PART3 : THE ORIGINS, DISPERSAL, AND DOMESTICATION OF TARO

In the preceding chapters (Part 2), I reported the survey and analysis of taro in New Zealand, a country located far from the likely geographical origins of the plant in Southeast Asia. Where exactly did the taro varieties growing in New Zealand and the Pacific Islands come from? To approach this question it was necessary to look westward to Australia, Papua New Guinea, and beyond into Asia. In Chapters Nine to Sixteen (Part 3), I describe the full geographical extent of wild taro in Australia for the first time. Analyses of ribosomal DNA variation were made after preparing recombinant plasmids with fragments of nuclear rDNA extracted from taro. In a survey of taro from Australia, Papua New Guinea, Asia, and the Pacific Islands, wide-ranging size variation was discovered among rDNA spacer fragments, in addition to the geographically widespread occurrence of what may be a single rDNA variant, in both diploid and triploid taro, from wild and cultivated habitats.

Investigations of chloroplast and mitochondrial DNA, and nuclear rDNA, and comparisons with other Colocasioid taxa, confirmed the taxonomic integrity of *C. esculenta*, consistent with suggestions for a single geographical origin of the species within mainland Asia. Restriction site mapping of the cloned rDNA fragments from taro, and *in situ* hybridisation to chromosomes, demonstrated that taro rDNA is structurally similar to rDNA in other eukaryote taxa. Ribosomal DNA variation in taro was therefore interpreted, in part, by analogy to other taxa. Variation in taro rDNA was also interpreted in terms of possible local and long-distance dispersal by humans and natural vectors, within and between Asia and the Pacific.

The possibly natural origins of wild taro in Australia and New Guinea are discussed and then related to a broader view of origins, dispersal, and domestication of the species. Part 3 is adapted from Matthews (1990).

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On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Nine
Taro in Prehistory

9.1 Vegeculture and the Origins of Agriculture in New Guinea

Colocasia esculenta (L.) Schott, commonly known as taro, belongs to the pan-tropical and monocotyledonous family Araceae. In prehistory, before the modern era of world exploration, the cultivation of taro extended to Japan and southern China in the North, New Zealand in the South, Africa and southern Europe in the West, and Polynesia in the East (Leon 1977; Petterson 1977; Cable 1984). Within these extremes taro was, and still is, associated with diverse forms of agriculture, from forest swiddens to permanent fields, in drylands and wetlands (deYoung 1960; Peralta 1982; Spriggs 1984; Thaman 1984). The introduction of taro to the Americas is attributed to European shipping from Asia during the nineteenth century (Plucknett 1976). Taro is one of many crops introduced to Africa from Southeast Asia in prehistory (Purseglove 1976). Production of taro by traditional methods has declined with the recent worldwide decline of subsistence agriculture and its replacement with cash-cropping (Wang 1983). Taro has not become a major cash crop, and on a broad scale the present geographical distribution of cultivated taro reflects mostly prehistoric processes, unaffected by very recent dispersal.

Wide debate exists regarding the relative antiquity of agricultural systems that depend mainly on the reproduction of crops by vegetative reproduction (vegeculture) and those that depend mainly on plants propagated by seed (seed-culture). This debate, reviewed by Harris (1972), has various expressions according to the different suites of crop species found in Africa, Southeast Asia, and South America. In tropical Southeast Asia, taro and yams (*Dioscorea* spp.) are the major vegetative staples, and rice (*Oryza sativa*) is the major seed staple.

Since the 1940s, a number of botanists and geographers have suggested for Southeast Asia that seed-culture dominated by rice replaced an older vegecultural system, characterised by a suite of root crops, fruit trees, and nut trees, still thriving in New Guinea and the South Pacific islands (Harris 1972; Spriggs 1982; Golson 1985). This view was questioned by Gorman (1977), who suggested that rice and taro were domesticated at the same time from wild progenitors in swampy habitats in mainland Southeast Asia.

The aspect of prehistory of central concern here is the relationship between taro and human settlement of Sahul, the continental plate to which New Guinea and Australia belong. Among the traditional food plants of the Australians and the New Guineans are a number of genera and species familiar as cultivated starch sources in Asia and Oceania (Golson 1971a; Yen 1985a, 1985b). These include *Musa* spp. (bananas), *Dioscorea* spp.

(yams), *Ipomoea* spp., and taro. In Australia, wild taro are utilised, according to ethnographic records, within a hunting and gathering context (Roth 1901; Levitt 1981; Crawford 1982; Jones and Meehan 1989). In New Guinea (Irian Jaya and Papua New Guinea) taro also are wild, but are best known ethnographically as cultivars within wetland and dryland agricultural systems.

Human settlement of the continent Sahul occurred by 40,000 years ago, in the late Pleistocene (Jones 1988; Allen 1989). Archaeological and other evidence from Kuk, a swamp site in the Western Highlands of Papua New Guinea, indicate a sequence of agricultural practices from a claimed date of 9000 years ago (Golson and Hughes 1980; Gorecki 1986; Golson 1989). Although no remains of recognisably cultivated plants were recovered from the earliest deposits, the present wild and cultivated flora of New Guinea and nearby islands provide many candidates for early cultivation.

Yen (1971, 1973, 1982, and 1990 in preparation) has developed the hypothesis that agriculture in New Guinea had an early and independent origin and elaboration, based on the domestication of a range of Melanesian plants that included basic staples, vegetables, and fruits able to sustain populations in various environments. With the subsequent arrival of colonists from Asia came the introduction of a number of already domesticated plants, including taro, various yam species, and the Eumusa section of bananas. The position of taro in this hypothetical sequence was noted by Yen (1982) as being equivocal. Although it has been generally accepted, on linguistic grounds, that taro entered the Pacific relatively recently (c. 4000 years BP) with Austronesian speakers of Asian descent (Pawley and Green 1973; Blust 1988), suitability for wet conditions made taro a candidate for earlier cultivation at Kuk swamp (Golson 1977). If direct palaeobotanical evidence is found for cultivation of taro at such an early date, this could reflect the introduction of cultivated taro before Austronesian speakers arrived, and/or the selection and domestication of taro already present in the pre-agricultural flora of New Guinea. Yen (1982) suggested that so-called feral taro in New Guinea and Northern Australia could in fact belong to the natural eastward extension of the Indo-Malaysian flora. The challenge here for botanical research is to determine whether or not taro contributed to an indigenous development of agriculture in New Guinea, and to assess the likely agricultural connections with Asia suggested by the presence of Austronesian speakers in coastal Melanesia and Oceania.

9.2 Early Botanical Observations

In what remains the most thorough general taxonomic treatment of the genus *Colocasia*, Engler and Krause (1920) described the home of *C. antiquorum* (synonym for *C. esculenta*) as being doubtless in island Southeast Asia (‘Ostindien’). They noted, however, that the species frequently becomes wild along stream banks, assuming the appearance of a natural part of the flora in tropical and subtropical regions. In June 1770, the botanist Joseph Banks made a detailed description of taro at the Endeavour River, Northeast Queensland (Banks 1770, see Appendix 12), and collected taro in Australia that year (Figure 9.1), probably also at the Endeavour River. These records, made before

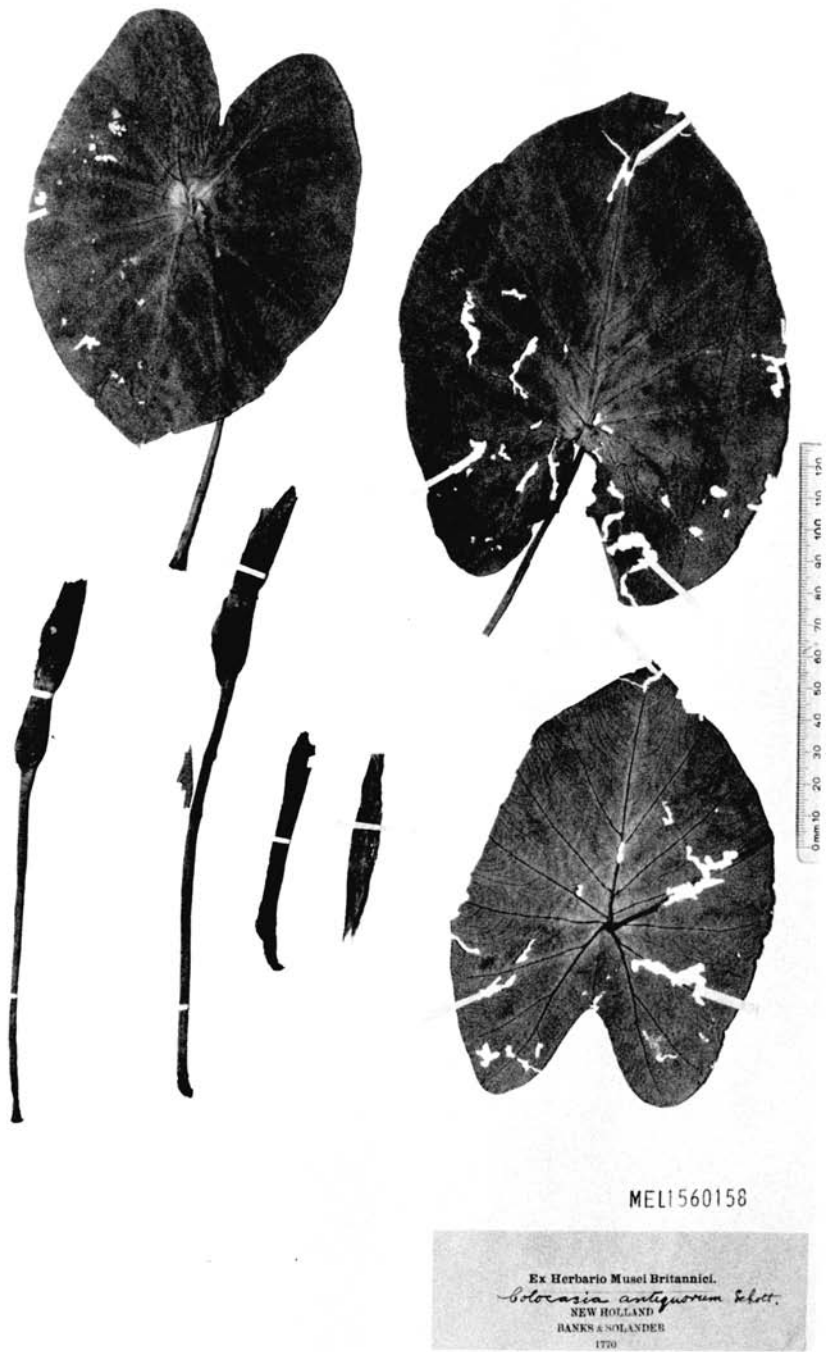

Figure 9.1 Australian specimens of *C. esculenta* collected by Joseph Banks in 1770. Note inflorescences on long peduncles, lower left. From the Melbourne Herbarium (an Australian specimen, collected by Banks in 1770, is also located at the Natural History Museum of Paris).

colonisation by Europeans, prove that taro was present in Australia in prehistory. The naming of taro within traditional Aboriginal classification systems also points to its antiquity in Australia (Jones and Meehan 1989: 127).

Ferdinand von Mueller (1865–1866), botanist at the Royal Botanic Gardens, Melbourne, described taro as 'apparently also indigenous in the warmer parts of East Australia', and Cheeseman (1900) reported taro as 'truly native in India and Malaya, and possibly also in some Pacific Islands'. Spier (1951) summarized previous observations as follows:

> Examination of the botanical literature reveals that little attention has been paid to the ultimate origins of many widespread plants. Taro (*Colocasia*) is, unfortunately, one of those so neglected. Even the great historical botanist, de Candolle, is vague about its origins, placing them in India, Polynesia, and the Malay Archipelago (de Candolle 1884). It seems probable, from other evidence, that we can rule out the last two of these, or at least Polynesia. Dispersion of the plant into Polynesia apparently followed human occupation of the region. This question hinges on the distribution of wild varieties of *Colocasia*. Burkill, in mentioning the presence of the wild plant in Polynesia, says that this is due to its robustness, not to its being a true native (Burkill 1935). Apparently here the "wild" form is not truly wild but escaped from cultivation. However, in continental Asia the case may well be somewhat different. Watt (1889), Balfour (1885), and Burkill (1935) all make references to wild varieties in tropical India: these are references to varieties of *C. antiquorum* and *C. esculentum*. In addition Watt states that *C. antiquorum* is found wild in Ceylon, Sumatra, and several islands of the Malay Archipelago .... I should be inclined to agree with Burkill's analysis of the situation and his statement that *Colocasia* is a native of India and elsewhere in Southeast Asia.

9.3 The Origins and Natural Geographical Range of Taro

In addressing the question of geographical origin, previous writers have not been explicit about whether they are referring to the origins of taro as a natural species, or to the origins of cultivated taro. If it is assumed that *C. esculenta* originated somewhere as a natural species, before humans were present, and that the geographical range was not extended by humans, then it follows that the origins of cultivated taro were within the natural geographical range.

Previous authors attempting to identify natural geographical range through observations of wild taro could not distinguish between plants ultimately derived from introductions, and of natural occurrence (early authors cited by Spier 1951, above; Hotta 1983; Matthews 1987, for example). The difficulty in identifying naturally occurring plants in the wild is compounded by the possibility that non-agricultural societies altered the distributions of the plant species they utilised (Ford 1985; Rindos 1984; Chase 1989; Yen 1989). Wild taro could have been introduced to wild habitats in areas beyond the natural geographical range of the species, before any association with agriculture. Contrary to the initial argument above, cultivated taro might not have originated within

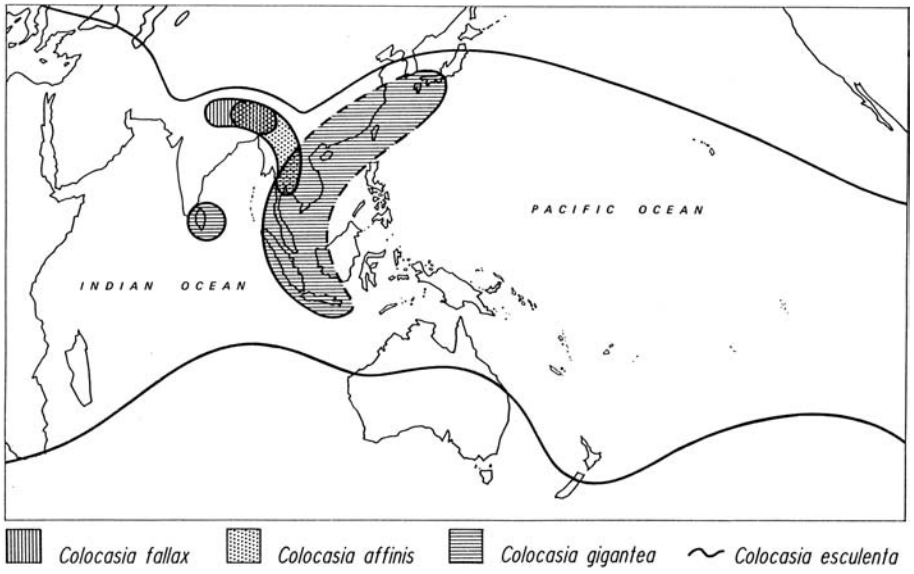

Figure 9.2 Distribution of *C. esculenta* (Africa to Americas), and other *Colocasia* species in Asia. Dotted lines indicate where the boundaries for *C. gigantea* are considered uncertain. See Appendix 13 for details of records.

the natural range of the species.

Practical and objective criteria for distinguishing indigenous and introduced taro are not obvious. In this thesis I describe progress towards establishing such criteria. Defining the natural geographical range of taro will be a major advance for investigating the origins of cultivated taro, and the origins of agricultural systems that were possibly based on taro cultivation.

9.4 The Origins of Taro as a Natural Species

Engler and Krause (1920) described seven species of *Colocasia*, but only four of them have been collected more than once, as revealed in a search of European herbaria in 1984 and 1985. These were *C. fallax* Schott; *C. affinis* Schott; *C. indica* (Lour.) Hassk., synonymous with *C. gigantea* Hook. f. (Hotta 1970); and *C. antiquorum* Schott, synonymous with *C. esculenta* (Linn.) Schott (Hill 1939). Figure 9.2 shows the distributions of these species based on herbarium records and published floristic accounts (Appendix 13). Records for the poorly known species, *C. gracilis* from Sumatra, *C. mannii* from Assam, and *C. virosa* from Bengal, are given in Appendix 14. Because all the other species of *Colocasia* are confined to Northeast India and Southeast Asia, it is proposed here that *C. esculenta* originated in this area. If phylogenetic relationships between the four species are determined, it will be possible to locate the origin of *C. esculenta* more closely within Southeast Asia, depending on which of the other species is

most closely related. For this reason, investigations with species of *Colocasia* other than *C. esculenta* were initiated and are described in this thesis.

The species *C. fallax*, *C. affinis*, and *C. gigantea* have much smaller geographical ranges than *C. esculenta*. *C. gigantea* is more widely distributed than the other minor species, which probably reflects its status as a lesser food crop of Southeast Asia. In southern Japan, *C. gigantea* is cultivated as a leaf vegetable (Hotta 1970), and the leaves are also eaten in Sri Lanka (V. Amarasinghe, pers. comm. 1985). Heyne (1927: 425; cited by Burkill 1966) reports that the fruit is used as a flavouring, and Li (1979) notes medicinal uses of *C. gigantea* stems. Although *C. gigantea* commonly bears fruit with many fully formed seeds (unpublished herbarium records) and might be easily dispersed by natural means, it is likely that humans have dispersed *C. gigantea* beyond its natural geographical range to some extent. The restricted ranges of *C. fallax* and *C. affinis* today are probably close to what are the natural ranges, since these species are little utilised. Li (1979) notes that *C. fallax* is used as a leaf vegetable in Yunnan, southern China, where the species is found in dense valley forest and shrublands, and *C. affinis* var. *jenningsii* is a rare ornamental in horticultural contexts outside Asia (personal observation). Dispersal of ornamental forms of *C. affinis* could have extended the geographical range of this species within Asia. No other records of the utilisation of *C. fallax* and *C. affinis* are known to the present author. Utilisation of *C. esculenta* as a food is commonly reported and all parts of the plant (corm, leaf, and inflorescence) can be eaten. Medicinal utilisation of taro is also common.

The above observations show that species of *Colocasia* other than taro are either not cultivated or are cultivated to only a minor extent, and assuming that this was the pattern in the distant past, then it is unlikely that *C. esculenta* first evolved within cultivation from one or more of the other species. Rather, it is proposed here that *C. esculenta* evolved as a natural species in Southeast Asia before utilisation of the genus by humans. This leaves open the question mentioned above, of what the natural distribution range of taro was before humans first utilised the species.

9.5 Dispersal Mechanisms: Sexual and Vegetative Reproduction

Herbarium specimens seen by the author, the author's own fieldwork, and published reports summarised and extended by Shaw (1975), Strauss et al. (1979), Sunell and Arditti (1983), and Nyman and Arditti (1985) support the view that wild taro produce flowers and viable seed abundantly throughout tropical areas of Asia and the Pacific. Experimental observations have demonstrated successful self-pollination (IITA 1978; Carson and Okada 1980), and cross-pollination (Wilder 1923; Patel et al. 1984). The morphology of inflorescences (male and female flowers separated by spathal constriction) and differential rates of male and female floral development encourage out crossing in taro (Jackson and Pelomo 1979), but self-pollination is probably also a normal event in wild populations, where pollination between flowering ramets (branches) of one clone is likely (author's observation). Inflorescences from Australian wild taro collected in 1770 can be seen in Figure 9.1. Fruit with seed, recently collected from wild taro in Australia

Figure 9.3 Inflorescences, fruit, and seeds of wild taro. *Top left*: a typical succession of inflorescences, produced from one shoot; in roadside ditch adjacent to secondary forest, Morobe Province, Papua New Guinea, June 1985. At left, a young inflorescence with spathe enclosing a spadix (not visible) which bears both female and male flowers. The upper part of the spathe turns bright yellow, releases a sweet scent, and opens slightly to admit pollinating insects. At right, an older inflorescence with upper part of the spathe completely unfurled, revealing the male portion of the spadix. At centre, an old inflorescence. The upper parts of the spathe and spadix have withered and fallen, and the remaining spathal chamber contains young fruit. Centimetre scale at base of plant. *Upper right*: young fruiting heads, one intact and the other dissected. At this stage, and at maturity, the fruit are green. Collected in rain forest at Behana Gorge, Northeast Queensland, Australia, by R. Hinxman, March 1989. *Lower left*: single ovary dissected to show the young seed within. From wild taro at Behana Gorge (details as above). *Lower right*: single mature seed taken from a herbarium specimen collected in a low-lying area of garden regrowth, near Ambunti, Sepik District, May 1966, by R. D. Hoogland and L. A. Craven (Rijksherbarium Leiden, specimen no. 226547). Note 500 um scale bar at side. Photographed at ANU with a Cambridge Model S360 scanning electron microscope; seed coated with gold, 20 nanometres thickness.

and Papua New Guinea, are shown in Figure 9.3 Although seed germination in the wild has not been described, it is likely that most sexual reproduction occurs in the wild, where flowers and fruit can reach full maturity most easily. Throughout recorded history taro has been a vegetatively propagated crop (Nyman and Arditti 1985), and seed might reach maturity and germinate in some cultivations. This does not occur frequently because most cultivated plants are harvested before flowers or fruit reach maturity. Haudricourt (1964) suggested that new clones of taro in New Caledonia are generated by plants flowering in fallow garden areas and in the wild.

The visibly predominant form of reproduction and dispersal in the wild is vegetative, and generative vegetative parts (corms, side-corms, and stolons) are frequently dispersed

down watersheds by water, from clumps of plants growing in or next to streams, rivers, and swamps (personal observation). Natural dispersal of pollen has not been observed, but drosophilid flies are believed to be vectors for taro pollen in Asia and New Guinea (Carson and Okada 1980). Hambali (1980) recorded the dispersal of taro seed by the common palm civet of Indonesia (*Paradoxuruxs hermaphroditus* Pallus, an omnivorous, frequently arboreal mammal). Marks left by the claws and teeth of the animal were found on the peduncle (stalk below the fruit), and germinating seed were seen in the excreta. Palm civets are widespread in Asia (Hambali 1980) and absent from Australia and New Guinea.

Nothing is known about the possible dispersal of fruit by birds, and specific topographical features that might be barriers or routes for long-distance natural dispersal cannot be identified, although distances alone suggest that the major Oceanic sea gaps are likely barriers. Natural long distance dispersal between watersheds must involve seed dispersal, because the vegetative parts of taro display no physical characters that suggest that animals could transport them intact.

The observations of natural reproduction and dispersal, although limited by the brevity of field surveys to date, are essential for interpreting the present geographical distribution of genotypic variation.

9.6 Analytical Approaches for Investigating the Origins and Dispersal of Taro

The combination of field surveys and laboratory analyses of genotype first began for taro in the 1950s with the application of cytological techniques that allowed direct visualisation of nuclear chromosomes. Surveys of chromosome numbers made by Yen and Wheeler (1968) and previous workers established that while diploid taro varieties (somatic chromosome number 2n = 28) are common in Asia and the Pacific, triploid varieties (2n = 42) are common only in Asia and, unexpectedly, in New Zealand. The nearest location of triploid taro outside New Zealand was found to be New Caledonia, but it was not known whether triploid varieties in these two countries were the same. Yen and Wheeler (1968) noted that triploid taro in New Caledonia and New Zealand could have been introduced from Asian sources following European colonisation in the Pacific. Matthews (1984, 1985) described the predominance of three phenotypically distinct triploid taro varieties in New Zealand today, but was unable to investigate the suggested outside sources.

Yen and Wheeler (1968) suggested that the general distributional pattern of diploids in Asia and the Pacific, and triploids in Asia, arose because cultivated diploids were introduced to the Pacific islands before cultivated triploids became common in Asia. They also suggested that the ultimate origin of the species was in India, because early reports indicated the presence of two chromosome number series in India, one of 2n =14, 28, and 42, and the other of 2n = 36, and 48 (see Darlington and Wylie 1955; Coates et al. 1988, for summaries of early reports). Surveys by Coates et al. (1988) and others have not confirmed the existence of the second chromosome number series, nor of the 2n

= 14 chromosome number, and the accuracy of the very early chromosome counts is now doubted (Coates, pers. comm. 1988).

Over the last four decades, analytical techniques for investigating genotypic variation have proliferated, with an accompanying progressive integration of old and new techniques. After cytological techniques achieved popularity, new electrophoretic and biochemical methods for investigating protein structure and enzymatic activity appeared, and iso-enzyme analysis became popular for evolutionary and biogeographical studies. Molecular genetic techniques developed during the last fifteen years make direct investigation of any kind of DNA possible, including genes for proteins previously investigated. Molecular and cytological techniques can be combined to visualise the positions of specific DNA sequences on chromosomes. A standardised range of molecular techniques is now available for investigating a far greater range of genotypic structure than was previously accessible by cytological or protein analysis alone.

Isozymes (Tanimoto and Matsumoto 1986) and corm proteins (Hirai et al. 1989) have been used to classify Japanese taro cultivars, but until such observations are extended beyond Japan, they cannot be easily related to the general history of the species. The present thesis represents the first application of molecular genetic techniques to the analysis of taro.

Applications of these techniques to basic questions about natural evolution, at one extreme, and for contemporary agricultural goals, at the other, are now common. The revolutionary rise of an international industry in modern agricultural genetics, with a large component of university research, has perhaps diverted attention from biogeographical studies of agricultural plants, although the two lines of work are perfectly complementary. There are many examples of molecular genetic studies of crop plant evolution exploring variation in diverse classes of DNA, notably ribosomal DNA, mitochondrial DNA, and chloroplast DNA. These studies have been based in agricultural research programmes, while the present investigation of taro is uniquely based in a programme of prehistorical research.

Molecular genetic studies with both evolutionary and geographical components have been carried out for only a few major crops. Notable examples are wheat, maize, rice, soybean, potato, and tomato, for which geographically extensive collections of germplasm were available. Much of the new information for these crops has not yet been related to social history. Prehistorians are interested in a very wide range of plant species significant as foods, medicinals, drugs, ornamentals, and sources of wood and fibre. While many of these are important crops today (taro, yam, banana, sugarcane, and sweet potato, for example), there are perhaps more which are not currently, or have never been, of major economic importance, and which are correspondingly unlikely to receive attention in agricultural research programmes.

9.7 Analysis of Ribosomal DNA

After preliminary experiments that are described in Chapter Eleven, ribosomal DNA analysis was chosen for the geographical surveys of genotypic variation in cultivated and

wild taro. Ribosomal DNA contains genes for a range of ribosomal RNA (rRNA) molecules that are incorporated into ribosomes (Figure 9.4). Ribosomes are the intracellular sites of protein synthesis, and are produced in large numbers in each cell. Often as much as 75 percent of the total cellular RNA is ribosomal RNA (Novikoff and Holtzman 1976). Most genes encoded by DNA sequences are transcribed into messenger RNAs, and these are later translated into protein sequences in synthetic reactions mediated by the ribosomes. Ribosomal DNA is thus essential to all cells in all living organisms. The nucleolus, an inclusion within the cell nucleus, is involved in the assembly of ribosome precursor particles. Nucleoli form at the chromosomal sites (loci) of the genes that code for the 18S, 5.8S, and 26S RNA molecules (Flavell and Martini 1982) and rDNA containing these genes is known as *Nor*-locus rDNA. Genes coding for rRNA were among the first genes isolated in pure form, and have been studied in detail in a very wide range of organisms (Appels and Honeycutt 1986).

In eukaryotes (which include flowering plants) one to five chromosomal locations (Nor-loci) contain extensive tandem arrays of repeated rDNA units, the total number of units ranging from 850 in rice to 9000 in maize. The number of rDNA units at a given locus is polymorphic within a species and in certain situations can change within a few generations. Within loci, individuals, and species, the nucleotide sequences of the ribosomal DNA repeat units are generally rather homogeneous as a result of intra-specific homogenisation processes, although the degree of homogeneity varies from one species to another. Repeat units vary in length and sequence through a range of mutational processes that include single base substitutions and sequence insertions and deletions. The units of the rDNA system are arranged in tandem, and appear to evolve in concert by as yet poorly understood mechanisms that include unequal crossing over, gene conversion, and sequence transposition between sister and non-sister chromosomes (Appels and Honeycutt 1986; Tautz et al.1987).

The rDNA unit has component sequences which evolve at different rates (Figure 9.5). The actual rRNA genes are very similar in distantly related taxa, while regions between genes, notably the large intergenic spacer region, may show virtually no similarity between distant taxa. Because of the different degrees of variability within different regions of the repeated DNA sequences, some kind of variation can be expected in any comparison of closely or distantly related taxa, and ribosomal DNA analysis has been used for many taxonomic and phylogenetic studies in both plants and animals (Appels and Honeycutt 1986; Hillis and Davis 1986; Rothschild et al. 1986). Assessment of evolutionary relationships within closely related taxa, using the more variable rDNA regions, is inevitably complicated by frequent mutation in such regions.

Among crop plant genera, rDNA variation between varieties and species, and variation in the chromosomal locations of rDNA tandem repeats, provide chromosomal, varietal, and species markers that can be used for crop-improvement breeding programmes (examples are May and Appels 1987 for wheat; and Zimmer et al. 1988 for maize). Appels and Honeycutt (1986) list seventy-three species or genera of flowering plants in which the structure and organisation of ribosomal DNA have been studied. Although most of these are cultivated species and their relatives, very few

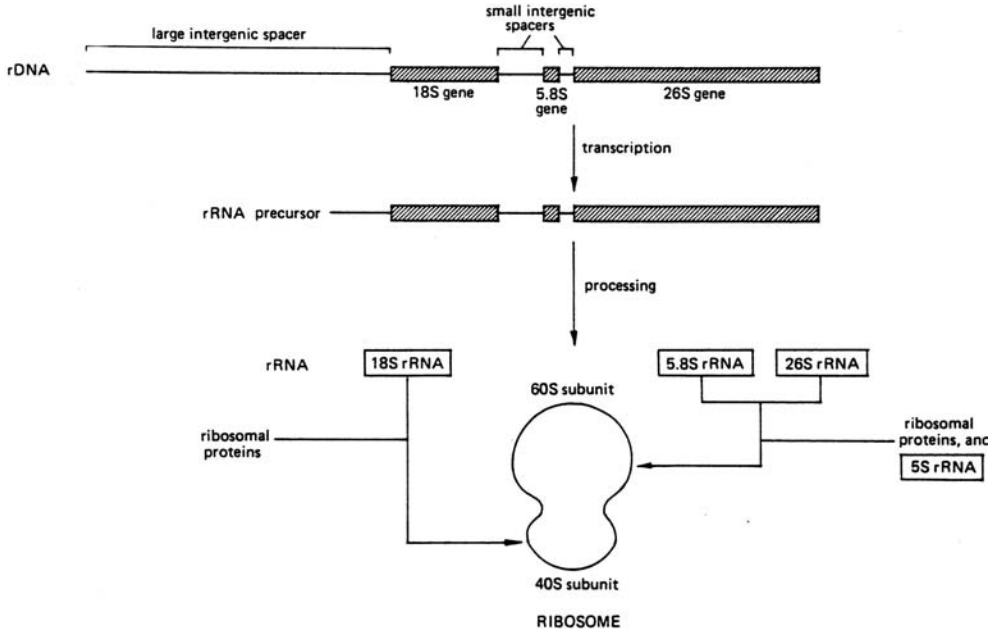

Figure 9.4 Structure and function of the *Nor*-locus ribosomal DNA repeat. Adapted from Hillis and Davis (1986)

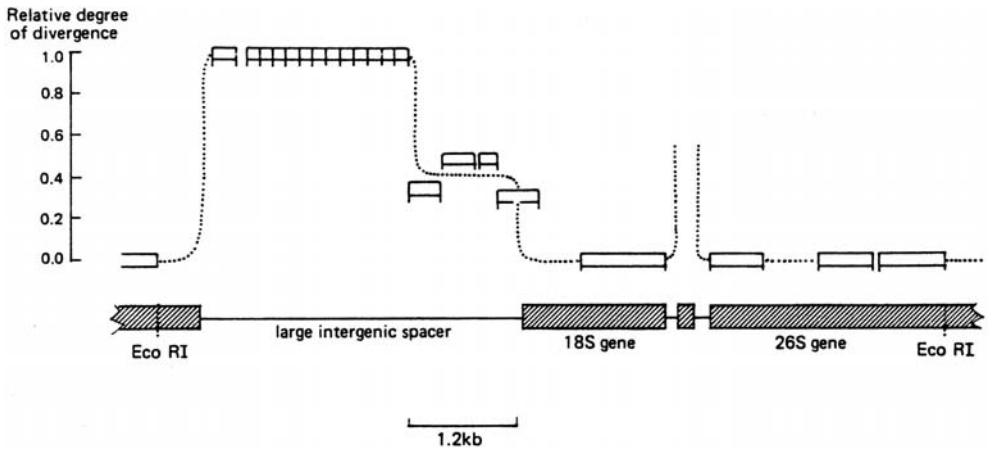

Figure 9.5 Summary of the relative degree of sequence divergence between cloned rDNA fragments from *Triticum aestivum* wheat (white blocks along upper dotted line) and four other species *Triticum*. Measures of sequence difference (thermal melting point data) were scaled relative to the maximally divergent sequences of the large intergenic spacer, and the minimally divergent sequences of the 18S and 26S rRNA genes. The asterisk, next to a break in the upper dotted line, marks a region where the exact position and degree of divergence were not determined. Adapted from Appels and Dvořák (1982b)

biogeographical studies of rDNA variation have been performed for cultivated species. The example of *Glycine* (soybean) and its relatives is of particular interest because it suggests and illustrates null hypotheses for the history of taro.

9.8 Soybean and its Relatives, and General Hypotheses for Ribosomal DNA Variation in Taro

The following summary follows Doyle and Beachy (1985). *Glycine max*, the cultivated soybean, is a major world crop today. The genus *Glycine* consists of nine species in two subgenera. The annual cultivated soybean and its wild progenitor, *Glycine soja*, belong to subgenus *Soja*. Both species are native to northeastern Asia and have diploid chromosome numbers of 2n = 40. Seven perennial species belong to subgenus *Glycine*, which has its centre of distribution in Australia. Chromosome numbers in most of these species are 2n = 40, but both diploids and polyploids (2n = 4x = 80) occur in *G. tabacina*, while a second species, *G. tomentella*, includes diploids, aneuploids (2n = 38, 78) and polyploids. Despite several bio-systematic studies on both subgenera, much remains to be learned about evolutionary relationships within the genus.

Ribosomal DNA was cloned from *G. max* and used to survey restriction enzyme digests of rDNA in collections of *G. max* and its relatives. Whereas heterogeneity for length and/or restriction sites was observed among small numbers of accessions in species of subgenus *Glycine*, no such variation was observed in over 40 samples of subgenus *Soja*. Sampling included accessions from throughout the native (sic = pre-modern c.f. natural range?) geographical ranges of *G. max* and *G. soja*, and a range of morphological and maturity-period types within each species. The absence of rDNA variation in subgenus *Soja* was thus thoroughly established. The species of both subgenera are inbreeders. The wild species of subgenus *Glycine* generally occur in small, isolated populations, and this, together with their breeding system, is presumably responsible for the variability observed amongst them. Human interest in the soybean, with a concomitant wide distribution of seeds and concerted efforts at plant breeding, possibly served as a homogenising force in the history of subgenus *Soja* (Doyle and Beachy 1985).

The history of taro might be similar, since putatively one species is involved, and has spread world-wide from an area of natural origin in Asia. If the general spread of taro were recent, with rapid dispersal by humans from a restricted natural distribution range, then the example of genus *Glycine* suggests that there should be little or no rDNA variation within cultivated taro. Alternatively, the natural distribution range of taro could be very wide, from eastern mainland Asia to the western Pacific, and ancient. Cultivated taro might possess very variable rDNA as a result of primary domestication processes over a wide natural distribution range in which ribosomal DNA variation was already present. Many other hypotheses can be imagined for contrast with general null hypotheses of (1) no ribosomal DNA variation in cultivated taro, (2) one area of origin of cultivated taro, and (3) a restricted natural distribution range, within the area of northeast India and Southeast Asia.

Initially, different kinds of DNA were assayed over a range of taro varieties, and in different species of *Colocasia*, using the methods described in Chapter Ten. These preliminary experiments, described in Chapter Eleven, demonstrated intra-specific variation in cultivated and feral taro, disproving the initial null hypothesis pointed above. The investigation of rDNA was therefore extended to include cultivated and wild taro from over a wide geographical range in Asia and the Pacific.

The structure and chromosomal location of ribosomal DNA in one variety of taro, described in Chapter Twelve, provide a reference point for subsequent observations of variation. Chapter Thirteen presents observations of rDNA that demonstrate the integrity of *C. esculenta* as a single taxonomic species. A survey of rDNA in diploid and triploid taro, from cultivated and wild habitats, and a wide geographical range, is described in Chapter Fourteen. In Chapter Fifteen, other kinds of biological evidence are considered in relation to the origins and antiquity of diploid taro in Australia and New Guinea, and triploid taro in Asia. Finally, in Chapter Sixteen, a general interpretation is given for the origins, dispersal, and domestication of taro.

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Chapter Ten
General Materials and Methods

This chapter describes the plants studied, sample field sources, and methods for DNA analysis, including the cloning of ribosomal DNA from taro and *in situ* hybridisation analysis of the rDNA chromosomal locus.

10.1 Identification, Description and Collection of Plant Samples

Plants used in the general survey of rDNA variation are identified individually in later chapters (detailed collection records for all the plants used were held at the Australian National University until their loss in an accidental archive fire). Here, the identification, description, and collection of taro and its relatives are outlined.

10.1.1 Identification of Colocasioid genera and species

The primary text for identifying members of Subfamily Colocasioideae is Engler and Krause (1920). A simple field guide to genera and species commonly encountered in the Pacific was provided by Massal and Barrau (1956), and the classification of Family Araceae to generic level has been revised by Bogner and Nicolson (1991) and Mayo et al. (1997). In the present study, different species of *Colocasia* were identified by reference to Engler and Krause (1920), and to herbarium specimens in European herbaria. Herbarium collections of *Colocasia* and other aroids have been extensively examined and their classifications checked, in recent years, by D. Nicolson of the Smithsonian Institution. Colour plates for *Colocasia* species analysed in the present study were prepared by the author at Photo Access Co-operative (Canberra), using the Cibachrome process, as follows: *C. esculenta* (Figure 10.1), *C. esculenta* var *fontanesii* (Figure 10.2), *C. gigantea* (Figure 10.3), *C. fallax* (Figure 10.4), *C. affinis* (Figure 10.5), and *Colocasia* sp, possibly a hybrid, *C. esculenta* x *C. affinis* (Figure 10.6).

10.1.2 Description of taro varieties

A protocol for the description and agronomic evaluation of taro varieties has been published by the IBPGR Executive Secretariat (1980), but this requires 115 separate items of information, many of which are not useful for distinguishing between varieties (Jackson and Breen 1985: 14). The simplified protocol used by Jackson and Breen (1985) also is designed for agronomic evaluation, and characters recorded include time to maturity, number of suckers (side-corms), corm shape, size, palatability, consistency, aroma, dry matter content, the edibility of leaves, and the incidence of pests and diseases.

None of these characters could be consistently examined in Canberra or in the limited time available during rapid field surveys. Ten colour characters for seven parts of the plant, and a simple categorisation of growth habit, were chosen for phenotype description (Figure 10.7) after experience in the field and with potted material indicated that the characters are stable:

1. Leaf

1.1 Blade veins: anthocyanin present or absent. Note if anthocyanin is located at junction with petiole.

1.2 Blade lamina (areas between the veins): anthocyanin present or absent.

1.3 Petiole — vertically graded colours: entirely green, yellow (rare), or anthocyanin pigmented; or a graded range from green to anthocyanin pigmented. Note if anthocyanin is located at junction with blade.

1.4 Petiole — variegation (stripes and/or flecks): present or absent. If present, the variegation may be green/white (only chlorophyll pigments involved), anthocyanin variegation against a background of green or white, or a complex mixture of chlorophyll and anthocyanin variegation.

2. Basal ring: white, green, or with anthocyanin. At the base of the petiole, under the sheath of the previous leaf, the dominant colour of the epidermis is usually white, leaving the basal ring distinct, but occasionally petiole epidermal colour extends to the base, obscuring the anatomically deeper colour or the basal ring.

3. Roots: white or with anthocyanin.

4. Skin (living epidermis of the corm below the outer layer of brown, decayed, epidermal and leaf tissue; scrape with knife to examine): white or with anthocyanin. 5. Cortex parenchyma (view in cross section of corm; the tissue between the skin and the zone of root initials): white, or with anthocyanin.

6. Core parenchyma (view in cross-section of corm; the entire region inside the zone of root initials): white, carrot orange, mustard yellow, or with anthocyanin. White includes faintly yellow, creamy colours, distinct from mustard yellow tints. Rarely, sectors of white and purple parenchyma were recorded, with a corresponding variegation of the corm skin.

7. Corm core fibres: colourless, white, yellow (usually very pale), or with anthocyanin.

8. Growth habit (lateral shoots): side-corms, direct shoots, or stolons. Stolons are defined here as side-shoots with at least one node and two internodes separating the mother corm and the lateral apex.

A range of presumed anthocyanin-based colours were observed: pink, red, purple, bronze (a mixture of purple and green), and black. This last, most intense, pigmentation was found only on petioles. Colour variants of taro arising during vegetative growth have been reported (e.g. Whitney et al. 1939: 46–47), but not in detail. Vegetative mutation of general colour pattern or morphology was not observed during the present study.

Detailed records of varietal differences in the size, shape, clustering, and number of central and lateral shoots were not made, since these are significantly affected by environmental conditions. Shoots growing directly from the central corm are not very

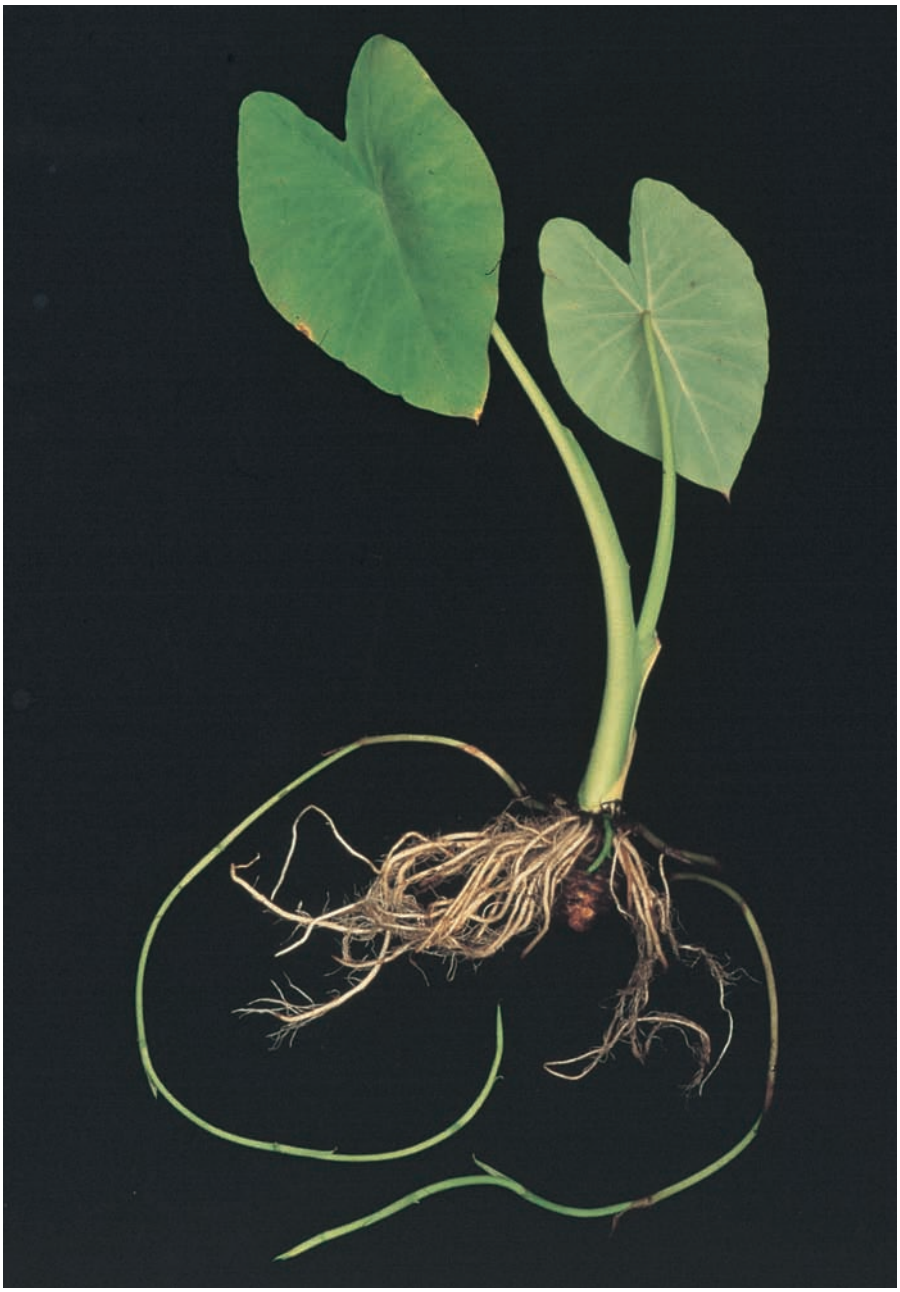


Figure 10.1 *C. esculenta* (L.) Schott from wild, tropical rainforest habitat, Russell River, northeastern Queensland, Australia. Phenotype typical for Australian wild taro: leaf blade with green veins and lamina; green petiole with no variegation; white basal ring; white roots; corm with white skin, white cortex parenchyma, white core parenchyma, yellow fibres, and small with a low density of starch: vegetative growth habit stoloniferous (potted plant maintained in Canberra). Chromosome number: 2n = 28. Wild taro in coastal Morobe Province, Papua New Guinea, displays a similar phenotype. Scale: plant approximately 0.5 m high (length of the larger petiole)

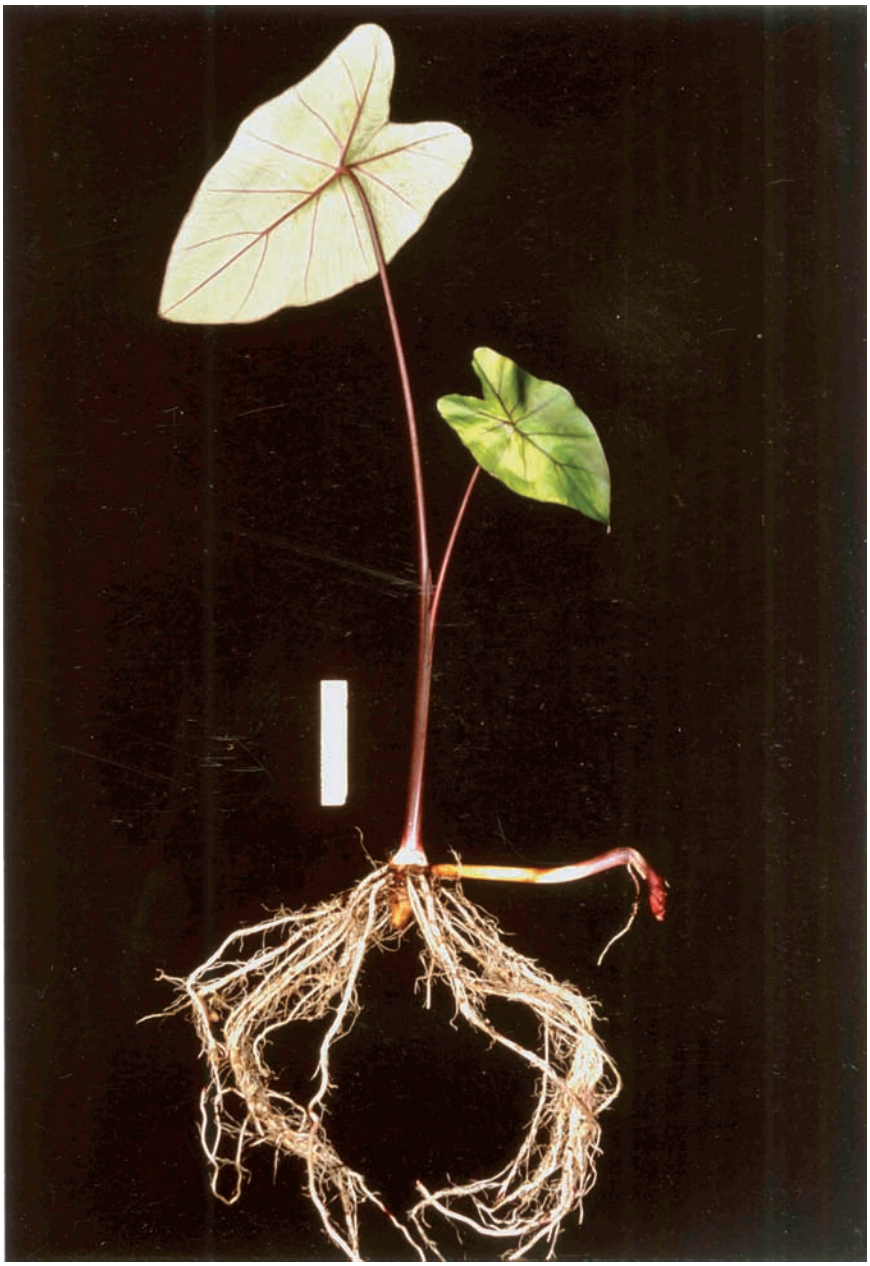


Figure 10.2 *C. esculenta*, var. *fontanesii* (Schott) A. F. Hill comb. nov., from wild lakeside habitat, inland mountain forest, Eliya, Sri Lanka (potted plant maintained in Canberra, T322). Not seen elsewhere in vicinity of the collection site; phenotype same as that of plants commonly grown as ornamentals in Australia and New Zealand: purple veins, purplish lamina, purple graded petiole colour, no variegation; white basal ring; white roots; corm with white skin, white cortex parenchyma, white core parenchyma, and yellow fibres; growth habit stoloniferous. The upper surface of the leaf blade has a distinctive shiny appearance and leathery texture. Chromosome number: 2n = 42. Scale bar: 5.5 cm

Figure 10.3 *C. gigantea* Hook, f., from Brisbane, source outside Australia not known. One-year-old seedling (T387.33) grown in Canberra from seed produced spontaneously, outdoors, in the Brisbane Botanic Gardens. White to green veins, green lamina, green petiole without variegation; white basal ring; white roots; corm with white skin, white cortex parenchyma, white core parenchyma, and yellow fibres, growth habit stoloniferous. Leaf veins in this species are more broadly spaced than is typical of *C. esculenta*, and the seed and inflorescence are also distinctive. Chromosome number: 2n = 28. See also Figure 18.1.

Figure 10.4 *C. fallax* Schott from Rockhampton, Australia, where it was grown as an ornamental (potted plant maintained in Canberra, T269; source outside Australia not known). Green veins, green lamina, purple petiole without variegation, white to green basal ring; white roots; corm with white skin, green to yellow cortex parenchyma, green to yellow core parenchyma, and colourless to yellow fibres; growth habit stoloniferous. The corm is a short narrow stem, almost woody, with a low density of starch. The lack of white colour probably reflects the lack of starch. The green to yellow colours probably represent environmentally induced variation in plastid development within the corm parenchyma. The upper leaf surface of this species has a matt sheen not found in *C. esculenta*, and the inflorescence is also distinctive. Chromosome number: 2n = 28

Figure 10.5 *C. affinis* Schott, var. *jenningsii* (Veitch) Engler, from cultivation in England (potted plant maintained in Canberra, T328; source outside England not known). Green veins, purplish black patches on lamina, green petioles without variegation, white basal ring; white roots; corm small with white skin; growth habit stoloniferous. The leaf colour pattern of this variety of *C. affinis* is not found in *C. esculenta.* Scale: 5.5 cm

Figure 10.6 *Colocasia* sp. (*C. affinis* x *C. esculenta*?), c.f. *C. marchalli Engler*; from Rockhampton, Australia, where it was grown as an ornamental (potted plant maintained in Canberra, T270; source outside Australia not known). Veins white to green, lamina purple edged with green, petiole green grading into purple without variegation, basal ring white; roots white; skin white, cortex white, fibres yellow; growth habit stoloniferous. Both the colour and the elongate shape of the blade distinguish T270 from *C. esculenta.* Chromosome number: 2n = 28 (P. J. Matthews count)

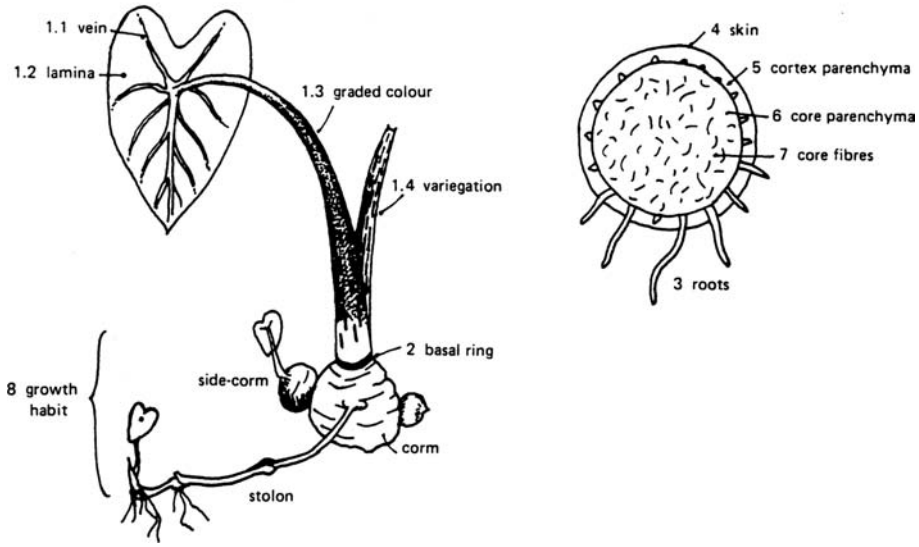


Figure 10.7 Characters for phenotype description of taro: 1.1–1.4 = leaf characters; 2 = basal ring; 3 = roots; 4–7 = central corm characters; 8 = growth habit (lateral shoots). The corm characters are illustrated in cross section (right). Side-corms and stolons are distinct character states which do not occur on the same plant. See text for descriptions of character states

common, and may or may not be environmentally induced. The two major categories of lateral shoot formation, side-corms and stolons, are consistently expressed under both field and glasshouse conditions.

Phenotypes were recorded (1) in the course of fieldwork, (2) for cross-checking the labels of accessions maintained in Canberra, and (3) to allow comparison of phenotypic and genotypic variation (Chapter Fourteen).

10.1.3 Field Surveys and collections

Exploratory fieldwork by the author was required to obtain wild taro from Morobe Province, Papua New Guinea (June–July 1985) and Queensland, Australia (September–October 1987). The geographical distributions and habitats of varieties collected in New Zealand were described by Matthews (1984, 1985). Further specimens of wild taro, mainly Australian, were received from various collectors, and most cultivated taro specimens were received directly from other collectors or from existing cultivar collections (Appendix 15). All plants received from outside Australia were introduced via the Australian Government Plant Quarantine Station at Weston Creek, Canberra, ACT.

Wild taro are widespread in Melanesia, but are rarely mentioned in ethnographic, agricultural, and botanical accounts. Treide (1967) discusses the role of wild plants as food in Melanesia generally, and cites Guppy (1887) for a record of taro growing wild

on river banks and in mountain ravines on islands in the Strait of Bougainville, Solomon Islands. Wild taro are common today in streams and swamps of the Solomon Islands generally (Patel et al. 1984; Spriggs pers. comm. 1989; Roe pers. comm. 1989). Henderson and Hancock (1988) note that the leaves and corms of wild taro, in the Solomon Islands, are usually too irritant to be edible. A wild variety on Rennell Island has an edible but fibrous corm, classed as a scarcity food, and the leaves of a wild variety in Malaita are collected as a vegetable. Within mainland New Guinea, wild taro have been reported across a wide geographic range. Bulmer (1968) notes feral *C. esculenta* as very plentiful in streamsides at 600 to 900 m and reaching 1500 m, among plants present in the Kaironk Valley region, Western Highlands, before European contact. Carson and Okada (1980) describe insects associated with wild taro which they found growing in rivers, streams, and along roadsides, near Lae, Morobe Province. Hyndman (1982) lists the leaves of wild taro, '*Colocasia* sp.', presumably *C. esculenta*, as a leaf vegetable obtained from foothill rain forest (500–1000 m) in the upper Fly River district of central New Guinea. In a survey of herbarium collections in Europe, Australia, and Papua New Guinea, only five records of wild taro in Papua New Guinea were obtained (Appendix 16), of which four were reported as being stoloniferous: on the Palmer River, Fly District; at Sogeri, near Port Moresby; at Nanokawari, West New Guinea; and at Buimo Creek near Lae, Morobe Province. The growth habit was not described for wild taro found near Mount Hagen and Mount Kuta, in the Western Highlands.

In 1985, wild taro were surveyed in the vicinity of Lae, Morobe Province, Papua New Guinea (Figure 10.8). In the lowland area surveyed, rainfall, temperature, and humidity are high throughout the year (McAlpine et al. 1983), and the wild taro occupy open, forest fringe habitats where both light and water are available (Figure 10.9). Although no clear distinction could be made between feral taro recently escaped from gardens and self-propagating wild taro, a high degree of phenotypic uniformity was noted among wild taro in sites that were not immediately adjacent to current gardens. Flowering was widespread. The commonly observed phenotype (Figures 10.1 and 10.10) consisted of a predominantly green blade and leaf, with a variable degree of purple colouring at the junction of blade and petiole, no variegation, a white basal ring, white roots, a vigorous stoloniferous growth habit, and a corm with very little starch (non-starchy). Throughout the area shown in Figure 10.8, village gardens contain various combinations of banana, sweet potato, yam, and taro. Under cultivation, the altitudinal range of taro in Papua New Guinea extends to 2700 m (Bourke 1982). The 1985 survey of wild taro was confined to altitudes of 1000 m and less, well below the potential altitudinal range for cultivated taro. It is thus possible that wild taro of the sort described above has a greater altitudinal range than recorded so far.

