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Population genetic structure of the endangered tree species *Taxus wallichiana* Zucc. in the Western Himalayas

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Abstract

***Taxus wallichiana* Zucc. (Taxaceae), commonly known as Himalayan yew, is a long-lived, dioeceous tree species distributed sparsely in the higher elevations of the Himalayan ranges. The species has become endangered and listed in the threat category of IUCN and Convention on International Trade on Endangered Species of Wild Flora and Fauna (CITES). Random Amplified Polymorphic DNA (RAPD) was used to study the genetic structure of severely restricted populations of this species, which would be useful in devising suitable conservation strategy. Analysis of molecular variance (AMOVA) revealed 89.95 per cent within population and 10.05 per cent between population components of variation. The Wright's fixation index (F_{ST}) or its analogue and migration rate (Nm) based on different methods revealed similar results. Rate of migration (Nm) varying from 1.00 to 1.30, indicated exchange of genetic material among the populations. However, FST that ranged from 0.16 to 0.21, suggested existence of genetic structuring. UPGMA dendrogram based on pair-wise OST values and Mantel's correlation (r = 0.47, p < 0.01) showed that genetic structure followed spatial distribution.**

Key words: *Taxus wallichiana*, RAPD, AMOVA, FST, gene flow

Introduction

Plant populations are not randomly arranged assemblages of genotypes but are structured in space and time. This structure may be manifested among geographically distinct populations, within a local group of plants or even progeny of individuals [1]. Manifestation of such genetic structure of populations of a species is determined by a set of intricately interacting processes such as life form, evolutionary history of the species, past geological events in the area, dispersion pattern, breeding behavior and genetic stochasticity including drift, migration, mutation and selection. Complex genetic phenomena resulting out of these processes often become difficult to resolve. A good understanding of the nature and amount of variation is therefore essential to devise suitable strategy for its domestication, conservation and sustainable management.

Genetic analysis of population structure have become commonplace with the advent of protein and DNA based markers that play an important role in the comprehension of genetic dynamics at different scales of time and space. RAPD markers have been successfully used in investigating genetic diversity in many plant species [2], because they are technically least demanding, large in number and applicable to a wide range of taxa without requiring prior information on the DNA sequence. Since, RAPD markers are dominant in nature, it is difficult to differentiate between dominant homozygote and heterozygote, which impairs accurate genotyping of individuals in a population. They are not directly amenable to various statistics used to estimate certain genetic parameters as compared to co-dominant markers. However, several strategies have been evolved [3-5] to address the problems associated with the estimates of parameters based on dominant markers. RAPD markers have proved useful in investigating the genetic structure of natural populations

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of various tree species and given results comparable to co-dominant markers such as isozymes, microsatellites and RFLP [6].

Taxus wallichiana Zucc. Syn. *Taxus baccata* Hook.f., more commonly known as Himalayan yew, is a small, evergreen and dioecious gymnosperm of the family Taxaceae. In India, it is distributed in drier, cooler and high altitude areas. Being shade demanding in nature, the species commonly occurs in patches as an under story canopy associated with either conifers namely *Abies pindrow, Picea smithiana* and *Cedrus deodara* or broad leaved tree species like *Aesculus indica, Quercus* spp. and *Juglans regia.* The species has greater economic and medicinal value than any other gymnosperms of the Himalayan region. In the Unani system of medicine, the extract from the bark and leaves is used for the preparation of a drug called *Zarnab.* The species got the world's attention because of its paclitaxol content, which has been found useful in the treatment of cancer [7]. Like any other medicinal plant, there is tremendous pressure on the natural populations of this species. Its scattered populations have declined considerably in recent decades due to exploitation of various plant parts for medicinal uses [8]. On the other hand, the regeneration of the species is very poor due to low seed germination, very long seed dormancy, slow growth (20-32 annual rings per inch of radius), seed destruction by animals and birds, which relish the fleshy aril, species got the world's attention because of its paclitaxol content, which WAX useful in poor survival and seedling establishment due to habitat destruction, thereby posing critical threat to the natural populations of the species to perpetuate. Keeping this in view, the government of India had put a ban on the trade, export and harvesting of its leaves, barks and other plant parts. The species was listed in IUCN Threat Category and Criteria LR 1c and appendix-II of the Convention on International Trade on Endangered Species of Wild Flora and Fauna (CITES). Therefore, it is necessary to understand the genetic structure of the populations in their nativity and devise appropriate strategies to conserve the genetic diversity still left in the natural habitats.

