Somatic Mutation Accumulation in Coast Redwood (*Sequoia sempervirens* (D. Don) Endl.)

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ABSTRACT:

Redwood (*Sequoia sempervirens* (D. Don) Endl.) is a long-lived, clonal, hexaploid tree species. Their longevity brings into focus questions about their genomic stability and integrity. Without a defined germline, mutations that accumulate in the branches of a plant may end up in sexual offspring, leading to possible decline of the population through propagation of deleterious mutations. To investigate how somatic mutations accumulate, we sequenced fourteen branches from a single 1400-year-old, 107-meter-tall redwood tree and germinated seedlings from cones from the two tops of this tree. Within the tree, the two tops had different fecundity (cone size, seed size, and seedling germination) attributable to a chromosomal deletion in one of them. Genomic and anatomical evidence suggests redwood trees have two coexisting cell lineages (tunica-corpus; L1, L2) within their usually stratified shoot apical meristems whose phenotypic expression is determined largely by normal branch ontogeny and parent branch selection. These separate cell lineages directly affect how mutations can accumulate in a redwood tree, which mutated lineages proliferate, and the extent to which any lineage contributes to sexual offspring. Redwoods usually show separation of cell lineages in their shoot apical meristems and different branch ontogeny which are shared with many other seed plants. Decoupling of stratified shoot apical meristem cell lineages through branching events leading to different fecundity between branches may well occur in other species, affecting per-generation mutation rate and genetic diversity in plant populations.

TABLE OF CONTENTS

INTRODUCTION…….....1

References……...3

CHAPTER 1……8

Abstract……8

Introduction……..9

Methods……...……...13

Results……16

Discussion……..20

Conclusion…….33

References…….34

Tables and Figures…….45

CHAPTER 2……..51

Abstract……..51

Introduction……51

Methods……..56

Results……65

Discussion……..72

Conclusion…….84

References……..85

Tables and Figures……...102

CONCLUSION……109

References……111

TABLE OF TABLES

Table 1.1 – Branch Origins……45

Table 1.2 – Cone Origins……...46

Table 2.1 – Reproductive Outcomes……102

Table 2.2 – Comparative Somatic Mutation Rates……..103

TABLE OF FIGURES

Figure 1.1 – Stratified Shoot Apical Meristem……..47

Figure 1.2 – Branch Origins……..48

Figure 1.3 – Cell Lineage Model of Redwood Sporophyte……...49

Figure 2.1 – T35 Redwood Genome Tree……104

Figure 2.2 – Loss of Heterozygosity……105

Figure 2.3 – Synteny Analysis……106

Figure 2.4 – Reproductive Outcomes……..107

INTRODUCTION:

Coast redwood (*Sequoia sempervirens* (D. Don) Endl., hereafter "redwood") is a hexaploid tree (Hirayoshi and Nakamura 1943; Stebbins 1948; Schlarbaum et al., 1984; Hizume et al., 2014; Scott et al., 2016; Ahuja 2022) that grows from 36-42º N latitude along the coast of North America. Redwoods can live for many centuries as single trunks (up to 2342 years; Sillett et al., 2022). During this time, redwoods undergo many mitotic divisions and regularly reach mature heights of 100 meters (maximum 116 meters, Sillett et al. 2021) and dry weights over 100 metric tons (maximum 350 Mg dry weight, Sillett et al., 2022). While redwoods reproduce sexually through seed, they also frequently reproduce asexually via sprouts from underground lignotubers (burls; Del Tredici 1998; Del Tredici 1999). These asexual sprouts make large genets (clones) consisting of up to 27 ramets (individual trunks) on the landscape (Douhovnikoff et al., 2004; Narayan 2015). These clones can be widespread with ramets of a single genet separated by up to 340 meters (Rogers 2000). Anywhere from 15-82% (average 61%) of ramets in a forest sampled at random constitute a single, unique genet (Narayan 2015). Redwood life history traits (clonality, longevity, physical dimensions, genome size, polyploidy) make the species a good candidate for studying genomic integrity, mutation accumulation, and intra-organismal genetic heterogeneity.

In most eukaryotic organisms, purifying (negative) selection through sexual reproduction is the process by which deleterious mutations are culled from a population. Redwoods are no exception. They make hundreds of thousands of seeds each year per tree (Bosch 1971). This excess redundancy and minimal investment in each individual seed enables selection to act on many offspring simultaneously. These redundancies also occur in the smaller subunits of a seed for selection to act earlier in process of sexual reproduction. Some ovules can have multiple

megagametophytes and each megagametophyte has many archegonia and eggs (Buchholz 1939). With multiple eggs in a single ovule, multiple fertilization events can happen, leading to simple polyembryony (dizygotic twins) and enabling competition between 'genets' within a seed. Similarly, cleavage polyembryony (monozygotic twins) occurs in conifer species. In redwood, a single zygote mitotically divides into four embryos (Buchholz 1939) where selection can act yet again, but this time between asexual 'ramets'. However, these strategies to purge deleterious alleles from the population are limited because sexual reproduction itself is limited. Also, because redwood megagametophytes are triploid (Narayan et al., 2015; Neale et al., 2022), not haploid, purifying selection is also limited (Orr 1995).

Polyploidy results in redundant copies of each homologous locus in a genome, which is essentially genome duplication as in the case of allopolyploid redwood (Scott et al., 2016). This gene duplication and genetic functional redundancy of polyploidy enables divergence between the sub-genomes to occur (Birchler and Yang, 2022). This results in both new gene expression due to dosage and potentially new functions of a gene (neofunctionalization, subfunctionalization; Birchler and Yang, 2022). Polyploidy also limits the effectiveness of purifying selection because increased ploidy masks the phenotypic effects of deleterious, recessive somatic mutations on an organism (Orr 1995). Beneficial mutations can be selected for if novel function or expression transcends the masking by wild-type alleles (Orr and Otto 1994; Otto 2007), a process known as neofunctionalization. These benefits of increased ploidy may be why polyploidy correlates with longevity of the genet (asexual reproduction and clonal growth; Otto and Whitton, 2000), although there is still debate about the causal relationship for this correlation (Van Drunen and Husband, 2019). Despite this causal uncertainty, increased longevity and the ineffectiveness of purifying selection (the accumulation of deleterious

mutations without phenotypic consequence) may theoretically result in greater intra-organismal genetic heterogeneity in polyploid species (Orr 1995).

This dissertation aims to answer questions about the sporophyte generation of redwood, and is split into two main chapters:

Chapter 1 addresses somatic cell lineages in the shoot apex and considers the following two questions. First, we ask to what extent does redwood have a stratified shoot apical meristem and can selection happen within or between the shoot apical meristems? Second, we ask to what extent are branches composed of different cell lineages and how deterministic or opportunistic this is?

Chapter 2 addresses modular growth more broadly and potential genetic implications of longevity and mutation rate by addressing the following two questions. First, we ask how genotypic variation through somatic mutation is organized within a single old tree? Second, we ask if reproductive success varies in different tree branches and if fecundity is affected by which cell lineage ultimately produces sexual offspring?

REFERENCES:

Ahuja, M.R. 2022. Origin and genetic nature of polyploidy in paleoendemic coast redwood (*Sequoia sempervirens* (D. Don) Endl.). *Silvae Genetica* 71: 54-64.

Birchler, J.A. and H. Yang. 2022. The multiple fates of gene duplication: deletion, hypofunctionalization, subfunctionalization, neofunctionalization, dosage balance constraints, and neutral variation. *The Plant Cell* 34: 2466-2474.

Bosch, C.A. 1971. Redwoods: a population model. Matrix methods may be used to model the growth, survival, and harvesting of California redwoods. *Science* 172: 345-349.

Buchholz, J.T. 1939. The embryogeny of *Sequoia sempervirens* with a comparison of the sequoias. *American Journal of Botany* 26: 248-257.

Del Tredici, P. 1998. Lignotubers in *Sequoia sempervirens*: development and ecological significance. *Madroño* 45: 255-260.

Del Tredici, P. 1999. Redwood burls: immortality underground. *Arnoldia* 59: 14-22.

Douhovnikoff, V., A.M. Cheng, and R.S. Dodd. 2004. Incidence, size, and spatial structure of clones in second-growth stands of coast redwood, *Sequoia sempervirens* (Cupressaceae). *American Journal of Botany* 91: 1140-1146.

Hirayoshi, I. and Y. Nakamura. 1943. Chromosome number of *Sequoia sempervirens*. *Botany and Zoology* 2: 73-75.

Hizume, M., K. Kaneko, and T. Miyake. 2014. A method for preparation of meiotic chromosomes of conifers and its applications. *Chromosome Botany* 9: 83-88.

Narayan, L. 2015. Clonal diversity, patterns, and structure in old coast redwood forests. Doctoral Dissertation, University of California, Berkeley.

Narayan, L., R.S. Dodd, K.L. O'Hara. 2015. A genotyping protocol for multiple tissue types from the polyploid tree species *Sequoia sempervirens* (Cupressaceae). *Applications in Plant Sciences* 3: 1400110.

Neale, D.B., Zimin, A.V., Zaman, S., Scott, A.D., Shrestha, B., Workman, R.E., Puiu, D., Allen, B.J., Moore, Z.J., Sekhwal, M.K., De La Torre, A.R., McGuire, P.E., Burns, E., Timp, W., Wegrzyn, J.L., and Salzberg, S.L. 2022. Assembled and annotated 26.5 Gbp coast redwood genome: a resource for estimating evolutionary adaptive potential and investigating hexaploid origin. *G3—Genes, Genomes, and Genetics* 12: jkab380.

Orr, H.A. 1995. Somatic mutation favors evolution of diploidy. *Genetics* 139: 1441-1447.

Orr, H.A. and S.P. Otto. 1994. Does diploidy increase the rate of adaptation? *Genetics* 136: 1475-1480.

Otto, S.P. 2007. The evolutionary consequences of polyploidy. *Cell* 131: 452-462.

Otto, S.P. and J. Whitton. 2000. Polyploid incidence and evolution. *Annual Review of Genetics* 34: 401-437.

Rogers, D.L. 2000. Genotypic diversity and clone size in old-growth populations of coast redwood (*Sequoia sempervirens*). *Canadian Journal of Botany* 78: 1408-1419.

Schlarbaum, S.E., T. Tsuchiya, and L. Johnson. 1984. The chromosomes and relationships of *Metasequoia* and *Sequoia* (Taxodiaceae): an update. *Journal of the Arnold Arboretum* 65: 251-254.

Scott, A.D., N.W.M. Stenz, P.K. Ingvarsson, and D.A. Baum. 2016. Whole genome duplication in coast redwood (*Sequoia sempervirens*) and its implications for explaining polyploidy in conifers. *New Phytologist* 211: 186-193.

Scott, A.D., A.V. Zimin, D. Puiu, R. Workman, M. Britton, S. Zaman, M. Caballero, A.C. Read, A.J. Bogdanove, E. Burns, J. Wegrzyn, W. Timp, S.L. Salzberg, D.B. Neale. 2020. A reference genome sequence for giant sequoia. *G3—Genes, Genomes, and Genetics* 10: 3907-3919.

Sillett, S.C., R.D. Kramer, R. Van Pelt, A.L. Carroll, J. Campbell-Spickler, M.E. Antoine. 2021. Comparative development of the four tallest conifer species. *Forest Ecology* 480: 118688.

Sillett, S.C., M.E. Antoine, A.L. Carroll, Mark E. Graham, A.R.O. Chin, R. Van Pelt. 2022. Rangewide climactic sensitivities and non-timber values of tall *Sequoia sempervirens* forests. *Forest Ecology and Management* 526: 120573.

Stebbins, G.L. 1948. The chromosomes and relationships of *Metasequoia* and *Sequoia*. *Science* 108: 95-98.

Van Drunen, W.E., and B.C. Husband. 2019. Evolutionary associations between polyploidy, clonal reproduction, and perenniality in the angiosperms. *New Phytologist* 224: 1266-1277.

CHAPTER 1

Shoot apical meristem stratification separates sexes of monoecious redwoods early in development due to different cell lineage origins of branches and cones

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ABSTRACT:

Shoot apical meristems are the ultimate origin of all sexual reproductive structures in vascular plants. For more than a century, the shoot apical meristem organization of redwoods (*Sequoia sempervirens*) has been debated as a zonate or a stratified shoot apical meristem. Using anatomical sections and chimeric clonal analysis, we endeavored to resolve the redwood shoot apex, branching origins, and the cell lineage origin of strobili. Redwood shoot apices are typically stratified into two coexisting cell populations, L1 and L2 (tunica corpus). Sylleptic and proleptic branches, growing concurrently with or delayed from the parent shoot, respectively, originate from different shoot apical meristem layers. These branches, therefore, can theoretically display different phenotypes if nonsynonymous somatic mutations occur in the layers (L1, L2) of the shoot apical meristem. Similarly, sylleptic and proleptic branches appear to make the megasporangiate and microsporangiate strobili, respectively. Therefore, theoretically, a somatic mutation in one layer (L1, L2) does not contribute equally to sperms and eggs, thus reducing the probability of homozygous zygotic fixation of a somatic mutation that has accumulated in the parent. Separate cell lineages in the redwood shoot apical meristem would directly affects how mutations can accumulate in a redwood tree, which mutated cell lineages proliferate, and the extent to which any cell lineage contributes to sexual offspring. Most seed

plants have stratified shoot apical meristems and/or different branch ontogenies (e.g. axillary vs. epicormic). If both conditions are present, as in redwood, our results may apply more broadly across many seed plant species.

INTRODUCTION:

Cell lineages are important tools for understanding organismal development. They can be used as tracers to determine which cells contribute to what tissues. Clearly not all cell lineages end up forming offspring (e.g. soma in animals; roots in plants). In animals, the cell lineages that form offspring are called germlines and typically constitute cell lineages of fewer divisions and fewer accumulated mutations (Walbot and Evans, 2001). Plants, however, use somatic cells to make offspring (Walbot and Evans, 2001), although they limit what cell lineages contribute to sexual offspring and new ramets and branches (Buss 1985; Klekowski 1988; Otto and Hastings 1998; Howe et al., 2022). The cell lineage origin of branches may depend on prior growth and environmental conditions of the parent branch (e.g. adventitious bud origin; Whitham and Slobodchikoff 1981; Klekowski et al. 1985; Otto and Orive 1995).

When a redwood branch dies back, epicormic branches, or branches that develop from dormant buds, emerge (Meier et al., 2012; Kramer et al., 2014). Epicormic branches can have two origins: from dedifferentiated cells or from residual meristematic cells (Meier et al., 2012). In redwood, new epicormic branches emerge from residual meristematic protoderm in the leaf axils (Fink 1984) whose cell lineages can remain separate from the rest of the trunk for >600 years (Peltier et al., 2023). Redwood is not unique in the morphological origin of its epicormic branches. Indeed, many families of seed plants develop epicormic branches from residual

meristematic origin (Meier et al., 2012) like the protoderm-derived epicormic branches in redwood (Fink 1984).

These branches may result in differential branch performance based on variation in how many mutations and what cell lineage ends up founding ("birthing"; Harper and Bell 1979; Buss 1983a; Cook 1985; Orive 1993) any new branch in a plant (Tian and Marcotrigiano 1993; Otto and Hastings 1998; Marcotrigiano 2000; Burian et al., 2016; Zahradníková et al., 2020). A developmental bottleneck, or the reduction in the cells needed to found a new branch, increases the likelihood that the new branch is made up of a single genotype (Otto and Hastings 1998; Reusch et al., 2021; Howe et al., 2022; Aanen et al., 2023). For example, if a branch develops on one side of the stem and that sector has a mutation not present on a different side of the stem, the branch will likely carry that mutation because it develops from a "bottleneck" or "subset" of all the cells in every sector of the parent branch. These bottlenecks reduce the potential genetic diversity of the new branch. The development of a branch can become more important if the cell lineages that found a branch remain separate for many cell divisions or over long periods of time, not only in secondary growth (Peltier et al., 2023) but in primary growth. If separated, cell lineages can accumulate different mutations independently (Klekowski et al., 1985). To examine this possibility, one must look at the origin of shoots themselves and how cell lineages are structured in the shoot apical meristem.

Seed plants have multiple apical initials in their shoot apical meristems. Cell lineages in the shoot apex separate in sectors lengthwise along the shoot axis, making semi-stable sectorial chimeras (Tilney-Bassett 1986). No single mutated genotype is stable because the apical meristematic initials frequently and stochastically mix (Klekowski and Kazarinova-Fukshansky, 1984a,b). This random mixture of cell lineages is consequential because it enables both intra-

meristematic selection and “somatic drift” (Reusch et al., 2021), or random fixation of a mutation within the asexual cell population in the shoot apical meristem. These processes of selection and drift work to homogenize the mutations within the entire shoot (Klekowski and Kazarinova-Fukshansky, 1984a,b). Thus, branches developing from different sides of the stem will have essentially the same genotype since all cells in the shoot have a relatively recent common ancestor cell. This would make different cellular origins of branches unimportant from a cell lineage or somatic mutation perspective since, without significant genotypic differences between different cells of a branch, where branches originate is strictly a developmental phenomenon without evolutionary consequence.

On the contrary, however, if cell lineages in a shoot apical meristem remain separate from each other, they can quickly diverge genetically accumulating different mutations within the layers of a single shoot (Klekowski et al., 1985; Pineda-Krch and Lehtilä 2002). This separation of cell lineages makes branch developmental origin important for which somatic mutations proliferate in which branches. Many seed plant shoot apices have a few discrete, periclinal (parallel to the surface) sub-populations or layers of cells that remain separate in primary growth of the shoot for many seasons of growth, perhaps indefinitely in some species (Tilney-Bassett 1986). The most internal layer of these meristems is the “corpus” and the additional external layer(s) is/are “tunica(s)” (Schmidt 1924). From the outside in, tunica(s) to corpus, these lineages can be described with Ln, where n increases centripetally (Satina et al., 1940). The outermost layer is the L1 and each internal layer increases in number. These shoot apical meristems are called “stratified”, referring to the periclinal strata of separate cell lineages present (Schmidt 1924). Stratified shoot apical meristems have subdivided intra-meristematic selection and drift in their sub-populations rather than across the whole meristem (Klekowski et al., 1985; Pineda-

Krch and Lehtilä 2002), thereby increasing the likelihood of the sub-populations accumulating mutations separately across long periods of time. Periclinal chimeras are examples of mutated cell lineages remaining separate and whose cell lineage composition is stable for long periods of time (Tilney-Bassett 1986). If redwoods have stratified meristems, then branches originating from the L1-derived protoderm (Fink 1984) could, potentially, have a different suite of accumulated somatic mutations than the L2-derived corpus.

Many gymnosperms typically have zonate shoot apical meristems without organized, stratified cell lineages. In these plants, accumulated mutations would homogenize within the single population of cells of the shoot apex. However, some gymnosperms in the Cupressaceae and Araucariaceae have a single tunica and overlaying corpus layers, L1 and L2, respectively. Stratified shoot apical meristems have been observed in the following genera: *Agathis* (Jackman, 1960), *Araucaria* (Guttenberg, 1961), *Calocedrus* (Korn, 2001), *Chamaecyparis* (Hejnowicz, 1959; Pohlheim, 1971; Korn, 2001), *Cupressocyparis* (cross of *Hesperocyparus* and *Callitropsis* species; Korn, 2001), *Hesperocyparus* (formerly *Cupressus*; Guttenberg, 1961), *Juniperus* (Hejnowicz, 1956; Hejnowicz 1959; Pohlheim, 1971; Ruth et al., 1985; Korn, 2001; Korn 2002), *Thuja* (Hejnowicz, 1959; Korn, 2002), and *Thujopsis* (Guttenberg, 1961). Some investigations have concluded that the redwood SAM has transient (little to no) stratification (Douliot, 1890; Crafts, 1943; Sterling, 1945). However, others have reported distinct stratification (Strasburger, 1872; Campbell, 1940; Cross, 1943). The matter is still considered to be unresolved. If stratification exists in redwood, it is theoretically possible for these cell lineages to mutate independently, indefinitely (Klekowski et al., 1985; Pineda-Krch and Lehtilä 2002), reducing the likelihood of mutational meltdown within an individual (Klekowski 2003). Stratified meristems could also result in branches of differential origins (Fink, 1984) composed entirely of different

genotypes separated as far back as the embryo itself (Klekowski 2003), and in long-lived organisms often across centuries and even millennia.

To test whether redwoods have stratified meristems, two approaches were used: anatomical sectioning (Crafts, 1943; Cross, 1943; Sterling, 1945) and chimeric morphological analysis (Hejnowicz, 1956; Pohlheim, 1971; Korn, 2001; Korn 2002), a novel approach for redwood. Chlorophyll-deficient chimeras can be used because chlorophyll is considered a neutral marker intra-meristematically (within the shoot apical meristem) since chlorophyll does not develop until cells are outside of the apical dome cells (Tilney-Bassett 1986). Chlorophyll-deficiency is not neutral when considering inter-meristematic (between branch) selection after the phenotype manifests, as chlorophyll-deficient foliage has completely different anatomy and physiology compared to green foliage (Peirce 1901; Pittermann et al., 2018; Moore, unpublished data). We used these approaches to answer the following questions: (1) are redwood shoot apical meristems stratified, (2) do redwood branches develop from different cell layers of the shoot apex, and (3) do redwood pollen and seed cones develop from different branch types?

METHODS:

Shoot tip sampling:

Fifty-six shoot tips from lateral branches were collected in September 2017 from the crown of a 107-meter tall, 1400-year-old redwood tree in the Santa Cruz Mountains (T131 in Sillett et. al. 2020; SESE104 in Neale et al. 2022; T35 in Sillett et al., 2022; Carroll and Sillett, 2023). The fifty-six samples covered the height gradient, from 14.5 meters (the lowest foliage) up to 106.5 meters (one meter below the top of the tree). Alongside these samples, another sixty-six shoot tips were collected from lateral branches of other young trees from the Santa Cruz

Mountains for a total of 122 shoot tips. These were fixed in 50% formalin-acetic acid-alcohol (FAA) solution and subsequently dehydrated through an ethanol (EtOH) series and tert-butanol (TBA) series and then finally embedded in paraffin (Jensen 1962). Once embedded, shoot tips were mounted on wood blocks and sectioned longitudinally at 7μm thickness with a rotary microtome. Sections were mounted on slides using Haupt's adhesive (Jensen 1962). The sections were triple stained using safranin, crystal violet, and fast green (Jensen 1962) and examined with a light microscope for periclinal divisions in the outermost cell layer (designated L1) to observe the relative extent of SAM stratification.

Chimeric Morphological Analysis:

Young redwood specimens that exhibited variegated periclinal chimerism (similar to patterns seen in Korn 2001; Korn 2002) were examined in a climate-controlled greenhouse setting in Volcano, California. Originally derived from five distinct cultivars, a total of ten specimens were vegetatively propagated and grown. Three of these five cultivars were F1 individuals from a cross pollination experiment conducted in the 1970s (Davis and Holderman, 1980; Stapleton and Holderman, 2016; Stapleton and Holderman, 2018a; Stapleton and Holderman, 2018b); one came from a planted redwood in Cotati, California with a spontaneous albino mutation, and one came from a naturally-occurring, privately-owned basal sprout along the Russian River in Sonoma County, California. All ten trees were measured for total stem length and branching phenotypes were characterized in each following the procedure below.

Branch phenotypes (proportion white or green) were characterized in a complete branching nodal inventory of each plant within the study. All visible nodes with branches along the main axis of a plant were numbered acropetally from base to tip. The distance from tree base

to each branching node on each main axis was measured to the nearest 0.5 cm and recorded. The numbers of green and white foliage leaves were used to determine a green percentage for each shoot emerging from each node. Most shoots were all green or all white, in which case individual foliage leaves to determine proportion of green to white were not counted and trees were recorded as 100% green or 0% green, respectively. Nodes with multiple branches were also recorded, with each branch recorded separately. In a similar manner, the nodal inventory was continued on the lateral branches.

