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QTL mapping reveals a two-step model for the evolutionary reduction of inner microsporangia within the asteracean genus *Microseris*

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Abstract The reduction of inner (adaxial) pollen sacs (microsporangia, MS) as a diagnostic character for the three asteracean species, *Microseris bigelovii, Microseris elegans* and *Microseris pygmaea*, was analysed in an interspecific cross between *Microseris douglasii* and *Microseris bigelovii* with 4 MS and 2 MS, respectively, using the average number of MS per plant as a quantitative character. A previous QTL (Quantitative Trait Locus) analysis had revealed one major QTL (3B) and three modifier QTLs (3A, 4A, 7A) with epistatic effects only on the homozygous recessive 2 MS genotype of QTL 3B. Here we performed a bulked segregant analysis on four 2 MS and four 4 MS DNA-bulks with 407 *Eco*RI/*Mse*I AFLP-primer combinations each. In this way additional AFLP markers were mapped close to QTL 3B and QTL 3A. Three of them were converted to SCAR (Sequence Characterized Amplified region) markers. All markers were tested in natural populations of the disporangiate (2 MS) species *M. bigelovii, M. elegans* and *M. pygmaea*, and in different populations of tetrasporangiate (4 MS) *M. douglasii*. The marker distribution suggests that locus 3B mutated in a progenitor of the disporangiate species. QTL 3A has evolved in the 2 MS background of the major gene in the disporangiate species. Since *M. pygmaea* and *M. bigelovii* are the sister group to *M. elegans*, the 4 MS genotype for (markers of) QTL 3A in *M. pygmaea* populations is most likely due to a back mutation to the 4 MS state and could explain the slight instability of the 2 MS phenotype in this species.

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Introduction

Taxonomically relevant (diagnostic) characters are rare within plants. Within the angiosperms such characters often concern the invariant number of organs or organ parts (meristic characters). The number of four pollen sacs (microsporangia, MS) per anther is characteristic for about 90% of all angiosperm species. Anthers with only two pollen sacs (disporangiate anthers, 2 MS) seem to have evolved independently from tetrasporangiate (4 MS) anthers in about 50 angiosperm families by partly different mechanisms (Endress and Stumpf 1990). Within the asteracean genus *Microseris* disporangiate anthers have evolved once within the group of autogamous annual species by the reduction of the inner (adaxial) microsporangia (Battjes et al. 1994). This evolutionary reduction is one rare example where the evolution and the genetics of a diagnostic character have been examined in detail (Battjes et al. 1994; Gailing et al. 1999). Molecular studies with chloroplast DNA (Wallace and Jansen 1995), RAPDs (Roelofs and Bachmann 1997a, b) and ITS (Roelofs et al. 1997) in addition to morphological studies (Chambers 1955) have shown the common derived position of *M. bigelovii, M. elegans* and *M. pygmaea* with 2 MS relative to *M. douglasii* with 4 MS. The reduction of the adaxial MS thus is a shared derived character (= a synapomorphy) for these three species.

In a previous study we performed a QTL study based on an AFLP (Amplified Fragment Length Polymorphism) marker map (Bachmann and Hombergen 1996) in the F6 inbred population of an interspecific cross between *M. douglasii* with 4 MS and *M. bigelovii* with 2 MS (Gailing et al. 1999). One major gene (QTL 3B) and four modifiers (QTL 1A, 3A, 4A and 7A) specifically segregated with the MS character. QTL genotypes could be predicted from sets of closely linked markers. Plants with the homozygous recessive genotype of QTL 3B showed a

bimodal distribution with about equal numbers of plants showing near 2 MS or near 4 MS per anther. Three of the modifiers (QTL 3A, 4A, 7A) acted only in the homozygous recessive background of the major gene to reduce the number of MS to 2. Thus our previous study suggested a two-step evolution of the disporangiate anther: (1) the mutation of a major gene exhibiting an unstable MS phenotype, and (2) the selection for the modifiers 3A, 4A and 7A in the 2 MS – background of the major gene 3B. The goal of the present research is (1) to identify additional markers that are closely linked to the previously identified QTL, 3B and 3A, by means of bulked segregant analysis (Michelmore et al. 1991), and (2) to use these markers to infer the distribution of the linked QTL in natural populations of *Microseris* that vary in the MS character.

This approach is based on linkage disequilibrium between multiple markers flanking the QTL with the relevant QTL alleles, and can only be seen as a first approximation to the population genetics of the genes affecting the MS phenotype. Molecular examination of naturally occurring alleles of the homeotic flowering gene CAULFLOWER (CAL) showed a high intraspecific variability that was correlated with differences in flower morphology between populations (Purugganan and Suddith 1998; Purugganan 2000).

Material and methods

Plant material

Plants from total of 85 populations of *M. douglasii, M. bigelovii, M. elegans* and *M. pygmaea* (Table 1) were tested for their QTL genotypes. All populations of *M. douglasii* have 4 MS per anther, while *M. bigelovii, M. elegans* and *M. pygmaea* possess 2 MS per anther. *M. pygmaea* accessions A92 and B95 and samples from Peru have trisporangiate anthers in at least some flowers of one flowering head and some adaxial MS are partly developed. While *M. douglasii, M. bigelovii* and *M. elegans* are distributed in North Western America, *M. pygmaea* occurs only in South America (Chile, Peru).

