Annals of Botany **112**: 589–602, 2013
doi:10.1093/aob/mct134, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Plastid DNA sequencing and nuclear SNP genotyping help resolve the puzzle of central American *Platanus*

Olga De Castro[1,*], Antonietta Di Maio[1], José Armando Lozada García[2], Danilo Piacenti[3], Mario Vázquez-Torres[4] and Paolo De Luca[1]

[1]*Dipartimento di Biologia, Università degli Studi di Napoli Federico II, Via Foria 223, Orto Botanico, 80139 Napoli, Italy,* [2]*Facultad de Biología, Universidad Veracruzana, Zona Universitaria, Circuito Gonzalo Aguirre Beltrán s/n, Xalapa, Veracruz, México,* [3]*Applied Biosytems, part of Life Technologies, Via G.B. Tiepolo, 20052 Monza, Italy and* [4]*Instituto de Investigaciones Biológicas, Universidad Veracruzana. Av. Dr. Luis Castelazo s/n Col. Industrial Animas, 91190 Xalapa, Veracruz, Mexico*
** For correspondence. E-mail olga.decastro@unina.it; odecastr@unina.it*

Received: 15 January 2013 Revision requested: 4 March 2013 Accepted: 23 April 2013 Published electronically: 24 June 2013

• *Background and Aims* Recent research on the history of *Platanus* reveals that hybridization phenomena occurred in the central American species. This study has two goals: to help resolve the evolutive puzzle of central American *Platanus*, and to test the potential of real-time polymerase chain reaction (PCR) for detecting ancient hybridization.
• *Methods* Sequencing of a uniparental plastid DNA marker [*psbA-trnH*$^{(GUG)}$ intergenic spacer] and qualitative and quantitative single nucleotide polymorphism (SNP) genotyping of biparental nuclear ribosomal DNA (nrDNA) markers [*LEAFY* intron 2 (*LFY*-i2) and internal transcribed spacer 2 (ITS2)] were used.
• *Key Results* Based on the SNP genotyping results, several *Platanus* accessions show the presence of hybridization/introgression, including some accessions of *P. rzedowskii* and of *P. mexicana* var. *interior* and one of *P. mexicana* var. *mexicana* from Oaxaca (= *P. oaxacana*). Based on haplotype analyses of the *psbA-trnH* spacer, five haplotypes were detected. The most common of these is present in taxa belonging to *P. orientalis*, *P. racemosa sensu lato*, some accessions of *P. occidentalis sensu stricto* (*s.s.*) from Texas, *P. occidentalis* var. *palmeri*, *P. mexicana s.s.* and *P. rzedowskii*. This is highly relevant to genetic relationships with the haplotypes present in *P. occidentalis s.s.* and *P. mexicana* var. *interior*.
• *Conclusions* Hybridization and introgression events between lineages ancestral to modern central and eastern North American *Platanus* species occurred. Plastid haplotypes and qualitative and quantitative SNP genotyping provide information critical for understanding the complex history of Mexican *Platanus*. Compared with the usual molecular techniques of sub-cloning, sequencing and genotyping, real-time PCR assay is a quick and sensitive technique for analysing complex evolutionary patterns.

Key words: Central America, genotyping, hybridization, introgression, internal transcribed spacer 2, *LEAFY* intron 2, *Platanus*, *psbA-trnH*$^{(GUG)}$ intergenic spacer, real-time PCR, sequencing, SNP.

INTRODUCTION

Recent studies examining the history of *Platanus* L. have revealed that complex hybridization phenomena have occurred in central American *Platanus* (Nixon and Poole, 2003; Feng *et al.*, 2005; Grimm and Denk, 2008, 2010; Denk *et al.*, 2012). The genus *Platanus* (Platanaceae) includes two major lineages, each corresponding to a subgenus: the monotypic *Castaneophyllum* J.F.Leroy (with *P. kerrii* Gagnep., endemic to Indochina) and subgenus *Platanus* J.F.Leroy, with all other species (Nixon and Poole, 2003). The most recent taxonomic treatments of the latter subgenus recognize six species distributed throughout the Northern Hemisphere, with a variable number of varieties (Nixon and Poole, 2003; Grimm and Denk, 2008, 2010; Denk *et al.*, 2012).

The fossil record of Platanaceae is excellent and well dated (leaves and reproductive structures date back to the Albian, late early Cretaceous) (Crane *et al.*, 1993; Friis and Crane, 1998; Kvaček *et al.*, 2001; Maslova, 2003). A historical biogeographic investigation recently carried out on *Platanus* by Feng *et al.* (2005) indicated that vicariance events have played a fundamental role in the present-day distribution of the genus. However, the recent history of *Platanus* is not entirely clear because the extant species (especially the Mexican taxa) show a considerable number of overlapping morphological characteristics, especially in regard to leaf shape, the number of capitula per inflorescence and various achene characters (Nixon and Poole, 2003). Cytological data for the genus are incomplete, and are almost entirely missing for the central American species. In the Old World species, $n = 21$ has always been observed (Ernst, 1963; Morawetz and Samuel, 1989; Oginuma and Tobe, 1991; Zemskova, 1993; Hanson *et al.*, 2001; Chen *et al.*, 2003; Ivanova, 2005).

In a comprehensive morphological and taxonomical revision of the central American *Platanus* spp. (Nixon and Poole, 2003), the authors suggested that interspecific hybridization and reticulation could have been important factors in the evolution of the genus, because of the intermediate morphologies and the high levels of interfertility detected. According to Nixon and Poole (2003), four species and six varieties are present in eastern Mexico and adjacent regions of the USA: *P. occidentalis* L. var. *occidentalis*, *P. occidentalis* var. *palmeri* (Kuntze) Nixon & Poole ex Geerinck, *P. mexicana* Moric. var. *mexicana*, *P. mexicana* var. *interior* Nixon & Poole,

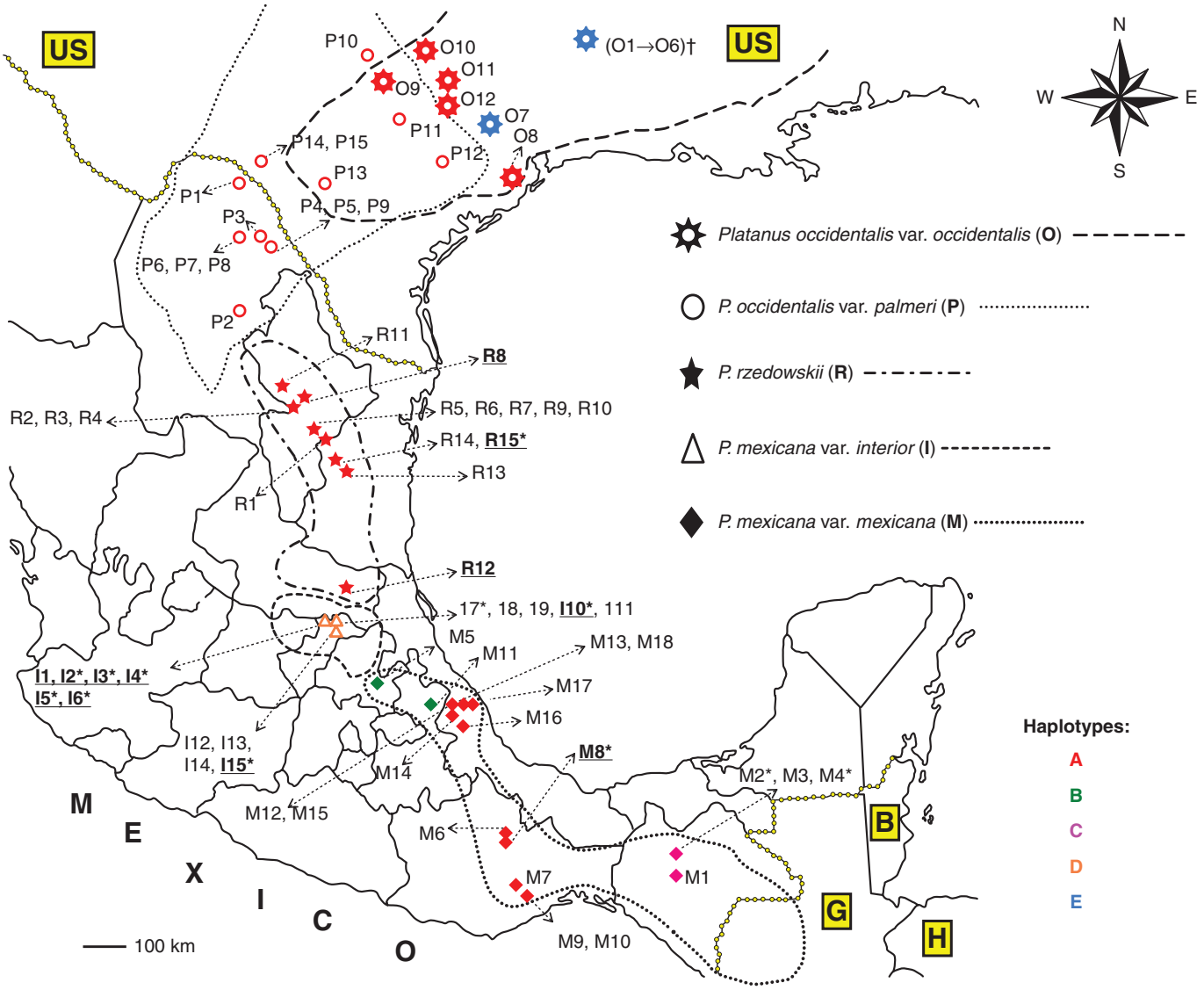

FIG. 1. Geographical range and localities of *Platanus* taxa analysed belonging to the ANA clade (Grimm and Denk, 2008) in eastern Mexico and the adjacent USA (US), Belize (B), Guatemala (G) and Honduras (H). Lines correspond to the geographical boundary of each *Platanus* taxon, except for the north-eastern distribution of *P. occidentalis* var. *occidentalis* which is partially shown [(O1 → O6)†]; symbols correspond to the *Platanus* taxa analysed. Information is also shown for plastid DNA haplotypes (coloured symbols) and nrDNA SNP data (alphabetical codes). Codes in underlined bold type and asterisks indicate specimens with heterozygosity patterns for ITS2 and *LFY*-i2 genotypes, respectively. For further details, see Fig. 5.

