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Development of Drought Associated AFLP and EST Candidate Gene Markers in *Fragaria* sp. as a Simple Genomic Model of *Rosaceae*

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ABSTRACT

Global warming exacerbates rainfall irregularity and causes a decline in water resources. Drought is one of the main limiting parameters in agricultural and fruit production. In this study, we established a standard method for measuring drought tolerance in *Fragaria* , the simplest genomic model in the *Rosaceae*, a plant family comprising important fruit species like almonds, apples, plums, etc. A quick screening method for monitoring *Fragaria* genotypes in response to dehydration involved conducting a short-term water deficit experiment. We measured two eco-physiological parameters associated with leaf water status, leaf relative water content (RWC), and leaf water loss rate (WLR) in a total of 20 strawberry cultivars (*Fragaria* × *ananassa* Duch.) from different breeding programs. The plants included two ecotypes of the European diploid species, *F. vesca*, and one American octaploid species, *F. chiloensis*. *Fragaria* genotypes responded to drought stress, as measured by WLR and RWC, and DNA fingerprints further described select *Fragaria* genotypes using AFLP and candidate gene EST markers. We revealed correlations among specific DNA markers, leaf WLR, and RWC while navigating the possibility of using association mapping in a small set of *Fagaria* accessions. The ultimate aim was to create a set of correlated markers to the physiological drought-involved traits in *Fragaria* . Using *F. vesca* extensively as a characterized *Rosaceae* mod e-l plant species in this study can ensure the benefits of drought charact e-rization and plant-based responses in other important *Rosaceae* fruit species.

Introduction

Plants endure numerous unfavorable climatic conditions during their growth cycles. Abiotic stress can cause primary diversions from usual plant life cycles that affect plant growth (Taiz and Zeiger, 1991; Younis et al., 2017; Farooq et al., 2020; Zulfiqar et al., 2020). Stresses can appear as biotic sources, including pathogen infection and insect attack, and abiotic sources, including heat, cold, drought, nutrient scarcity, higher salt levels, hazardous metals, and metalloid soils (Kang et al., 2017). Adverse temperatures (heat or frost), drought, and salt are the most frequently encountered climatic factors that reduce crop yields and fruit production (Fedoroff et al., 2010). Drought is a lack of available soil water but can also worsen by excessive evapotranspiration. Drought stress is due to an imbalance between soil water uptake and water loss through evapotranspiration (Lipiec et al., 2013). Plant

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adaptation mechanisms to drought stress are mainly determined by genetic and metabolic characteristics (Krasensky and Jonak, 2012). Plant responses to drought are extremely different according to their genetic background, hence descriptions of inter- and intra-species variations in drought tolerance (Rampino et al., 2006). Typical defense pathways are regulated by kinase enzymes and phytohormones. These pathways may include ion channels stimulated by jasmonic acid, abscisic acid, ethylene, and salicylic acid and through reactive oxygen species (ROS). These factors accumulate and reprogram the genetic and metabolic machinery (Taiz and Zeiger, 1991; Rejeb et al., 2014). To deal with the global drought situation, different strategies have been suggested, including breeding new drought-tolerant fruit varieties. Some wild germplasms and landraces may be better adapted to local ecological conditions and are valuable genetic resources for breeding toward drought tolerance (Rampino et al., 2006). The potential of such material and available variable gene pools for drought tolerance must be properly characterized at the physiological, morphological, and genetic level, as was previously reported for cowpea and wheat (Peleg et al., 2005; Rampino et al., 2006; Hegde and Mishra, 2009). Many molecular genomic markers are available for analyzing crops under drought stress. DNA markers facilitate marker-assisted breeding to enhance plant-driven tolerance to abiotic stress using advanced techniques and marker modification (Ullah, 2009; Younis et al., 2020). Furthermore, a focus on traits using single-gene single nucleotide polymorphisms (SNP) markers and EST candidate gene markers supports genetic mapping and the sequencing of stress-related traits in inbred lines (Ramzan et al., 2018; Younis et al., 2020). In tea plants (*Camellia sinensis*), a DNA marker association study for drought stress could provide a useful alternative for QTL mapping in a limited set of genotypes (Mishra and Sen-Mandi, 2004). Earlier studies reported on fruit responses to drought stress in *Rosaceae,* e.g., characterizing morphological responses in young seedlings of 5 Iranian almond species (*Prunus dulcis, P. eburnea, P. eleagnifolia, P. haussknechti*, and *P. scoparia* under polyethylene glycol-induced drought stress (Zokaee-Khosroshahi et al., 2014). Results indicated that drought generally caused a significant reduction in plant growth parameters such as fresh and dry leaf weight, leaf number, total leaf area, and leaf relative water content in all almond (*Prunus dulcis* Mill) species. However, there was a variation between species*, P. eburnea* had the highest relative water content among the species and showed the smallest decrease in the plant's fresh and dry weights but the greatest decrease in leaf number and total leaf area as an adaptive mechanism to drought stress. Other studies also evaluated the correlation between drought stress and fruit quality parameters in almonds and proved key quality parameters as markers of hydro-sustainable almonds (Lipan et al., 2022). These results presented positive correlations among the stress integral and dry weight, color coordinates, minerals (K, Fe, and Zn), organic acids (citric acid), sugars (sucrose, fructose, and total sugars), antioxidant activity, and fatty acids. Drought stress responses in *Fragaria* include a decrease in net photosynthesis and the leaf water potential at the cellular level and a reduction of leaf area and yield at the crop level. In addition, osmotic adaptation resulted in higher sucrose levels under drought stress in "Elsanta" strawberries (Razavi et al., 2008). Variations in drought tolerance were already assessed within the genus *Fragaria*, indicating *Fragaria chiloensis* as a more drought-tolerant species than *F. virginiana* (Zhang and Archbold, 1993). Interspecific variations were detected in the research, including higher solute accumulation and osmotic adjustment under water deficits in *F. chiloensis* compared to *F. virginiana*. Also, plant water relationship parameters like leaf water potential and relative water content (RWC) were variable between *Fragaria* species under water deficit (Archbold and Zhang, 1993). Other reports confirm that the response to a water deficit is species-specific; each particular *Fragaria* species shows a unique adaptive response to drought conditions (VanDerZanden and Cameron, 1996). Intraspecific variations in drought tolerance within *Fragaria chiloensis* clones were described in this study. So far, only a small set of genes known to be of interest for abiotic stress tolerance have been characterized in *Fragaria* (Schwab et al., 2009), and in particular, information about the genetic control of drought tolerance in *Fragaria* is missing. An improved understanding of the genome structure in *Fragaria* enables dissecting the structural and functional basis of adaptive traits like drought tolerance (Folta and Davis, 2006). *Fragaria vesca* has been intensively characterized as a model plant species for *Rosaceae* fruit trees (Shulaev et al., 2008). It is also considered an important genetic resource for introducing many important traits in cultivated strawberries. The genetic control of drought response in this simple characterized model is useful for characterizing drought response in the *Rosaceae* family. Thus, a study on the genetic background of different *Fragaria* genotypes can be informative, and this information can assist in

detecting DNA markers correlated to drought tolerance *in Fragaria* . To date, DNA marker techniques such as AFLPs, RAPDs, SSRs, and ISSRs have been employed to fingerprint *Fragaria* genotypes (Kashyap et al., 2005; Govan et al., 2008; Sargent et al., 2009). Also, gene-specific markers like STS (gene-specific sequence-tagged site), EST (expressed sequence tag) (Davis and Yu, 1997; Sargent et al., 2007), gene-specific intron-length polymorphisms, and cleaved-amplified polymorphic sequence (CAPS) have derived from functional genes coding sequences (Davis et al., 2007a, 2008b). These traits were already developed and applied for analyzing different traits of interest in *Fragaria* . Moreover, a few studies on *Fragaria* used EST-derived SSRs to determine linkage with candidate genes (Sargent et al., 2004; Gil-Ariza et al., 2006). EST markers developed in the coding region of functional genes have already proven to be transferable through genotypes and are powerful markers for fingerprinting in some crops (Scariot et al., 2007). However, these markers linked with drought tolerance are lacking in temperate fruits of the *Rosaceae* family and *Fragaria* sp. So far, an integrated physiological-molecular standard method for measurement of dehydration tolerance in temperate fruits from *Rosaceae* and *Fragaria* was not yet available; several parameters have applicability to define the dehydration tolerance of a plant (Verslues et al., 2006). Thus, the main objectives of this study were to develop a fast screening method for drought tolerance of *Fragaria* genotypes and to correlate the genetic structure of different *Fragaria* genotypes, assessing by expressed sequence tag (EST) and amplified fragment length polymorphism (AFLP) markers, with plant responses to drought stress. To evaluate the degree of drought tolerance in *Fragaria sp.,* a short-term water deficit experiment was performed based on already tested methods used on other species (Teulat et al., 2003; Suprunova et al., 2004; Rampino et al., 2006). We evaluated leaf-relative water content (RWC) and leaf water loss rate (WLR). The findings helped develop some associated AFLP and EST candidate gene markers for the genetic control and screening of drought resistance or sensitivity traits in *Fragaria* as a simple model in *Rosaceae* that might be helpful in exploring the genetic control of drought responses in other *Rosaceae* fruit species.

