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Full Length Research Paper

Nutrigenomic analysis of mulberry silkworm (*Bombyx mori* L.) strains using polymerase chain reaction - simple sequence repeats (PCR-SSR)

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The DNA marker use in assisting selection are safe method in breeding process and it is an important tool for authentication of new gene cascade in genome. In mulberry silkworm, the major economic and nutrigenomic traits are polygenic in nature. In the present study, we have utilized ten PCR-SSR microsatellite markers to gain better understanding on genotyping of certain nutrigenomic gene loci in nutritionally efficient silkworm breeds / hybrids. Results showed that a single yet varying size amplified band in all four parental silkworm strains (RMG_4, RMW_2, RBD_1 and RBO_2) and two clear amplified bands in the hybrids (RMG_4 × RBD_1 and RMW_2 × RBO_2) with different molecular weight from three PCR-SSR primers loci *viz*., F11139, F10429 and F10705. The PCR-SSR results demonstrated that homozygosity in newly evaluated nutritionally efficient parental silkworm strains and heterozygosity in hybrid. These investigations authentically confirmed the previous findings of heterotic nutritionally efficient silkworm hybrids with superior nutrigenomic traits. The developed molecular analysis in silkworm could be utilized for the benefit of the farmers in sericulture industry. In conclusion, these results would be useful in identification of nutrigenomic cascade of genes in silkworm and also emphasize the future prospects of silkworm functional mechanism in nutrigenomic studies.

Key words: Silkworm, breed, hybrid, nutrigenomics, PCR-SSR marker, homozygosity, heterozygosity, cascade of genes.

INTRODUCTION

The silkworm, *Bombyx mori* L. is the well-studied central Lepidopteron model system because of its rich repertoire, and well characterized mutations. It is an important source of livelihood for subsistence of farmers engaged in commercial silk production in many countries. Silkworm is a major insect model for research and the first Lepidopteron for which draft genome sequences became available (Tazima, 1978; Xia et al., 2004; Nguu et al., 2005). Silkworm with haploid nuclear genome size of 530 Mb broken into 28 chromosomes utilized as a framework genome to organize information for other Lepidopteron. Silkworm is known for its vast genetical, geographical diversity and the model animal for the utilization in the study of heterosis by crossing with deferent parents for commercial exploitation. Hundreds of such different geographical and mutant strains have been preserved in Japan, China, Korea, India, Italy, France and other countries (Goldsmith et al., 2005; Archak et al.,

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2007).

The microsatellite markers consist of an array of simple tandemly repeated mono, di-, tri- tetra-, penta or hexanucleotide repeats such as $(A)_n$, $(CA)_n$, $(GA)_n$, $(GTA)_n$, $(ATT)_n$, $(GATA)_n$, $(ATTTT)_n$, $(ACGTCG)_n$ which distributed across the genomes. These are ubiquitous in prokaryotic and eukaryotic genomes, randomly distributed both in protein coding and non-coding regions; these become highly informative and versatile classes of genetic markers (Tautz, 1989). The advantages of SSRs over other molecular markers: (i) multiple SSR alleles may be detected, (ii) SSRs are evenly distributed all over the genome, (iii) they are co-dominant, (iv) very small quantities of DNA are required for screening and (v) analysis may be semi-automated. Therefore, it is an imperative that these molecular markers are utilized for broad range of applications, such as genome mapping and characterization, phenotype at a single gene locus using a simple PCR-based mapping (Powell et al., 1996; Robinson et al., 2004). Recently, these microsatellite PCR-SSRs and ISSR markers has enabled researchers to investigate genetic linkage, polymorphism, homozygosity and heterozygosity analysis in gene loci among silkworm populations (Reddy et al., 1999a, b; Rao and Chandrashekharaiah, 2003; Shen et al., 2004; Li et al., 2005, 2006). Recently, RAPD markers linked to *nsd*-Z had been screened (Li et al., 2001) and the molecular linkage map of *nsd*-Z by SSR markers had been constructed in silkworm on monogenic trait against densonucleosis virus (Li et al., 2006; Muwang et al., 2007). However, not much is known about the pattern of gene expression, genomic organization, or molecular evolution of the silkworm breeds / hybrids on nutrition consumption and its metabolism in the recent trust area of nutrigenomics as these are the most important commercial parameter in sericulture industry.

