Phytogeographic and diversity of *Pinus tropicalis* revealed by analysis of chloroplast DNA sequences

Gretel Geada López[1] and Ko Harada[2]

[1]Universidad de Pinar del Rio, Cuba; [2]Ehime University, Japan

Abstract

Pinus tropicalis Morelet, the tropical pine, is an endemic endangered species restricted to the western part of the Cuban. We investigated the genetic diversity and population structure using cpDNA sequences. A total of 160 individuals from 7 natural populations were collected. Ten haplotypes were distinguishable based on nucleotide substitutions and indels in the sequences of the *trn*T-*trn*L spacer and *trn*L intron. Strong genetic differentiation at both the gene level (G_{ST} = 0.50) and nucleotide level (N_{ST} = 0.78) was found among populations. of the . Analysis of molecular variance (AMOVA) revealed the genetic structure of *P. tropicalis* (Φ_{ST} = 0.52; P < 0.05). The partitions created by AMOVA, when the population were divided into four region: North-Eastern (Galalon), Central (Viñales and Pilotos), North-western (La Jagua, San Juan, Bartolo, Mina Dora) showed that the significant differentiation was due to the high differentiation among geographical regions (Φ_{CT} = 0.499; P < 0.05) rather than the differentiation within the regions (Φ_{SC} = 0.077; P > 0.05), indicating that the gene flow via pollen is limited among geographically distant populations. The minimum-spanning network and neighbour-joining trees of haplotypes showed a strong geographical association among haplotypes, suggesting that historical events have played a major role in the current pattern of cpDNA variation. All the analyses hypothesize that population from the Central (Pilotos) could have served as the ancestral-Miocene refugia, the Galalon population was distantly related to the Central and North-western populations being a unique population at nuclear and cpDNA; therefore, this population should be considered as an Evolutionary Significant Unit (ESU).

INTRODUCTION

Genetic variation in natural populations is a major concern of evolutionary biologists because the amount and distribution of genetic variation is likely to affect the evolutionary potential of species and/or populations. In the last three decades, an enormous amount of data on genetic diversity in natural plant populations has accumulated, and correlations between genetic diversity and various attributes of species such as endemism, mating system and geographic distribution have been examined (Hamrick and Godt, 1989). Although such genetic data have been collected for a wide variety of wild plant species, data on tropical to subtropical and non-continental species are relatively limited (Hamrick and Godt, 1996b). Insular endemic plants have attracted the attention of many evolutionary biologists (Stuessy and Ono, 1998) because they often have unique characteristics that differ from their continental congeners relatives and because remarkable adaptive radiation is often found in insular endemic taxa (Fransisco-Ortega *et al,* 1997). In addition, island species are considered

to be more prone to extinction due to the genetic paucity in populations (Frankham, 1997). Island populations tend to have a lower level of genetic diversity than do continental populations, and from the viewpoint of conservation biology, it is therefore meaningful to examine genetic variation in island endemic species.

Tropical pine (*Pinus tropicalis* Morelet.) is an endemic insular species distributed naturally in Pinar del Río and in Isla de la Pinos in the western part of Cuba. *Pinus tropicalis* and *P. resinosa* represent relic species of the Eurasian lineage of *Diploxylon* pines (section *Pinus*) in North America (Geada *et al,* 2002), and their occurrence in diverse climates in America implies independent entries, probably at Early Paleogene to Late Cretaceous for the ancestor of *P. tropicalis* and at Miocene for *P. resinosa* in northeastern U.S. and Canada (Axelrod, 1986). Therefore, *P. tropicalis* signifies an endemic-aged taxon among the existing pines, but very little is known about the genetic variation in its natural populations (Price *et al,* 1998).

Despite the large range of distributions in Pinar del Río, the natural populations in Isla de Pinos have disappeared (Census of the Academia de Ciencias de Cuba, 1998). Historically, it was distributed in the northern part of Pinar del Río at lower elevation, but is currently only found as discrete populations located in mountainous areas at high altitudes in the northeastern, central and northwestern regions, where populations seem to be more continuous. Interest has recently been shown in the tropical pine because of its economical and ecological importance and because of its declining abundance over much of its range. The decline in its abundance is a consequence of the overexploitation of timber, invasion and plantation of *Pinus caribaea* var. *caribaea* and encroachment of grasses associated with fire exclusion. These phenomena seem to have altered the size and genetic structure of the natural populations and pose a serious threat to the development of mature woodland. As a result, most of the stands of *P. tropicalis* have been decimated; however, studies on the genetic variability of tropical pine have been limited to only few inherited quantitative traits. Knowledge of the genetic variation in this species is important for developing appropriate strategies for *in situ* conservation and regeneration of forests and also for determining whether genetic diversity will be lost through sampling for *ex situ* conservation, which is possible in other species (Hamrick and Godt, 1996a). In addition, for conservation planning for a single-species, it is important to identify the component of evolutionary lineages in order to retain maximum genetic diversity and in order to incorporate information on historical population processes (Moritz, 1994).

