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Genotype confidence percentage of SSR HRM profiles as a measure of genetic similarity in *Rosmarinus officinalis*

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ABSTRACT

Rosemary (*Rosmarinus officinalis* L.) is a highly allogamous plant with few genomic resources available. Among these, a discrete set of nuclear and cytoplasmic microsatellite markers is counted. High resolution melting (HRM) is a closed-tube technique widely used for sequence polymorphism detection. Until now, HRM data analysis in plants has been conducted reporting melting profiles to known allelic assets. Even for preliminary studies on general genetic diversity, laborious workflows have been proposed for HRM genotyping of microsatellite's indels as well as of SNPs. Genotype confidence percentage (GCP) of HRM curves is the most commonly used statistical transformation of Euclidean distance between HRM curves to determine whether two curves are identical or not. In the present study we tested an analysis workflow that implies the use of GCPs of HRM profiles as a direct measure of the genetic similarities on SSR loci, bypassing therefore genotyping. The comparison between capillary electrophoresis and HRM-GCPs data demonstrates the efficiency of this new technique in detecting genetic differences between rosemary populations. This method can be used for determining overall genomic similarity in phylogenetic and ecological studies. It is cost and time effective, suitable to complete automation and can be used in other allogamous plants as well.

1. Introduction

Rosemary (*Rosmarinus officinalis* L., Lamiaceae) is a medicinal and aromatic plant (MAP) and is both a wild and a cultivated Mediterranean species with high levels of heterozygosity (Hidalgo-Fernandez and Ubera-Jimenez, 2001). Rosemary biodiversity conservation and characterization is a strategic target for Italian Ministry of Agriculture and Forestry (Mi.P.A.F.) within "Convention on biological diversity" promoted by FAO (RGV-FAO project). In fact, notwithstanding its interesting medicinal properties, wild germplasm as well as cultivated varieties are not thoroughly characterized and rosemary cultivation is still a low input agronomic practice. Besides, genomic resources for *R. officinalis* in the nucleotide databases at NCBI (https://www.ncbi.nlm.nih.gov) that reported the species as *Salvia rosmarinus* (L.) Schleid., 1852 according to Drew et al. (2017), are limited to 105 sequences, including the complete chloroplast sequence and a discrete pool of nuclear and cytoplasmic simple sequence repeats (SSR) markers (Segarra-Moragues and Gleiser, 2009; Molecular Ecology Resources Primer Development Consortium et al., 2010; Mateu-Andrés et al., 2013).

High resolution melting (HRM) analysis of polymorphic SSR has been suggested by Ririe et al. (1997) and implemented in plants and other organisms during last 20 years. A recent review of applications in plants by Simko (2016) reports examples in 22 different plant genera. In all the works reported, HRM data analysis has been almost always conducted in order to report melting profiles to known allelic assets (Muleo et al., 2009; Distefano et al., 2012; Yan et al., 2012). In other cases, HRM has been proposed as a preliminary tool to detect polymorphism (Arthofer et al., 2011), or considered an instrument for visual comparison of the melting behaviour of few genotypes (Mackay et al., 2008; Hwang et al., 2011; Ganopoulos et al., 2015a,b; Xanthopoulou et al., 2014).

Genotype confidence percentage (GCP) is a statistical transformation of Euclidean distance between HRM curves that has been

Abbreviations: GCP, Genotype Confidence Percentage; SSR, Simple sequence repeats; HRM, High Resolution Melting; indels, Insertions and/or deletions; SNP, Single Nucleotide Polymorphism; PCoA, Principal Coordinates Analysis; MAP, Medicinal and aromatic plant; CE, Capillary electrophoresis; CE-SSR, Capillary electrophoresis of fragments containing simple sequence repeats; PDO, Protected designation of origin; UPGMA, Unweighted Pair Group Method with Arithmetic Mean; H_E, Expected heterozygosity; H_O, Observed heterozygosity; F_{ST}, Fixation index; G_{ST}, Genetic differentiation; HRM-GCP, Method for estimating genetic differences via Genotype Confidence Percentage of High Resolution Melting

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developed and optimized for normalizing diversity between melting curves and for determining whether two melting curves are identical or not. It has been especially optimized for minimizing differences among technical replicates and mark differences among different genotypes. Besides, GCP has already been used in bacteria as a direct measure of genetic diversity (Sakaridis et al., 2014). By this procedure, genetic diversity analysis was achieved more easily and concisely without direct genotyping.

