CRETAPLANT
A Pilot Network of Plant Micro-Reserves in Western Crete
Πιλοτικό Δίκτυο 'Μικρο-Αποθεμάτων Φυτών' στη Δυτική Κρήτη
(LIFE04NAT_GR_000104)

ΔΡΑΣΗ Α.5 - Καθορισμός της γενετικής ποικιλότητας και της πληθυσμιακής δομής φυτικών ειδών προτεραιότητας

ACTION A.5 - Determination of the genetic diversity and population structure for each of the 6 targeted species

ΕΝΔΙΑΜΕΣΗ ΕΚΘΕΣΗ

(Ιούνιος 2005 - Ιούνιος 2006)

Συντάκτες:
Έλλη Τζυρκαλλή (Υποψ. Διδάκτωρ), Γεώργιος Τσιάμης (Δρ Μοριακής Βιολογίας) και
Κώστας Μπούρτζης (Αναπλ. Καθηγητής Βιοχημείας και Μοριακής Βιολογίας)
Τμήμα Διαχείρισης Περιβάλλοντος και Φυσικών Πόρων, Πανεπιστήμιο Ιωαννίνων

ΠΕΡΙΛΗΨΗ ΣΤΑ ΕΛΛΗΝΙΚΑ

ΔΡΑΣΗ Α.5 - Καθορισμός της γενετικής ποικιλότητας και της πληθυσμιακής δομής φυτικών ειδών προτεραιότητας

1. Πρόσφατες πολυάριθμες μελέτες έχουν δείξει ότι η γενετική ποικιλότητα είναι σημαντική για τη διατήρηση των πληθυσμών, ειδικά πληθυσμών ειδών που έχουν «κατατμηθεί», έχουν υποστεί το «φαινόμενο της στενωπού» (bottlenecked) ή που έχουν χάσει γρήγορα τη γενετική ποικιλότητα και το εξελικτικό δυναμικό τους. Το μικρό μέγεθος πληθυσμών οδηγεί στην γενετική παρέκκλιση, σε φαινόμενα inbreeding («αιμομικτικές» διασταυρώσεις) και την απώλεια της γενετικής ποικιλότητας και της εξελικτικής δυνατότητας. Ο αριθμός, η προέλευση και η γενετική ποικιλότητα των πληθυσμών είναι τα κύρια σημεία που εξετάζονται κατά την αξιολόγηση των επιδράσεων των μετακινήσεων (translocations) πληθυσμών στη γενετική ποικιλότητα και τη διατήρηση πληθυσμών. Με τον αυξανόμενο αριθμό των απειλούμενων ειδών, είναι σημαντικό να ερευνηθούν τα αποτελέσματα των μετακινήσεων (translocations) στη γενετική ποικιλότητα. Ο κύριος στόχος της παρούσας Δράσης Α5 είναι ο προσδιορισμός της γενετικής ποικιλότητας και της πληθυσμιακής δομής για κάθε ένα από τα συγκεκριμένα φυτικά είδη προτεραιότητας (*Androcymbium rechingeri* Greuter, *Anthemis glaberrima* (Rech. F.) Greuter, *Bupleurum kakiskalae* Greuter, *Cephalanthera cucullata* Boiss. & Heldr., *Hypericum aciferum* (Greuter) N.K.B. Robson, *Nepeta sphaciotica* P.H. Davis καθώς και πληθυσμούς ειδών *Phoenix*), τα οποία αποτελούν πολύτιμες πληροφορίες για την ανάπτυξη ενός αποτελεσματικού σχεδίου διαχείρισης και διατήρησης αυτών των ειδών.

2. Στην παρούσα εργασία συμμετείχαν η υποψήφια διδάκτορας Έλλη Τζυρκαλλή, ο μεταδιδάκτορας Γεώργιος Τσιάμης και ο Αναπληρωτής Καθηγητής Κώστας Μπούρτζης του Τμήματος Διαχείρισης Περιβάλλοντος και Φυσικών Πόρων του Πανεπιστημίου Ιωαννίνων.

3. Τα δείγματα (φύλλα) από τα φυτικά είδη *Androcymbium rechingeri, Bupleurum kakiskalae* and *Phoenix theophrasti* συλλέχθησαν το Μάρτη του 2005, τα δείγματα από το *Nepeta sphaciotica* το Σεπτέμβρη του 2005 και από το *Hypericum aciferum* το Φεβρουάριο του 2006. Όλα τα δείγματα τοποθετήθηκαν άμεσα σε πάγο και στη συνέχεια στους -80 °C μέχρι τη χρησιμοποίησή τους για εξαγωγή DNA.

4. Η μεθοδολογία που ακολουθήθηκε στη συγκεκριμένη εργασία είναι η ακόλουθη: (α) απομόνωση DNA από όλα τα συλλεχθέντα δείγματα, (β) σχεδιασμός εκκινητών και η χρησιμοποίησή τους σε αντιδράσεις PCR για την ενίσχυση τμήματος των γονιδίων – γενετικών περιοχών *rbcL* και *matK* (χλωροπλαστικοί γενετικοί δείκτες) καθώς και της ITS *(*πυρηνικός γενετικός δείκτης), (γ) νουκλεοτιδική αλληλούχιση των PCR προϊόντων, (δ) ανάλυση των νουκλεοτιδικών αλληλουχιών, εκτίμηση της γενετικής ποικιλότητας και φυλογενετική ανάλυση, (ε) χρησιμοποίηση

μικροδορυφόρων για τη μελέτη της γενετικής ποικιλότητας στο είδος *Phoenix theophrasti* και (στ) απομόνωση μικροδορυφόρων στο είδος *Nepeta sphaciotica*.

5. Τα μέχρι στιγμής αποτελέσματα και βασικά συμπεράσματα της παρούσας μελέτης είναι τα εξής: (α) συλλογή δειγμάτων από τα είδη *Androcymbium rechingeri, Bupleurum kakiskalae, Hypericum aciferum, Nepeta sphaciotica* and *Phoenix theophrasti*, (β) επιτυχής απομόνωση DNA από όλα τα δείγματα, (γ) επιτυχής σχεδιασμός εκκινητών και ενίσχυση τμήματος των γονιδίων – γενετικών περιοχών *rbcL* (550 bp) για τα πέντε υπό μελέτη είδη, *matK* (730 bp) για όλα τα είδη πλην του *Hypericum aciferum* και ITS *(*περίπου 650-1150 bp) για τα είδη *Bupleurum kakiskalae, Hypericum aciferum* και *Nepeta sphaciotica*, (γ) η πλήρης νουκλεοτιδική αλληλούχιση των παραπάνω προϊόντων έδειξε ότι δεν ανιχνεύθηκε γενετική ποικιλότητα με τη χρήση των γενετικών δεικτών *rbcL* και *matK* στα δείγματα των υπό μελέτη ειδών ενώ η χρήση του γενετικού δείκτη ITS ανέδειξε την ύπαρξη γενετικής ποικιλότητας μόνο μεταξύ των δειγμάτων του είδους *Hypericum aciferum*, (δ) απουσία γενετικής ποικιλότητας στα δείγματα του είδους χρησιμοποίηση *Phoenix theophrasti* με τη χρήση ένδεκα (11) πυρηνικών μικροδορυφόρων ως γενετικών δεικτών, (ε) τρεις (3) μικροδορυφόροι αποτέλεσαν καλούς γενετικούς δείκτες για το διαχωρισμό δειγμάτων του είδους *Phoenix theophrasti* από αντίστοιχα δείγματα του είδους *Phoenix dactylifera*, (στ) κατασκευή δύο βιβλιοθηκών $(AG)_{12}$ και $(GAA)_8$ για την απομόνωση μικροδορυφόρων για διερεύνηση της γενετικής ποικιλότητας στο είδος *Nepeta sphaciotica*. Έχουν απομονωθεί 19 υποψήφιοι κλώνοι από την πρώτη βιβλιοθήκη ενώ την παρούσα στιγμή αναλύονται κλώνοι από τη δεύτερη βιβλιοθήκη.

6. Στο επόμενο χρονικό διάστημα προγραμματίζονται οι εξής εργασίες: (α) συλλογή δειγμάτων και απομόνωση DNA από τα είδη *Anthemis glaberrima* (Rech. F.) Greuter και *Cephalanthera cucullata* Boiss. & Heldr, αν αυτό καταστεί εφικτό, (β) ολοκλήρωση της χρησιμοποίησης PCR εκκινητών και ενίσχυσης τμήματος των γονιδίων – γενετικών περιοχών *rbcL* (550 bp) για τα πέντε υπό μελέτη είδη, *matK* (7300 bp) και ITS *(*1150 bp) για όλα τα υπό μελέτη είδη, (γ) ολοκλήρωση της πλήρους νουκλεοτιδικής αλληλούχισης των παραπάνω προϊόντων για την εκτίμηση της γενετικής ποικιλότητας των υπό μελέτη ειδών, (δ) προσπάθεια ολοκλήρωσης της απομόνωσης και χρησιμοποίησης μικροδορυφόρων για τη μελέτη της γενετικής ποικιλότητας στο είδος *Nepeta sphaciotica*.

