Chloroplast DNA Structural Variation, Phylogeny, and Age of Divergence among Diploid Cotton Species

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Abstract

The cotton genus (Gossypium spp.) contains 8 monophyletic diploid genome groups (A, B, C, D, E, F, G, K) and a single allotetraploid clade (AD). To gain insight into the phylogeny of Gossypium and molecular evolution of the chloroplast genome in this group, we performed a comparative analysis of 19 Gossypium chloroplast genomes, six reported here for the first time. Nucleotide distance in non-coding regions was about three times that of coding regions. As expected, distances were smaller within than among genome groups. Phylogenetic topologies based on nucleotide and indel data support for the resolution of the 8 genome groups into 6 clades. Phylogenetic analysis of indel distribution among the 19 genomes demonstrates contrasting evolutionary dynamics in different clades, with a parallel genome downsizing in two genome groups and a biased accumulation of insertions in the clade containing the cultivated cottons leading to large (for Gossypium) chloroplast genomes. Divergence time estimates derived from the cpDNA sequence suggest that the major diploid clades had diverged approximately 10 to 11 million years ago. The complete nucleotide sequences of 6 cpDNA genomes are provided, offering a resource for cytonuclear studies in Gossypium.

Introduction

Cotton is the most important fiber crop plant in the world. Four species were domesticated and remain under cultivation today, the New World allotetraploids G. hirsutum and G. barbadense (2n = 52), and the Old World diploids G. arboreum and G. herbaceum (2n = 26) [1–2]. The primary cultivated species is Upland cotton (G. hirsutum L.), which accounts for more than 90% of global cotton fiber output. Gossypium includes 52 species, including 6
allotetraploid species and 46 diploids [2]. The nascent allopolyploid spread throughout the American tropics and subtropics, diverging into at least six species, namely, G. hirsutum L. (AD1), G. barbadense L. (AD2), G. tomentosum Nuttalex Seemann (AD3), G. mustelinum Miersex Watt (AD4), G. Darwinii Watt (AD5), and G. ekmanianum (AD6) [1–2]. The diploid Gossypium species have been shown to comprise 8 monophyletic genome groups, A, B, C, D, E, F, G and K group [1,3–4].

Because of its economic importance and its value as a model for evolutionary studies, there is a rich history of molecular phylogenetic work in Gossypium (reviewed in [1–2]). These studies, although based mostly on a set of nuclear genes [5], or chloroplast DNA restriction sites [6], indicate low levels of divergence among species and even clades, and suggest a rapid, early diversification of the primary cotton lineages, such that many of the branch resolutions remain in question. Divergence among diploid clades was estimated to have occurred rapidly following an initial split around 6.8 MYA [5,7].

With the advent and rapid development of next-generation sequencing technologies [8–10], cotton genomics research has progressed rapidly in the last several years, such that nuclear genome sequences have now been published for model diploids D-genome [11–12], A-genome [13] and for the allopolyploids G. hirsutum [14–15], G. barbadense [16–17]. In addition, a large number of organelle genome sequences have been published [18–22]. Chloroplast DNA sequences have long been a major data source for plant phylogenetic inference [23–25], with both the relatively conserved coding and more highly diverged non-coding regions being useful at different levels [25–26]. Because of its abundance and relatively uniform size and organization [18–20,27], complete chloroplast (cp) genome sequences from Gossypium should be readily alignable and hence useful for phylogenetic analysis. As an initial step in this direction, Xu et al., [20] used complete nucleotide sequences of 12 cp genomes from four diploids and eight tetraploids to analyze the origin and evolution of allotetraploids.

To provide insight into divergence, phylogenetic relationships and cp genome structural variation across the entire genus, we performed a comparative analysis of 19 (13 unpublished) Gossypium cp genomes (2 from tetraploid species and 17 from diploids), including those from 6 diploids not previously sequenced. Phylogenetic analyses were performed using both nucleotide and indel data. Our comparative analyses of these 19 genomes provided detailed information on divergence within and between clades, including the age of divergence among species.

Materials and Methods

Plant materials and chloroplast isolation

Fresh leaves from six species representing four genome groups in Gossypium were collected for chloroplast extraction and sequencing. All materials were obtained from the National Wild Cotton Nursery, in Sanya, China, which were issued the permission by the authority: Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang, Henan, China. Chloroplast DNA was prepared following a previous published protocol [20,28]. Illumina libraries with paired-end, 90bp read, were generated using Illumina sequencing method on HiSeq2000 at Beijing Genomics Institute (BGI).

Chloroplast assembly and annotation

Raw reads were filtered using Bowtie2 [29] for possible nuclear and/or mitochondrial contamination by extracting only those reads that showed similarity with the published G. hirsutum (AD1) cp genome sequence. Chloroplast reads were subsequently assembled using a combination of Phrap [30] and Velvet [31] (hash length = 21, cov_cutoff = 30). Each inverted repeat (IR) region was specifically targeted using two long PCR reaction (each producing ~13 kb
fragments), whose products were purified for sequencing separately with Illumina. Chloroplast genes were annotated using an online DOGMA tool [32] using *G. hirsutum* (AD1) as a reference sequence. The sequences of identified tRNA genes were obtained using both DOGMA and tRNAscan-SE [33]. Genome maps were drawn with OGDRAW [34].

### Estimation of evolutionary divergence between sequences

The whole genome sequences were aligned with genome specific aligner: Alignathon [35]. Sequence alignments for each coding, intronic, and intergenic spacer regions were carried out by different alignment methods combining CLUSTALW [36], MUSCLE [37] and MAFFT [38] to address the alignment reliability, which demonstrate that using different alignment methods does not change the main results. The number of indels and substitutions were calculated by a custom Perl script. P-distances for any two genomes, genes, or non-coding regions were calculated with MEGA5.05 [39].

