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Evaluation of genetic variability in wild population of *Chironomids* of Manipur using SCoT marker

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Abstract
SCoT (Start Codon Targeted) is a novel method for generating plant DNA marker. It had been using to determine the genetic polymorphism or to delineate the species of plants. In the present study the primer has been utilized to evaluate the genetic variability of five *Chironomus* species for the first time from Manipur. The larvae from different breeding ground of aquatic habitats were collected and reared till adults emerged. The identification of the species was done from larvae, exuviate and adults. DNA were extracted from either larvae or exuviate or adults. The COX 1 primer used for the sequencing produced around 700 base pair. The primer of the SCoT I was used to run PCR and product were run gel in 2% agarose gel for one and half hour in duplicate. The countable bands were recorded for the analysis. The bands were recorded and analysed using NTSYS pc to construct the phylogenetic tree. This plant primer has been utilized in studying the polymorphism in insects *Chironomus* and SCoT I produces promising polymorphic bands as well as non-polymorphic bands. The future works should have focused on the increasing number of the primers (15 to 20) and the specimens for the study should be more so as to produce a promising results to evaluate delineate the species of the *Chironomus* more precisely and less laboriously as DNA sequencing and the phylogenetic studies. On the basis present results, it can be safely said the SCoT could be used for the evaluating the genetic variability of *Chironomus* in future.

Keywords: Manipur, SCoT primer, *Chironomus*, genetic variability, five species

1. Introduction
Recently, a molecular marker technique termed start codon targeted (SCoT) polymorphism, a simple and novel DNA marker technique, was developed by Collard and Mackill [1]. SCoT marker technique is a simple and novel targeted molecular marker tool which base on the short conserved region flanking the start codon (ATG) in plant genes; it can generate more information related to biological traits than random DNA markers [1]. SCoT markers employ longer primers (18-mer) producing high polymorphism which is reproducible [2], it is suggested that primer length and annealing temperature are not the sole factors determining reproducibility [1]. As a single primer amplification molecular marker technique, SCoT markers are technically simple, not time-consuming and not laborious, and requires no prior sequence information and targeting functional regions [3]. SCoT markers have been broadly and successfully used for evaluation of genetic diversity, phylogenetic, fingerprinting, variation, and differentiation since 2009 [2-4]. Start Codon Targeted (SCoT) is a novel method for generating plant DNA markers. This method was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT uses single 18-mer primers in polymerase chain reaction (PCR) and annealing temperature of 50 ^{0}C. PCR amplicons are resolved using standard agarose gel electrophoresis. This method was validated in rice using a genetically diverse set of genotypes and backcross population.
In the present study, the SCoT I primer had been utilized for the first time to study the polymorphism of the five different *Chironomus* species. It is observed that the primer is promising in and future many primers could be utilized to study more species of the *Chironomus.*

2. Materials and methods
2.1 DNA Extraction
Genomic DNA was extracted using the method described [5] with some modifications [6]; a number of 25 preserved larvae allow to dry were put in 1.5ml microcentrifuge tube and 100 µL

of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 1% SDS, 300 mM NaCl) were added. The larvae were then grind using a micro pestle (Tarson, India). Then another 300 µL of extraction buffer was added to the micro centrifuge tube. The mixture was kept in a water bath for 10 mins at 65 ^{0}C. Then an equal volume of chloroform: isoamyl alcohol (24:1) and tris-saturated phenol was added to the mixture (200 µL of chloroform: isoamyl alcohol and 200 µL of Tris-saturated phenol). After centrifuging for 1 min at 15000 RPM, the supernatant was collected in a new microcentrifuge tube. Then 800 µL of ice-cold isopropanol was added and mix by inverting the tube. Then the mixture was again centrifuged for 5 mins at 15000 RPM and the supernatant was discarded. The pellets were washed with 70% alcohol and dried. Then the pellets were dissolved in 50 µL TE buffer and stored at -20 ^{0}C.

2.2 Assay of DNA Yield and Purity

Absorbance (*A*) at 260 nm and 280 nm were measured using CE7200 7000 series UV spectrophotometer (Cecil, UK) for determining the yield and purity of the DNA.

2.3 Marker Genotyping and Data Analysis

PCRs were performed within a total volume of 10µL using a PTC-100 Thermocycler (MJ Research Model PTC100). PCR mixtures contained: PCR buffer (Promega; 20 mM Tris–HCl [pH8.4], 50 mM KCl), 1.5 mM MgCl2, 0.24 mM of each dNTP, 0.5 U of Taq polymerase (Promega), and 0.8µm of primer. Each reaction contained 25 ng of template DNA. A standard PCR cycle was used: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; the final extension was held for 5 min. All PCR amplification products were separated on 1.2% agarose gels in Tris–borate–ethylene diaminetetra acetic acid (TBE) buffer stained with ethidium bromide and visualized under UV light. Clear reproducible DNA markers(bands) were scored as binary data from gel photos (absent=0 or present=1). Calculation of the Jaccard similarity coefficient and construction of phylogenetic tree; were done using NTSYSpc applications.

Table 1: The table shows the number of species, habitat, location with GPS reading and materials collected.

