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QTL analysis of seed yield components in red clover (*Trifolium pratense* L.)

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Abstract Cultivars of red clover (*Trifolium pratense* L.), an important forage crop in temperate regions, are often characterised by an unsatisfactory level of seed yield, leading to high production costs. This complex trait is influenced by many components and negatively correlated with other important traits, such as forage yield or persistence. Therefore, seed yield has proven to be difficult to improve. Thus, the objectives of this study were to assess association among seed yield components and to provide the basis for identifying molecular markers linked to QTLs for seed yield components to assist breeding for improved red clover cultivars. A total of 42 SSR and 216 AFLP loci were used to construct a molecular linkage map with a total map length of 444.2 cM and an average distance between loci of 1.7 cM. A total of 38 QTLs were identified for eight seed yield components. The traits seed number per plant, seed yield per head, seed number per head, head number per plant and percent seed set were highly correlated with seed yield per plant, and QTLs for several of these traits were often detected in the same genome region. Head number per plant may present a particularly useful character for the improvement of seed yield since it can easily be determined before seed maturity. In addition, two genome regions containing four or five QTLs for different seed yield components, respectively, were identified representing candidate regions for further characterisation of QTLs. This study revealed several key components which may facilitate further improvement of seed yield. The QTLs identified represent an important first step towards marker-assisted breeding in red clover.

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Introduction

Temperate grasslands play an important role in agriculture, as they cover approximately 8% of the global land area (White et al. 2000). Red clover (*Trifolium pratense* L.), an outcrossing and diploid ($2n = 2x = 14$) species with a gametophytic self-incompatibility system, is an important component of permanent pastures and meadows as well as of grass-clover leys in temperate regions. Red clover is adapted to a wide range of environmental conditions, has a high nutritive value and, due to its ability to fix atmospheric nitrogen, red clover is of a high value to the environment (Taylor and Quesenberry 1996).

In the last decades, targeted selection has produced red clover cultivars that are considerably improved for traits such as forage yield and quality as well as resistance and persistence (Taylor and Quesenberry 1996). However, these improved cultivars often show an unsatisfactory seed yield that leads to high production costs and limits the success of cultivars in the marketplace (Taylor and Quesenberry 1996). Seed yield may be increased to a certain extent through improved management practices involving irrigation (Oliva et al. 1994) or selection of soil type (Belzile 1991). However, path coefficient analyses have revealed a causal relationship among several components associated with seed yield, highlighting the complexity of this trait (Montardo et al. 2003; Oliva et al. 1994). Although both studies consistently reported a significant effect of the number of heads per plant on seed yield, they were limited to the phenotypic observation of only a few seed yield components.

The negative correlation of seed yield with other important agronomic traits, such as forage yield (Steiner et al. 1997) represents a further impediment for

improving this trait. This negative correlation seems to be particularly pronounced for persistence, i.e. the ability to produce constantly high forage yield across several growing periods. For example, continuous selection from locally adapted Swiss ecotypes led to the development of cultivars with considerably improved persistence, which are commonly referred to as Mattenklee cultivars (Herrmann et al. 2005). These cultivars show significantly increased forage yield across three or four growing periods when compared to other red clover cultivars (Lehmann and Briner 1998). However, the seed yield of Mattenklee cultivars is considerably lower when compared to less persistent cultivars (Deneufbourg 2004).

Molecular dissection of complex traits and the development of molecular markers linked to genes and quantitative trait loci (QTL) controlling such traits may provide new tools for breeding, which can complement traditional breeding approaches (Newbury 2003). Identification and integration of QTLs in genetic linkage maps is a promising step towards the development of molecular markers for marker-assisted breeding. Several examples have been reported for forage crops. In white clover (*Trifolium repens* L.) QTLs for seed yield and other seed yield components were recently identified (Barrett et al. 2005; Abberton et al. 2005). In perennial ryegrass (*Lolium perenne* L.) a genome region associated with four herbage quality traits was located in the vicinity of genes involved in lignin biosynthesis and is therefore a candidate region for more detailed characterisation of QTLs controlling herbage quality (Cogan et al. 2005). In major crops, such as soybean (*Glycine max* L.) QTLs have been identified for a large number of traits including seed yield (Mansur et al. 1996), and in addition, molecular markers have been developed for application in breeding, for example to select for a soybean cyst nematode resistance allele (Cregan et al. 1999b). However, forage crops in general and red clover in particular have lagged behind in this rapid development. Currently, two linkage maps, a basic prerequisite for identification of markers linked to important traits, have been reported for red clover; one consisting of 157 RFLP markers (Isobe et al. 2003) and the other consisting of 1,357 SSR and 148 RFLP markers (Sato et al., submitted). However, so far there is no information available on QTLs controlling important traits, such as seed yield for red clover.

