Evaluation of intra-specific variability in *Avicennia marina* Forsk. using RAPD markers

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Random amplified polymorphic DNA (RAPD) markers for determining the genetic diversity among Mangrove species, *Avicennia marina* Forsk. were used. Individuals collected from several sites in India were analysed for their genetic relationship. Both inter- and intra-populational level polymorphisms were observed. The dendrogram arising out of the RAPD data shows prominent patterns of relationships that are discussed in the light of the respective habitats of the species distributed in India. The usefulness of RAPDs in biodiversity assessment and evaluation has therefore been tested.

UNDERSTANDING the genetic basis of diversity of plants helps utilize plant genetic resources effectively. Because of the long-term life cycle, tree genetics tends to receive less attention than annual crops. Among tree species, mangrove forest trees have not so far been subjected to detailed genetic analysis. Mangroves form an important group of plants for several of their characteristics, including their ability to withstand high levels of salinity, besides protecting coastal areas from seawater intrusion[1]. However, this group has suffered from lack of information on their genetics. They are distributed all over tropics and 59 species belonging to 29 families occur in India. Mangrove habitats now face serious threat due to extensive felling, changing coastal environment including lack of freshwater inflow[2]. The performance of each species differs in such an environment and differences are apparent[3]. Conservation of mangroves is thus a major priority in coastal area development. Of several cosmopolitan species of mangroves, *Avicennia marina* is an important species because it can withstand varying soil conditions, high levels of salinity, lack of freshwater inflow for considerable periods of time and pollution of varying forms[4]. Besides they have good rates of regeneration and show different adaptational features. Genetic characterization of mangroves is unfortunately limited owing to lack of concerted studies on identifying the genetic variability within and in-between species. There is no report on the genetic diversity of mangroves except for *Rhizophora* species[5]. Though isozyme studies have been carried out in mangroves their reproducibility is rather poor.

Genetic analysis of tree species, especially of forest trees, has not received much attention from molecular biologists until recently. Relatively few genetic maps are available for forest trees[6–8]. This is mainly due to lack of suitable pedigrees and basic genetic data like number of chromosomes, linkage groups and even population genetic analysis.

Mangroves are threatened all over the world today due to direct and indirect exploitation. Conservation of mangroves has thus become important not only to protect the coastal areas but also to protect the coastal communities from seawater intrusion and from the adverse impacts of potential changes in sea level rise. One of the suggestions for mangrove restoration is to select genetically superior germplasm and to introduce them into target sites[9]. But for this to happen it is important to understand the extent of diversity in individual species and the relationship these species have with their respective habitats. Diversities in structural formations of mangrove ecosystems can be witnessed along the latitudinal and longitudinal gradients. This may be due to the climate or soil or due to water availability[10]. Thus there is a necessity to evaluate the genetic diversity of these trees.

Random amplified polymorphic DNA (RAPD)[11] markers have been successfully used to assess the genetic diversity within several groups of plants, including tree species like conifers, Norway spruce, pine and others[12–14]. Besides this, RAPDs were also used for several other purposes like genome mapping, population genetics, introgression analysis[15]. RAPDs are better choice of markers for evaluation of genetic diversity in species where cloned markers are not available. Hence RAPD analysis was undertaken in mangrove species, *A. marina* which showed clear inter- and intra-populational variability from the collections made in India. The sites selected were from different habitats ranging from unpolluted Pichavaram to polluted sites like Ennore with petrochemical pollution and Adyar with inorganic pollution. Duplicate samples were collected from Ennore and Bombay so that they can represent the trueness of the genetic analysis. Genetic correlations and genetic distance analysis were done using RAPD data to relate them with the habitat and growth.

A. marina accessions were collected from different sites in India (from Bombay, Ganapatipule in Maharashtra, Pichavaram in Tamil Nadu, Ennore and Adyar in Madras). Seven accessions representing five locations were chosen to see if the RAPD markers were useful to study their genetic diversity. For detecting the variations a total of 25 primers were initially used, out of which 12 gave diagnostic polymorphisms of inter- and intra-populational variability.

