PaleoBios 29(3):60–75, June 1, 2010

Estimating polyploidy levels in fossil *Salix*: A critical review of cell size proxy methods

WALTER K. BUECHLER
1192 E. Braemere Rd., Boise, ID 83702; buecdoug@msn.com

Studies have used cell size as a proxy to estimate polyploidy levels, the number of chromosome sets in somatic cells, in modern and fossil plant species. This paper critically evaluates these methods by reviewing cell size- and polyploidy-related literature, and provides new cell size data from herbarium material and fossil remains of willow genus *Salix*. The 40 extant taxa used in the study include most of the polyploidy levels encountered in *Salix* (2n = 38, 76, 114, 152, 190). Diploid and tetraploid species were morphologically similar to the fossil specimens. Specimens from alpine and arctic regions, forms rarely found in the fossil record, were included to extend the range of polyploidy levels. Measurements taken for this study were on the petiole epidermal cells of fossil and herbarium specimens and the stomatal guard cell complexes of herbarium material. A literature review reveals cell size may not relate only to DNA content, but to a plant's age, nutritional state and the seasonal timing of organ development. Cell size measurements show that cultivated plants grown at elevations more than 800 m below their original place of growth have a significant increase in cell size. Leaf length-to-width ratio, infrageneric classification, and adaptation to dry or humid environments also correlate with cell size. Cell size proxies for estimating polyploidy levels in fossil willows provide only accurate results if morphologically similar modern plant material from natural habitats is used as a reference for comparison. Leaves should be similar in overall shape, base and apex shape, blade length and width, length-to-width ratio, petiole length, petiole-to-blade length ratio, venation pattern, and margin dentition.

INTRODUCTION

The extant genus *Salix* Linnaeus 1753 comprises one of the most diverse groups of woody plants. They range in habit from large trees, shrubs of all sizes, or may be trailing or mat forming. Leaf sizes range from leaf areas of only a few mm^2 to areas of more than 100 cm^2. Salix is also one of the few examples of a woody genus with a large number of polyploid species (Wilkinson 1944), with ploidy levels from diploid (2n = 2x = 38) to dodecaploid (2n = 12x = 228) (Argus, in press). To date, little is known about when changes in ploidy levels occurred in the course of the genus' evolutionary history. There are three types of events or mechanisms of change that are of interest: (1) the likely single transition from a hypothetical base number x = 9 or 10 to the modern base number x = 19, (2) multiple changes in polyploidy levels based on x = 19 throughout the evolutionary history of the genus, and (3) potential changes in DNA content through gene duplication and deletion without changes in chromosome numbers. Molecular sequence data may in the future answer some of these questions (e.g. see volume edited by Leitch et al. 2004). Cell size proxy methods, data on geographic distribution of fossil and modern species (Skvortsov 1999), and multivalent frequency during meiosis (Buechler, unpublished data) may then be used to calibrate the molecular clock and verify the molecular sequencing results.

Methods using cell size as proxies for estimating ploidy levels assume that the size of epidermal and stomatal cells (defined as overall length × width) strongly correlates with DNA content and that plants of the same species with closely matching cell sizes have the same polyploidy level (Stebbins 1950). In studies of fossil leaves, the cell sizes of morphologically similar extant species with known chromosome numbers are used for comparison. As examples, Miki and Hikita (1951) determined stomatal and epidermal cell sizes in both fossil and living *Sequoia* and *Metasequoia* species. They concluded that polyploidy levels (hexaploid and diploid, respectively) did not change between the Pliocene and present. Roth and Dilcher (1979) found a polyploid population within the Eocene species *Paleorubiaceophyllum eocenicum* (Rubiaceae) by comparing epidermal cell size. Masterson (1994) used stomatal guard cell size as an indicator for polyploidy levels in fossil Platanaceae, Lauraceae, and Magnoliaceae. These three families had mid-Cretaceous and early Cenozoic members with polyploidy levels lower than those of any extant species in these families. During my work on the Late Miocene Pickett Creek flora, I determined petiole epidermal cell size for 18 fossil *Salix* leaves from three Idaho Miocene floras. *Salix succorensis* Chaney and Axelrod (1959), *S. inquirenda* Knowlton (1926), *S. wildcatensis* Axelrod (1944), *S. desatoyana* Axelrod (1985), and two undetermined specimens all had small cell sizes and were therefore assumed to be diploids (Buechler 2000). The result for *S. wildcatensis* is of particular interest because its closest modern equivalent, *S. lasiolepis* Bentham (1857), is tetraploid.

The first use of cell size as a proxy for cellular DNA content and chromosome numbers was to test the results of artificially induced polyploidization in horticultural plants and food crops (Schwanitz 1952, 1953). The assumption was that for the cell to function correctly, a specific ratio between the volume of a cell's nucleus and its cytoplasm is required (Schwanitz 1952). In these applications, cuttings or seeds from the original diploid plant and those from the derived polyploid are grown under similar environmental and nutritional conditions, with cell size assumed to depend predominantly on DNA content. This assumption is, however, not necessarily valid when comparing material from plants of different genetic decent, from different species, or different environments. Recent work on extant *S. rotundifolia* Trautvetter (1832), an arctic dwarf willow with a diploid and a hexaploid variety (var. *dodgeana* (Rydberg) E. Murray 1983, and var. *rotundifolia* respectively), revealed that the polyploidy level of some populations or plants could not unambiguously be determined on the basis of stomatal cell sizes (Buechler, unpublished data). This finding suggests that cell size in *Salix* and perhaps in other genera may depend more strongly on other parameters than DNA content than previously thought.

The purpose of the present study is to draw attention to possible limitations of cell size related proxy methods and to present new data on petiole epidermal and stomatal cell size in *Salix.* Petiole epidermal tissue was chosen because it is more likely to be preserved in fossil *Salix* remains than epidermal cells or stomatal guard cells of the leaf blade. The study follows two routes: (1) a critical review of cell size- and polyploidy-related literature and (2) cell size measurements on *Salix* herbarium material of known polyploidy levels and on fossil remains of the same genus. As the two approaches are different, the literature review is aiming at a wider range of genera and of cell size influencing parameters than the cell size measurements, the two avenues are presented separately in this publication.

LITERATURE REVIEWS

The age of a plant, the position of an organ on the plant, and the seasonal timing of organ development may have an influence on cell size. Schwanitz (1952) published a critical review of cell size methods for use in plant breeding research. He found that stomatal cell size in young plants of *Sambucus nigra* L. averaged 145 µm in length, while in old plants the average length was only 122 µm. The corresponding ratio of 1.68:1 in cell volume between the two plants is close to the ratio of 2:1, a ratio one would expect after a doubling of the chromosome number. In *Symphoricarpos racemosus* Michx., guard cells of leaves on a one year old non-fruiting shoot were on average 103 µm long, whereas those on a two year old fruiting shoot of the same plant measured only 79 µm (corresponding to a volume ratio of 2.21). In *Verbascum thapsiforme* Schrad. pollen diameter changed from 24.7 µm at the beginning of the blooming period to 18.5 µm near the end (volume ratio 2.38:1).

Plant nutrition can influence the amount of nuclear protein, chromatin structure, chromosome volume, and potentially cell size. In root tip cells of *Viola conspersa* Reichenb., the chromosome volume (chromatin content of cells) increased from 1.92 µm^3 when plants were watered without fertilizer, to 4.75 µm^3 when watered with a "normal" phosphoric acid concentration, and to 7.39 µm^3 under an "over dosage" regime (Pierce 1937). Although no measurements of cell size were made, the author stated that cells grown without fertilizer "appeared noticeably smaller" than those grown with excessive phosphorus. Bennett and Rees (1969) acknowledged the importance of Pierce's findings and reported similar data for root tip meristems of *Secale cereale* L. and *Allium cepa* L. They found that the amount of DNA had not changed with the amount of fertilizer and that the increase in chromosome volume was due to an increase in chromatin protein content. Baluška and Kubica (1992) investigated the relationship between the amounts of nuclear proteins and DNA, chromatin structure, and cell size in tissues of the maize root apex. Average cell size correlated not only with the amount of nuclear DNA, but also with nuclear chromatin structure and basic nuclear proteins. While none of the above publications established a quantitative causal connection between plant nutrition and cell size, the combination of their findings suggests that such a relationship may exist. Therefore, it seems prudent to use only cell size data from naturally grown plants as reference for polyploidy estimates.

Schwanitz and Pirson (1955) compared chromosome volumes and cell sizes of cultivated diploid gigas plants (plant varieties with enlarged leaves or other organs) with those of closely related natural species of the same polyploidy level. In three out of 13 groups (*Allium*, *Vicia* and *Lycopersicum* sp.) chromosome volumes and cell sizes of gigas plants were significantly larger than in comparable natural species. In all the other groups no or insignificant increases in chromosome volume were observed, and the increase in leaf size was partially or entirely due to increases in cell number per leaf. The authors suggested changes in chromosome structure and polyteny (DNA replication without cell division) as possible reasons for the increases in chromosome volume and cell size. Cell size data from plants with unusually large leaves or other enlarged organs should not be used as reference for polyploidy estimates even though they fit the description of a species.

Improved techniques for water status measurements in living plant cells have led to a better understanding of water uptake mechanisms and plant responses to changes in environmental parameters. Studies show that cell elongation and therefore final cell size in higher plants can be limited by water deficiency, nutrient deficiency, and cold and heat stress (Nonami 1998). Turgor pressure (pressure of the cell content against the cell wall) is sustained in expanding cells by water taken up by the cells. Consequently, if there is insufficient water available to the plant, cell elongation is slowed down or stopped. Water potential and its response to environmental parameters play a central role in plant development and may explain some of the findings listed above.

Xiao (2001) investigated the influence of water availability on stomatal cell size in *Salix psammophyla* C. Wang & Chang Y. Yang in the semi-arid Maowusu Sandlands of Inner Mongolia. At an average yearly precipitation of 315 mm, the abaxial stomatal cell size (L × W) was 160 µm^2 and the adaxial cell size 498 μm². When precipitation was artificially lowered to 50%, cell size was reduced by 26% and 31% respectively. A doubling of the yearly precipitation resulted in an increase of cell size by 95% and 30% respectively. Despite the naturally occurring variation of 250–490 mm per year (Jiang and He 1999) being about half that of the variation during the experiment, the resulting change in cell size would still be large enough to obscure the estimation of polyploidy levels. However, this effect of variation in water availability on cell size and thus on ploidy estimates can be reduced by using average values of larger numbers of specimens from different deposition layers (fossil remains) and from different years (herbarium material) for comparison.

