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Original article

RAPD markers reveal genetic variation between *Cichorium spinosum* L. and *Taraxacum* sp.; a substantial medicinal plants of Greece

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Summary. Fifteen Random Amplified Polymorphic DNA (RAPD) markers were used to measure genetic diversity and genetic relationships between five endemic genera of Mediterranean basin (Greece). Three species of *Cichorium spinosum* were collected; two from island Crete (Greece) and one from island Kythnos, and other two species of *Taraxacom sp*. (Asteraceae); are from Orhomenos and Athens. Two hundred-forty amplified products and 163 RAPD bands were scored with an average of 67.91% of them revealing polymorphism across accessions. In this research OPD-05 primer with 24 bands showed the highest number of bands, while the OPM-18 and OPB-16, both of them with 8 bands showed the least number of bands. Also OPV-06 primer with 18 polimorphic bands showed the highest number of bands. The least number of polymorphic bandswere found in OPX-18. UBC-292, OPAN-01, OPB-16, OPM-18, OPD-05 primers. Subsequently, OPD-05 primer with 29.16% showed the least percentage of polymorphism degree, whereas OPM-18 and OPB-16 primers with 87.50% showed the highest percentage of polymorphism. UPGMA clustering based on data from polymorphic RAPD bands revealed two distinct group which joined to form one major cluster at 32% level of similarity. Also *Cichorium spinosum,* Crete and *Cichorium spinosum,* Kythnos, varieties with 100% similarity are synonyms. The similarity indices of the RAPD dendrogram ranged between 30% and 100% averagely high enough to suggest useful variability for genetic diversity and plant breeding.

Key words: *Cichorium spinosum* L., *Taraxacum sp.*, Genetic Variation, Greece, RAPD, medicinal plants.

Introduction

Cichorium spinosum L., 2n=18 (Stamnagathi) is a perennial species in the genus *Cichorium* which contains approximately 6 to 10 species (*C. intybus, C. endivia, C. pumilum, C. spinosum, C. calvum and C. bottae*) and belongs to Asteraceae family (Figure 1 and Table 1) (1). Stamnagathi can be found in Spain, Balearic Islands, Turkey, Italy (Sicily), South Greece (Crete) and Aegean islands. It appears that Crete, the southern-most island of Greece, has a unique flora, with more than approximately 1,800 plant species; among these approximately 180 are endemic to Crete (2, 3). *Cichorium spinosum* is known as one of these important native plants in Greece that is consumed as salad, raw, eitherfresh or boiled in water, and served with olive oil and lemon, or cooked in red sauce with lamb meat, (4, 5) Comparing to spinach, Stamnagathi has higher nutritional values and is better source of protein, antioxidants (vitamin C and E, beta carotene, alpha-tocopherol, glutathione, and phenols), minerals (K, Ca, Mg, Fe, Cu, Mn, Zn) and linolenic

acid (omega-3). The importance of antioxidants from wild plant (Stamnagathi) in the diet of Crete as decreasing both the risk and the death rate in patients with one episode of myocardial infarction.

Taraxacum sp. (x = 8, Asteraceae) is a short plant, usually with a yellow flower and notched leaves and exudes a milky sap when broken (Figure 1 and Table 1). The dandelion is also known by its generic name *Taraxacum* and is native to Europe, Mediterranean region, Asia, and has spread to many other places like America, Australia and New Zealand as weeds. Genetic variation in populations of apomictic *Taraxacum* species is much higher as expected (6). The genus *Taraxacum* is a complex of about 3000 species worldwide (7). Species in the genus *Taraxacum* represent a polyploid series of diploid (2n = 2x = 16) sexual species and polyploids. The most common polyploid is triploid (2n = 3x = 24) (8). While the dandelion is considered a weed by many gardeners, the plant has been widely utilized for medicinal and edible purposes. Dandelions are grown commercially at a small scale as a leaf vegetable. They are probably closest in character to mustard greens (9). A first reference to its application is reflected in its name, which is derived from the Greek words "taraxis" for inflammation and "akeomai" for curative. Taraxacin, taraxasterol, stigmasterol, chicoric acid, caffeic acid, scopoletin, inulin, esculin, guaianolide, desacetylmatricarin, ß-glucopyranosyl ester and sonchuside A are among the medicinally active compounds (10-12). Esculin is one of the most important components used in pharmaceutical industry (13). It is an elasticity giving and sealing material for blood vascular system (14). The leaves are high in vitamin A, vitamin C and iron, carrying more iron and calcium than spinach. Dried leaves and roots are available as herbal tea. Ground roasted dandelion root can be used as a coffee substitute. Drunk before meals, stimulate digestive functions (15). It is considered an excellent cleansing tonic for the liver. Whole plant extracts possess choleretic, diuretic, anti-inflammatory and anti-oxidative, anti-carcinogenic, analgesic, anti-hyperglycemic, anticoagulatory and prebiotic effects (16-19). However, outline of scientific results from the beginning of the last century, more detailed pharmacological and nutritional investigations of *Taraxacum* and *Cichorium spinosum* have become an issue of increasing interest in the past years.

