http://dx.doi.org/10.4314/ajtcam.v10i2.7

RAPD-PCR and real-time PCR HRM BASED GENETIC VARIATION EVALUATIONS of *Urtica dioica* PARTS, ECOTYPES and EVALUATIONS of MORPHOTYPES in TURKEY

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Abstract

Urtica dioica is an ethnobotanically and medicinally important Complementary and Alternative Medicine (CAM) plant worldwide and in Turkey; 90 % of herbal CAM applications depend on it in Turkey. It has a wide range of habitats in nearly all continents. It is found in all three phytogeographical regions in Turkey (Euro-Siberian, Irano-Turanian, Mediterranean) with high adaptivity to heterogeneous geographies such as climate, soil types and altitudes. This fact in relation to the assessment of chemical constituents of the plant and combining with further genetic and morphological variation data can assist and enhance the works for the utility and reliability of CAM applications in effect and activity of this plant species. In this work we have made some preliminary experiments with novel approaches to reveal the ecotypes and genetic variation of mighty ecotypes of *Urtica dioica* from different phytogeographical regions of Turkey (Euro-Siberian and Mediterranean). The ecotypes have heterogeneity in both its parts (leaf, stem, root) as revealed by Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) using random primers and High-resolution Melt (HRM) analysis using *Urtica dioica* specific primers and universal chloroplast DNA (cpDNA) primers and morphological traits such as phenolic contents and antioxidant capacities of plants' leaf infusions as used in medicinal applications in Turkey. This work will contribute a lot for the development of molecular markers to detect the genetic variation and heterogeneity of *Urtica dioica* to further relate with expected phenotypes that are most useful and relevant in CAM applications.

Key words: *Urtica dioica*, ecotype, phytogeography, Turkey, RAPD-PCR, real-time PCR, High-resolution melt analysis, sequence matching, cpDNA, agglutinin isolectin VII precursor (chia5.7.2) gene exon 3.

Introductıon

It is becoming increasingly interesting and popular to work with medicinal plants because the use of herbal materials for treatment and health maintenance as part of complementary and alternative medicine applications is on the rise and there is a trend of living in harmony with nature. The Turkish flora is remarkable for its diversity with more than 12000 vascular plant species, about 2000 are ethnobotanically used as especially medicine and spice. *Urtica dioica* is one of the 2000 taxa of plants evaluated for medicinal and aromatic purposes and still one of the 500-1000 taxa of plants used as traditional medical use.The use of *Urtica dioica* as a drug prepared from medicinal plants in Turkish traditional medicine dates back centuries ago and it has a big part in ethnobotanical usage as food in Turkey (Davis, 1965-1985; Davis et al.,1988; Güner et al., 2000, Türe et al., 2010; Öztürk et al., 2012).

U. dioica, is a herbaceous perennial flowering plant species, highly variable in size, branching, leaf and inflorescence form and degree of hairiness. Cultivation experiments carried out by Pollard & Briggs (1982) with individuals from nine populations covering a range of habitat provide evidence that phenotypic plasticity is important in *U. dioica*; for example, fewer hairs were produced on plant grown in the shade than on those in full sun. These experiments also support the conclusion that much of the variation (including the polymorphism in stinging-hair density) is genetically based and heritable (Pollard and Briggs 1982; Bharmauria, et al., 2009; Taylor, 2009).