Moreover, there is only limited information available for association among seed yield components, restricted to phenotypic observations of only few seed yield components. The analysis of additional traits, such as seed yield per head or time of flowering as well as the molecular dissection of these traits may help to elucidate the association of seed yield components and to develop new strategies for seed yield improvement.

The objectives of the study presented here were: (1) to characterise the association among eight seed yield components, (2) to identify components which are easy to score and thus allow for improved selection and (3) to identify genome regions containing QTLs for seed yield components for the future development of molecular markers for marker-assisted improvement of seed yield in red clover. For this purpose we established a red clover F_1 population segregating for seed yield components, investigated eight seed yield components in a field experiment, constructed a genetic linkage map using AFLP and SSR markers and performed QTL analysis.

Materials and methods

Plant material

A two-way pseudo-testcross population was created performing manual reciprocal pollinations and using two red clover genotypes with contrasting levels of seed yield. One genotype was selected from the cultivar Violetta (pV), a Belgian cultivar characterised by a high seed yield but displaying low persistence (CLO-DvP, Ghent, Belgium). The other genotype was selected from the Swiss Mattenklee cultivar Corvus (pC), which is characterised by excellent persistence but rather shows low seed yield (Agroscope FAL Reckenholz, Zurich, Switzerland).

Seeds were harvested from each maternal plant separately and a total of 400 seeds (200 per maternal plant) were germinated on wet filter paper (Schleicher and Schuell, Dassel, Germany) and transferred to soil-filled pots. Plants were grown for 3 months under long-day conditions [16 h light (≥100 $\mu Em^{-2}\ s^{-1}$)] in the greenhouse. To obtain plants with as many shoots as possible, they were then cultivated for 5 months under short-day conditions [9 h light (275 $\mu Em^{-2}\ s^{-1}$)] in the growth chamber. Clonal replicates were produced by cutting the plant in at least five parts, each containing equally sized longitudinal sections of the taproot. Roots were dipped in synthetic auxin (3–Indol Butyric Acid, Pokon and Chrysal International, Naarden, Netherlands) and regrown for 4 months in the greenhouse under long-day conditions as described previously.

Experimental conditions and phenotypic evaluation

In spring 2003, four clonal replicates of 280 genotypes (each parental plant serving as maternal genotype for a subset of 140 progenies) were planted in the field in a 4×4 lattice with the blocks arranged as a Latin square design, i.e. each genotype was represented once in each of the four rows and columns of the lattice, respectively. The field experiment was established at Agroscope FAL Reckenholz in Zurich, where temperature and rainfall average 8.5°C and 1,042 mm $year^{-1}$, respectively. Plants were established in a clay soil together with *Poa pratensis* cv. Compact sown at the time of planting.

Phenotypic evaluation of the seed yield components was performed after the first cut in summer 2004. Eight traits were investigated: Seed yield per plant (g; SYP), seed number per plant (SNP), seed yield per head

(g; SYH), seed number per head (SNH), head number per plant (HNP), thousand-seed weight (g; TSW), percent seed set (number of seeds per 100 florets; PSS) and time of flowering (days after first cut; TOF). TOF was defined as the day when three heads of a plant were flowering. On maturity, one head per plant was used to count florets and seeds in order to calculate PSS. Seed yield and number of seeds of 20 randomly picked heads, as well as seed yield of the remaining heads were determined to calculate SYP, SNP, SYH, SNH, HNP and TSW.

Analysis of variance was performed using the general linear model (GLM) of the STATISTICA software (version 6.1, StatSoft, Tulsa, OK, USA). Least square means were used for all further calculations. Heritability was calculated according to the formula $h^2 = \sigma_{g(mp)}{}^2/(\sigma_{g(mp)}{}^2 + \sigma_e{}^2/r)$, where $\sigma_{g(mp)}{}^2$ represented the variance component of the genotype nested within the maternal plant, $\sigma_e{}^2$ represented the variance component of the error term and r represented the number of replicates (Wricke and Weber 1986).

Genotyping

DNA of 254 genotypes was extracted from fresh or frozen leaf tissue using the DNeasy 96 plant kit (Qiagen, Hilden, Germany). AFLP analysis was performed as described by Herrmann et al. (2005) using 21 primer combinations. The primer combinations were named according to the standard primer combination code (Keygene, Wageningen, Netherlands; see Fig. 1). One hundred and seven SSR primer pairs were screened for polymorphism in the mapping population. SSR assays of primer pairs selected from TPSSR01 to TPSSR57 (Kölliker et al. 2005) were performed using the protocol of Kölliker et al. (2005) and 5′ pigtail addition to the reverse primer to promote non-templated adenylation of amplicons (Brownstein et al. 1996). Forward primers of SSR loci RCS004–RCS233 reported by Sato et al. (submitted) were modified by 5′ concatenation of the M13-18-mer 5′-TGTAAAACGACGGCCAGT-3′, which permitted concurrent fluorescence labelling of PCR products by a third primer (M13) with an incorporated fluorophore (Boutin-Ganache et al. 2001) and reverse primers carried the 5′ pigtail. PCR analyses were conducted in a total volume of 20 µl containing 15 ng DNA, 1× PCR buffer, 0.1 µM of forward primer, 0.4 µM of M13 and reverse primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 U *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions consisted of 4 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 50°C or 55°C depending on the primer pair, 30 s at 72°C, 10 cycles of 30 s at 95°C, 30 s at 53°C, 30 s at 72°C followed by a final extension of 10 min at 72°C.

