The Complete Chloroplast and Mitochondrial Genome Sequences of Boea hygrometrica: Insights into the Evolution of Plant Organellar Genomes

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Abstract

The complete nucleotide sequences of the chloroplast (cp) and mitochondrial (mt) genomes of resurrection plant Boea hygrometrica (Bh, Gesneriaceae) have been determined with the lengths of 153,493 bp and 510,519 bp, respectively. The smaller chloroplast genome contains more genes (147) with a 72% coding sequence, and the larger mitochondrial genome has less genes (65) with a coding faction of 12%. Similar to other seed plants, the Bh cp genome has a typical quadripartite organization with a conserved gene in each region. The Bh mt genome has three recombinant sequence repeats of 222 bp, 843 bp, and 1474 bp in length, which divide the genome into a single master circle (MC) and four isomeric molecules. Compared to other angiosperms, one remarkable feature of the Bh mt genome is the frequent transfer of genetic material from the cp genome during recent Bh evolution. We also analyzed organellar genome evolution in general regarding genome features as well as compositional dynamics of sequence and gene structure/organization, providing clues for the understanding of the evolution of angiosperms genomes in plants. The cp-derived sequences including tRNAs found in angiosperm mt genomes support the conclusion that frequent gene transfer events may have begun early in the land plant lineage.

Introduction

Plastid and mitochondria are essential organelles in plant cells. Chloroplasts conduct photosynthesis in the presence of sunlight and mitochondria indirectly supply energy within plant cells; together they form the powerhouses of the cell. Both chloroplasts and mitochondria possess their own genomes. The chloroplast (cp) genome and mitochondrial (mt) genomes are often used for the study of plant evolution [1,2]. From the information of all sequenced cp genomes, most of them range from 120 to 160 kb in length and have GC contents of 30 to 40%. The quadripartite organization is shared by almost all cp genomes, consisting of a large-single-copy region (LSC; 60–90 kb) and a small-single-copy region (SSC; 16–27 kb), as well as two copies of inverted repeats (IRs) of ∼20 to 28 kb in size. The gene content and structure of angiosperm cp genome is highly conserved [3,4]. Expansion and contraction of the IR as well as gene and intron losses have been documented in a wide range of angiosperms [3,6].

The mt genome plays crucial roles in plant development and productivity [7]. In comparison to other non-plant eukaryotes, plants have large and complex mt genomes [8,9]. Mt genomes of seed plants are unusually large and vary in size at least in an order of magnitude. Much of these variations occur within a single family [10]. Seed plant mitochondrial genomes are characteristic for their very low mutation rate [11], frequent uptake of foreign DNA by intracellular and horizontal gene transfer [12], and dynamic structure [13]. The evolving land plants have gained new mechanisms to facilitate more frequent gene exchanges between mt and cp genomes as well as between mt and nuclear genomes, which make mt genomes increase their sizes [14].

In the past several years, we have witnessed a dramatic increase in the number of complete organellar genomes, especially those of plants. Until now, there are 206 complete cp genomes and 47 mt genomes having been deposited in GenBank Organellar Genome Resources. With the emergence of next-generation sequencers, new approaches for genome sequencing have been gradually applied due to their high-throughput, time-saving, and low-cost advantages. With a new gene-based strategy and combining data from the two next-generation sequence platforms, pyrosequencing (Roche GS FLX) and ligation-based sequencing (Life Technolo-
The Organellar Genomes of Boea hygrometrica

gies SOLiD), we successfully assembled cp and mt genomes of resurrection plant Boea hygrometrica (Bunge) R. Br [13]. Boea hygrometrica or Bh is a small dicotyledonous, homochlorophyllous resurrection plant in the Gesneriaceae family, and it is widespread in China, inhabiting shallow rock crevices from humid tropical regions to arid temperate zones [16,17]. In this study, we analyze genomic features and structures of both cp and mt genomes of Boea hygrometrica. Through organellar genome comparison with other lower plants and angiosperms, we provide information for the better understanding of organellar genome evolution in land plants.

Results and Discussion

Features of Boea hygrometrica cp genome and mt genome

The Bh cp genome is 153,493 bp in length and has a GC content of 37.59%. Similar to those of other angiosperms [4,18,19], the Bh cp genome maps as a circular molecule with the typical quadripartite structure: a pair of IRs (25,450 bp, covering 16.6%) separated by the LSC (84,692 bp, covering 55.1%) and SSC (17,901 bp, 11.7%) regions (Figure 1). It encodes 147 predicted functional genes and 19 of them are duplicated in the IR regions. Among these 147 genes, we identified 103, 36, and 8 protein-coding, tRNA, and rRNA genes, respectively (Table 1 and Table 2). 38% of the genome is non-coding, including introns, intergenic spacers, and pseudogenes. All the rRNA genes (rrn16, rrn25, rrn5 and rrn4.5) and 7 tRNA (trnL-CAU, trnL-GAG, trnL-GAU, trnV-UGC, trnR-ACG, and trnV-GUU) genes are located in IR regions. Similar to other dicot species, Bh has two genes (trnI and trnH) located in the position of IR/LSC junctions. This is different in monocots, such as rice and maize, whose cpDNAs have a fully duplicated trnI gene in the IR/LSC junctions [18]. The average length of intergenic regions is 385 bp, varying from 1 to 2,291 bp. There are 4 cases of overlapping genes (psbD-psbC, ndhK-ndhC, atpE-atpB, and ycf1-ndhF), resulting in an average coding density (including conserved genes, unique ORFs and introns) of 1/1,058 bp. The cp genome has 19 intron-containing genes with 12 in protein-coding genes and 7 in tRNA genes. In terms of size, gene content, and intron composition, Bh cpDNA closely is mapped to Olea europaea cpDNA (155,872 bp, GC 37%) [20] among all angiosperms. Sequence alignment shows that 93% (142,189 bp) of the Bh cp genome sequence are covered by that of O. europaea with 95.4% identity (Figure S1). 36 tRNAs are detected, enabling B. hygrometrica cp genome to decode all 61 codons. All of 3 stop codons are present with UAA being the most frequently used (UAA 40%, UAG 33% and UGA 27%) (Table S1). Phylogenetic analyses, which were constructed by 63 protein-coding sequences from 12 cp genomes (one green algae as outgroup, one land and 10 seed plants) (Table S2), indicates that the phylogenetic position of Boea hygrometrica is closer and older than V. vinifera among the analyzed dicots (Figure S2).

