The Genetics and Embryology of Taiwan Fir (*Abies kawakamii* (Hayata) Ito)*

Andrej Kormutak[1,3] and Jenq-Chuan Yang[2]

1 Summary

The genetic study of Taiwan fir (*Abies kawakamii* (Hayata) Ito) was undertaken aiming to elucidate the genetic status of the species within the genus *Abies*, as well as to clarify the causes of extremely low quality of its seeds.

At the level of chloroplast and genomic DNAs, the species exhibits the closest relationship with *A. homolepis*, both of which belong taxonomically to the section *Homolepides*. Within the group of 15 *Abies* species compared so far, the PCR/RFLP profiles of cpDNA and RAPD amplification patterns of the above species deviate not only from the genetically uniform group of Mediterranean species, *A. alba*, *A. cephalonica*, *A. nordmanniana*, *A. cilicica*, *A. pinsapo*, and *A. numidica*, but also from the species *A. nephrolepis*, *A. sachalinensis*, *A. veitchii*, and *A. koreana* of the section *Elate*, all of which are of Asian origin. Being genetically heterogeneous, Asian firs resemble the North American species *A. concolor* and *A. grandis* of the section *Grandes* and *A. procera* of the section *Nobiles* which have also been found to be genetically differentiated.

The results of the DNA study have closely correlated the established crossability relationship between *A. kawakamii* and some other representatives of firs studied so far. The compatible hybridological relationship is characteristic only for the interspecific combination *A. kawakamii* × *A. homolepis*, as contrasted with a strong reproductive isolation of Taiwan fir from *A. lasiocarpa*, *A. concolor*, *A. alba*, *A. cephalonica*, and *A. cilicica*, respectively. The prezygotic hybridological barrier was found to be responsible for fertilization failures in the interspecific crossings *A. kawakamii* × *A. alba* and *A. kawakamii* × *A.cephalonica*. Taken together, the results of DNA study and artificial hybridization preferentially substantiate the delineation of individual sections within the genus *Abies* as proposed by Liu (1971).

At the intraspecific level, Taiwan fir seems to share a rather high degree of genetic diversity as evidenced by the mean number of 2.2 alleles per locus and the average heterozygosity, he, of 0.283. The coefficient of genetic distance based on the isozyme polymorphism of 2 *A. kawakamii* populations has accordingly been found to average 0.087 suggesting considerable intraspecific differentiation.

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[1] Institute of Plant Genetics, Slovak Academy of Science, Nitra, Slovakia.
[2] Taiwan Forestry Research Institute, 53 Nanhai Road, Taipei 100, Taiwan, ROC.
[3] Corresponding author whose air-tickets and living expenses during his visiting study at Taiwan Forestry Research Institute were supported by National Science Council.
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The process of sexual reproduction of *A. kawakamii* was cytologically investigated from the standpoint of both pollen and ovule development covering the period from differentiated pollen mother cells and megaspore mother cells until the stages corresponding to the shedding of mature pollen and seeds. As far as pollen formation is concerned, the developmental pattern is comparable with those observed in other species of firs including a high sensitivity of the species' microsporogenesis to abrupt declines of temperature. The same is true of the viability parameters of *A. kawakamii* pollen. By its average germinability of 85.7% and the length of pollen tubes averaging 379.7 μm, the pollen of Taiwan fir was found to be at least comparable to pollen fertility typical for other *Abies* species. The conclusion was therefore drawn ruling out low fertility of *A. kawakamii* pollen as a primary cause of the poor quality of its seeds. Considerable variation was observed between individual study trees with regard to both pollen body size and fertility of pollen grains, which was not, however, related to the elevational distribution of the trees.

The course of the fertilization process is illustrated with regard to both the prezygotic and postzygotic stages of ovule development with special reference to the nature of involved retardant factors. The high frequency of polyembryony has, in this connection, been shown to be the most remarkable feature of *A. kawakamii* embryogeny, shared by an overwhelming majority of the ovules processed. On the contrary, the abortive development of embryos was found to represent the most divergent aspect of the species' embryogeny by which Taiwan fir deviates strikingly from the other species of firs in which the process of embryogeny has previously been illustrated. Encompassing both the early and advanced stages of embryogenesis, abortion was shown to be primarily responsible for the low quality of *A. kawakamii* seeds. Except for this disturbance, the deterioration of female gametophyte as well as archegonia degeneration caused by the inhibition of pollen germination at the top of nucellus were found at the prezygotic stages resulting in abortion of a small portion of pollinated ovules.

Key words: Taiwan fir (*Abies kawakamii* (Hayata) Ito), karyological structure, embryology, isoenzyme polymorphism, DNA marker, microsporogenesis, microgametogenesis, pollination, embryogenesis, crossability.

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It is symbolic that the beams of sun, itself a symbol of Taiwan, meet the Island's surface at the elevations where Abies kawakamii *dominates making expressive the beauty of its stands which in spite of being apparent, remains mysterious in many respects.*

2 Introduction

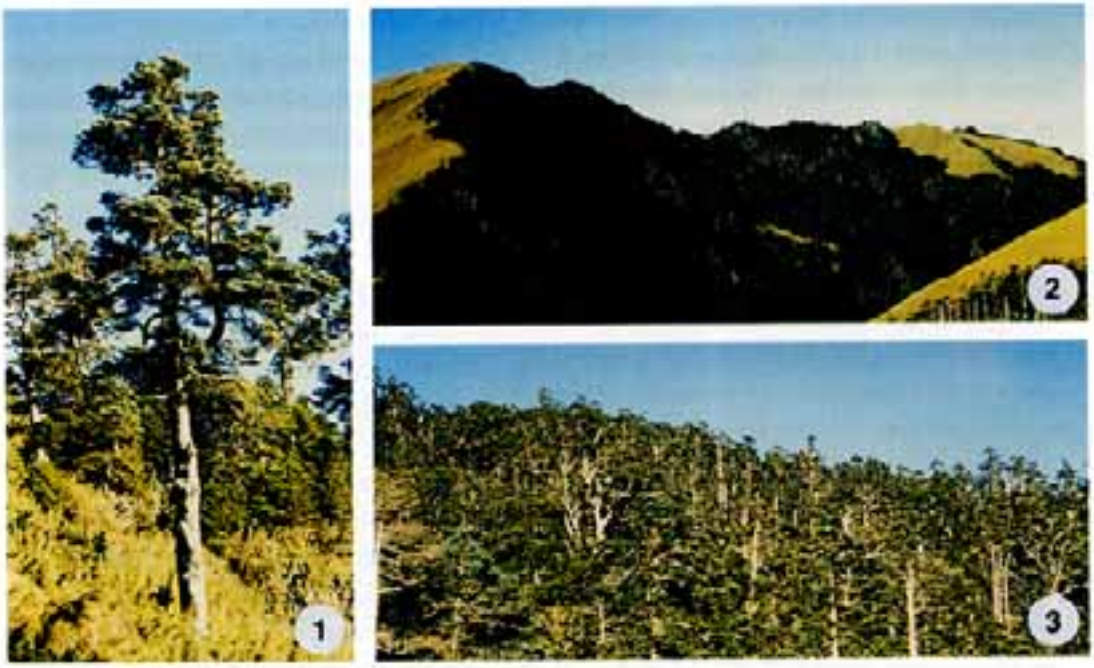
In their recent treatise, Flora of Taiwan (Li and Keng, 1994) have introduced the Taiwan fir (*Abies kawakamii* (Hayata) Ito) as a truly endemic species of Taiwan which dominates the subalpine vegetation zone throughout the entire length of the Central Mountain Range. Within a span of its elevational distribution between 2800 and 3600 m (Hsieh *et al.*, 1994), the species varies considerably with regard to height growth performance of individuals. At the lower boundary of the zone, trees usually reach 17-20 m in height and 1 m in diameter, while at their uppermost limit they are of dwarf appearance reaching only a few meters' height.

Taxonomically Taiwan fir has been dealt with since 1913 (Patschke, 1913), but its position within the genus does not seem to be settled

definitely. According to the most recent taxonomic account of the genus *Abies* by Farjon and Rushforth (1989), the species belongs to the subsection *Laterales* of the section *Balsamea* where it shares a common position with *A. sibirica*, *A. balsamea*, and *A. lasiocarpa*. On the contrary, Liu (1971) positioned Taiwan fir in the section *Homolepides* with *A. holophylla*, *A. homolepis*, and *A. mariesii* as co-members, thus separating *A. kawakamii* from *A. sibirica*, *A. balsamea*, and *A. lasiocarpa* which, according to the author's classification system, belong to the sections *Picita* and *Balsamea*.

In spite of the fact that the fiber of Taiwan fir was reported to be convenient material for the pulp and paper industry (Chen, 1967) , the species is presently considered to be of little practical or commercial importance, mainly due to its location at high elevations and the related difficulty in accessing its stands on steep mountain slopes. This circumstance has contributed a great deal to the preservation of Taiwan fir on the Island where it constitutes one of the principal components of the montane dendroflora. Contrary to the postulated low economic benefit of the species, the significance of its extensive forests in stabilizing the high mountain ecosystems can therefore hardly be denied (Figs. 1-3). As was emphasized by Krylov *et al.* (1986), the role of fir forests at these elevations remains indisputable in such ecologically important processes as water retention, soil protection, and maintaining slope integrity.

Sexual reproduction being the only way in which Taiwan fir regenerates naturally is of special interest, as knowledge of it is essential for revealing those mechanisms which govern the maintenance of the species at these elevations. As a matter of fact, our knowledge about the reproductive biology of the species is rather scanty, restricted mainly to data which refer to the



Figs. 1-3. Taiwan fir and its stands at Hohuan mt. Fig. 1. General view of a tree. Fig. 2. Taiwan fir stands at elevations of 3300 m and 3090 m (Fig. 3).

approximate period of flowering and quality of mature seeds (Chen, 1967; Lai, 1994).

With special reference to sexual reproduction, the finding of very low viability of Taiwan fir seeds is particularly distressing and suggests some disturbances during embryogenesis. As reported originally by Chen (1967), depending on the date of collection, the percentage of germinating seeds of the species varies within the limits of 3.7%-10.8% only. The corresponding figure reported recently by Lai (1994) was an average 12.5% germination rate in years of rich seed crops and 4.7% germinating rate in years of poor harvests. Together these data demonstrate unequivocally a relatively low potential of the species to reproduce sexually caused probably by problems which are encountered in development of its reproductive structures. This necessitates the study of those aspects of Taiwan fir reproduction directly related to development of its seeds and which determine their quality in a decisive way. In particular, this is true of the processes associated with pollen and ovule development as well as of the role which the viability of these structures plays in ensuring the normal course of embryo development.

Still another aspect worthy of consideration concerns the genetic status of Taiwan fir within the genus *Abies*, especially its relationships with other species of the genus which in the present study are evaluated from the standpoints of chloroplast and genomic DNAs variation, and crossability of the species with some representatives of European, North American and Asian firs, respectively. At the intraspecific level, the genetic variability of the species is illustrated within and between 2 populations studied as revealed by isozyme gene markers.

3 Karyological structure

The history of karyological investigation of conifers is relatively long reaching as far back as 1915 when the first attempt by Hutchinson (1915) was made to determine the chromosome counts in *Abies balsamea*. Using microtome sections of developing microsporocytes and fertilized ovules of the species and subsequent reconstructions of the parts separated by sectioning, the author however came to an erroneous conclusion, postulating the haploid and diploid chromosome numbers for *A. balsamea* to be 16 and 32, respectively. It was only in 1933 that Sax and Sax (1933) provided the 1st correct estimates of both chromosome counts and chromosome morphology for 56 species representing 16 genera of conifers. The basic chromosome number derived for most Gymnosperms was shown to be 12 with varying proportions of chromosomes which possessed median and submedian centromeres in individual genera compared.

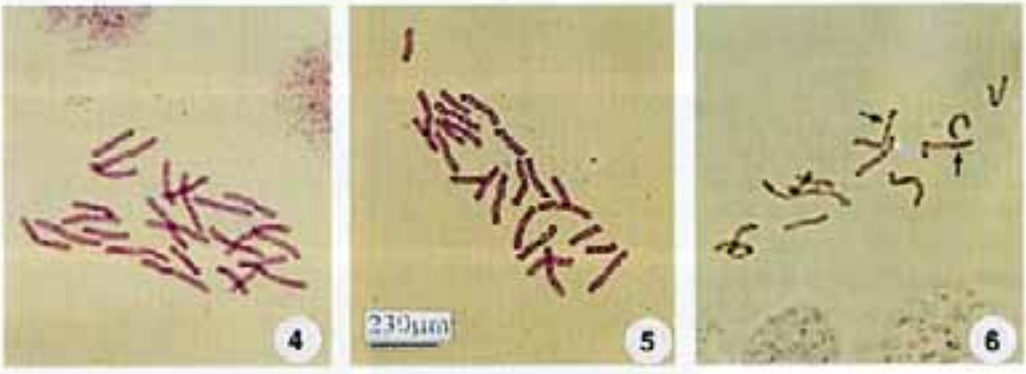
The genus *Abies* was represented in the 1933 study by *A. cephalonica* and *A. concolor*. Both of them were found to contain in their haploid chromosome complements 7 isobrachial chromosomes with median centromeres and 5 heterobrachial chromosomes with submedian position of their centromeres, as compared with only 1 heterobrachial chromosome in the complement of *Pinus*, 3 in *Picea* and 6 in the haploid complement of *Larix*.

The basic karyological formula referring to *Abies* species was confirmed later in a variety of additional species including those of *A. pindrow*, *A. spectabilis*, *A. alba*, *A. cephalonica*, *A. borisii-regis*, *A. numidica*, *A. pinsapo*, *A. concolor*, and *A. koreana* (Mehra and Khoshoo, 1956; Upadhaya, 1975, Moulalis and Illies, 1975; Kormutak, 1985). With special reference to *A. kawakamii*, Hsin (1972) reported on the karyological structure of 8 *Abies* species including *A. balsamea*, *A. fraseri*, *A. grandis*, *A. lasiocarpa*, *A. homolepis*, *A. kawakamii*, *A. mariesii*, and *A. sachalinensis*. In

the haploid sets of these species 6 to 7 chromosomes with median centromeres and 5 to 6 chromosomes with submedian or even subterminal centromeres were recognized. Also, according to Mergen and Burley (1964), the haploid chromosome sets of 6 North American species of firs are characterized by 3 chromosomes that are distinctly heterobrachial and 2 chromosomes in which submedian positioning of their centromeres is controversial. No species-specific differences in this respect were observed according to the authors.

A. kawakamii seems to conform to the classic karyological formula by Sax and Sax (1933) as well, rather than the karyological patterns derived by Hsin (1972) and Mehra and Burley (1964). Based on the cytological examination of 69 cells which originated from 21 root tip meristems, the karyological structure of the species was derived and involves 14 large chromosomes with median position of their centromeres and 10 shorter chromosomes with exclusively submedian location of their primary constriction (Figs. 4-5). At the haploid chromosome level, this structure was confirmed by examining young tissue of developing female gametophytes in cells of which 7 long isobrachial chromosomes and 5 short heterobrachial chromosomes were accordingly recognized (Fig. 6).

In contrast to root tip chromosomes, whose size and morphology were affected by pretreatments with 0.1% and 0.2% colchicine, the chromosome set of a female gametophyte may be considered to be more natural. It represents the corresponding characteristics of these constituents of the nucleus under normal circumstances as found during the early stages of female gametophyte development. It was this type of tissue that Sax and Sax (1933) preferred in their pioneer study on root meristems and which is believed to offer a better opportunity for distinguishing details in chromosome morphology and structure. In the present chromosome sets of *A. kawakamii*, it is the presence of secondary constrictions that are considered peculiar as revealed in non-pretreated chromosomes of female gametophytes but not in the chromosomes of root tip meristems. Fig. 6 shows that this structural feature is shared by the 2 long chromosomes of the species' haploid complement. This aspect of karyological structure of firs is a subject of persisting controversy. No secondary constrictions have, for example, been observed in *A. spectabilis* (Upadhaya, 1975) which is in contrast with findings by Mehra and Khoshoo (1956) who described 2 chromosome pairs of the isobrachial type with secondary constrictions in *A. pindrow*,

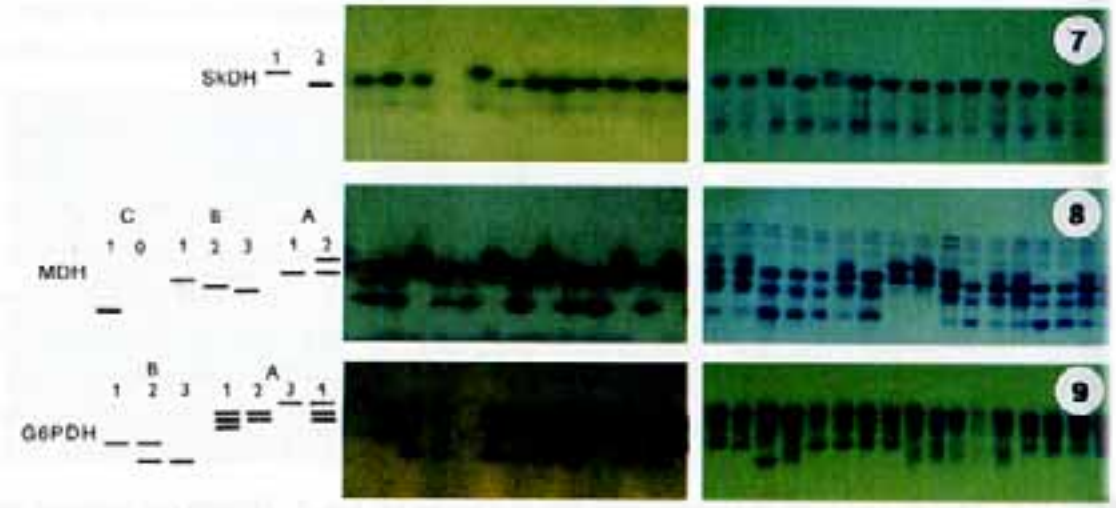


Figs. 4-6. Diploid and haploid chromosome sets of Taiwan fir. Fig. 4. Diploid chromosome set of root tip meristem pretreated with 0.1% colchicine. Fig. 5. Diploid chromosome set of root tip meristem pretreated with 0.2% colchicine. Fig. 6. Haploid chromosome set of female gametophyte with chromosomes containing secondary constrictions (arrows). The bar on Fig. 5 is for Figs. 4-6.

while the same conclusion was drawn in relation to *A. nordmanniana* by Todua (1979). Three chromosome pairs are postulated to possess secondary constrictions in the species *A. sachalinensis* (Mergen and Lester, 1961) as well as in those of *A. alba, A. cephalonica,* and in their hybrid form *A. borisii-regis* (Moulalis and Illies 1975). On the contrary, Mergen and Burley (1964) considered both the presence and position of the secondary constrictions to be variable chromosome markers, on the basis of which it is difficult to reveal substantial differences between firs at the species level. More thorough research is therefore needed to settle this problem definitively with as many species included as possible, and with a unified methodological approach applied.

4 Isoenzyme polymorphism

Among specific advantages of isoenzymes that contribute to their utilization as gene markers in population study of conifers, the codominant nature of the enzyme gene loci together with the absence of epistatic or environmental effects must be mentioned. This enables one to discriminate efficiently between the homozygous and heterozygous carriers of an allele, and has resulted in obtaining the basic characteristics of the population genetic structure of many species. From this it is possibile to assess the extent of genetic variability and its spatial distribution at levels encompassing genes, individuals, and populations, respectively (Hattemer, 1991). In *Abies* this approach has previously been applied to describe the population structural parameters of at least 15 species and 3 naturally occurring hybrids of the total number of 39 species and 9 natural hybrids characteristic of the genus (Liu, 1971). With special reference to Asian firs, the mode of isoenzyme inheritance in *A. sachalinensis* (Nagasaka and Koono, 1990), *A. veitchii,* and *A. homolepis* (Miyata and Ubukata, 1993) as well as the allozyme diversity of *A. sachalinensis* (Matsuura and Sakai, 1972), *A. koreana* (Chung and Lee, 1985), and *A. mariesii* (Suyama *et al.*, 1992) have been analyzed. The results obtained so far only partially corroborate the data of the kind reported for some representatives of European and North American firs, such as a relatively low degree of genetic diversity revealed in *A. mariesii* (0.071) and exceedingly high value of the same parameter



Figs. 7-9. Isozyme banding patterns found in diploid tissue of Taiwan fir with locus designation and allozyme numbers in shikimate dehydrogenase (Fig. 7); malate dehydrogenase (Fig. 8); and glucose-6-phosphate dehydrogenase (Fig. 9).

reported for *A. sachalinensis* (0.714).

Having undergone an isozyme study of 2 of its populations from Mt. Morrison (Yushan) and Hohuan mt., Taiwan fir has provided additional data on the isoenzyme polymorphism of firs extending the information relative to genetic diversity and differentiation of the *Abies* species in the region.

4.1 Allele frequencies

An overwhelming majority of analyzed loci were found to be polymorphic in the 2 populations compared (Figs. 7-9). The only exception was the locus IDH which showed a complete fixation of its IDH-1 allele in the Hohuan mt. population. Also the loci SkDH and MDH-C share their most common alleles occurring in the Mt. Morrison population at frequencies higher than 0.95. The remaining loci did not exhibit any population-specific polymorphism. The compared populations differ only slightly with respect to the allele frequencies of the IDH and MDH-C loci but profoundly regarding the allelic frequencies in the rest of the loci scored (Table1).

4.2 Genetic diversity parameters

The genetic diversity of *A. kawakamii* seems to be rather high. The proportion of polymorphic loci reached a level as high as 71.4% in the Mt. Morrison and 85.7% in the Hohuan mt. populations. The highest mean heterozygosity has as a rule been observed in those loci which exhibit the greatest variation between populations. In particular, this is true of the loci G6PDH-B and MDH-A, whose mean heterozygosities averaged 0.632 and 0.506, respectively. Comparable in this respect are the loci MDH-B (0.498) and G6PDH-A (0.492), whose mean heterozygosities did not, however, exceed 0.5. On the contrary, the loci MDH-C and IDH showed the lowest degree of heterozygosity with a mean value higher than 0.1 in the former and lower than 0.1 in the latter. At the population level, the Mt. Morrison population

Table 1. Allele frequencies and observed and expected heterozygosities for polymorphic loci in 2 populations of *A. kawakamii* [1)]

Locus	Alleles		Mt. Morrison	Hohuan mt.
G6PDH-A		1	0.588	0.875
		2	0.412	0.125
	observed	h	0.000	0.000
	expected	h	0.492	0.222
G6PDH-B		1	0.353	0.788
		2	0.176	0.112
		3	0.471	0.100
	observed	h	0.000	0.225
	expected	h	0.632	0.362
IDH		1	0.941	1.000
		2	0.059	0.000
	observed	h	0.118	0.000
	expected	h	0.112	0.000
MDH-A		1	0.529	0.275
		2	0.471	0.725
	observed	h	0.000	0.000
	expected	h	0.506	0.404
MDH-B		1	0.250	0.625
		2	0.714	0.344
		3	0.036	0.031
	observed	h	0.000	0.000
	expected	h	0.434	0.498
MDH-C		1	0.966	0.939
		2	0.000	0.000
		3	0.034	0.061
	observed	h	0.000	0.000
	expected	h	0.068	0.116
SkDH		1	0.150	0.050
		2	0.985	0.950
	observed	h	0.029	0.100
	expected	h	0.029	0.096
	Average	he	0.325	0.242

[1)] Vegetative buds collected early in the spring from individual trees at Mt. Morrison and Hohuan mt. were analyzed for their isozyme composition using the procedure by Cheliak and Pitel (1984); Sample size ranged between 28 and 34 in the Mt. Morrison and between 32 and 40 in the Hohuan mt. populations; The average heterozygosity per population is the arithmetic mean of the h_e value across all loci.

Table 2. Genetic diversity parameters in the 2 populations of *A. kawakamii* [1]

	Mt. Morrison	Hohuan mt.
Proportion of polymorphic loci	0.71	0.85
Average number of alleles per locus	2.3	2.1
Mean heterozygosity observed[2]	0.021	0.046
	(0.017)	(0.033)
Mean heterozygosity expected[2]	0.325	0.242
	(0.093)	(0.069)

[1] Genetic characteristics of populations were computed from the primary data comprising diploid genotypes by using the BIOSYS-1 computer program for analysing genetic structures from electrophoretic data (Swofford and Selander, 1981).
[2] SE is shown in parentheses.

was characterized by a mean heterozygosity of 0.325 differing considerably from the Hohuan mt. population at only 0.242. As to the total number of alleles in surveyed loci, 16 alleles were found in Mt. Morrison and 15 alleles in Hohuan mt. populations yielding an average of 2.3 and 2.1 alleles per locus in respective populations (Table 2).

In terms of average values, the genetic diversity of *A. kawakamii* is characterized by a 0.78% share of polymorphic loci among the total number of 16 alleles of 7 loci scored so fir with a mean value of 2.2 alleles per locus. The average heterozygosity accordingly averaged at he of 0.283 and ho of 0.033, respectively.

In the set of examined loci, Taiwan fir seems to have a lower number of alleles per locus but a higher mean expected heterozygosity than the overall average for Gymnosperms (A = 2.38; He = 0.169; Edwards and Hamrick, 1995). As fir as *Abies* species are concerned, *A. kawakamii* is comparable with *A. balsamea* and *A. cephalonica* with a reported 2.0-2.1 alleles per locus and an expected heterozygosity of 0.261-0.295 in the former (both parameters refer to 8 polymorphic loci; Neale and Adams, 1985), and with 1.6-2.1 alleles per locus and an expected heterozygosity 0.175-0.290 in the latter (referring to 9 polymorphic loci; Fady and Conkle, 1993). The other species analyzed so far seem to be genetically less diverse than *A. kawakamii* as evidenced by the corresponding parameters in *A. lasiocarpa* (A = 1.6; He = 0.125; 18 loci; Shea, 1987), *A. fraseri* (A = 1.4; He = 0.201-0.403; 4 loci; Diebel and Feret, 1991), *A. mariesii* (A = 1.83; He = 0.071; 23 loci; Suyama *et al.*, 1992), *A. bornmulleriana* (A = 1.8; He = 0.198; 13 loci; Fady and Conkle, 1993), and *A. alba* (A = 1.6; He = 0.169; 12 loci; Fady and Conkle, 1993). Additional data have been reported for *A. alba* which deviate from those given above with regard to both the mean number of alleles per locus (A = 2.89; He = 0.098-0.178; 18 loci; Longauer, 1995) and the He value (A = 1.63; He = 0.107; 13 loci; Vicario *et al.*, 1995). Also, a surprisingly high degree of genetic polymorphism was reported for *A. sachalinensis* (0.714; Nagasaka and Koono, 1990) which may be related to the proximity of the investigated populations to the center of the species' origin in Hokkaido as supposed earlier by Matsuura and Sakai (1972). Thus, except for the above-mentioned *A. sachalinensis*, only the mean number of alleles per locus in *A. alba* and the expected heterozygosity of *A. fraseri* surpass the corresponding characteristics of *A. kawakamii* suggesting a much higher level of genetic diversity in the latter. However, in order to provide more conclusive evidence of the kind, an increased number of loci as well as additional populations of the species should be involved in the comparisons. This species shows an obvious higher genetic diversity relative to the 2 other species of the conifers endemic to Taiwan, i.e., those of *Chamaecyparis formosensis* and *Chamaecyparis taiwanensis*. Both of them were reported to exhibit

much lower levels of heterozygosity than many other conifers. The phenomenon is hypothesized to be due to the bottleneck effect which occurred during the geological history of the Island drastically reducing the genetic variation of the trees (Lin *et al.*, 1994). *A. kawakamii* does not seem to conform to this notion.

4.3 Genetic differentiation

In comparison with some other species of firs, Taiwan fir also seems to be exceptional regarding the degree of genetic differentiation of its populations. This evidence comes from the mutual comparison of the coefficients of genetic identity and genetic distance which were calculated for the 2 populations of the species according to Nei (1978). The respective coefficients were found to reach 0.913 and 0.087, respectively, indicating a fairly high genetic differentiation between the populations at Mt. Morrison and Hohuan mt. In *A. mariesii*, genetic distances between its 3 stands have, for example, oscillated within the limits of 0.007-0.012 only (Suyama *et al.*, 1992), whereas in *A. alba* within the limits of 0.027-0.043 as based on 88 populations of the species located in the eastern part of its natural distribution (Longauer, 1995). More profound differentiation in *A. alba* has, however, been reported for 14 stands in Bavaria with genetic distance coefficients oscillating between 0.020 and 0.159 (Konnert, 1993).

The geomorphological heterogeneity of Taiwan offers a plausible explanation for such a profound differentiation of *A. kawakamii* stands. Its numerous mountains function as efficient barriers which prevent gene flow in the species, thus favoring genetic differentiation. The restriction of gene flow probably occurs irrespective of the occurrence of *A. kawakamii* on the top of the Central Mountain Range. It is highly probable that, in addition to this natural obstacle, the overlap of the rainy season with the period of flowering of the species in spring may be important as well.

5 Genetic status of *Abies kawakamii* as revealed by DNA markers

Molecular genetic markers derived from direct analysis of genetic polymorphism in DNA sequences represent a novel approach in evaluating genetic variability of forest trees. They share several advantages over isozyme markers of which the absence of tissue specificity and ontogenic variability together with stability towards environmental variation and potentially unlimited number are the most frequently mentioned (Neale *et al.*, 1992). With forest trees, more attention has been focused on the variation in their chloroplast DNA (cpDNA) rather than polymorphism in genomic DNA, mainly because of the occurrence of cpDNA in multiple copies which makes its detection easier. Also, the cpDNA molecule is smaller and structurally simpler than nuclear DNA which allows straightforward molecular interpretations of its polymorphism (Palmer *et al.*, 1988). With special reference to the chloroplast genome of conifers, cpDNA variants were found to be distributed non-randomly, with the vast majority being localized in one or a few "hot spots" only. These regions are likely to be composed of DNA that does not encode functional products owing to the fact that it may mutate with little deleterious effect (Straus *et al.*, 1992). In *A. alba* such a region has recently been detected between the genes *trnS* and *psbC*, as evidenced by the restriction site polymorphisms in the corresponding PCR products in 10 populations of the species (Ziegenhagen *et al.*, 1995). As far as *Abies* species are concerned, it is the 2nd

illustration of within-species variation of this kind, following the previous successful attempt by Tsumura *et al.* (1994) in describing the clinal variation of cpDNA in 7 populations of *A. mariesii*. Each of these populations has exhibited 2 frequency variations. No variation, on the other hand, was found in the 3 spacer regions between the t-RNA genes of cpDNA in *A. alba* and *A. nebrodensis* populations investigated by Vicario *et al.* (1995). Also, Tsumura *et al.* (1995) were not able to detect any substantial differences between the 4 *Abies* species native to Japan using RFLP analysis of 6 PCR-amplified genes of cpDNA. However, in their recent paper, the authors reported on the highly polymorphic nature of 2 mitochondrial genes revealed in 5 Japanese *Abies* species and their respective 46 populations (Tsumura *et al.*, 1996). Preliminary information has also been released postulating the existence of both within, and among-population variations in RAPD markers of *A. koreana* (Kim *et al.*, 1996).

Our approach, based on utilization of the universal primer by Demesure *et al.* (1995) in amplification of cpDNA together with the use of RAPD markers, proved efficient in determining the genetic relationships among 15 *Abies* species of diverse geographic origin, including *A. kawakamii*. The taxonomic status and geographic distribution of these 15 species are given in Table 3.

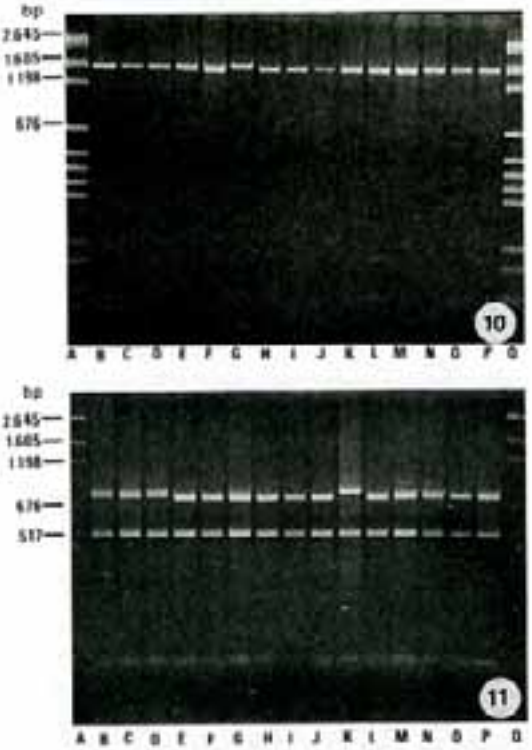
5.1 PCR/RFLP patterns of chloroplast DNAs in some *Abies* species

By using the primer pair of sequences 5' GGT TCG AAT CCC TCT CTC TC 3' and 5' GGT CGT GAC CAA GAA ACC AC 3' (Demesure *et al.*, 1995), the cpDNA fragment was amplified representing the flanking region between the genes *trnS* [tRNA-Ser(UGA)] and *psbC* (psII 44 kD) with an estimated size of about 1600 bp. No differences in size of the fragment were observed among individual species (Fig. 10). Following its digestion with 6 restriction endonucleases, a varying number of restriction fragments were obtained.