In the present investigation, genetic diversity in nine populations of *T. wallichiana* in its natural range in the western Himalayas was studied using RAPD markers. The major objectives were to quantify the amount and study the nature of distribution of different components of genetic variation using various diversity measures.

Materials and methods

Plant material

Nine geographically different populations of *Taxus wallichiana* Zucc. in the north western Himalayas were selected for the study (Table 1). In the sampling method, the number of tree samples marked in each population was proportional to the size of its stand. At least 50 meter distance was maintained between any two consecutive sample trees so as to eliminate the close relatives (mostly half-sibs and clones). Altogether, 72 trees were marked across nine populations. Fresh and healthy leaves from the young and tender shoots were collected and given a quick deep in liquid nitrogen. The samples were then stored at –70°C for extracting DNA.

Table 1. Geographical location of *Taxus wallichiana* populations sampled in the western Himalayas

SN	Population	Latitude	Longitude	Altitude (m above msl)
1	Nichar	31°30'N	77° 59' E	3000
2	Khadapathar	31°08'N	77°38'E	1900
3	Hatu	31° 15'N	77°27'E	3300
4	Nachan	31°36'N	77° 12' E	2900
5	Jalori Pass	31°33'N	77°20'E	3200
6	Patlikul	32° 11'N	77°02'E	2300
7	Manali	32° 16' N	77° 10' E	2050
8	Bharmour	32°20'N	76°37'E	2800
9	Dalhousie	32°30'N	76° 02' E	2600

DNA extraction and amplification

DNA was extracted following CTAB method as described by Doyle and Doyle [9] with little modification. Five gram of young leaf tissue was ground to fine powder in liquid nitrogen and incubated at 60°C in DNA extraction buffer (100mM Tris HCl, 4M NaCl, 20mM EDTA, 2% CTAB and 0.2% β-mercaptoehanol) for one hour with intermittent swirling. It was then emulsified with equal volume of chloroform:iso-amyl alcohol (24:1) followed by centrifugation. The aqueous phase was precipitated by addition of chilled iso-propanol; precipitated nucleic acid was spooled out, washed twice with 70% ethanol, dried under vacuum and dissolved in $T_{10}E_1$ buffer. RNA was removed by RNAse treatment whereas proteins and other impurities were removed by phenol-chloroform extraction. The upper aqueous phase was collected after centrifugation and mixed with 1/10th volume of 3 M Sodium acetate. DNA was precipitated by adding two volumes of chilled absolute alcohol, pelleted by centrifugation, dried in vacuum and dissolved in $T_{10}E_1$ buffer. DNA was quantified by running the purified DNA on 0.8% agarose gel alongside diluted uncut lambda DNA as standard. Final DNA concentration of 12.5ng/µl was made by making appropriate dilution in $T_{10}E_1$ buffer. For RAPD amplification [10], thirty decamer primers (OPA07, OPA08, OPA09, OPA10, OPA14, OPA17, OPA18, OPB05, OPB10, OPB17, OPB20, OPG01, OPG02, OPG06, OPG07, OPG08, OPP01, OPP02, OPQ02, OPQ07, OPQ08, OPQ17, OPAA01, OPAA02, OPAA03, OPAA04, OPAA06, OPAA07, OPAA08, OPAA10) of Operon Technologies Inc., CA, USA were used. The amplification reactions were carried out in a thermocycler (Perkin Elmer, Model 9600, USA). The amplified products were mixed with 10 X loading dye (containing Bromophenol blue and Xylene cyanol) and were separated by gel electrophoresis (1.2% agarose gel containing ethidium bromide) in 1X TBE buffer (pH 8.0) medium by applying a constant voltage @ 5 volts/ cm for 3 h. The size of the amplified fragments was determined by using size standards (100 bp DNA ladder plus, MBI Fermentas, Lithuania). DNA fragments were visualized under UV light and photographed using Polaroid photographic system for permanent records.

