The Structure and Identification of the Chromosomes of Pinus radiata D. Don

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Pndroduction

The advance of cytogenetic research in the conifers has been limited by difficulty in identification of individual chromosomes. In *Pinus*, particularly, the karyotypes of a representative range of species show a remarkable uniformity in length of the long metacentric chromosomes, and only one, or sometimes two, shorter submetacentrics can be ocularly distinguished (SAX and SAX 1933, NATARAJAN et *al.* 1961, SAYLOR 1961, 1964, YIM 1963). Measurements of chromosome length and centromere position from large scale photographs or projections have been used to determine the relative dimensions of each chromosome, but no one has shown how to ensure positive identification of individual chromosomes.

Secondary constrictions have been reported in conifer species. However, only in the work of SIMAK (1962) on *Larix* decidua has the use of constrictions been clearly demonstrated to distinguish between chromosomes of otherwise similar dimensions. In most accounts constrictions have been recorded as being too variable in appearance to be used as a diagnostic feature. No study of secondary constrictions in conifers has been published; little seems to be known of their function or behaviour.

In this paper a new development in technique is advanced which promises to allow new advances in conifer cytology. The female gametophyte or endosperm tissue which was first used by SAX and SAX (1933) has received little attention since. It will be shown that chromosome preparations from this haploid tissue may be studied at a premetaphase stage (before the chromosomes have attained the full condensation of metaphase), when constant structural features along the chromosome arms may be seen. These provide markers by means of which each chromosome may be distinguished and a detailed chromosome map made. Premetaphase chromosome preparations provide the evidence to explain the function and appearance of the secondary constrictions and smaller achromatic regions seen in the chromosomes from root meristem squashes.

A cytological diagnosis will be made on some plants whose chromosomes vary from the normal.

Review of Literature

1. Cytological Studies in *Conifers*. – The stages of development of the female gametophyte tissue have been well described by FERGUSON (1904) in her studies of the life history of two Pinus species. It is derived from the basal megaspore which undergoes a small series of divisions to attain a size of about 32 cells, and then enters a long dormant period. About a year later (a month after anthesis in Pinus radiata) it recommences growth, and during a month of great activity some thousands of cell divisions occur. It is this short stage of rapid cell division in a free nuclear state which is ideal for cytological study. Following the formation of cell walls, archegonia, egg cells, and fertilisation, divisions cease and cells mature as storage parenchyma to form the endosperm.

After the pioneer work of SAX and SAX (1933) little use of gametophyte tissue had been made till recently SANTAMOUR (1960), 'SARKAR (1963), and MERGEN and BURLEY (1964) used it for chromosome counts and karyotype analysis. The studies were based on metaphase chromosomes.

Root meristem tissue collected from germinating seeds or potted plants has been the usual material for karyotype studies, particularly since the discovery of colchicine or hydroxyquinoline as a pretreatment to contract the long chromosomes. Other tissues used include the cotyledons of germinating seed (TJIO and ÖSTERGREN 1954, YIM 1963), the meristem of expanding needle bases (MERGEN 1958), and shoot meristems (WINTON 1964), but foliar tissues are difficult to work with and seem suited mainly for chromosome counts on plants from which other tissues are not available.

SIMAK (1962) drew attention to small variations in the relative lengths of each chromosome as measured in squash preparations. These could result in "reversal of order" of certain chromosomes when the chromosomes of a cell were listed in order of length. Similarly he reported some cases of "brachial reversal" when the shorter arm of a metacentric chromosome was measured to be the longer.

SIMAK's data has drawn attention to the need for some characteristic additional to chromosome length and arm ratio for positive identification of individual chromosomes. Attempts to locate secondary constrictions of constant position in each chromosome to serve as structural markers have failed up to the present time because of inability to make consistent observations. An understanding of the nature and behaviour of secondary constrictions is therefore necessary for further progress in conifer cytology.

In the two references to cytological study of Pinus radiata root meristem tissue was used. MEHRA and KHOSHOO (1956) reported the chromosome number to be $2n = 24$, the prevailing number in the Pinuceae, and there were four secondary constrictions. RAO (1958) described the karyotype and reported five pairs of constricted chromosomes.

2. *Secondary* constrictions *and* nucleoli. – The occurrence of secondary constrictions is closely related to the formation of nucleoli in the cell, therefore a brief outline of the behaviour of these two features may be helpful. For longer reviews, see KAUFMANN (1948), VANDERLYN (1948), and VINCENT (1955).

Nucleoli are spherical bodies composed mostly of protein with some RNA, which usually arise at specific points on particular chromosomes at telophase, and which usually disperse prior to the metaphase of the following division. As the nucleolus enlarges, it displaces the portion of chromosome arm distal to it, which maintains continuity through the nucleolus by a chromonematic thread which

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may actually be a spiral of a very low order. Later, as the nucleolus diminishes in volume, the separated parts return together, although contact is not usually achieved, and its position is often revealed at metaphase by a clear achromatic gap, the secondary constriction.

Its width (or the length of the thread) depends partly on the size of the nucleolus but more on the completeness of return of the separated parts together after dispersion of the nucleolar matter. The prominence of constrictions at metaphase may therefore vary from a quite wide gap to a thin achromatic band, or perhaps may not be discernible at all. The point of attachment of the nucleolus to the chromosome has been shown in *Zea* and *Medeola* to be a small darkly staining heterochromatic region called a nucleolus organiser (McCLINTOCK 1934, STEWART and BAMFORD 1942).

In many plant species only one nucleolus is formed per genome and is often associated at the end of one chromosome, thus forming a near-terminal constriction with a small distal beadlike piece of chromosome called a satellite. However, in other species there are two or more nucleoli and the constrictions caused by each may be located at any position along the chromosome arms. In the latter cases a variable number of nucleoli may be present due to a tendency for them to fuse together although the amount of nucleolar matter remains similar from cell to cell. Under certain circumstances nucleoli may be formed on chromosomes or at positions other than the normal nucleolar forming ones.

