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ISOLATING BARRIERS BETWEEN THE CALIFORNIAN CLOSED-CONE PINES

by

A.G. Brown B.Sc.For.(Syd.); Dip. For. (Canb.)

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ABSTRACT

The strength and nature of factors preventing the interspecific and intraspecific hybridisation of the Californian closed-cone pines, *Pinus attenuata*, *P. muricata* and *P. radiata* were examined in cultivated material in the Australian Capital Territory.

Differences in flowering times were evident among some provenances of the main species, but were not significant. Flowering times of the major species were sufficiently different to be important, and only a very small overlap in flowering times occurred between *P. radiata* and *P. muricata*, the earliest and latest species respectively.

Yields of germinable seeds from intraspecific crosses involving *P. muricata* were low; the seeds which were obtained came from crosses utilising southern provenances of the species. The northern provenances yielded no viable seed and they also gave very low yields of viable seeds when crossed with southern trees of the same species. There was no evidence of comparable behaviour in the widespread, ecologically-differentiated *P. attenuata*. Low seed yields were largely due to reproductive failure at or near the time of fertilisation.

Strong evidence was obtained that Cedros Is. pine presently described as a variety of *P. muricata* belongs under *P. radiata*.

Growth rates of interspecific hybrid seedlings appeared to be intermediate between those of seedlings of the parental species.

Attempts to utilise properties of *P. attenuata* such as cold or drought resistance in any hybridisation programme with *P. radiata* should not be hampered by reproductive barriers. However the more attractive northern forms of *P. muricata* are so isolated that effective hybridisation would be very difficult.

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PREFACE

This study was carried out while the author was employed by the Forestry and Timber Bureau, Canberra; Bureau support for the project is gratefully acknowledged.

I would like to acknowledge the advice given by Professor S. Smith-White, who reviewed early proposals and progressive results of this investigation.

The study owes much to Dr. J. M. Fielding, who made himself freely available for discussion throughout the course of the work. His enterprise in establishing most of the plants used, in many cases from seed collected personally, provided an invaluable facility.

I am also grateful to Dr. R. D. Johnston, who critically reviewed the thesis manuscript and to Mr. R. Paton who competently carried out a large part of the technical work.

With the exception of the above assistance, the work of other authors specifically acknowledged in the text and some minor technical jobs performed by others at my direction, the results embodied in this thesis represent my personal, original investigations.

I. INTRODUCTION

The Californian closed-cone pines form a coherent taxonomic group of species, the natural distribution of which is limited to the State of California, U.S.A., the adjacent State of Oregon, the peninsula of Baja California, Mexico and several nearby islands. One member of the group, *Pinus radiata* Don, has been very widely planted as a forest tree in southern hemisphere countries (Scott 1960) and limited plantings have been made of some of the other species (Streets 1962).

Detailed studies of the variation within each of the species have been made (Duffield 1951; Fielding 1961 *a*; Newcomb 1962; Forde 1964 *a*, *b*, *c* and *d*) and the possible role of hybridisation in species formation or in adding to the amount of variation within species has been examined. Duffield (1951) concluded that there was some evidence that *Pinus radiata* had, by introgression, influenced some characteristics of *P. muricata* Don at one point in the range of the latter species. Newcomb (1962, p. 159) found little evidence that *P. radiata* had introgressed *P. attenuata* Lemmon and Forde (1964 *d*, p. 498) discounted the possibility that *P. radiata* had been influenced by either of the other species. The possibility of hybrid origin of any of the three main entities in the group was rejected by Duffield (1951, p. 46). Following field observations Stebbins (1950, 1955) and Duffield (1951) reported that natural hybrids do occur with low frequency in areas of contact between *P. attenuata* and *P. radiata* and between *P. muricata* and *P. radiata*.

Under conditions of cultivation, hybrids between all three species have been reported (for example Stockwell and Righter 1946; Duffield 1951, 1952; Bannister 1958 *a*).

The aim of this study was to obtain data on the efficiency of the mechanisms likely to be involved in maintaining the identity of the species and varieties in this group and to determine the relative significance of the possible isolating mechanisms, especially under conditions of cultivation when ecological and spatial barriers significant in the areas of natural occurrence are rendered ineffective. Such information may assist in elucidating taxonomic and phylogenetic problems in the group as well as being of value in planned hybridisation programmes.

The material available for the work comprised seedlings planted in 1955 or earlier in three localities in the Australian Capital Territory. Series of provenances of each of the species, mainly raised from seed collected by Dr J.M. Fielding, were set out, when sufficient material was available, in plots about one chain square containing about 80 plants. The planting sites are more fully described in Chapter VI.

The studies were primarily designed to examine barriers between groups at specific level. Some information was obtained concerning varieties while data at provenance level is in general insufficiently replicated to enable conclusions to be confidently drawn concerning variations here in the effectiveness of isolating mechanisms.

The general method adopted was to examine possible isolating mechanisms from the time of pollen maturation in the parental species until the resulting hybrids were about one year of age. Studies of possible weaknesses in the hybrids between this stage and the attainment of sexual maturity, and examination of hybrid fertility, were prevented by the generation time of the species involved. Finally, an assessment was made of the degree of isolation between and within the various species and the relative significance of the factors encountered.

II. TAXONOMY

Shaw, in 1914, arranged the recognised species of the genus *Pinus* in a series of sections, subsections and groups based "on the evolution of the cone and seed from the comparatively primitive conditions that appear in *Pinus cembra* to the specialised cone and peculiar dissemination of *Pinus radiata* and its associates". *Pinus muricata*, *P. attenuata* and *P. radiata*, the main entities in the Californian closed-cone pine group, were classified together in section Diploxylon (Hard Pines), sub-section Pinaster (species with a persistent fascicle sheath and an articulate, effective seed wing), group Insignes (cones persistent, sometimes serotinous). In the same group were 13 other species, some occurring in Europe, others in North America.

Pilger (1926) arranged the section (sub-genus) Diploxylon in eight sections and placed *P. muricata* in section 6, Banksia and the other two species in section 11, Taeda.

Duffield (1952) reconsidered the arrangement of species within the sub-genus Diploxylon, aided by results from interspecific crossing programmes and information on the turpentine chemistry of many species. He placed *P. radiata*, *P. muricata* and *P. attenuata* together in an unnamed group XIV, together with *P. greggii*, *P. pringlii*, *P. patula* and *P. oocarpa*, justifying this group in the following words "Group XIV remains rather unsatisfactory. It contains the (three) coherent California closed-cone pines and one Mexican species, *P. greggii*, which resembles the California closed-cone pines morphologically. Otherwise, for lack of new evidence and interpretation, this group constitutes the remains of Shaw's group Insignes". The only successful interspecific hybrids in Duffield's group XIV involved the three Californian species.

Subsequently, in 1954, there was a report from South Africa that a hybrid progeny of *P. patula* and *P. muricata* had been planted, but the identity of the trees has not been confirmed. Hyun (1956) reported that the cross *P. rigida* x *P. radiata* had been made with relative ease in Korea. However, Hyun and Yim (1964) found a complete breakdown in samples of ovules of the same cross: further examination is required to clarify the position. Fielding and Nicholson (1956) and Fielding (1960) have reported and verified the successful hybridisation of *P. patula* and *P. greggii*, but attempts to cross these species with the Californian species have continued to be unsuccessful (Brown, unpublished).

Recent studies have confirmed the presence of distinct sub-species within each of the major Californian species.

Duffield (1951) considered that *P. muricata* could be divided into four sub-species -

P. muricata Don var. *borealis* Duffield
P. muricata Don
P. muricata Don var. *remorata* (Mason) Duffield
P. muricata Don var. *cedrosensis* Howell

He applied the name *borealis* to those populations north of Inverness and regarded the mainland occurrences south of San Luis Obispo Co. as *muricata* var. *muricata*, leaving the occurrences at Inverness and Monterey indeterminate.

The variety *remorata* was confined to Santa Cruz and Santa Rosa Islands, while the variety *cedrosensis* was from Cedros Island. However, there is strong evidence (Newcomb 1959, Fielding 1961 *b*) further supported by the results to be given later in this thesis that these pines are probably identical with *P. radiata* var. *binata* (Engelm.) Lem. recorded from Guadeloupe Is. (Bannister 1958 *c*).

Forde and Blight (1964) examined the chemical constituents in turpentine samples collected from trees representing the first three of Duffield's sub-specific groups. It was found that three distinct groups could be recognised in the mainland collections of P. muricata. Two correspond to var. orientalis and var. muricata, while a third middle group contains the morphologically dissimilar populations from Inverness (Ridge) and Monterey. The samples from var. remorata are not very dissimilar from samples from adjacent mainland stands (var. muricata).

Newcomb (1962) reviewed the taxonomy of Pinus attenuata. He reaffirmed the identity of two sub-species -

Pinus attenuata Lemmon ssp. attenuata
Pinus attenuata ssp. acuta (Mayr) Newcomb

The northern populations (north of Cuesta Pass in San Luis Obispo Co.) belong to ssp. attenuata, while three southern populations (Cuesta Pass, San Bernardino Mtns and Santa Ana Mtns) belong to ssp. acuta. The remaining, southernmost, population at Ensenada, Baja California, is indeterminate.

Forde (1964 a, b, c and d) has recently studied the mainland populations of P. radiata in detail. She pointed out (1964 d) that while the southern (Cambria) population did differ in some respects from the other two, it could not be referred to var. macrocarpa, as suggested by Fielding (1961 a). The present position is that all mainland trees may be described as P. radiata Don var. radiata, while the trees on Guadeloupe Is. and possibly those on Cedros Is. are referable to var. binata (Engelm.) Lem.

III. NATURAL OCCURRENCE

All three species occur in discontinous stands on the western coast of temperate North America and some adjacent islands. Pinus radiata and P. muricata are essentially littoral, while P. attenuata has a more variable distribution, occurring at elevations of from 600 ft to 6000 ft and extending 180 miles inland. The places named in the following discussion are shown in Figure 1.

Pinus radiata - There are three small mainland occurrences of this species. At Point Ano Nuevo (Swanton), it is found in a few small patches having an aggregate area of several hundred acres, extending from sea level to about 500 ft in elevation and a mile and a half from the coast. The largest occurrence, further south at Monterey, is of over 6,000 acres, extending to just over 1000 ft in elevation in an area about nine miles long (N-S) by eight miles wide (E-W). About 90 miles south, the last mainland occurrence is found at Cambria within two miles of the coast at elevations of less than 300 ft. The area here is between 2,000 and 3,000 acres. Lindsay (1932 a) concludes his description of occurrence with the words "in general, the pine is found on gentle to moderate slopes, with a maximum elevation of around 1,100 ft and a maximum distance from the sea of about six miles. In the high rainfall area at Swanton it is found on all aspects, but both at Monterey and Cambria, which are distinctly drier, the hot and dry aspects do not appear to be favourable to the species".

P. radiata var. binata occurs in groves on the high narrow ridge at the northeast extremity of Guadeloupe Island (Harshberger 1911, p. 623).

The natural occurrence of P. muricata is described by Forde and Blight (1964) as follows - "The mainland distribution of the species is very discontinuous and can be divided into seven geographical sections, most of which consist of one or a few small stands. The location of these is approximately as follows -

(1) Trinidad Head, northern Humboldt Co., with a few individual trees reported as far north as Crescent City, Del Norte Co. (Mason 1949);

(2) from north of Fort Bragg, Mendocino Co., to Fort Ross, Sonoma Co., a more or less continuous strip some 80 miles long and the principal area of the species;

(3) Inverness Ridge, Marin Co.;

(4) Monterey Peninsula, Monterey Co.;

(5) San Luis Range near Coon Ck, San Luis Obispo Co.;

(6) La Purisima Ridge, Lompoc, Santa Barbara Co.;

(7) San Vicente, Baja California.

"Some of the more notable gaps in the distribution are (in round figures): between Trinidad Head and Fort Bragg - 120 miles; between Inverness Ridge and Monterey - 120 miles; between Monterey and Coon Ck - 100 miles and between La Purisima Ridge and San Vicente - 350 miles".

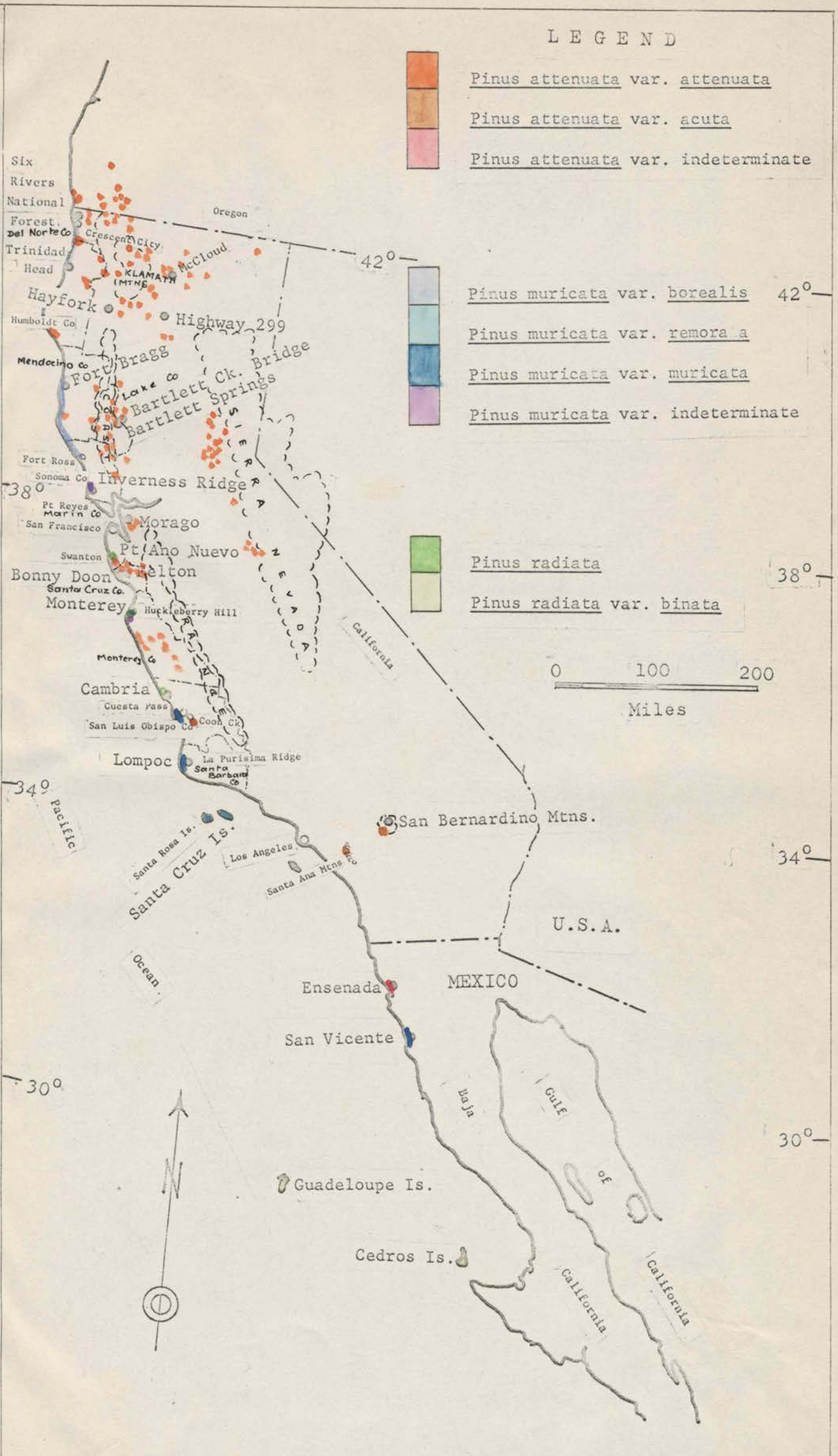


FIG.1 PORTION OF WESTERN NORTH AMERICA

McMillan (1956) noted that the species was confined to discontinuous, highly acid soils. Lindsay (1932 b, p. 7) writes "P. muricata is never found more than a few miles from the sea coast. The topography where the species is found varies considerably. In Humboldt Co. it is on flat to gently sloping country near the coast. Further south at Fort Bragg it occupies a coastal plain and extends to a very slight extent into the gently rolling country away from the coast. At Point Reyes (near Inverness) the topography is hilly with moderate to steep slopes. At Monterey it occupies a small area of shallow soils on the seaward slopes of Huckleberry Hill at an elevation of around 800 ft. Further south at Coon Creek (San Luis Obispo Co.) it is found on the moderate slopes of gullies, running seawards at an elevation of between 750 and 1250 ft". Jepson (1910) (cited by Lindsay 1932 b p. 7) writes "(the species occurs) between Ensenade and San Quentin.... in the San Vicente Valley about six miles from the coast and 250 ft above the sea...."

Duffield (1951) gives more detail of the natural distribution in the important mainland occurrences. He concludes (p. 28) "It seems probable that in the northern areas (Sonoma and Humboldt Counties) bishop pine is more abundant than it was before the days of logging, fires and widespread land clearing and that the present large populations are the offspring of a relatively small number of scattered individuals. The areas south of Huckleberry Hill (Monterey) differ strikingly in this respect - that the bishop pine here is unquestionably the dominant tree".

P. muricata var. remorata, on the Channel Islands, Santa Cruz and Santa Rosa Is., is discussed by Mason (1930, 1934, 1949).

P. muricata var. cedrosensis. - Harshberger (1911, p. 640) reports "the mountain summit (of Cedros Is.) is adorned with open groves of Pinus muricata". Fielding (1961 b) states "the pines......occur as small, scattered stands of trees on dry rocky slopes - probably relics of a forest that once was more widespread".

The natural occurrence of Pinus attenuata is described by Lindsay (1932 c) and Newcomb (1962). This species is able to tolerate harsher climates than either of the species described above. Populations tend to be found on the drier slopes and ridge tops. The eastern limit is the Sierra Nevada foothills, the western the coastal foothills (see Figure 1). The pattern of distribution varies from very scattered distinct populations in southern and Baja California and the southern Sierra foothills to a widespread distribution in Santa Cruz Co., and in Lake Co. northward into the Klamath Mtn area. Newcomb (1962) considers that the very interrupted pattern may be due to the apparent dependance of the species upon fire for satisfactory regeneration.

There is extensive fossil evidence that the closed-cone pine forests were in the Quaternary more extensive and more homogeneous than the present discontinuous remnant stands (Mason 1934; Langenheim and Durham 1963).

IV. NATURAL HYBRIDISATION AND INTROGRESSION

Pinus attenuata has a zone of contact with *P. radiata* at Pt Ano Nuevo. Here the two populations grow at times in adjoining or slightly mixed groups. The smaller population of *P. radiata* grows on the seawards-facing slopes and in the canyon bottoms while *P. attenuata* occurs over several square miles on the drier slopes behind (Duffield 1951).

In the zone of contact between the two species putative hybrids resembling the controlled cross *P. x attenuradiata* (Stockwell and Righter 1946) have been seen by Newcomb (1962) and others (Stebbins 1950). Stebbins (quoted by Bannister 1958 *a*) has reported that, of 1,000 trees which were examined in the immediate vicinity of the boundary line between the two communities, eight were found which were considered to be F_1 hybrids or back-cross derivatives. Stebbins gained the impression that all the *P. radiata* forest here differed from that at Monterey in the direction of *P. attenuata*, and might therefore consist wholly of derivatives of introgression. De la Puente (1960) examined samples of cones from each species in the area of contact and compared these with samples collected at a single point elsewhere in the range of each species. He concluded from the cone characteristics that there was probably a flow of genes from *P. attenuata* to *P. radiata* stronger than in the opposite direction.

Subsequent investigations by Bannister *et al*. (1962) of the turpentines of *P. radiata* and by Forde (1964 *d*) of morphological features failed to show that the *P. radiata* had been influenced by *P. attenuata*. Newcomb (1962) likewise found no obvious evidence that *P. attenuata* here had been introgressed by *P. radiata*: he concluded that if introgression had been important in the evolution of *P. attenuata* in the area concerned the recognition of it was very difficult.

Natural contact occurs between *P. radiata* and *P. muricata* in the Monterey area. The latter species occurs as a small enclave of about 500 acres in extent within the *P. radiata* forest. The two species appear to occupy different ecological areas and mingle only around the margins of the enclave and along the creeks which cross it (Lindsay 1932 *a*, *b*; Duffield 1951; Forde 1964 *d*). The *P. muricata* occurs on more elevated sites having shallow soils derived from shale and diorite.

Duffield (1951 p. 20) located a small number of putative hybrids as a result of a deliberate search, but he noted that the apparent frequency of hybrids recognisable as such in the field was quite low. However, neither Lindsay (1932 *a*) (cited in the following reference) nor Forde (1964 *d*) were able to locate any morphological evidence that *P. radiata* was introgressing *P. muricata*. A study of the turpentine of *P. muricata* by Forde and Blight (1964) produced no evidence to support Duffield's thesis. Forde (1964 *d*) was also unable to find evidence that *P. radiata* had been introgressed by *P. muricata*.

The hybrid *P. attenuata* x *P. muricata* has not been found in nature; nowhere do the parent species come in contact (Duffield 1951, p. 46). There are important ecological differences between the species which might limit such contact: *P. attenuata* is tolerant of and occurs on serpentine soils, whereas *P. muricata* occurs only on acid soils (McMillan 1956).

The various investigations considered above indicate that despite significant zones of contact in the natural occurrence of the three species recognisable hybridisation is limited and detectable introgression is absent in the populations concerned. As _P. radiata_ and _P. muricata_ were apparently associated in coastal forests during the Pleistocene (about one million years ago) (Mason 1934, p. 94) but have continued to maintain their identity the isolating mechanisms between them must be quite effective.

V. ISOLATING MECHANISMS

The segregation of a previously interbreeding population into two reproductively isolated segments tends to restrict the supply of genes available to each of these segments and to canalise them into certain paths of adaptation. Evolutionary specialisation is therefore greatly furthered by the process of speciation (Muller 1942, p. 122). There are in nature a great many mechanisms which restrict or prevent the continued pooling of genes of evolving races or species. These mechanisms hinder or prevent the formation of hybrid individuals in the first place or render any hybrids either inviable, infertile or selectively disadvantageous. Muller (1942, p. 84) considered that isolating mechanisms of the second type rarely developed except after bars to crossing have become fairly well established as he believed they could not form as a result of a single mutational step. However, the latter is not invariably true; for example an interspecific lethal gene is known in *Crepis tectorum* which causes the early death of hybrids with *C. capillaris* (Babcock 1947).

Classifications of different isolating mechanisms have been published by Muller (1942, p. 72), Dobzhansky (1947, p. 257), Stebbins (1950, p. 196), Davis and Heywood (1963) and others. That of Stebbins, reproduced in a modified form below, will serve as a basis for the present discussion.

EXTERNAL BARRIERS
- I. SPATIAL ISOLATION (= geographic isolation)

PARTIALLY EXTERNAL, PARTIALLY INTERNAL BARRIERS
- II. PHYSIOLOGICAL ISOLATION (=reproductive isolation)
  - A. Barriers between parental species
    - 1. Ecogeographical isolation
    - 2. Ecological (microgeographical) isolation of sympatric types
    - 3. Temporal and seasonal isolation

INTERNAL BARRIERS
    - 4. Mechanical isolation
    - 5. Prevention of fertilisation: gametic isolation
  - B. Barriers in the hybrids
    - 6. Hybrid inviability or weakness
    - 7. Failure of flowering in the hybrids
    - 8. Hybrid sterility (genic and chromosomal)
    - 9. Inviability and weakness of F2 and later segregates.

In any particular case a number of these isolating factors may be present; isolation may not depend upon any single one but upon the aggregate effect of a number of partial barriers (Dobzhansky 1947, p. 256).

SPATIAL ISOLATION

Mayr (1942, p. 155-185) cites a number of examples to show that in the animal kingdom geographic or spatial isolation is necessary for the maintenance of two closely related species as separate entities; species which cross readily have geographically separate natural occurrences. Stebbins (1950, p. 238) considers that such is also the case with plants. Geographical isolation alone is not sufficient for the formation of species or even sub-species. "It is not clear how physiologically isolated species develop in non-contiguous territories where opportunities for hybridisation are not available" (Dobzhansky 1947, p. 287). However, in many cases isolation may be accompanied by divergence in the development of the populations concerned, due to ecological adaptation, or, in small populations, to genetic drift or a combination of the two (Stebbins 1950). Ecological differences may not always be readily assessed when comparing habitats in widely separated geographic areas. Two species of *Platanus*, *P. occidentalis* and *P. orientalis*, occur respectively in the eastern United States and the eastern part of the Mediterranean region. Although morphologically distinct, the two species may be crossed to yield a vigorous, hardy and fertile hybrid (Sax 1933). Superficially this is a simple case of spatial (geographic) isolation, but closer examination indicates wide ecological divergence between the species in adaptation to the very different climates of the habitats concerned (Kendrew 1953).

PHYSIOLOGICAL OR REPRODUCTIVE ISOLATION

Whereas spatial isolation may arise from an historical accident, physiological or reproductive isolation is the result of the action of biological factors.

A. Barriers Between Parental Species

(1) Ecogeographical Isolation. - Stebbins (1950) considered that this represents the commonest type of isolating mechanism in the plant kingdom; it results from the existence of related forms in different geographic regions which differ in their respective ecological conditions. A good example is that of *Platanus*, quoted above; other examples are in *Picea* (Wright 1955) and perhaps in the white pines (Duffield and Righter 1953).

