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RESEARCH ARTICLE

Eco-morphology and molecular attributes of twisted and straight Chir pine (*Pinus roxburghii* Sarg.) growing in Uttarakhand: Central Himalaya of Indian Himalayan region

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Abstract

The hills of Uttarakhand in Indian Central Himalaya provide one of the best habitats of Chir pine (*Pinus roxburghii* Sarg.; Gymnosperm; 2n= 24) forests which play an important role in the economy. Based on the available literature of Civil & Soyam, Department of Forest, Almora, Chir pine represents 81.43% of the district's forest area. Five sites (Panwanaula, Majkhali, Matela, Dhaula Devi and Someshwar) in and around Almora have been analyzed for eco-morphological and segregating molecular profiles developed. Twenty trees of each trait (twisted vs straight) from dense pine forest (altitude range 1500-1900 m) were randomly selected. Two types of twisted characters were observed in the trees: (1) twist in anticlock direction which is initiated at the early stage of trees at an angle of approximately 7^0 and renders the plant not useful for commercial purposes, and (2) Clockwise twist is initiated at later stages. The ratio of twisted to straight pine trees in all the sites as observed in field surveys is about 1:1, and has also been recorded by Civil & Soyam, Department of Forest, Almora, was also observed in our field surveys. Genomic DNA of selected trees of each trait was isolated and used for amplification. A total of 40 RAPD and 10 ISSR primers were tested; more than 45% similarity was observed in both twisted and straight trees. When these data were used for construction of graphic phenogram, two separate groups of straight and twisted trees were observed. Interestingly some twisted trees (possessing approximately 7^o or lower twist in the stem) were grouped along with straight trees. The results of this study will be helpful in developing strategies to segregate twisted and straight trait in Chir pine at an early stage not only for the plantation programmes but also for management purposes.

1. INTRODUCTION

In India, pine forests are found in Uttarakhand, Jammu and Kashmir, Haryana, Himachal Pradesh, Uttar Pradesh, parts of Sikkim, West Bengal and Arunachal Pradesh. The total area under pine forests is estimated to be 8, 90,000 hectares and occurs between 450 m to 2300 m altitude. Chir pine is one of the most useful tree species in the Himalayan region. The upper limit of gregarious Chir forests is about 1950 m, though the species is found scattered on warm aspects up to 2300 m (Troup, 1921; Mirov, 1967). About 80% of chir pines have been reported to be twisted in Garhwal and Kumaun region (Kala, 2003). Many trees in the natural populations are twisted (spiral grain) and wood from the twisted plants is not useful for many purposes resulting in huge revenue losses to Government and private owners. Based on the intensive research, the fundamental cause of twisted trait /spiral grain angle changes in individual trees governed by different factors i.e environmental conditions like wind, rainfall, sun and even the rotation of earth and the most important factor is heritability (Harris, 1989). But no strict causal relationship between environmental conditions and fibre direction was observed because grain angle is highly heritable (i.e. under strict genetic control) although its expression may be dependent on different factors. This trait is governed by genes present in the genome and there is a possibility to believe that twistedness in pine is also controlled by some gene(s). In other report spiral grain in wood is often characterized by a very large inclination of fusiform cells even reaching 90° with respect to the stem axis (Hartig, 1895; Harris, 1989; Kubler, 1991). Based on several reports, the inclination of the cells (non-storied cambium) is caused by unidirectional oblique anticlinal divisions (Z or S) and intrusive growth entering the space between radial walls (Bannan, 1956, 1966; Hejnowicz, 1961, 1971; Jones, 1963; Harris, 1969, 1973, 1981; Kubler, 1991; Wloch et al., 2002).

