Characterization and differentiation of old *Camellia japonica* cultivars using simple sequence repeat (SSRs) as genetic markers

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INTRODUCTION

The ornamental camellia most widely cultivated is *Camellia japonica*, which comprises thousands of cultivars. Specimens of different cultivars are present in many historical gardens all over the world and have become elements of cultural heritage (Salinero et al., 2003, Accati et al., 2003) some of them are regarded as notable trees and are protected by special preservation orders (Rodríguez-Dacal and Izco, 1994; Decree 67/2007 of the Xunta de Galicia).

In the gardens of manor houses in Galicia (NW Spain) and Portugal grow camellias more than one century old, in most cases unidentified, even though the appropriate identification of genetic resources of ornamental cultivars of *C. japonica* is crucial for management of the garden collections. Thus, in the last years the specimens of artistic and historic interest growing in these gardens have been catalogued (Salinero and Vela, 2004; Salinero et al. 2007). Characterization was carried out using morphobotanic descriptors selected according to recently proposed standardized morphobotanical tests for the characterization of ornamental cultivars of the genus *Camellia* (Accati et al., 2000; Remotti, 2002; Salinero and Vela, 2004; Jiyuan et al. 2008). Specimens were compared to the information included in the references of the International Camellia Register (ICR) (Savige, 1993), and also to camellia drawings of old camellia publications and ancient nursery catalogues. When possible, plants were also compared to specimens belonging to different camellia collections, regarded as reference specimens on the basis of the existing written information about them.

On this basis, two groups of plants were first established, those assigned to cultivar 'Bella Romana' and those ascribed to 'Prince Eugene Napoleon' ('Pope Pius IX'), both originated in Belgium in the 19th century. 'Bella Romana'presents large flowers, petals mostly imbricated and arranged in several rows, pink marked with numerous streaks of crimson (Figure 1). 'Prince Eugene Napoleon' has large flowers, with numerous small vivid cherry petals, round and imbricated, (Figure 2).

Figure 1. L'Ilustration Horticole.1863, vol. 10

However, the identification of these plants casted some doubts. A number of morphological traits showed some variability among specimens grouped in the same cultivar, and this variability was also observed within a single plant. Morphological characteristics can be influenced by subjective visual assessment. Also environmental conditions can cause significant variability in features as the size, color or shape of the flower (Trehane, 2007). Considering this, it was not possible to establish

classes discriminatory and reliable for several morphological traits in these two groups of plants. In the last decade, the development of DNA simple sequence repeat (SSR or microsatellite DNA) as genetic markers in genus *Camellia* has provided a direct study of genotype (Ueno *et al.* 1999; Freeman *et al.* 2004; Abe et al. 2006; Hung et al. 2008; Zhao et al. 2008; Ueno and Tsumura 2009; Yang et al. 2009).

Figure 2: Verschaffelt, 1858-59, Nouvelle Iconographie, vol. 6

In this way, genetic structure of *C. japonica* Japanese old growth forest was investigated by Ueno *et al.* (2000, 2002) who identified wild individuals using four DNA microsatellite markers. But the cultivars with ornamental interest have never been investigated with microsatellite markers. DNA microsatellites markers add an important complement to the morphobotanic descriptors that can be used for cultivars identification since they are independent from environment. So combining the genetic profile with morphobotanic descriptors might implement the characterization of the ornamental camellia cultivars.

The aim of the present work was to characterize and differentiate two groups of ancient specimens of *Camellia japonica* grown in gardens of Galicia and Northern Portugal using microsatellite genotyping, that initially were assigned to the cultivars 'Bella Romana' and 'Prince Eugene Napoleon' by morphobotanic descriptors.

