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Molecular approach of genetic affinities between wild and ornamental *Platanus*

Guillaume Besnard[1], Abderrhamane Tagmount[1], Philippe Baradat[1], André Vigouroux[2] & André Bervillé[1,*]

[1]*INRA UR Génétique et Amélioration des Plantes;* [2]*INRA Laboratoire de Botanique, Biologie et Pathologie Végétale, 2 place P. Viala, F34060 Montpellier Cedex 1, France; (*author for correspondence; E-mail: berville@ensam.inra.fr)*

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Summary

Apart from the wild species *P. orientalis* and *P. occidentalis*, the cultivated plane trees constitute a wide and heterogeneous group, with uncertain genetic status and largely debated names. The recent canker stain problem in Europe makes it necessary at the present time to consider the genetic resources and to determine the genetic bases of all these trees. To attain this objective, a genetic molecular approach was used to analyze 60 trees of *P. orientalis* and *P. occidentalis*, different London planes (*P. hispanica* and *P. densicoma*), a few controlled *P. occidentalis* × *P. orientalis* hybrids and particular trees from arboreta and old parks. Molecular analysis involved thirty RAPD fragments generated with nine primers, PCR-RFLP in the 5S RNA genes and mitochondrial polymorphisms revealed by RFLP method. Clones were recognized among *P. hispanica* and *P. densicoma* trees. A Correspondence Analysis and a dendrogram constructed according to the genetic distances confirmed the supposed hybrid origin of *P. hispanica* and *P. densicoma* between *P. occidentalis* and *P. orientalis.* Contribution of *P. orientalis* to their constitution seems more important than that of *P. occidentalis*. Mitochondrial DNA polymorphisms indicated that crosses occurred in both directions. Moreover, *P. occidentalis* as female parent led to *P. densicoma* whereas *P. orientalis* as female parent led to *P. hispanica*. Low prevalence of pure species individuals and confusion risks with hybrid trees even for old trees are highlighted.

Introduction

In most European towns, in North America and other temperate countries, plane trees represent an important ornamental or lining tree. For example, they constitute 40% of tree plantations in Paris, more in London, and provide thousands of trees in all-important towns in Europe. Today, the canker stain, a grave fungal disease native to the USA (Walter, 1946), threatens the trees in Europe with heavy damages already present in the Mediterranean area (Anselmi et al., 1994). This led us to reconsider the genetic resources of the genus to look for resistance possibilities (Vigouroux, 1992; McCracken, unpublished) and to assess the genetic basis of the propagated ornamental trees.

Platanus includes nine to ten species, some doubtful (Santamour 1972). The barriers to hybridization between the two main species of the genus (*P. occidentalis* L. from the United States and *P. orientalis* L. from the Eastern Mediterranean Basin and Middle East) arose from ecogeographic isolation for at least 30 million years (Stebbins, 1950). However, man disturbed this isolation after the discovery of America and subsequent botanical material exchanges occurred. So, taken as a whole, European plane tree populations observed everywhere are often considered as hybrid products between the American and oriental species. In fact, this group of cultivated trees, all or a part of them referred as London plane, is not homogeneous. In the past, it has given rise to several studies

and different names were proposed. For instance, in 1919, working with old archives, voucher herbaria and numerous leaf and fruit measurements, Henry and Flood reconstituted a possible history of the London plane, for them *P. acerifolia* (Ait) Willd, presently named *P. hispanica* Moench. They argued its hybrid origin between individuals of *P. occidentalis* and *P. orientalis* gathered in the Oxford Botanical Garden in the seventeenth century. However, from this period onwards, many events might have occurred, suggested by a rather wide variability of London planes. In 1908, Dode distinguished among the cultivated planes a species he referred to as *P. densicoma*. It is very probably the *P. pyramidalis* (Rivers) Henry & Flood (1919) roughly described by Rivers in 1856. This type is rather well characterized with large and often solitary fruit balls, clearly conical achenes, moderately-lobed and wide leaves with a dense green, rugged trunk and erect bearing. Generally mixed in plantation with *P. hispanica*, it is frequent in northern France as in northern Europe. By 1970, it appeared impossible to Santamour (1970) 'to verify the hybrid origin of any individual London plane because it refers to an assemblage of trees that are generally intermediate between the two putative parents'. Vigouroux et al. (1997) have also proposed an hybrid origin between *P. occidentalis* and *P. orientalis* for *P. densicoma*. In contrast, Dode has suggested an American origin for this taxon. In fact a frequent confusion has been already made in the nineteen century with *P. occidentalis* (Loudun, 1854 and many people thought *P. occidentalis* frequent in Europe at this period as denounced by Hooker, 1856). Confusion is still frequent even in arboreta. This muddled situation states clearly the questions we would now answer with molecular markers. A possible hybrid status of *P. hispanica* and *P. densicoma* infers that genetic markers unique to each putative parental species should be found in their genome.

We used the PCR technology with 10-mer primers (RAPD) or specific 5S DNA primers and then the RFLP method with a mitochondrial gene as a probe. Indeed, these methods have been widely studied to point out genetic structures and possible hybrid origin in several tree species and to determine the direction of crosses: i.e. *Pawlownia* (Wang et al., 1994), *Gliricidia* (Dawson et al., 1996), and *Fraxinus* (Jeandroz et al., 1995, 1997). Using samples of plane trees from Greece, the Middle East, the USA and France, belonging to the taxa of *P. orientalis, P. occidentalis, P. hispanica* and *P. densicoma,* we computed multivariate analyses and estimated genetic distances to define relationship between them.

