Microhomologies are associated with tandem duplications and structural variation in plant mitochondrial genomes

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Abstract

Short tandem repeats (STRs) contribute to structural variation in plant mitochondrial genomes, but the mechanisms underlying their formation and expansion are unclear. In this study, we detected high polymorphism in the \textit{nad7-1} region of the \textit{Pinus tabuliformis} mitogenome caused by the rapid accumulation of STRs and rearrangements over a few million years ago. The STRs in \textit{nad7-1} have a 7-bp microhomology (TAG7) flanking the repeat array. We then scanned the mitogenomes of 136 seed plants to understand the role of microhomology in the formation of STR and mitogenome evolution. A total of 13,170 STRs were identified, and almost half of them were associated with microhomologies. A substantial amount (1197) of microhomoloiies was long enough to mediate structural variation, and the length of microhomology is positively correlated with the length of tandem repeat unit. These results suggest that microhomology may be involved in the formation of tandem repeat via microhomology-mediated pathway, and the formation of longer duplicates required greater length of microhomology. We examined the abundance of these 1197 microhomologies, and found 75% of them were enriched in the plant mitogenomes. Further analyses of the 400 prevalent microhomologies revealed that 175 of them showed differential enrichment between angiosperms and gymnosperms and 186 differed between angiosperms and conifers, indicating lineage-specific usage and expansion of microhomologies. Our study sheds light on the sources of
structural variation in plant mitochondrial genomes and highlight the importance of microhomology in mitochondrial genome evolution.

**Keywords:** structural variation, microhomology, short tandem repeat, mitochondrial genome, *Pinus.*
Significance Statement

Short tandem repeats are ubiquitous and play important roles in the evolution of plant mitogenomes, but the mechanisms underlying their origin and proliferation remain unclear. In this study, we revealed that tandem repeats were associated with microhomologies in seed plants mitogenomes, and the accumulation of microhomologies was lineage specific showing differential enrichment among angiosperm and gymnosperm. These results suggest that microhomologies may be involved in the formation of tandem repeat via microhomology-mediated pathway, and have undergone lineage-specific usage and expansion. Our results highlight the high prevalence of microhomology and its important role in mitogenome evolution.

Introduction

The plant mitochondrial genome (mitogenome) is characterized by an unusually low silent substitution rate along with extensive structural variation (Palmer, et al. 2000). In the vast majority of plant mitogenomes, synonymous substitution rates are up to 20 times lower than those of nuclear genomes (Drouin, et al. 2008; Wang and Wang 2014). By contrast, frequent length variation and rearrangements associated with repetitive sequences have been detected throughout mitogenomes (Cole, et al. 2018; Jaramillo-Correa, et al. 2013; Marechal and Brisson 2010), leading to multiple alternative isoforms (Kozik, et al. 2019; Unseld, et al. 1997). Short tandem repeats (STRs) account for a substantial proportion of repetitive content in mitogenomes and are lineage- and gene region-specific, leading to substantial structural differences.
among species and populations (Godbout, et al. 2005; Jaramillo-Correa, et al. 2013; Potter, et al. 2013; Wang and Wang 2014). In conifers, STRs serve as mutagenic hotspots with elevated substitution rates (Jaramillo-Correa, et al. 2013). Although STRs are ubiquitous and play important roles in the evolution of plant mitogenomes, the mechanisms underlying their origin and proliferation remain unclear.

Recent studies suggest that sequence duplications and rearrangements in plant mitogenomes are linked to the repair of double-strand breaks (DSBs) (Davila, et al. 2011; Gualberto and Newton 2017; Shedge, et al. 2007) via either non-homologous end-joining (NHEJ) or microhomology-mediated end-joining (MMEJ) pathways (Lieber 2010; McVey and Lee 2008). In the NHEJ pathway, broken DNA strands are usually processed by degradation of the 5’-end and subsequent blunt-end ligation, leading to insertions and/or deletions of variable lengths (Figure S1A) (Lieber 2010). In the MMEJ pathway, microhomologous sequences anneal to each other before the joining of broken ends, resulting in deletions and/or insertions flanking the breakpoints (Figure S1B) (Garcia-Medel, et al. 2019; McVey and Lee 2008). While MMEJ relies on substantial microhomology for the repair of DSBs, this is not essential for NHEJ. Other mechanisms, including fork stalling and template switching (FoSTeS), and microhomology-mediated break-induced replication (MMBIR), also generate structural variation and rely on microhomology (Hastings, et al. 2009; Ottaviani, et al. 2014; Taylor, et al. 2015). In the FoSTeS pathway, the lagging strand (which is formed by the stalling of the replication fork) disengages from the stalled fork, invades the other active replication fork by annealing to a
microhomologous sequence, and restarts synthesis at the invaded fork (Figure S1C) (Ottaviani, et al. 2014). The process of template switching continues until the lagging strand returns to the original replication fork, resulting in DNA deletion or duplication (Ottaviani, et al. 2014). In the MMBIR pathway, a DSB is created by the collapse of the replication fork, and the 3’ overhang at the broken end invades a new microhomologous template and re-initiates replication of the invaded template (Figure S1D) (Hastings, et al. 2009). As in the FoSTeS pathway, the extended end may switch to multiple new templates until annealing back to the original fork, leading to complex structural variation. These mechanisms have been investigated by comparative bioinformatics analyses of population genomic data of plant nuclear genomes (Vaughn and Bennetzen 2014), and examined by in vitro (Garcia-Medel, et al. 2019) and in vivo experiments in plant chloroplast (Kwon, et al. 2010) and nuclear genomes (Schiml, et al. 2016). However, little is known about the process of structural variation in plant mitogenomes although different hypotheses have been proposed (Christensen 2013; Davila, et al. 2011; Gualberto and Newton 2017; Palmer and Herbon 1988). Moreover, the relative contributions of various mechanisms underlying the formation of rearrangements have not been evaluated.

