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Genetic diversity within and among populations of the endangered species *Taxus fuana* (Taxaceae) from Pakistan and implications for its conservation

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Abstract

The West Himalayan yew, *Taxus fuana* Nan Li & R.R. Mill (Taxaceae), is an endangered species endemic to the Western Himalayas. An investigation of the genetic diversity of wild populations of *T. fuana* in Pakistan was undertaken. The genetic diversity and genetic structure was quantified using random amplified polymorphic DNA (RAPD) variation in 219 individuals of the 10 populations. Of the 32 universal primers screened 16 produced highly reproducible, clear RAPD bands. Using these primers, 193 discernible DNA fragments were generated, of which 164 (84.97%) were polymorphic. The statistical results indicated that there was a relatively low genetic diversity within populations (with percentages of polymorphic bands, PPB, ranging from 29.53 to 50.26%, with an average of 38.34% and a Nei's genetic diversity index (H_E) of 0.1165), and a high genetic differentiation among populations ($G_{ST} = 0.5842$, $\Phi_{ST} = 0.5685$) within these populations. The gene flow (N_m) was low with only 0.3558.

The distribution of genetic diversity revealed among the wild populations of *T. fuana* in Pakistan may result from their evolutionary history including habitat fragmentation, genetic drift combined with limited gene flow. The results are unexpected for an outcrossing, wind-pollinated and long-lived woody species. Based on these findings, conservation concerns are discussed together with possible strategies for implementing *in situ* and *ex situ* conservation.

Keywords: Gene flow; Genetic diversity; Pakistan; Population structure; RAPD; *Taxus fuana*

1. Introduction

Genetic variation is generally believed to be a prerequisite for the short- and long-term survival of a species (Schonewald-Cox et al., 1983; Lande, 1988), and the importance of preserving genetic diversity of wild and domesticated species is widely acknowledged today (Karron, 1991). Over the last decade, the preservation of wild species has become a focus of interest for conservation biologists. The extinction rates of wild species are increasing directly and

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indirectly in response to human activities, and conservation plans for endangered species have been suggested from various biological perspectives (Primack, 1993; Bowles and Whelan, 1994; Sutherland and Hill, 1995; Pickett et al., 1997). Forty percent of the forest area in Pakistan is composed of coniferous and scrub forests that are confined to northern hills and mountains. This small percentage of forests is in danger of declining at an alarming rate. Several timber species, e.g., *Ulmus*, *Quercus*, *Fraxinus*, *Taxus* and *Picea* are already endangered or vulnerable (Khan, 2003). The long-term survival and evolution of species depend on the existing diversity in its present habitat. The genetic diversity is a fundamental component of biodiversity and is closely related to the geographic distribution of genotypes that constitutes subspecies, races or ecotypes (Sreekumar and Renuka, 2006).

The West Himalayan yew, *Taxus fuana* Nan Li & R.R. Mill, is an evergreen nonresinous gymnosperm, endemic to the western Himalayas, i.e. Southwest Xizang (Tibet) of China, Nepal, North India and Pakistan (Li and Fu, 1997; Fu et al., 1999; Möller et al., 2007). *Taxus* specimens in Pakistan were variously identified as *Taxus baccata* L. or *Taxus wallichiana* Zucc. (Nasir and Ali, 1972, 1987) until Li and Fu (1997) described *T. fuana* as a new species based on specimens from Southwest Xizang, China. Based on the combined morphological and molecular data analysis, *T. fuana* was shown to represent a unique species in the western Himalayas (Shah et al., in press). *T. fuana* is dioecious, outcrossing, wind-pollinated, grows as tree or large shrub in the understory of mixed or *Pinus* forests along an elevational gradient ranging from 1800 to 3300 m. The seeds are surrounded by a unique red fleshy cuplike aril when mature which plays a key role in attracting birds and mammals that disperse the seeds (Wilson et al., 1996). *T. fuana* is an evergreen tree or large shrub. Generally, its height varies from 12 to 30 m. In Pakistan, the distribution of *T. fuana* is confined to the North-West Frontier Province (N.W.F.P.), the Federally Administered Tribal Area (F.A.T.A.) and the Pakistan territory of Kashmir (Azad Kashmir), occurring at elevations of 2500–3100 (–3400) m.

The yew plant has attracted the world's attention due to the cancer-inhibitory alkaloid Paclitaxel (Taxol). Its antitumor property was first reported in 1964 from the pacific yew, *Taxus brevifolia* (Wani et al., 1971), which was found to be very effective against murine leukemia cells (Appendino, 1993). Paclitaxel, the active of the extract, was later found also to be very effective in the treatment of ovarian and breast cancers (McGuire et al., 1989; Holmes et al., 1991).