Materials and methods

Plant material

Fifty trees (Table 1) were first analyzed, representing the four studied species (Or = *P. orientalis*, Oc = *P. occidentalis*, Hi = *P. hispanica,* De = *P. densicoma*) plus three controlled hybrids *P. occidentalis* × *P. orientalis* (Hy). The choice was based on morphological characteristics and on wild locations for *P. orientalis* and *P. occidentalis*. For the wild locations one or two individuals per site were analyzed since no actual primitive wild population still exists. The hybrid individual Hy1 has the *P. occidentalis* tree TyOc1 as female parent, a tree that grows beside a *P. orientalis* tree in the Angers arboretum (see below). Both hybrid plants, Hy2 and Hy3, were obtained from the same *P. occidentalis* female parent spontaneously hybridized by a *P. orientalis* tree interpenetrating its branches with the first one in a collection of AFOCEL[1]. In addition, ten trees in old French parks (generally from eighteenth century) and arboreta *a priori* being of pure species and referred as *P. orientalis* type (TyOr) or *P. occidentalis* type (TyOc) (Table 1) were compared with the previous plants.

Molecular methods

DNAs were prepared according to Gentzbittel et al. (1995) from 5 g of frozen leaves.

RAPD analysis

The RAPD amplification and electrophoresis procedures were described by Quillet et al. (1995). Eight primers (Bioprobe, France), previously screened by Vigouroux et al. (1997), were used on the DNA's from all individuals: A11, D11, D20, P11, P12, P17, P18, and P19. The presence or the absence of each fragment was noted. The homology of sequence of some RAPD fragments present in different species was verified by hybridization according to the protocol described by Besnard et al. (2002). This also allowed to verify whether a fragment could be detected even when undetected on the gel.

[1] 1 AFOCEL: Association Forêt Cellulose. Domaine de l'Etrançon. 77 370 Nangis, France.

Table 1. Origin, places and main traits of sampled *Platanus* trees

Species and individual code	locality, city, department and country	Typical characters
P. orientalis		
Or1	Trèbes (Aude, France) AFOCEL's collection[1]	
Or2	Trèbes (Aude, France) AFOCEL's collection[1]	
Or3	Trèbes (Aude, France) AFOCEL's collection[1]	Balls grouped
Or4	Thessaly (Greece)	by 4 or 5 with conical
Or5	Direct introduction from Platamonas (Greece)	achenes, leaves deeply
*Or6**	Direct introduction from Athenes (Greece)	lobed,
*Or7**	Direct introduction from Xania, Crete (Greece)	anthracnosis
*Or8**	Direct introduction from Samos Island (Greece)	resistance, spread
Or9	Direct introduction from Samos Island (Greece)	bearing
Or10	Direct introduction from Samos Island (Greece)	
*Or11**	Bakfaze, Oronte Valley (Syria)	
Or12	Bakfaze, Oronte Valley (Syria)	
*Or13**	IFAPO, Damas (Syria)	
*Or14**	Direct introduction from Hamadan (Iran)	
P. occidentalis		
Oc1	Trèbes (Aude, France) AFOCEL's collection[1]	
Oc2	Trèbes (Aude, France) AFOCEL's collection[1]	
Oc3	Trèbes (Aude, France) AFOCEL's collection[1]	Very shallowly lobed
Oc4	Trèbes (Aude, France) AFOCEL's collection[1]	leaves, solitary balls,
Oc5	Trèbes (Aude, France) AFOCEL's collection[1]	flat achenes,
Oc6	Trèbes (Aude, France) AFOCEL's collection[1]	anthracnosis
*Oc7**	Athens (Georgie, USA)	Susceptibility
*Oc8**	Direct introduction from Morgan (Illinois, USA)	
*Oc9**	Direst introduction from Sikestone (Missouri, USA)	
*Oc10**	Direct introduction from Stoneville (Mississipi, USA)	
*Oc11**	Direct introduction from Greenville (Mississipi, USA)	
*Oc12**	Direct introduction from Winona (Mississipi, USA)	
P. hispanica		
*Hi1**	Museum National d'Histoire Naturelle, Paris (Seine, France)	
Hi2	Bed of the Loire (France) (cutting)	
*Hi3**	Bed of the Loire (France) (cutting)	Balls grouped
Hi4	Montargis (Loiret, France)	by 2 or 3 with
Hi5	Montargis (Loiret, France)	spheroconical achenes,
Hi6	Restinclières, Prades-le-Lez (Hérault, France)	smooth
Hi7	Restinclières, Prades-le-Lez (Hérault, France)	trunk, more or
Hi8	Bonnier de la Mosson's park, Montpellier (Hérault, France) ($XVIII^{th}$ cent park)	less lobed leaves,
*Hi9**	ENSA, Montpellier (Hérault, France)	
*Hi10**	Lavalette, Montpellier (Hérault, France) ($XVIII^{th}$ cent. park)	
*Hi11**	Peyrou park, Montpellier (Hérault, France)	
*Hi12**	Peyrou park, Montpellier (Hérault, France)	
P. densicoma		
*De1**	ENSA, Montpellier (Hérault, France)	Frequently solitary
*De2**	ENSA, Montpellier (Hérault, France)	and
*De3**	Castle of Bionne, Laverune (Hérault, France) ($XVIII^{th}$ cent.)	large balls with
De4	A street in Montpellier (Hérault, France)	conical achenes –

