RESEARCH ARTICLE

Novel insights into Pinus species plastids genome through phylogenetic relationships and repeat sequence analysis

Umar Zeb 1, Xiukang Wang 2*, Sajid Fiaz 3*, Azizullah Azizullah 1, Asad Ali Shah 4, Sajjad Ali 5, Fazli Rahim 5, Hafiz Ullah 6, Umed Ali Leghari 7, Weiqiang Wang 2, Taufiq Nawaz 8

1 Department of Biology, The University of Haripur, Khyber Pakhtunkhwa, Pakistan, 2 College of Life Sciences, Yan’an University, Yan’an, Shaanxi, China, 3 Department of Plant Breeding and Genetics, The University of Haripur, Haripur, Pakistan, 4 Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Faisalabad, Pakistan, 5 Department of Botany, Bacha Khan UniversityCharsadda, Khyber Pakhtunkhwa, Pakistan, 6 Department of Botany, University of Chitral, Khyber Pakhtunkhwa, Pakistan, 7 Department of Agriculture, Mir Chakar Khan Rind University, Sibi, Balochistan, Pakistan, 8 Department of Food Science and Technology, The University of Agriculture Peshawar, Khyber Pakhtunkhwa, Pakistan

* wangxiukang@yau.edu.cn (XW); sfiaz@uoh.edu.pk (SF)

Abstract

Pinus is one of the most economical and ecological important conifers, model species for studying sequence divergence and molecular phylogeny of gymnosperms. The less availability of information for genome resources enable researchers to conduct evolutionary studies of Pinus species. To improve understanding, we firstly reported, previously released chloroplast genome of 72 Pinus species, the sequence variations, phylogenetic relationships and genome divergence among Pinus species. The results displayed 7 divergent hotspot regions (trnD-GUC, trnY-GUA, trnH-GUG, ycf1, trnL-CAA, trnK-UUU and trnV-GAC) in studied Pinus species, which holds potential to utilized as molecular genetic markers for future phylogenetic studies in Pinus species. In addition, 3 types of repeats (tandem, palindromic and dispersed) were also studied in Pinus species under investigation. The outcome showed P. nelsonii had the highest, 76 numbers of repeats, while P. sabiniana had the lowest, 13 numbers of repeats. It was also observed, constructed phylogenetic tree displayed division into two significant diverged clades: single needle (soft pine) and double-needle (hard pine). The outcome of present investigation, based on the whole chloroplast genomes provided novel insights into the molecular based phylogeny of the genus Pinus which holds potential for its utilization in future studies focusing genetic diversity in Pinus species.

Introduction

Pinus L. (Pinaceae) is an important genus of conifers with more than 230 species. It is a broadly distributed in temperate zones of Northern Hemisphere [1]. Pines include important tree species which are commercially used in pharmacology and wood pulp industries around the world. Genus Pinus is divided into two subgenera Strobus, (Haploxylon) and Pinus
and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

(Diploxylon) [2]. Moreover, anatomical, molecular, and morphological evidence strongly reinforced divergence of Strobus and Pinus, respectively [3]. Because of ecological importance and diversity, genus Pinus prove a best model for molecular study of conifers. Pinus genomes are extremely large (c. 20–40 Gb) and shown no evidence of recent polyploidy or chromosomal duplication. Pine chromosomes (2n = 24) are uniform both in number and appearance, owing to lack of major distinguishing physical features [4].

The phylogenetic tree displayed evolutionary relationships among different biological species based on similarities and differences for their maternal characteristics. Moreover, phylogenetic relationships in Pinus species are regularly studied through genome sequencing [5]. The whole chloroplast genome has numerous features e.g., small in size, conserved structure, maternal inheritance, and species identification is broadly applied for evolutionary studies [6].

