INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

www.scienceandnature.org

Review Article

ROLE OF MOLECULAR MARKERS (RAPD & ISSR) IN SILKWORM CONSERVATION

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ABSTRACT
The silkworm *Bombyx mori* which is one of the prominent molecular model system is being continuously evaluated through genetic approaches since several decades to better the best in silk production. Because of its structural simplicity exhibiting holometabolus type of metamorphosis, several molecular genetical approaches are very well established in many countries utilizing this insect. Added to these benefits the silk produced by this insect is considered as "Queen of textile fibres" and several breeding strategies with background knowledge of bio-molecular techniques, proteomics and genomics heralded a new avenue in the contemporary biology. Hence, RAPD & ISSR markers are ideal and most widely applied and probably because they do not require the knowledge of genome sequences and protocol is relatively simple, rapid and cost effective and also high reproducible in nature. Moreover, Current trends of application of DNA marker techniques have made revolution at molecular world. Hence, in this review survey, we focused on optimistic application of RAPD & ISSR molecular markers for the conservation of silkworm resources, insights into the dynamics of genome fingerprinting, genome mapping, genetic diversity and phylogenetic relationships, advantages and dis-advantages and future under investigations through utilizing RAPD & ISSR marker techniques, *etc* in silkworm *Bombyx mori* are herein discussed.

KEY WORDS: *Bombyx mori*, Conservation, RAPD & ISSR Techniques, Application.

INTRODUCTION

India is being a systematic cultural heaven in the world, where a step needs for the conservation of its wealth or biological resources (animals, insects and plants) is nowadays a big intricate problem due to plenty of poaching (hunting), deforestation by the human being itself. Henceforth, to enrich the knowledge of molecular genetics is get being fulfill the conservation of the wealth as this may reflects on "prevention is better than cure" or "be safe, don't sorry" because conservation or preservation of molecular genetics of inter and intra natural population is a first and foremost fundamental aim of conserving biological resources. The conservation of silkworm is too worthy because silkworm an insect is highly valuable than compare to the other all lepidopteran insects group. The reason behind it is, commercially valuable silk producing insect on which scores of farmers of tropical and temperate Asian countries rely for their revenue. Domesticated silkworm *Bombyx mori* (Insecta: Lepidoptera: Bombycidae) is a monophagous and feeds exclusively on the leaf of mulberry a hardy evergreen deep rooted plant belongs to the genus Morus (Family: *Moraceae*). However, silkworm germplasm encompass around 3000 genotypes having its origin in temperate and tropical countries (Nagaraju *et al.,* 2000). Silkworm gene pool is broadly categorized into low yielding strains are adopted to tropical conditions and are non-diapausing, while high yielding strains adopted to temperate climate and undergo embryonic diapauses. High yielding strains have higher cocoon weight, cocoon shell weight, shell ratio and better yarn qualities in comparison to low yielding strains (www.silkgermplasm.com) but are highly susceptible to diseases. India is being a tropical country utilizes low yielding native strains and breeds developed from Japanese and Chinese strains of *Bombyx mori* for the silk production through the different breeding strategies, which are mainly aimed to developing vigorous breeds and hybrids to meet twin demand of high survival and high production of quality silk.

Thus, it has resulted in enriching conservation of silkworm germplasm resources due to the efforts of silkworm breeders in the research of molecular genetics and breeding programmes of silkworm *Bombyx mori. S*ilkworm is using widely in basic research in biotechnology and molecular genetics as a model insect. It is worthy to say, due to adaptation of different breeding technologies as well as bio-molecular technologies through utilizing the sequential knowledge of proteomic and genomic trends to fulfill the age old dream of sericulture scientists (scions) to make silkworm become a "molecular model" in the advanced bio-molecular world. Hence, sericulture scientists are efficiently applying the different markers because, the markers have a key role in conservation study of silkworm genetic fingerprinting, variability, diversity and relationships *etc,* which helpful in the construction of linkage maps and in the tracking of individuals or lines carrying particular genes. The emergence of marker systems has closely followed

developments in biochemistry and molecular biology for the past 40 years (Hubby and Lewontin, 1966). The shortcomings of biochemically derived markers such as isozymes, drove the development of markers based on DNA polymorphism (Kan and Dozy, 1978).