Secondary constrictions have been found to be associated with nucleolus formation in at least one chromosome of all plants in a large study undertaken by RESENDE (SWANSON 1960). However some constrictions may have no association with nucleolus formation. Some causes ascribed to non-nucleolar constrictions include regions of differential spiralisation and differences in nucleic acid content (both being heterochromatic regions), and sites of special weakness, e. g. the junction of a translocation (KAUFMANN 1948).

Constrictions are thus a structural characteristic of chromosomes, and as a constant feature they can be used as distinctive markers to identify chromosomes of otherwise similar appearance (WARMKE 1945).

Techniques

1. Preparation of Female Gametophyte Tissue.

The female gametophyte tissue is an egg-shaped body situated in the ovules and is at the optimum stage for study when it has reached such a development that it can be readily dissected out without rupture of the enclosing membrane. In *Pinus radiata* it is then 1.0—1.5 mm. long. The material is in optimum condition for study for a period of about two weeks only. For *Pinus radiata* in central Victoria this occurs 3—4 weeks after anthesis, i. e. late September — early October, but for the later flowering *Pinus nigra* it is immediately following anthesis (mid November).

Fixation of collections should be undertaken only when the material is actively dividing, or the results may be very disappointing. The recommended procedure is to place intact conelets in a plastic bag with a little water and hold them under warm conditions, e. g. at 20° C — 25° C, for from eight hours to overnight prior to fixation. This warm treatment usually induces a rise in cell division frequency. In this way collections may be made in any weather, for cell division may cease entirely during cold periods. However, good collections can sometimes be obtained when fixation is undertaken immediately after collection in the field during fine warm weather, particularly following spring showers. The fixative used is freshly mixed absolute alcohol (3 parts) and glacial acetic acid (1 part).

The preparations may be stained with either acetocarmine or FEULGEN, but the best technique involves a combination of both. A gametophyte tissue is dissected from each and hydrolysed in 1 N HCl at 60° C for about 15 minutes, then transferred to FEULGEN solution and allowed to soak overnight. The gametophyte is then placed into 45% acetic acid for a short time prior to further staining. It is transferred to a drop of acetocarmine, ruptured, the enclosing membrane removed, and the cells spread in the stain. After addition of the cover slip, the slide is heated before squashing to achieve better flattening and spreading of the chromosomes. The slide is then made permanent by removing the cover slip under absolute alcohol and remounting with euparal.

Intensification of the acetocarmine stain with additional iron added from the instruments used may be undertaken in order to stain the nucleoli. Hydrolysis is unnecessary if staining with acetocarmine only is intended, however the combination stain described yields more intense and crisper results with minimum staining of the cytoplasm. An additional effect of the hydrolysis is to change the consistency of the thin gametophyte membrane so that it can be easily removed prior to squashing. Without hydrolysis it fragments on rupture of the gametophyte.

2. Preparation of Root Meristems.

The germinating seed with the root about 5 mm. long or the excised root from an actively growing potted plant, was transferred to a Petri dish containing filter paper wet with a 0.2% colchicine solution and left at room temperature for 24 hours. During this period cells accumulated at metaphase due to inhibition of spindle formation, and the chromosomes contracted in length. Roots were then fixed in 3 : 1 alcohol-acetic acid.

The best staining of root meristem preparations was also obtained with a combination of FEULGEN and acetocarmine. After hydrolysis in 1 N HCl at 60° C for 15 minutes the roots were soaked overnight in FEULGEN solution after which the brightly stained meristem was excised and squashed in acetocarmine.

3. Study of microsporogenesis.

Further evidence relating to chromosome structure was obtained from the pachytene stage of microsporogenesis. Shoots bearing staminate strobili were collected during late June-early July at Creswick, Victoria, and kept with their bases in water.

Duplicate collections of staminate strobili at the desired stage were made. One collection was fixed in fresh 3 : 1 alcohol : acetic acid, and the second in a similar fixative to which some ferric chloride had been added. The latter collection was suited to acetocarmine squash preparations, for with the additional iron the staining of the slender chromosomes was intensified, particularly the nucleoli. FEULGEN squash preparations were undertaken with the former collections in order to observe DNA and heterochromatic regions.

4. Measurement of chromosomes.

When the best plates of chromosomes were selected they were photographed on 35 mm. pan film. Prints were prepared at a magnification of about 1800. During the analysis of each photograph it was usual to refer back to the original plate under the microscope for clarification of fine detail. The following measurements and calculations were made: —

(a) total length of each chromosome arm, and total chromosome length. Curved chromosomes were measured by a series of of straight lines along the middle of the chromosome.
(b) arm ratio for each chromosome — ratio of long arm to short arm.
(c) relative length of each chromosome as a percentage of the average chromosome per cell complement.
(d) position of every constriction, whether a clear gap or lightly stained band. The distance from each constriction to the centromere was calculated as a percentage of the length of the chromosome arm. The relative prominence of each constriction was noted.

5. Conventions.

The chromosomes were designated 1—12 in order of decreasing total length.

A concise system of reference to each constriction was considered necessary. The information needed to designate any structural feature is as follows: —

— the particular chromosome (1—12)
— whether long or short arm involved (L or S)
— the position in that arm (denoted by percent distance away from the centromere).

Thus, 8 S 76, designates the prominent secondary constriction found on the short arm of chromosome 8 and situated 76% of the distance along that arm.

Table 1. — Relative lengths of the chromosomes for a range of species in the family *Pinaceae*. — The relative length of each chromosome has been calculated as a percentage of the average chromosome length for the species.