The foremost component of the present study to comprehend nutrigenomic analysis for homozygosity and heterozygosity on certain gene loci in newly developed nutritionally efficient silkworm strains. Parental polyvoltine (RMW_2 and RMG_4), bivoltine (RBO_2 and RBD_1) and the hybrid combinations such as $RMW_2 \times RBO_2$ and $RMG_4 \times RBD_1$ were analyzed by DNA fingerprinting with the assistance of ten different PCR-SSR primers for identify-cation of new nutrigenomic cascade of genes in silkworm.

MATERIALS AND METHODS

Silkworm strains and rearing

The two newly identified nutritionally efficient silkworm hybrids *viz.,* $RMW_2 \times RBO_2$ and $RMG_4 \times RBD_1$ and the parental breeds, polyvoltine (RMW_2 and RMG_4) and bivoltine (RBO_2 and RBD_1) were utilized for the study. All these silkworm strains were brushed and reared according to standard rearing methods adopted by Datta (1992). After cocooning, the healthy silkworm pupae were utilized for preparation of DNA for genomic analysis with PCR-SSR primers.

DNA extraction

Ten pooled silkworm pupae of two newly identified nutritionally efficient silkworm hybrids ($RMW_2 \times RBO_2$ and $RMG_4 \times RBD_1$) with its respective four parental breeds *viz.,* RMW_2, RMG_4 (polyvoltine) and RBO2, RBD_1 (bivoltine) were frozen in liquid nitrogen and the genomic DNA was prepared (Suzuki et al., 1972; Sambrook et al., 1989).

These frozen pupa was pulverized with a mechanical homo-genizer in a microcentrifuge tube and suspended in DNA extraction buffer (50 mmol/L Tris–HCI (pH 8.0), 100 mmol/L NaCl, 20 mmol/L EDTA) that contained 150 µg/ml proteinase K. After digestion with the proteinase K at 50°C for 8 – 10 h, phenol–chloroform extraction was carried out. The DNA was recovered by isopropanol precipitation and purified DNA molecule was dissolved in Tris-EDTA buffer (pH 8.0) and concentration was measured spectrophotometrically (Li et al., 2005, 2006).

Microsatellite SSR primers

Ten primer sequences of PCR-SSR repeat motif in silkworm were selected from the previously well characterized microsatellite repeats represented different gene loci *viz.,* Fl0429, Fl1139, Fl0548, Fl0316, Fl0568, Fl0705, Fl0650, Fl0665, Fl0664, Fl0537 (Li et al., 2005, 2006).

PCR amplification

The basic program used to amplify PCR-SSR DNA was performed on a thermal cycler PTC 100 (MJ Research). Polymerase chain reaction cycles for the SSR microsatellite loci included (i) an initial denaturation step at 95°C for 3 min, an annealing step at 63°C for 1 min and an extension step at 72°C for 1 min followed by (ii)14 cycles of 94°C for 30 s denaturation, a 14-step touchdown decreasing by 0.5°C at each step to 56°C (30 s) and an extension step at 72°C for 1 min. (iii) conditions for the last 24 cycles were 94°C for 0.5 min, 56°C for 30 s, and 72°C for 1 min followed by (iv) a final elongation step at 72°C for 10 min extension. The PCR was performed in a final volume of 15 µL containing 10 mmol Tris–HCl/L (pH 8.4), 50 mmol KCl/L, 1.5 mmol $MgCl_2$/L, 0.2 mmol each dNTP/L, 0.2 µmol each primer/L, approximately 20 ng of each silkworm parental breeds / hybrids genomic DNA, 0.5 U of Taq polymerase and distilled de-ionized water.

