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ORIGINAL PAPER

High-Throughput RAD-SNP Genotyping for Characterization of Sugar Beet Genotypes

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Abstract High-throughput single-nucleotide polymorphism (SNP) genotyping provides a rapid way of developing resourceful sets of markers for delineating genetic structure and for understanding the basis of the taxonomic discrimination. In this paper, we present a panel of 192 SNPs for effective genotyping in sugar beet using a high-throughput marker array technology, QuantStudio 12K Flex system, coupled with Taqman OpenArray technology. The selected SNPs were evaluated for genetic diversity among a set of 150 individuals representing 15 genotypes (10 individuals each) from five cytoplasmic male steriles (CMSs), five pollinators, and five commercial varieties. We demonstrated that the proposed panel of 192 SNPs effectively differentiated the studied genotypes. A higher degree of polymorphism was observed among the CMSs as compared to pollinators and commercial varieties. PCoA and STRUCTURE analysis revealed that CMSs, pollinators, and varieties clustered into three distinct subpopulations. Our results demonstrate the utility of the identified panel of 192 SNPs coupled with TaqMan OpenArray technology as a wide set of markers for high-throughput SNP genotyping in sugar beet.

Keywords Sugar beet · Genetic diversity · SNP genotyping · QuantStudio platform

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Introduction

Genotyping with molecular markers is a rapid and cost-effective strategy for assessing genetic variation, developing genome-wide association mapping approaches, establishing linkage maps and is useful in the development of cultivar-specific plant breeding programs (SyvŠnen 2005; Ganal et al. 2012). Previously, several types of molecular markers have been described and used effectively to describe population structure, although most of them are limited in their use because of the high cost of large-scale analyses. Among the various types of markers, single-nucleotide polymorphisms (SNPs) are recommended markers for mass-throughput genotyping (Mammadov et al. 2012). SNPs occur at a frequency of at least 1 % in a given population and, together with recombination, are the two main sources of genetic diversity (Ganal et al. 2009). SNPs as markers are abundantly distributed across the genome and can be found in coding as well as non-coding regions (Rafalski 2002). Among crops, variation in SNP frequency along the genome has been observed: maize has one SNP every 104 base pairs (bp) (Tenaillon et al. 2001), wheat has one SNP every 200 bp (Ravel et al. 2006), soybean has one SNP every 273 bp (Zhu et al. 2003), and sugar beet has one SNP every 130 bp (Schneider et al. 2001). In the past 10 years, various high-throughput SNP genotyping approaches have been developed (Gupta et al. 2008), the

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applicability of which depends on the number of samples and markers to be analyzed for population genomics.

Sugar beet is one of the world's most important crops currently supplying around 20 % of the sugar consumed worldwide (Biancardi et al. 2010). An estimation of the genome length on the basis of the *C* value is reported to be 714 to 758 million base pairs (McGrath et al. 2007), and most observed sugar beet genotypes are diploid ($2n = 2x = 18$). Currently, a loss in the genetic basis of the commercial sugar beet varieties has been observed, mainly due to the repeated use of a limited number of genotypes as parents in breeding programs (McGrath et al. 1999). A narrow genetic basis is likely to cause inbreeding depression and reduced genetic variability, which in turn can lead to genetic plateaus in sugar beet (Geidel et al. 2000). The release of the RefBeet_0.9 draft assembly of the whole genome sequence of KWS2320 genotype has allowed genome-wide mapping strategies, thus facilitating genotyping efforts (http://bvseq.molgen.mpg.de/index.shtml). Few studies so far have examined the genetic diversity of sugar beet parental lines and their progeny on the basis of the SNPs mapped to the available scaffolds of the sugar beet genome (Li et al. 2011; Simko et al. 2012). To increase resources for the effective discrimination of the underlying genetic basis in sugar beet breeding programs and to boost genetic improvement, a more detailed genetic characterization of germplasm collections and their genetic relationships is presently a matter of prime concern. The development and application of high-throughput genome-wide genotyping methods, such as SNP arrays, can significantly broaden the current germplasm screening capabilities and their subsequent evaluation in correlation to the parental lines. Life Technologies Inc. (LTI, Carlsbad, CA, USA) recently released a platform (QuantStudio 12K Flex system coupled with Taqman OpenArray technology) having key elements required for high-throughput SNP genotyping (Johnson et al. 2012), thus allowing for a rapid genotyping of large number of SNPs (up to 3,072) in many individuals (up to 480) in a relatively short time. In this paper, we introduced a novel high-throughput SNP genotyping approach, based on QuantStudio 12K Flex system, to assess the genetic diversity in sugar beet. In the light of the present goal, we evaluated the potential of 192 SNPs as markers for sugar beet genetic and genomic research.

