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Chloroplast DNA analysis in Tunisian date-palm cultivars (*Phoenix dactylifera* L.): Sequence variations and molecular evolution of *trn*L (UAA) intron and *trn*L (UAA) *trn*F (GAA) intergenic spacer

Hela Sakka[a,b], Ghada Baraket[a], Sonia Dakhlaoui Dkhil[a], Salwa Zehdi Azzouzi[a], Amel Salhi-Hannachi[a,*]

[a] *Laboratoire de Génétique Moléculaire, Immunologie et Biotechnologie, Faculté des Sciences de Tunis, Université Tunis El Manar, 2092 El Manar, Tunis, Tunisia*
[b] *Faculté des Sciences de Bizerte, 7021 Zarzouna, Tunisia*

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ABSTRACT

Sequences of *trn*L intron and *trn*L–*trn*F spacer of chloroplast DNA of date palm (*Phoenix dactylifera* L.) were analyzed to detect polymorphism and elucidate molecular evolution. The size of these non-coding regions ranged from 364 to 397 for the intergenic spacer, from 545 to 591 for the intron and from 897 to 981 base pairs for the combined sequences. The averages of GC-contents were 33.3%, 35.9% and 34.8% in the spacer, the intron and the combined data, respectively. The overall ration of transition/transversion *R* was of 2.19 for the intergenic spacer, indicating that transitions are more frequent than transversions. Haplotypic and nucleotide diversities showed high level of variation of chloroplast non-coding regions. Phylogenetic trees generated using neighbor joining and maximum parsimony analyses showed two groups. The structure of which is independent of the geographic origin or sex of trees. The observed mismatch distribution of pairwise nucleotide differences for cpDNA differed from the one predicted under a model of expansion, as the shapes of the functions was not unimodal. This deviation was also supported by tests of neutrality, which showed that Tajima's D and Fu's Fs values were mostly negative but always insignificant. These results rejected the hypothesis that there has been recent demographic population growth in this species. We may conclude that date palm chloroplast genome provides a new efficient opportunity to evaluate the genetic diversity and to examine the phylogenetic relationships in this crop.

1. Introduction

In North Africa, as in several tropical countries, oasis culture consists of date palm groves (*Phoenix dactylifera* L. $2n = 36$), which are major factors of social, environmental and economic stability in these regions. In Tunisia, this crop is of great socio-economic importance. In fact, date palm constitutes the principal financial resources and food supplies of oasis cultivators and contributes to the development of subjacent cultures (alfalfa, fig trees, pepper, tomato, etc.). For instance, about 10% of Tunisians depend on date palm and undercover cultures. Investigation on date palm patrimony led to identify more than 250 cultivars (Rhouma, 1994) and this gives evidence of the important genetic diversity in date palm groves. The date palm is highly diverse due to the existence of a large number of cultivars spread over different oases in Tunisia. Morphological characters diversity of date fruits and biochemical markers like isozymes and proteins (Baaziz and Saaidi, 1988; Rhouma, 1994; Ould Mohamed Salem et al., 2001) have earlier been employed for the identification of date fruits; however, these traits are greatly influenced by environmental factors as well as the developmental stages of the plant. Molecular markers have earlier been used for germplasm characterization of date palm cultivars and a considerable genetic diversity has been detected using random amplified polymorphic DNA (RAPD) (Sedra et al., 1998; Trifi et al., 2000), inter simple sequence repeats (ISSR) (Zehdi et al., 2004b; Abdulla and Gamal, 2010), microsatellites (SSR) (Zehdi et al., 2004a, 2012; Elshibli and Korpelainen, 2008), random amplified microsatellite polymorphisms (RAMPO) and the amplified fragment length polymorphism (AFLP) (Rhouma et al., 2011) markers.

