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Phylogenetic Analysis of Mexican Pine Species Based on Three Loci from Different Genomes (Nuclear, Mitochondrial and Chloroplast)

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1. Introduction

1.1 Broad outline of the genus *Pinus*

Pines are the most important group of conifers. The genus *Pinus* emerged between Late Jurassic to Early Cretaceous period more than 150 million years ago (Gernandt et al., 2008). Like other conifers, pine trees are characterized by naked ovules, i.e. not protected inside an ovary. Because of this trait, pine trees do not have true fruits, bearing instead structures called cones (Arber & Parkin, 1907; Cronquist, 1968). Pines are distinguished from other conifers by needle-shaped leaves (aciculae), usually in clusters of 2 to 5, forming structures called fascicles (Farjon & Styles, 1997).

Several groups of coniferous trees are well represented in Mexico (Rzedowski, 1978). According to Martínez (1948), Farjon (1996) and Farjon & Styles (1997), Mexico was a second center of diversity of the genus *Pinus*, this country having the largest number of records of species of this genus. Both Martínez (1948) and Mirov (1967) recognized 38 species and 25 varieties or forms of pines in Mexico, but the number is now higher – over 45 species are recognized at present (Farjon & Styles, 1997; Perry et al., 1998).

The genus *Pinus* has two well-defined subgenera: on the one hand, trees with leaves having a single vascular bundle and several external resin canals, as well as fascicles with a usually deciduous sheath. These form the subgenus *Strobus*, some 15 species of which occur in Mexico, classified in two sections and three subsections (Farjon & Styles, 1997). On the other hand is the subgenus *Pinus* that is characterized by leaves with two vascular bundles and fascicles with a usually persistent sheath while still attached to the tree; in some cases the sheath persists even after the fall of the fascicle (Farjon & Styles, 1997). In Mexico, 34 species of this subgenus are recognized which are classified in two sections and six subsections according to Farjon & Styles (1997).

Pine forests in Mexico are usually heterogeneous in terms of both species and age of the trees. The latter may attain 1.2 m in diameter and 50 m in height. In natural forest (Figure 1), however, the largest trees may measure up to 1.5 m in diameter and may exceed 250 years in age (Challenger, 1998).

Fig. 1. *Pinus ayacahuite* at the National Park "Lagunas de Zempoala", Mexico.

1.2 Selected species and reproduction

During the 20th century there was a huge increase in the number of pine plantations in many countries. Species were selected with care trying to know the origin of those to be used, and genetic knowledge of seed-producing trees was required (Le Maitre, 1998). Thus began the use in pines of traits of origin imposed by countries importing seed for tree plantings (France, Japan, Australia and New Zealand). The intent was to grow higher yielding trees in the soil types and climates of countries where they are not native (India, Brazil, Australia, South Africa and the Philippines) (Busby, 1991). One such case is the use of *Pinus tecunumanii* in South Africa (Chapman et al., 1995).

Advances in biotechnology and modern genetics have furthered *in vitro* cultivation. Similarly, the use of molecular tools to reconstruct the phylogeny of pines has helped improve the efficient use of certain species (Gernandt et al., 2005). This has increased the use of Mexican species including *P. patula, P. oocarpa, P. chiapensis, P. maximinoi* and *P. devoniana* (Le Maitre, 1998).

1.3 Expansion of the use of Mexican pines

In 1963, a broad study was initiated of the options available for reforestation of vast deforested areas in Kenya and South Africa. This study recommended *P. caribaea* for use in these countries, given its tropical distribution (Styles, 1998). From that time on, the British government has financed seed collection and began collaborating with the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecurias (INIFAP) and Universidad Autónoma Chapingo in the study of Mexican pines capable of growing

successfully in highly deforested areas within the British Commonwealth. Botanical and forestry studies have been focussed on the Forestry Institute (Oxford University Department of Plant Sciences) where much of the most significant information on the pines of Mexico and Central America is deposited, the book by Frajon & Styles (1997) being the fruit of this labor.

In forestry conferences held since 1963, the list of potentially usable pine tree species was enlarged, with *P. oocarpa*, *P. patula, P. tecunumanii, P. chiapensis, P. maximinoi* and *P. pseudostrobus* being recommended for reforestation or commercial exploitation in different countries. The use of these species has spread to other non-Commonwealth countries such as Argentina, Brazil, the Philippines, Puerto Rico and Colombia which now use Mexican pine species in their logging industry (Le Maitre, 1998).

