African Journal of Biotechnology Vol. 8 (13), pp. 2948-2958, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Genetic variation within and among fragmented populations of endangered medicinal plant, *Withania coagulans* (Solanaceae) from Pakistan and its implications for conservation

Syed Abdullah Gilani[1,2]*, Akira Kikuchi[1] and Kazuo N. Watanabe[1]

[1]Gene Research Center, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba Ibaraki, 305-8572, Japan.
[2]Department of Botany, Kohat University of Science and Technology (KUST), Kohat, Pakistan.

Accepted 14 May, 2009

Withania coagulans **is an under-utilized, endangered medicinal plant that can be found scarcely as fragmented populations in South Asia. Theoretically, the fragmented population should show low diversity within population and higher diversity among population with low rate of gene flow. To test this hypothesis, we conducted diversity analysis of 7 populations of *W. coagulans* from the districts of Kohat and Karak in NWFP (North West Frontier Province), Pakistan using PBA (P450 based analogue) markers. Our results showed contrary findings from the hypothesis for fragmented population. The findings showed higher diversity within population and lower diversity among population like larger unfragmented populations even though the population size was less than 100 in all populations. Percentage of polymorphic bands (PPB) in these populations was 50 to 71.95% with gene diversity (H) of 0.192 to 0.298. AMOVA (Analysis of molecular variance) test showed that there was low genetic diversity among populations (17%) and high genetic diversity within population (83%). F_{ST} value showed low genetic differentiation (0.0911) and high gene flow (*Nm* 2.494) among populations. Isolation by Distance Model revealed no correlation between genetic and geographic distances as well as ecotypes (soil variation) vs genetic distances. Presumably, the larger fragments, formed long time ago due to geological changes, were reduced into smaller fragments recently due to the human pressure. the fruits of the plant are exploited for commercial purposes and the whole plant is used as fodder and fuelwood thus threatening the current survival of the limited fragmented populations. Conservation measures for the species existence are also discussed and recommended.**

Key words: Among population, genetic diversity, conservation, population biology, within population, *Withania coagulans*, Kohat plateau, Pakistan.

INTRODUCTION

Withania coagulans (Stocks) Dunal of family Solanaceae is an under utilized endangered medicinal plant, distributed in mediterranean and south Asian regions (Negi et al., 2006). *W. coagulans* grows wildly in Kohat Plateau in Karak and Kohat districts of NWFP (North West Frontier Province) Pakistan. The local people use the plant against various diseases like small pox, stomach aches, teeth cleaning, emmenagogue agent for females and pain killer for swollen testis in males (Anonymous, 2003; Ishtiaq et al., 2005, 2007). Besides the consumption of plant for fuel purposes, the leaves are used as febrifuge, vegetables and fodder for camel and sheep (Anonymous, 2003; Gilani et al., 2008).

The district of Kohat lies between 33° - 04' to 33° - 34' north latitudes and 70° - 29' to 72° - 01' east longitudes (Anonymous, 1999) and Karak between 70° - 40' to 71° - 30' north latitudes and 32° - 48' to 33° - 23' east longitudes (Anonymous, 2000). Total populations of Kohat and Karak

*Corresponding author. E-mail: gilani_abdullah@yahoo.com.

districts are 562,640 (Anonymous 1999) and 430,795 (Anonymous, 2000), respectively. The local people in both the districts use local plants for various medicinal purposes. Recent studies have shown that there are more than 100 medicinal plants growing wildly in the district of Kohat (Ilahi, 2008). Most of these medicinal plants are under severe human pressure, which are used as herbal medicines and collected for commercial purposes also. The fruits and whole plant of *W. coagulans* are collected for commercial purposes and as a source of fodder in both the districts. It has also been observed that *W. coagulans* populations are fragmented and contain a limited number of individuals. It is possible that continuous human pressure may rapidly diminish the local flora if proper conservation measures are not taken in time. The other living organisms dependent on *W. coagulans*, for example pollinators, will also be adversely affected (Breinholt et al., 2009) as well as the local people will lose the sources of herbal remedies and income too. Before making any decision for conservation planning, it is important to evaluate genetic diversity and population structure of endangered plants (Breinholt et al., 2009).

Genetic diversity studies of *W. somnifera* have shown considerable genetic variations while using AFLP (Amplified fragment length polymorphism) and SAMPL (Selectively amplified microsatellite polymorphic Loci) primers (Negi et al., 2000, 2006). However, diversity studies of *W. coagulans* have not been carried out till date especially within and among populations. All kinds of markers such as isozymes, PCR-RFLP (PCR based restriction fragment length polymorphism), RAPD (Random amplification of polymorphic DNA), AFLP (Amplified fragment length polymorphism), SSRs (Simple sequence repeats) and ISSRs (Inter-simple sequence repeats) etc are used to evaluate diversity in genetically neutral regions (Yamanaka et al., 2003). However, these markers do not cover the entire genome (Karp, 2002). Previously, our lab had developed PBA (P450 based analogue) markers as the functional genomic markers based on cytochrome P450 (Yamanaka et al., 2003). Cytochrome P450 play an important role in the process of oxidative detoxification and biosynthesis of secondary metabolites in higher plants (Kessman et al., 1990; Donaldson and Luster, 1991; Song et al., 1993; Teutsch et al., 1993; Ohkawa et al., 1998). PBA markers showed higher genetic diversity in 51 intra and interspecific plant species including potato (both wild and domesticated), tomato, sweet pepper and eggplant of solanaceae family (Yamanaka et al., 2003) and also differentiated between Myanmar bananas and international cultivars (Wan et al., 2005). These markers were not only successful for genetic diversity studies but also proved valuable markers to construct genetic maps in potatoes (Yamanaka et al., 2005). Looking at the previous scientific findings and successful results, these primer sets were selected to assess genetic variation in *W. coagulans*.

It was necessary to evaluate the population structure in fragmented populations of *W. coagulans* in Kohat Plateau. Theoretically, there must be lower genetic diversity within fragmented populations and higher among these populations, as these populations diverge more rapidly than larger populations (Frankham et al., 2007). To test this hypothesis, we selected the fragmented populations of *W. coagulans* for the study. Besides, knowing the evolutionary process and mechanism, evaluation of population genetic diversity also depicts the scenario of biological conservation issues of the species (Schaal et al., 1991). The objectives of our study were to assess genetic diversity within and among populations of *W. coagulans* in Kohat Plateau, to determine their population structure and assess their conservation status.

