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TREE ROOT IDENTIFICATION USING DNA FINGERPRINTING

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TREE DIAGNOSTICS

1. Introduction

The most current and recognised technology available for tree root identification is microscopy. Root sections are viewed for comparison against a root anatomy atlas to enable root identification to genus level, excluding members of the Rosaceae family, and the *Salix* and *Populus* genera (Biddle 1998; Cutler *et al.* 1987). This technology is routinely applied when seeking to prove tree root damage to buildings and infrastructure for litigation or insurance claims. The increasing volume and expense associated with claims warrants investigation into improved technologies, offering precise identification at least to species level, and ultimately between individuals. Such techniques are based on the DNA of tree species rather than wood anatomy.

In the last twenty-five years, molecular genetic techniques have become increasingly sophisticated and accessible to offer DNA – based procedures for species identification, or in other words, genotypic rather than phenotypic analysis (Tingay & del Tufo 1993). Genetic comparison or identification becomes possible now that DNA can be accessed, copied and amplified to study its chemical structure and biological function.

In horticulture, DNA based technology has been widely applied in such areas as plant breeding, cultivar selection and plant conservation. Specifically, molecular tools have focused on improving our understanding of molecular evolution, phylogenic inference of species, genetic analysis and manipulation, genetic mapping, genetic breeding and phylogenetic relationships (Lee & Henry 2001; Morell *et al.* 1995). While there has been considerable research into genetic relatedness, improvement and cultivar identification, there has been little investigation or need for general plant identification. More recently, rhizosphere analysis and soil alteration has prompted interest in identification of below ground parts of plants to genus and often species level (Brunner *et al.* 2001; Linder *et al.* 2000; Bobowski *et al.* 1999; Jackson *et al.* 1999). There may be scope to apply these principles and technologies to achieve accurate, reliable tree root identification.

1.1. Tree Root Damage

Blame for damage to built structures and infrastructure is commonly attributed to trees even in the presence of equally probable other causes, and even in the absence of trees (Cutler 1995; Moore 1991). Incidents of alleged root damage continue to increase yet investigation and proof of damage relies on often inconclusive technology.

1.1.2 Causes of Tree Root Damage

Trees have the potential to directly or indirectly cause physical damage to structures in the following ways:

- The larger woody roots move structures when roots expand with age to physically lift or move structures (Wagar & Barker 1983)

- The uptake of soil moisture in dryer months or periods of drought may cause soil subsidence, leading to the flexure of footings in buildings and walls (O'Malley & Cameron 2001; Biddle 1998)
- Tree roots may proliferate near a water and / or nutrient source. Where pipes are leaking from poor installation or age, for example, tree roots will rapidly initiate at the moisture source. Root expansion or growth follows, leading to further pressure and opening of cracks or joins (Stal & Rolf 1998)
- Removal of trees can indirectly lead to damage as there is no longer the means for excess water uptake during wetter months (Driscoll 1983)

1.2 Tree Root Identification

1.2.1 Root Characteristics

The above ground characteristics of the tree (leaf shape and size, flowers, bark, form etc.) usually enable conclusive identification. Conversely, the root system offers few distinctive features. The characteristics of the root system where variation is systematic are: root diameter, colour, growth potential and texture (Fitter 1996). Few of the root system characteristics, however, offer sufficient variation to distinguish between individuals, species or even genera. Cutler *et al.* (1987) developed profiles of the internal anatomy of roots to enable identification to the level of genus. While this methodology has shortcomings, such as an inability to distinguish between species like Salix and Populus, it is most useful in verifying or disproving tree root damage claims at the genus level.

1.2.2 Existing Technology

Accurate identification of tree roots in the event of damage to buildings or infrastructure is essential as litigation over alleged damage is increasing (Hannah & Yau 1990). It must first be determined whether roots encountered are active and conducting water and that their presence is the cause of drying and shrinking of clays or damage to pipes. The existing identification technique requires microscopy and comparison with species presented in a root anatomy manual (Cutler *et al.* 1987).

