Stomatal Size in Fossil Plants: Evidence for Polyploidy in Majority of Angiosperms

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Three published estimates of the frequency of polyploidy in angiosperms (30 to 35 percent, 47 percent, and 70 to 80 percent) were tested by estimating the genome size of extinct woody angiosperms with the use of fossil guard cell size as a proxy for cellular DNA content. The inferred chromosome numbers of these extinct species suggest that seven to nine is the primitive haploid chromosome number of angiosperms and that most angiosperms (approximately 70 percent) have polyploidy in their history.

Polyploidy affects the basic biology and evolution of organisms because the addition of an extra set of chromosomes also increases cell size and the copy number of alleles (*1*). The ecological and evolutionary effects of these and associated changes have been widely debated (*2–5*), and even the frequency of polyploidy is controversial, with estimates for flowering plants ranging from 30 to 80% (*6–10*). Here I assess the frequency of polyploidy in angiosperms by inferring genome size in extinct species on the basis of cell sizes in fossil material (Fig. 1).

Stebbins (6) estimated that 30 to 35% of angiosperm species had a chromosome doubling in their history. He counted all of the chromosome numbers in a genus that are integer multiples of a lower chromosome number. This approach is conservative because even the lowest chromosome number in each genus could be a polyploid number if the ancestors of the genus were polyploid or if all the diploid members of the genus were extinct. Grant (*7, 8*) estimated that 47% of angiosperms are polyploid by arguing, first, that the peak in haploid chromosome number at n = 7 to 9 (Fig. 2) reflects the primitive state; and second, that for species with $n > 13$ there are 40% more even than odd chromosome numbers, which suggests that doubling of chromosomes is much more common than dysploidy (*11, 12*). Angiosperms with chromosome numbers in the n = 11 to 13 range were thus assumed to be ascending dysploids.

Stebbins (*2*) argued that most living angiosperms with $n > 11$ are polyploids with extinct diploid ancestors, so that the actual frequency of polyploidy in angiosperms is greater than 50%. Goldblatt (*9*) used cytological estimates of basic haploid numbers in angiosperm families (*13*) to suggest that all monocots with chromosome numbers greater than 10 (~70% of extant taxa) are polyploid.

A cladistic analysis of basal angiosperms found it equally parsimonious to assume n = 11 to 13 for the primitive haploid number (*14*) rather than the usual estimate of n = 7 to 9 (*15–18*). Another study suggested n = 11 to 13 for the primitive chromosome number of the angiosperms, based on the study's conclusions that paleoherbs are basal within angiosperms as a whole (*19*). If the basic chromosome number of angiosperms is n = 11 to 13, then only 30% of living angiosperms are polyploid.

In many angiosperm families, all three traditional methods (counting multiples of chromosomes, assuming polyploidy for $n > 13$, and assuming polyploidy for $n > 10$) converge on the same proposed estimates (Fig. 3, A and B). However, these methods give very different estimates for families with a series of critical chromosome numbers of n = 10 to 18. For example, the lowest known chromosome numbers in the living Platanaceae (n = 21), Magnoliaceae (n = 19, Fig. 3C), and Lauraceae (n = 12, Fig. 3D) have at least two possible evolutionary histories. First, these species could be diploids, which implies that most angiosperms acquired their present chromosome numbers through descending dysploidy. Alternatively, these species could be polyploids whose diploid ancestors are extinct.

Cell size is correlated with DNA content (*2, 4, 20*) and thus with chromosome number (*21*), so that the study of fossil guard cells (leaf epidermal cells that control the opening and closing of stomata) permits the estimation of ploidy levels (Fig. 4). Here, I use data from the fossil record of the angiosperm families Platanaceae, Lauraceae, and Magnoliaceae to determine the probable evolutionary history of the minimum chromosome numbers known in those families today.

To confirm the relation between cell size and ploidy levels in the study families, I measured guard cell sizes (*22*). Leaf samples came from different geographic areas, from

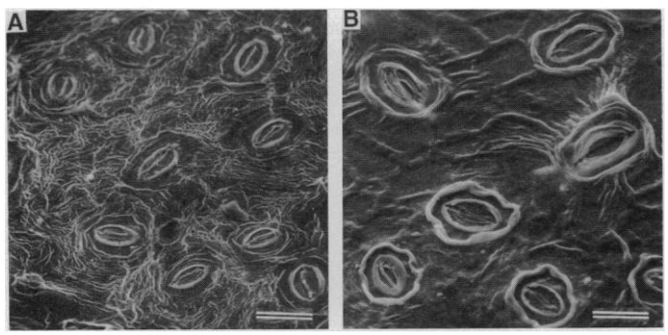

Fig. 1. Scanning electron micrographs for comparison of guard cells from (**A**) the Miocene Clarkia *Platanus* and (**B**) the Recent *Platanus acerifolia*. Scale bar: 20 μm.