Cone Origins:

From three trees planted in San Jose, CA, two sexually-reproductive branches were collected in September 2023. The branches were dated using tree rings or annual foliage growth increments. The branches also had different overall architectures due to light conditions. Those in full sun grew more upward vertically, some grew horizontally, and those in shade grew downward vertically (as in Kramer et al., 2014). We collected two branches in each light condition. Cones were counted and their branching type (proleptic or sylleptic) recorded. Sylleptic and proleptic branches are branches that elongate concurrently with the parent or delayed after the parent, respectively. Cones from proleptic branches at any point from base to cone were considered “proleptic” in origin. Similarly, cones from sylleptic branches that maintained syllepsis for the entirety of the branching from base to cone were recorded as “sylleptic” in origin. A total of 4865 cone origins (4528 pollen cones; 337 seed cones) were recorded. Proportions were recorded per branch of proleptic male, proleptic female, sylleptic male, and sylleptic female.

To determine on average which cell lineage gave rise to the meiocytes in pollen and seed cones, we used data from the average shoot apical meristem cell layer contribution to sylleptic and proleptic branches as determined by the percentage green foliage from the chimeric morphological analysis. These average proportion of cell lineage contributions (L1 or L2) to the type of branch was multiplied by the average numbers of the two possible types of pollen and seed cone branch origins (sylleptic or proleptic) to reach an average shoot apical meristem layer of origin determination (L1 or L2) in the cone products of meiosis (microspores or megaspores). This allowed the sporophytic cell layer origin of the gametes to be deduced.

RESULTS:

Mitotic divisions in the shoot tip:

Shoot tip sectioning (Figure 1.1) revealed the extent of shoot apical meristem stratification based on the relative abundance of periclinal divisions in the outermost layer (L1). In all 122 meristems sectioned longitudinally or transversely, no periclinal divisions in the apical dome that would interrupt stratification were observed. Thus, periclinal divisions are likely rare events in the redwood shoot apical meristems sampled for this study.

Branching:

Redwoods grow with two modes of branching (Fink 1984), syllepsis and prolepsis (Hallé et al., 1978). Sylleptic branches emerge concurrently with the parent branch, while proleptic branches delay timing of emergence (Hallé et al., 1978). These branching modes have been mostly used to describe tropical trees; this is the first study to apply these terms to redwoods.

Chimeric Morphological Analysis:

We observed the phenotypes of chlorophyll-deficient ("albino"; Peirce 1901; Douglas and Holdermann, 1980; Pittermann et al., 2018) and green periclinal chimeras with two different, reciprocal patterns of chimerism (Figure 1.2ab vs. Figure 1.2c). The plants were either GW (green-white; green=L1, white=L2; Figure 1.2ab) or WG (white-green; white=L1, green=L2; Figure 1.2c) based on the coloration of the branching morphology of the trees and the literature of periclinal chimeric conifers (Hejnowicz, 1956, Hejnowicz, 1959; Jackman, 1960; Guttenberg, 1961; Pohlheim, 1971; Ruth et al., 1985; Korn, 2001; Korn, 2002). The ten trees ranged in height from 51.0 to 163.0 cm, total stem length from 2.18 to 17.75 meters, and age from 1.5 to 4.5 years old. In the 66.36 meters of total redwood stem analyzed across the ten trees, 2666 total branches had developed from 2332 branching nodes (Table 1.1).

Because chlorophyll is not made in epidermal cells of redwood, white and green phenotypes could only be observed in the L2 layer of any given branch. Thus, the L1 phenotype was "invisible" to our macroscopic observations, although through pigment differences in branches, we could observe and quantify a pattern (Figure 1.2). A majority of sylleptic (concurrently growing with the parent shoot tip) branches phenotypically expressed the parental L2 (96.94%, n=1730), whereas a majority of proleptic (later emerging) branches phenotypically expressed the parental L1 (84.30%, n=936). Likewise, once the parental L1 phenotype was expressed in a proleptic branch, the branch never produced the parental L2 phenotype in subsequent branches, demonstrating "fixation" of the parental L1 genotype. For example, in GW chimeras (Figure 1.2ab) where the L1 was green and the L2 was white, sylleptic branches grew mostly white whereas proleptic branches grew mostly green. In GW chimeras (Figure 1.2ab), once a proleptic branch was green, it never displayed white again. Therefore, L1 remains

“invisible” until it founds an L2 layer in proleptic branches expressing visible pigment (chlorophyll in this example).

Therefore, we conclude that a majority of sylleptic branches develop maintaining the stratified structure of the parent axis (Figure 1.1), whereas a majority of proleptic branches develop from the parental L1 only, in effect removing any parental L2 genotype from the entire branch. We interpret this result as phenotypic evidence corroborating proleptic branches forming in the empty axil from residual meristematic protoderm (Fink 1984). The minority of shoots that contradicted this rule usually displayed a mix of the L1 and L2 phenotypes. These occurred due to periclinal divisions mixing the cell lineages in the cortical parenchyma (derived from L2) and protoderm (derived from L1) contributing to the founding of proleptic branches (Fink 1984).

A small minority of sylleptic and proleptic branches displayed phenotypes exactly opposite of the majority trend (parental L1 phenotype in sylleptic branches and parental L2 phenotype in proleptic branches). Only 0.58% of sylleptic branches (10 of 1730) and 7.69% of proleptic branches displayed exclusively parental L2 and L1 genotypes, respectively. The other 2.48% of sylleptic branches and 8.01% of proleptic branches demonstrated a chimeric (mixed) phenotype, indicating their L2 development from both layers (L1 and L2). Of these chimeric branch phenotypes, sylleptic and proleptic branches expressed 83.6% (n=43 std. dev.=19.1%) and 70.9% (n=75, std. dev.=28.0%) of the expected phenotype in the expected branches (Table 1.1). Therefore, proleptic branches are more likely than sylleptic branches to display mixed layers. This introduces more genomic variability in these branches due to mutations accumulated independently in the L1 and L2.

To account for both non-chimeric and chimeric branches, since chimeric branches still closely resembled the expected pattern in non-chimeric branches, proportions of cell layer

contributions were calculated per branch. On average, the L2 of sylleptic branches displays parental L2 phenotypes 99.0% of the time, while L2 of proleptic branches displays parental L1 phenotypes 90.0% of the time. This indicates that both branches follow a majority trend of displaying only one cell layer phenotype. It also indicates that sylleptic branches maintain the trend more than proleptic branches. These values also indicate that nearly always (96.9%, Table 1.1), sylleptic branches maintain the stratification of L1 and L2 with rare leakage events (periclinal division mixing cells between the layers), congruent with our anatomical data.

Cone Origins:

The types of branch that bore pollen and seed cones differed significantly ($p=2.99x10^{-3}$). Per branch, 73.4% (S.E. ±7.6%; n=337) of seed cones were borne on sylleptic branches, while 82.1% (S.E. ±2.4%; n=4528) of pollen cones were borne on proleptic branches. The variation between number of seed and pollen cones on any given branch (n=6) is because pollen cones are produced in greater abundance than seed cones, not due to sampling bias.

When the layer of origin percentages of proleptic and sylleptic branches are included in the analysis, and assuming L2 produces meiocytes in all branches (Tilney-Bassett 1986), differences in cell lineage represented in microspores and megaspores becomes apparent (Table 1.2). For microspores, 82.1% of microspores come from proleptic branches. Likewise, 90.0% of proleptic branch L2 tissues arise from the parental L1 cell lineage. Therefore, 73.9% of microspores derive from the parental L1. For megaspores, 73.4% of megaspores come from sylleptic branches. Likewise, 99.0% of sylleptic branch L2 tissues arise from the parental L2 cell lineage. Therefore, 72.7% of megaspores derive from the parental L2 cell lineage.

Microspores and megaspores directly contribute to sperm and egg, respectively, via mitosis. Therefore, we can assume gamete genotypes produced by the gametophyte stage are essentially the same as the microspore and megaspore genotypes, assuming no mutation. As a result, in any given tree, 72.7% of eggs will ultimately derive from the sporophyte L2 genotype, where 73.9% of sperm will ultimately derive from the sporophyte L1 genotype.

DISCUSSION:

Proleptic and Sylleptic Branches:

The definition of proleptic branches closely resembles that of epicormic branches, or branches that emerge in a different growing season from the parent (Meier et al., 2012). While there is a vast literature referring to redwood epicormic branches, we view proleptic branches as a more inclusive term of any delayed growth, not requiring branch emergence from a different growing season (Meier et al., 2012). In redwood, notably, proleptic branches can develop in the same growing season as the parent branch (Fink 1984), despite expansion still being delayed by at least four months (our data) after the parent shoot made the node. Often, we find, in cases where proleptic branches emerged during the same growing season, they shared an axil with a sylleptic branch. So one could define “proleptic” branches in redwood as “accessory and epicormic branches” to fully cover prolepsis. Similarly, in most temperate trees, sylleptic branching does not occur. To adequately describe the branching variation in this temperate redwood tree, syllepsis and prolepsis were the best choice.

The proportion of sylleptic and proleptic branches is different between trees and branches. Proleptic branches appear rare in the short term, with a vast majority of branches in a young, fast-growing redwood consisting of sylleptic branches (note proportions of total sylleptic

to proleptic branch numbers in each young tree of Table 1.1). However, after damage to the trunk or shoot tips (i.e. crown fire, wind damage, drought dieback, etc.), proleptic branches arise more regularly often replacing the damaged shoot (Fink 1984). These new proleptic branches often develop from the parental L1 layer, leaving the parental L2 layer "behind" in primary growth. An exception to this exists in the Korbel KT redwood cultivar (Libby and Tufuor 1984), where proleptic branches form regularly in almost every node creating a hedge-like habit. In either case, variation in the proliferation of proleptic versus sylleptic branches determines the cell lineage origins of primary growth in a tree and can vary widely between different circumstances, genetic or environmental. Proleptic branches are commonly called "epicormic branches" (Meier et al., 2012; Kramer et al., 2014), though not all epicormic branches are proleptic.

Sylleptic and proleptic redwood branches generally look the same morphologically and can only be distinguished by the knowledge of the timing of their release or their position relative to another branch from the same node. These branching types are distinct from the shoot dimorphism defined by mature shoot physiology and leaf anatomy (Chin et al., 2022). Not all axial or peripheral shoots correspond directly to sylleptic and proleptic branching, though some correlations between the two may exist. This may be a fruitful avenue of inquiry for future study. A proleptic branch generally develops in a seemingly "empty" axil (Figure 1.2c), though on occasion, up to 77.8% (n=45) of proleptic branches can develop as an accessory branch proximal to a sylleptic branch's point of attachment (Figure 1.2d, 1.2e) within a single tree. In other words, proleptic branches can be either primary or secondary axillary branches depending on whether or not a sylleptic branch had grown first in the axil. Therefore, it is insufficient to define proleptic branches as accessory branches or secondary axillary branches because they can also form as primary axillary branches.

Stratified Meristems:

After more than a century of debate and contradictory evidence, our shoot tip sections and chimeric analysis from eleven redwood trees support the earlier conclusion that redwoods typically have stratified shoot apical meristems (Figure 1.1). The old, tall T35 redwood tree (Sillett et al., 2022; Carroll and Sillett, 2023) and the ten chlorophyll-deficient periclinal chimeras studied maintain layered sub-populations within the shoot apical meristem with little to no cellular displacement between layers for many meters of stem growth. Consequently, the shoots we observed are always composed of two coupled populations, which only decouple from each other in proleptic branching events or rare periclinal divisions. Periclinal divisions mix the layers and occur very rarely per meter of stem growth as observed in the chlorophyll-deficient periclinal chimeras. Thus, intra-meristematic selection can only occur within the layers and rarely between them, and is an important feature of stratified meristems (Klekowski et al., 1985; Pineda-Krch and Lehtilä 2002). This spatial restriction of intra-meristematic selection or drift compared to ancestral, zonate meristems seen in most gymnosperms enables separation of lineages across time (Klekowski et al., 1985; Pineda-Krch and Lehtilä 2002). This leads to differential mutations accumulated and potentially different phenotypes, like chlorophyll-deficiency of trees (this study, Park et al., 2023).

Proleptic branching may be another source of both increased intra-meristematic and inter-meristematic selection. Usually founded by the parental L1, normal ontogeny of proleptic branches in redwood typically replaces the parental L2 genotype with only the L1 cells and genotype (Figure 1.2). However, in a minority of cases, proleptic branches result in a mixture of parental L1 and L2 genotypes. In these instances, intra-meristematic selection can still act

between the layers. While each shoot apical meristem has an L1 and L2 cell lineages, ontogeny determines which genotype remains more permanent over time (parental L1) and which genotype is more transient (parental L2) in primary growth regardless of any external selective pressures. Importantly, the parental L2 genotype is more permanent in secondary growth because that cell lineage founds cambia (Tilney Bassett 1986).

However, selection on branches (such as exposure to abiotic stresses like fire or wind or biotic stresses like herbivores and fungi) cannot be ignored, despite the difficulty of studying it experimentally. In a growing Cupressaceae stratified shoot, the L1 produces the epidermis only, where the L2 produces all other cells (Hejnowicz, 1956, 1959; Pohlheim, 1971; Ruth et al., 1985; Korn, 2002). In a shoot at any given time, selection may act on the L1 only via epidermal phenotype (cuticle, stomata) alone, whereas selection acts on the L2 in any of the other components of shoot (cortical, cork cambium, photosynthetic/chlorophyll, wood—heartwood, sapwood, vascular cambium, etc.). Therefore, we suspect that inter-meristematic (between-branch) selection is greater on L2 than on L1 simply due to the unequal number of total cells the L1 and L2 originate.

However, if branch replacement occurs due to selective pressure (pruning, frost damage, shading, stress; see review in Meier et al., 2012), they will be replaced by new proleptic branches (Fink 1984). Branch dieback promotes proleptic (epicormic) branching (Fink 1984; Kramer et al., 2014). In these new proleptic branches, our results suggest the parental L2 genotype will usually be lost and new branches replaced by mostly the parental L1 cells and genotype (Table 1.1; Figure 1.3). The frequent replacement of parental L2 genotypes with parental L1 genotypes in new proleptic branches, especially after shoot damage like fire or windfall, results in the parental L1 genotype contributing to meiocytes equally or more than the parental L2 genotype.

In effect, during branch dieback, the L1 invades the L2 through prolific proleptic branch formation. The alternative, as discussed above, is where the parental L2 genotype is mixed in the founding cells with the parental L1 genotype, enabling intra-meristematic selection. When the parental L2 genotype is lost in a proleptic branching event, the parental L1 genotype is exposed to the heightened selection experienced by the L2 cell layer. The parental L1 cells now make all components of the new branch (epidermis, cortex, cambia, pith, etc.). So while the L2 cell lineage experiences a majority of selection, the parental L2 genotypes consistently get replaced with the parental L1 genotypes in a majority of proleptic branches, exposing those branch-founding L1 genotypes to inter-meristematic, branch-level selection.

These L2 genotype replacement events with L1 through proleptic branching increase due to branch failure (Fink 1984). However, even without branch failure or conspicuous selection on branches, L2 genotype replacement due to proleptic branching may be genetically predisposed. If any genotypes increase proleptic branching (such as Korbel KT; Libby and Tufuor 1984), theoretically more mutations can be exposed to branch-level selection and offspring formation (Marcotrigiano 2000; Klekowski 2003). Organisms limit what cell lineages contribute to new ramets and sexual offspring (Buss 1985; Howe et al., 2022). Therefore, through environmental (abiotic or biotic) or genotypic variation, redwood trees may theoretically alter their branching phenotype (proportion of sylleptic or proleptic branches) thus altering their per-generation effective mutation rate (Otto and Orive 1995). Put another way, the number of somatic mutations that end up fixed in a branch (due to the prevalence of these fixed, proleptic branches in the single tree “population” of branches) is proportional to the number of mutations that, ultimately, persist in the sexual offspring.

To view this from a different perspective, we can trace a theoretical mutation in the L1 along a time series. Initially, a cell in the apical dome is mutated. If that cell can survive, the mutated cell lineage competes with the other L1 initials. From here, regardless of whether the mutated cell outcompetes (through selection or stochastic drift) the rest of the L1 initials (Klekowski and Kazarinova-Fukshansky, 1984a,b), its mutation most likely ends up fixed in a small, proleptic branch, founding both the L1 and L2 of this branch (Table 1.1). When this occurs, the mutated parental L1 has separated from the parental L2 lineage, and selection can act on the entire branch and each tissue (primary or secondary) that the mutated parental L1 genotype ultimately makes. Branch survival will determine whether the branch reproduces. In this way, mutations accumulated in redwood are progressively scrutinized by selection in increasingly emergent levels of organization: from cell, to cell layer, to meristem.

The composition and development of cell lineages in a plant may be a morphological way by which plants alter their effective mutation rate through intra-organismal selection of these lineages (Otto and Orive 1995). Where sexual reproduction acts as “instant” mutation fixation in the zygote, plant sporophytes utilize intra-organismal selection through series of chimeric steps across time before mutation fixation in a ramet or branch (Pineda-Krch and Fagerström 2001), and then potentially in a zygote. Ultimately, the genetic contribution of a genet to the next generation of genets derives from multiple factors: its original genotype, the mutations accumulated from cell lineage to branch to ramet, and the intra-organismal selection and drift acting on variation of module experiences across space and time (Harper 1985). Therefore, in modular organisms, the selection experienced by organizational sub-units (cells, cell lineages, branches, ramets) affects how mutations accumulate and are passed on to the next generation.

Plant “Germlines”

Whether plant germlines exist has been an ongoing topic of debate (Lanfear 2018; Burian 2021) and is still unresolved. A plant germline would need to be early-segregating and with a lower division and/or mutation rate compared to ‘typical’ somatic cells. While this study does not explicitly or experimentally find a germline, it does constrain where a germline may be situated in redwood. If a “functional germline” does indeed exist in redwood, it would be in the stratified shoot apical meristem (Wang et al., 2019; Ren et al., 2021; Burian 2021), as these meristems have segregated layers that create the meiocytes (Tilney-Bassett 1986). In angiosperms, especially, where these stratified meristems are typical across most of the taxa, studying cell lineage characteristics of the layers (development, division, and mutation rates) would be useful in addressing the germline problem.

In plants with stratified meristems, the meristematic sub-populations develop as early as the shoot apex does in the mature embryo. In redwood, this means that the L1 and L2 develop when the seedling is quite young and can remain separate for tens of meters of stem growth, perhaps indefinitely, until proleptic branching “removes” the L2 from further propagation by founding a new L2 from the parental L1. While some of the early shoot apical meristem papers contradict this separation via stratification (Douliot, 1890; Crafts, 1943; Sterling, 1945), we saw little evidence of this in our chimeric analysis data spanning over 66 meters of primary growth. In rare cases, a cell from the outer L1 layer would enter the L2 layer, but these sectors do not remain mixed for long, frequently reverting back to the original L2 phenotype. In redwood seedling development, a lignotuber, essentially a collection of underground buds embedded in wood and bark, develops in the first two nodes (Del Tredici 1998). The lignotuber helps separate new ramet-forming cell lineages early in genet development (Del Tredici 1998). Since the

parental L1 genotype founds a majority of proleptic branches (Table 1.1) and the parental L2 genotype can be lost in the primary growth of a tree, the L1 cell lineage could be considered analogous to a redwood germline.

A germline would be expected to have reduced division and/or mutation rate (Walbot and Evans, 2001; Lanfear 2018). In *Arabidopsis* and *Solanum*, somatic cell lineages that form new branches or sexual offspring divide less than the rest of the soma of the plant, irrespective of plant height and age (Burian et al., 2016; Groot and Laux 2016; Watson et al., 2016). These new branches, theoretically with fewer mutations, look very similar to the residual protoderm that initiates proleptic branches in redwood (Fink 1984) and other seed plants (Meier et al., 2012). These pockets of "young" cells that have not divided as much, irrespective of internodal stem length (Burian et al., 2016; Groot and Laux 2016; Watson et al., 2016), may help to explain why taller plant species seem to have slower rates of mutation accumulation compared to shorter plants (Lanfear et al., 2013). Our data showing branches originating from different meristem layers may help explain why the mutation rate is not always dependent on stem length, age, or branch location (Zahradníková et al., 2020; Schmitt et al., 2023). Some somatic cell lineages, like the L1, could contribute fewer accumulated mutations to new proleptic branches and meiocytes, also making the L1 a potential candidate for a functional germline in redwood. Interestingly, redwood L1 and L2 have different numbers of apical initials and give rise to vastly different numbers of cells (L1 makes apical dome and shoot surface area, while L2 fills in the apical dome and shoot volume). This suggests that L1 initials may divide less frequently since they are more numerous and make fewer offspring cell derivatives. This result also supports the notion that, compared to L2, L1 could be a potential "functional germline" in redwood.

An assumption made in this plant germline debate is that sexual offspring derive from either a hypothetical functional germline or from somatic cells, a limitation that does not correspond with our results. Our study indicates that meiocytes do not always derive from a single meristematic layer (Table 1.2). In redwood, instead, the two cell lineages provide redundancy and either could theoretically contribute to offspring. In this way, a redwood is a mosaic of cells derived from the parental L1 and L2 genotypes which become more genetically heterogenous over time through accumulated mutations. Both layers and their different mutations that accumulate can contribute to offspring, albeit unequally. The proportion of the parental L1 to parental L2 genotypes in meiocytes directly correlates with the proportion of proleptic to sylleptic branches, respectively, and therefore with the proportion of pollen and seed cones. Accordingly, a redwood tree could utilize different proportions of the parental L1 "proposed functional germline" cells or parental L2 "somatic" cells to found the new branches and sexual generations. This branching ratio can change depending on deterministic (e.g. inherent genotype; Libby and Tufuor 1984) or environmental factors (e.g. branch dieback due to biotic or abiotic factors).

Similarly, the separation of L1 and L2 from embryo to gametes is not absolute; specifically, L1 replaces L2 in most proleptic branches. Proleptic branching resembles, in a way, the initial embryonic separation of the L1 and L2 lineages (Figure 1.3). In the embryo, cell lineages have not had much time to accumulate different mutations, so it is highly likely that L1 and L2 are genetically identical at the initial separation. Over time, however, L1 and L2 can theoretically mutate independently, further separating until the time when a proleptic branch arises from parent L1 cells. In this case, a developmental bottleneck forces L1 and L2 of most proleptic branches to form from a single layer (and only a subset of sectors from this layer). This

genetically homogenizes the new shoot and its distal L1 and L2 layers, only for them to theoretically separate and accumulate mutations as the new proleptic branch grows further from its point of origin. Cone origins, therefore, matter the most when branching is sylleptic, with L1 and L2 layers separated further back into time and more likely to have different mutations within them (Figure 1.3).

Redwood reproduction, therefore, is perhaps more variable than simply reproduction from a hypothetical L1 germline. By growing with two layers and those two layers able to independently contribute to sexual offspring, a redwood tree can have two different sets of accumulated mutations in its offspring. A germline implies a single cell lineage that is genetically "static" or unlikely to change quickly. Redwoods may employ this strategy with respect to L1 but still use L2 to accumulate different mutations. The latter potentially incorporates any of these diverging genotypes in the meiocytes and in new embryos, increasing chances of heterozygosity of somatic mutations and thereby reducing genetic load.

Cell Lineage "Sex Determination"

Gymnosperms have poor pre-zygotic limits to inbreeding (Willson and Burley, 1983). This is likely why most gymnosperms are dioecious, with separate male and female individuals (Walas et al., 2018). Interestingly, while some families are entirely dioecious, some families like Cupressaceae are a mix of dioecious and monoecious species. Here, we present a new form of functional dioecy whereby the "males" and "females" are separated by somatic cell layers in the shoot apical meristem.

We find that, on average, microsporangiate strobili and megasporangiate strobili, and therefore the microgametophytes and megagametophytes they produce, ultimately derive from

different layers of the shoot apical meristem, main axis (parental) L1 and L2 respectively. We hypothesize that this separation of "sexes" early in development reduces the heritability of somatic mutations assuming inbreeding within the monoecious individual. Put another way, for redwood, we hypothesize that this system reduces risk of homozygous fixation of any somatic mutations through the separation of somatic mutations to majority male or female reproductive tissues (Figure 1.3). This is done through the separation of L1 and L2 in proleptic and sylleptic branches respectively. However, and interestingly, it does not eliminate the possibility of homozygous fixation of a beneficial, neofunctionalized somatic mutation assuming risk of deleterious somatic mutations is reduced through meiosis or polyploidy. In other words, the reproductive separation of the system is not 100%, but ends up being closer to 73% for both cones (Table 1.2), so homozygous fixation is possible, but it would be greatly reduced in most seeds.

