The Complete Chloroplast Genome of *Ginkgo biloba* Reveals the Mechanism of Inverted Repeat Contraction

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Abstract

We determined the complete chloroplast genome (cpDNA) of *Ginkgo biloba* (common name: ginkgo), the only relict of ginkgophytes from the Triassic Period. The cpDNA molecule of ginkgo is quadripartite and circular, with a length of 156,945 bp, which is 6,458 bp shorter than that of *Cycas taitungensis*. In ginkgo cpDNA, *rpl23* becomes pseudo, only one copy of *ycf2* is retained, and there are at least five editing sites. We propose that the retained *ycf2* is a duplicate of the ancestral *ycf2*, and the ancestral one has been lost from the inverted repeat A (IR_A). This loss event should have occurred and led to the contraction of IRs after ginkgos diverged from other gymnosperms. A novel cluster of three transfer RNA (tRNA) genes, *trnY-AUA*, *trnC-ACA*, and *trnSeC-UCA*, was predicted to be located between *trnC-GCA* and *rpoB* of the large single-copy region. Our phylogenetic analysis strongly suggests that the three predicted tRNA genes are duplicates of *trnC-GCA*. Interestingly, in ginkgo cpDNA, the loss of one *ycf2* copy does not significantly elevate the synonymous rate (*K*s) of the retained copy, which disagrees with the view of Perry and Wolfe (2002) that one of the two-copy genes is subjected to elevated *K*s when its counterpart has been lost. We hypothesize that the loss of one *ycf2* is likely recent, and therefore, the acquired *K*s of the retained copy is low. Our data reveal that ginkgo possesses several unique features that contribute to our understanding of the cpDNA evolution in seed plants.

Key words: *Ginkgo*, chloroplast genome, *trnSeC*, inverted repeat contraction, duplication.

Introduction

Ginkgo (*Ginkgo biloba* L.), also known as maidenhair tree, is a well-known living gymnosperm fossil with edible seeds, medicinal efficacy, and ornamental value (Pang et al. 1996). Fossil records suggest that during the late Mesozoic and early Tertiary era (ca. 120–60 Ma), the genus *Ginkgo* reached its highest species diversity and was widespread in the Northern Hemisphere (Gong et al. 2008). Today, ginkgo is the only living species left within the family Ginkgoaceae, and its natural habitat is restricted to small areas in China (Shen et al. 2005).

The presence of two large inverted repeats (IRs) is one of the most remarkable features in the chloroplast genomes (cpDNAs). In land plants, dynamic expansion/contraction of IRs has been previously reported in some lineages, such as Apioideae (Plunkett and Downie 2000), monocots (Wang et al. 2008), ferns (Wolf et al. 2010), and Pinaceae which have extremely reduced IRs (Lin et al. 2010). The fluctuating lengths of IRs contribute to increase/decrease of cpDNA sizes and can be utilized to address phylogeny but with the need of caution (Wolf et al. 2010).

Using gene mapping and cross-hybridization methods, Palmer and Stein (1986) constructed the first cpDNA map of ginkgo and reported its IR length of approximately 17 kb. Apparently, the IR of ginkgo is significantly shorter than those of most angiosperms (ca. 20–28 kb, Chumley et al. 2006) and *Cycas taitungensis* (ca. 25 kb, Wu et al. 2007), which indicates that ginkgo has experienced an IR contraction. However, the mechanism of IR contraction in ginkgo remains unclear.

Therefore, this study aimed to 1) elucidate the cpDNA organization of ginkgo with reference to other gymnosperms and 2) expand our understanding of cpDNA diversity and evolution as part of our long-term gymnosperm cpDNA

