Genetic variation in *Taxus baccata* (Himalayan yew) of central Nepal

Khem Raj Bhattarai[1], H. Hertel[2] and B. N. Prasad[3]

The present work was carried out to find genetic variation in different populations of *Taxus baccata* of central Nepal by isozyme analysis. This variation has been drawn on the basis of collection of fifty-eight different individual trees growing in different populations at central Nepal. Thirteen different enzyme systems were tested using horizontal starch gel and vertical polyacrylamide gel electrophoresis from its needles interpopulation heterozygosity was higher than within population. Highest genepool diversity was observed in Shivapuri population of Kathmandu Valley.

Keywords : *Taxus baccata*, electrophoresis, enzyme system, genetic diversity

Taxus baccata is one of the endangered trees of family - Taxaceae. It distributes widely from Europe to Asia. In Nepal, it sparcely occurs from east to west between 2000 to 2700 m. mostly associated with *Rhododendron spp., Tsuga dumosa, Abies spectabilis, Cedrus deodara, Picea smithiana and Betula alnoides.* Medicinal importance of this species is well known as a source of taxol, a novel diterpenoid which was first isolated from stem bark of *Taxus brevifolia* (Wani *et al.* 1971). Needle, stem and bark of several species of *Taxus spp.* including *T. baccta* have been reported to contain taxol and 10-deacetylbaccatin III (Witherup *et al.* 1990). Haphazard collection of this species in Himalaya to extract taxol have resulted a serious threats (Sarin, 1993). Thus it is urgent to characterise natural declining stands of *Taxus* of Nepal Himalaya by suitable markers to identify valuable gene for conservation.

Genetic variation refers to condition of heterogeneity. As single gene is unlikely to control adptability of individuals or even population to a changing environment the presence of different genetic variants in population must be responsible for their capacity to adapt changing environments (Hattmer 1991). Genetic variation has been found to be crucial for survival of plant population. A decline of this resource in forest has recently been observed (Scholz *et al*, 1989). Because of coincidence of temporal changes in environment and longevity, widespread genetic variation had to be expected in trees (Gregorius *et al.* 1979, Gregorius 1989). Trees like *Taxus* which grow very slow and live up to 2000–3000 years, variation can be observed even more as compared to short living trees.

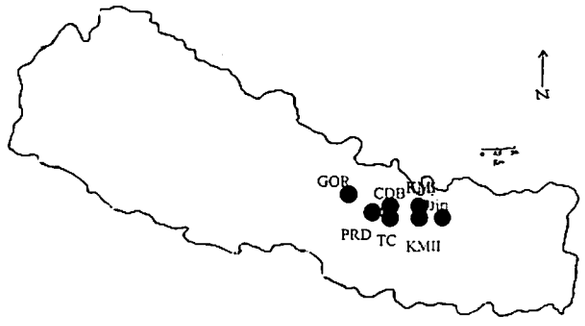
Isozyme analysis are presently most widely-used for study of forest plant genetics. They are independent of environmental factors and traits are mostly codominantly expressed in heterozygous genotypes (Hertel 1997). Isozymes provide a method for assaying levels of genetic variation in natural population, genetic markers for estimating mating systems, seed dispersal system, natural history of trees (Loveless 1992). On the other hand many indivdual trees possess genotype which can provide sufficient replicate genotype of particular location. For a few principal conifers, isozyme methods have been found quite successful to observe variation in plant population. Therefore in the present study also this method have been used to assess genetic variation present within and between some populations of *Taxus baccata* in central Nepal. This work is expected to be useful for breeding programme, for domestication and conservation of this valuable but endangered tree in Nepal.