P. racemosa Nutt. var. *racemosa*, *P. racemosa* var. *wrightii* Benson and *P. rzedowskii* Nixon & Poole.

Sympatry is documented only for the varieties of *P. occidentalis* from Texas (USA) (Fig. 1); however, Nixon and Poole (2003) suggested that contact zones probably occurred in the past for *P. rzedowskii* and *P. mexicana sensu stricto* (*s.s.*), *P. occidentalis* var. *palmeri* and *P. racemosa* var. *wrightii*, and *P. mexicana* var. *interior* and *P. occidentalis* var. *palmeri*.

Some synonyms of *P. mexicana* var. *mexicana* are still of questionable nomenclatural status, i.e. *P. chiapensis* Standl. (from Chiapas, Mexico) and *P. oaxacana* Standl. (from Oaxaca, Mexico). Their origin and distinctness have caused debate among taxonomists without arriving at a solution (Nixon and Poole, 2003; Feng *et al.*, 2005).

Recently, molecular analyses paying special attention to subgenus *Platanus* have provided a greater understanding of the evolutionary history of the genus (Feng *et al.*, 2005; Grimm and Denk, 2008, 2010). These authors agree that hybridization, rather than incomplete lineage sorting, has played a role in the evolution of the central American *Platanus* spp., and several speculations have been made about their evolutionary history.

In light of their molecular results [based on internal transcribed spacer (ITS) sequences], Grimm and Denk (2008) sub-divided subgenus *Platanus* into a Pacific North American–European (PNA-E) clade (*P. orientalis* L. and *P. racemosa* species aggregate) and an Atlantic North American (ANA) clade [*P. mexicana sensu lato* (*s.l.*), *P. occidentalis s.l.* and *P. rzedowskii*]. Successively, Grimm and Denk (2010)

established a framework for the evolutionary unfolding of *Platanus*, using evidence from three nuclear DNA regions [ITS, 5S-intergenic spacer (IGS) and *LEAFY* intron 2 (*LFY*-i2)]. Based on their results, they suggested an ancient hybridization between lineages ancestral to the modern *P. occidentalis* and *P. mexicana* complex. Specifically, *P. occidentalis* var. *palmeri*, *P. mexicana* var. *interior* and *P. rzedowskii* may be the result of hybridization. In addition, the last two taxa appear to have a complex history, including possible reticulation and gene flow among several other taxa. Accordingly, major contributors to *P. mexicana* var. *interior* could have been *P. rzedowskii* and *P. occidentalis* var. *palmeri*. Major contributors to *P. rzedowskii* could have been *P. mexicana s.s.* and *P. occidentalis* var. *palmeri*. However, *P. occidentalis* var. *occidentalis* may also have played a role in the formation of *P. rzedowskii*, as also reported by Nixon and Poole (2003) and Feng *et al.* (2005). Finally, Grimm and Denk (2010) underline the possibility that the PNA-E clade may have played a fundamental role in the evolution of the central American *Platanus* (ANA clade) and especially of *P. occidentalis s.l.* and *P. rzedowskii*.

The purpose of this study is to add new information to resolve speculations about the complex history of subgenus *Platanus* in eastern Mexico and adjacent areas in the USA. Here we address the following questions. (1) Were the PNA-E taxa (*P. orientalis* and *P. racemosa s.l.*) involved in the origin of *P. occidentalis s.l.* and *P. rzedowskii*, as hypothesized by Grimm and Denk (2010)? (2) Are the hybridization events recorded in the literature specific to *P. rzedowskii*, or do they represent a more widespread phenomenon in these taxa? (3) Do the problematic taxa *P. oaxacana* and *P. chiapensis* actually correspond to *P. mexicana s.s.*, as proposed by Nixon and Poole (2003)?

To address (1) we employed sequencing of a uniparental plastid DNA marker and, to address (2) and (3), qualitative and quantitative genotyping of single nucleotide polymorphisms (SNPs) of biparental nuclear ribosomal DNA (nrDNA) markers already tested by Grimm and Denk (2008, 2010; *LFY*-i2 and ITS).

A further goal of this study was to assess the potential of real-time polymerase chain reaction (PCR) for detection of ancient hybridization phenomena in plants. This technique has been employed for the last 20 years in a range of molecular biological applications (Van Guilder *et al.*, 2008) but is now being used increasingly for identification and hybrid recognition purposes (e.g. Werth *et al.*, 2010; Baerwald *et al.*, 2011; Culumber *et al.*, 2011; Harwood and Phillips, 2011; Matejusová *et al.*, 2012).

MATERIALS AND METHODS

Plant material and DNA extraction

Eighty-eight specimens of *Platanus* were collected in the field or obtained through the generosity of Guido Grimm, Thomas Denk and the herbaria US and TEX (Supplementary Data File S1). Some of the samples obtained from G. Grimm and T. Denk are the same as those described previously in their works (Grimm and Denk, 2008, 2010). Voucher specimens were deposited at NAP, S, US or TEX herbaria. The sampling for each taxon from the ANA clade was employed to reflect the real distribution as accurately as possible (Fig. 1). As maternal tissue, five achenes (pericarps) were analysed in the holotype *P. oaxacana* sheet (US 00888488, Fig. 2), with the exceptional permission of the Smithsonian Institution (US National Herbarium), gratefully acknowledged here.

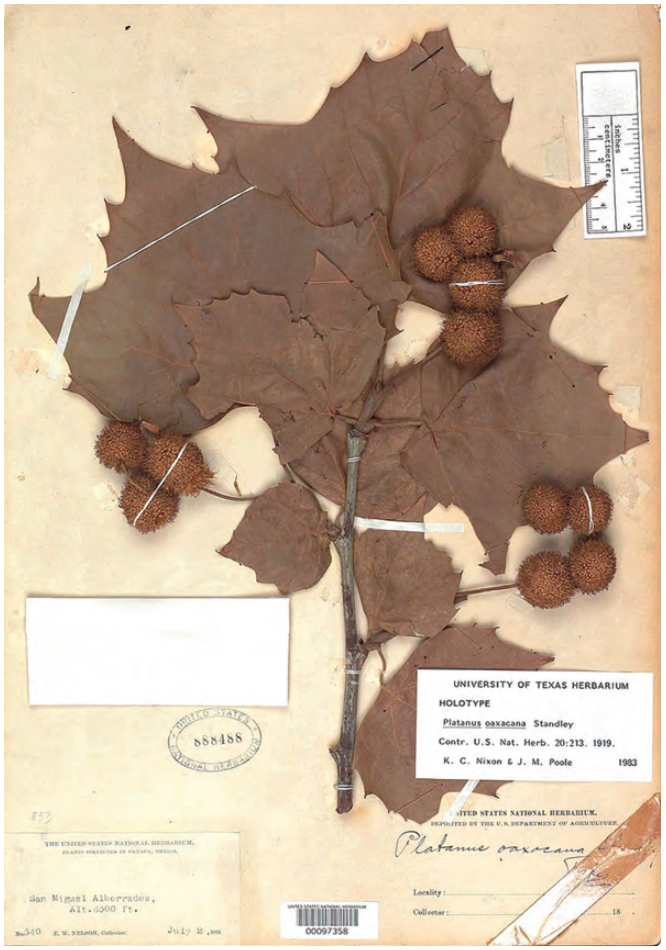

FIG. 2. Holotype of *Platanus oaxacana* Standl.; San Miguel Alborrados (Oaxaca, Mexico), 2 July 1894, E. W. Nelson 540 (US 00888488).

Total DNA was isolated according to the method of Doyle and Doyle (1987), using approx. 50 mg of dehydrated leaf tissue and a chloroform–isoamyl alcohol (24:1) purification step. The DNA was resuspended in 50 μL of sterile water. The concentration was estimated by a comparison of 1 μL of DNA with a DNA standard (Marker II, AppliChem GmbH) on a 0·8 % agarose gel containing 0·5 μg mL^{-1} ethidium bromide.

PCR amplification, sequencing and analysis of plastid DNA markers

Except in a few cases of documented biparental inheritance (Corriveau and Coleman, 1988; Hansen *et al.*, 2007), in most angiosperms the genome of plastids is inherited maternally through the seeds (Corriveau and Coleman, 1988); as a consequence, plastid DNA may provide insight into the fraction of maternal contribution (i.e. seed dispersal) to the genetic composition of the taxa investigated.

To discriminate among the 88 *Platanus* specimens investigated (Supplementary Data File S1), two types of non-coding plastid DNA markers were tested. The markers included introns [*rps16*

TABLE 1. *Primers used for PCR amplification and/or cycle sequencing of plastid and nuclear DNA regions of the* Platanus *taxa sequenced in the study*

Primer	Sequence (5′–3′)	Reference
Rps16 intron		
Forward: rpsF	GTG GTA GAA AGC AAC GTG CGA CTT	Oxelman *et al.* (1997)
Reverse: rpsR2	TCG GGA TCG AAC ATC AAT TGC AAC	Oxelman *et al.* (1997)
TrnL$^{(UAA)}$ intron		
Forward: c	CGA AAT CGG TAG ACG CTA CG	Taberlet *et al.* (1991)
Reverse: d	GGG GAT AGA GGG ACT TGA AC	Taberlet *et al.* (1991)
AtpB-rbcL intergenic spacer		
Forward: oligo 2	GAA GTA GTA GGA TTG ATT CTC	Manen *et al.* (1994)
Reverse: oligo 5	TAC AGT TGT CCA TGT ACC AG	Manen *et al.* (1994)
TrnT$^{(UGU)}$-*trnL*$^{(UAA)}$ intergenic spacer		
Forward: PL_T1	GTA AAC TAC TGA GAT CTT AGC	This study
Reverse: PL_T2	AGA TAT TGA TGA TTG ATA ATC G	This study
PsbA-trnH$^{(GUG)}$ intergenic spacer		
Forward: *psb*A3′f	GTT ATG CAT GAA CGT AAT GCT C	Sang *et al.* (1997)
Reverse: *trn*Hf	CGC GCA TGG TGG ATT CAC AAT CC	Tate and Simpson (2003)
Forward : PL_*psb*A3′f_int ~ 100	GTA AAA ACA TTA CTA CGG G	This study
Reverse: PL_*trn*Hf_int ~ 400	GGA ATT TCT ACC ATT CAT C	This study
LEAFY intron 2 (*LFY*-i2)		
Forward: Pla*LFY*5	GTA ACT GGA CAT CCG ATC TGA TAT CC	Feng *et al.* (2005)
Reverse: Pla*LFY*4	AGC ATA GCA GTG CAC ATA GTG TCT C	Feng *et al.* (2005)
Forward: PL_L-ex2_F internal	AGG TTG GTA TGT GCT TGG C	This study
Internal transcribed spacer (ITS)		
Forward: 18S (3′)	GGA GAA GTC GTA ACA AGG TTT CCG	Aceto *et al.* (1999)
Forward: 5·8S (3′)	TTG CAG AAT CCC GTG AAC CAT CG	Aceto *et al.* (1999)
Reverse: 26S (5′)	CCA AAC AAC CCG ACT CGT AGA CAG C	Aceto *et al.* (1999)
Reverse: 26S (5′) internal	TTC GCT CGC CGT TAC TAA GGG	This study

and *trnL*$^{(UAA)}$] and intergenic spacers [*trnT*$^{(UGU)}$-*trnL*$^{(UAA)}$, *atpB-rbcL* and *psbA-trnH*$^{(GUG)}$]. Molecular markers were amplified using both primers reported in the literature and also new primers designed in this study (Table 1). The volume of each amplification reaction was 25 μL, using 5 ng of template, 12·5 μL of DreamTaq™ PCR Master Mix (Fermentas, Thermo Fisher Scientific) and 0·125 μL of 50 μM primer. The cycling parameters were: initial denaturation at 95 °C for 3 min, followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing for 1 min as a function of the T_m of the primers used, and extension at 72 °C for 1 min. A final extension at 72 °C for 5 min was included.