Project: A Pilot Network of Plant Micro-Reserves in Western Crete

Action A.5: Determination of the genetic diversity and population structure for each of the 6 targeted species

Interim Report (June 2005 – June 2006)

Elli Tjirkalli, George Tsiamis and Kostas Bourtzis
Department of Environmental and Natural Resources Management
University of Ioannuna

July 2006

Contents

A. Genetic analysis of *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica, Hypericum aciferum* and *Phoenix theophrasti* based on the nucleotide sequences of the chloroplast *rbcL, matK* genes and the ITS region.

B. Characterization of nuclear microsatellite markes in *Phoenix theophrasti (Palmae*)

C. Isolation and characterization of microsatellite loci in *Nepeta sphaciotica* (*Lamiaceae)*

A Pilot Network of Plant Micro-Reserves in Western Crete

Abstract

Androcymbium rechingeri, *Bupleurum kakiskalae*, *Nepeta sphaciotica, Hypericum aciferum* and *Phoenix theophrasti* are endemic (the latter subendemic) to the island of Crete, Greece. Their limited population size and a number of threats make them critically endangered, and in need of active conservation. Moreover, these plants are of Community priority according to the Directive 92/43/EEC. Genetic surveys using genetic markers *rbc*L and *mat*K genes of *cp*DNA and internal transcribed spacers of ITS *nr*DNA were undertaken to investigate genetic diversity within each species to provide genetic data for the conservation programme LIFE-Nature 2004: *A Pilot Network of Plant Micro-Reserves in Western Crete (Chania Prefecture)*. PCR amplified products were used to analyze phylogenetic relationships among the species. No genetic variation was found at intraspecific level based on the *cp*DNA genes, however it was observed based on the ITS region in *Hypericum aciferum*. The conservation status of these species has been evaluated and it is recommended that additional surveys using microsatellites loci must be performed in order to further evaluate the genetic diversity of the species.

A. Genetic analysis of *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica, Hypericum aciferum* and *Phoenix theophrasti* based on the nucleotide sequences of the chloroplast *rbcL, matK* genes and the ITS region.

A. Introduction

The conservation of biodiversity (microorganisms, plants and animals and the ecosystems that they form) ultimately depends upon the conservation of genetic diversity within species. Conservation genetics is thus likely to play a vital role in developing a strategy for the short and long-term preservation of biodiversity. During the last two decades the role of genetics in conservation biology, and in ecology in general, has been greatly emphasized (Pertoldia and Topping, 2004). Also the loss of genetic diversity in species populations is a concern because reduced polymorphism also reduces the evolutionary potential (Lynch, 1996). Therefore, DNA analyses are increasingly used to estimate the extent and organization of genetic diversity in populations and the intelligent use of their genetic resources (Luikart and England, 1999). It is well known that molecular techniques serve as suitable surrogates for estimating genetic diversity and population genetic structures (Pritchard *et al.*, 2000). However, knowledge of the loss of variability that has actually taken place is often hampered by lack of information on the genetic composition of populations. A large body of techniques has thus been developed to investigate the genetic diversity based on species population structure (Cockerham and Weir, 1993; Slatkin, 1995; Pritchard *et al.*, 2000). In this study we evaluate the use of three genetic markes *rbc*L and *matK* genes and internal transcribed spacer 18SF-26SR of nuclear ribosomal DNA for studying the genetic diversity of *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica, Hypericum aciferum and Phoenix theophrasti.*

A.1. Crete

The island of Crete, Greece is located in the south eastern part of the Mediterranean region. The surface area of the island is 8336 km^2, excluding the small islets around and its population reaches 600,000 people. Crete has a mostly mountainous terrain with extreme elevations in the western part in which is located the Lefka Ori range and some of its peaks exceed 2453 m in height. The island is relatively well watered by numerous natural springs and several rivers. The climate of Crete is sub-humid Mediterranean with long hot and dry summer and relatively humid and cold winter as the main characteristics. (Chartzoulakis and Psarras, 2004). Crete, from the floristic point of view, is considered one of the richest territories in Europe. About 1800 taxa, species and subspecies, including some 186 endemics are occurring in a relatively small area. More than 10% of them are endemic in Crete and not found elsewhere (Damanakis and Matthas, 1987). This number is considered to be very high compared with other European territories with similar or even larger area. Regional, habitat and altitudinal distribution of endemics are very uneven. The geographical isolation of the island has been an important factor for species differentiation and evolution. Also the existence of high altitudes has provided sufficient isolation to ecotypes for independent evolution (Critopoulos, 1975).

Many endemic taxa are rare and consist of small populations which are restricted to one or few cliffs or mountain sites (Bergmeier, 1997). This is why 52 Cretan endemics are included in the Red Data Book of Greece (Phitos *et al.*, 1995). It has long been known that Crete is, within Greece, the

region where the concentration of rare endemics is highest. This underlines the importance of Crete area in a European context, with respect to the need for conservation of threatened plant species (Greuter, 1979). Specific rare endemics namely: *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica*, *Hypericum aciferum* and *Phoenix theophrasti* (Fig. A.1) 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, which are protected under the pan-European NATURA 2000 network, comprising Special Areas of Conservation (SACs) for threatened habitants and species (Council of Europe, 1992). The inclusion of these sites in the Natura 2000 network emphasizes the need to establish genetic and ecological data for improved protection and management of these areas.

The aim of the present study is to develop genetic markers for evaluating the conservation significance of species based first on the evaluation of genetic data and second on ecological data. 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) genes of chloroplast DNA (*cp*DNA) and internal transcribed spacers 18S-26S of nuclear ribosomal DNA (ITS) (ii) information's about their habitat, ecology, distribution, population trends and endangered status (Box A.1); (iii) to use available data and produce reasonable preliminary conclusions about the conservation status of the species. It must be emphasised that it is the first time for Greece that the genetic diversity of endangered species will be taken into account in the context of an integrated plan for species conservation and rational management (NIKUA-MAICh-FDC[1], 2004).

Box A.1:

Androcymbium rechingeri Greuter Phylum: *Angiospermae*, Classe: *Liliopsida* (Monocots), Subclasse: *Liliidae*, Order: *Liliales*, Family: *Liliaceae* (Lily Family), Genus: *Androcymbium*

Description: Corm single. Stem 2-7 cm long. Leaves 2-15 cm long, linear to lanceolate, glabrous. Flowers 1-6(-12), shortly pedicellate; perianth segments 20-25 mm long; limb lanceolate, acute, 3-4(-6) mm broad, white with pinkish veins. Filaments 4-5 mm long; anthers 1-1.8(-2) mm long. Fruit 6-10 mm long; obpyriform, occasionally glandular-punctate only at the apex. Seeds subspherical, glanulate. Flowering December to February (Phitos et al., 1995).

Habitat and Ecology: *Androcymbium rechingeri* is a winter-flowering, corn-bearing geophyte. It occurs close to the sea on stabilized sandy ground and is specialized to habitats in the transition zone between coastal dunes and inland phrygana. Therefore, co-occurring species include widespread phrygana shrubs such as *Coridothymus capitatus* and *Genista acanthoclada.*

Distribution and population trends: *Androcymbium rechingeri* is endemic to the Cretan area. On Crete, it is known from three sites, one near Falasarna, the two on the small offshore islets Elafonisi and Imeri Gramvousa. The species has also recently found to form small populations at two locations on Karpathos.

Endangered status: Endangered due to tourism since the plants grow next to sandy beaches, the populations are at risk of any activities related to beach tourism, such as construction work, road building, trampling, or mere disturbance. Collecting: The attractive *Androcymbium rechingeri* has frequently been collected by bulb enthusiasts as well as presumably by professional collectors due its horticultural value. Such impacts cause direct loss to the population and damage the habitat by trampling (NIKUA-MAICh-FDC, 2004).

Conservation measures taken: All three Cretan populations are included in the Natura 2000 sites GR4340001 (Imeri and Agria Gramvousa-Tigani and Falasarna-Pontikonisi) and GR4340002 (Elafonisos and Karpathos islet), but no effective measures for conservation have been taken at community or national level. Regarding the ex-situ conservation, a seed lot is stored in the Seed Bank of MAICh and the species is cultivated in the Botanic Garden.

[1] NIKUA: National and Kapodistrian University of Athens
MAICh: Mediterranean Agronomic Institute of Chania
FDC: Forest Directorate of Chania

Bupleurum kakiskalae Greuter Phylum: *Angiospermae*, Classe: *Magnoliopsida* (*Dicots*), Subclasse: *Rosidae* (*Rosiflorae*), Order: *Apiales*, Family: *Umbelliferae* or *Apiaceae*, Genus: *Bupleurum*

Description: Monocarpic perennial with a tap root. Sterile plants are up to 12 years old and consist of a singe stout unbranched woody stem 1 cm or more thick and about 12 cm long, carrying a tight rosette of 15-30 oblanceolate leaves up to 25 cm long. Fertile stems are up to 1 m high, arising fro previous year's rosettes, bearing a loose, freely-blanched panicle of umbels each with 4-6 rays; bracts and bracteoles 5-9 nerved, herbaceous, c. 3 mm long, obtuse or blunt, ligulate or spathulate; petals yellow; ovaries pruinose (with a waxy secretion or bloom on the surface), smooth. Ripe fruits unknown. Flowering July-September (Phitos *et al.*, 1995).