Electrophoresis of PCR products

The following PCR amplified product was mixed with 5 µl TE buffer and 2 µl 40% sucrose containing 0.5% bromophenol blue was loaded on to a 1.5% agarose gel along with the 100 bp ladder mole-cular weight marker (Promega) and run at constant voltage (150 V) for 2 h. After electrophoresis, the gels were stained with Ethidium Bromide, clear bands were UV visualized and photographed with digital scientific camera in gel documentation system (Hou et al., 2005). To authenticate repeatability of the results obtained, the PCR amplification and gel electrophoresis was repeated twice.

RESULTS

Morphological features nutritionally efficient silkworm strains

The newly identified nutritionally efficient silkworm strains

Table 1. Nutritionally efficient silkworm strains with origin and cocoon traits utilized for genomic analysis.

Parental breeds / Hybrids	Origin	Cocoon color	Cocoon shape	Cocoon built	Cocoon grains
RMW_2	Madagascar	White	Oval	Medium	Fine
RMG_4	Madagascar	Greenish Yellow	Oval	Medium	Medium
RBO_2	Exogenous	White	Oval	Medium	Fine
RBD_1	Exogenous	White	Dumbbell	Medium	Fine
RMW_2 x RBO_2	Hybrid	White	Oval	Hard	Fine
RMG_4 x RBD_1	Hybrid	Greenish Yellow	Hybrid	Hard	Fine

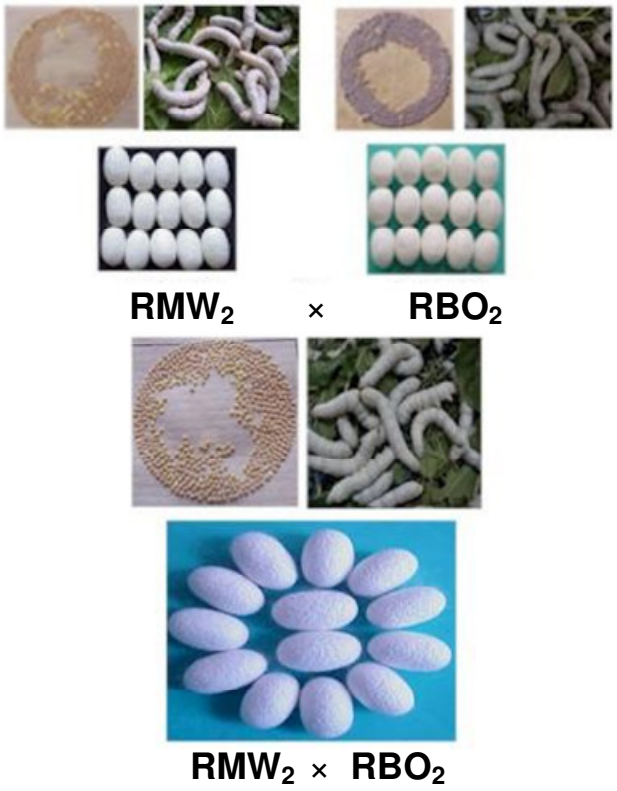

Figure 1. Nutritionally efficient silkworm hybrid RMW_2 × RBO_2 with its parental strains.