AFLP and SSR amplification products were analysed on an ABI Prism 3100 genetic analyzer using POP-4 polymer and 36 cm capillaries, and scored using the Genotyper 3.6 software (Applied Biosystems, Foster City, CA, USA).

Linkage mapping and QTL analysis

A genetic linkage map was established using JoinMap (version 3.0; Van Ooijen and Voorrips 2001) and the CP (cross-pollination) population type. For grouping, a LOD threshold of four or lower was obtained. The order of loci was determined at LOD larger than 1.0, REC smaller than 0.4 and a jump threshold of five using Kosambi's mapping function.

QTL analysis was performed based on least square means of the genotypes using MapQTL (version 5.0, Van Ooijen 2004). In order to reduce computing time necessary for calculations with the CP population type and the MQM (multiple QTL model) algorithm, the number of loci was optimised for each trait as follows:

In a first step two maps were calculated for each parental plant, i.e. one map was calculated with loci heterozygous in pV, the other map contained loci heterozygous in pC. QTL analysis was performed on these maps for all traits using composite interval mapping (CIM) of PlabQTL with the genotypes coded as a doubled haploid (DH) population type (Utz and Melchinger 2003). In a second step, loci near putative QTLs identified in step one on both maps, as well as most biparental SSR loci were included to construct one combined reduced map with an average locus distance of approximately 10 cM. QTL analysis was then performed on this reduced map for all eight traits using backward cofactor selection and MQM mapping. In a last step, separate maps were constructed for each trait with increased locus density in regions where putative QTLs were identified in step two. Final QTL mapping was performed with backward cofactor selection and MQM mapping using these maps optimised for each trait. QTLs were taken into account when the LOD score was higher than the LOD threshold derived from the respective map using 10,000 permutations.

Results

Establishment of the mapping population and phenotypic evaluation

Of the 200 seeds harvested from each parental plant, 176 and 172 produced viable genotypes from pV and pC, respectively. Of these, 82% (140 from pV and 145 pC) yielded at least four equivalent clonal replicates. To include the same number of genotypes from each maternal plant, the final mapping population consisted of 280 F_1 genotypes.

Analyses of variance of the phenotypic data revealed highly significant variation among the 280 genotypes for all eight seed yield components studied (Table 1). No significant effect of the maternal plants was observed, i.e. there was no difference between genotypes where seed was harvested from pV when compared to genotypes where seed was harvested from pC. The proportion of variance explained was highest (0.71) for TSW followed

