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Mitochondrial DNA capture and divergence in *Pinus* provide new insights into the evolution of the genus

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ABSTRACT

The evolution of the mitochondrial (mt) genome is far from being fully understood. Systematic investigations into the modes of inheritance, rates and patterns of recombination, nucleotide substitution, and structural changes in the mt genome are still lacking in many groups of plants. In this study, we sequenced >11 kbp mtDNA segments from multiple accessions of 36 pine species to characterize the evolutionary patterns of mtDNA in the genus *Pinus*. We found extremely low substitution rates and complex repetitive sequences scattered across different genome regions, as well as chimeric structures that were probably generated by multiple intergenomic recombinations. The mtDNA-based phylogeny of the genus differed from that based on chloroplast and nuclear DNA in the placement of several groups of species. Such discordances suggest a series of mtDNA capture events during past range shifts of the pine species and that both vertical and horizontal inheritance are implicated in the evolution of mtDNA in *Pinus*. MtDNA dating revealed that most extant lineages of the genus originated during Oligocene–Miocene radiation and subgenus *Strobus* diversified earlier than subgenus *Pinus*. Our findings illustrate a reticular evolutionary pathway for the mt genome through capture and recombination in the genus *Pinus*, and provide new insights into the evolution of the genus.

1. Introduction

Modes of inheritance, rates and patterns of recombination, nucleotide substitution, and structural changes are key elements that characterize genome evolution. The plant mitochondrial (mt) genome is maternally inherited and, in general, is characterized by an unusually low silent substitution rate along with rapid size and structural changes (Palmer et al., 2000). However, extremely high variations in the substitution rate and length variation in several angiosperm and gymnosperm genera have been reported (Cho et al., 2004; Jaramillo-Correa et al., 2013; Mower et al., 2007; Parkinson et al., 2005; Ran et al., 2010). These studies illustrate that patterns of mtDNA variation can be lineage- and gene region-specific. Genome capture and recombination induced by hybridization are two other evolutionary phenomena associated with the mt genome but, as of yet, are poorly investigated systematically (Barr et al., 2005; Galtier et al., 2009). Gene flow and introgressive hybridization are pervasive in plants (Abbott et al., 2013). The hybridization dynamics can be affected by the relative abundance of hybridizing species with the expectation that gene flow will be predominately from the more abundant species towards the less abundant one (Lepais et al., 2009). When a colonizing species spreads into an area already occupied by a related species, the invading species is initially rare, and thus a massive introgression of resident genes into the invader's gene pool would be expected (Currat et al., 2008). The asymmetric introgression from local species into invader is stronger for genome components with low migration ability and limited intraspecific gene flow, such as mtDNA in plant (Currat et al., 2008). The reason is that if intraspecific gene flow is low, genome components from the resident species that have introgressed into the invading species will not be diluted by migrants from other populations of the invading species, and would become rapidly fixed in the gene pool of the invader following demographic growth (Currat et al., 2008; Petit and Excoffier, 2009). In this context, hybridization between invading and local species during range shifts can promote introgression from local species into colonizing species, and resulting in invading species capturing the mt genome from local species (Petit and Excoffier, 2009). Hybridization could also promote inter-parental mtDNA recombination, mediated by occasional paternal leakage (Jaramillo-Correa and Bousquet, 2005; Städler and Delph, 2002; Wang et al., 2011). In some cases, the recombinants can colonize large areas (Wang et al., 2011), and may even replace the original mitotype during range shifts. The capture of mtDNA could produce phylogenies that differ from those of paternally or biparentally

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inherited genetic markers, while inter-parental mtDNA recombination can generate phylogenies that vary between mtDNA segments. However, the frequency and evolutionary consequences of mtDNA capture and recombination at the phylogenetic scale in different groups of plants are unclear.

The genus *Pinus* is one of the largest extant gymnosperm genera, with more than 100 species spread over the Northern Hemisphere (Mirov, 1967). Rates of mtDNA substitution are available for only a few species, representing a tiny fraction of this large and diverse genus (Mower et al., 2007). This sparse sampling severely limits our ability to detect historical changes in the mt genome at a phylogenetic scale. In *Pinus*, the mt genome is maternally inherited and dispersed via seeds, while the chloroplast (cp) genome is paternally inherited and transmitted via pollen and seeds (Neale and Sederoff, 1989; Wang et al., 1996). Pines are not strongly reproductively isolated: frequent introgressive hybridizations between current and historical parapatric taxa have been documented (Cullingham et al., 2012; Mirov, 1967; Senjo et al., 1999; Wang et al., 2011; Willyard et al., 2009). Historical lateral transfer and recombination of the mt genome has been reported in mtDNA and cpDNA based phylogeographical studies of *Pinus* (Godbout et al., 2012; Senjo et al., 1999; Tsutsui et al., 2009; Wang et al., 2011). However, only a few genome regions and taxa have been surveyed in previous studies, thus it is unclear whether the capture and recombination of mtDNA were widespread phenomena in the evolution of the genus *Pinus*.