Total genomic DNA was isolated from lyophilized leaf material. To 300–400 mg of powdered leaf tissue, 1000 µl of extraction buffer (1 M Tris-HCl, pH 8.0, 5 M NaCl, 0.5 M EDTA, 14 β mercaptoethanol, and 1% CTAB) was added and incubated at 65°C for 90 min with occasional mixing. The mixture was then cooled

to room temperature and 500 µl of chloroform : octonol (24 : 1) was added. After centrifuging at 10,000 rpm at 4°C for 10 min the supernatant was transferred to a fresh tube. To this 750 µl of ice-cold isopropanol was added. The DNA was washed with 500 µl of 70% ethanol and 750 µl of 3 M sodium acetate and again with 70% ethanol. The DNA was then air-dried and suspended in 1X TE buffer.

Decamer oligonucleotide primers were used in this study. Table 1 lists primers that were selected for the final analysis. Amplifications were done in volumes of 25 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each of dATP, dTTP, dGTP, and dCTP (Perkin Elmer, USA), 5 ng of genomic DNA, 0.5 µM of primer and 3 units of *Taq* DNA polymerase (Perkin Elmer, USA). The amplification was carried out on a Perkin Elmer 480 thermal cycler (Perkin Elmer, USA). Samples were amplified at one step of 2 min at 94°C, 40 repeats of the following cycles—one min at 94°C, one min at 37°C, two min at 72°C, and then a final extension time of 4 min at 72°C. The amplified products were then analysed on 1.8% agarose gels stained with ethidium bromide.

For each primer, bands were scored for their presence (+) or absence (–) of a amplified product. From these data a simple matching coefficient (SMC) analysis was carried out on a GENSTAT 5 program (licensed to Lawes Agricultural Institute, UK) to analyse the genetic distance and genetic relatedness. SMC takes into account the entire amplification products from various genotypes under study against all the primers scored for. Thus weightage is given as a shared presence or absence of bands also in calculating similarities and distance of different genotypes.

Table 1. List of amplification products generated by using arbitrary 10 base oligonucleotides, based on all accessions

Primer	Sequence	No. of fragments detected: within *A marina*	No. of fragments detected: between *A marina*
1.	3′ GTTTCGCTCC 5′	35	19
2.	3′ TGATCCCTGG 5′	14	14
3.	3′ CATCCCCCTG 5′	27	16
4.	3′ GGACTGGAGT 5′	22	13
5	3′ TGCGCCCTTC 5′	21	12
6.	3′ CCATTCCGAG 5′	2	2
7.	3′ CACCTGCTGA 5′	17	4
8.	3′ GTGAATGCGC 5′	4	1
9.	3′ CCTACACGGT 5′	15	5
10.	3′ CAGCCGAGAA 5′	11	3
11.	3′ GAAGGAGGCA 5′	21	8
12.	3′ TGAACCGAGG 5′	16	6

Isolation of DNA from Mangrove species has been a difficult problem until now. This is because they contain a number of secondary metabolites like phenolics, terpenoids, alkoloids and mucilage. The DNA extraction protocol followed here solved this problem. Of the 25 primers screened, 12 were found to be suitable for diagnosis. Amplified products could be detected from all primers used. A total of 205 amplified products were scored. Within an individual plant the primers detected RAPD patterns containing DNA bands within 0.1 to 2.3 kb.

Except for primer 4 (Table 1) all the others gave good variability profiles. Primer 4 was not able to detect

