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ORIGINAL PAPER

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Mapping gene *H* controlling cane pubescence in raspberry and its association with resistance to cane botrytis and spur blight, rust and cane spot

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Abstract Disease resistance is increasing in importance, as consumers require high-quality raspberry fruit at a time when chemical disease control is undesirable. Breeders have limited resources and rarely include a primary screen for each fungal disease. Marker-assisted breeding would facilitate the introduction of resistance into elite germplasm and breeding lines. An additional 20 simple sequence repeats have been added to the existing raspberry linkage map. Gene *H*, which determines cane pubescence (genotype *HH* or *Hh*), the recessive allele of which gives glabrous canes (genotype *hh*), has been mapped on to linkage group 2 and shown to be closely associated with resistance to cane botrytis and spur blight but not rust or cane spot. Other map regions on linkage groups 3, 5 and 6 associated with disease resistance are reported here.

Introduction

Red raspberry (*Rubus idaeus* L.) production is an important high-value horticultural industry in many cool temperate regions of the world. In Europe, most raspberry production is concentrated in the northern and central countries, although there is an increasing interest in growing cane fruits in southern Europe, e.g. Greece, Italy, Portugal and Spain. Stems and leaves of red raspberry and its close relatives are prone to infection by several fungal diseases, which have the potential to cause serious yield loss worldwide. Disease resistance is increasing in importance, as consumers require high-quality fruit at a time when chemical disease control is undesirable. Breeders have limited resources and rarely include a primary screen for each fungal disease. It has been reported that resistance to some fungal diseases are associated with distinctive morphological traits, most notable being cane pubescence (fine hairs). Pubescence is determined by gene *H* (genotype *HH* or *Hh*), the recessive allele of which gives glabrous canes (genotype *hh*). Gene *H* is rarely homozygous (*HH*) because it is linked with a lethal recessive gene (Jennings 1967). Raspberry cultivars and selections with fine hairs (pubescent canes) are reported to be more resistant to cane botrytis (*Botrytis cinerea*) and spur blight [*Didymella applanata* (Niessl) Sacc.] than non-hairy ones (Knight and Keep 1958; Jennings 1982a; Jennings and Brydon 1989) but more susceptible to cane spot (*Elsinoe veneta*), powdery mildew (*Sphaerotheca macularis*) and yellow rust (*Phragmidium rubi-idaei*) (Jennings 1962; Keep 1968, 1976; Jennings and McGregor 1988; Anthony et al. 1986). How gene *H* results in the large increase or decrease in disease resistance has not been determined. It has been suggested that it is due to linkage with major resistance genes or minor gene complexes that independently contribute to the resistance or susceptibilities of the six diseases affected. An alternative explanation is that the gene itself is responsible through pleiotropic effects on each of the resistances (Williamson and Jennings 1992). The gene is known to have other pleiotropic effects besides its main effect on cane pubescence: it is associated with a small increase in spine frequency and decrease in spine size (Jennings 1962; Keep et al. 1977). Hairs and spines are both outgrowths

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of epidermal cells and their early development is inter-related (Peitersen 1921). It would therefore seem likely that gene *H* acts early in development and affects several cell characteristics. Resistance to *B. cinerea* and *D. applanata* is highest in immature tissues and Williamson and Jennings (1992) have postulated that the gene increases resistance by delaying cell maturity and this delay may reduce resistance to *P. rubi-idaei* and *E. veneta* as these fungi only invade immature tissues. Alternatively cane hairiness itself could affect the ability of fungi to adhere and infect tissues (Jennings 1962).

Spur blight and cane botrytis occupy the same ecological niche on raspberry canes and often one or the other predominates. Spur blight infects mature or senescent leaves on young canes of raspberry, then grows within the petioles to the nodes where the disease lesions develop. Buds at infected nodes are retarded in growth compared to those at non-infected nodes, and infection usually results in failure of buds to develop into fertile lateral shoots the following spring (Pepin et al. 1985). Cane botrytis infects in a similar manner though with bud dwarfing more severe. Lateral shoot loss with these diseases is the most important factor for yield loss. It has been shown that a common resistance operates against these two pathogens (Williamson and Jennings 1986). Whether resistance is caused by a major gene, a complex of dominant minor genes or a major gene in combination with minor genes has yet to be determined. Field screening either with natural infection or with a simple wound inoculation method (Jennings and Williamson 1982) remains the best method for analysis of resistance status, as glasshouse inoculations do not result in characteristic disease symptoms.

Cane spot, also known as anthracnose, can be expressed in leaves, fruits and young canes but it is most easily recognised by the deeply penetrating sunken lesions it produces on second-year fruiting canes (floricanes) that lead to damaged vascular tissue and yield loss in susceptible cultivars. The causal agent, *E. veneta*, is intractable for use in resistance screening because its growth rate is exceptionally slow in vitro (Williamson and Jennings 1992). Natural infection of selections in field observation plots with a high disease incidence remains the most effective way of resistance breeding, though screening of cultivars by use of inoculum generated in vitro is now possible on a small scale (Williamson et al. 1989).

Gene *H* has been reported to be associated with high susceptibility to cane spot in European cultivars and originally it was thought that cultivars carrying the gene would inevitably be susceptible. However, several North American cultivars are good sources of resistance, despite carrying the gene. Single major gene resistance has been reported, but Jennings and McGregor (1988) have questioned this finding.

Yellow rust on raspberry has come to prominence over the last 20 years with some highly susceptible cultivars including Glen Moy, Glen Ample and Tulameen. The expansion of raspberry production under plastic tunnels has exacerbated the disease because of high humidities generated. Bait plants in commercial plantations in replicated field experiments have been used to assess genetic resistance. The American cultivar Latham is known to exhibit complete resistance transmissible through four generations and determined by gene *Yr* (Anthony et al. 1986). These authors also found incomplete resistance to rust in some of their crosses and reported that gene *H* was associated with susceptibility to rust.

Graham et al. (2004a) developed a genetic linkage map of raspberry, based on the progeny of a cross between the cultivars Latham and Glen Moy. This population segregates for gene *H* and for resistance to the above diseases. The overall objective of the research presented here was to identify map regions associated with resistance to cane botrytis, spur blight, cane spot and yellow rust and to explore the relationship between gene *H* and resistance to these diseases. The development of additional polymorphic co-dominant microsatellite markers and the determination of the map location for gene *H* are presented.

Materials and methods

Enhancement of genetic linkage map through development of further co-dominant markers

Library of nebulised DNA

Generation and screening of a library of nebulised genomic DNA library for simple sequence repeat (SSR) identification was carried out as follows: genomic DNA was extracted from the red raspberry cultivar Glen Moy using a 2% CTAB method (Graham et al. 2003). Ten microgram of DNA was re-suspended in 750 µl TE with glycerol at a final concentration of 10% v/v and nebulised using a disposable plastic nebuliser and a range of pressures/times from 10 to 30 psi and 60 to 120 s. Optimal shearing within the desired size range was generated at 20 psi for 90 s. Size-selected fragments between 400 bp and 2 kb were ligated into pGEM-T Easy (Promega) and transformed into *Escherichia coli* DH10B cells. Using a DIG nucleic acid detection kit (Roche), 9×384 well plates were screened with Dig-labelled oligomers of $AG_{(13)}$ as per manufacturer's instructions. Positive colonies were sequenced on an ABI Prism 377 automated sequencer using the BigDye terminator cycle sequencing reaction ready kit (Applied Biosystems). DNA sequences were quality scored using the Phred package (http://www.phrap.com). Primer pairs were designed using Primer 3 (Rozen and Skaletsky 1998) synthesised by MWG and tested on the cultivars Glen Moy and Latham. For SSR polymorphism, PCR reactions were set up in 25 µl reaction with 25 ng DNA, 2 µM each primer, 200 µM of each nucleotide, 1.5 mM of $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Roche) per reaction, in a Gene Amp PCR System 9700 thermal

cycler (Applied Biosystems) for 25 cycles with denaturation at 94°C for 45 s, annealing at 59°C for 45 s and extension at 72°C for 1 min, with a final extension step of 5 min at 72°C. Primer pairs were fluorescently labelled on the left primer with HEX, FAM or TET (MWG) and PCR products were prepared according to Macaulay et al. (2001) for analysis on the ABI Prism 377. Allele sizes were determined using GENESCAN software programme (Applied Biosystems) and GeneScan-350 (Tamra), as an internal size standard.