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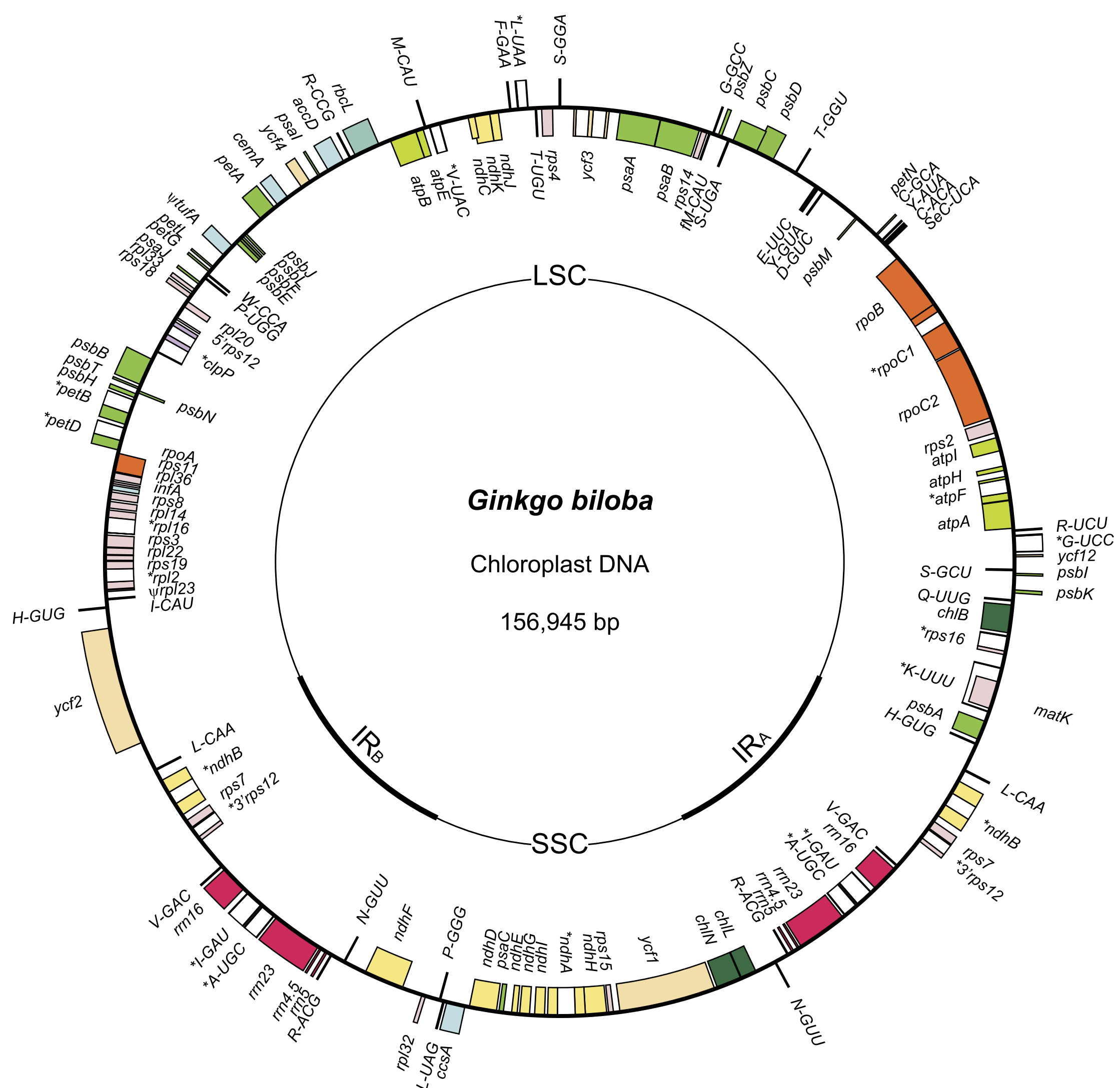


Fig. 1.—Complete cpDNA map of *Ginkgo biloba*. Genes inside and outside the circle are transcribed clockwise and counterclockwise, individually. *: genes with introns. Ψ: pseudogenes.

evolutionary study. Here, we report several unique characteristics of ginkgo cpDNA, propose the underlying mechanism of its IR contraction, and discuss the evolution of an unusual transfer RNA (tRNA) gene cluster.

