Patterns of accumulation of camptothecin, an anti-cancer alkaloid in *Nothapodytes nimmoniana* Graham., in the Western Ghats, India: Implications for identifying high-yielding sources of the alkaloid

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Camptothecin, a monoterpene indole alkaloid, is regarded as one of the most promising anticancer drugs of the twenty-first century. Among the various plant sources, the highest yields of the alkaloid are reported from *Nothapodytes nimmoniana* (Icacinaceae), a small tree distributed in the Indian subcontinent. Because of the enormous demand for the chemical worldwide, there has been an indiscriminate extraction of the trees from many parts of India, especially from the Western Ghats, a mega-diversity forest range along the western coast of India. Recently the tree has been assigned a vulnerable status. In an effort to conserve the remaining populations of the species and to identify high-yielding sources of the alkaloid, attempts are being initiated in chemically profiling the species. As a first step in this direction, we have attempted to establish the general patterns of accumulation of camptothecin in *N. nimmoniana* across individuals, plant parts, plant size and sex of plants, in the Western Ghats. Individual trees with as high as 100 per cent greater camptothecin content than hitherto reported have been found. The study indicates the potentiality of further screening populations of *N. nimmoniana* to identify high-yielding sources that can be used for developing *in vitro* production systems or for establishing high-yielding clonal populations.

Keywords: Camptothecin, chemical diversity, Icacinaceae, *Nothapodytes*, patterns of accumulation.

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CAMPTOTHECIN, a monoterpene indole alkaloid, is a promising plant-based metabolite known for its anti-tumour activity[1–4]. Irinotecan and topotecan, two water-soluble derivatives of camptothecin (CPT), have been approved by the Food and Drug Administration (FDA) of the United States of America for treating colorectal and ovarian cancer[5–7]. In fact, CPT is regarded as one of the most promising anticancer drugs of the twenty-first century[8].

CPT was first isolated from a Chinese deciduous tree *Camptotheca acuminata* Decaisne (Nyssaceae)[9]. Later it was isolated from a variety of plant species including *Merriliodendron megacarpum*[10,11] and *Nothapodytes nimmoniana*[12] (family Icacinaceae), *Ophirrohiza mungos*[13] and *O. pumila*[14] (family Rubiaceae), *Eravatamia heyneana* (family Apocynaceae) and *Mostuea brunonis* (family Loganiaceae)[10]. Among these, the highest concentration of CPT (about 0.3% on a dry weight basis) has been reported from *N. nimmoniana*[12].

N. nimmoniana Graham., formerly known as *N. foetida* (Wight) Sleumer and *Mappia foetida* Meirs, is a small broad-leaved tree commonly referred to as 'Stinking Tree'. It is distributed in the warmer regions of the Indian subcontinent in southern India, Sri Lanka, parts of eastern India in Assam, and in the Himalayan foothills in north India, Myanmar and Thailand[15]. In recent years, because of the enormous demand for the chemical worldwide, there has been an indiscriminate extraction of the trees from many parts of India, especially from the Western Ghats, a mega-diversity forest range along the western coast of India[16]. It is estimated that over the last decade there has been a 20% decline of the natural population of this species in the Western Ghats[16,17]. Owing to this threat, *N. nimmoniana* has been classified as a 'vulnerable' species[16].

Against this background, recently, there has been considerable interest in India to map the populations of *N. nimmoniana*, chemically characterize populations and to identify populations/individuals with high CPT yields. High-yielding sources can be used to produce material for clonal multiplication and to develop cell lines with high CPT yield. Such approaches may help in reducing the threat to the natural populations. Chemical profiling of the species can signal 'chemical hot-spots' of the species that could lead to the conservation of elite populations and lines for further use. As a first step in this direction, we have attempted to establish the general patterns of accumulation of CPT in *N. nimmoniana* across individuals, plant parts, plant size and sex of plants in the Western Ghats. We also evaluate the consistency of the CPT yields across sampling seasons.

The study was conducted on *N. nimmoniana*, Graham. (Icacinaceae). The species shows a wide array of breeding systems including male, female, hermaphrodite, monoecious, andromonoecious, gynomonoecious and trimonoecious individuals[15]. CPT is commonly extracted from the stem bark.