A DNA molecular marker in essence detects nucleotides sequence variation at a particular location in the genome. The genetic variation/diversity must be found between the parents of the chosen cross for the marker to be informative among their offspring and to allow its pattern of inheritance to be analyzed. DNA markers can generate fingerprints, which are distinctive patterns of DNA fragments resolved by agarose-electrophoresis and detected by staining or labeling. The advent of the PCR was a breakthrough for molecular marker techniques and made possible many fingerprinting methods. Among all marker techniques till even today studied by various sericulture scientists, the RAPD and ISSR marker techniques are most widely applied and probably because they do not require the knowledge of genome sequences and protocol is relatively simple, rapid and cost effective and also high reproducible in nature (Srivastava *et al.,* 2004 and Vijayan *et al.,* 2005). Hence, it is a high time for silkworm conservationists and geneticists to take appropriate measures to conserve the valuables resources available in the world (Table-1) as well as in CSGRI Hosur, India (Table-2), different research centers and Univesrsities from further degradation and extinction. Therefore in this review article it has elaborated the importance of RAPD and ISSR marker techniques application for the conservation of *Bombyx mori* insight into the genetic-diversity, fingerprinting, genetic mapping, genetic polymorphism, identification and relationships of silkworm genotypes and application of these markers system for the conservation of domesticated silkworm *Bombyx mori* L.

TABLE 1. The conservation of silkworm races in different countries

Sl. no	Country	Bivoltines	Multivoltines	Total
1	Japan	1542	30	1572
2	India	450	150	600
3	China	580	20	600
4	Russia	500	0	500
5	S.Korea	300	6	306
6	N. Korea	281	5	286
7	Bulgaria	183	0	183
8	Brazil	65	10	75
9	France	53	0	53
10	Iran	50	0	50
11	Italy	30	0	30
12	Thailand	25	5	30
13	Vietnam	20	5	25
Total		4079	231	4310

(Source: FAO Manual, 2003)

TABLE 2. The conservation of silkworm races in CSGRC Hosur, India which obtained from the different countries.

Sl. no	Country	Bivoltines	Multivoltines	Total
1	India	207	63	270
2	Japan	64	3	67
3	China	40	4	44
4	Russia	19	0	19
5	France	11	0	11
6	Thailand	4	0	4
7	Bangladesh	0	3	3
8	Brazil	3	0	3
9	Vietnam	3	0	3
10	Poland	3	0	3
11	Ukrain	2	0	2
12	S. Korea	1	0	1
13	Indonesia	1	0	1
14	Iraq	1	0	1
Total		359	73	432

RAPD Marker technique

Generally molecular genetics research on insect provides valuable information on silkworm population structure, speciation, gene flow and genetic diversity, relationship studies and explanation on insect diversity based on their interaction with environment factors, either biotic (including other biological species) or abiotic. Many times, this molecular markers data help to distinguish between different species for conservation purpose, where there is no other comprehensive way available to do so. Hence, RAPD marker method has been reported to be an efficient tool to differentiate geographically and genetically isolated silkworm population. It has been used to verify the existence of population of species that have

arisen either through genetic selection under different environmental conditions or as a result of genetic drift (Fuchs *et al.,* 1998). Apart, there are several disadvantages that must be taken into account when using this RAPD technique. The most easily counteracted drawback is the dominant mode of inheritance of RAPD bands, which reduces the information provided by locus. Because each primer can amplify several loci and there are many commercially available primers, the loss of imformation per locus can be easily balanced by using a high number of loci (Levitan and Grosberg, 1993).

However, the RAPD markers have been used in gene mapping to genetically characterize species to estimate genetic variability (Stewart and Excoffier, 1996) and to determine the genetic structure of populations of various organisms (De Sousa *et al.,* 1999). Ultimatum, the RAPD is particularly useful to study the genetic structure of silkworm genotypes because they reveal polymorphisms in non-coding regions of the genome (Vucetich et al., 2001) is one of the key tools for conservation of silkworm *Bombyx mori* L.

