3 per cent suggesting a possible influence of abiotic factors on their genetics for survival under stress conditions. It is evident that the *Trichogramma* specific ITS 2 region amplification and sequencing can be effectively used as a molecular method for identification of *Trichogramma* species. The ITS 1 and 2 region has been used extensively to examine the taxonomic status of species for diagnostic purposes. The species diagnostic differences in sibling species of mosquitoes of the genus *Anopheles* was determined (Collins and Paskewitz, 1996). Zhu and Greenstone (1999) utilized the ITS 2 region to distinguish species and strains of a hymenopterous parasitoid *Aphelinus*. In their study, the bands were monomorphic and could be used only for species

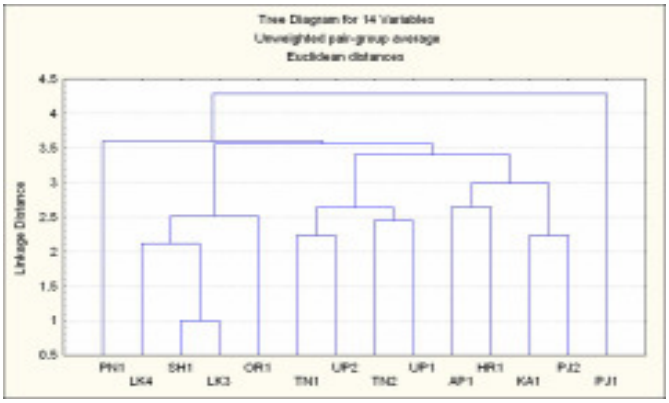

Fig 3.Dendrogram generated from RAPD marker analysis of 14 populations using Neighbor Joining method based on Dice distance

Table 3. ITS 2 sequence similarity of *Trichogramma* populations collected from the sugarcane cropping systems in various agroclimatic zones in India

POPULATIONS	Sequence similarity (%)													
	PJ1	PJ2	UP1	UP2	TN1	TN2	HR1	KA1	PN1	OR1	AP1	LK3	LK4	SH1
PJ1		100	99	99	99	99	99	100	94	100	84	96	99	80
PJ2			99	99	99	99	99	100	94	100	84	96	99	80
UP1				100	99	99	99	99	93	99	86	97	100	80
UP2					99	99	99	99	93	99	86	97	100	80
TN1						100	100	99	93	99	85	97	99	80
TN2							100	99	93	99	85	97	99	80
HR1								99	93	99	85	97	99	80
KA1									94	100	84	96	99	80
PN1										94	80	91	93	81
OR1											84	96	99	80
AP1												68	86	69
LK3													97	72
LK4														80
SH1														

identification. Similar observations on ITS 2 pattern were made in our study and the minor variations were detected only after sequencing the ITS 2 fragments.

The *Trichogramma* populations from different regions were compared for their diversity using 40 RAPD markers (Table 1), of which CCGCATCTAC, CCGCATCTAC, AGTCGTCCCC, TGCGTGCTTG, CTACGGAGGA, AACCGACGGG, TGCCCTGCCT, AGCACTTCGG were found to be polymorphic. The size of RAPD fragments ranged from 200 to 3000 bp. With 8 polymorphic RAPD markers, 1389 scorable bands were obtained that constitute 19% polymorphic markers. A dendrogram (Fig 3) was constructed based on the scored data. The dendrogram revealed that all the populations in the study are unique and the sub clusters are not in consonance with the geographical collection zones. This is an interesting observation and a detailed review of the introduction of these natural enemies in the Indian ecosystems can help explain this type of genetic associations. The populations SH1 (Uttar Pradesh) and LK 3 (Uttar Pradesh) appear to be genetically closely related populations forming a close sub cluster with minimum genetic distance. The wasp populations from Maharashtra (PN1) and Punjab (PJ1) appeared to be genetically diverse as they cluster to the extremes of the dendrogram. This looks plausible as they inhabit the ecosystems which vary significantly in temperature and humidity. The population from Andhra Pradesh (*T. pretiosum*) is genetically closely related to *T. chilonis* population from Haryana. This finding is surprising as these regions are geographically far apart and any physical migration of the wasps is not possible. It appears that these are genetically related introduced populations. The *Trichogramma* populations studied from Indian sugarcane cropping system are unique and they associate in different clusters based on genetic distances. The population specific RAPD markers have been recorded and can be used for identifying eight populations. These rare markers can be an indication of population sub division. Absence of rare or population specific markers indicate a genetic bottleneck in the recent history of population. This occurs when a population has undergone a drastic contraction and its numbers rebuilt from a small number of individuals, a common occurrence in insect colonies. This type of contraction is also a possibility in Indian populations as they are introduced parasitoids. The genetic variation of Hymenoptera as measured by isozyme electrophoresis is approximately one third of that of diploid insects (Berkelhamer, 1983; Unruh *et al*., 1986; Owen, 1993) though there are few reports of high genetic diversity (Sheppard and Heydon, 1986; Boato and Battisti, 1996). We have not measured the genetic variation index; however our genetic distance estimates endorse the reported scientific information. Further studies are carried out to correlate the temperature and pesticide tolerance levels and to link these to the genetic backgrounds of these populations. This information from Indian ecosystems will prove vital in effective biological control programs.

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