RAGEP Marker System (RAGEP) and its Validation: A Novel Approach to Assess Insect Biodiversity.

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Insects are omnipresent and encompass 3/4th of the whole animal kingdom. Their wide range of ecological adaptability makes them fittest in the extreme ecosystems. Therefore, there is a need to assess their biodiversity in terms of biological and biogeographical identities. We have devised a novel marker system called Random Amplified Genome Encoding Primer (RAGEP) which has been described to assess the migratory pattern and intra species diversity in H. puera. In the present study we propose to characterize the RAGEPs for its heritability, stringency, reproducibility, species specificity and development of species-specific tags and genome specific tags (nuclear and mitochondrial) using RAGEP marker system. We provide convincing results to accept RAGEP system as good marker system to address biodiversity in terms of its biological and biogeographical identities

Key words: RAGEP, insect, biodiversity.

Introduction

Biodiversity deals with biological and geographical entities such as genes, chromosomes, species, families, and habitats or biogeographic regions. Insects forms one of the most crucial components of the biodiversity. It comprise of 75% of total animal kingdom. Lepidoptera is the second largest order among insects comprising of butterflies, skippers, and moths that falls in 127 families and 46 super families described so far. Around 98% of species fall under Ditrysia in order Lepidoptera. They are omnipresent in all biotic environments. Their wide range of ecological adaptability makes them fittest in the extreme ecosystems. This means that in order to understand the nature of these insects it is essential that we have both distinction and description of biological and biogeographical entities. Therefore, there is a need to develop a precise, rapid, efficient and highly reproducible tool to address the biodiversity in terms of its biological and

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biogeographical identities.

DNA based marker system gained tremendous importance over the years as they display greater level of polymorphism in comparison to biochemical and protein marker system. Over the last two decades several DNA based marker system has been developed to understand the interspecies and intraspecies diversity. These DNA based marker system has been profoundly used in the field of population biology, phylogeny, genetic mapping and genome characterization in insects [1, 2 & 3]. PCR based RAPD (Random Amplified polymorphic DNA) technique had made easily scorable bands [4] but on the other hand this system suffers from poor reproducibility, making the marker system less popular in insects [5]. However, it has been extensively used for assessing the biogeographical patterns. The development of amplified fragment length polymorphism (AFLP) was adopted as a better alternative for RAPD because of its reliability, reproducibility and capability to resolve multiloci [6]. Inherent disadvantages of this method were that it was expensive and requires cumbersome procedure. This method could resolve biogeographical and biological patterns. Sequencing based methods are more precise which can address both biogeographical and biological patterns but are very expensive. We have devised a novel marker system called Random Amplified Genome Encoding Primer (RAGEP) which has been described to assess the migratory pattern and intra species diversity in H. puera [7]. This technique mimics RAPD which was PCR-based fingerprinting but instead of random primers we employed sequence specific primers. In this paper we propose to characterize the RAGEPs for heritability, stringency, reproducibility, species specificity and development of species-specific tags and genome specific tags (nuclear and mitochondrial) using RAGEP marker system.

**Materials and Methods**

**DNA Isolation and Polymerase Chain Reaction**

DNA extraction was performed with a minor modification of isolation and purification protocol as described earlier being extracted from whole larvae [8]. The quality of the DNA was checked spectrophotometrically by taking the absorbance ratios of 260/280 nm and the working stock for PCR template is diluted to 100ng/µl.

Both nuclear and mitochondrial RAGEP amplifications were performed in a total volume of 30 µl. Each reaction consisted of 1x Taq buffer with 1.5mM MgCl2, 1.2U of Taq polymerase (BG), 0.25 mM of dNTPs (Amersham) and 12 pM of primer per reaction. Primers are from the UBC universal primer kit for insects [9] and primer sequences are listed in Table-1. Amplifications were performed in similar cycling conditions in a Thermocycler (Biorad) programmed as follows: initial denaturation at 95°C for 5 min., followed by 45 cycles of cycle denaturation at 94°C for 1 min., annealing at 36°C for 1 min., in higher stringency 42°C for 1 min., extension at 72°C for 2 min. and final extension at 72°C for 5 mins. The amplification products were separated using 1.2% agarose gel in 0.5xTBE buffer with ethidium bromide staining to visualize the product separation using a Bio-Rad's Fluor S imager. The molecular weight of each band was estimated by comparing with a co-migrating 100-bp ladder (Amersham). RAGEP fingerprints were interpreted using Bionumerics software for heritability (Applied Maths Kortrijk Belgium, ver.3.5).

For species specific-tagging six representatives from Hyblaea puera population and six other lepidopterans Teak skeletonizer (E. machaeralis (Walk.)), Cotton Bollworm (Heliothis armigera (Hübner)), Fruit-Piercing Moth (Othreis fullonia
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Table-1 List of primer sequences used in the study.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primer Names</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>SR-J-14233</td>
<td>AAGAGCGACGGCGATGTGT</td>
</tr>
<tr>
<td>2</td>
<td>N4-N-8924</td>
<td>AAAGCTCATGTGAAAGCTCC</td>
</tr>
<tr>
<td>3</td>
<td>CB-N-10920</td>
<td>CCCTCAGATGATTTGCTCTCA</td>
</tr>
<tr>
<td>4</td>
<td>TK-N-3785</td>
<td>GTTTAAGAGACCAGTACTTG</td>
</tr>
<tr>
<td>5</td>
<td>CytC-B-3'</td>
<td>CATCTTGGTGCCCGGGATGTATTTCTT</td>
</tr>
<tr>
<td>6</td>
<td>CK6-5'</td>
<td>GACCACCTCCGAGCTATCTC(CG)ATG</td>
</tr>
<tr>
<td>7</td>
<td>EFS599</td>
<td>ATCTCCGGATGGGACCGG(CT)GACAA</td>
</tr>
<tr>
<td>8</td>
<td>C2-N-3661</td>
<td>CCACAAATTCTGAAACATTGACCA</td>
</tr>
<tr>
<td>9</td>
<td>Tub3-5'</td>
<td>GATTGGAGGCC(AGCT)GG(AGCT)ACCAGGA</td>
</tr>
</tbody>
</table>