1.2.2 Need for precise identification

McCombie (1995) presents the incidence of tree root damage to buildings in terms of cases by distance to the building. Whilst 75 % of cases revealed that the tree stood within 10 metres of the building, 25% stood beyond this distance. Cases ranged from 10 metres to a staggering 34 metres. It would be reasonable to assume the further the distance from the tree, the greater the need for definitive tree identification, particularly as there may be more than one tree of the same or different species.

Pohls (2001) survey of tree root blockage to pipes, reported that of the 117 sites visited, 298 trees were considered as blockage candidates standing 0.7 metres to 35 metres from the site of intrusion or damage to pipes. While there is no basis for alleging the trees ‘caused’ the damage, it would seem important to consider all species on site rather than the nearest tree or the biggest. Interestingly, melaleucas and eucalypts were found more frequently nearest to the blockage site, in around 40% of confirmed blockages.

1.2.4 Molecular techniques for plant identification

In horticulture and arboriculture, DNA marker technology offers considerable advantages over morphological comparison or microscopy for species classification, identification and plant breeding (Morell *et al.* 1995). The presence of DNA in the cells of all plants is the key to accurate, expeditious, reliable and inexpensive identification by providing a means for analysing a large number of genetic characters for comparison between species (Morell *et al.* 1995; Jorgensen & Cluster 1989).

1.2.5 Genetic information in plant cells

The genome represents the sum total of DNA in an organism's chromosomes. Organisms such as trees have more than 10,000 genes per genome (Knox *et al.* 2001). The difference between individual trees lies in the variation in the composition of their DNA genomes. Distantly related genera such as *Eucalyptus* and *Picea* will have conspicuous and multiple differences in their genes, whereas the genomes of *Eucalyptus globulus* (Tasmanian Blue Gum) and *Eucalyptus bicostata* (Southern Blue Gum) will have few differences. Differences between individuals of the same species will be fewer again. Nevertheless, there are sufficient differences to distinguish between individuals (Tobin & Morel 1997). Advances in molecular technology make it possible to sequence the genome enabling plant scientists to compare plants and determine their relationships (phylogeny).

1.2.6 DNA extraction and preparation

The first step in the process of plant identification or genetic analysis is extraction of DNA. The appropriate method of extraction is determined by the plant tissue type (roots, leaves, cambium, bark), the state of the tissue (fresh, frozen, dry), and the type of plant genotyping or profiling required (Henry 2001). Analysis of fresh tissue is preferred as there is little opportunity for fungal growth in tissues or degradation of DNA leading to possible contamination or poor DNA yields.

The extraction process involves three principal events. The first step is to break down the cell wall (lysation). This is achieved initially by grinding the sample to the consistency of a fine powder in a mortar and pestle with the assistance of liquid nitrogen. This is then completed with the addition of detergents and salt solutions to lyse the lipid cell membrane. The second step, occurring more than once during the procedure is disruption of the nuclear membrane by centrifugation. This action leads to the break down, not only of cell walls, but cell membranes and nuclear membranes. Finally, and critically, the DNA must be shielded from enzymes that potentially shear the DNA, by incubation at 60°C+ to denature the enzymes. The addition of other counteracting enzymes such as ribonuclease effectively removes contaminating RNA. Whilst extraction protocols vary widely (de la Cruz *et al.* 1997) they involve many stages before suspending the DNA in buffer and storing in an eppendorf tube (-20°C) for later use.

