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Identification case of evidence in timber tracing of *Pinus radiate*, using high-resolution melting (HRM) analysis

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ABSTRACT

Fast, accurate detection of plant species and their hybrids using molecular tools will facilitate assessment and monitoring of timber tracing evidence. In this study the origin of unknown pine samples is determined for a case of timber theft in the region of Araucania southern Chile. We evaluate the utility of the trnL marker region for species identification applied to pine wood based on High Resolution Melting. This efficient tracing methods can be incorporated into forestry applications such as certification of origin. The object of this work was genotype identification using high-resolution melting (HRM) and trnL approaches for *Pinus radiata* (Don) in timber tracing evidence.

Our results indicate that trnL is a very sensitive marker for delimiting species and HRM analysis was used successfully for genotyping *Pinus* samples for timber tracing purposes. Genotyping samples by HRM analysis with the trnL1 approach allowed us to differentiate two wood samples from the Pinaceae family: *Pinus radiata* (Don) and *Pseudotsuga menziesii* (Mirb.) Franco. The same approach with Pinus trnL wood was not able to discriminate between samples of *Pinus radiata*, indicating that the samples were genetically indistinguishable, possibly because they have the same genotype at this locus. Timber tracing with HRM analysis is expected to contribute to future forest certification schemes, control of illegal trading, and molecular traceability of *Pinus* spp.

Dear Editor,

Chile currently has 16.6 million hectares of forest, supporting an industry with exports worth more than 6 billion USD per year. Of this area, 13.6 million hectares consist of native forest, planted mixed forests and other formations. The total area of forestry plantations is approximately 2.8 million hectares, mainly *Pinus radiata* (Don) and eucalyptus (*Eucalyptus* spp.). Environmental and economic crimes in the forest sector are associated with plantations of introduced and native species: timber theft, firewood theft, illegal logging and theft of trunks, etc. National Forest Service information [1] shows that 495 environmental crimes involving forest products were reported in Chile between 2004 and 2010, mainly related to illegal logging of native species. Moreover, the prosecution service receives at least one complaint per week of theft of timber from forest land, 64% involving Insignis pine (*P. radiata*). The current problem is that a significant number of cases never come to trial because of the lack of evidence to support judicial proceedings, due to the lack of a routine, rapid method of tracing wood remnants for evidence.

High-resolution melting (HRM) analysis allows genotyping of wood species by differentiation of DNA sequence variants such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels), based on the shape of the melting transition curves (T_m) of real-time PCR products. HRM analysis can be applied not only to allele differentiation by targeting well-characterized SNPs, but also to screening for the existence of unknown sequence variations without a sequencing process.

High-resolution melting (HRM) analysis is a closed-tube method for rapid analysis of genetic variation within PCR amplicons [2]. Genetic variants with differences in the base composition present differences in their melting temperatures. These are detected by monitoring the fluorescence as the temperature is increased, and the species are differentiated by their characteristic melting curves, visualized by the loss of fluorescence as the DNA duplex melts.

Ganopoulos et al. [3] applied HRM curve analysis for differentiating leaf and wood samples of *Pinus* species and hybrids. One trnL marker was able to differentiate eight *Pinus* species by HRM curve analysis. The trnL region may not be regarded as the most variable region in the plastid genome, nevertheless it has proved to be a suitable base region for molecular identification, and showed that trnL is a very sensitive marker for delimiting species biodiversity. High-resolution melting (HRM) analysis was exploited as a molecular fingerprint method for fast, accurate differentiation of DNA sequence variants in *Pinus* spp. Applications can also be advanced through the application forensic of this approach. Finally, different short length regions of the plastid genome have been used as DNA molecular identification sites primarily for species identification [4]. The objective of this work

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was genotype identification of pine samples using the high-resolution melting (HRM) and trnL approaches for timber tracing evidence.

In this study the geographical location of unknown pine samples is determined corresponding to a case of theft of timber, forest plantations take place in La Araucanía region in southern of Chile. Samples of Insignis pine (*P. radiata*) and Oregon pine (*Pseudotsuga* menziesii) were used for trnL molecular analysis of Pinaceae wood. Samples of needles were also taken from the same plants. The codes used for these samples are PIA, PCU and PP for *P. radiata* (Don), and POA for *P. menziesii* (Mirb.) Franco (reference sample). DNA was extracted from wood and pine needles. The samples of wood were taken from the growth rings located in the transition between sapwood and heartwood. Genomic DNA was isolated by DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. PCR amplification, DNA melting and end point fluorescence level acquisition for the PCR amplifications were performed in a total volume of 15 μL on an Illumina real-time PCR Thermocycler (ILLUMINA-ECO^{TM} Real-Time PCR System). SYBR Green I was used to monitor the accumulation of the amplified product during PCR and subsequent product melting in the Illumina Thermocycler (Eco^{TM} Software v 4.1.2.0). The reaction mixture contained 20 ng genomic DNA, 300 nM forward and reverse primers (Pinus trnLWood-F: 5′-