Habitat and Ecology: *Bupleurum kakiskalae* is a monocarpic hemicryptophyte. It goes through a long sterile period and the plants die after flowering. It grows in crevices of a vertical rock face in a cliff system at 1450-1500 m. The cliff consists of Plattenkalk, a metamorphic calcareous, commonly thin-bedded and readily disintegrating rock. In its single locality co-occurring chasmophytes include other regional endemics such as *Campanula jacquinii* and *Crepis auriculifolia*.

Distribution and population trends: *Bupleurum kakiskalae* is only known from a single cliff at Kaki Skala in the Lefka Ori (White Mountains, Crete). A recent assessment by MAICh team raises the population to 30-40 flowering individuals but no exact assessment was possible.

Endangered status: Endangered due to small population size combined with the fact that it is restricted to one location make species vulnerable to any single destructive event and grazing-the wider area is a part of the traditional summer pasture for goats and sheep held by owners of the community of Sfakia (West Crete).

Conservation measures taken: the population is located at the boundaries of the Samaria National Park and is included in the proposed Natura 2000 site GR4340008 (Lefka Ori), but no effective conservation measures have been taken at community or national level. Regarding the ex-situ conservation, a small seed lot is stored in the Seed Bank of MAICh but the efforts for cultivation of species were unsuccessful (NIKUA-MAICh-FDC, 2004).

Nepeta sphaciotica P.H. Davis Phylum: *Angiospermae*, Classe: *Magnoliopsida* (Dicots), Subclasse: *Asteridae* (Sympetalae tetracyclicae), Order: *Lamiales*, Family: *Labiatae* or *Lamiaceae*, Genus: *Nepeta*

Description: Perennial herb with several stems 5-20 cm high from a woody rootstock. Leaves arranged in opposite pairs up the stems, pale green, oblong to ovate, softly hairy, 13-20 mm long, with coarsely crenate margins. Flowers in whirled clusters which are aggregated into a spike at the trip of the stem; calyx 7-9 mm long, grey-villous and glandular-hairy, ending in 5 pointed lobes; corolla white, with pink spots, 10-12 mm long, two-lipped with the upper lip two-loded and the lower lip three-lobed with an expanded, dentate, concave middle lobe. Flowering June-October.

Habitat and Ecology: *Nepeta sphaciotica* grows among metamorphic calcareous rock in a sheltered place on a summit area at 2200-2300 m altitude. It occurs on rocky slopes and stabilized scree and is a characteristic species of chasmophytic communities with *Arabis cretica* and *Sedum idaeum*.

Distribution and population trends: *Nepeta sphaciotica* is a local endemic of Lefka Ori. The single existing population is located at an inaccessible and windy site on the northern slope of Mt. Zvourichti. A recent estimation by MAICh team raises the population to 2000-3000 individuals.

Endangered status: Endangered due to grazing (Mt. Zvourichti belongs to the traditional areas of summer pasturage in the Lefka Ori) and its small population, combined with extremely local distribution make the species vulnerable to any local inadvertent impact and to random processes.

Conservation measures taken: The area of the population is included in the Natura 2000 site GR4340008 (Lefka Ori), but this has not yet effected any conservation measures. Regarding the ex-situ conservation, a small seed lot is stored in the Seed Bank of MAICh but the efforts for cultivation of species have been unsuccessful (NIKUA-MAICh-FDC, 2004).

Phoenix theophrasti Greuter Phylum: *Angiospermae*, Classe: *Liliopsida* (Monocots), Subclasse: *Arecidae* (*Spadiciflorae*), Order: *Arecales*, *Palmales*, *Principes*, Family: *Arecaceae* or *Palmae*, Genus: *Phoenix*

Description: A sizeable palm tree, with one, to several main trunks up to 10 m tall (including the foliage) and surrounded by dense clumps of shorter shoots arising as suckers at the base. Each trunk is crowned by a bunch of 3-5 m long, pinnate leaves forming a spherical crown. The larger (middle) pinnae may reach a length of 40 cm and are linear, folded lengthwise in the middle, and pungent-tipped; toward the base of the leaf the pinnae are transformed into stout, sharp spines that border the whole length of the petioles. Flowering April-May (Phitos et al., 1995).

Habitat and Ecology: Is found in coastal areas, either on steep calcareous cliffs and rocks within a few meters of the sea, or somewhat inland along moist valley floors, stream banks and rocky gullies. Occurrence of palms invariably indicates a water-source. Salt tolerance of the species enables it to survive combines pressures of exposure to coastal winds and sea water (Barrow, 1998).

Distribution and population trends: It is now recorded from nine coastal localities on the island (Turland *et al.*, 1993). At present, the species is known only from Crete and southwestern Turkey. All populations are included in the Natura 2000 sites P.Chania: Chryssoskalitisa (GR4340002), Drapano Georgoupolis (GR4340010), P.Rethimno:

Moni Preveli (GR4330003), Valley Phoenix west of ormou Plakia (GR4340012), P. Irakleion: Gkazi-Almiros (GR4310001), Stalida, Saint Nikitas-Tsoutsouras (GR4310005), Saintcannyon-Martsalo (GR4310004), P. Lasithiou: aesthetic forest Vai (GR4320009), Erimoupoli, Moni Toplou (GR4320006).
Endangered status: Vulnerable due to unrestrained tourism (camping, sea-shore tourism).
Conservation measures taken: The largest Known Greek population at Vai has been declared an «aesthetic forest preserve» in the late 1970s, and its core area was effectively protected by fencing in spring 1983. The preserve area covers 20 ha (NIKUA-MAICh-FDC, 2004).

Hypericum aciferum (Greuter) N.K.B. Robson Phylum: *Angiospermae*, Classe: *Magnoliopsida* (Dicots), Subclasse: *Dilleniidae*, Order: *Theales*, Family: *Guttiferae*, Genus: *Hypericum*
Description: Low, procumbent shrub. Leaves 5-12 x 0.6-1.4 mm, narrowly linear-spathulate, coriaceous. Flowers (1-)3, pedicellate, heterostylous. Sepals ±3.5 mm long, almost erect, elliptical. Petals c. 9mm, deciduous. Stamen fascicles 3, alternating with 3 fleshy fasciclodes. Overy with 2 ovules in each loculus. Seeds 1.5-1.7 mm long elongate, subcylindrical, and slightly carinate, with a fleshy caruncle. Flowering June-October (Phitos et al., 1995).
Habitat and Ecology: *Hypericun aciferum* is a typical chasmophyte, growing on vertical, coastal rocks, at altitudes between 4-50 m. The plants appear to be tolerant to frequent spraying by sea water. Accompanying species iclude other rare regional endemics such as *Allium bourgeauii* subsp. *creticum*, *Centaurea argentea*, *Dianthus fruticosus* subsp. *creticus*, *Origanum dictamnus*, *Ornithogalum creticum* and *Staehelina fruticosa.*
Distribution and population trends: *Hypericun aciferum* is a local endemic known (only two small populations) from the coastal area of the gorges Trypiti and Domata in south-west Crete, in the district of Sfakia. Previous population counts reported 35 individuals at Domata and 50-60 individuals at Trypiti. The MAICh team observed much higher numbers at both locations in 1997, but the exact estimation of the population size requires a more detailed study due to the inaccessibility of the sites.
Endangered status: Endangered due its limited population size that makes the species vulnerable to any local inadvertent impact to random processes.
Conservation measures taken: The two locations of the populations are included in the Natura 2000 site GR4340008 (Lefka Ori), but no special conservation measures have been taken. Regarding the ex-situ conservation, the species is cultivated (plants originating from wild origin graftings) at the Botanical Garden of MAICh and the Botanical Garden of TEI of Irakleion (NIKUA-MAICh-FDC, 2004).

Plant Species	Conservation and Protection Status			
	IUCN category			Natura 2000
Androcymbium rechingeri	Endangered	Greek Law[2] (P.D. 67/1981)	Bern Convention	Directive 92/43/EEC (Annex II*[3]/IV)
Bupleurum kakiskalae	Endangered	Greek Law (P.D. 67/1981)	Bern Convention	Directive 92/43/EEC (Annex II*/IV)
Nepeta sphaciotica	Endangered	Greek Law (P.D. 67/1981)	Bern Convention	Directive 92/43/EEC (Annex II*/IV)
Hypericum aciferum	Endangered	-	Bern Convention	Directive 92/43/EEC (Annex II*/IV)
Phoenix theophrasti	Vulnerable	Greek Law (P.D. 67/1981)	Bern Convention	Directive 92/43/EEC (Annex II/IV)

(Georghiou and Delipetrou, 2001)

[2] Greek Law-Presidential Decree 67/1981. «Protection of indigenous flora and wildlife, determination of the coordination process and inspection of their research» (includes 916 taxa).
[3] The species in Annex II along with a star, symbolize the Priority Species of the Directive 92/43/EEC, which means that for their protection and conservation on-side management measures, are needed (MAICh, 2002).