MATERIALS AND METHODS

Plant material consisted in specimens of *C. japonica* grown in several gardens in Galicia and northern Portugal where cataloging work has been made in recent years (Table 1). Camellia plants were assigned to two groups according to their morphological similarity: Group 1 'Bella Romana' and Group 2 'Prince Eugene Napoleon'. First plants were compared in each group among themselves, and then with reference plants of cultivars 'Bella Romana' and 'Prince Eugene Napoleon' grown in Galicia gardens. For molecular markers analysis, in each plant, DNA was isolated from 50 mg of leaves with EZNA HP Plant DNA Kit according to the manufacturer protocol (Omega Bio-Tek). A set of fourteen polymorphic DNA microsatellites sequences developed in *C. japonica* (Ueno et al., 1999; Abe et al., 2006) and *C. sinensis* (Freeman et al., 2004; Hung et al., 2008; Zhao et al., 2008) was tested (Table 2). DNA fragments were amplified using the tailed primer method (Shuelke, 2000). Polymorphisms of 14 microsatellite loci were assessed in a maximum of 95 plants from the living camellia collection of *Diputación Provincial de Pontevedra*. The reactions were performed in 25 µL total volume containing 50 ng of genomic DNA. Cycling parameters were as follows: 5 min at 95ºC, 25 cycles for 1min at 95ºC, 45 s of hybridization with corresponding Tm (Table 2), 1 min at 72ºC, 20 cycles for 1 min at 95ºC, 45 s of hybridization at 55ºC Tm, 1 min at 72ºC and a final step of 60 min at 72ºC. The fluorescent dye labels used were 6-FAM, NED, PET and VIC (Applied Biosystems). A volume of 1 µL of amplification products were added to 20 µL of formamide and 0.3 µL of Genescan 500 LIZ size standard. Mixed solution was denatured at 95ºC for 3 min. The samples were run on ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Allele scoring was performed using the GeneScan 3.5 (Applied Biosystems).

Table 1. Codes of the camellia specimens characterized in this work together with the location, municipality and the cultivar name abscribed by means of morphological descriptors.

Specimen	Location	Municipality	Cultivar
C0266	Vicenti garden	Pontevedra ((NW Spain))	'Prince Eugene Napoleon'
C0268	Fine Arts School garden	Pontevedra ((NW Spain))	'Prince Eugene Napoleon'
C0275	Herrería Square garden	Pontevedra (NW Spain)	'Prince Eugene Napoleon'
34 Mar	Pazo de Mariñan	Bergondo (NW Spain)	'Prince Eugene Napoleon'
578	Jardim Botânico do Porto	Porto (Portugal)	'Prince Eugene Napoleon'
C0277	Marín City Hall garden	Marín (NW Spain)	'Prince Eugene Napoleon'
C0106	**Pazo de Gandarón**	**Pontevedra (NW Spain)**	'Prince Eugene Napoleon' **reference specimen**
C0253	Pazo de Lourizán	Pontevedra (NW Spain)	'Bella Romana'
C0269	Pazo Quiñones de León	Vigo (NW Spain)	'Bella Romana'
S04	Soutomaior Castle	Soutomaior (NW Spain)	'Bella Romana'
116	Soutomaior Castle	Soutomaior (NW Spain)	'Bella Romana'
287	Estación Fitopatolóxica do Areeiro	Pontevedera (NW Spain)	'Bella Romana'
C0084	Liceo Casino de Pontevedra	Poio (NW Spain)	'Bella Romana'
176	**Estación Fitopatolóxica do Areeiro**	**Pontevedra (NW Spain)**	'Bella Romana' **reference specimen**

Table 2: DNA analysis of different camellia cultivars from living collection of *Diputación Provincial de Pontevedra* for 14 microsatellite loci. Allele size range, number of alleles detected (*A*), observed (*Ho*) and expected (*He*) heterozygosities.

Locus	Repeat motif	Tm (ºC)	Alleles size (bp)	*A*	*Ho*	*He*
MSCjaF25	$(CA)_8(AAAAAT)_4$	46	213-245	12	0.65	0.71
MSCjaH38	$(GA)_{14}$	55	344-370	10	0.69	0.87
MSCjaF37	$(AG)_{13}(GAA)_7$	58	343-362	8	0.78	0.89
MSCjaH46	$(GA)_{16}$	60	443-461	6	0.34	0.56
Ca01	$(CAG)_6...(CAG)_5$	55	157-176	4	0.18	0.48
Ca08	$(CT)_{10}$	55	154-187	6	0.52	0.73
MSCjaR02	$(CT)_8...(CT)_{11}$	55	200-224	6	0.56	0.77
Ca06	$(GT)_{12}(GA)_{10}$	55	237-262	8	0.67	0.78
Z641	$(AGAGA)_3$	55	149-209	4	0.22	0.45
Z496	$(AG)_{11}$	64	102-122	4	0.23	0.41
CamsinM5	$(GT)_{15}(GA)_8$	64	206-224	7	0.53	0.77
MSCjaQ11	$(GA)_{13}$	60	219-248	6	0.47	0.70
MSCjaT25	$(GA)_4...(GA)_{18}$	60	172-193	4	0.29	0.43
CamsinM11	$(CA)_{12}$	60	173-182	4	0.32	0.59