During our field survey, it was observed that the number of *T. fuana* populations in Pakistan was rapidly declining, not because of their exploitation for the extraction of taxol but mainly due to human pressure in the form of habitat destruction, widespread deforestation and over-exploitation for fuel, fodder, timber, and farming, especially in the Azad Kashmir range. If such anthropogenic harvesting pressures continues without sustainable management of the resource, the genetic diversity and natural abundance of this species will be at risk. Most of the extant populations of the species are small and scattered in isolated patches throughout this region. An understanding of the level and distribution of genetic variation of *T. fuana* populations is therefore an important prerequisite for an effective conservation of the species in this region.

T. fuana has not been studied intensively except for its taxonomic and nomenclatural treatment (Li and Fu, 1997). Nothing is known about the population genetics of this species in Pakistan. The present study is a step towards investigating the genetic diversity and population structure of *T. fuana* in this region, which represents the westernmost limit of the distribution of the species. Knowledge of the level of genetic diversity and the way in which it is maintained among populations are major aspects in conservation. For rare and endangered species this knowledge plays a significant role in the formulation of appropriate conservation management strategies (Milligan et al., 1994).

Random amplified polymorphic DNAs (RAPDs) have been widely used to investigate population structures and genetic diversities (Williams et al., 1990; Maki and Horie, 1999; Wang et al., 2004; Sreekumar and Renuka, 2006), and to propose phylogenetic hypotheses in plants (Rath et al., 1998). This technique is simple, PCR-based, require no prior sequence information, and a large number of putative loci may be screened quickly. RAPD markers are considered to be a rapid tool for assessing the genetic diversity at the molecular level (Williams et al., 1990, 1993) and their analysis provides a high resolution discrimination of samples and can be carried out on small amounts of DNA (Carter and Sytsma, 2001; Jolner et al., 2004). Some limitations of RAPDs, such as poor reproducibility in early RAPD analyses, has been largely overcome through improved, strictly applied laboratory techniques and scoring procedures (Nybom and Bartish, 2000). If used carefully, results obtained from RAPDs are reliable (Jolner et al., 2004), as shown by Collins et al. (2003) for *Taxus* species and hybrids.

The main objective of the present study was to quantify the genetic variation and genetic population differentiation of wild populations of the endangered species *T. fuana* in Pakistan. The following specific questions are addressed: (1) What are the levels of the genetic diversity within and among population of *T. fuana*? (2) Does the endangered species *T. fuana* exhibit weak population structure due to frequent pollen flow and seed dispersal? (3) Are patterns of genetic

variation consistent with other species in the genus *Taxus*? Implications of the results for devising an appropriate conservation strategy are discussed.

2. Materials and methods

2.1. Plant material

T. fuana is a dioecious, outcrossing, wind-pollinated tree species, which is endemic to western Himalayas. Pakistan presents the westernmost distribution of this species. During 2005 and 2006 leaves of 219 individuals were collected from 10 wild populations in North Pakistan, including one from Azad Kashmir, covering the overall distributional range of the species in Pakistan. Two populations were collected in the Federally Administered Tribal Area (F.A.T.A.), two from Punjab and five across the North-West Frontier Province (N.W.F.P.) (Fig. 1 and Table 1). In each of the 10 populations, random samples of 8–30 plants were analysed by RAPD. Young, healthy leaves were collected and dried in silica gel until DNA extraction. The voucher specimens were deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

2.2. DNA extraction and PCR amplification

Genomic DNA was extracted from dried leaves of all accessions using a CTAB method (Doyle and Doyle, 1987) modified by an additional ammonium acetate wash as described by Weising et al. (1995) for further purification (Collins et al., 2003). Total DNA was dissolved in 50–100 μL 1× TE (pH 8.0) and checked by electrophoresis on 1.0% agarose gels containing 0.5 mg/mL of ethidium bromide and visualized by ultraviolet light. DNA concentration was determined with a UV–vis spectrophotometer (TU-1800) and the DNA diluted to a final concentration of 20–30 ng/μL for subsequent use.

