Annals of Botany **89**: 483–489, 2002
doi:10.1093/aob/mcf047, available online at www.aob.oupjournals.org

Molecular Cytogenetic Analysis of *Podocarpus* and Comparison with Other Gymnosperm Species

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Received: 3 January 2001 Returned for revision: 24 February 2001 Accepted: 11 December 2001

DNA sequences have been mapped to the chromosomes of *Podocarpus* species from New Zealand and Australia by fluorescent *in situ* hybridization. Unlike other conifers, these species show only one pair of major sites of 45S rDNA genes, and two additional minor sites were seen in the Australian *P. lawrencei*. Unusually, 45S sequences collocalize to the same chromosomal region as the 5S rDNA. The telomere probe $(TTTAGGG)_n$ hybridizes to the ends of all chromosomes as well as to a large number of small sites distributed along the length of all chromosomes. Two other simple sequence repeats, $(AAC)_5$ and $(GATA)_4$, show a diffuse pattern of hybridization sites distributed along chromosomes. Southern blots using a variety of probes obtained from the reverse transcriptase of retroelements (*gypsy*, *copia* and LINE) from *P. totara*, *P. nivalis* and *Dacrycarpus dacrydioides* show that these retroelements are abundant and widespread in Podocarpaceae and also in others conifers. Some retroelements such as *copia* pPonty3 and *gypsy* pPot1li are more abundant in the genome of *Picea abies* and *Ginkgo biloba* than in the species from which they were amplified.

Key words: *In situ* hybridization, karyotypes, *Podocarpus*, rDNA, Pinaceae, retroelements, genome evolution.

INTRODUCTION

Modern gymnosperms are representatives of an ancient group of plants first recorded as fossils in the Upper Devonian (350 million years ago; Ma) (Biswas and Johri, 1997). They consist of two major groups, conifers and cycads. The monophyletic origin of the gymnosperms has been questioned (see Page, 1990) but recent molecular phylogenetic studies provide strong support for monophyly (Qiu *et al*., 1999; Soltis and Soltis, 2000). The gymnosperms are characterized by large genomes and relatively low chromosome numbers; polyploidy is rare (Khoshoo, 1959; Murray, 1998). Enigmatic features of the chromosomes of the gymnosperms include their characteristically large size and the remarkable constancy of number and karyotype amongst the species of most genera. Conspicuous examples of this are found, for example, in Pinaceae. Hizume (1988) has summarized much of the available information and has shown that 178 out of 180 species in nine genera have the chromosome number $2n = 24$. This uniformity must have been maintained for a very long period of time since in *Pinus* the same karyotype is found in different sections of the genus that were already present in the Cretaceous (90 Ma) (Stockey and Nishida, 1986). Support for the ancient nature of the sectional divisions of the genus also comes from the work of Krupkin *et al*. (1996) and Wang *et al*. (2000) using 'molecular clocks'.

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When more detailed studies are made using differential chromosome banding (MacPherson and Filion, 1981; Hizume *et al*., 1989, 1993, 1995; Davies *et al*., 1997) and *in situ* hybridization of repeat sequence DNA (Hizume *et al*., 1992; Brown *et al*., 1993; Doudrick *et al*., 1995; Lubaretz *et al*., 1996), a greater diversity of chromosome organization becomes apparent. Chromosome banding reveals that species differ in the number of banded chromosomes, size of the bands and their molecular composition (MacPherson and Filion, 1981; Hizume *et al*., 1989, 1993, 1995; Davies *et al*., 1997). Most of the *in situ* studies have investigated the distribution of ribosomal DNA (rDNA) genes. In *Pinus*, for example, the number of major sites of 45S rDNA ranges from ten pairs in *Pinus radiata* D. Don and *Pinus taeda* L. (Jacobs *et al*., 2000) to 12 pairs in *Pinus sylvestris* L. (Lubaretz *et al*., 1996). The 5S sites are less numerous but range in number from one to three pairs (Doudrick *et al*., 1995; Hizume *et al*., 1995) and, in relation to the 45S sites, may be either on different chromosomes (Hizume *et al*., 1995), on the same chromosome but on different arms (Doudrick *et al*., 1995) or closely adjacent (Lubaretz *et al*., 1996). Other highly repeated DNA sequences, such as retrotransposons, have been localized to the chromosomes of *Pinus elliottii* Engelm. (Doudrick *et al*., 1995; Kamm *et al*., 1996), and a satellite DNA family cloned from *Picea* species has been located on different subsets of chromosomes of *Picea glauca* (Moench) Voss and *Picea sitchensis* (Bong.) Carr. (Brown *et al*., 1998). The telomere repeat $(TTTAGGG)_n$ found in most angiosperms has been localized at the ends of gymnosperm chromo-