Recently, an extremely divergent region in plant plastome has been identified called "hotspot region" and served an useful genetic marker for phylogeny and evolutionary studies of genus Pinus [7]. Previous studies showed that the genus Pinus had shared several genomic sequence variations for cp DNAs owing to their recent divergence radiation, regular interspecific and introgression gene flow among species [8]. The low degree of genomic divergence among Pinus species has been attributed to a large number of molecular evolution taking place in related species [9]. Therefore, it hold integral importance to understand complete phylogenetic relationships of Pinus species to understand the underlying genetic mechanisms controlling its diverse features [10].

Complete chloroplast genome are circular DNA molecules, had a quadripartite shape with large single copy (LSC) region, a small single copy (SSC) region, and two inverted repeats (IRs) regions [6]. Previous studies had revealed that plastid DNA of gymnosperm plants were extremely maintained in genome structure, order and gene contents [11]. The repeat sequence analysis in plastome contributes to various cellular functions including RNA editing, gene mobility and gene evolution [12]. Repetitive sequences are categorized into three modules: local repeats (simple sequence repeats (SSRs) and tandem repeats), families of dispersed repeats (mostly transposable elements and retro-transposed cellular genes), and segmental duplications (duplicated genomic fragments). The large number of repetitive sequences involved during the process of evolution in plant genomes depending on their structure and mode of multiplications [13]. Moreover, long repeat sequences are spread throughout the chloroplast genomes of Pinus species. Recent studies have shown that most repeat sequences were positioned in the intergenic and intron regions whereas, limited repeat sequence were located in the coding regions of gymnosperm plastomes [1]. The diversity of the repeated sequences may provide valuable information for species adaptation to varying environmental condition.

In the present study, we will analyzed complete chloroplast genomes of or seventy-two Pinus species to identify structural variations and theircomparative genome analysis. We aimed toinvestigae comprehensive structural variations in Pinus genomes, examination of large repeat sequence variation in the plastid genome of Pinus, and reconstruction of phylogeny of major lineages of Pinus species based on complete chloroplast genome.

Materials and methods

Materials

The whole plastid genome dataset of seventy-two Pinus species and their three outgroups (Picea glauca, Abies koreana and Abies nephrolepis) were identified and downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/). The Pinus complete cp genomes sequencing was annotated and further utilized for analysis.
Chloroplast genome sequencing, annotation and divergence analysis

Data were used to generate a consensus sequence inside the software Geneious R v 8.0.2 (Biomatters Ltd., Auckland, New Zealand). Preliminary, the plastome annotation was turned using the program DOGMA (https://domainworld-services.uni-muenster.de/dogma/). The stop and start codons are manually adjusted in Geneious R v 8.0.2. The round plastid genome map was drawn with the Organellar Genome DRAW v1.1 (OGDRAW) [14]. The sequence rearrangement of seventy-two plastomes was undertaken on Mauve Alignment [15]. To display interspecific variation, the alignments of the plastid DNA of the seventy-two genus _Pinus_ were envisioned by mVISTA online software (https://genome.lbl.gov/vista/mvista/about.shtml) in the Shuffle-LAGAN mode and _P. squamata_ specie was used as reference. The percentages of variable characters for non-coding and coding regions were counted via procedure given by Zhang et al. [16].

Repeat sequence analysis

We found three types of repeats in complete chloroplast genome of seventy-two _Pinus_ species: dispersed, tandem, and palindromic whereas, web-based REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer/) programmed was used to investigate these repeat sequences. The dispersed and palindromic repeats were used on following condition; (1) sequence identity 90%; (2) Hamming distance = 1 (3) repeat size minimum = 30 bp [17]. Tandem motifs (>10 bp in length) was identified using online software Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) [18].