Thirty-two primers (Operon Technology, Alameda, California, USA) were screened initially on eight random samples for their ability to amplify polymorphic bands among the populations. Sixteen primers produced strong, clear and reproducible bands. These primers were selected for further study of the 219 individuals (Table 2). PCR reactions were carried out in a total volume of 25 μL reactions containing, 2.5 μL 10× PCR buffer, 2.5 μL $MgCl_2$ (25 mM), 2.0 μL

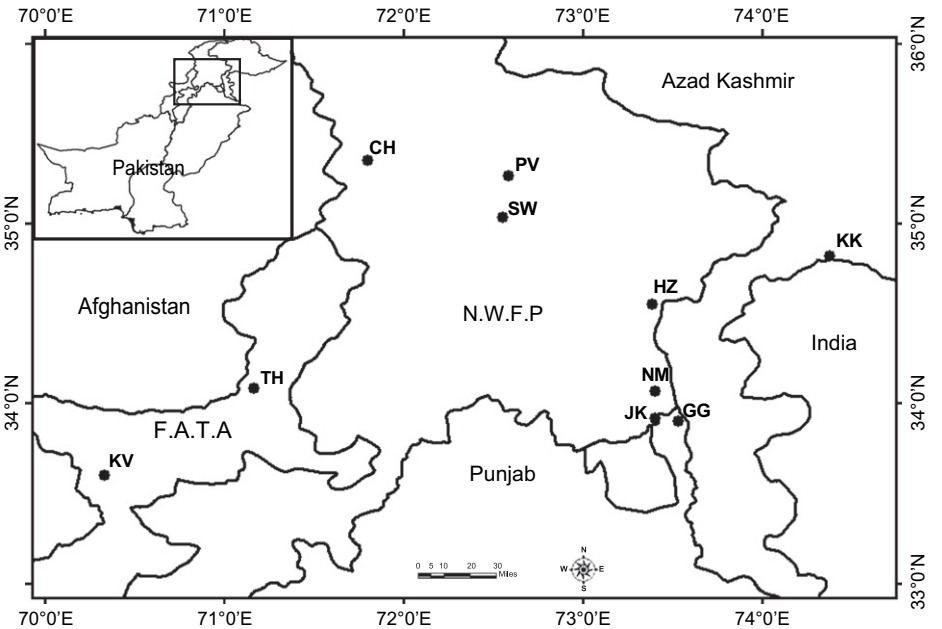

Fig. 1. Map showing the locations of the populations of *Taxus fuana* in North Pakistan sampled in this study. F.A.T.A. – Federally Administered Tribal Area; N.W.F.P. – North-West Frontier Province. Population abbreviations follow those in Table 1.

Table 1
Populations of *Taxus fuana* sampled for RAPD analysis

Population	Abbreviation	Locality	Sample size	Position	Elevation (m)	Voucher
Mukshpuri	NM	Pakistan, N.W.F.P., Mukshpuri	20	34°04 N, 73°25 E	2790	Amin-25031–25050
Hazara	HZ	Pakistan, N.W.F.P., Hazara	8	34°33 N, 73°23 E	2200	Amin-25204–25211
Neelam Valley	KK	Pakistan, Azad Kashmir, Neelam Valley	28	34°50 N, 74°22 E	2150	Amin-25061–25088
Jhika Gali	JK	Pakistan, Punjab, Jhika Gali	30	33°55 N, 73°24 E	2122	Amin-25116–25145
Galehra Gali	GG	Pakistan, Punjab, Galehra Gali	29	33°54 N, 73°32 E	2000	Amin-25147–25175
Swat	SW	Pakistan, N.W.F.P., Swat	22	35°02 N, 72°35 E	2200	Amin-25226–25247
Palas Valley	PV	Pakistan, N.W.F.P., Palas Valley	20	35°16 N, 72°35 E	2000	Amin-25251–25270
Chitral	CH	Pakistan, N.W.F.P., Chitral	20	35°21 N, 71°48 E	2050	Amin-25271–25290
Tirah	TH	Pakistan, F.A.T.A., Tirah	24	34°05 N, 71°10 E	2000	Amin-25291–25314
Kurram Valley	KV	Pakistan, F.A.T.A., Kurram Valley	18	33°36 N, 70°20 E	2439	Amin-25315–25332

dNTPs mixture (2.5 mM), 1.25 μL primer (10 μM), 0.5 μL formamide and 1.0 unit of Taq polymerase (TaKaRa Biotechnology Dalian Co., Ltd., Dalian, China), 13.25 μL double distilled water and 2 μL template DNA (50 ng genomic DNA). The RAPD-PCR amplifications were conducted in a GeneAmp PCR System 9700 Thermal Cycler (PerkinElmer, USA) and run through the following temperature profile: a hot start at 95 °C followed an initial denaturation of 2 min at 95 °C, this was followed by two cycles of 30 s at 95 °C, 1 min at 37 °C, and 2 min at 72 °C, then two cycles of 30 s at 95 °C, 1 min at 35 °C, and 2 min at 72 °C, thereafter followed 40 cycles of 30 s at 95 °C, 1 min at 35 °C and 2 min at 72 °C, and a final step of 10 min extension at 72 °C (Hollingsworth et al., 1998).

