

Paternal inheritance of chloroplast and mitochondrial DNA in huon pine (*Lagarostrobos franklinii*).

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Abstract

In conifers, plastid DNA is passed on through the male line (pollen grain). Transfer of mitochondrial DNA is maternal in the well-researched Pinaceae family (conifers I) and is considered mostly paternal in other conifer families (conifers II). However, few studies in conifers II have definitely established the mode of transfer using molecular markers within species. This is the first molecular genetic study on organelle transfer in the Podocarpaceae family. Two intraspecific crosses of a rare Tasmanian podocarp, *Lagarostrobos franklinii*, were established with the same iconic father, which is a vegetative clone of a 11,000 year-old tree. Polymorphic positions in the parents' genomes of one cross were identified using genome skimming and bioinformatics. Illumina data were compared with a published plastome and newly assembled mitochondrial DNA contigs representing about half of the father's genome. Derived PCR-based markers were used to genotype parents and offspring of both crosses. Plastid DNA and mitochondrial DNA were both paternally inherited. We discuss other molecular genetic studies on mitochondrial DNA transfer in conifers II, which all suggest paternal transfer. This could mean that this trait evolved only once, between 280 and 320 MYA, in the ancestor of this group.

Introduction

The mode of inheritance of organelle DNA influences population genetics. Paternal transfer disperses organelle DNA through both pollen and then with seeds, increasing effective population size and enhancing natural selection over genetic drift. Consequently organelle-encoded genes show greater genetic differences between populations (higher G_{st} values) in species with maternal inheritance compared to those with paternal inheritance (Petit et al. 2005). Additionally, the mode of inheritance can affect sex types. Cytotypes are selected to promote their own transfer (Burt and Trivers 2006). Maternal transfer of mitochondrial DNA (mtDNA) could lead to cytoplasmic females that coexist with cosexuals (gynodioecy), as observed in many angiosperms (Burt and Trivers 2006). Paternal transfer can potentially result in cytoplasmic males that coexist with cosexuals. The latter system can easily evolve toward dioecy, males and females in the same population (de Jong and Shmida 2024).

In angiosperms, organelle DNA is usually passed on through the egg cell (Mogensen 1996; Schneider 2023; Chung 2025). This is unsurprising since egg cells contain more organelles than sperm cells. However, additional mechanism further prevent paternal transfer. For instance, in *Arabidopsis thaliana*, plastids and mitochondria are eliminated from the generative cell in the pollen (Chung 2025). In conifers plastid DNA (ptDNA) is transferred paternally with the pollen (Adams 2019). The single known exception is the genus *Cunninghamia* (Cupressaceae), with two species in China and Taiwan that transfer ptDNA maternally (Lu et al. 2001). In the Pinaceae (conifers I) mtDNA is always maternally transferred (Petit et al. 2005; Adams 2019), while in the other conifer families (conifers II or cupressophytes: Araucariaceae, Cupressaceae, Podocarpaceae, Sciadopityaceae and Taxaceae) mtDNA transfer is considered mostly paternal (Mogensen 1996; Adams 2019; but see discussion).

Mogensen (1996) suggested that the mode of organelle DNA transfer for a single or a few species is representative of their larger phylogenetic group. This paradigm is generally accepted (e.g., Whittle and Johnston 2002). However, related species may have different modes of transfer. For example, ptDNA is maternally inherited in the angiosperm *Turnera sidoides* (Speranza et al. 2007) and paternally in *T. ulmifolia* (Shore and Triassi 1998). The *Turnera* genus, with about 100 species in the American subtropics, is an interesting subject for studying ptDNA transfer evolution. Paternal inheritance of mtDNA occurs in some *Cucumis* species but not in others (Havey et al. 1998). This variation within a genus is unknown for gymnosperms.