MATERIALS AND METHODS

Plant material collection

W. coagulans was collected from 7 different locations of Kohat and Karak districts of NWFP Pakistan (Table 1 and Figure 1). 5 leaves per plant were collected from 30 individuals per population covering an area of 10,000 m^2 per population. The fresh leaves were initially packed in paper envelop so that moisture is absorbed and then transferred to polyethylene bags with silica gel. The samples were labeled and stored at 4°C while the voucher specimens of the plant were submitted at the department of Botany, Kohat University of Science and Technology (KUST), Kohat, Pakistan.

Soil analysis

Soil was collected from 12 inches depth from each population site. The soil samples were packed in polythene bags and tested at soil testing laboratories of Agricultural Research Institute, Tarnab, Peshawar, Pakistan and Barani Area Research Station (BARS), Kohat, Pakistan.

DNA extraction

Genetic diversity studies were carried out at Gene Research Center, university of Tsukuba, Tsukuba, Japan. The method of Doyle and Doyle (1990) for genomic DNA extraction was followed with little modification. DNA was extracted from 187 individuals comprised of 7 populations. 0.2 g dried powdered leaves of *W. coagulans* were added into 700 µl micropreparation buffer (300 ul extraction buffer, 5% sarkosyl, 1% sodium bisulphite, 2% PVP (polyvinylpyrrolidone), 1% mercaptoethanol and 300 µl lysis buffer). The extraction buffer was prepared from 0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA (ethylenediaminetetraacetic acid) and lysis buffer from 0.2 M Tris, 0.05 M EDTA, 2 M NaCl (Sodium chloride), 2% CTAB (Cetyl Trimethyl Ammonium Bromide). The step of adding 700 µl chloroform /isoamylacohol (24:1) was repeated twice. DNA was precipitated with isoamyl alcohol and washed with 70% ethanol. DNA pellet was dissolved in 50 ul dDW. The quality and quantity of DNA was checked on 1% agarose gel and spectrophotometer at 260/280 nm.

PCR conditions and reaction

PCR conditions and reaction were followed as per protocol of Yamanaka et al. (2003) and Wan et al. (2005). 15 PBA primers sets were used for the current study (Tables 2 and 3) (Yamanaka et al., 2003). The reaction mixture of 25 µl contained 20 ng DNA, 1 X PCR

Table 1. Details of collection localities and total population size of *Withania coagulans* in Pakistan.

No.	Place	Pop. Size	Latitude	Longitude	Alt. (m)	Remarks
2007-7-07-3	Wanki Siraj Khel	<50	33°01'34.4"	71°05'48.2"	557	Plain, graveyard, bank of dried river
2007-7-07-3	Takht Nasrati	<50	33°00'29.8"	71°04'17.8"	531	Foothill of mountain, north facing slope
2007-7-08-3	Bahadar Khel	<50	33°10'06.8"	70°58'35.8"	558	North facing slope, plant found on soil of salt mountain
2007-7-08-5	Teri	<50	33°17'55.2"	71°02'50.5"	674	Small hill, north facing slop
2007-7-25-3	KDA	<100	33°36'44.4"	71°29'17.8"	541	Plain area, undeveloped part of residential area
2007-7-26-1	Sanda Fateh Khan	<100	33°23'44.2"	71°13'29.2"	581	South facing slope of mountain
2007-7-27-2	Ustarzai	<50	33°36'23.7"	71°13'23.9"	682	South facing slope of mountain

Pop. Size = number of individuals per 10,000 m^2 area. Alt. = Altitude.

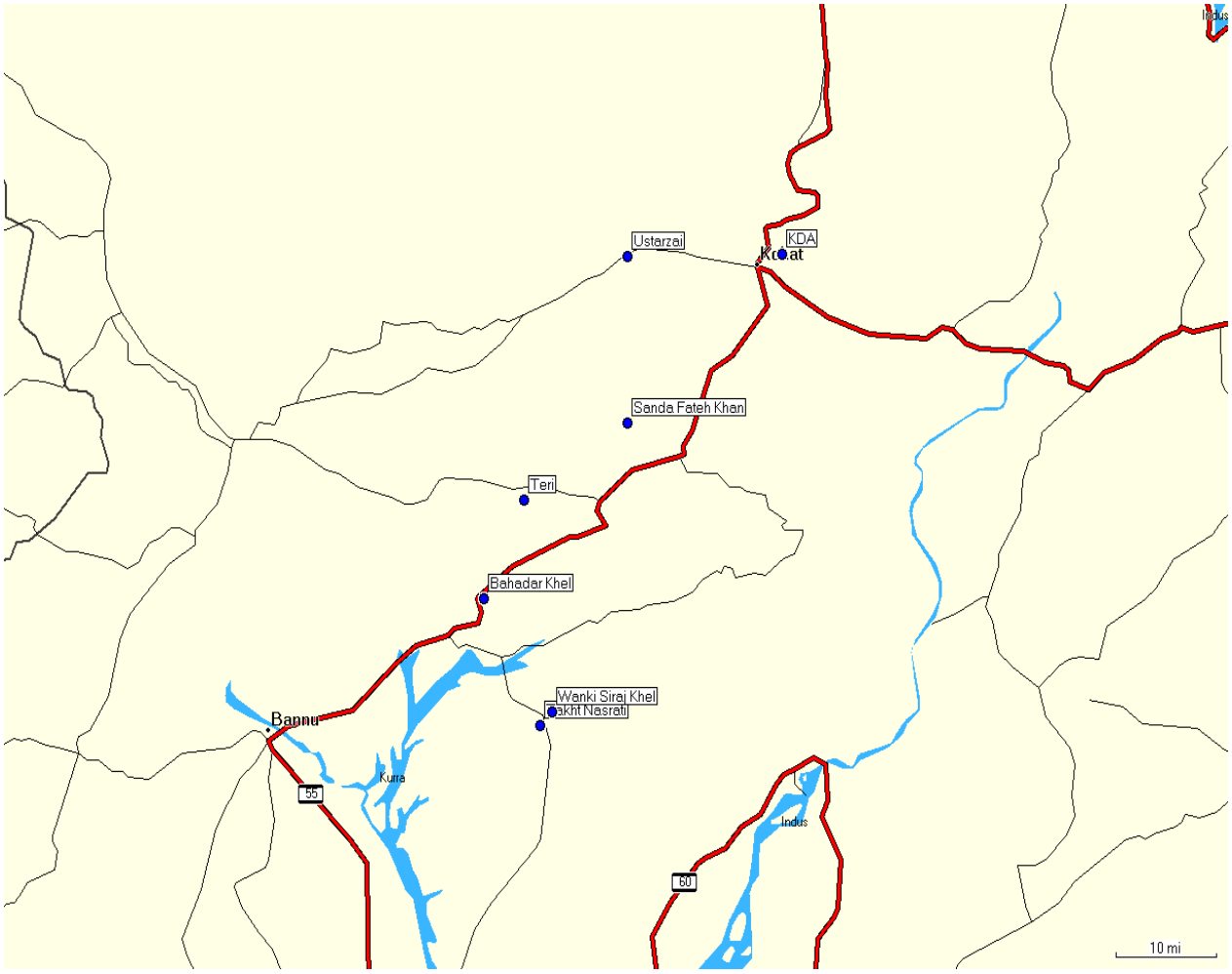

Figure 1. Map based on coordinates of collection localities for *W. coagulans* from 7 populations.

buffer (Takara, Japan), 0.16 mM of dNTPs, 1 mM of each primer and 1 unit of Taq polymerase (Takara, Japan). A total reaction of 32 cycles comprised of 1 min at 92°C, 2 min for annealing temperature and 3 min at 72°C in a thermal cycler (Yamanka et al., 2003). PCR

Table 2. PCR primers used for genetic diversity study.

