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Research Article

Integrated Chemometric Fingerprints of SSR Markers and Leaf Polyphenol Profile for Marker-Trait Associations in *C. Sinensis* Species

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Abstract

Integration of the genetic and metabolic finger printing can provide a new approach to differentiate among and within species. Therefore, in the present study, SSR based genetic finger printing and HPLC-based metabolic finger printing was used to successfully discriminate the five sweet orange populations. PCA analysis revealed that genomic and metabolic data generated similar pattern of clustering and highlighted a high intraspecific variability in *C. sinensis* species. Both data were integrated, leading to the identification of associated SSR alleles with secondary metabolites. In fact, Multiple Regression Analysis (MRA) identified seven SSR alleles associated with six metabolites which may provide clues for identification of the genotypes with higher values. Furthermore, the correlation network between SSR markers and metabolites revealed a complex correlation network, which indicated the special metabolic pathways and the regulation networks of citrus species. This study is reporting the benefits of correlating SSR markers to metabolic data in citrus with the aim to identify molecular markers associated to distinct quality traits which would be very valuable in the selection of appropriate parents for future breeding programs.

Keywords: *C. sinensis*; SSR markers; Leaf polyphenols; Marker-trait associations

Introduction

The characterization of germ plasm banks, genetic variation and the improvement of oranges and other citrus species, has not been successfully carried out due to traits related to the reproductive biology of these species [1]. In fact, understanding the taxonomy, phylogenetic relationships, and genetic variability in citrus is critical for the determination of genetic relationships, characterization of germplasm, control of genetic erosion, design of sampling strategies for core collections, establishment of breeding programs, and registration of new cultivars [2].

Sweet oranges *(Citrus sinensis L.)* is considered as the best orange in the world because of its important nutritional source for human health and its huge economic value where Tunisia is the sole producer and exporter in the world.

DNA genetic markers have successfully established the fundamentals of citrus phylogeny [3]. In citrus and related genera, microsatellite has been used for phylogenetic studies and assessment of genetic variability [4].

Because many antioxidant flavonoids are thought to have evolved in plants to protect the photosynthetic machinery from oxidative damage [5], they hypothesized that leaf tissue would contain significantly more antioxidant flavonoids than fruit tissues [6]. These results were confirmed in the study investigated by [7], who showed that leaf tissue of citrus had the maximum antioxidant potential. Developing an understanding of the distribution of phenolics in citrus and its related species will give an assessment of the diversity present in this important group of plants. Recent reports studied the distribution on the flavonoid of citrustissues [8]. All of these active researches were mainly focused on fruits; however, less information on flavonoid content in citrus leaves is available.

The genetic background might play a pivotal role in the biosynthesis of secondary metabolites highlighting the necessity to investigate the correlation between them using advanced statistical tools in managing germplasm collections, including characterizing and establishing systematic relationships. As mentioned above, independent studies of either genetic diversity or chemical profile of citrus have been conducted in recent years, but, to our knowledge, none of the previous works addressed the influence of genetic variability on metabolic composition. So, combined analyses of chromatographic finger printing and molecular markers might be effective methods which can reveal both chemical and genetic diversities of citrus. Therefore, a comprehensive exploration of the correlations between metabolic and genomic diversity, achieved by superimposing data from these two "omics" approaches, could provide information regarding both the broad and specific relationships between metabolo-genotypes. This information would aid the identification of genetic associations between the metabolic and/or visible phenotypes. To date, several attempts have been performed to find correlative relationships between genetic and metabolic diversity in various organisms. It is still unclear whether it is possible to discover such correlations between each metabolite and the polymorphic pattern found at each chromosomal location to explain natural variation. Combinatorial approaches of population genomics coupled with metabolic patterns should play a significant role in the exploration of the association

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of genetic variation with metabolic changes that have a significant impact on evolution and adaptation.

