Chloroplast Genome of *Lithocarpus Dealbatus*: Identification of Hotspots for Sequence Variability and Phylogeny Analysis in Quercoideae, a Sub-family of Fagaceae

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Abstract

Background

There has been phylogenetic ambiguity and species delimitation problem in *Lithocarpus* genus and the subfamily Quercoideae (Family: Fagaceae). *Lithocarpus dealbatus* is the dominant genus among the Quercoideae in India. Although several studies have been conducted on regeneration, ecological and economic significance of the species, limited information is available at genome scale. We sequenced and assembled the complete chloroplast genome of *L. dealbatus* and compared with other Quercoideae members to understand the sequence variations, rearrangements and its phylogenetic lineage in the Quercoideae.

Results

We assembled the 161,476 bp chloroplast genome of *L. dealbatus*, which has a large single-copy section of 90,732 bp and a small single-copy region of 18,987 bp, separated by a pair of inverted repeat regions of 25,879 bp. There were 133 genes in the cp-genome, including 86 protein coding genes, 39 transfer RNAs, and eight ribosomal RNAs. Analysis of repeat elements and RNA editing sites revealed inter-specific similarities within *Lithocarpus* genus. DNA diversity analysis identified highly diverged coding and non-coding hotspot regions in the genera *Lithocarpus*, *Quercus*, *Castanea*, and *Castanopsis*. These hot spots can be used as polymorphic barcodes to resolve the phylogenetic relationship at species level. We discovered five barcodes that could aid in species delimitation across the four genera of Quercoideae. The phylogenetic analysis among the Quercoideae established a monophyletic origin of *Lithocarpus*, and a closer evolutionary lineage with many *Quercus* species.

Conclusions

Our findings provide insights into the chloroplast genome of *L. dealbatus*, the mutational hotspot regions and repeat elements. These could be utilized as molecular markers/barcodes for detailed studies in population genetics.

Background

Lithocarpus dealbatus (Hook.f. & Thomson ex Miq.) Rehder., commonly known as stone oak, is a wild non-edible and nut-bearing tree belonging to subfamily Quercoideae under the family Fagaceae. The Quercoideae consists of seven genera viz., *Castanea*, *Castanopsis*, *Chrysolepis*, *Lithocarpus*, *Notholithocarpus*, *Quercus* and *Trigonobalanus*. The other subfamily Fagoideae is mono-generic with the genus *Fagus*. Quercoideae has 1088–1135 species, while Fagoideae has 11–14 species (The plant list, <http://www.theplantlist.org/>). *Lithocarpus* is the second largest genus of the family Fagaceae comprising ~ 330 species (The plant list, <http://www.theplantlist.org/>), most of which are distributed in the moist/wet evergreen forests of South-East Asia. In India, the genus *Lithocarpus* is represented by 17 species [1, 2]. *L. dealbatus* grows at an altitude range of 1,000–1,500 m asl and attains a height up to 30 m [3, 4]. It is found in the subtropical montane forests of the Eastern Himalayas and in the hills of north-eastern India [5]. The species is mostly used as fuelwood and timber, and its fruits (acorns) serve as a food for rodents. The hoarding of acorns by rodents helps the species in wide seed dispersal, maintenance of soil seed bank, and regeneration of the species in the forest [6–11].

Lithocarpus is closely related to *Quercus*, *Castanea* and *Castanopsis* genera of Fagaceae [12]. Inter-specific hybridisation within the family often creates ambiguity in species delimitation, thus necessitating to address their complexity in phylogeny [13–15]. The universal plastid markers cannot provide sufficient number of variable sites and phylogenetic signals to investigate the phylogeny in Quercoideae [16–18]. Previously, the use of nuclear markers in Fagaceae phylogeny inference yielded relatively low support for the monophyletic genus *Quercus* [18]. The results of earlier workers have showed that a single marker or even a combination of markers has very low discrimination rate [17]. Therefore, the chloroplast genome has been successfully used for phylogenetic analysis in *Castanea* and *Castanopsis* to resolve taxonomic discrepancies [16]. Chloroplast genome sequences have been widely used to resolve plant phylogenies due to their highly conserved structure and recombination-free uniparental inheritance [16]. In *Quercus*, Pang et al. [17] emphasized the species-level discrimination and the improvement of the phylogenetic resolution by using chloroplast genome sequences.

The chloroplast genome sequences have been also used to identify the mutational hot spot regions for designing the species-specific DNA barcodes. Unlike universal barcodes, the mutational hotspots can far better differentiate plant taxa down to the species level [15, 16]. Previously, Pang et al. [17] demonstrated that the newly discovered markers were more variable than the standard and universal plant DNA markers in *Quercus* species (e.g. *rbcL* and *matK*). Extremely low resolution of universal DNA barcode was also reported in the 12 Italian oak species [19]. As a result, several reports have been published highlighting the need to develop additional barcodes in Quercoideae species [17, 20]. Researchers have demonstrated that chloroplast genome mutations are clustered into hotspots, and these hotspot regions can be considered as real DNA barcodes [21]. Following the approach of mutational hotspots from the entire chloroplast genome, the species-specific barcodes in oak and *Castanea* species have been designed [17, 20]. Despite the fact that some specific barcodes with the highest discriminatory power have previously been reported in *Quercus*, additional markers are still required for the rest of the Quercoideae species considering their complex evolutionary background. Therefore, the availability of genomic information is essential to understand the evolutionary relationship of *Lithocarpus* genus and its species with other genera of Fagaceae.

Plant chloroplast genomes have circular quadripartite structure with sizes ranging from 107 to 218 kb [21]. In terms of structural organisation, gene content and layout, the chloroplast genome is extremely conserved [25]. Small single-copy (SSC) and large single-copy (LSC) sections are separated by two inverted repeats (IR) regions in the genome [25]. The chloroplast has 130 genes, including 80 protein-coding genes, 4 rRNA genes, and 35 tRNA genes [19]. The chloroplast genome is uniparentally inherited and highly conserved in nature, thus accumulating slow rate of evolutionary changes [22–26]. Despite the overall structural conservation, and contraction and expansion of the IR boundaries, the chloroplast genome can lead to variations in gene content resulting in genome size variation [27]. Besides, several mutational events happen in the genome due to insertion or deletion, single nucleotide polymorphisms (SNP), simple sequence repeats (SSRs) and tandem repeats [27, 28]. Such genome-scale variations open up the possibility of using these regions as molecular markers in diversity research, population genetics, and phylogenetic investigations [29–31]. To address the limited resolution of single-locus DNA barcodes, the entire chloroplast genome has recently been employed to obtain more reliable evolutionary evidence [30, 32].