Material and Methods

Plant Material

To evaluate the proposed SNP panel, we selected a set of 150 individuals representing 15 genotypes (10 individuals each) from five cytoplasmic male steriles (CMSs), five pollinators, and five commercial varieties (Table 1). CMS lines are monogermic, susceptible to diseases (e.g., rhizomania and cercospora), and are dominant lines for high sugar yield; on the contrary, pollinators are multigermic and resistant to rhizomania. The aforementioned genotyping lines were derived from an ongoing wide breeding program at CRA-Research Institute for Industrial Crops (Rovigo, Italy). Commercial sugar beet varieties, which are widely grown in Italy, are provided by BETA SCARL (Ferrara, Italy). Two of the five analyzed commercial varieties were resistant to nematodes (Variety_1 and Variety_5), and three were resistant to rhizomania (Variety_2, Variety_3, and Variety_4).

Automated Genomic DNA Isolation

Automated genomic DNA (gDNA) isolation was carried out using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) with the BioSprint 96 workstation (Qiagen) according to the manufacturer's instructions. In brief, 50 mg of leaf material was used as a starting material and was subsequently added to 2-ml tubes having a stainless steel bead suspended in 300 μl of RLT buffer (guanidine thiocyanate buffer under patent protection). For effective homogenization, TissueLyser (Qiagen) was used to homogenize 48 samples at a time with two 1-min shaking steps (30 Hz each). Subsequently, the samples were centrifuged at 6,000×*g* for 5 min. Following centrifugation, the pellet was discarded and the supernatant was used for the subsequent DNA isolation steps, which involve suspension with MagAttract magnetic particles allowing the binding of DNA to their silica surface. In downstream steps, DNA was purified by passing through four S-Block plates, the order of which is as follows: the first plate contained buffer RPW (guanidine thiocyanate buffer under patent protection) with isopropanol and RNase, second and third plates were loaded with 96 % ethanol, and the last one was loaded with 0.02 % (*v*/*v*) of Tween 20. Finally, the isolated DNA was suspended in 200 μl of nuclease-free water and stored at −20 °C until further use. For quality assessment and integrity check, quantification of the isolated DNA was done using a spectrophotometer at a 260-nm wavelength. A final yield of 21 ng μl^{-1} and A_{260}/A_{280} ratios ≥1.6 was obtained for further downstream analysis.

High-Throughput SNP Genotyping

The main goal of this research was to evaluate the potential of 192 SNPs as markers for research on sugar beet genetics and genomics. In view of the present goal, genotyping was carried out for 192 SNP markers mapped on the reference sugar beet genome (version RefBeet-0.9) downloaded from http://bvseq.molgen.mpg.de. The panel of 192 SNPs was identified using restriction-site associated DNA (RAD) sequencing of four individuals of a sugar beet pollinator (Pollinator_1). RAD sequencing was carried out at Floragenex Inc. according to the protocol described by Baird et al. (2008). Polymorphic

Table 1 Description of the sugar beet genotypes used in this study and average expected heterozygosity (H_E) estimated from SNP markers

Name	Selected trait	Monogermity or multigermity	Heterozygosity (H_E)
CMS_1		Monogerm	0.102
CMS_2		Monogerm	0.163
CMS_3		Monogerm	0.149
CMS_4		Monogerm	0.213
CMS_5		Monogerm	0.250
			0.175 a
Pollinator_1	Rhizomania	Multigerm	0.075
Pollinator_2	Rhizomania	Multigerm	0.068
Pollinator_3	Rhizomania	Multigerm	0.052
Pollinator_4	Rhizomania	Multigerm	0.102
Pollinator_5	Rhizomania	Multigerm	0.071
			0.074 c
Variety_1	Nematode	Monogerm	0.185
Variety_2	Rhizomania	Monogerm	0.124
Variety_3	Rhizomania	Monogerm	0.138
Variety_4	Nematode	Monogerm	0.175
Variety_5	Rhizomania	Monogerm	0.148
			0.154 b

Means (values in italics) within genotypes followed by a different letter are significantly different at the 0.05 probability level

markers were identified as per the procedure described by Baird et al. (2008). Briefly, reads were trimmed and cleaned, and reads with Ns and artifacts were removed. Polymorphic RAD tags were identified and were mapped to the reference genome of sugar beet and were scanned for the presence of single mismatches (Baird et al. 2008). The 192 SNPs showing a perfect match—with a single mismatch—to the reference genome were selected for evaluation as genotyping markers.

