15. Hombe Gowda, H. C., Vasudeva, R., Georgi P. Mathachen., Uma Shaanker, R. and Ganeshaiah, K. N., Breeding types in *Nothapodytes nimmoniana* Graham. *Curr. Sci.*, 2002, **83**, 1077–1078.
16. Ravi Kumar and Ved, D. K., *100 Red Listed Medicinal Plants of Conservation Concern in Southern India*, FRLHT, Bangalore, 2000, pp. 261–263.
17. Ved, D. K., Trade in medicinal plants – the state of our ignorance. *Amruth*, 1997, **1**, 2–8.
18. Ciddi, V. and Shuler, M. L., Camptothecin from callus cultures of *Nothapodytes foetida*. *Biotech. Lett.*, 2000, **22**, 129–132.
19. Thengane, S. R., Kulkarni, D. K., Shrikhande, V. A. and Krishnamurthy, K. V., Effect of thidiazuron on adventitious shoot regeneration from seedling explants of *Nothapodytes foetida*. *In vitro Cell. Dev. Biol.*, 2001, **37**, 206–210.
20. Fulzele, D. P., Satdive, R. K. and Pol, B. B., Growth and production of camptothecin by cell suspension cultures of *Nothapodytes foetida*. *Planta Med.*, 2001, **67**, 150–152.
21. Sundervelan, R. B., Desireddy and Veeresham, C., Camptothecine-A novel anti cancer agent from tissue cultures of *Nothapodytes foetida*. *Indian J. Pharm. Sci.*, 2002, **65**, 101–105.
22. Fulzele, D. P. and Satdive, R. K., Somatic embryogenesis, plant regeneration and the evaluation of the camptothecin content in *Nothapodytes foetida*. *Soc. In Vitro Biol.*, 2003, **39**, 212–216.
23. Thengane, S. R., Kulkarni, D. K., Shrikhande, V. A., Joshi, S. P., Sonawane, K. B. and Krishnamurthy, K. V., Influence of medium composition on callus induction and camptothecin(s) accumulation in *Nothapodytes foetida*. *Plant Cell Tiss. Org. Cult.*, 2003, **72**, 247–251.
24. Roja, G. and Heble, M. R., The quinoline alkaloids camptothecin and 9-methoxycamptothecin from tissue cultures and mature trees of *Nothapodytes nimmoniana*. *Phytochemistry*, 1994, **36**, 65–66.
25. Puri, S. C., Handa, G., Gupta, R. K., Srivastava, T. N., Somal, P. and Sharma, S. N., Quantitation of camptothecin in *Nothapodytes foetida*. *J. Indian Chem. Soc.*, 1999, **76**, 370–371.
26. Li, S., Yi, Y., Wang, Y., Zhang, Z. and Beasly, R. S., Camptothecin accumulation and variations in camptotheca. *Planta Med.*, 2002, **68**, 1010–1016.
27. Vasudeva, R., Hombe Gowda, H. C. and Raghu, H. B., Suitability of *Nothapodytes nimmoniana* as a profitable medicinal tree component for agroforestry systems of hill and transition zones of Karnataka. *My Forest*, 2004, **40**, 39–44.

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Genetic variation in ecoraces of tropical tasar silkworm, *Antheraea mylitta* D. using RFLF technique

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Antheraea mylitta **produces tasar silk and is an endemic species of the Indian subcontinent. Populations of this species show a certain degree of phenotypic variability for which they are designated as 'ecoraces'. In order to study the genetic variability and phylogenetic relationship among the different ecoraces, we have cloned and characterized a 281 bp *Mbo*I genomic DNA fragment, which has 75% identity at amino acid level with the 'reverse transcriptase' domain of TED retrotransposon of the lepidopteran insect, *Trichoplusia ni*. PCR-amplified *Mbo*I fragment from different ecoraces showed 99–100% sequence identity at nucleotide level. However, restriction fragment length polymorphism (RFLP) studies using this *Mbo*I fragment as probe have shown polymorphic pattern among the ecoraces. Phylogenetic relationships of different ecoraces obtained on the basis of RFLP pattern support the phenotypic and geographical relations.**

Keywords: *Antheraea* species, DNA, repetitive RFLP, retrotransposon, silkworm.