Table 3. List of species subjected to analysis of molecular phylogeny [1)]

Species designation	Species (Liu, 1971)	Section	Distribution
B	*A. homolepis* S. et Z.	*Homolepides*	Japan
C	*A. kawakamii* (Hay.) Ito	*Homolepides*	Taiwan
D	*A. nephrolepis* (Trautv.) Maxim.	*Elate*	Eastern Asia
E	*A. sachalinensis* (Fr. Schm.) Mast.	*Elate*	Sakhalin, Japan
F	*A. veitchii* Lindl.	*Elate*	Japan
G	*A. koreana* Wils.	*Elate*	Korean Peninsula
H	*A. alba* Mill.	*Abies*	Cent., South Eur.
I	*A. cephalonica* Loud.	*Abies*	Greece
J	*A. nordmanniana* (Stev.) Spach	*Abies*	Caucasus, Turkey
K	*A. cilicica* (Ant. et Kotschy) Carr.	*Piceaster*	Asia Minor
L	*A. pinsapo* Boiss.	*Piceaster*	Southern Spain
M	*A. numidica* DeLann.	*Piceaster*	Northern Africa
N	*A. procera* Rehd.	*Nobiles*	Western USA
O	*A. concolor* (Gord. et Glend.) Lindl.	*Grandes*	W. USA, N. Mex.
P	*A. grandis* (Dougl.) Lindl.	*Grandes*	Canada, W. USA

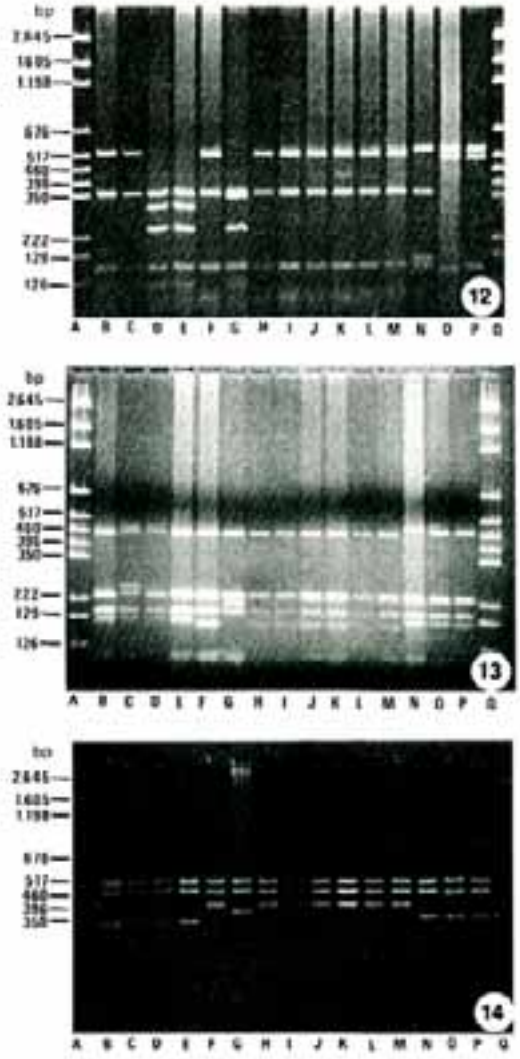
[1)] Individual species were represented in the experiment by only 1 tree growing in the arboretums of Mlynany and Kysihybel in Slovakia except for the A. alba and A. kawakamii trees representing natural stands of these species in Slovakia and in Taiwan, respectively.



Figs. 10-11. Size of PCR-amplified fragment of cpDNAs (Fig. 10) and its *Alu I* restriction fragment length profiles in individual species (Fig. 11). Designation of species as in Table 3.

We have not succeeded in cleaving the amplified fragment using *Rsa I* restrictase. The numbers of restriction fragments generated by the remaining restrictases differed considerably ranging from 2 in *Hpa I* and *Alu I* digests to as many as 6 fragments detected in *Hinf I* digest. One restriction site recognized by *Hpa I* and *Alu I* were identical in all 15 species compared as judged from the size of resulting fragments which were detected in agarose gels (Fig. 11). Species-specific restriction fragment patterns were generated by *Hinf I*, *Taq I*, and *Mva I* only. Of these, the *Hinf I* restriction fragment length profiles were found to be the most consistent with *Abies* taxonomy, reflecting relatively precisely the systematic status of individual species or their groups. As shown in Fig. 12, among Asian firs tested so far, the pair of *A. homolepis* (B) and *A. kawakamii* (C) of the section *Homolepides* is very distinct by its 3 restriction sites and 4 restriction fragments of identical size. This contrasts with the relatively heterogeneous group of species representing the section *Elate*, within which only the pair of species *A. nephrolepis* (D) and *A. sachalinensis* (E) shares identical profiles with 5 restriction fragments involved, while *A. veitchii* (F) and *A. koreana* (G) possess very diverse profiles with 3 and 4 restriction fragments detected, only one of which in each species differs conspicuously by its size. Accordingly, the number of restriction site variants ranges between 2 and 4 within a group. The European species as a whole are characterized by 2 restriction sites and 3 restriction fragments of identical size irrespective of the fact that *A. alba* (H), *A. cephalonica* (I), and *A. nordmanniana* (J) are taxonomically treated as members of the *Abies* section, while *A. cilicica* (K), *A. pinsapo* (L) and *A. numidica* (M) as members of the section *Piceaster*. The North American species *A. procera* (N) of the section *Nobiles* and *A. concolor* (O) and *A. grandis* (P) from the section *Grandes* share differentiated restriction fragment length patterns with 3 restriction sites and 4 fragments in the former and with 2 restriction sites and 3 restriction fragments of uniform size in the latter.

In comparison with the *Hinf I* digest, the banding patterns generated by *Taq I* and *Mva I* do not correlate so closely with the taxonomic position of investigated species. Figs. 13 and 14 illustrate that it is especially true of the species *A. homolepis* (B) and *A. kawakamii* (C) of the section *Homolepides*, whose *Taq I* profiles differ by 1 fragment with an approximate size of 227 bp which was found in *A. kawakamii* (C) but not in *A. homolepis* (B). The latter is indistinguishable by its 4 *Taq I* restriction fragments and 3 fragments of the kind generated by *Mva I* from *A. nephrolepis* (D) and *A. sachalinensis* (E) which belong



Figs. 12-14. Restriction fragment length profiles of PCR-amplified fragment of cpDNAs in individual species obtained after digestions with *Hinf I* (Fig. 12), *Taq I* (Fig. 13), and *Mva I* (Fig. 14).

taxonomically to different sections. Also, the uniform restriction fragment length patterns of *A. procera* (N), *A. concolor* (O), and *A. grandis* (P) derived by *Taq I* digestion make it difficult to discriminate not only between these North American representatives of firs mutually but to some degree also between them and the European species *A. alba* (H), *A. cephalonica* (I), *A.*

Table 4. Number and approximate size of restriction fragments generated for a PCR-amplified segment of cpDNA in individual species of firs by *Hinf I*, *Taq I* and *Mva I* [1]

	bp	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	517	+	+			+		+	+	+	+	+	+	+	+	+
	490														+	+
	350	+	+	+	+	+	+	+	+	+	+	+	+	+		
Hinf	290			+	+											
I	240			+	+		+									
	129													+		
	127	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	75	+	+	+	+		+									
	460	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	228		+													
Taq	222	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I	200						+									
	160	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	120	+	+	+	+									+	+	+
	110					+		+	+	+	+	+	+			
	530	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mva	480	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I	460				+	+	+	+	+	+		+				
	400	+	+	+							+		+	+	+	+

[1] Total DNA was extracted from 5 g of young leaves by a CTAB procedure (Murray and Thompson, 1980); Polymerase chain reaction was performed in a heat-sealed glass capillary using an Idaho Air Thermal Cycler. Each PCR reaction mixture consisted of 50 mM TRIS-HCl buffer, pH 8.5, 20 mM KCl, 2.5 mM MgCl2, 0.5 mg/ml BSA, 200 µg each of 4 dNTP, 60 ng of template DNA, 1.7 units of Taq DNA polymerase (Gibco BRL, Life Technologies GmbH Eggenstein), and 0.54 µ M of primer (Demesure *et al.*, 1995), respectively, in a total volume of 20 µl; Amplification conditions included a total of 45 cycles with template denaturation at 94 °C for 60 s , primer annealing at 37 °C for 7 s, and primer extension at 72 °C for 70 s. during the first 2 cycles. Time for template denaturation was reduced to 1 s. for the remaining 43 cycles. The reactions were further incubated at 72 °C for 4 min and the capillaries were stored at 4 °C before amplification products were digested; The PCR products were digested by *Rsa I*, *Hpa I*, *Alu I*, *Hinf I*, *Taq I*, and *Mva I* using 0.5 ng of amplified DNA, 3 units of respective endonuclease, and 2 µl of relevant endonuclease buffer in a total volume of 20 µl Digestion was performed overnight and restriction fragments generated were separated in 1.5% NuSieve 3:1 agarose (FMC Bioproducts) gel by electrophoresis with Tris-borate buffer; Designation of species is as in Table 3.

nordmanniana (J), *A. cilicica* (K), *A. pinsapo* (L), and *A. numidica* (M), respectively. Both groups have been shown to be differentiated only slightly with respect to the size of their 4 restriction fragments. However, with regard to the *Mva I* profiles, these groups were found to be profoundly differentiated. In light of the present *Taq I* and *Mva I* restriction fragment patterns, the diverse nature is indisputable of both *A. veitchii* (F) and *A. koreana* (G) species which deviate in their respective profiles from each other as well as from the remaining 2 members of the section *Elate* (*A. nephrolepis*-D and *A. sachalinensis*-E). A remarkable feature of *A. veitchii* (F) restriction profiles is their similarity with those of European species as revealed at the levels of *Hinf I*, *Taq I*, and *Mva I* digests. The results of PCR/RFLP analysis are summarized in Table 4.

Based on the number of common fragments, the values of the indexes referring to genetic distances between individual species were calculated (Table 5) and subsequent clustering of the species was done to illustrate the genetic relationships between them. As depicted in Fig. 15, a high degree of homology exists between the cluster pattern derived by *Hinf I* and that in which the combined data of *Hinf I*, *Taq I*, and *Mva I* digestions were taken into account. Conversely, the diverse nature of *Taq I* clustering is primarily due to the changed positions of *A. homolepis* and *A. kawakamii* species on the dendrogram.

The results of cluster analysis based on the combined data of all 3 digestions unequivocally prove close genetic relationships between *A. homolepis* and *A. kawakamii* of the section *Homolepides* and between *A. nephrolepis* and *A. sachalinensis* of the section *Elate* respectively. *Abies koreana*, an additional member of the section *Elate*, was shown to occupy relatively independent position. On the contrary, *A. veitchii* of the same taxonomic status surprisingly exhibited a higher affinity towards the European species than to the Asian firs. Owing to this divergence, it seems reasonable to deal with the above species of Asian firs as a relatively heterogeneous group with an incomparably higher degree of genetic differentiation displayed among its species than among those in Europe. The genetic uniformity of the latter appears to be the most contrasting feature of their cpDNAs in

Table 5. Genetic distances matrix generated by pairwise comparison of shared fragments between *Abies* species [1)]

	B	C	D	E	F	G	H	I	J	K	L	M	N	O
C	0.071													
D	0.200	0.250												
E	0.312	0.352	0.133											
F	0.333	0.375	0.470	0.375										
G	0.375	0.411	0.312	0.200	0.333									
H	0.333	0.375	0.470	0.375	0.000	0.333								
I	0.333	0.375	0.470	0.375	0.000	0.333	0.000							
J	0.333	0.375	0.470	0.375	0.000	0.333	0.000	0.000						
K	0.333	0.375	0.470	0.470	0.153	0.437	0.153	0.153	0.153					
L	0.333	0.375	0.470	0.375	0.000	0.333	0.000	0.000	0.000	0.153				
M	0.214	0.266	0.375	0.470	0.153	0.437	0.153	0.153	0.153	0.153	0.153			
N	0.437	0.470	0.470	0.555	0.588	0.611	0.588	0.588	0.588	0.588	0.588	0.500		
O	0.500	0.529	0.529	0.611	0.647	0.666	0.647	0.647	0.647	0.647	0.647	0.562	0.230	
P	0.500	0.529	0.529	0.611	0.647	0.666	0.647	0.647	0.647	0.647	0.647	0.562	0.230	0.000

[1)] Principal coordinate analysis (PCA) and UPGMA (Sneath and Sokal 1973) were employed to analyze the matrix of Nei's genetic distances; Genetic distance = 1 - genetic similarity. The latter is defined as the share of common fragments of the total number of fragments generated which occur in at least one of the compared pair of species; Designation of species is as in Table 3.

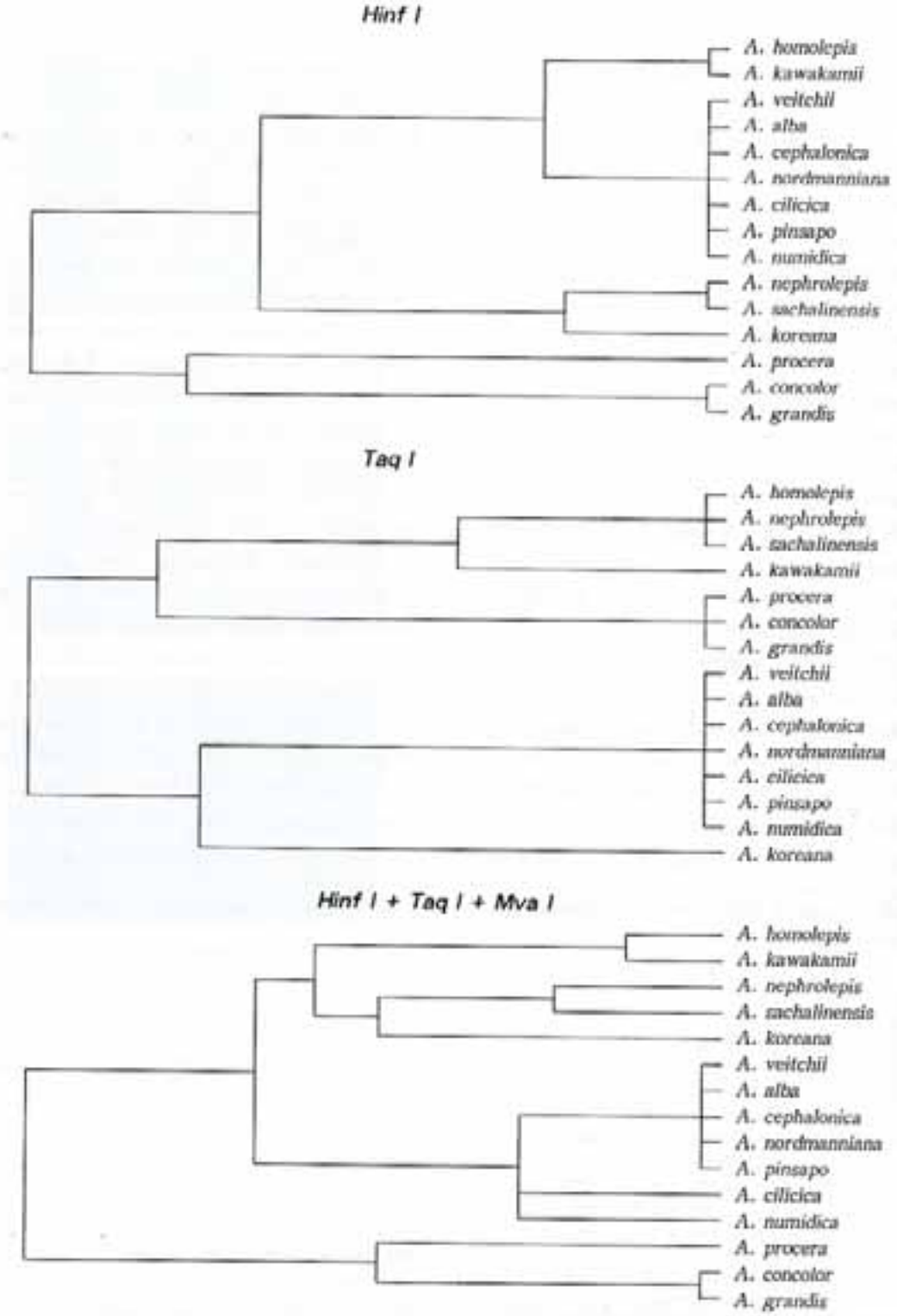


Fig. 15. Schemetic illustration of genetic distances between *Abies* species using the data of Hinf I and Taq I digestions separately as well as combined data of all 3 digestions with *Hinf I*, *Taq I*, and *Mva I*.

relation to both the Asian and North American species of firs. The differentiation ascertained within the North American firs was found to comply even more precisely to the pattern of taxonomic pertinence of the species concerned than in the case of Asian firs. *A. concolor* and *A. grandis* of the section *Grandes* accordingly were shown to occupy the same position which, however, differed considerably from that of the species *A. procera* belonging taxonomically to the section *Nobiles*.

5.2 RAPD amplification patterns in some *Abies* species

The relationships established between individual species on the basis of cpDNA have conclusively been confirmed at the level of genomic DNA as well. Using 12 primers with an arbitrary sequence of 10 nucleotides, the RAPD amplification patterns obtained show a high degree of consistency with the PCR/RFLP patterns depicted above, thus reinforcing the delineation of individual sections within the genus as proposed by Liu (1971).

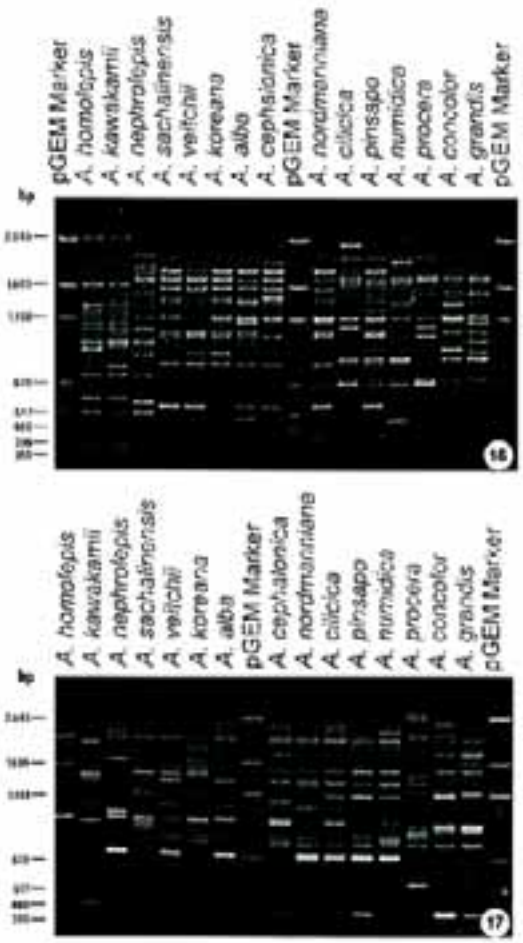
The primer sequences and the number of amplified DNA products of each primer for all the tested species are listed in Table 6. A total of 344 PCR products were scored, of which 315 (91.5%) were found to be polymorphic. The size of the amplified DNA products visualized in 1.5% agarose gels ranged from 350 to 2645 kb (Figs.16-17).

Similarity coefficients calculated from the shared fragments against the total number of amplified fragments in individual pairs of species compared varied widely, being the lowest between the pair of species *A. kawakamii* and *A. procera* (41.8%) and the highest between *A. pinsapo* and *A. numidica* (81.7%; Table 7). The magnitude of the differences observed within individual groups of firs between the highest and lowest coefficients of similarity reached 21.9% and 29% among Asian and North American species, respectively, and only 12.5% among European species. This may be taken as additional evidence supporting the heterogeneous nature of the former 2 groups of firs and the relatively homogeneous nature of the latter at the genomic level as well.

A remarkable feature of the relationships among Asian species was the highest degree of similarity between *A. veitchii* and *A. koreana* (74.6%), whose cpDNAs deviated so profoundly. Also, *A. nephrolepis* and *A. sachalinensis* exhibited a deeper mutual differentiation at the

Table 6. Nucleotide sequences of the arbitrary sequence primers used in the study and the number of monomorphic and polymorphic RAPD fragments scored for each primer

Primer	Sequence (5'-3')	Number of scored RAPD fragments		
		Polymorphic	Monomorphic	Total
OPE-03	CCAGATGCAC	22	3	25
OPE-04	GTGACATGCC	7	3	10
OPE-05	TCAGGGAGGT	37	2	39
OPS-01	CTACTGCGCT	32	3	35
OPS-02	CCTCTGACTG	37	1	38
OPS-03	CAGAGGTCCC	27	6	33
OPS-04	CACCCCCTTG	32	4	36
OPS-06	GATACCTCGG	30	0	30
OPS-11	AGTCGGGTGG	10	3	13
OPS-13	GTCGTTCCTG	11	2	13
OPS-15	CAGTTCACGG	46	0	46
OPS-18	CTGGCGAACT	24	2	26
	Total	315	29	344



Figs. 16-17. Polymerase chain reaction-amplified DNA polymorphisms in 15 species of fir using arbitrary primers OPS-06 (Fig. 16) and OPE-03 (Fig. 17).

genomic DNA level than at the level of cpDNA. Similarly, the tendency was more distinctly expressed among the Mediterranean species for differentiation between the group of species *A. alba, A. cephalonica,* and *A. nordmanniana* on the one hand and the group of species *A. cilicica, A. pinsapo,* and *A. numidica* on the other, according to their pertinence to the 2 different sections. Analogous to cpDNA, the pair of North American species *A. concolor* and *A. grandis* displayed a very high degree of mutual similarity in their genomic DNAs (78.8%), as well as a considerable divergence from *A. procera.* The latter was found to be profoundly differentiated from the overwhelming majority of species studied so far (Fig. 18).

With a special reference to Asian species, Tsumura *et al.* (1995) reported low percentages of nucleotide substitutions ranging between 0.19% and 0.94% as revealed in 4 *Abies* species native to Japan. Also, levels as low as 0-3 changes in the number of restriction sites were found to vary among individual species. Based on PCR/RFLP analysis of 6 chloroplast genes during which 58 digests were generated by 20 restriction endonucleases, the authors accordingly positioned the species *A. mariesii, A. sachalinensis, A. homolepis,* and *A. veitchii* on the same branch of a phylogenetic tree as a distinct group within *Pinaceae* which deviates considerably from the genera *Pinus* and *Picea.* The discrepancy which appears to exist between these data and the figure presented by us is primarily due to the different nature of the cpDNA regions involved in the comparison. For the species mentioned above, highly stable coding regions were scored representing 6 different chloroplast genes; these are considered to be preferably applicable in classific-

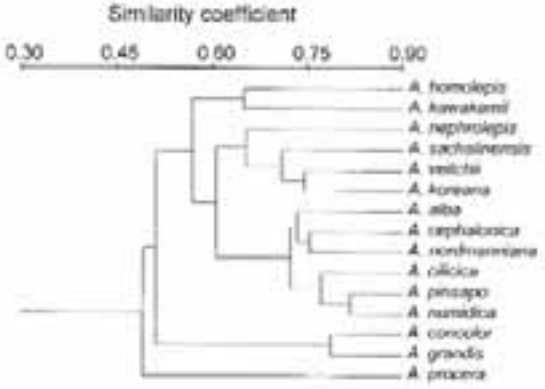


Fig. 18. Schematic illustration of genetic distances between *Abies* species based on similarity coefficients calculated from the percentage of shared amplified RAPD fragments against the number of total amplified fragments.

Table 7. Percent similarity coefficient matrix generated by pairwise comparison of shared RAPD fragments between *Abies* species [1)]

	B	C	D	E	F	G	H	I	J	K	L	M	N	O
C	65.0													
D	52.7	55.6												
E	55.1	54.9	66.4											
F	61.0	60.2	61.3	70.0										
G	58.4	56.7	68.7	72.2	74.6									
H	57.6	59.8	54.9	56.5	71.9	61.9								
I	55.8	57.9	55.4	51.7	64.0	59.3	72.2							
J	54.5	55.8	59.3	53.4	67.9	61.0	74.6	75.4						
K	56.3	56.9	60.2	59.6	67.9	64.7	74.4	76.6	72.7					
L	55.8	56.5	57.7	59.4	63.6	63.6	69.2	69.4	69.6	76.0				
M	59.2	56.9	57.3	57.5	65.7	61.9	73.0	73.8	72.7	78.5	81.7			
N	48.8	41.8	48.4	57.1	49.0	51.8	51.1	44.9	45.1	48.0	51.6	50.9		
O	50.2	54.2	51.9	54.1	53.1	52.8	54.3	46.6	53.6	53.9	51.9	49.3	49.8	
P	50.2	51.3	50.5	51.3	49.6	52.9	51.6	45.8	48.8	50.7	49.1	50.0	49.8	78.8

[1)] PCR conditions for RAPD were the same as those used in amplification of cpDNA except that a single 10-base primer (0.4 mM) was added in addition to the reaction mixture. The PCR products were separated in 1.5% NuSieve 3:1 agarose gel by electrophoresis in TBE buffer and stained with ethidium bromide. Because RAPDs were dominant markers, the character 1 was assigned to each of the scorable DNA fragments and 0 was given in the absence of a DNA fragment at the relative position of the same mobility. Similarity coefficients estimated from the proportions of shared amplified products by pairs of 2 species were calculated by Nei's estimate of similarity (Nei and Li, 1979); The designation of species is as in Table 3.

ation of plants at the genus or family level. On the contrary, the heterogeneous genetic relationships established among 6 Asian species by us refer exclusively to variations within the spacer segments between coding regions; this method is recommended for surveying relationships between closely related species of a given genus (Tsumura *et al.*, 1995).

In general, the degree of genetic affinity observed among tested representatives of firs at the level of DNA correlated positively not only with the systematic division of the genus *Abies* according to Liu (1971), but also with the data on crossability relationships between individual species of firs or between their groups. In the first place, the homogeneous nature of the Mediterranean species should be stressed. On the one hand, they share very similar profiles of their genomic DNAs and identical patterns of their chloroplast DNAs, and on the other, all species of the region uniformly exhibit mutually compatible relationships (Greguss, 1984). Conversely, the North American firs, despite a small number of species involved in the study, proved to be genetically more heterogeneous than the Mediterranean species, both groups of which were shown to be reproductively isolated (Kormutak, 1985). A close relationship established between *A. concolor* and *A. grandis* at the DNA level is in accord with a high hybridological affinity of these species observed under natural conditions in a sympatric zone in southwestern Oregon and in northwestern California (Martinez, 1948). This affinity has also been confirmed experimentally by numerous artificial crossing experiments (Larsen, 1934; Schlepitz, 1956; Ghaty, 1957; Duffield and Snyder, 1958; Rohmeder and Schönbach, 1959; Kantor and Chira, 1971; Frederick, 1977; Hawley and

DeHayes, 1985). Being members of the section *Grandes*, the DNA profiles of both the above species have simultaneously been shown to deviate strikingly from that of *A. procera* of the section *Nobiles*.

As far as crossability of investigated Asian species is concerned, there exist several lines of evidence postulating the compatible nature of the interspecific combinations: *A. sachalinensis* × *A. koreana*, *A. sachalinensis* × *A. homolepis*, *A. koreana* × *A. veitchii*, *A. koreana* × *A. homolepis*, and *A. veitchii* × *A. nephrolepis*, respectively. The compatible hybridological relationships have, in addition, been reported to exist between *A. veitchii* and the European species *A. alba*, *A. cephalonica*, *A. nordmanniana*, *A. cilicica* and *A. numidica* on the one hand and between the North American species *A. concolor*, *A. grandis*, and *A. balsamea* on the other. The same is true of the combinations of species *A. cephalonica* × *A. koreana*, *A. cephalonica* × *A. homolepis*, *A. nordmanniana* × *A. homolepis*, *A. procera* × *A. nephrolepis*, *A. procera* × *A. sachalinensis*, *A. balsamea* × *A. sachalinensis*, and *A. balsamea* × *A. homolepis*, respectively, all of which were found to intercross mutually (Rohmeder and Eisenhut, 1961; Klaehn and Winieski, 1962; Mergen *et al.*, 1964; Gaudlitz, 1983; Greguss, 1984). Owing to this diversity of intra- and inter-sectional crosses representing combinations of parental species of all 3 continents with both identical and strikingly diverging profiles of their DNAs, any conclusion concerning the relationship between DNA variation in Asian firs and the outlined crossability patterns seems to be premature.

6 Pollen development and viability

Among the external factors influencing development of pollen in forest trees, temperature and moisture obviously play the most important role (Stanley and Linskens, 1974). Early in the spring, heat sums are of decisive importance in triggering the process of meiotic division of pollen mother cells (Eriksson *et al.*, 1970), on the other hand, the relative constancy of temperature during the period which follows is essential for ensuring the normal course of such processes as microsporogenesis, maturation of strobili, and shedding of pollen. With special reference to firs, Ebel and Schmidt (1964) found significant relationships between pollen shedding of both subalpine and grand firs, and temperature and humidity of their surroundings.

Not only the entire process of pollen formation is affected in this way, but the available experimental evidence indicates that even all phases are temperature conditioned. According to Nekrasova (1976), in *Pinus sylvestris*, *P. sibirica* and *Abies sibirica*, the stages associated with division of nuclei start only at temperatures above 10 ℃ and the initiation of subsequent phases is accordingly determined by the relative total of effective temperatures above 5℃. Lower temperatures do not stop division of nuclei that has already begun but do retard it. *Abies sibirica* was shown in this connection to be less sensitive to low temperatures than was *Pinus sibirica*.

Also, meiotic irregularities, so profoundly affecting the viability of mature pollen, were shown to be conditioned not only genetically but even to a higher degree by temperature. Extremely deleterious in this respect are abrupt changes of temperature commonly occurring during late frosts which may induce a variety of chromosomal abnormalities in dividing microsporocytes. Under natural conditions, this phenomenon has been observed in *Larix decidua* by Christiansen (1960), while experimentally it has been illustrated in *Picea abies* by Johnson (1974). The same effect has been induced under laboratory conditions by

high temperatures leading to disturbances in pollen formation or even to the formation of diploid and tetraploid pollen grains in *Picea abies* (Eriksson *et al.*, 1970; Chira, 1972).

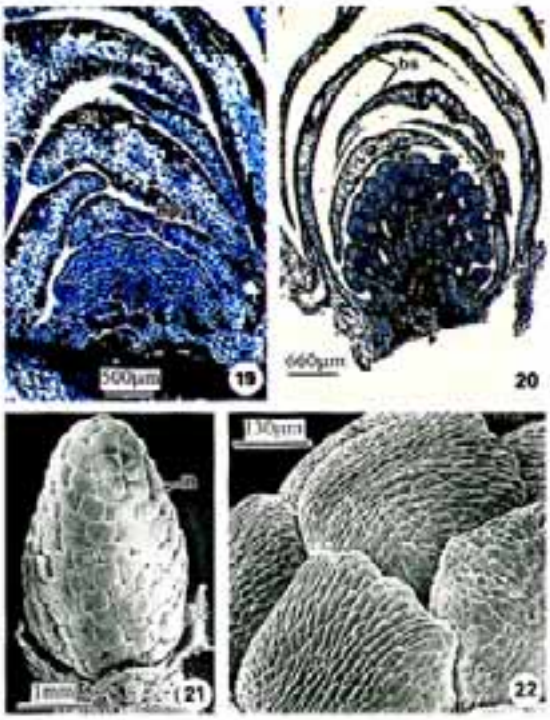
Temperature is believed to be responsible for the reduced yield of viable seeds in *Abies kawakamii* as well. An exceptionally profound effect is exerted in this respect by the absolute minimum temperature hindering the differentiation of flower buds during summer (Lai, 1994). There are reasons to suppose an equally sensitive response of reproductive structures of the species towards changes of temperature during their development in early spring when fluctuations of temperature in subalpine areas are exceptionally pronounced. Undoubtedly, this aspect of sexual reproduction of *A. kawakamii* is of considerable theoretical interest, mainly from the standpoint of its comparison with patterns of pollen development which have already been described in some other species. This list is rather short including only 6 North American, 4 European, and 4 Asian representatives of firs in which different aspects of pollen development were analyzed.