The determination of heritability and the assessment of genetic components of wood quality require that the contributions of environment be fully recognized and evaluated. This may be accomplished either by statistical procedures that allow for environmental variation or by experimental designs that hold environmental variation. Conventional selection and breeding programs for specific traits in forest trees is a long-term process. Hence molecular marker analysis of genetic diversity and construction of high density linkage maps of forest tree species helps in early selection of trees with economically important traits like wood density, tolerance to biotic and abiotic factors for incorporation in breeding programmes. Molecular marker has been utilized to identify markers linked to specific traits in forest trees (Devey et al., 1995). Grattapaglia et al. (1994) have developed linkage map for *Eucalyptus* using RAPD markers while Shepherd et al. (2003) have developed genetic maps for *Pinus* spp. using AFLP markers. The identification of marker linked traits such as twistedness in trees will help in early prediction of seedlings with the possibility of growing as twisted plants. Such screening system may help the foresters to select the plants from the seedlings population. In view of the problem on twisted pine, an attempt has been made to undertake an investigation with the following objectives: i) Comparison of twisted (T) with straight (S) pines at eco-morphological and genetic level; ii) Estimation of relationship among the environmental factor and site condition with morphological parameters in straight and twisted pine; iii) identification of segregating marker(s) between straight and twisted pine.

2. MATERIALS AND METHODS

2.1 Site selection and plant material

Based on the existing reports of Pine forest in Almora from Civil & Soyam, Department of Forest, Almora, five sites (Panwanaula, Majkhali, Matela, Dhaula Devi and Someshwar) of pine forest (1350-1900 m), where straight and twisted pine are growing in the ration of 1:1 were selected and eco-morphological and segregating molecular profiles were developed. Initially, only one district (Almora) has been selected for the study. Twenty trees of each trait from each site were randomly selected for morphological and molecular studies.

2.2 Eco-morphological data

Geographical location of study sites were recorded using GPS. Two types of twisted characters were observed in the trees: (1) twist in anticlock direction which is initiated at the early stage of trees at an angle of approximately 7o -15o and (2) Clockwise twist is initiated at later stages. Morphological data [i.e. tree height (TH; approx. in m; using Tangent method), DBH of drum width (DW in m; using Diameter at Breast Height method-DBH) number of branches of main trunk (all the branches; NB), number of twist in main trunk (NOT) and angle of twist with vertical axis (using protector from the base of the main trunk; AOT) were recorded (Fig 1).

2.3 DNA isolation and PCR

DNA was isolated (leaf samples of twenty trees of each tratits were pooled in to 10 i.e T1-T10 for twisted and S1-S10 for straight trees) following the procedure of Khanuja et al. (1999). Polymerase chain reactions (PCR) were carried out in 25 µl volume samples. A reaction tube contained 25 ng of DNA, 0.2 units of Taq DNA polymerase, 10 mM of each dNTPs, 1.5 mM MgCl2 and 5 pmol of RAPD (OPO, OPJ) and ISSR primers. The amplification was carried out using the DNA Engine thermal cycler (MJ Research, USA) using 94oC, 35oC and 72oC temperatures for 40 cycles. The DNA profile was analyzed following Nei and Li (1979) and the dendrogram of the genetic relatedness among the genotypes was constructed using UPGMA (unweight pair group method with arithmetic average) cluster analysis.

2.4 ***Data interpretation***

The number of polymorphic and monomorphic bands of RAPD and ISSR profiles were determined for each primer pair. Bands clearly visible in at least one line were scored (1 for presence, 0 for absence) and used in the data matrix. Fragment size was estimated by interpolation from the migration distance of marker fragments. Percent polymorphism was calculated as the proportion of polymorphic bands over the total number of bands. Variability for each band was measured using the polymorphic information content (PIC; Anderson et al., 1993).

$PIC=1-\Sigma pi^2$;

Where, *pi* is the frequency of the ith allele. The PIC was calculated for each primer combination. The marker index (MI) was calculated for each RAPD and ISSR primes as MI = PIC X ηβ, where PIC is the mean value of total PIC, η the number of bands, and β is the proportion of polymorphic bands (Powell et al., 1997).

Statistical analyses of morphological data were carried out using Statistica version 8.0 (software).