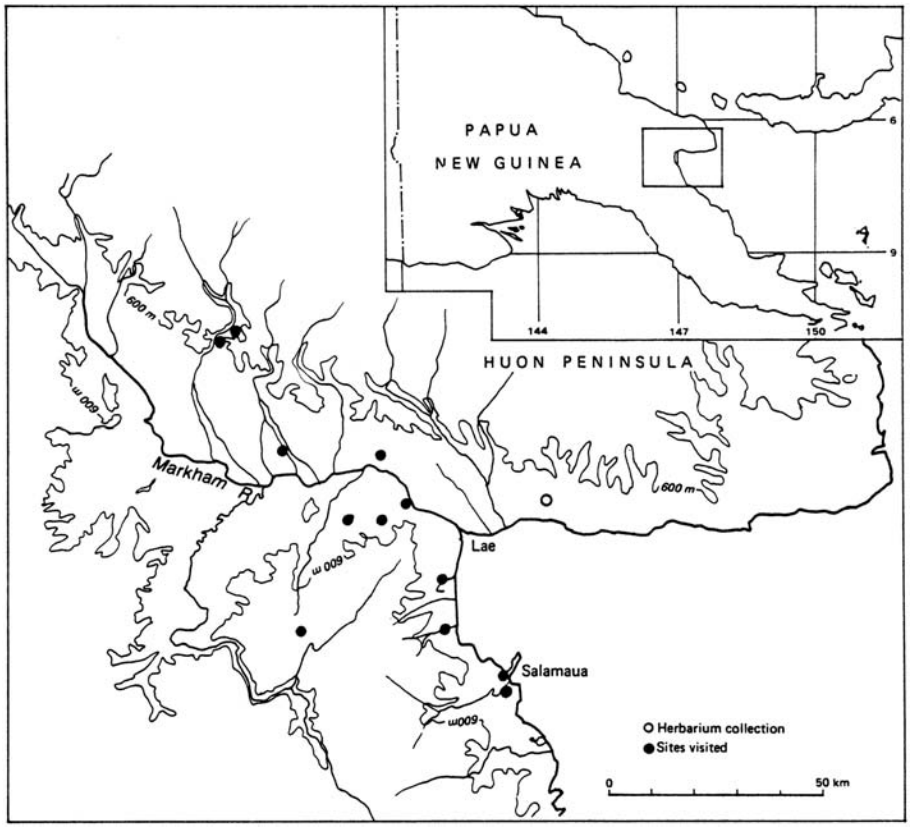


Figure 10.8 Locations of stoloniferous wild taro in Morobe Province, Papua New Guinea, July to August 1985. The search was limited to the vicinity of Lae, from Salamaua to the middle tributaries of the Markham River. One herbarium record is included (1964165 collection by A. Jenny). The sites are described in Appendix 16.

Figure 10.9 Wild taro, Morobe Province, Papua New Guinea (photos from Matthews 1987; see Appendix 16 for site details). Above: Pipi stream, near Labutali village, on coast between Lae and Salamaua, 16 July 1985; plants with long surface stolons, blade green with purple colour at junction to petiole, petiole green without variegation, basal ring white; inflorescences of various maturity, including some with young fruit and seed. Specimens of *Colocasiomyia pistilicola* Carson and Okada, a fly that probably pollinates taro, were collected from inflorescences at this site (see Figure 15.1). Below: Stream bank in forest near Nariyawan village, Leron River, inland tributary of the Markham River, 5 July 1985; a few metres from a foot track to mixed-crop gardens with taro. Phenotype like that described above, but not flowering (*in situ* photograph of Canberra live collection T225).

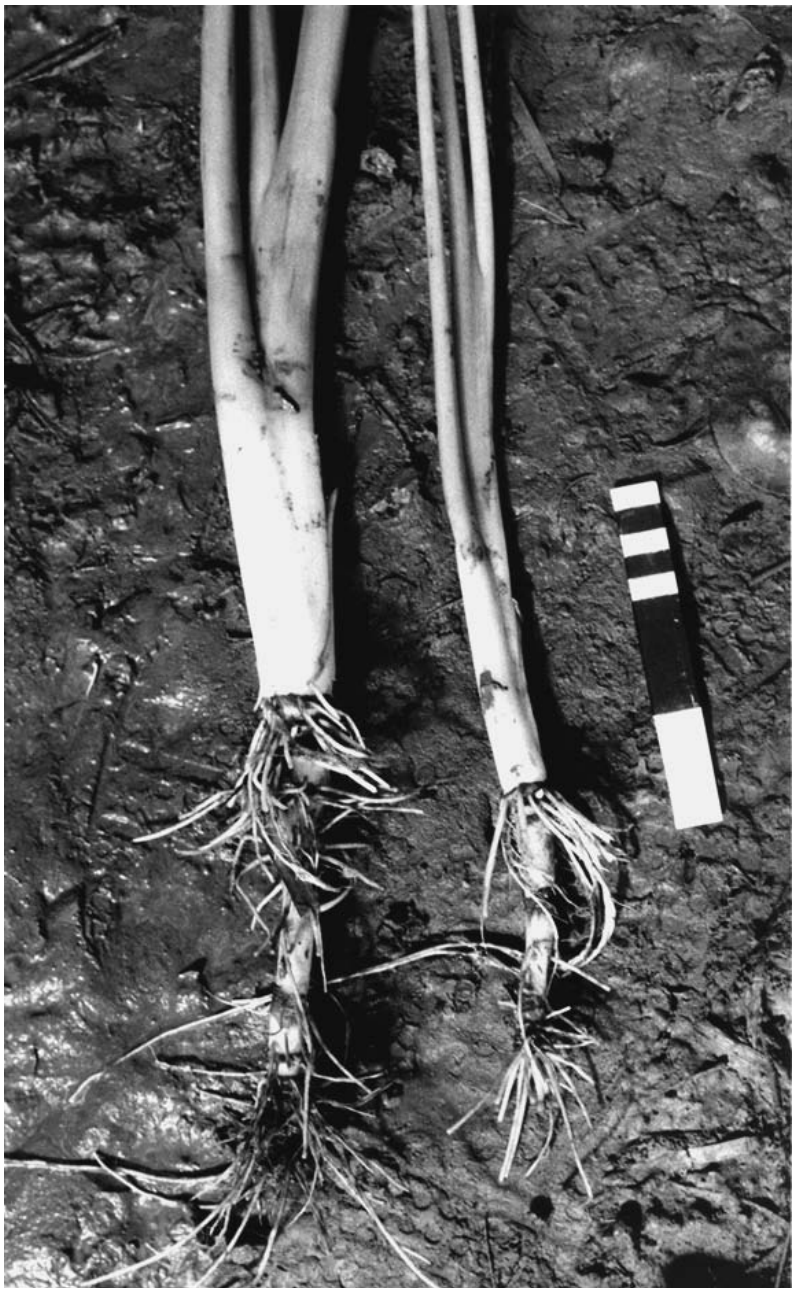


Figure 10.10 Wild taro photographed with alluvial mud at collection site, Rumu River, Morobe Province, Papua New Guinea; 29 June 1985 (*in situ* photograph of Canberra live collection T229). Blades and petioles green, basal ring white with coarse white roots, here cut short for the photograph, and non-starchy corms arising from stolons; wider than the base of the petiole. This phenotype is common among wild taro in Morobe Province, and wild taro in Australia display a similar phenotype. Scale bar: 15 cm

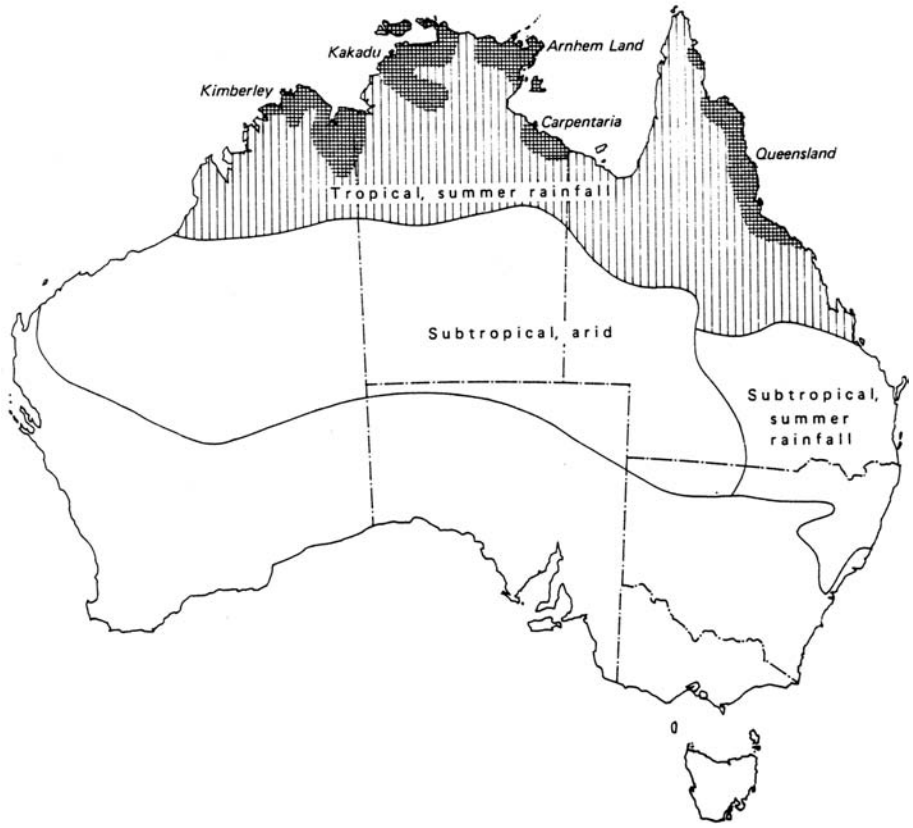


Figure 10.11 General distribution of wild taro in Australia, based on records from 1770 to 1989 (Appendix 17). Dark grey areas are where taro were recorded, and the light grey area indicates the possible full distribution range, within the climatic zone of tropical summer rainfall.

Records of wild taro in Australia are restricted to the climatic zone of tropical summer rainfall (Figure 10.11). The taro are widely distributed but generally rare, with highly localised patches associated with permanent water, in the Kimberley region of Western Australia (K. Kineally and S. Forbes pers. comm. 1985), Arnhem Land, Northern Territory (R. Jones pers. comm. 1989), and the Kakadu region, Northern Territory (Russel-Smith 1984). Exploration of the wet, tropical rain forest zone of northeast Queensland was undertaken because early ethnographic and botanical records and reports from correspondents indicated that wild taro is relatively common there (Appendix 17) and because access was easier than in the northern areas.

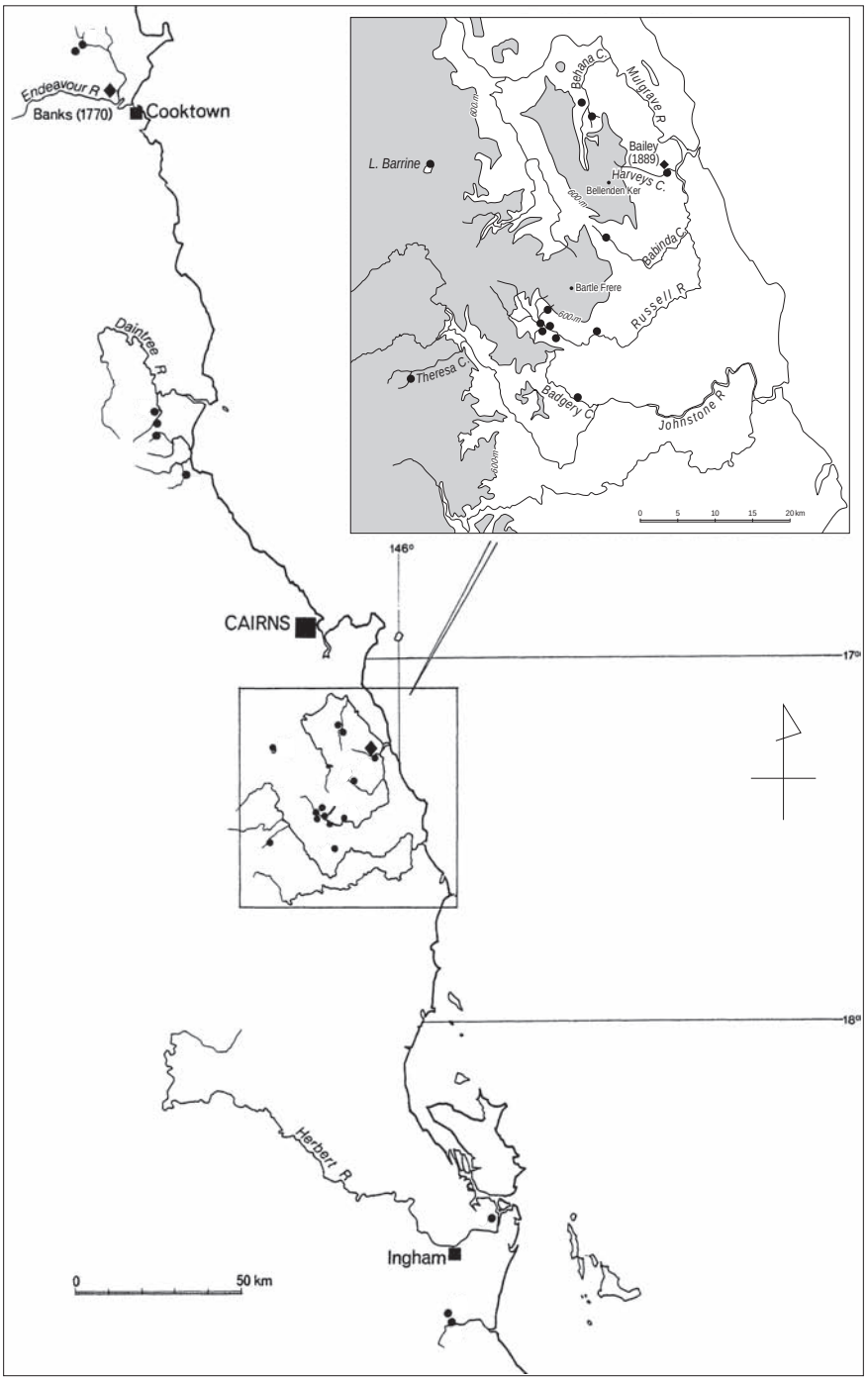


Figure 10.12 Wild taro in northeast Queensland: sites recorded between late 1987 and late 1989. One variety (Jiyer phenotype) was found in natural and disturbed habitats over the entire range shown. Historical records (Banks 1770, Bailey 1889) and site details are noted in Appendix 17.

Within the last twenty years (1970–1989), twice as many records have been made as in the previous 200 years (Table 10.1). The two least-accessible areas, Western Australia and Northern Territory, provide three-quarters as many records as the more accessible area, Queensland, although recent helicopter surveys by botanists are changing this balance (P. Latz pers. comm. 1989). During the last two years (late 1987 to late 1989) the specific search for taro in northeast Queensland yielded twenty-one records (Figure 10.12), as many as all previous records for this area. The small total number of records for Australia seems largely attributable to geographical remoteness. It is not known to what extent wild taro have been damaged by cattle and wild pigs. More damage is likely to be caused by cattle at permanent water holes in the drier northern areas.

The arid climatic zone (Figure 10.11) presents an unbroken southern barrier for taro in western and central Australia. In eastern Australia, the zone of subtropical summer rainfall does not impose any obvious, broad-scale, environmental limitation on the dispersal of taro. Three possible reasons for the lack of records south of Townsville can be suggested. First, natural short-distance dispersal and establishment could be prevented by the pockets of aridity that extend inland from the coast between Townsville and Bundaberg. Secondly, the ranges of animals and insects responsible for natural dispersal (if any) could be limited by climatic conditions, even though the plant is not. Thirdly, if humans have been responsible for dispersal, then unknown historical and cultural factors may have been involved which did not apply to the wide dispersal of taro across the top end of Australia. The area between Ingham and Cairns (Figure 10.12) competes with western Tasmania for the highest recorded rainfall in Australia, experiencing median annual rainfalls of 2400 to 3200 mm (Bureau of Meteorology 1986). The highest recorded altitude for a taro site in Australia, at c.980 m, is in this tropical, high-rainfall area at Cannabullen Falls on an inland tributary of the Tully River (B. Hyland collection, 27 March 1966; Appendix 17). Other sites approaching a similar altitude were recorded

Table 10.1 Summary of records for taro in Australia, 1770–1989, based on Appendix 17

	1770–1969	1970–1989	Totals
Western Australia	1	10	11
Northern Territory	4	17	21
Queensland	18	28	46
Unknown location	2	0	2
Totals	25	55	80

All or most of the records in this summary, and in Appendix 17, represent wild taro. Although most early reports do not indicate whether the plants were wild or cultivated, wild taro can generally be inferred because of an early date, prior to the institution of gardening by recent immigrants, or geographical remoteness. The summary excludes one uncertain but very early record by Leichardt (1847), and a repeated visit to one location by N. White (18 August 1983). Included in the summary are the different locations reported by Bailey (1889) and Roth (1901).

on the Atherton Tableland between Rockingham Bay and Cairns, during the period 1987 to 1988.

10.1.4 The Canberra Taro Collection

A living collection of taro was first established in Canberra in 1981 by D. E. Yen, with additions made continuously thereafter. Most of the collection was dispatched in 1989 to the Department of Botany, University of Selangor, Kuala Lumpur, Malaysia, and a representative range of Australian accessions was left with the Australian National Botanic Gardens in Canberra.

Taro plants are generally robust and survived well the process of field collection, transport, quarantine, and maintenance in Canberra. For international air transport, all soil was removed from the plants by washing in water, leaves were cut above the sheath of the outermost leaf, corms were cut two or more centimetres below the apex, and roots and rotting parts were removed. For transport, stolons were cut into conveniently sized sections, usually with at least two nodes each. Actively growing shoots were packaged in slightly damp cotton wool after dusting corm surfaces with sulphur. Dormant shoots (from temperate Japan) were packaged in dry paper. The shoots were wrapped with labels and placed in unsealed plastic bags with further labelling outside.

For maintenance in Canberra, the collection was kept heated and covered with glass or plastic at the Australian National Botanic Gardens and at the Research School of Biological Sciences. In summer, some plants were kept outside under shade cloth. The plants were grown in standard, sterile potting mixtures of peat, soil, pumice, and river sand, in plastic pots. Propagation was entirely by vegetative parts, generally corms, side-corms, direct side-shoots, or the nodes of stolons. Growth was strongest in summer and was encouraged by repotting, additions of fertiliser (Osmocote slow-release pellets), and by standing the potted plants in shallow trays of water. During the months of decreasing and short day-length leaves emerged slowly and decreased in size. Some accessions stopped growing completely. In winter, the plants were removed from trays and given less top watering and fertiliser. Fungal rots and garden slugs were the two most common causes for loss of accessions. Repotting, duplicate planting, encouraging active growth, and poison slug baits helped to reduce losses. White flies, aphids, and red spider mites were significant insect pests, particularly in late summer. Top watering and insecticide sprays achieved partial control of these. Plants that developed viral symptoms after quarantine were discarded after sampling for laboratory analysis.

10.2 Laboratory Methods

Sterile equipment and distilled water were used for all protocols. Frequently used stock solutions are described in Appendix 19.

10.2.1 Leaf harvest

For one DNA extract, leaves were harvested from one or more shoots of one clone. Clones were identified in the field by physical connections between shoots, and shoots

belonging to one clone were maintained in the Canberra collection under one accession number.

The best yields of DNA were obtained from leaves taken just before or just after emergence from the sheath of the preceding leaf (Figure 10.13). Leaves at this stage are usually yellow green or pale green, or visible by looking for the tip of the young leaf within the top part of a recently opened sheath. To remove the leaf, a thumbnail or blade was used to cut the petiole under and along the sheath, then to pull out the young leaf, detaching blade from petiole at the blade-petiole junction. Petioles yield little DNA and interfere later with smooth grinding of the frozen leaf blade. Very young leaves are yellow and yield less for their fresh weight. Older, emerged leaves also can be used, particularly any softer, immature portions, but the yield of DNA per gram of fresh tissue is less. It was not always possible to obtain an ideal leaf, and yields varied in the order of 100 to 1000 ug/g fresh weight. A good yield sufficient for several tests could be expected from a single young leaf 10–15 cm, in the immature rolled state, and weighing 1–2 g. After harvest, leaves were kept on ice for DNA extraction the same day. For longer storage (indefinitely) leaves were wrapped in aluminium foil with labels inside and out, and stored at -70°C or in liquid nitrogen (-176°C). Portable steel vacuum dewars were used for storing leaf samples in liquid nitrogen in the field (Queensland). For DNA extraction, the frozen leaves were removed from storage without allowing them to thaw. For sorting and unwrapping, deep-frozen samples were transferred to a small polystyrene container containing liquid nitrogen.

10.2.2 Extraction and purification of total DNA from leaves

A procedure similar to that described by Appels and Moran (1984) was used:

(1) Place 0.5–2 g of young leaf tissue in a mortar containing liquid nitrogen and approximately 1 g of acid-washed sand. Grind to a fine powder, using further liquid nitrogen to prevent thawing.

(2) Transfer powder to a second mortar, at room temperature, containing 6–8 ml of buffer (40 mM Tris.HCl, 80 mM NaCl, 160 mM Na EDTA, 0.5% SDS w/v, 0.05 mg proteinase-K/ml, pH approximately 7.5), freshly made by mixing 8 parts *stock leaf DNA extraction buffer*, 1 part 5% SDS (w/v H_2O), and 1 part fresh proteinase–K solution (0.5 mg/ml). Grind immediately but gently to ensure rapid penetration of the buffer.

(3) Transfer the viscous slurry to a 50 ml Nalgene polycarbonate tube, or other suitable tube, and incubate at 37°C for 1–3 hours while processing further samples.

(4) Add 1 g sodium perchlorate to each tube, dissolve by vortexing briefly.

(5) Balance tubes with either sand or *stock leaf-DNA extraction buffer*. Centrifuge to remove debris: 8000 rpm, Sorvall SS34 fixed-angle rotor, 5–8 minutes, RT or cold (e.g. 8°C).

(6) Transfer supernatant to clean tube, then gently add 9 ml of 70% ethanol saturated with sodium perchlorate (*EPR stock solution*), at RT. Mix gently with slow inversions to assist precipitation of DNA. The DNA precipitates may be white, yellow, or green, and stringy or cloudy. Stringy precipitates have DNA that is more intact, and are better than cloudy precipitates.

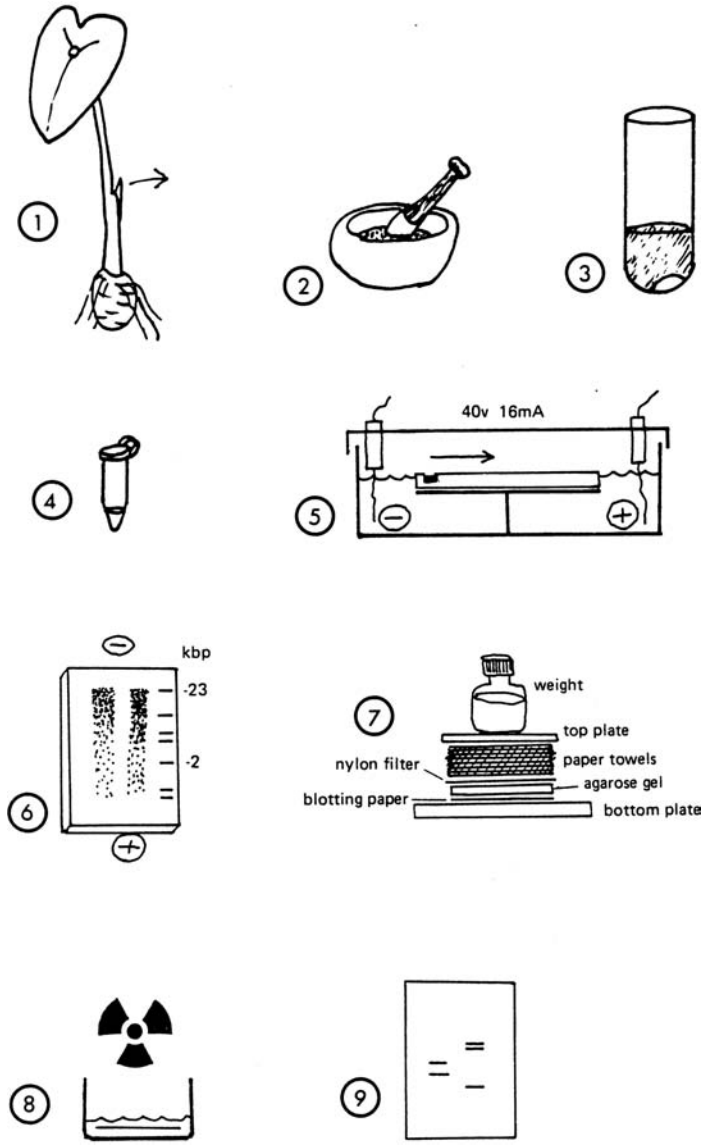


Figure 10.13 Schematic outline for DNA analysis: (1) harvest of young leaf, (2) reduction of tissue to powder by grinding in liquid nitrogen, (3) lysis with detergent and proteinase (enzyme), and further purification, (4) digestion of the DNA, in microgram amounts, with restriction enzymes, (5) electrophoresis in buffered salt solution after loading the reaction mixture onto an agarose, horizontal-slab gel, (6) visualisation of DNA samples and size standards (kilobase pairs) with ethidium bromide staining and UV light, (7) Southern transfer of DNA fragments to a nylon filter, by capillary fluid flow into paper towels, (8) incubation of the filter in hybridising solution with radioactively labelled DNA probe, (9) X-ray autoradiography, after washing to remove probe DNA that did not hybridise with the target leaf-DNA fragments.

(7) Remove stringy precipitates by winding on a sterile Pasteur pipette, retrieve cloudy precipitates by centrifugation: 8000 rpm, Sorvall SS34 fixed-angle rotor, 5–8 minutes, RT or cold.

(8) Resuspend the DNA in 0.4 ml (or multiples of 0.4 ml if pellet is large) of *stock 50 TE 20(8)*, in a 1.5 ml Eppendorf tube. Leave overnight at 4°C, if desired.

(9) To each 0.4 ml of resuspended DNA, add 0.2 ml *stock phenol* and 0.2 ml *stock chloroform*. To completely remove proteins and colour from the DNA, leave the mixture for several hours or overnight, at 4°C, without shaking. Spin 1 minute with bench centrifuge.

(10) Transfer the colourless, aqueous, upper phase to a fresh 1.5 ml Eppendorf tube and precipitate with 0.8 ml *stock ethanol and sodium acetate solution*, at RT. Store overnight or longer at 4°C or -20°C, if desired.

(11) Spin ethanol precipitate for 5–10 minutes with bench centrifuge. Discard supernatant and wash pellet with approximately 0.5 ml 70% ethanol. Dry moderately, then resuspend with 0.4 ml of *stock 10 TE 1(8)*. Leave overnight at 4°C and use Pasteur pipette to assist resuspension, if necessary.

(12) Add 2 ul of *stock boiled RNA'se* A (10mg/ml, Boehringer) to give a final concentration of 50 ug/ml. Incubate 20 minutes at 37°C.

(13) Add 45 ul of fresh proteinase-K solution (1 mg/ml) to give a final concentration of 100 ug/ml. Add 4.5 ul of 10% SDS to give a final concentration of 0.1% w/v. Incubate 20–30 minutes at 37°C.

(14) Add 0.2 ml *stock phenol* and 0.2 ml *stock chloroform*, shake vigorously, and spin immediately: 1 minute with bench centrifuge.

(15) Transfer aqueous phase to fresh 1.5 ml Eppendorf tube and precipitate with 0.9 ml *stock ethanol and sodium acetate solution*, store overnight or longer at 4°C or -20°C, if desired.

(16) Spin for 10 minutes with bench centrifuge. Discard supernatant and wash pellet with approximately 0.5 ml 70% ethanol. Dry moderately, then resuspend in an appropriate volume of *stock 10 TE 1(8)*, according to visible size of the pellet. Standardise resuspension volumes to assist routine estimation of DNA yield, later, e.g. 100 ul, 200 ul, or 400 ul.

10.2.3 DNA concentration and quality

Highly variable yields of lead DNA were obtained because the starting materials were not completely uniform. The bulk of total DNA extracted from leaves consisted of fragments in the 20–30 kbp size range, sufficiently intact for all requirements in the present study. DNA yields also varied with each bacterial culture if recombinant plasmid (methods described later). The following protocol was used to give DNA extracts of known concentration, and for quality checks:

(1) Estimate concentration by diluting 10 ul of each sample in 990 ul H_2O, and reading the optical density at 260 nm and 280 nm with a spectrophotometer. Calculate the approximate concentration for each sample, assuming that an extinction coefficient of OD 260 = 20 corresponds to 1 ug double-stranded DNA/ul (Maniatus et al. 1982: 458).

(2) Using the first estimate of concentration, electrophorese a standard quantity of each extract (e.g. 1 ug) on an agarose gel, stain with ethidium bromide, and photograph. Use visual comparison with a commercially supplied DNA sample of known concentration (e.g. intact lambda DNA) to check for under- or over-estimation. This step can also be used to check the degree of DNA degradation after extraction, and the amount of RNA accompanying the DNA. Estimate yields and adjust volumes with *stock 10 TE 1(8)* to give a final concentration of 1 ug DNA/ul. For extracts which require a smaller volume, precipitate the DNA with ethanol and sodium acetate, then resuspend.

Leaf DNA extracts which were yellow or yellow-brown after extraction and purification were often poorly cut by restriction enzymes. Because of the differential absorption of light by DNA and other compounds, the optical density ratio of OD 260/ OD 280 indicates the degree of nucleic acid purity. Pure DNA gives a ratio of 1.8 (Maniatus et al. 1982: 468). Contamination with RNA raises the ratio towards 2.0, while contamination with protein or phenol lowers the ratio. The protocol for lead DNA extraction, above, usually gave extracts with ratios in the range 1.6–1.9. Such extracts were reliably cut by restriction enzymes. Extreme values of c.a. 1.3 and 2.2 also were observed. Ratios above 1.8 indicate RNA, which does not interfere with restriction enzymes; ratios below 1.6 indicate contaminants, notably phenol, which may interfere with restriction enzymes; and at both extremes of optical density ratio, the amount of DNA present cannot be estimated accurately with the OD 260.

10.2.4 Propagation, extraction and purification of plasmid DNA

Various recombinant plasmids containing mitochondrial ampicillin resistance were propagated in *E. coli* on agar or in liquid, using Luria-Bertani (LB) medium with ampicillin. Mini scale extracts of plasmid DNA were prepared by alkaline lysis for screening plasmids after ligation with taro DNA fragments, and to create stocks of previously constructed and defined recombinant plasmids. Large-scale plasmid extracts were prepared by alkaline lysis of purification on caesium chloride gradients. Highly purified plasmid DNA was used to prepare radioactive probes and for restriction enzyme analysis. The protocols for plasmid propagation, extraction, and purification are essentially as described by Maniatus et al. (1982).

Mini-scale alkaline lysis

(1) Use a single, fresh bacterial colony from an agar plate to inoculate 2–5 ml of *stock LB medium* with ampicillin (50mg/l). Incubate for 5 hours to overnight at 37°C with vigorous shaking. Alternatively, streak a single colony onto a fresh LB agar plate, with ampicillin (50 mg/1), and incubate overnight at 37°C.

(2) Transfer 1.5 ml of liquid culture to a 1.5 ml Eppendorf tube and spin for 1 minute with bench centrifuge. Discard supernatant, and drain well or aspirate.

(3) Resuspend pellet by vortexing with 100 ul of ice-cold *stock GET solution* (50 mM glucose, 10 mM Na_2EDTA, 25 mM Tris.HCl, pH 8.0). Alternatively, take 1–2 platinum wire loops of cells scraped from an overnight agar plate culture, and resuspend in 100 ul of ice-cold *stock GET solution*. Stand tube at RT for 5 minutes.

(4) Add 200 ul of a freshly made solution of 0.2 MNaOH, 1% SDS, mix contents by inverting the tube rapidly two or three times, do not vortex. Stand tube on ice for 5 minutes.

(5) Add 150 ul of ice-cold *stock 3M potassium, 5M acetate*. Invert tube and vortex gently for a few seconds, until well mixed. Stand tube on ice for 5 minutes.

(6) Spin for 5 minutes with bench centrifuge, 4°C. Transfer supernatant to fresh 1.5 ml tube. Add 200 ul each of phenol and chloroform. Vortex briefly and spin 1 minute with bench centrifuge.

(7) Transfer upper aqueous phase to fresh 1.5 ml tube. Add two volumes of 100% ethanol at RT, vortex, and stand at RT for 2 minutes.

(8) Spin precipitate 5–10 minutes with bench centrifuge. Discard supernatant, then wash pellet with approximately 0.5 ml of 70% ethanol. Dry moderately, then resuspend with 50 ul of *stock 50 TE 1 (8).*

(9) Use 10 ul of this extract for further analysis, e.g. digestion with a restriction enzyme and electrophoresis. If small DNA fragments are expected, add *stock boiled RNA'se* A (10 mg/ml, Boehringer) to give a final concentration of 20 ug/ml, before or after restriction enzyme digestion. Removal of the RNA'se is unnecessary, unless the DNA concentration is to be estimated by spectrophotometry.

Large-scale alkaline lysis

Variations that allow different day/night work schedules are included in the protocol that follows.

(1) Inoculate 5 ml of *stock 13 medium* containing ampicillin (50 mg/1) with a single colony from an agar plate. Incubate at 37°C with vigorous shaking for 2–4 hours until cloudy, or overnight (starter culture).

(2) Inoculate 500 ml of *stock LB medium* containing ampicillin (50 mg/1) with 5 ml of the starter culture. Incubate during the day for 3–4 hours, until culture reaches an OD 600 of 0.8–1.0. Alternatively, inoculate the 500 ml in the evening and culture overnight. Note: amplification of plasmid copy number by chloramphenicol treatment was not used here; sufficient yields were obtained without it (c.f. Maniatus et al. 1982).

(3) Pellet cells by light centrifugation, e.g. 4,000 rpm, 4 minutes, in Sorvall GS-3 fixed-angle rotor, RT or cold.

(4) Discard supernatant and wash cells by resuspending with 100 ml of *stock 10 TE 1(8).* Pellet cells again as in step 3, and discard supernatant.

(5) Resuspend cells thoroughly in 8 ml of *stock GET* solution with freshly added lysozyme (50 mM glucose, 10 mM Na_2EDTA, 25 mM Tris.HCl, pH 8.0, lysozyme 5 mg/ml.). Use 10 ml pipette to assist resuspension, then transfer the cells to a 50 ml Nalgene polycarbonate tube, or other suitable tube.

(6) Add 16 ml of a freshly made solution of 0.2 M NaOH and 1% SDS, prepared from *stock 5M NaOH* and *stock 10% SDS*. Mix thoroughly but gently by inverting the tube two or three times, with parafilm cover. Stand tube on ice for 10 minutes.

(7) Add 12 ml of *stock 3M potassium, 5M acetate*. Mix by inverting the tube sharply several times, with parafilm cover. Stand tube on ice for 10 minutes.

(8) Centrifuge in either a Sorvall HB4 swing-out rotor, 9000 rpm (9,750 g), or a Sorvall SS34 fixed-angle rotor 10,000 rpm (10,400 g), 15 minutes, 4° G. The bacterial cell DNA and debris should form a tight pellet.

(9) Transfer supernatant to two 50 ml Nalgene tubes (approximately 18 ml to each). Add 0.6 volumes of isopropanol (12 ml) to each tube and mix well. Allow plasmid DNA to precipitate by standing tubes at RT for 15 minutes.

(10) Centrifuge in Sorvall SS34 rotor, 10,000 rpm, 30 minutes, at RT. Discard supernatant, then wash pellets with several ml of 70% ethanol. Dry moderately, and resuspend both pellets in a total of 8 ml *stock 10 TE 1(8)*. At this stage the pellets should have a translucent, colourless, rubbery appearance, and should dissolve readily.

Note: (1) to wash large (visible) DNA pellets it is not necessary to centrifuge them with the 70% ethanol, (2) plasmid DNA prepared in the manner above may be further purified by RNA'se treatment and phenol/chloroform extraction, or by centrifugation with caesium-chloride and ethidium bromide, as described next.

Purification of plasmid DNA by centrifugation with ethidium bromide in a caesium chloride density gradient

This method relies on the fact that ethidium bromide intercalates with double-stranded DNA in a way that depends on the conformation, supercoiled or relaxed, of the DNA. Plasmid DNA is circular and supercoiled when completely intact. The supercoiling reduces intercalation by ethidium bromide, so intact plasmid DNA retains a greater density than relaxed DNAs which bind more ethidium bromide (for example, nicked plasmid circles and linear fragments of nuclear, chromosomal DNA).

During centrifugation, caesium chloride (CsCl) solution forms a density gradient, and DNA molecules of different density migrate upwards or downwards to different positions according to the density of the surrounding gradient. The equilibrium position for each DNA molecule is where the density of the surrounding solution equals the density of the molecule, and the molecule migrates no further. Molecules of similar density are concentrated into one band. Centrifugation must be long enough for a gradient to form and for DNA molecules to reach their equilibrium positions. Excessive centrifugation compresses the gradient, and the DNA bands within it, towards the bottom of the tube. Only half the amount of ethidium bromide recommended by Maniatus et al. (1982) is required, a reduction that was found by trial to work and which is favoured because ethidium bromide is a potent carcinogen:

(1) Measure the volume of DNA solution (e.g. the dilute extract obtained by large-scale alkaline lysis) in a glass cylinder, and add 1 g CsCl for each millilitre. Cover with parafilm and mix gently by inversion. Stand cylinder for 1 hour, RT, to ensure that the CsCl dissolves completely. This solution may be stored at 4–8°C for at least a week, or may be frozen and stored indefinitely. CsCl will precipitate during storage, and larger protein aggregates may form. The latter may form immediately in any case, but are only an inconvenience (for loading through syringe, later). Protect the solution from light during storage.

(2) Add 0.4 ml of *stock ethidium bromide* solution (10 mg/ml in H_2O) for every 10

ml of CsCl solution. Mix well.

(3) Load the solution into a tube suitable for ultracentrifugation. For example, one 13.5 ml, polyallomer Beckman Quick-Seal tube will take the extract from one 500 ml cell culture that has been resuspended in 8 ml *stock 10 TE 1(8)* with 8 g CsCl and 0.32 ml *stock ethidium bromide* (10 mg/ml). A wide gauge disposable syringe fitted with a 10 ml barrel makes a convenient funnel. Pair tubes with DNA-CsCl-EthBr solutions of similar volume, or use CsCl blanks (1 mg CsCl/ml H_2O).

(4) Balance the paired tubes exactly with CsCl solution, and top with water-saturated paraffin oil. Check balance again, then seal.

(5) Centrifuge at 45,000 rpm for 36 hours, or 40,000 rpm for 40 hours, at 15°–20°C.

(6) Two bands may be visible in ordinary light, if the yield is high. Visualise faint bands with UV light. The upper band is linear bacterial DNA and/or nicked circular plasmid DNA. Insert a syringe into the top of the tube to let air in, then remove lower band with a second syringe. Lightly plug the upper syringe with tissue paper to prevent ethidium bromide solution squirting out. If only one band is found, take that. Record volume of the DNA fraction in the syringe (usually 1–2 ml), and transfer the fraction to a sterile glass tube.

(7) Add 1–2 volumes of water-saturated butanol, or pure isoamyl alcohol, cover tube, and shake to extract ethidium bromide. Repeat extraction four to six times, discarding the upper, non-aqueous phases.

(8) Place the lower, aqueous phase in a suitable ultracentrifuge tube, and dilute residual CsCl by adding *stock 10 TE 1(8)* (use 2x the original fraction volume). Beckman open-topped polyallomer tubes are suitable, 14 x 89 mm for SW41 Ti rotor, 25 x 89 mm for SW28 Ti rotor.

(9) To the new volume add two volumes of 100% ethanol. Mix well. If a large yield of DNA is expected, more than 50 ug for example, centrifuge immediately. For small yields, or if in doubt, stand tubes at -20°C for 1–2 hours. Some CsCl may precipitate, but will be removed by later steps. Centrifuge at 20,000 rpm, 1 hour, 4°C.

(10) Resuspend in 0.1–0.5 ml *stock 10 TE 1(8)*, depending on amount of DNA visible or expected, and the concentration desired. Use 10 ul diluted with 980 ul H_2O to estimate concentration by spectrophotometry (protocol 3, above). Check purity by agarose gel electrophoresis. There should be no high molecular weight bacterial DNA, and little low molecular weight RNA. Freshly extracted plasmids often form concatamers, resulting in more than one electrophoretic band. The extract is pure enough for most purposes, including restriction enzyme digestion and nick-translation.

10.2.5 DNA cleavage by digestion with restriction enzymes

Restriction enzymes (endonucleases) were obtained from Amersham, Bethesda Research Laboratories, Boehringer, New England Biolabs, and Pharmacia. Reactions mixtures for plant total-DNA were as follows: 2–8 ug DNA with 3–4 units of enzyme per ug DNA, in 20 to 50 ul of buffer comprised of DNA solution (DNA in 10 ul or less of *stock 10 TE 1(8)* or H_2O), 10x stock digestion buffer (10% of final total volume), and H_2O. Excess amounts of enzyme were added to allow for error in the estimations of DNA amount. To

establish many reaction mixtures at the same time, DNA was added to each tube in a fixed volume, plus or minus 2 ul, and mixed with an appropriately sized aliquot from a single pot of fresh, pre-mixed restriction enzyme and buffer, held on ice. This ensured uniform application of enzyme to each DNA sample, and reduced the manual effort. 10x buffers used were the *stock high, medium, and low salt* buffers recommended by Maniatus et al. (1982), or the *stock Tris-acetate (TA) buffer* of O'Farrell et al. (1980). TA buffer was satisfactory for *Bam* HI, *Eco* RI, *Hinf* I, *Kpn* I, *Rsa* I, *Taq* I, and *Xba* I, the enzymes most frequently used here. Incubation times were generally 1–3 hours, at 65°C for *Taq* I and 37°C for the other enzymes. Double digestions at 37°C were performed by incubating two enzymes at the same time in TA buffer. For double digestions, *Taq* I was added second and incubated at 65°C, after initial digestion at 37°C with a different enzyme. All reactions required for electrophoresis were stopped by adding a one-tenth volume of *stock bromophenol blue (BPB) running* dye (final concentrations in reaction mix: 0.01% w/v BPB, 6.8% v/v glycerol, 45 mM Na EDTA). Stopped reaction mixtures were sometimes stored at 4°C or -20°C, and thawed at RT or 60°C, before electrophoresis.

10.2.6 Agarose gel electrophoresis

DNA fragments were electrophoresed in horizontal-slab agarose gels (Figure 10.13) of various agarose concentrations and physical dimensions, chosen according to the expected fragment sizes (kbp) and amount (ug) DNA loaded. For rDNA analysis of total-DNA, gels were generally of 0.8–1.0% agarose, 13 cm wide, and 18 cm long from the loading slots. Reaction mixtures containing 4 ug total-DNA were loaded into slots 7 mm wide and electrophoresed overnight for approximately 12 hours at 16 mA and 40 V (fixed voltage), in TAE buffer (40 mM Tris-acetate, 1 mM Na_2 EDTA) prepared from *stock 50x TAE*.

To stain the DNA for UV light photography, each gel was immersed in approximately 200 ml H_2O before adding 5 ul of *stock ethidium bromide* (10 mg/ml H_2O). After gentle horizontal under a gentle flow of clean, cold tap water for 15–40 minutes, gels were illuminated from underneath with a 302 nm Transilluminator (UVP Inc.) and photographed in black and white with Polaroid positive/negative plates. DNA fragment sizes were determined by comparing their mobility with fragments of known size (size markers). Commercially supplied Lambda phage DNA (Boehringer) digested with *Hind* III was routinely used to provide markers. DNA fragments in total-DNA samples were only seen after Southern transfer from the gels and hybridisation probe analysis. Every gel was photographed immediately after electrophoresis to record the size markers, before Southern transfer, and also to record the success or otherwise of the restriction enzyme reactions.

10.2.7 Electroelution of DNA from agarose gels

Ribosomal DNA fragments were excised from recombinant plasmids with restriction enzymes, electrophoresed, visualised in UV light, and then electroeluted for sub-cloning (this chapter), restriction-site mapping (Chapter Twelve), and for preparing radioactive

DNA probes (Chapters 4–6). Two methods were used, essentially as described by Maniatus et al. (1982): electroelution from pieces of gel placed in a dialysis bag (Sartorius collodion bag), and electroelution into a trough cut in the gel immediately in front of the desired DNA band.

The eluted DNA fragments, in various volumes of electrophoresis buffer, were precipitated with two volumes of *stock ethanol and sodium acetate* solution, then centrifuged at high speed in Beckman SW28 or SW41 swing-out rotors, 20,000 rpm, for 1 hour, 4°C. To further remove gel contaminants, the fragments were resuspended in 0.2–0.4 ml stock *10 TE 1(8)* with addition of proteinase-K (final cone. 100 ug/ml) and SDS (final cone. 0.1%) and incubation at 37°C for 20 minutes. After one extraction with phenol and chloroform, the DNA was again precipitated, before final resuspension in *stock 10 TE 1(8)* to a convenient concentration (0.2–1.0 ug/ml), according to the expected yield, or the minimum volume needed for complete resuspension (50–100 ul).

10.2.8 Southern transfer

The following protocol, modified from Reed and Mann (1985) and Southern (1975), was used after electrophoresis to bind denatured (single-stranded) DNA to nylon filters.

(1) Place gel upside-down in a dry plastic box and submerge with *stock denaturing solution* (0.5 M NaOH, 0.5 NaCl). Cut away one corner for future orientation. Shake gently 30–60 minutes with horizontal motion.

(2) While the DNA denatures, cut and label a gel-sized piece of nylon filter (Gene Screen) with a ball-point pen, and cut away one corner for orientation. Soak the filter for at least 10 minutes in H_2O. Also prepare three gel-sized sheets of Whatman 3 mm chromatography paper. Soak one sheet in denaturing solution, drain excess liquid, and place the sheet on a flat plate. Later, this paper helps reduce lateral distortion of the gel.

(3) Place gel upside-down on the filter paper and blot excess liquid from the base plate. During electrophoresis, DNA migrates close to the underside, so DNA transfer upward is faster and more direct with this side of the gel upwards. The underside is also smoother, giving a better contact with the nylon filter, next.

(4) Lay wet nylon filter over the gel, orientating the cut corner with the cut corner of the gel. Line top edge along the leading side of the sample slots. Later, the edge of the filter provides a reference point for comparing migration of sample DNA fragments with the previously photographed DNA size markers.

(5) Briefly soak the second sheet of chromatography paper in H_2O and drain excess liquid. Place paper over the nylon filter, then use a Pasteur pipette to roll out air bubbles. Add the third sheet of chromatography paper, then stack 2–3 cm of paper hand towels on top. Cover with a flat plate and a centrally positioned weight of 200–500 g. Leave for at least 4 hours, or as much as 2 days if desired.

(6) Rinse the nylon filter for 1–5 minutes in 2x SSC at RT, blot lightly and place filter between two sheets of stapled blotting paper. Bake in tap-vacuum oven, 80°C, for at least 40 minutes and no more than 3 hours. Store at RT or cold, for up to at least 3 months if desired.

10.2.9 Preparation of radioactive probe by Nick-translation

Radioactive probes were made using a commercially prepared kit with convenient stocks of enzyme and non-radioactive nucleotide. Probes were usually made and used within a few days after arrival of fresh radioactive nucleotide. The following protocol (N. Contreras, pers. comm. 1986) is a modification of that recommended by the kit manufacturer (Bresa).

(1) To a 1.5 ml Eppendorf tube add the following in the order given: 0.5 ug DNA in 6 ul H_2O or *stock 10 TE 1(8)*, 4 ul buffer plus nucleotide cocktail (125 uM dATP, dGTP, and dTTP in 250 mM Tris.HCl, pH 7.6, 50 mM $MgSO_4$, 0.5 mM dithiothreitol and 250 ug/ml gelatin), 5 ul enzyme mix (5 units of *E. coli* DNA Polymerase I and 40 pg DNA'se I in a storage buffer of 40 mM potassium phosphate, pH 7.0, 1 mM MgCl, 0.2 mM DTT, 50% glycerol, and 200 ug/ml gelatin), 5 ul 2–^{32}P-dCTP (10 mCi/ml, specific activity 3000 Ci/mmole, Amersham). Vortex tube briefly, and spin for a few seconds in bench centrifuge.

(2) Incubate in 14°C water bath, 60 minutes.

(3) To remove unincorporated nucleotides, add 200 ul *stock 10 TE 1(8)* and apply the mixture to a Sephadex G-50 column, previously prepared in a disposable plastic column (Affini column, Amicon AF-100, diameter 8 mm, height 55 mm). Place column in 12 ml plastic tube (Falcon 2057) and centrifuge lightly in swing-out rotor, 3000 rpm (1,400 g), 3 minutes (Clements G-200 bench centrifuge). Discard column and store eluate at -20°C until required.

(4) Prepare hybridisation solution by mixing the radioactive probe with a maximum of 30 ml of fresh pre-hybridisation solution. This may be frozen stock thawed with a 50°–60°C water-bath, or new, and contains 0.1% SDS, 3x SSC, 5x Denhardts' solution, 50% formamide, 3 mM Tris.HCl; pH 8.0, and 0.3 mM Na_2EDTA prepared with 100% formamide (Fluka, highest purity) *stock 10% SDS*, *20x SSC*, *100x Denhardts'*, and *10 TE 1(8)*. Use minimum volume needed to just cover filter(s) in the hybridisation container.

During the nick-translation reaction, DNA'se I nicks the probe DNA, and DNA polymerase I catalyses incorporation of radioactive nucleotides into new DNA strands via the nicks (Old and Primrose 1981). The success of the reaction can be measured as the percentage incorporated radioactivity, (incorporated cpm/ total cpm) x 100. Incorporation (cpm) is measured by counting the number of radioactive emissions per minute from the probe DNA, after precipitation from an aliquot of the completed reaction mixture. Total cpm are measured directly from a second aliquot. The nick-translation protocol described above usually gives percentage incorporations of 30–60% (N. Contreras, pers. comm. 1988). Routine measurement of percentage incorporation was not needed because similar amounts of DNA were used in each reaction. Instead, the approximate incorporation (cpm) by the total eluted probe was routinely checked with a hand-held Geiger counter, for comparison with previous probes.

10.2.10 Hybridisation of radioactive DNA probe with DNA on a nylon filter

(1) Prehybridisation treatment: after Southern transfer (see above), soak filter in *stock pre-hybridisation solution* (0.1% SDS, 3x SSC, 5x Denhardts' solution, 50%

formamide, 3 mM Tris.HCl, pH 8.0, and 0.3 mM Na EDTA), 10 minutes –1 hour, 37°C, with gentle horizontal shaking. Fully immerse one or more filters individually in the pre-hybridisation solution, in a plastic food box covered to prevent evaporation. Just before the probe is ready to use, remove the filters and place them aside on the over-turned lid. After use, store the pre-hybridisation solution at -20°C, and re-use several times (but do not use for mixing with probes). Thaw in water-bath, 50–60°C, until precipitates of SDS and protein dissolve, giving a clear or slightly opaque solution.

(2) Boil probe to produce single strands: place probe solution (usually 25–30 ml) in a 100 ml conical glass flask and incubate with water-bath 95°–100°C, for 3–6 minutes. The probe DNA is now ready for hybridising to complementary single-stranded DNA bound on the filter.

(3) Remove and immediately pour probe into the empty box used for pre-hybridisation. The probe temperature should not be allowed to drop below 37°C, since this could promote a significant amount of reannealment by the probe (to the double-stranded state).

(4) Hybridisation: place up to three filters in the probe solution. Immerse one end of a single filter, then lower the rest of the filter carefully to prevent air bubbles being trapped. Before and after each filter, tilt the box to ensure complete coverage with probe. Cover box, and incubate at 37°C for at least 4 hours and as much as 18 hours, with gentle horizontal shaking. After hybridisation, return probe to the 100 ml conical glass flask, and store at 4°C or -20°C for reuse.

(5) To remove unbound or weakly bound probe, wash filters four times with a solution of 2x SSC, 0.1% SDS, pre-heated to 65°C, 10–15 minutes per wash, with gentle horizontal shaking. Cooling of the wash solution can be reduced by using a large volume (e.g. 250 ml per wash), which also lets the filters float separately. The number of washes seems to be more important than the time given to each. Filters can be left in the final wash solution at RT for at least a few hours, if desired, before autoradiography.

After the filter is washed, all DNA bound by Southern transfer remains, together with any probe DNA that has formed sufficiently strong complexes with similar or identical sequences on the filter. Low stringency washes separate only very weak complexes, and high stringency washes separate very strong complexes. The strength of each complex reflects the degree of sequence similarity between the probe and the target DNA. The stringency of the wash can be increased by decreasing SSC concentration, increasing formamide concentration, or by increasing the treatment temperature. The choice of conditions depends on the expected similarities between probe and target DNA fragments, and the desired sensitivity of detection.

The washing protocol given above was suggested by R. Appels (pers. comm. 1986) on the basis of work with wheat rDNA probes, and was routinely used here for taro rDNA probes hybridised with DNA from taro and its near relatives. Different conditions were used in preliminary experiments with chloroplast, mitochondrial, and rDNA probes from distantly related plant taxa (Chapter Eleven).

10.2.11 Reuse of probes and filters

Probes can be used a few times depending on the number of filters probed each time and the amount of probe removed by each filter. When severe non-specific binding occurs (artefactual spots and general smears), a large proportion of the probe may be lost (test probe solution with Geiger counter). A probe made with high incorporation of ^{32}P can be reused for at least two weeks, but will lose most radioactivity within a month because the half-life of ^{32}P is two weeks. Filters were cleaned for reuse by removing probe DNA with high stringency washes:

(1) Keep filter moist with water. It is more difficult to remove probe from dry filters.

(2) Probe stripping. Method 1 — wash filter 3 times with at least 200 ml H_2O, 85–95°C, 5 minutes each time, with horizontal motion shaking. Method 2 — wash filter once with hot water, as above, then wash for 10 minutes in 25 mM NaOH at RT. Wash twice more with hot water. Blot and store at RT or 4°C if desired.

(3) Treat as for a fresh filter.

10.2.12 Autoradiography

(1) After washing, blot radioactive filter lightly and seal within a very thin plastic bag.

(2) Place detector of a Geiger counter on the filter to locate and measure radioactive DNA bands. Choose an initial exposure time accordingly (30–60 minutes for bands emitting more than 500 cpm, 6 hours for emission of 50 cpm, 12 hours for emission of 10–20 cpm, 1–3 days if there is no clear signal).

(3) In a darkroom, sandwich the filter between two sheet of X-ray film (Fuji, NIF RX Medical), between two intensifying screens (Kodak) in a metal cassette: tape the filter and lower X-ray film to the lower intensifying screen, to prevent movement when the upper X-ray film is removed later. Mark both X-ray films to record the upper corners of the filter; these corners define the electrophoretic starting line. The exact position of the filter often becomes visible later, after development, as an outline against the area outside the filter.

(4) For short exposure, less than one hour, store the cassette at RT. For longer exposure, store cassette at -70°C. This gives sharper bands than long exposure at RT. Expose the upper sheet no longer than is thought necessary for the most radioactive bands on the filter. Replace the top sheet with a fresh sheet of film, develop the first film, and use this to guide exposure of the fresh film just added. Allow the lower film to over-expose with respect to the strongest bands, so that weak and possibly unexpected bands become visible.

10.2.13 Molecular cloning of taro rDNA

Taro *Eco* RI fragments were ligated into the tetracycline resistance gene of pBR322 and the ligation mixture used to transform *E. coli* strain RR1. Transformed bacteria, with ampicillin resistance conferred by pBR322, were immediately selected for by culturing the transformation mixture on agar plates with ampicillin. Transformants were replica-

plated directly onto agar plates with ampicillin, for maintenance, and onto nitrocellulose filters on ampicillin plates for further screening essentially as described by Grunstein and Hogness (1975). Six hundred transformant colonies were screened, and four were found with recombinant plasmids containing taro rDNA. The steps are described in detail below:

DNA preparation

Ribosomal DNA was partially purified by centrifuging total DNA to equilibrium in a CsCl/actinomycin-D density gradient, essentially as described by Gerlach and Bedrook (1979). *Eco* RI fragments were then prepared for cloning.

High molecular weight total-DNA was extracted from *C. esculenta* var AKL 34 using the protocol described above. The extract was quite pure (OD 260/280 = 2.0), with some RNA. Approximately 800 ug DNA was dialysed overnight against 25 mM sodium tetraborate, pH 9.0, giving a final volume of 2 ml. CsCl (58.07 g) was dissolved in 25 mM sodium tetraborate, pH 9.0, in a volume of 65 ml. The DNA solution and 400 ul actinomycin-D (0.5 mg/ml in 25 mM tetraborate, pH 9.0) were added, with further tetraborate solution to give a final volume of 68 ml (CsCl density 0.85 g/ml, weight actinomycin D/ weight DNA ratio 1: 4).

The solution was split between two polyallomer centrifuge tubes (Beckman Quick Seal), and centrifuged for 60 hours at 45,000 rpm. This produced a gradient with a clearly visible yellow band of actinomycin-D complexed with DNA, halfway down the tube. Fractions of 1 ml were taken from the bottom, and their OD 260 values determined (Figure 10.14).

Actinomycin-D binds to external guanosine residues on DNA double helices, forming complexes of lower density than DNA alone. Because rDNA repeats are relatively rich in guanosine, compared to most DNA in the extract, rDNA binds more actinomycin-D and reaches an equilibrium position of relatively low density. Aliquots (20 ul) from several fractions were tested for rDNA by dot blotting to a filter and hybridisation with a wheat rDNA probe, as follows. To each aliquot, 50 ul of *stock denaturing solution* (0.5 M NaOH, 0.5 M NaCl) was added. The mixture was incubated on ice for 5 minutes before adding 200 ul of ice-cold *stock neutralising solution* (3 M NaCl, 0.5 M Tris.HCl). After further incubation on ice (5–10 minutes), the mixtures were suctioned through a nylon filter, pre-soaked with H_2O and held in a perspex dot-blotting device with circular wells and attached to a tap-vacuum. The mixtures were suctioned through a nylon filter. Each well was rinsed with a further 100 ul of *stock neutralising solution*, before baking the filter in a vacuum oven.

