The Complete Chloroplast Genome Sequence of Tree of Heaven (*Ailanthus altissima* (Mill.) (Sapindales: Simaroubaceae), an Important Pantropical Tree)

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Abstract: *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae) is a deciduous tree widely distributed throughout temperate regions in China, hence suitable for genetic diversity and evolutionary studies. Previous studies in *A. altissima* have mainly focused on its biological activities, genetic diversity and genetic structure. However, until now there is no published report regarding genome of this plant species or Simaroubaceae family. Therefore, in this paper, we first characterized *A. altissima* complete chloroplast genome sequence. The tree of heaven chloroplast genome was found to be a circular molecule 160,815 base pairs (bp) in size and possess a quadripartite structure. The *A. altissima* chloroplast genome contains 113 unique genes of which 79 and 30 are protein coding and transfer RNA (tRNA) genes respectively and also 4 ribosomal RNA genes (rRNA) with overall GC content of 37.6%. Microsatellite marker detection identified A/T mononucleotides as majority SSRs in all the seven analyzed genomes. Repeat analyses of seven Sapindales revealed a total of 49 repeats in *A. altissima*, *Rhus chinensis*, *Dodonaea viscosa*, *Leitneria floridana*, while *Azadirachta indica*, *Boswellia sacra*, and *Citrus aurantiifolia* had a total of 48 repeats. The phylogenetic analysis using protein coding genes revealed that *A. altissima* is a sister to *Leitneria floridana* and also suggested that Simaroubaceae is a sister to Rutaceae family. The genome information reported here could be further applied for evolution and invasion, population genetics, and molecular studies in this plant species and family.

Keywords: *Ailanthus altissima*; chloroplast genome; microsatellites; Simaroubaceae; Sapindales

1. Introduction

*Ailanthus altissima* (Mill.) Swing, a deciduous tree in the Simaroubaceae family, is widely distributed throughout temperate regions in China. It grows rapidly reaching heights of 15 m (49ft) in 25 years and can tolerate various levels of extreme environments (e.g., low temperatures, sterile soils, arid land). Besides, it reproduces through sexual (seeds disperse by wind) or asexual (sprouts) methods [1]. Two hundred years ago it was brought to Europe and North America. *A. altissima* being an early colonizer can survive high levels of natural or human disturbance [2]. Therefore, in recent years, it is commonly known as an exotic invasive tree developed into an invasive species expanding on all continents except Antarctica [1]. While previous studies in *A. altissima* have mainly focused on discovering the biological features of this plant to prevent its expansion, there is limited information to
understand the impact of genetic diversity and evolution. Thus, genomic information of *A. altissima* is essential for further molecular studies, identification, and evolutionary studies.

Many studies have analyzed the genetic diversity of *A. altissima* using various markers, for example, chloroplast DNA [2,3], microsatellite primer [4,5]. These studies provided a detailed series of information about genetic structure and genetic diversity of *A. altissima* in native and invasive area. However, to understand the genetic diversity and population structure within *A. altissima* natural populations, more genetic resources are required.

It is well known that chloroplasts (cp) are key organelles in plants, with crucial functions in the photosynthesis and biosynthesis [6]. Current research shows that chloroplast genomes in angiosperms have highly conserved structure, gene content, organization, compared with either nuclear or mitochondrial genomes [7,8]. In general, cp genomes in angiosperms have circular structure consisting of two inverted repeat regions (IRa and IRb) that divides a large–single-copy (LSC) and a small-single-copy (SSC) regions [9]. Nevertheless, mutations, duplications, arrangements and gene loss have been observed, including the loss of the inverted repeat region in leguminous plants [7,10–12]. Some studies have applied plant plastomes to study population genetic analyses and basal phylogenetic relationships at different taxonomic levels [13], also to investigate the functional and structural evolution in plants [14–16]. At present, more cp genomes have been sequenced as a result of next-generation sequencing technologies advancement resulting in low sequencing costs.

More than 800 sequenced plastomes from various land plants have boosted our understanding of intracellular gene transfer, conservation, diversity, and genetic basis [17]. Although cp genomes have been sequenced in many trees such as *Castanea mollissima* [18], *Liriodendron tulipifera* [19], *Eucalyptus globules* [20], and *Larix decidua* [21], the plastome of *Leitneria floridana* (GenBank NC_030482) a member of Simaroubaceae has been sequenced but no analysis has been published at present despite the family containing many high economic value trees. Regardless of its potential use in crop or tree species improvement, studies on invasive species such as *A. altissima* which is also an important economic tree in the North China are too few. Chloroplast genome sequencing in invasive species could bring insights into evolutionary aspects in stress-tolerance related trait and genetic variation.