Either a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Life Technologies) or a My Cycler™-Thermal Cycler (Bio-Rad) was used for amplification. The amplified products were purified using a DNA Enzyme-free isolation Spin-Kit (AppliChem GmbH), and 50–100 ng of purified templates were sequenced according to the method of Di Maio and De Castro (2013), using the fluorescent dye (Big Dye™ Terminator Cycle Sequencing Kit ver. 3.1, Applied Biosystems, Life Technologies) and a 3130 Genetic Analyzer (Applied Biosystems, Life Technologies). Complete sequences of both strands of each PCR product were processed, aligned and visually checked using the AB DNA Sequencing Analysis version 5.2 (Applied Biosystems, Life Technologies), Sequence Navigator version 1.0.1 (ABI Prism, Perkin Elmer) and BioEdit version 7.0.9.0 software (Hall, 1999).

Haplotypes were identified based on the variation of the sequences of the markers analysed. A geographical map of the haplotypes was prepared and a network was created based on the median-joining algorithm (Bandelt *et al.*, 1999) to evaluate possible genetic relationships between haplotypes (Network version 4.6.0.0 software, available at http://www.fluxus-engineering.com/index.htm).

SNP qualitative and quantitative genotyping

In total, 75 specimens belonging to the ANA clade were analysed (Supplementary Data File S1): 18 for *P. mexicana s.s.*; 15 for *P. mexicana* var. *interior*; 12 for *P. occidentalis s.str.*; 15 for *P. occidentalis var. palmeri*; and 15 for *P. rzedowskii*.

LEAFY *intron 2. LEAFY* is a single-copy nuclear plant gene with a structure of three exons and two introns. According to Feng *et al.* (2005) and Grimm and Denk (2010), the 3′ region of the second intron of *LEAFY* (*LFY*-i2) presents considerable molecular variation in *Platanus* spp. Grimm and Denk (2010) used *LFY*-i2 and its variants (i.e. ancestral sequences) to detect ancient hybridizations between lineages ancestral to the modern American *Platanus* taxa.

After preliminary screening of a data set of all *LFY*-i2 sequences by direct-PCR and clones (including its ancestral variants) present in the EMBL Nucleotide Sequence Database (EMBL-Bank), which is based on the work of Feng *et al.* (2005) and Grimm and Denk (2010), we observed informative point mutations to discriminate between *P. mexicana s.s.* (except for two accession from Chiapas; direct-PCR sequences, AY706049–AY706050) and *P. occidentalis s.l.* (i.e. both

TABLE 2. *nrDNA SNP loci analysed with the qualitative assay and relative probes for* LEAFY *intron 2 (*LFY*-i2) and internal transcribed spacer 2 (ITS2)*

Code	SNP	bp	Probe (5′–3′)	Length (bp)	T_m
LFY-i2*					
LFY-1	G/A	421	T_{10}-CGT TTG GCA GCC ACC TG	27	56 °C
LFY-2	T/G	550	T_{10}-AGY TTT ACT TWA AAG CCT ACT AAA TA	36	56 °C
ITS2†					
ITS-a	T/G	163	T_5-CCC CCC GTG TGY CGT GT	22	58 °C
ITS-b	G/A	298	T_{16}-GCC CCG GAG ACC CCG	31	58 °C

Further details about SNP sequence position are shown in *Supplementary Data File S2 and †Supplementary Data File S3.

varieties), owing to the presence of two SNPs (positions 421 and 550 bp in Supplementary Data File S2). These positions present a guanine nucleotide (421 bp) and thymine nucleotide (550 bp) in *LFY*-i2 of *P. mexicana s.s.* and an adenine nucleotide (421 bp) and guanine nucleotide (550 bp) in *P. occidentalis s.l.*

After an analysis of nucleotide sequence composition near the SNP trough, by the Custom TaqMan® Genomic SNP Genotyping Assay Service (Applied Biosystems, Life Technologies), the two SNPs were suitable for a qualitative genotyping using a SNaPshot™ Multiplex Kit and end-point PCR.

For the SNP assay, *LFY*-i2 marker was amplified using the primers described in Feng *et al.* (2005; Table 1). Because the forward primer reported in the literature (Feng *et al.*, 2005) failed to produce efficient amplification for some taxa, we designed an internal primer that amplified the area of interest (Table 1). Amplification reactions were carried out employing the TITANIUM™ Taq PCR Kit (Clontech Laboratories, Inc.).

The probe sequences and their relative SNPs are shown in Table 2. Probe sequences were analysed for secondary structures, complementarity and specificity. The SNaPshot assay is based on a single nucleotide oligo (probe) extension using a fluorescently tagged ddNTP for sites on an amplicon that show species-specific polymorphism. As such, the end of a probe must be immediately adjacent to the SNP, which means that when optimizing primers, only length and directionality can be varied. To multiplex the SNPs, the probes must each be separated by 4–5 bp in length so that the fluorescent peaks can be individually interpreted. To achieve this separation, we added variable length poly(T) tails to the 5′ end of a probes to allow separation of SNaPshot products on the basis of size (Hurst *et al.*, 2009) (Table 2).

To remove unincorporated dNTPs and extra primers from *LFY*-i2 amplifications, enzymatic reactions were carried out with 5 U of shrimp alkaline phosphatase (SAP) (USB, Affymetrix, Inc.) and 2 U of exonuclease I (Exo I) (USB, Affymetrix, Inc.) at 37 °C for 60 min; the solution was then heated to 80 °C for 15 min to denature the enzymes.

The PCR amplification of SNaPshot probes followed the protocol presented in the Applied Biosystems manual with minor modification. Briefly, for each reaction (final volume 5 μL), we used 2·5 μL of SNaPshot reaction mix, 0·2 μM of each probe and 0·1 pmol of PCR template (*LFY*-i2 amplification). The reaction was then cycled 25 times for 10 s at 96 °C, 5 s at 56 °C and 30 s at 60 °C. We used a final SAP clean-up step, adding 1 U of SAP and incubating at 37 °C for 60 min and then heating at 75 °C for 15 min. Samples were electrophoresed on a 3130 Genetic Analyzer (Applied Biosystems, Life Technologies) with a fluorescently labelled internal size standard (GeneScan®-120 LIZ, Applied Biosystems, Life Technologies). Raw data were scored with the internal size standard using Peak Scanner version 1.0 software (Applied Biosystems, Life Technologies).

ITS. The internal transcribed spacer is a multicopy marker that can be prone to gene conversion and concerted evolution (Feliner and Rosselló, 2007). Grimm and Denk (2008) have shown that the homogenization in *Platanus* is incomplete owing to the presence of several highly variable ITS variants (functional, not pseudogenes). The rate of mutation is higher than the level of homogenization in these sequences. According to Grimm and Denk (2008, 2010), this marker has proved useful for inferring hybridization events in the evolutionary history of *Platanus*.

In this case, the SNP screening was similar to that reported for *LFY*-i2 (see above). After a preliminary screening of a large data set of ITS nrDNA sequences by direct-PCR and clones (including all ITS variants) present in the EMBL Nucleotide Sequence Database (EMBL-Bank), based on the work of Feng *et al.* (2005) and Grimm and Denk (2008, 2010), we chose the ITS2 marker (including its variants) because it discriminated well between *P. mexicana s.s.* and *P. occidentalis s.l.*, owing to the presence of three SNPs between the sequences (base positions 130, 163 and 298 – see Supplementary Data File S3). These positions presented a cytosine nucleotide (130 bp), thymine nucleotide (163 bp) and a guanine nucleotide (298 bp) in all the ITS2 accessions of *P. mexicana s.s.* and with a thymine nucleotide (130 bp), deletion with the next nucleotide was a guanine (163 bp), adenine nucleotide (298 bp) in *P. occidentalis s.l.*

After an analysis of nucleotide sequence composition near the SNP troughs, by the Custom TaqMan® Genomic SNP Genotyping Assay Service (Applied Biosystems, Life Technologies), only the first SNP was found to be suitable for quantitative genotyping using the TaqMan® MGB probes assay and real-time PCR, and the last two SNPs for a qualitative genotyping using a SNaPshot™ Multiplex Kit and end-point PCR.

For both SNP assays, ITS2 markers were amplified using the primers described in Aceto *et al.* (1999; Table 1). Amplification reactions were carried out employing the TITANIUM™ Taq PCR Kit (Clontech Laboratories, Inc.).

For the SNaPshot assay, the probe sequences and their relative SNPs are shown in Table 2. The SNaPshot method was the same as described above for *LFY*-i2, except for the annealing temperature of 58 °C.