Our understanding of the mitogenome structure and variation in gymnosperms is very limited. Only a handful of mitogenomes have been assembled to date, and the size of these genomes differs by more than 17-fold (Chaw, et al. 2008; Guo, et al. 2016; Kan, et al. 2020; Sullivan, et al. 2020) (0.35–5.99 Mb; Table S1). This size variation might be related to the abundance of repeats, including STRs, in each
genome. In this study, we report a highly variable region in \textit{nad}7-1 (NADH dehydrogenase subunit 7 intron 1) in the \textit{Pinus tabuliformis} mitogenome caused by complex rearrangements of STRs (Figure 1). The plant \textit{nad}7 gene contains five exons and encodes a subunit of respiratory complex I (NADH-ubiquinone oxidoreductase), which transfers electrons from NADH to ubiquinone (Pineau, et al. 2005). We detected a large number of haplotypes in 157 individual trees of \textit{P. tabuliformis}. To understand whether the observed high haplotype diversity was due to the retention of ancestral polymorphisms or the accumulation of new mutations following speciation, we examined the sequences in this region in 550 individuals of two closely related species and in multiple accessions of 21 additional pine species. We modeled the evolution of STRs in the region by close examination of sequence and structural variation among haplotypes. Finally, we scanned the mitogenomes of 136 seed plants to assess the role of microhomology in the formation of STRs and structural variation in plant mitogenomes.

Materials and Methods

Sampling and sequencing

\textit{Pinus tabuliformis} is a major coniferous forest species in northern and central China, with a range of 2,000 km from east to west and 1,200 km from north to south (Mao and Wang 2011; Ying, et al. 2004). Studies of the biogeography of \textit{P. tabuliformis} based on maternally inherited mitochondrial (mt) markers have provided valuable insights into its migration and colonization history (Chen, et al. 2008; Hao, et al. 2018; Xia, et
al. 2018). However, the few conserved mtDNA fragments used in previous studies limit our understanding of mitogenome evolution in this species.

In this study, we characterized diversity in a highly variable region, nad7-1, in 17 populations of *P. tabuliformis*, as well as in 23 and 11 representative populations of two closely related species, *P. densata* and *P. yunnanensis*, respectively. The distribution of the 17 sampled populations of *P. tabuliformis* is shown in Figure S2. The name, location, and sample size of all 51 populations are listed in Table S2. Additionally, we included 21 species representing all five recognized subsections of the subgenus *Pinus* for comparison (Gernandt, et al. 2005). For nine species, multiple accessions were collected from documented individuals grown by different institutions (Table S3). For other species, *nad7-1* sequences were obtained from previous publications (Table S3).

Genomic DNA was extracted from needles or seedlings using a Plant Genomic DNA Kit (Tiangen, Beijing, China). A sequence-specific primer pair was designed on the basis of a conserved region of *nad7-1* (Neale, et al. 2014) (*nad7-1F, 5’-GAGGGACAACCCTGGAATA-3’; nad7-1R, 5’-AAGGCCTCTCCATTGCAT-3’). The PCR amplified region of this primer pair was between 176,897 and 176,916 bp of the *P. taeda* mitogenome. The PCR products were examined by agarose gel electrophoresis (1.5% in TAE). The desired bands were cut from the gel, purified using a TIANgel DNA Purification Kit (Tiangen, Beijing, China), and sequenced using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).
**Phylogenetic analysis**

Sequences were aligned using ClustalX (Larkin, et al. 2007) and manually adjusted using BioEdit v. 5.0.9.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A median joining network of mitotypes was produced using Network 5 (Bandelt, et al. 1999), and a neighbor-joining (NJ) tree of mitotypes was constructed using MEGA for macOS (Stecher, et al. 2020). Topological robustness of the NJ tree was assessed using 1,000 non-parametric bootstrap replicates. Indels were coded as binary characters using gap-coder (Young and Healy 2003).

