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Research Article

Phylogenetic and evolutionary relationships in selected *Pinus* species using rbcL and matK chloroplast genes

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Abstract

The genetic diversity of *Pinus* plants has been assessed in various phylogenetic studies that reveal the polymorphism directly at DNA levels. The rbcL and matK are the most commonly used markers for phylogenetic studies of *Pinus* sp. that exhibit a diverse geophysical adaptiveness and geographical variations across different regions as a result of genotypic modifications. This study evaluated usefulness of rbcL and matK genes for molecular identification and phylogenetic study among various species of *Pinus*. Maximum Likelihood (ML) and Neighbor Joining (NJ) analysis of the data obtained from rbcL gene belonging to ten *Pinus* species revealed four clusters. First Cluster included *P. Wallichiana* (PW), *P.Elliottii* (PEI) *and P.Greggii* (PG). Second cluster included *P. Thunbergii* (PT), *P. Echinata* (PE) and *P.merkusii* (PM). Third cluster included *P.taeda* (Ptd) and *P. khasya* (PK) while fourth cluster included *P. Roxburghii*(PR) and *P. patula* (PP). Similarly ML and NJ analysis of the data obtained from matK gene belonging to ten *Pinus* species revealed four cluster and one outgroup. First Cluster included *P. Thunbergii* (PT), *P.Greggii* (PG) and *P.Elliottii* (PEI) Second cluster included *P. roxburghii*(PR) and *P.merkusii* (PM)*P. Echinata* (PE) and third cluster included *P. Wallichiana* (PW) and *P.taeda* (Ptd) while fourth cluster included *P. Patula* (PP) and *P. Khasya* (PK). *P.echinata* remained out clustered in this analysis.

Abbreviations

CTAB: Cetyl Trimethylammonium Bromide; PCR: Polymerase Chain Reaction

Introduction

Pinus (Pinaceae) is the largest genus of order Coniferales widely distributed throughout temperate zones in the Northern Hemisphere, and restricted to high elevation in the tropics and subtropics [1,2] Pines differ from other members of family Pinaceae and are easily identifiable by their dimorphic long and short shoots called fascicles. These fascicles bear long, narrow needle-like leaves mostly present in groups of two to five. Due to its great economical and ecological importance, phylogeny and systematics of this genus has received great attention based on morphology, anatomy, ethnobotanical values, karyotypic analysis, secondary products, isozymes, pharmacognosy and, most recently, molecular studies [2].

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Based on morpho-anatomical and molecular data, *Pinus* has been divided into two monophyletic subgenera: Haploxylon (subgenus Strobus, with single fibrovascular bundle in the needle, also known as "Soft pines") and Diploxylon (subgenus Pinus, with double fibrovascular bundles in the needle, also known as "Hard pines") [3-6]. These two subgenera namely Haploxylon and Diploxylon have further been divided into many sections and subsections [1,4].

In this study, we analyzed sequences from rbcL and matK, for ten species of the *Pinus* namely *P.merkusii, P.khasya, P.thunbergii*, *P.wallichiana*, *P.roxburghii*, *P.taeda*, *P.elliottii*, *P.echinata, P.patula* and *P.greggii*. Out of ten species selected for our study five species viz. *P.merkusii, P.khasya, P.thunbergii*, *P.wallichiana* and *P.roxburghii* are native to Indian subcontinent and grow luxuriantly in Indian Himalayas while remaining five viz. *P.taeda*, *P.elliottii*, *P.echinata, P.patula* and *P.greggii* are exotic species and have been introduced in Indian subcontinent. Our main objectives during the present study was to examine the phylogenetic relationships of selected species which are growing naturally from subgenus *Pinus* and *Strobus* at the inter- and intrasectional levels.

Material and methods

Collection of plant material

The leaves (needles) of 10 pine species were collected in September, 2016, from a wild population in the region of Ranikhet (located at 357 km NSE of New Delhi, latitude 29°39'52.2" (N); longitude 79°28'40.9" (E); altitude 1,727 m). The site received median rainfall, had very low winter temperature and low soil fertility. Voucher specimen of all the species selected for study was deposited in the herbarium of the National Botanical Research Institute, Lucknow (Table 1). Prior to extract preparation, study material was dried at room temperature and ground to fine powder in a blender. The collected plant material was brought to the laboratory and stored at -80°C.

