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Molecular polymorphism of cytoplasmic DNA in *Ficus carica* L.: Insights from non-coding regions of chloroplast DNA

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ABSTRACT

The *trnL* (UAA) intron and the intergenic spacer between the 3′ exon of *trnL* (UAA) and *trnF* (GAA) sequences were used as genetic markers for differentiating *Ficus carica* cultivars and establishing refined genetic relationships. The study was based on 20 fig cultivars, collected from south and centre of Tunisia. Since, the intron was thought to be more variable among close relatives than is the chloroplast spacer. The size of these non-coding regions varied from 554 to 589 and from 989 to 1022 bases pairs for the intron and the combined sequences correspondingly. The average of GC content was 33.9% and 34.6% in the intron and the combined intron and spacer respectively. High values of A+T contents were detected in both data sets and may explain the high proportions of transversions founded. The observed variation pattern of plastid DNA provides evidence of an important genetic diversity. The overall transition/transversion bias (R) was 0.202 in the intron and 0.27 in the combined regions. The RI index of 0.592 indicates that these combined sequences have clearly more homoplasy then the intron (RI = 0.705) and spacer (RI = 0.777) sequences separately. Phylogenetic trees were generated based on maximum parsimony (MP) and neighbor-joining (NJ) analysis of the chloroplast sequences data. Results proved that a typically continuous genetic diversity characterizes the local fig germplasm. In fact, relationships inferred from the cpDNA analysis suggest several clades, which do not show geographical or tree sex correspondence. Although the level of apparent diversity is considerable, we may conclude that non-coding regions of chloroplast genome provide a new and practical opportunity to evaluate genetic diversity and to discriminate fig cultivars. Revealed cytoplasmic DNA markers are reliable to elaborate a molecular data base to conduct management and breeding programs on local fig germplasm.

1. Introduction

Investigation on genetic diversity and relationships within cultivated species are necessary to highlight priorities for plant genetic resources conservation approaches. Molecular tools have been widely applied in many fields of plant genetics and evolution. Conservation in gene order, content and lack of recombination make the plastome an attractive marker for phylogeny analysis and genetic diversity studies. Chloroplast DNA was the most powerful tool for plant molecular systematics and cytoplasmic polymorphism studies over the last decade through the analysis of restriction site mutations, nucleotide substitutions, structural rearrangements and DNA sequencing (Taberlet et al., 1991; Gielly et al., 1996; Bakker et al., 1998; Molvray et al., 1999; Harding et al., 2000). Universal primers for amplification of several chloroplast introns, intergenic spacers and genes have been published, allowing to address a broad range of systematic and evolutionary questions at all taxonomic levels (Taberlet et al., 1991; Jordan et al., 1996; Hamilton et al., 1999; Cronn et al., 2002; Tate and Simpson, 2003). The intron *trn*L (UAA) and the intergenic spacer between the 3′ exon of *trn*L (UAA) and *trn*F (GAA) were broadly used for phylogenetic inference at intra and interspecific levels (Segraves et al., 1999). In fact, the non-coding regions provide the most practical source of data for phylogenetic inference at lower taxonomic levels (Morton and Clegg, 1993).

The fig tree, *Ficus carica* L. (Moraceae, $2n = 26$), is an old fruit tree associated with the beginning of horticulture in the Mediterranean basin. Cultivated forms with edible fruits were domesticated and selected, during a long history, from spontaneous figs occurring in the region. Being botanically gynodioecious and functionally dioecious, pollination in fig tree is made by the specific wasps (*Blastophagous psenes* L.).

In Tunisia, Fig cultivation is common and traditional and figs are mainly consumed fresh or dried. Fig tree is very often associated

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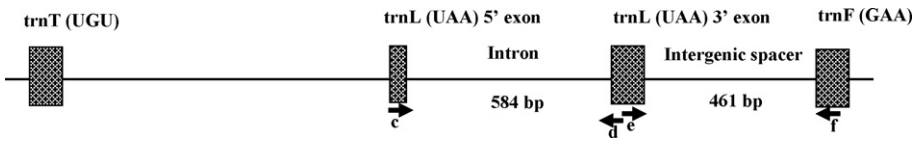

Fig. 1. Structure of the *trnT–trnF* region. Spacer and intron illustration, orientation and the approximate primer sites are presented according to Taberlet et al. (1991).

with other fruit species or as scattered trees in traditional orchards. Some regular plantations are recently established. All grown cultivars are local resulting from old selection and maintained by vegetative propagation. More than sixty cultivars are listed in various reports, but few ones are being propagated in commercial nurseries (Mars et al., 2008). The wide cultivars spread in different fig growing regions all over the country has resulted in ambiguity in their description and denomination. Consequently, problems of synonymy and homonymy may occur. Moreover, threat of genetic erosion, caused by biotic and abiotic stresses, became obvious. Thus, alternative strategies for fig genetic resources management were considered for their optimal use in present and future breeding programs. Morphometric and pomological analysis conducted on local fig resources revealed high level of genetic diversity and permitted to describe cultivars. More recently, molecular markers (RAPD, ISSR, RAMPO, SSR, AFLP and ITS) have been used to fingerprint and assess the genetic diversity and determine its structure and distribution (Salhi Hannachi et al., 2004, 2005, 2006; Chatti et al., 2007; Saddoud et al., 2007; Baraket et al., 2009a,b,c). In fact, the optimal use of genetic resources in future breeding requires studying genetic diversity and implementing strategies to conserve germplasm.