3. RESULTS

3.1 Eco-morphological analyses

Ratio of twisted and straight pine in the five selected sites was 1:1, recorded by Civil & Soyam, Department of Forest, Almora and revisited by authors. Eco-morphological analysis on 5 studied sites (altitudes 1390, 1460, 1652, 1826 and 1890 m asl) showed a wide range of variation in tree height. Height of trees ranged from 3-17m. Highest tree height (17.5m) in straight trait was observed in Majkhali while in Someshwar height ranged from 0.9-7.2 m. Among the twisted trees highest tree height (15.1 m) was observed in Panwanaula while lowest range was observed in Someshwar (0.55-9.7 m) and Dhaula Devi (2.3-7 m). Mean tree height among both the traits was 7.16 m. The range of drum width (DW) in straight trees was 0.14-1.82 m. Number of branches depends upon the altitude and tree height hence the range of number of branches was 3-22 in all study site. In Matela, Majkhali and Panwanaula the angle of twist was started at the angle of 7^o, 9^o and 15^o, while 30^o twist at the initial point was observed in Someshwar and Dhaula Devi (Table 1). Altitude showed positive and significant correlation with tree height (0.986**) and angle of twist (0.873*) respectively whereas negatively correlated with drum width (-0.773), number of branches (0.877*) and number of twist (-0.772). Angle of twist was negatively correlated with drum width (-0.84) and number of branches (-0.622; Table 2). As the altitude was dependent factor with all the parameters used in the analysis, tree height, angle of twist showed increasing trend with increased altitude while drum width, number of branches and number of angles showed decreasing trend with increased altitude in the twisted trees examined (Fig 2 a-e). Similarly in straight pine no significant relationship was observed in tree height with drum width (0.199).

3.2 Molecular analyses

A total of 40 RAPD and 10 ISSR primers were analyzed; only one primer showed segregating profile between Twisted (T) and Straight (S) pine. Approximate 800bp DNA band was present in the twisted pine and was confirmed (three times) by using different DNA samples (Fig 3 a-c). Based on the molecular profiles more than 45% diversity was observed. OPO primers showed 68% polymorphism while OPJ showed 48%. Among the used RAPD primers OPO primers showed 62.41 resolving power, 7.88 PIC (polymorphic information content) and 19.84 Marker index (Table 3). The highest genetic distance was 0.86 between T4 and S6 and lowest was 0.023. When these data were used for construction of dendrogram, two separate groups of straight and twisted trees were observed in the form of two major clusters: a) cluster I; and b) cluster II separated at 0.6-0.7 showing robust separation. Cluster I comprises all straight including 2 twisted trees and cluster II comprises all twisted trees. Interestingly some twisted trees (possessing approximately 7^o or lower twist in the stem) were grouped along with straight trees (Fig 4).

Table 1. Eco-morphological attributes of various analyzed individuals between twisted and straight Chir pine trees of selected studied sites.

S. No.	**Locations**	**Altitude (m amsl)**	**Trees trait**	**Tree height (m)**	**Drum width (m)**	**No. of branches**	**Angle of twisting (°)**	**No. of twist**
1	Matela	1430	Twisted Range Mean SD±SE	4.2-11.6 6.9 2.39±0.75	0.6-1.82 0.909 0.35±0.11	6-22 15.2 5.8±1.83	7-180 89 61.89±19.57	1-3 1.8 0.78±0.24
			Straight Range	5.8-16.2	0.4-1.15	6-18		