Using the standard protocols for hybridisation probe analysis (see above) the filter was probed with pTA250.2, which contains a wheat rDNA fragment spanning the 18S–26S genic region (courtesy R. Appels). After washing with 2x SSC and 0.1% SDS at 65°C, three peak rDNA fractions were detected (Figure 10.14), and pooled.

DNA from the pooled fractions was recovered by precipitation with 2 volumes of isopropanol saturated with NaCl, and centrifugation overnight in a swing-out rotor (20,000 rpm, Beckman SW 27). The precipitate was resuspended in 450 ul *stock 10 TE*

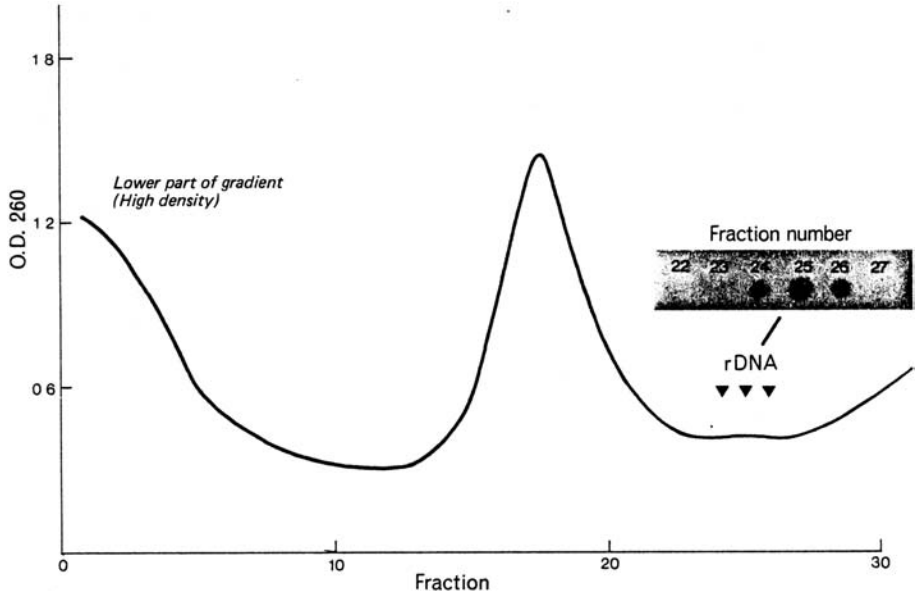


Figure 10.14 Partial purification of ribosomal DNA (enrichment procedure). After centrifuging total-DNA from AKL 34 to equilibrium, in a caesium chloride and ethidium bromide density gradient, 1 ml fractions were removed from the bottom of the tube, and aliquots diluted for optical density analysis at 260 run. Aliquots (20 ul) from lower density fractions above the DNA peak were transferred to a nylon filter and hybridised with a cloned wheat rDNA fragment (pTA250.2) to determine the peak rDNA fractions (24–26, inset).

1(8), and incubated with 0.1% SDS (4.5 ul *stock 10% SDS*) and 20 ug proteinase K (20 ul fresh solution, 1 mg/ml H_2O, at 37°C, 10 minutes. After one extraction with phenol and chloroform, the DNA was precipitated with ethanol and sodium acetate, and resuspended in 100 ul *stock 10 TE 1(8)*.

Preliminary experiments (see Chapter Eleven) established that taro rDNA possesses two *Eco* RI restriction sites. To create fragments suitable for cloning, 80 ul of the rDNA enriched extract was digested with approximately 30 units of *Eco* RI in a reaction volume of 100 ul (37°C, 1 hour, with appropriate buffer). After adding 30 ul of 5% SDS and incubating at 65°C for termination, the DNA fragments were stored at -20°C. The rDNA of *Eco* RI restriction fragments were subsequently fractionated by differential velocity centrifugation in a glycerol gradient (protocol of R. Appels, pers. comm. 1986), as follows:

The preliminary experiments showed that the *Eco* RI fragments lay within the 2–10 kbp size range, and to isolate fragments in this range, the *Eco* RI reaction mixture was thawed at 65°C, to dissolve SDS, and loaded onto a linear gradient of 10–40% glycerol. The gradient was prepared in a 13.2 ml polyallomer tube, then was allowed to stand 4 hours at 8°C to stabilise, before the DNA fragments were added. Centrifugation in a

swing-out rotor (Beckman SW 41, 27,000 rpm, 22 hours, 5°C) was terminated without braking after reducing speed to 2,000 rpm. Three fractions — the lower, middle, and upper thirds of the gradient — were taken from the bottom, and the DNA in each was precipitated with ethanol and sodium acetate.

Each fraction was processed further and the contents examined by electrophoresis, Southern transfer, and hybridisation with pTA250.2, to confirm the distribution of large and small *Eco* RI taro rDNA fragments. The desired fragments were expected in the middle fraction (2–10 kbp size range), and the following steps apply to this fraction. After centrifugation in a swing-out rotor (Beckman SW 27), 25,000 rpm, 3 hours, the precipitate was resuspended in 400 ul *stock 10 TE 1(8)*. This was incubated with SDS and proteinase K as previously, and extracted once with phenol and chloroform, with a further 50 ul of *stock 10 TE 1(8)* used to back-extract residual DNA from the phenol/chloroform phase. The fragments were again precipitated with ethanol and sodium acetate, spun for 20 minutes in a bench centrifuge, and washed with 70% ethanol, with a further 15 minutes of centrifugation. The precipitate was finally resuspended in 50 ul H2O, ready for cloning.

Ligation to pBR322

An unknown but small weight (less than 1 ug) of *Eco* RI fragments in 1, 5, or 10 ul of the final suspension (above) was placed in a 1.5 ml Eppendorf tube on ice, with addition of H_2O to a volume of 10 ul (if not already this volume), before mixing with 3 ul of commercially supplied pBR322 previously cut with *Eco* RI and dephosphorylated (0.1 ug/ul, BRL), 6.5 ul of ligation reaction mixture (fresh *stock solution* containing 8 parts *stock 5x ligase buffer*, 4 parts 10 mM ATP, and 1 part 1 M dithiothreitol), and 0.5 ul T4 DNA ligase (400 units/ul, BRL). This reaction mixture (final volume 20 ul) was incubated 4–20 hours at 14°C. The mixture was stored at -20°C without further treatment prior to transformation of competent cells.

Preparation of competent cells and transformation with the ligation products

One ml of overnight culture of *Escherichia coli*, strain RR1, was placed in 100 ml of fresh *stock LB medium* and incubated at 37°C with vigorous shaking until an OD 600 of approximately 0.7 was reached. Four 10 ml aliquots of cells were transferred to sterile plastic centrifuge tubes and centrifuged at 2000 rpm for 5 minutes. The supernatants were discarded, and the cells then resuspended in 5 ml cold $MgCl_2$ (0.01 M, approximately 5°C) per tube, and centrifuged (2000 rpm, 5 minutes). The supernatants were discarded. Each pellet of cells was resuspended in 5 ml cold $CaCl_2$ (0.05 M, approximately 5°C). The cells were resuspended and pooled in a total of 2.5 ml cold $CaCl_2$ (0.05 M, approximately 5°C). These cells, now competent for the uptake of plasmids, were held on ice for immediate transformation or stored for a few days at -20°C.

For transformation, 100 ul of competent cells were mixed with 38 ul cold $CaCl_2$ (0.05 M) and 2 ul of ligation mixture (containing 30 ng of the vector, pBR322). This was incubated on ice for 1 hour, then at 42°C for 2–3 minutes (heat shock). *Stock LB*

medium, 0.5 ml at RT, was added and the cells were incubated at 37°C for 1 hour, with shaking. The culturing step gives any plasmids that have entered cells an opportunity to replicate and express their antibiotic resistance genes. Three ml of top-agar (LB medium with agar, 45°C) was added to the final transformation mixture and poured onto one fresh LB agar plate (*stock LB medium* with 15 g Bacto-agar/1) with ampicillin (50 mg/1). After letting the top-agar set, the plate was inverted and incubated at 37°C overnight (Figure 10.15A).

RR1 is a fast-growing strain of *E. coli*, and embedding with top-agar constricted growth so that most colonies remained discrete. With a toothpick, individual colonies were transferred to a second ampicillan plate (Figure 10.15B) according to a paper grid sheet placed beneath the plate (100 colonies per plate). After overnight culture at 37°C, a brass stamp with 100 protrusions was used to replicate the colonies onto two ampicillin plates successively: first onto a nitrocellulose filter disk (Schleicher and Schuell, 82 mm diameter, 0.45 um pores) previously laid on one plate, and then directly onto the agar of a second ampicillan plate. Between different sets of colonies, the brass stamp was washed in ethanol and flamed with a Bunsen burner. Both plates were cultured overnight at 37°C (Figure 10.15C). After culturing, the second plate was stored at 4°C until further required. The filter was transferred to an agar plate (LB medium) with chloramphenicol (30 mg/1) and incubated at 37°C overnight, 14–16 hours (Figure 10.15D). This antibiotic inhibits cell division, but allows replication of the plasmid DNA, increasing the yield of plasmid DNA for each colony.

Screening colonies for plasmids with DNA inserts

Six hundred transformant colonies were obtained and cultured on six nitrocellulose filters. After chloramphenicol treatment (above) each filter was placed on a disk of Whatman No. 1 paper saturated to brimming with 3.5 ml of *stock denaturant* (0.5 M NaOH, 0.5 M NaCl), for 10–15 minutes (Figure 10.15E). This treatment lysed the cells and denatured the DNA simultaneously. After lysis, the filter was placed on blotting paper to remove excess denaturant, and then placed on a disk of No. 1 Whatman paper saturated with 3.5 ml *stock neutralising solution* (3 M NaCl, 0.5 M Tris.HCl), for 10–15 minutes (Figure 10.15F). The filter was laid on blotting paper to remove excess neutraliser, then put between two loose sheets of blotting paper for baking in a tap-vacuum oven (80°C). Several filters were placed in one 500 ml, deep glass beaker and incubated with pre hybridisation and hybridisation solutions as described above for Southern transfer filters. To detect plasmids containing rDNA inserts, wheat rDNA excised from pTA250.2 and electroeluted from agarose was used as a probe (Figure 10.16). Mini-scale plasmid DNA preparations of all apparently positive colonies were prepared and digested with *Eco* RI, then electrophoresed and transferred to filters by Southern transfer for hybridisation probe analysis, again with the rDNA fragment from pTA250.2. Three rDNA clones were detected. False positive colonies found in the initial colony screening were attributed to residual contamination of the probe rDNA fragments with pTA250.2 vector sequences, homologous to the vector used for cloning taro DNA (pBR322), or contamination with residual *E. coli* DNA. A fourth rDNA clone was

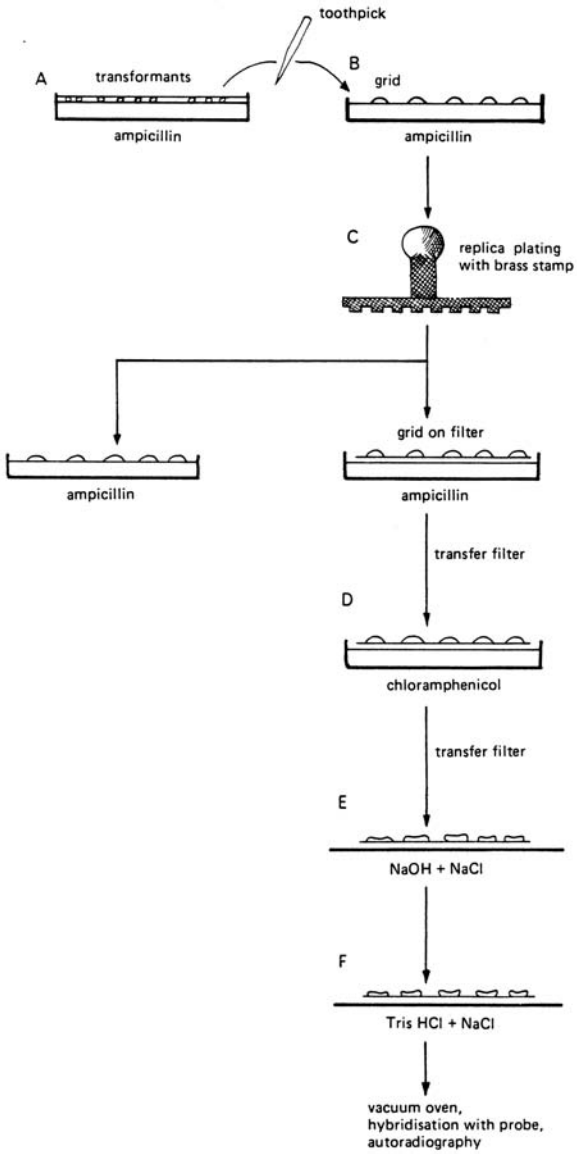


Figure 10.15 Screening for transformed cells and detecting recombinant clones by colony hybridisation, using the Grunstein Hogness method. See text for details. (A) Transformed cells of E. coli strain RR1 were selectively cultured in top agar on a medium containing the antibiotic ampicillin, for which the transforming plasmid pBR322 provides resistance. (B) Individual colonies were transferred by hand to a second agar plate according to a grid pattern, and then (C) replica plated with a brass stamp. One replica set of colonies was cultured then stored at low temperature, and a second was cultured on a nitrocellulose filter. (D) The filter was transferred to an agar plate with chloramphenicol, an antibiotic which stops bacterial cell growth but not the replication of plasmids in each cell. (E) To lyse the cells, the filter was transferred to blotting paper soaked with a strongly basic solution. After (F), treatment with a pH neutralising solution, (F) the filter was baked to help bind the released DNA, hybridised with a ^{32}P labelled rDNA probe, and autoradiographed (see Figure 10.16 for example).

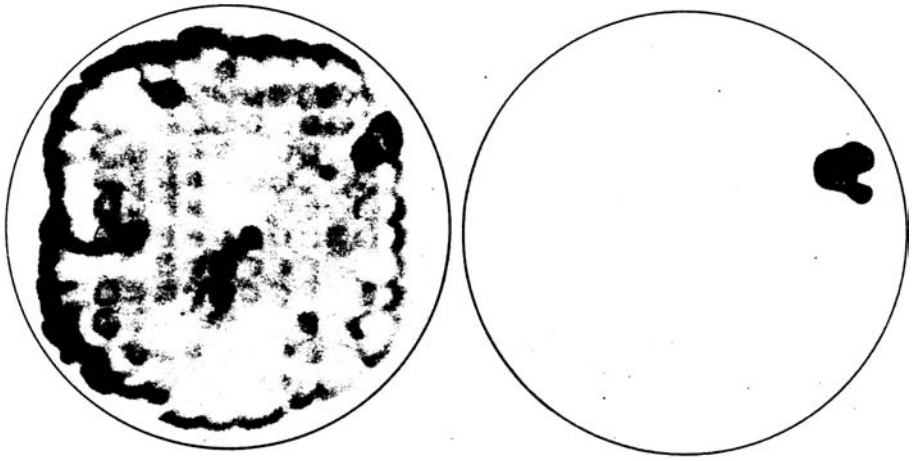


Figure 10.16 Colony hybridisation. A wheat rDNA insert purified from recombinant plasmid pTA250.2 was used to probe colonies of *E. coli* previously transformed with a ligation mixture of pBR322 and taro rDNA fragments (autoradiograph at left). The colonies were prepared on a filter as shown in Figure 10.15. Strongly hybridising colonies were analysed further after propagation from the stored replica date, plasmid extraction, agarose gel electrophoresis, Southern transfer, and hybridisation probe analysis with wheat rDNA from pTA250.2. Ribosomal DNA was detected only for the colony giving the strongest signal, upper right in the autoradiograph at left. The corresponding colony from the stored replica plate provided the recombinant plasmid pCe34.1.The taro rDNA insert from pCe34.1 was used to probe the filter again. Only the source colony for pCe34.1 contained DNA homologous to pCe34.1 (autoradiograph at right).

discovered when the original colony-blot filters were reprobed with the taro rDNA fragment excised from pCe34.1 (5.5 kbp *Eco* RI fragment), and pCe34.2 (4.0 kbp *Eco* RI fragment).

Subcloning to provide a probe specific for variable region of the rDNA large intergenic spacer

A 2.8 kbp *Taq* I fragment was subcloned from pCe34.1 as follows. After purifying a large-scale preparation of pCe34.1 by equilibrium density centrifugation, in caesium chloride and ethidium bromide, plasmid DNA was digested with *Taq* I, electrophoresed, and the 2.8 kbp fragments electroeluted. After extracting proteins with phenol and chloroform, and precipitation with *stock ethanol and sodium acetate solution*, the *Taq* I fragments were resuspended to a concentration of 0.2 ug/ul in *stock 10 TE 1(8)*.

Linearised vector molecules, with sticky ends suitable for ligation with *Taq* I fragments, were prepared by digesting pBR322 (Boehringer) with *Cla* I. After cleaning with phenol and chloroform, and ethanol precipitation, the vector molecules were de-phosphorylated essentially as described by Maniatus et a1. (1982). Approximately 5 ug pBR322 precipitate was resuspended in 44.5 ul H_2O, then mixed with 5 ul *stock Wx CIP buffer* (0.5 M Tris.HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$) and 0.5 ul Calf

Intestine Phosphatase (9.5 units; Boehringer). This mixture (50 ul total) was incubated at 37°C for 30 minutes, before adding 0.5 ul *stock 10% SDS* and incubating at 68°C for 45 minutes. After extracting proteins with phenol and chloroform, the vector molecules were precipitated with ethanol and sodium acetate.

For ligation, 0.2 ug of vector in 2 ul *stock 10 IE 1(8)*, and 0.4 ug of *Taq* I rDNA fragments in 2 ul *stock 10 TE 1(8)* were mixed with 10 ul H_2O, 4 ul *stock 5x ligase buffer*, 2 ul 10 mM ATP, 0.2 ul 1 M dithiothreitol, and 1 ul (40 units) of T4 DNA ligase (New England Biolabs). After incubation overnight at 16°C, the mixture was stored at -20°C prior to transformation of *E. coli* strain RR1, essentially as already described. Fifty-four transformants were obtained.

Since ligation involved only one rDNA fragment, there was a good chance that the desired fragment would be found in a small sample of transformants. For initial screening, 47 colonies were transferred to one LB agar plate with ampicillin (50 mg/ml) and one LB agar plate with tetracycline (16 mg/1), using the same grid for each plate. The *Cla* I site is in the tetracycline resistance gene in pBR322, and insertional deactivation of the tetracycline resistance gene was indicated by fourteen colonies which grew very slowly or not at all in the presence of tetracycline. Transformant colonies from the ampicillin plate were grown and lysed on a nitrocellulose filter, essentially as described for the initial cloning procedure (above), and the filter was probed with the *Eco* RI fragment from pCe34.1. All 14 tetracycline sensitive colonies contained the 2.8 kbp rDNA *Taq* I fragment. One was chosen and named pCe34.11.

10.2.14 *In situ* hybridisation of rDNA to chromosomal loci

In situ analysis using three different tritium labelled nucleotides gives probes of high specific activity, and autoradiographic exposures of such great resolution that hybridisation within a chromosome can be detected. The high resolution results from radioactive emissions of low penetrating power (beta particles), and detection requires the intensity provided by high specific activity.

Chromosome preparation

Procedure courtesy L. Mclntyre (pers. comm. 1987). The tips from several young roots were harvested (0.5 cm removed from roots 0.5–4 cm long) and placed in water on ice, overnight, or were *immersed with stock colchicine solution* (0.4% w/v in H90) for 2 hours at RT. These alternative treatments both serve to arrest cell division. Tips were fixed in a glass vial with a solution of ethanol and acetic acid (3:1) for between 4 and 24 hours. To soften the tissues, the fix solution was replaced with 1 M HC1 and the tips incubated at 60°C, 4 minutes. The terminal 1–2 mm were removed and then placed in a drop of 45% acetic acid on a very clean, dust-free slide, near one end of the slide (for later dipping treatments). A cylindrical brass rod with a flat end of approximately 2 mm diameter was used to gently squash the meristematic cell region out of the root cap, and then to macerate the meristematic cells. Visible clumps of cells and root-cap epidermal tissue, were removed with tweezers and a cover slide placed over the preparation. A piece of blotting paper was placed over the slide, and with heavy thumb pressure the

cells were squashed. After checking for the presence of metaphase chromosome spreads, with a light microscope, the slide was frozen by dipping in liquid nitrogen, and the coverslip flipped off with a razor blade. The slide was dipped in 100% ethanol, next in 70% ethanol, and was then air dried at RT.

Synthesis of ^{3}H copy-RNA probe from cloned rDNA

Procedure courtesy N. Contreras (pers. comm. 1987), modified from Pardue and Gall (1969). In an Eppendorf tube, 100 ul each of ^{3}H-CTP ([5-^{3}H] cytidine 5'-triphosphate, 28 Ci/mM, Amersham), ^{3}H-UTP ([5,6-^{3}H] uridine 5'-triphosphate, 32 Ci/mM, Amersham) and ^{3}H-ATP ([2,8-^{3}H] adenosine 5'-triphosphate, 41 Ci/mM, Amersham) were mixed and then dried in a rotary vacuum (Speedvac). The nucleotides were suspended in 15 ul *stock 10 TE 1(8)* (water is an alternative), with incubation at 37°C for 20 minutes to ensure complete suspension. Next, 0.2 ug of purified and proteinase K treated taro rDNA (insert from pCe34.1) was added in 10 ul H_2O, together with 10 ul of non-radioactive nucleotide, GTP (guanosine 5'-triphosphate, P-L Biochem). To this mixture were added 10 us of *stock 5x RNA polymerase buffer* (40 mM Tris.HCl, pH 7.9, 10 mM MgCl, 0.1 mM Na_2DTA, 150 mM KC1, 500 ug/ml bovine serum albumin), 0.5 ul of 1 M dithiothreitol, and 5 ul £. coll RNA polymerase (1 unit/ul, Boehringer) (50.5 ul final reaction volume). The RNA polymerase reaction was allowed to proceed for 30 minutes at 37°C, before removing 1 ul for estimating radioactive nucleotide incorporation. To remove DNA, 250 ul 0.05 M Tris, 10 ul DNA'se (0.2 mg/ml, Boehringer), 5 ul yeast RNA (10 mg/ml) and 5 ul $CaCl_2$ (0.01 M) were added and the mixture incubated a further 30 minutes at RT. The reaction was terminated fully by adding 25 ul Na_2DTA (0.25 M), 1 ul 5% SDS, 100 ul *stock phenol* and 100 ul *stock chloroform*. The phenol/ chloroform phase was washed with 200 ul of 0.05 M Tris and the pooled aqueous phases were precipitated with 2 volumes of 100% ethanol. For final storage at -20 C, prior to in situ hybridisation, the precipitate was resuspended in 200 ul of 6x SSC, giving approximately 35,050 cpm/ul (incorporated radioactivity).

Estimation of percentage incorporation

After incubation, 1 ul of the RNA polymerase reaction mixture was removed, as noted above, and added to 99 ul of H_2O. Ten microlitres of the dilution were spotted directly onto a glass fibre filter (Whatman) for estimation of total radioactivity. A further 10 ul was mixed with 50 ul of sonicated salmon sperm DNA (2 mg/ml) and 1 ml of 5% (w/v) trichloroacetic acid. The mixture was placed on ice for 5 minutes to allow precipitation, before being loaded onto a second glass fibre filter. The filter was washed twice with 2 ml aliquots of 0.1 M $Na_2H_2P_2O_7$ (Sigma) in 1 M HC1, and then with approximately 10 ml of 100% ethanol. This filter was used to measure incorporated radioactivity. Two replica filters were prepared for measuring total and incorporated radioactivity, four filters in all. The filters were dried and placed in glass scintillation vials with scintillant (0.06% 2,5-diphenyloxazole, Calbiochem, and 0.006% 1,4-di (2-[5-phenyloxazolyl] -benzene, Calbiochem, in tolulene). Radioactivity was measured with an automated scintillation spectometer, and the percentage of radioactive nucleotides incorporated into

copy-RNA was calculated to be 4.2%, corresponding to 7,010,000 cpm from the full reaction volume.

In situ hybridisation

Chromosome preparations (see above) were used for in situ hybridisation as described by Appels et al. (1978) with modifications recommended by N. Contreras (pers. comm. 1988). Several slides were processed at the same time, placed in Coplin jars for immersion. One hundred ml of 2x SSC was mixed with 0.1 ml pancreatic RNA'se A (2 mg/ml, final concentration 2 ug/ml, Boehringer) and preheated to 37°C. The slides were added and the jar incubated in a water bath for 30 minutes at 37°C. The solution was replaced with H_2O (37°C) to rinse the slides. To denature the DNA, the slides were incubated with 0.2 M HC1 at 37°C for 10 minutes, then washed with H_2O three times, once with 70% ethanol, once with 95% ethanol, and then air dried in a rack. The tritiated copy-RNA solution (6x SSC) was mixed with an equal volume of formamide (Fluka) to give a hybridisation solution containing 3x SSC and 50% formamide. The amount of mixture prepared was enough to give each slide approximately 5 ul, placed centrally in the area with chromosomes (exact amount just enough to fill the area beneath the coverslip). Approximately 90,000 cpm of incorporated radioactive nucleotide was received by each slide, the recommended amount being 100,000 cpm (N. Contreras, pers. comm.) or 70,000–700,000 (Arnold 1985: 46). An acid-washed coverslip was removed from storage in 95% ethanol and placed on each slide, then sealed with rubber cement (bicycle glue) which was allowed to partially set. The slides were placed in a 60°C air incubator for 2 minutes, to ensure that the probe remained single-stranded for hybridisation, and were then placed in an air incubator at 37°C overnight (20 hours). Before the extended incubation, each slide was checked for possible leaks and sealed with further glue if necessary. Incubation at 37°C for 4 hours is sufficient, and the temperature can be adjusted according to the expected similarity of the probe and target sequences (identical in the present example).

After hybridisation, glue was removed with tweezers, and the slides were placed in a Coplin jar containing 3x SSC and 50% formamide at 37°C, and incubated for 5 minutes, allowing the cover slips to float off the removal. The slides were incubated for two further periods of 10–15 minutes with fresh solutions of 3x SSC, 50% formamide, at 37°C, and were then incubated for three periods of 10 minutes with fresh solutions of 2x SSC, RT.

To completely remove unannealed copy-RNA sequences, the slides were incubated in a solution of pancreatic RNA'se A (Boehringer, 2 ug/ml in 2x SSC) for 30 minutes at RT. They were then rinsed with six periods of 10 minutes in fresh solutions of 2x SSC and 0.1% SDS, at RT, two periods of 15 minutes in 70% ethanol, and two periods of 15 minutes in 95% ethanol. The slides were air dried at RT.

To prepare slides for autoradiographic exposure, all operations were conducted in a dark room equipped with a light brown safe light (Ilford No. 902). Silver emulsion (Ilford Nuclear Research Emulsion, particle size K2, stored cold in a lead container and wrapped in aluminium foil) was prepared by incubating Ilford K2 paste at 45–50°C until liquified,

then mixing with an equal volume of preheated H_2O (45–50°C). Slides were placed back to back, with the chromosome preparations downwards, and dipped half-way into the emulsion, taking care not to touch the sides of the container. Excess emulsion was drained by tapping the slides on blotting paper, before standing them separately and vertically, emulsion upwards, in an open rack. The slides were transferred to a slide box and air dried for 1–2 days at RT, in darkness. The box was then sealed with black tape, wrapped in at least two layers of aluminium foil, and stored at 4°C. Slides to be used for an initial test exposure of 5 days were stored in a separate box. Successful exposures were obtained after 2 weeks.

For autoradiographic development, all materials were first brought to RT. The slides were developed for two minutes in fresh Kodak Dektol solution (1:1 dilution with H_2O, no more than six weeks old) and fixed for 4 minutes with Kodak fixative. After rinsing in H_2O, the slides were allowed to dry before staining in Giemsa solution (5 ml Giemsa mixed with 2 ml of 1 M $NaHPO_4/KH_2PO_4$ buffer, pH 6.8, and made up to 100 ml with H_2O) for 8 minutes at RT. The slides were rinsed in tap water, and while still wet were viewed under a light microscope to determine the extent of Giemsa staining. If de-staining was required, the slides were dipped in 100% ethanol then washed again with water. After satisfactory staining, the slides were dried and then made permanent with Depex mounting medium, using large rectangular coverslips so that the entire area of the chromosome preparation could be viewed. Photomicrographs were taken with a Zeiss light microscope and Kodak technical pan film (2415).

10.2.15 Thermal melting point analysis

The one-step protocol described here is similar to that described by Gill and Appels (1988), with minor modifications. ^{32}P labelled probes, made with the taro rDNA insert from pCe34.1, were incubated with Southern transfer filters in the usual manner, in 3x SSC, 50% formamide, 0.1% SDS, 5x Denhardt's, 3 mM Tris.HCl, pH 8.0, 0.3 mM Na_2EDTA, at 37°C, 4–18 hours, as described above. After the usual low-stringency washes in 2x SSC, 0.1% SDS, at 65°C, autoradiographs were made without allowing the filters to dry. The thermal melting step that follows (high-stringency hybridisation) was sufficient to remove 50% of the probe hybridised to internal control samples of identical DNA. Each filter was placed in a thick, clear plastic bag, double-sealed at three sides before adding approximately 25 ml of a simplified hybridising solution (3x SSC, 50% formamide), at RT, and enough to easily cover the filter. Air bubbles were squeezed out, and the bag was closed by double-sealing. A water-bath was preheated to the desired treatment temperature (e.g. 66°C), and the bag was incubated for 15 minutes with occasional turning and squeezing so that all parts of the filter received equal treatment. The filter was removed, washed under low-stringency conditions (2x SSC, 0.1% SDS, 65°C), blotted to remove excess fluid, and was autoradiographed.

10.2.16 Mitotic-cell chromosome counts from root tips

The protocol of Matthews (1984) was used (see Appendix 10.1).

Senri Ethnological Studies 88: 147–168 ©2014
On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Eleven
Preliminary Trials of Methods for Analysing Variation

11.1 Introduction

The study of taro began with trials of a range of different methods for detecting genotypic variation. Variable and invariant isoenzymes were detected by electrophoresis of crude leaf-protein extracts, followed by biochemical assays of the electrophoresed proteins. The study of isoenzymes was abandoned because it seemed logistically and interpretatively more difficult than investigating DNA variation. Attempts to prepare pure chloroplast DNA were unsuccessful because starch grains in the chloroplasts disrupted chloroplast membranes during centrifugation, and for other reasons not determined. To obtain pure chloroplast DNA, improvements are needed in the prior growing conditions and in the protocol for purifying chloroplasts. The initial results of isoenzyme analysis and chloroplast DNA purification are not presented.

To quickly obtain information about chloroplast, mitochondrial, and ribosomal DNAs, extracts of total DNA from taro were digested with restriction enzymes, electrophoresed, transferred to nylon filters, and probed with pre-existing clones of DNA from plant species other than taro. This approach is termed heterologous hybridisation-probe analysis. With chloroplast and mitochondrial DNA probes, from spinach and maize respectively, differences were observed between *C. esculenta* and *C. gigantea*, but not between varieties of *C. esculenta*. Tests with mitochondrial rDNA suggested, surprisingly, that mitochondria in *C. gigantea* are less closely related to mitochondria in *C. esculenta* than they are to mitochondria in *Alocasia brisbanensis*, another member of the tribe Colocasioideae. More investigation is needed to develop tests for chloroplast and mitochondrial variation within *C. esculenta*.

Tests with a 5S ribosomal DNA probe, from the nuclear genome of rye, demonstrated that the 5S rDNA locus in taro is structurally similar to 5S loci in other plants. Although not examined further, 5S rDNA has potential as marker of intraspecific genotypic variation.

Using 18S rDNA from the *Nor*-locus of wheat as a probe, variation was detected in an initial survey of a small number of taro varieties (variants)[1]. Very frequent mutation during vegetative reproduction, and amplification of new variants to detectable levels, would make *Nor*-locus rDNA variation unsuitable for investigating the long-term dispersal of taro. A variable, but not too variable, genotypic marker is required. Sexual reproduction by triploid taro in New Zealand is unknown (Matthews 1985), and the three phenotypically distinct variants found in New Zealand were tested with rDNA fragments cloned from taro (homologous hybridisation-probe analysis). No intravarietal differences

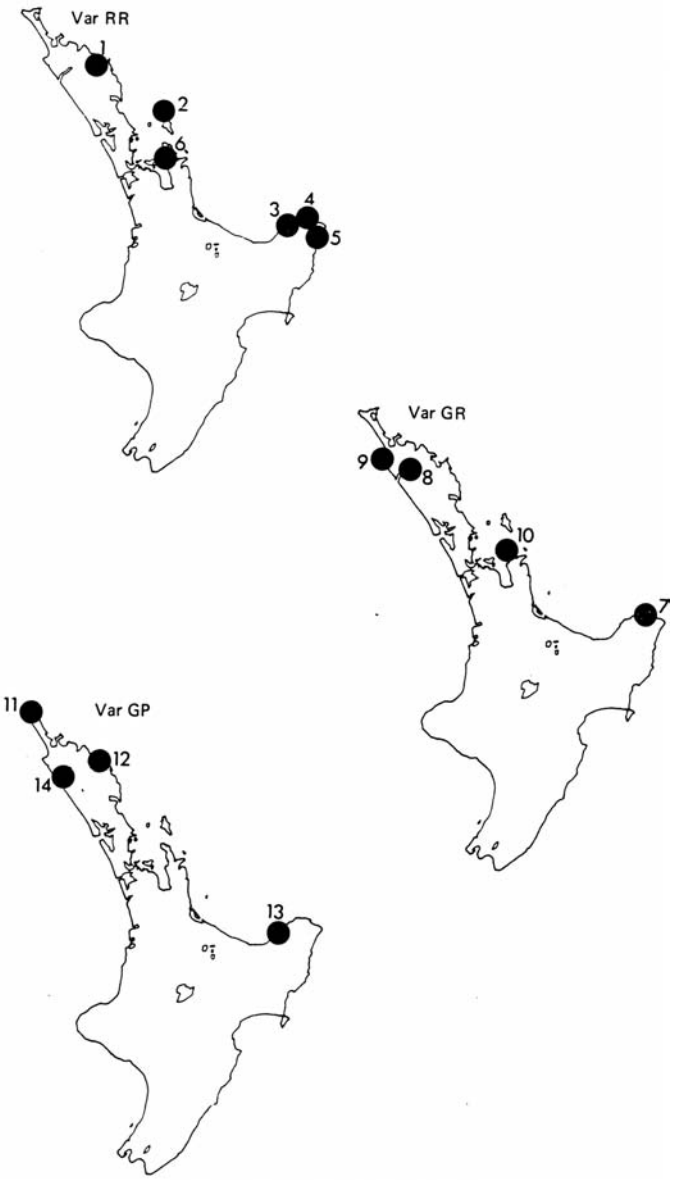


Figure 11.1 Sites of triploid taro variants RR, GR, GP from North Island, New Zealand, analysed with taro rDNA probes. For sites 1–14, the variant, Canberra accession number (T) with location, habitat, and chromosome number are as follows. Var. RR: 1 = T272, Okokako Road, Bay of Islands, wild; 2n = 42; 2 = T274, Whangapoua Beach, Coromandel Peninsula, wild, 2n = 42; 3 = T275, Putanga, East Cape, wild; 4 = T276, non-cultivated garden, Te Hekawa, East Cape, 2n = 42; 5 = T277, Hamana Stream, East Cape, wild, 2n = 42; 6 = T278, Port Charles, Coromandel Peninsula, garden, 2n = 42.Var. GR: 7 = T279, Te Hekawa, East Cape, garden, 2n = 42; 8 = T281, Reena, Hokianga, wild; 9 = T282, Waihopo, Aupouri Peninsula, garden, 2n = 42; 10 = T283, Colville, Coromandel Peninsula, garden, 2n = 42.Var. GP: 11 = T284, Kapowairua, North Cape, wild, 2n = 42; 12 = T 286, Te Arakanihi, Bay of Islands, wild, 2n = 42; 13 = T287, Rerepa Street, East Cape, wild, 2n = 42; 14 = T288, Reena, Hokianga, wild.

were found among geographically wide-spread samples of each phenotypic variety in New Zealand. The stability of rDNA variants, thus indicated, encouraged further investigation of rDNA variation on a wider geographical scale (see later chapters).

11.2 Materials and Methods

Materials and methods were largely as described in Chapter Ten. To test for intra- and interspecific variation, small numbers of diverse samples were surveyed first. If variation were found, then the same test combination of restriction enzyme and probe was applied to plants from a wide geographical range. The initial tests included diploid (2n = 28) and triploid (2n = 42) taro varieties from different locations (various Canberra collection numbers), another species (*C. gigantea*, Canberra collection T268, from an ornamental collection in Australia), and another genus (*Alocasia brisbanensis*, Canberra collection T222, wild from the Windsor Tableland, northeast Queensland, Australia).

A. brisbanensis A. Hay is an Australian species previously known by the name *A. macrorrhizos* (L.) G. Don, but separated by Hay and Wise (1989) into a supra-generic 'Ozarum group', distinct from the 'Macrorrhizos group' containing *A. macrorrhizos* (L.) G. Don and *A. flabellifera* A. Hay. The latter, known only from Papua New Guinea, also includes plants previously identified as *A. macrorrhizos* (Hay and Wise 1989). Samples of three New Zealand variants of *C. esculenta* were distributed in the field as shown in Figure 11.1.

11.2.1 Probes and wash conditions

During the preliminary experiments, a range of low to moderate stringency wash conditions were tried for filters after Southern transfer and hybridisation. The conditions recorded below do not represent the only successful wash conditions tried for each probe after hybridisation with taro DNA, and do not represent optimised conditions. Autoradiographic exposure times varied from hours to several days.

(1) Chloroplast DNA probe, *Sal* I-6 (Figure 11.2): *Sal* I fragment 6 (9 kbp) from the single copy region of the chloroplast genome of *Spinacia oleracea* (spinach), ligated with pBR322. Donated by P. R. Whitfield (C.S.I.R.O., Canberra, Australia). Washed twice with a solution of 2x SSC, 0.1% SDS, pre-heated to 55°C, 15 minutes per wash.

(2) Mitochondrial DNA probe, *Cox* I (Figure 11.3: 10 kbp *Bam* HI fragment from the mitochondrial genome of *Zea mays*, *cms*-c variety (maize), ligated with pUC19. Donated by C. S. Levings (North Carolina State University, Raleigh, U.S.A.). Contains the 1.5 kbp gene for subunit I of cytochrome oxidase (*Cox* I) and flanking regions (Isaac et al. 1985). Washed four times with a solution of 2x SSC, 0.1% SDS, pre-heated to 65°C, 15 minutes per wash.

(3) Mitochondrial DNA probe, 18–5S rDNA (Figure 11.3): 6 kbp *Bam* HI fragment from the mitochondrial genome of *Zea mays* (maize, variety not specified), ligated with pUC9. Donated by C. S. Levings. Contains 18S and 5S rDNA genes and flanking sequency (Chao et al. 1984). Washed two times with a solution of 2x SSC, 0.1% SDS, pre-heated to 65°C, 15 minutes per wash, and two times with a solution of 0.2x SSC,

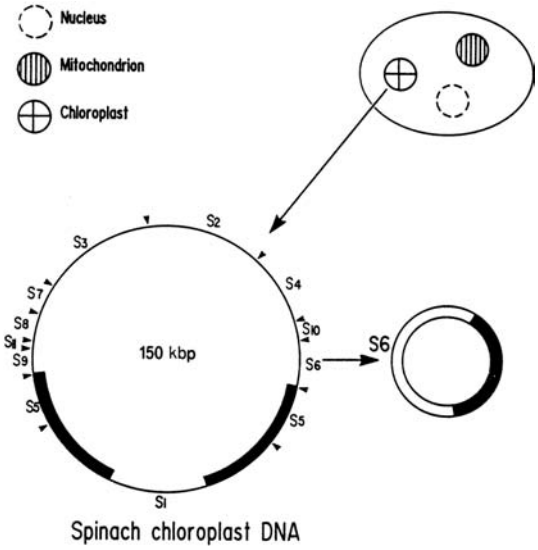


Figure 11.2 Derivation of a chloroplast DNA probe (*Sal* I-6) by ligation of *Sal* I fragment 6 (S6), from spinach chloroplast DNA, into a plasmid (lower right). Chloroplast restriction site map adapted from Herrmann et al. (1980).

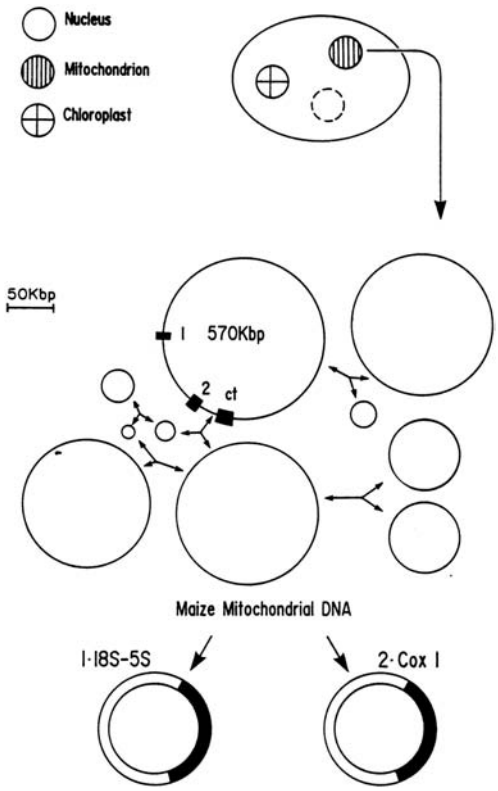


Figure 11.3 Derivation of mitochondrial DNA probe fragments ligated into plasmids (below) from *Zea mays* mtDNA (above). Cell components at top (schematic). *Cox* I = cytochrome oxidase subunit I gene; 18S-5S-chloroplast rRNA genes. Adapted from Lonsdale et al. (1984) and Dawson et al. (1986). The large circle of 570 kbp contains the entire sequence complexity of the genome. Intra-molecular recombination is believed to cause the breakdown of the large circle into a heterogeneous population of subgenomic circles. Mitochondria have a major role in ATP production and oxygen consumption (metabolic energy transfer), and every cell contains a population of mitochondria, the number varying between cell types. In most plants, each mitochondrion contains a heterogeneous population of mtDNA molecules.

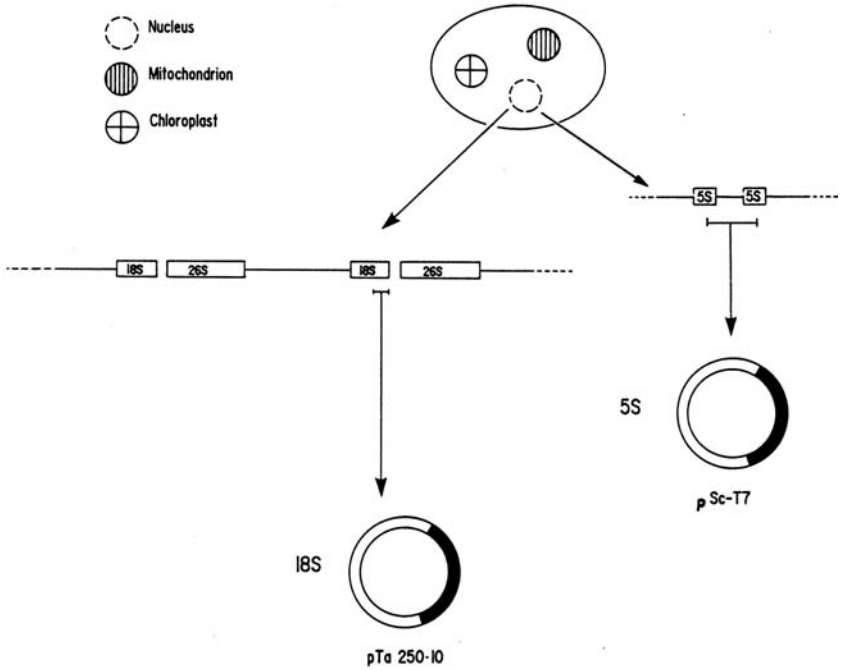


Figure 11.4 Derivation of nuclear rDNA probes. The plasmid pTA250.10 contains a fragment of 18S rDNA from the nuclear genome of *Triticum aestivum*. The plasmid pSc-T7 contains an entire 5S rDNA repeat from the nuclear genome of *Secale cereale*. Cell components at top (schematic) Ribosomal DNA diagrams not to scale. The 18S and 5S rRNA genes are located at different chromosomal loci within nuclear genomes.

0.1% SDS, pre-heated to 65°C, 15 minutes per wash.

(4) Nuclear 5S rDNA probe, Sc-T7 (Figure 11.4): 0.5 kbp *Bam* HI fragment containing an entire 5S rDNA repeat unit, from a nuclear chromosome of *Secale cereale* (rye), ligated with pBR322 (Lawrence and Appels 1986). Donated by R. Appels (C.S.I.R.O., Canberra, Australia). Washed four times with a solution of 2x SSC, 0.1% SDS, pre-heated to 65°C, 15 minutes per wash.

(5) Nuclear 18S rDNA probe, pTA250.10 (Figure 11.4): 1 kbp *Taq* I fragment from the 18S rDNA gene, from a nuclear chromosome *Nor*-locus of *Triticum aestivum* cv Chinese Spring (wheat), ligated with pBR322. Donated by R. Appels. Washed four times with a solution of 3x SSC, 0.1% SDS, pre-heated to 65°C, 15 minutes per wash.

(6) Nuclear rDNA probe pCe34.1: 5.5 kbp *Eco* RI fragment, from a nuclear chromosome of *C. esculenta* (taro), and containing the entire intergenic spacer region and flanked by parts of the 26S and 18S rDNA genes; ligated with pBR322 (cloning, see Chapter Ten; description, see Chapter Twelve). Washed four times with a solution of 2x SSC, 0.1% SDS, pre-heated to 65°C, 15 minutes per wash.

11.3 Results

The number of different DNA fragment classes, and their overall size ranges, are given in summary tables. This information is useful for choosing appropriate electrophoretic conditions and DNA size standards (individual estimates of DNA fragment sizes were not sufficiently accurate to justify detailed reporting).

(1) *Spinach chloroplast DNA probe, Sal I-6* (Table 11.1). With each of the five tests (*Bam* HI, *Eco* RI, *Hind* III, *Sal* I and *Xho* I) no differences were found between five varieties of *C. esculenta*. With *Bam* HI and *Eco* RI, *C. esculenta* gave restriction fragment patterns different from those of *C. gigantea*.

(2) *Maize mitochondrial DNA probe, Cox I* (Table 11.2, Figure 11.5). No differences were found between four varieties of *C. esculenta* or between *C. esculenta* and *A. brisbanensis* after *Eco* RI digestion. Only partial *Eco* RI digestion was achieved for *C. gigantea* (Figure 11.5), but the result nevertheless suggests that there is no difference between this and the other two species. Tests with *Msp* I and *Hae* II revealed differences between *C. esculenta*, *C. gigantea*, and *A. brisbanensis*, but no differences among seventeen accessions of *C. esculenta*. Tests with *Bam* HI also revealed no differences among the seventeen accessions of *C. esculenta*.

(3) *Maize mitochondrial DNA probe, 18–5S rDNA* (Table 11.3, Figure 11.6). Tests with *Bam* HI and *Hae* III revealed no differences between *C. gigantea* and *A. brisbanensis*, but did reveal differences between these species and *C. esculenta*. No differences were found among the eleven accessions of *C. esculenta* tested with *Bam* HI, and no differences were found among the fourteen accessions tested with *Hae* III.

(4) *Rye nuclear 5S rDNA probe, Sc-T7* (Figure 11.7). Two triploid varieties of *C. esculenta* produced regular ladders of *Bam* HI fragments based on multiples of 200 bp, indicating that the 200 bp sequence is a high copy-number unit tandemly repeated at one or more loci. Complete digestion of all repeats by *Rsa* I generated a single 200 bp band.

(5) *Wheat nuclear 18S rDNA probe, pTA250.10* (Figure 11.8). In an initial test with *Eco* RI, two varieties of *C. esculenta* produced a constant smaller band (4.0 kbp) and a variable larger band (5.5 and 6.0 kbp). No obvious size differences were found between two accessions of the phenotypic variety GP (T287, T284) from different locations. *C. gigantea* also produced a 4.0 kbp band, together with a large, 7.1 kbp band. Thirteen further accessions of *C. esculenta* from Papua New Guinea, Australia, the Philippines, and New Zealand were tested with *Eco* R.I. Variation of fragments larger than 4.0 kbp was found to be common, within the size range 5.5–6.0 kbp, and all the accessions displayed the 4.0 kbp size class. Because the experimental conditions for this preliminary survey gave poor resolution of size differences, details of the results are not presented.

(6) *Taro nuclear rDNA probe, pCe34.1*. Analysis of three triploid varieties of *C. esculenta* from New Zealand:

All accessions of var. RR and var. GR (six and three, respectively) gave the same pattern of *Taq* I fragments, different from the one pattern produced by the four accessions of var. GP (Figure 11.9). The *Taq* I fragments are generated from the intergenic spacer region of *Nor*-locus rDNA (see Chapter Twelve).

All accessions of var. RR and var. GR (six and four, respectively) gave the same pattern of *Rsa* I fragments after partial digestion, different from the one pattern produced by the four accessions of var. GP (Figure 11.10). The ladders of partial digestion products reflect the distribution of *Rsa* I sites throughout the rDNA repeat sequence. At least some of the variability of *Rsa* I fragments arose in the intergenic spacer region (see Chapter Twelve). In theory, variability also could have arisen in other parts of the rDNA repeat sequence. The survey of *Rsa* I partial-digestion products indicates that the overall rDNA repeat sequence is conserved between different accessions within each phenotypic variety (RR, GR, GP), and between varieties (RR, GR).

All accessions of var. RR and var. GR (six and four, respectively) gave the same pattern of *Bam* HI fragments, different from the one pattern produced by the four accessions of var. GP (Figure 11.11). The large and variable *Bam* HI fragments span the entire intergenic spacer region (see Chapter Twelve). *Bam* HI fragment length differences, between varieties (GP versus RR and GR) and between doublets within individual samples (RR and GR), appear directly correlated with the intergenic spacer variation exhibited by *Taq* I fragment length differences in the same set of samples (compare Figure 11.9 and 11.10).

Table 11.1 DNA restriction fragments from *C. esculenta* and *C. gigantea*, detected by hybridisation with spinach chloroplast DNA probe (*Sal* I-6). No variation was observed among varieties of *C. esculenta*. The two species gave different *Bam* HI and *Eco* RI patterns.
A. Number of different fragments, and their approximate size range in kbp, after digestion with five restriction enzymes (? = unclear results, – = no test).
B. Accessions tested (tests performed, T collection number, species and variety, somatic cell chromosome number, source, and collection date). Source abbreviations: Q = Queensland. NI = North Island. * = unprovenanced collections from botanical gardens or private collections.

A

	C. esculenta		*C. gigantea*	
Test enzyme	No. fragments	Size range kbp	No. fragments	Size range kbp
1. Bam HI	6	1.5–5.8	4	1.5–4.0
2. Eco RI	5	0.9–5.0	?	?–5.2
3. Hind III	2	8.5–10.8		
4. Sal I	3	6.4–16.0		
5. Xho I	3	6.2–13.0		

B

Tests	T. collection number	Name	2n=	Source	Collection date
1, 2	268	*C. gigantea*	–	Australia,Q.*	1985
2	110	*C. esculenta*	42	Indonesia, Timor	c.1963
1–5	277	*C.e.* var. RR	42	New Zealand, NI	1983
1–5	281	*C.e.* var. GR	42	New Zealand, NI	1983
1–5	284	*C.e.* var. GP	42	New Zealand, NI	1982
2	287	*C.e.* var. GP	42	New Zealand, NI	1983
1–5	289	*C.e.* var. AKL34	28	New Zealand, NI	1982

Table 11.2 DNA restriction fragments from *C. esculenta*, *C. gigantea*, and *A. brisbanensis*, detected by hybridisation with the *Cox* I mitochondrial DNA probe from maize. No variation was observed among varieties of *C. esculenta*. The *Eco* RI pattern for *C. gigantea* was not resolved (= ?) because of partial digestion, but was probably the same as for the other two species (see Figure 11.5). Different patterns were observed for each species with the enzymes *Msp* I and *Hae* III.
A. Number of different fragments, and their approximate size range in kbp, after digestion with four restriction enzymes.
B. Accessions tested (tests performed, T collection number, species and variety, somatic cell chromosome number, source, and collection date). Source abbreviations: Q = Queensland, PNG = Papua New Guinea, EHP = Eastern Highlands Province, NT = Northern Territory, WHP = Western Highlands Province, ESP = Eastern Sepik Province, NI = North Island. * = unprovenanced collections from botanical gardens or private collections.

A

	C. esculenta		*C. gigantea*	
Test enzyme	Fragments	kbp	Fragments	kbp
1. Eco RI	4	0.3–5.1	?	?
2. Msp I	6	0.4–2.1	5	0.4–3.4
3. Hae III	5	0.3–5.6	6	0.3–5.6
4. Bam HI	5	2.1–8.6	–	–

B

Tests	T. coll. number	Name	2n=	Source	Coll.date
1–3	222	*A. brisbanensis*	–	Australia, Q.	1985
2, 3	268	*C. gigantea*	–	Australia, Q.*	1986
2–4	11	*C. esculenta*	28	PNG, EHP	1982
2–4	21	*C. esculenta*	–	PNG, Moresby	1982
2–4	23	*C. esculenta*	28	PNG, Moresby	1982
2–4	31	*C. esculenta*	28	Australia, NT	1980
2–4	40	*C. esculenta*	–	Australia, Q.*	1981
2–4	42	*C. esculenta*	28	Australia, Q*	1981
2–4	49	*C. esculenta*	28	PNG, WHP	1982
2–4	50	*C. esculenta*	–	PNG, WHP	1982
2–4	152	*C. esculenta*	–	Philippines, Luzon	1985
2–4	225	*C. esculenta*	–	PNG, Moresby	1985
2–4	238	*C. esculenta*	–	PNG, ESP	1981
2–4	240	*C. esculenta*	–	PNG, ESP	1981
2–4	277	*C. e.* var. RR	42	New Zealand, NI	1983
2–4	281	*C. e.* var. GP	42	New Zealand, NI	1983
1–4	284	*C. e.* var. GP	42	New Zealand, NI	1983
1–4	287	*C. e.* var. GP	42	New Zealand, NI	1983
1–4	289	*C. e.* var. AKL34	28	New Zealand, NI	1982

Table 11.3 DNA restriction fragments from *C. esculenta*, *C. gigantea*, and *A. brisbanensis*, detected by hybridisation with the maize mitochondrial 18–5S rDNA probe. No variation was observed among varieties of *C. esculenta*. The *Bam* HI and *Hae* III patterns for *C. esculenta* differed from the patterns for *C. gigantea* and *A. brisbanensis*, and no differences were found between the latter two (see Figure 11.6).

A. Number of different fragments, and their approximate size range in kbp, after digestion with two restriction enzymes.

B. Accessions tested (tests performed, T collection number, species and variety, somatic cell chromosome number, source, and collection date). Source abbreviations: Q = Queensland, PNG = Papua New Guinea, EHP = Eastern Highlands Province, NT = Northern Territory, WHP = Western Highlands Province, ESP = Eastern Sepik Province, NI = North Island. * = unprovenanced collections from botanical gardens or private collections.

A

	C. esculenta		*C. gigantea*		*A.brisbanesis*	
Test enzyme	Fragments	kbp	Fragments	kbp	Fragments	kbp
1. Bam HI	3	3.3–8.6	3	3.3–11.6	3	3.3–11.6
2. Hae III	2	0.7, 2.1	2	0.7, 2.6	2	0.7, 2.6

B

Tests	T. collection number	Name	2n =	Source	Collection date
1, 2	222	*A. brisbanensis*	–	Australia, Q	1985
1, 2	268	*C. gigantea*	–	Australia, Q*	1986
2	11	*C. esculenta*	28	PNG, EHP	1982
1, 2	21	*C. esculenta*	–	PNG, Moresby	1982
1, 2	23	*C. esculenta*	28	PNG, Moresby	1982
1, 2	31	*C. esculenta*	28	Australia, NT	1980
1, 2	40	*C. esculenta*	–	Australia, Q*	1981
2	42	*C. esculenta*	28	Australia, Q*	1981
1, 2	49	*C. esculenta*	28	PNG, WHP	1982
2	50	*C. esculenta*	–	PNG, WHP	1982
2	225	*C. esculenta*	–	PNG, Morobe	1985
1, 2	238	*C. esculenta*	–	PNG, ESP	1981
1, 2	240	*C. esculenta*	–	PNG, ESP	1981
1, 2	277	*C. e.* var. RR	42	New Zealand, NI	1983
1	281	*C. e.* var. GR	42	New Zealand, NI	1983
1, 2	284	*C. e.* var. GP	42	New Zealand, NI	1982
1, 2	289	*C. e.* var. AKL34	28	New Zealand, NI	1982

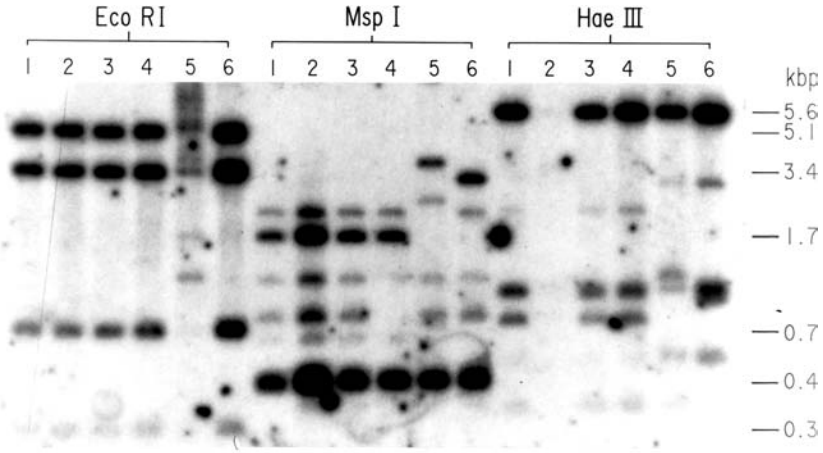


Figure 11.5 DNA fragments from *C. esculenta*, *C. gigantea*, and *A. brisbanensis* detected by hybridisation with the *Cox* I mitochondrial DNA probe from maize. Extracts of total DNA were digested with *Eco* RI, *Hsp* I, and *Hae* III. Lanes 1–4, *C. esculenta* (1–T289, 2–294, 3 = 277, 4–T281). Lane 5, *C. gigantea* (T268). Lane 6, *A. brisbanensis* (T222).