He= $1-\Sigma\ p_i^2$; p_i= frequency of the i^{th} allele

Ho= number of heterozygotes detected / total number of plants assessed

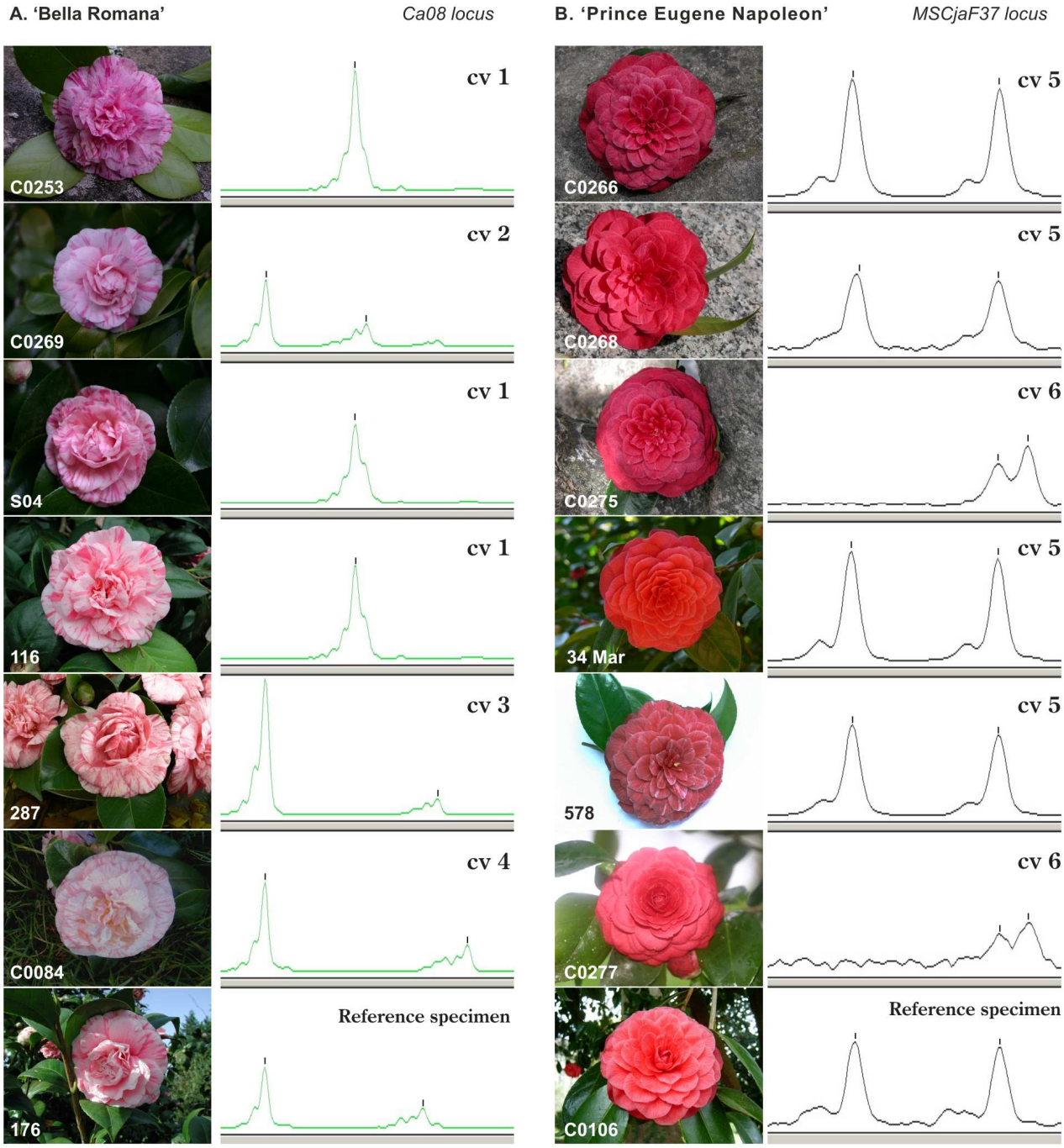

Figure 3. Flowers of the ancient camellia plants included in Group 1 'Bella Romana' (A) and Group 2 'Prince Eugene Napoleon' (B). Beside each specimen photograph there is a graph showing the allelic profile of the microsatellite which summarizes the genetic variation detected in the group, Ca08 in 'Bella Romana' and MSCjaF37 as regards 'Prince Eugene Napoleon'. Group 1 comprises 4 cultivars of *C. japonica* (cv1, cv2, cv3 and cv 4). Note that the profile of the studied cultivars of Group 1 is different from the reference cultivar 'Bella Romana' (176); as regards Group 2, there are 2 different camellia cultivars (cv 5, cv 6). Cultivar 5 is similar to the reference specimen of the cultivar 'Prince Eugene Napoleon'.

RESULTS

Group 1 'Bella Romana'

The genotyping of the six cultivars identified as 'Bella Romana' with 14 microsatellites revealed that they were 4 different cultivars of *C. japonica*. Cultivar cv1 comprises specimens C0253, S04 and 116, showing an identical genetic profile for the 14 microsatellites used. Each specimen C0269, 287 and C0084 corresponded to a different cultivar (cv2, cv3 and cv4, respectively) since their genetic profiles are different from each other and also from cv1. All studied specimens had a genotype different from the reference cultivar 'Bella Romana' (176). In group 1 the microsatellite named Ca08 covers all the genetic variability detected using the 14 microsatellites, since by itself is able to differentiate the studied cultivars and also separate them from the reference cultivar (Figure 3A).

Group 2 'Prince Eugene Napoleon'

The genetic profile of the six specimens belonging to Group 2 'Prince Eugene Napoleon' was also obtained with the selected microsatellites (Table 2). They revealed that they were two different cultivars of *C. japonica*. The first cultivar (cv5) grouped specimens C0266, C0268, 34Mar and 578 having the same results using the 14 microsatellites. The second cultivar comprises C0275 and C0277 (cv 6), having identical genetic profile but they differ from cultivar 5 in microsatellites MSCjaF25, MSCjaH38, MSCjaF37, MSCjaR02 and Ca06. In Group 2 specimens of cultivar 5 show the same genotype than the reference cultivar 'Prince Eugene Napoleon' (C0106). In Group 2 the microsatellite MSCjaF37 summarizes all the genetic variation detected using the 14 microsatellites; it is able to separate cultivars 5 and 6 and shows that cultivar 5 corresponds to the reference specimen 'Prince Eugene Napoleon' (Figure 3B).