Materials and Methods

Genomic DNA Extraction

Genomic DNA (gDNA) was extracted from fresh young leaves of a ginkgo plant in the greenhouse of Academia Sinica by use of a CTAB-based protocol (Stewart and Via 1993). The purity and integrity of the extracted gDNA were measured and judged by the OD 260/280 ratio and gel electrophoresis, respectively. The gDNA with a 260/280 ratio greater than 1.8 was collected for polymerase chain reaction (PCR) experiments.

Amplification and Sequencing

The cpDNA fragments were amplified using a long-range PCR method with LA Taq (Takara Bio Inc., Shiga, Japan) and specific primers (supplementary table 1, Supplementary Material online). Amplicons were purified (260/280 ratio = 1.8–2.0; 260/230 ratio > 2), then sequenced by use of an Illumina GA IIx sequencer (YOURGENE BIO SCIENCE Co., New Taipei City, Taiwan). We trimmed short reads (73 bp) of paired-end sequencing using CLC Genomic Workbench 4.9 (CLC Bio, Aarhus, Denmark) with an error probability <0.05 and then assembled these trimmed reads in the same software without any reference information. Regions with <200× coverage depth were trimmed off manually, and these trimmed regions were considered as gaps. Finally, the average coverage depth of contigs is approximately 2080×, which is greatly larger than the proposed minimum

coverage depth for cpDNAs (30×) (Straub et al. 2012). All gaps between contigs were filled with sequences of specific PCR products.

Annotation

We used DOGMA (Wyman et al. 2004) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to annotate protein coding, ribosomal RNA (rRNA), and tRNA genes. All tRNA genes were further verified by their structures predicted by tRNAscan-SE 1.21 (Schattner et al. 2005).

Examination of RNA-Editing Sites

RNA extraction and reverse transcription polymerase chain reaction experiments involved use of the Plant Total RNA Miniprep Purification Kit (Gene Mark Co., Taiwan) and the RevertAid First Strand cDNA Synthesis Kit (Fermentas Inc., Glen Burnie, MD), respectively. The obtained cDNAs were used as PCR templates for examining specific RNA-editing sites.

Estimation of Synonymous Rates (*K*s)

*K*s values of genes were estimated by use of PAL2NAL 1.3 (Suyama et al. 2006). *Amborella* genes were used as the reference.

Phylogenetic Analyses

Thirty-five tRNA sequences of ginkgo cpDNA were aligned by use of ClustalW (Thompson et al. 1994). The aligned sequences were used to construct a maximum-likelihood (ML) tree with a General time reversible + Gamma + Proportion Invariant (GTR+G+I) model and 1,000 bootstrapping analyses in MEGA 5 (Tamura et al. 2011).

Results and Discussion

Characteristics of *Ginkgo* cpDNA

The cpDNA of ginkgo (Accession number: AB684440) is a circular molecule of 156,945 bp with a pair of IRs separated by large single-copy (LSC) and small single-copy regions (fig. 1), which agrees well with the restriction mapping of Palmer and Stein (1986), although the total lengths slightly differ. We found that the shortened IR previously noted by Palmer and Stein (1986) is due to the complete loss of the *ycf2* from the IR_A. We identified 120 unique genes in ginkgo cpDNA: 81 protein-coding genes, 35 tRNA genes, and 4 rRNA genes. A total of 14 genes are duplicated, including three protein-coding genes, six tRNA genes, and four rRNA genes in the IR, as well as one tRNA gene in the LSC region. Thirteen protein-coding genes and eight tRNA genes have introns. The overall AT content is 60.4% (protein-coding genes, 61.1%; tRNA genes, 46.1%; rRNA genes, 44.7%; introns, 60.2%; intergenic spacers, 63.2%). We detected five C-to-U RNA-editing sites and experimentally verified them at the initial codons of *petL* and *rps8* and the terminal codons of *petL*, *rps4*, and *ndhC*.

Table 1
Comparison of cpDNA Features between *Ginkgo* and *Cycas*

Features	*Ginkgo biloba*	*Cycas taitungensis*
Size (bp)	156,945	163,403
LSC	99,221	90,216
SSC	22,258	23,039
IR	17,733	25,074
% AT content	60.4	60.5
% Coding genes	54.6	57.2
RNA-editing sites[a]	5	37[b] (Chen et al. 2011)
Total number of genes	134	133
Protein-coding genes	84	87
Duplicated genes	14	15
tRNA genes	42	38
rRNA genes	8	8
Genes with introns	21	21

NOTE.—SSC, small single copy.
[a] Editing sites with experimental verification.
[b] Partial editing sites are included.