Material and methods

Needles of *Taxus baccata* intact with 5-10 cm long twigs were collected from different natural forests and Botanical Gardens of Nepal (map). Sample collection was done in the begining of September 1998. After collection the twigs were wrapped in plastic bags and transported in fresh condition to the Laboratory of Institute of Forest Genetic and Forest Tree Breeding, Waldsieversdorf, Germany, for isozyme analysis. The collection represents two populations of Kavremahabharat forest and

[1] Department of Plant Resources, Plant Research Division Godawari, Lalitpur, Nepal
[2] Federal Research Centre for Forestry and Forest Product, Institute of Forest Tree Breeding Eberwalder Chaussee 3, D-15377 waldsieversdorf, Germany
[3] Central Department of Botany, TU, Kirtipur, Kathmandu, Nepal

Bhungkharkalek, (12 individual trees from Kavremahabharat I (KM I 1-12) and 11 individual trees from Kavremahabharat II, (KM II 1-11),) one population, five individual trees from Shivapuri Watershed area (Sh 1-5), one population of Gorkha,19 individual trees (Gor 1-19), one tree of Botanical Garden of Central Department of Botany, Tribhuvan University (CDB 1), nine different trees from arboreum and conservation section of Plant Research Division, Godawari, Lalitpur (PRD 1-9), one tree of Botanical Garden of Trichandra College (TC 1), and one population with 11 individual trees of Jiri (JR. 1-11).



Map : Collection of specimen from central Nepal

Homogenisation of needles

Needles (75-100 mg) were taken from each individual tree for extraction of protein in an already ice cooled mortar and homoginiate is made by grinding in a pestel with 300 µl extraction buffer (0.1M Tri-Borate-Buffer pH 7.4 with 1% (v/v) 2-mercaptoethanol, and 5% w/v polyvinylpyrolidone (PVP) (modified according to Lundkvist, 1974). In some cases where good banding pattern could not be observed another extraction, modifed Tris-buffer, (personal communication with Yanbaen) which contains 16% of sucrose with additional substances 1% diethyal-itheocarbamic acid, 1% PVP, 1% mercatoehanol, 0.02% NAD, 0.02% NADP, 0.02% NADH and 0.01% pyroxidol, were added to prevent degradation of protein. Sand particles were also added to facialiate grinding. Homoginiate was transferred into appendorf tube and applied for centrifugation at 5° C for 20 minutes at 15000 rpm.

Enzyme system

Both starch gel electrophoresis (SGE) separation system (Poulik 1957) and polyacrylamide gel electrophoresis (PAGE) separation system were used.

For IDH; MDH; FDH; GDH; 6PGDH, PGM and SKDH enzyme systems, Tris-Citratrate separation system was used. For ADH, LAP; PGI and MR enzyme (Ashton and Braden 1961) separation system and for MR and FLEST enzyme (Poulik 1957) separation system was used.

Enzyme visualisation

Thirteen different enzyme systems were visualised with slight modification of protocols described by Yeh and O' Malley (1980), Vallejos (1983), Cheliak and Pitel (1984).

Data analysis

Data were analysed by SAS package with modification by Thomas Stauber (1997).

Results and discussion

Seventeen alleles into eighteen gene loci were identified in thirteen enzyme systems tested. Some alleles were tested already by Thoma (1992), Lewandowski *et al,* (1992), and Hertel (1996) and some are interpreted on emperical basis. Two gene loci AAT-A and AAT-B were identifed in Aspartate aminotransferase, similarly LAP-A and LAP-B in Leuciniminopeptisae, PGI-A and PGI-B in Phpsphoglucoseisomerase while only one gene locus could be obseved in Alcoholdehydrogenase, Formatledehydrogenase, Fluoresentestarase, Gultamatedehydrogenase, Malatedehydrogenase, Phosphogluconatedehydrogenase, Phosphoglucomutase and Shikimatedehydrogenase structure of enzyme system. Gene loci, number of alleles and their banding pattern are presented in Table 1 and figure 1.

Aspartateaminotransferase (AAT) : There is no variation within and between population in locus AAT-A, it contains with homozygous genotype with allell 11. Similar result has been found by Thoma (1992) and Hertel (1996) in *Taxus baccata* growing in Germany and by Lewandowski *et al.* (1992) from *Taxus baccata* growing in Polland by isozyme analysis. While in case of AAT-B variation has been found with two different alleles 2 and 3. There is variation in AAT-B in the *Taxus* growing in Germany but allele 3 is not present (Hertel. 1996). The population of Shivapuri has variation at AAT-B, but there is no variation in the population of Kavremahabharat I and II and all population have allele 22. Similarly, no variation has been observed in population of PRD, Jiri, TC, CDB and GOR.

