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Brief Report

Analysis of genetic diversity of southern Spain fig tree (*Ficus carica* L.) and reference materials as a tool for breeding and conservation

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The common fig tree (*Ficus carica* L.) is a Mediterranean crop with problematic cultivar identification. The recovery and conservation of possible local varieties for ecological production requires the previous genetic characterization of the available germplasm. In this context, 42 lines corresponding to 12 local varieties and two caprifigs, in addition to 15 reference samples have been fingerprinted using 21 SSR markers. A total of 77 alleles were revealed, detecting a useful level of genetic variability within the local germplasm pools. UPGMA clustering analysis has revealed the genetic structure and relationships among the local and reference germplasm. Eleven of the local varieties could be identified and defined as obtained clusters, showing that SSR analysis is an efficient method to evaluate the Andalusian fig tree diversity for on-farm conservation.

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Food production and security are very dependent on the responsible use and conservation of the agrodiversity and the genetic resources. On-farm conservation is being promoted through international initiatives for the preservation of the genetic diversity of traditional varieties (Esquinas-Alcazar 2005). The common fig tree (*Ficus carica* L.) is a traditional crop along the Mediterranean basin, western and east Asia. Besides, the fig tree is naturally adapted to dry and arid climates with hot summers, developing a large root system to obtain water from soil many meters away from the trunk. Such kind of thermophilic trees may be useful to adapt to the consequences of the climate change and global warming, thus allowing to grow fig trees on hot and dry areas, where other species may not survive (Sugiura et al. 2007).

The fig tree has not been subjected to intensive plant breeding programmes, and thus many fig tree populations exhibit a rich genetic biodiversity, that can only be fully exploited once it is properly identified and classified. Traditionally, the plant germplasm characterization with the aim of its conservation has been carried out using morphological or agronomical traits. Nevertheless, and despite of the progress on the elaboration of descriptors, fluctuations among years, environments, or repetitions have made difficult its application until recently (Giraldo et al. 2010). These fluctuations are especially important in common fig tree germplasm, and consequently the cultivar identification is very difficult for this species. Particularly, a high level of vagueness and incongruence has been found in the local cultivated material in southern Spain. Recently, the development of DNA markers provides a direct analysis of the genotype independently from the environmental interference. Therefore, the cultivar characterization should be completed by integrating molecular markers.

Currently, simple sequence repeats (SSR) or microsatellites are the markers of choice for breeding programs (Hernandez 2005), due to their codominant nature, intraspecific polymorphism (which makes them highly informative), and easy automated detection by polymerase chain reaction (PCR). Genomic microsatellite markers have been developed for common fig tree in recent years (Khadari et al. 2001, Giraldo et al. 2005, Achtak et al. 2009) and were available for the present analysis.

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They have already been used to characterize ex situ conserved cultivars (Giraldo et al. 2005) as well as Moroccan (Khadari et al. 2005, Achtak et al. 2010), Tunisian (Saddoud et al. 2007) and Asian (Ikegami et al. 2009) local fig tree germplasm.

The present work has been approached as part of a preservation strategy for the conservation of both common fig tree germplasm and the traditional and local knowledge about its cultivation. The fig tree cultivation is a traditional activity for the sampled area of 'La Alpujarra Granadina', southern Spain, but at the moment this activity is threatened due to the area's depopulation, the poor condition of part of the plant material and the risk of introduction of plant material from other provenances without previously testing for adaptation to the area's local conditions. The past variability richness in the area and the organoleptic value of the figs is reported in the Spanish literature, for example as the 'famous figs' from Turón, both black and white types (Alarcón 1874). Together with the fig tree cultivation, the cultivation of almond, wheat and barley are mentioned for this village. At the present time, almond and fig tree cultivation coexist with grape cultivation, that was introduced during the twentieth century by immigrant farmers from the 'Levante' region, eastern Spain. With the aim to put into context the potential autochthonous genetic variability observed, as well as to detect possible introductions of exogenous material by these farmers, reference samples from ex situ collections from Levante and other provenances have been included in the analysis.

The present work is part of an effort for the characterization and on-farm conservation to generate value on the local fig tree germplasm, which is now threatened. We have fingerprinted a previously selected fig tree germplasm set and reference materials using microsatellite markers. The analysis of its genetic diversity using SSR markers has resulted an useful tool for guiding an in situ conservation strategy.