Consider the following thought experiment of a neutral mutation on a neofunctionalized, diploidized locus. Without cell lineage separation in the sporophyte, a neutral mutation accumulated in the sporophyte on any given locus will end up in 50% of gametes. Assuming inbreeding within the sporophyte, 25% of the next sporophyte generation will have the neutral mutation fixed in both copies while 50% will have one copy of the neutral mutation. Only 25% of new sporophytes will not have the mutated allele (Figure 1.3).

Now, if we assume the model suggested by our data (Figure 1.3), that most sperm and egg ultimately derive from different cell lineages of the stratified shoot apical meristem which are separated early in development, neutral mutation fixation is reduced by half. For example, if the L2 accumulates a mutation and the L1 does not, 50% of eggs will have this mutation while 0% of sperm will. Assuming the same inbreeding, none of the zygotes will be homozygous for

the mutation. It would result in 50% of the offspring heterozygous for the mutated allele and 50% of the offspring without the mutated allele. This halves mutation fixation for any given locus compared to the first example where sporophyte shoot apices are not stratified. Obviously for loci that are not neofunctionalized and diploidized and act truly as hexaploids, the chances of fixation of a mutation are 16.6% (one-sixth). However, expression underlying phenotypes of these hexaploid loci still may be affected by gene dosage effects (Birchler et al., 2005), making separation of accumulated somatic mutations in the two sexes still of critical importance to the survival of future generations. In summary, chances of mutation fixation are lower with the separation of cell lineages by sex early in development.

Similarly, beneficial mutations under this model are less-likely to be fixed as homozygotes. However, recall that this model is probabilistic, with only 73.9% for sperm and 72.7% for eggs deriving from L1 and L2, respectively (Table 1.2), due to probabilities of proleptic and sylleptic branches deriving from L1 and L2 (Table 1.1). This means that 26.1% of sperm and 27.3% of eggs do not follow this developmental pattern. This is due to variation in what branches produce cones and what cell lineages contribute to the branches. This variation should enable rare, beneficial mutations to fix. However, most beneficial mutations would only fix if beneficial in a heterozygous state, because deleterious alleles should accumulate in other portions of the "selfing" (same layer) genome and would be difficult to eliminate all of them through recombination alone.

The probability of fixing deleterious mutations is less than 0.5, as selection can happen in the gametophytes. Regardless of the existence of a "germline", simply the separation of cell lineages early in sporophyte development and the use of different sporophytic cell lineages for gamete production is enough to drastically reduce mutational load in the offspring. It is through

this mechanism, essentially functional cell lineage dioecy in a monoecious species, that Muller's ratchet (Felsenstein 1974), mutational meltdown from mutational load, is avoided in coast redwood.

Some experimental evidence suggests that separation of somatic cell lineages may be advantageous to offspring. Self-fertilized (inbred) annual *Erythranthe* (formerly *Mimulus*) seedlings grow better if crossed from flowers on different branches of the same plant rather than the same flower on the same plant (Cruzan et al., 2022). This suggested that the further away the flowers were from each other within a plant, the fewer same somatic mutations accumulated reducing the deleterious effects of inbreeding (Cruzan et al., 2022). We hypothesize that a similar phenomenon happens in redwood, but through somatic separation of the cell lineages early in development, rather than somatic separation of branches early in development as seen in *Erythranthe*.

While dioecy, or separation of sexes in separate individuals, is common in gymnosperm lineages, no other plant has been reported to show the modeled pattern of "sex determination" based on early separation of somatic cell lineages in the shoot apical meristem (Figure 1.3), though we hypothesize this may exist in other monoecious conifers with stratified shoot apices. Redwoods are not the only organism whose somatic cell lineages are correlated with the sex of their offspring. An ant species (yellow crazy ants; *Anoplolepis gracilipes*) has male individuals are chimeras of two divergent asexual cell lineages, W and R (Darras et al., 2023). These lineages are similar to the asexually divergent L1 and L2 lineages in redwood. The R lineage (like the L2) makes most of the somatic cells, where the W lineage (like the L1) makes very few. Interestingly, the somatic R lineage, when sexually reproductive, makes female offspring (like the L2), where the W lineage, when sexually reproductive, makes male offspring (like the L1).

However, like the L1 and L2 in redwood, the W lineage makes most of the sperm cells, whereas the R lineage makes very few sperm cells, respectively. Interestingly, the proportion is still random and close to the same value in both species. In our study, L1 genomes contribute to approximately 73.9% of male gametes; in yellow crazy ants, W haplotypes represent approximately 67% of male gametes (Darras et al., 2023). In summary, male crazy ants are like redwood branches whose two major somatic cell lineages propagate asexually and separately determine sex of the offspring for the lifetime of the colony, akin to the redwood tree.

There is another interesting commonality between redwoods and yellow crazy ants which warrants further study. The ant genomes have a cell division immediately following fertilization separating the W and R genomes. Redwoods are the only known conifer species reported to undergo cytokinesis after the first mitotic division of the zygote (Buchholz 1939). While unlikely, it is theoretically possible that L1 and L2 lineages separate in the first cell division. More work is needed to determine at which stage the two lineages separate.

CONCLUSION:

Redwoods typically have two sub populations of cells in their shoot apical meristems, the L1 and L2 (Figure 1.1). These divide the meristem into two, developmentally independent cell lineages which can mutate and diverge over time. As the meristem branches, the two sub-populations do not contribute equally to the new meristems equally. Sylleptic branches (concurrently growing with the parent; Hallé et al., 1978) are more likely to keep the cell lineage organizational status quo, while proleptic branches (delayed growth from the parent; Hallé et al., 1978) are more likely to replace the L2 with the L1 during branch formation, further subdividing the cell lineages within the tree (Figure 1.2). Along with the stratified subdivision of

meristematic layers, different branch types themselves are subdivisions of these two cell layers. The result is a redwood tree that separates out risk of transmitting accumulated mutations to the next generation over a wide swath of branches and their meristem layers.

Redwood longevity and genomic integrity, therefore, are integrally linked with the ability to compartmentalize theoretical mutations in separate cell lineages and sexes, thereby mitigating risk of mutation accumulation (Figure 1.3). These cell lineages (L1 and L2) are separated early in development, typically in the embryo. This means most sperm and egg from the same sporophyte may originate from layers that have accumulated different mutations throughout the life of that sporophyte (Figure 1.3). After meiotic recombination, even with inbreeding within the same sporophyte, somatic mutations are less likely to fix in the offspring due to the different cell lineage contributions to the meiocytes (Figure 1.3). This is a form of developmental selection (Buchholz 1922) within the soma (Klekowski 1988). At the same time, redwoods can utilize proleptic branches to expose L1 genotypes to selection, uncoupling their fate from the fate of the L2 (Table 1.1).

REFERENCES:

Aanen, D.K., A. van ’t Padje, and B. Auxier. 2023. Longevity of fungal mycelia and nuclear quality checks: a new hypothesis for the role of clamp connections in dikaryons. *Microbiology and Molecular Biology Reviews*: e00022-21.

Birchler, J.A., N.C. Riddle, D.L Auger, and R.A. Veitia. 2005. Dosage balance in gene regulation: biological implications. *Trends in Genetics* 21: 219-226.

Buchholz, J.T. 1922. Developmental selection in vascular plants. *The Botanical Gazette* 73: 249-286.

Buchholz, J.T. 1939. The embryogeny of *Sequoia sempervirens* with a comparison of the sequoias. *American Journal of Botany* 26: 248-257.

Burian, A. 2021. Does shoot apical meristem function as the germline in safeguarding against excess of mutations? *Frontiers in Plant Science* 12: 707740.

Burian, A., P. B. de Reuille, C. Kuhlemeier. 2016. Patterns of stem cell divisions contribute to plant longevity. *Current Biology* 26: 1385-1394.

Buss, L.W. 1985. The uniqueness of the individual revisited. In: J.B.C. Jackson, L.W. Buss, and R.E. Cook, eds. *Population Biology and Evolution of Clonal Organisms* 467-505. Yale University Press, New Haven and London.

Campbell, D.H. 1940. *The Evolution of the Land Plants (Embryophyta).* Stanford University Press. Stanford University, CA, USA.

Carroll, A.L. and S.C. Sillett. 2023. Overcoming crossdating challenges to generate ring-width chronologies for *Sequoia sempervirens* across its native range. *Dendrochronologia* 78: 126063.

Chin, A.R.O., P. Guzmán-Delgado, S.C. Sillett, J. Orozco, R.D. Kramer, L.P. Kerhoulas, Z.J. Moore, M. Reed, M.A. Zwieniecki. 2022. Shoot dimorphism enables *Sequoia sempervirens* to separate requirements for foliar water uptake and photosynthesis. *American Journal of Botany* 109: 564-579.

Crafts, A. S. 1943. Vascular differentiation in the shoot apex of *Sequoia sempervirens*. *American Journal of Botany* 30: 110-121.

Cross, G. I. 1943. A comparison of the shoot apices of the sequoias. *American Journal of Botany* 30: 130-142.

Cruzan, M.B., M.A. Streisfeld, and J.A. Schwoch. 2022. Fitness effects of somatic mutations accumulating during vegetative growth. *Evolutionary Ecology* 36: 767-785.

Darras, H., C. Berney, S. Hasin, J. Drescher, H. Feldhaar, L. Keller. 2023. Obligate chimerism in male yellow crazy ants. *Science* 380: 55-58.

Davis, D. F. and D. F. Holderman. 1980. *The White Redwoods:Ghosts of the Forest.* Naturegraph Publishers Inc., Happy Camp, California, USA.

Douliot, H. 1890. Recherches sur la croissance terminale de la tige des Phanerogames. *Annales des sciences naturelles: botanique.* VII. 11: 301-302 (in French).

Felsenstein, J. 1974. The evolutionary advantage of recombination. *Genetics* 78: 737-756.

Fink, S. 1984. Some cases of delayed or induced development of axillary buds from persisting detached meristems in conifers. *American Journal of Botany* 71: 44-51.

Groot, E.P. and T. Laux. 2016. How do long-lived plants escape mutational meltdown? *Current Biology* 26: R530-R532.

Guttenberg, H. von. 1961. Grundzüge der Histogenese höherer Pflanzen. II. Die Gymnospermen. *Handbuch der Pflanzenanatomie* 8 (in German).

Hallé, F., R.A.A. Oldeman, and P.B. Tomlinson. 1978. *Tropical trees and forests: an architectural analysis.* Springer-Verlag, Berlin, Germany.

Harper, J.L. 1985. Modules, branches, and the capture of resources. In: In: J.B.C. Jackson, L.W. Buss, and R.E. Cook, eds. *Population Biology and Evolution of Clonal Organisms* 1-33. Yale University Press, New Haven and London.

Hejnowicz, Z. 1956. First periclinal chimera among gymnosperms. *Acta Societatis Botanicorum Poloniae* 24:181-202.

Hejnowicz, Z. 1959. Eversporting periclinal chimeras. *Recent Advances in Botany* 2: 1446-1448.

Howe, J., J.C. Rink, B. Wang, and A.S. Griffin. 2022. Multicellularity in animals: the potential for within-organism conflict. *Proceedings of the National Academy of Sciences* 119: e2120457119.

Jackman, V. H. 1960. The shoot apex of some New Zealand gymnosperms. *Phytomorphology* 10: 145-157.

Jensen, W.A. 1962. *Botanical Histochemistry: Principles and Practice*. W.H. Freeman and Company, San Francisco and London.

Klekowski, E.J. Jr. 1988. *Mutation, Developmental Selection, and Plant Evolution*. Columbia University Press, New York, New York, USA.

Klekowski, E.J. 2003. Plant clonality, mutation, diplontic selection and mutational meltdown. *Biological Journal of the Linnean Society* 79: 61-67.

Klekowski, E.J., Jr., N. Kazarinova-Fukshansky. 1984a. Shoot apical meristems and mutation: fixation of selectively neutral cell genotypes. *American Journal of Botany* 71: 22-27.

Klekowski, E.J., Jr., N. Kazarinova-Fukshansky. 1984b. Shoot apical meristems and mutation: selective loss of disadvantageous cell genotypes. *American Journal of Botany* 71: 28-34.

Klekowski, E.J., Jr., N. Kazarinova-Fukshansky, and H. Mohr. 1985. Shoot apical meristems and mutation: stratified meristems and angiosperm evolution. *American Journal of Botany* 72: 1788-1800.

Korn, R.W. 2001. Analysis of shoot apical organization in six species of the Cupressaceae based on chimeric behavior. *American Journal of Botany* 88: 1945-1952.

Korn, R.W. 2002. Chimeric patterns in *Juniperus chinensis* 'Torulosa Variegata' (Cupressaceae) expressed during leaf and stem formation. *American Journal of Botany* 89: 758-765.

Kramer, R. D., S. C. Sillett, and A. L. Carroll. 2014. Structural development of redwood branches and its effects on wood growth. *Tree Physiology* 34: 314-330.

Lanfear, R. 2018. Do plants have a segregated germline? *PLoS Biology* 16: e2005439.

Lanfear, R., S.Y.W. Ho, T.J. Davies, A.T. Moles, L. Aarssen, N.G. Swenson, L. Warman, A.E. Zanne, and A.P. Allen. 2013. Taller plants have lower rates of molecular evolution. *Nature Communications* 4: 1879.

Libby, W. J. and K. Tufuor 1984. 'Korbel KT' USPP5345P.

Marcotrigiano, M. 2000. Herbivory could unlock mutations sequestered in stratified shoot apices of genetic mosaics. *American Journal of Botany* 87: 355-361.

Meier, A.R., M.R. Saunders, and C.H. Michler. 2012. Epicormic buds in trees: a review of bud establishment, development, and dormancy release. *Tree Physiology* 32: 565-584.

Neale, D.B., Zimin, A.V., Zaman, S., Scott, A.D., Shrestha, B., Workman, R.E., Puiu, D., Allen, B.J., Moore, Z.J., Sekhwal, M.K., De La Torre, A.R., McGuire, P.E., Burns, E., Timp, W., Wegrzyn, J.L., and Salzberg, S.L. 2022. Assembled and annotated 26.5 Gbp coast redwood genome: a resource for estimating evolutionary adaptive potential and investigating hexaploid origin. *G3—Genes, Genomes, and Genetics* 12: jkab380.

Otto, S.P. and I.M. Hastings. 1998. Mutation and selection within the individual. *Genetica* 102/103: 507-524.

Otto, S.P. and M.E. Orive. 1995. Evolutionary consequences of mutation and selection within an individual. *Genetics* 141: 1173-1187.

Park, H-S., J-H. Jeon, W. Cho, Y. Lee, J.Y. Park, J. Kim, Y.S. Park, H.J. Koo, J.H. Kang, T.J. Lee, S.H. Kim, J-B. Kim, H-Y. Kwon, S-H. Kim, N-C. Paek, G. Jang, J-Y. Suh, T-J. Yang. 2023. High-throughput discovery of plastid genes causing albino phenotypes in ornamental chimeric plants. *Horticultural Research* 10: uhac246.

Peirce, G.J. 1901. Studies on the coast redwood, *Sequoia sempervirens* Endl. *Proceedings of the California Academy of Sciences: Botany* 2: 83-106.

Peltier, D.M.P., M.S. Carbone, M. Enright, M.C. Marshall, A.M. Trowbridge, J. LeMoine, G. Koch, and A.D. Richardson. 2023. Old reserves and ancient buds fuel regrowth of coast redwood after catastrophic fire. *Nature Plants* (in press).

Pineda-Krch, M. and T. Fagerström. 2001. On the potential for evolutionary change in meristematic cell lineages through intraorganismal selection. *Journal of Evolutionary Biology* 12: 681-688.

Pineda-Krch, M. and K. Lehtilä. 2002. Cell lineage dynamics in stratified shoot apical meristems. *Journal of Theoretical Biology* 219: 495-505.

Pittermann, J., J. Cowan, N. Kaufman, A. Baer, E. Zhang, and D. Kuty. 2018. The water relations and xylem attributes of albino redwood shoots (*Sequioa* (sic) *sempervirens* (D. Don.) Endl.). *PLoS ONE* 13: e0191836.

Pohlheim, F. 1971. Untersuchungen zur Sproßvariation der Cupressaceae. I. Nachweis immerspaltender Periklinalchimären. *Flora* 160: 264-293 (in German with English abstract).

Ren, Y., Z. He, P. Liu, B. Traw, S. Sun, D. Tian, S. Yang, Y. Jia, and L. Wang. 2021. Somatic mutation analysis in *Salix suchowensis* reveals early-segregated cell lineages. *Molecular Biology and Evolution* 38: 5292-5308.

Reusch, T.B.H., I.B. Baums, B. Werner. 2021. Evolution via somatic genetic variation in modular species. *Trends in Ecology and Evolution* 36: 1083-1092.

Ruth, J., E.J. Klekowski, Jr., and O.L. Stein. 1985. Impermanent initials of the shoot apex and diplontic selection in a juniper chimera. *American Journal of Botany* 72: 1127-1135.

Satina, S., A.F. Blakeslee, and A.G. Avery. 1940. Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. *American Journal of Botany* 27: 895-905.

Schmidt, A. 1924. Histologische Studien an phanerogamen Vegetationspunkten. *Botanisches Archiv: Zeitschrift für gesamte Botanik* 8: 345-404 (in German).

Schmitt, S., P. Heuret, V. Troispoux, M. Beraud, J. Cazal, E. Chancerel, C. Cravero, E. Guichoux, O. Lepais, J. Loureiro, W. Marande, O. Martin, G. Vincent, J. Chave, C. Plomion, T. Leroy, M. Heuertz, N. Tysklind. 2023. Plant mutations: slaying beautiful hypotheses by surprising evidence. bioRxiv preprint https://doi.org/10.1101/2023.06.05.543657

Sillett, S.C., R. Van Pelt, A.L. Carroll, J. Campbell-Spickler, M.E. Antoine. 2020. Aboveground biomass dynamics and growth efficiency of *Sequoia sempervirens* forests. *Forest Ecology and Management* 458: 117740.

Sillett, S.C., M.E. Antoine, A.L. Carroll, Mark E. Graham, A.R.O. Chin, R. Van Pelt. 2022. Rangewide climactic sensitivities and non-timber values of tall *Sequoia sempervirens* forests. *Forest Ecology and Management* 526: 120573.

Stapleton, T. S. and D. F. Holderman 2016. 'Mosaic Delight' USPP26573 P3.

Stapleton, T. S. and D. F. Holderman 2018. 'Early Snow' USPP29217 P2.

Stapleton, T. S. and D. F. Holderman 2018. 'Grand Mosaic' USPP29606 P3.

Sterling, C. 1945. Growth and vascular development in the shoot apex of *Sequoia sempervirens*. I. Structure and growth of the shoot apex. *American Journal of Botany* 32: 118-126.

Strasburger, E. 1872. *Die Coniferen und die Gnetaceen. Eine morphologische Studie.* Jena, H. Dabis (in German).

Tian, H-C, and M. Marcotrigiano. 1993. Origin and development of adventitious shoot meristems initiated on plant chimeras. *Developmental Biology* 155: 259-269.

Tilney-Bassett, R.A.E. 1986. *Plant Chimeras.* Edward Arnold, Baltimore, Maryland, USA.

Walas, L., W. Mandryk, P.A. Thomas, Z. Tyrala-Wierucka, and G. Iszkulo. Sexual systems in gymnosperms: a review. *Basic and Applied Ecology* 31: 1-9.

Walbot, V. and M.M.S. Evans. 2001. Unique features of the plant life cycle and their consequences. *Nature Reviews Genetics* 4: 369-379.

Wang, L., Y. Ji, Y. Hu, H. Hu, X. Jia, M. Jiang, X. Zhang, L. Zhao, Y. Zhang, Y. Jia, C. Qin, L. Yu, J. Huang, S. Yang, L.D. Hurst, and D. Tian. 2019. The architecture of intra-organism mutation rate variation in plants. *PLoS Biology* 17: e3000191.

Watson, J.M., A. Platzer, A. Kazda, S. Akimcheva, S. Valuchova, V. Nizhynska, M. Nordborg, and K. Riha. 2016. Germline replications and somatic mutation accumulation are independent of vegetative life span in *Arabidopsis*. *Proceedings of the National Academy of Sciences* 113: 12226-12231.

Whitham, T.G., and C.N. Slobodchikoff. 1981. Evolution of individuals, plant-herbivore interactions, and mosaics of genetic variability: the adaptive significance of somatic mutations in plants. *Oecologia* 49: 287-292.

Willson, M.F. and N. Burley. 1983. *Mate choice in plants: tactics, mechanisms, and consequences.* Princeton University Press, Princeton, New Jersey, USA.

Zahradníková, E., A. Ficek, B. Brejová, T. Vinař, K. Mičieta. 2020. Mosaicism in old trees and its patterns. *Trees* 34: 357-370.

TABLES AND FIGURES:

Table 1.1 – Branch Origins

Tree	Total Stem Length (m)	Sylleptic Branch Phenotype		Proleptic Branch Phenotype	
		Parental L2	Other	Parental L1	Other
Cotati	6.185	164 (95.3%)	8 (4.7%)	2 (100.0%)	0 (0.0%)
Russian River	7.330	36 (100.0%)	0 (0.0%)	272 (80.0%)	68 (20.0%)
S1C1	7.015	175 (98.9%)	2 (1.1%)	86 (85.1%)	15 (14.9%)
S1C3	4.872	139 (97.9%)	3 (2.1%)	60 (80.0%)	15 (20.0%)
S1C4	5.111	98 (98.0%)	2 (2.0%)	112 (96.6%)	4 (3.4%)
S1C5	2.542	43 (100.0%)	0 (0.0%)	71 (89.9%)	8 (10.1%)
S1C6	4.710	166 (99.4%)	1 (0.6%)	32 (91.4%)	3 (8.6%)
S1C7	8.620	290 (97.3%)	8 (2.7%)	117 (81.3%)	27 (18.8%)
S2C1	17.753	495 (95.0%)	26 (5.0%)	14 (73.7%)	5 (26.3%)
S3C1	2.180	71 (95.9%)	3 (4.1%)	23 (92.0%)	2 (8.0%)
totals	66.328	1677 (96.9%)	53 (3.1%)	789 (84.2%)	147 (15.7%)
p-value		1.72×10^{-14}		1.00×10^{-7}	
		1.65×10^{-3}			

Table 1.1: Sylleptic and proleptic branch phenotypes are reported per tree, with number and percentages (in parentheses) of sylleptic branches showing L2 phenotype proleptic branches showing L1 phenotype (their respective majority "trends"). The table also includes the total stem length of each tree in meters. Besides the stem length column, all other reported values in each column are numbers of nodes per tree showing the following pattern. Sylleptic branches mostly replicate the L2 phenotype of the parent branch and proleptic branches mostly replicate the L1 phenotype of the parent branch. These are denoted as "Parental L2" and "Parental L1" columns, showing percentages of "trend-following" branches. The "other" column shows instances where the trend breaks, often with mixes of both phenotypes or complete reversion to the reciprocal phenotype. These instances are relatively rare. Student t-tests were used to calculate P-values comparing three different groups. In the top row, P-values are reported, indicating the certainty of the two branch types having a "trend" in their respective cell layer(s) of origin. The bottom row p-value demonstrates significant difference between two trend magnitudes. A less variable

trend (96.9%) of L2 phenotypes in sylleptic branches is significantly different from the more variable trend (84.2%) of L1 phenotypes in proleptic branches. This indicates that, compared to sylleptic branches, proleptic branches have significantly more variability in the cell layers that compose them.