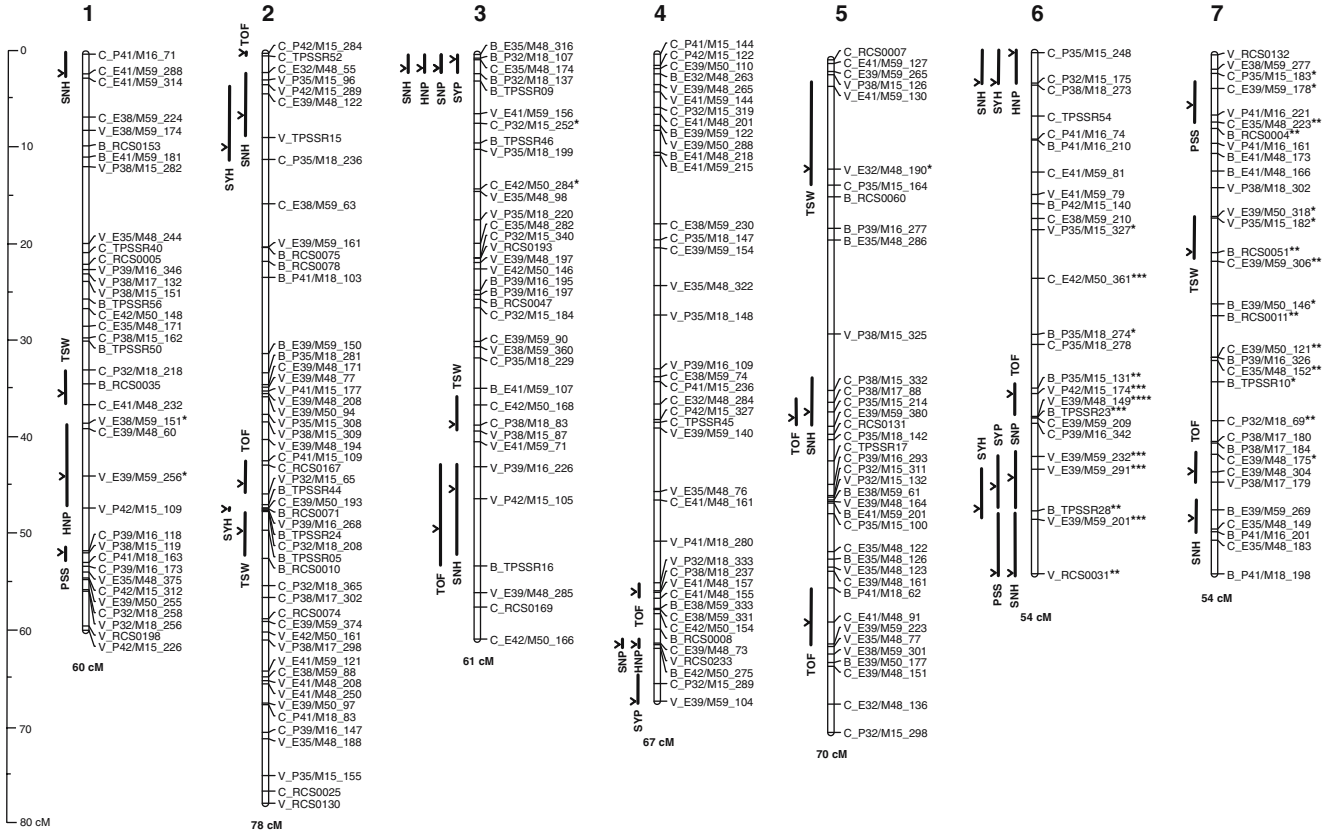

Fig. 1 Genetic linkage map of a red clover population based on 254 F_1 genotypes, 42 SSR and 216 AFLP loci. Locus names consist of a denomination of the origin of the parental alleles (B = bi-parental locus; C and V = mono-parental locus heterozygous in the parent from the cultivar Corvus and Violetta, respectively), followed by the locus name (standard primer combination code (Keygene, Wageningen, Netherlands) followed by the allele size in relative migration units for AFLP loci or the prefix TPSSR (Kölliker et al. 2005) and RCS (Sato et al., submitted) followed by an identification number for SSR loci, respectively). Significantly distorted loci are indicated by *asterisks* ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$). Positions of QTLs for eight seed yield components were calculated using MQM mapping, the optimised map for the respective trait and least square means of four replicates per genotype (*SYP* seed yield per plant; *SNP* seed number per plant; *SYH* seed yield per head; *SNH* seed number per head; *HNP* head number per plant; *TSW* thousand seed weight; *PSS* percent seed set; *TOF* time of flowering). The maximum LOD score position of each QTL is indicated with an *arrow* and a *bar* represents the interval between two positions obtained at LOD scores two units lower than the maximal score

by SNH (0.65) and TOF (0.64; Table 1). Heritability ranged from 0.51 for SYP to 0.85 for TSW (Table 2), whereas coefficients of variation ranged from 0.06 for TOF to 0.33 for SNP.

Pairwise comparison of SYP with the other seven seed yield components using product moment correlation revealed most of them to be highly significantly ($P < 0.01$) correlated with the exception of TOF which

Table 1 *F* values, level of significance and proportion of variance explained (R^2) of analysis of variance for eight seed yield components of a red clover population consisting of 280 F_1 genotypes assessed in a field experiment with four clonal replicates

	df	SYP	SNP	SYH	SNH	HNP	TSW	PSS	TOF
Maternal plant[a]	1	0.7^{NS}	1.9^{NS}	1.0^{NS}	2.2^{NS}	0.7^{NS}	1.6^{NS}	1.0^{NS}	0.4^{NS}
Genotype (maternal plant)	278	1.9^{***}	2.3^{***}	3.1^{***}	4.4^{***}	2.2^{***}	5.9^{***}	3.1^{***}	4.8^{***}
Column	3	18.5^{***}	18.0^{***}	19.1^{***}	23.0^{***}	10.7^{***}	4.8^{**}	11.1^{***}	5.5^{***}
Row	3	13.0^{***}	9.7^{***}	26.7^{***}	10.8^{***}	9.3^{***}	43.6^{***}	3.2^{*}	1.0^{NS}
Error	834								
R^2		0.47	0.50	0.58	0.65	0.48	0.71	0.56	0.64

SYP seed yield per plant; *SNP* seed number per plant; *SYH* seed yield per head; *SNH* seed number per head; *HNP* head number per plant; *TSW* thousand seed weight; *PSS* percent seed set; *TOF* time of flowering

[a] The population was based on reciprocal crosses where the parent from the cultivar Violetta (pV) served as maternal plant for one half of the genotypes and pC for the other half

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, *NS* not significant

showed only moderately significant ($P < 0.05$) correlation and TSW which was not significantly correlated to SYP (Table 3). Thereby, the correlation coefficients were highest for comparisons of SYP, SNP and HNP. Pairwise comparisons among the other seven seed yield components revealed two-thirds of them to be significantly ($P < 0.05$) correlated. For example, TSW and PSS as well as HNP and TOF showed a significantly negative correlation coefficient of −0.28 and −0.26, respectively (Table 3).

