Polyploidy in Gymnosperms: Revisited*

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Abstract

There are only a few natural polyploids in gymnosperms. These have been reported in Ephera spp. (Gnetales), and *Juniperus chinensis* 'Pfitzeriana' (2n = 4x = 44), *Fitzroya cupressoides* (2n = 4x = 44), and the only hexaploid conifer *Sequoia sempervirens* (2n = 6x = 66) (Coniferales). Sporadic polyploids and aneuploids occur at a very low frequency in nurseries in conifers, but most of them show growth abnormalities, remain dwarf, and may not reach maturity. One exception is an autotetraploid tree of *Larix decidua* (2n = 4x = 48) that has survived in a private estate in Denmark. Colchicine-induced polyploids (colchiploids) have been produced in a several genera of conifers, including, *Pinus*, *Picea*, and *Larix*. These colchiploids (Co) were hybridized to untreated diploids to produce C1 and C2 generations to investigate their chromosome behavior. The colchiploids showed a wide range of chromosome variability, ranging from diploids, triploids, and tetraploids, and many were mixoploids. The colchiploids also show growth retardation, remain dwarf, and their future potential applications in forestry remains uncertain. However, genetic variability in the colchiploids still offers prospects for isolating genetically stable new genotypes. Even though polyploidy is rare in extant conifers, is it possible that ancient polyploidy or paleopolyploidy, that is prevalent in angiosperms, has also played a role in the evolution of conifers. In this paper we shall review the current status of polyploidy in gymnosperms.

Key words: polyploidy, gymnosperms, conifers, coast redwood, alerce, 'Pfitzer' juniper, pines, genetic equilibrium, diploidization, colchiploids, ancient polyploidy.

Introduction

Polyploidy, that is presence of three or more genomes per nucleus, occurs both in plants and animals. It occurs at a relatively high frequency in plants as compared to animals. Polyploidy has been reported in animals that reproduce by parthenogenetic means, for example insects, amphibians, but occurs rarely in sexually reproducing animals, such as mammals (Otto and Whitton, 2000). Nevertheless, polyploidy has provided a rapid means for the evolution of new genes and speciation during the early evolution of both animals and plants, and still continues to be an important mechanism for speciation of plants (Stebbins, 1950; Ohno, 1970; Wendel, 2000). Polyploidy is widespread in plants, and recent estimates suggest that 50 to 80% of all angiosperms are polyploids (Masterson, 1994; Otto and Whitton, 2000). Many angiosperms may have experienced one or more episodes of polyploidization during their evolution (Soltis and Soltis, 1993; 1999; Wendel, 2000). In the Pteridophytes the incidence of polyploidy may be close to 95% (Grant, 1981). Although polyploidy is relatively common in the angiosperm trees, and perhaps all angiosperms had experienced polyploidy in their evolutionary history (Wendel, 2000), it is rather infrequent among gymnosperms (Khoshoo, 1959; Wright, 1976; Ahuja, 2001). The frequency of polyploidy may be close to 5% in the gymnosperms and about 1.5% in the conifers (Khoshoo, 1959).

So far there are only two reviews that have mainly dealt with polyploidy in gymnosperms, particularly conifers during the last century. One is an excellent review by Khoshoo in 1959, and the second a brief update on polyploidy in gymnosperms by Delevoryas in 1980. Polyploidy, as an avenue for mutagenesis and breeding has also been discussed in forest trees in several other papers (Gustafsson, 1960; Mehra, 1960; Gustaffson and Mergen, 1964; Khoshoo, 1963). We think it is time to have a fresh look at the polyploidy in gymnosperms in view of recent developments in the genomics of plants. In this paper we review the status of polyploidy, natural, sporadic and induced, in gymnosperms, particularly conifers, and discuss whether artificially-induced polyploidy has any future application in the genetic improvement of conifers. We also explore whether ancient polyploidy has played a role, if any, in the evolution of this group of mainly diploid gymnosperms.

Polyploidy in Gymnosperms

Gymnosperms consist of a widely divergent group of plants that have been variously placed in five or six orders or higher groups. Based on morphological and cytogenetic criteria, Khoshoo (1959, 1961) listed six orders in Gymnosperms: Cycadales, Ginkgoales, Gnetales, Welwitschiales, Ephedrales, and Coniferales. Recent molecular phylogeny studies based on chloroplast, mitochondrial, and nuclear genes, on the other hand, suggest that gymnosperms are divided into five different groups, namely: Cycadales, Ginkgoales, Gnetales (Gnetaceae, Ephedraceae, Welwitschiaceae), Pinaceae, and Coniferales II (comprising of all conifer families except Pinaceae) (Bowe *et al*., 2000; Chaw *et al*., 2000). Polyploidy has not been recorded in the Cycadales, and Ginkgoales, and lingering questions remain regarding the presence of polyploidy in Gnetales

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(*Gnetum*, n = 22, and *Welwitschia* (n = 21), (KHOSHOO, 1959, 1961). Polyploidy has been reported in *Ephedra* (n = 7), another member of Gnetales, and a few genera of Coniferales. In the following sections, we shall review the status of polyploidy in gymnosperms, but will discuss its occurrence and future in more detail in all Coniferales (Coniferales II and Pinaceae).

Gnetales

Ephedra

Polyploidy has been detected in ~50% species of *Ephedra* (n = 7), the most in any gymnosperm genus (KHOSHOO, 1959). Interspecific polyploidy (allotetraploids) (2n = 28) have been reported in *Ephedra altissima*, *E. intermedia*, *E. likiagenesis*, *E. saxatilis*, *E. sinica*, *E. americana* (=*E. andnia*). Sources of polyploidy remain undetermined in *E. breana* and *E. distacheae*, *E. viridis*, *fragilis*, and *E. monosperma* (FLORIN, 1932; MEHRA, 1946; HUNZIKER, 1953, 1955; KHOSHOO, 1959; 1961; DELEVORYAS, 1980).

