



## Estimation of genetic diversity in *Cupressus sempervirens* growing in different ecological zones of Malakand Division, KP, Pakistan

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### Abstract

The nature and magnitude of geographic genetic diversity among 11 genotypes of Pakistani *Cupressus sempervirens* was studied through Sodium DodecylSulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Five populations representing different ecological conditions were sampled. A total of five loci (bands) were detected in the collected germplasm of *cupresses sempervirens* genotypes. Only one locus (locus-1) was found monomorphic while the remaining were polymorphic loci. Out of polymorphic loci, locus-5 showed a high degree of intra-specific locus variation (*ISLV*) i.e. 54.54% followed by Locus-3 which indicated 36.36% *ISLV* in the collected germplasm of *C. sempervirens* genotypes. Intra specie locus contribution towards genetic dis-agreement (*LCTGD*) was 80%. It was found that the genotypes of each zone occupied separate cluster in the dendrogram. PCA was carried out to complement the cluster analysis information. SDS-PAGE profiling based on two-way cluster plotting successfully resolved the genotypes on the basis of zone distribution. For better understanding of the genetic structure of *C. sempervirens* further analysis using dominant molecular markers are recommended.

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## Introduction

*Cupressus sempervirens* is a conifer tree belongs to genus *Cupressus* of family Cupressaceae. It is a high altitude growing plant and mostly distributed in North America, the Mediterranean region and subtropical Asia (Rawat *et al.*, 2010). The tree of *Cupressus* is used ornamentally, fuel, shelter belt formation, boundary hedges, prevent soil erosion. A good quality timber and essential oils can be also obtained from it (Andreoli & Enopoulos 1990). Due to the importance of *C. sempervirens* all around the Mediterranean area and in other parts of the world, where this species has been introduced, there is considerable interest in the geographic patterns of genetic diversity (Korolet *et al.*, 1997). So many different methods were adapted for the study of geographic pattern of genetic diversity including morphological characterization and molecular techniques (Nisaret *et al.*, 2008). The emergence of molecular techniques got exceptional attention due to its increased use in crop improvement, selection of desirable genotypes for breeding crops and estimation of taxonomic relationship of many plant species. Among all molecular techniques, SDS-PAGE is a useful tool to evaluate genetic variability in several plant species (Wadood *et al.*, 2016 and Nisaret *et al.*, 2016). SDS-PAGE is a powerful technology for the study of geographic pattern of diversity in many plant species (Sultana *et al.*, 2006). It is a simple, reliable and now a day's extensively used biochemical technique to resolve the taxonomic and evolutionary problems of several plant species (Ladizinsky and Hymowitz, 1979; Badr 1995; Potokina *et al.*, 2000; Ghafoor and Arshad, 2008; Aytenet *et al.*, 2009; Nikolic *et al.*, 2010 and Nisaret *et al.*, 2016). Study of seed storage proteins profile through electrophoresis have been used in different systematic studies (Badret *et al.*, 2000; Zecevic *et al.*, 2000) and the importance of electrophoretic evidences in plant systematic have been discussed in detail by many workers (Kamel, 2005). Similarly the main advantage of the study of seed storage proteins as marker is the high polymorphism level, simple genetic control, environmental independence, and the economy, easiness and expeditiousness of their analysis.

Although the role of these proteins in forest species has been narrowly studied (Alvarez *et al.*, 2003). A few works have studied the genetic makeup of family Cupressaceae and mainly explored its biochemical compositions. Hence a study was deliberated to examine the level of genetic diversity in Pakistani *Cupresses sempervirens* based on SDS-PAGE. The main focus of the study is to investigate intra specific genetic diversity in Pakistani *Cupressus sempervirens* growing in different ecological zones of Malakand Division KP, Pakistan.

