High-Resolution Melting Analysis: a new molecular approach for the early detection of *Diplodia pinea* in Austrian pine

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Running title: High-Resolution Melting Analysis for the early detection of *Diplodia pinea*

Abstract

The differentiation of *Diplodia pinea* from closely related species, such as *Diplodia scrobiculata* and *D. seriata*, and its detection in plant tissue, represented a critical issue for a long time. Molecular screening tools have recently been developed to address this topic. In this study we applied one of the most sensitive and rapid diagnostic screening method so far developed, called High-Resolution Melting Analysis (HRMA), to detect *D. pinea* in Austrian pine (*Pinus nigra*). HRMA exploits differences in the melting behaviour of PCR products to rapidly identify DNA sequence variants without the need for cumbersome post-PCR methods. We developed a HRMA method to detect specific fungal sequences in the mitochondrial SSU rDNA gene. The reliability of this technique was firstly assessed on DNA extracted from pure cultures of *D. pinea* and closely related species. Amplicon differences were screened by HRMA and the results confirmed by direct DNA sequencing. Subsequently, HRMA was tested on DNA from symptomatic and symptomless pine shoots, and the presence of the fungus was also confirmed by both conventional and molecular quantitative approaches. The HRMA allowed the distinction of *D. pinea* from closely related species, showing specific melting profiles for the each pathogen. This new molecular technique, here tested in a plant-fungus pathosystem for the first time, was very reliable in both symptomatic and symptomless shoots. HRMA is therefore a highly effective and accurate technique that permits the rapid screening of pathogens in the host.

Key words: Botryosphaeriaceae, *Diplodia*, High-resolution melting analysis, mt SSU rDNA, *Pinus nigra*

Introduction

Diplodia pinea (Desm.) J. Kickx is a pathogenic fungus with a world-wide distribution that causes significant mortality and serious economic losses in pine plantations and nurseries (Stanosz *et al.* 2007; Swart & Wingfield 1991). The fungus is particularly injurious to Austrian pine (*Pinus nigra*

Arn.) (Blodgett and Bonello 2003). In southern European forests it has a role in the decline of Austrian pine, which occurs mostly under conditions of adverse climatic or environmental factors (Maresi *et al.* 2002, 2007).

The most common symptoms of the disease are crown wilt, tip blight, cankers and a blue stain of the timber, while on seedlings the fungus causes damping off and collar rot (Swart & Wingfield 1991). *D. pinea* can persist in asymptomatic pine tissue in latent phase for a long time, until unfavourable environmental factors predispose trees to fungal infections (Flowers *et al.* 2003; Stanosz *et al.* 2007). Studies examining the environmental conditions in nurseries and pine plantations have shown that stress factors, such as drought, enhance the susceptibility of the host, leading to an increase in the incidence and the severity of disease (Paoletti *et al.* 2001; Stanosz *et al.* 2001).

The taxonomy of *D. pinea* and species closely related to it had undergone significant revision in the past. Initially the fungus was most commonly named *Sphaeropsis sapinea,* which included four morphotypes (A, B, C and I) distinguished by their conidial and colony morphology, and by their aggressiveness to their host plants (de Wet *et al.* 2000, 2002; Smith & Stanosz 1995).

Some of these morphotypes were later elevated to species, with the A and C morphotypes together becoming *D. pinea*, and the B morphotype *D. scrobiculata.* The I morphotype became *Diplodia seriata* (= "*Botryosphaeria*" *obtusa*) (Burgess *et al*. 2001). *D. scrobiculata,* unlike *D. pinea*, is a relatively non-aggressive species that attacks the shoots of various conifers, including Austrian pine, causing canker lesions that are always however significantly smaller than those caused by *D. pinea* (Blodgett & Bonello 2003). *D. seriata* is a common pathogen of many fruit crops, on which it causes cankers and dieback (Phillips 2002; Slippers and Wingfield 2007; Úrbez-Torres & Gubler 2009). It has been identified in more than 30 host plants, including a number of pine species (Punithalingam & Walker 1973).

It is difficult to detect and distinguish these closely related species in the Botryosphaeriaceae. However, molecular tools, together with morphological techniques, have enabled the phylogenetic

relationships of these species to be revised (Crous *et al.* 2006; de Wet *et al.* 2003; Phillips *et al.* 2008). The molecular approach most commonly used to distinguish species within the Botryosphaeriaceae has been by comparing the DNA sequences from the internal transcribed spacer (ITS) regions of ribosomal RNA (rDNA) (Zhou & Stanosz 2001a). However, it is generally accepted now that in order to separate closely related or cryptic species it is necessary to compare sequences from multiple loci (Slippers *et al.* 2004; Phillips *et al.* 2008).

The choice of the target DNA region is an important consideration when designing molecular markers to detect and differentiate fungi (Khadempour *et al.* 2010). The ITS rDNA region is commonly employed for this purpose (White *et al.* 1990). However, in the case of *D. pinea* it is very difficult to use this region as a marker, since its sequence shows a very high level of homology to those of other closely related species such as *D. scrobiculata* and *D. seriata*. These species differ much more in the sequences of the mitochondrial small subunit ribosome gene (mt SSU rDNA) (Smith & Stanosz 2006; Zhou & Stanosz 2001b).

Sensitive techniques are needed so that a pathogen can be detected at an early stage of infection (Luchi *et al.* 2005a). For *D. pinea,* real-time quantitative PCR (qPCR) has been used for this purpose (Maresi *et al.* 2007). Recently, HRMA has also been very successful in clinical studies (Erali *et al.* 2008; Mancini *et al.* 2010). This method is very sensitive in genotype scanning and rapidly identifies DNA sequence variants, without needing cumbersome post-PCR techniques (Wittwer *et al.* 2003). HRMA exploits the fact that PCR products with different sequences have distinct melting profiles. The signal change that signifies the transition from a double to a single strand is generated by fluorescent dyes that actively intercalate double stranded DNA with very low interference with the PCR reaction. The high-resolution melting profile of a PCR product produces a specific, sequence-related curve that rapidly distinguishes between sequences even when they differ by only one nucleotide. Thus, the HRMA of PCR products can detect single-point mutations and distinguish single-nucleotide polymorphisms (SNPs) (Wu *et al.* 2009). HRMA being very

sensitive could conceivably serve to identify phytopathogenic fungi *in planta* even when they are present in very small amounts.