Primer	Sequence (5′to 3′)	References
CYP1A1F	GCC AAG CTT TCT AAC AAT GC	Inui et al. (2000), Yamanaka et al. (2003)
CYP2B6F	GAC TCT TGC TAC TCC TGG TT	Inui et al. (2000), Yamanaka et al. (2003)
CYP2C19F	TCC TTG TGC TCT GTC TCT CA	Inui et al. (2000), Yamanaka et al. (2003)
CYP1A1R	AAG GAC ATG CTC TGA CCA TT	Inui et al. (2000), Yamanaka et al. (2003)
CYP2B6R	CGA ATA CAG AGC TGA TGA GT	Inui et al. (2000), Yamanaka et al. (2003)
CYP2C19R	CCA TCG ATT CTT GGT GTT CT	Inui et al. (2000), Yamanaka et al. (2003)
Heme2B6	ACC AAG ACA AAT CCG CTT CCC	Kiyokawa et al. (1997), Yamanaka et al. (2003)
Heme2C19	TCC CAC ACA AAT CCG TTT TCC	Kiyokawa et al. (1997), Yamanaka et al. (2003)

Table 3. Details of 13 PBA primer-sets, which amplified polymorphic bands in 187 individuals of 7 *Withania* populations.

No.	Primer – set (Forward and Reverse)*	*Tm* (°C)*	Total Loci	No. of PB	PPB (%)
P 02	CYP1A1F/CYP2B6R	52.0	7	4	57.14
P 03	CYP1A1F/CYP2C19R	46.5	8	8	100.00
P 04	CYP1A1F/heme2B6	56.0	4	3	75.00
P 05	CYPIAIF/heme2C19	56.0	2	0	0.00
P 06	CYP2B6F/CYP1A1R	52.0	3	2	66.67
P 07	CYP2B6F/CYP2B6R	52.0	8	8	100.00
P 08	CYP2B6F/CYP2C19R	46.5	11	10	90.91
P 09	CYP2B6F/heme2B6	52.0	10	9	90.00
P 10	CYP2B6F/heme2C19	52.0	8	8	100.00
P 11	CYP2C19F/CYP1A1R	56.0	1	0	0.00
P 13	CYP2C19F/CYP2C19R	46.5	8	8	100.00
P 14	CYP2C19F/heme2B6	56.0	8	6	75.00
P 15	CYPI2C19F/heme2C19	56.0	4	3	75.00
Total			**82**	**69**	**71.52**

* Yamanaka et al. (2003).

products were electrophoresed on 2% agarose gel and then stained in ethydium bromide for 1 h. The bands were scored as ‘0’ for absent and ‘1’ for present and the data was recorded in MS Excel worksheet.

Statistical analysis

For calculating sample size and maximum number of individuals from the area (n), observed number of alleles (na), effective number of alleles (ne), Nei’s (1973) genetic diversity (H), shannon’s information index (I) and percentage of polymorphic bands, statistical software, POPGENE 1.31 (Yeh et al., 1999) was used assuming Hardy- Weinberg equilibrium.

To estimate variation within and among populations, AMOVA (Analysis of molecular variance) was also applied. AMOVA was calculated through another statistical package GenAlEx 6 (Peakall and Smouse, 2006). Estimation of the level of gene flow (*Nm*) and evaluation of population structure (F_{ST}) was also measured using AFLPSURV following Lynch and Milligan (1994). The gene flow among population was estimated by using the formula, $Nm = (1/F_{ST} - F_{ST})/4$ (Frankham et al., 2007). GenAlEx was also used for Principal Coordinate Analysis (PCA) and Mantel test to evaluate correlation between geographic and genetic distances and between soil variations and genetic variaton. Dendrogram was drawn based on Nei’s genetic distances using UPGMA (Unweighted pair group method with arithmatic mean) (Lynch and Milligan, 1994).

RESULTS AND DISCUSSION

Soil analysis

Altitudinal range of growing habitat of *W. coagulans* in Kohat Plateau was 531 - 682 m (Table 1). Soil analysis showed an arid habitat for *W. coagulans*. The results revealed that *W. coagulans* grows on variety of soils ranging from sandy to sandy loams with high level of potassium and pH (8 - 8.8) (Table 4). NPK (nitrogen, phosphorus, potassium) test showed that there was lesser amount of nitrogen (0.025 - 0.065 ppm), low to higher concentration of phosphorus (4.95 - 59.15 ppm) and higher amount of potassium (133 - 266 ppm) in the soils. The amount of organic matter was 0.51 - 1.31%

Table 4. Soil analysis of localities of Districts of Karak and Kohat in NWFP Pakistan.

Locality No.	Locality	Texture	Organic Matter (%)	Major Elements (ppm)			pH	EC (dSm^{-1})	TSS
				N	P	K	1:5 Ratio		%
District Kohat									
2007-7-25-3	KDA	Sandy Loam	1.20	0.060	59.15	266.0	8.20	0.10	0.032
2007-7-26-1	Sanda Fateh Khan	Sandy Loam	1.31	0.065	07.57	133.0	8.00	0.09	0.029
2007-7-27-2	Ustarzai	Sandy Loam	0.72	0.036	04.95	180.0	8.10	0.09	0.029
District Karak									
2007-7-07-3	Wanki Siraj Khel	sand	0.51	0.025	26.78	237.0	8.00	4.90	0.028
2007-7-07-3	Takht Nasrati	sand	0.72	0.036	28.39	180.0	8.20	0.12	0.038
2007-7-08-3	Bahadar Khel	sand	0.69	0.034	37.85	180.0	8.30	0.16	0.051
2007-7-08-5	Teri	silty clay loam	0.69	0.034	23.66	228.0	8.80	0.08	0.026

O.M. = organic matter, E.C. = electrical conductivity, N = Nitrogen, P = Phosphorus, K = Potassium, TSS = total soluble salts, dSm^{-1} decisiemens per meter.

which described its negligible amount in the soils. Salinity test showed that *W. coagulans* usually grows on low saline soils with EC (Electrical conductivity) 0.08 - 0.16 dSm^{-1} (decisiemens per meter) except the locality of Wanki Siraj Khel where there was comparatively higher salinity (4.9 dSm^{-1}).