Individuals of *N. nimmoniana* from three sites, viz. Sirsi, Joida and Ulvi (all in the Uttara Kannada district in the Western Ghats of Karnataka state, India) were randomly

sampled to assess the CPT content. At each site, individuals were sampled from an area of at least 5 hectares. Using a global positioning system (GPS), the latitude–longitude of the sites was recorded. The details of the sampling sites are given in Table 1. A total of 16 male and 16 female trees were sampled randomly from the Sirsi, Joida and Ulvi populations. From each tree, samples of wood, bark, root wood, root bark and matured leaf were collected. *N. nimmoniana* was also sampled from Biligiri Rangaswamy Temple Wildlife sanctuary (BR Hills) to examine the consistency in the yield of CPT across two seasons. Thirty individuals were randomly selected and labelled and samples drawn from five tissues (wood, bark, root wood, root bark and matured leaf) twice, in September (post-monsoon) 2004 and February (spring) 2005. Information on the sex of the individuals was not obtained for the BR Hills population. For each of the trees, the girth at breast height (gbh) was recorded.

All the samples were dried to constant moisture content at 60°C for 96 h in a hot air oven. The dried samples were ground to fine powder using a pestle and mortar. 0.1 g of fine tissue powder of each of the samples was extracted in 10 ml of 61% ethanol at 60°C for 3 h in a shaking water bath[3]. Initial studies relating the per cent recovery of CPT with time of incubation of the tissue in ethanol indicated an asymptote in the yield at 3 h (data not given). After cooling to room temperature, 1 ml of the extract was centrifuged at 10,000 rpm for 10 min at 10°C (HERMLE, Z-233 MK-2, Germany)[10]. The supernatant was passed through 0.2 µ filter (Tarsons, India) and analysed for CPT content using a HPLC. CPT accumulation was determined for (i) individuals from the various sites, (ii) male and female trees, (iii) plant parts including stem and root wood, stem and root bark and leaves, (iv) different size class of trees, (v) different geographical sites, and (vi) across sampling seasons.

Camptothecin was analysed by reversed phase HPLC (Supelco 516, LC-10AS, Shimadzu, Japan) on a C_{18} column (250×4.6 mm, 5 µm). The HPLC conditions were: 254 nm as the detector wavelength, 1.6 ml/min flow rate and 10 µl sample loop. The mobile phase was adjusted as follows: 40% acetonitrile and 60% water + 0.1% trifluro-acetic acid (TFA) in an isocratic mode[3,9]. A CPT (Sigma, 95% HPLC purified) standard sample was procured from Sigma Chemicals. The standard was prepared using DMSO and methanol in 1 : 50 (v/v) ratio respectively. The retention time of CPT was 3.5 min; for every five runs, the HPLC was re-standardized using the CPT standard. On an average, the coefficient of variation for the peak area for five consecutive runs of the standard CPT was 0.55%. The data were subjected to relevant statistical treatment using the Statistica version 4.0 software package.

Among the various tissues analysed, the root bark yielded the highest CPT content followed by stem bark. The average

Table 1. Sampling sites of *Nothapodytes nimmoniana* from the Western Ghats, India

Site	Latitude (°N)	Longitude (°E)	Altitude (m MSL)	Number of individuals sampled	Male	Female
Sirsi	14°36″.323	74°50″.948	679	8	4	4
Joida	15°10″.124	74°28″.910	560	18	9	9
Ulvi	15°5″.104	74°26″.815	530	6	3	3
BR Hills	14°50″.00	76°26″.00	900	30		

Geographical distance (km) between the sampling sites/populations is as follows: Sirsi to Joida = 67.9 km; Sirsi to Ulvi = 81.2 km; Sirsi to BR Hills = 343.8 km; Joida to Ulvi = 10 km; Joida to BR Hills = 412.9 km and Ulvi to BR Hills = 421.9 km.