Phylogenetic analysis

The complete dataset of _Pinus_ genome sequence was aligned using MAFFT V 7.0.0 programmed [19]. Phylogenetic analysis was carried out using the cpDNA of all seventy two _Pinus_ species (Table 1). These species were aligned with the Clustal W method of MEGA v7.0.18 software with manual inspection [20]. In addition, we included sequences from _Abies koreana_, _Abies nephrolepis_ and _Picea glauca_ as an outgroups. Maximum likelihood (ML) and maximum parsimony (MP) analysis were performed with the Akaike Information Criterion and an appropriate sequence evolution model selected by Model Test version 3.7. (AIC) [21]. Subsequently, one thousand (1000) bootstrap replicate was used to evaluate the support value of both ML and MP branches. PAUP* was used to calculate the phylogenetic reconstruction. Furthermore, the Bayesian phylogenetic analysis was operated using MrBayes v3.1.2 [22]. Markov Chain Monte Carlo (MCMC) was run over 3,000,000 generations, starting with an arbitrary tree and sampling topologies for every 100 generations. The first 2,500 trees (containing 25% of our samples) were burned (as recommended by MrBayes), and the remaining trees were used to build the 50% majority rule consensus tree and estimate Bayesian posteriors of nodal support probabilities.

Results

Genome features of seventy-two _Pinus_ species

The complete chloroplast genomes of seventy-two _Pinus_ species ranged in size from 114,087 ( _P. pumila_ ) to 121,976 bp ( _P. glabra_ ) (Table 1 and Fig 1). Plastid genomes had a quadripartite structure which present in most of the gymnosperm species. The complete genomes of _Pinus_ species comprised of a large single copy (LSC) region ranged from 64,415 ( _P. sylvestris_ ) to 65,610 bp ( _P. taeda_ ), and a small single copy (SSC) region ranged from 50,661 ( _P. sylvestris_ ) to 56,070 bp ( _P. glabra_ ), and inverted repeats (IRs) ranged from 244 ( _P. muricata_ ) to 492 bp ( _P. arizonica_ ) in size (Table 1). The whole plastome of GC content was comparable to the _Pinus_ species.
| Section | Species | Size (bp) | LSC (bp) | SSC (bp) | IR (bp) | Number of Protein Coding Genes | Number of rRNA Genes | Number of tRNA Genes | GC Contents (%) | Gene bank number |
|---------|---------|-----------|----------|----------|---------|--------------------------------|---------------------|---------------------|-----------------|-----------------|
| Double needle Section (Subgenera Pinus) | P. jaliscana | 119,697 | 64,805 | 54,092 | 403 | 75 | 4 | 37 | 38.5 | NC_035948 |
| | P. pringlei | 119,580 | 65,084 | 53,718 | 389 | 75 | 4 | 36 | 38.5 | JN854189 |
| | P. lawsonii | 119,411 | 65,135 | 53,498 | 389 | 75 | 4 | 36 | 38.5 | JN854188 |
| | P. oocarpa | 120,596 | - | - | - | 4 | 36 | 38.5 | NC_035949 |
| | P. palustris | 119,149 | 65,084 | 53,718 | 389 | 75 | 4 | 36 | 38.5 | JN854176 |
| | P. greggii | 119,480 | 64,849 | 53,853 | 389 | 74 | 4 | 36 | 38.5 | NC_035947 |
