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Genetic diversity among the morphs of *Antheraea assamensis* Helfer: Study using RAPD and internal transcribed spacer DNA1

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Antheraea assamensis Helfer, popularly known as Muga silkworm, the golden silk producer of Northeast India, is economically important and unique among the Saturniid silkworms. Muga silkworm is said to exhibit three semi-domesticated morphs i.e. green, blue, orange and one wild morph. In this study, the genetic diversity and phylogeny among the morphs of Muga silkworm collected from various geographical locations of Northeast India were investigated using RAPD and internal transcribed spacer DNA1 (ITS1) sequences. Thirty random primers generated 192 discrete bands; 123 of them were polymorphic (64.062%). The average amplicon per primer was found to be 6.4. In RAPD analysis, a wide range of genetic distance i.e. 0.0544-0.6228 was observed among the morphs. In the ITS1 sequence analysis, 35.35% of polymorphism and a range of genetic distance from 0.0024 to 0.2349 were observed. The phylogenetic trees based on RAPD and ITS1 sequences comprised of two major clades. The first clade comprised of the semi-domesticated morphs while the second clade included the wild morphs of different geographical origin. The information generated in this study can be used for conservation of the Muga silkworm through effective breeding programs.

Keywords: ITS1, Muga silkworm, RAPD

Morphological differences and similarities have been used to group and classify organisms into major taxonomic groups. However, discerning finer differences among strains, races, biotypes and morphs is usually difficult due to the influence of environment. DNA based molecular markers have been increasingly employed in diverse areas of biology including phylogenetic studies, evolution, ecology, population genetics and population dynamics in both plant and animal systems because of their abundant polymorphism and are independent of the environmental conditions[1]. Molecular markers *viz*. RAPD, AFLP, RFLP, ISSR, SSR, mitochondrial genes, etc. have been adopted to study the genetic diversity, evolutionary and phylogenetic intricacies in different groups of organisms including insects[2-5]. RAPD technique is a suitable tool which has proved to be an appropriate method for obtaining genetic markers for many different kinds of organisms, demanding no prior information about genomic organization. Due to these advantages, RAPD markers have been widely used to study intra or inter specific genetic diversity and genetic structure of animals[6]. Additionally, the internal transcribed spacer DNA1 (ITS1) is part of the eukaryotic cistron of ribosomal RNA genes, located between the genes coding for 18S and 5.8S rRNA[7]. It has been widely used in estimating phylogenies for many organisms at the population and species level due to its high level of sequence variation because of its noncoding structure[8,9].

The Northeastern region has been recognized as the centre of Seri-biodiversity in India. *Antheraea assamensis* Helfer, popularly known as Muga silkworm is a golden coloured silk producing insect, endemic to Northeast India. It is a polyphagous lepidopteran that feeds on 15 different host plant species. Unlike the other Antheraea species, this species has the lowest chromosome number (n=15) and ZZ/ZO sex chromosome system[10]. Muga silkworm is known for its production of quality silk with natural golden colour, lustrous, durability and resistant to UV radiation. It has better mechanical properties than other commercial silks which impart a wide range of use as a textile material[11]. Although

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Muga silkworm is a monotypic species, it has three semi-domesticated morphs *viz*. green, blue, orange, and one wild morph[12]. These morphs of Muga silkworm show variation in their phenotypic traits, such as larval colour, cocoon colour, larval weight, cocoon weight, shell weight, shell ratio, voltinism, etc. Due to deforestation activities, environmental pollution, disease and overexploitation for commercial uses the population of these morphs is declining rapidly. This depletion in population density may result in low genetic variation of this species. In order to develop a sustainable conservation program, assessment of genetic variation and phylogenetic relationship between and within species is a necessary prerequisite. Again, prior knowledge of genetic variation among different morphs of a single species can also be utilized in breeding program for improving the productivity. Many studies have been carried out to assess the genetic diversity of silkworms, but not for morphs of Muga silkworm[13-15]. Probably due to narrow and endemic distribution, the Muga silkworm has not drawn much attention to itself and only little information is available on the genetic diversity of this silkworm species in spite of their high economic value. Previously, the genetic diversity among different individuals of Muga silkworm collected from Northeast India was studied using SSR and ISSR markers[16,17]. In contrast, this manuscript added information on Muga silkworm as it tested the genetic diversity among the morphs.

