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A short phylogenetically informative cpDNA fragment for the identification of *Pinus* species

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ABSTRACT

The genus *Pinus* L. consists of ca. 110 ecologically and economically important species extending from the arctic zone to the tropics. Nevertheless, there is little information in the literature on DNA-based methods for the identification of pine species. Here, we identified a new cpDNA fragment (*trn*V-H/*x-h*) able to differentiate among pine species and correctly depict the phylogeny within the genus. The fragment was identified based on PCR-RFLP profiles and primers designed based on the sequences of six *Pinus* species naturally occurring in Greece (*Pinus brutia* Ten., *Pinus halepensis* Mill., *Pinus leucodermis* Antoine, *Pinus nigra* J.F. Arnold, *Pinus pinea* L., and *Pinus sylvestris* L.). We analyzed 90 highly similar pine sequences retrieved from the GenBank to investigate specificity of our marker and the haplotypes found showed to be specific to *Pinus* and able to differentiate among 39 different species. The phylogenetic tree constructed using these species, correctly depicted the phylogeny of the genus up to the subsection level. These characteristics together with its relatively small size (376–418 bp) make the *trn*V-H/*x-h* marker useful for pine identification even in contexts where DNA is degraded, such as in timber tracing, forensic botany and palaeobotanical investigations.

1. Introduction

Identification of plant species has been traditionally based on the analysis of morphological traits. However, in the last decades, DNA-based markers have shown to be very useful for species identification and a wide range of DNA markers has been identified in the three plant genomes (nuclear, mitochondrial, chloroplast), which are widely used for molecular identification at different taxonomic levels. The combination of *rbc*L + *mat*K markers has being proposed as a standard barcode for land plants (CBOL Plant Working Group, 2009), but the use of additional sequences for increased identification resolution in different research contexts is often necessary (Thomas, 2009; Hollingsworth et al., 2011).

Many plant research fields dealing with DNA analyses of ancient (fragmented) templates require the use of barcodes of short size. Forensic botany (e.g. Coyle et al., 2005; Craft et al., 2007; Ferri et al., 2009), molecular palaeoecology (e.g. Willerslev et al., 2007; Suyama et al., 2008; Parducci et al., 2013) and the fields involved in the control of illegal trade of timber or timber

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products, especially from threatened species, are just some examples. In the latter case, the use of molecular analyses, like metabarcoding, is an important identification tool, as morphological identification is not possible once the timber is processed (Deguilloux et al., 2003; Höltken et al., 2011; Muellner et al., 2011). Metabarcoding on environmental DNA extracted from lake and peat sediments has also shown to be an important and complementary tool for plant identification in classical palaeoecological analyses (Pedersen et al., 2015), where barcodes of short size (<400 bp) are necessary for successful amplification of degraded and fragmented DNA templates (Valentini et al., 2009 and references therein).

The genus *Pinus* L. consists of 111 species, all widely distributed in the Northern Hemisphere, from boreal to tropical landscapes (Price et al., 2000), and commercially exploited (e.g. for wood, paper, resin, pine-nuts). Although a number of different classification approaches have been proposed and used for studying the phylogeny of the genus (e.g. Strauss and Doerksen, 1990; Gernandt et al., 2005; Parks et al., 2009), there are still phylogenetic ambiguities at lower taxonomic levels and between closely related species [such as the Asian (Wang et al., 1999) or the Mediterranean group (Parks et al., 2009)]. In addition, despite the rapidly increasing amount of sequence data available for plant species, relatively little information is available for pine species identification (Parks et al., 2009, 2011; Armenise et al., 2012; Ganopoulos et al., 2013). In this work therefore, we aimed at identifying a single and short universal cpDNA fragment able to discriminate within *Pinus*, providing at the same time phylogenetic information on the genus.