Table 1 : Structure of enzyme system and Geneloci of *Taxus baccata*

Enzyme system	Enzyme subunit structure	Genelocus	Number of allele	Interpretation of banding pattern
Aspartate aminotransferase (AAT)	Dimer	AAT-A	1	Emperical
		AAT-B	2	Thoma 1992, Hertel 1996
Alcohol dehydrogenase (ADH)	Dimer	ADH-B	2	Hertel, 1996
Formate dehydrogenase (FDH)	Dimer	FDH	2	Emperical
Fluorosent esterase (FLEST)	Dimer	FLEST	3	Emperical
Gultamate dehydrogenese (GDH)	Hexamer	GDH-A	1	Emperical
Isocitrate dehydrogenese (IDH)	Dimer	IDH-A	2	Emperical
		IDH-B	4	Emperical
Leucine aminopeptidase (LAP)	Monomere	LAP-A	2	Emperical
		LAP-B	1	Lewandowski *et al*, 1992, Hertel 1996
Malatedehydrogenase (MDH)	Dimer	MDH	2	Emperical
Menadione reductase (MR)	Tetramer	MR-A	2	Lewandowski *et al*, 1992 and Thoma, 1992
		MR-B	1	
6-phosphogluconate dehydrogenase (PGDH)	Dimer	PGDH	1	Lewandowski *et al*, 1992, Hertel, 1996
Phosphoglucomutase (PGM)	Monomer	PGM-A	3	Thoma 1992, Hertel, 1996
Phosphoglucose isomerase (PGI)	Dimer	PGI-A	2	Emperical
		PGI-B	4	Thoma 1992, Lewandowski *et al*, 1992, Hertel 1996
Shikimate dehydrogenase (SKDH)	Monomer	SKDH	1	Thoma 1992, Lewandowski *et al*, 1992, Hertel 1996

Alcoholdehydrogenase (ADH) : Only one genelocus ADH-B could be observed in ADH enzyme system. There is variation with two different alleles 1 and 3, but 3 alleles 1, 2 and 3 were reported by Hartel (1996) from *Taxus baccata* growing in Germany, where as allele 2 has not been found Hialayan yew.

Formaledehydrogenase (FDH) : Two different alleles 1 and 2 were observed in with FDH enzyme system. The population of Shivapuri and Jiri is polymorphic with two different alleles 1 and 2 while population of PRD is hemozygous with alleles 22.

Fluorosentesterase (FLEST) : Only one genelocus have been observed in FLEST enzyme system. Polymorphic with three different alleles 1, 2 and 3 have been observed in this system. The population within Shivapuri and Jiri have variation but there is no variation within KM II, & PRD with FLEST enzyme system. The genelocus in FLEST is not tested yet by any authors.

Gultamatedehydrogenase (GDH) : There is no variation in GDH enzyme system only one allele 1 with monomorphic 11 is present in studied population but the *Taxus* growing in Germany is not tested with GDH yet by any authors.

Isocitratedehydrogenase (IDH) : Two gene loci IDH-A and IDH-B have been observed with polymorphic alleles. Only Shivapuri and KM I population is found varying while no variation in rest populations at IDH-A. Similarly, Shivapuri and KM I have been found varying while no variation is found in the rest of population at IDH-B. This enzyme system is not comparable with *Taxus baccata* growing in Germany, as it is not tested yet.

Leucineaminopeptidase (LAP) : Two geneloci LAP-A and LAP-B have been found in LAP enzyme system. Locus LAP-A is new for Nepali material it is not reported in the material of Germany (Hertel 1996) and Polland (Lewandowski et al, 1992). In LAP-B, there is no variation in Nepali material while two alleles with almost 50% of heterozygosity is reported by Hertel (1996) in the *Taxus* growing in Germany. In Himalyan yew, there is LAP-A present with heterozygous genotype alleles 1 and 2 but no such LAP-A could be found in material from Germany and Polland. While Himalayan yew is homozygous in locus LAP-B with only 1 allele.

Malatedehydroganase (MDH) : Only one emperical MDH locus could be identified and it is not comparable with other as it is not yet tested by pervious workers.