Here, we studied the genetic diversity and phylogeny among the morphs of Muga silkworm collected from various geographical locations of Northeast India using RAPD and internal transcribed spacer DNA1 (ITS1) sequences.

Materials and Methods

Sample collection

After a brief survey on habitat of Muga silkworm, four morphs were collected from various geographical locations of Northeast India and used in this study (Table 1). The morphs were green (GM), blue (BM), orange (OM) and wild (WM). The morphs were coded as GM01, GM02, GM03, GM04, GM05, GM06, BM01, BM02, OM01, WM01, WM02 and WM03 according to their geographical origin (Table 1). Ten numbers of individuals per morph per locality were used for the study. As these silkworms are cultured for economic purpose throughout Northeast India, no specific permissions were required for the collections and experiments. In this study, national park or other protected area of land or sea, endangered and protected species were not used.

The collected samples were preserved and reared at IASST's garden as well as in-house condition for their multiplication. Som (*Machilus bombycina* K.) plant was used for feeding the Muga silkworm throughout the experiment.

Phenotypic characters

Phenotypic characters like larval colour, cocoon colour, larval weight, cocoon weight, shell weight, shell ratio and voltinism of the morphs were studied. The colour of the larva and cocoon, larval markings and cocoon shape of each sample of Muga silkworm were observed visually and recorded (Table 2). The weights of the larvae, cocoons (with pupa inside) and shell were measured with the help of a single pan digital microbalance (Mettler Toledo, PB153-S). The values were expressed as Mean±SD. The shell ratio (%) was calculated by using the following formula:

$$\text{Shell ratio (\%)} = \frac{\text{Weight of the cocoon shell}}{\text{Weight of the entire cocoon}} \times 100$$

The values of shell ratio were expressed as Mean±SD.

DNA extraction

Genomic DNA was extracted from the larval tissue of each sample according to the standard procedure[18]. Briefly, one gram of larval tissue was ground in liquid nitrogen to make it fine powder and 10 mL of

Table 1 — Collection sites of Muga silkworm

Morph	Sample Code	Place	State	Position	Altitude (m)
Green	GM01	Khanapara	Assam	26°09′N 91°41′E	78
Green, Blue	GM02, BM01	Mangaldoi	Assam	26º43′N 92º03′E	34
Green, Blue	GM03, BM02	Udalguri	Assam	26°44′N 92°50′E	180
Orange	OM01	Howley	Assam	26°25′N 90°59′E	43
Green	GM04	Goalpara	Assam	26°17′N 90°32′E	44
Green	GM05	Titabor	Assam	26°16′N 93°51′E	104
Green	GM06	North Lakhimpur	Assam	27º15′N 94º90′E	101
Wild	WM01	Hahim	Assam	26º15′N 91º38′E	68
Wild	WM02	Tura	Meghalaya	25º30′N 90º16′E	478
Wild	WM03	Haflong	Assam	25°18′N 93°03′E	680

Table 2 — Phenotypic characters of the morphs of Muga silkworm

Morph	Larval colour	Larval marking	Cocoon colour	Cocoon shape	Larval weight (g)	Cocoon weight (g)	Shell weight (g)	Shell ratio (%)	Voltinism
GM01	Deep green	Plain	Bright golden brown	Oval, spindle	9.20±0.13	4.62±0.95	0.53±0.06	11.70±1.88	Multivoltine
GM02	Deep green	Plain	Bright golden brown	Oval, spindle	9.40±0.15	3.95±0.89	0.48±0.16	12.15±1.21	Multivoltine
GM03	Deep green	Plain	Bright golden brown	Oval, spindle	9.55±0.21	3.82±0.72	0.45±0.09	11.78±0.97	Multivoltine
GM04	Deep green	Plain	Bright golden brown	Oval, spindle	9.33±0.19	4.19±0.77	0.39±0.12	9.30±1.05	Multivoltine
GM05	Deep green	Plain	Bright golden brown	Oval, spindle	9.18±0.11	3.68±0.56	0.35±0.09	9.51±1.10	Multivoltine
GM06	Deep green	Plain	Bright golden brown	Oval, spindle	9.61±0.25	4.92±0.64	0.51±0.10	10.37±0.92	Multivoltine
BM01	Faint bluish	Plain	Whitish golden brown	Oval, spindle	8.80±0.13	5.36±1.11	0.48±0.08	9.09±1.30	Multivoltine
BM02	Faint bluish	Plain	Whitish golden brown	Oval, spindle	8.52±0.18	5.10±1.05	0.41±0.15	8.04±1.76	Multivoltine
OM01	Orange	Plain	Yellowish brown	Oval, spindle	7.80±0.12	1.59±0.56	0.22±0.06	14.50±3.70	Multivoltine
WM01	Green	Plain	Light golden yellow	Oval, spindle	9.90±0.23	6.82±1.09	0.61±0.19	8.98±2.12	Univoltine/ Bivoltine
WM02	Green	Plain	Light golden yellow	Oval, spindle	9.82±0.22	6.78±0.37	0.58±0.29	8.55±2.09	Univoltine/ Bivoltine
WM03	Green	Plain	Light golden yellow	Oval, spindle	10.65±0.11	7.12±1.61	0.72±0.09	9.66±3.15	Univoltine/ Bivoltine

