GENETIC AND ECOLOGICAL ANALYSIS OF PROTECTED-PRIORITY PLANTS (DIRECTIVE 92/43/EEC) IN CRETE

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INTRODUCTION

Crete, from the floristic point of view, is considered one of the richest territories in Europe. Specific rare endemics namely: *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica*, *Hypericum aciferum* and *Phoenix theophrasti* (Fig. 1) (the latter subendemic) are considered to be critically endangered due to tourism, farming, sheep and goat grazing, uncontrolled access resulting in trampling and plant collection, fires and finally habitat alteration through deforestation and drainage. All Cretan populations of the species are included in the Natura 2000 sites proposed for Greece. Within the framework of LIFE-Nature 2004 Project: *A Pilot Network of Plant Micro-Reserves in Western Crete (Chania Prefecture)-Action A.5* compose an effort for the estimation of the genetic diversity of these priority species. The specific objectives of this study are (i) determination of genetic diversity in light of the molecular sequencing data of *rbcL* (a gene encoding the large subunit of ribulose-1,5-bioshosphate-carboxylase), *matK* (encodes maturase K) genes of *cp*DNA and internal transcribed spacer ITS of nuclear ribosomal DNA, (ii) to use available data to produce reasonable preliminary conclusions about the conservation status of the species and to provide genetic data for the conservation LIFE-Nature 2004 project.

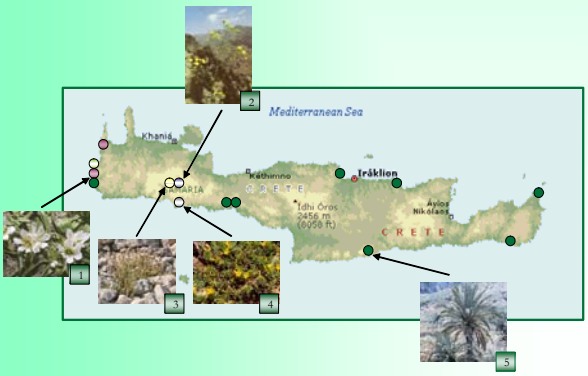

Fig.1 Map with the locations of the 4 plant species populations of Community priority (Directive 92/43/EEC). 1. *Androcymbium rechingeri*, 2. *Bupleurum kakiskalae*, *3. Nepeta sphaciotica*, *4. Hypericum aciferum* and *5.* the priority habitat type 9370 Palm groves of *Phoenix theophrasti.* The green points are the additional locations of *Phoenix theophrasti* populations.

MATERIALS AND METHODS

1. Plant samples

Samples of *Androcymbium rechingeri*, *Bupleurum kakiskalae* and *Phoenix theophrasti* were collected during March 2005, with further sampling of *Nepeta sphaciotica* being undertaken in September 2005 and *Hypericum aciferum* in February 2006. *Bupleurum kakiskalae* was not sampled during the fieldwork but from MAICh due to the inaccessibility of individuals.

2. DNA extraction

Total genomic DNA was extracted from leaf samples using a modified hexadecyl trimethyl ammonium bromide (CTAB) method Doyle and Doyle (1987). *Nepeta sphaciotica* DNA was extracted using a NucleoSpin® Plant Kit by Macherey-Nagel.

3. Polymerase chain reaction and DNA sequencing of the *rbcL* and *matK* genes and the ITS region

Approximately 650 bp of the *rbcL*, 730 bp of the *matK* gene and 350 bp from the ITS region were amplified from genomic DNA by polymerase chain reaction. The Qiagen ® Nucleotide removal kit was then used to purify the reaction products. The resulting purified DNA was then precipitated and the appropriate concentration was used for sequencing which was performed by Macrogen, Korea.

RESULTS

1. *rbcL* and *matK* gene-based genetic diversity and phylogenetic analyses

No intra-individual variation was detected within the species. Phylogenetic analysis was contacted using *Trillium undulatum* as an outgroup. A strict consensus tree of the *rbcL* gene and one with the *matK* gene with its bootstrap values is shown in Fig. 2 and 3.