The Bh master mt genome is assembled into a circular molecule of 510,519 bp in length (Figure 2) and has an average GC content of 43.27%. This size is bigger than the mt genome of Arabidopsis thaliana (366,924 bp) [21], but smaller than Vitis vinifera (773,279 bp) [12] among dicots. With only 12% of coding sequences, the largest part (88%) of this genome is non-coding, containing 1.45% repeat and 10.52% cp-derived sequences (Table 1). The mt genome has 65 genes, including 33 protein-coding, 4 tRNA, and 28 tRNA genes, and 10 genes have exon-intron structure. Similar to other angiosperms, the Bh mitochondrial uses the canonical genetic code. All 61 codons are present in mt genome, and UAA (46%) is the most frequently-used stop codon (Table S3). The known 33 protein-coding genes in mt genome are similar to other published angiosperm mt genomes (Table 2), such as 9 subunits of NADH dehydrogenase (complex I), 5 subunits of ATP synthase (Complex V), and 3 subunits of cytochrome c oxidase (complex IV). Compared to other angiosperms, we observed that there is one sdh3 and no sdh4 in Bh mt genome. There are 3 copies of sdh3 in mt genome of Neotinea tabacum [22], and both sdh3 and sdh4 are present in that of V. vinifera [12]. The Bh mt genome has an intron/exon structure that is unlike other higher seed plants (Table S4). There are two 5S rRNA (rn5) copies and one copy of rnl3 from its cp genome. The best sequence alignment score belongs to V. vinifera mt genome, with 23% (119,377 bp) of Bh mt genome being alignable to that of V. vinifera with 94.2% identity (Figure S3).

Plant cells often contain multiple clones or copies of cp and genomes, and thus the organellar genomes can be regarded as a population with genetic heterogeneity [4]. Polymorphic sites can be detected by aligning thousands of high quality reads to assembling of the cp or mt genome [23]. Our SNP analysis shows that there is no intravarietal SNPs (intraSNPs) found in Bh cp genome. However, we identified 729 SNPs in Bh mt genome (Table S5) and SNPs in mt genome occurred at a rate of 1 in 700 bp. We only detected 9 SNPs in gene regions with 2, 1, 1, and 5 in rnl5, rnl26, trnM-GAU, and rnl18, respectively (Table 3). There are no intraSNPs detected in known protein-coding gene regions. The intraSNPs have been demonstrated to be present in both cp and mt genomes of rice [25,24]. As an indicator for the heterogeneous nature of cp and mt populations, the intravarietal polymorphisms provide us useful markers for the future genetic studies on B. hygrometrica.

Structural dynamics of Bh mitochondrial genome in ontogeny

All previous studies on complete sequencing of flowering plant mt genomes are based on the master circle (MC) hypothesis [21,22,25–28]. Arrieta-Montiel et al. reported on the structural dynamics of the common bean mt genome [29]. The analysis of 10 recombinant clones supports existence of the MC molecule in wheat mt genome [7]. However, the result of field-inversion electrophoresis suggests that Physcomitrella mt genome does not consist of a multipartite structure, as seen in angiosperms [30]. As mitochondrial gene orders are significantly different between lower plants and higher flowering plants, the multipartite structures as seen among angiosperms may originate during the evolution of pteridophytes or seed plants [9,30]. Repeat prediction by REPutter shows that there are 14 repeat pairs in Bh mt genome (Table 4). Since not all the repeats are involved in recombination, from the mt assembly [15] we detected 3 repeat-specific contigs that are candidates for the recombination among the MC and other isomeric (IO) and subgenomic molecules, which have been confirmed by SOLiD sequencing [15]. Those 3 repeat contigs are the repeat pairs of two palindromic matches (1,474 bp and 843 bp) and one forward match (222 bp). By aligning all SOLiD long mate-pair reads to both ends of the repeats, we constructed the MC and 4 isomeric molecules (Figure 3). These subgenomic molecules are not discussed further because we have yet to find significant differences among the sequence reads.

