Determining the origin of macadamia kernels from market samples

Dr. Vasanthe Vithanage
CSIRO Plant Industry

Project Number: MC01002

MC01002

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the macadamia industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the Macadamia Industry.

ISBN 0 7341 1201 7

Published and distributed by:

Horticulture Australia Ltd

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50 Carrington Street
Sydney NSW 2000
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Final Report

Determining the origin of Macadamia Kernels from market samples

MC 01002
(June 2005)

Dr V. Vithanage and Dr A. Schmidt

CSIRO

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Chapter 1. Project details

1 **Project Details**

Project Title:	Determining the origin of Macadamia Kernels from market samples
HAL Project Number:	MC 01002

Project Leader:	DR V. Vithanage (Retired) and Dr C. A. McConchie CSIRO Plant Industry Queensland Bioscience Precinct 306 Carmody Rd St Lucia Qld 4067 Phone :07 3214 7789 Fax: 07 3214 2272 Email: cameron.mcconchie@csiro.au
Other Key Personnel:	Dr Adele Schmidt, and Kirrilee O'Connor

Purpose of the report:

The main objectives of the project were to investigate:

A. Establish DNA extraction procedures for raw, dry roasted and oil roasted kernel tissue of two known cultivars (e.g. A16 and Beaumont);

B. Develop a technique of identifying markers (STS, RAPD, RAF etc.) using the extracted DNA to be used in typing kernels;

C. Extend the marker system to cover all commercial cultivars both in Australia and overseas.

Funding sources:	HAL, AMS, CSIRO and collaborating Growers
Date of report:	June 2005

Chapter 2. Summaries

2.1 Media Summary

There were two parts to this project; one was to establish a quick and efficient method of extracting DNA from processed kernel; the other was to demonstrate that the extracted DNA could be genotyped using a robust fingerprinting method.

Using a commercial DNA extraction protocol (DNeasy Extraction Protocol, QIAGEN Pty Ltd) we have generated high quality DNA from raw and air-roasted kernel that can be readily and easily genotyped at microsatellite loci. Oil-roasted kernel samples generated significantly lower quantities of DNA, but it was still possible to genotype these samples at microsatellite loci.

Use of co-dominant microsatellite loci provides a range of advantages over previously employed dominant markers (Jarne & Lagoda 1996; Parker et al. 1998) and, when genotypes of processed kernel are compared to reference genotypes drawn from different growing regions, this will allow the identification of kernel source/origin.

2.2 Technical Summary

Current research has established a quick and efficient method of extracting DNA from raw and processed kernel, and generating DNA fingerprints from this DNA that can be used to identify kernel source/origin. Following investigation of a number of techniques, a combination of commercial DNA extraction procedure and genotyping at simple, single-locus microsatellite markers was identified as the most amenable to high-throughput analysis. The method works on raw, oil-roasted and air-roasted kernel.

2.3 Clarification of the methods used in typing kernels

Previous DNA marker research on macadamia has focused on isozyme analysis for cultivar identification (Vithanage and Winks 1992), and studies of genetic diversity using isozymes (Aradhya et al. 1997) or RAPD (random amplified polymorphic DNA) and STMS (sequence-tagged microsatellite site) markers (Vithanage et al. 1998). The recently devised RAF (randomly amplified DNA fingerprinting) marker system

(Waldron et al. 2002) readily generated sufficient numbers of reliable polymorphic markers for linkage mapping of macadamia (Peace et al. 2003), but these dominant markers are not ideal for studies that require identification of individual tree/kernel samples and/or populations (Jarne & Lagoda 1996; Parker et al. 1998).

Within the course of this study, early attempts to develop a reliable method for DNA extraction from raw and processed kernel (Chapter Three), failed to deliver DNA of sufficient quality for rapid high-throughput genotyping. A range of alternative DNA extraction protocols was employed to overcome this problem, including a pre-extraction treatment with solvent to remove excess oil. This protocol showed some initial success, but a range of technical and personnel issues prevented significant progress toward achievement of the project aims.

Following the appointment of a new research scientist in March 2005, an alternative commercial DNA extraction protocol was adopted. The method (DNeasy Extraction Protocol, QIAGEN Pty Ltd) allows for very high-throughput and produces high quality DNA from raw, oil-roasted and air-roasted kernel that is easily utilized in genotyping studies. Coupled with the development of a suite of new co-dominant microsatellite markers for use in a range of genetic studies in *Macadamia integrifolia* (and related species), the new DNA extraction procedure makes it possible to identify kernel source/origin from processed samples.

2.4 Recommendations for further improving the methods

The methodology developed here is designed to manually handle large numbers of samples. If the demand for this type of analysis is great and industry funding is forthcoming, then the methodology is readily amenable to automation at all stages:

- Using the current DNA extraction format, batches of up to 192 kernel samples can be processed in 1-2 hours.
- Amplification of microsatellite loci via PCR (polymerase chain reaction) can be performed in 1-2 hours for batches of up to 384 samples, with potential to amplify 2-5 loci in a single reaction.

- Analysis of PCR products can be fully automated through use of fluorescent labeling and laser detection systems.

The core aim of this project was to develop DNA extraction and genotyping methods that would, in future studies, allow the industry to determine the geographic origin of processed kernel. The successful identification of an efficient, reliable method for high-throughput DNA extraction and the development of a suite of co-dominant microsatellite markers, which are amenable to more meaningful analyses than previously employed dominant markers such as RAF (Jarne & Lagoda 1996; Parker et al. 1998) is critical. At present, there are 25 microsatellite markers available for genotyping of *Macadamia integrifolia* and related species and ongoing work within CSIRO Plant Industry should see this expanded to a suite of 100-200 markers over the next two years.

To make full use of the methodology and accurately determine the origin of kernel from processed samples, it will also be essential to identify reference genotypes of kernel from different geographic sources. This will require collection and genotyping of kernel from a variety of growing regions (both in Australia and overseas).

