Theor Appl Genet (2003) 107:123–129
DOI 10.1007/s00122-003-1220-5

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Identification of QTLs influencing agronomic traits in *Miscanthus sinensis* Anderss. I. Total height, flag-leaf height and stem diameter

Received: 26 June 2002 / Accepted: 25 September 2002 / Published online: 20 March 2003

Abstract We have developed the first quantitative trait locus (QTL) analyses for agronomic traits in a cross between $F_{1.1}$ (P1) and $F_{1.7}$ (P7) entries of *Miscanthus sinensis* Anderss. Both lines are offspring of the cross between MS-90-2 and MS-88-110. A map based on random amplified polymorphic DNA markers previously constructed was used to perform the QTL analyses. This map was developed using a new mapping strategy that has been designated offspring cross. Eleven QTLs were detected for height, panicle height and diameter using the programme MAPQTL 4.0 and the multiple QTL method. QTL significance was determined using several analyses, including Kruskal-Wallis analyses, empirical determination of LOD critical values using permutation tests, QTLs validation with field data over 2 years and co-localization of QTLs for correlated traits. The results obtained could be the first step in developing a marker-assisted selection programming in this species for biomass production.

Communicated by H.F. Linskens

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Keywords *Miscanthus* · QTL · Offspring cross · Agronomic traits · Biomass

Introduction

The European Union (EU) has set a target to minimise the greenhouse effect throughout the reduction of CO_2 emitted into the atmosphere. Consequently, it is promoting the use of renewable energy sources. One of these, biomass, is expected to contribute highly to the reduction of the greenhouse effect. According to Venendaal et al. (1997), four types of biomass resources may be considered: woody, herbaceous, oil and sugar crops. The genus *Miscanthus* Anderss. originates from Southeast Asia and consists of C4 grasses with a high potential as a biomass resource. This genus was introduced in 1935 from Japan to Denmark by Aksel Olsen (Nielsen 1990). Included in this genus is *Miscanthus* × *giganteus* Greef et Deu, the most cultivated species with respect to biomass production. *M.* × *giganteus* normally yields up to 25 t/ha (dry matter) from the third year onwards when harvested in the spring although huge differences in yield (from 2 t/ha to 44 t/ha) have been reported (Lewandowski et al. 2000). The great efficiency in nutrient acquisition of this species (Himken et al. 1997) has been explained by its great rooting depth and its high root density (Neukirchen et al. 1999). However, *M.* × *giganteus* has an extremely limited variability that hampers its breeding. In fact, it is a single clone vegetatively propagated. Nevertheless, the genus *Miscanthus* presents a high genetic variability distributed in four sections (Greef and Deuter 1993) with both *M. sinensis* Anderss. and *M. sacchariflorus* (Maxim.) Benth. the most interesting species to broaden the genetic base of *M.* × *giganteus* for breeding (Deuter and Abraham 1998).

M. × *giganteus* is a triploid and seed-sterile ($2n = 3x = 57$), resulting from a cross between *M. sinensis* ($2n = 2x = 38$) and *M. sacchariflorus* ($2n = 4x = 76$) (Greef and Deuter 1993; Linde-Laursen 1993). One of the two genomes of *M. sacchariflorus* was donated by *M. sinensis* while the other was inherited from an unidentified species

(Adati and Shiotani 1962). Consequently, *M.* × *giganteus* has two genomes with a high homology to *M. sinensis* and a third non-homologous genome (Greef and Deuter 1993; Linde-Laursen 1993). This makes *M. sinensis* the preferred species for mapping purposes, and the first genetic linkage map has been recently developed (Atienza et al. 2002), with the aim of using it for the detection of quantitative trait loci (QTLs) related to important agronomic traits such as total height, flag leaf height and stem diameter known to be important to biomass yield. Biomass yield and total height are correlated, while flag-leaf height may be an important factor since panicles may be lost dependent on the environmental conditions. Accordingly, the higher the flag-leaf height, the higher yield obtained if the panicles are lost. On the other hand, the stem diameter is an important character to avoid lodging of the plants. Full establishment of a *Miscanthus* field takes at least 3 years (Lewandowski et al. 2000; Clifton-Brown and Lewandowski 2002) and, therefore, the detection of molecular markers associated with traits of interest may be interesting for breeding of the species when integrated in a marker-assisted selection (MAS) programme.

