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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

were: *trn*V-H/*x*: 5′-AATGCGTTCTGGAACAAACA-3′, *trn*V-H/*h:* 5′-TGCAGGTTATGGGACAAAGA-3'. PCR amplifications of the *trn*V-H/*x-h* fragment were carried out in 25 μl reaction mixture containing 30 ng DNA, 3 mM $MgCl_2$, 0.4 mM of each primer, 0.15 mM dNTP's mixture, and 0.7 Units *Taq* polymerase (Thermo Scientific), using a PTC-100 thermal cycler (MJ Research) with the following conditions: t 94 °C for 4 min, 30 cycles of 1 min at 93 °C, 56 °C for 50 s, 72 °C for 2 min and a final step at 72 °C for 5 min. Successful amplification was checked on 2% agarose gel (Bio-Rad) and three to four individuals per species were purified with ExoSAP-IT® (Affymetrix) and sequenced using external sequencing service (Macrogen, Korea). DNA sequences were edited and aligned using BioEdit v. 5.0.9 (Hall, 1999) and compiled into a dataset (hereafter "Greek dataset") that included all the *trn*V-H/*x-h* sequences from the *Pinus* samples from Greece.

2.4. Universality analysis

Primer universality across pine species was tested by performing a BLAST search for highly similar sequences, using the *P. sylvestris* edited sequence as reference. From the resulted hits, we selected the respective *P. thunbergii* sequence (excerpt from D17510, including the primer regions) as reference and we performed a second BLAST search, resulting in a set of *Pinus* sequences, hereafter referred as "GenBank dataset" (the *trn*V-H/*x-h* sequences of the *Pinus* species acquired from the GenBank). Finally, we assessed species discrimination ability by using BLASTClust 2.17.0 (http://toolkit.tuebingen.mpg.de/blastclust), and setting the percentage of identical residues to 100. BLASTClust identifies and groups protein or nucleotide sequences based on similarity according to the values set in the stringency parameters. The universality of the primers was tested in all sequences by using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.5. Phylogenetic analysis

Sequences were compiled into a single file and aligned using ClustalX 2.1 (Larkin et al., 2007), using default parameters and the *Picea glauca* sequence (BT107316) as an outgroup. The best-fit model of nucleotide substitution was determined by the Akaike information criterion using jModelTest 2.0.1 (Posada, 2008). For phylogeny reconstruction, we employed Bayesian Inference (BI) using BEAST v1.6.1 (Drummond and Rambaut, 2007), and performed the analysis under the HKY + Gamma model (Hasegawa et al., 1985). We placed the Yule speciation prior on the phylogenetic tree and run two independent Markov Chain Monte Carlo (MCMC) analyses for 10 million generations with sampling every 1000 generations. Tracer v1.5 (Rambaut and Drummond, 2003) was used for diagnosing chain convergence and adequacy of mixing through inspection of the ESS (Effective Sample Size) parameter values (>200). We combined the data produced by the two independent runs with LogCombiner v1.5.2 and we calculated the posterior probabilities (PP) after burn-in of the first 2500 samples (of each run) using TreeAnnotator v1.5.2. Finally, we used FigTree v1.4 was used for tree visualization.

3. Results

3.1. PCR-RFLP variation and species discrimination

Single enzyme digestions of the *trn*V-*trn*H region from the six pine species from Greece, revealed a polymorphic fragment between *Xmn*I and *Hinf*I restriction sites. The double digestion with *Hinf*I and *Xmn*I restriction enzymes resulted in five distinct restriction profiles. Four profiles unique to *Pinus sylvestris*, *P. halepensis*, *P. brutia* and *P. pinea,* and one shared by *P. nigra* and *P. leucodermis*. We observed no intraspecific variation among the individuals (7–10) analyzed in each species.

3.2. trnV-H/x-h sequencing and species discrimination

PCR amplification with the *trn*V-H/*x-h* primers was successful in all 55 *Pinus* samples from Greece and size variation among species was visible on agarose gel. We obtained no PCR product for *A. cephalonica* and *P. abies*. The length of the PCR product in the different pine species ranged from ca. 380 bp (*P. brutia*) to ca. 420 bp (*P. sylvestris*). *P. leucodermis* and *P. nigra* had PCR products of the same size (ca. 390 bp) (Figure S1, Supplementary material).