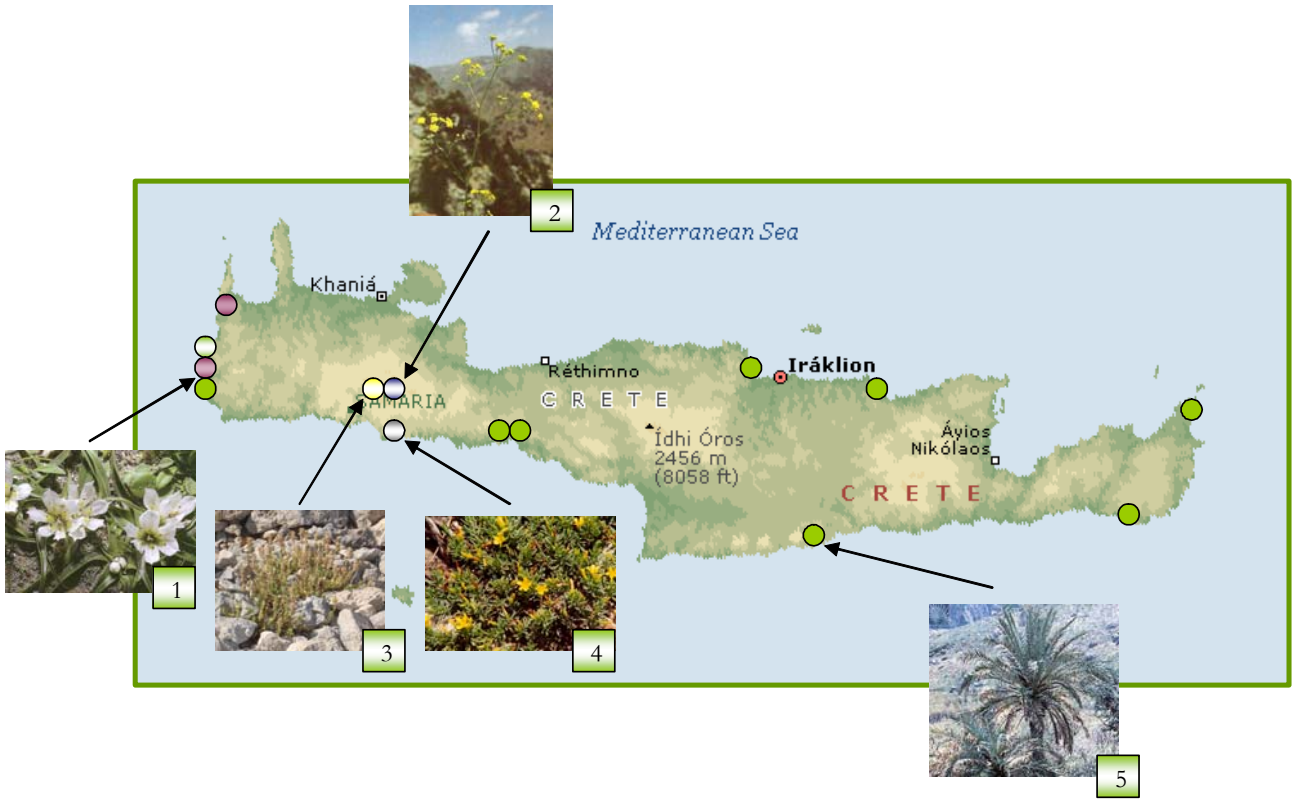

Fig.A.1 Map with the locations of the 5 plant species populations of Community priority (Directive 92/43/EEC). 1. *Androcymbium rechingeri* 2. *Bupleurum kakiskalae*, *3. Nepeta sphaciotica*, *4. Hypericum aciferum* and *5. Phoenix theophrasti.* The green points are the additional locations of *Phoenix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

A.2 Material and Methods

A.2.1. 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. All samples consisted of single leaves that immediately were placed in ice bags. One population of *Androcymbium rechingeri, Bupleurum kakiskalae, Nepeta sphaciotica, Hypericum aciferum* and *Phoenix theophrasti*, were sampled, consisting of 21, 16, 28, 30 and 21 individuals respectively. *Bupleurum kakiskalae* was not sampled during the fieldwork but from MAICh due to the inaccessibility of individuals. Not all of the plant individuals were sequenced using the *rbcL*, *matK* genes and the internal transcribed spacers ITS (Table A.1).

Table A.1 Samples included in the *rbcL*, *matK* and 18S-26S sequencing analyses

Species	Location	Site coordinate	Collector	Sequencing analyses of individuals		
				rbcL	***matK***	**ITS**
Androcymbium rechingeri	Crete: Elafonisi	23°31´E 35°16´N	E. Tjirkalli	6	6	-
Bupleurum kakiskalae	Crete: MAICh	24°03´E 35°29´N	E. Tjirkalli	6	6	6
Nepeta sphaciotica	Crete: Mt. Zvourichti	24°04´E 35°32´N	E. Tjirkalli	6	6	5
Hypericum aciferum	Crete: Trypiti	23°56´E 35°13´N	H. Remoutzou	6	-	6
Phoenix theophrasti	Crete: Chryssoskalitisa	23°31´E 35°18´N	E. Tjirkalli	6	6	-

A.2.2. DNA extraction

Total genomic DNA was extracted from leaf samples by using a modified hexadecyl trimethyl ammonium bromide (CTAB) method Doyle and Doyle (1987) from *Androcymbium rechingeri, Bupleurum kakiskalae, Hypericum aciferum* and *Phoenix theophrasti. Nepeta sphaciotica* DNA was extracted using a Plant Extraction Mini Kit by Macherey-Nagel.

A.2.3. Polymerase chain reaction

All plant individuals were PCR examined by amplifying their genomic DNA with a vast number of primers (Table A.2).

A.2.4. Polymerase chain reaction and DNA sequencing of the *rbcL* and *matK* genes

Exactly 550 bp of the *rbcL* and 730 bp of the *matK* gene were amplified from genomic DNA by polymerase chain reaction (PCR). These regions were amplified using primer pairs *rbcL*F: 5′-CTG ATA TCT TGG CAG CAT TCC GAG-3′, and *rbcL*R: 5′-CCC CAA GGG TGT CCT AAA GTT C-3', *matK*F: 5′-AAT TTA CGA TC(AT) ATT CAT TCA A(CT)A TTT C-3′ and *matK*R: 5′-TCG AAG TAT ATA CTT TAT TCG ATA C-3′. PCR amplifications were carried out in 50 µl reactions under standard conditions on a DNA Engine thermal cycler (MJ Research, Watertown, Massachusetts, USA) for 35 cycles. The amplification reaction mixture typically contained 2.5 µL of genomic DNA template (50-100 ng), 5 µL 10x $NH4^+$ buffer, 2 µL $MgCl_2$ (50 mM), 0.25 µL dNTPs (25mM), 0.25 µL Taq DNA polymerase, 1.25 µL of each primer (25pmol/L), and purified water to volume. Each cycle consisted of a denaturing step of 5 min at 94 °C, followed by 30 sec denaturation at 94 °C, an annealing step of 30 sec at 50°C and an extension step of 3 min at 72°C. After the last cycle, a final extension step (10 min, 72°C) was added. PCR products were analysed on 1% agarose gel to determine the presence, concentration and length of the amplified product. 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. The results were obtained by e-mail.

Table A.2 Base composition of amplification and sequencing primers used in this study

Primer	5′sequence 3′	
rbcL		
rbcLF	CTGATATCTTGGCAGCAT TCCGAG	
rbcLR	CCCCAAGGGTGTCCTAAAGTTC	
matK		
matKF	AATTTACGATC(AT)ATTCATTCAA(CT)ATTT C	
matKR	TCGAAGTATATACTT TATTCGATA C	
matKFN	CCAAAATCTCAGAATTTGAATTTAC	
matKFNN	GTC ATT GTG GAA ATT CCA TTC TTG C	These primers were designed within the conserved regions of the corresponding genes (*rbcL*, *matK* of *cp*DNA and 18SF-26SR of *nr*DNA) from species
matKLF	CAGAGGGGT TTGCTTTTATT	
ITS		
ITSFLam	TTGATATGCTTAAACTCAGCGGGT	
ITSRLam	CGTAGGTGAACCTGCGGAAGG	
Lam 18SF	GTAGGTGAACCTGCGGAAGAAG	
Lam 26SR	ATCCCGCCTGACCTGGGGTG	
Arec 18SF	GAAGTCCACTGAACCTTATC	

Arec 26SR	GGCC(AC)(AG)C(CT)(CT)GACC(CT)(GT)GGG	belonging to the
17SE	ACGAATTCATGGTCCGGTGAAGTGTTCG	Liliaceae,
ITS4	TCCTCCGCT TAT TGATATGC	Umbelliferae,
ITS_in_F	CTCGGCACCGGATATCTC	Labiatae, Guttiferae
ITS_in_R	ACTTGCGTTCAAAGACTCG	and Palmae families
ITS1F	TTAGAGGAAGGAGAAGT	respectively.
ITSR	GGCACGGCGAYGGGATC	
ITS2F	GTGAACCTGCGGAAGGAT	
18SF	ACGTCCCTGCCCTTTGTACACA	
26SR	GCGGTACTTGTTYGCTATCGGT	
5.8SF	TCGATGAARARYGYASYRAAVTG	
5.8SR	CABTTYRSTRCRYTYTTCATCGA	

A.2.5. Polymerase chain reaction and DNA sequencing of the internal transcribed spacers 18S-26S (ITS).

