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Inter Simple Sequence Repeat Markers Associated with Flowering Time Duration in the Chilean Strawberry (*Fragaria chiloensis*)

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ABSTRACT

The flowering and fructification period of the Chilean strawberry (*Fragaria chiloensis* (L.) Duch.) is restricted to approximately 2 mo, which seriously limits the commercial development of the species. The objective of the current investigation was to identify Inter Simple Sequence Repeat (ISSR) polymorphisms associated with flowering duration in accessions of *F. chiloensis*. The flowering duration data related to 41 accessions obtained over 3 years were analyzed, and a set of 40 ISSR primers tested. Two clusters were obtained through the Partitioning Around Medoids algorithm, with 23 *vs.* 18 accessions, and 64.1 vs. 95.6 days of flowering, respectively. Flowering duration, between the two groups, was significantly different. The years also revealed a significant effect, on flowering duration, between the two groups. Ten of the ISSR primers tested revealed reproducible and consistent banding patterns, displaying a total of 106 putative loci, of which 79 were polymorphic. Three ISSR loci (811_{779}, 844_{670}, 841_{980}) were identified owing to their significant contribution to the differentiation among the accessions. Similarly, three ISSR loci (811_{600}, 812_{1180}, 841_{980}) exhibited a significant correlation with the flowering duration variation. Locus 841_{980}, which presented the highest level of correlation with flowering duration, was isolated, cloned and sequenced, but it showed only a low level of homology with the relevant sequences published in the GenBank database. The identified loci showing high correlation with the flowering time could help build Quantitative Trait Loci (QTL) maps for selection and improvement programs in the *Fragaria* sp. genus or other related species.

Keywords: Association analysis, ISSR polymorphisms, Molecular markers.

INTRODUCTION

The duration of the flowering period is a highly relevant trait in strawberry production, because it impacts directly on fructification and productivity (Hancock et al. 1999). In the case of commercial strawberry (*Fragaria ananassa*), some of the current varieties are apt to flower and fructify continually and thereby greatly increase the commercial return of the crop (Darnell *et al.,* 2003).

In the cultivated form of the Chilean strawberry (*Fragaria chiloensis* ssp. *chiloensis f. chiloensis*), the flowering and fructification period is restricted to approximately 2 months, which seriously limits the commercial development of this

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species (Lavín and Maureira 2000; Retamales *et al.,* 2005). Fortunately, variation has been observed for this trait in accessions of the wild form of the species (*F. chiloensis* ssp. *chiloensis f. patagonica*) in a germplasm bank (Lavín and Maureira 2000; Becerra *et al.,* 2001). This variation appears to indicate that there exists a potentially heritable variation that could be exploited for Chilean strawberry genetic improvement programs. To date, however, in no study it has been attempted to obtain information regarding the genetic control of this trait in *F. chiloensis*.

Linkage analysis has been an important approach used to dissect such complex traits as flowering duration (Tanksley 1993; Long *et al.,* 1995; Yano and Sasaki 1997; Kearsey and Farquhar 1998; Mackay 2001; Mauricio 2001; Yano 2001; Doerge 2002). However, its main constraints are its low resolution (de la Chapelle and Wright 1998) and the analysis of narrow genetic diversity in a large experimental population (Doerge 2002). An alternative approach is association analysis, which has proven to be a powerful strategy for identifying the genetic polymorphisms in non-experimental populations (Risch and Merikangas 1996). However, association analysis requires detailed knowledge of the genetic relationships between the individuals studied, because the genetic structure must be specified to avoid false positives. In human populations, association analysis has resulted in the identification of important polymorphisms related to genetic defects with a high level of resolution (de la Chapelle and Wright 1998). The application of association analysis to plants has been on the increase over the past years (Thornsberry *et al.,* 2001; Buckler and Thornsberry 2002; Whitt *et al.,* 2002; Wilson *et al.,* 2004), especially after more powerful statistical methodologies becoming available (Pritchard *et al.,* 2000).

Inter-Simple Sequence Repeats (ISSR) are Polymerase Chain Reaction (PCR)-based molecular markers that have shown higher reproducibility, levels of variability and simplicity as compared with other dominant marker systems (Wolfe and Liston 1998). In plant genomes, moreover, some such microsatellite sequences as GA, CA and CT have been identified in transcribed regions, giving an additional value to ISSR (Li *et al.,* 2002, 2004; Morgante *et al.,* 2002). Inter-simple sequence repeats have proven useful for studying genetic diversity in a number of species and have resulted in the detection of statistical associations between specific bands and characteristics of commercial interest. Relevant examples are the ISSR bands associated with seed mass in flax germplasm (Wiesnerová and Wiesner 2004), silk yield in *Bombix mori* stocks (Chatterjee and Mohandas 2003) and oral cancer in human cells (Rai *et al.,* 2004).