Species	Chromosome												Chromosomes 1—11		Reference
	1	2	3	4	5	6	7	8	9	10	11	12	Range*	Standard Deviation*	
Pinus radiata	112	111	107	106	104	102	101	99	98	96	93	71	19	6.0	This paper
P. patula	114	110	108	105	104	102	102	100	97	94	93	72	21	6.5	PEDERICK (unpublished)
P. greggii	114	110	107	106	104	103	101	100	98	95	91	72	23	6.6	PEDERICK (unpublished)
P. taeda	113	110	106	105	104	104	102	101	98	94	89	75	24	6.8	SAYLOR (1961)
P. strobus	116	109	106	104	103	102	101	98	97	96	91	77	25	6.8	SAYLOR (1961)
P. virginiana	116	110	107	106	105	103	100	99	97	94	90	73	26	7.4	SAYLOR (1961)
P. palustris	115	112	108	105	104	103	101	100	96	94	88	73	27	7.9	SAYLOR (1961)
P. pinaster	115	114	110	108	106	104	100	99	97	89	88	70	27	9.1	PEDERICK (unpublished)
P. pinaster	117	112	108	107	105	103	102	101	95	90	85	74	32	9.4	SAYLOR (1964)
P. halepensis	115	111	108	106	105	105	103	100	97	89	87	73	28	8.6	PEDERICK (unpublished)
P. halepensis	117	113	109	105	104	102	101	101	97	93	86	73	31	8.8	SAYLOR (1964)
P. rigida	117	115	110	107	104	102	100	99	93	92	88	73	29	9.3	YIM (1963)
P. canariensis	116	113	109	106	105	104	101	100	98	92	86	71	30	8.7	PEDERICK (unpublished)
P. densiflora	119	112	109	106	106	103	103	101	99	90	84	68	35	9.7	SAYLOR (1964)
P. resinosa	119	112	109	108	107	103	102	99	98	93	83	68	36	9.8	SAYLOR (1964)
P. nigra	121	115	111	110	105	104	100	97	94	89	84	68	37	11.3	PEDERICK (unpublished)
P. nigra	118	113	110	107	106	105	103	98	98	90	81	71	37	10.5	SAYLOR (1964)
P. sylvestris	120	114	109	109	106	103	101	98	97	89	82	72	38	10.9	SAYLOR (1964)
P. sylvestris	120	114	109	107	107	106	101	101	97	90	79	70	41	11.3	NATARAJAN *et. al.* (1961)
*Picea abies***)	150	113	110	107	106	99	96	94	85	85	83	74	67	18.9	SAX and SAX (1933)
*Abies alba***)	139	127	111	111	108	96	95	88	88	87	80	70	59	18.4	MERGEN and BURLEY (1964)
*Abies nobilis***)	139	131	117	117	116	106	93	91	83	79	66	63	73	22.9	MERGEN and BURLEY (1964)
Larix decidua	144	128	124	118	116	106	88	86	79	74	71	67	73	24.7	SIMAK (1962)

* The range and standard deviation of the relative lengths of chromosomes 1—11 have been presented as statistics to indicate the relative uniformity of the chromosomes (excluding the short No. 12).

** Data for these species may be less accurate since the chromosome lengths were measured from published idiograms. Some chromosomes of *Picea*, *Abies* and *Larix* have high arm-ratios.

Results

1. Chromosomes at metaphase. — In root meristem preparations considerable difficulty was encountered in initial efforts to distinguish between the chromosomes. The small variation in length of the metacentric chromosomes undoubtedly was the main reason. Only the shortest, No. 12, could be ocularly distinguished by length. Subsequent data has shown that the variation in length between the remaining eleven chromosomes in *P. radiata* is less than in any other conifer species studied *(table 1)*. Arm ratio was also of limited use because of similarity between chromosomes, and it was also affected by distortions or non-uniform contraction (which sometimes caused reversal). Arm ratios were useful for separating chromosomes with a ratio greater than 1.10 from the others, but other evidence was essential.

Prominent secondary constrictions were observed as clear cut gaps of varying width in the chromosome arms with a frequency of 1 to 8 per cell. Smaller constrictions appeared as achromatic or light-staining transverse bands varying from only a little less prominent than the secondary constrictions down to barely discernible markings. There was often a slight narrowing or waist in the chromosome associated with these achromatic regions. The main difficulty in identification arose because the appearance of each constriction was not constant from cell to cell. For example, the most prominent constriction was situated on the shorter arm of chromosome 8. Two chromosomes carrying this constriction could be identified in most cells examined, but it varied from prominent to faint, and in some cells it was even difficult to locate. The lesser constrictions were even more variable, thus rendering the correct identification of the homologous pairs a matter of considerable difficulty, particularly since most constrictions occur in relatively similar positions, i. e. in the 40—65 region of the chromosome arms. Four chromosomes with prominent secondary constrictions could usually be identified from cell to cell — 8, 10, 9, 6 (in order of decreasing prominence), but prominent constrictions were sometimes seen in other chromosomes. A means of identifying all the chromosomes with confidence could not be determined. Achromatic or constricted regions were also observed in chromosomes not pretreated with colchicine *(fig. 2)* but a study of this material was not continued because it was difficult to find cells in which chromosomes were not bunched or superimposed, and stretching of chromosome arms was common.

Metaphase chromosomes from female gametophyte preparations *(fig. 3)* possessed the advantage of haploid material in that there was no chance of error due to incorrect identification of the homologous pairs, but the pattern of constrictions was similar to that found in root meristem preparations, and little further progress was made. The curved chromosomes were more difficult to measure.

2. Chromosomes at premetaphase. — The evidence needed to provide the key for chromosome identification and an understanding of chromosome structure was discovered when some plates of female gametophyte preparations were observed with well spread chromosomes not fully condensed to the metaphase state. The condensation had proceeded to the stage when they had separated sufficiently to observe the full length of each *(fig. 4)*. This stage is termed premetaphase. The length of these chromosomes from suitable plates ranged between 20—40 μ although some usable preparations were up to 70 μ. The shortened metaphase root chromosomes were only 10—15 μ long.

