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DEVELOPMENT OF THE IDENTIFICATION KEY FOR REFERENCE FIG *(FICUS CARICA* L.) VARIETIES FROM SLOVENE ISTRIA

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ABSTRACT

In Slovenia, the common fig is an underutilized fruit species grown in family yards in association with olives and other Mediterranean plants. Due to the functional properties of fig syconias, the interest in fig cultivation is increasing. In order to provide certified plant material in the region of Slovene Istria, the determination of reference varieties is required on the basis of the molecular marker system. The identification key containing the minimum primer pairs for unambiguous discrimination of reference fig varieties was developed with the choice of the most informative microsatellite loci. All recommended fig varieties for Slovenia were genetically differentiated by only 3 loci: FCUP008-2, FCUP013-7, and FCUP044-6.

Key words: *Ficus carica* L., reference variety, microsatellites, Slovene Istria

SVILUPPO DELLA CHIAVE DI IDENTIFICAZIONE PER LE VARIETÀ REFERENZIALI DI FICO COMUNE *(FICUS CARICA* L.) DELL'ISTRIA SLOVENA

SINTESI

In Slovenia il fico commune è una specie fruttifera sottoutilizzata che cresce in orti di famiglia, in associazione con gli ulivi e altre piante mediterranee. Vista l'elevata concentrazione di vitamine, minerali e biofenoli nei frutti di fico, l'interesse per la coltivazione di tale pianta è crescente. Al fine di fornire materiale vegetale certificato nella regione dell'Istria slovena, la determinazione delle varietà referenziali è richiesta con l'ausilio di sistemi molecolari di marker. La chiave di identificazione permette una discriminazione non ambigua delle varietà referenziali di fico, ed è stata sviluppata con la scelta dei loci microsatellitari più informativi. Tutte le varietà di fico raccomandate per la Slovenia sono stare geneticamente differenziate da soli 3 loci: FCUP008-2, FCUP013-7 e FCUP044-6.

Parole chiave: *Ficus carica* L., varietà referenziali, microsatelliti, Istria slovena

INTRODUCTION

The common fig *(Ficus carica* L.) is a typical fruit species of warm climate, widely spread in the Mediterranean Basin where its production is of great economic significance. In Slovenia, the cultivation of the common fig is limited to the coastal region of Slovene Istria, Goriška Brda and the Vipava Valley. Approximately 4,000 fig trees are planted on around 10 hectares of intensive orchards, and the production of fruits is estimated to total 50 tones per year (FAOSTAT, 2008). Yet large fig orchards are rare, and individual trees are frequently planted in family yards and gardens in association with olives and other Mediterranean plants. Fig fruits are prevailingly sold fresh at local markets. The economic potential of fig cultivation has been poorly addressed, and the fig tree is considered an underutilized fruit species in the region. Over the last few years, increasing attention and promotion have been paid to underutilized species whose reemergence could lead to higher agricultural diversification, greater use of marginal lands and a more balanced diet (IPGRI, 2002).

The functional properties of fig fruits, such as richness of fibers, biophenols, vitamins and minerals, contribute to their great demand among the consumers and encourage the use of fig fruits in the food-processing industry. In order to encourage fig growing in Slovenia, the research on variety structure and an inventory of fig genetic resources are required. Morphological survey was initiated at the national level, and preliminary results showed a great diversity of fig varieties (Podgornik *et al.*, 2008). The efficiency of fig growing revitalization is greatly influenced by variety choice, since not all varieties are suitable for commercial use. The following fig varieties are recommended for the Slovene cultivation area: 'Bela petrovka' and 'Miljska figa' as major varieties, and 'Zuccherina', 'Zelenka', 'Flazana', 'Pinčica', 'Laščica', 'Sivka' as minor varieties (Godec *et al.*, 2007). Cuttings for fig tree propagation are usually collected from individual trees at different locations, therefore mother trees ought to be determined and the identification system developed for propagation of certified plant material.

The objectives of the certification process are production and distribution of high quality plant material of superior varieties of established genetic identity and propagation of virus free plants. Even if morphological evaluation of varieties is traditional and well adopted, the use of phenotypic characteristics in the identification process is uncertain due to their high variation and dependence on environmental factors. Introduction of DNA molecular markers into the identification scheme of plant material has become a routine analytical approach, and among available marker systems, microsatellites or simple sequence repeats (SSR) are the most employed.

The recently developed microsatellites in the common fig (Khadari *et al.*, 2001; Giraldo *et al.*, 2005; Bandelj *et al.*, 2007) enabled introduction of these markers into the genotyping analysis of varieties. DNA fingerprinting of plants with microsatellites is reliable and fast once the most informative loci have been selected. Other advantages of the microsatellite marker system are the generation of non complex banding patterns, high polymorphism, and genotyping results comparable among different laboratories.