Approximately 1150 bp of the internal transcribed spacers 18SF-26SR were amplified from genomic DNA by polymerase chain reaction (PCR). This region was amplified using primer pairs 18SF: 5´-ACG TCC CTG CCC TTT GTA CAC A-3´, and 26SR: 5´-GCG GTA CTT GTT YGC TAT CGG T-3', 5.8SF: 5´-TCG ATG AAR ARY GYA SYR AAV TG-3´ and 5.8SR: 5´-CAB TTY RST RCR YTY TTC ATC GA-3´. PCR amplifications were carried out in three separated 20 μL reactions under standard conditions on a DNA Engine thermal cycler (MJ Research, Watertown, Massachusetts, USA) for 35 cycles. The amplification reaction mixture typically contained 1 μL of genomic DNA template (20-30ng), 2 μL 10x $NH4^+$ buffer, 0.8 μL $MgCl_2$ (50 mM), 0.1 μL dNTPs (25mM), 0.2 μL Taq DNA polymerase, 0.5 μL of each primer (25pmol/L) and purified water to volume.

A.2.6 Sequence alignment and phylogenetic analyses as revealed by *rbcL* and *matK* genes sequencing data

The final *rbcL*, *matK* and ITS sequences were aligned visually using DNAStar software. All phylogenetic analyses were conducted using Mega 3.1 software on PCs.

A.3. Results

A.3.1. rbcL gene-base genetic diversity and phylogenetic analyses

Partial sequences of *rbcL* gene (550 bp) were determined for 5 selected taxa, including *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica*, *Hypericum aciferum* and *Phoenix theophrasti*. No intra-individual variation was detected within the species. Phylogenetic analysis was contacted using *Trillium undulatum* as an outgroup. To obtain confidence limits, bootstrap analysis was conducted. Bootstrap values with 1000 replications were calculated. A strict consensus tree of the *rbcL* gene with its bootstrap values is shown in Fig.A.2. The tree showed 5 clades, consisting of 6 individuals of *Androcymbium rechingeri*, *Bupleurum kakiskalae*, *Nepeta sphaciotica*, *Hypericum aciferum* and *Phoenix theophrasti*.

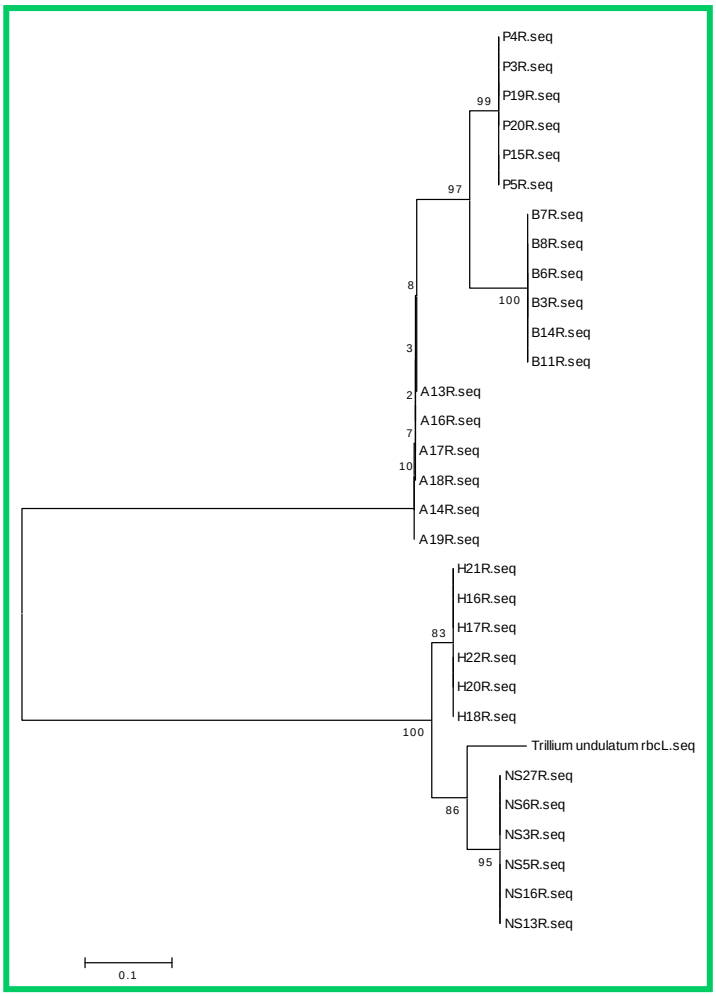

Fig.A.2 The consensus tree obtained from phylogenetic analysis of *rbcL* gene sequences for 5 taxa and *Trillium* as an outgroup (x 1000 replications). Numbers above branches are bootstrap values.

A.3.2. matK gene-base genetic diversity and phylogenetic analyses

No intra-individual variation was detected from the related sequences based on the *matK* gene. The results of phylogenetic analysis using the *matK* gene for 4 selected species, including *Androcymbium rechingeri, Bupleurum kakiskalae, Nepeta sphaciotica,* and *Phoenix theophrasti,* using *Trillium undulatum* as outgroup, clearly revealed the phylogenetic positions of the species. The *matK* tree obtained by the Mega 3.1 software is showed in Fig.3. The tree obtained for *matK* was very similar to the *rbcL* tree (Fig.A.2). Each clade consists of 6 individuals from the five studied plant species. Interpretations for the *rbcL* and *matK* sequence homogeny observed in both trees are currently under study.

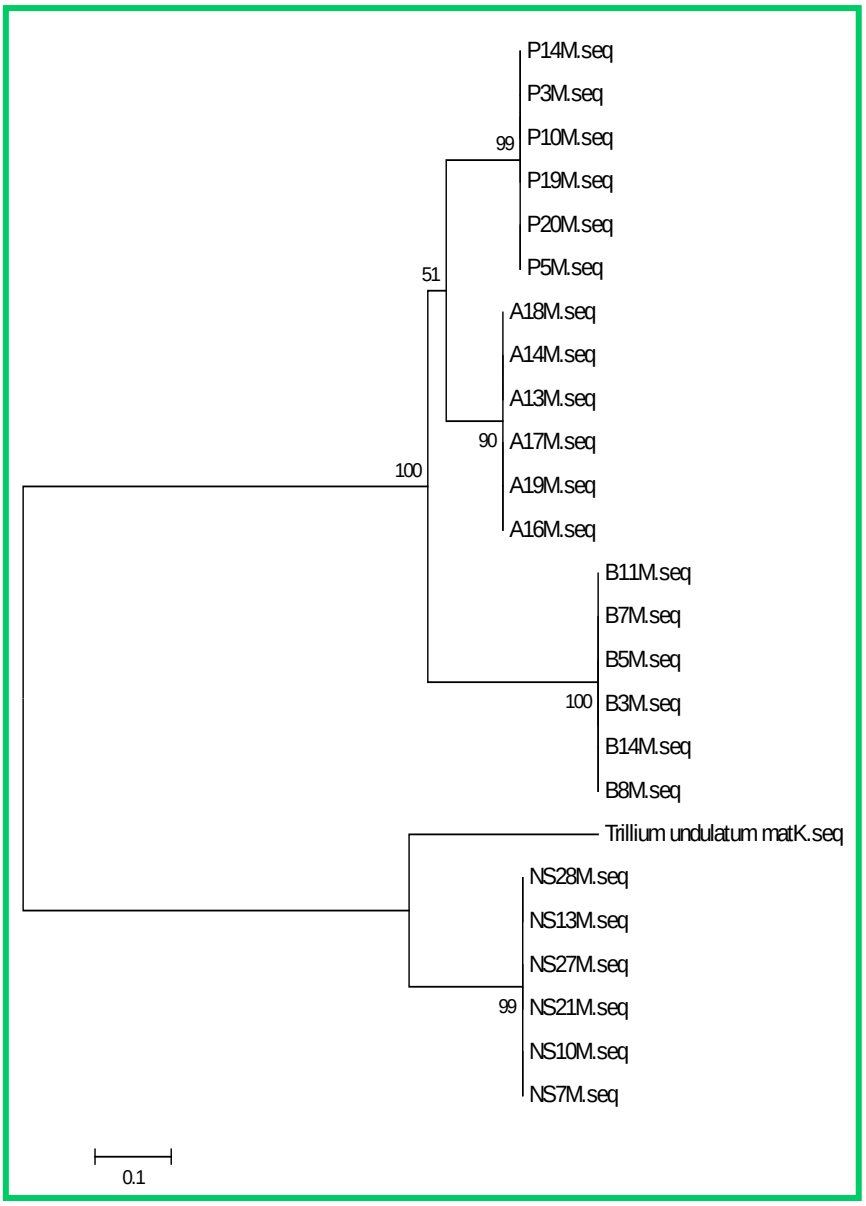

Fig.A.3 The consensus tree obtained from phylogenetic analysis of *matK* gene sequences for 4 taxa and *Trillium* as an outgroup (x 1000 replications). Numbers above branches are bootstrap values.

A.3.3. Internal transcribed spacers ITS base genetic diversity and phylogenetic analyses

Based on the results obtained by the related sequences from the study of 18S-26S region intra-individual variation was only detected in the plant species *Hypericum aciferum*. We obtained approximately 1100bp from *Bupleurum kakiskalae* and *Nepeta sphaciotica* and 650 bp from *Hypericum aciferum* samples. The results of phylogenetic analysis using the ITS region for *Bupleurum kakiskalae, Nepeta sphaciotica, Hypericum aciferum* is showed in Fig.A.4. The ITS tree was obtained by Mega 3.1 software. Pairwise sequence divergence values are presented in Table A.3.