DNA content of meristematic cells, and therefore cell size, may vary greatly within some genera. Bennet and Leitch (2005), in the Kew Gardens C-values database, list DNA amounts determined by Feulgen microdensitometry or flow cytometry from over 4,400 angiosperm species, representing about 1.7% of the global angiosperm flora. While these data may not be representative for all taxonomic groups, the following examples show that variation within a genus may be substantial. The Kew database lists 34 species of *Rosa*, out of more than 100 worldwide, showing DNA content (2C values) for diploids (18 species) ranging from 0.80 to 1.20 pg (± 20%), for tetraploids (10 species) from 1.85 to 2.30 pg (± 11%), and for hexaploids (2 species) from 1.30 to 2.90 pg (± 38%). The nine *Quercus* species (all diploid, out of about 450 worldwide) have DNA content values from 1.00 to 2.00 pg (± 33%), while the two *Betula* species (both diploid, out of about 60 worldwide) show one with an amount of 0.40 pg , the other with a much larger value of 1.50 pg. These examples demonstrate how important it is to use only data from the most closely related species as a reference.

Studies involving cell size and polyploidy often cite Stebbins (1950, pp. 302–303) as a reference for the validity of cell size proxy methods. Stebbins' comments, however, are very cautious about the way in which cell size can be used for estimating polyploidy levels. They are worth being included here in their original wording: "An increase in size of the individual cells is perhaps the most widespread effect of polyploidy. It often makes possible the use of measurements of certain cells of the plant, particularly the guard cells of the stomata and the mature pollen grains, to suggest the diploid or the polyploidy condition of plants represented only by herbarium specimens .... These measurements cannot be used indiscriminately and are valueless unless the sizes of stomata and pollen grains are known in individuals of the same or closely related species in which the chromosomes have actually been counted. Nevertheless, if these basic facts are known, and if the chromosomes of all of the diploid and the polyploidy species of a group are of about the same size, then measurements of the size of stomata and pollen grains can often provide valuable supplementary evidence on the relative geographic distribution of closely related diploids and polyploids ...." Stebbins emphasizes that polyploidy increases cell size primarily in meristematic tissue, and that the eventual size of organs depends as much on the amount of cell elongation as it does on the initial size of the cell. It is also important to note that Stebbins' statements concern only extant species and do not account for possible changes in DNA content due to gene duplication or deletion during genome evolution.

MATERIALS AND METHODS

The leaves of the extant *Salix* species used in this study were from herbarium specimens. Some of the chromosome numbers for the 87 leaf specimens used were determined either by the author (Büchler 1985, 1986, Buechler 2001, and unpublished data) or came from publications of other authors (see Table 1). The diploid and tetraploid species chosen were morphologically similar to the fossil specimens. Species from alpine and arctic regions, forms rarely found in the fossil record, were included to extend the range of polyploidy levels. Herbarium specimens were collected at natural habitats in Switzerland, in several natural locations in the United States, and from my collections of living plants in Wetzikon, Switzerland and in Boise, Idaho, USA. Wetzikon has an equable humid climate with a yearly average of 78% relative humidity and about 1300 mm of precipitation. The Wetzikon plants grew naturally without any artificial irrigation. Boise has a dry desert climate. During the growing season, relative humidity may fall to 20% or lower and monthly precipitation varies between 0 and 100 mm. Unlike the Wetzikon plants, the Boise plants were watered by drip irrigation.

Fossil material was used from the following sites: the Late Miocene Pickett Creek, Owyhee County, Idaho (Buechler et al. 2007); the Middle Miocene Clarkia, northern Idaho (Rember 1991); the Middle Miocene Emerald Creek, northern Idaho (Rember, unpublished); and petiole tissue of *S. hausruckensis* Kovar-Eder and Wójcicki (2001) from the Late Miocene of Hinterschlagen, Austria.

Petiole epidermal tissue from herbarium material was prepared using a method adapted from Traverse (1988). Specimen preparation included the following steps: (1) tissue acetolysed in nine parts acetic anhydride $[(CH_3CO)_2O]$ and one part concentrated sulfuric acid (H_2SO_4) for approximately one minute at 100°C; (2) object rinsed in water; (3) cuticular membrane removed with a needle and mounted in water or glycerin (fresh material should be dehydrated in alcohol and dried prior to acetolysis). Acetolysis destroys portions of the anticlinal epidermal cell walls but preserves cuticle, outer cell walls, and anticlinal wall bases.

Fossil leaves were prepared as follows: (1) pieces of cuticle removed from the petiole in chunks of 1–5 mm² and placed on a microscope slide; (2) objects cleared in a droplet of household bleach (Clorox®) for 1–3 minutes; (3) bleach removed with blotting paper and objects rinsed three times in large droplets of water; (4) cuticles dried and mounted in Eukitt (Kindler, Freiburg, Germany, synthetic polymer solution, refractive index 1.48–1.51) on a microscope slide.

Table 1. Petiole epidermal cell size and stomatal guard cell size of some fossil and modern *Salix* species. N: specimen from natural habitat; C: cultivated plant that originally may have come from a different elevation or climate; T: plant translocated from a different elevation or climate (the original location and elevation are known); F: fossil specimen; lit.: chromosome number from literature; Bue: chromosome number determined on the specimen plant by the author; est: polyploidy level estimated as a result of this study. Sample nos. 6, 8, 17, and 67 were omitted from the analysis because of insufficient numbers of stomata.

Sample number	Subgenus / section, species	Plant status	Herbarium specimen taken from / Original site of growth (if different)	Ploidy level, (2n, as multiple of base number x)(source)	Petiole cell size (µm²)	Guard cell size (µm²)
	***Protitea* / Humboldtianae**					
89	*S. humboldtiana* Willd.	N	Buenos Aires, Argentina, ~20 m	2 (lit.)		218
18	*S. nigra* Marsh.	N	Oberlin, Ohio	2 (lit.)	165	378
19	*S. laevigata* Bebb	N	Santa Inez Valley, California	2 (lit.)	118	521
21	*S. acmophylla* Boiss.	C	Wetzikon, Switzerland, 550 m / Bot. Garden, Cairo, Egypt, ~sea level	2 (Bue)	136	424
	***Protitea* / Wilsoniae**					
33	*S. chaenomeloides* Kimura	C	Boise, Idaho, 920 m / China, <1100 m	2 (Bue)	103	
	***Protitea* / Floridanae**					
80	*S. floridana* Chapman, leaf 1	N	Ocala National Forest, Florida	2 (Bue)	230	
81	*S. floridana* Chapman, leaf 2	N	Ocala National Forest, Florida	2 (Bue)	312	
	***Salix* / Unknown**					
32	*S. succorensis* Chaney and Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	135	
51	*S. succorensis* Chaney and Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	83	
53	*S. succorensis* Chaney and Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	116	
56	*S. succorensis* Chaney and Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	60.5	
58	*S. succorensis* Chaney and Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	92	
60	*S. succorensis* Chaney and Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	74.8	
29	*S. inquirenda* Knowlton	F	Miocene Pickett Creek, Idaho	2 (est.)	138	
54	*S. inquirenda* Knowlton	F	Miocene Pickett Creek, Idaho	2 (est.)	183	
55	*S. inquirenda* Knowlton	F	Miocene Pickett Creek, Idaho	2 (est.)	143	
57	*S. inquirenda* Knowlton	F	Miocene Pickett Creek, Idaho	2 (est.)	104	
59	*S. inquirenda* Knowlton	F	Miocene Pickett Creek, Idaho	2 (est.)	129	
61	*S. inquirenda* Knowlton	F	Miocene Clarkia, Idaho	2 (est.)	84.5	
70	*S. inquirenda* Knowlton	F	Miocene Clarkia, Idaho	2 (est.)	184	
71	*S. inquirenda* Knowlton	F	Miocene Clarkia, Idaho	2 (est.)	129	
	***Salix* / Subalbae**					
40	*S. babylonica* L.	T	Wetzikon, Switzerland, 550 m / Nepal-Himalaya, 3450 m	4 (Bue)	289	
	***Salix* / Salix**					
16	*S. alba* L.	N	Moenchaltorf, Switzerland	4 (Bue)	212	504
87	*S. fragilis* L.	N	Niederuster, Switzerland	4 (lit.)		761
15	*S. fragilis* L.	C	Schwerzenbach, Switzerland, 450m / not known	4 (lit.)	273	852
	***Salix* / Salicaster**					
34	*S. pentandra* L.	N	Urserental, Switzerland	4 (lit.)	269	
14	*S. pentandra* L.	N	La Punt, Switzerland	4 (lit.)	461	600
23	*Salix lasiandra* Benth. var. *lasiandra*	N	Silver Lake, Washington	4 (lit.)	264	556
24	*Salix lasiandra* var. *caudata* (Nuttall) Sudw.	N	Rocky Canyon, Boise, Idaho	4 (lit.)	359	589
25	*Salix lucida* Muhlenb.	N	Oberlin, Ohio	4 (lit.)	265	573

Table 1. (continued) Petiole epidermal cell size and stomatal guard cell size of some fossil and modern *Salix* species.

