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Flow Cytometric Analysis of DNA Content for Tropical and Temperate New World Pines

S. E. HALL†, W. S. DVORAK‡, J. S. JOHNSTON†, H. J. PRICE† and C. G. WILLIAMS*†

†*Genetics Program, Texas A&M University, College Station, Texas 77843-2135, USA and* ‡*CAMCORE, Box 7626, North Carolina State University, Raleigh, North Carolina 27695-7626, USA*

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Temperate pine species have unusually large, complex genomes which make genomic analysis problematic; it has been suggested that tropical pines might have smaller genome sizes than temperate pines. Laser flow cytometry (LFC) was used to measure genome sizes of 11 species from Mexico, Guatemala and Nicaragua, spanning latitudes 14°–37° N. These values were compared with previously reported LFC estimates for 17 subtropical and temperate species. Genome sizes in this study were large, varying 1·6-fold from 19·94 to 31· 76 pg/C. Genome size variation paralleled taxonomic classification more closely than latitudinal origin. Genome sizes of subgenus *Strobus* (soft pines) were larger, ranging from 27·36 to 31·76 pg/C; those of subgenus *Pinus* (hard pines) were smaller, ranging from 19·94 to 24·91 pg/C. The exception was hard pine subsection *Macrocarpae* which had larger genome sizes ranging from 26·33 to 28·35. Intraspecific variation was substantial for tropical hard pines *P. oocarpa* and *P. tecunumanii* yet negligible for tropical hard pine *P. patula*.

Key words: C-values, genome size, gymnosperms, conifers, *Pinus* spp., latitudinal variation, megagametophytes.

INTRODUCTION

Temperate pines have large genomes (>28 000 Mbp) composed of 90 % highly repetitive DNA (Kriebel, 1985; Wakamiya *et al*., 1993; Elsik and Williams, 2000); finding pines with smaller genome sizes would improve efficacy of genomics applications. Some tropical pines have been shown to have lower genome sizes than temperate or boreal species (Ohri and Khoshoo, 1986). This suggested ecogeographical factors related to latitude might have shaped genome size in pines as shown for some angiosperm families (e.g. Levin and Funderburg, 1979). The alternative hypothesis is that genome size variation parallels taxonomic relationships because divergence and speciation are often accompanied by changes in the amount of nuclear DNA (Price, 1976).

The genus *Pinus* is composed of over 100 species in the northern hemisphere spanning boreal to tropical latitudes, and half of all pine species are at tropical latitudes in Mexico and Central America (Perry, 1991). Two other centres of species diversity are recognized in the New World: California and western United States; and the southeastern United States (Farjon, 1996). Two subgenera, *Pinus* (hard pines) and *Strobus* (soft pines) are dispersed across centres of species diversity. These subgenera split between 136 and 190 million years ago (Miller, 1977) but share a similar karyotype of 12 pairs of metacentric chromosomes (Saylor, 1961).

Taxonomic classification is based on comparative morphology, supplemental crossability data and terpene analysis (Little and Critchfield, 1969; Perry, 1991). The two subgenera *Pinus* and *Strobus* are clearly separated by both morphology- and DNA-based phylogeny, but phylogenetic relationships within the subgenera have not been thoroughly studied. Phylogenetic studies of New World pines have been restricted to a small number of subsections or species (Strauss and Doerksen, 1990; Perez de la Rosa *et al*., 1995; Krupkin *et al*., 1996). The most comprehensive phylogenetic study (Liston *et al*., 1999) is based on ITS sequences but omits three of the seven subsections of pines used in this study. Taxonomy provides a stronger classification system than phylogeny at this time.

Of the New World species in this study, only classification of *P. coulteri* has some taxonomic ambiguity. *Pinus coulteri* has been placed in subsections *Oocarpae*, *Attenuate* and *Sabinanae* (see review in Farjon and Styles, 1997 pp. 45–46), but placement of *P. coulteri* in section *Macrocarpae* is supported by reproductive morphology (Little and Critchfield, 1969) and by chloroplast DNA restriction site analysis (Krupkin *et al*., 1996).