Data analysis

Only clear and reproducible DNA fragments were scored and used to generate a binary data matrix (1-presence, 0-absence). The allele frequencies were calculated under the assumption that each amplified band corresponded to a different RAPD locus. AMOVA procedure was followed to estimate the within and among population variance components with the help of ARLEQUIN software [11]. Pair wise population genetic distance (Φ_{ST}) was estimated for the study of genetic differentiation of populations. Test of significance for the Φ_{ST} values were estimated by non-parametric permutation approach [12]. The Φ_{ST} matrix was used to perform cluster analysis by UPGMA (Unweighted Pair Group Means on Arithmetic averages) and the dendrogram was constructed with the help of software NTSYSpc version 2 [13]. F_{ST} by Wright [14], θ (analogue of F_{ST}) by Weir and Cocknham [15] and F_{ST} by Lynch and Milligan [16] were estimated by following the assumptions, hypothesis and analysis as described for RAPD markers [4]. RAPDF$_{ST}$ [17] was used to find out these estimates. To see the presence of possible association of geographical origin of the populations with their genetic structuring, Mantel's correlation was calculated between the matrix of Φ_{ST} and the geographical distance among the populations.

Results and Discussion

RAPD profile

Genetic relationship based on RAPD markers has been successfully studied in a wide range of plant species [18]. In the present investigation, twenty-four out of thirty primers revealed polymorphism. Percent polymorphism, a ratio of the number of the polymorphic bands to the total number of bands amplified by a particular primer, varied from 14 to 75 %. Maximum polymorphism (75 %) was revealed by the primers OPQ02 and OPAA02. Altogether, 177 bands were amplified, out of which 76 (42.8%) were polymorphic.

Analysis of molecular variance and population genetic structure

ANOVA partitioned the total variation into within and between population components. Most of the variation (89.95 %) was attributed to the variation among individuals within the populations. However, there was presence of small (10.05 %) but statistically significant amount of variation among the nine populations (Table 2). F_{ST} values derived from ANOVA gave an insight into the inter population genetic relationship in the species (Table 3). All but five pairwise F_{ST} values (Nichar to Khadapathar, Khadapathar to Hatu, Khadapathar to Nachan, Khadapathar to Jalori Pass and Nachan to Dalhousie) were significant ($p < 0.05$) when individual pairs of populations were considered. Φ_{ST} between Bharmour and Manali (0.153) was found to be maximum followed by those between Bharmour and Khadapathar (0.147) and Bharmour and Hatu (0.144).

The relationship between the populations as depicted in the dendrogram revealed a fairly distinct pattern of the populations in accordance with their geographic locations (Fig. 1). Nichar, Hatu and Khadapathar populations, which were geographically contiguous, formed one group. Similarly, the populations of Chamba and Dalhousie, which were geographically closer to each other than the other populations were placed in one group. The only exception is Nachan population, which was clustered with Chamba and Bharmour although it was geographically closer to Patlikul and Jalori pass. Mantel's correlation between matrix of pairwise F_{ST} values and geographical distances was found to be statistically significant ($r = 0.47$, $p < 0.05$), which indicated that genetic structure was related to the spatial distribution of the populations. Therefore,

Table 2. ANOVA of RAPD variation for nine *Taxus wallichiana* populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of total variance	P value
Among populations	8	185.074	1.37436	10.05	<0.01
Within populations	63	775.301	12.30637	89.95	
Total	71	960.375	13.68073		

Table 3. Pairwise spatial and genetic distance among the populations of *Taxus wallichiana*

