

Complete Mitochondrial Genome of a Gymnosperm, Sitka Spruce (*Picea sitchensis*), Indicates a Complex Physical Structure

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Data deposition: The Oxford Nanopore MinION sequencing data are available at the SRA under accessions SRX5081713 and SRR8264752–SRR8264769. The Illumina HiSeq sequencing data have SRA accession SRR5028199. The Sitka spruce (*Picea sitchensis*) genotype Q903 complete mitochondrial genome is available at NCBI GenBank under accessions MK697696–MK697708.

Abstract

Plant mitochondrial genomes vary widely in size. Although many plant mitochondrial genomes have been sequenced and assembled, the vast majority are of angiosperms, and few are of gymnosperms. Most plant mitochondrial genomes are smaller than a megabase, with a few notable exceptions. We have sequenced and assembled the complete 5.5-Mb mitochondrial genome of Sitka spruce (*Picea sitchensis*), to date, one of the largest mitochondrial genomes of a gymnosperm. We sequenced the whole genome using Oxford Nanopore MinION, and then identified contigs of mitochondrial origin assembled from these long reads based on sequence homology to the white spruce mitochondrial genome. The assembly graph shows a multipartite genome structure, composed of one smaller 168-kb circular segment of DNA, and a larger 5.4-Mb single component with a branching structure. The assembly graph gives insight into a putative complex physical genome structure, and its branching points may represent active sites of recombination.

Key words: gymnosperms, Sitka spruce, organelle, genome assembly, sequencing, ABySS.

Introduction

Plant mitochondrial genomes are amazingly diverse and complex (Mower et al. 2012). Land plant mitochondrial genomes range in size from 66 kilobases (kb) for the parasitic angiosperm *Viscum scurruloideum* (Skippington et al. 2015) to >11 megabases (Mb) in the case of the flowering plant *Silene conica* (Sloan et al. 2012). Although their genome structure is often portrayed as a circle, the true physical structure of their genome appears to be a variety of circles, linear molecules, and complex branching structures (Backert et al. 1997; Backert and Börner 2000). Although many species have a single master circle representation of their mitochondrial

genome, others are composed of more than a hundred circular chromosomes (Sloan et al. 2012). The precise mechanism of how plant mitochondria replicate and maintain their DNA is not yet fully understood (Cupp and Nielsen 2014). It is hypothesized that recombination-dependent replication plays a role, giving a functional role to the repeat sequences often observed in mitochondria (Gualberto et al. 2014). This model does not fully explain how genomic copy number is regulated and maintained (Oldenburg and Bendich 2015), particularly in multipartite genomes (Vlcek et al. 2011). Although angiosperm mitochondrial genomes are well studied with numerous complete genomes available, gymnosperm mitochondrial

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genomes were scarcer until recently: One from each of the cycads (Chaw et al. 2008), ginkgos, gnetales (Guo et al. 2016), and conifers (Jackman et al. 2016). This year saw a number of mitochondrial genomes published in short succession (Guo et al. 2020; Kan et al. 2020; Sullivan et al. 2020). Although other gymnosperm mitochondrial genomes are smaller than a megabase, conifer mitochondrial genomes can exceed five megabases (Jackman et al. 2016), larger than many bacteria. Compared with other plants, conifers also tend to have very large nuclear genomes (De La Torre et al. 2014), particularly the spruce species (Birol et al. 2013; Nystedt et al. 2013; Warren et al. 2015). As plant mitochondrial genomes typically have <100 genes, what role this expanse of DNA serves, if any, remains mysterious.

Assembling plant mitochondrial genomes is difficult due to the presence of large (up to 30 kb) perfect repeats, which may be involved in active recombination, and hypothesized recombination-dependent replication (Gualberto et al. 2014). A hybrid assembly of both long reads, which are able to span most repeats, and accurate short sequencing reads, which correct indel errors, is well suited to tackle these challenging genome features. Hybrid assembly of long and short reads has been applied to assemble the plastid genome of *Eucalyptus pauciflora* (Wang et al. 2018), as well as plant mitochondrial genomes (Kovar et al. 2018; Kozik et al. 2019).