Table 1. Continued

Species and individual code	locality, city, department and country	Typical characters
*De5**	Chevreloup Arboretum, Roquencourt (Yvelines, France)	rugged trunk,
De6	Museum National d'Histoire Naturelle, Paris (Seine, France)	moderately lobed
De7	Avenue de Breteuil, Paris (Seine, France)	leaves, erect
*De8**	Avenue de Breteuil, Paris (Seine, France)	bearing
*De9**	Les Barres Arboretum, Nogent/Vernisson (Loiret, France)	
Hybrids *P. occidentalis* × *P. orientalis*		
Hy1	La Maulévrie Arboretum, Angers (Maine et Loire, France)	From seeds
Hy2	Trèbes (Aude, France) AFOCEL's collection	No morphological
Hy3	Trèbes (Aude, France) AFOCEL's collection	data
***Orientalis* types from old parks**		
TyOr1	Lavalette park, Montpellier (Hérault, France) (XVIIIth cent.)	Typical *P. orientalis*
TyOr2	Bonnier de la Mosson's park, Montpellier (Hérault, France) (XVIIIth cent.)	Typical *P. orientalis*
***Orientalis* types from old parks**		
TyOr3	Lamanon (Bouches du Rhône, France) (300 years?)	Rather moderately
TyOr4	Diane de Poitiers's castel, Les Clayes s/Bois (Seine, France) (XVIth cent.)	lobed leaves
TyOr5	Castel of Santenay (Sâone et Loire, France) (XVIth cent. park)	and balls often
TyOr6	Museum National d'Histoire Naturelle, Paris (Seine, France) (early XIXth)	grouped by 2 or 3.
TyOr7	Bagatelle Park, Paris (Seine, France) (XVIIIth cent.)	
***Occidentalis* types from arboreta**		
TyOc1	La Maulévrie arboretum, Angers (Maine et Loire, France)	*Occidentalis* aspect but
TyOc2	Les Barres Arboretum, Nogent/Vernisson (Loiret, France)	conical achenes and
TyOc3	Chevreloup Arboretum, Roquencourt (Yvelines, France)	anthracnosis tolerance

[1] Direct introduction of seeds from the USA (*P. occidentalis*) or from Greece or Turkey (*P. orientalis*).
* Means tree analyzed for the mitochondrial polymorphism.

We estimated the probability, $P_i|C_i \in G_k$, that the RAPD pattern of the i^{th} individual could be met in another individual belonging to the same group, G_k. That is the average probability of no-distinction of different genotypes on the basis of their RAPD profiles. This probability is the product of the average frequencies of presence, $(F_j|G_k)$ or absence, $(1 - F_j|G_k)$ of each marker within the considered group:

$$P_i|C_i \in G_k = \prod_{j=1}^{m} f_j|G_k$$

where m is the total number of markers over all groups, and $f_j|G_k$ is the average marker frequency, $F_j|G_k$, when present in the individual C_i, or $1-F_j|G_k$ if the marker is absent in this individual. This formula assumes an independent association between markers within the group of trees considered.

We looked for a possible grouping of individuals for each species using a Correspondence Analysis with the procedure CORRESP from SAS (SAS, 1992). We computed the Jaccard similarity (Jaccard, 1908) and we used the UPGMA algorithm (Benzécri, 1973) to construct the phenetic tree. A Discriminant Analysis on qualitative data was performed using the DISQUAL procedure (Saporta, 1990). It used as input data the coordinates of the individual trees from the four species of the first two axes of the Correspondence Analysis. This last analysis was done to test the significance of the average between group differences and to assess the possible origin of the ten Old French trees, which were processed as supplementary data.

Components of diversity for each species or taxa were determined. N represents the mean number of RAPD fragments for each group of individuals. To study the contribution of each parental species in the constitution of the hybrid forms we decomposed N into three components:

$$N = n_{Or} + n_{Oc} + n_{sp}$$

where n_{Or} is the mean number of specific markers to *P. orientalis*, n_{Oc} is the mean number of specific markers to *P. orientalis*, and n_{sp} is the mean number of markers present in both species.