The objectives of the research reported in this paper were to locate quantitative trait loci (QTLs) controlling variation in total height, flag leaf height and basal diameter. QTL mapping was performed in the map developed by Atienza et al. (2002) which consists of 257 RAPDs markers and constructed using the offspring cross mapping strategy.

Materials and methods

Plant material

A population of 89 F_1 hybrids from a cross between siblings $F_{1.1}$ (P1) and $F_{1.7}$ (P7) originating from a cross between MS-90-2 and MS-88-110 was used for mapping. Both parents are highly heterozygous and the grandparents are contrasting for the traits of interest. Plants were maintained in a greenhouse at the Instituto de Agricultura Sostenible (IAS-CSIC) of Córdoba, Spain.

Field trial and phenotypic measurements

The population was evaluated in a field trial at Córdoba (Spain) using a randomized block design with two replications. Each field plot consisted of three plants separated by 75 cm. The distance between plots was 100 cm and between rows 150 cm. The phenotypic characters measured were: (1) total height (H), in centimeters, – the average of ten shoots per plot; (2) flag-leaf height (PH), in centimeters – the average of ten shoots per plot; and (3) stem diameter (D), in millimetres – the average of ten shoots per plot (measured 5 cm above the soil surface). Means obtained from both replications were used in QTL analyses for these traits. Field data were collected for 2 consecutive years in order to perform the validation tests.

QTL analyses

QTLs were placed on a previous linkage map (Atienza et al. 2002). This map consists of 257 random amplified polymorphic DNA (RAPD) markers and was developed using the offspring cross-mapping strategy (Atienza et al. 2002).

Table 1 Model used to test the effect of QTL alleles (Knott et al. 1997) from Sewell et al. (2000, 2002)

Parental cross	$Q_1Q_2 \times Q_3Q_4 = Q_1Q_3$, Q_1Q_4, Q_2Q_3, Q_2Q_4
P1 effect	$(Q_1Q_3 + Q_1Q_4) - (Q_2Q_3 + Q_2Q_4)$
P7 effect	$(Q_1Q_3 + Q_2Q_3) - (Q_1Q_4 + Q_2Q_4)$
Interaction effect	$(Q_1Q_3 + Q_2Q_4) - (Q_2Q_3 + Q_1Q_4)$

QTL analyses were performed using the MAPQTL 4.0 package (Van Ooijen et al. 2000). This programme allows the detection of QTLs in cross-pollinating (CP) populations such as the one used in this investigation. A sequential procedure was used for QTL detection. First, we performed the non-parametric Kruskal-Wallis (KW) test (Lehmann 1975). This test does not use the map information, thereby enabling the detection of association between markers and traits individually. In a second step, interval mapping (IM) analyses was performed (Lander and Botstein 1989; Van Ooijen 1992). IM was used to select markers significantly associated with the trait to constitute an initial set of cofactors. A backward elimination procedure was applied to the initial set of cofactors. Only significant markers at $P < 0.02$ were used as cofactors in the multiple QTL method (MQM) (Jansen 1993, 1994; Jansen and Stam 1994) analysis for QTL detection. After the selection of cofactors, MQM analyses was performed. A mapping step size of 2 cM was used for both the IM and MQM analyses. The all-markers mapping approach (Knott and Haley 1992; Maliepaard and Van Ooijen 1994) was used to estimate the genetic information of not completely informative markers with respect to the four possible allelic combinations. Five neighbouring markers on each side of a marker were used for this purpose.

The significance thresholds for accepting the presence of potential QTLs in IM were determined using simulation tables (Van Ooijen 1999). The LOD critical values for MQM analyses were empirically determined by performing the permutation analyses (1,000 replications) (Churchill and Doerge 1994). According to Van Ooijen (1999) two different LOD thresholds can be considered: the chromosome-wide significance threshold (αc) and the genome-wide significance threshold (αg). Both thresholds are established using a significance level of 5%, which means that the probability of finding a LOD above the threshold within a chromosome (αc) or within the whole genome (αg) by chance is just 5%.