| | P. patula | 119,356 | 65,130 | 53,448 | 389 | 75 | 4 | 36 | 38.5 | JN854175 |
| | P. occidentalis | 119,826 | 65,204 | 53,844 | 389 | 75 | 4 | 36 | 38.5 | JN854177 |
| | P. taeda | 120,534 | 65,116 | 53,590 | 389 | 75 | 4 | 36 | 38.5 | NC_021440 |
| | P. pungens | 119,528 | 65,084 | 53,718 | 389 | 75 | 4 | 36 | 38.5 | JN854167 |
| | P. caribaea | 119,523 | 64,849 | 53,634 | 399 | 75 | 4 | 36 | 38.5 | JN854222 |
| | P. elliottii | 119,767 | 65,140 | 53,849 | 389 | 75 | 4 | 36 | 38.5 | JN854202 |
| | P. glabra | 121,976 | 64,936 | 56,070 | 485 | 75 | 4 | 36 | 38.8 | JN854199 |
| | P. muricata | 118,328 | 65,039 | 52,745 | 399 | 75 | 4 | 36 | 38.5 | JN854165 |
| | P. radiata | 119,678 | 65,155 | 53,736 | 399 | 75 | 4 | 36 | 38.5 | JN854215 |
| | P. coulteri | 119,829 | 64,830 | 53,129 | 485 | 75 | 4 | 36 | 38.5 | JN854161 |
| | P. sabiniana | 119,767 | 65,140 | 53,849 | 389 | 75 | 4 | 36 | 38.5 | JN854193 |
| | P. jeffreyi | 119,742 | 65,140 | 53,824 | 389 | 75 | 4 | 36 | 38.5 | JN854201 |
| | P. douglasiana | 119,624 | 65,076 | 53,658 | 444 | 76 | 4 | 36 | 38.5 | JN854205 |
| | P. arizonica | 119,965 | 64,899 | 54,084 | 492 | 75 | 4 | 37 | 36.4 | JN854216 |
| | P. devoniana | 119,688 | 65,116 | 53,794 | 389 | 75 | 4 | 36 | 38.5 | JN854208 |
| | P. montezumae | 119,181 | 65,103 | 53,255 | 523 | 75 | 4 | 35 | 38.5 | JN854183 |
| | P. hartwegii | 119,460 | 64,869 | 53,623 | 485 | 75 | 4 | 36 | 38.5 | JN854206 |
| | P. pseudostrobus | 117,391 | 64,712 | 51,901 | 389 | 74 | 4 | 35 | 38.5 | JN854178 |
| | P. clausa | 118,899 | 65,027 | 52,918 | 484 | 75 | 4 | 35 | 38.5 | JN854217 |
| | P. roxburgii | 119,409 | 64,886 | 53,776 | 384 | 75 | 4 | 36 | 38.6 | JN854162 |
| | P. pinea | 119,195 | 64,843 | 53,564 | 394 | 75 | 4 | 36 | 38.5 | JN854173 |
| | P. heldrichii | 117,823 | 65,065 | 51,952 | 406 | 75 | 4 | 35 | 38.6 | JN854195 |
| | P. halepensis | 118,947 | 64,750 | 53,237 | 394 | 75 | 4 | 36 | 38.5 | JN854197 |
| | P. brutia | 120,570 | 64,900 | 54,610 | 485 | 75 | 4 | 36 | 38.5 | JN854224 |
| | P. pinaster | 119,212 | 64,932 | 53,492 | 399 | 73 | 4 | 36 | 38.5 | FJ899583 |
| | P. latteri | 119,279 | 65,069 | 53,432 | 389 | 75 | 4 | 36 | 38.5 | JN854190 |
| | P. resinosa | 119,527 | 65,057 | 53,681 | 402 | 75 | 4 | 36 | 38.5 | FJ899556 |
| | P. tropicalis | 118,924 | 65,002 | 53,133 | 389 | 75 | 4 | 36 | 38.5 | JN854156 |
| | P. massoniana | 119,025 | 65,139 | 53,108 | 389 | 75 | 4 | 36 | 38.6 | NC_021439 |
| | P. sylvestris | 115,909 | 64,415 | 50,661 | 420 | 75 | 4 | 37 | 38.6 | KR476379 |
| | P. densiflora | 119,124 | 65,179 | 53,147 | 399 | 75 | 4 | 19 | 38.5 | JN854210 |
| | P. fragilissima | 119,038 | 65,143 | 53,097 | 399 | 75 | 4 | 36 | 38.5 | JN854200 |
| | P. kesiya | 118,986 | 65,179 | 53,009 | 399 | 75 | 4 | 36 | 38.5 | JN854191 |
| | P. hwanghanensis | 118,993 | 65,179 | 52,961 | 399 | 75 | 4 | 36 | 38.5 | JN854194 |
| | P. yunnanensis | 118,614 | 65,061 | 52,763 | 395 | 74 | 4 | 36 | 38.5 | JN854151 |