The length of recombinant repeats (222 bp, 843 bp, and 1,474 bp) of Bh is different from that of V. radiata, and demonstrates the recombination across short mt repeats [38–297 bp] [31]. The longer repeats are reminiscent of those found in other angiosperm mitochondrial genomes, which are involved in mt genome rearrangements and can result in stoichiometric
shifting of subgenomic mt genome topologies, occasionally beyond detection level for one (or more) of alternative DNA topologies [29,32]. The 3 recombinant repeats are located in gene-rich regions and split mt genome into 6 segments. Each segment has some essential conserved genes, such as \textit{nad1} and \textit{rps13} in segment A, and \textit{atp6}, \textit{nad2} e3-5 and \textit{cox6} in segment B. From this point, it is possible that the mt genome of \textit{Bh} do not have subgenomic molecules. There are 4 genes (\textit{nad1}, \textit{nad2}, \textit{nad5} and \textit{sdh3}) with exon-intron structure separated by the recombinant repeats (Table 5). The \textit{nad1} gene in MC molecule is cross-strand gene with exon 4 in positive strand and exon 1–3 in negative strand. Recombination involving introns might lead to rearranged molecules without loss of essential genes [30]. In all rearranged 4 isomeric molecules, the Trans-splicing genes are different, and the IO3 molecule has all 4 Trans-splicing genes. Trans-splicing status of group II intron widely distributes in the mt genome of higher plant [33–35]. We compared gene structures of 15 mt genomes from lower to higher plants, and found 3 conserved genes (\textit{nad1}, \textit{nad2}, and \textit{nad5}) as well as other higher plants contain trans-splicing intron (Table 6), while there is no intron in those genes of \textit{Chara}.

Figure 1. The circular-mapping chloroplast genome of \textit{B. hygrometrica}. Features on transcriptionally clockwise and counter-clockwise strands are drawn on the inside and outside of the outer circle, respectively. Genes belonging to different groups are color-coded (Y, pseudogene). The genome coordinate and GC content are shown in the inner circle. The thick lines indicate inverted repeats (IRA and IRB), which separate the genome into small (SSC) and large (LSC) single copy regions. The map was drawn by using OGDRAW [47].

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The Organellar Genomes of Boea hygrometrica

We compared 12 cp genomes ranging from green algae to angiosperm (Table S6). The GC contents of cp genome are lowest in lower plants (Charophyta and Bryophyta) but highest in Cycads. Monocots seem to have slightly higher GC contents than dicots among their cp genomes. For example, C. vulgaris (184,933 bp; Charophyta) has the largest genome while B. hygrometrica (184,933 bp) has 2 copies in IR regions except the 2 copies of rrn4.5. The GC contents of cp genome are also variable from 40 to 47%. There is a massive difference of coding sequences between lower and higher plants. The coding sequence in C. vulgaris is 90.7%, whereas it is 4.94% in Trispermum dactyloides. Repeat content ranges from 1 to 41% among seed plants and are smallest in B. hygrometrica, composed of only 1.45% of the genome. Both large (>1,000 nt) and small (<50 nt) repeats affect recombination in seed plants [7,31,37]. The protein-coding genes and tRNAs in mt genomes also vary largely because of the large number of function-unknown proteins or ORFs in mt genomes and frequent plastid DNA insertions including cp tRNA genes [26,38].

We also carefully examined conserved genes in different plant lineages (Table S4). First, there are 14 conserved core protein-coding genes shared among all lineages, including seven subunits of NADH dehydrogenase (Complex I), one subunit of ubiquinol cytochrome c reductase (Complex III), three subunits of cytochrome c oxidase (Complex IV), and three subunits of ATP synthase (Complex V). All these genes play important roles either in proton movement across the inner membrane of the mitochondrion or electron transfer reactions in the respiratory chain. However, the gene structures are not conserved among them, and only two genes (nad4 and cox2) have exon-intron structures in all mt genomes. For comparison, there are 9 genes (nad3, nad4L, nad6, nad7, cob, cox3, atp6, atp8, and cox1) in mt genomes without exon-intron structure in both seed and early land plants and with exon-intron structure at least in one lower plant. Intron structure in mt genes is common as we only detected 6 genes (sld3, sld4, atp4, atp6, cob, and cox1) in mt genomes without introns among all plants. Second, gene loss is more frequent in dicots than monocots, as genes in cytochrome c biogenesis are lost in dicots. Third, all four ribosomal protein genes are conserved in both seed and early land plants and with exon-intron structure at least in one lower plant. Intron structure in mt genes is common as we only detected 6 genes (sld3, sld4, atp4, atp6, cob, and cox1) in mt genomes without introns among all plants. Second, gene loss is more frequent in dicots than monocots, as genes in cytochrome c biogenesis are lost in dicots. Third, all four ribosomal protein genes are conserved in both seed and early land plants and with exon-intron structure at least in one lower plant.
aestivum and B. vulgaris). Copy number-variable mt genes are reported in wheat, rice, and maize [7]. In summary, since the gene coding fraction is much less among mt genomes as compared to cp genomes, even conserved genes are also variable in gene content, structure, and intron positioning [30].