2.5 Proposed Publication Schedule

Article	Proposed Submission date	Proposed Journal
Methods of identifying the origin of macadamia kernels from market samples	December 2005	AMS Bulletin
Isolation and Characterization of Microsatellite Loci from *Macadamia integrifolia*	February 2005	Molecular Ecology

2.6 Acknowledgements

We are indebted to Darren Burton for acting as project champion for the AMS and providing invaluable assistance and advice throughout the project. We also thank the AMS Industry Development Officer, Kim Jones of the AMS R & D committee for valuable advice at various stages of the project. We also thank Cameron McConchie and Cameron Peace for encouragement and advice throughout the project. Dr. Bernard Carroll's (Dept. of Biochemistry, University of Qld) valuable advice on methodology is gratefully acknowledged. We also thank the macadamia growers, Greg Sumner (Chevallum, Qld), Peter and Nancy Zeck (Nambukka Heads, NSW), Geoff Royal (Woolgoolga, NSW), Frank Hathaway (Lismore, NSW) and Peter Stace (Jiggi, NSW) for their co-operation and access to orchards and Marie O'Sullivan, CSIRO Mathematical and Information Sciences, for assistance in biometry. Further acknowledgements are given at the end of relevant chapters.

2.7 References

Aradhya, M. K., Yee, L. K., Zee, F. T. and Manshardt, R. M. 1997. Genetic variability in *Macadamia*. *Genet. Res. Crop Evol*. 44: 1-14.

Jarne, P. L., J.L. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution 11:424-429.

Klimyuk, V. Carroll, B. J., Thomas, C. M., and Jones, J. D. G. (1993) Alkali treatment for rapid preparation of plant tissue for reliable PCR analysis. *The Plant Journal* **3**, 493-494.

Parker, P. G., Snow, A.A., Schug, M.D., Booton, G.C., Fuerst, P.A. 1998. What molecules can tell us about populations: Choosing and using a molecular marker. Ecology 79:361-382.

Peace C, Vithanage V, Carroll BJ, Turnbull C (2003) A genetic map of macadamia based on randomly amplified DNA fingerprinting (RAF) markers. *Euphytica,* 134:17-26

Vithanage, V. and Winks, C. W. (1992). Isozymes as genetic markers for Macadamia. *Sci. Hort*. 49: 103-115.

Vithanage, V., Hardner, C., Peace, C., Anderson, K. L., Meyers, N. and McConchie, C. (1998) Progress made with molecular markers for genetic improvement of macadamia. *Proceedings of the International Symposium on Biotechnology of Tropical and Subtropical species.* October 1997. Brisbane, Qld. Australia.

Waldron J, Peace CP, Searle, IR, Furtado A, Wade N, Graham MW, Carroll BJ (2002) Randomly Amplified DNA Fingerprinting (RAF): a culmination of DNA marker technologies based on arbitrarily-primed PCR amplification. *J Biomed Biotech* 2:141-150.

Chapter 3: Recommendations

3.1 Extension/adoption of research outcomes

Preliminary results from this investigation have been presented at industry meetings organised by the AMS R & D committee and Annual General Meetings of the Australian Macadamia Society. Any laboratory that is equipped to carry out DNA handling work can carry out the methodologies used and developed in this study. Since the DNA extraction protocol uses a commercial kit (Dneasy, QIAGEN Pty Ltd) it will not be possible to obtain patent protection for this method. There is a publication planned for the end of this year, subject to clearance by the industry.

3.2 Directions for Future Research

3.2.1 History

Macadamia production in Australia is expanding and the drive for export promotion is ever increasing. Macadamia kernels are bulked at harvest, processed and sold as mixed kernels. Therefore, once the product leaves the farm, the identity of the kernel is lost. The ability to trace a retail pack to identify varieties found in it will enable the industry to maintain a strict quality control regime to safeguard against unwanted microbial and chemical contamination and potential product adulteration by mixing with overseas cultivars.

The ability to trace the product to its origin will enable the industry to guarantee food safety and use the Australian logo to gain maximum promotional benefits for the Australian Industry. When the quantitative breeding program advances, new improved cultivars will be available for cultivation and it may be advantageous to market these new products under the Australian Macadamia Logo to harness the specific benefits associated with each type of kernel. With the forces of change operating globally in the horticultural sphere, consumers are concerned about issues such as “Genetically Modified Food”, “Chemical Residues” “Organic Food” etc. The best method of answering or countering consumer concerns is to be able to trace the

origin of a product. This will not only help maintain quality standards, but also effectively trace the source of any outbreaks of microbial or chemical contamination and act as a deterrent to product adulteration by mixing with other cultivars to gain undue advantage through use of the Australian logo. A robust kernel typing system will assist in implementing a scheme for product identification. The technology will also be readily adaptable to check for the presence of GMO (genetically modified organism) material.

As part of the macadamia-breeding project, techniques have been developed to extract quality DNA from mature leaves that is required for genetic mapping (Vithanage and Peace 1999). Unlike leaf DNA, kernel DNA comes from the first set of leaves of the embryo and has only half the maternal DNA component. The other half could come from self-pollen or from any other pollen source. Therefore, the maternal parent needs to be identified only from half of the genetic make up. Preliminary tests carried out at our laboratories indicated that DNA could be extracted and detected from kernels dry roasted up to 180^{o}C for 12 minutes. However, visualisation of DNA from oil-roasted kernels could not be visualized using standard electrophoretic methods. Theoretically, DNA can be extracted not only from fresh tissue, but also from mummified fossil tissue and also long-stored museum/herbarium specimens (Houde & Brown 1988; Paabo 189; Paabo et al. 1989; France & Kocher 1996). In practice however, many refinements are needed to the existing methodologies to accommodate a different type of source material.

3. 2. 2 Key Features

Using a commercial DNA extraction protocol (DNeasy Extraction Protocol, QIAGEN Pty Ltd) we have generated high quality DNA from raw and air-roasted kernel that can be readily and easily genotyped at microsatellite loci. Oil-roasted kernel samples generated significantly lower quantities of DNA, but it was still possible to genotype these samples at microsatellite loci.

Use of co-dominant microsatellite loci provides a range of advantages over previously employed dominant markers (Jarne & Lagoda 1996; Parker et al. 1998) and, when genotypes of processed kernel are compared to reference genotypes

drawn from different growing regions, this will allow the identification of kernel source/origin.