(2) Ecological (Microgeographical) Isolation of Sympatric Types. - This form of isolation exists when sympatric groups are prevented from mingling by ecological factors; their boundaries do not overlap. When the ecotone is narrow, trans-pollination may occur. If the groups are interfertile, selection may eliminate most or all hybrids within each distinct habitat although the ecotone may be populated by intermediate forms.
Briggs (1962) described such a situation in *Ranunculus*: all nine species examined had similar karyotypes and natural hybrids showed normal meiosis and high fertility. The species differed in their ecological adaptations and the hybrids were rigidly restricted to narrow marginal zones between stands of the parental species. The role of selection in restricting gene flow was demonstrated by transplant experiments. The genus *Eucalyptus* contains numerous examples of such isolation (Pryor 1959, p. 467-8): hybrids may be absent or rare in sharp transition zones (such as those of edaphic origin) undisturbed by man, or relatively frequent in

graded habitats which have been disturbed by burning and partial clearing ("hybrid habitats" or "disturbed habitats"). In these habitats vigorous early growth may be followed by early death. Isolation of this type appears to exist between P. radiata and P. attenuata at Ano Nuevo Point (the latter species occupying the more elevated sites with shallower soils and probably lower precipitation) and between P. radiata and P. muricata at Monterey (the latter species also occurring on more elevated sites with shallower soils) as interbreeding may occur relatively frequently when the species are grown in mixture under cultivation (Bannister 1958 a).

(3) Temporal or Seasonal Isolation. - Temporal isolation exists when pollen is shed at different times of day. Seasonal isolation occurs when the plants of one group flower at a different time to those in the related group. Stebbins (1950) quotes examples from the genera Lactuca and Ixeris, as well as Pinus. P. radiata flowers somewhat earlier than P. attenuata and appreciably earlier than P. muricata: Stebbins (1950) and Forde (1964 d) suggest that seasonal isolation may be quite important in this group of species. Winton (1964) considered that a difference in heat requirement of black and white spruce both for initiation of meiosis and ultimately for pollen release was the probable, primary isolating mechanism in these sympatric species: the difference in requirements resulted in seasonal isolation.

(4) Mechanical Isolation. - This mechanism may act to prevent effective pollination in plants. It is particularly effective in groups with elaborate floral structures such as the orchids and asclepiads (Stebbins 1950). Doyle and Kane (1943) describe what may be a form of mechanical isolation in Picea orientalis. In this species alone of those examined the cones hang vertically downwards at pollination. The pollen grains sink in the pollination fluid, even though winged and thus reach the nucellus of the more-or-less upright ovules. The wings of the grain are relatively smaller and the stigmatic part of the integument more developed than in other species of Picea. Variations in the size of pollen grains of Pinus sp. have been recorded by Wodehouse (1935). It is possible that the large size of some pollen grains might prevent the pollen from floating up through the neck and arms of the micropyle to the nucellus in the ovules of species characteristically possessing pollen of small size.

(5) Prevention of Fertilisation: Gametic Isolation. - This may be due to non-germination of pollen, inadequate pollen tube development, or failure at fertilisation. Stebbins (1950) considered that this was rarely the primary cause of reproductive isolation. Many accounts may be found in the literature of isolating mechanisms of this type in angiosperms; some serve to prevent or minimise self-fertilisation (Stout 1945). The mechanisms involved may be either biochemical, as in the case below reported by McWilliam, or mechanical, as when the length of stylar tissue is too great to be traversed by the growing pollen tube before the ovary becomes non-receptive (Gardella 1950).

Stockwell (1939), McWilliam (1959) and Hagman and Mikkola (1963) considered that mechanisms of this type were significant in interspecific crosses in the genus Pinus. McWilliam found that pollen tube growth was completely inhibited in one cross which he investigated (P. elliottii Engelm. x P. nigra Arnold), while in other crosses (for example P. resinosa Ait. x P. nigra) pollen tubes were found to penetrate the nucellus in a small percentage of the ovules: the success of interspecific crosses in Pinus was considered to depend on the ability of pollen tubes to grow and function normally in the nucellar tissue of a foreign species. The production of fully grown but empty seeds in attempts to produce hybrid pines (for example Buchholz 1944; Wright 1953) indicates breakdown of the ovule at some stage close to fertilisation.

(6) Hybrid Inviability or Weakness; Failure of Flowering. - Even if fertilisation occurs successfully the hybrids may fail for many reasons before seed maturity is attained, or less commonly subsequently but before the hybrid is able to flower. These barriers are considered by Stebbins (1950 p.215) to be very important and the most widespread of those which separate plant species, although Davis and Heywood (1963, p. 442) argue that this view would be difficult to prove. (Stebbins, p. 199 also claims that spatial and ecogeographic is "the commonest type of isolation in....the plant kingdom"!)

Snyder (1950) found that within a single species, Elymus glaucus, different crosses between local populations failed at a variety of stages ranging from fertilisation to flowering of the F1.

If Pinus ovules are fertilised under natural conditions by a mixture of pollen from various species, one would expect that only the more vigorous embryos would survive the early intense competition within the ovules. Such a mechanism has been reported by Sarvas (1962, p. 172) to appreciably reduce the number of selfed seed produced in Pinus sylvestris. A modest reduction in the vigour of hybrid zygotes would probably likewise greatly reduce the incidence of hybrid seed.

Hagman and Mikkola (1963), crossing Pinus peuce with P. cembra, found that the hybrid zygote lived until division of the first four nuclei occurred and cell wall formation started - i.e. the hybrid nucleus was able to function as long as no organisation of the new zygote was necessary. They suggest that the abnormal nucleus does not permit the formation of certain substances required for morphogenesis: relevant chemical differences have been demonstrated between pines (McWilliam 1959).

Embryo culture may be used to overcome incompatibility reactions between the developing embryo and the endosperm or the seed coat. Stone and Duffield (1950) found that the seed produced by crossing P. lambertiana and P. armandii could be induced to germinate if excised when comparatively mature.

Under field conditions any reduction in fitness in the hybrid relative to the parents must seriously reduce the frequency with which hybrid plants will attain sexual maturity. Whitaker (1934) reported a spectacular case in which Nicotiana F1 hybrids form parenchymatous tumours in their vegetative parts due to genic disharmony, without impairing vegetative growth of the affected plants.

Pryor (1959, p. 468) noted that some eucalypt hybrids, such as E. cinerea x E. blakelyi and their segregates are at best weakly viable and present little challenge to the pure species.

(7) Hybrid Sterility. - This form of sterility is not due to any reduction in fitness of the organism as a whole, but rather to specific disharmonies between the parental gene complexes which act during the formation of reproductive structures, at meiosis or during the later development of the gametophytes or gametes (Stebbins 1950). Two general forms of hybrid sterility were recognised by Dobzhansky (1947) - genic sterility, the failure of sex organs to develop to the point where meiosis can take place or by genically controlled abnormalities in the chromosomes themselves and chromosomal sterility resulting either from lack of homology between the chromosomes of the parents of a hybrid, as in the Raphano - Brassica hybrid, or from segregational imbalance as in Primula kewensis (= P. floribunda x verticillata). In both cases the tetraploids are fertile (Darlington 1958, p. 43-4). Observations of meiosis in 2n hybrids can demonstrate meiotic breakdown (for example Snyder 1951; Hayman 1955). The determination of the type of sterility mechanism involved in interspecific hybrids can sometimes be made after allopolyploids have been produced; even then the position may not be clear as the failure to set seed may result from the effects of both types of sterility factors (Stebbins 1950). Spontaneous, successful and true-breeding allopolyploids are well known, but the likelihood of sterility being overcome by this means in Pinus seems remote because of the very constant nature of the chromosome complement in the genus (Sax and Sax 1933, Sax 1960).

Reduced pollen viability has been reported in Pinus hybrids by Duffield (1952), who stated that of ten F1 hybrids which had flowered at the Institute of Forest Genetics, none showed more than 40 to 50 percent pollen abortion. A much greater reduction in pollen viability has been observed by the present author in many trees of the hybrid P. patula x P. greggii described by Fielding and Nicholson (1956).

Although Stebbins (1942) claimed that partial loss of fertility is less important in perennial species than in annual ones, plants so affected are at a serious disadvantage; partially sterile organisms are subject to strong adverse selection irrespective of their generation time. Any population in which partly intersterile individuals can interbreed freely is at a disadvantage in competition with other populations in which only complete interferility or complete intersterility prevails (Sturtevant 1938).

Mather (1943, p. 52) pointed out that both isolating mechanisms and hybrid sterility are polygenic characters; therefore partial intersterility would be of frequent occurrence at boundaries between incipient species.

(8) Degeneration of Progeny of Hybrids. - Examples are known of vigorous F1 hybrids which set seed readily, but whose progeny are weak or disease-susceptible. Among woody plants, examples have been quoted by Syrach-Larsen (1937) in Larix gmelinii x L. kaempferi and by Johnson (1946) in Populus alba x P. grandidentata. The causes for this degeneration may be either genic or chromosomal; it is

difficult to determine which is operating in a particular case. Stockwell and Righter (1946) claimed that the F2 of *P. attenuata* x *radiata* displayed normal vigour.

SUMMARY OF POSSIBLE ISOLATING MECHANISMS IN THE CALIFORNIAN CLOSED-CONE PINES

Geographical isolation is important at the present time in the area of natural occurrence in only one of the possible three species combinations, i.e. *P. attenuata* x *P. muricata*. Stebbins (1950, p. 239-40) postulated that the three species owe their initial differentiation to ecogeographical isolation. Seasonal periodicity of growth suggests that *P. radiata* originated on the temperate central Californian coast, that *P. muricata* evolved much farther north on the coast and that *P. attenuata*, which exhibits pronounced winter dormancy, arose in cold inland areas of northern California where the largest and most luxuriant stands are found at the present time. The present regions of contact appear to have existed for some time: Duffield (1951 p. 21) cites reports that the Huckleberry Hill (Monterey) stand existed as such in 1846. However, there is little evidence of extensive hybridisation or introgression. Other mechanisms must therefore be involved in maintaining the species as separate entities at the present time. Ecological isolation may be significant: *Pinus radiata* occupies the better sites in each locality in which it contacts the other species and the edaphic requirements of *P. attenuata* and *P. radiata* differ. Such a barrier would not usually be effective in cultivated stands of two or more of the three species.

Some authors have suggested that differences in flowering time may reduce hybridisation, although the discovery of some hybrids in the field indicates that this barrier is not completely effective. The effect of topography upon flowering times is considerable: Duffield (1953) estimated that each increase of 1000 ft in elevation delayed flowering by about 8 days. (Evidence on this point is also given later in this thesis). Thus the pattern of occurrence of two species in an area of broken topography could minimise the value of small numbers of observations for assessing the effectiveness of temporal isolation. The relative difficulty of finding hybrids between *P. radiata* and *P. muricata* in the field may be due to the greater difference in flowering time between these two species (Duffield 1953) than between *P. radiata* and *P. attenuata*.

A mechanical barrier may exist if the pollen of the different species varies widely in size.

Genetic barriers may well contribute to the rare occurrence of hybrids in the field: it is difficult to assess their importance in relation to the factors above by observation alone. However, any genetic barriers are not complete, for apart from the few natural hybrids known, the production of artificial hybrids has been reported, for example by Stockwell and Righter (1946) and by Duffield (1952).

There is little evidence that hybrids are markedly less vigorous than their parents. Stockwell and Righter (1946), Duffield and Righter (1953) and Bannister (1958 *a* and *b*) report that both F1 and F2 hybrid progenies are quite vigorous. It is also unlikely that the fertility of the F1 is reduced to the point where this factor becomes of significance - in the ten F1 pine hybrids at Placerville Duffield (1952) reported seed yields as being usually 80 percent as good as those from the parent species.

The above evidence suggests that the following mechanisms are of importance in areas of natural contact or in artificially established mixtures -

1. Seasonal isolation
2. Possibly mechanical isolation
3. Gametic isolation
4. Possibly hybrid weakness.

VI. PLANTINGS USED IN THIS STUDY

Field work was carried out at three sites west of Canberra, in the Australian Capital Territory, where plantings of the species concerned have been made since 1930. The rainfall at the sites is about 38 ins per annum, more or less evenly distributed throughout the year; the mean maxima and minima for January are about 75°F and 53°F and for July about 47°F and 32°F respectively (from Pryor 1954). The westernmost two sites had, prior to clearing, a wet sclerophyll forest of species such as *Eucalyptus fastigata*, *E. viminalis* and *E. robertsoni*, while the other locality had a poorer dry sclerophyll forest of species such as *E. rossii*, *E. macrorhyncha* and *E. maculosa*. The conifers are growing quite well at each site, although there are some differences among the various provenances (see for example Fielding 1961 *a*). In general, growth rates are at least comparable with those recorded in the native habitats by Lindsay (1932 *a*, *b* and *c*) and Newcomb (1962, p.54). Figure 2 shows the location of the three sites and their topographical situation. Each site and the relevant plantings will be discussed below.

1. *Blundel Arboretum* (Figures 3, 4 and 5). This is situated in a broad valley, at about 2,500 ft A.S.L. The valley runs north and south; high ground to the west minimises the force of prevailing westerly winds; the site is quite frosty because of the accumulation of cold air at night. Species used were -

(1) *Pinus muricata*. - Seed for plot 46 came from Hamner Springs, N.Z., described as the "blue" form of the species. This "blue" form resembles var. *borealis* in the northern portion of the species' range, for example in Humboldt Co. The trees were 11 years old in 1960. Plot 63 was planted with seed collected by Dr J. M. Fielding from Trinidad Head and Fort Bragg (var. *borealis*), and Inverness (var. indeterminate) in the northern half of the species' range. The trees were 9 years old in 1960. Plot 113A contains a few trees of var. *remorata* raised from seed obtained from the arboretum at Mt. Burr, S.A. The Mt. Burr stand originated from seed collected on Santa Cruz Is. (Fielding 1961 *a*).

(2) *Pinus radiata*. - These plants are of uncertain provenance, but are likely to be from Monterey. Observations were made on three groups of trees - small trees, 5 years of age, in plot 116; tall trees (about 80 ft high) 30 years of age in plot 6, and a group of 5 year old trees to the south-west of the arboretum and at about the same elevation.

2. *Reid's Pinch Arboretum* (Figures 6, 7 and 8). This planting is situated on a south-facing site near the crest of a ridge; it is sheltered from the north and to a lesser extent from the west; cold-air drainage is unimpeded. Elevation is about 3350 ft A.S.L. The plantings run around rather than across the contours. Species used, grown from seed obtained by Dr J. M. Fielding and planted in 1950, were -

(1) *Pinus attenuata*. - Two plots from collections at Morago and Bartlett Ck - localities within the range of var. *attenuata*.

(2) *Pinus radiata*. - The three plots represent each of the mainland occurrences - i.e. Pt Ano Nuevo, Monterey and Cambria.

(3) *Pinus muricata*. - The six plots represent collection points fairly equally spaced on the mainland. Trinidad Head and Fort Bragg represent var. *borealis*, Inverness and Monterey represent populations the taxonomic status of which is indeterminate, and Lompoc and San Vicente represent var.

muricata.

3. Hall's Block Arboretum (Figures 9 and 10). This is situated on a gentle slope of eastern aspect sheltered from the west by higher topography, at an elevation of 2400 ft A.S.L. Cold air drainage is not very good. The important plantings here were made in 1954 and 1955 by Dr J. M. Fielding.

(1) Pinus attenuata. - A series of ten provenances represent most important areas of occurrence. These are Six Rivers N.F., McCloud, Hayfork, Highway 299, Bartlett Springs, Pt Ano Nuevo, Felton and Bonny Doon (var. attenuata); San Bernardino Mtns (var. acuta), and Ensenada (var. indeterminate).

(2) Pinus muricata. - This is a planting of clones derived from trees of the same stock as in plot 46, Blundel Arboretum - i.e. probably var. borealis, for example from Humboldt Co.

(3) Pinus radiata. - Plants of uncertain origin, but probably from Monterey. A group of trees from Cedros Is., supplied as P. muricata var. cedrosensis (Fielding 1961 b) but closely resembling P. radiata var. binata are also growing here (Plot 16).

Plot Size, etc. Where sufficient plants were available, plots about one chain square, nominally containing 81 plants at 8' x 8' spacing were established. Insufficient plants were available to replicate these plots; in some cases the number of plants available was not enough for one such plot.

The field plantings were usually made with open-rooted plants raised for one year in the nursery; age is taken from the time of field planting. Competition from the regrowth of natural vegetation was controlled by periodic slashing.

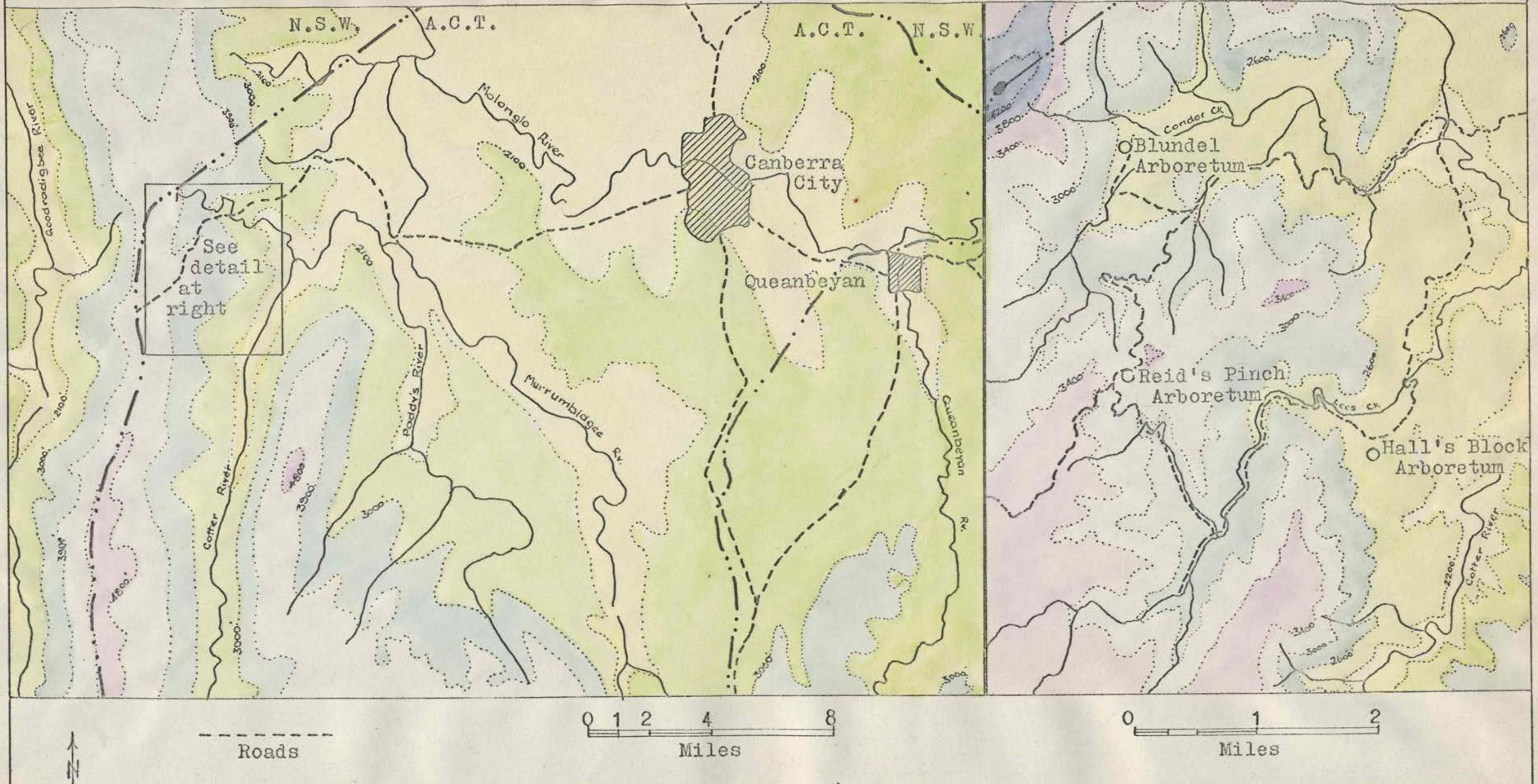
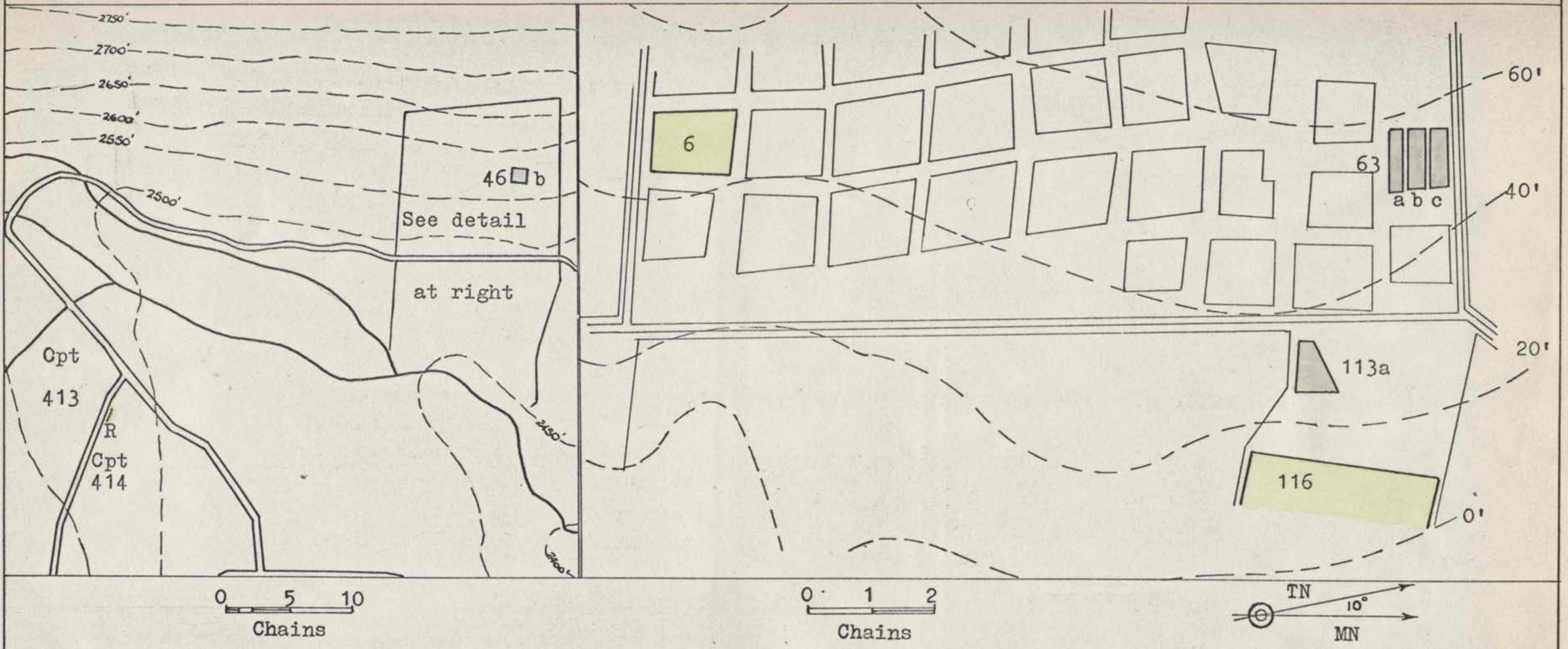


FIG. 2 LOCATION OF FIELD PLANTINGS IN AUSTRALIAN CAPITAL TERRITORY ARBORETA



🟩 Pinus radiata-probably from Monterey

6-Old, tall trees (planted 1930,1939)
116-Small trees(planted 1955)
R-Small trees in Cpt 414 (planted 1955)

▨ Pinus muricata

46b-Probably Humboldt Co.
63a-Fort Bragg
63b-Trinidad Head, Humboldt Co.
63c- Inverness Ridge

▨ Pinus muricata var. remorata

113a- Santa Cruz Is.

FIG. 3 PORTION OF BLUNDEL ARBORETUM AND THE ADJOINING PLANTATION AREA

FIG. 4 BLUNDEL ARBORETUM

A view from the north in 1965. See Figure 3 for the species in the numbered plots.