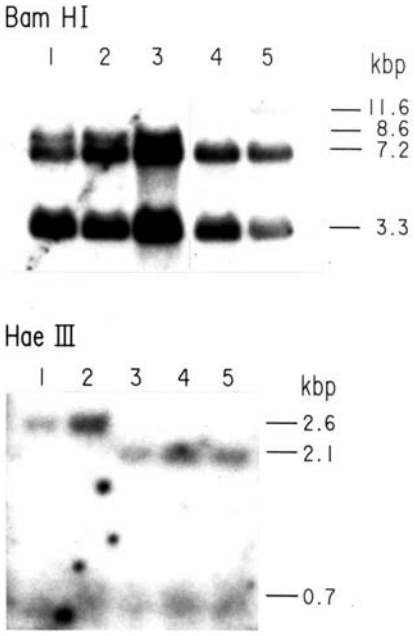


Figure 11.6 DNA fragments from *C. esculenta*, *C. gigantea* and *A. brisbanensis* detected by hybridisation with the mitochondrial 18–5S rDNA probe from maize. Total DNA extracts were digested with *Bam* HI and *Hae* III. Samples for *Bam* HI: lanes 13, *C. esculenta* (1 = T277, 2 = T43, 3 = T23); lane 4, *C. gigantea* (T268); lane 5, *A. brisbanensis* (T222). Samples for *Hae* III: lane 1, *C. gigantea* (T268); lane 2, *A. brisbanensis* (T222); lanes 3–5, *C. esculenta* (3 = T238, 4 = T31, 5 = T11)

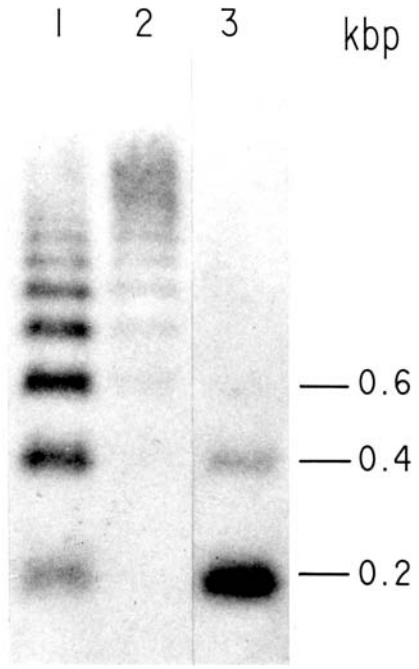


Figure 11.7 DNA fragments from two varieties of *C. esculenta*, detected by hybridisation with the 5S rDNA probe (Sc-T7) from *Secale cereale*. Lane 1: T281 (var. GR, 2n = 42, New Zealand) digested with *Bam* HI. Lane 2: T264 (var. *fontanesii*, 2n = 42, Australia) digested with *Bam* HI. Lane 3: T281 digested with *Rsa* I. The fragments in lane 1 (bp, approximate only) form a ladder of monomers (200 bp), dimers 400 bp), trimers (600 bp), and so on.

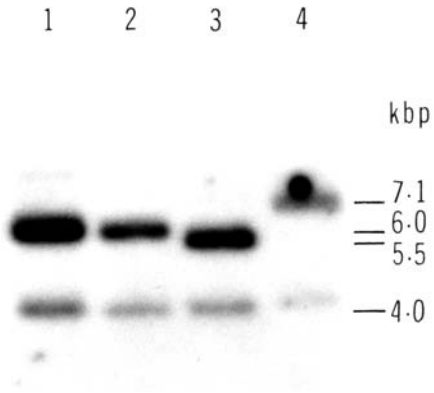


Figure 11.8 Taro DNA fragments detected with the nuclear rDNA, 18S gene probe from wheat (pTA250.10), after digestion of total DNA with *Eco* RI. Samples: (1) T287, *C. esculenta* var. GP, 2n 42, New Zealand, (2) T284, *C. esculenta* var. GP, 2n = 42, New Zealand, (3) T289, *C. esculenta* var. AKL 34, 2n = 28, New Zealand, (4) T268, *C. gigantea*, Australia.

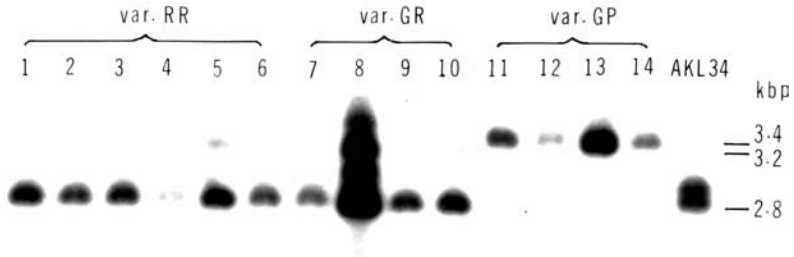


Figure 11.9 Ribosomal DNA spacer fragments from New Zealand triploid varieties RR, GR, and GP (sites 1–14), and the diploid AKL34. Autoradiograph of *Taq* I fragments after Southern transfer and probing with pCe34.1. One sample of var. GR, from site 8, gave partial digestion products because of excess DNA, together with the bands expected for this variety. See Figure 11.1 for site locations.

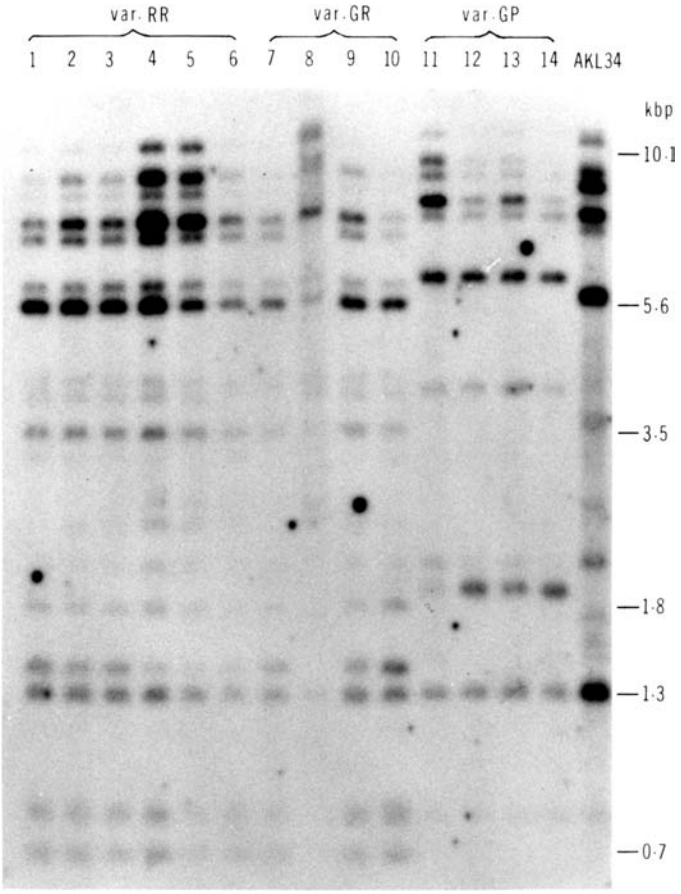


Figure 11.10 Ribosomal DNA spacer and genic fragments from New Zealand triploid varieties RR, GR, and GP (sites 1–14), and the diploid AKL34. Autoradiograph of *Rsa* I fragments (partial digestion products) after Southern transfer and probing with pCe34.1. See Figure 11.1 for site locations.

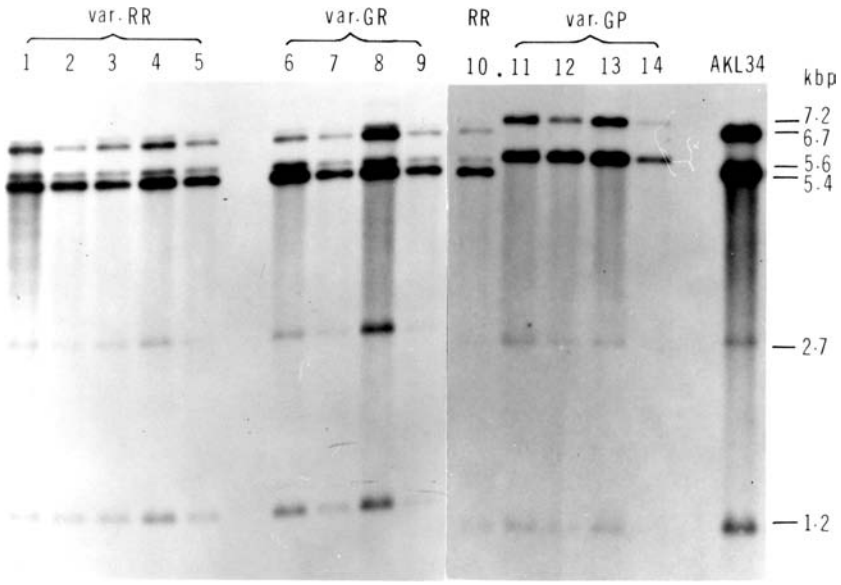


Figure 11.11 Ribosomal DNA variation in New Zealand triploid varieties RR, GR, and GP (sites 1–14), and the diploid AKL34. Autoradiograph of *Bam* HI fragments after Southern transfer and probing with pCe34.1. See Figure 11.1 for site locations. The large and variable fragments in the upper part of the autoradiograph span the intergenic spacer regions of rDNA repeats.

11.4 Concluding Discussion

After considering the identity of DNA fragments detected by hybridisation-probe analysis, comparisons are made between varieties of *C. esculenta*, and between *C. esculenta*, *C. gigantea*, and *A. brisbanensis*. The stability of rDNA in clonal lineages of taro is discussed in relation to the survey of vegetative clones from New Zealand.

11.4.1 The identity of fragments detected by hybridisation-probe analysis of total DNA

Within a broad classification of life forms, taro belongs to the plant eukaryotes, organisms with cells in which there are three kinds of organelle containing a DNA genome. The organelles are the nucleus, the mitochondrion, and the chloroplast. Total-DNA prepared from taro thus contains DNA from each of these organelles. In theory, DNA fragments from one or more organelle might be detected simultaneously by hybridisation-probe analysis of total-DNA extracts. In general it is most likely that probes derived from chloroplast, mitochondrial, and nuclear DNA hybridise most strongly to fragments from the chloroplast, mitochondrial, and nuclear genomes respectively, but cross-hybridisation between genomes is theoretically possible because of (1) common ancestry (endosymbiotic evolution) and (2) DNA exchange between genomes.

The endosymbiotic theory

Nuclear, mitochondrial, and chloroplast genomes are generally believed to have a common ancestry (Figure 11.12). Although the details of this ancestry are by no means clear, the serial endosymbiosis theory illustrated in Figure 11.12 is strongly supported by comparisons of the structure, biochemistry, and genotypic variation of cells and organelles in a broad range of life forms (Taylor 1979; Dayhoff and Schwartz 1980; Doolittle and Bonen 1981; Küntzel and Köchel 1981; Pace et al. 1986; Cavalier-Smith 1987). As a consequence of this evolutionary history, sequences similar to the probe sequence might have been inherited from a very ancient genome by more than one kind of organelle in taro. However, considering the time involved, hundreds of millions of years, only extremely stable sequences would be sufficiently similar for detectable cross-hybridisation between a probe from one genome and a target sequence from another.

Analyses of various highly conserved (stable) rRNA genes have been important for recent development of the endosymbiotic theory because rRNA genes occur in the genomes of each lineage involved — in the eukaryotic nuclei, in mitochondria and chloroplasts, and in the prokaryotes (Pace et al. 1986; Sogin and Gunderson 1987). For higher plants, the various studies have shown that, first, mitochondria and chloroplasts are both more closely related to bacterial prokaryotes than they are to plant nuclei; and second, that the mitochondria and chloroplasts represent separate, monophyletic lineages (Palmer 1985). In practice, under the kinds of experimental conditions described in this chapter, cross-hybridisation due to ancient shared ancestry is either absent, or too weak, to confuse identification of total-DNA fragments detected by hybridisation probe analysis, even when nuclear or mitochondrial rDNA probes are used.

The exchange of DNA sequences between genomes

Exchanges of various DNA sequences between nuclear, mitochondrial, and chloroplast genomes have been reported for a range of plant species (Stern and Palmer 1984; Whisson and Scott 1985), and are illustrated schematically in Figure 11.12. The phylogenetic history and molecular mechanisms for reported exchanges are unclear at present, but many different kinds of exchange are evident, and exchange in general is probably a continuing and common phenomenon in plants (Stern and Palmer 1984).

An example of particular interest is reported by Stern and Lonsdale (1982) who found, in *Zea mays*, a 12 kbp fragment of chloroplast-like DNA in purified mitochondrial DNA (Figure 11.3). This fragment has a base sequence more than 90% similar to the corresponding sequence in maize chloroplasts. Within the 12 kbp fragment, a sequence was found that hybridised strongly to chloroplast 16S rDNA, and weakly to functionally homologous, structurally similar, mitochondrial 18S rDNA. Detailed analysis confirmed the presence in maize mitochondria of a 16S rDNA sequence, which must have come from a chloroplast.

It is evident from the above that any probe DNA sequence has some chance of hybridising to fragments from any kind of genome in a total-DNA extract, regardless of how variable or stable that sequence might have been in the past. The genomic locations of fragments are not proven by hybridisation probe analysis of total-DNA extracts alone.

Genomic locations can be investigated by (1) analysis of total-DNA in conjunction with controlled breeding trials, using progeny analysis to determine the mode of genetic transfer, usually maternal for chloroplasts and mitochondria, and biparental for nuclear chromosomes, with Mendelian frequencies for alleles on nuclear chromosomes, (2) analysis of DNA purified from each organelle, and (3) *in situ* hybridisation. In Chapter

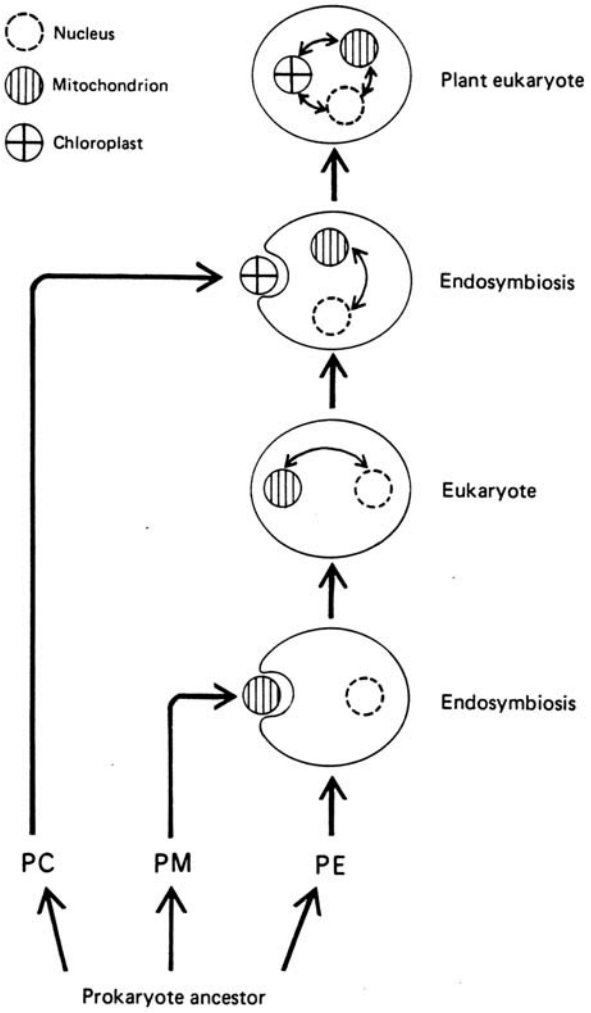


Figure 11.12 Evolutionary scheme (serial endosymbiosis hypothesis) for plant eukaryotes, adapted from Taylor (1976) and Doolittle and Bonen (1981). PM = protomitochondrial symbiont. PC = protochloroplast endosymbiont. PE = protoeukaryote. The early evolution of prokaryotes and of the nuclear genome are unresolved in this scheme. Arrows within cells indicate possible movements of DNA sequences between co-existing genomes. Symbioses may have occurred independently more than once for both kinds of endosymbiosis shown.

Twelve, *in situ* hybridisation of the taro rDNA probes to nuclear chromosomes is demonstrated.

11.4.2 Intraspecific comparisons

In recent years, much has been learned about the general qualities of variation in chloroplast and mitochondrial DNAs (for reviews, see Birky 1978; Palmer 1985, 1987; Sederoff 1987; Zurawski and Clegg 1987; Palmer and Herbon 1988). Both chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) undergo slow rates of base substitution. Plant mitochondrial genomes are generally composed of variously-sized circles (for example, see Figure 11.3) among which recombination occurs. These complex genomes evolve rapidly in structure, but slowly in sequence (Palmer and Herbon 1988). Palmer (1987) notes that chloroplast genomes are always composed of one large circular molecule (for example, see Figure 11.2), which evolves very slowly with infrequent rearrangements (inversions and transpositions). Insertions or deletions of extremely short sequences (one to ten base pairs) are the most common form of CPDNA mutation. The overall slow rate of change makes the CPDNA genome most valuable for studies aimed at the species level or above, although intraspecific variation has been reported for a few species (Palmer 1987; Neale et al. 1988). Phylogenetic analysis of plant mtDNA variation is severely limited by the high frequency of confounding rearrangements (Palmer 1987; Palmer and Herbon 1988). Nevertheless, the relatively frequent rearrangement of plant mtDNA improves the chance of detecting different maternal lineages within a species, and mapping their geographical distributions, with or without phylogenetic interpretation. For example, patterns of mtDNA variation in *Zea mays* subspecies trates (maize and teosinte) closely parallel those of whole plant and cytological variation (Timothy et al. 1979; Weissinger et al. 1983).

In the preliminary experiments with chloroplast and mitochondrial DNA probes, no variation was observed among different accessions, phenotypic varieties, and chromosome number variants of taro. Few tests were performed, so it is still quite possible that chloroplast and mitochondrial DNAs do vary in different lineages of taro. Two general strategies can be envisaged for future tests with heterologous probes. The first strategy is to continue tests with probes which have already revealed interspecific variation (see below). Further restriction enzymes could be used with these probes to localise the variable sequences, and to examine them closely in different varieties of taro. Locations of interspecific variation may also be locations at which intraspecific variation is functionally permissable and likely to occur. The second strategy is to continue testing previously untried combinations of probe and enzyme. According to the general views of chloroplast and mitochondrial DNA variation, noted above, restriction fragment differences between varieties of a species are most likely to reflect length differences (insertions, deletions), or inversions, rather than single base-pair changes. Any enzyme that cuts left and right of a length difference, or of an inversion break point, is potentially able to reveal the difference. Initially, therefore, it may be more profitable to try many new probes with just a few enzymes, rather than using a wide range of enzymes with each new probe.

The rye nuclear 5S rDNA probe and *Bam* HI were used to test two phenotypically distinct triploid varieties (Figure 11.7). Incomplete (partial) digestion with *Bam* HI generated ladders of similar periodicity for both varieties (Figure 11.7). Partial digestion by the methylation-sensitive *Bam* HI is attributed to 5-methylcytosine residues at the GGATCC *Bam* HI recognition sequences, as demonstrated for 5S rDNAs from other plant species (Goldsborough et al. 1982; Rafalski et al. 1982; Reddy and Appels 1989). Only the large multimers were produced by var. *fontanesii* (Figure 11.7), possibly as an artefact of reaction conditions. *Rsa* I digestion of taro 5S rDNA was complete and confirmed the monomer size of 200 bp. In other plants, single *Bam* HI and *Rsa* I sites occur in the highly conservative genic region of 5S repeat units, and the taro 5S rDNA unit can be aligned by analogy as shown in Figure 11.13.

Both intra- and interspecific variation have been detected in many grass species of the tribe Triticeae, but different species within one genus (*Secale*) could not be unambiguously differentiated even with extensive sequence analysis (Reddy and Appels 1989). Analysis of 5S rDNA could be useful for studying the evolution of taro, but it is not clear *a priori* what kind of information would be obtained.[2)]

The spacer sequences of 5S rDNAs accumulate mutations in the form of base

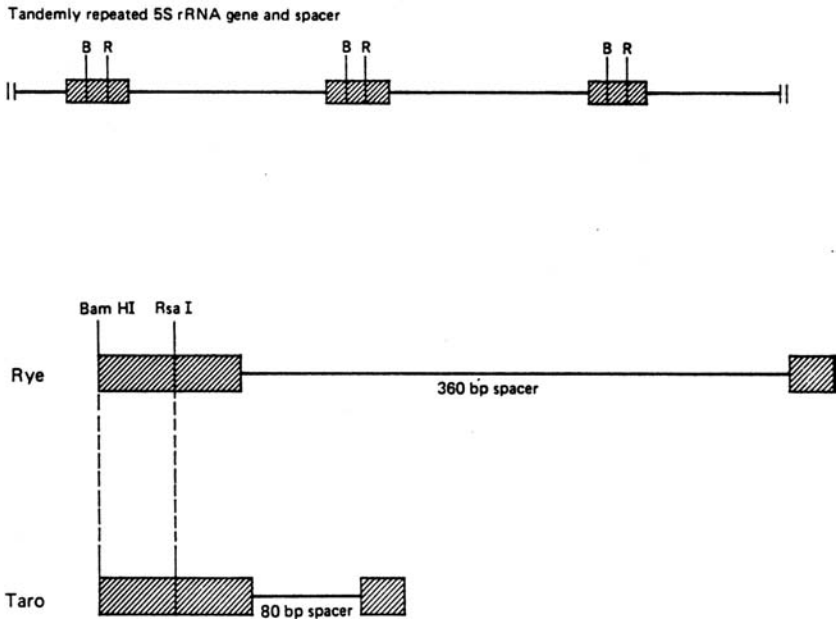


Figure 11.13 Alignment of *C. esculenta* (taro) and *Secale* cereales (rye) 5S rDNA repeat units, as suggested by the presence of single *Bam* HI and *Rsa* I restriction sites in taro 5S rDNA. The 5S rRNA gene of taro is assumed to be 120 bp long, as in rye and in plants and animals generally. Map for the rye repeat unit adapted from the full nucleotide sequence published by Reddy and Appels (1989).

substitutions, duplications, deletions, and insertions, with only minor heterogeneity in the gene region (see Reddy and Appels 1989, with references). The 5S rRNA gene of plants and animals is generally 120 bp in size (Scoles et al. 1988), and if the size estimate for the entire taro 5S repeat is correct, then a spacer sequence of only 80 bp can be inferred (Figure 11.13). This is very short compared to the 340 to 360 bp spacer sequences reported by Reddy and Appels (1989) for *Secale* sp. If 5S spacers are generally very short in *C. esculenta*, but are subject to insertions and deletions of similar size to those reported for the Triticeae, then finding phylogenetically informative base substitutions might be difficult. On the other hand, insertions and deletions could provide useful typological markers for different 5S rDNA lineages, with or without phylogenetic interpretation. Tests with the wheat 18S rDNA probe and *Eco* RI (Figure 11.8) provided the first indication of intraspecific variation, and for this reason, and because of their size (two fragments spanning a complete repeat sequence), *Eco* RI fragments were chosen as targets for cloning. Wheat rDNA probes were also used to screen *Eco* RI fragments from taro during the cloning process (see Chapter Ten). The cloned *Eco* RI rDNA fragments from taro were then used to test for variation among New Zealand triploid varieties (section 4, below).

11.4.3 Inter-specific comparisons

With the mitochondrial probe *Cox* I and the enzymes *Msp* I and *Hae* III, differences were found between each of the three species examined (Figure 11.5). With the mitochondrial 18–5S rDNA probe, similar *Bam* HI and *Hae* III fragments were detected for *C. gigantea* and *A. brisbanensis*, different from the fragments produced by *C. esculenta* (Figure 11.6). In each of these comparisons, some DNA fragments did not vary between species, and when *Eco* RI fragments were probed with *Cox* I, no variation was detected (Figure 11.5). The dendrogram constructed from these results (Figure 11.14) suggests that mitochondria in *C. gigantea* are less closely related to mitochondria in *C. esculenta* than they are to mitochondria in *A. brisbanensis*. The apparent close relationship of *C. gigantea* and *A. brisbanensis* could be due to convergent mutational events within the history of either species. Alternatively, if the classification correctly reflects the overall phylogeny of mitochondria in these taxa, then there may have been introgression of mitochondria from one species lineage to another, or variant mitochondrial lineages may have existed within ancestral populations prior to the differentiation of *Colocasia* and *Alocasia*.

It is perhaps significant that Engler and Krause (1920) placed *C. gigantea* Hook. f. (synonymous with *C. indica* [Lour.] Hassk) in Section 'Caulescentes', separate from three other commonly collected species, *C. esculenta*, *C. affinis*, and *C. fallax*. These three were placed together in the Section 'Tuberosae'. *C. gigantea* differs strikingly from the other *Colocasia* species in morphological aspects of the leaf, inflorescence, fruit, and seed (Engler and Krause 1920; and personal observation). Although *C. gigantea* is not obviously like *Alocasia* species phenotypically, its designation as a member of *Colocasia* should perhaps be questioned, considering the affinity between its mitochondria and those of *Alocasia*.

The kinds of mutation that created differences between *Colocasia* species and *A.*

brisbanensis cannot be described yet. Intraspecific rearrangements of a sequence immediately adjacent to the *Cox* I gene, in varieties of *Zea mays*, were reported by Isaac et al. (1985), and possible mechanisms and controlling factors for the rearrangements were suggested. These included specific mitochondrial sequences thought to promote recombination and undefined controls by nuclear genomes on the constitution of mitochondrial genomes. Given the rapid rate of plant mitochondrial DNA change (see Section 2 above), it cannot be assumed that the same recombination processes and nuclear influences operate in taxa that are distantly related, such as *Colocasia* and *Alocasia*. Using a maize *Cox* I mitochondrial DNA probe to survey total-DNA extracts, Breiman (1987) found intra- and interspecific variation among species of *Aegilops*, and no variation among species of Triticum (wheat) that shared the AB haploid genome

	Tests		DNA patterns		
	Probe	Enzyme	*C. esculenta*	*C. gigantea*	*A. brisbanensis*
1	Cox I	Eco RI	1.1	1.1*	1.1
2	Cox I	Msp I	2.1	2.2	2.3
3	Cox I	Hae III	3.1	3.2	3.3
4	18S - 5S	Bam HI	4.1	4.2	4.2
5	18S - 5S	Hae III	5.1	5.2	5.2

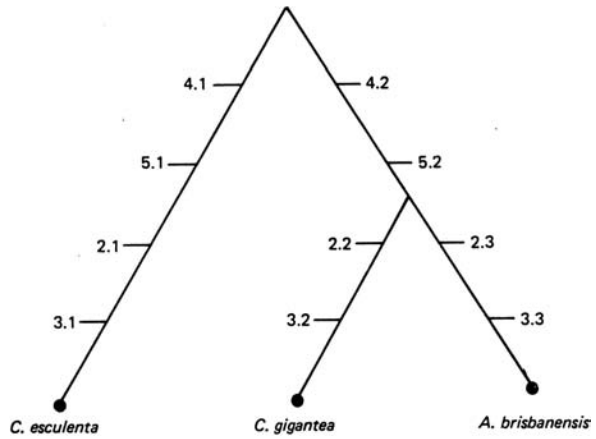


Figure 11.14 Dendrogram for species of *Colocasia* and *Alocasia*, based on restriction fragment patterns detected with mitochondrial DNA probes from maize. Results from Figures 11.5 and 11.6.

A. Five tests, each characterised by the probe and the restriction enzyme used. The different patterns that resulted are each identified by a test and pattern number. The tests represents characters, and the patterns are the observed character states. Asterisk: result same as for the other species, but interpretation based on partial digestion products.

B. Dendrogram based on the distribution of variable character states among species.

(nuclear chromosomal complement). Whatever the causes for variation in different taxa, it appears that the *Cox* I gene and its flanking regions will be useful generally for investigating intra- and interspecific evolution.

Palmer (1985) reviewed observations of chloroplast genomes in diverse taxa. In the relatively conservative genomes of chloroplasts, length mutation is the most common kind of mutation. Very short length mutations of a few base pairs occur predominantly in non-coding regions, and larger length mutations of 50–1200 bp are often found to cluster in relative 'hotspots', usually at the two ends of the large single copy region. The spinach chloroplast DNA probe *Sal* I-6 used here (Figure 11.2) is derived from one end of the large single copy region, and the different DNA patterns detected for *C. esculenta* and *C. gigantea* (Table 11.1) probably reflect length mutations in the same region of *Colocasia* chloroplast genomes.

11.4.4 Ribosomal DNA stability in clonal lineages

The generation of a new detectable and heritable rDNA variant requires that there be (1) mutation, (2) intracellular amplification within the genome, and (3) a positive bias in the reproduction of cells containing the variant (sorting out), in generative vegetative meristems and/or the germlines leading to gametes (eggs and pollen). These processes are poorly understood (see Chapter Nine), and when beginning the study of taro, little could be assumed about the stability of rDNA in clonal lineages.

The question of stability within clonal lineages is of concern for two reasons. First, mutation might occur after collection in the field, during the study, because of inherent genetic instability or in response to the study protocol (live field collections were maintained for up to four years by vegetative propagation, prior to testing). Second, the study is primarily concerned with the long term history of taro within the human era, and the vegetative propagation of cultivars is a major component of this history. If ribosomal DNA changed frequently during vegetative reproduction, then it would be of little use for tracing the long-term dispersal history of cultivated taro.

It is commonly thought that genomes do not change during clonal reproduction. This view is most commonly expressed as an implicit assumption (e.g., see Ellstrand and Roose 1987) or expressed summarily in textbook definitions of clonal reproduction (e.g., Weier et al. 1974; Briggs and Walters 1984; Mayo 1987). The observation that genomes in general are stable during clonal reproduction does not necessarily apply to specific components of a specific genome. Ribosomal DNA mutation during vegetative reproduction (mitosis in non-floral, generative apices) does not appear to have been the subject of direct experimental investigation, although this would be possible with the test system established by Durrant (1962) with *Linum usitatissimum* (cultivated flax). Environmentally induced and heritable effects on ribosomal DNA copy number were found by Cullis (1979) using varieties produced by Durrant (1962), but the observed effects cannot be ascribed to a particular stage in the flax life cycle because the original treatments (Durrant 1962: 29) spanned both pre-floral and floral stages. The opportunity to assess the stability of rDNA variants during vegetative reproduction was presented by New Zealand collections of *C. esculenta* varieties RR, GR, and GP, described by

Matthews (1984, 1985).

Ribosomal DNA was surveyed in three phenotypically distinct triploid varieties (RR, GR, GP) in accessions from a wide geographical range within New Zealand (Figure 11.1). No variation was observed in tests which examined the intergenic spacer region specifically (*Taq* I fragments, Figure 11.9), unmapped *Rsa* I recognition sites scattered through the spacer and genic regions of rDNA repeats (Figure 11.10), and the overall length of rDNA repeats (*Bam* HI fragments, Figure 11.11). The lack of rDNA variation within varieties is interpreted as evidence for rDNA stability during vegetative reproduction. This interpretation follows from observations of taro reproduction in New Zealand, and two assumptions discussed below.

Seed production by taro in New Zealand has never been reported, although flowering is common (Cooper 1969; Matthews 1984) and reproduction appears to be obligately vegetative. Likely reasons are the short summer growth period, which may not be long enough for seed development, and the fact that the varieties under consideration are triploid (Matthews 1985). In winter in New Zealand, the growth of taro slows down and frequently ceases completely (personal observation), and it is a general observation for plants that triploids are either completely or predominantly sterile (a single extra set of chromosomes results in irregular chromosome pairing during meiosis). The three phenotypically distinct varieties are therefore thought to represent three different clonal lineages.

The degree of clonal stability indicated by the observations of rDNA homogeneity depends on the interpretation of when the triploid varieties were introduced. If prehistoric, the introductions could have been as early as c. 800 to 1000 years ago, according to archaeological estimates of human occupation dates (Trotter 1982; Caughley 1988). Alternatively, it has been suggested that these varieties could have been imported from China by merchants supplying immigrant market gardeners, sometime between the turn of the century and the 1930s (Yen and Wheeler 1968). The wide distributions of the varieties (Matthews 1985), and local information supplied to this author at a number of field sites, make it certain that triploid taro arrived before World War II (1939). There is no evidence for variation arising within the course of the study, and ribosomal DNA patterns in New Zealand appear to have been stable for a period of at least fifty years.

Interpreting the results as a test of clonal stability requires two assumptions: (1) there were not multiple introductions of genotypically similar clones, derived from different sexual progeny produced outside New Zealand, and (2) homogeneity within the varieties distinguished here did not arise through the convergence of separate clonal lineages. If there were multiple introductions of a particular variety, then it seems more probable that they came from one clonal lineage, established in cultivations in the area of origin, than from separate but genotypically similar clonal lineages. The second assumption is made because it is hard to imagine how convergence could occur on all clonal branches of different lineages without the survival of intermediate and progenitor rDNA patterns in many branches.

To conclude, the survey of New Zealand triploid varieties indicated that rDNA is sufficiently stable for use as a genotypic marker for the dispersal of clonal lineages, and

attention was turned next to a wider survey of Asian and Pacific taro, described in the following chapters.

Notes

1) In this volume, the term ‘variant’ is used as an equivalent to ‘variety’ as an informal term, not as a formal botanical term.
2) Using 5S rDNA genic-region primers and the Polymerase Chain Reaction (PCR), Nakayama et al. (2008) amplified 5S repeats in a wild taro from Myanmar and found a simple ladder pattern like the one reported here. The basic repeat unit was found to be 210 bp, and the PCR products formed a 210, 420, 630 bp… ladder. The PCR products were sequenced, showed variation within a single population (represented by seeds from one location), and were used for *in situ* hybridisation to chromosomes, revealing the cytological locus of 5S repeats.

Senri Ethnological Studies 88: 169–178 ©2014
On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Twelve
The *Nor*-Locus Ribosomal DNA Repeat Unit of *C. esculenta*

12.1 Introduction

As described in the previous chapter, *Eco* RI digestion and hybridisation probe analysis with a wheat rDNA probe revealed large taro fragments (c.a. 5.6–6.0 kbp) that varied in size between taro varieties, and smaller fragments (c.a 4.0 kbp) that were invariant. One large and one small *Eco* RI fragment were cloned from AKL 34, a New Zealand diploid taro variety. The recombinant bacterial plasmids containing the taro rDNA fragments were named pCe34.1 and pCe34.2 respectively (see Chapter Ten). Restriction enzyme digests and double-digests of the cloned rDNA fragments, and of total-DNA extracts from AKL 34, were probed with previously defined wheat rDNA probes to locate the restriction enzyme sites and approximate gene positions in taro rDNA. These experiments confirmed the expectation that the large *Eco* RI fragments from taro contain the intergenic spacer region, and consistent with this were later observations that pCe34.1, and a 2.8 kbp *Taq* I fragment excised from pCe 34.1, could be used as probes to detect small and highly variable *Taq* I, *Hinf* I, and *Rsa* I fragments (see last part of Chapter Eleven; and Chapter Fourteen). Variation in the large intergenic spacer region was expected because it is generally observed within and between eukaryote species (see Chapter Nine). Restriction site mapping was also performed using pCe34.1 and pCe34.2, and the 2.8 kbp *Taq* I fragment excised from pCe34.1, to probe digests of the cloned rDNA fragments and digests of total DNA from AKL 34.

A cloned rDNA fragment from taro variety AKL 34 was hybridised *in situ* to chromosomes from mitotically dividing root-tip cells of AKL 34. Two major chromosomal loci were found associated with the microscopically visible clear areas within the cell nuclei, which indicate functionally active nucleoli where rDNA is transcribed to produce rRNA. The results described in this chapter provide a structural and functional basis for relating observations of rDNA variation in taro to the many reports of *Nor*-locus rDNA in other organisms. Taro rDNA does not appear at all unusual, and this simplifies the interpretation of rDNA variation in taro and its near relatives in the chapters that follow.

12.2 Materials and Methods

The diploid (2n = 28) taro variety AKL 34 was collected from a house garden in Northland, New Zealand, in 1982 and was described by Matthews (1984). Two *Eco* RI fragments spanning the taro rDNA repeat unit were independently cloned from AKL 34 using the vector pBR322 and *Eschericia coli* strain RR1. The cloning protocol, described

in Chapter Ten, was similar to that used by Appels et al. (1987), with minor modifications. Extraction of total-DNA and of plasmids containing the rDNA sequences, restriction enzyme digestion, electrophoresis, Southern transfer, and autoradiography were performed as described in Chapter Ten.

To map restriction enzyme sites and gene positions, the following enzymes and enzyme combinations were used, and the digestion products electrophoresed side-by-side: *Eco* RI, *Bam* HI, *Eco* RI + *Pst* I, *Bam* HI + *Pst* I, *Eco* RI + *Xba* I, *Bam* HI + *Xba* I, *Eco* RI + *Kpn* I, *Bam* HI + *Kpn* I, *Bam* HI+ *Eco* RI. In separate experiments, with the taro 2.8 kbp *Taq* I fragment from pCe34.1 as probe, the enzymes *Taq* I, *Hinf* I, *Taq* I + *Hinf* I, and *Rsa* I were used. Wheat rDNA clones from the PTA recombinant plasmid series, described by Appels and Dvorák (1982 a, b), were kindly supplied by R. Appels: pTA250.1 (4.4 kbp *Bam* EI/*Eco* RI fragment spanning the large intergenic spacer), pTA250.2 (3.6 kbp *Bam* HI fragment spanning most of the rRNA gene region), pTA250.3 (0.9 kbp *Bam* UI/*Eco* RI fragment from the 26S gene), pTA250.10 (1.0 kbp *Taq* I fragment from the 18S rRNA gene), and pTA250.11 (0.5 kbp *Taq* I fragment from the 26S rRNA gene). For restriction site mapping, digests of the cloned rDNA fragments and of total DNA from AKL 34 were probed with pCe34.1, pCe34.2, and the 2.8 kbp *Taq* I fragment excised from pCe34.1, resulting in the plasmid pCe34.11 (Chapter Ten) and providing probe rDNA for later surveys of intergenic spacer variation in taro (Chapter Fourteen).

For *in situ* hybridisation of a cloned rDNA sequence to chromosomes, the methods of Appels et al. (1978) and McIntyre (1987) were followed. Synthesis of ^{3}H-labelled copy-RNA probes from pCe34.1, preparation of chromosomes from mitotically dividing root-tip cells, hybridisation, and autoradiography were as described in Chapter Ten.

12.3 Results

12.3.1 Mapping

A 2.8 kbp *Taq* I fragment from pCe34.1 was mapped to the large intergenic spacer region as shown in Figure 12.1. When used as a probe, the 2.8 kbp *Taq* I fragment hybridised strongly to two doublets composed of *Bam* HI/*Pst* I fragments, generated by double digestion of total-DNA with *Bam* HI and *Pst* I. Weak hybridisation to the *Pst*/*Eco* fragment was seen with digests of the cloned insert, and no hybridisation to this fragment was detected with total-DNA digests. This may be because of either (1) a very small amount of overlap by the 2.8 kbp *Taq* I fragment, or (2) the presence of divergent sub-repeat sequences on both sides of the *Pst* I site.

The experiments with wheat and taro rDNA probes, and with cloned rDNA and native total-DNA, gave mutually consistent results, and these are summarised as a single map for an entire repeat unit (Figure 12.2). Because pCe34.1 and pCe34.2 were independently prepared from total-DNA extracts, they contain rDNA sequences that were almost certainly not from the same repeat unit *in vivo*. Mapping of the two cloned fragments and of rDNA in total-DNA extracts confirmed that pCe34.1 and pCe34.2 are typical for rDNA repeats in AKL 34. The rDNA fragments from these clones can

therefore be shown as adjacent to each other, for schematic purposes (Figure 12.2). By analogy to maps for restriction sites and rRNA genes in other genera (*Triticum* and *Pisum*), the boundaries of the large intergenic spacer and the rRNA genes of taro can be shown as in Figure 12.3.

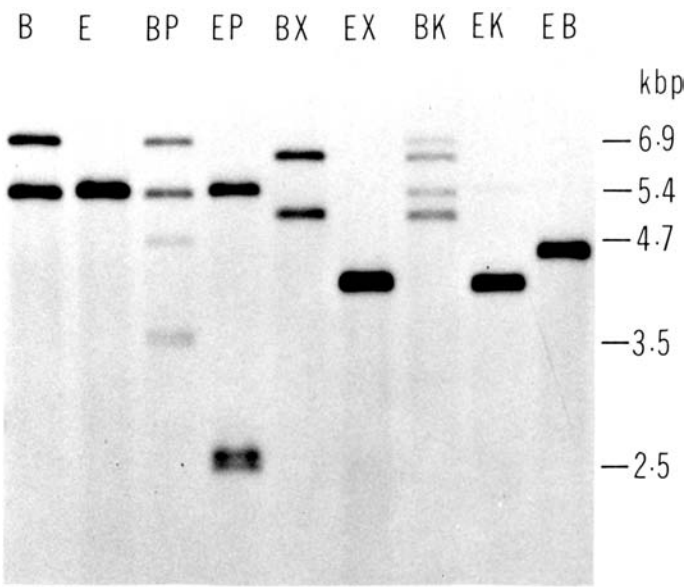


Figure 12.1 Mapping the position of the 2.8 kbp *Taq* I fragment from pCe34.1. This fragment was used to probe total DNA extracts from AKL 34, after digestion with the same set of enzymes used for restriction-site mapping. The position of the *Taq* I fragment is revealed by reference to the map shown in Figure 12.2. The enzymes used and the fragments detected are listed below. The 5′ to 3′ orientations of double-digestion fragments are indicated in brackets: the first letter indicates the 5′ restriction enzyme site, and the second letter indicates the 3′ restriction enzyme site (see Figure 12.2). Asterisks refer to the 26S gene *Bam* HI site which is not cut in all repeat units.

B = *Bam* HI; 6.9 kbp, 5.4 kbp.

E = *Eco* RI; 5.5 kbp.

BP = *Bam* HI + *Pst* I; doublet, approx. 4.7 kbp (BP); doublet, approx. 3.5 kbp (*BP); plus undigested *Bam* HI fragments.

EP = *Eco* RI + *Pst* I; doublet, approx. 2.5 kbp (EP); plus undigested *Eco* RI fragments.

BX = *Bam* HI +*Xba* I; 6.5 kbp (BX); 5.0 kbp (*BX).

EX- *Eco* RI +*Xba* I; 4.2 kbp (EX).

BK- *Bam* HI + *Kpn* I; 6.4 kbp (BK); 4.9 kbp (*BK); plus undigested *Bam* HI fragments, the results of partial digestion by *Kpn* I.

EK= *Eco* RI + *Kpn* I; 4.1 kbp (EK); plus faint band of undigested *Eco* RI fragments, the result of partial digestion of *Kpn* I.

BE = *Bam* HI +*Eco* RI; 4.4 kbp (EB).

The doublet *Bam* HI/*Pst* I fragments span the region from two 26S gene *Bam* HI sites, one of which is not cleaved in all repeat units, to the spacer region *Pst* I site (Figure 12.2). AKL 34 possesses two rDNA size variants which differ by approximately 100 bp, and the difference resides within the intergenic spacer region spanned by the *Bam* Hl/*Pst* I fragments. Similar size variation was seen after *Hinf* I and *Rsa* I digestion of total DNA from AKL 34, using the 2.8 kbp *Taq* I spacer fragment as probe. This probe also detected an approximately 1.5 kbp fragment in an *Rsa* I digest of the cloned sequence in pCe34.1, and fragments of 1.5 kbp and 1.6 kbp after *Rsa* I digestion of total DNA from AKL 34. The cloned sequence thus appears to be derived from the smaller rDNA variant found in AKL 34. The relative positions of *Taq* I and *Hinf* I intergenic spacer sites (Figure 12.4) were determined by single and double digestion of total DNA. The 5′ to 3′ orientation of these sites within repeat units, and their exact positions relative to *Rsa* I sites, have not been determined. The 100 bp spacer difference lies within the 2 kbp sequence shared by the *Taq* I and *Hinf* I fragments. The 1.5 1.6 kbp *Rsa* I fragments must be derived from entirely within the variable 2 kbp region, as suggested in Figure 12.4, or from partly within it.

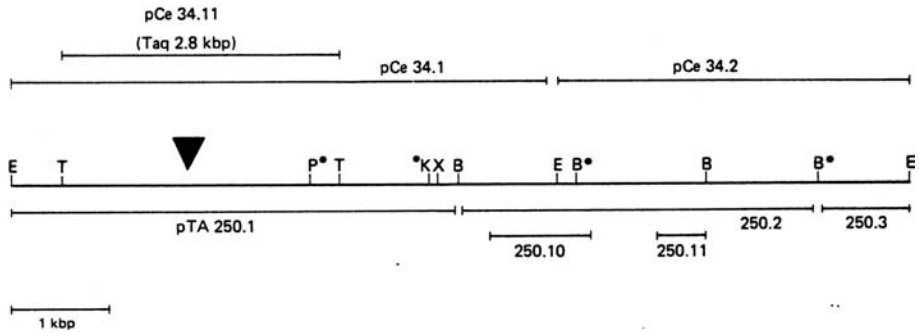


Figure 12.2 Restriction-site map for *Nor*-locus rDNA from taro variety AKL 34, orientation 5′ to 3′ (left to right), wheat determined with rDNA probes. Restriction endonucleases: E = *Eco* RI, T = *Taq* I, P = *Pst* I, K = *Kpn* I, X = *Xba* I, B = *Bam* HI. Asterisks mark sites which consistently failed to be cut in a high proportion of repeat units in total-DNA extracts. pCe = taro rDNA clone: the 2.8 kbp *Taq* I fragment was used as a probe after excision from pCe34.1. Wheat rDNA probes: pTA250.10 marks the 3′ end of the 18S rRNA gene, pTA250.11 marks the 5′ end of the 26S rRNA gene, and pTA250.3 is close to the 3′ end of the 26S rRNA gene.
Two rDNA variants detected in total-DNA extracts from AKL 34 are distinguished by an approximately 100 bp size difference located between the *Taq* I sites (arrow). The exact position is not known. *Taq* I sites 5′ and 3′ to the 2.8 kbp fragment give many very small fragments which have not been mapped. The 2.8 kbp *Taq* I fragment hybridised strongly to the *Eco*/*Pst* (5′–3′) fragment in digests of cloned insert and total DNA, and is therefore shown located over this fragment.

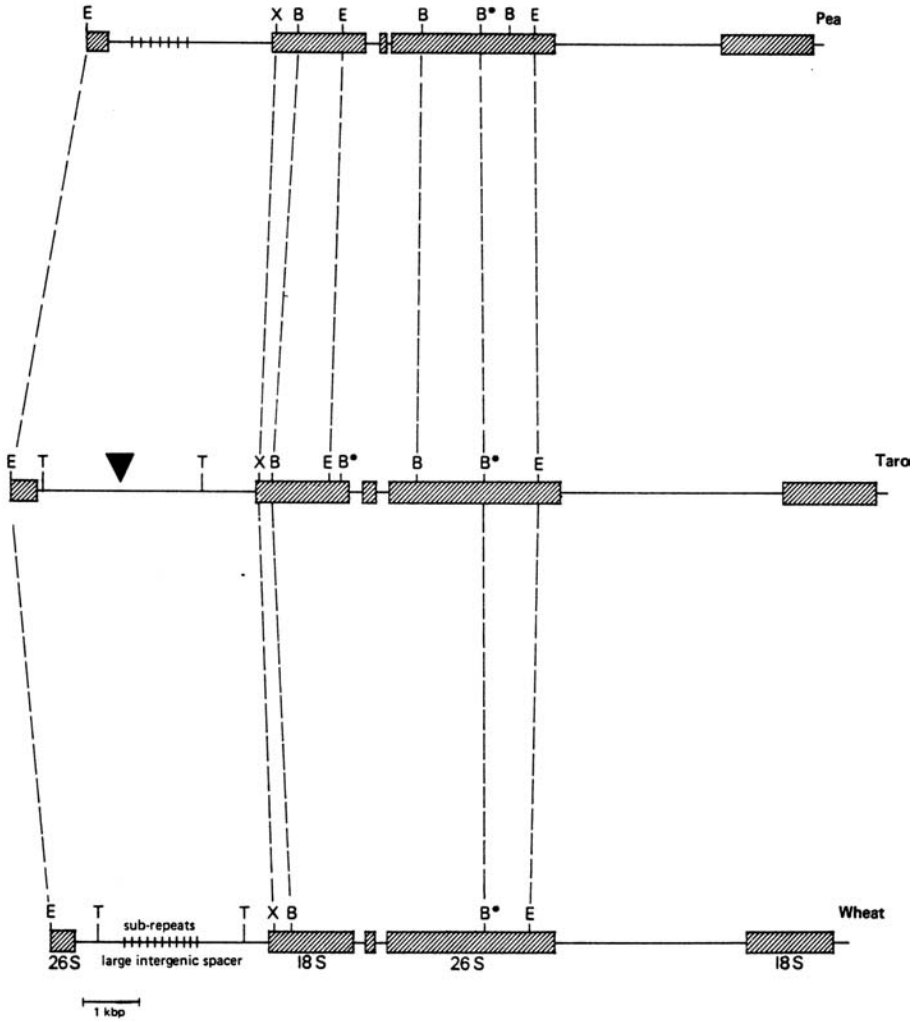


Figure 12.3 Alignment of the taro rDNA restriction site map with maps for *Pisum sativum* (pea) and *Triticum aestivum* (wheat).

The boundaries for the rRNA genes in taro are not known exactly, but are presumed to be similar to the boundaries indicated for pea and wheat, since the overall arrangement of rRNA genes, and the large intergenic spacer, is the same in these three species and eukaryotes generally. The dotted lines connect what are believed to be conserved (homologous) restriction sites. Asterisks mark sites which are consistently not cut in a high proportion of repeat units, in extracts of total DNA. This phenomenon has been reported in pea, wheat, and other plant species, and is a further indication of homology (see text). An arrow marks the variable region of taro rDNA, which correspond, in position, to the variable spacer sub-repeat regions of pea and wheat. The pea map is compiled from Jorgensen et al. (1987) and Kaufman et al. (1987), and the wheat map is compiled from Appels et al. (1980), Appels and Dvořák (1982a), and Jorgenson et al. (1987).

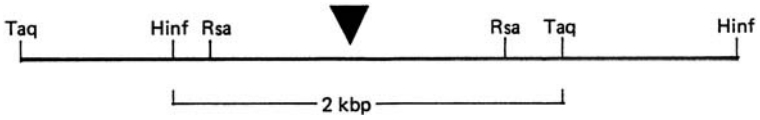


Figure 12.4 Restriction site map for a variable segment in the large intergenic spacer region, for taro variety AKL 34, 5′ to 3′ orientation not determined. The exact positions of *Rsa* I sites relative to *Taq* I and *Hinf* I sites are not known. The *Rsa* I fragment may lie within or overlap the central *Taq* I/*Hinf* I fragment. The arrow marks the approximate location of a 100 bp size difference observed between rDNA variants in total-DNA extracts from AKL 34. Within rDNA repeats, there are many other *Taq* I, *Hinf* I, and *Rsa* I sites outside the spacer segment shown here.

12.3.2 *In situ* hybridisation to chromosomes in the cells of root-tips

At interphase, during the cell life cycle, chromosomes are uncoiled and cannot be visualised as individual entities. However, nucleoli are visible at interphase and mark the sites of ribosomal RNA synthesis from rDNA (Novikoff and Holtzman 1976). After *in situ* hybridisation with copy rRNA from pCe34.1, autoradiographic grains were visible in the nucleoli of interphase cells (Figure 12.5). These grains were arranged in two distinct clusters, suggesting the existence of two separate chromosomal loci for rDNA.

Mitotic cell division is the basis for vegetative reproduction, and for the differentiation of roots and other plant parts. There are four phases during mitotic cell division: prophase, metaphase, anaphase, and telophase. During prophase, chromosomes are visible in partially condensed form, each having doubled during late interphase (the S period), giving one parent and one daughter chromatid. During metaphase the chromosomes condense further, become aligned on the mitotic spindle, and display clearly distinct chromatids. In subsequent phases, the chromatids separate and the division of the nucleus and cytoplasm produces two daughter cells. *In situ* hybridisation of the copy rRNA probe to prophase and metaphase chromosomes (Figure 12.5) revealed two major chromosomal loci for rDNA on separate chromosomes. These loci correspond to the two areas of dispersed grains seen in the nucleoli of interphase cells.[1)]

12.4 Concluding Discussion

Spacer length variation in taro is probably due to the presence, and variation in number, of sub-repeat units, as reported for pea (sub-repeat unit of 180 bp) and wheat (sub repeat unit of 130 bp). The size difference of approximately 100 bp in spacer fragments from AKL 34 is probably due to differences of one or very few sub-repeat units.

The *Taq* I sites that border highly variable regions in the spacer regions of taro and wheat (Figure 12.3) are the result of convergent (parallel) evolution. Spacer regions and their sub-repeat structure are conserved as structural components of *Nor*-locus rDNAs, but the base sequences within them vary greatly among diverse taxa (Appels and Honeycutt 1986). Ribosomal DNA sequence divergence between taxa is greatest in the sub-repeat arrays, relative to genic regions and other parts of the large intergenic spacer

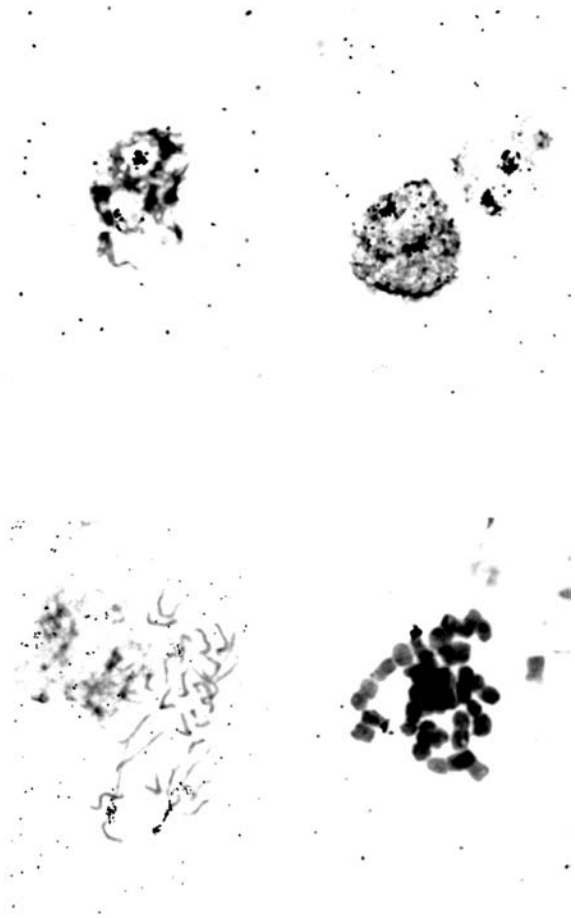


Figure 12.5 Taro rDNA probe from variety AKL 34 (5.5 kbp *Eco* RI fragment) hybridised *in situ* to mitotic chromosomes from the same variety. Silver emulsion autoradiographs of chromosomes. Clusters of silver grains (black dots) mark the locations of two rDNA loci in each cell. The upper frames show interphase cells. Two clear areas mark nucleoli, in the cell at left, and each nucleolus contains one rDNA locus. The lower left frame shows contents of prophase cell, with silver grains dispersed along two partly condensed chromosomes; the lower right frame shows one metaphase cell, with one rDNA locus visible on each of two fully condensed chromosomes (at bottom left and top centre).

(see Chapter Nine, Figure 9.5, from Appels and Honeycutt 1986). It is likely that most rDNA differences detected among different varieties of taro (Chapters 11 and 14) are due to varying numbers of sub-repeats.

The *Taq* spacer fragment is produced in tests with wheat because the sub-repeat sequence lacks *Taq* I sites (Appels and Dvořák 1982a). Sub-repeats are probably present in taro rDNA, and the same explanation is suggested for *Taq* I, *Hinf* I, and *Rsa* I spacer fragments from taro (Figure 12.4). These three enzymes require specific sequences of

only four nucleotides to cleave DNA, and the four-base recognition sequences are very common. Cleavage of rDNA with these enzymes generates many very small fragments from regions outside the variable spacer region. In the future, mapping the positions of such common sites in rRNA genes may be particularly useful for close characterisation of different rDNA variants, because many sites are recognised and this increases the chance of detecting variation in conserved genic regions. One *Bam* HI site in the 26S rRNA gene remained uncleaved in a large proportion of rDNA repeats, and two *Bam* HI autoradiographic bands of approximately equal intensity resulted (Figure 12.1). A similar result has been reported for the same *Bam* HI site in many plant species, and various explanations have been proposed. These involve either base sequence change within the *Bam* HI recognition sequence, or a base modification by methylation that makes the enzyme recognition sequence resistant to cleavage. The latter explanation is favoured by most writers, although it does not exclude the possibility of base sequence changes having occurred. The argument for methylation is considered next.

Resistance to cleavage by *Bam* HI can be explained (Siegel and Kolacz 1983) by postulating the presence of a guanosine residue adjacent to the 3′ cytosine in the *Bam* HI recognition sequence, GGATCC. The resulting CGG sequence would be a substrate for methylation. Jorgensen et al. (1987) noted that this explanation is consistent with the fact that the *Bam* HI site is detected in only about one half of the rDNA repeats in pea, as well as in wheat, barley, rye, corn, beans, and pumpkin. Taro and many other species can be added to this list. Gruenbaum et al. (1981) proposed that CCG sequences, when synthesised during DNA replication as copies of complementary GGC, will be methylated at one or other C at random, but not at both, resulting in 50% methylation at each cytosine. Since *Bam* HI is sensitive to methylation of the internal but not the external cytosine in its recognition sequence (McClelland 1983), Jorgensen et al. (1987) expected about half of the *Bam* HI sites adjacent to a G at the 3′ end to be resistant to cleavage.