The amplification products were separated via electrophoresis on 1.6% (w/v) agarose gels in 1× TBE buffer at 180 V for 1 h, followed by 120 V for 3 h. A 100-bp DNA ladder was used as a reference for sizing the fragments obtained. Staining was achieved with 1.5 μL ethidium bromide (10 ng/mL) added to each gel. They were then photographed with the Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories Ltd, UK). The gel images were evaluated using an image analysis software for gel documentation (Lab Works Software, Version 3.0; UVP, Upland, CA 91786, USA). A negative control reaction, in which template DNA was omitted, was added to every batch of PCR reactions in order to check that no self-amplification or DNA contamination occurred.

Table 2
Attributes of primers used for RAPD analysis from 219 individuals of *Taxus fuana* sampled from 10 populations

Primer	Sequence (5′–3′)	No. of bands scored	No. of PB	PPB (%)	Approx. band size range (bp)
OPA-04	AATCGGGCTG	11	10	90.91	500–1300
OPA-07	GAAACGGGTG	13	12	92.31	450–2000
OPA-10	GTGATCGCAG	17	13	76.47	550–2000
OPA-14	TCTGTGCTGG	13	11	84.62	500–1500
OPF-03	CCTGATCACC	15	13	86.67	450–2000
OPF-05	CCGAATTCCC	10	9	90.00	350–1500
OPF-14	TGCTGCAGGT	14	13	92.86	400–2500
OPF-15	CCAGTACTCC	12	11	91.67	600–2000
OPF-20	GGTCTAGAGG	11	9	81.82	600–2000
OPP-03	CTGATACGCC	11	8	72.73	500–1750
OPP-08	ACATCGCCCA	10	9	90.00	550–1500
OPP-11	AACGCGTCGG	13	12	92.31	600–2000
OPP-14	CCAGCCGAAC	13	11	84.62	500–2500
OPG-01	CTACGGAGGA	10	8	80.00	650–1750
OPG-12	CAGCTCACGA	7	4	57.14	650–1750
OPC-06	GAACGGACTC	13	11	84.62	500–2000
Total		193	164	84.97	

Note: PB, polymorphic bands; PPB, the percentage of polymorphic bands.

Table 3
Genetic variability within populations of *Taxus fuana* detected by RAPD analysis

Population	n	A_e	H_E	S	PPB (%)	G_{ST}	N_m	Φ_{ST}
NM	20	1.2245 (0.3425)	0.1299 (0.1886)	0.1958 (0.2698)	41.45			
HZ	8	1.1655 (0.3042)	0.0983 (0.1697)	0.1486 (0.2472)	29.53			
KK	28	1.1847 (0.3321)	0.1066 (0.1787)	0.1594 (0.2578)	30.57			
GG	29	1.1682 (0.2964)	0.1019 (0.1683)	0.1554 (0.2469)	32.12			
JK	30	1.2005 (0.3222)	0.1196 (0.1196)	0.1814 (0.2606)	38.34			
SW	22	1.2016 (0.3112)	0.1233 (0.1747)	0.1894 (0.2564)	39.38			
PV	20	1.2228 (0.3049)	0.1343 (0.1753)	0.2145 (0.2584)	46.11			
CH	20	1.2260 (0.3067)	0.1418 (0.1733)	0.2216 (0.2542)	50.26			
TH	24	1.2032 (0.3004)	0.1267 (0.172)	0.1961 (0.2539)	41.97			
KV	18	1.1141 (0.2149)	0.0776 (0.1315)	0.1273 (0.2023)	33.68			
Mean	21.9	1.1911 (0.3035)	0.1165 (0.1651)	0.1790 (0.2507)*	38.34			
Average species level		1.4760 (0.3501)	0.2812 (0.1780)°	0.4235 (0.2441)†	84.97	0.5842	0.3558	0.5685

n, Sample size; A_e, effective number of alleles per locus; H_E, Nei's genetic diversity; S, Shannon's index; PPB, the percentage of polymorphic bands; G_{ST}, genetic differentiation between populations estimated by using POPGENE 1.31; N_m, estimated gene flow; Φ_{ST}, genetic differentiation between populations estimated by using AMOVA. Standard deviations are shown in parentheses; *S_{pop}; †S_{sp}; °S_T.