ANTHERAEA MYLITTA D., a lepidopteran insect of the Saturniidae family produces tasar silk of commercial importance. This species is endemic and distributed in different geographical regions of India in the form of ecological races (Table 1). They show variation in their phenotypic traits such as fecundity, voltinism, cocoon weight, silk ratio and also in their host plant preference[1]. Two major problems of this non-mulberry silkworm are (1) gradually decreasing number of ecoraces and (2) their identification. Therefore, to understand the genetic closeness and also for the identification of the wild silkworm ecoraces, development of molecular marker is important. Several molecular markers have been developed in case of *Bombyx mori* like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR) analyses, fluorescent-dye-labelled nucleotide in addition to ISSR–PCR reaction (FISSR–PCR) and single nucleotide polymorphism (SNPs) for high throughput genotyping for divergent silkworm strains[2–6]. Mariner transposable element was used as a marker to classify the systematic positions of silk, producing insects[7]. In addition, retrotransposons were used as markers to study the genetic variability in several insects

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Table 1. Distribution and morphological characteristics of nine ecoraces of *Antheraea mylitta* D

Ecorace	Place of collection	State of collection	Nature of rearing	Cocoon colour	Latitude (°N)/longitude (°E)
Andhra Local	Warangal	Andhra Pradesh	Wild	Whitish-grey	18.0; 79.35
Bhandara Local	Chanda	Maharashtra	Wild	Grey	21.12; 79.4
Daba	Bankura	West Bengal	Semi-domesticated	Grey	24.17; 87.15
Modal	Mayurbhanj	Orissa	Wild	Blackish-grey	21.30; 86.1
Nalia	Sundergarh	Orissa	Wild	Grey	22.0; 86.46
Raily	Bastar	Madhya Pradesh	Wild	Blackish-grey	19.05; 82.25
Sarihan	Barsati	Bihar	Wild	Grey	24.17; 87.15
Sukinda	Sukindagarh	Orissa	Semi-domesticated	Yellow	20.47; 86.32
Tera	Midnapore	West Bengal	Wild	Grey	22.25; 87.21

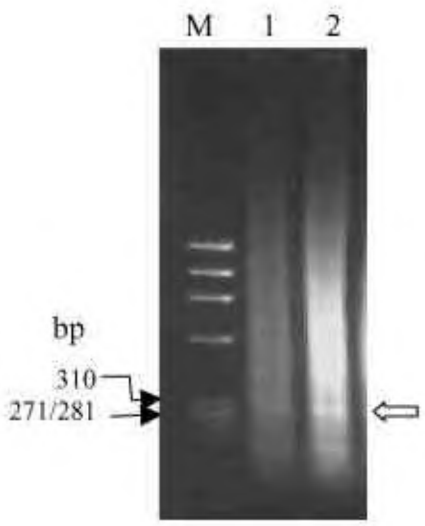

Figure 1. Agarose gel electrophoresis of genomic DNA from Andhra Local ecorace digested with *Mbo*I. Lane M, Molecular weight marker; lane 1 and 2, *Mbo*I-digested genomic DNA from two individuals. Black arrows indicate size in bp of the molecular weight marker and hollow arrow indicates high intensity *Mbo*I genomic fragment in the background of the smear.

and plants. No molecular marker of tasar silkworm has been reported to identify the genetic diversity at ecoraces level. We have identified and characterized an *Mbo*I-digested genomic DNA fragment, which has been used as RFLP marker for the ecoraces of tropical tasar silkworm.

Genomic DNA from Andhra Local ecorace was isolated and digested with *Mbo*I completely[8,9]. The *Mbo*I digestion produced a high-intensity band of about 300 bp size in the background of smear in agarose gel electrophoresis like repetitive DNA (Figure 1). That fragment was cloned in *Bam*HI site of pBluescript® II SK + vector (Stratagene, USA) and was transformed into DH5α strain of *Escherichia coli*. To verify the repetitive nature of this fragment, Southern hybridization was carried out for all the nine available ecoraces collected from several geographical regions. Genomic DNAs were isolated from all the ecoraces, digested completely with *Mbo*I, blotted onto a nylon membrane[10] and hybridized with P^{32}-labelled *Mbo*I fragment of Andhra Local. In case of all the ecoraces except Modal, the autoradiogram showed a single highlighted band of about 300 bp. Only in case of Modal, a ladder-like pattern was obtained along with the 300 bp band (Figure 2 *a*).

The *Mbo*I fragment of Andhra Local was sequenced using M13 universal primers. It was 281 bp long and had 63% AT and 37% GC content. The tblastx analysis[11] of *Mbo*I fragment sequence (DQ069899) showed 75% identity and 88% similarity at amino acid level with reverse transcriptase gene of *Trichoplusia ni* retrotransposon, TED, spanning from 764 to 859 amino acids and also with several other retrotransposable elements. TED, a lepidopteron retrovirus like retrotransposon, was initially identified as a single copy insertion in the genome of baculovirus during infection of cultured *T. ni* cells with baculovirus[12]. After tblastx analysis we designated our *Mbo*I fragment as 'TED-like element' or 'TLE', because of its maximum closeness towards TED.