Comparing the soil characteristics of district Kohat with Karak, sandy loamy soil was the characteristic of 3 localities in Kohat while in Karak, the soil was sandy in nature. Organic matter contents were neglibly low (0.51 - 1.31%) in all the 7 localities, however these were higher in Kohat as compared to district Karak. Salinity level in district Kohat was also low as compared to district Karak. The salinity level at Teri was lower (0.08 dSm^{-1}) than rest of the other collection localities of the species. Rest of the parameters of the soil showed mixed pattern in all the 7 habitats of *W. coagulans*. These results showed that *W. coagulans* is resistant to unfavourable conditions and can grow in such circumstances also.

An interesting distribution pattern of *W. coagulans* showed that it was growing on south facing slopes in Kohat, while Karak vegetation (Table 1) was growing on north facing slopes of hills/mountains thus confirming the geography and flora of Kohat Plateau that was formed in Eocene era (Meissner et al., 1974; Shah, 2003). This pattern shows that districts of Kohat and Karak form a valley of Kohat Plateau from north (Kohat) and extending towards south (Karak).

KDA is situated at the foothill area of Kohat range. Ustarzai is between Kohat and Samana ranges towards west. Sanda Fateh Khan, Teri, Bahadar Khel also known as Karak Mudstone (Saleemi and Ahmad, 2000), Takht Nasrati and Wanki Siraj Khel are regions of Surghar range. We also divided these 7 populations into 3 geological areas within Kohat Plateau, KDA as the foothill of Kohat range, Ustarzai as area between Kohat and Samana ranges and rest of the populations as Surghar range because of their closed vicinity. Ancient populations of endemic *Viola calaminaria* had higher variation in the heavy metals than the recent ones (Bizoux and Mahy, 2007). These 2 populations represented the pattern of clonal diversity and genetic structure in heterogeneous conditions of heavy metal stress (Bizoux and Mahy, 2007). However, in our case, we did not find this kind of pattern when we divided these populations into geological zones.

Genetic diversity within and among population

Among 15 primer sets, 11 primer sets gave amplification for *W. coagulans* populations (Table 3). Total detected loci were 82 and percentage of polymorphic bands for all primers was 71.52% (Table 3). Percentage of polymorphic bands (PPB) in 7 populations of *W. coagulans* was 50 to 71.95% with gene diversity (H) of 0.192 to 0.298 (Table 5). The populations of Takht Nasrati and Ustarzai had higher polymorhic bands of 60.98 and 71.95% within populations, respectively. While lowest polymorphic bands 50 and 54.88% were observed in Teri and Wanki Siraj Khel populations respectively. Shannon index (I) varied from 0.284 to 0.431. Overall gene diversity (Ht) was 0.3228 (Table 8). AMOVA test showed that there was low genetic diversity among populations (17%) and high genetic diversity within population (83%). Our results also showed lower F_{ST} value (0.0911) and higher rate of gene flow (*Nm*) (2.494).

Inflorescence of *W. coagulans* is polygamodioecious that is, pistillate and bisexual flowers grow on one plant and staminate and bisexual flowers on other plant (Clarke, 1916; Nasir, 1985; Hunziker, 2001). Self compatibility is the characteristic of *W. coagulans* and the common characteristic of Solanaceae (Whalen and Anderson, 1981). Self compatibility leads to gender dimorphism that promotes outcrossing and prevent inbreeding depression resulting from self fertilization (Lloyd, 1976; Charlesworth and Charlesworth, 1978 a, b; Anderson and Symon, 1989;

Table 5. Genetic variation within population of *Withania coagulans* in NWFP Pakistan.

Population	n*	na	ne	H	I	PPB (%)
Wanki Siraj Khel	20	1.549 (0.501)	1.408 (0.411)	0.228 (0.219)	0.330 (0.311)	54.88
Takht Nasrati	30	1.610 (0.410)	1.425 (0.413)	0.237 (0.216)	0.346 (0.304)	60.98
Bahadar Khel	17	1.586 (0.496)	1.389 (0.383)	0.224 (0.207)	0.331 (0.296)	58.54
Teri	30	1.500 (0.503)	1.331 (0.380)	0.192 (0.205)	0.284 (0.296)	50.00
KDA	30	1.586 (0.496)	1.390 (0.380)	0.226 (0.206)	0.333 (0.295)	58.54
Sanda Fateh Khan	30	1.634 (0.485)	1.443 (0.413)	0.248 (0.214)	0.361 (0.301)	63.41
Ustarzai	30	1.720 (0.452)	1.534 (0.390)	0.298 (0.203)	0.431 (0.287)	71.95
Average species level		1.829 (0.379)	1.477 (0.367)	0.278 (0.186)	0.417 (0.254)	59.75
Mean	187					

n = sample size and maximum number of individuals from the area; na = Observed number of alleles; ne = Effective number of alleles; H = Nei's (1973) gene diversity; I = Shannon's Information index; PPB = percentage of polymorphic bands.

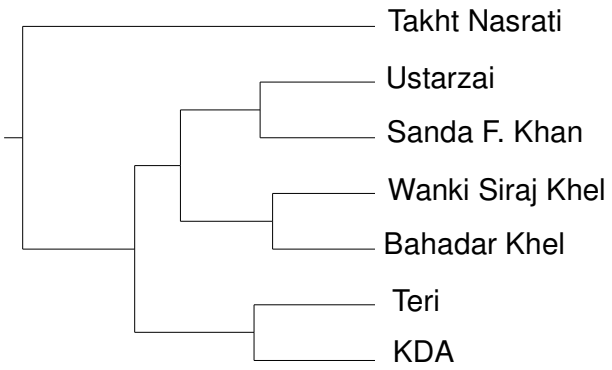

Figure 2. UPGMA dendrogram illustrating genetic relationships of 7 populations of W. coagulans based on Nei's genetic distance among different populations.

Sakai and Weller, 1999). Our results are also confirming that despite the human pressure on *W. coagulans* and limited population size (Table 1), within population genetic variation was high due to the outcrossing nature of the species.