The aim of the present study was to devise a sensitive and specific HRMA assay for the molecular detection of *D. pinea* in Austrian pine shoots. To assess the reliability of the assay, the following samples were tested: i) DNA extracted from a pure culture of *D. pinea* and some other phylogenetically closely related fungi; ii) a DNA mixture of pine DNA and the DNA of each fungus at a known concentration, and iii) DNA from pine samples, to find out whether fungal DNA could be detected *in planta* in symptomatic and asymptomatic pine shoots.

Materials and methods

Pine sample preparation, isolation and real-time qPCR

The occurrence of *D. pinea* on Austrian pine trees was investigated in two pinewoods in Tuscany, Italy. The first wood was located at Monte Morello (Florence; N 43° 51' 02.34''; altitude 580 m a.s.l.) where the fungus causes serious damage. This wood has previously been used by our team for other studies on the *D. pinea* – Austrian pine pathosystem (Feci *et al.* 2002). The second area was a young plantation of Austrian pine at Monte Senario (Florence; N 43° 53' 15.88"; E 11° 19' 55.09"; altitude 650m.a.s.l.) where tip blight is only rarely found.

Thirty pine samples were collected: 20 from the first site (10 symptomatic and 10 asymptomatic shoots); and 10 from the second site (5 asymptomatic pine shoots and 5 samples of fascicles of healthy needles). One sample was collected per tree.

All samples were surface-sterilised with 70% ethanol, 1.05% NaOCl and Tween 80 (following Stanosz *et al.* 2001) and rinsed three times in sterile water. Each shoot and each needle fascicle was divided into two parts; the first part was used for isolation on PDA, and the second for DNA extraction (Luchi *et al.* 2005b).

Fungal cultures from the plated fragments were identified on the basis of cultural and conidial characteristics. Colonies typical of *D. pinea* were incubated on a pine-needle agar medium to produce pycnidia *in vitro*, so that the identity of the colonies could be confirmed (Luchi *et al.* 2007). *D. pinea* in pine tissue was also quantified by qPCR as described by Luchi *et al.* (2005b). These DNA samples were subsequently used for the HRMA assay.

Fungal isolates

Eighteen *Diplodia* isolates were collected from different countries and hosts (Table 1), including some species closely related to *D. pinea,* such as *D. scrobiculata* and *D. seriata*. Out-group fungal species (from the Xylariaceae family) were used as negative controls (Table 1). All strains were stored at 4°C in the culture collection of the Department of Agricultural Biotechnology, University of Florence, Italy (DiBA). Each strain was grown on 300PT cellophane discs (Celsa, Varese, Italy) in 90 mm Petri dishes containing potato dextrose agar (PDA; Difco, Detroit, MI, USA) and maintained in the dark at 20-25°C. After 7 days, mycelium was scraped from the cellophane surface and stored in 1.5 ml microfuge tubes at -20°C. The mycelium was ground in liquid nitrogen with a mortar and pestle. DNA was then extracted using the EZNA™ Plant DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions.

HRMA to detect* D. pinea *in pine

Primer design

The mt SSU rRNA gene was chosen as the target gene because the sequence of this gene distinguishes between *D. pinea* and the species most closely related to it, *D. scrobiculata* and *D. seriata*, and because it is suitable for primer design (Smith & Stanosz 2006).

The mt SSU rRNA gene sequences of *D. pinea* (accession n. AF051636), *D. scrobiculata* (accession n. AF051637), and *D. seriata* (accession n. DQ023299) were obtained from GenBank

(www.ncbi.nlm.nih.gov) and aligned using a multiple sequence alignment program (T-Coffee software, Swiss Institute of Bioinformatics) to locate any differences between the species.

Primers for HRMA were selected using Primer3 software (Rozen & Skaletsky 2000). The oligonucleotide sequences were: a) forward primer *DpHRM_F* 5'-GCTACCTTGGAGTAAGGGACA-3'. and b) reverse primer *DpHRM_R* 5'-TTTCCATCTAGGAGCGAAAAT-3'. The PCR product was 89 bp long.

HRMA conditions

HRMA was performed on RotorGene™ 6000 (Corbett Research, Sydney, Australia) in a final volume of 20 µl containing: 1X PCR buffer (Applied Biosystems, Monza, Italy), 2.5 mM $MgCl_2$ (Applied Biosystems), 375 nM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 3.2 mM of each dNTP (Applied Biosystems), 1X EvaGreen™ dye (Invitrogen, Carlsbad, CA), 0.6U Ampli-*Taq* polymerase (Applied Biosystems), 30 ng fungal DNA (listed in Table 1). Each sample was processed in duplicate.

PCR was carried out in 9700 GeneAmp PCR System (Applied Biosystems) thermal cycler, starting with an initial denaturation step at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 57°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 15 min. The samples were denatured with an initial hold of 5 min at 95°C and 1 min at 40°C and a melting profile from 70-80°C with a ramping degree of 0.05°C. HRMA was performed using the software provided by the Rotor-Gene™ 6000.

Sequencing HRMA products

To verify the HRMA results, sequencing analysis was performed on all the samples of *Diplodia* strains (Table 1). After HRMA, the samples were purified with the PCR purification Kit (Qiagen, Italy) and then submitted to cycle sequencing using 2 µl of BigDye Terminator Ready Reaction Mix (Applied Biosystems, Italy) and 0.8 µM of the same primer that was used for HRMA in a final

volume of 10 µl. After purification with a DyeEx 2.0 Spin kit (Qiagen, Italy), the samples were analysed with an ABI Prism 310 Genetic Analyser (Applied Biosystems, Italy).

