

# Detection of favorable alleles for plant height and crown rust tolerance in three connected populations of perennial ryegrass (*Lolium perenne* L.)

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**Abstract** Plant height, which is an estimator of vegetative yield, and crown rust tolerance are major criteria for perennial ryegrass breeding. Genetic improvement has been achieved through phenotypic selection but it should be speeded up using marker-assisted selection, especially in this heterozygous species suffering from inbreeding depression. Using connected multiparental populations should increase the diversity studied and could substantially increase the power of quantitative trait loci (QTL) detection. The objective of this study was to detect the best alleles for plant height and rust tolerance among three connected populations derived from elite material by comparing an analysis per parent and a multipopulation connected analysis. For the studied traits, 17 QTL were detected with the analysis per parent while the additive and dominance models of the multipopulation connected analysis made it possible to detect 33 and 21

QTL, respectively. Favorable alleles have been detected in all parents. Only a few dominance effects were detected and they generally had lower values than the additive effects. The additive model of the multipopulation connected analysis was the most powerful as it made it possible to detect most of the QTL identified in the other analyses and 11 additional QTL. Using this model, plant growth QTL and rust tolerance QTL explained up to 19 and 38.6% of phenotypic variance, respectively. This example involving three connected populations is promising for an application on polycross progenies, traditionally used in breeding programs. Indeed, polycross progenies actually are a set of several connected populations.

## Abbreviations

PH Plant height  
PGR Plant growth rate

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## Introduction

Perennial ryegrass (*Lolium perenne* L.) is the most widely sown perennial forage grass in temperate regions of the world. It is of interest because it is fast-growing, has high nutritional value and has good persistency under grazing (Potter 1987; Wilkins 1991). Animal production is determined by the biomass available in pastures (McGilloway et al. 1999; Barre et al. 2006). Hence, improving vegetative yield is a major challenge. Genetic improvement, through phenotypic selection, has been obtained for annual herbage yield but is mainly due to an increase in yield in summer and autumn, rather than in spring (Sampoux et al. 2011). Moreover, herbage yield, herbage intake and forage quality

are dramatically affected by crown rust which is caused by *Puccinia coronata* Corda f. sp. *lolii* (Smit 2005). Herbage yield and rust tolerance are the main selection criteria for perennial ryegrass breeders.

Herbage yield is traditionally measured on sward, i.e. plots of about 10 m<sup>2</sup>, for at least 2 years. This measurement requires a large number of seeds, is expensive and time consuming. Therefore, efforts have been made to use different indirect measurements of herbage yield based on morphological traits. Studies on fescue and ryegrass species have shown that under infrequent cutting, herbage yield was correlated with plant characteristics such as leaf length and leaf elongation rate (LER) (Rhodes 1969, 1971; Horst et al. 1978; Reeder et al. 1984). Moreover, selection for lamina length in nurseries of spaced plants can be an effective means of modifying lamina length in dense canopies (Rhodes and Mee 1980; Hazard et al. 1996; Hazard and Ghesquière 1997). On spaced plant design, Barre et al. (2009) reported a correlation of 0.8 between lamina length and stretched plant height (PH) on 185 individuals coming from a cross between a short- and a long-leaved genotype of perennial ryegrass. Hence, PH measured in nurseries of spaced plants can be used to estimate herbage yield.

Like the majority of outbreeding species, which are often highly heterozygous, perennial ryegrass breeding is limited by several constraints. To cope with inbreeding depression and the inability to achieve large scale controlled crosses, perennial ryegrass breeders create synthetic varieties. Synthetic varieties are artificial populations resulting from the multiplication in a free pollination way of a given number of parents (from four to several hundred) chosen by phenotypic selection for their own agronomic value and their aptitude to combination (Gallais 1990; Bourdon et al. 2005). The high diversity level maintained in synthetic varieties results in slow genetic progress (Gallais 1990). To overcome this limitation, a decrease in the number of parents used in the polycross could be considered but inbreeding depression would severely reduce the agronomic value of the progeny. Marker-assisted selection (MAS) could be an interesting alternative as it should make it possible to increase the frequencies of favorable alleles without decreasing the overall diversity of the variety. The first step to use MAS is to identify regions of the genome involved in the variation of quantitative traits (QTL: quantitative trait loci), and more specifically the alleles that confer positive value to the traits.

QTL for traits linked to herbage yield (Table 1) and for rust tolerance (Dracatos et al. 2010) have been identified in perennial ryegrass. These studies highlight the fact that traits linked to herbage yield such as leaf and lamina length, LER and PH, are controlled by many genes with weak effects and with strong genotype x environment interactions. Concerning rust tolerance, both major and

minor QTL have been identified on all seven *Lolium* linkage groups (LG).

Most QTL studies are carried out on biparental populations, i.e. F1-derived populations. The advantage of the latter is that they offer the possibility of scanning the whole genome with a limited number of markers due to high linkage disequilibrium (LD). However, these populations are not easy to produce and their variability is low compared to synthetic varieties that are produced from many parents. Recently, to increase genetic diversity under study while keeping a high LD, it has been proposed to build crossing designs composed of biparental populations that are connected by common parents (Blanc et al. 2006). The main interest of detecting QTL in these connected multiparental populations is to extend the genetic diversity within the crossing design and the opportunity to compare the alleles of different parents within a single model.

There are different models for QTL detection in heterozygous plants. The first one is a model per parent which compares the two alleles of each parent in a biparental cross which corresponds to the pseudo-testcross strategy (Grattapaglia et al. 1995). It can be performed using any software for QTL detection which generally includes analyses for backcross such as QTL cartographer (Basten et al. 2007). Another model takes into account the four genotypic classes per locus expected in a biparental cross between heterozygous plants. This model can be used with MapQTL (Van Ooijen et al. 2002) or with the single-population analysis of the software MCQTL (Jourjon et al. 2005). Finally, all the parents of a connected multiparental population can be studied within a single model (MCQTL) that detects the QTL explaining a significant part of variation in the whole population. Using this method, Blanc et al. (2006, 2008) detected a higher number of QTL in the connected analysis than in single-population analyses. However, it has been reported that some QTL detected in the single-population analysis were not recovered in the multipopulation analysis (Billotte et al. 2010). This may be attributable to a small population size or to a dilution effect (Billotte et al. 2010). This dilution effect has also been reported for QTL with small effects by Pierre et al. (2008) in *Medicago truncatula* lines using QTL cartographer and MCQTL.

