



Gametophytic apomixis in a gymnosperm, *Larix decidua* Mill., results in normal male meiosis

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Abstract

An adult tree of *Larix decidua* Mill., European larch, was produced from doubling one haploid female gametophyte. Whether this tree can produce normal male meiocytes is the crucial question. This adult's pollen mother cells (PMCs), or male meiocytes, were squashed and stained. Male meiosis was normal and no abortive pollen grains were observed. This female gametophytic apomict of a conifer, a dihaploid adult, is 100% homozygous yet also reproductively competent with normal male meiosis and functional male pollen. Here we show that doubled female gametophytes can produce embryos and reproductively competent adult trees. This shows a way to gain rapid homozygosity and produce completely inbred lines for larch. This is a novel breeding shortcut reported for the first time for a conifer species.

Keywords

Apomixis; *Larix*; Meiosis; Pollen mother cells; Inbred line; Gynogenesis

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1 Introduction

Apomixis, or the asexual production of embryos, provides a breeding shortcut for producing plants that achieve 100% homozygosity in a single generation. What is not yet clear is whether female gametophytic apomicts can be bred. Breeding depends on reproduction with normal meiosis. Creation of such dihaploids has been possible for breeding food crops, such as maize, canola, onions and garlic (i.e. Havey 2004; Alan 2021) but no precedent exists for conifers and other forest trees. This could be a

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valuable approach for larch and other conifers given that inbreeding depression in larch and other conifers is too severe for inbred line development using conventional breeding methods.

Apomixis occurs often among ferns and angiosperms (Hörandl 2024) yet is rare among conifers and other long-lived gymnosperms. The best-known is the instance of paternal apomixis via natural androgenesis in Algerian cypress (*Cupressus dupreziana* A. Camus), which has abnormal meiosis in its pollen mother cells (PMCs): as much as 65 % of the pollen is dihaploid (Pichot and El-Maâtaoui 2000). After being delivered into eggs of another species, Mediterranean cypress (*C. sempervirens* L.), these male gametes multiply their chromosomal complement to become diploid embryos. The surrogate female parental species contributes no genes to the offspring (Pichot et al. 2001; Pichot et al. 2008). This suggests dihaploid breeding technologies could be developed to rapidly create homozygous lines (Bonga et al. 2010).

Artificial maternal apomixis is one way to produce dihaploids. A haploid female gametophyte is doubled using *in vitro* induction. So far, this has produced haploid embryos in a Norway spruce, *Picea abies* L. (Simola and Santinen 1990) and European larch, *Larix decidua* Mill. (Nagmani and Bonga 1985; von Aderkas and Anderson 1993). Of these, only larch grew into trees. These dihaploid trees are now reproductively competent adults. Whether these adult apomict larch trees have normal male meiosis and thus can be bred further is the question.

To answer this, we collected various stages of pollen male meiocyte development. These male meiocytes are more abundant (Williams 2009), easier to process (Rohr and Bonga 1983, Slobodnik and Guttenberger 2000) and have a lengthy development period (Kosinski 1986), which makes for easier sampling. Male development in *Larix* is a well-documented model system for conifer meiosis (Sax 1932, 1933; Ekberg et al. 1968; Owens and Molder 1971; Zhang et al. 2008). In this study we report that meiosis and microsporogenesis is normal for dihaploid (DH) larch trees derived apomictically by gynogenesis, or gametophytic apomixes.

2 Materials and methods

2.1 Plant materials

Pollen cone buds were collected from two mature trees of *Larix decidua*. The first was a mature apomictic tree, which developed female and male cones (Figure 1). The tree was derived from line 2110, a dihaploid line created *in vitro* via gynogenesis (Figure 2) from megagametophyte (haploid) cultures (von Aderkas and Anderson 1993). The second was a sexually mature tree of *Larix decidua* raised from seed. Buds were sampled at three sample points separated by five-day intervals beginning from mid-March to end of March.



Figure 1. a. Apomictic *Larix decidua* at time of anthesis and female cone receptivity. b. Female cone of apomict. c. Male cone of apomict. d. Apomictic *Larix decidua* that leafed out later the same spring.