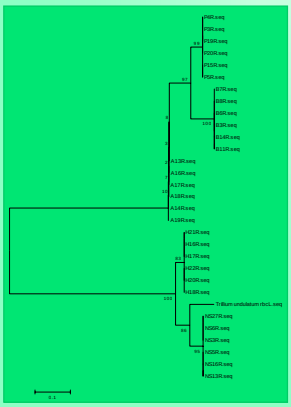

Fig.2 The consensus tree obtained from phylogenetic analysis of *rbcL* gene sequences for 5 taxa and *Trillium* as an outgroup (x 1000 replications). Numbers above branches are bootstrap values.

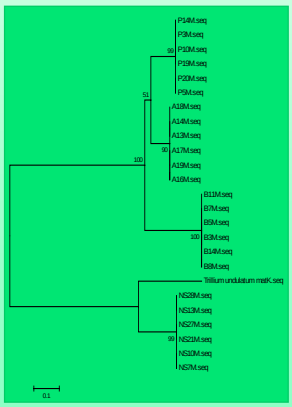

Fig.3 The consensus tree obtained from phylogenetic analysis of *matK* gene sequences for 4 taxa and *Trillium* as an outgroup (x 1000 replications). Numbers above branches are bootstrap values.

2. Internal transcribed spacer region genetic diversity and phylogenetic analyses

Based on the results obtained by the related sequences from the study of the ITS region intra-individual variation was detected only in *Hypericum aciferum*. The results of phylogenetic analysis using the ITS region for *Bupleurum kakiskalae* and *Nepeta sphaciotica* showed no genetic variation at all. The ITS tree is shown in Fig.4.

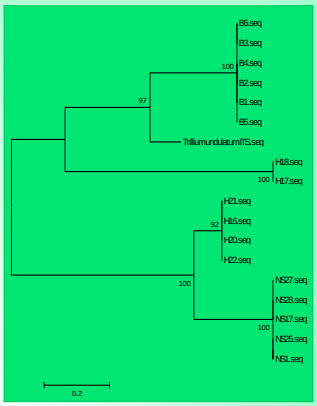

Fig.4 The consensus tree obtained from phylogenetic analysis of the ITS region sequences for 3 taxa using *Trillium undulatum* as an outgroup (x 1000 replications).