ISSR molecular markers have been widely utilized in strawberry for different applications. The genetic similarity among strawberry cultivars was assessed through ISSR markers (Debnath *et al.,* 2008; Morales *et al.,* 2011). Meanwhile, the genetic diversity and structure of the Chilean strawberry was also estimated through ISSR markers with acceptable levels of reliability (Carrasco *et al.,* 2007) and as well a molecular marker as based on ISSR being developed for the Seasonal Flowering Locus (SFL) in *Fragaria vesca* (Albani *et al.,* 2004). The objective of the current investigation was to identify ISSR polymorphisms associated with flowering duration in the non-cultivated form of *F. chiloensis*.

MATERIALS AND METHODS

Plant Material

Forty one accessions of *Fragaria chiloensis* L. Duch. f. *patagonica* (red fruit) from different origins, and for which complete flowering time data were available for at least 3 years were chosen for analyses on the basis of their potential commercial use. The germplasm bank was built with

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accessions collected from the Central-South Region of Chile (Table 1, Figure 1).

The germplasm bank was sited at the Experimental Substation of the National Institute of Agricultural Research (INIA, 35°58´S and 72°02´W), in Cauquenes, Chile. The plants are conserved in soil with three replications per accession under the same environmental conditions and agronomic practices.

Table 1. Identification, geographic origin of the collected accession and flowering time periods for 41 *Fragaria chiloensis* accessions.

ID[a]	Accession	Geographic origin of the accession			Flowering time period		
		LS[b]	LW[c]	Al[d]	2001	2002	2003
7	3HUE1A	35°05'	72°03´	50	55	59	83
18	93CHI1A	36°54'	71´°25'	1400	95	97	105
22	90SAU1A	36°25'	71°08´	Winf[e]	69	57	43
23	2BAK1A	47°20´	72°50´	305	79	71	92
25	2BAL1A	45°52´	71°51´	0	60	78	127
26	2BAL1B	45°52'	71°51'	0	52	97	64
31	2CAM1A	42°50'	72°53'	0	129	104	104
32	2CAM1B	50'42°	72°53'	0	79	52	62
49	2CPU1A	41°48'	73°43'	0	60	92	68
52	2FUT4A	43°08'	71°40'	Winf	44	77	35
60	2MAL1A	46°08'	72°06'	351	39	35	134
63	2PAL2A	43°30'	71°55'	183	96	35	71
85	3BUD1A	38°48"	73°23'	80	96	50	63
92	3CAY4A	37°47'	73°10'	580	73	97	134
93	3CAY5A	37°48'	73°09'	660	74	64	59
94	3CAY5B	37°48'	73°09'	660	79	42	57
95	3CAY5C	37°48'	73°09'	660	83	85	48
96	3CAY5E	37°48	73°09'	660	116	135	135
97	3CON1A	38°03'	73°16'	535	55	63	198
98	3CON1B	38°03'	73°16'	535	62	70	114
100	3CON3A	38°06´	73°16´	210	87	78	79
102	3COR1A	39°56´	73°13´	300	117	65	141
103	3CUN1A	38°51´	71°40´	410	137	97	70
108	3ELI1A	36°48´	71°40´	760	82	56	91
113	3FRI1A	37°46´	72°48´	775	98	43	107
124	3HAN1A	39°16´	73°10´	50	101	78	91
125	3HUI1A	39°56´	73°34´	5	79	97	84
129	3ICA4A	38°49´	71°23´	1285	96	78	99
136	3IDA1A	37°33´	73°21´	235	66	71	63
141	3LAJ2A	37°23´	71°24´	1020	125	90	134
148	3LOL2A	38°07´	71°24´	750	46	97	70
166	3LLE1A	36°52´	71°35´	1000	57	43	92
178	3MAI1A	40°12´	72°08´	Winf	62	71	94
183	3MAN1A	37°47´	72°51´	560	65	36	92
203	3NEH1A	38°46´	73°25´	30	64	50	50
206	3NIE1A	39°47´	73°23´	30	95	50	63
209	3NIT1A	38°07´	71°20´	805	53	22	92
221	3PUY1A	40°44´	72°05´	1060	62	97	95
225	3PUY5A	40°42´	71°56´	1290	62	97	88
227	3RAM1A	37°20´	73°13´	240	32	78	72
234	3RIN1A	Winf	Winf	80	66	15	8

[a] Identification Number; [b] Latitude South (°); [c] Longitude West (°); [d] Altitude (m); Flowering time period (days), measured from the formation of the first floral button to the last flower by each genotype, [e] No information was available in the germplasm bank regarding the geographic coordinate or altitude collection site for the accession.

