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Genetic Diversity among Indian Oak Tasar Silkworm, *Antheraea proylei* J. Revealed by ISSR Markers

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The Indian Oak Tasar silkworm, *Antheraea proylei* J. is a beneficial insect with great economic importance in India for its silk production. In this study, six populations of *Antheraea proylei* and *A. frithi* Moore (as an out group) were subjected to inter simple sequence repeat (ISSR) marker analysis in order to assess its genetic diversity. Fifteen ISSR primers produced 91 markers among different breeds of *A. proylei* and *A. frithi* of which 89 are polymorphic, generating 97.8% polymorphism. The dendrogram constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and cluster analysis made using Nei's genetic distance resulted in the formation of one major group containing four sub-groups separating the breeds. This result suggests that ISSR amplification is potentially useful for molecular characterization of oak tasar silkworm genotypes.

Key words: *Antheraea proylei*, ISSR, polymorphism, dendrogram

Introduction

India is one of the major countries producing oak tasar silk in the global context. *Antheraea proylei* Jolly which is commonly known as the Indian Oak Tasar silkworm is a semi-domesticated silkworm commercially exploited for the production of silk. In India, *A. frithi* Moore is also conserved in the germplasm bank along with some evolved lines of *A. proylei* for utilization in the production of oak tasar silk. Oak tasar culture plays an important role in the economic uplifting of the weaker section of the society who are inhabiting in the oak belt of the sub-Himalayan region of India extending to Manipur, Nagaland, Assam, Mizoram, Meghalaya and Arunachal Pradesh in the North-East and Jammu & Kashmir, Himachal Pradesh and Uttarakhand in the North-West region of Himalaya.

Assessment of genetic variation among silkworm is useful for predicting potential genetic gain in a breeding program and setting up appropriate cross breeding strategies. Traditionally, morphological and phenotypic characters have been used for this purpose. In recent years, DNA-based molecular markers such as random amplified polymorphic DNA (RAPD) (Nagaraja and Nagaraju, 1995), amplified fragment length polymorphism (AFLP) (Lu *et al.*, 2001), restriction fragment length polymorphism (RFLP) (Sethuraman *et al.*, 2002), simple sequence repeats (SSR) (Shen *et al.*, 2004) and inter simple sequence repeats (ISSR) (Chatterjee and Mohandas, 2003) have been widely adopted in silkworm genetic diversity studies. In general, these studies provided the positive information to enhance the understanding of silkworm phylogeny.

ISSR (Inter simple sequence repeat) markers have been extensively used for genetic analysis of plants and animals (Bornet *et al.*, 2002). The genetic variability analysis of silkworm was made by the ISSR markers (Li *et al.*, 2007). Further, the technique of ISSR amplification is sensitive enough to differentiate closely related individuals (Zeitkiewicz *et al.*, 1994).

Molecular characterization of *A. proylei* has been carried out by some workers like Arunkumar *et al.*, 2006; Chatterjee and Tanushree, 2004. However, there is no much more information about its breeds. Hence, attempts have been made to characterize *A. proylei* and its breeds using ISSR markers.

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Table 1. List of ISSR primers used and their polymorphism

Sl. No.	Primer no.	Primer sequence (5'→3')	No. of total bands	No. of polymorphic bands	Percentage of polymorphism
1	807	AG AG AG AG AG AG AGAGT	5	5	100.0
2	811	GAG AG AG AG AG AG AGAC	7	7	100.0
3	812	GA GA GA GA GA GA GA GAA	5	5	100.0
4	822	TCTCTCTCTCTCTCTCA	4	4	100.0
5	826	ACACACACACACACACC	8	8	100.0
6	827	AC AC AC AC AC AC AC ACG	7	6	85.7
7	828	TGTGTGTGTGTGTGTGA	6	6	100.0
8	830	TGTGTGTGTGTGTGTGG	6	6	100.0
9	835	AGAGAGAGAGAGAGAGYC	6	6	100.0
10	840	GAGAGAGAGAGAGAGAYT	7	7	100.0
11	844	CT CT CT CT CT CT CT CTRC	8	7	87.5
12	861	ACC ACC ACC ACC ACC ACC	3	3	100.0
13	881	GGGTGGGGTGGGGTG	6	6	100.0
14	884	HBHAGAGAGAGAGAGAG	6	6	100.0
15	885	BHBGAGAGAGAGAGAGA	7	7	100.0
		Total	91	89	97.8

Y=(A,G,C,T); H=(A,C,T); B=(C,G,T); R=(A,G)

Materials and Methods

Silkworm breeds

Moths of *Antheraea proylei*, its breeds namely PRP_2, PRP_3, PRP_5, PRP_{12}, RPP_4 and Blue were used for the present study. Here, *Antheraea frithi* was used as an out group. The moths were randomly collected from the germplasm bank of Regional Tasar Research Station, Imphal, Manipur (India) for the study.