Simulation tables (Van Ooijen 1999) were used to obtain a first estimation of αc and αg. Following this, the permutation test was performed, and αc and αg were calculated for each character, trait and linkage group (LG). When a QTL exceeded αc in its LG but did not rise above the αg threshold, the probability of obtaining its peak LOD just by chance in that LG was determined from the permutation test. QTLs above the αc were considered as potential QTLs. The significance of QTLs was evaluated using the LOD thresholds, KW analyses, validation test over 2 years and co-localization of QTLs for correlated traits.

The QTL positions were estimated as the position with the maximum LOD score on a linkage group. Uncertainty of the map position was indicated by a 2-LOD support interval (Conneally et al. 1985; Van Ooijen 1992).

Since in this population a QTL can segregate for four different alleles, i.e. parental mating type $Q_1Q_2 \times Q_3Q_4$, four different genotypes can be obtained. In this way three deviance effects can be calculated as described by Sewell et al. (2000, 2002) using the model proposed by Knott et al. (1997). Three effects were calculated: P1 effect (difference in effect of the alleles inherited from P1), P7 effect (difference in effect of the alleles inherited from P7) and the interaction effect (i.e. deviation from additivity, where a value of zero indicates complete additivity when there is genetic information from both parents) (Table 1; Sewell et al. 2000, 2002).

Table 2 Results from QTL analyses of height (H), flag-leaf height (PH) and stem diameter (D)

QTL	Year	LG	2-LOD support confidence interval[a]	CM[b]	LOD[c]	Effects[d]			% Var[e]
						P_1	P_7	I	
D1	2000	6	20.8 ← → 25.9	22.8	4.8	–0.45	–1.15	0.07	29.0
D2	2000	11	N ← → 28.0	12.9	3.7	9.67	6.31	–2.66	24.5
D3	2000	12	24.4 ← → 33.9	31.7	2.8	–2.63	6.72	4.54	14.5
D1	2001	2	19.9 ← → 28.9	24.5	2.9	–0.30	–0.79	0.19	14.0
H1	2000	4	30.4 ← → 32.3	30.6	4.1	–35.54	–48.26	–21.25	24.4
H2	2000	8	23.3 ← → 29.6	26.9	3.0	–13.71	–4.99	–16.15	10.4
H3	2000	9	36.8 ← → 67.9	42.4	4.0	–9.16	–10.01	–22.24	12.4
H4	2001	2	18.8 ← → 20.4	19.2	3.8	–29.76	–30.34	–58.67	24.1
H2	2001	8	23.7 ← → 29.5	26.9	4.2	–5.57	–34.55	–0.06	18.0
H5	2001	13	2.4 ← → 20.0	16.6	3.2	13.57	34.74	42.80	34.9
PH1	2000	5	37.5 ← → 45.0	39.2	5.0	8.60	–1.85	40.40	45.5
PH2	2001	1	38.4 ← → 41.5	38.5	4.5	22.46	–25.76	11.72	15.8
PH3	2001	8	14.4 ← → 23.6	20.1	5.3	–41.38	–104.84	–31.48	31.7

[a] N, End of linkage group (LG)
[b] Peak position in centiMorgans
[c] Maximum LOD
[d] Deviance effects (P1, P7 and interaction) as showed by Sewell et al. (2000, 2002)
[e] Percentage of phenotypic variance explained by the QTL

Results and discussion

Correlation between traits

The traits analysed in this work were correlated. A significant correlation ($P<0.05$) was found between traits for the years 2000 – $r_{D\text{-}PH} = 0.38$, $r_{D\text{-}H} = 0.48$, $r_{H\text{-}PH} = 0.91$ – and 2001 – $r_{D\text{-}PH} = 0.59$, $r_{D\text{-}H} = 0.57$, $r_{H\text{-}PH} = 0.67$. Likewise, data from 2000 and 2001 were correlated – $r_H = 0.42$, $r_{PH} = 0.43$, $r_D = 0.47$. Since D, H and PH are correlated, the localization of QTLs for these traits over the same genome positions would be a significant result. In the same way, the validation of results throughout 2 years of analyses constitutes another parameter for evaluating the significance of the QTLs.