The early stages of the process, including initiation of the pollen-cone buds, and their differentiation and development during the predormancy period, have, for example, been illustrated in *A. amabilis* (Owens and Molder, 1977a), *A. lasiocarpa* (Singh and Owens, 1981; Owens and Singh, 1982), and *A. grandis* (Owens, 1984). The post-dormancy development of pollen cones has been analyzed from the standpoint of morphological changes accompanying development of microstrobili in *A. balsamea* (Powell, 1970; 1977) and *A. veitchii* (Seido, 1979) as well as with regard to phenological patterns of pollen development in *A. procera, A. amabilis, A. grandis,* and *A. lasiocarpa* (Franklin and Ritchie, 1970). Very detailed accounts relative to cytological and morphological aspects of postdormancy development of pollen cones with emphasis on the initiation of meiotic division and structure of pollen mother cells, tetrads, and mature pollen grains have been provided for *A. amabilis* (Owens and Molder, 1977a) and *A. grandis* (Singh and Owens, 1982). Also, details of the internal structure of pollen grains during gametogenesis in *A. balsamea* were provided as early as 1914 (Hutchinson, 1914). Both the course and duration of meiotic division of pollen mother cells in *Abies* have been described in the 2 groups of firs with *A. sachalinensis, A. homolepis, A. nobilis,* and *A. borisii-regis* involved in the 1st group (Mergen and Lester, 1961), and *A. alba, A. nordmanniana, A. pinsapo, A. concolor, A. grandis,* and *A. koreana* in the 2nd group (Kantor and Chira, 1965a). Such investigations are also important in *A. kawakamii,* and in combination with viability tests of mature pollen, they may shed more light on the role which pollen grains eventually play in reducing the seed set of the species. It was tentatively suggested by Lowe (1974) that pollen viability and vigor may affect seed weight and that this, in turn, may affect their germination in *A. balsamea.* Neither this question nor the basic cytological and genetic aspects of pollen biology have yet been studied in *A. kawakamii*

6.1 Differentiation of pollen-cone buds

Pollen cones differentiate from the apices which initiate in the axils of leaves on the abaxial side of proximal shoots (Fig. 23). A series of microsporophylls began to differentiate acropetally around the dome-shaped pollen cone apices in the 2nd half of July 1996. At an elevation of 2955 m the process was initiated around July 24 when the microsporophyll primordia could be recognized as small protuberances encompassing the peripheral zone of apices (Fig. 19). All microsporophylls had initiated by the beginning of September.

Microsporophylls at the very top of a pollen cone share a spherical shape on longitudinal section, while those on the sides of a pollen cone are oval. Within each of them the sporogenous cells have already formed (Fig. 20) giving rise to the pollen mother cells which represent a prominent feature in the cytological structure of pollen cones during successive stages of their predormancy development. By late October, the microsporophylls had already considerably enlarged reaching the size and form which are typical for dormant pollen cones. In *A. kawakamii* they are flattened, and rectangular with obtuse tips, completely covering the cone apex (Figs. 21-22), as compared, for example, with the spherical microsporophylls of *A. lasiocarpa* (Owens and Singh, 1982). On longitudinal section, the microsporangia dominate the internal structure of microsporophylls at this time and consist of a group of densely packed pollen mother cells or microsporocytes that are surrounded by a unicellular layer of tapetum which is, in turn, enclosed by a 4-celled-layered microsporangial wall (Fig. 25). The pollen mother cells which have already reached the premeiotic stage are characterized by finely granulated cytoplasm and large nuclei with a variable number of darkly stained nucleoli (Fig. 26). The latter were reported to be a very variable characteristic in *Abies* varying in number between 3 and 8 in individual pollen mother cells, but in a majority of species investigated, Mergen and Lester (1961) found 3-5 nucleoli to be common. The same applies also for the study trees of *A. kawakamii* investigated by us.

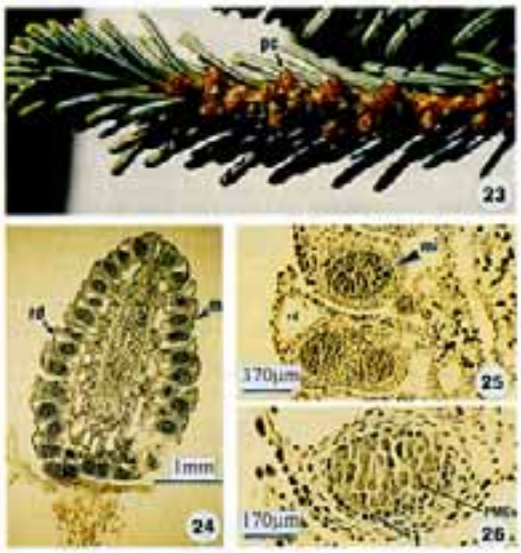


Figs. 19-22. Differentiation and early development of microsporophylls. Fig. 19. Initiation of microsporophylls in mid-July with protuberances of their primordia (mp) at the surface of a pollen cone. Fig. 20. Longitudinal section of a pollen-cone bud at the end of September with fully differentiated microsporophylls (m) and surrounding bud scales (bs). Fig. 21. Morphology and arrangement of microsporophylls (m) in pollen cone with bud scales removed as revealed by scanning electron microscope at the end of January. Fig. 22. Details of microsporophylls' surface structure.

Except for the microsporangia, the resin-ducts may be distinguished at the tips of microsporophylls. Their size is remarkably large representing perhaps another peculiarity in cytological structure of dormant pollen cones (Fig. 24). Since the time of their differentiation in July, the pollen cones were covered with bud scales which are very resinous in *A. kawakamii*.

Taking into account the time of pollen cone initiation and duration of their early development, it seems that the entire process of pollen cone differentiation, lasting in *A. kawakamii* about 65 days, is a little shorter than the corresponding process in *A. lasiocarpa*, *A. balsamea* and *A. amabilis* in which the pollen-cone buds were reported to attain their dormancy within a span of at least 75 days (Owens and Molder, 1977a; Powel, 1977; Owens and Singh, 1982).

6.2 Microsporogenesis and microgametogenesis

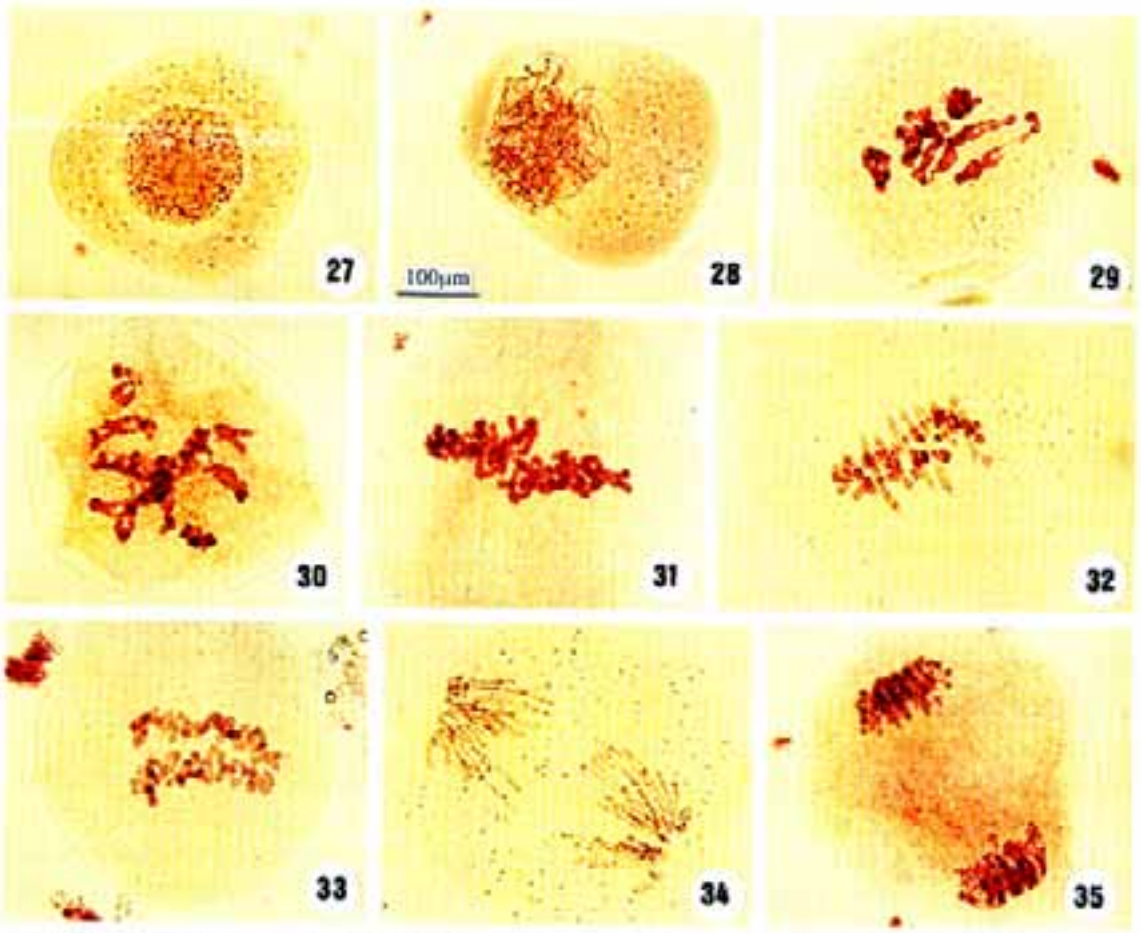


Figs. 23-26. Dormant pollen-cone buds and their internal structure. Fig. 23. Lower surface of pollen-cone- bearing shoot with dormant pollen-cone buds (pc). Fig. 24. Median longitudinal section of a dormant pollen cone with bud scales removed showing microsporophylls (m) with microsporangia and resin-ducts (rd). Fig. 25. Details of a microsporophyll showing microsporangia (mi) and a resin-duct (rd). Fig. 26. Details of a microsporangium with pollen mother cells (PMCs) and an enclosing layer of tapetum (t).

The period of pollen-cone dormancy in *A. kawakamii* extended from the beginning of November till the middle of February as judged by the initiation of meiosis in pollen mother cells. Near the lower boundary of the zone representing the natural distribution of the species (2850 m), the pollen mother cells had already entered meiosis on February 15, followed by a few days' interruption of the process due to a transient decline in temperature. In pollen cones, the beginning of meiosis is marked by a spatial separation of the pollen mother cells from each other within a mass of previously very compact cells of a microsporangium. At the level of single pollen mother cells the initiation of meiosis is marked by a change of their angular shapes to rounded ones, as well as by a gradual disappearing of the nuclear membrane and subsequent spiralization and contraction of the chromosomes. As a result, the microsporocytes passing early prophase could be distinguished in the developing cones throughout the 2nd half of February. Accordingly, a sieve-like structure of the nucleus with a rather diffuse state of chromonemata and more distinct chromomeres along them may be identified with the proleptonema stage (Fig. 27), while the tangled and intertwining chromosome threads with a higher degree of spiralization and excentric clumping in the cell are identified with the leptonema stage of prophase I (Fig. 28).

The stage corresponding to zygonema, during which the pairing of homologous chromosomes and crossing-over take place, is omitted in the illustrated sequence of meiotic events. However, the persistence of chromosome pairing may still be followed in the pachynema stage together with the longitudinal duality of the chromosomes, each of which consists of 2 chromatids (Fig. 29). The most conspicuous feature of this stage is the changed morphology of dividing chromosomes which, due to continuing contraction, become more compact and visible. As a result, the number and position of chiasmata in individual bivalents can be assessed. As far as the assessment can be made from Fig. 30, there are 1-3 chiasmata per bivalent in *A. kawakamii* which are evenly spaced along the bivalents. It is of interest to note at this point that the same number of chiasmata has also been observed in 6 other species of firs investigated by Kantor and Chira (1965a). The stage illustrated in Fig. 30 involves both non-termalized and termalized chiasmata sharing accordingly the features of the diplonema stage and diakinesis. Starch grains had disappeared at these stages. Their presence in the cytoplasm of pollen mother cells have been traced since the beginning of February. At the successive stage,

the bivalents which are still joined by the chiasmata move to the middle part of the cell constituting the metaphase plate (Fig. 31). Only now the chiasmata seem to achieve the highest degree of terminalization in bivalents (Fig. 32). Separation of homologous chromosomes of bivalents and their subsequent movement towards the opposite poles of the cell are highly synchronized throughout both early (Fig. 33) and advanced anaphase I (Fig. 34) during which a reduction of the number of diploid chromosome sets to haploid ones is achieved. In an overwhelming majority of dividing microsporocytes of *A. kawakamii*, the chromosomes seem to be partially despiralized during anaphase I, showing rather long and slender shapes with knobs at the ends of their chromatids. However, this condition is of transient duration only as evidenced by a compact appearance of the chromosomes resuming during late anaphase I (Fig. 35).



Figs. 27-35. Course of *A. kawakamii* microsporogenesis as illustrated by the 1st half of meiotic division of microsporocytes. Fig. 27. Pollen mother cell at proleptonema stage with a sieve-like structure of its nucleus. Fig. 28. Pollen mother cell at leptonema stage with intertwined chromosome threads. Figs. 29-30. Pachynema stage of prophase I showing chromosomes with terminalized chiasmata. Fig. 31. Metaphase I with chromosome bivalents arranged at the equatorial plate. Figs. 32-33. Early anaphase I. Fig. 34. Anaphase I with partially despiralized chromosomes containing knobs at their ends. Fig. 35. Late anaphase I with chromosomes having resumed their compact structure. The bar on Fig. 28 is for Figs. 27-35.

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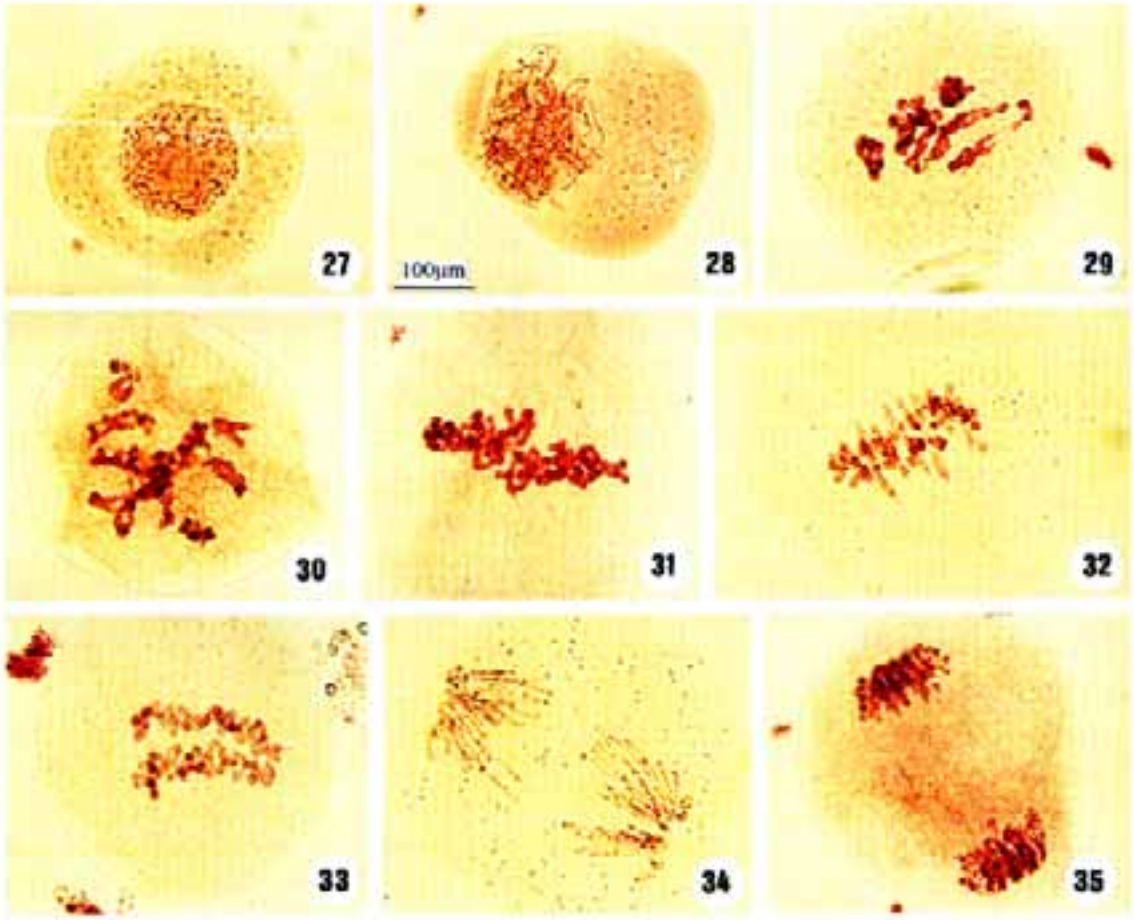

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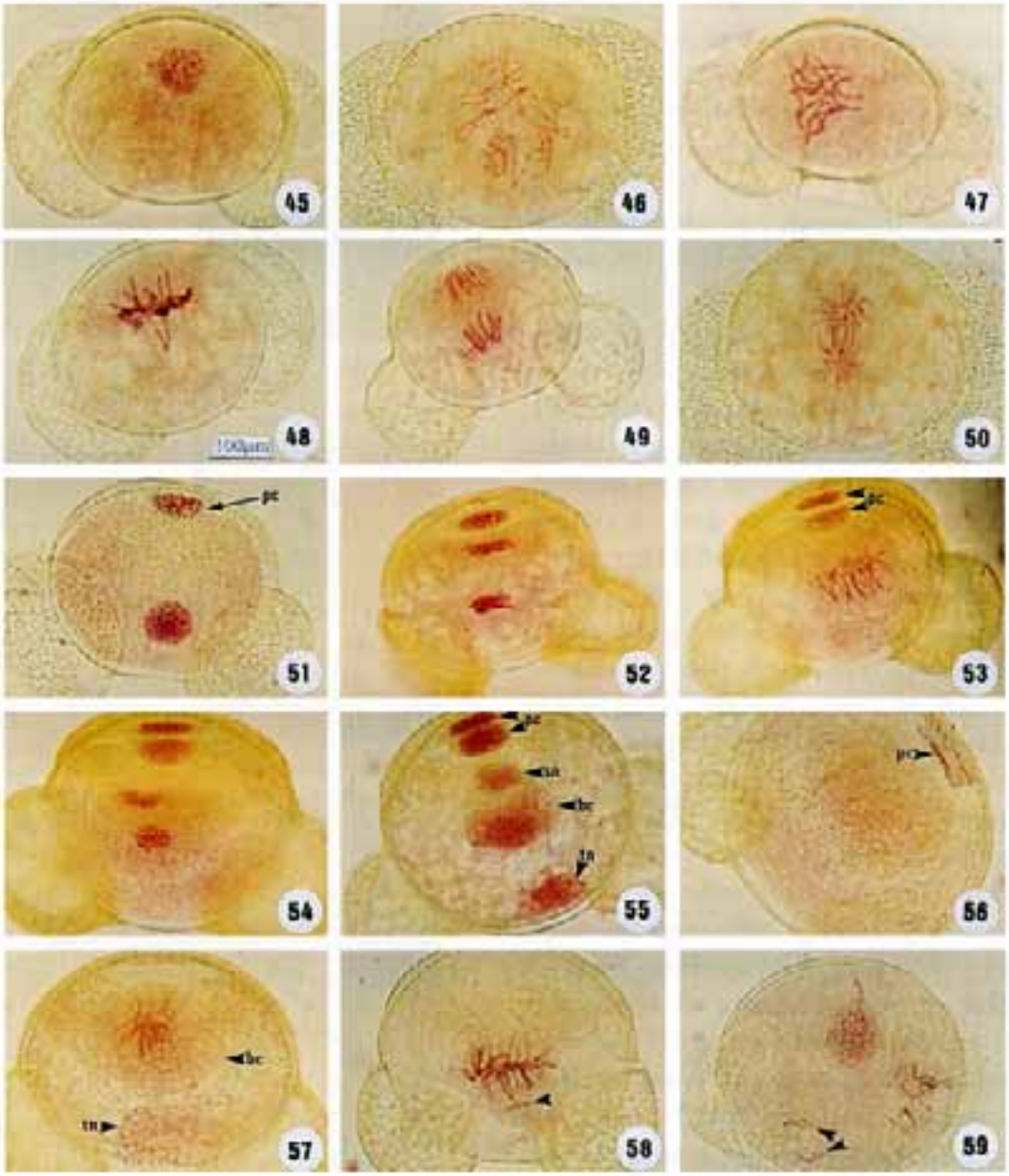
microspore becomes more apparent in the released microspores only (Figs. 43-44). The bladders had, as a rule, attained their typical size before microspores entered gametogenesis at the end of March (Fig. 45).

As far as the timing of meiosis in *A. kawakamii* is concerned, the longest duration was typical for prophase I, lasting 2 weeks at an elevation of 2850 m, i.e. from mid-February till the end of the month. Two additional weeks were necessary for dividing microsporocytes to pass the remaining meiotic stages including the release of microspores from the tetrads in mid-March. Throughout this period all meiotic stages could be revealed within a given tree, some of them in an overlapping sequence. A predominant occurrence of stages corresponding to metaphase I-telophase I was at the 1st week and the rest of the developmental stages at the 2nd week of the period.

The development of microspores following their release from the tetrads proceeded very rapidly. Only a few days later, completely differentiated pollen grains could be found in a single microsporangium, each of them consisting of fully developed air sacs and large pollen body with conspicuously sculptured exine and haploid nucleus (Fig. 45). The latter soon entered mitotic division thus initiating the process of microgametogenesis. On the background of its diffuse structure, the 2-3 nucleoli could be distinguished on March 23, suggesting an interphase nature of the nucleus. During early and late prophase which followed the extended and contracted chromosomes could accordingly be visualized (Figs. 46-47). This stage of microgametogenesis was considered by Mergen and Lester (1961) to be very convenient for the study of chromosome morphology. After metaphase (Fig. 48) and anaphase (Figs. 49-50), both of which are of short duration, the 1st mitotic division of the nucleus is completed yielding 2 nuclei or cells. At an elevation of 2850 m at Hohuan mt., the process took place on March 26. The cell situated near the pollen wall represents the 1st prothalial cell which is typified by its smaller size and flattened, lenticellular shape and which does not divide further (Fig. 51). A few days later, on April 2, the larger cell on the opposite side of a pollen grain underwent a 2nd mitotic division giving rise to the 2nd prothalial cell which is larger and of different form than the 1st one (Fig. 52).

In gymnosperms, the prothalial cells represent the vegetative tissue of the male gametophyte whose number and persistence varies in individual trees and species (Coulter and Chamberlain, 1910; Mergen and Lester, 1961; Mauseth, 1988). In *A. balsamea*, as many as 4 prothalial cells were, for example, observed by Hutchinson (1914), but 3 cells of this kind were reported to be more common. Mergen and Lester (1961), on the contrary, illustrated only 2 prothalial cells in 4 species of firs investigated by them as did Kantor and Chim (1965a) for *A. nordmanniana*. Two prothalial cells also seem to be typical for *A. kawakamii*.

Being overgrown by the intine, the prothalial cells are separated from the internal content of the pollen grain and degenerate in the further course of gametogenesis (Figs. 52-53). The central cell continues to divide mitotically. As a result, the generative nucleus and the tube nucleus are produced after the 3rd mitotic division (Figs. 53-54). The former subsequently divides into a smaller stalk nucleus and a larger body nucleus giving rise to the 5-celled structure of pollen grains; this condition in *A. kawakamii* was attained 10 days before shedding of its pollen, i.e. on April 13 (Fig. 55). The prothalial cells had by this time reached their maximum size, the tube nucleus had also moved to the opposite side of the pollen grain, while the stalk nucleus was found compressed into a cavity of the body cell, the condition which was previously recognized by Hutchinson (1914). One of the most remarkable features characterizing the final stages of pollen maturation in *A. kawakamii* concerns the progressive disintegration of both prothalial cells which occurred in the period April 13-23; the remains of these cells were incorporated into the thickening pollen wall (Fig. 56). The tube nucleus had meanwhile enlarged considerably,

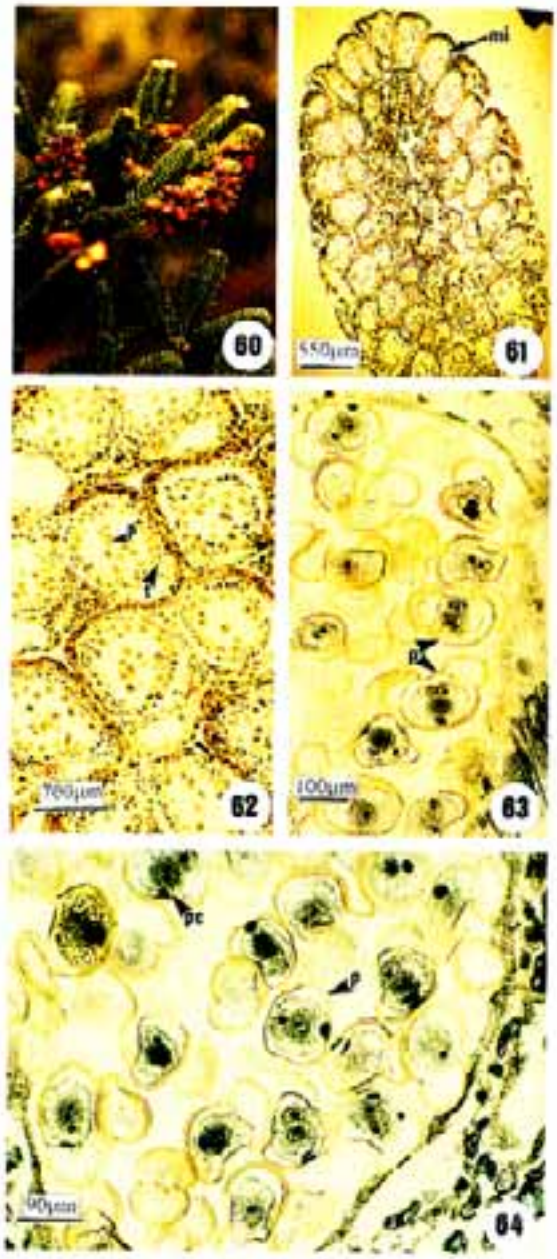


Figs. 45-59. Microgametogenesis and its disturbance in *A. kawakamii*. Fig. 45. Morphologically differentiated pollen grain containing interphase nucleus. Figs. 46-47. Early and advanced prophase of the 1st mitotic division of the pollen nucleus. Fig. 48. Metaphase. Figs. 49-50. Anaphase of the 1st mitotic division of the nucleus. Fig. 51. Prothalial cell (pc) and pollen nucleus after 1st mitotic division. Fig. 52. Anaphase of the 2nd mitotic division of the pollen nucleus giving rise to the 2nd prothalial cel. Fig. 53. Metaphase of the 3rd mitotic division of the pollen nucleus. Fig. 54. Anaphase of the 3rd mitotic division giving rise to the generative nucleus and the tube nucleus. Fig. 55. The 5-celled structure of a pollen grain after the 4th mitotic division of its nucleus containing 2 prothalial cells (pc), stalk nucleus (sn), body nucleus (bc), and tube nucleus (tn). Fig. 56. Remains of degenerating prothalial cells (pc). Fig. 57. Pollen grain at the time of pollen shedding with tube nucleus (tn) and dividing body cell (bc). Figs. 58-59. Disturbed microgametogenesis showing the chromosomes which have not been incorporated into metaphase plates during the 1st (Fig. 58) and 2nd mitotic divisions of the pollen nucleus (arrowheads - Fig. 59). The bar on Fig. 48 is for Figs. 45-59.

whereas the body cell was found to be in a state of mitotic division at the time of pollen shedding giving rise to the 2 sperm nuclei (Fig. 57).

Lacking prothalial cells, the pollen grains of *A. kawakamii* differ at this stage from pollen grains of such species as *A. balsamea, A. amabilis, A. lasiocarpa, A. grandis, A. sachalinensis, A. homolepis, A. nobilis, A. borisii-regis,* and *A. pindrow*, respectively, in internal structure of which all 5 nuclear derivatives of the haploid microspore nucleus were reported to be preserved (Hutchinson, 1914; Mergen and Lester, 1961; Mehra and Dogra, 1965; Owens and Molder, 1977a; Singh and Owens, 1981; Owens and Singh, 1982). As revealed at the level of sectioned microsporangia, in *A. kawakamii* such a value is an exception rather than the rule with the prevailing majority of pollen grains lacking prothalial cells (Figs. 63-64). Both body cells and tube cells are distinct in mature pollen by their darkly stained nucleoli, the number of which seems to vary between 1 and 4 in the former and between 1 and 3 in the latter. By the middle of April the microstrobili were very compact. Only shortly before shedding of pollen, they elongate considerably attaining their maximum length during pollination. They are either purple or yellow with typically elongated microsporangia on the longitudinal section (Figs. 60-61). The tapetal layer of microsporangia was maintained in *A. kawakamii* as late as early April (Fig. 62) with its remnants only detected at the time of pollen shedding (Fig. 64). The phenological pattern of pollen-cone bud development covering the period since their differentiation until maturity is schematically illustrated in Fig. 65.

The overall duration of meiotic division in *A. kawakamii*, starting from the early prophase of pollen mother cells until the release of microspores from the tetrads, was found to be restricted to a period of 26 days at an elevation of 2850 m. However, when referring to the time of pollen shedding, the period extends an additional 39 days



Figs. 60-64. Microstrobili of *A. kawakamii* shortly before pollen shedding. Fig. 60.Still-intact purple strobili. Figs. 61-62. Longisection of microstrobili showing microsporangia (mi) with pollen grains (p) and the remnant of the tapetum (t). Figs. 63-64. Detail of microsporangia at the time of pollen shedding showing pollen grains (p) lacking prothalial cells (pc) as well as those with preserved prothalial cells.

resulting in a total duration of the process of pollen

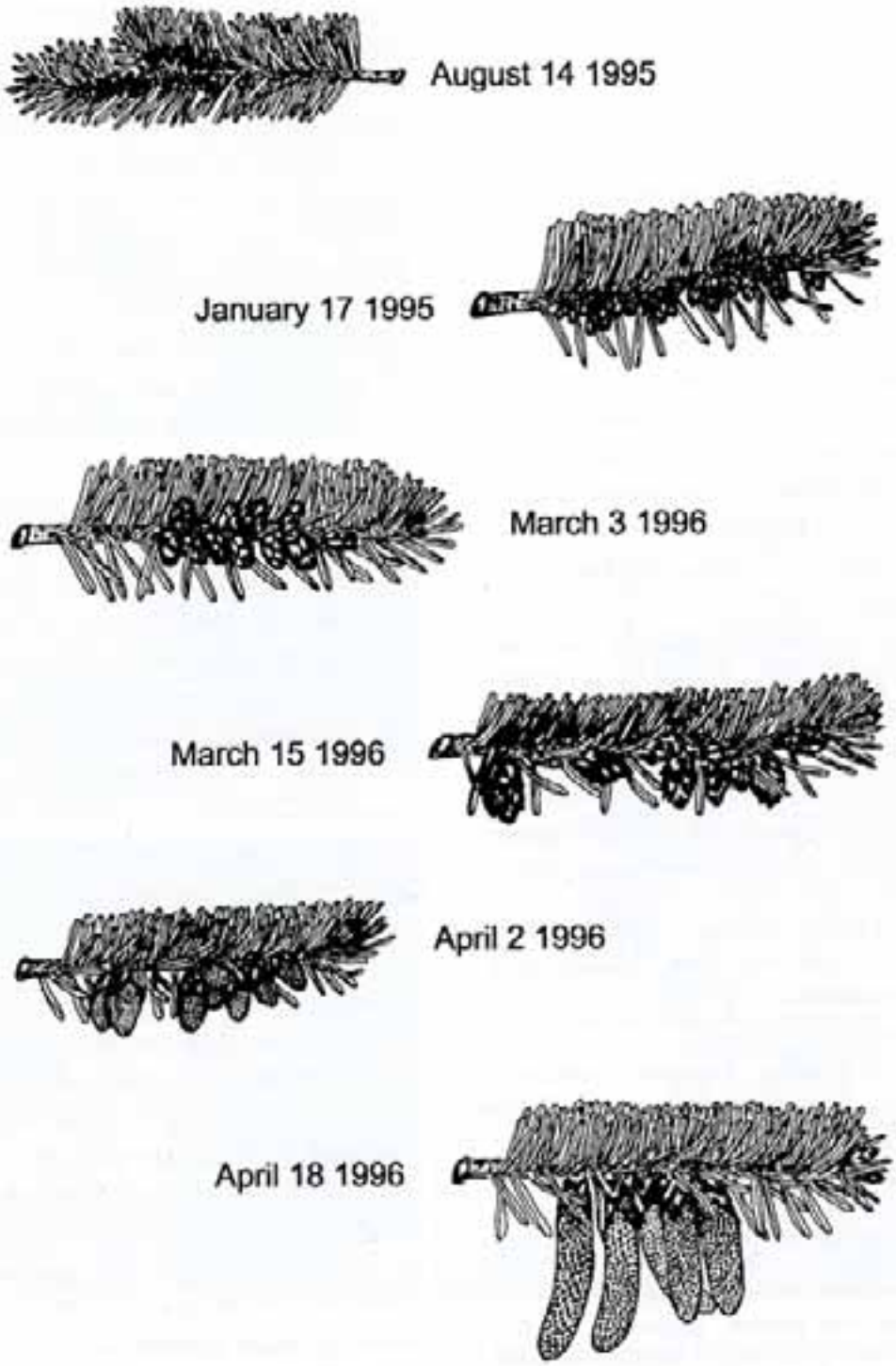


Fig. 65. Schematic illustration of microstrobili development in *A. kawakamii* covering the stages since pollen-cone bud differentiation until maturity of strobili (Drawing by Alzbeta Kormutak).

development of 65 days. It follows from Table 8 that there were apparently 2 opposite tendencies observed with increasing elevation, i.e., the delayed initiation of meiosis and shortened duration of pollen development.

At an elevation of 3130 m, the shift in the beginning of meiosis was represented by a delay of 19 days, whereas at 3230 m this delay was 1 month relative to an elevation level of 2850 m. Conversely, the extention of the process of pollen development at 3130 m was found to be 57 days as compared with only 50 days as ascertained at 3230 m. All these deviations may be accounted for by a later spring as well as by a larger sum of effective temperature at higher elevations due to the more intense sunshine representing a kind of species' adaptation to its high-elevation environment.