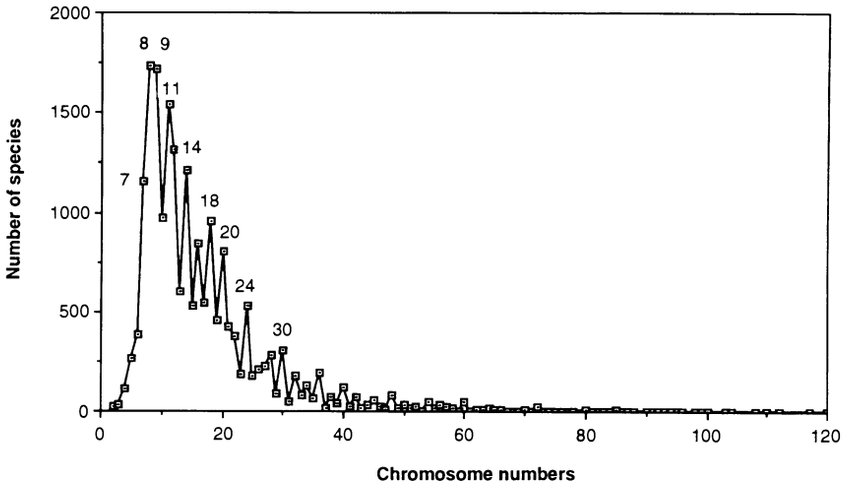

Fig. 2. Distribution of haploid chromosome numbers for 19,838 species (limited to $n < 120$), based on data compiled [J. Masterson, in (*10*)]. The pattern is comparable to that published by Latimer and Grant (*7, 8*) which was based on a sample of 17,138 species.

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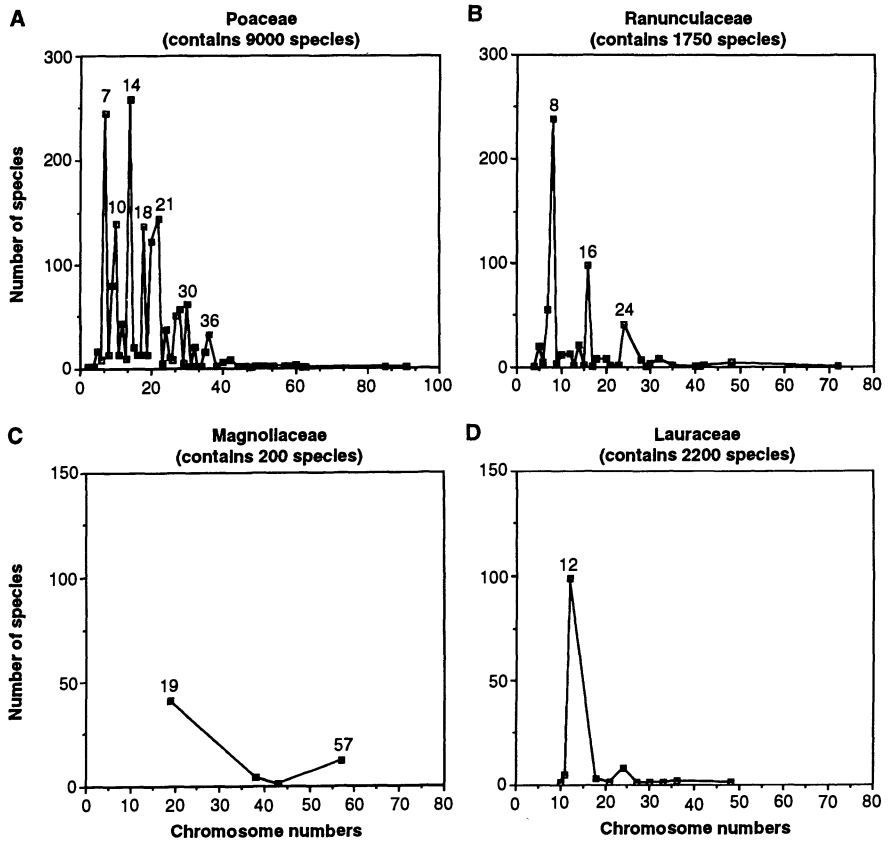
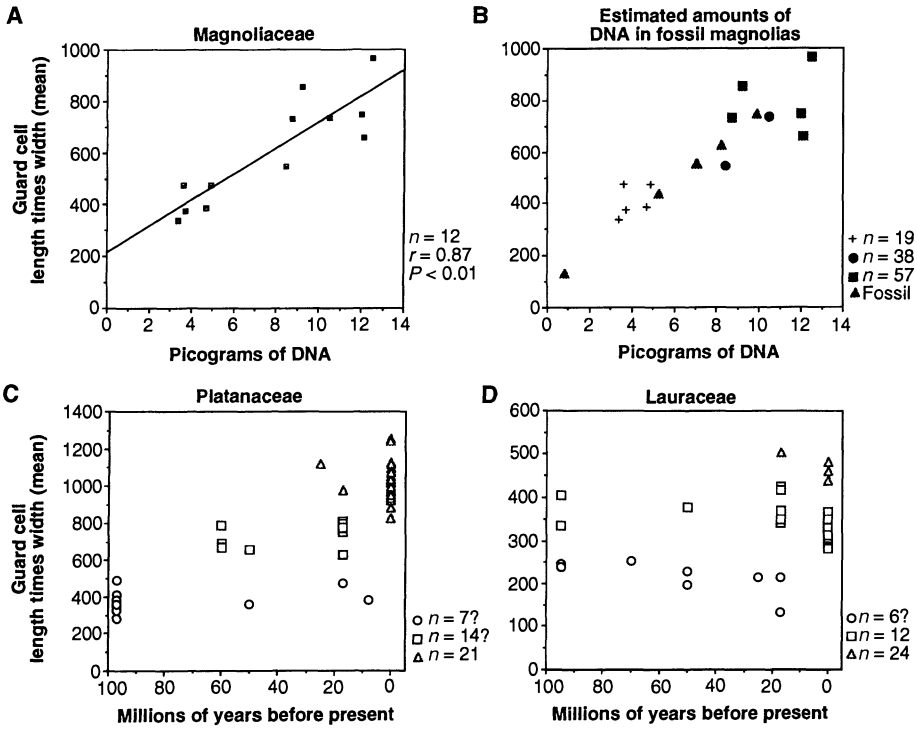