Further SSR development from PstI library

Further SSRs from the *Pst*I genomic library previously described (Graham et al. 2002, 2004a) were tested for utility. Previously 100 of the 258 SSRs were examined. Here a further 26 SSR loci were examined.

Mapping population and phenotypic analyses

The mapping population previously described (Graham et al. 2004a) consists of a full-sib family of 320 progeny generated from a cross between the European red raspberry cv. Glen Moy and the North American red raspberry cv. Latham. This population was planted in autumn 2001 at two different environmental locations in randomised complete block trials with three replicates of two plant plots at both locations (12 plants per genotype). The sites varied in altitude and slope. Latham has glabrous canes (hh) and is resistant to rust and cane spot. Glen Moy on the other hand has pubescent canes (Hh) and is resistant to cane botrytis and spur blight. Plants were designated Hh (pubescent) or hh (glabrous) by visual inspection of cane morphology.

Disease screening

Four diseases Cane botrytis (*B. cinerea*), spur blight (*D. applanata*), cane spot (*E. veneta*) and yellow rust (*P. rubi-idaei*) were scored for all experimental plots in both trials on each plant. Detailed descriptions and images of the disease symptoms can be found in the Compendium of Raspberry and Blackberry Diseases and Insects (Ellis et al. 1991).

Disease symptom scoring All four diseases were scored for presence or absence. For cane botrytis and spur blight a continuous severity score was made based on the degree of disease spread in the canes (Williamson and Hargreaves 1981).

Cane botrytis Disease was scored three times (BS1, BS2, BS3) during mid-summer (July–August) in each of two seasons and later in winter (BA1, November–January). Disease was scored if pale brown lesions were present in summer and grey/whitish lesions, watermarking and sclerotia were present in winter. When cane blight was present in winter, the extent of disease spread in the canes in the winter scoring was also given a severity score from 1 to 4 where 4 was given for canes showing widespread lesions from top to bottom over the whole cane, 3 being half of the cane showing lesions, 2 being a few lesions and 1 being a small isolated lesion.

Spur blight Here again three scorings were carried out in two seasons, a summer scoring where chestnut brown lesions were scored on the primocanes below a node and around the axillary bud (DS1, DS2, DS3), and in winter silvered floricane lesions covered with black pseudothecia were recorded (DA1). Again a severity score of 1–4 was recorded for the disease spread in winter based on the number and extent of lesion spread over the canes.

Spur blight/cane botrytis disease complex Symptom expression for both cane botrytis and spur blight varied greatly with cane phenotype and in this segregating population, wide variation in cane phenotype has been observed. In order to avoid mis-scoring one plant as resistant to one disease but susceptible to the other, an overall disease score was recorded based on the presence of symptoms of either or both diseases. This approach is probably more valid than assessing each disease individually because a common resistance mechanism is known to operate against both pathogens that occupy the same ecological niche (Williamson and Jennings 1986). These are recorded as MS1-3 and MA1-3 summer and winter scorings, respectively.

Cane spot Scorings of disease symptom expression was carried out four times in autumn. For cane spot, elliptical cavities and deep sunken lesions that developed on the fruiting cane were recorded.

Rust The presence of rust was monitored on four dates beginning in May when yellow pustules (aecia) were first visible on the adaxial leaf surface through to August, when yellow uredinia were present on the abaxial leaf surface.

Statistical analysis

Genetic linkage map construction

JoinMap V2.0 (Stam and Van Ooijen 1995) was used to construct the linkage map. Segregation data from 453 markers (93 SSRs, 9 EST-SSRs and 351 AFLPs) (259 previously published) which were polymorphic in the Glen Moy × Latham parents were analysed. Linkage groups were separated at a LOD score of 7.0. Map distances were calculated using the Kosambi mapping function.

Analysis of phenotypic data

Cluster analysis of the disease measurements was used to explore the lines for major groupings of resistant and susceptible individuals. This was based on a cityblock measure of similarity between pairs, suitable for both 0–1 presence/absence data and 0–4 disease severity data (Gower 1985).

Each trait was then analysed for possible QTLs on the marker map. Most methods for QTL interval mapping assume that the phenotypes can be modelled as a mixture of normal distributions. However, such an analysis is not appropriate for diseases scored as presence/absence. The disease data were therefore analysed using a generalised linear model with binomial errors and a logit link function (McCullagh and Nelder 1989). For each date, the number of replicates showing infection was analysed, as a proportion of the replicates assessed. For rust and cane spot, an overall measure of disease was also formed as the number of infected replicates, summed over all the dates. While this overall measure violates the binomial assumption of independence, in that lines assessed as diseased on one date should be assessed as diseased on subsequent dates, it is informative about the total burden of disease for each line.

As the linkage map was quite dense, little information was lost by fitting the generalised linear model to each marker in turn and assessing which markers were most significant. However, care must be taken to use a suitable threshold for significance. One approach is to control the familywise type I error rate (FWER), the probability of erroneously rejecting the null hypothesis of no significant effect for any of the markers assessed (Lander and Kruglyak 1995). However, as the number of markers increases, the threshold for significance becomes more and more stringent and the power of the test to detect true effects is reduced. An alternative approach is to control the false discovery rate (FDR) (Benjamini and Hochberg 1995), which is defined as the expected proportion of true null hypotheses that are rejected. Simonsen and McIntyre (2004) carried out a simulation study to assess the power of various statistics for detecting multiple QTL and their ability to control the FDR.

Here two methods from Simonsen and McIntyre (2004) were used. The first, denoted H, is the original method of Benjamini and Hochberg (1995), and is implemented as follows. Let $P_{(i)}$, $i=1,\ldots,m$, be the ordered significances of the generalised linear model relating a phenotypic trait to the marker i. If k is the largest value of i such that

$$P_{(i)} \leq \frac{i\alpha}{m},$$

then the k values $P_{(1)},\ldots,P_{(k)}$ are declared significant. This controls the FDR at a level $\leq \alpha$. Here, a value $\alpha=0.05$ is used. This test is quite stringent, partly because if one marker is associated with a trait, then linked markers will also show associations.

The second approach is to Eliminate Linked Markers (ELM). In this case any marker linked to a marker already found to be significant is excluded from consideration. The ELM/G approach combines this with a geometric α spending function, where the ith ordered test is tested with probability $\alpha_i=\alpha/2^i$. This controls the FWER $\leq \alpha$. This approach was found to be less stringent than method H, but to give higher power for detecting QTLs (Simonsen and McIntyre 2004).

Typically this approach indicated one or two chromosomes where markers were linked to the trait. A generalised linear model was fitted for both markers and their interaction to test if this was significant. The same model was also fitted to 100 bootstrap replicate samples (Efron and Tibshirani 1993) to test whether the markers were consistently significant.

