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Genetic variation of resistance to red needle cast in the RPBC Cloned Elites population

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REPORT INFORMATION SHEET

REPORT TITLE	GENETIC VARIATION OF RESISTANCE TO RED NEEDLE CAST IN THE RPBC CLONED ELITES POPULATION
AUTHORS	NATALIE GRAHAM, NARI WILLIAMS, MARI SUONTAMA, YONGJUN LI AND MARTIN BADER
CLIENT	MBIE / RADIATA PINE BREEDING COMPANY
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EXECUTIVE SUMMARY

Report Title: Genetic variation of resistance to red needle cast in the RPBC Cloned Elites population

Authors: Natalie Graham, Nari Williams, Mari Suontama, Yongjun Li and Martin Bader

The problem

The RPBC 2013/2014 Cloned Elites are the likely future of the New Zealand *Pinus radiata* breeding programme, but very little is known about the resistance of this germplasm to red needle cast (RNC), a disease caused by the pathogen *Phytophthora pluvialis.* Current heritability estimates for resistance have been assessed in limited numbers of genetic trials as the ability to screen targeted populations relies on these populations being located in trials with sufficient RNC exposure.

This project

Pinus radiata clones from the well-characterised RPBC Cloned Elites were screened for resistance to infection by *Phytophthora pluvialis* using a detached needle assay. Heritability of resistance was estimated.

Key Results

Quantitative differences in the susceptibility of *Pinus radiata* clones and families were observed, which has allowed for the selection of extreme phenotypes for further study.

Implications of Results for Client

The results of this study indicate a broad range in susceptibility/resistance of clones to red needle cast in the RPBC Cloned Elites. Initial heritability estimates indicate low to moderate heritability, which should allow genetic gains to be made for resistance to red needle cast in the breeding programme. With the potential to breed for improved resistance to RNC, industry should be able to target resistant germplasm to disease-prone sites.

Further Work

This work should be further validated with observations of these clones in the field. Heritability, cross-resistance analyses and implications for breeding and selection are being investigated further in conjunction with RPBC.

Genetic variation of resistance to red needle cast in the RPBC Cloned Elites population

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Introduction

Phytophthora pluvialis is a foliar pathogen of *Pinus radiata* (radiata pine) which has been shown to cause red needle cast (RNC) (Dick, Williams et al. 2014). It produces masses of sporangia on infected leaf tissues and causes the premature casting of radiata pine needles. A detached needle assay has been developed for screening radiata pine with *P. pluvialis* and applied to assays aimed at identifying potential genetic resistance to RNC and assessing responses to chemical treatments for RNC control.

To date, genetic variation of RNC resistance has been quantified in limited genetic trials using field-based observations (Dungey, Williams et al. 2014). Heritabilities were estimated for a small pilot screen for RNC in the Radiata Pine Breeding Company (RPBC) Cloned Elites, but this was only in 20 genotypes (Graham, Li et al. 2014). Structured populations with sufficient number of individuals with RNC observations are required to allow these heritability estimates to be confirmed. Lab-based screening using a detached needle assay would allow heritability estimates to be calculated using the well-characterised family structure of the RPBC Cloned Elites population. These Elites are also the likely future of the New Zealand *P. radiata* breeding programme, particularly as part of the training populations for the RPBC Genomic Selection Partnership. Little is known, however, about the resistance of this germplasm to infection by *P. pluvialis.* Quantification of this trait would allow for the targeting of resistant genotypes to disease-prone areas, and enable the identification of high-value parents for future breeding. This information will enable breeders to make informed assessments of the relative level of genetic gain that could be achieved through resistance-focused breeding programmes.

In parallel to the studies reported here, the RPBC Cloned Elites are being established in replicated trials across New Zealand, some of which will be exposed to RNC over the coming years, in addition to other foliar disease such as *Cyclaneusma* needle-cast and Dothistroma needle blight. An understanding of the susceptibility to all three of these diseases will provide an opportunity to investigate cross-resistance. This work is being reported to RPBC separately.

Objective

To screen *Pinus radiata* clones from the RPBC 2013/2014 Cloned Elites population for resistance to infection by *Phytophthora pluvialis*, and estimate heritabilities.

Materials and Methods

Plant material - Pinus radiata *clones*

The RPBC Cloned Elites consist of 63 families from 55 parents, with 24 full-sibs per family. A unique Clone ID (X_Y) was created by concatenating the family ID (X) with the Clone number (Y). In this study, a total of 392 unique genotypes were screened, at an average of 6.2 clones per family (see Appendix 1).

Plants were propagated from stool beds as bare-rooted cuttings, planted in Scion's nursery. Only individuals with at least five ramets available for testing were screened. Due to the scale of each screening and the inoculum requirements of each assay, forty-four clones were screened in each experiment (batch), with a further four reference clones included in every experiment to help assess between-experiment variations. These reference clones (03_16, 15_07, 23_18, and 29_22) are part of the RPBC Elites, selected to represent a range of susceptibilities based on their observed performance in the first experiment. A total of 392 clones were screened across multiple experiments over a period of 11 months (Jan 2014 – Nov 2014), comprising a total of 13 individual experiments.

