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Original scientific paper

THE MOLECULAR CHARACTERIZATION OF AN EXTENDED MULBERRY GERMPLASM BY SSR MARKERS

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The objective of this work is the molecular characterization using nuclear SSR markers of different mulberry (*Morus alba* L.) cultivars from different countries including Cuba, Costa Rica, Brazil, South Korea, Ethiopia, China, Japan, Italy and Spain to deep in the dissemination through the world of this species. Results established the value of SSR markers for distinguishing different genetic lineages and characterize an extensive and largely gene pool available to mulberry cultivars. The results revealed the presence of 53 different alleles from the 12 SSR analysed in the 37 assayed genotypes. The Japanese, Italian and Spanish cultivars formed a separated group in which only 5 genotypes were present in the Cuban germplasm bank. On the other hand the Cuban cultivars 'Cuba 2' and 'Cuba 3' propagated from Chinese parents were grouped in the same branch, which

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suggests that these cultivars retain the genomic characteristics of their selection. It was also possible to relate the genotypes of cultivars 'Tigriada' and 'Acorazonada' present in Costa Rica as well as to evaluate other cultivars for their use in silkworm feeding. Molecular results also evidenced the dissemination of the cultivated mulberry specie from China around the world, the introduction in South Korea and Japan in the first dissemination step and the later wide dissemination of modern mulberry to the Mediterranean area and later to South America and the Caribe area.

Keywords: Dissemination, Genetic diversity, Microsatellites, *Morus alba*, Origin

INTRODUCTION

Mulberry, (*Morus* L., Moraceae), is a deciduous, perennial tree, growing wild and under cultivation in many temperate regions in the world. Mulberry is believed to have originated in the northern hemisphere, particularly in the Himalayan foothills, and spread to the tropics of southern hemisphere in China and Japan (HOU, 1994; VIJAYAN *et al.*, 2011). In addition, recent molecular studies also revealed an early diversification of Moraceae in Eurasia and subsequent migration into the southern hemisphere to South America (ZEREGA *et al.*, 2005). However these results are not conclusive. VIJAYAN *et al.* (2011) suggested two main ways of dissemination, one to Korea and Japan and other to India, Persia, Europe and South and North America. To date the dissemination way of this important species through the world remains unclear. In addition, the taxonomy of *Morus* is also complex and disputed. Over 150 species names have been published, and although differing sources may cite different selections of accepted names, only 10–16 are generally cited as being accepted by the vast majority of botanical authorities (TIKADER *et al.*, 2008a; 2008b; 2009). Morus classification is even further complicated by widespread hybridization, wherein the hybrids are fertile. Of all the species, only *M. alba* is relevant for sericicultural purposes, and as a consequence, many efforts directed to the collection and improvement of its germplasm have been made in Asiatic countries.

The importance of these species arises from the fact that the leaves of the species *Morus alba* L. are the only food source of the silkworm *Bombyx mori* L. As a consequence, this species is an integral part of the sericicultural activity, still very important in countries from Asia, such as China, India, Bangladesh, Pakistan and others (VIJAYAN *et al.*, 2011). The mulberry fruit is an edible, multiple fruit, 2–3 cm long. Immature fruits are white, green, or pale yellow. In most species, the fruits turn pink then red while ripening, then dark purple or black and have a sweet flavour when fully ripe. In many countries like Turkey and Greece, mulberries are grown also for fruit production (ERCISLI, 2004; ERCISLI and ORHAN, 2007). Leaves of *M. alba* have a considerable nutritional value and proteins of high quality. They are used as a food supplement mixed with flour in India, and as a ruminant foodstuff (ERCISLI and ORHAN, 2007). A more recent but promising utilization of mulberry is the application of its products in the field of medicine. In this aspect, ethanolic and aqueous extracts of leaves and bark have showed some effects on control of hyperglycemia, skin hyperpigmentation, inflammation, atherosclerosis and neurodegeneration (BUTT *et al.*, 2008; VENTAKESH-KUMAR and CHAUHAN, 2008).