RAPD (Random Amplified Polymorphic DNA) markers are used to analyse the genetic diversity of an individual by using random primers (20-23). In cont-

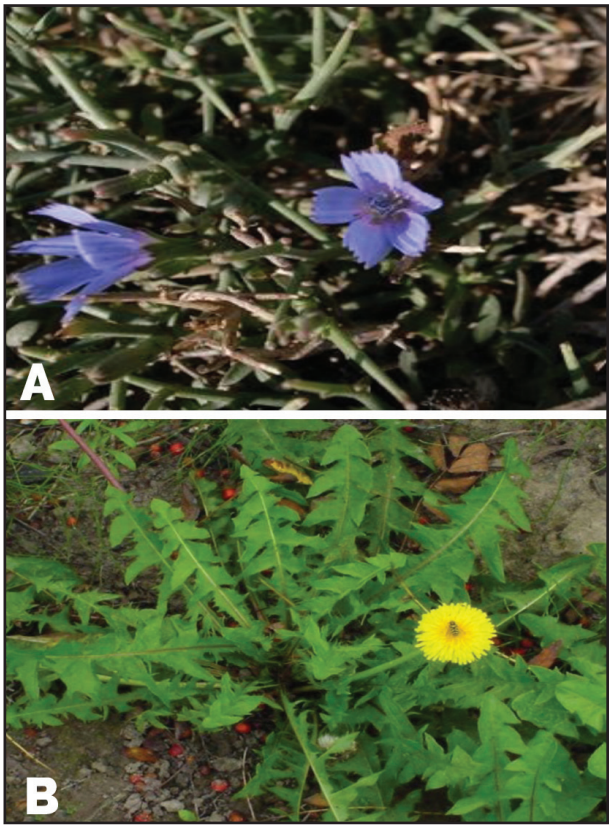

Figure 1. *Cichorium spinosum* (Stamnagathi), b: *Taraxacum* sp.

Table 1. Taxonomy of *Cichorium spinosum* (Stamnagathi) and *Taraxacum sp.*

	***Cichorium spinosum* (Stamnagathi)**	***Taraxacum***
Kingdom	Plantae	Plantae
Subkingdom	Tracheobionta	Tracheobionta
Division	Magnoliophyta	Magnoliophyta
Class	Magnoliopsida	Magnoliopsida
Order	Astrales	Astrales
Family	Asteraceae	Asteraceae
Subfamily	Cichorioideae	Cichorioideae
Tribe	Lactuceae	Lactuceae
Genus	Cichorium L.	Cichorieae
Species	*Cichorium spinosum*	*Taraxacum*

rast to *C. intybus* which has a wide geographic distribution and is the most variable species of *Cichorium*, any RAPD marker method on Stamnagathi with Taraxacum have not been performed to date. Therefore, current work suggests applying this type of marker for the first time and the result of such study could be of importance to breeders interested in broadening the gene pool of *Cichorium spinosum* L. and *Taraxacum* sp. The aim of the present study is to assess genetic variation within and among ecotypes of the species using RAPD markers. Another important aim is to contribute genetic information and a theoretical basis for protection of the species and also one step further, in breeding efforts and management activities to increase the value of efficiency and quality of spiny chicory in the future.

In this study, we successfully utilized the RAPD technique for rapid characterization of 5 plant species of *Cichorium spinosum* L., and *Taraxacum* sp. with 15 primers from two Greek islands (Kythnos and Crete). The determination of genetic variation and relationships between cultivars, as well as the application of the data obtained for ecotypes identification, is discussed.