Annotating plant mitochondrial genomes is also challenging, due to numerous features of plant mitochondria that are not typical of most organisms. For one, RNA editing of C-to-U is pervasive, and this process creates AUG start codons by editing ACG to AUG (Hiesel et al. 1989) or by editing GCG to GUG, an alternative start codon used by some plant mitochondrial genes (Sakamoto et al. 1997). RNA editing can also create stop codons in a similar fashion. Further complicating annotation using available bioinformatics pipelines, the typical GU-AG splice site expected by most splice-aware alignments tools is instead GNGCG-AY (Y denotes C or T) for group II introns (Lambowitz and Zimmerly 2011). Also, *trans*-spliced genes are common in mitochondrial genomes (Kamikawa et al. 2016; Guo et al. 2020), and no purpose-built software tool exists for identifying and annotating *trans*-spliced genes. To add further difficulty, *trans*-spliced exons may be as small as 22 bp, as is *nad5* exon 3 of gymnosperms (Guo et al. 2016) and other vascular plant mitochondria (Knoop et al. 1991). For these reasons, annotating a plant mitochondrial genome remains a laborious and manual task.

In this study, we report on the sequencing, assembly and annotation of the mitochondrial genome of Sitka spruce (*Picea sitchensis*, Pinaceae), a widely distributed conifer in the coastal regions of the Pacific Northwest of North America. We show that this mitochondrial genome is one of the largest among plants and exhibits a multipartite genome structure.

Materials and Methods

Genome Sequencing and Assembly

Genomic DNA was extracted from young Sitka spruce (*P. sitchensis* [Bong.] Carrière, genotype Q903) needles, as described in Coombe et al. (2016). We constructed 18 Oxford Nanopore 1D sequencing libraries, 16 by ligation of 1–7 µg of lightly needle-sheared genomic DNA and 2 by rapid transposition of 0.6 µg of unshredded genomic DNA, and sequenced these on 18 MinION R9.4 flow cells. This whole genome sequencing produced 98 Gb in 9.6 million reads (SRA accession SRX5081713), yielding 5-fold depth of coverage of the roughly 20 Gb nuclear genome, and 26-fold depth of coverage of the mitochondrial genome. We first obtained a rough but computationally efficient assembly using Miniasm (Li 2016), after trimming adapter sequences with Porechop (Wick et al. 2017a) and polished the resulting assembly with Racon (Vaser et al. 2017). We selected contigs with homology to the white spruce (*Picea glauca*, interior white spruce genotype PG29) mitochondrial genome (Jackman et al. 2016) using Bandage (Wick et al. 2015), retaining contigs with at least one ≥5-kb alignment to the white spruce mitochondrion by BlastN (Altschul et al. 1990). We then proceeded to align back the nanopore reads to our draft Sitka spruce assembly with minimap2 (Li 2018), segregate aligned reads and assemble them de novo first with Unicycler (Wick et al. 2017b), and then with Flye (Kolmogorov et al. 2019), as described (supplementary methods, Supplementary Material online). The final genome sequence was obtained by polishing with Pilon (Walker et al. 2014) using Illumina HiSeq sequencing reads of the same DNA extraction (supplementary fig. S1, Supplementary Material online).

Annotation

We annotated coding genes and non-coding rRNA and tRNA genes using automated methods where possible, and performed manual inspection to refine these automated annotations. We used Prokka (Seemann 2014), which uses Prodigal (Hyatt et al. 2010) to identify single-exon coding genes and open reading frames (ORFs). We used MAKER (Holt and Yandell 2011), which uses BLASTP and Exonerate (Slater and Birney 2005) to identify *cis*-spliced coding genes. Prokka used ab initio predictions from Prodigal. MAKER used evidence alignments only. Genes in GenBank from *Viridiplantae* mitochondria were used as evidence. The annotations of Prokka and Maker were combined, along with the manually annotated *cis*-spliced and *trans*-spliced genes. No species-specific TE libraries were used.

We used tRNAscan-SE (Lowe and Eddy 1997) and Aragorn (Laslett 2004) to identify tRNA. We used RNAmmer (Lagesen et al. 2007) and Barrnap (Seemann 2014) to identify rRNA. We used RNAweasel (Lang et al. 2007) and Infernal (Nawrocki

et al. 2009) to identify group II introns. RFAM motif Domain-V (RM00007) represents domain V of group II introns, and RFAM family Intron_gpII (RF00029) represents both domains V and VI (Kalvari et al. 2018).