Welwitschia

The only other gymnosperm which is morphologically and cytologically unique or rather bizarre is *Welwitschia mirabilis* (=*W. bainesii*), belongs to the order Gnetales. Based on the somatic chromosome number of 2n = 42 (FLORIN, 1932; FERNANDES, 1936), *Welwitschia* has often been interpreted to be a hexaploid because of the basic chromosome number of n = 7 in the allied genus Ephedra (KHOSHOO, 1959, 1961). Subsequently, detailed karyotype studies of KHOSHOO and AHUJA (1962, 1963) confirmed the somatic chromosome number of 2n = 42, and further revealed that: 1) all chromosomes are telocentric, having terminal centromere, 2) there was gradual transition from longest to the shortest chromosomes, the longest pair being 3.25 times longer than the shortest pair, and 3) there was only one pair of satellite chromosomes in the chromosome complement, and only two nucleoli were detected in the metabolic nuclei. Based on these observations, it would appear that *Welwitschia* karyotype does not have any qualitative or quantitative relationship with *Ephedra* (n = 7). That the chromosome number in *Welwitschia* is multiple of 7 does not necessarily imply that it is a hexaploid from *Ephedra*-like ancestors (KHOSHOO and AHUJA, 1962, 1963). On the other hand, the basic chromosome number in *Welwitschia* is indeed x = 7, derived from an extinct ancestor other than *Ephedra*-like, and that *Welwitschia* (a putative hexaploid) has presumably undergone diploidization to achieve the present diploid-like state. Alternatively, the present chromosome number 2n = 2x = 42 in *Welwitschia* originated by Robertsonian centric fissions in a diploid ancestral lineage (possibly x = 11), followed by chromosomal loss/rearrangements (SCHLARBAUM and AHUJA, unpublished). Nevertheless, the origin of the unique telocentric karyotype in *Welwitschia* still remains an enigma.

In recent years genome sizes (1C value) have been estimated in the three genera of Gnetales (LEITCH *et al*., 2001). The average genome size in the tetraploid *Ephedra* (2n = 4x = 28) is ~16,000 Mb, while in *Gnetum* (2n = 44), which may be another possible polyploid, the genome size is ~3,900 Mb, and in *Welwitschia* (2n = 42) it is ~7,000 Mb. Based on the genome size, it is difficult to draw any conclusions regarding the origin of *Welwitschia* karyotype, as *Welwitschia* has a genome size that is nearly half of the tetraploid *Ehedra*, but almost close to the diploid *Ephedra*, and two-times that of *Gnetum*. Clearly, there seems to be little correlation between chromosome numbers and genome sizes, and possible downsizing (LEITCH and BENNETT, 2004) and/or upsizing of genome size could account for the bizarre genome sizes in Gnetales. In my opinion, molecular cytogenetic studies involving genomic in situ hybridization (GISH) in members of Gnetales and other gymnosperms may shed light on the karyotype ancestry and polyploidy in *Welwitschia*.

Coniferales

Conifers, order Coniferales, are mainly a diploid group of plants with a highly conservative karyotype system. The haploid chromosome numbers are typically n = 12 in the family Pinaceae (exception: *Pseudotsuga menziesii*, n = 13), Taxaceae, Cephalotaxaceae; n = 11, in Cuppresaceae (including Taxodiaceae), n = 13 in Araucariaceae; n = 10 in Scidipityaceae, n = 9 in Phyllocladaceae, and a mixed bag ranging from n = 9-19 in Podocarpaceae (KHOSHOO, 1961; MURRAY, 1998). There are only a few natural polyploids among conifers (KHOSHOO, 1959; DELEVORYAS, 1980). We shall discuss polyploidy, natural, sporadic, or induced, in the two major families of conifers, namely, Cupressaceae and Pinaceae.

Cupressaceae (including the former Taxodiaceae)

Sporadic polyploidy (triploids and tetraploids) have been reported in otherwise diploid members (2n = 22) of Cupressaceae: *Cryptomeria japonica* (2n = 33, 44) (CHIBA, 1951), *Juniperus chinensis* (2n = 33, 44), *Juniperus chinensis* (2n = 33, 44), *J. squamat* (2n = 44), *J. virginiana* (2n = 33) and *J. sabina* (2n = 44) (HALL *et al*., 1979). However, these sporadic polyploids survive only in nurseries and private estates. In addition, colchicine-induced polyploidy has been also produced in two genera of (Cupressaceae). JENSEN and LEVAN (1941) produced a tetraploid in the diploid *Sequoiadendron giganteum* (2n = 22). The tetraploid seedlings and young plants had 2n = 44, grew slowly, and had shortened needles. We have also produced colchiploids in *Sequoiadendron giganteum* and *Sequoia sempervirens* (2n = 6x = 66) at the Institute of Forest Genetics, Placerville, CA, USA, in 1999 and followed them for 5 years till 2004 (AHUJA, unpublished). Two colchiploids (4x) in *Sequoiadendron* were similar in character to those produced by JENSEN and LEVAN (1941), but died due to unknown causes in the nursery. Two colchiploids in *Sequoia* (12x) had thick and shortened needle and grew slowly for the first year. Both these colchiploids became chimeric in the second year, and only the normal *Sequoia* (2n = 6x = 66) survived by the 5th year.

Natural polyploids occur in different genera in Cupressaceae. These include *Juniperus chinensis* 'Pfitzeriana', *Fitzroya cupressoides*, and *Sequoia sempervirens* discussed below.

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'Pfitzer' Juniper: an allotetraploid

The story of *Juniperus chinensis* 'Pfitzeriana' began in 1890 in the Späthe Nursery in Germany, where it was introduced as *J. chinensis* 'Pfitzeriana', named after the nursery propagator, Wilhelm Pfitzer. This is an evergreen conifer shrub with wide spreading branches. Male and female cones are borne on separate trees. Based on the presence of 22 pairs of chromosomes in the meiotic cells, SAX and SAX (1933) suggested that *J. chinensis* 'Pfitzeriana' was a tetraploid. Although the hybrid origin of *J. chinensis* 'Pfitzeriana' was proposed by PETER VAN MELLER, a New York plantsman, in 1947, this was not accepted by the horticultural community until recently (see DE LUC *et al*., 1999). The purported hybrid origin of *J. chinensis* 'Pfitzeriana' was resolved by molecular studies. By employing RAPDs as genetic markers, DE LUC *at al.* (1999) showed that *J. chinensis* 'Pfitzeriana' was an allotetraploid (2n = 4x = 44) derived by hybridization between *J. chinensis* (n = 11) and *J. sabina* (n = 11).