### Phylogenetic analyses and divergence time of *Gossypium* diploid clades

The most closely related and publicly available chloroplast sequence was determined via BLAST [40] against publicly available databases using *Gossypium hirsutum* as the query (outgroup = *Theobroma cacao*, Malvales, GI:342240206). Initially, a DNA substitution model for our data sets was selected using jModelTest version 2.1.4 [41] and the Akaike Information Criterion (AIC). Among the 88 models tested, the general time reversible (GTR) including rate variation among sites (+ G) and invariable sites (+ I) (= GTR + G + I) model was chosen as the best fit to our data sets, followed by the Transversional model + G + I and GTR + I models. Maximum likelihood (ML) trees were generated for all phylogenetic comparisons using either in MEGA5.05 [39], PhyML 3.0 [42] or RAxML [43], all using a General Time Reversible (GTR) model and a rate of Gamma distributed with invariant site (G+I) Bootstrap support (BS) values for individual clades were calculated by running 1,000 bootstrap replicates of the data. Gaps/missing data were evaluated both as complete deletions and as missing data, both of which gave the same topology in each case. Bayesian analysis of the ML trees was conducted by MrBayes [44] under GTR gamma with the following parameters: 3 runs with four chains for 10 million generations and using a burn-in fraction of 25%.

To evaluate phylogenetic signal present in the indel data, we coded gaps using modified complex coding [45] as implemented in SeqState [46]. The indel data was evaluated both separately and in conjunction with the substitution data using RAxML [43]. Again, a GTR model was invoked for the nucleotide substitution partition, while the MULTICAT model (as implemented in RAxML) was invoked for both the standalone and state-data partition of the combined analysis, and both trees were generated using 1000 alternative runs on distinct starting trees and rapid bootstrapping with consensus.

Divergence time was estimated for the 78 concatenated chloroplast protein-coding exons dataset using PhyloBayes 3.3f [47], using the autocorrelated Lognormal relaxed-clock mode [48] and the tree generated from the above dataset and CAT+GTR model. For the molecular clock analysis, a birth-death prior on divergence time and fossil calibrations with soft bounds were used, and we selected three fossil calibrations for *Gossypium* vs *Theobroma*, ancestors shared between A and D subgenomes and the split of A and AD genomes (S8 Table). The range of fossil age was collected from relevant literature on fossils [49] and a recent molecular calculation of the *Gossypium* clades [50–51]. We allocated 10% of the probability mass to lie outside each calibration interval. All calculations were performed by running 10,000 generations and sampled every 25 generations (after burn-in of 2,500 generations).
Results and Discussion

Size, content and structure of six new Gossypium chloroplast genomes

Gossypium chloroplast (cp) genomes from six diploid species were newly sequenced for this study, representing four of the eight cotton diploid genome groups (G. robinsonii C2, G. somalense E2, G. capitis-viridis B3, G. areysianum E3, G. populifolium K; GenBank accessions JN019791 to JN019795 and KP221924, respectively). These cp genomes (Table 1) show high identity and similarity in gene content and genome organization with each other and with previously published cotton cp genomes [20], with only minor differences in genome size and composition. The length of these six genomes range in size by only 521 bp, from the largest (G. robinsonii, C2, 159,726 bp) to the smallest (G. incanum, E4, 159,205 bp), with most of the size differences occurring in the large single-copy (LSC) region (Table 1 and Fig 1).

Notably, all are smaller than the previously published G. hirsutum cp genome [18] by more than 500 bp. All six cp genomes contain 112 genes, including 78 protein-coding genes, 4 ribosomal RNA genes and 30 tRNA genes, and 17 duplicated genes located in IR region (Fig 1, S2 Table). Both the length of the coding regions and the overall GC content vary minimally as well (<1% each; Table 1).

Nucleotide divergence among cp genomes of 19 Gossypium species

In addition to the six newly presented cp genomes, we also analyzed 13 previously sequenced cp genomes, including representatives of the A, B, C, D, E, F and G genome groups (S1 Table). Not surprisingly, the lowest levels of nucleotide divergence among these 19 species were detected within genome groups, some of which show remarkable uniformity. Within the E-genome, for example, the comparison of G. somalense (E2) and G. areysianum (E3) yielded only a single-nucleotide change in a protein-coding exon and a total of 10 nucleotide substitutions across all non-coding regions, a nucleotide distance of 0.000075; the distance within the A-genome was similarly low (0.000074; S3 Table). Low levels of divergence may not be uniform across genome group, however. For example, the distance between G. incanum (E4) and G. stocksii (E1) (0.000668) was about 8-fold higher than that of G. somalense (E2) and G. areysianum (E3) (S3 Table), making it larger than that found within the B-genome, 0.000284 for G. anomalum (B1) and G. capitis-viridis (B3) and smaller than D-genome, 0.001283 for G. raimondii (D5) and G. gossypioides (D6) that was, lower than for the other two comparisons, as expected based on previous cpDNA analyses [6]. All intra-genomic comparisons are performed. Interestingly, among the Australian cottons, G. sturtianum (C1) was more similar to G. bickii (G4) than to G. robinsonii (C2) and G. populifolium (K), supporting the proposal [52–53] that G. bickii has an
introgressive ancestry with a maternal donor from the *G. sturtianum* lineage. As expected, the divergence among genome groups was typically an order of magnitude larger, ranging from 0.003593 to 0.009612. The pairwise comparisons within A+AD, B, *G. sturtianum* vs *G. bickii* (C1 vs G1), D and E groups showed the divergence values less than 0.26% because of their highly close relationship during evolution. In addition, the distances, ranging from 0.26% to 0.53%, contains pairwise comparisons between close *Gossypium* groups, for example, distances between A+AD and F groups, and some comparisons within C + G + K groups (*G. populifolium* vs *G. robinsonii*, *G. populifolium* vs *G. bickii*). However, the distances more than 0.53% contained species compared that own a really distant relationship and come from different phylogenetic groups, such as the largest pairwise comparisons distances between C + G + K groups and other five groups. Interestingly, and consistent with the first published phylogenetic data using

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**Fig 1. A consensus map of six newly sequenced *Gossypium* chloroplast genomes.** Genes on the outside of the outer circle are transcribed in the clockwise direction and genes on the inside of the outer circle are transcribed in the counterclockwise direction. The inner circle delineates the inverted repeat regions (IRa and IRb), the small single-copy region (SSC), and the large single-copy region (LSC). Functional categories of genes are color-coded.