Simple sequence repeat (SSR) also called microsatellite markers are known to be more informative and versatile DNA-based markers used in plant genetic research [22]. These DNA markers are reliable molecular tools that can be used to examine plants genetic variation. SSR loci are evenly distributed and very abundant in angiosperms plastomes [23,24]. Chloroplast microsatellites are typically mononucleotide tandem repeats, and SSR in the fully sequenced genome could be used in plant species identification and diversity analysis. CpSSR in the fully sequenced plants plastomes such as; orchid genus *Chiloglottis* [25], *Cryptomeria japonica* [26], *Podocarpus lambertii* [27], *Actinidia chinensis* [28], have proven to be useful genetic tools in determining gene flow and population genetics of cp genomes. However, the lack of published plastome of *A. altissima* has limited the development of suitable SSR markers.

Here, we sequenced the complete chloroplast genome of *A. altissima*, and characterized its organization and structure. Furthermore, phylogenetic relationship using protein coding genes from selected species, consisting of 31 species from five families was uncovered for the Simaroubaceae family within the order Sapindales. Lastly, this resource will be used to develop SSR markers for analyzing genetic diversity and structure of several wild populations of *A. altissima*.

2. Results and Discussion

2.1. *Ailanthus altissima* Genome Size and Features

The *A. altissima* chloroplast genome has a quadripartite organizational structure with overall size of 160,815 base pairs (bp) including two copies of Inverted repeats (IRa and IRb) (27,051 bp each) separating the Large Single Copy (LSC) (88,382 bp) and Small Single Copy Region (SSC) (18,332 bp) (Figure 1). Notably, the genome content; gene order, orientation and organization of *A. altissima* is
similar to the reference genome and other sequenced Sapindales plastomes \cite{29,30} with genome size of about 160 kb. The overall guanine-cytosine (GC) content of the whole genome is 37.6\%, while the average adenine-thymine A + T content is 62.36\%. The relatively higher IR GC content and A + T bias in this chloroplast have been previously reported in genomes of relative species in order Sapindales \cite{31}. The GC content of the LSC, SSC and IR regions are 35.7, 32.2 and 42.6\% respectively. Moreover, the protein coding sequences had 38.3\% GC content.

The tree of heaven (A. altissima) chloroplast genome harbored a total of 113 different genes, comprising 79 protein coding genes (PCGS), 30 transfer RNA (tRNA) genes, and four ribosomal RNA (rRNA) genes (Table 1). All the 77 PCGS started with the common initiation codon (ATG), but rps19 and ndhD genes started with alternative codons GTG and ACG respectively, this unusual initiator codons have been observed to be common in other angiosperm chloroplast genomes \cite{32–34}. Of the 79 protein coding sequences, 60 are located in the LSC, 11 in the SSC and eight genes were duplicated in the IR region, while 22 tRNA genes were found in LSC, seven replicated in the IR region and one located in the SSC region.

Similar to some closely related plant species in order Sapindales, the chloroplast genome of A. altissima has maintained intron content \cite{35}. Among the 113 unique genes, the rps16, rpoCl, petB, rpl2, ycf15, ndhB, ndhA, atpF, six tRNA genes (trnG-GCC*, trnA-UGC*, trnL-UAA*, trnI-GAU*, trnK-UUU*, trnV-UAC*) possess one intron, and ycf3 and clpP genes harbored two introns. The rps12 trans-splicing gene has two 3′ end exons repeated in the IRs and the 5′ end exon located in the LSC region, which is similar to that in C. platymamma \cite{30}, C. aurantiifolia \cite{29}, Dipteronia species \cite{35}. The ycf1 gene crossed the IR/SSC junction forming a pseudogene ycf1 on the corresponding IR region. The rps19 gene in
A. altissima was completely duplicated in the inverted repeat (IR) region, which most other chloroplast genomes have presented [29,30].

Table 1. List of genes found in Ailanthus altissima Chloroplast genome.

| Functional Category | Group of Genes                  | Gene Name           | Number |
|---------------------|---------------------------------|---------------------|--------|
| Self-replication    | rRNA genes                       | rnr16, rnr23, rnr5, rnr5 | 4      |
|                     | tRNA genes                       | trnA-UGC, trnC-GCA, trnD-GUC, trnE-UUC, trnG-UCC, trnH-GUG, trnI-CAU, trnL-GAU, trnL-UAA, trnL-UGA, trnG-GCC, trnM-CAU, trnN-GUG, trnP-GGG, trnP-UGG, trnQ-IUG, trnR-ACG, trnS-GGA, trnS-UGC, trnT-UGC, trnV-GAC, trnV-UGC, trnW-CCA, trnY-GUA | 30     |
| Ribosomal small subunit | rps2, rps3, rps4, rps7, rps8, rps11, rps14, rps15, rps16, rps18, rps19 | 12     |
| Ribosomal large subunit | rpl22 | 9      |
| Photosynthesis       | Large subunit of rubisco          | rbcL                | 1      |
|                     | Photosystem I                    | rpsA, rpsB, rpsC, rpsD, rpsE, rpsF, rpsH, rpsL, rpsP, rpsT, rpsZ | 6      |
|                     | Photosystem II                   | psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbL, psbP, psbT, psbZ | 15     |
|                     | NADH dehydrogenase               | ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhI, ndhJ, ndhK | 11     |
|                     | Cytochrome b/f complex            | petA, petB, petD, petG, petL, petN | 6      |
|                     | ATP synthase                     | atpA, atpB, atpE, atpF, atpH, atpI | 6      |
|                     | Other                            | matK                | 1      |
|                     | Maturase                         | ctp, ctp*            | 1      |
|                     | Subunit of acetyl-CoA carboxylase | accD                | 1      |
|                     | Envelope membrane protein         | cemA                | 1      |
|                     | Protease                         | ctp, ctp*            | 1      |
|                     | c-type cytochrome synthesis      | ccsA                | 1      |
|                     | Functions unknown                | Conserved open reading frames (ycf) | 4      |
|                     |                                 | ycf1, ycf2, ycf4, ycf15 | 113    |
| Total               |                                 |                     | 113    |