Linkage mapping

Forty-two (39%) of the 107 SSR loci analysed yielded polymorphisms among the 254 F_1 genotypes used for mapping (Table 4). AFLP analysis yielded a total of 216 polymorphic loci. The nine AFLP primer combinations based on the restriction enzymes *Eco*RI/*Mse*I extended by three selective nucleotides (*Eco* + 3/*Mse* + 3) yielded 5–19 polymorphic loci each, with a total of 117. The 12 *Pst* + 3/*Mse* + 2 primer combinations yielded 4–13 with a total of 99 loci (Table 4).

All 258 loci were mapped onto seven linkage groups (LG; Fig. 1). The length of the LGs ranged from 54 cM (LG 7) to 78 cM (LG 2) with an average of 63.5 cM, resulting in a total map length of 444.2 cM (Fig. 1). Average distance between loci varied from 1.5 (LG 2) to 2.2 (LG 6) with an average of 1.7 cM across the entire map.

Of the 258 loci 58 (22.5%) were bi-parental, i.e. both parents were heterozygous for that locus, whereas the remaining 200 (77.5%) loci were mono-parental, i.e. heterozygous in one parent and homozygous in the other parent (Table 4). No gap larger than 10 cM between two loci across the linkage map and no clustering of loci depending on the marker system or the restriction enzymes used was observed (Fig. 1).

The percentage of distorted segregation ($P \leq 0.05$) ranged from 5% for the 19 mono-parental SSR loci to 26% for the 23 bi-parental SSR loci resulting in an average of 12% for the total of 258 loci (Table 4). Highly distorted loci ($P \leq 0.001$) were mainly observed on LG 6 where 48% of the loci were distorted. On LG 7, 47% of the loci were distorted but distortion was less severe ($P > 0.001$; Fig. 1).

Table 2 Key characteristics of eight seed yield components of a red clover population consisting of 280 F_1 genotypes assessed in a field experiment based on least square means of four replicates per genotype

	Mean	Minimum	Maximum	SD[a]	h^2 [b]
SYP[c]	10.0	0.9	19.3	3.1	0.51
SNP	5,722	713	11,309	1,870	0.58
SYH	0.14	0.07	0.21	0.02	0.70
SNH	79	48	110	11	0.79
HNP	72	12	140	21	0.57
TSW	1.75	1.27	2.21	0.15	0.85
PSS	0.76	0.51	0.93	0.08	0.70
TOF	49	38	62	3	0.80

[a] Standard deviation
[b] Heritability
[c] For description of seed yield components, see Table 1

QTL analysis

A total of 38 significant QTLs, with a LOD score higher than the LOD threshold calculated from the respective trait and the corresponding map, were detected across all eight seed yield components (Table 5; Fig. 1). Three to eight QTLs were found per trait explaining together 33.8–69.1% of the total variance with an average of 48% across all traits. Individual QTLs explained 2.1–33.7% of the variance with an average of 10%. Two QTLs explained more than 30% (TSW and PSS), two explained 20–30% (SNH and TOF), whereas eight QTLs explained 10–20% of the total variance (Table 5). Seven additional QTLs were identified with LOD scores only slightly lower than the LOD threshold, i.e. two QTLs for SYP (LG 5, 51 cM; LG 1, 44 cM), two for SNP (LG 5, 37 cM; LG 1, 45 cM), one for TSW (LG 6, 48 cM) and two for PSS (LG 2, 78 cM; LG 1, 0 cM; data not shown).

On each LG four to nine significant QTLs were detected and up to five QTLs for different traits were located within 10 cM (LG 6, 44–54 cM). QTLs of highly correlated traits (Table 3) were often detected within 1–10 cM (Fig. 1). For example, all QTLs for SYP and for SNP, traits which showed a correlation coefficient of 0.95, were identified within 1 cM (LGs 3 and 6) and 6 cM (LG 4), respectively. In addition, of the seven QTLs identified for SYP and HNP, traits which showed a correlation coefficient of 0.87, two sets of two QTLs were located within 6 cM (LGs 3 and 4). On the other hand, QTLs of insignificantly correlated traits (Table 3) were often located on separate LGs or at least were not identified in the same region (Fig. 1). For example six QTLs of SYP and TSW, traits which showed an insignificant correlation coefficient of only 0.08, were detected on separate LGs, whereas two QTLs were located 37 cM apart on LG 3 (Fig. 1).

