DNA BASED SAMPLING, DETECTION, QUANTIFICATION AND DAMAGE THRESHOLD OF *MELOIDOGYNE* SPP. IN GRAPEVINE

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To
My father Dr M A Quader
and
my mum Zahera Khatun

TABLE OF CONTENTS

CHAPTER THREE: DETECTION AND QUANTIFICATION OF RKN

CHAPTER FOUR: RKN QUANTIFICATION METHODS, SPECIES IDENTITY AND NON RKN IN VINEYARDS

CHAPTER SIX: GENERAL DISCUSSION

Summary

Studies were conducted to develop soil sampling, detection and quantification techniques for *Meloidogyne* spp. (root-knot nematodes, RKN) in vineyards. A survey was conducted in vineyards of South Australia (SA) to validate a DNA based quantification method for RKN and to determine the population structure of RKN across the grapevine growing areas of SA. The effect of different RKN densities on vine growth during their establishment was also determined.

The distribution pattern of RKN was studied in five vineyards in three locations in SA. Nematodes were extracted from soil samples at two depths (0-300 and 300-600 mm) for each of five positions; three along the vine row, one under cover crops and one adjacent to cover crops in the inter-row. RKN were found to be aggregated along the vine rows. The highest RKN population was found in samples taken close to vines, especially those at about 100 mm from the base of vine, and the second highest was in the row between two vines. No significant difference was found between RKN population levels at the two soil depths. RKN populations under cover crops were significantly lower than in the vine rows. It was concluded that core samples for quantification of RKN population in vineyards should only be collected along the rows. To standardise the collection procedure for RKN, it is recommended that soil samples should be collected about 100 mm from the vine to a depth of 300 mm. The effect of cover crops on RKN populations in vineyards is discussed.

The sampling method developed for RKN was tested for its suitability for ectoparasitic *Xiphinema* spp. and migratory endoparasitic *Pratylenchus* spp., nematodes that also affect grapevines. Using the same methodology, the horizontal, vertical and seasonal distribution of dagger nematodes (*Xiphinema* spp.) and root

lesion nematodes (*Pratylenchus* spp.) were monitored monthly for 12 months in a Barossa Valley vineyard of SA. Nematode densities were determined at five different horizontal positions from the vines, including rows and inter-rows, at two depths 0-300 and 300-600 mm. The dagger nematodes occurred mainly along the rows and at higher density at 300-600 mm. Whereas, root lesion nematodes were at similar densities in rows and inter-rows, but occurred at greater density at 0-300 mm. The population densities of both nematodes were greater in October and November and lowest in February (late summer). Based on these data and other reports, sampling near the vine to a depth of 600 mm in late spring is considered to be the best option for *Xiphinema* and *Pratylenchus* in SA vineyards.

Identification methods based on the North Carolina differential host test and DNA methods were assessed for their ability to distinguish a collection of SA populations of RKN from vineyards. The NC differential host test differentiated *M. incognita* but not *M. arenaria* race 2 from *M. javanica*. A combination of the NC host test and PCR amplification of mtDNA could differentiate between *M. arenaria, M. incognita* and *M. javanica*. A mtDNA based method was successfully used to differentiate *M. arenaria* from *M. incognita* and *M. javanica* by PCR amplification. However, subsequent RFLP analysis of PCR-mtDNA product did not differentiate between *M. incognita* and *M. javanica*. The PCR amplifications of D3 expansion region of 28S rRNA gene and intergenic sequences of ribosomal DNA (IGS-rDNA) were also made to distinguish *M. arenaria, M. incognita* and *M. javanica*. The identification of these species with D3 expansion region of 28S rRNA gene was not possible. The sequences of this region are highly conserved among the species, limiting the possibility of their identification based on this D3 expansion region alone. PCR amplification of IGS-rDNA of genomic DNA from a single female of each species produced distinct banding patterns that can differentiate the species

from each other. These species-specific banding patterns were reproducible across a range of individual nematodes of each species collected from different geographical locations of Australia. The method may also be applied to the examination of intraspecific variation of *Meloidogyne*.

A DNA based quantification method was evaluated under controlled condition on species of RKN from grapevines. A clear relationship was found when the DNA assay was applied to soil samples with addition of known numbers of RKN juveniles. A strong relationship was also found between the DNA assay and addition of nematodes for both *M. incognita* and *M. javanica*. The relationship between the DNA assay and number of nematodes added remained robust in both sand and clay soil types. In these experiments, the DNA assay could detect levels as low as 40 juveniles per 400 g soil. The DNA assay appears not only to be adequately sensitive but is consistent for the accurate estimation of both important species (*M. incognita* and *M. javanica*) in both clay and sandy soils, so it is likely that the method could be successfully applied to a range of soils occurring in Australian vineyards.

The sampling and identification methods developed were used to validate the DNA quantification method under vineyards condition and to survey vineyards of SA. A comparative study, based on extraction, bioassay and DNA methods, was performed for the quantification of *Meloidogyne* spp. in vineyards of SA. DNA based species identity and differences in the sequences of internal transcribed spacers (ITS-1 and ITS-2) of rRNA genes in individuals of *Meloidogyne* were also determined. The DNA method was consistently better than commonly used methods for quantification of RKN in various vineyard soils. Four species, *M. javanica, M. incognita, M. arenaria* and *M. hapla*, were recorded in vineyards of SA. The former three species were predominantly found in warmer and *M. hapla* in cooler regions. The DNA sequences in ITS regions of rRNA genes were highly conserved (<2%

divergence) among the individuals of the main species in SA vineyards. Variability in rRNA genes and its relation to the DNA based method for quantification of RKN is discussed.

A microplot experiment was conducted over two years (2000-2002) to determine the effect of *Meloidogyne incognita* population densities on the growth of grapevine during establishment. Four RKN population densities, 25, 154, 960 and 2400 per 1000 ml soil, were assessed on a susceptible cultivar Colombard and a moderately resistant cultivar Sultana. At the first assessment, there was a direct relationship between inoculum density and root gall number in Colombard, but no galls were found in Sultana roots. In the first growing season, RKN did not reduce the growth of either cultivar. However, in the second season, RKN population densities greater than 25 per 1000 ml soil significantly reduced the pruned weight of Colombard but increased pruned weight in Sultana. Therefore, the damage threshold of RKN for grapevines will vary between cultivars. However, for an apparently intolerant cultivar, such as Colombard, the damage threshold for RKN would be about 1 to 25 per 1000 ml soil at establishment. The damage threshold density was found to be 1.5 *M. incognita* per 1000 ml soil by the Seinhorst crop-loss model. This damage threshold for *M. incognita* on grapevines and its implication to the decision making process for the establishment of a vineyard is discussed.

Statement

I hereby declare that research work presented in this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institute and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Motiul Quader

Publications arising from this thesis

A. Refereed papers

Quader, M., Riley, I.T. and Walker, G.E. 2001. Distribution pattern of root-knot nematodes (*Meloidogyne* spp.) in South Australian vineyards. *Australasian Plant Pathology* 30:357-360.

Quader, M., Riley, I.T. and Walker, G.E. 2002. Damage threshold of *Meloidogyne incognita* for the establishment of grapevine. *International Journal of Nematology* 12, 1-6.

Quader, M., Riley, I.T. and Walker, G.E. 2003. Spatial and temporal distribution patterns of dagger (*Xiphinema* spp.) and root-lesion (*Pratylenchus* spp.) nematodes in a South Australian vineyard. *Australasian Plant Pathology*, (in press).

Quader, M., Riley, I. T., Asgari, S. and Walker, G. 2002. Quantity, identity and nucleotide polymorphism in rRNA genes of *Meloidogyne* spp. from South Australian vineyards. *Journal of Nematology*, (submitted).

Quader, M. and Riley, I.T. A simple rDNA based PCR method to distinguish *Meloidogyne incognita*, *M. javanica* and *M. arenaria* from grapevine. *Nematology* (submitted).

Industry journal articles

Quader, M., Riley, I., Herdina, Ophel-Keller K and Walker G. 2002. Root-knot nematode quantification for management options in grapevines. *The Australian and New Zealand Grapegrower and Winemaker* 458, 13-16.

Quader, M., Riley, I., and Walker G. 2002. Nematode pests in vineyards of South Australia. *The Australian and New Zealand Grapegrower and Winemaker* 464, 62-64.

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Abbreviations used

%	Per cent
Ac	Acetate
Bp	Base pair(s)
BSA	Bovine serun albumin
^{O}C	Degrees celcius
cDNA	Complementary deoxyribonucleic acid
conc.	Concentration
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleotide triphosphate
EDTA	Ethylenediamineteraacetic acid
ETS	External transcribed spacer
EtBr	Ethidium bromide
g	Gram
GFV	Grape fanleaf virus
IGS	Inter-genic sequence
IPTG	Isopropyle-bD-thiogalactopyranoside
ITS	Internal transcribed spacer
ITS1	Internal transcribed spacer-1
ITS2	Internal transcribed spacer-2
kb	Kilobase
kbp	Kilobase pairs
LB	Lura Bertani
M	Molar

µg	Microgram
µl	Microlitre
min	Minute
µM	Micromolar
mM	Milimolar
mRNA	Messanger RNA
NC	North Carolina
OD	Optical density
PCR	Polymerase chain reaction
pGEM-T	*E. coli* strain
PRLV	Peach rosette mosaic virus
RADP	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RKN	Root-knot nematodes
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Revolution per minute
SA	South Australia
SDS	Sodium dodecyl sulphate
s	Second
spp.	Species
ss	Single stranded
5.8S	5.8S sub unit of ribosome
16S	165S sub unit of ribosome
18S	18S sub unit of ribosome

28S	28S sub unit of ribosome
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-DETA
Tris	Tris-hydroxymethyl-aminomethane
U	Unit
Tween 20	Polyoxyethylenesoribitan monolarate
UC	University of California
UV	Ultraviolet
V	Volt
v	Volume
X-Gel	5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside

CHAPTER ONE
REVIEW OF LITERATURE

1.1 Introduction

There are about 60 known species under the genus *Vitis* (Winkler *et al.* 1974). Among them *Vitis vinifera* provides the main source of wine and table grapes. This species originated from the regions between and south of the Caspian and Black Seas in Asia Minor (Winkler *et al.* 1974). The first grapevine cuttings and seeds were brought to Australia by European settlers in 1788 and successfully established at Farm Cove, the site of the present Sydney Royal Botanic Garden (Gregory 1988). Since then viticulture has extended to all states and territories of Australia. Currently about two million hectares are under grapevine cultivation, which is likely to be increased the area in near future due to high demand (Anon. 1996). The Australian grape and wine industry is aiming to supply 6.5% of the value of world wine production by the year 2025 (Anon. 1996). To achieve this goal several strategies have been undertaken, including development of sustainable management practices for pest and diseases of grapevine, such as plant-parasitic nematodes.

1.1.1 Plant parasitic nematode

Nematodes are a complex, diverse group of roundworms that occur worldwide in most of environments. Many species are important parasites of plants and animals. Chinese literature as early as 235 BC includes descriptions that may refer to a white *Heterodera* female and attached egg mass on soybean roots (Noel 1992). The discovery of the Leeuwenhoek microscope in the early 17th century opened the possibility of nematode research for the first time in history. Indeed, nematodes were used to explore the capabilities of the recently developed microscope. The first microscopic based discovery of a nematode took place in 1743 with the observation of plant parasitic nematode *Anguina tritici* in wheat (Luc *et al.* 1990). Plant-parasitic nematodes in only 24 genera are regarded as economically

important pests of crop plants, causing loss of about 10% of world production, and about one third of the losses attributed to pests and diseases generally (Whitehead 1998). The worldwide financial loss caused by nematodes was estimated US$100 billion annually by Oka *et al.* (2000), and the loss in Australia is about $300 to 400 million annually (Anon 1999).

More nematologists place nematodes in the phylum Nematoda and into two classes: the Adenophorea and the Secernentea (Maggenti 1991), with 18 and 6 orders respectively. Plant parasitic nematodes are found mostly in the orders Dorylaimida (Adenophora) and Tylenchida (Secernentea), with the majority in the latter (Barker 1998).

Most species of plant-parasitic nematodes are 1 to 2 mm in length and may vary in shape from filiform to fusiform, pear-shaped or pyriform, lemon-shaped or kidney-shaped. Most plant-parasitic nematodes complete their lifecycle within 20 to 30 days at 18-27^{O} C, few Dorylaimid nematodes have a life cycle as long as 1 to 2 years (Ferris and Ferris 1998). Nematode movements in soil are limited to the existing soil pores where they swim in a film of moisture. Nematode movement is greatest when the mean soil particle diameter is equal to about one-third to one-half the length of the nematode (Wallace 1958a,b). Some plant-parasitic nematodes invade the aboveground portion of plants. For example, species of tylenchids, such as *Ditylenchus dipsaci*, are an internal parasite of bulbs, stems and leaves, and rarely attack roots (Ferris and Ferris 1998). A number of species of the genus *Aphelenchoides* are primarily bud and foliage parasites (Ferris and Ferris 1998). Nematodes in the genus *Anguina* cause leaf or stem galls and seed galls.

Some plant-parasitic nematodes, such as *Xiphinama americanum*, are ectoparasites, feeding on roots and injuring the root cortex and endodermis. Others, such as nematodes in the genus *Hoplolaimus*, may penetrate partially into a root and

are often called semi-endoparasites. Migratory endoparasitic nematodes, such as *Pratylenchus penetrans,* can enter and migrate intercellularly. The root-knot nematodes *Meloidogyne* spp., the citrus nematode *Tylenchulus semiendopenetrans* and the cyst nematode *Heterodora* and *Globodera* are all sedentary endoparasitic nematodes.

1.1.2 Plant nematode interaction

Plant nematode interactions are a complex phenomenon that depends on species, hatching stimuli, attraction to host, penetration and migration in host tissue, recognition of tissues suitable for feeding-site formation, and may lead to elaborate modification of host cells (Hussey and Williamson 1998). Nematodes use the stylet to penetrate the host, to inject secretions into host cells, to withdraw nutrients from cytoplasm, and to migrate within the host tissue. Secretions from oesophageal gland are important for the establishment of a feeding site. The sensory system of plant parasitic nematodes also plays a major role in parasitism (Dusenbery 1987; Perry 1994).

In response to nematode attack, host cells modify their function, metabolism and phenotype. The type of modification depends on the species of attacking nematode. For example: *Meloidogyne* and *Heterodera* feeding sites become a giant cell, while the feeding site of *semipenetrans* becomes a group of discrete nurse cells (Hussey and Williamson 1998). Biochemical responses, such as an increase of biochemical in cells, expression of gene(s), are common in nematode infested plants. Auxin and ethylene levels increase greatly in tomato roots infected by *Meloidogyne javanica* (Glazer *et al.* 1986). Ryan (1990) reported pathogenesis related proteins such as protein inhibitor proteins I and II, which are induced in leaves upon nematode infection. Hydroxyproline-rich glycoproteins, that form a major

component of plant cell walls, have been reported to be produced in plant-defence responses. Niebel *et al.* (1993) found that mRNA levels were significantly increased in galls induced by *M. javanica* one week after infestation and began to decrease after a further two weeks. Root-knot nematodes (*Meloidogyne* spp.) induced genes related to cell division in plant (Niebel *et al.* 1994).

1.1.3 Distribution pattern of nematodes in field

Nematodes are patchily distributed in soil (Goodell and Ferris 1980), making development of reliable sampling strategies more difficult (McSorley *et al.* 1985; McSorley and Parrado 1982). Even the best laboratory technique, to detect or quantify a nematode population in a soil sample, has little value if representative core samples are not taken accurately from the field. The irregular horizontal distribution of nematodes is probably the greatest obstacle to determine reliable number of nematode populations. Over a distance of a few meters, population densities in a field can differ significantly (Barker and Nusbaum 1971; Barker *et al.* 1985; Goodell and Ferris 1980).

Despite the effect of a wide range of biological and soil factors, stratification along plant rows is another important factor in the horizontal distribution pattern of nematodes (McSorley 1998). In annual crops, nematodes are often concentrated in the top 300 mm of soil, particularly the top 100 to 200 mm (Norton 1978). Therefore, a sampling depth of 200 to 300 mm is adequate for most situations (McSorley 1987). However, for both shallow and deep-rooted crops, nematode distribution may follow the root distribution of the host plants (Barker and Nusbaum 1971; Ferris and McKenry 1974). In such cases, deeper samples may be more useful for accurate estimation of field nematode populations. On the other, seasonal changes, such as temperature and moisture, may affect the vertical distribution

pattern of nematodes (McSorley 1987). Therefore, it is essential to understand the distribution of plant parasitic nematodes, such as root-knot nematodes, in cropping soils in order to understand and predict nematode population change and to use this knowledge to improve nematode management systems (Duncan and McSorley 1987; McSorley and Phillips 1993).

1.2 Root-knot nematodes and grapevine

The discovery in 1850 that root-knot nematodes (*Meloidogyne* spp.) caused galls on cucumber roots is considered an important milestone in the field of nematode research (Mai *et al.* 1968). Root-knot nematode species can be found world wide affecting many plant species. More than 60 *Meloidogyne* species have been described with different pathogenicity on different host plants (Esnard and Zuckerman 1998). Four *Meloidogyne* species (*M. incognita, M. javanica, M. hapla* and *M. arenaria*) are reported to most commonly affect grapevine yield (Stirling 1976; Stirling *et al.* 1992). *M. hapla* is predominant in cooler regions such as southern Australia (Sauer 1974; Starling and Cirami 1984), in the northern vineyards of California (McKenry 1992) and in France (Boubals 1992). *M. javanica* tends to predominate in areas with a hot summer climate, such as the Murray Valley of Australia, the Central Valley of California (McKenry 1992) and in South Africa's Western Cape Province (Loubser 1988). *M. incognita* can also be found in all of this area (McKenry 1992), while *M. arenaria* is present in France (Boubals 1979). However, all four of these species could be found together in one region. For example, all four were reported from the Barossa Valley of South Australia (Stirling 1976).

1.2.1 Lifecycle of root-knot nematodes in grapevine

The life cycle of root-knot nematode in grapevine has been described by Bird (1978) and by others (McKenre 1992; Brown *et al.* 1993; Nicol *et al.* 1999). Root-knot nematodes are sedentary endoparasites, which hatch from eggs as second-stage juveniles. These migrate through soil to grapevine roots, invade roots and establish a feeding site. The feeding sites eventually become giant cells. The surrounding root cortex of the feeding site swells to form a characteristic gall. Second stage juveniles develop into adults by moulting three more times. Most of the adults are female, but some develop into males, which then stop feeding, leave the roots and move freely within the soil. One gall may contain one or several females, each of which may lay up to 1500 eggs in a gelatinous matrix on the root surface. In grapevine, each generation takes just over a month under optimal conditions (Bird 1978) and several generations may be produced per season (McKenre, 1992; Brown *et al* 1993). Thus, a single juvenile can give rise to more than 125 million progeny in a season lasting 3-4 months (Nicol *et al.* 1999).

1.2.2 Root-knot nematodes in Australian vineyards, grape yield and quality

Australian grape production is mainly based on own-rooted *Vitis vinifera* (Nicol *et al.* 1999), which is highly susceptible to root-knot nematodes. Root-knot nematodes are found in all viticultural regions of Australia. Almost all vineyards on sandy soils are infested with these nematodes, and overall infestation levels are probably similar to those in other countries with similar climates (Seinhorst and Sauer 1956; Meagher 1960; Sauer 1962; Meagher *et al.* 1976; Stirling 1976; Harris 1984; McLeod and Gendy 1996; Nicol *et al.* 1999). In California, up to 65% of vineyards are infested with root-knot nematodes (Nicol *et al.* 1999), while in South

Africa's West Cape Province 77% of surveyed vineyards contained root-knot nematodes (Smith 1977).

The exact yield loss in grapevine due to root-knot nematode is difficult to determine. However, it is estimated that Australian viticulture loses 7% of production to nematodes (Stirling *et al.* 1992). Root-knot nematode alone can reduce grape yield up to 60% in severe cases (Nicol and Heeswijck 1997). In California, the annual estimated loss from root-knot nematodes is about 20% of grape production (Raski 1986) and in Washington 10% of grape production is lost due to root-knot nematode alone (Esnard and Zuckerman 1998). In addition to direct yield losses, high nematode populations at planting may result in establishment problems, delayed development and uneven vine performance (Raski 1954; McElroy 1972; Hardie and Cirami 1988). Mechanical injuries caused by root-knot nematodes favour the entry of microbial pathogens (Port and Khan 1993) including *Rhizoctonia solani, Phytophthora cinamomi*, *Phythyum ultimum, Verticilium dahliae*, and *Dematophora necatrix* (Chiarappa 1959; McGechan 1966; Van der Merwe *et al.* 1972; Walker 1995). Based on field observation, Walker (1994) reported that 86% of grapevine roots damaged by root-knot nematodes were infested by the fungal pathogen *Rhizoctonia solani* compared to 22% infestation without infestation by RKN. Walker (1997) also found in a pot experiment that the severity of fungal root rot caused by *R. solani* was increased by combined inoculation with the fungus and root-knot nematodes.

While, there is no direct evidence on how grape quality is affected by nematode infestation but various studies have shown that optimal requirements for quality grapes, such as respiration, photosynthesis, nutrient absorption and translocation, water relations, hormone balance and sugar accumulation can be affected by nematode infestation (Hussey and Williamson 1998). Nematode

parasitism in roots can disrupt physiological processes throughout the whole plant. Root damage affects nutrient and water uptake and translocation by roots causing stunted, usually chlorotic and low yield (Dropkin 1979).

1.2.3 Symptoms in grapevine caused by root-knot nematode

Poor or restricted vine growth, reduced yields, and off-coloured grapes are observed in fields heavily infested with root-knot nematodes (Raski 1988, Fig 1.1). The secondary root systems become severely deformed by the formation of galls where the nematodes have invaded, become sedentary and matured (Esnard and Zuckerman 1998, Fig. 1.2 and Fig. 1.3). Feeder roots are usually killed. Aboveground symptoms of root-knot infestation are most prominent in sandy soils where nutritional deficiencies and water stress are greatest. One species of root-knot nematodes (*Meloidogyne nataliei*) parasitises vines without inducing galls, but it is only known from vineyards in Michigan (Diamond 1994).

1.2.4 Quantification of root-knot nematodes

Many kinds of nematodes occur in association with plants but damage only results from high population densities of plant parasitic nematodes, rather than from mere occurrence. In a vineyard, accurate quantification of population density and potential for increase of a parasitic species is critical in anticipating crop damage (Duncan and Noling 1998). Unreliable quantification of nematodes will limit the definition of economic thresholds and the assessment of management options in grapevine. Recent developments in nematology have seen the provision of a commercial service for the quantification of nematodes based on DNA technologies in soil used for field crops (Hannam 1999). This technology is being used in quantification of root-lesion nematodes and some soil borne fungal diseases in

Figure 1.1 Restricted growth of grapevine in root-knot nematode infestation vineyard

Figure 1.2 Galled feeder roots of grapevine due to root-knot nematode infestation

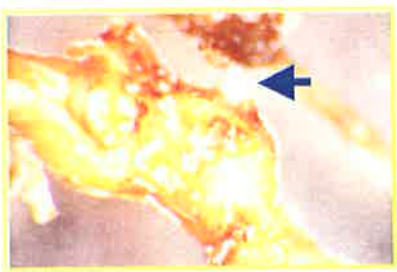


Figure 1.3 Egg mass from female in gall

cereals (Hannam 1999). It is considered to be more accurate and reliable than conventional methods for quantification of plant parasitic nematodes (Hannam 1999). The approach offers potential for viticulture to better define pest levels and to assess the applicability of various control strategies. However, despite availability of DNA probes, further development of the technology is needed before a commercial service can be offered to the viticulture industry.

1.2.4.1 Molecular based population study

The identification of nematodes has been based largely on morphological and physiological differences, to some extent reproductive isolation, general ecological differences, and quantification relies on counts under a microscope based on morphology (Hirschmann 1971; Luc *et al.* 1990). More recently, biochemical and molecular techniques have been used in the identification of nematodes (Curran and Robinson 1993; Ferris and Ferris 1992).

Many methods are available to identify species and biotypes of root-knot nematodes, such as study of morphology (Jepson 1978), differential host range (Taylor and Sasser 1978) and cytogenetics study (Triantaphyllou 1985). Most of these techniques are inaccurate, unreliable and/or time consuming (Stanton *et al.* 1997). McLeod and Steel (1999a) reported that identification of *Meloidogyne* spp. by perineal pattern, from 17 vineyards within five viticultural districts in NSW, was inconsistent with identification by mtDNA analysis. The advent of DNA based diagnostics offers an opportunity to overcome these problems.

Some biochemical tools have been used to identify species of *Meloidogyne*, such as isozyme patterns (Esbenshades and Triantaphyllou 1985). A number of molecular tools, such as random amplified polymorphic DNA (RAPD) (Cenis 1993; Baum *et al.* 1994), restriction fragment length polymorphism (RFLP) of amplified

sequences of mitochondrial DNA (Harris *et al.* 1990; Power and Harris 1993; Hugall *et al.* 1994; Stanton *et al.* 1997) and mtDNA analysis (Stanton *et al.* 1997) to generate DNA polymorphic marker(s) to differentiate species of nematodes are available. Recently, sequences of the internal transcribed spacer (ITS) region, located within the cistrons of rDNA genes have proved a powerful tool for species or subspecies identification of many organisms including root-knot nematodes (Powers *et al.* 1997; Zijlstra *et al.* 1997; Szalanski *et al.* 1997; Uehara *et al.* 1999; Goncalves and Rosto 2000). The versatility in the ITS as a genetic marker made this region attractive for a wide range of genetic studies such as diagnostics, phylogenetic study, evaluation of population level evolutionary process and molecular taxonomy (Cherry *et al.* 1997; Stanton *et al.* 1997; Uehara *et al.*1999; Goncalves and Rosto 2000).

1.2.4.2 Ribosomal DNA

Ribosomal RNA (rRNA) genes exist as tandem arrays or cistrons in the DNA (Noller 1984). DNA in each set of chromosomes may contain hundreds of cistrons (Fig. 1.4), a factor that is important when dealing with very small amounts of DNA. In most eukaryotes, the 5' to 3' organisation of a cistron is an external transcribed spacer (ETS), the 18S gene, an internally transcribed spacer one (ITS1), the 5.8S gene, an internally transcribed spacer two (ITS2), and 28S rRNA gene (Fig. 1.5; Brosius *et al.* 1981; Noller *et al.* 1980). Each cistron is separated from its neighbour cistron by intergenic sequences (IGS). The three genes are transcribed as a single unit then the external and internal transcribed spacers are spliced off to from the mature 16-18S, the 5.8S and the 26-28S rRNAs (Fig. 1.5 and 1.6; Michot *et. al.* 1984; Nomura *et al.* 1969). The mature rRNAs are bind together with ribosomal proteins to form ribosomes, the protein synthesis unit (Fig. 1.7). Ribosomes are

composed of two, a large and a small, sub-units (Fig. 1.7). These subunits are complex of proteins and structural RNA molecules (Fig. 1.6 and 1.7).

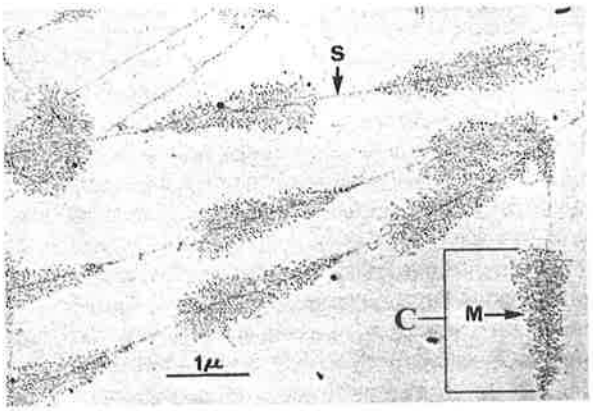


Figure 1.4 Electron microscopy of cistrons (C). Transcription of cistrons generates a series of matrices (M), each separated from next by non transcribed spacer or inter genic sequences (S). (After Lewin 1994)

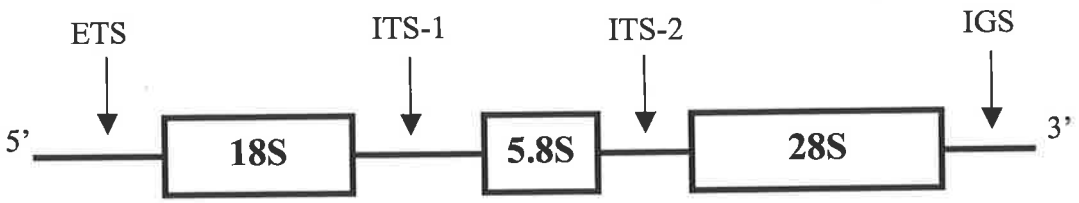


Figure 1.5 A cistron of ribosomal genes. External transcribed spacer (ETS), 18S gene, internally transcribed spacer one (ITS1), the 5.8S gene, internally transcribed spacer two (ITS2), 28S gene and intergenic spacer (IGS) region. (After, Lewin 1994).

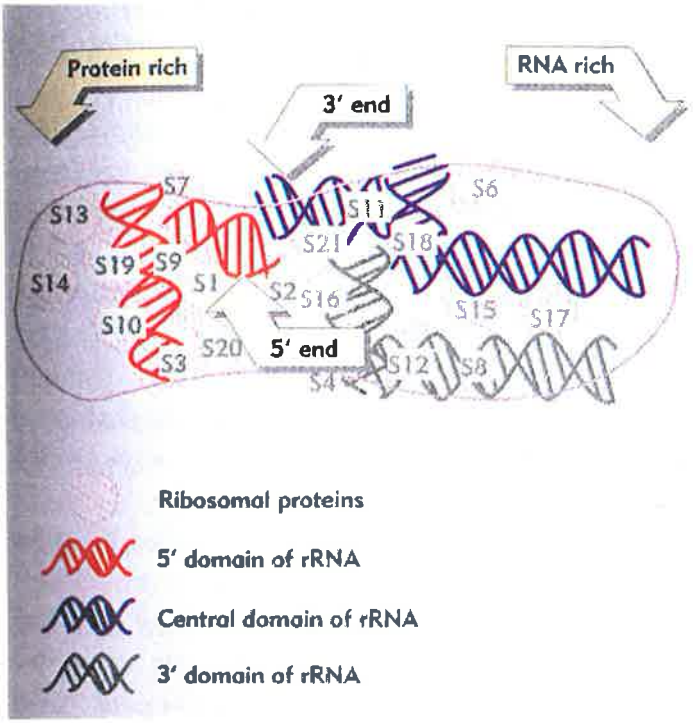


Figure 1.6 Two-dimensional projection of a three dimensional reconstruction of 30S subunit of a ribosome. (After, Lewin 1994).

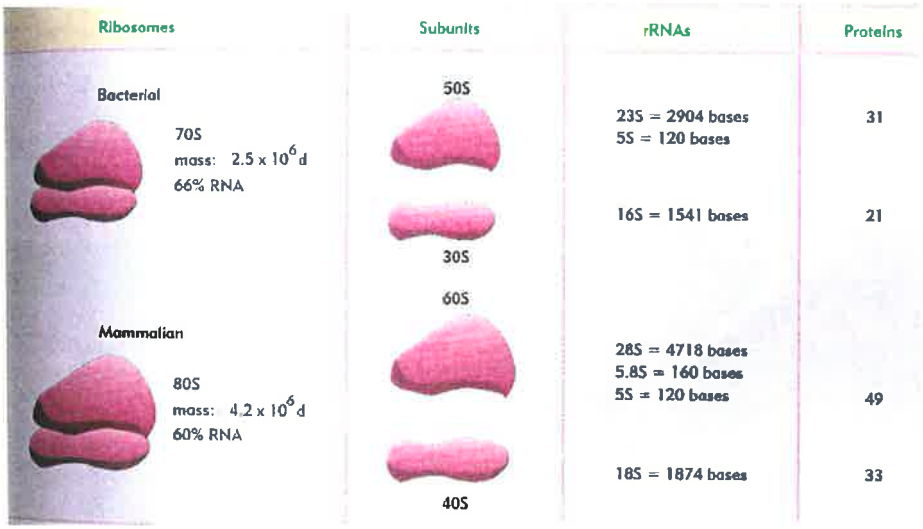


Figure 1.7 Structural elements of ribosome. (After, Lewin 1994).

1.2.4.3 Ribosomal DNA based detection and population structure

The rDNA, which include rRNA genes and ITS, is a mosaic of conserved and variable domains which allows the use of conserved PCR-primer sets to initiate PCR amplification from targeted domains in the ribosomal genes or to amplify regions between and within these gens (Thomas and Wilson 1991; Vrain *et al.* 1992). Vrain *et al.* (1992) designed two primers of 21 sequences each from the conserved sequences in 18S and 26S genes of ribosomal DNA of *Caenorhabditis elegans* to amplify partial sequence of 18S gene, complete sequences of ITS1, 5.8S, ITS2 and partial sequences of 28S gene of *X. americanum* to study population structure of this nematode. These two 21 nucleotide sequences were also homologous to the sequences found in rDNA of *Caenorhabditis elegans* and other nematode sequences in the GenBank (Ellis *et al.* 1986; Vrain *et al.* 1992). Currently these two primers have been widely used in the rDNA-based detection of many nematode species including root-knot (Power *et al.* 1997; Zijlstra *et al.* 1997). These primer sets can be directed to span regions of great variability, which lie between the conserved primer binding sites (Power *et al.* 1997). So far, the ITS region of most nematode species can be amplified with these universal primers (Powers *et al.* 1997).

In general, coding regions for genes are more conserved through the evolution than the less functional ITS region (Vrain *et al.* 1992; Zijlstra *et al.* 1997). Therefore, size variation or restriction length variation in ITS can be observed within or between texa (Zijlstra *et al.* 1997). The ITS based RFLP or sequence variations have been shown to be a good indicators of species identification and to study population variation of root-knot nematodes (Zijlstra *et al.* 1997; Powers *et al.* 1997).

1.3 Crop loss assessment in grapevines

Crop loss assessment in nematology is used to determine economically important species and their impact on growth and yield for rational management decisions (Duncan and Noling 1998). This information is important to evaluate any control method based on its ability to reduce the nematode population in the soil below the minimum density that inhibits growth (Barker and Olthof 1976).

Impact risk studies in grapevine have mostly been conducted in artificial environments to compare growth against different nematode population levels (Anwar and Gundy 1989; Walker 1997). Such information is often considered to be more useful when derived from field data because the physical conditions and special patterns of nematodes inoculated in pots differ from those in the field (Noe and Campbell 1985; Walters and Braker 1993). It is quite clear that the edaphic, biotic and climatic effects on plants and nematodes cannot be fully reproduced in pot studies. In contrast the relationship between nematode density and yield under field condition is influenced by patchiness in naturally infested soil (Noe 1993). Containerised, micro-plot field studies provide a compromise between the need for experimental control and natural conditions (McSorley *et al.* 1985).

Methods to conduct micro-plot field trials involve establishing nematode infested and nematode-free plots, or establishing plots with a range of nematode densities, through the use of nematicides or other means (Ferris 1984a). The micro-plot approach is especially useful to study cumulative effects of nematodes in grapevines because the cost of long-term studies in vineyard can be prohibitive (Duncan *et al.* 1999). In Michigan USA, Ramsdell *et al.* (1996) evaluated the effects of four species of plant parasitic nematodes including *M. hapla* on hybrid grapevines under micro-plot conditions for six years. No such study has yet been conducted in Australia to estimate the density dependent damage caused by root-knot nematodes.

1.4 Objectives

Based on this review of the literature, the following objectives were set as priority areas for investigation in grapevine nematology in South Australia:

- To develop sampling, detection and quantification techniques for RKN affecting grapevines.
- To assess population structure of RKN in vineyards to validate the DNA-based quantification technique.
- To determine damage thresholds for the root-knot nematode species affecting grapevines.