			Mean SD±SE	7.26 3.62±1.14	0.861 0.236±0.72	16.2 6.03±1.90		
2	Panwanaula	1630	Twisted Range Mean SD±SE	3.5-15.1 7.54 4.03±1.27	0.35-1.42 0.803 0.4±0.12	6-20 15.4 4.59±1.45	7-360 168.1 144.83±45.79	1-4 2 1.3±0.41
			Straight Range Mean SD±SE	5.1-13.2 9.95 2.89±0.91	0.31-1.45 0.954 0.34±0.10	5-19 11 4.13±1.30		
3	Majkhali	1826	Twisted Range Mean SD±SE	3.1-10.5 5.92 2.4±0.75	0.4-1.42 0.824 0.35±0.11	5-15 11.8 3.9±1.23	9-180 118 61.83±19.55	1-3 1.6 0.69±0.22
			Straight Range Mean SD±SE	5.5-17.5 9.21 4±1.26	0.3-1.53 0.992 0.416±0.13	5-17 10.6 4.42±1.39		
4	Someshwar	1890	Twisted Range Mean SD±SE	0.55-9.7 5.85 3.56±1.12	0.12-0.78 0.621 0.197±0.062	5-19 11.2 5.18±1.63	30-360 171 114.06±36.06	1-3 1.8 0.78±0.24
			Straight Range Mean SD±SE	0.9-7.2 4.99 2.55±0.80	0.14-0.64 0.444 0.184±0.058	3-14 8.7 3.91±1.23		
5	Dhauladevi	1805	Twisted Range Mean SD±SE	2.3-7 5.45 1.68±053	0.31-0.75 0.602 0.158±0.049	5-17 10.5 2.87±0.90	30-360 174 110.27±34.87	1-3 1.8 0.91±0.28
			Straight Range Mean SD±SE	1.8-6 4.39 1.54±0.48	0.23-0.69 0.507 0.147±0.046	8-16 11.6 2.98±0.94		

Note:SD- Standard deviation; SE- Standard error

Table 2: Correlation among eco-morphological parameters between twisted and straight trees

	ALTITUDE	TH	DW	NB	AOT	NOT
ALTITUDE	1					
TH T S	0.986** 0.857	1				
DW T S	-0.773 -0.109	-0.565 0.199	1			
NB T S	-0.877* -0.867*	-0.905* -0.941*	0.8 0.209	1		
AOT T	0.873*	0.883*	-0.84	-0.622	1	
NOT T	-0.772	-0.802	-0.06	0.819	-0.507	1

** Correlation is significant at 0.01%

*Correlation is significant at 0.05%

Note: T- twisted; S- Straight; TH- Tree height; DW- drum width; NB- Number of branches; AOT- Angle of twist; NOT- Number of twist

Table 3: Degree of polymorphism and quality of RAPD and ISSR data generated in the study

S.No	Primers	% polymorphism	Resolving power	PIC	MI
1	OPO	68	62.41	7.88	19.05
2	OPJ	48	21.49	6.83	9.01
3	ISSR	46	47.44	6.91	10.21

Note: PIC-Polymorphic Information Content, MI- Marker Index

Fig 1: Twisted Chir pine having clockwise twist (clock wise twist of 360 °)