Menadionereduetase (MR) : In MR, two locus MR-A and MR-B could be identified, for locus MR-B still needs confirmation. It may be either IDH-A or IDH-B. In MR-A there are two allele 1 and 2, similar result was reported by Hertel (1996) in *Taxus baccata* from Germany with nearly same level of heterozygosity. No variation in at MR-B in Himalayan Yew.

6-Phosphogluconatedehydroganase (PGDH) : In PGDH, there is variation in PGDH but with very low level of allelic frequency. Materials of Germany have also variation but all allele have nearly 50% frequency. In PGM-A, there is variation with three different alleles 1, 2 and 3 simalar heterozygosity with three different alleles has been observed by Hertel (1996) with high level of heterogygosity.

Shikimatedehydrogenase (SKDH) : In the enzyme system SKDH, no activities of enzyme have been found in Shivapuri, KMI, CDB and GOP population, while in the rest populations there is no vatiation and only one of the genotype with allele 22 was found, but alleles 3 and 2 is very common with 90% frequency and alleles 1 and 2 are rear in the *Taxus baccata* of Germany (Hertel, 1996).

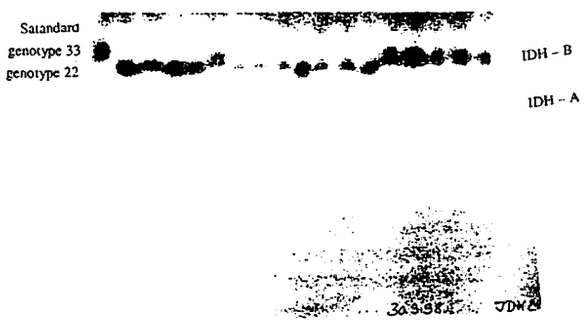


Fig. 1 : Banding pattern in IDH enzyme system

(In *T baccata* two geneloci IDH-A and IDH - B have been observed in Zymograme, in IDG -B there is a variation between different genotype while in IDH - A enzyme activities are very low so no clear band could be observed.)

Heterozygosity : The observed heterozygosity per population per genelocus is given in Table 2. The highest heterozygosity present in the population of Jiri. Higher the heterozygosity greater the inter changing of pollen between different populations.

Table 2 : Heterozygosity present in different populations

Observation	Populations	Mean heterozygosity
1	CDB	0.0833
2	GOR	0.0625
3	Jiri	0.2778
4	KM I	0.1667
5	KM II	0.0769
6	PRD	0.0588
7	Shivapuri	0.1647
8	TC	0.0625

The maxium heterozygosity found in the population of Jiri which is 0.2776 then second largest heterozygosity was observed in the population of KM I which is 0. 1667 and the least heterozygosity (0.0588) was observed in PRD. The highest mean genotype was found in the population of Shivapuri. The mean genotype present in different population is given in the following table.

Table 3 : Number of genotype per population

Population	Mean genotype per population
CDB	1.00
GOR	1.25
Jiri	1.00
KM I	1.23
KM II	1.00
PRD	1.00
TC	1.00
Shivapuri	1.27

Genetic distance : The genetic distance between two different populations at locus AAT-A is zero. While the genetic distance between two different populations at locus AAT-B is different than AAT-A. The genetic distance between CDB and GOR, CDB and KM I, CDB and KM II is 1, this indicate that these population are completely different. Similarly genetic distance between GOR and CDB, Jiri and GOR, PRD and GOR is 1 at locus AAT-B genetic distance between KM I and CDB, KM I and Jiri, KM I and PRD, KM I and TC also 1, the genetic distance between KM II and CDB, KM II and Jiri, KM II and PRD and KM II and TC also 1. The genetic distance between Shivapuri population and CDB, GOR, Jiri, KM I KM II, PRD and TC is less than 1. Among the *Taxus baccata* grown in Botanical Gardens, the genetic distance between TC and CDB, TC and PRD is equal to zero which indicate these genotype are completely similar at locus AAT-B.

The genetic distance between all population at locus ADH-B is less than 1, thus all population differ from each other at locus ADH-B. The genetic distance between all population at locus GDH is

also zero. In locus IDH-A, the genetic distance between CDB and KM II, KM II and Jiri, KM II and CDB, KM II and Jiri, KM II and PRD KM II and TC, KM II and TC is 1. While in IDH-B genetic distance is less than 1. The genetic distance between different population at locus, MR-B and LAP-B is zero.

