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Novel genetic code and record-setting AT-richness in the highly reduced plastid genome of the holoparasitic plant Balanophora - Huei-Jiun Su, Todd J. Barkman, Weilong Hao, Samuel S. Jones, Julia Naumann, Elizabeth Skippington, Eric K. Wafula, Jer-Ming Hu, Jeffrey D. Palmer, and Claude W. dePamphilis

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Significance

There are increasing numbers of reduced-size plastid genomes in parasitic plants. Investigating the plastid-genome divergence in Balanophora helps to understand selective and mutational forces that cause genomal evolution.

Abstract

The *Balaphonora* plastids are among the most reduced ever, with only 19 genes. They have no tRNA genes for protein synthesis, so they must all be imported. They also have a very high AT volume, which produces a bias in codons. For example, functions change, such as TAG being reassigned from a stop codon to tryptophan.

Mycoheterotrophic angiosperms and holoparasitic angiosperms are completely nonphotosynthetic plants, making them prime subjects for testing/observing the reduced plastid genome.

The plastomes of photosynthetic angiosperms have relatively similar characteristics among the species, in categories such as size and number of genes, but nonphotosynthetic angiosperms

have lots of variation. The proteins involved in photosynthesis and ATP synthesis are absent or are pseudogenes within nonphotosynthetic angiosperms, leaving very few genes that solely deal with protein synthesis and core processes.

Holoparasitic *Balanophoraceae* are biologically interesting within the context of this study. They are often mistaken as fungi and are morphologically extreme, containing the smallest flowers in the world. Their plastome is quite interesting, containing many overlapping genes, high compactness, and high AT-levels and codon-usage.

Results

Balanophora laxiflora and *Balanophora reflexa* were obtained. All but two of their 19 genes differed in size. *Balanophora* genomes are one of the smallest plastomes.

No photosynthetic, ATP synthase, RNA polymerase, or splicing factor genes are detectable within the plastomes. Only certain rRNA types were found, and only one type of tRNA was detected, but there could've been RNA genes so small that they were undetectable.

Due to certain structural changes, it's unlikely that *trnE* (the tRNA gene) is functional within both *Balanophora* species.

Both *Balanophora* plastomes are collinear with *Schoepfia* and *Nicotiana* (and most other angiosperms), except for the location of *rpl*14

Extremely Compact Genes and Genomes

The plastomes have high densities because of the overlap between genes. Photosynthetic angiosperms normally have 3 overlapping pairs, but *B. reflexa* has 5 overlapping pairs, and *B. laxiflora* has 4 overlapping pairs. Two overlaps are shared between the two - likely from a common ancestor, whereas the rest were from a divergence.

Only two of fifteen protein genes have the same size as homologs in hemiparasitic *Schoepfia*, a relative.

Extreme AT Base Composition and Codon-Usage Bias

Balanophora are highly compositionally biased (toward AT or GC). They also have the most compositionally biased proteins/protein genes because of the amino acids available.

Explanations of pressures by nitrogen deficiency as a cause for codon bias do not correlate with the behaviors between the nuclear genomes versus the plastome.

An Altered Genetic Code in Balanophora Plastomes

TAG, universally a stop codon for land plants, serves the normal function of TGG in *Balanophora*, but TGG is not present in either species. Along with other codonal alterations, the conclusion that the genetic code change occurred in the *Balanophora* ancestor was drawn.

The hypothesis that TAG codons act as premature stop codons doesn't hold up after scrutiny. The hypothesis that A->G RNA editing changes TAG to UGG doesn't hold up primarily because *B. laxiflora* has no RNA editing. The genetic change is thus the remaining theory.

Highly Divergent but Functional Genes

There's lots of variation. There's high variation in amino acid divergence within *Balanophora*, and the biased nucleotide composition of the genomes causes *Balanophora* to have a greater nucleotide identity than amino acid identity.

The high ds branches for *Balanophora* are indicative of a high mutative pressure, and the dN/ds ratio is much greater than that of other land plants. There's also evidence of purifying selection and continued evolution after the fact that allows for some genes to still be functional.

Transcription and Splicing of Balanophora Plastid Genes

Because all cDNA sequences were shown to be identical to the genome sequence, there was no evidence of RNA editing in the *B. laxiflora* plastid. This rules out the codon-editing theory.

Abundance of Oil Droplets and Presence of Elaioplasts in Balanophora Tissues.

After various tests on *Balanophora yakushimensis*, the researchers found that *Balanophora* use plastids to store lipids, not starches.

Discussions

Radical Evolution of the Balanophora Plastome.

Balanophora so radically evolves that no other species fully matches its characteristics

> Ex. Nothing matches its AT-richness, codon-usage bias, or novel genetic code

Similar to apicoplasts but apicoplasts have more ribosomal protein and tRNA genes and have very little gene variation

Novel Genetic Code in Balanophora Plastid DNA.

Balaphanora is the first in more than 2000 sequenced plastomes of land plants to make a code change.

TAG: stop ->tryptophan

TAA or TGA: stop

Possible explanation for TAG change from stop to tryptophan is AT bias of the genome

“Plastid-specific $tRNA^{Trp}$(Cua)” scenario

Extraordinary AT Content and Codon-Usage Bias.

Extreme Compaction of the Balanophora Plastome

High deletion rates likely causes the extreme compaction

Few others rival *Balaphonora* in compactness

Does genome compaction lead to shrinkage of plastid-encoded proteins?

Most compact plastomes are not AT-rich -> genome compaction forces likely distinct from AT

Function of the Balanophora Plastome.

Divergent genes would be riddled with TAA stop codons, but this is not the case with *Balanophora*, so it is functional

Very few plants have to import all tRNA for protein synthesis like *Balanophora*. *Balanophora* still has *trn*E, which functions in heme biosynthesis

The plastome’s role in lipid synthesis lines up with the masses of lipids found within them

Fate of the Balanophora Plastome

Balanophora nuclear genes, unlike the plastome genes, show very little bias, which doesn’t allow it to efficiently translate plastid genes transferred to the nucleus

Plastid dna may permanently lock in some genes

Prospectus

Study of slower-evolving species can uncover antecedent conditions to the change in genetic code and other radical *Balanophora* features.

Material and Methods

Genome Assembly and Validation

DNA Preparation from *B. laxiflora* influoresences & library generated from 250-bp paired-end Illumina MiSeq reads

Used BLASTn and BLASTx searches, verified with National Center for Biotechnology Information nonredundant (NCBI nr) database

plastome assembly of B. laxiflora was validated by PCR amplification of total DNA, using DreamTaq Green PCR Master Mix

Genome Annotation and Mapping

(codonw.sourceforge.net/index.html) was used to analyze GC content

Phylogenomic Analysis

RAxML version 8.1.17 (104) with the GTR+I+G model and 1,000 bootstrap replicates was used to calculate maximum-likelihood analyses

Evolutionary Rate Estimation

PAML version 4.8 package (107) was used to estimate dN and dS by using the tree topology of SI Appendix, Fig. S10

Questions

Have studies into the slower-evolving species shown evidence into the antecedent conditions of the radical changes seen in *Balanophora*?

Do other fast-evolving species corroborate the conditions seen in *Balanophora*, such as the genetic code change?

Problem to Solve

Similar fast-evolving species to *Balanophora* could show the same radical changes. However, slowly-evolving species could give an explanation as to how exactly these changes come about, so both must be investigated.