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The evolution of *rbcL*: A methodology to follow the evolution patterns of *Medicago* and *Sulla* (*Fabaceae*) genera

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ABSTRACT

The chloroplast *rbcL* gene is selected to be a standard barcode for the phylogenetic analyses of several land plants. Using universal primers pair, the 3' frame was used to assess the phylogeny of the two Legume genera *Medicago* and *Sulla* and the evolution of the translated protein. The multiple alignment exhibited high conservation rates (97.56% and 96.43% for *Medicago* and *Sulla* respectively), which might be a brake for the phylogenetic assessment. Nevertheless, the topologies of the cladograms drawn using Maximum Likelihood method, showed a low intraspecific divergence and a slightly high interspecific variation, which confirms the efficiency of *rbcL* for such characterization' analyses. Besides, the composition and the patterns of the Amino-Acid sequence and the deduced Codon Usage, highlighted a non-aleatoire distribution of the codons. Thus, the non-synonymous substitutions rates traduced a positive selection occurring in the rbcL of the *Medicago* and *Sulla* species.

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1. Introduction

The *rbcL* (*ribulose-1,5-bisphosphate carboxylase/oxygenase*) encodes for the large subunit 55-kDa of the *Rubisco* (EC 4.1.1.39), which transforms the CO_2 and ribulose-1,5-bisphosphate (RuBP) into two molecular 3-phosphoglyceric acid, catalyzing the first reaction of CO_2 fixation in the photosynthesis. Moreover, *RuBisCo* interferes in the photorespiration machinery (Krapralov and Filatov, 2007). In systematics and phylogenetics, this gene is cataloged by the *Consortium of Barcode Of Life* CBOL as a DNA barcode, able to identify between all land plants' genera and species. It was even recorded as a species-level discriminator at a variety of taxonomic levels (Kuzmina et al., 2012). Kress and Erickson (2007) designed a new reverse primer to amplify by PCR the valuable region and many phylogenetic analyses of angiosperms were undertaken. In fact, *rbcL* is known to be conservative, which led its investigation interesting in phylogenetic studies of green plants (Morton et al., 2002; Zhang et al., 2011). Besides, the understanding of the codons distributions allows to follow the evolution of the translated protein.

The *rbcL* gene is investigated herein among and between two forage crops *Sulla* and *Medicago,* originating from North Africa. The two genera belong to an important cluster (*Fabaceae*) of the Angiosperms that comprises almost 14000 taxons

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Abbreviation

AA	Amino Acid
CAI	Codon Adaptation Index
CBI	Codon Bias Index
CBOL	Consortium of Barcode of Life
dN	rate of Non-Synonymous mutations
dS	rate of Synonymous mutations
ENC	Effective Number of Codons
GC	Guanine-Cytosine content
ITS	Internal Transcribed Spacer
MAS	Multiple Alignment Sequences
ML	Maximum Likelihood Method
NS	number of Non-Synonymous mutations
ORF	Open Reading Frame
RSCU	Relative Synonymous Codon Usage
S	number of Synonymous mutations
w	Non-Synonymous/Synonymous rate

nested within the Legume family (Lewis et al., 2005). Even if many studies investigated *Medicago* L., the phylogeny of this genus still not well-defined. In fact, many polytotomic links were reported (Bena et al., 1998). The use of barcodes is then required to resolve these fogginess and to clarify the relationships between the species.

Regarding the *Sulla* genus, the new taxonomy of Choi and Ohashi (2003) based on morphological aspects was confirmed by several molecular markers such as ITS and SSR (Chennaoui et al., 2007; Zitouna et al., 2013). These studies converge to a recent and slow evolution of the species.

The purpose of the current study is to demonstrate the effectiveness of the barcode *rbcL*, on *Medicago* to resolve the polytomy, and on *Sulla* genus to precise the phylogeny.

In this context we specially investigated: i) the phylogeny using Maximum Likelihood method ii) the polymorphism in the nucleotides and Amino-Acids sequences and (iii) the factors that influence the pattern of the codon usage.

2. Material and methods

2.1. Study areas

The two analyzed genera are represented by six North African species. *Sulla* is composed by *Sulla coronaria* L., *Sulla carnosa* Desf., *Sulla capitata* Desf., *Sulla spinosissima* L., *Sulla flexuosa* L., and *Sulla pallida* Desf., while *Medicago truncatula* Gaertn., *Medicago scutellata* Mill., *Medicago murex* Willd., *Medicago polymorpha* L., *Medicago minima* L., and *Medicago orbicularis* (L.) Bartal., constituted the six annual species of *Medicago*.

The provenance of *Sulla* species and their characteristics are well described by Zitouna et al. (2013). The Morrocan seeds were provided from SARDI core-collection under accessions codes as reported by Zitouna et al. (2014). All *Sulla* and *Medicago* species are $2n = 16$, except *Medicago polymorpha* and *M. murex*.

2.2. PCR amplification and DNA sequencing

To test the efficiency of *rbcL*, an intraspecific study was carried on 10 individuals belonging to each of *Sulla coronaria* and *Medicago murex*. The other species were represented by a single sequence. Totally, we examined 33 specimens.

Small amounts of fresh seedlings were placed into racked sterile tube and ground into fine powder for DNA extraction following manufacturer protocols of the « Pure Link Plant-Invitrogen » commercial kit.

PCR reactions were carried on a GeneAmp-PCR System 9700. The PCR conditions followed the suggestions of the CBOL working group. Kress and Erickson (2007) created the new reverse oligonucleotide. Noting that the same primers were used on the sequencing step, carried with the Big Dye Terminator Ready Reaction Kit on a ABI PrismTM 3130 DNA automated sequencer (Applied Biosystems; HTDS, Tunisia).

2.3. Statistical analyses

The *rbcL* nucleotide and Amino-Acid (AA) sequences were prealigned with MUSCLE software and then refined on the Multiple Alignment step via SeaView version 4 software (Gouy et al., 2010). The non-alignable regions were excluded prior to efficient analyses and results. ORF regions were defined using *Open Reading Frame Finder* implemented on the NCBI webserver (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

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