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Molecular phylogeny and genetic diversity of Tunisian *Quercus* species using chloroplast DNA CAPS markers

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ABSTRACT

Chloroplast DNA variation was studied in five evergreen *Quercus* species from the Western part of Tunisia using Cleaved Amplified Polymorphic Sequence (CAPS) technique. Five primer pair/endonuclease combinations have been used. Chlorotypes of *Quercus* species have been identified. The enzyme *Hinf*I was more efficient in detecting polymorphism in oak species than *Taq*I. The phenogram showed five groups defining the five studied oak species: *suber* group, *afares* group, *coccifera* group, *canariensis* group and *ilex* group. The topology of phenogram showed that the classification depends only on species and independently of their geographic origin. The principal component analysis (ACP) corroborated the results of the tree branching and confirmed the existence of five species groups. Our results showed a genetic proximity between *Quercus afares* and *Quercus coccifera* species that may be due to temperature tolerance or the demographic history of these species. Nevertheless, a high value of G_{ST} calculated ($G_{ST} = 1$), suggesting that the maximum of variation is maintained among oak species. This result was confirmed by the low value of the genetic diversity within species ($h_S = 0$), the value obtained of the total genetic diversity ($h_T = 0.378$) and the absence of gene flow between species ($N_m = 0$). A high genetic proximity has been registered between *Q. afares*, *Quercus suber* and *Quercus canariensis*. Moreover, *Q. afares* shared the chlorotype of *Q. suber* and *Q. canariensis* which suggests its hybrid origin.

1. Introduction

Evergreen oaks (2n = 24) are diploid and they dominate most forest habitats in the Mediterranean basin (Takhtajan, 1986). Five species are particularly important in this region: holm oak (*Quercus ilex* L., 1753), the cork oak (*Quercus suber* L., 1753), canary oak (*Quercus canariensis*, Willd, 1809), Pomel oak (*Quercus afares*, Maire, 1933) and kermes or holly oak (*Quercus coccifera* L., 1753). Although their distributions and habitats are overlapping but ecological differences exist among them. *Q. suber* is an evergreen tree which has a great importance in the North-Western of Tunisia, for both economic and ecological values of cork (Passarinho et al., 2006) and plays a key role in maintaining biodiversity (Hidalgo et al., 2008). The distribution of *Q. suber* is limited to the Northern part of Tunisia and represents 10% of the total forest area in the North-West of Tunisia

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and 4.3% of the world's cork oak forest area. Although, the conservation of this forest genetic patrimony is very important. However, *Q. Ilex* dominates the forests of all kinds of substrates. The Holm oak acorns represent a major component in the feeding systems of many Mediterranean wild and livestock species. Moreover, it is the basic feed ingredient for domestically meat pigs (Ruperez, 1957).

Q. coccifera and *Q. suber* are more thermophilous than *Q. ilex* and appear to have contrasting environmental requirements. Kermes oak, is more frequent in arid and disturbance-prone environments, predominantly on limestone substrates (Tutin et al., 1993), while the cork oak has strict humidity and soil requirements being only found on acidic or decarbonated soils (Martínez-Ferri et al., 2004). These species are sympatric in many areas, but some differences in their ecological requirements produce distinct responses to environmental conditions and hence different evolutionary histories. Previous studies have shown differences in their genetic variation patterns in both nuclear and cytoplasmic levels (Belahbib et al., 2001; Lumaret and Jabbour-Zahab, 2009). Inter-specific gene flow has already been detected in the crosses *Q. coccifera*–*Q. ilex* and *Q. suber*–*Q. ilex* (Belahbib et al., 2001). However, *Q. suber* and *Q. canariensis* are sympatric with *Q. afares* over most of their geographical distribution.