DISCUSSION

Since the 19th century camellias have been extensively used in gardening and they have increasingly been gaining importance in the last years. At present, some of the specimens that were planted at that time they are still growing in the historical gardens, however there is not available information either of the origin or the name of the cultivar. The cataloguing of this material is a very difficult task even for technicians having an extensive experience and knowledge on camellias. In particular, there is not reliable information on the reference specimens of the two groups studied: 'Bella Romana' and 'Prince Eugene Napoleon'. These reference specimens were identified at the end of the 20th century by one of the pioneers in camellia cultivar cataloguing, Mr. Antonio Odriozola (Odriozola, 1986).

Molecular techniques have suceed in caracterising individuals in varied groups of organisms. In different plant genus have proved to be useful and complementary tools to morphobotanic descriptors for the characterization of cultivars (Varshney et al. 2005). As regards the specimens studied in the present work, the microsatelite markers have revealed a genetic varibility higher than using the morphobotanic descriptors alone. This fact points out the need to complement morphobotanic descriptors with microsatellite genetic markers, since they enhance the possibilities of discrimination among cultivars and even they would enable the identification of plants in growth stages different from the blooming period. However, due to their genetic variability that could be masked by the similarity of morphobotanic descriptors among the different cultivars, it is necessary to establish reference specimens to guarantee a proper and accurate identification.

The use of these microsatellite genetic markers is of special interest in those cultivars difficult to differentiate, such as these having formal double white flowers ('Rainha Santa Isabel', 'Alba Delecta', 'Alba Imbricata'), those double red-pink and a white band in the middle of the petal ('Cruciata', 'Bella di Firenze', 'Arciduca Carlo') as well as others being very similar in appearance.

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