Intraspecific variation in pine DNA content has not been determined for tropical pines using consistent methods such as laser flow cytometry (LFC) (Galbraith *et al*., 1983), the preferred method of genome size determination for pines (Wakamiya *et al*., 1993; Dolezel *et al*., 1998). Using laser flow cytometry, temperate pine *P. taeda* had a notable absence of intraspecific variation (Wakamiya *et al*., 1993) but introgression, hybridization and ongoing speciation in Mexico, a primary centre of species diversity (Perry, 1991; Farjon, 1996), may cause intraspecific variation in genome size. This study tested the following hypotheses: (1) genome size variation parallels taxonomic relationships rather than latitude; and (2) there is intraspecific variation in genome size among tropical Mexican pine species.

* For correspondence. Fax (979) 845-6049, e-mail claire-williams@tamu.edu

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TABLE 1. *New World hard and soft pine species reported by Wakamiya (1994)*

Region	Species
Western U.S. Subgenus *Strobus*	*P. flexilis* James *P. lambertiana* Douglas *P. monticola* Douglas *P. monophylla* Torr.
Western U.S. Subgenus *Pinus*	*P. jefferyii* Grev. & Balf. *P. coulteri* D. Don. *P. attenuata* Lemmon *P. muricata* D. Don. *P. radiata* D. Don. *P. torreyana* Parry *P. sabinana* Dougl.
Southeastern U.S. Subgenus *Strobus*	*P. strobus* L.
Southeastern U.S. Subgenus *Pinus*	*P. palustris* Mill. *P. elliottii* Engelm. *P. taeda* L. *P. echinata* Mill. *P. serotina* Michx. *P. clausa* (Chapman) Vasey *P. virginiana* Mill.

MATERIALS AND METHODS

Plant material and sample preparation

Seventeen New World pine species were added to this study (Table 1) from a previous study (Wakamiya *et al.*, 1993) because equipment and protocol were common to both studies. In our study, seed collections for 11 tropical pine species were made by the Central American and Mexican Coniferous Resources Cooperative (CAMCORE). Separate provenances were sampled separately for three of the 11 species (Dvorak and Donahue, 1992; Table 2) to obtain estimates of intraspecific variation. All seed collections represented at least 100 plants throughout each range or provenance. The two combined datasets represented four of the nine soft pines species and six of the ten hard pines species from the western United States. The southeastern United States was represented by its only soft pine species and by seven of its 12 indigenous hard pines. Mexico and Central America were represented by one of 15 soft pines and nine of the 29 hard pines.

Mean genome sizes were estimated from three separate megagametophyte samples randomly drawn from each accession. The haploid megagametophyte tissue was separated from the embryo of each mature seed and one megagametophyte was processed in each of the three runs. Using a protocol adapted from Price and Johnston (1996), the tissue was macerated in 6 ml preparation buffer; 1 l buffer consisted of 8·4 g sodium citrate, 4·2 g 3-[N-morpholino] propane-sulfonic acid, 4·26 ml of 4·9 M magnesium chloride, 1·0 ml of Triton X-100 and 100 µl of 10 mg ml^{-1} RNAase A. After adjusting the buffer to pH 7·2, propidium iodide was added to a concentration of 50 ppm during maceration. Macerated tissue in buffer was filtered through 50 µm nylon mesh then centrifuged at 1200 rpm for 3 min. The supernatant was replaced with 700 µl fresh buffer. Macerated *Pisum sativum* 'Minerva Maple' (9·64 pg/2C; Johnston *et al.*, 1999) leaves were added to each megagametophyte sample as the internal standard. A common seedlot for *Pinus eldarica* was used for estimating the replicate effect between Wakamiya (1994) and this study. No standard errors could be estimated from the estimates reported by Wakamiya (1994) because all five megagametophytes were processed together. In both studies, DNA content was determined using a Coulter Epic Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with a water-cooled laser tuned at 514 nm and 500 mW.

