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Identification of a Polymorphic Region in the Chloroplast Gene rbcL by Ecotilling

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Abstract: This study describes the application of the EcoTILLING method, using the denaturing High-Performance Liquid Chromatography (DHPLC) system, for the detection of a polymorphic region in the chloroplast gene *ribulose* 1, 5-bisphosphate carboxylase gene (*rbcL*) of *Eryngium* spp. Five *Eryngium* L. species: *E. maritimum*, *E. creticum*, *E. glomeratum*, *E. campestre*, *E. billardieri* and two ecotypes of *E. creticum* were collected from several locations in Syria and used in this study. Chromatographic data, confirmed by sequencing results, showed the presence of two SNPs in *E. glomeratum* (C/T and A/G) and one SNP in each of *E. campestre*, *E. creticum* eco. Slenfeh and *E. creticum* eco. Dara'a (A/G). The detected polymorphic region was tested for a successful separation of plant families and sub families. The procedure described in this paper enables low cost, highly sensitive and high-throughput sample screening which enables the assembly of information on candidate polymorphic regions in genes of interest to phylogenetics.

Key words: *Eryngium* spp., DHPLC, SNPs detection

INTRODUCTION

DNA polymorphism widely exists in various plant species and plays an important role in biological evolution. The variation in nucleotide sequences can be a major accountant for natural variation among plant species. Sources of variation can be natural or induced and the outcome can be phenotypic and/or functional variation among such species. Biologists and taxonomists have been involved in studying the natural variation among species and several projects have been launched to study the relations among species and the diversity of life on earth (Maddison *et al*., 2007). The exploitation of certain genes such as: rbcL, atpB, 18S rDNA, 26S rDNA, matK, ndhF and atpA in plant species phylogenetics has a rewarding knowledge in terms of analyzing relationships among plant species such as, the monocots (Chase *et al*., 2000, 2006; Soltis *et al*., 2000).

The ribulose-1,5-biphosphate carboxylase/oxygeanse (Rubisco), the most abundant protein on earth (Ellis, 1979), controls both the reduction of CO_2 and the oxygenolysis of ribulose-1,5-bisphosphate which indicates its agricultural and environmental significance (Xu and Tabita, 1996). Rbisco is composed of two types of subunits, the large (L) and small (S) subunits. Whereas, the rbcS vary among diverse organisms, the rbcL primary structure is relatively well conserved (Xu and Tabita, 1996). The rbcL gene has been used in inferring the phylogenies in a number of gymnosperms (Bousquet *et al*., 1992; Chase *et al*., 1993; Wang *et al*., 1999). rbcL gene has been found to provide a wealth of informative characters that resulted in the best resolved and supported trees obtained in studying the molecular phylogenetics of Meliaceae (Muellner *et al*., 2003).

The DHPLC detection system has been vastly exploited in discovering disease-causing mutations and SNPs. It has been proved to be a high throughput, sensitive, specific and robust platform for the detection of DNA variants (Yu *et al*., 2005; Omrani and Nordenskhold, 2005; Bennett *et al*., 2001). McCallum *et al*. (2000) introduced TILLING (Targeting Induced Local Lesions IN Genomes) and the use of DHPLC to detect base pair changes in plant genomes by heteroduplex analysis. The TILLING procedure is considered to be a high throughput, low cost and high accuracy approach compared with the other methods (McCallum *et al*., 2000; Colbert *et al*., 2001; Till *et al*., 2006; Elias *et al*., 2009). The TILLING technique combines the chemical (EMS) mutagenesis (Koornneef *et al*., 1982), the creation of mutant populations and mutation discovery in plants. Whereas, its derivative, the EcoTILLING technique, involves mainly the detection of natural variation in natural populations (Comai *et al*., 2004). EcoTILLING has been successfully conducted to survey genetic variation in several plant species using nuclease cleavage and DHPLC (Gilchrist *et al*., 2006; Rakshit *et al*., 2007; Elias *et al*., 2009; Till *et al*., 2010; Fusari *et al*., 2011).