Materials and Methods

Plant materials

In total, 20 strawberry cultivars (*Fragaria* × *ananassa* Duch.) from different breeding programs were used in this study: 6 European genotypes, 13 genotypes from the USA, and one genotype obtained from Turkey; two ecotypes of the European diploid species *F. vesca* and one American octaploid species *F. chiloensis* were also included (Table 1). *F. chiloensis* is drought tolerant (Zhang and Archbold, 1993), while *F. vesca* is probably less tolerant to drought, as can be assumed from its natural habitat in marshlands in Europe. Cultivars were selected from different breeding programs and different production regions, with an annual rainfall ranging from 497 mm (semi-arid Iran) to 1500 mm (temperate Western Europe). Several abiotic stresses cause similar responses at the cellular level; therefore, relevant information about abiotic stress tolerance is usually required for genotypes (Table 1). The parentage of the genotypes can also serve as helpful information. All plants were grown for three months in a greenhouse at Ghent University (51.3 N, 3.4 E) according to good horticultural practices for runner production. Daughter plants were cut from the stolons, and after a production cycle of 18 months, these plants were transferred to a growth chamber two weeks before the start of the experiment for preconditioning. They were well-watered and grown in an environment with a constant temperature (22 °C) and relative humidity (60%). A 16 h photoperiod (from 6 h00 to 22 h00) was provided, with 100 μmol m^{-2} s^{-1} PPFD during the day (Philips, Master HPI-T Plus, 400 Watts).

RWC and WLR measurements and dehydration conditions

The leaf water loss rate (WLR) and relative water content (RWC) of detached leaves were determined as part of a rapid screening of *Fragaria* genotypes, as described by Suprunova et al. (2004) and Verslues et al. (2006). Leaf samples were harvested between 8 and 9 a.m. The RWC was measured at two time intervals: immediately after detachment of the leaves (RWC at harvest) and after a dehydration time of 4 h (RWC after 4h). For RWC at harvest, young fully-expanded leaves were excised. Fresh weight (FW) was immediately recorded. The leaf samples were soaked for 4 h in distilled water at room temperature under constant light (40 μmol m-2 s-1) to determine the turgid weight (TW).

Table 1. Overview of the studied *Fragaria* genotypes, grouped according to their parentage. Data on abiotic stress tolerance are given.

Genotype	Abiotic stress tolerance	Abbreviation	Parentage	Place cultivar developed	*DNA code* [C]
Darselect[b]	Tolerant to winter or frost, tolerant to water deficit	Dar.	Elsanta x Parker No. Plant 10, 402	France, EU	7
Elsanta[b]	Sensitive to salinity, tolerant to cold	Els.	Gorella x Holiday[1]	The Netherlands, EU	11
Figaro[b]	Tolerant to high temperature (heat tolerant), low chilling requirement	Fig.	Elsanta x Pajaro No. pp18, 079P2	The Netherlands, EU	9
Honeoye[b]	Winter hardy, tolerant to cold	Hon.	Vibrant x Holiday[1]	New York, US	5
Lambada[b]		Lam.	(Sivetta x Holiday) x (Karina x Primella)[2]	The Netherlands, EU	3
Selva[b]	Fairly cold hardiness, tolerant to cold	Sel.	CA 70.3-177 (Sib of Brighton) x CA 71.98-605 [Tufts x 63.7-10 (parent of Pajaro)] or Brighton x (Turfts x Pajaro)[1]	California, US	1
Sonata[b]	Tolerant to high temperature (heat tolerant)	Son.	Elsanta x Polka No. US PP18, 000 P2	The Netherlands, EU	6
Aliso[a]		Ali.	CA 52.16-15x self ((CA 39.177-4 x 39.96-18) x self)[1]	California, US	19
Sequoia[a]	Low chilling requirement, average water need	Seq.	CAL 52.16-15 (a sib of Wiltguard and parent of Aliso) x CAL 51s1-1 (selected from a first generation selfed population of Lassen)[1]	California, US	21
Yalouva[a]	Tolerant to salinity	Yal.	Arnavutkoy x Aliso	Turkey, Middle East	17
Frezno[a]	Fairly tolerant to salinity, low chilling requirement	Frez.	Lassen x CAL 42.8-16 (Sib of Tioga)[1]	California, US	14
Tioga[a]	Fairly tolerant to salinity, tolerant to high temperature	Tio.	Lassen x CAL 42.8-16 (Sib of Frezno)[1]	California, US	13

No.: US plant patent number.
[a] Cultivars were obtained from the Agricultural Research Center, Kurdistan, Iran.
[b] Cultivars were obtained from *Fragaria* Holland, The Netherlands.
F. vesca ecotype 1 = accession nr 19911660, Botanical Garden of Ghent University, source: Ravels, Belgium.
F. vesca ecotype 2 = accession nr NPB 19790383, National Botanical Garden, Meise, Belgium source: Meise, Belgium.
F. chiloensis = accession nr NPB 20040573-82, National Botanical Garden, Meise, Belgium population original from the Channel Islands and was obtained through Chelsea Physic Garden, UK.
[C] DNA sample number in all expermental and data analyses.
[1] Parentage based on NCGR-Corvallis - *Fragaria* Germplasm, US.
[2] Parentage based on Plant Research International, Wageningen, the Netherlands.

Continued Table 1. Overview of the studied *Fragaria* genotypes, grouped according to their parentage. Data on abiotic stress tolerance are given.

Genotype	Abiotic stress tolerance	Abbreviation	Parentage	Place cultivar developed	*DNA code* [C]
Missionary[a]		Mis.	Hoffman x Lady Thompson or Michel or by a native wild *F. Virginiana*[1]	Virginia, US	20
Tennessee Beauty[a]	Susceptible to drought	Ten.	Missionary x Premier[1]	Tennessee, US	18
Camarosa[b]	Tolerant to salinity	Cam.	Douglas x CAL 85.218-605 No. Plant 8, 708	California, US	12
Catskill[a]	Average water need	Kat.	MarshallxHoward 17	New York, US	15
Diamante[b]		Dia.	CAL 87.112-6xCAL 87.270-1 No. Plant 10, 435	California US	8
Korona[b]	Sensitive to salinity less than Elsanta, tolerant to cold	Kor.	Tamella x Induka	EU	2
Kurdistan[a]		Kur.	Unknown (dominant cultivar in Iran)	France, EU	16
Ventana[b]		Ven.	CAL 93.170-606 x CAL 92.35-601 No. US pp13, 469 P3	California US	10
Fragaria vesca L. (Ecotype 1)		Ves.1	*Fragaria* sp.		22
Fragaria vesca L. (Ecotype 2)		Ves.2	*Fragaria* sp.		4
Fragaria chiloensis	Drought tolerant	Chl.	*Fragaria* sp.		23

No.: US plant patent number.

[a] Cultivars were obtained from the Agricultural Research Center, Kurdistan, Iran.

[b] Cultivars were obtained from *Fragaria* Holland, the Netherlands.

F. vesca ecotype 1 = accession nr 19911660, Botanical Garden of Ghent University, source: Ravels, Belgium.

F. vesca ecotype 2 = accession nr NPB 19790383, National Botanical Garden, Meise, Belgium source: Meise, Belgium.

F. chiloensis = accession nr NPB 20040573-82, National Botanical Garden, Meise, Belgium population original from the Channel Islands and was obtained through Chelsea Physic Garden, UK.

[C] DNA sample number in all expermental and data analyses.

[1] Parentage based on NCGR-Corvallis - *Fragaria* Germplasm, US.

[2] Parentage based on Plant Research International, Wageningen, the Netherlands.