**Plastid DNA insertions in mt genome**

One of the important events in determining mt genome size in angiosperms is the frequent capture of sequences from the cp genome [10,26,28,40]. A recently study demonstrates frequent DNA transfer from cp to mt genomes occur as far back as the common ancestor of the extant gymnosperms and angiosperms, about 300 MYA and the frequency of cp-derived sequence transfer is positively correlated with variations in mt genome size [41]. For instance, B. hygrometrica mt genome contains fragments of cp origin, ranging from 50 to 5,146 bp in length (Table S7). The total fraction of cp-derived sequences present in Bh mt genome is 53,440 bp, 10.5% of the whole mt genome. Most of these insertions are conserved, as evidenced from the observation that 45 out 80 insertions (over 50 bp) are identified in mt genomes of

| Table 2. Gene content of B. hygrometrica cp and mt genomes. |
|---|
| cpDNA | Photosystem I | psaA,psaB,psaC,psaI |
| Photosystem II | psbA,psbB,psbC,psbD,psbE,psbF,psbH,psbI,psbK,psbL,psbM,psbN,psbT,psu,psbT |
| Cytochrome b/f complex | petA,petB,petD,petE,petL,petN |
| ATP synthase | atpA,atpB,atpF,atpH,atpL |
| NADH dehydrogenase | ndhA,ndhB,ndhC,ndhD,ndhE,ndhF,ndhG,ndhH,ndhI,ndhJ,ndhK |
| Rubisco large subunit | rbcL |
| RNA polymerase | rpoA,rpoB,rpoC1,rpoC2 |
| Ribosomal proteins (SSU) | rps2, rps3, rps4, rps7, rps11, rps12, rps13, rps16, rps18, rps19 |
| Ribosomal proteins (LSU) | rpl2, rpl14, rpl16, rpl20, rpl22, rpl23, rps4, rps7, rps8, rps11, rps12, rps13 |
| Hypothetical chloplast reading frames | ycf1, ycf2, ycf4, ycf7, ycf10, ycf15, ycf33, ycf37, ycf41, ycf54, ycf68, ycf89 |
| ORFs | orf42, orf56, orf93 |
| Transfer RNAs | trnA-UGC, trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnG-UCC, trnH-GUG, trnL-CAU, trnM-GAU, trnR-ACG, trnR-UCU, trnS-GCU, trnS-UGA, trnT-UAG, trnV-ACG, trnV-GAC, trnW-CCA, trnY-GUA |
| Ribosomal RNAs | rrr4.5, rrr5, rrr16, rrr22 |

| mtDNA | Genes of Mitochondrial Origin |
|---|
| Complex I (NADH dehydrogenase) | nad1, nad2, nad3, nad4, nad5, nad6, nad7, nad9 |
| Complex II (succinate dehydrogenase) | sdh3 |
| Complex III (ubichinol cytochrome c reductase) | cob |
| Complex IV (cytochrome c oxidase) | cox1, cox2, cox3 |
| Complex V (ATP synthase) | atp1, atp4, atp6, atp8, atp9 |
| Cytochrome c biogenesis | ccmB, ccmC, ccmF, ccmFn |
| Ribosomal proteins (SSU) | rps2, rps3, rps4, rps7, rps12, rps13 |
| Ribosomal proteins (LSU) | rpl16 |
| Maturases | matR |
| Other genes* | BohyM-orf1, BohyM-orf2, BohyM-orf3 |
| Transfer RNAs | trnC-GCA, trnE-UUC, trnG-GCC, trnH-GUG, trnL-CAU, trnM-GAU, trnR-ACG, trnR-UCU, trnS-GCU, trnS-UGA, trnT-GGU, trnV-GAC, trnW-CCA, trnY-GUA |
| Ribosomal RNAs | rrr4.5, rrr5, rrr16, rrr22 |

| Genes of Chloroplast Origin |
|---|
| Genes with intact ORFs | psaA, petA, petB, petD, petE, petL, petN, psa3, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbK, psbL, psbM, psbN, psbT, psu, psbT |
| Pseudogenes† | accD, atpA, atpB, atpF, atpL, psbA, psbH, psbI, psbK, psbL, psbM, psbN, psbT, psu, psbT |
| Transfer RNAs | trnD-GUC, trnE-GAA, trnH-GUG, trnL-CAU, trnM-GAU, trnR-ACG, trnS-GCU, trnT-GGU |

*Genes that are conserve among other function unknown mitochondrial genes or ORFs.
†Genes that are transferred to mtDNA from cpDNA in fragments.
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other plants. The average GC content between old insertions (at least found one homolog in the mt genomes of other plants) and new insertions (no homologs found in other mt genomes) is obviously different (Figure S4). The average GC content of old insertions (41.59%) is distinct from that of mt genomes (43.27%) as well as the average GC content of new insertions (35.32%) is close to the GC content of cp genomes (37.59%; Table 8). The result suggests positive correlations between the GC content of cp genomes and new insertions (coefficient value $r^2 = 0.69$) and between the GC content of mt genomes and old insertions (coefficient value $r^2 = 0.64$). There is a significant difference between the GC content of old and new insertions ($t$ test: $P < 0.01$). All of cp-derived sequences in lower plants are old insertion, and it is strange to see there are no new insertions from cp genomes during the evolution of A. thaliana. Compared to other angiosperms, the most striking feature of Bh mt genome is the frequent sequence transfer from the cp genome in its recent evolution. Cp-derived sequence analyses may provide clues for the understanding functional indications of DNA insertions from cp in mt genomes.