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Chloroplast Genome Evolution of Diploid Gossypium

The phylogeny of Gossypium has been previously evaluated [5] using limited plastid and nuclear data. In the most recent analysis using both chloroplast and nuclear data, inconsistencies in the basal branching patterns of the genus were both observed and well-supported. Statistical analyses of incongruence provided greater support for the nuclear tree topology [5], as opposed to the cp-resolved topology. To revisit this inconsistency, we inferred phylogenetic relationships among the eight Gossypium genome groups using a concatenated analysis of all 78 chloroplast protein-coding genes and Theobroma cacao as an outgroup. The topology of the resulting tree (Fig 2) was congruent with that previously reported [5], which evaluated only four cp loci, two genes and two non-coding regions. To explore this further, we performed both a separate analysis for each of the 78 genes as well as an analysis of the molecule as a whole. Only one individual gene, ndhf, showed the same topology as the concatenated analysis, an unsurprising result given the low amount if divergence within each gene and hence the lack of resolution for many gene-clade combinations. When the entire cp genome was considered (gaps excluded), support for the topology increased, with a minor discrepancy in the placement of G. populifolium (K genome; S1 Fig). These observations are perhaps unsurprising, as the cp genome as a whole is subject to the same evolutionary influences as its smaller components (unlike the nuclear genome), yet it is notable that the results from the analysis of the entire genome are consistent with those previously reported for few loci (if better supported), which suggests that, at the phylogenetic level evaluated here, a small fraction of the chloroplast can adequately serve to represent the evolutionary history of the whole [5].
The resolution of intraclade relationships, however, was largely reliant on the substantial sequence information afforded by whole cp genome sequencing. Interestingly enough, the phylogenetic analyses conducted here indicate that this may be true for some intraclade relationships, which were far less distinct than others in the same genome group. In the E-genome, for example, of the four species evaluated, two (\textit{G. somalense} and \textit{G. areysianum}) were nearly identical in their chloroplast genomes, whereas the other two E-genome species (\textit{G. stocksii} and \textit{G. incanum}) species in E clade here had more distinct sequences. This high similarity was also present for \textit{G. africanum} (A1-a) and \textit{G. arboreum} (A2), which, as previously noted [4,20,54] are distinguishable morphologically, yet may still be in the initial stages of species differentiation (as indicated by the low level of sequence divergence). This indicates that, while limited sampling of the chloroplast molecule may be sufficient for interclade phylogenetics, more extensive sampling is required for adequate resolution at close specific relationships.

Structural variation among cotton chloroplast genomes

Insertion-deletion polymorphisms (indels) may be another useful source of phylogenetically informative characters [55–57]. Phylogenetic analysis of indel patterns has been broadly applied, from discerning interfamilial relationships among mammals [58], to reconstructing generic level plant phylogenies [56], to species recognition issues in \textit{Gossypium} [59]. The most recent phylogenetic analysis of relationships among diploid cotton genome groups [5], also used indel polymorphisms as a line of evidence; however, this dataset was restricted to few indels derived from both the nuclear and chloroplast genomes, in roughly equal proportions. To revisit this issue, we scored and evaluated the pattern for 1420 indels in the 19 \textit{Gossypium} and \textit{T. cacao} cpDNA protein-coding and non protein-coding regions (S6 Table).
IR junction polymorphisms are present, yet phylogenetically uninformative. Although the cp genomes studied here are extremely similar in structure, size, gene number and gene order, numerous small indels differentiate the genomes even among closely related species. Of the 1420 indels that differentiate these cp genomes, 69 (5 in coding and 64 in non-coding regions) are located in the IR region (S6 Table). Given that the IR region is the only place in the cp genome that recombination is expected, we analyzed the junction between these regions and the single copy regions separately.

When analyzed using *Theobroma cacao* as an outgroup, three IR junction types (I, II, and III) were detected (Fig 3), which differ in their placement of *rps19* and *trnH* at the IR-LSC junction site. In assigning an IR junction type (S1 Fig) to each species cp genome, it becomes readily apparent that, while there may be some phylogenetic signal in these IR junction polymorphisms, there must also exist a certain amount of fluidity in their expansions/contraction. For example, of the four E-genome species sampled, three belong to Type II, whereas the other belongs to Type I; the three D-genome species evaluated were likewise split between Types I, II and III. This is indicative of evolutionary fluidity of IR expansion/contraction within genome groups; when plotted against the phylogeny (S1 Fig), it becomes clear that the pattern of IR junction types observed here represents as many as 6 independent switches, independent of whether we invoke a sequential, two-step expansion [60] or if we allow the IR junction types to switch equally among the three. The potentially labile nature of the IR region is further underscored by the observations that: (1) the IR region in cotton has expanded (relative to *T. cacao*) to include part of *ycf1*, (2) the *T. cacao* IR region has expanded (relative to *Gossypium*) to include part of *ndhF*. Further analyses involving many related species and genera are necessary to understand the evolution of the IR junction.

**Phylogenetic signal in chloroplast indels supports the chloroplast phylogeny, is incongruent with nuclear data.** The utility of indels for phylogenetic purposes has been discussed, leading to the general conclusion that indel polymorphisms can be informative characters with low levels of homoplasy [57], often supporting or refining the inferences determined through substitution data [55–58]. The use of indel data for the most recent analysis of interclade relationships in *Gossypium* [5], however, presented a different scenario. That is, while the
chloroplast loci evaluated in that study resolved relationships that were also resolved here (Fig 2), the indel data presented there (Cronn 2002, Fig 4C) suggests an entirely different relationship among genome groups where the D-genome represents the basal-most branchpoint and the African B-genome is more closely related to the F-A genome clade than to the Australian species. This latter phylogeny has been the most widely accepted [2,4], in part due to the statistical analysis [5]; however, challenges to the branching order have been cited [54].