Along the length of each chromosome a number of discontinuities were observed. Some were large gaps some-

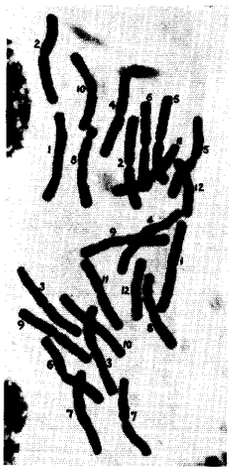

Fig. 1. — Metaphase in a root meristem cell, *P. radiata*, pretreated in 0.2% colchicine for 24 hours at room temperature (about 70°F). Stained with both Feulgen and acetocarmine. (×1000)

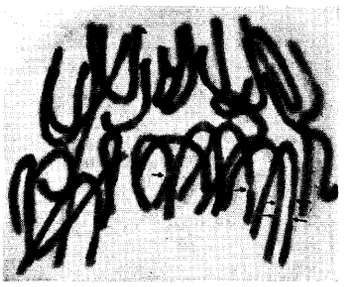

Fig. 2. — Metaphase in a root meristem cell, *P. radiata*. The chromosomes have not been shortened by a pretreatment. Arrows indicate the positions of some constrictions. Stained by Feulgen. (×1000)

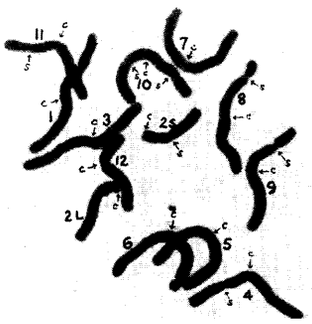

Fig. 3. — Metaphase chromosomes from female gametophyte tissue, *P. radiata*. Each chromosome has been identified. Chromosome 2 has mis-divided at the centromere, the two arms being independent (see text). The position of the centromeres *(c)* and some secondary constrictions *(s)* are indicated. Stained with acetocarmine only. (×1000)

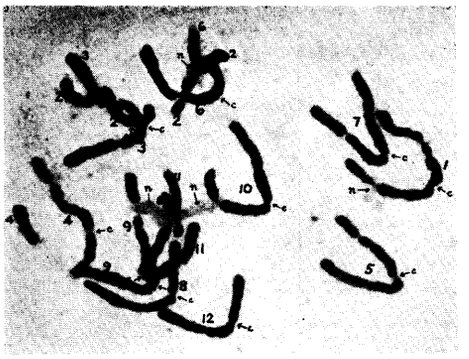

Fig. 4. — Premetaphase chromosomes from female gametophyte tissue, *P. radiata*. Each chromosome is identified. The position of centromeres *(c)* and nucleolar matter *(n)* is marked. Some constrictions are associated with nucleoli; many other constrictions are visible. Stained with acetocarmine. (×1000)

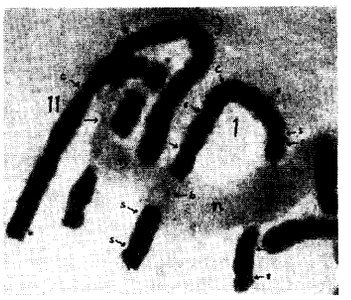

Fig. 5. — Premetaphase chromosomes from female gametophyte tissue showing the chromatin threads which traverse the nucleoli. Small bodies may be seen on the thread within the nucleolus *(b)*. Centromere denoted *(c)*, nucleolus *(n)*, small constrictions *(s)*. Stained with acetocarmine. (×1500)

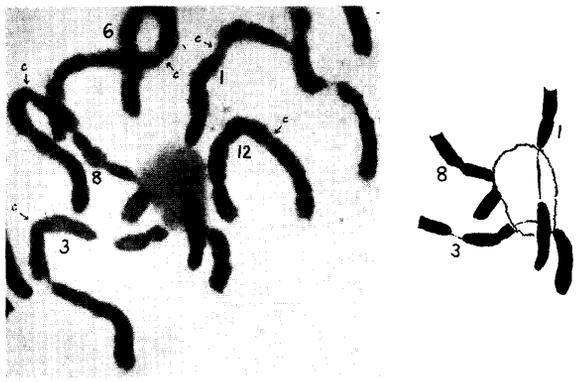

Fig. 6. — Premetaphase chromosomes from female gametophyte tissue showing three chromosomes associated with one nucleolus. The distal portions of these chromosomes have been displaced varying distances. Note that in this case the distal portion of chromosome 8 has only been displaced a short distance. Stained with acetocarmine. (×1500)

times connected by a fine chromatin thread, others appeared as small gaps, achromatic regions, lightly stained bands, or simply a short narrowing or "waist" in the chromosome. In iron-enriched preparations in which one or more nucleolar bodies were visible, some of the larger discontinuities were seen to be associated with the nucleoli. In these cases a fine chromatin thread traversed the nucleolus connecting the separated parts of the chromosome *(fig. 5)*. Occasionally this thread was observed to be double, i. e. a pair of fine parallel threads, indicating the replicated structure of the chromosomes at this stage. Sometimes one or more small chromatin grains were seen on the thread within the nucleolus *(fig. 5)*. Frequently more than one chromosome was associated with one nucleolar body and in such cases it was necessary to follow the connecting chromatin thread through the nucleolus to ensure correct identification of the separated parts of each chromosome *(fig. 6)*. The chromatin thread did not necessarily pass through the widest part of the nucleolus. Although the nucleolar constrictions were traversed by a chromonematic thread, no such thread was observed traversing the smaller discontinuities. If present, and not spiralled, such a thread could be too thin for resolution by the light microscope. The

number of nucleoli per cell varied from one to four or more. When more than one was present, the individual volumes were smaller than that of a single nucleolus. Sometimes there were some very small ones *(fig. 4)*. The position of the centromeres was usually easy to locate since the chromosomes usually bent at these regions; however they were not dissimilar in appearance to some of the other achromatic regions.

When the lengths and arm ratios of these premetaphase gametophyte chromosomes were compared with corresponding data from metaphase root meristem cells it was found that the position of each nucleolar constriction in the former corresponded to a secondary constriction in a chromosome of similar dimension from the latter. Chromosomes 8, 10, 9, and 6 could thus be identified in both tissues. Some of the non-nucleolar constrictions of premetaphase could be seen as the faint achromatic regions and "waists" in the best stained metaphase preparations. The ease of viewing constrictions declined as the degree of condensation of chromosomes advanced to metaphase. In relatively long chromosomes up to 10 constrictions could sometimes be seen, whereas approaching metaphase the number was usually limited to the 3—4 most prominent.