5S DNA analyses

Because no diagnostic RFLP marker was detected using 18S and 25S rRNA genes as probes coupled with 4

Table 2. Frequencies of RAPD fragments, of 5S ribosomal units, and of mitochondrial types in each species or group of individuals. *Or, Oc, Hi, De,* and *Hy*, mean *P. orientalis*, *P. occidentalis, P. hispanica*, *P. densicoma,* and *P. orientalis* × *P. occidentalis* hybrids, respectively. The number of genotypes characterized for each marker is given in brackets

Marker	Frequency in each taxon				
RAPD fragments	*Or* (14)	*Oc* (12)	*Hi* (9)	*De* (7)	*Hy* (3)
P18-950	0.43	0	0.67	1	0.33
P19-875	0.79	0	0.33	0.14	0
P11-1400	1	0	1	1	0.67
P11-1600	0.93	0	0.78	0.86	1
P12-600	0.29	0	1	0.86	0.67
D11-600	1	0	1	1	1
D11-1300	1	0	0.67	0.86	1
A11-300	0.79	0	0.89	1	1
A11-325	0.07	0	0.33	0	0
A11-400	0.71	0	0	0	0
A11-500	0.86	0	0.56	0.57	0.33
A11-600	1	0	0.89	0.86	1
P17-400	1	0	1	1	1
D20-1200	0	0.92	0.44	0.71	0.67
D20-475	0	0.33	0	0	0
D20-1600	0	1	0.89	0.71	1
P17-700	0	0.67	0	0.14	0
P11-1500	0	1	0.33	0.43	0.67
A11-350	0	0.42	0	0.14	0
A11-625	0	0.5	0.11	0	0.67
P19-850	0.29	1	1	0.86	1
P17-1000	0.86	1	1	1	1
D20-550	0.14	1	0.89	0.86	1
P11-550	0.71	1	0.89	1	0.67
P12-1450	0.71	1	1	0.86	1
P12-825	1	0.17	1	1	1
D20-1500	0.14	0.33	1	0.86	0.67
A11-650	0.21	0.92	0.33	0.29	0.33
D11-400	0.71	0.92	1	1	0.67
D11-375	0.71	0.33	0.67	0.29	0
5S units	*Or* (11)	*Oc* (12)	*Hi* (8)	*De* (6)	*Hy* (-)
Unit 1	0.55	1	0.88	1	–
Unit 2	0.64	0	0.38	1	–
Unit 3	0.36	0	0.5	0	–
Unit 4	0.45	1	0.38	1	–
Unit 5	0.64	0	0.5	1	–
Mitochondrial RFLP	*Or* (6)	*Oc* (6)	*Hi* (6)	*De* (6)	*Hy* (-)
*Hind*III-*atp6*-1500 presence	0	1	0	1	–
*Hind*III-*atp6*-1500 absence	1	0	1	0	–

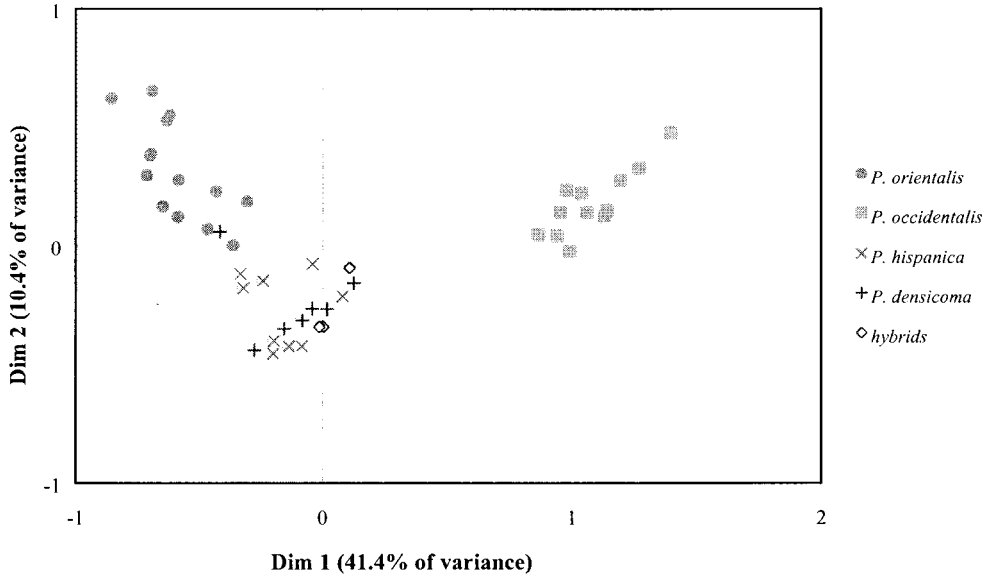

Figure 1. Correspondence Analyses based on RAPD data from *Platanus* trees.

restriction enzymes (A. Tagmount, Unpublished data), we chose to study the 5S rRNA gene variation. 5S DNA intergenic spacers were amplified by PCR using 40 ng of the pair of primers 5SF1 (REF 77180: 5′GGA TCC GAT CAT ACC AGC AC3′) and 5SR1 (REF 77181: 5′AGG ACT TCC CAG CTC AC3′) in 50 μl final volume containing 30–50 ng DNA, 0.4U *Taq* DNA Polymerase, 250 μM of each dNTP, and annealing at 55 °C with 35 cycles. The different 5S DNA units were revealed by restriction of the PCR product with *Bam*HI (30 U, at 37 °C, for 5 h) and electrophoresed onto 2.2% agarose gel at 2.5 V/ cm for 8 h.

Mitochondrial DNA polymorphism

This characterization was performed on only a few trees due to leaf quantity limitation on most of our samples. It was revealed by RFLP of the total DNA of 24 trees restricted separately by *Eco*RI or *Hind*III using the *atp6* fragment from maize (Dewey & Timothy, 1986) as a probe kindly provided by P. Saumitou-Laprade (Lille University). Maternal inheritance of mitochondrial DNA was checked in the progeny of the crossing Oc9 × Or8 onto 10 hybrid tree DNA samples.