Alerce: an autotetraploid

Alerce, *Fitzroya cupressoides*, is a rare conifer endemic to temperate forests in southern South America. It mainly grows in discontinuous populations in the coastal Cordillera and central depression of Chile, and on the western and eastern slopes of Andes in Chile and Argentina (PREMOLI *et al.*, 2000a). Alerce is a long-lived natural tetraploid with a chromosome number of 2n = 44 (HAIR, 1968). After bristlecone pine, *Pinus longaeva*, alerce is the second oldest living tree in the world reaching a mature age of more than 3600 years (LARA and VILLALBA, 1993). Recent molecular studies using RAPDs and isozymes have revealed that there is enormous (85–92%) genetic variability within the populations of alerce, and about 8% genetic variability between different populations of alerce (ALLNUTT *et al.*, 1999; PREMOLI *et al.*, 2000a). Analysis of allozyme polymorphic loci has suggested that total genetic diversity of alerce was nearly half (PREMOLI *et al.*, 2000b) of the typical levels of genetic diversity published in other conifers (HAMRICK *et al.*, 1992). Further, isozyme polymorphism studies indicate a tetrasomic inheritance pattern of allozymes, suggesting that alerce is an autotetraploid. In nature, alerce regenerates both by seed and vegetative propagation. Many of the stands of mature alerce trees lack regeneration capacity (VEBLEN and ASHTON, 1982; FRAVER *et al.*, 1999), perhaps because vegetative propagation occurs at a higher frequency in juvenile trees.

There are lingering questions regarding the origin of autotetraploidy in alerce, that is, when it became an autotetraploid, what is its reproduction strategy in nature, and whether all populations of alerce are still polyploids. Since there is only one study on the somatic chromosomes in alerce by HAIR in 1968, it appears that a detailed chromosome studies (both somatic and meiotic) in different population of alerce in Chile and Argentina would be necessary to ascertain the current status of polyploidy, whether it is still an autotetraploid or has become a partially diploidized autotetraploid, in nature.

Coast redwood: an hexaploid that did not diploidize

Coast redwood (*Sequoia sempervirens*) is the only conifer that is a hexaploid (2n = 6x = 66) (HIRAYOSH and NAKAMURA, 1943; STEBBINS, 1948; FOZDAR and LIBBY, 1968; SAYLOR and SIMONS, 1970; SCHLARBAUM and TSUCHIYA, 1984a; HIZUME *et al.*, 1988; TODA, 1996; AHUJA and NEALE, 2002). It belongs to the family Taxodiaceae (now included under Cupressaceae) that includes nine genera and 13 species. Redwood is a California endemic, restricted in its distribution to the fogbelt along the coastal northern California and southern Oregon border.

Based on the amount of nuclear DNA in hexaploid genome (64.27 pg or ~63,000 Mb) (HIZUME *et al.*, 2001) of *Sequoia sempervirens* (2n = 6x = 66), the genome size (1C-value) is 63,000/2 = 31,500 Mb, which is the DNA amount in the unreplicated gametic nucleus independent of the polyploidy. However, the mean basic genome size, that is, genome size in the ancestral diploid genome, in polyploids, according to the formula by BENNETT *et al.* (1998) and SOLTIS *et al.* (2003), is determined by the number of genomes in a polyploid. Based on this formula, the mean basic genome size in hexaploid Sequoia would be 63,000/6 = 10,500 Mb. Besides coast redwood, all other genera in Taxodiaceae are diploids (2n = 22), and some representative members have genome sizes (1C) around 10,000 Mb.

It is not known when the polyploid coast redwood evolved from its diploid ancestors and which are its putative progenitors, living or extinct. Cytogenetic studies, based on the presence of multivalents in the meiotic cells, have suggested that coast redwood may be an autoallopolyploid (AABBBB), or a segmental allopolyploid ($A_1A_1A_2A_2A_2A_2$ or $A_1A_1A_2A_2A_3A_3$) (STEBBINS, 1948; SAYLOR and SIMONS, 1970; SCHLARBAUM and TSUCHIYA, 1984A, 1984b) or even a partially diploidized autohexaploid (AAAAAA) (AHUJA and NEALE, 2002). Comparative morphological (STEBBINS, 1948; HIDA, 1957; TAKASO and TOMLINSON, 1992), and molecular phylogeny studies (GADEK *et al*., 2000; KUSUMI *et al*., 2000) support that *Metasequoia* and *Sequoiadendron*, both diploids with 2n = 22, are more closely related to coast redwood than other genera of Taxodiacea. Based on these and our studies (AHUJA and NEALE, 2002), we have speculated that coast redwood may contain one, two or three similar or different ancestral genomes that may have been possibly derived from some ancient species of *Sequoia*, or *Metasequoia*, *Sequoiadendron*, or other members of Taxodiaceae (AHUJA and NEALE, 2002). Whether any downsizing of polyploid genome (LEITCH and BENNETT, 2004) has occurred in *Sequoia* remains unknown because of lack of knowledge on the putative ancestors. It is also unknown whether there were one or more episodes of polyploidization in the evolution of coast redwood, a phenomenon not uncommon in plants (SOLTIS and SOLTIS, 1999; OTTO and WHITTON, 2000; WENDEL, 2000).

