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Sequence analysis of the internal transcribed spacers (ITSs) region of the nuclear ribosomal DNA (nrDNA) in fig cultivars (*Ficus carica* L.)

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ABSTRACT

Sequences of the internal transcribed spacers (ITSs) of nuclear ribosomal DNA (nrDNA) were analysed in a set of Tunisian fig (*Ficus carica* L.) cultivars. The size of the spacers sequences ranged from 200 to 279 bases for ITS1 and from 253 to 314 bases for ITS2. Variation of GC contents has been also observed and scored as 59–68% and 55–68% for ITS1 and ITS2, respectively. This data exhibited the presence of polymorphism among cultivars. The intra-specific variability level of the ITS sequences proved a variation both in the length and in the sequences studied. In fact, ITS1 and ITS2 sequences were considered as a useful tool to establish genetic relationships among cultivars. Our results indicate that the diversity detected among closely related genotypes supported strongly the efficiency of ITS sequences for establishing relationships between cultivars. ITS2 seems to be relatively more informative than ITS1 regarding length or GC contents. Considerable genetic diversity was observed among fig at intra and inter-cultivars levels. Two polyclonal varieties were identified. In addition, data proved that a typically continuous genetic diversity characterizes the local fig germplasm. The topology of the derived dendrogram strongly supported this assumption. In fact, genotypes are clustered independently from their geographical origin or the sex of trees suggesting a narrow genetic basis among the ecotypes studied in spite of their phenotypic distinctiveness. Implications of these results for management of fig germplasm collections are discussed.

1. Introduction

The common fig (*Ficus carica* L., 2*n* = 26 chromosomes) (Weiblen, 2000) belongs to the order of Urticales and the family of Moraceae, with over 1400 species classified into about 40 genera (Watson and Dallwitz, 2004). The *Ficus* species are gynodioecious, and functionally dioecious. Some of them are functionally female producing only a seed-bearing fruit, whereas others are functionally male and produce only pollen and pollen-carrying wasp progeny (Kjellberg et al., 1987; Janzen, 1979; Weibes, 1979). The symbiosis between figs and their pollinators is a prominent example of coevolution. A specific wasp species belonging to the Agaonidae family (Hymenoptera, chalcidoidea) is required for pollination in each of the 700 *Ficus* species (Berg, 2003). It should be stressed that, among these species *F. carica* is one of the oldest known fruit crop and used for fruit production (Beck and Lord, 1988; Kislev et al., 2006). Total world fig production is over 1 million tones. Turkey, Egypt, Greece, Iran, Morocco, Spain and USA yielded about 70% of total production (FAOSTAT, 2004). In Tunisia, fig cultivation consists of diversified and well-adapted ecotypes exhibiting the presence of a large array of genotypes that characterizes the local germplasm. Despite this phenotypic diversity, fig cultivation remains traditional and relatively limited since only a small part of the total area reserved to the fruit trees contains fig plantations yielding about 30,000 tons per year (DGPA, 2006). Fruits are consumed fresh or dried and for industrial production. Figs are also used traditionally for their medicinal properties. Moreover, the local germplasm is threatened by genetic erosion (Urbanization, absence of phytosanitary norms, rainfall irregularities, mono-varietal culture) and some biotic constraints such as the fig mosaic disease. Consequently, a lack of cultivars has occurred and constituted a constraint in the preservation and the improvement of the local fig phytogenetic resources. Therefore, it is imperative to establish strategies for preservation and conservation of local germplasm. For this purpose, studies have reported the use of morphometric, pomological traits and isozymes and permitted to evidence a large phenotypic variability (Mars et al.,

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1998; Rhouma, 1996; Hedfi et al., 2003; Salhi-Hannachi et al., 2003; Chatti et al., 2004a). However, these studies are less rewarding since the derived characterization is not suitable to establish reference genotypes for fig breeding programs. To overcome this inconvenience, large scale of PCR-DNA based methods such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), random amplified microsatellite polymorphism (RAMPO) and simple sequence repeats (SSR) have been successfully designed in this crop (Valizadeh et al., 1977; Khadari et al., 1995; Chessa et al., 1998; Elisiario et al., 1998; Cabrita et al., 2001; Chatti et al., 2004b, 2007; Saddoud et al., 2007; Salhi-Hannachi et al., 2004, 2005, 2006). Thus, it was assumed that the local germplasm is characterized by a typically continuous genetic diversity. In addition, despite the suitability of these techniques in the ecotypes' discrimination, several cases of synonymy have been scored and not well elucidated using the evidenced markers (Chatti et al., 2007; Saddoud et al., 2007). Therefore, we become interested in the development of additional markers in order to precise the genetic diversity organisation and to contribute to the molecular characterization of fig ecotypes. For this purpose, analysis of the internal transcribed spacer (ITS) of nuclear ribosomal DNA was investigated. Note worthy that the nrDNA organisation, high copies of tandem units in a single or multiple loci, offers several advantages over other parts of the genome (Rogers and Bendich, 1987; Hamby and Zimmer, 1992). (i) It is useful for phylogenetic studies at the intra specific level (Jorgensen and Cluster, 1988; Downie et al., 1994; Baldwin et al., 1995; Campbell et al., 1995; Susanna et al., 1995). (ii) This gene family undertakes rapid concerted evolution (Arnheim, 1983; Zimmer et al., 1980; Hillis et al., 1991). (iii) Its detection, amplification and sequencing are made easy by regards to the presence of highly conserved sequences flanking each of the two spacers. Therefore, analysis of the rDNA would provide an attractive approach suitable to generate efficient molecular markers to assess phylogenic relationships as well as the genetic diversity organisation in higher plants.