CHAPTER TWO
DEVELOPMENT OF SOIL SAMPLING METHOD

2.1 Distribution pattern of root-knot nematodes (*Meloidogyne* spp.) in vineyards and soil sampling method

2.1.1 Introduction

Like many other plant parasitic nematodes, root-knot nematodes (RKN, *Meloidogyne* spp.) are likely to be unevenly distributed in agricultural soil. This irregular distribution pattern, especially the horizontal distribution, of nematodes is probably the greatest obstacle to the reliable determination of nematode population density in agricultural soil (McSorley 1998). Few studies have been made of the distribution patterns of RKN in vineyards (Ferris and McKenry 1977; Rao *et al.* 1979) and more information is needed to standardise methods for sampling vineyards in Australia (Nicol *et al.* 1999). This information is essential for assessments of nematode population densities in the field to be meaningful (Araya *et al.* 1999). The main objective of this study was to determine the distribution of RKN in infested vineyards relative to the position of the vine, with a view to recommending a standardised sampling position.

2.1.2 Materials and methods

Five RKN infested vineyards in South Australia, two in New Residence, one in McLaren Vale and two in Padthaway, were selected for this study. The details of these vineyards are given in Table 2.1.1. The grapevines were showing symptoms such as restricted vine growth. Five vines per field were selected randomly from these affected vines. Soil samples were collected from these vines between August and October 2000. Ten soil samples were collected from five positions within about 100 to 1500 mm from the vine in row and inter-row. The positions were:

Table 2.1.1 Soil types, cultivars, vineyard age and cover crops in five vineyards in three locations of South Australia infested with root knot nematodes (*Meloidogyne* spp.).

Locations	Sites	Soil types	Cultivar	Vineyard age (years)	Inter-row cover crop	
					Common and scientific name	RKN host status
New Residence (34^0 22'S & 140^0 24'E)	1	Sandy loam	Merlot	2	Mustard (*Brassica* sp.)	Good host[a] (*M. javanica* & *M. incognita*)
	2	Sandy loam	Colombard	2	Mustard (*Brassica* sp.)	Good host[a] (*M. javanica* & *M. incognita*)
McLaren vale (35^0 13'S &138^0 32'E)	1	Sandy loam over sandy clay	Chardonnay	11	Oat (*Avena fatua*) cv. Swan/Wallaroo	Non host[a]
Padthaway (36^0 36'S &140^0 29'E)	1	Sandy clay loam	Pinot Nior 05V12	19	Capeweed (*Arctotheca calendula*) Sorrel (*Rumex acetosella*) Marshmallow (*Malva parviflora*) Sub clover (*Trifolium subterraneum*)	Good host[b] (*M. hapla*) Moderate host[c] (*M. arenari*) Good host[d] (*M. arenaria, M. incognita & M. javanica*) Good host[e] (*M. arenaria, M. incognita & M. javanica*)
	2	Light sand over red clay subsoil	Riesling	28	Cocksfoot (*Dactylis glomerata.*)	Good host[f] (*M. chitwoodi*)

[a]McLeod and Warren 1993; [b]Stirling and Wachtel 1985; [c]Tedford and Fortnum 1988; [d]Ibrahim *et al.* 1982; [e]Kouame *et al.* 1989; [f]Griffin *et al.* 1984

P1, in inter-row under the cover crop, midway between vines of neighbouring rows,

P2, adjacent to the cover crop on a line between P1 and the vine (about 300 mm from the vine),

P3, about 100 mm from the vine on a line between P1 and the vine,

P4, about 100 mm from the vine along the row,

P5, midway between vines along the row.

Soil samples (about 600 ml) were collected from 0-300 mm and 300-600 mm depths at each position using a 50 mm diameter auger. The soil was mixed gently and nematodes were extracted from 400 g soil at field moisture content for each sample for 5 days using Whitehead tray method (Whitehead and Hemming 1965). Nematodes were collected on a 20 μM aperture sieve and stored in water in closed containers at 5^{O} C until counted. Nematodes were counted in a Sedgewick Ratter Cell (Graticules Ltd, Tonbridge, UK) counting slide under compound microscope at 100X or 200X magnification. A sub-sample of 200 g moist soil was oven dried at 105^{O} C for 72 hours to determine the constant dry weight (Gardner 1968). This dry weight was used to estimate nematode population per 400 g of soil (Hooper 1986).

A log(x+1) transformation was performed for raw data. Data was analysed by analysis of variance using a nested block and treatment structures to accommodate spatially dependent elements of the experimental design and logarithmic transformation to adjust for non-normality of the raw data with the statistical packages GENSTAT 5 (Lawes Agricultural Trust, Rothamsted Experimental Station).

2.1.3 Results

The grapevines of all the vineyards studied were own rooted and in light-textured soils (Table 2.1.1). The mean RKN population across all vineyards was about 300 nematodes per 400 g soil (median 150 RKN/400 g) but the infestation of individual vines reached as much as 4000 nematodes per 400 g soil.

The mean RKN population density in the row was significantly higher than the population density in the inter-row under or next to cover crops (Figure 2.1.2). In addition, the RKN population density next to cover crops was significantly higher than that under cover crops (Figure 2.1.2). The RKN population density did not vary significantly along the rows (Figure 2.1.2). There was no significant difference in densities between the sampling depths, and no significant interactions between sample position and depth.

The RKN population density differed significantly with position across the three regions (Figure 2.1.3). The RKN population density under cover crops (P1) at Padthaway was significantly higher than at McLaren Vale and New Residence. RKN population densities next to cover crops (P2) were similar in all locations. At McLaren Vale the RKN population densities in the row were significantly higher than in the inter-row. In contrast, at Padthaway and New Residence at least two sampling positions from within the row were not statistically different from the population in the inter-rows next to the cover crop. Within each location, the RKN population density at the three sampling positions (P3, P4 and P5) within rows did not vary significantly.

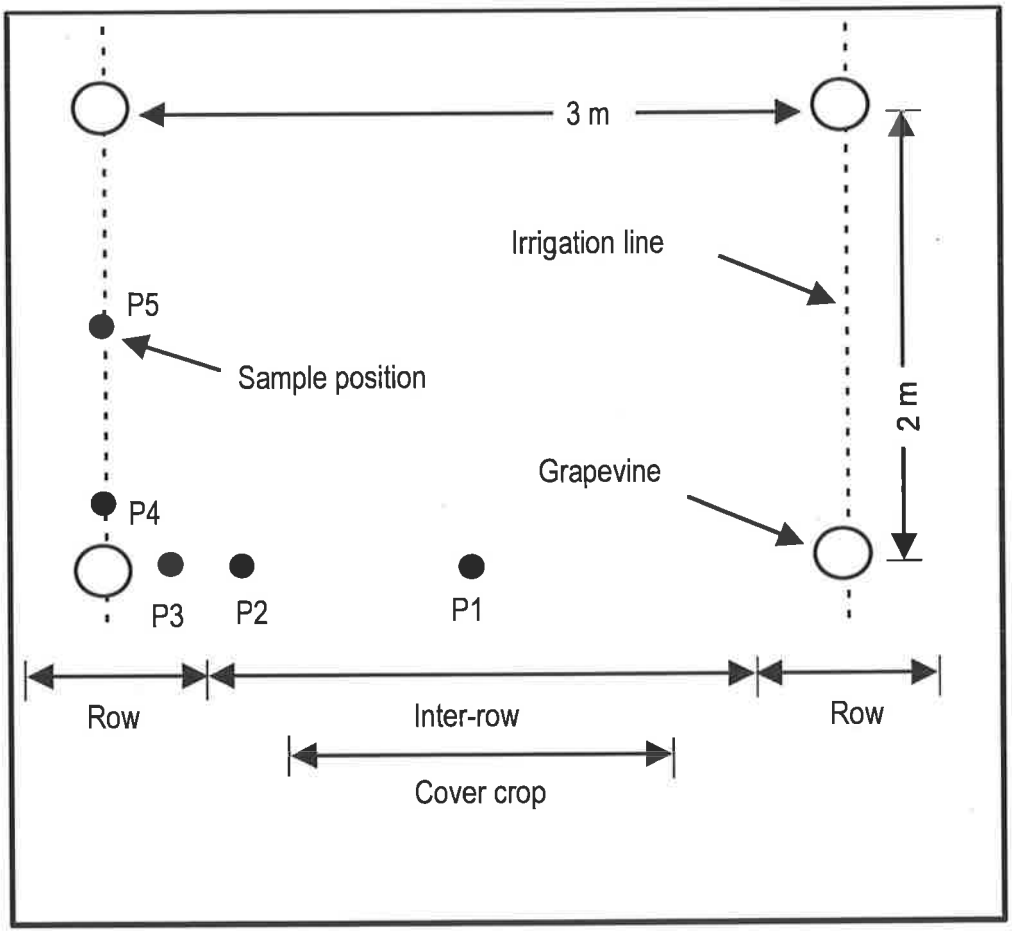


Figure 2.1.1 Schematic diagram of showing positions (P1-P5) sampled in South Australian vineyards relative to the grapevines and row spacing (dimensions approximate).

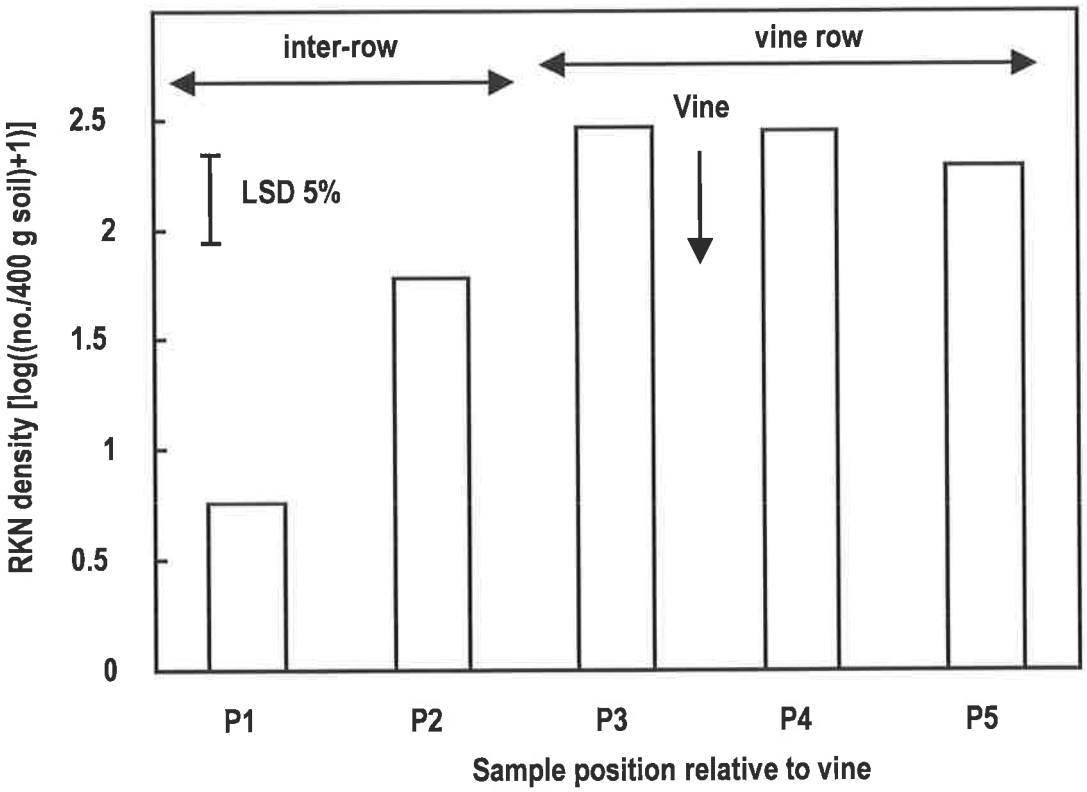


Figure 2.1.2. Mean population of root-knot nematodes (*Meloidogyne* spp.) positions in five infested vineyards in South Australia (details of sampling position given in the text and Fig. 2.1.1).

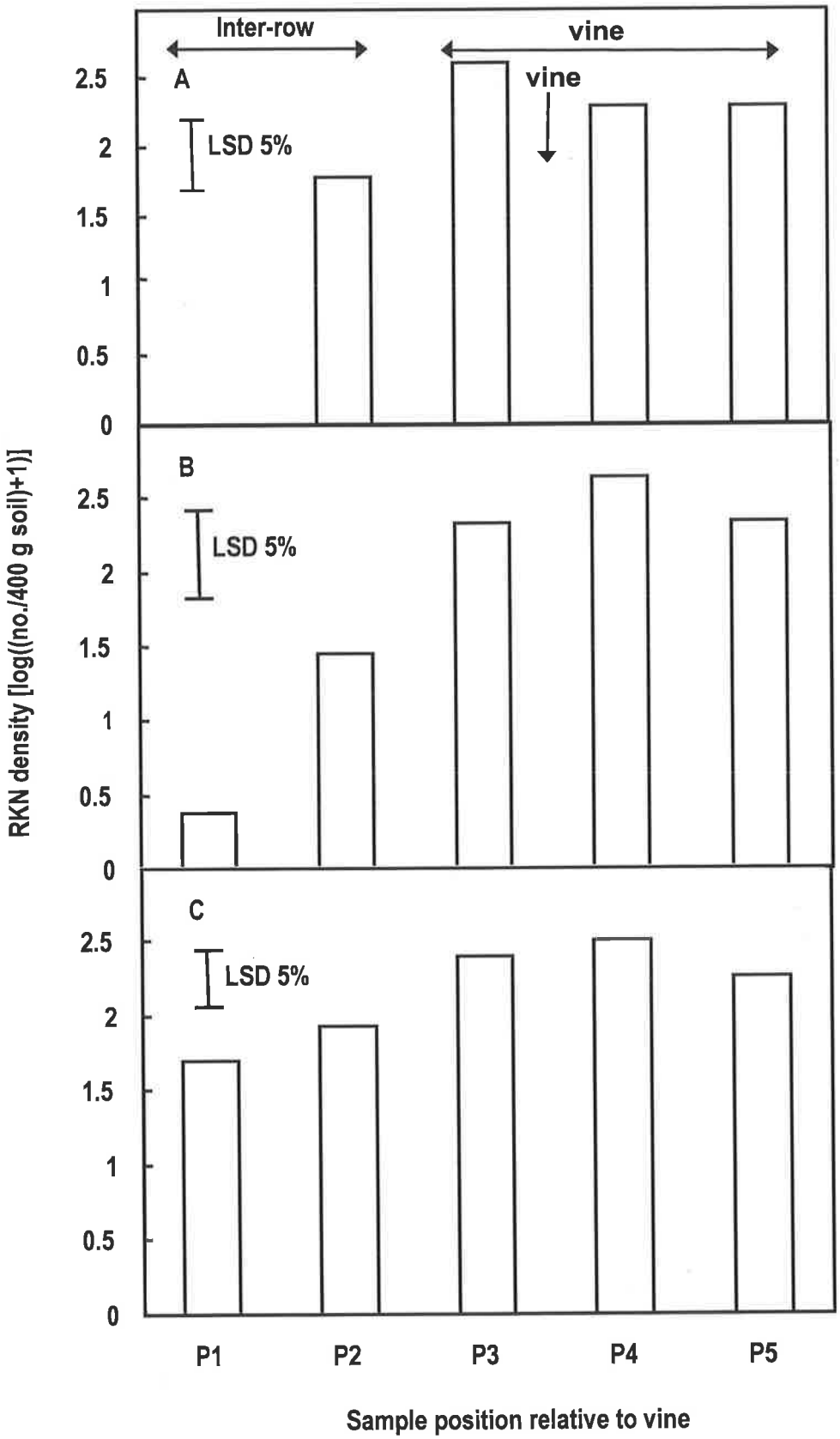


Figure 2.1.3 Mean population of root-knot nematodes (*Meloidogyne* spp.) at five positions in infested vineyards in three growing areas in South Australia (details of sampling position given in the text and Fig. 2.1.1, within region LSD shown). A. New Residence. B. McLaren Vale. C. Padthaway.

2.1.4 Discussion

The light soil types and susceptible cultivars grown on their own roots in vineyards studied provided conditions highly favourable for the multiplication of RKN. Verma *et al.* (1998) found that the reproduction rate of *M. incognita* was greatest in sandy soil, followed by loamy, and least in clay. In Australia, the occurrence of RKN in grapevines in sandy soil is more likely than in loamy clay or clay soils (Handreck 1972; Nicol *et al.* 1999). The population densities estimated here fell within the range (40-400 RKN per 200 ml of soil) considered to present a moderate risk of yield loss (Stirling *et al.* 1999).

The significantly higher population density of RKN in vine rows compared to inter-rows indicates that these nematodes are mainly aggregated within the root zone of the grapevine. In a two-year study of irrigated own-rooted grapevine (cv. Thompson seedless) in California, USA, *Meloidogyne* spp. were found only in the area in which the grapevine root system occurred (Ferris and McKenry 1977). Hunter (1998) found that the majority of vine roots are located within the in-row distances. This pattern of distribution is common in sedentary endoparasitic nematodes, such as RKN, which deposit all of their eggs at site of invasion, frequently in masses, leading to aggregated distribution (Ferris *et al.* 1990). The similarity of population densities found within the row indicates that core samples could be collected from anywhere in the rows. In an endeavour to establish a standardised sampling position for grapevines, it is recommended that samples be collected about 100 mm from the vine, given that higher counts tended to occur closer to the vine and vine spacing varies between vineyards. Also, the finding that high population densities were restricted to the vine rows is of considerable practical significance in that control measures should be concentrated in this area, greatly reducing chemical applications and the cost of other treatments (Rao *et al.* 1979).

The lack of interaction in population density at two depths indicates that vertical distribution pattern of RKN in the vineyards sampled did not vary significantly over these two depths. Ferris and McKenry (1977) also failed to find differences in RKN populations in the upper 600 mm of soils in Californian vineyards. This indicates that representative core sample for RKN can be collected from the top 300 mm of soil in vineyards. Despite of the inclusion of a range of cultivars of different ages (two to 28 years, Table 2.1.1), the consistent pattern of RKN distribution relative to the vines indicated that the distribution of the nematode was not greatly affected by the age of vines or cultivars. So it appears reasonable to conclude that the distribution pattern of RKN in SA vineyards (or at least similar vineyards) established for more that two years would be similar.

The low density of RKN in the inter-row occurred irrespective of the susceptibility of cover crops. This may result from a low density of vine roots in inter-rows and little contact between vine roots and roots of susceptible cover crops. Hunter (1998) found that the majority of grapevine roots were located within the vine row. Therefore, suppressive activity from the roots of cover crops or incorporated organic matter in inter-row may have minimal or no impact on RKN population density in the row. The highly compacted soil between the row and inter-row resulting from the regular movement of farm machinery may creates a barrier to any potential lateral movement of benefit associated with cover crops. Therefore, growing of non-host or nematode-suppressive cover crops in the inter-row may not produce useful control of RKN populations in vine rows. More research is needed to evaluate the possibility of growing suppressive plants close to the vines to achieve any improved control. Studies over six years showed that some cover crops, such as perennial ryegrass (*Lolium perenne* L.), did not significantly effect the incidence and severity of bacterial and fungal diseases, fruit yield and quality of tomato (McKeown

et al. 1998). However, a full investigation on the effect of plants grown close to grapevines is needed before drawing any conclusion.

On the other hand, care should be given to selecting cover crops, because presence of adjacent susceptible hosts might create an extra inoculum source for the vine row during inter cultural operations. Clearly more information is needed by growers for the selection of cover crops, because in all but one vineyard in this study had cover crops that are known to be moderate to good hosts of RKN species that damage grapevine. Care should also be taken in selecting cover crops that are hosts for other nematodes damaging to grapevine. In Florida, sorghum has been extensively used to reduce *Meloidogyne* spp. and to increase the amount of soil organic matter, but after a few years of sorghum cultivation sting nematode (*Belonolaimus longicaudatus*) had become a significant problem (Overman and Martin 1978). In Australia, there is little information on host status of cover crops to nematodes that infest grapevine (Nicol and Heeswijck 1997; Nicol *et al.* 1999; McLeod and Steel 1999b). However, more comprehensive study is needed to select plant species that are not hosts of major grapevine nematodes or other grapevine pests and that may have nematicidal properties.

2.2 Assessment of the RKN sampling technique for its ability to sample other importent nematodes in vineyard

2.2.1 Introduction

Knowledge of the vertical, horizontal and seasonal distribution of plant parasitic nematodes is important to determine an appropriate sampling procedure for nematode quantification for predictive and diagnostic purposes. These studies also help to identify factors that affect nematode population dynamics.

Dagger nematodes (*Xiphinema* spp.) are present in all major grapevine growing areas of the world (Raski 1988). They are commonly found in soil samples from vineyards in Australia and are probably an important component of the nematode pest complex of grapevines (Nicol *et al.* 1999). Three species, *Xiphinema index*, *X. americanum* and *X. pachtaicum* have been identified in Australian vineyards (Meagher *et al.* 1976; Harris 1980). *Xiphinema index* and *X. americanum* can transmit grape fanleaf virus (GFV) and peach rosette mosaic virus (PRLV), respectively (Hewitt *et al.* 1958). PRLV has not been recorded in Australia but GFV and its vector *X. index* are present in some grapevine growing areas (Harris 1980). The dagger nematode-virus complex causes significant economic damage in Californian vineyards (Raski 1988).

Root lesion nematodes (*Pratylenchus* spp.) have been associated with poor growth in grapevine (Raski and Krusberg 1984). So far six species, *P. vulnus*, *P. coffeae, P. jordanensis*, *P. neglectus*, *P. thornei* and *P. zeae* have been found in soil and root samples from vineyards of major viticultural regions of Australia (Meagher *et al.* 1976; Stirling 1976; Mcleod *et al.* 1994; Walker 2000a,b). It has been reported in California that about 70% of vineyards are infested with *Pratylenchus* spp. (Nicol *et al.* 1999). Pinochet *et al.* (1976) reported that the inoculation of the cultivar

Thompson Seedless in pots with 500 *X. index* or 1000 *P. vulnus* together, or individually, causes significant suppression of root and shoot growth.

There have been two separate studies of the seasonal changes in distribution of *Xiphinema* spp. (Harris 1980) and *Pratylenchus* spp. (Walker and Morey 2001) in Australia but no similar information is available for mixed populations of *Pratylenchus* and *Xiphinema.* Such information is important to standardise methods for sampling vineyards and for the development of effective management strategies (Nicol *et al.*1999). The objective of this study was to assess the horizontal, vertical and seasonal distribution pattern of *Xiphinema* and *Pratylenchus* spp. with a view to recommending a standardised sampling procedure in relation to vines. The rainfall and temperature records from station close to experimental site were used to support the interpretations of seasonal fluctuations in *Xiphinema* spp. and *Pratylenchus* spp.

2.2.2 Materials and methods

A commercial vineyard in Nuriootpa (34°28'S 138°59'E), South Australia, infested with *Xiphinema* spp. and *Pratylenchus* spp. was selected for the study. The site had a sandy loam (0-200 mm) over loamy clay sub soil planted with the grape cultivar Shiraz on own roots. The field was drip irrigated from late November to late March. Inter-row cover crop ryegrass (*Lolium perenne*) was sown in May 2000 and crop residues were incorporated into soil following the seed harvest in October. The cover crop was sown again in May 2001.

The study began in early July 2000. Initially, 20 soil samples (one sample/vine) were collected randomly, about 100 mm away from the vines within row at a depth of about 600 mm and assessed for nematode population density to locate vines with heavy infestations of dagger and lesion nematodes for further study.

Each of the sample locations in the vineyard was marked for future sampling. The data were not included in further analysis.

For the study of spatial (vertical and horizontal) distribution nine marked vines with the highest nematode population densities were selected. For each vine, ten soil samples were collected in August 2000 from five positions (P1-P5) within about 1500 mm from the vine within the row and inter-row (Fig.2.1.1 previous experiment of this chapter, p 36) and from two depths (0-300 and 300-600 mm) with an auger (50-mm diameter).

For the study of temporal (seasonal) distribution four vines from the nine selected above, were sampled throughout the year. Soil samples were collected approximately at monthly intervals from September 2000 to August 2001. At each sampling time, at total of four samples were collected from a position 100 mm away from a vine within the row (equivalent to P3 or P4) and two from the inter-row (P1) at two depths (0-300 and 300-600 mm). Subsequent samples were taken about 70 mm from earlier positions to avoid errors introduced by root damage during previous sampling.

Soil from each sample (about 600 ml) was mixed carefully and placed in a polythene bag. Soil was transported in insulated containers and processed within 6 h of collection. A sub-sample of 200 ml was processed for *Xiphinema* spp. by the method of Flegg (1967). Different growth stages of *Xiphinema* spp. were determined under the microscope on morphological characters such as body size, and the presence and position of female reproductive organs (Hunt 1993). Another 200 ml sub-sample was used to extract *Pratylenchus* spp. for seven days using the Whitehead tray method (Whitehead and Hemming 1965). The roots present in each sub-sample (about 0.5-3 g) were cut into pieces and macerated in a blender for one minute and added to the top of 200 ml soil placed on the same Whitehead tray for

Pratylenchus spp. extraction (Mani and Hinai 1996). Nematodes were collected with a 20 μm aperture sieve and stored in water in a closed container at 5^{O}C until counted. Nematodes were counted in a Sedgewick-Rafter Cell (Graticules Ltd, Tonbridge, UK) under a compound microscope at 100-200x magnification.

The temperature and rainfall data were obtained from the Bureau of Meteorology Australia for the Nuriootpa Viticultural Station (Station no. 023373, 34^{O}45'S 139^{O}00'E), South Australia (Table 2.2.1).

The statistical package GENSTAT 5 (Lawes Agricultural Trust, Rothamsted Experimental Station) was used to analyse all dada. Nematode population densities were transformed [$\log_{10}(x+1)$] to compensate for the non-normality of the raw data. All the transformed data were used to general analysis of variance. The transformed data were analysed by analysis of variance using nested treatment structures to accommodate the spatially dependent elements of the experimental design. For the spatial distribution data, depth was nested with position and for the seasonal distribution data, depth was nested within position, which was nested within sampling time. The correlations between the mean number of both nematodes enumerated at each position and depth (individually and combined) for each sampling time and the corresponding mean temperature and total rainfall for the month were calculated.

2.2.3 Results

2.2.3.1 Spatial distribution

Xiphinema spp. were mainly aggregated along the vine row, while *Pratylenchus* spp. were distributed more evenly within and between rows (Fig. 2.2.2). The mean population density of *Xiphinema* spp. (0-600 mm) was

significantly greater close to the vine (P3 and P4) than mid way between the vines (P5), and declined to the lowest level in the middle of the inter-row (P1).

Where *Xiphinema* spp. were numerous (P2-P5), the population density at 300-600 mm was significantly greater than at 0-300 mm. The mean population densities of *Pratylenchus* spp. (0-600 mm) were similar across all sampling positions. However, *Pratylenchus* spp. was significantly more numerous at 0-300 mm in all positions.

2.2.3.2 Temporal distribution

Mean population densities (0-600 mm) of both *Xiphinema* spp. and *Pratylenchus* spp. were greatest in October-November and least in February (Fig. 2.2.3). The overall population densities of both the nematodes started to increase again from March and continued to the end of the study period (Fig. 2.2.3). Throughout the year the population density of *Xiphinema* spp. in the row was significantly greater than in the inter-row (Fig. 2.2.4A). In contrast, the density of *Pratylenchus* spp. did not differ significantly between the row and inter-row (2.2.4B). Over the whole year the population density of *Xiphinema* spp. at 300-600 mm significantly exceeded the density at 0-300 mm, however, this difference was not found in all months (Fig. 2.2.4C). Conversely, *Pratylenchus* spp. was mostly more numerous at 0-300 mm but similar to *Xiphinema* spp. this pattern was not found at every sampling time (Fig. 2.2.4D).

There was no significant specific trend in the occurrence of the various developmental stages of *Xiphinema* spp. over time (Fig. 2.2.5). However, correlation analysis showed a significant positive relationship ($r = 0.60$, $p = 0.05$) between the number of adults and number of juveniles of *Xiphinema* spp.

Rainfall and temperature data are presented in Table 2.2.1. The correlation analysis only revealed a few significant negative relationships between temperature and *Pratylenchus* spp. population density, and only accounted 50% or less of the variation in the data (Table 2.2.2).

Table 2.2.1 Monthly rainfall and temperature during 2000-2001 in Nuriootpa viticultural district (Source: Bureau of Meteorology Australia).

Sampling time (2000-2001)	Total monthly rainfall (mm)	Mean daily temperature (^{O}C)		
		Maximum	Minimum	Mean
September	57.2	21	9	15
October	52.8	21	9	15
November	21.8	30	15	22.5
December	8.0	30	15	22.5
January	13.2	36	18	27
February	15.2	33	18	25.5
March	35.0	27	12	19.5
April	21.4	24	9	16.5
May	68.0	21	8	14.5
June	68.2	18	6	12
July	44.0	15	6	10.5
August	85.8	18	6	12

Table 2.2.2 Significant inter-relationships between occurrence of nematodes (*Xiphinema* spp and *Pratylenchus* spp.) and weather conditions (temperature).

Inter-relationship(n=24-48)	Value of correlation coefficient (r)
Temperature and total nematodes at 0-600mm	- 0.462*
Temperature and *Pratylenchus* spp. at 0-600 mm	- 0.540*
Temperature and *Pratylenchus* spp. at 0-600 mm in row	- 0.418*
Temperature and *Pratylenchus* spp. at 0-600 mm in inter-	- 0.479*
Temperature and *Pratylenchus* spp. at 300 mm	- 0.533*
Temperature and *Pratylenchus* spp. at 600 mm	- 0.525*

* = Significant at 5% level.

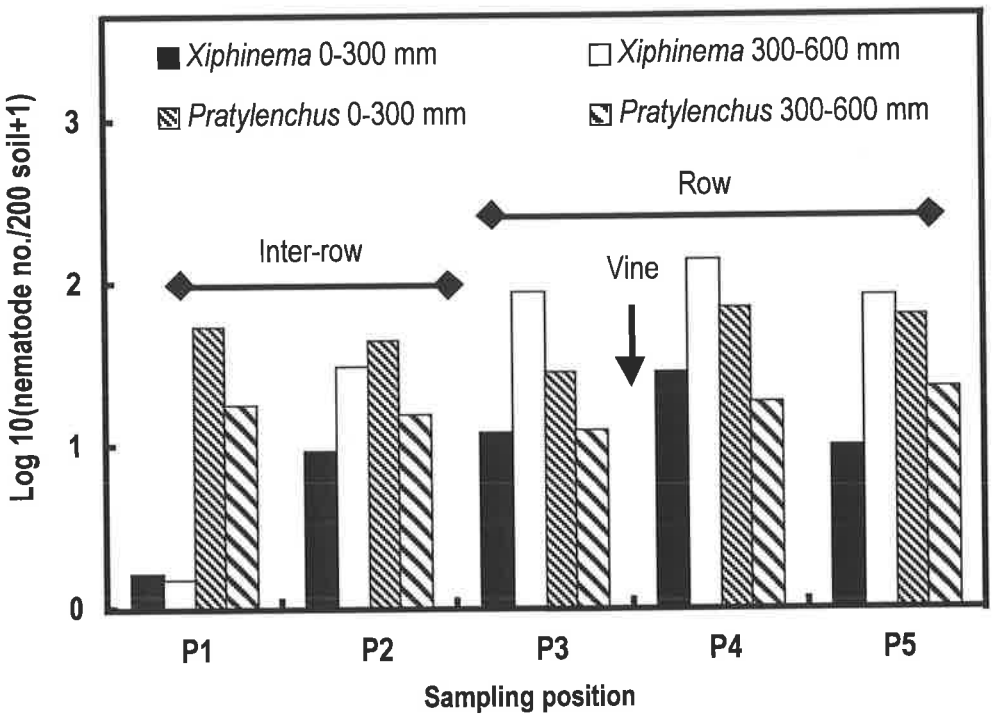


Figure 2.2.2 Mean populations of *Xiphinema* spp. and *Pratylenchus* spp. at two depths and five positions relative to grapevines in a vineyard near Nuriootpa, South Australia. (Positions P1-P5 described in Fig. 2.1.1).

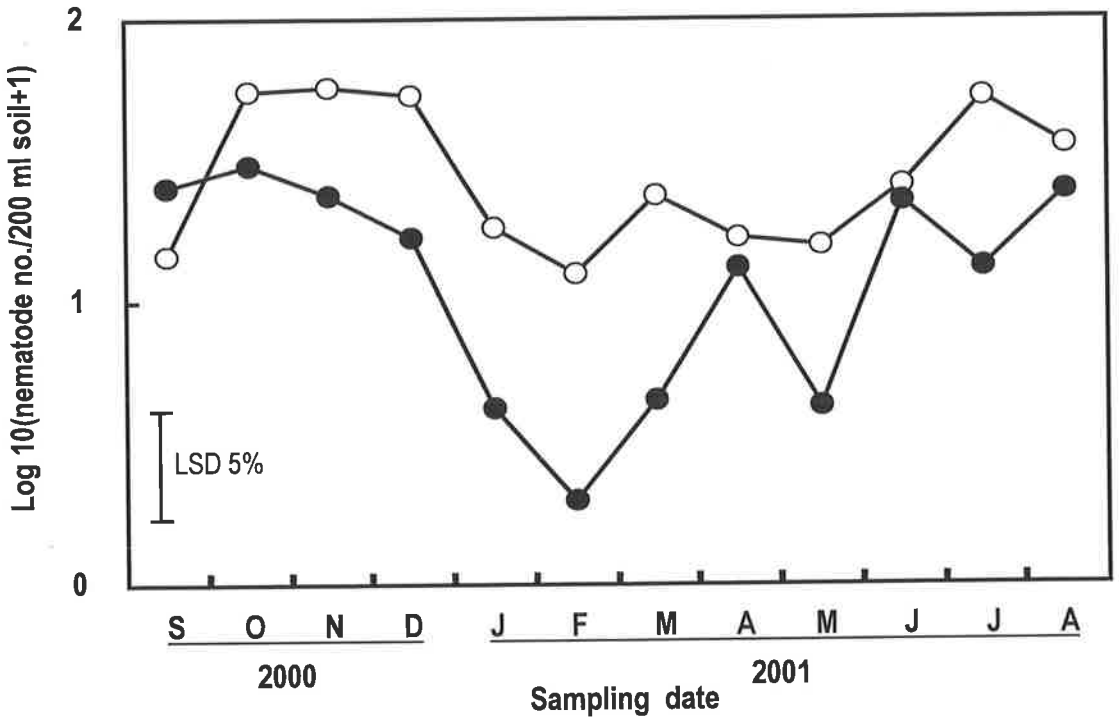


Figure 2.2.3 Mean population density of *Xiphinema* spp. ( ) and *Pratylenchus* spp. ( ) in a vineyard near Nuriootpa, South Australia, during a 12 months period.