HRMA assay sensitivity in host DNA

To assess the reliability and sensitivity of HRMA in detecting small amounts of *D. pinea* DNA against a background of host tissue DNA, serial dilutions of fungal DNA from *D. pinea* isolate 411 were mixed with DNA extracted from pine needles that had previously been assessed negative to *Diplodia* by qPCR. Fungal DNA was diluted in pine tree DNA at different concentrations (50%, 25%, 10%, 5%, and 1%) and tested. The same test was also performed on mixed samples of DNA from each of the other *Diplodia* species (*D. scrobiculata* or *D. seriata*) and pine DNA.

In addition, we examined whether the co-occurrence of *D. pinea*, *D. scrobiculata* and *D. seriata* in a sample in equimolar proportion (15 ng /µl each), together with pine DNA, affected the DNA melting profile of these species.

HRMA validation in pine samples

The capacity of HRMA to detect *D. pinea* directly in pine tissue was assessed. Total DNA concentration from symptomatic and asymptomatic shoots and pine needles was adjusted to 1ng/µl. Each sample was run in duplicate following the HRMA protocol described above. Reference DNA extracted from pure cultures were also included (*D. pinea,* 411; *D. scrobiculata,* 124; *D. seriata,* N144) (Table 1).

Results

Specificity of primers and HRMA

The designed primers were group-specific, detecting all the species in the Botryosphaeriaceae studied (Table 1), and none of the outgroup species (Table 1).

HRMA distinguished *D. pinea* from the other species studied, showing a distinctive melting curve for this species (Fig 1). The *D. pinea* melting profile formed a single cluster showing specific curve shifts on the x-axis and distinct from the melting curves of the closely related *D. scrobiculata* and *D. seriata*.

The melting temperature (T_m) of each species was indicated by the HRMA protocol, with *D. pinea* having the lowest T_m (75.96°C), *D. scrobiculata* the highest (76.24 °C) and *D. seriata* an intermediate T_m (76.06 °C) (Fig 1).

These melting profiles differed significantly from the melting curves of other species of Botryosphaeriaceae such as *D. corticola* and *Neofusicoccum luteum*.

The HRMA results were confirmed by direct sequencing. In all cases the sequencing showed the expected sequence variations between *D. pinea* (TGC) on the one hand and *D. scrobiculata* (ATG) and *D. seriata* on the other (AGC) (Fig 1-2).

Interestingly the mixed DNA sample of *D. pinea*, *D. scrobiculata* and *D. seriata*, together with pine DNA, had a melting profile intermediate between the melting profiles generated by each single strain (Fig 3).

HRMA reliability in host DNA

The reliability of HRMA in detecting fungal DNA in pine tissue was tested in mixed samples containing pine DNA plus the DNA of one of each fungal species. The amount of fungal DNA tested ranged from 1% to 50% of total DNA. HRMA detected the DNA of *D. pinea*, *D. scrobiculata* and *D. seriata* when these were separately mixed with pine DNA (Fig 4a,b). The

melting curve of each sample overlapped with the curve of the fungal reference DNA of the species in that sample.

The melting curves of the mixed samples of *D. pinea* DNA and pine DNA in varying proportions are shown in Figure 4c. A dilution of 1% was the detection threshold of this assay, corresponding to 3 ng of fungal DNA against the background of host DNA. The detection threshold of *D. scrobiculata* and *D. seriata* was the same as that for *D. pinea*.

As expected, co-occurring pine tree DNA had no effect on the fungal melting curves.

Detection of* D. pinea *in pine samples after isolation and real-time qPCR

Symptomatic pine shoots showed tip-blight, necrotic tissue and conidia of the pathogen, but symptomless shoots and needles did not show any fungal fruitbodies or necrotic tissue. Isolation on growth media confirmed the occurrence of *D. pinea* in all symptomatic pine shoots (10 out of 10) (Table 2). In the asymptomatic pine shoots, 6 out of 15 shoots yielded *D. pinea* after isolation, while the asymptomatic pine needles did not show any *D. pinea* mycelium (Table 2). *D. scrobiculata* and *D. seriata* mycelium was not found in any of the shoots or needles.

By qPCR, *D. pinea* DNA was detected in all symptomatic pine shoots (in amounts ranging from $7.0x10^4$ to $2.4x10^5$ pg/µl), and in 14 out of 15 symptomless pine shoots (Table 2). The amount of fungal DNA in symptomless shoots also differed between the sampling sites. At the Monte Morello site *D. pinea* levels of DNA ranged from 1.3 to 3.2 x 10^3 pg/µl, while at Monte Senario they were much lower (from 1fg to 0.05 pg/µl). At Monte Senario all needle samples were negative by real-time qPCR (negative control).

Diplodia pinea *detected by HRMA in pine shoots*

DNA samples after being subjected to qPCR were examined by HRMA to detect *D. pinea*. Results produced by qPCR and HRMA were the same for all symptomatic samples (Table 2).

HRMA also confirmed that *D. pinea* occurred in all asymptomatic pine shoots from Monte Morello, and in none of the shoot and needle samples from Monte Senario (Table 2).

Both symptomatic and asymptomatic pine shoots unambiguously indicated the melting curve of *D. pinea*. This curve overlapped with that of the *D. pinea* DNA used as reference in the assay (Fig 5).

Discussion

The present study reports the application of HRMA to detect fungal DNA in host plant tissue. The method is here applied to the *D. pinea* – Austrian pine pathosystem, which has been used in the past to study the effect of *D. pinea* on pine (Bonello & Blodgett 2003). The HRMA technique is here used to investigate a plant-fungus interaction for the first time.