In the study herein, we describe plastid DNA polymorphism in figs and report a comparative analysis of two chloroplastic regions. The intron of *trn*L (UAA) and intergenic spacer between the 3′ exon of *trn*L (UAA) and *trn*F (GAA) (Fig. 1) were sequenced and used for the reconstruction of substitution patterns. Polymorphism of fig germplasm inferred from the chloroplast DNA variation is compared with estimates based on data from the nuclear genome and completion of the two data sets will enable the establishment of genetic relationships among fig cultivars. Additionally, because studies of molecular polymorphism and molecular evolution are complementary, comparisons among different data sets are made to verify how each cpDNA region evolves and contributes to genetic differentiation. Previous studies (Olmstead and Sweere, 1994; Soltis and Soltis, 1998) showed that analysis of combined data sets provide increased phylogenetic signal, resulting in both higher internal support (e.g., bootstrap percentages) and more discriminated genotypes. The implications of these results for *ex situ* conservation of *F. carica* L. and improvement programs are also discussed.

2. Materials and methods

2.1. Plant material

Twenty fig cultivars, identified in centre and south of Tunisia were studied (Table 1). Considered cultivars (18 females and 2 males), represent the economically most important cultivars.

2.2. DNA extraction and amplification

Total genomic DNA was extracted from fresh leaves of single representative adult trees according to the Dellaporta protocol (Dellaporta et al., 1983). DNA concentration was determined by both spectrophotometry at 260 nm and by 0.8% agarose-gel electrophoresis according to Sambrook et al. (1989). Amplification of the *trn*L intron and the adjacent *trn*L–*trn*F spacer was achieved using the forward and reverse primers designed by Taberlet et al. (1991). The primer pairs, denoted 'c'(5′-CGAAATCGGTAGACGCTACG-3′), 'd' (5′-GGGGATAGAGGGACTTGAAC-3′) and 'e' (5′GGTTCAAGTCCCTCTATCCC-3′), 'f' (5′ATTTGAACTGGTGACACGAG-3′), were used to amplify the intron of *trn*L (UAA) 5′ exon and the *trn*L (UAA) 3′ exon (i.e. the intron *trn*L) and the spacer between the *trn*L (UAA) 3′ exon and the *trn*F (GAA) exon (i.e. the *trn*L–*trn*F spacer) respectively. The two non-coding regions of the chloroplast DNA were amplified by PCR in a DNA thermocycler (crocodile III QBIOgene, Illkirch, France). Conditions for PCR amplification were 25 mM of $MgCl_2$, 2 mM of dNTP mix, 1 μM of each primer and 1 unit of DNA *Taq* polymerase (Fermentas) with 20 ng of DNA in a final reaction volume of 25 μL. Cycling conditions were: 94 °C for 4 min as an initial denaturation step before entering 35 cycles each composed of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C. A final extension step of 10 min at 72 °C was usually programmed at the last cycle. Agarose-gel electrophoresis (1.5%) and ethidium bromide staining were used to check the PCR products.

2.3. DNA sequencing

Fig. cultivar's DNA was sequenced for the *trn*L intron and the adjacent *trn*L–*trn*F spacer. The purified PCR products for these regions were sequenced in both strands, by the automated fluorescent cycle sequencing method using the Big Dye Terminator Ready Reaction Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Primers c or d and e or f was used as sequencing primers for the intron and the spacer regions respectively.

2.4. Sequence analysis

Sequences of all the 20 annotated accessions were submitted to NCBI GenBank (Table 1) (Accessions numbers: EU191005–EU191024 for the intron and EU244613–EU244632 for the spacer).

The identity of *trn*L–*trn*F spacer and *trn*L intron sequences was confirmed through a BLASTN search in NCBI data base (Altschul et al., 1997). Aligned sequences in the DAMBE program (Xia, 2000) were analyzed with MEGA version 4.0.2 software (Tamura et al., 2007). Considering gaps as missing data, the data sets were analyzed to estimate polymorphisms and to infer genetic relationships among fig cultivars. For each sequence, length and proportion of GC content were estimated. The observed ratio of the mutational event was calculated. The alignment was manually checked and pairwise sequence divergence between cultivars in *trn*L intron and the combined *trnL* intron and *trnL–trnF* spacer was calculated according to the Kimura-2 method (Kimura, 1980). Phylogeny reconstruction was performed using neighbor-joining (NJ) and maximum parsimony (MP) methods applying MEGA version 4.0.2 software. NJ tree was obtained using the Maximum Composite Likelihood criterion while the MP tree was constructed using the Close-Neighbor-Interchange algorithm with search level three in which the initial trees were obtained with the random addition of sequences (10 replicates). In MP analysis all the characters were assigned equal weights at all nucleotide positions (Fietch, 1971). In both NJ (Saitou and Nei, 1987) and MP (Eck and Dayhoff, 1966) analyses, all posi-