Genetic Diversity of Portuguese Autochthonous Apple Cultivars

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Abstract

Apple (***Malus × domestica* Borkh.**) is one of the most widespread and commercially important horticultural crops in the world. Nowadays, there is an increasing interest in autochthonous apple cultivars for organic farming as measure of environmental sustainable practice, once they are a privileged material that by natural selection acquired resistance to diseases and pests. Moreover, many of the regional cultivars have particular interesting organoleptic characteristics. This work constitutes the first molecular approach using ISSR markers on regional apple material from Northwest Portugal. A set of fourteen accessions representing autochthonous apple germplasm was studied using nine ISSR primers in order to estimate their genetic diversity and relationships. The obtained data enabled to assess the high genetic variability of the restricted number of Portuguese autochthonous apple material analysed, to uncover possible synonyms and intracultivar variability and the detection of possible identification errors.

INTRODUCTION

Apple (*Malus × domestica* Borkh.) is economically the most important tree fruit crop in moderate climate zones (Goulão and Oliveira, 2001) and has been cultivated for a long time, with references dating back to Roman times (Adebayo et al., 2009). Although more than 7000 cultivars have been described from different countries, world's production is based on a limited number of cultivars (Patzak et al., 2012), contributing to the standardization of cultivars and consequently to the loss of diversity.

In the beginning of the twentieth century, Portugal had a high number of traditional apple cultivars (Dinis, 2007). However, due to a series of technical, political and socio-economic constraints these varieties were replaced over time by others, more productive, usually imported. Nonetheless, there is still a great diversity of apple landraces, mainly in the rural areas from the northern regions of the country.

The traditional cultivars most protected from erosion are those with high commercial value, particularly, *Bravo de Esmolfe, Riscadinha de Palmela*, both dispersed throughout the country and *Porta-da-Loja*, mainly confined to the Minho region, in Northwest Portugal (Veloso et al., 2008). Currently, only the regional cultivars, *Bravo de Esmolfe* and *Riscadinha de Palmela,* are cultivated under designation 'Protected Denomination of Origin' (DOP).

An accurate identification, characterization and conservation are essential for apple breeding programs, not only for development of new apple cultivars with enhanced desirable traits, but also to support the diversification of the gene pool and preservation of unique genetic traits available in these regional cultivars. Portuguese regional apple germ-plasm is still blurred with too many synonymies and homonymies.

Molecular techniques have been complementing traditional characterization and several studies on apple were performed using markers such as RAPD (Goulão et al., 2001;

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Adebayo et al., 2009), ISSR (Goulão and Oliveira, 2001; Smolik and Krzysztoszek, 2010) and SSR (Foroni et al., 2012; Urrestarazu et al., 2012).

The main objective of this study was to assess the level of genetic variability, using ISSR marker, in a set of Portuguese autochthonous apple cultivars, and estimate the similarities among them.

MATERIALS AND METHODS

Fourteen accessions of autochthonous apple cultivars, sampled at different locations on Minho province, in Northwest Portugal, and installed in the apple collection of the Polytechnic Institute of Viana do Castelo (IPVC), were studied (Table 1). DNA extraction was carried out from woody shoots following the protocol of the NucleoSpin® Plant kit (Macherey Nagel, Duren, Germany). Nine primers that produced clear, reproducible and polymorphic bands were selected from the UBC#100/9 set (University of British Columbia, Vancouver, Canada). The reaction mixture for 20 µL contained 0.15 mM dNTPs, 2 mM $MgCl_2$, 20 ng template DNA, 0.5 µM of a single primer and 0.8U *Taq* DNA polymerase in the manufacturer's buffer (MBI Fermentas, Lithuania). The PCR program comprised an initial denaturation step (94°C/4 min.), followed by 35 cycles of 94°C/30 s, 52°C/45 s and 72°C/2 min, with a final 5 min. extension at 72°C. PCR amplifications were carried out using a T-Professional Basic (Biometra GmbH, Göttingen, Germany) thermocycler. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels using TBE buffer and stained with ethidium bromide. Fragment sizes were estimated by comparison to a DNA molecular weight ladder (GeneRuler™ 100 bp DNA Ladder Plus, MBI Fermentas, Lithuania).

For the genetic similarity analysis, PCR amplification bands were scored as present (1) or absent (0) to produce a binary ISSR dataset. Genetic similarity matrices among genotypes were calculated according to the simple matching (SM) coefficient and a dendrogram was constructed using the Unweighted Pair Group Method of the Arithmetic Averages (UPGMA), as implemented in the software package NTSYS-pc, version 2.02g (Rohlf, 1998).