The postulated presence of guanosine adjacent to the 26S gene *Bam* HI site was confirmed in the rice 26S rDNA sequence published by Takaiwa et al (1985). Beginning at base number 1905, the sequence CGGATCCG was reported (*Bam* HI recognition sequence underlined). Because this is a palindrome, 50% of replications from either strand may generate an internal methylated cytosine in the *Bam* HI recognition sequence. The same eight base sequence is probably associated with the 26S gene *Bam* HI site (six bases) in taro.

A similar explanation to that just given may also apply to the *Bam* HI site in the taro 18S gene, and to the *Kpn* I recognition sequence (GGTACC), which is similar to that for *Bam* HI, and which is not detected in all taro rDNA repeats (Figure 12.1, *Kpn* I + *Bam* HI digest). The recognition sequence for *Pst* I (CTGCAG) contains methylation-sensitive cytosines and is located in the large intergenic spacer where the frequency of methylation may be related to regulation of rDNA transcription (von Kalm et al. 1986). In *Lilium henryi*, von Kalm et al. (1986) found a *Pst* I site in a consistently under-methylated region of the spacer, and a spacer *Kpn* I site, close to the 18S gene, was also detected in only a proportion of rDNA repeats from total-DNA extracts (Figure 12.6).

In situ hybridisation of the cloned 5.5 kbp *Eco* RI rDNA fragment demonstrated that

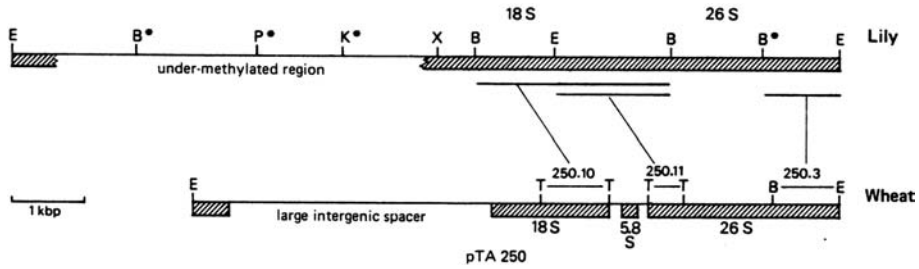


Figure 12.6 The rDNA repeat unit of *Lilium henryii* aligned with wheat rDNA probes from the pTA250 series (adapted from von Kalm et al. 1986). Asterisks mark sites for restriction enzymes that did not cleave the rDNA of all repeat units (see text for explanation). Restriction enzymes: E = *Eco* RI, B = *Bam* HI, K = *Kpn* I, X = *Xba* I, T = *Taq* I.

rDNA in the diploid taro variety AKL 34 is located in two major loci associated with nucleoli. The cloned rDNA is thus positively identified as the rDNA of nucleolar organiser regions (*Nor*-locus rDNA). Ribosomal DNA fragments detected in total DNA extracts with the cloned rDNA probes are mostly derived from the *Nor*-loci, since these are the major (large copy number) sites for rDNA. The two rDNA loci in AKL 34 are most simply interpreted as belonging to one homologous pair of chromosomes. However, it is possible for one of two homologous chromosomes to have no rDNA (a null locus), and a more complicated interpretation is that the rDNA loci in AKL 34 are on two heterologous chromosomes, with each locus paired by a null locus. The absence of *Nor*-locus rDNA within a chromosome complement (haploid set) has been reported for the toad *Xenopus* as a condition that is lethal in homozygous chromosomal combinations and viable in heterozygous combinations (Novikoff and Holtzman 1986: 335). Because cultivated taro is propagated vegetatively, non-lethal heterozygous conditions could have become common in cultivation, even if such conditions were rare in the wild because of selection against them.

In *Triticum dicoccoides* and *T. aestivum*, major rDNA loci are found on two pairs of homologous chromosomes, IB and 6B, which are readily distinguished by the possession of secondary constrictions and by their chromosome-arm length ratios (Appels and Dvorák 1982a). Initial karyotypic analysis of AKL 34 (Matthews 1984) revealed many chromosomes with similar morphology, making it difficult to identify homologous pairs. Whether or not the rDNA loci in AKL 34 are homologous could be addressed directly and perhaps most easily by *in situ* analysis of meiotic cell divisions. Homologous loci would be observed on paired chromosomes during meiotic metaphase I, and heterologous loci would be found on unpaired chromosomes.

To conclude, the structure of taro rDNA, its possible modification by methylation, and its association with nucleoli are consistent with reports for other plant species. Taro *Nor*-locus rDNA does not appear at all unusual, and in later chapters, variation in taro rDNA can be directly related to rDNA variation in other species.

Note

1) In later studies (Kokubugata and Konishi 1999, Nakayama et al. 2008), 45S rDNA probes were used for *in situ* hybridisation to chromosomes from taro, and confirmed the presence of two separate rDNA loci in plants with somatic chromosome numbers of 2n=28. Since the samples involved came from different countries, it is likely that the presence of two loci in the diploid complement of taro is general for the species.

Senri Ethnological Studies 88: 179–190 ©2014
On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Thirteen
C. esculenta (Taro) as Homogeneous Taxon

13.1 Hybridisation in the Indo-Malaysian Region?

The name *Colocasia esculenta* (L.) Schott is used today in a broad sense, and refers to a polymorphic species containing many phenotypic varieties, some of which have been given formal descriptions (Plucknett 1983). Little is known about sexual reproduction by Colocasioid genera and species (taxa in Subfamily Colocasioideae) in nature, and it cannot be said how well the existing taxonomy of genera, species, and varieties reflects biological barriers to interbreeding. Hybridisation between biological or taxonomic species has been important in the evolution of many crops, in diverse ways (Simmonds 1976, 1979), and could have contributed to the polymorphism evident in taro today, in theory.

During the present study, it was not difficult to distinguish varieties of taro from other Colocasioid species commonly encountered in the field (notably *Alocasia* spp. and *Xanthosoma saggitifolium*), and from the few available representatives of other *Colocasia* species (*C. affinis, C. fallax,* and *C. gigantea*). There was thus no immediate indication of interspecific hybridisation. On the other hand, difficult-to-identify herbarium specimens and mitochondrial DNA polymorphism did suggest the possibility of complex genetic origins.

There are many herbarium specimens of *Colocasia* which collectors, taxonomists, and the present author could not define at the species level, and it is notable that most of these were collected in the Indo-Malayasian region (Appendix 14), where the geographical ranges of the four better known *Colocasia* species overlap (see Chapter Nine, Figure 9.2). Hybridisation between species might therefore account, to some extent, for the difficulty in defining specimens collected there. Other reasons for poor identification include the absence of diagnostic plant parts at the time and place of collection.

Experiments described in Chapter Eleven suggested that the mitochondrial genome of *C. gigantea* is more closely related to that of *Alocasia brisbanensis* than that of *C. esculenta*. This implies that the populations giving rise to the genus *Colocasia* possessed a mixture of different mitochondrial types, or that an *Alocasia* species somehow hybridised with a *Colocasia* species. The latter event seems unlikely, given the major phenotypic differences between genera, but if the suggestion is correct, then there might not be any strong barriers to hybridisation between the phenotypically defined species of *Colocasia*.[1)]

In this chapter, two kinds of rDNA analysis show that plants identified as taro form

a homogeneous genotypic group that is distinct from other species of *Colocasia* and other Colocasioid genera. A methylatable *Bam* HI site was found in the 18S rRNA gene of all taro varieties tested, and is absent in other taxa. Thermal melting point (Tm) analyses of taro and other taxa show that the large intergenic spacer sequences of different *Colocasia* species have diverged, and that little if any sequence divergence has arisen among varieties of taro, despite the observation of size variation among taro spacer fragments. An apparent interspecific hybrid is identified by Tm analysis, but the general survey of rDNA in taro varieties does not indicate any wider occurrence of hybridisation in the history of taro.

13.2 Materials and Methods

Samples of taro and other species of *Colocasia* were collected and maintained as described in Chapter Ten. Protocols for DNA extraction from young leaves, restriction enzyme digestion, Southern transfer, preparation and hybridisation of radioactive DNA probes, and autoradiography are also given in Chapter Ten. *Bam* HI, *Taq* I, *Hinf* I, and *Rsa* I rDNA fragments were detected using taro rDNA probes prepared from pCe34.1 and pCe34.2, the recombinant plasmids described in Chapter Twelve.

The taxonomic and geographical distributions of a *Bam* HI site near the 3′ end of the 18S rRNA gene were examined, and a simple one-step thermal melting protocol (see Chapter Ten) was used for qualitative assessments of similarity between the rDNA spacer sequences of *C. esculenta* var. AKL 34 and other species, varieties, and genera.

For thermal melting point analyses, probes were prepared with either the taro rDNA sequence excised from pCe34.1 or the entire recombinant plasmid. The 5.5 kbp *Eco* RI rDNA fragment in pCe34.1 spans the entire large intergenic spacer, and therefore detected all the variable spacer fragments generated by *Taq* I, *Hinf* I, and *Rsa* I. After hybridisation of the radioactive probe to membrane-bound rDNA fragments at 37°C, a standard low-stringency wash was used to remove weakly bound or unbound probe DNA. The result was recorded by autoradiography, before placing the freshly probed membrane into a hybridising solution at a high temperature such as 66°C. Only probe fragments that were very similar in sequence to target rDNAs were still bound to the filter after the second, high-stringency treatment.

The temperature at which 50% of probe fragments dissociate, for any combination of probe and target DNA, is known as the thermal melting point (Tm). For the second, high-stringency hybridising treatment, a temperature was sought that was near, or at, the Tm of the probe hybridised to itself (i.e., 100% sequence similarity). Visual comparison of the DNA band intensities, before and after the Tm treatment, and allowance for differences in autoradiographic exposure times, allowed an approximate check of the amount of probe actually removed from test samples and a control sample of 100% similarity. High-stringency hybridisation was carried out within one or two days after low-stringency hybridisation, and radioactive decay was therefore negligible because the half-life of the probe isotope, ^{32}P, is two weeks.

Removal of probe by thermal melting is very sensitive to very small differences in

temperature and formamide concentration (Appels and Dvorák 1982a). If too little or too much probe is removed from the control sample of 100% similarity, then the differences in band intensity between these and less similar samples may be too little to detect visually. In some of the experiments summarised here, DNA from AKL 34 was the internal control of 100% similarity, used to assess the effectiveness of the high-stringency wash. No sequence difference was detected in the comparison of AKL 34 and J3A, a wild diploid taro sample from Queensland, and the latter also served as an internal control for some experiments.

13.3 Results

13.3.1 Analysis of Bam HI restriction fragments

The restriction-site map for taro variety AKL 34 (Chapter Twelve) provides a guide for interpreting the *Bam* HI rDNA fragments generated by varieties of taro and by other species and genera in the Tribe Colocasioideae. Of particular interest here are the smaller *Bam* HI fragments from the 18S to 26S rRNA genic region (Figure 13.1). Comparison of these fragments from *C. esculenta* and other Colocasioid species (Figure 13.2) shows that the latter do not possess a *Bam* HI restriction site at the 3′ end of the 18S rDNA. To see whether or not this site is diagnostic for *C. esculenta*, *Bam* HI fragments were examined in a wide taxonomic and geographical range of samples. Only *C. esculenta* possesses the 3′ 18S site, and it was found in all varieties examined (Table 13.1). In *C. esculenta*, the 2.7 kbp fragments observed are thought to be the result of methylation blocking *Bam* HI cleavage at the 3′ 18S site in a proportion of rDNA repeat units. This explanation was noted in Chapter Twelve and is discussed again later. With other Colocasioid species, 2.7 kbp fragments are found, and 1.2 kbp fragments are not found, because there is no *Bam* HI site at the 3′ end of 18S rDNA (Figure 13.1).

C. esculenta var. *fontanesii* has a very distinctive phenotype (see Chapter Ten, Figure 10.2), and prior to rDNA analysis, the species identification of this variety was not certain, in the opinion of the present author. The *Bam* HI rDNA fragments generated with this variety (accession T264, result not presented) demonstrated that the methylatable 18S *Bam* HI site is present, consistent with the identification of var. *fontanesii* as a variety of *C. esculenta*. The possibility that some rDNA in var. *fontanesii* is derived from another species is not excluded by the *Bam* HI analysis, but evidence against this possibility is provided by the thermal melting-point analysis described later.

A small range (300 bp) of size variation is evident in the 2.7 kbp *Bam* HI fragments (Figure 13.2), more clearly resolved after shorter exposures than shown here. The differences are probably located in the small intergenic spacers flanking the 5.8S rRNA gene (Figure 13.1). Relatively long exposures are shown here to maximise detection of the 1.2 kbp fragments. The genic region within the taro rDNA probe (pCe34.1) hybridises strongly to the genic 2.7 kbp fragments of Colocasioids of both Australasian origin (*Colocasia*, *Remusatia*, *Alocasia*) and South American origin (*Xanthosoma*, *Caladium*), providing an internal control for detection of the 1.2 kbp fragments.

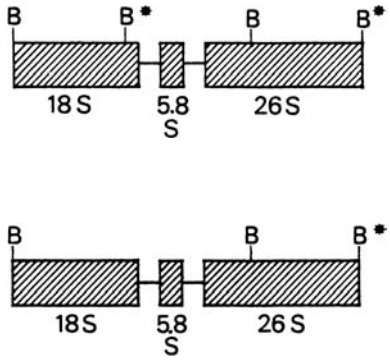


Figure 13.1 *Bam* HI restriction sites in the rDNA genic regions of *C. esculenta* (above) and other members of the Colocasioideae (below). Asterisks mark sites which are consistently not recognised by *Bam* HI in a large proportion of repeat units, probably because of methylation. Small c.1.2 kbp fragments are generated by *Bam* HI cleavage at the 3′ end of the 18S rRNA gene (second *Bam* HI site from left). These migrate as one band during electrophoresis, and are diagnostic for *C. esculenta*. When the 3′ end of the 18S gene is not cleaved by *Bam* HI, one large fragment of c.2.7 kbp is generated.

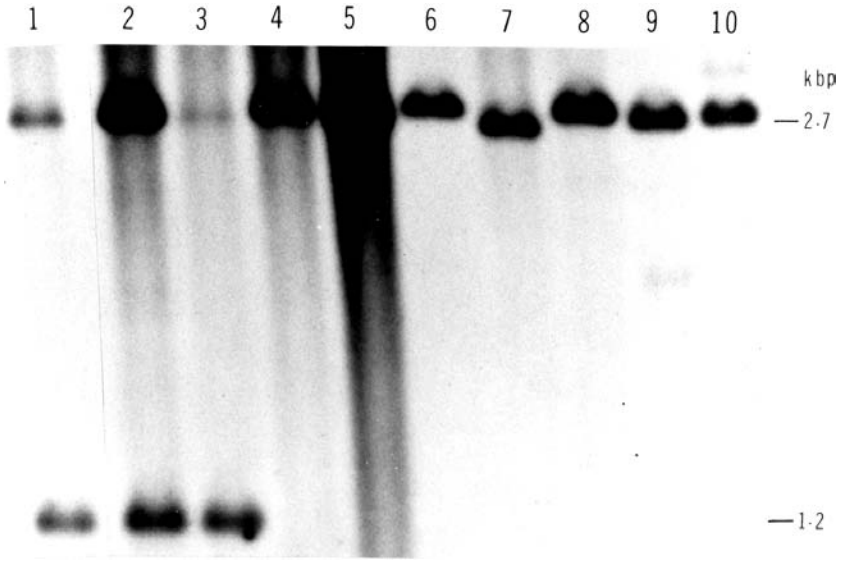


Figure 13.2 A diagnostic test for *C. esculenta* ribosomal DNA. *Bam* HI fragments from the rDNA genic regions of taro and its relatives, detected with the taro rDNA fragment from pCe34.1. Fragments detected include the c.1.2 kbp fragments characteristic of *C. esculenta* and the c.2.7 kbp, 18S-26S genic fragment found in all Colocasioids tested so far. Restriction-site maps for these fragments are given in Figure 13.1. Samples: 1 = *C. esculenta*; wild, Queensland, Australia (field coll. no. J3A). 2 = T270, putative hybrid (*C. esculenta* x *Colocasia* sp?); ornamental, Australia. 3 = T271, *C. esculenta*; ornamental, Australia. 4 = T328, *C. affinis var. jenningsii*; ornamental, Australia. 5 = T259, *C. fallax*; ornamental, Australia. 6 = T268, *C. gigantea*; ornamental, Australia. 7 = T221, *Remusatia vivipara*; wild, Cape York, Australia. 8 = *Alocasia brisbanensis*; wild, Queensland, Australia (field coll. Daly Park N1). 9 = T144, *Xanthosoma saggitifolium*; cultivated, Ifugao, Philippines. 10 = T317, *Caladium* sp; cultivated, Colombo city, Sri Lanka. All lanes are from the same Southern transfer filter, with an autoradiographic exposure of 53 hours.

Table 13.1 Taxonomic and geographical distribution of the methylatable *Bam* HI site (18S rRNA gene) within Subfamily Colocasioideae. (1) to (3): Numbers of collections tested. The area of origin is indicated in brackets for species known to be collected outside their natural or usual distribution range. (4): The number of different varieties of *C. esculenta* tested was less than 70 because there was replication of varieties among the collections. All 24 Australian collections were of the Jiyer phenotypic variety, from Queensland, and only four phenotypic varieties are represented by the 16 collections from New Zealand. Diploid and triploid taro collections were tested.

(1) *Colocasia esculenta* (all with methylatable 18S *Bam* HI site)		
Japan	12	
Philippines	9	
Timor	2	
Papua New Guinea	7	
Australia	24	
New Zealand	16	
(2) *Colocasia* species (all without the methylatable 18S *Bam* HI site)		
C. affinis	1	Australia (ex Asia)
C. fallax	1	Australia (ex Asia)
C. gigantea	4	Australia & Asia (ex Asia)
(3) Colocasioid genera (all without the methylatable 18S *Bam* HI site)		
Alocasia brisbanensis	9	Australia
Alocasia odora	1	Japan
A. cuprea x? (hybrid)	1	Auckland (ex Asia)
A. magnifica	1	Papua New Guinea
Remusatia vivipara	1	Australia
Xanthosoma sagittifolium	1	Philippines (ex South America)
Caladium sp. n.d.	1	Sri Lanka (ex South America)
(4) TOTALS (no. of varieties, species or genera followed by no. of collections, and presence or absence of the methylatable 18S *Bam* HI site).		
Colocasia esculenta varieties	<70 (70)	present
Other *Colocasia* species	3 (6)	absent
Other Colocasioid genera	4 (15)	absent

13.3.2 Thermal melting point (Tm) analysis

Temperatures of 66–67°C resulted in the loss by thermal melting of approximately 50% of the taro probe fragments hybridised to taro spacer fragments. The procedure demonstrated clearly that the spacer sequences of *Colocasia* species have differentiated with respected to the spacer sequence of *C. esculenta*, and the result for the putative hybrid T270 confirms that inter-species hybridisation within the genus *Colocasia* is possible (Figure 13.3; plant portrait in Figure 10.6). To determine whether or not such hybridisation has been important in the history of taro, thermal melting point analysis

was used to survey varieties of *C. esculenta* from a range of countries.

A total of thirty-three diploid and triploid taro were surveyed, including plants from Australia, Papua New Guinea, Japan, Madagascar, and Vanuatu (Table 13.2). J3A, an Australian field leaf sample, was from a plant of the Jiyer phenotypic variety (see Figure 10.1), known to be diploid from chromosome counts on plants from the Jiyer and other Queensland sites. It is very likely from previous studies that most of the Papua New Guinea accessions are diploid, and that most of the Japanese accessions are triploid.

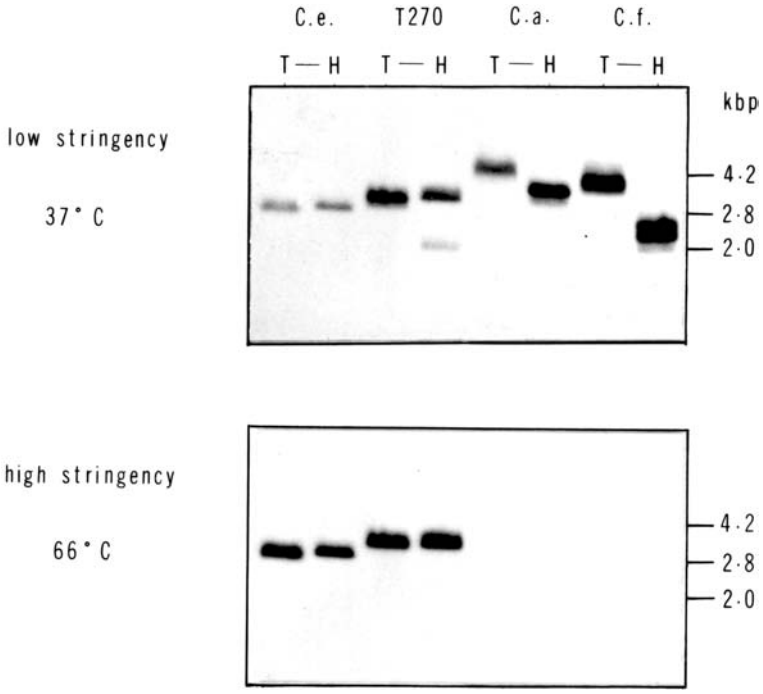


Figure 13.3 Thermal melting point analysis of rDNA spacer sequences in *Colocasia* species. After Southern transfer, *Taq* I (T) and *Hinf* I (H) fragments were probed with the rDNA *Eco* RI fragment from pCe 34.1, which contains an entire large intergenic spacer sequence from *C. esculenta* var. AKL 34. The standard low-stringency hybridisation (30-minute autoradiograph) was followed by a high-stringency treatment (66°C, 150-minute autoradiograph). C.e. = *C. esculenta* (Field coll. J3A, Queensland). T270 = a putative hybrid (*C. esculenta* x another species, possibly *C. affinis*). C.a. = *C. affinis* (T328). C.f. = *C. fallax* (T269).

Sequence similarity between the target spacer fragments of *C. esculenta* (J3A) and the probe from the same species is high, resulting in strong retention of the probe after thermal melting (high-stringency wash). Thermal melting removed probe from the 2.0 kbp *Hinf* I fragments of T270, but not from the larger (3.2 kbp) *Hinf* I fragments, confirming the hybrid origin of this accession. By comparison of the original autoradiographs for each wash of T270, it can be seen that weakly hybridising *Taq* I fragments migrated in the lower part of the c.2.9 kbp *Taq* I band. These fragments may be derived from the same rDNA repeats as the weakly hybridising 2.0 kbp *Hinf* I fragments. Sequence similarity between the probe from *C. esculenta* and the target spacer fragments of *C. affinis* and *C. fallax* is relatively low, resulting in weak retention of probe after thermal melting.

Among twenty-four of the plants surveyed, eight different rDNA spacer fragment patterns were recognised, and clear Tm results were obtained for a further nine collections despite poorly resolved rDNA patterns. The 5.5 kbp *Eco* RI rDNA fragment cloned from AKL 34, and contained in pCe34.1, hybridised as strongly to spacer fragments from other varieties as to the fragments from AKL 34. In other words, no differential loss of probe DNA was observed after sufficient thermal melting to remove approximately 50% of fragments hybridised to rDNA spacer sequences of 100% similarity, as illustrated in Figure 13.4.

Taq I and *Hinf* I spacer fragments from five accessions of *C. esculenta* var. *fontanesii* were also tested, using pCe34.1 and a thermal melting temperature of 67°C. As suggested above, the species identification of this variety was questionable. Each accession tested (T 264, 291, 330, 339, and 322) displayed the same pattern of rDNA spacer fragments (see Chapter Fourteen, Figure 14.11), and none of the fragments

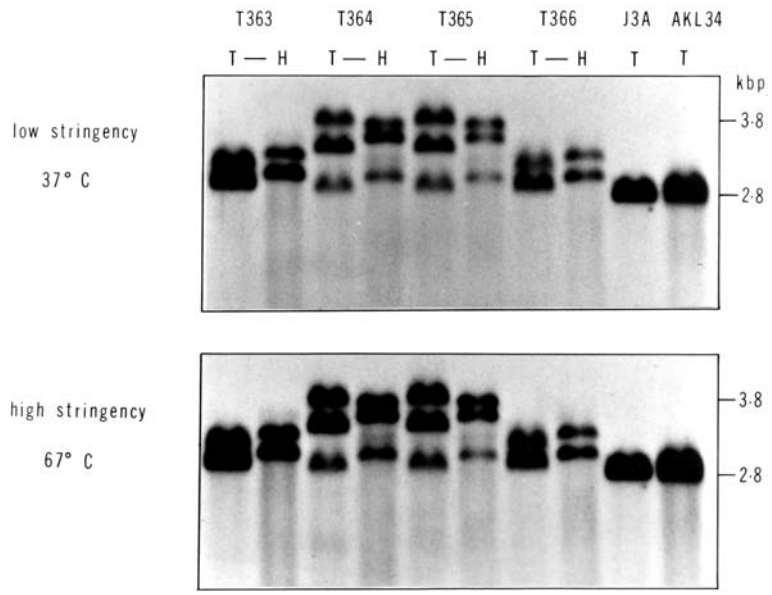


Figure 13.4 Thermal melting point analysis of rDNA spacer sequences in varieties of *C. esculenta*. After Southern transfer, *Taq* I (T) and *Hinf* I (H) fragments were probed with the rDNA *Eco* RI fragment from pCe34.1, which contains an entire large intergenic spacer sequence from *C. esculenta var.* AKL 34. The standard low-stringency hybridisation (3-hour autoradiograph) was followed by a high-stringency treatment (67°C, 6-hour autoradiograph). T363 to T366 are cultivars from Madagascar, J3A is a wild variety from Queensland, Australia, and AKL 34 is a cultivar from New Zealand.

The analysis of AKL 34 with its derivative pCe34.1 displays approximately 50% thermal melt-off under the condition of 100% sequence similarity between probe and target DNA. The second autoradiographic exposure was twice as long as the first, but gave rDNA bands of similar intensity, thus indicating that a 50% thermal melt-off was achieved. Retention of the probe by spacer fragments appears equally strong for J3A, and T363 to T366. Similar results from thermal melting point analysis were obtained for a wide range of taro samples (see Table 13.2).

Table 13.2 List of plants tested for thermal melting point analysis of rDNA spacer sequences in *C. esculenta*. Spacer fragments were generated with *Taq* I, *Hinf* I, or *Rsa* I, before Southern transfer and probing as illustrated in Figure 13.4. For each country from which plants were obtained, the classes of rDNA patterns are noted (Qld 1:1, Puk 1:2, etc; A-C: described in Chapter Fourteen) followed by the field or accession (T) numbers, and chromosome numbers if known.

Australia (n = 1, *Taq* I, *Hinf* I)	
Qld 1:1	J3A (2n= 28)
Papua New Guinea (n = 13, *Taq* I, *Hinf* I)	
Qld 1:1	T247, 258
Puk 1:2	T197, 198, 202, 205, 208
Buk 2:1	T203
Puk 2:3	T188, 190, 193
Not determined	T206, 207
Japan (n = 11, *Rsa* I)	
A	T294, 305
B	T296, 303
C	T298, 299 (2n = 42), 302 (2n = 42), 306
Not determined	T293 (2n = 42), 304, 326
Madagascar (n = 4, *Taq* I, *Hinf* I)	
Puk 2:3	T363, 366
Col 3:4	T364, 365
Vanuatu (n = 4, *Taq* I, *Hinf* I)	
Not determined	T377, 378, 379, 380

showed any lack of sequence similarity when the probe from *C. esculenta* var. AKL 34 was removed by thermal melting (data not shown). This supports the identification of var. *fontanesii* as a variety of *C. esculenta*, and suggests that no other species has contributed to the rDNA complement, assuming that differentiation among all *Colocasia* species is accompanied by a degree of rDNA differentiation similar to that seen in the comparison of *C. esculenta, C. affinis,* and *C. fallax*.[2)]

13.4 Concluding Discussion

Restriction site analysis of the 18S rRNA gene and thermal melting point analysis of the intergenic spacer region both indicate that *C. esculenta* is a homogeneous taxon, distinct from other species of *Colocasia*. Heterologous-probe analysis of mitochondrial DNA (Chapter Eleven) led to the same conclusion. The history of taro may therefore simply be the history of one species evolving in Asia and then gaining an extended distribution by natural dispersal and with the assistance of humans. There is no evidence that polymorphism within this species was generated by interspecific hybridisation. In the chapter that follows, differentiation within the species *C. esculenta* is illustrated by a

survey of variation attributed to length mutation in large intergenic spacers of rDNA.

Because the sequence of the 18S gene, methylatable *Bam* HI site is in a conserved region of rDNA, it is expected that a similar sequence exists in other species of *Colocasia*. As there are no sequence data for these, an inspection was made of 18S gene sequences published for distantly related plant taxa. Near the 3′ end of the 18S gene in rice, in a position that corresponds to the approximate map position of the taro *Bam* HI site, a nine-base pair sequence was found which matches the eight-base sequence predicted for taro (Figure 13.5). The taro sequence is predicted by assuming that methylation of the six-base *Bam* HI site occurs and requires the presence of flanking cytosine and guanosine residues, as suggested in Chapter Twelve. Dams et al. (1988) aligned the 18S sequences of diverse plant and animal taxa. The sequence for rice (Figure 13.5) may be regarded as a latent methylatable *Bam* HI site, in that loss of one guanosine residue would be sufficient to create the required sequence. It is proposed here that the *Bam* HI site in taro is a phylogenetically derived character state, since it is absent from other species of *Colocasia*, and from other genera such as *Xanthosoma* and *Caladium*, which are definitely phylogenetic outgroups. Latent *Bam* HI sites among near relatives of taro probably differ from the rice sequence, and the specific mutational event that gave rise to the *Bam* HI site in taro cannot be determined by comparison to rice.

Comparison of the aligned sequences for distantly related plant taxa (Figure 13.5) suggests a local hot spot of variability just where it is proposed that a single base deletion would transform the rice sequence into a *Bam* HI site. By analogy to the kinds of variability exhibited by the aligned sequences, it is proposed that the *Bam* HI site in *C. esculenta* arose by base substitution and/or deletion.

Of interest for future investigation is the slight size variation apparent among the 2.7 kbp *Bam* HI fragments from different species of *Colocasia* and different Colocasioid genera (Figure 13.2). The 2.7 kbp fragment spans the two small intergenic spacers and the 5.8S gene, between the 18S and 26S genes (Figure 13.1), a region which has been found by thermal melting analysis to vary in sequence among species of *Triticum* (Appels and Dvořák 1982b; see Chapter Nine, Figure 9.5). This region has not been intensively studied in plants, but a comparison of sequences in two species of toad (Furlong and Maden 1983) and a phylogenetic study of frog species (Hillis and Davis 1986) have shown that interspecific variation in the two small (transcribed) spacers involves insertions, deletions, and base substitutions.

The presence of a species-specific *Bam* HI site in the 18S gene provides a potentially useful target for species diagnosis of archaeological DNA residues using the polymerase chain reaction (PCR). PCR is currently regarded as an ideal tool to amplify very small numbers of, or even single, intact ancient DNA molecules present in a vast excess of damaged molecules (Pääbo et al. 1989). Because archaeological DNA residues will be highly degraded, if found, it is necessary for diagnostic characters to be located within very short sequences. More detailed analysis of the sequences immediately flanking the methylatable 18S *Bam* HI site might reveal other diagnostic features. These would make false identifications due to evolutionary convergence or technical artefacts less likely, should the PCR technique be employed in the future.

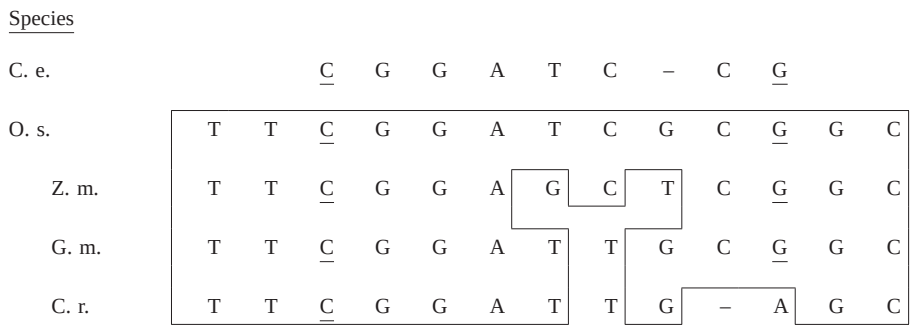


Figure 13.5 The methylatable *Bam* HI site from the 18S rRNA gene of taro, aligned with 18S rDNA sequences published for other plant taxa. C.e. = *Colocasia esculenta* (taro), O.s. = *Oryza sativa* (rice), Z.m. = *Zea mays* (corn), G.m. = *Glycine max* (soybean), C.r. = *Chlamydomonas rheinhardtii* (a green alga). Deletion of one guanosine residue (G) from a sequence like that reported for rice would create a methylatable *Bam* HI site like that predicted for taro. Conserved bases in the published sequences, relative to rice, are boxed. The recognition sequence for *Bam* HI, GGATCC, is in bold type; underlining marks bases which are required for cytosine methylation in the sequences shown, or their complements. The sequence for rice begins 105 base pairs from the 3′ end of the 18S rRNA gene, and at base position 3443 in the alignment for rice and other species compiled by Dams et al. (1988). Homology between the 12–13 bp sequences illustrated for rice, corn, soybean, and *Chlamydomonas* was established by aligning complete, approximately 1800 bp, 18S rDNA sequences (ibid).

Thermal melting analysis demonstrates that differences in the sizes of rDNA spacer fragments, within and between varieties, are not associated with major changes in sequence content (Figure 13.4). In Table 13.2, the rDNA classifications of the tested accessions represent different combinations of variously sized rDNA spacer fragments. These observations are consistent with the suggestion (Chapter Twelve, Figure 12.3) that spacer fragments from taro vary in size according to the copy number of a short repeated sequence (sub-repeat) within each spacer. The inferred sub-repeat sequence of taro varies little among rDNA variants and phenotypic varieties.

Sequence divergence among the rDNA spacers of *Colocasia* species is recorded in Figure 13.3. Exact thermal melting points for hybridisation of the taro probe to taro and other species were not determined, so the amount of sequence differentiation between species cannot be quantified. For a comparison of *Triticum* species, using *Triticum* probes, Gill and Appels (1988) regularly used high-stringency temperatures of 63–64°C, equal or close to the Tm of the 130 bp spacer sub-repeat from *T. aestivum cv* Chinese Spring, hybridised to rDNA of the same variety (Appels and Dvořák 1982a). Tm analysis for *Colocasia* was conducted without prior knowledge of the degree of differentiation between species, or of the Tm for the taro probe hybridised to taro rDNA, but the choice of temperature was guided by the previous Tm analysis of rDNA spacer sequences in wheat. Relatively high temperatures, compared to those used for wheat, were chosen to ensure that thermal melting would be observed. The internal controls (probe and target

DNA the same or very similar) indicated that temperatures of 66°C and 67°C were close to the Tm for taro DNA-taro DNA hybridisation. The exact temperatures realised in experiments set at 66–68°C may have differed by as much as plus or minus 0.5°C. In some experiments set at 67°C, and in one of 68°C, much more than 50% of the taro probe was removed from taro rDNA, and the results could not be relied upon to provide any discrimination between taro varieties. For maximal differentiation between *Colocasia* varieties and species, a high-stringency temperature less than 67°C is probably needed.

The hybrid variety T270 is phenotypically similar to *C. affinis* var. *jenningsii* in that it possesses decorative patches of anthocyanin between the secondary veins of the leaf blades (compare Figure 10.5 and 10.6, Chapter Ten), but under the conditions of maintenance in Canberra its growth is more vigorous, similar to that of *C. esculenta*. In Canberra, *C. affinis* var. *jenningsii* grows weakly in the glasshouse and loses its leaves during winter. Engler and Krause (1920: 68) described *C. marchallii* Engler as a possible hybrid of *C. affinis* and *C. antiquorum* (= *esculenta*), and noted that it is cultivated in glasshouses (in Europe) and that its origin is unknown. T270 and *C. marchallii* resemble each other in leaf shape and coloration, but a full comparison with the description by Engler and Krause (1920) is not possible because a complete inflorescence was not obtained from T270.

Assuming that T270 is a hybrid formed by *C. affinis* (variety not specified) and *C. esculenta*, then it might be a product of natural hybridisation within the relatively limited range recorded for *C. affinis* (Northeast India to Burma, including Northwest Thailand, see Chapter Nine, Figure 9.2). This is the same area in which most collections of indeterminate species identity have been collected. T270 is stoloniferous, and in the potted condition does not produce a starchy corm. This and other hybrids may have arisen naturally in the Indo-Malayasian region without generating varieties favoured by humans for cultivation and/or dispersal in the wild, as food plants. With the exception of T270, the rDNA analyses do not suggest that interspecific hybridisation has occurred, but other genotypic components from other species could have introgressed into populations of *C. esculenta*.

Alternatively, hybridisation between *Colocasia* species might require human intervention, for reasons beyond the scope of the present discussion. *C. affinis* var. *jenningsii* is widely, though not commonly, cultivated in glasshouses (Engler and Krause 1920; present author, personal observation). Although regarded by Engler and Krause (1920) as originating in tropical Himalaya, no definitely wild specimens of var. *jenningsii* have been located in European herbaria by the present author. If var. *jenningsii* is not of natural origin, and is a parent of T270 (= *C. marchallii*?), then T270 is certainly not a natural hybrid.

Notes

1) Experimentally, it has been shown that intergeneric crosses with *Colocasia* are possible, though the resulting offspring are sterile (see references cited in Ahmed et al. 2013). Ahmed (pers. comm. 2013) has found evidence of hybridisation between *C. esculenta* and wild *Colocasia*

species found in northern Vietnam. Analysis of chloroplast DNA sequences suggests that *C. gigantea* should be regarded as a separate genus, more closely related to *Alocasia* than *Colocasia* (Ahmed et al. 2013: Figure 2c).

2) *C. esculenta* var *fontanesii* (accession T322 from Sri Lanka) is shown in Figure 10.2. In recent years, numerous new species of *Colocasia* have been described in Southeast Asia (see Chapter 18).

Senri Ethnological Studies 88: 191–228 ©2014
On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Fourteen
Nor-Locus rDNA Variation in *C. esculenta*

14.1 Introduction

Ribosomal DNA was surveyed in a wide range of wild and cultivated taro from Asia and the Pacific. When taro DNA was digested with the restriction enzymes *Taq* I, *Hinf* I, and *Rsa* I, highly polymorphic fragments from the large intergenic spacer region of rDNA repeat units were generated. The fragments varied in their length (number of nucleotide base pairs) and were used to create an rDNA typology for taro. Many of the polymorphisms were correlated with differences in the overall length of rDNA repeat units. This indicates mutations involving insertions or deletions of nucleotide sequences, rather than single nucleotide substitutions in the recognition sequences of restriction enzymes used for analysis (an individual base substitution can cause a recognition site gain or site loss). The latter kind of mutation probably also contributed to the observed range of rDNA variants. The mutational events that caused variation in taro rDNA were not the immediate object of investigation, and remain largely unresolved. Analogy with rDNA variation studied in other taxa suggests that most rDNA variation in taro is related to increases or decreases in the number of sub-repeat sequences (a form of sequence insertion and deletion mutation) within the large intergenic spacer regions of rDNA repeats.

The analyses outlined above did not provide absolute measures of parentage or identity among individual plants, varieties, or vegetatively propagated lineages. Different mutational events, and reassortment of chromosomes during sexual reproduction in different locations, could have produced similar overall patterns of rDNA variants in different locations. *Taq* I and *Hinf* I analyses were conducted to establish a typology of rDNA variation. The ribosomal DNA patterns of wild and cultivated taro from different areas are compared in terms of this typology.

In the survey, the simplest rDNA pattern found was one that represented a single rDNA variant (Qld 1:1 class in the typology of *Taq* I and *Hinf* I restriction fragment patterns). This was the only variant found in wild taro over 400 km of the wet tropical rain forest zone of northeastern Queensland. The Queensland rDNA variant appears to be present in other wild populations in Australia and Papua New Guinea, and in cultivated taro from Asia, Papua New Guinea, and the Pacific Islands. Individual plants from wild populations in Australia (not including Queensland) and Papua New Guinea, and many plants from cultivations outside Australia, display mixtures of rDNA variants. Genotypic heterogeneity was detected among diploids and triploids, suggesting diverse origins (as sexual progeny) for the clonal lineages within each cytological group. A comparison of

phenotypic and rDNA variation suggests that the often-noted phenotypic diversity of cultivated taro is inversely related to genotypic diversity, as measured by rDNA analysis. This is thought to reflect positive selection by humans for variability in the colour characters used to measure phenotypic diversity.

Ribosomal DNA variation among a small number of Japanese triploid varieties is correlated with the previous classification of those varieties by Japanese botanists based on phenotypic characters. The temperate adapted Japanese cultivars lack the Queensland rDNA variant, which appears widespread elsewhere. The rDNA patterns displayed by the New Zealand triploid varieties RR and GR were identical (see also Chapter Ten), and were similar to those of the Japanese triploid taro group *Eguimo*, thought by Japanese botanists to have originated in China.

Cultivated taro from Madagascar, the Philippines, Papua New Guinea, Tonga, and the Society Islands shared a pattern of rDNA fragments identified in the typology as the Puk 2:3 class. The Col 3:4 class was found in taro accessions from Madagascar, Sri Lanka, and Japan. Both these classes represent mixtures of rDNA variants found within individual plants. Assuming that the shared rDNA patterns are in some sense monophyletic (of one origin, each rDNA variant individually or as a mixture of variants), then some kind of historical connection is suggested for very widely distributed lineages of cultivated taro. For each rDNA class, it is not known whether the combination of rDNA variants originated in one location, or whether it originated independently in many different locations from a widely distributed set of similar wild progenitors. For the New Zealand triploids, and for an ornamental triploid variety, var. *fontanesii*, historical records provide a basis for suggesting recent transfers between specific geographical areas.

The ribosomal DNAs of phenotypically similar wild taro in different regions of northern Australia and in Papua New Guinea are different. Within northern Australia, different rDNA classes are distributed homogeneously within different areas, demonstrating the existence of limits to past dispersal of taro within Australia by humans and by natural means.

14.2 Materials and Methods

Plants were collected and maintained as described in Chapter Ten. DNA extracts were prepared from fresh leaves or leaves stored in liquid nitrogen and analysed using the protocols described in Chapter Ten for restriction endonuclease digestion, agarose gel electrophoresis, Southern transfer to nylon filters, hybridisation with radioactive rDNA probes, and autoradiography. All probes were made as described in Chapter Ten using the taro rDNA clones described in Chapter Twelve. The clone pCe34.1 contains an approximately 5.5 kbp *Eco* RI fragment which spans the large intergenic spacer (IGS) region, from the 3′ end of the 26S gene to near the 3′ end of the 18S gene. The clone pCe34.2 contains an approximately 4 kbp *Eco* RI fragment which spans the genic regions from near the 3′ end of the 18S gene, to near the 3′ end of the 26S gene. pCe34.11 contains a subcloned 2.8 kbp *Taq* I fragment from the IGS region within pCe34.1, provided IGS-specific probes. The recombinant plasmids (vector plus insert) were either

used as probes directly, or as sources for purified rDNA inserts, which were also used as probes.

Size estimates, for fragments detected by autoradiography, were made by calibration with standard size markers as described in Chapter Ten. In addition, an internal control for experimental variation between gels was used, in the survey of *Taq* I and *Hinf* I restriction fragments, as follows. A standard sample was included with each set of test samples. This sample was a digest of total DNA from one plant (J3A) harvested in Queensland at the Jiyer Cave site. J3A gave one size of rDNA fragment when digested with either *Taq* I or *Hinf* I. Best estimates for these fragments were calculated as the mean of several independent estimates, i.e., separate gels. The mean for *Taq* I was 2779 bp, with a standard deviation (n-1) of 114 bp, based on 14 tests, and the mean for *Hinf* I was 2865 bp, with a standard deviation (n-1) of 118 bp, based on 7 tests. One or the other of these means was used to calculate a correction factor for each test gel: correction factor = mean/control. The previously obtained estimate for each autoradiograph band was multiplied by the correction factor, which was generally close to one.

All accessions tested with *Taq* I and *Hinf* I are listed in Appendix 20, with general location, habitat, chromosome number if known, and rDNA class if known.

14.3 Results

14.3.1 Spacer fragment variation within taro generally

Figure 14.1 summarises intergenic spacer variation detected in *C. esculenta* after *Taq* I and *Hinf* I analysis. Spacer fragment sizes represented by individual autoradiograph bands were estimated for all individuals, after normal autoradiographic exposure; that is, long enough for resolution of the major bands. The results for cultivated and wild taro, from Asia and the Pacific, are summarised in Table 14.1 for *Taq* I analysis and in Table 14.2 for *Hinf* I analysis. The variety *C. esculenta* var. *fontanesii* was not included in these summaries because its recorded distribution is largely due to historically recent dispersal as an ornamental (see below). For convenience, four Madagascan samples were included in the present summaries as Asian, because Madagascar was colonised by Asians by at least several hundred years ago (Battistini and Verin 1972, Bellwood 1985), and the plants almost certainly came from Asia.

The differences detected by *Taq* I and *Hinf* I are highly correlated because these enzymes cut DNA on each side of the same variable region within the large intergenic spacer (see Chapter Twelve). Figure 14.2 illustrates the correlation between differences among *Bam* HI and *Eco* RI fragments, which span the entire IGS region, and differences between fragments generated by *Taq* I and *Hinf* I. The correlations indicate that the restriction fragment polymorphisms are due to a length difference within the intergenic spacer region. Additional 2 kbp fragments generated by *Taq* I analysis of T294 are not associated with differences among the large *Bam* HI and *Eco* RI fragments, nor with differences among the *Hinf* I fragments, and indicate the presence of minor class (low copy number) of repeat units. The 2 kbp fragments presumably reflect some other kind of mutation, probably either an inversion or a base substitution.

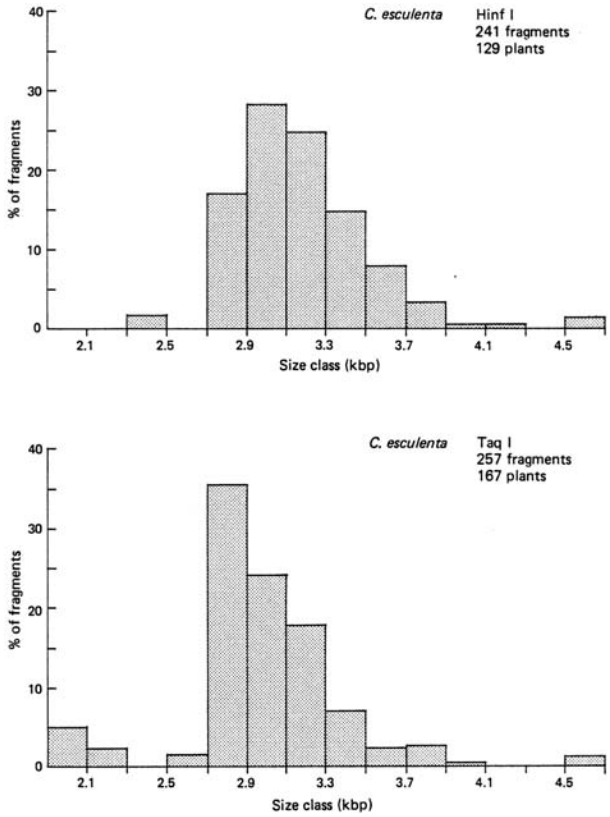


Figure 14.1 Percentage frequencies of *Taq* I and *Hinf* I rDNA intergenic spacer fragments in different size classes. Using *Taq* I, 257 fragments were detected in 167 plants (data from Table 14.1); using *Hinf* I, 241 fragments were detected in 129 plants (data from Table 14.2).

A provisional typology of rDNA spacer fragment patterns is advanced in Table 14.3. The mean size estimates for fragments in each class were not based on fully independent estimates for individual collections, since samples belonging to one class frequently occurred more than once on the same Southern filter, and were therefore subject to the same sources of estimation error. The essential defining criterion for admission to each class was the range of fragment sizes exhibited, rather than the intensity of bands containing the fragments. Band intensities were usually but not always consistent among the patterns assigned to the same class. The assignment of an rDNA pattern to an already recognised class was often based on a single test with each enzyme. Samples for which digests with *Taq* I and *Hinf* I were not electrophoresed on the same gel were not classified (except in the case of Queensland samples with single *Taq* I and *Hinf* I bands) because the relative positions of multiple *Taq* I and/or *Hinf* I bands were also assessed visually to confirm the similarity of restriction fragment patterns. The geographical

Table 14.1 Frequency distributions for rDNA spacer fragment size variants, detected after *Taq* I analysis of wild and cultivated taro from Asia, Papua New Guinea, Australia, and the Pacific Islands. The numbers of assays (plants used) for each area, and each habitat, are shown below, and the sum frequencies for fragment size variants are shown at right. On average, 1.5 different fragments (size variants) were detected per plant (257 fragments/ 167 plants).

Size class	Asia		P.N.G.		Australia		Pacific Is.		Sum Fragments			
kbp	Cult	Wild	Cult	Wild	Cult	Wild	Cult	Wild	Cult	Wild	All	%
1.9 - 2.09	8		1			4			9	4	13	5.0
2.1 - 2.29	2		2			2			4	2	6	2.3
2.3 - 2.49												
2.5 - 2.69	1		2				1		4		4	1.6
2.7 - 2.89	11	1	24	2		29	19	5	54	37	91	35.4
2.9 - 3.09	17	1	23	5		14	2		42	20	62	24.1
3.1 - 3.29	22	3	10	1			6	4	38	8	46	17.9
3.3 - 3.49	7	2	3			2		4	10	8	18	7.0
3.5 - 3.69	3		1			2			4	2	6	2.3
3.7 - 3.89	3	2	2						5	2	7	2.7
3.9 - 4.09	1								1		1	0.4
4.1 - 4.29												
4.3 - 4.49												
4.5 - 4.69				3						3	3	1.2
Sum Assays	33	4	50	6	0	44	21	9	104	63	167	

Table 14.2 Frequency distributions for rDNA spacer fragment size variants, detected after *Hinf* I analysis of wild and cultivated taro from Asia, Papua New Guinea, Australia, and the Pacific Islands. The numbers of assays (plants used) for each area, and each habitat, are shown below, and the sum frequencies for fragment size variants are shown at right. On average, 1.9 different fragments (size variants) were detected per plant (241 fragments/129 plants).

Size class	Asia		P.N.G.		Australia		Pacific Is.		Sum Fragments			
kbp	Cult	Wild	Cult	Wild	Cult	Wild	Cult	Wild	Cult	Wild	All	%
1.9 - 2.09												
2.1 - 2.29												
2.3 - 2.49						4				4	4	1.7
2.5 - 2.69												
2.7 - 2.89	11		15	3		3	8	1	34	7	41	17.0
2.9 - 3.09	9	1	27	5		17	9		45	23	68	28.2
3.1 - 3.29	27	2	22	3		1	5		54	6	60	24.9
3.3 - 3.49	16	3	12	3			2		30	6	36	14.9
3.5 - 3.69	7	3	6			2	1		14	5	19	7.9
3.7 - 3.89	6		1			1			7	1	8	3.3
3.9 - 4.09			1						1		1	0.4
4.1 - 4.29	1								1		1	0.4
4.3 - 4.49												
4.5 - 4.69				3						3	3	1.2
Sum Assays	36	4	46	6	0	21	15	1	97	32	129	

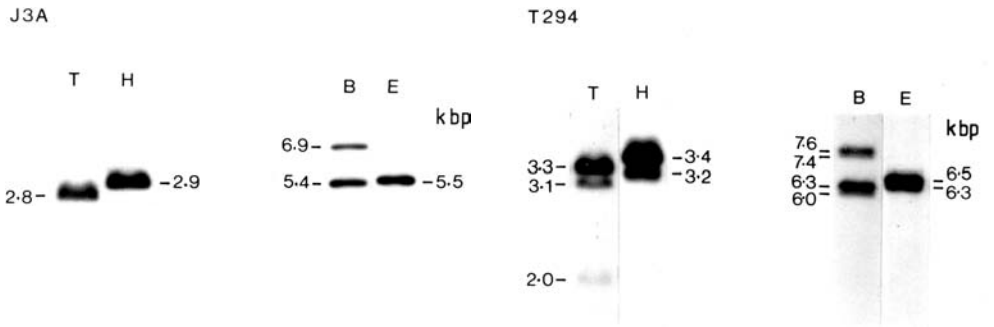


Figure 14.2 Contrasting examples that illustrate rDNA length variation within and between plants. The interpretations are guided by reference to Figures 12.2 and 12.4. The sample J3A produces single *Taq* I and *Hinf* I rDNA fragments, which span overlapping portions of the large intergenic spacer region. The combined pattern of *Taq* I and *Hinf* I fragments is identified as the Qld 1:1 class. The much larger *Bam* HI and *Eco* RI fragments, which span the entire spacer region, are also single, and J3A thus appears to contain a single rDNA length variant. This variant is shorter, in kilobasepairs, than the variants displayed by T294.
The *Taq* I and *Hinf* I spacer fragments from T294 form doublets with separations of approximately 200 bp, and doublets which reflect similar size differences are evident among the larger *Bam* HI and *Eco* RI fragments. The doublets are most simply attributed to one 200 bp length difference within the spacer region, rather than to independent losses or gains of *Taq* I, *Hinf* I, *Bam* HI, or *Eco* RI restriction sites. Although the origin the faint 2.0 kbp spacer fragment is not yet known, length mutation does not appear to be involved.
J3A is a wild diploid variety (Jiyer phenotype) from Jiyer Cave, Russell River, Queensland; T294 is a cultivated triploid variety (Shōgaimo phenotype group) from Tokushima, Japan.

distributions recorded for each rDNA class are summarised in Table 14.4. The integrity of the Puk 1:2 class is dubious. Replicate tests of Queensland-type rDNAs with *Hinf* I frequently generated a partial digestion product resembling the upper, low intensity *Hinf* I band recorded for the Puk 1:2 class. The numbers of individuals possessing only the Queensland rDNA variant, in Table 14.4, have probably been underestimated.

14.3.2 Wild taro in Australia and Papua New Guinea

Samples from coastal northeastern Queensland were collected over the entire recorded range of the Jiyer wild phenotype, between Ingham and the Endeavour River, a distance of approximately 400 km (Figure 14.3; for phenotype see Figure 10.1). Chromosome counts of 2n = 28 were obtained for three sites, from north to south: (5) Hope Vale; (10) Russell River; (8) Blues patch (Figure 14.3).

Twelve plants from sites 1 to 4, and one plant from site 13, were tested with *Hinf* I, and 35 plants from sites 1 to 13 were tested with *Taq* I (Figure 14.4). In replicated tests with the plant J3A (field number) from Jiyer Cave (site 10), *Taq* I and *Hinf* I digests were electrophoresed together, establishing the Qld 1:1 rDNA class (Table 14.3 Figure 14.2). In replicated tests with *Hinf* I and one DNA extract from J3A, a faint, upper *Hinf* I band was frequently but not always detected, indicating partial digestion. The faint, upper *Hinf* I bands in Figure 14.4 (sites 1 to 4) reflect the same partial digestion effect. The

Table 14.3 Estimates of rDNA spacer fragment sizes (bp) for twenty rDNA classes recognised for taro. The typology is provisional because rDNA classes were not defined by replication of a single type sample, with the exception of the Qld 1:1 class, which was primarily defined by replication of tests with J3A, a field sample from Jiyer Cave, Queensland.
T = *Taq* I, H = *Hinf* I. The standard deviations, calculated for classes represented by more than 10 collections, measure estimation error and small unresolved differences in fragment sizes (differences not resolved in assigning the results of different assays to the same rDNA class). The plants assayed are listed in Appendix 20.
The label for each rDNA class consists of an abbreviated name for the first source area or location, for the rDNA class, and numbers for the ratio of *Taq* I to *Hinf* I fragments. Qld = Queensland, Australia; Puk = Pukanti, Western Highlands Province, PNG; East = Easter Island; Buk = Mbukl, Western Highlands Province, PNG; Arn = Arnhem Land, Australia; Kim = Kimberley, Australia; Bay = Bayninan, Luzon, Philippines; Col = Colombo, Sri Lanka; Moro = Morobe Province, PNG; Kat = Katmandu, Nepal; Min = Mine, near Tokushima, Shikoku Island, Japan; Ruti = Ruti Valley, Eastern Sepik Province, PNG; and Eli = Eliya, Sri Lanka.

rDNA Class	Enzyme	Estimate bp	s.d.$^{n-1}$	n
Qld 1:1	T	2779	114	14
(J3A)	H	2865	118	7
Qld	T	2809	36	23
(all)	H	2905	46	23
Puk 1:2	T	2850	83	16
	H	3226	98	19
	H	2970	107	19
East 1:3	T	2812	–	2
	H	3119	–	2
	H	2991	–	2
	H	2868	–	2
Buk 2:1	T	2974	–	1
	T	2749	–	1
	H	2860	–	1
Arn 2:2	T	2806	–	3
	T	2036	–	3
	H	2878	–	3
	H	2328	–	3
Kim 2:2	T	3530	–	3
	T	2922	–	3
	H	3636	–	3
	H	3025	–	3
Bay 2:2	T	3173	–	2
	T	2808	–	2
	H	3259	–	2
	H	2895	–	2
Col 2:2	T	3837	–	1
	T	3266	–	1
	H	3598	–	1
	H	3337	–	1
Moro 2:2	T	4586	–	1
	T	2962	–	1
	H	4538	–	1
	H	2900	–	1
Moro 2:3A	T	4586	–	1
	T	2962	–	1
	H	4538	–	1
	H	3367	–	1
	H	3091	–	1
Moro 2:3B	T	2962	–	1
	T	2720	–	1
	H	3367	–	1
	H	3059	–	1
	H	2809	–	1
Puk 2:3	T	3163	76	12
	T	2904	65	12
	H	3526	79	12
	H	3283	98	12
	H	3018	91	12
Kat 2:3	T	3521	–	1
	T	2605	–	1
	H	3598	–	1
	H	2933	–	1
	H	2720	–	1
Min 3:2	T	3381	–	2
	T	3133	–	2
	T	2047	–	2
	H	3466	–	2
	H	3219	–	2
Ruti 3:2A	T	3592	–	1
	T	3167	–	1
	T	2904	–	1
	H	3523	–	1
	H	3244	–	1
Ruti 3:2B	T	3806	–	2
	T	2867	–	2
	T	2148	–	2
	H	3829	–	2
	H	2959	–	2
Moro 3:3	T	4537	–	1
	T	2932	–	1
	T	2749	–	1
	H	4538	–	1
	H	3125	–	1
	H	2932	–	1
Col 3:4	T	3810	–	3
	T	3422	–	3
	T	2880	–	3
	H	3748	–	4
	H	3511	–	4
	H	3230	–	4
	H	3003	–	4
Eli 3:5	T	4071	–	3
	T	3855	–	3
	T	2905	–	3
	H	4126	–	3
	H	3947	–	3
	H	3713	–	3
	H	3218	–	3
	H	2964	–	3
Kat 4:4	T	4094	–	1
	T	3716	–	1
	T	3446	–	1
	T	2100	–	1
	H	4183	–	1
	H	3838	–	1
	H	3559	–	1
	H	3196	–	1

Table 14.4 General geographical distributions of rDNA classes identified in 86 samples of cultivated and wild taro (including the Eli 3:5 class of var. *fontanesii*).