Fig. 3. Frequencies of chromosome numbers in two families in which similar estimates of percentage of polyploidy are given by all three methods [counting multiples of chromosomes (*6*), assuming polyploidy for $n > 13$ (*7, 8*), and assuming polyploidy for $n > 10$ (*9, 10*). (**A**) Poaceae (1674 samples tested, 235 genera sampled out of 635): Estimates are 74, 65, and 69%, respectively. (**B**) Ranunculaceae (568 samples, 39 genera sampled out of 58): Estimates are 42, 37, and 40%. (**C** and **D**) Two families in which the cutoff numbers give different estimates: Magnoliaceae (C) (58 samples, 7 genera sampled out of 7): Estimates are 61, 100, and 100%, and Lauraceae (D) (118 samples, 21 genera sampled out of 45): Estimates are 11, 15, and 99%.



leaves grown in sun and shade, from spring and fall growth, from freshly picked leaves, and from leaves that had been in an herbarium for 100 years (*23*). Despite the ecological and preservational differences, conspecifics with the same chromosome number almost always grouped together, as was the case in other studies that found only slight variations in guard cell size as a result of ecological conditions (*24*). This variation is not large enough to obscure the relation between cell size and amount of DNA, which was further confirmed by measurements of cellular DNA in the same taxa (Fig. 4A) (*25*). Fossil guard cell sizes, then, can be used as proxies for cellular DNA content in these families in order to track temporal changes in genome size (*26, 27*).

The presence of fossil guard cells that are smaller than those seen in living angiosperms with low chromosome numbers supports the higher estimates of the frequency of polyploidy in angiosperms. If no extinct species of Platanaceae or Magnoliaceae had guard cells smaller than those of living species, this suggests either that the diploid chromosome numbers in these families are $n = 19$ and $n = 21$, which supports the 30 to 35% estimate of the frequency of angiosperm polyploidy, or that the ancestors of these families were polyploid themselves. If the ancestors were polyploid or if extinct species exhibit smaller guard cells than living species, then species with $n = 19$ to 21 must be polyploid, which supports the higher (47 to 80%) estimates of the frequency of polyploidy in angiosperms. Similarly, if no extinct species of Lauraceae with guard cells smaller than those of living species exist, then $n = 12$ could be a diploid chromosome number, which supports the 47% estimate. Conversely, if the ancestor of Lauraceae were polyploid or if the extinct species have smaller guard cells than living species, this would support the 70 to 80% estimate.