Table 1. *Local germplasm sampled.*

Accession	Common name	Location
PA20	Higuera Blanca de Pasa	Murtas
PA21	Higuera Blanca de Pasa	Turón
PA17	Higuera Blanca de Pasa	Murtas
PA18	Higuera Blanca de Pasa	Turón
PA15	Higuera Blanca de Pasa	Turón
PA16	Higuera Blanca de Pasa	Turón
PA19	Higuera Blanca de Pasa	Turón
CC47	Higuera de Carne Colorá	Turón
HC49	Higuera de Cobre	Turón
HC50	Higuera de Cobre	Mecina Tedel
HY26	Higuera Ayuela	Turón
HY22	Higuera Ayuela	Murtas
HY35	Higuera Ayuela	Turón
HY25	Higuera Ayuela	Turón
HY36	Higuera Ayuela	Jorairátar
HY31	Higuera Ayuela	Turón
CB27	Calabacilla Blanca	Murtas
CB43	Calabacilla Blanca	Turón
CN22	Calabacilla Negra	Turón
CN24	Calabacilla Negra	Turón
CN29	Calabacilla Negra	Jorairátar
CN28	Calabacilla Negra	Turón
CN30	Calabacilla Negra	Murtas
HR51	Higuera de Regalo	Turón
HR52	Higuera de Regalo	Turón
BB3	Brevera Blanca	Murtas
BB1	Brevera Blanca	Turón
BB32	Brevera Blanca	Turón
BB5	Brevera Blanca	Turón
BB33	Brevera Blanca	Murtas
BN7	Brevera Negra	Murtas
BN6	Brevera Negra	Turón
BN34	Brevera Negra	Mecina Tedel
BM10	Brevera Morada	Murtas
BM11	Brevera Morada	Turón
BM12	Brevera Morada	Mecina Tedel
HP54	Higuera de Pascua	Turón
HP55	Higuera de Pascua	Turón
HR37	Higuera Roela	Murtas
HR38	Higuera Roela	Turón
CA41	Caprifig	Turón
CA40	Caprifig	Turón

MATERIAL AND METHODS

Plant material

The selection of the analyzed material was based on a previous morphological and agronomical characterization, including interviews with farmers to integrate the local knowledge about the traditional names (or local denominations) (Guzmán-Casado et al. unpubl.). It consisted of 42 selected trees belonging to twelve local denominations (Table 1). Reference materials were chosen among representative samples of previously analyzed germplasm pools using the same SSR markers (Giraldo et al. 2005). Additionally, samples of commercial and cultivated varieties from Spanish germplasm pools were tested (for example, varieties traditionally cultivated in the Levante area from which migration of farmers to the Contraviesa region is known). All the reference material are listed in Table 2.

DNA isolation and SSR analysis

The DNA was isolated from fig tree leaves using the cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson 1980), as modified by Hernández et al. (2001). The concentration of each sample was estimated by comparing band intensity with lambda DNA of known

Table 2. *Reference germplasm.*

Accession	Name in tree (Fig. 1)	Origin
Pingo de Mel-8P	Ping	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Col de Dame Gris-8-2	Cuell	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Kadotta	Kado	Estación Experimental Agraria de Elche, Spain
Dauphine VII 3	Daup	Conservatoire Botanique National Méditerranéen de Porquerolles
Brevera Blanca	BBCV	Local nursery at Córdoba, Spain
Marroco 19 (feral)	Marr	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Colar	Cola	Estación Experimental Agraria de Elche, Spain
Mission	Miss	Estación Experimental Agraria de Elche, Spain
Brown Turkey	Brtk	Estación Experimental Agraria de Elche, Spain
Albatera	Alba	Estación Experimental Agraria de Elche, Spain
Burjassote Noire-6-16	BurN	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Col de Dame Gris-8-2	Cdam	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Lampeira-8-1	Lamp	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Bellone-10-32	Bell	Conservatoire Botanique National Méditerranéen de Porquerolles, France
Marsellaise-9-23	Mars	Conservatoire Botanique National Méditerranéen de Porquerolles, France

concentrations under blue light using a DR195M "Dark Reader" transilluminator from Clare Chemical Research (Dolores, CO, USA), after 0.8% (w/v) agarose gel electrophoresis and staining with GelRed from Biotium (Hayward, CA, USA). Samples were extracted by triplicate for the local germplasm.