**Tandem repeat analysis**

To assess tandem repeats in mitogenomes of seed plants, assemblies of mitogenomes of 128 angiosperms and eight gymnosperms were retrieved from GenBank (Table S1). For species with multiple mitogenome assemblies, the assembly with the highest quality was used in this study. Tandem repeat sequences were identified using Tandem Repeats Finder (Benson 1999) with the following default parameters: maximum period size = 500 bp; detection matching probability \( Pm = 0.8 \); detection indel probability \( Pi = 0.1 \); and alignment weights of match, mismatch and indel = 2, 7 and 7, respectively. We filtered tandem repeat arrays with overall matches < 60% or alignment scores < 50. Among overlapped repeat arrays, the one with the highest alignment score was retained.

Delimiting a microhomology is not straightforward, and various thresholds are used in previous studies (Bhargava, et al. 2016; Ceccaldi, et al. 2016; Ottaviani, et al. 2014).
In this study, we defined a microhomology as a set of short (<70 bp) homologous DNA sequences at breakpoint junctions and flanking regions (Ottaviani, et al. 2014). To search for microhomology in tandem repeat regions, the sequence of the last unit of the repeat array was compared with the consensus sequence of repeats. The last unit was considered as a microhomologous sequence if its length was less than 70 bp, and less than half of the consensus repeat. For example, in a tandem repeat array with the last unit of length $n$ ($n < 70$ bp) and consensus sequence of length $m$, if $n < m/2$, the sequence of the last unit was regarded as a microhomology in the repeat array (Figure S3). Microsatellites (also known as simple sequence repeats [SSRs]) containing repeats of 1–6 bp were not included in microhomology analyses because they have complex mutation models (e.g., stepwise mutation and two-phase models) (Ellegren 2004; Takezaki 2017), which were not the focus of this study.

To quantify the enrichment of microhomologous sequences, we developed a statistic termed as enrichment of microhomology (ECH). First, we counted the observed number of microhomologous sequences ($\text{Obs}_{\text{MH}}$) on both DNA strands of the mitogenome (no mismatch allowed). Then, we shuffled (per base) the mitogenome 1,000 times, and randomly sampled the same number of bases (without replacement) at each shuffling. The expected number of microhomologous sequences ($\text{Exp}_{\text{MH}}$) on both strands was then counted in each replicate, and the mean and 95% confidence interval (CI) of the $\text{Exp}_{\text{MH}}$ were calculated across the 1,000 replicates. The ECH was defined as the ratio of $\text{Obs}_{\text{MH}}$ to the mean of $\text{Exp}_{\text{MH}}$ (ECH = $\text{Obs}_{\text{MH}}$/mean of $\text{Exp}_{\text{MH}}$). The significance of ECH was determined by comparing the value of $\text{Obs}_{\text{MH}}$
with the 95% CI of \( \text{Exp}_{\text{MH}} \); the ECH was considered significant \((P < 0.05)\) when \( \text{Obs}_{\text{MH}} \) was larger than the upper limit of the 95% CI of \( \text{Exp}_{\text{MH}} \). For each microhomology, we calculated the value of ECH and tested the significance of enrichment in the mitogenome.

To further test whether microhomologies were differentially accumulated among seed plants, we calculated the ECH values for 400 microhomologies in the mitogenomes of 136 seed plants, and examined the enrichment of these microhomologies among angiosperms, gymnosperms, and conifers (Table S4). Among these 400 microhomologies, 397 were common in plant mitogenomes (length \( \geq 6 \) bp; \( \text{Obs}_{\text{MH}} \geq 100 \) and ECH >1.00), one was detected in the \( \text{nad}7-1 \) region of \( P. \) tabuliformis (“TAAAGGT”; see Results and Discussion below), and two were previously reported in sugar beet (“CCATACT” in the \( \text{rrn}26 \) gene region) (Nishizawa, et al. 2000) and Norway spruce (“GAAGAA” in tandem repeats in the \( \text{mh}44 \) gene region) (Bastien, et al. 2003). The significance \((P\text{-value})\) of over-representation of microhomology was calculated using Mann-Whitney U-test and then corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) adjustment (Benjamini and Hochberg 1995).