Note: * Gene with one intron, ** Genes with two introns. (×2) Genes with two copies.

2.2. IR Expansion and Contraction and Genome Rearrangement

The angiosperms chloroplast genomes are highly conserved, but slightly vary as a result of either expansion or contraction of the single-copy (SC) and IR boundary regions [36]. The expansion and contraction of the IR causes size variations and rearrangements in the SSC/IRa/IRb/LSC junctions [37]. Therefore, in this study, exact IR boundary positions and their adjacent genes of seven representative species from different families in order Sapindales were compared (Figure 2). The functional ycf1 gene crossed the IRA/SSC boundary creating ycf1 pseudogene fragment at the IRb region in all the genomes. Besides, ycf1 pseudogene overlapped with the ndhF gene in the SSC and IRA junctions in four genomes with a stretch of 9 to 85 bp, but ndhF gene is located in SSC region in L. floridana, R. chinensis and A. altissima.

The rpl22 gene crossed the LSC/IRb junction in all the chloroplast genomes except in R. chinensis. Furthermore, this gene was partially duplicated forming a pseudogene fragment at the corresponding IRA/LSC junction in L. floridana and B. sacra, but completely duplicated in D. viscosa. In all the seven chloroplast genomes, the trnH-GUG gene was located in the LSC region, however this gene overlapped with rpl22 gene in D. viscosa. The results reported here are congruent with the recent studies which showed that the trnH-GUG gene was situated in the LSC region in some species from order Sapindales, while the SSC/IRa border extends into the protein coding gene ycf1 with subsequent
formation of a ycf1 pseudogene [29,30]. Despite the seven chloroplast genomes of Sapindales having well-conserved genomic structure in terms of gene order and number, length variation of the whole chloroplast genome sequences and LSC, SSC and IR regions was detected among these genomes. This sequence variation might have been as a result of boundary expansion and contraction between the single copy and IR boundary regions among plant lineages as suggested by Wang and Messing 2011 [38].

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Figure 2. Comparison of IR, LSC and SSC junction positions among seven Chloroplast genomes. The features drawn are not to scale. The symbol ϕ means pseudogene created by IRb/SSC border extension into ycf1 genes. Colored boxes for genes represent the gene position.

The mauve alignment for seven species revealed that all the genomes formed collinear blocks (LCBs). In particular, all the seven species; A. altissima, Leitneria floridana, Azadirachta indica, Citrus aurantiifolia, Boswellia Sacra, Spondias bahiensis and Dodonaea viscosa reveal a syntenic structure, however block two was inverted (from rpl20 to rbcL genes) compared to the reference genome (Aquilaria sinensis). The collinear blocks of the genes including ribosomal RNA, tRNA, and protein coding genes revealed that all the seven genomes were relatively conserved with no gene rearrangement (Figure 3). Some other studies have revealed homology in genome organization and no gene rearrangements, thus our findings support their conclusions [31,39,40].
2.3. Codon Usage and Putative RNA Editing Sites in Chloroplast Genes of A. altissima

In this study, we analyzed codon usage frequency and the relative synonymous codon usage (RSCU) in the A. altissima plastome. All the protein coding genes presented a total of 68,952 bp and 22,964 codons in A. altissima chloroplast genome. Of 22,964 codons, leucine (Leu) being the most abundant amino acid had a frequency of 10.56%, then isoleucine (Ile) with 8.54%, while cysteine (Cys) was rare with a proportion of 1.12% (Tables S1 and S2, Figure 4). Our study species genome is like other previously reported genomes which showed that leucine and isoleucine are more common [41–45]. Furthermore, comparable to other angiosperm chloroplast genomes, our results followed the trend of codon preference towards A/T ending which was observed in plastomes of two Aristolochia species [46], Scutellaria baicalensis [47], Decaisnea insignis [34], Papaver rhoas and Papaver orientale [48] Cinnamomum camphora [49], and Forsythia suspensa [41]. All the twenty-eight A/U—ending codons had RSCU values of more than one (RSCU > 1), whereas the C/G—ending codons had RSCU values of less than one. Two amino acids, Methionine (Met) and tryptophan (Trp) showed no codon bias. The results for number of codons (Nc) of each protein coding gene ranged from 38.94 (rps14 gene) to 58.37 (clpP gene).