Number and validity of QTLs detected

A total of 11 potential QTLs were detected when both years were considered (Table 2). Of these, five were detected for height (H1, H2, H3, H4 and H5), three for flag leaf height (PH1, PH2 and PH3) and three for diameter (D1, D2 and D3). The localization of these QTLs is shown in Fig. 1. All QTLs exceeded the chromosome-wide significance threshold (αc) calculated using permutations for their respective linkage groups (Table 3). However, most of them remained under the genome-wide significance threshold (αg). The significance of a QTL is normally decided using a LOD threshold. The determination of this threshold can be made using different criteria. Sometimes the LOD threshold is arbitrarily fixed and QTLs over this level are considered to be significant. This methodology has been used by several investigations (Tozlu et al. 1999a, b; Lespinasse et al. 2000; Li et al. 2000) but seems to be inadequate since a high proportion of false positives can be obtained. Van Ooijen (1999) developed simulation tables to improve the determination of the LOD threshold for QTL consideration. This methodology has been used by King et al. (2000) and Maliepaard et al. (2001). The LOD thresholds calculated from simulation tables were $\alpha c = 2.9$ and $\alpha g = 4.4$ in the present study, as shown in Table 3. The LOD threshold can be also empirically determined using the permutation test (Churchill and Doerge 1994). We used simulation tables (Van Ooijen 1999) and the permutation test (Churchill and Doerge 1994) to determine the LOD thresholds. The simulation tables were used to calculate the αc (2.9) in a first step to detect putative QTLs for the IM analyses. The permutation test was subsequently used to calculate αc and αg LOD thresholds since they seem to be more accurate than the ones calculated using simulation tables. Nevertheless,

Table 3 LOD significance threshold for H, PH and D using simulation tables and permutation tests

QTL	Year	LG	LOD	αc[a]	αg[b]	αc[c]	αg[d]	Significance[e]
D1	2000	6	4.8	2.9	4.4	3.1	4.4	99.9
D2	2000	11	3.7	2.9	4.4	2.7	4.4	99.2
D3	2000	12	2.8	2.9	4.4	2.8	4.4	94.3
D1	2001	2	2.9	2.9	4.4	2.9	4.4	94
H1	2000	4	4.1	2.9	4.4	3.1	5.1	99.1
H2	2000	8	3.0	2.9	4.4	3.0	5.1	95
H3	2000	9	4.0	2.9	4.4	3.6	5.1	98
H4	2001	2	3.8	2.9	4.4	3.2	4.2	98.7
H2	2001	8	4.2	2.9	4.4	2.5	4.2	100
H5	2001	13	3.2	2.9	4.4	2.6	4.2	98.8
PH1	2000	5	5.0	2.9	4.4	4.1	6.0	98.5
PH2	2001	1	4.5	2.9	4.4	3.6	4.4	
PH3	2001	8	5.3	2.9	4.4	2.8	4.4	

[a] αc calculated from simulation tables
[b] αg calculated from simulation tables
[c] αc determined using permutation test
[d] αg determined using permutation test
[e] Probability of certainty of a QTL considering αc[c]

