Biosecurity New Zealand
Tiakitanga Pūtaiao Aotearoa

MPI 18608 Project Report

Topic 1.4 — Initial identification of genetic markers linked to resistance

Biosecurity New Zealand Technical Paper No: 2019/36

Prepared for Ministry for Primary Industries
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ISBN No: 978-1-99-000851-1 (online)
ISSN No: 2624-0203 (online)

June 2019

New Zealand Government

Ministry for Primary Industries
Manatū Ahu Matua

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PFR SPTS No. 18040-1.4

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Contents

1 Introduction 1

2 Review of infection and phenotyping methods 2

3 Review of type of genetic markers 2

4 Review of detected loci 3

5 Review of genomics resources for Myrtaceae 3

6 Marker development for the Ppr1 locus in *E. nitens* 4

7 Marker development for the Ppr1 locus in mānuka and *E. nitens* 5

8 Recommendations 5

1 Introduction

Eucalyptus trees with resistance to the *A. psidii* strains present in Brazil have been developed using breeding and molecular systems since the 1970s and are grown commercially. Genetic loci associated with resistance have been identified in *E. grandis* in Brazil and in other Eucalypt species in Australia. Knowledge of the genetic control of resistance to myrtle rust is essential for establishing breeding strategies involving conventional as well as marker-assisted selection of resistance genes into breeding populations. A literature review of genetic resistance to *A. psidii* was performed to inform us about the potential genetic structure of resistance in Myrtaceae species (Table 1). Most studies on the genetic control of tolerance to myrtle rust have focused on Eucalyptus species (including *E. globulus*, *E. grandis*, *E. urophylla*, *E. pellita* and interspecific hybrids). Trait mapping was achieved in segregating populations, using linkage mapping with resistance treated as a single Mendelian locus (Junghans et al. 2003, Mamani et

al. 2010), quantitative trait locus (QTL) mapping (Butler et al. 2016, Rosado et al. 2010) and a genome-wide association study (GWAS; Resende et al. 2016). Additional GWAS and QTL studies have been conducted in *E. globulus* and *Corymbia* spp., respectively, and will be published soon. Some papers reported on the heritability and segregation of myrtle rust resistance in breeding populations (Teixeira et al. 2009, Santos et al. 2014, Miranda et al. 2012, Alves et al. 2012); however, they do not indicate what loci are involved, their location in the genome or the magnitude of their effect. More recently, transcription studies have highlighted differential expression in resistant versus susceptible individuals in *Melaleuca quinquenervia* and *Szygium luehmannii* (Hsieh et al. 2018; Tobias et al. 2018).

The first locus detected for resistance to myrtle rust was derived from *E. grandis*: Ppr1 for *P. psidii* resistance 1 (Junghans et al. 2003). Ppr1 was further confirmed and mapped to Eucalyptus chromosome 3 (Mamani et al. 2010). Alves et al. 2012 provided evidence for a complex genetic control of resistance in interspecific hybrids, detecting a locus in the same genomic region as Ppr1 as well as additional loci for resistance on chromosomes 1, 2, 5, 6, 7, 9 and 10 with evidence for both additive and epistatic interactions between loci. They suggested Ppr1 could be interacting with other loci with epistatic interactions. A GWAS analysis detected markers associated with myrtle rust resistance on chromosome 3 using a population of interspecific hybrids, which could also correspond to Ppr1 or an allele of Ppr1. Rosado et al. also found a QTL on chromosome 3 using a *E. grandis* x *E. urophylla* population.

2 Review of infection and phenotyping methods

Two methods were used for assessing Eucalyptus plants for their tolerance to myrtle rust: natural infection (Miranda et al. 2013; Teixeira et al. 2009) and inoculation from single pustule isolates (all others). The most commonly used methods are those developed by Junghans et al. (2003) and Pegg, Brawner, and Lee (2014). In brief, young seedlings are grown for 4–5 months in a greenhouse and then sprayed with a solution of *P. psidii* spores obtained from a single isolate. The tolerance is then scored 12 days after inoculation using a scale indicating the size of new pustules or hypersensitive response from the host plant. This will vary depending on species. In a recent study (not yet published), Pegg scored seedlings 20 and 25 days after inoculation, in *Corymbia* and *E. globulus*, respectively, because *E. globulus* took longer to show symptoms (J. Freeman, pers. comm.). The inoculation method is preferable to natural infection because the host-pathogen interactions may involve gene for gene relationships. Natural infection may occur by exposure to a mix of isolates and polymorphic pustules making it difficult to detect gene for gene interactions.