To assess the correlative relationship between metabolic and genomic diversity found in 5 citrus populations. To perform these analyses, we developed a robust procedure to explore the relationship between metabolic and genomic diversity. Therefore, the present study attempts to assess the genetic and phytochemical diversity of *C. sinensis* populations and to understand the association of SSR markerswith biochemical traits. As a result, we present the first correlation map of metabolic and genomic diversity of *C. sinensis* species.

Materials and Methods

Plant materials

C. sinensis mature leaves were collected from adult (10 to 15 years old) cultivated healthy trees, uniform in size and growth vigor, grown under the same pedoclimatic conditions and located in citrus orchards in the cap bon peninsula (36°45'0" N, 10°45'0" E). The samples were stored in dry ice after collection, transferred to the laboratory and immediately grinded into liquid nitrogen and kept at 80°C until analyzed. Biological replicates were collected for each tree.

DNA extraction and SSR genotyping

The isolated leaf material was used for genomic DNA extraction by using a citrus specific protocol. Leaf samples were ground with liquid nitrogen in separate 2 ml eppendorf tubes. Extraction buffer [100 mM Tris-Hcl, 50 mM EDTA, 500 mM NaCl, SDS (20%) and 140 mM B-mercaptoethanol] was added, vortexed and incubated at 65°C for 30 min. After that, potassium acetate (5M) was added in each tube and the mixture was incubated on ice for 30 min. Subsequently, all tubes were centrifuged at 12000 rpm for 30 min. Then, isopropanol was added to the aqueous layer; the DNA was pelleted by centrifugation for 20 min and washed with 80 % ethanol. The DNA was dissolved in TE buffer and quantified before a dilution step to obtain uniform concentration of 50 µg/ml.

Twelve markers were used for the diversity study [9]. PCR was performed in a final volume of 15 µl. Each PCR reaction consisted of 1.5 mM of $MgCl_2$, 2.5 mM of dNTPs, 0.2 µM of each primer and 1U of DNA Taq polymerase with 50 ng of DNA. Cycling conditions were: 94°C for 5 min as an initial denaturation step before entering 40 cycles each composed of 30 sec at 94°C, 30 sec at annealing temperature, 1 min at 72°C and a final extension step of 4 min at 72°C.

Determination of total phenolic, total flavonoid contents and antioxidant capacity

The grounded citrus leaf material (3g) of each sample was separately extracted by shaking with 30 ml of pure methanol for 30 min. The extracts were then kept for 24 h at 4°C, filtered through a Whatman filter paper (No. 4), dried under vacuum and stored at 4°C until their analysis as described by Mau et al. [10]. Total phenolic and flavonoid contents were determined according to Dewanto et al. [11]. Total phenolic content was assayed by adding 0.125 ml of the Folin-Ciocalteu reagent and 0.5 ml of deionized water was mixed well and immediately, the absorbance was measured at 510 nm using UV-Vis spectrophotometer.

The antioxidant ability of the citrus methanolic extract was assessed using the stable free radical 2,2- Diphenyl-1-PicrylHydrazyl (DPPH). The antiradical activity was expressed as IC50 (concentration required to cause a 50% DPPH inhibition: lg/ml). The inhibition percentage I% of radical scavenging activity was calculated as DPPH scavenging effect (%) = [(A0-A1)/A0] 9 100; where A0 and A1 are the absorbance's of the control and the sample at 30 min, respectively. The antiradical activity was expressed as IC50 (µgmL-1), a low IC50 value corresponding to a high antioxidant activity. BHT was used as a positive standard, and all samples were analyzed in triplicate.