## Materials and method

### Exploration and collection

During 2015 exploratory trips were made to different areas of District Dir (L) KP Pakistan. Small strands of *Cupresses sempervirens* were found in five different ecological zones (Zone-1 = Talash, Zone-2 = UOM, Zone-3 = Gulabad, Zone-4 = Munda and Zone-5 = Maidan) of the district. For the study of geographic patterns of genetic diversity through SDS-PAGE, three genotypes of *C. sempervirens* were sampled in Zone-1, one in zone-2, two in zone-3, two in Zone-4 and three genotypes in zone-5. Seeds collected from each genotype were shade dried and assess for further characterization.

### Biochemical characterization

For biochemical characterization (SDS-PAGE), the seed coat from each seed was removed and placed in oven over night at 37°C to remove the water content from seed. The dry seeds of each genotype were then grounded and poured 0.01 g in E tubes. 400 ml of protein extraction buffer (PEB) was added to the sample and vortexed (using Gyromix vortex) thoroughly to homogenize. The proteins were extracted at room temperature for 20 minutes. In order to purify, the homogenate samples were centrifuged at 13,000 rpm for 10 minutes at 25°C. The extracted crude proteins were recovered as clear supernatant and were transferred to a new 1.5 ml Eppendorf tubes and stored at 4°C until they were run on the polyacrylamide gel. The composition of protein extraction buffer, electrode buffer solution, staining solution, destaining solution and solution for gel

electrophoresis were according to the method adopted by Laemmli (1970). For reproducibility of protein bands three experiments were performed. Data from electrophoregram were scored 1 for presence and 0 for the absence of protein bands and put in Microsoft Excel 2007.

#### Data analysis

Data were processed through un weighted pair-group method (UPGMA) cluster analysis conducted using software STATISTICA. Principal Component Analysis (PCA) was conducted by using PC-ORD. This multivariate approach was selected to complement the cluster analysis information, because cluster

analysis is more sensitive to closely related individuals, whereas PCA is more informative regarding distances among major groups (Hauser and Crovello, 1982).

#### Results and discussion

Five reproducible bands were observed for SDS-PAGE among the genotypes that is presented on the Fig. 1. Intra specifically the genotypes showed variation in their protein profile, Maximum number of protein bands (5 bands) were detected in genotypes C-7 and C-8 belong to zone-4 (Munda) while minimum protein bands were observed in the genotype reported from zone-2 (UOM).

**Table 1.** Intra-specific locus variation in *Cupressus sempervirens* detected through SDS-PAGE.

S NO:	BANDS PRESENT	BANDS ABSENT	VARIATION%	STATUS	GENETIC DISAGREEMENT
LOCUS-1 (Band-1)	11(100%)	0 (0.00%)	0	mono	1
LOCUS-2 (Band-2)	8 (72.72%)	3 (27.27%)	27.27	poly	0.72
LOCUS-3 (Band-3)	7 (63.63%)	4 (36.36%)	36.36	poly	0.63
LOCUS-4 (Band-4)	8 (72.72%)	3 (27.27%)	27.27	poly	0.72
LOCUS-4 (Band-5)	5 (45.45%)	6 (54.54%)	54.54	poly	0.45

Locus contribution toward genetic disagreement 80  
 $GD = (\text{poly loci} / \text{total loci}) \times 100$

#### Cluster analysis and principal component analysis

The phylogenetic relationship among all the genotypes through dendrogram has been presented in the Fig. 2. One fourth linkage distance divided all the genotypes into two lineages, whereas these were further divided into four clusters. The cluster 1 is represented by three genotypes of *C. sempervirens*, collected from Zone-1 (Talash). Importantly; the cluster 2 have two sub clusters in which Sub cluster-1 (SC-1) represented by one genotype of *C. sempervirens* collected from Zone-2 (UOM) while SB-2 represented by two genotypes collected from Zone-3 (Gulabad). The cluster-3 consists of two genotypes of *C. sempervirens* collected from Zone-4 (Munda) and cluster 4 grouped 3 genotypes of *C. sempervirens* collected from Zone-5 (Munda). Importantly, In dendrogram the genotypes of each zone occupied separate cluster that revealed a clear cut differentiation in genotypes based on geographic distribution.