1.4 Classification of pines

In recent years a large number of phylogenetic studies have been made of the genus *Pinus*. Thus, Strauss & Doerksen (1990) analyzed the pattern of restriction fragments, including at least one species of each of the 15 subsections proposed for the genus in the 1969 study by Little & Critchfield. Karalamangala & Nickrent (1989) studied the relationships of 14 taxa of the subgenus *Pinus* using isozyme loci. Krupkin et al. (1996) conducted a phylogenetic analysis of species of the subgenus *Pinus*, based on RFLP of chloroplast DNA. Later, Liston et al. (1999) constructed a phylogeny of the genus using sequences of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. Gernandt et al. (2005) made a review of the genus, proposing that sections and subsections be classified based on morphological and genetic (the genes MatK and rbcL) characters. Recently, Parks et al. (2009) performed a reconstruction of several groups of pines using the full chloroplast genome and obtaining greater resolution than with the use of other biomarkers.

The genus *Pinus* is recognized as a naturally occurring group, and the two subgenera are also widely recognized (even though their names have been changed). However, subdivisions within each subgenus remain highly controversial, and a large number of classifications have been published since Shaw's in 1914. One of our purposes in the present this study was to determine which of the latest proposals more closely approaches the evidence of markers from different genetic regions.

The most generally accepted classifications of pines, including Mexican species, are found in three studies that make use of both morphological (Farjon & Styles, 1997) and molecular – MatK and rbcL genes (Gernandt et al., 2005) – information, as well as evidence from different sources including chemical, distribution, morphological and molecular data (Price et al., 1998). These classifications are shown in Table 1.

However, as Table 1 shows, despite all the studies undertaken, a number of ancestor-descendant relationships in Mexican species included within some subsections of the genus remain unclear, perhaps due to their recent origin.

With these antecedents as a background, the aim of the present study was to examine Mexican species of pines and reconstruct their phylogenetic relationships using three types of genetic material with very different characteristics. Thus, we seek to provide a summary of certain studies (including those of our study team) in order to establish the phylogenetic and evolutionary relationships of Mexican species of pines, as these are the most commonly used species in plantations outside their region of origin (Le Maitre, 1998).

	Proposed subsection		
Species	Farjon & Styles, 1997	Price et al., 1998	Gernandt et al., 2005
P. attenuata	Attenuata	Attenuatae	Australes
P. ayacahuite	Strobi	Strobi	Strobus
P. caribea	Australes	Australes	Australes
P. cembroides	Cembroides	Cembroides	Cembroides
P. coulteri	Attenuata	Ponderosae	Ponderosae
P. culminicola	Cembroides	Cembroides	Cembroides
P. devoniana	Pseudostrobi	Ponderosae	Ponderosae
P. douglasiana	Pseudostrobi	Ponderosae	Ponderosae
P. durangensis	Oocarpae	Ponderosae	Ponderosae
P. engelmannii	Ponderosa	Ponderosae	Ponderosae
P. flexilis	Strobi	Strobi	Strobus
P. greggii	Attenuata	Oocarpae	Australes
P. hartwegii	Ponderosa	Ponderosae	Ponderosae
P. herrerae	Contortae	Oocarpae	Australes
P. jeffreyi	Ponderosa	Ponderosae	Ponderosae
P. lambertiana	Strobi	Strobi	Strobus
P. lawsonii	Oocarpae	Oocarpae	Australes
P. leiophylla	Leiophyllae	Leiophyllae	Australes
P. lumholtzii	Pseudostrobi	Leiophyllae	Australes
P. maximartinezii	Parrayanae	Cembroides	Cembroides
P. maximinoi	Pseudostrobi	Ponderosae	Ponderosae
P. monophylla	Cembroides	Cembroides	Cembroides
P. montezumae	Pseudostrobi	Ponderosae	Ponderosae
P. muricata	Attenuata	Attenuatae	Australes
P. nelsonii	Nelsoniae	Cembroides	Balfourianae
P. occidentalis	Australes	Australes	Australes
P. oocarpa	Oocarpae	Oocarpae	Australes
P. patula	Oocarpae	Oocarpae	Australes
P. pinceana	Nelsoniae	Cembroides	Cembroides
P. ponderosa	Ponderosa	Ponderosae	Ponderosae
P. praetermissa	Oocarpae	Oocarpae	Australes
P. pringlei	Oocarpae	Oocarpae	Australes
P. pseudostrobus	Pseudostrobi	Ponderosae	Ponderosae
P. quadrifolia	Cembroides		Cembroides
P. radiata	Attenuata	Attenuatae	Australes
P. remota	Cembroides	Cembroides	Cembroides
P. rzedowskii	Parrayanae	Rzedowskianae	Cembroides
P. strobiformis	Strobi		
P. strobus	Strobi	Strobi	Strobus
P. teocote	Oocarpae	Oocarpae	Australes
P. tropicalis	Pinus	Pinus	Pinus

Table 1. Three infrageneric classification systems of Mexican pines. Columns show the subsection assigned by each group of authors to a given species.