The multiparental connected populations thus combine the advantage of wide diversity and powerful QTL detection. Consequently, using these populations would seem to be promising for the creation of synthetic varieties with a MAS approach. The objective of this study was to detect the best alleles for PH and rust tolerance among elite plants of perennial ryegrass using large connected populations and comparing two QTL detection methods: the pseudo-testcross strategy using QTL cartographer and the multipopulation connected analysis of MCQTL. Mapping

**Table 1** Summary of the QTL related to vegetative yield referenced in the literature

References	Traits	Linkage groups (LG)							Range of phenotypic variation explained	
Yamada et al. (2004)	Leaf length	5							5.4–38.0	
Armstead et al. (2008)		7								
Barre et al. (2009)	Plant height	1	2	4			6	7	5.3–43.0	
Kobayashi et al. (2011)		1	2	3	4	5	6	7		
Sartie et al. (2011)		1	2	3	4	7				
Yamada et al. (2004)		1	3							
Turner et al. (2008)		1	4			5				
Studer et al. (2008b)		1	2	3						7
Barre et al. (2009)		1	2	4		5	7			
Kobayashi et al. (2011)	1	2	3	4	5	6	7			
Turner et al. (2008)	Leaf elongation rate (LER)	2		3					11.1–13.6	
Barre et al. (2009)		4								
Sartie et al. (2011)		1	3			6		7		
Yamada et al. (2004)	Fresh weight	4			5					3.7–29.5
Turner et al. (2008)		1	4		5					
Anhalt et al. (2009)	Dry weight	2		3	5		7		3.6–32.6	
Anhalt et al. (2009)		2		3	5		7			
Sartie et al. (2011)		1	2					6		
Turner et al. (2008)	Dry matter	3							6.0–11.1	
Anhalt et al. (2009)		2	3	7						

populations and methods employed in this study are discussed for synthetic variety MAS.

## Materials and methods

### Plant material

Three elite genotypes (Nemo A, Nemo B, and Nemo H) were crossed with the same elite plant, Nemo F, considered as a “tester”, to produce three connected mapping populations, i.e. three full-sib families with Nemo F as a common parent. The four plants were chosen in 2005 in a nursery of the forage breeding program of the French company Jouffray-Drillaud. They belong to the late maturity group and were selected for vegetative yield, rust tolerance and forage quality. They were not related except for Nemo B and Nemo F which had the same mother plant. The plants were cloned and crosses were made in 2006. For each cross, a pollen-proof bag was set on the spikes of both parents to avoid contamination by foreign pollen. Due to self-incompatibility, self-pollination was expected to be limited. For each cross, seeds were separately harvested on each parent.

### Field experiment

For each cross, seeds harvested on each parent were sown in a greenhouse to obtain one hundred plants per parent. In spring 2007, 200 plants per progeny were transplanted in a nursery of spaced plants with 0.45 m between plants at the experimental station of Jouffray-Drillaud (Saint Sauvant, La Litière 46°23'N 0°05'E). For each cross, the 200 plants were distributed into five plots of 20 individuals originating from one mother plant and five plots of 20 individuals originating from the other mother plant. The 30 plots including all crosses were completely randomized. Each genotype was represented by a single plant following the recommendation of Knapp and Bridges (1990) and Moreau et al. (2000) who showed that it is more efficient to increase the population size rather than the accuracy of phenotypic evaluation for QTL detection. The parents were not available to be included in the experimental trial. The plants were cut to approximately 5 cm height on 20 September and 16 November 2007, and 14 March, 26 June and 26 November 2008, and 24 March 2009. Fifty kg/ha of nitrogen were applied on the nursery after each cut.

Crown rust susceptibility was scored once in summer 2007 by a single person after a natural infection, with a

1–9 scale where one is for a plant without symptoms and nine is for a highly susceptible plant based on the density of rust pustules on leaves. Rust susceptibility was not scored in the following seasons due to a lack of fungus attack.

Stretched plant height (PH) was measured using a modified HerboMETRE® (ARVALIS, Institut du Végétal) which made it possible to stretch the plant leaves and to record plant height from the ground to the top of the longest leaves. PH was measured on 19 September and 15 November 2007, and once a week from 27 March to 2 May, and 25 November 2008 and 6 May 2009.

### Genotypic data

For DNA extraction, leaves were collected in the field and stored at  $-80^{\circ}\text{C}$ . DNA was extracted using the large-scale DNA extraction protocol (CIMMYT 2005) adapted for 96-well plates.

The markers used were SSR from various sources (Jones et al. 2001; Kubik et al. 2001; Faville et al. 2004; Jensen et al. 2005b; Lauvergeat et al. 2005; Saha et al. 2005; Gill et al. 2006; Hirata et al. 2006; Studer et al. 2008a; Barre et al. 2009) except for the OSW marker which is an STS marker (Lem and Lallemand 2003). The four parents were genotyped using 283 markers to select, within each cross, markers (1) well spread over the genome, (2) heterozygous in at least one parent and (3) exhibiting different genotypes between the two parents. As the maps were not saturated with codominant markers, AFLP markers were added. *EcoRI/MseI* enzyme combination was used and eight primer pairs per cross generated a various number of markers per cross (Supplementary Material 1). Finally, the number of markers used for mapping was 58, 66 and 57 for Nemo A  $\times$  Nemo F, Nemo B  $\times$  Nemo F and Nemo H  $\times$  Nemo F, respectively, including 21 SSR or STS markers genotyped in the three mapping populations.

For all markers, PCR amplification was done on PTC-100, PTC-200 (MJ Research) or on Tetrad (Biorad) thermocyclers. For STS and SSR markers, a similar protocol as Barre et al. (2009) was used but with a final reaction volume of 10  $\mu\text{l}$  containing 1 $\times$  polymerase buffer, 0.325 U of MP Biomedicals polymerase, 0.2 mM of dNTP (Invitrogen), 0.1  $\mu\text{M}$  of forward primer with a M13 tail, 0.2  $\mu\text{M}$  of reverse primer, 0.1  $\mu\text{M}$  of M13 tail IRD700 or IRD800 labeled primer and 20 ng of DNA. For AFLP markers, the protocol described by (Vos et al. 1995) was used. A Li-Cor IR2 (Li-Cor Inc) sequencer was used to separate the labeled amplified DNA fragments on a 6.5% acrylamide gel. Marker segregation was scored using SAGA Generation 2 software (Li-Cor Inc) by two different persons and the results were compared.

### Data quality control

Molecular markers were used to discard 34 self-pollinated plants and five plants with foreign alleles. Nineteen plants died in the field and were not included in the analyses. Finally, the number of plants used for statistical analyses was 175 for Nemo A  $\times$  Nemo F, 178 for Nemo B  $\times$  Nemo F and 189 for Nemo H  $\times$  Nemo F.