Fossil records suggest that coast redwood may have originated during the early Tertiary Period (~65 mya) (MILLER, 1977). In spite of its antiquity, *Sequoia* has not undergone the evolutionary process of complete diploidization, involving both changes in cytological behavior and genic constitution, to achieve a diploid-like state. Meiotic studies have revealed the presence of a few to several multivalents, in addition to a majority of bivalents in *Sequoia* (HIRAYOSHI and NAKAMURA, 1943;

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STEBBINS, 1948; AHUJA and NEALE, 2002). Because of irregular meiosis the seed set is rather low, varying from 1 to 10% in *Sequoia*. Inheritance of isozymes in controlled crosses in coast redwood has indicated hexasomic segregation in megagametophytes, and precludes strictly disomic segregation of allozyme markers (ROGERS, 1997). Genetic diversity as measured by percentage of loci polymorphic (92%) was very high in coast redwood (ROGERS, 1997), and the mean number of alleles per locus observed (2.8) is the highest value reported in the western North American conifer species (MILLAR and MARSHALL, 1991).

The origin from the diploid ancestral species would most likely involve two (2R) rounds of doublings (polyploidization) to achieve the hexaploid state in *Sequoia* (AHUJA and NEALE, 2002). If we assume that some ancient species of *Sequoia*, or dawn redwood, *Metasequoia* and giant sequoia, *Sequoiadendron*, or some other genera of Taxodiaceae, all having a genome size of ~10,000 Mb, were involved in the ancestry of *Sequoia*, then after 1R of polyploidization and hybridization *Sequoia* would have a haploid genome size (1C) of ~30,000 Mb, and after another round (total 2R) it will have a hexaploid status of 2n = 6x = 66 (6C) of ~60,000 Mb (*Figure 1*). Since genome size (1C) in extant *Sequoia* is ~31,500 Mb, it is conceivable that ancestral species of *Sequoia* had a genome size close to ~10,000 Mb. Since the genome size in extant *Metasequoia* is ~11,000 MB and that of *Sequoiadendron* is ~10,000, it would appear that if ancient species of these two genera, or other genera, that were possibly involved in the ancestry of *Sequoia*, may have had a genome size rather similar to the extant genera. This is a purely a speculative model, and alternative models based on smaller or larger genome sizes of putative ancestors could be generated. At present, we do not know if the genome size in *Sequoia* has undergone any major changes (insertions or deletions of genomic sequences, retrotransposon amplification) during its evolution to hexaploidy over millions of years.

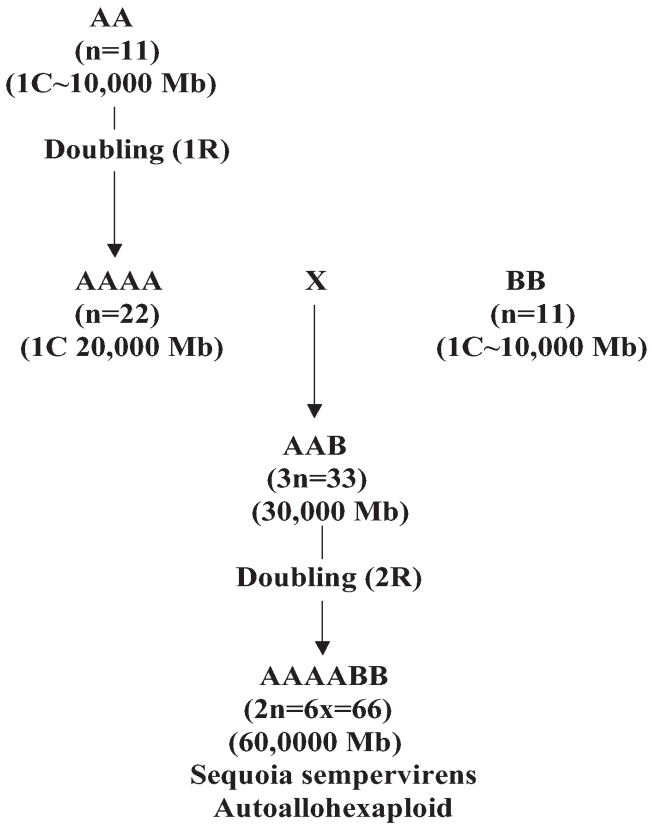

Figure 1. – Origin of hexaploid *Sequoia sempervirens* from diploid ancestral species having a genome size (1C) of ~10,000 Mb. After one round of polyploidization (1R) followed by hybridization, or hybridization followed by one round of polyploidization, the genome size of extant *Sequoia* (~30,000 Mb) is close to the expected 3 C genome size. After another round of polyploidization (total 2R) the 6C (hexaploid) genome size of ~60,000 Mb is attained in *Sequoia.* This model is based on the assumption that *Sequoia* is an autoallohexaploid. However, regardless of the nature of polyploidy in *Sequoia*, the same genome size could be achieved if the ancestral species had a genome size of ~10,000 Mb. Even if the genome sizes of ancestral species were larger or smaller than 10,000 Mb, deletions or accumulation of repetitive DNA sequences, for example, retrotransposons, could have still resulted in the current genome size in *Sequoia.*

Recent studies have indicated that diverse families of both *Gypsy*- and *Copia*-like retroelements are a major component of the gymnosperm genome, and are widespread across their chromosomes (FRIESEN *et al*., 2001; STAURT-ROGERS and FLAVELL, 2001; MURRAY *et al*., 2002). Although the proportion of transposable elements in the genome of *Sequoia* have not been investigated so far, it is not known to what extent retrotransposons, that are ubiquitous in the eukaryotes, have contributed to the evolution of genome size in *Sequoia*. Molecular biology studies involving comparative genome sequencing, or expressed sequence tag (EST) sequencing, using single copy nuclear genes for estimating the number of similar or different genomes may provide clues to the genome size dynamics and nature of polyploidy in *Sequoia*.

Had *Sequoia* reproduced exclusively by sexual means, it would have probably become extinct long ago during its evolutionary history because of competition from other tree species. However *Sequoia* evolved another strategy for survival: it also reproduces by vegetative means from the basal stem sprouts and burls (OLSON *et al*., 1990). Therefore, *Sequoia* reproduces by both sexual and asexual methods to maintain heterozygosity and adaptability for survival. But as a polyploid, *Sequoia* did not completely diploidize to become a paleopolyploid. Alternatively, it might have been nature's incomplete experiment in evolving polyploidy in a conifer with a caveat; there was a mid-course correction by establishing an additional mode of reproduction, the vegetative propagation, for the survival of a rare polyploid in a conifer! In that sense, *Sequoia* is a relict polyploid and not a paleopolyploid.

