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Validation of a Simple Molecular Method for Assessment of Clonal Heterogeneity in Kiwifruit (*Actinidia deliciosa* A. Chev.)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OM and CG designed the study. Authors MB and VI managed the analyses of the study. Authors MB and OM managed the experimental process, the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: The industry of the green fleshed kiwifruit over the world is based on the cultivar Hayward due to outstanding characteristics of its fruits. Mutations and improper labeling of introduced plants could give rise to clonal heterogeneity within kiwifruit cultivars. Therefore, the objective is to validate a simple molecular approach to evaluate the clonal purity of cultivar Hayward in micropropagation laboratories and nurseries.
Study Design: Molecular analysis of the genetic variability within cultivar.
Place and Duration of Study: Biotechnology Laboratory at the Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata – Estación Experimental Agropecuaria Balcarce, Instituto Nacional de Tecnología Agropecuaria, Argentina, 2013.
Methodology: Genomic DNA of four introductions and clone 8 of cultivar Hayward was amplified

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with randomly amplified polymorphic DNA markers. Cluster and principal coordinates analyses were performed.
Results: The analyses revealed that all introductions were genetically different from each other.
Conclusion: Small genetic differences within cultivars could be detected by this simple molecular approach when it is performed under well-controlled experimental conditions in each micropropagation laboratory or nursery.

Keywords: Cultivar Hayward; genetic variability; RAPD markers; cluster and principal coordinates analyses.

1. INTRODUCTION

Kiwifruit (*Actinidia* spp.) is native to Eastern Asia, but the greatest diversity of species is found in China [1]. This genus includes more than 60 species which are dioecious perennial and have different ploidy levels, from diploids (2n=2x=58) to octoploids (2n=8x=232). Fruits from different species differ in shape, size, pilosity, color, flavor and edibility. Three species have been commercially exploited to date, (1) *Actinidia chinensis* Planch, whose fruits have yellow flesh, smooth skin, sweet aromatic flavor and are high in vitamin C, (2) *Actinidia arguta* Franch. & Sav., which produces small fruits (baby kiwifruits) arranged in bunches that are eaten whole, with the skin, and (3) *Actinidia deliciosa* (A. Chev.) Liang & Ferguson, which produces bright-green fleshed fruits [2].

Kiwifruits are grown from clonally preserved and vegetatively propagated cultivars. The first cultivars of green fleshed kiwifruit (*A. deliciosa*) have been obtained from selections of wild populations. The most widely grown commercial cultivar Hayward is only two or three generations from the wild [3]. It is preferred over other cultivars because of the characteristics of its fruits (good size, flesh firmness, flavor, acidity/sugar ratio and exceptional shelf life).

Later on, green fleshed kiwifruit cultivars have been developed from selection of (a) seedlings derived from either open-pollination of cv. Hayward or controlled crosses between cv. Hayward and different pollinizers, (b) bud mutations of Hayward (cultivars Green Light®, Top Star®, Early Green® and Bo-Erica® [4]) and (c) strains of Hayward, known as clonal selections of Hayward, which have been chosen because they showed fewer fruit faults (excessive width or frequent “Hayward mark”). Most of these clonal selections have been selected in Italy (clones 8, K and Maeba®) and New Zealand (clone Kramer) [1,4].

Since each kiwifruit cultivar would be propagated from the original cultivar clone, growers expect that each of their orchards represents a single genotype with stable and uniform performance. For that reason, it is critical that only plants of correctly named cultivars be sold. Mislabeled plants introduced and propagated by nurseryman could result in production losses for several years, due to replanting or the need to topworking [5].

In Argentina, approximately half of kiwifruit local production is concentrated in Southeastern Buenos Aires, an area with optimal agroecological conditions for this fruit crop. All genetic materials of green fleshed kiwifruit planted in this country were introduced from New Zealand, Chile and Italy. Hayward is the most widely planted female cultivar, and the clone 8 is the clonal selection of cv. Hayward currently grown in the new orchards. This clone was selected from Hayward variants collected from the main kiwifruit-growing area in Italy. This variant differed in the percentage of cymes bearing more than one flower and in the low frequency of deformed fruit (presence of Hayward mark, flat and fan fruit) [4]. Interestingly enough, there are available introductions of cultivar Hayward in local nurseries whose identity with clone 8 has not been proved to the present.