Table 1.2 – Cone Origins

	Seed Cones		Pollen Cones	
	Sylleptic	Proleptic	Sylleptic	Proleptic
Tree 1, Branch 1	37 (75.5%)	12 (24.5%)	373 (18.3%)	1664 (81.7%)
Tree 1, Branch 2	11 (73.3%)	4 (26.6%)	76 (14.0%)	476 (86.0%)
Tree 2, Branch 1	31 (37.3%)	52 (62.7%)	72 (13.6%)	458 (86.4%)
Tree 2, Branch 2	38 (80.9%)	9 (19.1%)	124 (28.8%)	306 (71.2%)
Tree 3, Branch 1	99 (90.8%)	10 (9.2%)	111 (13.7%)	699 (86.3%)
Tree 3, Branch 2	28 (82.4%)	6 (17.6%)	19.1%	144 (80.9%)
mean percent of cone type	73.4%	26.6%	17.9%	82.1%
percent std. dev.	18.7%	18.7%	5.9%	5.9%
percent std. error	7.6%	7.6%	2.4%	2.4%
p-values	1.40×10^{-2}		2.09×10^{-5}	
	2.99×10^{-4}			

Table 1.2: Seed and pollen cone branch origins by number and percentage of cone type (in parentheses) from six large redwood branches from three trees. Student T-tests were conducted between the two sets to calculate p-values. P-values were reported for whether a trend existed with sylleptic and proleptic branches and which cones they produce. Sylleptic branches formed significantly more seed cones than pollen cones, and proleptic branches formed significantly more pollen cones than seed cones. The p-value in the lower row compares whether the two trends have significantly different magnitudes. Interestingly, they do, indicating seed cones have more variable branch origins (73.4%) than pollen cones (82.1%).

Figure 1.1 – Shoot Apical Meristem Stratification

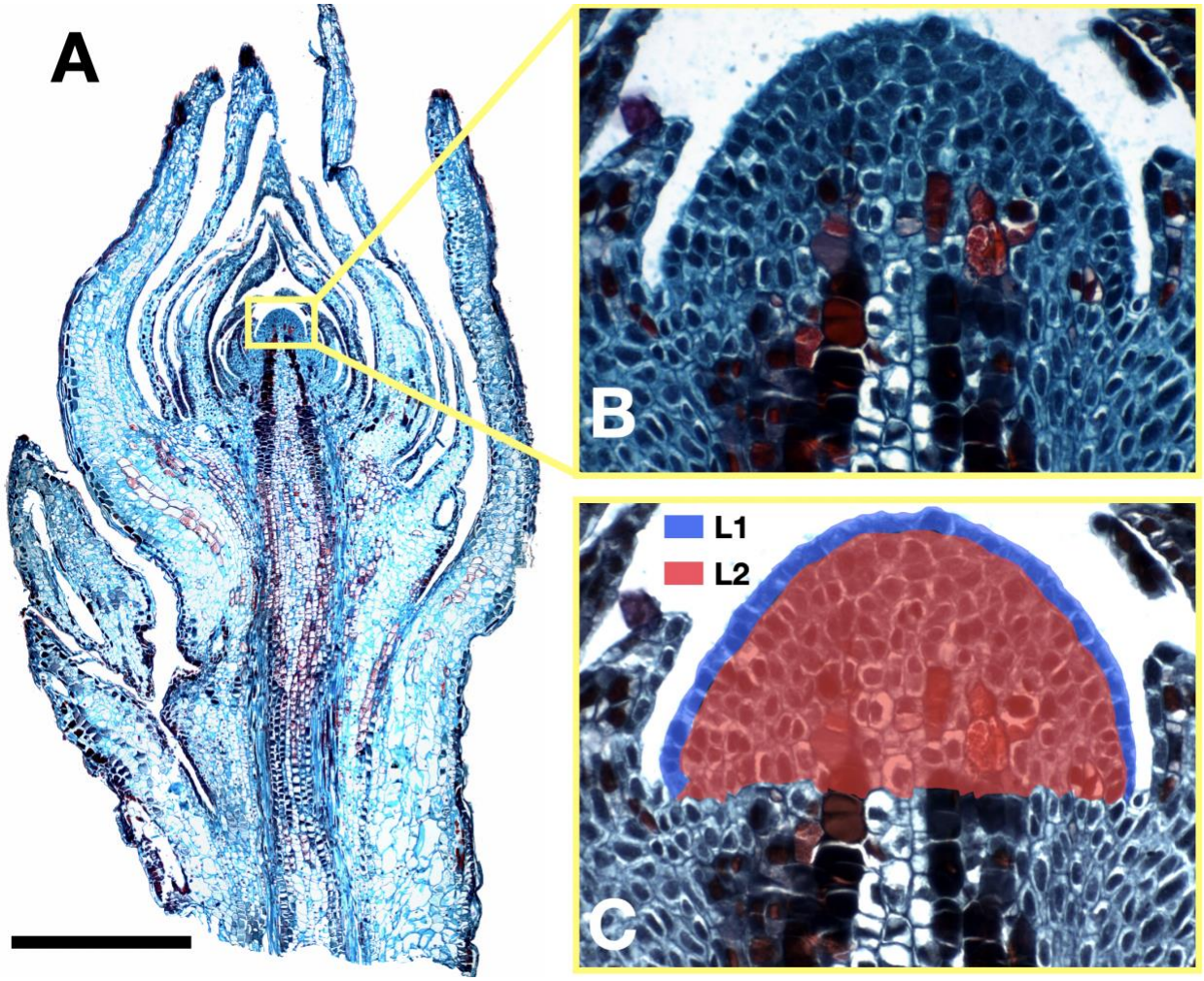

Figure 1.1: Median longitudinal section of a redwood vegetative shoot tip (A) and its corresponding shoot apical meristem in the inset (B). Scale bar is 1 mm. Panel C shows the same shoot apical meristem with the two stratified subpopulations of cells labeled in false color. The outer layer, colored blue, is the L1; the inner layer, colored red, is the L2. The shoot apical meristem has a stratified organization with distinct L1 (blue outer layer) and L2 (red inner layer). These two separate subpopulations which rarely mix enable differential mutation accumulation to occur between the two layers, allowing theoretical genetic divergence between the two layers.

Figure 1.2 – Branch Origins

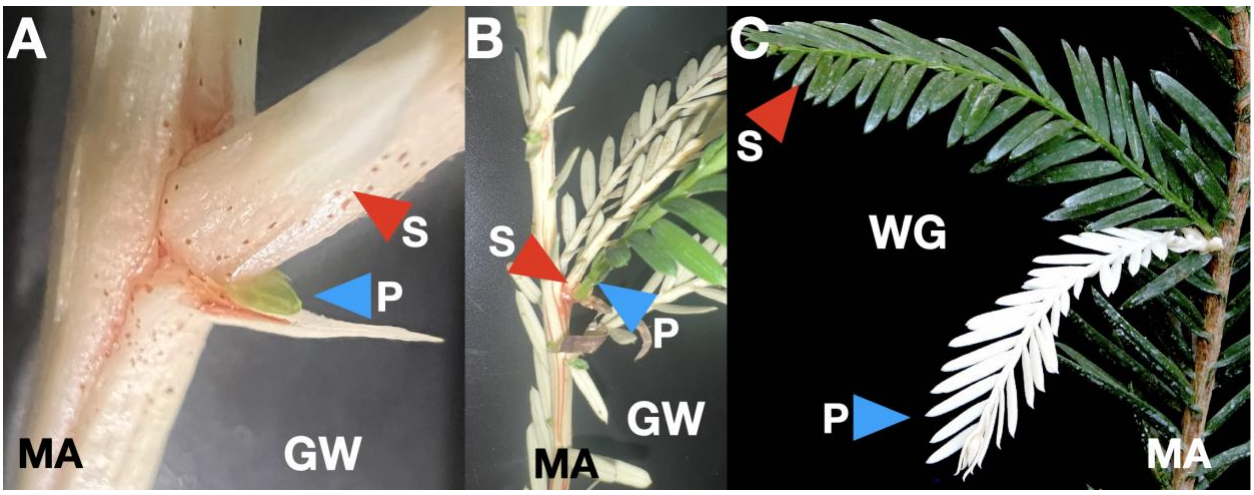

Figure 1.2: Sylleptic (red arrows; labeled S) and proleptic (blue arrows; labeled P) branches developing from single nodes on the main axis (labeled MA) on chlorophyll-deficient chimeras. Sylleptic branches grow concurrently with the parent branch and proleptic branches grow delayed. Sylleptic and proleptic branches have distinct developmental origins, with proleptic branches forming only from residual protoderm (Fink 1984). In periclinal chimeras, cells that produce the epidermis (L1; tunica) may be different in genotype than cells that produce the interior layers (L2; cortex). In chlorophyll-deficient periclinal chimeras, sylleptic and proleptic branches emerge with different colors. Plants can have a green L1 and white L2 (GW; A, B) or the opposite white L1 and green L2 (WG; C). Note that the parental L1 phenotypes make up the proleptic branches (green in A and B; white in C), indicating protoderm origin. Proleptic branches can occur by themselves in otherwise empty axils as seen in most of the green buds in (B) or as accessory branches, secondary to a sylleptic branch as seen with the arrows. Once branches have fully matured (C), sylleptic and proleptic branches appear very similar in form while retaining evidence of different cell lineage origins by their different colors in chlorophyll-deficient periclinal chimeras.

Figure 1.3 – Redwood Sporophyte Cell Lineage Model

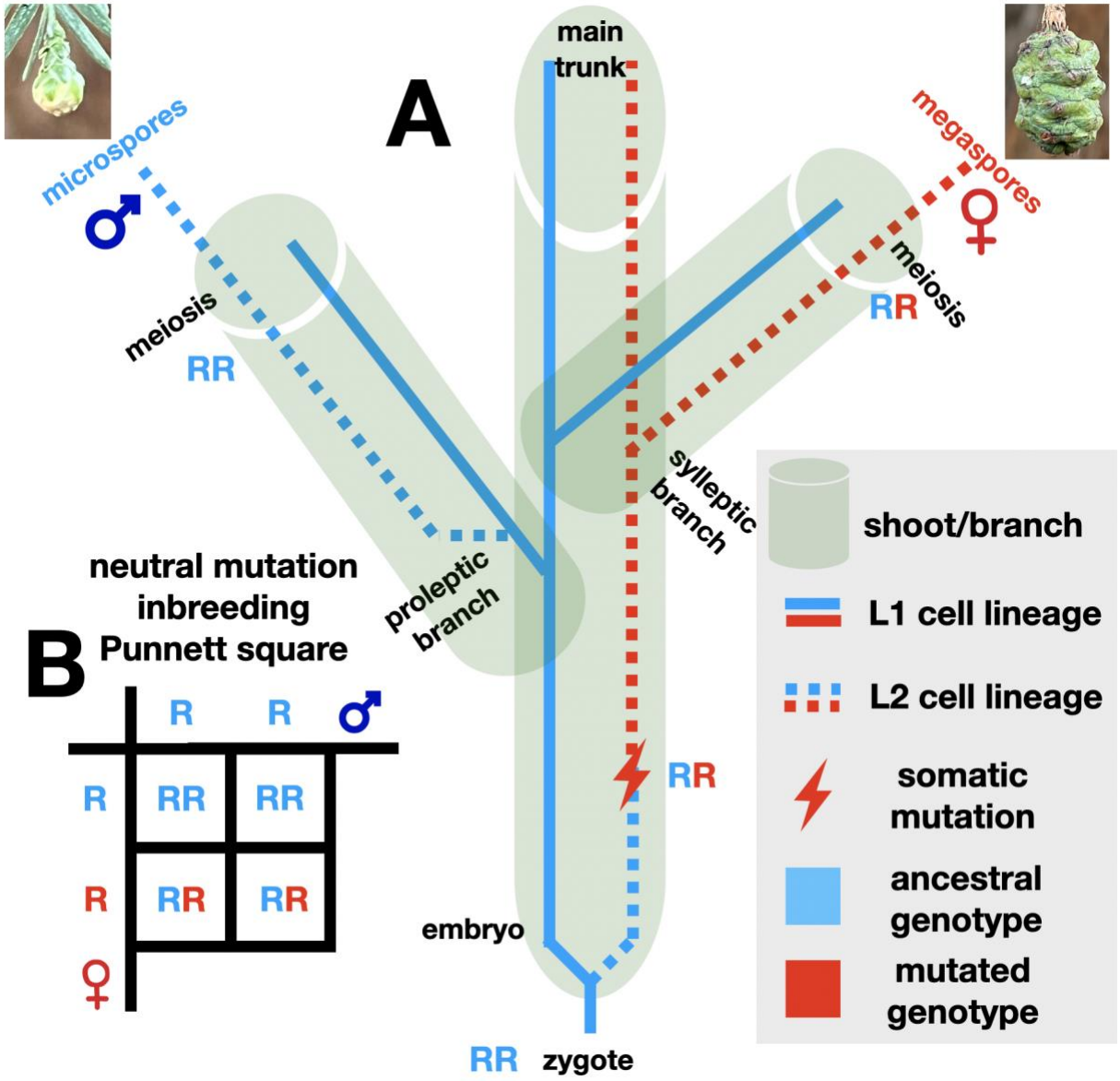

Figure 1.3: A diagrammatic representation of the redwood life cycle showing sporophyte from zygote to meiosis. This model shows the majority fate of two cell layers in the shoot apical meristem (L1 and L2), after two types of branching events (sylleptic and proleptic) and after a putative somatic mutation affects one of the cells in the shoot apex. A zygote of a single genotype grows into an embryo with a shoot apical meristem where two cell layers, L1 and L2, are set aside in development, represented by dotted and dashed lines respectively. In this

example, these separate lineages accumulate different mutations over time, with the divergence represented in red and blue colors. Timing of branch release correlates with branch developmental origin. Sylleptic branches emerge with the parent shoot as axillary branches and proleptic branches emerge later. The parent shoot apex layering is copied in sylleptic branches while proleptic branches are produced only from the parent L1 layer. Cells that undergo meiosis (meiocytes), forming spores, gametophytes, and ultimately gametes, are ultimately derived from different layers. Sylleptic branches and the parental L2 (red, dashed) make most seed cones and eggs. Proleptic branches and the parental L1 make most pollen cones (blue, dashed) and sperm. Sperm and egg genotypes, therefore, can come from cell lineages that have accumulated mutations separately for the life of the sporophyte. Therefore, if inbred, the gametes reduce the likelihood of fixation of most somatic mutations that accumulate during the lifetime of the redwood sporophyte, which can grow for millennia.

CHAPTER 2

Redwoods accumulate somatic mutations and maximize intra-organismal genetic diversity leading to variation in reproductive fecundity within an individual.

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ABSTRACT:

Redwoods are a long-lived, clonal, hexaploid tree species. Their longevity brings into question safeguards of genomic stability and integrity. Without a defined germline, mutations that accumulate in the branches of a plant can end up in sexual offspring, leading to possible decline of the population through propagation of deleterious mutations. To investigate how somatic mutations accumulate, we sequenced fourteen branches from a single 1400-year-old, 107-meter-tall redwood tree and germinated seedlings from the two tree tops. Per year and per generation somatic mutation rates in this tree were higher than previously recorded in other plants. One of the two tree tops experienced a loss of a chromosome, affecting its fecundity (cone size, seed size, and seedling germination) compared to the other top. We speculate that intra-organismal selection maintains both longevity of each generation and fecundity across centuries.

INTRODUCTION:

Intra-organismal genetic heterogeneity may be important, perhaps critical, to the survival of modular organisms whose sexual reproduction is infrequent (Klekowski et al., 1985; Gill et al.

1995; Orive 2001; Pineda-Krch and Lehtilä 2004). Modular organisms are defined as organisms whose growth consists of many sub-units, or modules, repeated in an indeterminate way (plants, fungi, corals; Harper 1977; Harper and Bell 1979). Branching modular organisms accumulate mutations within their branches through mitosis (Watson et al., 2016; Schmid-Siegert et al., 2017; Anderson et al., 2018; Plomion et al., 2018; Hanlon et al., 2019; Hiltunen et al. 2019; Wang et al. 2019; Hofmeister et al., 2020; López and Palumbi, 2020; Orr et al., 2020; Yu et al., 2020; Zahradníková et al., 2020; Perez-Roman et al., 2021; Ren et al., 2021; López-Nandam et al., 2022; Schmitt et al., 2023). These somatic mutations, as opposed to gametic ones, may be a major source of nucleotide variation in a lineage (Whitham and Slobodchikoff 1981; Antolin and Strobeck 1985; Orive 2001). This is due to theoretically large reservoirs of mutations accumulated overtime in the plethora of shoot apical meristems and branches that compose a tree (Antolin and Strobeck, 1985; Klekowski et al., 1985; Klekowski 1988; Tomimoto et al., 2023) and that these mutations are heritable (Plomion et al., 2018; Wang et al. 2019). This heritability of accumulated somatic mutations in sexual offspring, due to normal somatic cell contribution to meiocytes and asexual reproduction (e.g., somatic embryogenesis) in the branches, means per-generation mutation rates are likely to be higher in modular organisms than unitary organisms with germlines (non-clonal animals) (Buss 1983b; Whitham and Slobodchikoff, 1981; Hanlon et al., 2019). This is hypothesized because trees, unlike animals, may harbor beneficial mutations in their somatic cell lineages and pass on those mutations to the next generation (Whitham and Slobodchikoff 1981; Pineda-Krch and Lehtilä 2002; Plomion et al., 2018; Wang et al. 2019; Aanen et al., 2023).

However, without selection, most mutations are not beneficial. Most accumulated somatic mutations that affect fitness are disadvantageous, resulting in higher mutational load in

the population (Keightley and Lynch 2003). Muller's ratchet (Muller 1964) describes how disadvantageous mutations accumulate in a population without sexual recombination. Without recombination, a beneficial mutation could not fix in the haploid products of meiosis because of the overwhelming presence of masked, deleterious alleles that would be exposed and potentially lethal. Therefore, meiosis needs to occur regularly to avoid mutational meltdown (Lynch et al., 1993), or extinction by accumulation of deleterious mutations. Increased sexual reproduction, or short generation times, exposes mutations through a haploid phase where selection can act on each haplotype combination (Lynch et al., 1993; Mable and Otto 1998; Walbot and Evans 2001). The other way to reduce negative effects of Muller's ratchet is by having "infinite" population sizes where selection is a bigger influence than drift (Felsenstein 1974).

The "population size" of a redwood, or any modular or clonal organism, is difficult to calculate because of problems dependent on what constitutes an "individual" in the "population" (Harper 1977; Harper and Bell 1979; Harper 1985; Buss 1983a; Buss 1985; Orive 1993; Clarke 2012; Aanen et al., 2023). Criteria for individuality mainly concern the hierarchical organizational level (genet, ramet, branch, module, metamer) at which the "individual unit" is reproductively and physiologically autonomous, and genetically homogeneous for potential variation in fitness ("individuation criteria", Clarke 2012). A modular organism lives on a continuous spectrum for both criteria, making discrete concrete boundaries of what constitutes an individual sometimes difficult to define. Physiological autonomy varies across scale and is partitioned in smaller units of organization such as ramets or branches (Sprugel et al., 1991). Similarly, genet homogeneity varies across scales, where the original zygotic genome will mutate in ramets and branches of roots and shoots, leading to intra-organismal genetic heterogeneity across time and space (Genetic Mosaicism Hypothesis; Gill et al. 1995; Pineda-Krch and Lehtilä

2004). As such, selection must act on each of these organizational hierarchies (Lewontin 1970; Buss 1983b; Otto and Orive 1995; Otto and Hastings 1998; Pineda-Krch and Fagerström 2001; Yu et al., 2020; Reusch et al., 2021; Howe et al., 2022; Aanen et al., 2023; Schmitt et al., 2023) to avoid mutational meltdown in the genet and population (Klekowski 2003). Selection at various hierarchies of structural complexity has been called "developmental selection" (Buchholz 1922; Klekowski 1988), where genomes go through developmental bottlenecks (Howe et al., 2022; Aanen et al., 2023) enabling selection and drift to act. Therefore, to evaluate how selection operates at smaller levels of biological organization (e.g., cells, organs), one must consider how to define the "individual" units (Buss 1983a) of the otherwise larger "population" that evolves in redwoods.

First, one could consider the redwood individual on the genet or ramet level. Redwood forests consist of 24 to 129 genets (Narayan 2015) and 77 to 339 ramets (Van Pelt et al., 2016) per hectare. If a genet, or single fertilization and all its mitotic descendants, constitutes an individual, the total population of redwood genets is relatively small with infrequent zygotes achieving maturity. This relatively small population size and rare sexual recombination means that, according to Muller's ratchet, mutational load threatens redwood populations with extinction or extirpation. Another interpretation of a tree is as a "population" made up of "individual" branches (Harper 1977; Harper and Bell 1979; Buss 1983a). Some individual redwood ramets have complex crowns (Sillett and Van Pelt, 2001) that blur what constitutes a tree versus a forest (Sillett and Van Pelt, 2000). In this case, a large branch "population" would reduce the impact of Muller's ratchet and mutational load would not be a threat to the species (Felsenstein 1974).

In this view of conceptualizing branches as “individuals” and a tree as a “physiologically interconnected population of branches”, selection in these “individuals” plays a crucial role (Fagerström 1992; Pineda-Krch and Lehtilä 2002; Otto and Orive 1995). Interpreting a genet or ramet as a population of branches requires a demographic approach, describing changes in subunit (branch) growth as birth, competition, reproduction, and death of “individuals” in the asexual genet “population” (Harper and Bell 1979; Buss 1983a; Cook 1985; Orive 1993). Some branches may be selected against (branch dieback due to fire, wind, pests, shade, frost, water availability, etc.) or promoted (reiteration in sunlight; Kramer et al., 2014) in the crown of a tree, leading to differences in branch-level fitness (Whitham and Slobodchikoff 1981).

Finally, based on the developmental analyses (shoot tip sections and chimeric analysis) in Chapter 1, redwoods typically have stratified shoot apical meristems. This means they have two concentric layers in the shoot apical meristem dome, the L1 and L2 (tunica and corpus, respectively), which develop into separate parts of the soma (see Chapter 1). Redwoods have two different branches which typically develop from different layers. Proleptic branches, ones that delay emergence (e.g., epicormic branches; Hallé et al., 1978), entirely originate through a developmental bottleneck founded by cells from the L1 of the parent meristem (see Chapter 1). Sylleptic branches, those that grow concurrently with the parent branch (Hallé et al., 1978), maintain the original layering of the parent meristem, not going through the same developmental bottleneck of proleptic branches (see Chapter 1). These different layers may accumulate somatic mutations differently, so we use this to inform our sampling methods to accurately probe mutation rate in coast redwood.

We investigated how somatic mutations accumulate in the crown of a single very old, very large tree and measured how these mutations affect phenotypes of the sexual offspring from different branches within a tree.

METHODS:

Redwood Genome Tree (T131, SESE104, T35) Selection and Sampling:

Discovered in November 2016, T35 (Figure 2.1; T131 in Sillett et. al. 2020; SESE104 in Neale et al. 2022; T35 in Sillett et al., 2022; Carroll and Sillett, 2023) is the southernmost known ultra-tall redwood tree (>107 meters tall). It grows in a remote part of the Santa Cruz Mountains in California (San Mateo, Santa Clara, and Santa Cruz counties). Tree ring cores were collected across the height gradient from 10 meters to 101 meters. T35 has been dated to 1388±12 years old (Sillett et al., 2022) and has two tops that reach 107 meters after diverging at 80 meters (Figure 2.1). The tallest top grows out of a trunk break tree-ring dated to the year 1467, exactly 550 years prior to sampling (Carroll and Sillett, 2023). The second top comes from a branch that was below the break. Cones were collected from these two tops. DNA was sequenced from a megagametophyte from one seed at 100x coverage and assembled (Neale et al., 2022), to make downstream mapping more accurate given the hexaploid nature of the species. In addition to cones, in September 2017, nineteen foliage samples were collected spanning the height gradient (14.5-106.5 meters) with recorded heights and locations in the tree (Figure 2.1). Samples were kept at 4ºC for a week before flash freezing with liquid nitrogen and storage in a -80ºC freezer.

T35 Sampling for Sequencing:

Many seed plants can make periclinal chimeras that maintain distinct cell lineages throughout the life of a plant (Tilney-Basset 1986). Mutations accumulate independently in each lineage. If DNA is extracted from mixtures of these cell lineages, mutant allelic ratios could be minuscule and be considered false negatives and thrown out of the analysis (Yu et al., 2020; Schmitt et al., 2022; Schmitt et al., 2023). To avoid this, we strived to reduce possible chimeric mixtures of cell lineages that would confound mutation calling efforts through meticulous dissection of foliage samples.