Pinaceae

Although all the genera in the family Pinaceae (for example, *Pinus*, *Picea*, and *Larix*) are basically diploid (2n = 24), sporadic polyploids have been observed in some genera. The karyotype of *Pinus* species has been studied more extensively than other genera of conifers (SAX and SAX, 1933; MEHRA and KHOSHOO, 1956; SAYLOR, 1972, 1983; PEDERICK, 1970; BORZAN and PAPĔS, 1978; DOUDRICK *et al*., 1995; LUBARETZ *et al.*, 1996; HIZUME *et*

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al., 2002). All extant pines are diploids with a chromosome number of 2n = 24, and have a rather similar but not identical karyotypes (SAYLOR, 1972; 1983). Pines have large chromosomes over 10 µ in size, and the 11 chromosomes in the haploid genome are mainly metacentric and one or two chromosomes are submetacentric (PEDERICK, 1970; SAYLOR, 1972, 1983; HIZUME *et al*., 2002). Cytogenetic studies have not detected polyploidy in pines (KHOSHOO, 1959; MIROV, 1967). However, based on Giemsa-banding studies that revealed similar bands on different chromosomes in the pine genome (*Pinus resinosa*), DREWRY (1988) suggested that hidden polyploidy has played a role in the evolution of the pine genome. But superficial homology of Giemsa bands on different chromosomes may not necessarily be indicative of the duplicate segments on chromosomes and ancient polyploidy, without the DNA sequence analysis.

Pines not only have large chromosomes, they also have large genome size. It ranges from ~17,000 Mb in Pinus banksiana to 31,200 MB in *Pinus lambertiana* (MURRAY, 1998). By contrast, the genome size in the representative angiosperm trees is much smaller: 540 Mb in *Populus deltoids* (DHILLON, 1987), and 800 Mb in *Quercus robur* (OHRI and AHUJA, 1990). The genome size is 35-40-fold larger in conifers as compared to angiosperm trees. By and large, the genome size in angiosperms is considerably smaller than the genome size in gymnosperms (LEITCH *et al*., 2001). The question is how have pines achieved such large chromosomes and genome size during their evolution?

Sporadic polyploidy

Sporadic polyploidy has been observed in the seedlings and solitary trees of various genera of Pinaceae (2n = 24) with multiple and aneuploid chromosome numbers. These include a triploid (2n = 36) in a cross between *Larix deciduas* (2n = 24) x *L. occidentalis* (2n = 24) (SYRACH-LARSEN and WESTERGAARD, 1938), a tetraploid (2n = 48) in *Larix deciduas* (CHRISTIANSEN, 1950), a tetraploid (2n = 48) in *Picea abies* (2n = 24) (KIELANDER, 1950), a triploid (2n = 36) and a tetraploid (2n = 48) in *Pinus densiflora* (2n = 24) (ZINNAI, 1952), mixoploid seedlings with diploid (2n = 24), triploid (2n = 36), and tetraploid (2n = 48) tissues in *Pinus elliottii* (MERGEN, 1958), *Picea abies* (ILLIES, 1952, 1953, 1958), and *Pinus thunbergii* (NISHIMURA, 1960). The polyploid seedlings are generally chimeric and do not develop normally to become successful polyploids. They have been usually observed in the commercial nurseries or private estates. However, one exception was an autotetraploid *Larix decidua* (2n = 4x = 48) tree that survived to become a mature tree in an estate in Denmark (CHRISTIANSEN, 1950). Meiosis was highly irregular due to multivalent formation and the fertility was low in this tree. Because of growth problems, the sporadic polyploids or aneuploids may not be able to compete with trees of the same or other species, and therefore, would have little chance of survival in nature

Colchicine-induced polyploids (colchiploids)

Colchicine-induced polyploids (colchiploids) have been produced in a number of conifer species, particularly in the families Pinaceae, and Cupressaceae, by different investigators. Earlier studies based on seedlings and young trees in the genera of Pinaceae, namely, *Pinus ponderosa* (MIROV and STOCKWELL, 1939), *Picea abies* (KIELANDER, 1950), and *Pinus elliottii* (MERGEN, 1959), showed that the colchiploids were not stable, and became diploids via chimeras.

Since extensive research on colchiploids in conifers was carried out in Germany and Sweden extending across several generations over three decades, we shall discuss these studies in detail and draw conclusions regarding the application of colchiploids in forestry. Detailed research on the colchiploids was carried out by ILLIES (1951, 1957, 1966a, 1966b, 1969) in *Picea* and *Larix* over several generations at the Institute of Forest Genetics and Forest Tree Breeding, Grosshansdorf, Germany. She produced colchiploids (Co generation) in *Picea abies* in 1949 and examined somatic chromosome numbers in both root tips and bud meristems of two years old colchiploids. These colchiploids were mixoploids, ranging in chromosome numbers from 2n = 24 to 2n = 48) (ILLIES, 1951). In *Larix deciduas* (2n = 24), and *Larix leptolepis* (2n = 24) Illies, not only produced colchiploids (Co) generation in 1949 (ILLIES, 1951), but also crossed these (Co) with untreated diploids to produce C1 and C2 generations of auto- and allopolyploid (3n and 4n) and followed these polyploids over many years. Detailed cytological analyses of these colchiploids revealed that meiosis was irregular in the colchiploids, and most of them were mixoploids (chromosome numbers ranging from 24–48), and euploidy was rare in *Larix* colchiploid trees grown under field conditions (ILLIES, 1951, 1966a, 1966b, 1969). Since karyotypes of *Larix deciduas* and *L. leptolepis* differ from each other with respect to one chromosome (number 7) (SIMAK, 1962, 1964), it presented opportunities for distinguishing specific aneuploid genotypes. ILLIES (1969), speculated that some of the aneuploid variants in the Co, C1, and C2 generations, following chromosome rearrangements, could develop into stable genotypes similar to another member of Pinaceae, *Pseudotsuga menziesii* (2n = 26), and offer material for genetic improvement of forest trees.