Materials and Methods

Plant Samples

Seeds and plant tissues from the three ecotypes of *Cichorium spinosum* and two of *Taraxacum* were collected in situ from two Greek islands [two from Crete, a mountainous (sample 1) and a coastal ecotype (sample 2) and one from Kythnos (sample 3), as well as from two ecotypes of *Taraxacum* sp. collected in Votanikos, Athens (latitude 37° 98' 27", longitude 23° 70' 40", sample 4) and Orhomenos, Voiotia (latitude 38° 49' 55", longitude 22° 98' 88", sample 5). The seeds were implemented in order to propagate the plants and get the sample tissues for further analysis. Seeds were sown in disk trays containing peat moss on November 15th and put in an unheated greenhouse. The young seedlings (at the stage of 4-5 true leaves) were transplanted into 5 L plastic pots containing peat moss and perlite in a ratio of 1:1. Prior to transplantation a base dressing was applied, whereas during cultivation on a regular irrigation regime was applied (1-2 times per week depending on temperature) with 250 ml of tap water. Once a week fertigation was applied with a solution containing 300 ppm of nitrogen. Cultivation of the plants took place in two different experimental sites (Agricultural University of Athens and University of Thessaly, both in Greece). The young leaves from the three ecotypes, were collectedin order to be analysed for molecular character status. The samples were individually placed in sealable polyethylene bags, transported to laboratory and then kept under deep freezing conditions (-80°C) until DNA extraction.

Genomic DNA isolation

Genomic DNA extraction was performed from young 1–2 cm long leaf samples of plants. Total DNA was extracted and leaf tissue purified as described by DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). 100 mg of young leaves were ground in a sterile mortar to a fine powder in liquid nitrogen and homogenized. The powder was transferred to a new 2 ml polypropylene tube and 1 ml of DNA extraction buffer (50mM Tris-HCl [pH 8.0], 0.7mM NaCl, 10mM EDTA, 2% hexa decyl trimethylammonium bromide, and 0.1% 2-mercaptoethanol) and incubated in 65 °C for 15 min. Then centrifuge at 14000g for 10 min and re-suspended in 100 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 15 µg/ml RNAse and incubated for 30 min at 37°C. The pellet was dried at room temperature, re-suspended in 100 µl of TE and stored at 4°C. The purity and quantity of isolated DNA were determined spectrophotometrically (GeneQuant-1300; GE Healthcare, Buckinghamshire, UK). DNA quality also was checked by 1.5% agarose gel electrophoresis.

PCR amplification of DNA

Standard arbitrary 10-mer oligonucleotides (Operon Technologies Inc., USA) were tested for RAPD analysis. A total of 15 primers were initially screened on 5 cultivars of chicoryum. The effects of magnesium, template DNA concentrations, pH values, and length

of the denaturation stage of the amplification were all examined. Finally the best concentration for each variety PCR amplification was carried out in a total volume of 25 µl containing 200 ng genomic DNA, 4 µl 5X reaction buffer, 2µl of 25 mM $MgCl_2$, 0.5µl of 2.5 mM dNTPs, 200 ng primer and (0.5 unit) Taq DNA polymerase (Promega). The PCR program was started with an in by 35 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72 °C. Finally extension was performed at 72 °C for 8 min. Amplification products were separated on 2% agarose gels in TBE buffer and stained with ethidium bromide and photographed under UV light.

Data analysis

The electrophoretic patterns were visually analysed and Bands were scored '1' for its presence and '0' for its absence. Genetic similarity data among accessions relations between ecotypes were determined with respect to the similarity index method UPGMA and a dendrogram was generated by the NTSYS (1.8) computer programme (24).

Results

This research focuses on the genetic differentiation between three ecotypes of spiny Chicory (samples 1-3) and two ecotypes of *Taraxacum* sp. (samples 4 and 5). were analyzed using of 15 RAPD decamer primers. Degree of polymorphism and information content for 15 RAPD random primers along with their sequences, applied to all the samples (Table 2).

A total of 240 bands were amplified and 163 of them were polymorphic giving the average of 10 amplified bands per primer and the level of DNA polymorphism established among local cultivars was 67.91%. The size of amplified fragments ranged between 200 and 1800 bp. From the selected primers OPV-06 yielded the maximum number of polymorphic (18 bands) bands. The lowest number of polymorphic bands (7 bands) were obtained using OPD-05, OPM-18, OPB-16, OPAN- 01, UBC-292 primers. When the ratios of polymorphic bands are examined on the basis of primers, the highest ratio (87.50%) was determined by OPM-18 and OPB-16. The polymorphic information content was highest for the primer OPT-02 (85.71%) followed by the primer OPV-06 (81.81%). Also the lowest ratio (29.16%) was obtained with OPD-05. Figures 2.a., 2.b., show the sample gel images of RAPD patterns obtained with primers OPT-04, OPF-06, OPX-14, GLA-09, OPV-06, OPM-16.