2.3. Data analysis

Since RAPD markers are dominant, it was assumed that each band represents the phenotype at a single biallelic locus (Williams et al., 1990). Amplified fragments were scored as "1" for presence and "0" for absence of homologous bands, and a matrix of different RAPD phenotypes was established and used for statistical analysis. Only clear, unambiguous, consistent and reproducible bands between 350 and 2500 bp were considered for data analysis. Weak bands were excluded. The binary data matrix was analysed using the program POPGENE version 1.31 (Yeh et al., 1999)[1], assuming Hardy–Weinberg equilibrium, to estimate the following genetic diversity parameters at population and species level: the percentage of polymorphic bands (PPB), the effective number of alleles per locus (A_e), Nei's (1973) gene diversity (H_E, also called expected heterozygosity) and total population gene diversity (H_T). The genetic diversity of the variation was also estimated using Shannon's index (S) (Lewontin, 1972). It was calculated as $S = -\sum p_i \log_2(p_i)$, where p_i is the frequency of a given RAPD band. The Shannon's index was calculated at two levels: the average diversity within population (S_{pop}) and gene diversity within the species (S_{sp}, total diversity). To examine the population genetic structure, the gene differentiation (G_{ST}) was estimated among the populations.

An analysis of molecular variance (AMOVA) was applied to estimate the variance components for RAPD phenotypes, partitioning the variation within and among populations. Input data files for the AMOVA v. 1.55 (Excoffier et al., 1992) were generated using AMOVA-PREP (Miller, 1998). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations.

The level of gene flow (N_m) was measured following Nei's (1973) gene diversity statistics. Gene flow was indirectly estimated among the populations by using the formula: $N_m = 0.5(1-G_{ST})/G_{ST}$ (McDermott and McDonald, 1993).

To visualize the genetic relationship among populations, a dendrogram was constructed based on Nei's genetic distance (D) by an unweighted pair-group method of cluster analysis using arithmetic averages (UPGMA) using Tools for Population Genetic Analysis (TFPGA) version 1.3 (Miller, 1997). In order to test for a correlation between Nei's genetic distance (D) between populations (Nei, 1972) and geographical distances (in km) among populations, a Mantel test was performed using TFPGA with 1000 permutations.

[1] Since this paper went to press, a revision of *Taxus* has been published by Spjut [Spjut, R. W., 2007. Taxonomy and nomenclature of *Taxus* (Taxaceae). J. Bot. Res. Inst. Texas 1, 203–289.] that recognizes 21 named species and three other unnamed species, 12 of them occurring in China. The name *Taxus fuana* Nan Li & R. R. Mill is replaced by the earlier but long overlooked name, *T. contorta* Griffith, published in 1854.

Table 4
Analysis of molecular variance (AMOVA) within and among *Taxus fuana* populations

Source of variation	d.f.	SSD	MSD	Variance component	Total variance (%)	*P*-value[a]
Among populations	9	3645	405	18	56.85	<0.001
Within populations	209	2860	14	14	43.15	<0.001

d.f., degree of freedom; SSD, sums of squares; MSD, mean square deviations.
[a] *P*-values are the probabilities of having more extreme variance component than the observed values by chance alone. Significance tests after 1000 permutation.

3. Results

3.1. Genetic diversity

The 16 selected RAPDs primers generated a total of 193 bands (loci) that ranged in size from 350 to 2500 base pairs (bp) across all 219 individuals of the 10 populations of *T. fuana*. The primers yielded 7–17 bands, with an average of 12.1 bands per primer. Among the 193 bands, 164 (84.97%) were polymorphic (Table 2). The percentage of polymorphic bands (PPB) at the population level was 38.34% on average, ranging from 29.53% (HZ) to 50.26% (CH) (Table 3). The average effective number of alleles per locus (A_e) was 1.1911 and 1.476 at the population and species level, respectively. Assuming Hardy–Weinberg equilibrium, the average gene diversity was estimated to be 0.1165 (H_E) within populations and 0.2812 (H_T) at the species level (Table 3). The Shannon's index was 0.1790 (S_{pop}) and 0.4235 (S_{sp}) at population and species level, respectively. Among the populations, PV and CH exhibited high levels of variabilities (PPB = 46.11 and 50.26%; $A_e = 1.2228$ and 1.2260; $H_E = 0.1343$ and 0.1418; $S = 0.2145$ and 0.2216, respectively), whereas the populations HZ and KK exhibited low levels of variabilities (PPB = 29.53 and 30.57%; $A_e = 1.1655$ and 1.1847; $H_E = 0.0983$ and 0.1066; $S = 0.1486$ and 0.1594, respectively) (Table 3).