The potential RNA editing sites in tree of heaven chloroplast genome was done using PREP program which revealed that most conversions at the codon positions change from serine (S) to leucine (L) (Table 2). In addition, 15 (27.78%), 39 (72.22%), and 0 editing locations were used in the first, second and third codons respectively. One RNA editing site converted the amino acid from apolar to polar (proline (P) to serine (S). Overall, the PREP program identified a total of 54 editing sites in 21 protein coding genes, with ndhB and ndhD genes predicted to have the highest number of editing sites (9). Followed by ndhA, matK, rpoC2, and rpoB with four editing sites, whereas ndhF had three sites. Interestingly, fifty three of fifty four RNA editing conversions in the A. altissima chloroplast...
which also found that most RNA editing sites led to amino acid change from polar to apolar, resulting in increase in protein hydrophobicity [41,46,50].

Figure 4. Amino acid frequencies in A. altissima chloroplast genome protein coding sequences.

Table 2. Predicted RNA editing site in the A. altissima chloroplast genome.
Comparisons of RNA editing sites with other six species from other families revealed that *R. chinensis* and *D. viscosa* have high RNA editing sites (61 each distributed in 20 and 17 genes respectively) followed by *B. sacra* (57 in 20 genes), *A. altissima* (54 in 21 genes), *A. indica* (53 in 21 genes), *C. aurantiifolia* (52 in 21 genes), and *L. floridana* 48 in 20 genes. As shown in Table S3, these results are consistent with several studies in that all the RNA editing sites predicted among the seven species are cytidine (C) to uridine (U) conversions [41,50–52]. Majority of RNA editing occurred at the second positions of the codons with a frequency from 62.30% (38/61) in *D. viscosa* to 81.28% (39/48) in *L. floridana*, which concurs with previous plastid genome studies in other land plants [53,54]. All the species shared 19 editing sites distributed in twelve genes (Table 3), whereas the two species from Simaroubaceae family (*L. floridana* and *A. altissima*) shared 33 editing sites in 16 genes this implies that the RNA editing sites in these two species are highly conserved (Table S4). Like previous studies [41,51,55], the *ndhB* gene in most of species analyzed here have the highest number of editing sites. Notably, a RNA editing event was detected at the initiator codon (ACG) resulting in ATG translational start codon in the *ndhD* gene.

The cytidines marked are putatively edited to uredines.

Table 2. Cont.

| Gene  | Nucleotide Position | Amino Acid Position | Codon Conversion | Amino Acid Conversion | Score |
|-------|---------------------|---------------------|------------------|-----------------------|-------|
| ndhD  | 2 1                 | ACG ≥ ATG           | T ≥ M            | 1.00                  |
|       | 313 105             | CGG ≥ TGG           | R ≥ W            | 0.80                  |
|       | 383 128             | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 674 225             | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 878 293             | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 887 296             | CCT ≥ CTT           | P ≥ L            | 1.00                  |
|       | 1076 359            | GCT ≥ GTT           | A ≥ V            | 1.00                  |
|       | 1298 433            | TCA ≥ TTA           | S ≥ L            | 0.80                  |
|       | 1310 437            | TCA ≥ TTA           | S ≥ L            | 0.80                  |
|       | 290 97              | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 586 196             | CTT ≥ TTT           | L ≥ F            | 0.80                  |
|       | 1919 640            | CCT ≥ CTT           | A ≥ V            | 0.80                  |
|       | 166 56              | CAT ≥ TAT           | H ≥ Y            | 0.80                  |
|       | 320 107             | ATC ≥ ATA           | T ≥ I            | 0.80                  |
|       | 119 40              | CCT ≥ CTT           | P ≥ L            | 0.86                  |
|       | 77 26               | TCT ≥ TTT           | S ≥ F            | 1.00                  |
|       | 308 103             | TCA ≥ TTA           | S ≥ L            | 0.86                  |
|       | 830 277             | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 338 113             | TCT ≥ TTT           | S ≥ F            | 1.00                  |
|       | 551 184             | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 566 189             | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 2426 809            | TCA ≥ TTA           | S ≥ L            | 0.86                  |
|       | 41 14               | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 1681 561            | CAT ≥ TAT           | H ≥ Y            | 0.86                  |
|       | 2030 677            | ACT ≥ ATT           | T ≥ I            | 1.00                  |
|       | 2314 772            | CGG ≥ TGG           | R ≥ W            | 1.00                  |
|       | 4183 1395           | CTT ≥ TTT           | L ≥ F            | 0.80                  |
|       | 248 83              | TCA ≥ TTA           | S ≥ L            | 1.00                  |
|       | 209 70              | TCA ≥ TTA           | S ≥ L            | 0.83                  |
Table 3. List of RNA editing sites shared by the seven plastomes predicted by PREP program.