We also analyzed the inserted cp genomic sequences to mt genomes. First, there are 85 cp-derived fragments in the Bh mt genome. Most of them, especially protein-coding sequences, have no intact gene structure or have frameshifts/indels and the fact suggests that these cp-derived sequences are degenerated and lack functional constraints [41]. However, protein-coding genes such as

![Figure 2. The circular-mapping mitochondrial genome of B. hygrometrica. Features on transcriptionally clockwise and counter-clockwise strands are drawn on the inside and outside of the outer circle, respectively. The genome coordinate and GC content are shown in the inner circle. Genes belonging to different groups are color-coded. The map was drawn by using OGDRAW [47]. doi:10.1371/journal.pone.0030531.g002](image-url)
ribosomal proteins and tRNAs originated from cp genomes appear still functional in B. hygrometrica mt genome. Similar genes are also seen in other angiosperms [26,28]. Second, it is curious to investigate the locations of cp-derived sequences in cp DNAs to see if any particular regions in cp genomes are hotspots of DNA transfer. Among 80 transferred sequence fragments in B. hygrometrica cp genome, 45, 7, and 28 are from LSC, SSC, and IR regions, respectively. The numbers of fragments appear correlating to the lengths of LSC (34,692 bp), SSC (17,901 bp) and IRs (50,900 bp), and such correlations are also seen in other angiosperms, including maize, rice, wheat, and tobacco [41]. In conclusion, DNA transfer from cp genomes to mt genomes in angiosperms occurs randomly as it has been proposed earlier by Mastsuo et al in rice [42].

Table 3. Intravarietal polymorphisms (IntraSNPs) in the gene regions of B. hygrometrica mt genome.

| Gene    | Position | SNP    | Read Coverage |
|---------|----------|--------|---------------|
| trn5    | 234508   | C/T    | 273/122       |
| trn5    | 234560   | G/A    | 320/152       |
| trn26   | 289003   | A/G    | 366/86        |
| trnM-CAU| 339634   | A/T    | 415/868       |
| trn18   | 509408   | G/A    | 193/80        |
| trn18   | 509565   | C/T    | 191/39        |
| trn18   | 509941   | G/T    | 107/24        |
| trn18   | 509978   | T/C    | 148/90        |
| trn18   | 510096   | T/G    | 182/57        |

The Organellar Genomes of B. hygrometrica

To investigate whether mt genomes encode a full set of tRNAs necessary for protein synthesis in the organelle, we identified 28 tRNA genes from the complete B. hygrometrica mt genome based on tRNA structures and realized that all 61 codons are used by B. hygrometrica mt mitochondria (Table S3). However, the tRNA genes encoded by the mt genome alone are not sufficient to decode all codons; for instance, tRNA(misUAA) is missing in B. hygrometrica, and it suggests that the role of the missing tRNA is supplied by either cp or nuclear genomes [22,26,41]. tRNAs originated from plastids are called cp-derived tRNAs and their counterparts are native mt tRNAs. Half of the 28 mt tRNAs in B. hygrometrica are identified as cp-derived tRNAs (Table S8) and 19 amino acids are encoded by only one codon except for leucine (UAA and CAA) and serine (GCU, UGA, and GGA).

In contrast to the protein coding genes, mt tRNA genes appear constantly being transferred from cp genomes during the evolution of angiosperms Figure 4 and Table S8, and the proportion of cp-derived tRNAs in mt genomes increases from 0% in Charophyta to 55% in dicotyledonous plants. There are 17 cp-derived tRNAs and 14 mt native tRNAs in mt genome of V. vinifera, which has the most cp-derived tRNAs among dicotyledonous plants. Seven mt-native tRNA genes (trnD-GUC, trnE-UCC, trnL-CAU, trnK-UUU, trnM-CAU, trnS-GCU, trnS-UGA and trnW-GUA) and one cp-derived tRNA gene (trnF-GAU) are common to all 15 species. Compared to the mt native tRNA genes in lower plants, there are three tRNA genes (trnH-GUG, trnV-GGU, and trnW-GUA) integrated as part of large cp genomic fragments into mt genomes among angiosperms [26]. This indicates that frequent DNA transfer from cp to mt genomes occur as far back as the emergence of seed plants [41]. We detected two different tRNAs transfer events in seed plants. One is trnC-GCA transfer in monocots and the other involves two (trnD-GUC and trnQ-UUG) gene transfer events in dicots. Cp-derived tRNA genes replace their mt counterparts were identified in all sequenced angiosperms, even in gymnosperm C. m. mtDNA. But these replacement not occurred in Marchantia, Reclinomonas, Cyanidioschyzon, Nephroselmis, Chara, and Physcomitrella [43]. The mt-native tRNA gene (trnG-GCC) had all been lost in monocots and six mt-native genes (trnA-UGC, trnG-UCC, trnL-UAG, trnR-UCA, trnR-ACG, and trnT-GGU) are mostly lost in all angiosperms.