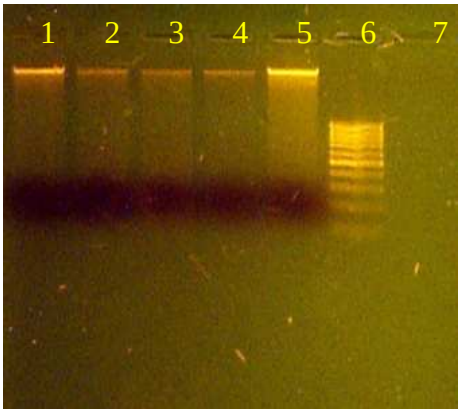

Figure 1.1 DNA Extraction by CTAB method showing DNA band: Lane 1 – *Corymbia henryi* (leaves); Lane 2 – *Corymbia henryi* (roots); Lane 3 *Corymbia maculata* 4 (leaves); Lane 4 – *Corymbia maculata* 3 (leaves); Lane 5 – *Corymbia maculata* (roots); Lane 6 – Molecular Ladder; Lane 7 – blank.

After extraction, a process of DNA replication and amplification is utilised to enable the DNA to be visualised. This is typically accomplished with the Polymerase Chain Reaction or PCR. Here, a segment of DNA is specified and extracted for replication, before being visualised as distinct bands by electrophoresis (Figure 1.1)(Innis & Gelfand 1990). The technology revolutionised access to new knowledge for investigation across scientific fields including medicine, agriculture and archaeology (De Cresce & Lifschitz 1993; Krawetz 1989). As there are many different DNA extraction protocols, so too are there many PCR protocols, often requiring optimisation for a positive result (Innis & Gelfand 1990). In some instances it may be necessary to purify the DNA sample (Steane *et al.* 1999; Tyson *et al.* 1998). This is common with a number of tree species as amplification may be hindered or masked by the large quantities of polyphenols and polysaccharides present in the cells (Henry 2001; de la Cruz *et al.* 1997). Problems may also be encountered with residual ribonucleosides interfering with primer attachment during thermal cycling (Porebski *et al.* 1997). Steenkamp *et al.* (1994) noted difficulties extracting DNA from plants indicating that methods for isolation may not be readily transferable from one species to another. DNA isolation for many tree species requires specialised techniques to obtain the requisite DNA yield and concentration. Bobowski *et al.* (1999) reported lower yields and higher incidence of contaminants when extracting DNA from root samples resulting in difficulties with amplification. Linder *et al.* (2000) found increasing difficulty in extracting amplifiable DNA as the growing season progressed. Towards the end of the growing season all attempts at extraction failed. All samples required purification to prevent interference with PCR amplifications. This presents obvious impediments to developing routine isolation of DNA from widely varying common tree species for profiling and identification.

1.3 DNA Profiling

1.3.1 The Polymerase Chain Reaction

Once the DNA has been successfully extracted, there are numerous techniques available for DNA profiling. This has become possible with PCR based technology. The Polymerase Chain Reaction (PCR) method enables researchers to make multiple copies of DNA segments between two defined nucleotide sequences using a DNA polymerase from the bacterium *Thermus aquaticus* (Tobin & Morel 1997). Synthetic polynucleotide sequences are created to

correspond precisely with sequences at each end of the segment to be copied. The polynucleotide sequences are comprised of about 20 nucleotides and are referred to as oligonucleotides. The oligonucleotides serve as primers for the DNA Polymerase. The process of multiple copying and amplification is achieved quickly and simply (Arcade *et al.* 2000). The PCR process is, therefore, the enzymatic amplification of a single region or fragment of DNA to produce multiple copies (Wolfe & Liston 1998; Morell *et al.* 1995). Firstly, the target DNA fragment is denatured by heat treatment to separate the double stranded region of the genome. A drop in temperature allows the oligonucleotide primers to anneal to complimentary base pair sequences. This results in a copy from each strand from the polymerase (enzyme) activity (Hoelzel & Dover 1991). Repeated cycles of denaturing, annealing and (where appropriate) extension result in exponential copies of the original DNA template that can be viewed following electrophoresis (Morell *et al.* 1995). Each application can then be optimised to obtain the requisite quality and quantity of amplified DNA. These include adjustments to the concentration of the template DNA, the primers, deoxynucleotide triphosphates (dNTPs) and magnesium chloride.