Total dry weight (DW) was recorded after drying for 24 h at 80 °C. Leaf RWC was measured after leaf dehydration for 4h (RWC after 4h) on a second set of leaves by applying the same protocol but allowing the leaves to dry for 4 h on filter paper (23 °C, 40 µmol m^{-2} s^{-1} and 30% relative humidity). The leaf RWC at both time points was calculated according to Barr and Weatherley (1962): RWC (%) = [(FW-DW)/(TW-DW)] ×100. The difference between leaf RWC after 4h and leaf RWC at harvest (ΔRWC) was calculated as leaf RWC at harvest - leaf RWC after 4h. The leaf water loss rate (WLR) was determined on a third set of young fully-expanded leaves. Fresh weight was documented after 0 (FW) and 4 h drying on filter paper (W4) (23 °C, 40 µmol m^{-2} s^{-1} and 30% relative humidity). Total dry weight (DW) was recorded after drying for 24 h at 80 °C. Leaf WLR was calculated according to Suprunova et al. (2004): WLR (g h^{-1} g^{-1} DW) = [(FW-W4)]/[DW× 4]. Each measurement was repeated three times, and the experiment was repeated twice.

DNA isolation and AFLP amplification

For DNA extraction from leaf material, the Qiagen DNeasy Plant Mini Kit was used. A modified AFLP protocol (Vos et al., 1995) was followed according to De Riek et al. (1999). Selective amplification was carried out using four fluorescent 6-FAM or HEX labeled *Eco*RI/*MseI* primer combinations with six selective bases: *Eco*RI-ACT/*MseI*-CGA (PC_1), *Eco*RI-AGG/*MseI*-CAA (PC_2), *Eco*RI-ACC/*MseI*-CAT (PC_3), *Eco*RI-ACC/*MseI*-CTA (PC_4). Of the final PCR product, 1 µL was mixed with 13.5 µL Hi-Di™ Formamide (Applied Biosystems) and 0.5 µL of the GeneScan™-500 Rox® Size Standard (Applied Biosystems). Products were denatured by heating for 3 min at 90 °C. Capillary electrophoresis and fragment detection were performed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Polymorphic bands were scored as present or absent (1/0) using the GeneMapper® 4.0 software (Applied Biosystems). The thresholds for marker frequency ($0.15<f<0.85$) and average marker peak height ($h>100$) were set according to De Riek et al. (1999).

EST marker development and amplification

Candidate genes for EST marker development were initially selected based on their putative function in plant drought tolerance (data not shown). Candidate gene-EST marker amplification and screening for polymorphic bands were conducted on a full set of selected *Fragaria* accessions (Table 2). EST marker analysis was performed as described by De Keyser et al. (2006). Polymorphic alleles of EST markers were scored as present or absent (1/0). Quantity One software (version 4.5.1) from BioRad (Hercules, CA, USA) was used for scoring and sizing all bands.

Table 2. Polymorphism rate of all markers in Fragaria genotypes.

Clone name	Acc No	Putative function	Polymorphism rate
EST-1	**AF159627**	APX18*	2/3
EST-2	**AF159629**	APX27*	2/3
EST-3	**AF159630**	APX42*	4/4
EST-4	**DY674761**	CAT	2/3
EST-5	**CO816707**	Unknown	7/8
EST-6	**CO381280**	MnSOD	7/7
EST-7	**CO817459**	Unknown	6/7
EST-8	**CX661438**	RAB18	10/12
EST-9	**CO817183**	DREB2a	3/4
EST-10	**DY674391**	Unknown	3/4
EST-11	**DV438427**	DREB3	4/5
EST-12	**DY670596**	Unknown	1/2
EST-13	**CO817580**	RD22	5/6
EST-14	**CO817057**	Unknown	3/4
EST-15	**CO816798**	Unknown	6/7
EST-16	**AY663110**	AKR*	4/4
EST-17	**CO816877**	Unknown	5/6
EST-18	**AF199508**	FaOLP1*	4/5
EST-19	**DQ325524**	FaOLP2*	3/4
EST-20	**DY668033**	P5CS	1/2
EST-21	**DY670745**	Unknown	9/10
EST-22	**AB275667**	FaSAI*	3/4
EST-23	**AB267868**	FaSPS1*	3/4
EST-24	**AB267869**	FaSPS2*	2/3

Statistical analysis

In the case of ESTs, we calculated the number of polymorphic alleles per locus: (n_p) and the average number of alleles per locus n_{av} that

equaled the number of polymorphic alleles (n_p) divided by the number of loci (L) (Belaj et al., 2003; Scariot et al., 2007). RWC and WLR were analyzed via one-way ANOVA and the cultivars were grouped using Tukey's test (P=0.01). The calculation of genetic similarities (Jaccard's similarity coefficient), canonical discriminant analysis, Principal Coordinate Analysis (PCoA), Kruskal-Wallis analysis (P≤0.05), correlation analysis (Pearson's coefficient), hierarchical cluster analysis (UPGMA), chi-square (X^2) test (P≤0.05), and biplot test (scatter dot graph) were performed using SPSS 11.01 on Windows (SPSS Inc., Chicago IL). To evaluate the reproducibility, bootstrapping (1000 permutations) was done using the Tree Con Package on Windows (version 1.3b). Mantel analysis (Mantel, 1967; Mantel Nonparametric Test Calculator for Windows, Version 2.00, 1999) was employed for testing correspondence between the matrices. Statistical significance among data was evaluated by permutations (1000×) and expressed as a probability (Smouse et al., 1986).

Results

RWC and WLR measurements

Genotypes varied in the RWC at harvest and the RWC after 4h. The RWC at harvest ranged from 90.2% in 'Sequoia' to 96.3% in 'Darselect' (data not shown). The RWC after 4h ranged from 44.2% in "*F. vesca*" to 67.1% in 'Figaro' (Fig. 1). The RWC decline rate (ΔRWC) showed significant differences among the genotypes and ranged from 26.3% in 'Figaro' to 47.7% in *F. vesca* (P=0.01) (data not shown). The rate of water loss as assessed by WLR varied significantly between the genotypes (P=0.01). WLR ranged from 0.15 g h^{-1} g^{-1} DW in 'Figaro' to 0.44 g h^{-1} g^{-1} DW in *F. vesca* (Fig. 2). Pearson's correlation study showed strong correlations among the RWC after 4h, WLR, and ΔRWC. However, the RWC at harvest correlated weakly with the RWC after 4h and WLR. No correlation occurred between the RWC at harvest and ΔRWC (Table 3). A canonical discriminant analysis was employed to maximize genotype separations. Three canonical discriminant functions were generated: canonical function 1 (Can1) was responsible for 63% of the separation, canonical function 2 (Can2) accounted for 24% of the separation while the third canonical function (Can3) explained a significant although rather low amount of the variation (only 13%). Can1 was tightly correlated with RWC at harvest (r=0.69) and RWC after 4h (r=0.67), Can2 was associated with ΔRWC (r=-0.70) and Can3 was dominated by WLR (r=0.82). Sorting along Can 1 suggested two classes: class a was defined as all genotypes which were placed below 0.00 (<0.00, group mean=- 0.8) with a lower RWC after 4h (<57.20%), a higher WLR (≥ 0.32 g h^{-1} g^{-1} DW). Class b defined all genotypes which reached above 0.7 (>0.7, group mean=1.85) with a higher RWC after 4h (>57.20%) and a lower WLR (<0.32 g h^{-1} g^{-1} DW) (Fig. 3). Class a was drought sensitive, and class b was defined as drought tolerant (Fig. 3). Hierarchical clustering (Euclidean distance coefficient) of each genotype was based on the canonical discriminant functions, suggesting that these two phenotypic classes are distinct (data not shown).

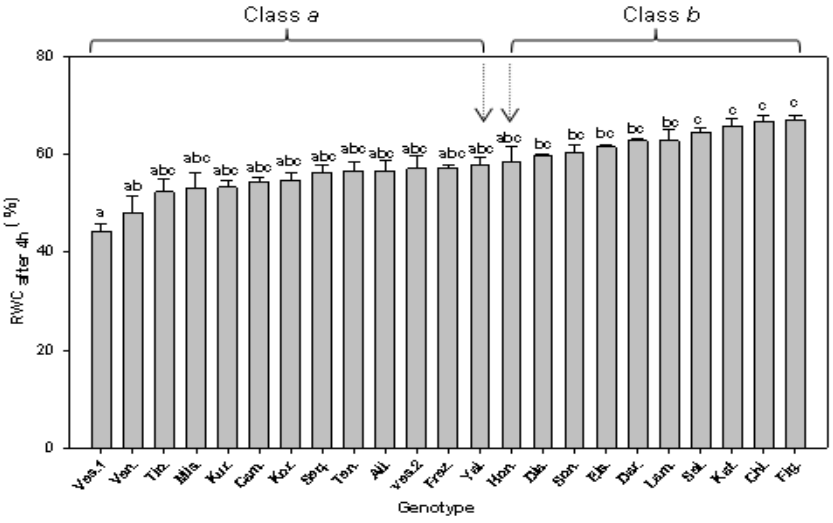

Fig. 1. Relative water content after a dehydration time of 4h (RWC after 4h) of the strawberry genotype (mean values ± STD). Letters a and b are significantly different according to Tukey's test (P≤0.01). Class a: genotypes defined as drought sensitive showing a high WLR and a low RWC after 4h. Class b: Genotypes defined as drought tolerant showing a low WLR and a high RWC after 4h.