Based on these observations, we may conclude that within the elevation range of 2850-3230 m, the process of pollen development in *A. kawakamii* including pollen shedding is completed within a period of 50-67 days. The species is comparable in this respect with *A. borisii-regis* and *A. grandis* with 57- and 60-day periods of pollen development reported (Mergen and Lester, 1961; Owens, 1982). On the contrary, it differs strikingly from the species *A. sachalinensis*, *A. nordmanniana* and *A. homolepis* in which the corresponding process takes only 21, 24 and 31 days, respectively (Mergen and Lester, 1961; Kantor and Chira, 1965a). The species *A. pinsapo* and *A. lasiocarpa* deviate less in this aspect of reproductive biology, sharing a 45-day period for completion of pollen development (Kantor and Chira, 1965; Singh and Owens, 1981).

Table 8. Timing of meiosis and pollen shedding in *A. kawakamii* trees growing at 3 different elevations[1)]

Altitude	Meiosis		Pollen
	Initiation	Cessation	Shedding
2850 m	February 15	March 15	April 23
3130 m	March 6	March 26	May 2
3230 m	March 15	April 2	May 14

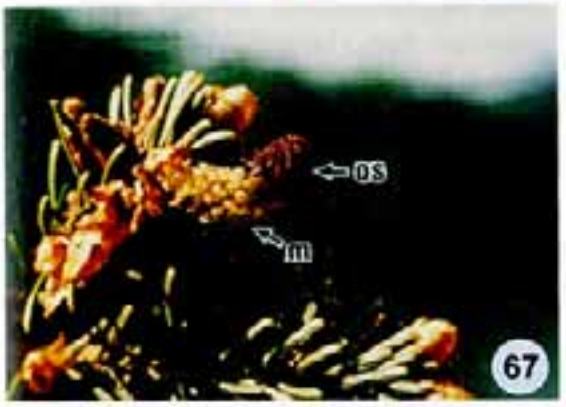
[1)] Samples of developing pollen and seed cones were collected at 5-8 days regular intervals from 9 trees of *A. kawakamii* growing in a natural stand at Hohuan mt. Specimens were fixed in FAA solution, dehydrated in ethyl alcohol series, embeded in paraffin, sectioned at 6 µm, and stained in iron-haematoxylin. The squash preparates of developing pollen mother cells were prepared in a 1% solution of acetocarmine.

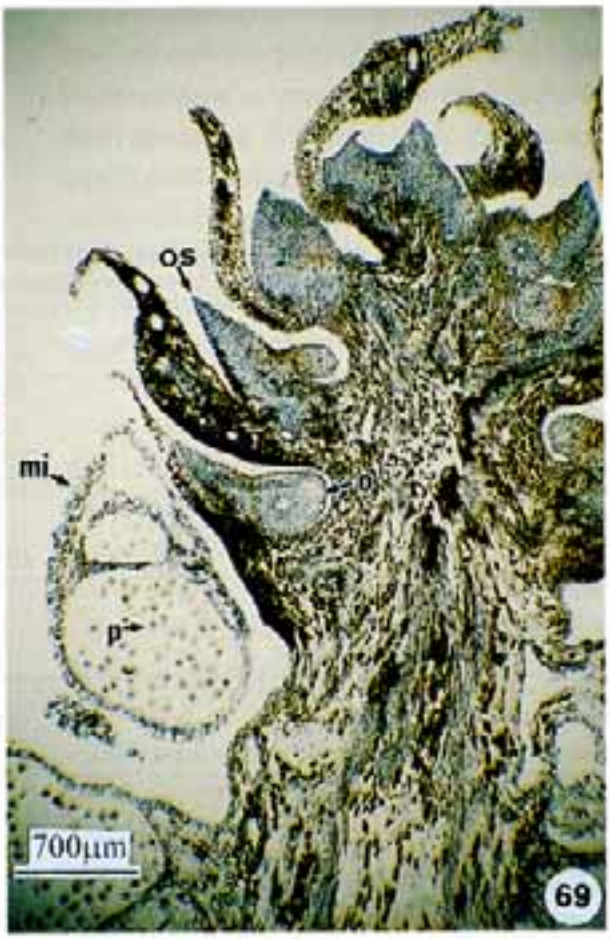
However, it is not only the duration of pollen development by which *A. kawakamii* deviates from other species compared so fur, but also the date of initiation of meiotic division which indicates a diverse timing of phenological events associated with development of the species' reproductive structures. As shown in Table 8, the species enters meiosis as early as mid-February, being comparable in this regard with *A. grandis* only, but differing profoundly from the remaining species mentioned above with corresponding dates situated in early April.

Like in other species of the genus, the microstrobili of *A. kawakamii* are clustered on the lower side of shoots exhibiting a relatively even distribution within a crown. At the highest whorls of branches, they occur on the same shoots with macrostrobili as is the case for the middle and bottom parts of a crown where macrostrobili are also very common in *A. kawakamii*. Pollen shedding is confined to a period of 12-14 days. In addition to the ordinary monosporangiate microstrobili of typical size and morphology, male strobili with modified shape were also found in *A. kawakamii* representing a rare example of anomalous reproductive structures.

At lower elevations trees were found, for example, with twinned microstrobili which had developed from a common bud (Fig. 66). Conversely, at an elevation of 3200 m, trees

bearing bisporangiate strobili were found with as many as 2-6 whorls of ovulate sporophylls at their tips and with more numerous whorls of staminate sporophylls beneath (Figs. 67-68). Longitudinal sections of such strobili revealed that their macrosporophylls possess morphologically fully differentiated ovules exactly as microsporophylls are characterized by their conspicuous microsporangia with fully developed pollen grains (Fig. 69). Pollen extracted from bisporangiate strobili was shown to be comparable in its viability with the quality of pollen produced by typical monosporangiate male strobili.





Figs. 66-69. Anomalous microstrobili of *A. kawakamii*. Fig. 66. Twinned (arrow) and normal microstrobili (below) of the species. Figs. 67-68. Bisporangiate strobili with prevailingly staminate sporophylls (m) and with ovular sporophylls (os) at their apex. Fig. 69. Median longitudinal section of a bisporangiate strobillus showing fully differentiated ovules (o) on the ovuliferous scales (os) and fully developed pollen (p) within microsporangia (mi).

6.3 Meiotic irregularities

The developmental pattern presented in the previous section represents a typical sequence of events that accompanies pollen development in *A. kawakamii* and which is supposed to be under strong genetic control. However, its highly coordinated nature may occassionally be disturbed by climatic factors which have also been shown to exert profound effects on the process of pollen development in conifers. Especially, the developmental stages associated with distribution of chromosomes to the poles of dividing microsporocytes were reported to respond very sensitively to climate variations (Mergen and Lester, 1961; Kantor and Chira, 1965a). Our data obtained from the comparative analysis of the process in 9 study trees of *A. kawakamii* distributed along the elevation gradient of 2850-3230 m at Hohuan mt. indicate that, except for the stages mentioned above, the meiotic phases which precede visualization of the chromosomes in pollen mother cells are equally affected by environmental influences. In particular, it is true of a small portion of pollen mother cells which failed to proceed in meiosis which they had already entered. The main reason of this failure was plasmolysis of their cytoplasmic content and a highly pycnotic condition of their nuclei (Fig. 70). The deviations are supposedly caused by occasional sub-zero night temperatures in the region early in February. Their frequency is relatively low varying between 0%-2.2% in individual trees (Table 9). The affected pollen mother cells seem to degenerate during the later stages of meiosis exerting only a small effect on mature pollen quality.

Table 9. Frequency of meiotic disturbances revealed during different stages of meiosis in individual trees of Taiwan fir growing at different elevations at the Hohuan mt.

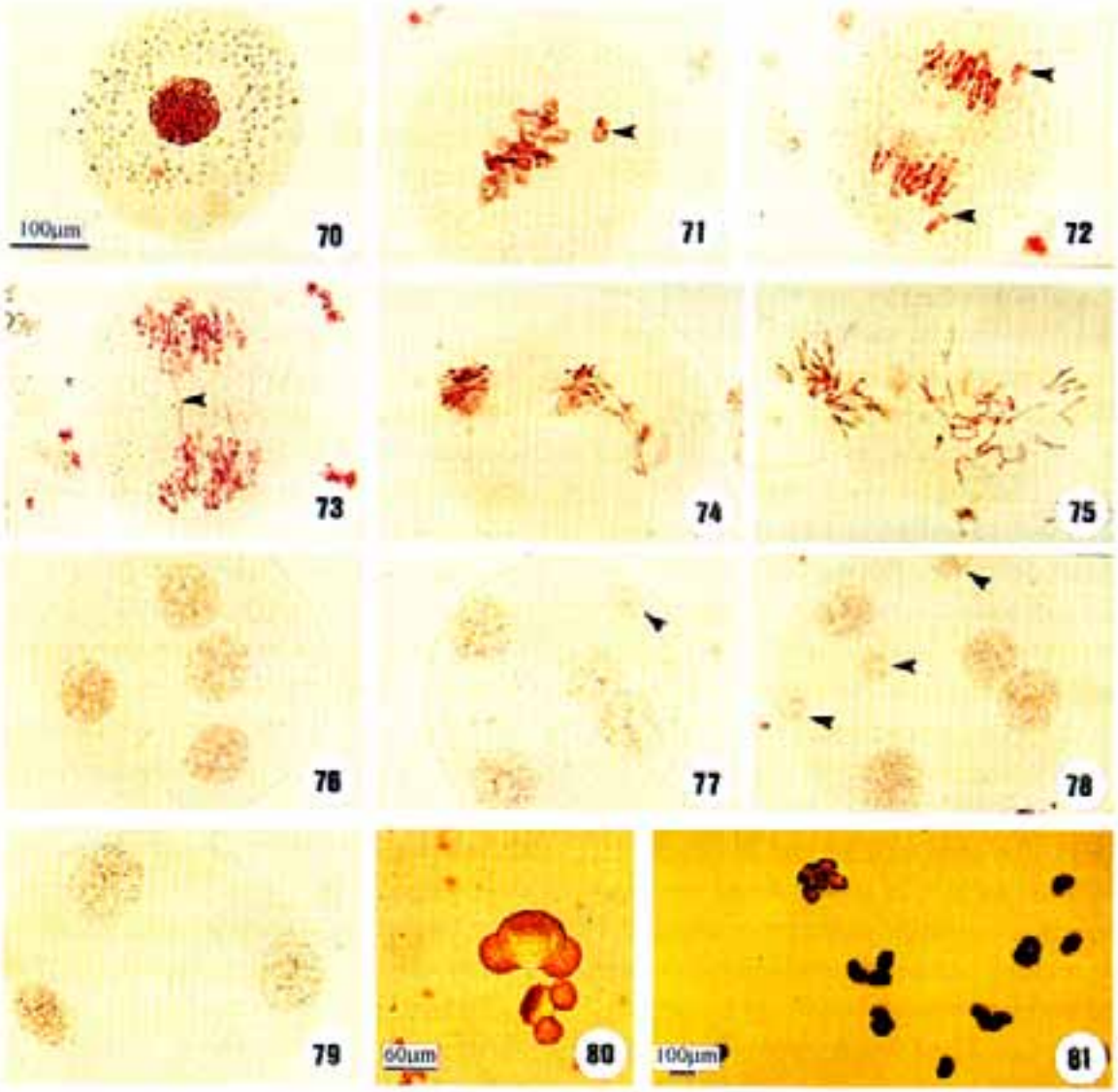
Tree no.		1	2	3	4	5	6	7	8	9	Average
Elevation		2850	3050	3130	3130	3160	3160	3160	3230	3230	
Plasmolyzed	a[1]	500	500	500	500	500	500	500	500	500	500
PMCs	b	6	8	7	10	6	7	4	3	4	6.1
	c	1.2%	1.6%	1.4%	2.0%	1.2%	1.4%	0.8%	0.6%	0.8%	1.2%
Pycnosis of	a	500	500	500	500	500	500	500	500	500	500
PMC nuclei	b	8	5	6	1	2	0	5	2	11	4.4
	c	1.6%	1.0%	1.2%	0.2%	0.4%	0	1.0%	0.4%	2.2%	0.8%
Metaphase I	a	74	319				236		130	185	188
with loose	b	27	28				22		17	36	26
chromosomes	c	36.4%	8.7%				9.3%		13.0%	19.4%	17.3%
Anaphase I	a	163	162				69		19	53	93.2
chromosome	b	33	7				4		2	18	12.8
bridges	c	20.2%	4.3%				5.7%		10.5%	33.9%	14.9%
Anaphase II	a						19			31	25
chromosome	b						1			20	10
bridges	c						5.2%			64.5%	34.8%
Abortive	a									531	531
tetrads	b									17	17
	c									3.2%	3.2%
Pollen germination		94.3%	92.8%	90.5%	84.6%	77.3%	64.1%	82.0%	92.8%	75.8%	83.3%
Pollen tube length (μm)		493	412	476	397	292	382	328	369	256	378

[1]a-total number of cells analyzed; b-number of cells with corressponding deviation; c-percentage of cells with corresponding deviation.

Meiotic irregularities with a more profound impact only occurred at advanced stages of meiosis and concerned the behaviour of individual chromosomes or whole sets of chromosomes during their metaphase orientation or anaphase distribution. Accordingly, chromosomes loosely situated in the cytoplasm at metaphase I not being incorporated into the metaphase plate represented the most extensive type of deviation occurring in 5 study trees, with frequencies ranging from 8.7% to 36.4%. The non-incorporated chromosomes were predominantly ring-shaped resembling either a bivalent with terminalized chiasmata or a true ring chromosome (Fig. 71). The 1st alternative seems however more probable as evidenced by a precocious and relatively independent movement of 1 chromosome to each of the poles of the spindle apparatus during subsequent anaphase I (Fig. 72). This type of meiotic irregularity has also been observed in *A. nobilis* (Mergen and Lester, 1961). The same trees affected in metaphase I were also found to be affected during anaphase I suggesting a common origin of persisting defects. At anaphase I, chromosome bridges had distorted the normal distribution of bivalents in study trees nos. 1, 2, 6, 8, and 9, with predominantly 1 or 2 chromosome pairs involved (Fig. 73). Their frequencies were mostly lower than, but to some degree correlated with, those observed during metaphase I (Table 9).

By the end of March, meiotic division had reached its completion in almost all study trees except for tree no. 9 in which the process was delayed for a few days. The tree had passed the 2nd meiotic division during frosty weather lasting for 4 days. As a result, anaphase II was severely affected by the low temperature with numerous chromosome bridges formed (Fig. 74) and widely scattered chromosomes exhibiting all the signs of spatial disorientation (Fig. 75). The frequency of these defects was remarkably high, reaching 64.5% and resulting in the formation of 2 types of abortive tetrads, i.e., those containing supernumerary nuclei (Figs. 77-78) and tetrads with reduced numbers of nuclei (Fig. 79) as compared with ordinary 4-nuclei tetrads of the species (Fig. 76). Their occurrence was not restricted only to meiotic stages of pollen development but persisted also during microgametogenesis, as illustrated by the chromosomes loosely situated or scattered in the cytoplasm of microspores during the 1st and 2nd mitotic divisions of their nuclei (Figs. 58-59). The micronuclei of abortive tetrads probably gave rise to pollen grains of reduced size (Fig. 80) which were never observed to germinate on artificial agar media (Fig. 103). In *A. sibirica* pollen grains of the same type were reported to be fully germinable (Plaksina, 1969). Still another type of pollen grain with distorted shape refers to a small portion of pollen grains with reduced air sacs and light color (Fig. 81) whose germinability was comparable with that of pollen with normal shape and coloration.

As to the quality of mature pollen in individual study trees, its viability was only partially correlated with frequencies of meiotic deviations given above (Table 9). In order to illustrate this, compare the 94.3% germination of pollen grains and the average length of pollen tubes of 493 µm ascertained in tree no.1 which had a relatively high frequency of meiotic disturbances with the reduced quality of pollen in tree no. 5 which had corresponding viability parameters averaging 77.3% and 292 µm, respectively, and a very low frequency of meiotic defects. Conversely, the reduced viability of pollen in trees no. 6 and 9 was closely correlated with the frequent occurrence of meiotic irregularities which, in addition, encompassed anaphase II. It seems therefore as if disturbances during the 2nd meiotic division have a more profound impact on the quality of mature pollen than those occurring during the 1st half of



Figs. 70-81. Meiotic irregularities accompanying pollen development in *A. kawakamii*. Fig. 70. Pollen mother cell with highly pycnotic nucleus which does not enter meiosis. Fig. 71. Chromosome detached from metaphase plate (arrowhead). Fig. 72. Precocious movement of chromosomes during anaphase I (arrowheads). Fig. 73. Chromosome bridge (arrow) at anaphase I. Figs. 74-75. Severely affected anaphase II and successive meiotic stages by an abrupt decline of temperature. Figs. 76-79. Normal tetrad with 4 haploid nuclei (Fig. 76) and anomalous ones with 1 (Fig. 77) and 3 (Fig. 78) supernumerary micronuclei (arrowheads) as well as an abortive tetrad with 3 haploid nuclei only (Fig. 79). Figs. 80-81. Anomalous pollen grains with reduced size (Fig. 80) and rudimentary air sacs (Fig. 81). The bar on Fig. 70 is for Figs. 70-79.

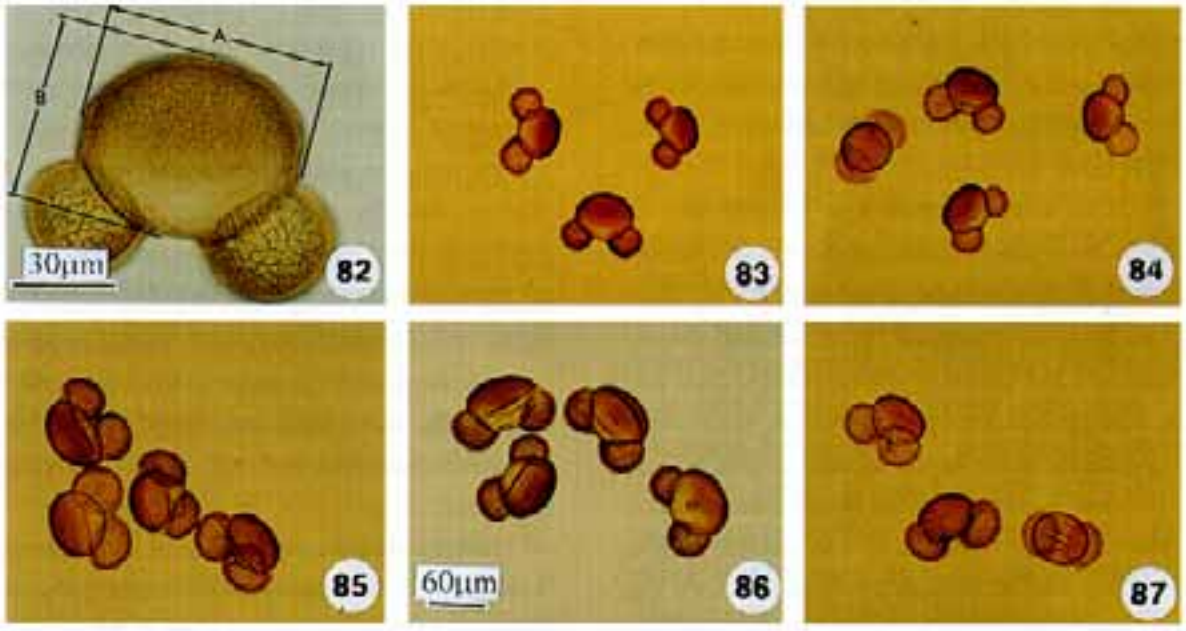
the process. The differential sensitivity of meiotic stages to low temperatures was also observed by Kantor and Chira (1965a). A short-term drop of temperature to 0-2 ℃ accordingly caused a 74% sterility of pollen in *A. pinsapo* when in prophase I but a complete loss of pollen viability in *A. koreana* when in either hetereotypic or homeotypic anaphase. Fluctuations in temperature, especially

those of an abrupt nature, are obviously one of the major sources of variation in quality of *A. kawakamii* pollen as well. However, to illustrate it more convincingly, further ecologico-genetic studies are needed.

6.4 Size of mature pollen grains

Evaluation of pollen size in *A. kawakamii* was performed with regard to the main body or the corpus of a pollen grain, completely neglecting the size of air sacs. The size measurements involved both the length and height characters of a corpus in sampled pollen grains as viewed from the equatorial and polar axes, respectively. The characters measured are illustrated in Fig. 82 together with samples of acetolyzed pollen of 5 *Abies* species.

Although it was reported previously by Liu (1971) that the corpus in *Abies* is mostly over 90 μm long, our data indicate that this estimate does not apply to *A. kawakamii*. Its pollen was found to be much smaller with a corpus length ranging between 65.76 and 81.00 μm with a mean value of only 72.8 μm . Accordingly, the height of pollen grain body was shown to vary in 11 study trees within the range of 50.44-65.72 μm, averaging 55.71 μm (Table 10). The reduced nature of these variables becomes even more apparent when compared with the corresponding characters of some other species. In *A. pinsapo* the equatorial axis averages 97.3 μm, whereas the polar axis averages 90.9 μm (Arista and Talavera, 1994). The corresponding characters of the corpus of *A. alba* range from 98.07 to 116.72 μm in length and from 80.83 to 90.05 μm in height depending on the provenance (Popnikola, 1971), whereas in *A. sibirica* they are within the limits of 90.90-99.30 μm and 77.4-80.0 μm, respectively, depending on elevation (Plaksina, 1969). Also, among 9 species of firs studied by Kantor and Chira (1965) the pollen corpus of *A. numidica*, *A. cephalonica*, and *A. nordmanniana* was characterized by such large



Figs. 82-87. Pollen size variation in 5 *Abies* species as revealed by acetolysis of their pollen. Figs. 82-83. Acetolyzed pollen of *A. kawakamii* with length (A) and height (B) parameters of its corpus designated. Figs. 83-86. Size of acetolyzed pollen in *A. kawakamii* (Fig. 83), *A. firma* (Fig. 84), *A. alba* (Fig. 85), *A. nordmanniana* (Fig. 86), and *A. lasiocarpa* (Fig. 87). The bar on Fig. 86 is for Figs. 83-87.

Table 10. Size of corpus in pollen grains of *A. kawakamii* as revealed in 11 individuals of the species growing at different elevations[1]

Tree no.	Elevation (m)	Length(A) (μm)	Height(B) (μm)	A : B	Duncan test[2] A	Duncan test[2] B
1	2850	71.44 ± 4.48	54.76 ± 4.13	1.30 ± 0.06	d	d e
2	2955	81.00 ± 4.29	65.72 ± 3.83	1.23 ± 0.06	a	a
3	2955	66.88 ± 5.27	52.72 ± 5.89	1.27 ± 0.11	e	f e
4	2965	76.92 ± 3.61	58.44 ± 4.35	1.32 ± 0.08	b	b b
5	3090	73.68 ± 4.80	56.20 ± 6.22	1.32 ± 0.11	c	d c
6	3090	76.60 ± 4.66	57.36 ± 5.63	1.34 ± 0.12	b	b c
7	3090	74.16 ± 4.79	54.84 ± 3.66	1.35 ± 0.08	c	d e
8	3225	72.72 ± 5.15	55.72 ± 5.55	1.31 ± 0.11	c	d c
9	3225	67.44 ± 3.28	50.44 ± 3.70	1.34 ± 0.09	e	g
10	3225	65.76 ± 3.39	51.92 ± 3.93	1.27 ± 0.08	e	f g
11	3225	74.20 ± 6.22	54.72 ± 6.75	1.37 ± 0.17	c	d e
Average		72.80 ± 6.39	55.71 ± 6.28	1.31 ± 0.11		

[1] Fifty pollen grains of each tree were sampled after their acetolysis in a mixture of glacial acetic acid and hydrochloric acid (4:1) followed by treatment with a mixture of acetic acid anhydride and sulphuric acid (9:1), both at 75 ℃ (5 min). After each treatment, samples were centrifuged and rinsed with distilled H_2O. The acetolysed pollen was mounted in glycerol and examined microscopically the same day.
[2] Means in a given column with the same letter do not differ significantly.

diameters as 112.4 μm, 107.7 μm, and 106.9 μm as well as by mean values of height of 99.3 μm, 94.8 μm, and 94.3 μm, respectively.

The flattened shape of the pollen body, so typical of conifers, is more or less distinctly expressed also in *A. kawakamii* as evidenced by the length-height ratio varying between 1.23 and 1.37 (Table 10).

Of no less importance than a description of pollen body size in quantitative terms is also the finding of its considerable variation in individual study trees. As confirmed by the Duncan test, as many as 5 individuals or groups of individuals can be distinguished among 11 study trees which differ significantly in the length of their pollen bodies. The same is also true of the height characteristics which seem, however, to be less variable (Table 10). This may be taken as evidence indicating the existence of individual variability in *Abies* species.

It is reasonable to mention in this connection that Popnikola (1971) also observed a variation in size of pollen grains in *A. alba* originating from purple and yellow male strobili. The pollen from purple strobili was found to be larger than that from yellow ones or from those exhibiting a combination of both color types. On the contrary, Gudeski (1974) was not able to confirm the validity of this relationship in either *A. alba* or *A. cephalonica* denying not only the correlative association between the color of male strobili and pollen size, but also the utility of the latter in distinguishing the above species. Also, Hanover (1973) studying morphological variation in pollen of some coniferous species including that of *A. homolepis*, concluded that there is little variation of the kind within species, and that pollen morphology is not particularly useful for the study of genetic variation in *Pinus*, *Picea*, and *Abies* species.

In light of these conclusions, it seems that *A. kawakamii* is an exception exhibiting very distinct variation between individual trees in both pollen body characters studied as evidenced also by the variation analysis (Table 11). This variation did not, however, display any relationship with the elevational distribution of individual study trees.

Table 11. Variance analysis of pollen body size within a group of *A. kawakamii* study trees

Length of corpus

Variance resource	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Trees	10	10921.60	1092.16	51.14	0.0001
Error	539	11510.40	21.35		
Corrected total	549	22432.00			
Height of corpus					
Variance resource	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Trees	10	8216.61	821.66	32.84	0.0001
Error	539	13486.00	25.02		
Corrected total	549	21702.61			

As shown in Table 10, trees nos. 2 and 3 growing only a few meters apart at elevation of 2995 m differ most markedly in the size of their pollen bodies; similar examples may be found at each of the elevations traced. This is in accordance with data published by Plaksina (1969) and Kirgizov (1978) who also observed considerable variation in pollen body size but little effect of elevation on the size of *A. sibirica* pollen. Rather than ecologically conditioned variation, the variability in pollen body length between individual study trees of the species was emphasized by the authors. Such an important character of pollen is undoubtedly conditioned genetically and the revealed variability between individual trees of *A. kawakamii* probably reflects the different nature of their genotypes.

The genetic background of pollen size variation is much more apparent at the species level in which conspicuous differences in pollen body size may be observed between species of different geographical habitats or of different taxonomic status. The data listed in Tables 12-13 refer to only 1 tree of each species and cannot therefore be considered to adequately reflect the extent of existing intraspecific variation. Nevertheless the value in featuring species-specific variations in pollen body size is obvious. It follows from the tables that pollen length varies in such a broad extent as 69.12 to 102.84 µm, being the shortest in *A. homolepis* and the longest in *A. cilicica*. Within this range, a group of Asian species, *A. homolepis, A. kawakamii,* and *A. firma* of the sections *Homolepides* and *Momi,* may be distinguished by sharing the smallest corpus. On the contrary, the largest pollen bodies were found in the European species *A. cilicica, A. nordmanniana, A. alba,* and *A. cephalonica* of the sections *Piceaster* and *Abies,* as well as in the North American species *A. concolor* of the section *Grandes*. *A. grandis* as an additional representative of the section *Grandes* on the North American continent occupies an intermediate position in this respect, while *A. lasiocarpa* of the section *Balsamea* with a natural distribution on the same continent possesses pollen comparable in size with that of Asian firs mentioned above. Height variables of pollen bodies have, as a rule, correlated with the described pattern of length variation also.

The relative differences in pollen size and morphology between individual species of firs as observed by scanning electron microscopy are illustrated in Figs. 88-97. It is evident that, owing to a high degree of morphological uniformity of pollen grains in *Abies,* differences in pollen size alone cannot serve as a reliable criterion for discrimination between individual species. This is the conclusion which had already been drawn earlier by Kantor and Chira (1965b) and Hanover (1973) but it seems reasonable to us to modify it in the sense that the established magnitude of these

Table 12. Size of corpus in pollen grains of some *Abies* species[1]

Species	Length(A) (μm)	Height(B) (μm)	Duncan test[2] A	B
A. homolepis	69.12 ± 7.63	52.36 ± 7.92	f	f
A. kawakamii	72.68 ± 6.01	58.48 ± 6.53	e f	e
A. firma	71.28 ± 3.69	58.72 ± 3.78	f	e
A. alba	95.16 ± 12.71	66.16 ± 9.82	b c	d
A. cephalonica	91.52 ± 10.79	72.64 ± 8.93	c	c
A. nordmanniana	98.24 ± 12.88	76.44 ± 11.68	b	b
A. cilicica	102.84 ± 11.7	80.12 ± 10.61	a	a
A. concolor	98.08 ± 10.49	73.76 ± 8.75	b	b c
A. grandis	83.56 ± 8.34	65.24 ± 10.07	d	d
A. lasiocarpa	76.16 ± 7.39	61.64 ± 8.55	e	e

[1] Data refer to 1 tree of each species only with 50 pollen grains per species used.
[2] Means in a given column with the same letter do not differ significantly.

Table 13. Variance analysis of pollen body size in 10 *Abies* species

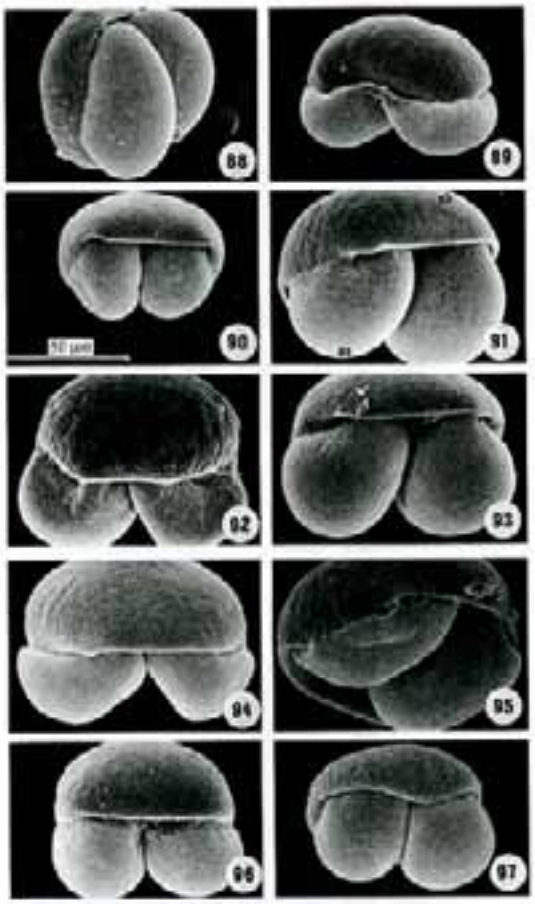
Length of corpus

Variance resource	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Species	9	73766.75	8196.30	88.57	0.0001
Error	490	45344.00	92.53		
Corrected total	499	119110.75			
Height of corpus					
Variance resources	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Species	9	36239.75	4026.63	50.53	0.0001
Error	490	39040.68	79.69		
Corrected total	499	75289.43			

differences at least provides a certain ground for delineation between the European and Asian species of firs. The contribution of data on pollen size variation in the field of palynology is indisputable, particularly in extending the information already available (Aytug, 1967; Elicin, 1967) relative to the details in pollen morphology and surface texture in some additional species of firs.

6.5 In vitro cultivation of pollen

Among the standard tests used in evaluating pollen viability, *in vitro* germination is supposed to be the most reliable test method. This holds true irrespective of the fact that compositions of artificial media do not usually satisfy the requirements for optimum growth conditions as found in the conducting tissues of female flowers. It is primarily the protrusion of a pollen tube from a pollen grain and its subsequent elongation as a vivid manifestation of the functional activity of the pollen which favors this method over non-germination assays. These assays are based on detection of functional enzymes by specific stains, and may give false positive results due to occasional non-specific binding of dyes with chemical elements on nonliving systems (Stanley and Linskens, 1974). A few attempts are known with rapid prediction of pollen quality in *Abies* species based on triphenyltetrazolium chloride staining (Chira, 1963) as well as on measuring the membrane integrity of pollen, carbohydrate, and amino acid content of the leachate (Ching and Ching, 1976) and adenosine-triphosphate content of air-dried pollen (Ching *et al.*, 1975). However, the results of these studies have usually been relat -



Figs. 88-97. Scanning electron microscope micrographs illustrating variations in size of pollen body (pb) and air saes (as) in 10 *Abies* species. Fig. 88. *A. homolepis*. Fig. 89. *A. kawakamii*. Fig. 90. *A. firma*. Fig. 91. *A. alba*. Fig. 92. *A. cephalonica*. Fig. 93. *A. nordmanniana*. Fig. 94. *A. cilicica*. Fig. 95. *A. concolor*. Fig. 96. *A. grandis*. Fig. 97. *A. lasiocarpa*. The bar on fig. 90 is for Figs. 88-97.

ed to the actual germination of pollen as found on agar medium. The latter is considered by Smirnov (1977) to be a standard which represents the most objective assessment of pollen fertility. Also, the results of *in vitro* assays, in which the share of filled seeds is used as the main criterion of pollen viability, are often questioned because of occurrence of incompatibility reactions during which the germination of viable pollen grains is inhibited partially or completely, depending on the intensity of the reaction. Therefore, keeping in mind the outlined limitations of both the above methods, we confined our studies on pollen viability in *A. kawakamii* completely to the *in vitro* germination test using agar as a supporting medium and sucrose as the sole source of carbohydrates.