Results and Discussion

Extremely high variation in \( \text{nad}7-1 \) of \( P. \) tabuliformis

Analysis of the \( \text{nad}7-1 \) region in 157 individuals of \( P. \) tabuliformis identified 19 haplotypes with lengths ranging from 782 to 1835 bp (Table S2; Figures 1 and S2;
Supplementary data S1). Sixteen of these haplotypes were population-specific, seven of which had frequencies of > 50% in the population. The high polymorphism in \textit{nad7-1} was in contrast to the low diversity observed in other mtDNA regions in this species, with fewer than five haplotypes detected over three segments (NADH dehydrogenase subunit 1 intron 2, NADH dehydrogenase subunit 4 intron 3, and NADH dehydrogenase subunit 5 intron 1) spanning a total length of 2800 bp in range-wide samples (Chen, et al. 2008; Hao, et al. 2018; Wang, et al. 2011).

The observed polymorphism could reflect either ancestral polymorphisms or new mutations that occurred after speciation. While ancestral polymorphisms are often shared by closely related species before complete lineage sorting, new mutations are usually lineage specific. To distinguish between these two alternatives, we further sequenced \textit{nad7-1} in 375 and 175 individuals of two closely related species, \textit{P. densata} and \textit{P. yunnanensis}, respectively (Table S2). Four mitotypes (H1, H9, H20, and H21) were detected in \textit{P. densata}, of which two (H1 and H9) were shared with \textit{P. tabuliformis} (Table S2). In the 175 samples of \textit{P. yunnanensis}, only two species-specific mitotypes (H21 and H22) were found, and none of these were shared with \textit{P. tabuliformis} (Table S2). We extended our survey to an additional 21 \textit{Pinus} species (Table S3) and found that none of the haplotypes in \textit{P. tabuliformis} were shared with other species. Additionally, the \textit{P. tabuliformis} haplotypes were highly divergent from those of other species, differing by multiple deletions and substitutions. Taken together, these results supported the hypothesis that variations in \textit{nad7-1} in \textit{P. tabuliformis} were formed after the divergence of the species.
Alternatively, mitotypes specific to *P. tabuliformis* could be explained by incomplete lineage sorting of ancestral polymorphisms during species diversification. However, this hypothesis is very unlikely considering the low rate of lineage sorting in the mitogenome of *Pinus*; mitotypes could be shared between pine species that diverged millions of years ago (Zhou, et al. 2010). The extensive population-specific haplotypes in *P. tabuliformis* are more likely to have originated recently, after the divergence of populations dated at 0.58–3.67 million years ago (MYA) (Xia, et al. 2018). High polymorphism has also been reported in the *nad7-1* region of three other pine species (Table S3): *P. banksiana* (14 haplotypes; Godbout, et al. 2005), *P. armandii* (11 haplotypes; Liu, et al. 2014), and *P. kwangtungensis* (nine haplotypes; Tian, et al. 2010). Unlike in *P. tabuliformis*, mitotypes in these species were usually shared with sister species, suggestive of incomplete sorting of ancestral polymorphisms.

*Tandem duplication in nad7-1 of P. tabuliformis*

Examination of sequence structure of the *nad7-1* region in the *P. tabuliformis* revealed two conserved blocks surrounding one highly variable block. The upstream conserved block was 223 bp with a single substitution in three haplotypes (H4–H6), and the downstream conserved block was 39 bp without any variation (Figure 1A). The variable block was characterized by a set of perfect or imperfect (differed by 1–4 indels or substitutions) tandem repeats with different copy numbers among haplotypes (Figure 1).
We identified seven motifs (R1a, R2a, R3a, R1b, R2b, R3b and Rs) in the variable block (Figure 1B). Because this highly variable block is absent from the mitogenomes of other pine species, we cannot infer the ancestral sequence based on homology with outgroups. The Rs motif was basic and shared among haplotypes (Figure 1B); it is thus possible that Rs (or its ancestral sequence) duplicated and one of the copies acquired substitutions. Among the motifs, R2b was most similar to Rs and thus likely evolved from Rs, followed by further changes to generate the other motifs (see our hypothetical evolutionary pathway for these motifs in Figure S4). Consistent with this hypothesis, H1 containing the RsR2b array is the most frequent and probably most ancient haplotype.

All haplotypes ended with the same R1aR1b array in the variable block (Figure 1). R1a and R1b differed by three substitutions and a 32 bp deletion. One plausible evolutionary pathway is that R1a was duplicated to generate R1b. Alternatively, R1b was derived from Rs, and R1a was derived from R1b. The evolutionary history of these motifs may be more complex than we described (Figure S4), and multiple pathways might have been involved in the formation of duplicates.