Results

SSR development

Seventy-five clones from the nebulised library hybridised to the AG oligomer and were sequenced. Primer pairs were designed to the flanking sequences of 16 of the repeat regions using Primer 3 and tested for polymorphism on the Glen Moy and Latham parents (Supplementary Data Table 1). Those showing polymorphism were used on the progeny for mapping, five of which were placed on the linkage map.

Pst genomic library

Of the 26 new primer pairs tested, 20 were heterozygous and suitable for mapping and 11 of these were confidently placed on the linkage map. The remaining six was homozygous or monomorphic (Supplementary Data Table 2).

A number of additional markers from Graham et al. (2004a) have now been placed on to the genetic linkage map through the addition of other newly developed markers. These were: Rub259f, LEAF102, FRUITE4 and FRUITG7.

Linkage map

The updated *Rubus* map (Fig. 1) has 349 markers and a total length of 669 cM. A further 55 markers are known to belong to the Latham map of linkage group 3, but are not shown here because of the difficulty of obtaining a reliable order with such densely spaced markers. Six linkage groups from the two parents can be paired by co-dominant and dominant bridging markers (segregating as aa:ab:bb, aa:b- or ac:ad:bc:bd). One group that was isolated on the previous map remained isolated at a LOD of seven, but joined another group at a LOD of six. However, there are still two groups, one from each

parent, that cannot be associated with each other or with any of the other groups, even at a much lower LOD score, and therefore there are eight linkage groups in total.

The gene controlling cane pubescence, gene *H*, was mapped to the Glen Moy parent on linkage group 2 at approximately 48 cM.

Disease scores

High disease incidence was recorded at both field sites for cane botrytis and spur blight whereas rust and cane spot were only identified at one site. Symptom expression for cane botrytis and spur blight varied among the progeny, thus the overall score for the disease complex is probably more accurate, as is the presence or absence score, rather than the assessment of disease severity which can be masked by cane morphology. A severity score was also attempted by Daubeny (1987); however, based on results over 2 years the low scores 1 and 2 were classed as resistant and scores of 3 and 4 as susceptible, low scores being inconsistent. Daubeny (1987) suggested from these data that two gene pairs were required for resistance, with the presence of at least two dominant genes necessary.

Cane spot symptoms were recorded as a presence and absence score, as only minor differences in the extent of disease symptoms on infected canes were observed. For rust, again a presence or absence score was given, as no evidence for any difference in disease severity on any of the progeny could be detected. However, the presence of rust on the Glen Moy parent was much more severe than that on any of the progeny where minor rust symptoms showing little variation across the progeny were seen.

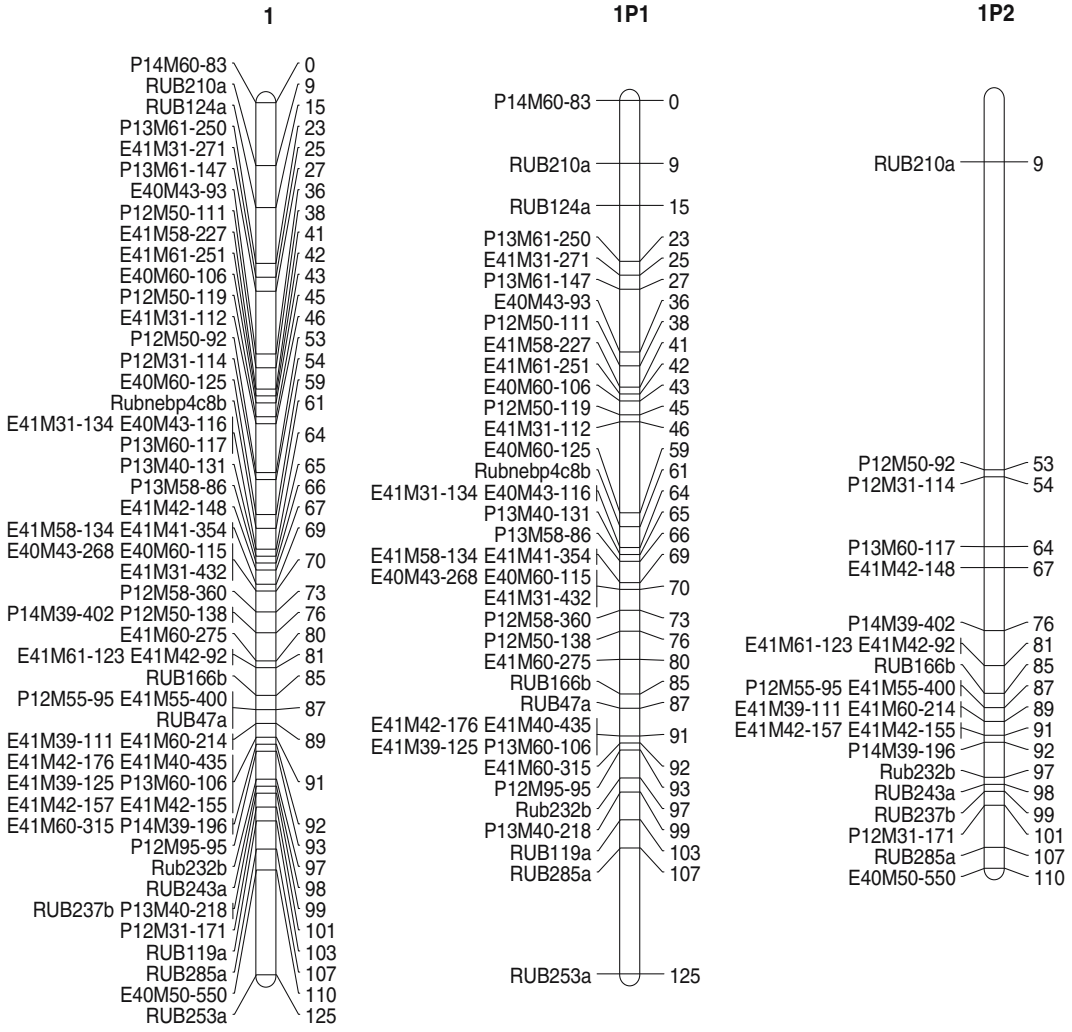


Fig. 1 Genetic linkage map of red raspberry showing the overall map and the maps for Latham (P1) and Glen Moy (P2). 1-LOD support intervals for the traits are shown by *bars*

Analysis of trait data

Cluster analysis of the offspring based on all the disease trait data (using average linkage cluster analysis and a cityblock measure of similarity) showed that the offspring fell into two separate groups (Fig. 2). A chi-squared test of independence was used to investigate which of the disease measurements was associated with this separation: this showed that there were no significant differences between the two groups of the offspring regarding presence of cane spot or rust, but that there were highly significant differences for all the measurements of cane botrytis and spur blight apart from the final summer scoring of overall disease, MS3. The cluster analysis was repeated using the data on cane botrytis and spur blight only. The 48 individuals in the upper cluster tend to show botrytis and/or spur blight in most of the disease assessments, while the 46 individuals in the lower cluster are less likely to show the disease.