(Clerck)), Leaf roller (*Sylepta derogata* (F.)), Leaf folder (*Cnaphalocrocis medinalis* (Guenée)) and the Silkworm (*Bombyx mori* (L.)) were used for this study. We selected monomorphic bands from the SR-J-14233, N4-N-8924, CB-N-10920 and TK-N-3785. These bands ranges from 1.2kb to 400bp in size and were cloned in pGEM-T Easy vector system (Promega). These cloned bands were sequence characterised and used as probe to confirm the species specificity. PCR products were separated in 1.2% agarose gels in 0.5xTBE buffer and the transfer was done overnight using upward capillary method on Hybond N+ nylon membrane (Amersham). The probes were radiolabeled by PCR using [$\alpha$-P$^{32}$] CTP. The membrane was hybridized at 65°C and washed with 0.1x SSC for high stringency. The membrane was exposed in phosphor imager screen and imaging was done on Bio-Rad’s Personal Molecular Imager FX.

**Results and Discussion**

In an earlier report we have shown the species specificity and reproducibility of RAGEP fingerprints (App-1 & App-2). In the present study we further evaluated the reproducibility by increasing the stringency of PCR conditions while species specific-

ity was further assessed to generate species specific tags and genome specific tags from mitochondrial genes and nuclear genes.

**Heritability of RAGEP markers**

Heritability of the RAGEP markers was done separately for each nuclear and mitochondrial marker. The field collected larval samples were reared in artificial diet in laboratory and were labeled as parental P1 generations (P1a & P1b). The parental P1 generations were reared for their F1 progenies (F1a & F1b). Pupation and emergence of the adults were monitored properly. The field collected samples were not used in the study because they were heterogeneous population. Thus heritability of the markers was estimated by comparing the banding pattern of the P1 with the F1 progenies. Only bands showing clear and reproducible patterns were included in the final analysis and these were scored. The Dice coefficient, Unweighted Pair Group Method (UPGMA) parameters were used to interpret the RAGEP fingerprints with 1000 replication of bootstrapping. The dendrogram establishes the reliability of RAGEP fingerprints in intra population’s studies (Fig. 1). The similarity index between parents and the progenies ranged from 50 to 60%. This clearly indicated that the progenies inherited
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This confirms the heritability of the RAGEP fingerprints.

**Stringency of RAGEP markers**

The stringency of the RAGEP fingerprints was evaluated by repeating the experiments with same sets of insect samples at lower stringency (36°C) (Fig. 2 Panel B) as well as at higher stringency annealing temperatures (42°C) (Fig. 2 Panel A). In spite of the fact that increasing the annealing temperature will result in reduction in non-specificity, in RAGEP fingerprints most of the prominent bands are reproducible at higher stringency. We repeated the experiments both in mitochondrial as well as nuclear RAGEP’s. The prominent scorable bands are the 50% characteristics from their parents. This confirms the heritability of the RAGEP fingerprints.
found in high and low stringent conditions which prove to validate our RAGEP marker system.

**Species-specific tags in *Hyblaea puera* using RAGEP Fingerprints**

In order to characterise species-specific tags in RAGEP fingerprints, we selected some of the prominent monomorphic bands. These monomorphic bands were cloned and sequenced. These sequence characterized bands were further used to screen for their species specificity. To check the species-specificity we selected six random individuals from the wild along with six other Lepidopterans. Both the gel image and the blots show that the RAGEP fingerprints generated for the individual species remains unique (Fig. 3A & 3B). Our results made obvious that bands generated randomly at lower stringency using RAGEP PCR are inimitable to the particular species. These species specific tags varied from 500 bp – 1.2kb. This provides the trustworthiness of RAGEP fingerprints in developing precise SCAR markers. We had acquired four sequence-specific tags for *H. puera* species.

In compared to other methods RAGEPs can not only be used for assessing the biological diversity but also for assessing the biological complexity. In an earlier study we have shown that RAGEP-PCR can enhance the understanding of insect population dynamics and aid in tracing the spread and cause of epidemics. In addition to intra species diversity it can also be used for inter species diversity and phylogenetics analysis. Both Mitochondrial and Nuclear RAGEP PCR fingerprints are derived from the randomness of RAGEP-PCR. Inspite of their randomness the major bands do not cross hybridize between different marker systems and inherits in a dominant pattern. However, it is difficult to predict with certainty that the bands are diagnostic feature of the any genome, but since RAGEP PCR uses gene specific primers, the PCR products could therefore be a result of amplification of homologous genes or pseudogenes of nuclear or mitochondrial origin or which could also represent nuclear mitochondrial DNA (NUMTs). Mitochondrial DNA sequences are frequently transferred to the nucleus giving rise to NUMTs, which are considered to be common in eukaryotes [10]. Very high rate of horizontal transfer between organelle and nuclear genomes has been reported in the brown mountain grasshopper, *Podisma*

![Fig. 3. Species-specific tags generated using RAGEP PCR, panel A shows the gel pictures and panel B represents the Southern blots.](image)
Acknowledgements

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