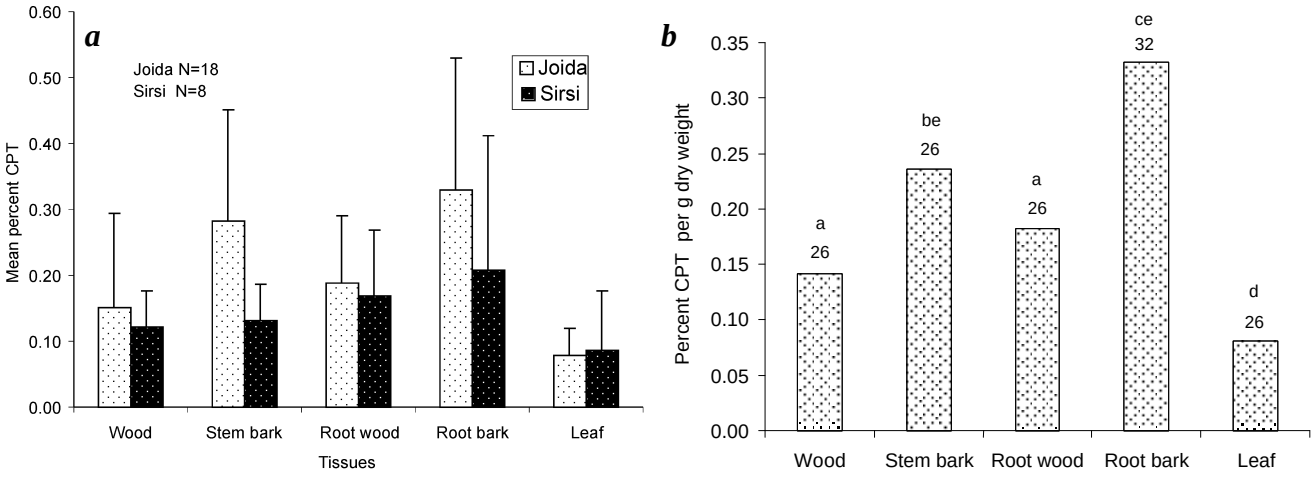

Figure 1 ***a, b.*** ***a***, Mean per cent CPT per g dry weight in different tissues of *Nothapodytes nimmoniana* from two sites in the Western Ghats; ***b***, Mean per cent CPT per g dry weight in different tissues at three sites in the Western Ghats. The numbers on the histograms indicate number of trees used in the analysis. Respective histograms with dissimilar letters indicate a significant difference in CPT content (*t* test $p < 0.05$).

Table 2. Two-way ANOVA for the CPT content (per cent CPT per g dry weight) in different tissues and trees of *Nothapodytes nimmoniana*

Source of variation	SS	df	MS	*F*	*p*-value	*F* crit
Trees	8.829	25	0.033	2.139	0.004	1.616
Tissues	0.694	4	0.174	11.189	0.000	2.463
Error	1.551	100	0.016			
Total	3.075	129				

SS = Sum of squares; df = Degrees of freedom; MS = Mean sum of squares; *p*-values ≤ 0.05 were regarded as significant.

Table 3. Two-way ANOVA for the CPT content (per cent CPT per g dry weight) in different tissues and sexes of *Nothapodytes nimmoniana*

Source of variation	SS	df	MS	*F*	*p*-value	*F* crit
Sexes	1.51E-06	1	1.507E-06	7.90E-05	0.99	3.92
Tissues	6.94E-01	4	1.735E-01	9.09E+00	0.00	2.44
Interaction (sexes × tissues)	9.07E-02	4	2.266E-02	1.19E+00	0.32	2.44
Within	2.29E+00	120	1.908E-02			
Total	3.07E+00	129				

SS = Sum of squares; df = Degrees of freedom; MS = Mean sum of squares; *p*-values ≤ 0.05 were regarded as significant.

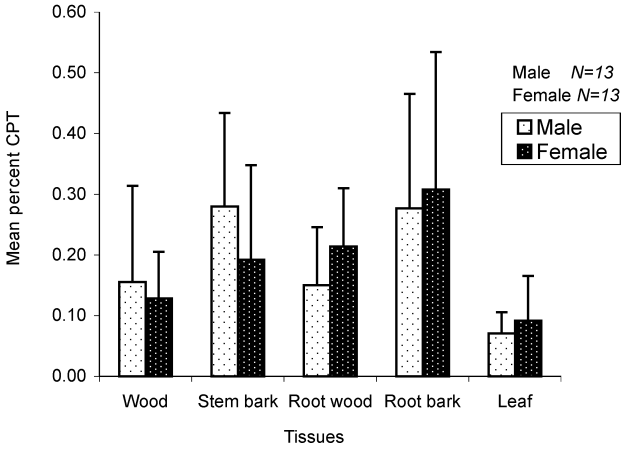

Figure 2. Mean per cent CPT per g dry weight in different tissues in male and female trees of *Nothapodytes nimmoniana*. Data for trees were pooled over two sites, Joida and Sirsi, in the Western Ghats.