A total of 10 ng of isolated DNA sample was mixed with 2.5 μl of TaqMan OpenArray Genotyping Master Mix in a 384-well plate. The samples were subsequently loaded onto the OpenArray plate using the QuantStudio 12K Flex OpenArray AccuFill System (LTI). After real-time PCR and allelic discrimination, the results were analyzed using TaqMan Genotyper v1.2 software (LTI).

Statistical Analysis

We estimated the following genetic parameters: linkage disequilibrium (LD) and average expected heterozygosity (H_E) in each genotype, and genetic distances (Dst) (Nei 1978) between genotypes, using ad hoc scripts and the package GenABEL (Aulchenko et al. 2007) in the R programming environment version 2.12.2. The average H_E in the three groups (CMSs, pollinators, and commercial varieties) was compared through analysis of variance followed by Duncan's test using the R package ASREML (Butler et al. 2007). To cluster the examined sugar beet genotypes, principal coordinate analysis (PCoA) was carried out using the program GenAlEx (Peakall and Smouse 2006). The Bayesian algorithm implemented in the program STRUCTURE 2.1 (Pritchard et al. 2000) was used to infer the most likely number of clusters (K) with the following parameters: number of iterations=10, length of burn-in period=10,000 replicates, number of replicates after burn-in=10,000, with true number of clusters (K) ranging from 2 to 6.

Results and Discussion

In the present research, we proposed a fingerprinting analysis of 15 sugar beet genotypes using an array of 192 SNP markers, with the aim of providing a SNP panel for the effective discrimination of sugar beet genotypes. We observed that the majority of the SNPs (95 %) were polymorphic across sugar beet genotypes, which support the use of the developed SNP marker panel for high-throughput SNP genotyping in sugar beet. The array of 192 SNPs identified in this study along with their corresponding mapping coordinates are available as supplementary material S1 (Table S1). The selected SNPs in the present study produced high-quality signals with a rate of undetermined results accounting for only 0.45 %, which is an important parameter for selecting suitable marker systems. Previously, a similar estimate of the undetermined rate (0.2 %) has been observed in sugar beet (Simko et al. 2012). We observed an average LD of 0.111, 0.080, and 0.075 in CMSs, pollinators, and commercial varieties, respectively, which is in line with the previously reported LD values in sugar beet (Viard et al. 2004; Arnaud et al. 2009).

To validate the effectiveness of the genetic discrimination using 192 SNP markers, we selected two population genetic parameters: average expected heterozygosity (H_E) and genetic distance (Nei 1978), which were estimated within and between sugar beet genotypes to determine the genetic diversity in the sampled population. Significant differences ($p < 0.05$) were found for heterozygosity in the different genotypes (Table 1). The average expected heterozygosity ranged from 0.052 to 0.250 across 15 genotypes. The overall expected heterozygosity in CMSs (H_E=0.175) was substantially higher as compared to pollinators and commercial varieties (H_E=0.074 and H_E= 0.154, respectively). Genetic distances between genotypes were also estimated, and the matrix of pairwise genetic distances is reported in Table 2. The highest value of genetic distance was found between CMS_3 and pollinator_1 (Dst= 0.445), and the lowest genetic distance was observed between pollinator_4 and pollinator_5 (Dst=0.043). The low genetic diversity observed among pollinators is probably a direct consequence of the breeding programs at the Institute for Industrial Crops of Rovigo, which all shared the same initial resistance source to rhizomania, 2281-R1 (Biancardi et al. 2002). A wide genetic basis is essential in sugar beet to select and to breed for disease resistance, to prevent inbreeding depression, and to allow for adaptation to changing environmental conditions (Biancardi et al. 2012).