The amplification reveals the sequence variation between species to enable phylogenetic and systematic analysis. Existing PCR based technology used for tree identification and phylogenetic studies that may be suited to tree root identification includes:

1. Sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (Steane *et al.* 1999)
2. Restriction Fragment Length Polymorphisms (RFLPs) (Bobowski *et al.* 1999; Brunner *et al.* 2001)
3. Amplified Fragment Length Polymorphism (Regner *et al.* 2001; Arcade *et al.* 2000)
4. Microsatellites (Hormanza 2001; Rossetto *et al.* 1998)
5. Inter-Simple Sequence Repeat (ISSR) (Arcade *et al.* 2000)
6. Random amplified polymorphic DNA (RAPD) (Hormanza 2001)

All of the above techniques were explored and trialled as potentially capable of tree root identification across common tree species. Successful identification was achieved using Inter-Simple Sequence Repeat markers (ISSR).

1.3.2 Inter-Simple Sequence Repeat markers (ISSR)

This DNA marker system is a progression of the microsatellite[1] technology enabling analysis and identification of the various genomes without prior knowledge of the loci characters (Godwin *et al.* 2000). Di and tri-nucleotide repeat characters are targeted in ISSRs using primers to locate highly variable microsatellite sequences and known base pairs at the 3' end of the DNA template. The single primer locates two microsatellite regions along the DNA strand enabling the PCR reaction to amplify the band between the two microsatellites (the

[1] Microsatellite loci are regions of DNA with high rates of mutation making analysis of closely related species feasible (Longato & Bonfante 1997). Microsatellites consist of tandem repeats of short DNA sequences of high variation distributed within the genome (Byrne *et al.* 1996). The advantages of microsatellites over other PCR applications are the presence of more readily detectable polymorphisms than are available with other PCR based techniques such as RAPDs and RFLPs. Microsatellite markers are therefore well suited to identification of species particularly where there are low levels of diversity (Byrne *et al.* 1996).

locus). Typically there are numerous microsatellite pairs generating many bands per reaction. Recent research, demonstrates the potential of ISSR technology to apply across a range of plant taxa as well as to distinguish between individuals of the same species.

Nagaoka & Ogihara (1997) suggest the high variability produced in banding profiles is superior to RFLP and RAPD reactions. Further, the technology offers repeatability and sufficient variability to investigate genetic variation among closely related individuals (Nagaoka & Ogihara 1997; Fang & Roose 1997). Wolfe *et al.* (1998) developed primers to work across a range of species and taxa. Therefore, it may be conceivable to modify known primers to broaden or narrow the host range whether across species or taxa, or between individuals (Zietkiewicz *et al.* 1994; Leroy *et al.* 2000). The versatility of these ISSR primers indicates potential for identification of a wide range of tree DNA samples with a small number of primers. The majority of studies have successfully amplified plant DNA from the northern hemisphere (Aranzana *et al.* 2001; Arnau *et al.* 2001; Regner *et al.* 2001; Arcade *et al.* 2000; Esselman *et al.* 1999; Wolfe *et al.* 1998; Fang & Roose 1997) with scope for application to plants of the southern hemisphere (Godwin *et al.* 2000) and Australian natives (Van der Nest *et al.* 2000).

The ISSR molecular marker system has compared favourably to other available PCR and non-PCR based technologies in one or more variables: cost, speed, detectable polymorphisms, cross-transferability of primers and reproducibility (Arnau *et al.* 2001; Regner *et al.* 2001; Leroy *et al.* 2000; Wolfe *et al.* 1998). Zietkiewicz *et al.* (1994) predicts the greater potential for ISSR markers to include taxonomic and phylogenetic comparison as well as gene mapping across organisms.

Irrespective of the method of molecular analysis, the objective is to determine or prove similarity or difference with samples. The closer the genetic relatedness, the more challenging differentiation becomes. Lee & Henry (2001) claim it is impossible to prove two DNA samples are the same without comparing the entire genomes. This is clearly not commercially viable, but there must be an accepted probability of demonstrated identity. New techniques such as ISSR potentially exhibit high levels of discrimination between samples with scope even to distinguish between clones (Robinson *et al.* 1997).

