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The genomic and physical organization of *Ty*1-*copia*-like sequences as a component of large genomes in *Pinus elliottii* var. *elliottii* and other gymnosperms

(genome evolution/*Pinus*/retrotransposon)

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ABSTRACT **A DNA sequence, *TPE*1, representing the internal domain of a *Ty*1-*copia* retroelement, was isolated from genomic DNA of *Pinus elliottii* Engelm. var. *elliottii* (slash pine). Genomic Southern analysis showed that this sequence, carrying partial reverse transcriptase and integrase gene sequences, is highly amplified within the genome of slash pine and part of a dispersed element >4.8 kbp. Fluorescent *in situ* hybridization to metaphase chromosomes shows that the element is relatively uniformly dispersed over all 12 chromosome pairs and is highly abundant in the genome. It is largely excluded from centromeric regions and intercalary chromosomal sites representing the 18S-5.8S-25S rRNA genes. Southern hybridization with specific DNA probes for the reverse transcriptase gene shows that *TPE*1 represents a large subgroup of heterogeneous *Ty*1-*copia* retrotransposons in *Pinus* species. Because no *TPE*1 transcription could be detected, it is most likely an inactive element—at least in needle tissue. Further evidence for inactivity was found in recombinant reverse transcriptase and integrase sequences. The distribution of *TPE*1 within different gymnosperms that contain *Ty*1-*copia* group retrotransposons, as shown by a PCR assay, was investigated by Southern hybridization. The *TPE*1 family is highly amplified and conserved in all *Pinus* species analyzed, showing a similar genomic organization in the three- and five-needle pine species investigated. It is also present in spruce, bald cypress (swamp cypress), and in gingko but in fewer copies and a different genomic organization.**

Retrotransposons that proliferate by reverse transcription of RNA intermediates are a feature of all eukaryotic genomes examined and the major class of mobile genetic elements in plants (1). Because of their structure, two classes of retrotransposons are distinguished: those flanked by long terminal repeats (LTR) and non-LTR retrotransposons. Since the first *Ty-copia* elements were detected in plants [*Ta*1 in *Arabidopsis thaliana* (L.) Heynh. (2), *Tnt*1 in *Nicotiana tabacum* L. (3)] they have been found across a broad phylogenetic spectrum and all major lineages of plants including Chlorophyta, Bryophyta, Pteridophyta, as well as Gymnospermae (1, 4, 5). Most of these *Ty*1-*copia* elements were identified by using a PCR assay designed to detect *copia*-like reverse transcriptase gene sequences. *Ty*1-*copia* reverse transcriptase gene sequences have been identified from *Pinus thunbergii* Parl. and *Pinus coulteri* D. Don by PCR (4, 5). A few *Ty*1-*copia* group elements have been characterized in detail: *Ta*1 of *A. thaliana*, *Tnt*1 of *N. tabacum*, *Tst*1 of *Solanum tuberosum* L. (6) *Bare*1 of *Hordeum vulgare* L. (7), and *Hopscotch* of *Zea mays* L. (8).

Sequence analyses of PCR fragments of reverse transcriptase genes revealed very high degrees of sequence heterogeneity even within a single species, which is put down to the high copy number of *Ty*1-*copia* retroelements detected in plants (9, 10), in contrast to the limited diversity and copy number seen in *Saccharomyces cerevisiae* and *Drosophila melanogaster* (11, 12). The degree of sequence divergence is linked generally to phylogenetic relationships, implying that sequence divergence during vertical transmission of *Ty*1-*copia* retrotransposons along evolving plant lineages has been a major factor in their evolution (1, 4, 5). Some significant exceptions indicate that horizontal gene transfer of *Ty*1-*copia* elements needs also to be considered (9, 13, 14).