Results

Polymorphism revealed between Platanus species using RAPD fragments

Thirty polymorphic and 13 monomorphic fragments were noted and coded (primer-size in bp). Several fragments enabled us to distinguish *P. orientalis* and *P. occidentalis* species (Table 2). Most of these fragments were present in *P. hispanica*, *P. densicoma* and controlled hybrids *P. occidentalis* × *P. orientalis.* No marker was specific or unique to *P. hispanica* and *P. densicoma* representatives. Seven RAPD markers (A11-650, D11-400, D11-600, D20-550, D20-1600, P17-700, P19-875) were used as probes to verify sequence homology between fragments of the same size in different species. This confirmed the readings, and thus we considered that the same size for two RAPD fragments corresponded to homologous fragments.

The extreme probabilities, *P*, of obtaining one of the RAPD profiles were relatively low in *P. hispanica* ($1.17\ 10^{-2}$ for Hi3 to $5.67\ 10^{-6}$ for HiCl1) and in *P. densicoma* ($1.4\ 10^{-2}$ for De1 to $8.41\ 10^{-9}$ for De4). Consequently, we can consider that two trees displaying the same profile, correspond, in all likelihood, to a clone. Thus, two clones were found in *P. hispanica*

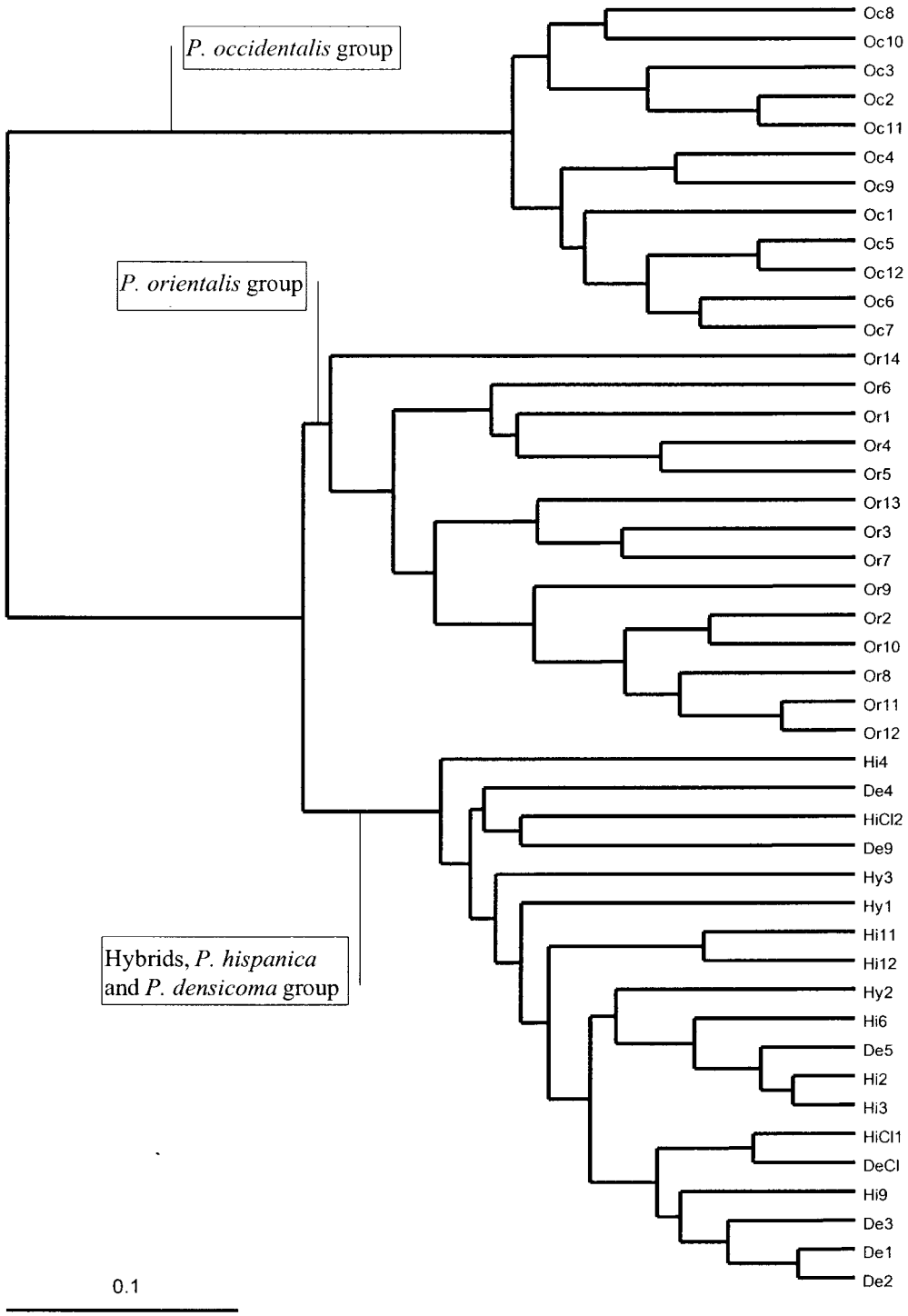

Figure 2. Dendrogram constructed with UPGMA on Jaccard dissimilarities between *Platanus* trees.