Statistical analysis

Genome sizes were compared with taxonomic classification (Little and Critchfield, 1969; Perry, 1991). A nested analysis of variance was used to evaluate differences in genome size between seeds within species and between species. In the case of the five Mexican species with intraspecific sampling (Table 2), a separate nested analysis was run for these five species using samples, provenances within species and species. Species and provenances within species were treated as random effects:

$$y_{ijkl} = \mu + S_i + P(T)_{j(k)} + T_k + \varepsilon_{ijkl}$$

where y_{ijkl} is the individual genome size measurement, μ is the experimental mean, S_i is the ith seed where $i = 3$, $P(T)_{j(k)}$ is the jth provenance within a species ($i \neq j$), and T_k is the kth species where $k = 11$ and ε_{ijkl} is experimental error. Scheffé's test was used for multiple comparisons. The regression model $y = \alpha + \beta x + \varepsilon$ described the linear relationship between genome size (y) and mean latitudinal origin (x). Latitudinal minutes were converted to decimal values. Regression analysis was based on Table 2 because exact sample locations were not reported for Wakamiya (1994); this did not skew the analysis because latitudinal extremes of 13 to 38° encompassed the central ranges for all 28 species. All statistical analyses were conducted using PROC GLM from SAS version 6.11 (SAS Institute, Cary, NC, USA).

RESULTS

Closely related pines had less variation in genome size compared to distantly related species (Fig. 1) and genome size reduction was not concomitant with latitudinal origin (Fig. 2). Soft pines had genome sizes between 27 and 32 pg/C, well outside the range of 20 to 22 pg/C found among most hard pines (Fig. 1). Hard pine section *Australes* had a cluster of species with the smallest genome sizes. The difference between these two subgenera was statistically significant at the 0·01 % level in the analysis of variance.

There was one notable exception to the smaller genome size trend in hard pines: subsection *Macrocarpae* had unusually large genome sizes (Fig. 1). Genome sizes were between 26·33 and 28·35 pg/C for these three species (Fig. 1). The gap between *Macrocarpae* and subsections of the subgenera *Pinus* was statistically different from other

TABLE 2. *Location of the Mexican and Central American seed collections and genome size measurements*

Species	Genome size ± s.e.	Provenance, Country	Latitude/Longitude
Pinus caribaea var. *hondurensis* (Senecl.) Barr. & Golfari	21·94 ± 0·17	Poptun, El Peten Guatemala	16°21′N/89°25′W