In recent times, the advancement of high throughput sequencing technologies resulted in increase in chloroplast genomes in the public domain. However, genomic information on *Lithocarpus*, particularly complete chloroplast

genome in the NCBI database is represented by only two species [33];<https://www.ncbi.nlm.nih.gov/nuccore/?term=Lithocarpus+chloroplast%2C+complete+genome>). Therefore, in the present study, we sequenced and assembled the complete chloroplast genome of *L. dealbatus*. The assembled chloroplast genome was successfully annotated and compared with other Quercoideae members to understand its structural variations and rearrangements. In addition, a phylogenetic tree was constructed to deduce the evolutionary relatedness of *L. dealbatus* with other members of Quercoideae clade. The highly divergent regions and the SSRs identified in the chloroplast genome will improve the understanding of the ecological significance with reference to its spatial distribution, adaptability, and evolutionary relationship of *L. dealbatus* within Fagaceae.

Results

L. dealbatus chloroplast genome assembly and structure

A total of ~ 47Gb data with more than 15,67,18,852 adapter clean short PE reads were used for *de novo* chloroplast genome assembly. The total length of the assembled *L. dealbatus* chloroplast genome was 161,476 bp with an average coverage of 1494 x (Fig. 1). The complete chloroplast genome exhibits a typical quadripartite structure, comprising a pair of IR (IRA and IRB) regions (25,879 bp) divided by an SSC region (18,987 bp) and an LSC region (90,732 bp). The overall GC content of the genome was 36.7%, while the GC content of LSC, SSC and IR regions were 34.6%, 30.9% and 42.7%, respectively.

Homology searched through the BLASTN program revealed a high sequence similarity of *L. dealbatus* Chloroplast genome with *Lithocarpus hancei* and *Lithocarpus balansae* Chloroplast genome. A high-quality chloroplast genome sequence was finally submitted to the Genbank, NCBI database under the accession number MZ322408.

Chloroplast genome encoding genes

The whole chloroplast genome of *L. dealbatus* encodes 133 genes, consisting of 86 protein-coding genes, 39 transfer RNA (tRNA) and 8 ribosomal RNA (rRNA) genes (Table 1). Among 133 genes, eight protein-coding genes (*ndhB*, *psbD*, *rpl2*, *rpl23*, *rps7*, *rps12*, *ycf1* and *ycf2*), nine tRNA and four RNA genes were duplicated in the genome (Table 2). Altogether, 13 protein-coding and eight tRNA genes were found to contain an intron, in which two genes (*ycf3* and *clpP*) harboured double intron. The *rps12* was identified as a trans-spliced gene in the genome. However, *trnk-UUU* has an intron encompassing the *matK* gene. The IR regions each harboured seven protein-coding genes, seven tRNA and four rRNAs.

Table 1
Details list of genes encoded by *L. dealbatus* chloroplast genome

Gene category	Gene name				
Ribosomal RNA genes	<i>rrn16</i> ^D	<i>rrn23</i> ^D	<i>rrn4.5</i> ^D	<i>rrn5</i> ^D	
Transfer RNA genes	<i>trnA-UGC</i> _{D*}	<i>trnC-GCA</i>	<i>trnD-GUC</i>	<i>trnE-UUC</i>	<i>trnF-GAA</i>
	<i>trnM-CAU</i>	<i>trnG-GCC</i> _{D*}	<i>trnH-GUG</i>	<i>trnI-CAU</i> ^D	<i>trnI-GAU</i> _{D*}
	<i>trnK-UUU</i> _*	<i>trnL-CAA</i> _D	<i>trnL-UAA</i> _*	<i>trnL-UAG</i>	<i>trnM-CAU</i>
	<i>trnN-GUU</i> _D	<i>trnP-GGG</i>	<i>trnP-UGG</i>	<i>trnQ-UUG</i>	<i>trnR-ACG</i> ^D
	<i>trnR-UCU</i>	<i>trnS-GCU</i>	<i>trnS-GGA</i>	<i>trnS-UGA</i>	<i>trnT-GGU</i> ^D
	<i>trnT-UGU</i>	<i>trnV-GAC</i> _D	<i>trnV-UAC</i> _*	<i>trnW-CCA</i>	<i>trnY-GUA</i>
Large subunit of ribosomal proteins (LSU)	<i>rpl2</i> _{D*}	<i>rpl14</i>	<i>rpl16</i>	<i>rpl20</i>	<i>rpl22</i>
	<i>rpl23</i> ^D	<i>rpl32</i>	<i>rpl33</i>	<i>rpl36</i>	
Small subunit of ribosomal proteins (SSU)	<i>rps2</i>	<i>rps3</i>	<i>rps4</i>	<i>rps7</i> ^D	<i>rps8</i>
	<i>rps11</i>	<i>rps12</i> _{D*}	<i>rps14</i>	<i>rps15</i>	<i>rps16</i> _*
	<i>rps18</i>	<i>rps19</i>			
DNA dependant RNA polymerase	<i>rpoA</i>	<i>rpoB</i>	<i>rpoC1</i> _*	<i>rpoC2</i>	
Photosystem I	<i>psaA</i>	<i>psaB</i>	<i>psaC</i>	<i>psaI</i>	<i>psaJ</i>
Photosystem II	<i>psbA</i>	<i>psbB</i>	<i>psbC</i>	<i>psbD</i> ^D	<i>psbE</i>
	<i>psbF</i>	<i>psbH</i>	<i>psbI</i>	<i>psbJ</i>	<i>psbK</i>
	<i>psbL</i>	<i>psbM</i>	<i>psbN</i>	<i>psbT</i>	<i>psbZ</i>
Cytochrome b/f	<i>petA</i>	<i>petB</i> _*	<i>petD</i>	<i>petG</i>	<i>petL</i>
	<i>petN</i>				
ATP synthase	<i>atpA</i>	<i>atpB</i>	<i>atpE</i>	<i>atpF</i> _*	<i>atpH</i>
☒ Genes with one intron.					
☒☒ Gene containg two introns.					
^D Gene harbouring duplicated copies.					