3.2.3 Questions Raised

The core aim of this project was to develop DNA extraction and genotyping methods that would, in future studies, allow the industry to determine the geographic origin of processed kernel. The successful identification of an efficient, reliable method for high-throughput DNA extraction and the development of a suite of co-dominant microsatellite markers, which are amenable to more meaningful analyses than previously employed dominant markers such as RAF (Jarne & Lagoda 1996; Parker et al. 1998) is critical. At present, there are 25 microsatellite markers available for genotyping of *Macadamia integrifolia* and related species and ongoing work within CSIRO Plant Industry should see this expanded to a suite of 100-200 markers over the next two years. To make full use of the methodology and accurately determine the origin of kernel from processed samples, it will be essential to establish reference genotypes of kernel from different geographic sources by collecting and genotyping kernel from a variety of growing regions (both in Australia and overseas).

The oil and air-roasting methods used in this study are common industry procedures and the successful isolation of high quality DNA from roasted kernel makes it possible to identify kernel origin. There is however, a range of alternative processing procedures and macadamia kernel is used in a variety of products (e.g. cakes, biscuits, confectionary). Having isolated quality DNA from kernel that has been roasted at high temperatures (125-135°C), it is likely that the DNA extraction procedure we have used will work on kernel processed using methods that are less destructive to nucleic acids, but the extraction procedure has not yet been tested on alternatively processed products.

References

France, S. C., and T. D. Kocher. 1996. DNA sequencing of formalin-fixed Crustaceans from Archival Research Collections. Molecular Marine Biology and Biotechnology 5:304-313.

Houde, P., and Brown, M.J. 1988. Museum specimens as a source of DNA for studies of Avian phylogeny. Auk 105:773-779.

Paabo, S., Higuchi, R.G., Wilson, A.C. 1989. Ancient DNA and the polymerase chain reaction: The emerging field of molecular archaeology. The Journal of Biological Chemistry 264:9709-9712.

Paabo, S. 1989. Ancient DNA: Extraction, characterization, molecular cloning and enzymatic amplification. Proceedings of the National Academy of Sciences of the United States of America 86:1939-1943.

Chapter 4: Development and Testing of Methods for Extraction of DNA from Roasted Macadamia Kernel and Genotyping using DNA Markers

A.L. Schmidt, K. O'Connor and V. Vithanage

4.1 Introduction.

Macadamia is a high value crop that is native to Australia, but grown in many countries. Macadamia kernels are bulked at harvest, and processed/sold as mixed kernels. Therefore, once the product leaves the farm, the identity of the kernel is lost. The ability to trace a retail pack to identify varieties found in it will enable the industry to maintain a strict quality control regime to safeguard against unwanted microbial and chemical contamination and potential product adulteration by mixing with other overseas cultivars. Such a system would also allow the industry to use the Australian Macadamia logo to capture maximum promotional benefits for the Australian industry. Consumer concerns about "Genetically Modified Food" and "organic" food can also be addressed if the industry can guarantee the origin of the kernel in retail packs.

Product adulteration has been identified as a major problem in the food industry, irrespective of whether the food product is solid or liquid. Examples include dilution of high cost vegetable oil and fruit juice with cheaper varieties, mixing of honey with other forms of sugar and combination of cows milk with other types of milk. Various methods of detecting such adulterations have been developed and these are critical in maintaining quality control of food products. Gas chromatography has been employed to detect differences in delta 13C values (arising through different modes of carbon fixation) and thereby: a) distinguish maize oil from other oils (Woodbury et al 1995) and; b) detect different types of sugars in fruit juices (Krueger, 1988). Detecting differences in the levels and type of tocopherols with the help of reverse-phase HPLC has also been an effective method of detecting adulterations of oils (Dionisi et al 1995). ELISA techniques have been successfully used to detect both the presence of almond components in processed foods (Hlywka, et al 2000) and the treatment of meat with casein (Smith, 1992) and sterol determination has been

developed as an effective method for detection of vegetable oil in ice cream (Sheppard, et al 1985).

The detection of food adulteration has important consequences for both human health, and the protection of various industries. With regard to protection of product quality, DNA based techniques represent a recently developed, but particularly powerful tool (Deroin et al 1999). For example, development of DNA marker technologies has allowed identification of the species origin in a variety of dairy products (Plath, et al 1997). However, discrimination between varietal differences of a single species of plant or animal in adulterated food has always been a difficult task. To develop and implement such a system requires the development of methods to:

a) Extract DNA from processed food products
b) Genotype that DNA in a manner that will allow identification of product source.

This study is concerned with developing methods that will allow identification of source cultivars from kernel, in both raw and processed states.

4.2 Materials and Methods

4.2.1 Kernel Material

Preliminary testing of DNA extraction and genotyping methods used kernel from a single cultivar, 849. Kernel was divided into three treatment groups:

a) Raw kernel
b) Oil-Roasted kernel (125°C for 12 minutes)
c) Air-roasted kernel (135°C for 12 minutes)

4.2.2 DNA extraction

The research project was conducted in two phases, under the control of two separate research scientists, meaning development of methodologies was also undertaken in two separate phases. The first method of DNA extraction, developed under the first research scientist, was based on a previously published 'Victor Prep' method (Klimyuk et al 1993) with some modifications, details of which are indicated below. During the second phase of the work, the Victor Prep method, which had performed inconsistently since 2001, was abandoned in favor of a commercial DNA extraction protocol.

4.2.2.1 Victor Preps

Victor Prep extractions were conducted in accordance with the published protocol (Klimyuk et al 1993), incorporating an additional solvent extraction of the lipid rich tissue. Several solvents and solvent mixes were tested and the preferred solvent was hexane:isopropanol. Other solvents tested included chloroform, hexane, chloroform:methanol, acetone and isopropanol. All of these solvents performed adequately, with the exception of isopropanol, which performed worse than a control treatment with distilled water.

The complete Victor Prep extraction protocol is summarized in Figure 4.1.