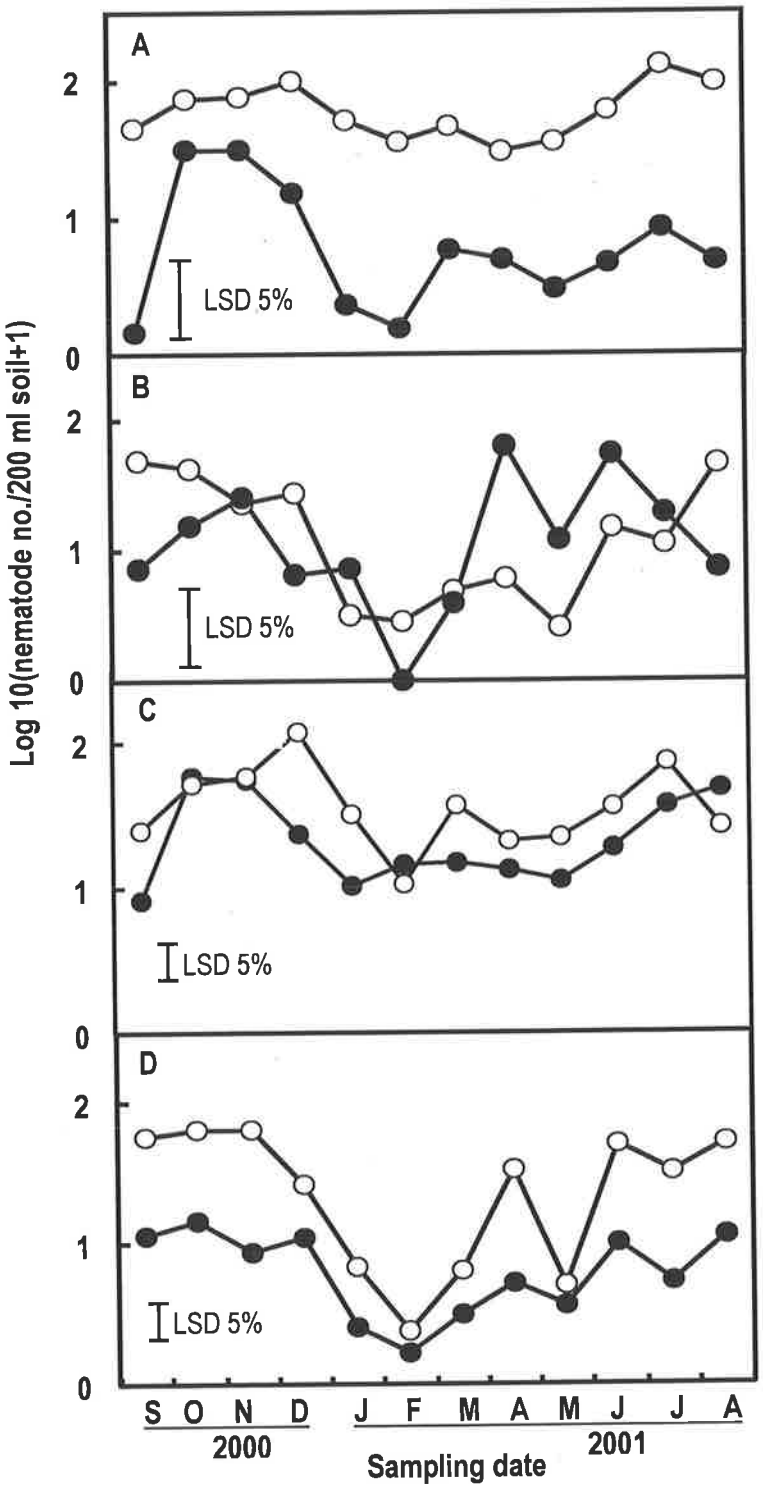


Figure 2.2.4 Mean population density of *Xiphinema* spp. and *Pratylenchus* spp. at 0-600 mm in rows, inter-rows and at two depths of a vineyard near Nuriootpa, South Australia. (A) *Xiphinema* spp. density in rows ( ) and inter-rows ( ), (B) *Pratylenchus* spp. density in rows ( ) and inter-rows ( ), (C) *Xiphinema* spp. density 300 mm depth ( ) and 600 mm depth ( ), (D) *Pratylenchus* spp. density at 300mm depth ( ), and 600 mm depth ( ).

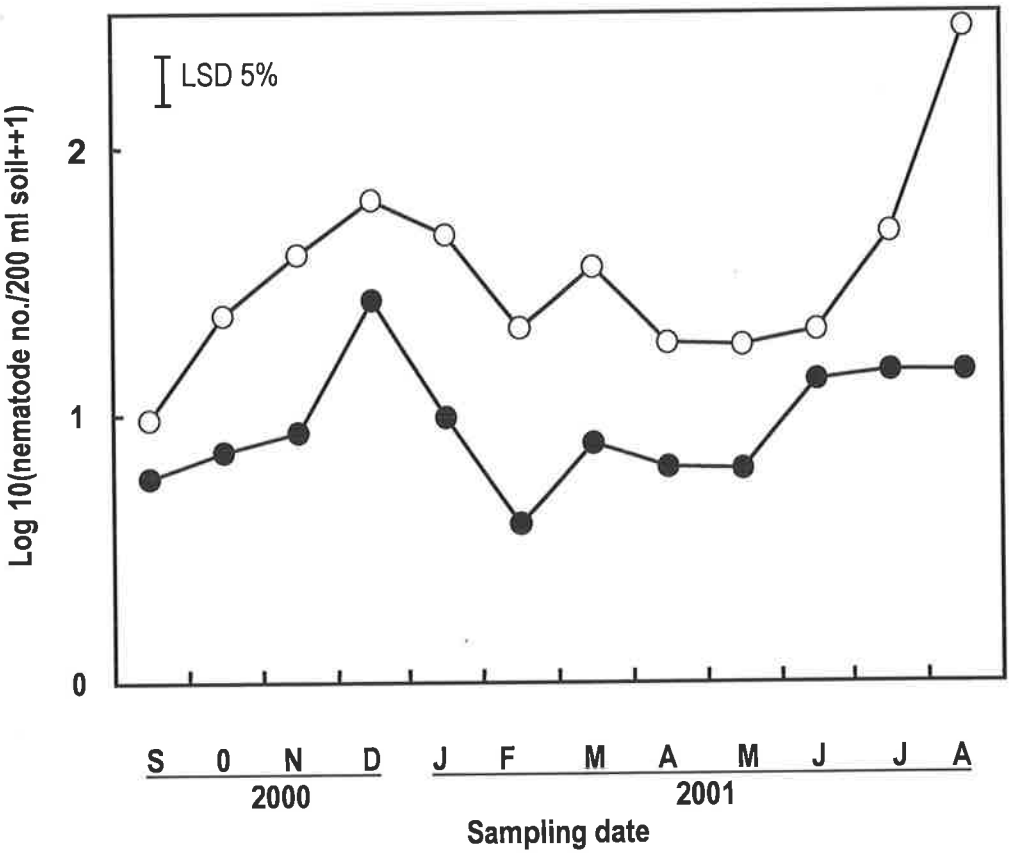


Figure 2.2.5 Mean density of juvenile ( ) and adult ( ) stages of *Xiphinema* spp. in a vineyard near Nuriootpa, South Australia, during a 12 month period.

2.2.4 Discussion

The greater population density of *Xiphinema* within the row compared to the inter-rows was probably due to the higher density of grapevine roots and moisture levels maintained by irrigation. Hunter (1998) found that the majority of grapevine roots are located within the vine row. Feil *et al.* (1997) also recorded that *Xiphinema* are distributed mainly in rows. The absence or reduced number of *Xiphinema* in the inter-row may have resulted from ryegrass, being a poor host. There is a report of reduction of *X. americanum* populations in soil by growing perennial ryegrass (*L. perenne*) as a cover crop (Boldyrev and Borzykh 1983). Also, Griffiths and Robertson (1988) reported perennial ryegrass to be a poor host of *X. diversicaudatum*.

We observed the higher density of *Xiphinema* in clay loam soil at a depth of 300-600 mm in this vineyard, but Harris (1979) recorded a higher *X. americanum* population density in the upper 0-150 mm of soil than 150-650 mm deep in a vineyard in north-eastern Victoria. Harris also found that the density was higher in sand than in sandy clay loam soil. Likewise, no consistent pattern in *Xiphinema* spp. distribution emerges from studies in other countries (Ponchillia 1972; Ferris and McKenry 1974; Sultan and Ferris 1991; Esmenjaud *et al.* 1992; Feil *et al* 1997). However, across all studies it appeaed that sampling to 600 mm will include the layers most populated by *Xiphinema* irrespective of the species, climate or soil type.

At our study site the *Xiphinema* population peaked in late spring to summer and the dropped during late summer. Across a range of local and international studies, seasonal patterns vary (Cohn 1969; Cotton *et al.* 1970; Ferris and McKenry 1974; Norton 1978; Harris 1979; Pinochet and Cisneros 1986), which is likely to be a function of many factors including the dominant *Xiphinema* species, cultivars, soils and climate. In Victoria (Harris 1979, the only other Australian study), numbers

peaked in late autumn to early winter when the grapevines were dormant. However, in SA the decline in population density in late summer may be a function of temperate given that the vines were watered and had not yet become dormant.

The relatively even horizontal distribution of *Pratylenchus* found in this study may indicate that both grapevine and the ryegrass cover crop support its reproduction. Watson *et al.* (1995) found significant numbers of *Pratylenchus* spp. in association with perennial ryegrass in New Zealand. Walker and Morey (2000) suggested that the *Pratylenchus* might multiply on susceptible cover crops, leading to continual reinvasion of grapevine roots, even if the grapevines themselves are poor hosts. The capability of *Pratylenchus* to move between soil and roots (Kimpinski and Welch 1971) along with the light soil type up to 200 mm deep in the vineyard is another possible contributor to its more even distribution.

Although at in this study *Pratylenchus* was found mostly in the upper soil profile, Walker and Morey (2001) found them in greater number at 300-600 mm. Such differences may be a function of soil type or perhaps the relative susceptibility of the shallow-rooted cover crops versus the deep-rooted grapevine to the *Pratylenchus* population in the vineyard. As with *Xiphinema* sampling to 600 mm will cover such variation.

This study found a peak in population density of *Pratylenchus* in October and a minimum in February. In a study of *Pratylenchus* in soil and roots, Walker and Morey (2001) likewise found a peak in numbers in the soil in October at the time of the main root flush. A peak in roots followed in December as a result of subsequent multiplication within the roots. These authors also observed smaller peaks in April and June for soil and roots respectively, corresponding with the smaller autumn root flush, but suggested the best time for sampling would be October for soil and December for roots (Walker and Morey 2001).

The significant negative correlation between *Pratylenchus* numbers and mean monthly temperature was likely to have resulted from absence of the susceptible cover crop during summer and drier soil. It is unlikely to be due to temperature *per se,* as all life-stages of *Pratylenchus* can invade roots and develop/reproduce between soil temperatures of 10-30^{O}C (Townshend 1991).

Given these findings, it is clear that factors driving variation in the population densities of these nematodes within the region is needed to develop a fully robust sampling method. In the absence of such data, it appears that sampling close to the grapevine, to a depth of 600 mm (especially in the deeper sandy soils) in mid to late spring will give an adequate representation of the exposure of the vine to *Xiphinema* and *Pratylenchus*. This is consistent with the proposed standard sampling method for *Meloidogyne*, however, the increased depth is suggested to compensate for the greater variation in vertical distribution.

CHAPTER THREE
DETECTION AND QUANTIFICATION OF RKN

3.1 Host test and DNA methods to distinguish *Meloidogyne incognita*, *M. javanica* and *M. arenaria*

3.1.1 Introduction

More than 60 *Meloidogyne* species have been described with different pathogenicity on different host plants (Esnard and Zuckerman 1998). Three species of RKN *(M. arenaria, M. incognita* and *M. javanica*) are the most common in Australian vineyards (Nicol *et al.* 1999). In order to develop efficient management strategies for *Meloidogyne* spp., it is essential to determine the species that cause significant threat to agricultural crops including grapevines (Stanton *et al.*1997; Hugall *et al.* 1994).

The North Carolina (NC) differential test (Taylor and Sasser 1978) relies on combinations of resistance and susceptibility reactions by nematodes of *Capsicum frutescens* L. (capsicum) cv. California Wonder, *Gossypium hirsutum.* (cotton) cv. Deltapine 16, *Arachis hypogae* (peanut) cv. Florunner, *Lycopersicon esculentum* (tomato) cv. Grosse Lisse, *Nicotiana tabacum* (tobacco) cv. NC95 and *Citrullus vulgaris* Schrad (watermelon) cv. Charleston Gray. The response of the NC differential host test to nematodes has been described as 'fairly reliable' for identification of the four common *Meloidogyne* species (Eisenback *et al.* 1981; Stanton and O'Donnell 1998).

The identification of RKN by DNA is more reliable than other methods (Powers and Harris 1993; Petersen and Vrain 1996; Zijlstra *et al.* 1995; 1997). There are several DNA methods to differentiate agriculturally important species of RKN (Powers and Harris 1993; Petersen and Vrain 1996; Zijlstra *et al.* 1995; 1997; Blok *et al.* 1997; Zijlstra 1997; Powers *et al.* 1997; Georgi and Abbott 1998). These methods are based on either direct PCR amplification or PCR followed by restriction

fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) or ribosomal DNA (rDNA) of RKN. The mtDNA methods of Powers and Harris (1993) and Stanton *et al.* (1997) can differentiate *M. arenaria* from *M. incognita* and *M. javanica* by direct size variation of PCR products while *M. incognita* can be distinguished from *M. javanica* by restriction digestion of amplified mtDNA product (Powers and Harris 1993; Stanton *et al.* 1997). *Meloidogyne hapla* and *M. chitwoodi* can be distinguished by digestion of their ITS products with a number of restriction enzymes (Zijlstra *et al.* 1995). Differentiation of *M. arenaria, M. incognita* and *M. javanica* by means of rDNA-RFLP patterns has not been achieved (Xue *et al.* 1993; Zijlstra *et al.* 1995; 1997). Identical sequences in internal transcribed sequences (ITS) regions indicate that identification by ITS- rDNA-RFLP is not possible for these three nematode species (Powers *et al.* 1997). However, Zijlstra *et al.* (2000) were able to differentiate these three species (*M. arenaria, M. incognita* and *M. javanica*) using sequence characterised amplified region based PCR assays. The method is very effective for the identification of concern species (*M. arenaria, M. incognita* and *M. javanica*) but it requires three pairs of primers and three different PCR conditions to identify each of the species. Hence, it is worthwhile to conduct further study on D3 expansion regions of 28S rRNA gene and intergenic sequences (IGS) of rDNA for discrimination between *M. arenaria, M. incognita* and *M. javanica.*

Duncan *et al.* (1999) were able to distinguish species of *Pratylenchus* based on the D3 expansion region of 28S rRNA gene, but this has not been attempted for *Meloidogyne* species. The IGS-rDNA regions is useful for discrimination between and within species of *Meloidogyne* and many other taxa (Crease 1995; Petersen and Vrain 1996; Castro *et al.* 1997; Georgi and Abbott 1998; Jackson *et al.* 1999, Reed and Phillips 2000). A rDNA cluster contains many transcription units, each

separated from the next by IGS regions. The IGS length varied widely between and within species of different taxa (Crease 1995; Castro *et al.* 1997; Jackson *et al.* 1999; Reed and Phillips 2000). This IGS length variation between species is an important genetic characteristic that can be used for species identification. Petersen and Vrain (1996) developed rDNA based primers that can amplify IGS length variation to discriminate between *M. chitwoodi*, *M. hapla* and *M. fallax*. Using PCR products from IGS of 5S and 18S rRNA genes, Blok *et al.* (1997) could differentiate *M. mayaguensis* from *M. arenaria, M. incognita* and *M. javanica* but not between *M. arenaria, M. incognita* and *M. javanica*. Given that there is no relatively simple molecular method available to distinguish *M. arenaria, M. incognita* and *M. javanica*, the three most common *Meloidogyne* species infesting grapevine in Australian, the aim of this study was to evaluate the potential of D3 expansion region of 28S rRNA gene and IGS-rDNA for this purpose.

3.1.2 Materials and methods

Root-knot nematodes were collected from five vineyards at four locations in South Australia and cultured in susceptible tomato plants in a glasshouse. Two known populations of *M. incognita* and *M. javanica* were sampled and cultured in tomato roots. A pure culture of each of the RKN collections was developed using single egg-masses in tomato plants. The preliminary species identity of each of these pure cultures was made using the NC differential host test (Hartman and Sasser 1985) and a mtDNA based method (Powers and Harris 1993). Due to several unsuccessful attempts to differentiate *M. javanica* from *M. incognita* with *Hinf*I (obtained from two different commercial sources, Promega and GeneWarks) digestion, as described by the Powers and Harris (1993), the species identity of *M. javanica*/*M. incognita* was double checked by SCAR based PCR method of Zijlstra

et al. (2000) (Table 3.1.1). The rDNA-IGS regions were then amplified from these identified DNA extracts. The reproducibility of species specific rDNA-IGS banding patterns were assessed using genomic DNA from individual RKN collected from different locations within Australia and identified by mtDNA (Powers and Harris 1993) and the SCAR (Zijlstra *et al.* 2000) methods (Table 3.1.2).

3.1.2.1 The *Hinf*I viability test

The *Hinf*I restriction enzyme was applied to mtDNA-PCR product of *M. incognita* and rDNA-PCR product of *Fusarium oxisporum* (amplified during nematode DNA amplification from galled roots) to verify the workability of restriction enzyme according to the instructions of the manufacturer. The PCR amplifications of mtDNA using the methods of Powers and Harris (1993) and Stanton *et al.* (1997), and rDNA PCR amplifications were made according to the methods of Zijlstra *et al.* (1997).

3.1.2.2 DNA extraction from single female

A modified phenol/chloroform extraction method was used for the DNA extraction from individual females (Sambrook *et al.* 1989). A female was squashed in 10 µl extraction buffer (100 mM EDTA,100 mM NaCl, 100 mM Tris pH 7.5, 0.5% SDS and 200 µg proteinase K) on a cover slip using sterile forceps under the microscopc and transferred immediately into a 1.5 ml chilled (–20^{O}C) centrifuge tube by pipetting. Tubes containing a squashed female were stored at –20^{O} C (2-4 h) until all selected females for a day were prepared. The final volume was adjusted to 100 µl by adding extraction buffer to each tube, and incubated at 58^{O}C overnight. After a brief centrifugation, 1 µl RNAase-A (10mg/ml) was added, and the contents were

mixed by flipping, and incubated at 37^{O} C for 15 min. A 100 µl mixture of phenol, chloroform and isoamyl alcohol (24:24:1) was added to the tube and incubated for 10 min at 55^{O}C in a water bath, then vortexed vigorously for one min. Tubes were centrifuged in a bench top centrifuge at 14000 rpm per min for 6 min. The supernatant was collected into a fresh tube to which 4 µl of 5 M NaCl was added, and the content were mixed gently. Two hundred microlitres of 100% ethanol was added to the tube, and the contents were mixed by inverting the tubes five times. They were then placed at –20^{O}C for at least one hour. Tubes were then centrifuged for 13 min at maximum speed, liquid was removed by pipetting and the pellet was washed with 250 µl of 70% ethanol. The pellet was air-dried and dissolved in 50 µl 1XTE.

3.1.2.3 DNA quantification

3.1.2.3.1 Spectrophotometry

PCR amplified DNA showing bright bands were quantified using spectrophotometer and calculated according to Sambrook *et al.* (1989).

3.1.2.3.2 Spot test

PCR products with faint or absent bands (<200 ng/µl) were quantified by spot test. A series of dilutions of these PCR products was spotted (1 µl) on to the surface of a 1% agarose slab gel containing ethidium bromide (0.5 µg/ml). Similarly, another series of known amount of DNA (eg 62.5, 125, 250, etc. 1 µg) was placed next to the unknown dilutions on same slab. The spots were allowed to dry and photographed under UV illumination. The amount of DNA was estimated by

comparing the intensities of the photographed of unknown DNA sample spots with the known one.

3.1.2.4 Restriction digestion of PCR products

PCR products of a *M. hapla*-type were digested with *Dra*I enzyme (2 µl sterile ddH2O, 8 µl PCR product, 1.2 µl 10X buffer and 1 µl enzyme, SIGMA, USA) overnight at 37^{O} C to confirm the species identity (Powers and Harris 1993).

3.1.2.5 Primers

The sequences of the primers used in this study are shown in Table 3.1.3. The primers were designed and constructed from the published sequence information using the commercial facility, GeneWorks, Adelaide, SA.

The approximate primer locations on rDNA of *Meloidogyne* spp. are presented in Fig. 3.1.1. Primers D1, 5'-GACCCCTCTTGAAACACGGA-3' and D2, 5'-TCGGAAGGAACCAGCTACTA-3' used in amplification of D3 expansion of 28S rRNA (Al-Banna *et al.* 1997; Duncan *et al.* 1999; Subbotin *et al.* 2000). IGS-rDNA amplification primers

G1, 5'-AAAGGGCAGGGACGTAATCAA-3' and

G2, 5'-TAGTAGCTGGTTCCTTCCGA-3' were designed based on their closest position to IGS regions of rDNA (Vrain *et al.* 1992; Al-Banna *et al.* 1997). These IGS-rDNA primer sequences are conserved in the 18S and 28S rRNA genes of *Caenorhabditis elegans* (Ellis *et al.* 1986) and many other nematodes including species of *Meloidogyne* (Vrain *et al.* 1992; Powers and Harris 1993; Zijlstra *et al.*, 1995; 1997; Zijlstra 1997; Powers *et al.* 1997; Al-Banna *et al.* 1997; Subbotin *et al.* 2000).

3.1.2.6 PCR amplifications

3.1.2.6.1 PCR amplification for mtDNA and Sequence Characterised Amplified Regions (SCAR) methods

PCR amplifications of DNA from females of the RKN were carried out using methods described by Powers and Harris (1993) for the species *M. arenaria, M. incognita*, *M. javanica* and *M. hapla*. The DNA producing a *M. incognita/M. javanica* type fingerprint(s) by the method of Powers and Harris (1993) was used in SCAR method to distinguish *M. javanica* from *M. incognita* (Zijlstra *et al.* 2000). DNA samples, which produced a *M. incognita/M. javanica* type band (1.7 kb DNA band) by the Powers and Harris (1993) method but did not produce a *M. javanica*-type fingerprint in replicated PCR amplifications were considered to be *M. incognita*.

3.1.2.6.2 PCR amplification of IGS-rDNA

DNA suspension (10 µL) was added to a PCR mixture containing 5 µl of 10X DNA Taq polymerse incubation buffer, 3 µl of 25 mM MgCl2, 1 µl of 10mM dNTP-mixture (Sigma), 0.8 µM of each primer (GeneWorks, Adelaide, Australia), 2.5 U Taq polymerase (Promega Corporation, NSW, Australia), and double distilled water to a final volume of 50 µL. Mineral oil (50 µl) was added on top of PCR mix. The mixture was placed in a DNA thermal cycler (MJ Research Inc., USA). In each PCR run a negative control without DNA template was included.

An initial steps of 94^{O} C for 2 min, 55^{O} C for 2 min, 72^{O} C for 2 min then forty cycles of amplification (94^{O} C for 1 min, 55^{O} C for 1 min., 72^{O} C for 2 min) followed by a final extension at 72^{O} C for 10 min, were performed. Following DNA amplification, 5 µl of PCR product was used for electrophoresis in 0.5X TBE buffer (Sambrook *et al.*, 1989) in 0.7% to 1.5% agarose gel (Sigma) stained with 0.5 µg/ml

ethidium bromide. A 100 bp (Sigma) and a 1 kb (Promega) DNA ladders were used as size markers. The gel was viewed on an UV transilluminator and photographed.

3.1.2.7 Gel electrophoresis and photography of gel

The PCR products and digested DNA fragments were separated by agarose gel electrophoresis (1.5%) at 100 V for 1 h. A 100 bp ladder DNA was used in each gel as a standard for all PCR products or digested DNA. DNA in gel was stained with ethidium bromide (10mg/ml) for 15 minutes followed by distaining in ddH2O for 5-10 min. The gel was photographed with Polaroid or digital camera over UV illumination.

3.1.2.8 DNA sequencing

The D3 expansion of 28S rRNA gene and the unknown PCR products (720 bp), obtained frequently during PCR amplification of RKN DNA from galled roots, were purified using PCR purification kit (Roche Diagnostics Corporation, USA). The purified products were sequenced directly without cloning using specific primers (D1, 5'-GACCCCTCTTGAAACACGGA-3'
and D2, 5'-TCGGAAGGAACCAGCTACTA-3') in an Applied Bio System 373 sequencer (USA). The sequence data, from chromatographs, showing strong signal without any background noises were only considered for further analysis (Fig. 3.1.2).

3.1.2.9 Data analysis

Estimation of DNA fragment lengths, based on relative mobility on the gel, were calculated using the computer program GEL (Schaffer and Sederoff 1981). The computer programs MacClade and PAUP were used to analyse the raw data generated from the banding patterns of individual nematodes (Shoshani and

McKenna 1998). Pairwise genetic distances between individual nematodes were calculated and a tree showing general relationships among the individual nematodes was constructed using the unweighted pair group method with arithmetic mean (UPGMA, Sneath and Sokal 1973). Sequences of D3 expansion regions of 28S rRNA gene of *M. arenaria, M. incognita* and *M. javanica* and unknown PCR products were analysed using different computer programs. Computer program SeqEd® was used to edit DNA sequences. Sequences were aligned using MegAlign program of LASERGENE® to estimate the sequence pair distances (using cluster method with weighted residue weight table). The basic local alignment search tool (BLAST) at National Centre for Biotechnology Information, USA, (web address www.ncbi.nlm.nhi.gov) was used for sequence similarity searches (Altschul *et al.* 1990). The coding and non-coding sequences in rRNA genes of RKN were identified by aligning with the known sequences in the GenBank.

3.1.3 Results

3.1.3.1 Species identity of RKN from vineyards

None of the tubes without template DNA produced any amplification during PCR. Six out of seven single egg-mass cultures (pure) of RKN showed *M. arenaria* race 2/*M. javanica* type reaction and one showed *M. incognita* type reaction to the NC differential hosts test (Table 3.1.3). The PCR amplification of these isolates by the method of Powers and Harris (1993) gave *M. arenaria* type 1.1 kb bands from three females and *M. incognita*/*M. javanica* type 1.7 kb bands from four females. Restriction digestion of 1.7 kb DNA with the enzyme *Hinf*I did not produce any fragmented DNA.

3.1.3.2 The *Hinf*I viability test

The PCR method of Stanton *et al.* (1997) produced 557 bp bands with DNA from the same sources of mtDNA analysis of Powers and Harris (1993) but again no restriction cut was found in subsequent use of enzyme *Hinf*I. The application of enzyme *Hinf*I to the 720 bp PCR-rDNA product of *Fusarium* sp. gave three bands of about 420 bp, 210 bp and 90 bp (Fig. 3.1.3 lane 2) while 760 bp PCR-rDNA products of *M. incognita* gave two bands of about 320 bp and 440 bp (Fig. 3.1.3, lane 3).

The *Fusarium* spp., amplified (720 bp) and identified by sequence alignment, was a by-product of the DNA suspension from RKN galled roots during rDNA amplifications using the method of Zijlstra *et al.* (1997).

3.1.3.3 D3 expansion based identification

The PCR amplification of the D3 expansion region of the large subunit of 28S rRNA gene for each species of *Meloidogyne* produced a single band of about 300 bp (Fig. 3.1.4A). Analysis of the sequence showed that the band consisted of 301 nucleotides. Sequence alignment, using a cluster method with weighted table, showed that the sequences in D3 expansion region of three species are the same, except the twelfth sequence position in *M. arenaria* (Table 3.1.4). Sequence alignment of these species, using the computer program BLAST 2.2.1, with GenBank sequences of D3 expansion of 28S rDNA of *M. arenaria* (GenBank accession number 1147729 and 1147726) and *M. javanica* (GenBank accession number 1870247) also revealed that the sequences are highly conserved among the species and differ by only one base addition at position 52 and one mismatch (G instead of A) at position 53 of the species studied.

3.1.3.4 IGS-rDNA based identification

The PCR products from single juvenile and genomic DNA of single female nematodes were identical in size. Primers gave five bands of about 2 kb, 1.65 kb, 1.33 kb, 1.27 kb and 0.98 kb for *M. arenaria,* six bands of about 1.65 kb, 1.27 kb, 0.98 kb, 0.69 kb, 0.053 kb and 0.388 kb for *M. incognita* and produced two bands of 1.65 kb and 0.53 kb for *M. javanica* (Fig. 3.1.4B). The 1.65 kb band was common to all the three species but the 0.053 kb band was found in *M. incognita* and *M. javanica*. The 2 kb and 1.33 kb bands were unique to *M. arenaria,* the 1.27 kb, 0.388 kb bands were unique to *M. incognita*, and the 1.27 kb and 0.98 kb bands occurred in both *M. arenaria* and *M. incognita.*

3.1.3.5 Identification of individual nematodes

The PCR amplification with a pair of mtDNA based primers (Powers and Harris, 1993) produced a 1.1 kb band in *M. arenaria*, and a 1.7 kb band in both *M. incognita* and *M. javanica*, (Fig. 3.1.4C), but we could not differentiate *M. incognita* from *M. javanica* following restriction digestion of their mtDNA-PCR products with enzyme *Hinf*I. No fragmentation of the product was achieved even using enzyme from two different commercial sources (Promega Corporation, NSW, Australia and GeneWorks, Australia). The mtDNA method of Stanton *et al.* (1997) also did not differentiate *M. incognita* from *M. javanica*. A band of about 0.557 kb was produced for the three species tested but no cut was found upon restriction digestion of this PCR product with enzyme *Hinf*I (Fig. 3.1.4C).

3.1.3.6 Reproducibility of IGS-rDNA based identification technique

The IGS-rDNA primers were able to reproduce species-specific banding patterns in many individuals of three species of RKN *M. arenaria, M. incognita* and

M. javanica (Fig. 3.1.6). Some genetic variability was also observed between individuals of each species. The primers gave 16 types of banding patterns across all the 52 individuals of the *Meloidogyne* species, of which two types were from seven individuals of *M. arenaria*, four from 26 individuals of *M. incognita* and 10 from 21 individuals of *M. javanica* (Fig. 3.1.5). Based on banding patterns generated by the primers G1 and G2, individuals of root-knot nematode were grouped into three main groups (Fig. 3.1.6). All individual RKN identified as *M. arenaria* by mtDNA technique were included in *M. arenaria* group in a genetic tree based on IGS-rDNA analysis (Fig. 3.1.6).

Table 3.1.1 The North Carolina differential host test, mitochondrial DNA (mtDNA) and Sequence Characterised Amplified Regions (SCAR) based species identity of isolates of *Meloidogyne* spp. from different locations of South Australia.

Locations	NC differential host test type	mtDNA type (Powers and Harris 1993)	SCAR based species identity Zijlstra *et al.* 2000
Winkie ($34^{O}18$'S $140^{O}31$'E)	*M. arenaria* race 2 / *M. javanica*	*M. arenaria*	-
New Residence ($34^{O}22$'S $140^{O}24$'E)	*M. arenaria* race 2 / *M. javanica*	*M. arenaria*	-
,,	*M. arenaria* race 2/ *M. javanica*	*M. incognita*/ *M. javanica*	*M. javanica*
McLaren Vale ($35^{O}13$'S $138^{O}32$'E)	*M. arenaria* race 2/ *M. javanica*	*M. arenaria*	-
Padthaway ($36^{O}36$'S $140^{O}29$'E)	*M. arenaria* race 2/ *M. javanica*	*M. incognita* / *M. javanica*	*M. javanica*
Adelaide *M. incognita* ($34^{O}93$'S $138^{O}59$'E)	*M. incognita*	*M. incognita* / *M. javanica*	*M. incognita*
Adelaide *M. javanica* ($34^{O}93$'S $138^{O}59$'E)	*M. arenaria* race 2 / *M. javanica*	*M. incognita* / *M. javanica*	*M. javanica*

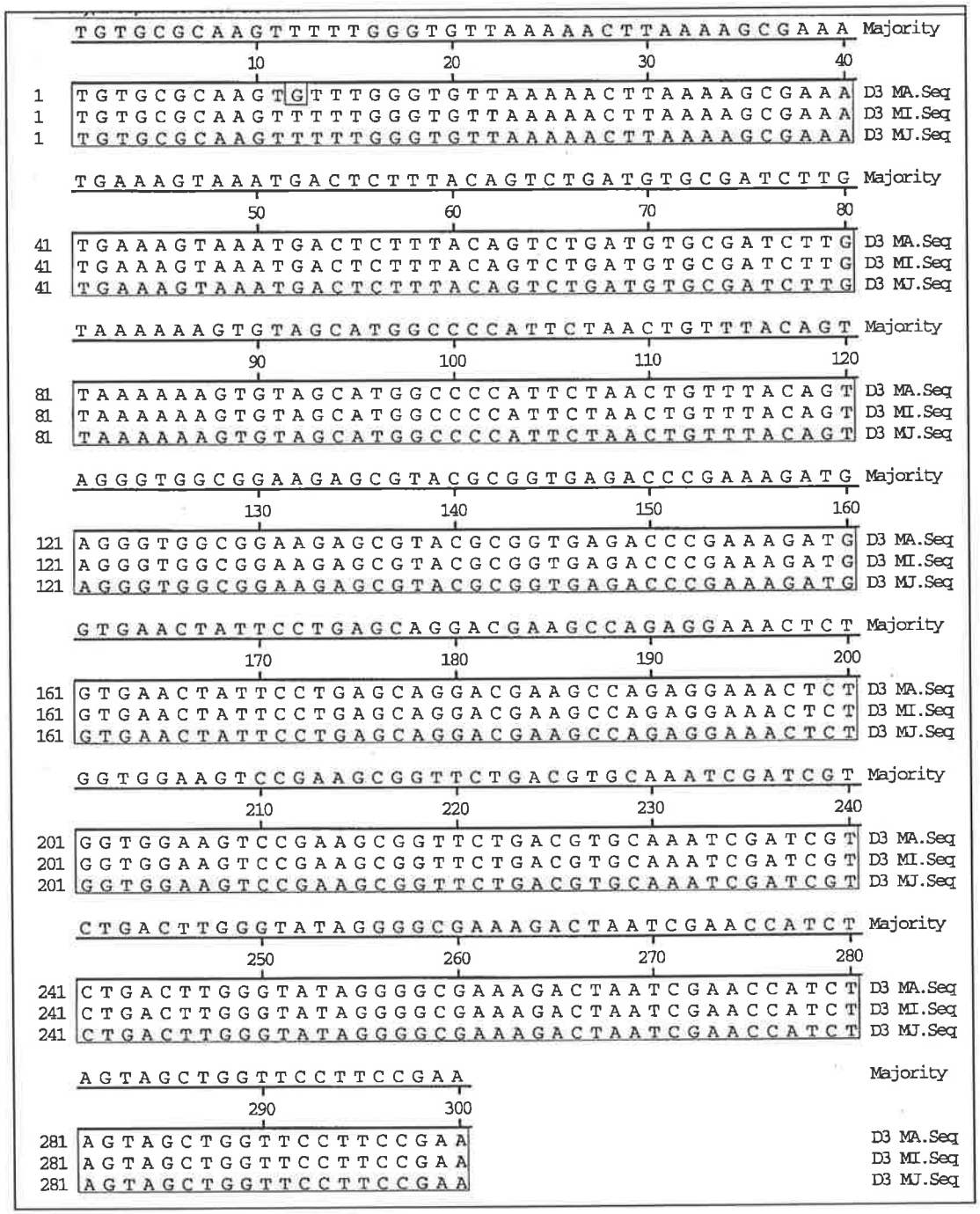
Table 3.1.2 Individual root-knot nematodes (*Meloidogyne* spp.) with source, host and species identity based on mitochondrial DNA (mtDNA) method.

Code number of individual nematodes	Source		Original host	mtDNA based identity (Powers and Harris 1993)
	Locations	Longitude/ Latitude		
1 to 4	Winkie, SA	34°18'S 140°31'E	Grapevine, cv. Colombard	*M. javanica/ M. incognita*
5 to 8	New-Residence, SA	34°22'S 140°24'E	Grapevine cv. Merlot	,,
9 to 11	,,	,,	Grapevine cv. Colombard	,,
12 to 21	Adelaide, SA	34°93'S 138°59'E	Grapevine cv. Unknown	,,
22 to 24	,,	,,	Unknown	,,
25, 26	McLaren Vale, SA	35° 13'S 138°32'E	Grapevine cv. Clombard	,,
27 to 29	,,	,,	,,	*M. arenaria*
30 to 32	Padthaway, SA	36°36'S 140°29'E	Grapevine cv. Pinot Nior	*M. javanica/ M. incognita*
33 to 35	,,	,,	Grapevine cv. Riesling	,,
36 to 39	Brisbane, QLD	27°5'S 152°98'E	Tomato	,,
40 to 49	South Johnstone, QLD	17°59'S 145°99'E	Tobacco	,,
50 to 52	,,	,,	,,	*M. arenaria*
53 (*M. arenaria*)	,,	,,	,,	,,
54 (*M. incognita*)	Adelaide, SA	-	Grapevine cv. Unknown	*M. javanica/ M. incognita*
55 (*M. javanica*)	,,	-		,,

Table 3.1.3 Primers sequence and approximate positions in the DNA of root-knot nematodes (*Meloidogyne* spp.).