and its phenotypic salient features were showed in Table 1. Both polyvoltine non-hibernating parental strains (RMW_2 and RMG_4) were of Madagascar origin with oval shape cocoon, RMW_2 with white color cocoon and RMG_4 with greenish yellow cocoon color. Both the breeds were with pigmented egg color and slender bluish larval color. Bivoltine parental breeds were hibernating in nature (RBO_2 and RBD_1) and exogenous origin with white color cocoon but one with oval shape and another with dumbbell shape cocoon respectively. The egg color of the both the bivoltine were grayish color. The cocoon built and grains of all parental breeds were fine except in RMG_4 where in medium cocoon grains were observed. The newly developed nutritionally efficient hybrid RMW_2 × RBO_2 was characterized with white color oval cocoon, pigmented egg color and robust bluish white larval color (Figure 1). Another hybrid, RMG_4 × RBD_1 was characterized with greenish yellow color cocoon with hybrid shape, pigmented egg color and bluish larval color (Figure 2). This selected silkworm strains were identified as nutritionally efficient based on nutrigenetic traits of few strains revealed from earlier study of Ramesha et al. (2010).

Characterization of SSR primers

Out of ten SSR primers selected and utilized, seven were $(CT)_n$ and three $(CA)_n$ repeat motifs with varying length. The PCR amplification were found in two $(CA)_n$ repeat

Figure 2. Nutritionally efficient silkworm hybrid $RMG_4 \times RBD_1$ with its parental strains.

motif viz., F10429 and F11139 and one $(CT)_n$ repeat motifs of F10705 in this study (Table 2).

Optimization of PCR-SSR amplification

The pooled DNA sample from ten - fifteen silkworm pupae from newly identified nutritionally efficient polyvoltine silkworm breed, RMW_2 was amplified from randomly selected SSR primer, F10429 and resulted in a series of discrete bands with varying intensity. The genomic DNA template concentration assays were carried out over a range from 5 - 50 ng (5, 10, 15, 20, 25, 35, and 50 ng) in a total volume 15 µl final PCR reaction mixture (Figure 3; Lanes A - G). Template DNA concentration of 15 - 35 ng (Lane B, C, D, E and F) was found to generate a consistent amplification profile but lower (Figure 3; Lane 'A' with 5 ng) and higher (Figure 3; Lane 'G' with 50 ng) concentration appeared to be unenthusiastic amplification. At optimum template DNA concentration (15 - 25 ng/15 µl of final reaction volume), the PCR-SSR DNA profile remained consistent and repeatable (Figure 3).

Homozygosity and heterozygosity specific gene amplification

Three primers yielded PCR-SSR amplification products in all six silkworm strains. The number and size of the amplified products varied depending upon the sequence of SSR primers and silkworm breeds utilized. Size of the amplified products ranged from 250 bp to 1 kb in the monomorphic loci (Figure 4). It was observed that primers F11139 gave 400 and 300 bp amplified products in two set of parents, RMW_2 and RMG_4 and RBO_2 and RBD_1 respectively. Whereas in hybrids ($RMW_2 \times RBO_2$ and $RMG_4 \times RBD_1$), both 300 and 400 bp products were observed (Figure 4A). Primer F10429 amplified a 250 bp product in all six silkworm breeds/hybrids (Figure 4B) in addition to 1 kb band in hybrids. Approximately 1 kb PCR amplified band was observed in all six silkworm strains from the primer F10705 and 300 bp product in hybrids in addition (Figure 4C). The PCR-SSR results revealed one band in parents and two different size bands in hybrid combinations from all three primers. The amplification reactions of PCR-SSR patterns were consistent and reproducible for the silkworm strains and PCR-SSR

Table 2. The repeat motif with primer sequence on SSR loci.