Conifers are commercially important and inherently interesting because they dominate many terrestrial ecosystems. Despite the enormous genome size of gymnosperms [>20000 Mbp for *Pinus* species (15, 16)], little is known about the structure and composition of the nuclear genome of any gymnosperm. With the exception of chromosome numbers, which are very conserved ($2n = 24$) (17), there are few investigations of the relatively high percentage of repetitive sequences in the genomes of gymnosperms. Reassociation kinetics data (18, 19) showed 75% of the genome to be repetitive DNA. A retrotransposon element, *IFG*7, was isolated from *Pinus radiata* D. Don and described. This *Ty*3-*gypsy* class element, showing a different gene order compared to the *Ty*1-*copia* elements, is highly amplified in the genome.§ Genetic linkage maps based on restriction fragment length polymorphism and random amplified polymorphic DNA markers have been constructed for different pine species (20–24).

In the present work, we aimed to examine the presence and genomic organization of *Ty*1-*copia* elements in *Pinus* species and particularly in slash pine.¶ We also aimed to investigate the importance of the retrotransposon as a component of the enormous and relatively conserved genomes of gymnosperms.

MATERIALS AND METHODS

Plant Materials, DNA Extraction, Cloning, and Sequencing. Total genomic DNA was extracted from needle tissue (10 g fresh weight) from the plant species listed with authorities in Table 1, following the protocol from Wagner *et al.* (25). Genomic slash pine DNA was shot-gun cloned into pUC18 (26), and highly repetitive sequences were isolated. Clones selected for investigation were sequenced in both directions on an automated 373A DNA sequencer (Applied Biosystems). One clone, named *TPE*1, had homology to *Ty*1-*copia* sequences (see *Results*) and was used as a probe.

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Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

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§Kossack, D. S., Barrios, M. & Kinlaw, C. S., International Union of Forestry Research Organizations Molecular Genetics Meeting, September 30–October 4, 1990, Lake Tahoe, NV (abstr.).

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. Z50750).

Table 1. Gymnosperm species used for experiments

Genus	Subgenus	Species	Common name	Source
Pinus L.	*Pinus*	*P. echinata* Mill.	Shortleaf pine	Harrison City, MS
		P. elliottii Engelm. var. *elliottii*	Slash pine	Harrison City, MS
		P. palustris Mill.	Longleaf pine	Harrison City, MS
		P. caribaea Morelet	Caribbean pine	Puerto Rico
		P. oocarpa Schiede		Puerto Rico
		P. banksiana Lamb.	Jack pine	Oneida County, WI
		P. massoniana Lamb.	Masson pine	Harrison City, MS
		P. resinosa Ait.	Red pine	Oneida County, WI
	Strobus Lemm.	*P. strobus* L.	White pine	Oneida County, WI
Picea Diet.		*P. abies* (L.) Karst.	Norway spruce	Oneida County, WI
		P. glauca (Moench.) Voss.	White spruce	Oneida County, WI
Taxodium Rich.		*T. distichum* (L.) Rich.	Baldcypress	Harrison City, MS
Gingko L.		*G. biloba* L.	Gingko	Harrison City, MS

All samples were from the collection of Southern Institute of Forest Genetics, U.S. Department of Agriculture, Saucier, MS.

DNA Labeling and Southern Hybridization. The nonradioactive chemiluminescence method ECL (Amersham) was used for DNA labeling, hybridization, and detection. Southern blots were prepared using standard protocols (27). The hybridization, with a DNA concentration of 10 ng/cm^2 of membrane, was done overnight with a stringency of 90%.

Fluorescent *in Situ* Hybridization. *TPE*1 was labeled with biotin-11-dUTP (Sigma) by PCR. pTa71, carrying rRNA-encoding DNA and intergenic spacers (28), was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation. Chromosome preparation and *in situ* hybridization were done by the procedures of Doudrick *et al.* (29). Briefly,