The interspecific cross (accession number H27) between the annual species *M. douglasii* (accession B14) with 4 MS and *M. bigelovii* (accession C94) with 2 MS was done by J. Battjes in 1991. Spontaneous selfing in a single F1 hybrid resulted in 106 F2 plants. The original linkage map based on AFLP (Vos et al. 1995) was constructed in 150 F5 plants derived from 82 plants of the original F2 (Bachmann and Hombergen 1996). F6 and F7 inbred generations were derived from individual F5 plants.

We determined the number of pollen sacs in all plants as follows: closed capitula of about 3 mm in diameter were harvested, immediately fixed in Craf III solution and dehydrated in a graded butanol series (Gailing et al. 1999). The flowering heads were embedded in glycol metacrylate (Feder and O'Brien 1968) and sectioned with a rotary microtome. Sections were stained with periodic acid-Schiff's reaction (PAS, Sigma) and toluidine blue (TBO, Sigma).

QTL mapping

In our former study (Gailing et al. 1999) QTLs for the MS character were mapped using simple regression methods and interval mapping options in the computer packages Qgene (Nelson 1997) and QTL cartographer (Basten et al. 1994, 1997). Homozygous and heterozygous QTL genotypes were predicted from linked markers with opposite polarity (i.e. from the one or the other parent). The individual QTL effects and their interactions were tested by an unpaired *t*-test. Epistasis was detected by testing the effects of the modifiers on the 2 MS and 4 MS genotypes of the major QTL 3B.

Bulked segregant analysis

In order to find additional markers closely linked to QTL 3B and QTL 3A we performed a bulked segregant analysis (Michelmore et al. 1991) based on the QTL genotypes of QTL 3B and QTL 3A (Table 2). DNA was isolated with the DNeasy plant mini kit (Qiagen) from 107 F7 plants derived by repeated spontaneous selfing from F5 plants. In order to map additional markers to QTL 3B and QTL 3A (Gailing et al. 1999) for each QTL genotype (2 MS = *M. bigelovii* genotype, 4 MS = *M. douglasii* genotype) three bulks of 10, 15 and 30 plants were prepared (a total of 6 bulks). The *M. bigelovii* allele frequencies in these six bulks are given for all QTLs in Table 2. Two additional DNA bulks were constructed from natural populations of *Microseris* that varied in the MS character. The 2 MS bulk was assembled from 16 populations representing the species *M. bigelovii, M. elegans* and *M. pygmaea* and the 4 MS bulk from 16 populations of *M. douglasii*. A total of 407 *Eco*RI/*Mse*I primer combinations were tested in each of the eight bulks. Diagnostic bands for the MS character in the "QTL bulks" and in the "population bulks" were tested in 16 disporangiate plants and in 16 tetrasporangiate plants, and mapped to linkage group 03 in the inbred population of 107 plants with Mapmaker Macintosh V2.0 using the Kosambi mapping function and the commands FIRST-ORDER, RIPPLE and TRY (Lander et al. 1987; modified by S. Tingey for Macintosh). The marker order on linkage group 03 was colinear with the map order generated with JOINMAP (Stam 1993). Markers not correlated with the MS phenotype but closely linked to QTL 3B and QTL 3A on the former linkage group did not map on that linkage group. Association between marker and MS phenotype was determined by simple linear regression analysis (Haley and Knott 1992), by multiple regression and interval mapping (Lander and Botstein 1989) with an interval size of 1 cM (Nelson 1997). In order to set significance thresholds, a permutation test (1,000 shuffles) proposed by Churchill and Doerge (1994) was performed with the software package Qgene (Nelson 1997). QTL effects were also tested by the application of non-parametric statistics [Spearman's rank correlation coefficient, U-test = Wilcoxon-Mann-Witney test; Qgene by Nelson (1997), and StatView from the SAS Institute]. The results were consistent with those obtained by parametric methods. With all test statistics the effects of QTL 3A and QTL 3B were highly significant (data not shown). Additionally the markers associated with QTL 3B and QTL 3A were tested in different *M. douglasii, M. bigelovii, M. elegans* and *M. pygmaea* lines.

AFLP

AFLPs were generated following the protocol of Vos et al. (1995) with slight modifications. We used *Eco*RI and *Mse*I as restriction enzymes and the appropriate primer combinations. PCR reactions were carried out in a Gene Amp PCR System 9700 thermocycler (PE Applied Biosystems) with a heated lid. The PCR profile for the preselective amplification was an initial cycle of 2 min at 72 °C followed by 20 cycles of 10 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C and a 30 min extension step at 60 °C. Four microliters of the diluted preselective AFLP (1:25 or 1:50 depending on the concentration of the preselective PCR product) were used in a selective amplification with E+3 and M+3 selective primers that carry three selective bases beginning with a 2 min denaturation step at 94 °C followed by 33 cycles with 10 s at 94 °C, a 30 s annealing step and a 2 min extension step at 72 °C. The initial annealing temperature of 65 °C was reduced subsequently in the first nine cycles by 1 °C. The reaction was continued at 56 °C for the last 24 cycles ending with

Table 1 Populations of *Microseris* used in this study. Most strains are from California (USA: California). Collections by K. Bachmann, J. Battjes and H. J. Price were in cooperation with K. L. Chambers