Discussion

The explanatory power of trait dissection and QTL analysis largely depends on a reliable assessment of the phenotypic variation for the traits under study. In outcrossing species, where homozygous lines are difficult to obtain, replicated field experiments often rely on clonal replicates of individual genotypes. However, for red clover such replicates are difficult to obtain (Cumming and Steppler 1961), because the tap root system is less suitable for cloning than the fibrous root system of grasses or the stoloniferous growth of white clover. Establishing plants under short-day conditions to enhance shoot formation and dipping cutlets in synthetic

Table 3 Product moment correlation coefficients for pairwise comparisons of eight seed yield components of a red clover population consisting of 280 F_1 genotypes

	SYP[a]	SNP	SYH	SNH	HNP	TSW	PSS
SNP	0.95^{***}						
SYH	0.42^{***}	0.32^{***}					
SNH	0.42^{***}	0.47^{***}	0.79^{***}				
HNP	0.87^{***}	0.89^{***}	0.02^{NS}	0.10^{NS}			
TSW	0.08^{NS}	-0.19^{**}	0.39^{***}	-0.20^{**}	-0.09^{NS}		
PSS	0.22^{***}	0.28^{***}	0.31^{***}	0.47^{***}	0.09^{NS}	-0.28^{***}	
TOF	-0.16^{*}	-0.23^{***}	0.11^{NS}	0.03^{NS}	-0.26^{***}	0.15^{*}	0.11^{NS}

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *NS* not significant
[a] For description of seed yield components, see Table 1

auxin to enhance root formation was highly successful with 82% of the genotypes producing four or more clonal replicates. This method provides a valuable prerequisite for further QTL analysis in red clover.

Besides reliable phenotypic data, a robust linkage map with evenly distributed markers is needed. The linkage map developed here consisted of seven linkage groups and was based on 258 SSR and AFLP loci (Fig. 1). As the map in this study and the map reported by Sato et al. (submitted) included common SSR loci, and the map reported by Isobe et al. (2003) in turn included RFLP loci present on the map of Sato et al. (submitted), a congruent numbering of LGs was used for all the three maps. The total map with a length of 444.2 cM was only slightly shorter than the 535.7 cM of the red clover map based on 157 RFLP loci reported by Isobe et al. (2003), but exhibited only half the length of a very recently developed map based on 1,505 SSR and RFLP loci (850.4 cM; Sato et al., submitted). The large difference in locus number may be one reason for the different map lengths, as was also reported for other species, such as perennial ryegrass (Armstead et al. 2002; Bert et al. 1999; Jones et al. 2002). Other factors, such as the heterogeneity of the parents, i.e. the particular parental genotype, may also play an important role. Since longer maps only rarely covered additional genome regions, when compared to shorter maps of the same species (Cregan et al. 1999a; Freyre et al. 1998), the 258 loci used in this study are likely to cover the majority of the red clover genome and to be sufficient for QTL analysis. However, complete genome coverage of the map presented here cannot be assumed.

For enhanced accuracy and power of QTL detection, increased number of genotypes rather than the number of loci used for analysis are crucial. A population size of at least 200 genotypes is needed to detect a QTL with an explained variance of 5% (Van Ooijen 1992). On the other hand, the power of detecting a QTL remains virtually the same no matter a map with an average locus distance of 10 cM or with an infinite number of loci is used (Darvasi et al. 1993). The average locus distance of 1.7 cM obtained in the present map therefore provided a good basis for QTL analysis.

In addition, the two marker systems used in this study complemented one another and allowed the construction of a meaningful map. On the one hand, the integrated SSR loci can be used to link the map with the other published red clover maps (Isobe et al. 2003; Sato et al., submitted) providing a valuable basis for further investigation of genome regions of interest. On the other hand, the AFLP loci offer a powerful tool to quickly fill gaps between SSR loci on linkage maps. One drawback of the AFLP marker system might be the clustering of loci in centromeric regions observed using the restriction enzyme *Eco*RI (Bert et al. 1999; Vuylsteke et al. 1999). Thus, to avoid clustering of AFLP loci but still ensuring coverage of centromeric regions, both rare cutting restriction enzymes, *Eco*RI and *Pst*I, were used. Although, the average distance between two loci of the map was comparable to maps in which clustering of

Table 4 Number of AFLP and SSR loci of a red clover linkage map based on 254 F_1 genotypes

	Bi-parental loci[a]			Mono-parental loci[b]		Total
	abxcd[c]	efxeg	hkxhk	lmxll (pC)	nnxnp (pV)	
SSR	13 (23%)	10 (30%)	–	12 (0%)	7 (14%)	42 (17%)
AFLP *Eco*/*Mse*[d]	–	–	19 (5%)	52 (15%)	46 (17%)	117 (15%)
AFLP *Pst*/*Mse*[d]	–	–	16 (13%)	47 (6%)	36 (8%)	99 (8%)
Total	13 (23%)	10 (30%)	35 (9%)	111 (10%)	89 (13%)	258 (12%)