Inoculum preparation

Zoospore inoculum was prepared in accordance with Scion's standard protocol (NM 2014). In brief, three active isolates of *P. pluvialis* were grown on carrot agar at 17 ^{o}C for three days. Plugs of agar and mycelium were taken from the leading edge of the colonies, flooded with clarified carrot broth in flat bottom flasks and incubated for three days at 17 ^{o}C (Erwin and Ribeiro 1996). The resulting mycelial mats were rinsed thoroughly for 8 hours with deionised water, drained and flooded with 50 ml sterile pond water. These were incubated at 17 ^{o}C in the dark for a further three days before zoospore release was induced with 45 minute intervals at 4 °C in the dark then at room temperature (21-22 ^{o}C) on a light box. Zoospore concentrations were determined using a haemocytometer and standardised to a minimum concentration of 5 x 10^3 zoospores per ml with sterile pond water. Zoospore suspensions were used within two hours of preparation.

Detached needle assay

Twenty healthy fascicles were collected from each plant with five fascicles assigned at random to two independent blocks (A and B) per treatment (H_2O and *P. pluvialis*-inoculated). Each tube was inoculated with either 4.5 ml of *P. pluvialis* zoospore suspension or sterile pond water overnight (18 hours). Fascicles were placed on trays moistened with wet paper towels and incubated in a controlled environment (17 ^{o}C, 65-70 % relative humidity, 14 h photoperiod) for 10 days. The needles within each fascicle were separated and lesions counted and measured.

Experimental Design

The experiment was set up as an alpha design with five super blocks (ramets) applying the inoculation treatment as whole-plot factor and the clone ID as sub-plot factor. The data set for a single experiment comprised 4800 observations.

Statistical analysis

Linear mixed effects models (LMM) fitted by restricted maximum likelihood were used to analyse the lesion length data and the average lesion number per fascicle and replicate (R version 3.1.2 (R Core Team, 2015) , R-package *nlme* (Pinheiro J 2015)). In order to assess the consistency of the four reference clones across experiments, the model used contained batch, inoculation treatment (H_2O control, pathogen-inoculated), clone identity, and their interaction as fixed effects. The nested random term contained tray nested within ramet

reflecting the alpha design. The batch-specific models were run in the same fashion with inoculation treatment, clone identity and their interaction as fixed term and the same random term as described above.

The significance of the fixed terms was assessed using a backwards selection procedure based on likelihood ratio tests (Zuur AF 2009). Graphical model validation tools were used to test the underlying assumptions of variance homogeneity and normality (plots of standardised residuals *vs.* fitted values and against all explanatory variables to evaluate variance patterns, quantile-quantile plots to assess the normality criterion). The residual plots of all models indicated strong heteroscedasticity. In the case of the reference clone model, the heteroscedasticity was modelled using a combination of an exponential variance function using the fitted values as variance covariate and a constant variance function (*varIdent*) with treatment as grouping factor. For the batch-specific models, the variance pattern was modeled using a constant variance function (*varIdent*) using inoculation treatment as a grouping factor.
The significant batch $\times$ treatment $\times$ clone interaction in the lesion length model for the reference clones was followed up applying a multiple comparison procedure using Tukey contrasts (R-package *multcomp*) (Hothorn, Bretz et al. 2008).

No pedigree information was overlayed in the analysis, and the lesion means were not adjusted for the genetics of the individuals.

Heritability estimation

The following genetic model was used to estimate variance components of the observed variables total lesion length, average lesion length and number of lesions:

$$\mathbf{y} = \mathbf{X}\boldsymbol{b} + \mathbf{Z}_1\boldsymbol{u} + \mathbf{Z}_2\boldsymbol{c} + \boldsymbol{e}$$

where $\mathbf{y}$ is the vector of phenotypes, $\boldsymbol{b}$ is the vector of fixed effects for the mean, the treatment, the interaction of tray within batch, and the scorer, $\boldsymbol{u}$ is the vector of random additive genetic effect of an individual tree, $\boldsymbol{c}$ is the vector of random clonal genetic effects (non-additive genetic effects) of an individual tree and $\boldsymbol{e}$ is the vector of random residual effects. Matrices $\mathbf{X}$, $\mathbf{Z}_1$ and $\mathbf{Z}_2$ are known incident matrices relating observations in $\mathbf{y}$ to the effects of $\boldsymbol{b}$, $\boldsymbol{u}$ and $\boldsymbol{c}$.

Genetic analysis was undertaken using ASReml –R. The total number of individuals in the pedigree was 532, with 392 clones having records in the data.

Narrow sense heritability (h^2) was estimated as $\sigma_a^2 / (\sigma_a^2 + \sigma_c^2 + \sigma_e^2)$ and broad sense heritability (H^2) was estimated as $(\sigma_a^2 + \sigma_c^2) / (\sigma_a^2 + \sigma_c^2 + \sigma_e^2)$, where σ_a^2 is the additive genetic variance, σ_c^2 is the clonal (non-additive) genetic variance and σ_e^2 is random residual variance component.

Results

Reference clone consistency

The consistency of the responses of four reference clones across batches was evaluated and a significant batch × treatment × clone interaction was observed (χ^2 = 152.7, df = 36, *P* < 0.001). As anticipated, the control treatment showed negligibly small lesions whereas the pathogen-inoculated samples showed substantial lesion development, at least in some of the reference clones (Fig. 1). Despite the large variation among batches, there was some degree of consistency among the four reference clones. Reference clones 29_22 (black) and 23_18 (dark grey) emerged in the majority of batches as more susceptible than clones 03_16 (white) and 15_07 (light grey).

