Genetic diversity and morphological variation of *Pinus gerardiana* along the environmental gradient from Zhob, Balochistan, Pakistan

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**Abstract.** Gul J, Saeed S, Ahmed A, Leghari SK, Basit A, Rehman A, Khan MZ. 2021. Genetic diversity and morphological variation of *Pinus gerardiana* along the environmental gradient from Zhob, Balochistan, Pakistan. *Nusantara Bioscience* 13: 121-128. *Pinus gerardiana*, an evergreen gymnosperm, is an economically- and ecologically valuable tree found in the Takht Suleman Mountain Range, Zhob northeastern edge of the Balochistan, Pakistan. The present study is based on the assessment of the impact of environmental gradients with special reference to altitudinal gradient and soil variables on morphological, phytochemicals and genetic variation of *P. gerardiana*. A total of 27 genotypes of *P. gerardiana* were collected from the three elevation zones ranging from 2000 - 3500 m above sea level. The genetic diversity was assessed by combined markers, the random amplified polymorphic DNA (RAPD) and the Inter Simple Sequence Repeat (ISSR). Polymorphic bands analyzed the data to generate a dendrogram, based on the unweighted pair group method with arithmetic mean (UPGMA). Morphological characters assessed the morphological characters. Phytochemicals were investigated; the total phenolic content and total flavonoid contents were estimated and compared amongst all accessions. Our results revealed variations along altitudinal gradients and related to soil characteristics. The populations at the Middle altitude zone have greater diversity than populations at lower and higher altitudes. The resulting altitudinal variation can be attributed to various geographical and environmental conditions. These results can help in conservation and cultivation of this economically important tree.

**Keywords:** Altitudinal gradient, soil variables, phytochemicals, *Pinus gerardiana*, Zhob Forest, Pakistan

**INTRODUCTION**

The *Pinus gerardiana* Wall. ex D.Don, locally known as "Chilgoza" or "neozia pine", is considered one of the most promising trees with ecological and economical values around the world. This tree plays a very important role in the economic progress and livelihood of communities living close to the Forest (Shalizi and Khurram 2016). The tree is utilized for food, medicine and timber by the native communities of the area and wildlife. The tree contributes to the local income and revenue, sustains the soil surface, sustains a suitable microclimate, provides shelter, and is a refuge for animals. In Pakistan, 20% of the forest consists of *Pinus* trees, which can survive in harsh, cold environmental conditions, including excessive drought, high wind, and severe cold. The mountains of Sulaiman hold the world’s largest expanse of Chilgoza (over 260 km²) (Ahmed et al. 2011). The Chilgoza forests are under constant threats and pressure from the timber Mafia. This species is listed as highly threatened due to excessive cutting. During their explorations, many different analysts have identified the adverse anthropogenic activities in the area with regards to this tree, such as collecting for fuelwood, overgrazing and burning, which are responsible for the significant destabilizing influence in the forests (Ahmed et al. 2009; Beg and Mirza 1984; Hussain 2013).

The environmental factors, soil variables and amount of precipitation have influenced the growth of the Chilgoza pine tree. Natural regeneration of Chilgoza pine is very poor or entirely lacking in this zone (Kumar et al. 2013; Kumar et al. 2016). Genetic diversity provides the template for adaptation and evolution of populations and species.

Genetic variation is the key factor for the conservation of biodiversity (Thomas et al. 1999). In recent years, PCR-based molecular markers have allowed the use of DNA sequences in genetic analyses to provide a better understanding of the genetic diversity and differentiation of natural populations (Malik et al. 2008). Genetic make-up plays an important role in diverse ecosystems (Meloni et al. 2006). Recent research identifies better the role of molecular markers to assess the genetic diversity amongst and within the population. Nowadays, different molecular primers are in use for analysis of genetic diversity. RAPD markers are quite suitable for DNA fingerprinting as they are rapid and easy to assess (Kernodle et al. 1993). The Inter Simple Sequence Repeat (ISSR) is also used efficiently as no prior sequence is required (Adams et al. 2003). Loss of genetic variability is a major problem in biological diversity conservation because it inhibits a species from responding to natural selection and limits its evolutionary potential. If the harmful source of genetic diversity is known, different resources can be used efficiently in conservation.

In view of the aforementioned, the main objective of this study was, therefore, to assess the genetic diversity and population structure of economically important tree *P. gerardiana*. This would aid in defining conservation
studies for the declining *P. gerardiana* population in Balochistan, Pakistan. Intraspecific variation at morphological and molecular level through morphological and molecular markers, assessment of ecological diversity of the area and its impact on the genetic diversity help for a better understanding of the genetic structure of the species.