To evaluate possible discrepancies between indel and substitution-derived data, we used maximum likelihood to reconstruct phylogenetic trees using both indel only data, and concatenated indel + substitution information. Again, both the indel-derived data and the indel + substitution data recovered a tree either identical (indel + substitution) or nearly identical (indel only) to that recovered by substitution data alone. This is in constrast to the indel data presented in Cronn et al. [5], but perhaps not surprisingly so. The indel data previously used was a combination of nuclear and chloroplast derived indels, in a roughly 50–50 proportion, with the resulting tree more closely resembling the nuclear gene tree than the chloroplast gene tree. That the nuclear and chloroplast data resolve a different, contrasting tree from the chloroplast indel data alone indicates a possible incongruence between the nuclear and chloroplast genomes of *Gossypium*. This may be partially explained by a hypothesis tentatively put forth by Cronn and Wendel over 10 years ago [53], which discussed the propensity for cotton species to experience cryptic introgressions among diverse species, often over great distances.

**Fig 4.** Inferred gains and losses of chloroplast genomic features during the evolution of *Gossypium* diploid species. Genomic characters were mapped on the tree. Gains and losses of characters are indicated by solid and hollow symbols, respectively. *: the indels length aligned with *G. hirsutum*. The number in parentheses represents the length of indels.

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Although cotton species typically exist as small, isolated populations, the genus has a remarkable tendency for long-distance dispersal and introgression among species that seem unlikely to geographically meet. This propensity for long-distance dispersal and introgression is well-discussed [53]; however, the observations most applicable to the present are those of multiple chloroplast introgressions among species. As mentioned above, the close inter-clade relationship between \textit{G. sturtianum} and \textit{G. bickii} can be attributed to introgression of a \textit{G. sturtianum}-like chloroplast into the \textit{G. bickii}, and a similar observation can be made between \textit{G. raimondii} and \textit{G. gossypioides}. More ancient introgression events can be difficult to readily pinpoint; however, the incongruence between data types (nuclear versus chloroplast), as well as morphological characters atypical of the genome group, suggest an ancient introgression between a B-like ancestor with an ancestor leading to the Australian (CGK) genome groups. Further, extensive nuclear sampling will be required to determine if the incongruence between these datasets supports these interclade introgression events.

**Phylogenetic placement of indels and implications for genome size.** Indel accumulation was primarily restricted to non-coding regions (S7 Table), which contained over 96% of the indels scored (S6 Table). Of the 1,420 indels that differentiate these cp genomes, only 55 occurred in gene regions, with the length of these rare indels typically occurring as a multiple of three (to preserve protein coding capacity). Interestingly, and as observed in other species [61–62], the terminal codon of \textit{rbcL} has undergone considerable variation among the species analyzed. Also notable are the multiple events that occurred in some \textit{ycf} gene family members, which is identical to previous results [63]. Indels in the non-coding regions were far more frequent and variable in size (S6 Table; S7 Table), ranging in length from 1 to 272 bp, with lengths 1, 5, and 6 bp occurring most frequently, an observation consistent with an earlier report [20]. To evaluate the rate of indel formation among related genome groups, we phylogenetically mapped the phylogenetic polarizable insertions and deletions onto the \textit{Gossypium} phylogeny produced here (Fig 4). As is perhaps expected by the types of mutational processes expected in the chloroplast (e.g. slipstrand mispairing), for any given branch, there were typically nearly equivalent numbers of insertions and deletions; however, two notable exceptions exist. In both the B- and E-genome lineages, the number of deletions was greatly increased and greatly outnumbered the insertions (Fig 4). For the B-genome, but not for the E-genome, this created a relative increase in the number of indel events (as compared to sister branches). For the B-genome, there were a total of 34 indels polarized (compared to 15 for the Australian CGK branch), whereas the number of polarized events in the E-genome lineage was similar to that of the lineages leading to F and A+AD (31 in E-genome, versus 34 and 37 in F and A+AD, respectively) (Fig 4).

Genome size evolution itself is a dynamic process involving counterbalancing mechanisms whose actions vary across lineages and over time [7]. While many of these mechanisms are more active and/or restricted to the nuclear and plant mitochondrial genomes, cpDNA intergenic regions are known to often exhibit substantial insertion/deletion (indel) polymorphism within and among plant species [64–67]. This propensity for deletion may, in part, explain the relatively small size of the B- and E-genome chloroplast genomes.

**Divergence times of major clades in \textit{Gossypium}** We used the data gathered here to reevaluate the divergence time for each of the species in this study, using \textit{T. cacao} as an outgroup and relaxed molecular clock analyses were performed for our dataset using three calibration points (S8 Table). Prior analyses have put the divergence time for \textit{Theobroma-Gossypium} at least 60 million years ago (mya) [49], A-genome diploids native to Africa and Mexican D-genome diploids diverged ~ 5–10 mya [51] and the formation
of the allopolyploid at $1\text{–}2 \text{ mya}$ [50]. The divergence time between each species represented was calculated (Fig 5) with variance around the age estimates (S2 Fig). The divergence time between *Gossypium* and *T. cacao* was estimated at $78.5 (56.8–130.8) \text{ mya}$, which is consistent with earlier estimates [49]. While we cannot estimate the formation of the genus itself adequately (without access to a more closely related outgroup), the earliest divergence (between the B+C+G+K-genome clade and the remainder of the genus) was estimated as occurring approximately $9.8 (6.7–13.6) \text{ mya}$, similar to the estimates of the age of the genus [1–2] and consistent with the notion of rapid radiation. Also consistent with prior analyses, which recovered short internodes for most branches, the majority of intraclade divergences fell in the range of $7\text{–}9 \text{ mya}$. Interestingly, and perhaps demonstrating yet again the peculiarities present in the B-genome, while this clade groups strongly with the Australian clade (C+G+K) phylogenetically, the estimate of divergence time between the B-genome and the remainder of the genus is typically $7.9 (5.0–10.0) \text{ mya}$ (Fig 5 and S2 Fig), which is similar to the radiation times calculated for the rapid radiation present in all other cotton clades, after divergence from the Australian cottons.