2. Genomes of *Pinus* used in this study

Three noncoding loci (ITS, MatK and Nad1) were used in the analysis for the present study. Thus, we have three DNA regions that are not subject to selective restrictions. These three noncoding regions were selected from three different genomes exhibiting different forms of inheritance in pines (Petit & Vendramin 2007). The first locus used is a region formed by the internal transcribed spacers (ITS1 and ITS2) and a fragment of the gene 5.8S. The latter is a nuclear gene transmitted by both parents to offspring (Soltis et al., 2000). The second fragment is a chloroplast mutase K (MatK) that in most pine species is inherited through the father, i.e. through pollen (Soltis et al., 2000). Finally, a fragment of the intron 2 of subunit 1 of NADH dehydrogenase present in the mitochondrion was used (Nelson & Cox, 2004). This locus is inherited exclusively through the mother and provides some idea of the inheritance of female functions in pines (Mitton et al., 2000). Table 2 lists the accession numbers for each sequence in each of the species.

2.1 Procedure for obtaining the sequence of the Nad1 gene

We amplified and sequenced a fragment of the intron 2 of the Nad1 gene, which as stated previously, is a region of the pine mitochondrion. The GenBank already had sequences for MatK (Gernandt et al, 2005), ITS1 and ITS2 (Liston et al., 1999), but reconstruction had not been made using this region. The procedure used to obtain the sequence is described below.

2.2 Extraction of total DNA

Extraction was performed using the 2% CTAB (cetyl trimethyl ammonium bromide) and chloroform-isoamyl alcohol method: 500 mg of foliage material was taken from each species and macerated with liquid nitrogen to obtain a fine powder, following the Doyle & Doyle (1987) protocol.

2.3 PCR amplification

To amplify a fragment of the intron 2 of the mitochondrial gene Nad1 by polymerase chain reaction (PCR), the primers designed by Mitton et al. (2000) and Vargas et al. (2006) were used. Both oligonucleotides have an annealing temperature (Tm) of 59.5 ºC. Amplification reactions were performed in a Biometra® T-Personal thermocycler under the following conditions: initial denaturation temperature 94 ºC for 5 min, and 30 cycles of denaturation at 92 ºC for 1 min, alignment at 59.3 ºC for 1 min, extension at 72 ºC for 3 min; and a final extension at 72 ºC for 5 min.

The final volume of the PCR reaction mixture was 50 µL and its composition was as follows: 5 µL buffer 10X, 3 µL $MgCl_2$ 3 mM, 4 µL dNTP 400 mM, 1.5 µL nad1G15 pM, 1.5 µL 730F 15 pM, 0.2 µL albumin at 2.95 mg/mL, 0.25 µL of 1.25 U Taq polymerase, 2 µL of DNA at 25 ng/µL, and finally 32µL H_2O.

2.4 Clonation

Clonation was performed with the TOPO TA Cloning ® Five-minute cloning of Taq polymerase-amplified PCR products kit (Invitrogen™) as described below.

2.4.1 Insertion of the DNA fragment

The clonation vector was the (linear) synthetic pCR 2.1-TOPO plasmid that has genes of tolerance to kanamycin and ampicillin, which were used as selection markers. This plasmid

also has the promoter of the Lac gene (Lac P) and the Lac Zα fragment where the gene in question is inserted, since a topoisomerase is covalently connected on its 3' extremes and an unpaired thymidine is attached on its thyroxine 274 residue via a bond with a phosphate group. The plasmid also has two origins of replication (ColE1 ori and f1 ori), which ensures vector replication.

Ligation occurs since Taq polymerase has terminal transferance activity, which adds a desoxyadenine on the 3' extreme of the PCR product. This desoxyadenine is complementary to the thymine residue present in the vector and through base complementarity the two are aligned, promoting rupture of the topoisomerase phosphate bond and the release of energy that is coupled by the topoisomerase ligating the DNA fragments.

Reaction conditions were as follows: in a microcentrifuge tube was placed 0.5 to 4 µL of fresh PCR product, 1 µL of saline solution, sterile water to attain a volume of 5 µL, and 1 µL of TOPO® vector to obtain a final volume of 6 µL. The reaction mixture was mixed gently prior to incubation at room temperature for 5 min. It was subsequently incubated in ice until transformation.

2.4.2 Procurement of competent cells (transformation)

The strains *Escherichia coli* DH5αTM – T1 R and *Escherichia coli* Mach1 TM –T1 R were provided by the manufacturer of the kit and had attained competent state as a result of chemical treatment by the supplier. They were kept frozen until transformation.