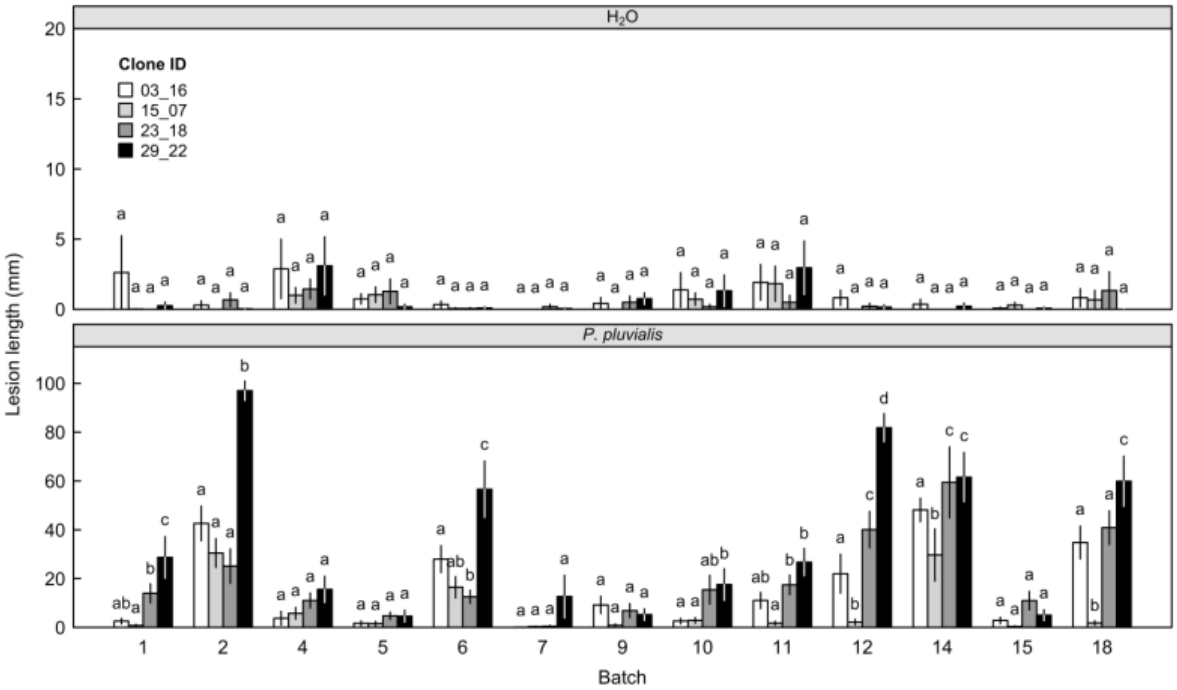

Fig. 1 Mean lesion length per fascicle in the four *Pinus radiata* reference clones across 13 batches. H_2O control (*upper panel*), *Phytophthora pluvialis* infected needles (*lower panel*). Different lower case letters within a batch indicate statistically significant differences at α = 0.05 (multiple comparison procedure using Tukey contrasts).

Batch-wise clone ranking based on lesion length

The maximum lesion length varied widely between batches (Fig. 2). However, there was some consistency with regards to the reference clones (RC), with clone 29_22 showing up as the one with the largest lesions followed by clone 23_18 in the majority of batches. In the later batches (beyond batch 6) RC 15_07 emerged as one of the least susceptible clones often with lesion lengths < 10 mm per fascicle. Similarly, RC 03_16 occurred amongst the less susceptible clones tested. All batch-specific mixed-effects models yielded a significant treatment × clone interaction, meaning that lesion length varied significantly across clones but these differences were modulated by the treatment (i.e. H_2O control vs. *P. pluvialis* inoculation; Appendix 2).