Objective: In this study, we sequenced two noncoding regions of cpDNA, *trn*T-*trn*L spacer and the *trn*L intron, and used them as markers to estimate the phylogeographic pattern and the genetic variation of *P. tropicalis*. Because the cpDNA does not recombine and paternally inherited in pines (Ennos, 1994) distinct lineages may be represented in data, including information about the dispersal of pollen and seed.

MATERIALS AND METHODS

Study sites and sampling: The seven natural populations of *P. tropicalis* in Pinar del Río, Cuba were surveyed in roder to evaluate the genetic diversity of the endemic plant. One population in the northeast of Pinar del Río (Galalón), two populations in the central region (Pilotos and Viñales), and four populations in the northwestern region (La Jagua, Bartolo, San Juan and Mina Dora) (Figure 1). Needle samples were collected from individual trees of the populations La Jagua, Pilotos, Galalón, Bartolo and Mina Dora,

and used seedlings from germinated seeds of the remaining two populations we collected. Total DNA was isolated by the CTAB method (Doyle and Doyle, 1990).

Sequence analysis: The *trn*T-*trn*L spacer and *trn*L intron were amplified by PCR using the universal primers described by Taberlet *et al* (1991). PCR products were directly sequenced after purification using a GENECLEAN KIT III (BIO101). DNA sequencing was performed using an ABI 310 Genetic Analyzer (Perkin Elmer) with an ABI BigDye Terminator Cycle Reaction Kit following the manufacturer's instructions. The sequences were aligned visually. Because the chloroplast genome is haploid and does not undergo recombination, it can be viewed as a single locus, and the two regions were thus combined in order to derive a haplotype of each individual. A neighbour-joining tree was generated with the identified haplotypes using MEGA ver. 2 (Kumar *et al,* 2001). The minimum spanning network was constructed with the option implemented in ARLEQUIN version 2.000 (Schneider *et al,* 2000).

Population genetic analysis of the cpDNA sequence variation: Inter- and intra-population genetic diversities were quantified in two: at the nucleotide levels using nucleotide diversity within populations (π_S) and overall populations (π_T) and the coefficient of nucleotide differentiation N_{ST}= (π_T - π_S/π_T), was estimated. At the gene level using gene diversity within a population (H_S), the gene diversity in the entire population (H_T) and the coefficient of gene differentiation (G_{ST}) were estimated, and the UPGMA tree was generated based on Nei's genetic distance (1978) among populations using the program GDA (Lewis and Zaykin 2001). The significance of genetic differentiation between populations and that among regions were tested by molecular analysis of variance (AMOVA) using ARLEQUIN version 2.000 (Schneider *et al,* 2000). The statistical molecular variance of Φ_{CT} (among regions, ie, between northeastern, central and northwestern regions), Φ_{SC} (among populations within regions), and Φ_{ST} (among populations) were estimated. The mean divergence time (T) and the 95% confidence intervals between the populations were estimated using nucleotide divergence between populations (D_{XY}) and the substitution rate per site per year (r) calculated by Geada *et al* (2002) using the method of Haubold and Wiehe (2001).