[Values are expressed as mean ± SD (n=10)]

extraction buffer was added. The mixture was incubated at 37°C for 2 h with occasional swirling. The extraction buffer contained 0.1 M Tris-HCl (pH 8), 0.25 M EDTA (pH 8), 0.01 M NaCl, 0.5% SDS and 100 µg/mL Proteinase K. The DNA was extracted twice with phenol: chloroform: isoamyl alcohol (24:24:1) and once with chloroform. The supernatant DNA was precipitated with 0.1 volume of 3M sodium acetate and two volumes of ice cold absolute ethanol. Following precipitation, the DNA was washed twice with 70% ethanol and dissolved in TE buffer. The RNAse treatment was done by adding RNAseA (100 µg/mL) and incubating at 37°C for 1 h. DNA was re-extracted with phenol-chloroform-isoamyl alcohol, chloroform and ethanol precipitation as described earlier. The extracted DNA was then checked on 0.8% agarose gel. Quantification of the DNA samples was done using UV-Vis Spectrophotometer (Eppendorf, Germany).

PCR amplification with RAPD primers

A total of 60 decamer random primers were obtained from Genei (Bangalore, India) and used for the study. But only 30 of these produced good amplification and hence were chosen for further analysis. The list of primers and their sequences has been given in Table 3. The PCR amplification was carried out in a thermal cycler (Applied biosystems, Germany) with 25 µL reaction mixture containing 2.5 µL buffer (10X Taq DNA polymerase buffer containing 15 mM $MgCl_2$), 2.5 mM dNTPs (from 10-mM stock), 20 pM primer (random decamer primer), 1 unit of Taq DNA polymerase and 30 ng of genomic DNA. The PCR profile was as follows: 1 cycle of 94°C for 5 min, 44 cycles of 94°C for

Table 3 — List and banding profile produced by RAPD primers for the morphs of Muga silkworm

RAPD primer	Nucleotide sequence (5′→3′)	No. of total bands	No. of polymorphic bands	Proportion of polymorphic bands (%)
DF1	AGCCTGAGCC	6	5	83.33
DF2	TGCCGAGCTG	9	9	100
DF3	AAGCGACCTG	5	2	40
DF5	GACCGACCCA	8	6	75
DF6	CATTCGAGCC	6	4	66.66
DF8	TGTCATCCCC	7	6	85.71
DF10	GTCCGGAGTG	5	2	40
DF12	CTCAGTGTCC	5	2	40
DF15	CAGGCCCTTC	5	5	100
DF18	AGGTGACCGT	8	5	62.5
DF19	GGGGTGACGA	6	3	50
DF20	CACAGGCGGA	6	3	50
DF22	AGGTCTTGGG	4	1	25
RPL2	CAGAAGCGGA	4	3	75
RPL5	CATCGCCGCA	8	5	62.5
RPL8	ACAGCCTGCT	5	4	80
RPL10	GGCTGCAATG	6	4	66.66
RPL11	GGCTGCGACA	8	2	25
RPL12	AGAGCCGTCA	6	4	66.66
RPL15	AGCAGCGCAC	7	6	85.71
RPL16	CAAACGTGGG	6	4	66.66
RPL18	AGACGATGGG	7	4	57.14
RPL22	GGGTCTCGGT	7	5	71.42
RPL23	GGTCGATCTG	5	3	60
RPL25	GGGCCAATGT	7	3	42.85
RPL26	TGTCATCCCC	7	5	71.42
RPL27	TGGGCGTCAA	4	1	25
RPL28	CTGTTGCTAC	9	6	66.66
RPL29	CAGGGAAGAG	8	5	62.5
RPL30	ACTGAACGCC	8	6	75
Total		192	123	
Average		6.4		64.06