Alignment the *trn*V-H/*x-h* sequences in the Greek dataset showed no intraspecific variation and revealed polymorphisms diagnostic for the six species, resulting in six species-specific haplotypes (Fig. 1). In particular, *P. brutia* and *P. halepensis* shared a 4-bp deletion at position 170 and a 8-bp deletion at position 331, *P. brutia* showed a species specific 4-bp deletion at position 321, *P. nigra* and *P. leucodermis* differed by a point mutation at position 187 (C > A), *P. sylvestris* showed a 22-bp duplication at position 234 and *P. pinea* showed a point mutation at position 207. All mutations were consistently found in all individuals sequenced in each species. The *trn*V-H/x-h Greek dataset is deposited at the GenBank (Accession numbers: KJ004079 to KJ004098).

3.3. Primer universality in the genus Pinus

From the BLAST search, we retrieved 100 highly similar sequences. After excluding duplicates and alignments with poor matching we assigned 96 sequences to 95 *Pinus* species, subspecies and varieties (GenBank dataset), and one to *P. glauca*. We assessed species discrimination ability of the *trn*V-H/*x-h* region based on the number of unique sequences found. Submission

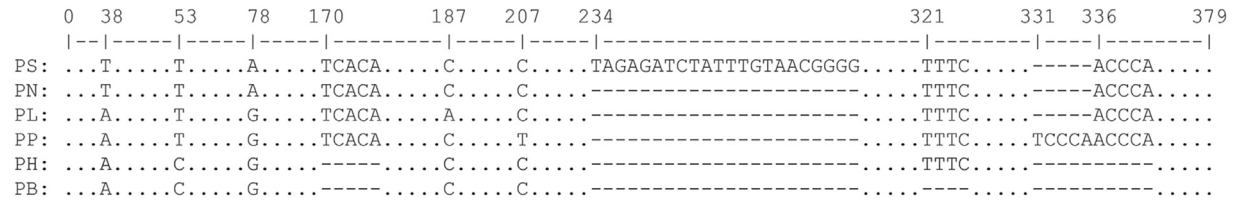

Fig. 1. Alignment of the *trn*V-H/*x-h* sequences. Point mutations and indels are noted. PL: *P. leucodermis*, PN: *P. nigra*, PS: *P. sylvestris*, PH: *P. halepensis*, PB: *P. brutia*, PP: *P. pinea*. Double column width.

of the sequences to BLUSTClust revealed 52 haplotype clusters. Forty haplotypes were assigned to a single *Pinus* species and one to *P. glauca*. The rest of the haplotypes were shared by two or more species. We found three haplotypes in *P. ponderosa* P. Lawson & C. Lawson and two in *P. pseudostrobus* Lindl, each representing a different variety (Table S1, Supplementary Material). The Primer BLAST search resulted in 90 hits belonging to pine species and corresponding to the BLAST results. We found missing bases at the beginning of the fragment in the sequences with no match, therefore it was not possible to resolve whether the forward primer could be annealed or not. The lengths of the BLAST-derived products ranged from 376 to 418 bp and 25 sequences showed from one to a maximum four nucleotide mismatches in the primer sequence (Table S1, Supplementary Material).

3.4. Comparison of datasets

Comparison of the two datasets (the Greek and the GenBank datasets) revealed no differences between the corresponding sequences for *P. brutia*, *P. halepensis*, *P. nigra, P. pinea* and *P. leucodermis*. It should be noted that *P. heldreichii* var. *leucodermis* (as referred in the GenBank dataset) is a synonym of *P. leucodermis* (included in the Greek dataset) (Christensen, 1997). This comparison, however, showed no match for *P. sylvestris* sequences. In particular, the *P. sylvestris* sequence from the Greek dataset matched with *Pinus densiflora* Siebold & Zucc. sequence from the GenBank (JN854210), both carrying a characteristic 22-bp duplication. Instead, the *P. sylvestris* sequence from the GenBank (JN854158) matched with *P. nigra* sequences from both datasets.

3.5. Phylogenetic analysis

We compared the phylogenetic tree constructed using all the sequences from both datasets with the *Pinus* phylogeny proposed by Gernandt et al. (2005) and discarded four entries due to extended gap regions. The tree shows two primary clades with posterior probabilities (PP) above 0.90, the first with sequences belonging to the subgenus *Pinus* and *Strobus,* and the second with sequences belonging exclusively to the subgenus *Strobus* (Fig. 2). We observed, two cases of paraphyly with *P. latteri* Mason and *P. merkusii* Jungh & de Vriese (of subsection *Pinus*) to be clustered with the Section *Pinaster*. Species of the subsection *Strobus* were not well differentiated and clustered in three different groups with the majority of the terminal branches showing low supporting values (Fig. 2).