Kiwifruit cultivars cannot be readily and reliably identified from visual plant characteristics, especially in the young vegetative stage [5] because *Actinidia* vines can be very variable morphologically, even between leaves and shoots from different parts of the one plant [3]. On the contrary, molecular markers - Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment-Length Polymorphism and microsatellites - are currently being applied in kiwifruit breeding for cultivar identification and somaclonal variation detection [6].

In this investigation we analyzed the genetic diversity of the introductions of cultivar Hayward using RAPDs markers in order to (1) determine if these introductions were genetically different from each other and if so, examine the relationship among them and (2) evaluate the potential of this

marker for detecting small genetic differences within cultivar Hayward. The reason why RAPD has been used in this study is that it can be the molecular marker chosen by commercial nurseries and micropropagation laboratories for testing simply, quickly and cheaply the clonal purity of their own propagated or imported plants, and eventually detecting new genetic variants.

2. MATERIALS AND METHODS

2.1 Plant Materials

Five introductions of cultivar Hayward were used in this analysis: “C”, “CC”, “H”, “8Ch” and “8I”. The last one, which is known for having been selected in Italy in 1993 [4], was introduced from an Italian nursery ten years ago. The rest of introductions came from the Chilean nurseries. These materials were introduced in Argentina as *in vitro* plantlets from inter-nursery exchanges of plant materials. These *in vitro* plantlets were clonally propagated through single-node cuttings by local nurseries generating the plants used in this study. Another commercial cultivar, male “M52”, was used in the analysis as an out-group.

2.2 DNA Isolation and PCR Amplification

Total genomic DNA of each introduction (clone) was extracted from newly emerged leaves of five plants having three true leaves using Dneasy Plant Mini Kit (Qiagen). DNA concentration was quantified using a digital spectrophotometer (SmartSpectTM 3000 BIO-RAD, USA) based on 260 nm absorbance, and DNA quality was inferred by 260 nm/280 nm ratio and checked on agarose gel. DNA amplification was performed in volumes of 25 µl, containing 0.4 µM primer, 200 µM dNTP mix, 2,5 mM $MgCl_2$, 10X PCR buffer, 2 U Taq polymerase (Platinum, Invitrogen) and 25 ng DNA as template. Amplifications were performed on a Thermal Cycler (Biometra) with the following program: 1 cycle at 92℃ for 2 min; 45 cycles at 94℃ for 30 s, 37℃ for 1 min, and 72℃ for 2 min, and 1 cycle at 72℃ for 7 min. Amplification products were resolved in 1.5% TAE agarose gels, stained with Sybr Safe and visualized with blue light transiluminator.

Nineteen primers of the Operon Technologies Inc. (OPC4, OPC6, OPC10, OPC16, OPC20, OPE3, OPE19, OPQ8, OPQ9, OPQ12, OPQ15, OPQ18, OPQ19, OPQ20, OPS2, OPS8, OPS9, OPS15 and OPS18), which had been used by [7], were firstly screened on two materials (clone “8I” and cultivar “M52”). Nine of them, which were selected for their consistent and reproducible electrophoretic patterns, were then used to analyze all the materials. To overcome the reproducibility problems of RAPDs markers, DNA isolated from the same set of plants belonging to each clone was amplified at two different times using a common stock of reagents and the same thermal Cycler.

2.3 Data Analysis

Each marker was scored as 1 (band present) or 0 (band absent) in each clone. Polymorphism Information Content (PIC) was calculated for each marker [8]. Statistic program InfoStat [9] was used to compute Jaccard´s coefficients of similarity and to perform Principal Coordinate Analysis (PCA), Minimum Spanning Tree and the dendrogram according to the UPGMA (unweighted pair group mean arithmetic) clustering algorithm. In addition, cophenetic correlation coefficient was obtained to measure goodness-of-fit of the dendrogram to the similarity matrix [10].