Relationship between trait and marker data

Cane spot

A generalised linear model analysis of the presence/absence of cane spot identified two locations of the genome as containing possible QTLs affecting cane spot, close to 95 cM on linkage group 2 (alleles from Latham) and close to 21 cM on linkage group 4 (alleles from Glen Moy). The results are summarised in Table 1. The more stringent criterion, H, identified markers from linkage group 2 for date 2 and overall cane spot, and bootstrap analysis confirmed the significance of these. Markers on linkage group 2 were identified for the other dates, but

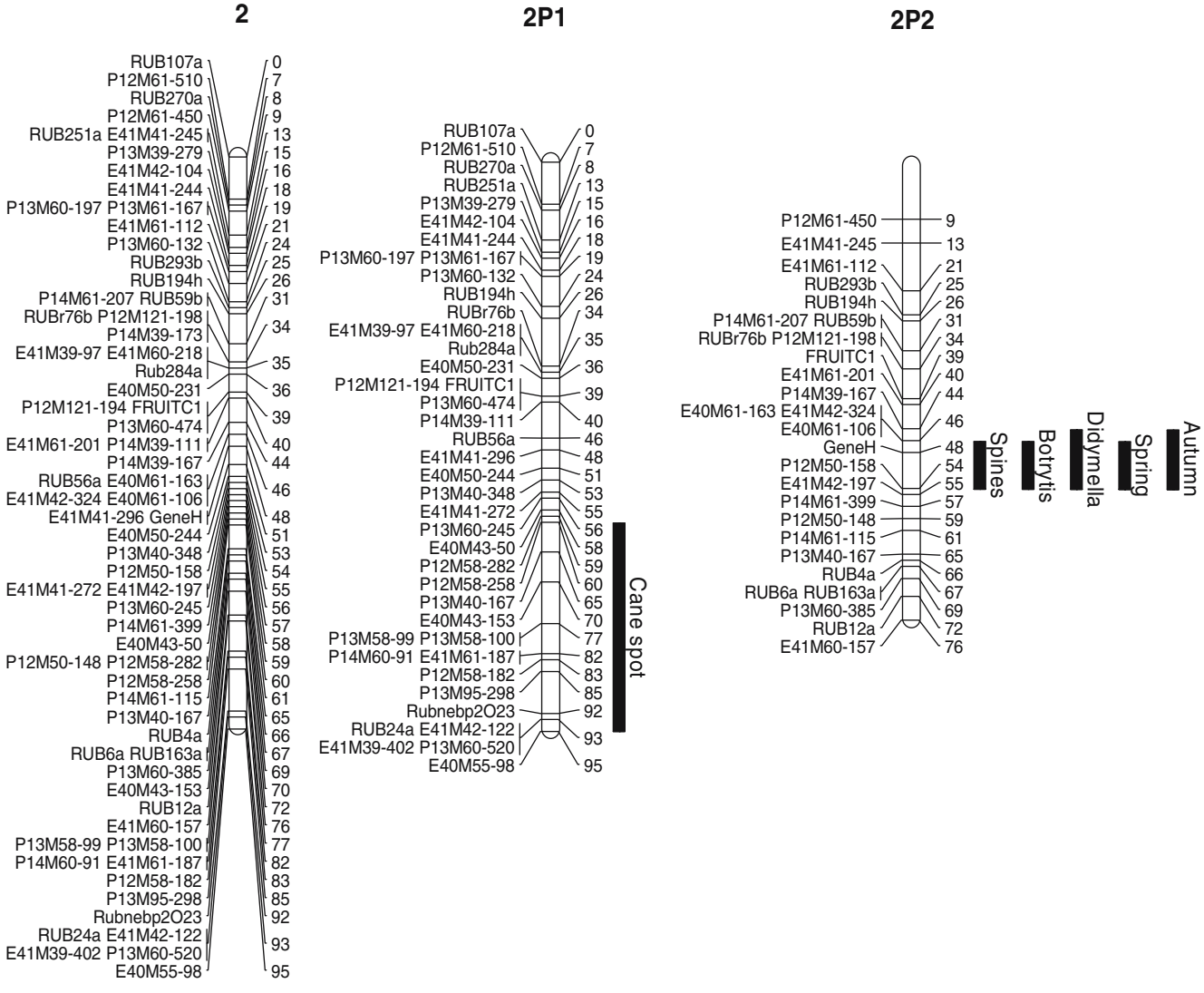
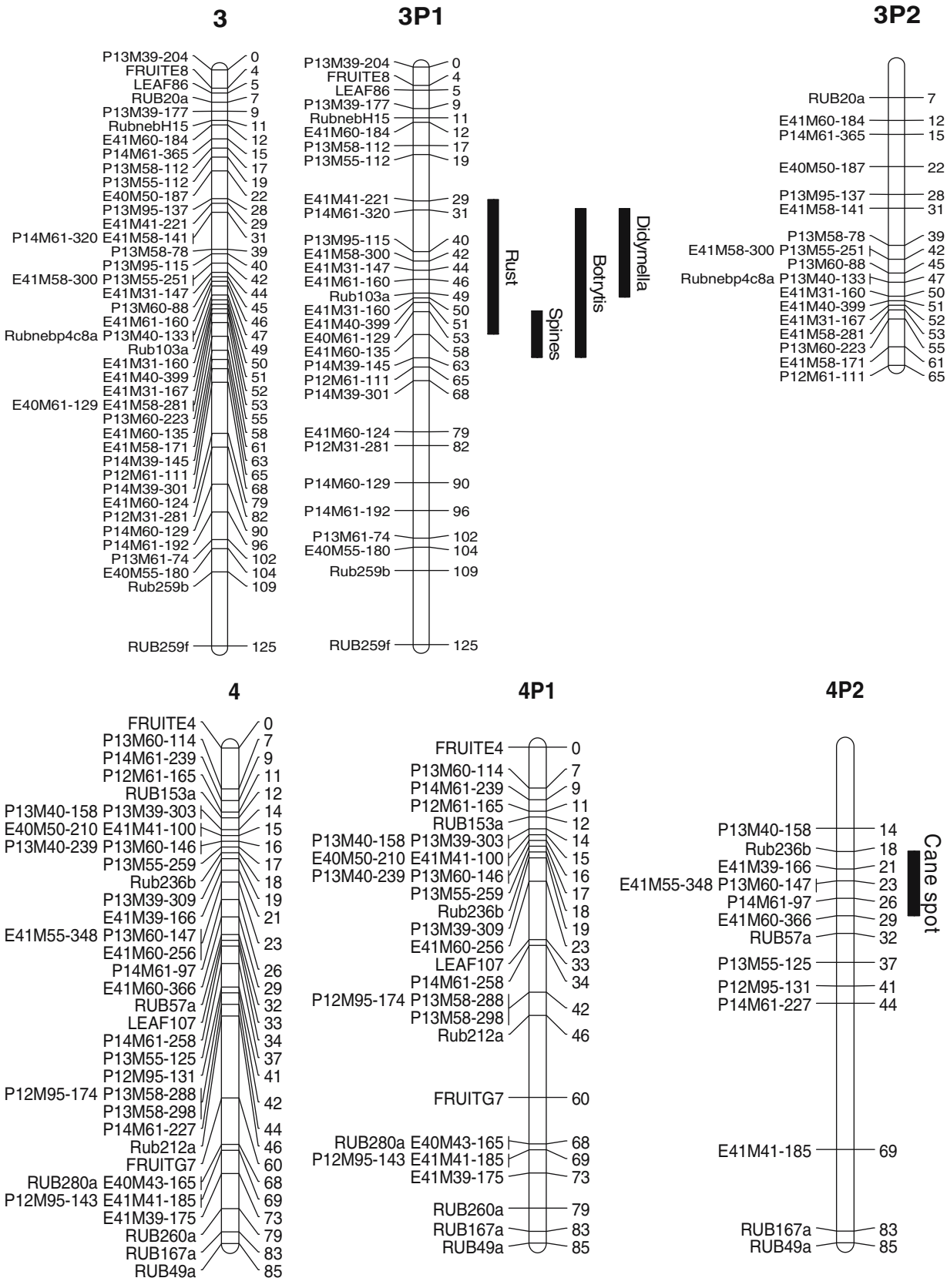


Fig. 1 (Contd.)