Results and Discussion

Complete Genome Assembly

The complete mitochondrial genome of Sitka spruce is 5.52 Mb assembled in 13 segments, and has a GC content of 44.7%. The assembly statistics are summarized in [table 1](#). The genome assembly is composed of two components: A 168-kb circular segment (labeled 10), and a larger 5.36-Mb component composed of 12 segments, as visualized in [figure 1](#) using Bandage ([Wick et al. 2015](#)). The eleven largest segments, ranging in size from 84 kb to 1.65 Mb, have similar depth of coverage, assumed to represent single-copy genomic segments. The two smallest segments (27 and 24 kb

labeled 12 and 13 in [figure 1](#), respectively, representing <1% of the mitochondrial genome) exhibit an estimated copy number of two based on their depth of sequencing coverage. No sequence variation is evident in these two repeats. An absence of variation in the repeat implies that they may be involved in active recombination ([Maréchal and Brisson 2010](#)). Though 10% of reads are >24 kb, no reads fully span these repeats.

The complete mitochondrial genome assembly of Sitka spruce assembled from Oxford Nanopore sequencing is composed of 13 contigs >20 kb with an N50 length of 547 kb. The use of long reads was critical in achieving this contiguity and completeness. In contrast, the draft mitochondrial genome assembly of white spruce was assembled from paired-end and mate-pair Illumina sequencing and is composed of 117 contigs >2 kb arranged in 36 scaffolds with a contig and scaffold N50 length of 102 and 369 kb, respectively ([Jackman et al. 2016](#)). The fragmented state of the white spruce mitochondrial assembly provided little information as to the structure of the mitochondrial genome, whereas the Sitka spruce assembly graph ([fig. 1](#)) suggests a multipartite genome structure.

The complete genome is composed of 1.7% (93 kb) of genes with known function, 28.0% (1,545 kb) of 6,806 ORFs (each of at least 90 bp), 3.7% (205 kb) of repeats, and 66.6% unclassified sequences. Of the ORFs, 1,039 are at least 300 bp (100 amino acids) in size and compose 7.2% (400 kb) of the genome. Aligning the ORFs with BLASTP, 63 ORFs (17 ORFs of at least 300 bp) have a significant

Table 1
Summary of Assembly Statistics

Assembly Statistics	
Number of contigs	13
Assembly N50	547 kb
Undetermined bases	None
Largest contig	1.65 Mb
Total reconstruction	5.53 Mb
GC content	44.7%

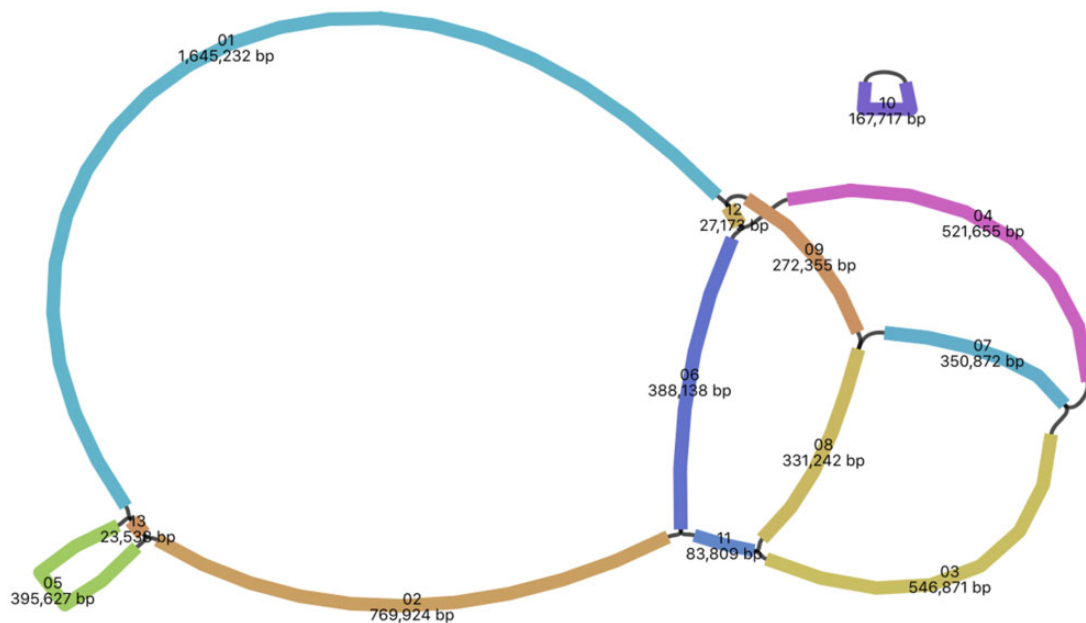


FIG. 1.—The assembly graph of the mitochondrial genome of Sitka spruce. Each colored segment is labeled with its size and named 01–13 by rank of size. Only segment 12 and 13 representations are inferred as repeats. All segment adjacencies are supported by the long reads, indicating a complex branching genomic structure.