ISSR Marker technique

Inter simple sequence repeat-PCR (ISSR) based technique, which involves amplification of DNA fragments present at an amplifiable distance in between two identical microsattlite repeate regions oriented in opposite direction. This technique targets simple sequence repeats (microsatellites) that are abundant and dispersed throughout the genome and reveal data that reflect the length variation between adjacent microsatellites. The use of SSR requires knowledge of the sequence of the region flanking the tandem repeats. ISSR in contrast does not require any prior knowledge of genome sequence. The ISSR-PCR uses a single primer composed of a di-or tri-nucleotide simple sequence repeat and it uses primers that are anchored at the 5' or 3' end of repeat region and extend into the flanking region. Generally single primers are used to generate fragments that are size-separated on either an agarose or polyacrylamide gel. The advantages of this ISSR technique include multiple polymorphic loci, high throughput and low cost. This technique is rapid and can differentiate between closely related individuals. This technique has provided a powerful tool for the investigation of genetic variation within and between the genotypes. Recently investigations have been carried out by several researchers through utilizing ISSR markers for characterization, genomic finger printing, genetic diversity and phylogenetic analysis, genome mapping, determination of SSR motif frequency gene tagging and use in marker assisted selection and evolutionary studies of mulberry silkworm Bombyx mori (Nagaraja and Nagaraju, 1995, Hussain *et al.,* 2000, Nagaraju *et al.,* 2001, Vijayan and Chatterjee, (2003),Vijayan *et al.,* 2004 and 2005, Mohanda *et al.,* 2004, Srivastava *et al.,* 2004, Awasthi *et al.,* 2004 and Sarala *et al.,* 2005).

Application of RAPD & ISSR to understand the genetic polymorphism

In the molecular marker system, the RAPD & ISSR markers has been efficiently utilizing till even today because mainly to resolve the intricate problems related to genetic polymorphism. The genetic polymorphism is an essential aspect in conservation biology because a fundamental concept of natural selection states that the rate of evolutionary change in a population is proportional to the amount of genetic diversity present in it (Fisher, 1930). In particular ISSR marker is a conceptually simple, easy than RAPD and not required the knowledge of genomic sequences for estimation of genetic polymorphism Chatterjee *et al.,* (2004) and Kar *et al.,* (2005). The detection and exploitation of naturally occurring DNA sequence polymorphisms are among the most significant developments in molecular biology. It is thereby a premier study to understand the genetic polymorphism or variation exist among silkworm genotypes because generally to realize the maximum heterosis of the silkworm races, it is essential to understand the variability among the silkworm genotypes because polymorphism/variability is basic requirement for the genetic improvement of a breed (Siddique, 1992).

This kind of higher polymorphism was reported by several sericulture scientists such as Chatterjee and Datta, (1992) and also Nagaraju, (1994) in his investigation utilizing the two races of silkworm *Bombyx mori* to understand the sensitivity of RAPD assay using arbitrary prmiers in nistari and NB series and Nagaraju and Singh, (1997) and also Nagaraju *et al.,* (2001) demonstrated that RAPD is one of the important tool to differentiate each genome without resolving to its physical formation where higher polymorphism exists. This kind of higher polymorphism among closely related insects of non-mulberry silkworms is too reported by Vijayan *et al.,* (2005) in *antheraea mylittae* through utilizing both RAPD as well as ISSR markers. Hence, to enrich genetic knowledge of polymorphism, the genetic conservation strategies are key through RAPD & ISSR markers have wide potential applications in silkworm genotypes improvement programmes.

Application of RAPD & ISSR to understand the phylogenetic relationships:

Central Sericulture Germplasm Resource Centre, Hosur is an important silkworm germplasm Bank in India where 432 different types of bivoltines, multivoltines and mutants both from indigenous as well as exotic races conservation is carrying out. It is very important to understand and identify the genetic relationships or back ground genomic knowledge of all the silkworm genotypes, which are conserving/maintaining not only in Indian germplsms but also in the World for different scientific research studies, academic studies, development of new breeds, *etc.* Therefore, the RAPD and ISSR markers are the potential to resolve the genetic architecture of the races and highly concerned to evolutionary geneticists. Many of these architectural issues can be addressed by analysis of a collection of tightly linked markers and the appropriate experimental design Walsh, (2001). In plants also several dominant RAPD and ISSR markers on agaroge gel were used to find out the genetic relationships, originality of broad range of silkworm germplasm stocks earlier studies revealed relative advantages of different algorithms based on grouping of maize breeds (Ajmona Marson *et al.,* 1992 and Mumm *et al.,* 1994). But silkworm strains used in primarily are of Asian origins. It is well know that most of strains were descent from China in the long past and

adopted to diverse climates, point to genetic closeness among them. This indicated a necessity of more than one algorithm to examine genetic relationship within the closely related silkworm populations. For instance, Nistari is an original tropical strain of Indian origin and its rearing has been practiced in Ganges River Valley since more than a century (Mukherjee, 1912). On the other hand PM (pure mysore) is a tropical, low yielding Indian strain. Low genetic distance and clustering of PM with Nistari reflected that these strains are genetically closer (Appukuttannair *et al.,* 2007). Such kinds of investigations have been done by several researchers through utilizing the RAPD & ISSR markers. Apart, the Dhanikachalam Velu *et al.,* (2008) has reported the genetic relationships of 20 mutants and has shown all the strains formed into one major cluster and 6 sub-clusters, showing that all the strains originated from same origin and similar voltinism. Similarly Reddy *et al.,* (1999 b) analyzed 13 silkworm strains from different origins and he has classified them into non-diapause and diapauses groups.