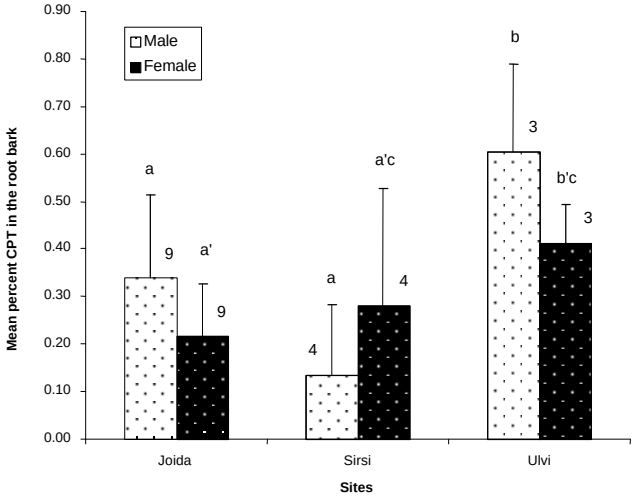

Figure 3. Mean per cent CPT per g dry weight in root bark of male and female trees from three sites in the Western Ghats. The numbers on the histograms indicate number of trees used in the analysis. Respective histograms with dissimilar letters indicate a significant difference in CPT content (*t* test $p < 0.05$).

content of CPT in the root bark was $0.333 \pm 0.213\%$ followed by $0.236 \pm 0.158\%$ in the stem bark. The root wood and the stem wood had respectively 0.182% and 0.142%. The leaves yielded the lowest CPT content of $0.081 \pm 0.057\%$. The pattern was consistent across individuals, sex and geographical sites of collection (Figures 1 and 2; Table 2). Seeds yielded $0.179 \pm 0.071\%$ CPT. Analysis of CPT in two-year-old seedlings indicated highest yields in root tips (0.4%) followed by matured (0.21%) and young (0.205%) leaves, wood (0.20%) and bark and root (0.16%).

Over sites, there was no difference in the CPT content between the male and female trees (Figure 2 and Table 3), though within sites, at Joida and Ulvi, the male trees had significantly higher CPT content compared to females, while

Table 4. Correlation of CPT content (per cent CPT per g dry weight) between different tissues

	Wood	Bark	Rwood	Rbark	Leaf
Wood	1.000	0.225	0.183	– 0.116	0.179
Bark		1.000	0.186	**0.483**	0.051
Rwood			1.000	**0.469**	0.295
Rbark				1.000	0.011

Correlation analysis was performed over 26 trees from two sites, Sirsi and Joida in the Western Ghats. Values in bold indicate significance at $p < 0.05$. Rbark = root bark. Rwood = root wood.

at Sirsi, the females had a significantly higher CPT content compared to the males (Figure 3). In both the sexes, the root bark (0.277 ± 0.189% in male and 0.308 ± 0.226% in female) had the highest CPT content followed by that in the stem bark (0.280 ± 0.154% in males and 0.192 ± 0.156% in females).

There was a significant variation in the CPT content among individuals collected from the three sites. For example, the mean CPT content of root bark of male trees (0.605 ± 0.183%) from Ulvi was significantly greater than that for the male trees in Sirsi or Joida (0.339 ± 0.175% in Sirsi and 0.135 ± 0.157% in Joida) (Figure 3). Similar differences were obtained for the CPT content in females across the sites.

To examine if there exists any allometric relation among the various tissues in the accumulation of CPT, we analysed the correlation of CPT content. The CPT content of root bark was significantly correlated with that of the root wood and stem bark ($r = 0.483$ and $r = 0.469$, $P < 0.05$; Table 4). Besides this, there was no significant correlation for the CPT content among the various tissues.