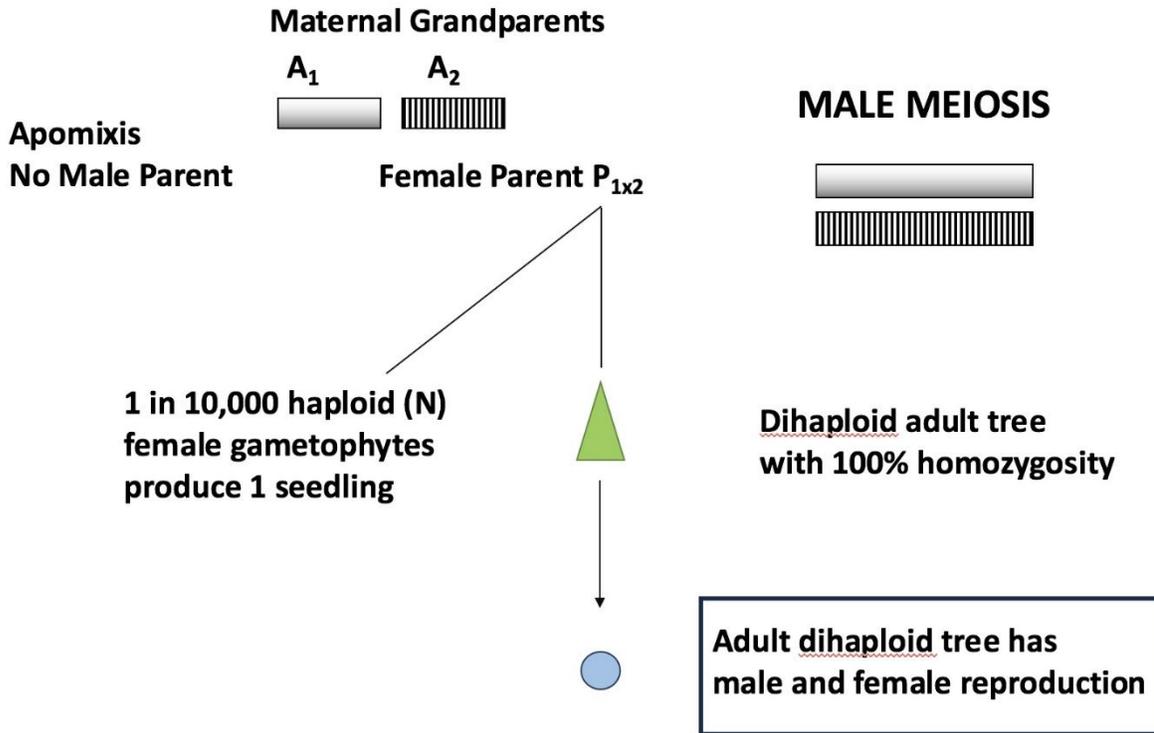


Figure 2. One of 10,000 female gametophytes removed from a *Larix decidua* parent produced a dihaploid seedling that developed into a mature reproductively competent adult tree.

2.2 Cytogenetics

We used two fixation methods. In the first, buds were fixed in 3:1 ethanol:glacial acetic, which was changed daily until the solution was clear and free of chlorophyll (Grimstone and Skaer 1972). In the second, meiocytes were fixed in a minimum of 10

times their volume of formaldehyde-aceto-alcohol fixative mix (FAA) combined with 10 % formalin (9:1 FAA:formalin).

Meiocytes and pollen stages were stained using acetocarmine in chloral hydrate, hematoxylin and iron according to Edwards and Miller (1972) and viewed on a Zeiss Axioplan microscope equipped with an M35 camera.

3 Results

3.1 Meiosis I and II

Our findings show that meiotic progression in an apomictic *Larix decidua* adult was similar to that in a normal tree. Male meiosis began in the autumn. Pollen mother cells (PMCs) passed through zygotene and leptotene before arresting in pachytene and diplotene. They remained in a diffuse diplotene stage for five or six months until early spring. With the return of warmer weather, meiosis completed relatively quickly in early spring with mature pollen being released in late March or early April. This apomictically-derived larch tree was able to complete Meiosis I and II as shown in Figure 3.