3. RESULTS AND DISCUSSION

Nine out of the 19 evaluated primers (47.37%) were informative generating 69 scorable bands (see example in Fig. 1). The average band number per primer was 7.66, with a minimum of 4 (OPQ9) and a maximum of 11 (OPQ8 and OPQ18) (Table 1). Considering all materials analyzed in this study, 34 out of 69 bands were polymorphic, which represented 50.72% of polymorphism. However, the polymorphic band number was reduced to14 (20.29%) when only the introductions of cultivar Hayward were considered.

In our study, three primers (OPC10, OPS8 and OPQ8) showed the highest levels of polymorphism, with PIC values higher than 0.6 (Table 1) whereas others had medium (OPQ18, OPQ15 and OPQ20) and low (OPS9, OPQ9 and OPC4) PIC values (Table 1). The average level of stable polymorphisms was very good, demonstrating that RAPDs markers were useful to discriminate all introductions of cultivar Hayward.

The dendrogram constructed with the complete set of data (Fig. 2), based on Jaccard´s similarity coefficient (Table 2), showed a clear distinction among the genotypes. The cophenetic correlation

coefficient was very high (0.982) indicating a good representation of the distance matrix by the dendrogram [10]. The greatest genetic distance was observed between the five introductions of cultivar Hayward and the cultivar "M52". This differentiation was also equally evident in the PCA (Fig. 3) in which the CP1, that explains 59% of the total variability observed among the genotypes, classified the Hayward introductions in the left quadrant and the cultivar "M52" in the right one. This result is expected to occur since cultivar "M52" is a pollinator unrelated to Hayward, and therefore, it supports its usefulness as an out-group. All introductions of the cultivar Hayward resulted different from each other (Fig. 2). This differentiation could also be visualized in the PCA (Fig. 3) in which the CP2, that explains 21.9% of the total variability observed among the genotypes, classified the five introductions in two groups delimited by the quadrants of the graph: clones "CC" and "H" in the left upper quadrant and clones "C", "8I" and "8Ch" in the left lower one.

The differentiation of the introductions became even clearer when the cultivar "M52" was excluded from the Principal Coordinates Analysis (Fig. 4). Both principal coordinates, CP1 and CP2, accounted for more than 80% of the total variability among Hayward introductions. The genotypes were divided in four groups by the axes of the coordinates: clones "8I", "CC" and "H" were plotted in different quadrants, while clones "8Ch" and "C" were grouped together. This major similarity between the last two introductions could also be visualized clearly in the Minimum Spanning Tree (Fig. 4).

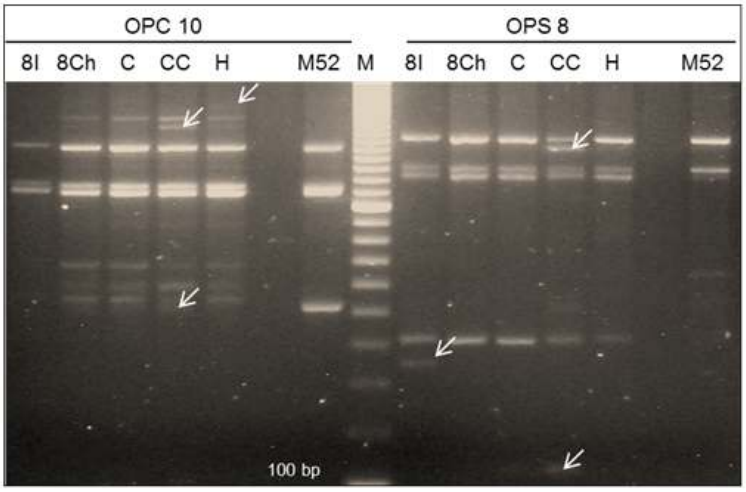

Fig. 1. Banding patterns of primers OPC 10 and OPS 8 in the five introductions of cultivar Hayward and cultivar "M52". M stands for DNA size markers/100 bp ladder. Arrows indicate additional bands in the introductions of Hayward

Table 1. Number and size range of amplified bands and Polymorphism Information Content (PIC) values of nine RAPD primers