The concentration of agar was set arbitrarily at 1.5% to comply with the 1%-2% content of agar in culture medium that was found to represent the optimum for culturing of both *A. veitchii* and *A. homolepis* pollen (Ito *et al.*, 1983; Saito *et al.*, 1983), and which is routinely used in assaying the germinability of coniferous pollen. Alternately, the optimum concentration of sucrose was established experimentally using a 4-grade scale within a 0%-28% sucrose gradient.

As follows from Table 14, a relatively broad extent of sucrose concentrations exitst, ranging from 4% to 20%, at which a high percentage of germinating pollen of *A. kawakamii* is attained. Within this range, as high as 87.0%-94.3% of

Table 14. Effect of sucrose concentration in culture medium on *in vitro* germination of *A. kawakamii* pollen [1)]

Sucrose conc. (%)	Pollen germination Mean (%)	Pollen tube length Mean (μm)
0	67.5 ± 5.6	139.93 ± 53.38
4	88.7 ± 0.6	493.06 ± 171.54
8	94.3 ± 1.3	487.73 ± 112.86
12	93.8 ± 2.1	354.75 ± 103.26
16	92.7 ± 2.5	235.20 ± 68.77
20	87.0 ± 3.8	196.13 ± 60.74
24	51.2 ± 6.3	123.28 ± 44.11
28	28.8 ± 8.3	100.44 ± 42.23

[1)] Each sample or variant used in the germination test was in triplicate. The percentage of germinating pollen of each replicate was estimated from samples of 200 pollen grains, while the length of pollen tubes used samples of 30 pollen grains. Samples were incubated at 25 ℃ for 48 h.

cultured pollen grains germinated differing significantly in this respect from the control variant without sucrose as well as from the variants with 24% and 28% concentrations of sucrose in culture medium, respectively. The 2 last mentioned variants were characterized by 51.2% and 28.8% proportions of germinating pollen which represents a considerably reduced figure also in relation to the control variant without sucrose with the 67.5% share of germinating pollen. This finding may be taken, therefore, as evidence indicating an inhibitory effect of higher sucrose concentrations on germination of *A. kawakamii* pollen.

Surprisingly enough, the tendency of pollen germinability is only partially paralleled by pollen tube growth potential of a tested sample. As seen from Fig. 98, the relationship between pollen tube growth and content of sucrose in medium is much more clear cut than the case of pollen germination. The maximum length of pollen tubes has as a rule been attained only on media with 4% and 8% sucrose, respectively, both of which are mutually comparable in this respect. On the other hand, the reduction of pollen tube growth registered in all the remaining variants is statistically highly significant, as confirmed by the Duncan test. However, in spite of a small shift in the requirements of both pollen viability parameters for optimal sucrose concentration, the medium with 8% sucrose was found to represent the optimum variant at which the highest percentage of germinating pollen grains (94.3%) and the longest pollen tubes (487.73 µm) were achieved. This medium was therefore used as a base in all subsequent tests of *in vitro* germination of *A. kawakamii* pollen.

It is interesting to point out in this connection that the optimum values of sucrose concentration found experimentally in some other species of firs are similar but not identical with that revealed in *A.kawakamii*. In *A. sachalinensis* (Muto, 1962), *A.*

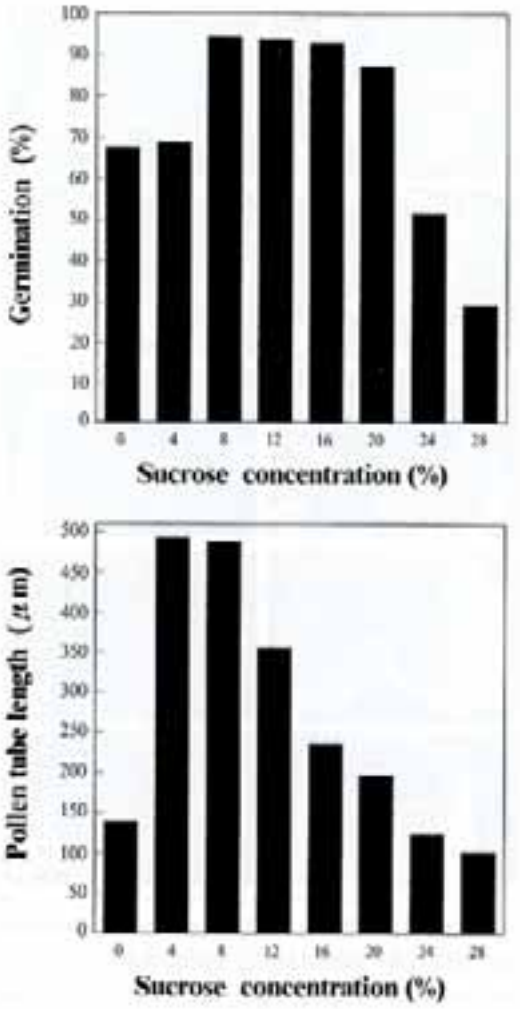


Fig. 98. Graphical illustration of the effect of sucrose concentration on *in vitro* germination of *A. kawakamii* pollen.

balsamea (Razmologov, 1964), *A. veitchii* (Saito *et al.*, 1983), and in 6 additional species of firs involving *A. alba, A. nordmanniana, A. grandis, A. pinsapo, A. concolor,* and *A. koreana* (Kantor and Chira, 1965), it was found that the best germinability of pollen was achieved with 10% sucrose in agar medium. On the contrary, in *A. homolepis* (Ito *et al.*, 1983) a broad range of 5%-20% sucrose concentrations was reported to be suitable for *in vitro* germination of its pollen. However, closer inspection of the figure graphically illustrating this relationship reveals that it was 10% sucrose in

culture medium at which the highest percentage of pollen germination was achieved. In spite of the deviation observed in *A. kawakamii*, the species also seems to conform the requirement for sucrose concentration in the culture medium as in the species mentioned above.

6.6 Pollen viability in individual trees

The main conclusions drawn from a comparative study on pollen viability in 21 trees of *A. kawakamii* growing within an elevation range of 2850-3230 m at Hohuan mt. concern a relatively high germinability of pollen as well as its considerable variation in individual study trees. It follows from the data given in Table 15 that the proportion of germinating pollen in tested trees ranged as widely as 61.0%-95.2% and averaged 85.8%. Although 4 study trees (nos. 7, 13, 15, 21) exhibited 61%-77% germinability of their pollen, in the prevailing majority of sampled trees the germination of pollen did not drop below 82% indicating a fairly high quality of *A. kawakamii* pollen. It is noteworthy that the group of 4 trees mentioned above predominantly involved trees with the highest frequency of meiotic irregularities observed (nos. 5, 6 - Table 9; nos. 13, 15 - Table 15) including trees whose microsporogenesis was affected so profoundly by the late frost (no. 9 - Table 9; no. 21 - Table 15). Statistically these trees represent the most conspicuous deviation from the average value, but differences in germinating capacity of pollen registered in the remaining trees also oscillated between less apparent and very profound differences illustrating the existence of distinct individual variability in fertility of *A. kawakamii* pollen. No relationship was observed between the germinability of pollen in individual study trees and their elevational distribution as shown schematically in Fig. 99 and confirmed stat-

Table 15. Pollen viability parameters revealed in individual study trees of *A. kawakamii* growing at different elevations[1)]

Tree no.	Elevation (m)	Pollen germination Mean (%)	Pollen tube length Mean (μm)
1	2850	94.3 ± 2.6	493.42 ±115.14
2	2870	89.5 ± 3.8	387.11 ±114.83
3	2955	87.7 ± 0.8	400.40 ±136.00
4	2955	95.2 ± 1.5	407.73 ±104.73
5	2965	84.2 ± 6.4	361.95 ± 88.99
6	3050	92.8 ± 1.6	412.26 ±119.04
7	3090	61.0 ± 9.3	370.48 ±105.89
8	3090	92.5 ± 0.9	357.95 ± 86.27
9	3090	84.2 ± 3.3	499.91 ±108.23
10	3090	89.3 ± 3.4	358.13 ± 81.27
11	3130	84.7 ± 4.7	397.95 ± 86.04
12	3130	91.7 ± 1.2	476.97 ±107.08
13	3160	77.3 ± 3.3	292.66 ± 98.40
14	3160	82.0 ± 5.4	328.44 ± 82.24
15	3160	64.2 ± 2.6	382.75 ± 80.44
16	3225	83.7 ± 1.6	299.33 ± 98.30
17	3225	90.8 ± 2.0	340.97 ±109.98
18	3225	93.8 ± 10.3	351.02 ± 87.97
19	3225	93.7 ± 0.8	427.91 ±109.92
20	3230	92.8 ± 0.3	369.82 ± 92.55
21	3230	75.8 ± 6.3	256.93 ± 77.55
Average		85.8 ± 3.0	379.71 ± 99.56

[1)] The number of estimated pollen grains and culture conditions were the same as those referred in Table 14 except only 8% sucrose was used throughout the experiment.

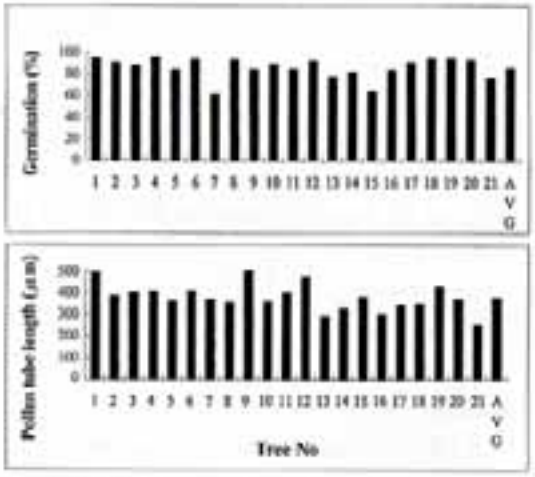


Fig. 99. Variation of pollen viability parameters in 21 study trees of *A. kawakamii* growing at different elevations; AVG - average values.

istically by the Duncan grouping.

Pollen tube growth exhibited a similar tendency with germinability, with the shortest pollen tubes being mostly typical for samples with the lowest share of germinating pollen, while the longest ones were from samples with the highest proportions of germinating pollen. In particular it is true of study trees nos. 13 and 21 whose low pollen germinability was paralleled by the slowest pollen tube growth reaching on average only 292.66 µm and 256.93 µm, respectively. It is in a sharp contrast with the longest pollen tubes of study trees nos. 1, 9, and 12 averaging 493.42 µm, 499.91 µm, and 476.97 µm, respectively (Table 15). In 2 additional samples of the bottom category regarding germinability (nos. 7, 15), this correlation was not, however, preserved, as was also seen in a few other trees in which the pollen viability characteristics were not so closely correlated . In general, the characteristic feature of pollen tube growth potential is its variable nature throughout the whole elevation range traced. A mutual comparison of the data illustrated in Fig. 99 reveals that the variability of this parameter was much more pronounced than was that of germinability, both of which, however, were shown to vary substantially in individual study trees (Table 16).

This type of individual variability was also observed in 56 trees of *A. alba* in Jugoslavia together with differences in pollen germination percentage between 4 localities compared by Popnikola (1971). Conversely, no variation of this kind was recently reported for *A. pinsapo* pollen, but the experiment involved only 2 trees (Arista and Talavera, 1994). As evidence supporting our finding relative to the lack of a relationship between fertility of *A. kawakamii* pollen and elevation of individual study trees, data published by Kirgizov (1978) may be taken. They indicate the absence of differences between morphological characters and fertility of *A. sibirica* pollen at the elevations of 800, 1200, and 1500 m in the Altai Mountains. Our study only encompassed an elevation difference of 380 m, but it seems reasonable to suppose that, except for the genetic constitution of a tree and climatic conditions, it is the position of a tree within a stand which exerts some effect on the course of microsporogenesis and the quality of the resulting pollen. The availability of sufficient amount of sun, together with shelter provided by neighboring trees or by terrain, as well as some other circumstances which contribute to the optimatization of external factors ensuring the normal course of microsporogenesis, may be looked upon as important sources of variation.

Cytologically, the process of pollen germination is remarkable by the presence of a very distinct tube nucleus of either a round shape

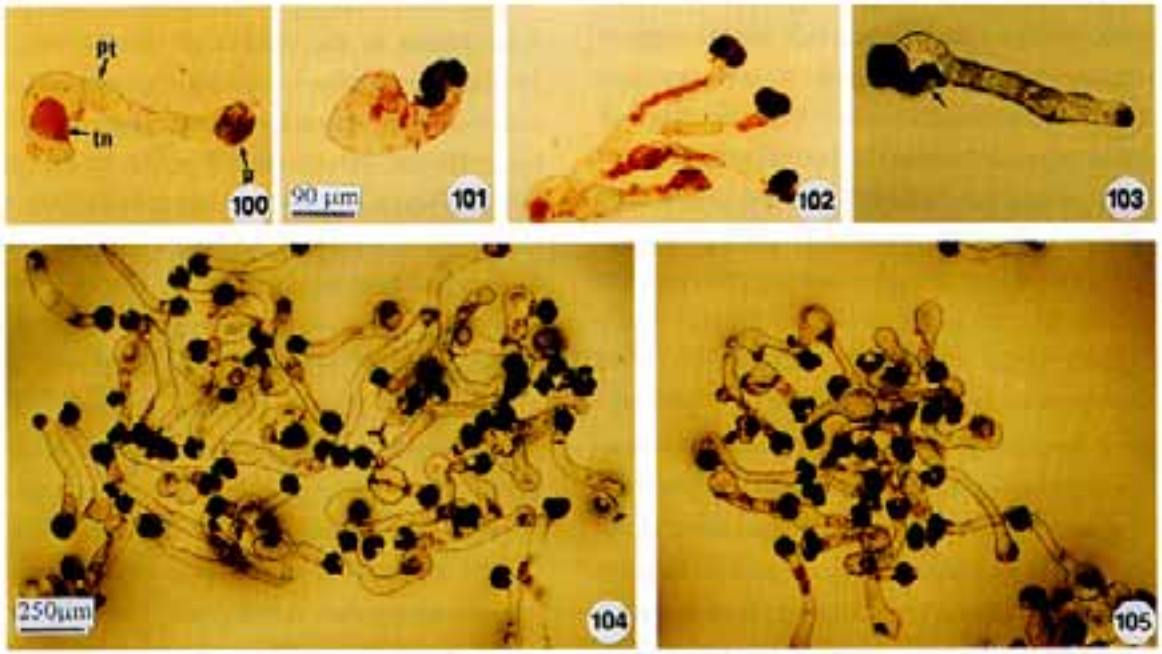
Table 16. Variance analysis of viability parameters of *A. kawakamii* pollen within a tested group of study trees

Pollen germination

Variance resource	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Trees	20	5399.57	269.97	19.24	0.0001
Error	42	589.33	14.03		
Corrected total	62	5988.90			
Pollen tube length					
Variance resource	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Trees	20	6959936.68	347996.83	34.32	0.0001
Error	1869	18951262.04	10139.79		
Corrected total	1889	25911198.72			

at the very tip of the growing pollen tube (Figs. 100-101) or by a diffuse form stretched along the middle part of the pollen tube (Fig. 102). However, not only the tube nucleus but also the pollen tubes themselves were found to vary in their morphology. As seen in Figs. 104-105 in samples collected from lower elevations, the rod-shaped tubes were typical, while in those derived from higher elevations the club-like form of pollen tubes prevailed. The latter also occurred frequently in samples of stored pollen irrespective of their origin.

In general, the viability of *A. kawakamii* pollen is relatively high as evidenced by the data presented above. Every pollen sample of *A. kawakamii* has exhibited an absolute germinability as reported for *A. pinsapo* pollen (Arista and Talavera, 1994) but with its average germinability of 85.8% and average length of pollen tubes of 379.71 µm the species is comparable with *A. sibirica A. veitchii,* and *A. alba* in which pollen germination rates ranged from 86.5% to 94.9%, 77% to 93% and 88% to 93% (Kirgizov and Mosin, 1980; Kormutak, 1995) respectively. The length of pollen tubes under comparable culture conditions as in case of *A. kawakamii* averaged in *A. veitchii* as low as 248 µm, while in *A. alba* at 326 µm (Saito *et al.*, 1983; Kormutak, 1995). According to the maximum length of pollen tubes, *A. kawakamii* samples nos. 1, 9, and 12 with their 493.42 µm, 499.91 µm, and 476.91 µm long tubes even surpassed the above-mentioned samples of *A. pinsapo* pollen with its corresponding pollen tube length averaging only 450 µm. Also, the average viability characteristics of *A. kawakamii* pollen represent higher values relative to both pollen germinability and pollen tube length than those ascertained in *A. alba, A. nordmanniana, A. pinsapo,* and *A. concolor* in which these variables oscillated within the extent of 25%-39% and 55-222 µm, respectively. The only exception in this respect was the fertility of *A. grandis* pollen characterized by 46% germi-



Figs. 100-105. In vitro pollen germination showing the round tube nucleus at the tip of the pollen tube (Figs. 100-101) or the diffuse one in the middle part of a tube (Fig. 102). Fig. 104. Rod-shaped pollen tubes. Fig. 105. Club-like pollen tubes. Fig. 103. Germinable pollen grain of normal size and non-germinable pollen grain of reduced size (arrowhead); p-pollen grain, pt-pollen tube, t-tube nucleus. The bar on Fig. 101 is for Figs. 100-103; the bar on Fig. 104 is for Figs. 104-105.

nability and by average length of pollen tubes as long as 518 µm (Kantor and Chira, 1965). A lower percentage of germinability than that established by us in *A. kawakamii* has been reported for *A. homolepis* (66%) also (Ito *et al.*, 1983).

In light of these comparisons, it is obvious that fertility of *A. kawakamii* pollen is high enough to ensure effective fertilization of ovules. Accordingly, the fertilization failures in *A. kawakamii* as indicated by an enormously high share of empty seeds regularly produced by the species can hardly be ascribed to low fertility or even to inviability of its pollen. It is clear that the causes of this phenomenon must be searched for in the sphere of either early ovule development or embryogenesis.

6.7 Longevity of pollen during storage

Of the external factors which are of decisive importance in maintaining pollen viability during storage, only the effect of temperature was tested. The relative humidity as well as the type of atmosphere surrounding the pollen were not taken into consideration because of the difficulties with their regulation inside the bottles with silica gel into which the vials with assayed pollen were inserted. The study involved the pollen sample of only 1 study tree (no. 1) whose capability to retain its fertility was tested throughout the period of 6 months under 4 temperature regimes, i.e., room temperature, +4℃, -20 ℃, and -70℃, respectively.

As expected, the most progressive loss of pollen viability was registered in samples stored at room temperature. The continuous nature of this process is well illustrated by the data presented in Table 17. However, it follows simultaneously from the table that this continuity was transitionally interrupted by an abrupt decrease of pollen viability at the very beginning of storage when pollen germination and pollen tube growth

Table 17. Changes in pollen viability parameters during storage of pollen at room temperature[1)]

Weeks	Pollen germination Mean (%)	Pollen tube length Mean (µm)
0	94.3 ± 2.6	493.42 ±115.14
1	71.8 ± 6.6	257.24 ± 92.51
2	89.3 ± 3.4	469.42 ±137.08
3	88.0 ± 1.3	413.24 ±113.42
4	85.2 ± 2.4	374.84 ± 95.38
5	78.2 ± 3.6	302.18 ± 93.29
6	66.0 ± 6.5	251.11 ± 73.98
7	33.2 ± 4.8	112.97 ± 59.32
8	29.3 ± 4.9	91.11 ± 48.05
9	20.2 ± 2.0	75.73 ± 42.26

[1)] The number of estimated pollen grains and culture conditions were the same as those referred in Table 14 except only 8% sucrose was used throughout the experiment.

dropped during the 1st week of storage to a level which corresponds to viability parameters registered 4-5 weeks later. This decline represents one of the most conspicuous features observed in the behavior of pollen under room temperature storage. According to Stanley and Linskens (1974), it may be related to the inactivation of enzymes and metabolic substrates essential for germination, or it may simply be an adaptive reaction of pollen to a sudden change of relative humidity in the bottle with silica gel. In the week which followed, pollen viability was restored reaching a level which was statistically comparable with the control but only with regard to pollen tube length (493.42 µm vs. 469.42 µm). Pollen germination at this stage averaged 89.3% only deviating significantly from the average germination of the control variant (94.3%). Only in the 3rd week of storage did pollen tube growth potential also drop below the level of statistical significancy. This tendency of decreasing pollen viability was clearly expressed throughout the period of the next 7 weeks when pollen germinability and pollen tube length declined to as low as 21% and 15%, respectively, relative to the control. Statistical significance of

these differences as well as the differences exhibited by the viability characteristics during all 9 weeks of storage were convincingly confirmed by the F-test (Table 18). The complete loss of pollen fertility, as a rule, occurred at the stage corresponding to the 10th week of storage which is a much longer longevity span than that reported for the 8 exotic species of firs introduced into Belarussia and which retained pollen viability under similar storage conditions for 5-12 days only (Kravchenko *et al.*, 1973).

The same reaction of pollen during room temperature storage was also typical for the initial phase of pollen storage at low temperatures. Irrespective of storage temperature of +4 ℃, -20 ℃, or -70 ℃, samples uniformly responded to lowered temperature by a decrease in germinability and pollen tube length below the level of the control. The decline in germination percentage was remarkably uniform averaging at 90.0%-90.3% in all 3 types of samples as compared with the 94.3% average germinability of the control variant (Table 19). The extent of reduction of pollen tube growth, on the other hand, differed slightly among the tested samples, being the most intensive at +4℃ (436.35 μm), intermediate at -20 ℃(452.08 μm), and lowest at -70 ℃ (479.82 μm) relative to the control (493.42 μm). In the sample stored at -20 ℃, the declining tendency in both pollen viability parameters persisted throughout the rest of the test period as contrasted with considerably variable values in samples stored at +4 ℃ and -70 ℃.

Following the initial decline in percentage of germinating pollen grains, the pollen sample at -20 ℃ progressively lost its viability during the subsequent 5 months of storage exhibiting germinability as low as 24.5% at the end of the 6-month period. The process was paralleled by a continuous reduction in the pollen tube length parameter as evidenced by the 452.08 μm length of pollen tubes found after 1 month of storage and by the 258.22 μm length of pollen tubes at the end of the test period. In relative terms, this decline reached 74.1% of pollen germination and 47.7% of pollen tube length relative to the control, with both viability parameters deviating statistically from the control after the 3rd and 2nd month of storage, respectively.

Contrary to the pollen sample kept at -20 ℃, the viability of pollen stored at +4 ℃ and -70 ℃ was comparable with the viability of the control throughout the entire period of storage. The pollen maintained at -70 ℃ even attained a higher germinability after 5 months of storage (94.8%) than the control (94.3%) and the same was true of the pollen tube parameter which averaged 528.17 μm at the end of the test period as compared with

Table 18. Variance analysis of viability parameters of *A. kawakamii* pollen during storage at room temperature

Pollen germination

Variance resource	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Replicates	2	199.85	99.92	11.83	0.0005
Weeks	9	20705.34	2300.59	272.47	0.0001
Corrected total	11	20905.19			

Pollen tube length

Variance resource	Degree of freedom	Sum of squares	Mean square	F-value	Prob.
Replicates	2	24979	12489	1.48	0.2277
Weeks	9	19363845	2151538	255.30	0.0001
Corrected total	11	19388825			

Table 19. Changes in pollen viability parameters during storage of pollen at 3 different low temperature regimes[1)]

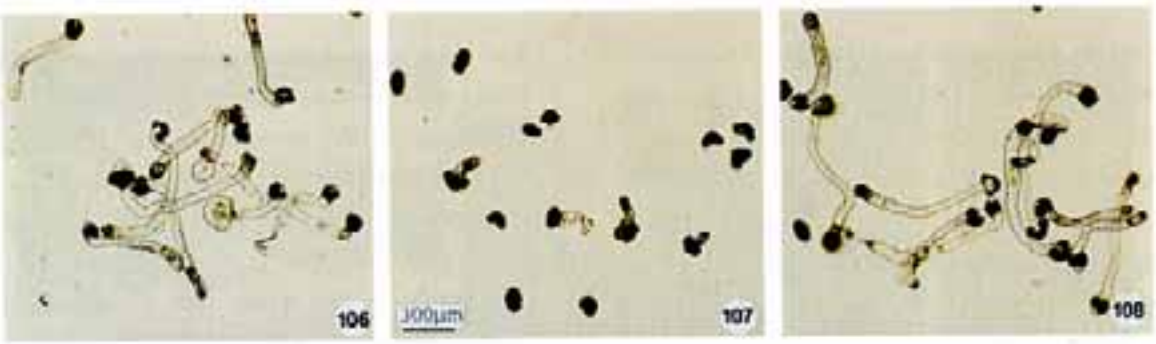
Temp.	Time (mo)	Pollen germination	Pollen tube length
		Mean (%)	Mean (μm)
Control		94.3 ± 2.6	493.42 ± 115.14
+4 ℃	1	90.3 ± 1.6	436.35 ± 113.41
	2	91.8 ± 2.8	335.91 ± 103.64
	3	93.8 ± 1.6	488.00 ± 94.47
	4	93.0 ± 0.9	467.11 ± 100.32
	5	91.7 ± 2.9	387.55 ± 81.56
	6	93.2 ± 0.3	441.52 ± 100.15
-20 ℃	1	90.3 ± 1.9	452.08 ± 112.26
	2	88.8 ± 3.3	374.46 ± 128.05
	3	79.2 ± 4.3	474.17 ± 102.91
	4	40.7 ± 5.3	311.86 ± 99.31
	5	28.8 ± 6.3	221.35 ± 75.74
	6	24.5 ± 6.2	258.22 ± 86.41
-70 ℃	1	90.0 ± 2.5	479.82 ± 123.53
	2	91.2 ± 3.3	323.64 ± 107.71
	3	94.3 ± 2.1	489.33 ± 101.47
	4	93.0 ± 0.9	468.71 ± 114.05
	5	94.8 ± 0.3	465.73 ± 90.28
	6	89.5 ± 3.3	528.17 ± 121.13

[1)] The number of estimated pollen grains and culture conditions were the same as those referred in Table 14 except only 8% sucrose was used throughout the experiment.

493.42 μm in the control. In general, the germinability of both variants at +4℃ and -70℃ was subject to small variations during storage oscillating between 90.3% and 93.8% at +4℃ and between 89.5% and 94.8% at -70 ℃. On the contrary, pollen tube length varied more widely ranging within such broad limits as 335.91 and 488.00 μm in the former and 323.64 and 528.17 μm in the latter. Also, in comparison with the pollen germination exhibiting a certain decline only during the 1st month of storage, the declining tendency was more profound regarding pollen tube growth and persisted for 2 months in both variants (Table 19). The average values of pollen tubes ascertained in these samples after 2 months of storage at +4℃ (335.91 μm) and -70℃ (323.64 μm) represent statistically significant deviations from the control (492.42 μm).

In light of these results, it is obvious that storage of *A. kawakamii* pollen at lowered temperatures is preferred to storage at room temperature. Among the low temperature regimes tested so far, storage at both +4℃ and -70℃ gave the best results with the viability of stored pollen comparable to that of freshly collected pollen of the control. This finding is consistent with data published by Popnikola (1971) and Dobrinov and Gagov (1975) postulating a temperature around +4 ℃ to be the most convenient for storage of *A. alba* pollen, but is in contradiction with the reported complete loss of pollen viability in *A. pinsapo* following 6-month storage at +4℃ (Arista and Talavera, 1994).

Within the context of the observed responses of stored pollen, the progressive decline of pollen viability at -20℃ storage represents a rather strange result as illustrated by the 24.5% proportion of germinating pollen ascertained in the sample at the end of the test period in sharp contrast with the 93.1% and 89.5% germination parameters of pollen kept at +4℃ and -70℃, respectively (Figs. 106-108). The only explanation for such a deviation is increased relative humidity within a vial with pollen which supposedly occurred during manipulation of the pollen following its storage, adversely affecting its germination during the subsequent period. Accordingly, the profound effect of temperature on germinability of half-year stored pollen revealed by the variance analysis refers exclusively to the reduced viability parameter of pollen at -20 ℃ (Table 20). However, as far as the pollen tube length is concerned, all 4 temperature regimes differed significantly from each other with the -20℃ sample deviating the most widely. In spite of the fact that overall duration of storage was restricted to a period of 6 months, length of storage has also been found to exert considerable effect on pollen viability chara-



Figs. 106-108. In vitro germination of *A. kawakamii* pollen after 6-months' storage at +4 ℃ (Fig. 106), -20 ℃ (Fig. 107) and -70 ℃ (Fig. 108). The bar on Fig. 107 is for Figs. 106-108.

Table 20. Variance analysis of pollen viability parameters during storage at 3 different temperature regimes

Pollen germination

Variance resource	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Replicates	2	11.23	5.61	0.06	0.9419
Months	5	2010.97	402.19	4.29	0.0027
Temp.	3	6297.90	2099.30	22.41	0.0001

Pollen tube length

Variance resource	Degrees of freedom	Sum of squares	Mean square	F-value	Prob.
Replicates	2	11619	5809	0.53	0.5885
Months	5	3928447	785689	71.71	0.0001
Temp.	3	4043479	1347826	123.02	0.0001
Mo. x Te.	10	4435550	443555	40.49	0.0001

cteristics. As in the case of temperature, pollen germination was shown to be affected to a lesser degree by prolonged storage than was pollen tube growth.

7 Ovule development and embryogenesis

As in the case of pollen grains, ovules of true firs have also been subjects of cytological investigations since the beginning of the century when Miyake (1903) gave a general account of fertilization in *A. balsamea* together with some details describing fertilization of its egg nucleus. Numerous studies have since been undertaken aiming to elucidate different aspects of the process in the species mentioned above as well as in some other firs. Hutchinson (1914, 1924) described the behaviour of such ovular structures as the ventral canal cell, egg nucleus, and chromosomes during fertilization of *A. balsamea* and cytological structure of the embryo during its early developmental stage. Studies that appeared since that time have covered some additional aspects of fertilization in firs including pollination mechanisms in *A. nordmanniana*, *A. homolepis*, *A. koreana*, *A. webiana*, and *A. balsamea* (Doyle and Kane, 1943; Powell, 1970), megasporogenesis and archegonial development in *A. pindrow* (Dogra, 1966), and embryo development in *A. pinsapo* (Buchholz, 1942), *A. firma* (Sugihara, 1947), and *A. pindrow* (Mehra and Dogra, 1975, 1977; Konar and Nagmani, 1980). Clarifying the nature and sequence of events encompassing a small part or a larger span of ovule development and embryo-

genesis, these studies have contributed enormously to the elucidation of general patterns of fertilization in *Abies*. It was shown that, in spite of a common occurrence of such events as pollination, fertilization, and maturation of seeds, different species or representatives of the same species may differ with regard to the initiation and the overall duration of these events depending on climatic conditions under which they grow (Singh and Owens, 1981). In order to illustrate climatic influences on reproductive variation, an approach based on a complete study of sexual reproduction in firs is preferred to an analysis of only partial aspects. The aim is to describe a species-specific course of fertilization as well as deviations which are supposed to be responsible for the relatively low and highly variable reproductive potential among *Abies* species.

In *A. amabilis* such an approach was applied to detect the primary reasons of its irregular cone-bearing capacity (Owens and Molder, 1977b), whereas it was used in *A. lasiocarpa* and *A. grandis* to search for the causes of their low seed sets (Singh and Owens, 1981, 1982). Low seed sets represent the most serious problem in the reproductive biology of *A.kawakamii* as well. Against a background of a relatively high viability of pollen, it is reasonable to suppose there are 2 mechanisms underlying the phenomenon, i.e., insufficient pollination or abortive ovular development.