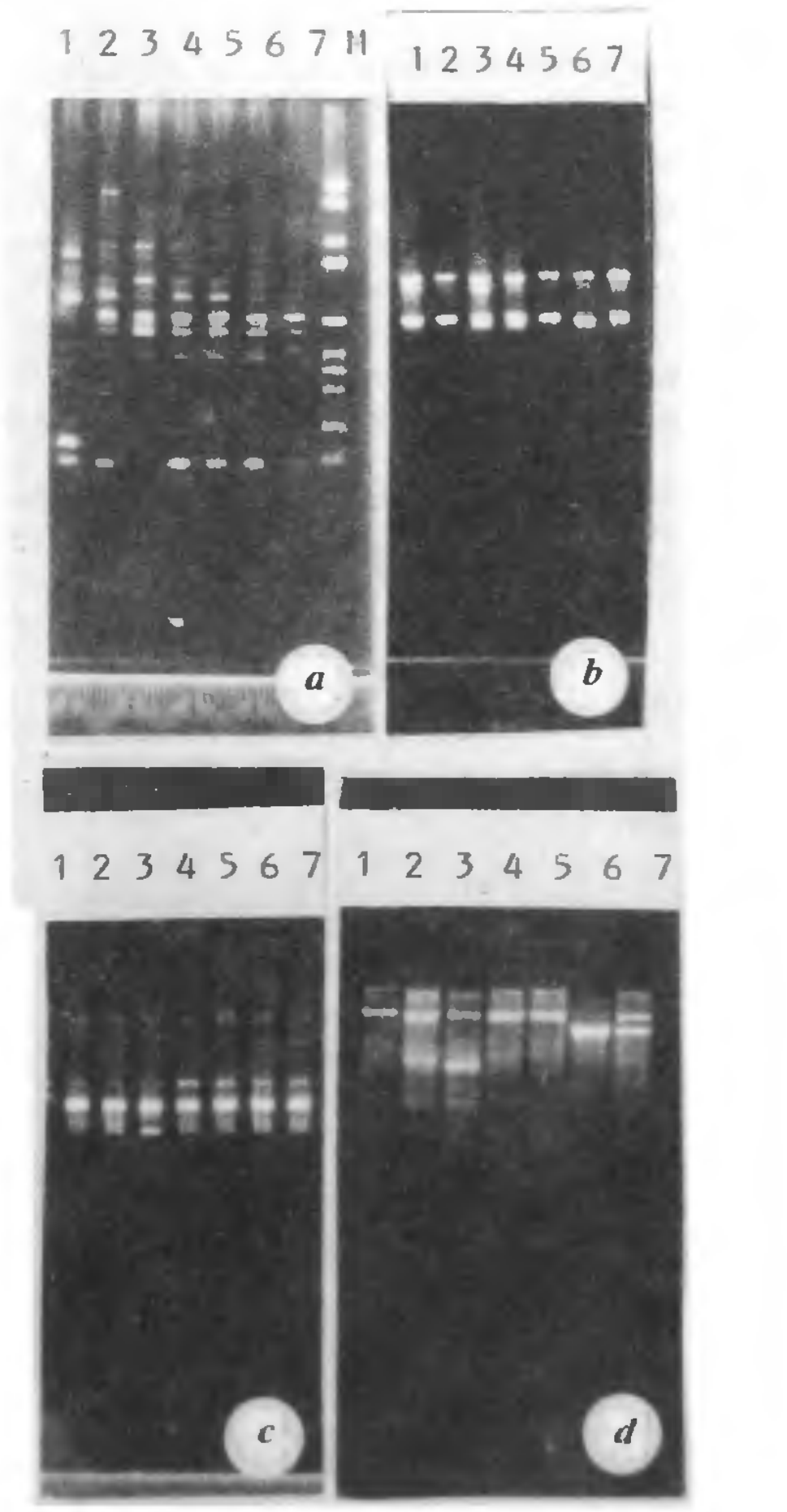

Figure 1. Example of amplified products from *A marina* populations collected from: Lanes 1 & 2, Bombay, 3, Ganapatipule; 4, Pichavaram, 5, Adyar, 6 & 7, Ennore *a*, Amplified products using primer 3′ GTTTCGCTCG 5′; *b*, Amplified products using primer 3′ TGATCCCTGG 5′; *c*, Amplified products using primer 3′ CATCCCCCTG 5′, *d*, Amplified products using primer 3′ GGACTCCAGT 5′.

any variation within the samples. This polymorphism was apparent with all the primers and was not only between samples but also within the samples collected from different sites (Figure 1). The dendrogram constructed based on the SMC analysis and represented in Figure 2 distributes the genotypes into different clusters. The amount of variability detected when the DNA sample was subjected to repeated RAPD analysis was the same, confirming the reproducibility of the analysis.

The two sites selected each in Ennore and Bombay showed considerable variations (Figure 1). All the primers except primers 4 and 8 detected variations within these sites and the dendrograms showed their relatedness (Figure 2). Genetic distance map was also constructed based on the RAPD data using SMC (Figure 3) to identify the distances. It was found that all the genotypes were distinctively different and separate based on amplified products.