**MATERIALS AND METHODS**

**Study site and ecological diversity of site**

Zhob Forest of Balochistan, Pakistan is part of the Sulaiman Mountain Range that located in the eastern edge of the Iranian Plateau, where the Indus River separates it from the subcontinent (Figure 1). Its elevation is approximately 3,380 m above sea level (Table 1). The study area exhibits a dry temperate climate characterized by long winters from October to April and short summers from June to August. Though rain is scarce, precipitation is received mostly in the form of snow during winter. The data pertaining to meteorological conditions of the area during the study period are presented (Figures 2.A-B).

| Sampling site          | Site no. | Code | Latitude (N) | Longitude (E) | Elevation (m asl.) |
|------------------------|----------|------|--------------|---------------|--------------------|
| Lower elevation zone   | 1        | L1   | 31.55°       | 69.91°        | 2000               |
|                        | 2        | L2   | 31.56°       | 69.92°        | 2050               |
|                        | 3        | L3   | 31.55°       | 69.92°        | 2090               |
| Mid semi-arid zone     | 4        | M1   | 31.58°       | 69.93°        | 2800               |
|                        | 5        | M2   | 31.57°       | 69.92°        | 2950               |
|                        | 6        | M3   | 31.57°       | 69.93°        | 2960               |
| Upper arid zone        | 7        | U1   | 31.53°       | 69.91°        | 3440               |
|                        | 8        | U2   | 31.57°       | 69.82°        | 3365               |
|                        | 9        | U3   | 31.54°       | 69.91°        | 3380               |

**Figure 1.** Map of study site in Zhob Forest, Balochistan, Pakistan

**Figure 2.** A. Temperature and B. Annual precipitation of the study site in 2018-2019
Sample collection
A survey was conducted during 2018-2019 to locate the populations of *P. gerardiana*. The whole distribution area of the species was divided into three zones, viz. lower elevation zone, mid semi-arid zone and upper arid zone. Data from three to five sites were recorded from each elevation zone. Soil samples were collected from each site for soil characterization. Fresh leaf samples for DNA extraction were taken from three randomly chosen trees in each site.

Ecological characteristics
Different ecological characteristics of the study sites were measured including microclimatic conditions and edaphic factors such as soil.

Elevation and aspects
Elevations of study sites were measured during sampling with the help of Global Positioning System (GPS). Aspects of the area were noted using Compass.

Temperature and annual precipitation
Temperature and annual precipitation data were collected for the years 2018-2019 from the metrological survey center, arid zone Quetta, Balochistan Pakistan.

Soil analysis
Composite soil samples were collected from the surface layer 0-30 cm depth. Samplings were made with systematically-randomized method (Zare et al. 2011). Samples were dried at room temperature, sieved with 2 mm sieve and then stored in zip bag for further analysis. Physical and other Characteristics were examined at “Peer Mehar Ali Shah Arid Agriculture University”, Rawalpindi, Pakistan.

Survey of price list
To check the economic importance and economic value of plant, market survey was carried out. Price list was compared with open market (Table 2).

Morphological diversity analysis
To analyze the morphological diversity, tree samples were collected from nine sites at three different elevation zones. Samples were pressed and mounted on herbarium sheets. Data were analyzed by using morphological characters (S1).

Phytochemical analysis
**Total flavonoid content (TFC)**
The TFC was estimated by Ordonez et al. (2006) technique using equation \( Y=0.0255x, \ R^2=0.9812 \) absorbance at 420 nm.

**Total phenolic content (TPC)**
The TPC was calculated by the method of Slinkard and Singleton (1977) at 765 nm absorbance and expressed as mg/g tannic acid equivalent using the equation \( Y=0.1216x, \ R^2=0.9365 \).

**Genetic diversity analysis**
Fresh pine needles were collected for DNA extraction and assessment of genetic diversity was undertaken by using molecular markers. The method of Saeed et al. (2017) was used for extraction and purification.

**PCR reaction**
A total of thirteen primers were used, including twenty RAPD and ten ISSR, amongst them thirteen were polymorphic (Table 3). The reaction mixture was used by also following the method of Saeed et al. (2017).

**Statistical analysis**
For molecular markers, the amplified bands were scored as 1 (present) and 0 (absent), and data were clustered (dendrograms) based on similarity matrices using the paired group method with the help of software NTSYS 2.10 (Rohlf 1998). For soil data and phytochemical parameters, Agglomerative hierarchical clustering was performed using Minitab software.