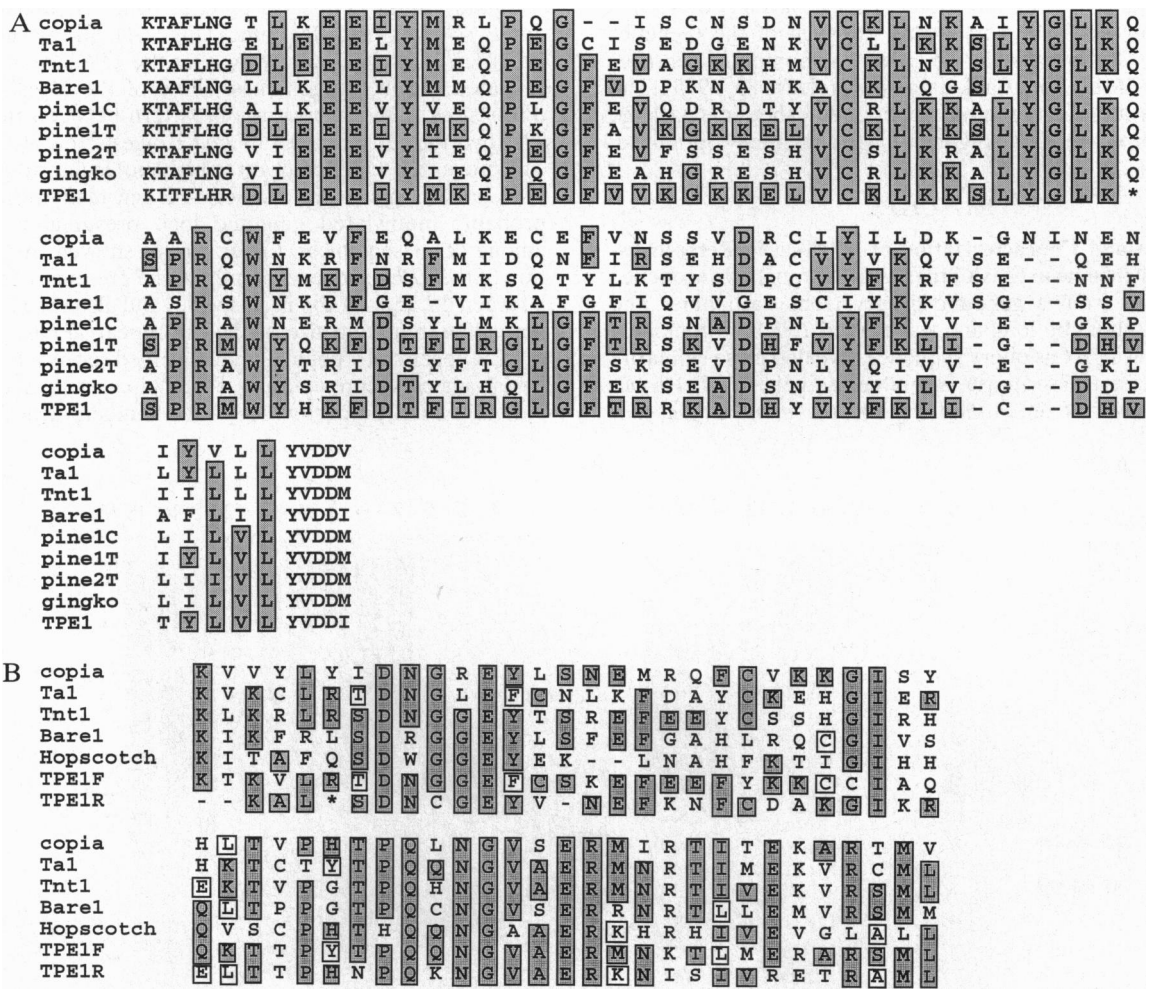

FIG. 1. Alignments of predicted amino acid sequences across conserved domains of different *Ty*1-*copia* retrotransposons. Dashes show gaps that were introduced to optimize the alignment. Stop codons are marked by asterisks (*). Homologous amino acids including the *TPE*1 sequence are boxed. (*A*) Alignment of the slash pine element *TPE*1 to reverse transcriptases of *Ty*1-*copia* retrotransposons from *Drosophila melanogaster* (copia), *Arabidopsis thaliana* (Ta1), *Nicotiana tabacum* (Tnt1), *Hordeum vulgare* (Bare1), *Pinus coulteri* (pine1C), *Pinus thunbergii* (pine1T, pine2T) and *Gingko biloba* (gingko). KTAFLHG und YVDDM sequences correspond to the oligonucleotide primers. (*B*) Alignment of two slash pine element sequences from *TPE*1 (*TPE*1F and *TPE*1R) to integrases of *Ty*1-*copia* retrotransposons from *D. melanogaster* (copia), *A. thaliana* (Ta1), *N. tabacum* (Tnt1), *H. vulgare* (Bare1), and *Zea mays* (Hopscotch).

seedling root tips for chromosome preparations were treated with colchicine and fixed in alcohol/acetic acid (3:1). An enzyme mixture containing cellulase and pectinase was used to soften the root tips that were then squashed on chromic acid-cleaned slides. The hybridization mixture containing the probe was denatured and added to the chromosome preparations; both were denatured in an Omnislide thermal cycling machine (Hybaid, Middlesex, U.K.) at 80°C for 8 min. After hybridization overnight at 37°C, washes were carried out with 82% stringency. Sites of hybridization were detected using streptavidin-Cy3 conjugate (Sigma) for biotin-labeled probes and fluorescein isothiocyanate-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) for digoxigenin-labeled probes. Slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole), mounted in antifade solution, and photographed with a Leica epifluorescence microscope with appropriate filters.

Northern Analysis. Poly(A)$^+$ RNA was isolated from pollen and needles of slash pine using oligo(dT)$_{25}$-coated magnetic beads according to the instructions of the manufacturer (Dynal, Oslo). Northern blots were prepared as described by Sambrook *et al.* (27). *TPE*1 was labeled by random priming with [^{32}P]dCTP.