1 min (denaturation), 36°C for 1 min (annealing), 72°C for 2 min (elongation) followed 72°C for 10 min. The PCR products were resolved on 1.5% agarose gel and the gel image was recorded by using Biodoc-lt imaging system (UVP, UK).

RAPD data scoring and analysis

RAPD markers were scored in a binary form as presence (1) or absence (0) of amplified bands for each sample. The genetic variation was analyzed on the basis of the banding pattern using parameters such as number of total bands, number of polymorphic bands, proportion of polymorphism (%), genetic distance and genetic similarity analysis[19] using TFPGA 1.3 software[20].

Cluster analysis was also performed based on the genetic similarity and genetic distance matrix by means of the unweighted pair group method with arithmetic mean (UPGMA) using TFPGA 1.3 software to study the genetic relationship among the morphs.

PCR amplification and sequencing of ITS1

A 450-bp of ITS1 region was amplified by PCR. The primers used for amplification were forward 5'-CGTCGCTACTACCGATTGAATG-3' and reverse 5'-GCGTTCGAAGTGTCGATGTT-3'. The primers were synthesized by Bangalore Genei (Bangalore, India). Amplification was carried out in a thermal cycler (Applied biosystems, Germany) with 25 µL reaction mixture containing 2.5 µL buffer (10X Taq DNA polymerase buffer containing 15 mM $MgCl_2$), 2.5 mM dNTPs (from10-mM stock), 10 mM each primer, 1 unit of Taq DNA polymerase and 30 ng of genomic DNA. The PCR reaction conditions were as follows: 1 cycle of 94°C for 5 min, 35 cycles of 94°C for 30 s (denaturation), 50°C for 1 min (annealing), 72°C for 1 min (elongation), followed by 72°C for 5 min. The amplified PCR products were resolved in a 1.5% agarose gel and documented in Biodoc-lt imaging system (UVP, UK). The PCR products were purified with the Geneipure Quick PCR Purification Kit (Merck millipore, India). The purified PCR products were sent to Bangalore Genei to sequence in both directions. Sequence primers were the same as used in PCR reaction.

Analysis of ITS1 sequences

The sequences of all samples were assembled using Contig Express software and aligned using ClustalX 1.83[21]. After alignment, invalid end sequences were trimmed. The sequences were subjected to BLAST (Basic Local Alignment Search Tool) to perform sequence similarity searches[22]. All sequences obtained in this study have been deposited in GenBank. Nucleotide frequencies, nucleotide pair frequencies (the rate that one nucleotide was substituted by another nucleotide) and transition/transversion ratio (R) (the number of transitions to the number of transversions for a pair of sequences) were calculated by using MEGA6 software[23]. The overall transition/transversion bias (R) was calculated, where

$$R = \frac{[A*G*k1 + T*C*k2]}{[(A+G)*(T+C)]}$$

All positions containing gaps and missing data were eliminated from the dataset (Complete-deletion option). InDels (Insertion-Deletion polymorphism) was estimated using DnaSP5 software[24]. The same software was used also to detect conserved, variable, parsimony informative and singleton sites.

In order to determine the extent of genetic variation among the morphs of Muga silkworm, the average number of pairwise nucleotide differences among DNA sequences (k) and nucleotide diversity (Pi), the average number of nucleotide differences per site between two sequences (K/m, where m is the length of sequences) were calculated. All these analyses were performed using DnaSP software. The phylogenetic tree based on ITS1 sequences were constructed by Neighbor-joining (NJ) method with Kimura 2 parameter model using MEGA6 software. Bootstrap analysis (1000 bootstrap replications) was employed to test the reliability of the topologies of phylogenetic tree. The sequence of *Bombyx mori* (DQ164788) was taken as out group in the phylogenetic tree.