Adequate representations of the species and mt genomes are required for reconstruction of the biogeographical history of the genus. A previous study based on cp and nuclear DNA data suggested a Cretaceous origin for the genus *Pinus* and Miocene divergence for most extant subsections (Willyard et al., 2007). However, the processes of divergence in the genus remain unclear. Distinct phylogenies may be obtained from genetic markers with different inheritance pathways, and a comparison of the three genomes will provide an opportunity to eliminate the ambiguities in the evolutionary history of *Pinus*. In addition, because of limited seed dispersal and the low substitution rate in the mt genome, the maternal phylogeographic structure of the genus *Pinus* could have persisted for longer than both the cp and nuclear DNA structures. Thus mtDNA data should help decipher the dispersal and speciation history of the genus.

In this study, we examined sequence variation in nine mtDNA regions in 36 pine species representing nine of the 11 subsections of the genus (Gernandt et al., 2005), and five outgroups representing three genera in Pinaceae. By using phylogenetic analyses and molecular dating, we investigated (1) the substitution rate and structural changes of mtDNA in the genus *Pinus*; (2) historical introgressions in the genus that resulted in mtDNA capture and recombination; and (3) the biogeographical history of the genus.

2. Materials and methods

2.1. Plant materials

We sampled 36 pine species to represent nine of the 11 subsections recognized by Gernandt et al. (2005). Most species were represented by multiple accessions, and a total of 173 individuals were analyzed (Table 1; Tables S1 and S2). *Picea abies*, *Picea koraiensis*, *Abies holophylla*, *Abies firma* and *Cathaya argyrophylla* were selected as outgroups because of their close relationships to *Pinus* (Wang et al., 2000). All samples for each species were collected either from documented individuals grown by different institutions or from natural stands (Fig. 1; Tables S1 and S2).

2.2. DNA isolation, PCR amplification, and sequencing

Genomic DNA was extracted from needles or seedlings using a Plant Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. Nine mtDNA regions: *matR*, *rrn*18 (18S ribosomal RNA), *cox*1, *nad*1-2 (*nad*1 intron 2), *nad*3-*rps*12 (the intergenic region between *nad*3 and *rps*12), *nad*4-3 (*nad*4 intron 3), *nad*5-1 (*nad*5 intron 1), *nad*5-4 (*nad*5 intron 4) and *nad*7-3 (*nad*7 intron 3), were selected for polymerase chain reaction (PCR) amplification. These regions were chosen because they contained reasonable amount of variation between pine species and can be aligned across the genus. The primers for these loci were obtained from previous studies (Dumolin-Lapegue et al., 1997; Soranzo et al., 1999; Wang et al., 2000, 2014) with the exception for *nad*7-3, for which the primers were designed based on *Pinus taeda* mt genome assembly (Neale et al., 2014). The primer sequences, positions relative to *P. taeda* mt genome, annealing temperature, and sizes of each product are listed in Table S3. The

Table 1
List of species sampled. *Pinus* classification follows Gernandt et al. (2005). Numbers of mitotypes identified in each species are in parentheses.

Subgenus *Strobus*			Subgenus *Pinus*		
Section and subsection	Species	Sample size	Section and subsection	Species	Sample size
Sect. *Parrya*			Sect. *Pinus*		
Subsect. *Balfourianae*	*P. aristata*	4 (2)	Subsect. *Pinus*	*P. densiflora*	4 (1)
	P. balfouriana	2 (2)		*P. merkusii*	4 (1)
Sect. *Quinquefoliae*				*P. sylvestris*	4 (1)
Subsect. *Gerardianae*	*P. bungeana*	43 (1)		*P. tabuliformis*	4 (3)
	P. gerardiana	3 (1)		*P. thunbergii*	4 (1)
	P. squamata	2 (1)		*P. yunnanensis*	4 (3)
Subsect. *Krempfianae*	*P. krempfii*	4 (1)	Subsect. *Pinaster*	*P. halepensis*	4 (1)
Subsect. *Strobus*	*P. armandii*	4 (1)		*P. pinaster*	4 (1)
	P. koraiensis	4 (1)		*P. canariensis*	6 (2)
	P. wallichiana	3 (3)		*P. roxburghii*	3 (2)
	P. parviflora	4 (2)	Sect. *Trifoliae*		
	P. albicaulis	3 (2)	Subsect. *Contortae*	*P. banksiana*	4 (1)
	P. strobus	2 (1)		*P. contorta*	4 (1)
	P. ayacahuite	4 (1)	Subsect. *Australes*	*P. taeda*	4 (1)
	P. pumila	1 (1)		*P. rigida*	4 (1)
	P. peuce	2 (1)	Subsect. *Ponderosae*	*P. maximinoi*	4 (1)
	P. cembra	2 (1)		*P. ponderosa*	4 (1)
	P. strobiformis	7 (3)	Outgroups	*Picea abies*	1 (1)
	P. monticola	5 (1)		*Picea koraiensis*	1 (1)
	P. lambertiana	5 (1)		*Abies holophylla*	1 (1)
	P. flexilis	4 (1)		*Abies firma*	1 (1)
				Cathaya argyrophylla	1 (1)