A multiplex qPCR assay for the identification of *Pinus palustris* and *Pinus elliottii*

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Abstract: Differentiating the wood of high-strength southern yellow pines (*Pinus palustris* and *P. elliottii*) from their lower-strength congeners (*P. taeda* and *P. echinata*) presents a substantial challenge once diagnostic external features, such as needles, cones, and bark are absent. This difficulty arises from their wood anatomical indistinguishability and low genetic resolution driven by highly conserved genome organization and extensive allele sharing. To address this, we developed and validated a multiplex qPCR assay utilizing SYBR Green chemistry and asymmetric primer concentrations, working with leaf and seed DNA. The assay targets short amplicons (<100 bp) to maximize the likelihood of amplification of the fragmented DNA typical of solid wood. The method achieved high linearity ($R^2 > 0.99$) and efficiency, successfully co-amplifying targets while maintaining clear discrimination through distinct mean melting temperatures (77.98°C [SD 0.11] and 69.25°C [SD 0.26], respectively). Sensitivity testing established limits of detection at 1 pg for *Pinus palustris* and 10 pg for *Pinus elliottii*. Validation across a panel of 84 individuals representing all four species yielded 100% accuracy, precision, and recall. This study establishes a rapid, gel-free molecular diagnostic tool that overcomes the limitations of wood anatomical identification. By validating a short-amplicon design on leaf and seed tissues, it provides a proof-of-concept for future applications on solid wood and offers a reproducible methodological framework for species verification.

Keywords: Multiplex qPCR; SYBR green; Southern yellow pine; Species identification.

Introduction

The commercial southern yellow pine (SYP) species group includes loblolly (*Pinus taeda*), shortleaf (*P. echinata*), longleaf (*P. palustris*), and slash (*P. elliottii*) pine. This group plays a central role in the United States' forest economy and is widely distributed throughout the southeastern region (Koch 1972; Gaby 1985; Barnett 2019). Although these species are often marketed collectively, this practice masks species-specific variations in wood properties. For example, longleaf and slash pine typically have higher mechanical properties, such as modulus of elasticity (MOE) and modulus of rupture (MOR), and wood density compared to shortleaf and loblolly pine (Senalik and Farber 2021) (Table 1). These relative rankings can vary

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Table 1. Wood specific gravity and mechanical properties of southern yellow pine (SYP) species at 12% moisture content (Senalik and Farber 2021).

Species	SG	MOR (kPa)	MOE (MPa)
P. elliottii (Slash)	0.59	112,000	13,700
P. palustris (Longleaf)	0.59	100,000	13,700
P. echinata (Shortleaf)	0.51	90,000	12,100
P. taeda (Loblolly)	0.51	88,000	12,300

SG specific gravity, *MOR* modulus of rupture (kPa), and *MOE* modulus of elasticity (MPa)

among studies not only because different mechanical properties are being evaluated (e.g., SG, MOR, MOE), but also due to biological, environmental, and geographic factors. These differences are consistent with their phylogenetic relationships; recent analyses indicate that *P. elliottii* and *P. palustris* form a sister-species pair that is distinct from the pair formed by *P. taeda* and *P. echinata* (Jin et al. 2021). While species such as sand pine (*P. clausa*) and Virginia pine (*P. virginiana*) occur regionally, they are minor contributors to commercial lumber streams and were not included in this study (Gaby 1985; Senalik and Farber 2021).

Species-specific differences in mechanical properties, in conjunction with other interacting factors, are especially relevant in high-value structural markets and grading systems, such as Machine-Stress-Rated (MSR) lumber, where specific minimum strength and stiffness values are required (Galligan et al. 1977; Western Wood Products Association 2008; Ross 2015). SYP MSR lumber represents a substantial market sector: during the strong construction cycle of 2005, SYP contributed 347.1 million board feet of the more than 2 billion board feet of MSR lumber produced; by 2018, SYP MSR production increased to 568.3 million board feet, making it the second largest MSR species group after Spruce-Pine-Fir (Entsminger et al. 2020). Market trends reflect increasing adoption of machine grading systems, with MSR usage among mills in the US South rising from fewer than 5% in 2004 to nearly 30% by 2020 (Entsminger et al. 2020).

The economic importance of MSR lumber, combined with species-linked differences in mechanical performance, creates strong incentives for mills to verify the species composition of the logs they purchase. In cases where mills are willing to pay a premium for higher strength species such as slash or longleaf pine, unexpectedly low MSR yields might indicate that the delivered logs were perhaps not (or not entirely) the species ordered. Species verification provides a means for mills to assess whether the raw material aligns with purchase agreements and expected product performance. A similar need arises in purchases of gatewood—logs not previously under supply contract purchased from independent logging firms upon delivery to the mill—where rapid screening of logs can help mills ensure they are procuring their desired species. These dynamics parallel broader trends in the forest products sector, where both landowners and mills increasingly engage in species- or quality-based valuation. Recent economic analyses show that landowners require price premiums to justify management intended to produce higher-quality pine sawtimber (Regmi et al. 2022) and that mills themselves are willing to pay premiums for higher-quality material (Regmi et al. 2022). In this context, a DNA-based identification tool offers a practical means of verifying species composition from suppliers, supporting adequate valuation, resolving disputes, and enhancing transparency in SYP procurement.

Identifying southern yellow pine species in standing trees relies on morphological characteristics such as needles, cones, and bark patterns, but most of these diagnostic features are lost once a tree is harvested. Species identification then depends on wood anatomical features, which can distinguish major subgeneric groups of *Pinus*, including the white, red, and yellow pine groups (Kukachka 1960; Panshin and Zeeuw 1980), but not individual species within the southern yellow pine group. The anatomical similarity among *P. elliottii*, *P. palustris*, *P. echinata*, and *P. taeda* has long been recognized (Kukachka 1960; Howard and Manwiller 1969; Eberhardt et al. 2022) (Figure 1) and reflects their close phylogenetic relationships (Gernandt et al. 2005; Jin et al. 2021; Xia et al. 2023).

The challenge of differentiating these genetically distinct but anatomically indistinguishable groups has prompted research into molecular tools that target subtle genetic differences. For instance, Olatinwo et al. (2020) developed a conventional polymerase chain reaction (PCR) method using a series of cpDNA markers to distinguish the four major SYP species specifically in the context of seed verification for reforestation efforts. Their method is unlikely to be suitable for our needs as conventional PCR depends on the successful amplification of relatively long DNA fragments, which is poorly suited for fragmented DNA typical of solid wood (Rachmayanti et al. 2009; Jiao et al. 2012, 2014; Höltken et al. 2012), and we intend to have a field-deployable test not dependent on gel electrophoresis.

In contrast, quantitative polymerase chain reaction (qPCR) is particularly advantageous for analyzing fragmented DNA because it targets short amplicons and utilizes fluorescence-

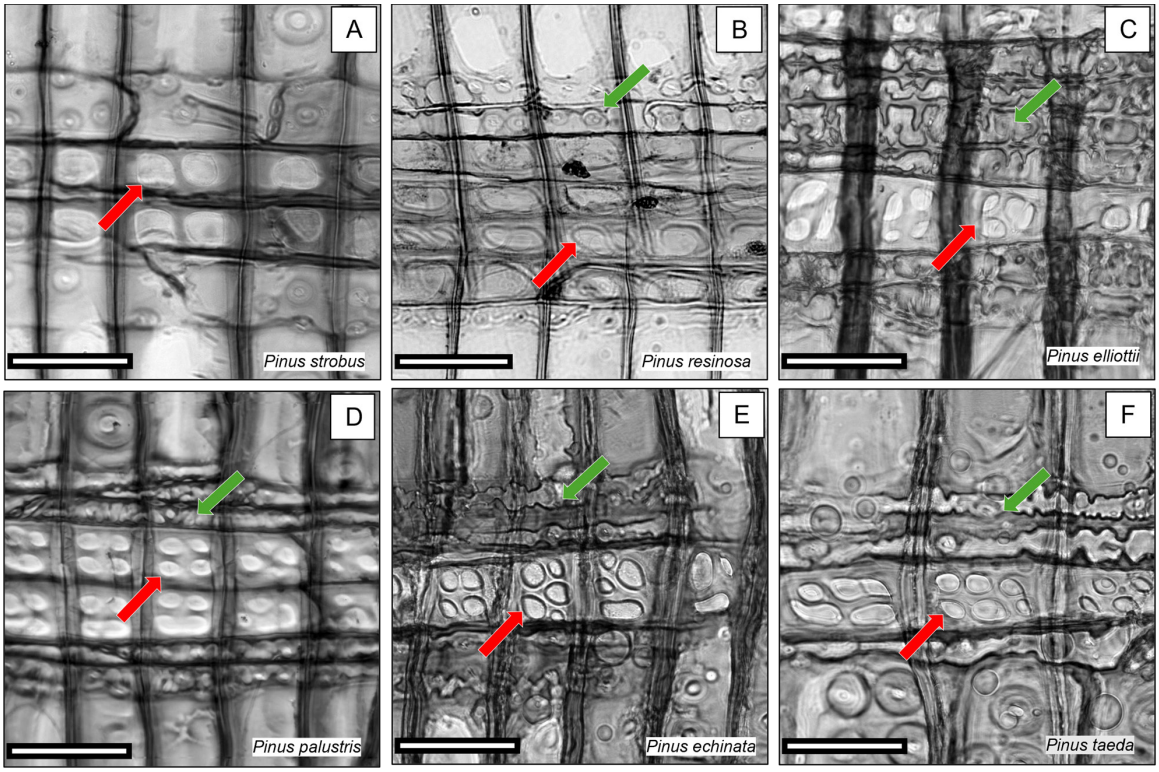

Figure 1. Radial wood anatomy comparison across six *Pinus* species. (A) White Pine Group (*P. strobus*): lacks dentate ray tracheids, with fenestriform cross-field pits; (B) Red Pine Group (*P. resinosa*): has dentate ray tracheids, with fenestriform cross-field pits; (C–F) Southern Yellow Pine species (*P. elliottii, P. palustris*, *P. echinata*, *P. taeda*): all show dentate ray tracheids and pinoid cross-field pits, illustrating the anatomical similarity within the group that precludes species-level identification. Green arrows point to ray tracheids; red arrows point to cross-field pitting; scale bar=50µm.