Regarding the situation in Europe, the sericicultural activity is active but quite reduced (as in the Balkan countries) or completely lost except for research (as in France, Italy and Spain). However, in these countries there are still germplasm collections of *M. alba*. These collections were formed mostly along the XIX and XX centuries, when silk industry was an important economic resource and specific institutions were developed for providing advanced technology

for the improvement of the industry (GONZÁLEZ-MARÍN, 2001). In the European context, Spain was one of the main producers of silk during the period of XVI to XX centuries. The activity was introduced by the Arabs and was mainly concentrated in the Mediterranean Coast, from Valencia to Granada. After the epidemic of *Nosema bombycis* of the 1850s that devastated most of the European industry, silk production in Spain remained concentrated in the Region of Murcia. However, the activity ended in the 1970s as a result of the lack of competitiveness in relation to silk produced in Asia (GONZÁLEZ-MARÍN, 2001). The predominant cultivars that could be considered more ancient in Spain are 'Valenciana precoz', 'Valenciana rizada', 'Cristiana', and 'Filipina'. All these cultivars produce leaves very apt for silkworm feeding and are tolerant to the dry and warm conditions of the climate of Southeast Spain (GONZÁLEZ-MARÍN, 2001). In Cuba, introduction of *M. alba* is more recent, in middle of the last XX century, with and interesting perspectives as viable alternative for animal and silkworm feeding (GARCÍA *et al.*, 2006; MARTÍN *et al.*, 2007).

Although the profitable production of textile silk is not possible in Spain or other European countries a new interest for sericiculture as arisen as a consequence of the new applications of silk biomaterials in the field of biomedicine (WANG *et al.*, 2006). This is now the situation of Cuban silk production with a recent introduction of *Morus* germplasm (GARCÍA *et al.*, 2006). In addition to this, *M. alba* products have a clear interest in the field of nutrition and health, based on a wide scientific literature. Consequently, the analysis or the traditional *M. alba* germplasm still in collection could provide a basis for sustaining the development of this group of new applications.

Apart of the traditional botanical, pomological and agronomical analysis, the field of breeding and collection of plant germplasm has been extremely benefited by the development of molecular markers. During the last two decades, a wide set of DNA-based technologies have been developed and applied to every wild or cultivated plant group. This application includes also the genus *Morus*, in order to document its genetic diversity (VIJAYAN *et al.*, 2004; 2005; 2006b; TIKADER *et al.*, 2009), its phylogeny (VIJAYAN *et al.*, 2004b) and its genome organization (KHURANA and CHECKER, 2011; VIJAYAN, 2010). Apart from preliminary studies with isozyme variation (RAO *et al.*, 2011), the genetic diversity of *Morus* populations has been analysed with techniques such as random amplified polymorphic DNA (RAPD) (IPECK *et al.*, 2012; ORHAN and ERCISLI, 2010; ORHAN *et al.*, 2007), sequence-related amplified polymorphism (SRAP) (ZHAO *et al.*, 2010), inter-simple sequence repeat (ISSR) (IPEK *et al.*, 2012; VIJAYAN and CHATTERJEE, 2003; VIJAYAN *et al.*, 2006a; WEIGUO *et al.*, 2006; 2007) and amplified fragment length polymorphism (AFLP) (SHARMA *et al.*, 2000; KAFKAS *et al.*, 2008).

All the mentioned markers are suitable for variability studies of wild populations. However, some of them are codominant, show low reproducibility or are technically complex. As a consequence, the technique of simple sequence repeat (SSR) (POWELL *et al.*, 1996; VENKATESWARLU *et al.*, 2006; VIJAYAN *et al.*, 2006b) based on the characterization of variation in genomic microsatellite regions is usually preferred. The application of SSR markers requires the previous characterization of a set of microsatellite loci of the genome of the species under analysis. However after the work of AGGARWAL *et al.* (2004) that characterized 6 microsatellite loci, and ZHAO *et al.* (2005), that described 10 loci specific of Morus, it is possible to make a complete description of Morus variability, genetic relationships and pedigrees with this type of marker. A complete linkage map of mulberry has been already developed through the integration of RAPD, ISSR and SSR markers (VENTAKESWARLU *et al.*, 2006), which is an invaluable tool