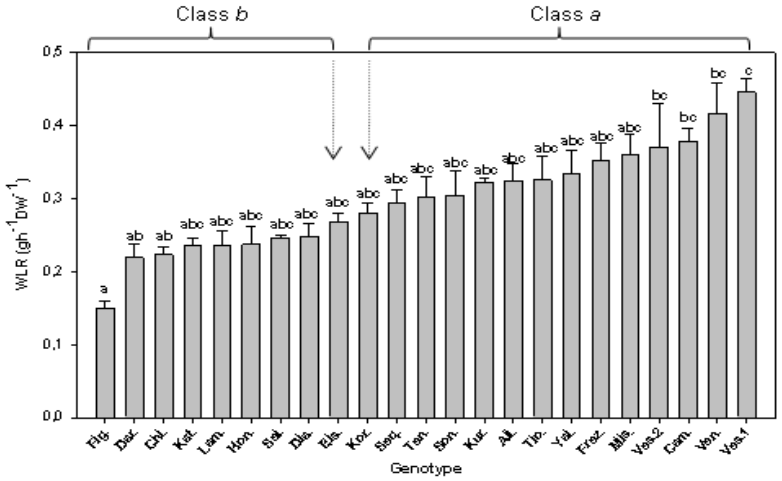

Fig. 2. Water loss rate (WLR) after a dehydration time of 4 h on the strawberry genotypes (mean values ± STD). Letters a and b are significantly different according to Tukey's test (P≤0.01). Class a: Genotypes defined as drought sensitive showing a high WLR and a low RWC after 4h. Class b: Genotypes defined as drought tolerant showing a low WLR and a high RWC after 4h.

Table 3. Correlation coefficients among eco-physiological traits in Fragaria genotypes according to Pearson's correlation coefficient.

	WLR	**RWC after 4h**	**Δ RWC**
RWC at harvest	-0.279**	0.262**	-0.014
WLR	-	-0.846**	0.797**
RWC after 4h	-	-	-0.969**

** indicates significant correlations (P≤0.01) (2-tailed).

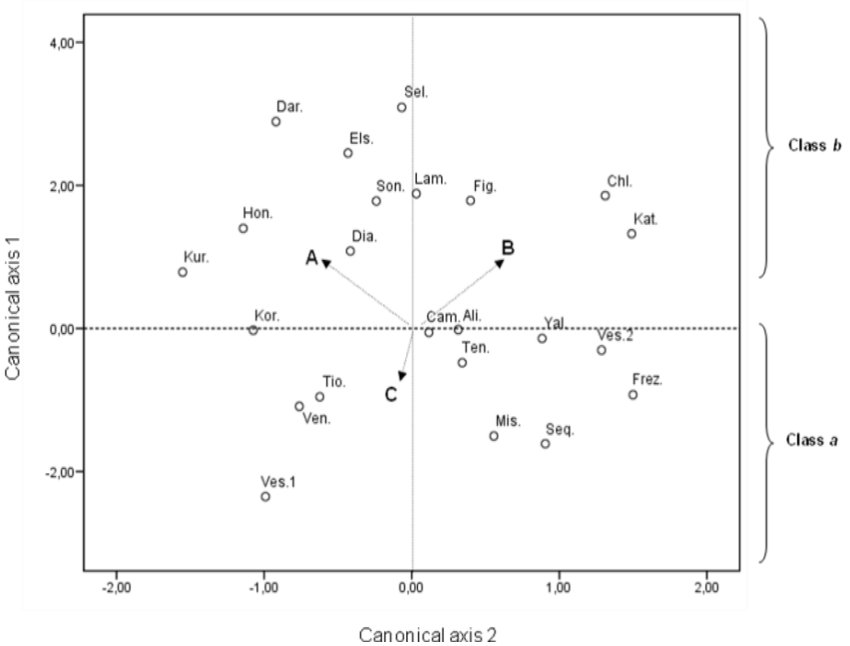

Fig. 3. Grouping of *Fragaria* genotypes based on discriminant factors of measured ecophysiological traits (Class a<0.00 and class b>0.7 on a reference line of canonical axis 1). Arrow A shows RWC at harvest, arrow B shows RWC after 4 h, and arrow C shows WLR, based on standardized canonical discriminant function coefficients. Class a: genotypes defined as drought sensitive showing a high WLR and low RWC after 4 h. Class b: genotypes defined as drought tolerant showing a low WLR and high RWC after 4 h. Two exceptions in class b are 'Kurdistan' with low RWC after 4 h and high WLR. 'Sonata' had high WLR.

Ultimately, 24 EST-based markers were available for genetic characterization (Table 2). In 23 *Fragaria* genotypes, 121 alleles appeared, 99 of which were polymorphic (Table 2). The total number of alleles per locus ranged from 2 (EST-12 and EST-20) to 12 (EST-8); on average, 78% of these markers were polymorphic in the dataset (Table 2). The number of polymorphic bands per genotype was variable between different ESTs and genotypes. For instance, in EST-8, a minimum of one and a maximum of nine polymorphic bands per genotype were scored in different accessions. In EST-20 and EST-12, one polymorphic band was only found in a limited number of genotypes. The average number of alleles per locus (nav) for an EST was four.

Genetic relationship as revealed by AFLP and EST markers

For both marker types, pairwise genetic similarities between individual plants and hierarchical cluster analysis revealed a general relationship among the genotypes. The diploid species (*Fragaria vesca*) was separated from the other genotypes both with AFLP (Fig. 4a) and EST (Fig. 4b). The octaploid species (*Fragaria chiloensis*) clustered with the other octaploids but was distant from the strawberry cultivars. AFLP confirmed the genetic similarity between some related genotypes like "Tennessee Beauty" and its parent "Missionary," the full-sibs "Tioga," "Frezno," and "Elsanta," and their descendants "Figaro" and "Darselect." However, the other descendant of "Elsanta," "Sonata," appeared to be very distant from this cluster (Fig. 4a). By hierarchical cluster analysis, EST markers confirmed the genetic similarity within studied strawberry cultivars such as "Tioga," "Fresno," "Tennessee Beauty," and "Missionary." 'Darselect', 'Sonata', and 'Elsanta' clustered together with 'Honeoye'. However, 'Figaro' was not in this group (Fig. 4b). ESTs appeared to be better to group cultivars adapted to semi-arid conditions, including 'Sequoia', 'Aliso', 'Tennessee Beauty', 'Missionary', 'Tioga' and 'Frezno'. Overall, clustering was generated from the AFLP and EST data and was generally in good agreement with the taxonomic classification of *Fragaria* genotypes. The Mantel test showed a significant ($P=0.001$) correlation among Jaccard matrices calculated from both marker techniques ($R=0.81$). Genetic relationships were also revealed and quantified by using PCO. Jaccard's similarity matrix of 23 genotypes was calculated based on combined AFLP and EST data and used as input for PCO analysis. Four component axes generated by this analysis indicated 76% of the variance in 23 *Fragaria* genotypes (Eigenvalues$\geq$1), where component axes 1 and 2, with the highest effect, were responsible for 66% of this variance. *Fragaria* species strongly separated on axes 1 and 2 (Fig. 4c).

Association testing of DNA markers with physiological traits

Kruskal-Wallis analysis ($P\leq0.05$) was applied to determine AFLP and EST markers linked to the individual measured physiological traits as well as to the canonical discriminant factors (Table 4). In AFLP, most of the markers correlated to the individual physiological traits and were linked to the discriminant factors of these traits. In ESTs, 10 markers linked to the measured physiological traits were also correlated to their derived discriminant factors while 9 ESTs were only linked to the traits and not to their discriminant factors (Tables 4 and 5). Hierarchical clustering of *Fragaria* genotypes showed that according to AFLP/EST markers linked to the WLR, RWC after 4h appeared at one side, and discriminant factors of all measured physiological traits were at the other side; the correlations between the obtained ordinations and the two main physiological classes of genotypes (tolerant/sensitive) were studied (Fig. 5a-c and Fig. 6a-c). The effect of the commonly correlated AFLP/EST markers on RWC after 4h was the opposite on WLR markers, and a negative correlation with WLR showed a positive correlation with RWC after 4h and vice-versa. This behavior was the same for all common correlated AFLP and EST alleles.