Pinus greggii Engelm.	20·68 ± 0·31	Mean	
Northern Mexico	20·61 ± 0·68	Los Lirios, Coahuila, Mexico	25°22′N/100°29′W
		Ojo de Agua, Nuevo Leon, Mexico	24°53′N/100°13′W
		La Tapona, Nuevo Leon, Mexico	24°43′N/100°10′W
Central Mexico	20·75 ± 0·02	El Madrono, Queretaro, Mexico	21°16′N/99°10′W
		Laguna Seca, Hidalgo, Mexico	21°02′N/99°10′W
		San Joaquin, Mexico	20°56′N/99°34′W
Pinus jaliscana Perez de la Rosa	21·78 ± 0·55	La Bulera	20°45′N/104°57′W
		Milpillas	20°44′N/104°53′W
		El Tuito, Mexico	20°21′N/105°12′W
		Purificacion	19°48′N/104°37′W
Pinus maximartinezii Rzedowski	26·75 ± 0·92	Zacatecas, Mexico	21°22′N/103°14′W
Pinus muricata D. Don	20·28 ± 0·25	Fort Bragg, California USA	39°26′N/123°48′W
		Sea Ranch, California USA	38°45′N/123°31′W
		Fort Ross, California USA	38°29′N/123°00′W
Pinus oocarpa Schiede	21·74 ± 0·31	Mean	
Central Mexico	21·11 ± 0·23	Ocotal, Mexico	18°15′N/99°52′W
Southeastern Guatemala	22·38 ± 0·18	La Mina, Chiquimula, Guatemala	14°48′N/89°25′W
		San Jose la Arada, Chiquimula, Guatemala	14°40′N/89°57′W
Pinus patula Schiede et Deppe	21·92 ± 0·11	Mean	
Northern Mexico	21·94 ± 0·07	El Cielo, Tamaulipas, Mexico	23°04′N/99°14′W
		Conrado Castillo, Tamaulipas, Mexico	23°56′N/99°28′W
Central Mexico	21·90 ± 0·24	Ingenio del Rosario, Veracruz, Mexico	19°31′N/97°06′W
		Corralitla, Veracruz, Mexico	18°38′N/97°06′W
Pinus patula var. *longipedunculata* Loock	20·90 ± 0·46	San Mateo Rio Hondo, Oaxaca, Mexico	16°08′N/96°28′W
Pinus pringlei Shaw	22·34 ± 0·31	Acaten, Michoacan, Mexico	19°17′N/101°19′W
		Santo Domingo, Oaxaca, Mexico	17°23′N/97°46′W
		El Guajolote, Guerrero, Mexico	17°09′N/99°56′W
		El Tlahuitoltepec, Oaxacta, Mexico	17°04′N/96°02′W
		Santa Maria Lachixio, Oaxaca, Mexico	16°44′N/97°03′W
		Sola de Vega, Oaxaca, Mexico	16°28′N/96°59′W
Pinus radiata D. Don.	22·43 ± 0·21	Ano Nuevo, California USA	37°06′N/122°17′W
		Monterey, California USA	36°36′N/121°54′W
		Cambria, California USA	35°34′N/121°05′W
Pinus tecunumanii (Schwertfedger) Equiluz et Perry	20·49 ± 0·39	Mean	
Eastern Guatemala	21·43 ± 0·15	El Pinalon, El Progresso, Guatemala	15°04′N/89°54′W
		San Jeronimo, Baja Verapaz, Guatemala	15°03′N/90°18′W
		Finca La Piedad, El Progreso, Guatemala	15°02′N/90°02′W
Belize	20·79 ± 0·27	Mountain Pine Ridge, Cayo, Belize	16°58′N/89°00′W
		San Pastor Ridge, Belize	16°40′N/88°57′W
Nicaragua	19·25 ± 0·67	Las Camelias, Nueva Segovia, Nicaragua	13°46′N/86°18′W
		Yucul, Matagalpa, Nicaragua	12°56′N/85°46′W
		Apante, Matagalpa, Nicaragua	12°54′N/85°56′W