Developments in molecular techniques (PCR based technologies) have radically improved phylogenetic analysis. Deficiencies in tree root identification by microscopy warrant investigation of more accurate identification technology between species and individuals. Molecular marking demonstrates excellent potential for tree identification based on successes in cultivar identification in agriculture, viticulture and horticulture (Hormanza 2001; Regner *et al.* 2001; Fang & Roose 1997; Thomas & Scott 1993)
The objective of this study was to investigate the potential to identify tree roots using molecular techniques.

2. CASE STUDY – TREE IDENTIFICATION TRIAL

The aim of this study was to investigate the use of ISSR technology to conclusively match unidentified tree roots to one of four possible trees.

2.1 Materials and methods

For this study, an unidentified root sample was presented as one of four possible (containerised) trees:

1. *Gleditsia tricanthos* 'Shademaster'
2. *Gleditsia tricanthos* 'Limegold'
3. *Quercus robur*
4. *Platanus* 'Digitata'

All trees were grafted with the exception of the *Quercus robur*. The root stock of the two *Gleditsia tricanthos* samples were of the same species. DNA was extracted from the unknown root sample and cambial tissue from all four containerised trees with the intention of matching the DNA of the roots with the corresponding cambial tissue. For the grafted samples, cambium was removed from below the graft union of the containerised tree to ensure a precise DNA match.

2.1.1. DNA Extraction

Plant tissue for each sample was ground to a powder using liquid nitrogen and, where necessary, a few grains of autoclaved sand were added to assist with tissue disruption. Genomic DNA was extracted from leaves, roots and stems (cambium) using DNeasy Plant Mini Kit (Qiagen) in accordance with the manufacturers instructions. A minor modification to the protocols was made to assist with removal of large amounts of precipitates through additional centrifugation. The presence of DNA was confirmed by gel electrophoresis (1.2% agarose and 1% ethidium bromide).

2.1.2 ISSR Analysis

PCR reactions of 20µL reaction mixture contained 0.0001 – 1.0µL of DNA, 10µL of HotStarTaq Master Mix (Qiagen), 9.0 – 9.999 µL of sterilised H_20 and 1µL (0.02-0.05 µM) of primer (Table 5.2). Di and tri-nucleotide anchored primers were used for amplifications[2]. The optimum annealing temperature of 36 - 44°C was determined for each primer.

Where adequate PCR product bands resulted, the successful primers were then selected for amplification of the four cambium samples. Three primers were selected for their potential to produce consistent bands. The DNA was then amplified from the 4 cambium samples as before using primers NHHB61, 64 and 65.

[2] **Primers used in ISSR case studies.**

Primer Code	Repeat Sequence	Citation
NHAW60	**CT**	**Maunder *et al.* 1999; Wolfe *et al.* 1998**
NHHB61-64	**CA**	**Culley & Wolfe, 2001; Arcade *et al.* 2000; Esselman *et al.* 1999; Wolfe *et al.* 1998**

All samples were then loaded alongside each other onto a 2% agarose stained with 1% ethidium bromide gel and run for 1 hour at 80 amps. The gel was then viewed under UV light conditions, photographed using the *Kodak Digital Science™ DC120 Zoom Camera* and analysed with the *Kodak Digital Science 1D™* Image Analysis Software.

2.2 Results and discussion

DNA from the root and cambium samples was successfully isolated (Figure 2.1).

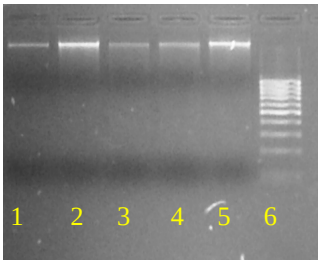

Figure 2.1. Single DNA bands from Lane 1 - Unknown root sample, Lane 2 – *Gleditsia tricanthos* 'Shademaster,' Lane 3 - *Gleditsia tricanthos* 'Limegold,' Lane 4 – Quercus robur, Lane 5 – Platanus 'Digitata', Lane 6 – Molecular Ladder.