Although DNA sequences data and barcoding are now well accepted global standards for species identification (Ali and Choudhary, 2011), they also have some limitations (Nock et al., 2011). However, the chloroplast DNA sequences alone or with combination of nuclear sequences are now being extensively explored

* Corresponding author. Tel.: +216 71 87 26 00; fax: +216 70 86 04 32.
E-mail address: Amel.SalhiHannachi@fsb.rnu.tn (A. Salhi-Hannachi).

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(Kress et al., 2005; Yao et al., 2009). Nevertheless, a number of molecular phylogenetic studies by chloroplast DNA analysis were reported in many species (Tsumura et al., 1995). The slow rate of chloroplast DNA sequence and his structural evolution provides opportunities for identifying genotypes at the species level and below (Palmer, 1987; Wolfe et al., 1987; Birky, 1988). The low frequency of structural changes in the chloroplast DNA (cp DNA) together with a conservative rate of sequence evolution make it an ideal target for plant phylogenetic study (Clegg and Zurawski, 1992; Olmstead and Palmer, 1994). In angiosperms, the chloroplast genome is maternally inherited (Corriveau and Coleman, 1988) and deeper knowledge about its structure, sequence variation, and evolution provides useful information for developing propagation technologies, such as cytoplasmic breeding and transgenic insertion. The date palm chloroplast genome is a typical circular double-stranded DNA molecule, and it shares a common quadripartite structure: a pair of IRs (27,276 bp) separated by the LSC (86,198 bp) and the SSC regions (17,712 bp) (Yang et al., 2010). In a previous study, we have performed the PCR-RFLP procedure to assess the date palm's cpDNA diversity within and between genotypes (Sakka et al., 2004). Thus, it has been assumed that this approach is reliable to determine genetic polymorphisms and examine the ecotypes genetic relationships in this crop. On the other hand, this methodology has allowed the molecular characterization of date palm genotypes. As a result, only 27 over 38 cultivars have been easily identified (Sakka et al., 2004). However, date palm cultivars differed mainly because of a particular agronomy due to mutational events that arise throughout domestication process. Consequently, a loss of information may happen by chloroplast DNA analysis since it corresponds to a small part of the date palm genome. In fact, comparative DNA sequencing seems particularly relevant. This technique is relatively fast, convenient, and offers a large data set of discrete characters. The sequence of a chloroplast gene such as *rbc*L, which has been widely used in inferring phylogeny in plants (Clegg and Zurawski, 1992), is likely to change too slowly to provide enough characters for phylogenetic analysis between congeneric species. Recent results indicate that chloroplastic non-coding regions such as the intron *trn*L (UAA) and the intergenic spacer between the *trn*L (UAA) 3′ exon and the *trn*F (GAA) gene can be used to address questions concerning relationships among closely related species or genera (Van Ham et al., 1994; Gielly and Taberlet, 1994). The intron and the intergenic spacer are useful because they hold a much higher mutation rate (Gielly and Taberlet, 1994; Manen and Natali, 1995; Baker et al., 1999; Jung et al., 2005; Tsai et al., 2006; Pirie et al., 2007; Geleta et al., 2010; Baraket et al., 2009, 2010a,b). Chloroplast DNA (cpDNA) sequence variation is widely used in systematics and phylogenetic inference at different taxonomic levels (Taberlet et al., 1991; Johnson and Soltis, 1994; Liang and Hilu, 1996; Hilu and Liang, 1997; Bayer et al., 2002; Shaw and Small, 2005; Crawford and Mort, 2005). Taberlet et al. (1991) reported that the *trn*T–*trn*L and *trn*L–*trn*F intergenic spacers and *trn*L intron are useful for evolutionary studies at low taxonomic levels and since then, these regions have been used extensively in phylogenetic studies. Here, we investigated the phylogenetic relationships among eight cultivars of date palm based on nucleotide sequences of two non-coding regions in the chloroplast DNA, i.e., the *trn*L (UAA) intron and an intergenic spacer between the *trn*L (UAA) and *trn*F (GAA). The intergenic spacer *trn*L–*trn*F and *trn*L intron evolved faster than codon region in the chloroplast genome (Neuhaus and Link, 1987; Gielly and Taberlet, 1994; Hilu and Liang, 1997) and are often used to study relationships among genera (Johnson and Soltis, 1994; Plunkett et al., 1997), among species (Gielly and Taberlet, 1994; Kajita et al., 1998), and also within species (Fujii et al., 1997). We sequenced these DNA regions hoping that we would get an even better resolution with more reliable technical support than our earlier PCR-RFLP study (Sakka et al., 2003). With these sequences, we could obtain a reliable phylogeny in this crop and allow the examination of its relationships with other *Phoenix* species.