based detection to bypass the limitations of agarose gels. Fluorescence monitoring offers significantly higher sensitivity than gel electrophoresis; studies on processed wood have demonstrated that samples yielding no observable bands on a gel due to low copy number can still be reliably detected and identified through real-time fluorescence thresholds (Mohamed et al. 2012). Furthermore, this method allows for species authentication via melt curve rather than amplicon size, enabling accurate identification even with minimal starting material (Lee et al. 2016). qPCR has successfully been used to amplify DNA from ancient wood, which is a source of highly fragmented DNA (Watanabe et al. 2015; Gómez-Zeledón et al. 2017), and it is widely applied in forensic species identification and environmental DNA monitoring (Davy et al. 2015; Borland and Kading 2021). Given these advantages, qPCR represents a particularly suitable platform for developing species-differentiation assays in southern yellow pine wood. In this study, multiplex SYBR® Green chemistry (a proprietary nucleic acid stain; Thermo Fischer Scientific, Waltham, MA, USA) was selected for its cost-effectiveness compared to probe-based methods, efficiency, and compatibility with melt curve analysis, which enables discrimination among closely related taxa without the need for post-PCR gel electrophoresis (Ponchel et al. 2003; Tajadini et al. 2014; Zhou et al. 2017; Naeem et al. 2024).

The objective of this study is to develop a qPCR-based multiplex assay capable of differentiating *Pinus elliottii* (slash pine) and *Pinus palustris* (longleaf pine) from one another and from the lower-strength species *Pinus echinata* (shortleaf pine) and *Pinus taeda* (loblolly pine). To demonstrate proof-of-concept, we validate this assay using DNA extracted from leaf and seed tissues, laying the groundwork for extension of this method to wood and wood products.

Materials and methods

Plant material and DNA extraction

Plant tissue was collected from four southern yellow pine species (*P. palustris*, *P. elliottii*, *P. echinata*, and *P. taeda*), including mature needles from field-grown trees, nursery seedlings, and commercially sourced seeds. Specimens were obtained from several regions within each species' native ranges, as detailed in Table 2. All leaves (needles) and seeds were stored with silica gel at −20°C until processing. To minimize the risk of cross-

Table 2. Sampling locations, tissue sources, and DNA parameters used for assay development.

Species	Sampling location	Developmental stage	Tissue source	Specimen (n)	Concentration (ng/µL)	A260/280	A260/230
P. palustris (Longleaf)	Mississippi	Trees	Needles	2	10.99	1.65	0.24
	Georgia	Seedlings	Needles	6	9.25	1.64	0.07
	Florida	Seedlings	Needles	10	9.14	1.70	0.06
	Louisiana	Seeds	Embryos	4	90.62	1.88	1.97
P. elliottii (Slash)	Mississippi	Trees	Needles	4	10.99	1.65	0.16
	Georgia	Seedlings	Needles	10	9.25	1.64	0.22
	Florida	Seedlings	Needles	8	9.14	1.70	0.32
P. echinata (Shortleaf)	Mississippi	Trees	Needles	5	45.59	1.62	0.48
	Missouri	Seedlings	Needles	9	16.70	1.94	0.38
	Arkansas	Seeds	Embryos	6	18.13	1.71	0.44
P. taeda (Loblolly)	Georgia	Seedlings	Needles	10	8.85	1.63	0.27
	Florida	Seedlings	Needles	10	13.26	1.55	0.25

contamination, work surfaces and tools were decontaminated with 10% bleach and 70% ethanol between samples. For DNA extraction, 50 mg of needle tissue was weighed per sample. For seeds, seed coats were removed, and a single embryo (mass not determined) was excised for each extraction using sterile tweezers and avoiding inclusion of endosperm tissue. Tissue disruption was performed in 2 mL tubes containing steel beads (DNeasy® Plant Pro Kit, QIAGEN, Germantown, MD, USA). Tubes were vortexed for 3 min, cooled on ice for 1 min, and the cycle was repeated (a total of 6 min vortexing). After addition of Solution CD1 (cell lysis buffer) and Solution PS (inhibitor removal reagent) from the kit, the vortex-cool cycle was repeated three additional times (9 min of vortexing with the solutions).

Total genomic DNA was isolated from the disrupted tissue following the DNeasy® Plant Pro Kit (Qiagen) manufacturer's protocol. DNA was eluted in 50 µL of elution buffer. DNA concentration (ng/µL) and purity (A260/280 and A260/230 ratios) were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA extracts were diluted with nuclease-free water to a final working concentration of 5 ng/µL for qPCR assays. All DNA extracts were stored at −20°C until further use.

Primer design and *in silico* evaluation

Complete and partial chloroplast genome sequences for *Pinus palustris* (Longleaf pine, LLP; JN854176.1), *P. elliottii* (Slash pine, SLP; NC_042788.1, JN854202.1, MK135066.1), *P. echinata* (Shortleaf pine, SFP; NC_021440.1, JN854204.1, NC_065458.1, MZ424449.1), and *P. taeda* (Loblolly pine, LBP; KC427273.1, KY964286.1) were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database. The sequences were aligned in Geneious Prime® software (version 2024.0.5) by using the multiple alignment using fast fourier transform (MAFFT) plugin with default parameters (Katoh and Standley 2013). Alignments were manually inspected to identify candidate diagnostic sequence polymorphisms located within potential primer binding sites that could distinguish between the four SYP species.

Primers targeting the diagnostic loci were initially designed using the Primer3 plugin in Geneious Prime®. Design parameters were set as follows: primer length of 18–30 bp, a product size of 75–150 bp, and an optimal melting temperature (T_m) of approximately 60°C with a maximum T_m difference of 2°C between primer pairs. This strict size constraint (<100 bp) was a deliberate methodological design choice to maximize the assay's future applicability to solid wood. Candidate primers were screened *in silico* to minimize secondary structures, such as hairpins and self-dimers. To improve primer performance and compatibility in multiplex reactions, sequences were manually refined beyond the initial Primer3 output. These refinements included optimizing G/C content at the 3' end (GC clamp), minimizing T_m differences between primer pairs, and reducing potential cross-dimerization. Final primer combinations were selected to generate amplicons with distinct melting temperatures, facilitating clear differentiation by melt curve analysis. Primer specificity was assessed *in silico* using NCBI Primer-BLAST against the nucleotide (nt) database restricted to *Pinus* (taxid:3318). Primer-BLAST results confirmed species-level specificity: LLP primers aligned only with *P. palustris*,

while SLP primers aligned with *P. elliottii* and *P. caribaea* (a non-regional species). Neither primer set aligned with *P. clausa*, *P. virginiana*, or any other species within the southern yellow pine group. This lack of complementarity indicates that the assay would fail to amplify these minor species, thereby preventing misidentification in mixed-species supply chains.

Final primer combinations were selected to generate amplicons with distinct melting temperatures, facilitating clear differentiation by melt curve analysis. The primer sequences, G/C content, and expected amplicon sizes are summarized in Table 3. To further evaluate the melting behavior of the amplicon products, *in silico* melt curve predictions were performed using uMELT v3 (https://www.dna.utah.edu/umelt/) using default parameters (Dwight et al. 2011). Each amplicon was assessed to ensure it produced a single, distinct melt peak, supporting its suitability for multiplexing. Predicted melting temperatures and complete amplicon sequences are shown in Table 4.

Simplex qPCR assay setup

Each primer pair was first validated individually in simplex qPCR assays under two distinct experimental conditions to assess performance and compatibility. First, assays were run under standard conditions following the master mix manufacturer's recommendations. These 10 µL reactions consisted of 5 µL PowerUp™ SYBR™ Green Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 5 ng of genomic DNA, and 0.5 µL each of forward and reverse primer (10 µM stock; Custom DNA Oligos Invitrogen), resulting in a final concentration of 500 nM per primer (forward and reverse, 1000 nM in total primer concentration). Reactions were conducted in triplicate. Thermal cycling included Uracil-DNA Glycosylase (UDG) activation (50°C, 2 min) and denaturation (95°C, 2 min), followed by 35 cycles of 95°C for 30 s and 60°C for 1 min. Annealing temperature was optimized using gradient PCR from 55°C to 65°C to confirm single-amplicon specificity and determine characteristic melting temperatures (T_m). Second, assays were run under multiplex-simulation conditions to evaluate performance under the constraints of the final multiplex design. These reactions used reduced primer concentrations (200 nM for LLP; 300 nM for SLP) and the optimized multiplex thermal profile (denaturation at 95°C for 15 s; annealing/extension at 61°C for 20 s).

Multiplex qPCR assay optimization

Following simplex validation, a multiplex SYBR Green qPCR assay was optimized. An asymmetric primer concentration matrix was employed to compensate for the higher efficiency of the LLP primer pair (see results section for simplex assays). Optimization started with a total primer concentration of 1000 nM (matching the simplex assay), representing an initial 600 nM/400 nM (SLP/LLP) split. A matrix of combinations was then tested, using total SLP concentrations of 600, 400, and 200 nM and total LLP concentrations of 400 and 200 nM. Each concentration combination was tested in triplicate. Optimal concentrations (Table 5) were selected to minimize the Cq difference between LLP and SLP, but this condition produced primer-dimer artifacts and overlapping melt peaks (~69.0°C),

Table 3. Primer sequences and characteristics for SYBR Green qPCR assays targeting southern yellow pines.