Most studies on genetic variation of forest trees have been carried out using nuclear markers (Belahbib et al., 2004–2005; Shiran et al., 2011). The chloroplast DNA, is a slow mutating, non-recombining, clonally inherited genome (Wolfe et al., 1987) which is maternally inherited in oaks (Dumolin et al., 1995) and typically shows low intra-specific variation. Although in the last decade, the development of new markers has allowed the detection of enough variation to assess phylogeographic patterns. More recently, chloroplast microsatellites (cp SSR) have been developed in French oak trees for haplotype discrimination of oak material (Deguilloux et al., 2004). Moreover, studies in *Quercus* species have been reported using PCR-RFLP or CAPS technique (Petit et al., 2002) to elucidate the postglacial recolonization history in Europe (Petit et al., 2003) and America (Grivet et al., 2006). This approach has been shown to be a powerful tool for phylogenetic reconstruction at both inter- and intra-specific levels and for identifying populations that have expanded from refugia during the last glacial period (Abbott et al., 2000). Palynological data suggest a southern origin for the European oaks (Huntley and Birks, 1983). Phylogeographic studies (Dumolin-Lapègue et al., 1997a) carried out using chloroplast DNA markers have demonstrated the existence of three glacial refugial areas localized in the three peninsulas: Iberia, Italy and the Balkans. Studies based on cp DNA-RFLP of cork oak (Lumaret et al., 2005) showed that this technique cannot differentiate between very small fragment-size changes (insertions/deletions) and are easily detected by PCR-RFLP. This technique was used successfully to analyze the variation of cpDNA fragments in *Q. suber*, *Q. ilex* and *Q. coccifera* populations sampled predominantly in Iberia and Morocco (Lumaret et al., 2005; López-de-Heredia et al., 2007). In the present work, cp PCR-RFLP analysis of five Tunisian *Quercus* species (*Q. canariensis*, *Q. suber*, *Q. ilex*, *Q. afares* and *Quercus cocciffera*) using several DNA fragment/endonuclease, was performed to identify phylogenetically informative characters based on small fragment changes, to identify oak chlorotypes, to establish the genetic relationships between them and to make a comparative study of the genetic structure of the three Tunisian oaks (*Q. canariensis*, *Q. suber* and *Q. afares*) in order to verify if *Q. afares* acts as species apart or as a hybrid species.

2. Material and methods

2.1. Plant material

Five oak species were used in this study: *Q. suber* (cork oak), *Q. coccifera* (kermes oak), *Q. canariensis* (Canry oak) *Q. afares* (Pomel oak) and *Q. ilex* (holm oak) and are collected from the North-Western region of Tunisia (Table 1). Distribution range and local species presence are governed by climatic and edaphic factors (Rameau et al., 1989). A total of 210 individuals from Tunisia of *Q. suber* (85 samples consisted of 17 populations), *Q. ilex* (25 samples represented by 5 populations), *Q. coccifera* (3 populations composed of 15 samples) *Q. canariensis* (14 populations consisted of 70 samples) and *Q. afares* (3 populations represented by 15 samples) covering the Western area of Tunisia were sampled. Moreover, samples of *Q. canariensis*, *Q. suber*, and *Q. ilex* from Morocco (5 individuals, for each species) have been used as controls for each species. In each stand, adult leaves were collected from 5 nonadjacent trees per species.

2.2. Extraction of DNA and amplification

The DNA was extracted using a modified protocol of Doyle and Doyle (1990) modified by Dumolin et al. (1995). The polymerase chain reaction (PCR) method was detailed in Demesure et al. (1995). Five pairs of primers were used to amplify the following chloroplast fragments: *trn*C[tRNA-Cys(GCA)]- *trn*D[tRNA-Asp(GUC)] (CD), *trn*T[tRNA-Thr(GUG)]- *trn*F[tRNA-Phe(UGU)] (TF), *trn*D[tRNA-Asp(GUC)]- *trn*T[tRNA-Thr(GGU)] (DT), *psa*A[PSI (P700 apoproteine A1)]-*trn*S[tRNA-Ser(GGA)] (AS) and *trn*S[tRNA-Ser(GCU)]- *trn*R[tRNA-Arg(UCU)] (SR) (Taberlet et al., 1991; Demesure et al., 1995; Dumolin-Lapègue et al., 1997b; Grivet et al., 2001).

2.3. Enzymatic digestion

The amplified fragments were digested with five units of either *Hinf*I or *Taq*I enzymes. Digestion products were separated by electrophoresis in polyacrylamide gels as described in El Mousadik and Petit (1996). Fragments were revealed with ethidium bromide method in order to check polymorphisms. Fragment sizes were estimated using a 1 kb ladder (Gibco BRL).