### Conclusions

Whole chloroplast genome sequencing has been on the rise [68–73], providing an abundance of information both for phylogenetic utility, as well as cytonuclear interactions and accommodation. Here, we report the generation of 6 new *Gossypium* chloroplast genomes, and compare these to 13 other cotton chloroplast genomes to evaluate the evolution of the chloroplast as a whole over the entire genus. The data presented here are congruent with prior chloroplast-based phylogenetic analyses, indicating that, in many cases, sequencing of few chloroplast loci...
may be just as effective as sequencing the entire molecule. The analyses here also revisit a perhaps underappreciated feature of cotton evolutionary history: the propensity for hybridization and introgression on different time scales and among species whose geographic distance renders the occurrence remarkable. The continued incongruence between the nuclear and chloroplast genomes warrants further exploration through increased nuclear representation. Finally, the sequences presented here represent a valuable resource for cytonuclear coevolution in the genus Gossypium, as well as future organelle-based studies.

Supporting Information
S1 Fig. Phylogenetic relationships of the nineteen species of Gossypium constructed by maximum likelihood based on the whole chloroplast in its entirety (excluding gaps), with IR junction type listed on the right. Numbers above node are the branch length. (Bayesian tree is similar, and therefore not displayed).
(TIF)
S2 Fig. Chronogram showing Gossypium phylogeny and divergence time variance around the age estimates with T. cacao as an outgroup. Consensus tree presenting divergence dates produces by the PhyloBayes analysis of the 78 concatenated chloroplast protein-coding exons dataset using three fossil calibration points (S8 Table), the autocorrelated Lognormal relaxed-clock mode, the site-heterogeneous mixture CAT+GTR substitution model, and soft bound 10%. A geological time scale is shown at the bottom. The arrows represent for three calibration points.
(TIF)
S1 Table. General features of other Gossypium cp genomes cited in this paper.
(DOCX)
S2 Table. Genes encoded by Gossypium chloroplast genomes. Note: * , ** gene containing a single or two introns, respectively. §, The gene has two copies.
(DOCX)
S3 Table. The overall nucleotide distance (coding + non-coding with an IR excluded, excluding indels) among the 19 cotton species. Note: A1 = G. herbaceum, A1-a = G. africanaum, A2 = G. arboreum, AD1 = G. hirsutum, AD2 = G. barbadense, F1 = G. longicalyx, E1 = G. stocksii, E2 = G. somalense, E3 = G. aresyianum, E4 = G. incanum, D1 = G. thurberi, D5 = G. raimondii, D6 = G. gossypioides, B1 = G. anomalous, B3 = G. capitis-viridis, C1 = G. sturtianum, C2 = G. robinsonii, G1 = G. bickii, K = G. populifolium.
(DOCX)
S4 Table. The nucleotide distance between 19 Gossypium species. Note: The upper triangle shows the number of substitutions in protein-coding exon regions and the lower triangle shows the number of substitutions in non-coding regions. The repeated sequences, naturally, sometimes complicate the alignment process, so we removed an IR region from all chloroplast genomes aligned here. A1 = G. herbaceum, A1-a = G. africanaum, A2 = G. arboreum, AD1 = G. hirsutum, AD2 = G. barbadense, F1 = G. longicalyx, E1 = G. stocksii, E2 = G. somalense, E3 = G. aresyianum, E4 = G. incanum, D1 = G. thurberi, D5 = G. raimondii, D6 = G. gossypioides, B1 = G. anomalous, B3 = G. capitis-viridis, C1 = G. sturtianum, C2 = G. robinsonii, G1 = G. bickii, K = G. populifolium.
(DOCX)
S5 Table. Mean nucleotide distances of protein-coding exons and non-coding regions among 19 Gossypium species. Note: yellow colors indicate the minimum distances, green
colors indicate the maximum distance and NA indicates that there exists overlap sequences between two genes. clpP and ycf3 both contain two introns, while we merged them into one. (XLSX)

S6 Table. Indel length description and Indels data matrix for phylogenetic analysis. Note: Indels were coded as unordered characters with binary states (in the case of simple presence/absence indels) or multistate characters (in the case of indels with variable length but one identical 5’ or 3’ end). (XLSX)

S7 Table. Indels that discriminate Gossypium cp genomes. Note: The upper triangle shows the number of indels in protein-coding exon regions and the lower triangle shows the number of indels in non-coding regions. The repeated sequences, naturally, sometimes complicate the alignment process, so we excluded the IR region from the analysis. A1 = G. herbaceum, A1-a = G. africanum, A2 = G. arboreum, AD1 = G. hirsutum, AD2 = G. barbadense, F1 = G. longicalyx, E1 = G. stocksi, E2 = G. somalense, E3 = G. aresyianum, E4 = G. incanum, D1 = G. thurberi, D2 = G. raimondii, D3 = G. gossypioides, B1 = G. anomalum, B3 = G. capitis-viridis, C1 = G. sturtianum, C2 = G. robinsonii, G1 = G. bickii, K = G. populifolium. (DOCX)

S8 Table. Calibrations with fossil taxonomic information, fossil age and references. (DOCX)

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Author Contributions
Conceived and designed the experiments: JH. Performed the experiments: ZC KF PL YW FL QX MS ZZ XC XW. Analyzed the data: ZC CEG PL. Contributed reagents/materials/analysis tools: YW KW JH. Wrote the paper: ZC CEG JFW KW JH.