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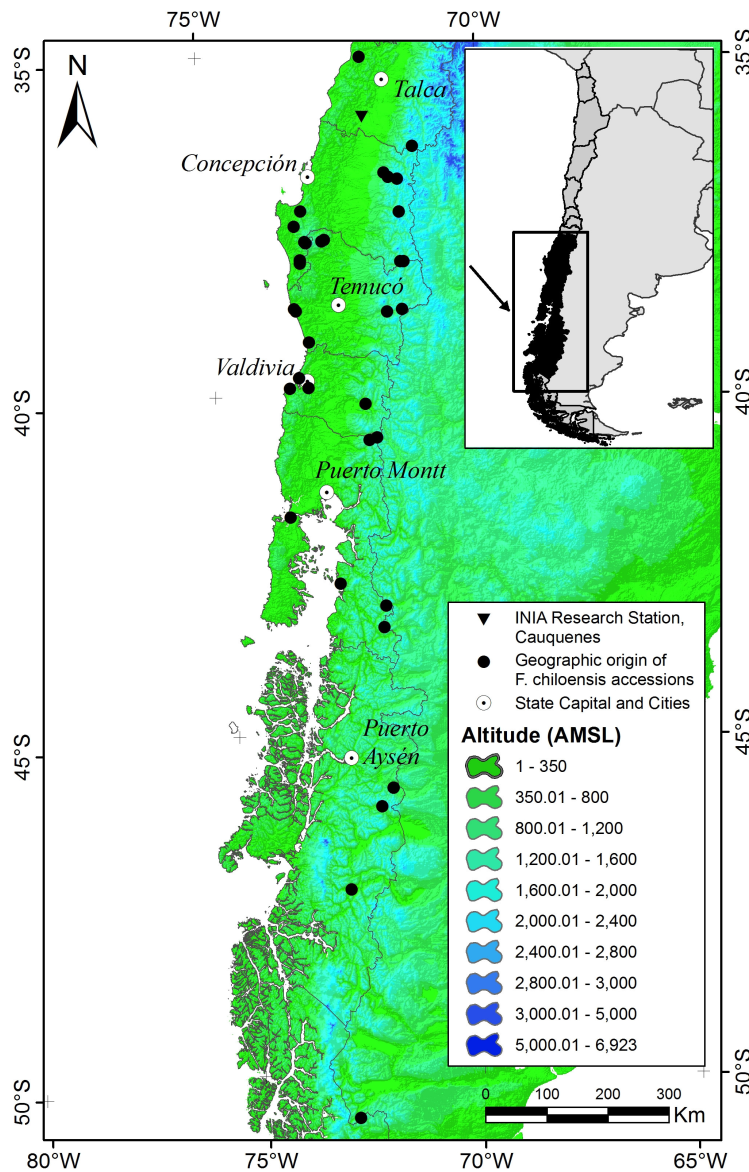

Figure 1. Geographic origin of the *F. chiloensis* accessions (red form) analyzed throughout the study, and sited at the INIA-Cauquenes Research Station.

Flowering Time Duration Data

The analysis was performed for all the plants belonging to each genotype (three plants per genotype). The whole germplasm bank was raised in a single plot of approximately 0.3 Ha,. For each genotype, flowering time was considered as the time period from the appearance of the first floral button until the last open flower observed. The flowers produced on both the main plant and on the runners (stolons), were taken into consideration. The study was carried out over the spring-summer seasons for a duration of three years. The temperature data for the study period were obtained from the INIA-Cauquenes weather station.