Early studies of organelle transfer used microscopy to track organelle presence and fate in pollen and egg cells during fertilisation and embryo development (Mogensen 1996). These studies are informative but give no definite answer when organelles are present in both sperm and egg cells. For instance, in *Passiflora*, cotyledons retained organelles from both parents, but only one type remained in the first leaves (Shrestha et al. 2021). Subsequent studies used interspecific crosses and examined the presence of species-specific markers in hybrids (Mogensen 1996). However, there is co-adaptation of organelle and nuclear DNA within a species (Greiner and Bock 2013), so interspecific crosses may give unexpected results. For instance, in crosses between *Pinus mugo* and *P. sylvestris*, hybrids received maternal ptDNA from *P. mugo*, while in the reciprocal cross hybrids received paternal ptDNA (Kormutak et al. 2018), as documented for all Pinaceae investigated so far.

Ideally, studies on organelle transfer involve experimental crosses within species. However, intraspecific nucleotide diversity of mtDNA or ptDNA is low (for instance, Clark and Carbone 2008 for *Lagarostrobos franklinii*), making it challenging to identify DNA sequence variants that discriminate between paternally and maternally derived organelles and to develop PCR-based markers for genotyping offspring. While plastid genome sequences are known for many species, mitogenomes are largely unknown for conifers II and need to be assembled first.

Leaves of established plants should be sampled rather than seeds. This procedure allows for competition between different cytotypes within the plant and the detection of the correct remaining type in the leaves (Shrestha et al. 2021; Chung 2025). In some gymnosperms, Podocarpaceae included (Kruijt, personal observation 2026), seeds can develop without fertilisation, containing only diploid nucellus tissue with organelles from the mother plant. Including such unfertilised seeds in the genetic analysis could mislead us into thinking that organelle transfer is maternal.

This paper examines the mode of transfer of ptDNA and mtDNA in *Lagarostrobos franklinii*, a conifer in the Podocarpaceae family. This ancient family of 194 species in 19 genera (Farjon 2001) originated in the south of Gondwana. Gondwana's breakup led to the distribution of specialised podocarps across Africa, Asia, Australia, New Zealand, Central and South America, and several South Pacific islands. Fossils of podocarps found on Antarctica show they were once a dominant part of the vegetation (Fontes and Dutra 2010). Most podocarps are dioecious (Walas et al. 2018). No molecular genetic studies on organelle inheritance have been done in the Podocarpaceae. The mode of transfer of both

ptDNA and mtDNA is unknown, and the review of Mogensen (1996) labeled this family with a question mark (“information fragmentary, no detailed studies available”).

Material and methods

Plant material

Lagarostrobos franklinii, native to Tasmania, can grow up to 25 m tall with a diameter at breast height of 150 cm. Tree rings indicated that many individuals were over 1,000 years old (Anker et al. 2001). In Pinetum Blijdenstein (Hilversum, the Netherlands) a male plant (accession number 950044, Thuenen-ID: Gym109) and a female plant (female 1, accession 950043, Thuenen-ID: Gym108) grow next to each other since 1998. The garden acquired both plants from the Botanical Garden of Atlanta (US). The male plant is a clone from a dominant tree over 11,000 years old (Shapcott 1997), growing on Mount Read, near Lake Johnston (Tasmania) at c.1000 meter above sea level. Female 1 originates from Mount Cleveland, Tasmania, where it was cloned from a tree growing at 370 meter above sea level. Another female (female 2, Thuenen-ID: Gym126) was acquired from the plant breeding company Esveld in the Netherlands. The male fertilised both females, with no other males nearby. Seeds were collected in 2019. Seed germination is slow in this species and germination percentages are low (Shapcott 1991). Seeds were kept for 36 weeks in wet sand and emerging seedlings were potted in late 2020. At harvest in March 2025, only 5 offspring (Thuenen-IDs: Gym122-Gym125, Gym127) remained for DNA analysis, with heights between 10 and 20 cm.

Preparation of DNA

DNA was extracted from leaves and stem as described in Ziegenhagen et al. (1993), modified only by adding 50 µl 1 M DTT (AppliChem GmbH, Darmstadt, Germany) and 20 µl Proteinase K (20 mg/ml; New England Biolabs, Frankfurt/Main, Germany) after the extraction buffer. DNA was then resuspended in 50 µl low TE buffer. NanoDrop and Qubit (BR Assay) measurements (Thermo Fisher Scientific) were used to analyse DNA quality and quantity.