Both strains are characterized by sensitivity to kanamycin and ampicillin. To each of the vials containing the strains was added 2 µL pCR-TOPO 2.1 vector with the insert. Contents were mixed gently and incubated in ice for 5 to 10 min, after which thermal shock was applied for 30 s at 42 ^{o}C. The vials were again placed in ice. Then 250 µL of SOC medium (supplied by the manufacturer) was added at room temperature. Vials were capped and centrifuged at 200 rpm for 1 h. Transforming cells (10-50 µL) were seeded in plates containing LB medium with 50 µg/mL of ampicillin or kanamycin, supplemented with 40 µL Xgal at 40 mg/mL and pre-incubated at 37 ^{o}C in duplicate (to ensure procurement of isolated colonies, 20 µL of SOC medium was added). The plates were incubated at 37 ^{o}C for 8 to 12 h.

2.4.3 Selection and storage of transforming cells

Transforming cells are white with a light blue tint and negative ones are a deep blue color as a result of Xgal hydrolysis.

Ten or more colonies were selected and cultured overnight in LB medium with 50 µg/mL of ampicillin or kanamycin. Subsequently, aliquots were taken and reseeded for 4 h. To optimize clonation results, several colonies were collected.

The colonies collected were inoculated in 1 to 2 mL of LB medium with 50 µg/mL of ampicillin or kanamycin and incubated to attain stationary phase. Then 850 µL of the culture was mixed with 150 µL sterile glycerol in a cryotube, storing the latter at –80 ^{o}C until further use.

2.5 Sequencing

Sequencing of the DNA fragment was carried out by the Sanger method in a Perkin Elmer ABI Prism 910 genetic sequence analizer using the PRISM TM dye terminator cycle sequencing reaction kit, at the Unidad de Biología Molecular of the Instituto de Fisiología Celular (Universidad Nacional Autónoma de México).

Species	Gene		
	Nuclear ITS	Chloroplast Mat K	Mitochondrial Nad1
P. ayacahuite	AF036981.1	AY497257.1	
P. caribea		AB063498.1	
P. cembroides	AF343994.1	AY115783.1	
P. coulteri	AF037013.1	FJ580103.1	
P. culminicola	AF343988.1	AY115776.1	
P. devoniana		DQ168622.1	JN225481
P. douglasiana	AF037012.1	FJ580063.1	JN225482
P. durangensis	AF037010.1	FJ580067.1	JN225483
P. engelmannii		FJ580070.1	AY761139.1
P. flexilis	AY430075.1	EF546711.1	
P. greggii		DQ166030.1	JN225478
P. hartwegii	AF037008.1	FJ580088.1	JN225484
P. herrerae		AB080943.1	JN225479
P. jeffreyi	U88040.1	FJ580107.1	JN225485
P. lambertiana	AF036990.1	DQ168638.1	
P. lawsonii		AB097784.1	JN225480
P. leiophylla	AF037017.1	AB081085.1	AY761136.1
P. lumholtzii	AF037026.1	AY497278.1	
P. maximartinezii	AF036994.2	DQ168631.1	JN225470
P. maximinoi		AB161010.1	JN225486
P. monophylla	AF343986.1	DQ168632.1	
P. montezumae	AF037009.1	FJ580090.1	AY761137.1
P. muricata		FJ580111.1	
P. nelsonii	AF343999.1	DQ168633.1	
P. occidentalis		AY497281.1	
P. oocarpa		DQ353710.1	JN225472
P. patula	AF037019.1	AY497284.1	JN225473
P. pinceana	AF343996.1		
P. ponderosa	AF037011.1	FJ580108.1	AF231325.1
P. praetermissa		DQ353711.1	JN225475
P. pringleii		AY497283.1	JN225474
P. pseudostrobus		FJ580102.1	JN225487
P. quadrifolia	AF343991.1	AY115771.1	
P. radiata		AB080934.1	
P. remota	AF343989.1	AY313936.1	
P. rzedowskii	AF036996.2	AY115791.1	
P. strobiformis		EF546726.1	AB455848.1
P. strobus	AY430064.1	AY497255.1	AB455849.1
P. teocote	AF037018.1	AY497285.1	AY761138.1
P. tropicalis	AF037005.1	AB080920.1	

Table 2. GenBank sequences of each of the regions analyzed, listed by species.

3. Data analysis

3.1 Phylogenetic reconstruction

Phylogenetic trees are mathematical structures or models showing the evolutionary history of the group under study. Such trees are formed of nodes interconnected by branches. Terminal nodes are operational taxonomic units (OTUs) and represent the genomes, species or sequences being studied. Internal nodes represent hypothetical ancestors, while the ancestor of all the nodes is the root of the tree. The number of branches adjacent to a given internal node determines the degree of resolution of the node. Thus, if there are more than three branches, the node is said to be unresolved (polytomy) and may represent synchronous divergence (simultaneous evolution of more than two descendant nodes) or poor certainty of the evolutionary relationships within this group of nodes (Hall, 2008).