Comparisons of *Ginkgo* and a *Cycad* cpDNAs

Because cycads and ginkgo are the two most ancient lineages of gymnosperms, we compared their cpDNA features. The cpDNA organizations of both ginkgo and *C. taitungensis* are similar (table 1), except that ginkgo has only a single copy (SC) of *ycf2* and its *rpl23* has become pseudo, and *Cycas* lost the *trnT-GGU* originally located between *psbD* and *trnE-UUC* in the LSC region (fig. 1 in Wu et al. 2007). These events led to a downsizing of ginkgo cpDNA. In addition, ginkgo cpDNA contains a specific cluster of three novel tRNA genes (*trnSeC-UCA*, *trnC-ACA*, and *trnY-AUA*) that are located between the *rpoB* and the *trnC-GCA* of the LSC region (fig. 1).

Pseudogenization of *rpl23*

In addition to the dysfunctional *tufA* reported by Wu et al. (2007), *rpl23*, which is retained in many land plants and is near the junction of IR_B and LSC regions, becomes pseudo in ginkgo (viz. Ψ*rpl23*). In gymnosperms, loss of *rpl23* was previously reported in gnetophyte cpDNAs (Wu et al. 2009). The Ψ*rpl23* of ginkgo has a truncated 5′ region as compared with the functional *rpl23* of *Cycas*. These data suggest that *rpl23* was independently lost from these two gymnosperm lineages.

trnH-GUG as an Evolutionary Footprint Caused by IR Contraction

As mentioned previously, loss of an *ycf2* copy downsized the IRs and the cpDNA of ginkgo. Therefore, two questions are raised: whether the IR_A of ginkgo originally had a syntenic *ycf2* copy, as is found in most seed plants, and if the ginkgo did lose the *ycf2* copy from the ancestral IR_A, to what extent the retained *ycf2* copy evolved. To answer these questions, we first compared the boundaries of IRs among two