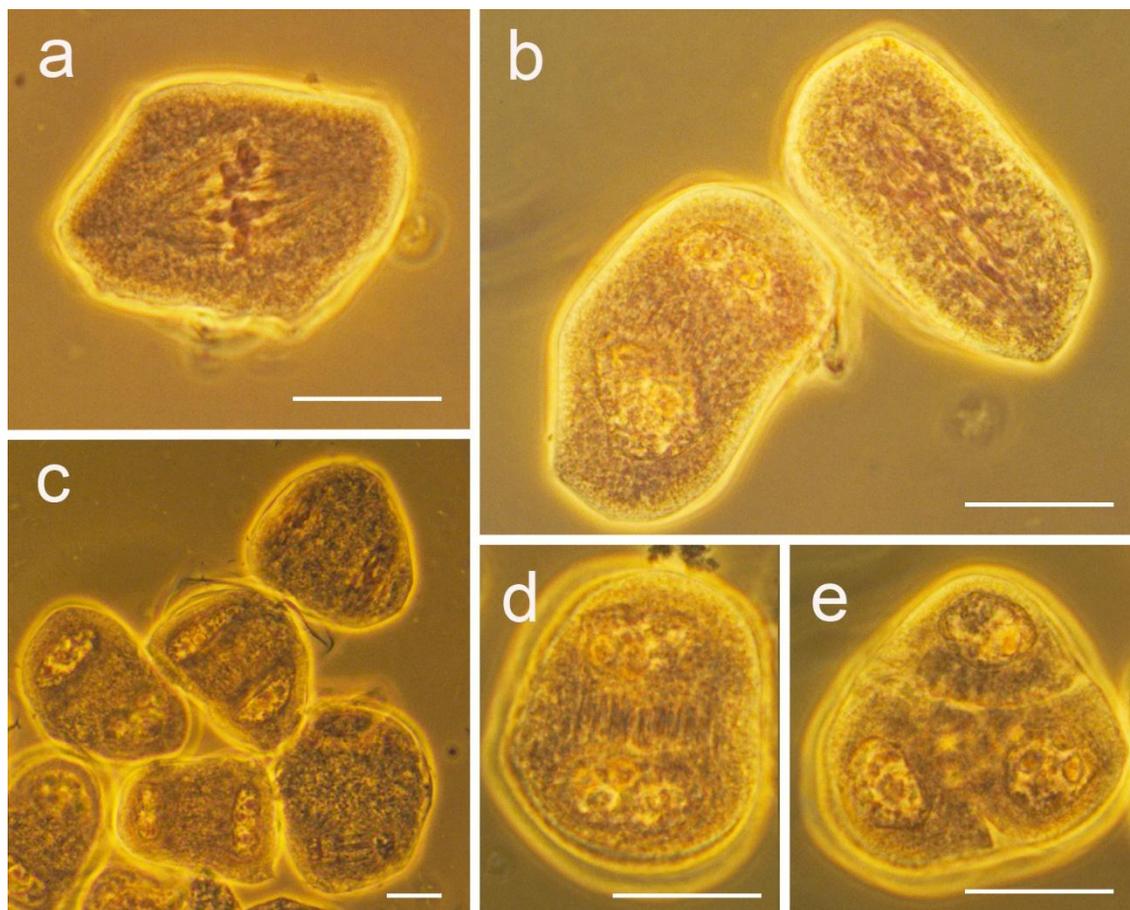


Figure 3. **a.** Metaphase I in normal *Larix decidua*. **b.** Metaphase I and Anaphase I in apomictic *L. decidua*. **c.** Anaphase I and Prophase II in apomict. **d.** Anaphase I in apomict. **e.** triad of apomict. Scale bars = 25 μ m.

Meiocytes that were sampled before March 20th were all at the diplotene stage (not shown). Meiosis within any adult larch typically showed some asynchrony (Tables 1 and 2). Adult larch showed different stages at a particular sampling point, e.g. 28th March, of the control tree buds (Table 1), in which most figures were in diplotene, but some were in metaphase I (Figure 3a). At some timepoints, samples collected, even when collected in relatively large numbers, resulted in odd data distributions, as certain stages were asynchronous (Figures 3b, 3c). For example, samples from the 27th March were mostly in prophase II in both trees, whereas samples taken the next day were mainly in Meiosis I, mostly at the diplotene stage. In one tree we found anaphase I, but none were observed in the control (Figure 3d). This is due to accidental bias in sampling, which may be reflected in our distributions of stages of meiosis over time. In our trees, the rate of meiosis appeared to be slightly faster by a day or two in the dihaploid compared with the control tree (*cf* Tables 1 and 2). We did not gather data on chiasma frequency as the transition through diplotene to diakinesis was very rapid.