The previously isolated DNA was amplified using the M13 protocol based on 21 microsatellite primer sequences developed by Khadari et al. 2001: MFC1, MFC2, MFC3, MFC4, MFC5, MFC6 and MFC8; and by Giraldo et al. 2005: LMFC12, LMFC13, LMFC14, LMFC15, LMFC18, LMFC19, LMFC20, LMFC21, LMFC24, LMFC26, LMFC27, LMFC30, LMFC31 and LMFC38. A M13 primer sequence was added to the forward primer to allow detection with a common fluorescently labeled (VIC or FAM) M13 primer as described by Tobias et al. 2005.

Amplification reactions were carried out in 25 µl volume containing 0.2 µM of each PCR primer, 200 µM of each deoxynucleotide triphosphate, 1.5 mM of $MgCl_2$, 1 unit of *Taq* DNA polymerase from Biotools (Madrid, Spain) and 50–100 ng of template DNA in the presence of 1 unit of *Taq* DNA polymerase, 50–100 ng of genomic DNA, 0.5 µM of marker-specific reverse primer, 0.033 µM markers-specific M13-tailed forward primer and 0.5 µm Hex- or FAM-labeled M13 primer, using a MyCycler Gradient thermocycler from Bio-Rad (Hercules, CA, USA). The PCR conditions were those described by Khadari et al. 2001 and Giraldo et al. 2005. Fragment sizes were resolved by capillary electrophoresis using an ABI 3130XL Genetic Analyzer (Carlsbad, CA, USA) and further analyzed with the GeneScan 3.7 software from the same manufacturer.

Genetic variability analysis

A total of 21 polymorphic microsatellite markers were used for the genetic variability study, using 55 common fig tree accessions. The statistical analysis, including the number of alleles per locus, allele frequencies, observed and expected heterozygosity (H_o and H_e) and polymorphism information content (PIC) values, was carried out using the application PowerMarker 3.25 (Liu and Muse 2005). The allele frequency data from PowerMarker was used to export the data in binary format. A genetic distance matrix using the Nei index (Nei and Li 1979) was calculated using PhylTools 1.32 (Buntjer 1997), and the unweighted pair group method with arithmetic mean (UPGMA) clustering was carried out using the Neighbor module in the Phylip 3.66 package (Felsenstein 1993). The cophenetic correlation coefficient (CCC) of the generated dendrogram was computed using the visual Basic program for Microsoft Excel 2000 CCC (Dighe et al. 2004). The CCC is the correlation coefficient (r) calculated from the linear regression between the corresponding values of the original distance matrix and the cophenetic matrix derived from the calculation of the UPGMA tree. The dendrogram was drawn using the Mega 3.1 software (Kumar et al. 2004).

RESULTS AND DISCUSSION

The molecular analysis of the 57 common fig tree accessions using 21 microsatellite markers detected a total of 77 alleles. The number of alleles per locus ranged from 2 (for loci MFC5, MFC8, LMFC18, LMFC27 and LMFC31) to 9 alleles (for locus LMFC30), with a mean of 3.6 alleles per locus and a mean PIC of 0.456. The highest and lowest PIC values were scored for the loci LMFC30 and MFC5, respectively. For the local germplasm, the observed heterozygosities (H_o) were similar to the expected (H_e) for most of the primer pairs tested (Table 3), whereas for five primer pairs the H_os were bigger than H_es and for four primer pairs the situation was

Table 3. *Summary of SSR genetic parameters for all the analyzed germplasm (local and reference samples). PIC: polymorphic information content, H_e: expected heterozygosity, H_o: observed heterozygosity*.