Species	Population	Description
M. douglasii	A26	USA: CA, Humboldt Co. Garberville. Coll. K. L. Chambers
M. douglasii	B13	USA: CA, Solano Co. Rio Vista Junction. Coll. K. L. Chambers
M. douglasii	B14	USA: CA, Fresno Co. Parkfield. Coll. K. L. Chambers
M. douglasii	B15	USA: CA, San Francisco Co. Presidio. Coll. K.L. Chambers
M. douglasii	B18	USA: CA, San Luis Obispo Co. Morro Bay. Coll. K. L. Chambers
M. douglasii	B21	USA: CA, San Francisco Co. Presidio. Coll. K.L. Chambers
M. douglasii ssp. *tenella*	B39	USA: CA, Monterey Co. Tularcitos Creek. Coll. K. L. Chambers
M. douglasii	B57	USA: CA, Fresno Co. Panoche Road. Coll. K. Bachmann
M. douglasii	B77	USA: CA, San Mateo Co. Woodside. Coll. K. L. Chambers
M. douglasii	C57	USA: CA, San Luis Obispo Co. Cayucos. Coll. K. L. Chambers
M. douglasii	D57	USA: CA, Santa Clara Co. Jasper Ridge. Coll. K. L. Chambers
M. douglasii	D68	USA: CA, San Luis Obispo Co. Cuesta Park. Coll. J. Price
M. douglasii	D81	USA: CA, Fresno Co. Parkfield-Coalinga Road. Coll. J. Price
M. douglasii	E34	USA: CA, Tehama Co. Corning Corner, Coll. J. Battjes
M. douglasii	E43	USA: CA, San Luis Obispo Co. Sycamore Ridge. Coll. J. Battjes
M. douglasii	E44	USA: CA, San Luis Obispo Co. Cripple Creek. Coll. J. Battjes
M. douglasii	E52	USA: CA, San Luis Obispo Co. Cholame-II. Coll. J. Battjes
M. douglasii	E55	USA: CA, Monterey Co. Jolon. Coll. J. Battjes
M. douglasii	E59	USA: CA, Alameda Co. Midway. Coll. J. Battjes
M. douglasii	E60	USA: CA, Solano Co. Rio Vista. Coll. J. Battjes
M. douglasii	E60A	USA: CA, Solano Co. Rio Vista. Coll. J. Battjes
M. douglasii	E63	USA: CA, Solano Co. Cook Lane. Coll. J. Battjes
M. douglasii	E68	USA: CA, Colusa Co. Cortina Ridge-II Coll. J. Battjes
M. douglasii	E68A	USA: CA, Colusa Co. Cortina Ridge-II Coll. J. Battjes
M. douglasii ssp. *platycarpha*	E73	USA: CA, Riverside Co. Alberhill Mountain. Coll. K. L. Chambers
M. elegans	A24	USA: CA, Humboldt Co. Garberville. Coll. K. L. Chambers
M. elegans	B33	USA: CA, Monterey Co. Jolon. Coll. K. L. Chambers
M. elegans	B36	USA: CA, San Diego Co. SanDiego. Coll. K. L. Chambers
M. elegans	B56	USA: CA, Fresno Co. Little Panoche. Coll. K. Bachmann
M. elegans	C48	USA: CA, Monterey Co. Jolon. Coll. H. J. Price
M. elegans	D02	USA: CA, Santa Clara Co. San Antonio Valley Road. Coll. K.L. Chambers
M. elegans	D03	USA: CA, San Luis Obispo Co. Cholame. Coll. K. L. Chambers
M. elegans	D04	USA: CA, San Luis Obispo Co. Parkfield Connector. Coll. K. L. Chambers
M. elegans	D05	USA: CA, San Joaquin Co. Corral Hollow. Coll. K. L. Chambers
M. elegans	D07	USA: CA, Fresno Co. Parkfield. Coll. K. L. Chambers
M. elegans	D08	USA: CA, Solano Co. Dixon. Coll. K. L. Chambers
M. elegans	D09	USA: CA, Solano Co. Rio Vista. Coll. K. L. Chambers
M. bigelovii	C93	USA: CA, Santa Barbara Co. Pt. Sal. Coll. K. L. Chambers
M. bigelovii	C94	CANADA: BC, Victoria, Uplands Park. Coll. M. C. Melburn
M. bigelovii	D33	USA: CA, San Luis Obispo Co. Co. Cambria. Coll. K. L. Chambers
M. bigelovii	D43	USA: CA, Mendocino Co. Ft. Bragg. Coll. K. L. Chambers
M. bigelovii	D46	USA: CA, San Mateo Co. Pescadero. Coll. K.L. Chambers
M. bigelovii	D52	USA: OR, Curry Co. Goat Island. Coll. L. R. Johnston
M. bigelovii	D58	USA: CA, Monterey Co. Laureles Canyon. Coll. J. Price
M. bigelovii	E22	CANADA: BC, Vancouver Island, Victoria, Uplands Park, Cattle Point. Coll. A. Ceska
M. bigelovii	F11	CANADA: BC, Vancouver Island, Victoria, Saxe Point. Coll. A. Ceska
M. pygmaea	A92	CHILE, Prov. de Santiago exact locality unknown
M. pygmaea	B95	(from Botanical Garden Berlin, originally Botanical Garden Nijmegen, The Netherlands)
M. pygmaea	C37	CHILE, O'Higgins Prov., Rancagua. Coll. C.E. Munoz
M. pygmaea	C95	CHILE, IV. Región (de Coquimbo), Prov. de Choapa, Panamericana Norte km 297. Huentelauquen. Coll. J. Grau
M. pygmaea	C96	CHILE, IV. Región (de Coquimbo), Prov. de Choapa; El Teniente. Coll. J. Grau
M. pygmaea	C97	CHILE, IV. Región (de Bernardo O'Higgins), Prov. de Colchagua, Camino del Cobre. Coll. J. Grau
M. pygmaea	C98B	CHILE, Región Metropolitana, Prov. de Santiago, Cuesta Barriga. Coll. J. Grau
M. pygmaea	C98S	same location as C98B
M. pygmaea	C99	CHILE, IV. Región (de Coquimbo), Los Vilos. Coll. J. Grau
M. pygmaea	G70	PERU: Canta, Lachaqui. Coll. G. Vilcapoma, 30 inbred lines from individual field-collected plants