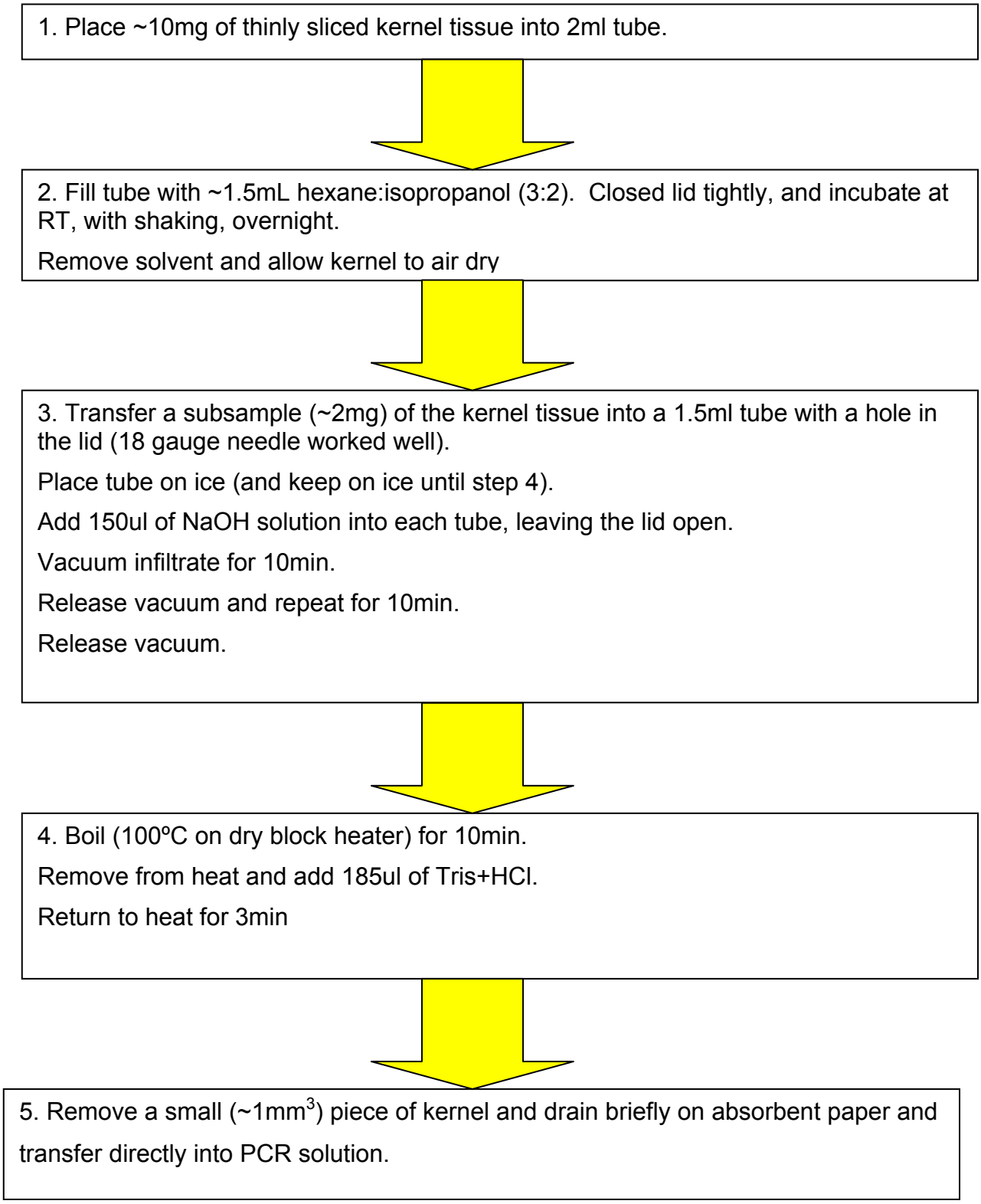


Figure 4.1: Flow Chart Summary of Victor Prep DNA Extraction Method

4.2.2.2 QIAGEN DNeasy® DNA Extraction

Following inconsistent results from a range of DNA extraction procedures, including Victor Preps and standard CTAB-lysis procedures, the QIAGEN Pty Ltd (Melbourne) DNeasy® DNA extraction protocol was selected from a range of commercial DNA extraction protocols for the following reasons:

- The method includes several steps that remove oils, detergents, proteins, polysaccarides and other problematic compounds found in plant tissues.
- Kits can be purchased in a high-throughput format that allows simultaneous processing of 96 samples within a single plate. This format is compatible with 96-well PCR machines.
- The method requires little or no specialized equipment and all required reagents are supplied with the DNA extraction kit.

The DNeasy extraction protocol is summarized in Figure 4.2.