Primer		Amplified band		PIC
Name	**Sequence**	**Number**	**Size range (bp)**	
OPC10	TGTCTGGGTG	10	2150 - 530	0.66
OPS8	TTCAGGGTGG	8	1650 - 130	0.66
OPQ8	CTCCAGCGGA	11	3000 - 600	0.72
OPQ18	AGGCTGGGTG	11	2000 - 500	0.5
OPQ15	GGGTAACGTG	5	1900 - 820	0.5
OPS9	TCCTGGTCCC	5	1700 - 710	0.28
OPQ9	GGCTAACCGA	4	1400 - 950	0.28
OPQ20	TCGCCCAGTC	6	2500 - 900	0.5
OPC4	CCGCATCTAC	9	2020 - 690	0.28

Table 2. Distance matrix of five introductions of cultivar Hayward and cultivar "M52" based on Jaccard similarity coefficient

	8Ch	8I	C	CC	H	M52
8Ch	0,00					
8I	0,32	0,00				
C	0,14	0,31	0,00			
CC	0,38	0,45	0,35	0,00		
H	0,26	0,38	0,24	0,33	0,00	
M52	0,64	0,65	0,61	0,66	0,64	0,00

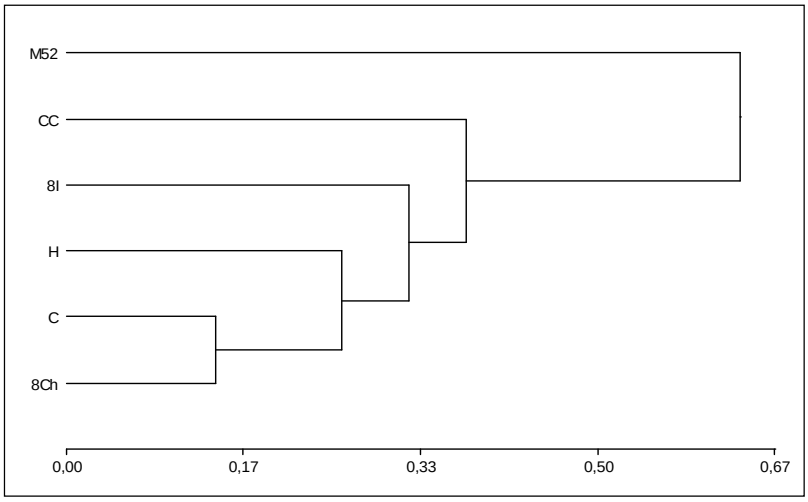

Fig. 2. UPGMA dendrogram of the five Hayward introductions and cultivar "M52"

It is reasonable to assume that the genetic variability found among introductions of Hayward was caused mostly by bud spontaneous mutations [1], and eventually, by mutations during the micropropagation stage [11]. The closest relationship between introductions "8Ch" and "C" (Fig. 4) could be explained by assuming that mutations occurred during the micropropagation of the clone 8 introduced from Italy to Chile ("8Ch") and gave rise to the clone "C". Particularly in the case of clones 8 from Italy and Chile, the distance between them (Table 2) was much higher than that expected considering clone "8Ch" as the result of successive agamic propagations of the original clone "8I". This result might be explained by the occurrence of undesirable mutations during the micropropagation stage, as discussed by [5,11]. Another explanation might refer to the mislabeling of plant material as "8Ch" when introduced in the nurseries. This fact appears to be occurring more often than what should be expected. Thus, Messina et al. [12] reported the presence of misidentified plants in imported materials and Prado et al. [13] identified some mislabeled Hayward clones in commercial orchards in Northeastern Spain. In accordance with these results, Ferguson [3] strongly advised that the identity of all materials entering a germplasm collection should always be checked and it should never be assumed that material is true to label. In this context, simple markers that can be used routinely in each micropropagation laboratory or nursery are extremely important.