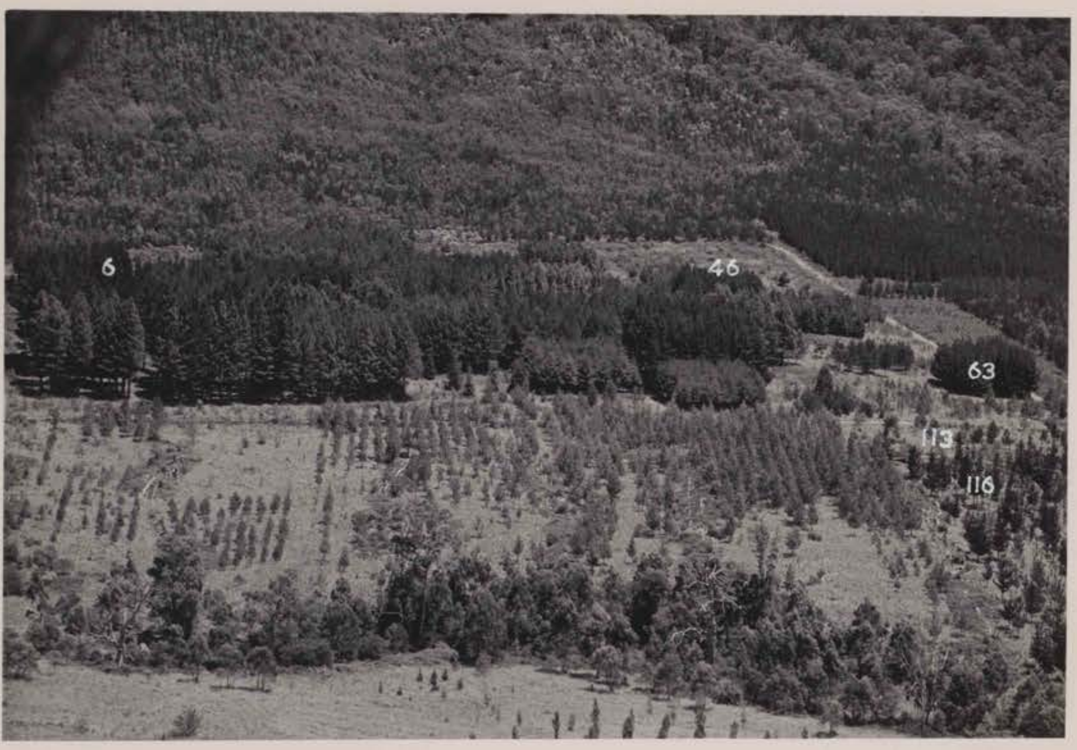


FIG. 5 BLUNDEL ARBORETUM

A view from the east in 1965. See Figure 3 for the species in the numbered plots.

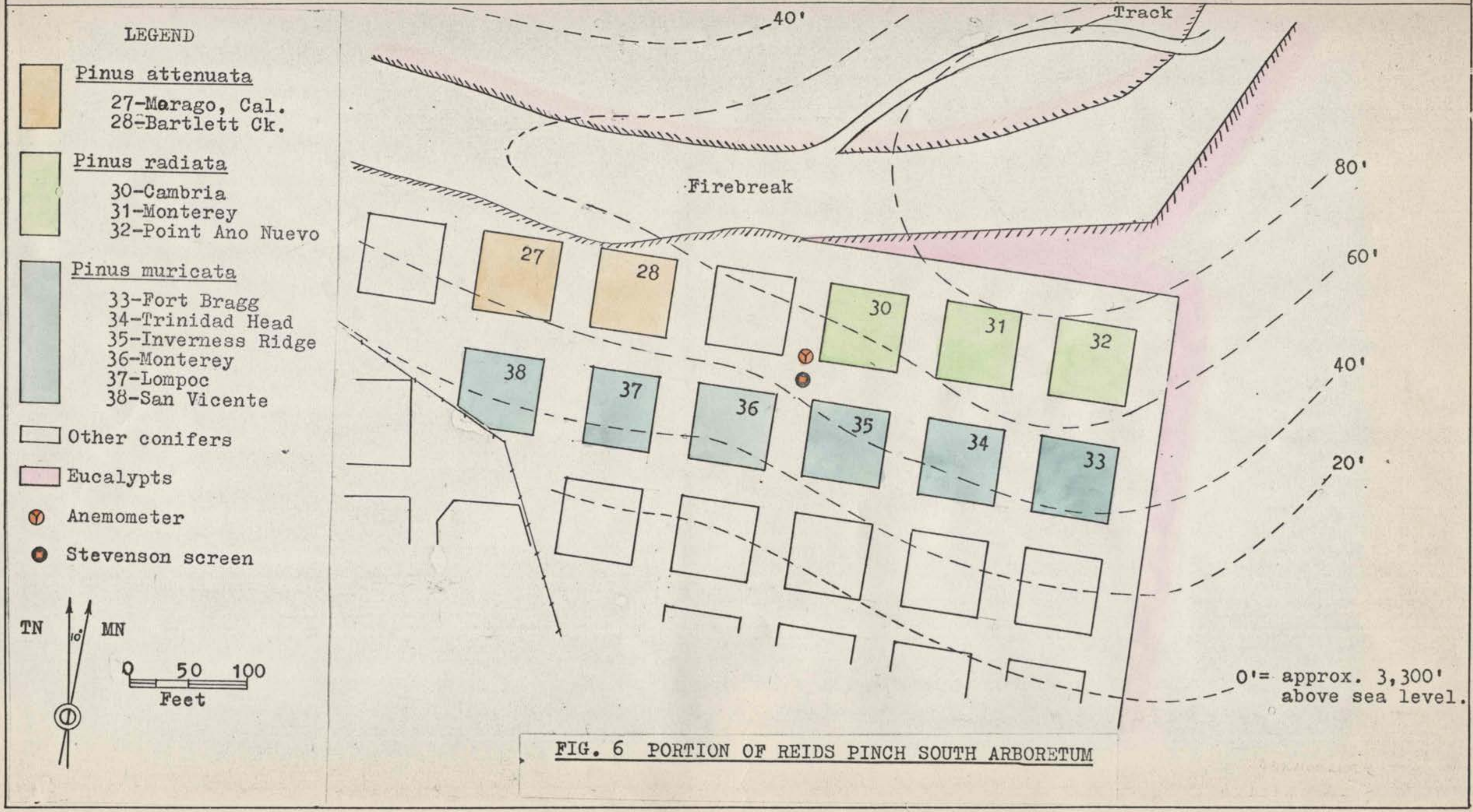


FIG. 6 PORTION OF REIDS PINCH SOUTH ARBORETUM

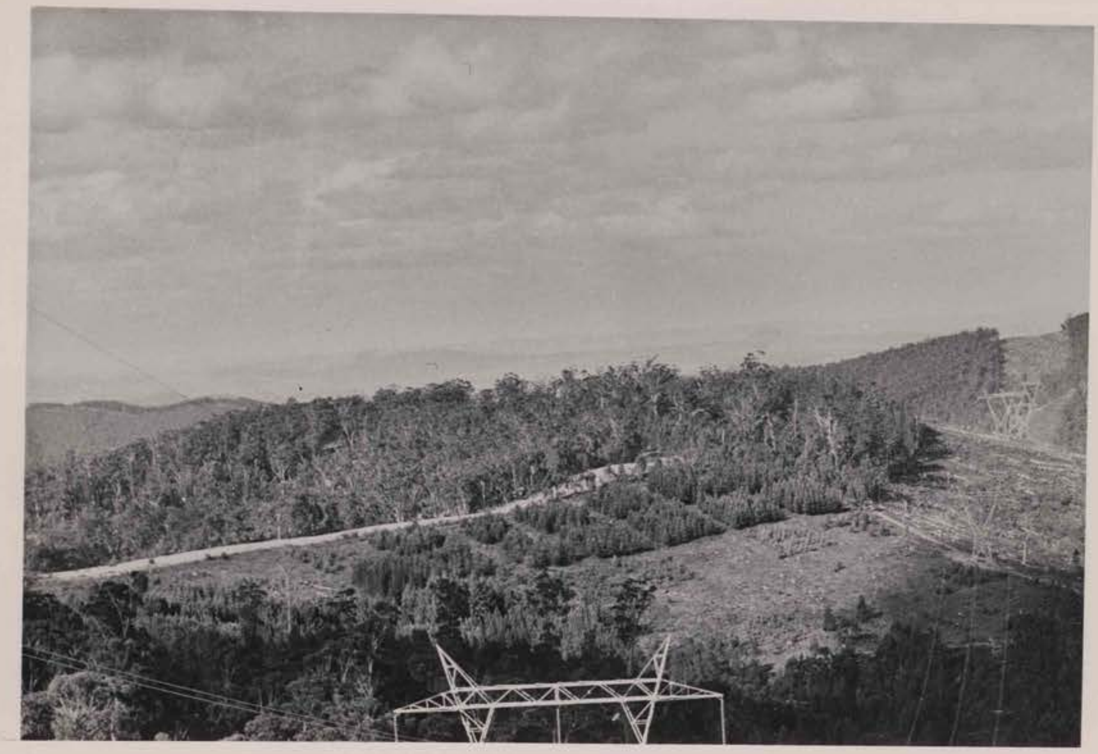


FIG. 7 REID'S PINCH ARBORETUM

A view of portion of the arboretum from the south-west in 1960.

FIG. 8 REID'S PINCH ARBORETUM

Looking eastwards through the arboretum in 1960. The plots on the right are of P. muricata; those on the left beyond the Stevenson screen are of P. radiata.

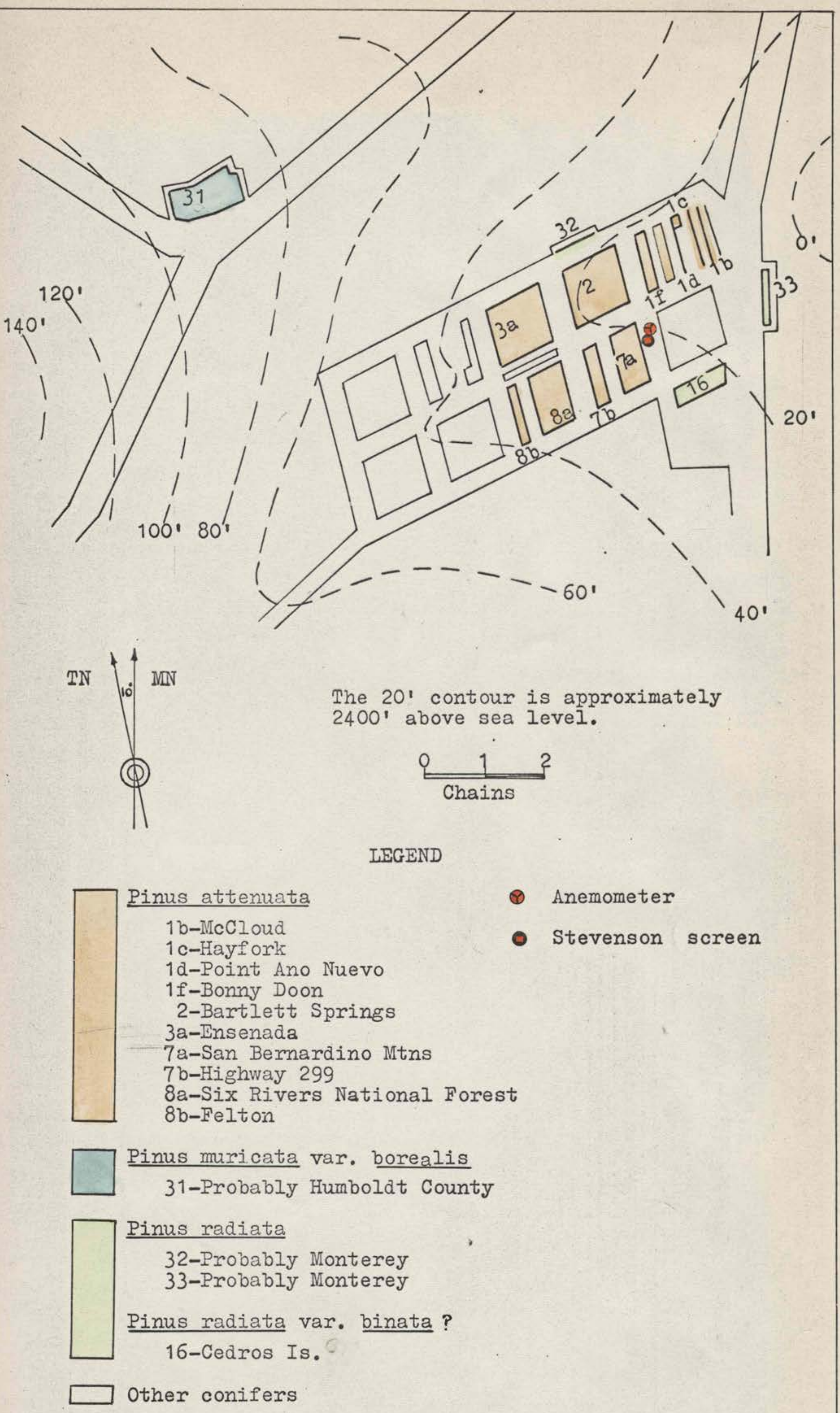


FIG. 9 PORTION OF HALL'S BLOCK ARBORETUM

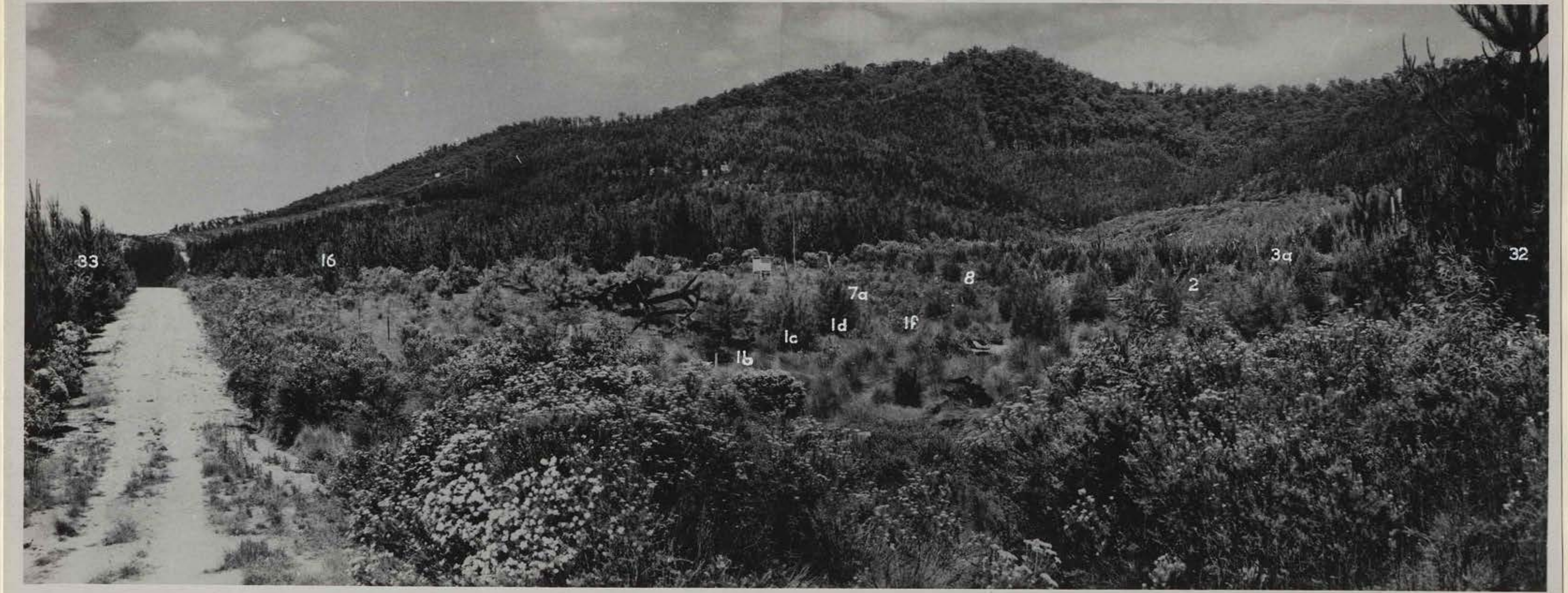


FIG. 10 HALL'S BLOCK ARBORETUM

A view from the north-east in 1960. See Figure 9 for the species in the numbered plots. Pollination bags are visible on the *P. attenuata* on the right-hand side of the photograph.

VII. INVESTIGATIONS OF SEASONAL ISOLATION

Several authors have published limited information on the flowering times of the Californian closed-cone pines. Stebbins (1950) states that at Monterey "*P. radiata* sheds its pollen in early February, while the pollen of *P. muricata* is not shed until April". Duffield (1953) lists the dates when pollen was ripe in *P. attenuata* (two points in the species range) and *P. muricata* var. *borealis* (one point) under natural conditions, and of all three species under cultivation in the Eddy Arboretum at Placerville. Both Millett (1944) and Fielding (1957) have reported the times of pollen dispersal in Australia of cultivated *P. radiata* of unstated origin. Fielding (1961 *a*) reported the dates at which pollen shed commenced in the *P. muricata* and *P. radiata* plots at Reid's Pinch.

METHOD

In the present study observations were made on times of pollen dispersal and flowering of provenances of all three species to determine the efficiency of phenological differences in isolating the species under conditions of cultivation; the value of differences in areas of natural occurrence may be inferred from the results obtained.

As climatic factors have a considerable influence upon times of pollen dispersal and flowering (Duffield 1953; Sarvas 1962 p.34) an attempt was made to measure some of these factors during the relevant period in 1960. Small meteorological stations were established at two of the three localities - Reid's Pinch and Hall's Block. These were equipped with maximum and minimum thermometers, weekly recording hygrothermographs and anemometers. At intervals of about one week throughout the period when observations were made humidity readings were taken at each meteorological station by means of an Assman hygrometer. These and the thermometric readings were taken to enable the values read from the record charts to be corrected. The thermometers were compared with each other and with a spare third set before readings commenced to ensure that the readings obtained were as comparable as possible. The thermometers and hygrothermographs were housed in standard Stevenson screens the locations of which are shown in Figures 6 and 9. The anemometers were placed on poles 25 ft and 18 ft above ground level at Reid's Pinch and Hall's Block respectively. Because of the quite different heights of the nearby trees at each locality, the absolute wind mileages recorded have little significance.

Observations on pollen dispersal and flowering were made usually at intervals of one week from late August 1960 until early December 1960. These were supplemented by further work in 1961 and 1963.

The presence of shedding pollen was detected (where possible) by shaking the trees or branches and watching for pollen dispersal or occasionally by observation alone from the ground. The abundance of pollen was recorded in the following subjective terms:

Absent

Rare - small amounts seen shedding on some trees

Common - some catkins on most trees

Abundant - numerous catkins shedding on all or most trees.

Quantitative estimates of the amount of pollen or other particles present in the atmosphere may be made with various types of equipment (see for example Durham 1946; Hirst 1952; Sarvas 1952, 1955, 1962; Speck 1953). Because of the relatively small size of most of the plots available for this study, the great influence of weather upon dispersal (Sarvas 1962), and the errors to which the simpler types of spore traps are subject (Gregory 1951, 1952) no effort was made to use such a technique on a large scale. However, in 1963 spore traps (Rack 1957) were set up in plots of each of the main species at Reid's Pinch. The trap in the P. radiata plot is illustrated in Figure 11. The receptive surface was a 3 ins x 1 in microscope slide coated with a high melting-point petroleum grease. The slides were changed at intervals of about seven days; sometimes the period was longer. (Sarvas (1962) found that spheres coated with petrolatum and exposed to the weather for one week then caught 95% of the pollen caught by freshly-coated ones). The mouth of the trap was about 10 ft above the ground; the trees in the plots were 25 ft to 40 ft tall at the time.

The stage of development of megasporangiate stroboli or flowers was usually observed from the ground either with the naked eye or with the aid of 10 x 50 binoculars, or more rarely by climbing the trees. The heights of the trees were in general less than 35 ft. The abundance of open flowers was recorded in the following terms:

Absent

Rare - a few flowers opening or open on some trees

Common - some open flowers on most trees, or most flowers open on some trees

Abundant - most flowers open on most trees.

The flowers were considered to be "open" when axial growth of the strobilus had caused the strobilus to emerge from the enveloping scales. This stage was observed to coincide with the megasporophylls opening sufficiently to permit pollen to reach the micropyle. Direct observation of this opening was possible only on some occasions because of the distance between the point of observation and the strobilus. Where possible the closing of the flowers was also noted.

RESULTS

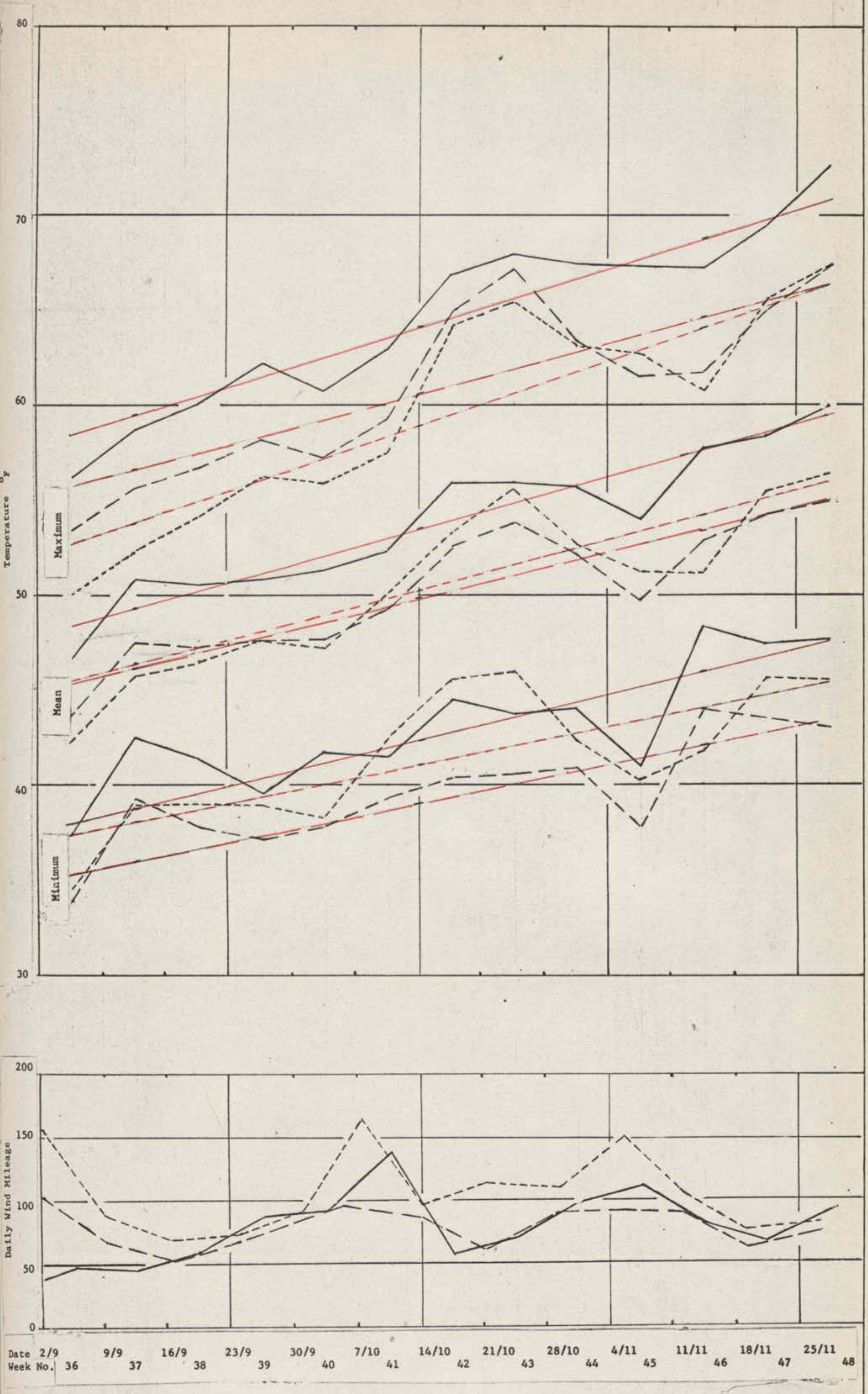
A summary of the corrected temperature records and anemometer readings for 1960 is shown in Figure 12. The weekly period for which each point was computed commenced at 9 a.m. on Fridays, the Forestry and Timber Bureau "weather week" for 1960. Corresponding data for Canberra (Forestry and Timber Bureau, Yarralumla) is also included in this figure.