Gene category	Gene name					
	<i>atpI</i>					
Protease	<i>clpP</i> ^{**}					
Rubisco	<i>rbcL</i>					
NADH dehydrogenase	<i>ndhA</i> [*]	<i>ndhB</i> ^{D*}	<i>ndhC</i>	<i>ndhD</i>	<i>ndhE</i>	
	<i>ndhF</i>	<i>ndhG</i>	<i>ndhH</i>	<i>ndhI</i>	<i>ndhJ</i>	
	<i>ndhK</i>					
Maturase	<i>matK</i>					
Envelop membrane protein	<i>cemA</i>					
Subunit of acetyl-CoA-carboxylase	<i>accD</i>					
C-type cytochrome synthesis gene	<i>ccsA</i>					
Conserved hypothetical chloroplast open reading frames	<i>ycf1</i> ^D	<i>ycf2</i> ^D	<i>ycf3</i> ^{**}	<i>ycf4</i>		
☒ Genes with one intron.						
☒☒ Gene containg two introns.						
^D Gene harbouring duplicated copies.						

Table 2
Comparative analysis of seven Quercoideae chloroplast genomes.

Species	<i>L. dealbatus</i>	<i>L. balansae</i>	<i>L. hancei</i>	<i>C. henryi</i>	<i>C. sclerophylla</i>	<i>Q. pannosa</i>	<i>T. doichangensis</i>
Genome size	161,476 bp	161,020 bp	161,304 bp	160,907 bp	160,497 bp	161,222 bp	159,938 bp
LSC	90,732 bp	90,596 bp	90,585 bp	90,527 bp	90,255 bp	90,522 bp	89,445 bp
SSC	18,987 bp	19,160 bp	18,959 bp	18,998 bp	25,675 bp	19,000 bp	19,295 bp
IR	25,879 bp	25,632 bp	25,880 bp	25,961 bp	18,892 bp	25,850 bp	25,600 bp
Protein coding genes	86	87	87	82	86	85	81
rRNAs	8	8	8	8	8	8	8
tRNAs	39	39	44	37	37	37	39
Duplicated genes	21	19	24	17	21	17	17
CDS	80,577 bp	80,142 bp	80,199 bp	77,685 bp	79,647 bp	78,852 bp	71,778 bp
NCDS	80,899 bp	80,878 bp	81,105 bp	83,222 bp	80,850 bp	82,370 bp	88,360 bp
GC%	36.7	36.7	36.7	36.7	36.8	36.9	37
CDS - coding sequence.							
NCDS - Non-coding sequence.							

Comparison of Quercoideae chloroplast genomes

Mauve alignment identified four locally collinear blocks in *L. dealbatus* and other six were compared with publicly available genomes (Fig. 2). Overall, the synteny of gene order was similar in all genomes except an inversion of around 275 bp in the LSC region of *T. diochangensis* (Figure S1). The inversion has explicitly occurred in the intronic region of the *atpF* gene. The mVista genome alignment showed that the genic regions were mostly conserved with a few exceptions (Fig. 3). Overall, the *Lithocarpus* genome showed a higher level of nucleotide identity in comparison to *Castanea*, *Castinopsis* and *Quercus*. In the present investigation, *ycf1* gene revealed significant variation among the seven compared genomes. Intronic regions revealed a higher level of divergence in contrast to the un-translated region (UTR) and genic regions.

The size variation in chloroplast genomes is often due to the expansion and contraction of IR junctions in higher plants. Hence, we analysed and compared the location of the IR border and their adjacent genes among

the compared chloroplast genomes (Fig. 4). In *L. dealbatus*, the *ycf1* gene was detected at IRB/SSC junction with 4607 bp inside SSC and 1083 bp inside the IRB region. On the other hand, the other partially duplicated copy of the *ycf1* gene was found at IRA/SSC junction with 1083 bp inside IRA and 20 bp inside the SSC region. A similar trend was observed in all the compared genomes except in *Quercus pannosa* and *T. diochangensis*, where *ycf1* was missing at IRA/SSC or IRB/SSC junctions. The size of the *ycf1* gene in *L. dealbatus* ranged from 1103 bp to 5690 bp. Conversely, the length of *ycf1* gene in the rest of the genomes varied between 795 bp in *T. diochangensis* and 5681 bp in *Q. pannosa* genome. The *ndhF* gene was positioned near IRA/SSC region in the compared genomes except that in *T. diochangensis*. Besides, *ycf1* overlapped with the *ndhF* gene at IRA/SSC junctions in three genomes such as *L. hancei*, *Castanopsis sclerophylla* and *Castanea henryi*. Two genes, *rps19* and *rpl2* were spotted completely inside the LSC and IRB regions on either side of the IRB/LSC junction. Other duplicated copy of *rpl2* gene was situated entirely inside the IRA region and was only absent in *T. diochangensis*. We observed the position of *trnH* gene within LSC region in all the seven compared genomes.

Repeat sequences

The chloroplast genome of *L. dealbatus* comprised 43 repeat regions, of which 17 were forward, 22 were palindromic, 3 were reverse, and one was a complement (Fig. 5A, B). Overall, the total number of tandem repeats varied between 34 in *Q. pannosa* and 49 in *Trigonobalanus doichangensis* (Fig. 5A). Comparative analysis showed that most repeats were palindromic, ranging from 19–26 in numbers in seven compared genomes (Fig. 5B). This was followed by forward repeats with the highest number in *C. henryi* (19) and lowest in *C. sclerophylla* and *Q. pannosa*, each with 12 repeats. The reverse repeats were absent in *C. sclerophylla*, while these were highest in *L. hancei* and *T. doichangensis* (5). Similarly, a limited number of complement repeats were observed in *L. hancei* (2), *C. henryi* (1) and *C. sclerophylla* (2), whereas it was absent in *L. balansae*, *Q. pannosa* and *T. doichangensis*. The majority of the repeats ranged from 30–34bp, while a negligible number of repeats belonged to the size category of 45–64bp (Fig. 5C).