Table 1. Base pairs differences (above diagonal) and number of base substitutions per site (below diagonal, calculated according to Kimura 2-parameter) of internal transcribed spacer (ITS) sequences from 3 taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. H18.seq		0.113	0.175	0.175	0.175	0.000	0.175	0.249	0.249	0.249	0.249	0.249	0.141	0.141	0.141	0.141	0.141	0.141
2. Trillium undulatum ITS.seq	0.075		0.167	0.167	0.167	0.113	0.167	0.157	0.157	0.157	0.157	0.157	0.042	0.042	0.042	0.042	0.042	0.042
3. H22.seq	1.367	1.275		0.000	0.000	0.175	0.000	0.039	0.039	0.039	0.039	0.039	0.170	0.170	0.170	0.170	0.170	0.170
4. H21.seq	1.367	1.275	0.000		0.000	0.175	0.000	0.039	0.039	0.039	0.039	0.039	0.170	0.170	0.170	0.170	0.170	0.170
5. H20.seq	1.367	1.275	0.000	0.000		0.175	0.000	0.039	0.039	0.039	0.039	0.039	0.170	0.170	0.170	0.170	0.170	0.170
6. H17.seq	0.000	0.075	1.367	1.367	1.367		0.175	0.249	0.249	0.249	0.249	0.249	0.141	0.141	0.141	0.141	0.141	0.141
7. H16.seq	1.367	1.275	0.000	0.000	0.000	1.367		0.039	0.039	0.039	0.039	0.039	0.170	0.170	0.170	0.170	0.170	0.170
8. NS28.seq	1.643	1.226	0.327	0.327	0.327	1.643	0.327		0.000	0.000	0.000	0.000	0.227	0.227	0.227	0.227	0.227	0.227
9. NS27.seq	1.643	1.226	0.327	0.327	0.327	1.643	0.327	0.000		0.000	0.000	0.000	0.227	0.227	0.227	0.227	0.227	0.227
10. NS25.seq	1.643	1.226	0.327	0.327	0.327	1.643	0.327	0.000	0.000		0.000	0.000	0.227	0.227	0.227	0.227	0.227	0.227
11. NS17.seq	1.643	1.226	0.327	0.327	0.327	1.643	0.327	0.000	0.000	0.000		0.000	0.227	0.227	0.227	0.227	0.227	0.227
12. NS1.seq	1.643	1.226	0.327	0.327	0.327	1.643	0.327	0.000	0.000	0.000	0.000		0.227	0.227	0.227	0.227	0.227	0.227
13. B6.seq	1.175	0.358	1.326	1.326	1.326	1.175	1.326	1.490	1.490	1.490	1.490	1.490	0.000	0.000	0.000	0.000	0.000	0.000
14. B5.seq	1.175	0.358	1.326	1.326	1.326	1.175	1.326	1.490	1.490	1.490	1.490	1.490	0.000	0.000	0.000	0.000	0.000	0.000
15. B4.seq	1.175	0.358	1.326	1.326	1.326	1.175	1.326	1.490	1.490	1.490	1.490	1.490	0.000	0.000		0.000	0.000	0.000
16. B3.seq	1.175	0.358	1.326	1.326	1.326	1.175	1.326	1.490	1.490	1.490	1.490	1.490	0.000	0.000	0.000		0.000	0.000
17. B2.seq	1.175	0.358	1.326	1.326	1.326	1.175	1.326	1.490	1.490	1.490	1.490	1.490	0.000	0.000	0.000	0.000		0.000
18. B1.seq	1.175	0.358	1.326	1.326	1.326	1.175	1.326	1.490	1.490	1.490	1.490	1.490	0.000	0.000	0.000	0.000	0.000	

The sequence boundaries of the ITS1 region and the 5.8S of nrDNA region in the 3 taxa included here were determined by comparison to those of Wurdack *et. al.* 2005, of Neves and Watson, 2004 and of Trusty *et. al.*, 2004 (unpublished data).

Together, these taxa form three different groups. *Nepeta sphaciotica* and *Bupleurum kakiskalae* individuals form two clades with a bootstrap value of 100%. *Trillium undulatum* form a supported clade with bootstrap value of 96 % along with *Bupleurum kakiskalae.* The topology of the neighbor-joining tree (Fig. 4) resulting from the ITS region are two informative sites, and four homoplasious.

DISCUSSION

In the present study the topologies of the NJ trees obtained for the *rbcL* and *matK* genes of *cp*DNA followed the same pattern (Figs 2 and 3) between the five taxa. The *rbcL* gene has evolved very slowly and its phylogenetic resolution was thus very limited and showed no sequence variation. The phylogenetic tree obtained by the *matK* gene sequence data showed identical results. However *matK* gene has evolved approximately three times faster than *rbcL* (Johnson and Soltis 1994, 1995; Liang and Hilu, 1996). Unlike *rbcL* and *matK* genes the ITS (internal transcribe spacer) region of ribosomal DNA, is a very rapidly evolving genetic region (Pryer *et al.*, 2001). According to our results genetic diversity at intraspesific level is occurring only within the individuals of *Hypericum aciferum* and thus enlightening our current study with significantly data about the genetic structure of the other four species.

Overall, the populations of the five species examined in the present study presented limited or no genetic variation at all based on the sequence information of both nuclear (ITS1) and chloroplast (*rbcL* and *matK*) genetic markers suggesting that they should be still considered as endangered species and special conservation strategies should be employed for the protection of their populations. Our future goal is to isolate and use microsatellite markers for a more thorough analysis of the genetic diversity of these Protected-Priority plants of Crete.

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