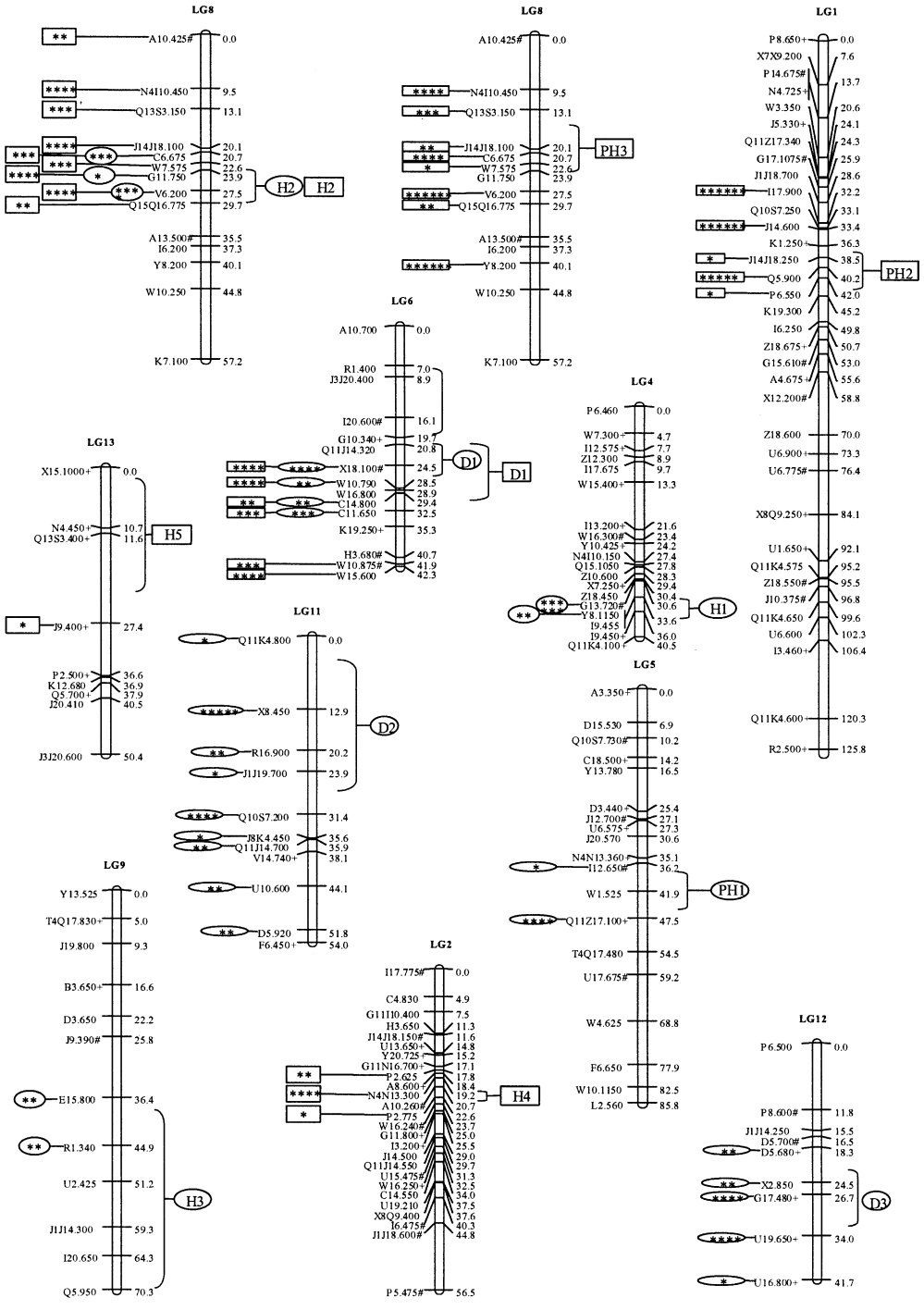

Fig. 1 Localization of QTLs for height (H), flag-leaf height (PH) and diameter (D) in the linkage map of *Miscanthus sinensis*. Distances in Kosambi (centi-Morgan) units, cumulative. QTL names and significance of Kruskal-Wallis analyses are identified with *ellipses* for the year 2000 and *boxes* for 2001. The significance of the Kruskal-Wallis analyses is: $*P \leq 0.1$, $**P \leq 0.05$, $***P \leq 0.01$, $****P \leq 0.005$, $*****P \leq 0.001$, $******P \leq 0.0005$, $*******P \leq 0.0001$. Two-LOD support interval is shown by a *bracket* at the *right side* of the linkage groups. Marker nomenclature is as follows. (1) Single primer: markers obtained from single primer reactions were named with the primer designation followed by the molecular weight of the marker. In this way, the marker P8.650 was obtained with the primer OP-P8 (OP, Operon Technologies) and its molecular weight is 650. (2) Pairwise primer combinations: markers obtained from the combination in a single PCR reaction with two primers were designated by a code for the both primers followed by the molecular size of the amplified fragment. Therefore, marker X7X9.200 was amplified from the combination of the primers OP-X7 and OP-X9 and has a molecular weight of 200. (3) Parental origin: a symbol was added to the marker name in order to distinguish mono- and bi-parental markers. Mono-parental markers from P1 are followed by the symbol $\underline{+}$, while those obtained from P7 were followed by $\underline{\#}$. The bi-parental markers have no symbol after their name

the thresholds were in most cases using the two different methods (Table 3).