3. Variation in constriction appearance. — In the course of these comparisons some important variations in the appearance of specific constrictions were observed. Although a nucleolus was always observed on chromosome 8 there was not always one associated with chromosomes 10, 9 and 6 at their typical positions. A small achromatic zone or waist was usually seen instead. Nucleoli also formed occasionally at other positions. Some sites have been observed with a nucleolus association only once or twice in several dozen cells analyzed. The frequency of nucleolus activity at different chromosome sites is listed in *table 2.*

Variation of the non-nucleolar constrictions was also observed. A particular constriction which was seen in some preparations as a clear gap was observed in other cases as a fine or indistinct waist and sometimes was not visible at all. There were many preparations in which only one of a pair of adjacent constrictions was visible. However in replicate preparations it was often likely to be the other constriction which was observed. It was therefore necessary to examine several replicates of the same chromosome in order to account for all constrictions.

4. Chromosome dimensions. — When measurements from several premetaphase cells were compared it was possible to identify similar chromosomes from cell to cell with confidence, using the constrictions as markers. In this way 12 chromosome patterns of relative length, arm-ratio, and constriction position were established. Then, with these patterns and a knowledge of constriction variation, it was possible to identify completely all chromosomes in cells from root meristem preparations. Only metaphase plates of optimal staining could be analysed since it was necessary that a number of minor constrictions be visible, particularly in chromosomes 3, 4, 5 and 7 which normally do not contain prominent constrictions.

The relative lengths and arm ratios of the chromosomes calculated from 29 female gametophyte plates from 4 trees are compared with similar data from 12 root meristem plates (from different plants) in *tables 3 and 4.* The relative length of each chromosome was first calculated in each plate as a percentage of the average chromosome of that plate. Relative length was therefore based on total length of chromosome per cell and not on the longest chromosome present which might be subject to stretching. The differences between the two estimates of relative length for each chromosome *(table 3)* were not statistically significant, therefore a combined average was determined. The similarity between the estimates of chromosome length from the two types of tissue suggests that the contraction of chromosomes from about 50 μ down to 10 μ takes place relatively uniformly along their length.

The limitations of the use of length alone for identification of chromosomes in *Pinus radiata* may be demonstrated by a hypothetical example of an unknown chromosome of relative length, say 104. The 95% confidence limits of chromosome length in preparations of good quality is approximately ± 5. Therefore the unknown could be one of the chromosomes with relative length between 109 and 99, i. e. chromosomes 3—7. The employment of constriction posi-

Table 2. — Chromosome constriction sites at which nucleolus formation has been observed during premetaphase, arranged in approximate order of frequency of formation.

Chromosome	Approximate frequency of nucleolus formation				
	Regular >95%	Usual 80—95%	Frequent 40—80%	Occasional 10—40%	Uncommon < 10%
1				1S58, 1S65	1L54, 1L60, 1L67
2				2S58	2S65, 2L70
3				3S64	
4					4L59, 4L68
5				5S64, 5L66	5S50 5L59
6		6S63			
7					7L53, 7L62, 7S63
8	8S76				
9		9L59		9L67	9S54
10		10S62			
11					11S48, 11S64, 11L56
12					

Table 3. — Relative lengths of *Pinus radiata* chromosomes.

Chromosome	Root Meristem		Female Gametophyte		Combined Mean
	Mean	Standard Error n = 24	Mean	Standard Error n = 29	
1	111.8	0.83	112.8	0.96	112.3
2	110.4	0.72	111.9	0.47	111.1
3	106.8	0.36	107.3	0.49	107.0
4	105.8	0.44	106.4	0.45	106.1
5	104.2	0.42	103.4	0.37	103.8
6	102.2	0.32	102.7	0.36	102.4
7	101.1	0.30	100.3	0.39	100.7
8	99.2	0.70	97.8	0.71	98.5
9	93.7	0.45	93.1	0.44	98.4
10	96.4	0.53	96.7	0.44	96.5
11	93.8	0.77	92.9	0.64	93.3
12	70.9	0.53	70.8	0.62	70.8

Table 4. — Arm ratio of *Pinus radiata* chromosomes.

Chromosome	Root Meristem		Female Gametophyte		Combined Mean
	Mean	Standard Error n = 24	Mean	Standard Error n = 29	
1	1.042	0.007	1.058	0.009	1.05
2	1.182	0.010	1.181	0.011	1.18
3	1.071	0.008	1.075	0.012	1.07
4	1.046	0.008	1.076	0.011	1.06
5	1.081	0.010	1.072	0.009	1.08
6	1.133	0.008	1.149	0.013	1.14
7	1.040	0.007	1.065	0.010	1.05
8	1.065	0.008	1.082	0.013	1.07
9	1.059	0.014	1.047	0.008	1.05
10	1.116	0.011	1.126	0.009	1.12
11	1.165	0.011	1.172	0.010	1.17
12	1.636	0.013	1.641	0.009	1.64

tions and arm ratios is therefore absolutely essential for correct identification.

Differences between the two estimates of arm ratio were also not significant, and a combined ratio was determined for each chromosome *(table 4)*. The similarity between the arm ratios of many of the chromosomes resulted in this property being of least diagnostic value. Its main use was as an aid to distinguish the chromosomes with a ratio greater than 1.10 from the others. Examples of brachial reversal, i. e. when the long arm appeared shorter in the specimen, were recorded in the chromosomes with an arm ratio nearest to 1.0, and even up to 1.07.

5. Preparation of a chromosome map. — An idiogram was prepared *(fig. 7)*, based on the estimates of relative length *(table 3)* and arm-length ratio *(table 4)*. The constriction positions were determined from over 30 female gametophyte plates prepared from 6 trees, each estimate having a precision of usually less than ± 1.0 length units. The 95% confidence limits for the positions of the well separated constrictions on the short arm of chromosome 8 are listed as follows: —

Constriction	Observations	Mean	95% Confidence Limits
8 S 30	27	29.85	± 0.75
8 S 42	18	42.11	± 1.16
8 S 60	23	59.65	± 0.82
8 S 76	35	75.74	± 0.69

The actual number of constrictions is undoubtedly greater than that shown in *fig. 7*. Only the more prominent ones have been depicted. Occasional observations of other very small constrictions in the central and distal portions of the chromosomes were made, but they were not mapped being too small for diagnostic purposes. It is also likely that in some cases observations from two adjacent sites made on different plates have been grouped together. This is most likely in cases where there are numerous small constrictions which do not vary much in degree of prominence, e. g. in chromosome 1.