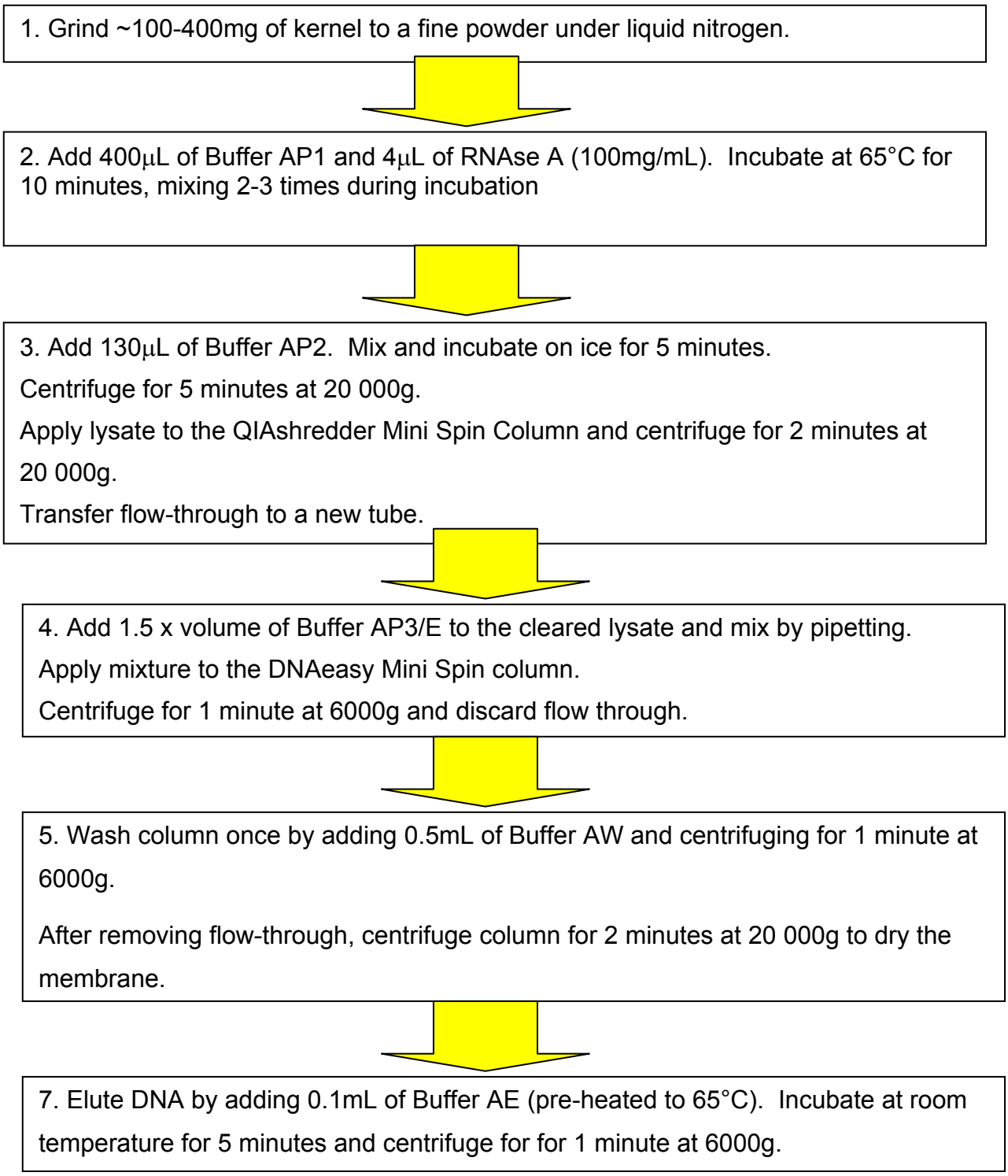


Figure 4.2: Flow Chart Summary of QIAGEN Pty Ltd DNeasy Extraction Method

4.3 PCR Method and separation of fragments

4.3.1 RAF Genotyping

The amplification reaction for RAF analysis (Peace et al 2003) was performed in volumes of 4.0 μl consisting of the smallest piece of kernel described above (20 ng of macadamia genomic DNA as control), 1.5U of Stoffel Fragment (PE Biosystems)), 1.0 μl of 10 x DAF buffer (25 mM Tris, pH 8.0, 25 mM KCl, 12.5 mM $MgCl_2$), 10 μmol of primer (Operon Technologies), 0.062 mM dNTP, 2.4 μl of water and 0.25 μl α-^{33}P-dATP. After 5 min at 94^0 C, 30 cycles of PCR were performed (denaturation 30 s at 94^0 C; annealing 1 min each at 57^0C, 56^0C, 55^0C and 54^0C ; and an extension 5 min at 72^0C) using a fast thermal cycler (Corbett PC-960).

The amplified products of PCR reactions were separated on a 5% denaturing polyacrylamide gel (Waldron et al 2002) for 2.75 – 3.0 h at 90 W per gel (Bio-rad apparatus). The gels were dried and exposed on Hyperfilm overnight.

4.3.2 Microsatellite Genotyping

Prior to 2005, the pool of microsatellite markers for use in *Macadamia integrifolia* consisted of two sequence tagged microsatellites and three RAF-derived microsatellites identified during previous projects (Table 4.1). To increase the power of genetic analysis and develop the capacity for further studies, additional microsatellite markers are currently being developed within CSIRO Plant Industry. As at June 2005, the pool of new microsatellite loci includes ~25 new markers and, for the purposes of this study, DNA samples were genotyped at four of these markers (Table 4.1).

Table 4.1: Summary of Microsatellite Markers for *Macadamia integrifolia*

The markers *2M13, 62M13, 143Mi, 172Mi* and *100Mi* were developed during previous studies, as indicated. *MinμS* markers were developed during the course of this project. It is expected that a suite of 50-100 *MinμS* markers will be available by January 2006. Note that primers for the MinμS0004 locus amplified two loci, scored as MinμS0004A and MinμS0004B.

Marker ID	Marker Description	Repeat Motif	Marker Source
2M13	Sequence tagged microsatellite	Unrecorded	Vithanage et al. 2002
62M13	Sequence tagged microsatellite	Unrecorded	Vithanage et al. 2002
143Mi	RAF-derived microsatellite	Unrecorded	Vithanage et al. 2001
172Mi	RAF-derived microsatellite	Unrecorded	Vithanage et al. 2001
100Mi	RAF-derived microsatellite	Unrecorded	Vithanage et al. 2001
MinμS0002	Enriched microsatellite library	(CA)n	Schmidt et al., expected publication early 2006
MinμS0004	Enriched microsatellite library	(GT)n	Schmidt et al., expected publication early 2006
MinμS0005	Enriched microsatellite library	Imperfect (GA)n	Schmidt et al., expected publication early 2006
MinμS0007	Enriched microsatellite library	(GA)n	Schmidt et al., expected publication early 2006

Amplification of microsatellite loci derived from the enriched genomic library was performed in 7μL reactions containing 1 x PCR buffer (FisherBiotech International Ltd), 1.5mM $MgCl_2$, 0.2mM lowC dNTPs (10mM dATP, 10mM dTTP, 10mM dGTP, 1mM dCTP), 9μCi ^{33}P-dCTP, 0.1μM of each of the forward and reverse primers, 0.15 units of *Tth Plus* DNA polymerase (FisherBiotech International Ltd) and 25-50ng (~2μL) of genomic DNA. Thermocycle conditions involved a basic cycle of 95°C for 15s; followed by 40 cycles of 95°C for 15s, annealing temperature (T_a, primer dependent) for 15 s, 72°C for 30s. For the locus *MinμS0004*, the basic cycle was modified to include a T_a of 48°C for the first 10 cycles and a T_a a 50°C for the remaining 30 cycles.

PCR products were visualised via electrophoresis through denaturing 5% polyacrylamide gels (6M urea, 5% acrylamide:bisacrylamide19:1, 1 x TBE). Alleles were scored to 1bp accuracy with reference to a pUC18 sequence ladder.