Primer ID	Sequence (5' to 3')	G/C content (%)	T_m (°C)	Target gene	Product (bp)
LLP_F	TCTCATCATT**[TTCCGA]**TTCCG	42.9	62	ycf2	99
LLP_R	TCAATTCAGGATATGGCAAGAAC	39.1	60		
SLP_F	CTTGTGTCAACTAAAAAGAAGTAAAAA**[TACC]**	29.0	60	trnL-trnF IGS	95
SLP_R	TTTTTATCATATTAGTGACTTCCAGATCG	31.0	61		

Primer IDs indicate species and orientation: LLP for *Pinus palustris* (Longleaf pine), SLP for *P. elliottii* (Slash pine), with F and R denoting forward and reverse primers, respectively. Diagnostic sequences are bracketed within the primer binding sites.

Table 4. Predicted primer and amplicon sequences and melt temperatures for *Pinus palustris* and *Pinus elliottii*.

Primer	Primer + amplicon sequence	Melt peak (°C)
P. palustris (Longleaf)	**TCTCATCATT[TTCCGA]TTCCG**AATGAGTTTCGCTCGGTCCTTG ATTCTTCGAGATCATCTCATCCTTCTTGTTCGT**GTTCTTGCCATA TCCTGAATTGA**	84
P. elliottii (Slash)	**CTTGTGTCAACTAAAAAGAAGTAAAAA[TACC]**TTTTTACTAGT TTTTAGAATGATCTTTATTATTTT**CGATCTGGAAGTCACTAATAT GATAAAAA**	76

Primer binding sites are shown in bold. Species-specific sequences are bracketed. Melt peaks were calculated using uMELT.

Table 5. Reaction components for the optimized multiplex SYBR Green qPCR assay.

Component	Stock concentration	Volume per reaction (µL)	Final concentration
PowerUp™ SYBR™ Green Master Mix	2X	5.0	1X
SLP Forward Primer	10 µM	0.3	300 nM
SLP Reverse Primer	10 µM	0.3	300 nM
LLP Forward Primer	10 µM	0.2	200 nM
LLP Reverse Primer	10 µM	0.2	200 nM
DNA Template	5 ng/µL	1.0	5 ng
Nuclease-Free Water	N/A	3.0	N/A
Total volume	**N/A**	**10.0**	**N/A**

interfering with species differentiation (see results section, Figure 4). To address this, thermal cycling conditions were optimized by increasing the annealing temperature to 61°C and shortening the annealing/extension time to 20 s, and the denaturation time to 15 s. Final 10 µL multiplex qPCR reactions contained 5 µL PowerUp™ SYBR™ Green Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), the optimal concentration of SLP and LLP primer pairs (Table 5), and 5 ng genomic DNA template. For extracts with a concentration below 5 ng/µL, 1 µL of the undiluted extract was used.

Thermal cycling and fluorescence data acquisition were performed on a BioRad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The optimized protocol consisted of an initial Uracil-DNA Glycosylase (UDG) activation (50°C, 2 min), followed by denaturation and polymerase activation (95°C, 2 min). Amplification was carried out for 40 cycles of denaturation (95°C, 15 s) and annealing/extension (61°C, 20 s), with data acquisition at each annealing/extension step. Following amplification, a melt curve analysis was performed by increasing the temperature from 61°C to 95°C, with 0.5°C increments per 10 s, to confirm amplicon specificity.

Assay validation

Validation of the qPCR assays followed the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). A MIQE compliance table is available as Table S1 in the supplement. The simplex assays were evaluated for specificity, efficiency and linearity, and sensitivity using a single representative specimen for each species. The multiplex assay was validated across all four validation steps, including screening performance:

- Specificity: Determined by testing the assay against genomic DNA from target and non-target *Pinus* species. The specificity of amplification products was further confirmed by analysis of melt curves for each reaction (Ririe et al. 1997), ensuring a single, distinct melting peak for each target amplicon.
- Efficiency and linearity: Established using serial dilutions of target DNA (10-fold, across six orders of magnitude) to generate standard curves and calculate efficiency from the slope.
- Sensitivity: Defined as the lowest DNA concentration at which amplification was reliably detected in ≥ 95% of at least 20 replicates (Huber et al. 2013; Broeders et al. 2014).
- Screening performance: Evaluated on a panel of 84 individuals (Table 2), to determine accuracy, precision, and recall.

Specificity

To evaluate specificity, genomic DNA was extracted from one mature leaf from one individual for each of the two target species (*P. palustris* and *P. elliottii*) and the two non-target species (*P. taeda* and *P. echinata*), all collected in Mississippi. For each species, triplicate multiplex qPCR reactions were prepared using 5 ng of extracted DNA. Following amplification, melt curve analysis was performed to detect the presence of single, target-specific peaks, indicating specific amplification. For further confirmation, 4% agarose gel electrophoresis was used to separate qPCR products, with gels stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA) and imaged under UV light with Bio-Rad Gel Doc XR+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Assay specificity was validated by the absence of off-target peaks in melt curves and non-specific bands on gels, and by verification of expected amplicon sizes for each target.

Efficiency and linearity

To assess quantitative performance, standard curves were generated separately for *P. palustris* and *P. elliottii*, using multiple

extractions of the same individuals used for specificity evaluation. For each species, a 6-point, 10-fold serial dilution series was prepared, ranging from 10 ng to 0.1 pg per reaction. Each dilution was tested in triplicate qPCR reactions.

Standard curves were generated by plotting quantification cycle (Cq) values against the logarithm (base 10) of the DNA concentration for each species. The coefficient of determination (R^2) was calculated to assess the linearity of the assay over the dilution range. Efficiency (E) was determined from the slope of the standard curve using Equation (1). Assay performance was considered valid only when the standard curves exhibited R^2 values ≥ 0.98 and efficiencies were in the 80%–120% range (Taylor et al. 2010; Broeders et al. 2014).

$$E = 10^{\left(\frac{-1}{Slope}\right)} - 1 \quad [1]$$

Sensitivity

Sensitivity was evaluated by determining the limit of detection (LOD) for each species. The lowest concentrations in the dilution series were analyzed using a minimum of 20 replicates from the same extraction (technical replicates) per concentration (Bustin et al. 2009, 2025; Bell 2016). The LOD was defined as the lowest DNA input at which ≥ 95% of technical replicates produced an amplification and a specific melt curve peak corresponding to the expected amplicon. All LOD testing included appropriate no-template controls to monitor contamination and false positives.

Screening performance

To assess the screening accuracy and specificity of the optimized multiplex qPCR assay, 84 individuals, as detailed in Table 2, were evaluated. Genomic DNA was extracted from leaf or seed tissue from each specimen using the previously described extraction protocol. Each DNA sample was analyzed with the optimized multiplex qPCR protocol. To minimize the risk of cross-contamination, individuals from different species were processed and amplified in separate qPCR runs. Negative (no-template) controls were included in each run to monitor contamination.

Scoring of assay results was performed as follows: A sample was classified as a true positive (TP) if the assay correctly identified it as either SLP or LLP based on the presence of the characteristic melt peak. A true negative (TN) was recorded when a non-target species (LBP or SFP), with template DNA at standardized template concentrations (5 ng), showed no amplification signal. False positives (FP) were defined as any amplifications of non-target species, while false negatives (FN) were defined as any failure to detect a target species. For each metric, results were tabulated across all samples. The screening accuracy (Eq. 2), precision (Eq. 3), and recall (Eq. 4) were calculated using the following standard formulas:

$$\text{Accuracy} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{TN} + \text{FP} + \text{FN}} \quad [2]$$

$$\text{Precision} = \frac{\text{TP}}{\text{TP} + \text{FP}} \quad [3]$$

$$\text{Recall} = \frac{\text{TP}}{\text{TP} + \text{FN}} \quad [4]$$

Results

Simplex assay results

Specificity

Amplification curves for *P. palustris* and *P. elliottii* were compared between the two experimental setups (Figure 2, Panels A–D). Under standard conditions (1000 nM primer concentration; standard cycling), amplification signals were strong and consistent. However, under multiplex-simulation conditions (primer concentration of 200 nM for *P. palustris*, 300 nM for *P. elliottii*; rapid cycling), amplification signals were weaker, and Cq values were higher. Melt curve analysis (Figure 2, Panels E–H) showed single peaks for each species with mean melting temperatures (T_m) of 77.83 ± 0.29°C (*P. palustris*) and 69.5 ± 0.0°C (*P. elliottii*).

Efficiency and linearity

Standard curves for both species under the two conditions are shown in Figure 3. Under standard conditions (Panels A, C), amplification efficiencies were 99.1% for *P. palustris* and 103.1% for *P. elliottii*, with R^2 values exceeding 0.99. Under multiplex-simulation conditions (Panels B, D), efficiencies decreased to 77.0% for *P. palustris* and 73.3% for *P. elliottii*, with R^2 values still above 0.98.