Genetic diversity : The highest genepool diversity was found in the population of Shivapuri which is equal to 1.2701 while the multilocous diversity also found in Shivapuri population is equal to 163.678.

Cluster analysis : Root-Mean-Square (RMS) distance between CDB and PRD is zero. This indicates that these population are identical. The highest RMS value is measured at 1.2621 between CDB and PRD and KM I/II and GOR. The RMS between observations is 0.3380. The RMS between different population is given in Table 4.

Table 4 : Average linkage cluster

NO. of Cluster	Cluster joined	Root mean square distance between population
7	CDB PRD	0.0000
6	KM I KM II	0.044824
5	GOR CL6	0.078542
4	Jiri TC	0.184862
3	CL4 SH	0.491503
2	CL7 CL3	0.771604
1	CL2 CL5	1.262170

Ten different genotypes have been observed among the studied population of *T. baccata* of central Nepal. The Shivapuri population, have maximum variability. Two types of genotype are present in the population GOR and KM. More variability can be expected upon examination of more individual trees It is the objective of the future research to analyse twenty different trees from each population. Phylogenetic dendrogramme (Fig. 2) shows that population of CDB and PRD are close to each other. Similarly the population of Giri and that of TC are close and Shivapuri is close to both.

Population of KM I and KM II are geographically near permitting can be interchange of pattern between each other. But geographically distant population of GOR is close to these KM I and KM II.

Acknowledgement

This work has been carried out under German Academic Exchange Service. I am grateful to Mr. M. S. Bista, Director General, Department of Plant Resources for encouragement and Dr. D. Ewald for inviting me to carry out the work. My thanks goes to K. J. Malla, B. Ray, Pura Sedain for necessary help in collection in Nepal Himalaya, and Mrs. Elke Ewald for her kind help during laboratory analysis in Germany.

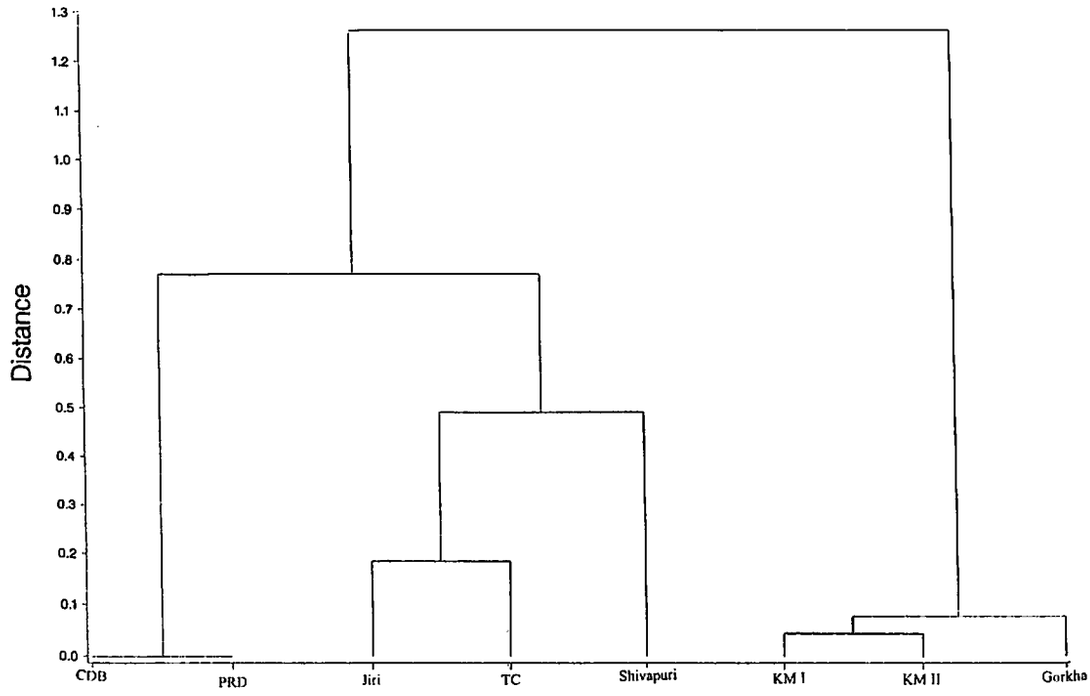


Fig. 2 : Phylogenetic dendrogram between different populations of *T. baccata*

References

Ashton, G.C. and Braden, A.W.H. 1961. Serum B-globulin polymorphism in mice. *Aust. J. Bio. Sci.*14, 248-254.