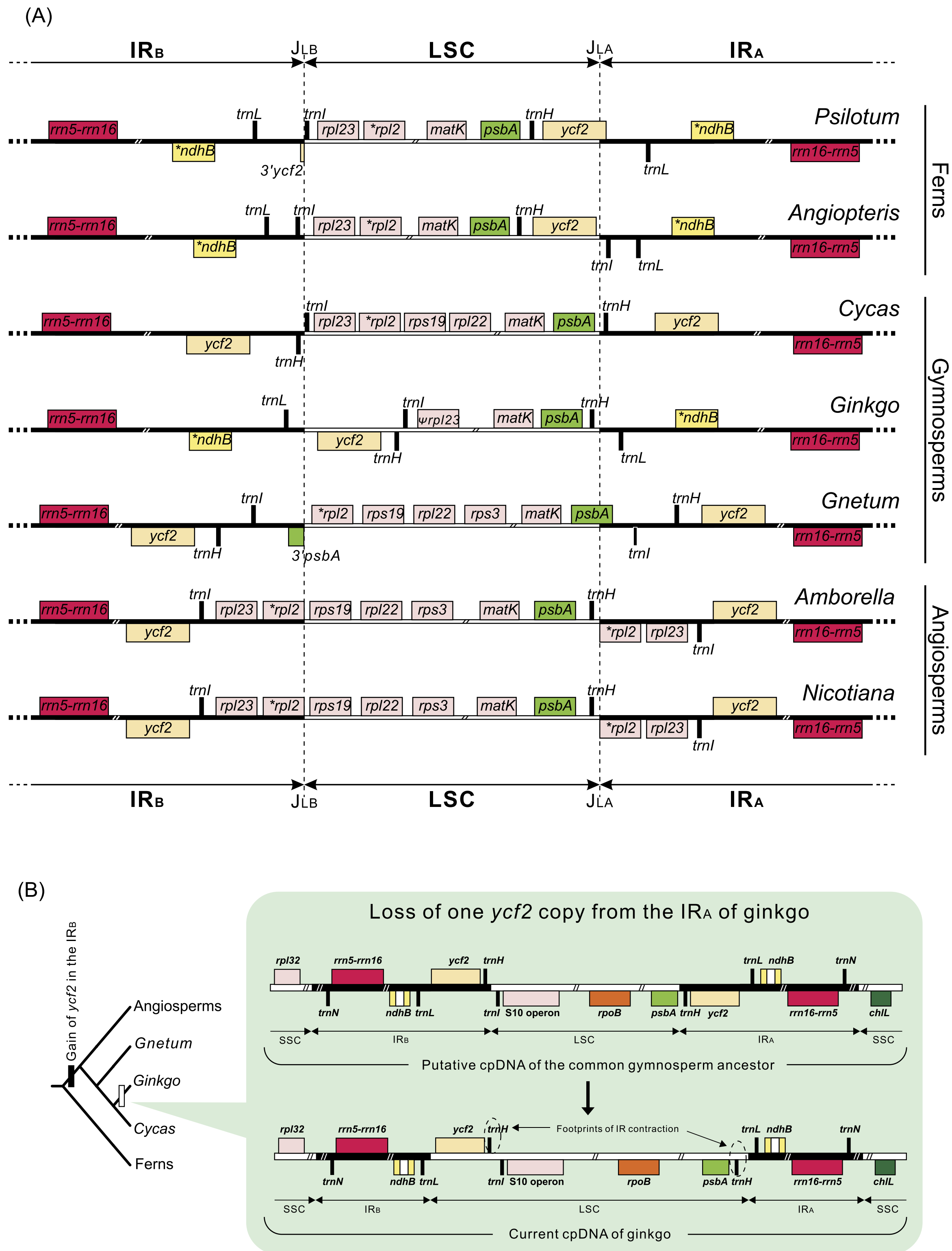


Fig. 2.—(*A*) Comparison of IR boundaries among two representative ferns (*Psilotum nudum* and *Angiopteris evecta*), gymnosperms (*Cycas taitungensis*, *Ginkgo biloba*, and *Gnetum parvifolium*), and angiosperms (*Amborella trichopoda* and *Nicotiana tabacum*). (*B*) Hypothetical scenario illustrating IR contraction in ginkgo cpDNA. The ancestral IRs of gymnosperms should have expanded to include a *trnH-GUG*, and then *ycf2* was lost from the ancestral IR_A during ginkgo evolution. The evolutionary footprints, two *trnH-GUG*, and IR contraction are indicated. The tree topology was modified from Wu et al. (2011). *: genes with introns. J_{LA}: junction between LSC and IR_A; J_{LB}: junction between LSC and IR_B regions.

representatives of ferns and five representatives of IR-containing seed plants, including *Cycas* (representative of cycads), ginkgo, *Gnetum* (representative of gnetophytes), *Amborella* (representative of basal angiosperms), and *Nicotiana* (representative of eudicots) (fig. 2*A*).

Because the IRs of leptosporangiate ferns (e.g., *Adiantum* and *Alsophila*) independently expanded to encompass *rps7*, *3′ rps12*, and *ycf2* (Wicke et al. 2011), two eusporangiate ferns (*Psilotum* and *Angiopteris*) that retain ancestral cpDNA organizations (Gao et al. 2011) were included to simplify evolutionary inferences. As compared with the IR boundaries of ferns, those of all seed plants, except ginkgo, expanded to include *ycf2* sequences (fig. 2*A*). This indicates that duplication of *ycf2* is a common trait among the cpDNAs of seed plants. Wu et al. (2007) proposed that the *ycf2* of IR_B was duplicated from that of IR_A. Of note, IRs of both cycads and gnetophytes retain a *trnH-GUG*. In contrast, this tRNA gene is absent from the IRs of both ferns and angiosperms, which suggests that duplication of *trnH-GUG* is gymnosperm specific. Because the cpDNA of ginkgo has two respective *trnH-GUG* sequences near its IR boundaries, each of the ancestral IRs of ginkgo should have expanded to include a *trnH-GUG* sequence, and subsequently the IRs were contracted by loss of at least the *ycf2* sequence from the IR_A (fig. 2*B*). As a result, the *trnH-GUG* that adjoins the current IR_A could be considered an evolutionary footprint due to the contraction of the ancestral IR_A.