TABLE 1. *List of* Podocarpus *species and hybrids used for* in situ *hybridization*

Species	2*n*	Site of original collection	Voucher
P. acutifolius Kirk	34	Unknown	AKU23710
P. lawrencei Hook. f.	38	Mt. Kosciusco, New South Wales, Australia	AKU23711
P. nivalis Hook.	38	Mt. Arthur, South Island, New Zealand	AKU23712
P. totara Benn. ex D. Don	34	Mt Hikurangi, North Island, New Zealand	AKU23713
P. totara 'Aureus'	34	Unknown	AKU23714
Hybrids			
P. hallii probable hybrid	36	Natural hybrid, male parent unknown, Ngaruroro River, North Island, New Zealand	AKU23715
P. nivalis × *P. totara*	36	Natural hybrid, Lake Lyndon, South Island, New Zealand	AKU23716
Podocarpus 'Country Park Flame'	36	Artificial hybrid of *P. lawrencei* × *P. acutifolius*	AKU23717
Podocarpus 'Country Park Fire'	38	Artificial hybrid of *P. lawrencei* × *P. nivalis*	AKU23718

TABLE 2. *List of species used for membrane hybridization*

Species
Podocarpus totara
P. nivalis
P. lawrencei
Dacrycarpus dacrydioides (A. Rich.) De Laub.
Dacrydium cupressinum Lamb.
Halocarpus bidwillii (Kirk) Quinn
Picea abies (L.) Karsten
Pinus elliottii Engelm.
Araucaria araucana (Molina) K. Koch
Ginkgo biloba L.
Cycas circinalis L.

somes. *Picea abies* (L.) Karsten and *Larix decidua* Mill. showed only terminal sites, but hybridization at centromeric sites was also seen in *Pinus sylvestris* (Fuchs *et al*., 1995; Lubaretz *et al*., 1996), and at a number of intercalary sites in *Pinus elliottii* (Schmidt *et al*., 2000).

In contrast to the uniformity of chromosome number and karyotype in most conifer families, Podocarpaceae are chromosomally variable (Hair and Beuzenberg, 1958; Hizume *et al*., 1988; Davies *et al*., 1997). Chromosome numbers range from $2n = 18$ to $2n = 38$ and the karyotypes consist of a mixture of different numbers of metacentric, acrocentric and telocentric chromosomes. There have been no previous molecular cytogenetic studies on the family, and the aim of the present work was to investigate the number and distribution of the repeat sequences in Podocarpaceae so that comparisons can be made with the other gymnosperm families.

MATERIALS AND METHODS

For *in situ* hybridization, five species of *Podocarpus* and a number of natural and artificially produced interspecific hybrids were studied (Table 1). Voucher specimens of all the plants are deposited in the herbarium of the University of Auckland (AKU). DNA from a range of gymnosperms (Table 2) was also used for analysis by Southern hybridization.