	Asia		PNG		Australia		E. Pacific		
r DNA class	Cult	Wild	Cult	Wild	Cult	Wild	Cult	Wild	Total
Qld 1:1			4			14	4		22
Puk 1:2			18				1		19
East 1:3							2		2
Buk 2:1			1						1
Arn 2:2						4			4
Kim 2:2						3			3
Bay 2:2	2								2
Col 2:2		1							1
Moro 2:2				1					1
Moro 2:3A				1					1
Moro 2:3B				1					1
Puk 2:3	3		6	1			2		12
Kat 2:3	1								1
Min 3:2	2								2
Ruti 3:2A			1						1
Ruti 3:2B			2						2
Moro 3:3				1					1
Col 3:4	3	1							4
Eli 3:5		1			3		1		5
Kat 4:4	1								1

absence of variation among the rDNAs of Queensland wild taro was further confirmed by analysis with *Rsa* I (31 samples from sites 1 to 12), *Bam* HI (34 samples from sites 1 to 12), and *Eco* RI (21 samples from sites 5 to 12).

For sampling within a site, samples were taken from physically separate shoots distantly located from each other within the site. This maximised the chance of detecting variation among sexual progeny (clones derived from different seed), and minimised the chance of collecting identical material from just one clone. The success of this strategy could not be measured because different clones could not be identified in the field. Despite the deliberate attempt to maximise the detection of variation, none was found over the entire range surveyed. At the time of sample collection (September and October 1987) flowering was recorded at sites 4, 7, and 10, and fruit with seed were found at site 5 (Hope Vale Mission), so taro probably do reproduce by seed in Queensland (see also Figure 9.3).

Live collections of wild taro from Kimberley and Arnhem Land, in northern

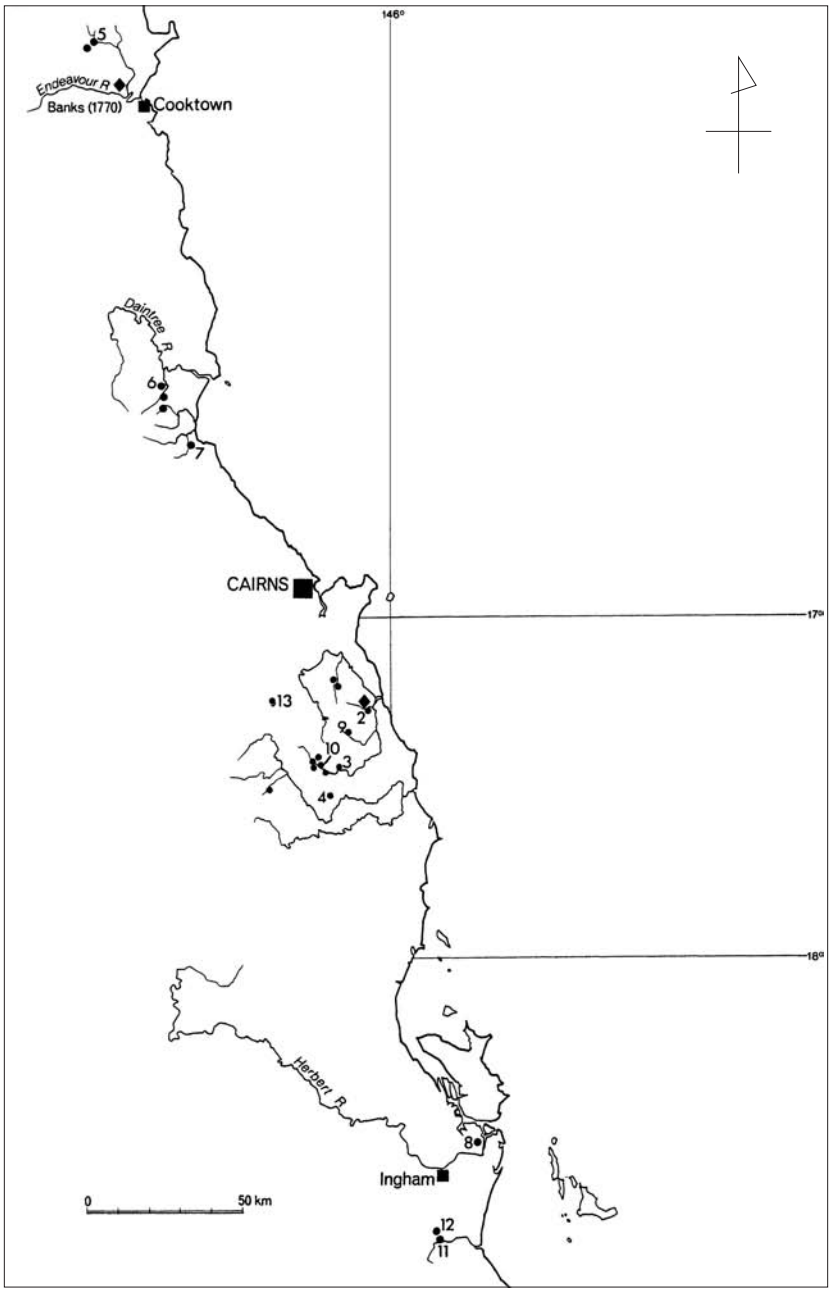


Figure 14.3 Locations of wild taro of the Jiyer phenotype (Figure 10.1) recorded in Queensland in 1987–88. The numbered sites are those for which rDNA analyses were carried out: (1) Whyanbeel Creek; (2) Harvey Creek, Bellenden Ker; (3) Combos Crossing, Russell River; (4) Badgery Creek, Johnstone River; (5) Hope Vale, Endeavour River; (6) Cassowary Creek, Daintree; (7) South Mossman River; (8) Blues patch, Herbert River; (9) Boulder Falls, Bellenden Ker; (10) Jiyer Cave, Russell River; (11) Gap Creek, Halifax Bay; (12) Gin Creek, Halifax Bay; (13) Lake Barrine, Atherton Tableland.

Australia, were received and tested with *Taq* I and *Hinf* I (Figure 14.5). Two further classes of rDNA pattern were recognised; Kim 2:2 from the Kimberley, and Arn 2:2 from Arnhem Land (Figure 14.5). Tests similar to those illustrated in Figure 14.5 were performed with *Taq* I and/or *Hinf* I, and with pCe34.1 or pCe34.11 as probes, for one plant per site from four sites in Kimberley (T335, 336, 337, and 386), and a total of six plants from five sites in Arnhem Land and the western coast of the Gulf of Carpentaria (T31, 331, 332, 333, 338, 376). Although *Taq* I and *Hinf* I fragments were not observed for every Kimberley and Arnhem Land sample, each restriction enzyme alone appears

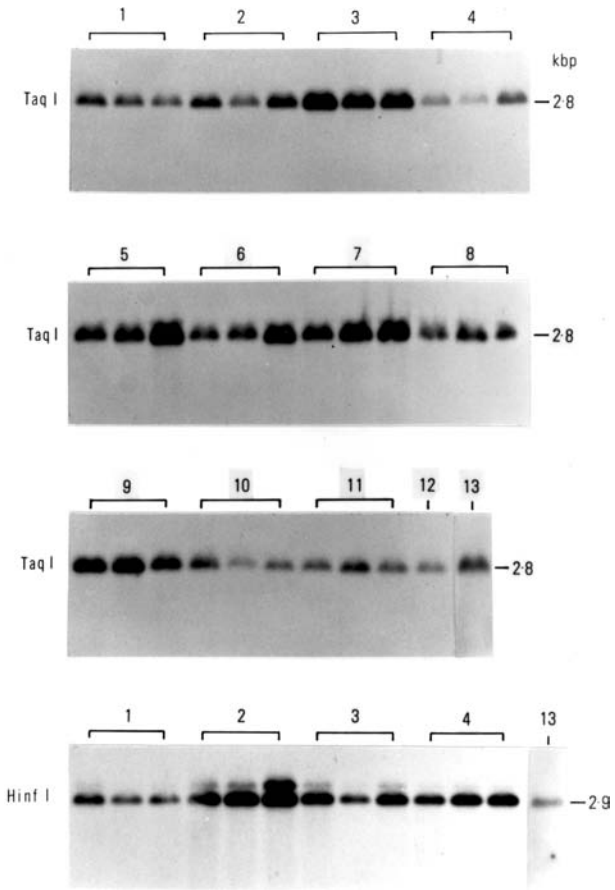


Figure 14.4 Ribosomal DNA analysis of wild taro in Queensland, Australia, sites 1–13 (see Figure 14.3). *Taq* I and *Hinf* I spacer fragments detected in autoradiographs after Southern transfer and probing with pCe34.1.
The results for *Taq* I are compiled from four radiographs, one for each group of sites: sites 1–4 (uppermost set), sites 58, sites 9–12, and site 13. The results for *Hinf* I (lowermost set) are compiled from two autoradiographs, one for sites 1–4 and one for site 13. The faint upper band observed with *Hinf* I is believed to be a partial digestion product.

diagnostic for the different rDNA classes within Australia. The ribosomal DNA patterns observed are homogeneously distributed in Kimberley and Arnhem Land, as well as in the more thoroughly surveyed Queensland area. The sample T376 (Gulf of Carpentaria) produced an rDNA pattern classified as Arn 2:2, according to the spacer-fragment size range, although the autoradiograph band intensities were not the same as for the Arnhem Land samples (result not presented).

Under potted conditions in Canberra, the plants from Kimberley and Arnhem Land were phenotypically very similar to the Queensland wild-type (Figure 10.1), though

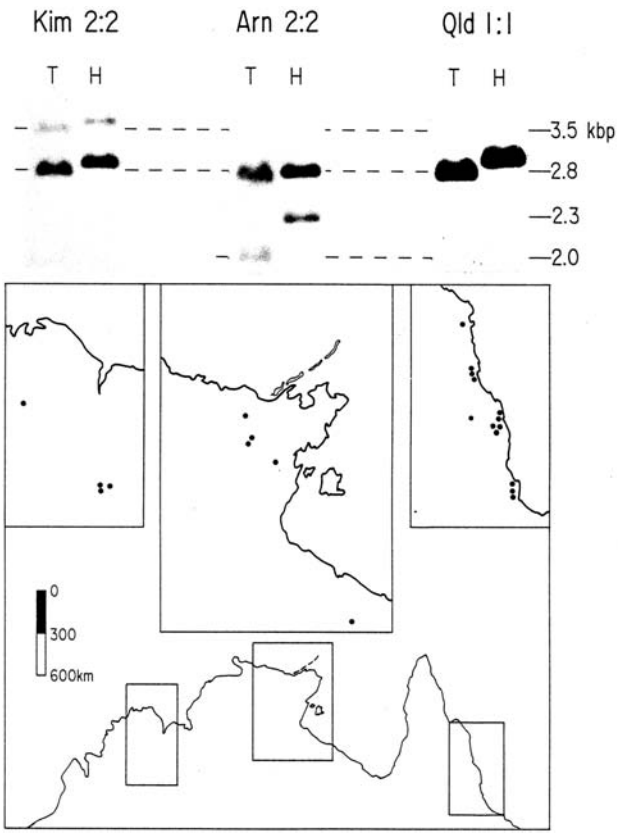


Figure 14.5 Ribosomal DNA in Australian wild taro. Upper panel: three different patterns of *Taq* I and *Hinf* I spacer fragments (classes Kim 2:2, Arn 2:2, Qld 1:1), detected in an autoradiograph of one filter after Southern transfer and hybridisation-probe analysis with pCe34.1. The three samples, left to right, are T336 (vic. Drysdale River, northern Kimberley, coll. S. Forbes 1984), T31 (vic. Maningrida, northeastern Arnhem Land, coll. R. Jones 1980), and J3A (Jiyer Cave, Russell River, northeastern Queensland, coll. P. Matthews 1987).
Lower panel: locations of all samples tested with *Taq* I and/or *Hinf* I. The plants in each region displayed rDNA spacer fragments characteristic for the rDNA patterns illustrated directly above, indicating regional homogeneity in the distribution of rDNA variation.

leaves from one Kimberley plant exhibited a small amount of red anthocyanin pigmentation, at the junction of the petiole and the blade. The Kimberley and Arnhem Land populations are identified here, provisionally, as possessing the Jiyer phentoype. Detailed phenotypic records were made during the Queensland field trip (dimensions of leaves and inflorescences) and it is hoped that similar observations can be made in northern Australia in the future.

The Kimberley rDNA spacer fragments are not extreme in terms of size, but exist in a combination not found elsewhere within or beyond Australia (Table 14.4). The Arnhem Land rDNA pattern (Arn 2:2) includes *Hinf* I fragments of 2.3 kbp, the smallest recorded for taro, and the recorded distribution of the Arn 2:2 pattern is also restricted to Australia (Table 14.4). The rDNA patterns displayed by Kimberley and Arnhem Land taro represent composites of more than one kind of rDNA variant. Each pattern includes fragments

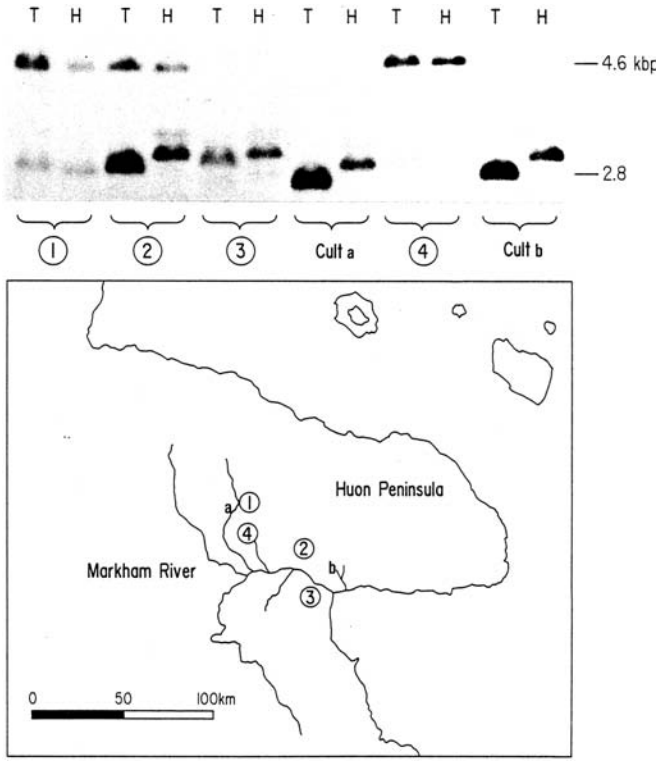


Figure 14.6 Ribosomal DNA in wild and cultivated taro, Morobe Province, Papua New Guinea. *Upper panel*: *Taq* I and *Hinf* I spacer fragments detected in an autoradiograph of one filter, after Southern transfer, and probing with the 2.8 kbp *Taq* I spacer fragment purified from pCe34.1. *Lower panel*: locations. Wild: (1) T225, Moro 2:2 pattern (2n = 28); (2) T226, Moro 2:3A pattern; (3) T227, Moro 2:3B pattern; (4) T229, Moro 3:3 pattern (2n = 28). Cultivated: (a) T228, Puk 1:2 pattern; (b) T234, Puk 1:2 pattern.

similar to those observed for Queensland, and the Queensland rDNA variant therefore appears common throughout northern Australia (Figure 14.5).

Side-by-side comparison suggests that the Queensland rDNA variant is also present in the rDNA patterns of wild and cultivated taro in Papua New Guinea (Figure 14.6). The sizes of *Taq* I and *Hinf* I spacer fragments generated by Queensland wild-type taro (2.8 kbp and 2.9 kbp) correspond closely to the modal frequency peaks for all fragments generated by these enzymes in the general survey of taro (Figure 14.1, Tables 14.1 and 14.2), and rDNA patterns were assigned to the Qld 1:1 class for samples from the eastern Pacific (Figure 14.7). The Qld 1:1 pattern is limited in distribution to the Pacific (Table 14.4), but spacer fragments similar in size to those produced by the Queensland rDNA variant are also common in Asia (Tables 14.1 and 14.2), and are possibly represented within rDNA patterns from tropical Asia (see results for widely distributed rDNA classes,

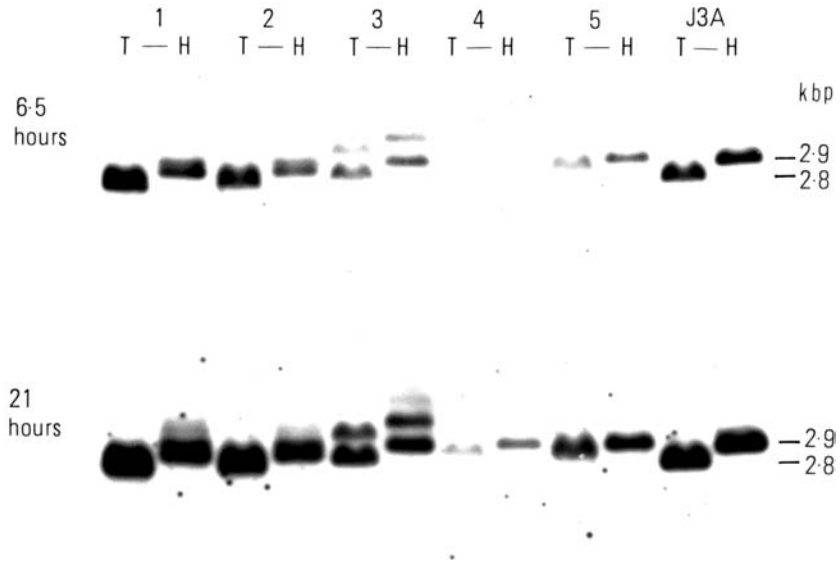


Figure 14.7 Cultivated taro from eastern Polynesia: rDNA spacer fragments detected after *Taq* I (T) and *Hinf* I (H) analysis. For each sample, the Canberra collection number, source, chromosome number, and rDNA class are as follows: (1) T117, Easter Island, 2n = 28, East 1:3; (2) T119, Easter Island, -, East 1:3; (3) T124, Huahine Island, 2n = 28, Puk 2:3; (4) T106, Hawai'i, -, Puk 1:2; (5) T104, Hawai'i, 2n= 28, Qld 1:1. J3A: wild diploid from Jiyer Cave, Queensland (type sample for Qld 1:1 rDNA class).
All the Polynesian samples produced major bands like those of the Queensland rDNA variant at right (J3A), and probably possess this variant. Two exposures of the same autoradiograph are shown to illustrate the difficulty commonly encountered in assessing faint bands. T104 was assigned to the Qld 1:1 class on the basis of the 6.5 hour exposure. The result for T106 was too faint for assessment after 6.5 hours exposure, and was assigned to the Puk 1:2 class after 21 hours exposure. The faint *Hinf* I bands displayed by the Polynesian samples might represent partial digestion products rather than other rDNA variants, a possibility which cannot be resolved without replication of the tests with *Hinf* I.

below). Without replication, side-by-side analysis, and a greater range of diagnostic tests (characters) for the Queensland rDNA variant itself, it is difficult to sustain identifications of this variant far beyond Australia, because convergent length mutation in the rDNA spacer region is a real possibility (see discussion).

Wild taro in Morobe Province, Papua New Guinea, were often phenotypically similar to those in Queensland (see Figure 10.1 and 10.10, Chapter Ten). Wild plants from three locations produced very large *Taq* I and *Hinf* I fragments (c.4.6 kbp, Figure 14.6), in addition to fragments similar in size to those from Queensland. The c.4.6 kbp *Taq* I and *Hinf* I fragments were the largest observed for the species, and were found nowhere else. The DNA of T225, from site 1 in Figure 14.6, was digested with *Bam* HI, *Eco* I, *Pst* I, *Xba* I, for restriction site mapping and comparison with the restriction-site map of AKL 34 (Chapter Twelve). The results were not interpretable simply in terms of insertion or deletion within the intergenic spacer region, and as no further investigation was carried out, details are not presented here. By contrast, the restriction-site map for the Queensland rDNA variant (sample J3A), prepared with the same enzymes, was fully consistent with the map already given for AKL 34 (Chapter Twelve), with minor size differences located within the large intergenic spacer region.

Flowering was observed by the author in Morobe Province in June and July 1985, in cultivations and in the wild, and it is likely that sexual reproduction contributes to the genotypic and phenotypic heterogeneity observed for wild plants in this area.

14.3.3 Ribosomal DNA and phenotypic variation

Some rDNA classes are associated with phenotypically diverse taro, and some phenotypes are associated with diverse classes of rDNA (Table 14.5). The phenotypic categories distinguished here rely on approximate descriptions of anthocyanin distribution, so generally incorporate more than one visually distinct phenotype. The number of plants incorporated in the survey was determined, at the time of writing, by the number for which it had been possible to describe both phenotype and genotype.

Among the phenotypically homogeneous wild taro of Australia, three different rDNA classes were found (Figure 14.5, discussed above). This suggests the possibility that rDNA diversity in general is not positively correlated with phenotypic diversity. A general negative correlation is suggested by inspection of Table 14.5, as follows. In this table, approximately half the samples are in the two categories with the least extensive pigmentation (0 or 1 part pigmented, including Australian wild plants), and approximately half are in the remaining eight categories (2 to 6 parts pigmented in different combinations). Fifteen different rDNA classes are represented among 23 plants in the first two categories, and only eight different rDNA classes are represented among 21 plants in the latter eight categories. The comparison suggests that rDNA diversity in the species is negatively correlated with phenotypic diversity. An explanation is proposed later.

Table 14.5 Classification of 44 samples of *C. esculenta* according to rDNA class and the distribution of anthocyanin pigments. No phenotype descriptions were available for the Moro 2:2, Ruti 3:2B rDNA classes reported in Table 14.3. The presence (+) or absence (-) of anthocyanins is shown for seven parts of the plant: (1) leaf, including blade and petiole; (2) basal ring; (3) roots; (4) corm skin; (5) corm cortex parenchyma; (6) corm core parenchyma; (7) corm core fibres. Ribosomal DNA classes are described in Table 14.3 and the phenotypic characters are described in Chapter 10.

PhenotypicClass							Ribosomal DNA Class																
Anthocyanin distribution							Old	Puk	East	Buk	Arn	Kirn	Bay	Col	Moro	Moro	Puk	Kat	Min	Ruti	Moro	Col	Eli	Kat
1	2	3	4	5	6	7	1:1	1:2	1:3	2:1	2:2	2:2	2:2	2:2	2:3A	2:3B	2:3	2:3	3:2	3:2A	3:3	3:4	3:5	4:4
–	–	–	–	–	–	–	2	1			1	2			1		2				1			
+	–	–	–	–	–	–		3					1	1		1	2	1	1			1	1	1
+	+	–	–	–	–	–																2		
–	+	+	–	–	–	–											1							
+	+	+	–	–	–	–																1		
–	+	+	+	–	–	–	1																	
+	+	+	+	–	–	–	1	6	2				1				2			1				
+	–	+	+	–	–	+												1						
+	+	+	+	–	–	+												1						
+	+	+	+	+	+	–				1														

14.3.4 Ribosomal DNA and chromosome numbers

Diploid and triploid taro display wide spacer-fragment size ranges (Table 14.6), relative to the ranges observed overall (Tables 14.1 and 14.2). Diversity is also apparent in the range of rDNA classes found among diploid and triploid taro (Table 14.7). Three known triploids gave rDNA patterns (Eli 3:5, and Kat 4:4) more complex than any shown by the known diploids. This is consistent with the greater potential for heterozygosity in triploids (three potentially different sets of chromosomes rather than just two), but complex rDNA patterns do not necessarily reflect chromosomal heterozygosity (see discussion).

14.3.5 New Zealand and Japanese Triploids

Triploid cultivars from Japan were tested with *Taq* I and *Rsa* I (Figure 14.8). In combination, these two enzymes define three classes of rDNA pattern (A to C), which show congruence with Japanese botanical group identifications (Table 14.8). The New Zealand triploid varieties RR and GR (generally cultivated) and GP (generally wild) were previously tested with *Taq* I, *Rsa* I partial digestion, and *Bam* HI (Chapter Eleven, Figures 11.9 to 11.11). In these tests, varieties RR and GR were indistinguishable from each other, and were distinct from var. GP. The grouping of varieties RR and GR and their separation from var. GP is also apparent after complete *Rsa* I digestion. Three *Rsa* I bands produced by var. RR and var. GR are clearly a sub-set of the *Rsa* I bands shown by Japanese varieties of the triploid *Eguimo* group (Figure 14.9). The variety GP may

Table 14.6 Size frequency distributions for *Taq* I and *Hinf* I rDNA spacer fragments in diploids (2n = 28) and triploids (2n 42). Although both diploid and triploid taro display a wide range of rDNA fragment sizes, most of the spacer fragments in each ploidy level are within or close to the modal size classes recorded for the species generally (see Figure 14.1, size classes 2.7–2.89 kbp for *Taq* I, 2.9–3.29 kbp for *Hinf* I). The number of assays (plants used) for each ploidy level is shown below. Appendix 20 lists the accessions for which both rDNA analyses and chromosome counts were made.

Size class	Taq I		Hinf I	
kbp	2n = 28	2n = 42	2n = 28	2n = 42
1.9 – 2.09	1	1		
2.1 – 2.29		1		
2.3 – 2.49			1	
2.5 – 2.69		1		
2.7 – 2.89	10	8	5	1
2.9 – 3.09	5	3	8	1
3.1 – 3.29	3	12	8	3
3.3 – 3.49	4	4	2	4
3.5 – 3.69	1	2	1	2
3.7 – 3.89		1	1	2
3.9 – 4.09		1		
4.1 – 4.29				1
4.3 – 4.49				
4.5 – 4.69	1		1	
Total Assays	16	17	13	6

Table 14.7 Ribosomal DNA classes in diploids (2n = 28) and triploids (2n = 42). The classes are listed in order of increasing complexity, that is, the number of different *Taq* I and *Hinf* I spacer fragments, as indicated by the ratios. Data from 11 accessions of taro.

rDNA class	2n = 28	2n = 42
Old 1:1	2	
East 1:3	1	
Kim 2:2	1	
Puk 2:3	2	
Kat 2:3		1
Moro 3:3	1	
Eli 3:4		2
Kat 4:4		1

also be related to *Eguimo*, but the apparent affinity is weaker because it depends on a comparison of only two rDNA bands (Figure 14.9, *Taq* 2.8 kbp probe).

The 1.6 kbp *Rsa* I fragment shared by samples 1 to 5 may be a conserved DNA sequence from a genic region flanking the intergenic spacer, and may also be produced

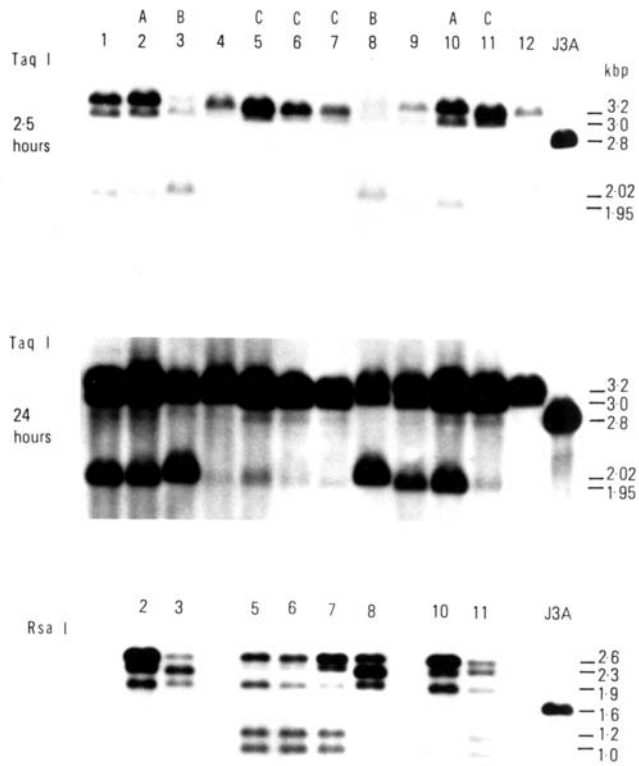


Figure 14.8 Japanese taro (samples 1–12), mostly cultivated: rDNA spacer fragments detected in autoradiographs after Southern transfer. The *Taq* I fragments were probed with pCe34.1; *Rsa* I fragments were probed with the 2.8 kbp *Taq* I spacer fragment excised from pCe34.1. Ribosomal DNA classes A–C, recognised here according to the combined results of *Taq* I and *Rsa* I analysis, are indicated at top. For each sample, the Canberra collection number, source, habitat, varietal group name, and chromosome number (if recorded) are as follows: (1) T293, Tokushima, cultivated, var. *Kuraimo*, 2n = 42; (2) T294, Takushima, cultivated, var. *Syogaimo*, 2n = 42; (3) T296, Kōchi, cultivated, var. *Erikake*; (4) T297, Kōchi, cultivated, var. *Eguimo*; (5) T298, Kōchi, cultivated, var. *Eguimo*; (6) T29-9, Kōchi, cultivated, var. *Eguimo*, 2n = 42; (7) T302, Nagano, wild, var. Kōbōimo, 2n = 42; (8) T3O3, Ehime, cultivated, var. *Erikake*; (9) T305, Kōchi, cultivated, var. Kuraimo; (10) T306, Kōchi, cultivated, var. *Eguimo*; (11) T326, Osaka, cultivated, vernacular var. *Ishikawawase*. J3A: wild diploid from Jiyer Cave, Queensland (type sample for Qld 1:1 rDNA class).
After 24 hours exposure, the Japanese taro display a faint *Taq* I band similar in size to the 2.8 kbp band that characterises the Queensland rDNA variant at right. This suggests that the Queensland rDNA variant is present in the triploid varieties in low copy number. More detailed analysis is required to confirm this.

Table 14.8 Summary of DNA and phenotype observations for eight accessions of Japanese taro, all from cultivations except T302, which was from the wild. Ribosomal DNA classes, A–C, are defined according to *Taq* I and *Rsa* I intergenic spacer fragments (see Figure 14.8). The phenotypic characters are described in Chapter Ten.
Group name = botanical name applied by the Japanese collectors. Vernacular name = name used by the growers. The chromosome numbers were provided by T. Kawahara, except for T294, which was examined by P. Gaffey. Asterisks indicate accessions, which belong to botanical varieties which are typically triploid (Kawahara pers. comm. 1985). Accession numbers are given for the Kyoto Plant Germplasm Institute (KPGI), and the Australian National University (ANU).

rDNA class	A	A	B	B	C	C	C	C
Group name	Shōgaimo	Kuraimo	Erikake	Erikake	Eguimo	Eguimo	Eguimo[1)]	Eguimo
Vernacular name	Yatsume	Kuraimo	Bon'imo	Erikake	Eguimo, Shimaimo	Shimaimo	Kōbōimo	?
Chromosome no.	42	*	*	*	?	42	42	?
Blade vascular	?	green	green	green	green	green	green	green
Blade laminar	?	green	green	green	green	green	green	green
Petiole graded	?	green	green/ purple	purple/ green	green/ purple	green/ purple	green/ purple	green/ purple
Petiole variegated	?	no	no	no	no	no	no	yes
Basal ring	?	white	white	white	white	white	white	white
Roots	?	white	white	white	white	white	white	white
Skin	?	white	white	white	white	white	white	white
Cortex	?	white	white	white	white	white	white	white
Core	?	white	white	white	white	white	white	white
Fibres	?	white	white	white	white	pale yellow	yellow	white
side-shoots	?	cormels	cormels	cormels	cormels	cormels	cormels	cormels
Locality	Tanima	Ōkage	Uriuno	Iwagaichi	Nakamine	Iwagara	Kutsukake-onsen	Kubo-Wakubo
Prefecture	Toku-shima	Kōchi	Kōchi	Ehime	Kōchi	Kōchi	Nagano	Kōchi
KPGI no.	7210	7308	7221	7304	7237	7245	7301	7309
ANUT no.	294	305	296	303	298	299	302	306

by J3A. The result for J3A is obscured by the presence of 1.6 kbp spacer fragments, as demonstrated by analysis with the *Taq* 2.8 kbp probe, below. All the other fragments are clearly diagnostic, and each of the New Zealand varieties display spacer fragments similar to those produced by the Japanese triploid variety *Eguimo*.

Very long exposure of the *Taq* I fragments from Japanese taro reveals a very faint 2.8 kbp band, suggesting that the Queensland rDNA variant is present in very low copy number (Figure 14.8).

This result, and the preceding comparisons, are discussed later with regard to the origins of the Queensland rDNA variant and the origins of New Zealand and Japanese triploid taro.

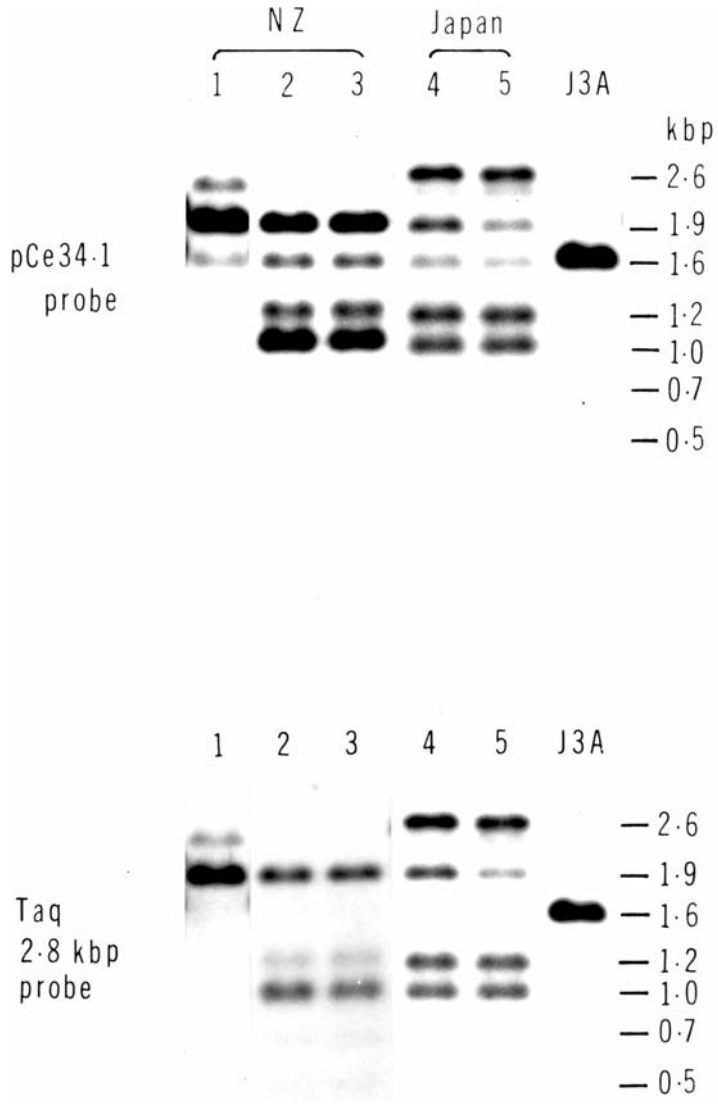


Figure 14.9 Comparison of triploid taro varieties from New Zealand and Japan. Autoradiographs of *Rsa* I fragments after probing with pCe34.1 (above) and the 2.8 kbp *Taq* I spacer fragment excised from pCe34.1 (below). For each sample, the Canberra collection number, source, habitat, variety name, and chromosome number (if recorded) are as follows: (1) T285, Bay of Islands, wild, var. GP, 2n = 42; (2) T272, Bay of Islands, wild, var. RR, 2n = 42; (3) T279, East Cape, from a garden, var. GR, 2n = 42; (4) T298, Kōchi, cultivated, var. *Eguimo;* (5) T299, Kōchi, cultivated, var. *Eguimo,* 2n = 42. J3A: wild diploid from Jiyer Cave, Queensland.

14.3.6 Widely distributed rDNA classes

Three rDNA classes, well defined by multiple bands of *Taq* I and *Hinf* I fragments, were found over very wide ranges. The type accessions and locations are T323, wild, from Colombo, Sri Lanka (Col 3:4 class), T188, cultivated at Pukanti, Papua New Guinea (Puk 2:3 class), and T322, wild near Eliya, Sri Lanka (Eli 3:5) class. The Col 3:4 class (Figure 14.10) was observed for accessions from Madagascar (cultivated), Sri Lanka (wild but probably feral, in urban area) and Japan (cultivated). The Puk 2:3 class (Figure 14.10) was observed for accessions from Madagascar (cultivated), the Philippines (cultivated), Papua New Guinea (cultivated and wild), Tonga (cultivated), and Huahine in the Society Islands, eastern Polynesia (cultivated). Analyses with *Rsa* I confirmed the similarity between accessions from Madagascar and Papua New Guinea (Figure 14.11), and the Philippines (results not presented because of poor autoradiographs).

The Eli 3:5 class (Figure 14.11) was observed for all accessions of *C. esculenta* var. *fontanesii* Schott, from Sri Lanka (wild), Australia (cultivated ornamental) and New Zealand (cultivated ornamental).

For each sample, the Canberra collection number, source, habitat, and chromosome number (if recorded) are as follows: Puk 2:3 class: (1) T1, Kuk, Western Highlands, cultivated, 2n = 28; (2) T167, Ruti, Western Highlands, wild; (3) T188, Pukanti, Western Highlands, cultivated; (4) T190, Pukanti, Western Highlands, cultivated; (5) T193,

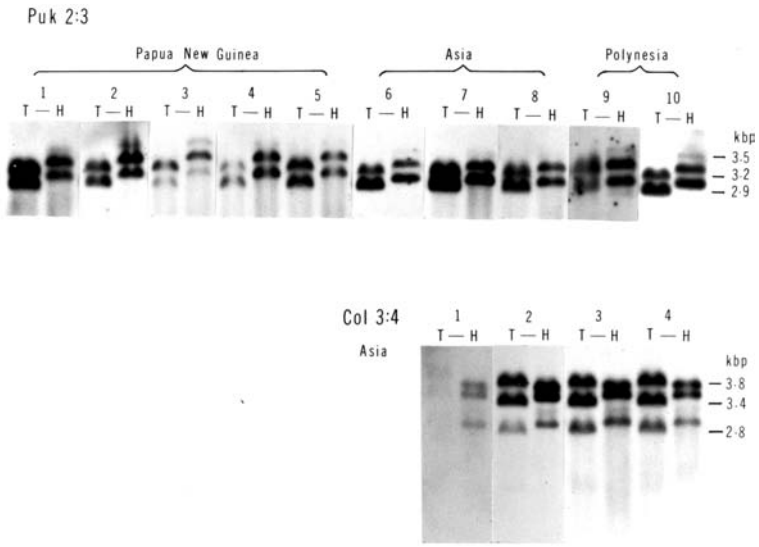


Figure 14.10 Widely distributed rDNA classes: Puk 2:3 and Col 3:4. *Taq* I (T) and *Hinf* I (H) spacer fragments detected in autoradiographs after Southern transfer and probing with pCe34.1 or the 2.8 kbp *Taq* I spacer fragment excised from pCe34.1. Results compiled from several separate autoradiographs, and size estimates based on the average for each rDNA class (Table 14.3). The uppermost *Hinf* I band is often faint and indistinct, and is possibly a product of partial digestion.

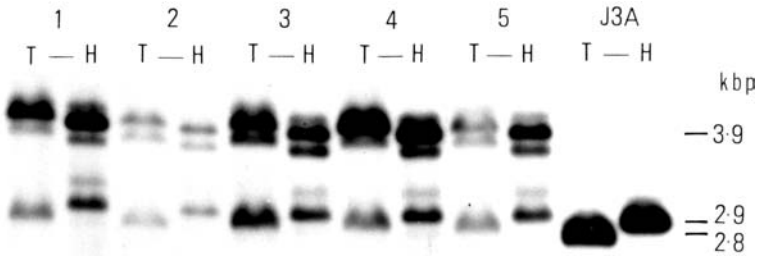


Figure 14.11 *C. esculenta* var. *fontanesii*: intergenic spacer fragments (samples 1–5). *Taq* I (T) and *Hinf* I (H) fragments detected with pCe34.1 after Southern transfer. No variation is observed between accessions. The combined pattern of *Taq* I and *Hinf* I fragments from this variety has been named Eli 3:5.
Samples: (1) T264, ornamental, Urunga, Australia, 2n = 42; (2) T291, ornamental, Kaitaia, New Zealand; (3) T330, ornamental, Sydney, Australia; (4) T339, ornamental, Melbourne, Australia; (5) wild, between Nuwara and Eliya, Sri Lanka, 2n = 42. J3A: wild diploid from Jiyer Cave, Queensland.

Pukanti, Western Highlands, cultivated; (6) T131, Luzon, Philippines, cultivated; (7) T363, Madagascar, cultivated; (8) T366, Madagascar, cultivated; (9) field collection only, Tonga, cultivated; (10) T124, Huahine Island, cultivated, 2n = 28.

Col 3:4 class: (1) T327, Kyoto markets, vern. *Akame-ebi-imo* possibly from Shikoku, southern Japan, cultivated (faint *Taq* I bands visible in original autoradiograph); (2) T323, Colombo, Sri Lanka, wild; (3) T364, Madagascar, cultivated; (4) T365, Madagascar, cultivated.

This variety, described by Engler and Krause (1920), is commonly cultivated as an ornamental in Australia and New Zealand (observation by P. J. Matthews). Cultivated plants collected in Australia and New Zealand were phenotypically identical to the wild plant collected from Sri Lanka (see Figure 10.2, Chapter Ten), and chromosome counts of 2n = 42 (triploid) were made for T322, from Sri Lanka, and T264 from Urunga, Australia.

Each of the widely distributed classes described above is in part characterised by spacer fragments similar in size to those produced by the Queensland rDNA variant, an observation of interest for discussing the possible origins of this variant, below.

14.4 Discussion

14.4.1 The Queensland rDNA variant

The survey of wild taro in Queensland established the existence of a single rDNA size variant in this area, characterised in part by analysis of *Taq* I and *Hinf* I fragments. The Queensland rDNA variant may be geographically widespread because *Taq* I and *Hinf* I spacer fragments, similar in size to those from Queensland, were observed for many taro from beyond Queensland, in other parts of Australia, and in Asia, and the Pacific. However, the possibility of convergent mutation makes it difficult to identify with

certainty the Queensland rDNA variant in plants from beyond Queensland.

It is a well-established general observation for diverse plant and animal taxa, and within species, that intergenic spacers vary in length because of deletions and insertions of short sequence motifs (sub-repeats) within the spacers (Appels and Honeycutt 1986; see also Chapter Nine). Such mutations can independently generate rDNA variants of similar length in diverse taxa, and in different genetic lineages within species. During the long-term evolutionary history of *C. esculenta*, rDNA variants of similar length to the Queensland variant may have become common because of convergent mutation and an as yet unknown selective advantage or molecular mechanism allowing them to proliferate within individuals, clonal lineages, and sexually reproducing populations.

The alternative to convergence, as a general explanation, is that the Queensland rDNA variant is monophyletic. The two major alternatives, polyphyletic versus monophyletic origin, cannot be resolved without closer characterisation of the Queensland rDNA variant itself. More diagnostic restriction enzyme tests for spacer and genic regions are needed so that rDNA variants are not distinguished solely in terms of intergenic spacer length. If convergent mutation did occur, its actual historical significance might not be great. Intensive studies of rDNA evolution in wheat (*Triticum* spp) have been carried out over recent years, and provide precedents for both hypotheses. It is sufficient here to note the precedents, without critical examination, as follows.

In Figure 14.12, the unimodal size frequency distribution for Taq I fragments from taro can be compared to the similar size frequency distribution (Flavell et al. 1986) for *Triticum dicoccoides*, a wild tetraploid wheat in Israel and neighbouring areas. Similar numbers of plants were examined, but the geographical range of *T. dicoccoides* is much smaller than that of taro. The DNA analyses for taro were conducted on the clonal offspring of vegetative shoots collected in the field, or directly on leaf samples from the field, while analyses for *T. dicoccoides* were conducted on individual seedlings taken from individual self-fertilised lines, established an unspecified number of generations previously with seed collected in the field. Flavell et al. (1986) are confident that the unimodal peak is indicative of the actual size frequency distribution for the species. The size frequency distribution for taro may also be representative for the species despite the likely inclusion of partial digestion products (as noted for the Puk 1:2 class, for example), and despite geographical sampling biases. The *Taq* I fragment that was most widespread among wheat populations was found in the least variable populations, and was in the most abundant size class (2.9–3.1 kbp, Figure 14.12). This variant was interpreted as representing a generally optimum spacer length maintained in high frequency by selection. Variance in the number of different spacer length variants, within populations, was found to be correlated with an index of climate, and it was suggested that spacer length polymorphisms represent regulatory adaptations to environmental factors, following indications elsewhere that the intergenic spacer regions are involved in regulating rRNA gene expression. Flavell et al. (1986) do not regard the present range of rDNA size variation as being related to founding effects, and therefore imply that rDNA size variation today is largely the product of frequent mutation coupled with differential selection. This sets a precedent for suggesting the polyphyletic origins of rDNA size

variants like the Queensland variant, and for the maintenance of such variants as a predominant class in the species by selection.

In another study of *Triticum* by Vinitsky et al. (1989), the nucleotide sequence of a large intergenic spacer from the *Nor-D3a* allele of *T. tauschii* (DD genomes) was compared to the sequence of a spacer from the same allele (rDNA variant) present in the hexaploid bread wheat, *Triticum aestivum* (AABBDD genomes). The species *T. tauschii* is regarded as one of the progenitors of *T. aestivum*. The two spacers compared were virtually identical in sequence, with an unchanged number of sub-repeats. In the period since the progenitor species contributed its *Nor-D3a* spacer length variant, at formation of the hexaploid, the intergenic spacers of the progenitor species and its descendant have not changed. These two species are believed to have been reproductively isolated from each other for at least 6,000 to 8,000 years (Vinitsky et al. 1989), so the *Nor-D3a* rDNA variant has been stably transmitted across sexual generations for at least this length of time. This example shows that the Queensland rDNA variant could be monophyletic and

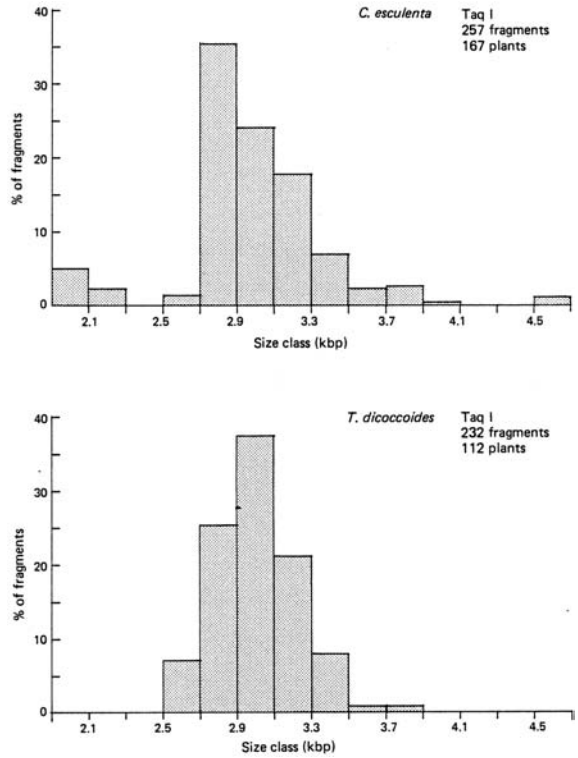


Figure 14.12 Percentage frequencies of rDNA intergenic spacer fragments in different size classes: *Taq* I fragments from *C. esculenta* (taro) and *Triticum diccoccoides* (a wild tetraploid wheat). For taro, 257 fragments were detected in 167 plants (data from Table 14.1); for wheat, 232 fragments were detected in 112 plants (data from Flavell et al. 1986).

ancient, and could have existed for thousands of years during the dispersal of taro through Asia and the Pacific by natural means or otherwise.

Ribosomal DNA variants represented in the patterns produced by Japanese triploid taro (Figure 14.8) and the triploid var. *fontanesii* from Sri Lanka (Figure 14.11) might prove on closer inspection to be identical to the Queensland rDNA variant. Assuming monophyletism, such a result would suggest that the Queensland rDNA variant existed in diploid progenitors shared by the Japanese and Sri Lankan triploids. The reduced copy number of the Queensland rDNA variant (not definitely identified) in the triploids from temperate Japan, and the apparent prominence of this variant in plants from tropical parts of Asia and the Pacific, suggest that taro in temperate and tropical areas may have diverged genetically. Closer characterisation of rDNA and more extensive geographical surveys beyond Queensland are needed to determine the ultimate genetic and geographical origins of the Queensland rDNA variant.

14.4.2 Taro in Australia

In order to clarify the status of wild taro populations in Queensland, some observations regarding cultivated taro are necessary.

Information on cultivated taro in Australia is either anecdotal or from personal observation in Queensland. Often it is stated that Melanesian sugar-cane workers were responsible for the introduction of taro to Queensland during the last 150 years (for example, Fatanowna 1989: 110) but there are other possible sources. These include Chinese growers and traders, first associated with gold mining last century (Choi 1975), missionaries, recent twentieth-century immigrants from Asia and the Pacific, plant collectors (working for botanical gardens, for example), and importers of ornamental plants.

In Queensland, cultivated plants were found on three properties, near Cairns, Ingham, and Mackay. These plants were phenotypically similar to each other and to taro found growing wild on two farms near Ingham and Mackay. The wild patch near Ingham is occasionally harvested by a local Aboriginal and Torres Strait Islander family (T. Palmis, Ingham, pers. comm. 1987). A small clump of wild taro, also similar phenotypically to those found cultivated, was found very near the remains of a major gold-mining camp on the lower Russell River. It is quite possible that the variety here was previously cultivated at the camp. R. Tucker (Townsville pers. comm. 1987) reports the existence of about thirty taro cultivars in North Queensland, none of which persist in the wild, in his experience.

Cultivated taro has been a very minor crop in Queensland, and despite introductions over the last 150 years there has been little dispersal of cultivated varieties into the wild. All the Queensland varieties cultivated for food and seen by the author were phenotypically distinct from the single wild phenotype that is common in Queensland. One plant, found wild on a farm near Mackay, was phenotypically similar to Queensland cultivated taro and yielded a *Taq* I pattern (result not presented) unlike the common pattern found in Queensland wild taro, and unlike the patterns of wild taro in Arnhem Land and Kimberley.

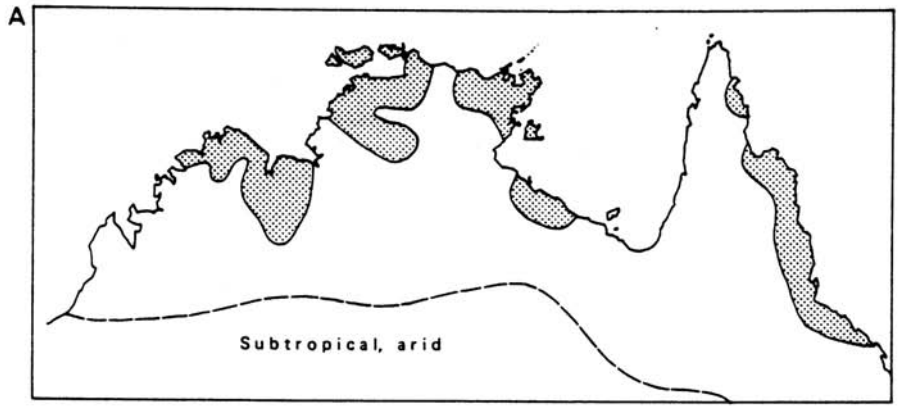
In Queensland, some wild patches in areas of rural settlement undoubtedly have a history of initial planting, occasional harvest, and replanting for maintenance, similar to the history of wild patches seen recently (Matthews 1985) in rural northern New Zealand.

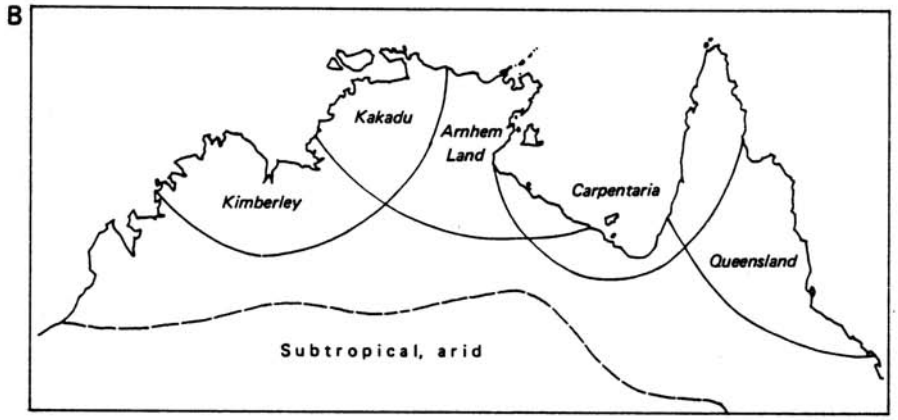
There is however no evidence for a general movement of recently introduced varieties into the wild in Australia. The following discussion refers to wild taro derived from wild populations that existed before European settlement. The early botanical records (Chapters 9 and 10) leave no doubt that such populations existed.

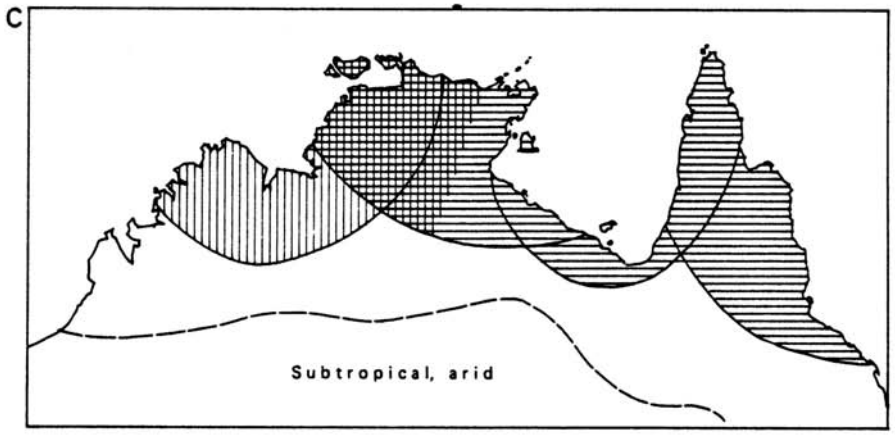
Neither natural dispersal nor humans appear to have caused wide-scale mixing of rDNA variants in wild taro across northern Australia (Figure 14.5). Natural animal vectors for fruit and seed have not been seen, but could be ground-feeding species of birds, rodents, or small marsupials, since the fruiting heads of taro droop towards the ground and eventually drop when mature (personal observation). Natural vegetative dispersal is limited to downstream displacement by water. Pollinating insects (also not yet identified) and fruit-eating animals could, in theory, facilitate gene flow across country between water systems. Limits to dispersal by humans may have been determined by environmental barriers to human movement, frequency of utilisation, transplantation practices, and/or social boundaries. Whatever the limits were, for the dispersal of taro, they must have changed over time according to changing natural and social circumstances. The existence today of different forms of wild taro in different areas, as detected by rDNA analysis, provides a starting point for biological approaches to determining the origins, antiquity, and dispersal of taro in Australia. The discussion below focuses on the analysis of genotypic variation within taro. In Chapter Fifteen, attention is given to other kinds of biological evidence relevant to the origins and dispersal of taro in Australia and New Guinea.

Figure 14.13 presents some purely hypothetical models for the distribution of genotypic variation in wild taro in Australia, based on the known distribution range of taro and on the known distribution of rDNA variation within that range. Future analysis of other aspects of genotype may detect variation that coincides in its geographical distribution with distribution of rDNA variation (Figure 14.13, A). This would confirm the absence of dispersal between areas. Alternatively, there may have been mixing of the known rDNA variants between geographical areas, with no intra-individual genotypic mixing (Figure 14.13, B). This would imply that no natural sexual breeding and dispersal occurred, and that vegetative dispersal by humans was prevalent and led to mixing between water systems.

If the distribution of other kinds of genotypic variation does not coincide with the distribution of rDNA variation (Figure 14.13, C), then some kind of natural gene flow would be indicated. Human dispersal of vegetative parts involves entire, non-segregating nuclear genomes, together with chloroplasts and mitochondria, whereas natural dispersal of seed and pollen provides opportunities for the differential movement of chloroplasts, mitochondria, and nuclear chromosomes. Nuclear chromosomes probably disperse more freely than chloroplasts and mitochondria, because these latter are not carried by pollen, if taro is like most other plants. If natural and differential gene flow is detected, then its nature and extent would have implications for the antiquity of taro in Australia (and New


A
Subtropical, arid


B
Kakadu
Arnhem Land
Kimberley
Carpentaria
Queensland
Subtropical, arid


C
Subtropical, arid

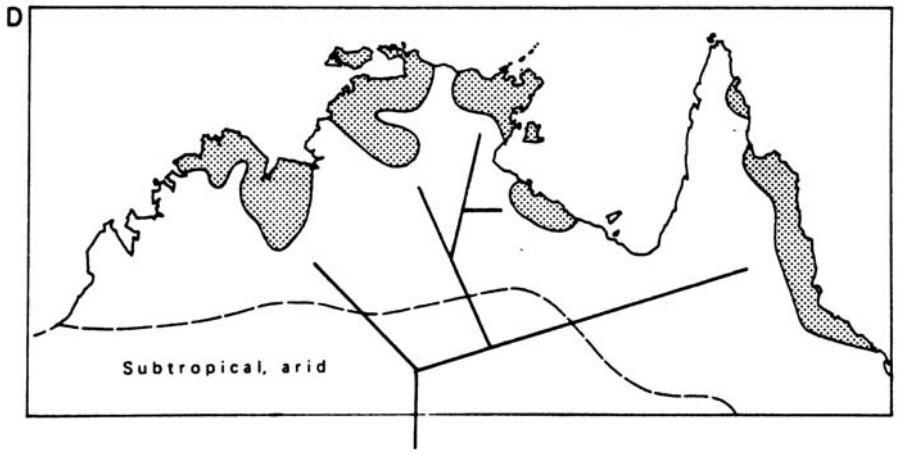


Figure 14.13 Wild taro in Australia: Hypothetical models relating possible dispersal processes in the past to outcomes that might, in theory, be observed in the present by combining field surveys and genotypic analysis.