RESULTS AND DISCUSSION

In the present study, ISSR molecular marker was used to analyze the genetic variability in accessions of Portuguese autochthonous apple cultivars. In some cases, for the supposedly same cultivar, accessions from different origins were analysed (Table 1). A total of 96 bands were amplified by nine ISSR primers, being 72 polymorphic (75%) (Table 2). The number of bands for each primer varied from seven (UBC 891) to 15 (UBC 810), with a mean of 10.7 bands per primer and the polymorphic bands ranged from three to 15, in the same primers (Table 2). An average of 2.7 exclusive bands was observed, revealing the UBC 889 primer the highest value (Table 2). The polymorphism ranged from 43.0% (primer UBC 891) to 100% (primers UBC 810, UBC 868 and UBC 873) (Table 2). The size of the amplified DNA fragments scored ranged from 200 to 2,500 bp, and UBC 848 primer revealed the highest amplitude of fragments size, from 290 to 2,500 bp (Table 2).

The dendrogram obtained based on SM similarity coefficient and using the UPGMA method, shows the discrimination of the accessions (Fig. 1). The 14 accessions studied have been clustered into two main groups, with a genetic similarity among them above 0.70. Although it may seem occur a narrow genetic pool in these regional apple cultivars it should be referred that for genetic similarity estimation all the bands (polymorphic and mono-morphic) were included, which increases the estimated genetic similarity values. Moreover, the geographic dispersion of the sampled accessions is quite limited and in some cases were included accessions supposedly corresponding to the same cultivar. Goulão and Oliveira (2001) in the analysis of 41 apple cultivars by seven ISSR primers detected a similarity coefficient between cultivars ranging from 0.71 to 0.92.

Two main groups were observed in the dendrogram (Fig. 1). One of them incorporated 78.5% of apple accessions and was composed by three clusters: in the first cluster Camoesa-de-Coura and Camoesa-Pedra accessions grouped with Verdeal; the

second cluster comprised three of the four Porta-da-Loja accessions analysed and the third cluster grouped accessions belonging to different cultivars (Espriega, Rajada Vermelha and Gilbarbela amarela) (Fig. 1).

Similarity values above 0.85 were observed inside groups of accessions with Camoesa (Camoesa-de-Coura/MTB-BRG and Camoesa-Pedra/ESAPL-PTL) and Porta-da-Loja (Porta-da-Loja RR/PTL, Porta-da-Loja AF/VVD and Porta da Loja P/TBR) designations, showing intracultivar variability. The remaining Porta-da-Loja accession, Porta-da-Loja/AF-TBR, was found in a different cluster, suggesting that this accession could be misidentified (Fig. 1). Results also suggest that Gilbarbeda accessions, Gilbarbeda-branca and Gilbarbeda-amarela, that differ in pulp colour (white and yellow, as the name indicates), may correspond to different cultivars (Fig. 1). Synonymies and homonymies are far from being completely clarified in apple as occurs in other woody species. Recently, Foroni et al. (2012) in 200 samples of apples cultivated in Azores islands, corresponding to regional cultivars, identified 60 synonyms and 32 homonyms, using ten SSR primers. Also Bassil et al. (2009) found synonyms and homonyms inside a group of 17 samples of apple and pear from the Azorean Terceira Island by use of nine SSR primers.

The variability analysis using ISSR markers allies the easiness of the procedure to the advantage of not need previous knowledge of the genome sequence to be amplified. So, their use in a first screen, in order to detect duplicated material, erroneous identifications or synonyms and homonyms identification, is recommendable. In the present study, the analysis of fourteen regional apple accessions from the Northwest of Portugal by nine ISSR primers allowed to identify a putative misidentification in one Porta-da-Loja accession and a synonym between two Camoesa accessions.

This study will proceed with the analysis of other molecular markers, particularly microsatellites, and will be extended to a broad number of regional cultivars accessions from different collections dispersed all over the country.

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Tables

Table 1. Plant material studied with respective designation and origin.

Accession	Origin (Municipality)
Camoesa-de-Coura/MTB-BRG	Quinta de Carcavelos (Braga)
Camoesa-Pedra/ ESAPL-PTL	Lourido (Ponte de Lima)
Espriega/AS-TBR	Moimenta (Terras de Bouro)
Gilbarbeda-amarela/AS-TBR	Moimenta (Terras de Bouro)
Gilbarbeda-branca/AS-TBR	Moimenta (Terras de Bouro)
Porta-da-Loja/RR-PTL	Quinta do Assento (Ponte de Lima)
Porta-da-Loja/AF-VVD	Lugar de S. José (Vila Verde)
Porta-da-Loja/AS-TBR	Costa do Além (Terras de Bouro)
Porta-da-Loja/P-TBR	Lajes (Terras de Bouro)
Perna-de-Pisco/AF-VVD	Quinta S. José (Vila Verde)
Rajada-vermelha/AS-TBR	Moimenta (Terras de Bouro)
Sangue-de-Boi/AS-TBR	Moimenta (Terras de Bouro)
Verdeal/ ESAPL	Quintela (Celorico de Basto)
Vermelha-de-Refóios/ ESAPL-PTL	Escola Superior Agrária- IPVC (Ponte de Lima)

Table 2. Number of total (TB), polymorphic (PB) and exclusive bands (EB), percentage of polymorphism (%P) and band sizes (bp) obtained with nine ISSR primers.