However, RAPD & ISSR marker techniques have not been used thoroughly in phylogenetic investigations based on relative similarity, inspite of their higher efficiency, cost effective and high reproducibility in silkworm molecular genetics and breeding programmes. In generally these have been effective in resolving problems relating to the phylogeny of Asian cultivated mulberry silkworm races and there is immense scope to use these powerful techniques in resolving domesticated, semi-domesticated and wild silkworm races status in many genus and in deciding the distinctness of different genera within and between the family of mulberry and silkworm genotypes.

Application of RAPD & ISSR to understand the genome mapping:

The silkworm *Bombyx mori* is an economically important insect and also well known excellent model genomic system. However, its genome analysis has been initiated since long period with emphasis objectives of obtaining genetic maps using different markers even today.

The RAPD & ISSR markers were too utilized in the genetic mapping programme for conservation of molecular genetics in *Bombyx mori* by several genetic conservationists are Promboon *et al.,* (1995), he has used RAPDs markers and Yasukochi, (1998) has used RAPD double primers and ISSR markers by Reddy *et al.,* (1999a) and most recently by Xia *et al.,* (2009) constructed a single-base pair resolution genetic map using genomic information from 40 domesticated and wild silkworm strains. With the help of 16 million single nucleotide polymorphism (SNP) markers, identified from the total genomic information, the domestication events and subsequent genetic differentiation in *B. mori* have been worked out.

Furthermore, the RAPD & ISSR markers were also utilized and utilizing efficiently in mulberry and other plant species. The ISSRs have also been used along with AFLP and RAPD markers in the mapping of Japanese and European genomes by Aracade *et al.,* (2000). According to an investigation by Wang *et al.,* (1998), 58 ISSR makers were mapped onto 18 RAPD linkage groups in soybean. The genetic linkage map of citrus was further saturated using 75 ISSR markers, which were dispersed among all the linkage groups (Sankar and Moore, 2001). However, also it was shown that, the level of segregation distortion of ISSRs is lower compare to RAPDs.

Application of RAPD & ISSR to understand the genome fingerprinting:

The genetic fingerprinting, is also called as genetic profiling, the main aim of this studies is identifying individual by DNA pattern through RAPD & ISSR and othere markers, which are use to run on Electrophoresis/Agarose-gel electrophoresis. The DNA fingerprinting unlike the usual fingerprinting which is based on the morphological features and primarily restricted to humans is revealing the identity of an organism at the molecular level. In fact this is the technique of finding the genetic identity. This is primarily based on the variation occurring at the molecular level that is on the base sequences of the genome. The fundamental techniques involved in genetic fingerprinting were discovered serendipitously in 1984 by geneticist Alec J. Jeffreys of the University of Leicester in Great Britain. The technique crossed the arena of the scientific frontiers mainly with the application in the forensics. With advent of time, development of various techniques paved way for the use of this technique in different fields giving newer dimensions to this Technique. The DNA profiling has been using in silkworms for conservation of bio-molecular genetics, identifying markers for traits, identification of gene diversity and variation *etc*. The most popular or widely used techniques used with relevant to silkworms are RFLP, RAPD, ISSR, SSR etc. Among all the markers, the RAPD & ISSR are potentially optimistic for the study on fingerprinting/profiling of silkworm genome. Several researchers have been made foundational investigations on genome fingerprinting of silkworms are Nagaraja and Nagaraju, (1995); Sharma *at el.,* (1999); Vijayan *et al.,* (2005); Pradeep *et al.,* (2006); *etc.*

Advantages and disadvantages of RAPD & ISSR markers for conservation of silkworm *Bombyx mori* L.