We also mined 125 simple and 30 compound SSRs in the chloroplast genome of *L. dealbatus* (Fig. 6A). Among 125 simple SSRs, 83 were mono-nucleotides (66.4%), 18 were di-nucleotides (14.4%), 8 were tri-nucleotides (6.4%), and 10 were tetra-nucleotides (8%). Besides, the number of penta-nucleotides was only 5 (4%), followed by a single copy of hexanucleotide (0.8%) (Fig. 6B). Comparative analysis revealed a total of 127, 130, 122, 116, 117 and 126 SSRs in *L. balansae*, *L. hancei*, *C. henryi*, *C. sclerophylla*, *Q. pannosa* and *T. doichangensis*, respectively. The majority of SSRs comprised of mono-nucleotides, followed by di-, tetra- and tri-nucleotides. These findings were consistent across all the compared genomes. The highest percentage of mono-nucleotide SSRs was found in *T. doichangensis* (73.8%), followed by *C. sclerophylla* (70.6%), while the least was recorded for *L. balansae* (63.7%). Similarly, the percentage of dinucleotide SSRs was most significant in *L. balansae* (15.7%), and the least was observed in *T. doichangensis* (10.3%). However, the number of hexa, hepta, octa, nona and deca-nucleotides ranged between 0–2 in the compared Quercoideae genomes. The majority of the mono-nucleotide SSRs had A/T rich sequences and rarely contained any C/G regions. The highest number of A/T rich repeats was found in *T. doichangensis* (91 nos), followed by *L. hancei* (82 nos), and the least was recorded in *L. balansae* and *Q. pannosa* with 75 repeats. Similarly, the di-nucleotide repeats were rich in AT/AT compared to AG/CT and were highest in *L. balansae* (16) and lowest in *T. doichangensis* (10) (Fig. 6C). SSR density was relatively greater in *C. sclerophylla* and *Q. pannosa* (with about 1.4 kb/ SSR) than the cp genomes of the other compared species. The least SSR density was recorded in *L. hancei* (with approximately 1.23 kb/ SSR) (Fig. 6D).

Nucleotide diversity

The comparative analysis of the coding and non-coding regions was carried out to determine the variations in the hotspot regions of *Lithocarpus*, *Quercus*, *Castanea* and *Castanopsis* chloroplast genomes. We found high divergence in non-coding regions than the protein-coding regions. The nucleotide diversity (P_i) for protein-coding regions ranged from 0.00059 (*ycf2*) to 0.09132 (*rpl33*) for *Lithocarpus* (Fig. 7A). Whereas in *Quercus*, the nucleotide diversity of protein coding regions ranged from 0.0002 (*rpl23*) to 0.03146 (*rpl36*), in *Castanea* it ranged from 0.000256 to 0.00533, and in *Castanopsis* it ranged from 0.000175 to 0.13913 (Fig. 7A).

Furthermore, *Castanopsis* had the highest average nucleotide diversity (0.006989) for protein-coding genes, followed by *Lithocarpus* (0.0066), *Quercus* (0.0034) and *Castanea* (0.001617). We observed five highly diverged coding regions in each members of Quercoideae such as *rpl33*, *petB*, *rpl32*, *ndhA* and *rpl22* in *Lithocarpus*, *rp136*, *ndhJ*, *petG*, *rps15* and *ndhF* in *Quercus*, *atpF*, *psaI*, *ndhF*, *psbI* and *matK* in *Castanea* and *rpl36*, *petB*, *atpF*, *ycf3* and *rpl22* in *Castanopsis*. However, across the studied four members of Quercoideae, the coding loci such as *rpl36*, *rpl33*, *ndhJ*, *atpF* and *ndhA* were highly diverge. The nucleotide diversity (P_i) on the other hand for non-coding regions ranged between 0.000278 (*ycf2-trnL-CAA*) and 0.04390 (*trnH-GUG-psbA*) for *Lithocarpus* (Figure S2a), 0.000536 (*ndhB-rps7*) and 0.013736 (*petA-psbJ*) for *Quercus* (Figure S2b), 0.000605 (*ycf4-cemA*) and 0.028562 (*trnH-GUG-psbA*) for *Castanea* (Figure S2c), and 0.000691 (*ndhG-ndhI*) and 0.027903 (*rbcL-accD*) for *Castanopsis* (Figure S2d). Overall, *Lithocarpus* had greater average nucleotide diversity (0.009315) of non-coding regions than *Castanopsis* (0.006118), *Quercus* (0.005901) and *Castanea* (0.005159). The highly diverged non-coding regions such as *trnH-GUG-psbA*, *rbcL-accD*, *cssA-ndhD*, *trnF-GAA-psbA* and *psbK-psbI* were found in *Lithocarpus*, followed by *Quercus* (*petA-psbJ*, *atpF-atpH*, *psaC-ndhE*, *ycf4-cemA* and *atpL-rps2*), *Castanea* (*ndhG-ndhI*, *rbcL-accD*, *psbC-trnS-UGA*, *ccsA-ndhD* and *psaJ-rpl33*) and *Castanopsis* (*rbcL-accD*, *ccsA-ndhD*, *psbK-psbI*, *psaJ-rpl33* and *psaI-ycf4*).

Substitution rate

The non-synonymous substitution (K_a) to synonymous substitution (K_s) ratios were calculated to understand the evolutionary pressure acting on the protein-coding sequences. A total of 79 shared protein-coding genes across all three *Lithocarpus* chloroplast genomes were utilised for calculating K_a/K_s ratios (Fig. 8). In the current analysis, the K_a/K_s ratio for 34 genes were zero. However, 37 genes revealed K_a/K_s ratio between the range of 0 and 1.0, which shows that these genes were under the purifying selection. The K_a/K_s ratios of eight genes (*accD*, *cemA*, *matK*, *ndhG*, *petB*, *rps2*, *rps3* and *rps12*) were greater than 1, indicating the positive selection acting on protein-coding genes. The average K_a/K_s ratio for 45 genes was 0.52.