Results

Phenotypic variation

The morphs of Muga silkworm showed variation in their phenotypic characters like larval colour, cocoon colour, larval weight, cocoon weight, shell weight, shell ratio and voltinism (Table 2).

RAPD analysis

RAPD profile generated with the primers for the morphs of Muga silkworm are depicted in Fig. 1. The total number of DNA fragments amplified, number of polymorphic bands and proportion of polymorphic bands (%) with individual primer are shown in Table 3.

Thirty random primers which yielded good amplification were chosen for fingerprinting the 12 Muga silkworm samples collected from various locations of Northeast India. Thirty selected primers generated 192 bands. One hundred twenty three of these were polymorphic (64.06%). Proportion of polymorphism (%) detected with each primer was as high as 100% in 2 primers (DF2 and DF15) to 25% in 3 primers (DF22, RPL11 and RPL27). The number of bands per primer ranged from 4 (DF22, RPL11 and RPL27) to 9 (DF2 and RPL28) with an average of 6.4 bands per primer. These bands were used for the genetic variability study followed by similarity and dissimilarity.

A wide range of genetic distance i.e. 0.0544-0.6228 was observed among the morphs (Table 4). The lowest genetic distance was observed between the green morph (GM02) and blue morph (BM01) collected from Mangaldoi which was 0.0544. The highest genetic distance was noticed between the green morph collected from North Lakhimpur (GM06) and wild morph collected from Halfong (WM03) which was 0.6228. Similarly, the genetic similarity was also calculated which ranged from 0.5364 to 0.9470. The maximum genetic similarity was found between GM02 and BM01 (0.9470). The minimum genetic similarity was detected between GM06 and WM03 (0.5364).

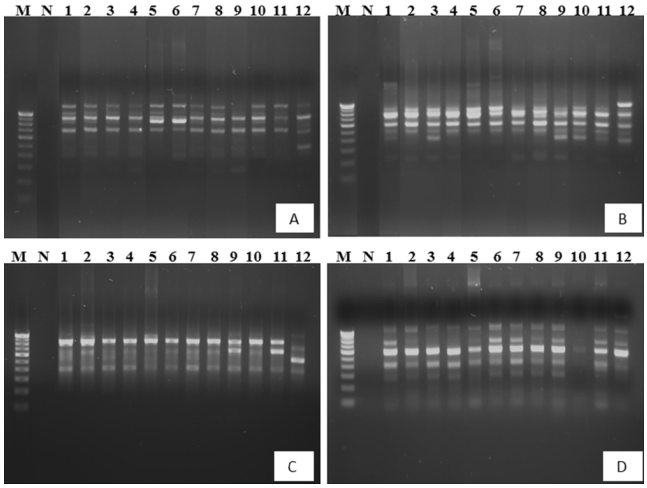

Fig. 1 — RAPD profile of Muga silkworm produced with primers (A) DF19; (B) RPL5; (C) RPL26 & (D) RPL30. [The lanes are M= DNA ladder of 100 bp, N= Negative control, 1= GM01, 2= GM02, 3= GM03, 4= GM04, 5= GM05, 6= GM06, 7= BM01, 8= BM02, 9= OM01, 10= WM01, 11= WM02 & 12= WM03]

Cluster analyzes based RAPD data

Both polymorphic as well as monomorphic bands were used to construct the dendrogram by using unweighted pair group method with arithmetic mean (UPGMA). Associations among the morphs revealed by UPGMA cluster analysis based on RAPD profile are presented in Fig. 2. The dendrogram comprised of two major clusters. One consisted of the semi-domesticated morphs. While the wild morphs i.e. WM01, WM02 and WM03 separated from the semi-domesticated morphs forming the other cluster. The first cluster was divided into two sub clusters: sub cluster I and sub cluster II. The morphs GM02,

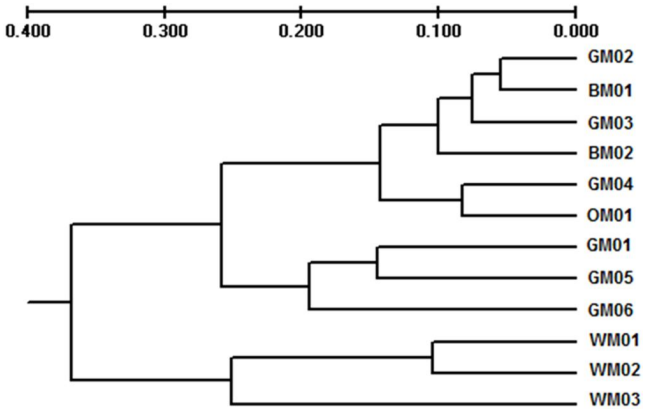

Fig. 2 — Dendrogram showing the genetic relationships among the morphs of Muga silkworm based on RAPD data.