The structural variation in *nad7-1* involved not only the stepwise expansion of a single motif and subsequent modification, as discussed above, but also a large duplication including multiple motifs. A sequence block (referred to as the DUP block), composed of one highly variable region (referred to as Rp hereafter) surrounded by three motifs, was duplicated in haplotypes H11–H19 (Figure 1A). The Rp motif varied among haplotypes with lengths ranging from 153 bp to 331 bp and
more than eight substitutions differentiating the most differentiated copies (Figure 1A). By contrast, the two copies of the Rp motif within each haplotype were identical, except for a 27 bp indel in the Rp motif of H13. This observation that two Rp copies within a haplotype were more similar to each other than to copies from different haplotypes suggests that the divergence of the Rp copies predated the duplication of the DUP block.

We found a 7-bp sequence (“TAAAGGT”; hereafter referred to as the TAG7) at the 5’ end of each motif as well as directly downstream of the variable block (Figure 1). The presence of this motif in tandem repeats seems consistent with models of microhomology-mediated sequence duplications, including MMEJ, FoSTeS, and MMBIR (McVey and Lee 2008; Ottaviani, et al. 2014). The MMEJ pathway may be important for the repair of DSBs, resulting in tandem duplications (McVey and Lee 2008), while FoSTeS and MMBIR can produce duplications and complex rearrangements by microhomology-mediated repair of broken replication forks (Ottaviani, et al. 2014). Slippage strand replication caused by the mispairing of existing microhomologous sequences can also account for the formation of tandem duplicates (Darmon and Leach 2014). These mechanisms have different genetic bases but are characterized by a similar signature of microhomology at the junction (Ottaviani, et al. 2014). NHEJ can also repair DSBs and generate tandem duplications, either through blunt end-joining or 1–4 bp microhomology (Lieber 2010), inconsistent with the length of the TAG7 observed in the nad7-1 region. In summary, we found complex structural variation due to the expansion and contraction of tandemly
arrayed duplicates in the *nad7-1* region of *P. tabuliformis*, and the TAG7 motif most likely served as a microhomologous sequence in the formation of structural variants. Short direct repeats flanking tandem arrays have been reported in other plant mitogenomes, and suggested to be involved in the generation of tandem repeat array (Bastien, et al. 2003; Nishizawa, et al. 2000). However, microhomology-mediated pathways cannot be reliably distinguished based only on the sequences surrounding breakpoint junctions. Future studies using highly reliable reporters are needed to elucidate the detailed biochemical and genetic underpinnings of sequence duplications as well as universal features associated with each of the underlying mechanisms.

*Tandem duplication associated with microhomology in plant mitogenomes*

To assess the importance of microhomology in tandem duplication and mitogenome evolution, we investigated the abundance of tandem repeats and their associations with microhomologies in 136 seed plants. The *in silico* search yielded 13,170 STRs, with 3–872 STRs per genome among angiosperms and 54–2358 STRs per genome among gymnosperms (Table S1). The median length of an STR unit was 22 bp, and median copy number was 2.2 per STR (Figure S5A and S5B). Both the length of the repeat unit and total length of the repeat array of tandem repeats were shorter than those of non-tandem repeats reported previously in plant mitogenomes (Chaw, et al. 2008; Guo, et al. 2016; Kan, et al. 2020; Wynn and Christensen 2019). Overall, tandem
repeats were more abundant in larger genomes, producing a positive correlation between genome size and STR length (Pearson’s $\rho = 0.708, P < 2.2e^{-16}$; Figure S5C).

The tandem repeats observed in mitogenomes were generally associated with microhomologies. In angiosperms, 52.6% of the tandem repeats ended in short homologies; this value was marginally higher than that in gymnosperms (42.6%) after controlling the genome size ($F_{1,133} = 3.622, P = 0.0592$; Figure 2A). It is worth noting that our estimation of microhomology abundance may be conservative because of two reasons. First, we did not consider sequences with lengths more than half of the repeat unit as a microhomology (see Materials and Methods). When considering all imperfect end-repeats as microhomologous, 79.4% and 77.8% of tandem repeats were associated with microhomologous sequences in angiosperms and gymnosperms, respectively (Table S1). Second, tandem repeats may have been lost or reduced in size to below the detection threshold during the repairing of DSBs via microhomology-mediated pathway or asymmetrical recombination (Davila, et al. 2011; Gualberto and Newton 2017).