The morphological identification of *U. dioica* is very straightforward, still the subpopulations and ecotypes due to heterogeneous environments they might live makes clearcut stratifications diffucult for intended usages. Taxonomy of *U. dioica* for ethnobotanical, especially complementary and alternative medicinal usages might have some complications due to wide range of habitats, and the heterogeneity of the morphological traits such as phenolic contents and antioxidant capacities of plants. Different parts of the *U. dioica* contain completely different chemicals which can be extracted in different ways such as the root of the *U. dioca* contains polysaccharides which are believed to be responsible for its anti-inflammatory effects and unidentified components present in certain aqueous, but not lipophilic extracts and/or similar chemicals with different concentrations in different parts of the same plant (Lapinskaya et al., 2008a, 2008b). To overcome various complications and difficulties in diagnosis of this species and to get rid of the need for personal expertise and experiences we designed a set of species specific primers for the *U. dioica* agglutinin isolectin VII precursor (chia5.7.2) gene, exon 3 (UDA) which encodes UDA protein that is a single-chain peptide found in roots and rhizome of *U. dioica* (Van Damme and Peumans, 1987) and used it for both molecular diagnostics and further to assess genetic variation using real-time PCR and HRM analysis in combination with universal, conserved chloroplast DNA sequence 16S rRNA gene (5'

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end) with Val-tRNA gene region (Al-Janabi et al., 1993; Brown, 2002). Advances in molecular biology and biotechnology have provided a range of techniques for direct examination of variation in DNA. Conventional PCR-based approaches with end-point detection techniques detect variation to an extend, whereas further variation can be detected by new technologies based on real-time PCR melting and high resolution melt analysis in a closed tube assay system (Ririe et al., 1997).

Digital PCR (dPCR)-based technologies rely on dilution of the PCR replicates in nano amounts can be furthered with next-generation sequencing is very important in high-throughput diagnostics and biotechnological applications. Levels of intra-specific and inter-specific variation and heterogeneity in *U. dioica* accessions and their parts by RAPD-PCR and real-time PCR HRM analysis, sequence matching function have been detected with dPCR's basic replicative dilution idea using gene sequences belonging to chloroplast genome, and *U. dioica isolectin VII precursor* gene (Uzonur et al., 2004a, 2004b, 2011).

In the first part of the work, the phenolic contents and antioxidant activities of the methanol extracts and leaf infusions of the *U. dioica* accessions that were also analyzed in the next two parts of the work have been compared to show the high variation that might be the reported variations in effect and activity of this plant. In the second part of the work the aim was to show the different heterogeneity levels starting with the intra-individual (leaf, stem, root) and extending to inter-individual levels that might be furthered to produce Sequence Characterized Amplified Region (SCAR) markers to relate with medicinally important traits of *U. dioica*. In the third part, two conserved genes of *U. dioica* accessions have been evaluated using real-time PCR based HRM analysis to detect the phytogeographic ecotypes of *U. dioica* in Turkey. This work can expand the detectability of variation that can not be detected by conventional methods or much more expensive with the others.

Materıals and Methods

Plant material

12 *U. dioica* accessions from various parts of Turkey as given in detail in Figure 1 have been collected in two different flowering seasons of the plant freshly and unless used freshly, kept at -20 ^{o}C until use. For all collection sites two sometimes three plants have been collected from the same locality/neighbourhood. Morphology based typing is done. The plant material has been divided into parts as leaf, stem and root for downstream applications such as preparation of the methanol extracts and infusions and isolation of DNA for PCR based work. DNA isolation has been done using MN Nucleospin Plant DNA isolation kit according to protocole 2. The DNA concentrations have been detected by Qubit flourometer.