### Phenotypic data analysis

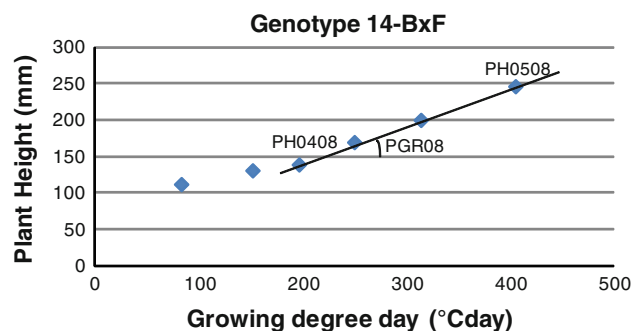
In spring 2008, plant growth rate (PGR08) was calculated using PH measurements from 11 April to 2 May corresponding to the linear growth phase (Fig. 1). For each plant, PGR08 was calculated as the slope of the linear function fitting PH against thermal time (base temperature of  $0^{\circ}\text{C}$ ). The studied variables are summarized in Table 2.

For each progeny, normality of traits was tested with the Kolmogorov–Smirnov test in the UNIVARIATE procedure of SAS (SAS Institute Inc. 2000). For each trait, the means of progenies were compared using a Student–Newman–Keuls test (GLM procedure of SAS). Pearson correlation coefficients were calculated by pairs of variables for each progeny (StatSoft 2009).

### Map construction and QTL detection

A  $\chi^2$  test was performed to test distortion segregation of the markers in each population. For the AFLP markers, only markers with a 1:1 segregation were chosen. LG numbers were given according to the *Lolium* reference map (Jones et al. 2002b).

For map construction and QTL detection, two methods were used. The first one was used to detect QTL for each parent (Nemo A, Nemo B and Nemo H) within each population (analysis per parent). In that case, map construction was performed per parent. Thus, for each parent (Nemo A, Nemo B and Nemo H), a map was built using Mapmaker 3.0 (Lander et al. 1987) following the two-way



**Fig. 1** Plant height kinetic in spring 2008 of genotype 14 from Nemo B  $\times$  Nemo F cross

**Table 2** Summary of the variables used for the study and their description

Variables	Score/unit	Description
Ru0807	1: resistant to 9: susceptible	Rust susceptibility scored on the 07 August 2007
PH0907	mm	PH measured 25 weeks after the transplantation
PH1107	mm	PH measured 4 weeks after the first cut
PH0408	mm	PH measured 4 weeks after the third cut
PH0508	mm	PH measured 7 weeks after the third cut
PGR08	mm/(°C day)	PGR calculated during the kinetic of regrowth in spring 2008
PH1108	mm	PH measured 23 weeks after the fourth cut
PH0509	mm	PH measured 7 weeks after the sixth cut

pseudo-testcross strategy (Grattapaglia and Sederoff 1994). The default parameters of the software were applied to define groups (minimum LOD score of 3.0 and a maximum distance between markers of 80 cM). The Haldane function was used. For each parent, a framework map was built by deleting some markers in the highly covered regions with the aim of creating a robust map with one marker every 5–10 cM. All co-dominant markers not included in the framework map were mapped using the “try” function.

QTL were detected using the software QTL cartographer version 2.5 (Basten et al. 2007). Composite interval mapping (CIM) was used on the three framework maps with the model 6 and the forward and backward regression for covariate selection. The analyses were done with a window size of 10 cM and a step of 2 cM. For each trait, the LOD score threshold was defined using 1,000 permutations according to Churchill and Doerge (1994). Confidence intervals were calculated using the one-LOD unit fall method (Van Ooijen 1992). In QTL cartographer, the additive effect of a quantitative trait is calculated as the difference between the means of the two groups based on alleles. In order to be consistent with MCQTL, the additive effects given by QTL cartographer were divided by two.

A second method was used to detect QTL in the three connected populations using a single model (multipopulation connected analysis). This method should increase the power of QTL detection. The tester parent Nemo F had to be included in the analysis as it played the role of connector between populations. Maps were built using the software Joinmap 3.0 which allowed to combine maps (Van Ooijen and Voorrips 2001). A map for each cross (Nemo A × Nemo F, Nemo B × Nemo F and Nemo H × Nemo F) was built and the linkage phase between marker alleles segregating from each parent was determined. For the three crosses, all LG grouped at a minimum LOD of 5. The Haldane function was used. Then, a consensus map of the three maps was built using the “Combine maps” function. A framework consensus map was built by deleting some markers in the highly covered

regions with the aim of creating a robust map with one marker every 5–10 cM. Nevertheless, SSR markers genotyped in all three crosses were all kept to have better links between maps. The linkage map was drawn using MapChart 2.1 (Voorrips 2002).

For this second method, QTL were detected using the software MCQTL with the *Outbred* module (Jourjon et al. 2005). The multipopulation connected analysis was used (Billotte et al. 2010) to detect QTL in the four parents within a single model. Both additive and dominance models were examined. The genotype probabilities were computed every 2 cM. The iterative QTL mapping (iQTLm) was used (Billotte et al. 2010) with a window of 15 cM around the putative QTL and forward stepwise method to select marker cofactors from the whole genome. The significant threshold of QTL was determined following the method given by Billotte et al. (2010) with 10,000 iterations on each trait for each model according to Churchill and Doerge (1994). Very similar *F* threshold values (range 2.93–3.02) were found for all traits with an average value of 3 with a type I error rate of 5%. This is the value chosen to perform QTL detection. The estimated model parameters recorded for each significant QTL were position, LOD-1 confidence region, percentage of phenotypic variation explained, and effects of the alleles for each parent. The percentage of phenotypic variance explained was calculated using the multipopulation connected analysis (Jourjon et al. 2005). This includes the effect of the four parents (Nemo A, Nemo B, Nemo H and Nemo F) and, therefore, is not consistent with the analysis using QTL cartographer which includes the effect of only one parent (Nemo A or Nemo B or Nemo H). At any given QTL, the sum of the two QTL allelic effects of each parent was null by constraint of the model. For each LG, effects were calculated relatively to the same homologous chromosome for all QTL. Finally, Student tests were performed to test the additive and dominance effects for the QTL with a type I error rate of 5%. The additive effect of a given QTL was tested using the following formula for each parent:



$$t = \frac{\alpha_i - \alpha_j}{\sqrt{\varepsilon \cdot (\sigma_i^2 + \sigma_j^2 - 2\text{cov}(i,j))}} = \frac{2\alpha_i}{\sqrt{2\varepsilon \cdot (\sigma_i^2 - \text{cov}(i,j))}}$$

with  $\alpha_i$  the additive effect of allele  $i$ ,  $\alpha_j$  the additive effect of allele  $j$ ,  $\varepsilon$  the residual variance of the model,  $\sigma_i^2$  the variance of allele  $i$  and  $\sigma_j^2$  the variance of allele  $j$ . For each cross, the dominance effect was tested using the following formula:

$$t = \frac{\delta}{\sqrt{\varepsilon \cdot \sigma_\delta^2}}$$

with  $\delta$  the dominance effect,  $\varepsilon$  the residual variance of the model and  $\sigma_\delta^2$  the variance of the dominance effect. All the parameters of the formulae were estimated by the MCQTL software.