DNA extraction, amplification and sequencing

DNA was extracted from 100 mg of dried needles using a modified CTAB method, treated with RNase, and purified by phenol. Two cpDNA regions (rbcL and matK) were sequenced using the primers (Table 2) designed by Wang, et al. [7]. PCR amplifications were accomplished at 95°C for 5 min for the initial denaturation followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 48°C (matK) and 52°C (rbcL) extension at 72°C for 2 min, and a final extension for 10 min at 72°C. The products from PCR were precipitated using ethanol and used as a template for the sequence reaction. The sequencing was carried out using an ABI 310 Genetic Analyzer (Applied Biosystems) with an ABI BigDye Terminator Cycle Reaction Kit following the manufacturer's instructions.

Alignments were performed using Clustal X [8]. The phylogenetic analyses were completed using single data sets. The phylogenetic and evolutionary analyses were performed with MEGA X using neighborjoining (NJ) and maximum likelihood (ML) algorithms. Gel electrophoresis representing DNA bands of rbcL and matK are represented in Figures 1,2 respectively.

Table 1: List of *Pinus* species selected for study.

Sample No.	Species	Collected from.	Herbarium No.
1.	*P.merkusii*	Kalika Forest, Ranikhet	304288(LWG)
2.	*P.khasya*	Kalika Forest, Ranikhet	304289(LWG)
3.	*P.thunbergii*	Kalika Forest, Ranikhet	304290(LWG)
4.	*P.wallichiana*	Kalika Forest, Ranikhet	304291(LWG)
5.	*P.roxburghii*	Kalika Forest, Ranikhet	304292(LWG)
6.	*P.taeda*	Kalika Forest, Ranikhet	304293(LWG)
7.	*P.elliottii*	Kalika Forest, Ranikhet	304294(LWG)
8.	*P.echinata*	Kalika Forest, Ranikhet	304295(LWG)
9.	*P.patula*	Kalika Forest, Ranikhet	304296(LWG)
10.	*P.greggii*	Kalika Forest, Ranikhet	304297(LWG)

Table 2: Nucleotide variation in Pine species from rbcL and matK of chloroplast genes.

Variable 1	rbcL 2	matK 3
Length of sequence (bp)	1351	1045
Number of species	10	10
Nucleotide composition	A=28.76 T=26.24 C=25.07 G=19.92	A=32.27 T=30.90 C=18.49 G=18.32

Results

Nucleotide variation

Composition of nucleotide from both genes was dominated of A-T (Adenine-Thymine) than GC (Guanine-Cytosine) and it was found to be A=28.76; T=26.24; C=25.07; G=19.92 and A=32.27; T=30.90; C=18.49; G=18.32 in rbcL and matK respectively (Table 2).

Phylogenetic relationships among selected species of Pinus

The results of the tree construction were midpoint rooting based on partial sequences of rbcL gene (Figure 3) and matK gene (Figure 4). The trees indicated that there were two major branches of group of selected pine species together. This study evaluated usefulness of rbcL and matK genes for molecular identification and phylogenetic study among various species of *Pinus*. Cluster analysis of the data obtained from rbcL gene belonging to ten *Pinus* species revealed four clusters. First Cluster included *P. Wallichiana* (PW), *P.Elliottii* (PEl) *and P.Greggii* (PG). Second cluster included *P. Thunbergii* (PT),*P. Echinata* (PE) and *P.Merkusii* (PM). Third cluster included *P.taeda* (Ptd) and *P. khasya* (PK) while fourth cluster included *P. Roxburghii* (PR) and *P. Patula* (PP) as shown in Figure 3. Similarly cluster analysis of the data obtained from matK gene belonging to ten *Pinus* species revealed four cluster and one outgroup. First Cluster included *P. Thunbergii* (PT), *P.greggii* (PG) and *P.Elliottii* (PEl) Second cluster included *P. Roxburghii* (PR) and *P.Merkusii* (PM),

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third cluster included *P. Wallichiana* (PW) and *P.taeda* (Ptd) while fourth cluster included *P. Patula* (PP) and *P. Khasya* (PK). *P.echinata* remained out clustered in this analysis (Figure 4).

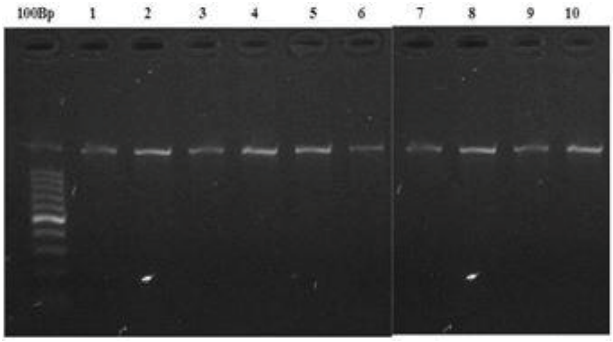

Figure 1: Gel electrophoresis showing amplicon of rbcL gene using ten species of *Pinus*..