| Gene   | A.A Position | Citrus aurantiifolia | Rhus chinensis | Dodonaea viscosa | Boswellia Sacra | Leitneria floridana | Azadirachta indica | Ailanthus altissima |
|--------|--------------|----------------------|----------------|-----------------|----------------|-------------------|------------------|-------------------|
|        |              | Codon (A.A) Conversion |                |                 |                |                   |                  |                   |
| atpF   | 31           | CCA (P) ≥ CTA (L)     | CCA (P) ≥ CTA (L) | CCA (P) ≥ CTA (L) | CCA (P) ≥ CTA (L) | CCA (P) ≥ CTA (L) | CCA (P) ≥ CTA (L) | CCA (P) ≥ CTA (L) |
| clpP   | 187          | CAT (H) ≥ TAT (Y)     | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) |
| MatK   | 358          | TCA (S) ≥ TTA (L)     | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) |
| ndhA   | 156          | TCA (S) ≥ TTA (L)     | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) |
|        | 196          | CAT (H) ≥ TAT (Y)     | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) |
|        | 249          | TCT (S) ≥ TTT (F)     | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) |
|        | 419          | CAT (H) ≥ TAT (Y)     | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) |
| ndhB   | 128          | ACA (T) ≥ ATA (I)     | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) |
|        | 107          | ACA (T) ≥ ATA (I)     | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) | ACA (T) ≥ ATA (I) |
| rpoA   | 278          | TCA (S) ≥ TTA (L)     | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) |
|        | 113          | TCT (S) ≥ TTT (F)     | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) | TCT (S) ≥ TTT (F) |
|        | 184          | TCA (S) ≥ TTA (L)     | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) |
|        | 809          | TCG (S) ≥ TTG (L)     | TCG (S) ≥ TTG (L) | TCG (S) ≥ TTG (L) | TCG (S) ≥ TTG (L) | TCG (S) ≥ TTG (L) | TCG (S) ≥ TTG (L) | TCG (S) ≥ TTG (L) |
| rpoC1  | 14           | TCA (S) ≥ TTA (L)     | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) |
| rpoC2  | 563          | CAT (H) ≥ TAT (Y)     | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) | CAT (H) ≥ TAT (Y) |
| rps14  | 27           | TCA (S) ≥ TTA (L)     | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) | TCA (S) ≥ TTA (L) |
2.4. Repeat Sequence Analysis

Microsatellites are usually 1–6 bp tandem repeat DNA sequences and are distributed throughout the genome. The presence of microsatellites was detected in the chloroplast genome of *A. altissima* (Figure 5). A total of 219 simple sequence repeats (SSRs) loci were detected, of which mononucleotide repeats occurred with high frequency constituting 190 (86.76%) of all the SSRs. Majority of mononucleotides composed of poly A (polyadenine) (39.27%) and poly T (polythymine) (47.49%) repeats, whereas poly G (polgyuanine) or poly C (polycytosine) repeats were rather rare (2.74%). Among the dinucleotide repeat motifs AT/AT were more abundant, while AG/CT were less frequent. One trinucleotide motif (AAT/ATT), five tetra- (AAAG/CTTT, AAAT/ATTT, AACT/AGTT, AATC/ATTG, and AGAT/ATCT) and two pentanucleotide repeats (AAAG/CTTTT and AATAG/ATTCT) were identified. Hexanucleotide repeats were not detected in the *A. altissima* chloroplast genome.

As shown in Figure 5, the SSR analysis for seven species showed that *Leitneria floridana* had the highest number of SSRs (256) while *Dodonaea viscosa* and *Rhus chinensis* had the lowest (186). In all the seven species, mononucleotide repeats were more abundant with A/T repeats being the most common repeats. This result is consistent with earlier studies in [31,35,46] which revealed that many angiosperm chloroplast genomes are rich in poly A and poly T. Moreover, in the seven analyzed species, hexanucleotide repeats were not detected, whereas *Azadirachta indica*, *Dodonaea viscosa* and *Leitneria floridana* had no pentanucleotide repeats.

The REPuter program revealed that *A. altissima* chloroplast genome contains 21 palindromic (p), 22 forward (f) and six reverse (r) repeats, however the complement repeats were not detected (Table 4). We notice that all the identified tandem repeats in *A. altissima* were more than 20 bp, while thirteen had length of more than 30 bp. Repeat analyses of seven Sapindales revealed a total of 48 or 49 repeats for each species, with all species containing forward, palindromic and reverse repeats.