for future breeding programs in the genus. SSR have been assayed in the introduction and spread of cultivated mulberry in the Indian subcontinent (KRISHNAN *et al.*, 2014a). More recently, similar molecular markers based on specific PCRs has been applied to mulberry characterization including inter-- Simple Sequence Repeat (ISSR) markers (KALPANA *et al.*, 2012; KRISHNAN *et al.*, 2014b; BANERJEE *et al.*, 2016) and sequence-related amplified polymorphism (SRAP) markers (BAJPAI *et al.*, 2014; HU *et al.*, 2015). SSRs, however, continue being the marker of choice in the molecular characterization of *Morus* species. These SSRs are being identified from expressed sequences using massive sequencing (CHECKER *et al.*, 2012; MATHITHUMILAN *et al.*, 2013; DAI *et al.*, 2015; SAEED *et al.*, 2016).

The objective of the present work is to make a genetic characterization by SSR markers of a collection of 37 *M. alba* genotypes from nine different countries including Cuba, Costa Rica, Brazil, South Korea, China, Japan, Italy, Ethiopia and Spain to clarify the dissemination of this cultivated species though the world.

MATERIALS AND METHODS

Plant materials and DNA extraction

Thirty seven mulberry genotypes from nine different countries including Cuba, Costa Rica, Brazil, South Korea, China, Japan, Italy, Ethiopia and Spain were included in the assay (Table 1; Figure 1). These genotypes are maintained at the Sericulture Program of CENSA (Havana, Cuba) and IMIDA of Murcia (Spain). Two g of leaf samples were powdered in liquid nitrogen to extract the DNA. Genomic DNA extraction was performed following the CTAB method described by DOYLE and DOYLE (1990). The concentration of DNA was investigated by measuring absorbance at 260 nm using a spectrophotometer (Bio- Photometer 6131, Eppendorf, Germany). DNA was quantified using a Biophotometer (Eppendorf, Barcelona, Spain).

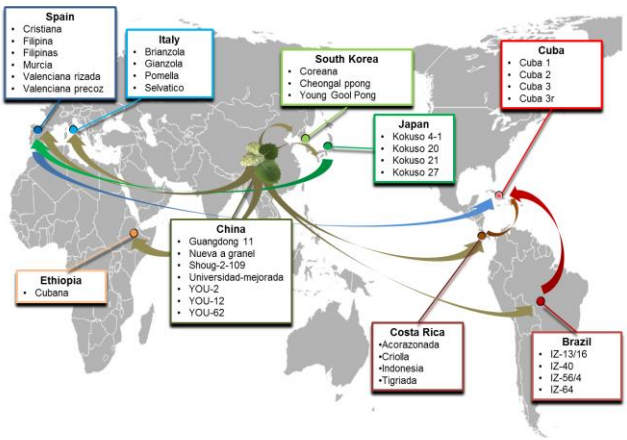

Figure 1. Dendrogram obtained by NJ cluster analysis based on the mean character difference distances among mulberry genotypes. Numbers below branches represent bootstrapping values. The scale bar represents simple matching distance.

Table 1. Origin and pedigree of the 37 mulberry genotypes assayed.