Table 1. The features of complete chloroplast genomes of seventy-two Pinus species.
**Table 1.** (Continued)

| Section | Species            | Size (bp) | LSC (bp) | SSC (bp) | IR (bp) | Number of Protein Coding Genes | Number of rRNA Genes | Number of tRNA Genes | GC Contents (%) | Gene bank number |
|---------|--------------------|-----------|----------|----------|---------|-------------------------------|---------------------|---------------------|-----------------|-----------------|
|         | *P. culminicola*   | 115,155   | 64,364   | 50,035   | 362     | 75                            | 4                   | 36                  | 38.7            | JN854213         |
|         | *P. discolor*      | 115,154   | 64,297   | 50,111   | 357     | 75                            | 4                   | 36                  | 38.7            | JN854207         |
|         | *P. cembroides*    | 115,919   | 64,394   | 50,581   | 460     | 75                            | 4                   | 36                  | 38.6            | JN854220         |
|         | *P. remota*        | 115,422   | 64,493   | 50,178   | 357     | 75                            | 4                   | 36                  | 38.6            | JN854164         |
|         | *P. quadrifolia*   | 115,508   | 64,385   | 50,367   | 362     | 75                            | 4                   | 36                  | 38.7            | JN854166         |
|         | *P. maximartinezii*| 115,620   | 64,575   | 50,269   | 382     | 75                            | 4                   | 35                  | 38.7            | JN854184         |
|         | *P. rzedowskii*    | 115,934   | 64,652   | 50,508   | 380     | 75                            | 4                   | 36                  | 38.6            | FJ899557         |
|         | *P. nelsonii*      | 116,210   | 64,604   | 50,845   | 367     | 74                            | 4                   | 35                  | 38.7            | EU998746         |
|         | *P. aristata*      | 116,918   | 64,251   | 51,707   | 480     | 75                            | 4                   | 35                  | 38.7            | FJ899567         |
|         | *P. bungeana*      | 116,751   | 64,311   | 51,490   | 475     | 75                            | 4                   | 36                  | 38.8            | NC_028421        |
|         | *P. gerardiana*    | 116,668   | 64,296   | 51,339   | 516     | 75                            | 4                   | 36                  | 38.7            | EU998741         |
|         | *P. strobusformis* | 116,200   | 64,230   | 51,108   | 474     | 75                            | 4                   | 36                  | 38.7            | JN854159         |
|         | *P. chiapensis*    | 116,197   | 64,524   | 50,895   | 392     | 75                            | 4                   | 36                  | 38.8            | JN854219         |
|         | *P. parviflora*    | 120,724   | 66,364   | 53,409   | 475     | 74                            | 4                   | 36                  | 38.6            | MG897304         |
|         | *P. wallichiana*   | 116814    | -        | -        | -       | -                             | -                   | -                   | -               | JN854154         |
|         | *P. squamata*      | 117,327   | 64,706   | 51,825   | 398     | 74                            | 4                   | 36                  | 38.7            | MG897303         |
|         | *P. lambertiana*   | 116,958   | 64,604   | 51,592   | 379     | 75                            | 4                   | 35                  | 38.8            | EU998743         |
|         | *P. pumila*        | 114,087   | 64,553   | 48,762   | 384     | 75                            | 4                   | 36                  | 38.7            | JN854168         |
|         | *P. dalatensis*    | 116,657   | 64,533   | 51,321   | 393     | 75                            | 4                   | 36                  | 38.8            | JN854211         |
|         | *P. armandii*      | 116,998   | 64,337   | 51,711   | 389     | 75                            | 4                   | 36                  | 37              | NC_029847        |
|         | *P. morrisonicola* | 116,636   | 64,104   | 51,770   | 381     | 74                            | 4                   | 36                  | 38.7            | MG897305         |
|         | *P. wangii*        | 118,073   | 65,598   | 51,521   | 476     | 74                            | 4                   | 36                  | 38.7            | MG897302         |
|         | *P. fenzeliana*    | 117,805   | 64,490   | 52,565   | 375     | 75                            | 4                   | 35                  | 36.8            | KX255674         |