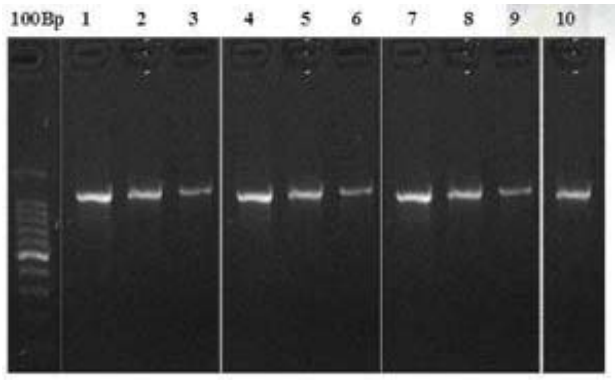

Figure 2: Gel electrophoresis showing amplicon of matK gene using ten species of *Pinus*.

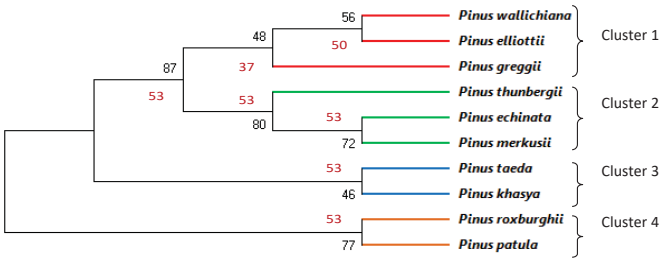

Figure 3: Phylogenetic tree of partial sequences of rbcL gene with 1000 bootstrap replications. Supporting values were indicated by color; Maximum Likelihood (black) and Neighbor Joining (red).

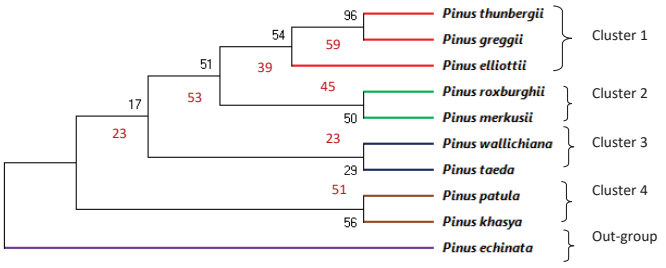

Figure 4: Phylogenetic tree of partial sequences of matK gene with 1000 bootstrap replications. Supporting values were indicated by color; Maximum Likelihood (black) and Neighbor Joining (red).

Discussion

Analyzing the amounts of nucleotide and patterns of nucleotide variation between and within species is important to understand the mechanisms of evolution by which processes of genetic polymorphisms within species become transformed into genetic divergence between species and genetic diversity is maintained. Such diversities are influenced by evolutionary processes, such as selection, recombination, mutation and population structures.

In one of the earliest studies by Govindaraju, et al. 1992 [9], phylogenetic study amongst 30 species of the *Pinus*, *P. taeda*, *P. echinata*, and *P. elliottii* were classified under the section *Pinea* and sub section *Australes*. The study also revealed that majority of the studies had maximum likelihood relationship with the species of *Pinus* . Majority of the species of *Pinus* fall under the lineage of Ponderosae, Oocarpae, Contortae, Australes, and Sabinianae. Wagner, et al.[10] also conducted phylogenetic study among *Pinus* sp., *Pinus echinata*, *Pinus elliottii*, *Pinus palustris*, and *Pinus taeda*. Phylogenetic studies on the basis of the study by Little and Critchfield [4] found that these species of *Pinus* had distinctive relations among them and the clades or sub sections mainly Australes and Ponderosae have common lineage. On the other hand, *P. wallichiana* was reported under the sub section *Strobus*. *P. gerardiana* under the sub section Gerardianae; *P. khasya* and *P. thunbergii* under *Pinus*, *P. merkusii* under subsection *Pinaster*, *P. roxburghii* under *Pineae*, *P. echinata* under *Australes*, *P. patula* under *Oocarpae* according to a phylogenetic study by [5]. The study found a distant relationship among the sub sections *Strobi*, *Pinus*, *Pineae*, *Pinaster*, *Oocarpae*, and *Australes*. However, *Oocarpae*, and *Australes* indicated the closest lineage amongst the *Pinus* sp. Furthermore, the phylogenetic study found that *Pinus*, *Pineae* and *Pinaster* subsetions were closely related to each other but distant relationship to Strobi for *P. wallichiana* and *Oocarpae*, and *Australes* for *P. echinata* and *P. patula*. However, these species were found to spread across North and Central American subsections to the Himalayas with common lineage that brings them under the same phylogeny.