There was significant variation in the CPT content across the individuals sampled from the three sites (Table 2). The frequency distribution of CPT content of the trees in the root bark was positively skewed (Figure 4). The CPT content ranged from as low as 0.04% to as high as 0.775%. The differences were consistent over repeated sampling of the tissues.

CPT content of stem and root bark was significantly correlated across the two seasons ($r = 0.52$; $P < 0.01$, $n = 30$ trees for stem bark and $r = 0.58$, $P < 0.01$, $n = 30$ trees for root bark; Figures 5 and 6). CPT content was not correlated with the size class of trees (Figure 7).

With the increased demand for camptothecin and its derived drugs, there has been an increasing pressure on naturally existing populations of *N. nimmoniana* from several parts of the country, especially from the Western Ghats[15–17]. Unfortunately because most of the extraction is illicit, there is little information on the magnitude of extraction. Because almost always the entire tree is felled, there is a serious threat to the species and its reproductive turnover.

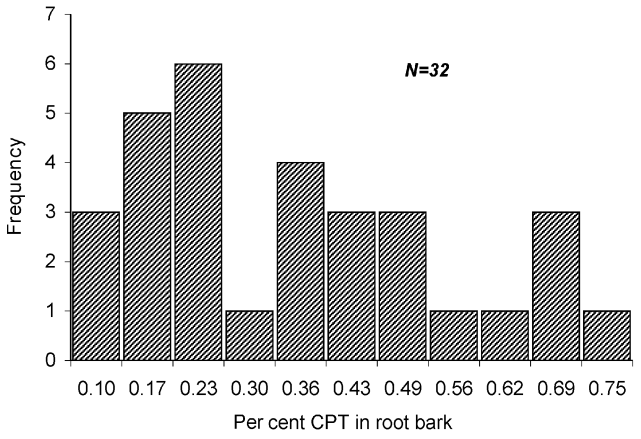

Figure 4. Frequency distribution of CPT (per cent CPT per g dry weight) content in the root bark of *Nothapodytes nimmoniana* trees from three different sites in the Western Ghats.

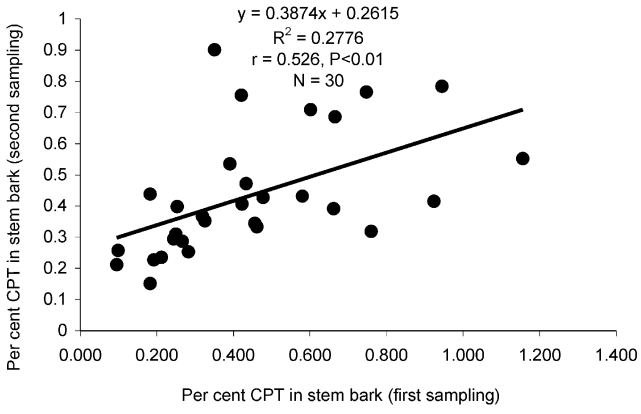

Figure 5. Correlation of per cent CPT in stem bark of *Nothapodytes nimmoniana* between two independent samplings (first sampling – September 2004 and second sampling – February 2005) at BR Hills, Western Ghats, India.

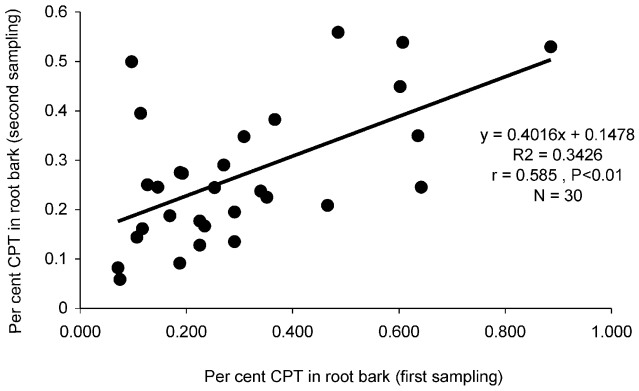

Figure 6. Correlation of per cent CPT in root bark of *Nothapodytes nimmoniana* between two independent samplings (first sampling – September 2004 and second sampling – February 2005) at BR Hills, Western Ghats, India.