Pinus species complete cp genome consisted of 114 functional genes, with 36 tRNA, 4 rRNA and 74 protein-coding. Among 114 genes, 11 genes for small ribosome subunits, 9 genes for large ribosome subunits, 4 genes for DNA-dependent RNA polymerase subunits and 50 genes fragments were related to self-replication. The translational initiation factor (infA) gene, 38 genes for photosynthesis, 6 genes for ATP synthesis, and 11 genes encoding subunits of photosystem I (Table 2).

**Repeat sequence variations and genome structure comparison**

In this study, we calculated three types of repetitions, i.e. dispersed, palindromic and tandem repeats. Among these repeat variations, a number of divisions and repeats were analyzed (S1 Table and Fig 2). We identified 5,943 repeats, among these repeats dispersed were most common with 2,612 (43.95%), followed by palindromic repeats with 1,921 (32.32%), and tandem repeats with 1,410 (23.72%) (Fig 1). Majority of repeats found circulated in intergenic regions and few were situated within generic regions. *P. nelsonii* were the most dispersed repeated sequences (76) followed by *P. pseudostrobus* (63) palindromic repeats whereas, *P. sabiniana* showed lowest number tandem repeats with only (13) tandem repeats (S1 Table).

For sequence identity analysis mVISTA was used with *P. squamata* sequence as reference (S1 Fig). It was observed that 72 Pinus species hold large number of sequence similarity however, lesser degree of variation was also observed. It is worthy to mention that non-coding regions displayed high levels of divergence compared to coding regions. The outcome helped
to identified hotspot divergent regions on Pinus cp genome (S1 Fig). The non-coding regions displayed sequence divergence, and percentage of variation ranged from 0 to 13.78% with an average of 4.96%, whereas, the percentage variation in coding region ranged from 0 to 9.98% with an average of 2.54% (Fig 3). Furthermore, we discovered that IR region has a lower number of mutations and is highly conserved in Pinus species. It noteworthy, we identified seven genes (\textit{trnD-GUC}, \textit{trnY-GUA}, \textit{trnH-GUG}, \textit{ycf1}, \textit{trnL-CAA}, and \textit{trnV-GAC}) at LSC and SSC region located within the non-coding regions showing greater levels of variation, with ability to act as divergence hotspot regions.

\textbf{Phylogenetic relationships of Pinus species}

The 72 \textit{Pinus} chloroplast genome sequences were used for phylogenetic analysis. Under the GTR+G+I model, we re-constructed three independent phylogenetic trees through different analytical methods: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) (Fig 4). Among investigated species, the phylogenetic analysis displayed congruent topologies, although the bootstrap value was kept slightly different for all phylogenetic trees. The phylogenetic tree further divided into two clades, single-needle section (subgenus
Strobus) and double-needle section (subgenus Pinus species) (Fig 4). We found that P. wangii, P. fenzeliana, P. morrisonicola and P. armandii posses close relationships and categorized in the single needle section. In addition, the P. parviflora, P. chiapensis and P. wallichiana were closely related to subgenus Pinus.

**Discussion**

**Features of cp genomes of Pinus species**

The chloroplast genome of higher plants is circular molecule with a length of 120–160 kb with approximately 130 genes [23]. The structure and organization of these genes found similar among the 72 Pinus species under investigation. Moreover, similar GC level for 72 Pinus species was observed which is less common for most of the terrestrial plants [23]. IRs contraction and expansion are extensively exhibited in many land plant species. The large IRs played a significant role in maintaining the constancy of whole plastome [24]. Small IR region may cause variations in genome structure and content of plastome [25]. Interestingly, in present study, we detected small IR regions in all investigated Pinus species (244 to 492 bp). Following