Table 1. Normal *Larix decidua*: counts of pollen mother cell counts and pollen during stages of meiosis (Abbreviations: D diplotene, Dia diakinesis, M metaphase, A anaphase, P prophase).

Collection Date	Meiosis I				Meiosis II		Tetrad	Total	
	D	Dia	M	A	P	M			A
March 22	53							53	
March 27					156			156	
March 28	100		9					109	
March 30					6	24	11	25	66
Total	153		9		162	24	11	25	384

Table 2. Dihaploid *Larix decidua*: counts of pollen mother cell counts and pollen during stages of meiosis (Abbreviations: D diplotene, Dia diakinesis, M metaphase, A anaphase, P prophase).

Collection Date	Meiosis I				Meiosis II		Tetrad	Pollen	Total	
	D	Dia	M	A	P	M				A
March 22	116		7		20				143	
March 27		1	6	7	243				257	
March 28			1	3	336	2			342	
March 30							3	8	45	56
Total	116	1	14	10	599	2	3	8	45	798

No obvious abnormalities were noted, such as univalents. We did not find out-of-plane bivalents, fragmented fusion among bivalents, stickiness, pycnosis, lagging chromosomes or chromosome bridges. Aneuploidy was not observed.

3.2 Pollen

Pollen produced from the apomictic *L. decidua* tree was more commonly observed in tetrads than in triads (Figure 3e). Meiosis was a little slower in the normal tree, which meant that our final sample was mostly meiosis II cells with few tetrads and lacked mature pollen. In contrast, the apomictic tree had 45 pollen grains on the last day of sampling (Tables 1 and 2). It should be noted that both trees later released ample pollen during anthesis.

4 Discussion

Our findings provide proof of concept that adult dihaploids of a conifer, *Larix decidua*, can produce viable, 100% homozygous male meiocytes. Progression through Meiosis I and II occurs normally as described by Sax (1932) for *L. decidua*. We did not detect any of the diversity of meiotic irregularities seen by Christiansen (1960) in PMCs of environmentally stressed *L. decidua*.

In both apomictic and normally sexual PMCs, progress through Meiosis I and II was similar. Stages of meiosis were, in every way, identical to those previously described in studies of larches including *L. decidua* (Kolwerzo-Lubnau et al. 2015), western larch, *L. occidentalis* Nutt. (Owens and Molder 1971) and Japanese larch, *L. kaempferi* (Lamb.)Carr. (Zhang et al. 2008). *Larix* was also similar to other pinaceous conifers, including *Abies*, *Picea* and *Pinus* (Luomajoki 1977). Generally, PMCs remain in diplotene for nearly half-a-year. PMCs do not appear to be in a dormant state. Instead, cells were shown both to have high levels of enzyme activity and to accumulate starch (Owens and Molder 1971). During this long diplotene phase chromosomes went through pronounced stages of reorganization (Zhang et al. 2008) as well as periods of transcriptional activity, e.g. RNA polymerase II and splicing factors U2, snRNA (Kolwerzo-Lubnau et al. 2015).

We noted some asynchrony during meiosis, but this is not unusual. Conifers may vary individually in the synchronicity of meiosis (Benkman et al. 1984). A high degree of meiotic asynchrony is normally found within trees of this species (Romanova and Tret'yakova 2005). The asynchrony that we observed within and between trees in late Meiosis I and throughout Meiosis II was likely due to variation in rates of cone maturation and may be due to chance selection of cones on a given sampling date. It is known that general cone development varies even within an individual tree of *L. decidua* (von Aderkas and Bonga 1988). Other larches, such as tamarack (*L. laricina* (Du Roi) K. Koch) exhibited asynchrony during meiosis (Hall 1982). Meiosis in larches is also known to vary by location and by environmental conditions (Ekberg et al. 1968).

With respect to pollen, Zhang et al (2008) noted that two types of pollen formation occurred in *L. kaempferi*. The first type, synchronous formation, was most common. However, asynchronous formation involving successive stages of cytokinesis were also seen, resulting in six different forms of pollen, e.g. triads, tetrads. We observed mostly tetrads. We did not observe any twin pollen or giant multinuclear microspores as have been reported occasionally (Slobodnik 2000).