	Nichar	Khadapathar	Hatu	Nachan	Jalori Pass	Patlikul	Manali	Bharmour	Dalhousie
Nichar	-	60	61	81	59	116	119	173	229
Khadapathar	0.038	-	25	72	56	133	143	180	232
Hatu	0.031	0.002	-	46	33	110	120	156	204
Nachan	0.122	0.057	0.086	-	22	68	80	110	159
Jalori Pass	0.083	0.086	0.064	0.077	-	78	88	127	180
Patlikul	0.082	0.107	0.106	0.111	0.057	-	16	59	117
Manali	0.111	0.131	0.104	0.143	0.137	0.079	-	64	120
Bharmour	0.140	0.147	0.144	0.096	0.119	0.100	0.153	-	57
Dalhousie	0.138	0.138	0.110	0.041	0.134	0.124	0.193	0.093	-

Values above diagonal are the geographical distance in km

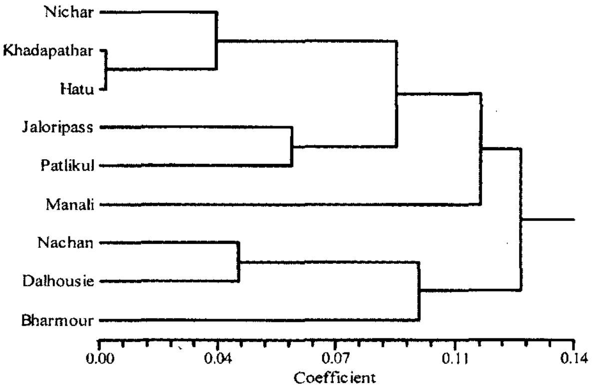

Fig. 1. UPGMA dendrogram based on pairwise F_{ST} values showing the genetic relationship among *Taxus wallichiana* population

estimates of F_{ST} and clustering pattern broadly followed spatial distribution of populations. Genetic structuring through isolation by distance, as explained by positive correlation between F_{ST} based genetic distance and spatial geographic distance among the populations, is more common in out crossing species [2]. Secondly, Himalayan yew has a restricted distribution characterized by highly fragmented and confined habitat occupancy. They are scattered in isolated patches in mid to high elevation areas that are separated by high-rise ridges of the Himalayan mountain ranges. A species having such habitat occupancy is likely to have some degree of genetic differentiation due to geographic isolation [19].

Inter-population sharing of genetic material was ascertained with the help of estimates of migration rate (Nm) and fixation indices (F_{ST} or its analogue) among the natural populations. FST value according to Wright's method was 0.19 and migration rate (Nm) was 1.10 individuals per generation. Based on Weir and Cockerham (1984), F_{ST} was 0.16 and Nm 1.30, while according to Lynch and Milligan (1994), F_{ST} was 0.21 and Nm 1.00. Irrespective of the methodology used, F_{ST} values were not appreciably different from each other, but deviated significantly from Zero ($p < 0.05$) in all the three cases. Predominantly outcrossing species generally show low value of F_{ST} and weak genetic differentiation as compared to predominant selfers. F_{ST} values in the present investigation were found close to those reported for populations of many out crossing species: 0.13 in *Pinus halepensis* [20], 0.11 to 0.18 in *Digitalis minor* [21] and 0.16 in *Ginkgo biloba* [22]. The observed F_{ST} values suggested that there was genetic structuring of the populations, although low in magnitude. This was supported by the estimated migration rates among the populations. The range of migration rates (Nm derived from F_{ST} of the three methods varying from 1.00 to 1.30) indicated exchange of genetic material among the populations. It supports the argument that some amount of differentiation could still be observed over a long period of time even in the presence of gene flow [23]. The seeds of *Taxus wallichiana* are embedded in attractive fleshy, sweet and non-poisonous aril which are relished by birds and mammals and are dispersed by ingestion [24]. It was also reported that the current distribution of Himalayan yew is supposed to be attained by long distance dispersal by birds [25]. Therefore, mode of seed dispersal could be one of the important factors responsible for the present inter-population genetic sharing among the yew populations like many other fleshy-fruited species that are dispersed by ingestion [26, 27]. However, the argument of the possibility of long distance gene flow through pollen can be discounted since the species is normally confined to the under-storey forest canopy, which does not come under the direct effect of wind. In such a situation the pollens, which are wingless, might not get enough opportunity for flight and had limited ability to disperse over a longer distance.