There are different methods for constructing phylogenetic trees, based on the known molecular or morphological information for each OTU, including distance (neighbor-joining), parsimony, maximum likelihood, and Bayesian methods (Hall, 2008). Nei & Kumar (2000) mention that selection of a particular method to reconstruct phylogeny often depends on the personal preferences of researchers or on their knowledge of a given area. Thus, researchers who are used to working with discrete morphological characters often use parsimony methods (Hall, 2008) while molecular biologists and geneticists prefer the use of analytical techniques such as maximum likelihood or Bayesian methods.

However, different authors have shown that there is no "the best method" for any one case in any one group of organisms (Nei & Kumar, 2000; Felsenstein, 2004; Hall, 2008), and the decision to use a particular method may therefore depend on software efficiency, speed of analysis, the data available, or the biological characteristics of the group being analyzed. Thus, in the end, this decision may be made based on the time available for calculation, the number of genes to be analyzed or the certainty of being able to recover the best evolutionary history of a group.

3.2 Phylogenetic reconstruction of pines using DNA sequences

To reconstruct the phylogeny of pines using the three loci from different genomes in the group of pines, it was decided to use the following strategy: first, each gene was independently analyzed and then, in order to recover the best phylogenetic signal, the three regions were combined. As these are noncoding regions, codons were not used as a weighting criterion (Nei & Kumar, 2000).

Sequences were aligned with Clustal X software taking the following parameters into account: gap opening, 15; gap extension, 6.66; and DNA weight matrix: Clustal W 1.6 (Thompson et al., 1997). After alignment, two methods of reconstruction were used: parsimony with PAUP 4.0 beta software (Swofford, 1999) and maximum likelihood with MEGA 5 software (Tamura et al., 2011).

Parsimony analysis was carried out with a branch and bound search with unordered data of equal weight and ACCTRAN branch length optimization was done. Gaps were treated as missing data. The maximum number of retained trees was 10,000. The branch support was tested with tree-bisection-reconnection (TBR) algorithm with a 1,000 resampling bootstrap using the 4.0 version of PAUP (Swoford, 1999).

Prior to maximum likelihood analysis, the MODELTEST v3.6 program (Posada and Crandall, 1998) was run in order to calculate the best substitute model for each locus and for combined analysis. The model with the highest Akaike information criterion (AIC) value

was used in each case. Once the model was selected, reconstruction was performed with a heuristic maximum likelihood method using the nearest neighbor interchange (NNI) algorithm with a bootstrap of 1000 replicates in MEGA v5 software (Tamura et al., 2011).

4. Phylogenetic analysis results

The number of species analyzed per genetic region was not homogeneous since some species could not be sequenced in all cases. Nevertheless, the number of species and of variant and informative sites in all loci was adequate for parsimony and maximum likelihood analysis (Table 3).

	Gene		
Statistics	Nuclear *ITS*	Chloroplast *Mat K*	Mitochondrial *Nad1*
Number of species analyzed	23	46	36
Number of sites	3171	1718	2564
Conserved sites	632	1616	231
Variable sites	571	102	1118
Parsi Informative sites	164	66	502
Singelton sites	119	36	502
CI	0.797	0.857	0.939
RI	0.938	0.982	0.992
Number of equally parsimonious trees	174	596	514
Length	128	70	132
Best substitute model	K2+I	T92+G	K2
Log-likelihood	-182.97	-1236.41	-917.17
Ts/Tv	1.986	1.163	0.885

Table 3. General data pertaining to phylogenetic reconstruction. Two-parameter (K2) model of Kimura; three-parameter T92 model of Tamura; G = with a nonuniform rate of evolution, following instead a discrete gamma distribution; I = with a fraction of invariant sites.
CI = consistency index; RI = retention index; Ts/Tv = transition/transversion ratio.

Analysis of each individual gene by each method revealed differences in formation of the groups, but the general pattern was always retained as shown in the combined analysis in Figure 1, in which reconstruction with maximum likelihood (best tree log-likelihood = -1197.22) is seen. It is evident from this phylogenetic tree that species of the subgenus *Pinus* were always separate from those of the subgenus *Strobus*. It can also be said that in the case of the subgenus *Pinus*, *P. tropicalis* is the most divergent species. If we consider some of the characteristics of this species, we see that it has a limited distribution (restricted to Caribbean islands) and is present below 700 m asl. Whether the species occurs in Mexico or not is unclear since, like *P. caribaea*, it is found only in small patches in the state of Quintana Roo.
In this same subgenus, high polytomy is seen in most species not within the *Australes* group of Gernandt et al. (2005), while the branch for this subsection is consistent and the sole difference is inclusion of *P. devoniana* (Figure 2).
As regards the subgenus *Strobus*, two well-supported groups with a bootstrap value of 99% are formed within the subsection *Strobi* of Farjon & Styles (1997) or *Strobus* of Gernandt el at. (2005); see Figure 2.