Identification of phenolic compounds

For HPLC analysis, 500 µl of BHT (ButylatedHydroxytoluene) (1 mg ml-1), as internal standard, was added to the methanolic extract. The phenolic compound analysis was carried out using an Agilent Technologies 1100 series Liquid Chromatograph (RP-HPLC) coupled with a UV-Vis multi-wavelength detector and equipped with a 250 9 4.6 mm, 4 µm Hypersil ODS C18 reversed phase column kept at ambient temperature. The mobile phase consisted of aceto-nitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was of 0.5 ml/min. The gradient program was as follows: 15% A/85% B 0-12 min, 40% A/60% B 12-14 min, 60% A/40% B 14-18 min, 80% A/20% B 18-20 min, 90% A/10% B 20-24 min, and 100% A 24-28 min. The injection volume was of 20 µl, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 lm membrane filter before injection. Phenolic compounds were identified according to their retention time as well as by spiking the sample with standards. Analyses were performed in triplicate.

Validation of quantitative analysis

Standard stock solutions of the used standards were prepared by dissolving 0.01 g in 10 mL methanol (1 mg/mL) and further diluted toappropriate concentrations for standard curve preparation. The peak areas were automatically measured by an integrator of HPLC instrument. Calibration curve was obtained by plotting the peak area against concentration. The Limit of Detection (LOD) and Quantification (LOQ) under the chromatographic conditions were determined by injecting a series of standard solutions until the signal-to-noise (S/N) ratio for each compound was 3 for LOD and 10 for LOQ.

Statistical analysis

The reproducible SSR alleles were scored and a binary matrix was constructed using each molecular marker. Polymorphism Information Content (PIC) values were calculated, for SSR loci, according to Smith et al. [12], using the algorithm for all primer combinations as follows: $PIC = 1 - \sum Pi^2$, where Pi^2 is the frequency of the ith allele. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed but also the relative frequencies of those alleles.

Analysis of variance was carried out for all of the chemical traits by SPSS v.21 software using one-way ANOVA. In order to explain the potentiality of these variables (chemical and SSR) and to depict relationships among individuals, Principal Component Analysis (PCA) was applied using PAST software. To check for possible correlations between genetic and chemical distances among cultivars, Mantel test was performed using XLSTAT-Pro 7.5.3 software.

Associations between molecular markers (as independent variables) and metabolite data (as dependent variables) were performed by a Multiple Regression Analysis (MRA) using "stepwise" method of "linear regression analysis" option of SPSS version21. The robustness of the multiple linear regression model in providing an adequate setting for association tests [13]. The analysis was

based on the following model: Y = a + b1m1 + b2m2 + ... + bjmj + ... + bnmn + d + e, which related the variation in the dependent variable (Y cultivar means for a quantitative trait) to a linear function of the set of independent variables mj, representing SSR markers (as first described by Virk et al. [14]. The bj terms are the partial regression coefficients that specify the empirical relationships between Y and mj, d represents between accession residuals, which is left after regression, and e is the random error of Y that includes environmental variation [14]. To select independent variables for the regression equation, F values with 0.045 and 0.099 probabilities were used to enter and remove, respectively. R^2 denotes the square of R, the multiple correlation coefficients. Selected markers were further tested independently with linear curve fitting using linear models for confirming the significance of β-statistics for each band identified by MRA. Beta can be defined as the standardized regression coefficient = BSx/Sy, where β is the regression coefficient or slope and Sx and Sy are the standard deviations of independent (x) and dependent (y) variables. Student's t test was performed to test significance between mean trait estimates of genotypes where specific markers were present and absent. Markers showing significant regression values were considered as associated with the trait under consideration. The criteria for correlation determination were correlation values of ≥0.5 or ≤-0.5 and p-values ≤0.05. Graphical visualization of the correlation network was performed using Cytoscape 3.0 (http://www.cytoscape.org/). The data used to build the correlation network were imported into the Cytoscape program as an EXCEL file containing the correlation values as well as a description of the different nodes: metabolites (circles) and SSR (triangles). Each pair of nodes, metabolite *vs*. SSR with a correlation coefficient ≥0.5 was connected by a line indicating a positive (+1) or negative (-1) correlation.