The average figures (°F) for the 14 weeks from August 27 - December 2 (No. 35-48 inclusive) were:-

Locality	Daily minima	Daily maxima	Mean daily
Canberra	42.4	64.2	53.3
Hall's Block	39.1	60.6	49.9
Reid's Pinch	41.1	59.2	50.2

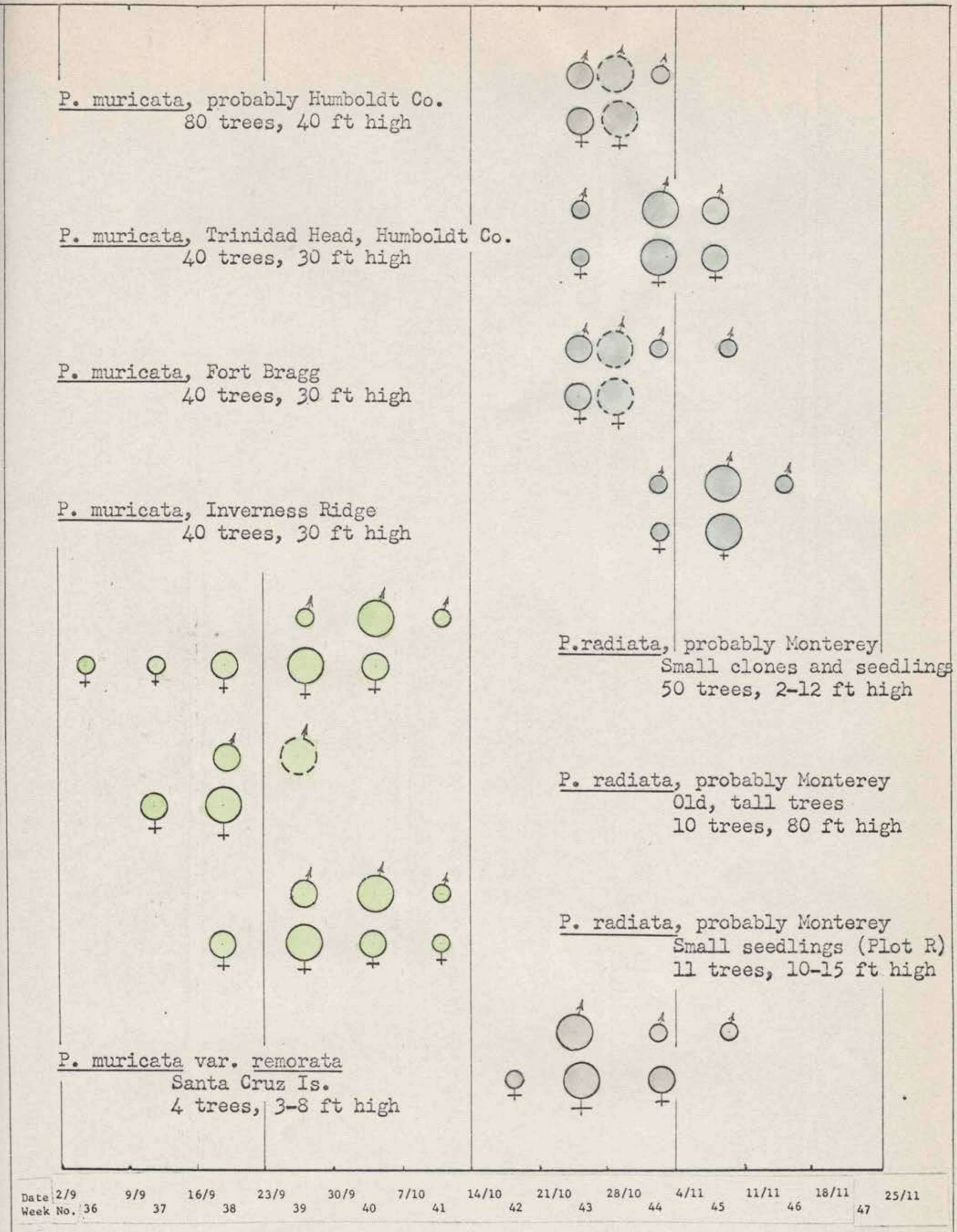
FIG. 11 SPORE TRAP, 1963

This trap was set up in Plot 30, P. radiata ex Cambria, at Reid's Pinch Arboretum.

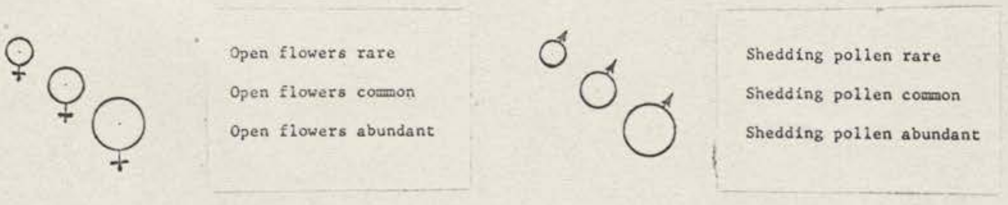


The lines in red are regression lines

FIG.12 TEMPERATURE AND WIND RECORDS, 1960



In this graph and following similar ones the plantings are arranged in order of natural occurance from north to south.



Actual observations are shown with solid lines
Interpolated points are shown with broken lines

FIG.13 TIMES OF POLLEN RELEASE AND FLOWERING
BLUNDEL ARBORETUM, 1960

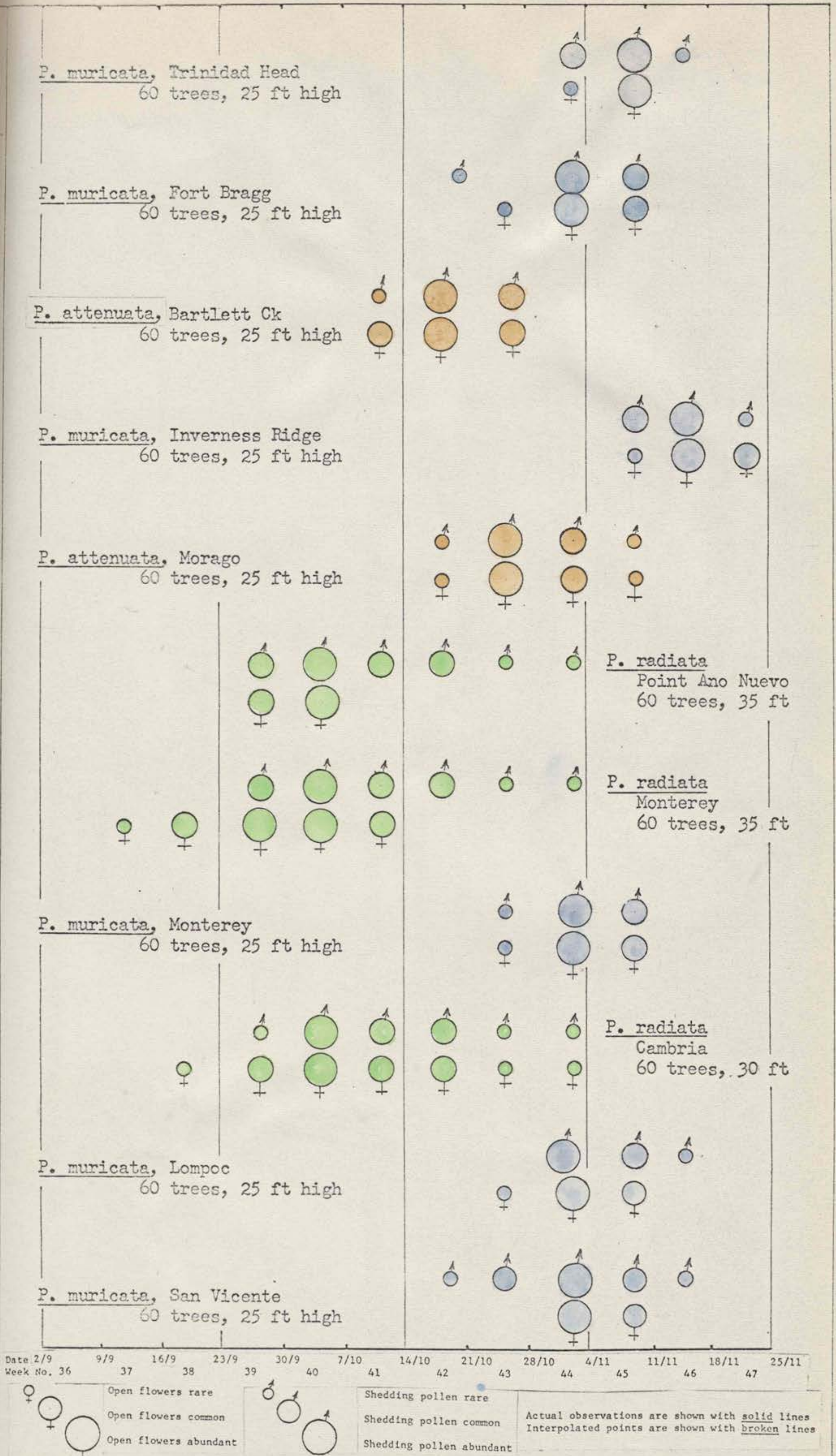


FIG.14 TIMES OF POLLEN RELEASE AND FLOWERING

REID'S PINCH ARBORETUM, 1960

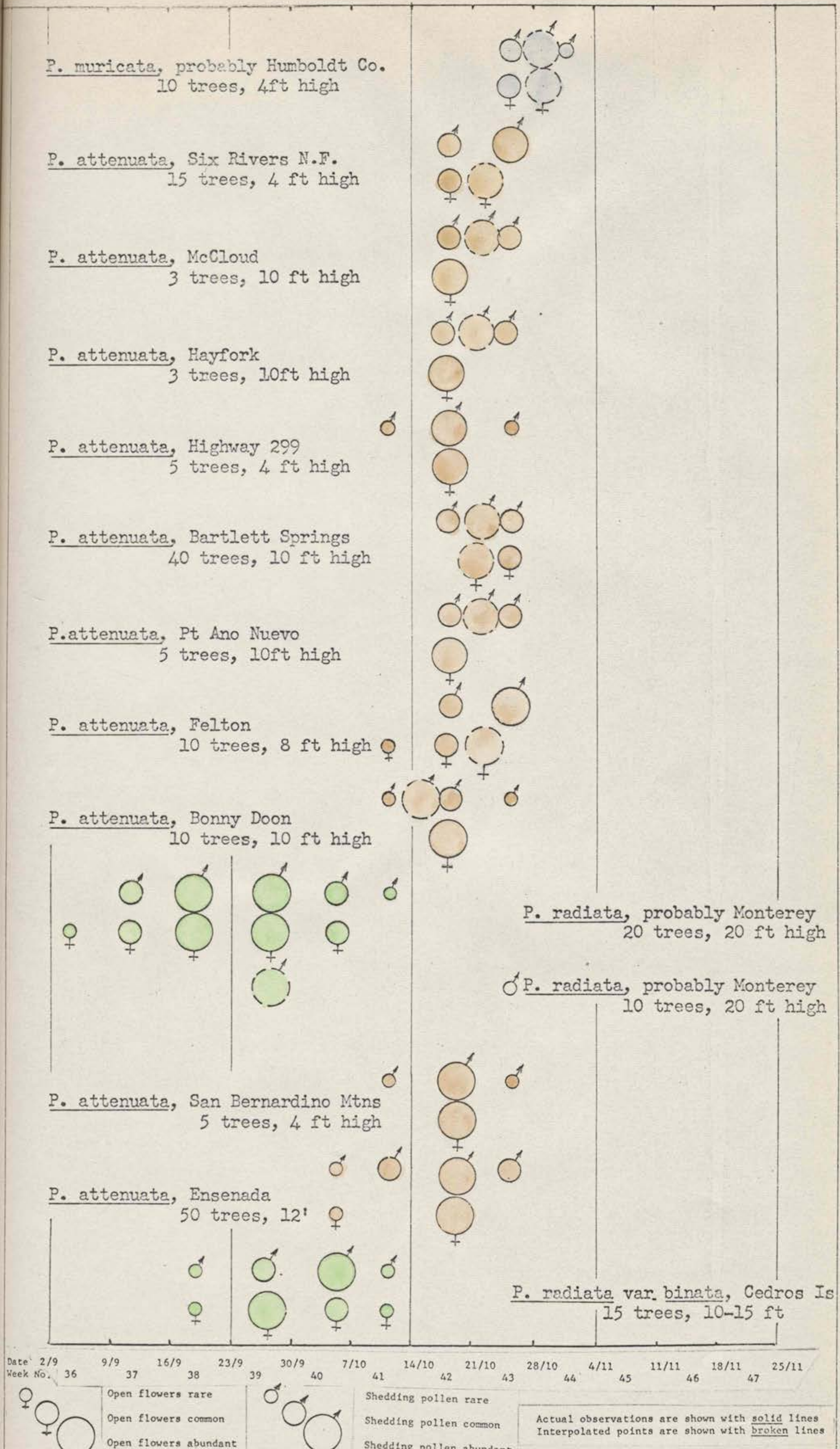


FIG.15 TIMES OF POLLEN RELEASE AND FLOWERING

HALL'S BLOCK ARBORETUM, 1960

Linear regressions of the maxima, minima and mean daily temperatures on time for this period were calculated for each site and are shown in Figure 12.

Although humidity has an important effect upon pollen dispersal (Fielding 1957; Sarvas 1962 p.56-7) the humidity data obtained are not presented. Because of slight errors in the clocks of the thermohygrographs, humidities at 9 a.m. and 3 p.m. could not be read with any accuracy; the only value which could be determined with certainty would be minimum daily relative humidity. As this is inversely correlated with temperature, the differences in the minimum humidity between the sites should be a reflection of temperature as discussed above.

The results of observations in 1960 on flowering and pollen dispersal at each planting site are depicted in Figures 13, 14 and 15. The number and approximate height of the trees in each sample is also recorded in these figures. Other minor results are given at appropriate points in the following discussion.

DISCUSSION

Pollen Dispersal. - A number of factors influence or extend the period during which pollen is dispersed. The growth process which permits pollen shed commences normally at the base of the catkin and extends acropetally; the catkins at the top of a shoot or tree usually mature first and maturation then extends basipetally to other catkins; the catkins on branches exposed to insolation mature before those in the shade (Fielding 1957): large trees growing in stands therefore have a much longer period of dispersal than do small scattered trees. Genotypic influence may also be important: in a group of 18 clones of P. radiata (apparently of a single provenance) Fielding (1960) reported that the date of the first observed pollen shed in each clone ranged from the 4th to the 25th of September. The time of pollen dispersal may also differ between different races when grown on a uniform site (see below).

The influence of temperature upon pollen-ripening and dispersal has considerable importance in the present study. Comparisons between the times of pollen maturation of trees growing in the three different sites cannot be made directly because of the differing environments prevailing at each locality. For example, P. radiata pollen was first observed shedding at Hall's Block on 12/9/1960, but none was observed until 27/9/1960 at Reid's Pinch, where the trees are growing on a cooler aspect at a higher elevation.

Temperature is probably the most important environmental factor varying appreciably between such sites. It influences pollen dispersal directly through the control which it exerts on the growth of the axis of the catkin (strobilus), and indirectly via humidity which at low levels causes the sporangia walls to rupture (Fielding 1957; Sarvas 1962 p.36). Fielding (1955) noted that pollen-bearing shoots showed a different seasonal growth pattern to vegetative shoots; it is not clear what aspect of the temperature regime is most important in pollen maturation. MacDougal (1938) found that active growth of the vascular cambium of P. radiata commenced when the mean cambial temperature rose to about 8°C (46°F).

The author has frequently observed that pollen matures appreciably earlier high in the crowns of tall trees than in smaller trees growing nearby; this is shown by the data on P. radiata from Blundel (Figure 13), where mature pollen was judged to be common in the crowns of tall trees in week 37, but rare or absent in smaller trees of the same species at this time. Temperature inversions are of frequent occurrence at night in this region. (Pryor (1954) gives the mean annual number of days of frost as 103 at Canberra and 140 at Bulls Head, a station at 4324 ft A.S.L. about 6 miles south of the planting sites). No records are known of air temperature gradients reaching to 100 ft above ground level in the Canberra area, but observations in other localities (Hough 1945; Daubenmire 1962 p.170) suggest that minimum temperatures may rise for hundreds of feet above ground level until the normal vertical temperature stratification is encountered; during the warmest part of the day the highest temperatures are encountered at ground level, although the temperature gradient then is not nearly as steep as at the coldest time of night. The delayed pollen maturation near ground level may be influenced by low night temperatures at this level. Growth (for example stem elongation) is in some plants mainly a night process (for example in Pinus sabiniana - Hellmers 1962 p.280); growth of the axis of the strobilus may thus be closely controlled by night temperature alone. Alternatively, the low night temperatures may exert their influence simply by depressing the overall temperature of the environment, thus having an effect even if axial growth is not confined to the hours of darkness.

A study of the temperature records from the Hall's Block and Reid's Pinch sites does little to clarify the position. It was previously noted that pollen dispersal in P. radiata was first observed 15 days later at the latter, higher (by 950 ft) site than at the lower one, although comparable provenances of both P. attenuata and P. muricata seem to show lesser differences, of perhaps 6 or 8 days. The variation between P. radiata and the other two species may be due to experimental error in the subjective observations and/or to the diminishing difference between the daily maximum temperatures at the sites during the period of observation (Figure 12).

Duffield (1953) found a mean difference of 8 days per 1000 ft elevation in Pinus ponderosa, and noted that Hopkins (1918) found a rate of change of 4 days for each 400 ft difference in elevation. Silen (1960) reported a delay of 13 days per 1000 ft increase in elevation for naturally-occurring Douglas fir; this decreased to four or five days when material from a single provenance was grown at different elevations.

The mean daily minimum for weeks 35-48 inclusive at Hall's Block (the lower site at which earlier flowering occurs) is two degrees less than at Reid's Pinch - that is, this evidence discounts the significance of minimum temperatures in influencing the process of pollen maturation. Some further support is also given to this by the relatively small delay (about a week, Figure 13) observed between the times of pollen dispersal in tall trees and small trees at Blundel Arboretum, a frosty site, relative to the difference in minimum temperatures one would expect between 10 ft and 100 ft above ground level (Geiger 1950, pp. 78 and 207) and the average rate of change of temperature per week (about 1.0°F per week, Figure 12).

Millett (1944) studied the relationship between temperatures and pollen dispersal of Pinus radiata. He found that the mean maximum air temperatures at Canberra and Mt Burr, S.A., for the fortnight prior to the first recorded pollen dispersal averaged 57.5°F and 54.7°F respectively for a five-year period, although varying by a few degrees from year to year. He also noted that because of the mild and rather cloudy spring weather at Mt Burr, after a milder winter than at Canberra, pollen shed at Mt Burr occupied a period more than twice as long as at Canberra: as pollen dispersal occurs earlier at Mt Burr than at Canberra (commencing on July 24 cf. September 8) the rate of change of mean daily air temperatures at Canberra during pollen dispersal is much greater than during pollen dispersal at Mt Burr.

It is apparent that while temperature does influence the time of pollen maturation and dispersal in pines, the exact nature of the control cannot be deduced from the evidence obtained in this study: heat sums, or some other more comprehensive method measuring the temperature regime of a site, may give a more satisfactory correlation with the time of onset of pollen dispersal than do simple daily maxima and minima or their means.

The following factors therefore influence the period during which pollen dispersal is observed in a species: tree size, the number of individuals in the sample observed, the degree of genetic diversity in the stand, the nature of terrain upon which the trees are growing, stand density and the climatic pattern prior to and during the period of pollen shed.

As the observations in this study were made on relatively small trees (usually below 35 ft in height) on fairly uniform sites, and on limited numbers of individuals, one would expect that the observed periods of pollen dispersal would be rather shorter than might be expected in extensive stands of large trees growing in an area of diverse topography.

Receptivity of Female Strobili. - The period during which the female strobili (flowers) are open in a species is affected by most of the factors which influence the period of pollen dispersal. On almost all P. radiata trees which have been closely observed in the A.C.T. the earliest flowers emerge from their enveloping scales before the tree begins to shed its pollen (Fielding 1960). Flowers may continue to emerge from higher "whorls" of buds for up to two months or more (Fielding 1953, p.36; 1960, p. 48). (In comparison, flowering in a stand of P. sylvestris occurs within 4 or 5 days - Sarvas 1962, p. 56).

The flowers of P. radiata are receptive soon after they begin to emerge from their enveloping scales (Pawsey 1961; Brown, unpublished data). At Canberra some flowers have produced seed when pollinated 17 days after emerging (Brown, unpublished data); Pawsey (1961) found that few flowers developed and that seed yields were low if pollination took place more than 14 days after the flower was open under the climatic conditions at Mt Burr. When pollination is effected the pollen is transported through the micropylar canal and onto the receptive surface of the nucellus by the micropylar fluid. Doyle and O'Leary (1935) and McWilliam (1958) considered that if pollen reached the nucellus the micropylar fluid would not reappear to transport any pollen which may later arrive at the arms or neck of the micropyle. However, Sarvas (1962, p.62 and 81) presents data which suggests that this is not invariably the case. McWilliam (1959) claimed that the closing of the micropyle is hastened by pollination. There seems little doubt that pollen first reaching a receptive flower is more likely to effect pollination than is pollen reaching the same flower later. This is supported by the observation of Sarvas (1962, p. 64) that the contraction of the micropylar canal, which occurs as the ovulate scales close, cuts off outside the pollen chamber of the ovule a great part of the pollen in the micropylar canal.

It thus appears that the greatest opportunity for cross-pollination between two species would exist when late pollen from an early-flowering species is able to reach the first emerging flowers of the later species, rather than the reverse.

Clinal Variation in Times of Flowering and Pollen Dispersal. -

Clinal variation in characteristics of forest trees is well known for example in *Pinus muricata* (Duffield 1951), *P. contorta* (Critchfield 1957) and *Eucalyptus* (Barber 1955; Pryor 1957). The reality of an apparent cline in the flowering time of cultivated material may be difficult to assess. Material collected from a number of points of differing latitude or altitude may flower simultaneously in the natural environment but show an apparent cline under cultivation due to altered photoperiodic or thermal conditions, or both; the converse may also be true.

Small but distinct differences were observed between provenances in all three species in the present study. The time of commencement of pollen shed is used as the criterion of "flowering time" in the following discussion; other details are given in Figures 13, 14 and 15. -

(1) *Pinus attenuata, Hall's Block*. - The earliest provenance was that from Ensenada (October 5), followed by Bonny Doon (October 11). All remaining eight provenances were shedding pollen by October 18. The earliest provenance was by far the southernmost of those examined. However, there was no clear pattern, either altitudinal or latitudinal, among the remainder, differences between which were relatively small.

(2) *Pinus muricata, Reid's Pinch and Blundel*. - There were distinct differences between some of the provenances in this species, but again no clear pattern was evident. At Reid's Pinch, the San Vicente provenance was shedding pollen on October 19, Fort Bragg on October 20, Monterey on October 25, Lompoc and Trinidad Head on November 2, and Inverness on November 9. Fielding (1961*a*) reported a similar sequence in 1961. The Fort Bragg, Trinidad Head and Inverness provenances at Blundel showed a similar pattern to those at Reid's Pinch. A few trees of var. *remorata* growing at Blundel appeared to be slightly earlier than the nearby Fort Bragg provenance which at Reid's Pinch was only slightly behind the earliest provenance in this species.

(3) *Pinus radiata*. - The differences among the three mainland provenances of this species at Reid's Pinch were not great. Pollen was common on both the Monterey and Pt Ano Nuevo provenances on September 27, but a lesser amount was present on the Cambria trees on this date. Taking into account the time of emergence of the flowers as well as information on pollen dispersal, it was concluded at the time when the observations were made that the order of flowering was Monterey, Pt Ano Nuevo and lastly Cambria at intervals of three or four days. Fielding (1961*a*) noted that the Monterey and Pt Ano Nuevo plots were both shedding on September 23, 1960, but that in 1961 the order was Pt Ano Nuevo, Monterey and Cambria on September 11, 13 and 15 respectively, reversing the order suggested above by the author for the two northern localities.

The Cedros Is. pine at Hall's Block has a flowering period not very different from *P. radiata*. Pollen of the latter species was first seen shedding on September 12, the first of Cedros Is. pine on September 19, and the first of *P. muricata* on October 25. This evidence supports Fielding's (1961*b*) suggestion that this variety is more properly placed under P. radiata than P. muricata.

Overlap between Species in Times of Flowering and Pollen Dispersal.-

(1) P. attenuata-P. radiata. - At Hall's Block the first open flowers on P. attenuata were seen on the San Vicente provenance on October 5; they were common by October 11. A few flowers were also out in the Six Rivers and Felton plots by this date. Open flowers were abundant on October 18 in the provenance from Pt Ano Nuevo where this species mixes with P. radiata. Pollen was common on P. radiata at Hall's Block on October 5, and small amounts were observed on shaded branches as late as October 25. Pollen on Cedros Is. pine was abundant on October 5.

An overlap of a few days was also observed at Reid's Pinch between P. radiata and P. attenuata from Bartlett Ck and Marago. To verify that the P. attenuata flowers were receptive when P. radiata pollen was available, flowers on six twigs of the Bartlett Ck provenance were pollinated and isolated to prevent the access of further pollen. The yields obtained are shown in Table 1.

TABLE 1

SEED YIELDS FROM P. ATTENUATA FLOWERS POLLINATED WHEN P. RADIATA POLLEN WAS SHEDDING

Pollen parent	No. flowers pollinated	No. cones collected	Seed yield per cone	Full seed per cone	Germination full seed
P.attenuata	14(plus 2 not open)	9	90	69	80%
P. radiata	6(plus 3 not open)	6	99	*	*

* There appears to be no reason why the cross with P. radiata should not have yielded viable hybrids. Most of the seed were full, but germination was not tested.

(2) P. muricata-P. radiata. - At Reid's Pinch the first open flowers on P. muricata were observed on October 25 in provenances from Fort Bragg, Monterey and Lompoc. This was also the last date on which pollen was observed on P. radiata.

In 1961 evidence of slight overlap was again obtained: on October 13, a few P. radiata catkins were found which had about one-third of the total pollen left. A germination test of the pollen showed that about 50% of the grains were viable; pollen tube growth seemed normal. On the same date, dispersing pollen was fairly common in the San Vicente provenance of P. muricata, and just starting in the Monterey group. Female flowers were just out in the Lompoc provenance. No open flowers or dispersing pollen was seen elsewhere, although Fielding (1961a) did note some pollen on the Trinidad Head trees on October 12. Three open flowers were collected from the Lompoc provenance on October 13, pollinated, kept under humid conditions for 12 hours, pollinated again, subjected to low humidity for a few hours, and fixed in formalin, acetic acid and alcohol (F.A.A.). A photograph of a section is shown in Figure 16: it is evident that these flowers were sufficiently advanced to receive pollen

FIG. 16 OVULES OF EARLY FLOWERS OF P. MURICATA

A few open flowers were found on 13.10.1961 on P. muricata from Lompoc growing at Reid's Pinch; small quantities of pollen were present on nearby P. radiata on the same day. To demonstrate the possibility of cross-pollination, a flower of P. muricata was collected, pollinated and sectioned. In the above photograph a pollen grain (arrowed) is shown in the micropylar canal.

of P. radiata on this day.

