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Start Codon Targeted (SCoT) markers provide new insights into the genetic diversity analysis and characterization of Tunisian *Citrus* species

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ABSTRACT

Start Codon Targeted markers were used to establish phylogenetic relationship among seven species from *Citrus* L. genus. Twelve SCoT primers were used for their ability to reveal polymorphism of the targeted codon of initiation. A total of 132 amplicons were generated and 93.9% of them were polymorphic. The polymorphism information content of 0.884 and the resolving power of 75.22 illustrate the efficiency of the tested SCoT primers in highlighting polymorphism. The average Nei's (1973) gene diversity (0.376), the Schannon's index (0.548) and the Gst parameter (0.346) describe an important polymorphism at the interspecies level in *Citrus* genus. Analysis of molecular variance suggested significant genetic differences within species. In fact, 84% of variance occurs within the species, whereas 16% of the variation was recorded among the species of *Citrus*. The limited gene flow (Nm = 0.941) was recognized as a major factor to explain the partition of the observed diversity. The principal coordinates analyses, Neighbor Joining and the Bayesian clustering approach based on the SCoT markers also confirm the discrimination of the species of *Citrus*. Our results confirm the relevance and suggest the effectiveness of the SCoT markers for assessing genetic diversity, characterization and identification of the species of *Citrus*.

1. Introduction

Citrus L. (Family: *Rutaceae*) is native to South-East Asia (Webber, 1967). The most important species are: citron (*Citrus medica* L.), lemon [*Citrus limon* (L.) Burm. F.], lime [*Citrus aurantifolia* (Christm.) Swing.], mandarin (*Citrus reticulata* Blanco), sour orange (*Citrus aurantium* L.), orange [*Citrus sinensis* (L.) Osb.] and grapefruit (*Citrus paradisi* Macf.). The differences between species are a matter of morphological and pomological characters such as size, shape, color and flavor of the fruit (Bonnassieux, 1988). In fact, two major classifications exist for the genus *Citrus* L.: Tanaka (1977) understands 162 species, whereas Swingle and Reece (1967) distinguish only 16 from it. Recently, Mabberley (1997) proposed a new classification of *Citrus* and suggests the presence of three ancestral species, namely *Citrus maxima* (Burm.) Merr. (pummelo), *Citrus reticulata* Blanco (mandarin) and *Citrus medica* L. (citron) and the hybrids of *Citrus* were obtained. The majority of *Citrus* is diploîd, only some natural polyploîd were identified (*Fortunella hindsii*. Tahiti lime) or artificially produced (Roose, 1988). Tunisia is

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considered one of the main *Citrus* producing countries in the Mediterranean basin (GIFruits, 2010) in which *Citrus* is a popular fruit crop of economic importance. In terms of production this sub-sector allows the supply of the local and international markets with fresh fruit for a period going six months a year. *Citrus* exports almost consist exclusively of Maltaise oranges. The *Citrus* resources are characterized by heterogeneity and diversity within and between major varietal, they represent a great potential for selection and breeding. It is thus imperative to use this potential for the conservation, improvement for better management and exploitation of these resources. In this contest, the development of molecular tools for the identification of local cultivars and the analysis of the genetic diversity of a national germplasm is necessary to develop varieties adapted to various environments and preserve our resources. Several molecular technics were used to generate nuclear molecular markers (Bréto et al., 2001; Barkley et al., 2006; Abkenar et al., 2007; Handaji et al., 2012; Garcia-Lor et al., 2013). The approach of chloroplast microsatellite has been demonstrated also for the evaluation of cpDNA in the *Citrus* L. genus (Deng et al., 2007). Recently, a novel and simple molecular technique termed Start Codon Targeted polymorphism (SCoT) has been demonstrated by Collard and Mackill (2009) and used to detect molecular markers in supplement of the popular markers RAPD and ISSR. This technique uses single 18-mer primers in single primer polymerase chain reaction (PCR) targeting different regions of the conserved region flanking the ATG start codon. Indeed, due to the simultaneous binding of the primer on both DNA strands, the sequence between the two binding sites is amplified. This technique has proven effective and reproducible markers and gave a high polymorphism correlated with traits of biological interest (Collard and Mackill, 2009). The goals of the present study, is to investigate the potential of the SCoT markers to reveal molecular polymorphism, to identify and investigate the genetic diversity of Tunisian *Citrus* germplasm represented by species of economical importance.