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Molecular Data

DNA Extraction, PCR Conditions and Visualization of PCR Products

Young buds of each accession were collected and immediately frozen in liquid nitrogen for DNA extraction. DNA extraction and PCR conditions were both carried out according to Carrasco *et al.* (2007). Throughout the present study, a set of 40 primers (set ISSR 100/8 from the University of British Columbia Biotechnology Laboratory) was tested (Table 2). All the PCR products were

Table 2. Inter Simple Sequence Repeat primers, originally from the Biotechnology Laboratory, University of British Columbia (Vancouver, British Columbia, Canada), tested for their association with the flowering time duration in *F. chiloensis*.

Primer ID	Sequence	Annealing temperature (ºC)
801	*atatatatatatatatt*	34
802	*atatatatatatatatg*	36
805	*tatatatatatatatac*	36
806	*tatatatatatatatag*	36
807	*agagagagagagagagt*	50
811	*gagagagagagagagac*	52
812	*gagagagagagagagaa*	50
814	*ctctctctctctctcta*	50
817	*cacacacacacacacaa*	50
818	*cac aca cac aca cac ag*	52
820	*gtgtgtgtgtgtgtgtc*	52
821	*gtgtgtgtgtgtgtgtt*	50
823	*tctctctctctctctcc*	52
826	*acacacacacacacacc*	52
828	*tgtgtgtgtgtgtgtga*	50
830	*tgtgtgtgtgtgtgtgg*	52
831	*atatatatatatatatya*	36
832	*atatatatatatatatyc*	38
833	*atatatatatatatatyg*	38
834	*agagagagagagagagyt*	54
839	*tatatatatatatatarg*	40
840	*gagagagagagagagayt*	54
843	*ctctctctctctctctra*	54
844	*ctc tct ctc tct ctc trc*	56
845	*ctc tct ctc tct ctc trg*	56
846	*cacacacacacacacart*	54
847	*cacacacacacacacarc*	56
861	*accaccaccaccaccacc*	60
864	*atgatgatgatgatgatg*	48
865	*ccgccgccgccgccgccg*	72
866	*ctcctcctcctcctcctc*	60
868	*gaagaagaagaagaagaa*	48
869	*gttgttgttgttgttgtt*	48
871	*tattattattattattat*	32
878	*cgatggatggatggat*	48
879	*cttcacttcacttca*	42
880	*ggagaggagaggaga*	48
892	*tagatctgatatctgaattccc*	60
895	*agagttggtagctcttgatc*	58
899	*catggtgttggtcattgttcca*	64

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checked through electrophoresis on 2% agarose gel, run in TAE 1X buffer and visualized by UV fluorescence after their being stained with ethidium bromide (0.25 µl ml^{-1} of staining solution).

Statistical Analysis

The Partitioning Around Medoids (PAM) algorithm was employed to cluster the accessions according to their flowering duration. This method searches for groups of representative objects called medoids, which display a minimum average of dissimilitude in relation with the other objects. Once the medoids are found, the data are classified in the nearest medoid (Kaufman and Rousseeuw, 1990).

The accuracy of PAM clustering was determined by means of logistic regression analysis, making it possible to estimate the degree of relationship of such binary variables as the patterns of a dominant marker (e.g. 0 and 1) with metric variables (e.g. flowering duration). Repeated measures of ANOVA were carried out to verify the average differences for flowering duration among the identified clusters. The statistical analyses for flowering duration were carried out using the STATISTICA v6.0 software (StatSoft, Uppsala, Sweden).

The grade of genetic differentiation between clusters, defined according to flowering duration, was studied by means of Φ_{st} and G_{st} incorporated into the Arlequin ver. 2.0 software (Analysis of Molecular Variance (AMOVA); Excoffier *et al.,* 1992; Schneider *et al.,* 2000). Additionally, the identity and Nei's genetic distance were assessed (Nei 1987). The genetic structure was analyzed by means of Structure ver. 2.1 software (Pritchard *et al.,* 2000). Through this Bayesian cluster method, the ancestry and membership coefficient of each individual is stimated. Linkage Disequilibrium (LD) analysis was carried out through POPGENE Software (Yeh and Boyle, 1996) and as well, Arlequin ver. 2.0 software (Schneider *et al.,* 2000). The significance level of each pair of alleles was determined through χ^2 test. To discard the LD produced by chance, the significance threshold was corrected through a Bonferroni test (α level [0.05]/number of allele combinations compared [3081]; P= 0.00002).

The association between polymorphic ISSR bands and flowering duration was assessed through a logistical regression using the STATISTICA ver. 6.0 software (StatSoft). This method identifies possible correlations among binary genetic markers (ISSR) and the containuing variables (flowering duration). A high level of correlation (high P-value) was considered as a sign of a strong association between ISSR frequency and the flowering duration.