## Results

### Phenotypic analysis

Basic statistics for all traits for each progeny are given in Supplementary Material 2. All traits showed normal distribution. Differences between crosses were significant for all traits except PH in 2009. The cross Nemo B  $\times$  Nemo F was more susceptible to crown rust and had higher PH and plant growth rate in spring 2008. All three progenies had a lower average and showed less variation for PH 7 weeks after defoliation in spring 2009 (PH0509) compared to PH 7 weeks after defoliation in spring 2008 (PH0508). Despite several significant correlations between traits (Supplementary Material 3), the majority was low (below 0.5) except for PH in spring 2008 measured 4 and 7 weeks after the third cut (PH0408 and PH0508). It is worthy of note that PGR08 was correlated with PH0508 but not with PH0408 expressing plant growth earliness after winter. So, high values of PH0508 could have resulted from either early plant growth after winter and/or high growth rate.

### Mapping

Segregation distortion for SSR and STS markers was observed in all progenies with Nemo A  $\times$  Nemo F (16.4%) and Nemo H  $\times$  Nemo F (12.5%) showing less segregation distortion than Nemo B  $\times$  Nemo F (36.5%). For crosses Nemo A  $\times$  Nemo F and Nemo H  $\times$  Nemo F, LG7 was the most affected while LG1, 2, 3 and 5 had the highest number of distorted markers in Nemo B  $\times$  Nemo F.

Using Mapmaker, one map per elite parent (Nemo A, Nemo B and Nemo H) was obtained consisting of seven linkage groups as expected, except for Nemo B in which LG2

was split in two unlinked segments (Supplementary Material 4). The maps of Nemo A, Nemo B and Nemo H covered 330 (38 markers), 541 (44 markers) and 501 cM (40 markers), respectively, with an average distance between markers of 8.7, 12.3 and 12.5 cM, respectively. The maximum distance between markers was 31.4 cM on LG2 for Nemo A, 42.1 cM on LG4 for Nemo B and 44.4 cM on LG3 for Nemo H.

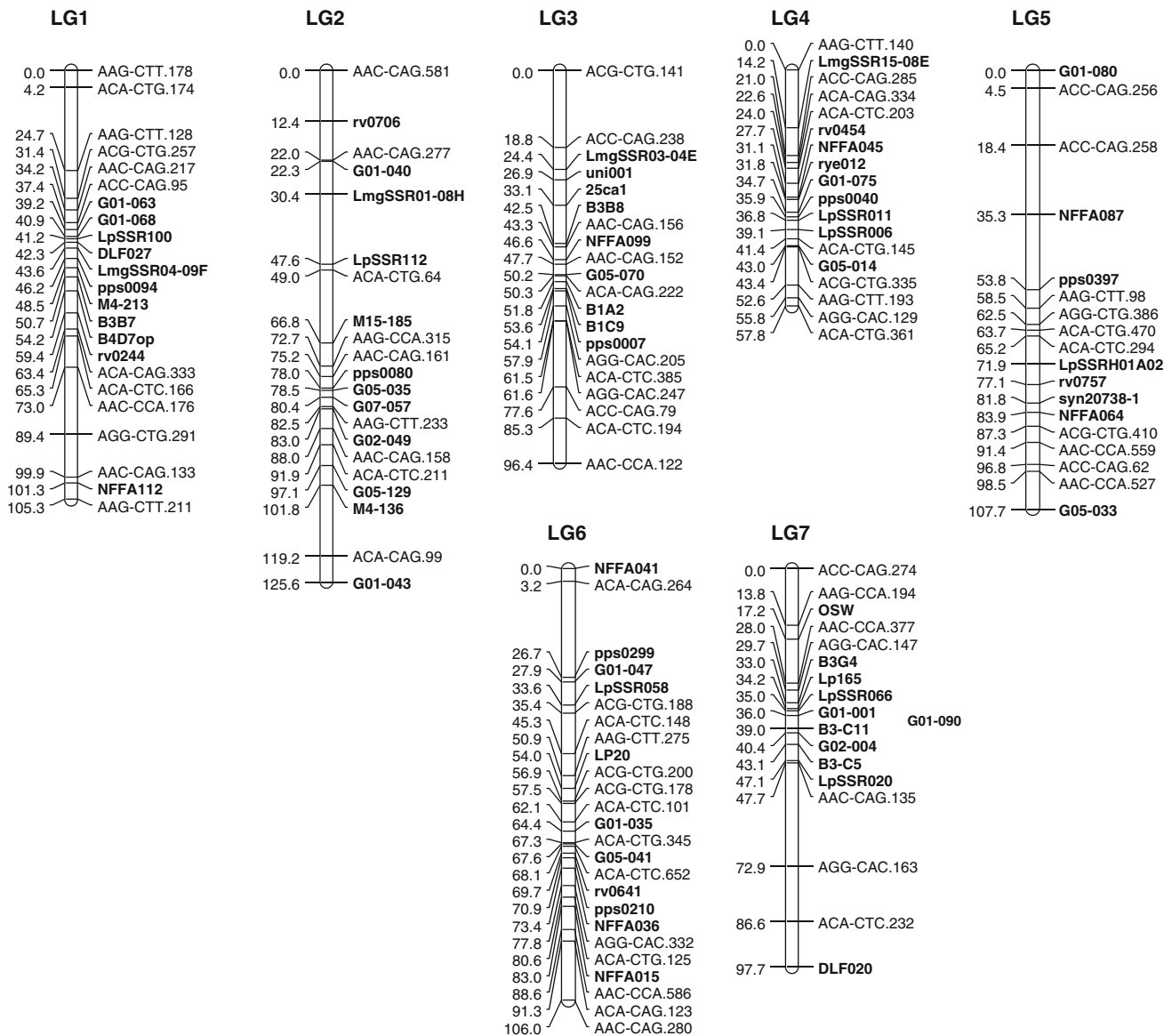
Using JoinMap, one map per progeny was built and then one consensus map was obtained (Fig. 2). The consensus map covered 696.5 cM with a total of 142 markers (72 AFLP, 69 SSR and 1 STS) and an average distance between markers of 4.9 cM. The maximum distance between markers was 25.2 cM and was obtained on LG7. The consensus map had 22 SSR markers in common with the map of Nemo A built with Mapmaker, 27 with Nemo B and 25 with Nemo H. A comparison between the three maps built with Mapmaker and the consensus map built with JoinMap (Supplementary Material 4) indicated that the distances between markers varied but the order of the markers was generally the same. Furthermore, several LG built with Mapmaker, especially for Nemo A, were quite short compared to the consensus map due to a lack of polymorphic markers. Nevertheless, the marker information derived from these LG was the same both in the analysis per parent and in the multipopulation connected analysis.