Chromosome preparations were made from root tips that were pretreated with 0·05 % colchicine for 24 h at 4 °C followed by a further 24 h in water at 4 °C before fixation in acetic acid : ethanol, 1 : 3. The fixed roots were digested for 30–40 min at 37 °C in a mixture of 2 % cellulase (Calbiochem, La Jolla, CA, USA), 4 % cellulase (Onozuka R10, Merck, Darmstadt, Germany), 2 % hemicellulase (Sigma, St Louis, MO, USA), 0·5 % pectolyase (Seishin, Tokyo, Japan) and 20 % liquid pectinase (Sigma) in 0·01M citrate buffer, pH 4·6, and squashed without heating on a slide.

The slides were hybridized with the following probes that were labelled with biotin and digoxigenin by nick translation, PCR or random priming as described in Schwarzacher and Heslop-Harrison (2000): (1) pTa71, which contains the 18S-26S rDNA (frequently called 45S rDNA) from *Triticum aestivum* L. (Gerlach and Bedbrook, 1979) labelled by nick translation; (2) pTa794, which contains a 410 bp *Bam*H1 fragment of the 5S rDNA from *T. aestivum* (Gerlach and Dyer, 1980) labelled by PCR; (3) pPot1li and pPongy2 which contain 416 and 419 bp fragments of reverse transcriptase of *gypsy*-like retroelements from *Podocarpus totara* and *P. nivalis*, respectively (Friesen *et al*., 2001; EMBL nucleotide database accession numbers AJ290646-AJ290647); and (4) synthetic oligonucleotides $(TTTAGGG)_5$, $(ACG)_7$, $(AAC)_5$ and $(GATA)_4$ labelled by random priming.

The hybridization mixture was prepared as outlined in Schwarzacher and Heslop-Harrison (2000) and the chromosomes were denatured at 90 °C for 8 min in a Hybaid Omnislide thermal cycler, hybridized overnight at 37 °C and washed, with the most stringent washes in 20 % formamide in 0·1 × SSC at 42 °C (allowing sequences with approx. 85 % identity to remain hybridized). Digoxigenin-labelled probes were detected with an FITC conjugated sheep anti-digoxigenin antibody (Roche, Basel, Switzerland) while biotin-labelled probes were detected with streptavidin conjugated to Cy3 (Sigma). Slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole), mounted in Citifluor AF1 (Agar Scientific, Stansted, UK) and observed with a Nikon epifluorescence microscope. Photographs were taken on Fuji Superia 400 colour print film, digitized and printed from Adobe Photoshop after contrast optimization using only functions affecting the whole image.