4.4 Results

4.4.1 Solvent Extracted DNA and RAF Markers

Solvent extraction of the oil in kernels prior to DNA extraction allowed genotyping at RAF markers. The best treatment was selected on the basis of a higher number of scorable bands (Fig 4.3). Results obtained with RAF markers are shown in Fig 4.4.

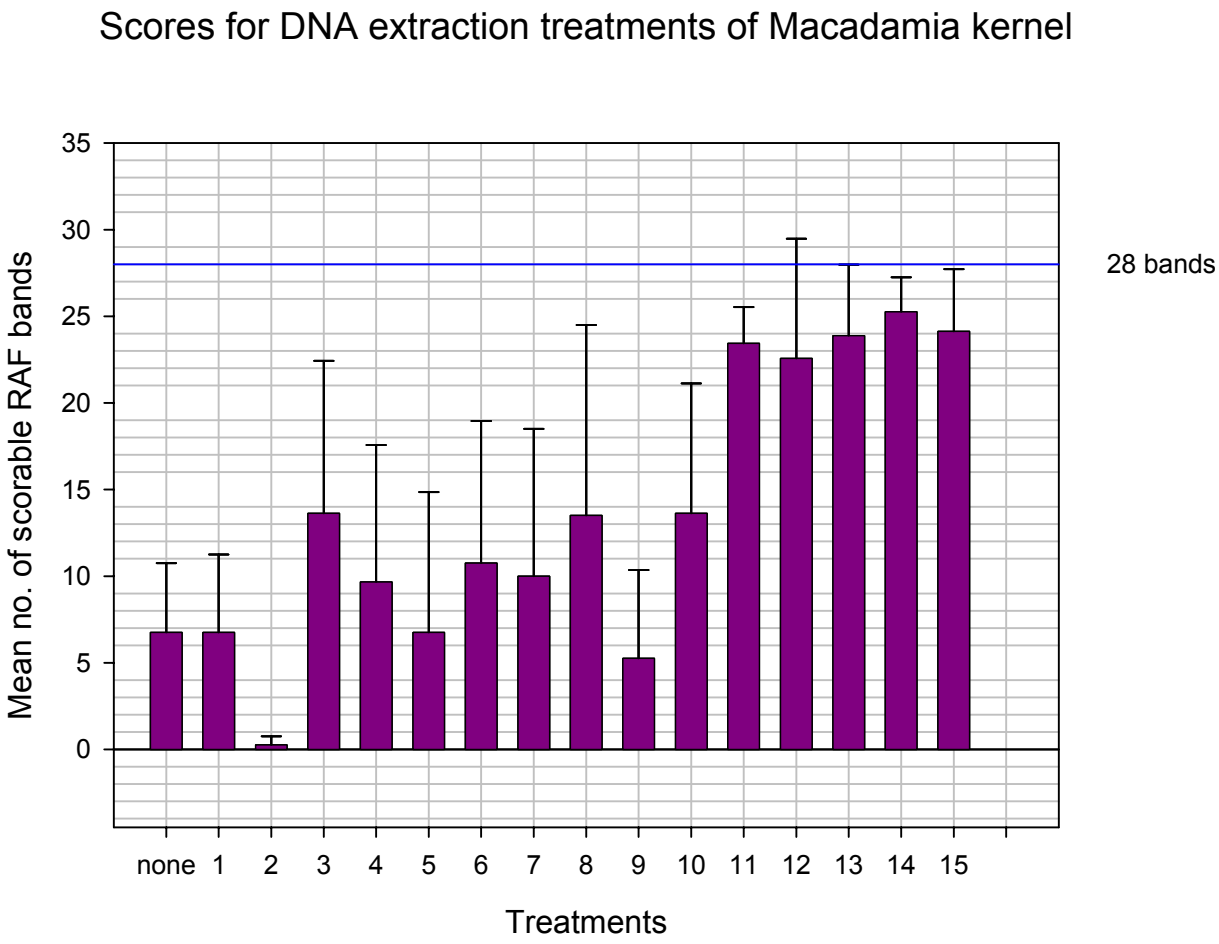


Figure 4.3: Mean number of scorable RAF bands after five different extraction treatments.

Each treatment was in triplicate. First histogram on the left is the control with water followed by isopropanol (1-3), chloroform (4-6), hexane (7-9), chloroform:methanol (10-12) and hexane:isopropanol (13-15).