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The genus of our study is *Eryngium* which represent a wide range of species adapted to harsh environmental conditions such as drought and saline conditions (Jawdat *et al*., 2010). *Eryngium* L. species are known for their importance in the field of medicinal plants research. Species such as *E. expansum*, *E. pandanifolium*, *E. rostratum*, *E. vesiculosum* (Brophy *et al*., 2003) and *E. bourgatii* (Pala-Paul *et al*., 2005) have been studied to assess essential oils. Other *Eryngium* L. species have been studied to evaluate the anti-inflammatory and antinociceptive activity (Kupeli *et al*., 2006). The genetic variation and characterization studies of *Eryngium* spp. (Apiaceae-subfamily Saniculoideae) have been conducted in several parts of the world (Jawdat *et al*., 2010; O'Leary *et al*., 2004; Worz, 2004; Andrada *et al*., 2001; Clausing *et al*., 2000; Gaudeul *et al*., 2000). The genus includes about 250 species in the world (Pimenov and Leonov, 1993; Worz, 2004). The distribution and phylogeny of *Eryngium* L. species in Syria were fully described in Jawdat *et al*. (2010) and were found to offer a rich source of genetic material of a great potential as medicinal plants that are adapted to extreme environmental conditions. This study investigates the efficiency of the DHPLC system in identifying polymorphic region (s) in the chloroplast rbcL gene of some *Eryngium* species in Syria by EcoTILLING. This paper also shows the utility of our findings in generating an examplary phylogenetic tree that separates plant families and sub-families into discrete clades.

MATERIALS AND METHODS

Regions of collection and identification of *Eryngium* spp. plants: Plants of the Family Apiaceae belonging to the genus *Eryngium,* were collected during the growing season in 2006 from different regions in Syria. Collection sites covered the northwest (Al Jaboul), west (sea shore and Slunfa) and south (Da'ara) of Syria and ranged from areas at the sea shore to mountainous areas 1400 above the sea level with rain falls ranging from 250 to 1000 mm (Jawdat *et al*., 2010). Seed collection, sampling and the morphological identification of plants were fully described in Jawdat *et al*. (2010).

DNA extraction: Total genomic DNA of collected plants was extracted from leaf material of each of the *Eryngium* L. species under study. DNA extraction was conducted following the protocol of Leach *et al*. (1986). Total DNA concentration was detected by Spectrophotometer (Gene quant, Amersham Biosciences, USA) and the concentration was adjusted to100 ng μL^{-1}.

Amplification of rbcL gene in *Eryngium* spp: Sequences of the rbcL gene of our studied *Eryngium* species were not available in the databases when commencing the project. Hence, the primer pairs were designed to in- silico amplify fragments of the *Eryngium giganteum* ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene sequence (gi|75758074|) using the Vector NTI (9.0.0) package. The primers were tested using blastn suite at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to detect homology to targeted gene. Primers were synthesized using the PolyGen DNA synthesizer (PolyGen DNA-Synthesizer, Germany). Three primer pairs (Table 1) succeeded in amplifying clear single bands with no primer dimers and were used to amplify the three gene fragments (amplicons) in our studied samples.

The iCycler PCR machine (BIO-RAD, USA) was used for the amplification of genomic DNA. The amplification reactions of total genomic DNA were performed in 50 μL reaction volumes, containing 10 X buffer minus Mg^{++}, 4 mM Mg^{++}, 0.4 mM dNTP mix, 0.4 μM of each forward and reverse primers, 1.25 units of Taq DNA polymerase (5 U μL^{-1}) Recombinant (Invitrogen, USA), 100 ng of DNA template and d.d.water to adjust to final volume. The cycle program included an initial 4 min denaturation at 95°C, followed by 30 cycles consisting of 45 sec at 95°C, 45 sec at 64°C for primer annealing and 1 min at 72°C with a final extension at 72°C for 7 min. Amplification fragments were separated on 1.5% agarose gels in 1X TAE buffer, stained with ethidium bromide and subjected to a UV transilluminator (BIO-RAD Documentation System, USA.). Amplicons were sequenced using ABI, 310 (USA) to verify results.