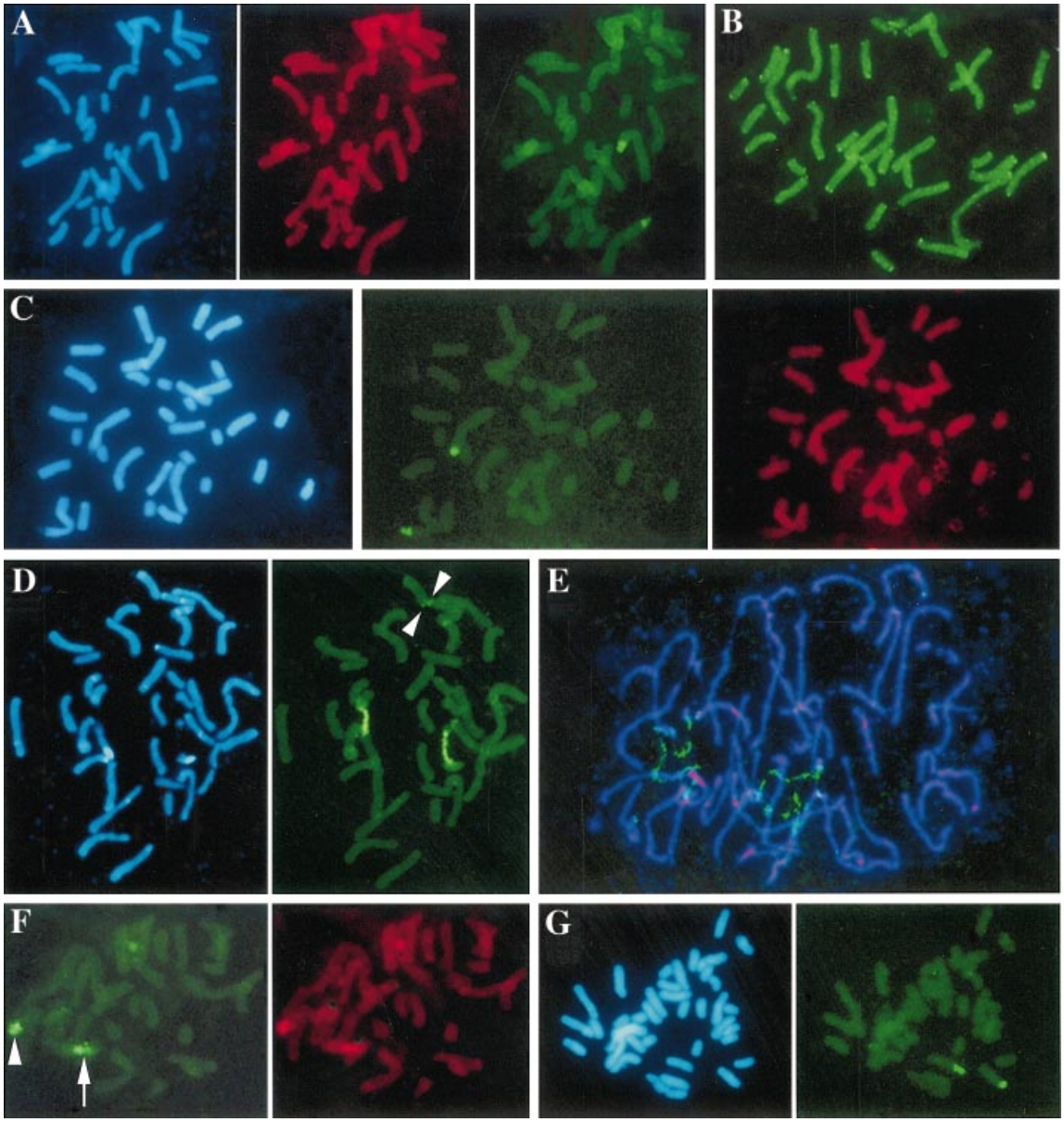

FIG. 1. Somatic chromosomes of *Podocarpus* species following *in situ* hybridization with repetitive DNA probes. Each figure part (A–G) shows one complete metaphase or prophase. A, *P. totara* metaphase. Left: stained with DAPI (light blue) but not showing any banding; centre: probed with the synthetic oligonucleotide $(TTTAGGG)_6$ (red) showing terminal and some intercalary hybridization sites; right: hybridization (green) of the 45S rDNA probe at one pair of sites. B, A *P. totara* metaphase hybridized with $(TTTAGGG)_6$ showing terminal sites on all chromosomes and some intercalary sites (green). C, A *P. totara* metaphase stained with DAPI (left), showing hybridization of 5S rDNA at one pair of sites (centre, green) and diffuse hybridization of $(GATA)_4$ (right, red). D, A metaphase of *P. lawrencei* showing conspicuous DAPI bands (left) and a major pair of interstitial 45S sites and terminal minor sites (arrowed) following *in situ* hybridization (right, green). E, A *P. lawrencei* prophase showing that the major 45S rDNA sites are decondensed. F, Metaphase of the hybrid 'Country Park Fire' (*P. lawrencei* × *P. nivalis*) showing collocalization of 45S rDNA (green, left) and 5S rDNA (red, right). The major sites are indicated by an arrow for the one derived from *P. lawrencei* and an arrowhead for that from *P. nivalis*. The minor sites are not clearly resolved on the plate. G, *P. nivalis* metaphase chromosomes stained with DAPI (left) and showing the single pair of sites of 45S rDNA hybridization on a medium-sized acrocentric chromosome pair (right, green).