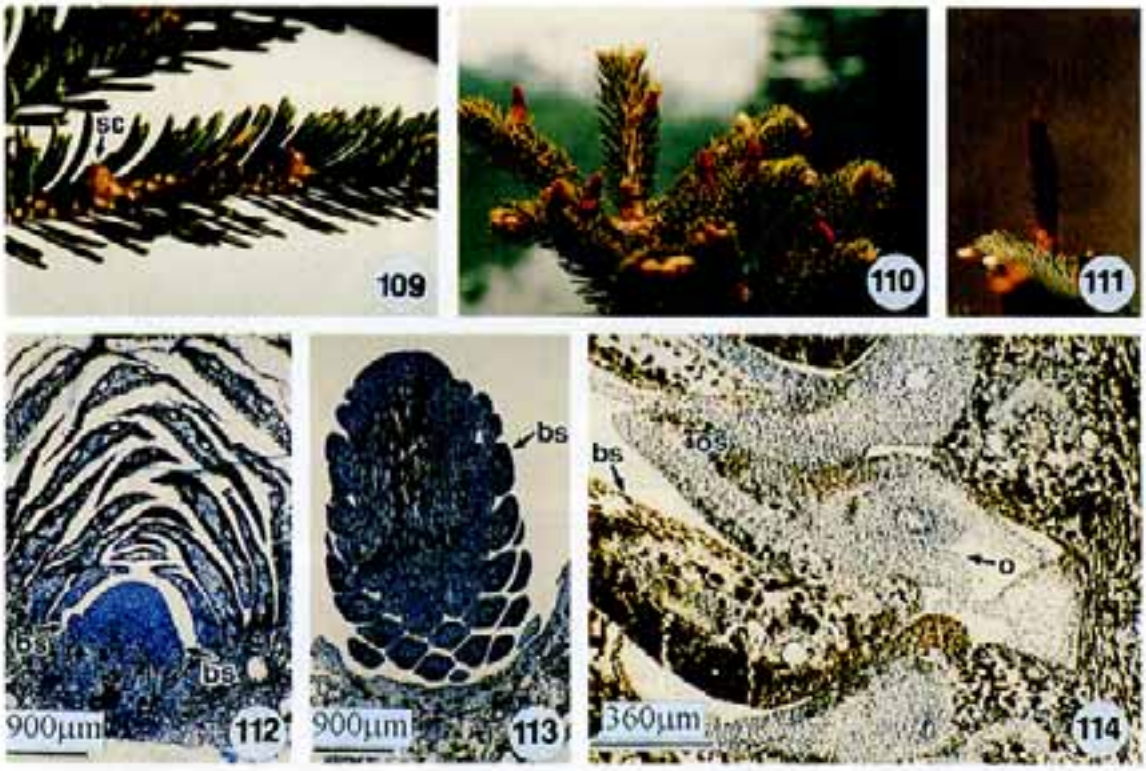
7.1. Prepollination and postpollination development of ovules and embryogenesis

7.1.1 Seed-cone bud initiation and postdormancy development of seed cones

Like other species of true firs, *A. kawakamii* shows a 1-year type of reproductive cycle with pollen-cone buds and seed-cone buds initiating in June and seeds achieving maturity during the period October-November of the following year, depending on the elevation (Chen, 1967; Liu, 1971). Seed-cone buds are axillary and occur on the upper side of the primary or secondary lateral shoots (Fig. 109). They are distributed more or less evenly in the crown with a predominant concentration on its upper whorls. During the time of bud-scale initiation, potential seed-cone buds are difficult to distinguish from vegetative lateral buds (Fig. 112). Seed-cone buds' bract scales and primordia of ovuliferous scales only became apparent in the beginning of September (Fig.113), achieving full differentiation by the end of the month.

Gross changes accompanying postdormancy development of seed-cone buds involved their swelling through mid-January to mid-March followed by bud bursting at the beginning of April (Fig. 110). A rapid elongation of the protruded megastrobili follows during the next 10-15 days. The female strobili reached their maximal receptivity on April 23 at an elevation of 2850 m and on May 1 at 2955 m. They were purple at this time and contained ovules with partially vacuolized megaspores and widely opened micropyles (Figs. 111 and 114). Schematically these changes are depicted in Fig. 115 together with the changes which accompany the morphogenetic transformation of pollinated female strobili into mature seed cones.

As mentioned already, abrupt declines in temperature occasionally occurred in the study area at Hohuan mt. during spring 1996 adversely affecting development of the reproductive organs in *A. kawakamii*. One such event occurred as late as April 22 coinciding with the period of flowering of the species in the region. Having completed its development by this time, pollen of *A. kawakamii* did not suffer from this climatic change as judged by its viability parameters, but a few days of frosty



Figs. 109-114. Prepollination development of female strobili. Fig. 109. Differentiated seed-cone buds on upper side of a shoot as found in July. Fig. 110. Megastrobili protruding from bud scales at the beginning of April of the next year. Fig. 111. Receptive megastrobillus in the 2nd half of April. Figs. 112-113. Longisections of developing megastrobili as found in the middle of June (Fig. 112) and at the beginning of September (Fig. 113) showing bract scales (bs). Fig. 114. Median longitudinal section of a small part of receptive megastrobillus showing bract scales (bc) and ovuliferous scales (os) with receptive ovules (o) at their bases.

weather combined with a snowfall and intense sunshine almost completely destroyed female strobili including those that were involved in artificial hybridization and which had therefore been protected by isolation paper bags. Only a small portion of these survived and subsequently developed into mature cones (Figs. 116-120). The excessive variation of climate in Taiwan's mountain regions encompassing a climatic range from subtropical to subarctic (Hsieh and Shen, 1994) may be therefore looked upon as a major factor responsible for the reduction of cone bearing capacity in *A.kawakamii*. This may eventually affect the frequency of rich cone crops, averaging 4.6 years in the species (Lai, 1994).

7.1.2 Cytological structure of *A. kawakamii* ovules

Development of ovules during the postdormancy period of seed cones was traced cytologically after the stage of megaspore mother cell. The latter differentiated from the sporogeneous cells of the cones and could be recognized at the end of March as an enlarged elongated cell which is centrally placed within a mass of sporogeneous cells and encircled by the 6-7-celled layer of parietal tissue (Fig. 121). The megaspore mother cell had entered meiosis on April 12 (Fig. 122) giving rise to 4 megaspores of which only the megaspore at the chalazal end of the nucellus develops further, while in the upper three meiotic products degen-

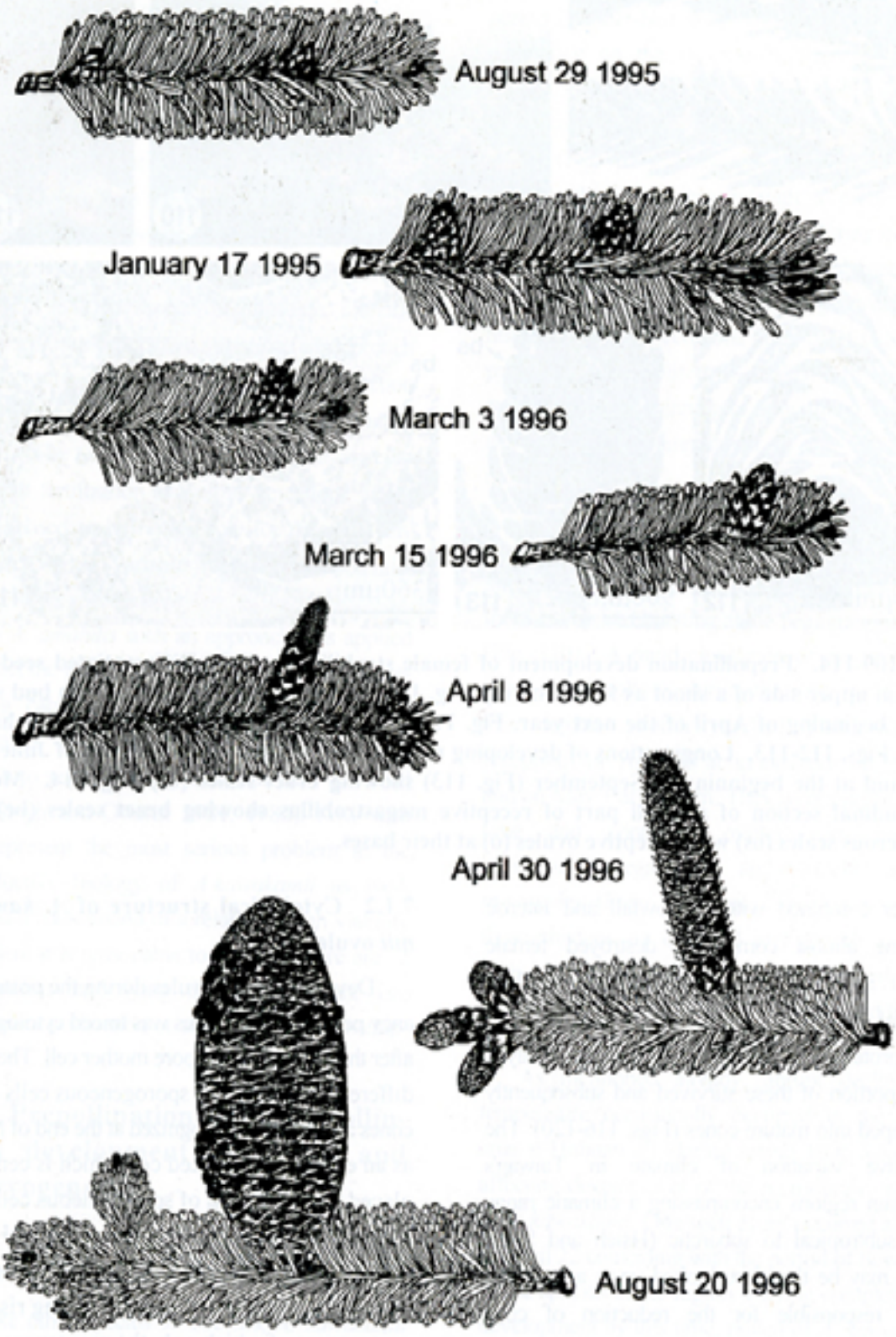
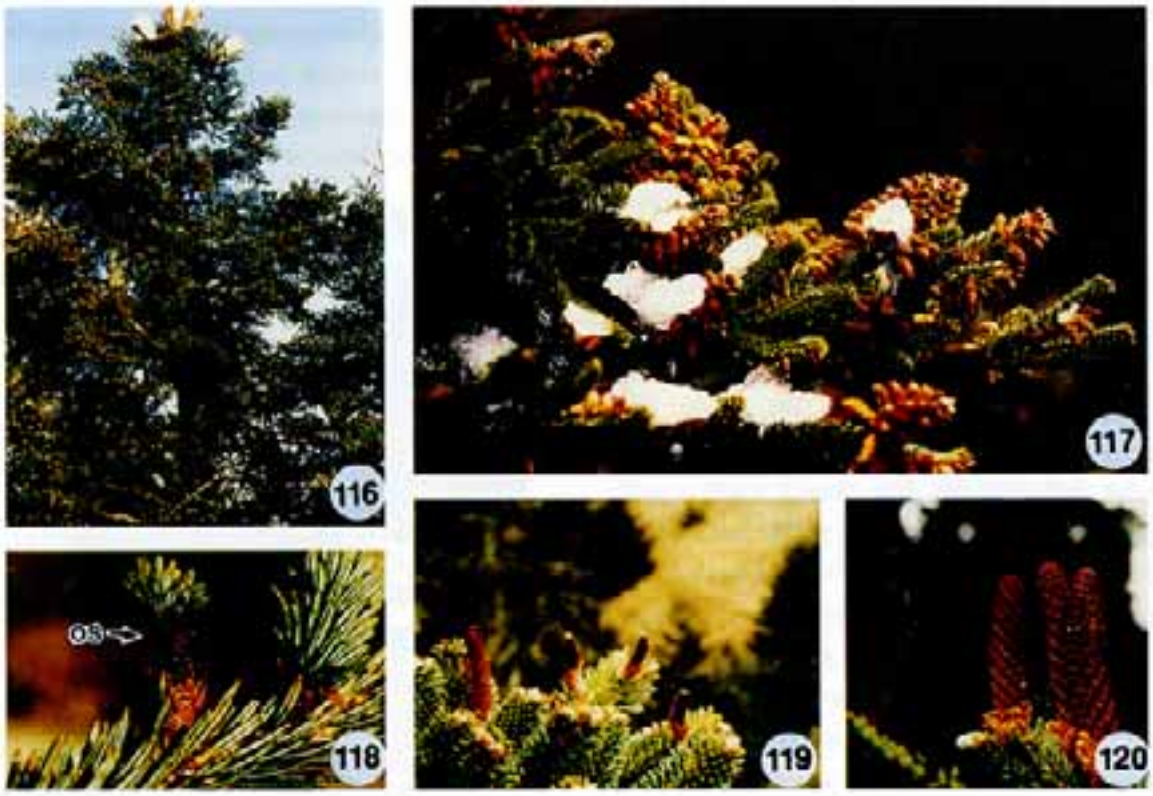
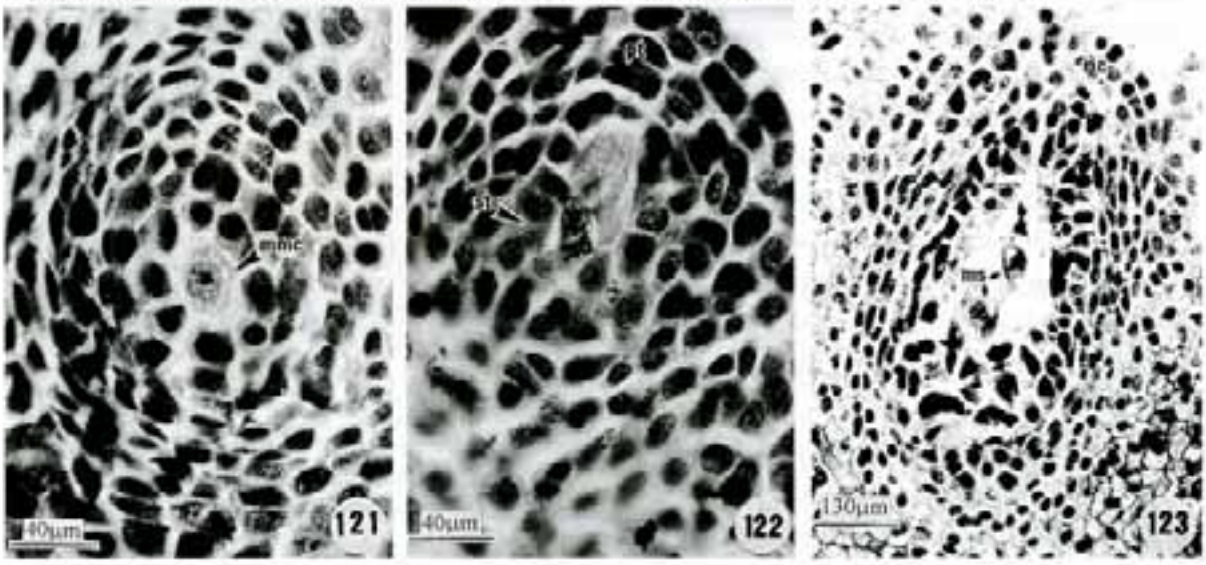


Fig. 115. Schematic illustration of seed-cone bud differentiation and its subsequent development into megastrobillus and mature cone (Drawing by Alzbeta Kormutak).



Figs. 116-120. Climatically conditioned damage to *A. kawakamii* megastrobili. Fig. 116. Maternal tree of the species with isolated megastrobili at its top. Fig. 117. Snowfall at the time of flowering of the species on April 23 resulting in extensive damage of its receptive female strobili (Fig. 119). Only some of them were left unaffected (Fig. 120). Fig. 118. Anomalous female strobillus of *A. kawakamii* consisting of ovuliferous scales (os) at its bottom part and needles on its tip.

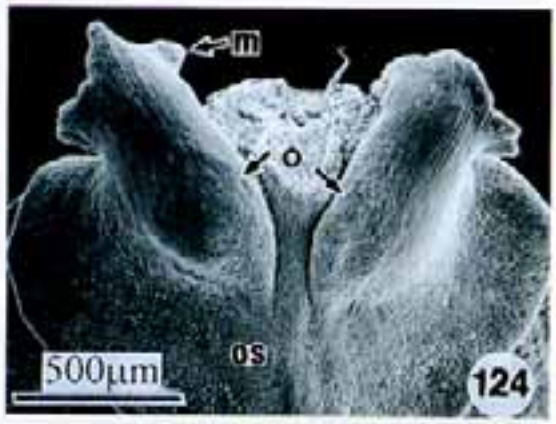


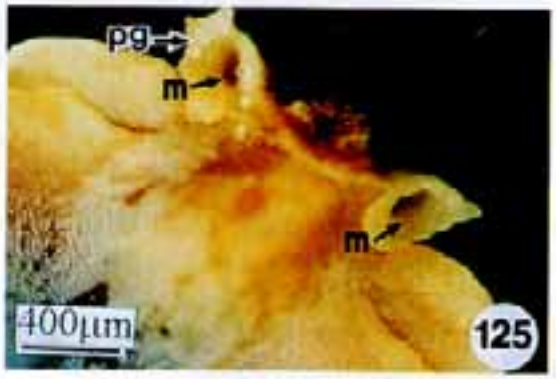
Figs. 121-123. Differentiated megaspore mother cell at the end of March (Fig. 121) and its meiotic division in mid-April (Fig. 122) giving rise to the 4 megaspores (ms) of which only one developed further (Fig. 123); pt-parietal tissue, st-spongy tissue, nc-nucellar cap.

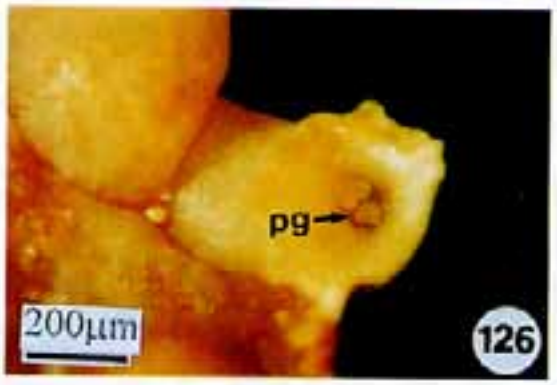
erate. The cells of the nucellar epidermis divide periclinally at this stage forming a thick nucellar cap (Fig. 123).

The receptive ovule is structurally differentiated into a relatively small megasporangium or nucellus with an enlarged and highly vacuolized megaspore within and surrounding the massive integument which is joined with the macrosporangium at its lower, chalazal end, and separated from it at its upper side forming a funnel-like micropyle with its free end (Fig. 127). The micropyle is widely opened during shedding of pollen and with its lobe-shaped margin well adapted for trapping air-borne pollen grains (Figs. 124-125). Both receptivity of female strobili and pollen shedding were synchronized in *A. kawakamii* persisting over the period of April 22-30 at an elevation of 2850 m. The pollen grains which sifted down between the bract and ovuliferous scales of a cone settled on the inner side of the micropylar funnel (Fig. 125) or entered the funnel and landed on the top of a nucellus (Figs. 126-127). Singh and Owens (1982) observed numerous microdroplets on the micropylar flange in freshly collected ovules of *A. grandis* which are supposed to make the micropylar surface sticky. The authors consequently ascribed secretory function to the epidermal and hypodermal cells of the micropyle. Kozubov *et al.* (1982) are also of the opinion that the nucellar cells are endowed with secretory ability which plays a significant role in adhesion of pollen to the top of the nucellus. The inner surface of the micropylar flange in *A. kawakamii* also seems to be moistened (Fig. 125).

Owing to the fact that the period of flowering in *A. kawakamii* overlaps the rainy season during the spring in Taiwan, an attempt was made to verify the efficiency of wind pollination of the species by inspecting still-receptive ovules under a stereomicroscope. Though there were only a few non-rainy days during pollen shedding at Hohuan mt., an overwhelming majority of inspected ovules was found to contain pollen grains within their micropylar openings (Fig. 126). This was true irrespective of the location of female strobili at the







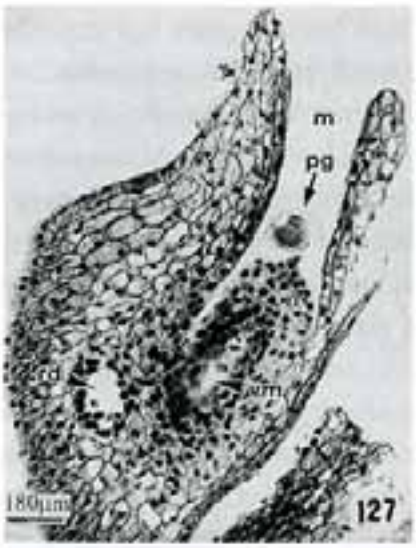
Figs. 124-126. Scanning electron and stereo microscope micrographs of receptive ovules. Fig. 124. Ovuliferous scale (os) containing 2 ovules (o) with a lobe-shaped margin of micropylar canal(m). Figs. 125-126. Pollinated ovules with pollen grains (pg) trapped on the inner side of micropylar funnel (Fig. 125) or within a funnel (Fig. 126).

top, middle, or bottom part of a crown suggesting that lack of pollination cannot be accepted as an explanation for low seed set in the species as was the case in *A. sibirica* (Okishev and Pugachev, 1983).

7.1.3 Female gametophyte and archegonia

At the time of pollination, the megaspore has already entered the stage of free nuclear division, thus initiating the process of female gametophyte development. Synchronous free nuclear division extends over a period of 2 months and is paralleled by a considerable enlargement in megaspore size. This is a much longer period than that reported for *A. amabilis, A. lasiocarpa,* and *A. grandis* in which the corresponding process was found to last 4-6 weeks only (Owens and Molder, 1977; Singh and Owens, 1981, 1982). The initial stage of female gametophyte formation is characterized by a few nuclei which are loosely scattered in the cytoplasm of the megaspore (Fig. 127) or ordered linearly in the form of a folded ribbon (Fig. 128). The stage is typified by its long duration, extending in *A. kawakamii* from the beginning of May till the middle of June. The coenocyte-like stage represented by the multitude of mutually interconnected haploid nuclei was only reached during the second half of June (Fig. 130). The more or less compact mass of a young megagametophyte is enclosed by the tapetal layer and consists of nuclei which are still free and very distinct from their nucleoli (Figs. 130-131). Cellularization of the free-nuclei gametophyte occurred in *A. kawakamii* at the end of June and marked the beginning of the next stage in female gametophyte development characterized by its conversion from the coenocyte state to the cellular state. The process is accompanied by a simultaneous initiation of archegonium initials from the superficial cells of the female gametophyte at the micropylar end of the ovule, which takes place irrespective of the fact that the gametophyte has only partially filled the megaspore by this time (Fig. 132). The conversion of archegonial initials into archegonia is preceded by a division of the former into neck initials and central cells followed by a tremendous enlargement of the central cells into archegonia. In *A. kawakamii,* the archegonia are elongate, exceptionaly vacuolate, and frothy in appearance (Figs. 133-135). The layer of cells adjacent to the archegonium divides transversely and differentiates into the archegonial jacket. Each archegonium is surrounded by its own jacket which is a unicellular type containing large and darkly stained nuclei (Fig. 133). Occasionally, 2 or 3 archegonia may be encompassed by a common jacket forming an archegonial complex (Coulter and Chamberlain, 1910; Chamberlain, 1935). In *A. kawakamii* this phenomenon, however, was not observed, and hence, its archegonia are of the separate type. The young female gametophyte with differentiated archegonia at its micropylar part is surrounded by a prominent megaspore wall (Figs. 133-135).

During the final stages of maturation of archegonia, the nucleus of the central cell divides giving rise to a small ventral canal cell (Fig. 135) and a large egg cell which is ovoid with finely granulated perinuclear cytoplasm (Fig. 136). Although the ventral canal cell was reported to disintegrate promptly in *Pinaceae* (Bold *et al.,* 1982), in *A. kawakamii* it persists until the penetration of the nucellus by germinating pollen grains (Fig. 138). The neck of the archegonium, a site where pollen tubes enter the archegonium, is elongated consisting of 2-4 cells which are arranged into 2 tiers and overlaid by the megaspore wall (Fig. 135). A frothy consistency of the egg cell cytoplasm so apparent during early developmental stages becomes dense and darkly stained in mature archegonia with numerous inclusions involved and a centrally placed egg nucleus (Fig. 136). The cytoplasm of the ventral



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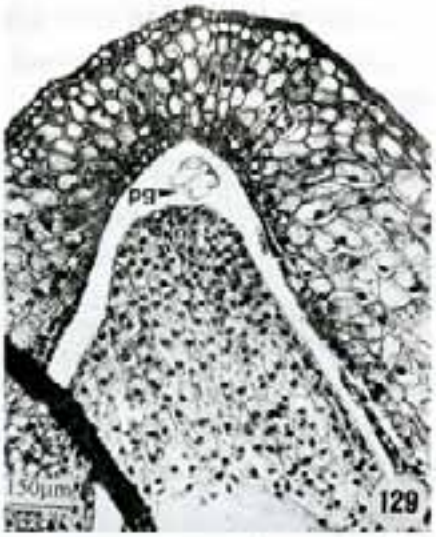

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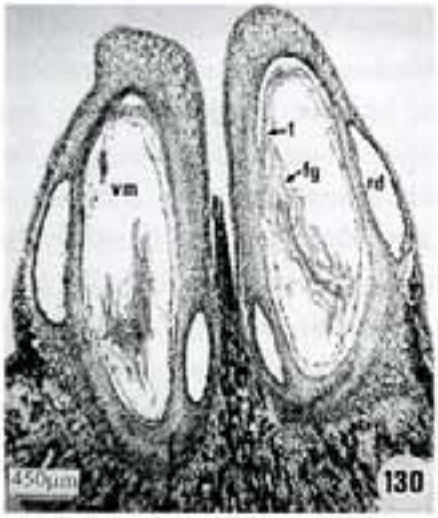

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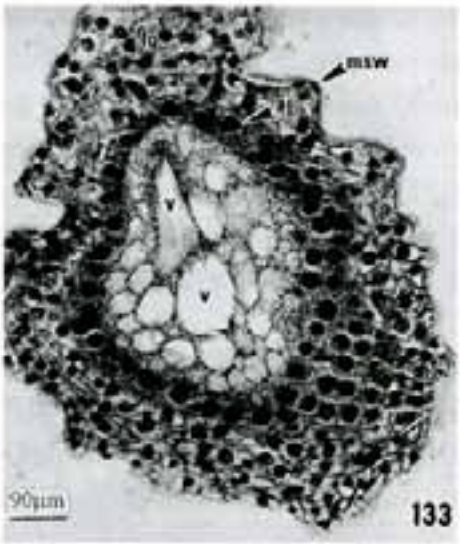

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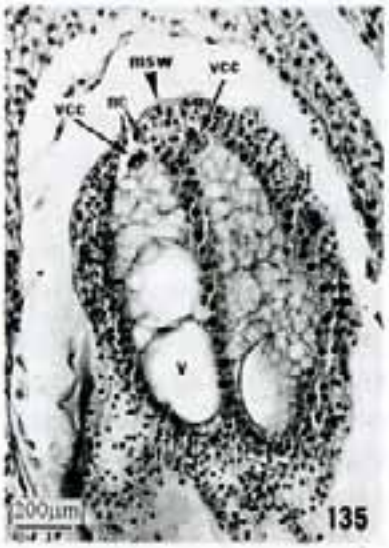

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Figs. 127-135. Longitudinal sections of pollinated ovules in different stages of development. Fig. 127. Ovule shortly after pollination consisting of nucellus (n), vacuolized megaspore (vm) and integument (i) with resin-duct (rd). Still-opened micropyle (m) contains pollen grain (pg). In the megaspore, the division of its nucleus has initiated the process of female gametophyte formation. Fig. 128. Later stage showing the ovule with enclosed micropyle and more advanced vacuolization of megaspore and female gametophyte (fg) formation. Fig. 129. Pollen chamber with pollen grain (pg) above the nucellus (n). Fig. 130. Highly vacuolized megaspore (vm) of the ovules in mid-June containing coenocyte-like female gametophyte (fg) enclosed by a tapetum (t). Resin-ducts (rd) around the megaspore are also distinct at this time. Fig. 131. Detail of a coenocyte-like megagametophyte (fg); t - tapetum. Fig. 132. Differentiation of archegonium (a) from superficial cells of female gametophyte (fg) at the end of June; n-nucellus, pg-pollen grain, rd-resin-duct. Fig. 133. Detail of archegonium with numerous vacuoles (v) within and surrounding the layer of archegonial jacket (aj); fg-female gametophyte, msw-megaspore wall. Fig. 134. Four archegonia (a) separated by archegonial jackets (aj); v-vacuoles, msw-megaspore wall. Fig. 135. Ventral canal cell (vcc) and neck cells (nc) of archegonia; v-vacuoles, aj-archegonial jacket, fg-female gametophyte, msw-megaspore wall.

canal cell has meanwhile protruded into the base of the neck cells, which is another condition which is recognized in *Abies* only shortly before fertilization of eggs (Singh and Owens, 1981). In *A. kawakamii* it was attained at the beginning of July (Fig. 138).

Archegonia occupy a special position among the reproductive structures described so far because their egg cells are fertilized giving rise to embryos whose presence or absence in developing seeds of a given tree depends on the extent of fertilized archegonia. The number of functional archegonia appears thus to act as a limiting factor which determines the yield of fully developed seeds in conifers. The low number of archegonia along with some additional reproductive failures have, for example, been shown to be the reasons for the low percentage of viable seeds in *A. amabilis* (Owens and Molder, 1977). As a matter of fact, this aspect of ovule biology is a subject of a certain variation in *Abies*. Except for the 2-3 archegonia commonly found in *Abies* species, Miyake (1903) occasionally observed 1 or 4 archegonia in *A. balsamea*. The same situation applies also for *A. kawakamii* where 1 or 4 archegonia were quite common (Figs. 133-134). In rare cases even as many as 5 archegonia were found.

7.1.4 Pollination mechanism

In 1996, the ovules were pollinated during 23-30 April, when the female gametophytes were in the early free nuclear stage (Fig. 127). The ovular micropyle remained open until the middle of May, then began narrowing due to the enlargement of cells forming the neck of the funnel (Fig. 128). By the end of May the micropyle was completely closed encompassing the pollen grains within the micropylar canal. The nucellus had meanwhile grown up filling the micropylar canal in its cap (Figs. 129 and 132). The space above the nucellus with the enclosed pollen grains is called the pollen chamber. It is a prominent feature of ovules not only during their pospollination developmental stages but also during early embryogenesis (Chamberlain, 1935; Bold *et al.*, 1982; Mauseth, 1988). Still another type of pollen chamber is represented cytologically by a shallow depression at the top of the nucellus formed by the disintegration of the apical cells toward the end of postpollination enlargement of the nucellus. As far as firs are concerned, it was illustrated in *A. balsamea*, *A. lasiocarpa*, and *A. grandis* (Powell, 1970; Singh and Owens, 1981, 1982). *Abies kawakamii* is characterized by possessing the 1st type of pollen chamber mentioned above with pollen grains located on the nucellar protuberance

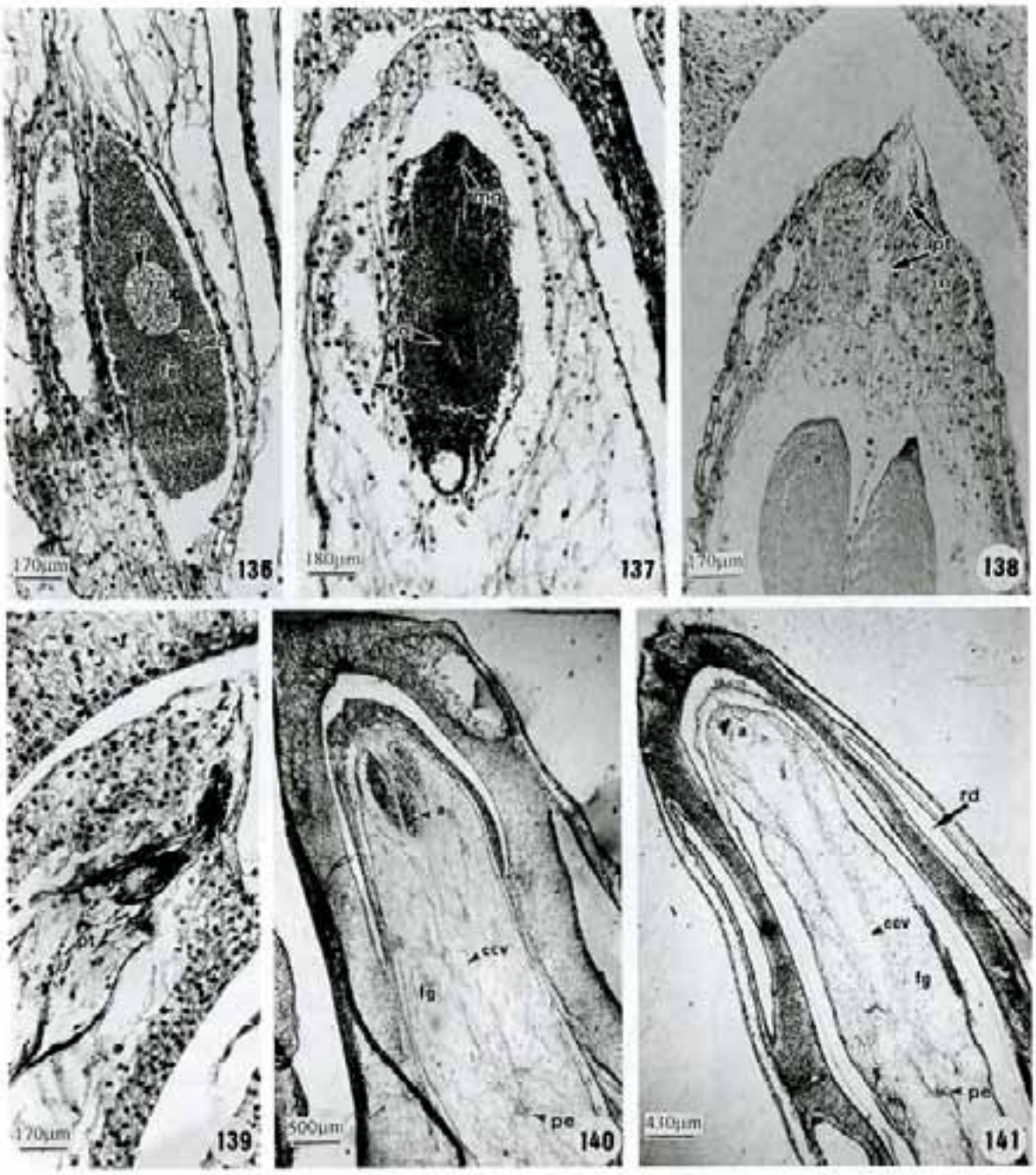
rather than in its depression (Figs. 138-139).