DNA extraction

Ten individual moths of each breed were collected and wings were cut. The moths were grinded into fine powder using liquid nitrogen and 10 mL of 2 PK lysis buffer (pH 8.0) was mixed with the powder and incubated at 37°C for 12 hrs after adding Proteinase-k (25 mg/mL). The standard procedure of phenol:chloroform:isoamyl alcohol method (Suzuki *et al.*, 1972) was followed and DNA was precipitated in ice cold alcohol after adding 3 M sodium acetate. DNA was dissolved in TE (Tris-EDTA) buffer (pH 8.0), diluted and quantified to a concentration of 10 ng per micro litre against uncut λ DNA (10 ng/μL).

PCR amplification of DNA with ISSR primers

A total of 24 ISSR primers synthesized from Eurofins Genomics India Pvt. Ltd. were used for the study, of which 15 primers showed high percentage of polymorphism (Table 1). PCR amplification was done in an MJ Thermal Cycler PTC 200, using 10 μL reaction mixture containing 30 ng DNA, 2 μL 10×PCR buffer, 10 mM dNTP, 25 mM $MgCl_2$, 25 pmol primer and 1.0 U of *Taq* DNA polymerase. The PCR schedule was 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min and a final extension of 10 min at 70°C.

Electrophoresis

The PCR products were resolved by electrophoresis using 1.5% agarose gel in Tris-boric acid/EDTA buffer (1×TBE). It was carried out with a constant voltage of 80 V for 2 h and stained with ethidium bromide (0.5 μg/mL) and photographed using the gel documentation system. DNA ladder of 1 kb was used as marker to estimate the size of PCR amplified products.

ISSR data scoring and analysis

Binary scoring of the profiles was done visually on the basis of presence (1) or absence (0) of bands. The genetic variability in the population was analysed on the basis of the banding pattern using parameters, such as percentage polymorphism and genetic distance analysis (Nei, 1978). The phylogenetic tree was constructed based on the UPGMA cluster analysis of Nei's genetic distance using TFPGA 1.3 software (Miller 1997).

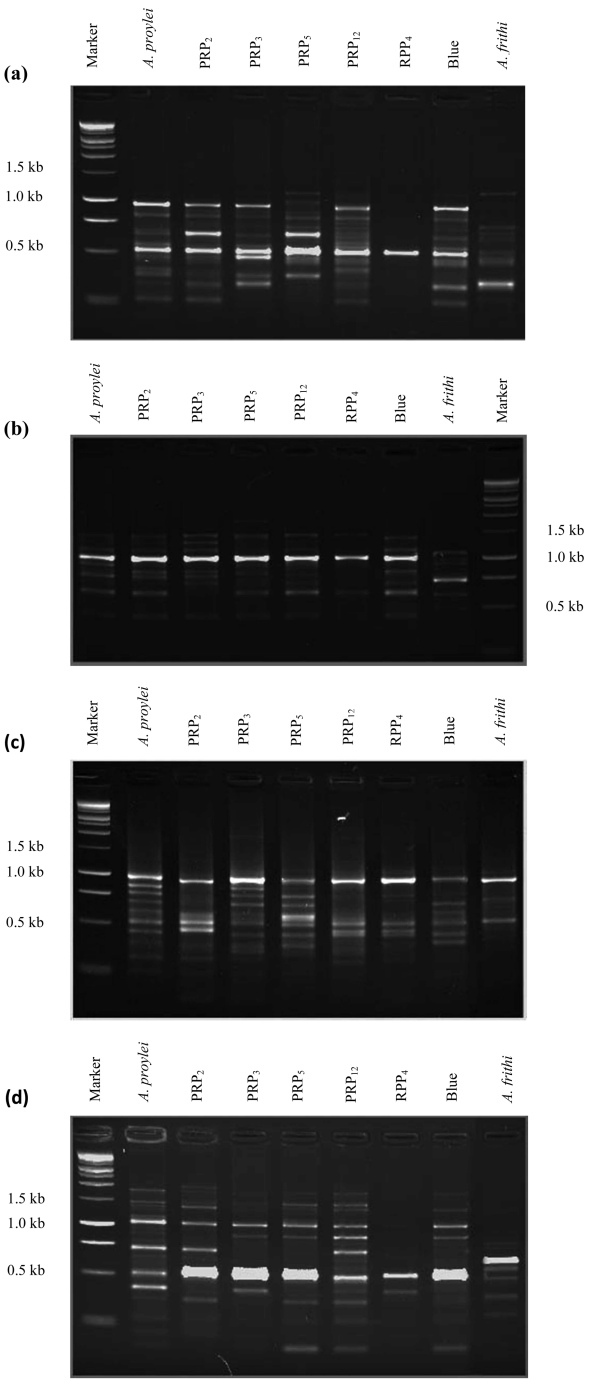

Fig. 1. PCR profiles generated with ISSR primers (a) UBC 807, (b) UBC 826, (c) UBC 827 and (d) UBC 885 for *A. proylei* and its breeds and *A. frithi*.