Cloning and Sequencing

The ISSR bands significantly associated with the flowering time were purified from agarose gels (2%), making use of EZNA kit, following the manufacture's recommendations (Omega Biop-Tek, Doraville, USA). The fragments were cloned into pCT2.1-TOPO vector (Invitrogen, Carlsbad, CA) using the cloning kit TOPO A (Invitrogen). Cloned fragments were sequenced in an ABI Prism 3100 sequencer (Perkin-Elmer, Boston, MA), using the BigDye Terminator v3.1 sequencing kit. The sequences were compared with those available from the databases and by means of the BLAST software (http:/www.ncbi.nlm.nih.go/BLAST).

RESULTS

Flowering Time Analysis

According to PAM analysis, the best clustering estimation occurred for the case of two groups. The logistical regression confirmed this classification (R^2= 0.6; P< 0.001). The first group consisted of 23 Chilean strawberry accessions showing an average flowering duration of 64.1 days. The second group was composed of 18 accessions

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with an average of flowering duration of 95.6 days.

The data presented homogeneous variances (data not shown), which allowed the application of repeated measures of ANOVA. Table 3 and Figure 2-A show that the average flowering duration differed significantly (P< 0.01) between the two groups for the 3 years study. These results were in agreement with the logistical regression. The years also showed a significant effect (Table 3; P< 0.01) on flowering duration between the two groups (Figures 2-B, -C and -D). This result was expected, because the inductive temperatures for flowering (< 15°C; Durner *et al.,* 1984) from March to August varied significantly (P= 0.002) among the 3 years analysis (data not shown). In contrast, the effect of the interaction of "group" versus "year" was not significant (P= 0.24),

Table 3. Analysis of variance for flowering period in 41 accessions of *F. chiloensis*, assessed in three different flowering seasons.

	Effects		Error			
	Df [a]	*MS* [b]	*Df*	*MC*	*F* [c]	*P* [d]
Cluster	1	30174,81	39	430,8836	70,03	0,000000 **
Year	2	3366,99	78	638,1367	5,27628	0,007093 **
Interaction	2	922,11	78	638,1367	1,44500	0,241986

[a] Degrees of freedom; [b] Mean Square; [c] Fisher's *F*-value, [d] Probability.
** = Highly significant, ns= Not significant.