### QTL detection and comparison across analyses

In this section, only the QTL of Nemo A, Nemo B and Nemo H will be considered. QTL results of Nemo F will be given in the tables of the multipopulation connected analyses, but they will not be described. Nevertheless, the possible use of QTL information of Nemo F will be discussed.

Single-population analyses (QTL cartographer) made it possible to detect six QTL for Nemo A, five for Nemo B and six for Nemo H, leading to 17 QTL in all (Table 3). Two QTL for rust susceptibility were detected, but only for Nemo A. They were on LG2 and 6 and explained 9.5 and 14.5% of the phenotypic variance, respectively. Fifteen QTL for plant growth parameter (PH and PGR) were distributed across all LG except LG1, all populations taken together. Their  $R^2$  ranged from 5.7 to 14.7. Four QTL were found for Nemo A, five for Nemo B and six for Nemo H.

A total of 33 QTL were identified for Nemo A, Nemo B and Nemo H using the additive model of the multipopulation connected analysis (Table 4). Seven QTL were found for rust susceptibility including three QTL for Nemo A, one for Nemo B and three for Nemo H. They were shared out between four locations on LG1, 2, 5 and 6. The percentages of variance explained by QTL for rust susceptibility were small except for the QTL on LG2 (32.5% of phenotypic variance) but in that case the main effect came from the tester parent, Nemo F. For PH and PGR, 26 QTL were detected



**Fig. 2** Consensus map of the three crosses Nemo A × Nemo F, Nemo B × Nemo F and Nemo H × Nemo F, built by JoinMap. SSR and STS markers are in *bold*

including six QTL for Nemo A, nine for Nemo B and 11 for Nemo H. All the LG were concerned. The percentage of phenotypic variance explained by the QTL ranged from 3.3 to 9.1. It was observed that generally two parents had a significant QTL at a given position.

A total of 21 QTL were identified for Nemo A, Nemo B and Nemo H using the dominance model of the multi-population connected analysis (Table 5). Six were found for rust susceptibility including three for Nemo A and three for Nemo H. No QTL was identified for Nemo B. These QTL were distributed along four regions on LG1, 2, 5 and 6. For PH and PGR, 15 QTL with significant additive effects were detected including three QTL for Nemo A, seven for Nemo B and five for Nemo H. All LG were

concerned except LG1. In addition, a total of ten dominance effects were identified with one for rust susceptibility (for Nemo H × Nemo F) and nine for PH (three for Nemo A × Nemo F, four for Nemo B × Nemo F and two for Nemo H × Nemo F). No dominance effect was identified for PGR. The values of dominance effects were generally smaller than the values of additive effects except for PH0907 for which Nemo A × Nemo F showed large dominance effects on LG3 and 7, and Nemo H × Nemo F showed large dominance effects on LG6. The percentage of phenotypic variance explained by the dominance model ranged from 5.4 to 15.4 for PH and PGR.

Out of a total of 39 QTL for all traits (only QTL with an additive effect), ten were common to the three QTL

**Table 3** QTL detected per parent by composite interval mapping with QTL cartographer for crown rust susceptibility and traits related to plant growth

Trait	Parents	LG	Closest marker	Position on the consensus map (cM)	Position (cM)	-1 LOD interval (cM)	Detection threshold	LOD score	R <sup>2</sup> (%)	Additive effect <sup>a</sup>
Ru0807	Nemo A	2	LmgSSR01-08H	30.1	0.0	0.0–18	2.1	4.2	9.5	0.5
		6	LpSSR058	33.6	22.0	10.0–36.1	2.1	4.8	14.2	0.6
PH0907	Nemo B	4	LpSSR011	36.8	65.3	57.3–75.6	2.4	2.8	7.9	14.8
	Nemo H	2	M4-136	101.8	90.3	82.3–90.3	2.4	2.5	5.9	9.8
PH1107	Nemo A	4	ACC-CAG.285	21.0	23.9	10.0–29.1	2.1	3.3	9.3	11.6
	Nemo H	7	LpSSR020	47.1	30.5	19.5–32.5	2.3	3.1	8.4	8.9
PH0408	Némo A	3	AGG-CAC.247	61.6	46.6	37.8–56.6	2.2	3.1	9.7	6.8
	Némo B	2a	LpSSR112	47.6	58.7	44.7–62.7	3.0	4.2	11.8	8.6
	Némo H	5	LpSSRH01A02	71.9	25.8	12.4–33.7	2.3	2.8	7.4	6.1
		7	Lp165	34.2	19.5	4.0–32.5	2.3	2.4	5.7	5.4
PH0508	Nemo A	3	B1A2	51.8	36.8	20.3–54.6	2.2	3.6	10.0	8.6
	Nemo B	2a	LpSSR112	47.6	58.7	48.7–62.7	2.7	6.1	14.7	12.2
		7	B3G4	33.0	37.9	23.3–46.6	2.7	4.4	8.9	8.5
	Nemo H	7	G02-004	40.4	23.5	12.0–32.5	2.2	4.5	11.1	10.9
PGR08	Nemo B	7	G01-090	37.9	46.6	31.4–56.6	2.5	3.0	8.9	0.02
PH1108	Nemo A	6	G01-035	64.4	55.3	45.2–62.8	2.2	2.5	6.6	5.5
PH0509	Nemo H	5	LpSSRH01A02	71.9	21.8	8.4–27.8	2.3	2.7	6.2	5.8

The position of the closest marker to the QTL on the consensus map has been added to compare the QTL detected in the different analyses

<sup>a</sup> Additive effects given by QTL cartographer were divided by two to be compared with additive effects of MCQTL

analyses (Fig. 3). Two QTL detected with the multipopulation connected analyses were considered as common when they had overlapping confidence intervals or when the position of the closest marker to a QTL detected by the analysis per parent was included in the confidence interval of the QTL detected in the multipopulation connected analyses. These common QTL generally had similar additive effects and had larger effects than the QTL which were not common. The additive model of the multipopulation connected analysis allowed (1) detection of all QTL detected in the analysis per parent except for four, (2) detection of all QTL with the dominance model of the multipopulation connected analysis except for two and (3) detection of 11 additional QTL detected neither by the analysis per parent nor by the dominance model of the multipopulation connected analysis (Fig. 3). Out of the 11 QTL, five were detected at the same location of QTL common to at least one of the other analyses. In the latter case, the effect of the QTL detected only with the additive model of the multipopulation connected analysis had a smaller effect than the QTL detected in another parent of another population. For example, for rust susceptibility on LG6 (Tables 3, 4, 5), the QTL for Nemo A was identified in the three analyses while the QTL for Nemo B had a lower additive effect and was only identified with the additive model of the multipopulation connected analysis. The six other QTL detected only by the additive model of the multipopulation connected analysis were identified

when several parents had low but significant additive effects at one location. For example, on LG6 for PH0509 (Table 4), Nemo A, Nemo B and Nemo H had additive effects with low but significant values and they were only detected in the additive model of the multipopulation connected analysis.