Table 2 *M. bigelovii* (2 MS) allele frequencies in the 4 MS and 2 MS DNA bulks

DNA bulks	QTL 3B	QTL 3A	QTL 7A	QTL 4A	QTL 1A
B2A (10 plants)	1	1	1	1	0.66
B2B (15 plants)	1	1	1	0.66	0.82
B2C (30 plants)	1	0.85	0.83	0.62	0.57
B4A (10 plants)	0	0.6	0.55	0.7	0.4
B4B (15 plants)	0	0.4	0.7	0.5	0.36
B4C (30 plants)	0	0.2	0.57	0.5	0.5

Table 3 SCAR primers for the number of MS in *Microseris*. The *Eco*RI respectively *Mse*I restriction sites are underlined. T_m-values were supplied by the manufacturer (metabion)

Primer name	Sequence (5′–3′)	*n*	T_m (°C)	% GC	Base pairs
E33/M69eco	GAATTCAAGATTCAAAATAAATGTTAG	27	54.3	22.2	185
E33/M69mse	TTAAGCGAGGTAAAGGTTTCG	21	55.9	42.9	185
E36/M52eco	GAATTCACCAAGTAGTTGATTTTGTG	26	58.5	34.6	145
E36/M52mse	TTAACCCATACACTGGTTTTTGAAAC	26	58.5	34.6	145
E37/M53eco	GTCATAGCCGAATCCATTTTATTC	24	57.6	37.5	170
E37/M53mse	GATAAGCAATAAGGCGGATTCC	22	58.4	45.5	170

30 min at 60 °C. The PCR reactions were carried out with *Eco*RI primers that were labelled with the fluorescent dyes 6- FAM (blue), JOE (green) or NED (yellow) with different emission maxima. GS 500 Rox (labelled with the fluorescent dye ROX) from Applied Biosystems was used as an internal size standard. Fragments were separated on an ABI PRISM 377 DNA sequencer (Applied Biosystems) in a multiplex analysis. AFLP gels were analysed with the GeneScan analysis and Genotyper 2.5 software from Applied Biosystems.

Development of SCAR markers from AFLP fragments

Sequence Characterized Amplified Region (SCAR) markers (Paran and Michelmore 1993) were developed from sequenced AFLP bands. In order to reduce the number of fragments, the unlabelled selective *Mse*I primers were elongated by one base (A, C, G or T) and used for a selective AFLP with the labelled *Eco*RI+3 primer. AFLP fragments were separated on a 10% denaturing polyacrylamide (PAA) gel and stained with silver nitrate according to Budowle et al. (1991). Bands of the estimated size were excised from the gel (with a scalpel) and diluted in 3 μl 1× TE buffer. Gel fragments were heated for 5 min in a 95 °C waterbath and 2 μl were used for the re-amplification of the fragment using the corresponding *Eco*RI/*Mse*I primers. The *Eco*RI primers were lablelled with the fluorescent dyes 6- FAM, NED, JOE, in order to check the fragment size in a subsequent ABI gel run. The PCR conditions were the same as in the selective AFLP protocol. PCR products were gel-purified from a 2% agarose gel or directly cloned into the pGEM-T-Easy-Vector-System (Promega). Successful transformation was tested by blue/white selection. Inserted fragments were amplified in a PCR reaction with the T7 and SP6 plasmid primers. Fragments of estimated size were sequenced on an ABI 377 PRISM DNA sequencer using the dideoxy mediated chain-termination method (Sanger et al. 1977) of the ABI PRISM dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were compared with sequences in the EMBL database using the program Fasta3 (Pearson and Lipman 1988). Primers were designed close to the 5′- end of the *Eco*RI or *Mse*I restriction site. The programs Oligo Calculator (http://mbcf.dfci.harvard.edu/docs/oligocalc.html) and oligo 3.4 (Rychlik and Rhoads 1989) were used for primer design. Primers were checked for self-annealing, dimer and hairpin formation. Specific primers were used for PCR amplification from genomic DNA in the F7 inbred population and in natural populations of *Microseris* that are polymorphic in the MS character. PCR probes were denatured at 95 °C for 3 min followed by 38 cycles at 95 °C for 30 s, T_m +2 °C for 30 s and 70 °C for 45 s, and an extension step at 70 °C for 8 min. The annealing temperature was chosen 2 °C above the melting temperature (T_m; Table 3).