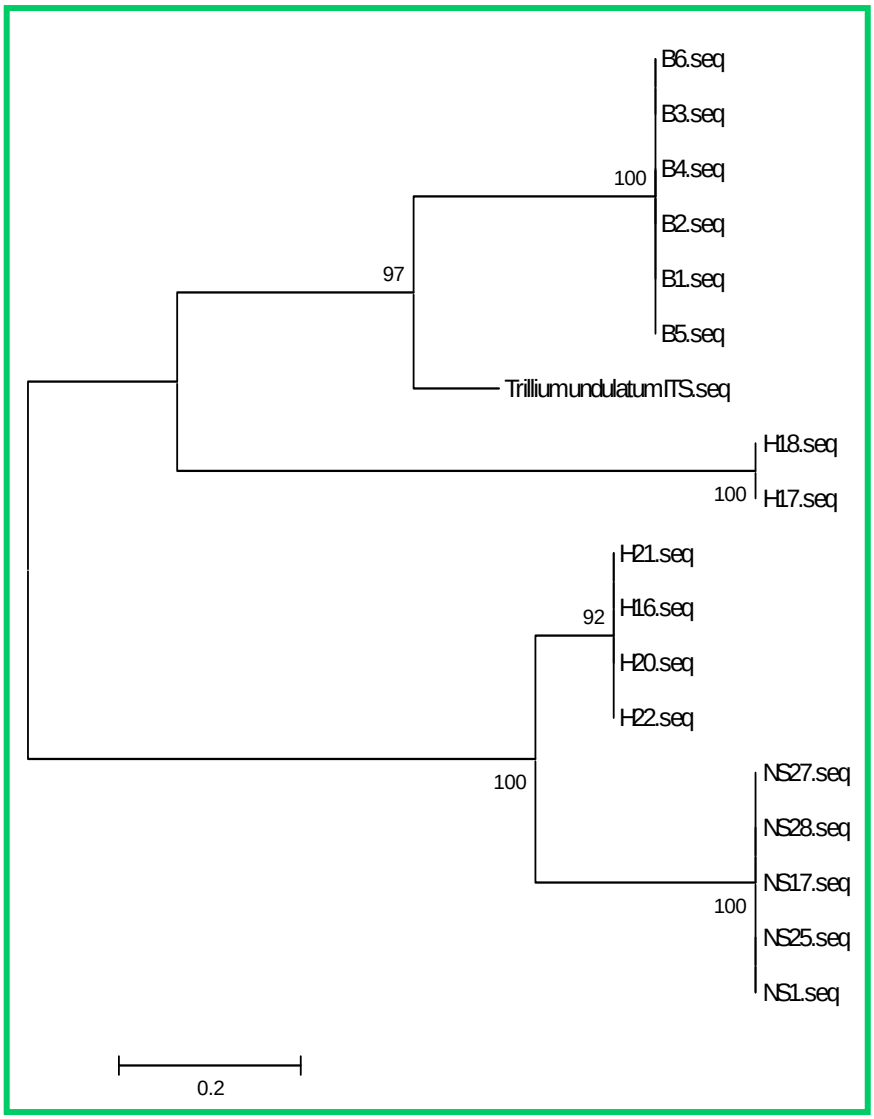

Fig.A.4 The consensus tree obtained from phylogenetic analysis of the ITS1 region and 5.8 gene sequences for 3 taxa. *Trillium* was used as an outgroup (x 1000 replications). The length of the obtained sequences after trimming using the MEGA 3.1 software is 346bp for each species.

	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	0.975		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	1.387	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	1.387	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	1.387	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	0.975	1.387	1.387	1.387		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	1.387	1.275	0.000	0.000	0.000	1.387		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
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
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	

Table A.3 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.

After trimming the resulting sequences from the 18S-26S region for the phylogenetic analysis of the three species, the actual length of the sequences decreased in 346bp accordingly. The sequence boundaries of one ITS region (ITS1) and one coding region (5.8S) of nrDNA 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. Together, these taxa form three different groups. *Nepeta sphaciotica* and *Bupleurum kakiskalae* individulas 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. A.4) resulting from the ITS region are two informative sites, and four homoplasious.

A.4. Discussion/Conclusions

In the present study the topologies of the NJ trees obtained for the *rbc*L and *matK* genes of *cp*DNA followed the same pattern (Figs 2 and 3) between the five taxa *Androcymbium rechingeri, Bupleurum kakiskalae, Nepeta sphaciotica, Hypericum aciferum* and *Phoenix theophrasti*. The *rbc*L gene has evolved very slowly and its phylogenetic resolution was thus very limited and showed no sequence variation and divergence rates within the species but at interspecifc level. It is considered to be highly conserved (Hayashi and Kawano, 2000). As other studies showed *rbc*L gene is appropriate for phylogenetic analysis at the familiar or generic level (Soltis *et al.* 1990; Shinwari *et al.* 1994; Kato *et al.* 1995; Tanaka *et al.*, 1997) and sometimes at the specific level (Yasui and Ohnishi, 1998).

The phylogenetic tree obtained by the *matK* gene sequence data showed identical results thus it showed also intrespesific variation between the species. The *matK* gene has evolved approximately three times faster than *rbc*L (Johnson and Soltis 1994, 1995; Liang and Hilu, 1996). It appears appropriate for analysis mostly at both the specific (Soltis *et al.* 1996; Osaloo *et al.*, 1999) and generic levels (Jonson and Soltis 1994; Liang and Hilu, 1996) situated in Osaloo and Kawano, 1999. Although we expected to see some intraspecific genetic variation among species using *matK* gene rather than *rbc*L gene which according to Olmstead and Palmer (1994) is known to have the highest overall substitution rate among the some 20 chloroplast genes, on the contrary we saw no genetic differentiation within the species from these two *cp*DNA molecular markes.

The ITS (internal transcribe spacer) region of ribosomal DNA, is a very rapidly evolving gene region (Pryer *et al.*, 2001) which is generally useful for plant systematics and genotyping (Schaal *et al.*, 1998). In comparison with the *matK* and *rbc*L genes the ITS spacer regions sequences evolves more rapidly than these coding regions (Baldwin 1992; Baldwin *et al.*, 1995) and thus we expected to see intraspecific genetic variation in all plant species. According to our results genetic diversity at intraspesific level is occurring only within the individuals of *Hypericum aciferum* enlightening our current study with significantly genetic data. Intraspecific genetic diversity of the nucleotide sequence of the chloroplast *rbc*L and *matK* genes and the ITS region cannot be observed from our genetic data except in *Hypericum aciferum*. Our study does not preclude the possibility that with more complete survey and the addition of evidence from other genes and microsatellites loci intraspecific genetic variability might be defined within the other four species.

A.4.1. Genetic variability and Conservation status

Giving account to our results we make a preliminary suggestion that *Androcymbium rechingeri, Bupleurum kakiskalae, Nepeta sphaciotica, Hypericum aciferum* and *Phoenix theophrasti* be used as flagship species-a symbol of conservation and awareness and also the survival of Crete's flora (Hughes *et*

al., 2003). The genetic results presented in this study indicate that the lack of genetic marker variation within the species is likely to be the result of low-level environmental adaptations of these plant species and thus emphasized the importance that the lack of molecular divergence have upon them. (Merila and Crnokrak, 2001; Simonsen, Pertoldi *et al.*, 2003). However the results of phylogenetic analyses of the 5 species have provided additional evidence concerning their phylogenetic relationships and the fact that the diversity among the species leads as to investigate for further genetic surveys developing microsatellites loci for each of the plant species, which will be most useful for our conservation biology studies.

Conducting molecular biological analyses and population genetic studies will be helpful for achieving further understanding the evolution of these species. Nevertheless for the protection of individual species and natural habitats Plant-Micro reserves[4] must be established in order to compare the conservation significance between endangered plant species in such areas and facilitate the decision making for their management (Botena *et al.*, 2004). The study of these endemic taxa is now more than ever judged to be so important and urgent in a conservational context in Crete.

[4] Plant Micro-Reserve are small land plots (up to 20 ha) of peak value in terms plant species richness endemism or rarity, given over to long term monitoring and conservation of plant species and vegetation types (Laguna *et al.*, 2004).

B. Characterization of nuclear microsatellite markers in *Phoenix theophrasti (Palmae*)

B. Introduction

Microsatellite markers are a valuable tool for plant genetics and breeding (Varshney *et al.*, 2005). Some preliminary results using microsatellite loci obtained by Billotte *et al.*, 2004 in *Phoenix theophrasti* population from Chryssoskalitisa, showed none genetic variation between the 21 plant samples. In addition to this we examined three more populations 1. from Vai aesthetic forest, 2. from a pale area in Vai and 3. from Moni Preveli (seed samples). Also in the present study we have examined another species of the genus of Phoenix and that is the known *Phoenix dactylifera* species.

B.2 Material and Methods

B.2.1. Plant samples

Fresh samples of *Phoenix theophrasti* were collected during March 2005, consisting of 21 individuals from Chryssoskalitisa. Seed samples were collected in 2004 and eight seeds from each population were planted in April 2006. One fresh sample of *Phoenix dactylifera* was collected from Chryssoskalitisa also.