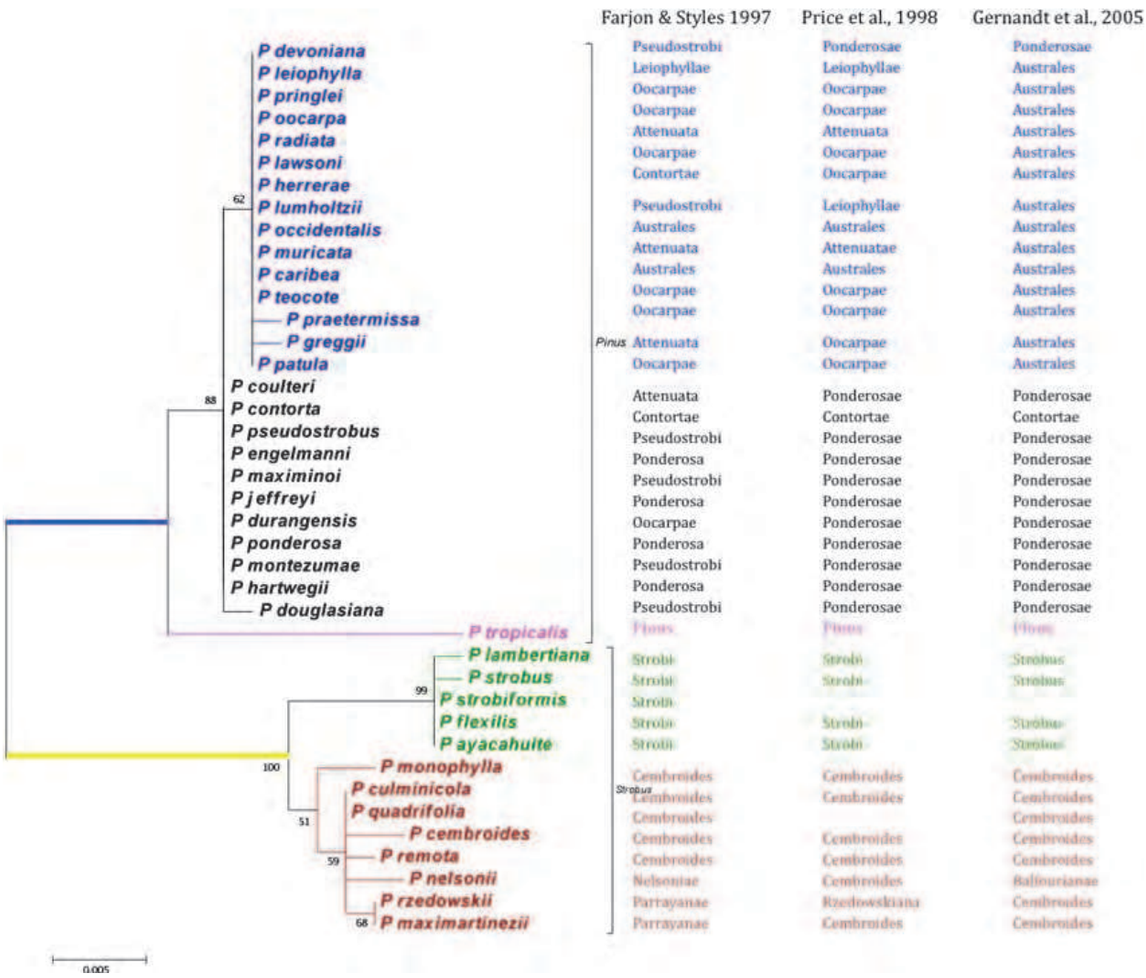

Fig. 2. Phylogenetic reconstruction by the maximum likelihood method with the T92 model (log-likelihood = -1197.22) using three loci (MatK, ITS and Nad1) from Mexican pine species. Bootstrap support is shown for each branch. Also shown are the subsections for infrageneric grouping of the pines of Mexico proposed by three groups of authors.

As regards the *Cembroides* group, *P. rzedowskii* and *P. maximartinezii* are seen to be closely related species that must be regarded as belonging to the same subsection. This branch also includes *P. nelsonii*, regarded as an independent group in all three classifications, but falling within the *Cembroides* group in the present reconstruction. The reasons for this species having been considered an independent group are to a great extent the morphological characteristics of its leaves (the three aciculae come together to form what looks like a single leaf), the shape of the scale of the seed, and a persistent peduncle in the female cone. It has a limited distribution and is therefore considered vulnerable (Farjon & Styles, 1997).