Sample number	Subgenus / section, species	Plant status	Herbarium specimen taken from / Original site of growth (if different)	Ploidy level, (2n, as multiple of base number x)(source)	Petiole cell size (μm^2)	Guard cell size (μm^2)
	***Salix* / Triandrae**					
20	*S. triandra* L. ssp. *concolor* Koch	N	Volketswil, Switzerland	2 (lit.)		752
22	*S. triandra* L. ssp. *discolor* Koch	C	Wetzikon, Switzerland, 550 m / Frambourg, Jura, France	2 (Bue)		349
	***Salix* / Magnificae**					
77	*S. magnifica* Hemsl.,leaf 1	C	Boise, Idaho, 825 m / W. Sichuan, China, 2100–3000 m	2 (lit.)	292	
78	*S. magnifica* Hemsl.,leaf 2	C	Boise, Idaho, 825 m / W. Sichuan, China, 2100–3000 m	2 (lit.)	269	
79	*S. moupinensis* Franch., leaf 1	C	Wetzikon, Switzerland, 550 m / W. Sichuan, China, 1500–3000 m	2 (Bue)	603	
82	*S. moupinensis* Franch., leaf 2	C	Wetzikon, Switzerland, 550 m / W. Sichuan, China, 1500–3000 m	2 (Bue)	536	333
	***Longifoliae* / Longifoliae**					
10	*S. interior* Rowl.	N	Oberlin, Ohio	2 (lit.)	65	263
48	*S. interior* Rowl.	N	Oberlin, Ohio	2 (lit.)	139	
49	*S. exigua* Nutt.	N	Bosque del Apache, New Mexico	2 (lit.)	172	
	***Chamaetia* / Setchellianae**					
7	*S. setchelliana* Ball	N	Denali Nat. Park, Alaska	2 (Bue)		1003
72	*S. setchelliana* Ball	N	Knik River, near Palmer, Alaska, leaf 1	2 (lit.)		790
73	*S. setchelliana* Ball	N	Knik River, near Palmer, Alaska, leaf 2	2 (lit.)		983
74	*S. setchelliana* Ball	N	Knik River, near Palmer, Alaska, leaf 3	2 (lit.)		959
75	*S. setchelliana* Ball	N	Knik River, near Palmer, Alaska, leaf 4	2 (lit.)	236	
	***Chamaetia* / Herbella**					
11	*S. serpyllifolia* Scop.	N	Gletschboden, Switzerland	2 (Bue)		378
12	*S. retusa* L.	N	Gletschboden, Switzerland	8 (Bue)	640	837
13	*S. herbacea* L.	N	Fluela, Switzerland	2 (lit.)		521
	***Chamaetia* / Glaucae**					
39	*S. glaucosericea* Flod. (male)	T	Wetzikon, Switzerland, 550 m / Fluela, Engadin, Switzerland, 2100 m	10 (Bue)	810	
50	*S. glaucosericea* Flod. (female)	N	Fluela, Engadin, Switzerland, leaf 1	10 (lit.)	454	
84	*S. glaucosericea* Flod. (female)	N	Fluela, Engadin, Switzerland, leaf 2	10 (lit.)		967
	***Vetrix* / Phylicifoliae**					
1	*S. phylicifolia* L.	T	Wattwil, Switzerland, 670 m / Lake Gjevil, Norway, ~600 m	6 (lit.)	501	517
44	*S. phylicifolia* L. (plant no.1)	N	Longmuir hills, Scotland	6 (lit.)	351	
45	*S. phylicifolia* L. (plant no.2)	N	Longmuir hills, Scotland	6 (lit.)	285	
46	*S. phylicifolia* L. (plant no.3)	N	Longmuir hills, Scotland	6 (lit.)	293	
47	*S. phylicifolia* L. (plant no.4)	N	Longmuir hills, Scotland	6 (lit.)	272	
62	*S. phylicifolia* L. (plant no.3, leaf 1)	N	Longmuir hills, Scotland	6 (Bue)	399	
63	*S. phylicifolia* L. (plant no.3, leaf 2)	N	Longmuir hills, Scotland	6 (Bue)	316	
64	*S. phylicifolia* L. (plant no.3, leaf 3)	N	Longmuir hills, Scotland	6 (Bue)	273	
65	*S. phylicifolia* L. (plant no.3, leaf 4)	N	Longmuir hills, Scotland	6 (Bue)	305	
91	*S. phylicifolia* L. (plant no.3, phyl 4)	T	Boise, Idaho, 900 m / Longmuir hills, Scotland, ~200 m	6 (Bue)		366
66	*S. phylicifolia* L. (plant no.3, leaf 5)	N	Longmuir hills, Scotland	6 (Bue)	423	
85	*S. phylicifolia* L. (plant no.3, leaf 6)	N	Longmuir hills, Scotland	6 (Bue)		507

Table 1. (continued) Petiole epidermal cell size and stomatal guard cell size of some fossil and modern *Salix* species.

Sample number	Subgenus / section, species	Plant status	Herbarium specimen taken from / Original site of growth (if different)	Ploidy level, (2n, as multiple of base number x)(source)	Petiole cell size (μm^2)	Guard cell size (μm^2)
36	*S. bicolor* Willd.	T	Wattwil, Switzerland, 670 m / Pyrenes, France, 1400–1700 m	6 (Bue)	318	
37	*S. hibernica* Rech. fil.	C	Wetzikon, Switzerland, 550 m / Benbulben, Ireland, ~400 m	6 (Bue)	277	
35	*S. hegetschweileri* Heer	T	Wetzikon, Switzerland, 550 m / Gletschboden, Switzerland, 1770 m	6 (Bue)	567	
4	*S. hegetschweileri* Heer	N	Urserental, Switzerland	6 (lit.)		526
9	*S. hegetschweileri* Heer	T	Wattwil, Switzerland, 670 m / Urserental, Switzerland, 1500 m	4 (Bue)	493	
69	*S. pulchra* Cham.	N	Bonanza Creek, Fairbanks, Alaska	4 (lit.)	226	
68	*S. planifolia* Pursh	N	Hell Roaring Creek, Stanley, Idaho	4 (lit.)	181	
3	*S. planifolia* Pursh	N	Bear Tooth Mountains, Wyoming	4 (lit.)	265	526
	***Vetrix* / Nigricantes**					
41	*S. mielichhoferi* Sauter	T	Wetzikon, Switzerland, 550 m / Seiseralp, Tirol, Italy, 1950 m	8 (Bue)	585	
42	*S. myrsinifolia* Salisb.	C	Wetzikon, Switzerland, 550 m / Wattwil, Switzerland, 620 m	6 (Bue)	281	
	***Vetrix* / Canae**					
86	*Salix elaeagnos* Scop. subsp. *angustifolia* (Cariot) Rech. fil.	C	Wetzikon, Switzerland, 550 m / Spain	2 (lit.)		135
	***Vetrix* / Helix**					
83	*S. purpurea* L.	N	Schwerzenbach, Switzerland	2 (lit.)	157	476
	***Vetrix* / Glabrella**					
43	*S. glabra* Scop.	T	Wattwil, Switzerland, 670 m / Ticino, Switzerland, 1600–1700 m	6 (Bue)	337	
	***Vetrix* / Caesiae**					
38	*S. caesia* Vill.	C	Appenzell, Switzerland, 1150 m / not known	4 (Bue)	202	
	***Vetrix* / Arbuscella**					
5	*S. foetida* Schleich.	N	Furka, Switzerland	2 (lit.)		295
2	*S. waldsteiniana* Willd.	C	Wetzikon, Switzerland / Appenzell, Switzerland, 1500 m	2 (Bue)		251
	***Vetrix* / Villosae**					
88	*S. helvetica* Vill.	N	Fluela, Switzerland, 1800 m	2 (lit.)		343
90	*S. helvetica* Vill.	T	Boise, Idaho, 900 m / Fluela, Switzerland, 1800 m	2 (lit.)		387
	***Vetrix* / Mexicanae**					
27	*S. lasiolepis* Benth.	N	Boise, Idaho	4 (lit.)	267	367
	Unknown affinity					
28	*S. wildcatensis* Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	106	
76	*S. hausruckensis* Kovar-Eder	F	Miocene Hinterschlagen, Austria	2 (est.)	256	292*
31	*S. desatoyana* Axelrod	F	Miocene Pickett Creek, Idaho	2 (est.)	52	
52	*Salix* sp.	F	Miocene Pickett Creek, Idaho	2 (est.)	123	
30	*Salix* sp.	F	Miocene Pickett Creek, Idaho	2 (est.)	142	

* average stomatal size from Kovar-Eder and Wójcicki 2001.

Stomatal guard cells from herbarium specimens were prepared as follows: (1) dense indumentum removed with a soft pencil eraser; (2) epidermal wax removed in acetone, approximately one hour; (3) tissue softened in isopropanol:water = 1:1 for one hour; (4) object cleared in household lye "Red Devil" (5 g lye dissolved in 100 ml water) for 1–2 hours or until translucent; (5) leaf rinsed several times in water and mounted in water.

Size of petiole epidermal cells was determined by tracing outlines of rectangular groups of cells with a camera lucida attached to a microscope. The area of the petiole cell group was then compared to a rectangle with the same area and divided by the number of included cells (Fig.1). Where possible, the cells of 2–5 groups from different parts of the petiole (viz. adaxial and abaxial side, distal and proximal part, 50–100 cells total) were measured and the overall mean cell size calculated.

Stomatal guard cell size was determined by measuring overall length (L) and width (W) of 20 stomata per specimen, each including the two paired guard cells and the opening between them (Fig.2), along a transect on the abaxial side near the broadest part of the leaf. The average stoma size (L × W) and standard deviation per leaf were calculated. The petiole epidermal cell and stomatal guard cell data obtained for the extant species came from tissue taken from mature, well-developed leaves, usually from the medial part of the branchlet.

A reference data set was compiled using cell size of modern species with known chromosome numbers. The polyploidy levels of the fossil species were estimated by comparing their cell size to the reference data. Trend lines (Figs. 3–6) of the form y = a × xb were calculated using Excel 2002. They represent the estimated central tendency of data points in a specific category.

RESULTS

Table 1 lists the cell size data, sample numbers, taxonomic affiliation, origin of plant material, and polyploidy levels. Specific data points in Figures 3–6 are labeled with the corresponding sample number. Table 2 shows examples of petiole epidermal and stomatal guard cell size variation within a leaf, between leaves on the same plant, between plants from the same location, and between plants of the same polyploidy level. These data emphasize the importance of using average values from adequate sample sizes from fossil and fresh plant material.

Figure 3 shows results of petiole epidermal cell size of fossil, cultivated, translocated, and plants from natural habitats, as a function of polyploidy level. Note that the data points for some of the plant status categories were shifted sideways for better visibility. Trend lines were calculated per subgenus (with subg. *Protitea* Kimura 1928 and *Salix* combined) for leaves from natural habitats using an average cell size for species with data from more than one leaf. If cell volume (Length × Width × Depth) is assumed to be proportional to

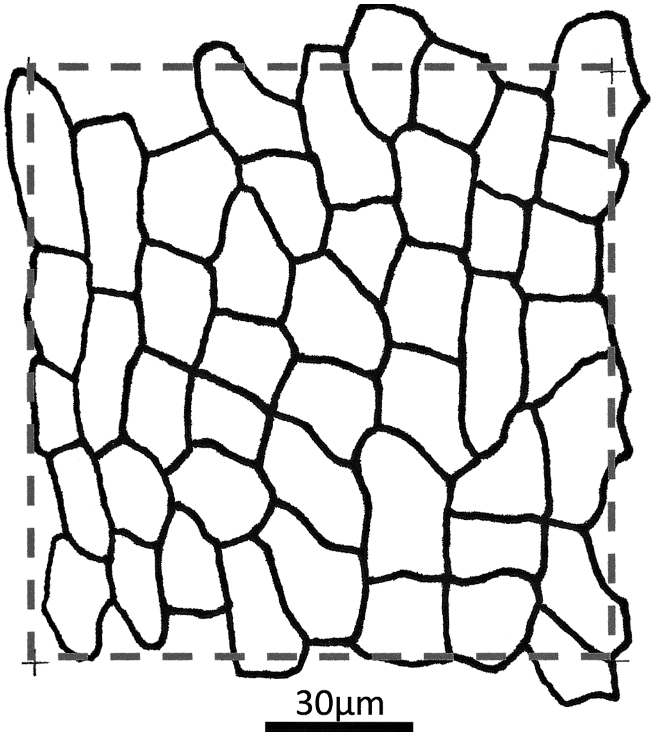


Figure 1. A group of 46 petiole epidermal cells of tetraploid *S. pentandra* (34) with an average cell size of 319 μm². The dashed line encloses the same area as the whole group of cells (see Methods).