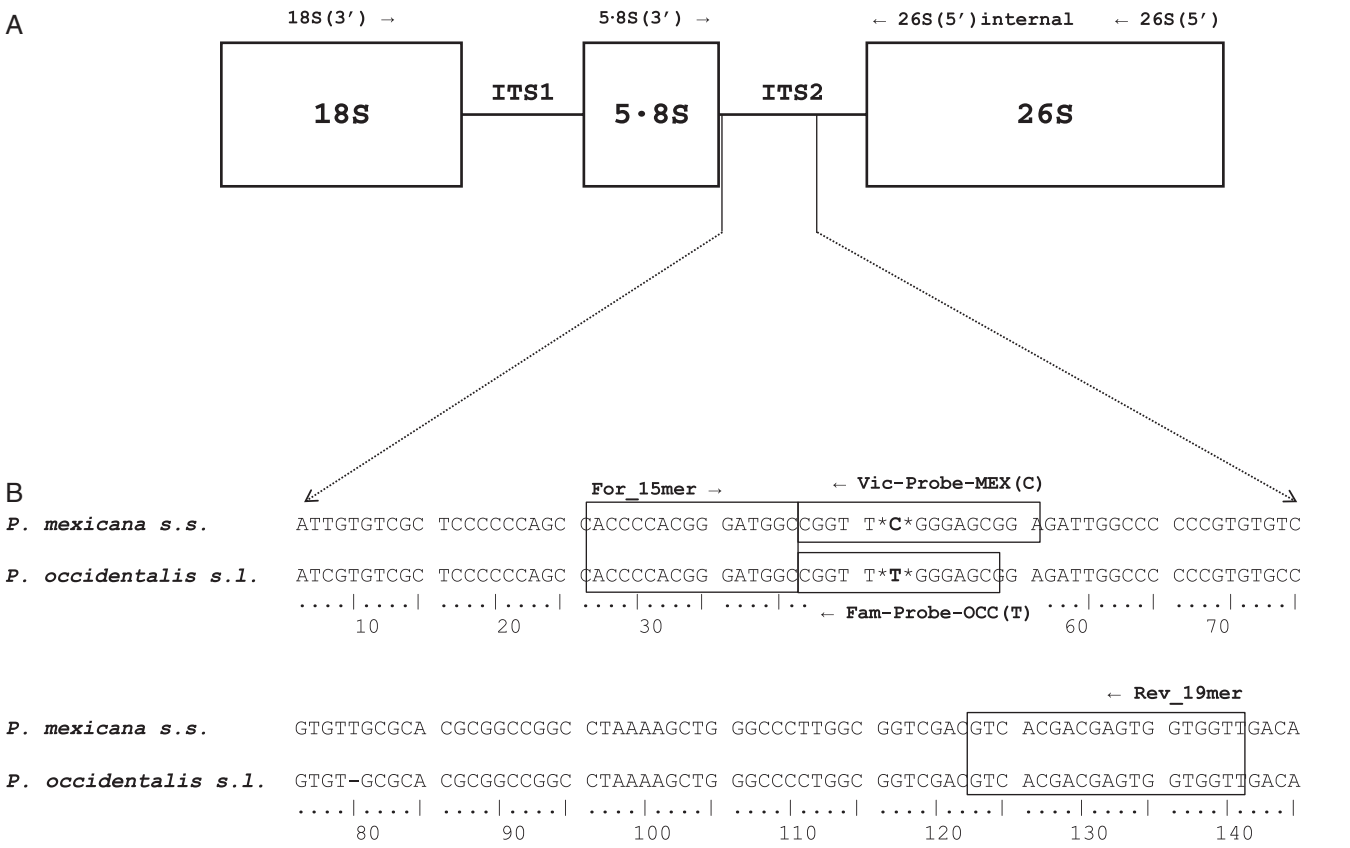

FIG. 3. (A) Outline of the nrDNA ITS and locations of oligonucleotides used as primers for PCRs and DNA sequencing in *Platanus* accessions. See Table 1 for the nucleotide sequence of each primer. (B) Alignment of partial sequences in ITS2 *P. mexicana s.s.* and *P. occidentalis s.l.* Boxes show the sequences used for the primers and probes. The nucleotides in the probes distinct for *P. mexicana s.s.* and *P. occidentalis s.l.* are indicated with asterisks. The SNP position corresponds to position 130 in the Supplementary Data File S3.

For the quantitative assay, the forward and reverse primers and the fluorescence-labelled probes for each species showing nucleotide polymorphism (*P. mexicana s.s.* and *P. occidentalis s.l.*) were designed using the Assay-by-DesignSM Service for the SNP Genotyping Assays (TaqMan® MGB Probes, FAM™ and VIC® dye-labelled) (Applied Biosystems, Life Technologies). These assays were designed for the genotyping of specific SNPs and each assay enables scoring of both alleles in a single well. The forward and reverse primer sequences were 5′-ACC CCA CGG GAT GG C-3′ and 5′-AAC CAC CAC TCG TCG TGA C-3′, respectively. The probes used were 5′-VIC-TCC GCT CCC GAA CCG-3′ for *P. mexicana s.s.* [MEX (C)] and 5′-FAM-CGC TCC CAA ACC G-3′ for *P. occidentalis s.l.* [OCC (T)]. The primer and probe positions are shown in Fig. 3. Genotyping was performed in 25 μL reactions including 30 pg of ITS2 template, 12·5 μL of 2× TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Life Technologies), 0·625 μL of 40× SNP Genotyping Assay Mix (Applied Biosystems, Life Technologies) and sterile water. The optimal starting template in each sample (standard included) was calculated by determining the threshold cycle (Ct) for several dilutions. The Ct was the cycle in which the first significant increase in fluorescence was detected. Two standards for both alleles were used, and each was serially diluted at intervals from 10^{-3} to 10^{-6}. The standards were obtained by sub-cloning the ITS2 fragments of *P. mexicana* var. *mexicana* (code M15) and *P. occidentalis* var. *occidentalis* (code O3) using the CloneJET™ PCR Cloning Kit (Fermentas Life Sciences). Positive and negative controls for both alleles were included in each genotyping plate.

The PCR cycling conditions were chosen according to the standard protocol recommended by the manufacturer (95 °C for 10 min; 40 repeated cycles of 92 °C for 15 s and 60 °C for 1 min). The reaction mixture for each sample was prepared in triplicate. All the PCRs were repeated twice to confirm the reliability of the results. Amplification was carried out using a 7500 Real Time PCR System (Applied Biosystems, Life Technologies) that provided a cycle by cycle measurement of the fluorescence emission from each reaction during the 60 °C annealing/extension step of each cycle. Fluorescence was analysed using 7500 System Sequence Detection (SDS) version 1.2.3 software (Applied Biosystems, Life Technologies) either with automatic calculation or manually set. PCR efficiency was calculated according to Smith *et al.* (2006) as $[(10^{-1/\text{slope}}) - 1] \times 100$.

Finally, to validate the SNP genotyping results, some assays were also sub-cloned using the CloneJET™ PCR Cloning Kit (Fermentas, Thermo Fisher Scientific). The sequencing method

was the same as described above for plastid DNA markers. The program Sequence Navigator version 1.0.1 (ABI Prism, Perkin Elmer) was used to compile 'contiguous sequences of each accession from the electropherograms generated by the automated sequencer. The DNA sequence of each *Platanus* sample was aligned with ClustalW, as implemented in the BioEdit version 7.0.9.0 software (Hall, 1999). The data matrix was verified against the original chromatogram files to ensure that all base assignments were valid at all possible positions.

RESULTS

Plastid DNA analyses

After a preliminary screening of several plastid DNA markers, we chose the *psbA-trnH*$^{(GUG)}$ intergenic spacer because it discriminated well among the taxa investigated here. The sequences of the *rps16* intron and *atpB-rbcL* intergenic spacer showed 100 % identity in all samples investigated. In contrast, the *trnL*$^{(UAA)}$ intron and *trnT*$^{(UGU)}$*-trnL*$^{(UAA)}$ intergenic spacer contained some SNPs but they were not diagnostic for the taxa in this study and were not geographically correlated.

Haplotypes were identified based on the variation of the polymorphic *psbA-trnH*$^{(GUG)}$ intergenic spacer; the differences were due to SNPs and gaps, as shown in Fig. 4 and Supplementary Data File S4. Because the reverse primer reported in the literature (Tate and Simpson, 2003) failed to produce efficient amplification for some taxa, we designed an internal primer that amplified the area of interest (Table 1).

Five different haplotypes were identified for the *psbA-trnH*$^{(GUG)}$ intergenic spacer: haplotype A was present in *P. mexicana s.s.* from Veracruz and Oaxaca (codes M6–M10 and M12–M18), some accessions of *P. occidentalis s.s.* from Texas (codes O8–O12), *P. occidentalis* var. *palmeri* (codes P1–P15) and *P. rzedowskii* (codes R1–R15); haplotypes B and C were present in *P. mexicana s.s.* from Hidalgo and Puebla (codes M5 and M11) and Chiapas (codes M1–M4), respectively; haplotype D was present in *P. mexicana* var. *interior* (codes I1–I15); and, lastly, haplotype E was present in *P. occidentalis s.s.* (codes O1–O7). The composition of the haplotypes is reported in Fig. 5 and their geographical distribution is shown in Fig. 1.

In the taxa belonging to the PNA-E clade (Pacific North America–Europe area), only haplotype A was identified in five accessions of *P. orientalis*, three of *P. racemosa* var. *racemosa* and four of *P. racemosa* var. *wrightii*. In addition, a hybrid accession was also analysed (*P.* × *hispanica* Mill. ex Muench, *P. orientalis* × *P. occidentalis s.s.*) and haplotype A was detected.

The haplotype network based on the median-joining algorithm showed a clear grouping pattern: haplotype A → haplotype B → haplotype C, with haplotypes D and E independently connected to haplotype A (Fig. 6).

SNP qualitative and quantitive genotyping

LEAFY *intron 2*. The two SNPs analysed using qualitative genotyping showed covariance (positions 163 and 298 bp in Supplementary Data File S2). SNaPshot analyses successfully identified the presence of SNPs in 12 of the accessions analysed (Fig. 5): specifically, in three accessions of *P. mexicana s.s.*

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        281        291        301        311        321        331        341        351        361
Haplotype A      TATTTAGAAA TATTTAAATA TATTAAATAT ATATATATAT TATGTAATAT ---------- ---------- GTAATATATT GTAATATATT
Haplotype B      TATTTAGAAA TATTTCAATA TATTAAATAT ATATATATAT TATGTAATAT ---------- ---------- GTAATATATT GTAATATATT
Haplotype C      TATTTCTAAA TATTTCAATA TATTAAATAT ATATATATAT TATGTAATAT ---------- ---------- GTAATATATT GTAATATATT
Haplotype D      TATTTAGAAA TATTTAAATA TATTAAATAT ATATATATAT TATGTAATAT ---------- ---------- -------ATT GTAATATATT
Haplotype E      TATTTAGAAA TATTTAAATA TATTAAATAT ATATATATAT TATGTAATAT ATATATATAT TATGTAATAT GTAATATATT GTAATATATT
                 *****  *** ***** **** ********** ********** **********                           *** **********