SNPs detection using DHPLC: This analysis was carried out on automated DHPLC system equipped with a DNASep® Cartridge supported with the Navigator™ software (v.1.5.2) (Trasngenomic, USA). About 5 μL of each PCR product containing 100 ng DNA was denatured for 4 min at 94°C and then gradually reannealed by decreasing the temperature from 94 to 40°C over a period

Table 1: Primer combinations used to amplify rbcL gene fragments in studied *Eryngium* spp.

Amplicon	Forward primer (5'-3')	Reverse primer (5'-3')
ErbcL 1	GGATTCAAAGCTGGAGTTAA	CGACCATACTTGTTCAATTTATCTC
ErbcL 2	GAGATAAATTGAACAAGTATGGTCG	CGAAAGTGCATACCATGATT
ErbcL 3	AGACAGAAGAATCATGGTATG	TCTCCTTCCATACCTCACAA

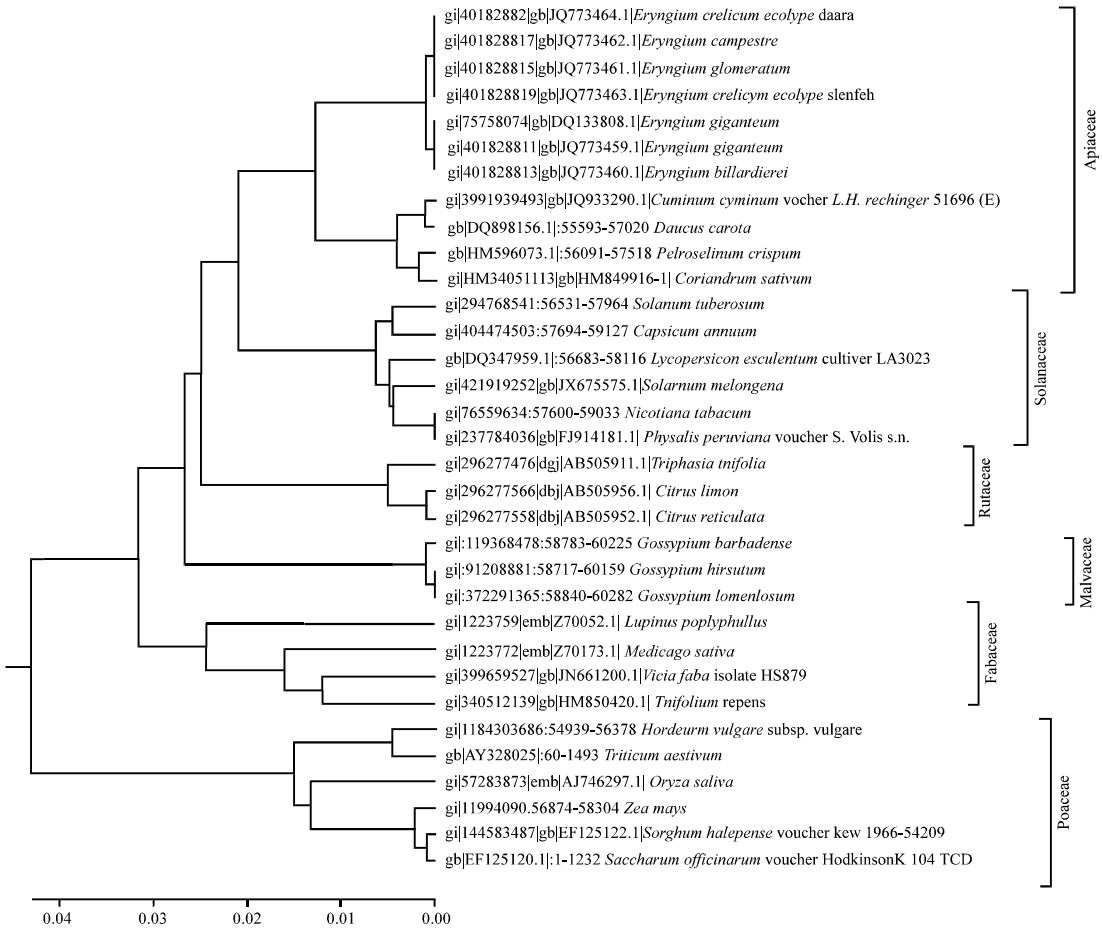

Fig. 1: Construction of the rbcL gene tree using the UPGMA method included in the MEGA (5.02) software. GenBank accession numbers precede species Latin names

of 50 min in order to enable the formation of heteroduplexes. The PCR products of all tested *Eryngium* species were then separated using DHPLC (flow rate of 0.9 mL min^{-1}) over a period of time (approx.10 min for each sample) through a linear acetonitrile gradient. The separated PCR products were analyzed against the corresponding rbcL amplicon sequence of the input data, *E. maritimum*. The column mobile phase consisted of a mixture of two buffers, buffer A (0.1 M triethylammonium acetate TEAA, pH 7.0) and buffer B (0.1 M TEAA, 25% (v/v) acetonitrile, pH 7.0). The mobile phase temperature required for the optimal resolution of homoduplex and heteroduplexes DNAs was determined experimentally by injecting one PCR product at increasing temperature until a significant decrease in sample retention time was observed. The predicted WAVE melting temperature for our amplicon ErbcL 2 was 57.4°C and the reliable DHPLC elution conditions are shown in Table 2. *E. maritimum* was used as a negative control (a reference sample) and chromatograms of all samples were overlaid with one from the negative control.

Table 2: Separation gradients and solution A and B percentages