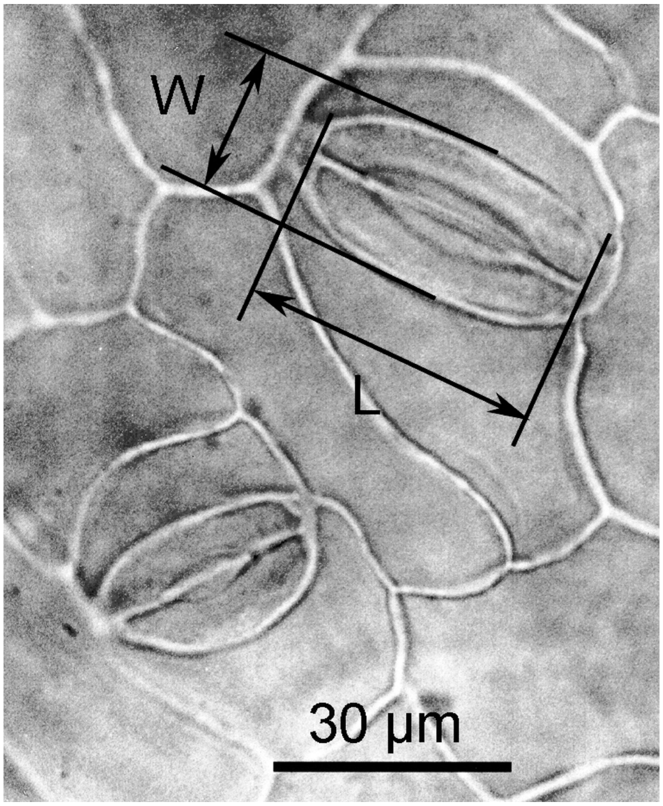
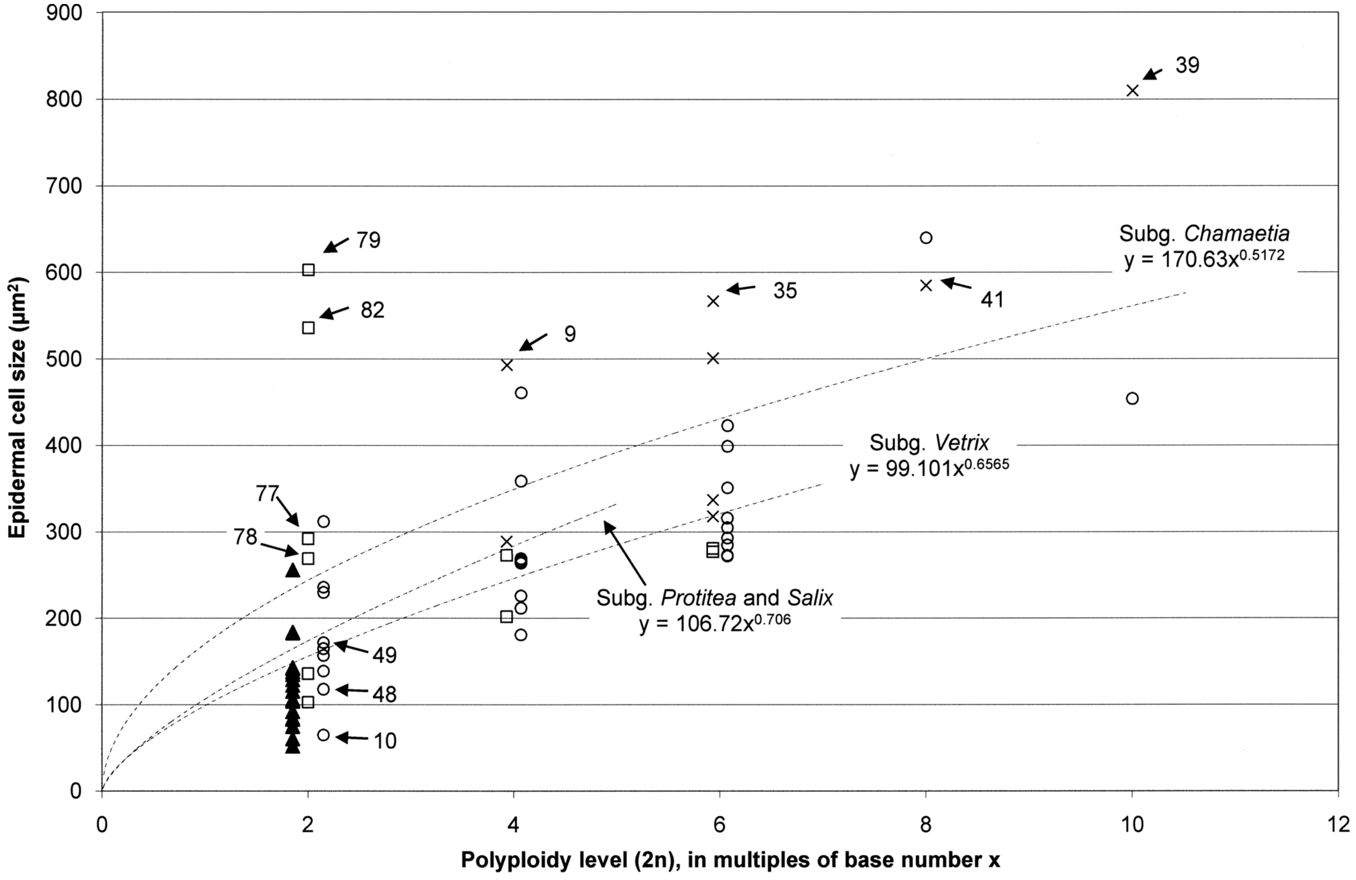


Figure 2. Stomata in tetraploid *S. lucida* ssp. *caudata* (24) with an average cell size (L x W) of 589 μm² (see Methods).

Table 2. Variation of cell size within several categories. Analyses are restricted to plants growing in natural habitats.

Variability within:	Example	Cell size, mean value and standard deviation
	Petiole epidermal cell size	
Cell groups	estimated typical values[1]	ratio = ~1 : 4
Group mean values of one petiole	*S. inquirenda* (fossil, no. 71), 8 cell groups	130 ± 20.7 µm^2 (± 16%)
Mean values of one plant	*S. phylicifolia* (modern, nos. 62–66), 5 leaves on the same twig	343 ± 64 µm^2 (± 19%)
Plant mean values of one habitat	*S. phylicifolia* (modern, nos. 44–47), 4 plants	300 ± 35 µm^2 (± 12%)
For variation within polyploidy levels see Fig. 1		
	Stomatal guard cell size	
One leaf (20 stomata per leaf)	*S. setchelliana* (diploid, no. 72)	790 ± 155 µm2 (± 20%)
	S. moupinensis (diploid, no. 82)	333 ± 64 µm^2 (± 19%)
	S. alba (tetraploid, no. 16)	504 ± 74 µm^2 (± 15%)
	S. phylicifolia (hexaploid, no. 85)	507 ± 54 µm^2 (± 11%)
One plant	*S. setchelliana* (3 leaves, prep. 72–74)	911 ± 105 µm^2 (± 12%)
For variation within polyploidy levels see Fig. 2		

[1] The size of individual cells has not been measured (see Methods).