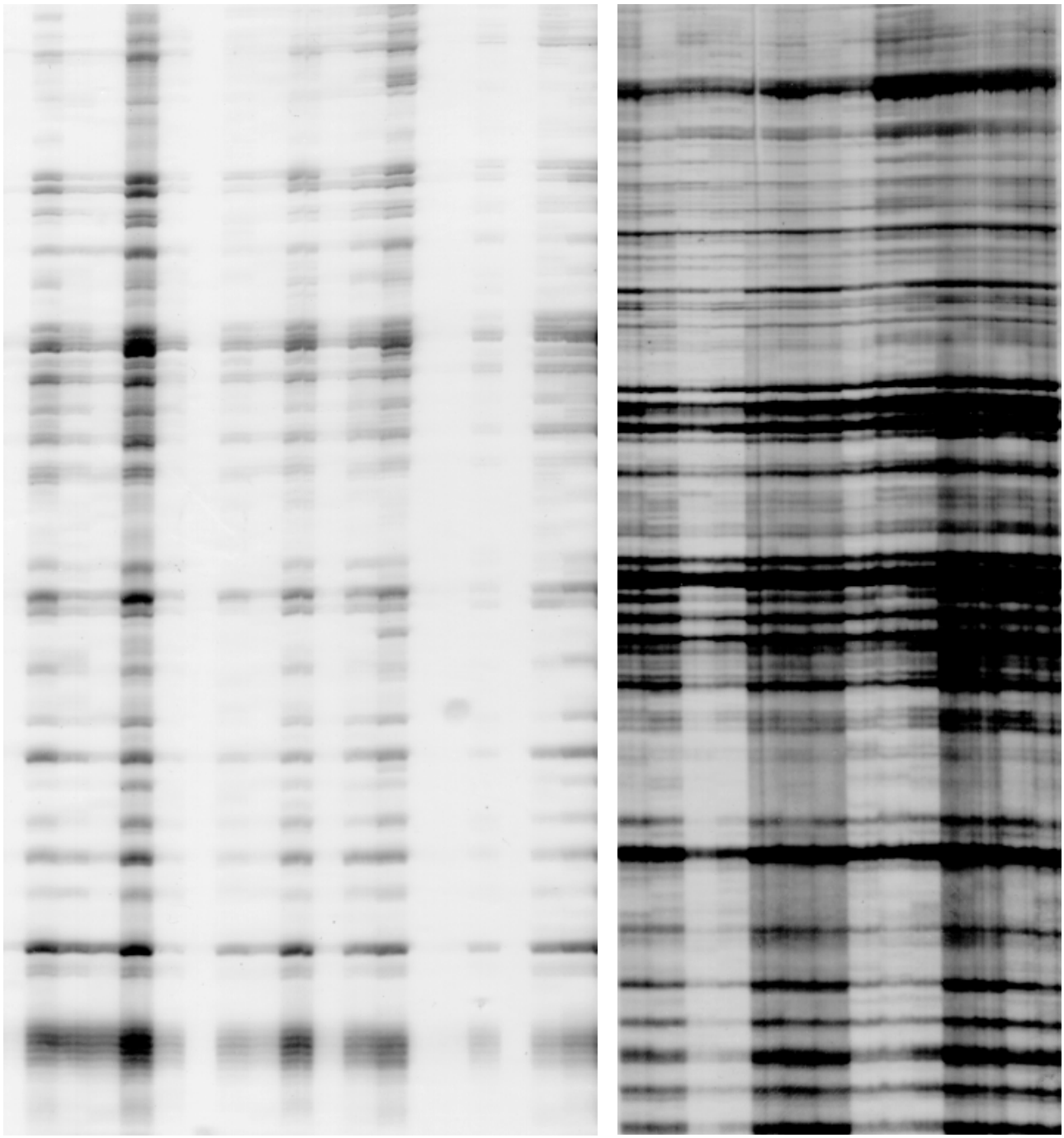


Figure 4.4: Results using RAF Markers

Autoradiograph images revealing the markers of fresh kernel tissue on the left and solvent extracted kernel tissue on the right.

4.4.2 DNeasy DNA Extraction and Single-Locus Microsatellite Genotyping

The DNeasy extraction protocol generated high molecular weight DNA that could be visualized via electrophoresis in agarose gels from both raw and air-roasted kernel (Fig. 4.5). Extractions from oil-roasted kernel could not be visualized via electrophoresis (Fig. 4, panel b). However, samples from all three treatment groups could be genotyped at microsatellite loci via polymerase chain amplification (Fig. 4.6).

Although preliminary tests were carried out using kernel from a single variety, high levels of polymorphism were observed in three of the four loci (MinμS0002, MinμS0004 and MinμS0007), indicating that these loci will be useful in identification of kernel source/origin.

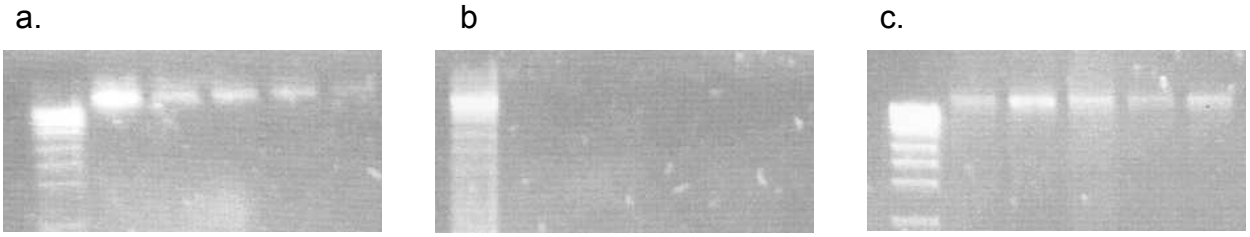
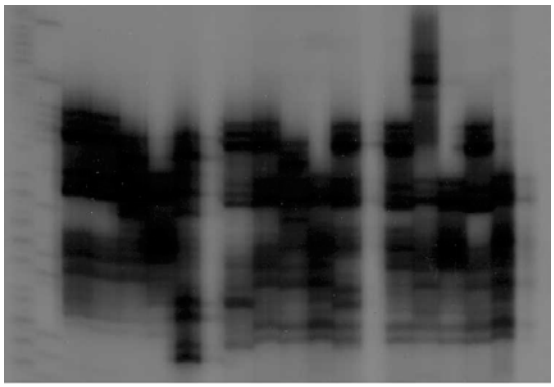


Figure 4.5: DNA Obtained using the DNeasy Extraction Procedure

10μL of extracted DNA was loaded onto 1.5% agarose gels, along with 3μL of a size/mass standard (MBI Fermentas MassRuler Mix). Note that DNA extractions from raw kernel (panel a) and air-roasted kernel (panel c) are of sufficient concentration to be visualized using this method, but extractions from oil-roasted kernel (panel b) are not.

a.



b.

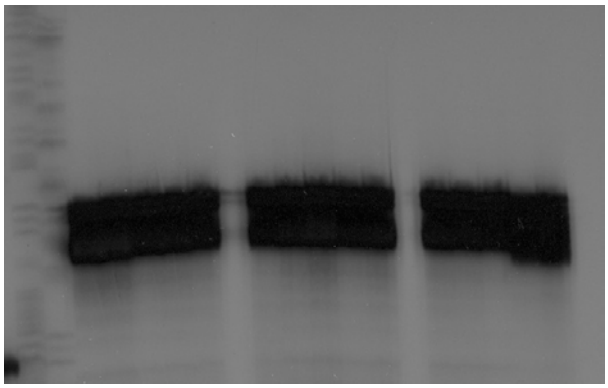


Figure 4.6: Microsatellite PCR Products from Raw, Oil-Roasted and Air-Roasted Kernel

Extractions from raw, oil-roasted and air-roasted kernel were amplified at the microsatellite loci:

a) MinμS0004: lanes 1-2 contain the pUC 18 size standard, lanes 3-7 contain samples from air-roasted kernel, lanes 9-13 contain products from oil-roasted kernel, lanes 15-19 contain products from air-roasted kernel and lanes 8, 14 and 20 contain non-template controls.
b) MinμS0005: lanes 1-2 contain the pUC 18 size standard, lanes 3-7 contain samples from air-roasted kernel, lanes 9-13 contain products from oil-roasted kernel, lanes 15-19 contain products from air-roasted kernel and lanes 8, 14 and 20 contain non-template controls.