Colchiploids were also produced in several conifer species and studied over a 30-year period in Sweden (JOHNSSON, 1975). These included *Pinus sylvestris*, *Pinus contorta*, *Picea abies*, and *Larix siberica*, all with 2n = 24. During the course of development, normal branches were removed so that all trees remained Co type. Chromosome determinations revealed that the colchiploids in these genera remained tetraploid after 30 years in Sweden. Flowering occurred after 30 years in these colchiploids, and the pollen was abnormal in these trees. As compared to Swedish colchiploids flowering occurred at earlier ages in colchiploids produced by others after: 7 years in Co *Pinus densiflora* in Japan (ZINNAI, 1953), 7 years Co *Larix decidua* and *Larix leptolepis* in Germany (ILLIES, 1957), perhaps due to environmental differences in respective countries. The Co *Pinus sylvestris*, *Pinus contorta*, *Picea abies*, and *Larix siberica* were crossed with their respective diploid parents to produce the C1 generation. The seed set was very poor

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in C1 generation of *Pinus sylvestris* and *Pinus contorta*, as 94–97% of the seeds were empty. The results were different in each C1. For example, in C1 *Pinus sylvestris*, about 80% seedlings were diploid (2n = 24), while the rest were mixoploids, while in C1 *Pinus contorta*, about 35% seedlings were diploid (2n = 24), 43% triploid (2n = 36), and the rest mixoploids. The C1 in *Picea abies* and *Larix siberica* there was practically no germination of C1 seed (JOHNSSON, 1975).

Ancient polyploidy?

Genomic studies have opened a new perspective on the question of neopolyploidy and paleopolyploidy and suggested that a number of animals and plants are paleopolyploids, that is, are ancient polyploids (WOLFE, 2001; RAMSEY and SCHEMSKE, 2002; BLANC and WOLFE, 2004). These paleopolyploids include humans (GIBSON and SPRING, 2000; MCLYSAGHT *et al*., 2002), fishes (VAN DE PEER, 2003), maize (GAUT and DOEBLEY, 1997; GAUT et al., 2000), *Arabidopsis* (**The Arabidopsis Genome Initiative**, 2000; KU *et al*., 2000), yeast (WOLFE, 2001), and tomato, cotton, soybean, (BLANC and WOLFE, 2004), that later became diploids by sequence divergence between duplicated chromosomes (WOLFE, 2001). These species have probably undergone ancient rounds of chromosome doubling followed by sequence divergence between duplicated chromosomes and deletions leading to gene loss. Further reduction or increase in genome size in these organisms may have occurred during the course of evolution by the interplay between the non-coding repetitive DNA and coding sequences. However, there are questions regarding the role of polyploidy and re-establishment of diploidy in eukaryotes: whether one or two rounds of polyploidization, or large scale segmental duplication of chromosomes, or both, played an important role in the evolutions of plant and animal species (WOLFE, 2001; SANKOFF, 2001; SEIOGHES, 2003). Recent genomic studies have provided an incentive to reexamine and redefine polyploidy in plants and animals.

Since polyploidy is absent in most extant pines in nature, is it possible that ancient polyploidy has played a role in the evolution of pines and other gymnosperms? In view of high incidence of polyploidy in angiosperms, it has been suggested that many if not all plant species have had at least one polyploid ancestor at some point during their evolution (WENDEL, 2000; BLANC and WOLFE, 2004). Are pines and other conifers exception to this rule? Or there are other mechanisms that could account for the evolution of conifers.

The origin of the genus *Pinus* is thought to be in early to middle Mesozoic (MILLAR, 1998). Fossil record suggests that ancient species of *Pseudoaraucaria* and *Pityostrobus* are closely related to pines, and may have contributed to the ancestral gene pool of pines (MILLAR, 1998). Although the genome size in prehistoric *Pseudoaraucaria* is not known, the extant *Araucaria* is ~10,000 Mb, nearly half the genome size in pines (average genome size 20,000 Mb). Is it possible that the pines are ancient polyploids derived by either: 1) hybridization between some ancient species of *Pseudoaraucaria*, *Pityostrobus*, or another ancient conifer, followed by one round (1R) of polyploidization and subsequent diploidization (*Figure 2*), or 2) one round of autopolyploidization (1R) in a putative pine ancestor, followed by diploidization, (*Figure 3*) or 3) large segmental duplications in a putative pine ancestor (*Figure 3*), leading to enlargement of genome size, followed by sequence divergence? Of the three different diagrammatic scenarios presented, two (*Figure 2*, *3 AA*) are based on the assumption that ancient polyploidy may have played a role in the pine evolution. While the third scenario does not involve polyploidy *per se*, but instead invokes large-scale segmental duplications (both gene and chromosomal segments) for the evolution of the pine genome (*Figure 3 BB*). However, at this stage it is difficult to predict which of the three scenarios presented in these models, or for that matter the presumed genome sizes in the pine ancestors, will be supported by the genomic data.