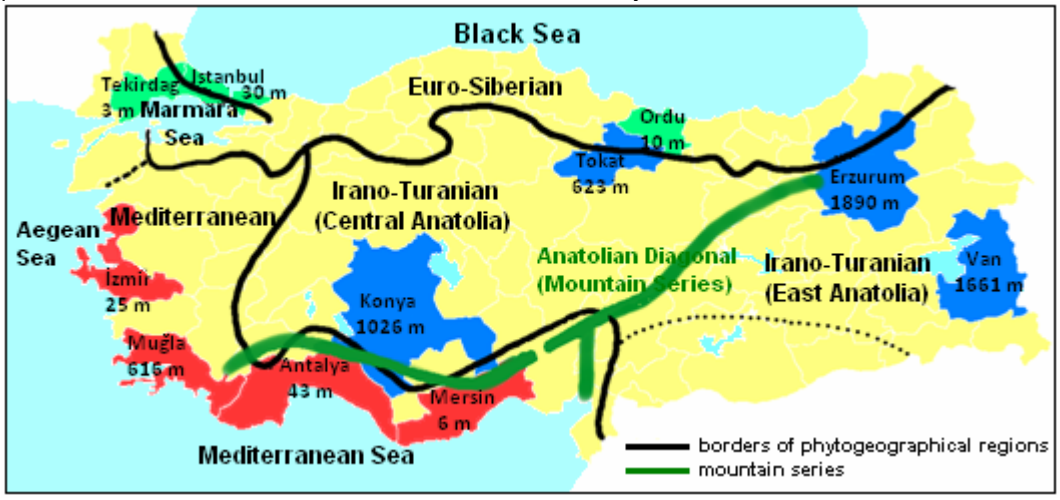

Figure 1: The map and approximate altitudes of Turkish cities where samples have been collected. The color codes are for the phytogeographical ecotypes: green for Euro-Siberian, red for Mediterranean and blue for Irano-Turanian. The sample collection cities and approximate altitudes are Antalya (43 m), Van (1661 m), Muğla (646 m), İstanbul (Anatolia) (30 m), Ordu (10 m), Tekirdağ (3 m), Erzurum (1890 m), Mersin (6 m), Tokat (623 m), İstanbul (Europe), İzmir (25 m), Konya (1026 m).

Evaluation of polyphenolic extracts and anti-oxidant capacity of plant parts' infusions and methanol extracts

For the evaluation of the antioxidant capacity and polyphenolic contents of the plant the different parts (leaf, stem, root) have been extracted/treated both with methanol and water and the extracts were evaluated with two different methods: CUPRAC method (Apak et al., 2004) and Trolox equivalent antioxidant capacity (TEAC) assay (Miller et al., 1993) for antioxidant capacity and Folin – Ciocalteu method for total polyphenolic content detection.

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Preparation of reagents and solutions:

The reagents for the CUPRAC method were; 1.0 x 10^{-2} M copper(II) chloride ($CuCl_2 \cdot 2H_2O$) in water, 1.0 M ammonium acetate (NH_4Ac) at pH = 7.0 in water, and 7.5 x 10^{-3}M neocuproine in 96 % ethanol (EtOH). The reagents for ABTS method were; 4.0 x 10^{-3} M ABTS in water, 1.2 x10^{-4} M hydrogen peroxide (H_2O_2) in 96 % EtOH, and 2.4 x 10^{-5} M HRP in a mixture (1:1, v/v) of EtOH and H_2O. Trolox standard solutions (1.0 x 10^{-3} M) were prepared in ethanol (used in both CUPRAC and ABTS assays as reference standards). The reagents for the Folin-Ciocalteu method were; 2 % (w/v) of Na_2CO_3 in 0.1 M NaOH (Lowry A), 0.5 % (w/v) of copper (II) sulphate ($CuSO_4$) in 1 % (w/v) of sodium potassium tartrate ($NaKC_4H_4O_6$) (Lowry B), Lowry C in a mixture of 50 ml Lowry A and 1 ml Lowry B, and Folin-Ciocalteu reagent (1:3, v/v) in water. The gallic acid standard solutions (1 x 10^{-3} M) were prepared in water (used in Folin-ciocalteu assay as reference standard).

Extraction Process

U. dioica leaves were extracted with aqueous 70 % methanol. 1 g-amount of the plant materials were extracted in stoppered flasks placed in an ultrasonic bath first with 10 ml solvent for 60 min, then with 10 ml solvent for 45 min, and finally with 5 more ml solvent for 15 min, the overall extraction taking 120 min. The three extracts were combined, brought to a final volume of 25 ml with aqueous 70 % methanol. The extracts were filtered through a GF/PET (glass fiber/polyethyleneterephtalate) 1.0/0.45 µm microfilter.