Locus symbol	Repeat motif	Primer sequence (5′→3′)
FI0316	$(CT)_9$	GCGATAAGACCGCCTATTGAAC GTGTATTAGGCACGAGAACTGACG
FI0429	$(CA)_{11}$	AAGGGATTCTCTACCAGTCAACCA TTTGACGCTGGCTTATAAATACTGTAT
FI0548	$(CT)_{12}$	ACAAAGTTCCCCAAAACGCTC TTCGGAATGAAACATCCTCAACTA
FI0568	$(CT)_7$	TCGTCCTACACTTGCGGGTT TGTTTCGTCAAGTCTGCTCGGT
FI0705	$(CT)_5$	GGGATAAGTGGGTCGTTTTGATT TGAGACCCAATAATGTCCCGAG
FI0650	$(CA)_6$	GAAAGCGGATGGTCCTACTCTG CTAAGTAAGAACCCAAGCTACACGA
FI0665	$(CT)_8$	TCCAAATGATTCTTGTCCACCTG TTCCTTTCTTTCAATTCTTCTGT
FI0664	$(CT)_{10}$	AAATTTCATACTCCTCCGTCCG CATTTCTACCACAGCCAAACGAT
FI0537	$(CT)_{15}$	CCATTTACAGGCTGGTATCCAT TAGCGATAAGACCGCCTATTGTA
FI1139	$(CA)_7$	CGGCACTTAAAAGTTTTCATATCAATC CTGACAGTGGTGAGTTAATAAAACAAAA

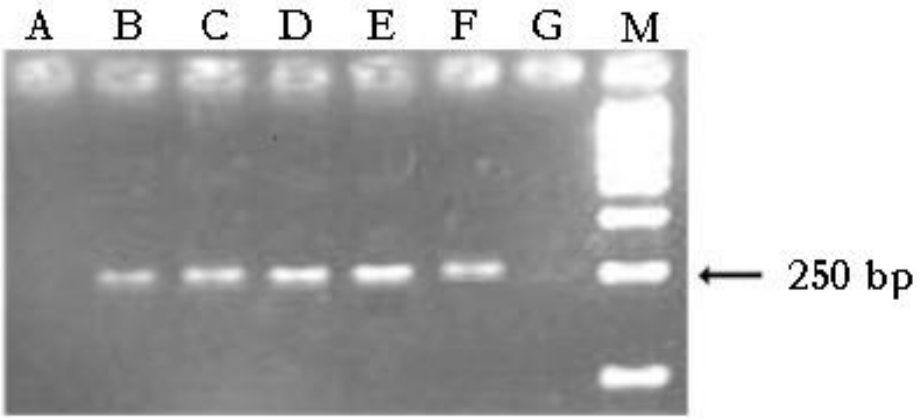

Figure 3. Optimization of PCR-SSR amplification with different concentration of of template DNA (Lane: A-G contains 5, 10, 15, 20, 25, 35 and 50 ng genome template DNA: M-Marker).

primer combinations.

DISCUSSION

The silkworm strains used for the current study were chosen based on the silkworm ten important commercial traits after been analyzed through general combining ability (GCA), specific combining ability (SCA), mid parent heterosis (MPH) and better parent heterosis (BPH) followed by nineteen nutrigenetic traits analysis. Dietary or nutritional factors and related metabolic interactions have direct and indirect influence on specific gene regulation and expression (Walker and Blackburn, 2004)