2. Materials and methods

2.1. Plant material

Plant material composed of young leaves, was kindly provided by the Centre de Recherches Phoénicicoles, INRA, Degache in the South of Tunisia. Cultivars were collected from Tozeur, Degache and Gabes localities (Fig. 1). We have analyzed a set of 8 accessions: 6 female trees (Deglet nour, Ftimi, Kentichi, Tazerzite jaune, Rhaimya and Bou Hattam) and 2 male trees: T156 and T159, four *Phoenix* species (*Phoenix reclinata*, *Pseudophoenix vinifera*, *Corypha taliera* and *Wallichia disticha*) and one *Quercus* species (*Quercus rubra*) used as an outgroup (Table 1).

2.2. DNA preparation

Total cellular DNA was extracted according to Dellaporta et al. (1984) with little modifications. Leaves were grounded in liquid nitrogen with a mortar and pestle and extracted for 30 min at 65 °C in an extraction buffer (100 mM Tris–HCl pH8, 50 mM EDTA, 500 mM NaCl, 10 mM CTAB). The use of CTAB is recommended to remove polysaccharides and polyphenolic products (Ait-Chitt et al., 1993; Lothi et al., 1994). After purification, DNA concentrations were estimated using a GeneQuant spectrophotometer

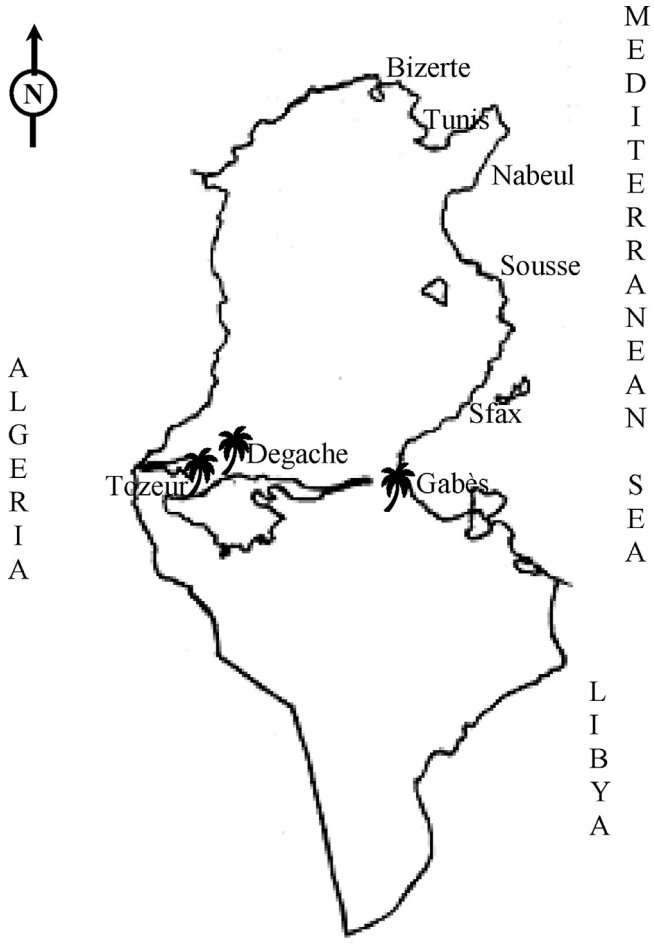

Fig. 1. Tunisia map showing the geographic origin of the analyzed date-palm ecotypes.