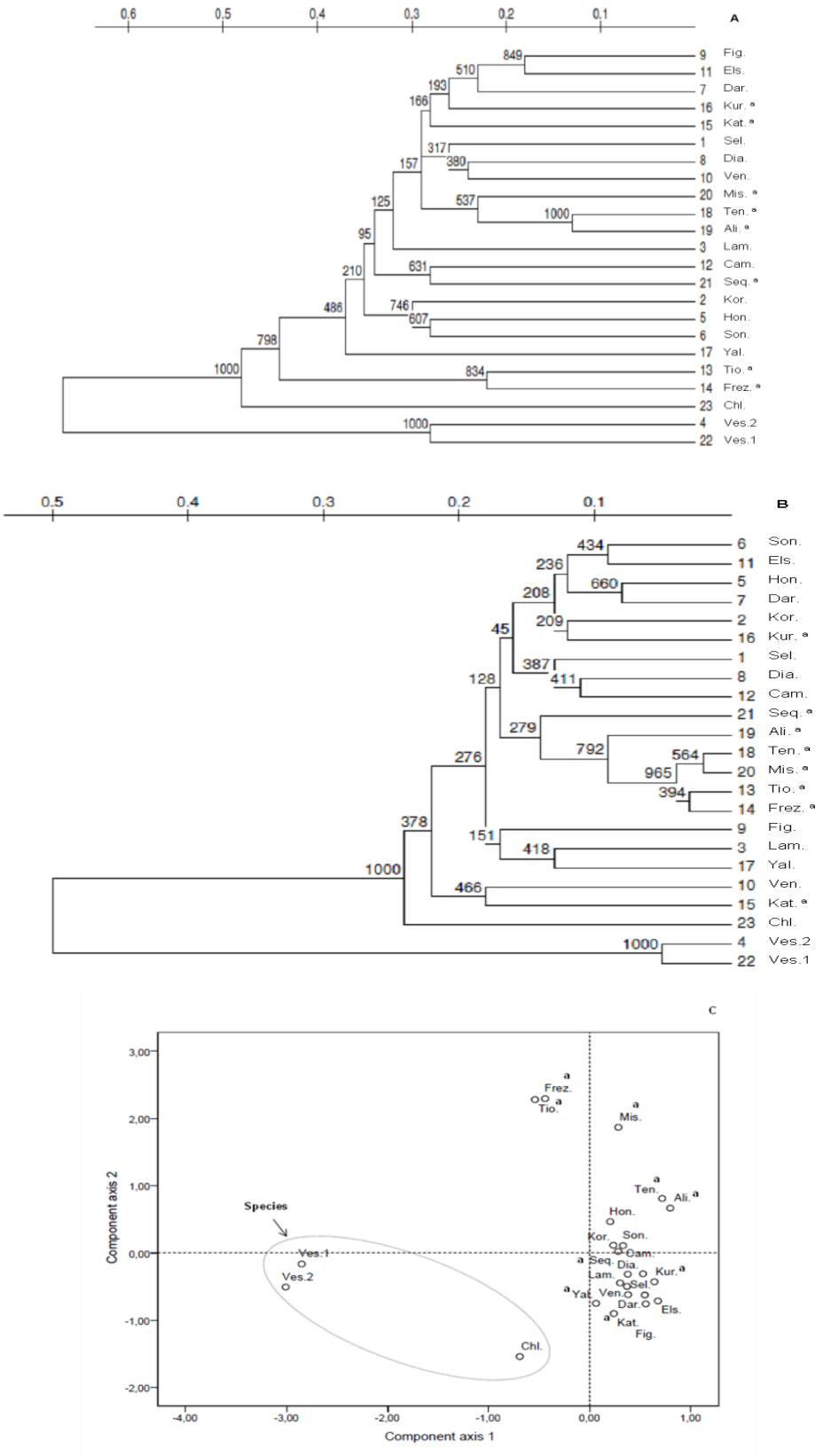

Fig. 4. a) Dendrogram of 23 *Fragaria* genotypes obtained via 369 AFLP markers, b) dendrogram of 23 *Fragaria* genotypes obtained using 24 EST markers (121 alleles), c) biplot of principal coordinate analysis (PCO) using combined AFLP and EST data (a Cultivars are adapted to Iranian climate conditions per the dendrogram, regarding Jaccard's similarity coefficient, UPGMA clustering, and bootstrap values from 1000 re-sampling cycles).

Mantel testing indicated a significant correlation (P=0.001, permutation 1000×) between similarity matrices of *Fragaria* genotypes that were made separately based on EST's linked markers and AFLP's linked markers to the WLR (R=0.78), RWC after 4h (R=0.43) and discriminant functions (R=0.79). Also, a significant correlation was proven between the Euclidean distance matrix of *Fragaria* genotypes, based on canonical discriminant functions, and the matrices, based on the linked AFLP/EST markers to the traits (P≤0.01) (Table 6). A low but still significant correlation (*P*=0.05) between the Euclidean distance matrix generated by canonical discriminant functions and the Jaccard similarity matrices generated by total AFLP/EST markers (Table 6). In both marker types, the reproducibility and effectiveness of markers correlated to the traits in the separation of physiological groups by comparison between the matrices generated by linked AFLP/EST markers and the ones generated by random markers (with the same allele number) due to their correlation with the matrices of total AFLP/EST markers. Results showed a lower correlation regarding linked markers to different traits than random (Table 6). Population structures affected these correlated markers, and the effects were tested by a non-parametric Kruskal-Wallis analysis. The individual scores of the 23 genotypes on PCO axes 1 and 2, with the most significant effects on genotype variation, were taken as input, and the correlations between these axes and all markers were investigated (*P*=0.05).

The results showed that both AFLP/EST and some correlated markers significantly differentiate for phylogenetic structure while some are neutral with no effect on genetic structure (Tables 4 and 5). The marker distribution or allele frequency of correlated markers among the 23 genotypes was determined using the chi-square test (X^2) test (P≤0.05). Allele frequencies indicating a lower presence or absence than 7/23 were considered a deviation from a 50/50 distribution (Table 4). The allele's effect on traits is indicated compared to the average of the total data set (%) ([0] band's absence and [1] band's presence). Regarding the canonical discriminant functions, only the significant loci were indicated as '*', *N*: the neutral effect (no significant effect) on genetic structure, as determined by Kruskal-Wallis analysis (P≤0.05), *N.R.*: neutral rare marker, *N.F.*: neutral frequent marker as determined by an X^2 test (P≤0.05).

Table 4. Number of markers correlating to the physiological traits and canonical discriminant functions as determined by Kruskal-Wallis analysis (P≤0.05). The number of frequent/rare neutral markers were determined by the X2 test (P≤0.05).

	RWC at harvest	**RWC after 4h**	**WLR**	**Δ RWC**	**Additive correlated markers [1]**	**Common correlated markers[2]**	**Canonical discriminant functions[3]**
AFLP (Total)	75	47	85	31	130	19	114
AFLP	24	15	18	11	37	6	41
(Neutral in G.S.)	[13/11]	[2/13]	[4/14]	[2/9]	[14/23]	[2/4]	[16/25]
EST	9	5	13	6	19	1	10
(Total)	(60 alleles)	(21 alleles)	(58 alleles)	(26 alleles)	(99 alleles)	(4 alleles)	(60 alleles)
EST	6	1	2	2	8	0	7
(Neutral in G.S.)	[5/1]	[0/1]	[1/1]	[0/2]	[5/3]		[4/3]

[1] All correlated markers which are at least linked to one of the measured traits.

[2] Common correlated markers which are linked to all measured traits.

[3] Markers correlating to the canonical discriminant functions.

Neutral in G.S.: no effect on genetic structure as determined by the Kruskal-Wallis analysis (P≤0.05).

Table 5. EST markers correlating to eco-physiological traits as determined by the Kruskal-Wallis analysis (P≤0.05). Putative functions occur between brackets.