Sequenced foliage samples were from small, proleptic branches, which delay their emergence, growing out of axils long after the parent shoot apical meristem has grown past. Not all redwood branches are proleptic (Fink 1984); however most branches in temperate trees are proleptic in the timing of their emergence (Hallé et al., 1978). In redwood, proleptic branches develop from residual protoderm in leaf axils (Fink 1984), a developmental bottleneck that limits the number of cells that found the new shoot apex. Sampling these branches would minimize which cell lineage contributes to the branch and its sequenced foliage. Likewise, if the shoot apical meristem in redwood is stratified (Strasburger, 1872; Campbell, 1940; Cross, 1943), only the mitotic descendants of the L1 would be sequenced in proleptic branches (see Chapter 1). While proleptic branches could potentially help reduce cell lineage mixtures due to hypothesized periclinal chimerism, it would not reduce cell lineage contributions due to sectorial chimerism. To avoid this, not only was foliage sampled after one or more proleptic branching events, but also only 1-3 needles were sampled emerging from the same sector along the proleptic branch to reduce cases of possible sectorial chimerism. This sampling protocol would reduce the

probability of potential chimerism in the sample, thereby making mutations easier to detect due to increased allele frequencies.

Ensuring higher allele frequencies could also be important due to the potential of hexaploidy to reduce these frequencies because of the near identity of multiple homologous copies. In cases where mapping does not separate out subgenomes, one mutated allele out of six homologous loci would would yield a variant allele frequency of one sixth and be hard to detect. If two cell lineages are equally represented in an extracted sample, the variant allele frequency in the resulting DNA preparation could be as low as one twelfth, a frequency that resembles noise from sequencing error.

Because increased ploidy and multiple separate cell lineages accumulating mutations separately could increase the noise and dilute the signal, it was critical to reduce the number of cell lineages sequenced in our samples. We did so by using single proleptic branches derived from protoderm (Fink 1984) and reducing the foliage sequenced to a few needles along a single sector of the proleptic branch.

DNA Sequencing:

A single redwood megagametophyte was sequenced using Illumina short reads at 100x coverage. These short reads were aligned to Oxford Nanopore long-reads, replacing any ambiguous bases with the single megagametophyte bases for a reference genome (Neale et al., 2022). In addition to the megagametophyte reference genome, fourteen foliage samples (of the nineteen collected from bottom to top of the tree; Figure 2.1) were selected for sequencing using exome capture following the same sequencing methods as De La Torre et al., 2021; De La Torre et al., 2022.

Foliage samples were removed from the -80º C freezer and lyophilized for 72 hours. One to three foliage leaves (needles) were selected for sequencing (see "DNA Sampling and Sequencing" section for more details). The tissue was mixed with PVP-40 and two glass beads and ground on a beat beater for two minutes at 3500 revolutions per minute. Genomic DNA was extracted using the Omega Biotek E-Z 96™ Plant DNA kit following the manufacturer protocol with a few adjustments, as follows. Proteinase K was added to the lysis buffer and the lysis was extended to 1.5 hours. DNA quality was assessed using a Qubit™ 2.0 Fluorometer (average concentration = 32.3 mg/µL), NanoDrop™ 8000 (average A260/280 = 1.82; average A260-A230 = 1.36), and gel electrophoresis (estimated fragment size = 7000 bp). Samples were normalized to 20 ng/µL in 50 µL Eppendorf tubes using an Eppendorf epMotion® 5075 automated liquid handling system (robot). The gDNA from the 14 samples was submitted to the University of California, Davis Genome Center for sonication, size selection, and library preparation.

Exome capture baits were designed using PacBio IsoSeq RNA data (De La Torre et al., 2022). After DNA extractions of presumed single-cell lineages, DNA libraries were bound to magnetic exome capture beads for exome enrichment before DNA sequencing. Illumina short read sequencing resulted in an average coverage of 41 reads per site. This same exome capture and sequencing was used for 92 other redwood trees collected from across the known redwood range (Kuser et al. 1995; De La Torre et al., 2021; De La Torre et al., 2022). Although the exome capture enrichment was for 41 Mbp, the total region sequenced and subsequently analyzed was 55 Mbp in size.

SNP Calling:

The fourteen foliage DNA libraries were processed using trim_galore 0.6.7 (Krueger et al., 2023) which uses cutadapt version 4.1. They were then mapped to the scaffold level reference genome using bwa mem 0.7.17 with a minimum mapping quality threshold of 40. The sam files were converted into sorted bam files using samtools 1.17 sort (Danecek et al., 2021). Then, using samtools mpileup, an *mpileup* file was created and adjusted to a human readable format (Lieberman and Henry, 2012), showing per site allelic frequencies and read counts for the fourteen branches. This *mpileup* file was queried for single nucleotide variants (SNVs) using a low-end cutoff of 5 reads required per allele.

To remove any bias from use of different upstream parameters, all population genetics work used the SNP calling pipeline following the methods published for the 92 trees (De La Torre et al., 2022).

Three methods for calculating mutation rate:

Mutation rates can be calculated in many ways, depending on how the samples were collected, various assumptions in the analysis, and different ways to interpret words like "generation", which can be imprecise in modular organisms (Harper 1977; Harper and Bell 1979; Cook 1985; Orive 1993). Even small adjustments into how mutation rate is calculated can change the results. We calculated mutation rate in three different ways, using different samples and assumptions detailed below. The first method used each sample to identify mutations that may not even be fixed in the branches themselves, but are only fixed in the meristematic cell populations of the L1 or L2. This method maximizes mutation detection, at the possible expense of including some false positives due to variant allele frequencies below 0.5. The second used

external population data (De La Torre et al., 2021; De La Torre et al., 2022) to avoid overcounting heterozygosity that could be explained by sexually-inherited alleles rather than somatically-inherited mutations. And the third method leveraged the tree structure of two independent tops that have been growing separately for at least 550 years (Carroll et al., 2023) to calculate mutation rate of fixed mutations separately in the two. All three of these methods had increasingly higher levels of scrutiny and *ad hoc* assumptions, thereby reducing measured mutation rates and increasing likelihood of false negatives.

1. *mpileup* Method:

The first mutation rate calculated was using an mpileup file generated from mapped reads from the fourteen branch samples from T35. The mpileup file was filtered so that at each site (locus), each sample (branch) had an allele with at least five reads. This value would serve as the total base pairs analyzed for calculating mutation rate. To count putative mutations, we looked for single nucleotide variations (SNVs) within T35. We also focused on gains of heterozygosity at each site (locus). This removed bias from the N, O, P, Q samples (Figure 2.1) that lost heterozygosity, since this may have come from a single event like the loss of a chromosome and not thousands of independent mutation events which would vastly increase our measured mutation rate. At each site (locus), we considered homozygosity to be where samples had only one allele at five reads depth, and heterozygosity to be where samples (branches) had two alleles both at five reads depth. To avoid false positives by counting heterozygosity present in the background (the heterozygosity of the original T35 zygote), we required that half or fewer of the samples (<7 of the branches) have the putative mutated allele. To reduce false positives from mapping errors due to polyploidy or repetitive regions, a maximum read depth of 150 base pairs per branch per site was used, with reads exceeding this number removed from the analysis.

SNVs were also filtered to include only regions with a gain of an allele, where homozygous sites became heterozygous or where one heterozygous base mutated to a different allele.

2. Population Genetics Method:

The second method for calculating mutation rate involved a much larger outside population data set of the 92 trees from across the redwood range (De La Torre et al., 2021; De La Torre et al., 2022). We used the same exome capture sequencing and downstream data analysis pipeline in the T35 samples as was used in the population samples (De La Torre et al., 2021; De La Torre et al., 2022). To determine how many unique SNVs were present in T35, we looked for alleles unique to T35 and not present in the broader population (see De La Torre et al., 2021; De La Torre et al., 2022). Any alleles present in the population were considered 'ancestral' alleles to T35 and therefore not somatic mutations that arose within T35. Therefore, we removed the SNPs present in both T35 and the broader population, so that our remaining SNPs were unique to T35 only. This left us with a small set of putative mutations which were unique to T35 as another measure of mutation rate.

3. Two Tops Consensus Method:

T35 has two tops, an unbroken top consisting of samples N, O, P, and Q and a twice broken top consisting of samples L, M, R, S (Figure 2.1). These two tops have been separate since T35's top broke 550 years ago (Carroll and Sillett, 2023), possibly much longer since the tree would have reached this height earlier than when the break occurred. Because of this top separation, however, mutation rates can be independently calculated for both the tops. To do this, we calculated a consensus genotype for the "zygote" based on the lower six samples, (ABEFHK; Figure 2.1) a method resembling the consensus method used in spruce (Hanlon et al., 2019). We then determined the consensus genotypes of NOPQ and LMRS separately and counted the

number of variants fixed in the two tops given any difference from the lower branch (ABEFHK) consensus. To avoid issues with the loss of heterozygosity SNVs present in abundance in NOPQ that we hypothesize are not independent mutations, we only analyzed SNVs that were homozygous in the lower branches (ABEFHK) and became heterozygous in the two tops (NOPQ and LMRS).

Chromosomal Synteny and Interpretation:

Unfortunately, the reference assembly for redwood (Neale et al., 2022) is not resolved at the chromosome level for it to be easy or possible to see chromosomal loss or account for other related copy number variations (CNVs) in the genome. However, chromosome-level assemblies exist for the two other extant redwood (Sequoioideae) species (*Sequoiadendron giganteum*, Scott et al., 2020; *Metasequoia glyptostroboides*, Fu et al., 2023) and another Cupressaceae species (*Cryptomeria japonica*, Fujino et al., 2023). While there have been two whole genome duplication events and tens of millions of years divergence time between these species and coast redwood (*Sequoia sempervirens*), there is evidence to suggest chromosome synteny remains relatively intact within the Cupressaceae (*Cryptomeria*, *Sequoiadendron*, and *Metasequoia*; Figure 2, Fu et al., 2023) and gymnosperms more broadly (*Ginkgo*, *Pinus*, *Sequoiadendron*; Supplementary Figure 1E, Niu et al., 2022).

There are regions of the T35 genome samples from branches N, O, P, and Q (Figure 2.1) that have lost heterozygosity present in the rest of the tree. These regions were tested to see if the loss of heterozygosity is due to loss of a chromosome, the simplest explanation, or due to something more complicated. We used loss of heterozygosity SNVs shared by N, O, P and Q (Figure 2.1) and aligned the reference contigs with any loss of heterozygosity SNV to all

Cupressaceae chromosomal assemblies (*Sequoiadendron*, Scott et al., 2020; *Cryptomeria*, Fujino et al., 2023; *Metasequoia*, Fu et al., 2023). We aligned these sequences using minimap2 (Li 2018) and dotplotly (Poorten, 2018) to visualize whether the loss of heterozygosity contigs in the T35 *Sequoia* sequences matched with larger *Sequoiadendron*, *Cryptomeria*, and *Metasequoia* chromosomes. In minimap2 (Li 2018), we used the -asm5 flag, where only sequences with 95% or greater alignment would map, thereby removing sequences where genome divergence exceeded 5%. This setting ensured high mapping quality between the sequences. Similarly, synteny between *Sequoiadendron*, *Cryptomeria*, and *Metasequoia* was also tested to see whether we can reliably use chromosomes in these three species to compare homologous chromosomes in *Sequoia*.

Similarly, to test whether the loss of heterozygosity was due to the large-scale deletions or loss of a chromosome, we plotted average read counts against average allele frequency. We hypothesized that the graph would show a negative correlation if the loss of heterozygosity was due to a large-scale deletion such as loss of a chromosome. If there was no correlation, then loss of heterozygosity was not a result of a copy number variation (CNV) such as a chromosome loss but rather a phenomenon such as uniparental disomy (UPD; Robinson 2000) or break induced replication (BIR; Kockler et al., 2021). A positive correlation would indicate a large-scale duplication of a chromosome, but loss of allele frequencies most likely would not result from a duplication.

Seedling Experiment:

Twenty seed cones from each top (LMRS; NOPQ; Figure 2.1), were weighed and seeds counted. All of the seeds (n=2562) from each cone (n=40) were divided into two groups. The

first group was dissected manually, checking visually for megagametophyte vitality inside the seed coat. Megagametophyte vitality required it to look white or pale yellow, not brown or black (indicating a dead megagametophyte). The second group was planted in a tray of soil in an alternating grid plot (each plot from one tree top adjacent to plots from the other tree top). This grid plot design was implemented to remove any measurement bias in the tray due to differences in water availability, soil depth, proximity to tray wall, light availability, or any other possible environmental gradient that could skew results. The sowed seeds were surveyed at two separate time points (18 and 71 days after planting) to evaluate seedling performance quantitatively (e.g., number of germinated seedlings) and qualitatively (e.g., observations of unusual seedling phenotypes).

RESULTS:

Mutation Rates:

1. *mpileup* Method

The first method describes single nucleotide variations (SNVs) that had more than 5 reads and were present in fewer than seven branches to avoid counting standing heterozygosity in mutation rate. This method found 891,852 mutated sites among 55,348,803 total sites, indicating a mutation rate of 1.15×10^{-3} per site per branch of the 1388 year old T35 tree. Since branches can be considered 'individuals' of modular organisms (see Introduction), we normalized the mutation rate across all branches and used the inferred age of the zygote (1388 years) to normalize our values (thus dividing the tree mutation total by total sites, total branches, and total age in years). Importantly, we cannot confirm that T35 is the first and only ramet produced from the zygote 1388 years ago or if it is a newer-generation "ramet" derived from a

basal sprout 1388 years ago. Therefore, since 1388 years is a measurement of ramet age, it is a minimum age estimate for the genet, thereby making any per year mutation rate a maximum estimate for the genet. When accounting for tree (ramet) age and averaging across all branches, the mutation rate was, at maximum, 8.29×10^{-7} per site per branch per year.

Surprisingly, many branches had roughly the same number of mutations, regardless of where they were positioned on the tree or tree topology. We had hypothesized that tree branching topology would correspond with mutation accumulation, where greater genetic differences would be seen in branch pairs at greater physical or temporal distances from each other. In other words, greater spatial or temporal separation in the tree should correlate with greater genetic divergence. For example, in higher distal branch pairs PQ and RS (Figure 2.1), both pairs are separated by small divergence times (33 and 7 years respectively) and small physical distances in the tree (1.4 and 0.8 meters respectively). We hypothesized these pairs would be relatively closely related in their accumulated mutations due to their closeness to each other spatially and temporally. However, they are as genetically different as other pairs of branches separated by greater distances and time within the tree. The maximum distances, both temporally and spatially, between any branch pair in the tree were between the lowest and highest samples, branches A and S (Figure 2.1; 14.1 meters and 106.5 meters, respectively). The physical and temporal separation of this pair of samples was ~2700 years and 94.8 meters, and yet these samples were as closely genetically related as samples R and S separated by only 7 years and 0.8 meters. In some instances, such as above the break in samples NOPQ and LMRS (Figure 2.1), we see branching-specific mutations fixed in all the tops (see Two Tops Consensus Method below), but a vast majority of mutations using this *mpileup* method did not show this segregation by branching.

These differences may be attributed to different cell lineages from different sectors founding the foliage sampled for sequencing, or they could be false positives. Because of this discrepancy, this calculation of mutation rate can be considered a maximum mutation rate with the real value likely lower than this. Therefore, we used two other methods to calculate mutation rate with differing assumptions to minimize false positives.

2. Population Genetics Method:

The second method to calculate mutation rate used population genetics and data from the 92 trees sampled throughout the redwood population (see Introduction). This method identified 27,277 mutated sites among 41,241,410 total sites, which is calculated to be 6.614×10^{-4} mutations per site within this single sporophyte. When accounting for tree age (using the age of the tree as "age of zygote") and averaging across all branches, the mutation rate was 3.40×10^{-8} per site per branch per year, which is very similar to the value obtained using the *mpileup* method. We suspect that the real value for coast redwood is on this order of magnitude and somewhere between the first and second values.

3. Two Tops Consensus Method:

The third method of calculating the mutation rate used a more stringent protocol that calculated the consensus genotypes across multiple branches. When we required that samples ABEFHK (all below the break; Figure 2.1), samples NOPQ (unbroken top), and samples LMRS (broken top) all had the same genotype at each site within each group, a lower mutation rate was found. This is expected, as the branches are what harbor genetic diversity (see Introduction) and requiring 4-6 branches to be "the same" removes real genetic variation that may only be present in a subset of the branches. We designated the lower branches ABEFHK as the "zygote" genotype, and enumerated the mutations found in LMRS and NOPQ. Using the known age of

branch separation (550 years; Carroll and Sillett, 2023) to calculate mutation rates, there were 7.82×10^{-9} mutations per site per year in NOPQ and 5.98×10^{-9} mutations per site per year in LMRS. Therefore, the average mutation rate when calculated using this method was 6.90×10^{-9} mutations per site per year. We suspect this is the minimum possible value for redwood somatic mutation accumulation rate.

Chromosomal Synteny and Interpretation:

Most branches within the tree displayed unique single nucleotide variations (SNVs), with a majority of sites monoallelic on a majority of branches and biallelic in a minority of branches. This was interpreted as homozygosity and heterozygosity, respectively. The majority allele was considered the zygotic (wild type or ancestral) allele while the minority allele was considered a somatic mutation. While most variation within the tree involved monoallelic sites becoming biallelic in one or a few branches (interpreted as a homozygous to heterozygous mutation), we found a large, notable exception of SNVs (n=9102) in one of the two tree tops. These SNVs were biallelic in a majority of branches and monoallelic in a single top comprised of four sampled branches (N, O, P, Q; see Figure 2.1). These four branches (N, O, P, Q) are found on one reiterated trunk all above 80 meters. This trunk (N, O, P, Q) was unbroken in comparison with the other trunk (L, M, R, S; Figure 2.1) which had two breaks: one at 79.5 meters and the other at 90.8 meters. The unbroken trunk and its branches (N, O, P, Q; Figure 2.1), were found to be monoallelic (homozygous) in contigs where the rest of the tree was biallelic (heterozygous). If these losses of heterozygosity were distributed equally across the genome, we would expect them to be independent mutation events. However, these losses of heterozygosity appeared in dense, contiguous clusters with some contigs having no loss of heterozygosity and others having many

instances. Because of this, the loss of heterozygosity was unlikely to be from rare, independent single-nucleotide mutation events, but rather from a single or multiple chromosomal aberrations.

Without a chromosomal genome assembly for redwood, we used three species in the Cupressaceae with chromosome-level assemblies to provide a comparison. The closest-related species to redwood (Liu et al., 2022), *Sequoiadendron* (Scott et al., 2020), another redwood *Metasequoia* (Fu et al., 2023), and another Cupressoid, *Cryptomeria* (Fujino et al., 2023). Using minimap2 (Li 2018) and dotplotly (Poorten, 2018), we plotted the redwood T35 contigs where NOPQ branch samples had a loss of heterozygosity to the assemblies of the other two redwood species (*Sequoiadendron* and *Metasequoia*; Figure 2.2a-e). We compared these contigs of interest with a control of contigs with SNVs that were a gain of heterozygosity (Figure 2.2a-d). The T35 redwood loss of heterozygosity mapped to a portion of chromosome 1 and chromosome 3 in *Sequoiadendron* (Figure 2.2a) compared to the control (Figure 2.2b). Similarly, the T35 loss of heterozygosity mapped to chromosome 3 in *Metasequoia* (Figure 2.2c) compared to the control (Figure 2.2d). Comparison of *Sequoiadendron*, *Metasequoia*, and *Cryptomeria* genomes to each other revealed synteny between chromosomes (Figure 2.3) consistent with the synteny described between Cupressaceae species (Fu et al., 2023) and gymnosperms more broadly (Niu et al., 2022).

To test whether haplotype loss caused the loss of heterozygosity, we plotted loss of heterozygosity SNV read counts and frequencies. A negative correlation between read count and allele frequency was observed in SNVs displaying loss of heterozygosity (Figure 2.2f), indicating the loss of heterozygosity was due to a loss of a copy of the allele rather than base change mutations. The simplest explanation for these results is a single chromosome loss rather

than >9000 independent base change mutations on contigs homologous to single-chromosomes of closely-related species (chromosome 3 in *Metasequoia*, *Cryptomeria*).

Comparison of the chromosome-resolved assemblies of three Cupressaceae indicates that a portion of chromosome 3 in *Metasequoia* and *Cryptomeria* translocated to chromosome 1 in the *Sequoiadendron* lineage only (Figure 2.3; Fu et al., 2023). Our data suggesting single chromosome loss in *Sequoia* (redwood) suggests that this species did not have the chromosome 3 to 1 translocation seen in its sister species *Sequoiadendron* (Figure 2.3).

Cell Lineages in T35 Samples:

We sampled proleptic branches, which delay their expansion and growth (Hallé et al., 1978), aiming to sequence the L1 cell lineage only. However, as seen in Chapter 1, cell lineages do not always perfectly correspond to branch ontogeny. The ancestral L1 genotype, as represented in most of our samples (A, B, E, F, H, K, L, M, R, S –Figure 2.1), is not represented in N,O,P,Q which comprise one segment and top of the tree T35. Because NOPQ samples are so different, even with a loss of a chromosome, we hypothesize that NOPQ is likely a rare instance where ancestral L2 genotype replaced the ancestral L1 genotype. We hypothesize that, as evident in the sampled NOPQ L1 genotypes, L2 cells below the break founded the entire shoot apical meristem (L1 and L2 cells) in NOPQ.

Similarly, evidence of some loss of heterozygosity are seen in samples H (proximal to NOPQ; Figure 2.1), as well as L and K (on the other top, adjacent to NOPQ; Figure 2.1), though with very low read counts. Samples H, L, and M have the highest allele frequency to read count ratio of the loss of heterozygosity alleles (Figure 2.2f). These results could be due to L2 leakage into the L1 through natural variation in proleptic branch ontogeny (see Chapter 1). The loss of

heterozygosity is observed at 60 meters in Sample H, below the break at 80 meters, indicating monosomic chimerism in the sample and the trunk below the break. Likewise, NOPQ shows fixation of the lost chromosome, indicating a complete loss of the ancestral L1 genotype since proleptic branches were intentionally sequenced, enriching L1 genotypes in all samples. Since NOPQ's L1 does not match the L1 of the rest of the tree, and loss of heterozygosity was observe in Sample H at 60 meters, we conclude it must be a fixed ancestral L2 in NOPQ.

Proleptic branches are synonymous with epicormic buds that can develop from a tree trunk (Meier et al., 2012; see Chapter 1). These buds emerge and often replace trunks after damage (Fink 1984), as has occurred twice on the "broken" top of T35 consisting of samples L, M, R, and S (Figure 2.1). Therefore, we conclude that a proleptic branch replaced the main top that broke for branches L and M, and then a second proleptic branch replaced the main top that broke before making branches R and S. Trunk damage is not apparent in the "unbroken" top consisting of samples N, O, P, and Q (Figure 2.1). This difference between the morphology and genotypes of the broken and unbroken tops (LMRS and NOPQ, respectively; Figure 2.1) neatly corresponds with the branch cell lineage origin results from Chapter 1 (Table 1.1).

Multiple breaks in samples from top R, S (Figure 2.1) most likely resulted in replacement by the L1 due to proleptic branching (see Chapter 1). Sequencing from this top also indicates no loss of heterozygosity (S has the lowest allele frequency to read count ratio in Figure 2.2f). We hypothesize that seeds from R, S top (Figure 2.1) originate from the ancestral L1 genotype. Conversely, in NOPQ (Figure 2.1), because of lack of visible physical breaks and fixation of the loss of one chromosome in top P, Q (Figure 2.1), we hypothesize that the ancestral L2 genotype makes the seeds that originate from this top. This may explain our seedling results in the next section.

Seedling Experiment:

A number of seed traits were all statistically significantly different between the two tops of T35. These include weight of seeds per cone, average seed weight, megagametophyte viability (dissected seeds), root emergence (sown seeds), cotyledon emergence (sown seeds), and seedlings that reach >5cm in height. Seeds from the broken top (branch samples L, M, R, S; Figure 2.1) had larger values compared to the unbroken top (branch samples N, O, P, Q; Figure 2.1) for the measurements listed above (Figure 2.4). Each of these values also correspond with fecundity of the two tops. The fecundity of the unbroken top had values approximately twice that of the broken top (Figure 2.4, Table 2.1).