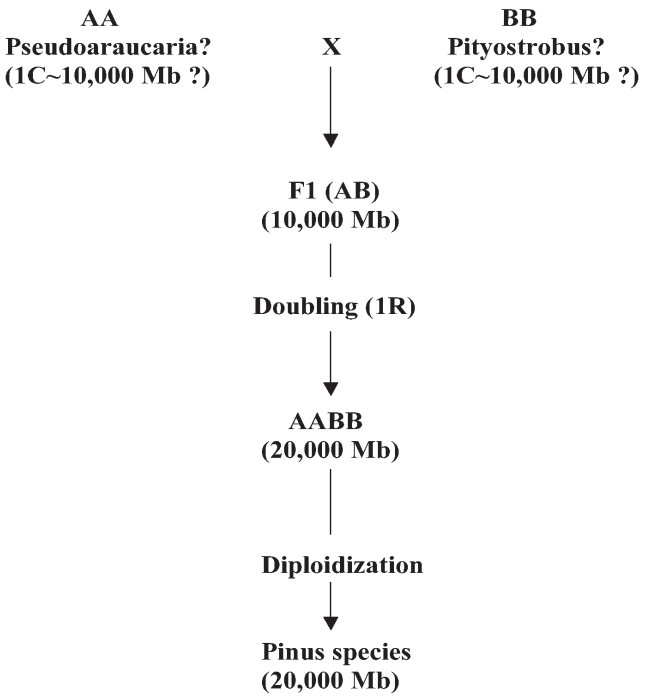

Figure 2. – Evolution of pines from their putative ancestors, possibly involving prehistoric genera *Pseudoaraucaria* and *Pityostrobus* with a 1C genome size of ~10,000Mb. Following hybridization and one round of polyploidization (1R) the present day pines with a 1C genome size (20,000 Mb) presumably evolved ~200 million years ago (early to middle Mesozoic) by divergence of genomic sequences, gene silencing, loss, and mutational events leading to the present day diploid-like state. In that sense, the extant pines may be ancient polyploids or paleopolyploids. Even if the genome sizes of ancestral species were larger or smaller than 10,000 Mb, deletions or accumulation of repetitive DNA sequences, for example, retrotransposons, could have still resulted in the current genome size in pines.

The current consensus map of loblolly pine, *Pinus taeda*, has not provided convincing evidence for the presence of duplicated syntenic regions (SEWELL *et al*., 1999) to support ancient polyploidy in pines. As it turns out, paleopolyploidy is rather difficult to detect because: 1) time erases the traces of duplication, 2) majority (70–90%) of duplicated genes formed during the millions of years of polyploid evolution may return to single copy state, thus reestablishing disomic segregation, for

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example, as in *Arabidopsis* and yeast, and 3) chromosomal rearrangements relocate duplicate segments around the genome, which further scramble the intragenomic synteny (OTTO and WHITTON, 2000; BLANC and WOLFE, 2004). Nevertheless, genome sequencing of pines, and chromosome painting (KATO *et al*., 2004), involving fluorescence in situ hybridization (FISH and GISH) (SCHWARZACHER, 2003) would be necessary to resolve the issue of paleopolyploidy in pines and other conifers.

Rarity and Future of Polyploidy in Gymnosperms

Why is polyploidy rare in conifers is a fascinating question? Is it possible that after the advent of ancient polyploidy, if it occurred, in the evolution of gymnosperms in particular conifers, there was no further need for the presence of polyploidy in this group of stable diploids that have reached a genetic equilibrium? A number of hypotheses have been proposed during the last century to explain the infrequency of polyploidy in gymnosperms (see KHOSHOO, 1959, 1961). These include: 1) higher frequency of interstitial chiasmata at meiosis, particularly in autopolyploids (SAX and SAX, 1933; ANDERSSON, 1947), 2) absence of double fertilization in conifers (MÜNTZING, 1933) (but not in all gymnosperms, see KHOSHOO, 1959), 3) nuclear-cytoplasmic ratio (DARLINGTON, 1947), and 4) ecospecific differentiation of conifer species (KHOSHOO, 1959, 1961).

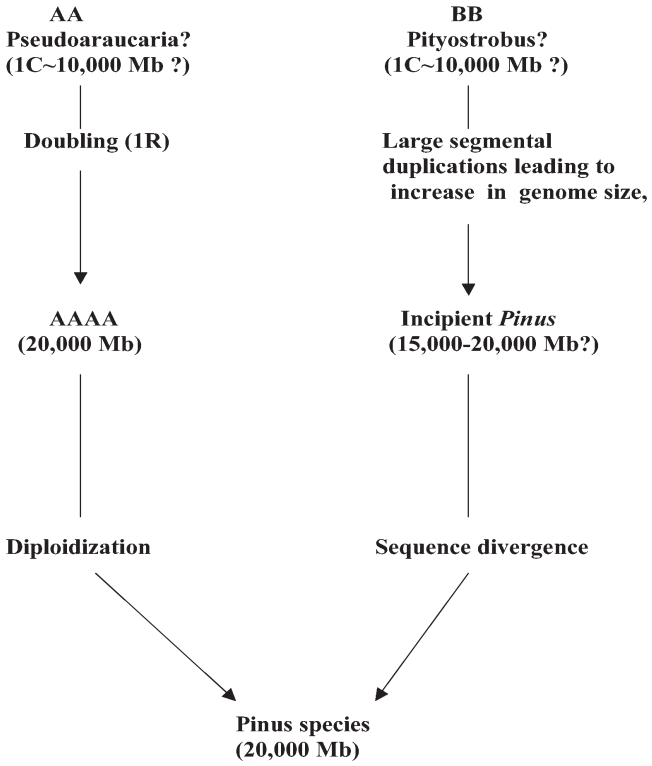

Figure 3. – Evolution of pines from their putative ancestors, possibly involving prehistoric genera *Pseudoaraucaria* and/or *Pityostrobus* with a 1C genome size of ~10,000Mb. Pathway one (AA) involves one round of autopolyploidization (1R). followed by diploidization. Pathway two (BB) involves large segmental duplications. The present day pines with a 1C genome size (20,000 Mb) presumably evolved ~200 million years ago (early to middle Mesozoic) by divergence of genomic sequences, gene silencing, loss, and mutational events leading to the present day diploid-like state. According to pathway one (AA), pines may be paleopolyploids. However, pathway two (BB) does not involve polyploidization *per se* for the origin of pines.

One major factor limiting polyploidy may be the number of large chromosomes and cell size (nuclear-cytoplasm ratio) as in plants like *Lilium* and *Fritillaria* (Liliaceae), which have already reached a "perfect equilibrium" (DARLINGTON, 1937) and lack polyploidy. Darlington extrapolated that conifers may have reached the 'upper limit" in this nucleo-cytoplasmic relationship and any change in this relationship by polyploidy would be deleterious. The question remains, how conifers have reached this limit during their evolutionary process, whether via ancient polyploidy or large scale chromosomal segmental duplications, in concert with the accumulation of retrotransposons?