Although αc and αg thresholds are important parameters for evaluating the significance of a QTL, they were not considered to be unique parameters in our investigation since dominant markers such us RAPDs (Williams et al. 1990; Welsh and McClelland 1990) and AFLPs (Vos et al. 1995) considerably reduce the detection power in QTL analyses. This would lead to lower peak LOD values of the QTLs being detected in our work. In addition, high peak LODs, could appear in this population when combined with dominant markers (http://www.joinmap.nl). However, these artefacts would be detected since markers within its confidence interval will not show association with the trait in the KW analysis. On the contrary, the permutation test will include them as real QTLs and therefore the LOD calculated using permutations would be higher than it should be. Therefore, several analyses were considered for evaluating the significance of each QTL. First, the KW analysis was considered to be

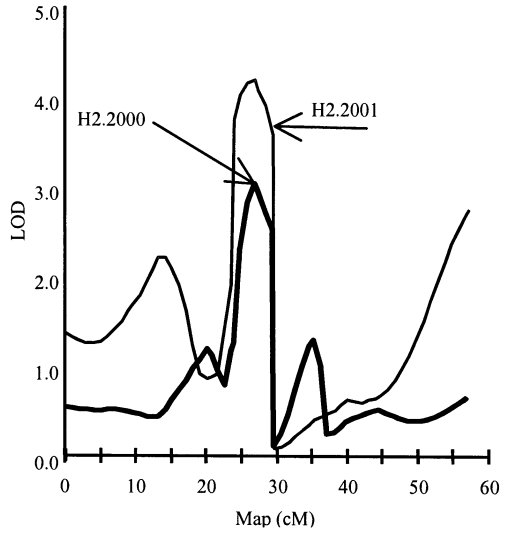

Fig. 2 LOD profile of H2 (over both years of analyses)

essential for QTL significance. In this way, QTLs were not considered when the markers in its confidence interval did not show association with the trait in the KW analysis. Likewise, the co-localization of QTLs of different traits would suggest that these QTLs are significant since H, PH and D were correlated. In addition, temporal replication of analyses constitutes an efficient validation method. In conclusion, we have considered the KW analyses, the LOD thresholds calculated using simulation and permutations, the co-localization of QTLs for different correlated traits and validation over 2 years for evaluating QTL significance.

Out of 11 QTLs reported (Table 2) H2 and D1 were validated and therefore considered to be significant QTLs. The LOD profile for H2 over both years is practically identical, as shown in Fig. 2. PH3 was co-localizated with H2 in 2001, which suggests that PH3 is also a real QTL since both traits were correlated. The remainder of the QTLs were detected in a single year. Of these, PH2 has a peak LOD of 4.5, which is higher than the LOD threshold (4.4) (Table 3). Although the rest of the QTLs remained under their respective LOD thresholds (Table 3), some can be considered to be significant based on the results obtained with the KW analysis, while other QTLs may be false positives. For example, the markers associated with H3 and H5 (Fig. 1) did not show significant association with the trait in the KW analysis. This lack of association suggests that H3 and H5 are not real QTLs since they did not reach the LOD threshold. On the contrary, H1, H4, PH1, D2 and D3 were located between markers highly associated with the trait in the KW analysis and, therefore, these QTLs seem to be real.