After being trapped in the micropyle, pollen grains remain quiescent above the nucellus for a period of 1 month after which they begin to germinate. According to Doyle and Kane (1943) such a long period of pollen dormancy may adversely affect its viability as the humid environment within the seed cone may not be ideal for pollen storage over 4-6 weeks in *A. amabilis* or 7-8 weeks in *A. nordmanniana*. Together with a relatively unspecialized pollination mechanism resulting in a low percentage of efficient pollination of the ovules, this dormancy may partially explain the low seed set generally observed in *Abies* (Doyle and O'Leary 1935, Dogra, 1964; Franklin, 1968; Allen and Owens, 1972). In *A. kawakamii*, pollen grains were found to have started germinating on the tip of the nucellus at the beginning of July. Several pollen tubes may eventually penetrate its elongated apex (Figs. 138-139) releasing their contents into archegonia. This in turn resulted in the fertilization of the egg cell by one of the sperm cells during the 1st week of July (Fig. 137). The details of these stages of the fertilization process as found in *A. balsamea* and *A. lasiocarpa* were illustrated by Hutchinson (1924) and Singh and Owens (1981), respectively.

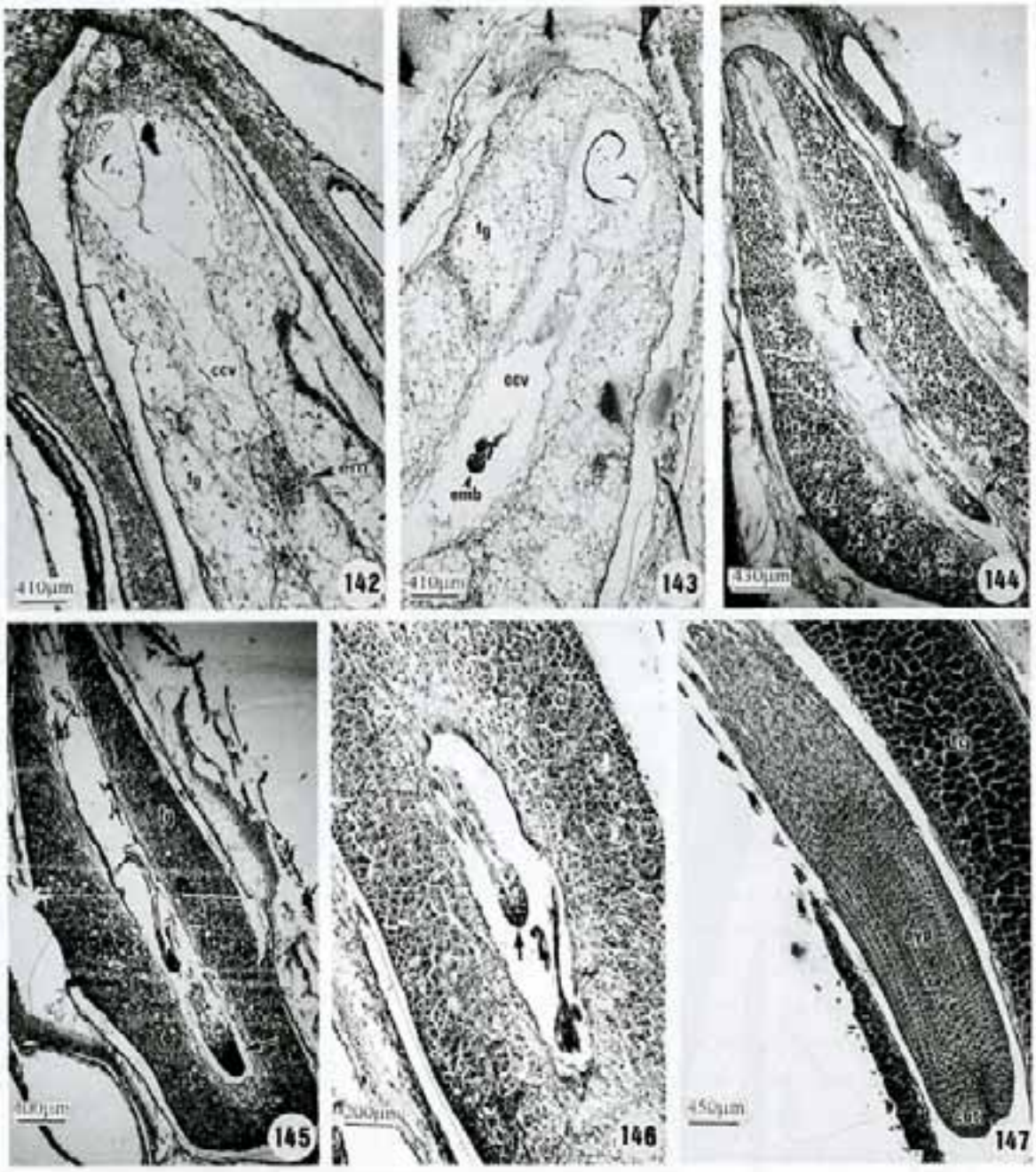
7.1.5 Embryogenesis

The initial stage of embryogenesis in *Abies* is represented by a zygote and its first 4 nuclei, all of which occur within the bounds of the archegonia. In *A. kawakamii*, these stages were not scored, mainly because of the relatively long intervals at which the developing ovules were sampled. As stated already by Owens and Molder (1977), studies of this kind should involve both daily collections and larger samples of collected cones.

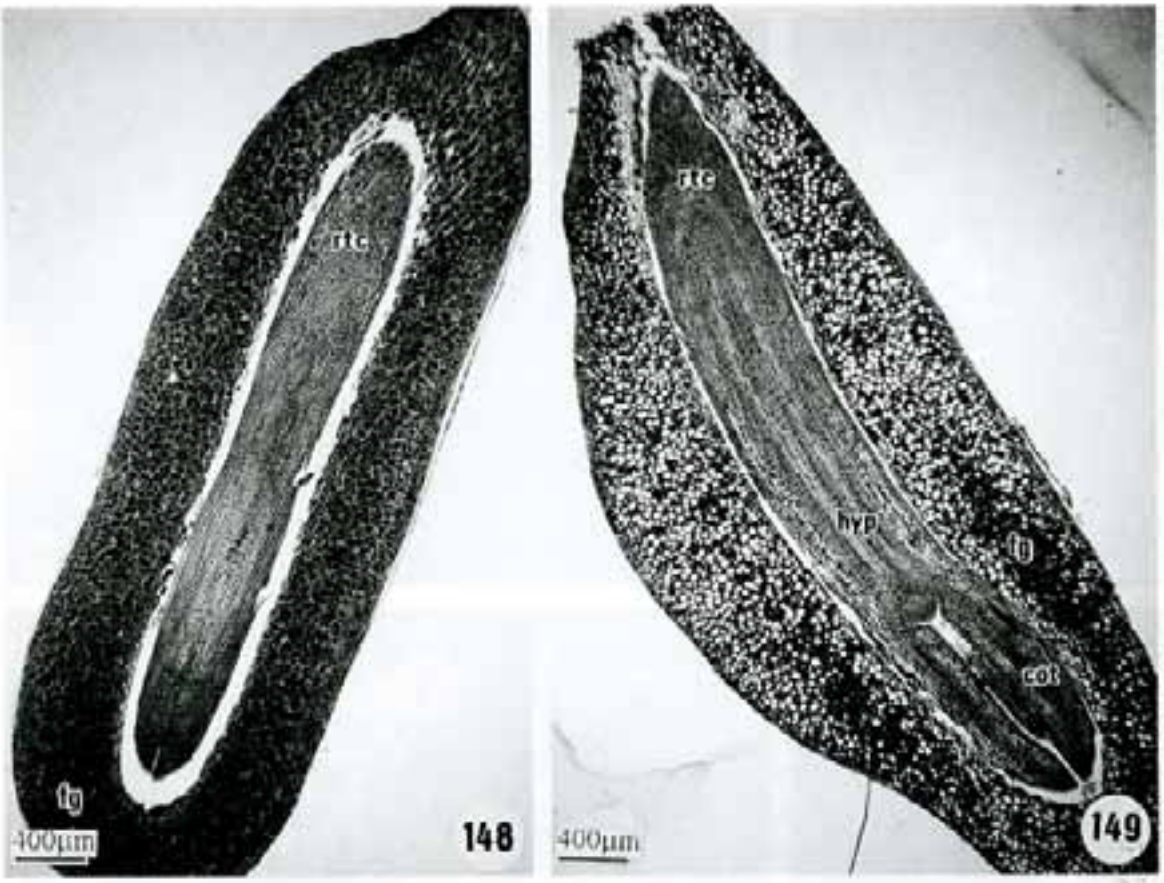
The first available stage of embryogenesis involves formation of a corrosion cavity beneath the archegonia and its subsequent extension within the female gametophyte, which is paralleled by disintegration of the archegonia soon after fertilization. At the chalazal end of the corrosion cavity, the 4 terminal cells of the proembryo, which have been pushed down by the elongating cells of primary and secondary suspensors, may be distinguished (Figs.140-141). The terminal embryonal cells divide transversely or longitudinally giving rise to the embryonal mass. The elongated cells of the adjoining secondary suspensor show a strong tendency at this stage towards splitting apart longitudinally, thus separating the cells of the embryonal mass they carry on their tips (Fig. 142). This splitting ultimately results in development of 2 or more young embryos within a common corrosion cavity. In conifers, this process is generally recognized as cleavage polyembryony. Its high frequency represents one of the most remarkable features of *A. kawakamii* embryogeny as well, with almost all ovules analyzed sharing it. The phenomenon may be observed also at the stage of the globular embryonal mass (Fig. 143). Intensive lytical activity of the growing young embryos resulted in the solubilization of the neighbouring cells of the female gametophyte and subsequent enlargement of a corrosion cavity during the 2nd half of July. Of the 2 or 3 embryos commonly occurring in a cavity during this period only one acquired a club-like shape and it prevailed in its further development over the remaining embryos which soon degenerate (Figs. 144-146). At the level of the female gametophyte, this developmental stage is characterized by a considerable accumulation of starch grains in its cells. The process of embryo elongation which took place during the next 2-3 weeks was very rapid and resulted in the formation of a massive embryo with differentiated cotyledons and a hypocotyl in the middle of August (Fig. 147), and with a root cap distinguished 1 week later (Fig.



Figs. 136-141. Longitudinal sections of developing ovules showing fertilization and early embryogenesis. Fig. 136. Mature archegonium containing egg nucleus (en) and perinuclear cytoplasm (pec); fg-female gametophyte. Fig. 137. Fertilized archegonium at the beginning of July containing egg nucleus (en) and male gamete (mg). Figs. 138-139. Longisections of the nucellus shortly before fertilization showing penetration of pollen tubes (pt) throughout the nucellar tissue (n); a-archegonium. Fig. 140. Proembryo (pe) at the bottom of corrosion cavity (ccv); fg-female gametophyte, a-archegonium. Fig. 141. The same as in Fig. 140 showing disintegration of archegonia (a); fg-female gametophyte, rd-resin-ducts.



Figs. 142-147. Longitudinal sections of female gametophyte (fg) showing early and advanced stages of embryo development. Fig. 142. Embryonal mass (em) in corrosion cavity (ccv) showing longitudinal separation of its cells. Fig. 143. Cleavage polyembryony at the stage of globular embryonal mass illustrated by the 2 globular embryos (emb) in a common corrosion cavity (ccv). Figs. 144-146. Polyembryony illustrated by the club-like shape of one of the present embryos (arrows) and degeneration of the supernumerary embryos. Fig. 147. Advanced stage of embryo development in mid-August with differentiated cotyledons (cot) and hypocotyl (hyp).



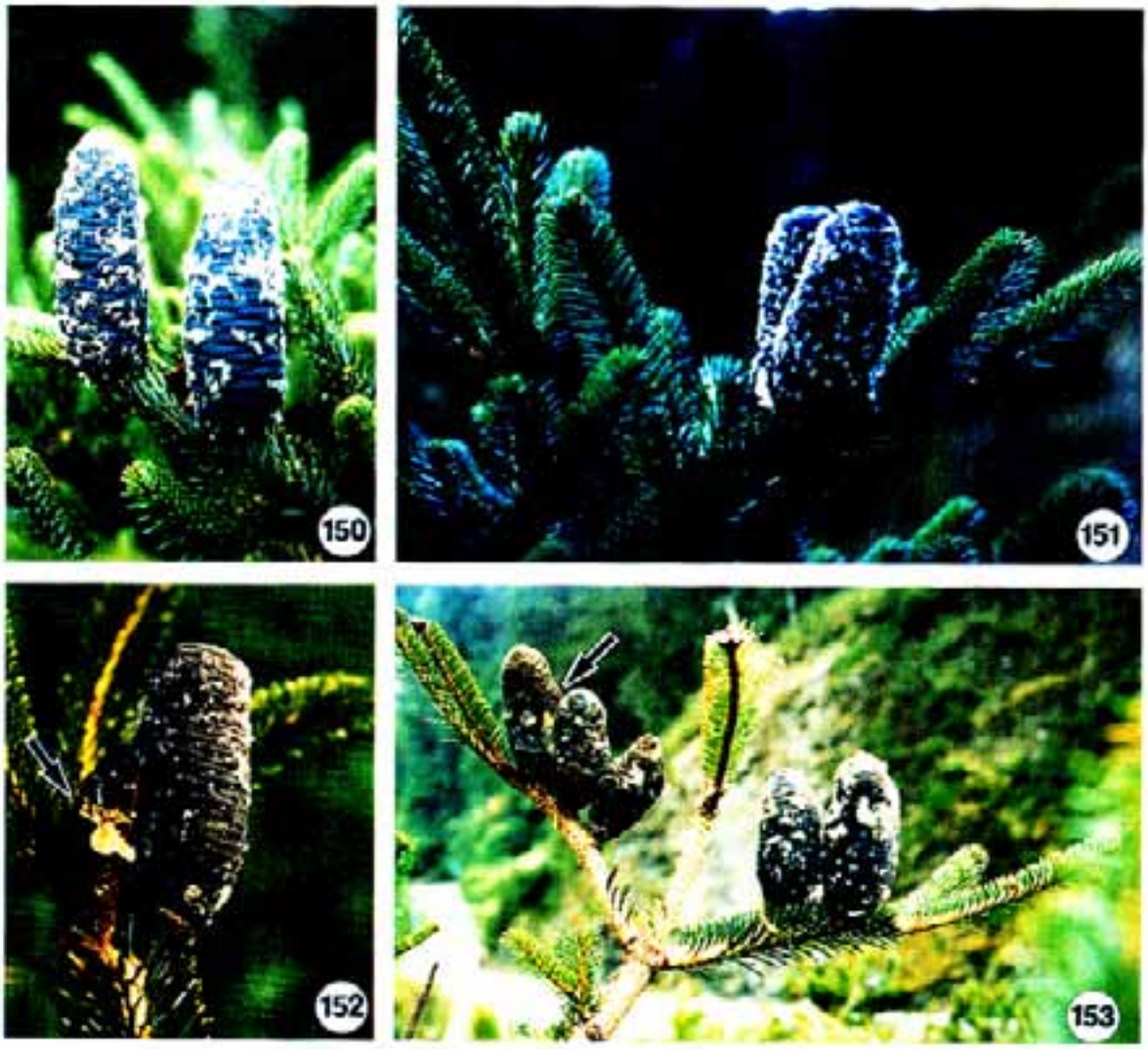
Figs. 148-149. Final stages of embryo development showing root cap (rtc) differentiation at the end of August (Fig. 148) and completely differentiated embryo in mid-September (Fig. 149); cot-cotyledons, hyp-hypocotyl, rtc-root cap, fg-female gametophyte.

148). The morphological differentiation of an embryo was completed by the middle of September followed by a period of its physiological maturation which, at an elevation of 2850 m, extended until the end of October (Fig. 149). The presence of densely packed lipoprotein and protein bodies within the cells of megagametophyte was observed, after the middle of August.

At the level of intact strobili, the development of ovules is accompanied by a gradual transformation of receptive female strobili into young cones. The process involves the thickening of female strobili rather than their noticeable elongation, as depicted schematically in Fig. 115. The fully developed mature cones of *A. kawakamii* are resinous (Figs. 150-153). This feature of their morphology is very pronounced and is characteristic not only of mature cones but could be traced visually since the conversion of megastrobili into young cones in June. Structurally it is expressed in the formation of resin-ducts in the receptive ovules and in their enlargement during further development of the ovules and seeds (Figs. 127, 130, 141, 146).

7.2 Disturbances accompanying ovule and embryo development

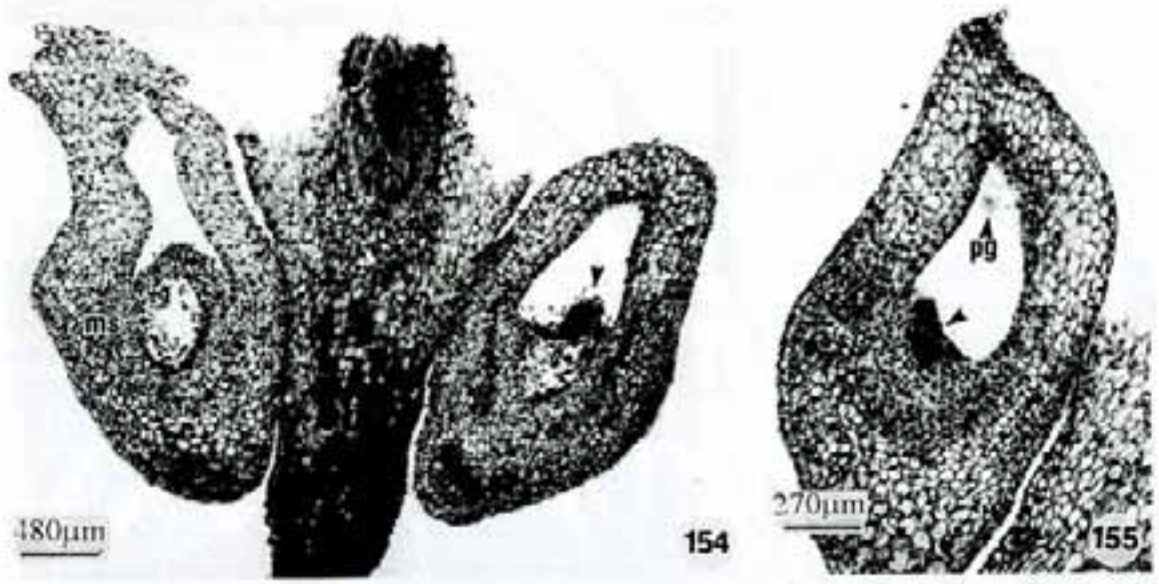
The structural and morphogenetic changes of ovules presented above represent the normal sequence of events leading to the development of fully differentiated and viable seeds of *A. kawakamii*. The pattern should be considered normal but far from typical in Taiwan fir. Only 2% of openly pollinated ovules of the species, for



Figs. 150-153. Morphology of fully developed and underdeveloped cones of *A. kawakamii*. Fig. 150. Young cones at the end of July. Fig. 151. Developing cones in August. Figs. 152-153. Fully developed mature cones in mid-September together with those which stopped their development at the stage of pollinated female strobili (arrow, Fig. 152) or in the middle of July (arrow, Fig. 153).

example, followed this pattern in 1996, which is a much-reduced value than the generally acknowledged degree of seed viability in *A. kawakamii* during years of poor harvests (Lai, 1994). Except for the reduced developmental potential of a certain fraction of receptive ovules, deviations from the normal course of the ovular development were due to alterations at both prezygotic and postzygotic stages of the fertilization process.

The 1st structural symptoms of developmental disorder were revealed in the ovules of the upper portion of female strobili soon after their pollination. The nucellar apex of these ovules began to degenerate on April 30 irrespective of the presence or absence of pollen grains in their pollen chambers (Figs. 154-155). A few days later, these symptoms were recognizable visually as browning spots in the central part of the ovular integuments resulting in partial or complete abortion of the respective fraction of ovules. Conversely, the alterations which occurred during further development of the ovules concerned their internal structures,



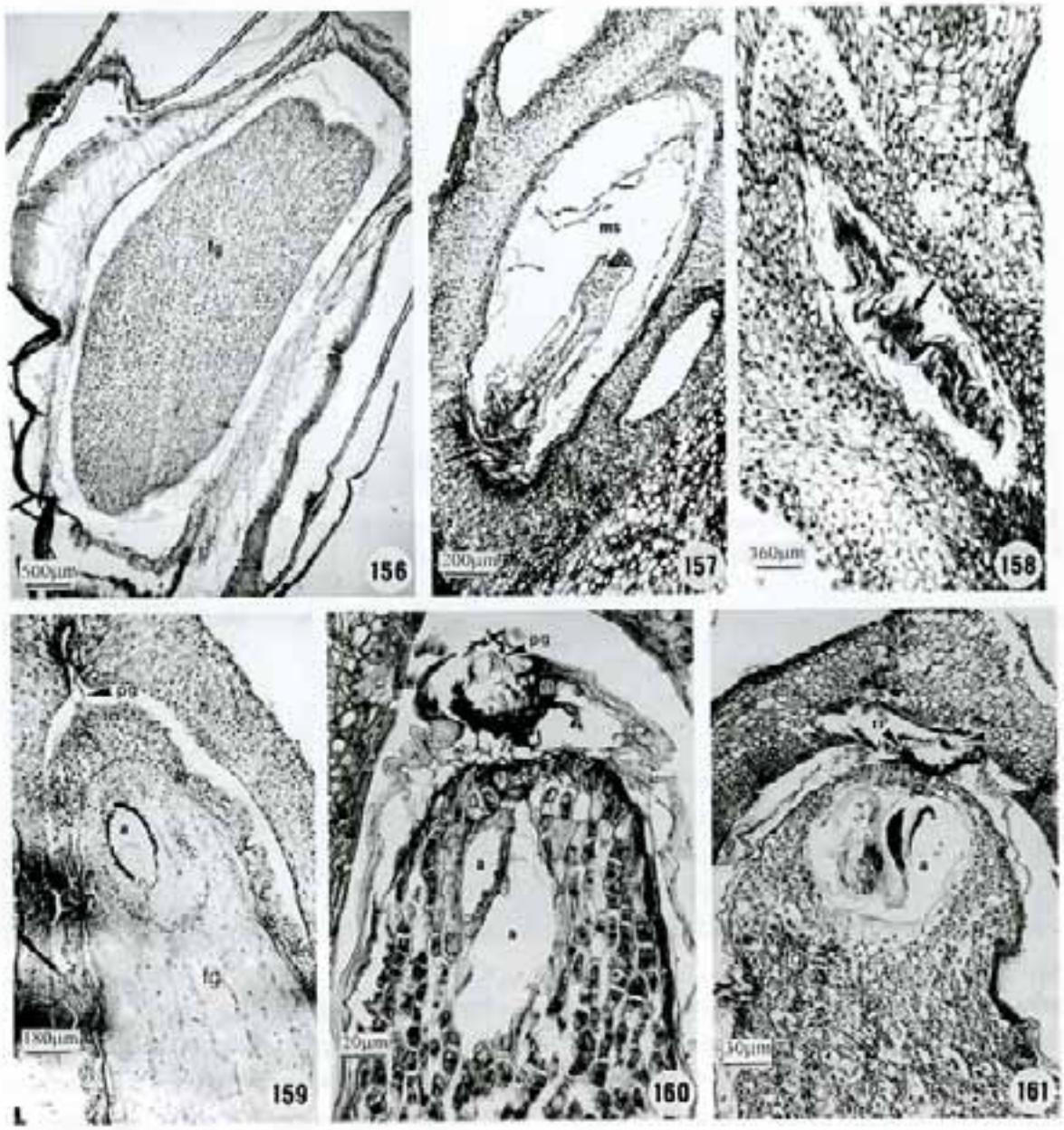
Figs. 154-155. Early degeneration of ovules at the upper part of female strobili due to deterioration of their nucellar apexes (arrows); ms-megaspore, pg-pollen grain.

leaving unaffected the marginal parts of the ovules. It is reasonable to suppose that this is primarily responsible for the high share of empty seeds produced by Taiwan fir.

At the stage of advanced female gametophyte formation, degeneration of the respective tissue occurred in a small fraction of ovules examined. The process began at the chalazal end of a megaspore and later encompassed the entire megagametophyte, thus eliminating the structural basis for subsequent archegonia differentiation (Figs. 157-158). At the beginning of July, a shrivelled mass of tissue could be found in the megaspore of affected ovules instead of a fully developed megagametophyte typical of ovules with a normal developmental pattern (Fig. 156).

Deterioration of mature archegonia represents another type of prezygotic deviation that had also been described in *A. lasiocarpa* but which predominantly concerned non-pollinated ovules of the species (Singh and Owens, 1981). In *A. kawakamii* it was a truly post-pollination phenomenon showing a close association with ovules which contained dormant pollen in their pollen chambers. The available cytological evidence indicates that the inhibition of pollen germination at the top of a nucellus, and the resulting absence of corresponding stimuli which are necessary at this stage for further development of archegonia, are the primary causes of their abortion. The dormant pollen grain revealed at the nucellar apex on July 5 (Fig. 159) could also be found during the next 15 days, this time enclosed within the darkly stained remnant of the already degenerated nucellus (Figs. 160-161). However, the most serious implication of such a long persistence of dormant pollen over the nucellus is a parallel degeneration of archegonia starting in the archegonial jacket and quickly spreading their internal structure. Both the sequence of events and nature of structural changes share features typical of gametophytic incompatibility.

The most extensive developmental disturbances occurred in *A. kawakamii* during postzygotic stages of ovule development encompassing practically the entire process of embryogenesis. Even early structures such as the embryonal mass manifested symptoms of structural disintegrity,

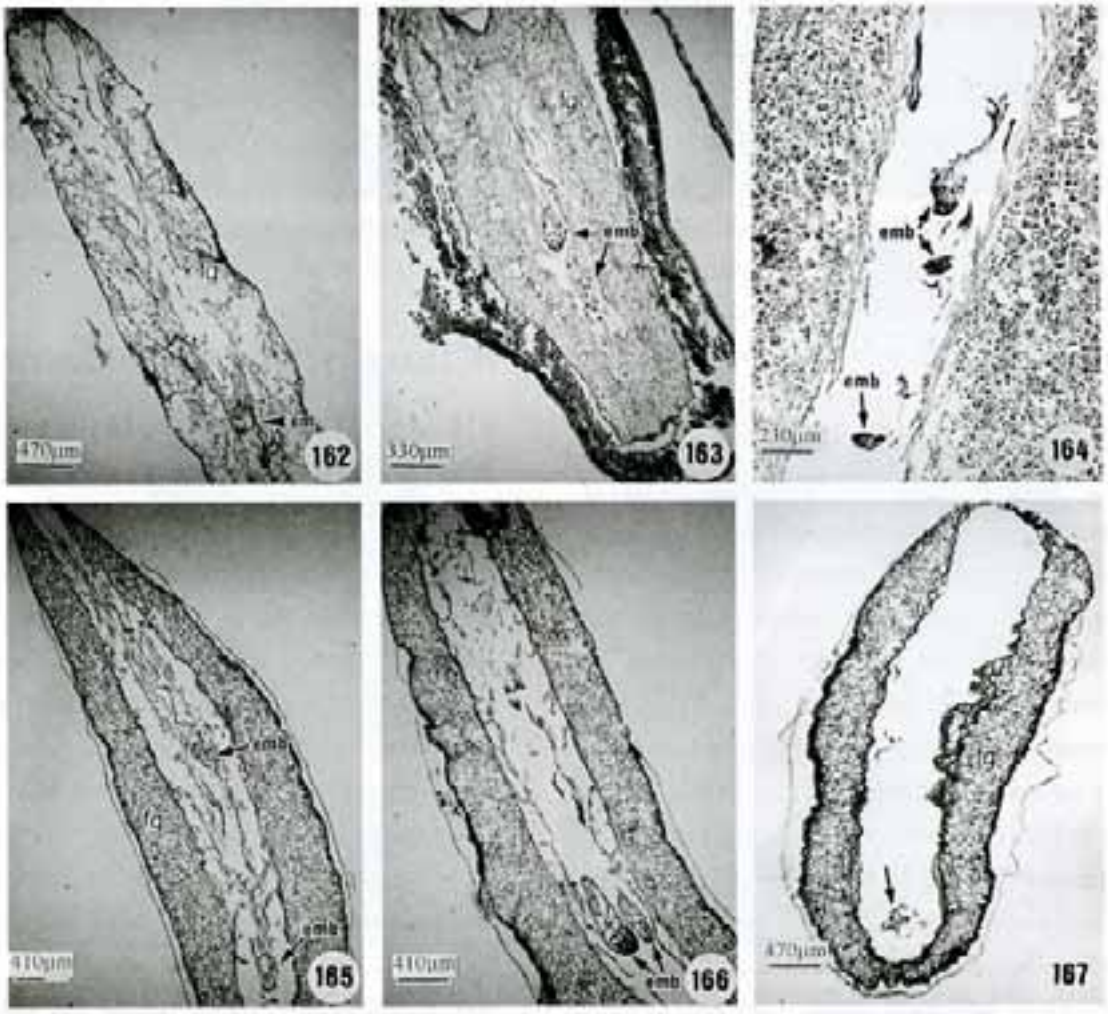


Figs. 156-161. Ovule degeneration at the stages of female gametophyte formation and mature archegonia. Fig. 156. Ovule with normal developmental pattern containing megaspore filled with female gametophyte (fg). Figs. 157-158. Interruption of the process of female gametophyte formation at the chalazal end of the megaspore (ms) (Fig. 157) and subsequent shrivelling of the tissue already formed (arrow, Fig. 158). Figs. 159-161. Inhibition of pollen germination at the top of the nucellus and the result of this degeneration of mature archegonia as found on July 5 (Fig. 159) and July 20 (Figs. 160-161); pg-pollen grain, n-nucellus, a-archegonium, fg-female gametophyte.

consisting of terminal tier cells which were not as compact as those in normally developing ovules (Fig. 162). The same was true of young embryos whose cells seemed to be structurally empty and

very transparent at the beginning of August (Fig. 163). The darkly stained nuclei still distinct at this time disappeared by mid-August when only remnants of the club-shaped embryos and suspensor cells could be found in the corrosion cavity (Figs.164-165). As far as distinctions could be made, abortion took place at the 8-celled stage of embryo development and concerned all 2 or 3 embryos present in a common embryo cavity (Figs.164-166). The process deviates by this feature from typical polyembryony during which 1 embryo usually outstrips the others and further continues its development. The early embryos were completely aborted by the end of August when, in developing seeds, only empty corrosion cavities could be recognized within a narrow envelope of degenerating megagametophytes (Fig. 167).

Except for the degenerating embryos, the ovules showing abortive development, also differred from those with normal developmental pattern by their female gametophyte. Throughout the entire period investigated, the megagametophyte of such ovules was rather thin exhibiting a low



Figs. 162-167. Embryo abortion during early embryogenesis. Fig. 162. Degenerative symptoms at the level of embryonal mass (em) and female gametophyte (fg). Figs. 163-166. Early symptoms of abortive development of 2 (Figs. 163, 165) or all 3 embryos (emb) present in a common corrosion cavity (Figs. 164, 166). Fig. 167. Remnants of aborted embryo (arrow) in a corrosion cavity as found in August.

capacity for accumulation of reserve substances as compared with the female gametophyte of normally developing ovules which was uniformly gorged with lipoprotein bodies at the corresponding developmental stages (Figs. 144-145).