Along with quantitative measurements, seedling phenotypes were categorized qualitatively as well. Of the 48 viable seedlings produced, the NOPQ top produced two chlorophyll-deficient (albino) seedlings, one isomorphic twin pair (both same size), one anisomorphic twin pair (one normal sized, one small), one seedling with a malformed split cotyledon, one triple cotyledon seedling, and two twisted hypocotyl seedlings (possibly twins that fused together). Of the 87 viable seedlings produced, the LMRS top produced one anisomorphic twin pair, one twisted hypocotyl seedling, and one triple cotyledon seedling. Compared to the LMRS broken top, the NOPQ unbroken top with chromosome loss had a much higher proportion of qualitatively abnormal seedlings (20.8% to 4.6% respectively).

DISCUSSION:

We investigated the role somatic mutations play in an old tree and how the accumulation of these mutations may affect its offspring. Notably, large-scale genomic variation can occur

within a single individual, with observed reductions in reproductive fitness depending on what mutations are present and absent in the parent branch. Below, we discuss some of the interpretations and implications of this work.

Mutation Rates:

Redwood tree T35 has accumulated a high number of mutations within the crown and a high proportion of mutations per site per branch per year. We calculated the mutation rate using three different methods giving, respectively, the following rates: 8.29×10^{-7}, 3.40×10^{-8} and 6.90×10^{-9} per site per branch per year. Mutation rate can be reported in different ways, including per generation, per year, or per cell division. Our mutation rate is the highest ever reported per generation. Also, to our knowledge, this is the oldest organism sequenced in a next generation sequencing mutation study. The values above would be multiplied by 1388 years (the minimum age of the T35). Using this method, the mutation rate for redwood generations is 1.15×10^{-3}, 4.71×10^{-4}, and 9.58×10^{-4} mutations per site per 1388-year generation. These values do not align closely with what would be expected in most biological organisms (between 10^{-7} to 10^{-10} mutations per site per generation; Lynch et al., 2016). However, a generation-based mutation rate is odd for a long-lived clonal tree species, mainly because there is no good definition of a "generation" for a tree that may sexually reproduce in its first century of life and every year thereafter until death often millennia later. Because of this, our annual rate of mutation is a better measure because of it can be compared easily between organisms (Table 2.2).

Each of our methods differed depending on the thresholds by which we identified ("called") mutations. The highest mutation number resulted when we assumed that variants present in a minority of branches were mutations; the lowest when we assumed that the variants

fixed in all four samples above the break on either top were mutations. The middle value resulted when we used variants present in the external population to filter out heterozygosity present in the zygote due to inheritance from the population versus heterozygosity due to somatic mutation accumulation. Each of these methods has drawbacks, but we assume that the real value is likely to be somewhere between the maximum and minimum value reported here. In terms of genetic functional unit, since branches of a tree mutate as individual cell populations, mutation rate ought to be measured on a per-branch basis. One could go further and measure mutation rate on a cell lineage basis, though with further subdivision of samples, more mutations that are not fixed in the larger subunits should become apparent.

There are three reasons why our mutation rate might be higher than expected given experimental results of other somatic mutation studies (Watson et al., 2016; Schmid-Siegert et al., 2017; Anderson et al., 2018; Plomion et al., 2018; Hanlon et al., 2019; Hiltunen et al. 2019; Wang et al. 2019; Hofmeister et al., 2020; López and Palumbi, 2020; Orr et al., 2020; Yu et al., 2020; Zahradníková et al., 2020; Perez-Roman et al., 2021; Ren et al., 2021; López-Nandam et al., 2022; Schmitt et al., 2023): (a) underestimation of genet age; (b) polyploid organisms accumulate more mutations; (c) sampling individual cell lineages increases mutation discovery.

First, the tree age value of 1388 years is based on a linear regression of age estimates at different heights along the main trunk (see Sillett et al., 2022 for detailed methods). While this age estimate works well for the ramet, it assumes that the ramet developed directly from a zygote. While we think this is likely, it may instead be a second-generation or older ramet that developed from a basal sprout. If this is the case, the T35 zygote (and therefore genet) may have been substantially older than the T35 ramet. Since multiple cell lineages can found a new ramet, it is possible that parental L1 and L2 cell lineages can be older and separated longer than 1388

years. There is no way to know with certainty whether T35 is a genet and ramet that is 1388 years old, or a 1388-year-old ramet of a much older genet. If the genet is older than the 1388-year ramet, the per year mutation rates would be overestimates while the per generation mutation rates would be unchanged.

Second, somatic mutation accumulation has not, to our knowledge, been studied in a polyploid organism this old. Polyplody enables higher mutation rates due to the masking of alleles (Orr 1995; Tsai et al., 2013). It is also possible that the hexaploidy of T35 inhibited proper mapping, leading to an underestimation of the mutation rate. We went to great lengths, however, to use the same individual as the reference genome and a high mapping quality threshold to ensure this problem did not occur. Similarly, we did not use sites with reads higher in abundance than a few times the average read count to avoid areas where mapping to all homeologs or repetitive regions could confound results. Therefore, we are confident that a vast majority of these putative mutations are likely to be real despite the challenges that come with polyploid bioinformatics.

Finally, our experimental results differ from most others working on somatic mutations by a few orders of magnitude. We made great efforts to sequence leaves that ultimately derived from one cell lineage, not multiple lineages, through sampling of L1-derived proleptic branches (Chapter 1). Detecting mutations may be difficult unless cell lineage bottlenecks fix those mutations. The use of chimeric tissues would likely lead to an underestimate of mutation rate in modular organisms (Anderson et al., 2018; Hiltunen et al., 2019; Yu et al., 2020; Ren et al., 2021; Schmitt et al., 2023). When sampling whole willow leaves irrespective of cell lineage composition, Ren et al. (2021) found that many somatic mutations had lower allelic frequencies. They hypothesized that the leaves developed from multiple progenitor cell lineages, as periclinal

chimeras of typical eudicots indicate (Tilney-Bassett 1986). A similar study on seagrass found that cell lineages in their samples were mixed and only detectable with deep sequencing (Yu et al., 2020). Finally, a study of two tropical tree species (Schmitt et al., 2023) describes inherited mutations at low allelic frequencies in the somatic branches of the parent. An explanation for the low number of mutations found in oak (Schmid-Siegert et al., 2017) may be due to sampling multiple cell lineages and removing low abundance single nucleotide variants (SNVs) due to mixtures of multiple cell lineages that greatly reduce mutated allele frequencies. Indeed, a reanalysis of oak somatic mutation data (Schmid-Siegert et al., 2017; Plomion et al., 2018) reveals that somatic mutations may have been significantly underestimated (Schmitt et al., 2022).

In spruce, two samples were taken from opposite sides of the trunk base, whose consensus genotype determined zygote genotype, and the tree top, whose consensus determined "mutated" genotype (Hanlon et al., 2019). The spruce study design should find any variants that are fixed in the top and not fixed at the base but ignores mutations fixed in sub-populations of cells in the shoot apex that could be heritable in the zygotes. Spruce, however, may not have any stratification in their meristems, indicating genetic homogeneity in the branches (Klekowski 1984a). If spruce shoot apices are stratified, this study likely underestimated mutation rate while still simultaneously finding the largest per-generation mutation rate until this present redwood study. In the third approach to calculate mutations in T35, we used consensus genotypes for the two tops to be able to compare spruce (Hanlon et al., 2019) and redwood following similar assumptions of meristematic genetic homogeneity. This indeed gave us the lowest rate of our three methods of calculating mutation rate, although it also helped us parse out L1 and L2 mutations that were fixed below the break. Because of redwood shoot apical meristem stratification, branch-level fixation of mutations is likely slow (except for L1-derived proleptic

branches), so consensus genotypes are not useful since the layers mutate independently of one another. For this reason, our third method using consensus genotypes represents a low bound for somatic mutation rate in redwood.

If cell lineages are not carefully considered in a sampling regime, many mutations that arise in only one of the typical three stratified layers of a eudicot meristem will not have allele frequencies sufficiently high to be counted. The minor alleles would be considered false positives due to sequencing error even if this is not the case (Yu et al., 2020; Ren et al., 2021; Schmitt et al., 2023). These mutants could only be detected with deep sequencing and high read counts (Yu et al., 2020), and with programs attuned to low allelic frequency detection (Schmitt et al., 2022; Schmitt et al., 2023). If visible chimeras are not available as they were in this study to enable sampling of a single cell lineage, one needs to account for these allelic variations in their mixed samples (Yu et al., 2020; Ren et al., 2021; Schmitt et al., 2023). One could look for low-abundance alleles and see if these allele frequencies "match" across multiple sites. To parse out clonal heterogeneity, one could use genetic heterogeneity across many sites to model proportions of each cell lineage in the mixed sample.

Parsing out cell lineages prior to sequencing to increase allele frequencies and mutation detection may also be relevant for the study of somatic mutation accumulation in animals. In branching corals, a recent study of somatic mutations indicated that sexually-reproductive cell lineages may derive from "a common ancestor stem cell lineage that self-renews and proliferates throughout a colony and that eventually generates into both germ and soma" (Lopez-Nandam et al., 2022). Our results suggest a similar phenomenon happening in redwoods; old trees renew their L2 lineages through proleptic (delayed; Hallé et al., 1978) branching and founding new L2 lineages from ancestral L1 lineages that change less frequently. They also reported that mutations

found in coral branches do not always match mutations found in coral gametes, indicating that normal somatic cells are not always part of the germline (Lopez-Nandam et al., 2022). These topics of cell lineage separation, meiocyte determination, and mutation accumulation will be an important and growing area of future research in developmental biology as these directly affect the fitness and evolution of many modular, branching organisms.

Our results indicate that to properly evaluate somatic mutation accumulation in plants, one must consider the branch or even sub-branch level of the more permanent cell lineages and try to isolate them or use proportional number of reads in the genome to predict how much L1, L2, and/or L3 a given sample contains based on minor allele frequencies. Without this, mutational load will always be underestimated.

We cannot definitively say how mutation rate compares between L1 and L2 since we only sequenced a single individual tree of one species. However, in another polyploid plant that emerges from underground tubers (like lignotubers in redwood), potato has been found with differential mutation rates between its L1 and L2 layers (Amundson et al., 2023). In potato, L2 has a faster mutation rate than L1, which we hypothesize is opposite those results in redwood. In T35, the parental L2 genotype appears to have had the largest-scale mutation (chromosome loss in N,O,P,Q—see Figures 2.1, 2.2) compared with the parental L1. We also compared the SNVs that accumulated between the NOPQ top and the LMRS top and found that the NOPQ top had 30.7% more than the LMRS top. We hypothesize that the cell lineages comprising NOPQ and LMRS derive from the ancestral L2 and L1, respectively, due to their respective unbroken and broken nature (see Chapter 1). If this is correct, these layers could have been separate at least since ramet formation 1388 years ago. These data indicate that, at least in T35, L2 appears to have mutated more than L1. Further work, especially involving more trees trying to determine if

L1 and L2 have differential mutation rates, would be required to assess whether L1 has more ancestral alleles with reduced genetic variance for establishing new proleptic branches in coast redwood, although our data does suggest that this is a reasonable possibility.

Genetic Variation:

There are limits to what can be deduced from a single redwood tree and the sequences of 14 branches, an extremely small subset of the total branches in T35. However, we have amassed evidence that this tree (T35) harbors a reservoir of genetic diversity within its crown. For example, T35 has a higher mutation rate than seen in other tree species (Schmid-Siegert et al., 2017; Plomion et al., 2018; Hanlon et al., 2019; Wang et al., 2019; Hofmeister et al., 2020; Orr et al., 2020; Ren et al., 2021; Zheng et al., 2022), and differential fecundity of the two tops of the tree due to chromosome loss (Figures 2.2, 2.4). It would not be surprising to find other old redwood trees with similar genomic variation in their crowns. This coincides with theoretical models of tree branches harboring mutations (Antolin and Strobeck, 1985), with far more within-tree genetic diversity in dense, tall forests (Tomimoto et al., 2023).

Importantly, this genetic variation is not always adaptive, but rather exists due to the accumulation of somatic mutations that can be beneficial, neutral, or deleterious. Any given diversity, therefore, still needs to undergo selection during the gametophytic phases of sexual reproduction before being fixed in the next generation of sporophytes (Buchholz 1922; Mable and Otto 1998; Walbot and Evans 2001). A good example of redwood variation in a subset of a genet that fails to be inherited by the next generation is chlorophyll-deficiency (albinism; Peirce, 1901; Douglas and Holdermann, 1980; Pittermann et al., 2018). While chlorophyll-deficient branches and ramets can exist and grow, a new chlorophyll-deficient sporophyte cannot survive

long after exhausting the nutritive tissue of the seed (the megagametophyte). Chlorophyll-deficiency, therefore, is a phenotype (Park et al., 2023) unable to persist in the sexual life cycle.

Genomic variation within a single plant can affect fitness of inbred individuals. In *Erythranthe* (formerly *Mimulus*), a usually annual, herbaceous plant, offspring selfed on the same branch from the same flower were less fit than selfed offspring from a flower on a different branch of the same plant (Cruzan et al., 2022). This result suggests that the genetic differences (i.e. somatic mutations) between the two branches accumulated since syngamy (less than a year) are enough to have different reproductive outcomes in the offspring. Our result, therefore, of different seedling fecundities from the two tops (Figure 2.4) demonstrates a similar phenomenon on a larger spatial and temporal scale. The branches that comprise the two tops (NOPQ vs. LMRS tops; Figure 2.1) split prior to the year 1467 (Carroll and Sillett 2023), indicating a minimum of 550 years of separate evolution on each top. Megagametophytes from one top had significantly different weights and viability compared to the other top (Figure 2.4), likely due to the chromosome loss, a major somatic mutation, in the NOPQ top (Figure 2.2).

Seedling Experiment:

The two tops (LMRS and NOPQ; Figure 2.1) appeared to have different fecundities based on different measures of reproductive fitness (Figure 2.4; Table 2.1). The broken top (LMRS; Figure 2.1) had heavier cones, heavier seeds, more living megagametophytes, more germinated seeds (Figure 2.4; Table 2.1) compared to the unbroken top (NOPQ; Figure 2.1). Similarly, NOPQ had proportionally more abnormal seedlings than LMRS, indicating an overall lack of reproductive fitness for that top. Seed weight is usually indicative of how capable the triploid spore can survive and successfully grow into a megagametophyte. Megagametophyte

viability indicates to what extent the triploid genome has the genes and regulation it needs to make a functional megagametophyte (Mable and Otto 1998). Dead megagametophytes reflect a lack of effective genetic expression, development, and overall function. This result directly and empirically highlights how sexual reproduction is helpful for purifying selection to purge somatic mutations that accumulate in the soma. While we did not sequence any of the offspring, we have both quantitative and qualitative phenotypic indications that the more mutated top (SNVs and chromosome loss; NOPQ top; Fig. 2.1) has worse reproductive output. We infer that these increased mutations and chromosome loss in NOPQ is the likely culprit that explains its poor fecundity.

Chromosome Loss and Mosaic Aneuploidy:

While sexual reproduction, going through unicellular haploid bottleneck of the spore, is the most common way plants purge mutations from a population (Mable and Otto 1998; Walbot and Evans, 2001), we may have inadvertently uncovered a strategy used by redwood to reduce the amount of deleterious genotypic variation within the sporophyte through increased selection on various alleles.

Purifying selection can most effectively operate on a eukaryotic genome in its haploid phase, when each allele is exposed (Mable and Otto 1998; Walbot and Evans, 2001). For species with long generation times, like redwood, new genets forming through normal sexual reproduction (meiosis and subsequent syngamy) are infrequent. Many years in the sporophyte phase can result in deleterious mutation accumulation, especially in a polyploid organism like redwood, due to allelic masking (Orr 1995). Reduced ploidy through sexual reproduction, and

the process of diploidization in polyploids, should reduce this possibility of mutational meltdown in populations (Muller 1964; Felsenstein 1974).

Diploidization, or the process of a polyploid organism restoring bivalent chromosome pairing during meiosis and returning to the status of diploid, occurs as sequences in homologous chromosome diverge from each other due to accumulated mutation (Li et al., 2021). Even though hexaploid, redwoods exhibit some level of diploidization, which is evidenced by diploid pairing, a trait demonstrated by common bivalent chromosome pairing in meiotic figures (Hirayoshi and Nakamura 1943; Stebbins 1948; Schlarbaum et al., 1984; Hizume et al., 2014). This is to be expected given how old polyploidy is in this lineage (>1.5 million years ago; Scott et al., 2016). Diploidization indicates that sequences likely have diverged enough for neofunctionalization (Birchler and Yang, 2022). Neofunctionaliztion and diploidization work together to reduce the frequency of deleterious alleles in products of meiosis by promoting purifying (negative) selection on the exposed, no-longer-redundant gene functions (Otto 2007; Birchler and Yang, 2022).

Just as diploidization can reduce genetic masking and therefore proliferation of deleterious alleles in offspring, T35 appears to use a complementary approach to the problem of mutational meltdown. In this study, T35 has two very different tops: one that derived from the ancestral L1 and the other that derived from the ancestral L2. The top derived from the ancestral L2, the unbroken top encompassing samples N, O, P and Q (Figure 2.1) displays loss of heterozygosity likely due to the loss of a chromosome. Assuming sufficient diploidization, this chromosomal loss resulted in monosomy, or aneuploidy resulting in only one of the two chromosome copies in the unbroken top. For genes where neofunctionalization occurred, hemizygosity (i.e. the presence of only one of two neofunctionalized alleles) would result in this

unbroken top. Hemizygosity should enable purifying selection on any single copies of neofunctionalized alleles, just as it would on the whole genome of the gametophyte. We see evidence of this purifying selection in the reduced fecundity (lower seed weight and seedling germination; Figure 2.4) in the NOPQ top as compared with the LMRS top (Figure 2.1). Similar purifying selection measured by percent microgametophyte (pollen) germination in monosomic *Gossypium* (Sanamyan et al., 2011) parallels the loss in megagametophyte viability (Figure 2.4) we report from the monosomic branch (NOPQ; Figure 2.1) in T35.

The phenomenon of cells with different chromosome number present in the same plant, mosaic aneuploidy or aneusomaty, is relatively common in plants (aneusomaty; D'Amato 1995; D'Amato, 1997). While our results are limited because T35 is only one tree, this is not the only occurrence of mosaic monosomy in a redwood. The Korbel KT clone (Libby and Tufuor 1984) is a second example of a redwood accession displaying a monosomic branch in an otherwise normal tree. With such limited redwood genetic sampling and copy number variation analysis, for two samples to both have mosaic monosomy is surprising. Along with other known aneuploid and chromosomal anomalies in entire redwood populations (accessory and satellite chromosomes; Fozdar and Libby 1968; Saylor and Simons 1970), mosaic aneuploidy in redwood may turn out to be relatively common, especially in old genets.

Mosaic aneuploidy has interesting evolutionary consequences. This phenomenon has been studied in a microbial eukaryote, *Leishmania* (Sterkers et al., 2011; Sterkers et al., 2012; Negreira et al., 2022). *Leishmania* is a genus of unicellular, inbred parasites that grows rapidly when transferred to a new host. This asexual reproduction results in large clones each with different chromosomal copy number (mosaic aneuploidy; Sterkers et al., 2012; Negreira et al., 2022). By effectively changing the copy number of any given chromosome, *Leishmania* clones

benefit simultaneously from “polysomy” in some chromosomes and “haploidy” in others (Sterkers et al., 2012). This organism benefits from the possibility of increased mutations with increasing copy number while simultaneously purging deleterious mutations through purifying selection with decreasing copy number. This is thought to increase functional diversity within the clone (Negreira et al., 2022) and enable rapid adaptation in varied environments (Sterkers et al., 2012).

In redwood, hexaploidy confers the evolutionary benefits of polyploidy, but due to the infrequency of sexual offspring, mosaic monosomy may be an important way redwood trees increase purifying selection on univalent chromosomes separated in branches or ramets of long-lived genets.

CONCLUSION:

This study investigated how mutations accumulate in a 1388-year-old coast redwood (*Sequoia sempervirens* (D. Don) Endl.). We report one of the highest eukaryotic mutation rates known and found that within a single individual, different branches can have different fecundities. We note that, indeed, as theoretical papers have modeled (Antolin and Strobeck, 1985; Klekowski et al., 1985; Klekowski 1988; Tomimoto et al., 2023), old modular organisms harbor significant genetic diversity within their branches. Inadvertently, we may have uncovered a method of genomic scrutiny in plants that does not require sexual reproduction and is seen in other eukaryotes due to improper meiosis. Overall, this study demonstrates ways redwoods maintain healthy, robust populations over long periods of time and evade mutational meltdown. It also highlights how difficult studying standing forest diversity is, as even single samples per tree do not adequately capture the potential adaptive alleles that may be present from somatic

mutations and within-tree selection. Future studies that investigate long-lived modular organisms may uncover other novel approaches to organismal longevity through hierarchical and mosaic selection within their branches.

REFERENCES:

Aanen, D.K., A. van ’t Padje, and B. Auxier. 2023. Longevity of fungal mycelia and nuclear quality checks: a new hypothesis for the role of clamp connections in dikaryons. *Microbiology and Molecular Biology Reviews*: e00022-21.

Amundson, K.R., M.P.A. Marimuthu, O. Nguyen, K. Sarika, I.J. DeMarco, A. Phan, I.M. Henry, and L. Comai. 2023. Differential mutation accumulation in plant meristematic layers. bioRxiv preprint https://doi.org/10.1101/2023.09.25.559363

Anderson, J.B., J.N. Bruhn, D. Kasimer, H. Wang, N. Rodrigue, and M.L. Smith. 2018. Clonal evolution and genome stability in a 2500-year-old fungal individual. *Proceedings of the Royal Society B* 285: 20182233.

Antolin, M. F. and C. Strobeck. 1985. The population genetics of somatic mutation in plants. *The American Naturalist 126*: 52-62.

Birchler, J.A. and H. Yang. 2022. The multiple fates of gene duplication: deletion, hypofunctionalization, subfunctionalization, neofunctionalization, dosage balance constraints, and neutral variation. *The Plant Cell* 34: 2466-2474.

Buchholz, J.T. 1922. Developmental selection in vascular plants. *The Botanical Gazette* 73: 249-286.

Buss, L.W. 1983a. *The Evolution of Individuality*. Princeton University Press, Princeton, New Jersey, United States.

Buss, L.W. 1983b. Evolution, development, and the units of selection. *Proceedings of the National Academy of Sciences* 80: 1387-1391.

Buss, L.W. 1985. The uniqueness of the individual revisited. In: J.B.C. Jackson, L.W. Buss, and R.E. Cook, eds. *Population Biology and Evolution of Clonal Organisms* 467-505. Yale University Press, New Haven and London.

Carroll, A.L. and S.C. Sillett. 2023. Overcoming crossdating challenges to generate ring-width chronologies for *Sequoia sempervirens* across its native range. *Dendrochronologia* 78: 126063.

Clarke, E. 2012. Plant individuality: a solution to the demographer's dilemma. *Biology and Philosophy* 27: 321-361.

Cook, R.E. 1985. Growth and development in clonal plant populations. In: J.B.C. Jackson, L.W. Buss, and R.E. Cook, eds. *Population Biology and Evolution of Clonal Organisms* 259-296. Yale University Press, New Haven and London.

Crafts, A. S. 1943. Vascular differentiation in the shoot apex of *Sequoia sempervirens*. *American Journal of Botany* 30: 110-121.

Cross, G. I. 1943. A comparison of the shoot apices of the sequoias. *American Journal of Botany* 30: 130-142.

Cruzan, M.B., M.A. Streisfeld, and J.A. Schwoch. 2022. Fitness effects of somatic mutations accumulating during vegetative growth. *Evolutionary Ecology* 36: 767-785.

D'Amato, F. 1995. Aneusomaty *in vivo* and *in vitro* in higher plants. *Caryologia* 48: 85-103.

D'Amato, F. 1997. Role of somatic mutations in the evolution of higher plants. *Caryologia* 50: 1-15.

Danecek, P., J.K. Bonfield, J. Liddle, J. Marshall, V. Ohan, M.O. Pollard, A. Whitwam, T. Keane, S.A. McCarthy, R.M. Davies, H. Li. 2021. Twelve years of SAMtools and BCFtools. *GigaScience* 10: giab008.

Davis, D. F. and D. F. Holderman. 1980. *The White Redwoods:Ghosts of the Forest.* Naturegraph Publishers Inc., Happy Camp, California, USA.