On October 22, 1963, some pollen was found on the P. radiata from Monterey, and open flowers were common on the Lompoc provenance of P. muricata.

(3) P. attenuata-P. muricata. - At Reid's Pinch the last pollen was observed on P. attenuata from Morago on November 9, 1960. By this date open flowers were present in all P. muricata provenances; in some plots they were abundant. In 1963 on October 22, pollen and open flowers were abundant on the P. attenuata from Bartlett Ck, while on P. muricata some pollen was shedding from the San Vicente trees and open flowers were common in the Lompoc trees.

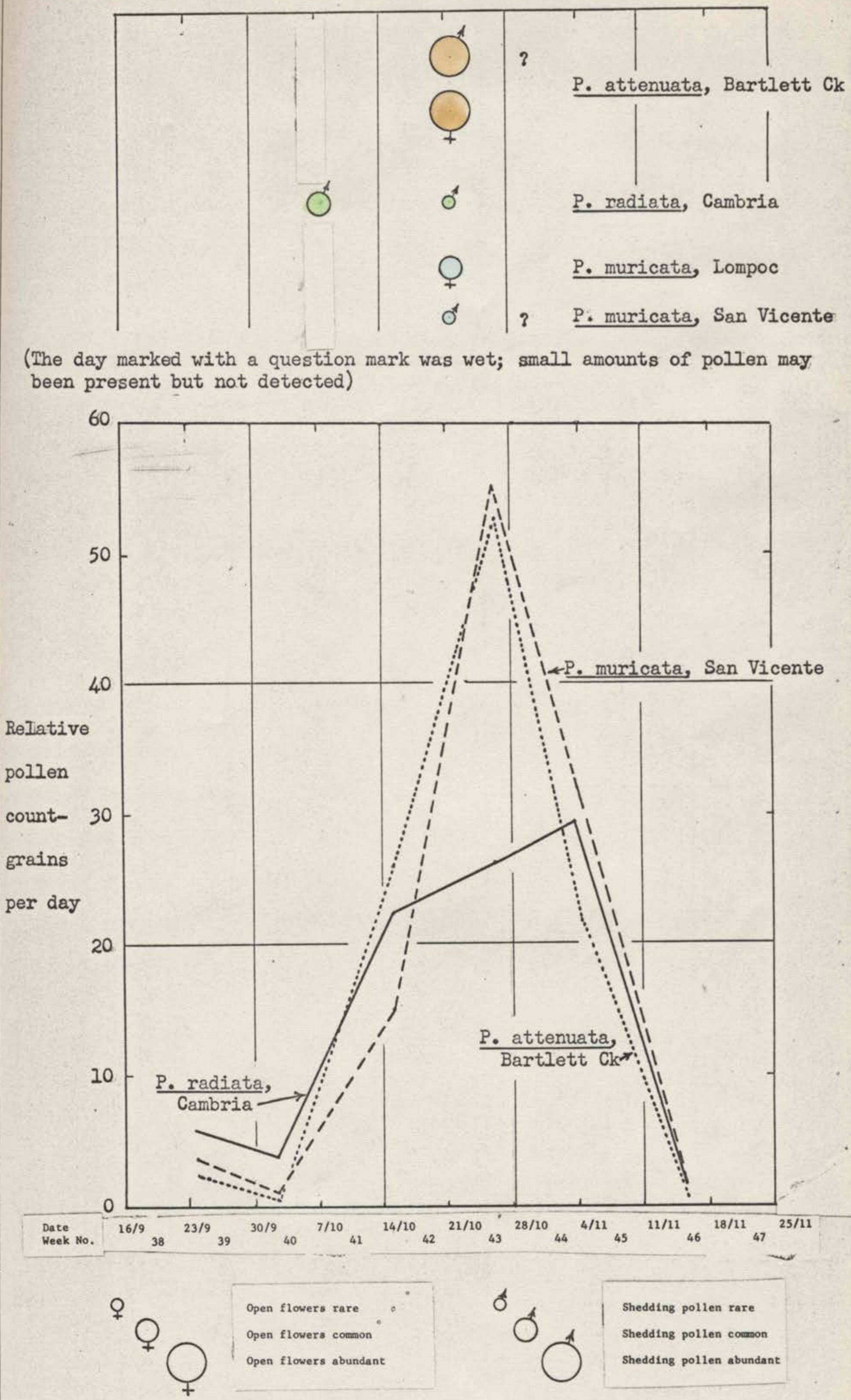
The results of the 1963 work with spore traps are shown in Figure 17. The trap in the P. radiata plot caught a relatively large amount of pollen during the period September 20 to October 22, but apart from this there is no appreciable difference between the three traps. A much more sensitive sampling design, perhaps in larger plantings, would be required to show differences of a few days in times of pollen dispersal. Fielding (1957), Sarvas (1962, pp. 35, 53) and many others have pointed out the great influence of weather upon pollen dispersal from day to day; observations in a single year are greatly influenced by the prevailing weather pattern which may be quite variable - see the temperatures and wind records in Figure 12.

CONCLUSIONS

Evidence from this study is that under cultivation overlap in the times of pollen dispersal and flower opening does occur between all the members of the Californian closed-cone pine group, although the frequency with which P. radiata pollen would be expected to pollinate flowers of P. muricata is not high. One would expect the overlaps observed to be minimal rather than maximal: each site studied was relatively uniform, sample size was limited and the trees were fairly small.

In the field the likelihood of interspecific pollination occurring would depend upon the disposition and number of the trees of each species present. If the trees of the later-flowering species were relatively few, tall and scattered on a warm site such as a north-facing slope, and the earlier species was plentiful and growing on a sheltered, south-facing slope at higher elevation there would be maximum overlap in flowering times, although the inefficiency of the pollen-dispersal mechanism in Pinus (for example Wright 1952; Pawsey 1961) would reduce the amount of late pollen from the later-flowering species reaching the earliest flowers of the earlier-flowering species. In the areas of natural contact between P. radiata and the other two species, P. radiata does occupy the more sheltered and cooler, although lower, sites. In view of the small gene flows which Dobzhansky (1947) postulates are sufficient to destroy the identity of (incipient) species, it is difficult to believe that seasonal isolation will be the principal or in the case of P. radiata and P. attenuata even an important factor in maintaining indefinitely the identity of the species under cultivation. A similar conclusion could be drawn concerning the areas of contact between the species in their natural occurrence.

The pattern of clinal variation in flowering time produces no evidence that there has been any strong selection to increase the differences in flowering time, thereby strengthening seasonal isolation, at the points of natural contact of the species.



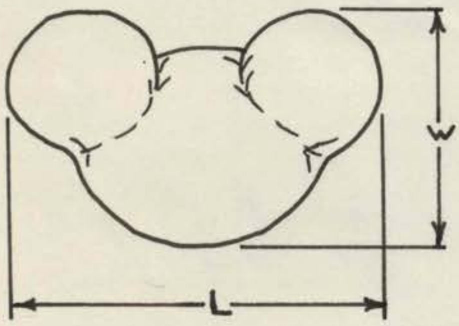
(The day marked with a question mark was wet; small amounts of pollen may been present but not detected)

FIG.17 TIMES OF POLLEN RELEASE AND POLLEN COUNTS FROM SPORE TRAPS
REID'S PINCH, 1963

VIII. INVESTIGATIONS OF MECHANICAL ISOLATION

Mechanical isolation by differences between species in the size of pollen grains or the micropylar canals could be detected by measurement of either of these structures. In this study pollen was chosen because of the relative ease with which an adequate sample can be examined.

Twenty measurements were made of length (L), width (W) and thickness (T) in grains from each pollen sample. The normal shape of a pollen grain and the dimensions which were measured are shown in the diagram below. The results obtained are shown in Table 2.



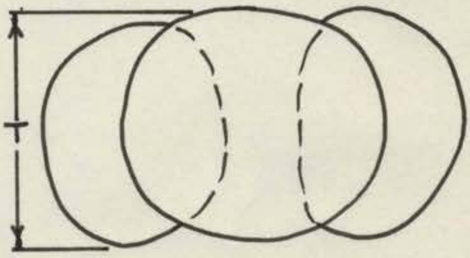


TABLE 2

DIMENSIONS OF POLLEN GRAINS (MICRONS)

	L		W	T
Species	Mean	S.D.	Mean	Mean
(1) *P. attenuata*	81.5	± 6.2	53.5	55.0
(2) *P. muricata* var. *borealis*	74.5	± 7.3	51.5	55.5
(3) *P. muricata* var. *remorata*	78.5	±11.6	52.5	56.0
(4) *P. radiata*	79.5	± 5.1	52.0	53.0
(5) Cedros Is. pine	73.5	± 5.5	53.0	52.5
Differences	**		NS	**

Although some differences in grain length and thickness are significant, appreciable overlap occurs between all the samples examined. It seems most unlikely that differences in pollen grain size would be an effective isolating mechanism.

The origins of the above samples were -

(1) Several trees in Plot 3, Hall's Block (Ensenada)

(2) Several trees in Plot 31, Hall's Block

(3) Mt. Burr Arboretum (supplied by Mr C.K. Pawsey)

(4) Five trees in the A.C.T. commercial plantings - probably Monterey provenance

(5) Six trees in Plot 16, Hall's Block

IX. INVESTIGATIONS OF THE FAILURE OF FERTILISATION (GAMETIC ISOLATION) AND EMBRYONIC LOSS

METHOD

The efficiency of isolating mechanisms at this stage of the life cycle was investigated by making both interspecific and intraspecific pollinations; the progress of fertilisation and seed development was followed on a non-quantitative basis by making periodic collections of ovules for microscopic examination and quantitatively by assessing cone yield, seed yield and seed germination.

The work was planned to provide information on as many of the possible interspecific combinations as possible. Controls were provided in the form of intraspecific pollinations, unpollinated strobili, and naturally-pollinated ("open-pollinated") strobili. Replication was in some cases limited by the small numbers of suitable strobilus-bearing twigs available; when material was plentiful twenty or more twigs were used for each cross. Where replication was possible on any scale a randomised-block type of layout was employed: each block contained all the pollen treatments being applied to the flowers of the species being used as the female parent. No deliberate attempt was made to test all possible provenances or varieties of a species, but in many cases several provenances were involved in each interspecific cross and the associated controls. The pollens used were mixtures prepared from collections of five trees within a provenance.

A separate test was made of the interfertility of trees in provenances of *P. muricata*. The design was an incomplete diallele cross using varying numbers of trees in four of the six provenances at Reid's Pinch.

Pollinations were carried out using techniques similar to those of Mergen *et al* (1955). The bags used to isolate the strobili were coated with aluminium paint on one side which was oriented to the north to minimise heating by insolation within the bag. In some cases the buds were washed with distilled water before isolation to minimise contamination by pollen adhering to the scales enclosing the unopened strobili. Pollen was usually applied twice (at an interval of about seven days) to each bag.

Pollen germination was checked as soon after collection as possible, and sometimes subsequently, if use was prolonged, in a hanging drop of distilled water. Chambers were made by cementing glass rings to slides and sealing with a cover-glass (McWilliam 1960). It was sometimes necessary to use pollen for receptive strobili before the results of these tests were available; if the results showed that the germination percentage of a pollen sample was not high the lot in question was replaced. An attempt was made to ensure that the pollens used had a germination of at least 70%.

The development of strobili in these crosses was checked by inspection during January and February, 1961, a few months after the isolating bags were removed, and again in February 1962. Mature cones were collected in December 1962.

In the spring and summer of 1960/1961 periodic measurements were made of year-old strobili to obtain accurate information on the pattern of growth, and thus the time of fertilisation which takes place when cones attain maximum size (McWilliam and Mergen 1958).

During the period between pollination and maturation strobili were collected at increasing intervals from the more important crosses so that the progress of the ovules could be studied in the laboratory.

The ovules were fixed in F.A.A. as soon as they were cut from the shoot. After some time the surplus tissue was cut away and the development of the ovules was assessed with the aid of a low-power dissecting microscope, or, in later collections when the material was larger, by eye.

Following fixation they were transferred to 70% alcohol and placed under refrigeration until dehydrated with tertiary butyl alcohol (Johansen 1940, p. 130) and embedded in paraffin. Sections were cut (usually at 8μ), stained with safranin and fast green or with chlorazol black E (in an attempt to provide material best suited to photomicrography) and mounted in balsam. Not all of the collected material was sectioned: emphasis was placed upon material from crosses which were considered less likely to succeed (for example P. muricata x radiata; P. muricata x attenuata) together with suitable controls. The ovules sectioned were selected from the central portion of the strobilus to avoid any possibility of including rudimentary ovules from scales in the apical or basal portions (Sarvas 1962, p. 104). The number of ovules sectioned from each strobilus varied; the average was about six. Further details of the material examined are given later in Table 15.

Following collection, the cones were stored in calico bags at room temperature until seed extraction was carried out in the early part of 1963. Cones were opened by placing them on wire trays in an oven fitted with a circulating fan and heated to 130-140° F. Seeds and wings were shaken from the opened cones and the wings broken from the seeds.

The seed was cleaned in a small "Clipper" hand-operated winnowing machine. The efficiency of this machine was checked by treating batches of open-pollinated seed of each species; seed collected in the various fractions was then subjected to a cutting test. The results obtained are shown in Table 3.

TABLE 3

SEED RECOVERED FROM WINNOWING MACHINE

Species	Initial No. of seed	Fraction					Total No. of seed recovered
		1		2	3	4	
		Full	Empty	Empty	Empty	Empty	
P.attenuata	245	168	16	45	15	-	244
P.muricata	300	284	-	8	8	-	300
P.radiata	287	278	8	1	-	-	287

Only one seed was lost in the air-blast. Fractions 2, 3 and 4 contained only empty seed; fraction 1 contained both full and empty seed. This fraction was regarded as "clean" seed for subsequent work.

The clean seed obtained from each twig was counted.

Samples of the seed obtained were subjected to germination tests. Five tests were carried out; as the method of sampling and other details varied in each case they will be described individually.

Germination Test No. 1. - Female parent *P. radiata*

When available, seed from 10 twigs from each cross were used. Within each twig, seeds were selected on two bases -

(1) Two random samples, each of 20 seeds, were drawn from each seed lot to enable an estimate of clean seed viability to be made; these samples were weighed.

(2) The remaining seeds were sorted into weight classes with the aid of a glass-fibre balance (Duffield 1960). It was hoped to obtain 40 seeds from each seed lot in a 2 mgm weight-class common to all seed lots, thus eliminating the influence of seed size upon the early growth of the resulting seedlings. It was soon evident that the weight-class distributions of the various seed lots did not always overlap; this is shown in the distributions for random samples from three twigs in the upper part of Figure 18. The weight sorting was therefore used to obtain a sample of full seed which was as homogeneous as possible; mean seed weight was determined subsequently by weighing the selected fraction. The weight-sorting did eliminate empty seed from the samples and it reduced the variation in weight of the selected full seed (Figure 18). The extent of the reduction in variation achieved depended upon the number of seeds available for sorting: when few seeds were present, it was necessary to include seeds from several weight-classes in each sample to obtain the required 20 seeds.

All seed samples were placed on filter paper over vermiculite in petri dishes (all previously sterilised) in an incubator at 21-22°C on 20/9/1963. The dish contents were kept moist with distilled water.

The germination test was discontinued on 9/12/1963.

Germination Test No. 2. - Female parents principally *P. attenuata* and *P. muricata*

Seeds from ten twigs from each treatment (male x female species) were chosen at random for testing when sufficient seed lots were available. All seed lots with 20 or more seeds were considered eligible for selection. If fewer than ten suitable seed lots were available in a given cross a second sample was drawn from sufficient of the available seed lots to give a total of ten samples. In some cases there was even insufficient seed for this: where possible some samples of 20 seeds were used, or occasionally even smaller samples.

The "full" seed were separated from the "empty" ones in each sample with the glass fibre balance, placing the dividing line at about 10 mgms. In most cases clear separation was obtained (see the bimodal nature of the weight-class distribution in the upper three histograms of Figure 18). In a few instances, apparently when the embryo was poorly developed or recently dead, marginal cases occurred: doubtful seed were placed in the "full" category. The mean seed weight in each fraction of each sample was determined.

Further samples, up to 12 in number, were taken from seed in crosses which yielded few "full" seeds in the first sampling; these were sorted as before.

Stratification of the "full" seed from most samples commenced on 8/7/64; treatment of a few lots commenced on 17/7/1964. The seeds were moistened with water and placed between moist plugs of cotton wool in test tubes at 3.5°C.

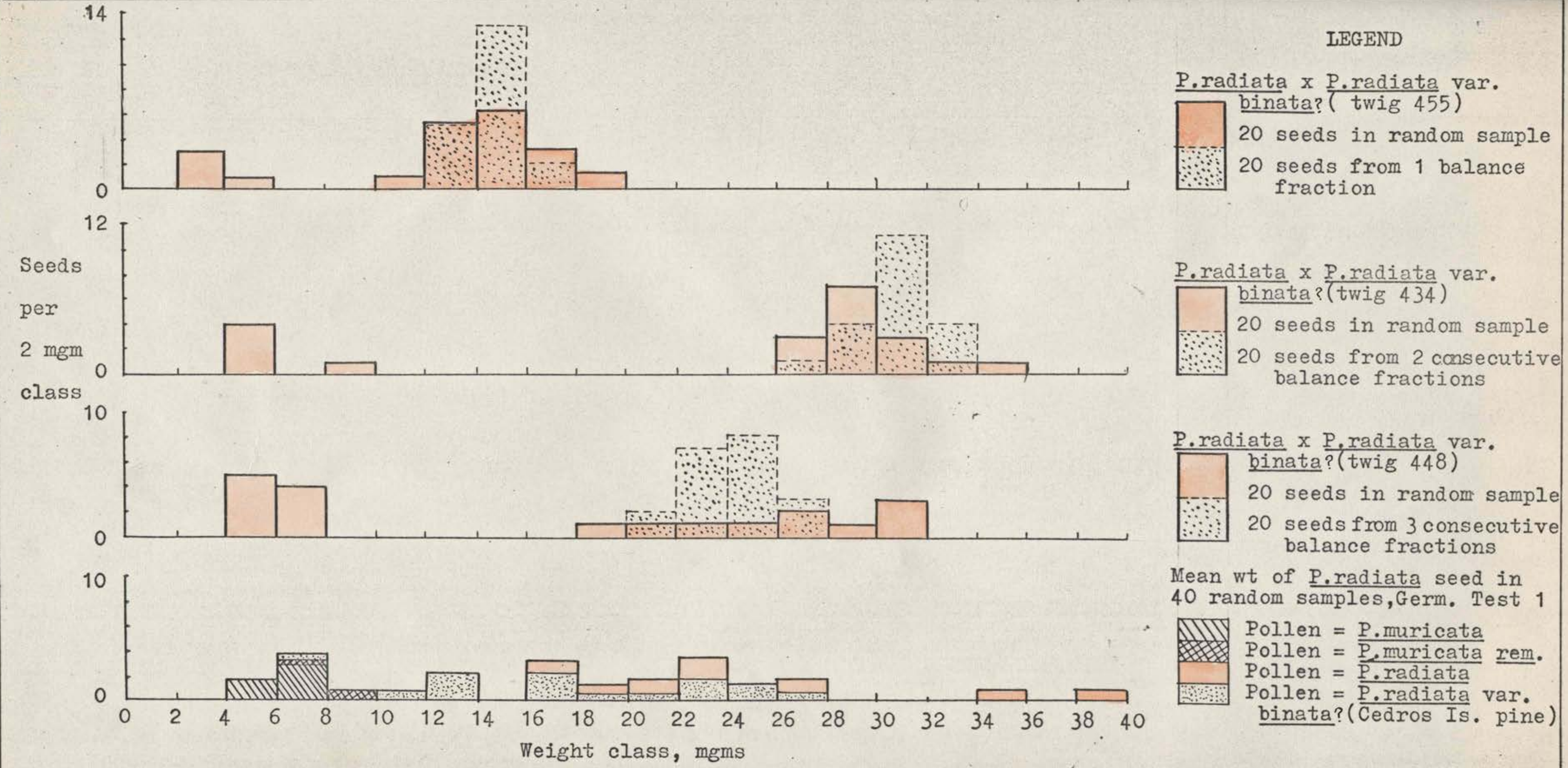


FIG. 18 THE FREQUENCY DISTRIBUTION OF SEEDS IN WEIGHT CLASSES

The germination test commenced at 21°C on 13/8/1964; media used were as in Test 1. Fungal growth commenced on the seeds almost immediately; an attempt was made to control it from 14/8/1964 with a solution of "Panodrench" (cyano (methyl mercuri) guanidine) at 1/1000 concentration of the commercial solution. This appeared to arrest the development of fungi but did not eliminate them.

The germination test was discontinued on 19/9/1964.

Germination Test No. 3 -

The results of Test 1 and preparatory work in Test 2 showed that interspecific crosses including P. muricata as one parent were unlikely to produce many filled seeds. All remaining seeds of such parentage were therefore tested in Test 3 to obtain the best possible data on the production of viable seed.

This seed was stratified and incubated as in Test 2 without prior separation into "full" and "empty" fractions or weighing.

Germination Test No. 4 -

This was carried out to examine the interfertility of northern and southern provenances of P. muricata. Random samples of 20 seeds were taken from 20 of the possible 36 combinations in a diallele design involving six trees in all. The seeds were stratified from 18/2/1965 to 15/3/1965 in petri dishes prepared as in Test 1. Before stratification they were soaked for 20 minutes in "Panodrench" at 1:20; a 1:200 solution was used for moistening the petri dish contents initially and a 1:500 solution was used for subsequent additions. Germination was carried out at 22°C between 15/3/1965 and 12/4/1965.

Germination Test No. 5 -

This was carried out to obtain more information on the interfertility of P. muricata provenances. Random samples of 20 seeds were taken from 19 of the possible 45 combinations in a diallele design involving nine trees from four provenances. The seeds were stratified at 4°C from 15/10/65 to 2/11/65 in petri dishes prepared as in Test 1. "Panodrench" at 1:500 was used for moistening the petri dish contents originally and for subsequent additions. Germination was carried out between 2/11/1965 and 22/11/1965 at 20.5°C.

SEED YIELDS AND GERMINATION TESTS: RESULTS AND DISCUSSION

(i) Cone Yields

The cone yields from the major portion of the pollination work are shown in Table 4. The unbracketed figures are the number of twigs involved in each cross; the percentages in brackets are the fraction of the strobili, apparently developing when the isolating bags were removed, which developed into mature cones (allowance was made for strobili collected for dissection).

TABLE 4

NUMBER OF TWIGS USED IN EACH CROSS AND % STROBILI MATURING

Male Parent	Female Parent				
	P.attenuata	P.muricata		P.radiata	
		ex mainland	var. remorata	ex mainland	ex Cedros Is.
P.attenuata	17(40%)	11(80%)	-	-	-
P.muricata ex mainland	13(54%)	11(96%)	-	9(60%)	1(0%)
P.muricata var. remorata	23(60%)	8(90%)	-	3(40%)	1(100%)
P.radiata ex mainland	27(74%)	15(82%)	1(100%)	-	7(49%)
P.radiata ex Cedros Is.	26(53%)	9(88%)	1(100%)	17(54%)	2(43%)
Open pollinated	19(54%)	-	-	-	-
Unpollinated	13(4%)	6(0%)	-	7(0%)	-

There is no superficial evidence in this table that the species of pollen applied has affected the proportion of treated strobili which reach maturity. Analyses were made of the proportion of treated flowers on P. attenuata and P. muricata which developed into mature cones. The species of pollen used was found to have no significant effect upon this proportion in either case. Similarly Wright (1953, p. 29) noted that within the five-needled white pines and usually within the series Lariciones pollination by any species from within the relevant group resulted in the development of normal cones containing full or empty seed.

It is of interest that one cone was obtained from an isolated but unpollinated twig of P. attenuata. Vegetative parthenocarpy (development of the cone with no stimulation from pollen) has been recorded in Pinus by Forshell (1953), Wright (1953, p. 29) and Ehrenberg and Simak (1956) although it did not occur in the work of McWilliam (1959). In addition to the 26 twigs listed in the last line of the above table, the author had previously treated 29 other twigs in this manner, but in only this one instance did a cone mature. In 1963 four twigs were isolated on the tree which had earlier produced the cone, but no strobili developed to maturity. It is possible that the strobilus which did produce the mature cone was accidentally pollinated, but the manner in which this may have occurred is not obvious. The apparently unopened buds were washed (using a laboratory wash-bottle) before bagging on 29/9/1960; all were observed to be open on 10/10/1960. The cone which developed was located on the upper whorl of the two whorls of flowers on the twig - that is it would have become receptive at a later date than those lower down. Pollen, perhaps from earlier-flowering P. radiata growing nearby, may have lodged on the twig above the flower before bagging and fallen on to the flower when the latter became receptive; or there may have been a small undetected hole in the isolating bag, or parthenocarpy may have occurred. No developed seed, either full or empty, were found in the developed cone. Although this one cone was obtained, the results from the treatment do indicate the efficiency of the isolating technique.

(ii) Seed Yields

An analysis of the yield of "clean" seed was made for two of the female parents - P. muricata and P. attenuata. Data from the other parents were inadequate to enable satisfactory tests to be carried out.

As the number of cones on each twig varied considerably, yields from a sufficient number of twigs to total about 10 cones were added together to give a unit of data for analysis. The figures in Tables 5 and 6 refer to the mean number of clean seed obtained per cone.