Infusion Process

Infusions *of U. dioica* leaves, stems and roots were prepared separately. 1.0 g of *U. dioica* leaves, bodies and roots with 25 mL deionised water were heated to 95 °C for 8 min. The infusions were filtered through a GF/PET (glass fiber/polyethyleneterephtalate) 1.0/0.45 µm microfilter.

Determination of total antioxidant capacity
CUPRAC Method

The CUPRAC method as described by Apak et al. (2004) was applied as follows: A mixture comprised of 1 mL copper(II) chloride, 1 ml neocuproine solution and 1 mL ammonium acetate buffer at pH 7.0 was prepared, x mL sample solution and (1 - x) mL distilled water were added, and well mixed (total volume: 4.0 mL). This final mixture in a stoppered test tube was let to stand at room temperature for 30 min. At the end of this period, the absorbance at 450 nm was measured against a reagent blank. Trolox was used as the reference standard in this method and the molar absorptivity of trolox is ε =1.62 x 10^4 L $mol^{-1}cm^{-1}$. The trolox equivalent molar concentration of the *U. dioica* samples in the final solution could be found by dividing the observed absorbance by the ε for trolox. The total antioxidant capacity in CUPRAC method was expressed as trolox equivalents (TR) in millimoles per gramme dry material (mmol trolox g^{-1} fw). The trolox equivalent of total antioxidant capacity of *U. dioica* samples in CUPRAC assay was calculated using the following equation:

$$TAC_{CUPRAC}\,(\text{mmol Trolox/g}) = \frac{A_{Extract}}{\varepsilon_{Trolox}} * \frac{V_{Final}}{V_{Sample}} * \frac{V_{Extract}}{m} * \upsilon$$

where: $A_{Extract}$ is the absorbance of extract, ε_{Trolox} is the molar extinction coefficient of trolox at 450 nm, V_{Final} is the final volume of the system, V_{Sample} is the volume of sample added to the system, $V_{Extract}$ is the final volume of the extract, m is the gramme amount of the *U. dioica* samples and v is the dilution factor of the extract.

ABTS Method

ABTS/HRP method using the horseradish peroxidase (HRP)-catalyzed oxidation of ABTS with a slight modification (Karaman et al., 2010) of the method devised by Cano et al. (1998) has been used as follows: The reaction mixture contained 1.0 mL each of ABTS and H_2O_2. The reaction was started by the addition of 1.0 mL of HRP. After 5 min when the formed ABTS radical was stabilized, x mL sample and (1.0 - x) mL EtOH were added to the mixture. After 5 min when the final absorbance was stabilized, the absorbance at 730 nm was recorded against EtOH. The absorbance difference (ΔA) was found by subtracting the absorbance of the antioxidant sample from that of the reagent blank. Trolox was used as the reference standard in this method and the molar absorptivity of trolox is ε =2.75 x 10^4 L $mol^{-1}cm^{-1}$. The trolox equivalent molar concentration of the *U. dioica* samples in the final solution could be found by dividing the observed absorbance by the ε for trolox. The total antioxidant capacity in ABTS method was expressed as trolox equivalents (TR) in millimoles per gramme dry material (mmol trolox g^{-1} dw). The trolox equivalent of total antioxidant capacity of *U. dioica* samples in ABTS assay was calculated using the following equation:

$$TAC_{ABTS}\,(\text{mmol Trolox/g}) = \frac{A_{Reference} - A_{Extract}}{\varepsilon_{Trolox}} * \frac{V_{Final}}{V_{Sample}} * \frac{V_{Extract}}{m} * \upsilon$$

where: $A_{Reference}$ is the absorbance of the ABTS solution, $A_{Extract}$ is the absorbance of the extract, ε_{Trolox} is the molar extinction coefficient of trolox at 730 nm, V_{Final} is the final volume of the system, V_{Sample} is the volume of sample

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added to the system, $V_{Extract}$ is the final volume of the extract, m is the gramme amount of the *U. dioica* and v is the dilution factor of the extract.