The percentage of distorted loci is indicated in parentheses
[a] Both parents were heterozygous for these loci
[b] Heterozygous in one parent [from the cultivar Corvus (pC) or Violetta (pV)] and homozygous in the other parent
[c] Segregation types according to JoinMap (Van Ooijen and Voorrips 2001)
[d] AFLP primer combinations based on the restriction enzymes *Eco*RI and *Mse*I or *Pst*I and *Mse*I, respectively

Table 5 Position and description of QTLs identified using MQM mapping, the optimised map for the respective trait and least square means of eight seed yield components of a red clover population consisting of 254 F_1 genotypes

Trait	Linkage group	Position (cM)	Closest neighbouring locus	Maximum LOD score[a]	Percentage variance explained
SYP[b]	3	0.6	C_E35/M48_174[c]	10.37	15.3
	4	67.3	V_E39/M59_104	4.90	7.0
	6	45.3	V_E39/M59_291	7.60	11.5
					Total 33.8
SNP	3	1.6	B_P32/M18_137	9.89	14.0
	4	61.4	V_RCS0233	6.02	8.1
	6	44.3	V_E39/M59_291	9.65	14.4
					Total 36.5
SYH	2	9.8	V_TPSSR15	6.56	8.1
	2	47.6	B_TPSSR24	7.60	6.4
	6	3.2	C_P32/M15_175	3.88	7.7
	6	47.3	B_TPSSR28	11.05	17.5
					Total 39.7
SNH	1	2.1	C_E41/M59_288	8.86	8.2
	2	6.2	C_E39/M48_122	4.79	2.6
	3	1.6	B_P32/M18_137	3.94	3.4
	3	44.9	V_P42/M15_105	5.49	5.4
	5	36.6	C_P35/M15_214	5.04	2.1
	6	3.2	C_P32/M15_175	4.81	7.0
	6	54.2	V_RCS0031	21.74	25.7
	7	48.3	C_E35/M48_149	11.25	8.5
					Total 62.9
HNP	1	43.9	V_E39/M59_256	5.35	6.7
	3	1.6	B_P32/M18_137	10.55	13.2
	4	61.4	V_RCS0233	7.92	9.9
	6	0.0	C_P35/M15_248	4.34	7.7
					Total 37.5
TSW	1	35.3	B_RCS0035	5.77	5.5
	2	49.6	B_TPSSR05	28.23	32.0
	3	38.5	C_P38/M18_83	4.35	4.0
	5	11.4	V_E32/M48_190	12.50	12.3
	7	20.6	B_RCS0051	5.17	4.7
					Total 58.5
PSS	1	51.9	V_P38/M15_119	4.63	5.1
	6	54.2	V_RCS0031	18.34	33.7
	7	5.5	V_P41/M16_221	3.93	4.7
					Total 43.5
TOF	2	0.0	C_P42/M15_284	9.56	8.4
	2	44.8	V_P32/M15_65	26.26	23.8
	3	49.2	V_P42/M15_105	7.50	6.4
	4	56.0	V_E41/M48_157	7.16	5.2
	5	37.6	C_E39/M59_380	5.17	4.5
	5	58.5	C_E41/M48_91	8.46	7.1
	6	35.5	V_P42/M15_174	12.52	10.5
	7	43.4	C_E39/M48_304	4.47	3.2
					Total 69.1

[a]Significant LOD threshold was 3.6 except for SNH where it was 3.7
[b]For description of seed yield components, see Table 1
[c]For description of loci, see Fig. 1

*Eco*RI loci was observed (Bert et al. 1999), we observed no such clustering (Fig. 1).

Due to the manifestation of deleterious recessives, inbreeding depression may lead to substantial segregation distortion and may negatively influence the stability of genetic maps as well as the accuracy of QTL analyses (Brummer et al. 1993; Echt et al. 1994). The use of non-inbred F_1 populations may be an effective way to avoid distortion in self-incompatible species, such as red clover (Tavoletti et al. 1996). Indeed, the proportion of significantly ($P \leq 0.05$) distorted loci observed (Table 4) was only 12%, which is considerably lower than the 37% observed in a backcross population of red clover (Isobe et al. 2003). Self-incompatibility may be another reason for segregation distortion and may result in a clustering of distorted loci around a self-incompatibility locus (Bert et al. 1999). Thus, the region on LG 6, where highly distorted loci were observed, might correspond to the single self-incompatibility locus reported in red clover (Lawrence 1996). Segregation distortion based on self-incompatibility can only occur if the parents share a common self-incompatibility allele. If this were the case, not all of the three resulting genotypes would have the same probability to be successfully pollinated. If pollen

is scarce, this may lead to incomplete pollination of florets resulting, first of all, in a negative effect onto PSS. As a consequence, ghost QTLs for seed yield components might be observed near the self-incompatibility locus. However, a lack of pollen is very unlikely for the present study since three quarters of the 1,120 individuals flowered within 10 days (data not shown) and PSS was comparable to other studies (Oliva et al. 1994). Moreover, the theoretical proportion of compatible pollen in an isolated progeny of a cross between two parents sharing one self-incompatibility allele varies only between 31.25 and 37.5% for the handicapped and favoured genotypes, respectively.