In order to study the TLE in other ecoraces of *A. mylitta* at sequence level, polymerase chain reaction (PCR) was carried out using TLE-specific forward 5′ GATCAAATAGCTAAATTC 3′ and reverse 5′ GATCGGATATGATGG 3′ primers. The PCR yielded single product from all ecoraces. The amplified products were cloned in pCR 2.1 TOPO TA vector (Invitrogen, USA) and sequenced using M13 universal primers. Multiple sequence alignment of TLEs of different ecoraces using ClustalW showed significant homology (>98–100%; DQ69900-DQ69904)[13]. As the TLE is almost identical at nucleotide level and present in all *A. mylitta* ecoraces, it was used as probe for RFLP analysis. Genomic DNA of all nine ecoraces was completely digested with *Mbo*I, *Hin*dIII, *Cfo*I or *Eco*RI and then blotted onto nylon membrane. Andhra Local TLE labelled with P^{32} was used as probe (Figure 2 *a–d*). *Hin*dIII and *Cfo*I showed significant difference in RFLP pattern between ecoraces. Combination of both can be easily used to identify the respective ecorace. In case of *Mbo*I digestion only Modal can be identified. *Eco*RI pattern is less informative, though there is some variation. From the Southern blot (Figure 2), it is concluded that the isolated locus belong to interspersed repeat in the genome and the obtained RFLP pattern may be the result of independent mutation in the different copies of the gene due to rele-