Analysis of molecular variance apportioned a major portion (89.16 %) of total variation to the variation among individuals within the populations, which might have resulted due to a variety of factors such as life form, ecological and life history traits, geographic range, successional status and breeding system. Geologic events such as uplifting of the Himalayas and intense glacial activities were believed to have taken place during Cenozoic era especially during latest tertiary and Pleistocene leading to extreme climatic changes [28, 29]. Current disjunct occurrence of *T. wallichana* in South-East Asia possibly had attained maximum diversity in ecological species isolation by the time of mid Miocene, the epoch when the present natural habitat of the species was subjected to intense geological events [25]. Therefore, historical gene flows among the populations and/or shared common ancestry during Pleistocene might have resulted in the availability of high within and low among population variation.

Occurrence of high within population variation in *Taxus wallichiana* may be explained by its successional status as late successional taxa often reveal higher within population variation than the early or mid successional taxa [2]. High within population variation at RAPD and isozyme loci was also reported in many other populations of the genus *Taxus* such as *T. baccata* [18] and *T. brevifolia* [30], which occupy similar habitats with similar life form and breeding behavior.

In conclusion, basic genetic information concerning natural populations of *T. wallichiana* was obtained using RAPDs in this study and geographic patterns in genetic variation among populations were detected. It was revealed that *T. wallichiana* had low between population and high within population genetic variation in spite of its low frequency and scattered distribution in isolated populations. Significant amount of genetic exchange among the populations was observed. Genetic structuring was also evident, which followed the geographical distribution of the species. Pattern of distribution of genetic diversity among the populations was consistent with the breeding behavior, life history and ecological behavior of the species. These genetic findings have important implications for the conservation and management of available genetic variation of this highly endangered medicinal tree species.

References

1. **Loveless M. D. and Hamrick J. L.** 1984. Ecological determinants of genetic structure in plant populations. Ann. Rev. Ecol. Syst., **15**: 65-95.
2. **Nybom H. and Bartish I. V.** 2000. Effect of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspect. Plant Ecol. Evol. Syst., **(3/2)**: 93-114.
3. **Lynch M. and Milligan E. G.** 1994. Analysis of genetic structure with RAPD markers. Mol. Ecol., **3**: 91-99.
4. **Apostol B. L., Black W. C. IV., Reiter P. and Miller B.** 1996. Population genetics with RAPD-PCR markers: the breeding structure of *Aedes aegypti* in Puerto Rico. Heredity, **76**: 325-334.
5. **Stewart C. N. and Excoffier L.** 1996. Assessing population genetic structure and variability with RAPD data: application to *Vaccinium macrocarpon* (American Cranberry). J. Evol. Biol., **9**: 153-171.
6. **Bouvet J. M., Fontaine C., Sanou H. and Cardi C.** 2004. An analysis of the pattern of genetic variation in *Vitellaria paradoxa* using RAPD markers. Agroforestry Syst., **60**: 61-69.
7. **McGuire W. P., Rowinsky E. K., Rosonshein N. N., Grumbine F. C. Ettinger D. S. and Armstrong O. K.** 1989. Taxol: A unique anti-neoplastic agent significant activity in advance ovarian epithelial neoplasmas. Ann. Int. Med., **111**: 273-279.