De La Torre, A.R., M.K. Sekhwal, and D.B. Neale. 2021. Selective sweeps and polygenic adaptation drive local adaptation along moisture and temperature gradients in natural populations of coast redwood and giant sequoia. *Genes* 12: 1826.

De La Torre, A.R., M.K. Sekhwal, D. Puiu, S.L. Salzberg, A.D. Scott, B. Allen, D.B. Neale, A.R.O. Chin, and T.N. Buckley. 2022. Genome-wide association identifies candidate genes for drought tolerance in coast redwood and giant sequoia. *The Plant Journal* 109: 7-22.

Fagerström, T. 1992. The meristem-meristem cycle as a basis for defining fitness in clonal plants. *Oikos* 63: 449-453.

Felsenstein, J. 1974. The evolutionary advantage of recombination. *Genetics* 78: 737-756.

Fink, S. 1984. Some cases of delayed or induced development of axillary buds from persisting detached meristems in conifers. *American Journal of Botany* 71: 44-51.

Fozdar, B.S. and W.J. Libby. 1968. Chromosomes of *Sequoia sempervirens*; 8-hydroxy-quinoline-castor oil pretreatment for improving preparation. *Stain Technology* 43: 97-100.

Fu, F., C. Song, C. Wen, L. Yang, Y. Guo, X. Yang, Z. Shu, X. Li, Y. Feng, B. Liu, M. Sun, Y. Zhong, L. Chen, Y. Niu, J. Chen, G. Wang, T. Yin, S. Chen, L. Xue, F. Cao. 2023. The *Metasequoia* genome and evolutionary relationship among redwoods. *Plant Communications* 4: 100643.

Fujino, T., Y. Katsushi, T.T. Yokoyama, T. Hamanaka, Y. Harazono, H. Kamada, W. Kobayashi, T. Ujino-Ihara, K. Uchiyama, A. Matsumoto, A. Izuno, Y. Tsumura, A. Toyoda, S. Shigenobu, Y. Moriguchi, S. Ueno, and M. Kasahara. 2023. A chromosome-level genome assembly of a model conifer plant, the Japanese cedar, *Cryptomeria japonica* D. Don. bioRxiv preprint https://doi.org/10.1101/2023.02.24.529822

Gill, D.E., L. Chao, S.L. Perkins, J.B. Wolf. 1995. Genetic mosaicism in plants and clonal animals. *Annual Review of Ecology, Evolution, and Systematics* 26: 423-444.

Hallé, F., R.A.A. Oldeman, and P.B. Tomlinson. 1978. *Tropical trees and forests: an architectural analysis.* Springer-Verlag, Berlin, Germany.

Hanlon, V.C.T., S.P. Otto, and S.N. Aitken. 2019. Somatic mutations substantially increase the per-generation mutation rate in the conifer *Picea sitchensis*. *Evolution Letters* 3: 348-358.

Harper, J.L. 1977. *Population Biology of Plants*. Blackburn Press, Caldwell, New Jersey, USA.

Harper, J.L. 1985. Modules, branches, and the capture of resources. In: In: J.B.C. Jackson, L.W. Buss, and R.E. Cook, eds. *Population Biology and Evolution of Clonal Organisms* 1-33. Yale University Press, New Haven and London.

Harper, J.L and A.D. Bell. 1979. The population dynamics of growth form in organisms with modular construction. In: Anderson, R.M., B.D. Turner, and L.R. Taylor, eds. *Population Dynamics: the 20th Symposium of the British Ecological Society* 29-52. Blackwell Scientific Publications, Oxford, London, Edinburgh, Melbourne.

Hiltunen, M., M. Grudzinska-Sterno, O. Wallerman, M. Ryberg, and H. Johannesson. 2019. Maintenance of high genome integrity over vegetative growth in the fairy-ring mushroom *Marasmius oreades*. *Current Biology* 29: 2758-2765.

Hirayoshi, I. and Y. Nakamura. 1943. Chromosome number of *Sequoia sempervirens*. *Botany and Zoology* 2: 73-75.

Hizume, M., K. Kaneko, and T. Miyake. 2014. A method for preparation of meiotic chromosomes of conifers and its applications. *Chromosome Botany* 9: 83-88.

Hofmeister, B.T., J. Denkena, M. Colomé-Tatché, Y. Shahryary, R. Hazarika, J. Grimwood, S. Mamidi, J. Jenkins, P.P. Grabowski, A. Sreedasyam, S. Shu, K. Barry, K. Lail, C. Adam, A. Lipzen, R. Sorek, D. Kudrna, J. Talag, R. Wing, D.W. Hall, D. Jacobsen, G.A. Tuskan, J. Schmutz, F. Johannes, and R.J. Schmitz. 2020. A genome assembly and the somatic genetic and epigenetic mutation rate in a wild long-lived perennial *Populus trichocarpa*. *Genome Biology* 21: 259.

Howe, J., J.C. Rink, B. Wang, and A.S. Griffin. 2022. Multicellularity in animals: the potential for within-organism conflict. *Proceedings of the National Academy of Sciences* 119: e2120457119.

Keightley, P.D. and M. Lynch. 2003. Toward a realistic model of mutations affecting fitness. *Evolution* 57: 683-685.

Klekowski, E.J. Jr. 1988. *Mutation, Developmental Selection, and Plant Evolution*. Columbia University Press, New York, New York, USA.

Klekowski, E.J. 2003. Plant clonality, mutation, diplontic selection and mutational meltdown. *Biological Journal of the Linnean Society* 79: 61-67.

Klekowski, E.J., Jr., N. Kazarinova-Fukshansky. 1984a. Shoot apical meristems and mutation: fixation of selectively neutral cell genotypes. *American Journal of Botany* 71: 22-27.

Klekowski, E.J., Jr., N. Kazarinova-Fukshansky, and H. Mohr. 1985. Shoot apical meristems and mutation: stratified meristems and angiosperm evolution. *American Journal of Botany* 72: 1788-1800.

Kockler, Z.W., B. Osia, R. Lee, K. Musmaker, and A. Malkova. 2021.Repair of DNA breaks by break-induced replication. *Annual Review of Biochemistry* 90: 165-191.

Kramer, R. D., S. C. Sillett, and A. L. Carroll. 2014. Structural development of redwood branches and its effects on wood growth. *Tree Physiology* 34: 314-330.

Krueger, F., F. James, P. Ewels, E. Afyounian, M. Weinstein, B. Schuster-Boekler, G. Hulselmans, and sclamons. 2023. FelixKrueger/TrimGalore v.0.6.6. https://doi.org/10.5281/zenodo.7598955

Kuser, J.E., A. Bailly, A. Franclet, W. J. Libby, J. Martin, J. Rydelius, R. Schoenike, and N. Vagle. 1995. Early results of a range wide provenance test of *Sequoia sempervirens*. *Forest Genetic Resources* 23: 21-25.

Lewontin, R.C. 1970. The units of selection. *Annual Review of Ecology and Systematics* 1: 1-18.

Li, H. 2018. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 34: 3094-3100.

Li, Z. M.T.W. McKibben, G.S. Finch, P.D. Blischak, B.L. Sutherland, M.S. Barker. 2021. Patterns and processes of diploidization in land plants. *Annual Review of Plant Biology* 72: 387-410.

Libby, W. J. and K. Tufuor 1984. 'Korbel KT' USPP5345P.

Lieberman, M. and I. Henry. 2012. Mpileup Generation and Parsing Tools. https://github.com/Comai-Lab/mpileup-tools

Liu, X-Q, X-M Xia, L. Chen, and X-Q Wang. 2022. Phylogeny and evolution of Cupressaceae: updates on intergeneric relationships and new insights on ancient intergeneric hybridization. *Molecular Phylogenetics and Evolution* 177: 107606.

López, E.H. and S.R. Palumbi. 2020. Somatic mutations and genome stability maintenance in clonal coral colonies. *Molecular Biology and Evolution* 37: 828-838.

López-Nandam, E.H., R. Albright, E.A. Hanson, E.A. Sheets, and S.R. Palumbi. 2022. Mutations in coral soma and sperm imply lifelong stem cell renewal and cell lineage selection. *Proceedings of the Royal Society B* 290: 20221766.

Lynch, M., R. Bürger, D. Butcher, and W. Gabriel. 1993. The mutational meltdown in asexual populations. *Journal of Heredity* 84: 339-344.

Mable, B.K. and S.P. Otto. 1998. The evolution of life cycles with haploid and diploid phases. *BioEssays* 20: 453-462.

Muller, H.J. 1964. The relation of recombination to mutational advance. *Mutation Research* 1: 2-9.

Narayan, L. 2015. Clonal diversity, patterns, and structure in old coast redwood forests. Doctoral Dissertation, University of California, Berkeley.

Neale, D.B., Zimin, A.V., Zaman, S., Scott, A.D., Shrestha, B., Workman, R.E., Puiu, D., Allen, B.J., Moore, Z.J., Sekhwal, M.K., De La Torre, A.R., McGuire, P.E., Burns, E., Timp, W., Wegrzyn, J.L., and Salzberg, S.L. 2022. Assembled and annotated 26.5 Gbp coast redwood genome: a resource for estimating evolutionary adaptive potential and investigating hexaploid origin. *G3—Genes, Genomes, and Genetics* 12: jkab380.

Negreira, G.H., P. Monsieurs, H. Imamura, I. Maes, N. Kuk, A. Yagoubat, F. Van den Broeck, Y. Sterkers, J-C Dujardin, and M.A. Domagalska. 2022. High throughput single-cell genome sequencing gives insights into generation and evolution of mosaic aneuploidy in *Leishmania donovani*. *Nucleic Acids Research* 50: 293-305.

Niu, S., J. Li, W. Bo, W. Yang, A. Zuccolo, S. Giacomello, X. Chen, F. Han, J. Yang, Y. Song, Y. Nie, B. Zhou, P. Wang, Q. Zuo, H. Zhang, J. Ma, J. Wang, L. Wang, Q. Zhu, H. Zhao, Z. Liu, X. Zhang, T. Liu, S. Pei, Z. Li, Y. Hu, Y. Yang, W. Li, Y. Zan, L. Zhou, J. Lin, T. Yuan, W. Li, Y. Li, H. Wei, H.X. Wu. 2022. The Chinese pine genome and methylome unveil key features of conifer evolution. *Cell* 185: 1-14.

Orive, M.E. 2001. Somatic mutations in organisms with complex life histories. *Theoretical Population Biology* 59: 235-249.

Orr, A.J., A. Padovan, D. Kainer, C. Külheim, L. Bromham, C. Bustos-Segura, W. Foley, T. Haff, J-F Hsieh, A. Morales-Suarez, R.A. Cartwright, and R. Lanfear. 2020. A phylogenomic approach reveals a low somatic mutation rate in a long-lived plant. *Proceedings of the Royal Society B* 287: 20192364.

Otto, S.P. 2007. The evolutionary consequences of polyploidy. *Cell* 131: 452-462.

Otto, S.P. and I.M. Hastings. 1998. Mutation and selection within the individual. *Genetica* 102/103: 507-524.

Otto, S.P. and M.E. Orive. 1995. Evolutionary consequences of mutation and selection within an individual. *Genetics* 141: 1173-1187.

Park, H-S., J-H. Jeon, W. Cho, Y. Lee, J.Y. Park, J. Kim, Y.S. Park, H.J. Koo, J.H. Kang, T.J. Lee, S.H. Kim, J-B. Kim, H-Y. Kwon, S-H. Kim, N-C. Paek, G. Jang, J-Y. Suh, T-J. Yang. 2023. High-throughput discovery of plastid genes causing albino phenotypes in ornamental chimeric plants. *Horticultural Research* 10: uhac246.

Peirce, G.J. 1901. Studies on the coast redwood, *Sequoia sempervirens* Endl. *Proceedings of the California Academy of Sciences: Botany* 2: 83-106.

Perez-Roman, E., C. Borredá, and A.L-G. Usach. 2021. Single-nucleotide mosaicism in citrus: estimations of somatic mutation rates and total number of variants. *The Plant Genome* 15: e20162.

Pineda-Krch, M. and T. Fagerström. 2001. On the potential for evolutionary change in meristematic cell lineages through intraorganismal selection. *Journal of Evolutionary Biology* 12: 681-688.

Pineda-Krch, M. and K. Lehtilä. 2002. Cell lineage dynamics in stratified shoot apical meristems. *Journal of Theoretical Biology* 219: 495-505.

Pineda-Krch, M. and K. Lehtilä. 2004. Costs and benefits of genetic heterogeneity within organisms. *Journal of Evolutionary Biology* 17: 1167-1177.

Pittermann, J., J. Cowan, N. Kaufman, A. Baer, E. Zhang, and D. Kuty. 2018. The water relations and xylem attributes of albino redwood shoots (*Sequioa* (sic) *sempervirens* (D. Don.) Endl.). *PLoS ONE* 13: e0191836.

Plomion, C., J-M Aury, J. Amselem, T. Leroy, F. Murat, S. Duplessis, S. Faye, N. Francillonne, K. Labadie, G. Le Provost, I. Lesur, J. Bartholomé, P. Faivre-Rampant, A. Kohler, J-C Leplé, N. Chantret, J. Chen, A. Diévart, T. Alaeitabar, V. Barbe, C. Belser, H. Bergès, C. Bodénès, M-B Bogeat-Triboulot, M-L Bouffaud, B. Branchi, E. Chancerel, D. Cohen, A. Coloux, C. Da Silva, C. Dossat, F. Ehrenmann, C. Gaspin, J. Grima-Pettenati, E. Guichoux, A. Hecker, S. Herrmann,

P. Hugueney, I. Hummel, C. Klopp, C. Lalanne, M. Lascoux, E. Lasserre, A. Lemainque, M-L Desprez-Loustau, I. Luyten, M-A Madoui, S. Mangenot, C. Marchal, F. Maumus, J. Mercier, C. Michotey, O. Panaud, N. Picault, N. Rouhier, O. Rué, C. Rustenholz, F. Salin, M. Soler, M. Tarkka, A.Velt, A.E. Zanne, F. Martin, P. Winker, H. Quesneville, A. Kremer, and J. Salse. 2018. Oak genome reveals facets of long life span. *Nature Plants* 4: 440-452.

Poorten, T 2018. dotPlotly https://github.com/tpoorten/dotPlotly

Ren, Y., Z. He, P. Liu, B. Traw, S. Sun, D. Tian, S. Yang, Y. Jia, and L. Wang. 2021. Somatic mutation analysis in *Salix suchowensis* reveals early-segregated cell lineages. *Molecular Biology and Evolution* 38: 5292-5308.

Reusch, T.B.H., I.B. Baums, B. Werner. 2021. Evolution via somatic genetic variation in modular species. *Trends in Ecology and Evolution* 36: 1083-1092.

Robinson, W.P. 2000. Mechanisms leading to uniparental disomy and their clinical consequences. *BioEssays* 22: 452-459.

Sanamyan, M.F., J.E. Petlyakova, E.A. Sharipova, I.Y. Abdurakhmonov. 2011. Cytogenetic characteristics of new monosomic stocks of cotton (*Gossypium hirsutum* L.) *Genetics Research International* 2011: 273642.

Saylor, L.C. and H.A. Simons. 1970. Karyology of *Sequoia sempervirens*: karyotype and accessory chromosomes. *Cytologia* 35: 294-303.

Schlarbaum, S.E., T. Tsuchiya, and L. Johnson. 1984. The chromosomes and relationships of *Metasequoia* and *Sequoia* (Taxodiaceae): an update. *Journal of the Arnold Arboretum* 65: 251-254.

Schmid-Siegert, E., N. Sarkar, C. Iseli, S. Calderon, C. Gouhier-Darimont, J. Chrast, P. Cattaneo, F. Schütz, L. Farinelli, M. Pagni, M. Schneider, J. Voumard, M. Jaboyedoff, C. Fankhauser, C.S. Hardtke, L. Keller, J.R. Pannell, A. Reymond, M. Robinson-Rechavi, I. Xenarios, and P. Reymond. 2017. Low number of fixed somatic mutations in a long-lived oak tree. *Nature Plants* 3: 926-929.

Schmitt, S., T. Leroy, M. Heuertz, and N. Tysklind. 2022. Somatic mutation detection: a critical evaluation through simulations and reanalyses in oaks. *Peer Community Journal* 2: e68.

Schmitt, S., P. Heuret, V. Troispoux, M. Beraud, J. Cazal, E. Chancerel, C. Cravero, E. Guichoux, O. Lepais, J. Loureiro, W. Marande, O. Martin, G. Vincent, J. Chave, C. Plomion, T. Leroy, M. Heuertz, N. Tysklind. 2023. Plant mutations: slaying beautiful hypotheses by surprising evidence. bioRxiv preprint https://doi.org/10.1101/2023.06.05.543657

Scott, A.D., N.W.M. Stenz, P.K. Ingvarsson, and D.A. Baum. 2016. Whole genome duplication in coast redwood (*Sequoia sempervirens*) and its implications for explaining polyploidy in conifers. *New Phytologist* 211: 186-193.

Scott, A.D., A.V. Zimin, D. Puiu, R. Workman, M. Britton, S. Zaman, M. Caballero, A.C. Read, A.J. Bogdanove, E. Burns, J. Wegrzyn, W. Timp, S.L. Salzberg, D.B. Neale. 2020. A reference genome sequence for giant sequoia. *G3—Genes, Genomes, and Genetics* 10: 3907-3919.

Sillett, S.C. and R. Van Pelt. 2000. A redwood tree whose crown is a forest canopy. *Northwest Science* 74: 34-43.

Sillett, S.C. and R. Van Pelt. 2001. A redwood tree whose crown may be the most complex on earth. *L'Arbre 2000*: 11-18.

Sillett, S.C., R. Van Pelt, A.L. Carroll, J. Campbell-Spickler, M.E. Antoine. 2020. Aboveground biomass dynamics and growth efficiency of *Sequoia sempervirens* forests. *Forest Ecology and Management* 458: 117740.

Sillett, S.C., M.E. Antoine, A.L. Carroll, Mark E. Graham, A.R.O. Chin, R. Van Pelt. 2022. Rangewide climactic sensitivities and non-timber values of tall *Sequoia sempervirens* forests. *Forest Ecology and Management* 526: 120573.

Sprugel, D.G., T.M. Hinckley, and W-Schaap. 1991. The theory and practice of branch autonomy. *Annual Review of Ecology and Systematics* 22: 309-334.

Stebbins, G.L. 1948. The chromosomes and relationships of *Metasequoia* and *Sequoia*. *Science* 108: 95-98.

Sterkers, Y., L. Lachaud, L. Crobu, P. Bastien, and M. Pagès. 2011. FISH analysis reveals aneuploidy and continual generation of chromosomal mosaicism in *Leishmania major*. *Cellular Microbiology* 13: 274-283.

Sterkers, Y., L. Lachaud, N. Bourgeois, L. Crobu, P. Bastien, and M. Pagès. 2012. Novel insights into genome plasticity in eukaryotes: mosaic aneuploidy in *Leishmania*. *Molecular Microbiology* 86: 15-23.

Strasburger, E. 1872. *Die Coniferen und die Gnetaceen. Eine morphologische Studie.* Jena, H. Dabis (in German).

Tomimoto, S., Y. Iwasa, and A. Satake. 2023. Branching architecture affects genetic diversity within an individual tree. bioRxiv preprint https://doi.org/10.1101/2023.10.02.560431

Tsai, H., V. Missirian, K.J. Ngo, R.K. Tran, S.R. Chan, V. Sundaresan, and L. Comai. 2013. Production of a high-efficiency TILLING population through polyploidization. *Plant Physiology* 161: 1604-1614.

Van Pelt, R., S.C. Sillett, W.A. Kruse, J.A. Freund, and R.D. Kramer. 2016. Emergent crowns and light-use complementarity lead to global maximum biomass and leaf area in *Sequoia sempervirens* forests. *Forest Ecology and Management* 375: 279-308.

Walbot, V. and M.M.S. Evans. 2001. Unique features of the plant life cycle and their consequences. *Nature Reviews Genetics* 4: 369-379.

Wang, L., Y. Ji, Y. Hu, H. Hu, X. Jia, M. Jiang, X. Zhang, L. Zhao, Y. Zhang, Y. Jia, C. Qin, L. Yu, J. Huang, S. Yang, L.D. Hurst, and D. Tian. 2019. The architecture of intra-organism mutation rate variation in plants. *PLoS Biology* 17: e3000191.

Watson, J.M., A. Platzer, A. Kazda, S. Akimcheva, S. Valuchova, V. Nizhynska, M. Nordborg, and K. Riha. 2016. Germline replications and somatic mutation accumulation are independent of vegetative life span in *Arabidopsis*. *Proceedings of the National Academy of Sciences* 113: 12226-12231.

Whitham, T.G., and C.N. Slobodchikoff. 1981. Evolution of individuals, plant-herbivore interactions, and mosaics of genetic variability: the adaptive significance of somatic mutations in plants. *Oecologia* 49: 287-292.

Yu, L., C. Boström, S. Franzenburg, T. Bayer, T. Dagan, and T.B.H. Reusch. 2020. Somatic genetic drift and multilevel selection in a clonal seagrass. *Nature Ecology and Evolution* 4: 952-962.

Zahradníková, E., A. Ficek, B. Brejová, T. Vinař, K. Mičieta. 2020. Mosaicism in old trees and its patterns. *Trees* 34: 357-370.

TABLES AND FIGURES:

Table 2.1 – Reproductive Outcomes

Mean values per cone	Broken top (LMRS)	Unbroken top (NOPQ)	p-value
Cone fresh weight (g)	2.38	1.87	**3.92×10^{-5}**
Seed weight (g)	0.371	0.271	**6.75×10^{-4}**
Total seeds (n)	67.5	60.6	1.09×10^{-1}
Live megagametophytes (n)	5.95	3.05	**5.78×10^{-3}**
Dead megagametophytes (n)	27.8	27.3	4.17×10^{-1}
Root emergence (n)	4.35	2.40	**7.37×10^{-3}**
Cotyledon emergence (n)	6.15	3.35	**2.61×10^{-3}**
>5 cm seedlings (n)	5.50	2.80	**3.71×10^{-3}**

Table 2.1: Fecundity values for the two tree tops of T35: the broken top comprising samples LMRS and the unbroken top comprising samples NOPQ. All values are per cone means of values indicating overall reproductive success. Student T-tests were conducted between the two groups, with p-values indicated comparing the fecundity values of the two tops. Bolded p-values are below a 0.05 threshold, indicating significant differences. Note, for most values, the broken top had significantly higher fecundity. The two values where this difference does not exist has to do with number of ovules initiated (hexaploid megasporangia and integuments, for instance) and not successful megagametophyte or seed development. Many values are almost double in the broken top compared to the unbroken top.

Table 2.2 – Comparative Somatic Mutation Rates

Lineage	Species	Mutation rate (per site per year)	Publication (Year)
Gymnosperms	*Sequoia sempervirens*	8.29×10^{-7}	this study, upper bound
		3.40×10^{-8}	this study, middle bound
		6.90×10^{-9}	this study, lower bound
	Picea sitchensis	8.03×10^{-10}	Hanlon et al. (2019)
Monocots	*Brachypodium distachyon*	6.13×10^{-9}	Wang et al. (2019)
	Oryza sativa	9.01×10^{-9}	Wang et al. (2019)
Eudicots	*Arabidopsis thaliana*	4.35×10^{-9}	Wang et al. (2019)
	Citrus clementina	4.40×10^{-10}	Perez-Roman et al. (2021)
	Eucalyptus melliodora	6.18×10^{-9}	Orr et al. (2020)
	Fragaria vesca	1.58×10^{-8}	Wang et al. (2019)
	Populus alba	9.24×10^{-9}	Zheng et al. (2022)
	Populus trichocarpa	1.33×10^{-10}	Hofmeister et al. (2020)
	Prunus mira	1.30×10^{-10}	Wang et al. (2019)
	Prunus mume	2.28×10^{-9}	Wang et al. (2019)
	Prunus persica	8.17×10^{-10}	Wang et al. (2019)
	Prunus mume	2.28×10^{-9}	Wang et al. (2019)
	Quercus robur	2.01×10^{-10}	Schmnid-Siegert et al. (2017)
	Salix suchowensis	2.58×10^{-9}	Wang et al. (2019)
	Salix suchowensis	4.32×10^{-9}	Ren et al. (2021)
Basidiomycete	*Armillaria gallica*	4.32×10^{-9}	Anderson et al. (2018)
Cnidarian	*Acropora hyacinthus*	4.90×10^{-9}	Lopez et al. (2020)

Table 2.2: A table of the somatic mutation papers that report a per-year mutation rate.