PCR Assay. The internal domain of reverse transcriptase genes from gymnosperm species was amplified using flanking primers and PCR programs described by Flavell *et al.* (30).

Computer Analysis. The FASTA program of the Genetics Computer Group package was used for homology searches within the EMBL/GenBank data base (release 83, 1995). The putative peptide sequence was generated by the same package using the program MAP. Alignments were manually optimized.

RESULTS

Isolation and Characterization of a *Ty*1-*copia* Retrotransposon Sequence from Slash Pine. A highly repetitive sequence was isolated from a genomic library from slash pine. The sequence is 1663 bp long and was named *TPE*1. A homology search using *TPE*1 as query sequence revealed close similarity to the reverse transcriptase and integrase genes of *Ty*1-*copia* retrotransposons from *P. thunbergii, A. thaliana, N. tabaccum* and others. Fig. 1 presents alignments of parts of the putative *TPE*1 peptide sequence with some previously determined reverse transcriptase and integrase sequences of *Ty*1-*copia* plant retrotransposons such as *Ta*1, *Tnt*1, *Bare*1, Hopscotch, and sequences isolated from *P. thunbergii, P. coulteri*, and *G. biloba* (4), as well as the *copia* element from *D. melanogaster*. In general, most of the identity was found at positions that were conserved in the majority of the compared retroelements. *TPE*1 can be identified as an internal part of a *Ty*1-*copia* retrotransposon, carrying reverse transcriptase and integrase gene sequences. We infer that a recombination event within *TPE*1 led to a compound structure of this element. The reverse transcriptase gene, following the integrase gene within the same reading frame, is destroyed by insertion of a partial integrase gene sequence, encoded on the opposite strand of *TPE*1 and, hence, lying in inverted orientation. This result indicates that the element cloned in *TPE*1 is defective. Furthermore, putative stop codons were found within the *TPE*1 sequence, and the introduction of frameshifts was required to enable an alignment with peptide sequences of other *Ty*1-*copia* retrotransposons.