SL. No.	Species	Location	Material
1.	A	Iroisemba (24^{0}48.765', 93^{0}53.367')	Larvae, Pupa
2.	B	Iroisemba (24^{0}48.570', 93^{0}53.546')	Larvae, Pupa
3.	*Chironomus plumosus*	Iroisemba, Lousitpat (24^{0}48.498', 93^{0}53.367')	Larvae, Pupa, Adult
4.	*Chironomus striapenis*	Iroisemba (24^{0}48.619', 93^{0}53.535'), Lamboikhongnangkhong (24^{0}48.313', 93^{0}54.361)	Larvae, Pupa, Adult
5.	*Chironomus circumdatus*	Canchi Indane, Kwakeithel, Keishamthong (24^{0}47.800, 93^{0}53.933)	Larvae, Pupa, Adult

3. Results

Genomic DNA was run using the COX universal primer of the insect to decide the identity of the Dipteran insect and later the PCR product will be sequenced for exact identification of the species as a downstream study.

In the present study, a single 18-mer SCoT I primer (5' CAACAATGGCTACCACCA 3') was used as a forward and reverse primer with an annealing temperature of 50°C and the amplicons were resolved using standard agarose gel electrophoresis. It resulted 12 clear, unambiguous, consistently reproducible uniform and scorable bands of which 91.67% bands were polymorphic while 8.3% bands were monomorphic.

Jaccard similarity coefficient is highest between *C. striapenis* and *Sp. B* (0.714); lowest between *C. circumdatus* and *Sp. B* (0.182). From the dendrogram, Fig. 3 the species can be grouped into two clusters based on their similarities Cluster I and Cluster II. Cluster I have 3 species, *C. plumosus*, *Sp. A* and *C. circumdatus* while Cluster II have two species, *Sp. B* and *C. striapenis.*

Table 2: Shows the Jaccard Similarity coefficient of the five different species.

	C. plumosus	***Sp. A***	***Sp. B***	***C. stripenis***	***C. circumdatus***
C. plumosus	1				
Sp. A	0.571	1			
Sp. B	0.375	0.250	1		
C. stripenis	0.444	0.333	0.714	1	
C. cirumdatus	0.400	0.444	0.182	0.250	1

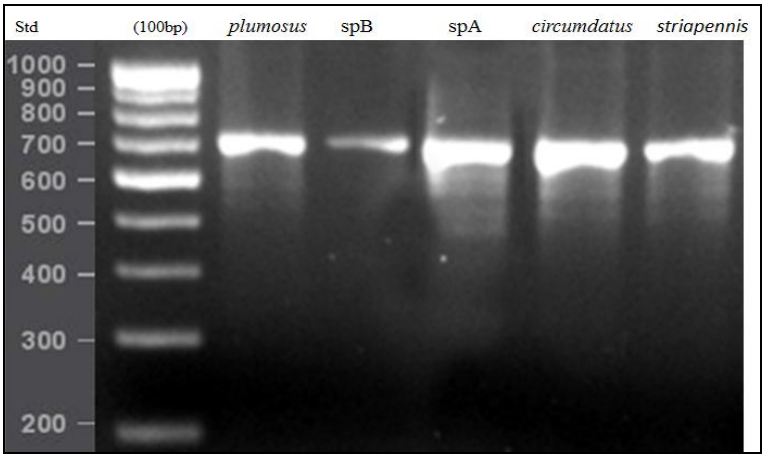

Fig 1: The gel plate showing the COX 1 PCR products of approximately 700 bp as instructed.

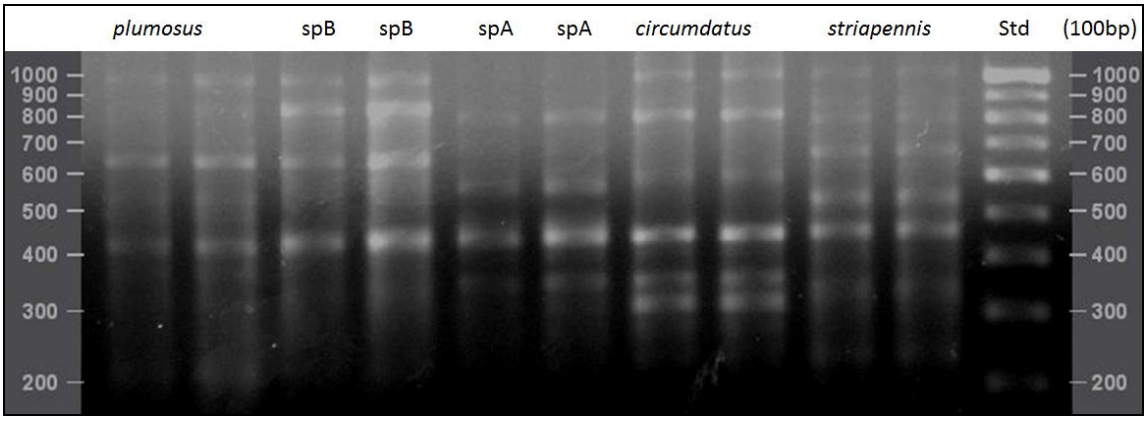

Fig 2: The amplification profile of SCoT primer I showing the polymorphic and non-polymorphic bands of the five species in duplicate of each species.