Formerly it was difficult to distinguish *D. pinea* from *D. scrobiculata* and *D. seriata* because most loci of these species have a high degree of homology, as have many other cryptic species in the Botryosphaeriaceae. However these species are kept apart by their distinct life histories: *D. scrobiculata* has a more limited host range and distribution than *D. pinea* (Bihon *et al.* 2010), and *D. seriata* is rare in most pines (de Wet *et al.* 2003; Flowers *et al.* 2003), though it is common in many other hosts such as grapevine (Urbez-Torres & Gubler 2009).

When HRMA was used on fungal DNA from pure culture, it distinguished *D. pinea* from its cryptic sister species *D. scrobiculata* and *D. seriata*, from other related species such as *D. corticola,* and from phylogenetically more distant species such as *N. luteum* (Phillips *et al.* 2008). This distinguishing power of HRMA incidentally shows the utility of the mtSSU rDNA locus in discriminating between species in the Botryosphaeriaceae. This locus has been shown to vary considerably in its nucleotides between *D. pinea*, *D. scrobiculata* and *D. seriata*, and has been used to design specific primer-pairs for each of these species (Smith & Stanosz 2006). With HRMA, however, these species were distinguished using only a single primer-pair, considerably reducing the cost of oligonucleotide synthesis and PCR validation.

In the present study HRMA also detected and identified *D. pinea* in Austrian pine, illustrating the usefulness of this tool to detect plant pathogens at an early stage. HRMA permits the rapid scanning of isolates or plant material in one tube and in one step, and with a single machine, without the need for sequencing. The efficiency of this technique is mainly due to the chemistry employed in detection, which uses third-generation fluorescent dsDNA dyes such as Eva Green, with low "toxicity" to the PCR amplification products, which makes it highly effective in measuring fluorescent signals (Wittwer *et al.* 2003). These characteristics give greater melt sensitivity and higher resolution melting profiles, making it possible to specifically detect small nucleotide variations between different samples within the same target region. Until now, HRMA has mostly been applied in clinical studies of gene mutations in cancer cells (Simi *et al.* 2008; Jones *et al.* 2008), and also to detect viruses (Varillas *et al.* 2010) and bacteria causing lethal food-borne infections in humans (Wang *et al.* 2010). In plants HRMA has been experimentally used to detect novel alleles in barley (Hofinger *et al.* 2009), to analyse olive germplasm (Muleo *et al.* 2009), to differentiate grapevine and olive cultivars (Mackay *et al.* 2008), and to identify bilberry samples (Jaakola *et al.* 2010).

In the present study, *D. scrobiculata* was not detected in sampled pine shoots by either isolation or HRMA. This is consistent with other studies in which the more aggressive *D. pinea* was found to be more common than the less aggressive *D. scrobiculata* (Palmer 1991; Smith & Stanosz 2006). Little is known however about what happens when *D. pinea* and *D. scrobiculata* co-occur in the same sample (Smith & Stanosz 2006). Though the occurrence of more than one fungus is only rarely reported, a mixed sample of *D. pinea*, *D. scrobiculata* and *D. seriata* DNA in this study produced a melting profile which is intermediate between the profiles generated by the single strains.

HRMA was also highly sensitive in detecting *D. pinea* in both symptomatic and asymptomatic plant material as compared with isolation and qPCR. In dilution tests to determine the sensitivity of HRMA in host tissue, amounts as little as 3 ng of *D. pinea* were detected. However, in symptomless

pine shoots HRMA detected *D. pinea* only when the fungal DNA concentration was higher than 3 pg/µl. When fungal DNA concentrations in symptomless pine shoots were lower (1 fg to 0.05 pg/µl) *D. pinea* was not detected by this method. In symptomless pine tissue the sensitivity of HRMA was comparable to that of conventional PCR, for which Smith & Stanosz (2006) reported a detection threshold of 1 pg/µl. Variation in the amount of fungal DNA in symptomless pine tissue may be related to variations in the levels of latent infections of *D. pinea*, due to discontinuous colonization of hyphal aggregates on the shoots (Flowers *et al.* 2006).

A combination of qPCR and HRMA has been proposed as a low-cost and rapid method to test for *D. pinea* when the number of samples is very large (Rouleau *et al.* 2009). Such a combination may also be the best way to detect *D. pinea* DNA, ensuring both specificity and accuracy. When a quantitative determination is not required, HRMA alone detects the fungus and identifies it. The HRMA technique meets all the conditions required for early detection of *D. pinea* in the pine host. It is a rapid, close-tube, highly efficient and low-cost post-PCR approach that permits a rapid screening of the pathogen before any symptoms appear in the tree. For this reason it could also be used to evaluate the effectiveness of treatments against *D. pinea* and other plant pathogens, and as a tool to examine planting stock or seeds for quarantine purposes, since the commercial shipment of infected plant material from nurseries is believed to be a major cause of the world-wide spread of the *D. pinea.*

Acknowledgements

The authors acknowledge the following collectors for kindly providing pure cultures of fungal isolates: P. Bonello (Department of Plant Pathology, The Ohio State University, USA); M. Jaspers (Faculty of Agriculture and Life Sciences, Lincoln University, New Zealand); J. Luque (Departament de Protecció Vegetal, Institut de Recerca i Tecnologia Agroalimentàries, Spain); L. Mugnai (Department of Agricultural Biotechnology, University of Florence, Italy); S. Savocchia (School of Agricultural and Wine Sciences, Charles Sturt University, Australia); G.R. Stanosz

(Department of Plant Pathology, University of Wisconsin-Madison, USA); J.R. Úrbez-Torres (Plant Pathology Department, University of California, USA); M.J. Wingfield (Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa). This work was supported by grant to Nicola Luchi from the Consorzio Interuniversitario Biotecnologie (CIB, Italy) "Borse CIB di Ricerca e Formazione Avanzata - Caratterizzazione di Specie Fungine Fitopatogene della Vite e del Pino mediante High-Resolution Melting Analysis".