RNA editing

The RNA editing sites in the *L. dealbatus* chloroplast genome were predicted using the program PREP suite. Overall, the programme predicted 55 editing sites in the 23 protein-coding genes (Fig. 9). All the substitution sites were from nucleotide base C to T. The analysis showed that most conversions were observed from the amino acid serine to leucine, followed by proline to leucine. The highest substitutions viz., 42 and 13 occurred at the second and first codon positions, whereas no editing was detected at a third base location of the codon. Of the 55 editing sites, we observed the highest editing sites in *ndhB* (10 sites), followed by *ndhD* (5 sites) and *rpoB* (5 sites) genes. Fifty two RNA editing sites in the *L. dealbatus* chloroplast genome were responsible for

hydrophobic amino acid changes, like isoleucine, leucine, proline, phenylalanine, methionine, valine, tyrosine, cysteine and tryptophan.

Comparative analysis among the Quercoideae genomes revealed the presence of RNA editing sites in *L. balansae* (53 sites in 21 genes), *L. hancei* (54 sites in 20 genes), *C. henryi* (54 sites in 20 genes), *C. sclerophylla* (53 sites in 20 genes), *Q. pannosa* (55 sites in 21 genes) and *T. doichangensis* (55 sites in 21 genes) (Fig. 9). Meanwhile, some of the compared genomes showed gene-specific RNA editing sites such as *psal* gene in *L. dealbatus*, *ycf3* gene in *Q. pannosa*, *rpl20* gene in *L. balansae* and *psbB* gene in *T. doichangensis*. However, the *L. dealbatus* genome showed a greater number of RNA editing genes than the other compared genomes, even though most editing sites were nearly similar (Table S1).

Phylogenetic analysis of Quercoideae species

In this study, a phylogenetic tree was constructed based on 95,904 bp nucleotide alignment by employing the GTR + G + I as a best-fitting model. The topology of phylogenetic tree was computed by ML method. The chloroplast genomes of 63 Quercoideae members were included by keeping two *Fagus* species as an out-group (Fig. 10). The phylogenetic tree showed that three *Lithocarpus* species made separate group sharing a common node with Clade-I of *Quercus* species. Among the three *Lithocarpus* species, *L. dealbatus* was closer to *L. hancei* than *L. balansae*. We observed that *Quercus* genus was the most diverse among the Quercoideae forming four different clades across phylogenetic tree. On the other hand, *Castanopsis* and *Castanea* formed two separate monophyletic groups and shared a common node. *T. doichangensis* was present at the basal position in the Quercoideae subfamily.

Molecular clock analysis suggests that the estimated lineage (stem) divergence time for *Quercoideae* members such as *Lithocarpus-Quercus-Castopsis-Castanea* was around 37.28 Mya (Figure S3). However, *Lithocarpus* started evolution around 22.80 Mya from one clade of *Quercus* while *Castanea-Castanopsis* evolved around 23.63 Mya from *Lithocarpus-Quercus* (1st clade). The estimated lineage divergence time for *Castanea* was 21.02 Mya. The other clades of *Quercus* (2nd, 3rd, and 4th) were having the lineage divergence times more than 23.90 Mya (Figure S3).

Discussion

Lithocarpus, a major Quercoideae genus in the Fagaceae family, contributes significantly to ecosystem structure and function of many sub-tropical forests, being the most dominant species [37]. It also provides renewable firewood/charcoal resources for the local communities [1]. Although it is an important species from ecological and economics perspective, a limited number of studies have been conducted on the species [6, 7, 9–11], particularly no genomics information on *L. dealbatus* is available. However, availability of chloroplast genomic resources is crucial to unravel the genetic architecture. In this context, we assembled the *L. dealbatus* chloroplast genome and carried out a comparative analysis with six other members of Quercoideae to comprehend the structural architecture of the chloroplast genome.

The size of the *L. dealbatus* chloroplast genome was 161,476 bp, which fell in the expected range (107–218 kb) of most angiosperm chloroplast genomes [21]. The typical quadripartite structure of the *L. dealbatus* chloroplast genome was consistent with the characteristics of the angiosperm chloroplast genomes in general

[18]. The *L. dealbatus* chloroplast genome displayed a low GC content (36.7%); this finding is similar to other reported genomes from Fagaceae family [17, 18].

The IR regions, on the other hand, have a greater GC content (42.7%) than both the LSC and SSC regions, owing to the comparatively high GC content of rRNA genes [17, 18]. Although gene content is nearly conserved in the analysed *Lithocarpus* chloroplast genomes, some subtle variations did exist.

The presence or absence of protein-coding genes constituted a notable difference amongst the genomes studied. We observed the loss of the *psbZ* gene in *L. balansae* and *L. hancei* genomes. Similar loss of *psbZ* gene was also identified in *C. sclerophylla*, *T. doichangensis* and other land plants [38]. In contrast, no loss of protein coding gene was noticed in *L. dealbatus* chloroplast genome. Similar observation was made in case of *Striga hermonthica* genome earlier [39]. Various studies mentioned the absence of the *infA* gene in the angiosperm chloroplast genomes [40]. In the present investigation too, the *infA* gene was absent in *C. henryi* and *T. doichangensis*. However, on several occasions, the missing chloroplast genes have been reported to be integrated into the nuclear genome [21, 25].

The chloroplast genome is known to be conserved in land plants although some structural variations are reported in genome like inversions that occur due to occasional rearrangement, which bring forth crucial data [41]. Our analysis reported no significant inversion in compared genomes except the small 275 bp size of inversion in the *T. doichangensis* genome. However, such small inversions are ubiquitous in angiosperm chloroplast genomes [35]. The mVISTA investigation showed that the non-coding regions were more variable than protein-coding regions, a regular trend observed in other chloroplast genomes [16, 43, 44]. The IR region showed a higher level of conservation than the LSC and SSC region, a similar finding noticed in other angiosperms [16]. The level of divergence in *Lithocarpus* genomes was calculated using nucleotide diversity (*Pi*) analysis. Consistent with the divergence pattern observed in most angiosperms, greater divergence was detected in *Lithocarpus* non-coding regions [29]. The majority of the hotspot divergence regions have been previously utilised to develop potential molecular markers [29]. Our analysis indicates that these highly divergent regions could be very informative in discriminating the *Lithocarpus* species.