The dendogram generated by RAPD analysis showed that the five samples (three of spiny chicory and two of *Taraxacum* sp.) are divided into two major groups. The first group includes three samples (1,4 and 5), while the second one contains samples (2 and 3) (Fig. 3). The relativity rate between genotypes (1 with 4 and 5) based upon coefficient of similarity in the first group, (samples 4 and 5) *Taraxacum* species, showed a high genetic similarity (83%), and it is so interesting because (sample 1) the mountanous type of spiny chicory is also showed higher similarity (66%) with samples (4) and (5) *Taraxacum* species, instead of the other ecotypes of spiny chicory. It shows that

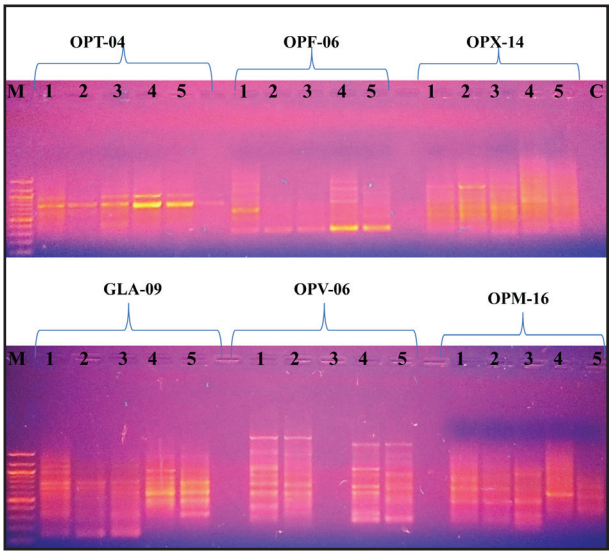

Figure 2. A) Shows the sample gel images of RAPD patterns obtained with primers OPT-04, OPF-06, OPX-14, M: Molecular weight marker (bp), C: Negativ control. 1: Cichorium spinosum,Crete (mountain), 2: Cichorium spinosum,Crete (plain field), 3: Cichorium spinosum, Kythnos, 4: Taraxacum sp. (Athens), 5: Taraxacum sp. (Orhomenos).
B) Shows the sample gel images of RAPD patterns obtained with primers GLA-09, OPV-06, OPM-16. 1: Cichorium spinosum,Crete (mountain), 2: Cichorium spinosum,Crete (plain field), 3: Cichorium spinosum,Kythnos, 4: Taraxacum sp. (Athens), 5: Taraxacum sp. (Orhomenos).

Table 2. Degree of polymorphism and information content for 15 RAPD random primers along with their sequences, applied to 3 *Cichorium* and *Taraxacum* species.

Number	Primer name	Sequence	Number of amplified fragments		
			Total (T)	Polymorphic %	Polymorphism (POL/T × 100)
1	OPD 05	5'-TGAGCGGACA-3'	24	7	29.16
2	OPV 06	5'-ACGCCCAGGT-3'	22	18	81.81
3	OPM 18	5'-TGAGTGGGTG-3'	8	7	87.5
4	GLA 09	5'-GGGTAACGCC-3'	22	14	63.63
5	OPX 14	5'-ACAGGTGCTG-3'	20	11	55
6	OPX 18	5'-GACTAGGTGG-3'	15	11	73.33
7	OPM 16	5'-CACACTCCAG-3'	16	12	75
8	OPY 18	5'-GACTAGGTGG-3'	13	10	76.92
9	OPT 02	5'-GGAGAGACTC-3'	14	12	85.71
10	OPB 16	5'-TTTGCCCGGA-3'	8	7	87.50
11	OPAN 01	5'-ACTCCACGTC-3'	10	7	70
12	OPA 02	5'-AATCGGGCTG-3'	17	12	70.58
13	OPT 04	5'-CACAGAGGGA-3'	22	13	59.09
14	UBC 292	5'-AAACAGCCCG-3'	11	7	63.63
15	OPF 06	5'-GGGAATTCGG-3'	18	15	83.33
	Sum		**240**	**163**	**67.91**