Multiplex assay results

Assay optimization

The initial multiplex qPCR assay used a primer concentration of 300 nM for *Pinus elliottii* and 200 nM for *Pinus palustris* in one reaction, based on the starting point of a systematic asymmetric primer concentration matrix. Melt curve analysis of the no-template control (NTC) showed a low-amplitude peak at

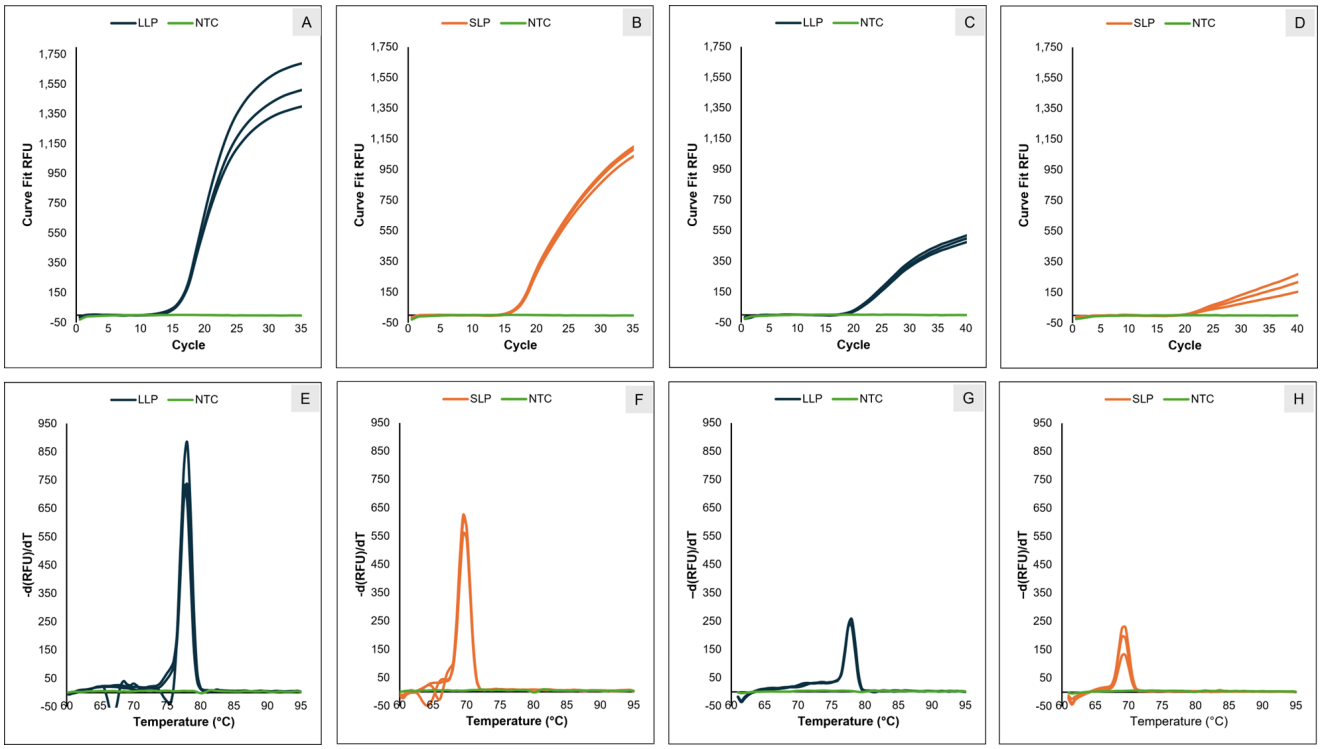

Figure 2. Amplification and melt curves for simplex qPCR assays under two conditions. (A–B) Amplification curves for *P. palustris* and *P. elliottii* at 1000 nM primer concentration (standard conditions); (C–D): Amplification curves for *P. palustris* (200 nM) and *P. elliottii* (300 nM) under multiplex-simulation conditions; (E–H) Melt peaks remained consistent (78.0 °C for *P. palustris*, 69.0 °C for *P. elliottii*) across both conditions.

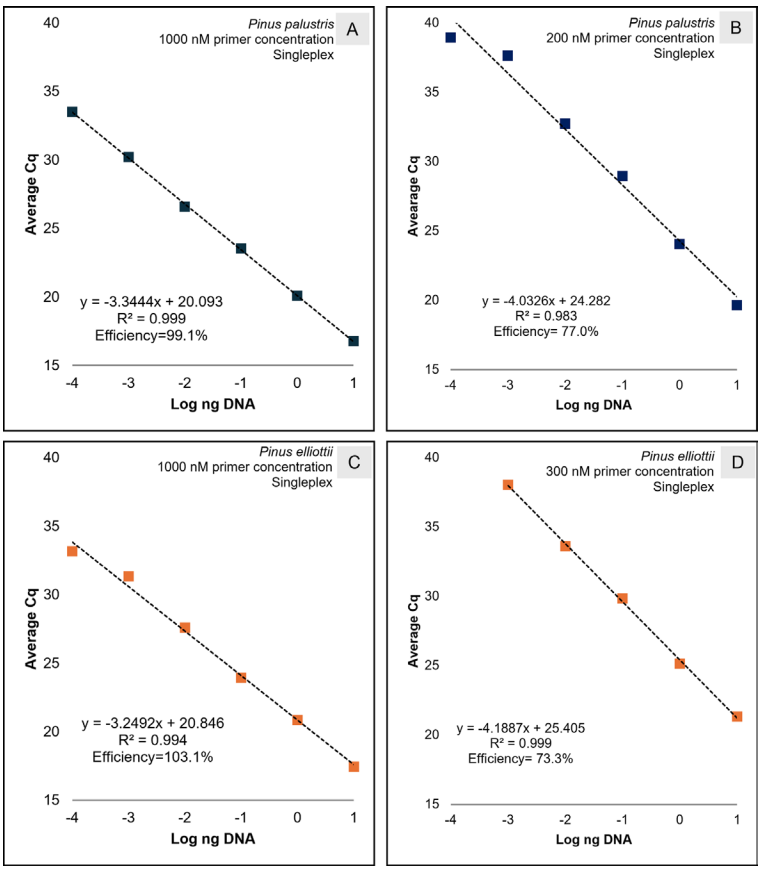

Figure 3. Standard curves for simplex qPCR assays under two conditions. (A, C): Standard conditions (1000 nM primers); (B, D): Multiplex-simulation conditions (200/300 nM primers). Amplification efficiencies were 99.1% and 103.1% under standard conditions, and 77.0% and 73.3% under multiplex-simulation conditions.

approximately 69.0°C. Agarose gel electrophoresis revealed low-molecular-weight bands in the NTC. These observations were recorded during initial testing with the above primer concentrations. Figure 4 presents the optimized multiplex qPCR assay results, including amplification curves (Figure 4A), melt curve profiles, and an inset gel electrophoresis for amplicon size confirmation (Figure 4B).

Specificity

The optimized multiplex qPCR assay used a primer concentration of 300 nM for *P. elliottii* and 200 nM for *P. palustris* in a single reaction, an annealing/extension temperature of 61°C, and shortened cycling time to 20 s. Under optimized multiplex conditions, amplification was observed only in the target species. Melt curve analysis showed distinct peaks with mean melting temperatures (T_m) of 77.67 ± 0.29°C for *P. palustris* and 69.0 ± 0.0°C for *P. elliottii*. No amplification was detected in non-target species (*P. taeda*, *P. echinata*) or in the no-template control (NTC). Agarose gel electrophoresis confirmed single bands of expected size for both target species and the absence of products in non-target species and the NTC (Figure 5).

Efficiency and linearity

Standard curves were generated for *P. palustris* and *P. elliottii* using 6-point, 10-fold serial dilutions of genomic DNA ranging from 10 ng to 0.1 pg of input DNA per reaction. The coefficients of determination (R^2) exceeded 0.99 for both species, indicating high linearity across this range. Amplification efficiency (E) was calculated as 86.2% for the *P. palustris* assay and 81.6% for the *P. elliottii*, based on the slope of the standard curves (Figure 6).

Sensitivity

The limit of detection (LOD) was defined as the lowest DNA concentration at which ≥95% of technical replicates produced a positive amplification and a specific melt curve peak. For *P. palustris*, the LOD was 1 pg (0.001 ng), and for *P. elliottii*, it was 10 pg (0.01 ng). At these concentrations, all 21 technical replicates amplified successfully (100%) and displayed a single melt peak at the expected melting temperature (T_m). The mean quantification cycle (Cq) values were 34.03 ± 0.90 for *P. palustris* and 33.20 ± 0.64 for *P. elliottii*, with all values below the MIQE-recommended threshold of 35 cycles.

Screening performance

The optimized multiplex qPCR assay was evaluated on a panel of 84 individuals representing both target and non-target species (Table 6). All 44 individuals representing target species (*P. palustris* and *P. elliottii*) amplified successfully and produced single melt peaks at the expected melting temperatures, whereas no amplification was observed in any of the 40 individuals of the non-target species (*P. echinata* and *P. taeda*) or in no-template controls (NTC). Based on these results, the

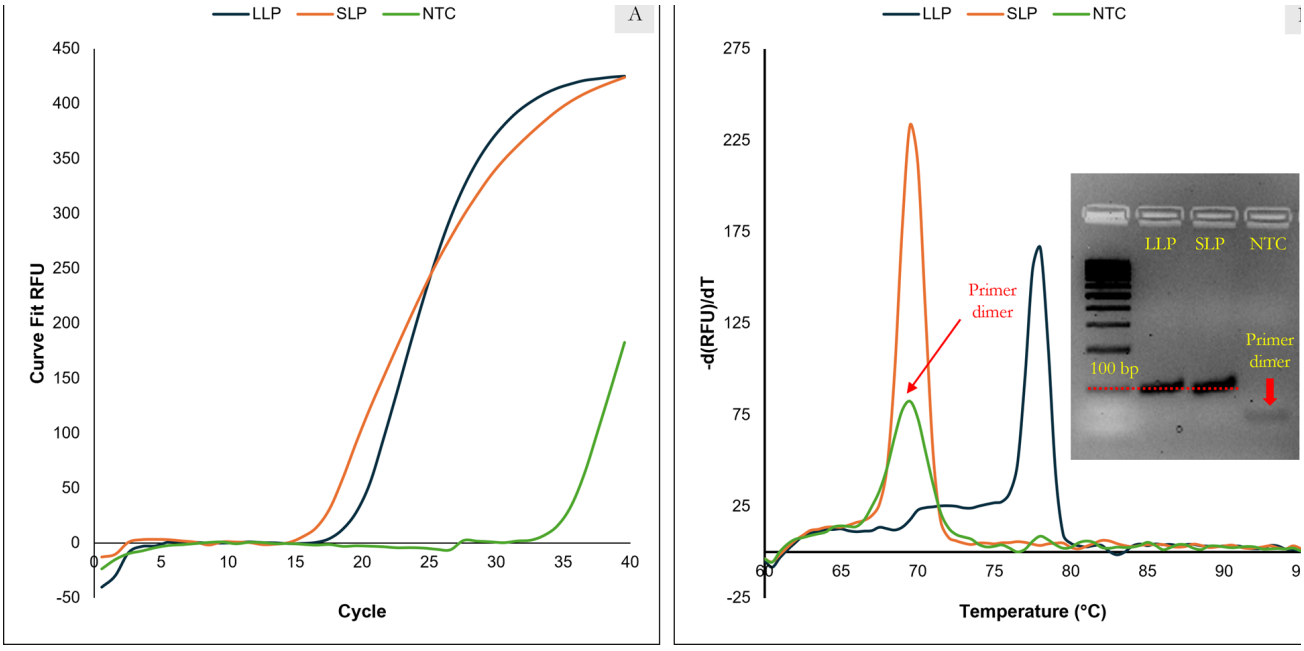

Figure 4. Multiplex qPCR assay results showing amplification and melt curve profiles with an inset gel electrophoresis. (A) Real-time amplification curves for *Pinus palustris* (LLP) and *P. elliottii* (SLP) under optimized multiplex conditions; (B) Melt curve analysis with distinct peaks at 78.0 °C (LLP) and 69.0 °C (SLP), confirming species differentiation. Inset: Agarose gel electrophoresis showing expected amplicon sizes for target species and absence of products for non-targets and NTC.