![Figure 5. Simple sequence repeat (SSRs) type, distribution and presence in A. altissima and other representative species from Sapindales. (A) Number of detected SSR motifs in different repeat types in A. altissima Chloroplast genome. (B) Number of identified repeat sequences in seven chloroplast genomes. (C) Number of different SSR types in seven representative species. F, indicate (forward), P (palindromic), R (reverse), and C (complement), while P1, P2, P3, P4, P5 indicates Mono-, di-, tri-, tetra-, and penta-nucleotides respectively. F: forward; P: palindromic, R: reverse; C: complement.](image-url)
Compliment repeats were not identified in other species except for *Azadirachta indica* and *Citrus aurantiifolia* which had one and three repeats respectively. *Citrus aurantiifolia* had the highest number of reverse repeats but also lowest number of forward repeats. Most of the repeat lengths were less than 50 bp, however *Boswellia sacra* chloroplast had seven forward repeats with length of between 65 to 251 bp. Besides, we found out that almost all the repeat sequences were located in either IR or LSC region.

| Number | Size | Position 1 | Type | Position 2 | Location 1 (2) | Region |
|--------|------|------------|------|------------|----------------|--------|
| 1      | 48   | 95,957     | F    | 95,975     | ycf2           | IRa    |
| 2      | 48   | 153,174    | F    | 153,192    | ycf2           | IRb    |
| 3      | 37   | 103,326    | F    | 125,821    | ycf2           | IRa    |
| 4      | 30   | 95,957     | F    | 95,993     | ycf2           | IRa    |
| 5      | 30   | 153,174    | F    | 153,210    | ycf2           | IRb    |
| 6      | 29   | 50,944     | F    | 50,972     | trnL-AAA*      | LSC    |
| 7      | 29   | 58,040     | F    | 58,078     | rbcL           | LSC    |
| 8      | 28   | 119,434    | F    | 115,460    | ycf1           | SSC    |
| 9      | 26   | 39,399     | F    | 39,625     | psbZ/trnG-UCC  | LSC    |
| 10     | 25   | 71,153     | F    | 71,178     | rplP-GGG/pal   | LSC    |
| 11     | 23   | 47,036     | F    | 103,323    | ycf3**         | IRa    |
| 12     | 23   | 112,456    | F    | 112,488    | rrn4.5/rrn5    | IRa    |
| 13     | 23   | 136,686    | F    | 136,718    | rrn5/rrn4.5    | IRb    |
| 14     | 22   | 11,749     | F    | 11,771     | trnR-UCL/atrPA | LSC    |
| 15     | 21   | 248        | F    | 270        | trnH-GUC/pal   | LSC    |
| 16     | 21   | 9541       | F    | 38,293     | trnS-GCU(trnS-UAG) | LSC |
| 17     | 21   | 41,956     | F    | 44,180     | psaB(psaA)     | LSC    |
| 18     | 21   | 49,678     | F    | 49,699     | trnL-AAA*      | LSC    |
| 19     | 20   | 47,036     | F    | 1965       | trnK-UIUI      | LSC    |
| 20     | 19   | 1945       | F    | 92,503     | atpH/atpI(ycf2) | LSC |
| 21     | 20   | 47,036     | F    | 125,821    | ycf3**(ycf15)  | IRa    |
| 22     | 20   | 88,907     | F    | 160,270    | rpl2           | IRa/IRb |
| 25     | 48   | 31,790     | P    | 31,790     | petN/pal      | LSC    |
| 26     | 48   | 95,957     | P    | 153,174    | ycf2          | IRa/IRb |
| 27     | 48   | 95,957     | P    | 153,192    | ycf2          | IRa/IRb |
| 28     | 37   | 125,821    | P    | 145,834    | ndhA*         | SSC/IRb |
| 29     | 36   | 30,970     | P    | 30,970     | petN/pal      | LSC    |
| 30     | 30   | 72,117     | P    | 72,117     | rrn33/rrn18    | LSC    |
| 31     | 30   | 95,957     | P    | 153,174    | ycf2          | IRa/IRb |
| 32     | 30   | 95,993     | P    | 153,210    | ycf2          | IRa/IRb |
| 33     | 27   | 542        | P    | 571        | trnH-GUC/pal   | LSC    |
| 34     | 25   | 11,403     | P    | 11,430     | trnS-GCU(trnR-UCL) | LSC |
| 35     | 24   | 4867       | P    | 4897       | trnK-UIUI/rrn16 | LSC |
| 36     | 24   | 9535       | P    | 48,164     | trnS-GCU(psaA/yef3) | LSC |
| 37     | 23   | 47,036     | P    | 145,831    | ycf3**(ycf15)  | IRa/IRb |
| 38     | 23   | 51,804     | P    | 119,066    | trnF-GAA/ndhB(rrn32/trnL-UIAG) | LSC/SSC |
| 39     | 23   | 112,456    | P    | 136,686    | rrn4.5/rrn5   | IRa/IRb |
| 40     | 23   | 112,488    | P    | 136,718    | rrn4.5/rrn5   | IRa/IRb |
| 41     | 22   | 39,195     | P    | 39,195     | psbZ/trnG-UCC | LSC    |
| 42     | 20   | 15,166     | P    | 156,674    | atpH(ycf2)    | LSC/IRb |
| 43     | 20   | 38,361     | P    | 48,100     | trnS-GCU(trnS-GGA) | LSC |
| 44     | 20   | 88,907     | P    | 88,907     | rpl2          | IRa    |
| 45     | 20   | 107,097    | P    | 107,130    | rrn16/trnL-UIA | IRa    |
| 46     | 23   | 39,184     | R    | 39,184     | psbZ/trnG-UCC | LSC    |
| 47     | 21   | 9751       | R    | 9751       | trnS-GCU(trnR-UCL) | LSC |
| 48     | 21   | 51,281     | R    | 51,281     | trnL-UIA/trnF-GAA | LSC |
| 49     | 21   | 85,055     | R    | 85,055     | rps8/rpl14    | LSC    |
| 50     | 20   | 53,712     | R    | 53,712     | ndhC           | LSC    |
| 51     | 20   | 9385       | R    | 13,356     | psbI(atpA/atpF) | LSC    |