Size distribution of microhomologies showed that 34.9% and 26.3% of all microhomologies in angiosperms and gymnosperms, respectively were longer than 6bp (Figure 2C). A previous study has suggested that homologous sequences with lengths of $\geq 6$ bp could form stable loops and result in structural variations (Montgomery, et al. 2013). Organellar DNA polymerases can effectively repair DSBs using microhomologous sequences as short as 6 bp in Arabidopsis thaliana (Garcia-Medel, et al. 2019). An in vitro study has also shown that the minimum primer length
for extension is 6 bp for DNA polymerase Klenow fragment (Zhao and Guan 2010). Additionally, we found that microhomology length is positively correlated with the length of the repeat unit (Pearson’s correlation $r = 0.634$, $P < 2.2e^{-16}$ in angiosperms; and $r = 0.746$, $P < 2.2e^{-16}$ in gymnosperms; Figures 2B and S6), consistent with the expectation that the longer the duplications, the greater the length of microhomologous sequences is needed to stabilize the annealed end before ligation (Ottaviani, et al. 2014; Vaughn and Bennetzen 2014). In summary, these results suggest that microhomology may be involved in the generation of tandem duplications, probably via microhomology-mediated repairing of DSBs (McVey and Lee 2008; Ottaviani, et al. 2014) or slippage strand replication (Darmon and Leach 2014). The prevalence of tandem repeats associated with microhomologous sequences in plant mitogenomes differs from the finding in the rice nuclear genome, in which tandem duplications rarely associate with microhomology (Vaughn and Bennetzen 2014). In the case of rice nuclear genome, tandem duplications are suggested to have formed through the repairing of DSBs via NHEJ (Vaughn and Bennetzen 2014). Future studies should evaluate whether the low abundance of microhomologous sequences is a rule in plant nuclear genomes, and whether different models govern tandem duplications in mitochondrial and nuclear genomes. Such studies would require both genome-wide and population-level scans of representative lineages.

*Abundance of microhomologous sequences in plant mitogenomes*
If the presence of microhomology can facilitate tandem duplication, it is then necessary to investigate the abundance of microhomologous sequences across plant mitogenome, and the relationship between microhomologies and genome sizes. In this study, we focused on 1,197 microhomologous sequences with length ≥ 6 bp, because these sequences were more likely to facilitate the formation of tandem duplicates than the shorter sequences (see above). These microhomologies were found in 1,716 STRs, which tend to be more abundant in intergenic regions (1,211 STRs), followed by RNA genes (60 STRs), and lastly exons (41 STRs) and introns (38 STRs) of protein-coding genes (Table S5). These results suggest that STRs and the associated microhomologies are more prone to accumulation in intergenic mtDNA, most likely because structural variations in intergenic regions are less functional constrained while such variations in coding regions will likely be removed by selection (Christensen 2014, 2013). Additionally, the structural variations in intergenic regions could be due to different processes of replication, recombination and repair that participate in the maintenance of plant mitogenomes stability, considering that plant mitogenomes are very different in isoforms (lineal, branched and circular) in contrast for example with human mitogenome (completely circular) (Oldenburg and Bendich 2015).

We noticed that most microhomologies were found in lineage- or mtDNA region-specific STRs. Of the 1197 screened microhomologies, 1,066 (89.1%) were species specific, found only in STR(s) of one species, and 998 occurred in only one STR (Table S5). Eighteen microhomologies showed high abundance (involved in 5–42 STRs) in mitogenomes of four species, including *Nymphaea colorata* (12
microhomologies), *Cucumis sativus* (three microhomologies), and *Physochlaina orientalis* (two microhomologies) (Table S5). Studies suggest that these three species experienced recent expansion of STRs (Alverson, et al. 2011; Dong, et al. 2018; Gandini, et al. 2019). The lineage- and region-specific use of microhomology is probably determined by availability of microhomologies around breakpoints or secondary structure that determine microhomology usage, and supports fast structural evolution of mitogenome after speciation.

We further examined the enrichment of microhomologies across plant mitogenomes, and found 75% of the 1,197 tested microhomologies were more abundant than expected in at least one mitogenome where the microhomology sequences formed STRs (Table S5). Among the 400 prevalent microhomologies (see Materials and Methods for details), 175 showed differential enrichment between angiosperms and gymnosperms and 186 differed between angiosperms and conifers (*P* < 0.05; Mann-Whitney U-test with Benjamini-Hochberg FDR adjustment; Figure 3, Table S4). Moreover, microhomologies identified in angiosperm STRs were more abundant in angiosperm species than in gymnosperm, and vice versa. For example, the TAG7 microhomology identified in the *nad7-1* region of *P. tabuliformis* was more highly enriched in conifers (mean ECH = 1.72) than in angiosperms (mean ECH = 0.867; *P* = 0.0427, Mann-Whitney U-test with Benjamini-Hochberg FDR adjustment; Figure 3A, Table S4). The estimated ECH value (3.40) was the highest for the mitogenome of *P. taeda*, with 595 observed TAG7 copies, which was 3-fold greater than that expected by chance (175 copies). These results suggest that
microhomologies have undergone lineage-specific expansion likely caused by microhomology-mediated tandem duplications in the mitogenome. Previous studies also show that expansion of STRs can occur in a group of closely related species (Gandini, et al. 2019). Consider all these findings, PCR amplification of mtDNA segments using primers derived from a reference genome can be unstable and unpredictable due to the high rate of structural variation in plants.