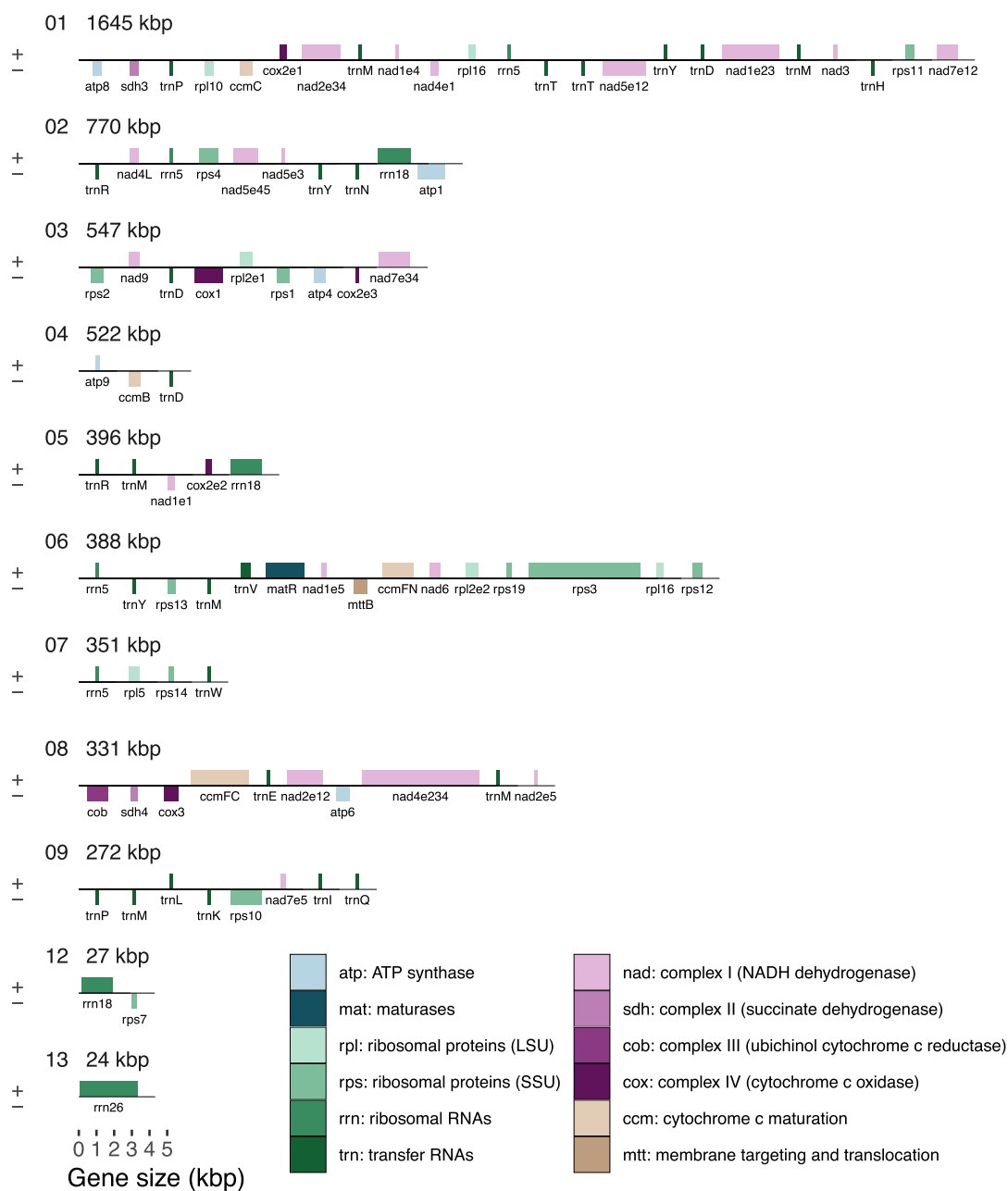


Fig. 2.—The order, orientation, and size of the genes within the Sitka spruce mitochondrial genome. Each box is proportional to the size of the gene including introns, except that genes <200 nucleotides are shown as 200 nucleotides. Intergenic regions are not to scale. Contigs 10 and 11 did not contain any annotated genes and are not shown.