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Figure Captions

Fig 1 - High-resolution melting analysis (HRMA) of mtSSU rRNA from DNA of the *Diplodia* species used in the study. HRMA profiles of the *Diplodia* species (centre). The Tm values of each species are given in the table (bottom), from the highest to the lowest. Electropherograms of sequencing analysis (on the right), showing differences in the nucleotides between *Diplodia pinea* and the two most closely related species, *D. scrobiculata* and *D. seriata*.

Fig 2 - Alignment of the nucleotide sequences of mtSSU rRNA from the *Diplodia* species used in the study. The forward (*DpHRM*_F) and reverse (*DpHRM*_R) primer annealing sites are indicated on top. Nucleotide differences between *Diplodia pinea*, *D. scrobiculata* and *D.seriata* are shown in bold inside the box. Fungal species: *D. pinea* (Dpin), *D. pinea* 'C' morphotype (DpinC), *D. scrobiculata* (Dscr), *D. seriata* (Dser), and *D. corticola* (Dcor). Strains are shown in parentheses.

⋆ = no differences between species

Fig 3 - Selected melting profiles showing the differences between the DNA from single reference species and a mixed sample ("Mixed DNA") containing, in the same tube, DNA from *Diplodia pinea*, *D. scrobiculata* and *D. seriata* mixed together with pine DNA in equimolar proportion.

Fig 4 - Sensitivity of high-resolution melting analysis (HRMA) in detecting *Diplodia pinea*, *D. scrobiculata* and *D. seriata* DNA when these species are mixed singly with pine DNA at concentrations of 50, 25, 10, 5 and 1%. All fungal melting curves were normalized on pine DNA (line parallel to the x-axis). **a.** High resolution melting curves of *D. pinea*, *D. scrobiculata* and *D. seriata* diluted in pine DNA; **b.** Difference plots for the data represent in **a**.; **c.** Particular of a difference plot for *D. pinea,* showing the highest (50%) and the lowest (1%) fungal DNA concentration in the total DNA.

Fig 5 - High-resolution melting curve showing *Diplodia pinea* DNA in both symptomatic (brown line) and asymptomatic (green line) Austrian pine shoots. Melting curves of DNA from pure-culture strains of *D. pinea* (overlap with pine shoots), *D. scrobiculata* and *D. seriata* are also shown.

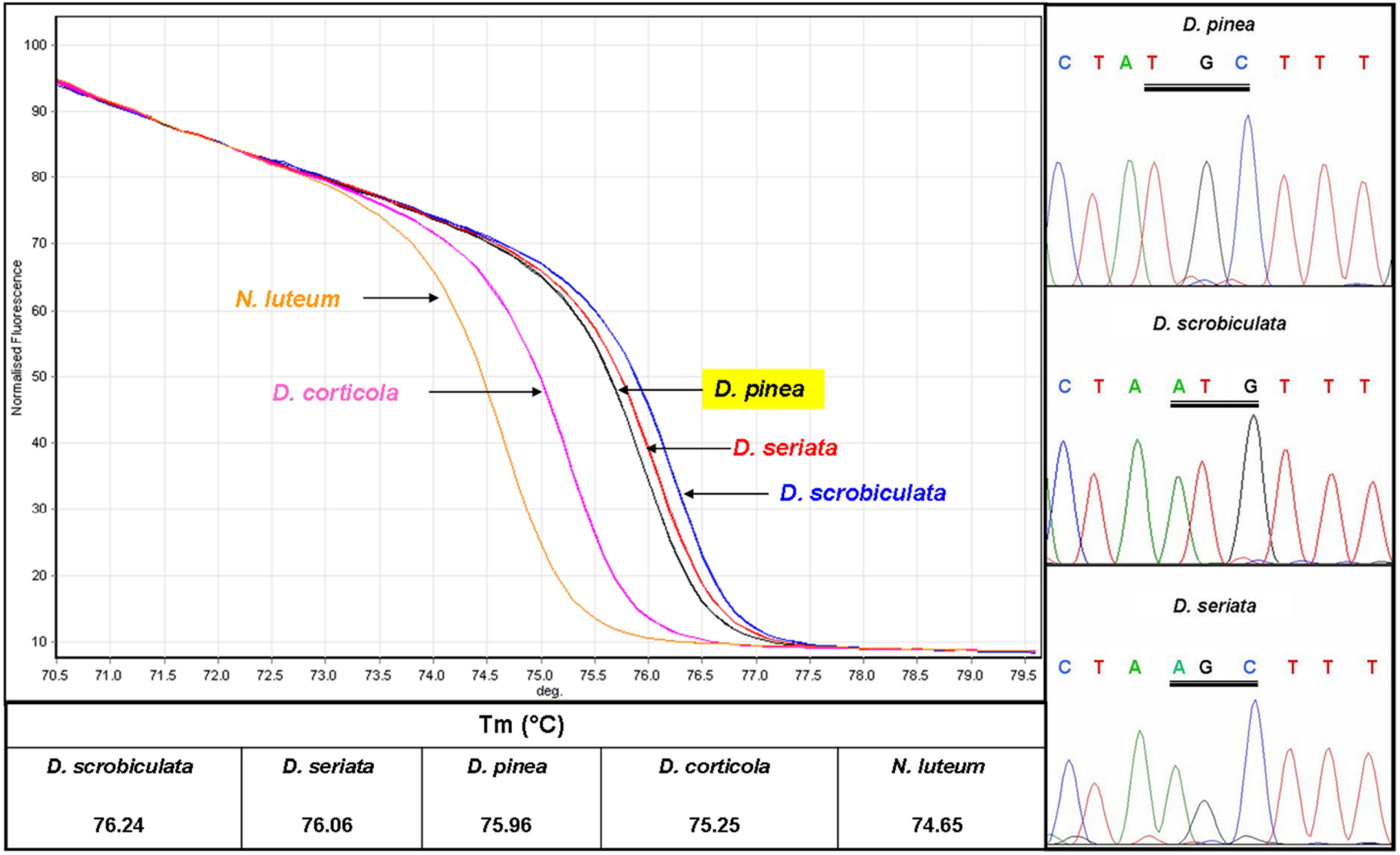
Table 1 - Fungal species studied.