Table 2. Market survey of *Pinus gerardiana* during 2018-2019

| Year | Price in Zhob market | Price in open market |
|------|----------------------|----------------------|
| 2018 | 1500-3000 PKR/kg (10-20 USD/kg) | 6000-6500 PKR/kg (37-41 USD/kg) |
| 2019 | 2500-3600 PKR/kg (16-24 USD/kg) | 6200-7600 PKR/kg (39-48 USD/kg) |

Table 3. Details of polymorphic primers used in the study of *Pinus gerardiana*

| Primer name | Sequence 3′—5′ | TA (°C) | TB | P | PB % |
|-------------|---------------|--------|-----|---|-----|
| OPA-2 | TGCCGAGCTG | 36 | 11 | 7 | 0.64 |
| OPA-3 | AGTCAGCCAC | 34 | 7 | 3 | 0.43 |
| OPA-4 | AATCGGGGCTG | 34 | 12 | 5 | 0.42 |
| OPA-7 | GAAACGGTGTG | 34 | 6 | 3 | 0.50 |
| OPA-8 | GTGACGTAGG | 36 | 4 | 2 | 0.50 |
| OPA-13 | CAGCACCAC | 36 | 11 | 8 | 0.73 |
| OPA-17 | GACCGTGTGT | 36 | 10 | 7 | 0.70 |
| OPA-20 | GTTGGCAGATCC | 36 | 7 | 6 | 0.86 |
| UBC-810 | (GA)8T | 52 | 6 | 2 | 0.33 |
| UBC-832 | (AT)7 TYC | 46 | 12 | 8 | 0.67 |
| UBC-844 | (CT)8RC | 47 | 10 | 7 | 0.70 |
| UBC-850 | (GT)8YC | 47 | 7 | 4 | 0.57 |
| UBC-857 | (ACA)5CYG | 52 | 6 | 4 | 0.67 |
| Total | | 109 | 66 | 66 | 0.61 |

Note: TA (°C): annealing temperature, T: Total bands, P: Number of polymorphic bands, PB: percentage of polymorphism.
RESULTS AND DISCUSSION

This is the first comprehensive report on morphological, chemical and genetic diversity of an economically important tree, *Pinus gerardiana*, from the Zhob Forest, Balochistan, Pakistan. *P. gerardiana* is an ecologically- and economically valuable species, distributed in different parts of the world. In Pakistan, it is distributed in high mountainous zones of the Pakistani-Afghan border, Sulaiman Mountain Range, and Kashmir.

Economic importance

*Pinus gerardiana* is used as edible nuts and as medicinal plants in folk medicines by the local community of the study area. Economically, it is a very important nut. The local communities of the study area are categorized in the low-earning income bracket. No proper source of income has been identified, except for the collection of pine nuts, cutting of pine trees, and grazing in order to sustain their livelihood. The study area is in a remote, difficult geographical zone of the mountain, with a low literacy rate and under the threat of floods and cross-border disturbance. Moreover, no proper health facilities are available. A price survey was made in the year 2018-19. In 2018, it ranged from 1500-3000 PKR/kg (10-20 USD/kg) and increases to 2500-3600 PKR/kg ((16-24 USD/kg) in 2019. The price of nuts in the open market of Quetta and the rest of Pakistan is almost double that of the local market.

Environmental characteristics

The environmental parameters of the study sites varied along the altitudinal gradient (Table 4). The pH ranges from 6.64 to 7.45 from high elevation to low elevation. The total organic matter (2.63 ± 0.11%) was highest at high elevation. Soil at mid-elevation has high sand content (52.12 ± 4.18%), high elevation has high percentage of silt and clay (36.15 ± 2.65 and 18.12 ± 2.66, respectively). Sodium, potassium, and nitrogen were also reported highest at the high elevation zone. The concentrations of heavy metals, nickel and zinc (0.10 ± 0.04 and 0.04 ± 0.00 mg kg⁻¹, respectively) were high at the low elevation zone.