The IR regions are considered as the most conserved region in chloroplast genomes [45]. In spite of this, several studies explained the role of contraction and expansion of IR regions in chloroplast genome size variations and rearrangement in plants [41, 45, 46]. Our comparative analysis revealed certain similarities and differences at the IR junctions in the compared genomes. Overall, IR regions were mostly conserved across the *Lithocarpus* genomes which was more or less consistent with previous reports pertaining to *Castanopsis* and *Quercus* genomes [16, 47]. However, the absence of the *ycf1* gene was noticed at IRA/SSC and IRB/LSC junctions in *Q. pannosa* and *T. doichangensis* species, respectively. Several workers have documented the loss of *ycf1* gene at the IR junctions in many land plants [45, 48]. Among the compared genomes, we observed extensive rearrangements at the IR junctions in *T. doichangensis*. These variations are known to contribute significantly towards the evolution of chloroplast genome structure. Long and complex repeats play a pivotal role in genome rearrangement and divergence [49]. Due to high sequence variations, tandem repeats are ideal for developing physical and genetic maps [50, 51]. Our results of repeat analysis revealed variations in total number of tandem repeats among the compared genomes, which is consistent with the earlier observations in Plantaginaceae and Schisandraceae chloroplast genomes [45, 52]. Moreover, minor variation was noticed in the distribution of tandem repeats regarding type and length, similar to the distribution patterned described in other plants [45, 52].

SSRs are widely distributed in the chloroplast genome and are found associated with sequence rearrangement and polymorphism [45, 53]. In the current study, SSR density moderately varied in the compared genomes. The highest density of SSR was observed in *C. henryi* (1SSR/1.31 kb) followed by *Q. pannosa* (1SSR/1.37 kb) and *C. sclerophylla* (1SSR/1.38 kb). The density of SSRs detected in the present investigation was greater in comparison to rice and the members of Solanaceae family [54, 55]. Of the total SSR repeats, mononucleotides were the most abundant repeats followed by dinucleotide and tri-nucleotide repeats as stated in the earlier studies [56, 57]. Interestingly, the population of A/T repeats was far more significant than G/C repeats in mononucleotide repeats. A similar distribution of SSRs was noticed in the Fagaceae species and other angiosperms [56, 57]. These findings suggest that the repeats mined in the current study can be utilised to develop molecular markers for studying population genetics, phylogeny, and differentiation of *L. dealbatus* and other members of Fagaceae.

RNA editing is an essential post-transcriptional mechanism observed in land plants. Identifying RNA editing sites in chloroplast genes help us to comprehend the underlying regulatory process(es) and its biological significance [46, 58]. We detected 53–55 RNA editing sites in 20–23 protein-coding genes across the compared genomes. The *L. dealbatus* chloroplast genome exhibited the highest RNA editing genes (in 23 genes) compared to closely related *L. balansae* (in 21 genes) and *L. hancei* (in 20 genes) genome. In a broad sense, the number of RNA editing genes and sites are variable in chloroplast genomes [27]. We found three gene-specific RNA editing sites in the present investigation. However, the loss or gain of editing sites and their frequencies is an independent even that arises through mutations in RNA editing factors or stress interference [58–60]. Consistent with the previous reports, the highest number of editing sites were detected in the *ndhB* (10 sites) and *ndhD* (8 sites) genes [27, 58]. We noticed frequent conversions at the second base position of the codon that may change the corresponding amino acid leading to alterations in protein primary, secondary or tertiary structures. Consequently, such modifications can play a crucial role in protein functioning [46]. Generally, most RNA editing conversions result in hydrophobic amino acid change, thus influencing the protein structure [27, 46]. Therefore, comprehensive investigation of RNA editing sites is an inevitable exercise that requires further attention in future.

The occurrence of point mutations through synonymous and non-synonymous nucleotide substitutions is crucial for gene evolution [61]. The calculation of Ka/Ks ratios has been extensively used to detect evolutionary pressure acting on protein-coding genes [53, 62]. Most protein-coding genes from *Lithocarpus* showed Ka/Ks ratios less than 1, which is consistent with the previous reports [50, 63]. This suggests that the majority of genes are under purifying selection. However, 34 genes showed Ka/Ks ratio of 0; such ratios were observed when the Ks values were either very low or no substitution present between the aligned sequence [28]. We observed positive selection in eight *Lithocarpus* chloroplast genes (*accD*, *cemA*, *matK*, *ndhG*, *petB*, *rps2*, *rps3* and *rps12*) with Ka/Ks values greater than 1. These genes have also been reported for positive selection in other species [64]. Overall, the average Ka/Ks ratio (0.52) of *Lithocarpus* clade reported in the present investigation was greater than that observed in the *Cordylis* species (0.26) [64], suggesting a possible evolutionary change in certain genes.

DNA barcoding is a fast and accurate method for identifying species by employing short piece of genomic DNA. However, as such no single universal barcode is available hitherto, which can discriminate the taxa up to species level [15, 17]. Very recently, researchers have employed novel strategies to overcome these issues

through two approaches i.e. DNA hotspot regions and super-barcoding [31, 32, 48, 57, 65]. Here, we have used the first approach to suggest the DNA divergent regions for resolving taxonomic conflicts and proposed a DNA barcode in four members of Quercoideae. We suggest five highly diverged coding (such as *rpl33*, *petB*, *rpl32*, *ndhA* and *rpl22* in *Lithocarpus*; *rp136*, *ndhJ*, *petG*, *rps15*, *ndhF* in *Quercus*; *atpF*, *psaI*, *ndhF*, *psbI*, *matK* in *Castanea*, *rpl36*; and *petB*, *atpF*, *ycf3*, *rpl22* in *Castanopsis*) and non-coding regions (*trnH-GUG_psbA*, *rbcl_accD*, *cssA_ndhD*, *trnF-GAA_psbA*, *psbK_psbI* in *Lithocarpus*; *petA_psbJ*, *atpF_atpH*, *psaC_ndhE*, *ycf4_cemA* and *atpL_rps2* in *Quercus*; *ndhG_ndhI*, *rbcl_accD*, *psbC_trnS_UGA*, *cssA_ndhD*, *psaJ_rpl33* in *Castanea*, and *rbcl_accD*, *cssA_ndhD*, *psbK_psbI*, *psaJ_rpl33*, *psaL_ycf4* in *Castanopsis*) from each genus by comparing chloroplast genome sequences. However, comparative study among the four genera revealed five highly diverged coding regions (*rpl36*, *rpl33*, *ndhJ*, *atpF*, and *ndhA*), which could be used to resolve inter-generic discrepancies in the aforementioned genus. Similar experiments were carried out in other studies, with the exception of a different number of highly variable regions [31, 48, 57]. The majority of the discovered barcodes in the current study were not reported as universal markers in previous studies, implying that all loci are not phylogenetically effective in resolving taxonomic discrepancies [15, 17, 60]. Therefore, the designing of intra- and inter-generic barcodes would be helpful to achieve greater phylogenetic resolution.