6. Observations of pairing of chromosomes at pachytene. — At the pachytene stage of microsporogenesis the chromosomes are characteristically very long paired threads entwined together into a 'ball'. A paired chromomere arrangement is evident. Studies of chromomere arrangements almost seem to be beyond the limit of current techniques. However, exceptionally clear staining of the chromomeres was obtained with an acetocarmine preparation from one particular tree, and after close scrutiny a number of examples of short lengths of 3—4 chromomeres were observed in which the chromomere sequence was different on each of the paired chromosomes. However, for lack of further evidence these differences were interpreted as an artifact of fixation and slide preparation rather than non-homologous chromomere arrangements.

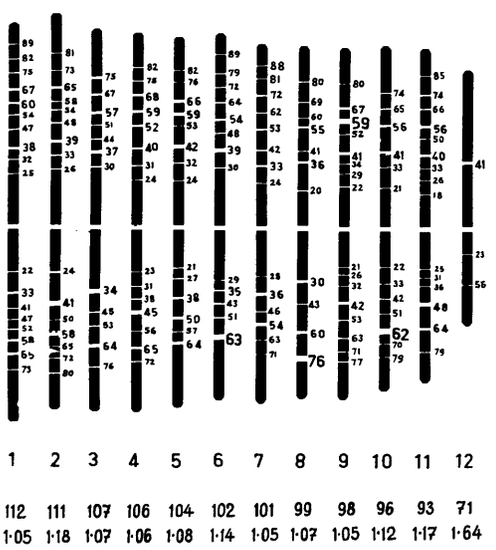

Fig. 7. — Idiogram of *Pinus radiata* chromosomes, including the position of constrictions. The width of each constriction has been drawn to indicate its relative prominence.

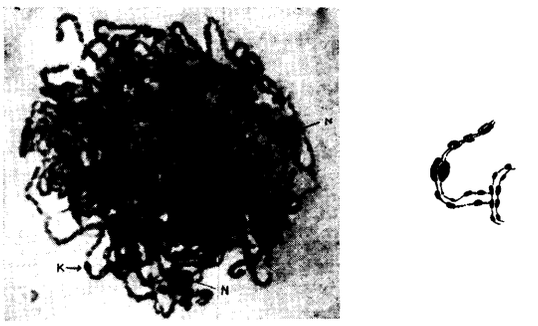

Fig. 8. — Pachytene stage of microsporogenesis, stained with acetocarmine. The paired chromomeric structure of the chromosomes is visible. A dark staining knob *(K)* and two nucleoli *(N)* are indicated. — On the right: A line drawing showing closer detail of the knob structure. (×1000)

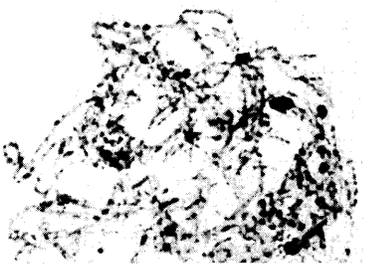

Fig. 9. — Chromosomes at the pachytene stage of microsporogenesis. Stained with Feulgen. The fine paired chromomeric structure can be seen, as well as numbers of dark staining heterochromatic regions. The intersection of chromosomes through the larger heavily stained regions indicates the temporary fusion of the heterochromatin. (×1000)

However, some densely stained paired bodies 5 to 10 times larger than chromomeres were observed similar in appearance to the 'knobs' of maize *(figs. 8, 9)*. In Feulgen preparations many such bodies were seen in each nucleus and it appeared that these heterochromatic regions tended to adhere together to form chromocentres. It was not possible to determine where these heterochromatic regions were situated in their respective chromosomes.

7. Identification of chromosomes in cytologically aberrant plants. —

(1) *Aneuploids:* — Three plants with 25 chromosomes have been found.

(a) A 14 year old tree growing at the Forest Research Institute, Canberra, was recorded as being of atypical appearance when planted. The female gametophyte tissues were found to segregate for 12 and 13 chromosomes. Analysis of premetaphase cells with 13 chromosomes revealed the tree to be trisomic for chromosome 11.

(b) When making root squashes from germinating seed, one seed was found to have cells with 25 chromosomes.

Identification of the trisomic chromosome was simple — it was number 12, since three shorter chromosomes with submedian centromeres were present.

(c) A one year old seedling raised from an open pollinated seed lot was slower growing than others of the same progeny grown from seeds of the same weight. Positive determination of the trisomic chromosome from root meristem preparations was very difficult since it was necessary to produce a perfectly stained and spread plate with clear constrictions in all 25 chromosomes. This could not be achieved due mainly to an inability to observe sufficient constrictions in some chromosomes. It is thought that the trisomic chromosome is 3 or 4 or 5.

Case (c) exemplifies the limitations encountered in critical cytological analyses in *P. radiata* when only root meristems are available for study, whereas in case (a) when the female gametophyte tissue was available a positive diagnosis could be made.

(2) *Chromosome misdivision at the centromere:* — A 30 year old tree was found to have one chromosome separated at the centromere *(fig. 3)*. This condition was permanent and was inherited in a Mendelian fashion by half its progeny. Examination of constriction positions and chromosome lengths in premetaphase gametophyte plates revealed that the two separated arms belong to chromosome 2. It appears that the misdivision has occurred directly through the centromere because at metaphase of meiosis in microspore mother cells both arms have terminal centromere behaviour and orientate towards the same pole. Furthermore, each separated arm must have sufficient centromere activity to survive.

A second example of centromere misdivision was found in another tree in which case chromosome 4 was affected.

(3) *A large deletion:* — A slow growing seedling with very short bunched foliage collected in a forest nursery was found to be diploid when root tissue was examined. However, one chromosome was very short, with a centromere near one end *(fig. 10)*. It was identified as a chromosome 2 with most of the long arm missing, since the constriction pattern of the complete arm corresponded to the short arm of chromosome 2, and only one other chromosome 2 was present. The aberration was present in all roots examined.