The aim of this work was to establish the identification key for recommended fig varieties in Slovenia by using microsatellite genotyped data. The identification key will serve for testing and confirmation of genetic identity of mother plants grown in nurseries for the purpose of propagation. The results of this work can serve as the basis for the establishment of the national fig collection and will be also used for fig genetic resources management in Slovenia.

MATERIALS AND METHODS

Plant material

The genotyping analysis included 8 recommended fig varieties for the Slovene cultivation area. From 1 to 4 fig

Tab. 1: List of fig varieties included in the genotyping analysis, number of analysed samples and location of sampling.
Tab. 1: Seznam sort fig, vključenih v genotipizacijo, število analiziranih vzorcev in lokacija vzorčenja.

Variety name	No. samples	Sampling location
'Bela petrovka'	4	Izola, Lucija, Glem, Dekani
'Miljska figa'	4	Seča, Osp, Glem, Dekani
'Zuccherina'	1	Šalara
'Zelenka'	1	Padna
'Pinčica'	1	Seča
'Laščica'	2	Padna, Nova vas
'Sivka'	1	Smokvica
'Flazana'	1	Goriška Brda
Sum of samples	**15**	

trees were sampled per variety on the basis of previous morphological description (Vrhovnik & Kodrič, 2004; Podgornik *et al.*, 2008). Fig leaves were collected from individual trees at different locations in Slovene Istria and Goriška Brda. The names of genotyped fig varieties and locations of sampled plant material are listed in Table 1.

Fig DNA isolation and amplification of microsatellites

Fig DNA was extracted from leaves by modified CTAB method, and amplification of microsatellites with fluorescence-based detection was performed as previously reported by Bandelj *et al.* (2007). Four primer pairs for fig microsatellite loci (FCUP008-2, FCUP013-7, FCUP044-6, FCUP068-1) were used in the genotyping analysis. For fluorescent detection, short primers of the developed pair were elongated for the M13(-21) 18 bp sequence according to Schuelke (2000). Amplification reactions were carried out in a total volume of 10 µl, containing 20 ng of fig DNA, 1X supplied PCR buffer (Promega), 0.2 mM of each dNTP, (Roche), 0.25 unit of *Taq* DNA polymerase (Promega), 0.2 µM of each locus specific primer and 0.075 µM of M13(-21) primer labelled at the 5′ end with Cy5 (MWG Biotech). Amplification was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems), and the conditions of the two-step PCR amplification were as follows: 94 °C (5 min), then 5 cycles of 45 s at 94 °C, 30 s at the initial annealing temperature (57 °C for loci FCUP008-2 and FCUP013-7, 60 °C for loci FCUP044-6 and FCUP068-1), which was lowered by 1 °C in each cycle, and the extension at 72 °C for 1 min 30 s. The second step of amplification passed though 25 (FCUP008-2, FCUP013-7, FCUP044-6) or 28 (FCUP068-1) cycles with the same cycling conditions except for the constant annealing temperature of 52 °C (FCUP008-2, FCUP013-7) or 55 °C (FCUP044-6, FCUP068-1). The reactions ended by 8 min extension at 72 °C. The amplification products were separated on a 7.5% polyacrylamide denaturing gel, containing 7 M urea. Electrophoresis was performed on an automated ALFexpressII sequencer (Amersham Biosciences), and the length of alleles was determined with the aid of an external standard (50–500 bp, GE Healthcare) and internal standard using Allele Locator 1.03 software.

RESULTS

In order to establish the database of reference fig varieties for Slovenia, genotyping with microsatellite markers was performed. The database of genotyped varieties was used as the basis for development of the identification key that includes the minimum primer pairs for distinguishing all varieties in case of propagation of certified plant material in nurseries. Development of the identification key enables fast and reliable determination of identity of mother plants, cuttings and young plants. For DNA genotyping analysis, 4 published microsatellite loci (FCUP008-2, FCUP013-7, FCUP044-6, FCUP068-1) were chosen (Bandelj *et al.*, 2007).

The microsatellite markers were successfully amplified in all 15 samples with 4 primer pairs used. PCR products were separated by polyacrylamide gel with high resolution using ALFexpressII sequencing instrument and detected automatically by fluorescence. Microsatellites lengths were determined automatically using a computer software package.

Altogether, 17 alleles were amplified at 4 loci in 8 fig varieties. 6 alleles were observed at locus FCUP008-2, 4 alleles were amplified at loci FCUP044-6 and FCUP068-1, and only 3 alleles were found at locus FCUP013-7. The varieties 'Bela petrovka', 'Miljska figa' and 'Laščica', represented by 4 and 2 samples respectively, showed identical DNA profiles, and no intra-variety polymorphism was found. The number of observed genotypes was 17, the highest number (5) was observed at locus FCUP008-2, while 4 different genotypes were found at other three loci. At 4 analyzed loci, 6 unique genotypes were observed. They were characteristic of the following varieties: 'Bela petrovka' [AE(160:180)], [NR(178:206)], 'Miljska figa' [BF(162,184)], 'Zuccherina' [PR(198:206)], 'Laščica' [DF(178:184)], and 'Flazana' [HH(208:208)] (Tab. 2).