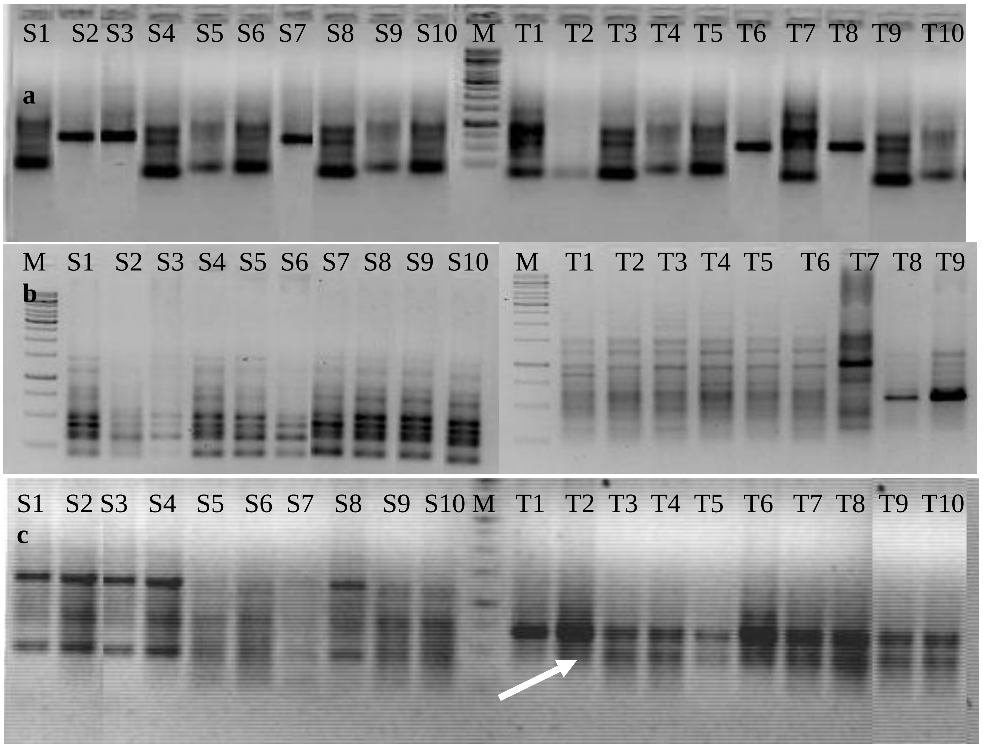

Fig 3 a-c: Genetic diversity and segregating DNA profiles of twisted and straight chir pine trees

Note: M: Markers; S1-S10- Straight chir pine samples; T1-T10- Twisted chir pine;
→ 800bp fragment segregating band in twisted pine.