Determination of total phenolic content
Folin-Ciocalteu Method

Total phenolic content was estimated by the Folin–Ciocalteu colorimetric method, based on the procedure of Singleton et al. (1999) was applied as follows: A mixture comprised of x ml sample, (2 - x) mL distilled water and 2.5 mL Lowry C was prepared and well mixed. This mixture was let to stand for 10 min at room temperature and then 0.25 mL diluted Folin-Ciocalteu's phenol reagent was added and this mixture was incubated 30 more min. At the end of this period, the absorbance at 750 nm was measured against a reagent blank. Gallic acid was used as a reference standard in this method and the molar absorptivity of gallic acid is is ε =3.25 x 10^3 L $mol^{-1}cm^{-1}$. The gallic acid equivalent molar concentration of the *U. dioica* samples in the final solution could be found by dividing the observed absorbance by the ε for gallic acid. The total phenolic content was expressed as gallic acid equivalents (GAE) in millimoles per gramme fresh material (mmol gallic acid g^{-1} dw). The gallic acid equivalent of total phenolic content of *U. dioica* samples in Folin-ciocalteu assay was calculated using the following equation:

$$\text{Total phenolic content (mmol Gallic acid/g)} = \frac{A_{Extract}}{\varepsilon_{Gallic\,acid}} * \frac{V_{Final}}{V_{Sample}} * \frac{V_{Extract}}{m} * \upsilon$$

where: $A_{Extract}$ is the absorbance of extract, $\varepsilon_{Gallic\,acid}$ is the molar extinction coefficient of gallic acid at 750 nm, V_{Final} is the final volume of the system, V_{Sample} is the volume of sample added to the system, $V_{Extract}$ is the final volume of the extract, m is the gramme amount of the *U. dioica* and v is the dilution factor of the extract.

RAPD-PCR Analysis

15 random 10mer oligonucleotide primers have been used to assess the intra and inter-individual genome wide variation of *U. dioica* accessions (Table 1) according to RAPD-PCR components and thermal cycling conditions given in Table 2 using Techne TC517 gradient thermal cycler. The RAPD-PCR results were analyzed using 2 % Agarose gel electrophoresis qualitatively by making replicative comparisons of the samples to show innate or acquired DNA band variations as appearance of bands, loss of bands, increase/decrease in band intensities compared for the same or different plants (Atienzar and Jha, 2006; Uzonur et al., 2004a, 2004b, 2011; Alpsoy et al., 2010; Kekeç et al., 2010)

Table 1: RAPD-PCR primer list and sequence information used to assess levels of genome wide variation of *U. dioica* accessions and parts.

Primer name	5'-3' sequence	Primer Name	5'-3' sequence
OPA-08	5'-GTGACGTAGG-3'	**OPB-08**	5'-GTCCACACGG-3'
OPA-09	5'-GGGTAACGCC-3'	**OPB-10**	5'-CTGCTGGGAC-3'
OPA-14	5'-TCTGTGCTGG-3'	**OPB-11**	5'-GTAGACCCGT-3'
OPA-18	5'-AGGTGACCGT-3'	**OPB-12**	5'-CCTTGACGCA-3'
OPB-01	5'-GTTTCGCTCC-3'	**OPB-14**	5'-TCCGCTCTGG-3'
OPB-05	5'-TGCGCCCTTC-3'	**OPB-17**	5'-AGGGAACGAG-3'
OPB-06	5'-TGCTCTGCCC-3'	**OPB-18**	5'-CCACAGCAGT-3'
OPB-07	5'-GGTGACGCAG-3'		

Table 2: RAPD-PCR components and thermal cycling conditions.