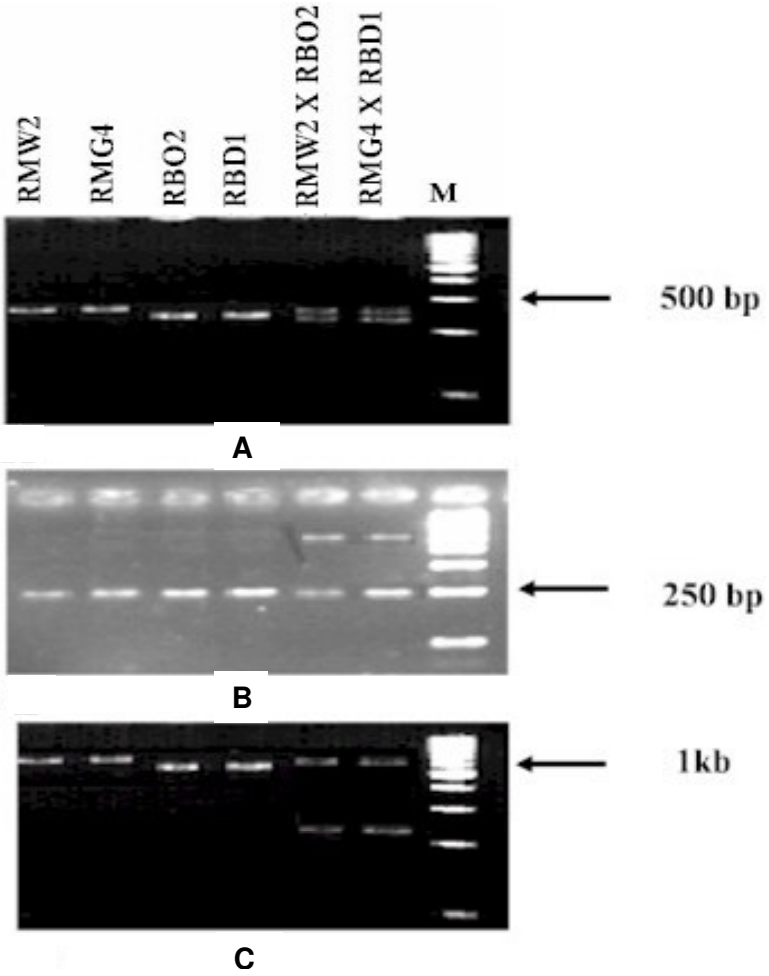

Figure 4. PCR-SSR profile on certain gene loci of homozygosity and heterozygosity in nutritionally efficient silkworm parental breeds and hybrids (A. F11139; B. F10429; C. F10705).

and such interactions and variation in the field of nutrigenetics applied to choose the silkworm strains based on their nutritional efficiency traits as 'biomarkers'. Our previous work has revealed insight on nutrigenetic traits difference amongst silkworm strains while suggesting as nutritionally efficient (Ramesha and Raju, 2009; Ramesha et al., 2010).

Though tremendous achievements have been made in silkworm improvement by exploring the genome at exponential growth in the field of molecular biology envisage the pattern of gene expression in silkworm. PCR-SSR technique proved to be valuable in uncovering genomic variability in silkworm, since past such variability was estimated only through morphological, biochemical and yield attributes (Chatterjee and Datta, 1992). In the past 20 years, the major effort in animal breeding has changed from quantitative to molecular genetics with emphasis on quantitative trait loci (QTL) identification and marker assisted selection (MAS). With the recent advent of PCR based approach, gel free visualization with automation in genomic analysis and genotyping methods have resulted in a rapid expansion of the power of molecular markers to address individual's identity authentically. The microsatellites emerged as the most popular and versatile marker for markers assistance selection (MAS) in silkworm breeding technology. It was observed that these markers revealed genomic variation among silkworm strains and results obtained in the present study were in accordance with the principle that the number of individuals used to estimate average heterozygosity could be very small if a large number of loci studied (Nei, 1978; Lewontin and Hubby, 1996). Two SSR markers, Fl0316 and Fl0568 showed from *nsd*-Z gene expression (Li et al., 2006) and Muwang et al. (2007) detected the homozygosity in the NILs of *nsd*-Z to inspect the replacement of the linkage groups of the donor parent on SSR markers in silkworm densonuleosis virus. Our results also suggested homozygosity in

parents and heterozygosity for hybrid by three viz., F11139, F10429 and F10705 out of ten SSR primers utilized on certain gene loci.