The ISSR-PCR reaction of the root sample produced good banding with all primers attempted (Figure 2.2). Amplification of microsatellites by PCR can often be complicated by the appearance of 'stutter' or 'shadow' bands (Armour *et al.* 1998, in Goldstein). These are highly pronounced PCR artefacts often associated with long repeats of dinucleotide sequences. Slippage can occur during the amplification process however this can be eliminated by anchoring the repeat sequences at the 3' or 5' end of the microsatellite region with one, two or three nucleotides (Wolfe *et al.* 1998). Any non-reproducible, very feint or shadow bands should be eliminated or not scored (Fang & Roose 1997). Polymorphism can then be scored visually as either absent or present (Arnau *et al.* 2001).

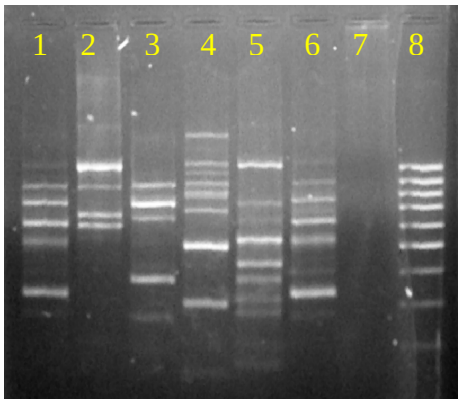

Figure 2.2. Unidentified root DNA (1µL) with primers: Lane 1 - NHHB61, Lane 2 - NHHB62, Lane 3 - NHHB63, Lane 4 - NHHB64, Lane 5 - NHHB65, Lane 6 – Diluted (unknown) root DNA (0.5µL) with primer NHHB61, Lane 7 – Negative control sample (0µL DNA) and Lane 8 – Molecular Ladder.

Primers NHHB61, 64 and 65 were selected for their superior banding in terms of quantity and clarity, and used in the ISSR-PCR reactions with the four cambium samples. Once characterised alongside the root sample with primer NHHB61 (Figure 2.3), it was immediately apparent the root sample was distinctly different from both the *Quercus robur* and the *Platanus* 'Digitata' samples. It therefore suggested the DNA was from one of the Gleditsia samples (Figure 2.3).

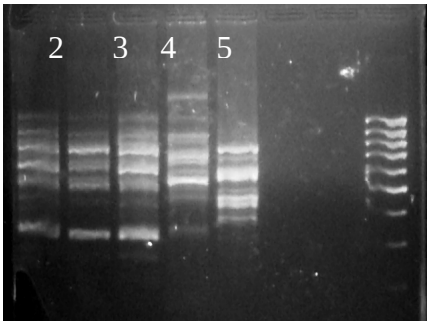

Figure 2.3. Primer NHHB61 amplified with: Lane 1 – unknown root sample; Lane 2 – *Gleditsia tricanthos* 'Shademaster;' Lane 3 – *Gleditsia tricanthos* 'Limegold;' Lane 4 – *Quercus robur*; Lane 5 *Platanus* 'Digitata'; Lane 6 – Negative Control; Lane 7 – Blank; Lane 8 – Molecular Ladder.

The root DNA and Gleditsia samples were then amplified using primers NHHB64 & 65 to ascertain if either primer could show variation between the individuals. Whilst the samples are referred to by their scion, (i.e. Limegold and Shademaster) it should be noted that the root stock were both *Gleditsia tricanthos* seedlings. Of the primers attempted, each indicated some variation between the two samples, but only one primer could conclusively separate the two individuals – Primer NHHB65. Figure 2.4 shows an absent band in Lane 2 as highlighted by the arrow. The clear bands depicted in Lanes 1 and 3 could not be considered stutter bands (Armour *et al.* 1999). The absence of the band in sample 2 would reasonably infer divergence at the primer site (Wolfe & Liston, 1998) or the ISSR product in Lanes 1 and 3 have been amplified from the same DNA (*Gleditsia tricanthos* 'Limegold.').