Table 4. Repeat pairs predicted based on REPuter in B. hygrometrica mt genome.

| Repeat length(bp) | Start Position | Match direction* | Repeat length(bp) | Start Position | E-value |
|-------------------|----------------|------------------|-------------------|----------------|---------|
| 1474              | 51709          | P                | 1474              | 232397         | 0.00E+00 |
| 843               | 42273          | P                | 843               | 108092         | 0.00E+00 |
| 222               | 153742         | F                | 222               | 405380         | 3.56E-118|
| 202               | 44767          | F                | 202               | 395750         | 1.77E-111|
| 180               | 449174         | F                | 180               | 504383         | 4.53E-93 |
| 165               | 156474         | P                | 165               | 272697         | 4.08E-84 |
| 131               | 25864          | P                | 131               | 291976         | 3.89E-66 |
| 119               | 449235         | F                | 119               | 504499         | 5.92E-59 |
| 116               | 42870          | F                | 116               | 246903         | 3.70E-57 |
| 116               | 108222         | F                | 116               | 246903         | 3.70E-57 |
| 109               | 300367         | F                | 109               | 308910         | 1.74E-55 |
| 120               | 403184         | P                | 120               | 496901         | 3.15E-55 |
| 102               | 126580         | P                | 102               | 462677         | 1.32E-44 |
| 101               | 521            | F                | 101               | 173834         | 5.13E-44 |

Note: the 3 recombination of repeat pairs for mt genomes are shown in bold.

*F and P stand for forward and palindromic matches, respectively.

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Table 5. The segments and organization of multipartite structures in B. hygrometrica mt genome.

| Mt segment | Start position | End position | Length (bp) | Exon of cross-strand gene |
|------------|----------------|--------------|-------------|---------------------------|
| A          | 43116          | 51708        | 8592        | nad1 e4                   |
| B          | 53183          | 108091       | 54908       | nad2 e3-5; nad5 e1        |
| C          | 108935         | 153741       | 44806       |                           |
| D          | 153964         | 232396       | 78432       | nad2 e1-2; nad5 e2-3; nad1 e2-3 |
| E          | 233871         | 405379       | 171508      | nad5 e4-5; sdh3 e2        |
| F          | 405602         | 42272        | 147189      | sdh3 e1; nad1 e1          |

Molecule | Organization | Cross-strand gene |
|----------|--------------|-------------------|
| MC       | ABCDEF       | nad1              |
| IOS1     | BACDEFG      | nad2; nad5        |
| IOS2     | BEDACF       | nad2; nad5        |
| IOS3     | ADEBCF       | nad1; nad2; nad5; sdh3 |
| IOS4     | ADCBEF       | nad1; nad5        |

Note: segments or exons in negative strand are underlined.

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Figure 3. Production of various molecular structures from the MC molecule by intra-molecular recombination between 3 different repeat pairs. A, B, C, D, E, and F are 6 segments separated by repeat pairs. Underlined segments are the negative strand as compared to the MC molecule. Three repeat pairs are shown in different colors. The keys show repeat name, length, and matching direction. F and P stand for forward and palindromic matches, respectively.

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Gene gain and loss in plant organelle genome

Starting from Bh organellar genomes, we have analyzed in a systematic way representative cp and mt genomes of various lineages and our results provide information for a better understanding of organellar genome evolution and function. Sequence-based phylogenetic analysis clearly supports the conclusion that Bh is much close to V. vinifera. Structural dynamics of Bh mt genome suggest that the multipartite structures may have started during the evolution of seed plants [30]. However, mechanisms for rapid mt genome rearrangement and expansion among plant lineages remain enigmatic. Based on eleven known cp and mt genomes of different lineages, we showed a strong relationship between the changing organellar genomes among angiosperms, and some of the lineage-associated gene gain and loss may provide excellent markers for phylogenetic studies (Figure 5). For instance, there are 9 cp and 4 mt genes lost during the evolution from green algae to lower land plants. It seems that monocots have a faster rate of evolution than dicots in organellar genomes in our study, because 3 cp and 9 mt genes are lost in monocots and only 2 mt genes are lost in dicots. In addition, gene structures and positioning of cp and mt genomes are also very informative for the understanding of land plant evolution. In agreement with the results of several previous studies, most of the transferred angiosperm sequences from cp to mt genomes become degenerated and are regarded as junk sequences, whereas some of the cp-derived tRNAs are still functional in mt genomes [26,29,41]. As more plant organellar genome sequences become available, the evolution of plant organellar genomes will unveil its details and mechanisms.

Materials and Methods

Genome sequencing and assembly

We developed an efficient procedure for Bh organellar genome sequencing and assembly using whole genome data from 454 GS FLX sequencing platform [15]. Briefly, we collected fresh leaves and extracted genomic DNA for 454 GS FLX sequencing (see manuals of GS FLX Titanium for detail). In order to validate genome assembly and to make sure for the assembly of the master circle or MC, we construed two mate-pair libraries (2 × 50 bp) for Genome sequencing and assembly using whole genome data from 454 GS FLX sequencing platform [15]. Briefly, we collected fresh leaves and extracted genomic DNA for 454 GS FLX sequencing (see manuals of GS FLX Titanium for detail). In order to validate genome assembly and to make sure for the assembly of the master circle or MC, we construed two mate-pair libraries (2 × 50 bp) for SOLiD 4.0 sequencing platform with insert sizes of 1–2 kb and 3–4 kb by following the SOLiD Library Preparation Guide. The method for assembling organellar genome was based on correlation between contig read depth and copy number in the genome [44]. We first filtered cp reads from the raw data

Note: “<” or “>” shows exons located in positive or negative strands, respectively.