Considering QTL of all traits on all parents, four genomic regions were involved in rust susceptibility (LG1, 2, 5 and 6) and ten genomic regions were involved in plant growth (Fig. 4). Three regions were common to both spring and autumn plant growth (LG3, 6 and 7). Two regions were specific to plant growth in spring (LG2 and 5). Five regions were specific to plant growth in autumn (LG1, 2, 4, 5 and 6). QTL were detected for all three elite parents whatever the analysis used. Moreover, using MCQTL, it was possible to identify the best allele among Nemo A, Nemo B and Nemo H when they shared the same QTL. For example, the allele of Nemo B on LG4 was the most favorable for PH0907 (Table 4). Each parent carried the best allele for at least one region.

## Discussion

### Plant growth traits

The QTL of PH measured at different periods were mostly at different QTL positions. This absence of consistency of



**Table 4** Quantitative trait loci (QTL) identified in the additive model of the multipopulation connected analysis with MCQTL

Trait	LG	Position on the consensus map (cM)	Closest marker	−1 LOD interval (cM)	<i>F</i> test	<i>R</i> <sup>2</sup> (%)	Global <i>R</i> <sup>2</sup> (%)	Additive effects			
								Nemo A	Nemo B	Nemo H	Nemo F
Ru0807	1	43.7	LmgSSR04-09F	24.2–49.8	3.9	4.3	38.6	<b>−0.2</b>	0.1	<b>0.3</b>	<b>−0.2</b>
	2	32.4	LmgSSR01-08H	28.2–35.3	42.6	32.5		<b>−0.4</b>	−0.2	<b>−0.3</b>	<b>1.0</b>
	5	81.1	syn20738-1	74.0–103.5	3.9	4.3		−0.2	0.0	<b>−0.4</b>	<b>−0.1</b>
	6	45.4	ACA-CTC.148	20.2–54.9	5.3	5.5		<b>−0.5</b>	<b>−0.3</b>	−0.1	0.1
PH0907	1	31.4	ACG-CTG.257	3.0–62.0	2.6	3.3	15.2	<b>11.9</b>	0.4	<b>−7.1</b>	−3.5
	4	36.8	LpSSR011	35.6–37.9	6.3	6.9		<b>7.7</b>	<b>−14.9</b>	−3.7	<b>5.9</b>
	5	107.7	G05-033	92.3–107.7	3.2	3.9		<b>8.2</b>	6.0	<b>−7.4</b>	<b>5.0</b>
	7	34.2	Lp165	25.3–35.5	3.7	4.4		−3.0	−2.3	<b>9.5</b>	<b>−6.6</b>
PH1107	4	24.0	ACA-CTC.203	16.4–28.6	5.5	6.0		<b>11.8</b>	−1.8	−3.1	<b>5.2</b>
PH0408	2	53.0	ACA-CTG.64	37.3–75.0	5.4	5.9	12.1	−1.1	<b>−9.7</b>	<b>−4.1</b>	1.7
	5	69.2	LpSSRH01A02	42.3–80.4	3.8	4.4		−2.3	<b>−4.2</b>	<b>−6.5</b>	1.4
	7	36.0	G01-001	0.0–41.0	3.0	3.7		1.7	3.0	<b>5.8</b>	1.1
PH0508	2	75.3	AAC-CAG.161	53.4–84.4	4.3	4.8	19.0	4.0	<b>−9.8</b>	<b>−4.3</b>	0.1
	3	42.5	B3B8	35.3–52.1	4.8	5.3		<b>−10.9</b>	<b>−4.8</b>	−0.8	1.2
	6	106.0	AAC-CAG.280	87.2–105.0	3.6	4.2		7.2	<b>−7.3</b>	−3.5	<b>−5.3</b>
	7	38.0	B3-C11	34.2–41.7	9.0	9.1		−0.2	<b>10.3</b>	<b>10.3</b>	0.9
PGR08	7	39.1	B3-C11	37.1–42.6	3.3	3.9		0.00	<b>0.02</b>	<b>0.01</b>	0.00
PH0509	5	77.1	rv0757	61.3–79.8	3.2	3.9	7.5	1.3	−0.3	<b>−5.3</b>	<b>2.6</b>
	6	77.8	AGG-CAC.332	60.1–106	3.4	4.1		<b>4.8</b>	<b>−5.5</b>	<b>−3.9</b>	1.5

Significant effects of the alleles are given in bold at a significance level of 5%. For each parent, the additive effects of a LG are given for the same homologous chromosome

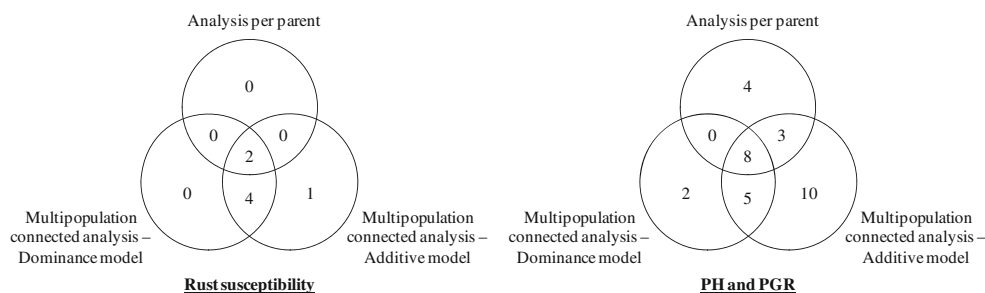
QTL for plant morphogenesis parameters in different environments is often observed in perennial ryegrass. Kobayashi et al. (2011) reported different QTL at the different periods of measurement of plant length and leaf length in their field experiment. Moreover, QTL detected in their field experiment were generally absent in the pot experiment and vice versa. These results suggest strong genotype × environment interactions and that different genes are involved in regulation of plant growth depending on the environment. Nevertheless, in our study, some co-localizing QTL for PH were observed in the different seasons and years studied, as on LG5 between QTL of PH in spring 2008 and 2009 and on LG7 with QTL of plant growth in spring 2008 co-localizing with QTL of PH in autumn 2007. In 2009, Barre et al. also reported QTL of lamina length measured in October 1999 which co-localized with QTL of plant height measured in April 2000 on LG7. The same year, Anhalt et al. reported co-localizing QTL between their greenhouse and field experiments on LG2, 3 and 7 for biomass traits. Those QTL seem stable in different environments and should be given a priority in the breeder's selection. However, those examples are less frequent than those where genotype × environment interactions occur.