3 Review of type of genetic markers

Junghans et al. (2003) used random amplified polymorphic DNA (RAPD) markers, Butler et al. (2016) used diversity array technology (DArT) markers and Resende et al. (2016) used single nucleotide polymorphism (SNP) markers. The rest of the papers used simple sequence repeats (SSR). RAPD are not a marker of choice as they are not reproducible between populations like the other types of markers. SSR are a suitable type of marker; however, they are not as high throughput as SNPs or DArT.

4 Review of detected loci

The first locus detected for resistance to myrtle rust was derived from *E. grandis*: Ppr1 for *P. psidii* resistance (Junghans et al. 2003). Ppr1 was further confirmed and mapped to Eucalyptus chromosome 3 (Mamaní et al. 2010). Alves et al. (2011) provided evidence for a complex genetic control of resistance in interspecific hybrids, detecting a locus in the same genomic region as Ppr1 as well as additional loci for resistance on chromosomes 1, 2, 5, 6, 7, 9 and 10 with evidence for both additive and epistatic interactions between loci. They suggested Ppr1 could be interacting with other loci with epistatic interactions. A GWAS analysis detected markers associated with myrtle rust resistance on chromosome 3 using a population of interspecific hybrids, which could also correspond to Ppr1 or an allele of Ppr1. Rosado et al. (2010) also found a QTL on chromosome 3 using a *E. grandis* x *E. urophylla* population. Four new QTL different from Ppr1 and named Ppr2 to Ppr5 were detected in *E. globulus* on chromosomes 3, 7, 6 and 9 (Butler et al. 2016), providing further evidence of the complexity of the genetic basis of resistance to myrtle rust. We recommend that the genomic region orthologous to the Ppr1 locus from *E. grandis* and confirmed in Eucalyptus hybrids should be the target of initial genetic marker development to test if this region is associated with resistance in other species, such as *E. nitens* (used for breeding at Scion) and mānuka.

5 Review of genomics resources for Myrtaceae

When undertaking comparative marker discovery work, such as proposed in this programme, access to genomic resources is of huge value. Despite the global importance of myrtles as commercial and iconic indigenous species, it appears relatively few genome assemblies for Myrtaceous species are available in the public domain. One of the first high quality genome assemblies to be completed in forest trees, the genome of *E. grandis*, is now in a second draft (Myburg et al. 2014). Of interest to New Zealand researchers will be the *Metrosideros polymorpha* genome published in 2016 (Izuno et al. 2016). The indigenous Hawaiian name for *M. polymorpha* is Ohi'a and it is a close relative of New Zealand's own pōhutakawa. Like pōhutakawa, Ohi'a is highly susceptible to *A. psidii*, which is known as Ohi'a Rust in Hawaii. Two other publically available genomes are for crape myrtle (*Lagerstroemia indica*) (Wang et al. 2015) and *Eucalyptus camalendulensis*.

Other genome assemblies close to public release include: high quality assemblies in mānuka (*Leptospermum scoparium*) from the New Zealand Institute for Plant and Food Research Limited, and *Corymbia citriodora* ssp. *variegata* (Shepherd et al. 2016). Additional genome projects in various stages of completion include: *Melaleuca alternifolia* from Meryn Shepherd (Calvert, Baten, Butler, Barkla, & Shepherd, 2018) and *E. globulus* (Rigault et al. 2012). In addition, Scion has skim Genotype-By-Sequence data sets, as yet unassembled, for *E. nitens*, *E. fastigata* and *E. regnans*, the three most commercially significant eucalypts in New Zealand.

Another notable genomic resources is the EuCHIP60K utilising the Illumina Infinium technology (Silva-Junior et al. 2015). This SNP Chip was designed to capture polymorphisms in 12 species of eucalypts as well as a single Corymbia species. We have successfully utilised this resource to genotype a number of eucalypts. Markers already genotyped within our eucalypt genomics selection programme will be investigated for polymorphic loci within close proximity to the resistance QTL of interest.

6 Marker development for the Ppr1 locus in *E. nitens*

Based on the literature review, we recommended that the genomic region orthologous to the Ppr1 locus from *E. grandis* and confirmed in Eucalyptus hybrids should be the target of initial genetic marker development to test if this region is associated with resistance in other species, such as *E. nitens* (used for breeding at Scion) and mānuka.