Genotype	Origin	Germplasm bank[1]	Year of introduction	Nature
Acorazonada	Costa Rica	SP-Cuba	1995	Traditional cultivar
Brianzola	Italy	IMIDA-Spain	1920	Traditional cultivar
Cheongal ppong	South Korea	SP-Cuba	2005	Traditional cultivar
Coreana	South Korea	SP-Cuba	2005	Traditional cultivar
Criolla	Costa Rica	SP-Cuba	1995	Traditional cultivar
Cristiana	Spain	IMIDA-Spain	S. XIX	Traditional cultivar
Cuba 1	Cuba	SP-Cuba	1990	Seedlings
Cuba 2	Cuba	SP-Cuba	1990	Seedlings
Cuba 3	Cuba	SP-Cuba	1990	Seedlings
Cuba 3r	Cuba	SP-Cuba	1990	Seedlings
Cubana	Ethiopia	SP-Cuba	1990	Clone (traditional cultivar)
Filipina	Spain	IMIDA-Spain	1920	Traditional cultivar
Filipinas	Spain	SP-Cuba	1995	Traditional cultivar
Gianzola	Italy	IMIDA-Spain	1920	Traditional cultivar
Guangdong 11	China	SP-Cuba	2011	Traditional cultivar
Indonesia	Costa Rica	SP-Cuba	1995	Traditional cultivar
IZ-13/6	Brazil	SP-Cuba	2000	Cultivated hybrid
IZ-40	Brazil	SP-Cuba	2000	Cultivated hybrid
IZ-56/4	Brazil	SP-Cuba	2000	Cultivated hybrid
IZ-64	Brazil	SP-Cuba	2000	Cultivated hybrid
Kokuso 4-1	Japan	IMIDA-Spain	1920	Clone (traditional cultivar)
Kokuso 20	Japan	IMIDA-Spain	1920	Clone (traditional cultivar)
Kokuso 21	Japan	IMIDA-Spain	1920	Clone (traditional cultivar)
Kokuso 27	Japan	IMIDA-Spain	1920	Clone (traditional cultivar)
Murcia	Spain	SP-Cuba	2011	Clone (traditional cultivar)
Nueva a granel	China	SP-Cuba	2011	Clone (traditional cultivar)
Pomella	Italy	IMIDA-Spain	1920	Traditional cultivar
Selvatico	Italy	IMIDA-Spain	1920	Traditional cultivar
Shoug-2-109	China	SP-Cuba	2011	Clone (traditional cultivar)
Tigriada	Costa Rica	SP-Cuba	1995	Traditional cultivar
Universidad-mejorada	China	SP-Cuba	2011	Clone (traditional cultivar)
Valenciana precoz	Spain	IMIDA-Spain	S. XIX	Traditional cultivar
Valenciana rizada	Spain	IMIDA-Spain	S. XIX	Traditional cultivar
YOU-12	China	SP-Cuba	2011	Cultivated hybrid
YOU-2	China	SP-Cuba	2011	Cultivated hybrid
YOU-62	China	SP-Cuba	2011	Cultivated hybrid
Young Gool Pong	South Korea	SP-Cuba	2005	Traditional cultivar

[1]SP: Sericulture Program, Havana, Cuba

SSR analysis

Extracted mulberry DNA was PCR-amplified using 12 nuclear SSR markers of primers flanking SSR sequences previously characterized and developed for *Morus* (AGGARWAL *et al.*, 2004) (Table 2). These SSRs are also referenced in the online database for mulberry microsatellites (MulSatDB) (http://btismysore.in/mulsatdb/) (KRISHNAN *et al.*, 2014c). PCR reactions were performed in a 25 µl volume and the reaction mixture contained 16 mM $(NH4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 2 mM MgCl2, 0.2 µM of each primer, 0.1 mM of each dNTP, one unit of Taq DNA Polymerase (Ecogen S.R.L.), and 90 ng of genomic DNA. Cycling parameters were: one cycle of 95°C for 3 min.; 35 cycles of 94°C for one min., annealing temperature for one minute, and 72°C for one min; followed by 10 min at 72°C. PCR reactions were carried out in a 96-well block Eppendorf Mastercycler. Amplified PCR products were separated depending on the differences in the sizes of the segregating alleles: if the difference was more than 5 bp we used 3% Metaphor® Agarose gel electrophoresis (Biowittaker, Maine, USA) stained with ethidium bromide (0.5 µg/ml), and visualized under UV light.