A PCoA was performed in order to gain further insights into the genetic similarity of the analyzed genotypes (Fig. 1). The first two principal coordinates of PCoA accounted for 36 and 22 % of the variance, respectively, thus jointly accounting for 58 % of the total variation in the dataset. The first principal coordinate (PC1) differentiated between commercial varieties and pollinators, whereas the second principal coordinate (PC2) was able to identify CMSs. PCoA revealed the formation of two distinct clusters in commercial varieties and pollinators while CMS genotypes were split into four distinct clusters. In general, low genetic diversity was found among sugar beet parental lines and commercial varieties, as previously reported (McGrath et al. 1999; Saccomani et al. 2009). However, the present study clearly demonstrates that the most genetically diverse genotype was the CMS_5, which is in agreement with its known genetic background, derived from unselected breeding lines (E. Biancardi, personal communication, 2013). In sugar beet, breeding for disease resistance within a narrow germplasm pool, together with the use of cytoplasmic male sterility and monogermity for the production of commercial seed, can potentially lead to loss of heterozygosity and consequent increase of homozygosity (Biancardi et al. 2010). The incorporation of novel wild beet germplasm into domestic sugar beet likely will lead to the broadening of sugar beet germplasm as suggested previously by Frese (2010) and Stevanato et al. (2013). In general, identification of the resources to augment the broad genetic basis is a prerequisite for breeding programs (Panella and

Table 2 Pairwise Nei's genetic distances among sugar beet genotypes

	CMS_1	CMS_2	CMS_3	CMS_4	CMS_5	Pollinator_1	Pollinator_2	Pollinator_3	Pollinator_4	Pollinator_5	Variety_1	Variety_2	Variety_3	Variety_4
CMS_2	0.295													
CMS_3	0.379	0.297												
CMS_4	0.334	0.192	0.268											
CMS_5	0.196	0.271	0.208	0.203										
Pollinator_1	0.413	0.300	*0.445*	0.216	0.403									
Pollinator_2	0.439	0.349	0.401	0.199	0.395	0.054								
Pollinator_3	0.359	0.278	0.386	0.204	0.359	0.184	0.154							
Pollinator_4	0.376	0.287	0.397	0.109	0.321	0.130	0.109	0.145						
Pollinator_5	0.414	0.287	0.379	0.104	0.357	0.156	0.151	0.144	*0.043*					
Variety_1	0.349	0.330	0.275	0.261	0.205	0.345	0.346	0.342	0.307	0.310				
Variety_2	0.369	0.386	0.299	0.284	0.220	0.374	0.367	0.350	0.326	0.325	0.046			
Variety_3	0.355	0.296	0.293	0.257	0.198	0.292	0.315	0.276	0.277	0.288	0.160	0.182		
Variety_4	0.360	0.275	0.297	0.259	0.246	0.295	0.316	0.240	0.259	0.250	0.175	0.208	0.057	
Variety_5	0.438	0.377	0.340	0.291	0.222	0.346	0.335	0.368	0.304	0.311	0.169	0.196	0.092	0.107

The highest and lowest values of average genetic distance are highlighted in italics