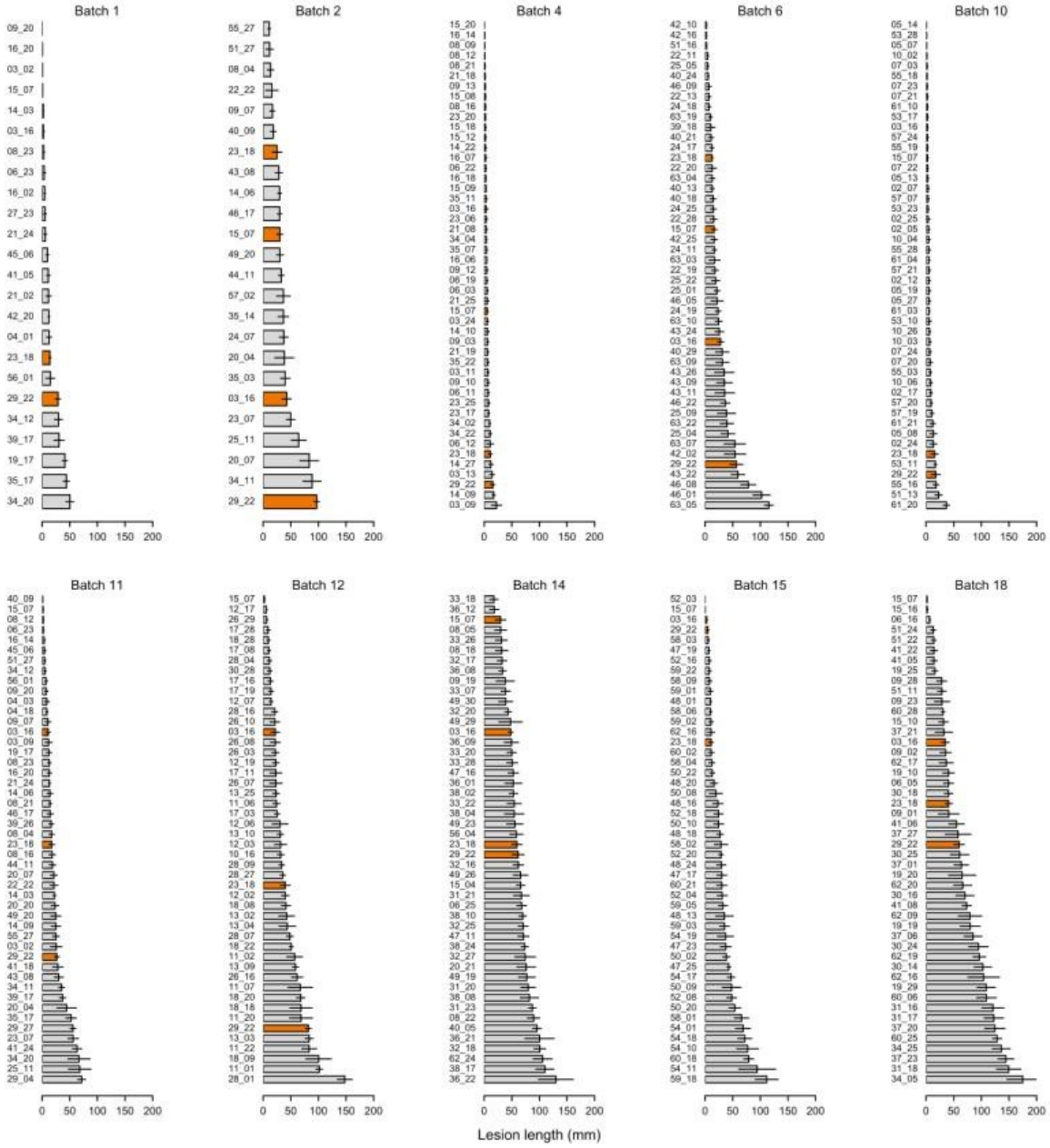

Fig. 2 Within-batch clone means based on lesion length (only pathogen-inoculated samples are shown). The four reference clones were present in all batches and are highlighted in orange.

Batch-wise clone ranking based on lesion number

Average lesion counts per fascicle did not exceed five, which reduced the resolution power for ranking using this variable. However, overall rankings using lesion counts were similar to what was observed with lesion length (Fig. 3). All batch-specific mixed-effects models produced a significant treatment × clone interaction (Appendix 3).