F: forward; P: palindromic; R: reverse* intron or ** introns.
2.5. Phylogenetic Tree

The phylogenetic position of *A. altissima* within Sapindales was carried out using 75 protein coding sequences shared by thirty-one taxa from Sapindales (Table S5). Three remaining species were from Thymelaeaceae family (*Aquilaria sinensis*) and Malvaceae (*Theobroma cacao* and *Abelmoschus esculentus*) from order Malvales selected as outgroups (Figure 6). The maximum likelihood (ML) analysis produced a phylogenetic tree which fully supported *A. altissima* to be closely related with *Leitneria floridana* with 100% bootstrap value. The ML resolved 26 nodes with high branch support (over 98% bootstrap values), however six nodes were moderately supported perhaps as a result of less samples use (59 to 95%). Concerning relationships among families within Sapindales order, family Simaroubaceae early diverged and formed a sister clade/relationship with a 95% bootstrap support to Rutaceae family. Interestingly, the placement of families within Sapindales in our phylogenetic tree supports the one reported by previous studies [30,56,57] based on some chloroplast and nuclear markers. The families Anacardiaceae and Burseraceae formed a sister clade/group, this clade further branched forming a sister clade with families Sapindaceae, Meliaceae, Simaroubaceae and Rutaceae analyzed in our study. Therefore, it is crucial to use more species for better understanding of Simaroubaceae phylogeny and evolution. This study provides a basis for future phylogenetic of Simaroubaceae species.

![Phylogenetic tree](image)

**Figure 6.** Phylogenetic tree of 31 Sapindales species with three outgroup Malvales species inferred from ML (Maximum likelihood) based on common protein coding genes. The position of *A. altissima* is shown in bold, while bootstrap support values are shown at each node.

3. Materials and Methods

3.1. Plant Materials and DNA Extraction

Fresh leaves of *Ailanthus altissima* were collected in Wuhan Botanical Garden, Chinese Academy of Sciences in China. Total genomic DNA isolation was carried out using MagicMag Genomic DNA Micro Kit (Sangon Biotech Co., Shanghai, China) based on the manufactures protocol. The quality and integrity of DNA were checked and inspected using spectrophotometry and agarose gel electrophoresis respectively. The voucher specimen (HIB-LZZ-CC003) has been deposited at the Wuhan Botanical Garden herbarium (HIB) Wuhan, China.
3.2. The Tree of Heaven Plastome Sequence Assembly and Annotation

Library preparation was constructed using the Illumina Hiseq 2500 platform at NOVOgene Company (Beijing, China) with an average insert size of approximately 350 bp. The high-quality data (5 Gb) were filtered from raw sequence data (5.2 Gb) using the PRINSEQ lite v0.20.4 (San Diego State University, San Diego, CA, USA) \[58\] (phredQ \( \geq 20 \), Length \( \geq 50 \)), followed by de novo assembling using NOVOplasty \[59\] with default sets (K-mer = 31). The seeds and reference plastome used was from the closely related species *Leitneria floridana* (NC_030482) with high coverage of chloroplast reads \(~1500 \times\). Lastly, one contig of *Ailanthus altissima* was generated and mapped with reference plastome using GENEIOUS 8.1 (Biomatters Ltd., Auckland, New Zealand) \[60\]. Finally, online web-based server local blast was used to verify the inverted repeat (IR) single copy (SC) junctions.