Data analysis

Polymorphic alleles were scored as present or absent (1/0). Band scoring was analysed using the GeneTools gel analysis software (SYNGENE, Cambridge, UK). Expected genetic heterozygosity (He) was calculated as 1-∑pi2 where pi is the frequency of the ith allele (NEI, 1978). Observed genetic heterozygosity (Ho) of each genotype or SSR marker was calculated as the number of heterozygous genotypes divided by the total number of markers or genotypes. Power of discrimination of each SSR marker was calculated as PD=1-∑gi2 where gi is the frequency of the ith genotype (KLOOSTERMAN *et al.*, 1993; VIJAYAN *et al.*, 2006b). Mean character difference distances were calculated for all pairwise comparisons with MEGA 6.06 software (TAMURA *et al.*, 2013), which was used to construct neighbor-joining (NJ) dendrogram depicting the phenetic relationship among genotypes. Relative support for the branches in each dendrogram was assessed by NJ bootstrap analysis (2000 replicates). Genetic distance estimation was also performed using the Maximum Composite Likelihood (MCL) method (TAMURA *et al.*, 2004).

RESULTS AND DISCUSSION

Polymorphism and heterozygosity of SSR markers

The results revealed the presence of 68 different alleles in the 37 assayed genotypes (Table 2). Twelve SSR primer pairs developed for mulberry and representing different regions of its genome were tested in 37 accessions of *M. alba* from the IMIDA and CENSA collections. In three (SS01, SS06 and SS17) of these loci no amplifications were observed. The other nine primer pairs had different levels of amplified bands, the size of which ranged from 113 to 398 bp. The number of alleles observed at each locus ranged from 2 (SS19) to 19 (MulSTR3), with a total of 68 for the nine loci and an average of 7.55 bands per locus. In all loci, the effective number of alleles was lower than observed, and varied from 1.177 (MulSTR2) to 5.415 (MulSTR3) (Table 2). These differences between the number of effective and observed alleles indicate the presence of rare alleles that exist in a few genotypes and could be used for their identification.

The observed of SSR markers heterozygosity ranged from 0.0 (SS19) to 0.91 (MulSTR3), with a mean of 0.52. The Power of Discrimination values were positive for all loci. The most informative locus was MulSTR3, with a PD of 0.99 and the least one was SS19 with a PD of 0.28 (Table 2). The number locus ranged from 2 (SS19) to 19 (MulSTR3) with a total of 68 for the nine loci.

Table 2. Simple sequence repeat (SSR) markers assayed and polymorphism obtained in the bulñberry genotypes assayed.

SSR marker	Nº of Alleles[1]	Size range	Heterozygosity	Power of discrimination
MulSTR1	8	160-212	0.86	0.82
MulSTR2	7	191-206	0.67	0.77
MulSTR3	19	113-398	0.91	0.99
MulSTR4	3	125-144	0.86	0.57
MulSTR5	6	160-190	0.53	0.73
MulSTR6	7	136-172	0.31	0.69
SS01	na	--	--	--
SS04	8	187-227	0.35	0.75
SS06	na	--	--	--
SS17	na	--	--	--
SS19	2	342-347	0.00	0.28
SS20	8	295-346	0.21	0.68
Average	*7.55*	*113-398*	*0.52*	*0.72*

[1]na: No Amplification

The assayed genetic pool has been isolated from the main genetic pool used in Asia and other countries for mulberry breeding projects. The cultivars and genotypes from Spanish origin, after centuries of selection, can provide interesting traits for palatability of leaves for feeding the silkworm, and also for rusticity and tolerance to dry and hot Mediterranean climates. As a consequence, this is a genetic material worth to consider for such breeding projects, and further studies on its genetic relationship with a wider collection of genotypes of international origin is justified.

In addition, the high molecular diversity observed using SSR and ISSR has also been described in the analysis of mulberry cultivars from South Korea and India (KALPANA *et al.*, 2012; KRISHNAN *et al.*, 2014a; BANERJEE *et al.*, 2016) and wild mulberry populations from India and China using SRAP markers (BAJPAI *et al.*, 2014; HU *et al.*, 2015). The propagation system through seed (GARCÍA *et al.*, 2006) should be the responsible of this great diversity.