Results

Bulked segregant analysis

In the DNA bulks, 65 AFLP markers showed reproducible amplification. Testing of these markers in the 32 individual 2 MS and 4 MS F7 plants resulted in 20 AFLP markers, or about 30% confirmed to discriminate between the genotypes. The confirmed markers were then mapped in the F7 inbred population relative to QTL 3B and QTL 3A of our former study (Gailing et al. 1999) providing 16 new AFLP markers on linkage group 03 (Fig. 1). One marker from *M. douglasii* (E41/M60 – 227 bp) and one marker from *M. bigelovii* (E36/M75 – 160 bp) were unlinked but significantly correlated with the number of MS ($R^2 = 0.26$; $R^2 = 0.22$). The new AFLP markers are named according to the *Eco*RI/*Mse*I primer combinations (nomenclature according to Keygene). Fragments from which SCAR markers have been developed are printed in boldface (Fig. 1). The QTL effects are characterized by the coefficient of determination, R^2. Threshold values for R^2 are indicated by different grey shades, and a LOD score value of 3.0 is given as reference line. Markers with the strongest effect on the MS character, map close to the strongest modifier gene QTL 3A of our former study ($R^2 > 0.30$, $R^2_{max} = 0.36$). The mean difference between plants with the *M. douglasii* and the *M. bigelovii* genotypes of this QTL is 0.93 MS (the average of 3.58 MS for the *M. douglasii* allele minus an average of 2.65 MS for the *M. bigelovii* allele). New markers associated with the previously identified major QTL 3B showed weaker effects than those of QTL 3A in the present analysis (Fig. 1, $R^2_{max} = 0.19$; 2.99 MS vs 3.64 MS). In accordance with our previous results, the new markers for QTL 3A have only a significant effect on the *M. bigelovii* (2 MS) allele of the "major gene" QTL 3B

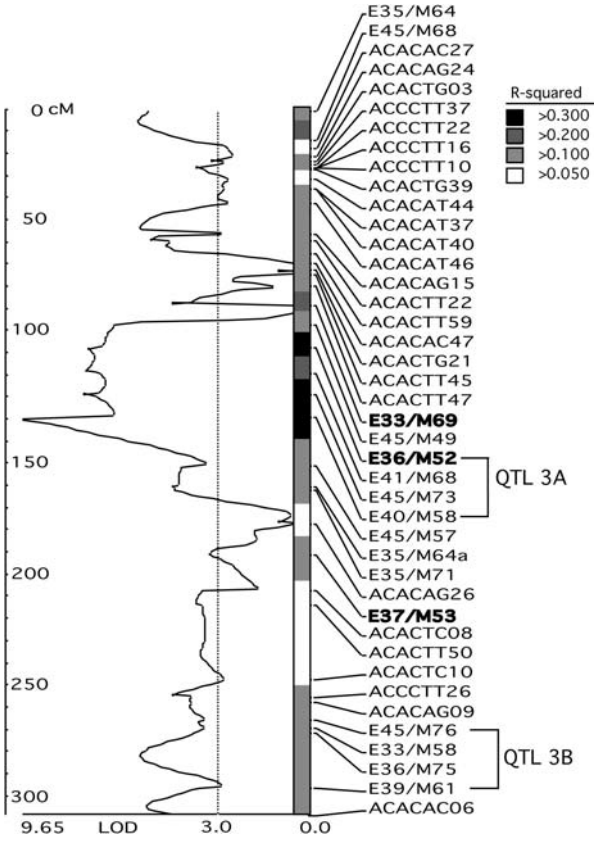

Fig. 1 Marker order of linkage group 03 with QTL 3B and QTL 3A. R^2 values of regression analysis are indicated in different grey shades. Dark regions indicate R^2 values above 0.30. Corresponding LOD scores of the interval analysis are shown on the left site. A LOD score of 3.0 is used as threshold. For bold-typed loci SCAR markers have been developed. All markers mapped in this study are named according to the primer combinations used to amplify the fragment (for example E40/M58, the nomenclature follows the standard list for AFLP primer, http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html). The remaining markers were mapped in a previous study and were designated by the selective *Eco*RI and *Mse*I bases (Bachmann and Hombergen 1997; Gailing et al. 1999)

E33/M69-185 bp (5' – 3')

GACTGCGTACGAATTCAAGATTCAAAATAAATGTTAGAAATGAGTTGTATGTTGC
ATATCTAAACTCATACCAATGAAATGTTCTCAAAAGAAGTTAGAATTTGGGTTTT
ACTGGTGAATACAAAACCGAATTGAGGCATTGGGAGTGAAATCGAAACCTTTAC
CTCGCTTAATCAGGACTCATC

E36/M52 – 145 bp (5' – 3')

GACTGCGTACGAATTCACCAAGTAGTTGATTTTGTGCTAAAATTGGAAAGAACGC
CATCACACACAATTCCAAGATTTGTGCATTATTGTAATTCATAACTTGGCAGGTTT
CAAAAACCAGTGTATGGGTTAATCAGGACTCATC