Genomic Organization and Heterogeneity Within *Pinus* Species. The genomic organization of *TPE*1 was analyzed by Southern hybridization to genomic DNA digests of three related three-needle *Pinus* species [Section *Pinus* Subsection *Australes Pinus* (31)], slash pine, *P. palustris* (longleaf pine), and *P. echinata* (shortleaf pine) (Fig. 2*A*). Strong signals were observed in all digests showing that the *TPE*1 family is highly repeated within the genomes. None of the digests revealed differences in the hybridization pattern between the species. The hybridization pattern in *Apa* I digests revealed a strong smear over the whole track up to high molecular weights (lanes 7–9) indicating the presence of *TPE*1 in many different and probably methylated genomic loci, presumably dispersed among other sequences. Other digests showed the conservation of the *TPE*1 sequence family by the presence of fragments between 0.2 and 4.8 kbp in all species and also showing that 4.8 kbp is the minimum size of the full-repeat *TPE*1.

PCR generated a population of diverged reverse transcriptase gene fragments representative of the *Ty*1-*copia* elements in slash pine. The PCR product was used for Southern hybridization to

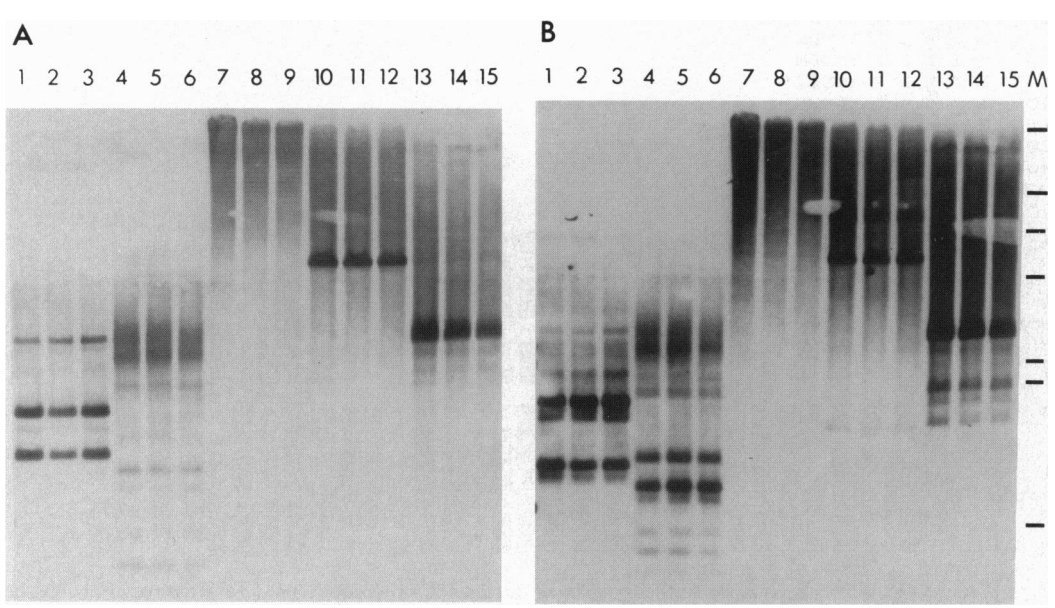

FIG. 2. Genomic organization of *Ty*1-*copia* elements within the genome of shortleaf pine, slash pine, and longleaf pine. (*A*) Genomic organization of the *TPE*1 family. Southern blots of genomic DNA digested with *Hae* III (lanes 1–3), *Hin*fI (lanes 4–6), *Apa* I (lanes 7–9), *Bam*HI (lanes 10–12), *Eco*RI (lanes 13–15) were probed with *TPE*1. Lambda *Hin*dIII-digested DNA was used as DNA size marker (M). (*B*) Rehybridization of the Southern blot described above *A* with a population of diverged reverse transcriptase gene sequences of *Ty*1-*copia* elements from slash pine, isolated by PCR.