(Hi5 and Hi7 = HiCl1; Hi1, Hi8 and Hi10 = HiCl2) and another in *P. densicoma* (De6, De7, De8 = DeCl1).

Correspondence analysis

The first two dimensions (explaining 41.4% and 10.4% of the variance, respectively) of the Correspondence Analysis onto species enabled the separation of *P. orientalis* individuals from *P. occidentalis* ones (Figure 1), whereas *P. hispanica, P. densicoma* and *P. occidentalis* × *P. orientalis* hybrids belonged to the same cluster and appeared to be intermediate between *P. occidentalis* and *P. orientalis*. Along the first dimension, *P. occidentalis* trees were separated from trees of all other species (*P. densicoma*, *P. orientalis* and *P. hispanica*), whereas the second dimension separated *P. orientalis* trees from *P. hispanica, P. densicoma* and *P. occidentalis* × *P. orientalis.*

Dendrogram

The dendrogram displayed two main branches with *P. occidentalis* and *P. orientalis* groups at the extremities, whereas *P. hispanica, P. densicoma* and the *P. occidentalis* × *P. orientalis* hybrid individuals were sister group to *P. orientalis* (Figure 2).

Discriminant analysis

Platanus hispanica and *P. densicoma* centroids were close (Figure 3, Table 3) and each cloud of points representative of these trees overlapped, meaning that these two denominations probably correspond to one taxon only. Old park trees clustered with the hybrids, suggesting they do not originate from a pure species, excepted TyOr1, which appears as a pure *P. orientalis* (Table 3). TyOr3 might be assigned to *P. hispanica, P. densicoma* or *P. orientalis*. It probably belongs to an introgressed population originating from crosses between hybrids and *P. orientalis*. The three arboretum individuals (TyOc) might also be the result of an introgression of *P. densicoma* or *P. hispanica* by *P. occidentalis* (Figure 3).

Components of diversity

Twenty RAPD fragments out of 30 can be considered as diagnostic markers in the distinction of *P. occidentalis* (7 markers) from *P. orientalis* (13 markers). Eighteen of these markers were found in *P. hispanica* and *P. densicoma* (Table 2). The genetic diversity (*N*) is larger in *P. hispanica* (19.4) and *P. densicoma* (19.1) than in *P. orientalis* (15.1) and *P. occidentalis* (12.4) (Table 4). Moreover, the components of this genetic diversity showed that *P. hispanica* and *P. densicoma* carried more markers from *P. orientalis* than from *P. occidentalis* and consequently were closer to *P. orientalis* than to *P. occidentalis*.

5S DNA

Polymorphisms in the 5S DNA were revealed between the 2 species (Table 2). Thus, 5 length variants were detected: Unit 1 (340 bp), Unit 2 (355 bp), Unit 3 (370 bp), Unit 4 (390 bp) and Unit 5 (420 bp). In our sample, we did not find any unit specific for *P. occidentalis*. In contrast, the 5S units 2, 3 and 5 were specific to *P. orientalis* and were present in *P. hispanica* and *P. densicoma*. No variation was revealed in *P. densicoma,* which always displayed the 4 units 1, 2, 4 and 5. TyOc1, TyOc2 and TyOc3 carried the units 2 and 5, which are specific to *P. orientalis* (data not shown). This result suggests that these trees have a hybrid status, which even displays the main typical traits of the *P. occidentalis* species (but they have conical achenes).

Mitochondrial DNA polymorphism

A polymorphism was detected with *atp6* gene as a probe (Figure 4). A single additional band of approximately 1,500 bp enabled us to distinguish *Platanus. occidentalis* trees from *P. orientalis* trees (Table 2). In the cross (Oc9 × Or8), we verified that the progenies carried the mitochondrial DNA type of the maternal tree Oc9. *P. hispanica* and *P. densicoma* trees displayed either the *P. orientalis* or the *P. occidentalis* mitotype, respectively (Table 2); this infers that hybridization has occurred in both directions. Moreover, the direction of the cross seems to lead to *P. hispanica* or *to P. densicoma* when *P. orientalis* or *P. occidentalis* plays the role of maternal parent, respectively.

Discussion

We recognized without ambiguity the two wild species using molecular markers. Moreover, *P. occidentalis* displayed a lower molecular genetic variability than *P. orientalis* that might be due to an intense regression of the population size during glaciation as already shown for most North American forest trees (Huntley & Webb, 1989).

Until now, the intermediary state of the morphological features (Bean, 1976), the vigor and the large