Variety specific or unique alleles were amplified in two varieties, 'Bela petrovka' (allele A: 160 bp at locus FCUP008-2) and 'Zuccherina' (allele P: 198 bp at locus FCUP068-1).

The allelic polymorphism allowed the discrimination of all analyzed varieties. The presence of individual alleles generated by 4 primer pairs in 8 fig varieties is shown in Table 2. A minimum number of 3 microsatellite markers were chosen for rapid varietal identification of recommended fig varieties. Specific allele profiles at locus FCUP008-2 were first assigned to three varieties: 'Bela petrovka', 'Miljska figa' and 'Laščica', the next three varieties, 'Sivka', 'Flazana' and 'Zuccherina', were differentiated by FCUP013-7, and the remaining two varieties, 'Zelenka' and 'Pinčica', were additionally genotyped by FCUP044-6. The identification key for the 8 fig varieties is presented in Table 2.

Tab. 2: Identification of 8 recommended fig varieties in Slovenia by microsatellite markers. The presence of alleles in specific genotype/variety is marked by symbol +. All varieties were differentiated by 3 microsatellite loci: FCUP008-2, FCUP013-7, FCUP044-6, which form the molecular identification key for fig varieties.
Tab. 2: Identifikacija 8 priporočljivih sort fig v Sloveniji z mikrosatelitskimi markerji. Zastopanost alelov v specifičnem genotipu/sorti je označena s simbolom +. Vse sorte ločimo s tremi mikrosatelitskimi lokusi: FCUP008-2, FCUP013-7, FCUP044-6, ki oblikujejo molekulski identifikacijski ključ za sorte fig.

Locus	Allele designation and allele in bp		'Bela petrovka'	'Miljska figa'	'Zuccherina'	'Zelenka'	'Pinčica'	'Laščica'	'Sivka'	'Flazana'
FCUP	A	160	+							
008-2	B	162		+	+	+	+			
	C	166							+	+
	D	178						+	+	+
	E	180	+		+	+	+			
	F	184		+				+		
FCUP	G	196				+	+	+	+	
013-7	H	208						+	+	++
	I	212	++	++	++	+	+			
FCUP	J	208					+	+	+	+
044-6	K	210							+	+
	L	217		++		++				
	M	219	++		++		+	+		
FCUP	N	178	+						+	+
068-1	O	196		++		++	++	++	+	+
	P	198			+					
	R	206	+		+					
Observed genotypes			AE, II, MM, NR	BF, II, LL, OO	BE, II, MM, PR	BE, GI, LL, OO	BE, GI, JM, OO	DF, GH, JM, OO	CD, GH, JK, NO	CD, HH, JK, NO

DISCUSSION AND CONCLUSIONS

In the Mediterranean Basin, the cultivated fig is a widely spread fruit species with a large number of local varieties whose identity has been poorly studied. The identification of reference varieties is important especially to nurseries where certified plant material is propagated. The accurate identification system of varieties is also important to growers when they plan to establish new permanent orchards as the quality of the crop is greatly influenced by variety choice. A reliable identification technique of plant material is also of significant importance for the establishment of germplasm collection and prevention of plant mislabelling during plantation. Long juvenile stage of vegetatively propagated fig trees and strongly expressed heterophylly prevent the determination of young trees' identity on the basis of morphological characteristics. The development of DNA markers has brought about new approaches in varietal analysis. DNA based identification procedures enable plant identification by generating genotype specific DNA banding profiles. Among available DNA markers, randomly amplified polymorphic DNA (RAPD) has been the most employed in *Ficus carica* L. germplasm characterization (De Masi *et al.*, 2005; Salhi-Hannachi *et al.*, 2005; Sadder & Ateyyeh, 2006), and the recent development of fig microsatellites enabled the inclusion of these markers into diversity studies of common figs (Khadari *et al.*, 2001; Giraldo *et al.*, 2005; Bandelj *et al.*, 2007).

In order to establish an accurate identification system for recommended fig varieties in Slovenia, DNA profiling of 15 fig samples belonging to 8 reference varieties was performed. Among the 3 published sets of fig microsatellite markers, we chose 4 FCUP primer pairs on the basis of their good polymorphic characteristics exhibited in our previous diversity study of cultivated figs (Bandelj *et al.*, 2007). The highest number of amplified markers was displayed by loci FCUP008-2, FCUP044-6, FCUP068-1 and FCUP70-2, with the criteria for marker choice also being high polymorphic information content value and low probability of identity. The choice of the most appropriate markers is an important step in the identification procedure of varieties as it affects the rapidity and extent of molecular analysis and, consequently, the cost of the procedure.