Cheliak, W.M. and Pitel, J.A., 1984. Techniques for starch gel electrophoresis of enzymes from forest tree species. Information report PI-X-42. Petawawa National Forestry Institute, Canadian Forestry Service, Canada.

Gregorius, H.R.1989. Genetic multiplicity for tolerance of atmospheric pollution. In: F.Scholz, H. R: Gregorius, and D.Rudin (eds). Genetic Effects of air pollutants in Forest Tree Populations. Springer, Berlin, Hiedelberg Pp.163-172.

Harttemer, H.H. 1991. Genetic Analysis and Population Genetics. S. Fineschi, M.E. Malvolti, F.cannata, and H. H. Hattemer (eds). Biochemical Markers in the Population Genetics of Forest Trees. SPB Academic Publishing bv, The Hague. pp.3-20.

Hertel, H. 1996. Genetic variation and geographical structure of English yew *(Taxus baccata L.)* in Mecklenburg-Verpommern (Germany). *Silvea Geneticae* 45:5-6, 290-294.

Hertel, H.1997. Inheritance of isozyme markers in English yew (*Taxus baccata* L). *Silvea Geneticae* 45:284-290.

Koveless M.D. 1992. Isozyme variation in tropical trees. Pattern of genetic organization In: Adams., W.T., Straus, H., Steven, Copes, L., Donald, and Griffin, A. R. Kluwer Academic Publishers, Boston, London. pp.67-94.

Lewandowski, A; Burchzyk, J. and Mejnartowicz, L. 1992. Inheritance and Linkage of Some Allozynes in *Taxus baccata* L. *Silvae Genetica* 41:42-347.

Lundkvist, K. Inheritance of leucine aminopeptidase isozymes in *Picea abies* K. Hereditas 76, 91-96, 1974.

Maurer, R. 1968 Disc-Electrophoresis. Walter de Gruyter and Co. Berlin.

Poulik, M.D. 1987. Starch gel electrophoresis in a discontinous system of buffers. *Nature* 180: 1477.

Sarin, Y.K. 1993. Need to conserve *Taxus baccata* trees. *Indian Forester.* 199 : 83-85.

Scholz, F.; Gregorius, H.-R.and Rudin, D. (1989). Genetic Effects of Air Pollutants in Forest Tree Populations. Springer, Berlin, Heidelberg,

Stauber, T. 1997 Macro und Prozedurensammlung fur SAS. Institute for Forest Genetics and Forest Tree Breeding. Eberwalder Chaussee 3, D-15377. Walsiversdorf, Germany.

Vallejos, C. E. 1983 Enzyme activity staining. Isoenzymes in Plant genetics and breeding. Part, I, ed. S.D. Tanksley, J.T: Orton, Elsevier Science publishing, Amsterdam, 469-516.

Wani M. C., Taylor H. L.; Wall M. E., Coggon P; and McPhail A T 1971. Plant Antitumour Agents. VI. The isolation and structure of taxol, a novel antileukemic antitumour agent from *Taxus brevifolia. J. Am Chem Soc.* 93:2325-2327.

Witherub K. M..' Look S. A.; Stasko M. W.; Ghiorzi, T. J.; Muschik G. M. and Cragg G. M. 1990 *Taxus spp.* needles contain amouns of taxol, taxol comparable to the bark of *Taxus brevifolia*: analysis and isolation. *J. Nat. Prod.* 53:1249-1255.

Yeh, F.C.and O'Malley, D. 1980. Enzyme variation in natural populations of Dauglas-fir, *Pseudotsuga menziessi* (Mirb.) Franco, from British Columbia.1. Genetic variation patterns in coastal populations. *Silvea Genetica* 29, 83-92.