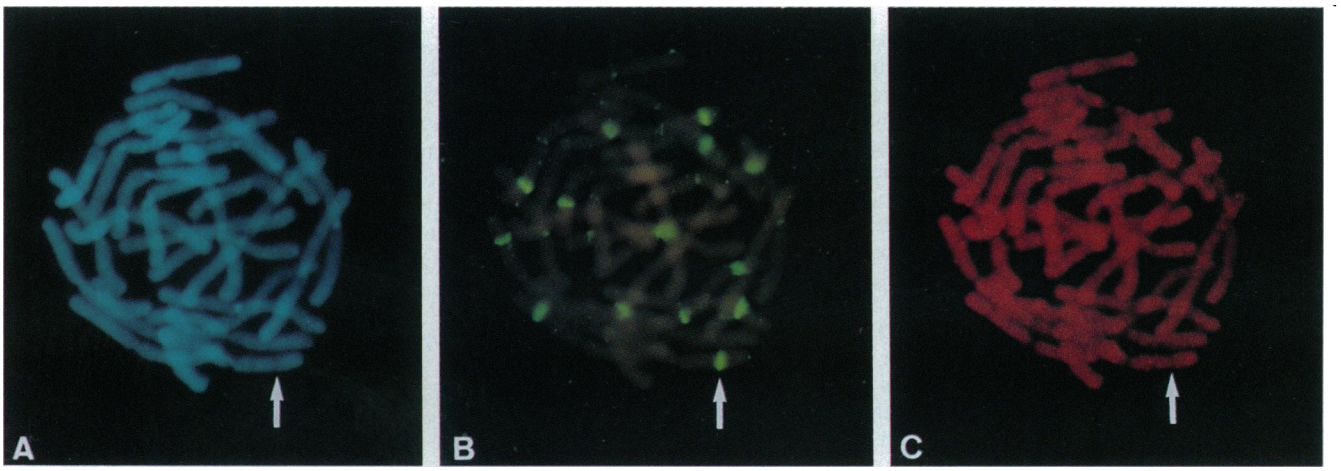

FIG. 3. Localization of a *Ty1-copia* retrotransposon family and the 18S-5.8S-25S rRNA genes along chromosomes of slash pine by fluorescent *in situ* hybridization. (*A*) DAPI staining of metaphase chromosomes of slash pine ($2n = 2x = 24$). (*B*) The same metaphase after *in situ* hybridization with 18S-5.8S-25S rRNA genes visualized by yellow-green fluorescence. (*C*) Detection of the *Ty1-copia* retrotransposon *TPE1* (red fluorescence) on the same metaphase chromosomes. Arrow shows an example of the relatively large exclusion from DAPI-negative intercalary region (arrow in *A*) harboring 18S-5.8S-25S rRNA genes (arrowed in *B*).

investigate the heterogeneity of the *TPE1* family in three three-needle pine species. Hybridization revealed a strong and complex pattern (Fig. 2*B*), indicating that *Ty1-copia* retrotransposons are a large component of the genomes of the three pine species. The *TPE1* Southern hybridization pattern (Fig. 2*A*) is a subset of the pattern revealed by a heterogeneous population of reverse transcriptase gene sequences. The most prominent bands are shared by *TPE1* and PCR-amplified sequences from the internal part of the reverse transcriptase gene. Hence, it was evident that *TPE1* represents a major family of *Ty1-copia* retrotransposons forming one large subgroup of heterogeneous *Ty1-copia* retrotransposons in slash, longleaf, and shortleaf pines. In addition, bands >4.8 kbp were found, indicating a larger repeat size of other families of *Ty1-copia* elements than found for the *TPE1* sequence family.