In this investigation nine RAPDs markers were able to unequivocally differentiate all introductions of cultivar Hayward. It is worth recalling, at this point, that the same nine RAPDs markers were successfully used to distinguish among *Actinia* species and cultivars within *A. deliciosa* [7]. By using the same type of marker, Palombi and Damiano [11] detected somaclonal variation in micropropagated kiwifruit. Although the reproducibility is a technical limitation associated with the RAPDs markers which hinders their transferability among the laboratories, it has been demonstrated in this investigation that the RAPD approach continues being useful to analyze the variability within cultivars of *Actinidia deliciosa.* Currently, there is a number of examples of how RAPDs markers continue providing valuable information such as detection of somaclonal

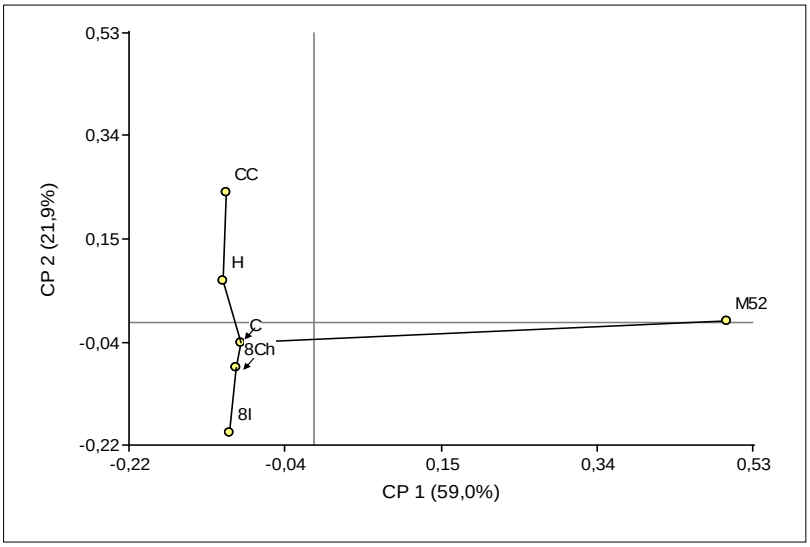

Fig. 3. Principal coordinates analysis and minimum spanning tree considering the five Hayward introductions and cultivar "M52"

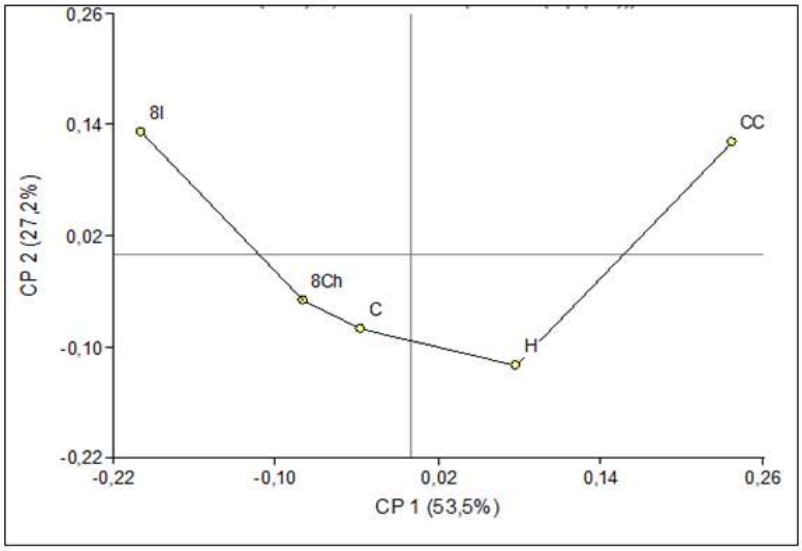

Fig. 4. Principal coordinates analysis and minimum spanning tree considering only Hayward introductions

variation in *Cereus peruvianus* [14], characterization of populations in *Echium italicum* [15] and pre-breeding material in *Medicago sativa* [16], assessment of genetic diversity in *Citrus* [17] and confirmation of the genetic fidelity of the regenerated plantlets and mother plant of *Aglaonema* 'Valentine' [18].

4. CONCLUSION

Clonal heterogeneity was detected within the cultivar Hayward. Therefore, RAPD approach is still a valuable alternative to analyze the variability within cultivars of *Actinidia deliciosa,* and eventually detect mislabeling and mutations of a given genotype when it is performed under well-controlled experimental conditions in each laboratory.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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