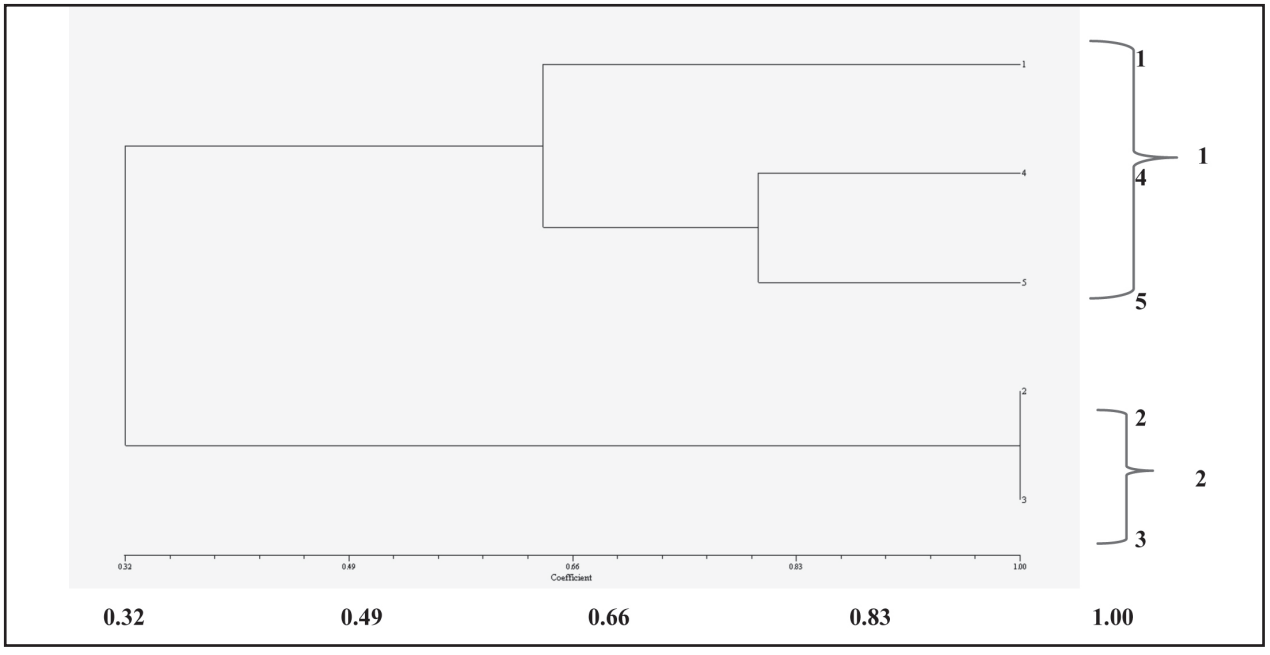

Figure 3. The UPGMA dendogram depicted by NTsys software based on RAPD data derived from similarity coefficients, showing the relationship of five cultivars of Cichorum based on all 15 primers. 1: *Cichorium spinosum,* Crete (mountain), 2: *Cichorium spinosum,* Crete (plain field), 3: *Cichorium spinosum,* Kythnos, 4: *Taraxacum* sp. (Athens), 5: *Taraxacum* sp. (Orhomenos).

the genotype number 1 (*cichorium spinosum*) had a near similarity (66%) with *Taraxacum* species. In group two, (sample 2 and 3) the similarity percentage was (100%) indicating the two ecotypes were synonyms. Moreover, it could be understood that group 1 with group 2 has a 32% of genetic similarity. The OPX, OPY and OPM series revealed the highest amplification probably due to high homology between the primers and the chicory DNA fragments.

This work as one of the first significant studies on *Cichorium* and *Taraxacum* species from Greece based on RAPD technique is a sensitive and efficient tool for genomic analysis in native species, that may be useful in future studies, by assigning new unclassified germplasm accessions to specific taxonomic groups, as well as for implementation in breeding projects.

Discussion

Molecular data was used to identify high priority populations in conservation programs and allow the selection of a minimum number of populations and should be preserved to modulate the loss of genetic diversity of a threatened species (25).

Genetic markers are useful tools for species demarcation. Nevertheless, each genetic marker has its own properties that has to be taken into account. Random Amplified techniques such as RAPD (21) are high resolvent and screen nuclear DNA regions throughout the genome. The simplicity of laboratory assay for RAPD markers makes them an attractive method for obtaining intraspecifc distinctions. Recently, random amplified polymorphic RAPD (a dominant DNA marker) has been widely used in many kinds of genetic analysis of vegetative crops because of their ease of use and speed and the wide availability of universal primers (26). Previously many authors have reported the influence of several molecular markers reactions for varietal identification and determination of parentage in chicory (27-31).

On the other hand, the preservation of genetic diversity within the species is a major target of conservation, because loss of genetic variation is thought to reduce the ability of populations to adapt to environmental change for survival (32-34).

RAPD method can also discriminate successfully among all the plant species, therefore in this research the chicory and Taraxacum ecotypes were chosen regarding their natural habitat and also as valuable folkloric medicines for treatment of diverse diseases and anti-inflammatory remedi. Furthermore, applying this marker is highly informative and could be used to disolve the probable problems associated with the controversy between the gene and the species applied. Consequently, the information generated from this study gives a clear picture of the mentioned species genetic relationship which in turns implies importance for genetic diversity conservation within and among populations and management policies as well.

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