The number of “++” indicates genes that lack exon-intron structures.

Table 6. Structural comparison of nad1, nad2, and nad5 genes in plant mt genomes.

| Species             | nad1 | nad2 | nad5 |
|---------------------|------|------|------|
| Chara vulgaris      | -*   | +*   | +*   |
| Marchantia polymorpha| +*   | ++   | ++   |
| Megaceros aenigmaticus | ++++ | ++++ | ++++ |
| Cycas taitungensis  | ++++ | ++++ | ++++ |
| Triticum aestivium  | ++++ | ++++ | ++++ |
| Oryza sativa        | ++++ | ++++ | ++++ |
| Sorghum bicolor     | ++++ | ++++ | ++++ |
| Tripasum daeystoloides | ++++ | ++++ | ++++ |
| Zea mays            | ++++ | ++++ | ++++ |
| Beta vulgaris       | ++++ | ++++ | ++++ |
| Brassica napus      | ++++ | ++++ | ++++ |
| Arabidopsis thaliana| ++++ | ++++ | ++++ |
| Nicotiana tabacum   | ++++ | ++++ | ++++ |
| Vitis vinifera      | ++++ | ++++ | ++++ |
| Boea hygrometrica   | ++++ | ++++ | ++++ |

Table 7. Comparison of basic features among 15 mt genomes.

| Scientific Name | Size (bp) | GC (%) | Coding (%) | Repeats (%) | Protein-coding genes | tRNAs | rRNAs |
|-----------------|-----------|--------|------------|-------------|----------------------|-------|-------|
| Chara vulgaris  | 67,737    | 40.9   | 90.7       | 3.2         | 46                   | 27    | 3     |
| Marchantia polymorpha | 186,609  | 42.41  | 20.3       | 10.1        | 76                   | 29    | 3     |
| Megaceros aenigmaticus | 184,908  | 46.01  | 17.9       | 2.6         | 48                   | 18    | 3     |
| Cycas taitungensis | 414,903  | 46.92  | 10.1       | 15.1        | 39                   | 26    | 3     |
| Triticum aestivium | 452,528  | 44.35  | 8.6        | 10.1        | 39                   | 25    | 9     |
| Oryza sativa     | 490,520   | 43.85  | 11.1       | 28.8        | 53                   | 22    | 3     |
| Sorghum bicolor  | 468,628   | 43.73  | 6.69       | 13.1        | 32                   | 18    | 3     |
| Tripasum daeystoloides | 704,100  | 43.93  | 4.94       | 40.6        | 33                   | 18    | 3     |
| Zea mays         | 569,630   | 43.93  | 6.2        | 11.4        | 163                  | 29    | 4     |
| Beta vulgaris    | 368,801   | 43.86  | 10.3       | 12.5        | 140                  | 26    | 5     |
| Brassica napus   | 221,853   | 45.19  | 17.3       | 5.5         | 79                   | 17    | 3     |
| Arabidopsis thaliana | 366,924  | 44.77  | 10.6       | 7.8         | 117                  | 21    | 3     |
| Nicotiana tabacum| 430,597   | 44.96  | 9.9        | 10.8        | 156                  | 23    | 4     |
| Vitis vinifera   | 773,279   | 44.14  | 4.98       | 1.71        | 74                   | 31    | 3     |
| Boea hygrometrica| 510,519   | 43.27  | 12.19      | 1.45        | 33                   | 28    | 4     |

Note: Parts of the dataset are available from published data [53].

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according known plant cp genome sequences and then assembled the “clean” read into cp genome into the major segments: large-single-copy (SSC), small-single-copy (SSC) and inverted repeats (IRs) regions. The mt genome assembly is more complicated than that of cp genomes. We filtered the contigs including mt conserve genes (such as NADH dehydrogenase and succinate dehydrogenase) and removed the contamination of cp sequences. The gene-based method for assembling mt genome has been reported earlier [7]. Mapping all the SOLiD mate-pair reads to mt contigs with Bioscope, we obtained the major contig relationship map in the repeat regions to assemble the MC.

Genome annotation

The cp genome was annotated by using the program DOGMA (Dual Organellar GenoMe Annotator) [45] coupled with manual corrections for start and stop codons. Protein-coding genes are identified by using the plastid/bacterial genetic code. Codon usage is predicted by using CodonW (http://codonw.sourceforge.net/). We construct a custom-designed amino acid database for protein-coding genes and nucleotide databases for rRNA and tRNA genes, compiled from all previously annotated plant mt genomes available at the organelle genomic biology website at NCBI (http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/organelles.html). NCBI BlastX and BlastN searches of the mt genome against the databases allow us to find protein and RNA genes, respectively. All BlastN and BlastX searches are carried out by using the default settings with e-value 1e-10. Putative RNA editing sites are inferred to create proper start and stop codons as well as to remove internal stop codons. We also used tRNAscan-SE [46] to corroborate tRNA boundaries identified by BlastN. The annotated GenBank files of the cp and mt genomes of Bh are used to draw gene maps using OrganellarGenome DRAW tool (OGDRAW) [47]. The maps were then examined for further comparison of gene order and content.