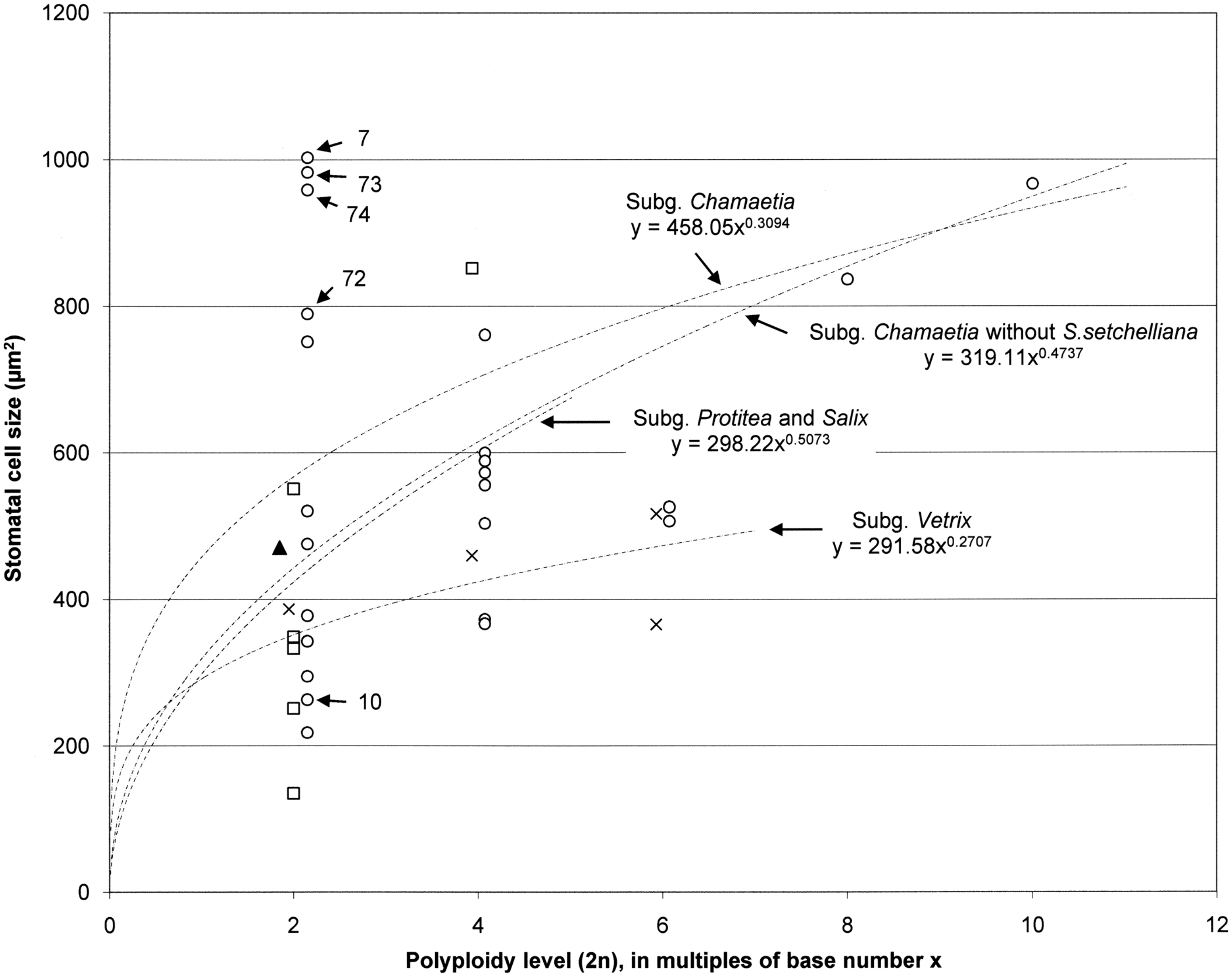


Figure 4. Stomatal guard cell size as a function of polyploidy level. All data points are shown (○ plants from natural habitats; ☐ cultivated plants; × translocated plants; ▲ fossil specimens (diploid level assumed). Trend lines are by subgenera (subg. *Protitea* and *Salix* combined) and only for plants from natural habitats. For species with more than one leaf the average cell size was calculated and used for trend lines.

DNA content and thus polyploidy level (x), and cell shape (L:W:D ratio) is constant, then $L \times W = a\,x^{2/3}$. Note that trend line exponents in Figure 3 are relatively close to this theoretical value of 0.666. Some of the upper outlier data points in Figure 3 are from cultivated and translocated plants that had been moved more than 800 meters to lower elevations. These data suggest that environmental parameters may also influence cell size. Fossil plants (diploid level assumed) are in the lower cell size range for diploids, together with three members of the extant subgenus *Longifoliae* (Andersson) Argus 1997 (sample numbers 10, 48, 49), a group of species adapted to dry air environments.

Figure 4 shows the results for stomatal guard cell size of fossil, cultivated, translocated, and plants from natural habitats, as a function of polyploidy level. Again, data for subg. *Protitea* and subg. *Salix* were combined. Some of the upper outlier data points (7, 72–74) belong to *S. setchelliana* Ball 1934, an arctic dwarf willow adapted to a humid microclimate. The trend lines calculated per subgenus were done using only leaves from natural habitats. Because of the

◄ **Figure 3.** Petiole epidermal cell size as a function of polyploidy level. All data points are shown (○ plants from natural habitats; ☐ cultivated plants; × translocated plants; ▲ fossil specimens (diploid level assumed). Trend lines are by subgenera (subg. *Protitea* and *Salix* combined) and only for plants from natural habitats. For species with more than one leaf the average cell size was calculated and used for trend lines. Upper outlier data points marked with sample numbers are from plants that have been translocated to lower elevations by more than 800 m.

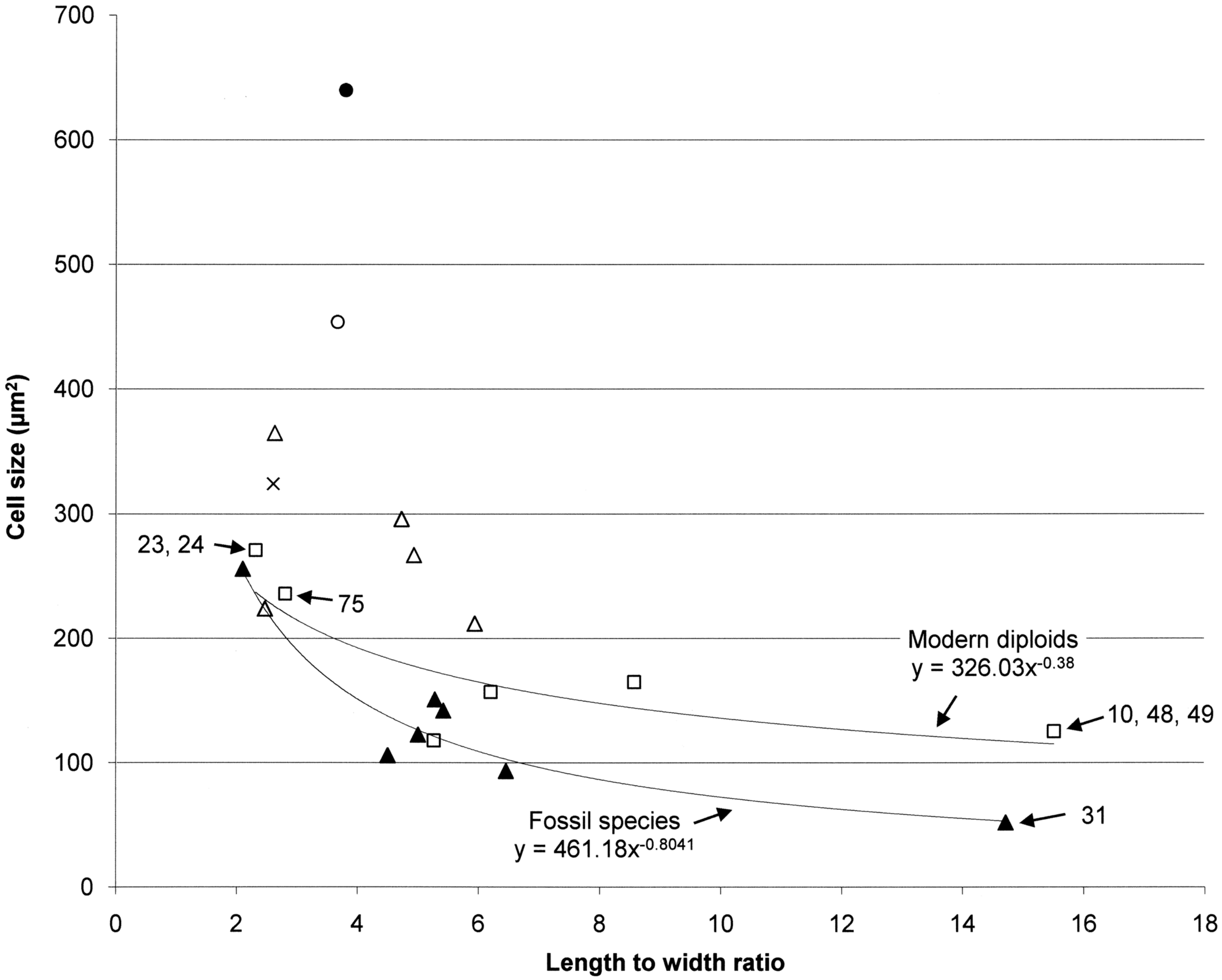


Figure 5. Petiole epidermal cell size in relation to leaf length-to-width ratio. Analysis restricted to plants growing in natural habitats. For species with more than one leaf the average was calculated and used for the graph. ▲ fossil diploids (ploidy level assumed); □ modern diploids; △ modern tetraploids; × modern hexaploids; ● modern octoploids; O modern decaploids.

outlier status of *S. setchelliana*, the trend line calculation for subgenus *Chamaetia* (Dum.) Nasarov 1936 was done with and without that species. Note that some trend line exponents are distinctly smaller than in Figure 3, indicating that stomatal guard cells vary less in size with polyploidy level than do petiole epidermal cells. Because of overlapping cell size ranges (Figs. 3 and 4) accurate estimates of polyploidy levels are not possible without additional information about the specimens and species involved.

Figure 5 shows petiole epidermal cell size in relation to leaf length-to-width ratio of fossil plants and extant plants from natural habitats, including trend lines for extant diploids and fossil species. Because of small sample sizes and a limited range in length-to-width ratio, there are no trend lines shown for higher ploidy levels. Note that fossil species have in general smaller cell sizes than extant diploids (Figs. 3, 5) and that modern leaves with length-to-width ratios higher than 6 are all from diploid species.

Figure 6 shows stomatal guard cell size in relation to length-to-width ratio of plants from natural habitats and one data point for fossil *S. hausruckensis* from the Late Miocene of Hinterschlagen, Austria. Because of generally small sample sizes per polyploidy level and the absence of narrow leaves at higher polyploidy levels, only a trend line for extant diploids was calculated. Data in Figures 5 and 6 suggest that length-to-width ratio should be closely matched between fossil and extant reference specimens.

DISCUSSION

Epidermal cell size has been used as a proxy to estimate ploidy levels in extant and fossil plants (e.g., Miki and Hikita 1951, Roth and Dilcher 1979, Masterson 1994). In order