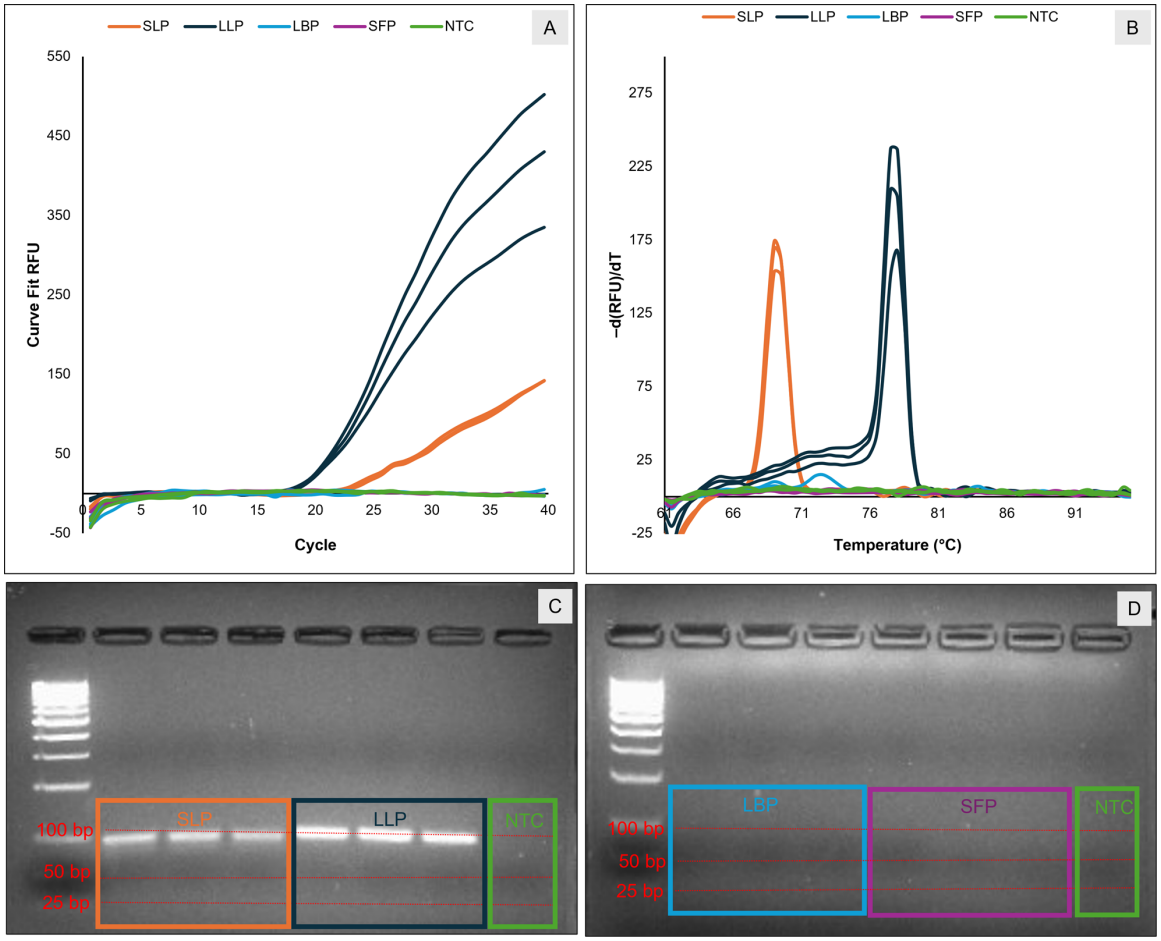

Figure 5. Analytical specificity of the optimized multiplex qPCR assay. (A) Real-time amplification curves for *Pinus elliottii* (SLP) and *P. palustris* (LLP) under optimized multiplex conditions. Non-target species (*P. taeda* (LBP), *P. echinata* (SFP)) and no-template control (NTC) showed no amplification; (B) Melt curve analysis showing distinct melting temperature (T_m) peaks at 69.0 °C for SLP and 78.0 °C for LLP, confirming species differentiation; (C) Agarose gel electrophoresis for target species showing expected amplicon sizes for SLP and LLP; (D) Agarose gel electrophoresis for non-target species and NTC showing absence of amplification products.

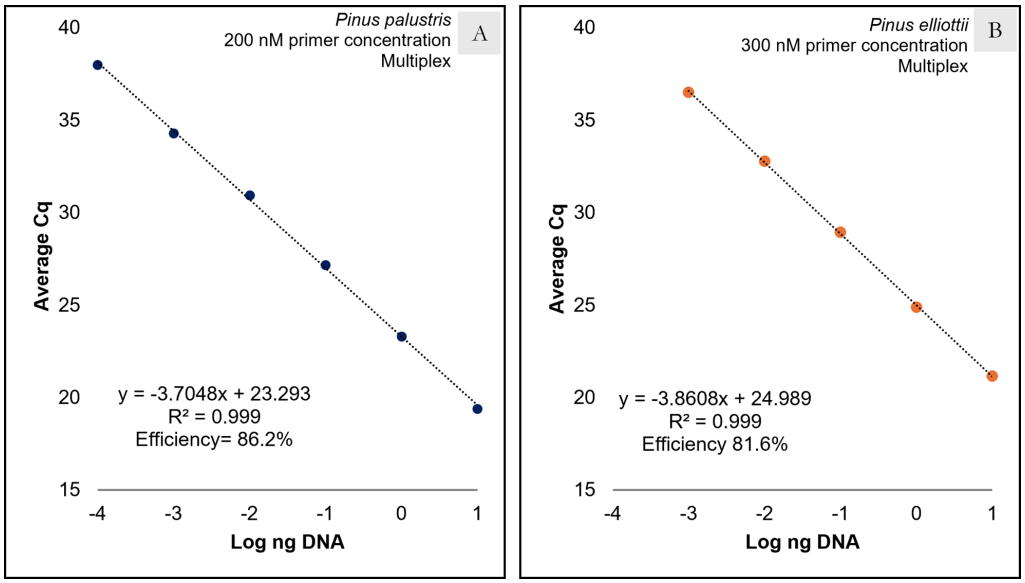

Figure 6. Standard curves for multiplex qPCR assays of *Pinus palustris* (A) and *P. elliottii* (B). Six-point, 10-fold serial dilutions (10 ng–0.1 pg) were amplified under optimized multiplex conditions. x-axis: $\log_{10}$ DNA concentration (ng); y-axis: quantification cycle (Cq). The linearity (R^2) exceeded 0.99 for both species; amplification efficiencies (E) were 81.6% (*P. elliottii*) and 86.2% (*P. palustris*).

Table 6. Quantification cycle (Cq) values for *Pinus palustris* and *P. elliottii* samples.

Species	Origin	Samples (n)	Cq (mean ± SD)
P. palustris (Longleaf)	Florida (FL)	10	21.6 ± 1.74
	Georgia (GA)	6	24.3 ± 0.50
	Mississippi (MS)	2	22.5 ± 0.70
	Louisiana (LA)	4	16.8 ± 0.59
P. elliottii (Slash)	Florida (FL)	9	23.2 ± 3.10
	Georgia (GA)	9	27.2 ± 4.29
	Mississippi (MS)	4	22.6 ± 2.07
P. echinata (Shortleaf)	Mississippi (MS)	5	NA
	Missouri (MO)	9	NA
	Arkansas (AR)	6	NA
P. taeda (Loblolly)	Florida (FL)	10	NA
	Georgia (GA)	10	NA

Values are presented as mean ± standard deviation for each origin. NA indicates no amplification in the multiplex qPCR assay.

screening performance metrics were calculated as follows: True Positives (TP) = 44, True Negatives (TN) = 40, False Positives (FP) = 0, False Negatives (FN) = 0, yielding 100% accuracy, precision, and recall.

Quantification cycle (Cq) values for the target samples (all at template concentration of 5 ng/µL) are summarized in Table 6, which shows mean Cq values ranged from 16.8 (Louisiana) to 24.3 (Georgia) for *P. palustris*, while *P. elliottii* ranged from 22.6 (Mississippi) to 27.2 (Georgia). All values remained below the MIQE-recommended threshold of 35 cycles. Figure 7 shows representative amplification curves and melt curve profiles for individuals from Florida, Georgia, Mississippi, and Louisiana, highlighting the distinct mean melting temperatures (T_m) (77.98 ± 0.11°C for *P. palustris* and 69.25 ± 0.11°C for *P. elliottii*) that enable clear species differentiation.