The QTL regions that were detected in this study appear to map in genomic regions where QTL related to plant growth had already been identified. However, the lack of common markers reduces the accuracy of the comparisons between studies. Nevertheless, an interesting example is on LG7. Many QTL were identified in our study close to the marker “OSW” which has been mapped in most published studies. This region co-localized with (1) QTL of fresh weight, dry weight and dry matter in the study of Anhalt et al. (2009), (2) QTL of plant length in spring, summer and autumn, and QTL of leaf length in summer in the study of Kobayashi et al. (2011) (R7 region), (3) QTL of leaf length in spring and autumn and QTL of plant height in spring in the experiment carried out by Barre et al. (2009), and (4) QTL of plant height in the study of Studer et al. (2008b). However, in the latter experiment, plant height was measured at harvest when the plants were headed while measurements were made during the vegetative phase in the other studies. Furthermore, on several studies, QTL of heading date were found in the same region of LG7 (Armstead et al. 2004, 2008; Studer et al. 2008b; Barre et al. 2009; Byrne et al. 2009). In our study, QTL of heading date were also located in this region (data not shown). This region of LG7 of perennial ryegrass shows a

**Table 5** Quantitative trait loci (QTL) identified in the dominance model of the multipopulation connected analysis with MCQTL

Trait	LG	Position on the consensus map (cM)	Closest marker	-1 LOD interval (cM)	F test	R <sup>2</sup> (%)	Global R <sup>2</sup> (%)	Additive effects				Dominance effects		
								Nemo A	Nemo B	Nemo H	Nemo F	AF	BF	HF
Ru0807	1	46.2	pps0094	0.0–69.6	3.2	5.6	41.3	<b>-0.2</b>	0.2	<b>0.2</b>	<b>-0.2</b>	0.0	0.0	0.1
	2	28.3	LmgSSR01–08H	28.0–36.0	35.5	31.4		<b>-0.4</b>	-0.2	<b>-0.4</b>	<b>0.9</b>	-0.1	0.0	0.0
	5	79.1	syn20738–1	66.1–93.0	3.4	5.8		-0.2	0.0	<b>-0.4</b>	-0.1	-0.2	-0.1	0.1
	6	26.7	pps0299	16.3–47.7	4.7	7.2		<b>-0.5</b>	-0.2	0.0	0.1	0.0	0.0	<b>-0.2</b>
PH0907	3	61.6	AGG–CAC.247	46.7–74.3	2.9	5.7	31.0	-2.7	4.5	<b>9.1</b>	-1.9	<b>-14.6</b>	-2.0	2.1
	4	36.8	LpSSR011	34.7–37.2	12.4	15.4		2.9	<b>-14.7</b>	-4.1	<b>4.7</b>	-5.1	<b>6.6</b>	-5.6
	5	107.7	G05–033	89.1–107.7	2.8	5.6		<b>8.7</b>	2.4	-5.5	<b>5.3</b>	-0.6	11.9	5.3
	6	35.4	ACG–CTG.188	28.6–40.6	3.9	6.9		-3.6	<b>-9.6</b>	-2.2	-2.0	-5.3	5.6	<b>-9.9</b>
PH1107	7	47.1	LpSSR020	45.0–58.6	4.2	7.2		2.0	-3.6	<b>7.1</b>	<b>-5.0</b>	<b>-11.7</b>	<b>7.6</b>	2.1
	4	26.0	rv0454	19.6–28.6	5.9	8.5		<b>11.1</b>	-2.0	-3.2	<b>5.0</b>	-5.0	0.0	<b>-5.4</b>
	PH0408	2	55.0	ACA–CTG.64	42.6–61.4	4.7	7.4	12.3	-1.2	<b>-9.9</b>	-3.4	<b>2.9</b>	3.2	<b>4.8</b>
PH0508	5	67.2	ACA–CTC.294	44.3–73.4	3.8	6.4		-2.6	<b>-4.1</b>	<b>-6.5</b>	1.3	3.4	3.2	-2.2
	3	51.8	B1A2	38.9–60.4	3.7	6.2	14.4	<b>-8.5</b>	<b>-4.8</b>	-0.1	-0.6	<b>-5.2</b>	<b>-4.3</b>	-2.5
PGR08	7	35.1	LpSSR066	29.2–41.3	6.8	9.4		-1.9	<b>10.3</b>	<b>10.5</b>	0.4	2.7	2.2	-1.9
PGR08	7	40.4	G02–004	37.5–46.6	3.0	5.4		0.00	<b>0.02</b>	<b>0.01</b>	0.00	0.00	0.00	0.01

Significant effects of the alleles are given in bold at a significance level of 5%. For each parent, the additive effects of a LG are given for the same homologous chromosome



**Fig. 3** Number of QTL detected for rust susceptibility on the *left* and PH and PGR on the *right*, for the analysis per parent and both additive and dominance models of the multipopulation connected analysis. Only the number of additive effects was compared. The number of QTL was determined by considering that two QTL detected in the

multipopulation connected analyses with overlapping confidence intervals were the same, or when the position of the closest marker to a QTL detected by the analysis per parent was included in the confidence interval of the QTL detected in the multipopulation connected analyses

high degree of synteny with the region of rice on LG6 which contains the Hd3 and Hd1 heading date QTL and their controlling genes *Hd3a* and *Hd1* (Armstead et al. 2004, 2005). Hence, there may be a close link between heading date and traits linked to plant growth. This link was also observed by Hazard et al. (2006) who showed a negative correlation between heading date and plant height. The genes *Hd3a* and *Hd1* could be good candidate genes for both heading date and vegetative plant growth.

#### Rust tolerance

In our experiment, several QTL for rust susceptibility were identified with the multipopulation connected analysis. Crown rust susceptibility has an average heritability in perennial ryegrass (Hayward 1977; Schejbel et al. 2007).

Therefore, a unique scoring should be reliable even if several scores would have been preferable. Moreover, QTL detected with the multipopulation connected analyses were identified in several parents. In addition, the QTL found on LG1 co-localized with the one detected by Schejbel et al. (2007), mapped between markers “DLF027” and “M4-213”. The other QTL detected in our study mapped to genomic regions where QTL have already been identified either in perennial ryegrass or Italian ryegrass. However, the lack of common markers between studies prevents us from giving accurate co-locations.