Conclusions

This study investigated the structural evolution of mitogenomes in pines and other plant taxa. We show that STRs can result in high polymorphism characterized by complex structural variation. These variants are species- and population-specific and form rapidly following speciation and during subsequent divergence. We illustrate that tandem repeats contribute to mitogenome expansion in plants, and that many repeats are associated with microhomology. A substantial portion of microhomologies in plant mitogenomes are long enough to result in structural variation, and the length of microhomology is positively correlated with the length of repeat units. These results suggested that microhomology are involved in the formation of tandem duplication. Most of the STR-associated microhomologies are differentially enriched between angiosperms and gymnosperms, indicating the lineage-specific usage and expansion of microhomology. Our results highlight the high prevalence of microhomology and its important role in generating structural variation in mitogenomes.
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Data Availability

All sequences obtained in this study were deposited in the GenBank database under the accession numbers MT792927–MT792949.

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**Figure Legends**

**Figure 1** Sequence structure of 19 haplotypes identified in *nad7*-1 of *Pinus tabuliformis*. (A) The two conserved blocks are indicated by a black rectangle; seven
motifs are indicated by pentagons with colors corresponding to those used in (B); the highly variable motif Rp is indicated by gray pentagons; and the TAG7 microhomology is indicated by a blue open triangle. Three insertions specific to a subset of motifs are indicated by yellow, light blue, and black arrows. A large sequence block (DUP) duplicated in H11–19 is outlined in red. (B) Alignment of the seven motifs.

**Figure 2** Tandem repeats associated with end homology in plant species. (A) Frequency of tandem repeats associated with end homology in angiosperm and gymnosperm species. In each box, horizontal lines from top to bottom refer to the first quartile, median, and third quartile. Each red, green, and blue dot represents an angiosperm, conifer, and gymnosperm (excluding conifers), respectively. (B) Length of end homology against repeat units. Correlations were evaluated by Pearson’s correlation coefficient tests. (C) Distribution of end homology lengths. Values of ≥ 6 bp suggest microhomology-mediated tandem duplication.

**Figure 3** Differential enrichment of microhomologies in mitogenomes of angiosperms, gymnosperms and conifers. (A) Microhomology “TAAAGGT” (TAG7); (B) “ATATACG”; (C) “AGCAAGC”; (D) microhomology “AGTCTTC”. Each red, green, and blue dot represents a representative angiosperm, conifer, and non-conifer gymnosperm, respectively. *P*-values of Mann-Whitney U-tests after Benjamini-Hochberg correction for ECH values of angiosperm vs. gymnosperm and angiosperm vs. conifer are shown in each boxplot. The top, middle, and bottom...
horizontal lines in each boxplot indicate the first quartile, median, and third quartile, respectively.

Supporting Information

Figure S1 Schematic representation of double-strand break (DSB) repair pathways that lead to structural rearrangements. (A) Non-homologous end-joining (NHEJ) pathway. The Ku heterodimer binds to the ends of a DSB induce the deletion/insertion of a few nucleotides (red line). (B) Microhomology-mediated end-joining (MMEJ) pathway. Microhomologous sequences (green box) anneal with broken ends before joining, resulting in deletions/insertions. (C) Fork stalling and template switching (FoSTeS) pathway. The lagging strand (blue dashed line) caused by replication fork stalling invades the other active replication fork by annealing with a microhomologous sequence (green box), resulting in deletion/insertion. (D) Microhomology-mediated break-induced replication (MMBIR) pathway. A DSB is created by the collapse of a replication fork, and the 3’ overhang of the broken end invades a new microhomologous template (green box) and re-initiates replication of the invaded template until annealing back to the original fork, leading to complex structural variation. This figure was modified from McVey and Lee (2008) and Ottaviani et al. (2014).