3.2. Population genetic structure

Significant genetic differentiation was observed among the populations of *T. fuana*: the genetic differentiation among populations (G_{ST}) was estimated as 0.5842, indicating that 58.42% of the genetic variability was distributed among populations, or most of the total genetic diversity (H_T) in *T. fuana* was distributed among populations. Nei's genetic distance (D) varied from 0.018 to 0.3514 which suggested a high level of genetic differentiation among pairs of populations. Significant genetic differences ($P < 0.001$) among populations were also detected by AMOVA analysis. Of the total molecular variance, 56.85% variation was attributed to among population diversity and the rest (43.15%) within populations (Table 4). The overall level of inferred gene flow (N_m) was estimated as 0.3558 individuals per generation among populations, indicating a low gene flow and limited pollen and seed dispersal between populations. The result of the Mantel test revealed that the genetic divergence of populations was not significantly correlated with geographic distance (Mantel test, $r = 0.3134$, $P = 0.350$) (Table 5). This indicates that an 'isolation by distance' model was not supported.

Table 5
Geographic distance (km) (above diagonal) and Nei's (1972) genetic distance (below diagonal) among populations of *Taxus fuana* analysed

Pop ID	NM	HZ	KK	GG	JK	SW	PV	CH	TH	KV
NM	****	54	123	17	23	133	153	205	206	288
HZ	0.0340	****	96	70	74	93	108	170	210	300
KK	0.1446	0.1823	****	135	128	168	171	242	306	396
GG	0.2461	0.2687	0.2267	****	13	147	168	217	207	286
JK	0.2381	0.2689	0.1999	0.2732	****	155	175	226	219	298
SW	0.2745	0.3242	0.2276	0.2720	0.2380	****	26	77	165	259
PV	0.2156	0.2631	0.2225	0.3063	0.2509	0.2109	****	72	185	278
CH	0.2114	0.2589	0.2255	0.3011	0.2505	0.2122	0.0181	****	153	237
TH	0.2490	0.3076	0.2443	0.3514	0.2492	0.2086	0.1091	0.1093	****	94
KV	0.2578	0.3157	0.2877	0.3359	0.2830	0.2385	0.1794	0.1940	0.1680	****

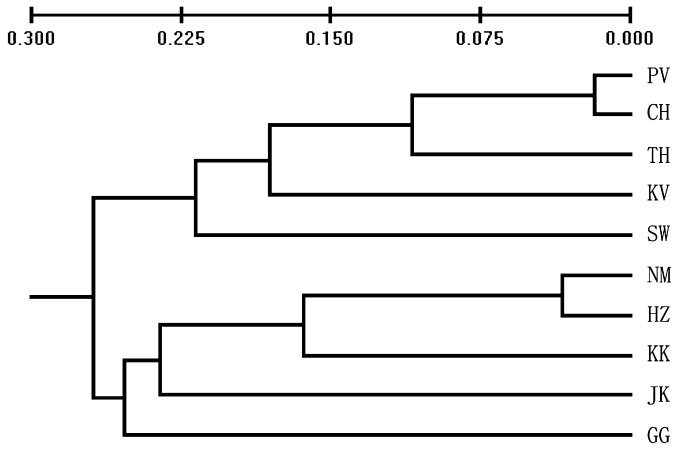

Fig. 2. UPGMA dendrogram based on Nei's (1972) genetic distance between the populations of *Taxus fuana* sampled in this study. Population abbreviations follow those in Table 1.

The UPGMA tree (Fig. 2) based on Nei's (1972) genetic distance (D) revealed that the 10 populations were separated into two clusters. One cluster included the five eastern populations (NM, HZ, KK, GG and JK) and the other cluster the remaining populations from central and western areas of North Pakistan (SW, PV, CH, TH and KV). Finescale spatial geographic patterns were not reflected in the groupings of populations in the two clusters (Figs. 1 and 2).