                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        371        381        391        401        411        421        431        441        451
Haplotype A      AATATATCTT CCTCGAAATC TTTTGACTTT TTTTTAAGTC ATTTTTGTGT ACAGTTACAG GATTGGCATT TTATGTTTAA GATCTGTATT
Haplotype B      AATATATCTT CCTCGAAATC TTTTGACTTT TTTTTAAGTC ATTTTTGTGT ACAGTTACAG GATTGGCATT TTATGTTTAA GATCTGTATT
Haplotype C      AATATATCTT CCTCGAAATC TTTTGACTTT TTTTTAAGTC ATTTTTGTGT ACAGTTACAG GATTGGCATT TTATGTTTAA GATCTGTATT
Haplotype D      AATATATCTT CCTCGAAATC TTTTGACTTT TTTTTAAGTC ATTTTTGTGT ACAGTTACAG GATTGGCATT TTATGTTTAA GATCTGTATT
Haplotype E      AATATATCTT CCTCGAAATC TTTTGACTTT TTTTTAAGTC ATTTTTGTGT ACAGTTACAG GATTGGCATT TTATGTTTAA GACCTGTATT
                 ********** ********** ********** ********** ********** ********** ********** ********** ** *******

Haplotype A = Platanus orientalis, P. × hispanica, P. racemosa s.l.   Haplotype B = P. mexicana s.s. (Hidalgo, Puebla)
              P. mexicana s.s.                                        Haplotype C = P. mexicana s.s. (Chiapas)
              P. occidentalis s.s. (Texas)                            Haplotype D = P. mexicana var. interior
              P. occidentalis var. palmeri                            Haplotype E = P. occidentalis s.s.
              P. rzedowskii
```

FIG. 4. Alignment of partial sequences of the *psbA-trnH*$^{(GUG)}$ intergenic spacer and haplotypes present in the *Platanus* taxa.

				GENOTYPE COLOUR = *PLATANUS MEXICANA s.s.* AND *P. OCCIDENTALIS s.l.*							
			SEQUENCING [*psbA-trnH*$^{(GUG)}$ spacer]	SNP QUALITATIVE ASSAY (*LFY-i2* and ITS2)				SNP QUANTITATIVE ASSAY (ITS2)			
Code	Taxon	Locality	Haplotype	*LFY*-1	*LFY*-2	ITS-a	ITS-b	Probe MEX (C)	Probe OCC (T)	Ct M	Ct O
M1	*Platanus mexicana* Moric. var. *mexicana* (= *P. chiapensis* Standl.)	Ixtapa, Chiapas, México	C	A	G	T	G	+	–	25·84	Undet
M2	*P. mexicana* var. *mexicana* (= *P. chiapensis*)	Pantepec, Chiapas, México	C	A/G	G/T	T	G	+	–	25·39	Undet.
M3	*P. mexicana* var. *mexicana* (= *P. chiapensis*)	Pantepec, Chiapas, México	C	G	T	T	G	+	–	27·01	Undet.
M4	*P. mexicana* var. *mexicana* (= *P. chiapensis*)	Pantepec, Chiapas, México	C	A/G	G/T	T	G	+	–	27·81	Undet.
M5	*P. mexicana* var. *mexicana*	Molango, Hidalgo, México	B	G	T	T	G	+	–	24·17	Undet.
M6	*P. mexicana* var. *mexicana*	Choapam, Oaxaca, México	A	G	T	T	G	+	–	24·29	Undet.
M7	*P. mexicana* var. *mexicana*	Santa María Ecatepec, Oaxaca, México	A	G	T	T	G	+	–	24·47	Undet.
M8	*P. mexicana* var. *mexicana* (= *P. oaxacana* Standl.)*	San Miguel Alborrados, Oaxaca, México	A	A/G	G/T	G/T	A/G	+ (66.37%)**	+ (33.63%)**	25·35	24.73
M9	*P. mexicana* var. *mexicana*	Río Otate, Oaxaca, México	A	G	T	T	G	+	–	28	Undet.
M10	*P. mexicana* var. *mexicana*	Río Otate, Oaxaca, México	A	G	T	T	G	+	–	27	Undet.
M11	*P. mexicana* var. *mexicana*	Apulco, Puebla, México	B	G	T	T	G	+	–	24·53	Undet.
M12	*P. mexicana* var. *mexicana*	Chiconquiaco, Veracruz, México	A	G	T	T	G	+	–	26·79	Undet.
M13	*P. mexicana* var. *mexicana*	Colipa, Veracruz, México	A	G	T	T	G	+	–	26·05	Undet.
M14	*P. mexicana* var. *mexicana*	Las Minas, Veracruz, México	A	G	T	T	G	+	–	26·22	Undet.
M15	*P. mexicana* var. *mexicana*	Misantla, Veracruz, México	A	G	T	T	G	+	–	26·11	Undet.
M16	*P. mexicana* var. *mexicana*	Xalapa, Veracruz, México	A	G	T	T	G	+	–	26·2	Undet.
M17	*P. mexicana* var. *mexicana*	Vega de Alatorre, Veracruz, Mexico	A	G	T	T	G	+	–	26·37	Undet.
M18	*P. mexicana* var. *mexicana*	Yecuatla; Veracruz, México	A	G	T	T	G	+	–	24·92	Undet.
I1	*P. mexicana* var. *interior* Nixon & Poole	Ahuacatlán, Querétaro, México	D	G	T	T	G	+ (93.46%)	+ (6.54%)	24·49	27·34
I2	*P. mexicana* var. *interior*	Ahuacatlán, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (83.09%	+ (16.91%)	22·18	28·69
I3	*P. mexicana* var. *interior*	Ahuacatlán, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (74.37%)	+ (25.63%)	25·62	25·28
I4	*P. mexicana* var. *interior*	El Llano to San Pedro, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (40.3%)	+ (59.7%)	24·82	22·05
I5	*P. mexicana* var. *interior*	El Llano to San Pedro, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (54.45%)	+ (45.55%)	23·48	24·66
I6	*P. mexicana* var. *interior*	El Llano to San Pedro, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (22.24%)	+ (77.76%)	27·8	25·5
I7	*P. mexicana* var. *interior*	El Trapiche, Querétaro, México	D	A/G	G/T	G	A	–	+	Undet.	25·80
I8	*P. mexicana* var. *interior*	El Trapiche, Querétaro, México	D	G	T	T	G	+	–	24·33	Undet.
I9	*P. mexicana* var. *interior*	El Trapiche, Querétaro, México	D	G	T	T	G	+	–	27·43	Undet.
I10	*P. mexicana* var. *interior*	El Trapiche, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (48.61%)	+ (51.39%)	28·29	26·80
I11	*P. mexicana* var. *interior*	El Trapiche, Querétaro, México	D	G	T	T	G	+	–	25·08	Undet.
I12	*P. mexicana* var. *interior*	Jalpan, Querétaro, México	D	G	T	T	G	+	–	24·19	Undet.
I13	*P. mexicana* var. *interior*	Jalpan, Querétaro, México	D	G	T	T	G	+	–	27·48	Undet.
I14	*P. mexicana* var. *interior*	Jalpan, Querétaro, México	D	G	T	T	G	+	–	27·65	Undet.
I15	*P. mexicana* var. *interior*	Jalpan, Querétaro, México	D	A/G	G/T	G/T	A/G	+ (44.58%)	+ (55.42%)	27·75	24·65

FIG. 5. Plastid DNA haplotypes [*psbA-trnH*$^{(GUG)}$ intergenic spacer] and nrDNA SNP genotypes [*LEAFY* intron 2 (*LFY*-i2) and internal transcribed spacer 2 (ITS2)] present in the *Platanus* taxa using qualitative and quantitative genotyping. The green genotype corresponds to the nucleotides present in specimens of *P. mexicana s.s.* and the blue genotype corresponds to the nucleotides present in specimens of *P. occidentalis s.l.* *Maternal tissue of five achenes. **$\pm$ 0·1 s.e.

O1	*P. occidentalis* L. var. *occidentalis*	Ontario, Canada	E	A	G	G	A/A	–	+	Undet.	25·02
O2	*P. occidentalis* var. *occidentalis*	Cave in Rock, Illinois, USA	E	A	G	G	A	–	+	Undet.	23·97
O3	*P. occidentalis* var. *occidentalis*	Squaw Creek Ames, Iowa, USA	E	A	G	G	A	–	+	Undet.	24·4
O4	*P. occidentalis* var. *occidentalis*	Missouri Botanical Gardner, Missouri, USA	E	A	G	G	A	–	+	Undet.	25·47
O5	*P. occidentalis* var. *occidentalis*	San Luis, Missouri, USA	E	A	G	G	A	–	+	Undet.	25·45
O6	*P. occidentalis* var. *occidentalis*	North Caroline, USA	E	A	G	G	A	–	+	Undet.	25·51
O7	*P. occidentalis* var. *occidentalis*	La Grange, Fayette Co, Texas, USA	E	A	G	G	A	–	+	Undet.	24·87
O8	*P. occidentalis* var. *occidentalis*	Dickinson, Galveston Co, Texas, USA	A	A	G	G	A	–	+	Undet.	25
O9	*P. occidentalis* var. *occidentalis*	6.5 km W of Hwy 87 & West Cherry Spring road, Gillespie Co, Texas, USA	A	A	G	G	A	–	+	Undet.	26·15
O10	*P. occidentalis* var. *occidentalis*	Crawford, McClennan Co, Texas, USA	A	A	G	G	A	–	+	Undet.	25·37
O11	*P. occidentalis* var. *occidentalis*	10 km NW of Hwy 620 on Hwy 71, Travis Co, Texas, USA	A	A	G	G	A	–	+	Undet.	24·63
O12	*P. occidentalis* var. *occidentalis*	Brushy Creek, Williamson, Texas, USA	A	A	G	G	A	–	+	Undet.	25
P1	*P. occidentalis* var. *palmeri* (Kuntze) Nixon & Poole ex Geerinck	Acuña, Coahuila, México	A	A	G	G	A	–	+	Undet.	25·54
P2	*P. occidentalis* var. *palmeri*	Monclova, Coahuila, México	A	A	G	G	A	–	+	Undet.	24·72
P3	*P. occidentalis* var. *palmeri*	Nueva Rosita, Coahuila, México	A	A	G	G	A	–	+	Undet.	24·79
P4	*P. occidentalis* var. *palmeri*	Río Sabinas, Coahuila, México	A	A	G	G	A	–	+	Undet.	25·40
P5	*P. occidentalis* var. *palmeri*	Río Sabinas, Coahuila, México	A	A	G	G	A	–	+	Undet.	24·76
P6	*P. occidentalis* var. *palmeri*	Río San Juan, Coahuila, México	A	A	G	G	A	–	+	Undet.	24·1
P7	*P. occidentalis* var. *palmeri*	Río San Juan, Coahuila, México	A	A	G	G	A	–	+	Undet.	24·69
P8	*P. occidentalis* var. *palmeri*	Río San Juan, Coahuila, México	A	A	G	G	A	–	+	Undet.	24·91
P9	*P. occidentalis* var. *palmeri*	Río Sabinas, Coahuila, México	A	A	G	G	A	–	+	Undet.	25·10
P10	*P. occidentalis* var. *palmeri*	9 km NW of spur 191 on Hwy 71, Burnett Co, Texas, USA	A	A	G	G	A	–	+	Undet.	24·89
P11	*P. occidentalis* var. *palmeri*	Austin, Travis Co., Texas, USA	A	A	G	G	A	–	+	Undet.	25·49
P12	*P. occidentalis* var. *palmeri*	De Witt Co., Texas, USA	A	A	G	G	A	–	+	Undet.	26·10
P13	*P. occidentalis* var. *palmeri*	Garmel State Paul, Uvalde Co, Texas, USA	A	A	G	G	A	–	+	Undet.	26·23
P14	*P. occidentalis* var. *palmeri*	N of Caustreche, Val Verde Co., Texas, USA	A	A	G	G	A	–	+	Undet.	24·55
P15	*P. occidentalis* var. *palmeri*	Rio Grande, Val Verde Co., Texas, USA	A	A	G	G	A	–	+	Undet.	25·61
R1	*P. rzedowskii* Nixon & Poole	Aramberri, Nuevo Léon, Mexico	A	A	G	G	A	–	+	Undet.	26·11
R2	*P. rzedowskii*	Cola de Caballo, Nuevo León, México	A	A	G	G	A	–	+	Undet.	25·88
R3	*P. rzedowskii*	Cola de Caballo, Nuevo León, México	A	G	T	G	A	–	+	Undet.	24·08
R4	*P. rzedowskii*	Cola de Caballo, Nuevo León, México	A	A	G	G	A	–	+	Undet.	24·99
R5	*P. rzedowskii*	El Ebanito, Nuevo León, México	A	G	T	G	A	–	+	Undet.	24·98
R6	*P. rzedowskii*	El Ebanito, Nuevo León, México	A	A	G	G	A	–	+	Undet.	24·86

Fig. 5 *Continued*

(=*P. chiapensis*, codes M2 and M4; =*P. oaxacana*, code M8); in eight accessions of *P. mexicana* var. *interior* (codes I1–I7, I10 and I15); and in one accession of *P. rzedowskii* (code R15). The nucleotides from the other accessions presented a taxon-specific genotype (*P. mexicana s.s.* or *P. occidentalis s.l.*) (Fig. 5) except for one accession of *P. mexicana s.s.* from Chiapas (=*P. chiapensis*, code M1) and for *P. rzedowskii* where seven accessions showed the *P. mexicana s.s.* genotype