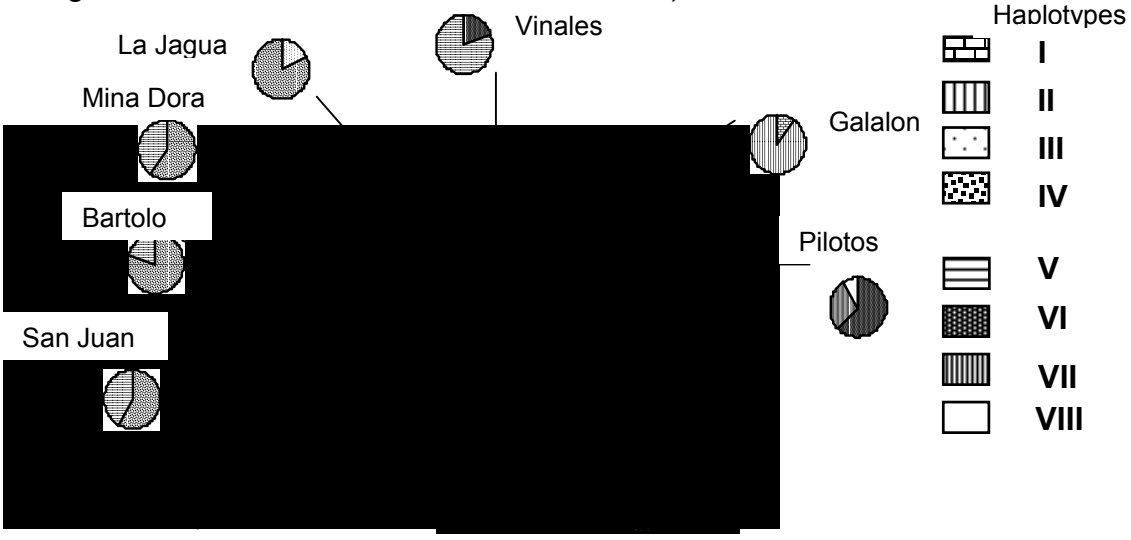

Figure 1. Location of *Pinus tropicalis* populations and haplotypes frequencies per populations.

RESULTS AND DISCUSSION

CpDNA sequence analysis

CpDNA sequences of the two non-coding regions and in all of the individuals from the five populations were determined. Differences of one length differences in the *trn*T-*trn*L spacer, with sizes of 456 to 466 bp, caused by the insertion/deletion of a 10-bp of 5'- AGAAGGGGAG - 3' were detected. No length variation was detected in the *trn*L intron, and the length total of sequences was 524 bp. Four nucleotide substitutions were detected from the completed aligned sequences of the *trn*T-*trn*L spacer and *trn*L intron (980-990 bp). The sequences have been deposited in the DDBJ database under the accession numbers AB097059-AB097066 for the *trn*T-*trn*L spacer and AB097067-AB097074 for the *trn*L intron.

Eight haplotypes were identified according to the numbers of substitutions and indels (Table 1). Haplotypes II, III and VIII were derived from haplotypes I, IV and VII, respectively, differing solely by the repeat motif (5'- AGAAGGGGAG - 3'). Haplotype I was predominant in the population Galalón. Haplotype IV was present in the populations La Jagua, San Juan, Mina Dora and Bartolo. Haplotype III was found at low frequency only in the population La Jagua, while haplotype V was found in the populations San Juan, Mina Dora, Bartolo and Viñales. The haplotype VI was found in the populations Viñales and Pilotos, while haplotypes VII and VIII were found only in the population Pilotos.

Table 1. Distribution of the *cp*DNA haplotypes among populations

Haplotype	**Position**					**Populations**							**Total**
	*trn*T-*trn*L spacer			*trn*L intron		**LJ**	**Ga**	**Ba**	**MD**	**Pi**	**Vi**	**SJ**	
	49	115	349	451	990								
I	A	G		A	C	9							9
II	A	G	*	A	C	1							1
III	A	A	*	T	T		2						2
IV	A	A		T	T		9	7	6			16	22
V	C	A		T	T			5	5		17	4	10
VI	A	A		T	C					15	3		15
VII	A	G		T	C					7			7
VIII	A	G	*	T	C					2			2

Note. The asterisk represented the absence of minisatelitte (-AGAAGGGGAG-) in the haplotype sequence

To improve the accuracy of the estimation of genealogical relationships among haplotypes, a minimum spanning network was constructed by linking sequences in a

hierarchical manner based on mutational changes between them (Figure 2). Within the minimum spanning network, the most closely related chlorotypes were linked by single mutations. The network showed that haplotypes VI and VII were in the interior nodes of the network, serving as linkers to haplotype I with haplotypes IV. According to the network, each haplotype can be detected by one step of mutation. Likewise, the neighbour-joining tree generated from the haplotype sequences (Figure 2) showed two well-defined groups, one of them for haplotypes I, II, VII and VIII and the other for haplotypes III, IV, V and VI. Within the first group, haplotypes II and I were separated from haplotypes VII and VIII, while within the second group, haplotype VI was the first to diverge followed by the divergence of haplotypes III, IV and V.