In order to undertake a wide characterization of genetic diversity within and between rosemary natural populations, we looked for an automated, rapid, simple and low cost strategy that could lead us to manipulate a great number of samples using as many as possible of the available markers. So we tested the average GCP computed on different SSR loci as a direct measure of similarity between genotypes in our diploid highly allogamous rosemary genotypes. According to previous studies on rosemary (Segarra-Moragues et al., 2016), genetic difference within population is minor than between populations so that genotypes of the same population are expected to group preferentially together. We decided to compare population grouping performances in principal coordinates analysis (PCoA) of data from HRM-SSR by GCPs to performances of data from traditional capillary electrophoresis of the same SSR (CE-SSR) by a standard genetic similarity index.

2. Materials and methods

A total of 25 rosemary genotypes were collected in groups of five per five natural areas: two in Sardinia (populations named as SANT, at Sant'Antioco, 39.03739°N and 8.41842°E and GON, at Cala Gonone 40.28035°N and 9.62717°E), two in Corsica (PER, at Capo Pertusato 41.366°N and 9.18083°E and CAN, at Punta di Canelle, 42.83329°N and 9.30974°E) and one in Tuscany (CASPE, at Castiglione della Pescaia, 42.76389°N and 10.87502°E). Per each population, genotypes were selected at random, at a minimum distance of 30 m from each other. DNA isolation was performed by DNeasy Plant Mini Kit (Qiagen, Germany) on lyophilised leaves.

Eight microsatellite primer pairs were selected from literature: two were built on rosemary chloroplast (ccmp6 and ccmp10; Mateu-Andrés et al., 2013), four were specifically built on rosemary genomic sequences (Roff101, Roff335, Roff424, Roff438; Segarra-Moragues and Gleiser, 2009) and two were built on *Salvia officinalis* L. (SoUZ002 and SoUZ007; Molecular Ecology Resources Primer Development Consortium et al., 2010). The last two already showed polymorphism in rosemary (Radosavljević et al., 2011).

For capillary electrophoresis (CE), a two-step PCR was performed using M13-tailed and M13 fluorescent primers. CE was performed in pool of four differently coloured markers (FAM, VIC, NED and PET; Applied Biosystems) so that a total number of 50 runs were performed by an external service using 500LIZ (Applied Biosystems) as size standard. Data were then read and edited by Peak Scanner (Peak Scanner™ Software; Version: 1.0; Applied Biosystems).

For HRM analysis, reactions were built up by Corbett CAS 1200 robotic instrument in 10 µl reactions. SsoFast™ EvaGreen® Supermix (Biorad - USA) was used according to manufacturer's instructions. On Rotor Gene 6000 mastercycler (Qiagen - Germany), a touchdown amplification protocol was performed. Melting was conducted after denaturing at 90 °C and renaturing PCR products at 50 °C for 2 min. Acquisition was made during cycling amplification and in the denaturation phase, acquiring luminescence each 0.1 °C. The Rotor Gene 6000 proprietary software (version 1.7.87) was used to visualize results. Normalization regions 1 °C wide were chosen as near as possible but not overlapping the region of melting. Data were then exported in R (R Core Team, 2016) where, for each microsatellite, a similarity matrix was built up in which each genotype was compared to all the others. Genotype confidence percentages (GCPs) were used as similarity values (S) and computed using the formula:

$$S_{rt} = 1,05^{\left[-0.02\times\sum_{i=a}^{z}(f_{ri}-f_{ti})^2\right]}$$

where $\boldsymbol{f_{ri}}$ and $\boldsymbol{f_{ti}}$ are the fluorescence values detected at temperature $\boldsymbol{i}$ for the $\boldsymbol{r}$ and $\boldsymbol{t}$ compared samples; $\boldsymbol{a}$ is the temperature at the starting melting point and $\boldsymbol{z}$ is the temperature at the end. A triangular similarity matrix was build up for each marker analysed and then transformed in the complementary dissimilarity matrix where $D_{rt} = (1\text{-}S_{rt}) \times 100$.