E37/M53 – 170 bp (5' – 3')

GACTGCGTACGAATTCACGTAAGTCATAGCCGAATCCATTTTATTCTTATAGGTA
ATATACCGTCGTGATGCTCGAACATTTCTCCAGTGCCCTGATTCCACACGCATTGA
TAGACAATACGTTCGTTTGGAATCCGCCTTATTGCTTATCCGGTTAATCAGGACTC
ATC

Fig. 2 Sequences of cloned AFLP fragments. The *Eco*RI respectively *Mse*I restriction sites are *underlined*

(new marker) (R^2 = 0.33). Neither QTL 3B or the other modifiers QTL 4A, 7A and 1A (Gailing et al. 1999) have any effect on either genotype of the new QTL 3A. The epistatic interactions between QTL 3B and the modifiers QTL 4A and QTL 7A were confirmed in this study. QTL 4A and QTL 7A showed only significant effects on the *M. bigelovii* (2 MS) genotype of QTL 3B (R^2 = 0.19; R^2 = 0.34).

Development of SCAR markers

No significant sequence identity of the AFLP markers to any known sequence could be detected. Primers were designed with the 5′ end on/close to the *Eco*RI and *Mse*I restriction sites (Table 3, Fig. 2). Reactions were most stringent when annealing temperatures were 2 °C above T_m values. Absence or presence of the band is dependent on sequence polymorphism in the primer binding sites, or large insertions/deletions (indels) between primer sites. This method is only successful if primers are designed in a region with few or no indels, and high sequence polymorphism between the 2 MS and 4 MS genotypes.

Distribution of AFLP and SCAR markers associated with QTL 3A and QTL 3B in natural populations of *Microseris*

AFLP and SCAR markers were tested in natural populations of *M. douglasii* (4 MS) and in *M. bigelovii, M. elegans* and *M. pygmaea* (2 MS) (Fig. 3). In populations A92 and B95 of *M. pygmaea* and in all samples from Peru, some flowers of one flowering head also possess anthers with 3 MS (trisporangiate anthers).

AFLP marker E39/M61 – 105 bp from *M. douglasii* that is associated with the 4 MS genotype of QTL 3B is present in all *M. douglasii* populations apart from population B21, and is absent in all populations of the disporangiate species *M. bigelovii, M. elegans* and *M. pygmaea*. The adjacent marker E45/M76 – 329 bp shows a similar pattern, but is amplified in one *M. elegans* and in one *M. pygmaea* line (Fig. 3a).

Markers associated with QTL 3A show the 4 MS genotype (absence of marker) in all *M. douglasii* populations apart from population B21 and the 2 MS genotype in most populations of *M. bigelovii* and *M. elegans*. In *M. pygmaea*, these markers show an inconsistent pattern (Fig. 3b). The SCAR marker E36/M52 – 145 bp with the strongest phenotypic effect from *M. bigelovii* (C94) is present in eight out of nine populations of disporangiate *M. bigelovii* (exception: population D58) and all 12 populations of *M. elegans*, but is absent in all 25 tetrasporangiate *M. douglasii* populations (except B21) and in all 39 accessions of *M. pygmaea* (including 30 plants from Peru), that possess disporangiate or di- and tri-sporangiate anthers. A similar distribution can be observed for the AFLP marker E40/M58 – 138 bp (QTL 3A). AFLP markers E41/M68 – 150 bp and E45/M73 –

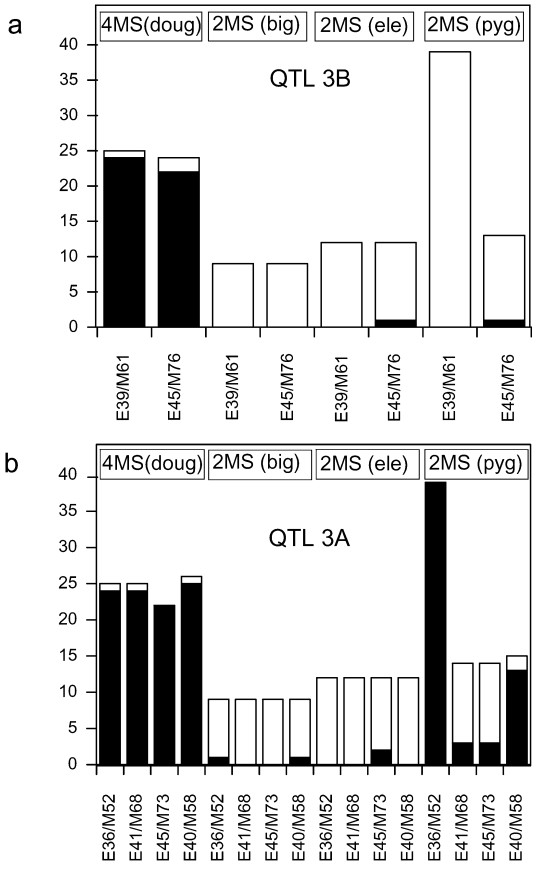

Fig. 3 QTL (marker) genotypes of QTL 3B and QTL 3A in natural populations of *M. douglasii* with 4 MS and *M. bigelovii, M. elegans* and *M. pygmaea* with 2 MS. Dark bars indicate the number of 4 MS (*M. douglasii*) genotypes and white bars the number of 2 MS (*M. bigelovii*) genotypes in each bulk

242 bp show a reverse pattern in *M. pygmaea* lines (Fig. 3b).