Time (min)	Solution A (%)	Solution B (%)
0.0	47	53
0.5	42	58
5.0	33	67
5.1	0	100
5.6	0	100
5.7	47	47
6.6	47	47

Samples with extra peaks (one, two or more), or with a difference in peak appearance, were scored as positive.

rbcL cholorplast gene tree: Sequences of amplicon 2 of our studied samples were aligned against the corresponding region in rbcL gene of other plant species that belong to different families using the CLUSTAL W method embedded in MEGA (5.02) software (Tamura *et al*., 2011). The construction of the rbcL gene tree was performed using the UPGMA method (Sneath and Sokal, 1973) included in the MEGA (5.02) software. Sequences used in building this rbcL tree and their GenBank accession numbers are included in Fig. 1.

RESULTS

The present study illustrates the efficient application of EcoTILLING method combined with the use of DHPLC, a mutation detection system, in identifying polymorphic regions in the highly conserved rbcL gene sequence.

Three amplicons were produced (ErbcL 1, ErbcL 2 and ErbcL 3) in all our studied samples and sizes were confirmed, by sequencing, to be 426, 460 and 453 bp, respectively.

The presence or absence of chromatographic alterations was investigated when screening studied samples individually against the initial input data sequence used for this study, the rbcL amplicon sequences of *E. maritimum*. No chromatographic alterations were detected in the amplicon ErbcL 2 in all studied species. On the other hand, ErbcL 1 and ErbcL 3 amplicons showed varied chromatographic alterations when analyzed against the input data of *E. maritimum*.

Further SNP analysis on ErbcL 2 amplicon was conducted by pooling in pairs, the reference PCR product sample; *E. maritimum* and each of the other samples to uncover possible polymorphisms. The pooling phase and the detection system succeeded in discriminating samples with chromatographic alterations against *E. maritimum* except for *E. billardieri* which showed a similar chromatogram. An example of chromatographic alterations is illustrated in Fig. 2.