A. No dispersal, natural or otherwise, occurred between the different areas known to possess wild taro (shaded areas, from Chapter Ten, Figure 10.11), or between the regions defined by the distribution of rDNA variation (see Figure 14.5). The spatial patterns of genotypic variation arising within each region are entirely derived from local reproduction and dispersal, subsequent to separate colonisations by taro.

B. The present range of taro resulted from the dispersal of vegetative parts by humans and natural dispersal via sexual reproduction did not occur. The distributions of rDNA variants (indicated here by curves) overlap, but intra-individual mixtures of rDNA classes are absent, proving that sexual reproduction did not occur (i.e., all heterozygosity is fixed).

C. Natural long-distance dispersal occurred (this would necessarily involve sexual reproduction). The overlapping distributions of rDNA classes are indicated by curves, and the distributions of other kinds of genotypic variation are represented by horizontal and vertical lines. The different kinds of genotypic variation do not coincide in their geographical distributions, and a lack of fixed heterozygosity proves that sexual reproduction occurred. The genetic effects of humans transporting and planting vegetative parts are obscured by natural breeding and dispersal.

D. Endemic differentiation occurred and the phylogenetic relationships of the different varieties that resulted are correlated with their geographical distributions (shaded areas). The species was present long enough for mutation, and dispersal by natural or other means was not sufficient to prevent regional differentiation.

Guinea also, due to its proximity within the continent of Sahul).

Future studies of rDNA and other aspects of genotype, and wider surveys outside Australia, might show that the rDNA variants observed here are endemic to Australia, together with other genotypic traits. The degree of endemic differentiation would, in itself, be some measure of antiquity, and a large degree of differentiation would be strong evidence that taro colonised Australia by natural means, before humans. If the endemic traits also allow phylogenetic interpretation (Figure 14.13, D), then relating geography and phylogeny would strengthen interpretations of endemism, antiquity, origins, and dispersal.

There already exist two potential tests for detecting sexual reproduction and natural gene flow in Australian wild taro. The Kim 2:2 and Arn 2:2 classes may each represent a simple heterozygous condition, i.e., each may possess a single rDNA locus, associated with one homologous pair of chromosomes and two alleles. If, for example, this is the case in Arnhem Land, and sexual reproduction occurs, then it should be possible to find two homozygous patterns, one composed of small *Taq* I and *Hinf* I fragments, and one composed of fragments like those found in Queensland (Qld 1:1 class). If only fixed heterozygosity is found, this would suggest the purely vegetative dispersal of a heterozygous clone.

The other potential test is cytological. Coates et al. (1988) recorded the presence of a single acrocentric chromosome among the metacentrics of a diploid wild taro from Australia (cytotype I-1). This heterozygous cytotype was recorded in Queensland (Yen pers. comm. 1988). If sexual reproduction was important for the dispersal of taro within Queensland, then it should be possible to find cytotypes that are homozygous acrocentric, homzygous metacentric, and heterozygous aerocentric, and heterozygous metacentric.

If dispersal in Queensland began in the north and then proceeded southwards, then this might be evident in a decline of heterozygosity in local populations from north to south. Alternatively, if fixed (100%) heterozygosity is found in all populations, this would be a strong indication that all reproduction was vegetative (and all seed non-viable), and that human dispersal was therefore entirely responsible for the movement of wild taro between water systems within Queensland.[2)]

14.4.3 Taro in Papua New Guinea and Oceanic Pacific Islands

Two main points of interest arise from the survey of taro in Papua New Guinea. First, the wild taro of Morobe Province are phenotypically similar to wild taro in Australia, but display an rDNA pattern that is distinct from the three Australian rDNA patterns and distinct from all cultivated taro tested for Asia and the Pacific. There may have been natural and endemic differentiation of wild taro in Papua New Guinea, as was proposed for Australia above. Secondly, assuming that the Queensland rDNA variant is monophyletic and ancient, then comparisons of *Taq* I and *Hinf* I spacer fragments suggest that this variant occurs in cultivated and wild taro in Papua New Guinea. If the Queensland variant proves to have originated in Sahul, then it will provide a marker for the indigenous cultivation and domestication of natural and/or new wild varieties originating in New Guinea.

If the Queensland rDNA variant is monophyletic and ancient within the species, with an origin in mainland Asia, then its occurrence in Pacific island cultivars would not necessarily have resulted from indigenous selection and domestication in New Guinea. The comparisons of *Taq* I and *Hinf* I fragments suggest that the Queensland rDNA variant is present in Pacific island cultivars, but the significance of this for the origins of oceanic island taro will not be known until the ultimate origin of the Queensland rDNA variant is known.

Two key areas for investigating the origins of wild taro and the Queensland rDNA variant in Sahul are the southern coast of Papua New Guinea and the islands of

Indonesia, west of Wallace's line. It is predicted that wild taro in the Fly River district, close to Northeast Queensland, will be found to possess the Queensland rDNA variant. If the Fly River taro are identical or most closely related to Queensland wild taro, this would confirm the shared recent evolutionary history of taro in Australia and New Guinea. Indonesia is probably the most recent ancestral area for wild taro in Sahul. If the wild taro of Indonesia are different, this would be good evidence for endemism in Sahul, unless the immediate origins of taro in Sahul lie beyond Indonesia. If wild taro in Sahul are more closely related to wild taro elsewhere than Indonesia, in Asia, then long-distance dispersal by humans might explain the apparent biogeographical disjunction.

14.4.4 Ribosomal DNA and phenotypic variation

Clawson (1985) suggested that positive selection for colour variation has been a general phenomenon for cultivated plant species, and this might also be true for *C. esculenta*. Anthocyanin pigments were not totally absent from wild taro in Australia and New Guinea. Red or purple colouring at the junction of the petiole and the blade was sometimes evident in the wild, not always on all the leaves of one plant displaying these pigments. Among taro collections generally, red or purple pigments were noted in responses by true roots (not the corms) to wounding or cramping when potted. The petioles of *C. fallax* were purple (Figure 10.4) and the blades of *C. affinis* displayed purple patches (Figure 10.5). Whether or not these are natural phenotypes for *C. fallax* and *C. affinis* remains to be verified in the field, as the available examples were unprovenanced horticultural curios. With these examples of pigmentation in the possibly natural wild taro varieties, and in species closely related to taro, the ability to produce anthocyanins cannot be completely excluded as a primitive trait for taro.

In Figure 14.14 a simplified historical scheme is proposed, based on the apparent inverse relationship between rDNA variability and phenotypic variability (see Table 14.5, results) and two assumptions. The assumptions are that less pigmented phenotypes represent a primitive condition within the species, and that most rDNA diversity evolved over a long period of time before selection and propagation by humans. A possible explanation is that selection by humans promoted variability in the expression of already existing anthocyanin genes, leading to the diverse colour patterns as observed in cultivars today. Whatever the details of the actual process, the evident outcome for cultivated taro is that more extensively and variably coloured varieties, as a group, exhibit less rDNA variation than less extensively pigmented varieties.

The same inverse relationship might apply to genes of agronomic significance. Colour variation is commonly used in the field to recognise different varieties, but as a guide for field sampling, it may fail to maximise sampled variation for genes affecting agronomic characters. In other words, there may be no general rule for relating colour variability and the variability of genes for other agronomic characters. The inverse relationship with colour variation may apply more generally to genes which have not been of agronomic importance.

Ribosomal DNA spacer variability in taro may or may not have been of agronomic importance in the past. This question is beyond the scope of the present thesis. However,

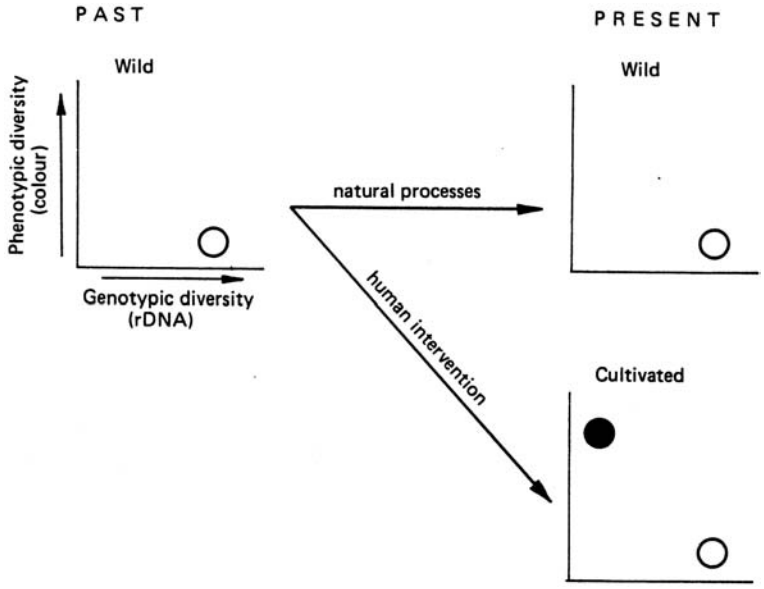


Figure 14.14 Historical model for taro illustrating the origins of an inverse relationship between phenotypic diversity and genotypic diversity, as measured by rDNA analysis. Open circles represent varieties with few anatomical parts (one or less) pigmented with anthocyanins, black circles represent varieties with more than one part pigmented. The model is derived by extrapolating the observations for wild taro in the present to the wild progenitors of cultivated taro in the past. The suggested result of human intervention (selection and propagation) is the creation of a phenotypically diverse but genotypically homogeneous class of highly pigmented, cultivated varieties.

if cultivars with relatively little pigmentation are considered to be primitive (close to wild types), then the detection of rDNA variability might be maximised by sampling primitive varieties from diverse geographical locations.

14.4.5 Ribosomal DNA and chromosome numbers

Ribosomal DNA variants are usually distributed in high copy number at chromosomal loci on one or more homologous pairs (diploids) or triplets (triploids) of chromosomes. Figure 14.15 illustrates, for a hypothetical homologous pair of chromosomes, how both homozygous and heterozygous chromosomes can have homogeneous or heterogeneous rDNA loci. Diploid and triploid plants displaying complex rDNA patterns could be the heterozygous progeny of homozygous diploid parents with homogeneous loci, or they could be the homozygous progeny of heterozygous diploids with heterogeneous loci. Between these two extreme cases, many other possibilities can be imagined.

In situ analysis of the diploid variety AKL 34 (Chapter Twelve) detected two rDNA loci, presumably one per haploid complement (i.e., on one pair of chromosomes). In other diploid and triploid taro, the number of loci per haploid complement may vary, contributing to the range and complexity of rDNA patterns observed. In *Triticum* spp

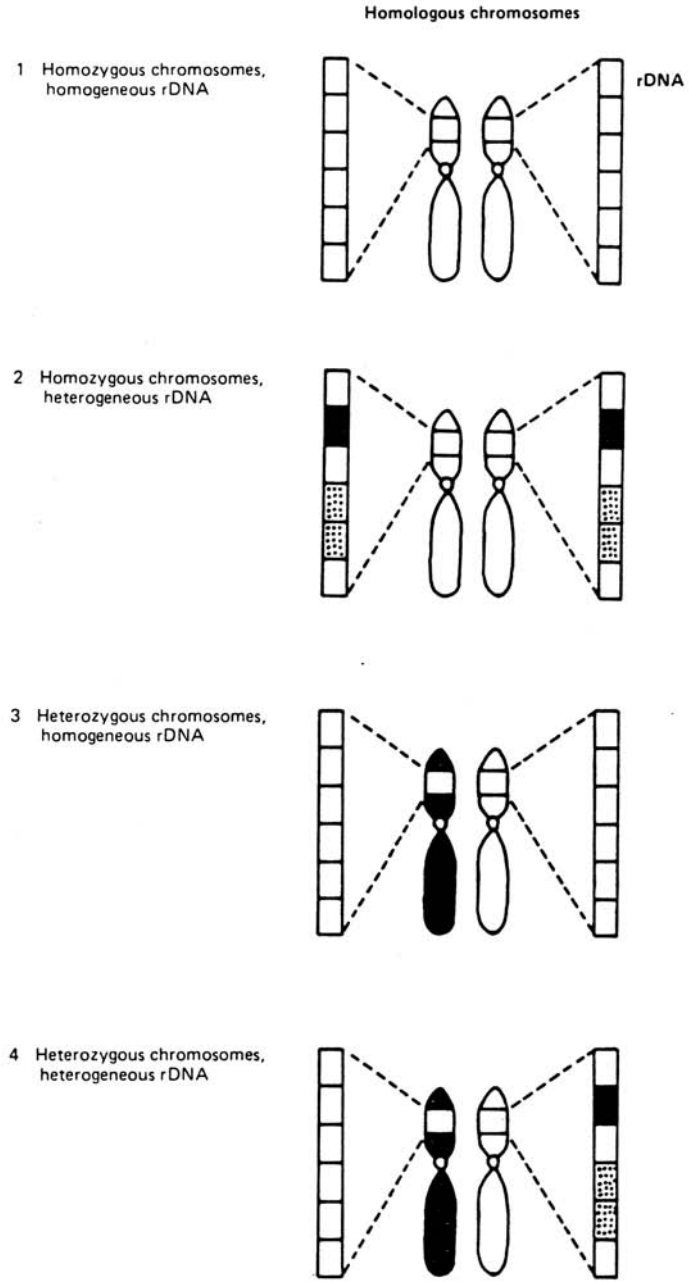


Figure 14.15 Diagram illustrating the distribution of rDNA repeat units on homologous chromosomes (four possibilities). A single rDNA locus is shown on each chromosome. Within rDNA loci, repeat unit variants are indicated as black, grey, or white. This diagram illustrates variation of just two homologous chromosomes, as would be found in a diploid plant, but essentially the same possibilities exist for triploid plants. Homologous chromosome pairs other than those bearing the rDNA loci may be heterozygous or homozygous, and there may be rDNA loci on more than one pair of homologous chromosomes.

(wheat) two or three loci per haploid complement have been recorded (Dvořák 1989). In *Triticum* and *Hordeum* (oats), homogeneity of rDNA spacer lengths within a locus is the prevalent pattern, but in several plant species, *Vicia faba* for example, intra-locus heterogeneity in spacer lengths is also evident. These examples show that for taro, no assumptions can be made about how the rDNA patterns relate to chromosomal homozygosity and heterozygosity, or to intra-locus homogeneity and heterogeneity. Studies of chromosomal morphology provide evidence for structural homozygosity and heterozygosity in taro (Coates et al. 1988), but such variation cannot be directly correlated with rDNA variation without further efforts to identify the chromosomes bearing rDNA loci.

Triploids can be assumed to be entirely or predominantly sterile. If offspring are produced, then these are not necesarily also triploid, because gametes produced by triploids may be haploid, diploid, or aneuploid. The natural dispersal of triploids is therefore limited to vegetative growth and the dispersal of vegetative parts by fresh water. In order to become widely distributed and common in Asia, as observed by Yen and Wheeler (1968) and others, triploids must have been transported by humans, and such dispersal was probably largely an agricultural phenomenon.

The surveys of rDNA in the New Zealand triploids (Chapter Eleven) and in *C. esculenta* var. *fontanesii* (this chapter) indicate that rDNA is stable within clonal lineages. The dispersal of what is believed to be one rDNA variant throughout Queensland must have taken much more than 220 years, the period since taro was first recorded in Queensland by Joseph Banks (see Chapter Nine). The diploid taro in Queensland probably dispersed by vegetative and sexual reproduction, indicating that rDNA in taro is stable over long periods involving sexual generations. Nevertheless, opportunities for rDNA mutation must be greater when both sexual and vegetative reproduction are possible. Most rDNA variants in taro may have originated over a very long period, in the course of vegetative and sexual reproduction by naturally occurring diploids. Ribosomal DNA variants in triploids today could have been inherited, in most cases, from diploid populations with much longer genetic histories than the triploids.

In theory, humans could have produced the present distribution of triploid plants by vegetative propagation of just one triploid clone, but it is unlikely that all rDNA variation in triploids is due to mutation within clonal lineages within the period of agricultural propagation and dispersal of triploids. The best evidence for separate polyploidisation events in the past would be the experimental reconstruction, by breeding, of the different triploids, using diploid varieties with the same range of rDNA variants as found in the triploids. Identifying triploid rDNA variants in diploids would in itself provide good circumstantial evidence for the parentage of triploids.

In the discussion that follows, comparisons of rDNA patterns and historical records are used to interpret the origins of New Zealand and Japanese triploid taro and the recent dispersal history of the triploid *C. esculenta var. fontanesii*.

14.4.6 The origins of New Zealand and Japanese triploid taro

Triploid taro were first reported in New Zealand by Yen and Wheeler (1968), and three

phenotypically distinct triploid varieties were described in detail by Matthews (1984, 1985). Two varieties, RR and GR, are common garden varieties, phenotypically very similar (rounded leaves, starchy corms and side-corms), and distinguished only by petiole colour. Var. GP possesses a distinct phenotype, including poor food qualities and long stolons, and is generally found wild. Variation in chromosomal morphology is correlated with the phenotypic variation: varieties RR and GR display cytotype II-3 and are distinct from var. GP, which displays cytotype I-3 (Coates et al. 1988, and Gaffey pers. comm. 1988). Ribosomal DNA variation detected with *Taq* I (Chapter Eleven, Figure 11.9), and *Rsa* I (Figures 11.10, 14.9) is also correlated with the phenotypic variation: varieties RR and GR display the same pattern of rDNA fragments and are distinct from var. GP.

The New Zealand triploid varieties RR and GR produce *Rsa* I rDNA spacer fragments similar to those in four Japanese accessions, T298, 229, 302, and 306 (Figure 14.9). These accessions display rDNA class C, as defined with *Taq* I and *Rsa* I (Figure 14.8). Accessions T298, 299, and 306 belong to the botanical group *Eguimo* (T. Kawahara pers. comm. 1985), one of the fourteen botanical groups of *C. antiquorum* (=*C. esculenta*) described for Japan by Kumazawa et al. (1956). The ribosomal DNA analyses (Figure 14.8) suggest that T302 and T326 (var. *Ishikawa-wase*) also belong to the *Eguimo* group. The growth habit and colouring of the New Zealand triploid var. RR (Figure 5.2), is very similar to that recorded for *Eguimo* in Table 14.8.

The *Eguimo* group is described by Takayanagi (1986) as triploid, and a chromosome count of 2n = 42 has been recorded for T299 (T. Kawahara, pers. comm. 1985). Matsuda (1950, cited by Yoshitake 1986) examined the morphology and growth of 205 taro varieties (158 from Japan, 38 from Taiwan, seven from China, one from Korea, and one from Sulawesi), and described 22 varietal groups, 15 of which were recognised as old or native Japanese groups. Six of these groups, *Eguimo* among them, included varieties that appear identical to varieties from China or Taiwan. Kumazawa et al. (1956) revised the classification of Matsuda (1950), retaining a similar number of varietal groups, including the *Eguimo* group. Takayanagi (1986, citing Kumazawa 1956) reviewed historical records for varietal names within each of the groups recognised in 1956. The earliest record for the use of the name *Eguimo* in Japan is in the late 17th century. A synonym, *Ao-imo*, was also recorded in Japan in the late 17th century, and in China in AD 560 (Chia, Ssu-hsieh, 6th century, cited by Takayanagi 1986). The historical records are thus consistent with the botanical observation that varieties of the *Eguimo* group are present in China. Varieties in this and other varietal groups are generally believed to be introductions from China. Takayanagi (1986) notes that introductions of taro to Japan could have taken place many different times, and that it is unclear exactly when and how introductions took place.[3)]

Kotani (1981) has reviewed evidence for early plant use in Japan, during the Jomon era of approximately 7000 BP–2300 BP. Nakao (1966, 1967; cited by Kotani 1981) proposed what has become known as the luciphyllous forest hypothesis, which suggests that slash-and-burn agriculture (shifting agriculture) evolved during the Jomon era in the evergreen broad leaf forests of western Japan. Five stages are proposed for the utilisation of plant resources (from earliest to most recent): (1) the gathering of wild nuts and roots,

(2) the selection and improvement of edible species, (3) cultivation of various root crop species, including taro, using slash-and-burn techniques, (4) cultivation of various seed crop species, including rice, with slash-and-burn techniques, and finally (5) wet rice agriculture, associated with irrigation and permanent paddy fields. Kotani (1981) noted that despite the popularity of this hypothesis for many years, no root crop remains had been recovered from the Jomon cultural context, and no convincing evidence for the practice of shifting agriculture had been obtained. Sasaki (1986; cited by Yoshitake 1986) has recently suggested that triploid taro varieties, being more temperate adapted, were introduced to Japan via the Yangtze River, and that diploid varieties may have entered on a warmer route, from Taiwan via the Ryukyu Islands. Sasaki (1986) estimates the date of the first introduction of taro to Japan as approximately 2500 BP, late in the Jomon era (the details of Sasaki's argument are not known to the present author). Yoshitake (1986) notes that taro varieties found in Japan today are not necessarily the same as the earliest introductions.

Yen and Wheeler (1968) recorded no triploid taro in tropical Polynesia, commonly accepted as the area of origin for the Māori. The absence of triploids in this area has been confirmed by later surveys (Sakamoto et al. 1976; Coates et al. 1988). The nearest field location of triploid taro recorded outside New Zealand has been New Caledonia (Yen and Wheeler 1968). The possibility that triploid taro were introduced from Melanesia to New Zealand in prehistoric times was (Yen and Wheeler 1968) and still is (Matthews 1985) contentious. Yen and Wheeler (1968) noted that 'with the wanderings of traders and whalers who took on native crew members in the nineteenth and twentieth centuries, the possibilities of plant transfers, while difficult to document, must be recognised'. They also reported the claim of a Chinese market gardener that a variety then grown in Auckland was imported from China. Morphologically, as judged by experimental cultivation, the market garden specimen was identical with two specimens of taro from near Māori settlements in North Auckland and the Bay of Plenty, and all three possessed similar chromosome numbers (presumably triploid, not directly stated). It was suggested that Chinese taro were imported in the period from the turn of the century until the 1930s by merchants supplying immigrant market gardeners.

The ribosomal DNA comparison, and the historical and botanical records for Japanese and Chinese taro, support the proposition by Yen and Wheeler (1968) that New Zealand triploid taro were introduced from China. The evidence is not proof because triploid taro are widespread in mainland Asia and varieties belonging to the *Eguimo* group may have existed in countries other than China at the time of introduction to New Zealand. It is also quite possible, given the close similarity of varieties RR and GR, that one of these is a colour variant of the other arising by vegetative mutation before or after introduction to New Zealand. Yen and Wheeler (1968) noted that Indochinese (Tonkin) migrants to New Caledonia could have introduced triploid taro to that country. It is likely that many taro varieties are shared by China and neighbouring Indo-Chinese countries.

Migration from the French colonies in Indochina to the colony in New Caledonia predates the turn of the century, and introductions from New Caledonia to New Zealand could have been made in the late nineteenth or early twentieth century. However, New

Zealand trade links with New Caledonia in this period were probably not as strong as with China, and the proposition of a Chinese origin for New Zealand taro (varieties RR and GR) is favoured here. In the absence of any strong affinities with taro outside New Zealand, no suggestions are made here regarding the origin of triploid variety GP.

Accepting that New Zealand triploid varieties originated in China has important implications for taro cultivation in New Zealand. There is no doubt that taro were present in New Zealand in prehistoric times, because of Māori traditions and botanical observations by early European observers (Best 1976). Assuming that the triploid variety GP is also a recent introduction, pre-European taro were probably all diploid varieties introduced from Polynesia. These appear to have been rapidly and almost completely displaced by the triploid varieties, RR and GR, within the last two hundred years. Only two of the nine plants reported by Yen and Wheeler (1968) were diploid. Matthews (1985) found diploid varieties at two sites and triploid varieties (RR, GR) at 193 sites.

Various contributing factors can be suggested to explain the apparently rapid displacement of diploid cultivars: Chinese triploid taro are probably temperate adapted and are probably easier to maintain in New Zealand than tropical Polynesian cultivars. Trials of Polynesian and Melanesian varieties have shown that the tropical varieties do not grow well in Japan, compared with Japanese varieties (Takayanagi 1986). In Japan the climate is temperate and strongly seasonal, as it is in New Zealand. Social factors are also important. Although agriculture has been dominated by European commercial crop species during the last two centuries, the tradition of taro cultivation has survived in Māori communities. Matthews (1985) suggested that the near absence of diploid taro in New Zealand was because of insufficient sampling or because Polynesian diploids were obligate cultivars in the temperate climate of New Zealand. The latter explanation is now favoured.

14.4.7 Widely distributed rDNA classes

The few but widely distributed records of the Col 3:4 and Puk 2:3 rDNA classes are difficult to interpret, since similar rDNA patterns could have been independently generated by similar diploid parents, among indigenous and/or introduced wild populations, in diverse locations. The observations permit the general suggestion that the clones tested, and/or their progenitors, are widely distributed in Asia and the Pacific, but do not allow close historical connections and routes of dispersal by humans to be identified. Better knowledge of geographical distribution, and closer characterisation of the rDNA variants represented in each rDNA class, would remove the problem of possible polyphyletism and strengthen arguments for particular historical connections and dispersal routes.

Ribosomal DNA variants can be monophyletic or polyphyletic, as noted previously for the Queensland rDNA variant, and combinations of rDNA variants can also be monophyletic or polyphyletic, depending on whether or not sexual processes (segregation and reassortment, recombination, and less well-known processes) produce the same combination of variants independently in different areas. Useful and not useful genotypic markers are characterised, in theoretical prescriptions, in Table 14.9. These prescriptions

also apply to conventional alleles and combinations of alleles, those characterised by iso-enzyme analysis for example.

The Col 3:4 and Puk 2:3 classes are probably monophyletic in some sense (Table 14.9, A1 to A6), but without closer analyses it cannot be assumed with complete certainty that they are not polyphyletic combinations of polyphyletic variants (Table 14.9, B2). In the case of *C. esculenta* var. *fontanesii*, discussed next, available historical records, and the fact that this variety is triploid, support the suggestion that the samples examined possess a monophyletic combination of rDNA variants (Table 14.9, A2, A3, or A4).

Examples of *C. esculenta* var. *fontanesii* from Sri Lanka (wild), New Zealand (cultivated ornamental) and Australia (cultivated ornamental) displayed identical phenotypes and rDNA patterns (Eli 3:5 class). Engler and Krause (1920) noted that var. *fontanesii* had been cultivated for a long time in hothouses in Europe, and according to Schott (cited by Engler and Krause 1920) it was cultivated in Holland around 1680 or 1690 as *'Arum Colocasia'* or *'zeylanicum'*, and the latter name suggests that the variety was introduced to Europe from Sri Lanka (formerly Ceylon). Because Australia and New Zealand were colonised primarily by Europeans, in the recent historical period, it is likely that var. *fontanesii* was introduced to these countries directly from Europe rather than from Sri Lanka. However, if the variety is also widespread in Asia in cultivation or in the wild, then it might well have been introduced directly from Asia. So far the variety has only been recorded in Sri Lanka. If it was not further introduced to Europe from Asia after 1690, and if Europe really was the source of the New Zealand and Australian examples, then the rDNA variants represented by the class Eli 3:5 must have existed for at least 300 years, following the 1690 date for Holland. Chromosome counts for plants from Sri Lanka and Australia show the variety to be triploid, so it is probably fully or mostly sterile. The phenotype and ribosomal DNA of var. *fontanesii* have probably been stable for at least 300 years of vegetative propagation as an ornamental (Figure 14.16 illustrates the historical reconstruction). Examples of var. *fontanesii* in Europe today are predicted to be identical to the plants recorded here for Sri Lanka, Australia, and New Zealand.

Table 14.9 Prescriptions for genotypic markers (rDNA complements) for investigating dispersal history.

Useful	
A1	Monophyletic variant
A2	Monophyletic combination of monophyletic variants
A3	Monophyletic combination of polyphyletic variants
A4	Monophyletic combination of monophyletic and polyphyletic variants
A5	Polyphyletic combination of monophyletic variants
A6	Polyphyletic combination of monophyletic and polyphyletic variants
Not useful	
B1	Polyphyletic variant
B2	Polyphyletic combination of polyphyletic variants

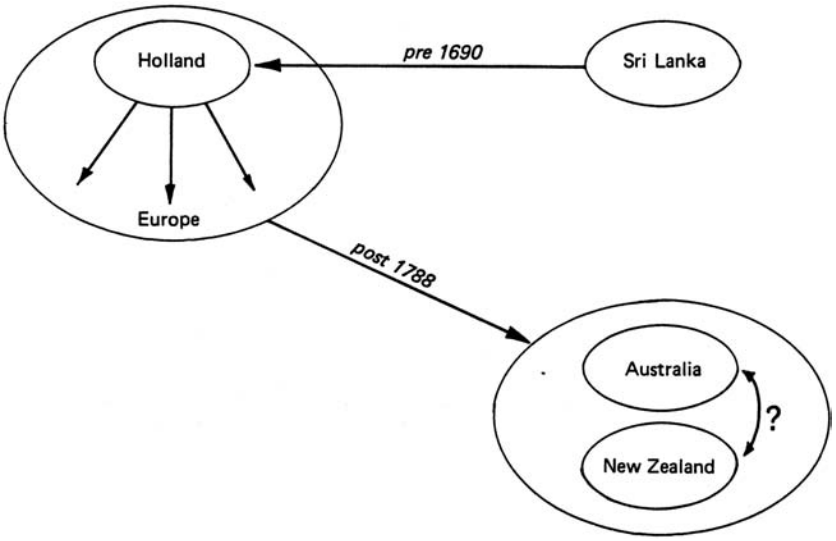


Figure 14.16 The possible recent origins of *C. esculenta* var. *fontanesii* in Australia and New Zealand. In both countries, it is now cultivated as an ornamental.

If the triploid var. *fontanesii* is indigenous to Sri Lanka, then diploid descendents of its diploid progenitors might exist there now. Distinctive phenotypic qualities of var. *fontanesii* (very shiny, leathery leaves, see Figure 10.2) have not been noted among any cultivated taro from Asia and the Pacific. The nearest diploid relatives of var. *fontanesii* may be genetically isolated in some way, perhaps because of a geographically isolated or very localised distribution.[4)]

14.4.7 Concluding statements

The present, non-intensive analysis of rDNA, with few restriction enzymes, appears better suited to locally intensive geographical surveys than to comparisons between few samples separated by very large geographical distances. Further intensive characterisation of the Queensland rDNA variant is recommended because this may be an ancient variant within the species, and may also be characteristic for taro in the tropical region of Asia and the Pacific.[5)]

Notes

1) This variety has been recognised as a member of the Egu-imo Group (Takeshita et al. 1991)
2) Heterozygosity and breeding at a Queensland taro site were recently demonstrated by Hunt et al. (2013), using samples collected during the 1987 field survey.
3) Further characterisation of rDNA variation in Japanese taro varieties was carried out by Matthews, Matsushita et al. (1992). Matsuda (2002), and Matsuda and Nawata (2002),

extended the rDNA survey into China and northern Vietnam, and suggested that most Japanese cultivars are derived from common Chinese cultivars.

4) A contrary interpretation is now possible: The wild species *Colocasia lihengiae* (Long and Liu 2001) has shiny, leathery leaves that are distinctly angular in outline, and was recently found in Arunachal Pradesh, India (Gogoi and Borah 2013) (shiny leaves indicate a lack of waxy leaf hairs; the leaves of *C. esculenta* are mainly dull in appearance because of waxy hairs that repel water). The widespread *C. lihengieae* might have crossed – in India or elsewhere – with *C. esculenta*, leading to production of var. *fontanesii* as a polyploid hybrid. This speculative suggestion can be tested through genetic analysis.
5) Matroud et al. (2012) have described a nested tandem repeat (NTR) structure present in large intergenic spacer region of taro rDNA. Their preliminary analysis suggests that changes in the NTRs of taro have been occurring on a 1000 year time scale, and that anlaysis of NTRs may be useful for studying plant populations.

Senri Ethnological Studies 88: 229–247 ©2014
On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Fifteen
Origins and Antiquity of Diploid Taro in Australia and New Guinea, and Triploid Taro in Asia

The major question raised in Chapter Nine was whether or not taro provided a basis for the indigenous development of agriculture in New Guinea. Taro could have been of ancient natural occurrence in Australia and New Guinea (Sahul), or could have been a pre-agricultural introduction by humans. The distinction is important because pre-agricultural dispersal of taro by humans, to Australia and New Guinea, could have established wild populations that were better or worse material for domestication than natural wild populations elsewhere. The antiquity of taro in Sahul, and the possibilities of natural occurrence versus introduction, were not resolved by the surveys of rDNA variation, but future examination of rDNA and other aspects of genotype could be informative, as discussed in Chapter Fourteen.

In the present chapter, other kinds of biological evidence are discussed with an emphasis on the possible pre-agricultural occurrence of diploid taro in Sahul. Some original field observations are briefly reported, as illustration. In the second part of the chapter, the possible origins and antiquity of triploid taro in Asia are discussed.

15.1 Diploid Taro in Australia and New Guinea (Sahul)

15.1.1 Archaeological and natural fossil remains

The soft herbaceous parts of taro are not very likely to be preserved as fossils, although they could be preserved in waterlogged swamp sites or as impressions in volcanic deposits (Spriggs 1982). Fossil taro seed have not been reported, and although pollen have been identified in archaeological sites in New Guinea, the identifications are not considered secure (Spriggs 1982). Charred remnants of taro, the outcome of cooking, may be a more likely source of archaeological fossils. The taxonomic characterisation of charred root crop remains is in progress (Hather 1991).

The possibility of recovering taro seed from archaeological contexts is indicated by the production of abundant seed in taro fruit (Figure 9.3). The general rarity of flowering within cultivations today suggests that pollen is unlikely to be found in the archaeological remains of cultivations (Spriggs 1982). A palynological analysis of deposits from a currently cultivated taro pond-field on Futuna did not reveal taro pollen because of the lack of flowering and/or because taro pollen did not survive the recovery techniques used (A. di Piazza, S. Haberle, pers. comm. 1989). In Australia, flowering by wild taro is common in Queensland (P. J. Matthews, field notes, and R. Hinxman, pers. comm. 1987). This area provides opportunities for testing recovery techniques in sites where pollen is

known to be produced, unrestrained by human actions. Direct observations of pollen deposition and initial degradation or preservation would be useful for assessing pollen recovery from archaeological deposits. Ancient natural sediments in Queensland are a potential source of direct evidence for the pre-human or pre-agricultural antiquity of taro in Sahul, and are perhaps more accessible than suitable deposits in New Guinea.[1)]

Starch grains and calcium oxalate raphides have been discovered on stone tools from the Solomon Islands, dating from 28,000–20,000 BP (Loy et al. 1992). After comparisons with the morphology of contemporary starch- and raphide-producing genera and species, the archaeological remains have been interpreted as probably coming from *Colocasia esculenta* and *Alocasia*. The evidence does not indicate whether cultivated or wild plant sources were involved, and sheds no light on whether or not taro is indigenous to the Solomon Islands. Fossil remains from a pre-human context are needed for direct proof that taro is indigenous. Nevertheless, if the interpretations are correct, the archaeological observations are the first direct evidence for taro in any part of Asia or the Pacific, much earlier than any date recorded for agriculture.

Spriggs (1982) noted the possibility of archaeological preservation of insects associated specifically with taro, giving *Papuana* spp. (taro beetles) and *Tarophagus proserpina* (taro leaf hopper) as examples. Whether or not these insect species are associated exclusively with *C. esculenta* is not yet clear. In the following discussion of insects associated with taro, the need to investigate host specificity is emphasised. It is an issue with double significance — for interpreting the evolutionary history of taro, and for interpreting archaeological remains of insects associated with taro.

15.1.2 Organisms that require taro as a host

A biological approach to estimating the antiquity of taro in Sahul can be made by examining organisms that require taro as a host. Endemic differentiation of organisms associated with taro in Sahul might suggest pre-human antiquity for taro, regardless of whether or not taro itself underwent endemic differentiation. The suggestion is qualified here, because taro could have been introduced as long ago as 40,000 years, and differentiation of an associated organism might have occurred within this period. Exploring this last possibility is beyond the scope of the discussion that follows.

Drosophilid flies

During fieldwork by the present author, drosophilid flies were found swarming in and around inflorescences of wild taro alongside a coastal forest stream between Lae and Salamaua in Morobe Province, Papua New Guinea, in July 1985 (Figure 15.1). Specimens taken from within an inflorescence were later identified by M. Arura (PNG Department of Primary Industries, Bubia) as *Drosophilella* (*syn. Colocasiomyia*) *pistilicola* Carson and Okada (Diptera: Drosophilidae)[2)]. This and another species, *Colocasiomyia stamenicola* Carson and Okada (1980), were first described by Carson and Okada after collecting flies in approximately the same area in the period August to December 1977. These workers investigated the ecology of drosophilid flies in this area, and examined numerous species associated with a wide range of plants. Their conclusions

regarding the specific association of drosophilid flies with flowers of *C. esculenta* are thus highly significant. Carson and Okada (1980: 28) write:

> The infloresence of *Colocasia esculenta* is a complex microcosm with four members of the family Drosphilidae of three genera breeding in it in the Morobe District of Papua New Guinea. The most thoroughly adapted for life in this specific type of inflorescence are the two species of *Drosophilella* [syn. *Colocasiomyia*]. Large number of adults of both species enter the fresh flower just as it is opening…these two species share the inflorescence niche in an extraordinarily precise manner. This reflects a set of highly refined adaptations in the case of the *Drosophilella* species; indeed, these species are apparently highly restricted to their host plant, *Colocasia esculenta*. As will be reported in a later paper…they do not visit even the quite similar inflorescences of other species of Araceae growing in very close proximity of *Colocasia*…. Although there are very strong similarities between *D. stamenicola* and *D. pistilicola*, they should not be described as

Figure 15.1 *Colocasiomyia* on a spathe of wild taro in Morobe Province, Papua New Guinea, July 1985. The flies shown may include both *C. pistilicola* and *C. stamenicola*. (This plant was growing on the bank of a stream, in a forest, opposite the plants shown in Figure 10.9).

> sibling species. Any one of a number of specific morphological differences can be used to separate them. Furthermore, strong morphological similarities are found between *stamenicola* and *alocasiae* and between *pistilicola* and *colocasiae*. The systematic and ecological relationships of these new species to others found in closely related hosts should serve as an interesting topic for further study…. Further investigation of the insect associates of *Colocasia esculenta* may aid in an estimation of the ancestral home of this important food plant.

If particular species of *Colocasiomyia* are associated exclusively with taro and are endemic in Papua New Guinea, then the degree and nature of endemic differentiation by the insect species might provide circumstantial evidence for taro being indigenous. The available evidence for *Colocasiomyia* from within and beyond Papua New Guinea does in fact suggest that this is the case, as follows.

C. pistilicola and *C. stamenicola* breed microallopatrically on the same spadix of *C. esculenta* in Papua New Guinea (Carson and Okada 1980). The microallopatry is exhibited by the larval life stages of these species, with larvae of *C. pistilicola* occupying the pistillate (female flower) zone within the taro infloresence, and larvae of *C. stamenicola* occupying the staminate (male flower) zone. These species are described by Carson and Okada (1982a) as 'synhospitalic', a term indicating the occurrence of two or more related parasitic species on the same host species or individual. Two other species, *C. xenalocasiae* Okada 1980 and *C. alocasiae* Okada 1975, share the spadix of *Alocasia odora* C. Koch in Taiwan and Okinawa (Okada 1975; Carson and Okada 1982a: 14). The phyletic relationships among these four *Colocasiomyia* species were analysed taximetrically using twenty diagnostic characters (Okada 1980). The analysis shows that *C. pistilicola*, the pistillate species on *Colocasia* in Papua New Guinea, is very similar to *C. xenalocasiae*, the pistillate species on *Alocasia* in Taiwan. In a reciprocal manner, *C. stamenicola*, on *Colocasia* in Papua New Guinea, closely matches *C. alocasiae*, its staminate counterpart on *Alocasia* in Taiwan. A fifth species has been described, *C. colocasiae* Duda 1924 from Java, collected from *C. esculenta* (Okada 1981: 279). From limited information (not all characters could be observed), this species is interpreted as being closest to *C. pistilicola* (Carson and Okada 1982a). Okada (1980) makes the prediction that another species will be found in Java which is synhospitalic with *C. colocasiae* and closely related to *C. stamenicola*. Carson and Okada (1982a: 14) suggest the following evolutionary scheme:

> It may be supposed that a couple of ancestral species of *Drosophilella* [syn. *Colocasiomyia*] had established synhospitalism on some plant of Araceae in a certain area of the Oriental Region, probably the southern part of Angaraland (mainland Asia). Tightly keeping synhospitalism, they then evolved into *alocasiae* and *xenalocasiae* and dispersed in the direction of Taiwan and Okinawa on the one hand and into *colocasiae* and an unknown species in Java as well as into *stamenicola* and *pistilicola* in New Guinea in a more easterly direction. The process of synhospitalic evolution and dispersal of *Drosophilella* probably retained an intimate association with the evolution and dispersal of

> the host plants. Furthermore, the process might well be correlated with land elevation in the southern islands through eustatic movement of the sea level during the early Neogene. Establishment of the present status of the distribution of these species was probably not much concerned with vicariance of primarily widely distributed ancestral species…. Rather, this case appears to have a more simple dispersal from a certain center of origin towards geologically newer lands.

Carson and Okada (1982a) thus suggest that the differentiation and dispersal of *Colocasiomyia* species was linked with the differentiation and dispersal of *Alocasia* and *Colocasia* species at a time which corresponds to the Miocene convergence (Whitmore 1981) of Laurasia and Gondwanaland. Little is known about the rate, degree, and location of genotypic differentiation in the insect and host species, and it cannot be assumed that the postulated evolutionary processes required as much as the 20–25 million years since the early Neocene. Jackson and Pelomo (1980) report that *Colocasiomyia* sp. nr. *seminigra* Duda is often present in large numbers in taro flowers in the Solomon Islands. The observation raises several questions. For example, do endemic *Colocasiomyia* species exist in the Solomon Islands? Do *Colocasiomyia* species occur any further eastwards, on remote oceanic islands? Such occurrences would suggest that the insect is an associate of human dispersal of taro.

Investigations of insects associated with *A. brisbanensis* Hay and *C. esculenta* in Australia would help to illuminate the evolutionary history of associations between drosophilid flies and taro. Although *Colocasiomyia* species have not been reported in Australia (Bock 1982), species of other drosophilid genera, *Drosophila* and *Styloptera*, have been[3]. Specimens of *Drosophila specensis* Bock, examined by Bock (1976), were not accompanied by information on particular plant hosts, but the species was clearly associated with rainforest habitats over a wide range within northeast Queensland. It may well be associated with *A. brisbanensis* and/or *C. esculenta* in this region, since another species of *Drosophila*, *D. metatarsalis* Okada and Carson, is associated with *Alocasia* and *Colocasia* in Papua New Guinea. *D. metatarsalis* was described for the first time after being collected from *A. macrorrhizos* (*A. nicolsonii* Hay, Hay pers. comm. 1989) and *C. esculenta* in Morobe Province, Papua New Guinea. Most examples found by Okada and Carson (1980) were in or near inflorescences of *A. nicolsonii*, but one female was reared from an inflorescence of *C. esculenta*. The authors noted that *D. metatarsalis* resembles *D. specensis* Bock 1976 from Queensland, Australia.

Two new species of *Styloptera* Duda were described by Okada and Carson (1980) and Carson and Okada (1980) from collections made in Morobe Province in 1977. One of these, *S. alocasiae* Okada and Carson, was found only on *Alocasia* spp., and the other species, *S. repletoides* Carson and Okada, was found only on *C. esculenta*. Apart from *S. formosae* Duda, recorded in Taiwan, only two other species of *Styloptera* are known, *S. striata* Bock and *S. wheeleri* Bock. Both are Australian and are known from one site each in northeast Queensland, within the known range of wild *C. esculenta* and *A. brisbanensis*. The 1967 collection site for *S. styloptera* (Bock 1982) was a waterfall location known as The Boulders (site 9 in Figure 14.3), where wild taro was collected in

1987. If, as seems quite likely, *Styloptera* spp. are specifically associated with taro in Papua New Guinea and Australia, and the insect species are endemic within Sahul, then this would support the suggestion that taro is indigenous to Sahul.

The insect associations of *Colocasia* and similar aroid hosts, notably *Alocasia* spp., represent a barely touched field of investigation. Okada and Carson (1980) describe, on the basis of collections within one small area of Morobe Province, Papua New Guinea, fourteen new species of Drosophilidae associated with the flowers of *A. macrorrhizos* (*A. nicolsonii* Hay?). This plant was described by Carson and Okada (1982b: 682) as a 'veritable zoological garden' for drosophilids. Studies of *A. brisbanensis* in Australia show that the garden of *Alocasia* contains more than just drosophilids. *Neurochaeta inversa* McAlpine is a new genus and new family (Neurochaetidae) known only in association with *A. macrorrhizos* (i.e. *A. brisbanensis* Hay), in or near rainforest habitats (McAlpine 1978; Shaw and Cantrell 1983a). Insects from families Aphididae, Ceratopgonidae, Chloropidae, Formicidae, Muscidae, Nitidulidae, Phoridae, and Staphylididae were also observed on *A. macrorrhizos* (i.e. *A. brisbanensis* Hay), in Australia (McAlpine 1978; Shaw et al. 1982; Shaw and Cantrel 1983b). These associations may or may not be host specific.

Less is known about insects associated with wild taro in Australia. During fieldwork in Queensland in September 1987, no drosophilids were seen on inflorescences. However, flowering in Queensland spans at least seven months, from September (personal observation 1987) to March (Hinxman pers. comm. 1989), and drosophilids might only become apparent at a certain time within this period.

Delphacid planthoppers and viruses

Adult specimens of *Tarophagus colocasiae* Matsumura (stat. rev. and comb. n. Asche and Wilson 1989) (Homoptera: family Delphacidae) were collected by the present author in September 1987 from the leaves of wild taro at Jiyer Cave on the Russell River (site 10, Figure 14.3). The specimens were later identified by Dr J. Donaldson, Queensland Department of Primary Industries, and are the first record of *T. colocasiae* in Australia (Donaldson, pers. comm. 1989)[4].

A nymph of what may be *Tarophagus* sp. was subsequently found on a plant collected by R. Hinxman approximately 2 km north of Jiyer Cave, at Moochoopa Falls, in August 1989 (Figure 15.2). It is difficult to identify nymphs because the available taxonomic descriptions for *Tarophagus* are based on adult morphology (Donaldson, pers. comm. 2002). The genus *Tarophagus* has been revised, and is recognised as containing three species (Asche and Wilson 1989). The known geographical distributions of these species differ, but overlap in the eastern part of mainland New Guinea (Figure 15.3).

Tarophagus is one of two insect genera known to transmit Alomae and Bobone viruses, which are apparently endemic to Papua New Guinea and the Solomon Islands (Mitchell and Maddison 1983). If these viruses are restricted to taro as a host, then their endemic differentiation in the New Guinea area might support the suggestion that *C. esculenta* is indigenous to New Guinea. Another virus, Dasheen Mosaic Virus (DMV), is

common worldwide among cultivated aroids (Zettler and Hartman 1986), and has been reported for cultivated aroids and wild *Alocasia* in Queensland (Greber and Shaw 1986). The spread or otherwise of virus diseases between wild *Alocasia* and *Colocasia* in Queensland would provide a measure of the host specificity of insects associated with taro. If any viruses are associated specifically with wild taro in Australia, then their

Figure 15.2 Nymph from a leaf of wild taro collected by R. Hinxman, 8 October 1989, in rainforest at Moochoopa Falls, Bellenden Ker National Park, Northeast Queensland. Tentatively identified as *Tarophagus* Zimmerman (Asche and Wilson 1989), species not determined. Upper photo: view of entire insect. Lower photo: front of head. Scale bars 100 um (0.1 mm) in both photos. The antennae, head structure, number of abdominal plates, and the foliate spur on the rearmost leg appear the same as shown for the adult stage of *Tarophagus*, described by Asche and Wilson (1989). Photographed with a Joebel scanning electron microscope, by D. Rentz, Department of Entomology, C.S.I.R.O., Canberra.

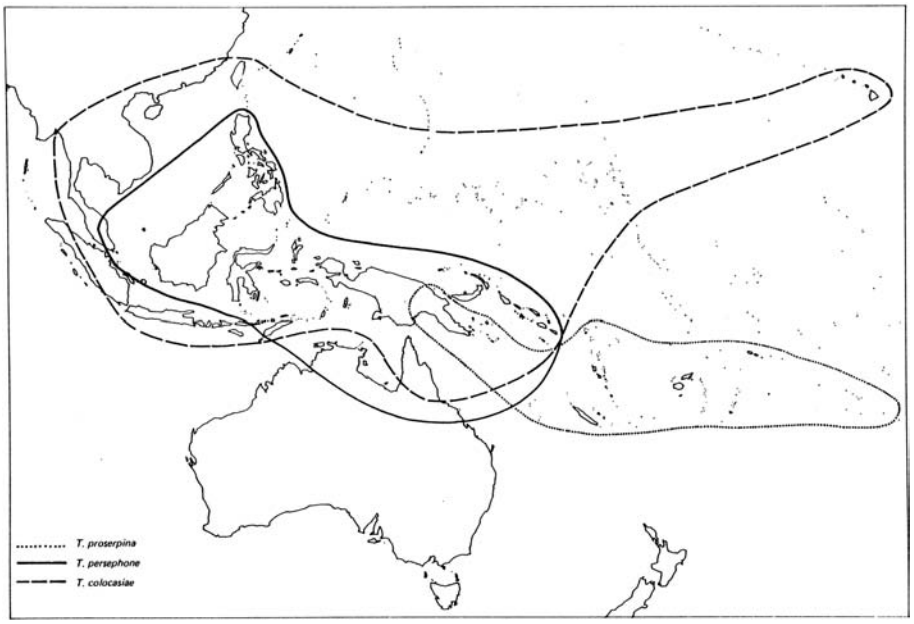


Figure 15.3 *Tarophagus* species in Southeast Asia and the Pacific. Adapted from Asche and Wilson (1989) with the addition of one record (collection by author) of *T. colocasiae* in Queensland.

distribution and differentiation within Australia would have significance for the possible origins of Australian taro.

Mitchell and Maddison (1983) noted *Alocasia* spp. as alternative hosts for *Tarophagus proserpina* (information source not given), but no distinction was made at that time between the three species of *Tarophagus* recognised now. It is therefore still possible that one or two species of *Tarophagus* are associated with *C. esculenta* exclusively. The only information available regarding *Alocasia* as an alternative host is the unsourced comment by Mitchell and Maddison (1983), and it is only implied by Asche and Wilson (1989) that all three species of *Tarophagus* are associated with *C. esculenta*. The host specificity of *Tarophagus* remains an open question. The extensive survey of Asian and Pacific insect records and collections by Asche and Wilson (1989, and Figure 15.3) shows that *T. proserpina* is endemic to the South Pacific. If *T. proserpina* is associated exclusively with taro, this would support the suggestion that taro is indigenous to New Guinea. The distribution of *T. proserpina* is intriguing because it suggests that cultivated taro was dipersed from Melanesia eastwards to Polynesia, but not west to Asia or north to Micronesia. According to Waterhouse and Norris (1987), *Tarophagus* was first established in Hawai'i in 1930, when its population rose and caused major crop damage. Presumably the species involved then was *T. colocasiae*, the only species recorded for Hawai'i by Asche and Wilson (1989). Plant transfer during the last

two centuries may well have increased the range of *T. colocasiae* and *T. proserpina* in the Pacific, but this does not affect the possible prehistorical significance of the absence of *T. proserpina* from Asia.

15.1.3 Lake Carpentaria and land connections between Australia and New Guinea

Taro may have reached Sahul before, during, or after the last period of low sea level, in the late Pleistocene, by natural means or as an introduction by humans. Until more is known about the natural vectors of taro fruit and seed, and the differentiation of taro within and beyond Sahul, it will be impossible to relate the present distribution of wild taro to particular geomorphological episodes. It is nevertheless of interest to consider the possible significance for taro of Lake Carpentaria and adjacent land areas between Australia and New Guinea during the late Pleistocene. During the last period of low sea

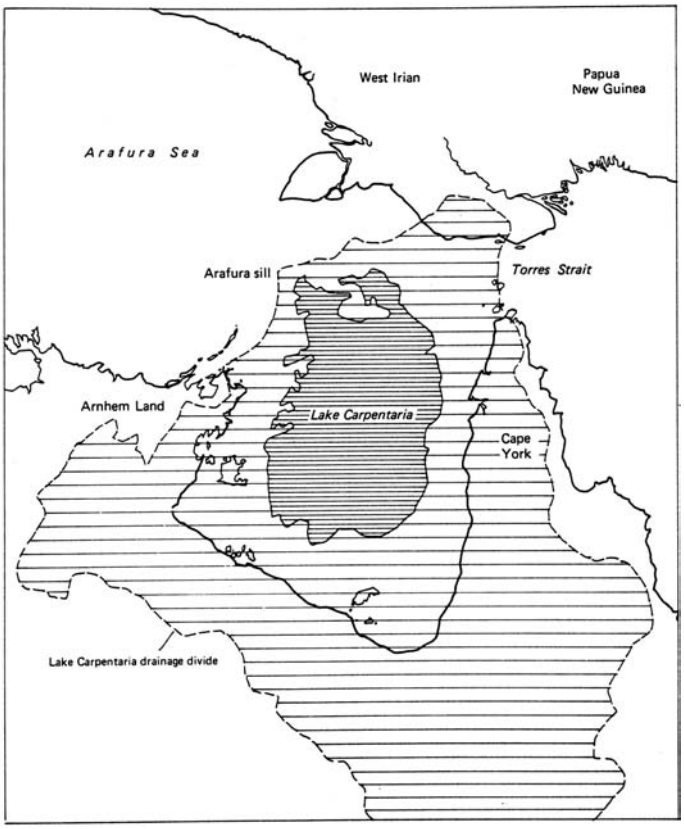


Figure 15.4 The Carpentaria drainage basin showing the maximum extent of Lake Carpentaria Basin, juxtaposed with the present coastlines of Australia and New Guinea. Lake Carpentaria is believed to have existed throughout the period of approximately 35,000 BP to 12,000 BP, with a maximum depth of only 10 m, and fresh-to-brackish water. Adapted from Figure 1a, Torgersen et al. (1988).

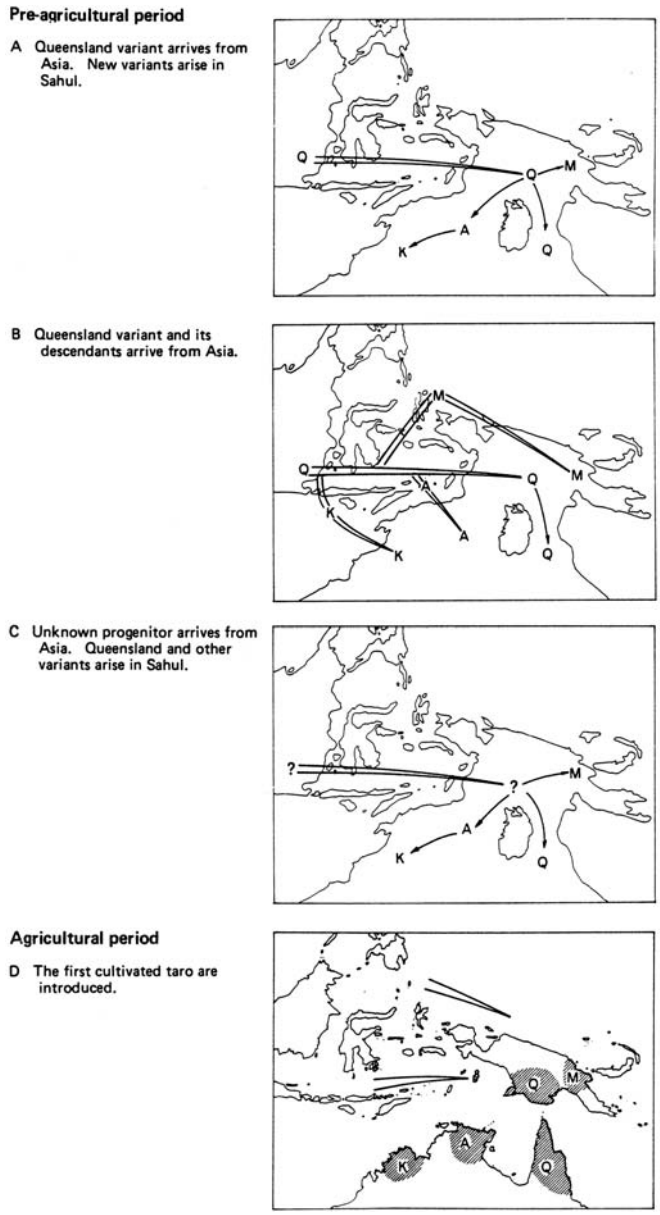


Figure 15.5 Possible origins and dispersal of diploid taros that display different rDNA patterns, in Sahul (panels A-G). Q = Queensland rDNA variant, Qld 1:1 class; K = Kim 2:2 class; A = Arn 2:2 class; M = Morobe Province rDNA patterns, with *Taq* I and *Hinf* I spacer fragments of c.4.6 kbp. The continent of Sahul is shown with Australia and New Guinea connected by land and with Lake Carpentaria, in the pre-agricultural period, before 8,000 years BP. The full separation of Australia and New Guinea by rising sea levels occurred at about 8,000 years BP. Panel D illustrates a possible scenario for the distribution of wild taro, and the possible introduction of cultivated taro from Asia, at an unspecified time after 8,000 BP, in the agricultural period. Agriculture may have begun in New Guinea with the introduction of cultivars from Asia, or it may have begun earlier, with selection from a range of wild taro already present.

level, from approximately 35,000 to 12,000 years BP, three main geographical features lay between Australia and Papua New Guinea: Lake Carpentaria, the Arufura Sill, and the Cape York/Torres Strait landbridge (Torgersen et al. 1988, and Figure 15.4). The shoreline and floodplains around Lake Carpentaria may have been unsuitable for taro if they were alternately subject to wet-season inundation and dry-season drought, as suggested by Torgersen et al. (1988). Permanent water sources suitable for taro may have existed above the floodplains, on the Arafura Sill and along what is now Cape York, and these areas may have provided routes for dispersal between the two sub-continents during the Pleistocene. A route or routes of dispersal might become evident from a detailed survey of genotypic differentiation among wild taro in the areas that once surrounded Lake Carpentaria. Surveys in Wallacea, the island region in central Malesia which lies between Wallace's line and Weber's line (Whitmore 1981), and in adjacent areas of Indonesia west of Wallace's line, are also needed to determine the history of wild taro in Sahul. The same areas would also be of pivotal interest for investigating the insects associated with taro in Sahul.