Primer sequences	Priming position/name	References
5'-GGTCAATGTTCAGAAATTTGTGG-3'	COOII gene	Powers and Harris 1993
5'-TACCTTTGACCAATCACGCT-3'	lrRNA gene	,,
5'-TTGATTACGTCCCTGCCCTTT-3'	18S rRNA gene	Vrain *et al.* 1992
5'-TTTCACTCGCCGTTACTAAGG-3'	28S rRNA gene	,,
D1, 5'-GACCCCTCTTGAAACACGGA-3'	,,	Al-Banna *et al.* 1997
D2, 5'-TCGGAAGGAACCAGCTACTA-3'	,,	,,
5'-GGTGCGCGATTGAACTGAGC-3'	SCAR	Zijlstra *et al.* 2000
5'-CAGGCCCTTCAGTGGAACTATAC-3'	SCAR	,,
5'-TGAATTTTTTATTGTGATTAA-3'	tRNA gene	Stanton *et al.* 1997
5'-AATTTCTAAAGACTTTTCTTAGT-3'	lrRNA gene	,,
G1, 5'-AAAGGGCAGGGACGTAATCAA-3'	18S rRNA gene	Vrain *et al.* 1992
G2, 5'-TAGTAGCTGGTTCCTTCCGA-3'	28S rRNA gene	Al-Banna 1997

Table 3.1.4 The alignment (using cluster method with residue weight table) of the DNA sequences from the D3 expansion region of rRNA genes of *Meloidogyne arenaria* (D3MA), *M. incognita* (D3MI) and *M. javanica* (D3MI).



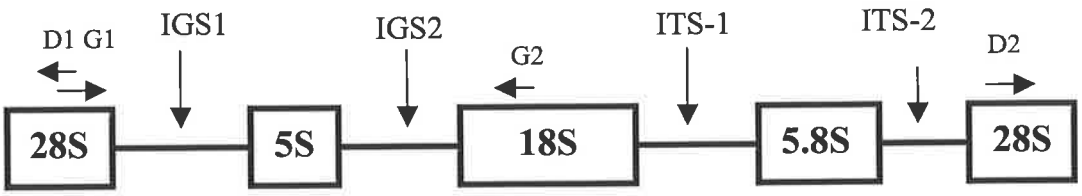


Figure 3.1.1 Diagram of the ribosomal cistron and intergenic regions (IGS) of *Meloidogyne* spp. Arrows indicate approximate position and direction of primers used (after Blok *et al.* 1997).

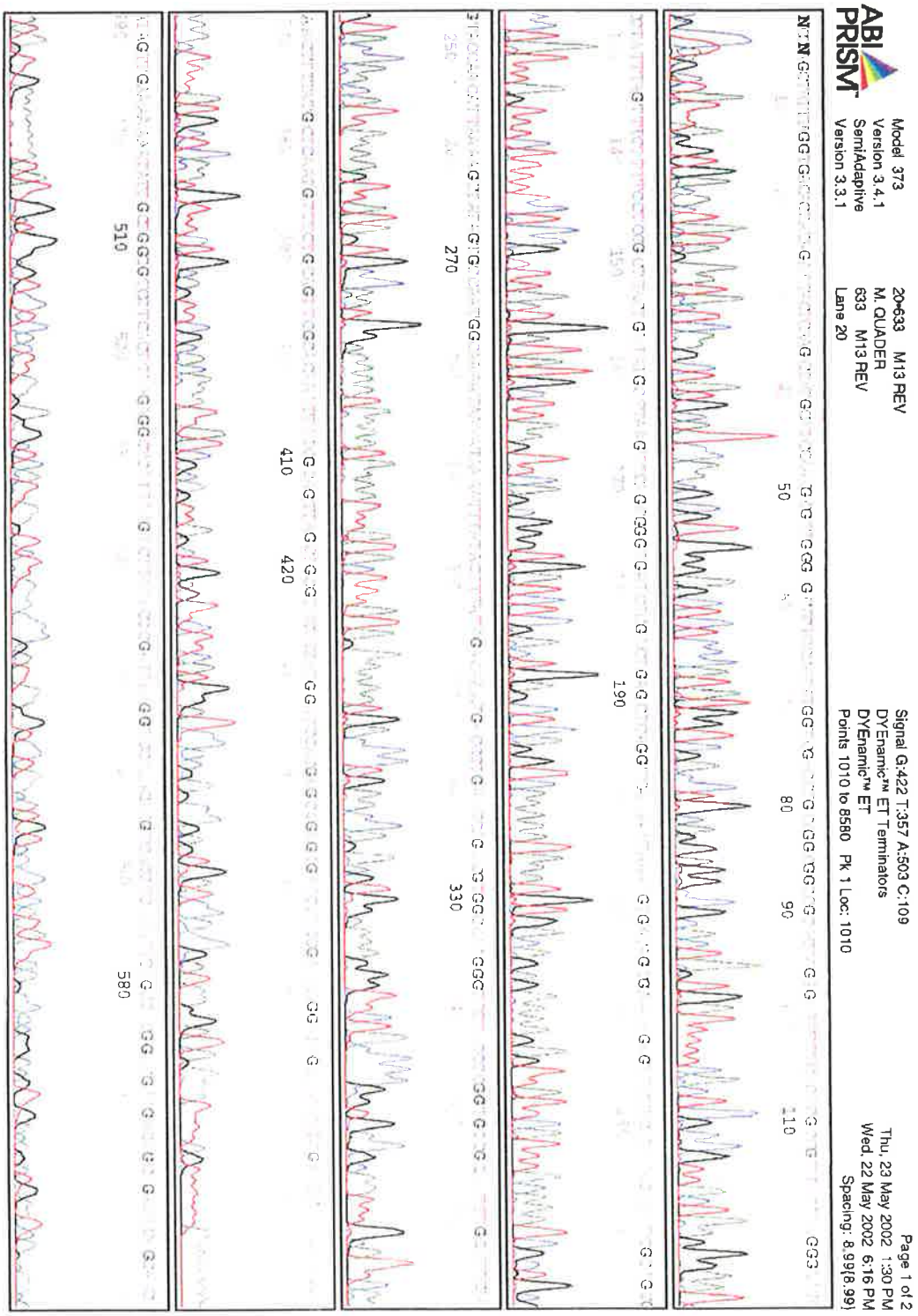
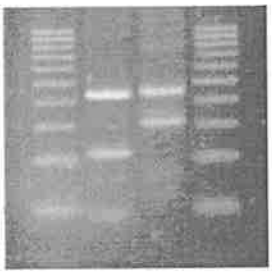


Figure 3.1.2 An example of a chromatograph for sequences used in the analysis of PCR products of ribosomal DNA (rDNA) of *Meloidogyne* spp. (chromatograph above showing 100% accurate calls/results to 450 bases).



ST *F* *Mi* ST

Figure 3.1.3 Restriction digestion of PCR-rDNA products of *Meloidogyne incognita* (*Mi)* and *Fusarium* sp. (*F*) amplified from galled tomato roots.

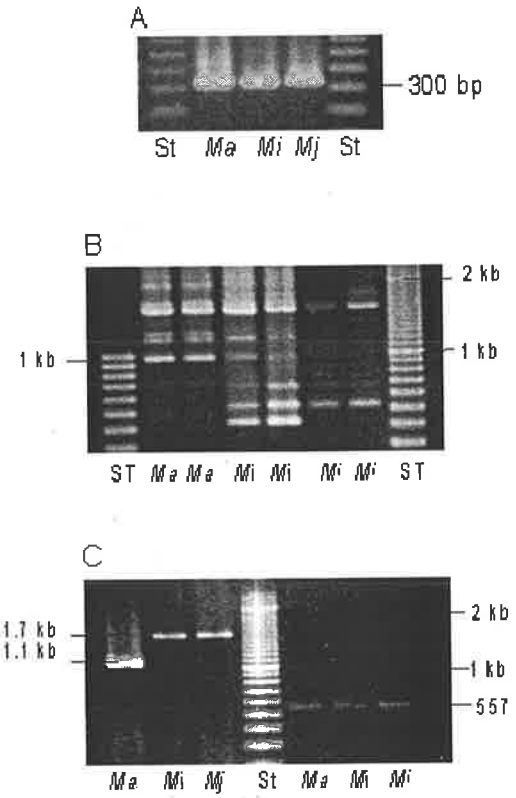


Figure 3.1.4 PCR products of (A) D3 expansion region of 28S rRNA gene, (B) IGS-rDNA and (C) mtDNA (lanes 1 to 3 and 5 to 7 are PCR products for primers described by Powers and Harris (1993) and Stanton *et al.* (1997) respectively) of *Meloidogyne arenaria* (*Ma*), *M. incognita* (*Mi*) and *M. javanica* (*Mj*). Lanes labelled St are 100 bp DNA ladder.

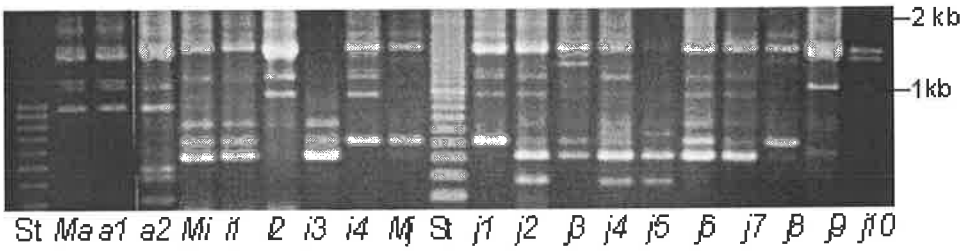


Figure 3.1.6 IGS-rDNA variants of *Meloidogyne arenaria* (*Ma*), *M. incognita* (*Mi*) and *M. javanica* (*Mj*). Lanes *a*1 to *a*2, *i*1 to *i*4 and *j*1 to *j*10 are IGS-rDNA variants of *Meloidogyne arenaria* (*Ma*), *M. incognita* (*Mi*) and *M. javanica* (*Mj*) respectively. Lanes labelled St are 100 bp DNA ladder.

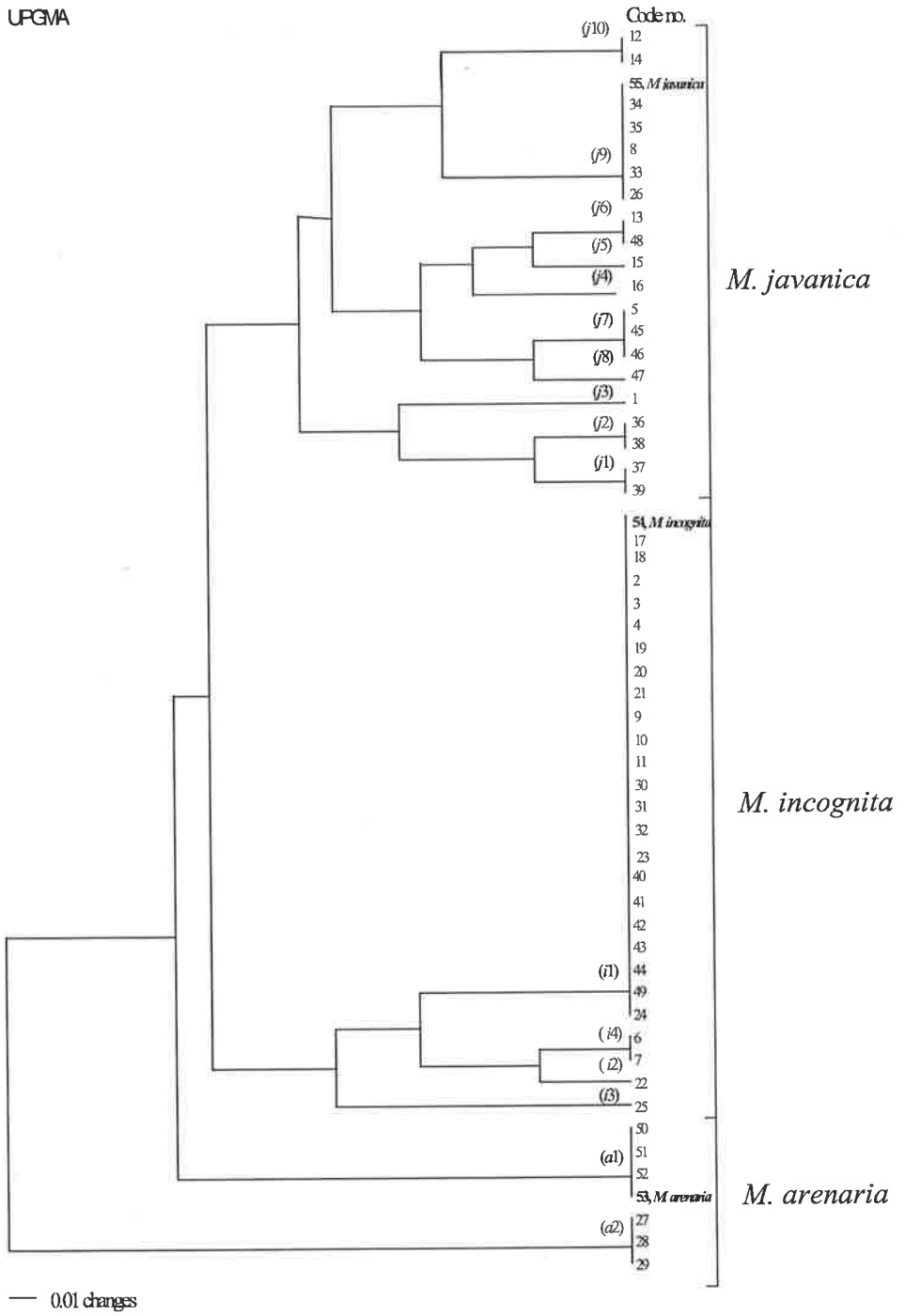


Figure 3.1.6 Dendrogram illustrating IGS-rDNA based general relationships of individual root-knot nematodes with *Meloidogyne arenaria*, *M. incognita* and *M. javanica*. Number within parenthesis correspond to genetic types in each species.

3.1.4 Discussion

3.1.4.1 Methods for species identity

Use of the NC differential host is useful to obtain an indication of species identity, and sometimes corresponded with mtDNA type identity of RKN (eg *M. incognita* in this study) but there may be variability in reaction of hosts to RKN that may make the test unreliable. One of the important sources of such variable may arise from the inability of differential host test to differentiate between *M. arenaria* race 2 and *M. javanica*. Stanton and O'Donnell (1998) also found some discrepancy between results when they assessed 40 Australian RKN populations for their host race status using the NC differential host test. Basically, the NC differential host test was intended for use in combination with perineal patterns of adult RKN females (Hartman and Sasser 1985), but these perineal patterns are also variable and unreliable as an indicator of species (Hugall *et a*l. 1994; McLeod and Steel 1999a). McLeod and Steel (1999a) found that the identification of *Meloidogyne* spp., from 17 vineyards within five viticultural districts in NSW, by perineal pattern was inconsistent with identification by mtDNA analysis. However, in the work described here, the NC differential host test method in combination with mtDNA type from PCR amplification of Powers and Harris (1993) method was good for the identification of *M. arenaria*, *M. incognita* and *M. javanica*. This combination eliminates the need for subsequent RFLP of PCR products from mtDNA to differentiate between *M. incognita* and *M. javanica*. The long time requirement (at least three months) for the identification of *Meloidogyne* species by the NC differential host test is a potential obstacle to the routine use of this method in species identification.

The use of DNA methods alone has been proved more reliable and less time consuming for the identification of many organisms, including RKN. The appropriate DNA method(s) is not only reliable for identification of RKN species, it can also

detect small changes in genetic make-up within species that are yet to be expressed physiologically or by morphological character(s). For example: previous reports (Powers and Harris 1993; Hugall 1994; Stanton *et al.* 1997) indicated the presence of restriction sites in mtDNA but so far we have not found any restriction site in mtDNA of a selection of RKN populations from South Australia. These changes in DNA did not affect the current relationship between the RKN populations and the NC differential host.

This study showed that the PCR products of either mtDNA or rDNA were the same size as described in the literature (Powers and Harris 1993; Zijlstra *et al.* 1995; Stanton *et al.* 1997), but restriction fragment length polymorphism were either absent or different (eg *Hinf*I digest of rDNA-PCR product of *M. incogn*ita) from those in the published descriptions. This discrepancy in position or absence of restriction site(s) in mtDNA or rDNA of RKN indicates that the RKN populations of selected vineyards of South Australia are different in their mtDNA makeup from those found elsewhere. The absence of restriction cut was not due to lack of activity of the enzyme or the procedure used, as positive results were obtained when the same enzyme *Hinf*I was tested on PCR-rDNA product of both *Fusarium* sp. and *M. incognita*. Williamson *et al.* (1994) also indicated that the failure to obtain any restriction digestion product from PCR based amplified mtDNA of *M. javanica* might be due to lack of activity of the enzyme when they attempted to apply the method, but no fragmentation of the product was achieved even using resctriction enzyme *Hinf*I from two commercial sources (Promega Corporation and GenWarks). However, there are several examples of the presence of mtDNA and rDNA restriction site variants within the species of different organisms including RKN (Bekal *et al.* 1997; Munechika *et al.* 1997; Newton *et al.* 1998; Whipple *et al.* 1998). Restriction site variants could be found in RKN populations, even within a single location. For example, in this study the

mtDNA variants found in *M. incognita/M. javanica* were different from variants described by Hugall *et al.* (1994) even though the nematodes were collected from locations very close to their study area in South Australia. Hence, it is reasonable to conclude that the variation in restriction sites in DNA could occur frequently in RKN populations of different geographical locations. Therefore, it is most unlikely to develop a universal RFLP based identification technique without prior knowledge of all possible variations in targeted species found locally and around the world. Thus, although PCR-RFLP or RFLP is good for phylogenetic studies, it is less applicable for species or haplotype identification, probably because it is based on single or few nucleotides, which may be more vulnerable to evolutionary processes compared to a piece of DNA or a whole gene.

The direct PCR amplification of a piece of mtDNA as a species-specific diagnostic marker (eg *M. arenaria*, Powers and Harris 1993) was the same size in all populations assessed locally and elsewhere (eg Harris *et al.* 1990; Hugall *et al.* 1994; Williamson *et al.* 1994; Stanton *et al.* 1997). Therefore, greater attention should be given to development of diagnostic DNA marker(s) based on PCR amplification of reasonably long piece(s) of species specific DNA sequences from organisms including RKN.

It appears from this study that during the PCR amplification of targeted DNA from galled roots, crude sources, the non-targeted band(s) may be found in the PCR product. This non-targeted band could come from different organisms including nematodes and *Fusarium* spp., as the primer sequences used for RKN are also complementary to rDNA of many nematode species (Powers *et al.* 1997).

3.1.4.2 D3 expansion based identification

Use of the D3 expansion region of 28S rRNA gene is unlikely to identify *Meloidogyne* species from grapevine in Australian as it is highly conserved among the species studied. It was useful in the identification of species of *Pratylenchus* (Al-Banna *et al.* 1997; Duncan *et al.* 1999) but not for *Globodera* (Subbotin *et al.* 2000). The findings here that sequences of D3 expansion regions are highly conserved, along with other studies on structure and sequences of rDNA (Powers *et al.* 1997; Zijlstra *et al.* 1995, 1997; Zijlstra 1997), indicate that discrimination of the *Meloidogyne* species concerned is not possible based on ITS-rDNA-RFLP.

3.1.4.3 IGS-rDNA based identification

The IGS-rDNA based PCR approach is able to reveal DNA polymorphism to differentiate *M. arenaria, M. incognita* and *M. javanica*. It is likely that the primers used amplified part of the 18S rDNA, part of 28S rDNA, the entire 5S rDNA and entire intergenic regions of rDNA from the genomic DNA of *M. arenaria, M. incognita* and *M. javanica*. The amplification of IGS region using primers located at the end of rRNA genes is common practice in identification and phylogenetic studies (Pendas *et al.* 1994; Suzuki *et al.* 1994; Sajdak *et al.*1998; Jackson *et al.*, 1999). The non-transcribed sequences (NTS) in IGS of rDNA were used in the identification of species and strains of the dermatophyte fungi *Trichophyton rubrum* (Jackson *et al.* 1999). Sajdak *et al.* (1998) amplified a portion of 5S rDNA and the entire IGS from total genomic DNA by PCR. Their primers gave four DNA bands of discrete size for each individual of the species *Coregnonus artedi* (Coregonid fish). Sequence analysis of these fragments revealed that differences in length of these amplified fragments resulted from differing number of a 130 bp repeat sequences found within IGS regions. Pendas *et al.* (1994) also produced two NTS length variants using primers on

5S rDNA of Atlantic salmon. Suzuki *et al.* (1994) were able to distinguished subspecies of mice (*Mus musculus domesticus* and *M. m. musculus*) using sequence differences in NTS-IGS region between 5S and 26S rDNA.

The multiple rDNA-IGS fragments sizes of each species and individuals might have come from either various rDNA-IGS length variations or/and from single IGS-rDNA due to the presence of a termination codon within an IGS. Zhuo *et al.* (1995) identified termination-like sequences in rDNA-IGS of lake trout fish (*Salvelinus namaycush*). The mtDNA or SCAR analysis of individuals of *Meloidogyne* species identity indicated that most of the IGS-rDNA variants occurred within the species, but a few, such as some variants in *M. arenaria*, were not clustered in a single clade, and variation can be found in the rDNA-IGS pattern. Moreover, there are several examples of new rDNA variants, formed due to mutation in IGS regions, that did not affect the stability of species (Dvorak *et al.* 1987, Sajdak *et al.* 1998; Reed and Phillips 2000). However, further study of structure and sequences of these amplified fragments of IGS-rDNA is needed to confirm this posibility.

The requirement of only two primers, as used in this study, eliminates the necessity to use several primers in distinguishing between species. The use of too many primers in a PCR may cause the formation of chimaeras due to competition between amplicons and limitation of substrates. The method also reduces identification time and cost as it does not require restriction digestion to discriminate between species.

Another important aspect of the technique is that the escape of any individual that might not have binding sites is most unlikely. This is because binding sites of primers used in this study are situated within the rRNA genes, which are highly stable against mutational processes, incomparison to IGS. Use of IGS based primers (eg Petersen and Vrain, 1996) in the identification PCR may prove to be unreliable, as the

primer binding sites may not occur in all individuals of the species concern (Georgi and Abbott 1998). The specificity of primers to particular conserved region(s) also eliminates the possibility of problem associated with random amplified polymorphic DNA analysis, such as reproducibility.

3.1.4.4 Reproducibility of IGS-rDNA identification technique

The species specific amplified DNA fragment size variations along with the reproducibility across a number of individuals of RKN from different geographic locations indicates that the technique may be used for rapid identification of the Meloidogyne species concerned. The discriminating capacity of the IGS-rDNA analysis, even between individuals of the species could be used to monitor the genetic consistency between individuals of a species in cost-effective way. The differentiation between race and/or haplotypes is important for nematode management, given that may have been different host ranges and be equally common in Australian agriculture (Hugall *et al.* 1994).

3.2 DNA method for the quantification of root-knot nematodes in vineyards

3.2.1 Introduction

Many kinds of nematodes occur in association with plants but damage only results when population densities of the plant parasitic species are high. In a vineyard, accurate quantification of population densities of plant parasitic nematodes and their potential for increase is critical in anticipating crop damage (Duncan and Noling, 1998). Unreliable quantification of nematodes will limit the definition of economic thresholds (the level at which control costs equal benefits) and the assessment of suitable management options in grapevines. Recent developments in Australian nematology have seen the provision of a commercial service (Root Disease Testing Services, now marketed as "PreDicta B", C-Qentec, Diagnostics, Aventis CropScience, for field crops) for the quantification of some nematode species in soil used for field crops based on DNA technology (Ophel-Keller *et al.* 1999; Hannam 1999; Hollaway *et al.* 2001). This technology is being used in quantification of root lesion nematodes, cereal cyst nematode and some soilborne fungal diseases of cereals. The approach offers promise for viticulture to better define pest levels and to assess the applicability of various control strategies. Its proponents consider the technology to be more accurate and reliable than the methods currently used for quantification of parasitic nematodes of grapevine, such as *Meloidogyne* spp.

Although DNA probes are available for the detection of RKN (Stirling *et al.* 2001), further work is needed before a commercial service can be offered to viticulture. Therefore, a study was undertaken to assess the potential to extend the commercially available DNA based method for the quantification of root-knot nematodes in vineyard soils.

3.2.2 Materials and methods

Eight soil samples were collected from a vineyard infested with RKN at New Residence, in the Riverland Region of South Australia. Each sample was mixed carefully and two subsamples of 400 g were taken. The population of RKN juveniles in one subsample was estimated under a microscope after extraction using the Whitehead tray method (Whitehead and Hemming, 1965). Nematodes were collected on a 20 μm sieve, and were then examined under a microscope. The RKN juveniles were counted. The RKN population in the other subsample was assayed using a DNA method used for cereal root disease testing (Ophel-Keller *et al.*, 1999). The DNA quantification procedures can not be detailed as they are commercial-in-confidence. The quantification principle of the method is PCR amplification of rDNA of root-knot nematodes (Ophel-Keller *et al.* 1999). However, the provision of this method commercially allow the work to be repeated as needed.

The following experiments were conducted to validate the DNA based quantification for RKN in vineyards (the DNA method was applied at various times, as the method is not influenced by the time of assessment):

Experiment 1. Known numbers of RKN juveniles were added to 400 g soil and then the DNA method was used to estimate the number of this nematodes. Eight replicate samples were used, to which 0, 12, 37, 111, 333 and 1000 juveniles (mixed species extracted from field soil) respectively had been added per 400 g of soil.

Experiment 2. A known number of eggs of RKN was added to 400 g soil and then estimated by DNA method. Three replicated samples with about 0, 500, 2500, 5000, 7500 and 10000 eggs per 400 g soil were assessed.

Experiment 3. Known numbers of two important species *M. incognita* and *M. javanica* were added to 400 g soil individually and then estimated by DNA method. Four replicate samples with 0, 25, 100 and 400 juveniles of *M. incognita* or *M. javanica* added per 400 g of soil were assessed.

Experiment 4. Known numbers of *M. incognita* were added to each of two soil types i) light sand, from the sandy part of an alkaline yellow duplex type soil at Padthaway and ii) clay from a hard alkaline red duplex type soil at Nuriootpa, SA and then assessed by the DNA method. Sixteen replicate samples each of sand and clay soil were assessed, with 0, 5, 40 and 320 juveniles added per 400 g soil.

Vineyard soils from which RKN could not be extracted were used in all assessments.

3.2.3 Results

On first examination of naturally infested soil samples, there appeared to be a poor relationship between the DNA method and the numbers of active nematodes extracted (Fig. 3.2.1). The DNA method gave an estimate of the mean RKN population about 11 times higher than that obtained by nematode extraction. This result prompted a more detailed assessment of the DNA method, as it was possible that the results over-estimated the nematode DNA due a lack of specificity, or the nematode extraction method may underestimated the actual population in some samples. However, clear relationships were found when the DNA assay was applied to soil samples with addition of known numbers of RKN juveniles (Fig. 3.2.2) and known number of RKN eggs (Fig.3.2.3). A strong relationship was also found between the DNA assay and addition of nematodes for both *M. incognita* and a *M.*

javanica (Fig. 3.3.4). The relationship between the DNA assay and number of nematodes added remained robust even in the two soil types (Fig. 3.3.5).

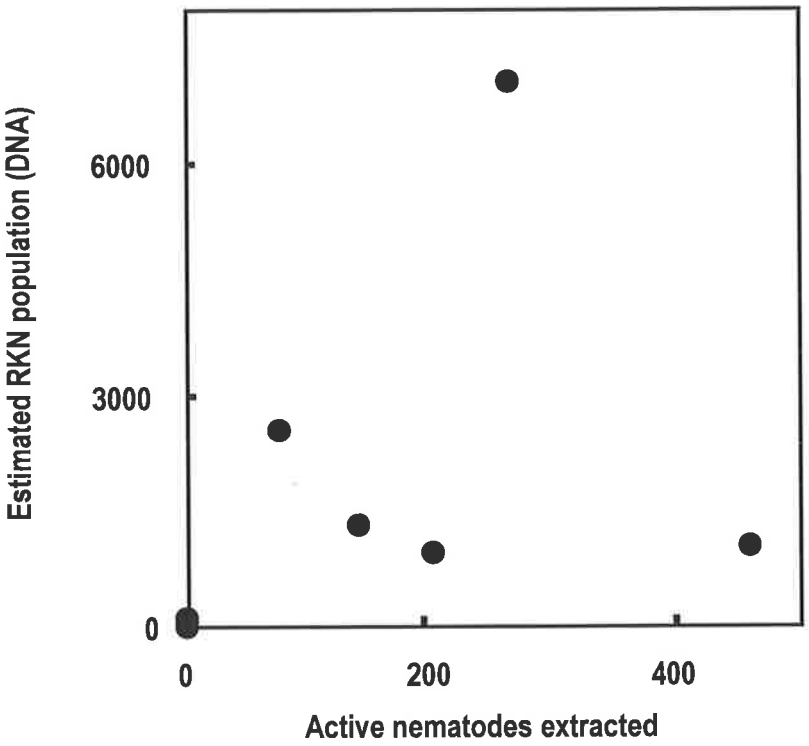


Figure 3.2.1 Relationship between root-knot nematode populations in naturally infested soil estimated by DNA method and extraction of active nematodes (400 g soil).

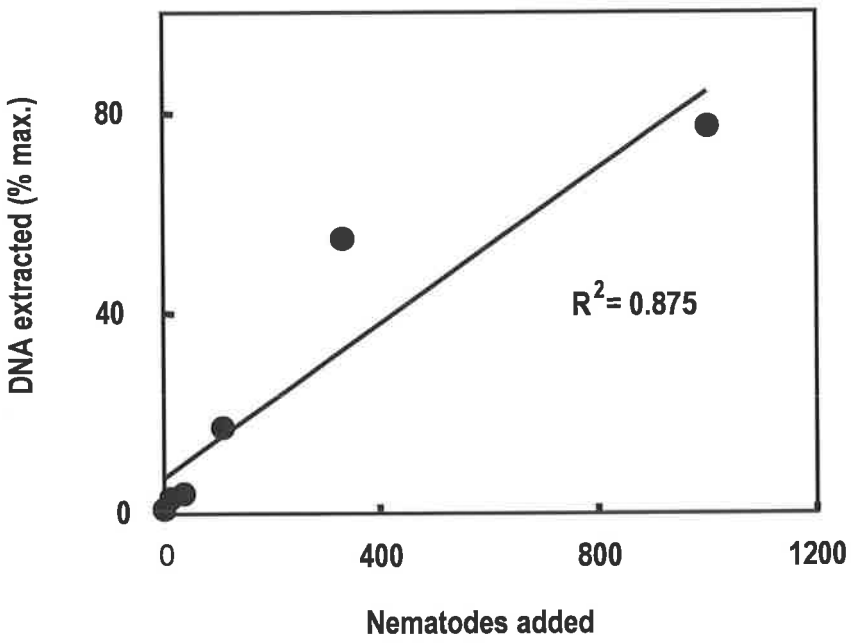


Figure 3.2.2 Relationship between root-knot nematodes DNA extracted and number of individuals added to soil (400 g)

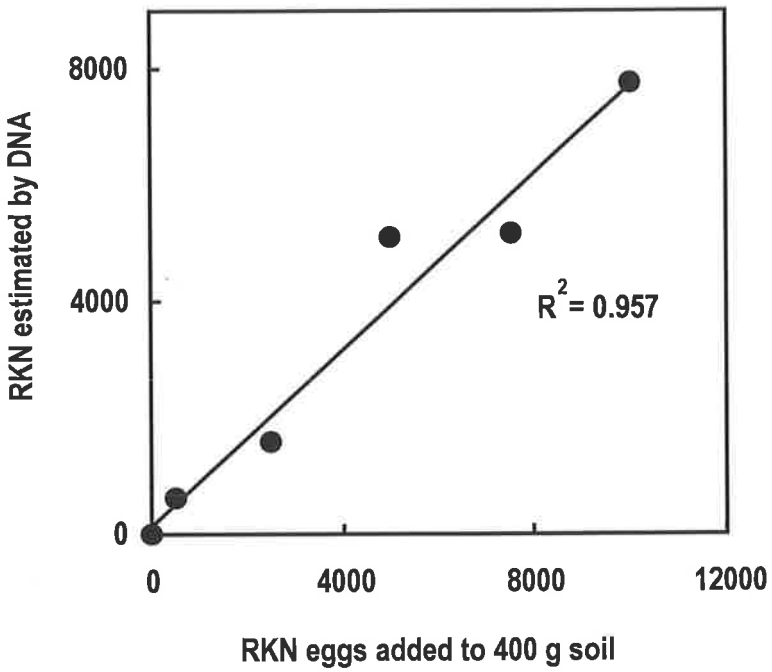


Fig. 3.2.3 Relationship between root-knot nematodes DNA estimated and eggs added to soil (400 g).

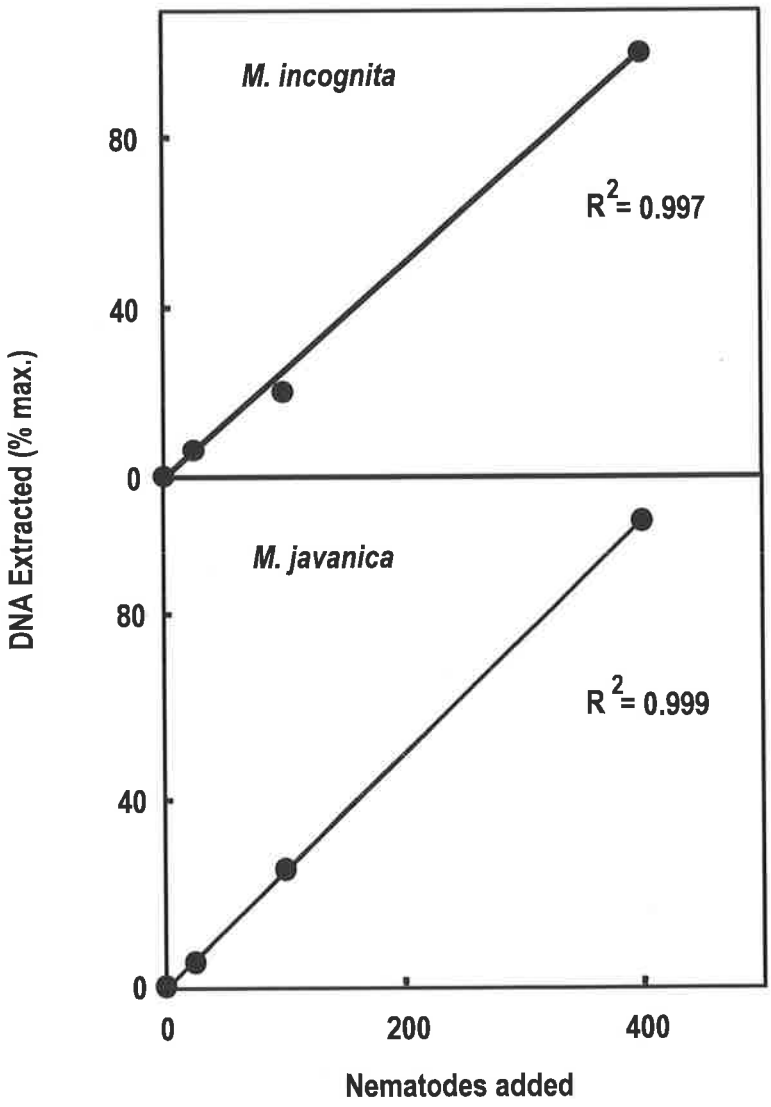


Figure 3.2.4 Relationship between DNA extracted (%) and number of juveniles of root-knot nematodes added to soil (400 g) for two root-knot nematode species.