Whole genome sequencing of the male and female 1 individual and trimming of the Illumina reads

Genomic DNA libraries were created from ~ 1.0 µg of total DNA of each sample and sequenced on an Illumina NovaSeq platform to create 150 bp paired-end reads (GENEWIZ Germany GmbH, from Azenta Life Sciences; Leipzig, Germany). In total, 13.18 million reads (3.95 Gbp data output) were generated from the individual female 1 and 10.36 million reads (3.11 Gbp) from the individual male (Kersten 2025). These data sets each correspond to a coverage of 0.27X (female 1) or 0.21X (male) in relation to the approximate size of the haploid nuclear genome of 14.896 Gbp estimated based on the C-value of *L. franklinii* analysed by Davies (1996) using flow cytometry (C-value provided by the Plant DNA C-values Database of Kew Royal Botanic Gardens; <https://cvalues.science.kew.org/>).

Illumina reads from both individuals were trimmed using the tool *trim reads* version 3.0 of the CLC Genomics Workbench (CLC-GWB) version 24.01 applying the following parameters: trim using quality scores = yes; quality limit = 0.05; trim ambiguous nucleotides = yes; maximum number of ambiguities = 2; automatic read-through adapter trimming = no; trim adapter list = yes; remove 5'/3' terminal nucleotides = no; minimum length = 80 bases; save broken pairs = yes.

De novo assembly of reads from the male and selection of mitochondrial contigs containing protein-coding genes

To remove plastome-derived reads prior to assembly, trimmed reads of the male (Gym109) were mapped to the *L. franklinii* ptDNA sequence (MW470983) (Stull et al. 2021) keeping unmapped reads as described previously (Kersten et al. 2022), but with the following modified mapping parameters: length fraction = 0.95; similarity fraction = 0.97. Unmapped paired-end reads were assembled by the tool *de novo assembly* v.1.5 of CLC-GWB (parameters: mapping mode = create simple contig sequences; automatic bubble size = yes; minimum contig length = 1,000; automatic word size = yes; perform scaffolding = yes; auto-detect paired distances = yes). The assembly resulted in 2,501 contigs with N50 of 1,752 bp and an accumulated contig length of about 4.99 Mbp. In total, 12 mtDNA contigs that include protein-coding genes were identified by blastN searches of the *L. franklinii* contigs against coding sequences retrieved from mitochondrial scaffolds of *Podocarpus macrophyllus* (MW354416-28) (Kan et al. 2021) with an expected threshold below e^{-10} and a greatest identity above 89%. The GC content of these contigs was calculated using the tool *create sequence statistics* of the CLC-GWB.

Identification of DNA sequence variants in the plastome and mitochondrial genome of female 1 and the male

Trimmed Illumina reads of the respective individual were mapped to both the *L. franklinii* ptDNA sequence (MW470983) (Stull et al. 2021) and the 12 mitochondrial contigs generated in this study (used as a combined reference). Mapping was performed using the tool *map reads to reference* version 1.9 of CLC-GWB (parameters: match score = 1; mismatch cost = 2; cost of insertions and deletions = linear gap cost; insertion cost = 3; deletion cost = 3; length fraction = 0.95; similarity fraction = 0.97; global alignment = no; auto-detect paired distances = yes; non-specific match handling = map randomly; execution mode = standard; minimum seed length = 15).

Individual SNPs and small InDels in ptDNA and mtDNA sequences were identified by analysing the mappings with the CLC-GWB tool *basic variant detection* version 2.6 (parameters: ploidy = 1; ignore positions with coverage above = 100.000; ignore broken pairs = yes; ignore non-specific matches = regions; minimum read length = 20; minimum coverage = 8; minimum count = 8; minimum frequency = 95%; no further filters set). Shared ptDNA variants between female 1 and male were identified using the CLC-GWB tool *identify shared variants* version 1.3. Shared variants were removed from the variant tables of female 1 and male. A non-redundant list of polymorphic variant positions (comparing female 1 and male) was established and genotype information was joined from the individual variant tables.