Population genetic analysis of cpDNA sequence variation

Genetic variation varied among populations, the population Galalón showing the lowest level of gene diversity (0.203) and the population La Jagua showing the next-lowest level of gene diversity (0.411) but with no nucleotide diversity (π = 0.00000), because the two present haplotypes in each population varied only in the repeat motif. The populations Viñales, San Juan, Bartolo, Mina Dora and Pilotos showed higher value of gene diversity (0.268, 0.442, 0.530, 0.533 and 0.539, respectively) and nucleotide diversity (0.00027, 0.00045, 0.00053, 0.00054 and 0.00049, respectively). Most of the genetic variation was restricted to the northwestern and central populations. The nucleotide diversities, π_T and π_S, were 0.00124 and 0.00026, respectively, whereas the gene diversity, H_T and H_S, were 0.762 and 0.417, respectively, indicating that there was a high nucleotide and gene differentiation among the populations (N_{ST} = 0.79 and G_{ST} = 0.45). The UPGMA tree generated from pairwise Nei's distance among populations (Figure 3) showed that the Galalón population was distant to the other populations, while the central populations (Pilotos and Viñales) occupied the basal position followed by the northwestern populations (La Jagua, San Juan, Bartolo and Mina Dora). The nucleotide divergence based on combined sequences was calculated to be 0.00247 $\pm$ 0.0013 between La Jagua and the other populations and 0.00168 $\pm$ 0.0012 between the central populations (Pilotos and Viñales) and the northwestern populations. Using these values, the age of population differentiation was estimated to be 33 MYA with 95% confidence intervals of 10 MYA and 45 MYA between La Jagua and the populations and 5 MYA with confidence intervals of 1 MYA and 18 MYA between central and northwestern populations.

Significant (P < 0.01) genetic differentiation was detected among populations by AMOVA (Table 2). Of the total molecular variance, 52% was attributed to population divergence and 48% was attributed to individual differences within populations. When the total variance was partitioned into three geographical regions, ie, northeastern (Galalón), northwestern (La Jagua, Bartolo San Juan, and Mina Dora) and central (Viñales and Pilotos), the 58% of the total molecular variance was attributed to the differences among regions (Φ_{CT} = 0.5841; $P < 0.01$), 39% was found to individual differences within population (Φ_{ST} = 0.607; $P < 0.01$), and 3% was attributed to populational differences within regions (Φ_{SC} = 0.0570; $P > 0.05$). As a result, significant genetic differentiation was found only among regions and within populations. This indicates that individuals of each population in the northwestern region and central region were more similar to their co-members than to individuals of other populations.

Table 2. Analysis of Molecular Variance (AMOVA)

Sources of Variation	d.f	Variance Component	Φ-statistics (*P*)
Among populations	6	0.2148 (V_a)	Φ_{ST} = 0.5202 (*P* < 0.01)
Within populations	100	0.1981 (V_b)	
Among regions	2	0.2951 (V_a)	Φ_{CT} = 0.4997 (*P* < 0.01)
Among population within regions	4	0.0120 (V_b)	Φ_{SC} = 0.0570 (*P* > 0.05)
Within populations	100	0.1981 (V_c)	Φ_{ST} = 0.6070 (*P* < 0.01)
Total	106	0.5052	

Genetic structures of populations

Allozymes studies have demonstrated that species with a restricted discontinuous distribution or an endemic endangered species often has a low level of genetic diversity relative to the levels of genetic diversity of widespread species with similar life histories (eg, Hamrick and Godt, 1989; Hamrick *et al,* 1992; 1996a). On the other hand, Hamrick and Godt (1996a,b) reported that pines often maintain relatively high levels of genetic variation at the nuclear level (ie, allozymes) and display little genetic differentiation among populations. However, the values of genetic variation found in natural populations of *P. tropicalis* in cpDNA (H_T = 0.762 and H_S = 0.417) are similar to those found in widespread pines such as *P. pinaster* (Ribeiro *et al,* 2001; 2002) and *P. sylvestris* (Provan *et al,* 1998) as well as those found in Californian closed-cone pines (Hong *et al,* 1993) and *P. resinosa* (Walter and Epperson, 2001) with limited distributions. Alternatively, polymorphism can be determined in terms of nucleotide diversity (π), which is the heterozygosity at the nucleotide level (Nei and Kumar, 2000). This value is expressed as $2N_e\mu = \theta$ under a mutation and drift equilibrium, indicating 'genetic health' of a population (Fu and Li, 1999). There have been few studies on nucleotide variation of organelle DNA in out-crossing, long-lived plants at populational levels (Matos and Schaal, 2000), and such plants are expected to have lower values of nucleotide diversity and nucleotide differentiation among populations (Dvornyk *et al,* 2002). However, we observed a high level of nucleotide diversity in *P. tropicalis* (π_T = 0.00124 and π_S = 0.00026), similar to the values reported for *P. radiata* and *P. muricata* (Hong *et al,* 1993) and *Pinus montezumae*-complex (Matos and Schaal, 2000). Hong *et al* (1993) found that nucleotide diversities within populations of *P. radiata* were as low as 0.002 and were from 0.0011 to 0.0033 in *P. muricata*, while Matos and Schaal (2000) calculated the nucleotide diversities in *P. hartwegii* and *P. montezumae* to be in the range of 0.0018 to 0.0025.