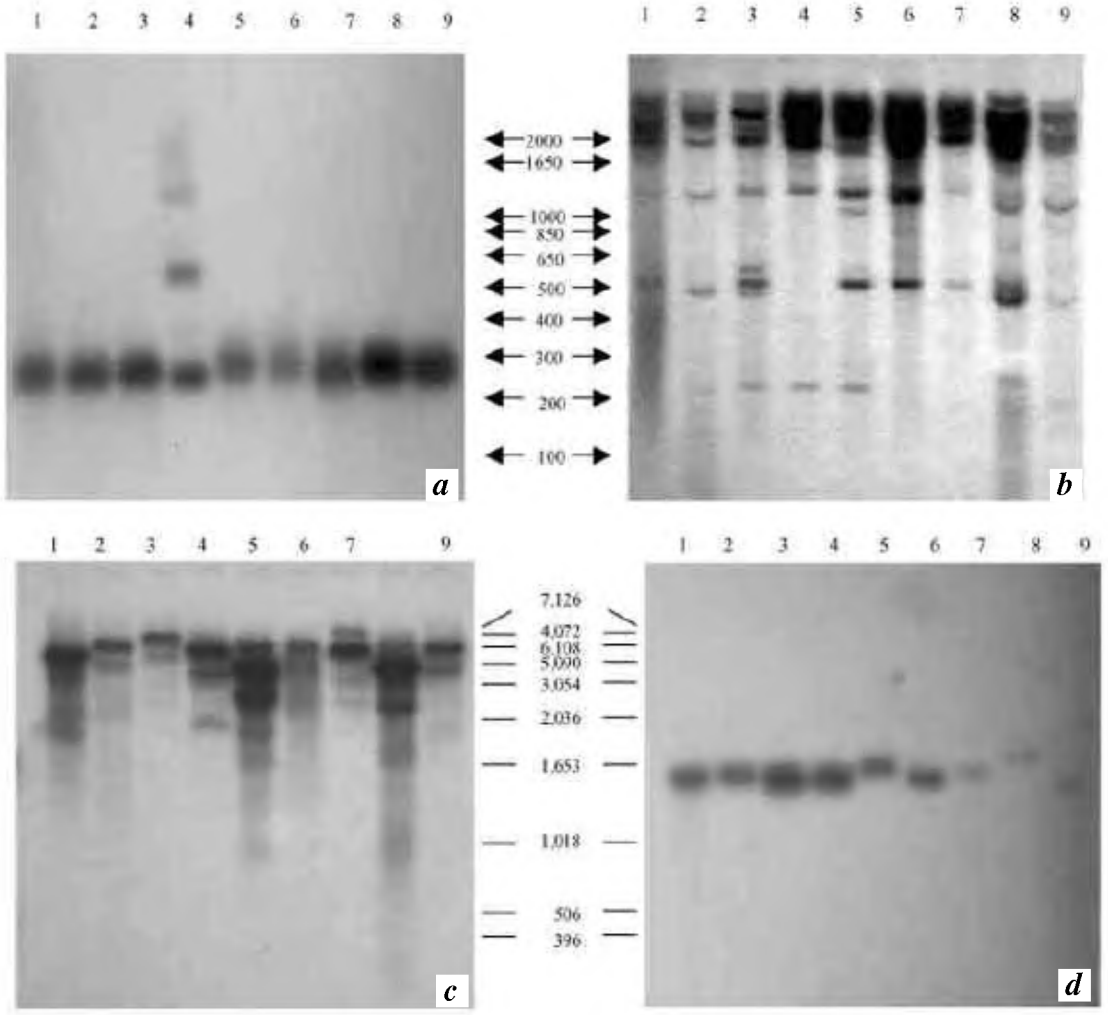

Figure 2. Southern blot analysis of total genomic DNA of different ecoraces of *A. mylitta*. Twenty micrograms of DNA was digested completely with (***a***) *Mbo*I, (***b***) *Hin*dIII, (***c***) *Cfo*I and (***d***) *Eco*RI restriction enzymes and hybridized with 281 bp of *Mbo*I fragment of Andhra Local. Lanes 1 to 9 show the digested genomic DNA of Andhra Local, Bhandara Local, Daba, Modal, Nalia, Raily, Sarihan, Sukinda and Tera ecoraces. Numbers indicate the bp of standard molecular weight marker.

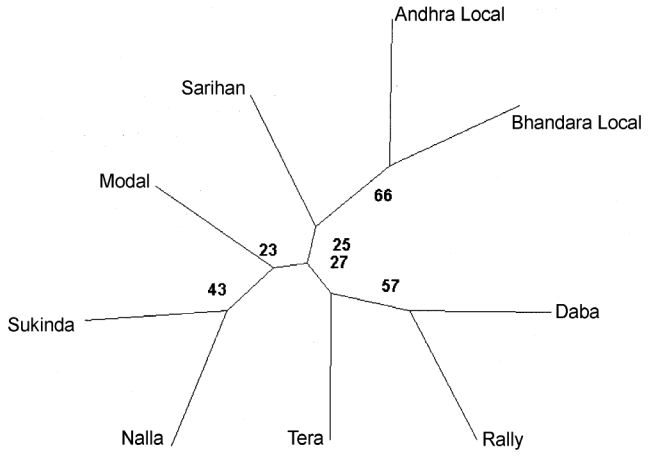

Figure 3. A heuristic parsimony analysis of *Mbo*I repetitive fragment between the ecoraces of tasar silkworm was carried out using PAUP*4.01. Maximum parsimony analysis yielded a single tree with a length of 59, CI = 0.644 and RI = 0.447. Values at nodes refer to the number of times the node occurred in 1000 bootstrap replicates.

vant selection pressure. This element can be used as DNA marker for assessing the genetic differentiation of stocks/populations[14].

Phylogenetic relation between ecoraces was determined[15] based on RFLP by constructing Maximum Parsimony (MP) tree with bootstrap values of 1000 replicates using PAUP* version 4.0b 10. The fragments that appeared on autoradiogram were scored as diallelic for each assigned locus (1 = band present; 0 = band absent), compiled into single matrix across the nine (ecoraces) and considered as input file. Phylogeny analysis indicates that the ecoraces clustered mostly according to their morphological characters and follow the geographical distribution (Figure 3). The Daba ecorace clusters with Raily, Modal with Nalia and Andhra Local with Bhandara Local and Sarihan. Their relationships are consistent with a neighbourhood structure of randomly mating population and the geographically closely situated populations tend to be genetically more similar.

It can be concluded from this study that the 281 bp *Mbo*I-digested genomic DNA segment can be used as an RFLP marker to distinguish the closely related ecoraces of tropical tasar silk-producing insect *A. mylitta* D.