IR Contraction Has No Effect on the Substitution Rate of the Retained *ycf2*

Perry and Wolfe (2002) discovered that in IR-containing legumes, the synonymous rates (*K*s) of IR genes are 2.3-fold lower than those of SC genes, whereas in IR-lacking legumes, the mean *K*s of formerly IR-residing genes are 1.3-fold higher than those of the remaining genes. The authors concluded that in IR-lacking cpDNAs, decreased copy number rather than intrinsic properties directly elevates the *K*s of genes formerly residing in IRs.

With the conclusion of Perry and Wolfe (2002), one should expect an accelerated *K*s in the retained *ycf2* of ginkgo cpDNA. Figure 3 shows comparisons of the *ycf2* and the rest of the IR genes among seven available IR-containing gymnosperm cpDNAs. The *K*s values are largely variable among lineages, with the highest in *Ephedra* of the gnetophytes. To exclude the lineage effect, the *K*s of *ycf2* was divided by the mean *K*s of the rest of the IR genes in respective lineages (the obtained ratios for *Cycas* 2.74; *Bowenia* 3.24; *Zamia* 2.79; *Ginkgo* 3.38; *Ephedra* 3.03; *Welwitschia* 3.79; *Gnetum* 3.57). Two-tailed *Z*-test results revealed no difference between ratios for ginkgo and other gymnosperms ($P = 0.29$). Therefore, in ginkgo cpDNA, the event of losing an *ycf2* copy is likely recent, and the retained copy accumulates few mutations.

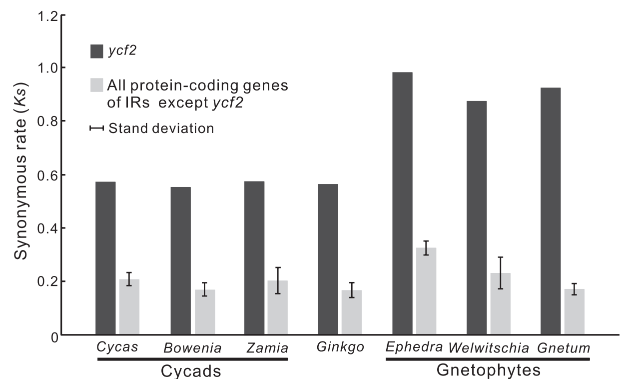


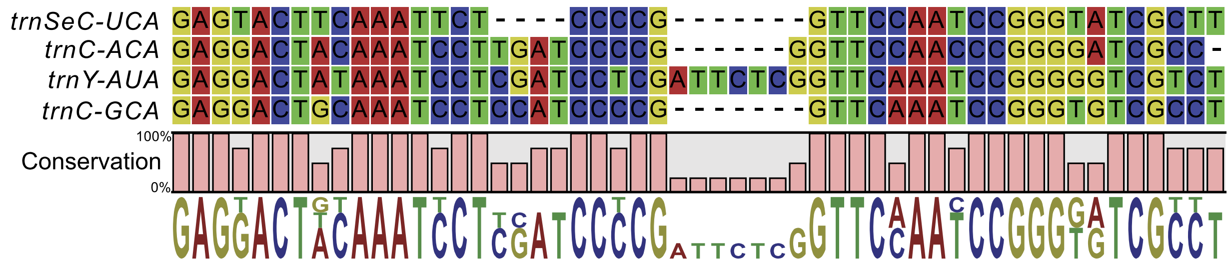
Fig. 3.—Comparison of synonymous rates (*K*s) between *ycf2* and the remaining IR genes. Three genes (*ndhB*, *rps7*, and *3′ rps12*) in cycads and ginkgo were selected to represent the remaining IR genes, and only *rps7* and *3′ rps12* were sampled from gnetophytes because *ndhB* was lost from the IRs of gnetophytes.