Principal coordinate analysis (PCoA) for each dissimilarity matrix obtained from HRM analysis was performed using SYN-TAX 2000 (Podani, 2001). A scattergram of PCoA for data of each of the genomic markers was built where dissimilarity matrices were gained by HRM data and points were labelled using allele calling gained by CE.

Two average dissimilarity matrices were then built up, one for CE data (by Dice coefficient) and one for HRM data and were both analysed and plotted after PCoA by SYN-TAX 2000.

3. Results

In capillary electrophoresis (CE), polymorphism was observed among 25 analysed samples by six of the eight primer pairs used. Amplification products for primers ccmp6 and ccmp10 seemed monomorphic as they produced bands of 84 bp and 120 bp respectively (very similar to what expected according to literature). Anyway, when analysed by HRM, even these fragments resulted polymorphic as better specified later. In Table 1, numbers of allele observed per each marker, band sizes and number of genotypes observed in the 25 accessions are reported. For each marker, alleles corresponding to peaks have been named from A to Q as the fragment size increased. Only 2% of peaks were not readable and were therefore scored as not available data.

In HRM, by each primer pair, all samples were amplified with the same threshold cycle and the melting curves (Wittwer et al., 2003) had all similar Tm and shape (raw data in Mendeley data). According to PCoA results, we report that both the scattergrams concerning the two chloroplastic microsatellites (ccmp) present a certain clustering of the five populations (Supplementary Fig. S1). Overall, the PCoA results of each single marker show a clustering somewhat intermediate between allele based clustering (as read in CE) and population based clustering

Table 1
Results of genotyping by capillary electrophoresis.

Marker	# observed alleles	Bands (sizes observed)	# observed genotypes
ccmp6	1	A (84)	1
ccmp10	1	A (120)	1
Roff101	17	A (169), B (172), C (175), D (178), E (180), F (184), G (186), H (189), I (192), J (195), K (200), L (203), M (206), N (212), O (220), P (226), Q (229)	20
Roff335	4	A (117), B (119), C (121), D (123)	5
Roff424	12	A (149), B (150), C (151), D (153), E (155), F (157), G (161), H (163), I (165), J (169), K (171), L (175)	16
Roff438	13	A (93), B (100), C (102), D (104), E (106), F (109), G (114), H (118), I (120), J (123), K (130), L (133), M (143)	19
SoUZ002	12	A (57), B (89), C (91), D (92), E (105), F (123), G (149), H (166), I (172), J (179), K (183), L (205)	12
SoUZ007	7	A (182), B (184), C (186), D (189), E (205), F (209), H (228)	7