RAPD-PCR Components		Step	Temp (°C)	Time	Cycles
DreamTaq™ PCR Mastermix (2X)	12.5 µl	**Initial Denaturation**	95	1–3 min	1
Primer Mix	0.1–1,0 µM	**Denaturation**	94	30 sec	25–40
Template DNA	10 pg - 1 µg	**Annealing**	Tm–5	30 sec	
Nuclease Free Water	Up to 25 µl	**Extension**	72	1 min/kb	
Total Volume	25 µl	**Final Extension**	72	5–15 min	1

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Real-time PCR HRM Analysis and sequence matching function applications

Two different sets of oligonucleotide primers have been used (Table 3): 1. Choloroplast genome specific primers; the conserved primers used to show various variation levels in our work with *U. dioica* for amplification of a 297 bp chloroplast DNA sequence 16S rRNA gene (5' end) with Val-tRNA gene region (Al-Janabi et al., 1993; Brown, 2002) and
2. *U. dioica* Agglutinin isolectin VII precursor (chia5.7.2) gene exon 3 specific primers designed by our group.
Species specific primer design: A set of primers were designed for species specific amplification of UDA gene of *U. dioica* using NCBI programs and specificity of primers were tested by BLAST analysis in non-redundant databases: GenBank+EMBL+DDBJ+PDB to confirm the 100 % specificity to detect *U. dioica* with the already available nucleotide data.
Real-time PCR HRM has been done for all the samples to show various levels of variation using the components and conditions given in Table 4, with Rotor-Gene 6000™ platform and its program efficienct in the application of genotype auto-calling function. This platform, with suitable ready to use Type-it® HRM™ PCR Mix (Qiagen) can characterize samples using the auto-calling function of the program Rotor-Gene Q Series Software 1.7 (Build 94) to auto-call the genotypes that match the controls with the highest confidence percentage clustering similar curve shapes automatically into groups representing different genotypes with high accuracy. From this automatic grouping the analysis has been furthered to detect the ecotypes of *U. dioica* belonging to Mediterranean and Euro-Siberian (Uzonur and Karabulut, 2012; Uzonur et al., 2011; Katmer et al., 2011; Özsoy et al., 2011).

Table 3: Primer sets used for real-time HRM analysis.

Primer	Sequence	Tm(˚C)	GC%	Amplicon size (bp)
Ch (R)	5'-AGTTCGAGCCTGATTATCCC-3'	59.43	50	297
Ch (F)	5'-GCATGCCGCCAGCGTTCATC-3'	68.87	65	
UD (R)	5'-GCATGTCGCAGTACCTCTTG-3'	61.56	55	463
UD (F)	5'-GCCTTGTGGTTCTGGATGTC-3'	61.45	55	

Table 4: Real-time PCR components and thermal cycling and HRM conditions.

Real-time PCR Components		Step	Temp (ºC)	Time	Cycles
2X HRM PCR Mastermix	12.5 µl	**Initial Denaturation**	95	5 min	1
Primer Mix	0.75µl	**Denaturation**	95	10 sec	25–40
Template DNA	20ng - 20µl	**Annealing**	55	30 sec	
Nuclease Free Water	Up to 25 µl	**Extension**	72	10 sec	
Total Volume	25 µl	**HRM**	65-95	2 sec	1

Results and Discussion

12 city accesions from 11 cities (2 accessions are from Istanbul-Europe and Istanbul-Anatolia) of Turkey have been collected during spring and autumn blooms of *U. dioica* in 2009. The analysis done on the collected samples was to discover the fact that although the accessions belong to the same species the amounts of antioxidants and phenolic substances show a very high degree of variation that can not be correlated with geographical parameters (Table 5 and Figures 2-5).

Various environmental factors like rainfall, temperature, altitude, dosage of the UV rays, soil type, climate, environmental pollution can be responsible for appreciable variation in the species, ecotypes should be determined in this respect with careful observations. Spatial and genetic variations are often assumed to result from environmental heterogeneity and different selection pressures as also stated in previous research (Bharmauria et al., 2009).