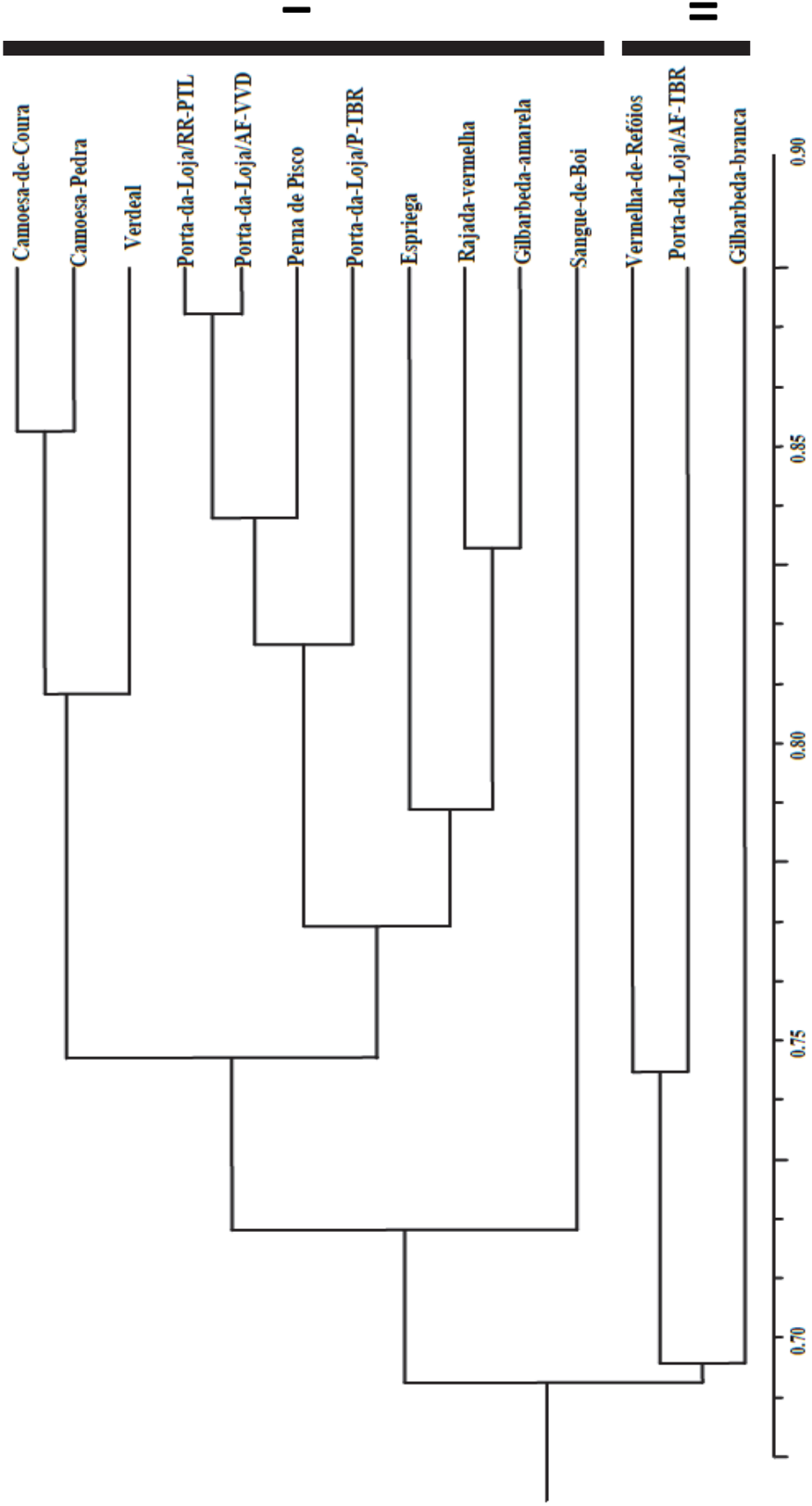| Primer	TB	PB	EB	%P	Size (bp)
UBC 810	15	15	0	100	290-1200
UBC 845	9	5	4	56	250-1800
UBC 848	14	11	3	79	290-2500
UBC 868	9	9	0	100	450-1500
UBC 873	9	9	0	100	330-1500
UBC 880	11	8	3	73	200- 900
UBC 889	11	5	6	45	300-1200
UBC 890	11	7	4	64	290- 900
UBC 891	7	3	4	43	350-1000
Total	96	72	24		
Mean	10.7	8.0	2.7	73.2	

Figures

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Fig. 1. UPGMA dendrogram based on SM similarity coefficient, representing phenetic relationships among the fourteen Portuguese apple accessions analysed by ISSR markers.