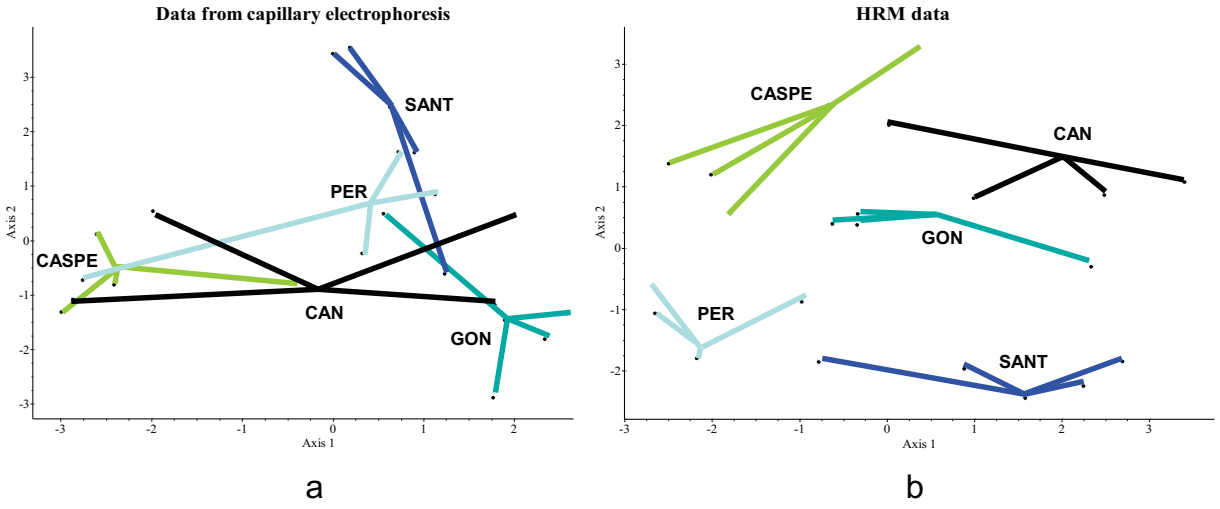

Fig. 1. Scattergram deriving from PCoA of average dissimilarity. Genotypes being part of the same population are connected each other by coloured lines: (a) data derived from capillary electrophoresis, (b) data derived from HRM-GCPs. (A different colour has been used per each population)

(data not shown). This is compatible with the hypothesis that some alleles of the same size are present in multiple variants that differ for one or more SNPs and that are prevalently similar inside each population examined.

Concerning the PcoA made on average similarity matrices (Fig. 1), HRM data on these eight polymorphic markers result in a more defined clustering of populations as all the five individuals of each of the five populations can be easily linked to each other and are localized in a specific, continuous and exclusive area of the scattergram (Fig. 1b). By contrast, CE data give no rise to a similar clear clustering, as the area of the scattergram including genotypes from a population is not continuously exclusive of that population (Fig. 1a).

The contribution of diversity explained by the first two axes in the scattergram is 31.88% (Fig. 1a) and 41.38% (Fig. 1b), respectively. The contribution of diversity explained by the third axis is 12.33% for Fig. 1a and 12.19% for Fig. 1b. A tri-dimensional scattergram of CE-SSR data (explaining 44.21% of the variability), did not improve clustering of genotypes in populations (data not shown). Even if ccmp polymorphisms detected by HRM analysis are not considered, a better clustering can be gained when using HRM data on the six genomic polymorphic traits (Supplementary Fig. S2).

4. Discussion

Workflows previously suggested for analysing SSR by HRM in plants, always included comparisons with CE and sequencing data implying the acquisition of a high level of knowledge of the polymorphic sites analysed (Simko, 2016). When analysing a great number of samples, with few reference data, the need of a workflow for classifying HRM curves as known or new has been evidenced and resolved by Mader et al. (2008). Nonetheless, the suggested workflow requires a visual analysis of the melting curves and the building of a reference group of samples to which each new sample had to be compared in order to establish if it was new or already known. If new, the sample had to be visually classified as heterozygous or not and then, if heterozygous, compared to artificial heterozygous samples to verify if it was made of known or new alleles. In rosemary analysed alleles were expected to be highly heterozygous due to high allogamy of this specie. The consequent lack of homozygous genotypes would have made HRM results interpretation through the suggested workflow even more laborious. Besides, as suggested by our data, an additional component of polymorphism was detected in HRM analyses and not detected in CE, making comparative analyses of results and consequent genotyping almost impossible. The presence of this kind of additional polymorphism is compatible with results of Segarra-Moragues and Gleiser (2009), who used the Roff-series SSR in a population study. As previously made in tomato by Ranc et al. (2008), SSR were used in rosemary for testing the Hardy-Weinberg equilibrium and conclusions about population structure were made. For Roff101 and other four alleles, the authors observed a heterozygote deficiency in their population and hypothesized the presence of null alleles. In plants with few available genomic resources, the assumed absence of polymorphism in flanking regions may reveal incorrect and a genotype showing a single peak, commonly treated as homozygous, could actually be heterozygous due to presence of undetected SNPs. Results coming from our HRM-SSR experiment suggest that SNPs are present in flanking regions of Roff101 and that the detected heterozygote deficiency could be due to the intrinsic limit of capillary electrophoresis of SSR traits (CE-SSR).