2. Materials and methods

2.1. Sampled material

Our analyses focused initially on six pine species (subgenus *Pinus*) occurring in Greece, Balkans, Europe or Eurasia (*Pinus brutia* Ten., *Pinus halepensis* Mill., *Pinus leucodermis* Antoine, *Pinus nigra* J.F. Arnold, *Pinus pinea* L., *Pinus sylvestris* L.) (Table 1). We sampled and analyzed 10 individuals per species (except for *P. nigra* and *P. leucodermis* were we sampled 8 and 7 individuals, respectively) from natural populations (naturalized in the case of *P. brutia*). We also used ten individuals of *Abies cephalonica* Loudon and six individuals of *Picea abies* (L.) Karst. from Greece, to test primer specificity. All sampled specimens showed typical morphological characteristics of their species, according to Christensen (1997) and were deposited in the TAU Herbarium (Aristotle University of Thessaloniki, Greece) (voucher numbers are given in Table 1). Fresh needles from the sampled material were stored at −20 °C until DNA extraction.

2.2. DNA extraction and PCR-RFLP analysis

Genomic DNA was extracted following the CTAB protocol by Doyle and Doyle (1990) with minor modifications. We selected the cpDNA intergenic region *trn*V-*trn*H (Parducci and Szmidt, 1999), and screened it using PCR-RFLP analysis. PCR amplifications were carried out using primers F: 5′-GCTCAGCAAGGTAGAGCACC-3′ and R: 5′-CTTGGTCCACTTGGCTACGT-3′ and following PCR conditions as in Parducci and Szmidt (1999). All amplifications were carried out on a PTC-150 thermal cycler (MJ Research).

The PCR products (up to 2600 bp) were screened for species-specific PCR-RFLP polymorphism. Restriction enzymes were selected based on the *Pinus thunbergii* Parl. sequence (Accession No.: NC_001631, Wakasugi et al., 1994), using the web software REMAP (http://emboss.bioinformatics.nl/cgi-bin/emboss/remap). Based on results from single-enzyme digestions, we performed double digestions with *Hinf*I (Thermo Scientific) and *Xmn*I (New England BioLabs) enzymes. The digestions were performed in 20 μl according to the manufacturer's instructions, including at least 500 ng of PCR product. The double digestion with *Hinf*I and *Xmn*I enzymes was carried out using Tango Buffer (Thermo Scientific). Digestion profiles were visualized on 4% agarose gel (SeaKem LE and Metaphor, LONZA), stained with Ethidium Bromide.

2.3. Primers design, PCR amplification, and sequencing

Based on the PCR-RFLP profiles from the six pine species we selected the polymorphic fragment between the *Xmn*I and a *Hinf*I restriction sites and designed two internal primers (*trn*V-H/*x, trn*V-H/*h*) flanking this fragment. The primers were designed using the Primer3 software (Untergasser et al., 2012), based on the *P. thunbergii* sequence. The primer sequences

Table 1
List of species sampled in Greece, their location and sample size and the TAU vouchers of the samples.

Species	Location	No of individuals	TAU vouchers
Pinus brutia Ten.	Seich-Sou forest, Thessaloniki	11	2011-A.Drouzas-Pb-01 to 2011-A.Drouzas-Pb-11
Pinus halepensis Mil.	Kassandra peninsula	10	2010-A.Drouzas-Ph-01 to 2010-A.Drouzas-Ph-10
Pinus leucodermis Ant.	Mt.Olympos	7	2010-A.Drouzas-Pl-01 to 2010-A.Drouzas-Pl-07
Pinus nigra Arn.	Mt.Pieria (6), Mt. Pindos (1), Mt. Olympos (1)	8	2010-A.Drouzas-Pn-01 to 2010-A.Drouzas-Pn-08
Pinus pinea L.	Sithonia peninsula	10	2010-A.Drouzas-Pp-01 to 2010-A.Drouzas-Pp-10
Pinus sylvestris L.	Mt.Pieria	10	2011-A.Drouzas-Ps-01 to 2011-A.Drouzas-Ps-10
Abies cephalonica Loud.	Mt. Chelmos	10	2011-A.Drouzas-Ac-01 to 2011-A.Drouzas-Ac-10
Picea abies (L.) Karst.	Mt. Rodopi	6	2011-A.Drouzas-Pa-01 to 2011-A.Drouzas-Pa-06