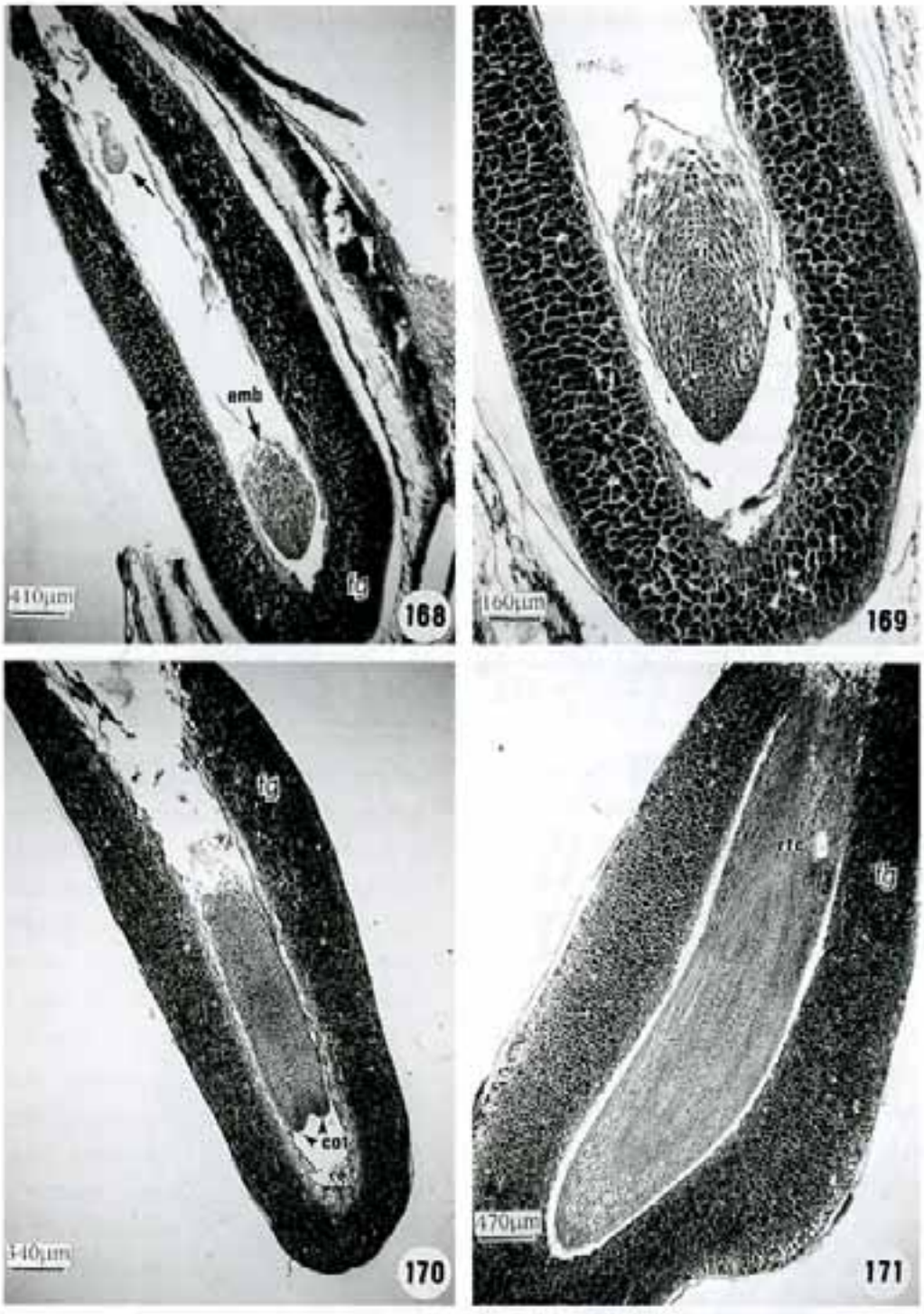
The phenomenon of embryo degeneration also persisted to some degree during the period of advanced embryo development, which in *A. kawakamii* extended throughout September. In contrast with early embryo abortion, the process proceeded this time under conditions of a fully developed female gametophyte and was coupled with the phenomenon of delayed embryo development. In the middle of September the aborting supernumerary embryos could still be recognized in a corrosion cavity together with embryos that had passed the club-shaped stage but which had failed to develop (Fig. 168). Such segmentary embryos are histologically characterized by differentiated root initials only and may persist eventually until maturity of seeds, representing the lowest category of underdeveloped seeds in *A. kawakamii* (Fig. 169). Conversely, the rudimentary embryos of the species found at the end of September were more elongated and, in addition, showed cotyledonary primordia differentiation (Fig. 170). Together with the embryos that had attained their full size but which failed to differentiate their cotyledons (Fig. 171), they represent another class of underdeveloped seeds of very common occurrence in *A. kawakamii*.

In light of the present cytological evidence, it is reasonable to consider disturbed embryogeny as a major cause of reduced viability of *A. kawakamii* seeds. The abortion of developing embryos occupies a prominent place, contributing in a decisive way to the extensive reduction in the amount of fully developed seeds in the species. However, of no less importance in this regard is the infestation of receptive megastrobili by pathogenic insects. Receptivity of female strobili lasting more than a week makes insect penetration into ovules not only possible but under prevailing strong infection pressure also very probable. From eggs laid at this time, larvae soon develop whose presence in a corrosion cavity could be traced from the end of June (Fig. 172). By a gradual consumption of the internal content of developing seeds, larvae attained their mature size in mid-August filling up the entire space of hollow seeds (Figs. 173-175). In *A. grandis* insect infestation was reported to be the most important factor in loss of seeds (Singh and Owens, 1982). The enclosed illustration of an x-ray analysis of seeds from open pollination suggests that this aspect of seed biology must be taken into account in explaining the poor quality of *A. kawakamii* seeds as well (Fig. 176).

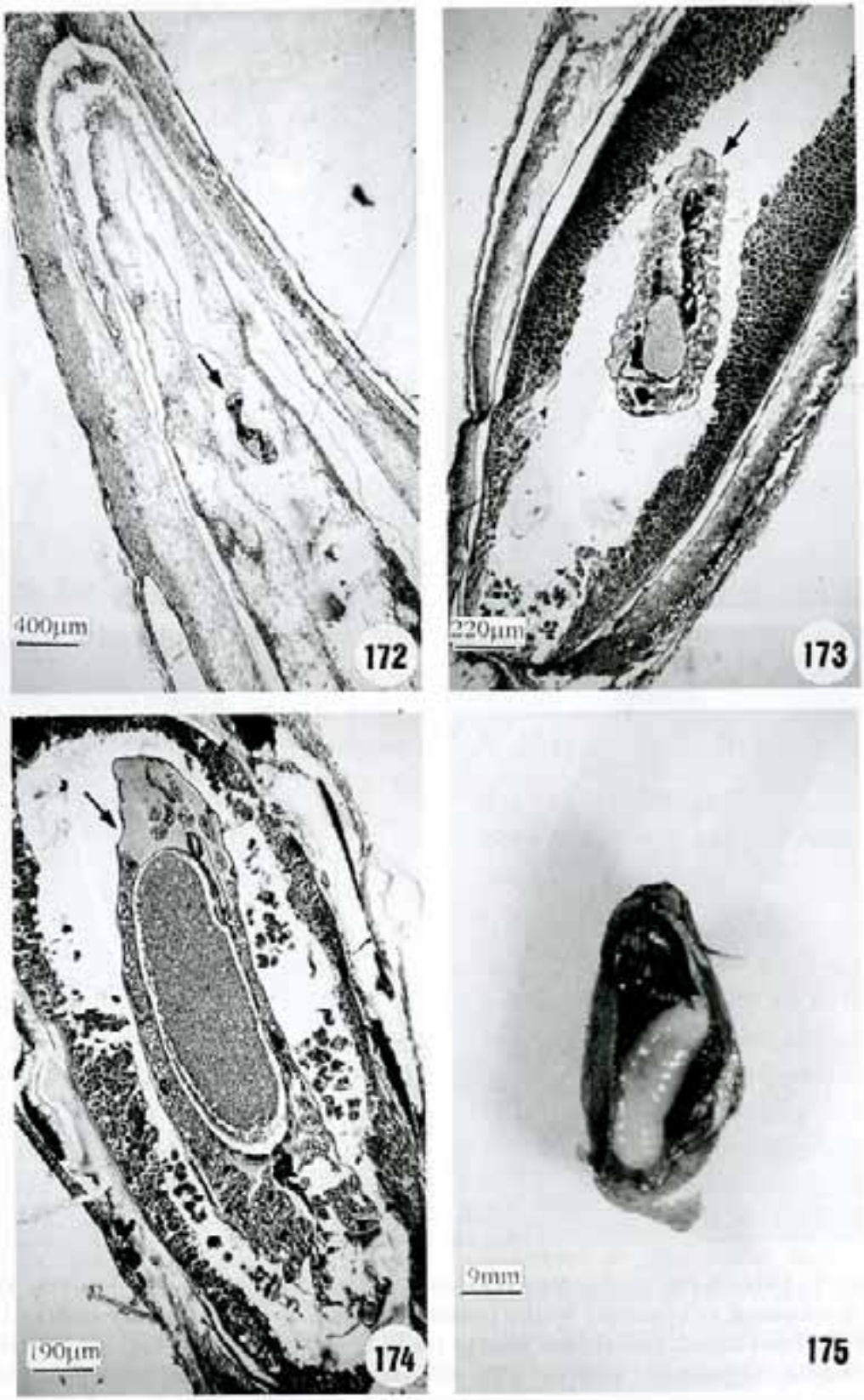
8 Interspecific hybridization

8.1 Crossability relationships betw-een *Abies* species with special em-phasis on Asian firs

Based on a high degree of karyological similarity of conifers which was originally established between their 53 species by Sax and Sax (1933), the conclusion has been drawn postulating that "species differences seem to be based primarily on genic changes, which, in many cases, do not prevent compatible species hybrids". As a consequence, many species are supposed to be maintained as distinct units solely by geographic or physiological isolation. With special references to *Abies*, Klaehn and Winieski (1962) and Wright (1953) also attributed the speciation of firs to geographic rather than genetic isolation. This aspect of *Abies* phylogeny has



Figs. 168-171. Delayed embryo development during advanced embryogenesis. Fig. 168. Delayed embryo development as evidenced by the presence of aborting supernumerary embryo (arrow) alongside underdeveloped, club-shaped embryo (emb) in mid-September. Fig. 169. Detail of an underdeveloped, segmentary embryo with differentiated root initials (ri) only. Fig. 170. Rudimentary embryo with differentiated cotyledonary primordia (cot) at the end of September. Fig. 171. Underdeveloped embryo without cotyledons at the end of September; fg-female gametophyte, rte-root tip cap.



Figs. 172-175. Development of ovules infested by pathogenic insects (arrows) covering the period from the end of June (Fig. 172) until September (Fig. 175).

Fig. 176. X-ray analysis of *A. kawakamii* seeds from open pollination showing fully developed seeds (dark) and hollow seeds with aborted internal content (light) and with infested larvae of Diptera.

subsequently been reinforced by the finding that there are no reliable diagnostic differences in chromosome size or structure of its 7 species (Mergen and Burley, 1964). These circumstances, together with the prevailingly compatible relationships established between *Abies* species during artificial hybridization experiments, contributed to the general acceptance of the view postulating "little difficulty in hybridizing fir species although the percentage of success was higher for crosses from the same geographic region than from inter-regional crosses" (Mergen *et al.*, 1964).

In their compilation of crossability relationships within the genus *Abies*, Klaehn and Winieski (1962) stated that a wide scale of transitions exist ranging between complete compatibility and complete incompatibility of individual pairs of species. The Mediterranean species were shown to be especially prone to hybridize, a fact that has been amply illustrated by artificial hybridization experiments (Rohmeder and Eisenhut, 1961; Greguss, 1984). On the contrary, there was only an "indication that many species crosses can be accomplished between the North American species" (Klaehn and Winieski, 1962). Hawley and DeHayes (1985) have in this connection proved a high degree of mutual crossability between the species *A. balsamea*, *A. fraseri*, and *A. balsamea* var. *phanerolepis* of the section *Balsamea*, as well as their low crossability with the species *A. concolor* of the section *Grandes*, thus illustrating the crossable nature of intrasectional hybrids and only partially compatible or incompatible status of intersectional crosses. The authors concluded that *Abies* species are perhaps genetically more recently differentiated than other *Pinaceae* genera, postulating the existence of barriers to crossability within *Abies*. Barriers of crossability of *Abies* hybridological behavior have convincingly been illustrated by Critchfield (1988) in his study on *Abies* crossability involving 12 species of firs of North American, Mediterranean, and Asian origin. It was shown that, although crosses between North American sections are sometimes possible, the firs of the eastern and western hemispheres may be isolated from each other by genetic barriers that are almost or completely insurmountable. Thus the claim that firs as a group virtually lack any crossing barrier is no longer tenable.

As far as Asian firs are concerned, the lack of hybrids in Klaehn and Winieski's (1962) survey was ascribed to either an incomplete study or the lack of respective species in those parts of the world where hybridization attempts had been undertaken. The latter reason seems to be more justified as evidenced by only 1 paper referring to crossing experiments with *A. myriana* and *A. homolepis* which were performed at the Sapporo Forest Breeding Experiment Station (Husao and Kenichi, 1951). Nonetheless, the list of artificial hybrids with Asian firs involved as parental species is surprisingly long. All of these have, however, been obtained in the numerous arboretums in the USA and Europe to which Asian firs were introduced in the past (Table 21).

Table 21. List of artificial hybrids of Asian species of firs compiled according to Husao and Kenichi (1951), Rohmeder and Eisenhut (1961), Rohmeder (1961a), Klaehn and Winieski (1962), Mergen *et al.* (1964), Gaudlitz (1983), Greguss (1984), and Greguss and Paule (1988)

A. alba × *A. veitchii* (1962, 1984)

A. cephalonica × *A. firma* (1984)
A. cephalonica × *A. veitchii* (1984)
A. cephalonica var. *apollinis* × *A. homolepis* (1964)
A. cephalonica var. *apollinis* × *A. koreana* (1964)
A. cephalonica var. *apollinis* × *A. recurvata* (1964, 1988)

A. nordmanniana × *A. homolepis* (1984)
A. nordmanniana × *A. firma* (1984)
A. nordmanniana × *A. veitchii* (1961, 1962, 1983, 1984)

A. cilicica × *A. firma* (1984)
A. cilicica × *A. veitchii* (1984)

A. numidica × *A. firma* (1984)
A. numidica × *A. veitchii* (1984)

A. concolor × *A. sibirica* (1962)
A. concolor × *A. veitchii* (1961, 1962, 1983, 1984)

A. grandis × *A. veitchii* (1962)

A. procera × *A. mariesii* (1964)
A. procera × *A. nephrolepis* (1983)
A. procera × *A. recurvata* (1962)
A. procera × *A. sachalinensis* (1964)

A. balsamea × *A. homolepis* (1962)
A. balsamea × *A. recurvata* (1962)
A. balsamea × *A. sachalinensis* (1962)
A. balsamea × *A. veitchii* (1962)

A. lasiocarpa × *A. sibirica* (1983)

A. mariesii × *A. cephalonica* var. *apollinis* (1964, 1988)
A. mariesii × *A. lasiocarpa* (1988)
A. mariesii × *A. firma* (1964, 1988)
A. mariesii × *A. homolepis* (1964, 1988)
A. mariesii × *A. sachalinensis* (1964, 1988)
A. myriana × *A. homolepis* (1951)

A. firma × *A. mariesii* (1964)
A. firma × *A. sachalinensis* (1964)

A. homolepis × *A. alba* (1961, 1961a, 1962)
A. homolepis × *A. cilicica* (1984)
A. homolepis × *A. concolor* (1961a, 1962)
A. homolepis × *A. grandis* (1961a, 1962)
A. homolepis × *A. lasiocarpa* (1988)
A. koreana × *A. firma* (1964, 1988)
A. koreana × *A. homolepis* (1984)
A. koreana × *A. lasiocarpa* (1964, 1988)
A. koreana × *A. veitchii* (1962)

A. sachalinensis × *A. alba* (1962)
A. sachalinensis × *A. cephalonica* (1962)
A. sachalinensis × *A. cephalonica* var. *apollinis* (1964 1988)
A. sachalinensis × *A. firma* (1964, 1988)
A. sachalinensis × *A. homolepis* (1962)
A. sachalinensis × *A. koreana* (1964)
A. sachalinensis × *A. lasiocarpa* (1964, 1988)
A. sachalinensis × *A. mariesii* (1964, 1988)
A. sachalinensis × *A. recurvata* (1962)

A. sibirica × *A. veitchii* (1962, 1983)

A. veitchii × *A. alba* (1961, 1962, 1983)
A. veitchii × *A. concolor* (1962)
A. veitchii × *A. grandis* (1962)
A. veitchii × *A. nephrolepis* (1983)
A. veitchii × *A. nordmanniana* (1962)
A. veitchii × *A. sibirica* (1983)

Such a long list of interspecific hybrids together with the nature of species combinations involved give a strong impression of a high crossability among the Asian species of firs as well as of a considerable degree of their hybridological affinity with the North American representatives of the genus. This impression is also reinforced by the occurrence of 2 spontaneous hybrids in the region. The hybrid *Abies* × *sibirico-nephrolepis* Taken. *et* Chien originated from a natural crossing between *A. sibirica* and *A. nephrolepis* and was only discovered in Northeastern China in 1954 (Takenouchi and Chien, 1957), whereas the hybrid *Abies* × *umbellata* Mayr. had arisen in a contact zone of the species *A. firma* and *A. homolepis* in Japan.

On the contrary, the lower number of hybrids between Asian and Mediterranean species reflects the phylogenetic divergency of both these groups of firs, the mutual intercrossing of which was shown to yield only a negligible percentage of viable seeds (Mergen *et al.*, 1964). The only exceptions in this respect are *A. veitchii* and perhaps also *A. firma* and *A. homolepis* which show compatible relationships with Mediterranean firs.

8.2 Crossability relationships between Taiwan fir and some other species of genus *Abies*

As follows from Table 21, Taiwan fir has not been involved in any crossings attempted so far. Consequently, data referring to its crossability with 6 other species of firs which are presented in Table 22 represent the 1st information of this kind.

Of the tested combinations, only the interspecific crossing *A. kawakamii* × *A. homolepis* proved to be compatible, while the remaining interspecific combinations yielded only empty seeds suggesting strong reproductive barriers between the parental species. The proportion of fully developed seeds in *A. kawakamii* × *A. homolepis* combination averaged at only 5.2% as compared with 8.7% filled seeds in controlled intraspecific crossing of *A. kawakamii* and with 2.0% seeds of the same category obtained from open pollination. It is reasonable to suppose that yields of sound seeds in all the 3 variants were considerably reduced by unfavorable climatic conditions during flowering of the species. A negligible content of filled seeds in the offspring from open pollination which did not even reach the level typical of the species' poor crops (3.7% - Chen, 1967; 4.7% - Lai, 1994) seems to corroborate this assumption. Also, the weather-caused reduction in number of mature cones was drastic as compared with the amount of pollinated female strobili and obviously posed a serious obstacle in providing more conclusive evidence relative to the crossability relationships between tested species. Even those macrostrobili which survived the frosty weather in April 1996 underwent abortion when reaching the stage of young cones (Fig. 152). Still another portion of cones stopped their development in July giving rise to underdeveloped cones with only empty seeds (Fig. 153). Nevertheless, though preliminary, the established crossability pattern correlates very closely with the PCR/RFLP profiles of chloroplast DNAs and RAPD amplification spectra of genomic DNAs of compared species, substantiating thus the taxonomic division of the genus *Abies* as proposed by Liu (1971). Of significance in this respect is the crossable hybridological status of *A. kawakamii* and *A. homolepis* species, both of which share a common position within the section *Homolepides*, as well as an incompatible relationship of the former with *A. lasiocarpa* of the section *Balsamea*. This is in sharp contradiction with the taxonomic account of the genus by Farjon and Rushforth (1989) in which *A. kawakamii* and *A. lasiocarpa* were included in the section *Balsamea*, while *A. homolepis* occupied a separate position within the section *Momi*.

Except for *A. lasiocarpa*, Taiwan fir was found to be isolated reproductively also from another representative of the North American firs, i.e., from *A. concolor* as well as from the Mediterranean species *A. alba*, *A. cephalonica*, and *A. cilicica* (Table 22). The available cytological evidence indicates that it is a prezygotic hybridological block which prevents the normal course of fertilization in *A. kawakamii* × *A. alba* and *A. kawakamii* × *A. cephalonica* combinations leading to abortion of their ovules at the mature archegonium stage (Figs. 177-182). The same stage was reported to be of critical importance also for unpollinated ovules of *A. lasiocarpa* which degenerated because of lack of fertilization (Singh and Owens, 1981). In the interspecific crossings mentioned above, all the prerequisities necessary for development of the ovules including the presence of pollen grains in the pollen chambers exist, but abortion still takes place due to the inability of pollen to germinate on top of the nucellus. Throughout the 1st half of July when wind-pollinated ovule fertilization and

Table 22. Results of artificial hybridization of Taiwan fir with some other species of firs[1)]

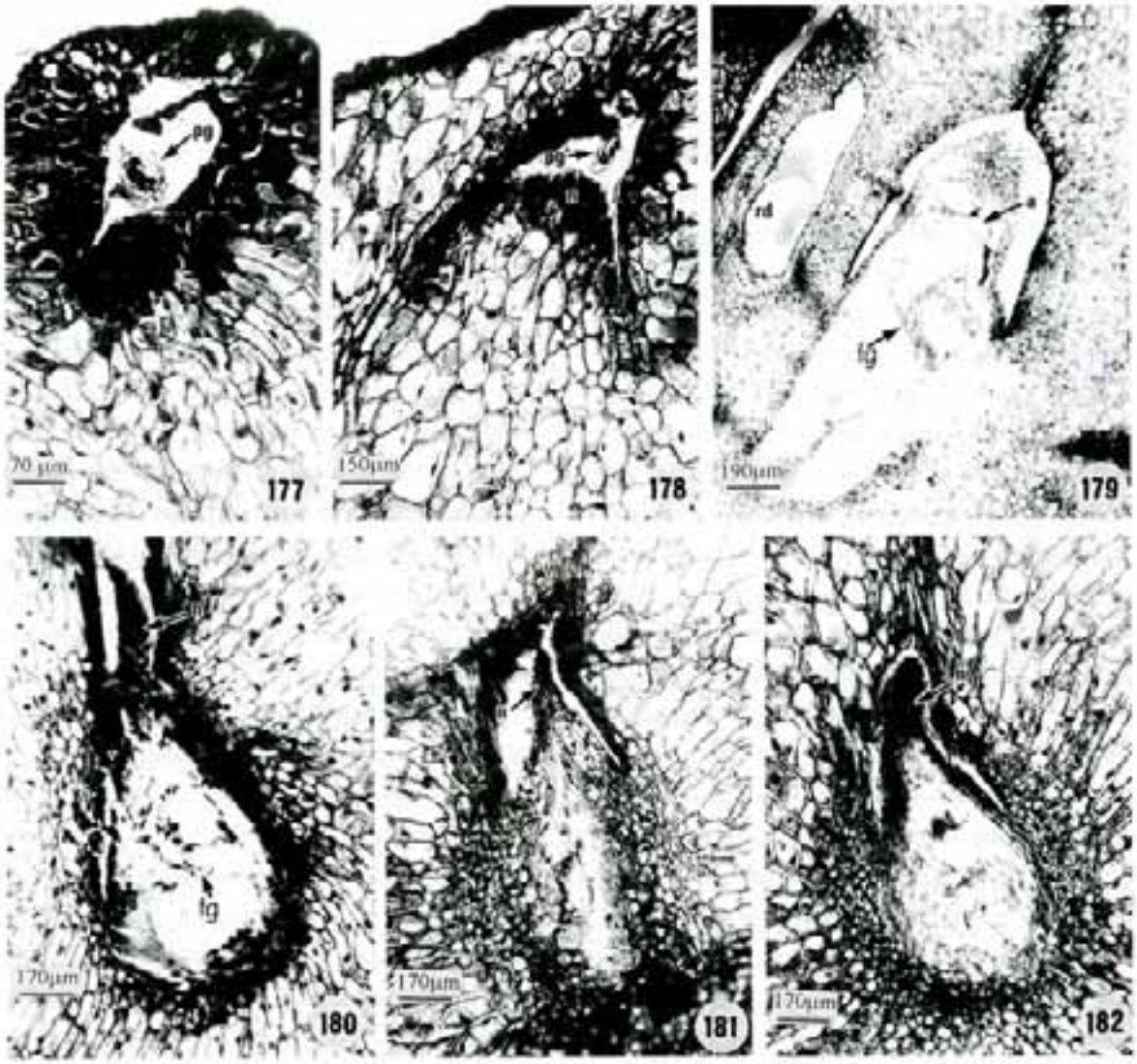
Combinations tested	Number of pollinated female strobili	Number of collected mature cones	Number of filled seeds per 400	Percent filled seeds
A. kawakamii open pollination		7	8	2.0
A. kawakamii × *A. kawakamii*	28	5	35	8.7
A. kawakamii × *A. homolepis*	45	6	21	5.2
A. kawakamii × *A. lasiocarpa*	32	4	0	0
A. kawakamii × *A. concolor*	35	4	0	0
A. kawakamii × *A. alba*	25	3	0	0
A. kawakamii × *A. cephalonica*	18	6	0	0
A. kawakamii × *A. cilicica*	30	5	0	0

[1)] The experiment was carried out using freshly collected pollen of *A. kawakamii* and pollen of other species stored for 1 year whose germinability had not fallen below 50%. Three maternal trees of *A. kawakamii* were used within which the seven variants of controlled pollinations had been done including that of *A. kawakamii* × *A. kawakamii*. The latter represents the intraspecific crossing of two different individuals of the species. To avoid uncontrolled pollination, the paper bags were placed on twigs with macrostrobili a few days before the receptivity of the latter was achieved. The microstrobili were still very compact structures at this period lacking any symptoms of pollen shedding. The mature microstrobili have subsequently been collected and allowed to dry at room temperature. The pollen obtained by sieving of the dried microstrobili was used in artificial pollination of receptive macrostrobili. The maternal tree situated at an elevation of 2850 m was pollinated on April 23, while the two additional mother trees located at an elevation of 3130 m on May 2. The paper bags were removed from the twigs two weeks later when the ovuliferous and bract scales of the macrostrobili were closed completely. The mature cones were collected in middle of October. The quality of hand-extracted seeds was estimated separately for each variant of crossings using x-ray analysis.

early embryogenesis occurred, in the ovules of both these combinations only dormant pollen grains could be found above the nucellus suggesting a strong incompatible reaction between them and the nucellar tissue of the ovules (Figs. 177-178). The absence of pollen tubes within the nucellar tissue and corresponding physiological stimuli led to the appearance of the 1st symptoms of ovule degeneration involving the abortion of the nucellar apex and disintegration of the archegonia (Figs. 178-179). Ten days later, degeneration had already encompassed the entire nucellus, also affecting the internal content of a megaspore (Figs. 180-182) and resulting in development of empty seeds. The latter may therefore be looked upon as a consequence of the gametophytic incompatibility between *A. kawakamii* on the one hand and *A. alba* and *A.cephalonica* on the other.

9 Conclusions

Although the history of taxonomic classification of firs goes as far back as 1842 when the 1st attempt at generic systematization of these trees was made by Spach (1842), Taiwan fir only first appeared in a taxonomic account by Patschke in 1913. The author included it in the subsection *Laterales* of the section *Centralis* together with *A. firma, A. fargesii, A. squamata, A. veitchii, A. mariesii,* and *A. homolepis.* The position of the species within valid taxonomic accounts which have been proposed since then varies considerably, depending on criteria used by individual authors. Franco (1950), for example, positioned Taiwan fir into the section *Pichta* among the species *A. sachalinensis, A. veitchii, A. nephrolepis,* and *A. koreana,* while Liu (1971) placed it into the section *Homolepides* together with *A. holophylla, A. homolepis,* and *A. mariesii.* Still another



Figs. 177-182. Incompatible symptoms in *A. kawakamii* × *A. alba* and *A. kawakamii* × *A.cephalonica* crossings. Figs. 177-178. Inhibition of pollen grain (pg) germination on the nucellus (n) found in mid-July in interspecific combinations *A. kawakamii* × *A. alba* (Fig. 177) and *A. kawakamii* × *A. cephalonica* (Fig. 178). Fig. 179-182. Abortive ovular changes resulting from lack of fertilization and involving archegonia disintegration (a) (Fig. 179) and degeneration of a nucellus (n) and female gametophyte (fg).

classification proposed by Landry (1984) treats Taiwan fir as a co-member of such morphologically and ecologically diverse species as *A. guatemalis, A. holophylla, A. homolepis, A. lasiocarpa, A. mariesii, A. pindrow, A. pinsapo, A. sibirica, and A. magnifica* all of which were included in the section *Piceaster*. Contrary to this classification system, Krylov *et al.*(1986) considered Taiwan fir to be taxonomically the most closely related to the species *A. mariesii*, both of which represent in their account the independent series *Mariesianae*. As mentioned already in the Introduction, in the most recent taxonomic classification of firs published by Farjon and Rushforth (1989), Taiwan fir occupies the common position within the section *Balsamea*

with *A. sibirica*, *A. balsamea*, and *A. lasiocarpa*.

The presented results of comparative study on chloroplast and genomic DNAs in 15 species of firs preferentially substantiate the taxonomic account of the genus by Liu (1971) within which Taiwan fir was shown to exhibit the highest similarity with *A. homolepis* of the section *Homolepides*. Of particular importance in this connection is also the established compatible hybridological relationship between these species which may serve as additional evidence illustrating a high genetic affinity between them. On the contrary, the species *A. lasiocarpa*, though postulated by Farjon and Rushforth (1989) to be closely related with *A. kawakamii*, proved to be reproductively isolated and hence genetically more divergent from the latter than *A. homolepis*. The same is true of the crossability relationships between *A. kawakamii* on the one hand and the Mediterranean firs *A. alba*, *A. cephalonica*, and *A. cilicica* on the other, indicating such degree of genetic differentiation between firs of different continents which exceeds the level of mutual compatibility. However, owing to the fact that this assumption is only based on a few mature cones obtained within the respective crossings of *A. kawakamii*, additional experiments on artificial hybridization of firs are necessary to prove it more conclusively. The same is true of studies on cytology of the fertilization process in some crosses of *A. kawakamii* with European and North American firs, which may provide more detailed information relative to the nature of existing hybridological barriers.

Taking into consideration the existence of a fairly close correlation between the crossability relationships of Mediterranean and North American species of firs and their PCR/RFLP profiles of chloroplast DNAs, the heterogeneous hybridological relationships may a priori be expected to prevail among Asian firs as well. The latter group was found to exhibit considerable differentiation at the DNA level between the compared sections *Homolepides* and *Elate* as well as between species of the *Elate* section, resembling thus the North American firs rather than the genetically uniform group of the Mediterranean species.

Reproductively Taiwan fir behaves like other species of firs, but being typically a high-elevation species, it simultaneously shares some features in its reproduction cycle which are more pronounced than in other representatives of firs studied so far, especially in species occurring at lower elevations. The extremely reduced seed set is obviously one of these. The phenomenon is related exclusively to the development of macrostrobili and may be conditioned climatically or genetically, both types of retardation having been illustrated in the present study. Accordingly, the amount of mature cones or even the periodicity in cone production may be affected adversely in *A. kawakamii* by the occasional occurrence of late frosts partially or completely damaging its receptive macrostrobili. The prezygotic hybridological barrier, cytologically represented by the inhibition of pollen germination on the nucellar tissue of an ovule, is supposed to occur exclusively in selfed ovules, accounting partially for the drastically reduced yields of filled seeds in the species. However, the evidence relative to its occurrence in *A. kawakamii*, though very expressive, remains to be verified as it contradicts the generally held view which postulates the absence of any kind of mechanisms preventing self-pollination in conifers (Koski, 1973; Forshell, 1974).

At the level of developing seeds, the most serious losses result from a high frequency of abortive embryos which represent not only a very conspicuous but also a considerably deviating aspect of *A. kawakamii* embryogeny, and encompasses the entire process of embryogenesis. In conifers, this phenomenon is usually ascribed to

the large extent of natural self-pollination resulting in homozygotization of sublethal or lethal genes in zygotes and their subsequent degeneration at different stages of development (Tiilila, 1967; Koski, 1973; Forshell, 1974; Sorensen, 1982). In light of this conclusion, it is reasonable to relate all the problems associated with low yields of viable seeds in *A. kawakamii* primarily to the high degree of selfing as a prevailing type of pollination supposedly occurring within its stands under natural conditions. The only means which may provide experimental evidence to validate this assumption are comparative studies on the efficiency of both selfing and outcrossing in the species, together with isozyme analysis of seed progenies from wind pollination.

Extensive degeneration of developing embryos of *A. kawakamii* takes place irrespective of the frequent occurrence of polyembryony, which is generally looked upon as an alternative mechanism to selfing which favors outbreeding in gymnosperms (Hagman, 1975). In *A. kawakamii*, it represents an additional feature which is very characteristic of the species' reproductive behaviour as evidenced by the exceptionally frequent occurrence of 2 or more embryos within a common corrosion cavity. It is interesting to note in this connection that some authors have attributed this phenomenon in conifers to harsh climatic conditions prevailing at high elevations and in the Arctic region (Doyle, 1957; Dogra, 1967; Simak, 1973). The available cytological evidence indicates that in *A. kawakamii* these embryos are of monozygotic origin probably representing genetically identical twins, which have arisen by longitudinal splitting of the same proembryo in the process of cleavage polyembryony. However, by sharing identical genotypes, they are not endowed with a selective advantage, which is typical for genetically heterogenic embryos of polyzygotic origin, and respond uniformly to the existing genetic load. The numerous examples of simultaneous abortion of 2 or more embryos of a given seed at early or advanced stages of their development seem to corroborate this idea. Thus, though very common in *A. kawakamii*, cleavage polyembryony does not present a special advantage for the species in preventing its embryos from degenerating. The only solution to its low-viability seed problems seems to be a supplemental mass pollination using pollen of distant and supposedly more differentiated populations of the species to avoid the existing profound inbreeding depression at the embryonal level. Though very preliminary, the data obtained on the isozyme polymorphism in 2 *A. kawakamii* populations substantiate such an approach in increasing the yields of viable seeds in the species.

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