Locus	Repeat motif	Alleles		PIC	H_e/H_o
		Number	Length		
MFC1	$(CT)_{13}$	3	174–192	0.409	0.51/0.77
MFC2	$(AC)_{18}(AT)_7$	5	158–172	0.635	0.69/0.67
MFC3	$(AC)_{15}TC(AC)_8(AT)_7$	6	122–134	0.707	0.74/0.67
MFC4.1	$(AT)_4(AC)_{11}$	2	192–192	0.263	0.00/0.00
MFC4.2		2	218–222	0.365	0.48/0.39
MFC5	$(GA)_{13}$	2	128–140	0.239	0.28/0.30
MFC6	$(TAA)_3,(GT)_8$	6	292–318	0.598	0.66/0.72
MFC8	$(CA)_9TA(CA)_{14}(TA)_6$	2	170–174	0.374	0.50/0.84
LMFC12	$(CT)_{55}$	4	349–377	0.582	0.65/0.50
LMFC13	$(GA)_{28}$	3	263–285	0.523	0.59/0.75
LMFC14	$(GA)_{16}$	3	210–214	0.406	0.45/0.44
LMFC15	$(TC)_{22}$	3	180–204	0.435	0.51/0.51
LMFC18	$(GA)_9$	2	116–122	0.375	0.50/0.56
LMFC19	$(AT)_{11}(AG)_{12}$	3	300–308	0.302	0.33/0.35
LMFC20	$(AAG)_9(AG)_{18}$	3	134–138	0.350	0.43/0.00
LMFC21	$(TC)_9$	3	258–266	0.384	0.42/0.21
LMFC24	$(CT)_{10}$	3	271–275	0.445	0.55/0.79
LMFC26	$(GA)_{15}$	3	220–232	0.470	0.55/0.00
LMFC27	$(TG)_{17}(AG)_6$	2	182–192	0.374	0.50/0.79
LMFC30	$(CT)_{18}(CA)_6$	9	226–256	0.798	0.82/0.79
LMFC31	$(GA)_{15}$	2	225–239	0.335	0.43/0.58
LMFC38	$(CT)_{20}$	6	212–222	0.667	0.72/0.40
Total		77			

the opposite. These figures indicate a high level of genetic diversity in the analyzed germplasm revealed by the microsatellite markers. This is in agreement with the fact that the fig tree has not been subjected to intensive plant breeding programmes, as previously indicated.

The dataset was analyzed in search for diagnostic markers for the traditional varieties. Alleles MFC3-122 and LMFC15-204 were discriminant for the local variety 'Brevera Blanca', whereas alleles LMFC26-222 and LMFC30-238 for 'Higuera de Pascua' and alleles MFC1-186 and LMFC30-220 were only present in 'Higuera Roela'. These results confirm the utility of the microsatellite markers for the characterization of this germplasm.

A dendrogram of the analyzed germplasm based on the 21 microsatellite markers was constructed using the Dice coefficient and the UPGMA clustering method (Fig. 1). The CCC analysis, the parameter that measures the correlation between similarity values calculated during tree building and the observed similarity, was found to be high ($r = 0.85$). The UPGMA dendrogram (Fig. 1) revealed three main clusters: cluster I (4 accessions), cluster II (26 accessions) and cluster III (25 accessions).

The UPGMA analysis shows the relationships among the local germplasm and with the reference varieties. The traditional denominations are largely descriptive therefore is not surprising the correlation among the denominations and the molecular relationships derived from the cluster analysis. The most genetically distinct cluster (shown as 'cluster I') includes the caprifigs and also the local denomination 'Higuera Roela' and does not include any reference variety. The local denomination 'Higuera Roela' has been found in cool vegetable gardens. In the past, it was abundant at the Jorairátar riverside. Its juicy fruits are highly appreciated for fresh consumption. Under irrigation they reach big sizes without flavor loss. This germplasm is therefore candidate for on-farm conservation.

The rest of the local germplasm can be divided into two main clusters (cluster II and cluster III in Fig. 1). Cluster II includes the early figs and the 'Higuera de Pascua' local denominations. This latter, like the early figs, produces two crops, but they are they are both produced on current seasons growth and take place after the summer. Its figs are not especially appreciated for its flavor, but the germplasm is interesting to produce almost until the end of the year. Additional local denominations like 'Valenciana' or 'Franciscana' suggest it may be a recent introduction. At the molecular level, it is very close to the Portuguese variety 'Lampeira'.