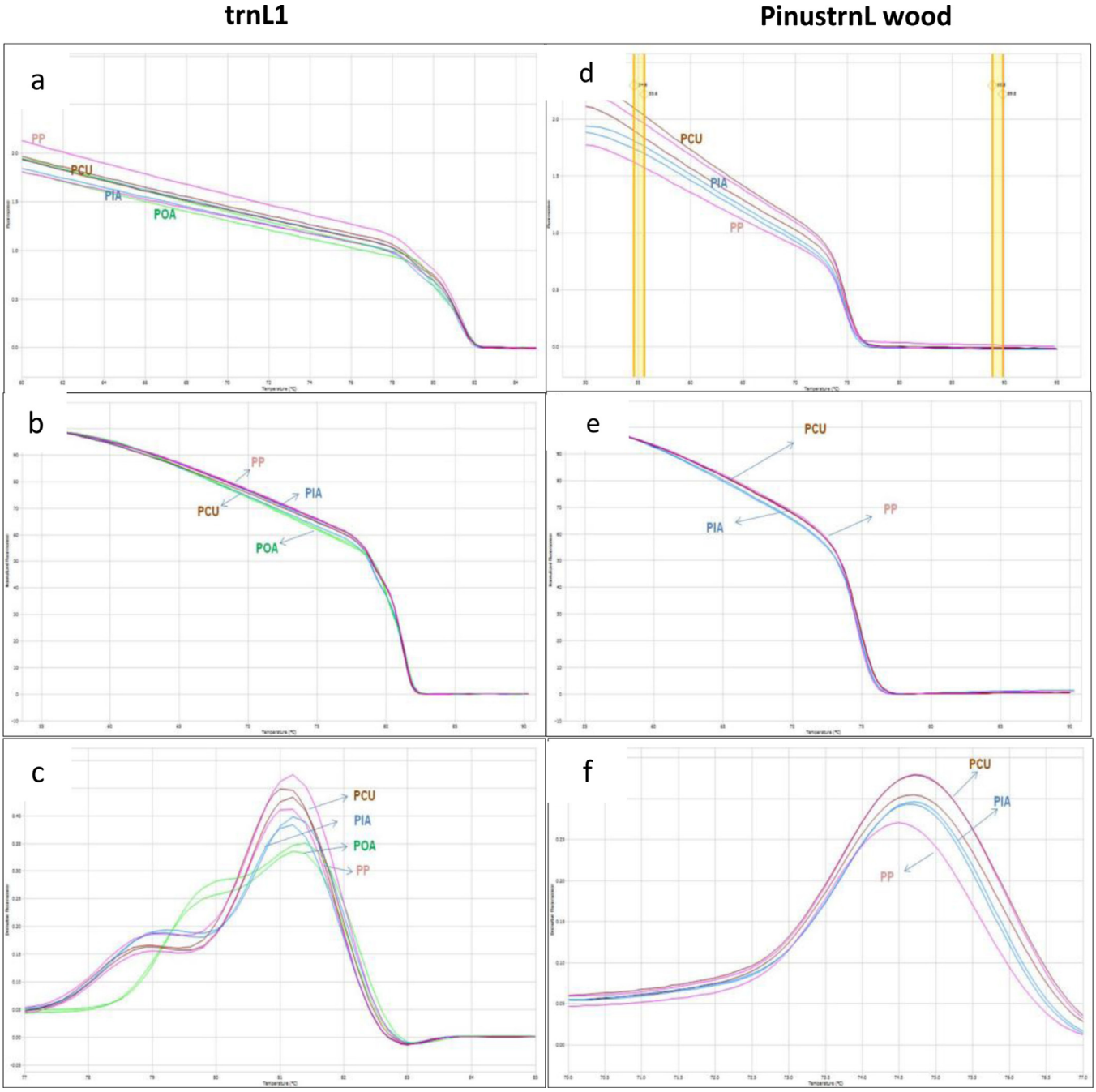

Fig. 1. (a and d) Raw melting, (b and e) normalized melt curve, (c and f) derivative melt with high-resolution melting (HRM) and trnL1-*Pinus* trnL wood approaches for *Pinus* sp. trnL1 samples: *Pinus radiata*: PIA, PCU, PP; *Pseudotsuga menziesii:* POA. *Pinus* trnL wood samples: *Pinus radiata*: PIA, PCU, PP.

Table 1
Mean ± standard deviation (SD) of the points for the melting peak of the amplicons result from pine sample, with trnL1 and *Pinus* trnLwood followed by high-resolution melt (HRM) curve analysis at a ramp of 0.1° s^{-1}.