| Gene group                     | Gene name       | Gene name       | Gene name       | Gene name       | Gene name       |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ribosomal RNA genes           | rrn16           | rrn23           | rrn5            | rrn4.5          | trnL-CAU        |
| Transfer RNA genes            | trnL-CAU        | trnL-UAA        | trnL-GAU        | trnL-UAG        | trnL-CAU        |
|                               | trnR-UCU        | trnR-ACG        | trnA-UGC        | trnW-CCA        | trnE-UUC        |
|                               | trnV-GAC        | trnV-UAC        | trnT-UGU        | trnF-GAA        | trnT-GGU        |
|                               | trnF-CAU        | trnF-UGG        | trnG-GCC        | trnP-GGG        | trnS-GGA        |
|                               | trnS-UGA        | trnS-GCU        | trnD-GUC        | trnC-GCA        | trnN-GUU        |
|                               | trnE-UUC        | trnY-GUA        | trnQ-UUG        | trnK-UUU        | trnH-GUG        |
|                               | trnG-GCC        | trnM-CAU        |                 |                 |                 |
| Small Subunit of ribosome     | rps2            | rps3            | rps4            | rps7            | rps8            |
|                               | rps11           | rps12           | rps14           | rps15           | rps18           |
|                               | rps19           |                 |                 |                 |                 |
| Large Subunit of ribosome     | rp12            | rp114           | rp116           | rp120           | rp122           |
|                               | rp123           | rp132           | rp133           | rp136           |                 |
| DNA-dependent RNA polymerase  | rpoA            | rpoB            | rpoC1           | rpoC2           |                 |
| Translational initiation factor| infA            |                 |                 |                 |                 |
| Subunits of photosystem I     | psaA            | psaB            | psaC            | psaL            | psaL            |
|                               | psaM            | ycf1            | ycf2            | ycf3            | ycf4            |
|                               | ycf10           |                 |                 |                 |                 |
| Subunits of photosystem II    | psbA            | psbB            | psbC            | psbD            | psbE            |
|                               | psbF            | psbH            | psbI            | psbJ            | psbL            |
|                               | psbM            | psbN            | psbT            |                 |                 |
| Subunits of cytochrome        | petA            | petB            | petD            | petG            | petL            |
|                               | petN            |                 |                 |                 |                 |
| Subunits of ATP synthase      | atpA            | atpB            | atpE            | atpF            | atpH            |
|                               | atpI            |                 |                 |                 |                 |
| Large subunit of Rubisco      | rbcL            |                 |                 |                 |                 |
| Maturase                      | matK            |                 |                 |                 |                 |
| Protease                      | clpP            |                 |                 |                 |                 |
| Subunit of acetyl-CoA         | accD            |                 |                 |                 |                 |
| C-type cytochrome synthesis   | ccsA            |                 |                 |                 |                 |

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https://doi.org/10.1371/journal.pone.0262040.t002
results displayed that in certain genes have variations for structure and contents compared to whole cp genome of *Pinus* species [26].

Previous investigations has exhibited that repeat sequences have performed significant roles in genome re-organization and recombination [27]. Among 72 *Pinus* species *P. nelsonii* genome had large numbers of repeats (76), whereas, *P. pseudostrobus* genome have (63) repeats. In contrary, *P. sabiniana* displayed lowest number of (13) repeats (S1 Table and Fig 2). However, the tandem, dispersed, and palindromic repeats distributions were comparable for

![Fig 2. A histogram of the number of repeats found in the seventy-two Pinus chloroplast genomes. (a) The number of repeats in subgenus Pinus (b) Number of repeats in subgenus Strobus.](https://doi.org/10.1371/journal.pone.0262040.g002)

![Fig 3. Variable characters percentage in homologous regions of Seventy-two Pinus species of chloroplast genome (a) Coding region (b) Non coding region. The homologous regions are oriented according to their locations in the chloroplast genome.](https://doi.org/10.1371/journal.pone.0262040.g003)
all *Pinus* species. A large number of repeats could maintain cp genomes constants, similar results reported by Zhang et al [16]. The repeat sequence displayed similar genes function rearrangement for further study in population genetics and *Pinus* species evolution [2].