In 2005, Muylle et al. (2005b) showed that QTL for rust resistance on LG1 and 2 of *Lolium* are syntenic to regions on group A and B chromosomes of oat on which crown rust resistance genes have been identified. Those candidate genes could be used to identify causal mutation for rust



**Fig. 4** Position on the consensus map of the QTL detected with the different methods for the three elite plants

resistance which would allow a more efficient MAS. As for Dracatos et al. (2008), they used sequence annotation against template genes from related *Poacea* species to identify candidate disease response genes in perennial ryegrass and map SNPs in those genes. Two SNPs were mapped close by the marker “pps0299” on LG6 close to which QTL of crown rust susceptibility were identified in our study. Those SNPs were identified in genes having a catalase or a cytosolic glutathione reductase function in wheat. They could be involved in the defense response to crown rust in *Lolium perenne*. In addition, other studies on crown rust resistance cite nucleotide binding site-leucine rich repeat (NBS-LRR) genes as promising genes involved

in the defense response. They are involved in pathogen recognition and signal transduction (Dangl 1995; Dangl et al. 1996; Dangl and Holub 1997; Schejbel et al. 2007; Dracatos et al. 2010). Some of these genes were mapped by Dracatos et al. (2009) on all LG but the lack of common markers prevents us from comparing their position to our QTL except for one mapped close to “pps0299” on LG6.

Interest of the multipopulation connected design

In our experiment, using a multipopulation connected analysis allowed us to detect more QTL than in the analysis per parent. It was previously shown on maize that the

power of detection in a multipopulation design is maximal when the populations studied share common parents (Blanc et al. 2006, 2008). In contrast, Billotte et al. (2010) observed less QTL using the multiparental connected design than using the analysis per parent. They explained that this result could be due to small population size which was not the case in our study. One drawback of the multipopulation connected analysis is that it is possible to miss QTL present in only one population due to dilution effect. The latter was observed in the study of Pierre et al. (2008). In our study, this dilution effect was observed only for four QTL with small additive effects detected in only one parent in the analyses per parent.

The multipopulation connected analysis allows a global comparison of allelic effects at each detected QTL and the best ones can be identified (Blanc et al. 2006, 2008). In our study, generally two parents had a significant QTL at a given location and the best alleles were not invariably from the same parent, depending on the QTL. In contrast, in the analysis per parent, most QTL were detected in a single population. This revealed the power of multipopulation connected analysis to detect QTL in different parents even for those with weak effects. On studies using pseudo-testcross in perennial ryegrass, QTL were generally not common to both parents of the mapping populations. In a study of Barre et al. (2009), there was no common QTL between the parents. In another study on waterlogging tolerance, Pearson et al. (2011) detected only two QTL common to the parents significant for both simple interval mapping and composite interval mapping. Several individuals can be used to collect different favorable alleles and then be included into a polycross.

Using a multiparental design allowed us to detect QTL in three parents and also in the tester parent within a single experiment. Up to now, all QTL studies on perennial ryegrass only involved biparental populations. Their parents could be chosen with opposite phenotypes for the trait of interest as for crown rust resistance in the F1 populations (Dumsday et al. 2003; Muylle et al. 2005a, b; Pfender et al. 2011), for flowering time with the F1 population studied by Byrne et al. (2009), for leaf length (F1 population, Barre et al. 2009), for biomass in the F2 population in the study of Anhalt et al. (2008) or for sugar content (Jones et al. 2002b). The parents could also be chosen for their diverse origin: geographical as in the F1 population studied by Faville et al. (2004) or in the F2 population described by Jensen et al. (2005a), or genetic as in the population p150/112 coming from the cross between a hybrid F1 and a doubled-haploid plant (Jones et al. 2002a). There is only one article in which two disconnected mapping populations were studied simultaneously to identify QTL for fertility traits

(Armstead et al. 2008). In all those studies, the idea was to maximize the genetic and phenotypic differences between parents to be sure to succeed in mapping and in QTL identification. Nevertheless, because of the very low diversity present, it was almost impossible to directly perform MAS in the progenies due to inbreeding depression expected in a polycross consisting of F1 or F2 population plants. These studies could be used to find the genes under the QTL and to study their diversity (allelic sequencing) in relation with traits (Paran and Zamir 2003). In that case MAS would be applied directly on genes. In our case, four elite plants were used for QTL detection to be able to perform MAS directly on the progenies. Even if the number of four parents is limited considering that two parents were related (Nemo B and Nemo F), this study made it possible to test the methods to evaluate QTL in multiparental connected populations using heterozygous plants. To our knowledge, using such multiparental design among heterozygous species is original as there is only one other published study on palm oil (Billotte et al. 2010).

#### Implications for selection

Parents of mapping populations are traditionally chosen with contrasted features to have a higher degree of polymorphism and more powerful QTL detection. In our study, elite parents selected for the same traits over several generations could have led to a small number of QTL detected. On the contrary, a relatively large number of QTL was detected either on rust susceptibility or plant growth. The cycles of phenotypic selection did not lead to the fixation of favorable alleles at a homozygous level. The high number of QTL detected in elite plants could be explained by the accumulation of favorable alleles at a heterozygous level. This phenomenon has also been observed in the study of Barre et al. (2009) in which QTL were detected in a F1 pseudo-testcross population between plants obtained after three cycles of divergent selection. The detection of several QTL of lamina length in this study implies that alleles of QTL were not fixed within each parent despite the strong selection. Moreover, in our study, few dominance effects were identified and they generally had smaller values than the additive effects suggesting that most of the genetic value is determined by additive effects. It is better not to take them into account at the QTL detection step as demonstrated by Rebaï and Goffinet (1993) to have a greater power of detection. Together, these elements lead to the possibility of increasing the frequency of favorable alleles using MAS starting from elite material.

To achieve this aim, several strategies could be used. Dolstra et al. (2003) gave a score to each individual as a function of the number of favorable alleles at QTL of

nitrogen-use efficiency in perennial ryegrass. The individuals having the highest scores were selected. The latter strategy could be applied in our study by choosing several individuals within each progeny to have sufficient diversity. The favorable alleles of Nemo F would therefore be included in the choice even if their effects could not be compared to those of the three other parents. This method would take all QTL with the same weight. But a weight could be included as in the selection index presented by Lande and Thompson (1990) or by Moreau et al. (1998) where both phenotype and effect of QTL are taken into account and weighted.

Another strategy would be the pyramiding of QTL as suggested by Hospital and Charcosset (1997) where QTL are monitored separately and then favorable alleles at different QTL are accumulated. Potential effects of repulsion should be taken into account and the individuals carrying out the good recombination should be selected.

Finally, the best strategy could be to combine the abovementioned strategies using a selection index which would consider both phenotype and QTL and break the link between QTL in repulsion.

## Conclusion

In our study, we detected QTL of rust susceptibility and plant growth parameters in perennial ryegrass using different QTL models. The additive model of the multi-population connected analysis was the most powerful. This analysis successfully applied on three connected mapping populations involving four parents seems promising for implementation in a design involving a larger number of populations with a higher variability to include them into a MAS strategy. However, biparental populations are difficult to produce at a large scale and they are not often used in forage species breeding schemes. Nevertheless, the breeder's material generally consists of polycross progenies. In those populations, using molecular markers makes it possible to identify the parents of each individual. This progeny is actually made up of several biparental connected populations in which the multipopulation connected analysis could be performed. Hence, MAS could be carried out directly in the breeder's material.

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