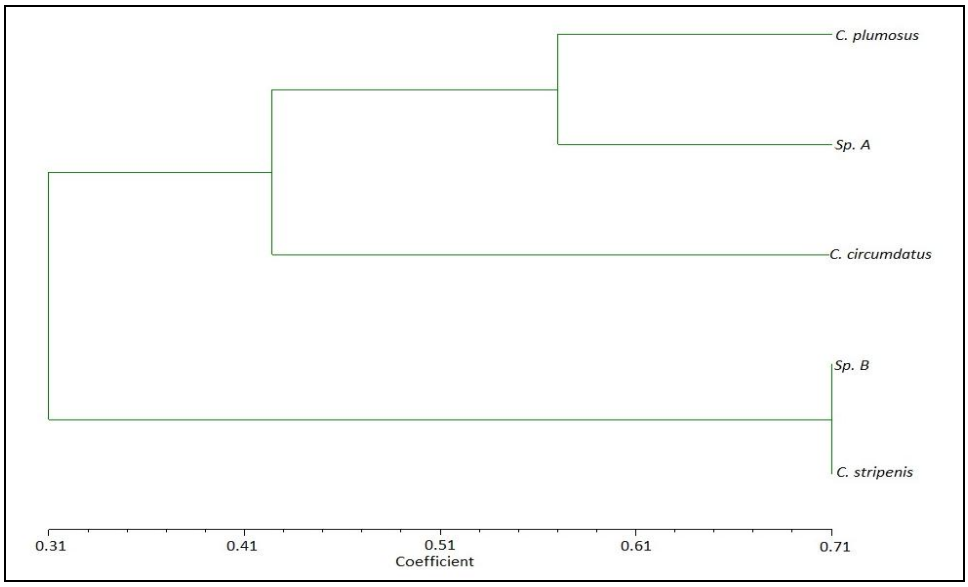

Fig 3: The phylogenetic tree/Dendrogram of the five species obtained from NTSYS pc applications of the SCoT I primer.

4. Discussion

SCoT markers are PCR-based single primers developed from the short conserved sequence of plant genes flanking the start codon ATG [1], these regions are conserved among all the genes. Despite being similar to other single primer (used both as forward and reverse primer) based techniques like RAPD and ISSR, SCoT markers are more reliable (annealing temperature 50 °C compared to 37– 42 °C), reproducible (target genes instead of arbitrary or non-coding regions) and easy to design. SCoT polymorphism detection is less cost and labour intensive compared to AFLP and other similar techniques (SAMPL, FIASCO). The utilization of the primer for the first time for the study of the polymorphism in the insect particularly the *Chironomids* species. a single 18-mer SCoT I primer (5' CAACAATGGCTACCACCA 3') used as a forward and reverse primer with an annealing temperature of 50°C and later resolved using standard agarose gel electrophoresis. It resulted 12 clear, unambiguous, consistently reproducible uniform and scorable bands of which 91.67% bands were polymorphic while 8.3% bands were monomorphic. This shows that the primer could be utilized for study of the insect polymorphism other the plant materials. SCoT markers have been broadly and successfully used for evaluation of genetic diversity, phylogenetic, fingerprinting, variation, and differentiation since 2009 [2-4]. Start Codon Targeted (SCoT) is a novel method for generating plant DNA markers. This method was developed based on the short conserved region flanking the ATG start codon in plant genes[1] is similarly applicable to animals had been proved by the present study. SCoT uses single 18-mer primers in polymerase chain reaction (PCR) and annealing temperature of 50 ^{0}C. PCR amplicons are resolved using standard agarose gel electrophoresis. This method was validated in rice using a genetically diverse set of genotypes and backcross population. Taxonomic classification is generally based on morphological traits, but the availability of novel and powerful molecular techniques is helping in modifying/refining the existing systems of classification and establishing phylogenetic relationships based on DNA/protein sequences especially in morphologically identical/related species. PCR-based molecular markers have contributed significantly in establishing phylogenetic relationships.

The future works should have focused on the increasing number of the primers upto 15 to 20 and the specimens for the study should be more so as to produce a promising results to evaluate delineate the species of the *Chironomus* more precisely and less laboriously as DNA sequencing and consequently the phylogenetic studies.

5. Conclusion

SCoT primers are easy and cost effective molecular approach and had been utilized. SCoT markers have been broadly and

successfully used for evaluation of genetic diversity, phylogenetic, fingerprinting, variation, and differentiation since 2009. Start Codon Targeted (SCoT) is a novel method for generating plant DNA markers. This method was validated in rice using a genetically diverse set of genotypes and backcross population. This plant primer has been utilized in studying the polymorphism in insects *Chironomus* and SCoT I produces promising polymorphic bands as well as non-polymorphic bands. The future works should have focused on the increasing number of the primers (15 to 20) and the specimens for the study should be more so as to produce a promising results to evaluate delineate the species of the *Chironomus* more precisely and less laboriously as DNA sequencing and the phylogenetic studies.

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