References
1. Wendel JF, Cronn RC. Polyploidy and the evolutionary history of cotton. Adv Agron. 2003; 78:139–186. doi: 10.1016/s0065-2113(02)78004-8
2. Wendel JF, Grover CE. Taxonomy and evolution of the cotton genus. In: Fang D and Percy R, editors. Cotton, Agronomy. Madison, WI: Monograph 24, ASA-CSSA-SSSA; 2015. in press.
3. Grover CE, Kim H, Wing RA, Paterson AH, Wendel JF. Microcolinearity and genome evolution in the AdhA region of diploid and polyploid cotton (Gossypium). Plant J. 2007; 50(6):995–1006. doi: 10.1111/j.1365-313X.2007.03102.x PMID: 17461788.
4. Wendel J, Brubaker C, Seelanan T. The origin and evolution of Gossypium. In: Physiology of Cotton. Edited by Stewart J, Oosterhuis D, Heltholt J, Mauney J: Springer Netherlands. 2010;1–18.
5. Cronn RC, Small RL, Haselkorn T, Wendel JF. Rapid diversification of the cotton genus (Gossypium: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. Am J Bot. 2002; 89(4):707–725. doi: 10.3732/ajb.89.4.707 PMID: 21665671.
6. Wendel JF, Albert VA. Phylogenetics of the cotton genus (Gossypium): character-state weighted parsimony analysis of chloroplast-DNA restriction site data and its systematic and biogeographic implications. Syst Bot. 1992; 17:115–143.
7. Grover CE, Yu Y, Wing RA, Paterson AH, Wendel JF. A phylogenetic analysis of indel dynamics in the cotton genus. Mol Biol Evol. 2008; 25(7):1415–1428. doi: 10.1093/molbev/msn085 PMID: 18400789.
8. Hudson ME. Sequencing breakthroughs for genomic ecology and evolutionary biology. Mol Ecol Resour. 2008; 8(1):3–17. doi: 10.1111/j.1471-8286.2007.02019.x PMID: 21585713.
9. Shendure J, Ji H. Next-generation DNA sequencing. Nat Biotechnol. 2008; 26(10):1135–1145. doi: 10.1038/nbt1486 PMID: 18846087.

10. Ansorge WJ. Next-generation DNA sequencing techniques. N Biotechnol. 2009; 25(4):195–203. doi: 10.1016/j.nbt.2008.12.009 PMID: 19429539.

11. Wang K, Wang Z, Li F, Ye W, Wang J, Song G, et al. The draft genome of a diploid cotton Gossypium raimondii. Nat Genet. 2012; 44(10):1098–1103. doi: 10.1038/ng.2371 PMID: 22922876.

12. Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, et al. Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibres. Nature. 2012; 492(7429):423–427. doi: 10.1038/nature11798 PMID: 23257886.

13. Li F, Fan G, Wang K, Sun F, Yuan Y, Song G, et al. Genome sequence of the cultivated cotton Gossypium arboreum. Nat Genet. 2014; 46(6):567–572. doi: 10.1038/ng.2987 PMID: 24836287.

14. Li F, Fan G, Lu C, Xiao G, Zou C, Kohel RJ, et al. Genome sequence of cultivated Upland cotton (Gossypium hirsutum TM-1) provides insights into genome evolution. Nat Biotechnol. 2015; 33(5):524–530. doi: 10.1038/nbt.3208 PMID: 25893780.

15. Zhang T, Hu Y, Jiang W, Fang L, Guan X, Chen J, et al. Sequencing of allotetraploid cotton (Gossypium hirsutum L. acc. TM-1) provides a resource for fiber improvement. Nat Biotechnol. 2015; 33(5):531–537. doi: 10.1038/nbt.3207 PMID: 25893781.

16. Liu X, Zhao B, Zheng HJ, Hu Y, Lu G, Yang CO, et al. Gossypium barbadense genome sequence provides insights into the evolution of extra-long staple fiber and specialized metabolites. Sci Rep-Uk. 2015; 5:14139. doi: 10.1038/Srep14139 PMID: 26420475.

17. Yuan DJ, Tang ZH, Wang MJ, Gao WH, Tu LL, Jin X, et al. The genome sequence of Sea-Island cotton (Gossypium barbadense) provides insights into the allopolyploidization and development of superior spinnable fibres. Sci Rep-Uk. 2015; 5:17662. doi: 10.1038/Srep17662 PMID: 26634818.

18. Lee SB, Kaittanis C, Jansen RK, Hostetler JB, Tallon LJ, Town CD, et al. The complete chloroplast genome sequence of Gossypium hirsutum: organization and phylogenetic relationships to other angiosperms. BMC Genomics. 2006; 7:61. doi: 10.1186/1471-2164-7-61 PMID: 16553962.

19. Ibrahim RI, Azuma J, Sakamoto M. Complete nucleotide sequence of the chloroplast (Gossypium barbadense L) chloroplast genome with a comparative analysis of sequences among 9 dicot plants. Genes Genet Syst. 2006; 81(5):311–321. doi: 10.1266/Ggs.81.311 PMID: 17159292.

20. Xu Q, Xiong G, Li P, He F, Huang Y, Wang K, et al. Analysis of complete nucleotide sequences of 12 Gossypium chloroplast genomes: origin and evolution of allotetraploids. PLoS ONE. 2012; 7(8):e37128. doi: 10.1371/journal.pone.0037128 PMID: 22876273.

21. Liu G, Cao D, Li S, Su A, Geng J, Grover CE, et al. The complete mitochondrial genome of Gossypium hirsutum and evolutionary analysis of higher plant mitochondrial genomes. PLoS ONE. 2013; 8(8):e69476. doi: 10.1371/journal.pone.0069476 PMID: 23940520.

22. Tang M, Chen Z, Grover CE, Wang Y, Li S, Liu G, et al. Rapid evolutionary divergence of Gossypium barbadense and G. hirsutum mitochondrial genomes. BMC Genomics. 2015; 16:770. doi: 10.1186/s12864-015-1988-0 PMID: 26459858.

23. Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, et al. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J Bot. 2005; 92(1):142–166. doi: 10.3732/ajb.92.1.142 PMID: 21652394.

24. Fu Y-B, Allaby R. Phylogenetic network of Linum species as revealed by non-coding chloroplast DNA sequences. Genet Resour Crop Evol. 2010; 57(5):667–677. doi: 10.1007/s10722-009-9502-7

25. Martin G, Baurens FC, Cardi C, Aury JM, D'Hont A. The complete chloroplast genome of banana (Musa acuminata, Zingiberales): insight into plastid monocotyledon evolution. PLoS ONE. 2013; 8(6):e67350. doi: 10.1371/journal.pone.0067350 PMID: 23840670.

26. Gielly L, Taberlet P. The use of chloroplast DNA to resolve plant phylogenies—noncoding versus rbcL sequences. Molecular Biology and Evolution. 1994; 11(5):769–777. PMID: 7968490.

27. Yang JB, Tang M, Li HT, Zhang ZR, Li DZ. Complete chloroplast genome of the genus Cymbidium: lights into the species identification, phylogenetic implications and population genetic analyses. BMC Evolutionary Biology. 2013; 13(1):84. doi: 10.1186/1471-2148-13-84 PMID: 23597078.

28. Gong XS, Yan LF. Improvement of the purification of chloroplast DNA from higher-plants. Chinese Science Bulletin. 1991; 36(19):1633–1635.

29. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012; 9(4):357–359. doi: 10.1038/Nmeth.1923 PMID: 22388286.

30. Machado M, Magalhaes WC, Sene A, Araujo B, Faria-Campos AC, Chanock SJ, et al. Phred-Phrap package to analyses tools: a pipeline to facilitate population genetics re-sequencing studies. Investig Genet. 2011; 2(1):3. doi: 10.1186/2041-2223-2-3 PMID: 21284835.
31. Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly de Bruijn graphs. Genome Res. 2008; 18(5):821–829. doi: 10.1101/gr.074492.107 PMID: 18349386.

32. Wyman SK, Jansen RK, Boore JL. Automatic annotation of organellar genomes with DOGMA. Bioinformatics. 2004; 20(17):3252–3255. doi: 10.1093/bioinformatics/bth352 PMID: 15180927.

33. Lowe TM, Eddy SR. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 1997; 25(5):955–964. doi: 10.1093/nar/25.5.955 PMID: 9023104.

34. Lohse M, Drechsel O, Bock R. OrganellarGenomeDRAW (OGDRAW): a tool for the easy generation of high-quality custom graphical maps of plastid and mitochondrial genomes. Curr Genet. 2007; 52(5–6):267–274. doi: 10.1007/s00294-007-0161-y PMID: 17957369.

35. Earl D, Nguyen N, Hickey G, Harris RS, Fitzgerald S, Beal K, et al. Alignathon: a competitive assessment of whole-genome alignment methods. Genome Res. 2014; 24(12):2077–2089. doi: 10.1101/gr.174920.114 PMID: 25273068.

36. Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics. 2002; Chapter 2:Unit 2 3. doi: 10.1002/0471250953.bi0203s00 PMID: 18792934.

37. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32(5):1792–1797. doi: 10.1093/nar/gkh340 PMID: 15034147.

38. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30(4):772–780. doi: 10.1093/molbev/msr306 PMID: 23329690.

39. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28(10):2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546353.

40. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215(3):403–410. doi: 10.1016/S0022-2836(05)80360-2 PMID: 2231712.

41. Santorum JM, Darriba D, Taboada GL, Posada D. jmodeltest.org: selection of nucleotide substitution models on the cloud. Bioinformatics. 2014; 30(9):1310–1311. doi: 10.1093/molbev/mst010 PMID: 24451621.

42. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010; 59(3):307–321. doi: 10.1093/sysbio/sys010 PMID: 20525638.

43. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014; 30(9):1312–1313. doi: 10.1093/bioinformatics/btu332 PMID: 24451623.

44. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19(12):1572–1574. doi: 10.1093/bioinformatics/btg180 PMID: 12912839.

45. Simmons MP, Ochoterena H. Gaps as characters in sequence-based phylogenetic analyses. Syst Biol. 2000; 49(2):369–381. doi: 10.1080/10635150075000539 PMID: 12118412.

46. Muller K. SeqState: primer design and sequence statistics for phylogenetic DNA datasets. Appl Bioinformatics. 2005; 4(1):65–69. 418. PMID: 16000015.

47. Lartillot N, Lepage T, Blanquart S. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. Bioinformatics. 2009; 25(17):2286–8. doi: 10.1093/bioinformatics/btp368 PMID: 19535536.

48. Thorne JL, Kishino H, Painter IS. Estimating the rate of evolution of the rate of molecular evolution. Mol Biol Evol. 1998; 15(12):1647–57. PMID: 9886200.

49. Carvalho MR, Herrera FA, Jaramillo CA, Wing SL, Callejas R. Paleocene Malvaceae from northern South America and their biogeographical implications. Am J Bot. 2011; 98(8):1337–1355. doi: 10.3732/ajb.1000539 PMID: 21821594.

50. Wendel JF. New world tetraploid cottons contain old-world cytoplasm. P Natl Acad Sci USA. 1989; 86(11):4132–4136. doi: 10.1073/pnas.86.11.4132 PMID: 16594050.

51. Senchina DS, Alvarez I, Cronn RC, Liu B, Rong J, Noyes RD, et al. Rate variation among nuclear genes and the age of polyploidy in Gossypium. Mol Biol Evol. 2003; 20(4):633–643. doi: 10.1093/molbev/msg065 PMID: 12679546.

52. Wendel JF, Stewart JM, Rettig JH. Molecular evidence for homoploid reticulate evolution among Australian species of Gossypium. Evolution. 1991; 45(3):694–711. doi: 10.2307/2409921.