**Comparative analysis of the genomic structure**

The complete chloroplast genome of *Pinus* species displayed a very low genetic divergence. Sequence alignment of 72 plastids genomes were compared, and used for sequence identity analysis via mVISTA programe, keeping *P. squamata* as a reference speicie (S1 Fig). The
similarity analysis exhibited a high sequence comparison across the plastid genomes having sequence identities below 90%. However, a low divergence region identified in LSC and less mutation rate in IRs region. In addition, the divergent hotspot regions (trnD-GUC, trnY-GUA, trnH-GUG, ycf1, trnL-CAA, trnK-UUU and trnV-GAC) were found in non-coding regions of some tRNA sequences. Several repetitive sequences were equally distributed in the divergence hotspot regions. These hotspot regions can be utilized for phylogenetic study and provide DNA barcoding for future evolutionary studies of gymnosperm species [28].

**Phylogenetic relationships of Pinus species**

The whole plastome phylogenetic analysis has been commonly undertaken in land plants [29]. During recent decade, a study has revealed phylogenetic relationship and comparisons of numerous protein-coding genes present in the chloroplast genomes [30]. That improved our understanding for phylogenetic relationship and molecular studies among Pinus species [31].

The current study used phylogenetic analysis based on entire cp genome sequence of 72 Pinus species having P. glauca, A. nephrolepis, and A. koreana serving as outgroups. Using ML, MP, and BI methods, we created a concurrent phylogenetic tree with a wide range of supported values (Fig 4). The phylogenetic tree of Pinus species was divided into two groups that corresponded to single needle sections and double needle sections. Among these sequenced species, single-needle section species i.e., P. morrisonicola and P. wangii categorized in the same clade, showing a close relationship with each other. Moreover, these two species showed a high similarity in their chloroplast genome sequences [31]. In addition, the phylogenetic tree revealed P. bungeana and P. gerardiana has a close relationship with each other [32]. The phylogenetic results exhibited P. clausa showed a sister clade to the Pinus species [33].

**Conclusion**

The present study determined the whole chloroplast genome a rich source to understand the evolutionary history. The cp genomes of Pinus species, genome structure and order were similar in nature. Moreover, the location and distribution of repeat sequences were determined, and common pairwise sequence divergences among cp genomes of interrelated species were identified. The whole genome sequencing proved to be a significant knowledge for plant taxonomic positioning. The main findings based on complete chloroplast genome of Pinus species divided into two sections, single needle sections and double-needle sections of Pinus species. The phylogenetic relationships dependent on the cp genome greatly developed our understanding on phylogeny of Pinus species. Comparative analyses of plastid genome sequences provide DNA markers for easy identification and classification. These results will provide portable confirmations and prove a solid basis for the improvement of chloroplast genome in Pinus species.

**Supporting information**

S1 Fig. Sequence alignment of chloroplast genomes of Pinus species. mVISTA-based identity plots viewing identity between seventy-two Pinus species cp genomes. The vertical scale indicates the percentage identity, ranging from 50% to 100%. Divergent hotspot refers to the places with more variable sites compared to another region. (DOCX)

S1 Table. Repeat sequences analysis in seventy-two Pinus species based on complete chloroplast. (DOCX)
Author Contributions

Data curation: Umar Zeb.
Formal analysis: Sajjad Ali, Hafiz Ullah.
Funding acquisition: Xiukang Wang.
Investigation: Azizullah Azizullah.
Methodology: Asad Ali Shah, Fazli Rahim.
Writing – review & editing: Sajid Fiaz, Asad Ali Shah, Hafiz Ullah, Umed Ali Leghari, Weiqiang Wang, Tauﬁq Nawaz.

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