Altitute of growth has been an important parameter for assessing the medicinal value of *U. dioica*. It is observed that *U. dioica* from high altitudes have more medicinal value that might be due to more stinging hairs and the amounts and variability of antioxidants and phenolic contents of *U. dioica* according to previous researches (Pollard and Briggs, 1982) whereas with our limited samples we could show that Erzurum accessions from the highest altitute (1890 m) has the highest total antioxidant and phenolic contents (Table 5) of leaf infusions when compared with the other city accessions (Figure 3). It is only for the leaf infusions not for methanol extracts and infusions of stems and roots of Erzurum accessions (data not shown) come first that is to be further confirmed with more samples with more controlled growth parameters. The previous work in the lipophilic fractions of homeopathic matrix tinctures of *U. dioica* L. whereas infusions of leaves is the homeopathic matrix tincture in CAM application preferences in Turkey (Yener et al., 2009).

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RAPD-PCR

Although in literature RAPD-PCR method is an underestimated controversial method in its reliability that is because of misusages of incompetent researchers that puts the blame on the method unfairly. This method is a sound method, coherent and reliable and unbeliavably informative when you use it with the right controls and in a replicative manner resembling the idea of digital PCR. It can show you any details at any level and you can further the RAPD work to confirm any kind of variation in DNA.

Table 5: Total antioxidant activity (using CUPRAC and ABTS/HRP methods) and phenolic substance (using Folin-Ciocalteu method) results of leaf methanol extracts and infusions of *U. dioica* accessions (cities).

Method	CUPRAC mmol Troloks/g		ABTS/HRP mmol Troloks/g		Folin–Ciocalteu mmol Gallic acid/g	
City	Methanol	Infusion	Methanol	Infusion	Methanol	Infusion
Antalya	$1,11x10^{-2}$	$1x10^{-2}$	$0,37x10^{-2}$	$0,43x10^{-2}$	$2,98x10^{-2}$	$3,6x10^{-2}$
Van	$0,37x10^{-2}$	$1,13x10^{-2}$	$0,24x10^{-2}$	$0,45x10^{-2}$	$1,51x10^{-2}$	$3,9x10^{-2}$
Mugla	$0,51x10^{-2}$	$1,1x10^{-2}$	$0,28x10^{-2}$	$0,44x10^{-2}$	$2,02x10^{-2}$	$2,6x10^{-2}$
Istanbul (Anatolia)	$0,17x10^{-2}$	$0,18x10^{-2}$	$0,04x10^{-2}$	$0,35x10^{-2}$	$0,47x10^{-2}$	$0,93x10^{-2}$
Ordu	$0,44x10^{-2}$	$0,98x10^{-2}$	$0,27x10^{-2}$	$0,42x10^{-2}$	$1,89x10^{-2}$	$3,7x10^{-2}$
Tekirdag	$0,94x10^{-2}$	$0,75x10^{-2}$	$0,33x10^{-2}$	$0,37x10^{-2}$	$2,53x10^{-2}$	$2,8x10^{-2}$
Erzurum	$2,08x10^{-2}$	$11,5x10^{-2}$	$0,57x10^{-2}$	$0,99x10^{-2}$	$4,34x10^{-2}$	$25x10^{-2}$
Mersin	$0,5x10^{-2}$	$2,2x10^{-2}$	$0,23x10^{-2}$	$0,60x10^{-2}$	$1,69x10^{-2}$	$4,6x10^{-2}$
Tokat	$2,62x10^{-2}$	$1,72x10^{-2}$	$0,75x10^{-2}$	$0,52x10^{-2}$	$5,76x10^{-2}$	$4,9x10^{-2}$
Istanbul (Europe)	$0,2x10^{-2}$	$0,31x10^{-2}$	$0,14x10^{-2}$	$0,36x10^{-2}$	$0,89x10^{-2}$	$2,7x10^{-2}$
Izmir	$0,42x10^{-2}$	$1,2x10^{-2}$	$0,25x10^{-2}$	$0,48x10^{-2}$	$1,98x10^{-2}$	$4x10^{-2}$
Konya	$0,33x10^{-2}$	$1,75x10^{-2}$	$0,15x10^{-2}$	$0,59x10^{-2}$	$0,93x10^{-2}$	$4,12x10^{-2}$