Genome size measurements were based on three megagametophytes and units are picograms per haploid nucleus.

hard pines except *Pinus jeffreyi* in section *Ponderosae*. Genome size of *P. jeffreyi* may reflect shared coancestry via interspecific hybridization because *P. jeffreyi* and *P. coulteri* naturally hybridize (Zobel, 1951).

Regression analysis of latitude and DNA content (Fig. 2) had an R^2 value of 0·001 % and a low slope value of 0·0084 ± 0·15. The slope value was not statistically different from zero. This lack of a latitudinal effect was further supported by the fact that tropical hard pines *P. pringlei* (22·34 pg/C) and *P. tecunumanii* (20·49 pg/C) were within the upper and lower extremes for the temperate hard pines; *P. clausa* was the lowest with 19·94 pg/C and *P. sabiniana*

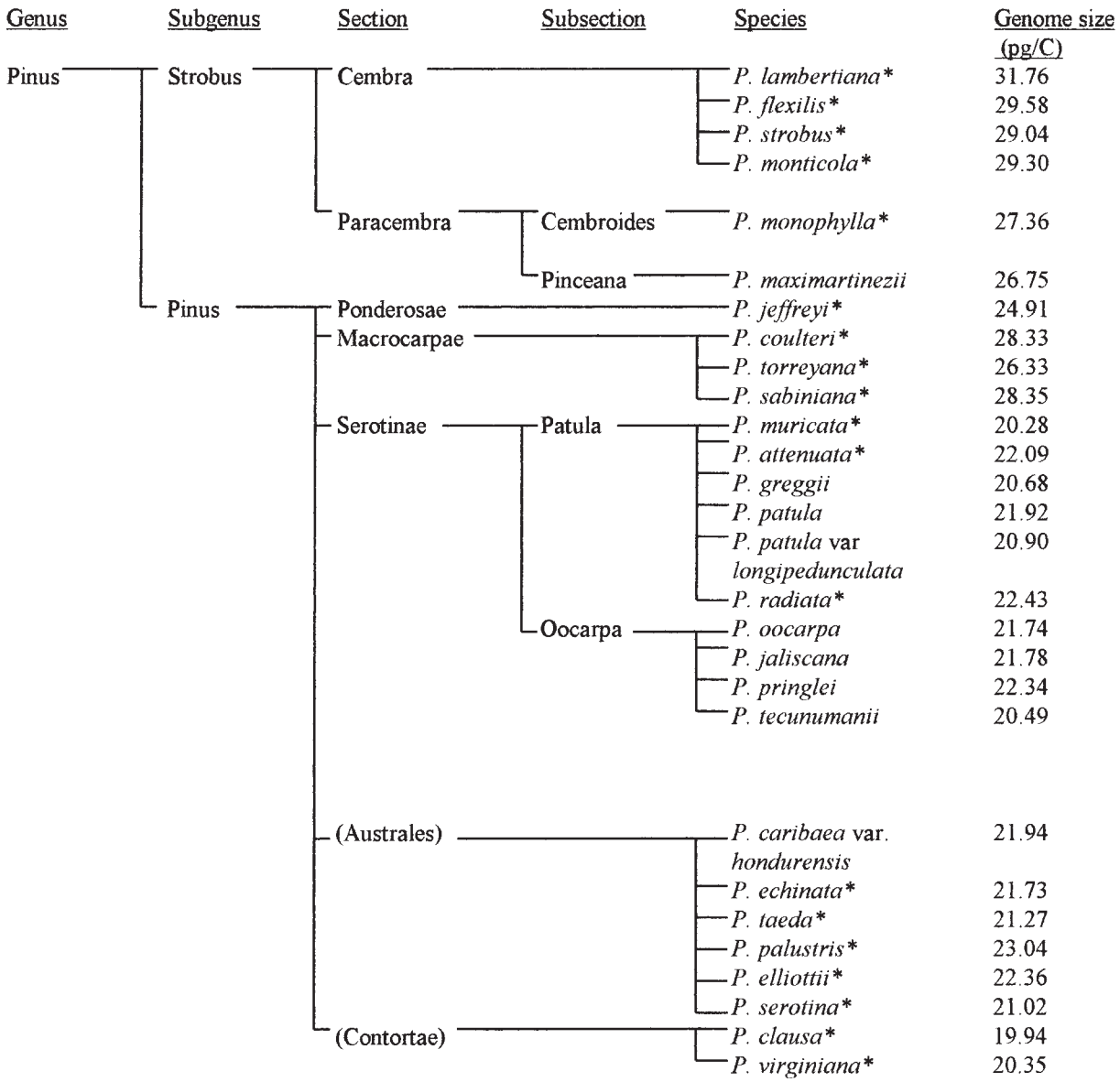

FIG. 1. Patterns of variation in *Pinus* genome size aligned with taxonomic classification based on Perry (1991). Parentheses at the section level indicate supplemental taxonomic classification from Little and Critchfield (1969). Genome size values for temperate and tropical species are indicated with an asterisk (*) and are taken from Wakamiya (1994). *P. radiata* and *P. muricata* were common to both studies; genome size values are from the tropical study.

was the highest with 28·35 pg/C (Fig. 1). Species in the hard pine subsection *Oocarpa* had genome sizes close to 22 pg/C, while soft pines in section *Cembra* had genome sizes closer to 29 pg/C (Fig. 1). Tropical soft pine *Pinus maximartinezii* (26·75 pg/C) had a slightly smaller genome size than temperate soft pines such as *P. strobus* (29·04 pg/C) but there was no latitudinal trend. Temperate soft pine *P. lambertiana* and temperate hard pine *P. clausa* represented the largest difference among the 28 species ($\Delta = 11{\cdot}82$ pg/C) (Fig. 1).

Contrary to trends with temperate pines (Ohri and Khoshoo, 1986; Murray, 1998), intraspecific variation for two of the three tropical *Pinus* species exceeded variation among species and the difference was statistically detectable at the 0·01 % level. Tropical hard pines *P. oocarpa* and *P. tecunumanii* had large intraspecific differences ($\Delta = 1{\cdot}27$ and 2·18 pg/C, respectively) (Table 2). Tropical hard pine *P. patula* showed negligible intraspecific variation but differed from *P. patula* var. *longipedunculata* ($\Delta = 1{\cdot}02$) (Table 2).