Two genomics experiments using material from within the New Zealand *E. nitens* breeding programme and genotyped with the EuCHIP60K (Silva-Junior et al. 2015) were examined for the numbers of markers collocating to specific Myrtle rust resistance loci reported in the literature.

The first experiment was a pilot study looking at parentage reconstruction in New Zealand breeding orchards for *E. nitens* (Telfer et al. 2015). The second was a larger genomic selection experiment across 72 *E. nitens* families being progeny tested for wood quality traits (Klápště et al. 2018).

Chromosome 3 of the *E. grandis* v1.0 genome was downloaded in FASTA format from the European Nucleotide Archive (KK198755). The *E. grandis* v2.0 genome was downloaded in FASTA format from Phytozyme. The v1.0 and v2.0 sequences for chromosome 3 were aligned using Mauve. There were minimal changes in the v1.0 and v2.0 sequences; however, the assembly of the sections of the chromosome was quite different. Large sections of sequences were located in different areas, and orders, along the chromosome, with some also undergoing reversal of orientation (Bartholomé et al. 2015). Because of this reorganisation of large sections of sequence, in order to map the SNP regions between v1.0 and v2.0, we used the section boundaries to investigate SNPs within QTL loci.

The Ppr1 region of interest from Resende et al. (2016) was a QTL region covering the sequence 54520115 – 59692264 on v1.0 of the genome. This was with a section on the v1.0 genome (51190772 – 60664100) that aligned as a complete block to a section of the same length on the v2.0 genome (55057630 – 64530958). Any SNP within this region on the EucSNPCHIP 60K therefore can be mapped to the v2.0 genome position by adding the offset of 3866858 to the v1.0 genome position.

The remaining (QTL) loci were all identified based on the v2.0 genome. As for the Ppr1 region, they were all identified within sections of the v2.0 genome that aligned to the v1.0 genome as discrete blocks. However, most of these were not of equivalent length between the v1.0 and v2.0 genome sequences, and some were also of the opposite orientation, therefore mapping within these regions was not quite so straight forward. For regions of equivalent length, but opposite orientation, a simple transformation could be performed to determine the v1.0 position. For those with different lengths, substring searches within the v1.0 section for a 200bp sequence (100bp either side of the SNP) were performed to determine the v1.0 position (Table 2)

For the QTL that were identified on the v2.0 genome, we were interested in finding all SNPs from the *E. nitens* EucSNP chip 60K within 1Mbp either side of the QTL peak that were polymorphic in the 96 parents. Because of the high degree of reorganisation of the v2.0 assembly versus the v1.0 chromosome, the boundaries of the discrete sequence block were used to search for all SNPs. The SNPs within the v1.0 range of the aligned block were isolated from the parent EucSNP chip results and any polymorphic SNPs were mapped back to the v2.0 genome position using the methods described above (Table 3).

7 Marker development for the Ppr1 locus in mānuka and *E. nitens*

The genomic regions orthologous to the Ppr1 locus were identified in the mānuka genome assembly. Blastn search for SSR from Mamadi et al. (2010) were performed on the mānuka genome assembly pseudo-chromosomes. Genetic markers were designed within these regions using the High Resolution Melting (HRM) technique. A total of nine PCR primer pairs were designed and screened in mānuka DNA samples (Table 4). Testing of genetic markers linked to Ppr1 in mānuka was done using PCR and HRM. The markers successfully amplify PCR products and are polymorphic in the tested accessions. However, we now need phenotypic data to compare the marker profiles with resistance/susceptibility.

8 Recommendations

The identification of polymorphic SNP in regions of the genome homologous to those in which markers have been associated with variation in resistance in other eucalypts provides a significant step toward identifying genetic markers for myrtle rust resistance in *E. nitens* and mānuka.

The EuCHIP60K genotyping tool for Eucalyptus, in combination with phenotyping for rust resistance, could be used to rapidly identify genetic markers for resistance in *E. nitens* based on those discovered in other species in the eucalypt group (*Eucalyptus* and *Corymbia*). The genomic data could then be used to predict the level of resistance in trees without phenotyping.

The genetic markers designed for mānuka will be screened over DNA samples from the seedlings inoculated with *A. psidii*. Marker-trait association will be performed to verify the linkage between the Ppr1 locus and tolerance to myrtle rust in *Leptospermum*.