3
P13M39-204 0
FRUITE8 4
LEAF86 5
RUB20a 7
P13M39-177 9
RubnebH15 11
E41M60-184 12
P14M61-365 15
P13M58-112 17
P13M55-112 19
E40M50-187 22
P13M95-137 28
E41M41-221 29
P14M61-320 E41M58-141 31
P13M58-78 39
P13M95-115 40
E41M58-300 P13M55-251 42
E41M31-147 44
P13M60-88 45
E41M61-160 46
Rubnebp4c8a P13M40-133 47
Rub103a 49
E41M31-160 50
E41M40-399 51
E41M31-167 52
E40M61-129 E41M58-281 53
P13M60-223 55
E41M60-135 58
E41M58-171 61
P14M39-145 63
P12M61-111 65
P14M39-301 68
E41M60-124 79
P12M31-281 82
P14M60-129 90
P14M61-192 96
P13M61-74 102
E40M55-180 104
Rub259b 109
RUB259f 125
3P1
P13M39-204 0
FRUITE8 4
LEAF86 5
P13M39-177 9
RubnebH15 11
E41M60-184 12
P13M58-112 17
P13M55-112 19
E41M41-221 29
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P14M39-301 68
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P14M60-129 90
P14M61-192 96
P13M61-74 102
E40M55-180 104
Rub259b 109
RUB259f 125
Rust
Spines
Botrytis
Didymella
3P2
RUB20a 7
E41M60-184 12
P14M61-365 15
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P13M95-137 28
E41M58-141 31
P13M58-78 39
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E41M31-167 52
E41M58-281 53
P13M60-223 55
E41M58-171 61
P12M61-111 65
4
FRUITE4 0
P13M60-114 7
P14M61-239 9
P12M61-165 11
RUB153a 12
P13M40-158 P13M39-303 14
E40M50-210 E41M41-100 15
P13M40-239 P13M60-146 16
P13M55-259 17
Rub236b 18
P13M39-309 19
E41M39-166 21
E41M55-348 P13M60-147 23
E41M60-256
P14M61-97 26
E41M60-366 29
RUB57a 32
LEAF107 33
P14M61-258 34
P13M55-125 37
P12M95-131 41
P12M95-174 P13M58-288 42
P13M58-298
P14M61-227 44
Rub212a 46
FRUITG7 60
RUB280a E40M43-165 68
P12M95-143 E41M41-185 69
E41M39-175 73
RUB260a 79
RUB167a 83
RUB49a 85
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Rub236b 18
P13M39-309 19
E41M60-256 23
LEAF107 33
P14M61-258 34
P12M95-174 P13M58-288 42
P13M58-298
Rub212a 46
FRUITG7 60
RUB280a E40M43-165 68
P12M95-143 E41M41-185 69
E41M39-175 73
RUB260a 79
RUB167a 83
RUB49a 85
4P2
P13M40-158 14
Rub236b 18
E41M39-166 21
E41M55-348 P13M60-147 23
P14M61-97 26
E41M60-366 29
RUB57a 32
P13M55-125 37
P12M95-131 41
P14M61-227 44
E41M41-185 69
RUB167a 83
RUB49a 85
Cane spot

Fig. 1 (Contd.)

only using the less stringent ELM/G criterion. Markers from linkage group 4 were also identified using the ELM/G criterion: bootstrap analysis confirmed the significance of marker E41M39-166 on linkage group 4 for date 1. A further location at 44 cM on linkage group 3 is also indicated for date 4, but only by the less stringent ELM/G criterion. When the most significant markers from linkage groups 2 and 4 were analysed together, both were significant but there was no significant interaction between them. Table 2 shows the probabilities of cane spot given the genotype at the most significant markers, based on the overall data.

Rust

A generalised linear model for the presence/absence of rust indicated associations with markers from Latham on linkage groups 3 and 5, as summarised in Table 3. The most significant markers were on linkage group 3, but the location of the most significant marker varied with the date on the measurement, from 29 to 58 cM. There was also an indication of an association with markers from Latham, linkage group 5. This was significant by the Benjamini–Hochberg criterion H for date 2 and for the overall analysis, and significant by the less stringent ELM/G for date 3. When the most significant markers from both linkage groups 3 and 5 were fitted together, there was a significant interaction ($P = 0.026$). However, only the marker from linkage group 3 was consistently significant following bootstrap analysis. The predicted probabilities of developing rust are shown in Table 4. There is a low probability of developing rust for lines carrying genotype aa at marker E41M31-147, regardless of the genotype at P13M39-147. However, for lines with genotype ab at marker E41M31-147 there is a significantly higher probability of developing the disease if the line also has genotype ab at marker P13M39-147.

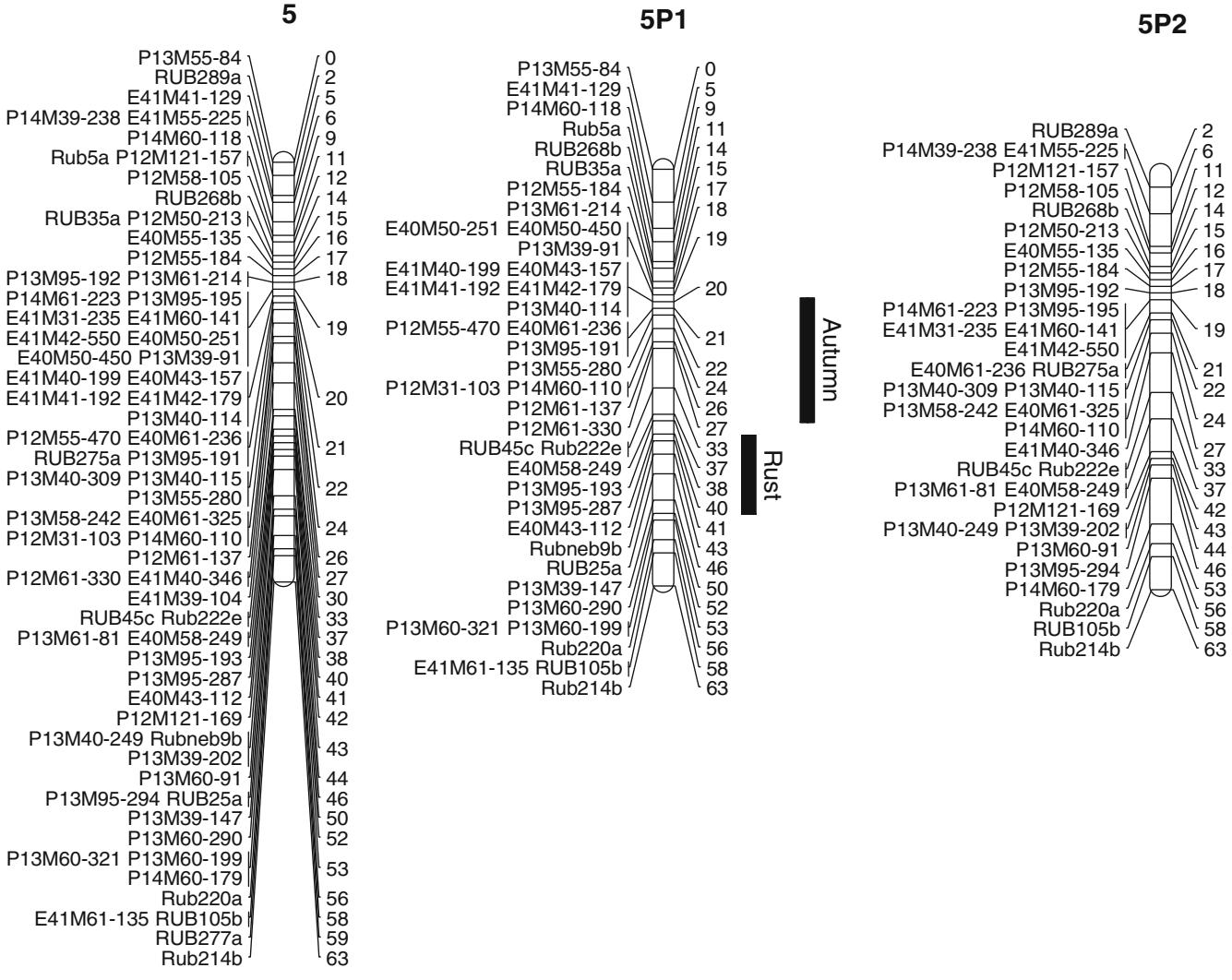

Fig. 1 (Contd.)