PBA markers have already been studied in various species (Yamanaka et al., 2003, 2005; Wan et al., 2005) that showed high diversity in these species. Another set of P450 genes (CYP28A1) showed considerable genetic diversity in *Drosophila mettleri* collected from different host plant species (Bono et al., 2008). The idea of selection of marker sets shall support relevantly for the genetic diversity study.

The populations of *W. coagulans* showed high level of genetic diversity within population (Table 8). Similarly, the gene flow was also high among population of *W. coagulans* (2.494 *Nm*). In addition, the results of AMOVA showed significant differences within and among population variation (Table 7). Smaller populations should diverge more rapidly than larger populations which can be observed from higher F_{ST} values and low *Nm* value. F_{ST} above about 0.5 is considered to be an indication of significant differentiation among fragments (Frankham et al., 2007). Our results showed lower F_{ST} value (0.0911) as compared to outcrossed animal pollinated (0.22) (Hamrick and Godt, 1989) and outcrossed wind pollinated plant species (0.10) (Hamrick and Godt, 1989). The rate of gene flow (*Nm*) was high (2.494). Contrary to our findings, Shah et al. (2008) had observed high genetic differentiation among population and low gene flow (*Nm* 0.3558) in fragmented *Taxus fuana* populations from Pakistan.

Cluster analysis (Figure 2) and Principal Coordinate Analysis (PCA) (Figure 3) showed that population of Takht Nasrati was separated from the rest of the populations. The population of Wanki Siraj Khel made cluster with neighbouring area, Bahadar Khel, which is at the distance of 19.6 km, but not with Takht Nasrati which was only 2.8 km far away from it (Table 6). The cluster analysis and PCA separated the populations into two major clusters. One of the clusters showed population of *W. coagulans* from Takht Nasrati as a distint group. Takht Nasrati population is distinct from the rest of the population. This population may be the part of other populations possibly dispersed from other neighbouring geographic zones into Kohat Plateau. The current geopolitical situation in the country does not allow to conduct ellaborated research on these populations. However, in future, if the conditions are favorable, the research may be further extended to other geological zones of the country.

Isolation by Distance Model

Mantel test with lowest R^2 value (0.0112) showed that there was no correlation between genetic and geographic distances (Table 6 and Figure 4). We also compared the soil characteristics as ecotypes with genetic variations among populations of *W. coagulans* using Mantel test (Data not shown), however, no correlation between ecotypes and genetic variation could be sorted out.

Mantel test confirmed that there was no correlation between geographic distance and genetic variation among populations of *W. coagulans*. Comparing soil com-

Table 6. Nei's genetic distance (below diagonal) and geographic distance in km (above diagonal).

Population	KDA	Sanda FK	Ustarzai	Wanki SK	Takht Nas	Bahad. K	Teri
KDA	0.000	34.4	24.7	74.6	77.3	68.4	54.2
Sanda FK	0.052	0.000	23.6	42.6	45.0	33.9	19.9
Ustarzai	0.045	0.011	0.000	65.5	67.8	53.7	38.3
Wanki SK	0.032	0.020	0.041	0.000	2.8	19.6	30.3
Takht Nas	0.079	0.064	0.045	0.064	0.000	20.2	32.0
Bahad. K	0.047	0.016	0.035	0.008	0.073	0.000	15.3
Teri	0.015	0.047	0.045	0.030	0.069	0.042	0.000

Table 7. Analysis of Molecular Variance (AMOVA) within and among 7 populations of *W. coagulans.*

Source	df	SS	MS	Estimated variance	% age	F -Value	P value
Among Populations	6	277.722	46.287	1.469	17%		
Within Populations	180	1315.343	7.307	7.307	83%	0.167	0.010
Total	186	1593.065	53.594	8.776			

Df = degree of freedom, SS = sum of squares, MS = Mean square.

Table 8. Gene diversity within 7 populations of *W. caogulans.*

N	Ht	Hw	Hb	Fst
7	0.322800	0.293400	0.029400	0.0911
Standard Error	0.013750	0.005679	0.184083	
Variance	0.000189	0.000032	0.033887	

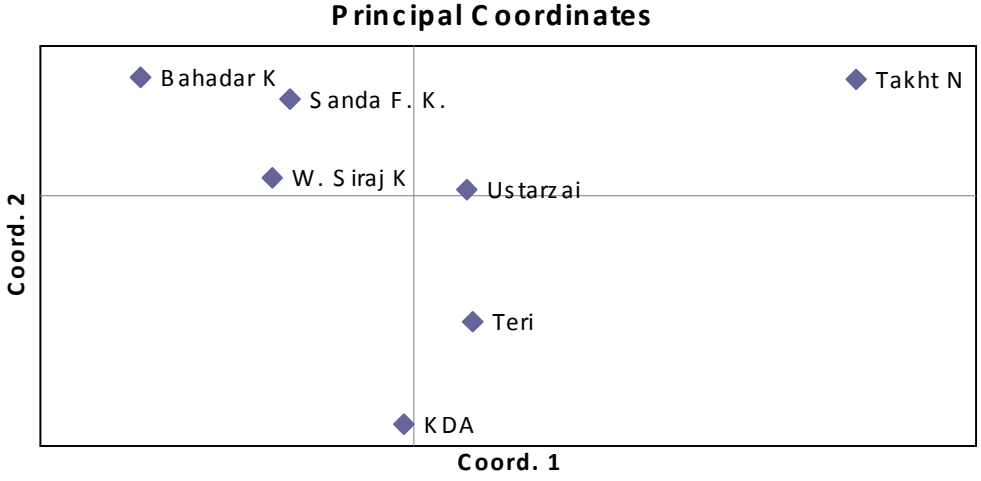

Figure 3. Principal Coordinate Analysis illustrating genetic differences among 7 populations of *W. coagulans.* Coordinate 1 is accounted for 34.23% and coordinate 2 for 25.77% of the total variation among populations.

ponents with genetic distances of populations, Mantel test also showed no correlation.