Table 4 — Genetic distance (below diagonal) and genetic similarity (above diagonal) based on RAPD banding pattern

	GM01	GM02	GM03	GM04	GM05	GM06	BM01	BM02	OM01	WM01	WM02	WM03
GM01	*****	0.7881	0.8013	0.7748	0.8657	0.8013	0.7881	0.7682	0.7881	0.6887	0.6821	0.5762
GM02	0.2382	*****	0.9338	0.8940	0.8126	0.7616	0.9470	0.9007	0.8808	0.7682	0.7483	0.6821
GM03	0.2215	0.0685	*****	0.8675	0.7861	0.7483	0.9205	0.9139	0.8675	0.7947	0.7483	0.6424
GM04	0.2551	0.1120	0.1421	*****	0.7993	0.7219	0.8808	0.8609	0.9205	0.7417	0.7483	0.6556
GM05	0.1442	0.2075	0.2407	0.2240	*****	0.8458	0.8259	0.7529	0.7728	0.6998	0.7065	0.6003
GM06	0.2215	0.2723	0.2899	0.3259	0.1675	*****	0.7748	0.7682	0.6821	0.6490	0.6424	0.5364
BM01	0.2382	0.0544	0.0828	0.1269	0.1913	0.2551	*****	0.9007	0.8675	0.7947	0.7483	0.6424
BM02	0.2637	0.1046	0.0900	0.1497	0.2838	0.2637	0.1046	*****	0.8212	0.7748	0.7020	0.6225
OM01	0.2382	0.1269	0.1421	0.0828	0.2577	0.3826	0.1421	0.1970	*****	0.7417	0.7616	0.6821
WM01	0.3729	0.2637	0.2298	0.2988	0.3569	0.4323	0.2298	0.2551	0.2988	*****	0.9007	0.7815
WM02	0.3826	0.2899	0.2899	0.2899	0.3475	0.4426	0.2899	0.3538	0.2723	0.1046	*****	0.7748
WM03	0.5514	0.3826	0.4426	0.4222	0.5103	0.6228	0.4426	0.4740	0.3826	0.2466	0.2551	*****

BM01, GM03, BM02, GM04 and OM01combined together to form sub cluster I. The sub cluster II comprised of the semi-domesticated morphs GM01 and GM05 followed by GM06.

Analysis of ITS1 sequences

The complete ITS1 region was sequenced for all the samples of Muga silkworm. The size of the fragment analyzed was 413 bp. The sequences were submitted to GeneBank and the accession numbers are given in Table 5. The sequences showed no InDels, the variation among the sequences due to nucleotide substitutions. Out of the 413 bp, 267 sites were conserved and 146 (35.35%) were polymorphic sites. Of these 146 variable sites there were 68 singleton sites and 78 parsimony informative sites. The nucleotide base compositions are shown in Table 5. The average A, T, C and G frequencies were 19.4, 28.0, 25.2 and 24.7%, respectively. The transition/transversion rate ratios were *k1* = 1.537 (purines) and *k2* = 1.64 (pyrimidines). The overall transition/transversion ratio was *R* = 0.793, where

$$R = \frac{[A*G*k1 + T*C*k2]}{[(A+G)*(T+C)]}$$ (Table 6).

The distance matrix obtained from the analysis of alignment of all sequences revealed a range of genetic distance from 0.0024 to 0.2349 (Table 7). The lowest genetic distance was found between GM02 and BM01 (0.0024). The highest genetic distance was observed between GM05 and WM03 (0.2349). The average number of pairwise nucleotide differences (k) was 42.48 and the nucleotide diversity i.e. the average number of nucleotide differences per site between two sequences (Pi) was 0.1029.