Overall botrytis/spur blight

The cluster analysis gave an overall division of the offspring into resistant/susceptible to botrytis and/or spur blight. Chi-squared tests of independence were used to test for associations between this grouping from cluster analysis and the molecular markers. Most of the markers on linkage group 2 from Glen Moy were identified by the H criterion as showing highly significant associations with the grouping, but the most significant association was with the 'hairy' gene, gene *H* ($\chi_1^2 = 51.2$, $P < 0.001$). Of the 48 individuals in the upper cluster, which were mainly susceptible, 42 had the non-hairy homozygous genotype *hh*, while 39 out

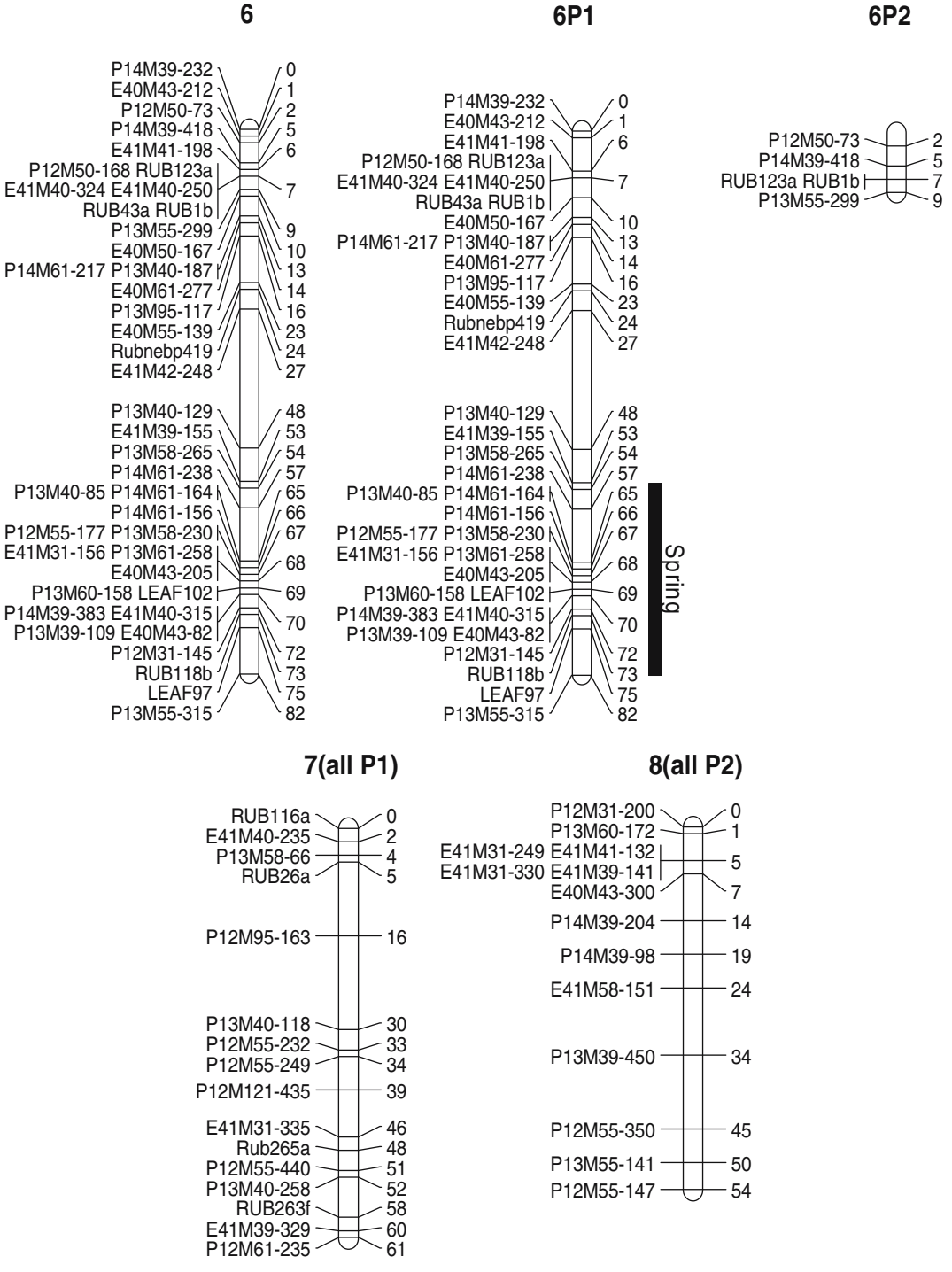

Fig. 1 (Contd.)