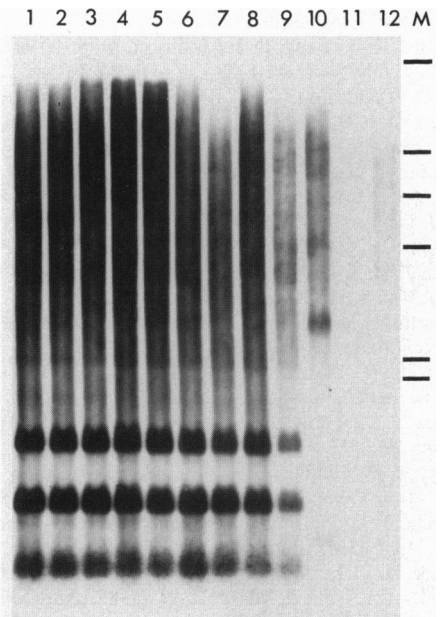

FIG. 4. Distribution of *TPE1* in several species of *Pinus* and other gymnosperms. Southern blot of *Dra* I-digested genomic DNA of *P. echinata* (lane 1), *P. elliottii* var. *elliottii* (lane 2), *P. palustris* (lane 3), *P. caribaea* (lane 4), *P. oocarpa* (lane 5), *P. banksiana* (lane 6), *P. massoniana* (lane 7), *P. resinosa* (lane 8), *P. strobus* (lane 9), *Picea abies* and *Picea glauca*, mixed (lane 10), *T. distichum* (lane 11) and *G. biloba* (lane 12) was hybridized with *TPE1*. Lambda *Hin*dIII-digested DNA was used as DNA size marker (lane M).

Chromosomal Localization of *Ty1-copia* Retrotransposons in the Genome of Slash Pine. From Southern hybridization it was evident that there has been a substantial amplification of the *TPE1* family in the slash pine genome. The chromosomal distribution of *Ty1-copia* elements in slash pine was investigated by fluorescent *in situ* hybridization to metaphase chromosomes ($2n = 2x = 24$) using biotin-labeled *TPE1* as a probe (Fig. 3*C*). Hybridization revealed that this element is dispersed relatively uniformly over all 12 chromosome pairs and represents a major component of the slash pine genome. It is largely excluded from DAPI-negative centromeric and intercalary regions harboring the major and minor 18S-5.8S-25S rRNA genes, as visualized by double *in situ* hybridization with digoxigenin-labeled rRNA genes (Fig. 3*B*).

Distribution in Different *Pinus* Species and Gymnosperms. The distribution of *TPE1* within different gymnosperms was investigated by Southern analysis. *TPE1* was used for Southern hybridization of digested DNAs from various *Pinus* and *Picea* species, *Taxodium distichum* (bald cypress or swamp cypress) and *G. biloba* (gingko). Fig. 4 shows that *TPE1* is highly amplified in all species of pine analyzed (lanes 1–9). A strong smear over the whole range with three strong bands could be detected. It is noteworthy that, although *TPE1* revealed the same structure and dispersed genomic organization within all pine species, the strength of hybridization differed. While all two- and three-needle pine species (Section *Pinus*) show strong hybridization and a very similar pattern (lanes 1–8), significantly less signal was observed in *P. strobus* (Section *Strobus*), a five-needle pine species (lane 9). *TPE1* is relatively highly amplified also in spruce, but much less hybridization signal could be detected in bald cypress and gingko, indicating either many fewer copies or considerable lower homology in these species. *TPE1* hybridization also showed a different genomic organization in spruce, bald cypress, and gingko, so we verified the presence of *Ty1-copia* elements among these species by a PCR assay. Sequences of the expected size (≈260 bp) were amplified (data not shown). No differences in size were detected, indicating the presence and conservation of the reverse transcriptase domain of *Ty1-copia* retroelements in the species.

DISCUSSION

We have isolated a highly repetitive DNA sequence, *TPE1*, from slash pine and used fluorescent *in situ* hybridization to map physically these elements on slash pine chromosomes.

Alignments of parts of the putative *TPE*1 peptide sequence with known *Ty*1-*copia* plant retrotransposons revealed identity at most positions that were conserved in the majority of the retroelements compared, and hence *TPE*1 was identified as a retroelement of the *Ty*1-*copia* type from slash pine, carrying reverse transcriptase and integrase gene sequences (Fig. 1).