($E < 0.001$) hit to the nr database. Plastid-derived sequences compose 0.25% (14 kb) of the genome spread across 24 segments.

The nuclear repeats LTR/Gypsy compose 51% of the repeat sequence, and LTR/Copia compose 7%. The genome also has non-transposable element repeats; simple repeat sequences compose 34%, low complexity sequences compose 3%, and 5% are other repeat sequences. The 36-bp Bpu repeat sequence is present in roughly 500 copies in *Cycas taitungensis*

and roughly 100 copies in *Ginkgo biloba* (Guo et al. 2016). We find only a single full-length copy with four mismatches in Sitka spruce, similar to *Welwitschia mirabilis*.

Genes

The mitochondrial genome of Sitka spruce has 41 distinct protein coding genes with known function, 3 distinct rRNA genes (supplementary table S1, Supplementary Material

online), and 27 distinct tRNA genes representing 18 distinct anticodons (Supplementary table S2, Supplementary Material online). The relative order and orientation of these genes are shown in figure 2. The 41 known protein coding genes found in the gymnosperm mitochondria *C. taitungensis* (Chaw et al. 2008) and *G. biloba* (Guo et al. 2016) are also found in Sitka spruce. The 29 introns, 16 *cis*-spliced, and 13 *trans*-spliced, are found in 10 protein coding genes, two pseudogenes, and one plastid-derived tRNA (Supplementary table S3, Supplementary Material online).

Four ORFs (937 [Q903MT_gene558], 507 [Q903MT_gene3172], 371 [Q903MT_gene4186], and 221 [Q903MT_gene4187] amino acids) contain an organellar DNA polymerase type B (DNA_pol_B_2) Pfam family (El-Gebali et al. 2019). The largest one also contains a segment homologous to a putative cell-wall binding lipoprotein (YkyA) Pfam domain. These ORFs have homology to DNA polymerase genes found in the mitochondria of *P. glauca* and the angiosperms *Cocos nucifera*, *Daucus carota*, *Helianthus annuus*, and *Silene vulgaris*. Two ORFs (781 [Q903MT_gene4185] and 560 [Q903MT_gene6929] amino acids) contain an RNA polymerase (RNA_pol) domain. These ORFs have homology to DNA-dependent RNA polymerase genes found in the mitochondria of *P. glauca* and the angiosperms *Beta vulgaris*, *C. nucifera*, *D. carota*, and *Phoenix dactylifera*. The two largest genes of the Sitka spruce mitochondrial genome are these putative DNA and RNA polymerase genes.

The *matR* gene [Q903MT_gene5018] and three additional ORFs (476 [Q903MT_gene679], 197 [Q903MT_gene3613], and 163 [Q903MT_gene5889] amino acids) contain a reverse transcriptase, or RNA-dependent DNA polymerase, (RT_like) NCBI conserved protein domain with similarity to the group II intron reverse transcriptase/maturase (group_II_RT_mat) NCBI conserved protein domain (Marchler-Bauer et al. 2017). We hypothesize that these ORFs may be additional maturases involved in splicing (Matsuura 2001). These ORFs have homology to mitochondrial genes of *P. glauca* and the angiosperm *Utricularia reniformis*.

Conclusion

The 5.5-Mb mitochondrial genome of Sitka spruce is among the largest ones in plants, and is the largest complete mitochondrial genome reported to date for a gymnosperm. It follows the trend seen for spruce and conifer nuclear genomes, which are also among the largest in plants (De La Torre et al. 2014). The physical structure of the Sitka spruce mitochondrial genome is not the typical circularly mapping single chromosome, but multipartite. The larger component of the assembly graph exhibits a rosette-like structure, mirroring the rosette-like structures observed in electron micrographs of mitochondrial DNA (Backert and Börner 2000). The intricate structure and the discovery of large repeat

elements suggest the presence of active sites for hypothesized recombination-dependent replication of the mitochondrial genome (Gualberto et al. 2014; Sullivan et al. 2020). Considering heteroplasmy resulting from naturally hybridizing species of spruce and paternal leakage of mitochondria, intermolecular recombination would result in interspecific hybridization of the mitochondrial genome, which has been reported to occur in natural spruce populations (Jaramillo-Correa and Bousquet 2005). The complete mitochondrial genome of Sitka spruce, a gymnosperm, should prove invaluable to future investigations into the genome structure and mechanism of replication of conifer mitochondrial genomes.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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