Measurements indicate that about 85% of the long arm has been lost, equivalent to about 45% of chromosome 2. The genic unbalance caused by this loss appears to have affected the plant in a manner similar to the genic unbalance seen in some *P. radiata* auto-tetraploids found among forest nursery stock.

Fig. 10. — Metaphase in root meristem of a plant with a deletion of most of long arm of chromosome 2. The chromosomes have all been identified. Stained with both Feulgen and acetocarmine. (×1000)

Discussion

The female gametophyte of *Pinus* provides an excellent tissue for cytological study when collected under optimum conditions. Similar well spread premetaphase plates have never been seen in root meristem preparations. The superiority of the former appears due to a slower rate of chromosome condensation to metaphase so that numbers of cells can be fixed with chromosomes only partly condensed, and also to the free nuclear state which allows the chromosomes to spread well when squashed.

Observations on the position and frequency of nucleoli in female gametophyte chromosomes at premetaphase have provided evidence needed to understand the variable nature of the secondary constrictions seen at metaphase in root meristem chromosomes. When the nucleoli disperse at the onset of metaphase the separated parts of the chromosomes tend to return together, although contact is not usually achieved at metaphase and the gap remains traversed by a chromonematic thread, appearing as a secondary constriction. The width of the constriction may indicate the amount of chromosome displacement caused by the nucleolus, but it does not necessarily indicate the size of the nucleolus since the thread sometimes passes across one side of the nucleolus. The same constriction can therefore be of different widths in sister cells.

The nature of the small chromatic grains often seen on the middle of the nucleolar thread is not known. It may be just an incompletely despiralled remnant of the thread. Similar observations have been made in other tissues, e. g. in mouse tumour cells (MELANDER 1963). They do not appear to be chromomeres or nucleolar organisers.

The nucleolar matter of a cell is not concentrated in one circular mass as in *Zea mays*, but may occur in several separate portions, each of different size, which may flatten out into irregular shapes. Two or more chromosomes may be associated with the same portion of the nucleolus.

Nucleoli are not always formed at the same positions. There appears to be a gradation of frequency of nucleolus association at the various constriction sites. Thus, 8 S 76 always has a nucleolus, 10 S 64 usually has a nucleolus, but other sites e. g. 11 S 48, only rarely are associated with a nucleolus. Since nucleoli occasionally form at atypical positions which would be seen at metaphase as secondary constrictions, confusion in identification of chromosomes from root cells would be likely unless other structural features were also evident. The variation in the positions of nucleolus formation may be due to competition between sites for nucleolus formation. After telophase the nucleolar material formed over the chromosomes gradually aggregates. A variety of sites for nucleolus aggregation appears to be available and those sites with the greatest capacity would tend to organise nucleoli most often. Such a competition has been suggested by Ho (1964) in the case of *Ginkgo biloba*.

The structural discontinuities are usually situated at regular intervals along the chromosomes, e. g. in chromosome 2 there is a constriction every 6 to 8 units of length. Whereas the larger discontinuities are associated with nucleolus formation, the function of the smaller ones is not known. Probably they also have a nucleolus-organising function but of a very low capacity and rarely carry out this function, although they might function as such during the early stages of nucleolus aggregation in telophase. However, some of them are visible in well stained metaphase preparations as achromatic gaps of varying promi-

nence and sometimes by a narrowing or waist in the chromosome. On account of their small size they may be termed tertiary constrictions. Although thread connections have not been seen to traverse the latter sites in the longer gametophyte chromosomes it is likely that chromosome continuity is maintained.

The distribution of the constrictions is not at random. The more prominent ones tend to occur in the central portion of the chromosome arms, in the regions 40 to 65, the one major exception being at 76 in chromosome 8 S.

There is likely to be a significant reason for this non-random distribution, probably related to efficiency in nucleolus formation (or some other function). A similar type of non-random distribution was described by LONGLEY (1939) for the location of heterochromatic knobs on the chromosomes of maize for which no adequate reason was advanced.

In *Pinus radiata* there are no satellites in the traditional sense of small terminal bodies connected by a SAT filament or thread on which the nucleolus is organised. From the literature it appears that the secondary-constricted nature of *P. radiata* chromosomes is typical of other *Pinus* species and also throughout the *Pinaceae*. It is certainly not typical of all gymnosperms, however, for *Ginkgo biloba,* the last surviving species of the ancient order *Ginkgoales,* has two pairs of satellited chromosomes (Ho 1964). However, observations by the present writer indicate that *Ginkgo* also has tertiary constrictions.

Since the larger constriction sites, and probably also the smaller sites, are structurally of differential spiralisation at premetaphase they are regarded as composed of heterochromatin.

Small heterochromatic regions have also been seen in pachytene chromosomes, at which stage the heterochromatin is in a condensed phase appearing as small dark-staining knobs when the bulk of the chromosome is despiralled. Since it is not possible to determine the position on the chromosomes occupied by the pachytene knobs there seems no way to determine whether there is any relation between the knobs and the constrictions. However, it has been observed that there are large numbers of both knobs and constrictions, in both cases there being a variation in size. Also, the proportion of the total chromosome length taken up by both knobs and constrictions is very small. It is possible that the condensed heterochromatic knobs seen at pachytene are in fact the same as the constriction sites seen at premetaphase as dispiralled threads contrasting with the condensed euchromatin, but this would imply a complete reversal of the condensation phase, or negative pycnosis, of the heterochromatin at all stages. If the knobs were situated adjacent to the constrictions reversal of condensation would only have to take place at one stage in the cell cycle in each case. The knobs seen at pachytene probably correspond to the small heterochromatin "dots" observed in resting nuclei.

There are no large blocks of heterochromatin in the chromosomes of *P. radiata* as in some other species of plants and animals. Rather, there is a series of small units spaced at fairly regular intervals along the chromosome. Although heterochromatin was once thought to be genetically inert, there is now increasing evidence from studies on many organisms that it has important functions. In *P. radiata* it is obviously connected with nucleolus formation in the cell. It may also have some effect in controlling the positions at which crossing-over takes place, limiting recombination, since crossing-over seems to be supressed in heterochromatic areas (ROBERTS 1965). Heterochromatin is known to have modifying effects on the expression of neighbouring genes (LEWIS 1950, MCCLINTOCK 1951).