4. Discussion

In this study we identified a short cpDNA fragment (*trn*V-H/*x-h*) able to both discriminate pine species and provide phylogenetic insight within the genus.

The *trn*V-H/*x-h* marker showed length variation already after electrophoresis on agarose gel in *P. sylvestris*, *P. brutia*, *P. halepensis* and *P. pinea* and was universal across the genus, providing therefore a single and simple-to-use tool for quick pine identification. Sequencing analysis revealed distinctive haplotypes in six pine species from Greece and all sequences matched the corresponding GenBank sequences, except for *P. sylvestris.* The similarity we observed between *P. sylvestris* and *P. densiflora*, could be due to sample misidentification, intra-specific variation, homoplasy or ancestral shared polymorphism, as the latter species occurs naturally in NE China, Korea and Japan (Critchfield and Little, 1966) and show no reproduction barriers with *P. sylvestris*, since the two species hybridize spontaneously where their distribution ranges overlap in NE China (Joung et al., 2013). Analysis of additional provenance samples from both taxa is however necessary to clarify this issue.

With our marker we could achieve an accurate discrimination in six species from Greece and in-silico discriminate 33 additional pine species of global distribution. Compared to the *ycf*1c-d fragment developed for pine species identification (Handy et al., 2011; Parks et al., 2011) spanning up to 2.7 kb, and to other approaches recently employed by Armenise et al. (2012) and Ganopoulos et al. (2013), our short marker offers the opportunity of being particularly useful in contexts where the DNA templates are degraded or of low-quality (e.g. herbarium specimens).

The phylogenetic tree generated using our marker is in agreement with the recent *Pinus* classification proposed by Gernandt et al. (2005), with the exception of three species of the subgenus *Strobus* (*P. fenzeliana* Hand.-Mazz., *P. wallichiana* A.B.Jacks., *P. koraiensis* Siebold & Zucc.) assigned instead to the subgenus *Pinus* (PP > 0.9), a finding that reflects pine geographical distribution in East Asia. The weak cohesion observed among the species of the subgenus *Strobus* could be