Phylogenetic analysis among the morphs of Muga silkworm based on ITS1 sequences

The phylogenetic relationships among the morphs of Muga silkworm was also studied using ITS1 sequences. A phylogenetic tree was constructed and the sequence of *Bombyx mori* was taken as an out group (Fig. 3). The topology of the tree was tested by bootstrapping and supported by high bootstrap values. The tree based on ITS1 sequences formed of two major groups- Group I and II. Group I consisted of two subgroups. The first subgroup comprised of GM02 and BM01 followed by BM02. The second subgroup included GM03, GM05 and GM06. Group II included WM01, WM02 and WM03. The samples

Table 5 — Sequence variation details and nucleotide base composition of ITS1 sequences among the morphs of Muga silkworm

Morph	GenBank Accession no.	Total length (bp)	Poly-morphic site	A (%)	T (%)	C (%)	G (%)
GM01	KU366521			19.4	28.6	24.7	27.4
GM02	KU366522			18.9	28.3	24.7	28.1
GM03	KU366523			18.9	29.1	24.2	27.8
GM04	KU366524			19.6	28.1	24.9	27.4
GM05	KU366525			18.2	32.2	19.6	30.0
GM06	KU366526			18.6	31.0	21.3	29.1
BM01	KU366527	413	146	18.6	28.6	24.7	28.1
BM02	KU366528			19.1	27.6	24.9	28.3
OM01	KU366529			19.1	28.3	24.9	27.6
WM01	KU366530			22.3	21.8	29.3	26.6
WM02	KU366531			21.3	23.7	28.8	26.2
WM03	KU366532			18.9	29.3	29.8	22.0
Average				19.4	28.0	25.2	27.4

Table 6 — Frequency of nucleotide substitutions in the ITS1 sequences among the morphs of Muga silkworm

	A	T	C	G
A	-	*7.81*	*7*	11.72
T	*5.4*	-	11.49	*7.62*
C	*5.4*	12.81	-	*7.62*
G	8.31	*7.81*	*7*	-

[Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in straight and those of transversional substitutions are shown in *italics*]

Table 7 — Pair wise genetic distances based on ITS1 sequences

	GM01	GM02	GM03	GM04	GM05	GM06	BM01	BM02	OM01	WM01	WM02	WM03
GM01	0.0000											
GM02	0.0121	0.0000										
GM03	0.0145	0.0073	0.0000									
GM04	0.0266	0.0194	0.0218	0.0000								
GM05	0.0823	0.0751	0.0726	0.0944	0.0000							
GM06	0.0630	0.0557	0.0533	0.0751	0.0242	0.0000						
BM01	0.0145	0.0024	0.0097	0.0218	0.0775	0.0581	0.0000					
BM02	0.0218	0.0097	0.0169	0.0291	0.0847	0.0654	0.0121	0.0000				
OM01	0.0291	0.0218	0.0291	0.0363	0.0969	0.0775	0.0242	0.0315	0.0000			
WM01	0.1816	0.1840	0.1913	0.1695	0.2276	0.2034	0.1864	0.1792	0.1768	0.0000		
WM02	0.1550	0.1574	0.1646	0.1429	0.2034	0.1792	0.1598	0.1525	0.1501	0.0896	0.0000	
WM03	0.1864	0.1937	0.2010	0.1792	0.2349	0.2107	0.1961	0.1889	0.1816	0.1671	0.1283	0.0000

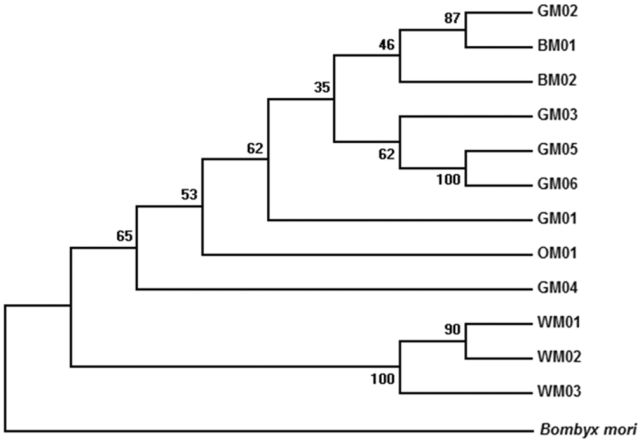

Fig. 3 — Phylogenetic tree based on ITS1 sequences. Numbers at the nodes indicate the bootstrap values generated by 1000 replications. The sequence of *Bombyx mori* was taken as out group.