Figure S2 Distribution of 19 mitotypes of Pinus tabuliformis. (A) Pie charts showing the proportions of mitotypes (H1–H19) in each of the 17 populations. (B)
Mitochondrial (mt) DNA network of 22 mitotypes. H1–H19 were observed only in *P. tabuliformis*; H20–H22 were found in *Pinus densata*; Hsyl was observed in *Pinus sylvestris*. Links with more than one mutational step are indicated by the number above, and the circle size is proportional to the frequency of mitotypes over all populations. The 12 mitotypes occurring multiple times are shown in separate colors. (C) Neighbor-joining tree of *nad7-*1 mitotypes. *P. sylvestris* mitotype was used as an outgroup. Bootstrap values are shown at branch nodes.

**Figure S3** Microhomology in tandem repeats of *Pinus taeda* mitogenome. Three repeat units were identified (indicated in red). The last unit (12 bp; 182,023–182,034 bp) was less than half the length of the consensus sequence (37 bp), and was identified as a microhomologous sequence. The sequence homologous to this 12 bp microhomology in each repeat unit is underlined. The 10 bp sequences before (181,939–181,948 bp) and after (182,035–182,044 bp) the repeat region are shown.

**Figure S4** Evolutionary network to hypothesize the origin of seven repeat motifs in *nad7-*1 of *P. tabuliformis*. “Del” denotes deletion; “Ins” denotes insertion; “Sub” denotes substitution. The complex replacement is indicated by the length of the sequences before and after the change, e.g., 13 bp → 3 bp indicates that a 13 bp sequence was replaced by a 3 bp sequence with a different composition. Dashed lines indicate possible reversed pathways according to repeat arrays.
**Figure S5** Tandem repeats in mitogenomes of 136 seed plants (128 angiosperms and eight gymnosperms). (A) Length of repeat unit in angiosperm and gymnosperm species. (B) Copy number of repeats in angiosperms and gymnosperms. (C) Correlation between mitogenome size and length of tandem repeats (Pearson’s $\rho = 0.708$, $P < 2.2e^{-16}$).

**Figure S6** Length of end homology against repeat units in groups of species with different mitogenome sizes. (A-C) gymnosperms, (D-G) angiosperms. Correlations were evaluated by Pearson’s correlation coefficient tests.

**Table S1** Number and length of tandem repeats in mitogenomes of 136 seed plants.

**Table S2** Geographic locations, sample sizes ($N$), and mitotype frequencies of 51 populations of three pine species.

**Table S3** Sample size and number of nad7-1 haplotypes identified in various *Pinus* species.

**Table S4** Comparisons of ECH values of microhomologies between angiosperms and gymnosperms, and between angiosperms and conifers. $P$-values were estimated by Mann-Whitney U-test and corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) adjustment.
Table S5 Genomic positions of tandem repeats with microhomologies ≥ 6 bp. The observed and expected number of microhomologous sequences on both DNA strands in the mitogenome (Obs$_{MH}$ and Exp$_{MH}$, respectively) were counted, and ECH (= Obs$_{MH}$/Exp$_{MH}$) of each microhomologous sequence (detected in tandem repeats) was calculated. ECH was not calculated when Exp$_{MH}$ = 0.

Data S1 Alignment of 23 nad7-1 mitotypes.
**Figure 1** Sequence structure of 19 haplotypes identified in *nad7-1* of *Pinus tabuliformis*.

(A) The two conserved blocks are indicated by a black rectangle; seven motifs are indicated by pentagons with colors corresponding to those used in (B); the highly variable motif Rp is indicated by gray pentagons; and the TAG7 microhomology is indicated by a blue open triangle. Three insertions specific to a subset of motifs are indicated by yellow, light blue, and black arrows. A large sequence block (DUP) duplicated in H11–19 is outlined in red. (B) Alignment of the seven motifs.
Figure 2 Tandem repeats associated with end homology in plant species. (A) Frequency of tandem repeats associated with end homology in angiosperm and gymnosperm species. In each box, horizontal lines from top to bottom refer to the first quartile, median, and third quartile. Each red, green, and blue dot represents an angiosperm, conifer, and gymnosperm (excluding conifers), respectively. (B) Length of end homology against repeat units. Correlations were evaluated by Pearson’s correlation coefficient tests. (C) Distribution of end homology lengths. Values of $\geq 6$ bp suggest microhomology-mediated tandem duplication.
Figure 3 Enrichment of the microhomology between angiosperm, gymnosperm and conifer mt genomes. (A) microhomology TAG7 (“TAAAGGT”). (B) “ATAACGT”. (C) “AGCAAGC”. (D) microhomology “AGTCTTC”. Each red, green, and blue dot represents one representative angiosperm, conifer, and non-conifer gymnosperm, respectively. P-values of Mann-Whitney U-tests after Benjamini-Hochberg correction for ECH values of angiosperm vs. gymnosperm and angiosperm vs. conifer were show on each plot. In each box, horizontal lines from top to bottom refer to the first quartile, median, and third quartile, respectively.