Although strong gene differentiation was found in the *cp*DNA of *P. tropicalis*, this value is not surprising for *Pinus*; for example, strong differentiation was found in *P. muricata* with G_{ST} = 0.87 (Hong *et al,* 1993) and in *P. resinosa* (θ = 0.56) (Walter and Epperson, 2001). G_{ST} in *P. ponderosa* was calculated to be 0.67 (Latta and Mitton, 1999), and G_{ST} in Douglas-fir was estimated to be 0.20 (Hong *et al,* 1995). Nevertheless, lower values of differentiation have been reported among seven populations of *P. flexilis* (G_{ST} = 0.013) (Latta and Mitton, 1997), *P. attenuata* and *P. radiata* (G_{ST} = 0.00 - 0.011) (Hong *et al,* 1993), *P. pinaster* (G_{ST} = 0.023 and 0.038) (Ribeiro *et al,* 2001; 2002), *P. resinosa* (G_{ST} = 0.12) (Echt *et al,* 1998), *P. albicaulis* (G_{ST} = 0.046) (Richardson *et al,* 2002), *P. banksiana* (G_{ST} = 0.020) and *P. contorta* (G_{ST} = 0.018) (Dong and Wagner, 1994). In contrast, no genetic differentiation was found among populations of Canadian Douglas-fir (Viard *et al,* 2001) and *P. torreyana* (Waters and Schaal, 1991). Therefore, genetic diversity and population structure vary considerably within and among species (Hamrick *et al,* 1992). The chief differences among pines involve geographic range, degree of spatial isolation of populations, and successional stage of their habitats.

Our analyses demonstrated that the genetic structure in the cpDNA variation of the tropical pine is strongly correlated with the geographical distribution of its haplotypes, which somehow characterized each region and populations. Templeton *et al* (1995) suggested that three factors mainly cause significant spatial/temporal association of haplotype variations: restricted gene flow, past fragmentation events and range expansion events. Schaal *et al* (1998), on the other hand, reported that geographical structure of the genetic variation in a population of plants is mainly the result of isolation and genetic drift, while gene flow between populations counteracts differentiation. However, those factors are not mutually exclusive alternatives but under certain conditions create to some degree a geographical and/or genetic structure of populations (Templeton, 1998).

In the model of isolation by distance (Slatkin and Maddison, 1990), restricted gene flow prevents newly arisen mutations (ie, haplotypes) from spreading, and these mutations will reside as “private” alleles or haplotypes of the original populations. On the other hand, the ancestral haplotype is older than its mutation offshoots, and must therefore exist at higher frequency and be more widely distributed. This explains the low frequency and limited distribution of haplotypes II, III and VIII as well as their external positions in the minimum-spanning network (Figure 2). Past fragmentation events will lead to the fixation of some distinctive haplotypes in each population, particularly because of the reduction in the effective population size and the effect of genetic drift. We believe that these factors have been involved in the current cpDNA distribution pattern of *P. tropicalis*. It is likely that past fragmentation events occurred during the Miocene-Pliocene (López, 1982) and subsequently restricted gene flow among distantly located populations. Range expansion events might have played a minor role in the present distribution of *P. tropicalis*, because no haplotypes were shared among geographically distant populations and the ancestral haplotypes reside in a restricted area.

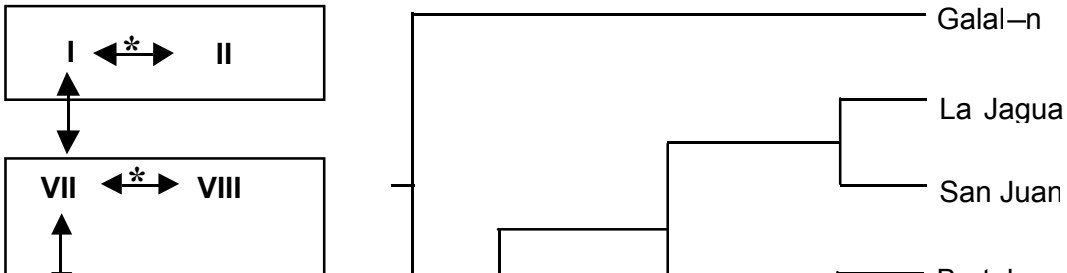

Figure 2. Relationships among haplotypes and populations. Left: Minimum-spanning network of the haplotypes detected in *P. tropicalis* using the *trn*T-*trn*L spacer and *trn*L intron. The asterisks represent the absence of minisatelitte -AGAAGGGGAG- in the haplotype sequence. Right: UPGMA tree generated on the pairwise distance among populations based on Nei's distance 1978.