GM01, GM04 and OM01 came out separately forming three different isolates.

Discussion

In the present study, random amplified polymorphic DNA (RAPD) and internal transcribed spacer DNA1 (ITS1) sequences were used to study the intraspecific variability and the phylogenetic relationships among the morphs of Muga silkworm collected from different geographical locations. These morphs represented a high degree of divergence with respect to geographic origin, morphological, qualitative and quantitative and biochemical characters[12]. Combined use of two markers with the same set of insect populations can improve the reliability of information obtained for developing robust conservation strategies.

Since RAPDs are random selection of DNA sequences, it was sensitive enough to detect differences among different morphs of a species. Wide genetic variation was observed among individuals of different morphs examined here. In the dendrogram, there was clear demarcation between the semi-domesticated and the wild morphs of Muga silkworm. The silkworms were referred as semi-domesticated owing to the fact that only cocooning and grainage operations were conducted indoor and reared on outdoor host plants. Individual moths of the semi-domesticated morphs i.e. green, blue and orange collected from Khanapara, Mangaldoi, Udalguri, Howley, Goalpara, Titabor and North Lakhimpur of Assam combined together to form a group. While the wild morphs collected from Hahim, Tura and Haflong formed another group.

ITS1 region from 12 samples of Muga silkworm was sequenced and analyzed. The sequences of ITS1 region have been widely adopted for inferring insect phylogenies. Polymorphism in the sequences of ITS1 region may arise when concerted evolution is not fast enough to homogenize repeats in face of high rates of mutation[25,26]. The higher polymorphism with RAPD than ITS1 region is due to the random annealing of the primers across the genome. The phylogenetic relationships based on ITS1 region showed similarities with those based on RAPD analysis. Phylogenetic tree constructed based on these sequences revealed two major clades each having high bootstrap values.

The geographic distance was the possible factor influencing genetic variation observed between the semi-domesticated and wild morphs which further supported the contention that the wild moths do not migrate far[27]. Limited migration among wild populations of insects was reported earlier[28]. Reasons behind the closer relationships exist among the semi-domesticated morphs are unclear, but could be due to inbreeding and genetic mixing among them[29]. The genetic variation observed between GM02 and BM01 is very narrow though they are morphologically different. The high genetic diversity for wild morph indicated the need to preserve the forest areas in Northeast India as repositories of diverse germplasm of Muga silkworm. In this study, even though the numbers of samples tested were few, it showed that the morphs were diverse and showed some geographical clustering, as has been suggested by previous study[16].

Knowledge of genetic variation within a species provides essential information in the formulation of appropriate management strategies directed towards their conservation and the establishment of effective and efficient breeding programs[30]. For harnessing hybrid vigor in silkworms, cross breeding strategies have been extensively used. The selection of genetically pure and divergent parents is critical to the success of a hybridization program in silkworm. It was shown that the parental homozygosity, also known as genetic purity, had a distinct influence on the degree of manifestation of hybrid vigour[31].

Moreover, in conservation genetics, information on genetic variation and relatedness between individuals is important in breeding programs in order to minimize inbreeding and the loss of genetic variation.

It is reported that a decline in genetic variation reduces the ability to adapt to environmental changes and decreases its long term survival. The loss of genetic diversity also results in lower individual fitness and poor adaptability[32]. Inbreeding is known to decrease genetic diversity and to reduce reproductive and survival rates which lead to increased extinction risk. Knowledge and studies on genetic diversity can reduce the extinction risk by helping to develop appropriate management programs that can minimize the risks implied through inbreeding.

Conclusion

This study suggests that RAPD and ITS1sequences are suitable enough for resolving genetic variation and phylogenetic relationships among the morphs of Muga silkworm. From this study, it is clear that these morphs not only differ in their phenotypic traits but also in their genetic makeup. The phenotypic and genetic variation revealed in this study might support in conservation of the natural biodiversity present among the morphs of this unique silkworm in Northeast India through effective breeding program and thereby provide efficient measures for germplasm conservation and silkworm breeding.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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