2. Materials and methods

2.1. Plant material and DNA extraction

The plant material consists of seven Tunisian species of *Citrus* L. genus, including 54 cultivars and each variety is represented by two trees. *Citrus aurantium* L. and *Citrus paradisi* L. species including 10 cultivars originated from the Interprofessional Group Fruit (GIF) located in the region of Cap Bon. *Citrus sinensis* L., *Citrus clementina* L., *Citrus reticulata* L., *Citrus* × *tangelo* L. (Hybrid: Tangerine × pomelo) and *Citrus limon* L. species including 44 cultivars originating from the technical center of *Citrus* situated in Zaouiet Jdidi (Cap Bon) (Table 1). The extraction of total genomic DNA was performed by the automat of extraction (QIAGEN kit) from lyophilized young leaves (20 mg). The quality of the extracted DNA was checked on a 1% agarose gel. The concentration of the DNAs obtained was estimated using a Qubit^{R} fluorometer (Molecular Probes™ invitrogen detection technologies).

2.2. Oligonucleotides and PCR assays

Twelve SCoT primers were used (Collard and Mackill, 2009) to amplify regions that frame ATG start codon in *Citrus* cultivars (Table 2). The solution of 25 μL contained 2.5 μL of Taq polymerase buffer (10X), 20 mM of dNTP, 25 mM of $MgCl_2$, 10 mM of primers, 0.5 U of Taq DNA polymerase, 25–30 ng of DNA and 25 μl of H_2O MiliQ, QSP. PCR reactions were performed in the PTC-100 thermal cycler (Programmable Thermal Controller). The amplification reaction begins with a phase of denaturation at 94 °C for 3 min followed by 35 cycles (denaturation at 94 °C for 1 min, specific appropriate melting temperature for each primer (Tm, °C) for 1 min, elongation at 72 °C for 2 min) with a final extension at 72 °C for 5 min.

2.3. Data analysis

The size of the amplified fragments was estimated on 1.5% agarose gel. The amplicons were coded linearly as the following: the number 1 (present) or 0 (absent). Pairwise genetic dissimilarities of genotypes using Jaccard coefficient (1908) were calculated using DARwin version 5.0.158 (Perrier and Jacquemoud-Collet, 2006). The dissimilarity coefficients were used to perform principal coordinate analyses (PCoA) and construct Neighbor Joining trees (Saitou and Nei, 1987) with a bootstrapping value of 10,000 replications using DARwin version 5.0.158. Molecular variance (AMOVA) analysis was also carried out on SCoT dataset using GenAlEx 6.51 (Peakall and Smouse, 2006). The AMOVA components were used as an estimation of molecular diversity at the hierarchical level among and within the species of *Citrus*. The parameters of the genetic diversity such as the average Nei's (1973) gene diversity (H), the Schannon's index (I) and the effective number of alleles (Ne) (Maruyama and Kimura, 1980) were calculated by the POPGENE software version 1.32 (Yeh et al., 2000). The total genetic diversity (Ht) and the mean genetic diversity within species (Hs) were calculated using POPGENE software version 1.32 (Yeh et al., 2000). The Nei's Gst (Nei, 1977) and Nm parameters (Nei, 1987), a measurement of the inter-specific diversity and the gene flow were calculated. The Shannon's index (I) was calculated for each locus (Lewontin, 1972). The polymorphism information content (PIC) was estimated using the formula of Smith et al. (1997). The resolving power of primers (Rp) was calculated based on the formula of Gilbert et al. (1999).