Although *ycf2* is essential for plants (Drescher et al. 2000), retaining two *ycf2* copies seems unnecessary because several lineages, such as lower land plants, lycophytes, eusporangiate ferns, conifers, and legumes, have only one *ycf2* copy. Of note, natural ginkgo populations show a low level of genetic variance (Shen et al. 2005), which suggests that ginkgo had experienced population bottlenecks in the past. Thus, loss of an *ycf2* copy might initially occur in an individual of ginkgo, but the bottleneck effect that ginkgo experienced later homogenized the genomic content of all individuals, with only one *ycf2* retained. Further studies are needed to investigate whether retaining two *ycf2* copies in a cpDNA is advantageous or not.

Duplications of *trnC-GCA* Occurred at Least Twice

We detected three adjacent tRNA genes (*trnY-AUA*, *trnC-ACA*, and *trnSeC-UCA*) in the same orientation, as well as *trnC-GCA* (a syntenic tRNA gene of all land plant cpDNAs), in the region between *petN* and *rpoB* of the LSC in ginkgo. We exclude the possibility that the three clustered tRNA genes derived from horizontal transfers because of no DNA-importing system in chloroplasts (Smith 2011). Intriguingly, these three clustered tRNA genes have high sequence similarity with *trnC-GCA* (fig. 4*A*): the sequence similarities between *trnC-GCA* and *trnY-AUA*, *trnC-ACA*, and *trnSeC-UCA* are 82.1%, 85.1%, and 80.8%, respectively. The ML tree depicted in figure 4*B* shows that almost all synonymous tRNA species are clustered with each other or to one another and that *trnY-AUA*, *trnC-ACA*, *trnSeC-UCA*, and *trnC-GCA* are grouped as a monophyletic clade (bootstrapping value = 70%), in which *trnC-ACA* and *trnSeC-UCA* form a subclade (bootstrapping value = 64%). This result suggests that the three clustered tRNA genes are duplicates of *trnC-GCA*, and they might derive from at least two duplication events. The tandem

(A)



(B)