The overall aim of this study was not only to identify QTLs for the development of molecular markers linked to seed yield, but also to elucidate the association among seed yield components. According to the correlation coefficients, SNP and HNP showed the largest effect on SYP (Table 3). This result is congruent with path coefficient analyses for seed yield in red clover, for which the number of heads was identified as the primary component affecting seed yield (Montardo et al. 2003; Oliva et al. 1994). Some of the seed yield components were not determined independently and showed some mathematical causality, partially explaining their correlation. However, detailed QTL analyses confirmed these associations since QTLs for SNP, HNP, SNH, SYH and PSS were detected in the same regions ($\leq$ 10 cM) like the three QTLs for SYP (Fig. 1; Table 5). Although all five factors substantially influence SYP, only HNP offers an advantage for phenotypic selection compared to SYP. HNP is comparatively easy to determine and can be assessed in the field earlier before seed maturity. Therefore, the selection for increased HNP may present a valuable strategy to improve seed yield in red clover.

On the other hand, TSW showed insignificant correlation with HNP and SYP (Table 3), which was in congruence with path coefficient analysis in red clover where the influence of thousand-seed weight was minor (Montardo et al. 2003; Oliva et al. 1994), or with white clover where thousand-seed weight was not correlated with seed yield and inflorescence density (Barrett et al. 2005). Since only two of a total of nine QTLs for HNP and TSW were detected in the same region, successful selection for TSW may be possible independently of the proposed selection for HNP. The QTL located on LG 2 is of particular interest for further investigations to improve TSW, as it explains 32% of the variation.

We were able to identify two regions covering less than 10 cM, where five (LG 6) and four (LG 3) QTLs of different seed yield components were clustered, respectively (Fig. 1). All but one QTL in these two regions explained more than 10% of the total variation (Table 5). However, QTL analysis based on segregating populations derived form parents with contrasting phenotypes has several limitations. The precision and accuracy of QTL detection depends on a large population size. Small populations lead to an underestimation of the number of QTLs and an overestimation of the explained variance (Schön et al. 2004) as well as to a limited precision regarding the QTL position (Visscher and Goddard 2004). This is particularly true when assessing traits influenced by a high number of QTLs with small effects (Schön et al. 2004). Moreover, in contrast to more recent approaches like association mapping, only two alleles at a given locus can be studied simultaneously. However, association mapping relies on information about the nature of linkage disequilibrium within the genome of the respective plant species (Flint-Garcia et al. 2003), which is currently not available. Taken these limitations into account, the presented results may still serve as a valuable base for further molecular dissection of seed yield in red clover. For future studies, the following approaches may be considered. Fine mapping of the detected QTL, using the information of the recently published high-density SSR map (Sato et al., submitted), offers a promising possibility to identify closely linked markers for marker-assisted breeding or even the identification of genes involved in the control of seed yield by map-based cloning. The recently published EST resources of the model legume barrel medic (*Medicago truncatula;* Cannon et al. 2005) offer an additional possibility to further explore genetic control of seed yield in red clover. A prerequisite to apply such information is a certain degree of synteny between the target species (i.e. red clover) and the species for which genetic information is available. The existence of syntenic relationships between a number of legume species including barrel medic, alfalfa (*Medicago sativa*), soybean, pea and birds foot trefoil (*Lotus japonicus*) has been demonstrated (Choi et al. 2004). Thus, comparative techniques such as comparative anchor marker tag sequences (CATS; Schauser et al. 2005) or single nucleotide polymorphisms (SNP; Andersen and Lübberstedt 2003) may be alternative approaches for future investigations. Based on genes and QTLs associated with seed yield in other species, such as barrel medic (Cannon et al. 2005) or white clover (Barrett et al. 2005), these approaches may help to elucidate genetic control of seed yield in red clover. In conclusion, with the stable linkage map obtained using SSR and AFLP loci and 254 genotypes of a F_1 population as well as with the field analysis based on four clonal replicates, a solid basis for QTL analysis for seed yield components was provided. A total of 38 QTLs were detected for the eight seed yield components. The associations among seed yield components allowed the identification of head number per plant as an easy to determine, indirect character to select for seed yield. Furthermore, two genome regions rich in QTLs for seed yield components were identified with great potential for future characterisation and the development of markers closely linked to seed yield components. To the best of our knowledge, this is the first report on QTL analysis in red clover, which presents an important first step towards marker-assisted selection and may help to implement new breeding strategies to complement breeding for complex traits, such as seed yield.

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