TABLE 5

FEMALE PARENT: P. ATTENUATA - AVERAGE YIELD OF CLEAN SEED PER CONE

Male Parent	P.muricata var.remorata	Pinus muricata	Pinus attenuata	Cedros Is. pine	Pinus radiata
Yield	41.7	38.7	74.0	77.5	103.4

Differences between the various male parents were significant at $P < .05$; the means underlined by the same line do not differ significantly from each other; any two means not underlined by the same line are significantly different at $P < .05$ (using Snedecor's (1956) modification of Tukey's comparison among means)

TABLE 6

FEMALE PARENT: P. MURICATA - AVERAGE YIELD OF CLEAN SEED PER CONE

Male Parent	Cedros Is. pine	Pinus attenuata	Pinus radiata	Pinus muricata	P.muricata var.remorata
Yield	15.7	27.7	28.2	43.0	61.0

Differences between the various male parents were significant at $P < .01$; the significance of differences between means is as above.

Examination of these data suggests that the species and varieties concerned fall into two groups, between which differences are only partially significant in the above tests -

(1) P. attenuata, P. radiata and Cedros Is. pine, pollens of which give relatively high yields of "clean" seed when applied to P. attenuata flowers, and low yields when applied to P. muricata;

(2) P. muricata and P. muricata var. remorata, pollens of which give relatively low "clean" seed yields when applied to P. attenuata, and high yields when applied to P. muricata.

(iii) Germination Test Results

Test No. 1 : Female parent P. radiata

(1) Random samples from each cross. - The crosses involving pollens of P. radiata and Cedros Is. pine gave a much higher germination than those involving P. muricata. Mean figures are given in Table 7.

TABLE 7

FEMALE PARENT: *P. RADIATA* - GERMINATION OF CLEAN SEED

Male Parent	Pinus radiata	Cedros Is. pine	Pinus muricata	P.muricata var.remorata
Germ. (%)	69.5	50.7	2.5	0.0
Twigs sampled	5	10	4	1

The means differed significantly at $P < .001$; the means underlined by the same line were not significantly different at $P < .05$. (Percentages were transformed to arcsines for analysis)

(2) Samples from known weight classes. - As the number of filled seed in crosses involving pollens of *P. muricata* or *P. muricata* var. *remorata* was very low or zero, the attempt to obtain samples of filled seed of this parentage was abandoned; tests made involved seed from *P. radiata* and Cedros Is. pine only. Mean figures are given in Table 8.

TABLE 8

FEMALE PARENT: *P. RADIATA* - GERMINATION OF FULL SEED

	P. radiata	Cedros Is. pine
Germination (%)	64.7	82.0
Twigs sampled	10	5

The means did not differ significantly ($P < .2$).

The results of this test indicate that a very poor yield of germinable seed is obtained when pollen of *P. muricata* or its variety *remorata* is applied to flowers of *P. radiata*. This appears to be largely due to a breakdown of ovule development at some stage close to fertilisation: the seeds from the interspecific cross are of normal size and appearance, but the contents are degenerate. This is reflected in low seed weights; the mean weight of random seed samples of various parentages is shown in the lower histogram of Figure 18.

Test No. 2 : Female parents principally *P. attenuata* and *P. muricata*

The yield of "full" seed per random sample, mean seed weight and germination results are given in Table 9. The proportion of empty seed is high in interspecific crosses between *P. muricata* and the other two species; there is slight evidence that the empty seeds produced are also lighter than those of other parentage. However, the germination figures for the "full" seed fraction do not show a corresponding trend. When the germination figures for whole samples are calculated (in the last column), it is again apparent that *P. muricata* is clearly separated from the other species.

If these germination figures are now applied to the seed yields per cone in the same samples, figures for the number of germinable seed per cone are obtained. The results of this calculation are shown in Tables 10 and 11; the values given are germinable seeds per cone for seed lots containing 20 or more seeds.

TABLE 9

RESULTS OF GERMINATION TEST NO. 2 ON CLEAN SEED

Female Parent	Male parent	No. twigs tested	No. samples tested	Empty seed		Full seed			Germ. % of whole sample
				No. per sample (1)	Mean Wt. mgm	No. per sample (1)	Mean Wt. mgm	Germ. %	
P. attenuata	P. attenuata	6	8	1.1	7	18.9	22	51	48
	P. radiata	10	10	6	9	14	30	73	51
	Cedros Is. pine	10	10	6	7	14	24	79	55
	Open pollinated	5	7	1.6	11	18.4	21	50	46
	P. muricata	6	10	19.9	7	0.1	23	100	0.5
	P.muricata var.remorata	10	10	17.9	6	2.1	23	81	8.5
P. muricata	P. muricata	7	7	8	4	12	13	86	52
	P.muricata var.remorata	5	10	7	5	13	15	76	49
	P. attenuata	6	14	20.0	3	0.0	-	-	0.0
	P. radiata	9	17	19.2	4	0.8	21	23	0.9
	Cedros Is. pine	4	14	17.2	3	2.8	14	2.5	1.7
P.muricata var.remorata	P. radiata	1	2(2)	14	5	2	17	0.0	0.0
	Cedros Is. pine	1	2	17	8	3	21	83	1.2

(1) Sample = 20 seeds chosen at random from "clean" seed

(2) One sample contained only 10 seeds.

TABLE 10

FEMALE PARENT : *P. ATTENUATA* - GERMINABLE SEED PER CONE

Male parent -	
Open pollinated	57.9
P. radiata	44.7
P. attenuata	43.7
Cedros Is. pine	37.1
P. muricata var. *remorata*	3.3
P. muricata	0.2

The yields were analysed by averaging the mean yield per cone for each three consecutive twigs within each treatment: these figures were transformed to log (x + 1) and examined by analysis of variance and Snedecor's modification of Tukey's test for differences between means. Differences between means were significant at $P < .001$; means not significantly different at $P < .05$ are marked by a common line.

TABLE 11

FEMALE PARENT : *P. MURICATA* - GERMINABLE SEED PER CONE

Male parent -	
P. muricata var. *remorata*	29.2
P. muricata	25.9
P. radiata	0.6
Cedros Is. pine	0.2
P. attenuata	0.0

An analysis was made as above; differences between means were significant at $P < .001$; means not significantly different at $P < .05$ are marked by a common line.

The proportion of twigs which yielded 20 or more seeds in each treatment was examined. It was found that when *P. attenuata* was used as the female parent, 84% of all twigs yielded 20 or more seed; differences between male parents were not significant ($P < .2$) when tested by the chi-square method; when *P. muricata* was used as the female parent 77% of all twigs yielded 20 or more seed; differences between male parents were not nearly significant. Thus the figures for germinable seeds per cone given above may be regarded as indicating the relative germinable seed production from all cones collected from these two species; absolute values can be calculated by reducing the figures given by factors of 0.84 and 0.77 respectively.

Examination of the germination results for *P. attenuata* cones by provenances is not very rewarding. Most of the pollinations were concentrated in Plots 2 and 3, Hall's Block, from Bartlett Springs and Ensenada respectively; no evidence that these provenances behaved differently in either interspecific or intraspecific crosses was obtained.

However, the situation in *P. muricata* is interesting, although the data are somewhat limited. The results from the *male parents* will be examined in turn -

(1) *P. muricata* - Figures are germinations per sample of 20 seeds; figures in brackets indicate the number of twigs sampled. The provenances of *P. muricata* used for female parents are listed in order of occurrence from north to south.

Trinidad Head, Humboldt Co x Humboldt Co. - 11(1)
Monterey x Monterey - 20(1)
Monterey x Humboldt Co. - 11(2)
Lompoc x Humboldt Co. - 0(2)

(2) *P. muricata* var. *remorata* - Figures as in (1)

Monterey x Santa Cruz - 1(2)
Lompoc x Santa Cruz - 15(2)
San Vicente x Santa Cruz - 16(1)

(3) *P. radiata* - Figures as in (1)

Trinidad Head x Monterey - 0.0(1)
Monterey x Monterey - 0.0(5)
Lompoc x Monterey - 0.0(2)
San Vicente x Monterey - 1.5(1)

(4) Cedros Is. pine - Figures as in (1)

Monterey x Cedros Is. - 0.0(3)
Lompoc x Cedros Is. - 0.5(1)

(5) *P. attenuata* - Figures as in (1)

Trinidad Head x (Bartlett Springs + Ensenada) - 0.0(2)
Monterey x (Bartlett Springs + Ensenada) - 0.0(4)

In each case it appears that the northern populations of *P. muricata* are distinct from the southern ones. The latter seem to cross somewhat more readily with other species or with var. *remorata*. However, one cannot discount the possibility that this apparent cline is confounded with some maternal effect, due for example to differences in flowering times in the provenances. Corroborative evidence from reciprocal crosses is desirable to confirm the above results.

Test No. 3 : Remaining seed from less successful interspecific crosses

The germination obtained from "clean" seed is shown in Table 12.

TABLE 12

GERMINATION OF REMAINING SEED OF LESS SUCCESSFUL CROSSES

Female parent	Male parent	No. twigs	No. seeds	Germ. %
P.attenuata	*P.muricata* (Humboldt Co.)	6	344	0.0(1)
P.attenuata	*P.muricata* var. *remorata*	13	1197	4.8(3)
P.muricata	*P.attenuata*	6	1176	0.0(1)
P.muricata	*P. radiata*	12	1746	3.3(3)
P.muricata	Cedros Is. pine	3	1280	.15(2)
P. radiata	*P. muricata*	2	c.600	0.0(1)
P. radiata	*P. muricata* var. *remorata*	1	C. 70	0.0(1)

(1) No evidence of viability, such as splitting of testas, was seen in any of the seeds.

(2) No evidence of viability was seen apart from the two seeds which germinated.

(3) Apart from the germinated seed, a small additional number (seven and ten respectively) were seen to grow for a short time before dying, due either to fungal attack or intrinsically low vigour.

The germinable seed in the cross *P. attenuata* x *P. muricata* var. *remorata* were spread in small numbers through 10 of the 13 twigs tested. Most of the germinable seed (53/58) in the cross *P. muricata* x *P. radiata* came from a single twig in the Lompoc provenance of the female parent.

The results of this test are consistent with and support those of Test 2.

Test No. 4 : Seed from inter-provenance crosses of *P. muricata*

The germination obtained is shown in Table 13. Each entry is based upon a test of 20 seeds from a single twig; the figures show germination percentage of "clean" seed.

TABLE 13

GERMINATION OF CLEAN SEED OF INTRASPECIFIC CROSSES OF *P. MURICATA*

Male parent		Female parent					
		Plot 33-Fort Bragg			Plot 37 - Lompoc		
	Tree number	12	13	14	10	11	15
Fort Bragg	12		0	60	5		0
	13				0		0
	14	70	20		0		0
Lompoc	10	0		0		55	65
	11			0	0		80
	15		0	0	85		

The mean values are:

	Fort Bragg	Lompoc
Fort Bragg	37.5%	0.8%
Lompoc	0.0%	57.0%

Clearly crosses between trees within these provenances yield much more germinable seed than do those between trees in different provenances; this design eliminates possible confounding due to maternal effects noted in Test 2.

<u>Test No. 5</u> : Additional seed from inter-provenance crosses of <u>P. muricata</u>

The germination obtained is shown in Table 14. The figures show germination percentage of "clean" seed.

TABLE 14

GERMINATION OF CLEAN SEED OF FURTHER INTRASPECIFIC CROSSES OF <u>P. MURICATA</u>

		Plot 33 Fort Bragg			Plot 34 Trinidad Head		Plot 36 Monterey	Plot 37 Lompoc	
Plot	Tree No.	1	2	3	4	5	8	10	11
Fort Bragg	1	75	90			95	50		
	2		95	80	70	95	4		
	3				100				
Trinidad Head	4				100	100			0
	5					75	55		
Monterey	8						95		
Lompoc	10							37(1)	60(2)
	11								60

(1) This figure is the mean of two 20-seed samples.

(2) This figure is the mean of three 20-seed samples.

All other figures are the result of a single 20-seed sample.

Crosses between trees of the same provenance have yielded a high proportion of germinable seed, even with the large number of selfings involved. Crosses within the northern blue forms (var. <u>borealis</u> from Fort Bragg and Trinidad Head) have given seed with a high germination. Crosses between these trees and those from Monterey, in the centre of the species' range, show a reduction of about 50% in seed germination; with trees from Lompoc, further south still, the germination falls to very low levels. The data are not adequate to show whether the gradient in interfertility is a smooth cline or a series of steps.

SEED YIELDS AND GERMINATION TESTS: SUMMARY AND CONCLUSIONS

No evidence was obtained that the species of pollen used for pollination affects the proportion of treated strobili which reach maturity. In the absence of pollination strobili usually die within a matter of months. One mature cone was obtained from such a treatment but the possibility of a small amount of pollen having accidentally reached the strobilus cannot be excluded. The cone contained no seed.

The yields of "clean" seed (both full and empty seed) per cone from interspecific crosses involving P. muricata were about half of those obtained from other crosses. This depression in seed yield was only partially significant.

Very few full seed were obtained from interspecific crosses involving P. muricata. However, the full seed which were obtained gave reasonable germination. When the number of germinable seed per cone was calculated, it was obvious that P. muricata was quite distinct from the other species: interspecific crosses between P. radiata and P. attenuata gave germinable seed yields not significantly different from the intraspecific controls, but yields from crosses involving P. muricata and either of the other species were very poor; this difference was highly significant.

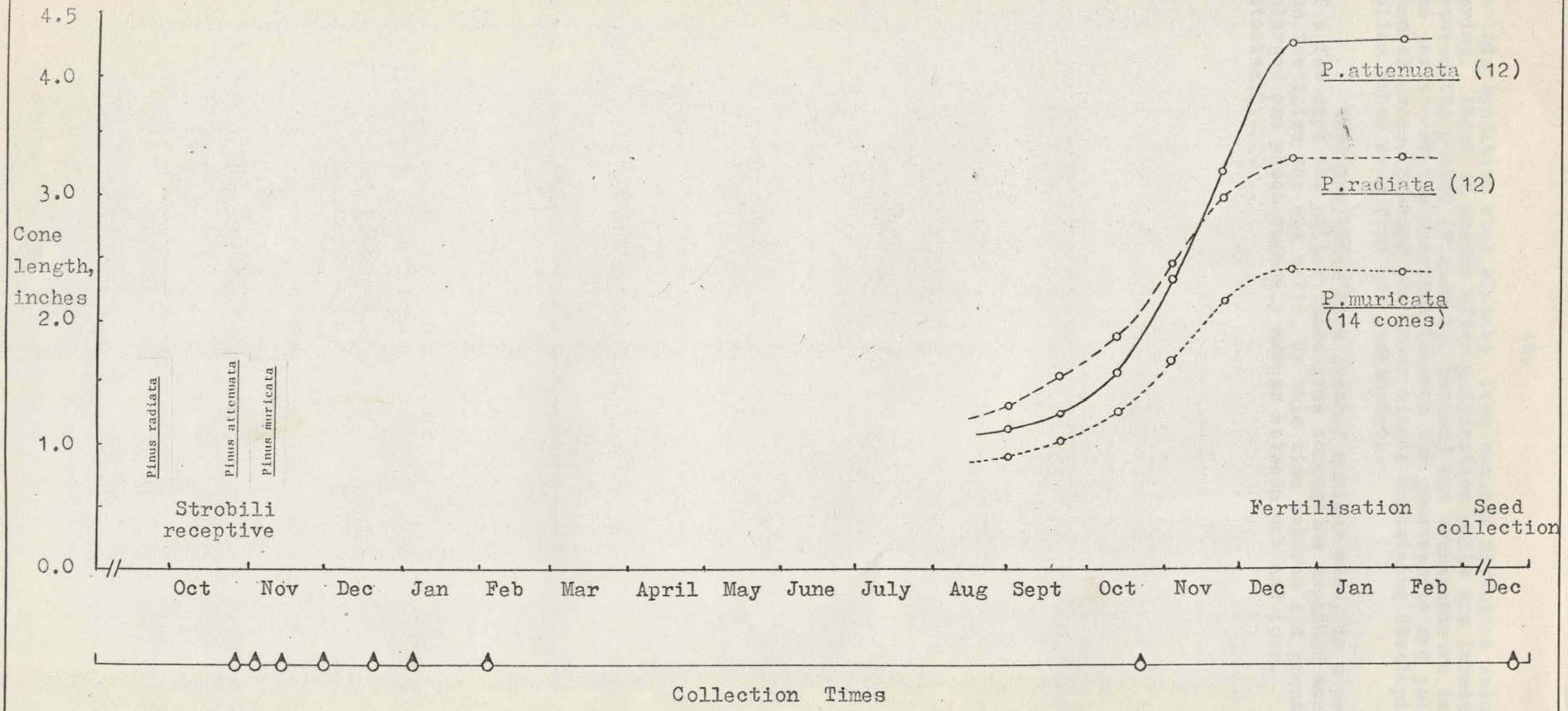
There is good evidence that P. muricata is highly differentiated insofar as intraspecific fertility is concerned. The more northerly forms seem to be genetically isolated from the southern ones to a much greater extent than P. attenuata is isolated from P. radiata. There is also evidence that the southern forms cross more readily with other species in the group than do the northern ones.

PROGRESS OF OVULE DEVELOPMENT: RESULTS AND DISCUSSION

The times of collection of developing ovules for examination and the growth in length of year-old conelets are shown in Figure 19.

The results of examination of sectioned material are shown in Table 15. Before discussing these, the sequence of events in the normal development of the seed of most pines will be outlined (from McWilliam and Mergen 1958; McWilliam 1959 and Sarvas 1962).

At the time of flowering a group of cells having large nuclei are present in the centre of the nucellus; one of these, the megaspore mother cell, is larger than the remaining surrounding cells, termed "spongy tissue". Several weeks after pollination the megaspore mother cell divides to give a linear tetrad of haploid megaspores, three of which normally degenerate almost immediately. Usually the megaspore furthest from the micropyle survives, growing vigorously for a period of weeks (Figure 20, centre). Later divisions result in a number of free nuclei scattered irregularly in the cytoplasm or oriented along the walls of the female gametophyte. By this time the pollen tubes have penetrated almost half the distance from the tip of the nucellus to the developing female gametophyte (Figures 21, top; 22, top). The ovule remains



Length measurements were made at Hall's Block and Reid's Pinch arboreta in 1960

FIG.19 COLLECTION TIMES OF OVULES AND SEEDS IN RELATION TO DEVELOPMENT OF FEMALE STROBILI in this condition over winter; free nuclear divisions resume in spring. About 12 months after pollination walls are formed between the nuclei (Figure 23, bottom) and archegonia are initiated; the portion of the nucellus between the gametophyte and pollen chamber grows, pushing the older tissue containing developing pollen tubes away from the gametophyte.

When the cones have reached maximum size, in a period of a few days the pollen tubes grow through the expanded nucellus and fertilize the egg cells, by this time matured and greatly enlarged and which commonly number between one and four. (Stockwell........

TABLE 15

OVULE DEVELOPMENT IN DIFFERENT TREATMENTS - RESULTS FROM SECTIONING

Date of Collection	Serial No.		No. Ovules Examined	See Figure No.	Comment
25.10.61	10	P. radiata x P. muricata	6		Pollen grains present in pollen chamber
2.11.61	12	P. radiata, unpollinated	6	20	No pollen present; enlarged megaspore evident in some ovules; one megaspore degenerating
15.11.60	11	P. attenuata x P. attenuata	14	20, top	Pollen present in pollen chamber
15.11.60	15	P. muricata x var. remorata	12		Pollen present. Megaspore evident, but not as advanced as in No. 12
1.12.60	26	P. radiata, unpollinated	3		Incipient degeneration of cells at the tip and in the centre of the nucellus
1.12.60	28	P. radiata, open pollinated	4		Limited degeneration of cells at tip of nucellus due to pollen tube growth
20.12.60	37	P. muricata unpollinated	7		Incipient degeneration in centre of nucellus
5. 1.61	39	P. radiata, unpollinated	8		Conelet almost dead; all ovules necrotic
5. 1.61	40	P. attenuata, unpollinated	12	21, bot.	All ovules showing some degeneration, but not as advanced as in No. 39 above
5. 1.61	41	P. radiata, open pollinated	7	21, top	Pollen present in pollen chamber; no sign of degenerate tissue
4. 2.61	42	P. attenuata, unpollinated	8	22, bot.	Incipient degeneration of cells at the tip and in the centre of the nucellus
3. 2.61	45	P. attenuata x P. radiata	3		Pollen present; tissues healthy
6. 2.61	52	P. muricata x P. attenuata	6		as above
7. 2.61	51	P. muricata x P. radiata	11	22	Pollen present; two of the ovules are showing incipient degeneration in the centre similar to that seen in unpollinated strobili. In these pollen is present but there is no pollen tube growth

Date of Collection	Serial No.		No. Ovules Examined	See Figure No.	Comment
13.10.61	55	*P. attenuata* x var. *remorata*	7		Pollen present; pollen tube growth not yet complete; contents of large central vacuole largely lost
13.10.61	56	*P. attenuata* x *P. attenuata*	10		As above
13.10.61	57	*P. attenuata*, open pollinated	3	23, top	As above
13.10.61	58	*P. muricata*, open pollinated	7		As above
13.10.61	61	*P. muricata* x Cedros Is. pine	9		As above
13.10.61	64	*P. attenuata* x *P. muricata*	9		As above
13.10.61	66	*P. muricata* x *P. radiata*	7		As above
13.10.61	65	*P. muricata* x *P. attenuata*	8	23, bot.	Pollen present and developing; megaspore structure preserved more satisfactorily than in Nos. 55-64 above
Dec. 1962	75	*P. muricata* x *P. radiata*	5		All seeds empty; embryo and endosperm necrotic
Dec. 1962	76	*P. attenuata* x var. *remorata*	5		As above
Dec. 1962	77	*P. radiata* x *P. muricata*	3		As above
Dec. 1962	78	*P. attenuata* x *P. attenuata*	3		One seed as above; two show normal embryos and endosperm
Dec. 1962	79	*P. muricata* x *P. muricata*	5		Three seeds full; two empty. One seed was from crosses between northern provenances; the remainder from crosses between northern and central provenances
Dec. 1962	80	*P. radiata* x Cedros Is. pine	5		All seeds full - embryo and endosperm normal

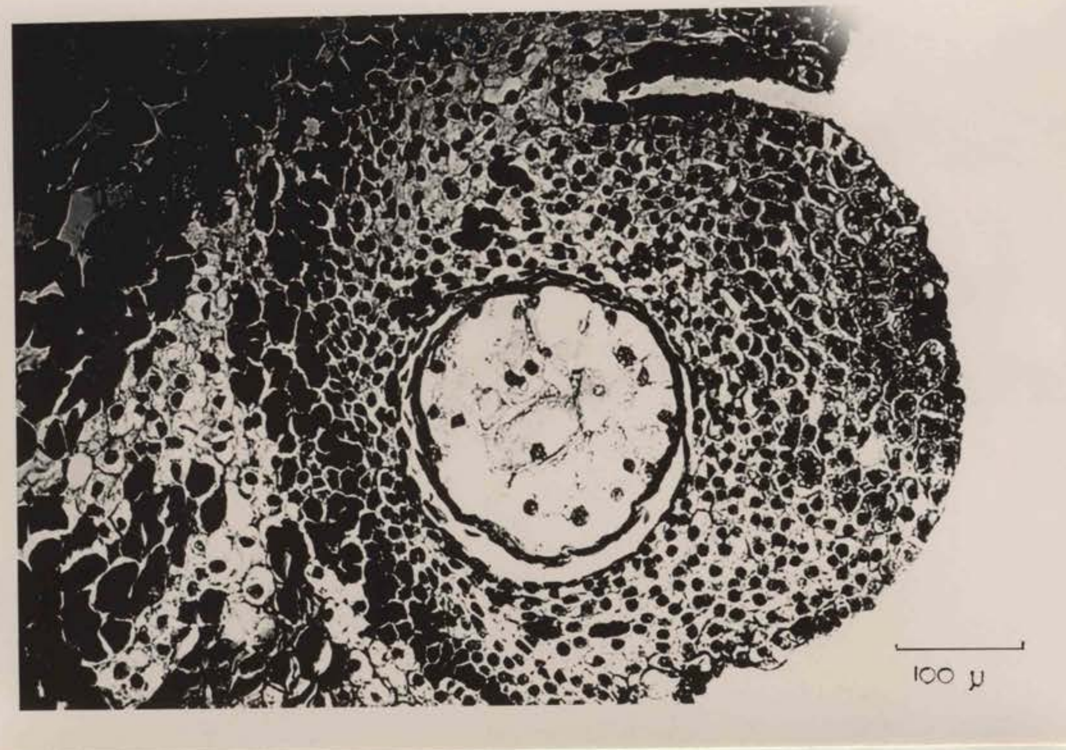
Top - P. attenuata x P. attenuata. Pollinated 12-27.10.1960; collected 15.11.1960. Normal development. Pollen grains fill the pollen chamber and pollen tubes have penetrated the nucellus. The micropylar canal has been closed by rapid growth of the now large cells above the pollen chamber. The section of the megaspore is not median. (Twig 11 A2/3.6)