QTL effects

The outbreeding characteristics of *M. sinensis* plus its auto-incompatibility make the genetic studies of this species more similar to those on forest trees than to herbaceous crops. As pointed out by Sewell et al. (2000), few QTL analyses in forest trees have attempted to determine the gene action for QTLs. However, Sewell et al. (2000, 2002) used an outbreed QTL model (Knott et al. 1997) which allows calculation of an interaction effect as well as two parental effects (Table 1). In this way, an interaction effect of zero means that alleles are additive, when both parents are heterozygous at that QTL. This was the case of D1 (year 2000) and H2 (year 2001) with interaction effects near zero (Table 2), whereas both QTLs were stable across years. However, the rest of the QTLs showed non-zero interaction, which suggests a dominance or epistatic effect. Likewise, they were detected in a single year. As pointed out by García et al. (2000), some authors consider the lack of stability across different years or environments as an indication of false QTLs. However, these QTLs may indicate a genotype × environment interaction. Similarly, the detection of QTLs in a single year has been interpreted as QTLs acting in different developmental phases of the crop. Both considerations could be applied in *Miscanthus*. On one side, the complete establishment of *Miscanthus* crop takes up to 3 years in northern European countries (Lewandowski et al. 2000) and, therefore, different genes may be expressed at establishment and at maturity. The establishment period of our field trial was shorter due to the warmer temperatures of southern Spain. Consequently, the establishment period is considered to be finished after the first year in contrast to the field trials conducted under northern conditions where at least 2 years is needed for *Miscanthus* establishment. On the other side, *Miscanthus* is a perennial rhizomatous crop and will grow within a variety of environmental conditions during its life cycle. Therefore, different QTLs may act in a variety of environments. We therefore consider that QTLs detected in a single year in this work could be QTLs showing genotype × environment interaction or QTLs expressed under different developmental stages since the final potential of genotypes is not fully shown during the establishment period. These types of QTLs can be found in several reports on forest or fruit trees (Asins et al. 1994; Plomion et al. 1996; Verhaegen et al. 1997; Conner et al. 1998; Marques et al. 1999; García et al. 2000; Sewell et al. 2000). In addition, other studies have not found evidence for the same QTLs over different growing seasons (Kaya et al. 1999).

The estimated effects of the QTLs for H2 and D1 varied from year to year (Table 2). The change in the effects of the alleles of a QTLs has been reported in QTL stable across years in *Pinus* (Lerceteau et al. 2001). This suggests environmental or epistatic interactions.

Potential use of QTLs in MAS

The detection of QTLs expressed in a particular environmental or physiological conditions could be of interest. In this way, the inclusion of these QTLs plus the stable ones (such as H2 or D1) in a breeding programme could be a good breeding strategy for developing genotypes adapted to a wide range of environments. One of the agronomic problems in *Miscanthus* cultivation is the overwintering during field establishment. *M. sinensis* has an improved cold hardiness compared with *M.* × *giganteus* (Clifton-Brown and Lewandowski 2002). The initial vigour of genotypes could be an important factor to reduce its mortality during field establishment. Plants with a higher development rate in the first year would accumulate a higher quantity of reserves, and therefore, it would be possible that their cold hardiness is increased. Therefore, the detection of QTLs during the first year of this work could be useful for this purpose. Likewise, the detection of QTLs when a *Miscanthus* crop is fully established could be an indication of QTLs expressed in a second stage of development. These QTLs could be responsible for plant growth after an initial phase, in which other QTLs were regulating the growth. Consequently, the development of genotypes with both types of QTLs could be of interest to obtain genotypes with a good establishment and a good development after the first year.

The QTLs detected for PH could be interesting to minimize another problem. *Miscanthus* leaves and tops may be lost during the winter under some environmental conditions. This leads to a yield reduction. In this way, the QTLs for PH could be of interest for selection of higher plants without consideration of top length, and it could be possible to obtain higher plants with smaller tops by combining the QTLs for H and PH since some QTLs for H may be controlling the top height.

Diameter is also an important trait related to the lodging of the plants. Plants with thicker stems would be stronger and the logging would be avoided. Consequently, the QTLs detected in this work could be of interest in the development of a MAS programme aimed at reducing lodging.

According to Marques et al. (1999) several authors have found evidence of homologous chromosome regions carrying similar QTLs in different species. Therefore, the results obtained in this work could be used in future research when investigating the genetic relationships within *Miscanthus*. In this way, our results could be expected to be of application in other species of this genus throughout the development of appropriate markers. Likewise, comparative mapping between *Miscanthus* and other cereals would be useful in a second step. For example, QTLs for height detected in this work such us H2 could be homeologous of wheat dwarfing genes. This could be investigated using comparative mapping. Comparative mapping would also be very useful for search genes or QTLs for application in a MAS programme for biomass production.

However, the utilization of the QTLs detected in this work should be made with care since the alleles and their effect could be different in other genetic backgrounds. Therefore, this study should be considered as a preliminary work to initiate a MAS programme including these traits in *Miscanthus*.

Acknowledgements This research was supported by project FAIR CT98-3571. The population used in this study was developed by K.K. Petersen and K. Kristiansen (Danish Institute of Agricultural Sciences). The first author gratefully acknowledges Consejería de Educación y Ciencia of the Junta de Andalucía for a pre-doctoral fellowship.

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