B.2.2. DNA extraction

Total genomic DNA was extracted from leaf samples by using a modified hexadecyl trimethyl ammonium bromide (CTAB) method Doyle and Doyle (1987).

B.2.3. Polymerase chain reaction

All plant individuals were PCR examined by amplifying their genomic DNA with a vast number of microsatellites loci (Table B.1). PCR products were analysed on 4% agarose gel to determine the presence and length of the amplified product.

B.3. Results

According to our results from the PCR screening that was based in the microsatellite loci on *Phoenix theophrasti* populations no genetic variation was visually detected, thus the samples had the same size. One remarkable feature was that we manage to detect genetic variation between the fresh and the seed samples of *Phoenix theophrasti* samples with the sample of *Phoenix dactylifera.*

B.4. Discussion/Conclusions

Genetic variability of *Phoenix theophrasti* populations in Crete was not detected using 11 pairs of microsatellite loci obtained by Billote *et. al*, 2004 (Table B.1). However 3 pairs of microsatellite loci (MdPdCIR3, MdPdCIR8 and MdPdCIR10) showed that *Phoenix dactylifera* sample had different size from *Phoenix theophrasti* samples (fresh and seeds) and that is presented in Fig.B.1.This is the first time to discriminate efficiently two species *Phoenix theophrasti* and *Phoenix dactylifera* originated from Crete using these three specific microsatellite markers(MdPdCIR3, MdPdCIR8 and MdPdCIR10).

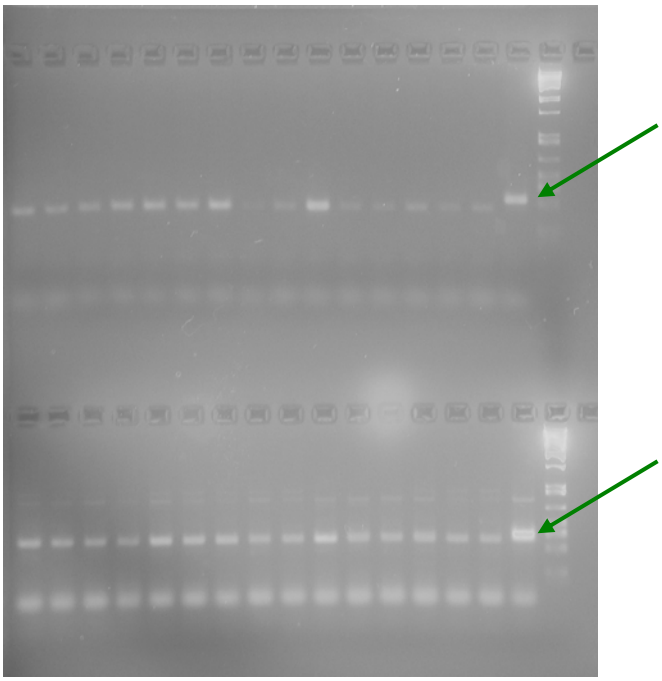

Fig.B.1 Picture form the gel electrophoresis of PCR products using in the upper row the MdPdCIR8 primers and in the down row MdPdCIR10 primers in 15 samples of *Phoenix theophrasti* and in 1 sample of Phoenix dactylifera. The arrows show the different size (higher, MdPdCIR8 ~ 300bp and MdPdCIR10 380~ bp) of Phoenix *dactylifera* sample.

Table B.1 Microsatellites described by Billote *et al.*, 2004

Locus	5´sequence 3´	Allele size	Alleles	Repeat motif
MdPdCIR1	F: ACCCCGGACGTGAGGTG R: CGTCGATCTCCTCCTCCTTTGTCTC	118-161	13	$(GA)_{22}$
MdPdCIR2	F: ACAAACGGCGATGGGATTAC R: CCGGCAGCTCACCTCTTCTAT	175-221	11	$(GA)_{15}$
MdPdCIR3	F: AGCTGGCTCCTCCCTTCTTA R: GCTCGGTTGGACTTGTTCT	120-156	12	$(GA)_{15}$
MdPdCIR4	F: CGAGACCTACCTTCAACAAA R: CCACCAACCAAATCAAACAC	156-192	12	$(GA)_{32}$
MdPdCIR5	F: GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC	281-332	6	$(GA)_{19}$
MdPdCIR6	F: GAGAGAGGGTGGTGTTATT R: TTCATCCAGAACCACAGTA	152-183	18	$(GA)_{29}$
MdPdCIR7	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG	153-184	15	$(GA)_{16}$
MdPdCIR8	F: GCAGTCAGTCCCTCATA R: TGCTTGTAGCCCTTCAG	142-175	10	$(GA)_{26}$
MdPdCIR9	F: CTTTTATGTGGTCTGAGAGA R: TCTCTGATCTTGGGTTCTGT	121-156	8	$(GA)_{17}$
MdPdCIR10	F: CAAGACCCAAGGCTAAC R: GGAGGTGGCTTTGTAGTAT	182-204	12	$(GA)_{17}$
MdPdCIR11	F: AGCGGGAAATGAAAAGGTAT R: ATGAAAACGTGCCAAATGTC	130-138	5	$(GA)_{14}$

C. Isolation and characterization of microsatellite loci in *Nepeta sphaciotica* (*Lamiaceae*)

C. Introduction

Nepeta sphaciotica is a perennial herb that grows among metamorphic calcareous rock in a sheltered place on a summit area at 2200-2300 m altitude. Is a local endemic of Lefka Ori and the single existing population is located at an inaccessible and windy site on the northern slope of Mt. Zvourichti. It is an endangered plant species due to grazing that combined with the extremely local distribution (small population) makes it vulnerable to any local inadvertent impact and to random processes. Knowledge about the genetic structure of *Nepeta sphaciotica* through the isolation of microsatelllite loci and their characterization in the population of the species will be valuable for its conservation.

C.2 Material and Methods

C.2.1. Plant samples

Samples of *Nepeta sphaciotica* were collected during September 2005, consisting of 28 individuals.

C.2.2. DNA extraction

Total genomic DNA was extracted from leaf samples using a DNAeasy Plant Mini Kit by Qiagen®.

C.2.3. Microsatellites

Two libraries enriched for $(AG)_{12}$ or $(GAA)_8$ repeat sequences were prepared using biotinylated probes and streptavidincoated magnetic beads. For each library approximately 10 μg of genomic DNA were isolated from a pool of *Nepeta Sphaciotica* using a DNAeasy Plant Mini Kit by Qiagen® and digested with *AluI* restriction enzyme. Five hundred nanograms of blunt-end DNA fragments of 300–800 bp were ligated to 100 pmol of *AluI* adapter. Purified adapter ligated DNA (300 ng) and PCR product (300ng) was heat denaturated (95 °C for 15 min) and hybridized to 100 pmol of a 3' biotinylated probe [$(AG)_{12}$ or $(GAA)_8$] in 0.5X saline sodium citrate buffer (SSC) at 57 °C and at 55°C for 2 h 15 min accordingly. The hybrids were added to 600 μL Streptavidin MagneSphere® Paramagnetic Particles (Promega), prepared according to manufacturer's instructions, and incubated at room temperature for 20 min. Two steps of washing in 300 μL of 0.1X SSC at 50 °C were performed followed by two washes in 300 μL of 0.1X SSC at room temperature.

Captured fragments were eluted from the beads by suspension in 100 μL of double-distilled water pre-heated to 50 °C. Ten microlitres of the microsatellite enriched product were amplified and made double-stranded in a 50 μL polymerase chain reaction (PCR) using 5 μm of the 21-mer linker as primer. After purification, PCR products were ligated into pGEM®-T Easy vector (Promega) and the plasmid transformed into DH5α cells (Invitrogen) following standard procedure (Sambrook *et al.* 1989). Recombinant clones were screened for the presence of microsatellite repeats by a three-primer PCR amplification test using two vector primers plus a repeat-specific primer $(AG)_{12}$ or $(GAA)_8$. DNA template was obtained as follows: white colonies were transferred to 180 μL Luria-Bertani (LB) broth containing 100 μg/mL ampicillin in 96-well microplates and incubated at 37 °C, 150 rpm overnight. Ten microlitres of each culture were then

added to 100 μL of LB containing 100 μg/mL ampicillin and allowed to grow for 4 h in a shaking incubator (Tsagkarakou *et al.* 2003).

Plasmid extraction was performed by heat denaturation at 99 °C for 5 min followed by immediate cooling on ice. Five microlitres of the plasmid extract were used as template in a 20-μL PCR containing 0.2 mm dNTPs, 1.5 mm $MgCl_2$, 0.5 μm of each primer, 1 unit Taq polymerase (Minotech)
and 1X enzyme buffer (Minotech). The PCR program was carried out on a Perkin-Elmer 9600 with one cycle of denaturation at 94 °C for 2 min followed by 30 cycles of 45 s at 93 °C, 45 s at 55 °C and 2 min at 72 °C. Plasmid DNA from clones that yielded two or more bands was extracted using the Nucleospin Plasmid kit (Macherey-Nagel). The miniprep products were sent to Microgen (Korea).