We recommend expanding the study to cover the full genome of host species as additional resistance loci other than Ppr1 could be found on other chromosomes. Genetic markers linked to these loci will then be useful for efficient selection of Myrtaceae seedlings carrying resistance to *A. psidii*.

Table 1. A literature review of genetic resistance to *A. psidii* was performed to inform us about the potential genetic structure of resistance in Myrtaceae species.

Title	Author	Year	Species	Inoculation method	Isolate	Type of analysis	Population type and size	#loci detected	% variance phenotypic explained	Chr list	Marker type
Genetic mapping provides evidence for the role of additive and non-additive QTLs in the response of inter-specific hybrids of Eucalyptus to Puccinia psidii rust infection	Alves et al.	2012	Interspecific Eucalyptus	inoculated with a single pustule isolate of Puccinia psidii and evaluated 20 days after inoculation	UFV-2	Segregation for resistance in families and QTL mapping	Several pop of varying size	1 + 3 epistatic QTLs	11%	chr3 + chr1, 2, 5, 6, 7, 9, 10	Microsatellites
Evidence for different QTL underlying the immune and hypersensitive responses of Eucalyptus globulus to the rust pathogen Puccinia psidii	Butler et al.	2016	E. globulus	Described in Pegg 2014		QTL mapping	F2 218 seedlings	4 loci (Ppr2 to Ppr5)	6.9 to 27.2%	3, 7, 6, 9	Microsatellites
Heritability for resistance to Puccinia psidii Winter rust in Eucalyptus grandis Hill ex Maiden in Southwestern Brazil	Miranda et al.	2012	E. grandis	Natural infection		Segregation for resistance and heritability	OP populations from provenances	na	na	na	na
Few Mendelian genes underlie the quantitative response of a forest tree, Eucalyptus globulus, to a natural fungal epidemic	Freeman et al.	2008	E. globulus	Natural infection		QTL mapping and validation	3x F2 populations	5 loci	6.2 to 36%	1, 2, 3, 7, 8	Microsatellites
Resistance to rust (Puccinia psidii Winter) in Eucalyptus: mode of inheritance and mapping of a major gene with RAPD markers	Junghans et al.	2003	E. grandis	Glasshouse inoculation	Several isolates used on parent	Linkage mapping	11x full sibs families	1 locus (PPpr1)	Mendelian locus	3	RAPD
Positioning of the major locus for Puccinia psidii rust resistance (Ppr1) on the Eucalyptus reference map and its validation across unrelated pedigrees	Mamani et al.	2010	E. grandis	inoculated with a single pustule isolate of Puccinia psidii and evaluated 20 days after inoculation	UFV-2	Linkage mapping	same as Junghans et al. 2003	2 locus (PPpr1)	Mendelian locus	3	Microsatellites
Regional heritability mapping and genome-wide association identify loci for complex growth, wood and disease resistance traits in Eucalyptus	Resende et al.	2016	Interspecific Eucalyptus	same as Junghans et al.		GWAS	Hybrid breeding population of 768 trees	1 locus (PPpr1?)	4.10%	3	SNP
Detection of QTL associated with rust resistance using IBD-based methodologies in exogamic Eucalyptus spp. populations	Rosado et al.	2010	E. granis x urophylla	inoculated with a single pustule isolate of Puccinia psidii and evaluated 20 days after inoculation	UFV-2	QTL mapping	F1 population s 131 indv	1 locus (allelic to Ppr1?)	37%	3	Microsatellites
Resistance of Eucalyptus pellita to rust (Puccinia psidii)	Santos et al.	2014	E. pellita	same as Junghans et al.		Segregation	4x F1 families	na	na	na	na
Análise da herança da resistência a Puccinia psidii em progênies de híbridos interespecíficos de eucalipto avaliadas sob condições naturais de infecção	Teixeira et al.	2009	Interspecific Eucalyptus	Natural infection		Segregation	15x progenies	na	na	na	na

Table 2. Location of markers genotyped in *E. nitens* that collocate with myrtle rust resistance in *E. grandis* genome assemblies V1 and V2.