To confirm results and identify the SNPs, sequencing of ErbcL 2 was conducted in all samples. The SNPs found in our work were both transitions; purine-purine (C/T) and pyrimidine-pyrimidine (A/G). *E. glomeratum* showed a synonymous SNP (C/T) which gave the same amino acid serine as in the reference sample *E. maritimum*. However, a nonsynonymous SNP (A/G) produced a substitution of an isoleucine for a valine residue at position 246 in all studied species except in *E. maritimum* and *E. billardieri*. The latter transition mutation may be functionally neutral due to the same nonpolar nature of both amino acids. A replacement of isoleucine by valine has been observed in *Flaveria* spp. rbcL gene and was reported in Kapralov *et al*. (2011).

Our sequences were submitted to GenBank at the National Center for Biotechnology Information (NCBI) available at http://www.ncbi.nlm.nih.gov/genbank/using BankIt submission tool. GenBank has assigned the following accession numbers for our sequences: JQ 773459 (*E. maritimum*), JQ 773460 (*E. billardieri*), JQ 773461 (*E. glomeratum*), JQ 773462 (*E. campestre*), JQ 773463 (*E. creticum* ecotype: Slenfeh) and JQ 773464 (*E. creticum* ecotype: Da'ra).

This detected polymorphic region, ErbcL 2, was tested for a successful separation of plant families and sub families. Sequences of amplicon 2 (460 bp) of our studied samples were aligned against the corresponding region in the rbcL gene of 27 plant species that belong to

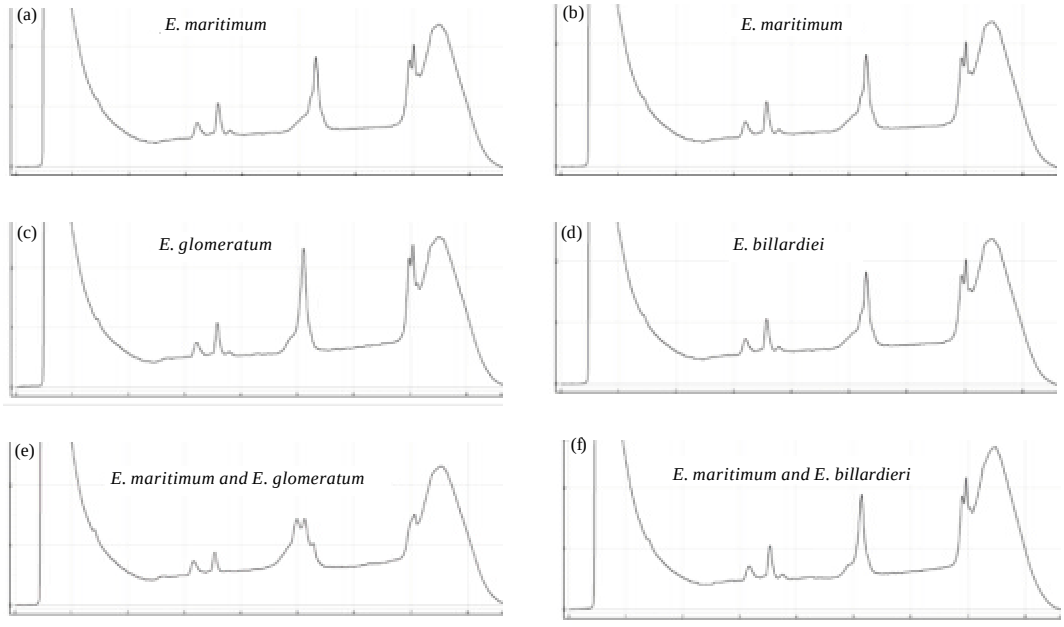

Fig. 2(a-f): DHPLC Chromotographic output of individual ErbcL 2 amplicons of (a,b) *E. maritimum*, (c) *E. glomeratum*, (d) *E. billardieri*, (e) pool of *E. maritimum* and *E. glomeratum* and (f) pool of *E. maritimum* and *E. billardieri*