In the combined analysis of sequences, the classification proposed by Gernandt et al. (2005) may be considered to best fit the evidence derived from the three genomes of pines. Thus, the *Australes* group is consistent with a branch supported by a bootstrap value of 62%. Similarly, the *Strobus* group is well resolved since its bootstrap support is 99%. The infrageneric groups proposed by Farjon & Styles (1997) obtained the least support in this analysis, followed by those of Price et al. (1998).

5. Final considerations

5.1 Commercial use of pine trees

Trees have played a major role in the provision of goods for human communities (Le Maitre, 1998). Even today they are prevalent in agro-industry, particularly pine trees which dominate the trade in forest products (Dove, 1992).

Economic profits are so important in the lumber and related products industry that massive cultivation of pine trees has spread to more than 50 countries, most of which did not have in their native forests these species, and now being grown. In fact, Lavery & Mead (1998) say that during the 1990s more than 4 million hectares worldwide were planted with *P. radiata*, a record for this type of plant. At a cost of 35 to 45 dollars/m^3 of wood, this makes logging one of the most profitable businesses (Nelson, 2006).

In many tropical countries, the massive planting of pines has been used as a model for development. However, there are other places where pines are not considered a good crop, as these trees do not generally bear edible fruits (except for pinyon nuts) nor can they be used to feed livestock (Guggenberger et al., 1989).

Successful programs in developing countries have mixed pines with food-producing trees or grazing crops. Thus, Chile has developed management programs in which *P. radiata* is grown alongside chestnut trees (*Castanea dentata*) and pastureland for sheep (Peñaloza et al., 1985). In countries with a strong forest tradition, such as the US and Denmark, the use of mixed stands to prevent soil loss and control exploitation is very common (Adlard, 1993; Le Maitre, 1998).

The idea of sustainable and sustained development has been disseminated for a long time in societies that make use of forest resources. This vision demands not only that lumber production is not seen as an only variable but also that biodiversity in natural localities is preserved (Kumar & Nair, 2004). Thus, many areas degraded in the past by bad management are now being proposed for forest recovery with pine trees (Hof & Joyce, 1992).

However, the road to be traveled is long since the tree-farming industry continues to use large tracts of land (as in Oaxaca, Mexico), causing irreparable damage to native forests.

5.2 The importance of pine trees

Trees of the genus *Pinus* are of great ecological importance as primordial members of temperate forests. They are also economically important, being a source of wood and resins. Since earliest times humans have used pine forest products as food, medicinal remedies, building materials and fuel (Styles 1998). However, the forested areas available to the logging industry represent a small part of the planet's surface and some of them are intensively used. The United States and Canada allocate large extents of forest to the logging industry, while Mexico and Central America have fewer forest areas available (Chalenger, 1998; Perry et al., 1998).

Reduced availability of forest areas for exploitation has stimulated tree plantings for commercial purposes. In some countries such as Canada, the Russian Federation, the Philippines and Chile important natural areas have been used to grow commercially valuable species. Altered areas and even unaltered natural areas have been ravaged (eliminating flora and fauna diversity) and sown with economically profitable plants. This increases productivity while posing a risk to biodiversity and soil conservation. The same problem generated by monoculture in agriculture is now being reproduced in forest areas. Native species are being replaced or eliminated and single crops are extending into areas that were once natural ecosystems or forests (Le Maitre, 1998).

Improved market access and elimination of international borders have made many developing countries consider assimilating these methods of production, replacing their natural forests with cultivated tree stands to solve resource generation. Over 56 million hectares in 90 tropical and subtropical countries are reported to be given over to forest plantings (Le Maitre, 1998). The alternative of exploiting altered resources in natural areas in order to increase production versus allocation of large tracts to forest monoculture leaving small parcels of unaltered land is still under debate. However, in many countries this question has made governments decide for exploitation – with unpredictable consequences. Evidently, negative impacts can be prevented only if such decisions are supported with management and reforestation programs backed by adequate knowledge of the species and populations that make up the resource (Styles, 1998; Rodríguez-Banderas, 2005).
In this sense, Kimminis (1997) has described four stages in the management of forest resources that are associated with past human practice. Thus, the first stage involves solely exploitation. This is when only timber-yielding resources are extracted from a locality without any type of control or regulations. Next comes a regulatory stage in which legal and political mechanisms are created in order to establish regulations to control the rate and patterns of forest exploitation. This stage is followed by one of sustainable management, in which an ecological and evolutionary vision of forest management prevails, with timber-yielding amounts being as important as species conservation for future generations in the environment in which they have evolved. Finally, there is a social stage in which the link between local community and social interests and forest-related activities is strong, and decisions concerning forest use are made jointly and by common consent with forest owners.