Marker development and genotyping of parents and offspring

We developed simple PCR-based markers to distinguish between ptDNA- and mtDNA-genotypes from male and female 1, based on selected polymorphic variant positions. The same markers were applied to female 2. PCR primers flanking selected SNPs/InDels (size range of PCR-products: 166 bp–554 bp; shorter lengths preferred for poor quality DNA) were design using Eprimer3, available online at the biotools page of GabiPD (<https://www.gabipd.org/biotools/>) (Usadel et al. 2012). Primer specificity was checked by BlastN analysis (CLC-GWB; tool *BLAST* version 1.2) versus the *L. franklinii* ptDNA sequence (MW470983) (Stull et al. 2021) and the 12 mitochondrial contigs generated in this study (used as a combined reference). Information on the developed markers and related primers is provided in Table S1.

PCR reactions contained ~ 50 ng template DNA, 1x PCR buffer, 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM of each primer, 0.3 μL DMSO (100%; Solis BioDyne, Tartu, Estonia) and 1 Unit DCSPol DNA polymerase (DNA Cloning Service, Hamburg, Germany) in a total volume of 25 μl. PCR was carried out in a Sensoquest Thermocycler (Göttingen, Germany) with a pre-denaturation step at 94°C for 4 min, followed by 35 cycles at 94°C for 45 sec, at a suitable annealing temperature for each primer combination (see Table S1) for 45 sec, at 72°C for 60 sec and a final elongation at 72°C for 7 min. PCR amplification products were checked relative to a Smart ladder (Eurogentec, Seraing, Belgium) on a 1.3% agarose gel stained with Roti-Safe GelStain (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

For Sanger sequencing, 1–6 μl PCR product (volume depending on PCR product concentration estimated from the related band at the agarose gel) and 1 μl of 10 μM forward or reverse primer solution, respectively in a total volume of 7 μl (completed with H₂O) were sent off to StarSEQ (Mainz, Germany). Sanger sequences were mapped to the ptDNA and mtDNA reference sequences using the tool *assembly sequences to reference* of the Sanger sequencing analysis toolbox of CLC-GWB. Genomic positions of interest were manually inspected in the mappings and related genotype information was extracted.

Results

The transfer of ptDNA and mtDNA in *L. franklinii* was analysed in offspring from two intraspecific crosses involving the same male and two females (1 and 2). Polymorphic ptDNA/mtDNA-positions were identified comparing the parents of the first cross (female 1 X male) based on Illumina sequencing of total DNA using the genome-skimming strategy (Straub et al. 2012; Dodsworth 2015) and subsequent bioinformatic analyses. Since only ptDNA sequences (Sudianto et al. 2019; Stull et al. 2021), but no mtDNA sequence of *L. franklinii* have been published, a draft assembly based on the male's Illumina data was performed. Out of 2501 contigs, 12 mtDNA contigs were selected, including protein-coding genes, with an accumulated contig length of 880,497 bp (size range: 45,117–125,264 bp) and a mean GC content of 46.81% (Kersten et al. 2025). These 12 contigs represent about half of the total mtDNA sequence of *L. franklinii*, considering the 1.54 Mb size of the mtDNA genome of *Araucaria cunninghamii* (Kan et al. 2021). To identify polymorphic ptDNA sequence positions, the Illumina data of female 1 and the male were compared to a published complete ptDNA sequence of *L. franklinii* (Stull et al. 2021).

Eight single nucleotide variants (SNVs) were found between the parents of the first cross. Analysis of the data of female 1 against the 12 mtDNA contigs of the male identified 30 SNVs, multi nucleotide variants or insertion/deletion variants. The F1-individuals of both crosses were genotyped at four ptDNA and four mtDNA-positions by PCR-based markers (Table S1) and Sanger sequencing (available at Kersten et al. 2025).

All markers in the offspring from cross 1 (female 1 X male) matched the genotype of the father (Tables 1 and 2). Female 2, the mother of cross 2, was genetically distinct from female 1 for one ptDNA marker (Table 1) and three mtDNA markers (Table 2). Female 1 and 2 are therefore different genotypes, not clones. At all three ptDNA positions where the male and female 2 differed, offspring from cross 2 showed the genotype of the father (Table 1). At the single mtDNA position where the male and female 2 differed, offspring had the genotype of the father (Table 2). Thus, transfer of ptDNA and mtDNA was paternal in all five offspring of the two intraspecific crosses analysed.