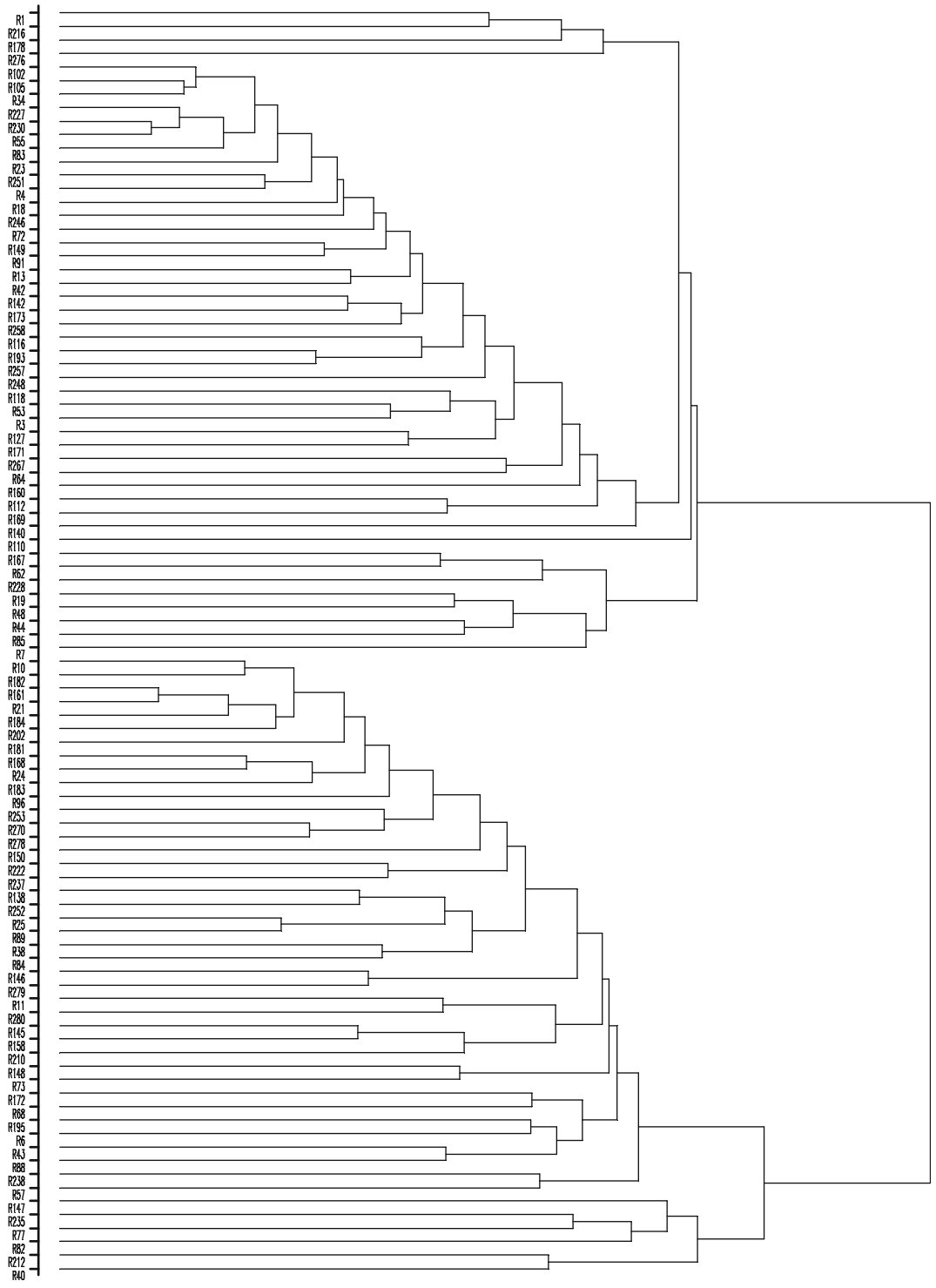

Fig. 2 Cluster analysis of the disease data demonstrating an overall division of the offspring into resistant/susceptible to botrytis and/or spur blight. The 48 individuals in the upper cluster tend to show botrytis and/or spur blight in most of the disease assessments, while the 46 individuals in the lower cluster are less likely to show the disease

of 45 individuals in the lower cluster (which showed more resistance) had the hairy heterozygous *Hh* genotype. (The gene *H* status for one individual could not be determined.)

Individual measurements of botrytis and spur blight

As above, generalised linear models were used to relate the presence/absence of botrytis and spur blight to the

Table 1 Significant associations of markers with cane spot measurements

Trait	H		ELM/G		Bootstrap
	Marker	LG	Marker	LG	
Date 1	–	–	E41M39-166	4, M, 21	E41M39-166
			P13M95-298	2, L, 85	
Date 2	E40M55-98	2, L, 95	–	–	E40M55-98
Date 3	–	–	E41M42-122	2, L, 93	
			E41M39-166	4, M, 21	
Date 4	–	–	E41M42-122	2, L, 93	
			E41M31-147	3, L, 44	
Overall	E41M42-122	2, L, 93	P13M60-147	4, M, 23	E41M42-122

H shows the most significant marker association by the Benjamini–Hochberg criterion H, using an FDR of 0.05. ELM/G shows any further markers added by the ELM/G criterion. Bootstrap indicates which of these markers are significant in 95% of bootstrap resamples. For the linkage groups: *L* Latham, *M* Glen Moy. The position is shown in centiMorgan

Table 2 Probability of overall cane spot presence, given the marker genotypes at E41M42-122 on LG2 and P13M60-147 on LG4

		LG4: P13M60-147	
		aa Probability	ab Probability
LG2: E41M42-122	aa	0.45 (0.071)	0.25 (0.054)
	ab	0.74 (0.054)	0.54 (0.066)

Standard errors are shown in brackets

marker data, for each measurement in turn. Table 5 shows the significance of gene *H* and those markers that were significant by the H criterion, the ELM/G criterion and by bootstrapping. Apart from spring scoring MS3, all measurements of cane botrytis and spur blight showed highly significant associations with gene *H*, with low disease scores for the *Hh* genotypes and higher disease for the *hh* genotypes. Gene *H* is clearly a major gene affecting resistance to both diseases: in order to test for further genes, the presence of disease was modelled for each other marker *i* in turn as a function of the genotype at gene *H*, the genotype at marker *i* and an interaction between the two genotypes.

Unlike gene *H*, most of the selected markers were from Latham and from linkage groups 3, 5 and 6. There were no markers clearly associated with cane botrytis only or spur blight only, but we note that the markers from linkage group 5 were significant only for measurements made in autumn and not for any of the measurements made in spring. The markers from linkage groups 3 and 5 had additive effects, while the markers selected from linkage group 6 for spring disease scores MS1 and MS2 interacted with gene *H*, as shown in Table 6. For MS1 measurement, the lines with genotype *aa* at LEAF102 showed a much great difference between gene *H* genotypes in the probability of showing disease than the lines with genotype *ab* at LEAF102. The MS2 measurements showed a similar pattern, but the most significant association was with LEAF97 in this case (Table 6).

Spines

In view of the changes to the map, the spines data presented in Graham et al. (2004a) were re-analysed. None of the offspring showed the spine-free phenotype of Glen Moy, but the degree of spininess varied and was scored on a 1–5 scale. A linear model was used to relate the average spine score across the six replicates to the marker data. The most significant marker was gene *H* ($P < 0.001$), explaining 36.0% of the trait variance. As

Table 3 Significant associations of markers with rust measurements

Trait	H		ELM/G		Bootstrap
	Marker	LG	Marker	LG	
Date 1	–	–	–	–	–
Date 2	E41M41-221	3, L, 29	–	–	E41M41-221
	P13M39-147	5, L, 50	–	–	–
Date 3	E41M60-135	3, L, 58	E40M50-450	5, L, 19	E41M60-135
Date 4	E41M31-147	3, L, 44	–	–	E41M31-147
Overall	E41M31-147	3, L, 44	–	–	E41M31-147
	P13M39-147	5, L, 50	E40M50-550	1, M, 110	–

H shows the most significant marker association by the Benjamini–Hochberg criterion H, using an FDR of 0.05. ELM/G shows any further markers added by the ELM/G criterion. Bootstrap indicates which of these markers are significant in 95% of bootstrap resamples. For the linkage groups: *L* Latham, *M* Glen Moy. The position is shown in centiMorgan

Table 4 Overall probability of developing rust, given the marker genotypes at E41M31-147 on LG3 and P13M39-147 on LG5

		LG5: P13M39-147		
		aa Probability	ab Probability	Combined Probability
LG3: E41M31-147	aa	0.09 (0.028)	0.05 (0.029)	0.07 (0.021)
	ab	0.26 (0.056)	0.50 (0.048)	0.39 (0.038)

Standard errors are shown in brackets

for botrytis and spur blight, every other marker was tested for a significant effect in addition to the effect of gene *H* and a significant interaction with gene *H*. The results are shown in the last line of Table 5. Two further markers were identified, both from Latham. One, E41M60-135, was from linkage group 3, in an area also associated with resistance to botrytis and spur blight and close to an area associated with rust. The other, P12M58-282, was on linkage group 2, in a location close to that of gene *H*. However, further markers bridging the parental maps would be necessary to tell if there is a single locus on linkage group 2, with alleles from both parents affecting the degree of spininess, or two loci. There was some evidence for an interaction between gene *H* and P12M58-282 ($P = 0.03$) but this interaction was not consistently present in bootstrap samples. Table 7 shows the predicted degree of spininess using the main effects of gene *H*, P12M58-282 and E41M60-135.