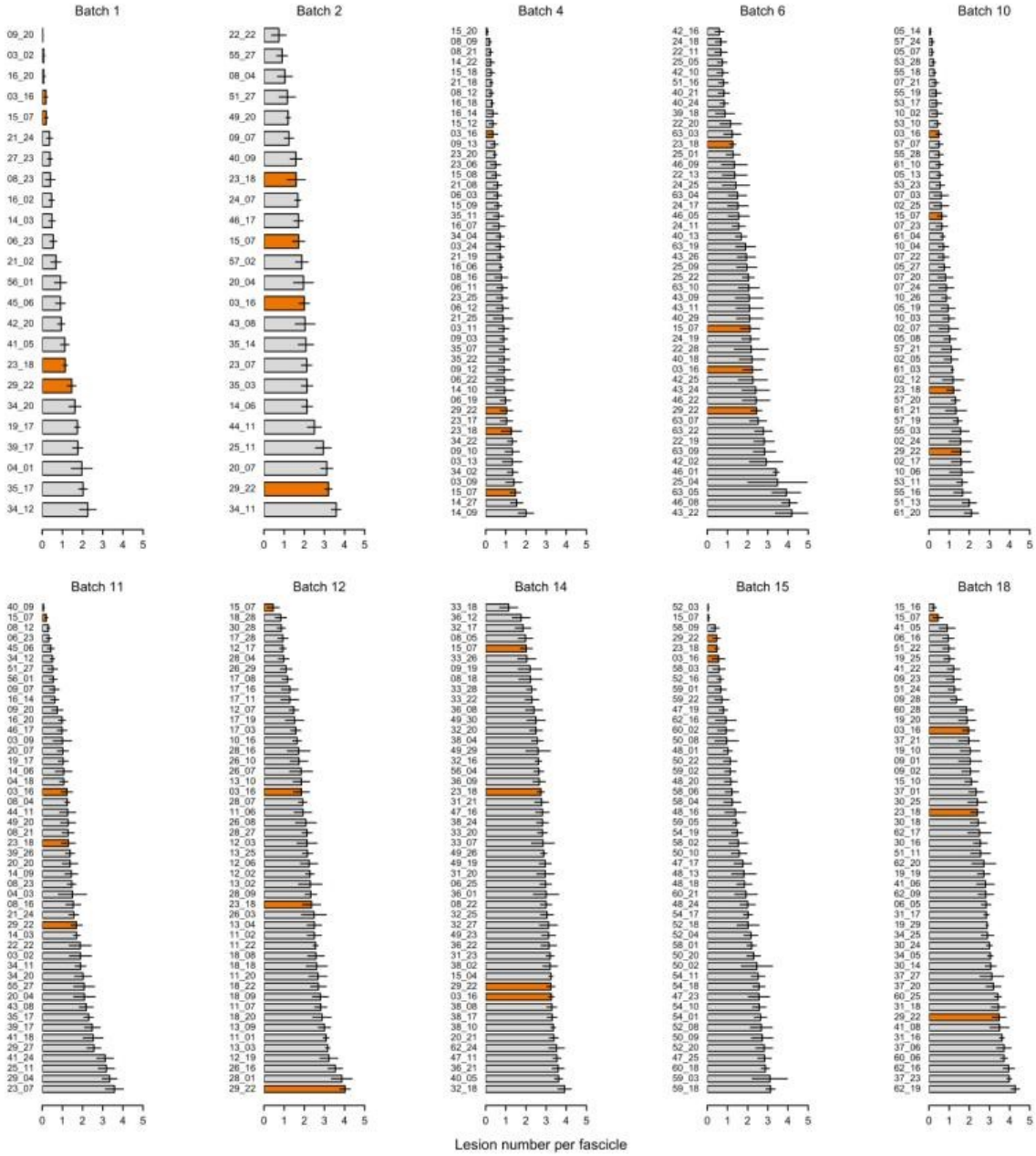

Fig. 3 Within-batch clone means based on the average lesion number per fascicle (only pathogen-inoculated samples are shown). The four reference clones were present in all batches and are highlighted in orange.

Overall clone and family ranking

Lesion length data were combined across experiments and used to produce both individual clone and family rankings (Fig 4 and Fig 5). These rankings indicate clones and families that are more resistant than the average across all clones/families.

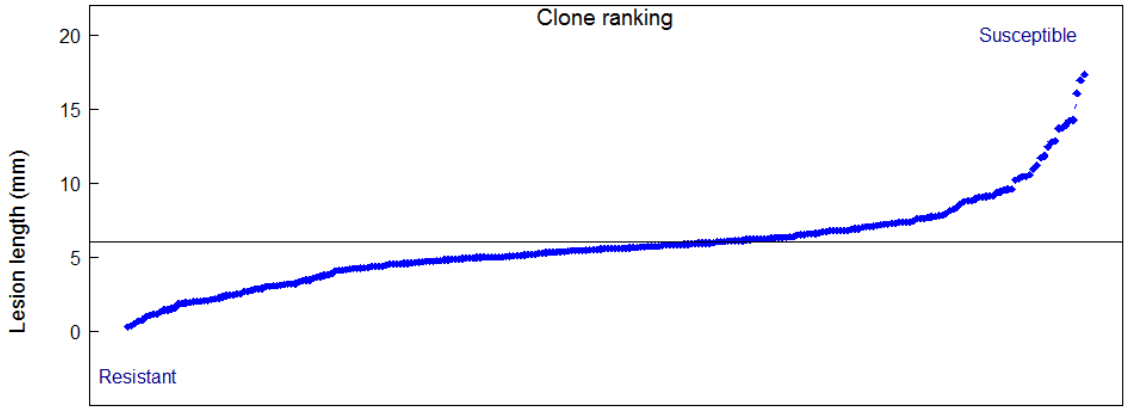

Fig. 4 Ranking of clones using average lesion length (mm) showing the average lesion length across all clones.

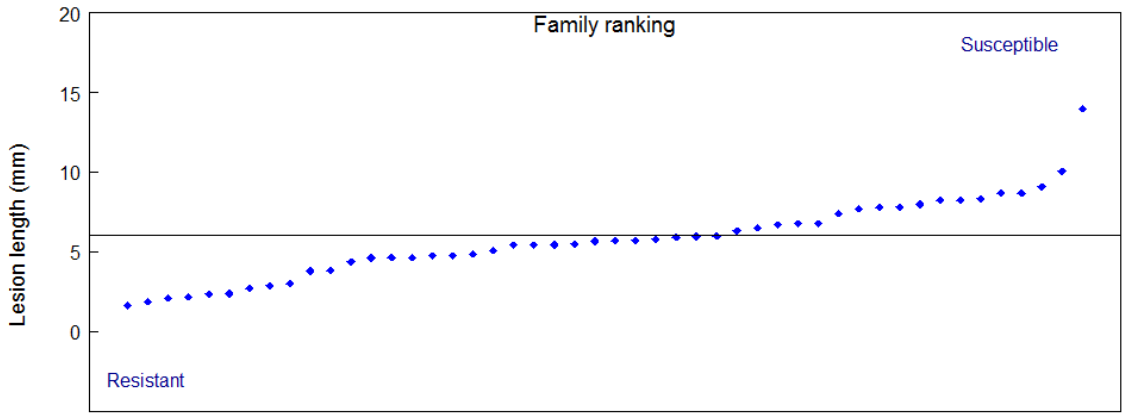

Fig. 5 Ranking of families using average lesion length (mm) showing the average lesion length across all families.

Heritability

All fixed effects were highly significant for the traits ($P < 0.0001$). Narrow sense heritability estimates were low for total lesion length (0.12) and average lesion length per fascicle (0.14). Broad sense heritability for lesion length was moderate (0.33) but lower for average lesion length (0.23). The number of lesions had a low estimate of narrow sense heritability (0.05) but somewhat higher broad sense heritability (0.16). Heritability estimates indicated that selection for the lesion length is possible. Heterogeneity of variance was evident in residual plots and may have some effect on estimated heritability.