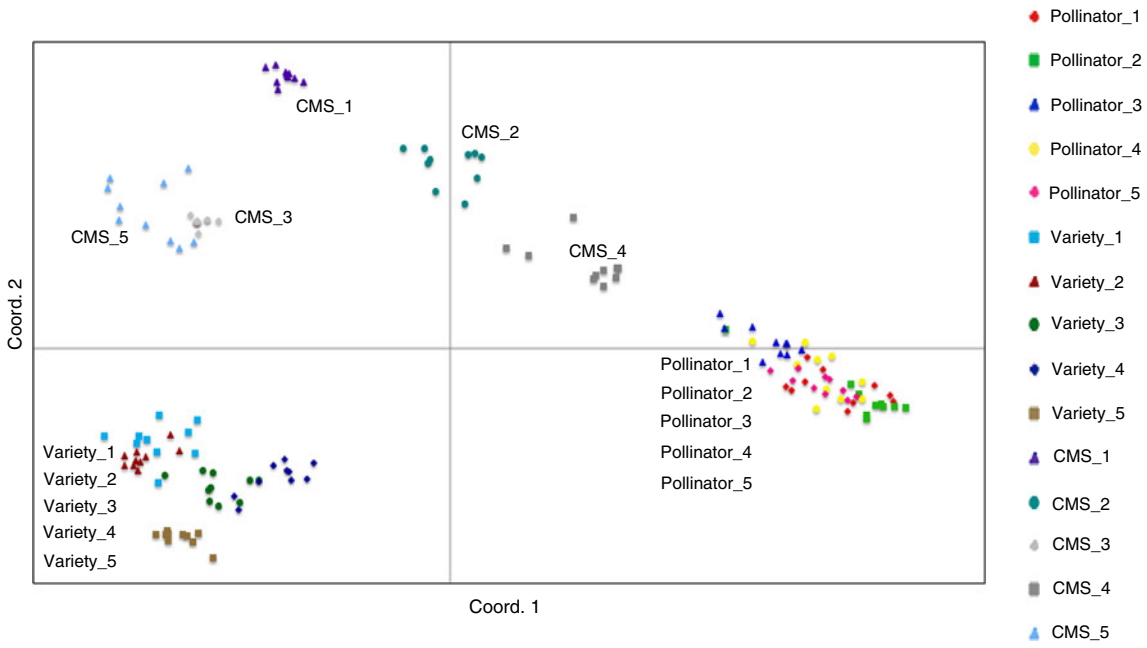

Fig. 1 Two-dimensional PCoA based on 192 SNPs of sugar beet genotypes (*CMSs*, *Pollinators*, *Varieties*). *Each dot* represents one individual. The first two principal coordinates of PCoA accounted for 58 % of the total variation

Lewellen 2007). The PCoA plot clearly illustrates the fine-scale genetic structure of sugar beet genotypes and allows effective discrimination among CMSs, pollinators, and commercial varieties.

In order to further investigate the potential of the selected panel of SNPs for effective sugar beet fingerprinting and to gain deeper insight into the genetic structure of the population, we further analyzed each group (CMSs, pollinators, and commercial varieties) using the Bayesian clustering algorithm of STRUCTURE, with varying K values (number of subpopulations) (Kaeuffer et al. 2007). The Bayesian analysis revealed that $K=3$ was the base value for the number of best supported clusters, thus classifying CMS, pollinator, and commercial variety genotypes each into three distinct clusters (Fig. 2). Our results showed that the clusters defined with the algorithm in STRUCTURE were similar with those revealed by PCoA. The observed results are in perfect agreement with the previously reported results by Li et al. (2011), which supports the observation of 90 % correspondence between the population structure defined by PCoA and STRUCTURE.

Conclusion

The present study proposed a wide repertoire of genome mapped RAD-SNPs markers for efficient characterization of

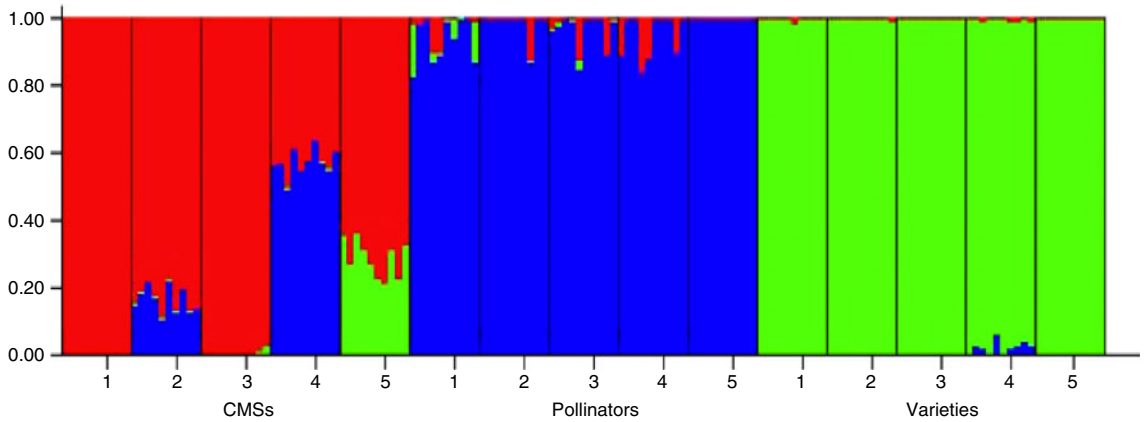

Fig. 2 Cluster analysis of sugar beet genotypes within each of the three groups (*CMSs*, *Pollinators*, *Varieties*) based on 192 SNP using the STRUCTURE software ($K=3$). Each individual is represented by a *vertical line*, which is partitioned into *colored segments* that represent the individual membership to the clusters

genetic diversity and population structure in sugar beet. The results of the present experimental layout clearly indicate that the proposed panel of 192 SNPs is a suitable resource for the effective discrimination of genetic diversity in sugar beet. In addition, the wide repertoire of SNPs evaluated in this study could serve as a potential source for the estimation of genetic relationships among sugar beet parental lines and varieties, with relevant impact on breeding program decisions.

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