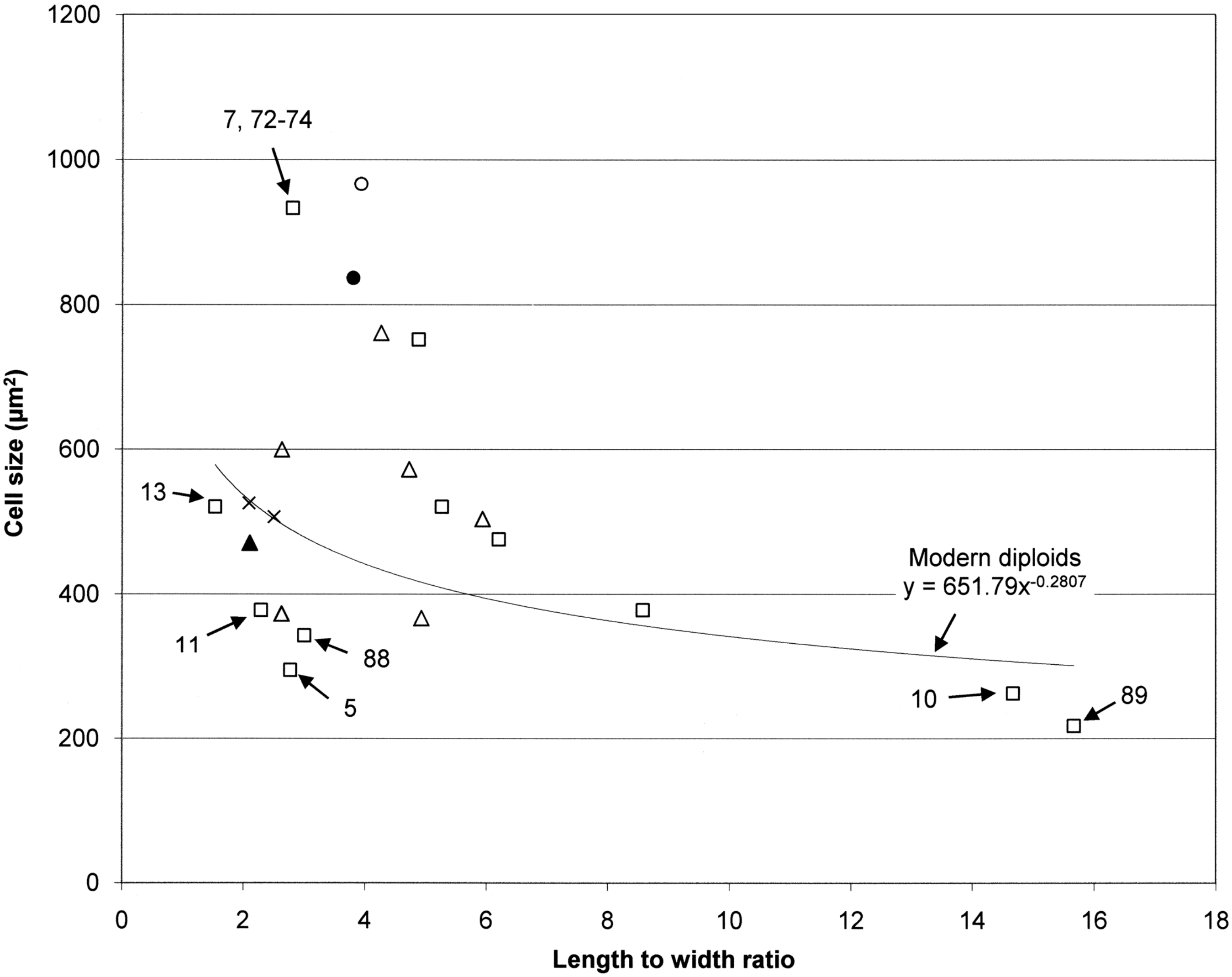


Figure 6. Stomatal guard cell size in relation to leaf length-to-width ratio. Of modern species only those from natural habitats were used. Of species with more than one leaf the average was calculated and used for the graph. ▲ fossil diploids (ploidy level assumed); □ modern diploids; △ modern tetraploids; × modern hexaploids; ● modern octoploids; ○ modern decaploids.

to determine if factors other than ploidy levels and DNA content affect cell size, e.g., infrageneric classification, leaf shape, climate, and elevation, the epidermal cells of fossil and extant *Salix* species were measured and statistically analyzed. Results show that the distribution of data points over a wide cell size range and the overlap between polyploidy levels (Figs. 3, 4) do suggest that cell size may depend on more than just the number of chromosome sets. Outlier data points provide clues about other parameters that may be involved.

For petiole epidermal cell size (Fig. 3), the upper cell size range for diploids, tetraploids and hexaploids is dominated by cultivated and translocated plants that had been moved to elevations more than 800 meters lower, indicating that elevation or related environmental parameters likely influence cell size. Two diploid species from W. Sichuan, China grow at very high elevations in their natural habitats: *S. moupinensis* Franchet 1887 (79, 82, Fig. 3) between 1500 and 3000 m, and *S. magnifica* Hemsley 1906 (77, 78, Fig. 3) between 2100 and 3000 m (Fang Zhenfu et al. 1999). The following outlier plants represent translocations from higher to lower elevations: *S. hegetschweileri* Heer 1840 (9, Fig. 3) by 830 m, *S. hegetschweileri* (35, Fig. 3) by 1220 m, *S. mielichhoferi* Sauter 1849 (41, Fig. 3) by 1400 m, and *S. glaucosericea* Flod. 1943 (39, Fig. 3) by 1550 m. Although no material of these plants was available from their natural habitat, their exceptionally large cell sizes are evidence of a possible influence of elevation or associated environmental parameters on cell size. However, it should be noted that most of these cultivated and translocated plants would not survive without horticultural care at their new place of growth. As long as leaves from natural habitats are used as reference, the influence of elevation on epidermal cell size will be smaller and potentially negligible. Even with the data points of cultivated and translocated plants removed, cell size ranges of adjacent polyploidy levels still overlap. Therefore, petiole epidermal cell size does not accurately estimate polyploidy levels without additional information on taxonomic affiliation and morphological characteristics of the specimens and species involved.

Stomatal guard cell size (Fig. 4) does not depend as much on changes in elevation as petiole epidermal cell size. *Salix setchelliana* (subg. *Chamaetia*, 7, 72, 73, 74, Fig. 4), a willow growing on glacial river gravel bars in Alaska, has the largest stomata of all species examined in this study. Species of subg. *Chamaetia* are small shrubs or mat forming, trailing plants that grow in a humid microclimate. With their generally small, roundish leaves, they represent a counterpart to the narrow-leaved members of subg. *Longifoliae*. *Longifoliae* species are adapted to xeric (dry air) conditions and exhibit some of the smallest cell sizes (10, 48, 49, Fig. 3; 10, Fig. 4). Trend lines in Figs.3 and 4 confirm that species of subg. *Chamaetia* have in general larger cells than those of other subgenera. As noted above for petiole epidermal cell size, stomatal cell size does not allow an accurate estimate of polyploidy levels without additional information about taxonomical affiliation and morphological characteristics of the specimens and species involved.

Cell size data of genetically identical plants grown at different elevations show some influence of elevation on cell size. The leaf samples from a plant of *S. phylicifolia* L. 1753 (85, Table 1) growing in a humid environment at ca. 200 m above sea level in Scotland had a stomatal cell size of 507 μm^2. After translocating cuttings to a dry air environment at 900 m in Boise, Idaho, the same plant (91, Table 1) had a stomatal cell size of only 366 μm^2. The leaf samples from a plant of *S. helvetica* Vill. 1789 (88, Table 1) growing in its natural habitat on the Fluela Pass in Switzerland (1800 m) had a stomatal cell size of 343 μm^2. However, when the plant was moved to Boise, Idaho (900 m), by cuttings, its stomatal cell size was 387 μm^2.

The mechanism by which cell size is affected by elevation is not obvious. UV-B radiation (Barkan et al. 2006, Wargent et al. 2009) and water potential and its response to environmental parameters during cell elongation (Nonami 1998) seem, however, good candidates for explaining why cell size in *Salix* increases when plants are moved to lower elevations.

A correlation was found between leaf length-to-width ratio and cell size. Petiole epidermal cell size (Fig. 5) and stomatal guard cell size (Fig. 6) increase significantly in wider leaves of diploid species. For species with higher ploidy levels, such a relationship is less obvious, partially because this study did not include enough high-level polyploids, and partially because leaves of high-level polyploids are not really that narrow. Species that typically grow in humid environments such as *S. floridana* Chapman 1860 (80, 81, Table 1) and members of subgenus *Chamaetia* like *S. setchelliana* (7, 72–75, Table 1), S. herbacea L. 1753 (13, Table 1), and *S. serpyllifolia* Scop. 1772 (11, Table 1) are found at the "wide" end of the length-to-width axis. Most species of the subgenus *Chamaetia* are low growing or mat-forming plants with leaves close to the moist ground. At the "narrow" end of the length-to-width axis are species with small cells that are adapted to seasonal xeric conditions, such as members of subgenus *Longifoliae* (10, 48, 49, Table 1) and *S. humboldtiana* Willd. 1806 (89, Table 1). Among fossil species, *S. desatoyana* (31, Table 1) has the narrowest leaves and the smallest petiole epidermal cells of all specimens included in this study. It is morphologically closest to modern *S. humboldtiana*.

In modern floras, leaf characters such as margin dentition (entire, toothed, or lobed), blade size, apex and base shape, length-to-width ratio, and overall shape, correlate in varying degrees with climatic parameters (Wolfe 1993). Because of the potential connection between environmental parameters and cell size found in this study, Wolfe's results underscore the importance of carefully matching leaf characters of fossil specimens and modern reference material.

Figures 3 and 4 show that Miocene *Salix* leaves in general have smaller petiole epidermal cell sizes than modern diploids. Because of the small sample size and the different subgeneric composition of fossil and modern species groups used in this study, the difference in cell size is not convincingly attributed to a change in the number of entire chromosome sets. Over geologic time an increase in DNA content of meristematic cells could have occurred through repeated gene duplication. Thibault (1998) examined the DNA content of 17 *Salix* species and hybrids growing in riparian habitats in Southeastern France using flow cytometry. DNA content in eight diploid species (2n = 38) varied from 0.79 to 0.96 pg/nucleus, and from 1.62 to 1.80 pg/nucleus in six tetraploid species. If a variation of ± 10% can occur between extant species from the same habitat type, we have to assume that DNA content, and thus cell size, could significantly change over a time scale of millions of years by mechanisms other than multiplication of chromosome numbers. In a study of 154 *Salix* genotypes Trybush et al. (2008) found that interspecific differences in DNA amount were relatively large and may have affected the estimation of polyploidy levels for some of the plants used in their study.

During cell elongation and cell differentiation, cell size may change differently depending on species and cell type. Evidence for this is suggested by the difference in the ratios between the size of stomatal complexes, which comprise two cells and the opening between them, and the petiole epidermal cells. For the leaves collected from plants growing in natural habitats, the average ratio between the two cell types is 2.29 (calculated from data in Table 1). Diploids range from 2.29 to 4.42 (four leaves, average 3.45), tetraploids from 1.30 to 2.38 (seven leaves, average 1.92), and the only octoploid leaf has a ratio of 1.31 (12, Table 1). The ratio decreases with higher polyploids because stomatal cell size changes less with polyploidy level than epidermal cell size (see also trend line exponents in Figs. 3 and 4).

Proxy methods using cell size to estimate ploidy levels assume that DNA content is proportional to cell volume (L×W×D). However, with a microscope only the two dimensions, cell L and W, are measured easily and accurately. Proxy methods further assume that cell shape does not change with cell volume and that cell size (L×W) is a valid input parameter for determining polyploidy levels. Skvortsov and Golysheva (1966) published images of transverse sections through blades of 45 *Salix* leaves. The publication includes leaves from all subgenera and polyploidy levels from diploid to octoploid. Because of their small size, these images do not allow a precise quantitative correlation analysis between cell depth and transverse cell width. However, they confirm that the three dimensional shape of epidermal cells does not systematically change with cell volume.