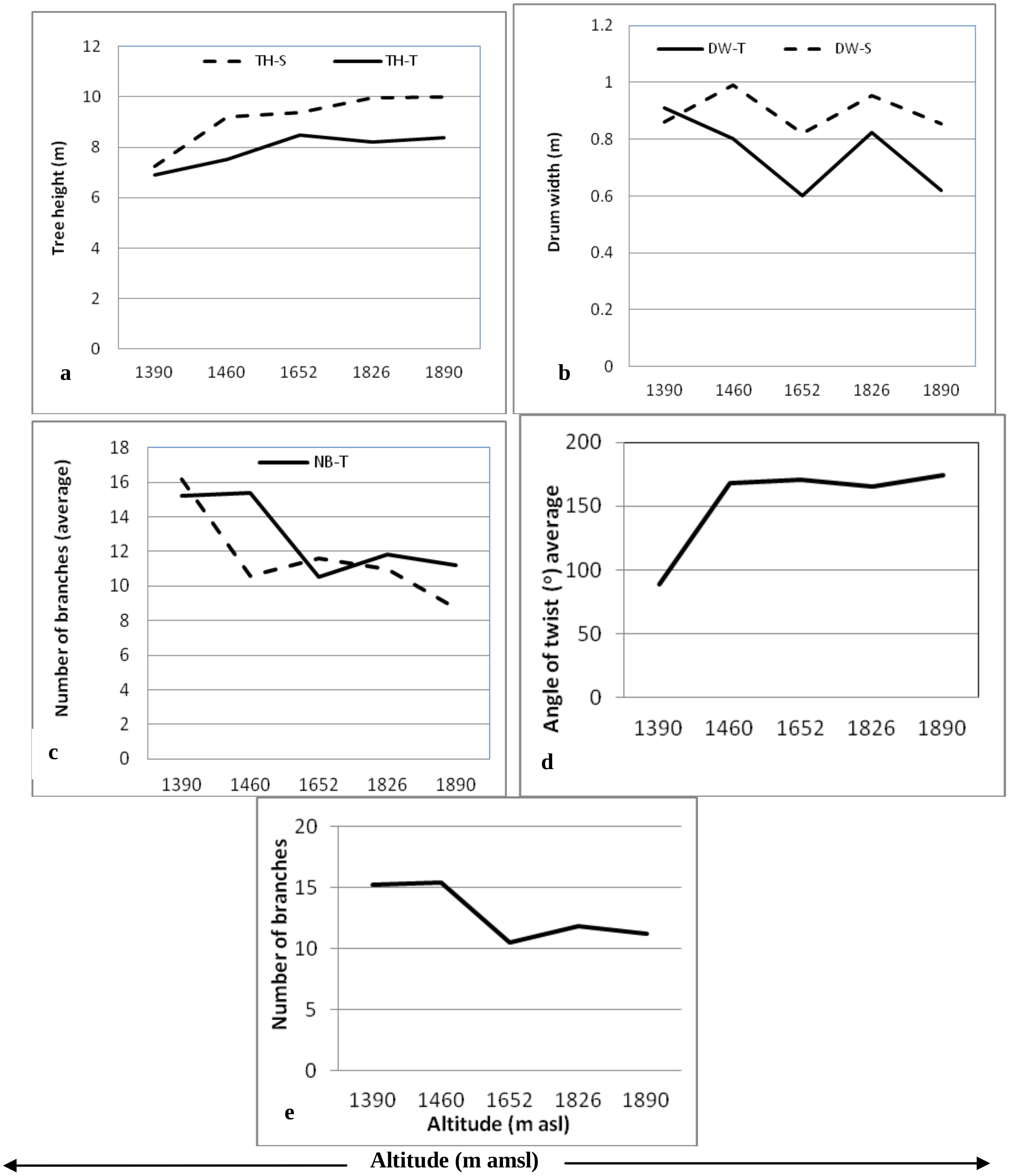

Fig 2a-e: Relationship of eco-morphological parameters with altitude in twisted (T) and straight bole (S) Chir pine trees; data shown in Fig1d and e don't apply in respect of chir pine trees with straight bole
Note: Tree height (TH); Drum width (DW); Number of branches (NB); Number of Twist (NOT); Angle of twist (AOT)

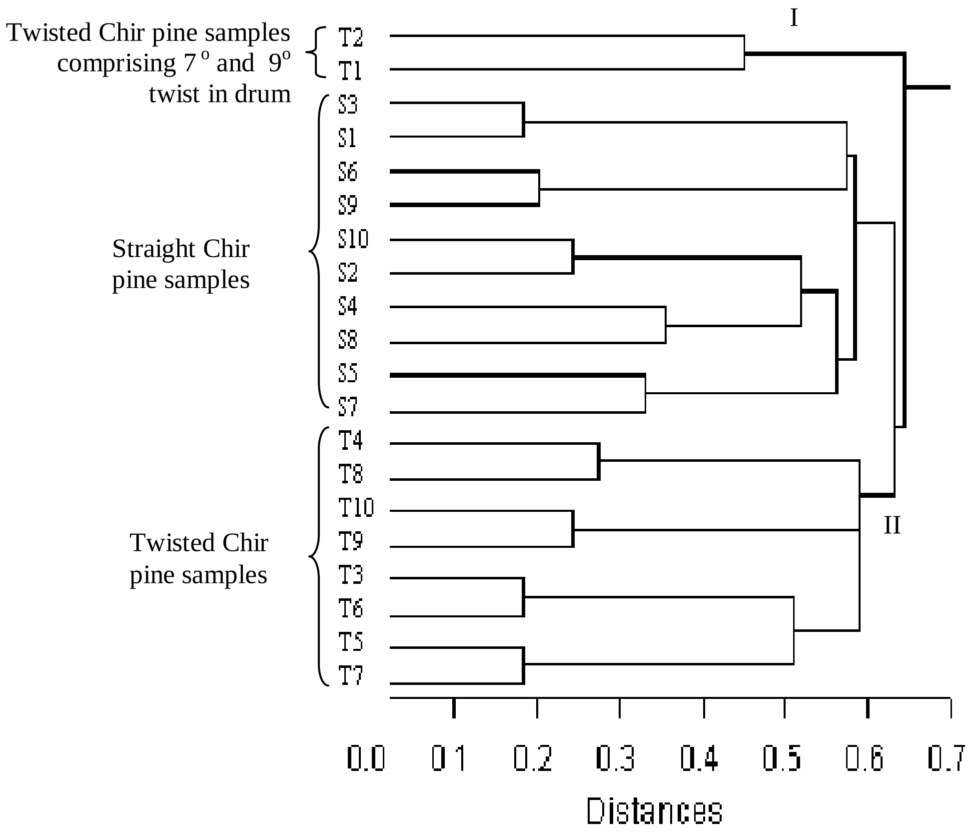

Fig 4: Dendrogram depicting segregating groups of twisted (T) and straight bole (S) chir pine trees twisted