Results and Discussion

Genetic diversity

Ten SSR primers were used to study the genetic diversity of five *C. sinensis* populations and the results are summarized in Table 1. Twenty-three alleles were scored, ranging from 110 to 300 bp, with a mean of 2.3 alleles per locus. The observed Heterozygosity (Ho) ranged from 0.110 (TAA27) to 0.750 (TAA15) with a mean of 0.81. The expected Heterozygosity (He) ranged from 0.115 (ATC09) to 0.660 (TAA) with a mean of 0.364. The detected level of heterozygosity within this study was in accordance with what has been reported in previous work [15]. The PIC ranged from 0.265 for the locus TAA27 to 0.6 for TAA1, with an average of 0.435, indicating a good level of polymorphism for the used loci. Generally, the sweet orange populations considered within this study showed a moderate level of polymorphism. In fact, similar results were obtained using SSR markers of sweet orange [16]. Based on these SSR data, the Principal Component Analysis (PCA) revealed that 88.86% of the variation was explained by the first two axes (axis1-71.75%, axis2-17.11%), where sweet orange populations were separated into three main groups (Figure 1). The first group (I) contained the three populations (Ta6, Su1 and KP3). The population SP2 and CS1 formed the second (II) and the third (III) group, respectively.

Total phenolic, total flavonoid content and antioxidant activity

For sweet orange populations, the TP levels in the leaf extracts ranged from 112.85 mg GAE/g DW (pop SP2) to 183.6 mg GAE/g DW (pop Ta6) (Figure 2a). These amounts exceeded those obtained in other citrus tissues [17,18]. The TF levels fluctuated between

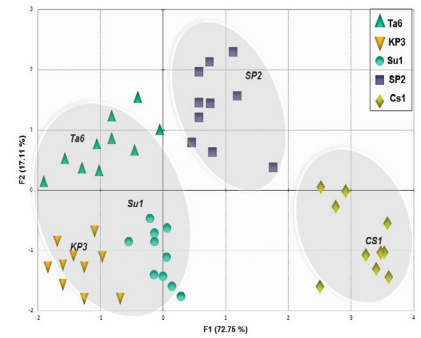

Figure 1: Principal Component Analysis (PCA) of *C. sinensis* populations based on SSR markers.

12.63 and 66.67 mg EC/gDW, KP3 had the lowest levels, while Ta6 had thehighest content. GafsiB2 and F15 had significantly higher TFcontents than the other genotypes (Figure 2b).

Additionally, the leaf extracts from *C. sinensis* were screened for their antioxidant activity using DPPH scavenging methods. The results highlighted an important *in vitro* antioxidant activity, which varies significantly according to the studied population (Figure 2c). The highest DPPH-radical scavenging activity was observed for SP2 population extract with an IC50 value of 0.45 mg/mL, while the population Ta6 displayed the lowest antioxidant activity with an IC50 value of 5.12 mg/mL. These results highlighted that citrus leaves may be considered as an important source of substantial secondary metabolites and as natural antioxidants.

RP-HPLC-DAD fingerprint analysis of *C. sinensis* leaves

Phenolic identification and quantification were set against authentic standards analyzed under identical analytical conditions. Variance analysis revealed highly significant differences between sweet orange populations for all the quantitative variables (Table 2). Overall, eight compounds from different phenolic categories (phenolic acids and flavonoids) were identified and were distinctly distributed among the studied populations (Table 2). Individual phenolic investigation depicted that the major compounds of *C. sinensis* leaves were naringin (69.82 µg/g DW), ferulic acid (52.92 µg/g DW) and naringenin-7-o-glucoside (51.8 µg/g DW). The populations Cs1 had the highest naringin (35.2µg/gDW), ferulic acid (15.32 µg/g DW) and naringenin-7-o-glucoside (21.52 µg/g DW) content Flavone and rutin were also detected in appreciable amounts in sweet orange leaves with 23.9 and 20.04 µg/g DW, respectively. These findings are in accordance with previous works that reported similar findings regarding the abundance of naringin in different citrus species [18,19]. In order to maximize the difference and easily discriminate among the samples, a PCA was implemented by performing singular value decomposition on the data array of the fingerprints. The first two axes of PCA plot accounting for 69.69%. In the PCA score plot, the five populations of *C. sinensis* were differentiated into four groups (Figure 3). Group I consisted of samples purchased from the populations Ta6 and Su1. The population SP2, KP3 and Cs1 formed the second (II), the third (III), and the fourth group (IV), respectively.