Table 1

Plastome genotype of offspring from two crosses involving the same male and two females (DNA markers and related ptDNA positions in Table S1)

Plant ID	Genotype/allele at four ptDNA positions analysed by markers ptDNA_M1-M4			
	ptDNA_M1	ptDNA_M2	ptDNA_M3	ptDNA_M4
Male	T	G	A	C
Female 1	G	T	G	A
Offspring 1.1	T	G	A	C
Offspring 1.2	T	G	A	C
Offspring 1.3	T	G	A	C
Offspring 1.4	T	G	A	C
Female 2	T	T	G	A
Offspring 2.1	T	G	A	C

Table 2

Mitochondrial genotype of offspring from two crosses involving the same male and two females (DNA markers and related mtDNA positions in Table S1)

Plant ID	Genotype/allele at four mtDNA positions analysed by markers mtDNA_M1-M4			
	mtDNA_M1	mtDNA_M2	mtDNA_M3	mtDNA_M4
Male	C	G	T	CAAA
Female 1	A	T	G	-
Offspring 1.1	C	G	T	CAAA
Offspring 1.2	C	G	T	CAAA
Offspring 1.3	C	G	T	CAAA
Offspring 1.4	C	G	T	CAAA
Female 2	C	G	G	CAAA
Offspring 2.1	C	G	T	CAAA

Discussion

Mechanisms behind paternal transfer

Mechanisms of paternal organelle transfer were reviewed by Mogensen (1986). In Pinaceae the pollen releases numerous plastids and mitochondria and two male nuclei in the egg cell. Female plastids in the egg cell migrate to the periphery and are not included in the newly formed cytoplasm surrounding the zygote. Female mitochondria occur throughout the egg cell and are abundant in the new cytoplasm surrounding the zygote. This new cytoplasm may also contain some male mitochondria (c. 10% in *Pinus monticola*, Owens 2004). Some of these male mitochondria may be successful, which results in some paternal leakage of mtDNA. This pattern applies to all Pinaceae.

Mechanisms in conifers II vary between families and have been studied in less detail (Mogensen 1986). The pollen releases two sperm cells, rather than nuclei, in the egg cell. The sperm nucleus remains surrounded by its own cytoplasm containing specialised plastids and mitochondria. Female organelles are distributed throughout the cytoplasm of the egg cell, but female mitochondria and/or plastids degenerate at some stage. Mogensen (1986) pointed out that this mechanism is also leaky when degeneration of female organelles is incomplete, in which case they can be included in the cytoplasm of the embryo. Such cases of maternal leakage have been documented in conifers II.

A microscopic study (Wilson and Owens 2003) on *Podocarpus totara* revealed that maternal plastids in egg cells showed signs of degeneration and appeared non-functional, suggesting paternal transfer of ptDNA. However, both female and male mitochondria appeared to be intact in the pro-embryo, suggesting that mtDNA transfer could be biparental. Wilson and Owens (2003) emphasised that

molecular genetic methods are needed to resolve this issue. Our study is the first to document strict paternal inheritance of both ptDNA and mtDNA for a member of the Podocarpaceae family. The mechanisms, especially the elimination of female plastids and mitochondria, remain a topic for future study.

Paternal transfer of ptDNA in all conifers

All conifers investigated so far, with the exception of *Cunninghamia*, transfer of ptDNA is paternal. In cycads (Cafasso et al. 2001; Zhong et al. 2011), *Ginkgo biloba* (Feng et al. 2023) and gnetophytes (Mogensen 1996) ptDNA transfer is maternal. Angiosperms also show maternal transfer with few exceptions (Mogensen 1996). The evolution of paternal transfer of ptDNA in conifers may have occurred after they branched off from the other major plant groups around 340 MYA (Stull et al. 2021) and before different conifer families emerged around 300 MYA (Leslie et al. 2018). *Cunninghamia*, the most basal Cupressaceae genus, diverged from other conifers around 157–171 MYA (Leslie et al. 2018). In this time schedule *Cunninghamia* is a reversion from paternal to maternal ptDNA transfer.