DISCUSSION

There is a strong relationship between genome size and taxonomic classification in New World pines. The previous report of a latitudinal trend in genome size for pines (Ohri and Khoshoo, 1986) may be due to imbalanced experimental design. In their study, 20 pine species were randomly

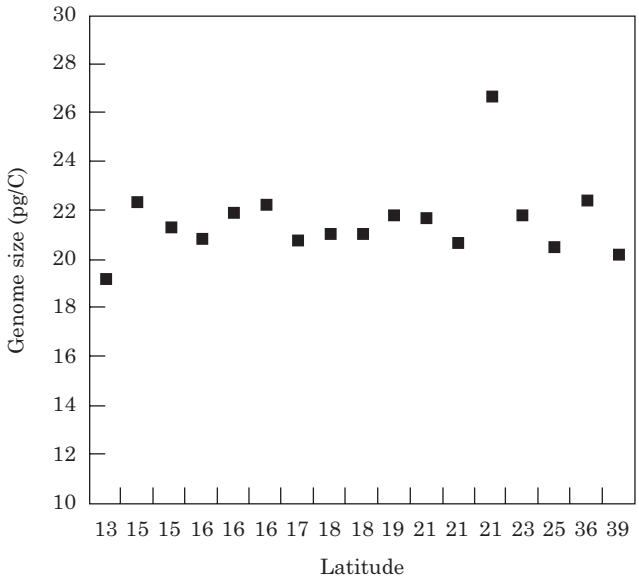

FIG. 2. New World *Pinus* spp. genome size is not explained by latitudinal origin. Data were based on the species and provenances in Table 2.

sampled from America, Europe and Asia. Of the temperate pines sampled, two species were soft pines (Asian pines *P. gerardinana* and *P. wallichiana*), and the other 16 species were hard pines. There were no tropical soft pines; the two pines of tropical origin (New World species *P. patula* and Asian species *P. roxburghii*) were both hard pines. Thus, their tropical *vs*. temperate comparison may be confounded by large genome size differences between hard and soft pines. If so, one would predict that *P. wallichiana* would have large genome sizes relative to hard pines worldwide. A wider test of the taxonomic classification hypothesis is needed for Asian relatives of these New World pine species.

The absence of a latitudinal trend in New World pines can also be attributed to historical movement across latitudes. Pines have historically large populations, hybridize freely with related species and are often invasive colonizers. There has been extensive movement of temperate pine species into tropical montane refugia during the climatic changes of the Eocene (Axelrod, 1986; Millar, 1993) and to a lesser extent during the Pleistocene (Barry, 1983). Contemporary classification of pines as tropical or temperate masks a history of ancient displacement events.

Two modest 1·6-fold difference in genome size for New World pines in this study was consistent with karyotypic orthoselection at the genus level. Over 30 pine species have been karyotyped and there are no apparent structural rearrangements in the pine karyotype and a clear absence of recent polyploidy (e.g. Sax and Sax, 1933; Pederick, 1967, 1970). All species have 12 chromosomes, 11 of which are isobrachial and the twelfth slightly heterobrachial (Saylor, 1961; Pederick, 1970; Dial and Stalter, 1980; Ohri and Khoshoo, 1986). The less conserved aspects of pine karyotypes include variable numbers of rRNA sites ($n > 7$) (Hizume *et al*., 1992).

Karyotypic orthoselection is not an attribute shared by all gymnosperm genera (Murray, 1998). Southern hemisphere gymnosperms, such as the podocarps (*Podocarpus* spp.), have more variability in their chromosome morphology and chromosome number (Davies *et al*., 1997). Genome sizes are similar; for example *Podocarpus acutifolius* has a genome size of 8·2 ± 0·5 pg/C, compared to *Podocarpus totara* with a genome size of 11·2 ± 1·3 pg/C (Davies *et al*., 1997).

Intraspecific variation in tropical pines may be due to the dynamic process of speciation at the primary centre of *Pinus* species diversity. Within-species genome sizes tended to decrease as the species colonized new regions, so that there was a slight tendency for larger genome sizes to occur near the oldest provenances. For *P. tecunumanii*, the northern range has the largest genome size and the smallest genome size is in the southernmost extreme, Nicaragua. Colonization may lead to increased interspecific hybridization which in turn raises genome size variation within parental species. Hybridization cannot explain the presence of interspecific variation among two tropical pines and the notable absence of intraspecific variation in the temperate regions because interspecific hybridization freely occurs among related pine species at all latitudes.

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