The same pattern of phylogenetic relationship was

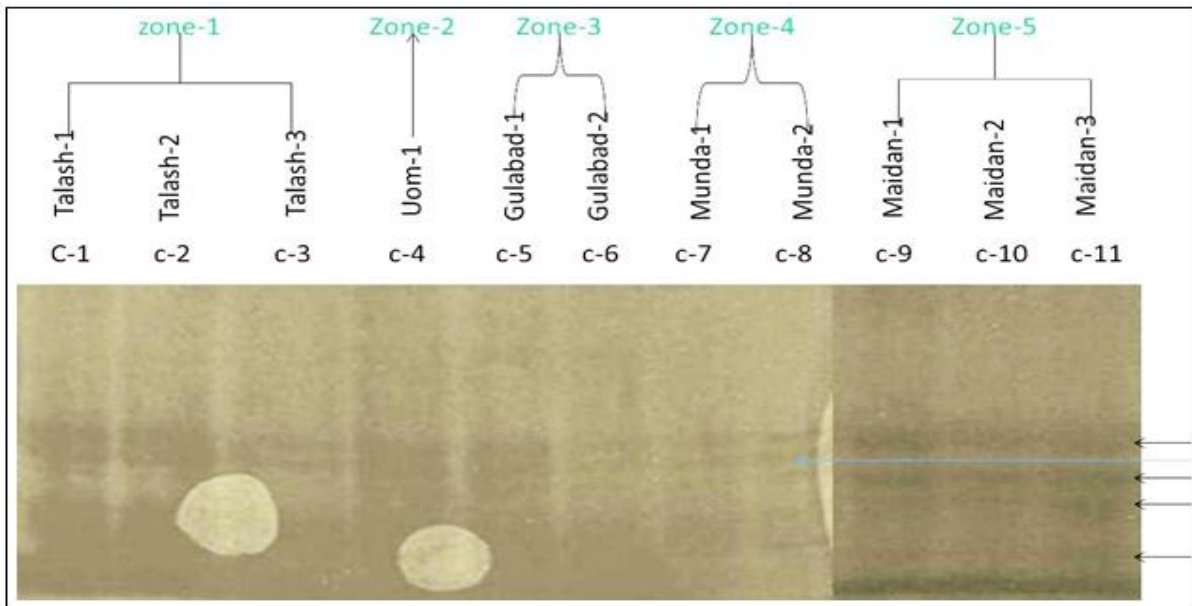
complimented by Principal component analysis (PCA). All the genotypes were grouped into four Clusters. Genotypes reported from Zone-1 were enfolded in Cluster-1. Genotypes reported from Zone-2 and Zone-3 were gathered separately into two sub-clusters in cluster-2. Similarly genotypes of Zone-4 and Zone-5 were enfolded in cluster-3 and Cluster-4 respectively.