The following five examples demonstrate the usefulness and limitations of estimating polyploidy levels in fossil specimens. Fossil *Salix hausruckensis* (76, Table 1) resembles most closely modern *S. floridana* (80, 81, Table 1, diploid) (Kovar-Eder and Wójcicki 2001). Both have wide elliptical lamina, rounded to cordate leaf bases, acute to slightly acuminate apices, and petiolar glands on some leaves (Figs. 7, 8). Their average cell sizes, 256 and 271 μm² respectively, are so similar that a diploid chromosome number is inferred for *S. hausruckensis*. Leaves of extant *S. magnifica* (77, 78, Table 1) and *S. moupinensis* (79, 82, Table 1), both diploid, also resemble those of *S. hausruckensis* in size and length-to-width ratio, but not in overall shape, apex form, and margin dentition. With a cell size of 280 μm², *S. magnifica* is very close to *S. hausruckensis*, while the cell size of *S. moupinensis* (570 μm²) is distinctly higher.

Salix inquirenda compares favorably to the modern diploid *S. bonplandiana* Kunth (Humboldt et al. 1817) (Buechler 2008), a species very closely related to *S. laevigata* Bebb 1874 and the tetraploid *S. lasiandra* Benth. 1857 (Axelrod 1992). A comparison of petiole epidermal cell sizes among the three species shows that *S. inquirenda* (137 μm²) is very close to *S. laevigata* (118 μm²) and distinctly smaller than the

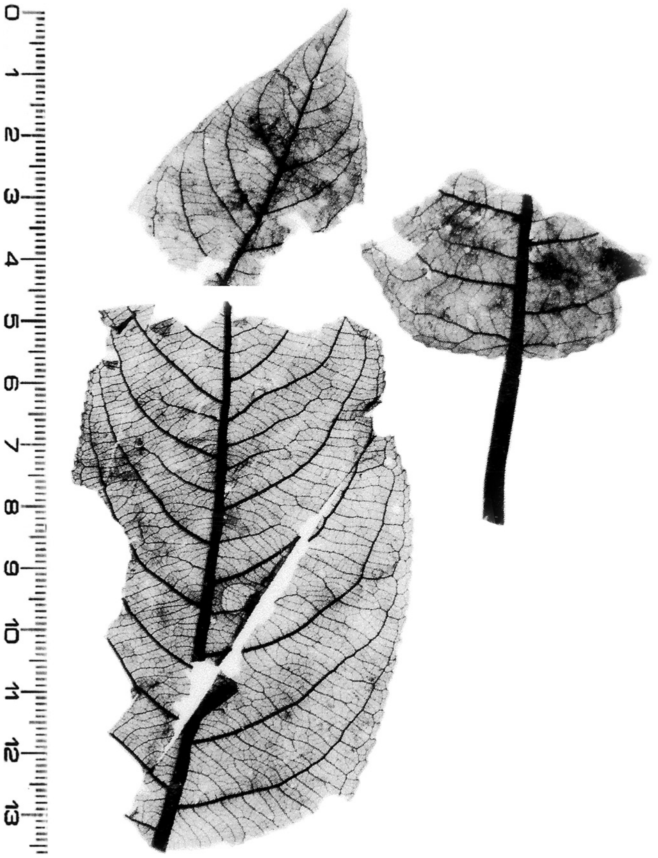


Figure 7. Fragments of three *S. hausruckensis* leaves. Note the similarity in venation pattern and leaf shape with extant *S. floridana* (Fig.8). Image courtesy Johanna Eder and Natural History Museum, Vienna, Austria.

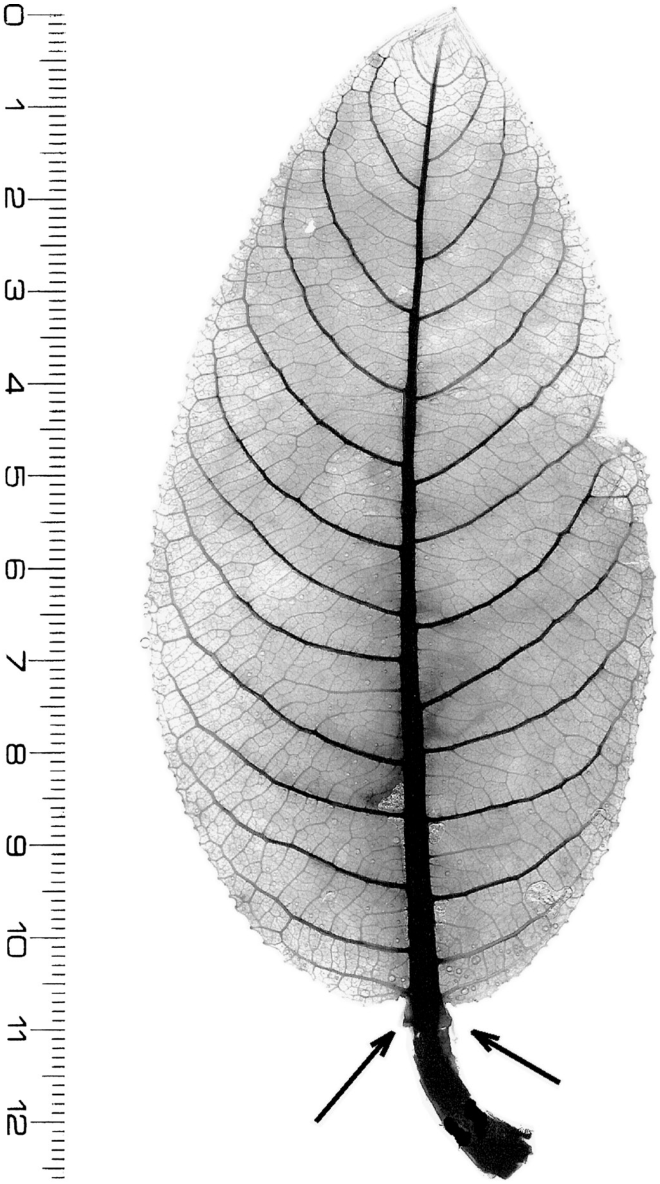


Figure 8. A cleared leaf of extant *S. floridana*. Note the similarity in venation pattern and leaf shape with fossil *S. hausruckensis* (Fig. 7). Arrows point at petiolar glands near the leaf base.

tetraploid *S. lasiandra* var. *lasiandra* (264 µm²). Therefore, it seems reasonable to consider *S. inquirenda* a diploid species.

Salix succorensis is morphologically similar to modern members of section Humboldtianae (subgenus *Protitea*). The petiole epidermal cells of *S. succorensis* average 93.5 µm² in area. They are smaller than the three comparable extant species of the section that were included in this study (see Table 1, *S. nigra* Marsh. 1785, 18; *S. laevigata*, 19; *S. acmophylla* Boiss. 1846, 21). However, the difference as compared to *S. laevigata* (118 µm², no. 19) is small enough to suggest that *S. succorensis* is also a diploid with base number 19.

Salix desatoyana (31) has the smallest epidermal cell size (52 µm²) of all the species examined, fossil and extant, followed by the extant diploid *S. interior* Rowlee 1900 (10) (65 µm²), which has an equally narrow leaf. It is reasonable to conclude that *S. desatoyana* is also an x=19 diploid as the difference in cell size between the two species is relatively small.

Salix wildcatensis is very similar if not indistinguishable from the modern tetraploid *S. lasiolepis* (Axelrod 1944) in overall leaf shape, size, length-to-width ratio, entire margin, and occasional irregular pattern of the secondary venation. Petiole epidermal cell size of a *S. wildcatensis* specimen from Pickett Creek (Buechler et al. 2007) is 106 µm². As this is less than half of what was determined for the tetraploid *S. lasiolepis* (267 µm²), it suggests that *S. wildcatensis* is diploid.

CONCLUSIONS

A review of the literature relating the amount of DNA content to cell size, and the comparative analysis of cell size measurements presented in this study, reveals that cell size in fossil and extant *Salix* may depend on parameters other than the number of chromosomes. As shown here for fossil willows, one should be cautious when using cell size as a proxy for estimating polyploidy levels. Results for fossil *Salix* species will be meaningful only if modern leaf material used for reference is from morphologically similar extant plants grown in natural habitats. Leaves should be similar in overall shape, base and apex shape, blade length and width, length-to-width ratio, petiole length, petiole-to-blade length ratio, venation pattern, and state of the margin (entire or toothed). If possible, several extant species should be included for reference in the analysis. Conclusions about polyploidy level changes in fossil *Salix* should agree with evidence from the geographic distribution of closely related modern species, and molecular sequence data. Clear evidence of a change in polyploidy level should be based on cell size (cell length × width) increases of about 60% , corresponding to a doubling of the cell volume, within a short geological time interval.

Some findings in this study may apply to other woody angiosperm genera. Others may be specific to *Salix* due to this genus' exceptionally large range of polyploidy levels in the extant species, frequent hybridization and gene introgression, and by the wide latitudinal distribution of the genus and related adaptations to different environments. Such characteristics could result in an unusually high variability of DNA content. A similar situation may, for example, exist in *Betula* (2n = 28, 56, 70, 84, 112, 140; Furlow 1990) and *Rosa* (2n = 14, 28, 35, 42, 56; Goldblatt and Johnson 2008).

The results of this study provide a representative overview of the cell size variation within the extant genus *Salix* in a sample size of 87. Differences between and within subgenera and the influence of elevation and leaf shape on cell size are based on smaller sample sizes and allow therefore only qualitative assessments. This study points out some of the limitations of cell size proxy methods and shows how uncertainties may be minimized. Future studies need to consider one fossil species at a time, investigate cell size variation over time, and cell size in comparable extant species in greater detail compared to this study.

ACKNOWLEDGEMENTS

I wish to thank several people for their help. George W. Argus contributed herbarium material of the *S. rotundifolia* complex, discussed various polyploidy-related issues with me, provided help with the bibliography, and critically read the manuscript. Dorothy A. Douglas, Biology Department, Boise State University, read the manuscript and made valuable suggestions. Johanna Kovar-Eder, Naturhistorisches Museum, Vienna, Austria, contributed several petioles of fossil *Salix hausruckensis* specimens. William C. Rember, University of Idaho, Moscow, Idaho, provided fossil material from Miocene Clarkia and Emerald Creek sites and discussed numerous aspects of this study with me. Special thanks go to Diane M. Erwin and Jeff Myers for reviewing and improving the manuscript.