R7	*P. rzedowskii*	El Ebanito to Las Cruciatas, Nuevo León, México	A	A	G	G	A	–	+	Undet.	24·87
R8	*P. rzedowskii*	El Cercado, Nuevo León, México	A	G	T	G/T	A/G	+ (23·16%)	+ (76·84%)	26.21	25·99
R9	*P. rzedowskii*	~ 8 mi E of Iturbide, Nuevo Léon, Mexico	A	A	G	G	A	–	+	Undet.	24·93
R10	*P. rzedowskii*	Las Crucitas, Nuevo León, México	A	A	G	G	A	–	+	Undet.	24·78
R11	*P. rzedowskii*	Monterrey, Nuevo León, México	A	G	T	G	A	–	+	Undet.	24·74
R12	*P. rzedowskii*	Tamasopo, San Luis Potosí, México	A	G	T	G/T	A/G	+ (84·62%)	+ (15·38%)	25.63	26·60
R13	*P. rzedowskii*	North of Ciudad Victoria, Tamaulipas, México	A	G	T	G	A	–	+	Undet.	24·91
R14	*P. rzedowskii*	Río Corona, Tamaulipas, México	A	G	T	G	A	–	+	Undet.	24·5
R15	*P. rzedowskii*	Río Corona, Tamaulipas, México	A	A/G	G/T	G/T	A/G	+ (18·55%)	+ (81.45%)	28·35	23·83

Fig. 5 *Continued*

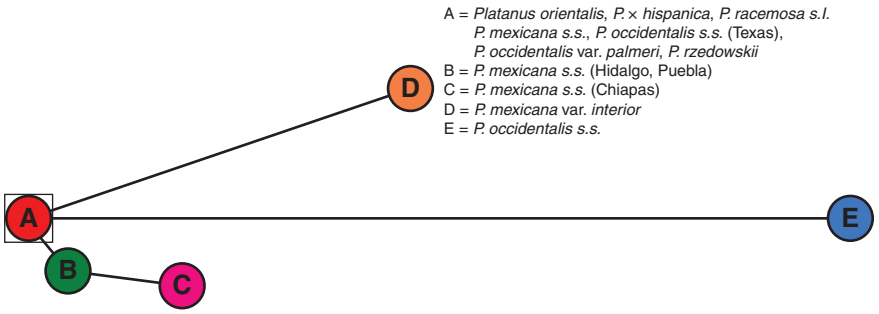

FIG. 6. *Platanus* plastid DNA haplotype network based on the median-joining algorithm.

(codes R3, R5, R8 and R11–R14) and the remainder showed the *P. occidentalis s.l.* genotype (codes R1, R2, R4, R6, R7, R9 and R10.)

ITS. The three SNPs analysed showed covariance. However, a test for DNA secondary structure using the RNAstructure version 5.4 software (Reuter and Mathews, 2010) indicated that these nucleotides are not part of any stem–loop structure.

A qualitative analysis was carried out on two SNPs using SNaPshot probes with ITS2 nrDNA (positions 163 and 298 in Supplementary Data File S3) from each *Platanus* taxon as a template (Table 2). The specimens analysed were the same as those used for SNP genotyping in *LFY*-i2 (see above). Except for in some accessions, the results were highly congruent with the *LFY*-i2 results (see Discussion).

Our SNaPshot analyses successfully identified the presence of an SNP in 12 of the accessions analysed (Fig. 5): in one accession of *P. mexicana s.s.* (= *P. oaxacana*, code M8), in three of *P. rzedowskii* (codes R8, R12 and R15) and in seven of *P. mexicana* var. *interior* (codes I2–I6, I10 and I15). The nucleotides from the other taxa had a taxon-specific nucleotide, except for an accession of *P. mexicana* var. *interior* (code I7) which had nucleotides corresponding to *P. occidentalis s.l.*

A quantitative analysis of a single SNP was carried out using TaqMan MGB probes with ITS2 template (position 130 in Supplementary Data File S3 and Fig. 3) from the same data set used in the previous SNaPshot assay (Fig. 5). The real-time PCR efficiencies were similar among specimens, ranging from 94 to 100 %, and the slopes ranged from −3·5 to −3·3 (R^2 >0·99). Sample Ct values were between 22·18 and 27·81, and showed good amplification/quantification of the template (Fig. 5).

Our real-time PCR analyses identified the presence of SNPs in the same specimens analysed with the SNaPshot assay for the ITS2, except for an accession of *P. mexicana* var. *interior* (code I1) (Fig. 5). The SNP was not detected in this sample by the SNaPshot assay. This is probably due to the higher resolution of real-time PCR which can detect low concentrations of probe (Fig. 5). This also explains the *LFY*-i2 SNP result in accession I1. The genotype frequency was variable in *P. rzedowskii* and especially so in *P. mexicana* var. *interior*, as shown in Fig. 5. Several individuals of *P. mexicana* var. *interior* [notably, samples from the El Llano to San Pedro population (codes I4–I6)] have either comparable amounts of genotypes or show a preponderance of the *P. occidentalis s.l.* genotype; individuals from Ahuacatlán show a preponderance of the *P. mexicana s.s.* genotype (codes I1–I3); the population from El Trapiche has two individuals which have the *P. mexicana s.s.* genotype (codes I8 and I9), one with a comparable amount and one (code I7) which, although identified phenotypically as *P. mexicana* var. *interior*, has only the *P. occidentalis s.l.* genotype. As expected from currently available information, the sequence from the other taxa presented a taxon-specific nucleotide. For example, *P. occidentalis s.l.* accessions presented a unique fluorescence corresponding to their own nucleotide (i.e. thymine/adenine).

As reported above, only one accession of *P. mexicana* var. *interior* (code I7) showed a genotype corresponding to that of *P. occidentalis s.l.*

Finally, some assays used in this study were validated by sub-cloning both ITS2 and *LFY*-i2. The sub-cloned samples were: one *P. mexicana* (=*P. oaxacana*, code M9), one *P. mexicana* var. *interior* (code I15), one *P. mexicana* from Veracruz (code M15), one *P. occidentalis* var. *palmeri* (code P1) and two *P. rzedowskii* PCR products (codes R14–R15). Twelve positive colonies for each amplification were selected for sequencing. Depending on the sample selected, colony sequencing confirmed the presence of a single nucleotide or an SNP.

DISCUSSION

Our results emphasize that hybridization has played a significant role in the evolution of the central American *Platanus* spp. and demonstrate the advantages of analysing plastid sequences and nuclear DNA SNP genotyping to help resolve several suppositions concerning this taxonomic puzzle. We improved both the usefulness and the accuracy of the real-time PCR allelic assay to better understand the complex systematic position of these taxa. According to our SNP DNA results and published data on ITS and *LFY*-i2 DNAs (Grimm and Denk 2008, 2010), two types of *LFY*-i2 and ITS DNA occur in our *Platanus* taxa. One of these distinguishes all accessions of *P. occidentalis s.l.* and one distinguishes the taxa belonging to *P. mexicana s.s.* The 3′ region of *LFY*-i2 (low-copy nuclear marker) and ITS2 (multi-copy nuclear marker) discriminate among taxa because of fixed mutations, permitting adequate differentiation of these *Platanus* spp. The SNP results were congruent except for a few exceptions (see below for *P. rzedowskii and P. chiapensis*) as expected from the literature (Feng *et al.*, 2005; Grimm and Denk, 2010).

On the whole, our results confirm the earlier hypotheses of Nixon and Poole (2003), Feng *et al.* (2005), Grimm and Denk (2008, 2010) and Denk *et al.* (2012), in which the evidence can be interpreted as being a consequence of hybridization in these central American *Platanus* spp.

Our plastid analyses ratify the results of Grimm and Denk (2010), who proposed that the lineage relating to the modern PNA-E taxa (*P. orientalis* and *P. racemosa s.l.*) forms one source of genetic material in the origins of *P. occidentalis s.l.* and *P. rzedowskii*. As shown in the haplotype median-joining network (Fig. 6), great relevance is assigned to haplotype A in the genetic relationships. This haplotype is present in the taxa belonging to the PNA-E clade (*P. orientalis* and *P. racemosa s.l.*), in five accessions of *P. occidentalis s.s.* from Texas, in *P. occidentalis* var. *palmeri*, in *P. mexicana s.s.* and in *P. rzedowskii*, and appears to be ancestral to haplotype E, present in *P. occidentalis s.s.* and haplotype D in *P. mexicana* var. *interior*. We propose that *P. occidentalis* var. *palmeri* and *P. mexicana s.s.* originate from a common ancestor included in the PNA-E taxa and that, over time, their nuclear markers accumulated several SNPs causing specific genotypes (see the Results and Fig. 5). We have also detected the presence of haplotype A in several accessions of *P. occidentalis s.s.* in the contact zone with *P. occidentalis* var. *palmeri* (Texas). This leads us to suppose that gene flow has occurred, or is occurring, between these taxa. These results confirm those of Nixon and Poole (2003), who showed that a morphological clinal pattern was present, which indicates possible extensive or secondary intergradations between these species. This hypothesis also finds support in Grimm and Denk (2010). Here, the authors used an evolutionary perspective to distinguish between *P. occidentalis* var. *palmeri* from northern Mexico and *P. occidentalis s.s.* from the north-eastern USA. In light of their molecular results, they treated *P. occidentalis* var. *palmeri* at species level as *P. palmeri* and they demonstrated a relatedness of this species to western Eurasian *P. orientalis* and to the *P. racemosa* species aggregate, which are considered its ancestral complex.

According to our study, *P. occidentalis* var. *palmeri* also appears to be independent of the *P. mexicana s.l.* lineage, with the *P. mexicana* complex presenting both distinctive morphological characteristics and also unique ITS and *LFY*-i2 DNA. Analysing the haplotype pattern, we observe that three different haplotypes have developed from *P. mexicana s.s.* (haplotype A): one is exclusive for *P. mexicana* var. *interior* (haplotype D) and the other two (B and C) are evolutionarily connected. Haplotype B was present in one accession from Puebla and Hidalgo; haplotype C was found in four specimens of *P. mexicana s.s.* from Chiapas (= *P. chiapensis*). Nixon and Poole (2003) were cautious in the taxonomic arrangement of this taxon and synonymized *P. chiapensis* with *P. mexicana s.s.*, although they observed that some morphological trends in Chiapas specimens were apparent from Veracruz and nearby areas: more acute leaf lobes, a whiter leaf vestiture and more leathery leaves than in *P. mexicana s.s.*