Morphological diversity

Morphology of the studied samples revealed that no variation was found in the studied population. In qualitative characters, no differences were found. The cone shape was nearly spherical, and cone color at maturation was dark brown. Seed shape is like a banana and the seed color was light to dark brown. Leaves were modified into needles that are dark green in color. Seed color varied along the altitudinal gradient. Seeds of high elevation were dark brown to black, while at low elevation, their color was light brown to reddish. Leaves were in fascicles of three needles. Quantitative characters showed a variation that may be due to the age of the tree or may be due to the impact of other environmental factors (biotic and abiotic factors). The height of tree was up to 40 ft (13 m) approximately. DBH of tree was up to 4 m. Leaf length 13 cm. Fruit size was 5 cm in length and 10 mm in width. The cone size ranged from 12 cm in length to 7 cm in width.

On the basis of quantitative characters (S1), cluster analysis was performed for all collected samples from three elevations. The cluster analysis revealed that there was a variation among the samples (Figure 2). This variation may due to anthropogenic activities or may be due to the impact of other environmental factors (biotic and abiotic factors). The age of the tree is the major factor in the variation of collected samples. Environmental factors and the soil type may also influence the size or growth of tree. As the moisture increases, the growth ring size also increases.

Genetic diversity

In the present results, 18 out of 30 primers produced clear bands in all collected samples. Two different marker systems, i.e., RAPD and ISSR were used for the first time on *P. gerardiana* from three elevation zones of Sulman Mountain Range. The amplified band size ranged from 200 to 1500 bp for RAPD and 150 to 1600 bp for ISSR (Figures 3 and 4). We used combined RAPD and ISSR markers to generate a dendrogram by cluster analysis. A combined marker system approach to detect polymorphism could be useful in removing errors and targeting various sites of genome as was previously used by (Saeed et al. 2020; Saeed et al. 2017).

Table 4. Mean values of environmental parameters along altitudinal gradient

| Variables                | Low elevation (Mean ± S.E) | Mid elevation (Mean ± S.E) | High elevation (Mean ± S.E) |
|--------------------------|----------------------------|----------------------------|----------------------------|
| **Topographic variables**|                            |                            |                            |
| Elevation (m)            | 2000 ± 50                  | 2650 ± 45                  | 3200 ± 45                  |
| Slope                    | 32.50 ± 2.32               | 44.50 ± 5.75               | 48.45 ± 4.35               |
| **Edaphic variables**    |                            |                            |                            |
| pH                       | 7.45 ± 0.12                | 7.23 ± 0.14                | 6.64 ± 0.17                |
| Total organic matter (%) | 1.62 ± 0.54                | 2.37 ± 0.14                | 2.63 ± 0.11                |
| Sand (%)                 | 50.3 ± 2.47                | 52.12 ± 4.18               | 45.32 ± 3.36               |
| Silt (%)                 | 33.21 ± 2.61               | 32.01 ± 2.57               | 36.15 ± 2.65               |
| Clay (%)                 | 16.07 ± 1.35               | 15.36 ± 1.45               | 18.12 ± 2.66               |
| Sodium (mg kg⁻¹)         | 11.45 ± 1.37               | 15.36 ± 2.34               | 17.33 ± 1.45               |
| Potassium (mg kg⁻¹)      | 6.74 ± 1.77                | 8.73 ± 1.75                | 11.18 ± 1.35               |
| Nitrogen (%)             | 3.17 ± 0.44                | 3.28 ± 0.08                | 3.81 ± 0.74                |
| Nickel (mg kg⁻¹)         | 0.10 ± 0.04                | 0.08 ± 0.00                | 0.09 ± 0.03                |
| Zinc (mg kg⁻¹)           | 0.04 ± 0.00                | 0.02 ± 0.04                | 0.03 ± 0.01                |

Note: S.E.: standard error
Out of the twenty-five markers from the tested primers, thirteen primers had amplified polymorphic bands. Eight were from RAPD and five from ISSR and exhibited polymorphism, showing reproducible bands amongst nine *P. gerardiana* accessions. Table 4 identifies the characteristics of banding patterns obtained from the primers. Thirteen markers amplified 109 total bands and 66 were polymorphic (61% polymorphism). The total number of RAPD and ISSR bands scored per primer also varied. The overall data revealed an average of 8.38 bands obtained per primer.

Based on the UPGMA tree (Figure 5), the cluster is delimited into three main clusters showing the variation within the species along the altitudinal gradient. The Middle elevation zone retains the highest genetic diversity. It may be due to better environmental conditions and fewer anthropogenic activities.

The UPGMA clustering has been based on RAPD and ISSR populations. The cluster consists of two groups cluster A and cluster B. Cluster A consists of two subgroups of populations from the High elevation and Middle elevation zones, while cluster B comprised of populations from the low elevation zone.