DNA was extracted from young leaf material of different gymnosperm species using the Qiagen DNAeasy Plant mini kit or the 2 × CTAB method. Isolated DNA was used directly for PCR amplification or digestion with the restriction enzymes *Hin*dIII and *Hae*III. Digested DNA (5 μg) was separated on 1·2 % agarose gels, blotted, and probed with the following clones that were all labelled by PCR: (1) pDdgy1, a 417 bp fragment of reverse transcriptase of *gypsy*-like element from *Dacrycarpus dacrydioides* (A. Rich.) De Laub.; (2) pPongy2 and pPonty3, a 419 bp fragment of reverse transcriptase of a *gypsy*-like element from *P. nivalis*; (3) pPot3li, which contains a 257 bp fragment of reverse transcriptase of a *copia*-like retroelement (accession number AJ2(0731) from *P. nivalis*,

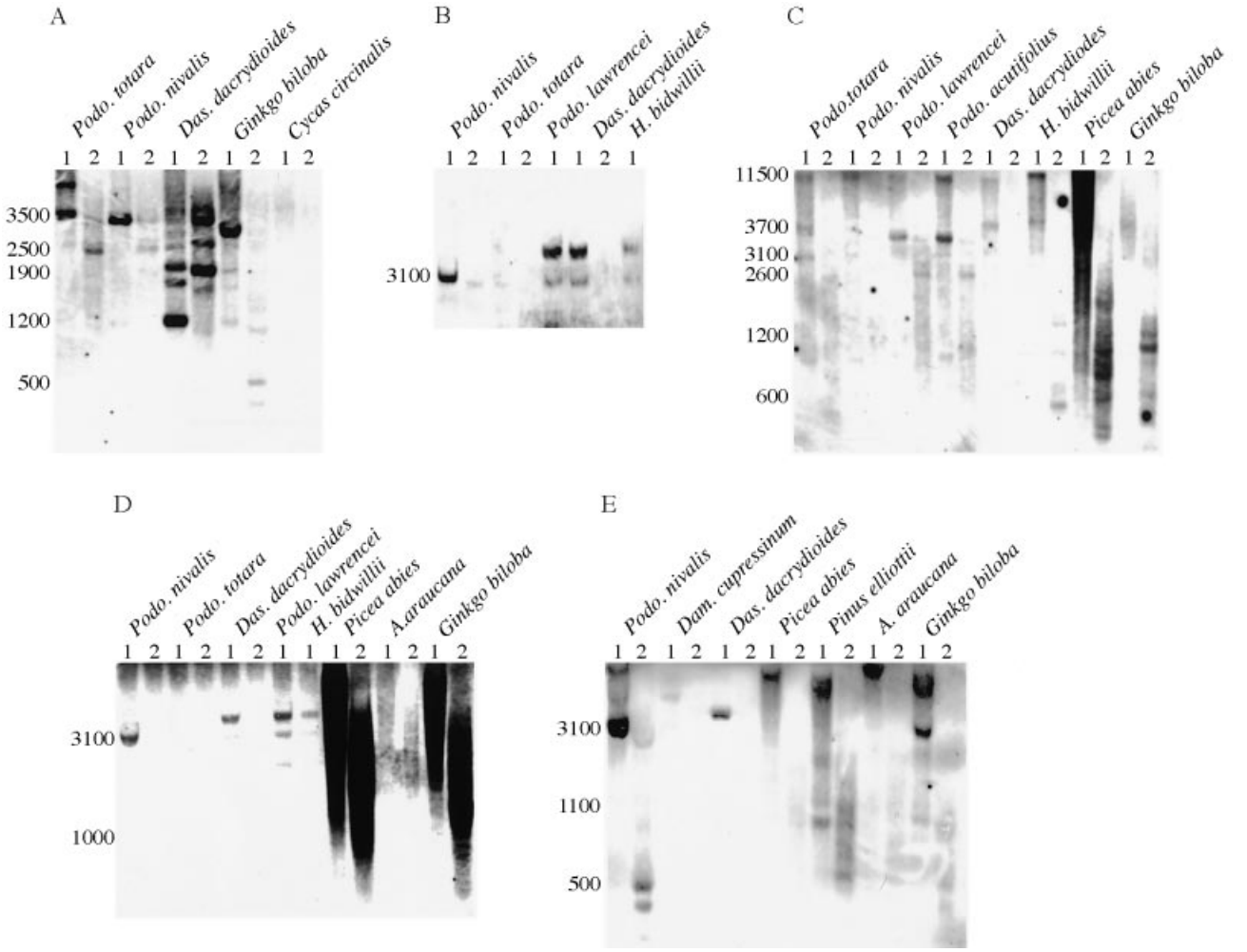

FIG. 2. Genomic organization and abundance of retrotransposon families in various gymnosperms analysed by Southern hybridization of genomic DNA digests. Luminographs show DNA from gymnosperm species digested with (1) *Hin*dIII and (2) *Hae*III probed with (A) *gypsy* pDdgy1, (B) *gypsy* pPongy2, (C) *gypsy* pPot3li, (D) *copia* pPonty3 and (E) LINE pDdli1. Podo, *Podocarpus*; Das, *Dacrycarpus*; Dam, *Dacrydium*; H, *Halocarpus*; A, *Araucaria*.

labelled by PCR; and (4) pDdli1, a fragment of a LINElement isolated from *D. dacrydioides*, using the non-radioactive chemiluminiscence method Alk-Phos Direct (Amersham, Little Chalfont, UK).

RESULTS

Four of the five *Podocarpus* species, those from New Zealand, showed a single pair of hybridization sites with the 45S rDNA probe. In *P. totara* (Fig. 1A), *P. hallii* and *P. acutifolius* the site was terminal on the largest acrocentric pair whereas in *P. nivalis* it was terminal on the second largest acrocentric pair (Fig. 1G). In the Australian species *P. lawrencei*, there was a major interstitial site on one of the small chromosome pairs and minor sites in terminal positions on two other pairs of chromosomes (Fig. 1D). In prophase