1. Sinha, A. K., Sinha, R. K., Goel, A. K., Sinha, B. R. R. P. and Thangavelu, K., A review on the breeding and genetic aspects of tropical tasar silkworm, *Antheraea mylitta*. D. In Proc. of the conference on cytology and genetics, 1994, vol. 4, pp. 7–16.
2. Yasukochi, Y., A dense genetic map of the silkworm, *Bombyx mori*, covering all chromosomes based on 1018 molecular markers. *Genetics*, 1998, **150**, 1513–1525.
3. Tan, Y. D. *et al.*, An amplified fragment length polymorphism map of the silkworm. *Genetics*, 2001, **157**, 1277–1284.
4. Reddy, K. D., Abraham, E. G. and Nagaraju, J., Microsatellites of the silkworm, *Bombyx mori*: abundance, polymorphism and strain characterization. *Genome*, 1999, **42**, 1057–1065.
5. Nagaraju, J., Kathirvel, M., Muthulakshmi, M., Subbiah, E. V. and Kumar, L. D., FISSR–PCR: a simple and sensitive assay for high throughput genotyping and genetic mapping. *Mol. Cell. Probes*, 2002, **16**, 67–72.
6. Cheng, T. C. *et al.*, Mining single nucleotide polymorphisms from EST data of silkworm, *Bombyx mori*, inbred strain Dazao. *Insect Biochem. Mol. Biol.*, 2004, **34**, 523–530.
7. Prasad, M. D. and Nagaraju, J., A comparative phylogenetic analysis of full-length *mariner* elements isolated from the Indian tasar silkmoth, *Antheraea mylitta* (Lepidoptera: Saturniidae). *J. Biosci.*, 2003, **28**, 443–453.
8. Datta, A., Ghosh, A. K. and Kundu, S. C., Differential expression of fibroin gene in developmental stages of silkworm *A. mylitta* (Saturniidae). *Comp. Biochem. Physiol. Part B*, 2001, **129**, 197–204.
9. Padhi, B. K., Ghosh, S. K. and Mandal, R. K., Characterization of *Mbo*I satellites in *Cirrhina mrigala* and *Clarias batrachus* (Pisces). *Genome*, 1998, **41**, 34–39.
10. Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001, 3rd edn.
11. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J., Gapped BLAST and PSI–BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, 1997, **25**, 3389–3402.
12. Rohrmann, G. F. and Karplus, P. A., Relatedness of baculovirus and gypsy retrotransposon envelope proteins. *BMC Evol. Biol.*, 2001, **1**, 1.
13. Thompson, J. D., Higgins, D. G. and Gibson, T. J., ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 1994, **22**, 4673–4680.
14. Wright, J. M., DNA fingerprinting in fishes. In *Biochemistry and Molecular Biology of Fishes* (eds Hochachka, P. and Mommsen, T.), Elsevier, Amsterdam, 1993, vol. 2, pp. 57–91.
15. Swofford, D. L., PAUP* Phylogenetic Analysis Using Parsimony (* and other methods), Ver. 4.0b 10. Sinauer Associates, Sunderland, Massachusetts, 2003.

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Effect of carbon source concentration and culture duration on retrievability of bacteria from certain estuarine, coastal and offshore areas around peninsular India

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Improved culture-based approaches efficiently compliment the limitations encountered in molecular methods to delineate the diversity and functions of uncultured organisms. The present study endeavours to reduce the anomaly between total counts (TC) and retrievable plate counts as colony forming units (CFU). Retrievability was improved by decreasing nutrient concentration in the culture medium. Maximum retrievability of 10^4 CFU ml^{-1} was obtained on 0.1% nutrient strength (100% corresponds to 8 g l^{-1} nutrient broth with 1.5% agar-agar) with samples from the Bay of Bengal offshore and coastal waters. However, in the Arabian Sea, retrievability was maximum (10^2 CFU ml^{-1}) on 33 and 100% nutrient strength. By varying the nutrient concentration, retrievability could be enhanced to 24% of TC in the estuaries, 3–14% in coastal waters and 5% in offshore waters. Bacteria from relatively more dynamic estuarine systems seemed less resilient compared to the coastal and offshore populations, as they were best retrieved (10^5 CFU ml^{-1}) only on 1% nutrient strength.

Keywords: Bacterial retrievability, coastal and offshore waters, colony forming units, estuaries, nutrient strength.

MANY decades ago it was realized that nutrient concentration in the commonly used media is several fold higher than that present in the environment[1–3]. For oligotrophic environments, plate counts on nutrient-poor media are several fold higher than those obtained on conventional media[4,5]. In the last decade, a number of molecular techniques have been employed to assess the bacterial diversity independent of cultural methods. Estimates on data compiled for culturability indicate that in sea water and unpolluted estuarine water, percentage culturability can reach up to 0.1 and 3% of total counts (TC) respectively, whereas from activated sludge[6] it can go up to 15. However, studies generally reveal that 99% or more of the bacterial diversity remains uncultured and unexplored[7].

Given that the molecular approach can only tell us about the existence and potential functions of microorganisms, it is of utmost importance to be able to retrieve, cultivate and

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