Pine sample	Species	Source of DNA	Peak 1 (°C) ± SD
trnL1			
PIA	*Pinus radiata* (Don)	Wood	81.15 ± 0.0707
POA	*Pseudotsuga menzie*sii (Mirb.) Franco	Wood	81.30 ± 0.00
PCU	*Pinus radiata* (Don)	Pine needles	81.10 ± 0.00
PP	*Pinus radiata* (Don)	Pine needles	81.15 ± 0.0707
Pinus trnLwood			
PIA	*Pinus radiata* (Don)	Wood	74.70 ± 0.00
PCU	*Pinus radiata* (Don)	Pine needles	74.70 ± 0.00
PP	*Pinus radiata* (Don)	Pine needles	74.60 ± 0.141

CTTATGAATAAAATGCTTGGAACG-3′; *Pinus* trnLWood-R: 5′-ATAA-CATCAGACAAAACTGG-3′; trnL1-F; 5′-CGAAATCGGTAGACGCTACG-3′; trnL1-R; 5′-GGGGATAGAGGGACTTGAAC-3′) [10], Fast PCR Master MIX SYBR green I 2X (KapaBiosystems, USA). The trnL-PCR protocol was performed using an initial denaturing step of 94 °C for 3 min followed by 30 cycles of 95 °C for 20 s, 54 °C for 30 s and 72 °C for 40 s, then a final extension step of 72 °C for 2 min. The fluorescence data were acquired at the end of each extension step during PCR cycles. Before HRM, the products were denatured at 95 °C for 5 s, and then annealed at 50 °C for 30 s to randomly form DNA duplexes. To trace the geographical location of wood by HRM, we used the primer sequence information obtained for trnL [4]. HRM was performed as follows: pre-melt at the first appropriate temperature for 90 s, and melt at a ramp of 10 °C in an appropriate temperature range with 0.1 °C increments every 2 s. Finally, the 2-resolution melt curve (HRM) of trnL markers was obtained using 95 °C for 15 s, 50 °C for 15 s and 95 °C for 15 s. The melting pattern of corresponding sequences of trnL1 and trnLwood amplicons from all *Pinus* samples were analyzed using the computer program Eco™ Software v4.1.2.0. The trnL1 locus produced polymorphic melting curves for the *Pinus* species based on HRM analysis. The DNA melting profile was analyzed to investigate whether the polymorphism in the trnL1 region of different *Pinus* samples was detectable in derivative melt and normalized melting curves (Fig. 1a–c). The melting peak temperatures for all pine samples are presented in Table 1. Peaks were evident for each sample within the range 79.00–82.00 °C. All profiles of *P. radiata* (PIA, PCU and PP) produced one peak, which was different from *P. menziesii* (POA). This enabled the unknown samples (evidence samples: PIA and POA) from the fire wood collection site to be identified, from which it was concluded that there was a mixture of species at the site.

Analysis of the normalized HRM curves with the trnL1 locus revealed that the two species could be distinguished visually, as the HRM curves obtained are highly characteristic for each amplicon. Because some peaks produced overlap, corresponding to duplicate samples, the HRM melting curves are characteristic for each species based on shape. They are dependent on the interplay between GC content, length of amplified product and sequence, even when they define the same T_m values. Furthermore, closer examination of the HRM difference curves, using Oregon pine (*P. menziesii)* as the reference sample (baseline), revealed part of the curve outside the 90% confidence interval curve, suggesting that all the samples examined using HRM curves are different species. By assigning *P. menziesii* as a reference genotype, we were able to estimate the confidence value of the similarity between *P. menziesii* and *P. radiata* used in the study. The *Pinus* trnL wood locus did not produce polymorphic melting curves among the *P. radiata* samples studied by HRM analysis (Fig. 1d–f). The melting peak temperatures for all pine samples are presented in Table 1. Peaks were evident for each sample within the range 74.25–74.50 °C. All the profiles produced only one maximum. Analysis of the normalized HRM curves with the trnL wood marker revealed that just one species is present (*P. radiata*). The HRM melting curves are similar, defining the same T_m values; the overlapping of some peaks corresponds to duplicate samples. HRM analysis with *Pinus* trnL wood was not able to differentiate between samples of *P. radiata*, indicating that the samples are genetically indistinguishable and therefore may correspond to the same genotype. The *P. radiata* samples, PIA, PCU and PP, all presented ΔT_m below 0.1 °C, which is too small for differentiation. We found that the trnL1 marker is a potential region for distinguishing different pine species. The graph of the normalization data (Fig. 1b) shows more consistent melting profiles in the pine samples evaluated. This allowed better grouping of the curves of different samples in the graph, and improved differentiation between *P. radiata* and *P. menziesii*. Differentiating pine species samples by trnL locus, based on the sequenced portion of the plastid trnL and HRM analysis, was effective in defining the taxonomic status of the species evaluated; these were taken from a fire wood collection site. We concluded that the site presented a mixture of two species: *P. radiata* and *P. menziesii*. The melting curves are similar in shape, but the T_m of *P. radiata* is approximately 0.15–0.20 °C lower than that of *P. menziesii*. Furthermore, the melting curve of Oregon pine shows a gradual transition over a wide temperature range, Fig. 1b (normalized melt) and c (derivative melt). In our case, pine identification using the trnL approach with HRM analysis was successful, and agrees with the results obtained in other plant species [3,5–7]. HRM analysis with the *Pinus* trnL wood approach was not able to differentiate different samples of *P. radiata.* This result suggests that the samples are genetically indistinguishable. In this study, we used HRM analysis with trnL markers to genotype pine samples originating from a timber theft. Timber tracing with HRM analysis is expected to contribute to future forest certification schemes, control of illegal trading, and molecular traceability of *Pinus* spp.

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