All three families have fossil species with guard cells that are smaller than those

Fig. 4. (**A**) A simple linear regression of guard cell size (length times width, each measured in micrometers) against picograms of DNA, which shows the relation between guard cell size and chromosome number. (**B** through **D**) Graphs showing study families with fossil and living members. Putative diploid taxa include (*26*) (B) *Magnolia* (PP44203) (Miocene); (C) platanoid 1 (G 191), *Sapindopsis* (G129-3, G115, G113, G123-3, G127-2, G84), *Platanus* (K) (Cretaceous), *Platanus* (UCMP) (Eocene), and *Platanus* (PP44204 and UCMP204) (Miocene); and (D) *Cocculophyllum* (K) and *Dombeyopsis* (YPM8198 and YPM8201) (Cretaceous). two laurels (genus uncertain) (D) (Eocene) and *Daphnogene* and laurel (genus uncertain) (PP44205) (Miocene). Each point represents 40 guard cell measurements.

of living species. This suggests that Platanaceae, Magnoliaceae, and Lauraceae all had mid-Cretaceous and early Cenozoic members with ploidy levels lower than those of any extant species (Fig. 4, B through D) (*28*). The inferred chromosome numbers of these extinct species support the hypothesis that 70% of all angiosperms are polyploid and suggest that $n = 7$ to 9 is the primitive chromosome number of angiosperms. Duplicated isozymes (16 to 21%) in species of Lauraceae ($n = 12$) and Calycanthaceae ($n = 11$) exceed frequencies expected for diploid species (*29*) and thus also support the claim that species with chromosome numbers in this range are polyploid (*30*). These data support Stebbins's paleopolyploid hypothesis (*18*), which suggests that many of the woody dicots (chromosome numbers $n \geq 12$) are polyploids. The data contradict Grant's aneuploid-polyploid hypothesis (*8*), which states that most $n = 12$ to 13 species are products of dysploidy.

Further work is needed to test the generality of these results. An absolute threshold between diploid and polyploid numbers cannot be drawn—chromosome numbers increase by a variety of complex processes, so that exceptions to any cutoff value will always occur (*12, 31*). Some isozyme studies have failed to find the expected duplications in plant species with chromosome numbers above $n = 10$, but this work must be reexamined to test for the possibility of diploidization (gene silencing, which results in diploid expression of a polyploid genome) (*32*). Further molecular analyses that search for nonfunctional copies of single-copy genes are needed to determine whether these groups really are exceptions to the $n = 10$ cutoff number (*33*). Finally, when a consensus is reached on a taxonomically restricted basal group or groups of angiosperms, data on the DNA amounts, isozyme numbers, and single-copy genes of a number of species in these taxa would be valuable (*34*).

Fossil cell size is a reliable source of data on the genome size of extinct plants. It can also be used to test hypotheses about the historical timing of changes in ploidy level (*35*) and about how genome size has affected the diversification and divergence of angiosperms.

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23. Herbarium samples came from the Field Museum (Chicago, IL), Missouri Botanical Garden, and Morton Arboretum (Lisle, IL). Fresh leaves came from the Missouri Botanical Garden, Chicago Botanical Garden, Morton Arboretum, Denver Botanical Garden, Strybing Arboretum (San Francisco, CA), University of California (Riverside and Berkeley), Huntington Botanical Gardens (San Marino, CA), and the Wilbur D. Mays Arboretum and Botanical Gardens (Reno, NV).
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27. Fossil cuticle was separated from matrix with HF, then oxidized using bleach, hydrogen peroxide, or CrO_3.
28. Several lines of evidence indicate that the fossilization process is not a seriously confounding factor in this study. First, a single locality often has two confamilial species with different guard cell sizes. Second, some Miocene [20 million years ago (Ma)] and Eocene (50 Ma) species plot together in all three families, which suggests that guard cell sizes are not simply a function of geologic age. Finally, some specimens of *Platanus neptuni* (Oligocene and Miocene) plot with the Recent species of Platanaceae, whereas others plot with the Eocene-Miocene species. Unless this species has a chromosome number higher than that of the extant members of the family, shrinkage due to fossilization is probably not a problem in this group.
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Differential Complementation of Bcr-Abl Point Mutants with c-Myc

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A complementation strategy was developed to define the signaling pathways activated by the Bcr-Abl tyrosine kinase. Transformation inactive point mutants of Bcr-Abl were tested for complementation with c-Myc. Single point mutations in the Src–homology 2 (SH2) domain, the major tyrosine autophosphorylation site of the kinase domain, and the Grb-2 binding site in the Bcr region impaired the transformation of fibroblasts by Bcr-Abl. Hyperexpression of c-Myc efficiently restored transformation activity only to the Bcr-Abl SH2 mutant. These data support a model in which Bcr-Abl activates at least two independent pathways for transformation. This strategy may be useful for discerning signaling pathways activated by other oncogenes.