Genetic relationships within the mulberry collection

The genetic relationships among the 37 mulberry accessions appear depicted in the dendrogram of the Figure 2. The whole dendrogram could be roughly subdivided in three main groups, designed from A to C, subdivided in seven subgroups. The group more distant of the rest (C2) is constituted by three Brazilian accessions together with Chinese cultivar 'Nueva a granel'. This is a singular group, clearly separated from the rest and close to the group of accessions from

Costa Rica (‘Tigriada’, ‘Acorazonada’ and ‘Indonesia’). The other 30 accessions are distributed in two great subdivisions, one including the groups A1and A2, and the other, the groups B1, B2 and B3. The group A1 includes accessions considered to be cultivated from ancient times in Murcia: ‘Valenciana rizada’, ‘Cristiana’ and ‘Filipina’ together with the Italian group (‘Brianzola’, ‘Gianzola’, ‘Selvatico’ and ‘Pomella’), the Japanese group (‘Kokuso 21’, ‘Kokuso 41’ and ‘Kokuso 20’) and the Chinese cultivar ‘Young-Gool-Pong’. The group B is constituted only by three subgroups B1 (Brazil accession ‘IZ-13-6’, Cuban accession ‘Cuba 1’ and Ethiopian accession ‘Cubana’); B2 (South Korean accessions ‘Cheongal’ and ‘Coreana’ and Spanish accessions ‘Filipinas’ and ‘Murcia’) and B3 (Chinese accessions ‘Youn-12’, ‘YOU-62’ and ‘Guangdong 11’; Cuban accessions ‘Cuba 2’ and ‘Cuba 3’, and Costa Rica accession ‘Criolla’).

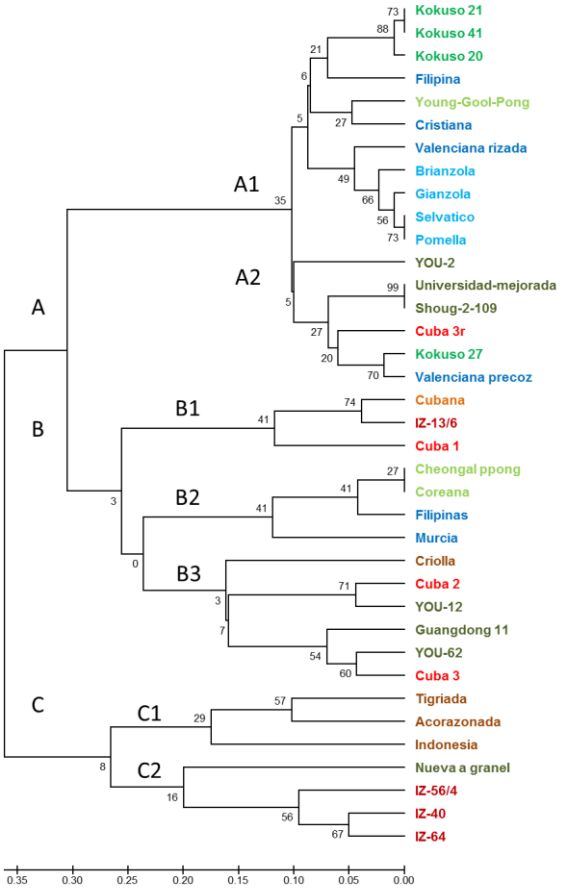

Figure 2. Location of the origin of the assayed mulberry genotypes and proposal dissemination ways from the origin in China.

The Japanese and Italian cultivars formed separated subgroups whereas the rest of accessions from the same country were clustered in different subgroups. On the other hand the Cuban cultivars 'Cuba 2' and 'Cuba 3' propagated from Chinese parents were grouped in the same branch, which suggests that these cultivars retain the genomic characteristics of their selection. It was also possible to relate the genotypes of cultivars 'Tigriada' and 'Acorazonada' present in Costa Rica as well as to evaluate other cultivars for their use in silkworm feeding.