Molecular markers may be broadly divided into two classes based on the method of their detection: **(1)** Hybridization-based markers and **(2)** Polymorase chain reaction (PCR)-based (Gupta *et al.,* 1999). The RAPD and ISSR markers have many advantages and dis-advantages and ISSR markers offer many improvements in silkworm *Bombyx mori* over RAPD and other available techniques are ***(i)*** small amounts of DNA may be used; ***(ii)*** small reaction volumes and amounts of enzyme are needed for PCR; ***(iii)*** the highpervariability of banding patterns; ***(iv)*** fresh or large quantities of material for DNA extraction are not required; ***(v)*** banding patterns are easily scorable and ***(vi)*** primers should be selected that will not yield overlapping results. On the other hand, the silkworm is a cold blooded insect, wherein higher annealing temperatures used for ISSR reactions may reduce the amount of template-primer mis-match artefacts than may be encountered with RAPD markers, which generally rely on lower annealing temperatures. Many researchers who have compared RAPD and ISSR methods have found that ISSR markers exhibit higher levels of polymorphism and/or reproducibility compared to RAPD markers in

mulberry and non-mulberry silkworms respectively (Srivastava *et al.,* 2004 and Vijayan *et al.,* 2005). Apart, advantages of the RAPD technology too include ***(i)*** suitability for work on anonymous genomes, ***(ii)*** applicability to problems where only limited quantities of DNA are available, ***(iii)*** efficiency and low expense, *etc.* In addition, where a direct cost comparison was made among RFLP, RAPD and ISSR techniques, the latter was found to be the most economical per polymorphism observed ($29, $57 and $10 respectively; Yang *et al.,* 1996).

The dis-advantages of ISSR marker is similar to those encountered in the use of RAPD marker; ***(i)*** clean DNA template and similar concentrations among accessions are required for standardization of reactions; ***(ii)*** optimization of initial reactions is needed; ***(iii)*** bands are scored as dominant markers and ***(iv)*** genetic diversity estimates are based on diallelic characters. Even with these few limitations, we believe that ISSR markers will provide an attractive alternative to RAPD markers and a technique that is much more easily implemented than amplified fragment length polymorphism (AFLP; reviewed in Wolfe and Liston, 1998). It is very important to understand the genetic structure of silkworm for the conservation of silkworm resource, it is clear that, the RAPD and ISSR markers have great potential advantages for application in silkworm.

Application of RAPD & ISSR markers in future for development of the silkworm, *Bombyx mori*

We note here several additional applications currently under investigations for advanced development of silkworm *Bombyx mori*.

1. **Sex determination:** In silkworm molecular (as well as agricultural insects and other) applications it would be convenient to have available markers that were sex-specific. We expect that little difficulty will be encountered in developing RAPD and ISSR markers with this characteristic.
2. **Generation of specific PCR primers for anonymous genomes:** A major limitation in the application of RAPD & ISSR-PCR to molecular problems is the absence of sequence information for the vast majority of genotypes. We suggest that this difficulty may be overcome for many applications by using a RAPD & ISSR-based strategy for developing 'designed PCR primers. Specifically, RAPD & ISSR primers with an embedded restriction site may be used to detect fragments showing the desired properties (e.g. detecting a particular taxon). These fragments may then be cloned and sequences used to develop specific 'designed' PCR primers for diagnostic markers.
3. **Quantitative analysis of mixed biosamples:** Analogous to the analysis of mixed paternity samples in silkworm, analysis of field samples of different genotypes/climate may be performed.
4. **Phylogeny:** RAPD & ISSR markers may prove to be useful characters for cladistic analysis, etc.

CONCLUSION

Molecular markers paved way for understanding how genotypes are stable or variable, thereby the breeders succeed in breeding different new varieties. the evidence of markers and their application now shows that they make important contribution to conservation of races/species. However, many silkworm genotypes now require benign human intervention to ensure their genetic originality through molecular and genetical approaches. It is to be admitted that the present review is also a venture in the field of technological developments of molecular genetics in the silkworm *Bombyx mori*. It may be leads to extensive use of molecular markers such as RAPD & ISSR for the conservation of silkworm resources. This has allowed assessment of the impact of genetic and molecular of silkworm genotypes and further much more similar innovative studies will be required in sericulture industry to understand the significance and application of RAPD & ISSR markers to innovate new concepts for conservation of molecular genetics in silkworm *Bombyx mori*. Hence, in this review, we have focused on how and where the application of molecular markers will efficiently helpful for the conservation of resources, insights into the dynamics of genome fingerprinting, genome mapping, genetic diversity and relationships, advantages and dis-advantages of markers techniques, *etc* in silkworm *Bombyx mori*.

It is worthy to reiterate with summarizing review, it may be said the present communication can be considered as a little foundation work based on which a great deal of study can be planned for detailed research investigations on proper application of RAPD & ISSR markers efficiently for the conservation of domesticated, semi-domesticated and wild silkworm resources in sericulture industry in India and elsewhere in the world.

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