Discussion

Simplex assay performance

The simplex assays confirmed that high primer concentrations under standard conditions produced strong amplification and near-ideal efficiency, consistent with MIQE guidelines for qPCR performance (Bustin et al. 2009). When reactions were run under multiplex-simulation conditions with lower primer concentrations and shortened denaturation and annealing time, amplification efficiency decreased, but melt curve profiles remained unchanged. This stability indicates that specificity was maintained despite reduced amplification, supporting the reliability of melt curve analysis for differentiating the species (Ririe et al. 1997; Chabi et al. 2019). The reduced efficiency under multiplex-simulation conditions was expected because lower primer concentrations and shorter extension steps limit primer-template interactions and amplification kinetics (Kralik and Ricchi 2017). This trade-off between efficiency and multiplex compatibility is widely reported in multiplex qPCR studies (Safdar and Junejo 2015; Sarkar et al. 2022).

Multiplex assay optimization

The initial multiplex reaction assay resulted in primer-dimer formation, evidenced by a low-amplitude melt peak near 69.0°C in the no-template control (NTC), and a low-molecular-weight band in agarose gels. To address this, the annealing temperature was increased to 61°C, and denaturation/annealing time was shortened to 15 s and 20 s, respectively. These adjustments are consistent with established qPCR optimization strategies, which show that higher annealing temperatures reduce non-specific primer binding and primer-dimer formation, while shorter annealing time prevents unintended amplification (Safdar and Junejo 2015; Kralik and Ricchi 2017).

Melt curve analysis was critical for detecting these artifacts because SYBR Green binds all double-stranded DNA, and primer dimers typically melt at a lower temperature than target amplicons (Ririe et al. 1997). Balancing amplification efficiency in multiplex reactions is a common technical hurdle. We demonstrate that optimizing primer asymmetry (300 nM vs. 200 nM) effectively mitigates competition between targets without sacrificing sensitivity (Sarkar et al. 2022).

Specificity

The Primer-BLAST search confirmed species-level specificity: (1) LLP matched only *P. palustris*, (2) SLP primers matched *P. elliottii* and *P. caribaea*, a non-regional species, minimiz-

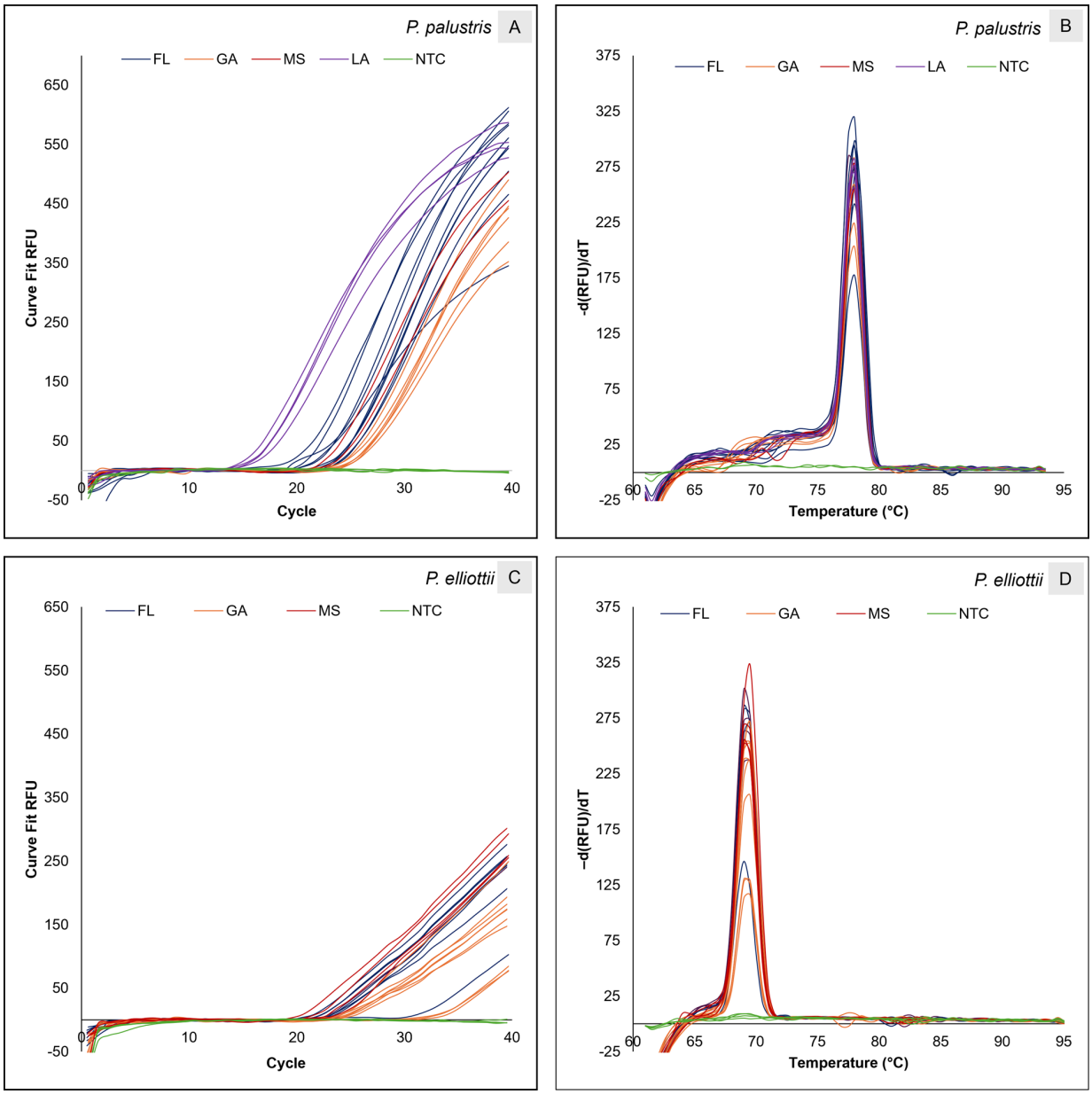

Figure 7. Screening oerformance of the multiplex qPCR assay for *Pinus palustris* and *P. elliottii*. (A) Amplification curves for *P. palustris* (Longleaf pine) from Florida (FL), Georgia (GA), Mississippi (MS), and Louisiana (LA); (B) Melt curve analysis for *P. palustris* showing a single peak at 78.0 °C; (C) Amplification curves for *P. elliottii* (Slash pine) from FL, GA, and MS; (D) Melt curve analysis for *P. elliottii* showing a single peak at 69.0 °C.
No-template control (NTC) showed no amplification in all panels.

ing practical risk of misidentification. (3) No matches were found for other regional species not typically found in trade (*P. clausa* or *P. virginiana*). Predicted melt temperatures from uMELT supported the expectation of distinct peaks, although observed values deviated slightly, likely due to experimental factors such as salt concentration and ramp rate (Dwight et al. 2011). The ~9°C separation between peaks provided strong species discrimination (100% accuracy, precision, and recall) under multiplex conditions, a strategy validated for SYBR Green assays (Zhou et al. 2017; Chabi et al. 2019).

Efficiency and linearity

The assay demonstrated high linearity under both simplex and multiplex conditions, with R^2 values exceeding 0.99 across six orders of magnitude of DNA concentration, meeting MIQE guidelines for qPCR performance (Bustin et al. 2009). Under standard conditions (1000 nM primers), amplification efficien-

cies were near ideal (99.1% for *P. palustris* and 103.1% for *P. elliottii*), indicating strong quantitative performance. When reactions were run under multiplex-simulation conditions with reduced primer concentrations and shortened cycling time, efficiencies decreased to 77.0% and 73.3%, respectively.

In the optimized multiplex conditions, efficiencies improved to 86.2% for *P. palustris* and 81.6% for *P.elliotti*, confirming that primer concentration balancing and thermal adjustments mitigated some of the trade-off between multiplex compatibility and amplification kinetics (Kralik and Ricchi 2017; Sarkar et al. 2022). These values fall within the 80%–120% range recommended for diagnostic screening, supporting the assay's reliability for species identification, rather than precise quantification (Broeders et al. 2014). The lower efficiency for the *P. elliottii* target may be affected by the lower G/C content of its primers and amplicon (Kralik and Ricchi 2017). This issue could not be solved by redesigning the primers in our case, due to the location of the target sequences, inherent interspecific DNA sequence similarities of cpDNA in *Pinus*, and sequence availability in NCBI.

Sensitivity and DNA Quality

The multiplex qPCR assay demonstrated high sensitivity, detecting as little as 1 pg of DNA for *P. palustris* and 10 pg for *P. elliottii*, with all technical replicates producing specific melt peaks and Cq values below the MIQE threshold of 35 cycles (Bustin et al. 2009; Forootan et al. 2017). These limits of detection indicate the assay's suitability for low-template scenarios, which is critical for future application to solid wood, where DNA quantity and quality are often compromised (Rachmayanti et al. 2009; Costa et al. 2022).

DNA extracted from leaf and seed tissues showed low A260/280 and A260/230 ratios, suggesting the presence of proteins, polysaccharides, and phenolic compounds—known PCR inhibitors in plant tissues (Demeke and Jenkins 2010). Despite these challenges, sensitivity testing confirmed reliable amplification at picogram-level DNA inputs, supporting the robustness of short amplicons (<100 bp) for material with low quantity and low-quality DNA, a strategy validated for forensic wood identification (Dormontt et al. 2015; Debode et al. 2017).