The silkworm breeds/hybrids with superior quantitative traits are by utilizing genetically divergent breeds through breeding programs. Most of the inbred lines or stocks of silkworm available today were generated by successive selection for specific traits of economic importance. Accurate estimation of genetic traits within inbred lines and relativity between them is important for both maintenance of inbred lines and hybrid preparation (Ramesha et al., 2009). Such estimates could be obtained by exploring the power of DNA fingerprinting or molecular analysis of silkworm genomic. In the present study, primers for di-nucleotide repeat motif of PCR-SSR markers were selected because of its abundance and estimated that the silkworm genome of 530 Mb accounted for 1.63 Mb of microsatellite repeats, equivalent to 0.31% of the genome (Dharma et al., 2005). In the achievement of reproducibility and strong signal in the PCR-SSR analysis, the significant parameter was the genomic template DNA concentration. PCR-SSR optimization procedures were carried out with varying concentration of silkworm genomic template DNA in order to ease the analysis on PCR-SSR amplification by only visible clear DNA bands on the gel and observed a reliable amplification with template DNA concentration between 10 - 35 ng (Figure 3). In this study, 20 ng of silkworm genomic template DNA was utilized for efficient PCR amplification and is in concurrence with the earlier studies. Among these selected ten SSR primers utilized for the study, seven $(CT)_n$ and three $(CA)_n$ microsatellite repeat motifs sequence (Rozen and Skaletsky, 2000).

The study examined genetic variability within and among the nutritionally efficient silkworm strains by DNA fingerprinting and the result obtained was consistent with voltinism and morphological differences. It is noticed that in silkworm, both dominance and epistasis are important for obtaining heterosis in traits for productivity of silk (Nagaraju and Kumar, 1995). When compared to closely related strains, genetically distant strains were more likely to have different fixed alleles at the same loci and hence their crosses should give higher degree of heterosis. Furthermore, heterosis was higher if both the parental strains are homozygous. The recent trend of genomic analysis led to the development of the PCR-SSR analysis promise to become a valuable tool for genomic analysis, led to generating molecular markers for specific trait's heterozygosity and homozygosity at allelic nature in gene locus than any other marker system. Apart from being the source of informative genetic markers, microsatellites attracted a lot of attention with respect to their origin, distribution, expansion, mutation and disintegration. The highest microsatellite content in genomic size of 397.71 Mb in *B. mori* among other full genome sequenced five insect species viz., *Drosophila melanogaster*, *Anopheles gambiae, Apis mellifera* and *Tribolium castaneum*. It represented about 0.72% of total silkworm genome with a maximum number of 111.006 repeats and of about 280 numbers of microsatellites per Mb genome (Dharma et al., 2005; Archak et al., 2007). Moreover, it was well established that of hereditary properties of PCR-SSR marker in silkworm and demonstrated that the PCR-SSR markers techniques could be successfully applied to the silkworm to reveal gene loci with many PCR-SSR primers producing patterns and potential to serve as molecular markers (Reddy et al., 1999a; Robinson et al., 2004; Li et al., 2006; Miao et al., 2005, 2007). The well characterized and accessible information on ten PCR-SSR markers in silkworm was utilized to investigate its utility in the molecular nutrigenomic analysis for the homozygosity and heterozygosity in newly identified nutritionally efficient silkworm parental strains of polyvoltine (RMW_2, RMG_4) and bivoltine (RBO_2, RBD_1) with its hybrids viz., $RMW_2 \times RBO_2$ and $RMG_4 \times RBD_1$ for the certain gene loci. PCR amplicon was found in two $(CA)_n$ repeat motif viz., F10429 and F11139 and one $(CT)_n$ repeat motifs of F10705. These observations strongly suggest $(CA)_n$ repeat motifs are closely linked in heterozygosity and homozygosity than $(CT)_n$. It was revealed that single varying size amplified DNA band in parental silkworm strains and two clear amplified bands in hybrids from three different gene loci (Figure 4). The result obtained in the present study was in concurrence with PCR-SSR primers utilized in the homozygosity and heterozygosity profile analysis for the *nsd* - Z gene (Chandrashekharaiah et al., 2006; Li et al., 2006). The PCR-SSR results suggested that the newly identified nutritionally efficient silkworm hybrids were genotypically heterozygous for certain nutritional gene loci. This heterozygosity nature in silkworm hybrid in contrast to the homozygous nature of parental silkworm breed was important for commercial exploitation of heterosis in sericulture industry (Nagaraju and Kumar, 1995; Ramesha et al., 2009).