According to SNP data from *LFY*-i2 and ITS2, our accessions from Chiapas are to some degree anomalous. This is also deduced by Feng *et al.* (2005) who admitted that *P. mexicana s.s.* from Chiapas (= *P. chiapensis*) could be phylogentically distinct from *P. mexicana s.s.* on the basis only of *LFY*-i2 results. Our data partly confirm this conclusion, demonstrating overlap with the *P. mexicana s.s.* genotype (Fig. 5). Feng *et al.* (2005) analysed only two specimens and with direct sequencing of PCR templates, whereas in our study highly informative molecular approaches have been applied to this taxon. However, we agree with Feng *et al.* (2005), admitting that *P. mexicana* from Chiapas presents a particular evolutionary history probably caused by a vicariance event during the late Miocene because of the tectonic deformation of the Isthmus of Tehuantepec. These geological phenomena formed a barrier between the Gulf of Mexico and the Pacific Ocean (Barrier *et al.*, 1998; Suárez, 2000) which probably influenced gene flow among accessions of *P. mexicana s.s.* (Chiapas and Veracruz-Oaxaca) determining the particular molecular genotypes (plastid and nrDNA) observed in *P. mexicana* from Chiapas. These distinctions create the opportunity for an exhaustive study both to determine whether *P. chiapensis* should be reclassified and to better understand its haplotype relationships with accessions from Puebla and Hidalgo. However, this would require extensive sampling because, if *P. chiapensis* were indeed to be a distinct species, it would be the first taxon to occur sympatrically with another species.

The ranges of *P. mexicana s.s.* and *P. occidentalis s.l.* are currently separated by about 700 km and this area is occupied mainly by *P. rzedowskii*, a species first described by Nixon and Poole (2003), and, according to recent molecular data, characterized by a complex evolutionary history (Grimm and Denk, 2008,

2010). Our plastid results indicate that the *P. rzedowskii* accessions have the same haplotype (A) found in *P. occidentalis* var. *palmeri*, in some accessions of *P. occidentalis s.s.* from Texas (contact zone with *P. occidentalis* var. *palmeri*) and in some *P. mexicana s.s.* (Figs 1 and 5). According to SNP data, *P. rzedowskii* may be the product of hybridization between *P. occidentalis* var. *palmeri* (or an ancestral taxon) and *P. mexicana s.l.* The SNP genotyping results of *LFY*-i2 DNA in *P. rzedowskii* were mostly congruent with those of ITS2 except for some accessions (see Fig. 5). In the ITS phylogenetic tree (Feng *et al.*, 2005), *P. rzedowskii* grouped with *P. occidentalis s.l.*, but with *LFY*-i2 it was placed in both *P. mexicana s.l.* and *P. occidentalis s.l.* This conflict between the ITS2 and *LFY*-i2 SNP results can be explained by the different evolution of these molecular markers and also by the presence of hybridization and introgression among these *Platanus* taxa. Other authors (Grimm and Denk, 2010) have previously suggested that *P. rzedowskii* may have been the product of recent hybridization between *P. occidentalis* var. *palmeri* (or an ancestral taxon) and *P. mexicana s.l.* In contrast, on morphological grounds, Nixon and Poole (2003) concluded that it is a distinctive species, with no complete sympatry and no intermediates with *P. occidentalis* var. *palmeri.*

Notably, other *Platanus* taxa have shown the presence of both genotypes (Fig. 5): some accessions of *P. mexicana* var. *interior* and one of *P. oaxacana* (= *P. mexicana* var. *mexicana*). In the case of *P. mexicana* var. *interior*, it is important to note that the presence of both genotypes is not constant; in fact <50 % of specimens are pure *P. mexicana s.s.* in terms of their genotypes (Fig. 5). These results can be explained by admitting that *P. mexicana* var. *interior* is affected by lateral gene flow (introgression) from *P. rzedowskii* and/or *P. occidentalis* var. *palmeri*. According to Nixon and Poole (2003), the leaves, capitula, habitat and range of *P. mexicana* var. *interior* could indicate past contact with and introgression from only *P. occidentalis* var. *palmeri*. Finally, in a recent article, Denk *et al.* (2012) proposed that var. *interior* should be discarded if new genetic data confirm the hybrid (or introgressive) nature of this taxon.

As far as molecular data in *Platanus* are concerned, our results indicate that *P. rzedowskii* has neither a distinctive plastid DNA haplotype nor a specific ITS/*LFY*-i2 genotype, and that *P. mexicana* var. *interior*, albeit having a characteristic haplotype, does not show any specific ITS/*LFY*-i2 genotype. Both taxa show individuals of mixed ITS/*LFY*-i2 ancestry, more abundant in *P. mexicana* var. *interior*. Indeed, *P. rzedowskii* and *P. mexicana* var. *interior* as a whole seem to represent an almost complete series of introgressants. From the above, it is reasonable to admit that hybridization and successive introgression have been key evolutionary factors in the origins of both *P. rzedowskii* and *P. mexicana* var. *interior*.

In this regard, the qualitative and quantitative heterozygous patterns obtained for the accessions of *P. mexicana s.s.* from Oaxaca (= *P. oaxacana*) are also interesting (Fig. 5). Additional information on the *P. oaxacana* specimen is necessary. This unique specimen of this taxon is the holotype deposited in the Smithsonian Institution, Department of Botany (USA) (Fig. 2). According to Nixon and Poole (2003, p. 126), this sample is unique from a morphological point of view. These authors defined it as an '. . . aberrant specimen in terms of the degree of toothing of the leaves but otherwise is consistent with *P. mexicana* var. *mexicana* in all important diagnostic characters.' However, because it is a single sample from the type location, the authors considered it as a synonym of *P. mexicana s.s.* Standley (1919) pointed out the evident relationships of *P. oaxacana* with *P. mexicana* from Veracruz (= *P. lindeniana* Mart. & Gal.), but at the same time admitted that in that species, the leaves were narrower, rounded and decurrent at the base, with a loose whitish tomentum, and the long, narrow lobes were commonly entire (Fig. 2).

For this reason, in September of 2009, we undertook a botanical expedition to sample *P. oaxacana* specimens, but no samples were found as a result of the strong anthropogenic impact in the area (deforestation), which has compromised the habitat of this taxon. Therefore, the authors obtained permission to analyse some fruits of the holotype to understand its relationship with the other *Platanus* taxa. According to our molecular data, this specimen can be seen as another example of hybridization, for which the parental contributors could be primarily *P. mexicana s.l.* (as shown by the frequencies reported in Fig. 5 and by morphology), but also *P. rzedowskii* or even other northern Mexican *Platanus* spp. In this case, the possible hybrid nature of *P. oaxacana* (which may be extinct) might have important biogeographic implications, suggesting that, in the past, the *P. oaxacana* population(s) was (were) connected to those of northern *Platanus*. Unfortunately, these speculations cannot form the basis of a hypothesis without additional data and/or specimens of *P. oaxacana*.

Based on our molecular analyses, the only assumption that we can make with confidence is that hybridization and/or introgression occurred or continues to occur in Mexican *Platanus* and this is why some specimens present both genotypes. We cannot exclude *P. occidentalis s.s.* from this gene exchange, because, even though it is distributed over a wide geographical area and has distinctive morphological characteristics (Nixon and Poole, 2003), it has both a shared plastid DNA haplotype (A, some accessions from Texas) and one exclusive haplotype (E) (Figs 1 and 5).

Dating these events is quite difficult. The upper time limit, according to Feng *et al.* (2005), is mid- to late Miocene (16·4–5·32 Mya), which is the estimated divergence time between *P. mexicana s.s.* and *P. occidentalis s.s.*, and the lower time limit could be the late Pleistocene (0·025–0·012 Mya), where the possible restriction of forests could have played a fundamental role in *Platanus* speciation (van der Hammen, 1974; Whitemore and Prance, 1987). The Pleistocene climate fluctuations may have resulted in greater closeness or a partial overlap of the ranges of *P. occidentalis s.l.*, *P. rzedowski* and *P. mexicana s.l.*, which, due to their moist habitat preferences, may have been confined to lowland refugia during the Pleistocene glacial maximum. Palynological studies have shown that the distribution of tropical forest was affected by climate change in the late Pleistocene and many species were restricted to refugia near the Equator where changes in rainfall and temperature were not as extreme (e.g. in riparian forests) (Haffer, 1969; van der Hammen, 1974; Prance, 1982; Bush *et al.*, 1992; Aide and Rivera, 1998). At present the *Platanus* spp. tend to be distributed in allopatric populations separated by xeric expanses (Nixon and Poole, 2003) and, therefore, present-day gene flow is unlikely.