Genetic diversity pattern was not in uniformity with geological zones. Geographic distances, soil factors as ecotypes and geological zones showed no correlation with genetic diversity in *W. coagulans*. In contrast, Lin et al. (2007) had found considerable correlation between ecotypes (soil water contents and soil salt contents) and

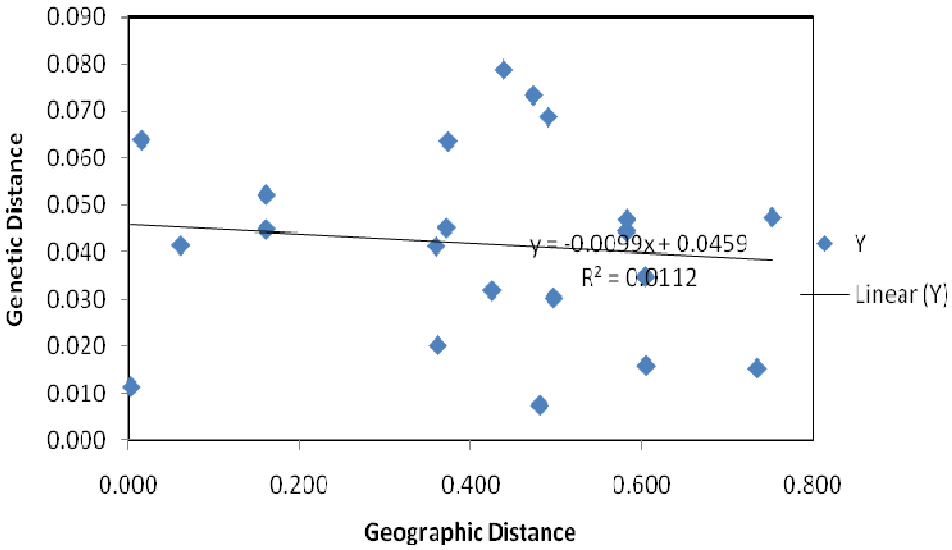

Figure 4. Graph showing correlation between geographic (x-axis) and genetic distances (y-axis) of *W. coagulans* populations in Kohat Plateau, Pakistan.

genetic distances of reeds (*Phragmites communis* Trin.). Zhang et al. (2007) found remarkable differentiation between soil-watery ecotypes in *japonica* rice than in *indica* rice. Similarly ecological - genetic microniche correlation analysis in wild barley showed differentiation between stressful sun rock microniche and least stressful shade soils microniche (Owuor et al., 2003).

Despite the small hills everywhere, the larger fragmented populations in past might have high gene flow between populations because of closed vicinity. Human pressure caused reducing the population sizes. White et al. (1999) also proposed the same when they got similar results from *Swietenia humilis* populations. Muir et al. (2004) also reported low genetic differentiation among population and high gene flow in *Quercus petraea* from Ireland. They reported a common phylogeographic history and absence of founder effect in these populations. They proposed that approximately 100 generations separated the current cohort of trees from the first post-refugial colonizers to Ireland. Roosa and Pia (2005) also observed low variation among populations (F_{ST} 0.052) of a herb, *Vincetoxicum hirundinaria*, in the southwestern Archipelago in Finland.

Our results showed no proper genetic structure between populations that is, low genetic differentiation among population and lack of relationship between genetic and geographic distances (including soil factors). The high diversity within population and low diversity among population is common in larger populations (Frankham et al., 2007). But our case is different in terms that our populations are fragmented with limited total size of less than 100 and 50 individuals (2 populations with 20 and 17 individuals only (Table 5), the case should be *vice versa* for fragmented populations (Frankham et al., 2007).

All of these populations seem to be descendents of a single population. However, there is possibility that there might be a single large population of *W. coagulans* in Kohat Plateau which converted into larger fragments that persisted for a long time due to geological changes in area (Muir et al., 2004). The current study and our field survey reveal that mostly these larger fragments reduced again into smaller fragmented populations due to the human pressure (White et al., 1999) on collection of the fruits of *W. coagulans* for the commercial purposes, thus not allowing the plant to produce progenies. Similarly, the whole plant is utilized by the local people for fuelwood thus not allowing the existence of current vegetation which is also one of the factors for fragmentation of *W. coagulans* populations.

Conservation status

Occurrence of *W. coagulans* in Kohat has been reported in 1908, as a general flora of Peshawar district when Kohat and Karak were administrative parts of Peshawar district (Anonymous, 1908). Currently, *W. coagulans* is exploited for its fruits which are sold commercially at herbal shops and the whole plant is used as fodder and fuelwood. Due to these factors the frequency of plant population is decreased. More than 10 years ago, one could see its distribution everywhere especially in North West Frontier Province (NWFP) of the country. But astonishingly its population is declined and it can be seen only at few places. Anonymous (2002) studied the phytosociology of the same belt of district Kohat, and analysed 26 stands selected from different places, *W. coagulans* was recorded in two plots only with relative frequency of 1.85 and 8.1%. In another study (unpublished), we observed that *W. coagulans* usually

forms community either with *Rhazya stricta* and/or *Justicia adhatoda*. During our field survey, we selected 18 hotspots to collect populations of *Withania* from Kohat and Karak but astonishingly we could find 7 populations only (Table 1) from all these 18 localities (Figure 1), 3 from Kohat and 4 from Karak. The rate of declination of *W. coagulans* is high in Kohat Plateau. We did not find the vegetation of *W. coagulans* near the roads where it was expected and present few years ago.

Current findings of distribution pattern of *W. coagulans* on north and south facing slopes in Kohat and Karak districts of Kohat Plateau (Table 1) shows the Kohat and Karak as valleys of Kohat Plateau where it was frequently distributed. Now the vegetation is confined only towards slopes and vanished from the plains of the valleys (Kohat Basin). While counting individuals in these fragments, it was noted that all of these populations were not more than 100 individuals per population (Table 1). In 2 populations of Wanki Siraj Khel and Bahadar Khel, the total number of individuals was 20 and 17 respectively (Table 5). Such a critical small number of populations and rapid fragmentation of the habitat is an alarming sign of probable extinction of the species in the near future, if such practice of local people of exploiting the plant is continued. The ultimate consequences of habitat fragmentation will be loss of genetic diversity and population extinction due to increased mating chances between closely related individuals and high genetic divergence among population (Hunter, 1996; Qian et al., 2001; Zhao et al., 2008).

Possibly human pressure has reduced larger stable fragmented population of *W. coagulans* into limited sized populations. The local people are collecting all the fruits from these plants so that the populations have less chances to propagate their seeds for regeneration. Similarly, the whole plant of *W. coagulans* is used as fodder and fuelwood, so the existing population is also in danger of extinction. Wide range of habitat from the soil analysis confirms that the species is resistant to grow in unfavorable arid conditions. Therefore, it may be concluded that human pressure might be one of the possible major factors of fragmentation and reduction of population size of *W. coagulans* instead of arid harsh environment of both the districts of Kohat and Karak.