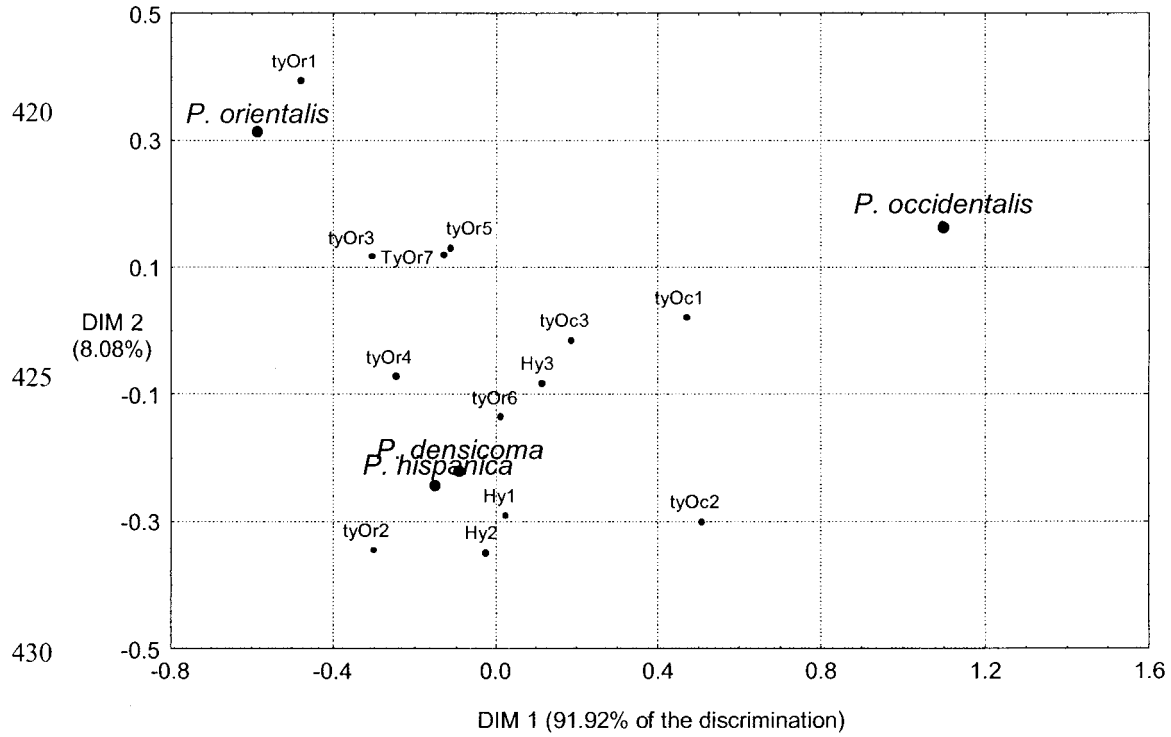

Figure 3. Discriminant Analysis based on RAPD data showing hybrid state of some old French trees. The larger points correspond to the centroid of the four groups of individuals: *P. occidentalis*, *P. orientalis*, *P. hispanica* and *P. densicoma*.

Table 3. Assignation of French old trees and hybrids to *Platanus* species from Mahalanobis distances . First row: F test with 2 and 38 d.f. Second row: Significance level or probability of greater value

	P. orientalis	*P. occidentalis*	*P. hispanica*	*P. densicoma*
P. occidentalis	393.94 ***			
P. hispanica	50.53 ***	193.65 ***		
P. densicoma	43.63 ***	143.56 ***	0.28 *0.761*	
TyOr1	0.32 *0.732*	49.88 ***	7.61 **	7.66 **
TyOr2	7.47 **	42.21 ***	0.61 *0.552*	1.02 *0.371*
TyOr3	2.08 *0.137*	38.93 ***	2.24 *0.118*	2.32 *0.110*
TyOr4	4.29 *	36.46 ***	0.60 *0.559*	0.74 *0.510*
TyOr5	4.92 **	28.94 ***	1.88 *0.164*	1.58 *0.218*
TyOr6	9.85 ***	24.54 ***	0.63 *0.542*	0.29 *0.752*
TyOr7	4.65 *	29.79 ***	1.77 *0.183*	1.51 *0.232*
TyOc1	23.35 ***	8.11 ***	8.25 ***	6.63 **
TyOc2	28.95 ***	9.88 ***	8.29 ***	6.78 **
TyOc3	13.32 ***	16.90 ***	2.81 *0.071*	1.98 *0.151*
Hy1	12.34 ***	25.66 ***	0.58 *0.573*	0.31 *0.740*
Hy2	12.23 ***	28.56 ***	0.43 *0.662*	0.30 *0.748*
Hy3	11.84 ***	20.03 ***	1.63 *0.208*	1.02 *0.372*

*** Significant at the 0.1% level; ** Significant at the 1% level; * Significant at the 5% level; probabilities corresponding to not significant difference ($p > 0.01$) are given is italic characters.