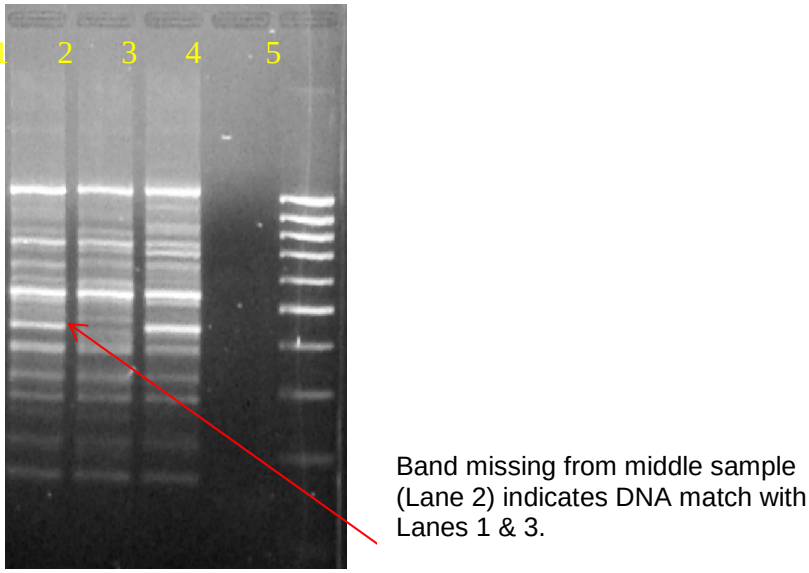

Figure 2.4 Primer NHHB65 amplified with: Lane 1 – unknown root sample; Lane 2 – *Gleditsia tricanthos* 'Shademaster' root DNA; Lane 3 – *Gleditsia tricanthos* 'Limegold' root DNA; Lane 4 – negative control sample, Lane 6 – molecular ladder

Using ISSR primer NHHB65 the unknown root sample was determined as *Gleditsia tricanthos* 'Limegold' and this was confirmed as the species. The technique proved sensitive enough to separate individuals with the same rootstock.

2.3 Conclusion

Previous studies have demonstrated that amplification is consistently repeatable irrespective of the plant part or the method of DNA extraction (Arnau *et al.* 2001). Further, target primers can be applied across a range of taxa (Zietkiewicz *et al.* 1994). To date, the vast majority of the studies in ISSR have focussed on mapping of the genome or genetic diversity of a single or closely related species (Hess *et al.* 2000; Arcade *et al.* 2000; Culley & Wolfe 2001; Deshpande *et al.* 2001).

This study has shown ISSR primers can be used to distinguish between genera and individuals. The study findings offer great promise for identification of invasive tree roots where the genetic analysis extends across taxa. Further, the methodology employed is relatively cheap quick efficient and accurate. Future investigation will increase familiarity with the DNA of tree species and their optimum ISSR primers. Improvement in DNA quality over time through new technology will do much to improve banding profiles and band numbers, although current quality is adequate.

ISSR technology coupled with professional, arboricultural site assessment and root treatment not only offers accurate root identification in tree root damage disputes, but also the elimination of false or erroneous claims. The technology as it stands is unique, reliable and has the potential to offer a valuable commercial service.

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APPENDIX 1

DNA Sequence data for:

1. *Corymbia maculata*
2. *Corymbia maculata*
3. *Platanus x acerifolia*
4. *Platanus x acerifolia*
5. *Melia azedarach*
6. *Melia azedarach*
7. *Platanus* 'Digitata'
8. *Platanus* 'Digitata'
9. *Tristaniopsis laurina*