The network of haplotypes provides insights into the migration history and distribution of genetic variation in *P. tropicalis*. According to our results, haplotypes are tightly clustered in the network and in concordance with the geographical region, without any missing intermediate haplotypes. This can be interpreted as isolation by distance rather than a past fragmentation events (Schaal *et al,* 1998). The presence of only two haplotypes in the northwestern populations is also evocative of an ancient major bottleneck or that those populations could be originated from one ancestral haplotype probably from Pilotos. Although the current northwestern populations are geographically distant, they might have been a full-size population at chloroplast levels until Pleistocene. The contact among those populations can be maintained by strong winds with a preferential southwest direction (Samek and Del Risco, 1990).

Historical factors

Pinar del Río is Cuban Jurassic-Cretaceous orogen whose constitution and evolution differ from the other Greater Antilles and is acknowledged as an edaphically distinct unit (Lewis and Draper, 1990; Kerr *et al,* 1999). The pine forests are the paraclimax *sensu* Texün (Borhidi, 1996) vegetal formation in Pinar del Río that is like one formation whose succession was broken or stopped by particular edaphic factors, especially by the impossibility of maturation of the soils (Samek and Del Risco, 1990).

According to palynological data (Areces, 1987) and sequence data (Geada *et al,* 2002), the ancestor of the *P. tropicalis* entered Cuba during Oligocene from the north of Pinar del Río. The paleogeography of this area during Oligocene-Early Miocene (Kerr *et al,* 1999) shows that Pinar del Río was divided into two emerged land areas: 1) the northeastern area including the Bahia Honda unit (ie, where Galalón is located) and small a section of the current central Pinar del Río (ie, where Pilotos is located) and 2) and the Alturas de Pizarra, but isolated from each other by the sea. Thus, the

establishment of *P. tropicalis* in Alturas de Pizarra could not have been possible until Late Pliocene (Borhidi, 1996; Areces, 1987). One possible scenario is that the founder population of *P. tropicalis* resided in the central-north part of Pinar del Río, as suggested by Samek and Del Risco (1990), and the current Pilotos population may represent a relic population of the ancestral one (see the minimum spanning network.). Not only the haplotype network but also the higher degree of haplotype diversity in Pilotos supports the idea of this population being a founder rather than the notion of it being a population formed by an admixture of haplotypes of west and east. However, the only available information is reduced to the recognition of morphologically diverged groups in the natural population of *P. tropicalis*, the northeastern populations (including Pilotos) and the northwestern populations (López, 1982; Padilla, 1999). The ancestral population could have had a wide-ranging of distribution, expanding from the central to northeast area (see Figure 1; Samek and Del Risco, 1990, pp.13). According to that, La Jagua and Pilotos populations were continuous during Miocene and up until late Pliocene. However, the further formation of Sierra del Rosario mountain system constituted a natural barrier, preventing a homogenizing effect of gene flow and allowing genetic divergence between the two groups of populations, Galalón in the northeastern area and Pilotos in the central area. Moreover, during Pleistocene, with invasion of *Pinus caribaea* and the complete emergence of Sierra de Cajálbana restricted more the east-west exchange between populations and imposed a strong bottleneck, thus leading to the fixation of different haplotypes in each population. For example, haplotypes II and I are present only in the population Galalón, while Haplotypes VI, VII and VIII were found only in Pilotos.

The land connection between Alturas de Pizarra and northeast of Pinar del Río at the end of Pliocene favoured the migration and colonizations of new areas in the northwest. The current pattern of variation in cpDNA in the northwestern populations is a result of the expansion of *P. caribaea* to low-to-middle elevations, which confined *P. tropicalis* to the most remote and extreme sites, particularly during Pleistocene. Habitat fragmentation and disturbance due to cleaning invasion of meadow grass and anthropogenic activities during the last four centuries could also have resulted in reductions in population sizes and could have led to genetic isolation; however those effects were less pronounced in long-lived species with a long generation time. Thus, cpDNA reflects the very ancient historical events (ie, founder or bottleneck) rather than actual events.