LITERATURE CITED

Argus, G.W. 1997. Infrageneric classification of *Salix* (Salicaceae) in the New World. *Systematic Botany Monographs* 52:1–121.

Argus, G.W. In press. *Salix*. *In* Flora of North America, Vol. 7. Oxford University Press, New York and Oxford.

Axelrod, D.I. 1944. The Mulholland flora. *Carnegie Institution of Washington Publication* 553:103–146.

Axelrod, D.I. 1985. Miocene floras from the Middlegate Basin, west-central Nevada. *University of California Publications in Geological Sciences* 129:1–279.

Axelrod, D.I. 1992. The middle Miocene Pyramid flora of western Nevada. *University of California Publications in Geological Sciences* 137:1–50.

Ball, C.R. 1934. New or little known west American willows. *University of California Publications in Botany* 17:399–433.

Baluška, F., and Š. Kubica. 1992. Relationship between the content of basic nuclear proteins, chromatin structure, rDNA transcription and cell size in different tissues of the maize root apex. *Journal of Experimental Botany* 43(252):991–996.

Barkan, L., M.A. Evans, and G.E. Edwards 2006. Increasing UV-B induces biphasic leaf cell expansion in *Phaseolus vulgaris*, suggesting multiple mechanisms for controlling plant growth. *Photochemistry and Photobiology* 82:1612–1620.

Bebb, M.S. 1874. A new species of willow from California, and notes on other North American species. *The American Naturalist* 8(4):202–203.

Bennett, M.D., and I.J. Leitch. 2005. Angiosperm DNA C-values database (release 5.0, Dec. 2004), available online at http://www.kew.org/cvalues/homepage.html.

Bennett, M.D., and H. Rees. 1969. Induced and developmental variation in chromosomes of meristematic cells. *Chromosoma* 27:226–244.

Bentham, G. 1857. Plantae Hartwegianae, Gulielmus Pamplin, London. 393 pp.

Boissier, P.E. 1846. Diagnoses plantarum orientalium novarum 7. Lipsiae. 130 pp.

Büchler, W. 1985. Neue Chromosomenzählungen in der Gattung *Salix. Botanica Helvetica* 95(2):165–175.

Büchler, W. 1986. Neue Chromosomenzählungen in der Gattung *Salix.* 2. Teil. *Botanica Helvetica* 96(2):135–143.

Buechler, W. 2000. Estimating polyploidy levels using cell size in *Salix* leaves (abstract). *American Journal of Botany* 87(6):80–81.

Buechler, W. 2001. IOPB Chromosome Data 17, Salicaceae. *IOPB Newsletter* 33:1–35.

Buechler, W.K., M.T. Dunn, and W.C. Rember. 2007. Late Miocene Pickett Creek flora of Owyhee County, Idaho. *Contributions from the Museum of Paleontology, the University of Michigan, Ann Arbor* 31:305–362.

Buechler, W. 2008. Revision of fossil willows: *Salix hesperia—S. inquirenda* and *Salix* subgenus *Longifoliae* groups. *PaleoBios* 28(2):62–75.

Chaney, R.W., and D.I. Axelrod. 1959. Miocene floras of the Columbia Plateau, part II. *Carnegie Institution of Washington, Publications*, 617:135–237.

Chapman, A.W. 1860. Flora of the southern United States. Ivison, Phinney & Co., New York. 621 pp.

Floderus, B. 1943. The continental *Salix glauca* species. *Svensk Botanisk Tidskrift* 37:169–172.

Franchet, M.A. 1887. Plantae Davidianae ex sinarum imperio, 2^{me} partie, plantes du Thibet oriantale**.** *Nouvelles Archives du Muséum national d'Histoire naturelle.* 233 pp.

Furlow, J.J. 1990. The genera of Betulaceae in the southeastern United States. *Journal of the Arnold Arboretum* 71:1–67.

Goldblatt, P., and D.E. Johnson (eds.). Index to plant chromosome numbers 1979–2008. Missouri Botanical Garden, St. Louis. Available online at http://mobot.mobot.org/W3T/Search/ipcn.html (accessed 10/23/2008).

Heer, J. 1840. *In* Hegetschweiler, Flora der Schweiz. Fr. Schulthess, Zürich. 1008 pp.

Hemsley, W.B. 1906. Some new Chinese plants. *Bulletin of Miscellaneous Information Royal Botanic Gardens, Kew* 5:147-163.

Humboldt F.A., A. Bonpland, and C.S. Kunth. 1817. Nova genera et species plantarum 2. Lutetiae Parisiorum. 323 pp.

Jiang, G.M., and W.M. He. 1999. Species- and habitat-variability of photosynthesis, transpiration and water use efficiency of different plant species in Maowusu sand area. *Acta Botanica Sinica* 41(10):1114–1124.

Kimura, A. 1928. Ueber *Toisusu*, eine neue Salicaceen-Gattung und die systematische Stellung derselben. *Botanical Magazine* (Tokyo) 42:287–290.

Knowlton, F.H. 1926. Flora of the Latah Formation of Spokane, Washington, and Coeur d'Alene, Idaho. *US Geological Survey Professional Paper* 140-A:1–55.

Kovar-Eder, J., and J.J. Wójcicki. 2001. A Late Miocene (Pannonian) flora from Hinterschlagen, Hausruck lignite area, Upper Austria. *Acta Paleobotanica* 41(2):221–251.

Leitch, A.R., D.E. Soltis, P.S. Soltis, I.J. Leitch, and J.C. Pires (eds.). 2004. Biological relevance of polyploidy: Ecology to genomics. *Biological Journal of the Linnean Society* 82:409–700.

Linnaeus, C. 1753. Species Plantarum. Holmiae. 1200 pp.

Marshall, H. 1785. *Arbustum Americanum.* Philadelphia. 174 pp.

Masterson, J. 1994. Stomatal size in fossil plants: Evidence for polyploidy in majority of angiosperms. *Science* 264:421–424.

Miki, S., and S. Hikita. 1951. Probable chromosome number of fossil *Sequoia* and *Metasequoia* found in Japan. *Science* 113:3–4.

Murray, E. 1983. Notae Spermatophytae No. 2. *Kalmia* 13:2–32.

Nazarov, M.I. 1936. *Salix.* Pp. 23–216 *in* V.L. Komarov (ed.), Flora of the USSR 5. Izdatel'stvo Akademii Nauk SSSR, Moskva, Leningrad.

Nonami, H. 1998. Plant water relations and control of cell elongation at low water potentials. *Journal of Plant Research* 111:373–382.

Pierce, W.P. 1937. The effect of phosphorus on chromosome and nuclear volume in violet species. *Bulletin of the Torrey Botanical Club* 64:345–355.

Rember, C.W. 1991. Stratigraphy and paleobotany of Miocene lake sediments near Clarkia, Idaho. Ph.D diss. University of Idaho, Moscow, ID. 180 pp.

Roth, J.L., and D.L. Dilcher. 1979. Investigations of angiosperms from the Eocene of North America: Stipulate leaves of the Rubiaceae including a probable polyploid population. *American Journal of Botany* 66(10):1194–1207.

Rowlee, W.W. 1900. North American willows. *Bulletin of the Torrey Botanical Club* 27:247–257.

Sauter, A. 1849. Mathias Mielichhofer. *Flora* (Regensb.) 32(2):655–667.

Schwanitz, F. 1952. Einige kritische Bemerkungen zur Methode der Bestimmung der Polyploidie durch Messung der Pollen- und Spaltöffnungsgrösse. *Der Züchter* 22(9):18.

Schwanitz, F. 1953. Die Zellgrösse als Grundelement in Phylogenese und Ontogenese. *Der Züchter* 23(1/2):17–44.

Schwanitz F., and H. Pirson. 1955. Chromosomengrösse, Zellgrösse und Zellenzahl bei einigen diploiden Gigaspflanzen. *Der Züchter* 25(7/9):221–229.

Scopoli, J.A. 1772. Flora Carniolica, ed. 2, vol. 2. Krauss, Viennae. 496 pp.

Skvortsov, A.K. 1999. Willows of Russia and adjacent countries: Taxonomical and geographical revision. University of Joensuu Faculty of Mathematics and Natural Sciences Report Series 39. Joensuu, Finland. 307 pp.

Skvortsov, A.K., and M.D. Golysheva. 1966. A study of leaf anatomy in *Salix* in relation to the taxonomy of the genus (in Russian). *Acta Botanica Academiae Scientiarum Hungaricae* 12:125–174.

Stebbins, G.L. 1950. Variation and Evolution in Plants. Columbia University Press, New York. 643 pp.

Thibault, J. 1998. Nuclear DNA amount in pure species and hybrid willows (*Salix*): A flow cytometric investigation. *Canadian*

Journal of Botany 76:157-165.

Trautvetter, E.R. 1832. De Salicibus Frigidis Kochii. *Nouveaux Mémoires de la Société Impériale des Naturalistes de Moscou*, 2:279-310.

Traverse, A. 1988. Paleopalynology. Unwin Hyman Ltd., London, UK. 600 pp.

Trybush, S., S. Jahodová, W. Macalpine, and A. Karp. 2008. A genetic study of a *Salix* germplasm resource reveals new insights into relationships among subgenera, sections and species. *Bio Energy Research* 1(1):67-79.

Villars, M. 1789. Histoire des plantes de Dauphiné 3:759-787. Grenoble.

Wargent, J.J., J.P. Moore, A.R. Ennos, and N.D. Paul 2009. Ultraviolet radiation as a limiting factor in leaf expansion and development. *Photochemistry and Photobiology* 85:279-286.

Wilkinson, J. 1944. The cytology of *Salix* in relation to its taxonomy. *Annals of Botany* 8(30/31):269-284.

Willdenow, C.L. 1806. *Salix*. Pp. 653-710 *in* Species Plantarum 4(2). G.C. Nauk, Berolini (Berlin).

Wolfe, J.A. 1993. A method of obtaining climatic parameters from leaf assemblages. *U.S. Geological Survey Bulletin 2040*. 71 pp.

Xiao, C.W. 2001. Effect of different water supply on morphology, growth and physiological characteristics of *Salix psammophylla* seedlings in Maowusu sandland, China. *Journal of Environmental Sciences* 13(4):411-417.

Zhenfu, F., Z. Shidong, and A.K. Skvortsov. 1999. Saliceae. Pp. 139-274 *in* W. Zheng-yi and P.H. Raven (eds.), Flora of China. Missouri Botanical Garden Press, St. Louis.