Although eleven of the chromosomes are very similar in length and centromere position, a different arrangement of constriction sites has been established for each chromosome, and this has provided the means for identification of individual chromosomes within a cell complement. Premetaphase plates from haploid female gametophyte tissue provided the most suitable material for chromosome identification. The limitation of this tissue is that plants must have attained flowering size. Careful attention to the conditions and timing of collection is essential. Analysis of plates is time consuming and replicates should be analysed in order to study all constrictions and determine their average prominence. However because much detail can be clearly seen on these chromosomes this tissue will allow analyses of a much more critical nature than root meristems, in which the tertiary constrictions are difficult to see. Chromosomes 4, 5 and 7 are the most difficult to identify, since they usually have no prominent constrictions, and there is little difference in length and arm ratio.

A number of cytological aberrants have been located and described in this paper. It seems likely that now with an improved technique for identification and analysis of particular chromosomes, it will be possible to find and study many more cytological variants. The presence of the numerous small constrictions will provide the cytologist with a useful series of markers along the length of each chromosome. It will be possible to compare the chromosomes of this and other related species in order to determine the extent and nature of evolutionary changes in the karyotype, as well as the cytological basis of species relationships. Such a comparison has already been undertaken between *P. radiata, P. patula,* and *P. greggii,* and it has been possible to identify similar constriction patterns in the chromosomes of the three related species (they are all in the group *Insignes* [SHAW 1914]), thus enabling a study of structural modification of the chromosomes from species to species. Some evidence of variation in the position and prominence of some tertiary constrictions has been found between different trees of *P. radiata.* The results will be reported elsewhere.

Conclusions

1. Chromosomes of *Pinus* at the premetaphase stage prepared from female gametophyte tissue are longer and exhibit details of constriction structure not visible in those at metaphase in root tissue.

2. There is little variation in the length of chromosomes in the genus *Pinus* and those of *P. radiata* are the most uniform of a wide sample of species. Positive identification of each chromosome from *P. radiata* root cells has been achieved only with the knowledge of constriction characteristics determined from the female gametophyte tissue.

3. There are many constrictions in the chromosomes of *P. radiata,* and their positions have been mapped. Their size ranges from that of the prominent secondary constrictions to very small tertiary constrictions which are often difficult to see, and many of which may not have been recorded. They are distributed at fairly regular intervals along the chromosomes, but are more prominent in the centre of the chromosome arms.

4. The prominent secondary constrictions are caused by nucleolus formation at particular constriction sites. Sometimes nucleoli are organised at alternative sites, which

gives rise to variation in the position of secondary constrictions from cell to cell. The heterochromatic structure of constrictions has been discussed.

5. A cytological diagnosis of a number of variants has been achieved. Chromosomal aberrations described include — a deletion, misdivision of the centromere (2 cases), 3 cases of aneuploidy (trisomy).

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Summary

Incompletely contracted chromosomes (at premetaphase) found in the haploid female gametophyte tissue of *Pinus radiata* have been used to study the arrangement of secondary and tertiary constrictions which are difficult to see at metaphase in both gametophytic and root meristem cells. Evidence for the structure and function of constrictions has been presented together with an explanation for their variable appearance at metaphase. A chromosome map has been prepared.

With this new knowledge it has been possible to identify all the chromosomes from root meristem preparations using a combination of chromosome length, arm ratio, and the position of constrictions. It has been shown that *Pinus radiata* has less variation in chromosome length than any other conifer for which data is available.

The identification of aberrant chromosomes from certain plants has been described.

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Zwei Funde von Zwittrigkeit an Pappeln der Sektion Aigeiros

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Einleitung

Angaben über das Vorkommen von Zwitterblüten innerhalb der Pappel-Sektion *Aigeiros* existieren bis jetzt nicht allzu häufig; die veröffentlichten Beschreibungen beziehen sich insbesondere auf *Populus thevestina* Dode (Jovanović und Tucović 1959, 1962, Ničota 1961, Žufa 1962). Aber auch an *Populus deltoides* Marsh. und *Populus nigra* L. wurden bereits hermaphrodite Blüten beschrieben (Campo 1963, May 1959, Žufa 1962). Wegen der theoretischen und praktischen Bedeutung von Zwittrigkeit für die Züchtung (s. Seitz 1953 und 1954) soll hier auf zwei weitere Fälle, einmal bei *Populus nigra* var. *betulifolia* (Pursh) Torr. und zum anderen bei einem Klon, der vermutlich aus einer Rückkreuzung einer Wirtschaftspappel mit *Populus nigra* L. entstanden ist, aufmerksam gemacht werden (Sauer 1956/57).

Pflanzenmaterial und Methode

Beide Mutterbäume wurden von Sauer (1955, 1956/57) für Züchtungszwecke ausgelesen. Der eine Baum, eine *Populus nigra* var. *betulifolia* wird hier unter Dornberg 6 geführt und stockt auf Auenlehm im weiteren Überschwemmungsgebiet des Oberrheins. Er galt bis vor kurzem als weiblich. Das zweite zwittrige Exemplar stammt aus der Umgebung von Zagreb/Jugoslawien aus dem Saveschwemmland mit schnellem Wechsel von kiesigen, lehmigen und sandigen Ablagerungen (Zagreb 1). Beide, als Kreuzungspartner benutzte Bäume sind in der Sammlung des Instituts als Pfropflinge vorhanden, von denen Zagreb 1 seit drei Jahren regelmäßig mehr oder weniger stark blüht. Für die Untersuchungen an Dornberg 6 wurden Blütenzweige des Altstammes benutzt. Dazu wurden je 5 Zweige im Zustand der Nachruhe aus der mittleren Stamm- oder Pfropflingsregion entnommen und in Wasserkultur zum Aufblühen gebracht. Die Kätzchen wurden im Spitzentrieb des Zweiges, im er-

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