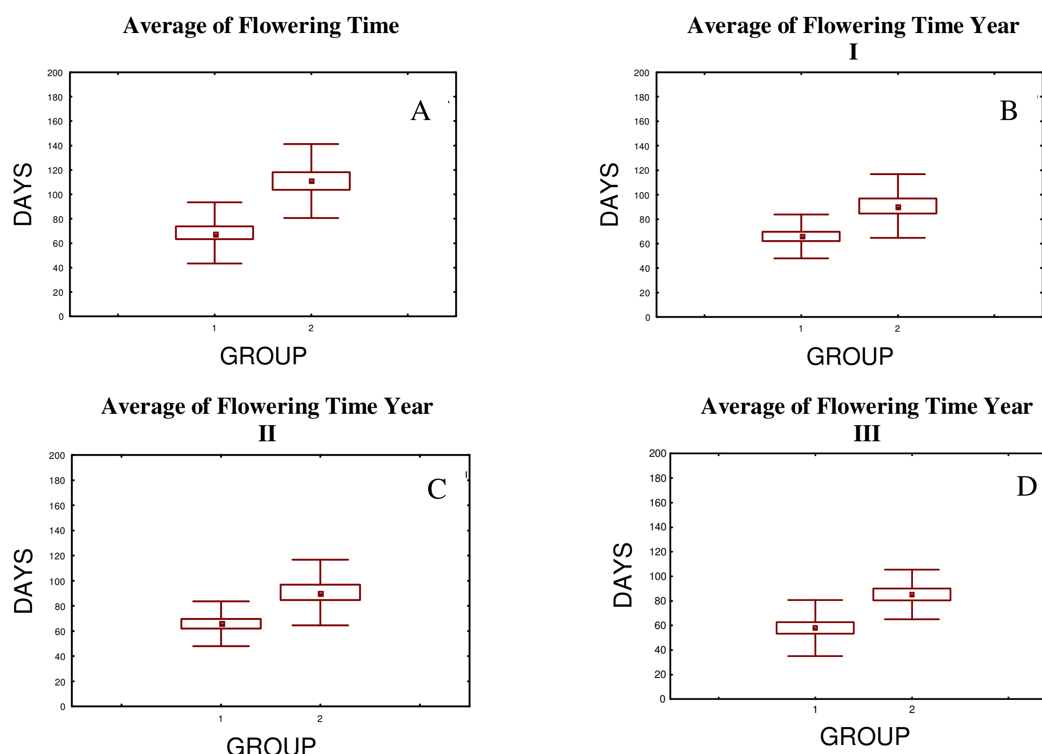

Figure 2. A comparison of the Analysis of Variance of flowering times between two Chilean strawberry groups: Group 1 (23 accessions; average= 64.1 days) and group 2 (18 accessions; average= 95.6 days). (A) Flowering time average for the three studied years; (B) Flowering time average for year I (2001); (C) Flowering time average for year II (2002), (D) Flowering time average for year III (2003).

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indicating that the magnitude of the variation among the accessions was constant over the different years analyzed.

Genetic Structures and Differentiation

Out of the 40 primers analyzed, only 10 revealed reproducible and consistent with ISSR band patterns. These 10 primers showed a total of 106 putative loci, of which 79 were polymorphic (75%) and 27 monomorphic (25%). A high proportion of the ISSR variability was observed among the accessions in each group. The average gene diversity was high (H= 0.21±0.18). Genetic differentiation was low between the two groups (ϕ_{st}= 0.009; G_{st}= 0.03) after 1000 permutations. In agreement with these results, the accessions revealed a high genetic identity (I= 0.99). Three ISSR loci contributed significantly to the observed differentiation: 811_{779} (ϕ_{st}= 0.12; p= 0.05), 844_{670} (ϕ_{st}= 0.18; p=0.04) and 841_{980} (ϕ_{st}= 0.35; p= 0.004). Interestingly, the same loci were the most important when the differentiation was assessed by means of G_{st}.

The Bayesian cluster analysis suggests that the 41 accessions under study lack subgroups. According to Pritchard *et al.* (2000), and Pritchard and Wen (2003), two important observations reinforce this conclusion: the high variability of the alpha values (0.02 at 0.14) once the Markov chain converges, and the symmetrical assignment of each individual to only one cluster.

Patterns of Linkage Disequilibrium and Association Analysis

The patterns of non-random association (LD) were analyzed for 3081 pairs of allelic combinations in the two flowering duration groups. In both groups, following the Bonferroni correction applied, only 3% of significant LD (P≥ 0.0002) was observed. When all the accessions considered together, the level of LD increased marginally to 4.6%.

The associations between ISSR molecular markers and flowering duration were analyzed by means of logistical regression. Out of the 79 polymorphic loci studied, only three revealed significant correlations with flowering duration variation: 811_{600} (R^2= 0.19; P= 0.04), 812_{1180} (R^2 = 0.20; P= 0.02), and 841_{980} (R^2= 0.25; P= 0.01).

Because locus 841_{980} presented the highest level of correlation with variation in flowering, the 980-pb band was isolated, and cloned to identify its sequence (Figure 3) and compared against sequences in the GenBank database (www.ncbi.nlm.nih.gov). The analysis showed high similarities with *Medicago truncatula* vascular protein (MTR_8g079820) (NCBI Accession number (AN) XM_003629543.1) with an *E-value*= 7^{-17}, along with *Arabidopsis lyrata subsp. lyrata* transducin family protein (AN: XM_002875992.1) with an *E-value*= 1^{-14}, and as well with other sequences stored in the database (Table 4).

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1    gcccttaatttgacaaccttggtactctttcanaaatcgctttggatgagtttagatcgaaagtcactttatcaaactcaaaattctaatcttcgctatgggcacag
112  gtgctaataatgataaaactatatattgtgcagcatctgttttgaaagtaaagctttcatcgttgggttcatctggaatattagctgagcatcaacctcaagctca
223  actacaaaagcatcatatgaaagggtgagatcaacatttttgtgaagcaggttcattatcacgaccatttcttgtgttcagttaatttggttgtattcagaggccttt
335  gtcnatggcttcnngtcnaaatggttggattttgtcaaatacaaatgcaaacatattccttctagtaaaccatatacataatccctgacaacatgtttcaacttca
447  actactacttttagattatgctaatttgcttgtttagagaaaatgagagatttgatggggnccatggttaccaatgctttagcgtttttgaattcccctttgagataat
558  cacatagcatctntatccacgcattaatttgtttgcaccaacttcactagatcaatcaactaatcttcgacatcttcaatcaatattgggaacttgctattcgatcat
670  ccccctgcagtaagtcagatgaaatgaatgatattttcaactctaggatataaatgagtttggttcaaaagttgtaggaggtacgatcgttgtaggagttgcaat
779  aacttcaaagcaacgcaaagaattcatgtgttctttcaaaaaagaattcatgtgttatttccactcttcaaattggatggaaggcattcaagtaatagcagaac
889  accactatcctgattggttttcctgagaaaggactgaaaaacgagatnntagataacancgtagcaaactagccgttgtcgatcaacgaggtctttgaggtg
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Figure 3. Sequence of 841_{980} locus obtained from a cloned band amplified using the ISSR primer 841.