Table 1. Estimates of variance components, narrow and broad sense heritability for lesion length, average lesion length and number of lesion counts.

	Lesion length$_1$	Average lesion length$_2$	No. of lesion counts
σ_a^2	64.00	7.78	0.03
σ_c^2	79.05	3.16	0.07
σ_e^2	-370.76	45.8	0.53
h^2	0.12±0.05	0.14±0.04	0.05±0.02
H^2	0.33±0.04	0.23±0.03	0.16±0.02

σ_a^2 = additive genetic variance, σ_c^2 = clonal variance, σ_e^2 = residual variance, h^2 = estimate of narrow sense heritability, H^2 = estimate of broad sense heritability, lesion length$_1$= lesion length (mm), Average lesion length$_2$= lesion length/no. of lesions observed.

Discussion

The results of this study indicate a broad range in susceptibility/resistance of clones to red needle cast in the RPBC's Cloned Elites. This is promising for the potential to breed for improved resistance to RNC. Heritability of lesion length were low to moderate at 0.12, 0.14 (narrow-sense) and 0.23,0.33 (broad-sense), but comparable to what has previously been reported from field-based assessments, which showed narrow sense heritability of RNC in the field to be between 0.21-0.31 and clonal heritability of 0.23-0.59 (Dungey, Williams et al. 2014). The additive genetic variation for lesion length in this present study suggests that the detached needle assay can detect genetic variation of resistance to RNC. As a result, lesion length assessments could be used in in breeding programmes to improve resistance to red needle cast in *P. radiata*. The range of heritabilities reported for disease resistance in *P. radiata* have been shown to vary considerably between trials and traits, but have most consistently been observed in the 0.1-0.4 range (Dungey, Low et al. 2006).

The vast majority of studies in which tree species have been screened for resistance to *Phytophthora* have shown clonal responses (see Table 2 for summary), but have not necessarily tracked or reported the heritability of resistance across a defined population. These studies have also focused on root-infecting species of *Phytophthora* with no resistance screening of aerial *Phytophthora* species that would be directly comparable to needle infection of *P. pluvialis* in *P. radiata.* In the few studies which have reported heritability estimates for *Phytophthora* resistance to soil-borne pathogens in trees, heritability has ranged from h^2 0.5-0.9, which are significantly higher than in our studies.

Table 2: Heritability estimates associated with tree species which have shown segregation in susceptibility to infection by *Phytophthora* pathogens (adapted from Dungey, Williams et al. (2014)).

* Clonal variation observed in screening and selection trials but no heritability reported

Host Common Name	**Latin Name**	***Phytophthora* species**	**Reference**	**Heritability estimate**
Port Orford Cedar	*Chamaecyparis lawsoniana*	*P. lateralis* *P. cinnamomi*	E.M. Hansen et al. 1989 Green et al. 2013 (McWilliams 2000)	Heritability not provided* Heritability not provided* h^2 0.61, H^2 = 0.98
Shortleaf pine	*Pinus echinata*		Zentmyer 1980	Heritability not provided*
Jarrah	*Eucalyptus marginata*	*P. cinnamomi*	McComb et al. 1991	
Apple	*Malus domestica*	*P. cactorum*	Utkhede and Quamme 1988	Heritability not provided*
Avocado	*Persea americana*	*P. cinnamomi*	(Douhan, Fuller et al. 2011)	Heritability not provided*
Radiata pine	*Pinus radiata*	*P. cinnamomi*	Butcher et al. 1984	h^2 = 0.86-0.90
Fraser fir Canaan fir Nordmann fir Trojan fir Turkish fir	*Abies fraseri* *Abies balsamea* var. *phanerolepis* *Abies nordmanniana* *Abies equi-trojani* *Abies bornmuelleriana*	*P. cactorum* *P. cinnamomi* *P. drechsleri*	Hoover 2013 (Frampton, Isik et al. 2013)	Heritability not provided* h^2 0.62 +/- 0.162 H^2 = 0.97 +/- 0.011 h^2 0.50 +/- 0.102 H^2 = 0.96 +/- 0.01
Coast Live Oak	*Quercus agrifolia*	*P. ramorum*	(Dodd, Huberli et al. 2005)	Heritability not provided*

Leaf age can affect disease susceptibility; therefore, care was taken to select fully formed needles that best represent those observed to be most affected in the field. However, due to the time interval it took to screen all 392 clones, the physiological age of the material and progressive crowding of plants in the stool beds differed from the first experiments to the final experiment, which was undertaken 11 months later. In addition, seasonal effects could be further affecting the residual variance being observed. This again highlights the need for between-experiment controls to account for these variations. Performance of individual clones should therefore be made with regard to the four reference clones in each assay-run.

Recent validation work comparing detached needle assays with on-plant inoculations suggests that the detached assay may be less useful at differentiating more subtle differences in susceptibility, such as those arising from multi-genic effects (Williams and Graham 2015). In general, the detached needle assay challenges plant material with high infection pressure which may limit the resolution of subtle differences in the susceptibility of individual clones. This may also contribute to the lower heritability observed in this study compared to field-based observations reported previously. However, the detached needle assay gives a tool for carrying out primary screening of a broad range of material not yet exposed to RNC in the field and with consistent inoculum pressure. Quantitative range of responses to infection, shown across the 13 assays presented here, demonstrate the utility of the assay in identifying clones with greater relative resistance.