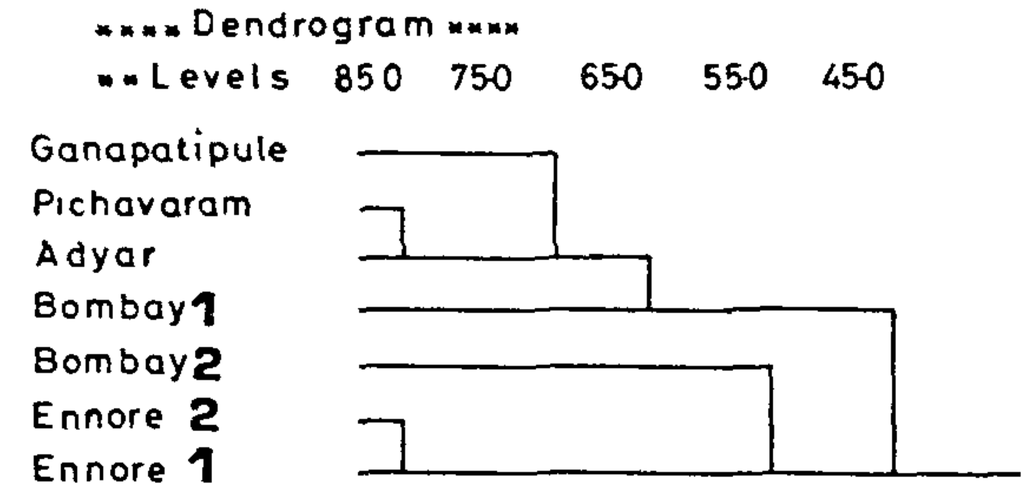

Figure 2. Dendrogram showing genetic relationships between *A marina* populations constructed using simple matching coefficient analysis based on RAPD analysis.