Scenarios for the origins and dispersal of the Queensland rDNA variant (Qld 1:1 class, Chapter Fourteen) are presented in Figure 15.5, where it is assumed that taro arrived before agriculture began in New Guinea, during an unspecified period of low sea level. It is also assumed that the other rDNA classes recorded for wild taro in Australia and New Guinea are derived from the Queensland rDNA variant. The possible polyphyletic origins of rDNA variants and classes are discussed in Chapter Fourteen. If taro reached Sahul very early, before Australia and New Guinea were fully separated by rising sea levels (c. 8000 BP, Torgersen et al. 1988: 259), then taro with the Queensland rDNA variant may have dispersed north and south of the Carpentaria basin as suggested in Figure 15.5A, B. Figure 15.5C illustrates the possible evolution of the Queensland rDNA variant within Sahul, in Queensland. The locations shown for the origin of the Queensland rDNA variant, Figure 15.5A, B, C, are purely hypothetical in each case. Figure 15.5D shows one of many possible scenarios for the differentiation and distribution of wild taro in Sahul, after the separation of Australia and New Guinea by sea. Cultivated taro are shown as introduced from Asia, but they may have also originated locally, from already existing wild populations, as discussed next.

15.1.4 Origins of cultivated and wild taro in Sahul

Given the various indications that taro reached Australia and Papua New Guinea in ancient times, in the pre-agricultural period, the major question that then arises is whether or not wild taro like those found today were alone sufficient for the evolution of cultivated taro in Papua New Guinea. This question could be addressed by phylogenetic analyses of wild and cultivated taro, using any parts of the nuclear, mitochondrial or chloroplast genomes that are informative for intra-specific phylogeny. Alternatively, the possible derivation of cultivated varieties from local wild populations could be investigated by examining genes (and their products) that directly affect qualities favoured by cultivators. Examples are genes affecting the synthesis and degradation of stored starch, or the production of enzymes such as trypsin inhibitor, which inhibits

digestive enzymes, and thus reduces the digestibility of taro for animals and humans (Bradbury and Holloway 1988: 109).[5] Wild taro in Australia and Papua New Guinea produce very little starch compared to cultivated or known feral varieties (personal observation).

Whether or not wild taro in Sahul are of pre-agricultural origin remains unresolved. To summarise the discussions here and in the preceding chapter, four general approaches can be suggested for future research: (1) a search for endemicity in wild taro genotypes, using typological and phylogenetic analysis, and geographical surveys within and beyond Sahul; (2) a search for evidence of long-term and differential gene flow between wild populations in different parts of Australia and New Guinea; (3) a search for ancient archaeological and natural remains, and (4) investigation of the possible host specificity and endemicity of organisms associated with taro.

15.2 Origins and Dispersal of Triploid Taro

15.2.1 Genetic mechanisms that create triploids

Mitotic chromosome doubling in a normal diploid ramet (the separate shoot or branch of a clonal lineage) could produce a tetraploid ramet (Figure 15.6). Vegetative proliferation of such a ramet would create a tetraploid clonal lineage with the potential to flower and produce many diploid (abnormal) gametes by normal meiotic processes. Such a clone could, in theory, produce many tetraploid offspring by self-pollination. This is illustrated in Figure 15.6 by the seedling with a somatic chromosome number of 2n = 56. Triploid offspring, with a chromosome number of 2n = 42, could also be produced by cross fertilisation with diploid shoots from the same clone (Figure 15.6), or different clones.

Alternatively, triploid taro might arise after rare, unreduced, diploid gametes were produced by abnormal male or female meiosis, or by aberrant pre-meiotic mitosis within floral cell lineages. Rare unreduced gametes would usually fertilise, or be fertilised by, normal haploid gametes. Tetraploids produced by the combination of rare unreduced gametes would be very rare or absent (Figure 15.7).[6] This theory is consistent with the absence of tetraploids in chromosome number surveys to date (Yen and Wheeler 1968; Sakamoto et al. 1976; Kuruvilla and Singh 1981; Coates et al. 1988).

From the above discussion it is concluded that sexual reproduction was essential for the creation of triploid varieties of taro, and that most triploids originated in the wild, where sexual reproduction is unrestrained. Pollen is produced by an inflorescence before seed, and cultivated plants are more likely to produce pollen than seed before being harvested. Occasionally, pollen from cultivated taro might be carried to nearby wild plants by species of *Colocasiomyia* (Figure 15.1), drosophilid flies, which are likely pollinators for taro (Carson and Okada 1980). In this way, aberrant sexual processes in cultivars could have led to the production of triploid seedlings in the wild, as suggested in Figure 15.7.

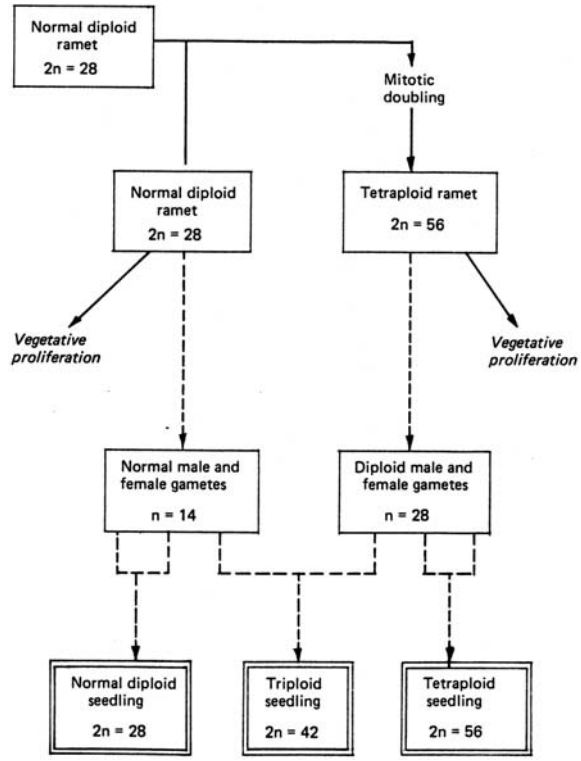


Figure 15.6 Scheme for the production of triploid taro by aberrant vegetative reproduction

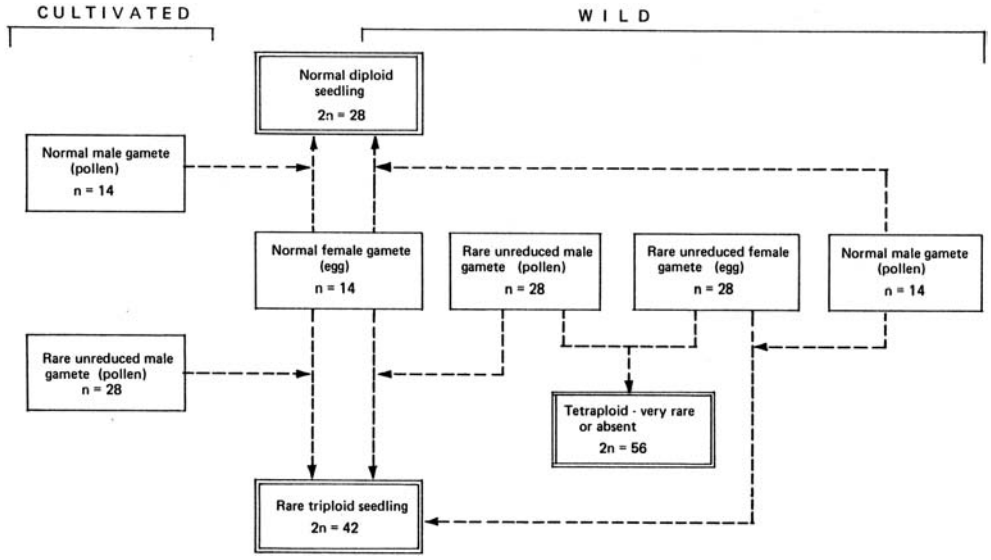


Figure 15.7 Scheme for the production of triploid taro by aberrant sexual reproduction

15.2.2 Evidence for the multiple origins of triploid taro

The multiple origins of triploid taro from different seed are indicated by the accumulated circumstantial evidence for diversity among triploids. Extreme differences in phenotype are exhibited, for example, by the New Zealand triploids var. RR (starchy with side-corms), var. GP (non-starchy with stolons), var. *fontanesii* (extremely dark anthocyanin pigmentation, very waxy leaves, non-starchy with stolons). Ribosomal DNA variation is recorded in Chapter Fourteen, and variation in chromosome morphology has been described by Coates et al. (1988).

Two triploid lineages are recognised by Coates et al. (1988), one characterised by the possession of three sets of acrocentric chromosomes (cytotype II-3) and the other by two sets of metacentric chromosomes with a third set containing metacentric and acrocentric chromosomes (cytotype I-1). The identification of homology between the various metacentric and acrocentric chromosomes is based on entire chromosome lengths and on the ratios of chromosome-arm lengths within each chromosome. In taro, these morphological characters are not very distinctive, and Coates et al. (1988) adopted the simplest possible interpretation by aligning chromosomes in a way that minimised the number of different arrangements recognised among the plants analysed. This reasonable but conservative approach may underestimate variation within the species. In all, eight different combinations of rDNA variants have been recorded for triploids in tests with *Taq* I, *Hinf* I, and *Rsa* I: two from New Zealand (Figures 11.9, 11.10, 14.10), three from Japan (Figures 14.8, 14.9), two from Nepal (Appendix 20) and one from Sri Lanka (Figure 14.11). As noted in Chapter Fourteen, proof that the different triploid lineages have separate sexual origins requires the identification of diploid progenitor genotypes (see Briggs and Walters 1984: 216–47 for some discussion of methods for identifying the progenitors of polyploids). For the purposes of further discussion, I will assume that triploid clones of taro originated many times from separate seed.

15.2.3 Geographical range and dispersal of triploids

Triploids occur mostly in Asia, and few have been reported for the Pacific Islands (Yen and Wheeler 1968; Sakamoto et al. 1976; Kuruvilla and Singh 1981; Coates et al. 1988). Recent historical explanations for the occurrence of triploids in New Zealand and New Caledonia were suggested by Yen and Wheeler (1968) and are discussed in Chapter Fourteen. Recent introductions of taro from Asia to the Pacific have been widespread. McKnight and Obak (1960: 5–6) reported that a taro variety was introduced to Palau (Micronesia), directly from Japan, by Japanese in the present century, and that this variety was largely cultivated by non-Palauans at the time of writing (late 1950s). Whitney et al. (1939: 7) reported that Chinese immigrants commercialised taro production in Hawai'i on a large scale from the latter part of the nineteenth century. One variety (*Bun-long-woo*) from China and three from Japan (*Akado*, *Tsurunoko*, and *Miyako*) were described (Whitney et al. 1939: 9, 24). It is likely that triploids were included among these recent introductions to the Pacific.

As noted in Chapter Fourteen, because triploids are sterile, the wide geographical range of triploid taro within Asia must be the result of dispersal by humans, and dispersal

by humans was probably largely an agricultural phenomenon. Before discussing the general absence of triploid taro in the Pacific Islands, explanations for the origins and prevalence of triploids in Asia are considered.

Within Asia, triploid cultivars are common in temperate Japan (Sakamoto et al. 1976: 44; Kawahara 1978) at high altitudes in Nepal (Kawahara 1978) and in the northeastern hills of Meghalaya, India (Kuruvilla and Singh 1981). Kawahara (1978) suggested that diploid and triploid taro may differ in their physiological responses to high altitude or high latitude, and Kuruvilla and Singh (1981) suggested that triploids evolved in response to the climate of the Meghalaya hills. Because of their sterility, evolution by triploids would be restricted to somatic mutation within clonal lineages. Adaptation to high altitude or temperate (high latitude) climates may have required prior breeding and natural selection among wild diploid populations. Cultivated diploids are not completely absent from Nepal, the Meghalaya hills or temperate Japan. If wild diploids were also present in any of these areas, and did reproduce by sexual means, then triploids could have been created that were adapted to higher altitudes and temperate climates. Such triploids, even if very rare in their original occurrence, could have become common in many areas of similar climate in the hands of agriculturalists. Triploidy, in itself, may have conferred some advantage for cultivation at high altitudes or latitudes, and no particular advantage in tropical areas.

The evolutionary response to climate suggested by Kuruvilla and Singh (1981), noted above, can also be read as implying that triploids originated because of conditions in the Meghalaya hills. If climates in hill areas of northeastern India are marginal for sexual reproduction, then environmental stresses may have induced irregularities in gamete production and the subsequent production of triploids. Marginal conditions for sexual reproduction might exist in many areas. In Japan, it appears that taro does not reproduce sexually because of the low autumn temperatures (Hirai et al. 1989), but conditions may be marginal for sexual reproduction in the warmer Ryukyu Islands and in the hill areas of southern China. Triploids may be common in marginal areas not because of any particular advantage for cultivation, but because they are more likely to be created there.[7)]

15.2.4 Absence of triploids in the Pacific

Several explanations can be suggested for the apparent absence of triploid taro in the Pacific before the recent historical period: (1) no taro were introduced by humans, from Asia to the Pacific; (2) few triploids were introduced because few taro, overall, were introduced; (3) triploids were not introduced because human dispersal of taro from Asia occurred before triploids became common in Asia; (4) the source areas within Asia for introductions to the Pacific were areas that lacked triploids; (5) triploids were not created in the Pacific; and (6) triploids did arise in the Pacific in the wild, but did not enter into cultivations to become sufficiently common for detection in surveys of chromosome numbers.

Yen and Wheeler (1968: 265) argued that the common occurrence of both diploids and triploids in Asia, and the lack of triploids in the Pacific, might reflect early dispersal

of diploids from Asia to the Pacific, before triploids became common in Asia (see (3), above). The earlier dispersal of diploids from Asia into the Pacific was regarded as being associated with agriculture (Yen and Wheeler 1968: 263), and it was thus implied that cultivated triploids became common in Asia relatively recently. This is consistent with the argument (previous section, above) that the wide geographical range of triploids was the result of agricultural dispersal. Subsequently, Coates et al. (1988) suggested that diploid taro first arrived in the Pacific as part of the natural eastward extension of the Indo-Malaysian flora into Sahul (after the early Miocene). This implies that the initial Pacific appearance of diploid taro was prehuman, and thus preagricultural. The likely pre-agricultural and possibly prehuman introduction of taro into the Pacific was discussed in the first part of this chapter. If it is accepted that the triploid taro group has multiple origins, and that diploids are indigenous in both Asia and the Pacific, then other explanations can be given for the absence of triploids in the Pacific, in addition to the possibility that triploids were of recent origin in Asia. In view of these two considerations, it is necessary to examine the above arguments (1) to (6) more closely.

No taro were introduced

Taro may be indigenous to Australia and New Guinea, so it cannot be assumed that all cultivated diploids were introduced from Asia. It remains to be seen whether or not Pacific cultivars are more closely related to wild taro in New Guinea than wild taro in Asia.

Few taro, overall, were introduced

If few taro were introduced from Asia during prehistory, then the statistical chance of triploids not being included may have been significant, even if triploids were present in the source areas. The great phenotypic diversity of diploid cultivars in the Pacific does not exclude the possibility that few introductions were made. Even a very few early introductions could have generated many new, diverse varieties in the Pacific by breeding in feral populations and by crossbreeding with wild populations of pre-agricultural origin. The overall rate of introduction from Asia may have never been very great. If introductions from Asia were made only very recently, within the agricultural period of Pacific prehistory, then there might have been too little time for Asian diploids and triploids to spread in the Pacific. Most diploids cultivated in the Pacific Islands could have originated in New Guinea, either directly or through subsequent breeding in feral populations beyond New Guinea.

Dispersal from Asia occurred before triploids became common in Asia

This argument, by Yen and Wheeler (1968), is noted above.

If the occurrence of triploids is time dependent, and results from genetic aberrations independently of environment or genotype, then most triploids were probably created during the long period of natural evolution in Asia, before human utilisation of the species. The possible environmental induction of triploids was noted above. Here, consideration is given to two further possibilities: firstly, the creation of triploids might

have been dependent on the existence of particular diploid genotypes, and secondly, the generation of suitable diploid genotypes may have been promoted by human actions.

For many polyploid crop species, polyploidy appears to have been a consequence of hybridisation between different species (Simmonds 1979: 284). The genomes of two different diploid species can be represented as AA and BB. The hybrid genotype, AB, produced by two such species, may well be highly infertile if there is insufficient homology between the A and B genomes for proper pairing at meiosis. Often, instead of unbalanced haploid meiotic products, a very small but significant percentage of unreduced gametes (AB) may be produced (Briggs and Walters 1984: 181). Rare unreduced gametes would usually encounter normal haploid gametes (A or B) after self- or cross-pollination, resulting in triploid plants (ABB or AAB).

The rDNA analyses described in Chapter Thirteen indicated that *C. esculenta* is genotypically homogeneous, and may therefore represent one biological species. It nevertheless remains quite possible that the genomes of some taro varieties are sufficiently different from each other for there to be some degree of infertility, and irregularity in cell division, among the allo-diploid progeny of cross-matings. An acceleration in the dispersal of taro varieties may have started when humans first began to use taro, perhaps before agriculture developed and certainly afterwards. If the varieties carried by humans, within and beyond the natural geographical range of taro, were brought into contact with very different varieties, then the generation of allo-diploids and allo-triploids may also have accelerated.

This argument suggests that most triploid taro are allo-triploids, and this is consistent with the complex rDNA patterns noted for triploids in Chapter Fourteen (Figures 14.8 and 14.9). Although homozygous, non-alloploid plants can display complex rDNA patterns, as discussed in Chapter Fourteen, mixtures of different rDNA variants and complex rDNA patterns are, in general, likely to reflect alloploidy. Coates et al. (1988) present cytological evidence for the existence of auto-alloploid (heterozygous) and autoploid (homozygous) triploid lineages.

During the pre-agricultural and agricultural periods, the absolute numbers of sexually reproducing taro probably increased dramatically following human dispersal of wild and cultivated taro and the establishment of feral populations in the vicinity of cultivations. Even without genetic mixing effects, increased absolute numbers of wild taro may have increased the overall rate of triploid production. At the same time, presumably, agricultural traditions of variety selection, trial, and propagation became widespread, providing opportunities for sterile triploids to enter into cultivation and the dispersal routes of cultivars.

The processes suggested above are equally plausible for Asia and the Pacific. The particular genotypes required to produce triploids may have arisen only in Asia, or other factors may have been limiting for the occurrence of triploids in the Pacific. The suggestion of causal relationships between human actions (dispersal and propagation of taro) and triploidy provides, for the first time, an explanation for the possible recent production of triploids within the agricultural period. Most triploid varieties adopted by cultivators in Asia may have been derived partially or entirely from feral diploid

cultivars.

If triploids in Asia came into existence recently, sometime within the agricultural period, then diploid varieties of Pacific origin could have been transported to Asia at earlier times (probably also within the agricultural period), and could have been the progenitors of triploids in Asia. This could explain the occurrence of what appears to be the Queensland rDNA variant in Japanese taro (see Chapter Fourteen), if it did not originate early in the evolution of the species in Asia.

The source areas in Asia lacked triploid taro

All or most triploids could have been located outside the source areas for introductions to the Pacific. Although triploids have been found throughout tropical Asia, in the Ryukyu Islands, Taiwan, India, Philippines, Borneo, and Timor (Yen and Wheeler 1968; Sakamoto et al. 1976; Kuruvilla and Singh 1981), the reports suggest that triploids are abundant in areas of high altitude and high latitude and less abundant in other areas. If evidence is found for the promotion of triploid production in climates that are marginal for sexual reproduction, then this would support the suggestion that triploids were not evenly distributed in Asia in prehistory[7].

Triploids were not created in the Pacific

Triploids may not have been created in the Pacific because suitable diploid progenitors were absent. This suggestion is contradicted by the observation (Coates et al. 1988) that one lineage of triploid taro (I-13 cytotype) is possibly derived from the diploid cytotype I-1, recorded for two wild plants from Australia and two cultivars from Papua New Guinea. The I-13 cytotype was discovered in the New Zealand triploid variety GP (Yen, pers. comm. 1988), a variety which is acrid, produces little starch, is stoloniferous, and lacks anthocyanin pigmentation (Matthews 1984). Phenotypically, this triploid is rather similar to the wild diploid taro recorded for Australia and New Guinea, which is consistent with the suggestion of a Pacific origin. Nevertheless, it remains possible that the wild diploid progenitors of var. GP were located in Asia, an area for which wild taro remain poorly known.

Triploids originating in the Pacific have not been detected

If triploids produced in the wild (via sexual reproduction) lacked the qualities desired by cultivators, then they are not likely to be detected in general surveys of cultivated taro. In the wild, they are likely to be rare with very localised distributions. Few wild plants have been tested in surveys published to date, so it is not surprising that few wild triploids have been encountered.

Notes

1) Haberle (1995) reported various distinguishing characteristics of taro pollen, and then found taro pollen in lake sediments dating from 9,000 to 6,000 years BP approximately, at Lake Euramoo in northeastern Queensland (Haberle 2005). This period corresponded to a period of

arrival and expansion of many rainforest taxa in the lake vicinity.

2) The genus name is hereafter given as *Colocasiomyia*, following Sultana et al. (2006) and authors cited therein.
3) Hunt et al. (2013) have since reported the presence of *Colocasiomyia* in Queensland, based on identification of an empty pupal cocoon collected in 1987. Further study is needed to identify the species present and determine its host range in Australia.
4) This and further finds of *Tarophagus* in Australia were reported by Matthews (2003).
5) Comparison of the biochemical defences of wild and cultivated taro remains a very promising area of study for understanding the domestication history of taro (Matthews 2010). The heat stability of taro trypsin inhibitor during cooking has been studied (Bradbury et al. 1992). Also of special interest are the heat resistance of lectins in taro corms (Seo et al. 1989), the cyanide content of the leaves and stems of edible aroids (Bradbury et al. 1995), and the acridity associated with calcium oxalate raphides (Bradbury and Nixon 1998).
6) This route of polyploid formation was later confirmed experimentally by Otsuka et al. (1995) and Isshiki et al. (1995).
7) Zhang and Zhang (1990, 2000) studied the distribution and abaundance of diploid and triploid taros in China, and found them to be more abundant at higher altitudes and higher latitudes. They attributed this to greater hardiness, and noted that the process of polyplodization must have been important for the (subsequent) spread of taro into new areas (under cultivation), namely: Yangtze River area, central and north China. They also noted the widespread occurrence of wild taros in southern China, and suggested that triploids may have arisen in south and central China druing a warmer climate period in that region (between 4000 and 1000 BC).

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On the Trail of Taro: An Exploration of Natural and Cultural History
Peter J. Matthews

Chapter Sixteen
Origins, Dispersal, and Domestication

In Chapter Fourteen, field surveys and ribosomal DNA analyses were discussed, and possible directions for future genotypic analysis suggested. In Chapter Fifteen, further sources of biological information were described, and possible approaches for investigating the origins and antiquity of diploid taro in Australia and New Guinea were summarised. Particular attention was also given to the origins of triploid taro. Here, in Chapter Sixteen, I conclude the thesis by taking a broad view of the origins, dispersal, and domestication of taro.

16.1 Taro as a Highly Dispersive, Colonising Species

In contrast to cultivated soybean, which, like taro, is recognised as one taxonomic species, taro has been shown to possess a range of ribosomal DNA variants. Cultivated taro therefore does not appear to have originated and spread from a single homogeneous wild population or race, as may have occurred with soybean. Extremely heterogeneous natural populations of wild taro might exist where the species originated, in mainland Asia. If not, then the variability of cultivated taro probably reflects some degree of prior differentiation among wild taro populations outside the area of species origin. It is unlikely that rDNA variation arose *only* within the agricultural period of prehistory. The available evidence, from all sources, suggests that wild taro in Australia and New Guinea may have arrived before agriculture began in New Guinea, but whether or not the species arrived very early, before human colonisation in the late Pleistocene, is not yet known.

With its capacities for both vegetative and sexual reproduction, each affording alternative means of survival and dispersal, taro appears to be a highly dispersive, colonising species. If taro was able to spread naturally from mainland Asia to Sahul, then its natural range in mainland Asia might also be very wide, perhaps from peninsular India to eastern China. Indigenous selection and domestication of taro could have occurred in New Guinea, and over a broad geographical range in Asia. Without denying social reasons for the absence of intensive agriculture (e.g., Chase 1989), the genotypic and phenotypic homogeneity of taro locally (see Chapter 14) may have acted against its domestication in Australia.

The following discussion of origins, dispersal, and domestication focuses on the starch-producing capacity of taro, a trait directly subjected to human selection during domestication, and the evidence from rDNA analysis for the differentiation of diploid populations in different geographical areas. Focus on a phenotypic character directly subject to selection by humans is required in order to explain the dispersal of taro by

humans. Although variation in rDNA may affect phenotypic variation in characters important to humans, rDNA itself has never been the immediate object of selection through human preference or intention. The phenotypic effects of rDNA variation, and intergenic spacer variation in particular, are currently under investigation (e.g., Cluster et al. 1987) but are beyond the scope of the present discussion. Ribosomal DNA has significance here simply as an indicator for the genetic differentiation or otherwise of wild taro, and as an indicator of sources for taro introduced to the Pacific islands, beyond the possible natural range of the species.

16.2 Natural Variation, Geographical Range, and Prerequisites for Cultivation

Members of *Colocasia* Section Tuberosae Engl. (*C. esculenta*, *C. fallax*, and *C. affinis*) may have evolved naturally from their most recent common ancestor within the region of northeastern India, southwestern China, northern Thailand, and Burma. *C. gigantea*, in Section Caulescentes Engl., may have originated south or east of the other species, since its extant distribution is mostly south and east of *C. fallax* and *C. affinis*. If the fruit and seed of *C. esculenta* were readily dispersed by birds and small mammals, then this species may have spread naturally through coastal regions of mainland and island Southeast Asia, as far east as Australia and New Guinea. Wild varieties and probably races evolved in partial geographic isolation from each other, and in diverse ecological circumstances. The distributions of different rDNA genotypes in the wild taro of Australia and New Guinea suggest such differentiation (Chapters 6 and 7). Natural differentiation would have created different conditions for domestication and other forms of plant-human interaction in different regions.

C. affinis, *C. fallax*, *C. gigantea*, *C. esculenta*, *C. esculenta* var. *fontanesii*, *C.* sp. and wild taro common in Australia and Papua New Guinea share the stoloniferous growth habit (Figures 10.1 to 10.6) and it is proposed here that this habit is a primitive condition for the genus. The stoloniferous wild taro varieties of New Guinea and northern Australia, with very little starch compared to modern cultivars, could be similar to the earliest natural forms of *C. esculenta*. The other *Colocasia* species noted above also produce very little starch.

Was starch production the only prerequisite for initial cultivation of taro by humans? Other traits, such as medicinal properties or edible leaves, may have enhanced the usefulness of taro, or may have been sufficient in their own right. For example, if plants were initially cultivated for their leaves, then small yields of starch from corms might have attracted attention as a secondary product, leading eventually to the selection of varieties with preferred starch qualities. Alternatively, wild taro could have been managed in the wild for multiple purposes, including the provision of starch, before cultivation began. Cultivation is defined here as the creation of an artificial environment specifically for the propagation of the desired plants. If the first selections from the wild became the sole basis for subsequent vegetative propagation in cultivations, then little new genotypic variation would have been available for selecting increased starchiness effectively,

whatever the initial quality of starch production by the selected wild varieties. Cycles of breeding (sexual reproduction) and selection would have been necessary for humans to promote starchiness effectively.

If early forms of agriculture resembled modern swiddening systems, then there were probably ample opportunities for cycles of breeding and selection. Swidden systems readily generate feral populations when gardens are not completely harvested before being left to fallow, because unrestrained breeding occurs in fallow areas. Actively cultivated gardens may have been major sources of pollen for nearby feral populations, and for any nearby natural wild populations, if suitable pollen vectors were present. The likely pollinating insects for taro in Papua New Guinea were discussed in Chapter Fifteen.

Occasional harvests and replanting of feral and other wild varieties, or protection of useful wild varieties when old and new garden areas were cleared, would have brought sexual progeny with new genotypes (the products of segregation and reassortment) into assemblages of vegetatively maintained cultivars. In this way, swiddening systems probably provided ideal circumstances for breeding in the history of vegetatively propagated crops such as taro. Varieties producing more starch could have originated within an agricultural context, even if the species was first cultivated for reasons other than starch production.

Explanations for the evolution of starchiness in nature lead to suggestions about where wild varieties with desirable starch qualities might have existed before humans began using taro. In the literature on the evolution of starchy root, tuber, and corm crops (each differing according to the anatomical region in which starch is deposited, but collectively known as root crops), starch storage has been explained as a common and natural evolutionary response by diverse taxa to either seasonally dry and/or seasonally cold climates.

In a discussion of South American root crops, Hawkes (1989: 482) suggests that tropical root crops originated not in the equatorial, Amazonian rainforests, where there is no strong selection for the development of underground organs, but in areas north and south of the equator, where there are well-marked dry seasons. Sauer (1952: 40, cited by Hawkes 1989: 482) states that underground starchy reserves help plants to survive the dry season and to regenerate quickly when rains return. Hotta (1983) suggests that root crops in eastern Asia developed where species hibernated naturally in response to either seasonal dry periods or seasonal cold periods. Starchy cultivated varieties of taro, from the tropical region of Southeast Asia and the Pacific, including the southern islands of Japan, are intolerant of cold and are morphologically distinct from cold-tolerant varieties of temperate eastern Asia (Hotta 1983). Hotta suggests that there are several separate genealogies of cultivated taro, originating among diverse starchy wild types within a natural distribution range limited to mainland Southeast Asia (Figure 16.1).

The significance of human dispersal of taro in the temporally unlimited pre-agricultural period remains completely unknown. We cannot yet assess, in terms of genetic variation and geographical distribution, the role of early humans in establishing suitable circumstances for domesticating taro. Under the hypothesis of domestication over

a broad geographical range, a restricted natural geographical range remains as strong a theoretical possibility as a wide natural geographical range. A wide geographical range could have been the result of dispersal by humans before agriculture began, and there may have been time subsequently for differentiation of the wild populations from which cultivated taro were derived. The following discussion focuses on the possibility of wide natural dispersal.

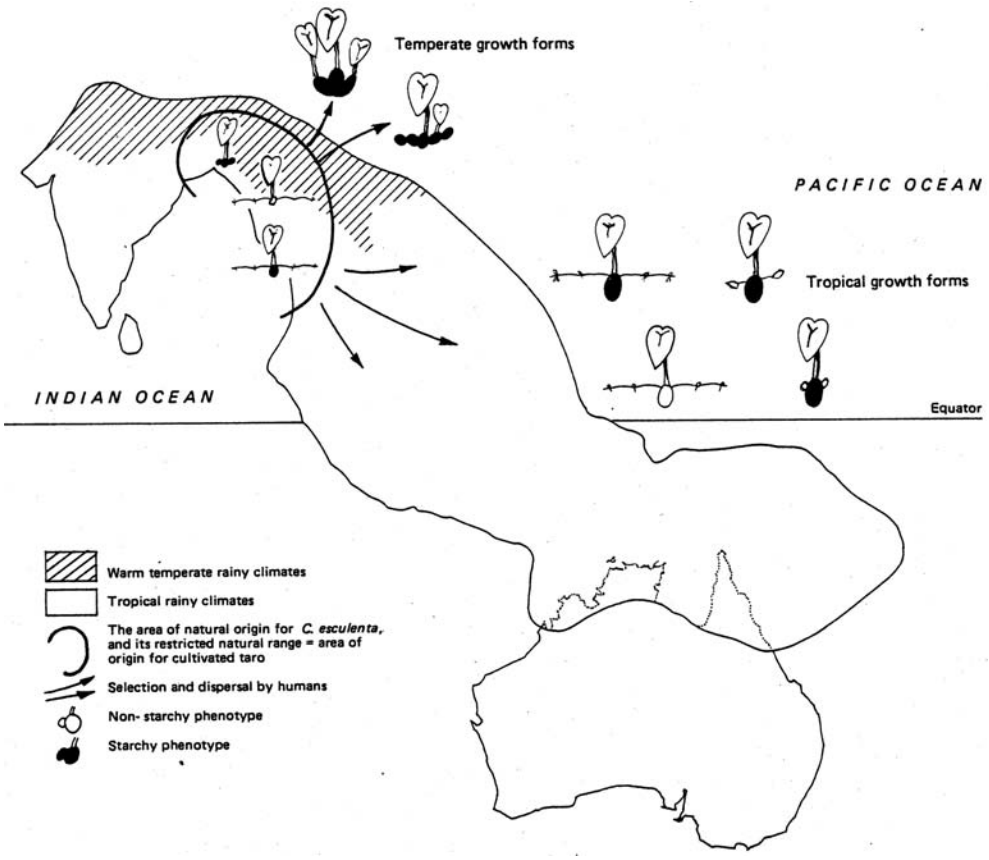

Figure 16.1 The possible restricted natural range of taro and a scheme depicting a single area of origin for cultivated taro. According to this scheme, the selection and dispersal of taro by humans began with starchy wild-types that evolved and dispersed naturally within a range restricted to mainland Southeast Asia. The theoretically possible natural range is also the likely area for the natural origin of the species. The large area outlined, encompassing India, northern Australia, and the area between, is the maximum theoretically possible range (see Figure 16.2).

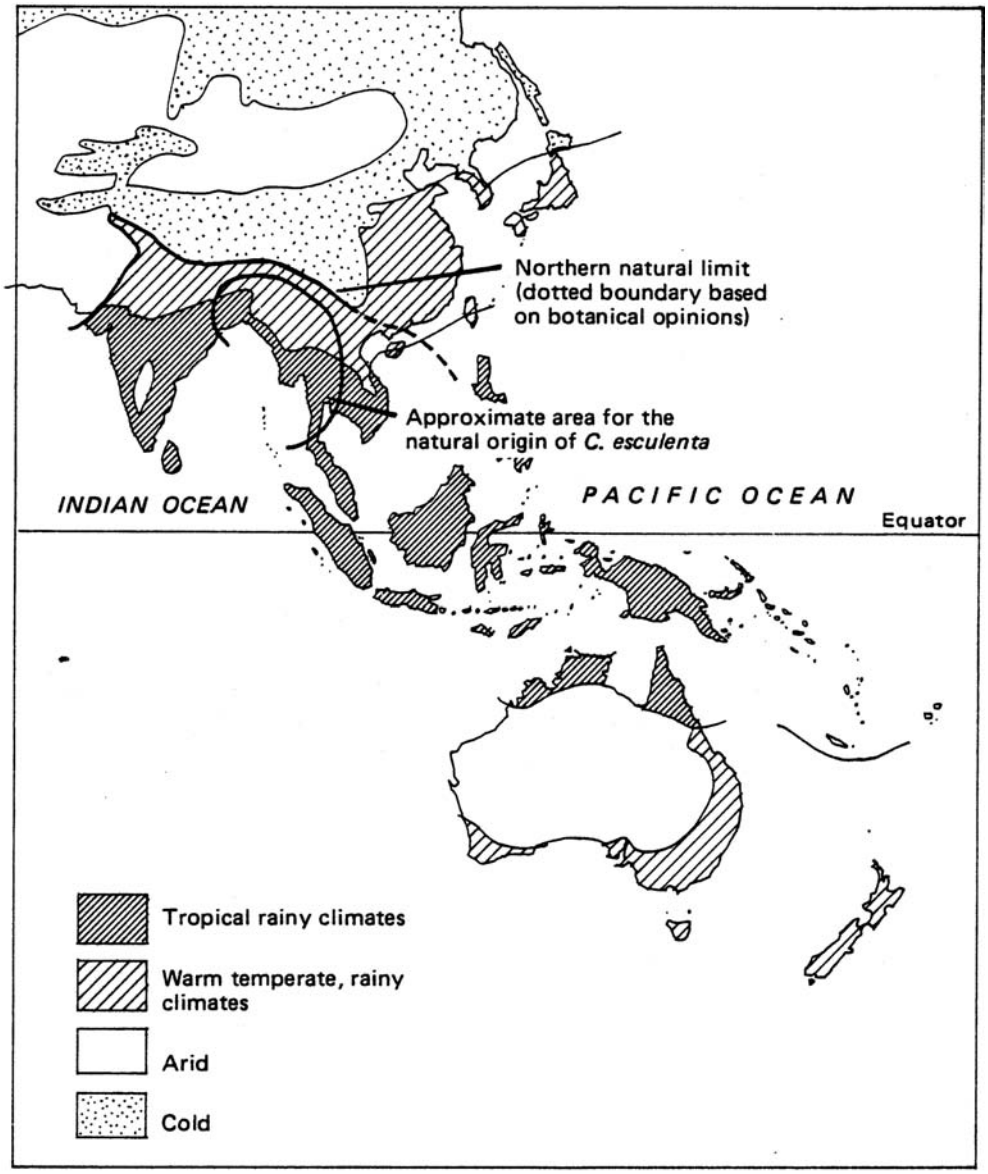

Figure 16.2 Climatic zones in Asia and the western Pacific. The origin of *C. esculenta* as a natural species is probably somewhere within the partly circled area. The maximum natural range considered possible, in theory, is limited by the Indian and Pacific oceans, by extreme cold and aridity in India and China, and by aridity in Australia. In eastern China, the natural range of taro may have reached its maximum extent within the warm temperate zone. Map adapted from Poynter and Atkinson (1984). Within the tropical rainy climate zone, seasonal shifts in surface winds produce the northern (summer) and southern (winter) monsoons, and bring heavy rainfall.

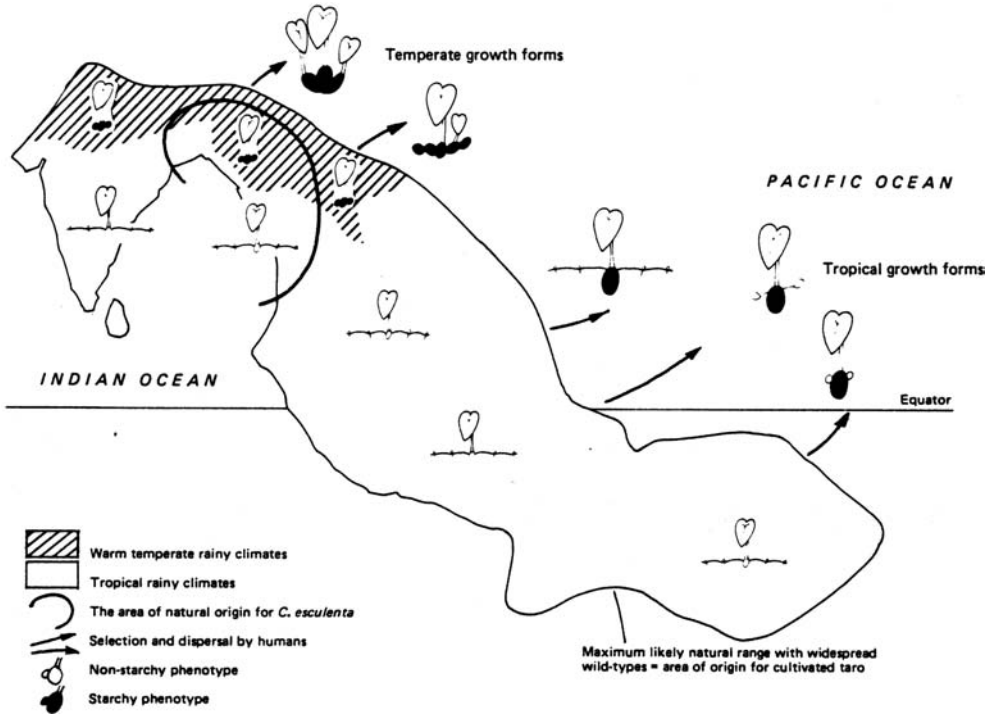

Figure 16.3 The maximum possible natural range for taro in Asia and the Pacific, and a scheme depicting the possible multiple geographical origins of cultivated taro. According to this scheme, the selection and dispersal of taro by humans began with non-starchy and starchy wild-types of wide natural occurrence, in the tropical and warm temperate zones respectively. The likely area for the natural origin of the species is also shown. The natural range outlined is based on the map shown in Figure 16.2.

16.3 The Maximum Possible Natural Geographical Range

The present geographical distribution of cultivated taro follows two major geographical and climatic axes (Figure 16.2). On a west to east axis, from India to Sahul, tropical rainy climates are found. These include perhumid climates in which wet tropical rainforests predominate, close to the equator, and monsoon climates with progressively longer seasonal dry periods, further north and south of the equator (Whitmore 1984: 55). In northern Australia, the summer monsoon rains follow a cooler dry period of about nine months.

On a south to north axis, from southern India and Southeast Asia to northern India and northern China, climates range from the tropical rainy types just described, through temperate rainy climates, to cold temperate rainy climates beyond the northern limit of cultivated taro.

Four major barriers define the maximum possible natural range of taro (Figure 16.3)

on the two geographical and climatic axes described above: (1) dry steppe and desert climates of northwestern India and central Australia; (2) the perpetually cold, high altitudes of the Himalayan mountains; (3) cold temperate climates of far western and northern China; and (4) the Indian and Pacific Oceans. Hay (1986: 3) notes that the diversity of the Pacific aroid flora (the botanical family of *Colocasia*) tails off eastwards very sharply at New Guinea, and that New Caledonia is devoid of endemic aroid genera, with only one, dubious, endemic species (*Rhaphidophora* sp.). The natural range of taro may extend as far as New Guinea, or somewhere farther east. The Malesian floristic region, in which many aroids occur, extends eastward as far as the Bismarck Archipelago, a limit that Whitmore (1984: 5) defines arbitrarily because no major forest-type boundary occurs between New Guinea and the Pacific islands. East of the Solomons, ocean gaps are almost certainly major barriers for natural dispersal by taro.

An absence of botanical records of taro in northern China (Li 1979), and the general belief that Japanese taro originated in southern China (see Chapter Fourteen), suggest that the northernmost natural limit for taro in China is within the temperate region (Figure 16.2). No wild taro, apart from those resulting from recent introduction, are known in the arid or temperate zones of Australia (see Chapter Ten, Figure 10.11), despite a history of extensive botanical exploration in Australia, and the possible southernmost natural extent of taro seems relatively well defined.

Vegetative growth habits and starch storage traits that might have evolved within the natural range, before human utilisation of taro, now become the subject of attention. The general relationships between latitude, longitude, and climate are assumed, for simplicity, to be broadly similar to those observed today, since the origins of the species in geological time and space are not known exactly.

16.4 Tropical Growth Forms

If the natural primitive form of *C. esculenta* possessed stolons and minimal starch reservoirs (as discussed above), then such a form could have arisen within the monsoonal region between India and Malaysia. In northern Australia, where a strongly seasonal dry/wet monsoonal climate prevails, the stoloniferous habit is undoubtedly important for survival. The stolons of wild taro can extend at least two metres, and probably more, with nodes from which new shoots grow and produce further stolons (personal observations in far northern Queensland). Stolons are water-seeking generative organs, and as such are an alternative to starch storage and dormancy for survival in monsoonal regions.

Among varieties of *C. esculenta*, stolons and side-corms are the major alternative forms of vegetative reproduction and dispersal, both forms of lateral shoot being readily dislodged and dispersed by water (a general field observation by the author). Stoloniferous wild plants also survive well in the wet tropical rainforests of northeastern Queensland and in Papua New Guinea, so it cannot be assumed that the stoloniferous habit evolved in response to monsoonal climate dry periods. However, if the stoloniferous habit is the primitive state for *C. esculenta*, then its retention may have lessened or

removed the need for starch storage as a response to seasonal aridity. Both rainforest and monsoonal forest regions could have accommodated the natural spread of taro, assuming that sufficient permanent water sources were available in monsoonal areas.

Stoloniferous wild taro synonymous with *C. esculenta* var. *aquatilis* (Hassk.) Kitamura (Hotta 1970) are known in Bengal, Malaysia, the Ryukyu Islands, and Java (Engler and Krause 1920; Hotta 1970), and it is proposed here that the wild stoloniferous taro of Australia and Papua New Guinea also belong to this varietal group. Haines (1924) presents the type description for *C. antiquorum* Schott var. *stolonifera* Haines, which he regarded as similar to the Javan var. *aquatilis* Hassk. The variety described by Haines was found wild and flowering along rivers near Ranchi and Palaman, Bihar Province, eastern India, in 1918 (H. H. Haines, Kew Herbarium sheets 4381, 4382), and is characterised as having green leaves and numerous long stolons, up to three metres in length. Mitra (1958) reports that *C. antiquorum* var. *nymphaeifolium* (Vent.) Engl. (syn. *C. esculenta* var. *aquatilis* [Hassk.] Kitamura in Hotta 1970) is wild throughout eastern India and Bengal, and it seems likely that var. *stolonifera* Haines, is an Indian form of var. *aquatilis* (Hassk.) Kitamura.

Although some occurrences of *C. esculenta* var. *aquatilis* may be primitive cultivars or represent naturalised (feral) lineages (Hotta 1970), var. *aquatilis* appears to be a widespread natural wild-type of the tropical region.

16.5 Temperate Growth Forms

If the non-starchy, stoloniferous habit is the primitive condition of *C. esculenta*, then starch deposition and side-corm formation are presumably derived (modern) traits, whether of natural origin or the outcome of domestication. At higher altitudes, growth is limited more by low temperatures and shorter day lengths than by water shortage, and stoloniferous varieties may have spread less successfully there than varieties with seasonally dormant starchy corms and side-corms. Winter dormancy improves survival in cold temperatures by corms and side-corms, whether in the field or as a stored harvest, and starch deposition provides shoots with reserves for renewed growth in spring. In mainland Asia, where *C. esculenta* is thought to have originated as a species, tropical rainy climates and warm temperate climates merge over short latitudinal and altitudinal distances (Figure 16.2). In this area, the differentiation and spread of different varieties under different climatic regimes could have begun early in the history of the species. The distribution of possibly natural wild taro does not extend to temperate southern Australia, and there is no reason to propose the differentiation of temperate adapted forms at this geographical extreme.

Hotta (1970) grouped all cultivars under the name *C. esculenta* var. *esculenta*. Within this variety diverse cultivar groups are differentiated according to chromosome number and morphology. *C. esculenta*. var. *esculenta*, as described by Hotta (1970), is essentially a broad convenience category for most cultivated taro. Diverse cultivar groups were also described by Whitney et al. (1939) for Hawai'ian cultivars; Kitamura (1949), Hotta (1970), and Hirai et al. (1989), for Japanese cultivars; and Ghani (1984) for

Malaysian cultivars. The classifications embrace starchy phenotypes that could have originated in either temperate or tropical regions, as natural wild-types or as products of domestication. Starchy and temperate adapted natural wild-types have yet to be identified in the field, and may be difficult to find since human activities could have resulted, directly or indirectly, in the loss of most natural populations of Asian taro.[1)]

16.6 The Multiple Origins of Cultivated Taro

It is proposed here that indigenous selection and domestication of taro occurred over a wide geographical range and involved genotypically and phenotypically diverse natural wild varieties. This proposition is illustrated in Figure 16.3 and discussed below.

16.6.1 Climatic and geographical extremes

At the temperate geographical extreme, starchy varieties evolved naturally in response to seasonal periods of low temperature and shorter day length. Subsequent selection and dispersal within traditional agricultural systems, which fostered genetic interactions between cultivated and wild populations, promoted fast summer growth, survival of winter storage, and improved starch production (quantity and quality).

At the tropical extreme, there is not yet any direct evidence for taro that starchy wild-types evolved in response to seasonal dry periods. Rather, there is a widespread stoloniferous wild-type, *C. esculenta* var. *aquatilis*, which in Australia and Papua New Guinea produces small acrid corms with very little starch. Where local wild populations of this variety expressed genotype-dependent variation in starch production, selection by humans in the wild and within cultivations could have transformed the relatively non-starchy wild-type into the cold-sensitive and starchy, stoloniferous, short-stoloned, and non-stoloniferous varieties now common in tropical areas (for example, the Mikashiki and Oyaimo cultivar groups described by Hotta 1983).

In the survey of rDNA variation, triploid cultivars from Japan and diploid wild varieties from Australia and Papua New Guinea represent opposing extremes in terms of geographical location, climate, and habitat. Contrasts established by these samples are discussed below.

16.6.2 Temperate Asia

Although many triploid taro varieties may have arisen through sexual processes in natural wild populations, the wide distribution of triploids in Asia largely reflects human agricultural activities (see discussion, Chapter Fifteen). The inherent sterility of triploids surely prevented them from having any major role in natural pre-agricultural evolution of the species. Among triploids, variations in rDNA (Chapter Fourteen), chromosome morphology (Coates et al. 1989), and phenotypes all point to the origins of triploids among diverse diploid varieties. The progenitors of triploids in temperate Japan and China were presumably temperate-adapted and starchy wild diploids, since genotypic change among triploid cultivars was limited to vegetative mutation. The agriculturally favoured qualities found among the diploid progenitors probably arose through a

combination of prior natural evolution and subsequent selection within agricultural systems. The starchy and temperate adapted natural wild-types predicted here (Figure 16.3) have yet to be identified in the field, and might be difficult to find since human activities could have resulted, directly or indirectly, in the loss of natural populations of Asian taro[1].

16.6.3 Tropical Pacific (Australia and New Guinea)

The absence of intensive taro cultivation in Australia is undoubtedly related to cultural factors, since contacts between the northern inhabitants and agricultural outsiders like the Macassans, if only within the past 400 years (MacKnight 1986), and the Torres Strait Islanders (Harris 1977; Barham and Harris 1983), for a certainly much longer time period, did not result in the transfer of agricultural techniques.

Despite the exploitation in northern Australia of many plant taxa used in agriculture in southeastern Asia, all Aboriginal groups routinely pursued hunter-gatherer modes of subsistence (Golson 1971b; Chase 1989: 51). Chase suggested that with the development of highly complex and deeply integrated religious beliefs and practices, societies such as those in Aboriginal Australia may have passed a critical threshold for receptivity to the agricultural practices of neighbours or visitors. In the Torres Strait, north of the islands of the Prince of Wales group, yams appear to have been the principle root crop, with sweet potato, taro, banana, and sugarcane also raised as staples (Harris 1977: 442). Taro is not recorded as a traditional crop for the islands closer to Cape York, where horticulture was less firmly established (Harris 1977: 442; Tucker pers. comm. 1987). Further southwards, in the Lockhart area of eastern Cape York, wild taro is regarded locally as native, and until recently was harvested as a staple food (Harris 1977: 433; R. Tucker pers. comm. 1987) (see Appendix 18).

From the above, it is clear that Australian wild taros were geographically isolated, and therefore genetically isolated, from agricultural populations of taro. Ribosomal DNA in Australian wild taro is homogeneous within sites and within regions, and is only known to vary between regions (Chapter Fourteen). Genotypic homogeneity on a local scale may have prevented effective selection by humans for improved starch production. Restricted dispersal of taro varieties within Australia, whether by natural means or by humans, and isolation from potential genetic sources outside Australia, may have preserved the genotypic integrity of wild taro populations that were unsuitable for domestication. Taro were possibly not domesticated in Australia for three reasons: cultural barriers to adoption, relative remoteness from potential sources, and the lack of suitable genotypes among the wild plants available locally within different regions of northern Australia.

Domestication is a synergistic process, in which human selectivity and environmental modification by humans (e.g., cultivation) are combined over time. This combination can lead to dramatic changes in plant productivity that could not arise from selection entirely in the wild, or simply by environmental modification without selective propagation from a range of genotypic variants. A detailed characterisation of what is a domesticated plant, the ‘domestication syndrome’ (Hanelt 1986), cannot be applied to all the cultivated taxa

commonly regarded as domesticated. Hanelt (1986) therefore defines the domestication syndrome as the crop-specific combination of characters which evolved in response to natural and artificial selection, under growing conditions essentially shaped by human activities. It is in this sense that cultivated starchy taro varieties are regarded here as domesticates.

If future genetic investigations define the genotypic basis for traits favoured during the domestication of taro outside Australia, it should be possible to determine whether or not the genotypic basis existed in Australia for similar domestication processes. If such a basis *did* exist, and wild populations do prove to be of sufficient antiquity to have allowed domestication, then purely cultural and social explanations for the lack of domestication could be advanced.

According to the general model proposed in Figure 16.3, taro was domesticated in New Guinea. Although the wild taro located in Morobe Province during the present study are phenotypically similar to the Australian wild taro, rDNA analysis proved them to be different genotypically. Other genotypic variants of stoloniferous wild taro probably occur in other, as yet unsurveyed, parts of New Guinea. If genotypic homogeneity partly explains why domestication did not occur in Australia, then genotypic heterogeneity may partly explain why domestication did occur in Papua New Guinea. Wild taro in parts of Papua New Guinea may possess greater local variability than has been observed in Australia. (cf. Hunt et al. 2013).

16.6.4 Bridging the temperate-tropical divide

The origin of the Queensland rDNA variant described in Chapter Fourteen is not known. It appears widespread among tropical cultivated and wild taro, from Southeast Asia to the eastern Pacific islands, and may be a distinguishing trait for taro indigenous to the tropical region (Chapter Fourteen). This proposition can be tested by future surveys of taro from temperate Asia (identification of the Queensland rDNA variant as a minor component is only tentative for triploid cultivars from temperate Japan, Chapter Fourteen). It remains to be seen whether or not any one rDNA variant is typical of temperate forms of taro.

Within Asia, between the temperate and tropical geographical extremes, genotypically mixed populations and varieties may have arisen during the natural and agricultural history of the species. To investigate effectively the proposed evolutionary division of *C. esculenta* into a temperate and a tropical group, and possible interactions between the two, it will be necessary to survey phylogenetically informative DNA sequences associated with different organelles (chloroplast, mitochondria, and nuclei) and different modes of genetic transmission.

16.7 Beyond the Natural Geographical Range

Beyond the northern limits of the natural range of taro, survival of the species depends on agriculture.[2)] Most new cultivars in northern regions originated as introductions from areas where sexual reproduction allowed genetic interactions between cultivated and wild

populations, and within wild populations. Some new varieties undoubtedly arose by vegetative mutation within cultivation, both within and beyond the natural geographical range. The stability of rDNA variants in clonal lineages (New Zealand rDNA survey, Chapter Eleven; *C. esculenta* var. *fontanesii*, Chapter Fourteen) and the stability of rDNA in Queensland over a long history of vegetative and sexual reproduction (Chapter Fourteen) are consistent with the general view that new plant varieties arise more readily by sexual reassortment among existing varieties than by new mutation in either vegetative or gamete-producing cell lineages.

On the tropical axis of distribution, sexually reproducing feral populations were easily established within and beyond the natural geographical range of taro, where water was sufficient. It is probable that new varieties were generated in the wild throughout the tropical Pacific.

If cultivated taro accompanied the Austronesian speakers who moved from Southeast Asia into the Pacific, then breeding between Asian introductions and indigenous Pacific taro probably has occurred in the western Pacific, where close contacts were made between speakers of Austronesian and speakers of other languages. The human interactions involved are currently under investigation by anthropologists and linguists (Pawley and Green 1985; Blust 1988; Ross 1988), archaeologists (Allen 1984; Gosden et al. 1989), and human biologists (Hill and Serjeantson 1989). Sorting out the genetic history of taro, and the associated human history, will be complicated if breeding between introduced and indigenous taro varieties was common, if cultivars came from parts of Asia where temperate and tropical gene pools were mixed, and if the movement of taro from the western Pacific was bidirectional, westward into Asia and eastward into the Pacific. Nevertheless, when cultivated and wild taro in Asia are better known, it might be possible to distinguish introduced and indigenous genetic lineages in the western Pacific, and the derivations of varieties introduced to the far oceanic islands of the Pacific.

Some indirect inferences regarding taro on the Pacific islands can be made from the observations of taro in Aotearoa (New Zealand). In Aotearoa, the historically recent disappearance of tropical Pacific (Polynesian) diploid cultivars was accompanied by an equally recent proliferation of introduced, cold-tolerant triploid varieties (see discussion in Chapters Eight, Eleven and Fourteen and Matthews 1985). Ribosomal DNA analysis and historical records indicate that the cultivated triploid varieties RR and GR have affinities with cultivated taro in temperate Asia (probably China, Chapter Fourteen). The rapid loss of traditional diploid varieties in Aotearoa suggests that introductions from the tropical Pacific islands, during prehistory, were cold-sensitive and only survived in cultivation. Cold-tolerant, diploid varieties in Aotearoa would have survived in the wild, as cold-tolerant triploid varieties do today, because special care would not have been required to maintain them. Assuming that the (now absent) diploid taro varieties in Aotearoa were typical of tropical Pacific varieties, then it appears that temperate-adapted Asian varieties did not reach (or did not survive in) the Pacific islands, before taro first reached Aotearoa.

During colonisation of the Pacific, migrants of Asian descent (the Austronesian speakers) may have acquired tropical, cold-sensitive diploid taro from the New Guinea or

nearby islands, and/or from the tropical region of Southeast Asia. This is consistent with interpretation of the Queensland rDNA variant (Chapter Fourteen) as a monophyletic variant, distributed throughout tropical Asia and the Pacific, and possibly absent in temperate Asia.

Notes

1) In China, 'loss of most natural populations' now seems less likely given the genetic and morphological diversity of wild taros reported there (Zhu et al. 2000), though it is still not clear which wild populations have natural origins, and which are derived from naturalised (feral) cultivars.
2) In particular, survival depends on the ability of farmers to keep planting materials alive despite cold winter temperatures. This is achieved through a variety of storage methods, and by trade between warmer and cooler locations (Matthews 2002).