Discussion

Fruit breeders have limited resources and rarely include a primary screen for fungal diseases in their selection process (Jennings, personal communication). The development of a saturated robust genetic linkage map is a major step in moving towards a marker-assisted breeding programme. The existing map has been revised through the addition of further 20 SSR markers, 5 from the nebulised library, 11 from the *Pst*I library and these markers brought in an additional four markers that could not previously be placed. Six groups from the parents can now be paired; the seventh group still remains to be completed. Map locations associated with resistance have been identified for all four diseases investigated.

The map location for gene *H* has been determined on linkage group 2 and associations with resistance to the cane botrytis and spur blight complex confirmed. In contrast to cane botrytis and spur blight, no significant associations between gene *H* and rust or cane spot were determined. Most markers on linkage group 2 from cv. Glen Moy, the resistant parent, showed high associations with botrytis and spur blight, but the most significant association was with gene *H* itself. For individual disease scores gene *H* was again highly significant.

A factor from Latham was identified on linkage group 3 which is associated with resistance to rust as well as resistance to spur blight, botrytis and increased number of spines. For rust the most significant markers were from Latham, the resistant parent, and situated on linkage group 3 with marker E41M31-147 at 44 cM having the most significant association. Anthony et al. (1986) have studied the inheritance of complete and incomplete resistances to rust in a half diallel cross including Boyne, which derives complete resistance from Latham. They found that crosses of Boyne to susceptible varieties all segregated for complete resistance and proposed that Boyne was heterozygous for a single resistance gene, designated *Yr*, which was derived from Latham. As the Latham × Glen Moy population also segregates for resistance to rust, we propose that Latham is also heterozygous for *Yr*, and that this lies on linkage group 3 close to E41M31-147. Anthony et al. (1986) also found variation in the degree of susceptibility among offspring of Boyne without complete resistance

Table 5 Analysis of the measurements of the presence of botrytis and spur blight

Trait	Disease	Season	Gene *H* dev ratio	Other markers
MA1	Mixed	Autumn	200.9***	P12M61-111, LG3, 3:1, 65^{B}, RUB59b, LG2, M, 31
MA2	Mixed	Autumn	24.1***	P12M61-330, LG5, L, 27^{B}
MA3	Mixed	Autumn	17.2***	P12M61-137, LG5, L, 26^{B}
MS1	Mixed	Spring	89.6***	LEAF102, LG6, L, 21^{BI}
MS2	Mixed	Spring	43.0***	LEAF97, LG6, L, 27^{BI}, P13M55-299, LG6, M, 9^{I}
MS3	Mixed	Spring	4.3*	–
BS1, DS1	Mixed	Spring	10.9***	–
BS2	Botrytis	Spring	53.0***	P14M61-238, LG6, L, 9
BS3	Botrytis	Spring	49.9***	E41M60-135, LG3, L, 58^{HB}
BA1	Botrytis	Autumn	19.0***	E41M41-129, LG5, L, 5^{B}
DS2	Spur blight	Spring	24.8***	–
DS3	Spur blight	Spring	18.0***	P13M95-115, LG3, L, 40^{B}, P13M61-250, LG1, L, 23
DA1	Spur blight	Autumn	54.2***	P14M39-383, LG6, L, 19^{B}
Spines			52.7***	E41M60-135, LG3, L, 58^{HB}, P12M58-282, LG2, L, 59^{HB}

Apart from gene *H*, the markers shown were generally selected by the ELM/G criterion. ^{H} indicates that the marker was also selected by the H criterion; ^{B} indicates that the marker was consistently significant in bootstrap resamples; ^{I} indicates that there was a significant interaction with gene *H*

*$P < 0.05$, ***$P < 0.001$

Table 6 Probability of disease at scorings MS1 and MS2, as a function of the genotypes at gene *H* and LEAF102, LEAF97, respectively

Gene *H*	MS1: LEAF102		MS2: LEAF97	
	aa	ab	aa	ab
Hh	0.87 (0.041)	0.65 (0.051)	0.90 (0.043)	0.76 (0.048)
hh	0.10 (0.035)	0.25 (0.052)	0.28 (0.056)	0.55 (0.069)

Standard errors are shown in brackets

Table 7 Predicted degree of spininess, given the genotypes at Gene *H*, P12M58-282 and E41M60-135

		LG3: E41M60-135	aa Spines	ab Spines
LG2:	Gene *H*	P12M58-282		
	aa	aa	2.4 (0.11)	2.0 (0.11)
		ab	2.1 (0.11)	1.7 (0.10)
	ab	aa	3.3 (0.11)	2.9 (0.12)
		ab	2.9 (0.11)	2.5 (0.11)

Standard errors are shown in brackets

and concluded Boyne to also be a source of incomplete resistance. In the Latham × Glen Moy cross, there is some evidence, although not highly significant, for a gene on linkage group 5, also from Latham, affecting the susceptibility of the offspring that do not carry the 'resistant' allele on linkage group 3 and this needs to be investigated further in the whole population as a possible source of incomplete resistance. This area on LG5 is also implicated in spur blight/botrytis resistance. There was no evidence, however, of gene *H* being related to incomplete resistance in this cross. None of the offspring were as susceptible to rust as the Glen Moy parent. One explanation of this is that there is another resistance gene, for which Latham is homozygous RR and Glen Moy is rr. In this case all offspring would be Rr and so more resistant than Glen Moy, but this gene would not segregate in this particular cross and cannot be mapped. It may be that the presence of this R gene overcomes any effect of gene *H*. We aim to investigate this by intercrossing the offspring to see if a further resistance gene can be mapped.

Gene *H* and a marker from LG3 associated with resistance to rust were also associated with spines. Glen Moy is spine-free, with genotype *ss* at the gene S for spininess, but all offspring have spines to some extent suggesting that Latham is *SS*. If Latham was *Ss* then offspring would segregate as in Glen Prosen by Glen Clova cross in Anthony et al. 1986. Therefore gene *S* cannot be mapped in our population. We can, however, map variation in the number of spines and this association with Gene *H* agrees with that found by other researchers (Jennings 1988). Hairs and spines are both outgrowths of epidermal cells and their early development is inter-related, hence such an association is not unexpected (Peitersen 1921). It does seem likely therefore that the gene itself is having some effect on resistance to *Didymella* and *Botrytis* and also spine number by its action early in development affecting several cell characteristics. Jennings (1982b) found that water run-off was more rapid on hairy canes than non-hairy ones and had suggested that bud infection therefore occurred less often on canes with hairs. However, by direct inoculation of cane tissues the effects on disease symptom expression could be demonstrated up to a year after infection. For cane spot again, no association with gene *H* could be detected; however, markers on linkage group 2 from Latham were shown to be highly significant. Markers from the Glen Moy parent on linkage group 4 were also identified. Jennings and McGregor (1988) scored the degree of cane spot in 15 segregating families using a 0–5 scale, which equated to a range of 093 spots per cane. Resistance to rust was highly correlated with resistance to cane spot in five of these families. However, none of these families had the complete resistance to rust derived from Latham. There is no evidence of any relationship between resistance to rust and resistance to cane spot in our data.

We now intend to saturate the map region surrounding gene *H* with RAPD and AFLP markers by creating bulks of additional progeny with the *Hh* and *hh* genotypes. Any markers identified as being close to gene *H* will be used to screen clones from a large insert library constructed from Glen Moy (Graham et al. 2004b) allowing us to clone gene *H*. Additionally further progeny will be screened for the diseases to examine regions of interest in more detail.

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