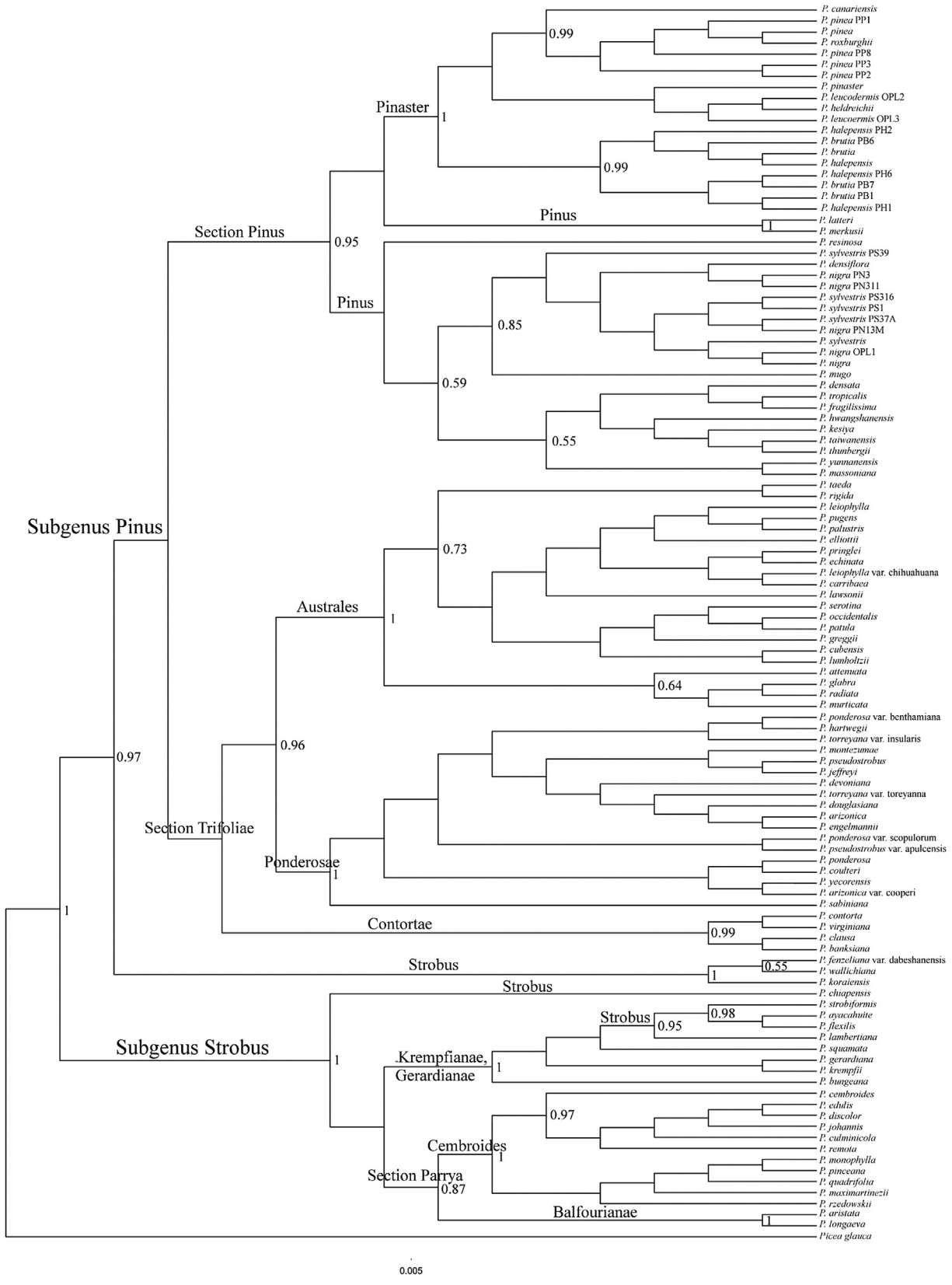

Fig. 2. Phylogenetic tree constructed using Bayesian Inference based on all *trn*V-H/*x-h* sequences. Numbers on nodes are Posterior Probabilities (PP) and only those above 0.50 are shown. Double column width.

attributed to non-monophyly already noted by Syring et al. (2007) within this subgenus, or to our limited sample size (9 out of 20 species of the section). The phylogenetic analysis using our marker however, shows strong support for the division in two subgenera (*Pinus* and *Strobus*) (PP > 0.99), as well as for the classification at the section level in both subgenera, with an exception in the section *Pinus* where *P. merkusii* and *P. latteri* are grouped in the section *Pinaster*. This however is not surprising, as *P. latteri* has been considered a synonym of *P. merkusii*, and affinities of the two species to the section *Pinaster* have been previously reported by Farjon (2001). Previous phylogenetic studies have also shown that, *P. merkusii* forms a monotypic clade diverging from the other members of the section *Pinus* (Wang et al., 1999; Gaeda Lopez et al., 2002; Parks et al., 2012). Subsections and geographical clusters within the genus *Pinus* were all well supported (PP > 0.50) but less so at the species level (PP < 0.50).

On the whole, despite its short length, our *trn*V-H/*x-h* marker provides concrete phylogenetic and discriminatory information in the genus *Pinus*. Compared to previous multi-locus approaches, the use of a single short marker is both cost- and time-efficient for a large number of applications, particularly when routine identification of pine species is required or when the DNA templates are degraded (timber species identification of logs and processed timber products). Another potential application of the *trn*V-H/*x-h* is in forensic botany. Since pines are a dominant component of the forests across the northern hemisphere, identification of pine species from plant traces like pollen grains, needles or tissue remains, can be very useful in crime scene investigations and forensic analyses (Craft et al., 2007; Wesselink and Kuiper, 2008). Classical morphological analysis of *Pinus* pollen allows often classification at subgenus (haploxylon & diploxylon) and family level while amplification of the *trn*V-H/*x-h* region directly from grains may allow identification at the species level. Finally, given its short size, the *trn*V-H/*x-h* fragment may be also used for metabarcoding ancient sediments in palaeoecological investigations (Parducci et al., 2005, 2012, 2013) or finally, due to its paternal inheritance (Neale and Sederoff, 1989), in hybrid species identification and paternity analyses studies in pines.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2016.03.001.

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