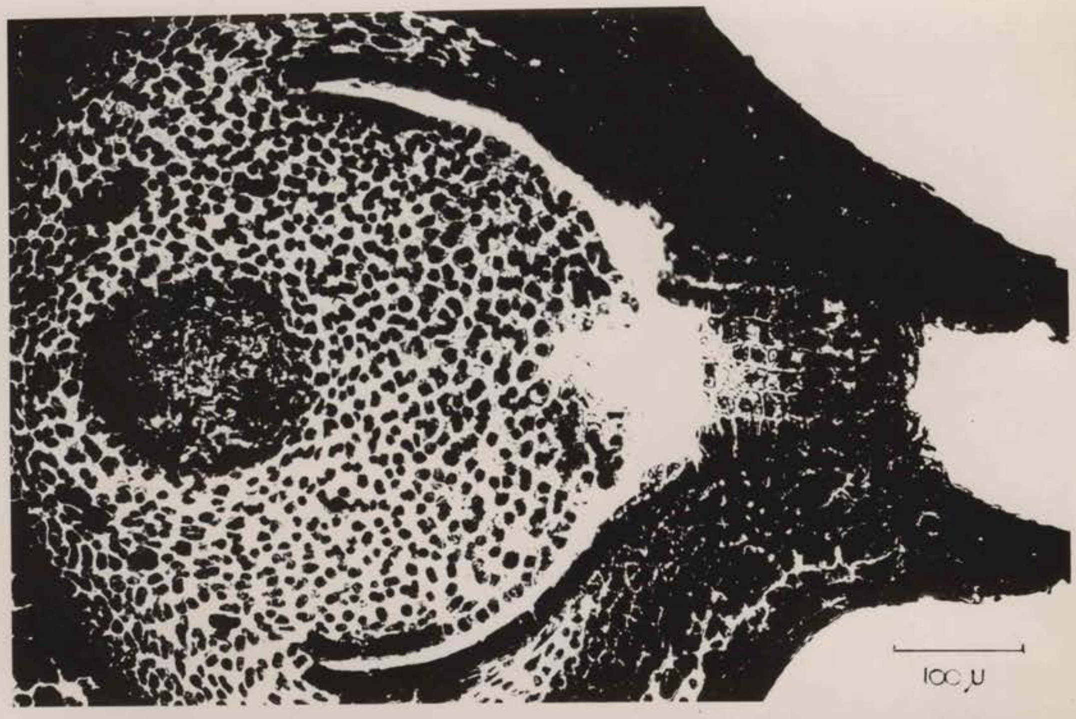
Centre - P. radiata, unpollinated. Receptive early October; collected 2.11.1960. The megaspore is well developed in the centre of the nucellus - nuclei of the cells are prominent; the walls inconspicuous. The tissue (tapetum) surrounding the megaspore is derived from the spongy tissue. (Twig 12 A2/1.1)

Bottom - Strobilus details as above. Pollen chamber empty; the concave tip of the nucellus through which pollen tube penetration normally occurs is evident. The megaspore is darkly staining, due to incipient degeneration. (Twig 12 A2/2.7)

FIG. 20 OVULES IN EARLY NOVEMBER SOON AFTER FLOWERING


100µ

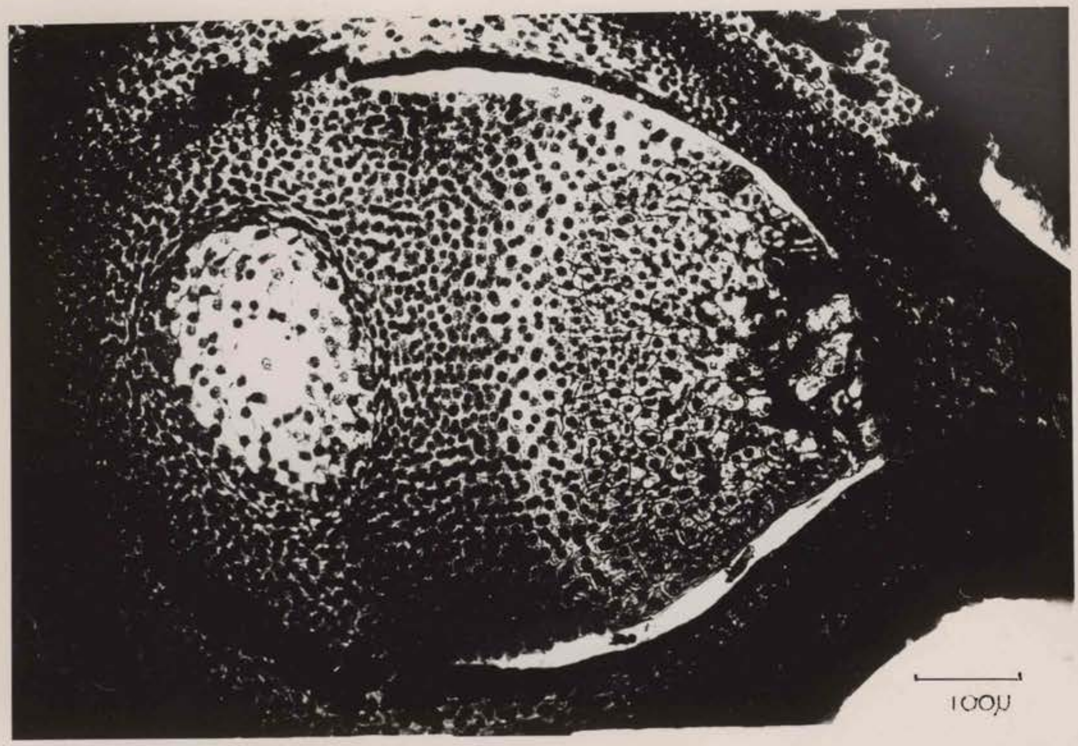

100 µ


100 µ

Top - P. radiata, open pollinated in early October, collected 5.1.1961. Some darkly-staining tissue at the tip of the nucellus is becoming degenerate, possibly due to damage caused by vigorously-growing pollen tubes. The megaspore is enlarging normally. (Twig 41 B1/1.7)

Bottom - P. radiata, unpollinated. Collected 5.1.1961 Tip of the nucellus and megaspore show degenerate tissue. (Twig 40 A1/3.1)

FIG. 21 OVULES IN EARLY JANUARY THREE MONTHS AFTER FLOWERING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100µ

Top - P. muricata x P. radiata. Pollinated 1-9.11.1960; collected 7.2.1961. Development is proceeding normally - pollen tubes are growing; there is no sign of megaspore degeneration. (Twig 51 B2/1.1)

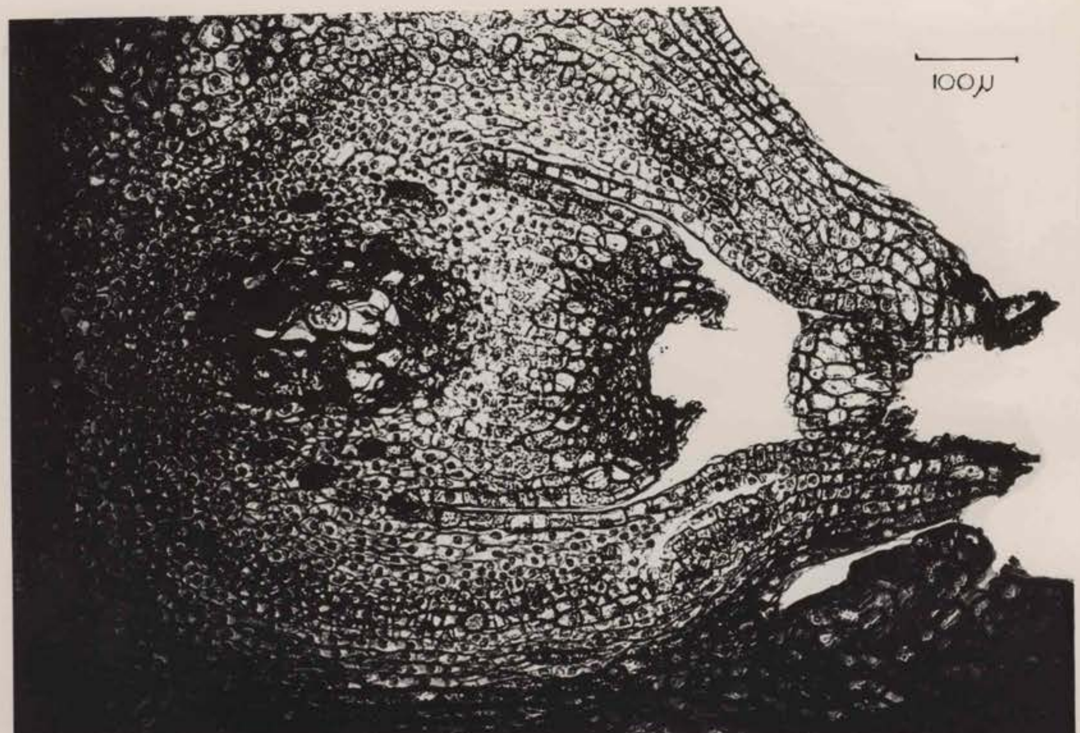
Centre - Strobilus details as above. Development of the left ovule seems normal; the right one contains ungerminated pollen in the pollen chamber and the megaspore is degenerating. (Twig 51 B2/1.4)

Bottom - P. attenuata, unpollinated. Receptive late October 1960, collected 4.2.1961. Nucellar tip and megaspore degenerating (compare with right-hand ovule above). Almost all other unpollinated conelets were dead by this time. (Twig 42 A1/1.5)

FIG. 22 OVULES IN EARLY FEBRUARY


100μ


100μ

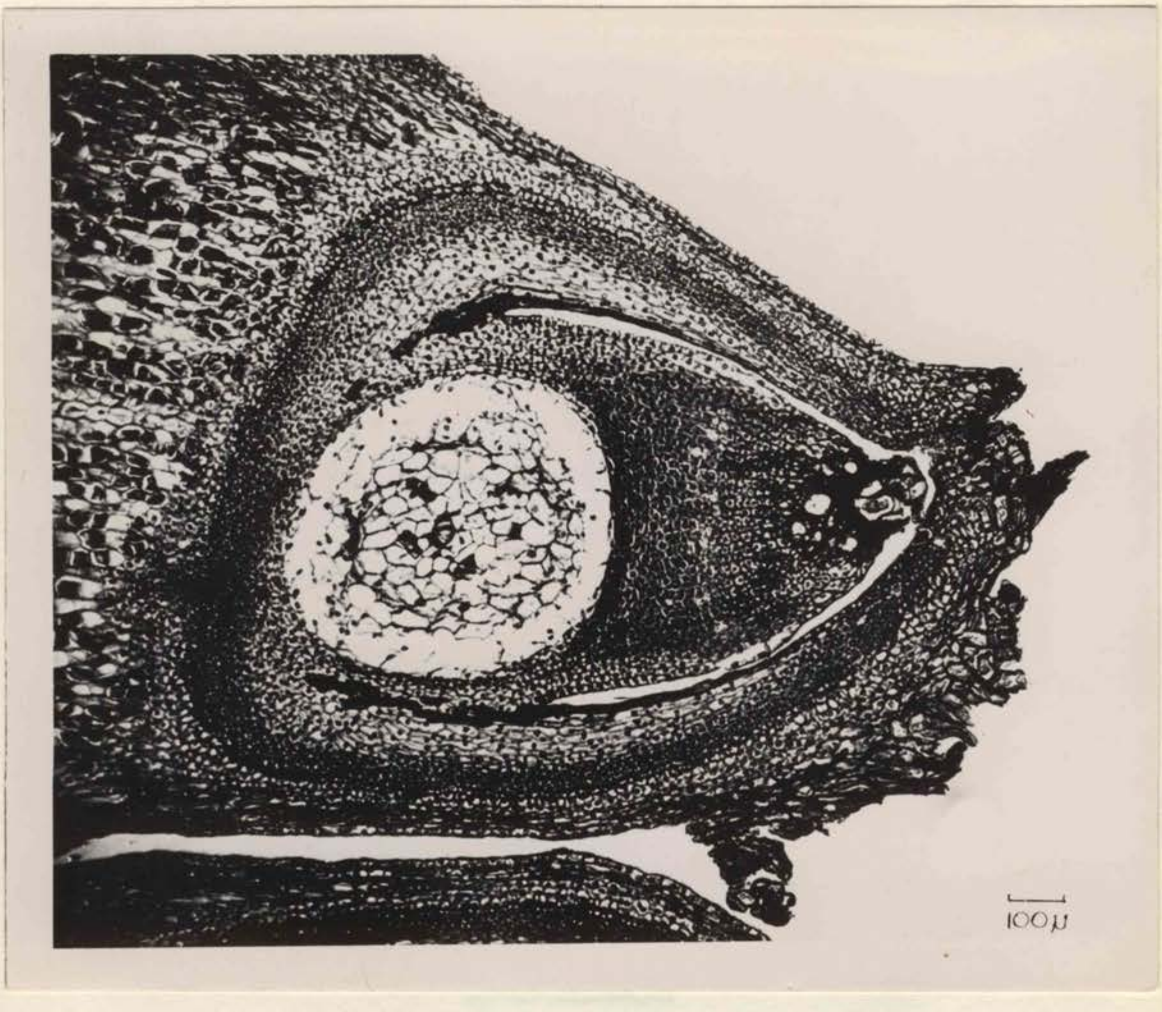

100μ

Top - *P. attenuata*, open pollinated, collected 13.10.1961. Pollen tubes have ramified through the nucellar tip but new growth of the nucellus separates developing tubes from the megaspore. The latter is probably still in the free nuclei stage prior to the formation of archegonia but details have been poorly preserved. (Twig 57 A3/1.1)

Bottom - *P. muricata* x *P. attenuata*. Pollinated 1-9.11.1960; collected 13.10.1961. Cell walls are appearing in the gametophyte. The development of the ovules in this and other relatively incompatible crosses collected on the same day is very similar to that of the fertile control crosses. (Twig 65 A2/2.1)

FIG. 23 OVULES IN OCTOBER ONE YEAR AFTER POLLINATION

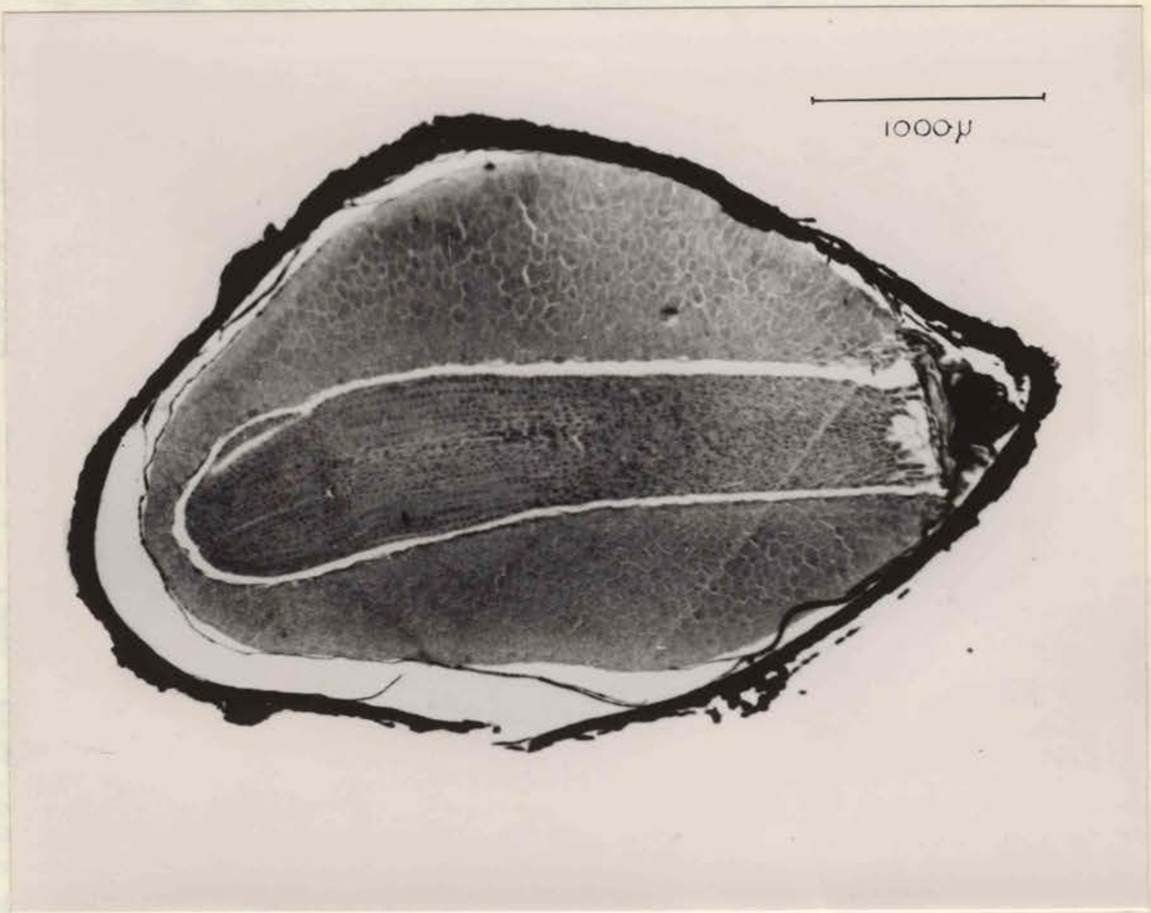

100 µ


100 µ

Top - P. muricata x P. muricata Normal full seed. The micropyle and remains of the nucellus are at the right-hand end of the section.

Bottom - P. attenuata x P. muricata var. remorata. Empty seed typical of most of the interspecific crosses involving P. muricata. They also occurred frequently in some inter-provenance crosses of the same species, and less frequently or rarely in other crosses.

FIG. 24 SEEDS IN DECEMBER 1962 TWO YEARS AFTER POLLINATION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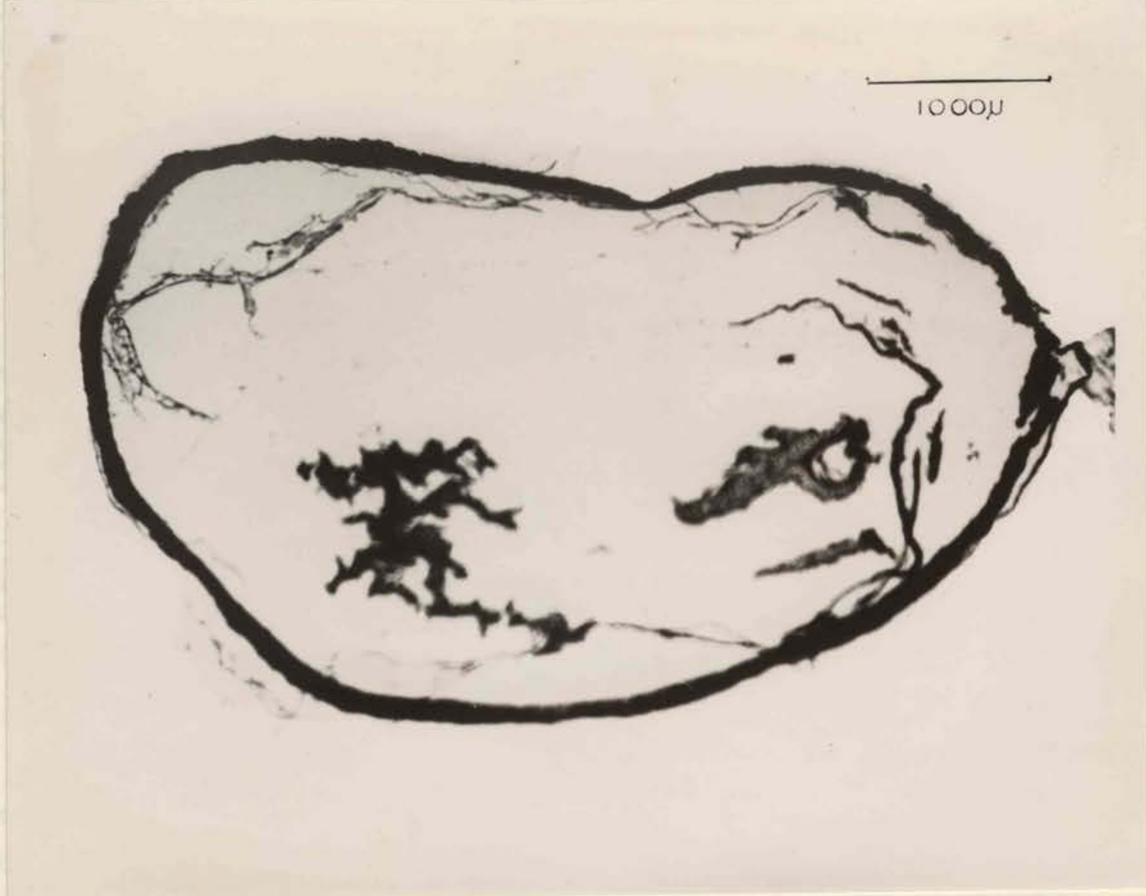
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(Stockwell (1939) reported archegonia to number commonly from six to twelve at the time of formation, falling to six or more at maturity). A pro-embryo forms in the base of each archegonium; each forms a further series (commonly four to six) of primary embryos. Normally only a single embryo matures (Figure 24, top).

In the material examined strobili which were isolated but unpollinated showed degeneration of the tissue in the centre of the nucellus in December, January and February following flowering (Table 15, Serial Nos. 26, 37, 39, 40 and 42; Figures 20, 21 and 22); most of the uncollected strobili died during the same period (Table 16). The unexpected development of one strobilus to maturity was discussed previously (page 31).

TABLE 16

LONGEVITY OF ISOLATED, UNPOLLINATED STROBILI

Event	Species			Total
	P. radiata	_P. attenuata_	_P. muricata_	
Bags removed	13-27/10/60	23/11-1/12/60	28/11/60	
No. healthy strobili present when bags were removed	32	32	22	86
Nett No. after collections	29	30	21	80
No. living strobili, 5-6/1/61	0	17*	?	?
Nett No. after collections	0	16*	?	?
No. living strobili, 3-6/2/61	0	2	0	2
Nett No. after collections	0	1	0	1
No. mature cones collected in December 1962	0	1	0	1

* These figures included 3 dying strobili.

With the exception of three ovules of _P. muricata_ x _P. radiata_ (Table 15, Serial No. 51, collected 7.2.1961, 3 months after pollination) all pollinated ovules examined in collections made up to and including October 1961, about 12 months after flowering, developed in a normal manner. The three exceptional ovules showed incipient degeneration of tissue in the centre of the nucellus (Figure 22, centre), similar to that in isolated unpollinated ovules (for example Figure 22, bottom). Pollen grains were present in the pollen chamber of each ovule, but no pollen tube growth was seen. McWilliam (1959) noted comparable degeneration in ovules of Austrian pine, P. nigra Arnold, when pollen of the incompatible red pine, P. resinosa Ait., was applied: pollen germination was poor and most of the ovules broke down between the second and fourth month following pollination; affected ovules became necrotic and shrunken. In the present case the species involved proved to have very low germinable seed yields (Table 9) and the total seed yield (full plus empty) was considerably lower than from relevant intra-specific controls (Table 6). It seems likely that this lowered seed production is due to a process similar to that seen by McWilliam and observed in this study in these three ovules. The number of affected ovules found is somewhat smaller than might have been expected from the magnitude of the reduction in seed yields in the less successful crosses (Tables 5 and 6), but this may be due to the relatively small sample of ovules sectioned.

The strikingly low yield of viable or full seed in inter-specific crosses involving P. muricata and some inter-provenance crosses of the same species (Tables 9, 12, 13 and 14) appears to be due to failure of development at or about the time of fertilisation. The material collected in October 1961, not long prior to fertilisation (Figure 19) showed no abnormalities (Table 15, Serial Nos. 37, 39, 40, 41). However, many seeds in the above crosses which reached full size a few weeks after this collection proved to be empty (Table 15 Serial Nos. 75-80; Figure 18; Figure 24). Collections were not made with sufficient frequency to enable the point of failure to be fixed accurately. The pollen tubes may have been incapable of the usual rapid development through the nucellus just prior to fertilisation; fertilisation itself may have failed or embryos may have formed and degenerated in the months before the seed matured and the final collection was made.

PROGRESS OF OVULE DEVELOPMENT: SUMMARY AND CONCLUSIONS

Degeneration of ovules in unpollinated strobili commenced within 6-8 weeks of the strobili becoming receptive; in all cases it was first seen in the megaspore and tip of the nucellus, although the latter was affected in some normally-pollinated controls. Almost all unpollinated strobili were dead by early January, 9-16 weeks after flowering.

A small number of ovules in the relatively incompatible cross P. muricata x P. radiata were found to be breaking down in February, 13 weeks after flowering. Such breakdown would depress seed yields as was noted in this and similar crosses (Tables 5 and 6).

Most of the low yields of viable seeds in crosses such as P. muricata x P. radiata seem to be due to failure of the reproductive process just prior to, at, or after the time of fertilisation. The barriers encountered at this time are more significant, judged on the condition of ovules sectioned and yields of full and empty seeds, than those operative in the early months following pollination.

X. GROWTH OF HYBRID SEEDLINGS

METHOD

The plants resulting from the germination tests were transferred from petri dishes to soil in pots, where subsequent measurements were made of growth. Pots about 8" in diameter were used, and more than one plant was usually put in each pot. In the final assessment differences in stocking in each pot and difference in mean seed weight between twigs were eliminated by co-variance analysis.

Test 1. - Plants were transferred to pots between 27/9/1963 and 9/12/1963; a steam-sterilised alluvial soil fertilised with John Innes Base (2 oz/2 gals of soil) was used. Final measurements were made on 5/6/1964.