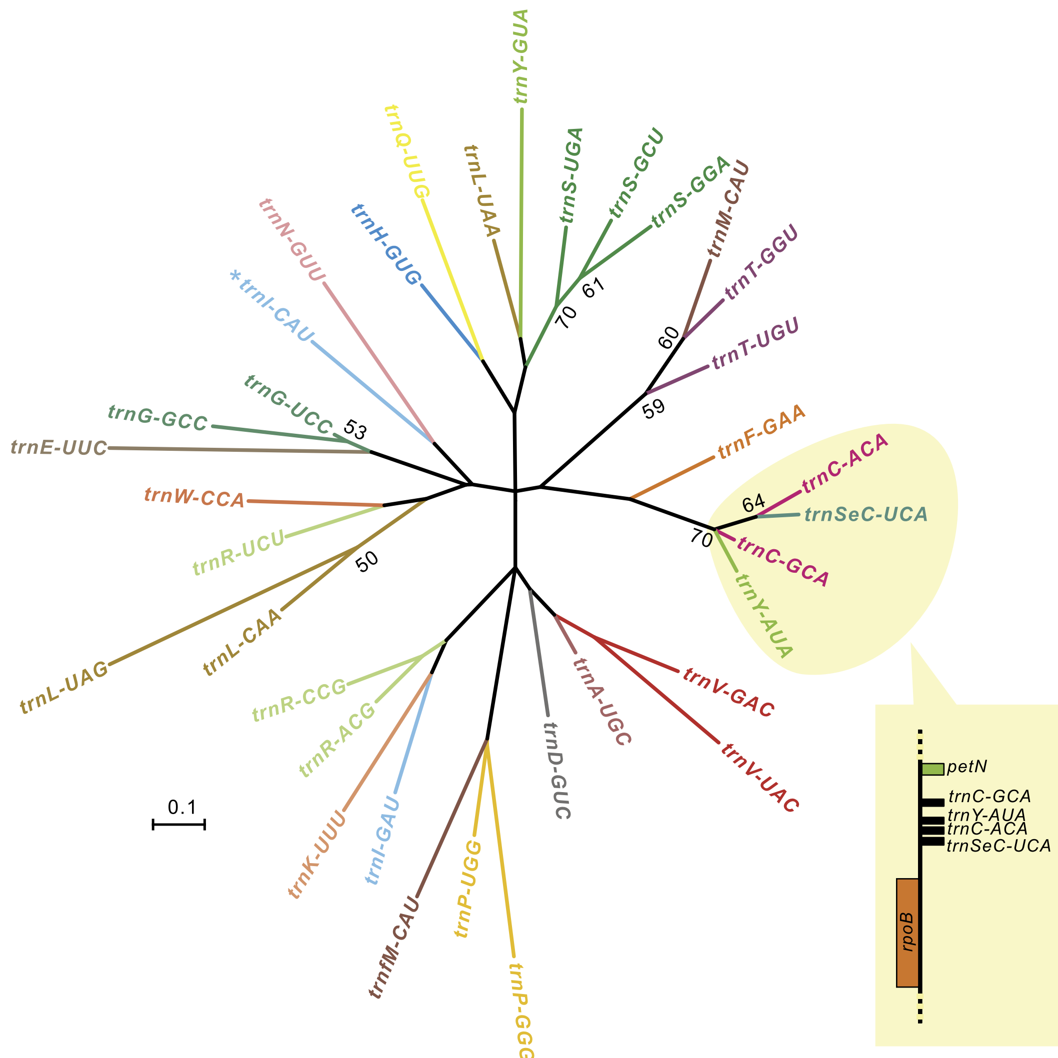


Fig. 4.—Phylogenetic relationships of the three clustered tRNA genes uniquely found in ginkgo cpDNA. (*A*) Alignment of *trnC-GCA*, *trnY-AUA*, *trnC-ACA*, and *trnSeC-UCA* sequences. (*B*) Unrooted ML tree based on all 35 tRNA genes encoding in ginkgo cpDNA. The *trnC-GCA* and the three clustered tRNA genes are in yellow shadow. Tree branches leading to synonymous tRNA species have the same colors. Values along branches denote bootstrapping values estimated from 1,000 replicates (only values ≥50% are shown).

duplicated *trnF-GAA* copies found in the cpDNAs of Brassicaceae were characterized by several parallel gains and losses (Koch et al. 2005). However, the duplicated tRNA genes that we reported here may not be specific to ginkgo or inherited from the common ancestor of ginkgophytes because cpDNAs of extinct ginkgo lineages, for example, *G. adiantoides* and *G. yimaensis* (Zhou and Zheng 2003), are unavailable. Interestingly, *trnSeC-UCA* was also annotated in the cpDNA of *Adiantum* (Wolf et al. 2003), a leptosporangiate fern, but it is not syntenic with that of ginkgo cpDNA. Gao et al. (2009) proposed that in the *Adiantum* cpDNA, *trnR-CCG* was substituted by *trnSeC-UCA* because the former is not essential. In contrast, in ginkgo cpDNA, *trnC-GCA* coexists rather than is replaced by its duplicates, possibly because *trnC-GCA* is vital for plant cell development (Legen et al. 2007). In addition, whether *trnSeC-UCA* of ginkgo cpDNA is functional and what is its evolutionary significance require further scrutiny.

Conclusions

We elucidated that the shortened IR of ginkgo cpDNA is a consequence of IR contraction, and the contraction mainly resulted from loss of one *ycf2* copy from the IR_A. The presence of two *trnH-GUG*, one near the junction of LSC-IR_A and the other upstream of *ycf2*, are considered as footprints of IR contraction. Unexpectedly, the *K*s of the retained *ycf2* copy is nonaccelerated, which suggests that the loss might be recent in ginkgo evolution. Moreover, we found a unique cluster of three tNRA genes upstream of *trnC-GCA* in ginkgo cpDNA. The duplicated relationships between the three clustered tRNA genes and *trnC-GCA* are evident on the basis of their high sequence similarity and phylogenetic evaluation. However, the evolutionary impact of this tRNA gene cluster needs further investigation.

Supplementary Material

Supplementary table 1 is available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

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