53. Cronn R, Wendel JF. Cryptic trysts, genomic mergers, and plant speciation. New Phytologist. 2004; 161(1):133–142.

54. Li P, Li Z, Liu H, Hua J. Cytoplasmic diversity of the cotton genus as revealed by chloroplast microsatellite markers. Genet Resour Crop Evol. 2014; 61(1):107–119. doi: 10.1007/s10722-013-0018-9
Oddou-Muratorio S, Petit RJ, Le Guerroué B, Guesnet D, Demesure B. Pollen-versus seed-mediated gene flow in a scattered forest tree species. Evolution. 2001; 55(6):1123–1135. doi: 10.1554/0014-363. mec859. PMID: 1135. doi:10.1554/0014-363. mec859. PMID: 1135.

56. Müller K, Borsch T. Phylogenetics of Utricularia (Lentibulariaceae) and molecular evolution of the trnK intron in a lineage with high substitutional rates. Plant Syst Evol. 2005; 250(1–2):39–67. doi: 10.1007/s00606-004-0224-1

57. Muller K. Incorporating information from length-mutational events into phylogenetic analysis. Mol Phylogenet Evol. 2006; 38(3):667–766. doi: 10.1016/j.ympev.2005.07.011 PMID: 16129628.

58. Luan PT, Ryder OA, Davis H, Zhang YP, Yu L. Incorporating indels as phylogenetic characters: impact on interfamilial relationships within Arctoidea (Mammalia: Carnivora). Mol Phylogenet Evol. 2013; 66(3):748–756. doi: 10.1016/j.ympev.2012.10.023 PMID: 23147269.

59. Grover CE, Zhu X, Grupp KK, Jareczek JJ, Gallagher JP, Szadkowski E, et al. Molecular confirmation of species status for the allopolyploid cotton species, Gossypium ekmanianum Wittmack. Genet Resour Crop Evol. 2015; 62(1):103–114. doi: 10.1007/s10722-014-0138-x

60. Mardanov AV, Ravin NV, Kuznetsov BB, Samigullin TH, Kolganova TV, et al. Complete sequence of the duckweed (Lemma minor) chloroplast genome: structural organization and phylogenetic relationships to other angiosperms. J Mol Evol. 2008; 66(6):555–564. doi: 10.1007/s00239-008-9091-7 PMID: 18463914.

61. Rodman J, Karol K, PRice R, Conti E, Systma K. Nucleotide sequences of rbcL confirm the capparlean affinity of the Australian endemis Gyrostemonacem. Australian Systematic Botany. 1994; 7(1):57–69. doi: 10.1071/SB940057

62. Randle CP, Wolfe AD. The evolution and expression of rbcL in holoparasitic sister genera Harveya and Hyobanche (Orobanchaceae). American Journal of Botany. 2005; 92(9):1575–1585. doi: 10.3732/ajb.92.9.1575 PMID: 21646175.

63. Handy SM, Parks MB, Deeds JR, Liston A, de Jager LS, Luccioli S, et al. Use of the chloroplast gene ycf1 for the genetic differentiation of pine nuts obtained from consumers experiencing dysgeusia. J Agr Food Chem. 2011; 59(20):10995–11002. doi: 10.1021/jf203215v PMID: 21932798.

64. Muloko-Ntouteume N, Petit RJ, White L, Abernethy K. Chloroplast DNA variation in a rainforest tree (Aucoumea klaineana, burseraceae) in Gabon. Mol Ecol. 2000; 9(3):359–363. me6359. PMID: 10736033.

65. Oddou-Muratorio S, Petit RJ, Le Guerroué B, Guesnet D, Demesure B. Pollen-versus seed-mediated gene flow in a scattered forest tree species. Evolution. 2001; 55(6):1123–1135. doi: 10.1554/0014-3820(2001)055[1123:PVSMGF]2.0.CO;2 PMID: 11475048.

66. Hamilton MB, Braverman JM, Soria-Hernanz DF. Patterns and relative rates of nucleotide and insertion/deletion evolution at six chloroplast intergenic regions in new world species of the Lecythidaceae. Mol Biol Evol. 2010; 27:240–256. doi: 10.1093/molbev/mes054 PMID: 20624729.

67. Njuguna W, Liston A, Cronn R, Ashman TL, Bassil N. Insights into phylogeny, sex function and age of Fragaria based on whole chloroplast genome sequencing. Mol Phylogenet Evol. 2013; 66(1):17–29. doi: 10.1016/j.ympev.2012.08.026 PMID: 22982444.

68. Ci van P, Foster PG, Embley MT, Seneca A, Cox CJ. Analyses of chlorophyte chloroplast genomes help characterize the ancestral chloroplast genome of land plants. Genome Biol Evol. 2014; 6(4):897–911. doi: 10.1093/gbe/evu061 PMID: 24682153.

69. Walker JF, Zanis MJ, Emery NC. Comparative analysis of complete chloroplast genome sequence and inversion variation in Lasthenia burkei (Maddieae, Asteraceae). American Journal of Botany. 2014; 101(4):722–729. doi: 10.3732/ajb.1400049 PMID: 24699541.

70. Wu Z, Ge S. The whole chloroplast genome of wild rice (Oryza australiensis). Mitochondrial DNA. 2014; 1–2. doi: 10.3109/19401736.2014.928668 PMID: 24960559.

71. Carbonell-Caballero J, Alonso R, Ibáñez V, Terol J, Talon M, Dopazo J. A phylogenetic analysis of 34 chloroplast genomes elucidates the relationships between wild and domestic species within the genus Citrus. Mol Biol Evol. 2015; 32(8):2015–2035. doi: 10.1093/molbev/msv082 PMID: 25873589.

72. Nguyen PAT, Kim JS, Kim JH. The complete chloroplast genome of colchicine plants (Colchicum autumnale L. and Gloriosa superba L.) and its application for identifying the genus. Planta. 2015; 242(1):223–237. doi: 10.1007/s00425-015-2303-7 PMID: 25904477.