SCAR markers E33/M69 – 185 bp and E37/M53 – 170 bp from *M. bigelovii* with smaller phenotypic effects in the periphery of QTL 3A show a different distribution pattern. E37/M53 – 170 bp shows the 2 MS genotype (marker presence) in all *M. bigelovii* and *M. elegans* populations, the 4 MS genotype in all 25 *M. douglasii* populations (except B21) and weak bands in 37 out of 38 plants of *M. pygmaea*. Marker E33/M69 – 185 bp shows the 2 MS genotype (marker presence) in all *M. bigelovii, M. elegans* and *M. pygmaea* populations apart from *M. pygmaea* C97, but also in six out of 25 *M. douglasii* populations (B21, B39, C57, D57, D68, E68).

M. douglasii population B21 differs from other *M. douglasii* populations in that it possesses the alleles found in *M. bigelovii* (2 MS) in five out of six markers tested that are linked to QTL 3A and one out of two markers linked to QTL 3B.

Discussion

Mapping of diagnostic characters

Most reliable taxonomically relevant (diagnostic) characters show discrete alternate character states. In plants, these are often meristic characters (counts) concerning constant numbers of organs or organ parts. The number of MS per anther is a typical example of such a character. It is typically invariant in natural populations of *Microseris* that have either 4 MS per anther (the plesiomorphic or ancestral state) or 2 MS (the apomorphic or derived state) (Battjes et al. 1994; Gailing et al. 1999). In interspecific crosses between plants with 4 MS and plants with 2 MS the character becomes variable, and molecular markers can be detected that cosegregate with the average number of MS in a plant. Unlike metric characters such as plant height, meristic characters such as the number of MS often show a bimodal F2 distribution with most plants approaching one or the other parental character state (Battjes et al. 1994; Gailing et al. 1999). Visscher et al. (1996) could show that such characters can be mapped with the QTL strategy originally developed for continuous characters without a significant loss of efficiency. Our results confirm that significant QTL effects can be detected using simple phenotype/marker regression (Haley and Knott 1992) even if the character distribution deviates significantly from a normal distribution.

Major gene effects

According to the classical model originally formulated by Fisher (1930) the evolution of major phenotypic differences is the result of gradual divergence resulting from mutations of many genes with individual small and additive effects (Dobhansky 1937; Coyne and Lande 1985; Orr and Coyne 1992). With the advent of molecular marker techniques it became possible to map QTLs on molecular linkage maps and to determine their individual effects on the phenotype in a growing number of wild species (Vlot et al. 1992; Bradshaw et al. 1995; Hombergen and Bachmann 1995; Lin and Ritland 1997; Bradshaw et al. 1998; Gailing et al. 1999; Hill and Doebley 1999; Kim and Rieseberg 1999; Hurme et al. 2000; Lauter and Doebley 2002). In many of those QTL studies on qualitative and quantitative character differences, one QTL with a major effect on the phenotype (measured as the phenotypic variance explained by its segregation, a "major QTL") and additional modifiers with smaller phenotypic effects could be detected (Hombergen and Bachmann 1995; Lin and Ritland 1997; Bradshaw et al. 1998; Gailing et al. 1999; Gailing and Bachmann 2000; Moritz and Kadereit 2001). Based on the classical model of Fisher (1930), Orr (1998, 1999, 2001) developed a simulation model for the evolution of adaptive character differences that agrees with the fixation of one major gene and additional modifiers during the response to selection. However, the definition

of a "major QTL" by an arbitrary threshold value, e.g. one accounting for 25% of the phenotypic variance (Bradshaw et al. 1995) is somehow artificial, since the visible effect of a QTL is dependent on the environment and on the genetic background (Battjes et al. 1994; Gailing et al. 1999). Especially in meristic characters concerning the number of organs or organs parts – such as the number of microsporangia – strong epistatic interactions between QTLs seem to be the rule (Vlot et al. 1992; Doebley et al. 1995; Gailing et al. 1999; Lauter and Doebley 2002). In the present case, different genotypes of three modifiers (3A, 4A, 7A) affect the phenotype only in the homozygous recessive (2 MS) genotype of the major gene and can compensate the effect of the major gene (Gailing et al. 1999). In this case, the mutation of the major gene alone has no visible phenotypic effect (Gailing et al. 1999).

When our new markers are used to identify genotypes, QTL 3A, the modifier gene with the strongest effect on the MS phenotype, explains 36% of the total phenotypic variance, while the "major gene" QTL 3B explains only 19%. However, QTL 3A has only a significant effect on the "permissive" (2 MS) genotype of QTL 3B. The suggestion that the 2 MS allele of QTL 3A arose and was selected in plants with a homozygous (2 MS) genotype for the major gene, QTL 3B, is supported by the observation that the 4 MS allele of the strongest marker, E39/M61 – 105 bp (from *M. douglasii*), for QTL 3B is absent in all populations of the disporangiate species *M. bigelovii, M. pygmaea* and *M. elegans* while the 2 MS allele of the strongest marker, E36/M52 – 145 bp, for QTL 3A has evolved in *M. bigelovii* and *M. elegans*.