different families (Apiaceae, Solanaceae, Rutaceae, Malvaceae, Fabaceae and Poaceae). The aligned sequences were used to build a phylogenetic tree using the UPGMA method (Fig. 1). Results indicated the presence of two main clusters, where the smaller cluster contained plant species from the Poaceae family and the other families were joined in the bigger cluster. The Poaceae cluster was divided into a cluster that contained two species (*Hordeum vulagre* L. and *Triticum aestivum* L.) from the Pooideae subfamily and another cluster that included three species (*Zea mays* L., *Sorghum halepense* (L.) Pers. and *Saccharum officinarum* L.) from the Panicoideae subfamily. *Oryza sativa* L., which belongs to the Ehrhartoideae subfamily, formed a bigger cluster when joined along with the Panicoideae cluster.

The dicots entries where joined in a big main cluster that has separated plant families (Apiaceae, Solanaceae, Rutaceae, Malvaceae and Fabaceae) into sub clusters. The genus *Eryngium*, which belongs to the Apiaceae family and the subfamily Saniculoideae, was separated from the other Apiaceae species which belong to the subfamily Apioideae, the leafy Parsley and coriander and both carrot and cumin. The polymorphic region succeeded in joining both carrot and cumin (subtribe: Daucinae), in one small sub-cluster. It has also joined both parsley and coriander, which belong to different tribes under the same subfamily, in another sub-cluster. Understandably, the number of input species is small, but it serves as an exemplary testing of polymorphisms in separating plant families and subfamilies.

DISCUSSION

Our study took the advantage of using rbcL gene, taking into consideration that the coding rbcL gene is easily amplified and sequenced in most land plants and has an impact in phylogeny investigations by providing a reliable placement of a taxon into a plant family and genus (Kress and Erickson, 2007). The easiness in amplifying rbcL gene will be of help in diminishing any technical drawbacks when analyzing the amplified samples using the DHPLC apparatus. However, the latter study does not encourage the use of rbcL gene singly to discriminate, due to the low level of mutation rate that distinguish this gene in particular and other plastid coding regions (Adams and Palmer, 2003). The rbcL gene is vastly utilized in plant phylogenies and evolution studies. The gene is known for its slow synonymous nucleotide substitution rate and its functional constraint (Wolfe *et al*., 1987). First suggestions that rbcL gene sequence was appropriate to use in phylogenetics studies were from Ritland and Clegg (1987) and Zurawski and Clegg (1987). Small scale phylogeny studies based on rbcL sequences were followed (Doebley *et al*., 1990; Kim *et al*., 1992). However, the first collaborative large-scale phylogenetic analysis using collected rbcL sequence data for a broad sampling of seed plants was conducted by Chase *et al*. (1993). The rbcL gene sequence alone and along with other chloroplast and nuclear DNA sequences have been extensively used to resolve plant species phylogenies and evolution.

The literature is now enriched with publications that support the appliance of multi-locus barcodes to maximally discriminate among plant species (Kress and Erickson, 2007; CBOL Plant Working Group, 2009). The major preference of the Plant Working groups of the Consortium for the Barcode of Life (CBOL) was to recommend a core-barcode consisting of portions of two plastid coding regions, rbcL+matK (CBOL Plant Working Group, 2009). The first plastid coding region, rbcL barcode, is easy to amplify, sequence and align in most land plants, despite it having only modest discriminatory power (Newmaster *et al*., 2006; Hollingsworth *et al*., 2011). This compensates for the difficulty in amplifying the powerful discriminately region of matK, which is the most rapidly evolving coding region of the plastid genome (Hilu and Liang, 1997).

EcoTILLING has proved practical when surveying SNPs in multi loci genes in large numbers of individuals compared to the accurate but expensive sequencing method (Gilchrist *et al*., 2006; Wang *et al*., 2007).

Our current study focuses on the practicality, the high efficiency and the potential of the DHPLC detection system in EcoTILLING for the discovery of polymorphic regions in highly conserved genes. The current work provides a potential service to large and medium-scale phylogeny projects that are using plastid regions such as the rbcL gene sequence in terms of reducing unnecessary sample sequencing and the coverage of a large number of samples per day.

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