In order to get a better understanding of how these results would translate into field-based observations, the phenotypic extremes from this study have been propagated via cuttings. These are being grown in pots and will be placed in infected forests where they will be naturally exposed to RNC in due course. This will provide useful information on how the detached needle assay compares with field infection. The final step will then be to assess the response of more mature plants in the field and correlate this with the detached needle assay observations. This should be resolved in future field trials, as the Cloned Elites families have been planted out as part of the RPBC's operations across multiple field sites in 2013 and 2014, some of which are in RNC-affected areas. Some of these trial sites will also likely experience exposure to other foliar diseases, such as *Cyclaneusma* needle-cast and Dothistroma needle blight. When further field-based phenotypic information is available on these other needle diseases we will be able to use the information from this report to seek genotypes with quantitative resistance to multiple pathogens.

Acknowledgements

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Appendix 1: List of clones screened per family

Family	No screened	Clone IDs screened										
1	5*	1_3	1_6	1_7	1_8	1_10						
2	6	2_5	2_7	2_12	2_17	2_24	2_25					
3	6	3_2	3_9	3_11	3_13	3_16	3_24					
4	5*	4_1	4_3	4_8	4_9	4_18						
5	6	5_7	5_8	5_13	5_14	5_19	5_27					
6	9	6_3	6_5	6_11	6_12	6_16	6_19	6_22	6_23	6_25		
7	6	7_3	7_20	7_21	7_22	7_23	7_24					
8	9	8_4	8_5	8_9	8_12	8_16	8_18	8_21	8_22	8_23		
9	11	9_1	9_2	9_3	9_7	9_10	9_12	9_13	9_19	9_20	9_23	9_28
10	6	10_2	10_3	10_4	10_6	10_16	10_26					
11	6	11_1	11_2	11_6	11_7	11_20	11_22					
12	6	12_2	12_3	12_6	12_7	12_17	12_19					
13	6	13_2	13_3	13_4	13_9	13_10	13_25					
14	6	14_3	14_6	14_9	14_10	14_22	14_27					
15	9	15_4	15_7	15_8	15_9	15_10	15_12	15_16	15_18	15_20		
16	6	16_2	16_6	16_7	16_14	16_18	16_20					
17	6	17_3	17_8	17_11	17_16	17_19	17_28					
18	6	18_8	18_9	18_18	18_20	18_22	18_28					
19	6	19_10	19_17	19_19	19_20	19_25	19_29					
20	7	20_4	20_7	20_8	20_20	20_21	20_22	20_23				
21	6	21_2	21_8	21_18	21_19	21_24	21_25					
22	6	22_11	22_13	22_19	22_20	22_22	22_28					
23	6	23_6	23_7	23_17	23_18	23_20	23_25					
24	6	24_7	24_11	24_17	24_18	24_19	24_25					
25	6	25_1	25_4	25_5	25_9	25_11	25_22					
26	6	26_3	26_7	26_8	26_10	26_16	26_29					
27	5*	27_3	27_4	27_23	27_27	27_28						
28	6	28_1	28_4	28_7	28_9	28_16	28_27					
29	6	29_4	29_9	29_22	29_24	29_26	29_27					
30	6	30_14	30_16	30_18	30_24	30_25	30_28					
31	6	31_16	31_17	31_18	31_20	31_21	31_23					
32	6	32_16	32_17	32_18	32_20	32_25	32_27					
33	6	33_7	33_18	33_20	33_22	33_26	33_28					
34	8	34_2	34_4	34_5	34_11	34_12	34_20	34_22	34_25			
35	6	35_3	35_7	35_11	35_14	35_17	35_22					
36	6	36_1	36_8	36_9	36_12	36_21	36_22					
37	6	37_1	37_6	37_20	37_21	37_23	37_27					
38	6	38_2	38_4	38_8	38_10	38_17	38_24					
39	6	39_2	39_3	39_13	39_17	39_18	39_26					
40	7	40_5	40_9	40_13	40_18	40_21	40_24	40_29				
41	7	41_5	41_6	41_8	41_18	41_22	41_23	41_28				
42	5*	42_2	42_10	42_16	42_20	42_25						
43	6	43_8	43_9	43_11	43_22	43_24	43_26					
44	5*	44_2	44_11	44_16	44_23	44_24						
45	6	45_3	45_5	45_6	45_19	45_20	45_22					
46	6	46_1	46_5	46_8	46_9	46_17	46_22					

Family	No screened	Clone IDs screened							
47	6	47_11	47_16	47_17	47_19	47_23	47_25		
48	6	48_1	48_13	48_16	48_18	48_20	48_24		
49	6	49_19	49_20	49_23	49_26	49_29	49_30		
50	6	50_2	50_8	50_9	50_10	50_20	50_22		
51	6	51_11	51_13	51_16	51_22	51_24	51_27		
52	6	52_3	52_4	52_8	52_16	52_18	52_20		
53	5	53_10	53_11	53_17	53_23	53_28			
54	6	54_1	54_10	54_11	54_17	54_18	54_19		
55	6	55_11	55_16	55_18	55_19	55_27	55_28		
56	6	56_1	56_2	56_4	56_20	56_21	56_25		
57	6	57_2	57_7	57_19	57_20	57_21	57_24		
58	6	58_1	58_2	58_3	58_4	58_6	58_9		
59	6	59_1	59_2	59_3	59_5	59_18	59_22		
60	6	60_2	60_6	60_18	60_21	60_25	60_28		
61	5*	61_3	61_4	61_10	61_20	61_21			
62	6	62_9	62_16	62_17	62_19	62_20	62_24		
63	8	63_3	63_4	63_5	63_7	63_9	63_10	63_19	63_22

*These families did not have additional members with sufficient ramets to allow for further screening within that family

Appendix 2: ANOVA table of the batch-specific models for lesion length (DF_{num} = numerator degrees of freedom, DF_{den} = denumerator degrees of freedom, F = F-value, P = P-value).