This DH apomictic adult is in itself unique. *Picea abies* produced apomictic embryos via haploid embryogenesis from female gametophytes, yet its embryos failed to reach the seedling stage (Simola and Santanen 1990). Similarly, Siberian larch (*L. sibirica* Lebed.) megagametophyte-derived cultures produced callus but no embryos; callus tissue proved to be cytogenetically unstable (Krutovsky et al. 2014). Compared with naturally occurring paternal apomictic conifers, such as *Cupressus dupreziana* (El Maâtaoui and Pichot 2001; Pichot et al. 2001), for which meiotic aberrations were observed, we saw no meiotic aberrations.

That DH apomixis can be experimentally achieved in a gymnosperm adds one more rare example of this phenomenon in this clade. Polyploidization, including spontaneous diploidization, though generally uncommon in gymnosperms, does occur. It may also occur during hybridization (Ohri 2021), and it may be experimentally induced using colchicine –treated *L. decidua* (Illies 1966). Unfortunately, even when polyploids

such as those of *L. sibirica* develop into sexually mature trees, meiosis and pollen development is abnormal (Johnsson 1975).

Larch and other conifers suffer severe inbreeding depression (Williams and Savolainen 1996). Over a 25-year period, Orr-Ewing (1976) created successive inbred lines (to S3) for Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco). He wrote despairingly that several more generations of inbreeding were required if uniform lines were to be produced. His surviving lines found today at Cowichan Lake Research Station, Mesachie Lake, Canada are unable to produce male or female cones (personal observation). By comparison, this novel process of creating trees via induced apomixis could shorten the process by generations and decades. A further advantage of tissue-cultured material can be cryopreserved and stored for decades, thus avoiding the necessity of keeping orchards or field-planted stocks. Our findings suggest early potential for a novel breeding method; further research is needed as follows.

Developing haploid-derived lines in long-lived conifer species are an alternative to selfed lines which is rapid and efficient. Since gametophytes are haploid, the low rate of induction in tissue culture, i.e. 1 in 20,000, is likely due to purifying selection (Williams and von Aderkas 2025). If it works in *Larix*, it should work in vitro gynogenesis may well work as it does for somatic embryogenesis for the closely related *Pseudotsuga* (Lelu et al. 1994; Kong and von Aderkas 2011). Other sources of tissue in the ovule could be attempted. It may also be possible to induce apomixis from the nucellus, which is sporophytic. In *Citrus*, apomixis via nucellus occurs (Ranganath 2004). Various genes important to the process have been identified (i.e. Wang et al. 2017). A similar level of understanding may be possible for conifers.

Other reproductive experiments become possible with this type of tree. The apomictic *L. decidua* genotype also has potential for testing meiotic recovery rates. It lends itself to study of genic-genotype-homokaryotype effects that are often confounded. Since having a homokaryotype allows comparison with non-homokaryotypes, studies using an apomict can be used to test meiotic drive in larch and other conifers. Combined with meiotic study, it may be used to answer questions related to chromosome stability, such as synteny, and paracentric inversions (Williams 2009).

Applications for larch breeding programs are premature without further research. The 100% homozygous inbred line produced in our study in one generation has normal male meiosis and therefore we suggest inbred lines could have potential for larch breeding programs. What is yet to be tested is as follows: 1) Is this pollen from DH adults capable of fertilizing a seed? 2) Is its female meiosis normal and do its fertilized eggs grow into seeds which germinate and grow into reproducing adults? 3) Do two different DH adults, when crossed as DH1 x DH2, yield the same offspring genotypes as DH1 x DH1 or DH2 x DH2 crosses? Only if these questions are answered can one begin applied tree breeding programs.

Our larch findings here offer a starting point towards developing homozygous genomes fix genes that may otherwise be lost during normal meiotic re-shuffling, or sexual recombination.

5 Conclusions

A dihaploid larch tree derived from tissue cultures grew into adulthood and became a reproductively competent tree exhibiting normal male meiosis and fully

developed pollen grains. This coniferous tree has a doubled haploid chromosomal complement, the only reported case of a 100% homozygous inbred line produced in a single generation.

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