Our results suggest that the primary phenotypic effect of a major mutation may be no more than a slight instability of the previously highly constant phenotype that permits selection of modifiers involved in the stabilization of the phenotype and to accumulate modifiers stabilizing an alternative phenotype. In our case the 2 MS genotype of the major gene QTL 3B is not expressed in the phenotype of plants (still) having the 4 MS alleles of all modifier genes. These modifier genes maintain the 4 MS phenotype through genetic redundancy. In order to reconstruct the sequence of evolution of the diagnostic MS character, it is necessary to map our QTL alleles on a phylogenetic tree constructed from neutral markers (see below).

Evolution of disporangiate anthers

The mode of evolution suggested by our QTL model (Gailing et al. 1999) is supported by our present study of the distribution of the marker genotypes in natural populations. One of the principal results of the former mapping study (Gailing et al. 1999) was that the effects of the modifiers 3A, 4A and 7A were only visible in the homozygous recessive (2 MS) genotype of the major QTL 3B. This result was confirmed in our present analysis. We suggested that the three modifiers have evolved in a plant homozygous-recessive for the major gene and that the disporangiate anther has evolved by a mutation in the major QTL and a subsequent selection for the three modifier QTLs. The possibility that phenotypically invisible and presumably selectively neutral polymorphisms in the modifiers were present in natural populations of *Microseris* before the mutation of the major gene cannot be ruled out completely. Our present results, however, support the first sequence of events.

Marker E39/M61 with the strongest effect of markers associated with QTL 3B shows the *M. bigelovii* (2 MS) genotype in all populations of the disporangiate species *M. bigelovii, M. elegans* and *M. pygmaea* but the 4 MS genotype in nearly all *M. douglasii* populations, while E36/M52 –145 bp as the strongest marker for QTL 3A has the 2 MS genotype in virtually all *M. elegans* and *M. bigelovii* populations and the 4 MS genotype in the *M. douglasii* and *M. pygmaea* populations (except B21). However, none of the markers can detect the 2 MS or the 4 MS phenotype with absolute accuracy. Especially in *M. pygmaea* markers associated with QTL 3A show an inconsistent pattern. Also *M. douglasii* population B21 with 4 MS shows the *M. bigelovii* genotype in nearly all markers associated with QTL 3B and QTL 3A. These results suggest that the linkage disequilibrium between the markers and the MS locus is not strong enough to permit the identification of the alleles of the MS loci associated with them in natural populations. Still, the distribution patterns found here suggest that QTL 3B has mutated in a common ancestor of *M. bigelovii, M. elegans* and *M. pygmaea*. The 2 MS allele of QTL 3A has evolved later in the disporangiate species. Since *M. pygmaea*/*M. bigelovii* is a sister group to *M. elegans* (Roelofs and Bachmann 1997a, b; Roelofs et al. 1997), a back mutation in QTL 3A in the South American species *M. pygmaea* (see Table 1) is the most probable explanation for the marker genotypes found. Polymorphisms in QTL 3A are visible in the phenotype only in the 2 MS background of QTL 3B. In contrast to *M. bigelovii* and *M. elegans, M. pygmaea* shows some anthers with 3 MS and sterile loculi rather than a stable absence of adaxial MS in all florets. A similar phenotype could be observed in our F6 inbred population for plants with the 4 MS genotype in only one of the modifiers in an otherwise 2 MS genetic background (Gailing et al. 1999; Gailing and Bachmann 2000). The observation from our QTL study (Gailing et al. 1999), that mutation in the major QTL 3B may be without effect on the MS phenotype since the modifiers 3A, 4A and 7A can compensate for the major gene effect (= genetic redundancy), might be an explanation for the 2 MS genotype in markers associated with QTL 3A and QTL 3B in the tetrasporangiate *M. douglasii* population B21. Support for such a system of genetic redundancy for the MS character could come from the direct access to the genes involved in the evolution of the MS character. Recent QTL studies reported such kinds of epistatic interactions in discrete (meristic) characters (Vlot et al. 1992; Gailing et al. 1999; Lauter and Doebley 2002) where the mutation of the major gene alone has no visible phenotypic effect. In this way hidden genetical variation might escape selection

(Gailing et al. 1999; Lauter and Doebley 2002). Since the effects of redundant genes are similar but not identical, functionally redundant genes can be preserved in evolution (Nowak et al. 1997). Similar cases of genetic redundancy have been described in the literature; for example for the homeotic MADS box genes CAULIFLOWER, APETALA1 and FRUITFULL (Bowman et al. 1993; Pickett and Meeks-Wagner 1995; Kempin et al. 1995; Ferrandiz et al. 2000) or for Class-I knox genes KNAT 1 and SHOOT MERISTEMLESS in regulating stem cell function (Byrne et al. 2002). The mutation of the CAULIFLOWER gene in *Arabidopsis* has no effect on the phenotype since its effect can be compensated by the paralogous gene APETALA1. Only double mutants produce the CAULIFLOWER phenotype in *Arabidopsis* (Bowman et al. 1993). Genetic redundancy is hypothesized to be a mechanism to protect natural populations against mutation in regulatory genes with strong phenotypic effect (Pickett and Meeks-Wagner 1995). It is likely that a similar mechanism may act in genes for meristic characters that were determined as QTLs, but further studies are required.

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