The present study portrays the achievement of nrDNA in a set of fig cultivars to analyse genetic diversity and establish a molecular database for fig breeding programs and the rational management of the local fig germplasm conservation.

2. Materials and methods

2.1. Plant materials

Twelve fig cultivars, collected from traditional plantations and *ex situ* conserved at the ISA (Chott Mariam), were used in this study (Table 1). The experimental material consisted of 11 common fig cultivars and 1 caprifig that represent the main cultivated forms in the Sahel region of Tunisia. These were chosen according to their attractive fruit qualities and their economic importance. *Punica granatum* is used in this study as an out-group sequence to root the tree.

Table 1
Tunisian fig (*Ficus carica*) cultivars studied with their localities of origin and fruit characteristics.

Label	Cultivar	Geographic origin	Variety type	Fruit colour
1	Besbessi 1	Mesjed Aîssa	San Pedro	Yellowish green
2	Besbessi 2	Mesjed Aîssa	San Pedro	Yellowish green
3	Besbessi 3	Mesjed Aîssa	San Pedro	Yellowish green
4	Zidi	Mesjed Aîssa	Smyrna	Black
5	Soltani	Ouardanine	Smyrna	Purple
6	Bidhi 1	Kalaa Kebira	Smyrna	White yellow
7	Hemri	Ghadhabna	Common	Reddish
8	Bidh beghal	Mesjed Aîssa	Smyrna	Dark Purple
9	Jrani[a]	Ghadhabna	Caprifig	–
10	Bidhi 2	Khmara	Smyrna	White yellow
11	Delgane	El Alia	Smyrna	Yellowish
12	Bither abiadh	Khmara	San Pedro	Yellowish

[a] Caprifig: male tree.

2.2. DNA purification

Total cellular DNA was extracted from 3 g of green tissues (leaves) according to Dellaporta et al. (1983). The DNA concentration was spectrophotometrically estimated and its integrity was performed by 0.8% analytic agarose gel electrophoresis (Sambrook et al., 1989).

2.3. Primers and ITS assays

The internal transcribed spacers region was amplified using the polymerase chain reaction (PCR) method (Saiki et al., 1988). For this purpose, the following primers identified as 5′-AAGGTTTCCG-TAGGTGAAC-3′ for the 3′ end of 18S nrDNA and 5′-TATGCT TAAACTCCAGCGGG-3′ for the 5′ end of 26S nrDNA (Fig. 1) were used as reported by Weiblen (2000).

Amplifications were carried out in a 25 μL reaction mixture volume containing 2.5 μL of 10× Taq polymerase reaction buffer, 1 U of Taq DNA polymerase (QBIOgene, Illkirch, France), 200 μM of each dNTP (DNA polymerization mix, Pharmacia), 6 pM of each primer and 20 ng of total cellular DNA. The reaction mixture was overlaid with a drop of mineral oil to prevent evaporation during thermal cycling. PCRs were performed in a DNA thermocycler (Crocodile III QBIOgene, Illkirch, France) as follows: reaction mixtures were heated at 94 °C for 5 min as an initial denaturation step before entering 35 cycles consisting of 45 s at 94 °C for denaturation, 1 min at primer appropriate temperature (45 °C) for annealing and 1 min 30 s at 72 °C for elongation. A final extension step of 10 min at 72 °C was performed at the last cycle.

Amplification products were separated using 1.5% agarose gel electrophoresis in 0.5× TBE buffer, stained with ethidium bromide and visualized under UV light (Sambrook et al., 1989). Excess of primers and dNTPs after amplification were removed by purification using the Wizard SV Gel PCR Clean-Up System kit according to the manufacturer's instructions (Promega, WI, USA). Sequencing

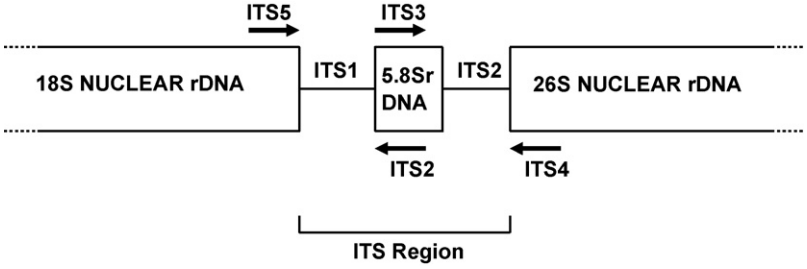

Fig. 1. Organisation of the internal transcribed spacer (ITS) region. The orientation and the approximate primer sites are presented according to Baldwin et al. (1995).