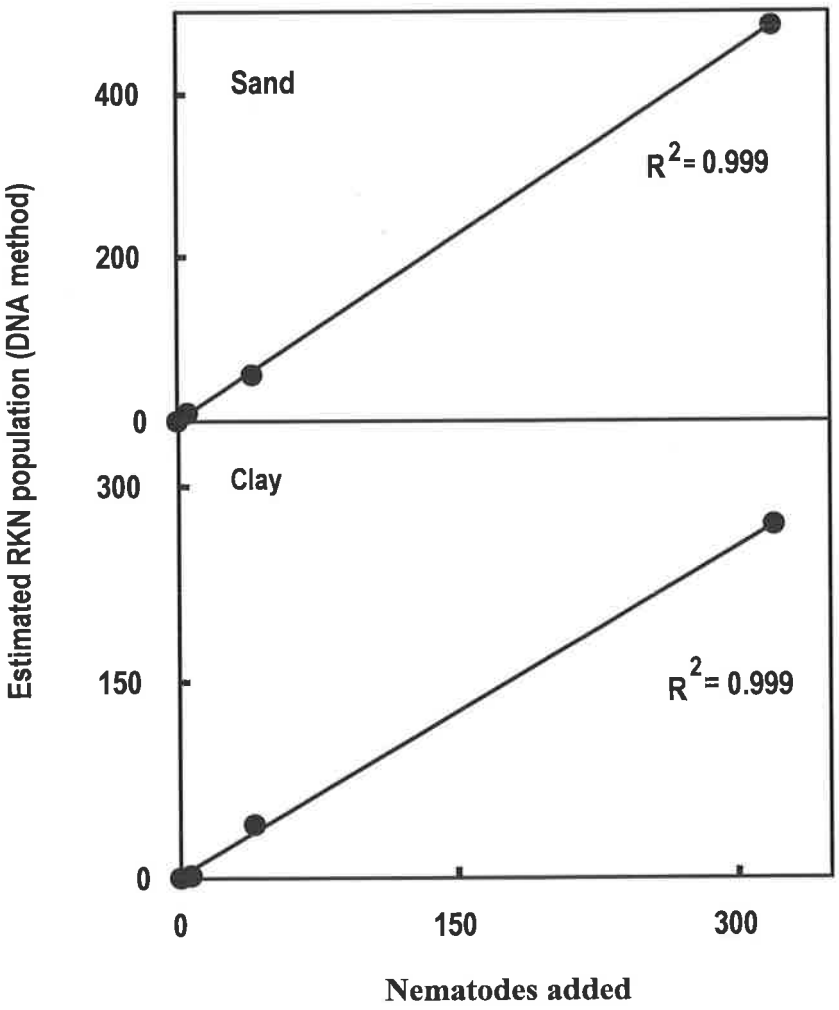


Figure 3.2.5 Relationship between DNA extracted and number of juveniles of root-knot nematodes added to two soil types (400 g soil)

3.2.4 Discussion

The disparity between the DNA results and nematode extraction from naturally infested soils may be due to the presence of RKN eggs The nematode extraction method only estimated the population of active nematodes and even then, extraction is unlikely to be complete. Hollaway *et al.* (2001) also found that the DNA method gave higher estimates of root lesion nematode populations than those obtained by extraction of active nematodes. Subsequent tests showed that the DNA method could estimate RKN eggs present in soil. In these experiments, the DNA assay could detect levels as low as 40 juveniles per 400 g soil, equivalent to the estimated damage threshold for RKN in grapevine (Stirling *et al.* 1999). The DNA assay appears not only to be adequately sensitive but is consistent for the accurate estimation of both important species (*M. incognita* and *M. javanica*) in both clay and sandy soils, so it is likely that the method could be successfully applied to a range of soils occurring in Australian vineyards. However, further work, including validation of the DNA method to estimate number of RKN present in a range of vineyards, was needed for the technology to become a useful vineyard management tool. This work is described in Chapter 4.

CHAPTER: FOUR

RKN QUANTIFICATION METHODS, SPECIES IDENTITY AND NON RKN IN VINEYARDS

4.1 Root-knot nematode (*Meloidogyne* spp.) quantification methods and identity revealed by DNA and nucleotide polymorphism in rRNA genes

4.1.1 Introduction

The estimation of population densities of *Meloidogyne* spp., (RKN) is performed mainly by extraction of live juveniles from soil and occasionally by bioassay involving growing host plants in infested soil. These methods are time-consuming and subject to considerable variability. In addition, the extraction method does not estimate populations of RKN eggs in soil. A DNA-based method is commercially available as "PreDicta B" (C-Qentec, Diagnostic, Aventis CropScience) for the quantification of root-lesion nematodes in cereals (Ophel-Keller *et al.* 1999; Hollaway *et al.* 2001) and can be used for the quantification of RKN in tomato (Stirling *et al.* 2001). Preliminary studies showed that the method is also useful in quantification of RKN affecting grapevines. However, before offering this test as a routine service for the viticulture industry, further study was needed to determine RKN genetic diversity and to verify the ability of the DNA method in quantifying the range of RKN populations found in grape growing areas. In addition, the area under viticulture has increased greatly since the survey of plant parasitic nematodes in 1976 conducted in South Australia (Stirling 1976). Knowledge of the quantity and identity of RKN populations in vineyards is also important for the development of an effective management strategy against RKN.

Molecular tools, such as specific amplification of mitochondrial DNA (mtDNA) followed by restriction fragment length polymorphism (Powers and Harris 1993; Hugall *et al.* 1994; Stanton *et al.* 1997), rDNA analysis (Powers *et al.* 1997; Zijlstra *et al.* 1997, 2000) have successfully been used to identify *Meloidogyne* spp. from various sources. In addition, the versatility in the internal transcribed sequences (ITS) of rRNA genes as a genetic marker has made this region attractive for a wide

range of genetic studies including variability studies in nematodes (Cherry *et al.* 1997; Stanton *et al.* 1997; Szalanski *et al.* 1997; Uehara *et al.*1999; Goncalves and Rosto 2000).

Therefore, the aims of this study were (1) to detect and quantify RKN in vineyards and (2) to determine the variability in rRNA genes of a selection of RKN populations in order to validate the available DNA quantification probes.

4.1.2 Materials and methods

4.1.2.1 Sampling vineyards

Twenty-one locations in three areas (Riverland, Fleurieu Peninsula and South-East) of South Australia (SA) were selected on the basis of being major grape growing areas in 2000, obtained from the Australian Bureau of Statistics (Fig. 4.1.1). In each location 1-5 vineyards (one vineyard/1000 ha) were sampled randomly. A total of 49 vineyards were sampled from these grape-growing areas of SA. A composite soil sample of 15-20 cores was collected from about 0.4-0.6 ha of a vineyard. Each core sample was collected about 100 mm from the vine up to 300 mm deep. The composite sample of about 2000 ml was placed in a plastic bag, transported in an insulated container and stored in a fridge until assessed. The samples were processed within one to three days of collections. Three sub-samples of 400 ml each were taken from a composite sample to estimate RKN number using (1) extraction, (2) DNA and (3) bioassay methods.

4.1.2.2 Quantification methods

4.1.2.2.1 Extraction method

The population of RKN juveniles in one sub-sample was estimated under a microscope after extraction by spreading the 400 ml soil in a thin layer on facial tissue

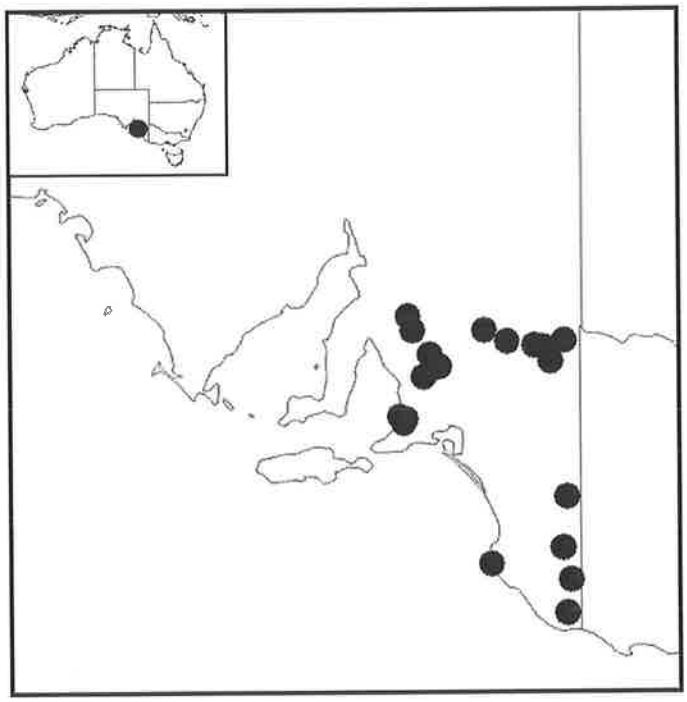


Figure 4.1.1 Soil sampling locations for root-knot nematode (*Meloidogyne* spp.) in vineyards from South Australia.

over a mesh support standing in water at room temperature for five days (Whitehead and Hemming, 1965). Nematodes were collected on a 20 μM sieve, and counted in a Sedgewick Ratter Cell (Graticules Ltd, Tonbridge, UK) counting slide under compound microscope at 100X or 200X magnification.

4.1.2.2.2 DNA method

A sub-sample of 400 ml soil was dried on a plastic tray at room temperature for about 10-13 days and submitted to the Root Disease Testing Service at South Australian Research and Development institute for their DNA test (PreDicta-B®). The DNA method can not be detailed due to commercial in-confidence but it is based on PCR amplification of rDNA of root-knot nematodes (Ophel-Keller *et al.* 1999).

4.1.2.2.3 Bioassay

Soil sub-samples of 400 ml each were added to 100 mm diameter plastic pots. Susceptible tomato seedlings (cv Roma) raised in a nematode-free steam sterilised UC potting mix (composition and preparation of UC soil is described in Chapter 5.1.2.1, Baker 1957) were transplanted in the pots and grown in a glasshouse. Four weeks after re-planting, the roots were carefully washed from soil (Barker 1985). The galls in the roots were counted using illuminated magnifier. The heavily galled roots were indexed using a 0-5 scale as follows: 0, no gall; 1, 1-24% of the roots galled; 2, 25-49%; 3, 50-74%; 4, 75-99%; 5, all roots galled (Stirling 1982).

The texture of the moist soil in pots were estimated from the observation of the changes in a small handful of soil worked into a ball and pressed between thumb and forefinger (Forge 1995).

4.1.2.3 RKN in galls of bioassay plants

After gall enumeration or scoring, a selection of galled roots (10-15 galls/selection/sample) were placed in water in Petri dishes for three days at room temperature to soften the roots. Galls were dissected under a microscope and female nematodes were counted from each gall as described above. Remaining root systems of bioassay plants were dried at room temperature to determine their weight per bioassay plant.

4.1.2.4 Determination of species identity of RKN

4.1.2.4.1 RKN preparation

Ten to fifteen RKN females were extracted from galls of the infested tomato plants used in the bioassay in soils from four regions (Riverland, Fleurieu Peninsula and South-East) to determine the species identity by DNA method.

4.1.2.4.2 DNA Extraction from single female

Described in Chapter 3.1.2.2.

4.1.2.4.3 PCR amplification

DNA suspension (10 µl) was added to 0.5 ml PCR tube containing PCR muster mix of 5 µl of 10X DNA Taq polymerase incubation buffer, 3 µl of 25 mM MgCl2, 1 µl of 10mM dNTP-mixture (Sigma), 0.8 µM of each primer (GeneWorks, Adelaide, Australia), 2.5 U Taq polymerase (Promega Corporation, NSW, Australia), and double distilled water to a final volume of 50 µl. Seventy µl mineral oil was added into each tube containing PCR mix. The mixture was placed in a DNA thermal

cycler (MJ Research Inc., USA). In each PCR run a negative control without DNA template was included.

Initially, all female DNA extracts were amplified using the reaction conditions described by Powers and Harris (1993). DNA extracts producing a *M. incognita/M. javanica* type fingerprint by the method of Powers and Harris (1993) were used in Sequence Characterised Amplified Regions (SCAR) method to distinguish *M. javanica* from *M. incognita* (Zijlstra *et al.* 2000). DNA samples produced *M. incognita/M. javanica* type band (1.7 kb DNA band) by the Powers and Harris (1993) method, but did not produce a *M. javanica*-type fingerprint in replicated PCR amplifications, were considered as *M. incognita*.

4.1.2.4.4 Restriction digestion of PCR products

PCR products of a *M. hapla*-type were digested with *Dra*I (2 µl sterile ddH2O, 8 µl PCR product, 1.2 µl 10X buffer and 1 µl enzyme, SIGMA, USA) at 37^{O}C overnight to produce restriction fragments that were confirmed the identity of the species *M. hap*la (Powers and Harris 1993).

4.1.2.4.5 Gel electrophoresis and photography of gel

Described in Chapter 3.1.2.7.2.

4.1.2.4.6 Primers

The sequences of the primers used in this study are shown in Table 4.1.1. The primers were designed and synthesised based on published sequences using the commercial facility GeneWorks, Adelaide, SA.

4.1.2.5 The ITS-1, 5.8S and ITS-2 of rRNA genes in RKN of SA

DNA isolated from two to three individuals of each identified species was used in PCR for the amplification of ITS-1, 5.8S and ITS-2 of the rRNA genes using primers described by Vrain *et al.* (1992) (Table 4.1.1). The amplified 760 bp PCR products were purified using a DNA purification kit (Roche Diagnostics GmbH). The high yielding purified PCR products were sequenced directly without cloning using specific primers

(18S, 5'-TTGATTACGTCCCTGCCCTTT-3' &

26S, 5'-TTTCACTCGCCGTTACTAAGG-3') while the low yield PCR products obtained from samples were cloned before sequencing.

4.1.2.5.1 Cloning of PCR products

The low yield purified PCR products were adjusted to a volume of 100 µl with double distilled water (ddH_2O) and precipitated by adding 20 µl of 0.3 M sodium acetate (pH 5.3) and 200 µl absolute ethanol followed by centrifugation at 14000 G for 7 min. DNA was re-suspended in 15 µl ddH_2O. The purified DNA fragments were cloned into plasmids vector (pGEM®–T-Easy vector system), transformed the plasmids into high efficiency competent cells (JM109) and colonised onto LB agar plates according to the instructions of the manufacturer (Promega Corporation, USA).

4.1.2.5.2 Plasmid DNA preparation

Three to four white colonies (bacterial cells transformed with nematode rDNA inserted plasmid) were isolated using blue/white selection and multiplied in liquid LB medium. Plasmid DNA were extracted from bacterial cells (JM109) using slightly modified mini-preparation method of Sambrook *et al.* (1989). An RNAase digestion

step was introduced before phenol:chloroform extraction step. The RNAase (DNAase free) was added at the rate of 20 μg/ml and mixed by flipping tubes then incubated at 37^{O} C for 20 min. In addition, the phenol:chloroform extraction step was only performed when the high quality preparations were needed.

In some cases (due to poor transformation) high yield and quality plasmid DNA was needed. In such case, plasmids were isolated from bacteria using Wizard® Plus SV Minipreps DNA Purification System according to the instructions of the manufacturer (Promega Corporation, USA).

The isolated plasmid DNA was tested for the insert by PCR amplification with M13 forward and reverse primers.

4.1.2.5.3 DNA quantification

Described in section 3.1.2.3.

4.1.2.5.4 DNA sequencing

DNA sequencing of cloned nematode rDNA was performed with M13 reverse and forward primers using in an Applied Bio System 373 sequencer (USA).

4.1.2.6 Data analysis

The computer program Bio-link® was used to generate a map of SA and sample locations. The program log-Linear Models of GENSTAT®-5 for the analysis of contingency table (Table 4.1.3) was used to determine accumulated analysis of varience (Table 4.1.4). As each devience, in the accumulated analysis of varience table (Table 4.1.4), is distributed as a chi-square on the associated degrees of freedom therefore, the terms in this models were tested (described in discussion) to determine

the relative proportions of RKN incidence in vineyards and for the interactions between the risks for RKN and methods used to determine the risks in vineyards of SA. A student's t-test (t-test) analysis was performed to test the null hypothesis that there were significant differences in dry root weight of individual bioassay plant and also in RKN numbers per 10 galls. The correlation between RKN number per 10 galls and total galls per bioassay plant roots was also determined using GENSTAT®-5. Computer program SeqEd® was used to edit DNA sequences. This program was used to remove the vector and primer sequences and to do simple overlap analysis of clones. Sequences were aligned using the MegAlign program of LASERGENE® to estimate the sequence pair distances (using a cluster method with weighted residue weight table) and construction of a phylogenetic tree. The BLAST at National Centre for Biotechnology Information, USA, was used for sequence similarity searches (Altschul *et al.* 1990). The coding and non-coding sequences in rRNA genes of RKN were identified by aligning with the known sequences in the GenBank. The GenBank rRNA genes sequences from *Meloidogyne* spp. (accession numbers U96301 to U96305 and AF248477), which showed maximum similarity with the sequences of this study, were used as inner group controls and sequences from a different nematode genus (eg *Heterodera glycines*, accession number AF216579) were used as an out-group control in sequence similarity analysis and the construction of a phylogenetic tree.

4.1.3 Results

4.1.3.1 Occurrence of RKN in vineyards and evaluation of quantification methods

Of 49 vineyards sampled, 26, 24 and 14 were found infested with RKN using DNA, extraction and bioassay methods respectively (Table 4.1.2). The DNA and

bioassay methods showed similar results in 33 vineyards (12 infested and 21 uninfested), DNA and extraction methods showed similar results in 34 vineyards (17 infested and 17 uninfested), whereas, extraction and bioassay methods showed similar results in 29 vineyards (9 infested and 21 uninfested). Combined DNA, extraction and bioassay methods showed similar results in only 25 vineyards (9 infested and 16 uninfested). The DNA method gave higher RKN counts over bioassay and extraction methods in 26 and 17 vineyards respectively, whereas the extraction method gave higher RKN counts over bioassay and DNA methods in 20 and 15 vineyards respectively. The bioassay method never estimated a higher count than DNA but gave higher counts over the extraction method in seven vineyards (Table 4.1.2).

On average of three extraction methods about 33% vineyards pose low to medium risk (14% low and 19% medium) while 11% vineyards showed in high-risk category (Table 4.1.3). The chi-square test for the relative proportions of the RKN infested vineyards detected by the three methods showed significant interactions between DNA x extraction methods and DNA x bioassay methods, but no significant interaction was found between extraction and bioassay methods (Table 4.1.4). The DNA and extraction methods showed similar ability to detect each of the risk categories estimated for the RKN infested vineyards, except in four cases where the bioassay underestimated of the risk category (sample no 25, 26, 36 and 37) (Table 4.1.5).

Variation was found in populations of RKN in infested vineyards with different soil types and the methods used (Table 4.1.5). The highest mean infestation was recorded in sandy soils and the lowest in loamy clay and clay, while a moderately high number of infested vineyards were found within clay soil with occasional limestone (Table 4.1.5). The DNA method had the highest detection ability in all soils except loamy caly.

4.1.3.2 RKN in galls of bioassay plants

One to three RKN females per gall were found in roots of tomato plants used in the bioassay method (Table 4.1.6). The majority of the galls contained a single female but several females were found in some galls. In general, there was a trend towards more females per gall in heavily infested soils (Fig. 4.1.2). The tomato plants grown in clay soil produced significantly fewer amounts of roots than plants grown in sandy soil (Table 4.1.6).

4.1.3.3 Species identity of RKN

DNA samples from RKN females amplified using mtDNA specific primers (Table 4.1.1, no. 1 and 2) produced the banding pattern of *M. arenaria* (1.1 kb) or *M. incognita/M. javanica* (1.7 kb) or *M. hapla* (0.52 kb). Whereas, primers No. 5 and 6 (Table 4.1.1) produced the *M. javanica*-type banding pattern (0.67 kb) (Fig. 3A). The restriction digestion of *M. hapla* PCR product (0.52 kb) produced two 0.29 kb and 0.23 kb fragments (Fig. 4.1.3B). This confirms the species identity of *M. hapla* because the 0.52 kb DNA can also be amplified from *M. chitwoodi, M. marylandi, M. naasi* and *M. nataliei* but restriction sites of these species differ from those of *M. hapla* (Powers and Harris 1993). The vineyard RKN populations in this study consisted of either one or a mixture of two or more *Meloidogyne* species (Table 4.1.7). Vineyards in Renmark and Loxton areas had more *M. incognita* and *M. javanica* than *M. arenaria* and *M. hapla*, but in Robe (South-East region) the species, *M. hapla,* was only found in a single infested vineyard. A vineyard in Barossa valley (Nuriootpa) contained a mixture of four nematode species (Table 4.1.7).

3.1.3.4 Nucleotide identity in rRNA genes

A 760 bp band was amplified across all individuals of the species studied when specific primers (Table 4.1.1, No. 3 & 4) were used in the PCR reaction (Fig. 4.1.3C). The sequences of this DNA fragment from different species were submitted to the GenBank (appendix B, accession numbers AF510057 to AF510064 and AF516721 to AF516723). The sequence alignment report is given in appendix C. It appeared from these sequence analysis that the sequences from ITS-1, 5.8S gene and ITS-2 of rRNA genes of *M. arenaria, M. incognita* and *M. javanica* were highly conserved among the individuals of these species from different regions of SA. The highest similarity (91 to 100%) was found between the sequences from the individuals of *M. arenaria, M. incognita* and *M. javanica* (Table 4.1.8). The similarity between the individuals of *M. hapla* and *M. arenaria, M. incognita* and *M. javanica* was 61-68% and the similarity between *M. hapla* and *M. chidwoodi* was 67-67%. *M. artiellia* from Italy was the most distally related inner group of *Meloidogyne* spp. analysed (Mart-GenBank.seq in Table 4.1.8).

The sequences from two individuals of *M. arenaria* from the same location were identical but some variation was observed between individuals from different locations (Fig. 4.1.4). However, all the individuals of *M. arenaria*, including one existing GenBank sequence, were grouped into one sub-group. The individuals of the species *M. javanica* and *M. incognita* were grouped into four sub-groups. Individuals of *M. hapla* were grouped into two sub-groups. As expected, none of the inner-group members were clustered with out-group species *Heterodera glycines*. Individuals of one species sub-group were included into another species sub-group (Fig. 4.1.4).

Table 4.1.1 Primers used in PCR reactions and their binding sites in DNA.

No	Primer sequences	Primer position/name	References
1	5'-GGTCAATGTTCAGAAATTTGTGG-3'	COOII gene	Powers and Harris 1993
2	5'-TACCTTTGACCAATCACGCT-3'	LrRNA gene	„
3	5'-TTGATTACGTCCCTGCCCTTT-3'	18S rRNA gene	Vrain *et al.* 1992
4	5'-TTTCACTCGCCGTTACTAAGG-3'	28SrRNA gene	„
5	5'-GGTGCGCGATTGAACTGAGC-3'	SCAR	Zijlstra *et al.* 2000
6	5'-CAGGCCCTTCAGTGGAACTATAC-3'	SCAR	„

Table 4.1.2 Estimation of root-knot nematode numbers per 400 g soil samples by different methods from vineyards of South Australia.

Locations	Longitude/Latitude	Sample No.	Root-knot nematodes no. estimated by three methods		
			DNA	Bioassay	Extraction
Auburn	34°31'S 138°41'E	1	6	0	0
		2	825	0	16
		3	0	0	23
		4	0	0	0
Clare	33°51'S 138°37,E	5	0	0	0
		6	0	0	0
Morgan	34°02'S 139°40,E	7	3104	0	210
		8	110	23	0
Waikeire	35°11'S' 139°59'E	9	20	0	462
		10	0	0	0
		11	355	0	280
		12	0	0	23
Kingston	34°14'S 140°21'E	13	0	0	0
Barmera	34°15'S 140°28'E	14	0	0	0
		15	213	0	36
		16	115	0	170
Renmark	34°10'S 140°45'E	17	0	0	88
		18	0	8	0
		19	100	98	8246

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Continued

Locations	Longitude/Latitude	Sample No.	Root-knot nematodes no. estimated by three methods		
			DNA	Bioassay	Extraction
Loxton	34°27'S 140°34'E	20	214	0	143
		21	0	1	24
		22	308	232	231
Kapunda	34°21'S 138°55'E	23	0	0	0
		24	5	0	0
Barossa	34O15'S 138O50'E	25	2508	Scale 4	176
		26	1255	Scale 3	54
Tanunda	34°32'S 138°58'E	27	163	78	0
		28	0	0	78
		29	0	0	0
Angaston	34°31'S 139°03'E	30	43	6	495
		31	22	1	0
		32	0	0	0
Noarlunga	35°11'S 138°30,E	33	0	0	0
		34	13	0	0
McLaren Flat	35°13'S 138°55'E	35	372	0	452
		36	2698	Scale 5	6102
		37	510	Scale 3	5140
Willunga	35°16'S 138°33'E	38	0	0	0

Continued to next page

Continued

Locations	Longitude/Latitude	Sample No.	Root-knot nematodes estimated by three methods		
			DNA	Bioassay	Extraction
Tatiara	36°16'S 140°47'E	39	7	0	0
		40	53	0	20
		41	0	0	8
Naracoorte	36°57'S 140°44'E	42	15	2	158
		43	0	0	0
		44	0	0	0
MtGambier	37°50'S 140°47'E	45	0	0	0
		46	0	0	0
Penola	37°23'S 140°47'E	47	0	0	0
		48	3	0	0
Robe	37°10'S 139°45'E	49	130	108	351

Table 4.1.3 Vineyards in different risk categories for root-knot nematodes (RKN, *Meloidogyne* spp.) estimated by three quantification methods.

[1]RKN risk category	Root-knot nematode infested vineyards estimated by different quantification methods (%)			
	Extraction	Bioassay	DNA	Average
Low	14	12	16	14
Medium	23	8	25	19
High	12	8	12	11
Total	**49**	**28**	**53**	**44**

1 = Nematode extraction efficiency <40, 40-400, >400 root-knot nematodes/400 ml soil represent low, medium and high risk situations respectively (Stirling *et al.* 1999).

Table 4.1. 4. Table of accumulated analysis of varience (AANOVA).

Change	df	Deviance	Mean deviance	Deviance ratio	Approx chi probability
Extraction	1	0.184	0.184	0.18	0.668
Bioassay	1	11.232	11.232	11.23	0.001
DNA	1	0.020	0.020	0.02	0.886
Extraction x bioassay	1	0.3583	3.583	3.58	0.058
Extraction x DNA	1	11.216	11.216	11.22	0.001
Bioassay x DNA	1	6.408	6.408	6.41	0.011
Residual	1	0.396	0.396		
Extraction x bioassay x DNA	1	0.396	0.396	0.4	0.529
Total	7	33.039	4.720		

Table 4.1.5 Comparative ability to detect root-knot nematodes (*Meloidogyne* spp.) by three methods in different vineyard soils.

Soil	Percent infested vineyards detected by different quantification methods			
	Extraction	Bioassay	DNA	Mean
Clay	38	6	50	31
Loamy clay	14	29	14	19
Clay with limestone	29	43	57	43
Sandy loam	10	50	75	45
Loamy sand	60	20	20	33
Sand	78	56	89	74
Mean	38	34	51	41

Table 4.1.6 *Meloidogyne* galls per plant, females per ten galls and dry root weights of bioassay plants (tomato) grown in pots with soils from vineyards, infested with root-knot nematodes.

Sample No.	Galls/plant/400 g soil	[1]RKN females per 10 galls	[2]Dry root wt./plant (g)	Soil type
8	23	13	0.37	Clay with limestone
18	10	10	0.52	Loamy clay
19	98	12	1.68	Sandy loam
22	232	16	1.5	Sand
25	Scale 4	21	0.65	Sand
26	Scale 3	19	0.81	Sandy loam
27	78	13	1.17	Sand
26	Scale 5	18	0.43	Sand
37	Scale 3	17	0.34	Loamy clay
49	108	16	1.42	Sand

1) $t = -2.16$, 2) $t = -1.27$ in student's t-test

Table 4.1.7 DNA based species identity of root-knot nematodes from vineyards in South Australia.

Vineyard locations	Number of pecies identified				
	Total females tested	*M. incognita*	*M. javanica*	*M. arenaria*	*M. hapla*
Renmark	8	0	8	0	0
Loxton	6	6	0	0	0
Nuriootpa	7	2	3	1	1
Tanunda	5	0	0	5	0
McLaren Vale	7	2	1	0	4
Robe	6	0	0	0	6

Table 4.1.8 Sequence pair distances of similarity metrix, using cluster method with weighted residue weight table.

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		93.7	97.9	97.3	65.8	67.1	66.6	91.2	98.1	96.5	96.6	97.9	97.7	98.1	97.9	95.4	66.8	47.4	40.7	1	Ma-NewRes.seq
2	1.9		94.8	93.3	61.9	62.9	63.0	88.9	94.2	92.5	93.0	94.2	94.0	94.4	94.4	91.6	63.9	47.3	40.5	2	Ma-Tanunda1.seq
3	1.0	1.9		97.9	66.2	67.3	67.2	92.2	99.0	97.1	97.7	98.8	98.5	99.0	99.0	96.2	67.7	49.6	40.6	3	Ma-Tanunda2.seq
4	0.4	2.1	0.8		66.9	68.1	67.4	91.8	98.8	99.2	97.3	98.3	98.1	98.5	98.5	98.3	67.9	43.3	43.8	4	Ma-GenBank.seq
5	21.6	23.2	22.0	21.6		97.9	94.6	63.5	67.5	66.7	67.1	66.2	66.7	67.1	67.4	67.4	65.4	45.0	45.4	5	Mh-Barossa.seq
6	21.3	22.9	21.6	21.3	0.8		94.8	63.7	68.3	68.1	67.5	67.3	67.7	68.1	68.1	68.3	66.7	44.4	46.1	6	Mh-Rob.seq
7	21.6	23.3	22.0	21.6	1.1	0.8		62.7	68.1	67.4	67.1	67.2	67.6	68.1	67.8	67.2	66.6	47.6	46.3	7	Mh-GenBank.seq
8	1.9	3.4	2.1	1.3	22.9	22.5	22.9		93.1	91.2	91.4	93.1	92.7	93.1	92.7	90.8	63.7	44.7	37.9	8	Mi-Adelaide1.seq
9	0.8	2.3	1.0	0.2	21.3	21.0	21.3	1.3		98.3	98.7	99.4	99.6	100.0	100.0	97.5	68.4	48.9	43.7	9	Mi-Adelaide2.seq
10	1.0	2.7	1.5	0.6	21.6	21.3	21.2	1.7	0.4		96.8	97.5	97.7	98.1	98.1	99.0	68.1	44.2	43.5	10	Mi-GenBank.seq
11	0.8	2.3	1.1	0.2	20.8	20.5	20.8	1.3	0.0	0.4		98.1	98.3	98.7	98.7	95.8	68.4	48.9	42.6	11	Mj-Adelaide.seq
12	0.8	2.3	1.0	0.2	21.8	21.5	21.9	1.5	0.4	0.8	0.4		99.0	99.4	99.4	96.7	67.9	48.5	43.8	12	Mj-Renmark1.seq
13	1.3	2.7	1.5	0.6	21.8	21.4	21.8	1.7	0.4	0.8	0.4	0.8		99.6	99.6	96.9	68.1	48.5	43.3	13	Mj-Renmark2.seq
14	0.8	2.3	1.0	0.2	21.3	21.0	21.4	1.3	0.0	0.4	0.0	0.4	0.4		100.0	97.3	68.5	49.0	43.8	14	Mj-Barossa1.seq
15	0.8	2.3	1.0	0.2	21.4	21.0	21.5	1.3	0.0	0.4	0.0	0.4	0.4	0.0		97.1	68.8	49.2	43.9	15	Mj-Barossa2.seq
16	1.0	2.7	1.5	0.6	21.6	21.3	21.2	1.7	0.4	0.0	0.4	0.8	0.8	0.4	0.4		67.8	49.5	43.8	16	Mj-GenBank.seq
17	20.3	21.9	20.4	19.8	21.5	21.6	21.6	20.9	19.7	19.4	19.6	19.8	20.0	19.6	19.6	19.4		50.5	44.7	17	Mc-GenBank.seq
18	32.0	32.8	31.9	31.7	32.5	32.5	32.8	33.0	31.6	31.6	31.0	31.9	32.1	31.7	31.6	31.6	34.0		43.1	18	Mart-GenBank.seq
19	32.6	33.5	33.1	32.7	36.7	36.1	36.7	32.9	32.6	32.8	32.1	32.4	32.9	32.5	32.4	32.8	37.1	35.3		19	Hg-GenBank.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Ma = *Meloidogyne arenaria*, Mh = *M. hepla*, Mi = *M. incognita*, Mj = *M. javanica*, Mc = *M. chitwoodi*, Mart = *M. artiellia*, Hg = *Heterodera glycines*

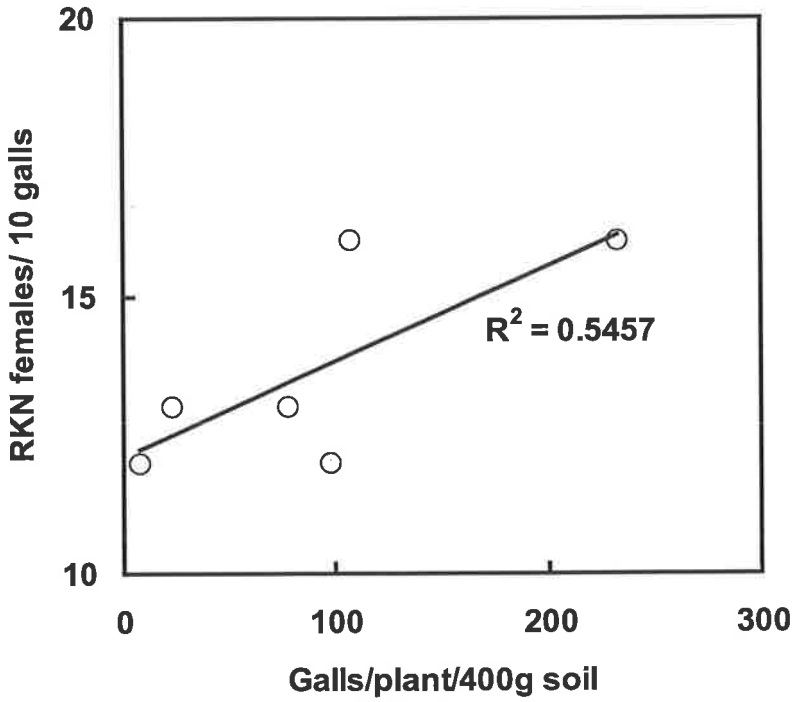

Figure 4.1.2 Relationship between number of female RKN per 10 galls and number of galls per plant per 400 ml soil

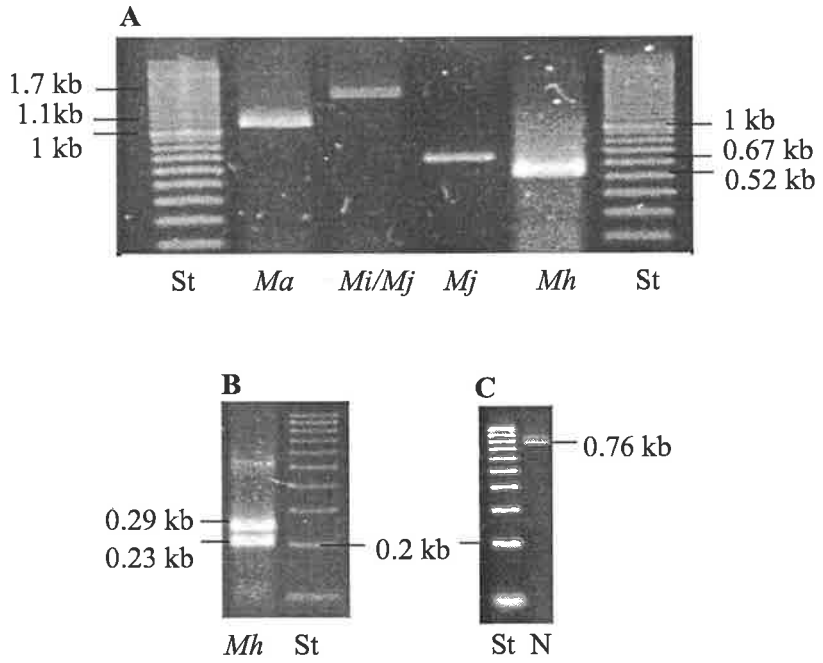

Figure 4.1.3 A) DNA markers for the identification of root-knot nematodes, Lane (L) 1 DNA ladder, L2, L3 and L5 mtDNA-PCR products of *M. arenaria* (*Ma*), *M. incognita* (*Mi/Mj*) and *M. hapla* (*Mh*) respectively, L4 PCR (SCAR) product of *M. javanica* (*Mj*); B) Restriction digestion (*Dra*I) products of PCR-mtDNA of *M. hapla*; C) PCR amplification of a rDNA fragment in *Meloidogyne incognita*. This band was detected in all species tested (not shown here).