4. Discussion

4.1. Low genetic diversity within populations

The population genetic analyses of the RAPD data revealed low levels of genetic diversity within populations of *T. fuana* from Pakistan analysed here. As long-lived dioecious gymnosperms, the level of genetic variation in *Taxus* should theoretically be relatively high within populations and relatively low between populations (Hamrick et al., 1992). However, in the present study, the 10 populations of *T. fuana* here showed the opposite trend. Their genetic diversity within populations ($H_E = 0.1165$; $S = 0.1790$) was lower than observed for regionally distributed species ($H_E = 0.21$) or outcrossing species ($H_E = 0.27$) using the same data source (Nybom and Bartish, 2000; Nybom, 2004). The same was true for the average Shannon index, which was much lower ($S_{pop} = 0.179$) than the average value for other gymnosperms ($S_{pop} = 0.386$) or other long-lived perennial plants ($S_{pop} = 0.242$) determined in previous studies (Nybom and Bartish, 2000). Also *T. fuana* possessed a much lower PPB (average of 38.34%) at the population level compared to another *Taxus* species, *T. baccata* (PPB = 71%, Hilfiker et al., 2004). On the other hand, similarly low genetic population level variation as in *T. fuana* was found in other *Taxus* species, such as *T. wallichiana* from Northeast India using RAPDs (Saikia et al., 2000), *Taxus canadensis* (Senneville et al., 2001) and *T. brevifolia* (El-Kassaby and Yanchuk, 1994) although using allozyme markers. Hamrick and Godt (1989) found much higher variation for a further 55 gymnosperms species using allozyme markers (PPB = 70.9%), suggesting that *Taxus* species in general possess a low within population variation. The different values for *T. baccata* may partly be explained by the use of different RAPDs primers, rather than a difference in their reproductive biology, as *Taxus* are all wind-pollinated and bird or vertebrate dispersed, or ecology. A low within population genetic diversity has also been found in relict species, such as *Cycas guizhouensis* (Cycadaceae) (Xiao et al., 2004), *Ammopiptanthus mongolicus* (Fabaceae) (Ge et al., 2005b) and *Monimopetalum chinense* (Celastraceae) (Xie et al., 2005), with different mating and dispersal systems. Taxaceae are a relict family as well, which apparently originated in the lower or middle Jurassic judged from fossil evidence (Florin, 1958; Harris, 1979). Thus the presence of the low intra-population genetic diversity appears to be common among relict plants, independent from their reproductive biology, the reason for which is unclear. In general, geographically widespread species tend to possess higher genetic polymorphisms within populations than restrictedly distributed species (Hamrick and Godt, 1996). The distribution of *T. fuana* is rather restricted and the populations

collected for the present study originate from a narrow region of North Pakistan, from the westernmost area of distribution of the species. This scattered distribution in isolated populations may thus explain the low within population variation (Ge et al., 2005a).

4.2. Significant population differentiation among populations

The genetic structure of plant populations reflects the interactions of various evolutionary processes including the long-term evolutionary history, such as shifts in distribution, habitat fragmentation, and population isolation, mutation, genetic drift, mating system, gene flow, and selection (Schaal et al., 1998). Analysis of RAPD data using different statistics (POPGENE and AMOVA) demonstrated similar interpretations of the genetic structure of *T. fuana* in Pakistan. The values of genetic differentiation detected among populations of *T. fuana* ($G_{ST} = 0.5842$, $\Phi_{ST} = 0.5685$) were much higher than the average for outcrossing plant species ($G_{ST} = 0.22$, $\Phi_{ST} = 0.27$) and for gymnosperms ($G_{ST} = 0.18$, $\Phi_{ST} = 0.24$) (Nybom and Bartish, 2000; Nybom, 2004). The highly significant genetic differentiation among populations found in *T. fuana* is very unusual for outcrossing, wind-pollinated, and long-lived woody species with vertebrate dispersed seeds (Hamrick and Godt, 1996). However, in *T. fuana* this may be mainly due to limited gene flow and genetic drift. With the patchy distribution of the yew populations, and because of their scattered position in the forest understory, where turbulence and wind velocity are reduced, the incoming gene flow is likely to be reduced because of low pollen dispersal (Wheeler et al., 1995). In addition, most of the populations are restricted to distant, isolated mountains with intervening mountains and valleys which may reduce gene exchange between populations. These landscape will limit, but not completely prevent, among population gene flow (Hilfiker et al., 2004) and contribute to the low level of gene flow and the high level of inter-populational differentiation (Wallace, 2002). The low estimates for gene flow among populations of *T. fuana* ($N_m = 0.3558$) indicate that the range of wind-mediated pollen dispersal may not be as far as generally expected as illustrated in *Taxus* (Allison, 1990) and recently in pines (Dyer and Sork, 2002; Walter and Epperson, 2004). Similar genetic structures with above-average values of population genetic differentiation were detected in isozyme studies of the North American yew species *T. brevifolia* (El-Kassaby and Yanchuk, 1994; Wheeler et al., 1995) and *T. canadensis* (Senneville et al., 2001), and an ISSR study of the *Amentotaxus argotaenia* complex (Ge et al., 2005a). The same holds for the genetic partitioning of RAPD variation in the South American conifer *Pilgerodendron uviferum* (Allnutt et al., 2003) which like *Taxus*, is wind-pollinated, but unlike *T. fuana*, is wind-dispersed and monoecious, with a scattered distribution. Genetic drift can affect the genetic structure and increase differentiation among populations of a species with small and isolated populations (Barrett and Kohn, 1991; Ellstrand and Elam, 1993). As a result of habitat fragmentation, large, continuous populations are split into small and isolated entities, which might result in limited gene flow between the entities (Lande and Barrowclough, 1987), loss of genetic diversity within entities and increase in the genetic differentiation from other entities (Buza et al., 2000). The small, patchy and isolated populations of *T. fuana* are likely to have been subjected to such strong genetic drift. The limited gene flow between the populations of this species ($N_m = 0.3558$) was not enough to overcome the diversifying effects of random drift. The Mantel test and the UPGMA dendrogram indicated that genetic differentiation among populations of *T. fuana* seems to be not correlated with geographic distance among populations, which provides further evidence of genetic drift (Dodd and Helenurm, 2002). As discussed in other studies (e.g., Fischer et al., 2000), low or absence of such a correlation suggests an important role of genetic drift as in *T. fuana*, which is in line with the observed pronounced differentiation among populations.