cells, these sites had a somewhat reticulate appearance with brightly fluorescing regions separated by non-fluorescing ones, most notable in *P. lawrencei* (Fig. 1E). In the F_1 hybrid 'Country Park Fire' (*P. lawrencei* × *P. nivalis*), there were clear differences in the degree of reticulation of the two sites; one was similar to those in *P. lawrencei*, whereas the other was less diffuse and consisted of three clear foci.

The 5S rDNA probes hybridized to similar sites to the 45S sites in all the taxa studied, a result that was shown by both single-target hybridization to chromosomes with characteristic morphology and double-target hybridization. In Fig. 1A and C, the longest acrocentric chromosomes of *P. totara* were labelled with the two rDNA probes. Collocalization was also seen in the hybrid 'Country Park Fire' (Fig. 1F), although the chromosomes with the rDNA hybridization sites were of different sizes and the exact site location also differed between the two labelled chromosomes. At prophase, the hybridization pattern with the 5S rDNA probe appeared very similar to that with 45S rDNA.

The telomere probe showed clear hybridization sites at the ends of all chromosomes of all taxa although the size of the sites did appear to vary both between and within

chromosomes (Fig. 1A and B). The telocentric chromosomes were clearly labelled at both ends. There were a number of interstitial sites that were distributed along the chromosome arms of most of the chromosomes (Fig. 1A and B). Two simple sequence repeats, $(AAC)_5$ and $(GATA)_4$, showed a diffuse pattern of hybridization sites along the length of all chromosomes (GATA; Fig. 1C). No hybridization was seen with the $(ACG)_7$ probe or with the two *gypsy*-like retroelements obtained from *P. totara* and *P. nivalis*.

The *in situ* hybridization protocol revealed up to four pairs of DAPI bands in some metaphases of *P. lawrencei* and its hybrids (Fig. 1D). None of the other species showed DAPI banding of their chromosomes (Fig. 1A, C and G).