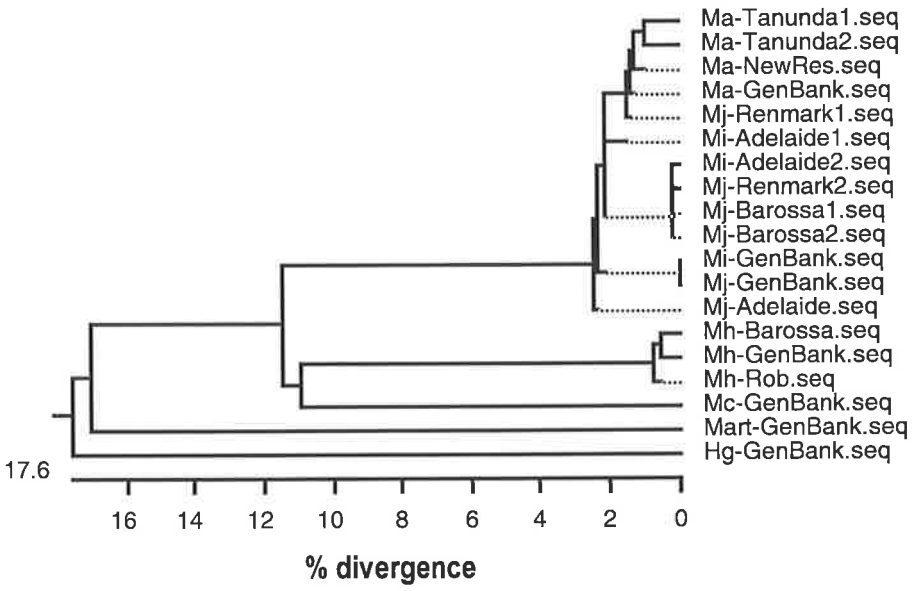

Figure 4.1.4 Dendrogram illustrating phylogenetic relationships among the individuals of root-knot (RKN, *Meloidogyne* spp.) nematodes from different locations of South Australian vineyards (SA) and GenBank sequences. *Ma = M. arenaria, Mi = M. incognita, Mj = M. javanica, Ma = M. hapla, M. chit = M. chitwoodi,* and *Hg = Heterodera glycines*

4.1.4 Discussion

4.1.4.1 Occurrence of RKN in vineyards and evaluation of quantification methods

The significant interaction between the DNA and extraction methods, and DNA and bioassay methods, but no interaction between extraction and bioassay methods indicated that the relative proportion of RKN infested vineyards detected by DNA method is significantly higher than the proportions detected by extraction and bioassay methods.

The better ability of DNA method to estimate RKN in all vineyard soils is supported the previous findings that the DNA method will remains robust in all types of vineyards soils , while the bioassay method has limitations in clay soil. This study supports the findings of Stirling (1982) that RKN are common in vineyards in SA. Stirling (1982) found RKN infestation in almost every vineyard of SA during a distribution study for the parasites of RKN. Like many earlier reports (Sauer 1962; Nicol *et al.* 1999), the current study also found that the RKN are significantly more common in sandy soils than in clay. The comparatively lower RKN detection ability of DNA method in sandy loam soil (Table 2.1.5) may be due to the lower number of RKN in sub-soil samples used in DNA test, as DNA test has a minimum detection limit of 40 RKN per 400 g soil.

4.1.4.2 RKN in galls of bioassay plants

The comparatively low ability of the bioassay method to detect RKN in clay type soils probably due to the textural effect of clay soil on RKN survival and/or invasion, since nematodes were detected in these soils by the other methods. The significantly lower amounts of tomato roots in clay soil during the bioassay also indicate the structural effect of the clay soil on the indicator plant. Hunter (1998)

found that RKN survival is significantly lower in clay soil than in sandy soil. However, further study on relationships between vineyard soil types and RKN invasion ability to bioassay plant roots is needed before drawing any conclusion.

On the other hand, the presence of more than one female in some galls could underestimate the RKN population density in vineyard soil. Therefore, in addition to the lengthy time required for the bioassay method, this method may be inaccurate in some soil types, such as clay. However, given the presence of low RKN density in vineyard soil and inaccuracy of DNA method to detect low RKN density in soil, the bioassay was effective in sandy soil, where it could detect as few as 8 nematodes per 400 ml soil in samples where none were detected by extraction and DNA methods.

4.1.4.3 Species identity

The species *M. incognita*, *M. javanica* and *M. arenaria* can be found in the vineyards of the comparatively warmer north and north-east regions of SA. In contrast, the occurrence of *M. hapla* is more likely in the vineyards of South-East regions of South Australia, where the temperatures are lower. This occurrence in cooler regions is characteristic of *M. hapla* (Trudgill *et al.* 1994; Forge and Macguidwin 1992). Stirling (1976) also reported similar distribution pattern of these *Meloidogyne* species in vineyards of south Australia. Therefor, despite the increased areas under viticulture since the last survey in 1976 (Stirling 1976), no significant change in the occurrence of species of RKN in SA vincyards.

The consistent results of three RKN quantification methods (Table 4.1.2, SN No. 49) for *M. hapla* again confirm the usefulness of the DNA method to quantify RKN species associated with grapevines in SA. This study also indicated that at least four species of RKN occur in grapevines of SA. Therefore, it is reasonable to

conclude that the DNA quantification method will be able to estimate the major species affecting grapevines in Australia.

4.1.4.4 Nucleotide identity in rRNA genes

The close positions of all individuals of each of the *Meloidogyne* species in phylogenetic tree and the high similarity in the sequences of ITS1, 5.8S gene and ITS2 indicated that the rRNA genes of individuals of the species *M. arenaria, M. incognita* and *M. javanica* are highly conserved. Powers *et al.* (1997) also found high similarity in the sequences of rRNA genes in these three species in the USA. The rRNA genes of *M. hapla* contained some dissimilar sequences from other species studied. However, the ability of the DNA method to quantify the RKN population which consisted only of *M. hapla* indicates that the difference observed is not present in the sequences used in the DNA based quantification method. Therefore, this DNA quantification method appears to be effective to quantify all RKN species in vineyards of the South Australia. The highly conserved sequences in rRNA genes of local and overseas populations of the three main species also indicates that the possible effectivess of the DNA method used for the quantification of RKN in USA. However, a large-scale diversity study on rRNA genes in worldwide RKN populations is needed before drawing further conclusions on the applicability of this DNA for all RKN populations. The insignificant change in ITS1, 5.8S gene and ITS2 of individuals of each species studied supports the view that the rRNA genes in individuals of RKN are highly stable. Woese (1987) reported that the sequence of these genes can remain similar over billions of years and the genetic crossing-over in sequences of these rRNA genes can take place only between highly related organisms, resulting in very little or no variation within the organism in these regions. Therefore, it is desirable that the DNA quantification probe is equally effective for all RKN populations found

in grapevines of South Australia and probably also the virulent RKN population, which can overcome the resistance of Ramsey rootstock (Walker 1997). However, a study on the sequence variability in ITS1, 5.8S gene and ITS2 of this virulant population is needed for confirmation.

4.2 Other parasitic nematodes in vineyards of South Australia

4.2.1 Introduction

Survey of parasitic nematode populations is important to provide information on the occurrence of damaging numbers and therefore the need for efficient management strategies. Surveys can also be used to collect vineyard populations to study virulence, resistance, plant nematode interaction and host range. In South Australia, the area under viticulture has increased greatly during last five years due to high demand of Australian wine in world market. Therefore, a survey for the incidence of nematode pests in South Australian vineyards was carried out in the 2001-2002 grapevine growing season to provide an up-to-date picture of populations and their distribution.

4.2.2 Materials and methods

The extraction method used was described in Chapter 4.1.2.2.1. The results for RKN incidence were included in this study for comparison with numbers of other plant parasitic nematodes found in vineyards of South Australia. The risk catagories for each nematode species were determined based on a published classification (McKenry 1992, Table 4.2.1).

4.2.3 Results

All but three vineyards (94%) were infested with at least one and up to five plant parasitic nematodes known to affect grapevines (Fig 4.2.1). Root-knot (RKN), Root lesion and Pin Nematodes were found in all regions (Riverland, Fleurieu Peninsula and South-East), and Ring and Dagger Nematodes were found in the Riverland and South-East regions of South Australia (Fig. 4.2.1). The relative

frequencies of occurrence of all these nematodes were higher in vineyards of the Riverland than in the Fleurieu Peninsula and South-East regions (Fig. 4.2.1).

Root-knot nematode was found in 59% of vineyards surveyed. About 39% of these RKN-infested vineyards had densities posing a high risk while 18% were at medium risk for grape yield loss (Table 4.2.2). The incidence of root lesion (*Pratylenchus* spp.) nematode was second highest (53%), followed by pin (*Paratylenchus* spp., 38%), ring (*Criconemella* spp., 22%) and dagger (*Xiphinema* spp., 10%) nematodes (Fig. 4.2.1 and Table 4.2.2). Forty-nine percent, 22%, 18% and 8% of the vineyards surveyed were in the medium to high-risk categories for root lesion, pin, ring and dagger nematodes respectively (Table 4.2.2).

Table 4.2.1 Relationship between number of nematodes in roots and soil and estimated damage potential in Vitis vinefera grapevines (McKenry 1992).

Nematode species	Risk category	Nematode/400 ml soil in summer
Root-knot	Low	<12
(*Meloidogyne* spp.)	Medoum	100
	High	>100
Lesion	Low	<10
(*Pratylenchus* spp.)	Medium	10-50
	High	>40
Pin	Low	<40
(*Paratylenchus* spp.)	Medium	50-500
	High	>500
Ring	Low	<12
(*Criconemella* spp.)	Medium	12-125
	High	>125
Dagger	Low	<10
(*Xiphinema* spp.)	Medium	10-100
	High	>100

Table 4.2.2 Estimated risks of yield loss from infestations of nematode pests in vineyards of South Australia during 2001-2002 grape-growing season.

Risk Category (McKenry 1992)	Nematode infested vineyards (%) for each risk category				
	[1]Root-knot	Root lesion	Pin	Ring	[1]Dagger
Low	2	4	16	4	2
Medium	18	37	18	16	8
High	39	12	4	2	0
Total	**59**	**53**	**38**	**22**	**10**

1 = Risk category may vary between extraction methods

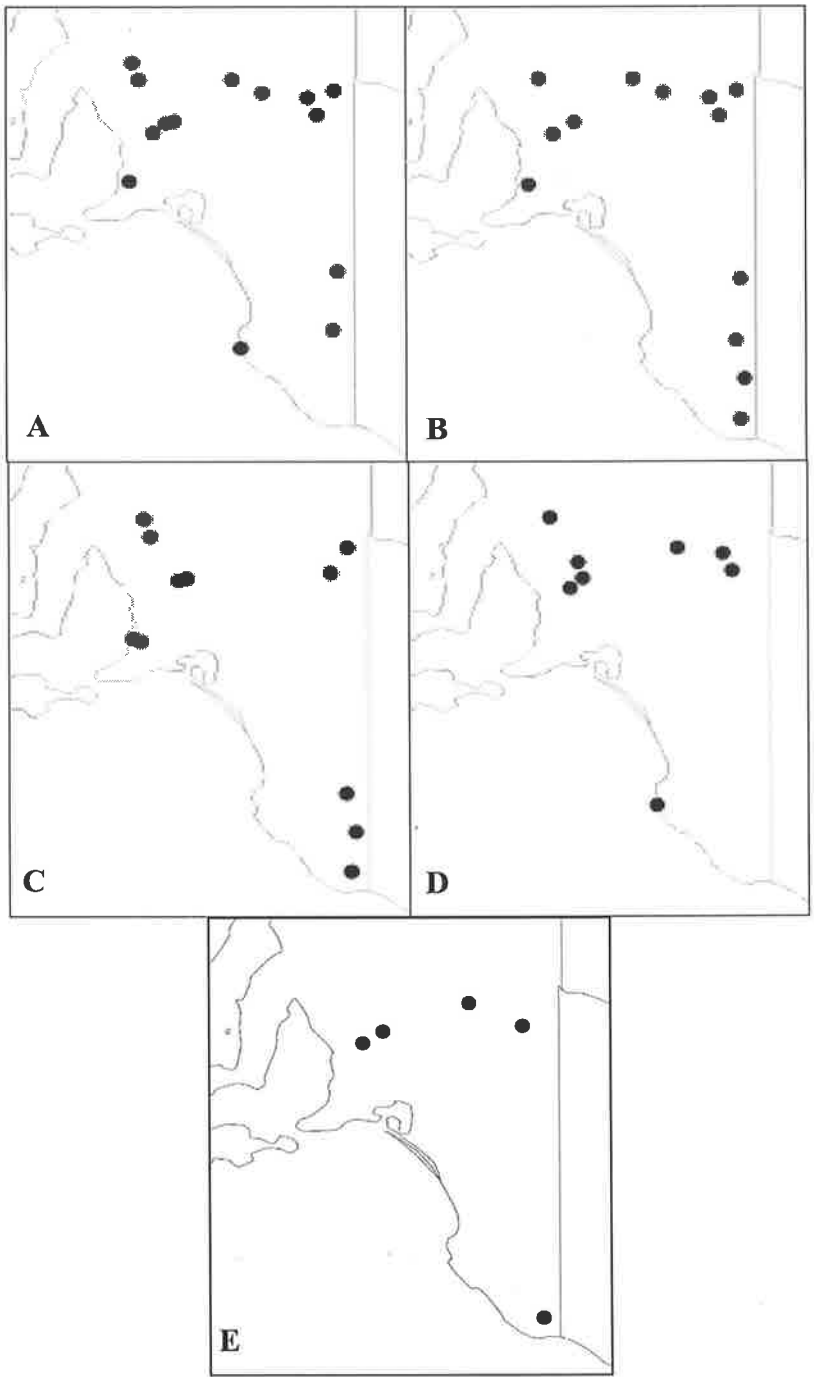

Figure 4.2.1 The occurrence (•) of plant parasitic nematodes in vineyards of South Australia. A) Root-knot, B) Root lesion, C) Pin, D) Ring and E) Dagger nematodes.

4.2.4 Discussion

The findings of this study support those of previous reports that RKN are the most common plant parasitic nematodes found in vineyards of South Australia. Stirling (1982) found RKN infestation in almost every vineyard of South Australia during a distribution study for the parasites of RKN. Like earlier reports (Sauer 1962; Nicol *et al.* 1999), the current study also found that RKN are significantly more common in sandy than in clay soils.

Despite a long grapevine growing history in Australia, few studies have been examined plant parasitic nematodes other than RKN. Root-lesion nematodes have been studied (Walker and Morey 2000; Walker 2001a,b; Walker and Morey 2001) but the influence of other plant parasitic nematodes, especially pin and ring (*Criconemella* sp.) nematodes, grapevine production in Australia has been little examined. Studies in other countries have shown that these nematodes can cause considerable damage in grapevines (Ramsdell *et al.* 1996; Belair *et al.* 2001). The current study indicates the potential for yield loss, as medium and high infestations of these nematodes was found (McKenry 1992). The dagger nematode, *Xiphinema* index, was not found in the vineyards sampled. This is an important nematode because of its ability to carry the damaging virus disease grapevine fan leaf mosaic virus in grape growing areas worldwide, including certain parts of Australia (Meagher *et al.* 1976). The dagger nematode *X. pachtaicum* was identified from soils of South Australia vineyards. The nematode *X. pachtaicum* does not carry any virus but it is not well understood how this nematode has been influenced the growth of grapevines.

CHAPTER FIVE

DAMAGE THRESHOLD FOR RKN ON GRAPEVINES

5.1 Damage threshold for root-knot nematode (*Meloidogyne incognita*) on establishment of grapevine

5.1.1 Introduction

Establishment of a vineyard is a critical phase in grapevine cultivation. Establishment may be unacceptably impaired if the field is infested with parasitic nematodes, such as root-knot nematodes (RKN, *Meloidogyne* spp.). The need to control this pest depends on knowledge of nematode population density in the field and potential effect on growth and yield (Ferris, 1978). This knowledge is important to evaluate the ability of a control method to reduce the nematode population in the soil below the damage threshold (Barker *et al.* 1976).

Damage threshold studies are considered to be more useful when derived from field data, but under field conditions the relationship between nematode population density and yield is influenced by patchiness of nematode distribution (Noe 1993). Containerised, micro-plot field studies provide a compromise between the need for experimental control and natural conditions. Despite some disadvantages in microplot experiments, such as their expense, difficulties in employing standard cultural practices and lack of full interaction between soil flora and fauna, data from microplots have provided valuable information and have been used extensively (Barker *et al.* 1976; Ramsdell *et al.* 1996; Viane and Abawi 1996). The significant advantage of microplots is reduction of variability that is inherent in field-plot data. In Michigan, Ramsdell *et al.* (1996) evaluated the effects of four species of plant parasitic nematodes, including *M. hapla*, on hybrid grapevines under micro-plot conditions. No such study has been conducted in Australia in order to estimate the population density dependent damage caused by *M. incognita*, a major species in Australian vineyards, for the establishment of grapevines.

Therefore, the objective of this study was to determine the damage threshold of RKN for the establishment of grapevines.

5.1.2 Materials and methods

5.1.2.1 Microplot preparation

The experimental field was at the Waite Campus of the University of Adelaide, South Australia. The field soil was a stony red brown earth, sometime with lime sub soil. In June 2000, the soil was tested for RKN by direct extraction (Whitehead and Hemming 1965) and no RKN was found. Microplots (600 mm deep and 450 mm in diameter) were constructed using a post hole digger (Fig. 5.1). The microplots were laid out at 2.1 m centres in rows 3 m apart. The nematicide Nemacur-50G® (Bayer, 50 g fenamiphos/kg) was applied at the rate of 50 g per m^2 in each hole and watered. The holes were lined with 0.45 mm thick black plastic sheeting about 15 days after nematicide treatment. Each hole was filled with pasteurised University of California (UC) mix, a 4:3 sand:peatmoss and balanced fertilizer mixture (Baker 1957). The mix was prepared as follows: the washed sand (four parts) was steamed at 100^{O}C for 30 min, then peatmoss (three parts) was added and mixed for ten seconds. The temperature of the combined sand/peat mix dropped to about 80^{O}C. After about 10 minutes cooling the fertilizers were added and mixed thoroughly. The pH of the mix was 6.8.

5.1.2.2 Grapevines

The grapevine cultivars Colombard and Sultana were selected based on their susceptible and moderately resistant responses to RKN respectively (Ferris and Hunt 1979; Ferris *et al.* 1984). The rootlings were purchased from a commercial nursery

and tested for RKN infestation in roots by visual and microscopic examination. The rootlings were grown for about one month in pots containing steamed UC mix.

5.1.2.3 Preparation and inoculation of RKN

The RKN was collected from a vineyard at New Residence ($34^{O}22'S140^{O}24'E$), Riverland, South Australia and cultured on susceptible tomato (cv. Grosse Lisse) plants in glasshouse conditions. Pure cultures from this RKN population were developed using single egg-masses on tomato plants. The species identity of each of these pure cultures was determined using the North Carolina differential host test (Hartman and Sasser 1985) and a mtDNA based method (Powers and Harris 1993), as earlier study showed that at least two identification methods are necessary to confirm the species identity of RKN from vineyards.

Inoculation was made one month after first bud-burst by pipetting a suspension of second stage juvenile nematodes (J2) into the hole around the vines (Melakeberhan and Ferris 1989). Vines were inoculated with three-day-old J2 of *M. incognita*. Four inoculum densities/treatments of 25, 154, 960 and 2400 J2/1000 ml soil were applied to ten replicated vines. All vines, together with soil from the pots were transplanted into microplots one month after inoculation and in a completely randomised design. The microplots were irrigated by drip irrigation system at the rate of 4 L/h as required. Standard cultural operations, such as fertiliser application, fungicide spray for powdery mildew, were performed for the vines in microplots except weeding, which was done by hand.

5.1.2.4 Nematode sampling and vine growth measurement

The roots (2-5 g) were collected from four sides of a vine three months after transplantation (March 2001) and the number of galls assessed visually. After

assessment, the roots were dried at 70^{O}C for 72 hours to determine the number of galls per gram of dry roots. The pruning and training were done according to recommended practice (Davidson 1992). First pruning was done at the end of June 2001 by cutting vine shoots back to two buds position. The length and diameter (top, middle and bottom) of these one year old wood vines were recorded. In the second season, during vine training, the excessive side branches (<6 mm diameter) were removed for two times in a season (December 2001, February 2002) and weighed. The trained canes were trimmed to 10–12 nodes. The trimmed canes of individual vines were weighed. The RKN population densities were assessed at the end of the second grapevine growing season in June 2002 by the DNA method using the commercial facilities of root-disease testing service of South Australian Research and Development Institute Ophel-Keller *et al.* 1999). The soil samples for DNA analysis were collected 100 mm away from a vine stem in a microplot up to 300 mm deep

5.1.2.5 Data analysis

GENSTAT 5 (Lawes Agricultural Trust, Rothamsted Experimental Station) was used for the statistical analysis. Logarithmic [log10 (x+1)] transformations were made for all data to adjust the non-normality of the raw data. Correlation analyses among all parameters of each cultivar were performed to determine possible dependency among the variables. Regression analysis was performed to explain and predict the probable relationship between nematode population density and growth. Analysis of variance (ANOVA) was also performed to determine the treatment effects on length, diameter and weight of one year old pruned of grapevine cultivars. The crop-loss model of Seinhorst (1965, 1998) was also fitted to a data set consisting of initial population density of nematodes and weight of pruning using the computer program SeinFit (Viaene *et al.* 1997). The model is described by

$y = y_m \cdot m + y_m \cdot (1\text{-}m) \cdot z^{(x-1)}$, for $x > t$

where y is the fresh weight of vine prunings, x is the nematode population density, t is the nematode population density below which growth reduction cannot be measured (the tolerance limit), y_m is the mean growth where the nematode population density is below the tolerance limit t, m is a constant, usually between zero and one, such that $y_m \cdot m$ is the pruned weight at the highest possible nematode population density and z is the slope determining parameter (between zero and one). The analysis program 'Double Partial Derivative Method' of Ferris *et al.* 1981 in the SeinFit was used to calculate the Seinhorst equation.

5.1.3 Results

Neither galled roots nor RKN in soil were detected in uninoculated microplots. Galled roots were detected in Colombard during the first grapevine-growing season (2000-01), whereas galls were not found in Sultana roots even after re-inoculation with RKN. The correlation coefficient values for all possible relationships between nematode population densities and growth parameters are presented in Table 5.1. Initial *M. incognita* population density was positively correlated with galling and final population densities (Fig. 5.2B) in Colombard but negatively correlated with pruned weights of Colombard (Table 5.1A). Whereas, initial nematode population densities were positively correlated with pruned weight of Sultana (Table 5.1B). The final RKN population density was negatively correlated with the length and diameter of pruned canes of Colombard (Table 5.1A), whereas, no such relationship was found among these parameters in Sultana (Table 5.1B). As expected, significant positive relationships were found between growth parameters, such as length and diameter of canes, of Colombard and Sultana (Table 5.1A and 5.1B).

Despite a significant positive relationship between inoculum densities and number of galls per gram roots of Colombard, none of the RKN population densities reduced the vine growth (cane length and diameter of prunings) of either cultivar during the first season (2000-2001). The mean of log-transformed cane length and diameter of both cultivars were the same, 1102 mm and 5.52 mm (backtransformed) respectively. The initial RKN population densities over 25 J2 per 1000 ml soil produced significantly higher number of galls per g roots than initial population densities of 25 J2 per 1000 ml soil (Fig. 5.2A) whereas, no significant difference was found among the final RKN population densities in microplot soil (Fig. 5.2B). RKN more than 25 J2 per 1000 ml soil reduced pruned weight of Colombard significantly in the second season (2001-02) but at the same time the RKN population density 2400 J2/1000 ml soil increased the grapevine growth in Sultana (Fig. 5.2C). As expected, pruning weights, in a season (2001-2002), increased with grapevine age (results not shown). The Seinhorst's damage threshold density (t) for RKN was 1.52 for the reduction of pruned weight after two year.

Table 5.1 Relationships among initial *M. incognita* (RKN) population densities (*Pi*), final RKN population densities (*Pf*), galls per gram roots, cane length, cane diameter and pruned weight in two grapevine cultivars (n = 50).

	Pi	*Pf*	Galls	Cane length	Cane diameter	Pruned Wt.
			A. Colombard			
Pi	1.00					
Pf	0.50**	1.00				
Galls	0.53**	0.32*	1.00			
Cane length	-0.20	-0.48**	-0.18	1.00		
Cane diameter	-0.23	-0.32*	0.06	0.54**	1.00	
Pruned Wt.	-0.49**	-0.41**	-0.47**	0.37**	0.31*	1.00
			B. Sultana			
Pi	1.00					
Pf	-0.09	1.00	-			
Cane length	-0.06	0.07	-	1.00		
Cane diameter	-0.01	0.09	-	0.71**	1.00	
Pruned Wt.	0.38**	-0.04	-	0.47**	0.32*	1.00

*= Significant at 5% level, **= Significant at 1% level, - = not found

Figure 5.1 Microplot

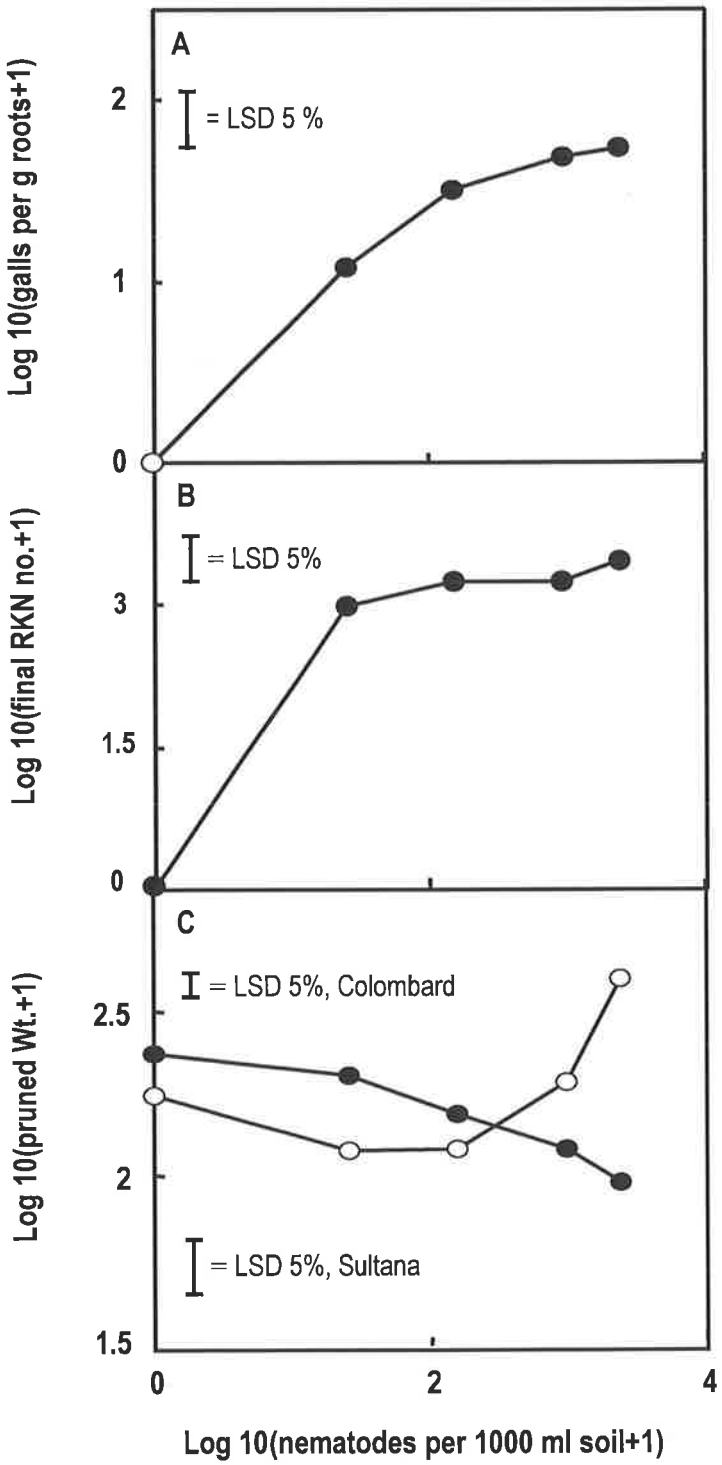

Figure 5.2 Treatments effect of initial RKN densities on galling (A), final population (B) and pruned weights (C); ( ) Colombard (O) Sultana.

5.1.4 Discussion

The correlation analysis indicated that the initial RKN population density has a significant negative relationship with the growth of the susceptible cultivar Colombard, but a positive relationship with the moderately resistant cultivar Sultana. Seinhorst (1968) also showed that the nematode damage/increase function is an essential linear relationship between plant damage/increase and log-transformed nematode population densities. The highly significant positive relationship between initial RKN population densities and galling or final population densities in microplots confirmed the nearly proportional multiplication of RKN in grapevine roots.

The analysis of treatment effects on vine growth indicated that population densities in excess of 25 J2 of *M. incognita* per 1000 ml soil will reduce growth significantly. Therefore, the damage threshold for RKN to establish grapevines lies between 1-25 J2 per 1000 ml soil. The Seinhorst's model estimated about 2 J2 per 1000 ml soil tolerance limit for the susceptible cultivar Colombard. The RKN multiplication patterns, such as slight difference among root galling and no difference among final RKN populations in soil (Fig. 5.2A and 5.2B), also indicates the possible presence of a very low tolerance level in a susceptible grapevine to RKN. This very low tolerance level indicates the possible severity of RKN infestation in grapevines and therefore supports the value of a zero RKN level in soil at planting for the establishment of a vineyard given the potential longevity of grapevines. However, even two RKN per 1000 ml soil at planting is difficult to detect with available methods including the DNA method Stirling and Nikulin, 1993). In such cases, use of resistant rootstocks would be a safe way of managing RKN in grapevines. The use of resistant rootstocks is recommended especially for prospective vineyards in sandy soils, as research has shown that the survival, invasion ability and damage potential of RKN is much higher in sandy than clay soil (Verma *et al.* 1998).

On the other hand, the identification of the RKN species, if present within detection limits of quantification methods in a prospective vineyard, should also be included in management strategies in grapevines. More than 60 RKN species have been recorded in different crops and weeds worldwide (Esnard and Zuckerman 1998), but only six and predominantly two species (*M. incognita* and *M. javanica*) affect Australian grapevines (Hugall *et al.* 1994; McLeod and Khair 1973; Stirling 1976). Therefore, fields for prospective vineyards may contain RKN species, which may not be virulent to grapevines, from previous crops or weeds, and these will also be estimated by the available quantification methods including DNA method, if the RKN population densities fall within current quantification limits. In practice none of the quantification methods includes identification of *Meloidogyne* at species level. In addition, the DNA-based quantification method was developed from a DNA probe common for all species of the genus *Meloidogyne* (Ophel-Keller *et al.* 1999). Further development of the DNA method is needed to develop species specific probes and to increase the sensitivity to about 1 RKN per 1000 ml soil and research is also required on the effect of biotic and abiotic factors in the estimation of economic thresholds for RKN in Australian viticulture. The collection of yield data for at least four more years may be needed to draw a strong conclusion on the relationship between nematode population density and yield in grapevines. An earlier study (Ramsdell *et al.* 1996) in the USA indicated that conclusive results can be found from combined growth and yield data over six years from planting.

The work reported here also demonstrated that RKN infestation might not have an immediate effect on growth of grapevines, as no growth reduction was found in Colombard in the first grapevine growing season despite high inoculum densities and presence of galls in roots. This response might be due to the ability of grapevines to produce large numbers of feeder roots within a short period of time (during the

growing season), sufficient to maintain growth in the first season but not enough against the higher nematode population densities in subsequent seasons. Similar results were found during a study to determine the damage threshold for *M. javanica* on pineapple, where higher populations and galls in roots did not reduce yields and growth in the first 12 months of infestation whereas, the yields from subsequent ratoon crops declined significantly (Stirling and Kopittke, 2000). This finding also supports the value of 'zero tolerance' for RKN in soil during the establishment of a vineyard because even the presence of a single nematode per 1000 ml soil at planting may significantly reduce the growth and yield of susceptible grapevines within a few years.

The higher RKN number in soil and the presence of galls in roots of Colombard and no or little infestation in Sultana are in agreement with previous findings (Ferris and Hunt 1979; Ferris *et al.* 1984). This also indicates that the RKN damage threshold may vary from cultivar to cultivar depending on the pattern of grapevine-RKN interaction in the field. In a similar microplot study, Ramsdell *et al.* 1996, observed variable growth and yield reductions in French-American hybrid grapevine cultivars in Michigan. Hence, the damage threshold of RKN for grapevines should be determined individually for each cultivar under a range of conditions. These differential RKN-cultivar responses can also be used to study the nature of resistance to RKN in grapevine. Such knowledge, especially the biochemical and molecular basis of RKN resistance in Sultana could be exploited to develop RKN resistance in other cultivars.

The finding of increased growth in Sultana supports Seinhorst's (1968) hypothesis that nematodes might have two mutually independent effects on plant species, both dependent on nematode population density. Thus, the higher nematode

population density caused growth reduction in Colombard but the same population density increased growth in Sultana. The increased pruned weight in Sultana for higher RKN population densities may be due to stimulation in plant growth caused by plant-nematode interactions. Wallace (1971) also found increased shoot weight of cultivar Cabernet Sauvignon inoculated with 4000 J2/vine in a glasshouse experiment. The opposite (reduction or stimulation) interaction of RKN on growth of grapevines also indicated the necessity to consider the RKN tolerance level of individual grapevine cultivar in determining economic threshold.

CHAPTER SIX
GENERAL DISCUSSION

6 General discussion and future directions

6.1 Soil sampling for parasitic nematodes

This study showed that the densities of plant parasitic nematodes in vineyards are higher in positions close to vines in the row, but vary with soil depths and types. The sedentary endoparasite RKN is equally distributed to 600 mm deep in all soil types, whereas the migratory endoparasitic root lesion nematodes can be distributed up to 300 to 600 mm deep depending on the presence of sandy soil in a vineyard, and the ectoparasitic dagger nematode is present in higher number between 300 and 600 mm deep. Results of this and other studies showed that the plant parasitic nematodes that affect grapevines were located close to the vines in row and up to 600 mm deep (Ferris and McKenry 1974; Harris 1980; Feil *et al.* 1997; Walker and Morey 2001). Therefore, to standardise a soil sampling method for all nematodes in relation to vines, it is recommended that the core sample should be collected 100 mm away from the vine to a depth of up to 600 mm, but specific sampling methods should be used for studies of particular nematodes. This soil sampling method can provide an overall picture of abundance of soil nematodes affecting grapevines, including non-parasitic and beneficial nematodes in the rootzone, which is useful for determining vineyard soil health. However, further studies, such as distribution studies for pin and ring nematodes in grapevines, are needed before drawing strong conclusions for a common soil sampling method for parasitic nematodes in grapevines.