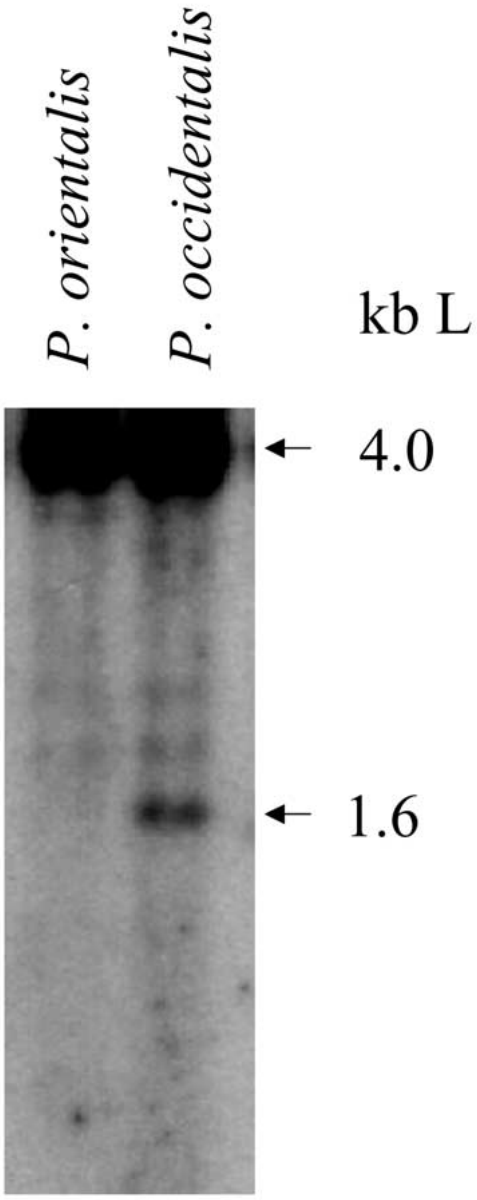

Figure 4. Mitochondrial RFLP of total DNA restricted by *Xba*I hybridized with the *atp6* sequence as a probe.

diversity of trees in one plantation sustained the hybrid origin of *P. hispanica*. Dode (1908) suggested the American origin for *P. densicoma* according to some indications of Michaux 'père' (*Flora Borealis Americana* cited by Dode, 1908) based on morphological characteristics and it was also supported by Rivals (1979) but not by other American authors (Santamour & McArdle, 1986). Correspondence Analysis and cluster analysis strongly support the evidence that

Table 4. Indexes of genetic diversity determined on RAPD data. *N*: Mean number of RAPD fragments by individual in each taxon. n_{or}, n_{oc}, n_{ns}; average number of markers from *P. orientalis*, *P. occidentalis* or both species, respectively

Species	N	n_{Or}	n_{Oc}	n_{ns}
Or	15.07	9.86	0	5.21
Oc	12.42	0	4.83	7.58
Hi	19.44	9.11	1.56	8.78
De	19.14	9	2.14	8
Hy	18.33	8	3	7.33

P. hispanica and *P. densicoma* appeared as a result of advanced generation hybrid, segregating and back-crosses to American and Eurasian species. In the phenetic tree, the intermediate position of *P. hispanica* and *P. densicoma* trees together with *P. occidentalis* × *P. orientalis* hybrids argues for their hybrid origin between *P. occidentalis* and *P. orientalis* as already shown by Vigouroux et al. (1997) on a smaller sample. Furthermore, the genetic diversity that was found to be wider in *P. hispanica* and *P. densicoma* than in the pure species is logically explained by recent hybridization. We easily found specific RAPD markers for *P. occidentalis* and *P. orientalis* but none for *P. densicoma* and *P. hispanica.* These two species displayed any markers present both in *P. occidentalis* and *P. orientalis*. All these results clearly support the hybrid origin of *P. densicoma* and *P. hispanica*. Although the extent of the 5S DNA polymorphism is slight when compared with RAPD fragments, this gene family also enabled us to demonstrate that the hybrid origin of *P. hispanica* and of *P. densicoma* has occurred in different genetic backgrounds since these two denominations were distinguished according to the unit frequency.

Since the discovery of America, *P. occidentalis* and *P. orientalis* have been introduced into gardens in Europe and in North America, respectively. Thus, crosses between them might have occurred several times and might have led to several hybrid types (advanced hybrid). In fact, only two diagnostic markers of the parental species out of 20 were absent from the two hybrid forms studied here. This means that they display a wide genetic diversity in comparison with the pure species and that several crosses could be implicated in the constitution of the ornamental or lining forms. The small number of generations certainly

explains the low genetic drift since the formation of the first hybrids. However, a more important contribution of *P. orientalis* is shown in the constitution of the hybrids, which matches up with phenol composition, closer to that of *P. orientalis*, observed by Hsiao & Li (1975). This phenomenon could be due to more frequent backcrosses by *P. orientalis* than by *P. occidentalis* in Europe, probably because of the disappearance of *P. occidentalis* in early nineteenth century (Hooker, 1856; Dode, 1908; Bean, 1976). The mitochondrial data support the existence of different directions for crosses in *P. hispanica* and *P. densicoma* with *P. orientalis* or *P. occidentalis* as female parent, respectively. However, this has to be verified on a wider sample of trees. Stebbins (1950) has noted that the F1 hybrids were perfectly viable, whereas low viability of the F2 progeny was observed. We can suppose that parental allele combinations were selected in the hybrid progenies, leading to different types of trees, but nuclear cytoplasmic interactions, probably implicated, remain to be studied. In the same way, the absence of some RAPD markers from *P. occidentalis* and *P. orientalis* (see A11-400 and D20-475) in the hybrid species might result either from a foundation effect or from negative interactions between alleles of the two parental species leading to the elimination of regions associated with some 'against' selected alleles or lineage sorting (Rieseberg et al., 1996).

In conclusion, as a practical consideration, our results revealed a hybrid status for most of the old remarkable French Park trees. Using only RAPD fragments, this feature was not revealed by Vigouroux et al. (1997) who have considered these trees as belonging to wild species. These trees were not suspected to be hybrid due to their wide growth and bearing suggesting they were older than the America discovery. This points out the extraordinary developmental potential of hybrid plane trees and the major possibility of confusion between pure species and hybrids.

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