Five retroelement clones, pDdgy1 from *Dacrycarpus dacrydioides*, pPongy2 from *Podocarpus nivalis*, pPotli3 from *P. totara*, pPonty3 from *P. nivalis* and pDdLi1 from *D. dacrydioides*, were used for Southern hybridization to DNA digests from *Podocarpus*, *Dacrycarpus*, *Halocarpus* and other representative gymnosperm genera (Table 2; Fig. 2). Each clone showed unique hybridization patterns and revealed differences in organization of different retroelements between genera, although most sequences tested were present in several genera. Probe pDdgy1 (Fig. 2A) was abundant in all Podocarpaceae species and also hybridized to DNA from *Ginkgo biloba*, but no hybridization was detected to *Cycas circinalis*. Probe pPongy2 (Fig. 2B) showed no hybridization to *P. totara* DNA or to the *Hin*dIII digests of *P. nivalis* and *D. dacrydioides* but showed distinct, though different, bands with the other taxa. Probe pPot3li (Fig. 2C) showed weak hybridization signal to all Podocarpaceae species, but strong signal with *Picea abies* and *Ginkgo biloba*. Similar results were obtained with Southern hybridization with the *copia* and LINE retroelement clones pPonty3 (Fig. 2D) and pDdli1 (Fig. 2E). pPonty3 showed low abundance in Podocarpaceae species, but strong hybridization to *Picea abies* and *Ginkgo biloba* DNA. pDdli1 showed only one fragment with *Hin*dIII digested DNA from *Dacrycarpus* (from which it was isolated) but strong hybridization to digested DNA from *Podocarpus nivalis*, *Ginkgo biloba* and *Picea abies*.

DISCUSSION

Unlike most other genera in the conifers, *Podocarpus* shows extensive karyological variation: species have chromosome numbers that include n = 9, 10, 11, 17 and 19, and karyotypes that comprise various combinations of metacentric, acrocentric and telocentric chromosomes (Hair and Beuzenberg, 1958; Davies *et al*., 1997). At the molecular level, our *in situ* hybridization results highlight both variable and constant features of the karyotypes. There is variation in the location and number of rDNA sites in the genus. All the species examined have only one major 45S rDNA site, but this is located on morphologically different chromosomes: in three of the New Zealand species it is terminal on the longest acrocentric chromosome, but in the fourth it is terminal on the small acrocentric chromosome. In the Australian species *P. lawrencei*, the major 45S site is interstitial and it has small additional terminal sites on two other pairs of chromosomes.

The 5S rDNA sites were tightly linked to the 45S sites in all *Podocarpus* species examined, with 5S and 45S signal collocalizing on the chromosomes. This collocalization has not been found in other gymnosperms although the 45S and 5S sites are adjacent in species such as *Pinus sylvestris*, *Picea abies* and *Picea sitchensis* (Lubaretz *et al*., 1996; Brown and Carlson, 1997). In two bryophytes, one liverwort (*Marchantia polymorpha* L.) and one moss (*Funaria hygrometrica* Hedw.), the 5S rDNA has been found to be inserted into the 45S rDNA repeat (Sone *et al*., 1999). Sone *et al*. (1999) suggest that this arrangement of rDNAs evolved after the separation of the bryophytes from other land plants. The molecular organization of the two rDNA repeats is unknown in *Podocarpus*, but understanding the organization of these major gene clusters has consequences for interpretations of the phylogeny of the group.

With respect to the 45S rDNA, the organization of the clusters on chromosomes in *Podocarpus* differs from that found in *Pinus*, *Picea* and *Larix*. In *Pinus* and *Picea*, the number of major 45S rDNA sites is in the range of six to ten pairs (Hizume *et al*., 1992; Brown *et al*., 1993; Doudrick *et al*., 1995; Jacobs *et al*., 2000) and there are three major pairs in *Larix* (Lubaretz *et al*., 1996). Several studies have reported the presence of additional minor sites, some of which are found at or near the centromere (Doudrick *et al*., 1995; Friesen *et al*., 2001).