5.3 Uses of Mexican pines

5.3.1 Potential management

It has been said that to have efficient management programs requires, among other things, a basic knowledge of the resource in question. This must be accompanied by the ecological characterization of species, an evolutionary knowledge (fully resolved phylogeny) of the groups to be managed in order to understand their adaptations; as well a social diagnosis of the human groups that interact with the forest (Arriaga et al., 2000).
Although the logging industry in Mexico is not as developed as in other countries, it has an important role in the national economy. Pines of the subgenus *Pinus* such as *P. patula, P. oocarpa, P. pseudostrobus* and *P. herrerae* are regarded as the most important trees for pulp and cardboard production, since their xylem forms long fibers that are useful in the manufacture of these products (Styles, 1998).
Pine trees are also important in the production of resins, particularly alpha-Pinene for cleaning products (known commercially as "Pinol"), as well as in the turpentine and cosmetics industries. In states such as Michoacán, Oaxaca, Jalisco, Chiapas and Nuevo León the use of *P. leiophylla, P. oocarpa, P. montezumae* and *P. teocote* for resin marketing is an important part of the local economy (Styles, 1998).
As regards the use of pines as a source of food, *Pinus cembroides* is the most important species, particularly in northern Mexico because of pine nut production. The latter are commercially valuable and are used to prepare diverse dishes and confectionery goods. The pink pinyon nut, obtained from Mexican species, is currently considered to have a better flavor and to be of higher quality than the white pinyon nut (Styles, 1998). *P. pinceana* and *P. nelsonii* have also been used for pinyon nut production. It is common to see the local population in Nuevo León near the end of summer and into autumn gathering pine cones in the forest and shelling the final product which is put up for sale in local markets.

5.4 Problems concerning areas of exploitation

As pines are of great importance in the forest industry, their use increases each year and has taken place indiscriminately in some localities in Mexico. Since the sawmill industry uses preferably the straightest trees with no knots, used areas of exploitation are left with remnants of poorer quality, smaller trees. This is assumed to lead to the genetic impoverishment of populations. Thus, the less healthy trees are left for forest regeneration. The result are stands with irregular crowns, winding trunks and low seed production, as has happened with *P. patula*, one of the first species to be widely used (Ledig, 1998; Styles, 1998).

Another more widespread problem in different forests in Mexico is the changeover of tracts of land with natural vegetation to agricultural areas (Perry et al., 1998; Salazar et al., 2010). This circumstance is more severe in areas where demographic pressure is strong (states such as Chiapas, the State of Mexico, Hidalgo and the Federal District).

The pressure that is exerted on certain pine species with a more limited distribution in Mexico is worth mentioning, particularly in the cases of *P. maximartinezii*, which forms a small forest of some 8 km² in area in Zacatecas, and *P. caribaea* in Quintana Roo, which is represented by vegetation patches covering only a few hectares (Styles, 1998). Both species are exploited locally beyond their capacity for regeneration and are likely to disappear, this circumstance being all the more serious in the case of *P. maximartinezii* as this is the only area at world level where it is found.

Our analysis indicates that most pine species with conservation problems fall within the group comprised by the subsection *Cembroides* (Farjon & Styles, 1997). To a great extent this may be due to the fact that they are pinyon pine species, the exploitation of which hampers recruitment in natural forests.

On the other hand, species belonging to the *Australes* group can potentially be used for reforestation or extensive plantings. Thus, the species *P. oocarpa, P. leiophylla* and *P. patula* are among those that have been successfully used in the lumber industry as well as for resin extraction (Styles 1998).

Fig. 3. Seedling of *Pinus* growing in a gap of a Natural Forest, Mexico.

The history of pines in Mexico goes back to ancient times, since species of the genus *Pinus* have been present in this area since the Late Cretaceous period. The presence of so many species may be due to climatic fluctuations and the vulcanism sustained by this region since that time (Miller, 1977).

Finally, we consider that studies such as this one are fundamental for the subsequent integral management of forests. Knowing which species are more closely related and which groups show a higher diversity enables such species to be considered as those of greatest potential use. Also evident are the species that are at higher risk and must be considered for adequate management and future conservation.

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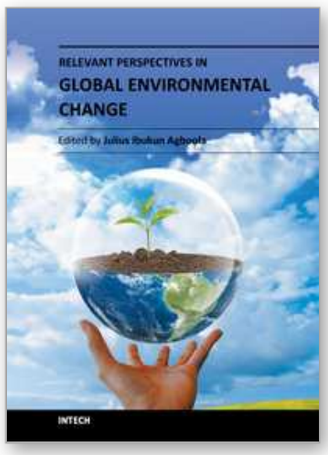

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