Results and Discussion

Genetic variability revealed through ISSR markers

The 15 ISSR primers generated 91 bands of which 89 are polymorphic showing 97.8% polymorphism. ISSR profile generated with primers UBC 807, UBC 826, UBC 827, UBC 885 are depicted in Fig. 1(a, b, c and d respectively). The total number of DNA fragments amplified and the number of polymorphic bands with individual primers are shown in Table 1. Percentage of polymorphism detected with each primer was as high as 100% in 13 primers (UBC-807, UBC-811, UBC-812, UBC-822, UBC-826, UBC-828, UBC-830, UBC-835, UBC-840, UBC-861, UBC-881, UBC-884 and UBC-885) to 85.7% detected by UBC-827. The number of bands per primer ranged from 3 (UBC-861) to 8 (UBC-826 and UBC-844). All these polymorphic bands were used for hierarchical clustering and analysis of genetic diversity following Nei's statistics. The number of amplification fragments generated per reaction varies with the primer-template combination but are generally scored easily using agarose gel technology (1.5%). High level of polymorphism was revealed by this study.

Cluster analysis

The genetic distance values based on the Nei's genetic distance are presented in Table 2. The distance between the breeds ranges from 0.2624 to 1.6205. The lowest distance was observed in PRP_2 and *A. proylei* with a value of 0.2624; whereas, the highest distance with a value of 1.6205 was observed in *A. proylei* and out group member *A. frithi*. However, among the breeds, highest distance was observed in *A. proylei* and RPP_4 with a value of 0.6397.

The dendrogram generated by UPGMA with Nei's genetic distance matrices for all the breeds is presented in Fig. 2. One major cluster was formed comprising of four groups. Group I comprised of *A. proylei* and PRP_2 at a genetic distance of 0.2624. Group II consisted of two sub groups, first sub group with PRP_3 and Blue at a genetic distance of 0.2768, and second was PRP_5, an isolated sub group. Group III consisted of PRP_{12} and RPP_4 at a genetic distance of 0.3062; *A. frithi* is in Group IV used as an out group. The ISSR technique is fast, reliable, providing an almost inexhaustible supply of genetic markers, and requiring little template DNA. This study indicated that ISSR-PCR methods are suitable to study intra- and inter-specific variation in this group of insects. ISSR-PCR was already known as a very effective method to understand intra-specific and genetic structure of population (Fang *et al.*, 1997; Nagaraju *et al.*, 2001; Zhou *et al.*, 1999), to generate species-specific genomic fingerprints (Zietkiewicz *et al.*, 1994; Wu *et al.*, 2005). It is reported that ISSR markers can be effectively utilized to analyse phylogenetic relationship and heterozygosity in silkworm (Velu *et al.*, 2008).

The study reveals the phylogenetic relationship of *A. proylei* and its breeds. The genetic distances among the breeds are also varied. Here, *A. proylei* and PRP_2 may be

Table 2. Nei's genetic identity (below diagonal) and genetic distance (above diagonal)

Population	*A. proylei*	PRP_2	PRP_3	PRP_5	PRP_{12}	RPP_4	Blue	*A. frithi*
A. proylei	*****	0.2624	0.3212	0.3520	0.4165	0.6397	0.4504	1.6205
PRP_2	0.7692	*****	0.3677	0.3677	0.4000	0.5406	0.2768	1.2150
PRP_3	0.7253	0.6923	*****	0.3062	0.5035	0.6190	0.2768	1.2150
PRP_5	0.7033	0.6923	0.7363	*****	0.4333	0.5035	0.4000	1.1436
PRP_{12}	0.6593	0.6703	0.6044	0.6484	*****	0.3062	0.3365	0.8473
RPP_4	0.5275	0.5824	0.5385	0.6044	0.7363	*****	0.5406	0.7497
Blue	0.6374	0.7582	0.7582	0.6703	0.7143	0.5824	*****	1.1436
A. frithi	0.1978	0.2967	0.2967	0.3187	0.4286	0.4725	0.3187	*****

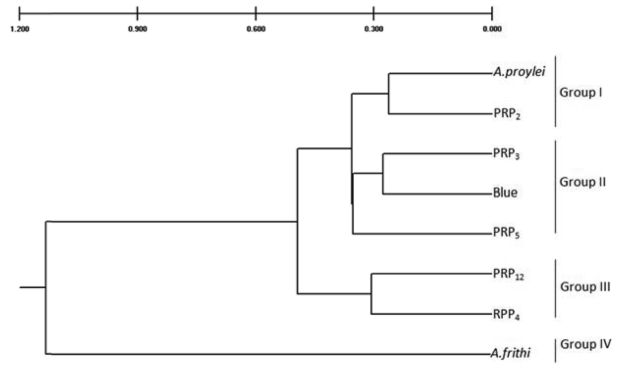

Fig. 2. Dendrogram illustrating phylogenetic relationship among the different breeds (using *A. frithi* as an out group) based on the UPGMA cluster analysis of Nei's genetic distance (1978).

closely related compared to *A. proylei* and RPP_4 which are distantly related as per observed genetic distance. The 15 selected ISSR markers have generated very high degree of polymorphism among the breeds. This study indicates that ISSR markers are useful for molecular characterization of oak tasar silkworm genotypes. Further, the findings made in this study are of much importance for germplasm conservation as well as breeding of these economically important silkworms.

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