Krupkin, et al. [11] also conducted phylogenetic analysis among 18 species of *Pinus* and used Wagner parsimony analysis for exploring the phylogeny of the *Pinus* sp. The study found that the clades Contortae, Ponderosae, Sabinianae, Australes, and Oocarpae consisted of majority of the *Pinus* sp. such as *P. taeda*, *P. echinata*, *P. gregii*, *P. patula*, *P. elliotti* and others. The species of these sub sections were in close parsimony with respect to anatomical structures and genetic structures. The study also found that the species under the sub section *Contortae*, shared distinctive relations with *P. taeda* or the other *Pinus* sp. of *Ponderosae* and *Australes*. Furthermore, the study argues the phylogenetic distribution of few *Pinus* species by [4] like Leiophyllae clade was found to have close lineage with *Ponderosae*, *Oocarpae*, *Contortae*, *Australes*, and *Sabinianae*.

Lately, [12] and [13] used matK, rbcL, and other genes to understand the phylogeny of the *Pinus* sp. According to [12] *P. taeda*, *P. echinata*, *P. greggii*, *P. patula*, and *P. elliottii* were all

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classified into the sub section *Australes* of the *Pinus*. *P. greggii* was the only species that had minimal genetic linkage to *P. taeda, P. echinata*, *P. patula*, and *P, elliottii*, whereas *P. taeda* and *P. patula* were closely related and *P. echinata* and *P.elliottii* showed close relation as they all belonged to the North and Central American regions. However, *P. thunbergii* was classified under the sub section Contortae and had low genetic linkage to *P.taeda*, *P.echinata*, *P.greggii*, *P. patula* and *P.elliottii* as they were found in the Himalayan region. Similarly, *P. greggii* and *P.patula* were positioned under *Australes* subsection with high genetic lineage, whereas *P.thunbergii* was positioned under Contortae.

Singh and Thapliyal [14] analyzed seed sources for genetic variation amongst exotic and indigenous *Pinus* sp. and the result presented a significant diversity in height, length, the width of cone and seed. The overall study result presented the heritability values of seed weight associated with maximum genetic gain. Such genetic traits disclosed a strong genetic control that revealed phenotypic and genotypic variations among the seed and cone are a result of genetic diversity [14]. The genetic variation occurred due to genotypic and environmental interaction. This concluded that the Indian species *P. roxburghii, P. wallichiana, P. kesiya, P. gerardiana, P.merkusii, P. thunbergii,* and *P. clausa* are closely related to each other with respect to needle anatomy and morphology as well as on the basis of genetic structure. In this study, out of the 10 taxa examined nine belong to subgenus *Pinus* and one to subgenus *Strobus*. With respect to anatomical structure of the leaf, two subgenera were easily distinguishable by the number of vascular bundles in their needle. Although various morpho-anatomical classification schemes have been proposed for the sections and subsections of subgenus *Pinus* as well as subgenus *Strobus*, the relationships among the subsections and their evolutionary processes are still being debated [3,14-18]. Due to large number of morpho-anatomical characters and presence of high levels of homoplasy in many of these morpho-anatomical characters, several differences among classification schemes have been encountered. Thus, a comprehensive treatment of phylogenetic analysis of both the subgenus is hampered by the inadequacy of discrete characters, which are also scarce in the genus *Pinus* compared to other plant groups [15]. Several molecular studies have been conducted on the species of both subgenus *Pinus* and subgenus *Strobus* and these studies revealed a large genetic dissimilarity between the two subgenera and within various species of these subgenera. as well as little genetic variation in subgenus *Pinus* [7,9,11,19]. However, these studies have been limited in terms of taxonomic sampling and/or geographic scope, particularly in subgenus *Pinus* and pine species growing in Europe and American continents. The study by [11] using chloroplast DNA (cpDNA) analyzed 18 Diploxylon pines species of North America. Other studies such as analysis of ITS sequences by [5] involved a broad sampling of *Pinus* subsections and covered a wide range of geographic regions. Emphasis on native species of Eurasian pines and their phylogenic relationship with other species growing in their vicinity after their introduction from other continents due to their economic interest is missing. However ITS data generated in the study strongly supported the hypothesis of the presence of a distinctive group of North American pines.

Conclusion

Further studies involving as many as species possible using multiple marker, including both subgenera and all their sections as well as subsection are need to be studied to develop a complete database that can be used for a full proof classification and identification of *Pinus* up to species level.

Authors contribution

LS was a major contributor in writing the manuscript, GS and PCV proposed the research idea, conceptualized the research design and prepared the final manuscript including the original experimental work, and data recording. PJ, RS, SP and AS was involved with material collection and identification.

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