Note that our values for coast redwood are relatively high compared to the others on the table. However, also note the variability withing species and genera that are orders of magnitude different from each other. This table demonstrates that mutation rate is difficult to measure, highly variable, and measurements depend largely on assumptions made during the analytic process.

Figure 2.1 – T35 Redwood Genome Tree

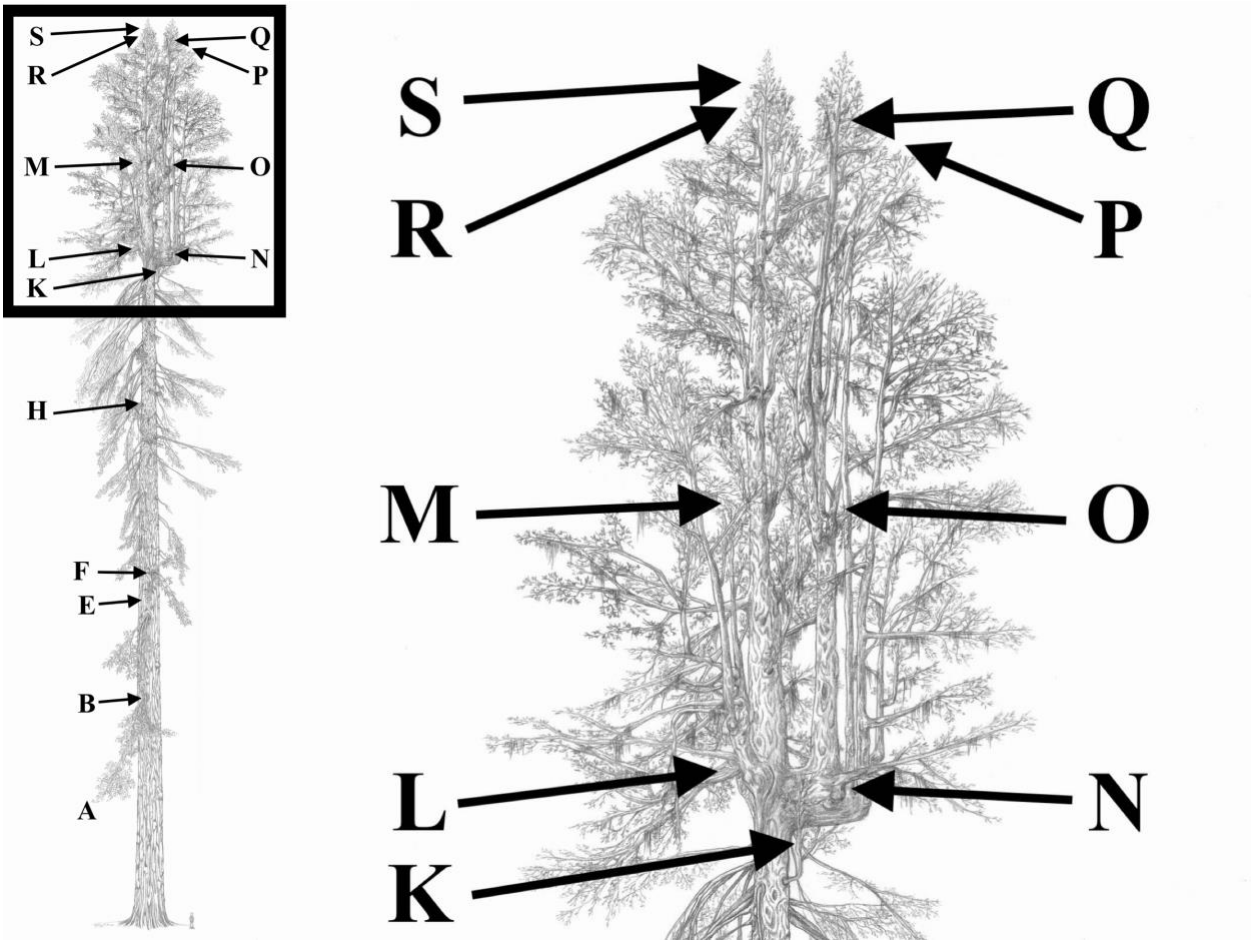

Figure 2.1: Artist's rendition of the redwood reference genome tree, T35, (Sillett et al., 2022; SESE104, Neale et al., 2022), drawn here to scale based on trunk diameter and mapping measurements (Sillett et al., 2022). T35 is 1388 years old, 107.5 meters tall, and is calculated to weigh 98 metric tons dry weight (Sillett et al., 2022). Its main trunk broke at 79.5 meters above the ground, 550 years ago (Carroll and Sillett, 2023), at sample L, resulting in multiple reiterated trunks and branches from just below the break (sample K; see enlarged panel). Letters indicate the different foliage samples collected for sequencing and sectioning. The broken top are samples L, M, R, and S were collected from a top that was twice broken in the past at 79.5 m and 90.8 meters, while samples N, O, P, and Q were from a second top that was not broken.

Figure 2.2 – Loss of Heterozygosity

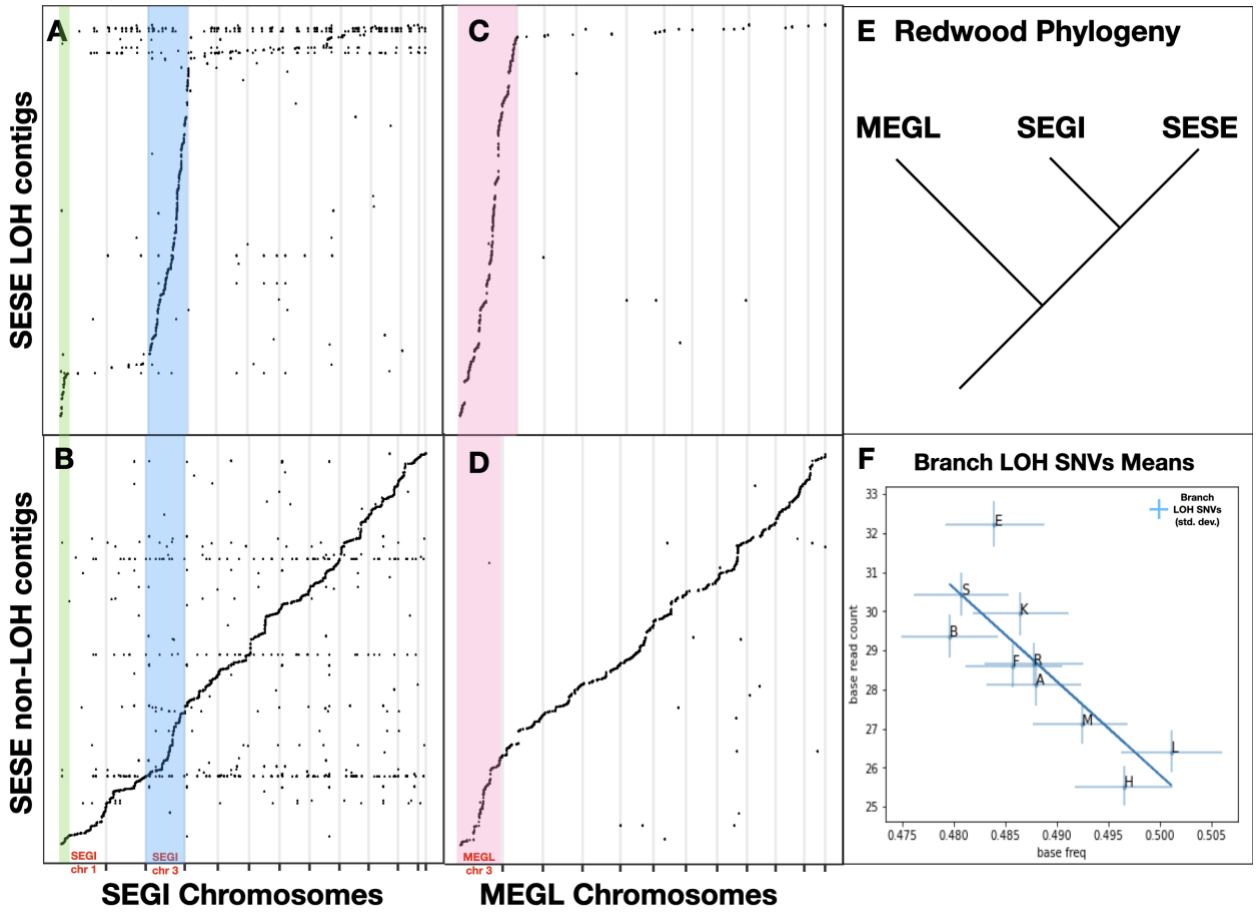

Figure 2.2: Redwood contigs from T35 (*Sequoia sempervirens*; labeled SESE; A-E) were mapped against *Sequoiadendron giganteum* (labeled SEGI; A, B) and *Metasequoia glyptostroboides* (labeled MEGL; C, D) chromosomal assemblies. The contigs with loss of heterozygosity (LOH) in branches N, O, P, Q are plotted in panels A and C. The loss of heterozygosity (LOH) contigs are homologous to SEGI and MEGL chromosome 3 (highlighted blue and pink respectively) and a small portion of SEGI chromosome 1 (highlighted green). As a control, all other contigs without loss of heterozygosity were plotted in panels B and D. They align fairly evenly across the SEGI or MEGL genome, without enrichment to any particular chromosome(s). Generic relationships between the redwoods are indicated in panel E (Liu et al., 2022). To see if loss of heterozygosity (LOH) was due to large scale loss of genomic material

(such as a chromosome) rather than independently mutated events, we tested whether homozygous single nucleotide variant (SNV) allele frequency and allelic read counts correlated negatively. We plot this in panel F showing the expected negative correlation, indicative of a large-scale chromosome loss causing the loss of heterozygosity. Further, this is evidence that small traces of the loss can be seen in the other samples, with samples lower and further right on the graph having more potential mixture of cell lineages with the chromosome loss.

Figure 2.3 – Synteny Analysis

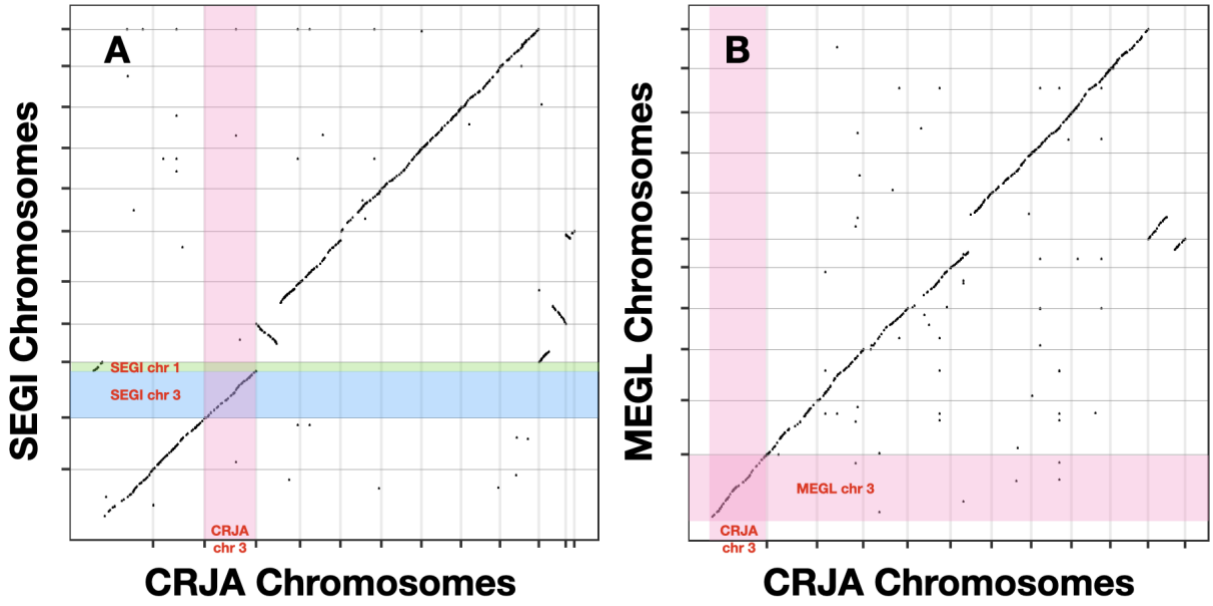

Figure 2.3. Synteny plots of Cryptomeria, Metasequoia, and Sequoiadendron chromosomal genome assemblies. All highlighted regions of the graph are homologous to the missing chromosome in the NOPQ samples of T35 (see Figure 2.1). The pink colors are for a single chromosome (chromosome 3) in Cryptomeria and Metasequoia. The end of this chromosome is found in chromosome 1 of Sequoiadendron, indicated by the green highlights. Because of the overall chromosomal synteny within the Cupressaceae family, we hypothesize T35 is missing a single chromosome.

Figure 2.4 – Reproductive Outcomes

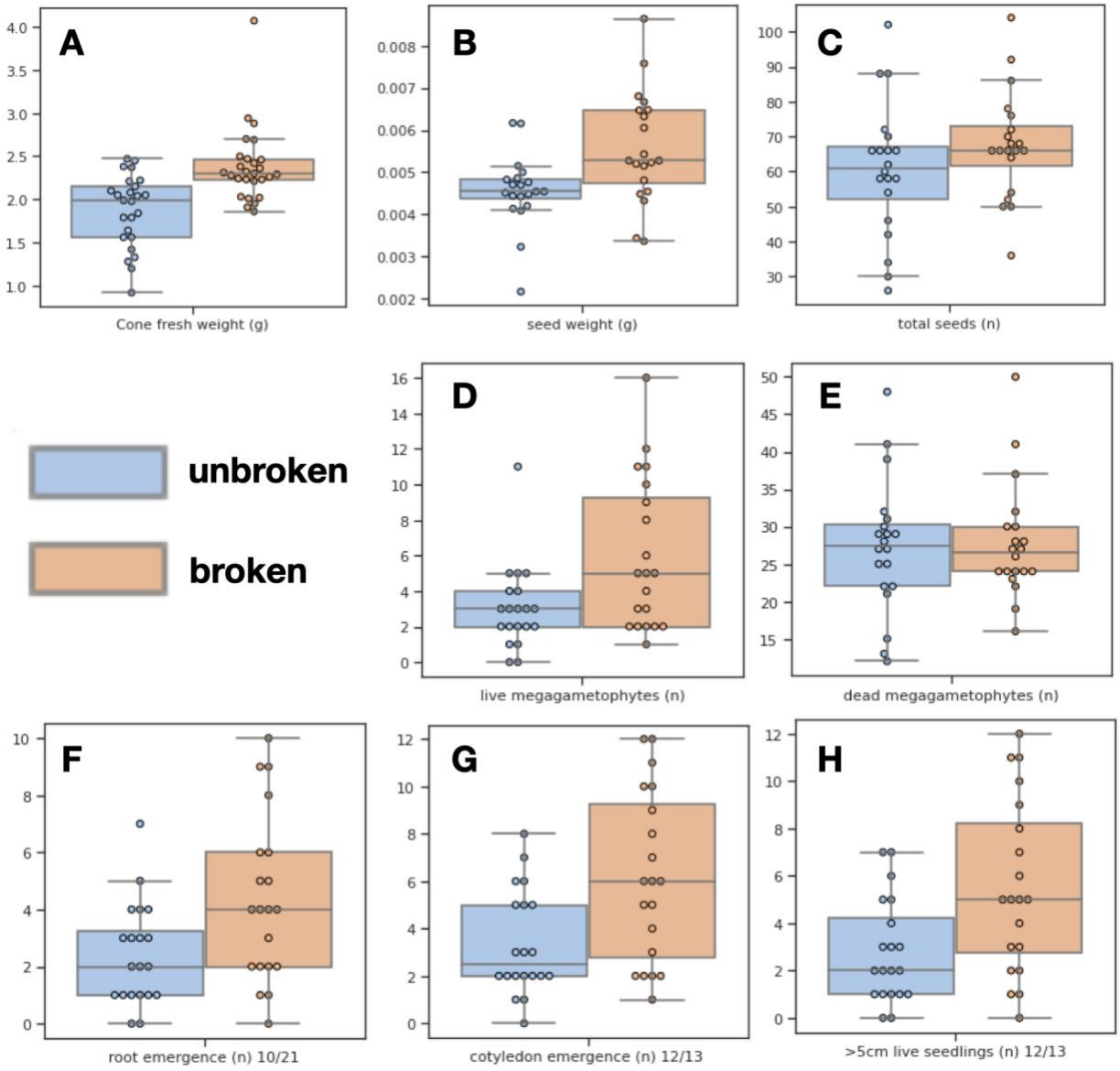

Figure 2.4: Cones were collected from the unbroken and broken tops of T35 (samples R,S vs. P,Q respectively; colored blue and orange respectively). The unbroken top had a loss of a chromosome which may explain some of the differences seen in this figure. For each graph, each dot represents one cone. The top row shows seed cone fresh weights (a), seed weights (b), and total seed number (c), each of which was higher on average in the broken top. Half of the seeds per cone were subject to dissections while the other half were grown in soil. The middle row

shows results from the dissections, indicating number of live (d) and dead (e) seeds per cone. Note that number of dead seeds were roughly the same between the tops, but number of live seeds were substantially higher in the broken top. The bottom row indicates seedling growth development chronologically, from root emergence (germination; f) to cotyledon emergence (g) and >5 cm height of seedlings (h), all indicators of potential seedling success in a natural environment.

CONCLUSION:

Across different scales of a redwood sporophyte genet, redundancy increases the likelihood of variation, variation increases the likelihood of selection or drift, and selection or drift increases the likelihood of fixation of that variation.

At the molecular level (see Chapter 2), hexaploidy is the redundancy that increases the likelihood of variation to accumulate through mutations across redundant, homologous alleles (Orr 1995). Diploidization (Li et al., 2021), neofunctionalization (Birchler and Yang, 2022), and mosaic monosomy (Sterkers et al., 2012) enables selection to act at this scale to fix alleles that are necessary for the survival of the next generation.

At the cell lineage (cellular subpopulations of the shoot apex) level (see Chapter 1), redundant copies (Figure 1.1) of shoot apical initials enable genetic variation to accumulate and fix along different sectors of the shoot through intra-meristematic selection and random drift (Klekowski et al., 1985). Through developmental selection bottlenecks in proleptic branches (Fink 1984), these mutated alleles, especially if in the L1, can become fixed in an entire branch (Figure 1.2).

At the branch (meristem, ramet) level (see Chapters 1 and 2), increased meristem number enables accumulation of variation between different branches through somatic mutation (Antolin and Strobeck, 1985; Klekowski et al., 1985; Klekowski 1988). This inter-meristematic variation in separate branches (Figure 2.2) enables inter-meristematic selection to occur, altering mutated allele abundance in distal sections of the plant.

This hierarchical selection (Buss 1983; Yu et al., 2020; Reusch et al., 2021; Howe et al., 2022; Aanen et al., 2023) within individual redwood sporophytes (zygotic products and their asexual descendants) occurs at the genome, cell lineage, and meristem levels. At the genome

level, the loss of a chromosome in T35 (Figure 2.1) and NOPQ (Figure 2.2) becomes noticeable in the fecundity of that tree top (Figure 2.4). At the cell lineage level, chimeric trees mix layers infrequently but can result in sectorial chimeras enabling intra-meristematic selection to occur (Figure 1.2). At the meristem level, new meristems are founded by different cell lineages (Figure 1.2), resulting in clear fixation of different genetic variation in ten chimeric trees and within T35 and its 14 branches.

The cell lineages and genomics of redwood correspond with each other to be used synergistically to deal with mutational load and enable increased longevity in the process. Redwoods have two shoot apex sub-populations, the L1 and L2 (Figure 1.1). These divide the meristem population into two, genetically independent cell lineages. As the meristem branches, the two sub-populations do not found the new meristems equally. Sylleptic branches are more likely to keep the cell lineage organizational status quo, while proleptic branches are more likely to replace the L2 with the L1, further subdividing the cell lineages within the tree (Figure 1.2). Along with the sub-population subdivision, branches themselves are subdivisions of these two cell lineages. The result is a tree that separates out risk of accumulated mutations over a wide swath of sexual cell populations (branches and sub-populations of shoot apices).

The genomic situation is the opposite. If one traces a mutation, it might be more likely to be fixed in a proleptic branch where selection may act on it independently of the other branches of the tree. Likewise, if a loss of a chromosome occurs, any neofunctionalized alleles of the univalent chromosome will be exposed to selection. The compartmentalization of risk seen in the branching form of a redwood tree happens also from polyploidy which minimizes the effects of any one mutation.

Redwood longevity and genomic integrity, therefore, are integrally linked with the ability to compartmentalize mutations in separate chromosomes and in separate branches, thereby mitigating risk. This is a form of developmental selection (Buchholz 1922) within the soma (Klekowski 1988). At the same time, redwoods can utilize proleptic branches to expose L1 genotypes to selection, uncoupling their fate from the fate of the L2. Similarly, neofunctionalized alleles that have gone through the process of diploidization can be uncoupled through mosaic monosomy, exposing any deleterious mutations by unmasking them from their functional alleles. In this way, redwoods can accumulate mutations, promoting beneficial ones and masking deleterious ones all in their somatic bodies before meiosis or sexual reproduction has taken place.

REFERENCES:

Aanen, D.K., A. van ’t Padje, and B. Auxier. 2023. Longevity of fungal mycelia and nuclear quality checks: a new hypothesis for the role of clamp connections in dikaryons. *Microbiology and Molecular Biology Reviews*: e00022-21.

Antolin, M. F. and C. Strobeck. 1985. The population genetics of somatic mutation in plants. *The American Naturalist 126*: 52-62.

Birchler, J.A. and H. Yang. 2022. The multiple fates of gene duplication: deletion, hypofunctionalization, subfunctionalization, neofunctionalization, dosage balance constraints, and neutral variation. *The Plant Cell* 34: 2466-2474.

Buchholz, J.T. 1922. Developmental selection in vascular plants. *The Botanical Gazette* 73: 249-286.

Buss, L.W. 1983. Evolution, development, and the units of selection. *Proceedings of the National Academy of Sciences* 80: 1387-1391.

Fink, S. 1984. Some cases of delayed or induced development of axillary buds from persisting detached meristems in conifers. *American Journal of Botany* 71: 44-51.

Howe, J., J.C. Rink, B. Wang, and A.S. Griffin. 2022. Multicellularity in animals: the potential for within-organism conflict. *Proceedings of the National Academy of Sciences* 119: e2120457119.

Klekowski, E.J. Jr. 1988. *Mutation, Developmental Selection, and Plant Evolution*. Columbia University Press, New York, New York, USA.

Klekowski, E.J., Jr., N. Kazarinova-Fukshansky, and H. Mohr. 1985. Shoot apical meristems and mutation: stratified meristems and angiosperm evolution. *American Journal of Botany* 72: 1788-1800.

Li, Z. M.T.W. McKibben, G.S. Finch, P.D. Blischak, B.L. Sutherland, M.S. Barker. 2021. Patterns and processes of diploidization in land plants. *Annual Review of Plant Biology* 72: 387-410.

Orr, H.A. 1995. Somatic mutation favors evolution of diploidy. *Genetics* 139: 1441-1447.

Reusch, T.B.H., I.B. Baums, B. Werner. 2021. Evolution via somatic genetic variation in modular species. *Trends in Ecology and Evolution* 36: 1083-1092.

Sterkers, Y., L. Lachaud, N. Bourgeois, L. Crobu, P. Bastien, and M. Pagès. 2012. Novel insights into genome plasticity in eukaryotes: mosaic aneuploidy in *Leishmania*. *Molecular Microbiology* 86: 15-23.

Yu, L., C. Boström, S. Franzenburg, T. Bayer, T. Dagan, and T.B.H. Reusch. 2020. Somatic genetic drift and multilevel selection in a clonal seagrass. *Nature Ecology and Evolution* 4: 952-962.