In many works (Mader et al., 2008; Montgomery et al., 2007), the attribution of a curve to a known type is done visually and the error connected is estimated as negligible. When the HRM has been proposed for commercial use in a test for authenticity of protected designation of origin (PDO) products (Ganopoulos et al., 2011), the needing of reliability and transferability was well marked so that setting of cut-off values for GCPs was proposed to establish if two melting curves are identical or different. More accurately, needing for a clear distinction between virus isolates, Hewson et al. (2009) underlined that setting the same cut-off value for GCPs in any kind of HRM experiment is not correct but a cut-off value should be specifically computed for each experiment by analysing the variance of GCPs inside each group of biological replicates and between different genotypes. Again, in this case, previous knowledge on existing variants and a good quantity of reference samples was required and used in order to correctly asset the experiment.

Since now, in plant studies, even when markers were used for general studies on genomic diversity, authors have always translated HRM results in a detailed genotyping panel before on-going similarity analysis as neighbour-joining method (Distefano et al., 2013; Wu et al., 2008), UPGMA clustering (Ganopoulos et al., 2011; Ganopoulos et al., 2015a,b; Hwang et al., 2011) or principal component analysis (Mader et al., 2010). Dice coefficient is commonly preferred to coefficients specifically built for microsatellite diversity analysis as Goldstein distance (Goldstein et al., 1995), in which diversity between genotypes is proportional to diversity in repetition numbers at each locus.

In this work we directly used the average GCPs values as a measure of genetic similarity as already done in bacteria by Sakaridis et al. (2014). The approach was based on the assumption that, approximately, the more diverse the sequences of two fragments are, the more

diverse their melting curves are. While in bacteria each marker corresponds to a single DNA fragment, in our allogamous genotypes each amplification product was expected to be composed of two different DNA fragments and their heteroduplex that produces an additional flex of melting at the lower temperatures. The method has many known sources of error and GCP is not linearly proportional to sequence similarity (Chagné, 2015; Hewson et al., 2009), nonetheless, the results gained from our pipeline were compared with another one that has always been accepted as a good tool for estimating genetic distances (CE-SSR) and proved to be more sensitive and accurate in remarking differences between populations. In fact, according to previous studies made by eleven genomic CE-SSR on 18 rosemary populations of 96 individuals each (Segarra-Moragues et al., 2016), we expected a positive correlation between geographical distances and genetic distances and, in particular, a good clustering of genotypes in populations.

As HRM-GCP analysis does not imply genotyping, many elaboration made in population genetics by CE-SSR and based on expected heterozygosity (H_E) and observed one (H_O), such as fixation index (F_{ST}) and genetic differentiation (G_{ST}), are not feasible. Besides, other possible analysis as structure and bootstrap, are still not implemented directly on dissimilarity matrices. Nonetheless, once a consistent group of HRM data will be collected, Mantel correlogram will be computable and pattern of geographic distribution will be estimable. Last but not least, HRM-GCP data can be used to build core collections using Max-length subtree method (Perrier et al., 2003).

In conclusion, we can state that HRM-GCPs can be adopted with good results for studies on general genetic diversity as well as phylogenetic studies in rosemary and in other allogamous plants. The new implemented pipeline is cost and time effective, suitable to complete automation, closed-tube and it allows estimating phylogenetic global similarities with enough accuracy to make the management of germplasm collections smarter and more efficient.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2018.04.006.

Data archiving statement

The data consisting of 200 normalized melting profiles of eight SSR fragments of 25 individuals are provided in Nunziata, Angelina; De Benedetti, Laura; Cervelli, Claudio (2018), “Melting profiles of simple sequence repeats”, Mendeley Data, v1 http://dx.doi.org/10.17632/4ccp5djpzp.1

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