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Table 4. Similarity between the sequenced 841_{980} (isolated from *F. chiloensis*) and sequences in the GeneBank database (www.ncbi.nlm.nih.gov).

Accession number	Description	Species	*E*-value
XM_003629543.1	Vascular protein	*Medicago trucantula*	7^{-17}
XM_002875992.1	Transducin family of proteins	*Arabidopsis lyrata subsp. lyrata*	1^{-14}
XM_003524537.1	Predicted uncharacterized proteins LOC100805443	*Glycine max*	2^{-18}
XM_003550025.1	Predicted uncharacterized proteins LOC100804284	*Glycine max*	2^{-17}
XM_003517240.1	Predicted uncharacterized proteins LOC100787845	*Glycine max*	9^{-16}
emb AM432638.2	Chromosome or whole genome sequences (contig VV78X211074.7)	*Vitis vinifera*	1^{-25}
gb AC235825.2	Chromosome or whole genome sequences clone GM_WBb0011N19	*Glycine max*	3^{-21}
gb AC232771.1	Chromosome or whole genome sequences	*Solanum lycopersicum*	2^{-18}
emb|CT573051.1	Chromosome or whole genome sequences	*Medicago truncatula*	7^{-17}
dbj|AP005707.3	Chromosome or whole genome sequences	*Oryza sativa* Japonica	2^{-11}

DISCUSSION

Association studies can be applied in natural populations and germplasm banks, but they not only require a deep knowledge of LD level and the genetic structure of the individuals but also an accurate characterization of the trait under study. All this information allows more precise association analysis and makes it possible to avoid false positives (Remington *et al.,* 2001; Flint-Garcia *et al.,* 2003).

The appropriate phenotypic characterization of samples grown under similar environmental conditions is one of the basic aspects for the genetic analysis of complex traits (Falconer and Mackay, 1996; Buckler and Thornsberry, 2002). The systematic collection of data from a germplasm collection maintained under homogeneous conditions made it possible to identify two groups of Chilean strawberry accessions, the flowering durations of which differed significantly. These results are not surprising, given that Lavín *et al.,* (2000), and Hancock *et al.* (2003) pointed out that the morphological and physiological differentiation observed in this species could correspond to the presence of different ecotypes along with its wide natural geographic distribution.

Additionally, the magnitude of LD in the strawberry and the appropriate management of the genetic structure would suggest that association analysis could be a very useful tool for identifying the genetic polymorphisms associated with the traits of agricultural interest. In the red-fruit Chilean strawberry, the level of LD (3-4.6%) was similar to that observed in the other such open-pollinated species as *Pinus contorta* ssp. *latifolia* (3,8%; Epperson and Allard, 1987), but inferior to that observed in corn (9,7%; Remington *et al.,* 2001). Open pollination (alogamy) has been highlighted as an important factor that reduces the LD in plants (Flint-Garcia *et al.,* 2003) and could partially explain the low LD observed in the Chilean strawberry. Another factor that can influence the LD observed, is the statistical correction employed to decide what actually a significant LD is. It is accepted that the Bonferroni correction is a very conservative method of avoiding false positives (Remington *et al.,* 2001).