Tests 2 and 3. - Plants were transferred to pots between 18/8/1964 and 19/9/1964. A steam-sterilised mixture of peat, sand and loam was used for the potting medium. Heights were measured and analysed on 8/1/65 and 3/6/1965.

RESULTS

Test 1. - The maternal parent in this test was P. radiata; only the male parents P. radiata and Cedros Is. pine produced a useful number of plants. One plant did germinate from P. muricata pollen, but it died shortly after germination. Mean height/pot at about 6 mo. was -

	P. radiata x P. radiata	P. radiata x Cedros Is. pine	Sig. of diff.
Unadjusted means	9.2"	6.9"	$P < .01$ (t-test)
Adjusted means	8.9"	7.1"	$P < .05$

Mean seed weight varied between 13 and 32 mgms; the regression of growth on seed weight was not significant ($P < .2$). The number of plants per pot varied from one to ten; the negative correlation between height and the number of plants per pot was not significant. ($P < .6$).

Test 2. - The material in this test fell into two groups, one group having P. attenuata as the maternal parent; the other P.muricata. Mean heights per pot at about 4 mo. and 9 mo. are shown in Table 17.

TABLE 17.

HEIGHT GROWTH OF SEEDLINGS FROM GERMINATION TESTS 2 AND 3

Female parent	Male parent	No. pots	Mean No. plts/pot	Ht. at 4 months, inches	Ht. at [illegible] months, inches*	
				Unadjusted	Unadjusted	Adjusted
P. attenuata	P. attenuata	6	6.7	4.3	5.6	5.4
P. attenuata	P. radiata	8	7.3	4.6	6.8	6.2
P. attenuata	Cedros Is. pine	9	8.5	4.0	5.2	5.5
P. attenuata	P. muricata	1	1.0	5.5	8.1	7.9
P. attenuata	P. muricata var. remorata	4	3.0	4.5	5.9	5.7
P. radiata	P. radiata	3	7.0	4.5	6.3	5.5
P. muricata	P. muricata	4	11.3	2.9	4.4	6.0
P. muricata	P. muricata var. remorata	8	6.8	4.0	5.7	6.5
P. muricata	P. radiata	1	2.0	4.8	7.0	6.6
P. muricata	Cedros Is. pine	1	1.0	6.4	8.9	8.9

* At 9 mo. differences between mean progeny heights were significant at $P < .05$. Regression significance was $P < .1$ for seed wt and $P < .6$ for plants per pot. Least significant differences between means at $P < .05$ were -

for 1 pot in each progeny - 1.6";
for 1 and 4 pots in each progeny - 1.3";
for 1 and 8 pots in each progeny - 1.2".

DISCUSSION

The growth of the presumed hybrids is not appreciably different from that of the parental needlings.

In the first test only the maternal parent was included as a control, and the growth of the hybrids is less than that of these parents. The growth of Cedros Is. pine male parents in the field has been less than that of P. radiata and the seedling growth reflects this tendency.

In the second test, two hybrids have shown considerably better growth than the parents, but in each case the cross is represented only by a single plant, while the parents are represented by about 40 plants. The analysis, based upon mean plant height in each plot, did not make due allowance for these very small samples. It is probably unsound to claim that this growth is an example of hybrid superiority under the condition of the test.

On the other hand, there is no evidence that hybrid growth is appreciably poorer than that of the parents, even in those cases in which one parent is P. muricata and in which very low yields of germinable seed were obtained. There is no evidence that these seedlings would not grow on to reach reproductive maturity.

XI. VERIFICATION OF HYBRIDS

The identity of hybrid pines has been confirmed on numerous occasions, often with techniques developed from those of Anderson (1936). Discriminant analysis has been used successfully by Mergen and Furnival (1960), Kriebel (1962) and others, to place identification on a statistical basis. Some of the features most useful for taxonomic purposes in Pinus are applicable only to plants older or larger or both than those available in this study. These include phenological characteristics such as time of flushing in spring and time of pollination, chemical characteristics of turpentines and morphological characteristics such as the nature of the winter bud, the shape and size of the mature cone, cone scales and seeds, and other features of the reproductive structures.

Needle structure, used as a basis for some specific keys (Sutherland 1934; Harlow 1947) has been found by both Critchfield (1957) and Newcomb (1962) to be less valuable for examining cultivated seedlings than for naturally-occurring parents, although it has been used with success in seedlings of white pines by Kriebel (1962).

Attempts were made to verify some putative P. muricata x P. radiata seedlings by use of the following characters:

1. Number of stomates per unit length of row -

Mergen (1958) successfully used this feature with hybrids of slash pine, and in 1959 he found it useful for three out of five other hybrid groups. In the present study, single secondary needles were taken from each individual plant in three progeny lots from each parental group; a segment about 1 cm long was cut from each needle 1 cm from the base, and the stomates present were counted with a binocular microscope (x 64). Figures for incomplete rows were discarded. The counts were converted to stomata per mm of row. The results are shown in Table 18.

TABLE 18

DISTRIBUTION OF STOMATES ON NEEDLES

	Progeny	No. of plants	No. of rows	Stomates/mm
P. radiata x P. radiata	1	8	28	9.00
	2	7	28	9.08
	3	5	25	10.01
P. muricata x P. radiata	1	2	33	8.72
	2	2	39	9.49
	3	10	35	8.62
P. muricata x P. muricata	1	10	48	9.54
	2	5	45	9.31
	3	9	32	8.30

The mean values and distributions are almost coincident; it is obvious that the species are not different in this characteristic

2. Number of serrulations per unit length of needle -

This feature was used by Bannister (1958b) for P. attenuata and P. radiata, and by Fowler and Heimburger (1958) for white pines. In the present study, a primary needle was collected from about one inch above the cotyledons from the largest three plants in each progeny group; three progenies were used for each parental group. The number of teeth per microscope field were counted half way along each needle. The results are shown in Table 19.

TABLE 19

NUMBER OF TEETH PER UNIT LENGTH OF PRIMARY NEEDLES

Parents	Progeny	Needle length, in.	No. of teeth
P. radiata x P. radiata	1	1.4	11.3
	2	1.2	9.8
	3.	1.0	12.2
P. muricata x P. radiata	1	1.4	10.5
	2	1.1	10.2
	3	1.3	12.0
P. muricata x P. muricata	1	0.9	15.3
	2	0.7	17.2
	3	0.8	15.7

Here the parental species seem to be distinct, but the putative hybrid is indistinguishable from the male parent.

3. Internal needle structure -

The primary needles used for the study above were sectioned at their mid-point after dehydration and embedding in paraffin. Characteristics frequently used for diagnostic purposes were found to be remarkably constant in all sections, e.g. -

Resin canals : Two per section; external; adjacent cells thin walled.

Hypoderm : Absent.

Endodermis : Cells of uniform shape and wall thickness.

Stomata : Modified epidermal cells arch over the stomatal entrance in one sample of P. muricata; the conspicuous over-arching in P. radiata described by Harlow (1947), was absent in this material.

Photomicrographs of some of the sections are shown in Figure 25.

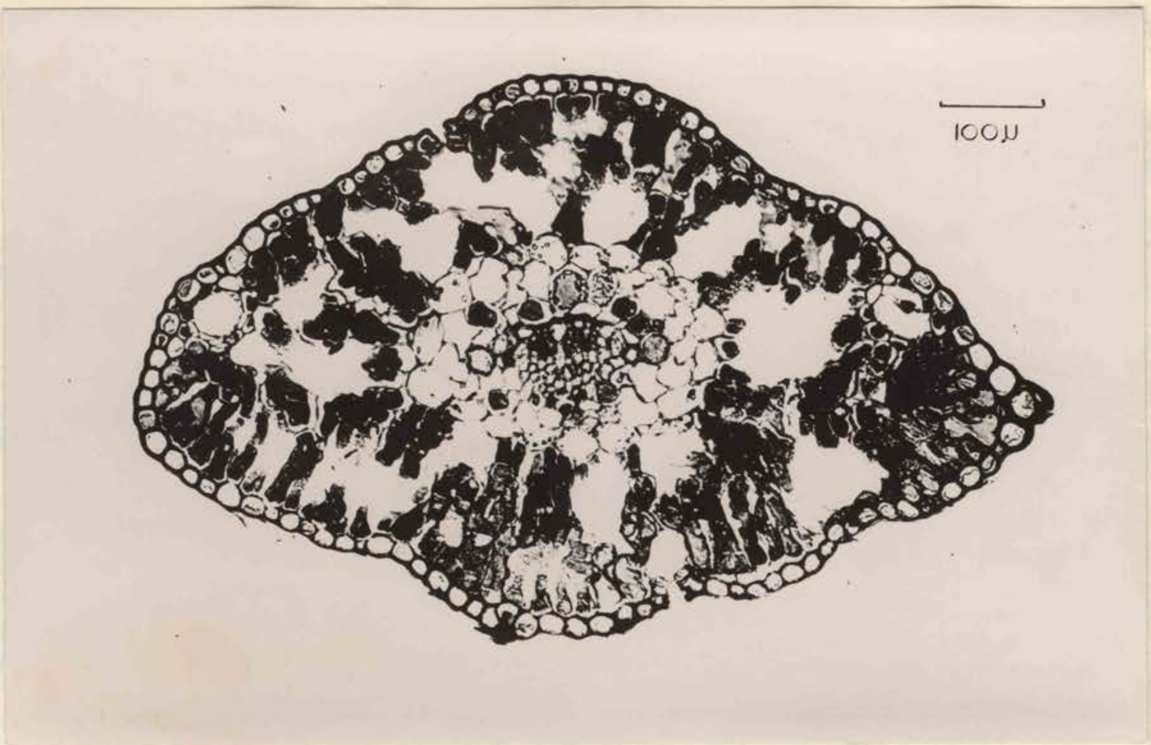
Top - P. muricata

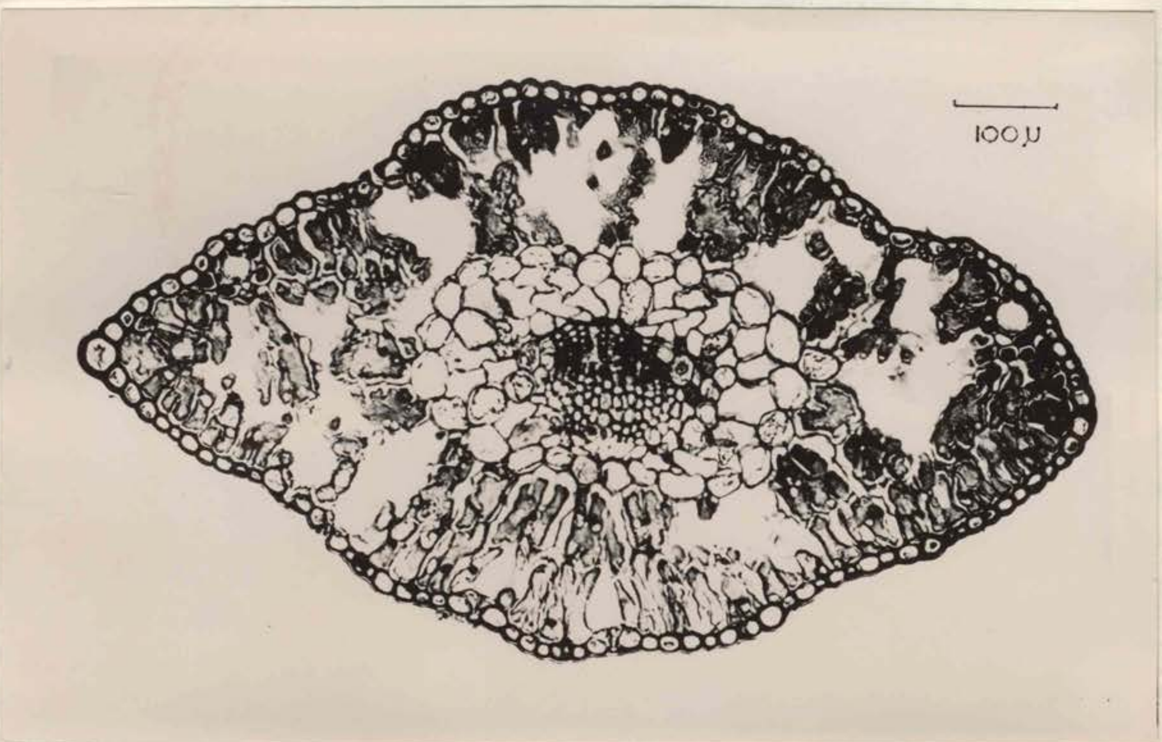
Centre - P. muricata x P. radiata

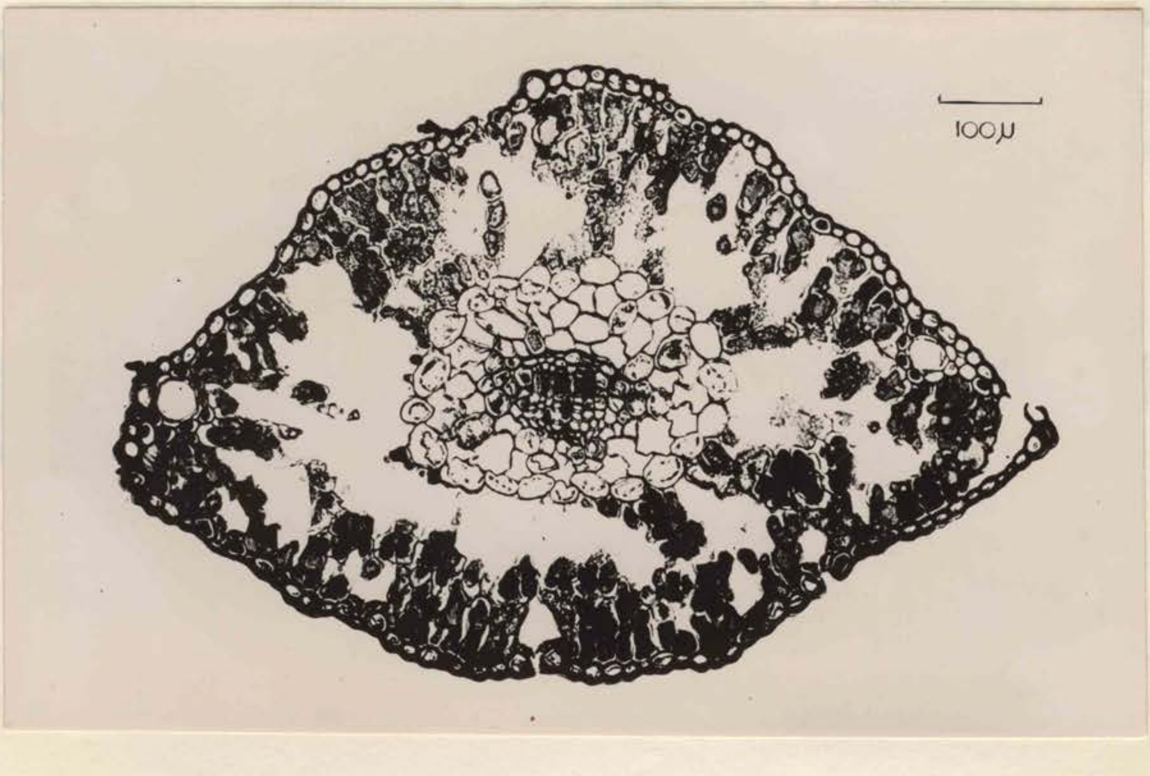
Bottom - P. radiata

FIG. 25 TRANSVERSE SECTIONS OF PRIMARY NEEDLES OF P. MURICATA, P. RADIATA AND THEIR PUTATIVE HYBRID

The sections are very similar; the features most frequently used for taxonomic purposes do not differ among them.


100μ


100μ


100μ

All available seedling groups were examined for the following features:-

1. Number of secondary needles per fascicle -

As the plants aged, secondary foliage appeared and it was possible to use additional features for verification purposes. P. muricata and Cedros Is. pine have a high proportion of mature fascicles bearing needles in groups of two, while P. attenuata and mainland P. radiata have needles typically in groups of three.

Each parental group (species or hybrid) was sampled by taking six pots (if available) containing different progeny lots. The largest four seedlings in each pot were assessed by counting the number of needles present in the oldest ten fascicles.

In some plants fewer than ten fascicles were present. It was noticed that the number of needles per fascicle often decreased in the later-formed fascicles. Plants which resulted from Germination Test 1, having P. radiata as the maternal parent, which were planted in the field in 1964, were also examined in this way. Results of this assessment are given in Table 20.

TABLE 20

NUMBERS OF SECONDARY NEEDLES PER FASCICLE

Parents	No.plts examined	% of needles in groups of -				
		1	2	3	4	5
P. muricata x P. muricata	17	0	56	42	2	0
P. muricata x P. muricata var. remorata	9	2	72	26	0	0
P. muricata x P. radiata	8	1	14	68	17	0
P. muricata x Cedros Is. pine	6	0	67	33	0	0
P. attenuata x P. muricata var. remorata	17	0	13	86	1	0
P. radiata x P. radiata	12	0	5	77	17	1
P. attenuata x P. attenuata	23	0	23	75	2	0
P. attenuata x P. attenuata	(19)	(0)	(3)	(95)	(2)	(0)**
P. attenuata x P. radiata	20	0	1	86	12	1
P. attenuata x Cedros Is. pine	24	0	11	88	1	0
P. radiata x P. radiata*	25	0	0.5	99	0.5	0
P. radiata x Cedros Is. pine*	25	0	2	97.5	0.5	0

* = Older plants growing in the field.

** = Bracketed figures exclude one atypical progeny.

There is a relatively high proportion of needles in pairs in the P. muricata parental group, and a low proportion in the P. radiata parental group. The putative hybrid, P. muricata x P. radiata is not very similar to either parent.

Crosses involving Cedros Is. pine show a higher proportion of needles in pairs than do similar ones with the predominantly three-needled mainland form. The cross *P. attenuata* x *P. attenuata* yielded an unexpectedly high number of two-needled fascicles; these were concentrated in a single pot. If the figures for this parental group are recalculated, omitting the deviant plants, a relationship is obtained for the *P. attenuata* x *P. muricata* var. *remorata* hybrid and its parents which is very similar to that for *P. muricata* x *P. radiata* and parents.

Thus, there is some evidence from secondary needle numbers that crosses of *P. muricata* (or its variety) or Cedros Is. pine with *P. attenuata* or *P. radiata* of mainland origin have produced truly hybrid progeny.

2. *Foliage and twig colours* - Seedlings of some pines develop distinctive colouring during winter months; some seedlings are also distinctly glaucous while others are green or yellow-green (for example Bannister 1958 *b*). On 10/9/1965, when the secondary needles per fascicle were counted, a record was made of foliage and twig colours on the same seedlings.

Crosses involving only *P. muricata* and *P. radiata*, or their varieties, were found to have green or yellow-green foliage, with light green, non-glaucous shoots, whereas those including *P. attenuata* as a parent frequently exhibited glaucousness or a reddish colouration of the needle tips and twigs, or both.

Rate of Germination may be used to distinguish hybrids from parental seedlings. (Righter and Duffield 1951 *a* and *b*). Examination of data from Germination Test 2 gave no indication that this feature would be of value in the present case.

Construction of Indices - Simple indices were prepared with the above data, for the two groups of hybrids which contained sufficient plants and of which parental seedlings were available for comparison.

(1) For the species *P. attenuata* and *P. radiata*, foliage and shoot colour was used as the distinguishing characteristic. Four features typical of *P. radiata* were each allotted a score of zero, while alternatives typical of *P. attenuata* were given a score of unity -

Foliage	- green = 0	or glaucous = 1
	- red colouration absent = 0	or red colouration present = 1
Shoot	- green = 0	or glaucous = 1
	- red colouration absent = 0	or red colouration present = 1

Results are given in Table 21.

TABLE 21

INDEX VALUES FOR P. ATTENUATA AND P. RADIATA

Percentage of plants having various scores

Seedlings	Score					Total
	0	1	2	3	4	
P. radiata x P. radiata	100	-	-	-	-	100
P. attenuata x P. radiata	19	52	29	-	-	100
P. attenuata x P. attenuata	17*	13	17	22	31	100

* The plants which made up this entry were atypical in needle numbers, having many of their secondary needles in twos.

(2) For the species P. muricata and P. radiata, secondary needle number was used as the distinguishing characteristic. A score of one unit was given for each fascicle among the first ten per plant having one or two needles. Results are given in Table 22.

TABLE 22

INDEX VALUES FOR P. MURICATA AND P. RADIATA

Percentage of plants having various scores

Seedlings	Score											
	0	1	2	3	4	5	6	7	8	9	10	Total
P. muricata x P. muricata	12	-	12	6	6	6	6	17	17	6	12	100
P. muricata x P. radiata	14	43	29	-	14	-	-	-	-	-	-	100
P. radiata x P. radiata	59	33	8	-	-	-	-	-	-	-	-	100

CONCLUSION

The young age of the plants involved and their morphological similarity made it difficult to verify the putative hybrids, but the few characters which proved useful do suggest that the groups examined were hybrids. Positive identification should be possible when the foliage and buds attain greater maturity.

XII. CONCLUSIONS

The times of flowering of the pines studied was appreciably influenced by topographic situation, almost certainly via temperature of the environment. The exact aspect of temperature which controls pollen maturation could not be deduced from field observations alone: solution of this problem will probably depend upon the use of controlled-environment facilities.

Small distinct differences were observed between the times of flowering of some provenances of the individual species. There was a tendency for the southern provenances to flower before the northern forms, but considerable variation from this simple clinal pattern occurred.

In the arboreta studied there was appreciable overlap in flowering times between P. attenuata and the later P. muricata, and a small amount of overlap between P. radiata and P. muricata. This latter was probably minimal for conditions of cultivation because of the small size and uniform nature of the stands examined.

Mechanical isolation proved to be unimportant: although some mean dimensions of pollen grains of the different species differ statistically, the differences are of little practical significance.

Cone yields from controlled pollinations were not affected by the species of pollen present. In the absence of pollination, rapid abortion occurred.

Yields of "clean" seed, containing full and empty seeds, were reduced by about 50% in interspecific crosses between P. muricata and either of the other species, in comparison with appropriate controls. The statistical significance of this reduction was marginal. "Full" seed yields from such crosses were low: 12% of all seed, compared with 84% for the intraspecific controls. However, germination of such full seed was as high as 100% in some lots.

Most of the breakdown in the reproductive processes following cross-pollination seems to occur at or near the time of fertilisation, although some ovules degenerate much earlier - a few months after pollination. The seed yields discussed above are a reflection of the timing of the incompatibility reaction which seems to be similar in all the incompatible crosses examined.

No evidence was obtained that growth of the hybrid seedlings was other than intermediate between that of seedlings of the parental species.

Because of the lack of suitable hybrid plants of known origin which had attained reproductive maturity (for example 5 - 10 years of age) no information was gathered on hybrid fertility.

Intraspecific isolating mechanisms affecting seed production were found to be remarkably well-developed in P. muricata, confirming a report from California to this effect (California Pacific Southwest Forest and Range Experiment Station 1963). The southern forms of this species cross with difficulty with P. attenuata and P. radiata, but the northern blue-foliaged provenances are highly isolated both from these other species and from the southern provenances. Too few seed lots were available to enable the geographic pattern of the intraspecific barriers to be clearly defined, but it seems that crosses between the northern provenances and trees from Monterey succeed more readily than those between the northern provenances and trees from the more southerly stand at Lompoc. It is possible that a reproductive discontinuity occurs at the sharp transition from the blue-foliaged northern form to the green southern type described by Duffield (1951) and which Forde and Blight (1964) suggested might coincide with a large change in turpentine composition between central and northern stands. The additional more southerly barrier suggested by limited data may also coincide with the transition between the central and southern chemical races.

What is the likelihood of the interspecific barriers breaking down in the areas of natural occurrence? There are few obvious barriers to the hybridisation of P. radiata and P. attenuata - they occur in intimate contact, their flowering times overlap and viable seed yields from manipulated crosses are high, but there is little natural hybridisation or introgression. The chances of P. muricata and P. radiata hybridising are much more remote. Flowering times are more widely separated, although a small degree of overlapping was observed in cultivated stands and yields of viable hybrid seed from manipulated crosses is very low. The opportunity for the third interspecific combination, P. attenuata and P. muricata to produce natural hybrids does not occur, although the trees do come within a mile or two of each other south of Fort Bragg. If in the recent past the two species did contact each other, there is little doubt that the barriers present would have effectively prevented much hybridisation.

The reproductive differentiation of P. muricata was not anticipated: no similar instance has been reported in Pinus although some species such as P. ponderosa Dougl. ex Laws. have very great natural ranges and show considerable morphological and ecological variation. If one applies the test of reproductive isolation alone as a measure of taxonomic status as in the "biospecies" of Cain (1954, p. 128), there is little doubt that the northern forms of P. muricata warrant specific rank: they are much more clearly separated from the southern provenances than are the "morphospecies" P. radiata and P. attenuata from each other. The circumstances leading to the establishment of such barriers within a species showing no outstanding variation in other characteristics and apparently free from steep selection gradients such as were described by Barber and Jackson (1957) are a matter for speculation.

A more complete understanding of the isolating mechanisms operating in the Californian closed-cone pines, together with results of detailed studies of morphology and chemical constituents, may eventually enable the taxonomic and phylogenetic status of the components of this group to be accurately evaluated.

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