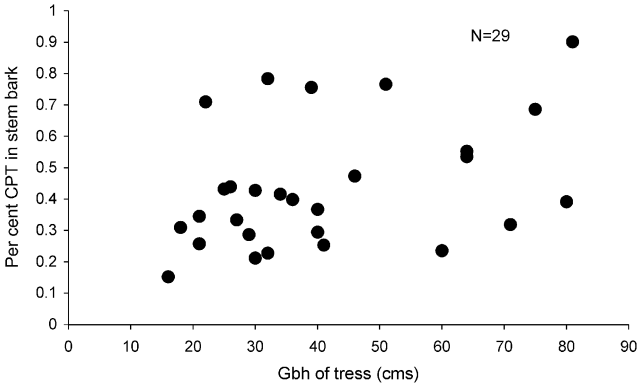

Figure 7. Correlation of gbh (cm) of stems and per cent CPT content in the stem bark of *Nothapodytes nimmoniana* at BR Hills, Western Ghats, India.

In the wake of these threats, it has been realized that efforts should be initiated to conserve and offer protocols for a sustained and managed utilization of the tree. A major initiative in this regard is to identify high-yielding sources of CPT such that these trees can be used as sources for either clonal multiplication or for developing *in vitro* production systems. Towards this direction few studies have been reported from India[18–24].

The present study was designed with the ultimate objective of mapping the populations of *N. nimmoniana* in the Western Ghats, and identifying high-yielding sources of CPT. Our study has reaffirmed the finding of Govindachari and Viswanathan[12] who reported highest yields of CPT from roots. Traditionally, CPT has been extracted from root, root bark and fruits. Fairly good amounts (0.10%) of the alkaloid are also reported from the seeds[25]. However, in *C. acuminata*, from where CPT was first extracted, CPT content was highest in leaves. Further still, CPT content was at least 10-folds higher in younger leaves than in older leaves[3]. The high concentration of CPT in the leaves has reportedly led to the poisoning of goats that browse on the leaves and even the honey bees foraging on the floral rewards[3]. However in trees of *N. nimmoniana*, the leaves contained very little CPT, though in the seedlings the CPT content in the leaves was as high as that in the wood. It is likely that CPT is synthesized in the leaves and because of its toxicity sequestered in the old and dead tissues. Roja and Heble[24] reported about 0.075% CPT in shoots of mature trees.

The relatively high level of CPT in the root bark was also reflected by the contents in the root wood and stem bark but not in the leaves (Table 3). There were no consistent differences in the CPT content between the sexes; in fact a pooled analysis over the sites indicated no significant differences between male and female trees. However significant differences in the CPT content were noticed across individuals and across the sites of collection. Individuals from Ulvi had the highest average CPT content ($n = 6$, CPT = $0.508 \pm 0.16\%$). The differences among the sites could not be attributed to either the geographical location (latitude) or the altitudinal differences. It is likely that the differences in the levels of CPT are a function of both the genetic and environmental backgrounds of the population. Few trees yielded about 0.775% amounting to about 100% greater CPT content than is currently reported. Our studies have indicated a fairly reliable degree of consistency between independent sampling and estimation. The CPT yields were relatively consistent across independent samplings over two seasons. Besides, the CPT yields were largely unaffected by the size class of stems beyond 16 cm gbh, a result that seems to be confirmed by the findings of Li *et al.*[26] in *C. acuminata*. Our results hold important implications for chemically profiling the populations of the species in the larger landscape of the Western Ghats in search of 'chemical hot-spots' of CPT. Our studies indicate the promising potentiality of exploring the natural variability in CPT content among individuals across a wider geographical range and identifying high-yielding lines. Individuals with high CPT content could be a potential source for raising plantations of high CPT yielding trees as a part of agro forestry systems without disturbing the natural populations[27]. In fact, driven by the huge demand for CPT, several captive plantations of the tree have already been initiated in the states of Karnataka and Tamil Nadu. Identification of high-yielding trees and their clonal multiplication could facilitate the cultivation of such trees in the plantations. Finally, the identified high-yielding trees could be used in developing cell lines and other *in vitro* production systems from which CPT could be commercially harvested.

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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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