CONCLUSION

The results presented here, clearly show that *Pinus tropicalis* populations are highly genetic structured due to mainly the strong geographical distribution of the genetic variation in terms of haplotypes diversity. The specie retains a large diversity at gene as well as nucleotide levels. The historical factor, during the formation of Pinar del Río province and the marine introgressions during the Quaternary have a crucial importance in the current pattern of diversity. On the other hand, the limited gene flow among the populations has also helped to enforce this phytogeographic pattern. The population of Galalón was identified as unique population, thus could be treated like an Evolutionary Significant Unit.

REFERENCES

Areces A (1987). Consideraciones sobre la supuesta presencia de *Pinus sylvestris* L. en el Oligoceno de Cuba. Publicación Centro Investigaciones del Petróleo. *Serie Geológica* **2**, 27-40.

Axelrod DI (1986). Cenozoic history of some western American pines. *Ann Missouri Bot Gard* **73**: 565-641.

Betancourt A (1987). *Silvicultura especial de árboles maderables tropicales*. Científico-Técnico: Havana. Cuba.

Borhidi A (1996). *Phytogeography and Vegetation Ecology of Cuba*. Akademiai Kiado: Budapest, Hungary.

Dong J, Wagner DB (1994). Paternally inherited chloroplast polymorphism in *Pinus*: estimation of diversity and population subdivision, and test of disequilibrium with a maternally inherited mitochondrial polymorphism. *Genetics* **136**: 1187-1194.

Doyle J, Doyle L (1990). Isolation of plant DNA from fresh tissue. *Focus* **12:** 13-15.

Dvornyk V, Sirvio A, Mikkonen M, Savolainen O (2002). Low nucleotide diversity at the *pal*1 locus in the widely distributed *Pinus sylvestris*. *Mol Biol Evol* **19:** 179-188.

Echt CS, De Verno LL, Anzidei M, Vendramin GG (1998). Chloroplast microsatellite revealed populations genetic diversity in red pine, *Pinus resinosa* Ait. *Mol Ecol* **7**: 307-316.

Ennos RA (1994). Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* **80:** 584-593.

Frankham R (1997). Do island populations have less genetic variation than the mainland populations? *Heredity* **78:** 311-327.

Fransisco-Ortega JA, Crawford DJ, Santos-Guerra A, Jansen RK (1997). Origin and evolution of *Argyranthemum* (Asteraceae: Anthemideae) in Micronesia. In: Givnish TJ, Systma KL (eds) *Molecular Evolution and Adaptive Radiation*, Cambridge University Press: UK. pp 407-432.

Fu YX, Li WH (1999). Coalescing into the 21st century: An overview and prospects of coalescent theory. *Theor Popul Biol* **56:** 1-10.

Geada- López G, Kamiya K, Harada K (2002). Phylogenetic relationships of *Diploxylon* pines (subgenus *Pinus*) based on plastid sequence data. Int J Plant Sci **163:** 737-747.

Hamrick JL, Godt MJW (1989). Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS Plant (eds) *Population Genetics, Breeding and Germplasm Resources*, Sinauer Associates: Massachusetts. pp 43-63.

Hamrick JL, Godt MJW (1996a). Conservation genetics of endemic plant species. In Avise JC, Hamrick JL (eds) *Conservation Genetics: Case Histories from Nature*, Chapman and Hall: London. pp 281–304.

Hamrick JL, Godt MJW (1996b). Effects of life history traits on genetic diversity in plant species. *Phil Trans Roy Soc London Biol Sci* **351:** 1291-1298.

Hamrick JL, Godt MJW, Sherman-Broylers SL (1992). Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6:** 95-124.

Haubold B, Wiehe T (2001). Statistics of divergence time. *Mol Biol Evol* **18:** 1157-1160.

Hong YP, Hipkins VD, Strauss SH (1993). Chloroplast DNA diversity among trees, populations and species in the Californian closed-cone pines (*Pinus radiata, P. muricata and P. attenuata*). *Genetics* **135:** 1187-1196.

Hong YP, Ponoy B, Carlson JE (1995). Genetic diversity and phylogeny in Douglas-fir based on RFLP and RAPD (DAF) analysis of nuclear, chloroplast and mitochondrial genomes, In: Baradat PH, Adams WT, Muller-Starck G (eds) *Population Genetics and Genetic Conservation of Forest Trees*, Academic Publishing: Amsterdam. pp 276-266SPB.

Kerr AC, Iturralde-Vinent MA, Saunders AD, Babbs TL, Tarney J (1999). A new plate tectonic model of the Caribbean: Implication from a geochemical reconnaissance of Cuba Mesozoic volcanic rock. *Geol Soc Amer Bull* **111:** 1581-1599.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* **17**: 1244-1245.