EST marker	$RWC_{at\ harvest}$	WLR	$RWC_{after\ 4h}$	Δ RWC	Canonical discriminant functions
EST-1 (*APX 18*)		[0]=36.64 [1]=-3.5	[0]=-2.95 [1]=8.36	[0]=4.60 [1]=-13	
EST-3 (*APX 42*)			[0=-2.52 [1]=9.07	[0]=3.67 [1]=-13.23	*
EST-4 (*CAT*)		[0]=36.64 [1]=-3.5	[0]=-2.81 [1]=8 N.R.	[0]=3.8 [1]=-10.8 N.R.	*N.R.
EST-11 (*DREB3*)	[0]=0.55 [1]=-0.72 N.F.	[0]=36.64 [1]=-3.5			
EST-7	[0]=-0.65 [1]=0.85 N.F.				*N.F.
EST-9 (*DREB2A*)		[0]= 36.64 [1]= -3.5			
EST-22 (*FaSAI*)	[0]=-0.84 [1]=0.92	[0]=11.37 [1]=-12. 4			*N.R.
EST-23 (*FaSPS1)*	[0]=-0.25 [1]=2.63 N.R.				*N.R.
EST-16 (*AKR*)	[0]=0.81 [1]=-1.25	[0]=-7.45 [1]=11.59	[0]=3.27 [1]=-5.1	[0]=-3.31 [1]=5.15	*
EST-12		[0]=36.64 [1]=-3.5			
EST-17		[0]=28.31 [1]=-4.25			
EST-18 (*FaOLP1*)		[0]=17.2 [1]=-6 N.R.		[0]=11.23 [1]=-4 N.R.	*N.F.
EST-20 (*P5CS*)		[0]=36.64 [1]=-3.5			
EST-21	[0]=-1.1 [1]=1 N.F.	[0]=6.25 [1]=-14.28 N.F.			*N.F.
EST-8 (*Rab18*)	[0]=-1.1 [1]=0.48 N.F.				*N.F.
EST-13 (*RD22*)		[0]=36.64 [1]=-3.5			
EST-14		[0]=36.64 [1]=-3.5			
EST-15	[0]=-0.58 [1]=0.76				
EST-6 (*MnSOD*)	[0]=-0.62 [1]=0.81 N.F.		[0]=-1.4 [1]=14.41	[0]=1.11 [1]=-24.5	*

Allele effects on traits were indicated compared to the average of the total data set (%), including [0] band's absence and [1] band's presence.

Regarding the canonical discriminant functions, only the significant loci were indicated as '*'.

N.: neutral effect (no significant effect) on genetic structure as determined by the Kruskal-Wallis analysis (P≤0.05), N.R.: neutral rare marker, N.F.: neutral frequent marker as determined by the X^2 test (P≤0.05).

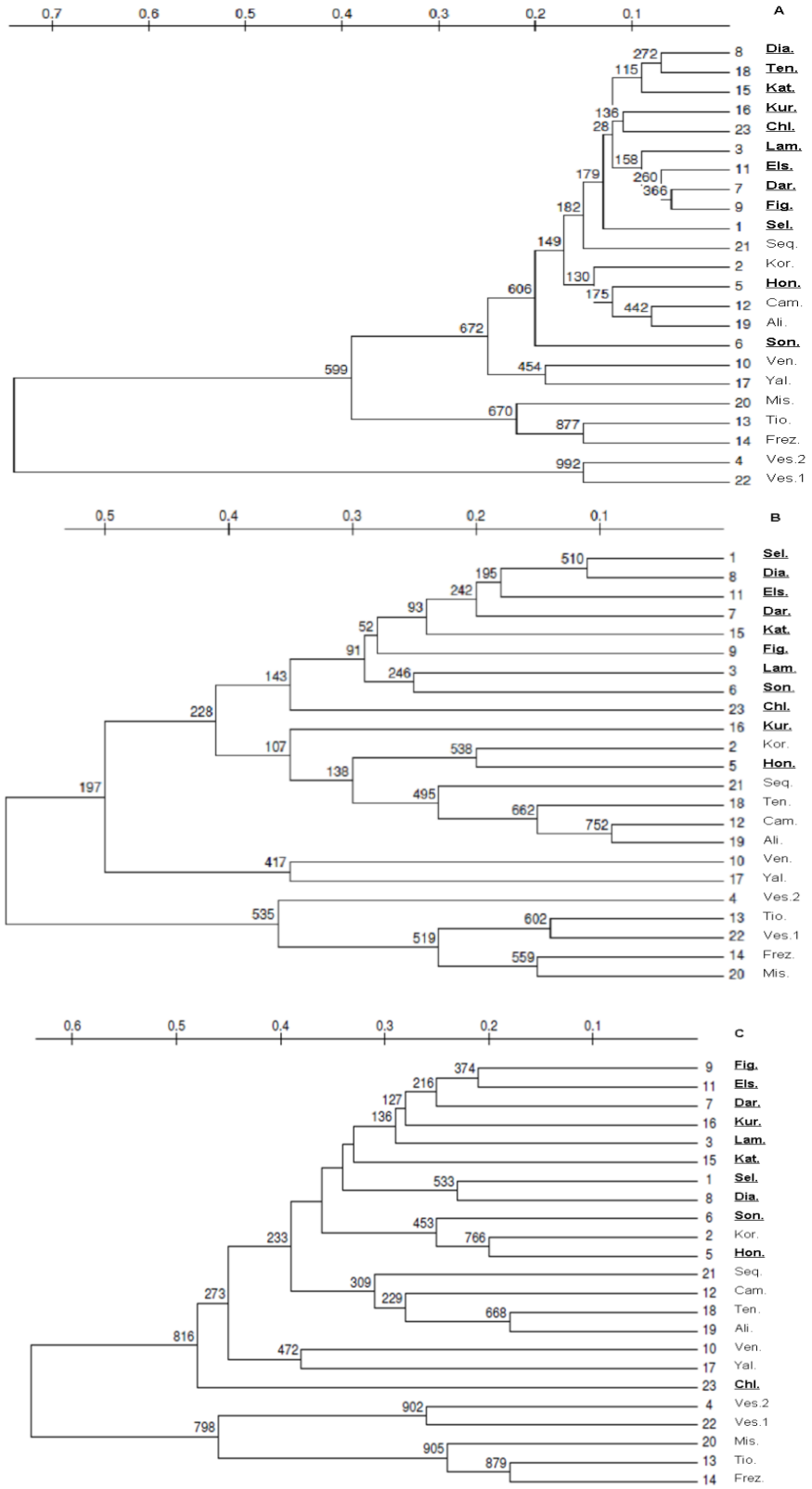

Fig. 5. Dendrograms of the 23 *Fragaria* genotypes obtained via: a) 85 AFLP markers correlating to the leaf WLR, b) 47 AFLP markers correlating to the leaf RWC after 4h, c) 114 AFLP markers correlating to the canonical discriminant functions derived from measured physiological traits by canonical discriminant analysis (i.e., Jaccard's similarity coefficient, UPGMA clustering, and bootstrap values from 1000 re-sampling cycles). Class b is marked as underlined bold names.