6.2 The occurrence of RKN in vineyards

There are more than 60 RKN species that affect plants around the world, but only a few affect grapevines. It is not known how many species occur in vineyards of Australia, but four (*M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*) were found

in vineyards of South Australia. Other species, such as *M. thamesi* and *M. hispanica* have been recorded in NSW (McLeod and Khair 1973) were not detected in vineyards of South Australia. However, a DNA-based species survey is needed in vineyards of other parts of Australia to determine species identity in grapevines and cover crops, to provide more precise information.

On the other hand, viticultural practices, such as cover crops in the inter-row and the presence of off-season weed species, will increase the likelihood of occurrence of other *Meloidogyne* species not necessarily virulent in grapevines. Therefore, accurate RKN species identification should be a part of the overall RKN management strategy. This recommendation is supported by evidence of variations in RKN invasion rates and their relationship to the interaction between species and grapevine cultivars (Lider 1954; McKenry 1992; Cain *et al.* 1984; Mckenry and Kretsch 1995; Walker 1997).

6.3 Identification methods for RKN species

The species *M. arenaria, M. incognita*, *M. javanica* and *M. hapla* can not be accurately differentiated by available detection methods. The differentiation between *M. incognita* and *M. javanica* is not possible, or very difficult, by mtDNA and rDNA analysis, but sequence characterised amplified regions (SCAR) method can differentiate these two species. The requirement of individual PCR reaction conditions for each species is a limiting factor for the SCAR method, especially when dealing with a small amount of DNA from a female or single juvenile. The IGS-rDNA method can be used easily to differentiate three main species (*M. arenaria, M. incognita* and *M. javanica*) in vineyards, but also generates polymorphism within the individuals of RKN. This polymorphism may be used to develop race specific DNA markers. The use of RFLPD method may not produce reliable identifications for the

RKN species, as the restriction sites may vary in RKN of different geographical origin or even from the same origin. The North Carolina differential host test can differentiate *M. incognita* from *M. arenaria* and *M. javanica*, but not *M. javanica* from *M. arenaria*. Therefore, none of the available methods can differentiate these four species with a single PCR reaction or host test. Hence, more research is needed to develop a single PCR based highly specific DNA method to differentiate these four species or at least for *M. incognita* and *M. javanica* in vineyards of Australia. In the mean time, it is recommended to use at least two different detection methods to confirm the RKN species identity from vineyards of Australia. The best results would come from DNA, such as a combination of mtDNA (Powers and Harris 1993) and SCAR (Zijlstra *et al.* 2000) methods, for these four species in South Australian vineyards.

Research is also needed to develop RKN species-specific quantification probes, because some species may be present in cover crops and weeds in the vineyard, that may not be virulent to grapevines, but would be recognised during quantification of RKN. More than 60 RKN species have been described with considerable variation in pathogenicity and (Esnard and Zuckerman 1998). The available DNA based quantification method is genus *Meloidogyne* specific, so this DNA method will estimate all RKN present in vineyards. However, for the soil sampling strategy it is highly desirable that the DNA method will quantify only RKN affecting grapevines, as the sampling strategy focuses on the grapevine root zone.

6.4 Other plant parasitic nematodes in vineyards

RKN remains the most common nematode pest in South Australian vineyards, and root lesion nematode is the second most common damaging nematode. Therefore, further research on management of parasitic nematodes other than RKN is needed

before they become a major threat to viticulture. This possibility is highlighted by reports of damage to grapevine by pin and ring nematodes in other countries and by the Australian cereal industry, where root lesion nematode has become a significant yield-limiting pest. An appropriate response would be for grape-growers to check vineyard soil for the incidence of all nematodes that affect grapevine. During the survey, I found that many growers did not know that their vineyard contained plant parasitic nematodes at levels high enough to cause yield loss during the season 2001-2002.

6.5 RKN quantification and DNA method

In controlled environment and field studies, the DNA method was shown to be an effective method for the quantification of RKN in vineyards. The method is equally effective for all RKN populations found in vineyards of South Australia. One of the important aspects of the DNA method is its simple sample processing procedure. The collected soil sample(s) for DNA method can be dried at room temperature and stored for a long time, which is a significant advantage of the DNA method over commonly used methods. Growers do not need to take extra care for soil samples, which can be sent to DNA testing laboratory by ordinary post. DNA testing will also help greatly to improve the quality of the nematode quantification test, as most of the procedures are robotic. Nicol *et al* (1999) highlighted the need to improve quality in laboratories providing nematodc diagnostic and quantification services. Therefore, it is reasonable to conclude from this study that the sampling strategy and DNA method will provide an effective tool for the quantification of RKN in vineyards. The number of core samples per vineyard should be collected according to recommendation in "Advisory service for nematode pests, operational guides", pp. 17-

20 (Stirling *et al.* 1999). A rule of thumb would be to spend 1% of the value of a crop on nematode sampling (Stirling *et al.* 1999).

6.6 RKN densities and quantification methods

RKN densities more than 25 per 1000 ml soil at vineyard establishment could cause significant damage after two years. In such case, soil treatment is essential prior to transplanting the vines. RKN densities less than 25 per 1000 ml soil may not produce significant damage in the first one or two season(s), but given favorable conditions for RKN (such as continuous availability of the host and sandy soil) densities could increase to levels at which they could damage grapevines within three or more years. Therefore, the only acceptable RKN density in soils for prospective vineyards, especially in sandy soils, would be zero or use of resistant rootstock.

This study also showed that the RKN tolerance density for susceptible grapevine cultivars is about 2 RKN per 1000 ml soil. This RKN density is almost impossible to detect by available quantification methods, including DNA method (Starling and Nikulin 1993). However, DNA assays will provide comparatively accurate information compared with other methods because of its ability to estimate RKN eggs from all types of vineyard soils and its insensitivity to soil processing conditions. In sandy soil, the bioassay method would provide better estimation than other quantification methods but the long time requirement is a limiting factor for the method.

6.7 Urgent needs

It appears from this study and studies worldwide that RKN remain a potential threat for own-rooted grapevines (Boubals 1992; Loubser 1988; McKenry 1992; Sauer 1974; Stirling 1976; Starling and Cirami 1984; Stirling *et al.* 1992). This threat is

much more likely for the viticulture industry in Australia, as grape production in Australia is predominantly based on own-rooted *Vitis vinifera*, a species of grapevine highly susceptible to RKN. In recent years, this threat has extended to vines on RKN resistant rootstock, such as Ramsey, as they become susceptible to certain RKN populations in South Australia (Walker 1997). These RKN populations might have evolved during the repetitive applications of nematicides/pesticides as a pest management strategy or selected by the use of rootstock. This loss of resistance is another reminder of the urgent need to develop sustainable management practice for pests including parasitic nematodes. This need is justified by the high incidence of RKN in vineyards along with the withdraw of highly effective nematicides, such as methyl bromide, to control RKN and the lesser efficiency of several of the few remaining nematicides, which are compromised by accelerated microbial degradation (Davis *et al.* 1993; Noling and Becker 1994; Sanday 2000).

On the other hand, the growth of cover crops in the inter-row, sometimes used as a cultural method of controlling RKN in vineyards, has no effect on RKN within the vine row. Therefore, at present no sustainable option is available for control of RKN in vineyards. Thus, there is an urgent need to develop sustainable RKN control method(s) for RKN affecting grapevines. Research, such as identification of plants that have RKN suppressive or nematicidal properties that can be grown in or delivered as mulch to the vine row and identification of and use of micro-organisms that are parasitic to RKN, is needed as part of sustainable RKN management practice in vineyards. Evaluation of individual and combined effects of suppressive plants and parasite(s) in controlling RKN, grape yield and quality in naturally RKN infested vineyards is also needed. Genetic approaches, such as the possible use of Bt toxin and other genes involved in resistant to root pests/disease, should be included in research to develop sustainable management practices for nematode pests. Therefore, an

integrated nematode management system involving common and molecular approaches should be considered to develop a sustainable management system for parasitic nematodes in vineyards. Collaborative work involving researchers from different crops including overseas collaborations would help to achieve this ambitious goal.

APPENDICES

APPENDIX A: DNA recipes and protocols

Extraction buffer

100 mM EDTA

100 mM NaCl

100 mM Tris pH 7.5

0.5% SDS

200 µg proteinase K

3 M Sodium acetate

4.081g Na-acetate was dissolved in 80 ml water. Adjust to pH 5.2 with glacial acetic acid (or to pH 7.0 with dilute acetic acid). Adjusted to 100 ml with water and sterilise by autoclaving.

1M Tris HCl (pH 8, for 100 ml)

12.11 g Tris (hydroxymethyl) aminomethane was dissolved in 80 ml deionised (ddH_2O) water and pH was adjusted to 8. The final volume was adjusted to 100 ml by adding ddH_2O water and autoclaved.

0.5M EDTA Stock (for 100 ml)

18.61 g EDTA (Ethylenediaminetetraacetic acid) was dissolved in 80 ml ddH_2O and pH was adjusted to 8 by concentrated sodium hydroxide. Volume was then adjusted to 100 ml and sterilise by autoclaving. (EDTA will not dissolve unless pH is equal to 8).

1X TE buffer (pH 8)

10 mM Tris HCl at pH 8

1 mM EDTA at pH 8

RNAase stocks

10 mg of powdered RNAase A bovine pancreas was dissolved in 10ml of 10mM Tris.Cl,15mM NaCl. Boil for 15 mins to denature any DNAase. Aliquot out into labelled tubes in 1ml lots and freeze.

2. PCR RECIPES

Commercially available PCR buffer, dNTP mix MgCl2 and *Teq* polymerase were used in DNA studies (described in respective experiments earlier).

3. GEL ELECTROPHORESIS RECIPES

10X TBE STOCKS (for 1L)

108.0 g Tris, 55.0 g Boric acid and 40 ml 0.5 M EDTA (pH8) were dissolved in 500-600 ml ddH_2O and then brought solution to 1L. *Note*: A precipitation may forms when concentrated solutions of TBE are stored for long periods of time. To avoid such problems, solution was stored at root temperature and any TBE solution with precipitation was discard.

0.5X TBE (1 to 20 dilution of stock) was used in agarose and gel electrophoresis tank. The agarose gel (0.5 to 2.5%) in 0.5X TBE was prepared in a 500 ml bottle by heated in a microwave until dissolved and then allowed to cool and sate into a gel-caster.

APPENDIX B: DNA sequences of *Meloidogyne* spp.

1. Accession No. AF510057. *Meloidogyne javan*...[gi:21217518]

LOCUS AF510057 480 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne javanica* isolate Ren1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510057
VERSION AF510057.1 GI:21217518
FEATURES Location/Qualifiers
source 1..480
/organism="*Meloidogyne javanica*"
/isolate="Ren1"
/db_xref="taxon:6303"
/country="Australia: Renmark"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>480
/product="internal transcribed spacer 2"
BASE COUNT 138 a 79 c 100 g 162 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttta agacctaatg agcctcttaa gtgnagccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaattctacc cttatcggtg gatcactagg
241 ctcgtggatc gatgaagaac gcagcaaact gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gttttgaacg caaatggccg cattgaggtc aaactctttg caacgtctgg
361 ttcagggtca ttttctctta tagcggaagc tttaatttct ataatgatgt tgttgcttta
421 tattttaaaa ggatttttgt ttattcatgt attaaatcta actgtgaaaa tcaaacaatt

2. Accession No. AF510058. *Meloidogyne javan*...[gi:21217519]

LOCUS AF510058 480 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne javanica* isolate Ren2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510058
VERSION AF510058.1 GI:21217519
FEATURES Location/Qualifiers
source 1..480
/organism="*Meloidogyne javanica*"
/isolate="Ren2"
/db_xref="taxon:6303"
/country="Australia: Renmark"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>480
/product="internal transcribed spacer 2"

BASE COUNT 138 a 77 c 103 g 162 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttta agacctaatg agcctcttaa gtgaggccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaattctatc cttatcggtg gatcactagg
241 ctcggggatg gatgaagaac gcagcaaact gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gttttgaacg caaatggccg cattgaggtc aaactctttg caacgtctgg
361 ttcagggtca ttttctctta tagcggaagc tttaatttct ataatgatgt tgttgcttta
421 tattttaaaa ggatttttgt ttattcatgt attaaatcta actgtgaaaa tcaaacaatt

3. Accession No. AF510059. *Meloidogyne javan*...[gi:21217520]

LOCUS AF510059 480 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne javanica* isolate Bar1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510059
VERSION AF510059.1 GI:21217520
FEATURES Location/Qualifiers
source 1..480
/organism="*Meloidogyne javanica*"
/isolate="Bar1"
/db_xref="taxon:6303"
/country="Australia: Barossa"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>480
/product="internal transcribed spacer 2"
BASE COUNT 138 a 78 c 101 g 163 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttta agacctaatg agcctcttaa gtgaggccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaattctatc cttatcggtg gatcactagg
241 ctcgtggatc gatgaagaac gcagcaaact gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gttttgaacg caaatggccg cattgaggtc aaactctttg caacgtctgg
361 ttcagggtca ttttctctta tagcggaagc tttaatttct ataatgatgt tgttgcttta
421 tattttaaaa ggatttttgt ttattcatgt attaaatcta actgtgaaaa tcaaacaatt

4. Accession No. AF510060. *Meloidogyne javan*...[gi:21217521]

LOCUS AF510060 478 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne javanica* isolate Bar2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510060
VERSION AF510060.1 GI:21217521
FEATURES Location/Qualifiers
source 1..478
/organism="*Meloidogyne javanic*a"

/isolate="Bar2"
/db_xref="taxon:6303"
/country="Australia: Barossa"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>478
/product="internal transcribed spacer 2"
BASE COUNT 138 a 78 c 101 g 161 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttta agacctaatg agcctcttaa gtgaggccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaattctatc cttatcggtg gatcactagg
241 ctcgtggatc gatgaagaac gcagcaaact gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gttttgaacg caaatggccg cattgaggtc aaactctttg caacgtctgg
361 ttcagggtca ttttctctta tagcggaagc tttaatttct ataatgatgt tgttgcttta
421 tattttaaaa ggatttttgt ttattcatgt attaaatcta actgtgaaaa tcaaacaa

5. Accession No. AF510061. *Meloidogyne arena*...[gi:21217522]

LOCUS AF510061 479 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne arenaria* internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510061
VERSION AF510061.1 GI:21217522
FEATURES Location/Qualifiers
source 1..479
/organism="*Meloidogyne arenaria*"
/db_xref="taxon:6304"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>479
/product="internal transcribed spacer 2"
BASE COUNT 138 a 80 c 103 g 158 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttca agacctaatg agcctcttaa gtgaggccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaattctacc cttatcggtg gatcactagg
241 ctcgtggatc gatgaagaac gcagcaaact gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gtttgaacgc aaatggccgc attgaggtca aactctttgc aacgtctggt
361 tcagggtcat tttctcttat agcggaagct ttaatttcta taatgatgtt ggtgctttat
421 attttaaaag gattttggtt tattcatgta ttaaatctaa ctgtgaaaat caaacaatt

6. Accession No. AF510062. *Meloidogyne arena*...[gi:21217523]

LOCUS AF510062 482 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne arenaria* isolate Tan1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and

internal transcribed spacer 2, partial sequence.
ACCESSION AF510062
VERSION AF510062.1 GI:21217523
FEATURES Location/Qualifiers
source 1..482
/organism="*Meloidogyne arenaria*"
/isolate="Tan1"
/db_xref="taxon:6304"
/country="Australia: Tanunda"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>482
/product="internal transcribed spacer 2"
BASE COUNT 137 a 82 c 105 g 158 t
ORIGIN
1 ctttatgtga tgttcaaatc atgaattcgc aatgaaatga tcgttgtgaa acggctgtcg
61 ctggtgtcta agtgttgctg atacggttgt gaacgtccgt gggtgtatat gtggtgacat
121 gttaggacta taatgagttt aacacctact gaccctctta gtgaggccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaatcctacc cttatcggtg gatcactagg
241 ggctcgtgga tcgatgaaga acgcagcaag ctgcgataat tattgcgaac tgcagaagta
301 ttgagcacaa aagttttgaa cgcaaatggc cgcattgagg tcaaactctt tgcaacgtct
361 ggttcagggt cattttctct tatagcggaa gctttaattt ctataatgat gttggtgctt
421 tatattttaa aaggattttg gtttattcat gtattaaatc taactgtgaa aatcaaacaa
481 tt

7. Accession No. AF510063. *Meloidogyne arena*...[gi:21217524]

LOCUS AF510063 480 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne arenaria* isolate Tan2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510063
VERSION AF510063.1 GI:21217524
FEATURES Location/Qualifiers
source 1..480
/organism="*Meloidogyne arenaria*"
/isolate="Tan2"
/db_xref="taxon:6304"
/country="Australia: Tanunda"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>480
/product="internal transcribed spacer 2"
BASE COUNT 136 a 79 c 104 g 161 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttta agacctattg agcctcttaa gtgaggccgc cagcaacctt
181 ttttttctct acatttgaaa aaaaaaacta aaattctacc cttatcggtg gatcactagg
241 ctcgtggatc gatgaagaac gcagcaagct gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gttttgaacg caaatggccg cattgaggtc aaactctttg caacgtctgg

361 ttcagggtca ttttctctta tagcggaagc tttaatttct ataatgatgt tggtgcttta
421 tattttaaaa ggatttttgt ttattcatgt attaaatcta actgtgaaaa tcaaacaatt

8. Accession No. AF510064. *Meloidogyne incog*...[gi:21217525]

LOCUS AF510064 481 bp DNA linear INV 27-MAY-2002
DEFINITION *Meloidogyne incognita* isolate Adel1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.
ACCESSION AF510064
VERSION AF510064.1 GI:21217525
FEATURES Location/Qualifiers
source 1..481
/organism="*Meloidogyne incognita*"
/isolate="Adel1"
/db_xref="taxon:6306"
/country="Australia: Adelaide"
misc_RNA <1..213
/product="internal transcribed spacer 1"
rRNA 214..371
/product="5.8S ribosomal RNA"
misc_RNA 372..>481
/product="internal transcribed spacer 2"
BASE COUNT 138 a 78 c 101 g 164 t
ORIGIN
1 ctttatgtga tgttcaaatt tgaattcgca atgaaatgat cgttgtgaaa cggctgtcgc
61 tggtgtctaa gtgttgctga tacggttgtg aacgtccgtg gctgtatatg tggtgacatg
121 ttaggactct aatgagttta agacctaatg agcctcttaa gtgaggccgc cagcaacctt
181 ttttttctct acattttaaa aaaaaaacta aaattctatc cttatcggtg gatcactagg
241 ctcgtggatc gatgaagaac gcagcaaact gcgataatta ttgcgaactg cagaagtatt
301 gagcacaaaa gttttgaacg caaatggccg cattgaggtc aaactctttg caacgtctgg
361 ttcagggtca ttttctctta tagcggaagc tttaatttct ataatgatgt tgttgcttta
421 tattttaaaa ggatttttgt ttattcatgt attaaatcta actgtgaaaa tcaaacaatt
481 t

9. Accession No. AF516721. *Meloidogyne hapla*...[gi:21591758]

LOCUS AF516721 767 bp DNA linear INV 26-JUN-2002
DEFINITION *Meloidogyne hapla* isolate Barossa1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION AF516721
VERSION AF516721.1 GI:21591758
FEATURES Location/Qualifiers
source 1..767
/organism="*Meloidogyne hapla*"
/isolate="Barossa1"
/db_xref="taxon:6305"
rRNA <1..189
/product="18S ribosomal RNA"
misc_RNA 190..402
/product="internal transcribed spacer 1"
/note="ITS-1"

rRNA 403..561
/product="5.8S ribosomal RNA"
misc_RNA 562..669
/product="internal transcribed spacer 2"
/note="ITS-2"
rRNA 670..>767
/product="28S ribosomal RNA"
BASE COUNT 206 a 148 c 171 g 242 t
ORIGIN
1 ttgattacgt ccctgccctt tgtacacacc gcccgtcgct gcccgggact gagccatttc
61 gagaaacttg gagactgttg atctaatttt tttaagttac tttgatggaa accaatttaa
121 tcgcagtggc ttgaaccggg caaaagtcgt aacaaggtag ctgtaggtga acctgctgct
181 ggatcattac tttttgtgat gttcaaattc gaatagtctc aacgtttatc gttgtgaacg
241 gctgtcgctg gtgtctaggt gttgctgatt cagctgtcaa cgtccgtggc tgaatatgag
301 gtgacatgtt aggaccttaa tcgggtttaa gacttaatga gcctcttaag tgaggacgcc
361 agcaatattt tttcaactat tttttttaaa aaacgaaaat ttttatccct atcggtggat
421 cactcggctc gtggatccat gaagaacgca gctaactgcg ataatttgtg cgaactgcag
481 aaacattgag cataaaagtt ttgaatgcaa attgcggcac tggggtagaa ccctttgcca
541 cgtctggttc agggtcattt ttctataaag tataaatttt attttatttt gccattggca
601 ctataacttt taatgtcggt acgcagcgat ttgtaaatga ataactcttt tcgctgtcac
661 atttattttt gacctgagct cagtcgagat cacccgctga acttaagcat atcagtaagc
721 ggaggaaaag aaactaaata ggattcccct agtaacggcg agtgaaa

10. Accession No. AF516722. *Meloidogyne hapla*...[gi:21591759]

LOCUS AF516722 768 bp DNA linear INV 26-JUN-2002
DEFINITION *Meloidogyne hapla* isolate Robe 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION AF516722
VERSION AF516722.1 GI:21591759
FEATURES Location/Qualifiers
source 1..768
/organism="*Meloidogyne hapla*"
/isolate="Robe"
/db_xref="taxon:6305"
rRNA <1..189
/product="18S ribosomal RNA"
misc_RNA 190..392
/product="internal transcribed spacer 1"
/note="ITS-1"
rRNA 393..562
/product="5.8S ribosomal RNA"
misc_RNA 563..666
/product="internal transcribed spacer 2"
/note="ITS-2"
rRNA 667..>768
/product="28S ribosomal RNA"
BASE COUNT 205 a 148 c 172 g 243 t
ORIGIN
1 ttgattacgt ccctgccctt tgtacacacc gcccgtcgct gcccgggact gagccatttc
61 gagaaacttg gggactgttg atctaatttt tttaagttac tttgatggaa accaatttaa
121 tcgcagtggc ttgaaccggg caaaagtcgt aacaaggtag ctgtaggtga acctgctgct
181 ggatcattac tttttgtgat gttcaaattc gaatagtctc aacgtttatc gttgtgaacg

241 gctgtcgctg gtgtctaggt gttgctgatt cagctgtcaa cgtccgtggc tgaatatgag
301 gtgacatgtt aggaccttaa tcgggtttaa gacttaatga gcctcttaag tgaggacgcc
361 agcaatattt ttttcaacta tttttttaa aaaacgaaaa tttctatcct tatcggtgga
421 tcactcggct cgtggatcca tgaagaacgc agctaactgc gataatttgt gcgaactgca
481 gaaacattga gcataaaagt tttgaatgca aattgcggca ccggggtaga acccttgcc
541 acgtctggtt cagggtcatt tttctataaa gtataaattt tattttattt tgccattggc
601 actataactt ttaatgttgg tacgcagcga tttgtaaatg aataactctt ttcgctgtca
661 catttatttt tgacctgagc tcagtcgaga tcacccgctg aacttaagca tatcagtaag
721 cggaggaaaa gaaactaaat aggattccct tagtaacggc gagtgaaa

11. Accession No. AF516723. *Meloidogyne incog*...[gi:21591760]

LOCUS AF516723 765 bp DNA linear INV 26-JUN-2002
DEFINITION *Meloidogyne incognita* isolate Adelaide2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION AF516723
VERSION AF516723.1 GI:21591760
FEATURES Location/Qualifiers
source 1..765
/organism="*Meloidogyne incognita*"
/isolate="Adelaide2"
/db_xref="taxon:6306"
rRNA <1..190
/product="18S ribosomal RNA"
misc_RNA 191..301
/product="internal transcribed spacer 1"
/note="ITS-1"
rRNA 302..558
/product="5.8S ribosomal RNA"
misc_RNA 559..666
/product="internal transcribed spacer 2"
/note="ITS-2"
rRNA 667..>765
/product="28S ribosomal RNA"
BASE COUNT 222 a 137 c 170 g 236 t
ORIGIN
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61 gagaaatttg gggaccgttg atttaatttt tctaaattac tttgatggaa accaatttaa
121 tcgcagtggc ttgaaccggg caaaagtcgt aacaaggtag ctgtaggtga acctgctgct
181 ggatcattac tttatgtgat gttcaaattt gaattcgcaa tgaaatgatc gttgtgaaac
241 ggctgtcgct ggtgtctaag tgttgctgat acgggtgtga acgtcgtggc tgtattatgt
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361 caaacctttt ttttctctac attttaaaaa aaaactaaaa ttctaacctt atcggtggat
421 cactaggctc gtggatcgat gaagaacgca gcaaactgcg ataattattg cgaactgcag
481 aagtattgag cacaaaagtt ttgaacgcaa atggccgcat tgaggtcaaa ctctttgcac
541 gtctggttca gggtcatttt ctcttatagc ggaagcttta atttctataa tgatgttgtt
601 gctttatatt ttaaaaggat ttttgtttat tcatgtatta aatctaactg tgaaaatcaa
661 acaattttga cctgaactca gtcgagagca cccgctgaac ttaagcatat cagtaagcgg
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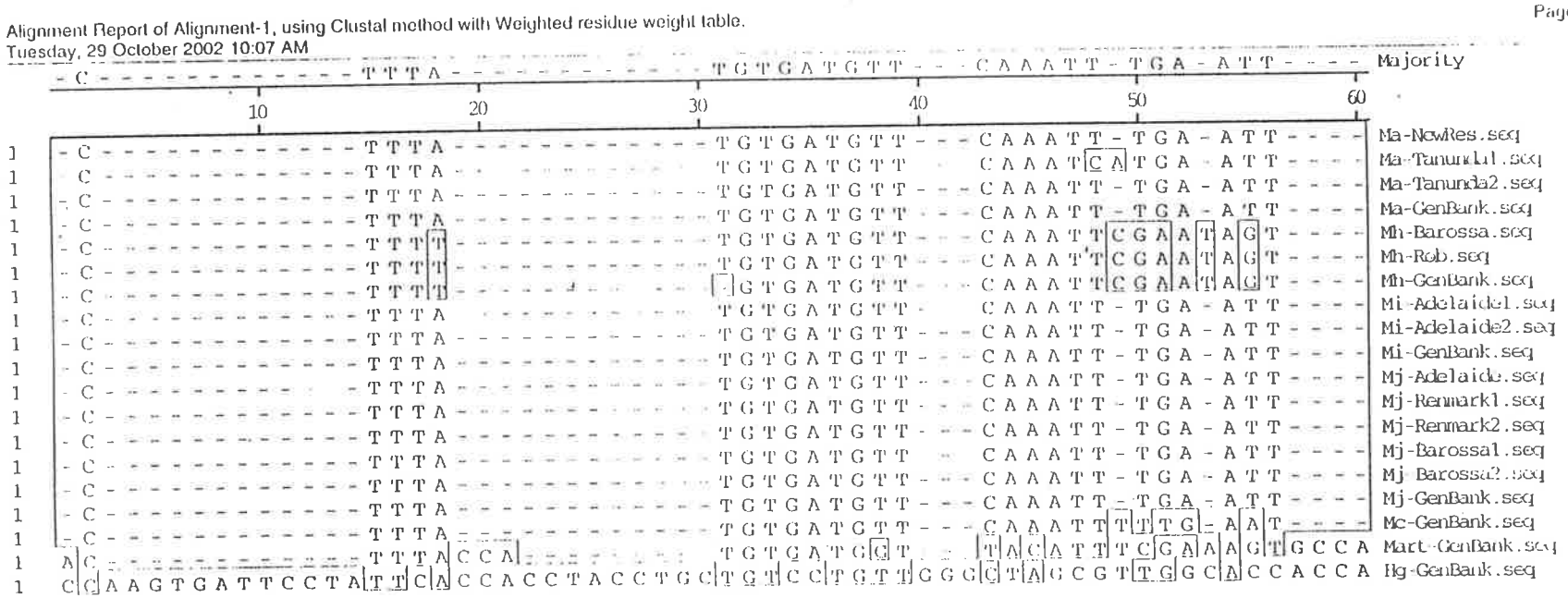
Appendix C: DNA sequence alignment report



Alignment Report of Alignment-1, using Clustal method with Weighted residue weight table.
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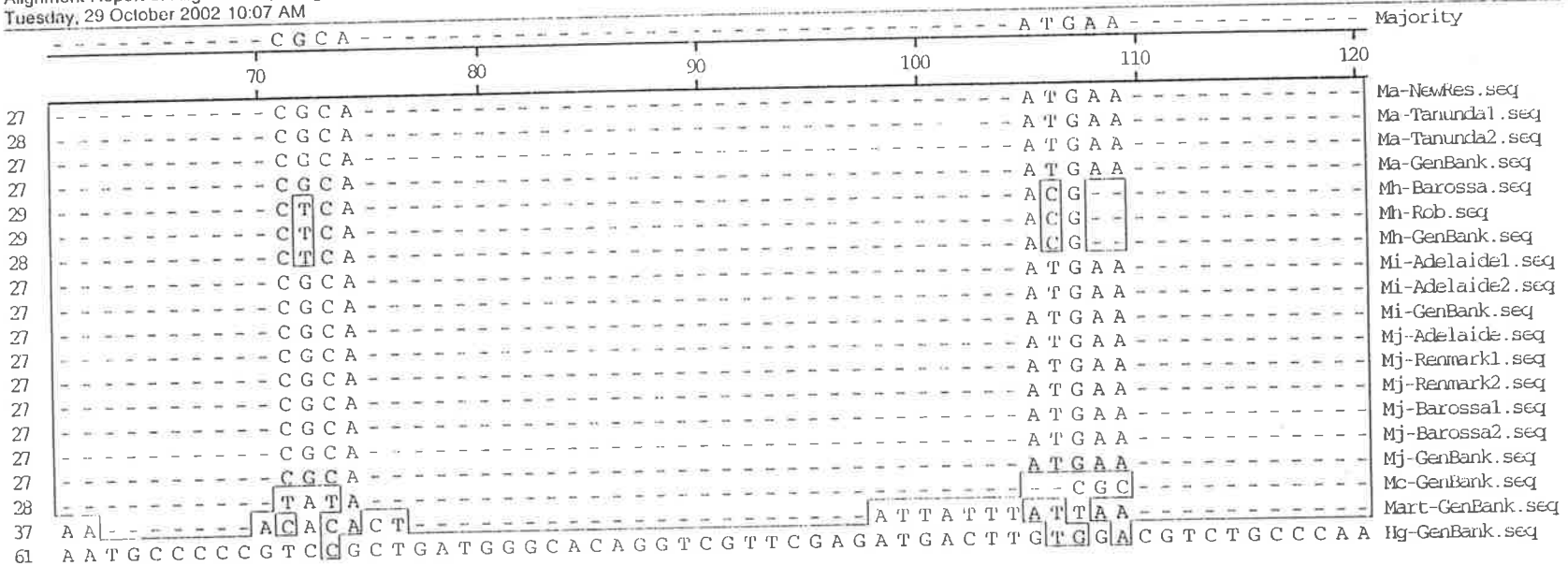

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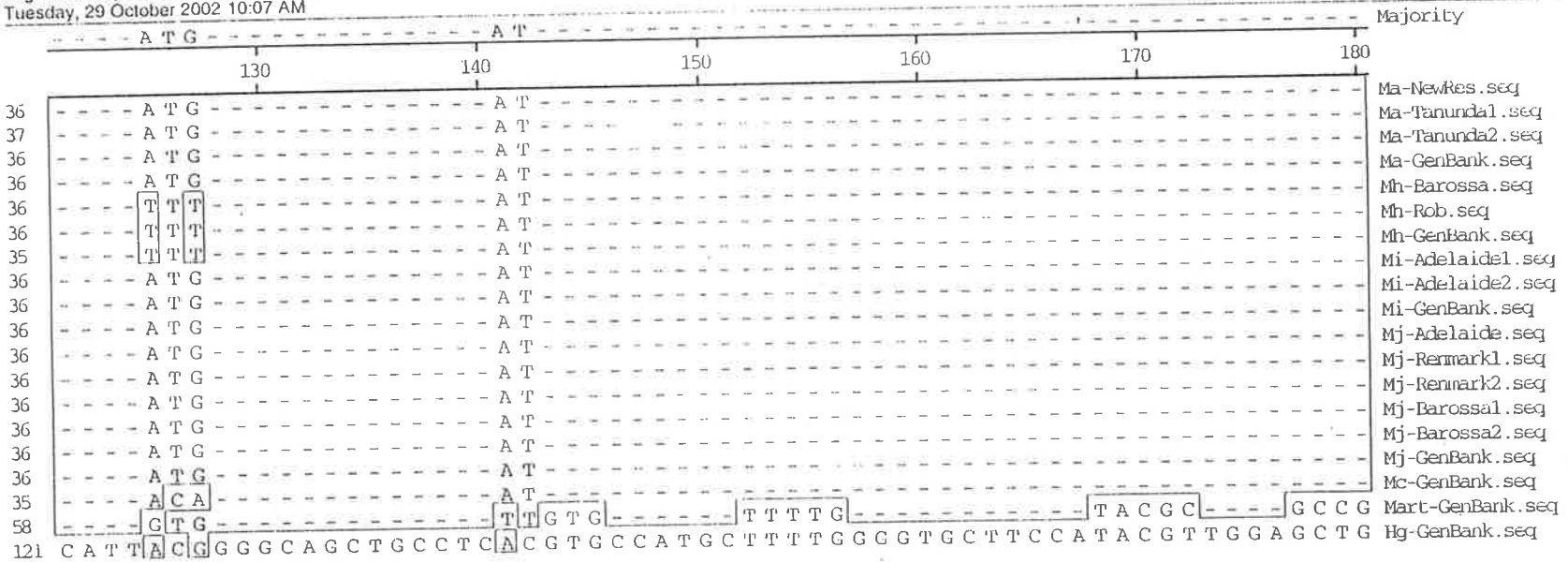