Although locus MinμS0005 shows little polymorphism in the example presented, it should be noted that kernel samples were derived from a single tree and, despite this, high levels of polymorphism were observed in three of the four loci (MinμS0002, MinμS0004 and MinμS0007), indicating that these loci will be useful in identification of kernel source/origin.

4.5 Discussion

The project was successful in refining the protocols for extracting DNA from oil rich kernel tissue of macadamia and generating PCR products that can be used in microsatellite genotyping procedures that will allow the identification of kernel source/origin. Although the Victor Prep method of DNA extraction showed initial success, results were inconsistent and the commercial DNA extraction procedure is preferred because it is more robust and readily amenable to high-throughput and/or automation.

The ongoing development of additional microsatellite loci within CSIRO Plant Industry will further increase the power of the methodology and, with further automation of techniques, will allow rapid, efficient screening of large numbers of samples. Use of co-dominant microsatellite loci provides a range of advantages over previously employed dominant markers (Jarne & Lagoda 1996; Parker et al. 1998) and, when genotypes of processed kernel are compared to reference genotypes drawn from different growing regions, this will allow the identification of kernel source/origin.

The ability to trace the product to its origin will enable the industry to guarantee food safety and use the Australian logo to gain maximum promotional benefits for the Australian Industry. When the quantitative breeding program advances, new improved cultivars will be available for cultivation and it may be advantageous to market these new products under the Australian Macadamia Logo to harness the specific benefits associated with each type of kernel. With the forces of change operating globally in the horticultural sphere, consumers are concerned about issues such as “Genetically Modified Food”, “Chemical Residues” “Organic Food” etc. The best method of answering or countering consumer concerns is to be able to trace the origin of a product. This will not only help maintain quality standards, but also effectively trace the source of any outbreaks of microbial or chemical contamination and act as a deterrent to product adulteration by mixing with other cultivars to gain undue advantage through use of the Australian logo. A robust kernel typing system will assist in implementing a scheme for product identification. The technology will also be readily adaptable to check for the presence of GMO (genetically modified organism) material.

The oil and air-roasting methods used in this study are common industry procedures and the successful isolation of high quality DNA from roasted kernel makes it possible to identify kernel origin. There is however, a range of alternative processing procedures and macadamia kernel is used in a variety of products (e.g. cakes, biscuits, confectionary). Having isolated quality DNA from kernel that has been roasted at high temperatures (125-135°C), it is likely that the DNA extraction procedure we have used will work on kernel processed using methods that are less

destructive to nucleic acids, but the extraction procedure has not yet been tested on alternatively processed products.

4.6 Acknowledgements

We thank the macadamia growers, Greg Sumner (Chevallum, Qld), Peter and Nancy Zeck (Nambukka Heads, NSW), Geoff Royal (Woolgoolga, NSW), Frank Hathaway (Lismore, NSW) and Peter Stace (Jiggi, NSW) for their co-operation and access to orchards and Marie O'Sullivan, CSIRO Mathematical and Information Sciences, for assistance in biometry. This study was partially funded by the Australian Macadamia Society and Horticultural Research and Development Corporation.

4.7 References

Deroin P., Breton F., Rosier F., Monnier AL., Koenig R., Hinfray J., Chevassus-au-Louis N., Philipon P. and Haseltine W. (1999). The new technological niches. [Les nouvelles niches technologiques.] Biofutur. 194, 79-99.

Dionisi F ; Prodolliet J ; Tagliaferri E. (1995). Assessment of olive oil adulteration by reversed-phase high-performance liquid chromatography/amperometric detection of tocopherols and tocotrienols. Journal of the American Oil Chemists' Society. 72, (12), 1505-1511.

Hlywka, J.J., Hefle, S.L., and Taylor, S.L. (2000). A sandwich enzyme-linked immunosorbent assay for the detection of almonds in foods. Journal of Food Protection, 63, (2), 252257.

Jarne, P. L., J.L. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution 11:424-429.

Klimyuk, V. Carroll, B. J., Thomas, C. M., and Jones, J. D. G. (1993) Alkali treatment for rapid preparation of plant tissue for reliable PCR analysis. The Plant Journal 3, 493-494.

Krueger DA (1988). Applications of stable isotope ratio analysis to problems of fruit juice adulteration. In: Adulteration of fruit juice beverages. Marcel Dekker, Inc. New York, 109-124.

Parker, P. G., Snow, A.A., Schug, M.D., Booton, G.C., Fuerst, P.A. 1998. What molecules can tell us about populations: Choosing and using a molecular marker. Ecology 79:361-382.

Peace C, Vithanage V, Carroll BJ, Turnbull C (2003) A genetic map of macadamia based on randomly amplified DNA fingerprinting (RAF) markers. Euphytica, 134:17-26

Plath A ; Krause I ; Einspanier R (1997). Species identification in dairy products by three different DNA - based techniques . Zeitschrift fur Lebensmittel-Untersuchung und -Forschung . 205, (6) , 437-441.

Sheppard AJ ; Shen CSJ ; Rudolf TS (1985) Detection of vegetable oil adulteration in ice cream. Journal of Dairy Science . 68, (5), 1103-1108.

Smith CJ. (1992) Applications of immunoassay to the detection of food adulteration . An overview. In. (Morgan MRA; Smith CJ; Williams PA (ed.) Food safety and quality assurance: applications of immunoassay systems, Elsevier Applied Science . London , UK. p13-32

Waldron J., Peace, C.P., Searle I.R.,Furtado A., Wade N.,Graham M. W. and Carroll B. J. (2002) Randomly amplified DNA fingerprinting: A culmination of DNA marker technologies based on arbitrarily-primed PCR amplification. Journal of Biomedicine and Biotechnology, 2, (3); 141-150

Woodbury SE ; Evershed RP ; Rossell JB ; Griffith RE ; Farnell P. (1995). Detection of vegetable oil adulteration using gas chromatography combustion/isotope ratio mass spectrometry. Analytical Chemistry (Washington), 67, (15), 2685-2690.