Preliminary gene annotation of assembled genome was done using the program DOGMA (Dual Organellar GenoMe Annotator, University of Texas at Austin, Austin, TX, USA) \[61\], and BLAST (http://blast.ncbi.nlm.nih.gov/). The positions of start and stop codons together with position of introns were confirmed by comparing with homologous genes of other chloroplast genomes available at the GenBank database. Moreover, tRNA genes were verified with tRNAscan-SE server (http://lowelab.ucsc.edu/tRNAscan-SE/) \[62\]. The chloroplast genome physical circular map was drawn using program OGDRAW (Organellar Genome DRAW) \[63\] Max planck Institute of Molecular Plant Physiology, Potsdam, Germany) accompanied by manual corrections. The chloroplast genome sequence of *A. altissima* was deposited in the GenBank database, accession (MG799542).

3.3. Genome Comparison and Gene Rearrangement

The border region between Inverted repeat (IR) and large single copy (LSC), also between inverted repeats and small single copy (SSC) junction were compared among seven representative species from Sapindales order. Additionally, alignments of seven chloroplast with one reference genome to determined gene rearrangements was carried out using Mauve v.4.0 \[64\].

3.4. Repeat Analysis in *A. altissima* Chloroplast Genome

Microsatellites were identified in the tree of heaven chloroplast genome and other selected representative genomes belonging to order Sapindales using an online software MicroSatellite (MISA) \[65\]. The minimum number of repetitions were set to eight repeat units for mononucleotide SSR motifs, five repeat units for dinucleotide SSRs, four for trinucleotide SSRs and three repeat units for tetra-, penta-, and hexanucleotide motifs. The REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer) program \[66\] (University of Bielefeld, Bielefeld, Germany) with default parameters was used to identify the location and sizes of forward, palindromic, complement and reverse repeat sequences in *A. altissima* chloroplast genome.

3.5. Codon Usage and RNA Editing Sites

CodonW1.4.2 (http://downloads.fyxm.net/CodonW-76666.html) was used to analyze codon usage. Subsequently, possible RNA editing sites in *A. altissima* protein coding genes were predicted using the program predictive RNA editor for plants (PREP) suite \[67\] with the cutoff value set to 0.8. PREP server uses 35 genes as reference for potential RNA editing sites prediction by comparing the predicted protein genes to homologous proteins from other plants.

3.6. Phylogenetic Analysis

Seventy five protein coding sequences present in 31 species from order Sapindales and three species from Thymelaeaceae (*Aquilaria sinensis*), Malvaceae (*Theobroma cacao* and *Abelmoschus esculentus*) as outgroups were used for the phylogenetic reconstruction. These species chloroplast genomes were downloaded from GenBank (Table S5). The protein coding sequences alignment was done using GENEIOUS v8.0.2 (Biomatters Ltd., Auckland, New Zealand) \[60\]. Maximum likelihood (ML) analysis
was carried out using RAxML version 8.0.20 (Scientific Computing Group, Heidelberg Institute for Theoretical Studies, Institute of Theoretical Informatics, Karlsruhe Institute of Technology, Karlsruhe, Germany) [68] with 1000 replicates for bootstrap test. Lastly, the jModelTest v2.1.7 [69] was used to select the best substitution model (GTR + I + G).

4. Conclusions

In this study, we present the plastome of tree of heaven from family Simaroubaceae which contains about 22 genera and over 100 species. *A. altissima* chloroplast genome possess circular and quadripartite structure which is well conserved similar to other plants chloroplast genomes. Nonetheless, the plastome showed slight variations at the four boundary junctions due to expansion and contraction in SC and IR borders. About 219 SSR loci and 49 repeats sequences were identified in *A. altissima* genome, this provides genetic information for designing DNA molecular markers for analyzing gene pool dynamics and genetic diversity of *A. altissima* natural populations aiming dispersal mechanism of this invasive tree. The phylogenetic analysis performed using 75 protein coding genes of 34 species available at the GenBank database, comprising 3 outgroup species from Malvales and 31 species representing families from order Sapindales. The two species from family Simaroubaceae formed a cluster and were group together with other families to form a single clade (Sapindales). In addition, the RNA editing analysis in *A. altissima* genome identified a total of 54 possible editing sites in 21 chloroplast genes with C-to-U transitions being the most. The availability of this chloroplast genome provides a tool to advance the study of evolution and invasion in *A. altissima* in order to address present evolutionary, ecological and genetic questions regarding this species.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/4/929/s1.

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Author Contributions: Yi-Ying Liao conceived and designed the experiment; Josphat K. Saina, Zhi-Zhong Li and Andrew W. Gichira assembled plastome sequence and revised the manuscript. Josphat K. Saina performed the experiments, analyzed data and wrote the manuscript. Yi-Ying Liao and Zhi-Zhong Li collected the plant material. All the authors read and approved submission of the final manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interest.

Abbreviations

| SC  | Single copy |
|-----|-------------|
| LSC | Large single copy |
| SSC | Small single copy |
| IR  | Inverted repeat |

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