Rapid declination of population of *W. coagulans* also suggests planning and recommending conservation measures. Keeping in view all of these circumstances, it is clear that all the populations have critical population sizes and need equal attention but Ustarzai population contains high genetic diversity (71.95%) therefore, this population needs attention to be conserved as *in situ* conservation. Forest department and NGOs in Pakistan are working for the protection of forests, they must collaboratively give proper attention to under-utilized crops also and select *W. coagulans* as the case study to propagate its vegetation in the area. Group efforts for conservation from every discipline from biosciences to legal advisors and policy makers are necessary as recommended by WHO/IUCN/ WWF (World Health Organization, International Union for Conservation of Nature, World Wide Fund for Nature) guidelines for conserving medicinal plants (Anonymous, 1993). Though *ex situ* conservation is not the replacement of *in situ* conservation but it may be considered as the compelementary effort to conserve maximum genetic diversity from all the populations of *W. coagulans* in the country (Anonymous, 1993). The germplasm needs also to be preserved in the gene bank (Anonymous, 1993) and to be cultivated in national parks (Protected Areas) and institutional botanical gardens of the country (Anonymous, 1993; Shah et al., 2008). However, for sustainable harvesting of *W. coagulans*, it is necessary to provide complete guidelines to the local communities in a published form for proper plant collection, harvesting of fruits and drying and storage methods to protect the habitat from the over-exploitation of natural resources (Gilani et al., 2007).

Conclusion

Kohat Plateau is also an important territory from geological point of view as it is the part of the Himalayan range in Pakistan and was formed in Eocene period. It is also important due to the geopolitical situation with boundaries attached to southern tribal belt and Afghanistan. *W. coagulans* is threatened medicinal plant in Kohat Plateau due to human pressure for herbal medicines and commercial exploitation. Therefore the population size of *W. coagulans* was decreased more critically less than 100 and all the test populations were fragmented. The general hypothesis for fragmented populations is high genetic differentiation and low gene flow among populations and lower diversity within population. Our results showed contrary findings to the general hypothesis of fragmented populations. Despite the limited size, all the populations showed high genetic diversity within population, high gene flow and low genetic differentiation among populations like normal populations. There might be a larger population which might have converted first into larger fragments with closer boundaries so gene flow was high. Recently, these populations might have reduced into limited number of individuals due to human pressure, therefore, the divergence among populations is not seen. However, in future, with the loss of populations, this divergence may be very clear, if no conservation measures are taken in time. It is recommended that *W. coagulans* may be considered as endangered medicinal plant and be conserved on the basis of sustainable harvesting.

ACKNOWLEDGEMENTS

This research work was conducted at University of Tsukuba, Tsukuba, Japan as a part of the doctoral degree

research of the principal author. The principal author was financially supported by Japanese government MEXT scholarship during his whole stay in Japan. All the travel and field expenses were sponsored by Kohat University of Science and Technology (KUST) under the joint collaborative project titled, “Establishment of Linkages with University of Tsukuba (Japan) for Joint Research and Education Program”, between KUST and University of Tsukuba which was funded by Higher Education Commission (HEC) Pakistan.

REFERENCES

Anderson GJ, Symon DE (1989). Functional dioecy and andromonoecy in *Solanum*. Evol. 43: 204-219.

Anonymous (1908). The Imperial Gazetteer of India. Vol. XX. Pardi to Pusad. New Ed., Oxford, Clarendon Press.

Anonymous (1993). Guidelines on the conservation of medicinal plants. WHO/IUCN/WWF document. Website http://www.wwf.org.uk/filelibrary/pdf/guidesonmedplants.pdf. [Accessed 5 June 2008].

Anonymous (1999). District Census Report of Kohat. Publication Census Organization, Statistics Division, Government of Pakistan, Islamabad.

Anonymous (2000). District Census Report of Karak. Publication Census Organization, Statistics Division, Government of Pakistan, Islamabad.

Anonymous (2002). Survey of the status, distribution and home range requirements of the Punjab Urial. WWFP Pakistan. Website http://www.wwf pak.org/sgp/pdf/species/4 urial project. pdf. [Accessed 3 June 2008].

Anonymous (2003). Wealth of India. Council of Scientific and Industrial Research, New Delhi, India. 10: p. 580.

Bizoux JP, Mahy G (2007). Within-population genetic structure and clonal diversity of a threatened endemic metalophyte, *Viola calaminaria* (Violaceae). Am. J. Bot. 94: 887-895.

Bono JM, Matzkin LM, Castrezana S, Markow TA (2008). Molecular evolution and population genetics of two *Dropsophila mettleri* cytochrome P450 genes involved in host plant utilization. Mol. Ecol. 17: 3211-3221.

Breinholt JW, Buren RV, Kopp OR, Stephen CL (2009). Population genetic structure of an endangered Utah endemic, *Astragalus ampullarioides* (Fabaceae). Am. J. Bot. 96: 661-667.

Charlesworth B, Charlesworth D (1978a). A model for the evolution of dioecy and gynodioecy. Am. Naturalist 112: 975-998.

Charlesworth D, Charlesworth B (1978b). Population genetics of partial male sterility and the evolution of monoecy and dioecy. Heredity 41: 137-154.

Clarke CB (1916). Solanaceae. In Hooker JH (ed) Fl. Brit. Indian 4: p. 240.

Donaldson RP, Luster DG (1991). Multiple forms of plant cytochromes P450. Plant Physiol. 96: 669-674.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Frankham R, Ballou JD, Briscoe DA (2007). Introduction to Coservation Genetics. Cambridge University Press, UK.

Gilani SA, Kikuchi A, Shinwari ZK, Khattak ZI, Watanabe KN (2007). Phytochemical, Pharmacological and Ethnobotanical Studies of *Rhazya stricta* Decne. Phytother. Res. 21: 301-307.

Gilani SA, Fujii Y, Watanabe KN (2008). Monograph on *Withania coagulans* (Stocks) Dunal. Plant Genetic Resources Monograph Series (PGRM) 6. Mimatsu Corporation Tokyo, Japan.

Hamrick JL, Godt MJW (1989). Allozyme diversity in plant species. In Brown AHD, Clegg MT, Kahler AL (eds) Plant Population Genetics, Breeding, and Genetic Resources. Sinauer, Sunderland, MA, pp. 43-63.

Hunter ML (1996). Fundamentals of Conservation Biology. Blackwell Science, London.

Hunziker AT (2001). *Genera Solanacearum*: the genera of the Solanaceae Illustrated, arranged according to the new system. A. R. G. Gartner, Verlag K., Ruggel, Liechtenstein.

Ilahi I (2008). Ethnobotanical studies and problems associated with regeneration of herbals in Kohat region. Pak. J. Bot. 40: 1743-1753.

Inui H, Komada T, Ohkawa Y, Ohkawa H (2000). Herbicide metabolism and cross-tolerance in transgenic potato plants co-expressing human *CYP1A1, CYP2B6* and *CYP2C19*. Pestic. Biochem. Physiol. 66: 116-129.