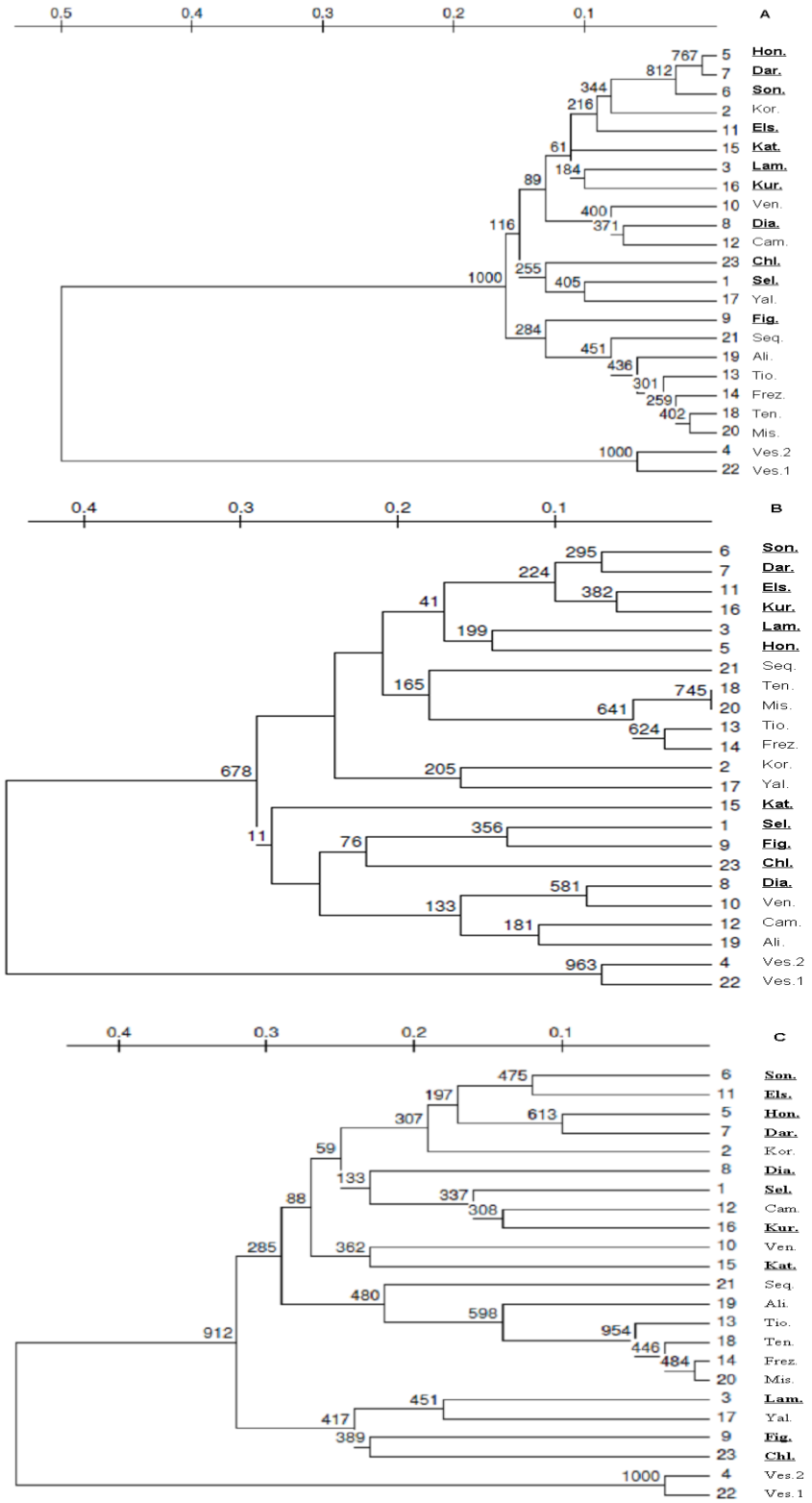

Fig. 6. Dendrograms of the 23 *Fragaria* genotypes obtained via: a) 13 EST markers correlating to the leaf WLR, b) 5 EST markers correlating to the leaf RWC after 4h, c) 10 EST markers correlating to the canonical discriminant functions derived from the measured physiological traits by canonical discriminant analysis (i.e., Jaccard's similarity coefficient, UPGMA clustering, and bootstrap values from 1000 re-sampling cycles). Class b is marked as underlined bold names.

Table 6. Correlation (R) between similarity matrices of Fragaria genotypes based on EST/AFLP markers linked to each trait (canonical discriminant functions). R1: euclidean distance matrix of the Fragaria genotypes based on canonical discriminant functions, R2: Jaccard's similarity matrices of the genotypes based on total EST/AFLP markers (P=0.001).

Marker type	Trait of linked markers	R1	R2	
			Specific linked markers	Random markers (with the same allelle's number)
AFLP	**WLR**	0.32**	0.77	0.99
	RWC at harvest	0.46**	0.80	0.99
	RWC after 4h	0.51**	0.72	0.99
	Δ RWC	0.53**	0.62	0.99
	Additive correlated markers[1]	0.39**	0.87	0.99
	Canonical discriminant functions[2]	0.48**	0.86	0.99
	Total alleles/markers	0.25*		
EST	**WLR**	0.28**	0. 84	0.99
	RWC at harvest	0.30**	0.90	0.99
	RWC after 4h	0.24**	0.54	0.99
	Δ RWC	0.24**	0.57	0.99
	Additive correlated markers[1]	0.26**	0.98	0.99
	Canonical discriminant functions[2]	0.30**	0.71	0.99
	Total alleles/markers	0.25*		

* indicates significance at P= 0.05, ** indicates significance at P= 0.01.
[1] All correlated markers which are at least linked to one of the measured traits.
[2] Canonical discriminant functions derived from all measured physiological traits by canonical discriminant analysis.

Discussion

Phylogenetic relationships

AFLP has been described as an effective marker technique for genetic analyses and fingerprinting of strawberry cultivars (Degani et al., 2001). As a multi-locus but dominant marker technique, it has effectively revealed genetic relationships. Clustering *Fragaria* genotypes based on AFLP or EST markers was in good agreement with their pedigree backgrounds (Table 1; Fig. 4a, b). Both marker techniques proved highly effective in discriminating the 23 different genotypes. Some ESTs, such as EST8 and EST21, with higher polymorphic detection capacity, performed much better than others and can be considered more appropriate EST markers in phylogenetic studies of *Fragaria* genotypes (Table 3). However, these EST markers reveal differences in intron length, and by their nature, they might encompass point mutations (SNPs) that are not differentiated. Only null alleles (no amplification because of mutations in the primer binding sites) were revealed, but it is unlikely that they would appear homozygously in a polyploid species. Overall, both ESTs and AFLPs are comparable and appropriate markers for finding paternity relationships, discriminating between ancestry groups of *Fragaria* genotypes as already mentioned in other genera (Gupta et al., 2003; Buhariwalla et al., 2005; Scariot et al., 2007). The Mantel test showed a high correlation between Jaccard's similarity matrices of genotypes generated by all AFLP markers and the complete EST dataset. This finding confirms a general fit to the overall genetic relationship revealed by each marker technique. Some differences in details can be interpreted due to the dominant and co-dominant nature of these two markers and the different types of polymorphism detection (Belaj et al., 2003). EST markers target expressed coding regions more likely to be conserved across genotypes and species than non-coding regions (Scariot et al., 2007). In our study, EST markers were more capable of differentiating drought-adapted genotypes than AFLPs (Fig. 4b). The combination of marker data sets and PCO analysis

separated species from cultivars. The groups resulting from this analysis confirmed the classifications induced by each marker type (Fig. 4c).

Plant water relations in response to drou ght stress

To enable a fast screening of *Fragaria* genotypes for drought tolerance, two parameters, WLR and RWC, as indicators of leaf water status, were measured. RWC can be considered an integrated measure of the plant water status and is an indicator of the metabolic activity in leaf tissues (Flower and Ludlow, 1986), and WLR estimates the rate of leaf water loss during exposure to drought. Genotype differences were established for RWC at harvest, although this time point is considered less indicative of drought tolerance. Therefore, ΔRWC was calculated to integrate the initial differences in RWC. The high correlations between RWC after 4h, WLR, and ΔRWC indicate that these three parameters refer to a similar reaction mechanism of the plant to water deficit (Table 2). Canonical discriminant analysis, aiming at maximizing the genotype differences for these three physiological measurements, revealed a drought-tolerant and sensitive class (Fig. 3). Drought tolerance in plants is related to its ability to maintain higher relative water content in the leaves and reduce water loss in drought conditions (Suprunova et al., 2004; Rampino et al., 2006; Lipiec et al., 2013; Rejeb et al., 2014). Rates of leaf water loss are associated with an altered accumulation of ABA and changes in stomatal conductance (Verslues et al., 2006; Rejeb et al., 2014). Abiotic stress signal transduction by ABA resulted in various genes activated and involved in cell homeostasis (Zhu, 2002; Rejeb et al., 2014). WLR measurements using detached leaves have been applicable to characterize drought tolerance in crops like wheat, wild barley, and sunflower (Teulat et al., 2003; Suprunova et al., 2004; Rampino et al., 2006). RWC was a relevant screening tool for drought tolerance in cereals (Teulat et al., 2003; Muthurajan et al., 2010) and castor beans (*Ricinus communis* L.) (Wei et al., 2010). The genotype characterization by leaf WLR and RWC after 4h confirms that *F. chiloensis* is drought-tolerant. This finding confirms earlier reports (Zhang and Archbold, 1993). An obvious difference was observed between the two *F. vesca* genotypes in their RWC after 4h. One clone was classified as drought-susceptible, but the others showed an intermediate response. Within-species variations in response to dehydration are most likely. Indeed, VanDerZanden and Cameron (1996) indicated a variation among eleven *Fragaria chiloensis* clones while studying water deficit stress.

Associations among random markers (AFLP), EST markers, and physiological parameters

Breeding drought-tolerant crops is valuable for crop production under deficit irrigation. Functional analysis enables correlating genetic variation in DNA markers or candidate genes with the physiological traits related to drought tolerance (Ramzan et al., 2018; Younis et al., 2020). One of the main goals for present and future research is to find the association between variation in drought-related quantitative traits like RWC and WLR and the effects of these traits on drought tolerance (Cattivelli et al., 2008). Association mapping can be a valuable tool in such exploratory efforts. Relevant results were recently documented regarding pine (Gonzalez-Martinez et al., 2006), maize (Yu and Buckler, 2006), wheat (Rhone et al., 2007), barley (Kraakman et al., 2006; Comadran et al., 2008), sorghum (Hamblin et al., 2004), etc., for drought tolerance or other different types of traits. This approach is valuable for distinguishing drought tolerance in a segregating population when linkage mapping cannot be easily generated (Tuberosa and Salvi, 2006). Genetic association mapping, or linkage disequilibrium mapping (LD), is a method that relies on linkage disequilibrium to study the relationship between phenotypic variation and genetic polymorphisms (Gupta et al., 2005; Breseghello and Sorrells, 2006). LD-based association analysis locates quantitative trait loci (QTL) based on the strength of the correlation between a trait and a marker (Sorkheh et al., 2008; Ramzan et al., 2018; Younis et al., 2020). Instead of biparental crosses between contrasting genotypes, a collection of cultivars, lines, or landraces are genotyped with densely spaced markers (Sorkheh et al., 2008). Association mapping based on LD seeks to establish a statistical association between allelic (or haplotype) variation at a locus and the phenotypic value of a trait across a large enough sample of unrelated accessions (Thornsberry et al., 2001). Significant regression between the trait data and the individual marker genotypes will identify the markers associated with the phenotype (Remington et al., 2001). In tea (*Camellia sinensis*) as an alternative to linkage mapping, fingerprinting of close clones by AFLP and RFLP techniques generated DNA markers associated with APX and SOD enzyme activities involved in drought tolerance (Mishra and Sen-

Mandi, 2004; Younis et al., 2020). In our case, first, we needed a reliable approach to analyze the association between AFLP and EST markers and measure physiological traits in a small set of *Fragaria* genotypes. We developed a technique based on Kruskal-Wallis analysis for testing the association between various markers and physiological traits, with their canonical discriminant factors.