Correlation analysis

Mantel test was used to evaluate the existing correlation between of the genomic and chemical data matrices. A significant correlation was found with the Mantel test (r = 0.618; P = 0.04) and the p-value

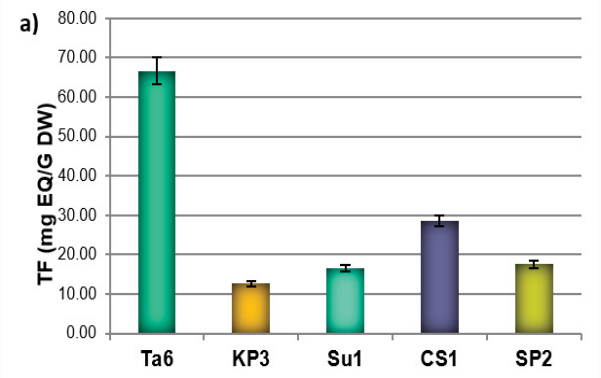

Figure 2a: Total Phenolic content (TP).

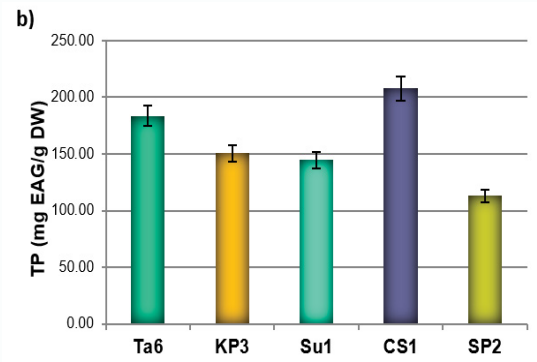

Figure 2b: Total Flavonoid content (TF).

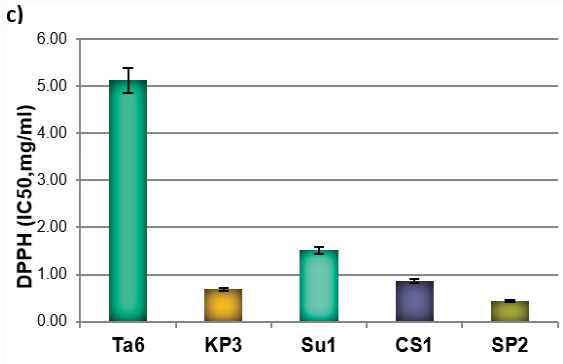

Figure 2c: DPPH scavenging activity of C. sinensis leaf extracts.

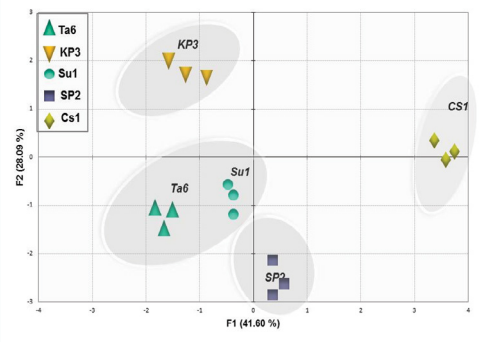

Figure 3: Principal Component Analysis (PCA) of C. sinensis populations based on polyphenol profiles.

Table 1: Parameters characterizing the ten microsatellite markers used in genotyping.