We noticed that the silkworm breeds/hybrids chosen for the present study reveal marked differences for genetical traits such as origin, voltinism, fecundity, body weight, larval duration, cocoon and shell color and weight, silk filament length etc. in addition to nineteen nutrigenomic traits such as ingesta, digesta, excreta, approximate digestibility (AD), reference ratio (RR), consumption indices (CI), relative growth rate (RGR), respiration and metabolic rate (MR), efficiency conversion of ingesta (ECI) and efficiency conversion of digesta (ECD) for larva, cocoon and shell, ingesta per gram (I/g) and digesta per gram (D/g) for cocoon and shell (Ramesha et al., 2010). The PCR-SSR results in the present study on nutritionally efficient silkworm are similar to the observation made in various other systems (Pejic et al., 1998; Lima et al., 2006; Moyib et al., 2007). Microsatellite molecular analysis reflected the geographical and morphological relations of nutritionally efficient silkworm strains.

The two newly identified nutritionally efficient hybrids

were distinctively high yielding than its relative parental silkworm breeds. The unique PCR-SSR profile in the hybrids led to identification of nutritional related cascade of genes families. Several such different gene cascades were identified, categorized and cloned in silkworm through molecular approaches (Li et al., 2006; Muwang et al., 2007; Kanginakudru et al., 2007; Roller et al., 2008; Chai et al., 2008; Pan et al., 2009).

From the present microsatellite nutrigenomic analysis on molecular markers (PCR-SSR) was noticed to be an ideal tool and play an imperative role for marker-assisted selection (MAS) in silkworm breeding programs. This study also indicated that marker assisted selection could be actualized to new nutritionally efficient silkworm breeds/ hybrids. Simple sequence repeats often have flanking regions highly conserved in related species, which allows the use of the same primer pairs in related genomes.

These linked molecular markers would be very useful in marker-assisted screening, because the co-dominant markers offer information on homozygous and heterozygous genotypes and save time in the breeding program of silkworms with authenticity. Furthermore, not much is known about the pattern of gene expression, genomic organization or molecular evolution of the silkworm breeds / hybrids on nutrition consumption and its metabolism as it is the most important commercial parameter in sericulture industry involving over 60% of the cost for production of mulberry leaf. Therefore, these results led to identification of nutrigenomic related cascade of genes in silkworm and more emphasize the future prospects of silkworm functional mechanism in nutrigenomic studies after recent completion of total silkworm genome sequencing.

Conclusion

This study demonstrated a single amplified band in four parental silkworm strains of RMG_4, RMW_2, RBD_1 and RBO_2 whereas two clear amplified bands in the hybrids of $RMG_4 \times RBD_1$ and $RMW_2 \times RBO_2$ with varying molecular size from three PCR-SSR primers gene loci viz., F11139, F10429 and F10705. Therefore, it was predicted strongly that the indication of homozygosity in newly evaluated nutritionally efficient parental silkworm strains and heterozygosity in its hybrid. Ultimately, these results can be used in marker assisted selection or gene transmission in silkworm breeding programs and emphasizes the future prospects of silkworm functional mechanism in nutrigenomic studies.

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Abbreviations: PCR-SSR, Polymerase chain reaction-simple sequence repeats; **bp,** base pairs; **TE,** tris- EDTA; **DNA,** deoxyribonucleic acid; **EDTA,** ethylene diamine trichloro acetic acid; **MAS,** markers assistance selection; **GCA,** general combining ability; **SCA,** specific combining ability; **MPH,** Mid parent heterosis; **BPH,** better parent heterosis, **AD,** Approximate digestibility; **CI,** consumption index; **RGR,** relative growth rate; **MR,** metabolic rate; **ECI,** efficiency conversion of ingesta; **ECD,** efficiency conversion of digesta.

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