The Chilean strawberry displays an important genetic differentiation between the two botanical forms found in Chile. Thus, to avoid false associations between ISSR markers and flowering time, the 41 accessions considered here were all of *F. chiloensis* f. *patagónica* (red fruit). The Bayesian clustering and AMOVA did not

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show any evidence of any subgroup between the studied accessions. The strategy of considering individual groups, genetically homogeneous, to study the LD, has led to the identification and isolation of several genes in human populations (Pritchard and Przeworski, 2001) and some crop plants (Flint-Garcia *et al.,* 2003). In the present study, logistical regression led to the identification of three ISSR loci of significant correlations with the flowering duration. This confirms the utility of the ISSR markers to study complex traits. Other investigations also have revealed ISSR polymorphisms related to flowering in *Fragaria vesca*. Cekic *et al.* (2001) identified some ISSR bands linked with the Seasonal Flowering Locus (SFL), an important locus involved in genetic control of flowering, and Albani *et al.* (2004) characterized the bands in use that locus as a specific marker. Similar to the results obtained by the latter group of researchers, the ISSR sequence associated in the present study with flowering duration in Chilean strawberry did not show any similarity with other sequences available in the GenBank. This could be expected considering that no previous description of ISSR randomly amplified regions have been reported. Besides that, the 841_{980} region might not only be associated with flowering time but also with other reproductive functions and it probably could show a high degree of polymorphism.

Interestingly, the ISSR bands associated with flowering duration were produced by dinucleotide primers GA, singled out as one of the most abundant microsatellites in plant genomes (Morgante *et al*., 2002), including the highly closed species *F. virginiana* (Ashley *et al*., 2003). Moreover, GA, CA and CT microsatellites have been associated with some transcribed regions of plant genomes (Morgante *et al*., 2002; Li *et al*., 2002; 2004).

The results presented here indicate the ongoing study as one of the few ones using association analysis to identify genetic polymorphisms related to complex traits in non-experimental populations, as is the case of the strawberry germplasm bank screened in the study. This is particularly noteworthy given the singular genetic-evolutionary characteristic of the Chilean strawberry (Hancock, 1999). Lastly, it is necessary to point out that these results constitute the first step toward an attainment of useful molecular markers for marker-assisted selection and as well for the identification of candidate genes involved in flowering time duration in *F. chiloensis.* In the future studies, more intensive must be carried out to characterize more sequences and to analyze their effects on flowering, while making use of segregating populations.

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شناسایی نشانگر های ISSR همراه با طول زمان گلدهی در نمونه های توت فرنگی شیلی (*Fragaria chiloensis*)

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چکیده

طول دوره گلدهی و میوه دهی در توت فرنگی شیلی (*Fragaria chiloensis* (L.) Duch.) محدود به دو ماه می باشد که این مورد توسعه تجاری گونه را به شدت محدود می کند. هدف از تحقیق حاضر شناسایی نشانگر های چند شکلی ISSR پیوسته با طول گلدهی در نمونه های *F. chiloensis* می‌باشد. برای این منظور داده های مربوط به طول گلدهی در طی ۳ سال در ۴۱ نمونه به همراه نتایج ۴۱ پرایمر ISSR مورد بررسی قرار گرفتند. با استفاده از الگوریتم PAM داده ها در دو گروه جداگانه قرار گرفتند که به ترتیب شامل ۲۳ نمونه در مقابل ۱۸ نمونه و روز تا گلدهی ۱.۶۴ در مقابل ۹۵.۶ بودند. بطوریکه طول گلدهی بین دو گروه معنی دار بود. از طرف دیگر اثر سال نیز برای صفت طول گلدهی بین دو گروه معنی دار بود. ۱۰ مورد از پرایمر های ISSR مورد استفاده الگوی باندی تکرارپذیری را ایجاد نمودند، بطوریکه در مجموع از ۱۰۶ مکان ژنی۷۹ مکان چند شکل بدست آمد. سه مکان ژنی ISSR (811_{779}, 844_{670}, 841_{980}) نقش عمده ای را در ایجاد تمایز بین نمونه ها نشان دادند. همچنین سه مکان ژنی ISSR ((811_{600}, 812_{1180}, 841_{980})) همبستگی معنی داری را با تنوع طول گلدهی نشان دادند. مکان ژنی 841_{980} که بالاترین همبستگی را با طول گلدهی نشان می داد جداسازی، همسانه سازی و تعیین توالی گردید ولی همولوژی کمی با توالی های موجود در بانک اطلاعاتی GenBank نشان داد. مکان ژنی که دارای همبستگی بالایی با تاریخ گلدهی دارد می تواند در ایجاد نقشه کنترل کننده صفت کمی (QTL) به منظور انتخاب و بهبود خصوصیات گونه های جنس *Fragaria* و یا حتی گونه های نزدیک به این جنس مورد استفاده قرار گیرند.

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