Another viewpoint on the rarity of polyploidy has been put forth by KHOSHOO (1959, 1961). According to KHOSHOO's hypothesis, there is an ecospecific differentiation between all the compatible taxa of conifers, that is, pairing of chromosomes is normal to a great degree following hybridization between the species (DUFFIELD, 1952; CRITCHFIELD, 1975, 1988). Therefore, any polyploids resulting from hybrids between such genera would largely be either autopolyploids or segmental allopolyploids in nature. These types of polyploids would have irregular meiosis and consequently large sterility problems. In the absence of vegetative propagation, these polyploids would not be able to survive and compete in nature with other plant/tree species. The rarity of natural vegetative propagation in conifers may be a bottleneck to the success of auto- and segmental allopolyploidy in conifers (KHOSHOO, 1961). This is precisely the reason for the survival of hexaploid coast redwood, and seems to have occurred perhaps only once in the history of conifers. However, this explanation would not apply to the allopolyploids, of which there are a few cases in gymnosperms.

There are a number of factors that seem to promote the establishment of polyploidy in plants. Successful establishment seems to depend on selfing, asexuality, and perenniality (STEBBINS, 1971; RAMSEY and SCHEMSKE, 1998; OTTO and WHITTON, 2000). Highest percentages of polyploids are found within the surveyed genera in perennial herbs, and the lowest in annuals, and the woody genera were intermediate (STEBBINS, 1971). There is empirical data that suggests that selfing helps the establishment of polyploids and outcrossing makes it more difficult (RODRIGUEZ, 1996). In gymnosperms, which are mainly outcrossing, polyploidy is rare and only 5% of gymnosperms and 1.5% conifers are polyploids. On the other hand, angiosperms are more often self-fertilized and polyploidy is considerably higher (50–8%) (STEBBINS, 1971; RODRIGUEZ, 1996).

Since polyploidy is rare in gymnosperms, and induced polyploids in conifers exhibit growth abnormalities, and generally remain dwarf, most forest geneticists have expressed doubts regarding the future of polyploidy in conifer improvement, particularly in the commercially important family Pinaceae, (KHOSHOO, 1959, 1963; MEHRA, 1960; LIBBY *et al*., 1969; JOHNSSON, 1975). On the other hand, polyploidy occurs in some genera in the

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family Cupressaceae, and may offer future possibilities. In angiosperm trees artificially induced triploids have been produced in *Populus tremula*, and other *Populus* spp. from the section Aigeiros (JOHNSSON, 1945; ZHANG *et al*., 2004), *Alnus glutinosa* (JOHNSSON, 1950), and *Betula verrucosa* (JOHNSSON, 1956). Of these triploids, only triploids in *Populus* show better growth and development. Whether triploidy has any future in different genera, namely, *Cryptomeria*, *Pinus*, *Picea*, or *Larix*, of conifers remains to be fully investigated.

Nevertheless, there may be a silver lining in the induced polyploidy research in gymnosperms, particularly commercially important conifers. New genotypes or species, that are desirable in forestry, may arise, following chromosomal rearrangements, from the euploid, mixoploid or aneuploid genotypes common in the colchiploids (Co) and their hybrid derivatives (C1, C2 generations). Mixoploids and aneuploids are genetically unstable and provide a genetic substrate for the occurrence of chromosomal rearrangements. Douglas fir (*Pseudotsuga menziesii*, 2n = 26) seems to be one such example, which has probably originated from another member of Pinaceae (2n = 24) ancestor by centromeric fission of a small chromosome (GUSTAFSSON and MERGEN, 1964; THOMAS and CHING, 1968). Recent genomic mapping data indicates synteny between two linkage groups in *Pseudotsuga menziesii* with one of the linkage groups in *Pinus taeda*, suggesting two syntenic linkage pairs representing that two different chromosomes in *Pseudotsuga* are possibly derived from one of *Pinus* (KRUTOVSKY *et al*., 2004). *Pesudolarix amabilis* (2n = 44) is another conifer, which was at one time considered a polyploid, but based on its karyotype consisting of 20 pairs of acrocentric and two pairs of metacentric chromosomes it appears to have arisen from an ancestor with n = 12 by centric fissions of 10 chromosomes (KHOSHOO, 1959; MERGEN, 1961). That is not to say, that these new species originated as a consequence of polyploidy, but rather that induced polyploidy can serve as a resource for mutagenesis. Therefore, even if polyploidy is a roadblock in conifers, production of artificial polyploidy offers possibilities for isolating new genotypes. At the same tine, more genomic research is necessary to understand the limits of polyploid dynamics in gymnosperms, particularly conifers.

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Reproductive Success of Pollen Derived From Selected and Non-Selected Sources and its Impact on the Performance of Crops in a Nematode-Resistant Japanese Black Pine Seed Orchard

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Abstract

The reproductive success of pollen derived from selected and non-selected sources and its impact on the performance of orchard crops were evaluated, using five pairs of microsatellite markers, in a Japanese black pine (*Pinus thunbergii* Parl.) clonal seed orchard consisting of 16 nematode-resistant clones. The paternity of each open-pollinated seed was determined by comparing the genotypes of seeds from six clones (24 trees) with genotypes of the 16 orchard clones and two trees (N1, N2) representing other genotypes that had been inadvertently included in the orchard. Out of 384 seeds examined, the paternity of 316 seeds (82.3%) was assigned to the clones within the seed orchard. On average, the male reproductive success of orchard clones varied from 0.0% to 10.5%, and was significantly related to the male-flowering fecundity of each clone. It was not related to the synchrony of flowering phenology between mates. The expected proportions of seeds produced by clonal trees as a result of pollination by orchard clones, and by contaminating pollen originating from internal and external sources were estimated at 86.8%, 3.3% and 9.9%, respectively. Nematode-resistant seedlings of Japanese black pine were produced from surviving 2-yr seedlings that had previously been inoculated with pinewood nematode (*Bursaphelenchus xylophilus*). Without pollen contamination, the survival rate of seedlings produced by mating between resistant clones is expected to be 62.4%. However, in this orchard the figure was reduced to 57.5%, due to pollen contamination from both internal and external sources.

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