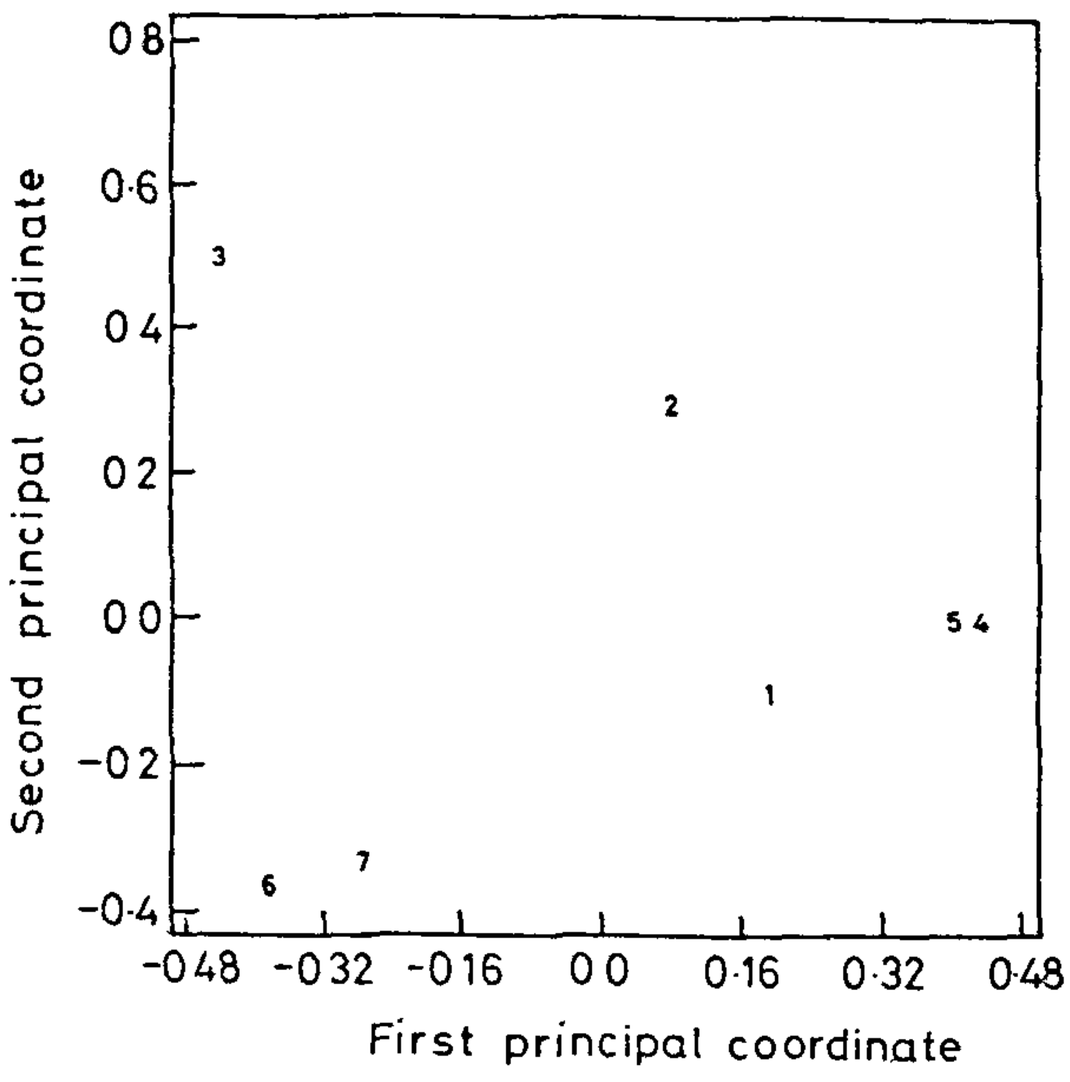

Figure 3. Genetic distance analysis of *A marina* revealed by RAPD markers and analysed using simple matching coefficient analysis 1 & 2, Populations from Bombay; 3, Ganapatipule; 4, Pichavaram; 5, Adyar, 6 & 7, Ennore

With an aim to understand the genetic diversity and the relatedness of mangrove species, *A. marina* was taken as a model. This is because of its several and varied adaptational and distributional patterns. RAPD was analysed on seven populations and the variability patterns were scored on the basis of amplified products. The analysis was carried out not only on a pairwise combination of variability but also accounted for the entire information given by all the genotypes against all the primers used. This comparison is based on the presence or absence of a band (amplified product) and give weightage as a shared presence in calculating similarities[16].

The distribution of the genotypes observed on the dendrogram is represented in Figure 2 and shows that the RAPD data can be a powerful tool in assessing the genetic diversity. With regard to intra-site variability, there is variability within *A. marina* collected from the same sites. This is surprising because the species have evolved from the same habitats for a long period of time. The frequent inundation of seawater might have helped cross pollination to account for this polymorphism. Though the dendrogram is quite revealing the groups are considered different with respect to sites. The similarity between the material collected from Bombay and Ennore may be due to similarities in soil conditions like hypersalinity and the type of pollution[17]. Ganapatipule and Pichavaram are considered to be reasonably less polluted and also received copious amounts of freshwater inflows, while Adyar falls between these two groups. This relatedness is also established in the genetic distance and similarity analyses.

The RAPD technique contributes to a better understanding of genetic characterization than the analysis of isozymes. For developing conservation methodologies it is important to evaluate the genetic diversity within a given species. Intra-populational level variation helps to identify suitable genetic material also. This kind of data *vis-à-vis* the other physiological, edaphic factors are taken into account when identifying suitable clones for introduction into different areas. Also, this study proves the utility of RAPDs in biodiversity measurement.

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Carbon and oxygen isotope trends in late Precambrian–Cambrian carbonates from the Lesser Himalaya, India

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$\delta^{13}C$ and $\delta^{18}O$ records of the Late Precambrian–Cambrian (Pc–C) carbonates from the Lesser Himalaya are reported here. The data depict two distinct cycles of ^{13}C maxima–minima and one distinct ^{18}O maxima for these carbonates. We suggest that isotopic variations across Pc–C stages relate to marked changes in the carbon and oxygen fluxes.

CARBON isotope signatures of the Precambrian–Cambrian boundary carbonates have been studied from a number of localities of the world[1–6], but their implications are still controversial. The preservation of original $\delta^{18}O$ records[3] in Pc–C boundary carbonates has also been questioned. We present here the result of a carbon and oxygen isotope study of two marine carbonate successions of Late Precambrian–Cambrian age from the Lesser Himalaya. The isotopic data are evolved in relation to sedimentary carbon budget and atmospheric oxygen level (?) changes etc.