#### Locus variation

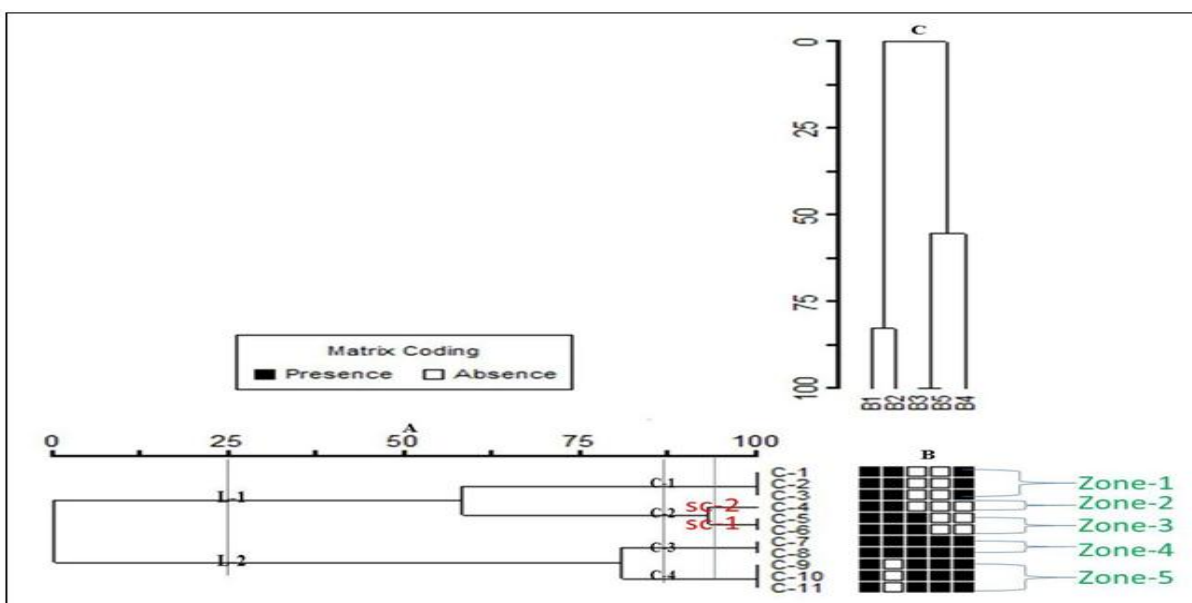
Intra-specific locus variation in eleven genotypes was also detected through SDS-PAGE. Among all loci, locus-1 was monomorphic and marked as species specific locus for *Cupressus sempervirens*. It indicates that regardless of climatic changes the genotypes of *C. sempervirens* maintained species specific identity at different ecological zones of Malakand Division Pakistan. Variation was found in L-2, L-3, L-4 and L-5. In Locus-2 (L-2) bands were missing in genotypes C-9, C-10 and C-11 and thus showed 27.27% genetic diversity. The genetic disagreement of the locus was 0.72%. In Locus-3 protein bands were present in

genotypes C-5, 6, 7, 8, 9, 10 and 11. This locus showed 36.36% variation due to the missing of protein bands in genotypes C-1, 2, 3 and 4 while the genetic disagreement of the locus was 0.63%. Similarly loci-4 and 5 shows 27.27% and 54.54% gentic diversity respectively. Intra-specific locus contribution toward genetic disagreement was 80% (Table-1).

Genetic studies on *C. sempervirens* with isozyme markers showed that genetic variation was higher in natural populations than in domesticated ones (Korol *et al.*, 1997; Papageorgiou *et al.*, 2005; Raddi and Sumer, 1999). Genetic markers have also been used to study genetic diversity in other cypress species in the Mediterranean (Bartel *et al.*, 2003; Bechir *et al.*, 2004).