	Locus	N	Ho	He	Pic
1	TAA1	3	0.6	0.53	0.6
2	TAA27	2	0.11	0.138	0.265
3	GT03	2	0.38	0.45	0.45
4	CAG01	2	0.733	0.42	0.461
5	ATC09	3	0.22	0.115	0.39
6	CT21	2	0.31	0.23	0.28
7	cAGG9	2	0.652	0.288	0.58
8	CAC15	2	0.4	0.32	0.3
9	CAC23	2	0.65	0.49	0.52
10	TAA15	3	0.75	0.66	0.5
	Mean	2.3	0.481	0.364	0.435

was calculated from the distribution of r (AB) estimated from 10,000 permutations (Figure 4). Thus, a correlation network was drawn using the whole data sets (Figure 4). Analytical results revealed the existence of 28 pairs of significant correlations among the identified metabolites. The highest positive correlation was detected between rutin and naringin ($r = 0.957$, $p< 0.001$), however, a strong negative correlation was observed between hesperetin and naringin ($r = -0.802$, $p< 0.001$). Moreover, the partial correlation, based on MRA analysis, revealed 7 SSR markers (alleles) showing statistically significant correlation and in association with some of the identified metabolites (Table 3). The proportion of metabolic variation (R^2) accounted by each marker was calculated (Table 3). Based on the obtained results, one to several metabolites seemed to be controlled and regulated by some locus represented by the SSR markers. The strongest correlation was that of CAG01165 with hesperetin ($r = 0.954$; $\beta = -0.954$; $P = 0.00$). CT21190 marker was associated with rutin and ferulic acid, showing a negative beta values ($\beta = -0.557$ and $\beta = -0.584$, respectively). Conversely, TAA27130marker was positively associated with naringenin7-o-glucosides ($\beta = 0.585$) and flavone ($\beta = 0.533$). These types of associations highlighted positive correlations between the pairs of metabolites rutin/ferulic acid, and naringenin 7-o-glucosides/flavone (Table 3). Some of the SSR markers were found to be associated with more than one metabolite in MRA. In fact, such associations may arise due to pleiotropic effect of the linked QTL on different traits. The markers cAGG9220 and CAG0 170 were found to be negatively associated with flavonose ($\beta = -0.628$ and $\beta = -0.611$, respectively). The marker ATC09180 displayed a negative association with ferulic acid ($\beta = -0.584$, $t = -2.590$, $P = 0.022$), and lastly, the marker cAGG9200 showed a strong negative correlation with flavone ($\beta = -0.714$, $P=0.003$). The identification of these highly associated SSR markers point out that these markers could be closely linked and located on the same chromosome region and may possibly represent a region controlling and/or regulating many metabolites. Thus, such important markers could be used for QTL analysis and for Marker-Assisted Selection (MAS) breeding programs [20-22].

Conclusion

The integrated genetic and metabolic fingerprinting approaches were validated as a potential method for discriminating differentC. sinensis populations. The SSR-based fingerprinting can identify the genotypes unequivocally, while the metabolic fingerprinting is reliable for rapid analysis of a large number of plant materials, revealing not only the varieties of genome and the environment, but also potential differential metabolites. Basically, the two methodselicit similar results based on the two data sets (SSR and phenolic compounds) and represent the optimal approach to verify inter-population diversity. The correlation network among metabolites

Table 2: Retention Times (RT), linear relationships among peak areas and analyte concentrations, test ranges, LOD and LOQ values of major phenolic compounds in *C. sinensis* leaf extracts.