Parameter	DF_{num}	DF_{den}	F	P	
Batch 1					
Intercept	1	394	26.48	< 0.001	***
Treatment	1	34	172.06	< 0.001	***
Clone	23	394	1.64	< 0.033	*
Treatment × Clone	23	394	8.87	< 0.001	***
Batch 2					
Intercept	1	393	7.54	< 0.006	**
Treatment	1	34	522.37	< 0.001	***
Clone	23	393	1.18	0.263	
Treatment × Clone	23	393	8.58	< 0.001	***
Batch 4					
Intercept	1	786	52.57	< 0.001	***
Treatment	1	74	123.69	< 0.001	***
Clone	47	786	1.56	0.011	*
Treatment × Clone	47	786	2.48	< 0.001	***
Batch 6					
Intercept	1	786	41.06	< 0.001	***
Treatment	1	74	563.00	< 0.001	***
Clone	47	786	1.99	< 0.001	***
Treatment × Clone	47	786	9.92	< 0.001	***
Batch 10					
Intercept	1	786	48.98	< 0.001	***
Treatment	1	74	229.55	< 0.001	***
Clone	47	786	1.71	0.011	*
Treatment × Clone	47	786	4.74	< 0.001	***
Batch 11					
Intercept	1	780	32.20	< 0.001	***
Treatment	1	74	504.87	< 0.001	***
Clone	47	780	1.62	0.006	**
Treatment × Clone	47	780	7.69	< 0.001	***
Batch 12					
Intercept	1	786	14.26	< 0.001	***
Treatment	1	74	935.08	< 0.001	***
Clone	47	786	1.04	0.402	
Treatment × Clone	47	786	11.35	< 0.001	***
Batch 14					
Intercept	1	779	3.10	0.079	
Treatment	1	74	1359.66	< 0.001	***
Clone	47	779	0.89	0.676	
Treatment × Clone	47	779	4.56	< 0.001	***
Batch 15					
Intercept	1	784	9.94	0.002	**
Treatment	1	74	617.06	< 0.001	***
Clone	47	784	1.34	0.069	
Treatment × Clone	47	784	9.03	< 0.001	***
Batch 18					
Intercept	1	777	7.25	0.007	**
Treatment	1	74	1440.91	< 0.001	***
Clone	47	777	1.29	0.097	
Treatment × Clone	47	777	14.00	< 0.001	***

Appendix 3: ANOVA table of the batch-specific models for lesion count (DF_{num} = numerator degrees of freedom, DF_{den} = denumerator degrees of freedom, F = F-value, P = P-value).

Parameter	DF_{num}	DF_{den}	F	P	
Batch 1					
Intercept	1	394	83.22	< 0.001	***
Treatment	1	34	271.49	< 0.001	***
Clone	23	394	1.34	0.138	
Treatment × Clone	23	394	9.14	< 0.001	***
Batch 2					
Intercept	1	393	19.42	< 0.001	***
Treatment	1	33	943.61	< 0.001	***
Clone	23	393	1.20	0.242	
Treatment × Clone	23	393	6.10	< 0.001	***
Batch 4					
Intercept	1	786	52.00	< 0.001	***
Treatment	1	74	47.98	< 0.001	***
Clone	47	786	1.89	< 0.001	***
Treatment × Clone	47	786	2.48	< 0.001	***
Batch 6					
Intercept	1	786	268.56	< 0.001	***
Treatment	1	74	449.13	< 0.001	***
Clone	47	786	2.15	< 0.001	***
Treatment × Clone	47	786	3.90	< 0.001	***
Batch 10					
Intercept	1	786	86.46	< 0.001	***
Treatment	1	74	85.90	< 0.001	***
Clone	47	786	2.21	< 0.001	***
Treatment × Clone	47	786	4.12	< 0.001	***
Batch 11					
Intercept	1	786	218.06	< 0.001	***
Treatment	1	74	243.50	< 0.001	***
Clone	47	786	3.40	< 0.001	***
Treatment × Clone	47	786	6.71	< 0.001	***
Batch 12					
Intercept	1	786	408.24	< 0.001	***
Treatment	1	74	798.77	< 0.001	***
Clone	47	786	1.56	0.011	*
Treatment × Clone	47	786	6.98	< 0.001	***
Batch 14					
Intercept	1	783	115.69	< 0.001	***
Treatment	1	74	3423.64	< 0.001	***
Clone	47	783	1.01	0.451	
Treatment × Clone	47	783	2.87	< 0.001	***
Batch 15					
Intercept	1	786	57.48	< 0.001	***
Treatment	1	74	1112.87	< 0.001	***
Clone	47	786	1.35	0.061	
Treatment × Clone	47	786	6.97	< 0.001	***
Batch 18					
Intercept	1	780	610.08	<]0.001	***
Treatment	1	74	1852.04	< 0.001	***
Clone	47	780	1.39	0.045	*
Treatment × Clone	47	780	10.81	< 0.001	***