In situ hybridization with the telomeric probe gave similar results in all the *Podocarpus* species. In addition to terminal hybridization sites, there were large numbers of interstitial sites distributed along the length of all chromosomes. Other works have shown that *Pinus sylvestris* chromosomes have terminal and interstitial telomeric sequence sites, some of which were centromeric (Fuchs *et al*., 1995), while Schmidt *et al*. (2000) found multiple, major intercalary sites in *Pinus elliottii*. In *Picea abies* and *Larix decidua*, hybridization signals were confined to chromosome ends (Lubaretz *et al*., 1996). Variability in the interstitial sites and copy number of the telomeric sequences seems to be characteristic of gymnosperms and their distribution is mirrored by that of the other simple sequence repeats that we have studied. No distribution pattern was evident on the chromosomes.

The presence of distinct DAPI bands in the Australian species *P. lawrencei* suggests differences in genomic organization from the New Zealand species. Previous studies reported that DAPI bands were absent from the karyotypes of the New Zealand species of *Podocarpus* (Davies *et al*., 1997), as confirmed here. One of the few northern hemisphere species, *P. macrophyllus* (Thunb.) Lamb., also shows conspicuous DAPI bands on approx. two-thirds of its large telocentric chromosomes (Hizume *et al*., 1988). These differences in banding patterns between species from different areas may be taxonomically significant as the status of the New Zealand and Australian species of the genus is under review (Molloy pers. comm.).

The retroelements that we isolated from *Podocarpus* and *Dacrydium* species were found to hybridize to DNA from a wide variety of other gymnosperms (Fig. 2); similar widespread hybridization has been reported in several

other groups of plants (Kamm *et al*., 1996; Brandes *et al*., 1997). The patterns of membrane hybridization of PCR amplified retroelement probes reflected differences in copy number and organization between different species. Some of these results are surprising. For example, a *copia* probe from *P. nivalis* showed no hybridization to the DNA of its close New Zealand relative *P. totara*, but showed two distinct bands with *P. lawrencei* from Australia and strong hybridization to *Picea abies* and *Ginkgo biloba*. Similar results were seen with the LINE and *gypsy* probes: pPot3li from *P. totara* showed little hybridization to *P. nivalis* and greatest hybridization to *Picea abies* and *Ginkgo biloba*, and pPongy2 from *P. nivalis* did not hybridize to *P. totara* DNA (Fig. 2B). The absence of clear signals following *in situ* hybridization with the LINE and *gypsy* probes on the chromosomes of several of the *Podocarpus* species and hybrids is not unexpected given the low level of hybridization of the same or similar probes on the gels. These results suggest that most families of retrotransposons were present in ancestral gymnosperms before the diversification of the recognized families, and that during the evolution of the different taxonomic lines different families of retrotransposons were amplified and multiplied (Friesen *et al*., 2001).

As a major and often dominant vegetation type in both northern and southern hemispheres, the differentiation and evolution of genomes and chromosomes in the gymnosperms are important elements in understanding the phylogeny, diversity and evolution of the species themselves. In Pinaceae, the level of polymorphism of genetic markers in the group is generally considered to be low (Nelson *et al*., 1993) and their karyotypes exhibit considerable uniformity in number and chromosome morphology. Study of the conifers as a whole shows that the repetitive DNA sequences differ widely within the group, as in other groups (Heslop-Harrison, 2000). These sequences provide useful diversity, and hence the study of their molecular cytogenetics at both the sequence and karyotypic level provides valuable information for determining the relationships, evolution and diversity of the group.

ACKNOWLEDGEMENTS

B.G.M. thanks the University of Auckland for sabbatical leave. We thank Graham Hutchins, Country Park Nursery, Hornchurch, Essex for the supply of plants and Dr Brian Molloy, Landcare Research, Lincoln, N.Z. for information on Podocarpus taxonomy. We thank EU Framework IV grant PL 962125 for support of this project to J.S.H-H.

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