Recently, researchers proposed the utility of the whole chloroplast genome as a “super-barcode”, which has provided a new perspective in plant identification and species delimitation [57–58]. Previous studies in *Quercus* revealed low phylogenetic resolution using the universal barcodes such as *rbcl*, *matK*, and *trnH-psbA* [15, 17]. Thus, increasing number of investigations are employing the whole chloroplast genome to overcome the low resolving power of single-locus markers for evaluating phylogenetic relationships [36, 43]. In the present study, resolving the phylogeny between *Lithocarpus* and other Quercoideae species could give insights into the evolutionary relationship in Fagaceae family. *Lithocarpus* genus formed a monophyletic group in which *L. hancei* and *L. balansae* were closely associated. The loss of *psbZ* gene in *L. hancei* and *L. balansae* genome is also confirming such phylogenetic relationship.

L. dealbaatus was more closely related to *L. hancei* than *L. balansae*, which is well-supported by sequence homology analysis. The *Lithocarpus* clade shared a common node with the *Quercus* species of clade-I, which is consistent with the high coverage and sequence similarity between some *Quercus* and *Lithocarpus* species. At the same time, molecular dating analysis revealed the divergence time for *Lithocarpus* from *Quercus* clade at 22.80 Mya in the early Miocene Epoch period. A near-similar divergence time was estimated for the separation of the *Quercus* and *Lithocarpus* genus based on the five genes by Larson-Johnson (2016). Furthermore, phylogeny analysis suggests that *Castanea* and *Castanopsis*, two closely diverged genus, spitted 21.02 Mya, which is in conformity with the previous morphological and molecular studies [30, 68]. On the other hand, *T. doichangensis* present at the basal position and showed as an early diverged genus in the Quercoideae subfamily. This is well supported by the previous fossil records [69]. Besides this, *Quercus* appeared as the most diverse species forming four distinct clades across phylogenetic tree [30, 70]. This could be because of endemism and allopatric speciation among *Quercus* species that may have amplified genetic and morphological variations in the course of species evolution [18]. However, the phylogenetic relationship of oak is complex owing to a considerable level of hybridisation, introgression, incomplete lineage sorting and convergent evolution [71]. In general, our tree topology (*Fagus*, *Trigonobalanus*, *Quercus*, *Castanopsis*, *Castanea*, *Lithocarpus*, and *Quercus*) is more or less similar to the earlier phylogenetic outcome [33].

Conclusions

In conclusion, the present investigation successfully assembled and annotated the complete chloroplast genomes of *L. dealbatus* with the MGI technology. The gene contents and synteny of the *Lithocarpus* chloroplast genomes were nearly conserved. A significant sequence homology was found among *L. dealbatus*, *L. balansae* and *L. hancei*. Analysis of repeat elements revealed a minor level of variations in the total number of repeat elements across analyzed genomes. The ML tree clearly showed the formation of monophyletic group for *Lithocarpus* species, and the whole clade of *Lithocarpus* was closely related to *Quercus* species. The comparative analysis of chloroplast genomes of *L. dealbatus* with that of other members of Quercoideae strengthened our understanding of evolutionary lineage. In addition, the mutational hotspot regions mined in the current investigations could be helpful to discriminate the closely related species in Quercoideae. We recommend that the highly mutational diverge regions should be used as a potential barcode for better resolution of species in various genera of Quercoideae. The newly proposed barcodes *in silico* identified in this study is required to be validated their extent of resolution power for species delimitation and phylogenetic reconstruction. This study has provided crucial insights into the characteristic features of chloroplast genome of *L. dealbatus*, the divergence hotspot regions and repeat elements, which can be utilized as molecular markers for further studies in population genetics.

Methods

DNA isolation and DNB sequencing on MGI platform

Fresh leaf samples of *L. dealbatus* were collected from Shillong, Meghalaya and stored immediately in liquid nitrogen. DNA extraction was performed from the stored leaf samples using DNeasy Plant Pro and Plant Kits (Qiagen). The quality of the extracted genomic DNA was determined in 0.8% agarose gel and further quantified through NanoDrop™ One/OneC Micro volume UV-Vis Spectrophotometer (ThermoFischer). The genomic library was constructed following the MGI's instructions (ILS Pvt. Ltd). DNBSEQ™ – G400 Genetic Sequencer (MGI) platform at Imperial Life Sciences Pvt. Ltd was used to generate PE150long reads.

Chloroplast genome assembly

The quality of MGI reads was checked through the FastQC program (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The clean reads were further used to *denovo* assemble the whole chloroplast genome through Novoplasty assembler by keeping the default parameter except Kmer length 39 and *rbcL* gene as a seed sequence from *L. balansae* genome (KP299291.1) [34]. The assembled chloroplast genome was confirmed by performing BLASTN against the non-redundant nucleotide database at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Annotation and map drawing of chloroplast genome

The chloroplast genome was further annotated using the Chlorobox Geseq program, keeping *L. balansae* and in build the Geseq MPI-MP genome set as a reference genome (<https://chlorobox.mpimp-golm.mpg.de/geseq.html>). Annotations of tRNA genes were conducted through the tRNAscan-SE tool. Manual adjustment of start and stop codon and exon-intron junctions were made in the NCBI ORF finder. Finally,

annotations and structural features of the chloroplast genome was visualised through the OGDRAW program [35].