8. **Molur S. and Walker S.** 1998. Conservation Assessment Management Plan workshop report for selected medicinal plants of northern, northeastern and central India. (CAMP). Organized by Zoo Outreach Organization CBS, Coimbatore and NBFGR, Lucknow, 22-26th Sep. 1997. 156 p.
9. **Doyle J. J. and Doyle J. L.** 1987. Isolation of plant DNA from fresh tissue. Focus, **12**: 13-14.
10. **Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A. and Tingy S. V.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., **18**: 6531-6535.
11. **Schneider S. Roessli D. and Excoffier L.** 2000. ARLEQUIN Ver 2.000. A software for population genetics data analysis. Genetics and Biometry Laboratory. Department of Anthropology and Ecology. University of Geneva.
12. **Excoffier L., Smouse P. E. and Quattro J. M.** 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial restriction data. Genetics, **131**: 479-491.
13. **Rohlf F. J.** 1998 NTSYS-pc, ver 2. Exeter Software, Setauket, NY.
14. **Wright S.** 1951. The genetical structure of populations. Ann. Eugen., **15**: 323-354.
15. **Weir B. S. and Cockerham C. C.** 1984. Estimating F-statistics for analysis of population structure. Evolution, **38**: 1358-1370.
16. **Lynch M., Pfrender M., Spitze K., Lehman N., Hicks J., Alien D., Latta L., Ottene M., Bogue F. and Colbourne J.** 1999. The quantitative and molecular genetic structure of a subdivided population. Evolution, **53**: 100-110.
17. **Black IV W. C.** 1998. FORTRAN programs for the analysis of RAPD-PCR markers in populations. Colorado State University, Ft. Collins, CO 80523.
18. **Hilfiker K., Holderegger R. P. and Gugerli F.** 2004. Dynamics of genetic variation in *Taxus baccata:* local versus regional perspective. Can. J. Bot., **82**: 219-227.
19. **Gonzalez-Astorga J. and Nunez-Farfan J.** 2001. Effect of habitat fragmentation on the genetic structure of the narrow endemic *Brongniartia vazquezii.,* Evol. Ecol. Res., **3**: 861-872.
20. **Gomez A., Alia R. and Bueno M. A.** 2001. Genetic diversity of *Pinus halepensis* Mill. Populations detected by RAPD loci. Ann. for. Sci., **58**: 869-875.
21. **Sales E., Nebauer S. G., Mus M. and Segura J.** 2001. Population genetic study in the Baleric endemic plant species *Digitalis minor* (Scrophulariaceae) using RAPD markers. Am. J. Bot., **88**: 1750-1759.
22. **Fan X. X., Shen L., Zhang X., Chen X. Y. and Fu C. X.** 2004. Assessing genetic diversity of *Ginkgo biloba* L. (Ginkgoaceae) Populations from China by RAPD markers. Biochemical Genet., **42**: 269-278.
23. **Gavrilets S.** 2000. Waiting time to parapatric speciation. Proc. R. Soc. Lond. B, **267**: 2483-2492.
24. **Gupta V. K., Varshneya Y. P. and Stevenson K. A. P.** 1962. An introduction to Gymnosperms. Kedarnath Ramnath, Meerut, India, 78-93 pp.
25. **Spjut R. W.** 2003. Introduction to Taxus: Methodology, Taxonomic relationship, leaf and seed characters, physiographical relationship, cultivation and chemistry. The World Botanical Associates Web Page. *www. worldbotanical.com.*
26. **Graham J., Squire G. R., Marshall B. and Harison R. E.** 1997. Spatial dependent genetic diversity within and between colonies of wild raspberry, *Rubus edaeus,* detected using RAPD markers. Mol. Ecol., **6**: 1001-1006.
27. **Bartish I. V., Jeppsson N., and Nybom H.** 1999. Population genetic structure in the dioecious pioneer plant species *Hippophae rhamnoides* investigated by random amplified polymorphic DNA (RAPD) markers. Mol. Ecol., **8**: 791-802.
28. **Conkle M. T.** 1992. Genetic diversity-seeing the forest through the trees. New For., **6**: 5-22.
29. **Owen L. A., Gualtieri L., Finkel R. C., Caffee M. W., Benn D. I. and Sharma M. C.** 2001. Cosmogenic radionuclide dating of glacial landforms in the Lahul Himalaya, northern India: defining the timing of Late Quaternary glaciation. J. Quarternary Sci., **16**: 555-563.
30. **Wheeler N. C., Jech K. S., Masters S. A., O'Brien C. J., Timmons D. W., Stonecypher R. W. and Lupkes A.** 1995. Genetic variation and parameter estimtes in *Taxus brevifolia* (Pacific yew). Can. J. For. Res., **25**: 1913-1927.