Species	Isolate	Host	Geographic origin	Collector
Diplodia pinea	S79	*Pinus nigra*	Florence, Italy	N. Luchi
D. pinea	S91	*P. pinea*	Castelfusano, Rome, Italy	N. Luchi
D. pinea	411	*P. resinosa*	Clearwater Co., Minnesota, USA	M.A. Palmer
D. pinea	128	*P. resinosa*	Grant Co., Wisconsin, USA	M.A. Palmer
D. pinea	3AP	*P. nigra*	Columbus, Ohio, USA	P. Bonello
D. pinea ('C' morphotype)	CMW4879	*P. patula*	Habinsaran, South Africa	M.J. Wingfield
D. scrobiculata	215	*P. resinosa*	Douglas Co., Wisconsin, USA	M.A. Palmer
D. scrobiculata	124	*P. banksiana*	Jackson Co., Wisconsin, USA	M.A. Palmer
D. scrobiculata	CMW189	*P. resinosa*	USA	M.A. Palmer
D. scrobiculata	CMW4898	*P. greggii*	Mexico	M.J. Wingfield
D. seriata	UCD352	*Vitis vinifera*	Monterey County, California, USA	J.R. Úrbez-Torres
D. seriata	UCD244	*V. vinifera*	Madera County, California, USA	J.R. Úrbez-Torres
D. seriata	B121	*V. vinifera*	Blenheim, New Zealand	M. Jaspers
D. seriata	N144	*V. vinifera*	Nelson, New Zealand	M. Jaspers
D. seriata	WP-J10	*V. vinifera*	Eden Valley, South Australia, Australia	S. Savocchia
D. seriata	WP-DO4	*V. vinifera*	Murrambateman, New South Wales, Australia	S. Savocchia
D. corticola	333	*Quercus suber*	Los Barrios, Spain	A. Trapero
D. corticola	155	*Q. ilex*	Sant Feliu de Buixalleu, Spain	J. Luque
Neofusicoccum luteum	PVFi-FL	*V. vinifera*	Montepaldi, Florence, Italy	L. Mugnai
Outgroup species				
Biscogniauxia marginata	CBS 279.61[a]	unknown	Morgantown, West Virginia, USA	H.L. Barnett
B. mediterranea	CBS 101016[a]	*Q. robur*	Bremmert-Kootwijk, The Netherlands	L. Bos
Hypoxylon fragiforme	CBS 801.72[a]	*Fagus sylvatica*	Baarn, Groeneveld, The Netherlands	-
Kretzschmaria deusta	CBS 163.93[a]	*Tilia petiolaris*	Hamburg, Germany	R. Schröer

[a] strains from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

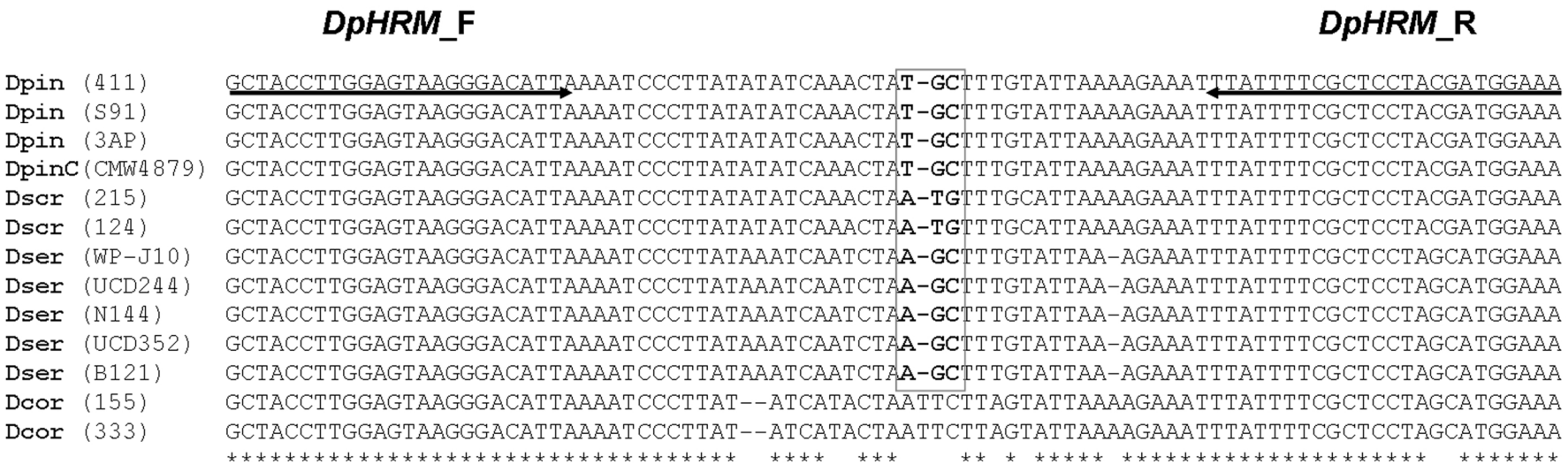
Table 2 - Detection of *D. pinea* on Austrian pine samples after isolation, real-time quantitative PCR (qPCR) and high-resolution melting analysis (HRMA). The number of total samples tested is shown in parentheses.