Screening Performance

The optimized multiplex qPCR assay achieved 100% accuracy, precision, and recall across 84 individuals, with no false positives or false negatives observed. All samples of target species exhibited single melt peaks at the expected temperatures (78.0°C and 69.0°C), while samples of non-target species (*P. taeda* and *P. echinata*) and no-template controls showed no amplification. This performance aligns with MIQE-based validation principles emphasizing accuracy, reproducibility, and specificity for quantitative real-time PCR assays (Broeders et al. 2014; Bustin et al. 2025). The absence of misclassification (i.e., false positives and false negatives) demonstrates that melt curve analysis provided sufficient resolution for species differentiation, even in specimens with variable DNA purity—a known challenge in plant molecular diagnostics (Demeke and Jenkins 2010; Schrader et al. 2012). Compared to conventional PCR methods, such as the one reported by Olatinwo et al. (2020), which requires post-PCR gel electrophoresis, this multiplex qPCR approach offers a faster workflow by detecting and separating two species in a single reaction (Sarkar et al. 2022). Furthermore, the use of SYBR Green chemistry provides a more cost-effective alternative to hydrolysis probe-based assays (Tajadini et al. 2014; Naeem et al. 2024). Most critically, our assay's short amplicons (<100 bp) are designed for low-quality and low-quantity DNA, which is expected to be a key feature for future work with solid wood (Debode et al. 2017).

Although all target samples were standardized to 5 ng/µL DNA input, Table 6 shows considerable variation in Cq values across geographic origins, ranging from 16.8 (Louisiana) to 24.3 (Georgia) for *P. palustris* and from 22.6 (Mississippi) to 27.2 (Georgia) for *P. elliottii*. These differences likely reflect biological and/or chemical factors. First, variation in parent material quality, such as the number of viable cells per unit tissue, can influence the effective copy number of chloroplast genomes, which likely affects amplification kinetics (Rowan et al. 2009). Second, residual PCR inhibitors (e.g., polysaccharides, phenolic compounds) commonly present in pine tissues may not have been fully removed by the commercial extraction kit, reducing amplification efficiency despite equalized DNA concentrations (Demeke and Jenkins 2010). Both factors are plausible explanations for the observed Cq range and have been widely reported in plant qPCR studies (Brunner et al. 2004; Kontanis and Reed 2006; Rowan et al. 2009; Demeke and Jenkins 2010; Die et al. 2016). While these differences did not compromise species identification, they underscore the importance of considering DNA purity and inhibitor removal in future applications.

In this study, true negatives were defined by the lack of amplification in non-target species assayed at standard template concentrations of 5ng/µL DNA. However, in practical applications involving an unknown template DNA concentration (as

would be true in the field with solid wood), the absence of an amplification signal cannot necessarily be interpreted as the absence of the target species. As noted in studies involving solid wood (Mohamed et al. 2012; Lee et al. 2016), reaction failure can be a result of a failed extraction protocol, degradation of target DNA in the specimen, or an extracted DNA concentration below the detection threshold. One approach to overcome these concerns would be to incorporate an internal amplification control (IAC) (Hoorfar et al. 2004). If the IAC fails to amplify, it suggests that there are one or more problems with the PCR conditions, indicating that the results of the test are inconclusive. Future work on solid wood should develop empirical diagnostic criteria for interpreting results based on rates of false negatives, false positives, and amplification failures when using wood as the DNA source.

Target plant genomes, marker development for *Pinus* Identification

The development of molecular screening tools for southern yellow pines is constrained by the close phylogenetic relationship among the species, which limits the number of differentiating genetic markers (Jin et al. 2021), especially in the chloroplast genome (plastome), where few sequence polymorphisms suitable for species discrimination have been identified. The plastome of *Pinus* is moderately A/T-rich (about 60% A + T overall) and contains simple sequence repeats, many of which are mononucleotide tracts composed of A or T bases (Powell et al. 1995). Such low-complexity, A/T-rich, or repetitive regions reduce the number of suitable primer-binding sites, requiring careful design to avoid non-specific amplification. The limitations of chloroplast-based markers highlight the importance of considering the distinct characteristics of the three plant genomes—chloroplast, mitochondrial, and nuclear—for species identification (Hollingsworth et al. 2011). The chloroplast genome in *Pinus*, a small, circular chromosome, is typically inherited paternally (Neale and Sederoff 1989; Chen et al. 2002) and is present in high copy number per cell (Sakamoto and Takami 2018), but its low rate of nucleotide substitution limits its utility for distinguishing closely related species (Wolfe et al. 1987), especially when considering the limited number of NCBI sequences available to assess sequence variability within and between taxa.

The mitochondrial genome in conifers is maternally inherited (Neale and Sederoff 1989) and present in high copy number per cell (Wolfe et al. 1987). Although it evolves at a slower nucleotide substitution rate compared to nuclear and chloroplast genomes (Drouin et al. 2008), this does not imply a lack of informative variation. In fact, studies have shown that conifers, due to their ancient lineage and long generation times, can accumulate substantial mitochondrial diversity over evolutionary timescales (Wu et al. 1998). Mitochondrial haplotypes have been successfully used to distinguish populations in *Pinus* (Potter et al. 2013), although their utility for fine-scale species discrimination may be limited compared to chloroplast markers (Fang et al. 2009). The mitochondrial genome is also structurally complex, often consisting of multiple large, recombining circular molecules (Gualberto and Newton 2017; Jackman et al. 2020). In contrast, the nuclear genome is organized into large, linear, biparentally inherited chromosomes and, despite its single copy number per cell, its enormous size in *Pinus* (often exceeding 20 Gb) (Neale et al. 2014; Zimin et al. 2014) provides a vast reservoir of comparatively rapidly mutating non-coding regions expected to contain an abundance of targets suitable for high-resolution assays (Eckert et al. 2010). The selection of a genomic target for species identification thus requires balancing genetic resolution with expected DNA source quantity and quality. For plant parts or products with high-quality and quantity DNA, the nuclear genome inevitably provides the most promise for high-resolution diagnostics (Eckert et al. 2010; Neale et al. 2014). For plant parts or products with inherently low quantity, and probably also low-quality DNA, such as wood, recovering the single-copy nuclear DNA can be challenging, even for small amplicons, in part due to the low copy number, and in part as a result of the comparatively high environmental lability of DNA in a linear chromosome (Watanabe et al. 2015). In these cases, high-copy-number organellar DNA, often with circular chromosomes, is a more practical target, as its abundance increases the likelihood of successful amplification in low-yield and/or low-quality material (Dumolin-Lapegue et al. 1997).

This genomic comparison highlights a direction for future research. Expanding marker discovery efforts beyond the highly constrained plastome (Liu et al. 2021) and into the nuclear genome is an alternative approach to enhance screening capabilities (Särkinen and George 2013). Exploring these larger and more diverse genomic regions offers the potential to uncover more diagnostically informative sequences, which would provide much-needed flexibility for assay design and could enable the development of higher-capacity multiplex assays for more comprehensive species identification (Hollingsworth et al. 2011). Such an approach would call for more reliable DNA extraction protocols—regardless of the targeted plant genome—that can overcome the low-yield DNA nature of wood and wood products (Costa et al. 2022).

Conclusions

The successful validation of this assay on leaf and seed tissue serves as a critical proof of concept for its application to solid wood. When coupled with a rapid, field-deployable DNA extraction protocol for wood and comparably field-deployable hardware, with lyophilized reagents, this assay could serve as a highly specific screening tool for the Southern yellow pine industry, where distinguishing anatomically similar species is a known, significant challenge. Ultimately, this work establishes a robust molecular workflow that integrates short-amplicon design with high-resolution melt curve analysis. While validated here for *Pinus*, this method offers an exemplar for developing high-throughput diagnostic tools for other plant taxa where morpho-anatomical identification is insufficient. Future validation using DNA extracted from solid wood will require systematic assessment, particularly as DNA yield and integrity are expected to be substantially lower than from needles or seeds. Wood-derived DNA often exhibits extensive degradation and co-extracted PCR inhibitors; furthermore, processing methods—such as drying from green to kiln-dried—introduce significant variability. Future research should quantify detection performance across these material types, specifically measuring amplification success rates to evaluate whether the assay maintains required sensitivity under these constraints.

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Appendix - Supplemental tables

Table S1. MIQE checklist compliance.

Item to check	Importance	Checklist
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	Materials and Methods
Number within each group	E	Table 2
Assay carried out by core lab or investigator's lab?	D	NA
Acknowledgement of authors' contributions	D	NA
SAMPLE		
Description	E	Materials and Methods
Volume/mass of sample processed	D	Materials and Methods
Microdissection or macrodissection	E	Materials and Methods
Processing procedure	E	Materials and Methods
If frozen - how and how quickly?	E	Materials and Methods
If fixed - with what, how quickly?	E	NA
Sample storage conditions and duration (especially for FFPE samples)	E	Materials and Methods
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	E	Materials and Methods
Name of kit and details of any modifications	E	Materials and Methods
Source of additional reagents used	D	NA
Details of DNase or RNAse treatment	E	NA
Contamination assessment (DNA or RNA)	E	NA
Nucleic acid quantification	E	Materials and Methods
Instrument and method	E	Materials and Methods
Purity (A260/A280)	D	Table 2
Yield	D	Table 2
RNA integrity method/instrument	E	NA
RIN/RQI or Cq of 3' and 5' transcripts	E	NA
Electrophoresis traces	D	NA
Inhibition testing (Cq dilutions, spike or other)	E	ND
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	NA
Amount of RNA and reaction volume	E	NA
Priming oligonucleotide (if using GSP) and concentration	E	NA
Reverse transcriptase and concentration	E	NA
Temperature and time	E	NA
Manufacturer of reagents and catalogue numbers	D	NA
Cqs with and without RT	D*	NA
Storage conditions of cDNA	D	NA

NA = Not applicable; ND = Not determined; E = Essential; D = Desired

* Testing for DNA contamination with a no-reverse-transcription assay is essential when RNA is first extracted. After DNA-free status has been verified, the control is recommended but no longer required.

** Reporting probe sequences is strongly encouraged to support transparency and reproducibility, but it cannot be required because some commercial predesigned assays do not provide this information.

Table S1. MIQE checklist compliance (*continued*).