Latta RG, Mitton JB (1997). A comparison of population differentiation across four classes of gene markers in limber pine (*Pinus flexilis* James). *Genetics* **146:** 1153-1163.

Latta RG, Mitton JB (1999). Historical separation and present gene flow through a zone of secondary contact in ponderosa pine. *Evolution* **53:** 769-776.

Lewis JF, Draper G (1990). Geology and tectonic evolution of northern Caribbean margin. In: Dengo G, Case JE (eds) *The Caribbean Region: Boulder, Colorado.* Geological Society of America: USA. Vol. H, pp 77-138.

Lewis PO, Zaykin D (2001). Genetic Data Analysis: Computer Program for the Analysis of Allelic Data, Version 1.0 [d16c]. Available at http://lewis.eeb.uconn.edu/lewishome/software.html

López A (1982). Variabilidad del género *Pinus* (Coniferales: Pinaceae) en Cuba. *Acta Botánica Cubana* **12:** 1-32.

Matos JA, Schaal BA (2000). Chloroplast evolution in the *Pinus montezumae* complex: a coalescent approach to hybridization. *Evolution* **54:** 1218-1233.

Moritz C (1994). Defining evolutionary significant unit for conservation. *Trends Ecol Evol* **9:** 373-375.

Nei M, Kumar S (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press: New York.

Padilla TG (1999). Tablas dasométricas para plantaciones de *Pinus tropicalis* Morelet. Ph.D. Thesis, Pinar del Río University.

Price RA, Liston A, Strauss SH (1998). Phylogeny and systematics of *Pinus*. In: Richardson DM (ed) *Ecology and Biogeography of Pinus*, Cambridge University Press: Cambridge. pp 49-68.

Provan J, Sorano N, Wilson NJ (1998). Gene-pool variation in caledonian and European Scots pine (*Pinus syvestris* L.) revealed by chloroplast simple-sequence repeats. *Proc Roy Soc London, Serie B-Biological Science* **265:** 1697-1705.

Ribeiro MM, Mariette S, Vendramin GG, Szmidt AE, Plomion C, Kremer A (2002). Comparison of genetic diversity estimates within and among populations of maritime pine using chloroplast simple-sequence repeat and amplified fragment length polymorphism data. *Mol Ecol* **11:** 869-877.

Ribeiro MM, Plomion C, Petit R, Vendramin GG, Szmidt AE (2001). Variation in chloroplast single-sequence repeats in Portuguese maritime Pine (*Pinus pinaster* Ait.). *Theor Appl Genet* **102:** 97-103.

Richardson BA, Brunsfeld SJ, Klopfenstein NB (2002). DNA from bird-dispersed and wind-disseminated pollen provides insights into postglacial colonization and

population genetic structure of whitebark pine (*Pinus albicaulis*). *Mol Ecol* **11:** 215-227.
Samek V, Del Risco E (1990). Los pinares de la provincia Pinar del Río, Cuba. Ed. Academia, La Habana. Cuba.
Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998). Phylogeographic studies in plants: problems and prospects. *Mol Ecol* **7:** 465-474.
Schnieder S, Rosseli D, Excoffier L (2000). *Arlequin ver. 2.000: A software for Population Genetic Analysis*. Genetic and Biometry Laboratory. University of Geneva, Switzerland. Available at http://anthro.unique.ch/arlequin
Slatkin M, Maddison WP (1990). Detecting isolation by distance using phylogenies of gene. *Genetics* **126:** 249-260.
Stuessy TF, Ono M (1998). *Evolution and speciation of island plants.* Cambridge University Press: Cambridge.
Taberlet P, Gielly L, Pautou G, Bouvet J (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol Biol* **17:** 1105-1109.
Templeton AR (1998). Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Mol Ecol* **7:** 381-397.
Templeton AR, Routman E, Phillips CA (1995). Separating population structure from population history: a cladistic analysis of geographical distribution of mitochondria DNA sequence data III Cladogram estimation. *Genetics* **140:** 767-782.
Virad F, El-Kassaby YA, Ritland R (2001). Diversity and genetic structure in population of *Pseudotsuga menziesii* (Pinaceae) at chloroplast microsatellite loci. *Genome* **44:** 336-344.
Walter R, Epperson BK (2001). Geographic pattern of genetic variation in *Pinus resinosa*: area of greatest diversity is not the origin of postglacial populations. *Mol Ecol* **10:** 103-111.
Waters ER, Schaal BA (1991). No variation is detected in the chloroplast genome of *Pinus torreyana*. *Can J For Res* **21:** 1832-1835.