Locus	SNP_ID Range	Name	[1]Location V1	[2]Location V2	[3]Orientation
E. globulus Qmyco5	12316 - 12550	EuBR03s16468115 EuBR03s18565224	16465124-18578014	16880084-18993283	+
E. globulus Sosr3	13246 - 13876	EuBR03s27576929 EuBR03s31513597	27576273-31528258	27580465-31532450	-
E. globulus Ppr2 QTL	14290 - 15085	EuBR03s35426530 EuBR03s42394462	35402671-42430585	36436850-43464964	+
CCV[4] *Rust_CT18*	14290 -15085	EuBR03s35426530 EuBR03s42394462	35402671-42430585	36436850-43464964	+
E. grandis Ppr1 GWAS	16390	EuBR03s56400715	56400715	60267573	+
E. grandis Ppr1 QTL	16244 - 16706	EuBR03s54520115 EuBR03s59692264	54520115-59692264	58386973-63559122	+
E. globulus Qmyco8	17532 - 17823	EuBR03s69554620 EuBR03s72357392	69550806-72366047	74628063-77443304	+
CCV[4] *QSB1_CT18*	17976 - 18152	EuBR03s74358083 EuBR03s75855829	74341305-75884442	82408840-83952244	-

[1] Location of SNP within the *E. grandis* Chromosome 3 Version 1 assembly
[2] Location of SNP within *the E. grandis* Chromosome 3 Version 2 assembly
[3] Orientation section delineated by the SNP markers in the V2 assembly compared to the V1 assembly
[4] CCV = *Corymbia citriodora ssp. Variegate.*

Table 3. Summary of markers genotyped in *E. nitens* that collocate with myrtle rust resistance in other Myrtaceae.

Locus	Reference	Number of marker	% captured	% polymorphic	SNP_ID range
E. globulus Qmyco5	Butler et al. 2016	235	94	38	12316 - 12550
E. globulus Sosr3	Butler et al. 2016	410	90	48	13426 - 13876
E. globulus Ppr2 QTL	Butler et al. 2016	796	88	28	14290 - 15085
CCV[2] *Rust_CT18*	Butler et al. in prep	638	89	23	14290 -15085
E. grandis Ppr1 GWAS	Resende et al. 2016	1	100	100	16390
E. grandis Ppr1 QTL	Resende et al. 2016	411	87	38	16244 - 16706
E. globulus Qmyco8	Butler et al. 2016	249	91	30	17532 - 17823
CCV[2] *QSB1_CT18*	Butler et al. in prep	145	91	25	17976 - 18152

Table 4. PCR primer pairs designed and screened in mānuka DNA samples.

Marker name	Locus targeted	Location in mānuka genome	Forward primer	Reverse primer
Ls3_6.03	Ppr1	Chr 3 - 6.03 Mb	GTCCCAAATTTGTTGAGCA	GTATTGACGGCTCCTCCTGA
Ls3_6.04	Ppr1	Chr 3 - 6.04 Mb	TGTCTGCCTTAGGGACTTCG	TGGTAAAGCTGCAGTTCGAG
Ls3_6.05	Ppr1	Chr 3 - 6.05 Mb	AAGGTCACCCCTTGATTTCC	CGCTATGGCCTCGATAAGTT
Ls3_6.13	Ppr1	Chr 3 - 6.13 Mb	CGATGTCACACGGAAAAATG	ATTAGGCTCCTGGCAAGTCC
Ls3_6.12	Ppr1	Chr 3 - 6.12 Mb	AAAGGGCCTATTTCCTCTGC	TGTTTGCAAAGGGTCTTTCTC
Ls3_6.09	Ppr1	Chr 3 - 6.09 Mb	AAGAAACTCCCGCCAAACTC	CTCCGATTCCACTCCCAAT
Ls3_6.49	Ppr1	Chr 3 - 6.49 Mb	AGCTAGCCCACAACCAGAAA	TGTGTATGCATTTAGACACGTGAG
Ls3_6.60	Ppr1	Chr 3 - 6.60 Mb	AGCGTCACAAGTCCAGCTCT	GCTGTCAATCCAGCTGTCAA
Ls3_6.69	Ppr1	Chr 3 - 6.69 Mb	CAGCGGAAGGAACCAATAGA	TGTTTTTGAGAGCCAAGACG

Confidential report for:

Ministry for Primary Industries
Client ref: 18608

PUBLICATION DATA

Chagne D, Telfer E. June 2019. Topic 1.4 – Initial identification of genetic markers linked to resistance. A Plant and Food Research report prepared for: MPI. Milestone No. 77454. Contract No. 34575 & 35604. Job code: P/313056/01 & P/340203/01. SPTS No. 17807-1.4.

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This report has been prepared by The New Zealand Institute for Plant and Food Research Limited (Plant & Food Research).
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