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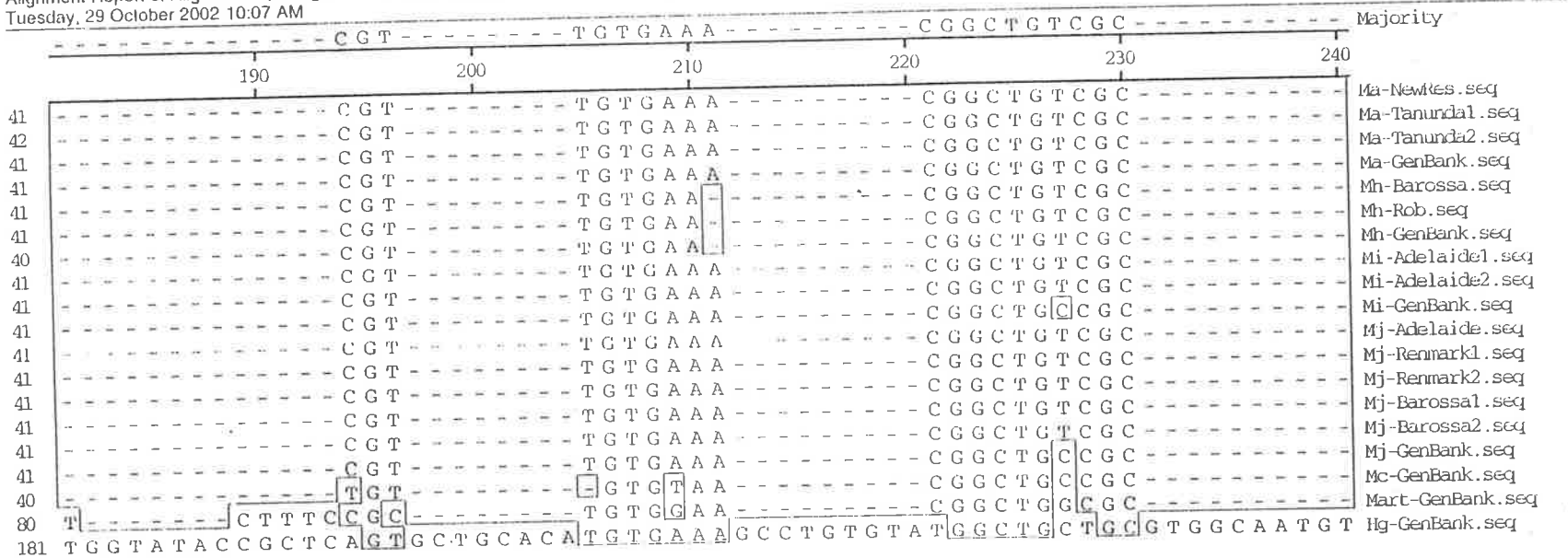

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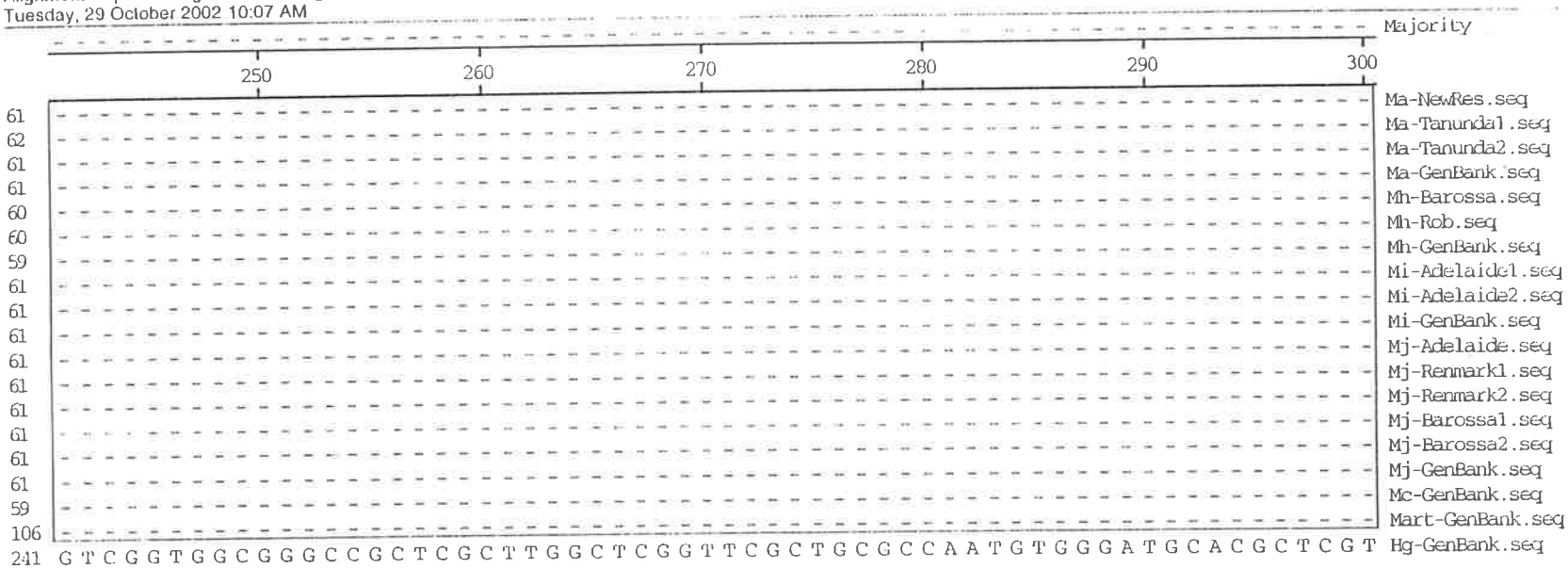

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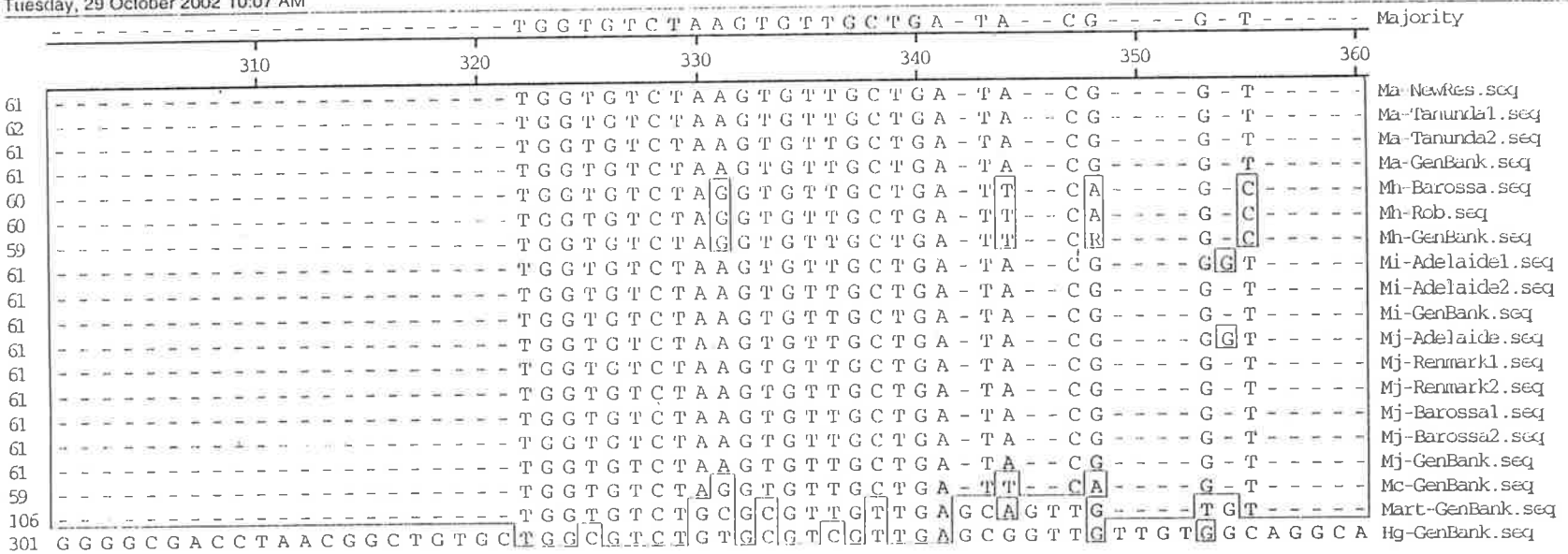

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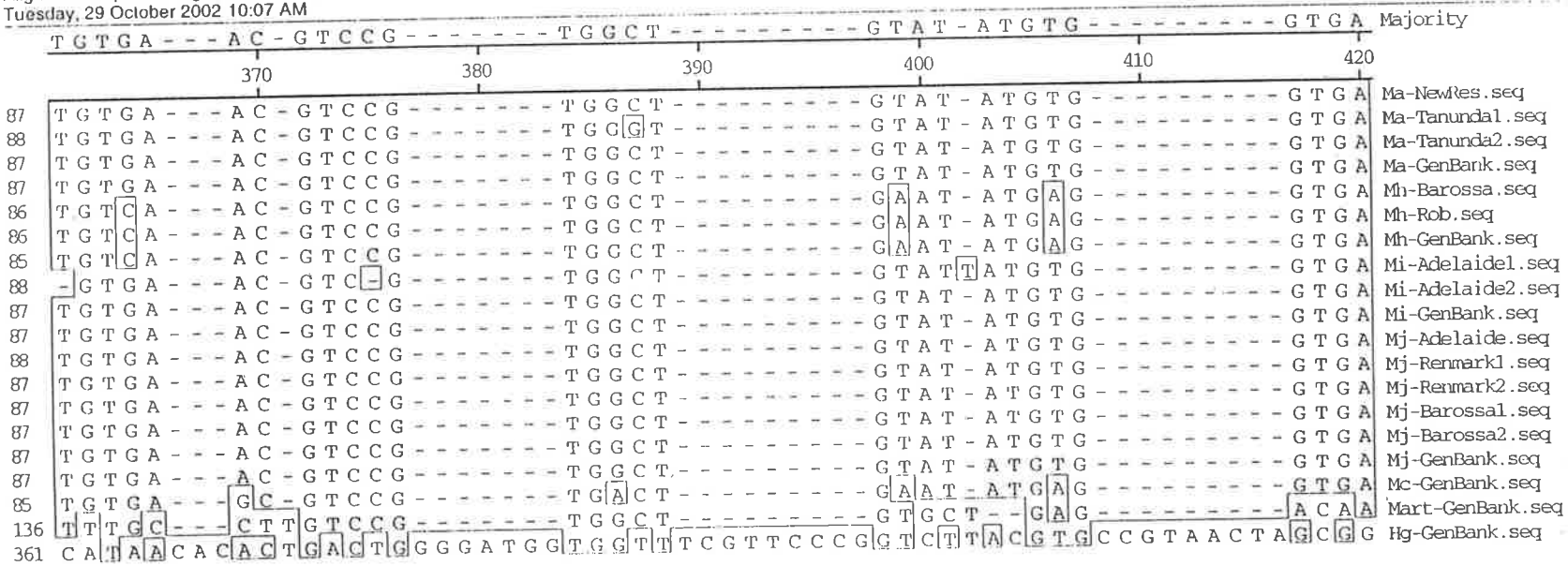

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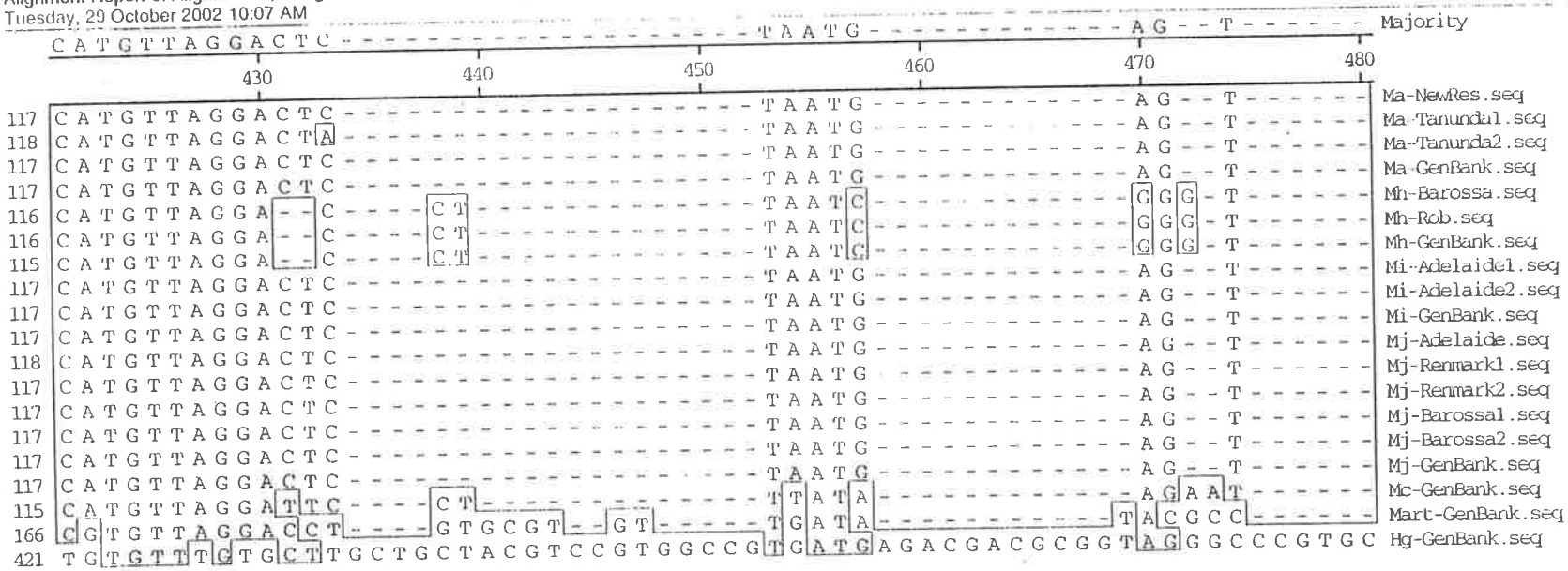

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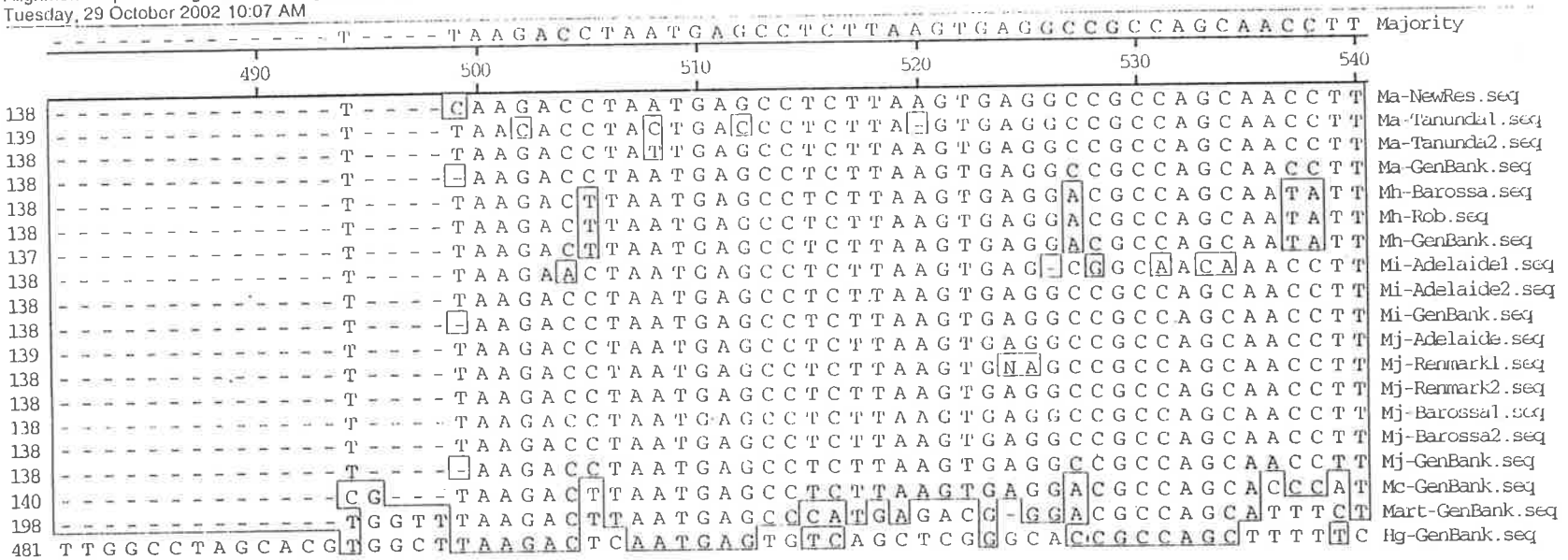

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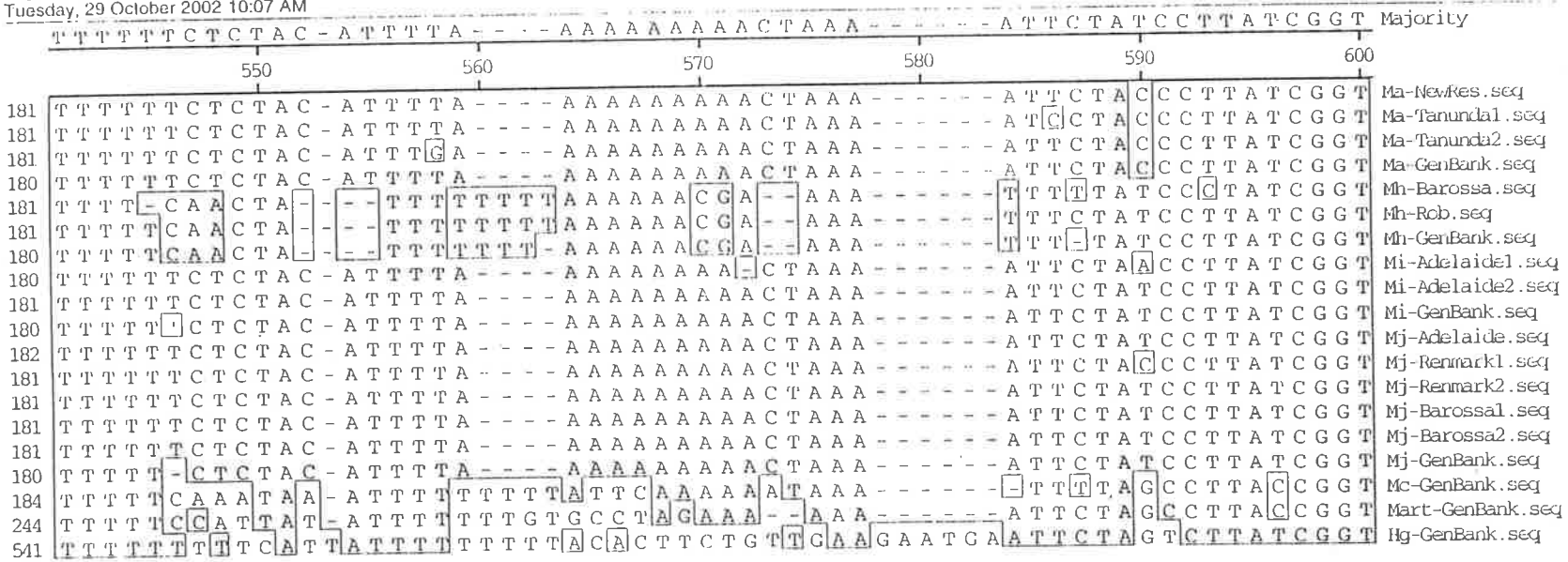

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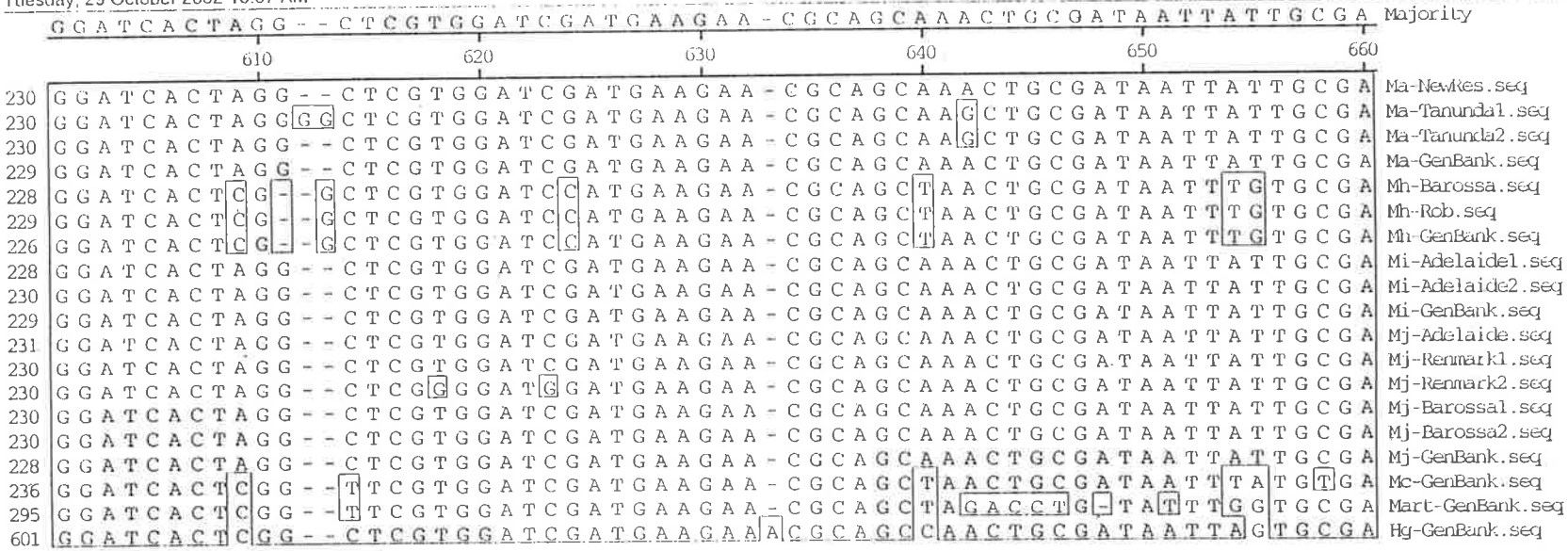

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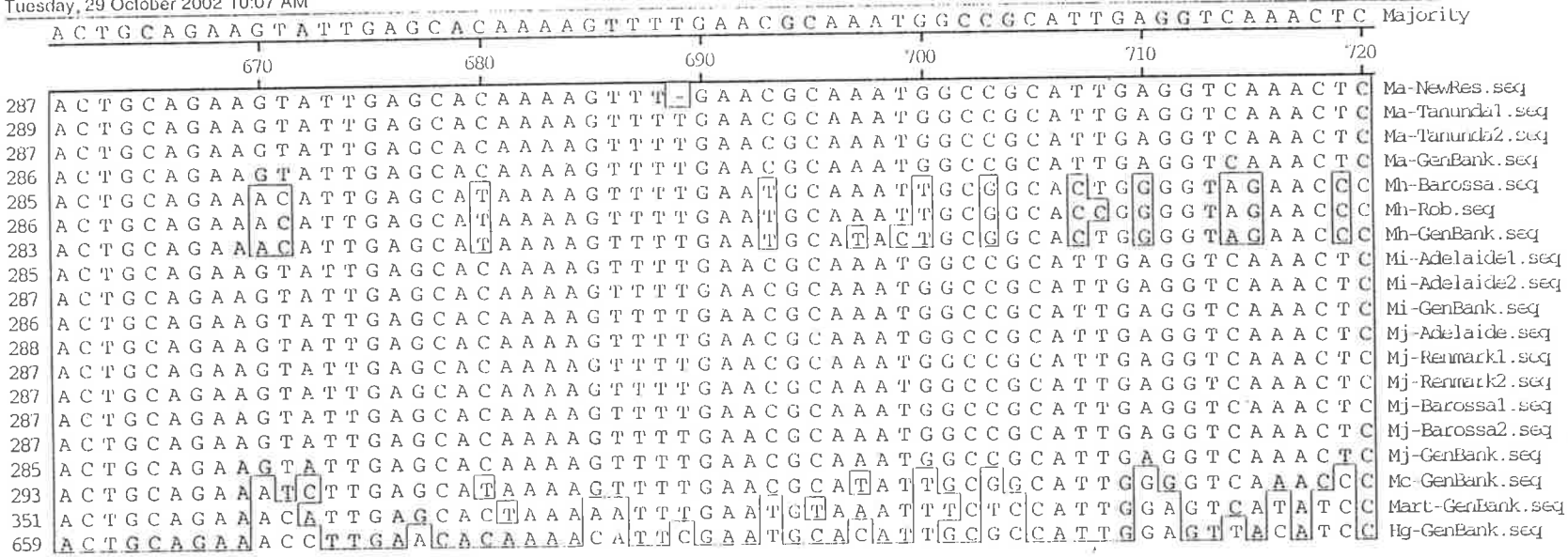

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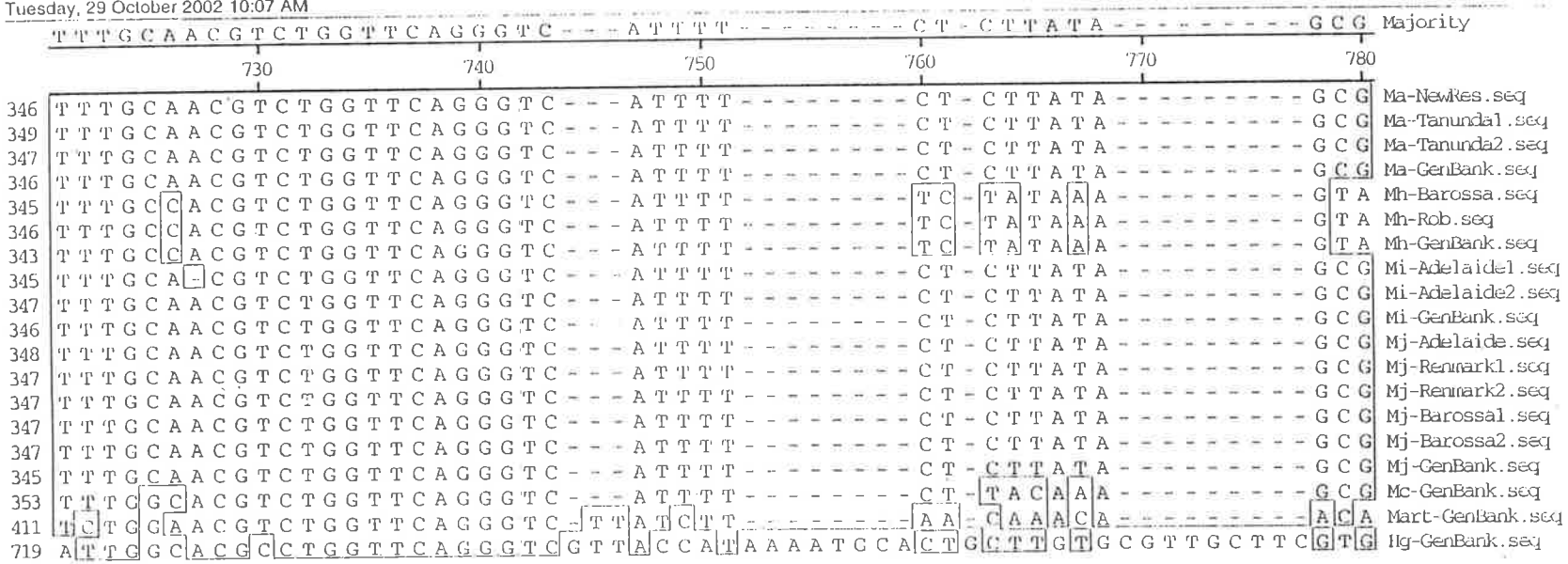

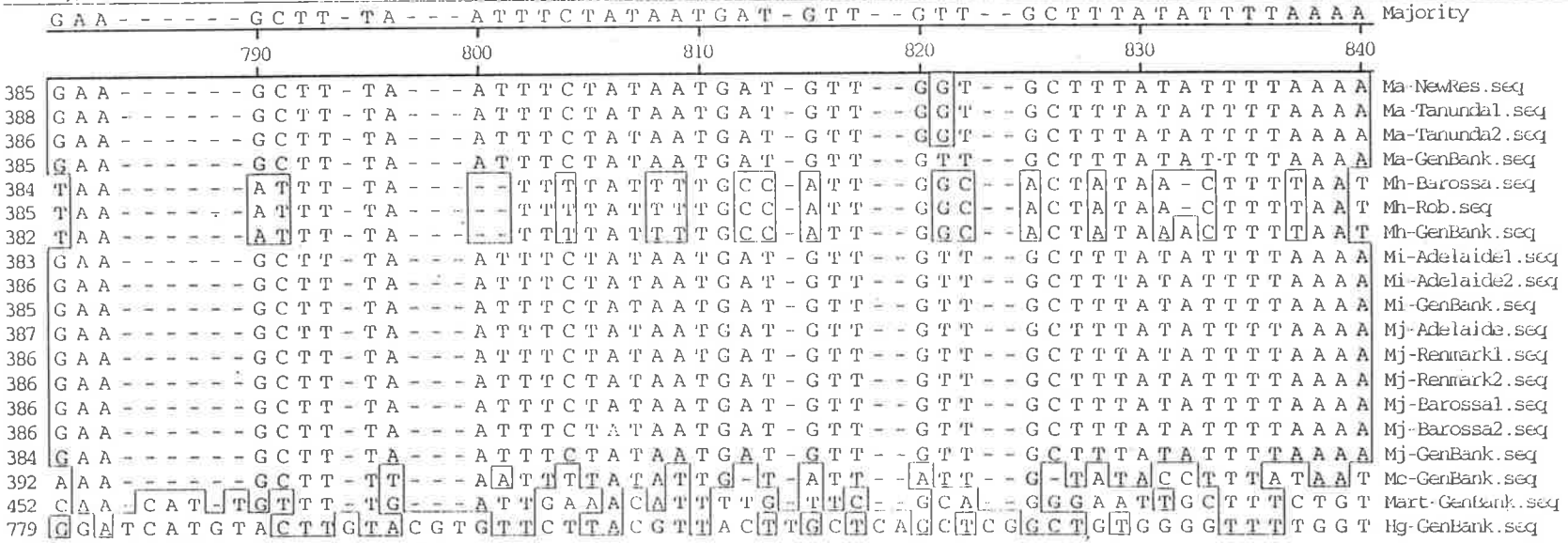
Majority
790
800
810
820
830
840
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Ma-Tanunda1.seq
Ma-Tanunda2.seq
Ma-GenBank.seq
Mh-Barossa.seq
Mh-Rob.seq
Mh-GenBank.seq
Mi-Adelaide1.seq
Mi-Adelaide2.seq
Mi-GenBank.seq
Mj-Adelaide.seq
Mj-Renmark1.seq
Mj-Renmark2.seq
Mj-Barossa1.seq
Mj-Barossa2.seq
Mj-GenBank.seq
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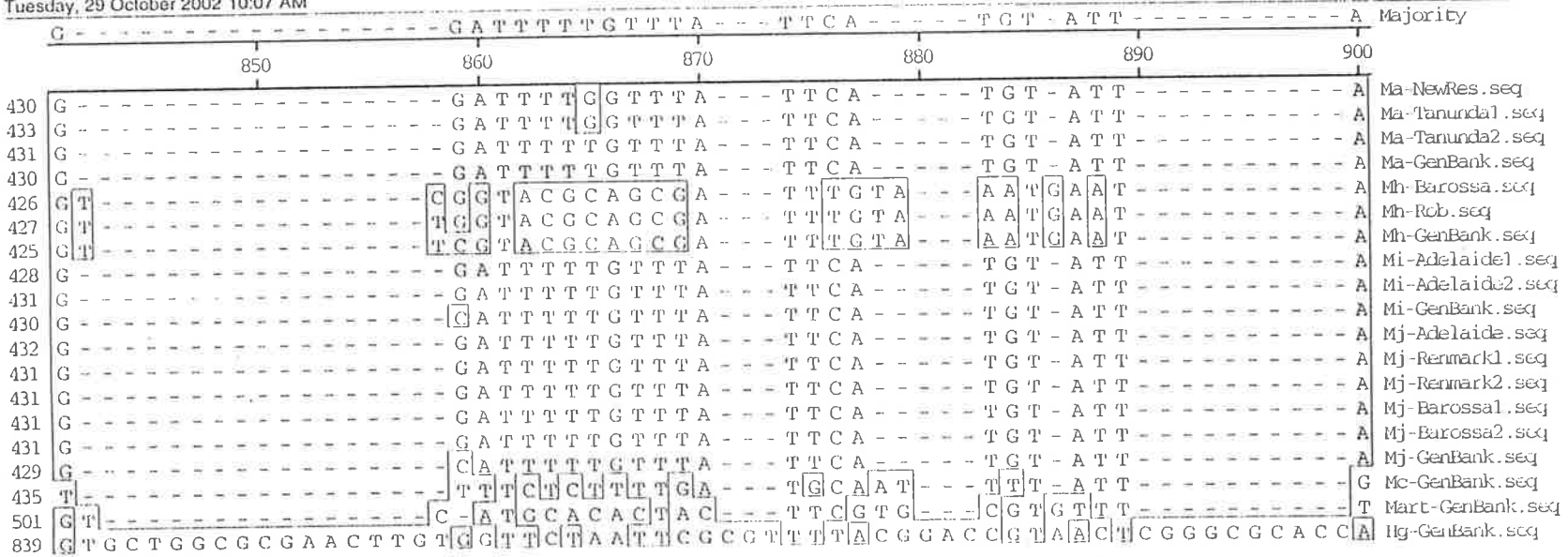
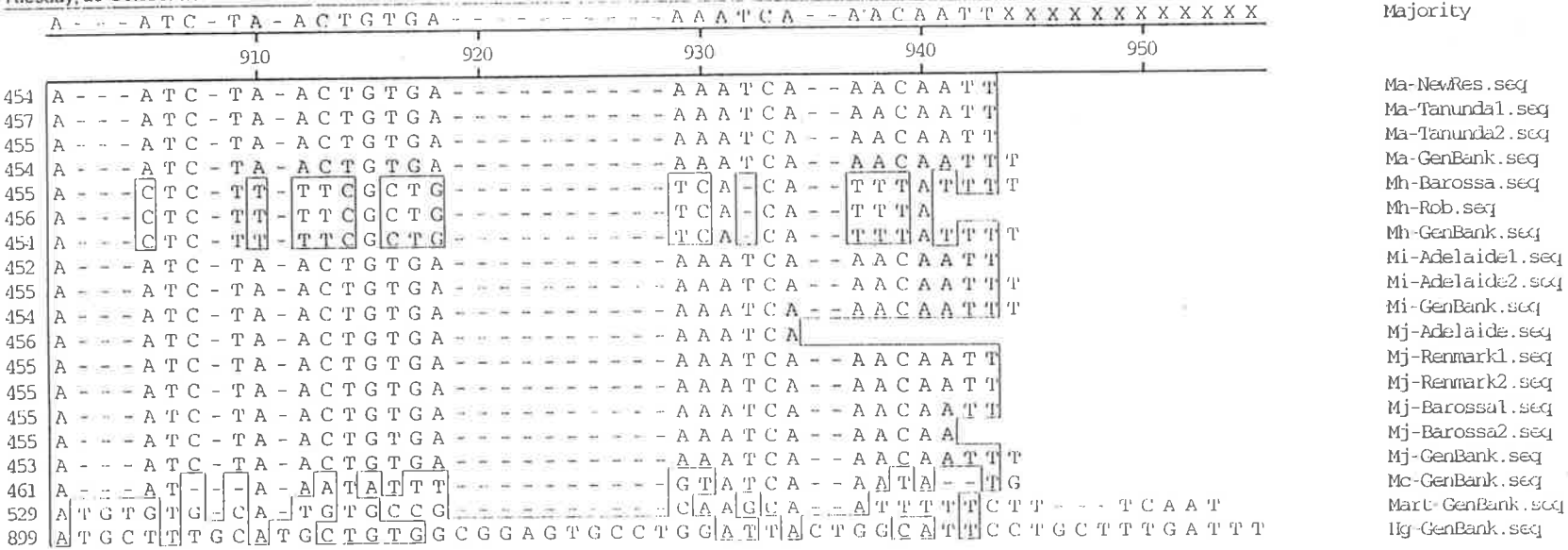
Mart-Genbank.seq
Hg-GenBank.seq

Alignment Report of Alignment-1, using Clustal method with Weighted residue weight table.
Tuesday, 29 October 2002 10:07 AM





Decoration 'Decoration #1': Shade (with black at 10% fill) residues that match the Consensus exactly.

Decoration 'Decoration #2': Shade (with solid black) residues that match the Consensus exactly.

Decoration 'Decoration #3': Shade (with white at 10% fill) residues that match the Consensus exactly.

Decoration 'Decoration #4': Box residues that match the Consensus exactly.

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