This finding yielded a set of correlated AFLP and functional EST markers (Table 4). Clusters of genotypes based on these correlated markers corresponded better to a class of tolerant and sensitive genotypes, defined by the physiological characterization (Fig. 5a-c and 6a-c; Table 6). For each marker technique, the validity of clusters generated by the linked markers proved not to result from the effect of data set reduction compared to randomly resampled alleles (Table 6). The higher correlation between matrix based on canonical discriminant functions and similarity matrices based on the linked ESTs and on AFLPs, compared to the non-selected marker data sets, indicates that these linked markers better reveal the underlying functional genetic structure related to drought tolerance in the studied *Fragaria* genotypes (Table 6). Also, the occurrence of common EST/AFLP markers linked to both WLR and RWC after 4h indicates the presence of some common genetic loci. Moreover, a low but significant correlation ($R=0.25$; $P=0.05$) occurred among the non-selected EST or AFLP data sets and the physiological measurements (Table 6). Although not directly selected, drought tolerance has probably always been critical in selecting productive genotypes. Thus, a general fit of the genetic structure revealed by markers to the functional diversity of *Fragaria* genotypes considering drought tolerance can be accepted. The general role in many association genetic studies is to use unlinked and putatively neutral markers to characterize genetic variation in the accessions used in the mapping study and to account for population structure (Hall et al., 2010; Ramzan et al., 2018; Younis et al., 2020). Therefore, the analysis of genetic structure is a prerequisite for successfully implementing association mapping approaches in an admixed population. To control this problem, we used the PCO analysis to estimate population structure or genetic relationship via a mixture of AFLP and EST data. We evaluated the resultant axes for association with correlated markers via Kruskal-Wallis analysis ($P\leq0.05$). The applied approach to check the effect of population structure operated through commonly used methods in association mapping research (Hall et al., 2010; Ramzan et al., 2018; Younis et al., 2020). With the ignorance of rare alleles ($X2$; $P\leq0.05$), results indicated that only some of the frequently correlated markers to physiological traits are neutral with no effect on phylogenetic structure, while many correlated markers can significantly affect genetic structure as well (Table 4). This finding shows how the genetic structure affected the plant population in this research and discovered associated markers relevant to the traits. Despite this and due to the low number of our accessions, these introduced markers, including those with significant effects on the genetic structure or the neutral ones with a low frequency, might be considered valuable markers that deserve further evaluations with more accessions. Therefore, the DNA fragments associated with physiological parameters in this study appear to be good candidates for further differentiating *Fragaria* genotypes as tolerant or sensitive to drought, especially in the case of EST markers. Overall, the approach applied in this study resulted in valuable functional and non-functional markers that require further evaluations in future research on association mapping analyses. These markers can analyze more accessions for marker-trait association analysis and MAS for drought tolerance. Hence, our research was an optimization study that examined a marker-trait association method for the preliminary dissection of QTLs involved in drought tolerance in a *Fragaria* collection, with no crossing and segregation history.

Candidate genes from known metabolic pathways behind drought tolerance, EST markers, and association with eco-physiological parameters

Although drought tolerance is a quantitative trait, single genes, such as those controlling physiological drought-tolerance-related traits and osmotic adjustment (OA), may have important roles in adaptation to drought-prone environments (Tuberosa and Salvi, 2006). Functional markers developed based on these genes are perfect sources for marker-trait association analysis and investigation of QTLs involved in drought tolerance (Andersen and Lubberstedt, 2003; Gupta et al., 2005; Younis et al., 2020). In this experiment, 16 of 24 developed EST markers were candidate genes for drought stress response (Table 3). The salient role of antioxidant defense systems, sucrose metabolism, and osmotic adjustment in regulating plant responses to drought stress is well known. Also, previous results elucidated the role of sucrose as a primary factor of osmotic adjustment under water deficit conditions in

strawberries (Razavi et al., 2008). Therefore, we can accept a direct functional linkage for these ESTs to genes regulating *Fragaria* responses to drought stress from sucrose metabolism, osmotic adjustment, and antioxidative mechanisms. We can infer a possible similar function for the EST markers developed from uncharacterized homologues in *Fragaria* to the functional genes in other plants. To this group belong homologue sequences of *CAT* and *MnSOD1* as antioxidant and detoxification enzymes, *DREB2A* and *DREB3* as the transcriptional regulatory factors in drought conditions, and *RD22* as the responsive gene to dehydration. *P5CS* is a key enzyme in proline biosynthesis, and Rab18 is a dehydrin-protective protein (Table 5). The last ESTs group were unknown EST sequences of *Fragaria* generated by cold, salicylic acid, or heat stress. These *Fragaria* sequences require further elucidation about their function in plant responses to drought stress. Nevertheless, the fact that they were generated by other abiotic stress in *Fragaria* and appear to correlate with drought stress in this experiment suggests their general role in stress response.

The generation of molecular–linkage maps based on linkage disequilibrium and candidate genes (molecular–function maps) is one reliable way to identify the genetic determinants of QTLs in plants without a mapping population despite the time-consuming and fine mapping that represents a worthwhile shortcut to QTL cloning (Causse et al., 2004). For specific traits of interest like drought tolerance, candidate-gene association mapping is one of the most salient categories for association mapping studies (Zhu et al., 2008). Many association studies based on candidate genes have concluded using tens to hundreds of markers in mapping populations consisting of a few hundred individuals (Hall et al., 2010; Younis et al., 2020). Due to the small size of the plant population in this study, the correlated ESTs generated based on *Fragaria* homologues of involved candidate genes in drought tolerance can be considered a valuable source for future mapping analysis in a larger population (with a higher number of individuals) or even in other genera of *Rosaceae* for drought tolerance (Table 5). In this regard, the ESTs with neutral effects on the genetic structure of *Fragaria* genotypes but frequently correlated with the phenotype, like EST6, EST7, EST8, EST21, and EST18, can be more valuable as functional markers, although they still need to be further investigated (Table 5). Therefore, EST markers developed based on functional genes in our study are useful for future marker-trait association studies to identify QTLs involved in drought tolerance in *Fragaria* .

Conclusion

In a limited set of *Fragaria* genotypes, it was possible to reach an integrated method, combining fast screening tools for plant leaf dehydration and associated markers from random AFLP or candidate gene ESTs. Phenotypic classes of plants grouped according to their drought response better corresponded to groupings made on correlated markers. This study elucidated some potential candidate genes involved in *Fragaria* drought tolerance that were previously identified but needed further characterization. As plant responses to drought stress are a complex quantitative phenomenon, higher drought tolerance could be attributed to the combination of different factors that cannot be genetically analyzed as a monogenetic character. Therefore, mapping them as QTLs can assist researchers in identifying regions involved in regulating this trait (Suprunova et al., 2004). The correlated markers identified in this study need further evaluations for their association with plant function in drought conditions and LD mapping in more unrelated genotypes. A more powerful approach would be to analyze these markers and check their trait association and neutrality in genetic structure. This finding is probably not so easy for the highly bred and narrow gene pool of *Fragaria* × *ananassa*. However, the linked markers identified in this study are still a good starting point for using this approach. Eventually, these markers might be applicable in germplasm screening for drought tolerance in *Fragaria* sp. Here, wild *F. chiloensis* and *F. vesca* ecotypes could be a good alternative. *F. vesca* is better characterized as a model plant species for *Rosaceae* (Shulaev et al., 2008) and is also considered a valuable genetic resource for introducing many salient traits in cultivated strawberries. The expression analysis of the genes evaluated in the present study can give more information about the genetic control of drought response in *Fragaria* sp.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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