Ishtiaq M, Khan MA, Shah A (2005). Plants used for family planning and sex disease treatment in Samahni Valley Pakistan. Ethnobotanical Leaflets International Web Journal. Website http://www.siu.edu/~ebl/leaflets/. [Accessed 4 April 2008]

Ishtiaq M, Hanif W, Khan M, Ashraf M, Butt AM (2007). An ethnomedicinal survey and documentation of important medicinal folklore food phytonims of flora of Sahahni Valley (Azad Kashmir), Pakistan. Pak. J. Biol. Sci. 10: 2241-2256.

Karp A (2002). The new genetic era: will it help us in managing genetic diversity? In Engels JMM, Rao VR, Brown AHD, Jackson MT (eds) Managing plant genetic diversity. Wallingford, CAB Publishing, pp. 43-56.

Kessman H, Choudhary AD, Dixon A (1990). Stress responses in alfalfa (*Medicago sativa* L.). III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts. Plant Cell Report 9: 38-41.

Lin W, Chen L, Zhu X (2007). An analysis of genetic diversity of different ecotypes of reed by molecular marker techniques. Zhiwu Shengli Yu Fenzi Shengwuxue Xuebao 33: 77-84. In Chinese.

Lloyd DG (1976). The transmission of genes via pollen and ovules in gynodioecious angiosperms. Theor. Pop. Biol. 120: 571-585.

Lynch M, Milligan BG (1994). Analysis of population genetic structure with RAPD markers. Mol. Ecol. 3: 91-99.

Meissner CR, Master JM, Rashid MA, Hussain M (1974). Stratigraphy of the Kohat Quadrangle, Pakistan. U.S. Geological Survey Professional Paper 716-D: p. 89.

Muir G, Lowe AJ, Fleming CC, Vogl C (2004). High nuclear genetic diversity, high levels of outcrossing and low differentiation among remnant populations of *Quercus petraea* at the margin of its range in Ireland. Annals Bot. 93: 691-697.

Nasir YJ (1985). Solanaceae In Nasir et al. (eds.) Flora Pak. 168: 1-61.

Negi MS, Singh A, Lakshmikumaran M (2000). Genetic variation and relationship among and within *Withania* species as revealed by AFLP markers. Genome 43: 975-980.

Negi MS, Sabharwal V, Wilson N, Lakshmikumaran MS (2006). Comparative analysis of the efficiency of SAMPL and AFLP in assessing genetic relationships among *Withania somnifera* genotypes. Curr. Sci. 91: 464-471.

Nei M (1973). Analysis of gene diversity in subdivided populations. PNAS 70: 3321-3323.

Ohkawa H, Imaishi H, Shiota N, Yamada T, Inui H, Ohkawa Y (1998). Molecular mechanisms of herbicide resistance with special emphasis on cytochrome P450 mono-oxigenases. Plant Biotechnol. 15: 173-176.

Owuor ED, Beharav A, Fahima T, Kirzhner VM, Korol AB, Nevo E (2003). Microscale ecological stress causes RAPD molecular selection in wild barley. Genet. Res. Crop Evol. 50: 213-224.

Peakall R, Smouse PE (2006). GENALEX6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6: 288-295.

Qian W, Ge S, Hong DY (2001). Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. Theor. Appl. Genet. 102: 440-449.

Roosa L, Pia M (2005). Population history, mating system, and fitness variation in a perennial herb with a fragmented distribution. Conserv. Biol. 19: 349-356.

Sakai AK, Weller SG (1999). Gender and sexual dimorphism in flowering plants: a review of terminology, biogeographic pattern, ecological correlates and phylogenetic approaches. In Geber MA, Dawson TE, Delph LF (eds) Gender and sexual dimorphism in flowering plant, Springer-Verlag, New York, New York, USA pp. 1-31.

Saleemi AA, Ahmad Z (2000). Mineral and chemical composition of Karak mudstone, Kohat Plateau, Pakistan: implications for smectite-illitization and provenance. Sediment. Geol. 130: 229-247.

Schaal BA, Leverich WJ, Rogstad SH (1991). Comparison of methods for assessing genetic variation in plant conservation biology. In Falk and Holsinger (eds) Genetics and Conservation of Rare Plants, Oxford University Press, New York, pp. 123-134.

Shah S (2003). A comparative study of structural styles in the Kohat Plateau, NW Himalayas, NWFP, Pakistan. Ph.D. Thesis. National Centre of Excellence in Geology, University of Peshawar, Pakistan.

Shah A, Li DZ, Gao LM, Li HT, Moller M (2008). Genetic diversity within and among populations of the endangered species *Taxus fuana* (Taxaceae) from Pakistan and implication of its conservation. Biochem. System. Ecol. 36: 183-193.

Song WC, Funk CD, Brash AR (1993). Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. PNAS 90: 8519-8523.

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier JM, Jeltsch JM, Durst F, Reichhart DW (1993). Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. PNAS 90: 4102-4106.

Wan Y, Watanabe JA, YI SS, Htaik T, Win K, Yamanaka S, Nakamura I, Watanabe KN (2005). Assessment of genetic diversity among the major Myanmar banana landraces. Breed. Sci. 55: 365-369.

Whalen MD, Anderson GJ (1981). Distribution of gametophytic self-incompatibility and infrageneric classification in *Solanum*. Taxon 30: 761-767.

White GM, Boshier DH, Powell W (1999). Genetic variation within a fragmented population of *Swietenia humilis* Zucc. Mol. Ecol. 8: 1899-1909.

Yamanaka S, Suzuki E, Tanaka M, Takeda Y, Watanabe JA, Watanabe KN (2003). Assessment of cytochrome P450 sequences offers a useful tool for determining genetic diversity in higher plant species. Theor. Appl. Genet. 108: 1-9.

Yamanaka S, Ikeda S, Imai A, Luan Y, Watanabe JA, Watanabe KN (2005). Construction of integrated genetic map between various existing DNA markers and newly developed P450-related PBA markers in diploid potato (*Solanum tuberosum*). Breed. Sci. 55: 223-230.

Yeh FC, Yang RC, Boyle T (1999). Microsoft Windows-based freeware for population genetic analysis. Release 1.31. Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada.

Zhang H, Sun J, Wang M, Liao D, Zeng Y, Shen S, Yu P, Mu P, Wang X, Li Z (2007). Genetic structure and phylogeography of rice landraces in Yunnan, China, revealed by SSR. Genome 50: 72-83.

Zhao NX, Gao YB, Wang JL, Ren AZ (2008). Population structure and genetic diversity of *Stipa grandis* P. Smirn, a dominant species in the typical steppe of the northern China. Biochem. Syst. Ecol. 36: 1-10.