Item to check	Importance	Checklist
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay	E	Results
Sequence accession number	E	Materials and Methods
Location of amplicon	D	Materials and Methods
Amplicon length	E	Table 4
In silico specificity screen (BLAST, etc.)	E	Results
Pseudogenes, retropseudogenes or other homologs?	D	NA
Sequence alignment	D	NA
Secondary structure analysis of amplicon	D	Results, Figure 4
Location of each primer by exon or intron (if applicable)	E	NA
What splice variants are targeted?	E	NA
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	Table 3
RTPrimerDB Identification Number	D	NA
Probe sequences	D**	NA
Location and identity of any modifications	E	Table 3
Manufacturer of oligonucleotides	D	Materials and Methods
Purification method	D	NA
qPCR PROTOCOL		
Complete reaction conditions	E	Table 5
Reaction volume and amount of cDNA/DNA	E	Table 5
Primer, (probe), Mg++ and dNTP concentrations	E	Table 5
Polymerase identity and concentration	E	Table 5
Buffer/kit identity and manufacturer	E	NA
Exact chemical constitution of the buffer	D	NA
Additives (SYBR Green I, DMSO, etc.)	E	Materials and Methods
Manufacturer of plates/tubes and catalog number	D	NA
Complete thermocycling parameters	E	Materials and Methods
Reaction setup (manual/robotic)	D	NA
Manufacturer of qPCR instrument	E	Materials and Methods
qPCR VALIDATION		
Evidence of optimization (from gradients)	D	Figure 2
Specificity (gel, sequence, melt, or digest)	E	Figure 2, 4, 5, 7
For SYBR Green I, Cq of the NTC	E	Figure 2, 4, 5, 7, Tables S2, S3, S4
Standard curves with slope and y-intercept	E	Figure 3, 6
PCR efficiency calculated from slope	E	Figure 3, 6
Confidence interval for PCR efficiency or standard error	D	ND
r2 of standard curve	E	Figure 3, 6
Linear dynamic range	E	Figure 3, 6
Cq variation at lower limit	E	Table S5
Confidence intervals throughout range	D	ND
Evidence for limit of detection	E	Table S5
If multiplex, efficiency and LOD of each assay	E	Results

NA = Not applicable; ND = Not determined; E = Essential; D = Desired

* Testing for DNA contamination with a no-reverse-transcription assay is essential when RNA is first extracted. After DNA-free status has been verified, the control is recommended but no longer required.

** Reporting probe sequences is strongly encouraged to support transparency and reproducibility, but it cannot be required because some commercial predesigned assays do not provide this information.

Table S1. MIQE checklist compliance (*continued*).

Item to check	Importance	Checklist
DATA ANALYSIS		
qPCR analysis program (source, version)	E	Materials and Methods
Cq method determination	E	Materials and Methods
Outlier identification and disposition	E	ND
Results of NTCs	E	Figure 2, 4, 5, 7
Justification of number and choice of reference genes	E	NA
Description of normalization method	E	NA
Number and concordance of biological replicates	D	Table 6, Table S4
Number and stage (RT or qPCR) of technical replicates	E	Tables S2 and S3
Repeatability (intra-assay variation)	E	Results
Reproducibility (inter-assay variation, %CV)	D	ND
Power analysis	D	ND
Statistical methods for result significance	E	ND
Software (source, version)	E	Materials and Methods
Cq or raw data submission using RDML	D	NA

NA = Not applicable; ND = Not determined; E = Essential; D = Desired

* Testing for DNA contamination with a no-reverse-transcription assay is essential when RNA is first extracted. After DNA-free status has been verified, the control is recommended but no longer required.

** Reporting probe sequences is strongly encouraged to support transparency and reproducibility, but it cannot be required because some commercial predesigned assays do not provide this information.

Table S2. Cq values and melting temperatures (T_m) for technical replicates of the species-specific simplex assay.

Simplex assay 1000nM					
Fluor	Type	Identifier	Replicate #	Threshold Cycle (Cq)	Melting temperatures (Tm)
SYBR	Pos Ctrl	LLP	1	16.15	77.50
SYBR	Pos Ctrl	LLP	2	16.36	78.00
SYBR	Pos Ctrl	LLP	3	16.22	78.00
SYBR	Pos Ctrl	SLP	1	17.88	69.50
SYBR	Pos Ctrl	SLP	2	17.82	69.50
SYBR	Pos Ctrl	SLP	3	18.06	69.50
SYBR	NTC	WTR	1	N/A	N/A
SYBR	NTC	WTR	2	N/A	N/A

Table S3. Cq values and melting temperatures (T_m) for technical replicates of the species-specific multiplex assay.

Multiplex assay					
Fluor	Type	Identifier	Replicate #	Threshold cycle (Cq)	Melting temperatures (T_m)
SYBR	Pos Ctrl	LLP	1	21.25	77.50
SYBR	Pos Ctrl	LLP	2	20.97	77.50
SYBR	Pos Ctrl	LLP	3	21.48	78.00
SYBR	Pos Ctrl	SLP	1	28.56	69.00
SYBR	Pos Ctrl	SLP	2	28.13	69.00
SYBR	Pos Ctrl	SLP	3	28.03	69.00
SYBR	Neg Ctrl	LBP	1	N/A	N/A
SYBR	Neg Ctrl	LBP	2	N/A	N/A
SYBR	Neg Ctrl	LBP	3	N/A	N/A
SYBR	Neg Ctrl	SFP	1	N/A	N/A
SYBR	Neg Ctrl	SFP	2	N/A	N/A
SYBR	Neg Ctrl	SFP	3	N/A	N/A
SYBR	NTC	WTR	1	N/A	N/A
SYBR	NTC	WTR	2	N/A	N/A
SYBR	NTC	WTR	3	N/A	N/A

Table S4. Cq values and the melting temperatures for the biological replicates in screening performance.

P. elliottii (SLP)				*P. palustris (LLP)*				*P. echinata (SFP)*				*P. taeda (LBP)*			
Location	Samples	Cq	T_m	Location	Samples	Cq	T_m	Location	Samples	Cq	T_m	Location	Samples	Cq	T_m
Florida	1	20.74	69.5	Florida	1	19.52	78.0	Arkansas	1	N/A	N/A	Florida	1	N/A	N/A
Florida	2	22.70	69.0	Florida	2	22.78	78.0	Arkansas	2	N/A	N/A	Florida	2	N/A	N/A
Florida	3	22.92	69.0	Florida	3	18.55	77.5	Arkansas	3	N/A	N/A	Florida	3	N/A	N/A
Florida	4	22.77	69.5	Florida	4	20.34	78.0	Arkansas	4	N/A	N/A	Florida	4	N/A	N/A
Florida	5	22.48	69.5	Florida	5	22.37	78.0	Arkansas	5	N/A	N/A	Florida	5	N/A	N/A
Florida	6	21.69	69.5	Florida	6	23.41	78.0	Arkansas	6	N/A	N/A	Florida	6	N/A	N/A
Florida	7	30.65	69.0	Florida	7	23.59	78.0	Mississippi	7	N/A	N/A	Florida	7	N/A	N/A
Florida	8	21.71	69.0	Florida	8	23.08	78.0	Mississippi	8	N/A	N/A	Florida	8	N/A	N/A
Georgia	9	24.23	69.0	Florida	9	20.97	78.0	Mississippi	9	N/A	N/A	Florida	9	N/A	N/A
Georgia	10	24.40	69.5	Florida	10	21.23	78.0	Mississippi	10	N/A	N/A	Florida	10	N/A	N/A
Georgia	11	23.07	69.5	Georgia	11	24.86	78.0	Mississippi	11	N/A	N/A	Georgia	11	N/A	N/A
Georgia	12	23.82	69.5	Georgia	12	23.71	78.0	Missouri	12	N/A	N/A	Georgia	12	N/A	N/A
Georgia	13	32.86	69.0	Georgia	13	23.75	78.0	Missouri	13	N/A	N/A	Georgia	13	N/A	N/A
Georgia	14	23.35	69.0	Georgia	14	24.52	78.0	Missouri	14	N/A	N/A	Georgia	14	N/A	N/A
Georgia	15	24.76	69.0	Georgia	15	24.37	78.0	Missouri	15	N/A	N/A	Georgia	15	N/A	N/A
Georgia	16	31.78	69.5	Georgia	16	24.78	78.0	Missouri	16	N/A	N/A	Georgia	16	N/A	N/A
Georgia	17	32.33	69.5	Mississippi	17	22.00	78.0	Missouri	17	N/A	N/A	Georgia	17	N/A	N/A
Georgia	18	31.71	69.5	Mississippi	18	22.99	78.0	Missouri	18	N/A	N/A	Georgia	18	N/A	N/A
Mississippi	19	19.78	69.5	Louisiana	19	16.78	78.0	Missouri	19	N/A	N/A	Georgia	19	N/A	N/A
Mississippi	20	22.53	69.0	Louisiana	20	16.85	78.0	Missouri	20	N/A	N/A	Georgia	20	N/A	N/A
Mississippi	21	24.66	69.0	Louisiana	21	17.46	78.0								
Mississippi	22	23.41	69.0	Louisiana	22	16.03	78.0								
Mean		24.93	69.25	Mean		21.54	77.98								
Standard deviation (SD)		4.05	0.26	Standard deviation (SD)		1.83	0.11								

Table S5. Cq values for technical replicates in the limit of detection (LOD) analysis.

Replication	*P. elliottii* (SLP)	*P. palustris* (LLP)
1	32.50	33.05
2	32.46	32.85
3	33.57	33.46
4	33.82	35.16
5	33.75	32.96
6	32.73	36.52
7	34.14	34.95
8	33.83	33.89
9	34.13	34.40
10	33.71	33.82
11	33.85	33.67
12	33.81	33.90
13	33.01	33.89
14	33.09	35.20
15	33.17	33.18
16	32.23	33.14
17	32.22	33.61
18	32.79	34.01
19	33.11	34.68
20	32.75	34.12
21	32.47	34.09
Mean	33.20	34.03
Standard deviation	0.64	0.90