The early figs cluster shows two main divisions: the white early figs ('Breveras Blancas'), separated from the black early figs ('Breveras Negras') and the purple

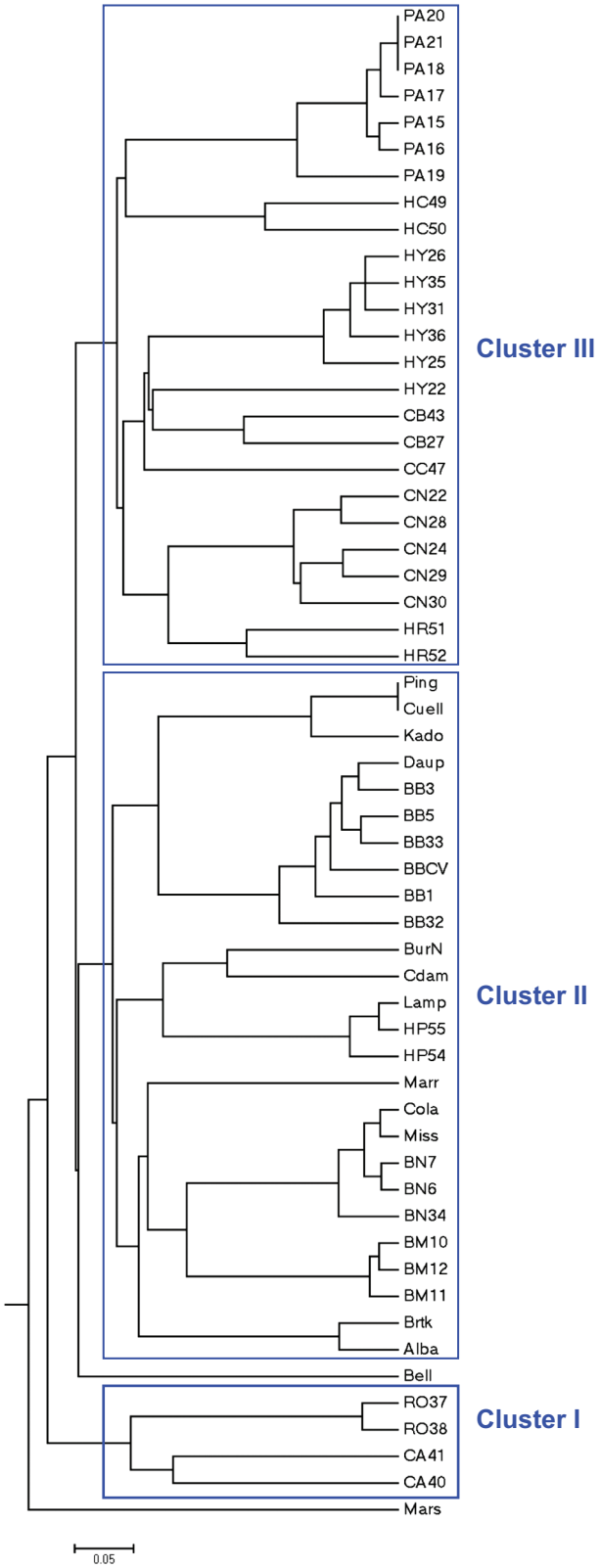

Fig. 1. UPGMA dendrogram analysis showing the genetic relationships among southern Spain local fig germplasm and reference varieties based on SSR marker analysis.

early figs ('Breveras Moradas'). The black early figs are genetically related to the varieties 'Colar' and 'Mission', whereas the white early figs show genetic similarity with a commercial white early fig tree and with the French variety 'Dauphine'. The early fig tree cluster shows a level of variability and relationships that can be used to guide conservation strategies.

The cluster III shows the genetic relationship among the seven remaining local denominations. No reference samples have been grouped in this cluster, suggesting that it includes most probably autochthonous germplasm. It includes one sample of the most threatened local variety that could be more abundant in the area in the past: 'Higuera de Carne Colorá'. Only a few isolated trees have been found, in poor condition, for such variety, being very old trees or regrowths. Thus, measures for on-farm conservation of this germplasm are recommended.

In conclusion, the present study has demonstrated how the integration of morphological, agronomical, ethnobotanical and genetic analysis can complement the analysis of agrobiodiversity of a traditional farm system that has developed varieties in situ, and put it in value. The resolutive power of the SSR marker system has been enough to distinguish and to establish genetic relationships among the germplasm classes found. This information will be used to guide the on-farm conservation measures to be undertaken in the area.

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