So far, little is known about the transposition activity of plant *Ty*1-*copia* retrotransposons because it is difficult to assess their transposition and mobility. Numerous mutations within the *TPE*1 sequence such as putative stop codons, interrupted reading frames, and a disrupted reverse transcriptase gene caused by recombination lead to the assumption that this element is defective. Moreover, most of the *Ty*1-*copia* elements are inactive in terms of retrotransposition in slash pine—at least in needle tissue because no transcripts of *TPE*1 could be detected by Northern analysis. This result reflects the common situation observed for most plant retrotransposons that were found to be transcriptionally inactive. In contrast to yeast and *Drosophila*, where transcription of retrotransposons occurs in most tissues during the normal life cycle, plant *Ty*1-*copia* elements are usually transcribed poorly. In plants, transposition of the *Ty*1-*copia* retroelements *Tnt*1 and *Tto*1 from tobacco has been detected under some conditions but seems to be strongly regulated by control of transcription (6, 32, 33).

Few investigations have shown the chromosomal distribution of retroelements. *Bis*1 shows quite uniform hybridization along all barley (4400 Mbp) chromosome arms, but it is absent or relatively rare in the centromeric heterochromatin and nucleolus organizer regions (34). A similar distribution was detected for the *Ty*1-*copia* retrotransposons in *Vicia faba* (13,000 Mbp) (10), whereas a less uniform pattern with absence or presence at a reduced density at some chromosomal regions, in particular at centromeric and intercalary heterochromatin and rRNA loci, was observed for the *Tbv Ty*1-*copia* elements from *Beta vulgaris* (758 Mbp) (35). Exclusion from heterochromatic and nucleolus organizer regions, as also found with *TPE*1, seems a feature of many plant *Ty*1-*copia* retrotransposon.

Detailed studies of elements within individual species revealed that, despite maintenance of the overall structure, a population of many different, but related, sequences are present within its genome (36, 37). Flavell *et al.* (30) have characterized 31 *Ty*1-*copia* clones in potato that could be clearly grouped into six related subfamilies, and diversities between them up to 75% have been observed. The degree of sequence heterogeneity shows no correlation with plant divisions, and therefore the source of this heterogeneity cannot be a property of any division (1). Theoretical studies, proposing that sequence heterogeneity is positively correlated with copy number of elements, were confirmed by investigations within the genus *Vicia* (10).

The two sections of the genus *Pinus, Pinus* and *Strobus*, had become distinct taxa by the early Cretaceous period [136 million yr ago (38)], so the relatively high conservation of the *TPE*1 element is noteworthy. The position of *P. resinosa* is of interest: it is native to North America but now normally placed in Subsection *Sylvestres* Loud. with *P. massoniana* and other Eurasian pines. The distinct differences in Southern hybridization between the two and the similarity of *P. resinosa* to the North American pines suggests that taxonomic affinities based on morphology, cone serotiny, and crossing experiments for Subsection *Sylvestres* might need reconsideration. Klaus (39) has proposed creating Subsection *Resinosae*, in Section *Pinus* for *P. resinosa*, a suggestion supported by the retrotransposon hybridization data. *TPE*1 is highly amplified in spruce, but in many fewer copies in bald cypress and gingko, and a different genomic organization was observed in these species than in pine species: the accepted phylogeny of the species correlates with the order of similarity of signal pattern and intensity for *TPE*1.

From Southern and *in situ* hybridization it was evident that there has been amplification of the *Ty*1-*copia*-like sequences in the genomes of all the *Pinus* species analyzed (genome sizes typically 20,000–25,000 Mbp). The high amplification and genomic distribution of the *TPE*1 family, dispersed among other sequences but excluded from particular chromosomal regions, is consistent with the amplification of *Ty*1-*copia* elements seen in the angiosperms. Within the gymnosperms the divergence of the *Ty*1-*copia* sequences follows taxonomic groupings, as in angiosperm groups where chromosome number is less conserved and genome size is both smaller and more variable.

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