4.3. Implications for conservation

Knowledge of the level and distribution of genetic variation is a prerequisite for the establishment of effective and efficient conservation practices (Ge et al., 1998). The generally low level of genetic variation of *T. fuana* apparently results from its past evolutionary processes, as well as some recent losses due to human activity. The pattern of population structure in *T. fuana* has important conservation implications. Loss of individuals at certain locations may not cause immediate loss in genetic diversity, but more damage may occur in terms of long-term genetic consequences due to reduced number of individuals and smaller population size. The conservation of endangered species depends upon maintaining a substantial population size (Carson, 1990). A major goal of conservation is the maintenance of genetic diversity and evolutionary processes in viable populations in order to prevent potential extinction (Falk and Holsinger, 1991; Godt and Hamrick, 1995). Loss of genetic diversity could lead to a decline in a species' ability to cope with

changing environment and demographic fluctuations both in the short and long term (Ellstrand and Elam, 1993; Milligan et al., 1994; Reisch et al., 2003).

Knowledge of the distribution of genetic variation of populations of rare and endangered species plays a key role in the formulation of appropriate conservation strategies (Falk and Holsinger, 1991; Francisco-Ortega et al., 2000). Considering the low genetic variation within population and significant genetic differentiation among population of *T. fuana* in Pakistan, preservation of only low genetic diversity populations would be insufficient to conserve all the genetic variation in this species. Thus, priority must be given to all the extant populations *in situ* and prevent further anthropogenic destruction. *Ex situ* conservation should be adopted and samples should be collected from as many populations as possible, especially those harbouring relatively high genetic diversity, such as population CH, NM in the current study. Because of its long life cycle, seed and germplasm collections in botanical gardens or other institutions should be of practical value for the conservation of genetic diversity in *T. fuana*. It is strongly recommended that seeds for propagating should be collected from different subpopulations to preserve as much genetic diversity as possible.

The present study shows that the patterns of genetic diversity of *T. fuana* found in the Western Himalayas are quite unique, with low within and high among population variation. *In situ* conservation measures and sustainable forest utilization should take this uniqueness into account. Given the low of allelic diversity within stands, the absence of obvious trends related to geography, special attention should be given to maintaining high genetic diversity of the species at landscape level, to ensure its long-term viability.

5. Conclusion

Through RAPD markers, the low level of genetic diversity within population and significant genetic differentiation among populations were revealed in 10 wild collected population of *T. fuana* in Pakistan, which may be the response to its evolutionary history, including habitat fragmentation, genetic drift and limited gene flow. The distribution of genetic variation and genetic structure of *T. fuana* populations was unexpected for this outcrossing, wind-pollinated and long-lived woody species, but may reflect its habitat preference as scattered understory tree. Further studies, including samples form the entire range of this species eastwards into Central Nepal, are required to fully understand the genetic make-up of this species, to locate hot spots of genetic diversity that may suggest Pleistocene refugia or the origin of this species.

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