Paternal transfer of mtDNA in conifers II

Conifers II was previously considered a mix of species with maternal and paternal mtDNA transfer (Mogensen 1996; Adams 2019). However, this view may need revision. First, studies on hybrids between species may not be representative. Second, in some cases results from modern DNA studies differ from those suggested by microscopy. An electron microscopic study of *Taxus baccata* (Pennell and Bell 1988) showed the presence of mitochondria in both pollen and egg cell. Based on this study Mogensen (1996) suggested that mtDNA transfer was maternal. However, molecular methods showed 98% paternal mtDNA transfer in *T. baccata* (Chybicki et al. 2016) and 100% paternal mtDNA transfer in *T. cuspidata* (Su et al. 2018). Bross et al. (2025) showed that the interspecific hybrid between *T. baccata* and *T. cuspidata* received both ptDNA and mtDNA markers from its paternal parent. Paternal transfer of both mtDNA and ptDNA may be the rule in Taxaceae.

Third, some new data have become available. Worth et al. (2014) documented paternal transfer of both mtDNA and ptDNA in the Japanese endemic *Sciadopitys verticillata*.

After excluding hybrids and correction of *Taxus* 8 species remain from the summary Table 1 in Adams (2019), all with paternal mtDNA transfer. Adding the new data of *S. verticillata* (Worth et al. 2014) and our *L. franklinii* data gives 10 species in conifers II, for which paternal transfer of ptDNA has been confirmed with molecular genetic methods. There is no known exception.

This current dataset is limited. The Araucariaceae family is most closely related to the Podocarpaceae, diverging around 260 MYA (Leslie et al. 2018). Both mtDNA and ptDNA transfer in Araucariaceae are considered paternal (Mogensen 1996), based on a single microscopic study on *Agathis* (Kaur and Bhatnagar 1984). Further data are clearly needed before any conclusion can be drawn about this family.

Species in conifers II that reversed to maternal transfer would be valuable for understanding the evolution of mode of transfer. The genus *Chamaecyparis* (Cupressaceae) (5 species) could be a candidate for further research. In *C. obtusa* 97.5% of the offspring had the same ptDNA as their father, 2.5% had the same ptDNA as their mother, and heteroplasmy was observed in seeds (Shiraishi et al. 2001). Natural selection could change the balance between paternal and maternal inheritance in these species, so it would be interesting to study genetic variation in this trait in natural populations.

If further studies show no exceptions and transfer of mtDNA is paternal in all conifers II, it suggests a single evolutionary event. The event would have occurred after the split between conifers I and II around 320 MYA, and before the diversification of conifers II into different families around 280 MYA (Leslie et al. 2018). Mogensen's (1996) suggestion that mode of inheritance of organelle DNA is a conserved trait, stands firm for conifers. The question remains why so few reversions from paternal to maternal inheritance are observed in conifers.

Abbreviations

ptDNA

plastid DNA

mtDNA

mitochondrial DNA

MYA

million years ago

CLC-GWB

CLC Genomics Workbench

SNV

single nucleotide variant

conifers I

Pinaceae

conifers II

other conifer families (Araucariaceae

Cupressaceae

Podocarpaceae

Sciadopityaceae and Taxaceae)

Declarations

Conflict of interest

The authors declare no conflict of interest.

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Author Contribution

BK: Methodology, writing. RK: Making crosses. AS: Coordination. TdJ: Writing.

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Data Availability

The Illumina data from DNA sequencing (genome skimming) of female 1 and male are available at the Sequence Read Archive (SRA) at National Center for Biotechnology Information (NCBI) (Kersten 2025). DNA sequences of the 2501 contigs from genome skimming and the 12 selected mtDNA contigs of the iconic *L. franklinii* male as well as all Sanger sequences generated in this study are accessible at an Open Science Framework (OSF) project (Kersten et al. 2025).

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