Cancer is a progressive disease caused by the accumulation of mutations, some of which result in abnormal signal transduction events (*1*). Because oncogenes are mutated forms of normal cellular counterparts, it is conceivable that oncogenesis results from the amplification or alteration of a normal signal, the generation of multiple signals, or a combination of these mechanisms. In this report, we examine signaling in mammalian cells by Bcr-Abl.

The *bcr-abl* chimeric oncogene is generated from a reciprocal translocation between chromosomes 9 and 22, which results in the fusion of *bcr* sequences upstream of the second exon of *c-abl*. Two Bcr-Abl proteins can be generated, P185 and P210, which are most commonly associated with acute lymphocytic and chronic myelogenous leukemias, respectively (*2*).

We developed a strategy to define genetically the minimum number of signals needed for oncogenesis. Point mutations were generated in domains of Bcr-Abl that are expected to participate in connecting its tyrosine kinase signal to downstream effector molecules. If the mutation impaired the transforming activity of Bcr-Abl by uncoupling a critical signaling pathway, then the overexpression of a downstream molecule would be expected to restore transforming activity. If all mutants were rescued by the downstream gene, then the same signaling pathway would likely have been inactivated by the different mutations. Complementation of one point mutant, but not another, would signify that each mutation blocks a different pathway.

Point mutations in the major tyrosine autophosphorylation site of the kinase domain (*3*) and in the tyrosine phosphorylation site at position 177 in the Bcr region (*4*) impair transformation activity of Bcr-Abl. A mutation at position 177 in P185 prevents association with Grb-2 (*4*), an adapter molecule involved in Ras activation (*5*).

We created a point mutation in the phosphotyrosine binding site of the Src–homology 2 (SH2) region of Bcr-Abl. The most highly conserved sequence in the SH2 domain is the FLVRES motif (*6*), where the Arg residue makes direct contact with the phosphate group of a phosphotyrosine moiety (*7*). Mutations in the FLVRES motif of transforming mutants of mouse type IV c-Abl and Src decrease their transformation potential in mammalian cells (*8*). In the FLVRES motif of P185 Bcr-Abl, Arg^{552} was substituted with Leu to create P185 R552L (*9*).

Wild-type (WT) P185, P185 R552L (FLVRES mutant), P185 Y813F (autophosphorylation mutant), and P185 Y177F (Grb-2 binding mutant) were transfected into 293T cells and analyzed by immune-complex kinase assays to assess autophosphorylation activity (Fig. 1). Although the mutation at Tyr^{177} had no effect on in vitro autophosphorylation, the FLVRES mutant and the autophosphorylation mutant had activities one-third and one-half that of WT P185, respectively.

Cells of the rat-1 line expressing transforming forms of the Abl kinase exhibit tyrosine phosphorylation of a protein doublet in the 62- to 65-kD range (*10*). To assess intracellular tyrosine phosphorylation, WT P185 and point mutants were introduced into rat-1 cells by retroviral infection (*10*). The expression of Bcr-Abl in cells was determined 48 hours after infection by protein immunoblotting. Viral titers for different Bcr-Abl variants were equivalent. No change in protein stability was detected in the point mutants as compared to WT P185. Although mutant and WT P185 proteins were expressed in approximately equal amounts, immunoblot analysis with antibodies to phosphotyrosine revealed that cells expressing the FLVRES mutant had severely reduced amounts of intracellular phosphotyrosine (Fig. 1). Although mutant P185 proteins exhibited ty-

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Table 1. Differential rescue of Bcr-Abl point mutants with Myc (*22*).

Retrovirus	Neo colonies			Myc colonies		
	Number	Size	Acidification	Number	Size	Acidification
Neo	0		–	2	S	–
WT P185	92	L	+	135	L	+
P185 R552L*	8	S	–	93	L	+
P185 Y813F†	3	S	–	12	S	–
P185 L552-F813‡	0		–	1	S	–
P185 Y177F§	5	S	–	7	S	–

*FLVRES mutant. †Autophosphorylation mutant. ‡Double mutant. §Grb-2 binding mutant.