Comparative analysis of *L. dealbatus* with Fagaceae chloroplast genome for structural rearrangement, similarity, expansion and contraction of IR and tandem repeat

Comparative analysis of the *L. dealbatus* chloroplast genome was performed using the previously published genomes such as *L. balansae* (KP299291.1), *L. hancei* (MW375417.1), *C. henryi* (MH998384.1), *C. sclerophylla* (NC_044680.1), *Q. pannosa* (NC_050963.1) and *T. doichangensis* (KF990556.1). Structural rearrangement among the chloroplast genomes was recognised through MAUVE alignment (<http://darlinglab.org/mauve/mauve.html>). The mVISTA program was employed to determine the similarity between the compared chloroplast genomes using the Shuffle-LAGAN model by keeping *L. dealbatus* as a reference (<http://genome.lbl.gov/vista/mvista/submit.shtml>). The expansion and contraction of IR junctions in chloroplast genomes were analysed and displayed through the IRscope tool (<https://irscope.shinyapps.io/irapp/>). Tandem repeats were identified through the REPuter program using the minimum repeat size 30 and hamming distance 3 (<https://bibiserv.cebitec.uni-bielefeld.de/reputer/>). MISA tool was employed to recognise SSRs with the minimum repeats 10 for mono-nucleotide, 5 for di-nucleotide, 4 for tri-nucleotide, 3 for tetra-, penta-, hexa-, septa-, octa-, nona- and deca- nucleotide, respectively (<http://pgrc.ipk-gatersleben.de/misa/misa.html>). We employed the Predictive RNA Editor for Plants (PREP) program to recognise the RNA editing in 35 reference genes (<http://prep.unl.edu/>).

DNA diversity and Ka/Ks analysis in *Lithocarpus*

Divergence across the coding and non-coding regions in the *Lithocarpus*, *Quercus*, *Castanea* and *Castanopsis* genus was determined using DnaSP v5.0 (Universitat de Barcelona, Barcelona, Spain) (<http://www.ub.edu/dnasp/>). MEGA X tool was used to determine the Ka/Ks ratio of protein-coding regions (<https://www.megasoftware.net/>).

Phylogenetic analysis and estimation of the divergence time

Phylogenetic study was performed using the chloroplast genomes of Quercoideae genomes available in the public database. We selected two chloroplast genomes from the genus *Fagus* namely *F. crenata* and *F. japonica*, as outgroups. Direct alignment of chloroplast genomes is challenging to exercise due to structural rearrangements and gene content. Hence, we employed the HomBlocks tool to determine locally collinear blocks (LCBs) present in chloroplast genomes for alignment [36]. Unaligned sequences were trimmed using the Gblocks program embedded in the HomBlocks pipeline. The best substitution model was calculated by Model test in the MEGA tool suggested by the Akaike information criterion (AIC). Finally, the GTR + G + I model was chosen to construct the phylogenetic tree using the maximum likelihood (ML) method in the MEGA X tool (<https://www.megasoftware.net/>). The branch support values for each were calculated based on 500 bootstraps. The phylogenetic tree was coloured and represented using the iTOL web server (<https://itol.embl.de/>).

A timetree was inferred using the ReltimeML-option in MEGA X. The same species used in our previous analysis such as *F. crenata* and *F. japonica* were constrained to be the outgroup in this divergence tree analysis. The reference node age was obtained by the divergence time of *Quercus ciliaris*- *T. diochangensis* (11.1–57.9 million years ago) and *Q. ciliaris*-*C. henryi* (6.0–49.0 million years ago) (<http://www.timetree.org/>).

Declarations

Ethics approval and consent to participate:

Manuscript number allotted by CSIR ethical committee: CSIR-NBRI_MS/2022/01/03

Consent for publication:

Not applicable

Availability of data and materials:

The assembled genome can be accessed in NCBI database with accession number MZ322408.

Competing interests:

The authors declare that they have no competing interests.

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Author contributions:

Conceptualization: SKB and SNJ; Sample collection and DNA isolation: BJ, PPS, GT and DA; genome assembly and annotation: RGS; experiment designing and data analysis: SKB, SNJ, RGS and RPB; manuscript draft preparation: RGS, RPB, and SNJ; Supervised the study and critical revision of the manuscript: SK

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Figures

Figure 1

Circular map of *Lithocarpus dealbatus* chloroplast genome representing the annotated genes in different colours according to their specific functions. The genes present on the inner circular map were transcribed clockwise, and those situated outside were transcribed anti-clockwise. The darker grey colour inside the circle shows the GC content, while the lighter grey colour indicating the AT content. The chloroplast genome borders were demarcated as LSC, SSC, IRA and IRB regions

Figure 2

MAUVE alignment of *L. dealbatus* chloroplast genome against some of the representative species from Quercoideae. Within each of the alignments, the local colinear blocks indicate the identical conserved regions represented by the same colour and are joined by lines

Figure 3

Sequence alignment of Quercioideae chloroplast genomes by mVISTA, with annotation *L. dealbatus* chloroplast genome as the reference

Figure 4

Details of junction positions between inverted repeat regions and small and large single-copy regions across the compared Quercoideae genomes. Each gene is depicted in different colours. The genes present on top are transcribed on the positive strand, whereas those present below are transcribed on the negative strand. The complete genome size of each species is mentioned on the left side

Figure 5

Comparative analysis of tandem repeats across compared genomes, A. Distribution of tandem repeats based upon their type in the chloroplast genome, B. Frequency of tandem repeats based on their size in the chloroplast genome

Figure 6

Category of SSRs in the compared Quercoideae genomes. A. Distribution of SSRs in simple and compound form, B. Representation of SSRs based on their unit size, C. Distribution of SSRs based upon the repeat motifs.

Figure 7

Comparison of the DNA diversity (P_i) values of protein-coding regions in *Lithocarpus*, *Quercus*, *Castanea* and *Castanopsis*

Figure 8

The comparative analysis of Ka/Ks ratio from *Lithocarpus* chloroplast genomes for individual genes

Figure 9

Distribution of RNA editing sites in genes across compared genomes

Figure 10

ML phylogenetic tree construction based on whole chloroplast genome Quercoidae species by keeping *Fagus* species as an out-group

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