4. DISCUSSION

Pine is one of the important forest trees in northern India, Nepal and Pakistan and has been widely planted for timber and resin. But these traits are largely affected by spiral grain (twisted traits). It is also apparent that the local environment strongly modifies wood quality by its influence on the overall growth pattern of the tree. A specific environment will bring forth a fairly definite outward expression of the basic genetic constitution of the tree in terms of wood quality. But the genetic potential of a tree permits a rather wide range of phenotypic expression, and every minute change in the environment will result in a different growth response and, consequently, a slightly altered wood quality. Thus, a particular genotype may exhibit an array of wood quality assessments when grown under a range of environments. When one considers the multiplicity of genotypes and environments that exist in nature within a species range, the potential diversity in wood quality can be readily appreciated. Inter population genetic diversity analyses among 72 accessions were carried out in four *Pinus roxburghii* populations of India using ISSR markers. The study showed 0.3-0.4 genetic distance (Parasharami and Thengane, 2012) while our result showed 0.6-0.7 genetic distance. Diversity in two species, i.e in *P. radiata* and *P. pinaster* at two specific gene (a glycin-rich protein homolog and CesA3, a cellulose synthase) levels indicated the potential susceptibility of the physiological role in these species (Pot et al., 2005). Broad sense of heritability was also reported for seedling height coupled with genetic advancement and subsequent gain in *Pinus sylvestris* L (Ghildiyal et al., 2009).

Another wood quality for which many studies have shown possibilities for genetic improvement is spiral grain.

Reports of Champion (1945) and coworkers in *Pinus roxburghii* showed possible genetic control of spiral grain. Self-pollinated, twisted *P. roxburghii* parents produced seedlings that were 68 to 82 percent twisted, observable in the cotyledon stage, leading Kadambi and Dabral (1955) to conclude that twist is a dominant character. Schmucker (1956) and Northcott, (1957) concluded that spiral grain in *Fagus sylvatica* and 6 conifer are dominant character because they rules over the other characters, therefore, probably results from inherent factors this result is well supported by our result of segregating molecular marker of twisted and straight trait. The concept of strong inheritance of spiral grain is challenged by Paul (1953), who feels that the spiral is caused by some factor other than heredity. In his opinion, interlocked grain is more likely to be strongly controlled genetically than spiral grain.

Geographic variation in natural stands does not indicate anything about inheritance per se but it shows the extent and kind of variation patterns in *P. elliottii. P. taeda* and *P. serotina* (McElwee and Zobel, 1963) while our investigations indicated that geographical site may affect the spiral grain parameters like number of twist (NOT) and angle of twist (AOT). It has also been observed that spiral grain character was prominent in early stage rather than later stage. Phenotypic correlations were observed between grain angle and the bow, crook and twist developed in 316 sawn and dried boards. All the phenotypic parameters were widely affected by environmental factors (like weather conditions stand structure, site quality, geographic locality etc.; Hallingbäck et al., 2010). Similarly in the present investigation geographical site (altitude) and drum width were negatively correlated with number of twist and highest correlation of altitude with tree height and number of twist [0.709 (T), 0.949 (S) and 0.633].

Although genotype and environment are inextricably united, in the sense that no tree can be grown completely devoid of its environment, it is possible to evaluate genotype "independent" of environment by suitable experimental designs and statistical controls.

5. CONCLUSION

In the present study considering the economic value of pine, eco-morphology and molecular analyses for twisted vs straight trait may play important role in the commercialization of pine. Based on the correlation studies altitude is negatively correlated with the number of twist suggesting that at higher altitude the possibility of this trait may become low but not absolute negative. This may be due to physiological and ecological conditions. However, loci in the chromosome of twisted pine give information of trait to screen the plants at nursery stage as per the requirement of the forest department and other related agencies. Research in genetic control of phenotypic characters and timber quality has scarcely scratched the surface and is indeed needed. The results of this study will be useful in developing strategies to segregate twisted and straight trait in Chir pine at an early stage not only for the plantation programmes but also for management purposes.

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Conflict of Interest

Authors state that they have no conflict of interest.

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