The selected microsatellite loci allowed the discrimination of all 8 fig varieties. The average number of amplified alleles per locus was 4.25, thus being identical to the number reported by Khadari *et al*. (2001) in their diversity study of 14 fig varieties. Using LMFC set of microsatellites, Giraldo *et al*. (2005) detected a lower number of alleles per locus (3) in their genotyping analysis of 15 fig varieties. The number of detected alleles is probably dependent on characteristics of loci and genetic background affecting similarity of varieties included into the analysis. In our study, high genetic similarity was observed between the varieties 'Flazana' and 'Sivka', as well as between 'Zelenka' and 'Pinčica'. Differences in allelic profiles of the above-mentioned varieties were found only at loci FCUP013-7 and FCUP044-6 respectively, while identical alleles were observed at other three loci.

The comparison of allelic profiles of samples within the varieties 'Bela petrovka', 'Miljska figa' and 'Laščica' revealed no differences, which testified to the homogeneity of the analyzed varieties and the absence of different clones. In other studies, genetically heterogeneous varieties were identified by means of inter simple sequence repeat, microsatellites (Khadari *et al*., 2005), RAPDs and AFLPs (Cabrita *et al*., 2001).

Discrimination of varieties by DNA markers is usually performed in the following three ways: by using unique markers, unique genotypes, and the combination of DNA profiles in different DNA regions. Our study revealed only 2 unique alleles characteristic of the varieties 'Bela petrovka' and 'Zuccherina'. These two varieties could be immediately identified by genotyping loci FCUP008-2 and FCUP068-1. Unique genotypes were found at all loci except at locus FCUP044-6 where two by two varieties shared the same allelic profile. By applying the combination of 3 loci (FCUP008-2, FCUP013-7 and FCUP044-6), we were able to distinguish all 8 reference fig varieties. Giraldo *et al*. (2005) distinguished 9 fig varieties by only 2 microsatellite loci. The number of primer pairs required for the discrimination analysis depends on genetic background and relatedness of analyzed varieties. The choice of locus is also an important factor in the discriminating process. In our previous study, locus FCUP068-1 generated high variability information (Bandelj *et al*., 2007), while in the present analysis it revealed identical genotypes for the varieties 'Miljska figa', 'Zelenka', 'Pinčica' and 'Laščica'. On the basis of this result, it can be concluded that locus FCUP068-1 is not suitable for variety identification of Slovene reference figs.

The present study provides the first identification key that includes the minimum number of primer pairs required for unambiguous discrimination of recommended fig varieties in Slovenia. Microsatellites proved to be a valuable molecular tool for varietal identification of figs, and the results of this work could be successfully implemented for confirmation of varietal identity of propagated fig plants in nurseries, for identification of fig trees of unknown identity in Slovene cultivation area, as well as for screening and managing of fig genetic resources in collections.

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RAZVOJ IDENTIFIKACIJSKEGA KLJUČA ZA REFERENČNE SORTE FIG *(FICUS CARICA* L.) SLOVENSKE ISTRE

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POVZETEK

Figa je v Sloveniji poznana kot manj razširjena in uporabljena sadna vrsta. V Slovenski Istri in toplejših predelih Vipavske doline in Goriških Brd uspeva na družinskih vrtovih v asociaciji s tipičnimi sredozemskimi kulturami. Visoka vsebnost vlaknin, biofenolov, vitaminov in mineralov v plodovih fige prispeva k njenemu večjemu povpraševanju na trgu ter k vključevanju sadežev v živilsko-predelovalno industrijo. Za pospeševanje gojenja fig v Primorju je pomembno zagotoviti sadilni material referenčnih in priporočenih sort. Z razvojem molekulskih markerjev identiteto sort v sadjarstvu ugotavljamo na nivoju genoma. Namen razvoja molekulskih identifikacijskih ključev v

sadjarstvu je določiti visoko informacijske molekulske markerje za posamezno sadno vrsto, s katerimi po opravljeni genotipizaciji sorte genetsko hitro ločimo, zmanjša pa se tudi obseg laboratorijskih analiz. Referenčne sorte fig, ki so predstavljene v sadnem izboru za Slovenijo, smo ločili s tremi visoko informativnimi lokusi mikrosatelitov (FCUP008-2, FCUP013-7 in FCUP044-6), ki ponazarjajo identifikacijski ključ oziroma najmanjše možno število lokusov, na osnovi katerih lahko priporočene sorte fig genetsko ločimo.

Ključne besede: *Ficus carica* L., referenčna sorta, mikrosateliti, Slovenska Istra

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