On the other hand, pairwise genetic distances among the 37 mulberry genotypes calculated by the MCL method present an overall value of 0.570 (Table S1). These genetic distances in general confirmed the phenetic relationships shown in Figure 2. The lowest values were reported for related cultivars such as 'Universidad-mejorada' and 'Shough-2-109' (0.0) or 'Coreana' and 'Filipinas', and 'Pomella' and 'Selvatico' (0.01). The highest genetic distances were observed between 'Cuba-1' and different cultivars including 'IZ-40', 'IZ-56-4', 'IZ-64', 'Universidad Mejorada', 'Shough-2-109', and others (1.0). In addition, between 'Cuba-2' and 'Criolla' with different cultivars (Table S1).

The present work has being based in the analysis of two collections of a wide geographic scope. As a consequence, there is a wider context of foreign genotypes for a deep comparative analysis of results. In general, the information on genetic diversity and relationships among European genotypes is scarce in comparison with the abundance of published studies on populations from India and China cited in the literature. In particular, there were not studies at all of Spanish germplasm before the present work. Moreover, the absence of crossed analysis of European and Asiatic cultivars prevents the perception of a global context of the diversity of the genus. However, a fact that appears clearly after the present study is that mulberry cultivars and genotypes from Murcia constitute a discrete genetic group in comparison with Japanese and even Italian genotypes (excluding some hybridization events).

The results described indicate that the accessions of the Spanish and Cuban collections of mulberry germplasm can be correctly discriminated with the allelic combinations at nine microsatellite loci. In the other hand, regarding the genetic relationship of genotypes, it seems apparent that there is a distinct clustering of the accessions of Spanish origin in relation with the Japanese cultivars and also, although at a lower level, with Italian cultivars. This conclusion is sustained by the fact that two distinct branches of the dendrogram (A and D) are constituted exclusively by accessions originated in Murcia. However, there are two branches, B and E that include accessions of both Murcian and Italian origin.

This can have several explanations. First, it cannot be excluded the possibility of events of misclassification. After a long period of about 30 years when the sericicultural activity at the IMIDA disappeared, it is possible that mistakes in the documentation of the material happened. This is compounded by the fact that some varietal denominations are imprecise and the assignation of varietal types to the genotypes could be vague and inaccurate. Another explanation could be the existence of hybridization events between genotypes from Murcia and Italy, as suggested by the intermediate position of group B. The bulk of the collection was formed years after the material from Italy was introduced and adapted to the Region. To clarify this, it would be advisable a study of Italian germplasm still maintained in collections of that country. There is no a clear explanation for the fact that the two groups of genotypes of Spanish origin, A and B, that include both similar genotypes such as 'Valenciana rizada', 'Valenciana precoz', 'Cristiana' and 'Filipina' cultivars by one side and 'Murciana' and 'Filipinas' by other

side, appear in two clearly separated clusters. This is an example of the great diversity of the different national ecotypes due to the seed propagation system.

Table 3. Heterozygosity level of bulberry genotypes assayed.

Genotype	Heterozygosity
Acorazonada	0.55
Brianzola	0.33
Cheongal ppong	0.00
Coreana	0.55
Criolla	0.71
Cristiana	0.12
Cuba 1	0.66
Cuba 2	0.66
Cuba 3	0.50
Cuba 3r	0.57
Cubana	0.62
Filipina	0.51
Filipinas	0.52
Gianzola	0.50
Guangdong 11	0.28
Indonesia	0.25
IZ-13/6	0.42
IZ-40	0.50
IZ-56/4	0.57
IZ-64	0.33
Kokuso 4-1	0.57
Kokuso 20	0.25
Kokuso 21	0.57
Kokuso 27	0.11
Murcia	0.62
Nueva a granel	0.37
Pomella	0.37
Selvatico	0.44
Shoug-2-109	0.75
Tigriada	0.50
Universidad-mejorada	0.50
Valenciana precoz	0.28
Valenciana rizada	0.44
YOU-12	0.57
YOU-2	0.50
YOU-62	0.50
Young Gool Pong	0.20
Average	*0.45*

Previous relationships studies in mulberry has been only focused in located germplasm form a concrete region of country including South Korean (KALPANA *et al.*, 2012; KRISHNAN *et al.*, 2014a; Indian (BAJPAI *et al.*, 2014; BANERJEE *et al.*, 2016) and Chinese (HU *et al.*, 2015) germplasm. Then these results great enlarge the existent data about the relationships of mulberry accessions and cultivars from different countries.