Analyses on SNPs, repeats, and cp-derived sequences

We identified intra-specific SNPs in both cp and mt genomes. Using BioScope, we mapped two runs of SOLiD mate-pair reads to both cp and mt genomes (BioScope Software User Guide). We carried out repeat sequence analysis using the REPuter web-based interface (http://bibiserv.techfak.uni-bielefeld.de/reputer/) [48], including forward, palindromic, reverse and complemented repeats with a minimal length of 50 bp. Transposable elements and other repeated elements were mapped with RepeatMasker Web Server (http://www.repeatmasker.org/cgi-bin/WEBRepeat-
Figure 5. Phylogenetic distribution of gene gain and loss from chloroplast and mitochondrial genomes in plant lineages. The genes that are gained independently in different lineages are underlined. Genes of chloroplast and mitochondrial origins are indicated with green and red, respectively. Phylogenetic relationships of the plants are determined based on the conserved genes of chloroplast genomes described in Figure S2.

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Masker) running under the cross_match search engine. Cp-derived sequences are identified with BlastN search of mt genomes against Bh annotated cp genomes (Identity ≥80%, E-value ≤1e-5, and Length ≥50 bp). The cp-derived sequences were then aligned to all known plant mt genomes by using BlastN (Identity ≥80%, E-value ≤1e-5, and Coverage ≥50%). tRNAs transferred to the mt genome were identified by aligning to all tRNAs in the cp genome of the same species by using BlastN (Identity ≥80%, E-value ≤1e-5, and Coverage ≥50%).

Phylogenetic Analysis

We compare the Bh cp genome with other plant organellar genomes, and use the homologous protein-coding sequences to construct phylogenetic tree. Sixty-three cp protein sequences (psaA, psaB, psaC, psaI, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, rbcL, rpoA, rpoB, rpoC1, rpoC2, rpl12, rpl23, rpl22, rpl32, rps14, rps18, rps2, rps3, rps4, rps7, rps8, rps11, rps14, rps18, rpl2, rpl36, cemA, and ycf4) from 12 different organisms (Table S2) are aligned and concatenated into a dataset of 196,313 amino acids.

We align amino acid sequences from individual genes using MUSCLE v3.8.31 [49], remove ambiguously aligned regions in each alignment using GBLOCKS 0.91b [50], and concatenate the aligned sequences. We use maximum likelihood method and PhyML v3.0 [51] under Jones-Taylor-Thornton (JTT and gamma distribution of rates across sites with four categories) model of sequence evolution to construct phylogenetic trees. Confidence of branch points is estimated based on 100 bootstrap replications. We obtained the best tree after heuristic search with the help of Modelgenerator [52].

Accession Numbers

The GenBank accession numbers for the sequences mentioned in this article are as follows: Chara vulgaris, NC_008097 and NC_005255; Marchantia polymorpha, NC_001319 and NC_001660; Megaceros aenigmaticus, NC_012651; Cycas taitungensis, NC_009618 and NC_010303; Triticum aestivum, NC_002762 and NC_007579; Oryza sativa, NC_001320 and NC_011033; Sorghum bicolor, NC_008602 and NC_008360; Tribusacum dactyloides, NC_008362; Zea mays, NC_001666 and NC_007982; Beta vulgaris, NC_002511; Brassica napus, NC_008285; Arabidopsis thaliana, NC_000932 and NC_001284; Nicotiana tabacum, NC_001689 and NC_006581; Vitis vinifera, NC_007957 and NC_012119; Olea europaea, NC_013707; Boea hygrometrica, JN107811 and JN107812.

Supporting Information

Figure S1 Chloroplast genomic alignment between Boea hygrometrica and Olea europaea. Alignments with direct match are shown in red and reverse match are shown in blue. Obviously, alignments of two IR regions are indicated in blue.

(DOC)

Figure S2 Molecular phylogenetic analysis based on Maximum Likelihood method by using JTT matrix-based model (Chara vulgaris as outgroup). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of selected plants. The dataset is composed of sixty-three conserved cp proteins concatenated to 14,894 positions from 12 plant cp
organisms. Nodes receive over 80% bootstrap replicates are indicated at phylogenetic positions and 

**Figure S3** Mitochondrial genomic alignment between *Boea hygrometrica* and *Vitis vinifera*. Alignments with direct match are shown in red and reverse match are shown in blue.

**Figure S4** The GC distribution between new and old cp-derived sequences in mitochondrial genome of *Boea hygrometrica*. Hits stand for the results of homologous alignments with other known mitochondrial genomes.

**Table S1** Table Codon usage table of the chloroplast genes.

**Table S2** cp and mt genomes of 15 plants comparison in this study.

**Table S3** Codon usage table of the mitochondrial genomes.

**Table S4** Gene content and characteristic comparison of 15 mt genomes.

**Table S5** Intravarietal single nucleotide polymorphisms (intraSNPs) in mt genome.

**Table S6** Gene content and characteristic comparison of 12 cp genomes.

**Table S7** Blast result of chloroplast-derived DNA segment in mitochondrial genome of *Boea hygrometrica*.

**Table S8** tRNAs comparison of both cp and mt genomes among 11 pant.

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**Author Contributions**

Conceived and designed the experiments: JY XD SH. Performed the experiments: XW XZ. Analyzed the data: TZ YF. Contributed reagents/materials/analysis tools: XD. Wrote the paper: TZ YF.

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