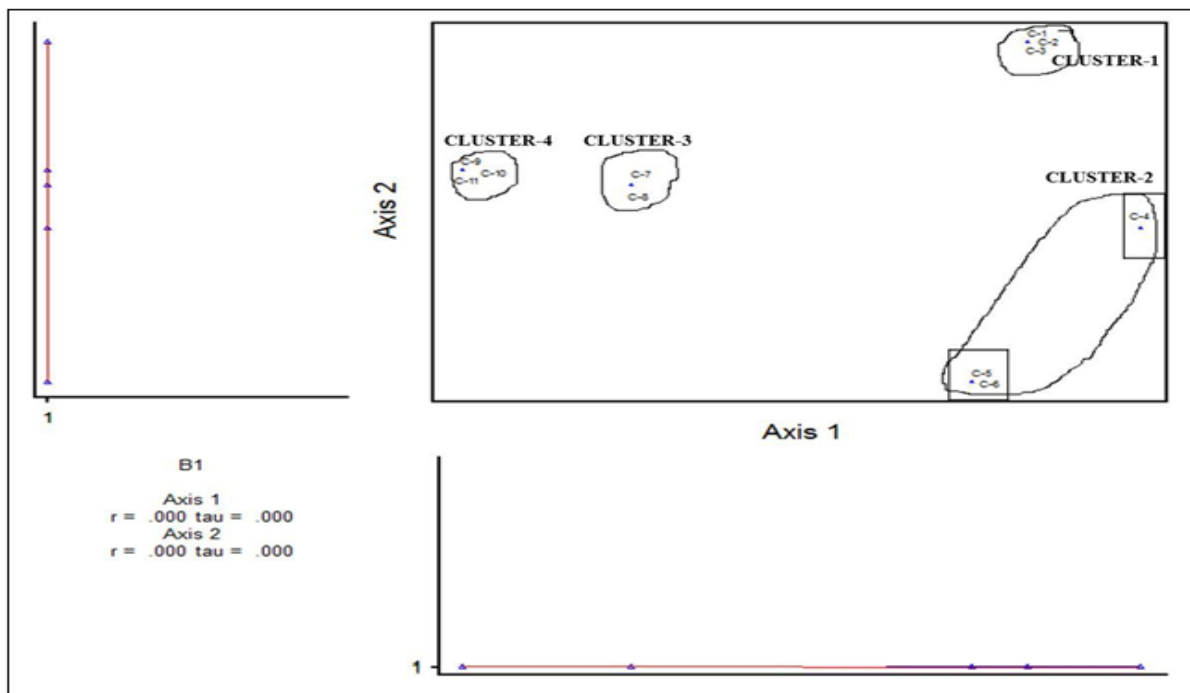
**Fig. 1.** Electrophoregram showing inter and intra-species variation in 11 genotypes of *C. sempervirens* collected from Dir, Khyber Pakhtunkhwa, Pakistan.



**Fig. 2.** Two way Cluster Analysis of Molecular Traits Matrix coding indication the presence and absence of protein bands using PCA: **[A]**; Cluster analysis of 11 genotypes of *C. Sempervirens* are. **[B]**; Zygomorph of 5 bands reported in *C. sempervirens* are. **[C]**; Genetic polymorphism based on protein polypeptide distributed in 11 genotypes of *C. Sempervirens* are.

Rushforth *et al.*, 2003). However, the genetic structure of *C.sempervirens* has not yet been investigated with SDS-PAGE at the wide-range scale. The conservation of what remains of this species should be based on the knowledge of its genetic diversity and characteristics (Shahroodian *et al.*,

2011). In the present investigation, SDS-PAGE was surveyed in natural populations of *Cupressus sempervirens* located in five regions at Dir (L) of Pakistan to achieve genetic information useful for gene conservation.



**Fig. 3.** A scattered plot grouped 11 genotypes of *C. sempervirens* into four cluster by using Principal Component Analysis (PCA).

According to the result revealed by SDS-PAGE the genotypes of same ecological zone showed similarity in their protein banding pattern. The numerical analysis (Phylogenetic tree and cluster plotting) sorted all the genotypes into four clusters. It was found that each cluster had slight discriminative protein banding. Variation within the genotypes was found based on their ecological distribution and each cluster in the dendrogram was represented by the genotypes belong to same ecological zone. Among all the genotypes high degree of genetic diversity (60%) was found in a single genotype reported from Zone-3 UOM followed by the genotypes of Zone-1 and Zone-3 which indicated 40% genetic diversity. Similarly the locus-5 showed high degree (54.54%) of genetic polymorphism followed by locus-3 which represented 36.36% variation. Importantly locus-1 was monomorphic which indicated that the genotypes of

*C.sempervirens* maintained their species specific identity at different areas of Dir (L) regardless of environmental fluxes. The results obtained in this study make a connection with the works previously published by (El-Kassaby, 2000) and (Batoul *et al.*, 2014).

### Conclusion

The present study indicated that diversity was present in the genotypes of *C. sempervirens* at molecular level. Seed storage proteins profile maintained species specific identity regardless of different environmental and climatic conditions of two regions and SDS-PAGE method presented here could be an important tool for geographic pattern of genetic diversity in *C.sempervirens*. To increase knowledge about the genetic structure of *C. sempervirens* we also recommend further analysis using dominant

molecular markers to have a better resolution power.

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