On the other hand, the observed heterozygosity level of bulberry genotypes ranged from 0.11 ('Kokuso 27') to 0.75 ('Shoug-2-109'), with an average value of 0.45 (Table 3).

Dissemination of Cultivated Mulberry

Considering that the silk trade has existed for a long time and that mulberry is also cultivated for its fruit and for animal feed, its germplasm has a very wide distribution range in Asia, Europe; Africa and in the North and South America.

The origins of most cultivated mulberry varieties are believed to be in the China/Japan area and in the Himalayan foothills. China germplasm is located in most of the cluster identified, indicating the great influence of the native germplasm in the rest of spread mulberry cultivars around the world. These molecular results also evidenced the dissemination of the cultivated mulberry specie from China around the world, the introduction in South Korea and Japan in the first dissemination step and the later wide dissemination of modern mulberry to the Mediterranean area and later to South America and the Caribe area. In addition, alternative ways where also observed from Japan to Spain and from Spain to Cuba (Figure 1).

SSR have been assayed in the introduction and spread of cultivated mulberry but only in the Indian subcontinent (KRISHNAN *et al.*, 2014a). However, our results assaying germplasm from many countries complete previous findings from VIJAYAN *et al.* (2011) showing additional ways of dissemination from Mediterranean area and later to South America and the Caribe area to those described by these authors, a first way from China to Korea and japan and other second way from China, India, Bangladesh, Pakistan and others. In addition, alternative ways where also observed from Japan to Spain and from Spain to Cuba not described by VIJAYAN *et al.* (2011). Our results also complete previous findings from ZEREGA *et al.* (2005) describing an early diversification of Moraceae in Eurasia and subsequent migration directly into the southern hemisphere to South America. In this sense, we have to note that these authors only assayed 12 accessions from *M. alba* from India, China and Brazil.

CONCLUSION

Molecular characterization of mulberry germplasm using SSR markers is a powerful methodology for genetic characterization and relationships among studied accessions. Our results complete previous findings describing an early diversification of Moraceae in Eurasia and subsequent migration directly into the southern hemisphere to South America. These results evidenced the dissemination of the cultivated mulberry specie from China around the world, the introduction in South Korea and Japan in the first dissemination step and the later wide dissemination of modern mulberry to the Mediterranean area and later to South America and the Caribe area.

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MOLEKULARNA KARAKTERIZACIJA PROŠIRENE GERMPLAZME DUDA NA OSNOVU SSR MARKERA

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Izvod

Cilj ovog rada je molekularna karakterizacija pomoću nuklearnih SSR markera različitih sorti duda (*Morus alba* L.) iz različitih zemalja, uključujući Kubu, Kostariku, Brazil, Južnu Koreju, Etiopiju, Kinu, Japan, Italiju i Španiju. Rezultati su pokazali mogućnost primene SSR markera za razlikovanje različitih genetičkih rodova i karakterisali su obiman genski fond sorti duda. Rezultati su pokazali prisustvo 53 različita alela iz 12 SSR u 37 analiziranih genotipova. Japanske, italijanske i španske sorte formirale su odvojenu grupu u kojoj je samo 5 genotipova bilo prisutno i u kubanskoj banci germplazme. S druge strane, kubanske sorte "Kuba 2" i "Kuba 3", koje su razmnožene od kineskih roditelja, grupisane su u isti klaster, što sugeriše da su ove sorte zadržale svoje genomske karakteristike. Takođe je bilo moguće povezati genotipove kultivara „Tigriada“ i „Acorazonada“ prisutne u Kostariki, kao i proceniti druge sorte za njihovu upotrebu u ishrani svilene bube. Molekularni rezultati pokazuju i širenje kultivisane vrste duda iz Kine širom sveta, uvođenje u Južnoj Koreji i Japanu u prvom koraku širenja i kasnije širenje modernih sorti duda u mediteransko područje, a kasnije u Južnu Ameriku i područje Kariba.

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