Finally, the use of real-time PCR assays allowed the generation of results that contribute usefully to our understanding of the history of Mexican *Platanus*. The speed with which useful results can be obtained is greater than with other methods. Using the two PCRs (end-point and quantitative), we obtained results comparable with and more accurate than those of Grimm and Denk (2010), who used the time-consuming technique of sub-cloning and sequencing of the PCR products. Our approach may indeed be useful for mapping potential hybridization zones between eastern North American and Mesoamerican *Platanus*. The technique has some difficulties (e.g. suitable probes and primers must be designed), but real-time PCR is a sophisticated method which has potential in a wide variety of applications in plant research due to its sensitivity, specificity and quantitative output. In our experience, real-time PCR has become an almost routine tool and, due to its proven reliability, applications based on it will probably increase in number.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. File S1: vouchers and EMBL nucleotide database accession numbers. File S2: file.nex alignment of 32 sequences of *LEAFY* intron 2 (*LFY*-i2) from GenBank. File S3: file.nex alignment of 133 sequences of internal transcribed spacer 2 (ITS2) from GenBank. File S4: file.nex alignment of five haplotypes of *psbA-trnH*$^{(GUG)}$ intergenic spacer.

ACKNOWLEDGEMENTS

We thank Katherine Rankin (former Supervisory Museum Specialist, US National Herbarium) for her help, and Guido Grimm and Thomas Denk (Departement of Palaeobotany, Swedish Museum of Natural History, Sweden) who provided specimens and made very helpful suggestions. The authors are also grateful to Luca Paino (Department of Biology, University of Naples Federico II, Italy) for the sequencing service.

'*Whoever has, at least once, observed the beauty of nature with his own eyes is not destined for death but for nature itself . . .*' Konrad Lorenz.

In memory of Dr Gioacchino Vallariello.

LITERATURE CITED

Aceto S, Caputo P, Cozzolino S, Gaudio L, Moretti A. 1999. Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. *Molecular Phylogenetics and Evolution* **13**: 67–76.

Aide TM, Rivera E. 1998. Geographic patterns of genetic diversity in *Poulsenia armata* (Moraceae): implications for the theory of Pleistocene refugia and the importance of riparian forest. *Journal of Biogeography* **25**: 695–705.

Baerwald MR, Schumer G, Schreier BM, May B. 2011. TaqMan assays for the genetic identification of delta smelt (*Hypomesus transpacificus*) and wakasagi smelt (*Hypomesus nipponensis*). *Molecular Ecology Resources* **11**: 784–785.

Bandelt H-J, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.

Barrier E, Velasquillo L, Chavez M, Gaulon R. 1998. Neotectonic evolution of the Isthmus of Tehuantepec (south-eastern Mexico). *Tectonophysics* **287**: 77–96.

Bush MB, Piperno DR, Colinvaux PA, *et al*. 1992. Paleological profile of a lowland tropical lake in Panama. *Ecological Monographs* **62**: 251–275.

Chen RY, Song WQ, Li XL, Li MX, Liang GL, Chen CB. 2003. *Chromosome atlas of major economic plants genome in China*, Vol. 3: *Chromosome atlas of garden flowering plants in China*. Beijing: Science Press.

Corriveau JL, Coleman AW. 1988. Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 species. *American Journal of Botany* **75**: 1443–1458.

Crane PR, Pedersen KR, Friis EM, Drinnan AN. 1993. Early Cretaceous (early to middle Albian) platanoid inflorescences associated with *Sapindopsis* leaves from the Potomac Group of eastern North America. *Systematic Botany* **18**: 328–342.

Culumber ZW, Fisher HS, Tobler M, *et al*. 2011. Replicated hybrid zones of *Xiphophorus* swordtails along an elevational gradient. *Molecular Ecology* **20**: 342–356.

Denk T, Grimm G, Roseler A-K. 2012. When field botany meets history: taxonomy of *Platanus mexicana* in Mexico. *Willdenowia* **42**: 99–115.

Di Maio A, De Castro O. 2013. SSR-patchwork: an optimized protocol to obtain a rapid and inexpensive SSR library using first-generation sequencing technology. *Applications in Plant Sciences* **1**: 1200158.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin, Botanical Society of America* **19**: 11–15.

Ernst WR. 1963. The genera of Hamamelidaceae and Platanaceae in the southeastern United States. *Journal of the Arnold Arboretum* **44**: 193–210.

Feliner GN, Rosselló JA. 2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Molecular Phylogenetics and Evolution* **44**: 911–919.

Feng Y, Oh S-H, Manos PS. 2005. Phylogeny and historical biogeography of the genus *Platanus* as inferred from nuclear and chloroplast DNA. *Systematic Botany* **30**: 786–799.

Friis EM, Crane PR. 1989. Reproductive structures of Cretaceous Hamamelidae. In: Crane PR, Blackmore S. eds. *Evolution, systematics, and fossil history of the Hamamelidae. Introduction and 'lower' Hamamelidae*, vol. 1. The Systematics Association Special Volume No. 40A. Oxford: Clarendon Press, Oxford University Press, 155–174.

Grimm GW, Denk T. 2008. ITS evolution in *Platanus*: homoeologues, pseudogenes, and ancient hybridization. *Annals of Botany* **101**: 403–419.

Grimm GW, Denk T. 2010. The reticulate origin of modern plane trees (*Platanus*, Platanaceae): a nuclear marker puzzle. *Taxon* **59**: 134–147.

Haffer J. 1969. Speciation in Amazonia forest birds. *Science* **165**: 131–137.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hansen H, Escobar LK, Gilbert LE, Jansen RK. 2007. Paternal, maternal, and biparental inheritance of the chloroplast genome in *Passiflora* (Passifloraceae): implications for phylogenetic studies. *American Journal of Botany* **94**: 42–46.

Hanson L, McMahon KA, Johnson MAT, Bennett MD. 2001. First nuclear DNA C-values for another 25 angiosperm families. *Annals of Botany* **88**: 851–858.

Harwood AS, Phillips RB. 2011. A suite of twelve single nucleotide polymorphism markers for detecting introgression between cutthroat and rainbow trout. *Molecular Ecology Resources* **11**: 382–385.

Ivanova D. 2005. Mediterranean chromosome number reports 15 (1445–1456). *Flora Mediterranea* **15**: 719–728.

Kvaček Z, Manchester SR, Guo Z-H. 2001. Trifoliolate leaves of *Platanus bella* (Heer) comb. n. from the Palaeocene of North America, Greenland, and Asia and their relationships among extinct and extant Platanaceae. *International Journal of Plant Sciences* **162**: 441–458.

Manen JF, Natali A, Ehrendorfer F. 1994. Phylogeny of the Rubiaceae-Rubieae inferred from the sequence of a cpDNA intergene region. *Plant Systematics and Evolution* **190**: 195–211.

Maslova NP. 2003. Extinct and extant Platanaceae and Hamamelidaceae: morphology, systematics, and phylogeny. *Paleontological Journal* **37**: S467–S590.

Matejusová I, Bland F, Hall AJ, *et al*. 2012. Real-time PCR assays for the identification of harbor and gray seal species and sex: a molecular tool for ecology and management. *Marine Mammal Science* **29**: 186–194.

Morawetz W, Samuel MRA. 1989. Karyological patterns in the Hamamelidae. In: Crane PR, Blackmore S. eds. *Evolution, systematics, and fossil history of the Hamamelidae*. *Introduction and 'lower' Hamamelidae*, vol. 1. Oxford: Clarendon Press, Oxford University Press, 129–154.

Nixon KC, Poole JM. 2003. Revision of the Mexican and Guatemalan species of *Platanus* (Platanaceae). *Lundellia* **6**: 103–137.

Oginuma K, Tobe H. 1991. Karyomorphology and evolution in some Hamamelidaceae and Platanaceae (Hamamelididae; Hamamelidales). *Botanical Magazine* **104**: 115–135.

Oxelman B, Lidén M, Berglund D. 1997. Chloroplast *rps*16 intron phylogeny of the tribe *Sileneae* (Caryophyllaceae). *Plant Systematics and Evolution* **206**: 393–410.

Prance G. 1982. *Biological diversification in the tropics*. New York: Plenum Press.

Reuter JS, Mathews DH. 2010. RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinformatics* **11**: 129.

Sang T, Crawford DJ, Stuessy TF. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* **84**: 1120–1136.

Smith CJ, Nedwell DB, Dong LF, Osborn AM. 2006. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environmental Microbiology* **8**: 804–815.

Standley PC. 1919. Tropical American phanerogams, no. 3. *Contributions from the United States National Herbarium* **20**: 173–220.

Suárez G. 2000. Reverse faulting in the Isthmus of Tehuantepec: backarc deformation induced by the subduction of the Tehuantepec ridge. In: Delgado-Granados H, Aguirre-Díaz G, Stock JM. eds. *Cenozoic tectonics and volcanism of Mexico*. Boulder, CO: Geological Society of America Special Paper 334, 263–268.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.

Tate JA, Simpson BB. 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany* **28**: 723–737.

Van der Hammen T. 1974. The Pleistocene change of vegetation and climate in tropical south America. *Journal of Biogeography* **1**: 3–26.

Van Guilder HD, Vrana KE, Freeman WM. 2008. Twenty-five years of quantitative PCR for gene expression analysis. *BioTechniques* **44**: 619–626.

Werth S, Cornejo C, Scheidegger C. 2010. A species-specific real-time PCR assay for identification of three lichen-forming fungi, *Lobaria pulmonaria*, *Lobaria immixta* and *Lobaria macaronesica*. *Molecular Ecology Resources* **10**: 401–403.

Whitemore TC, Prance GT. 1987. *Biogeography and Quaternary history in tropical America*. Oxford: Clarendon Press.

Zemskova EA. 1993. O kariotipe Platanus kerrii (Platanaceae) [On the karyotype of *Platanus kerrii* (Platanaceae). *Botanicheskii Zhurnal* **78**: 15–17.