**Chemical diversity**

All accessions of *P. gerardiana* were characterized as having significantly varied levels of total phenolic content (TPC), expressed as tannin equivalent and flavonoids contents as quercetin equivalent within and amongst populations (Figure 6). The TPC ranged from 40 to 58 mg\(^{-1}\) within the population. Flavonoids also showed a diverse pattern amongst all sites, ranging from 104 to 123 mg g\(^{-1}\). Based on combined data of TPC and flavonoid contents, the dendrogram generated two main clusters: A and B. Cluster A comprised the population from high. Cluster B comprised of accessions from the middle elevation zone and low elevation zones (Figure 7).

**Discussion**

*Pinus gerardiana* grows in the tropical to subtropical regions of Pakistan. The seeds are used as edible nuts and medicine by the local people in the vicinity of the study sites. The *P. gerardiana* tree is facing the threat from anthropogenic activities like other conifers and conservation action needs to be undertaken on an urgent basis. The tree grows in high elevation cold climatic zones (Kumar et al. 2016). The present study aimed to investigate the genetic variability and the impact of environmental gradient with special reference to altitude and soil for this economically important tree. Molecular, phytochemical, and morphological variations were assessed on different samples collected along three elevation zones. From earlier reports, no evidence had been found on the genetic variation of *P. gerardiana* by using markers from a comparison of three different elevations. The regeneration pattern was studied for conservation as well as the population structures by (Akbar et al. 2014; Aziz et al. 2017).
Figure 6. Patterns of phytochemical variations amongst different populations along elevation gradients. Site codes as: L: Low elevation, M: Middle, H: High.

Figure 7. Dendrogram constructed using phytochemical diversity of samples from different populations along elevation gradients. Site codes as: L: Low elevation, M: Middle, H: High

Due to the importance of this valuable seed, it is greatly in demand, not only in the local market, but also from national and international markets (Peltier and Dauffy 2009). Earlier reports on an open market survey showed its prices to be approximately US$ 20-30 per kg. Open market prices are very high compared with the local market and best quality seed from the area is exported to other parts of the country and around the world (Akbar et al. 2013; Khan et al. 2015). In the global market, Pakistan, India and Afghanistan contribute the largest quantities of nut (Akbar et al. 2014; Kumar et al. 2014). In the present research shows intra-species genetic variation of *P. gerardiana* using RAPD and ISSR marker techniques. Eighteen genotypes from three elevation zones were assessed. Thirteen markers amplified 109 total bands and 66 were polymorphic (61%). The results suggest that RAPD markers show high genetic polymorphism in their capacity for producing polymorphic amplicons. Similar results were obtained by (Sinha et al. 2013) on the genetic polymorphism of *Pinus roxburghii*. Their study also justified the importance of genetic variations in both ex-situ and in situ conservations. In the present study, we found that chemical variation along altitudinal gradients may be a response to UV radiation. This may suggest the species adaptation to strong UV radiation and low-temperature environments at higher elevations. Flavonoid content is the main source for plant protection from UV
radiation, and protects plant tissues. Moreover, many studies have provided new evidence that UV light induces the synthesis of flavonoids (Berli et al. 2010; Saeed et al. 2018).

Population structure of P. gerardiana is affected by different ecological factors like elevation, climate and soil variability. Similar findings were reported earlier (Kumar et al. 2013; Kumar et al. 2016; Sáenz-Romero et al. 2011; Sharma 2005) for P. gerardiana. In the present study, high genetic diversity within populations may be attributed to the effect of the environmental factors (Hahn et al. 2012; Sani et al. 2018) and anthropogenic activities in agreement with (Saeed et al. 2017). Climatic factors are associated with micro-geographical genetic differences, which may cause the phenotypic plasticity buffers against environmental changes over a plant’s life cycle. Further, it weakens over time as climatic event changes.

Measurement of genetic diversity through molecular markers is difficult as it shows the adaptations of environment and other conditions. (Jump and Penuelas 2014). Earlier (Aziz et al. 2017) suggested the conservation of P. gerardiana for the future of this valuable Pine forest. This condition would have further broader implications, both for the ecosystem and livelihood of the local people. In conclusion, based on our findings, it is concluded that there is an important genetic and phytochemical variation along the altitudinal gradient among the P. gerardiana populations. Such patterning of genetic and phytochemical differentiation could result from the environmental (temperature, precipitation and soil characteristics) and the human disturbance variation along the altitudinal gradient. Our findings could help to design a conservation program that should include implementation of sustainable management plans, considering the large ecological and economic local importance of this pine species.

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