In the northwestern Himalaya, two major carbonate-bearing successions, namely, the Deoban Formation of Lower to Middle Riphean age and the Krol Formation of Late Vendian (Ediacaran) age are present[7–9] (Figure 1). The Deoban Formation contains an approximately 1000 m thick succession of carbonate rocks (stromatolitic dolomites, dolomitic limestones, cherty limestones an oolitic limestones) with intercalated beds of shales. It is followed by a shallow marine sequence of the argillo-siliciclastic deposits known as the Simla/Jaunsar Group which comprises of Mandhali, Chandpur and Nagthat formations. The Simla/Jaunsar Group is overlain by the Blaini Formation of early Vendian (Varanginian) age, consisting of diamictite beds and minor deposits of microbial dolomites, siltstones and shales. The Krol Formation of Vendian age includes an ~2000 m thick succession of stromatolitic dolomites, cherty limestones, shales, sedimentary breccia, oolites and grainstones. It

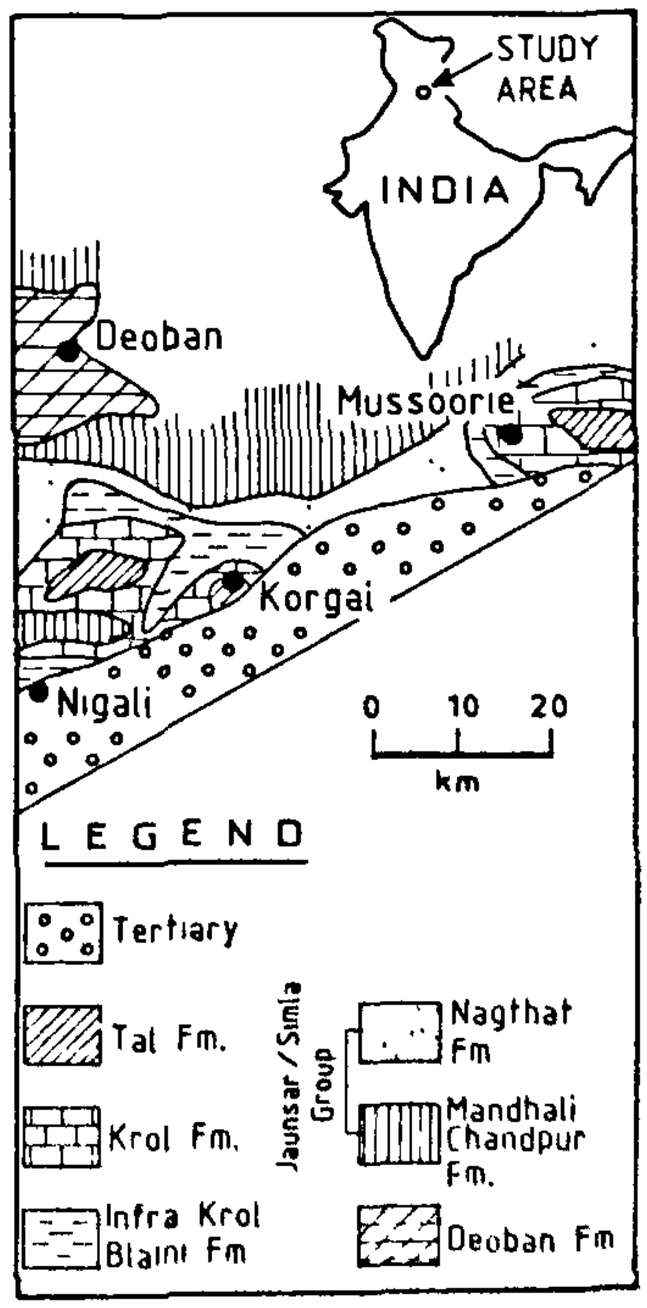

Figure 1. Geological sketch of the Deoban, Blaini and Krol-Tal sedimentary succession of the Lesser Himalaya, showing location of the study area (map after Chaudhri and Kalia[22])