Collection Site	**Pine samples**	**N°. of samples with *D. pinea***		
		Isolation	**qPCR**	**HRMA**
Monte Morello	Symptomatic shoots (10)	10	10	10
	Asymptomatic shoots (10)	6	10	10
Monte Senario	Asymptomatic shoots (5)	0	4	0
	Asymptomatic needles (5)	0	0	0



Tm (°C)				
D. scrobiculata	*D. seriata*	*D. pinea*	*D. corticola*	*N. luteum*
76.24	76.06	75.96	75.25	74.65

Fig 1 - High-resolution melting analysis (HRMA) of mtSSU rRNA from DNA of the *Diplodia* species used in the study. HRMA profiles of the *Diplodia* species (centre). The Tm values of each species are given in the table (bottom), from the highest to the lowest. Electropherograms of sequencing analysis (on the right), showing differences in the nucleotides between *Diplodia pinea* and the two most closely related species, *D. scrobiculata* and *D. seriata*.



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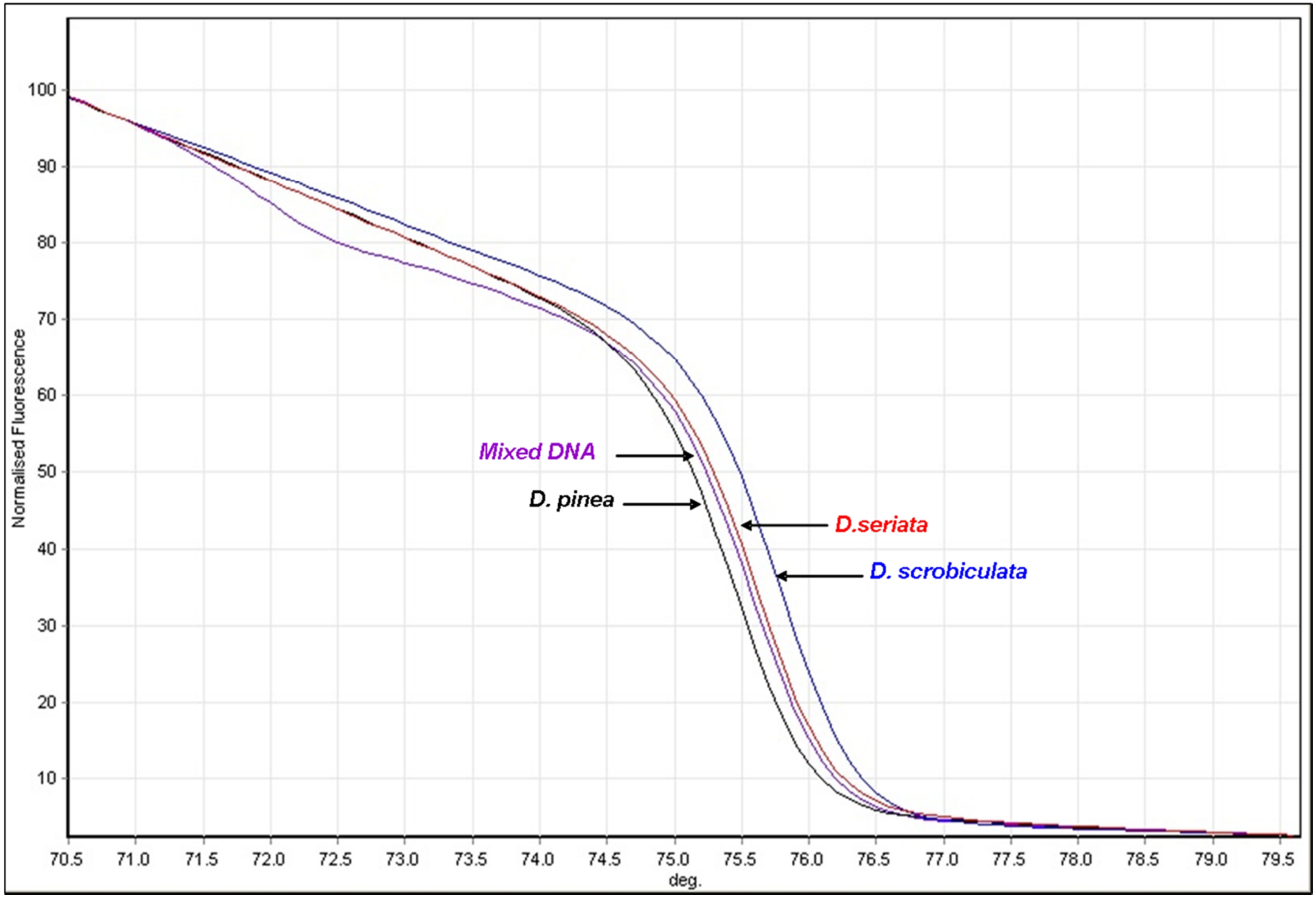