Analytes	RT (min)	Standard curve	R2	Test range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Concentration (mg/g DW)				
							Su1	Ta6	KP3	CS1	SP2
Ferulicacid	20.23	y=30.635x + 8.155	0.9994	0.2-20	0.04	0.12	10.12 ± 1.15	8.64 ± 0.01	14.26 ± 0.175	15.32 ± 6.04	4.58 ± 0.184
Rutin	21.65	y = 4.25x + 19.82	0.9998	5-40	0.693	2.31	2.36 ± 0.02	0.18 ± 0.08	1.39 ± 0.002	9.91 ± 5.32	6.2 ± 0.3
Hesperetin	19.81	y=12.405x + 6.755	0.9991	5-40	4.994	16.648	3.2 ± 0.5	2.9 ± 0.045	5.26 ± 0.006	1.7 ± 2.58	1.5 ± 0.025
Quercetin	24.2	y = 9.58x – 7.47	0.9988	2.5-20	0.269	0.896	1.28 ± 0.26	7.65 ± 2.16	0.97 ± 0.32	3.78 ± 3.83	0.35 ± 0.061
Naringin	18.2	y = 23.46x – 259.8	0.9975	5-25	0.268	0.894	1.17 ± 0.131	1.9 ± 0.27	2.62 ± 0.43	35.2 ± 8.66	28.93 ± 0.99
Flavone	22.64	y = 20.01x – 62.93	0.9994	2.5-20	2.353	7.843	10.6 ± 0.017	0.64 ± 0.135	0.84 ± 0.003	11.75 ± 3.53	0.14 ± 0.015
Naringenin-7-o-glucoside	19.16	y = 1.590x + 0.047	0.9991	0.3-75	0.05	0.15	0.31 ± 0.045	2.1 ± 1.12	25.2 ± 0.051	21.52 ± 5.11	2.67 ± 0.21
Flavonose	23.78	y= 2.976x + 0.214	0.9998	0.3-30	0.04	0.13	0.6 ± 0.006	1.59 ± 0.009	0.1 ± 0.011	2.65 ± 0.49	0.24 ± 0.007

Table 3: Molecular markers associated with phenolic compounds in *C. sinensis* as revealed using the multiple regression analysis.

Metabolite	SSR markers (alleles)	r	R^2	β	t	P
Flavone	cAGG9200	0.714	0.51	-0.714	-3.676	0.003
	TAA27130	0.533	0.284	0.533	2.27	0.041
Ferulicacid	ATC09180	0.584	0.341	-0.584	-2.59	0.022
	CT21190	0.584	0.341	-0.584	-2.59	0.02
Flavonose	cAGG9220	0.628	0.391	-0.628	-2.9	0.012
	CAG01170	0.611	0.373	-0.611	-2.78	0.016
Rutin	CT21190	0.557	0.31	-0.557	-2.417	0.031
Hesperetin	CAG01165	0.954	0.911	-0.954	-11.51	0
A-2, hydroxyphenylacetic	CAG01165	0.858	0.736	-0.911	-9.43	0
Naringenin7-o-glucosides	TAA27130	0.585	0.342	0.585	2.598	0.022

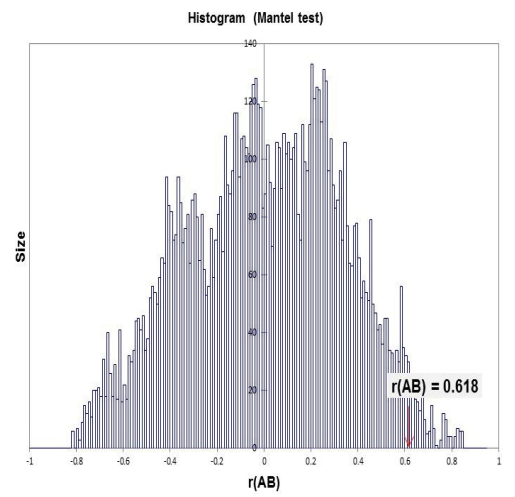

Figure 4: Histogram of correlation of the mantel test assessing the relationship between genetic and metabolic dis¬tances for the five C. sinensis populations.

and DNA markers provided a basis for deeper insight into metabolic pathways and the regulation networks.Hence, the molecular marker–traits associationidentification could be of great use for the breeder toidentify promising seedling/hybrids at an early stage ofthese plants and to improve the conservation and management of the relevant genetic resources.

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