C.3. Results

Recombinant clones were screened for the presence of microsatellite repeats. Two hundred and forty two clones were screened by PCR from the $(AG)_{12}$ library and 68 were positive. Of the 68 clones sequenced, 49 were discarded because either the repetition pattern was too short, i.e. less than eight repeats or the regions flanking the repeated sequence were not suitable for primer design and because of many PCR duplicates. From the 19 positive clones primers will be determined shortly. From the $(GAA)_8$ library 242 clones were screened by PCR and another 20 are being checked for positive clones.

References

Baldwin B. G. (1992). Phylogenetic utility of the internal transcribed spacer of nuclear ribosomal DNA in plants: An example from the Compositae. *Molecular Phylogenetics and Evolution*, 1: 3–16.

Baldwin B. G., Sanderson M. J., Porter J. M., Wojeciechowski M. F., Campbell C. S. and Donoghue M. J. (1995). The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. *Annals of Missouri. Botanic Garden*, 82: 247–277.

Barrow C. S. (1998). A Monograph of *Phoenix* L. (Palmae: Coryphoideae). The Herbarium Royal Botanic Gardens, Kew, Richmond, Survey, TW9 3AB, U.K.

Bergmeier E. (1997). Are Cretan endemics threatened by grazing;. Museum of Natural Histroy, Crete, Greece.

Billotte N., Marseillac N., Brottier P., Noyer J. -L., Jacquemoud-Collet J.-P., Moreau C., Couvreur T., Chevallier M.-H., Pintaud J.-C. and Risterucci A.-M. (2004). Nuclear microsatellite markers for the date palm (*Phoenix dactylifera* L.): characterization and utility across the genus *Phoenix* and in other palm genera. *Molecular Ecology Notes*, 4: 256-258.

Boteva D., Griffiths G. and Dimopoulos P. (2004). Evaluation and mapping of the conservation significance of habitants using GIS: an example from Crete, Greece. *Journal for Nature Conservation*, 12: 237-250.

Chartzoulakis K. and Psarras G., (2004). Global change effects on crop photosynthesis and production in Mediterranean: the case of Crete, Greece. *Agriculture, Ecosystems and Environment*, 106: 147–157

Cockerham C. C. and Weir B. S. (1993). Estimation of gene flow from F-statistics. *Evolution*, 47: 855–863.

Council of Europe (1992). Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and wild fauna and flora. *Official Journal of the European Communities*.

Critopoulos P. (1975). The endemic taxa of Crete. Eds. Problems of Balkan flora and vegetation. Publishing house of the Bulgarian Academy of sciences, Institute of Botany, Sofia. pp.: 169-177

Damanakis M. and Matthas U. (1987). XIN International Botanical Congress. Excursion no. 60. Endemism and Island Botany: the flora and vegetation of Crete (Greece). Excursion Guide. Berlin. (pp. 5-10).

Doyle J.J., Doyle J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19: 11-15.

Georgiou K. and Delipetrou P. (2001). Eds. Threaten endemic flora species in South Greece. Crete District- District Fund, Museum of Natural History Crete. Iraklion.

Greuter W. (1979). The Endemic flora and the significance of its protection. Practical of Conference of the protection of fauna-flora and biotypes. Hellenic Society for the Protection of Nature.

Hayashi K. and Kawano S. (2000). Molecular systematics of *Lilium* and allied genera (*Liliaceae*): phylogenetic relationships among *Lilium* and related genera based on the *rbcL* and *matK* gene sequence data. *Plant Species Biology*, 15: 73–93.

Hughes M., Hollingsworth M.P. and Miller G. A. (2003). Population genetic structure in the endemic Begonia of the Socotra archipelago. *Royal Botanic Garden Edinburgh*, 113: 277-284.

Johnson L. A. and Soltis D. E. (1994). *MatK* DNA sequences and phylogenetic reconstruction in Saxifragaceae s. *str. Systematic Botany* 19: 143–156.

Johnson L. A. and Soltis D. E. (1995). Phylogenetic inference in Saxifragaceae sensu stricto and Gilia (Poleminiaceae) using matK sequences. *Annals of the Missouri Botanical Garden*, 82: 149–175.

Kato H., Terauchi R., Utech F. H. and Kawano S. (1995). Molecular systematics of the *Trilliaceae sensu lato* as inferred from *rbcL* sequence data. *Molecular Phylogenetics and Evolution*, 4: 184–193.

Laguna E., Deltoro I.V., Pèrez-Botella J., Pèrez-Rovira P., Serra LI., Olivares A., Fabregat C. (2004). The role of small reserves in plant conservation in a region of high diversity in eastern Spain. *Biological Conservation*, 119: 421-426.

Liang H. and Hilu K. W. (1996). Application of the *matK* gene sequences to grass systematics. *Canadian Journal of Botany*, 74: 125–134.

Luikart G., and England, P. R. (1999). Statistical analysis of microsatellite DNA data. *Trends in Ecology and Evolution*, 14: 253–256.

Lynch M. (1996). A quantitative genetic perspective on conservation issues. In J. C. Avise, and J. L. Hamrick (Eds.). Conservation Genetics: Case Histories from Nature (pp. 457–501). New York: Chapmann and Hall.

MAICh (2002). Threaten Plants of Crete according with the European Directive 92/43/EEC for the conservation of nature habitants and wild fauna and flora. Crete. Chania.

Merila J. and Crnokrak P. (2001). Comparison of Genetic Differentiation at marker loci and quantitative traits. *Journal of Evolutionary Biology*, 14: 892–903.

NIKUA-MAICh-FDC (2004). Cretaplant «A Pilot Network of Plant Micro-Reserves in Western Crete». LIFE-Natura 2004.

Olmstead and Palmer J. D. (1994). Chloroplast DNA systematics: A review of methods and data analysis. *American Journal of Botany*, 81:1205-1124.

Osaloo S. K. and Kawano S. (1999). Molecular systematics of Trilliaceae II. Phylogenetic analyses of Trillium and its allies using sequences of *rbc*L and *matK* genes of *cp*DNA and internal transcribed spacers of 18S–26S nrDNA, *Plant Species Biology*, 14: 75–94.

Osaloo S. K., Utech F. H., Ohara M. and Kawano S. (1999). Molecular systematics of *Trilliaceae* I. Phylogenetic analyses of *Trillium* using *matK* gene sequences. *Journal of Plant Research*, 112: 35–49.

Pertoldia C. and Topping C. (2004). The use of agent-based modelling of genetics in conservation genetics studies. *Journal for Nature Conservation*, 12: 111-120.

Phitos D., Strid, A., Snogerup, S. and Greuter, W. (1995). The Red Data Book of rare and threatened plants of Greece. K. Michalas S. A. Athens.

Pritchard J. K., Stephens, M. and Donnelly, P. J. (2000). Inferences of population structure using multilocus genotype data. *Genetics*, 155: 945–959.

Pryer K.M., Schneider H., Smith A.R., Cranfill R., Wolf P.G., Hunt J.S., and Sipes S.D. (2001). Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. *Nature*, 409: 618-622.

Schaal B. A., Hayworth D. A., Olsen K.M., Rauscher, J. T. and Smith, W. A. (1998). Phylogeografic studies in plants: problems and prospects. *Molecular Ecology*, 7: 465-474.

Shinwari Z. K., Kato H., Terauchi R. and Kawano S. (1994). Phylo- genetic relationships among genera in the *Liliaceae–Asparagoideae–Polygonatae* sensu lato inferred from *rbc*L gene sequence data. *Plant Systematics and Evolution*, 192: 263–277.

Simonsen V., Pertoldi C., Madsen A. B. and Loeschcke V. (2003). Genetic differentiation of foxes (*Vulpes vulpes*) analyzed by means of craniometry and isozymes. *Journal for Nature Conservation*, 11: 109–116.

Slatkin M. (1995). A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, 139: 457–462.

Soltis D. E., Sotis P. S., Clegg M. T. and Durbin M. (1990). *rbc*L sequence divergence and phylogenetic relationships in Saxifragaceae sensu lato. *Proceedings of the National Academy of Sciences*, 87: 4640–4644.

Soltis D. E. Kuzoff R. K. Conti E., Gornall R. and Ferguson K. (1996). *MatK* and *rbc*L gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. *American Journal of Botany*, 83: 371–382.

Tanaka N. Setoguchi H. and Murata J. (1997). Phylogeny of the family Hydrocharitaceae inferred from *rbc*L and *matK* gene sequence data. *Journal of Plant Research*, 110: 329–337.

Tsagkarakou A., Roditakis N. (2003). Isolation and characterization of microsatellite loci in Bemisia tabaci (Hemiptera: Aleyrodidae). *Molecular Ecology Notes*, 3: 196-198.

Turland N.J., Chilton L. and Press J. R. (1993). Flora of the Cretan Area. Annotated Check List and Atlas. The Natural History Museum, HMSO, London. pp. 439.

Varshney K. R., Graner A. and Sorrells E. M. (2005). Genic microsatellite markers in plants features and applications. *Trends in Biotechnology*, 23: 48-55.

Yasui Y. and Ohnishi O. (1998). Interspecific relationships in *Fagopyrum* (Polygonaceae) revealed by the nucleotide sequences of the *rbcL* and *accD* genes and their intergenic region. *American Journal of Botany*, 85: 1134–1142.