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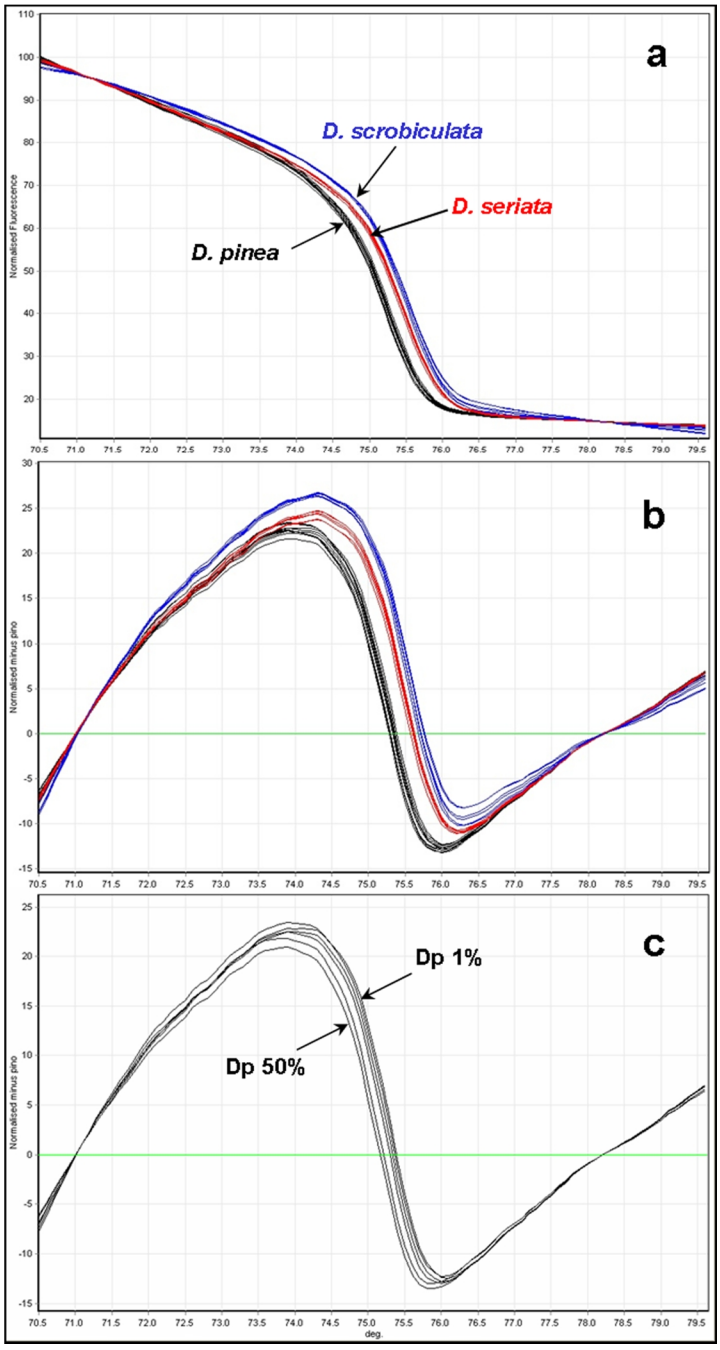

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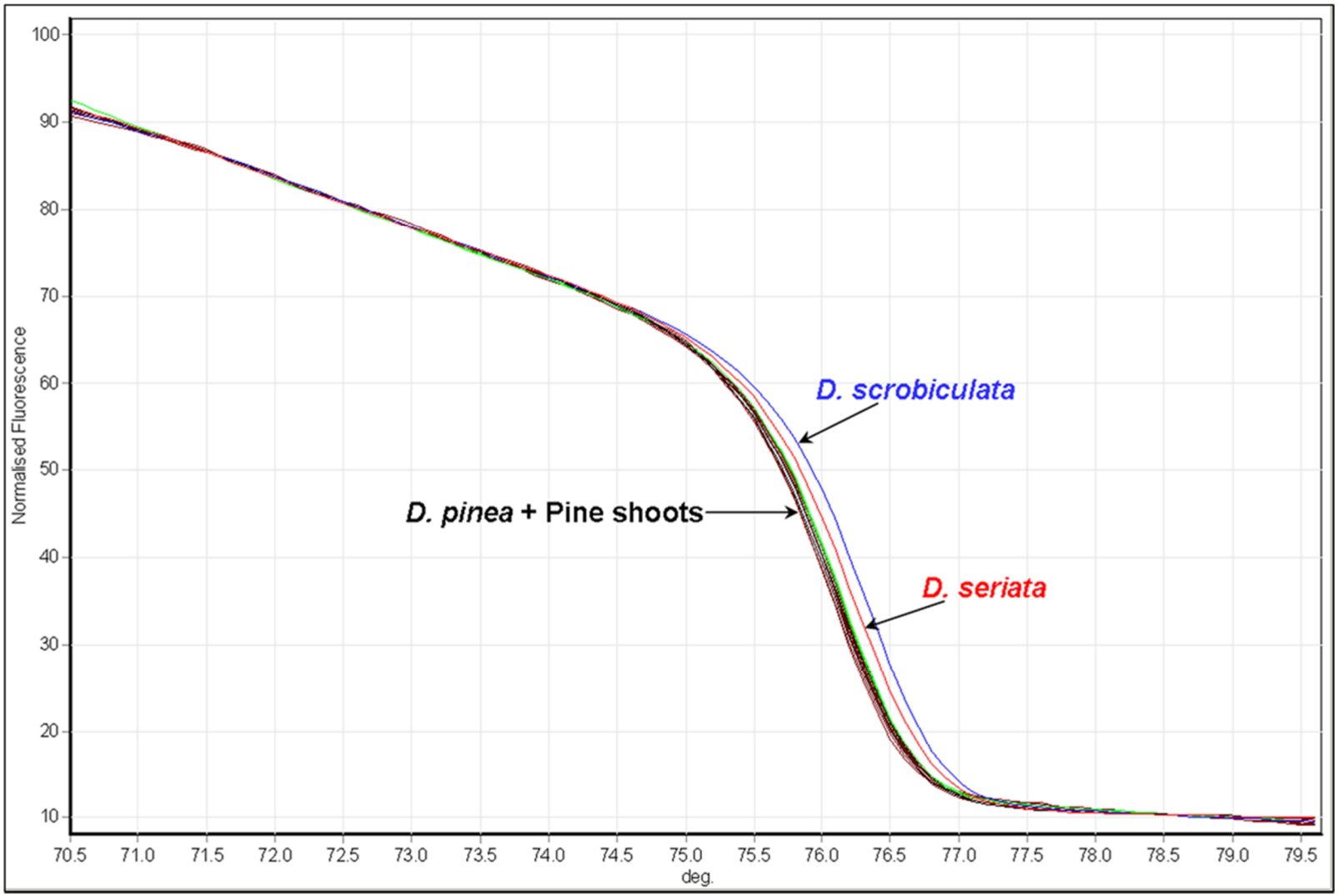

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