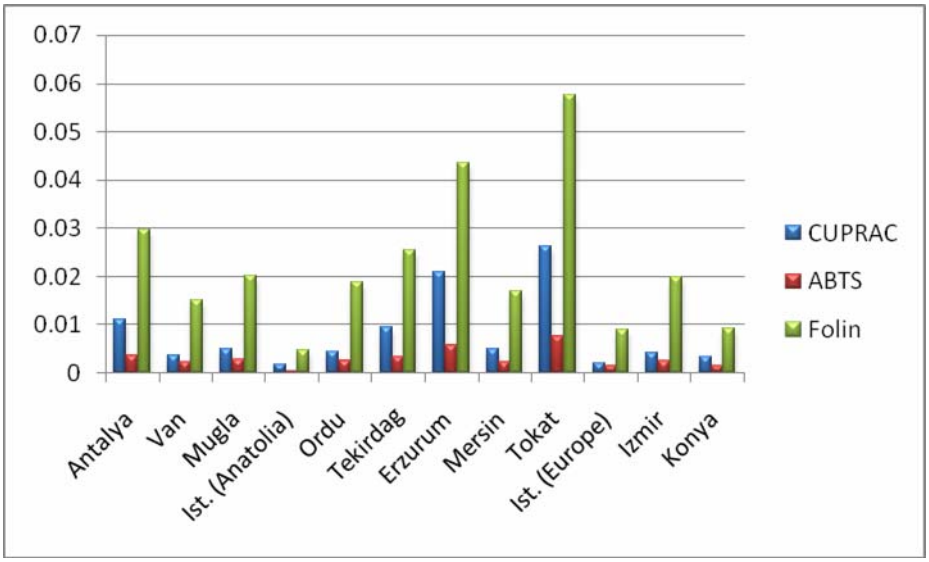

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Figure 2: Total antioxidant (CUPRAC and ABTS methods) and phenolic content (Folin- Ciocalteu method) results of 70 % methanol extracts of leaves of *U. dioica* accessions.

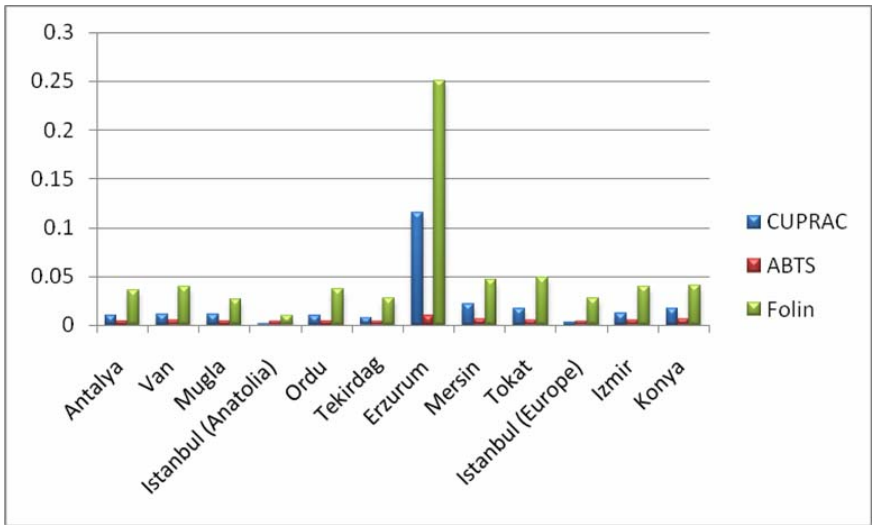

Figure 3: Total antioxidant capacity (CUPRAC and ABTS methods) and phenolic content (Folin-Ciocalteu method) results of infusions of leaves of *U. dioica* accessions.

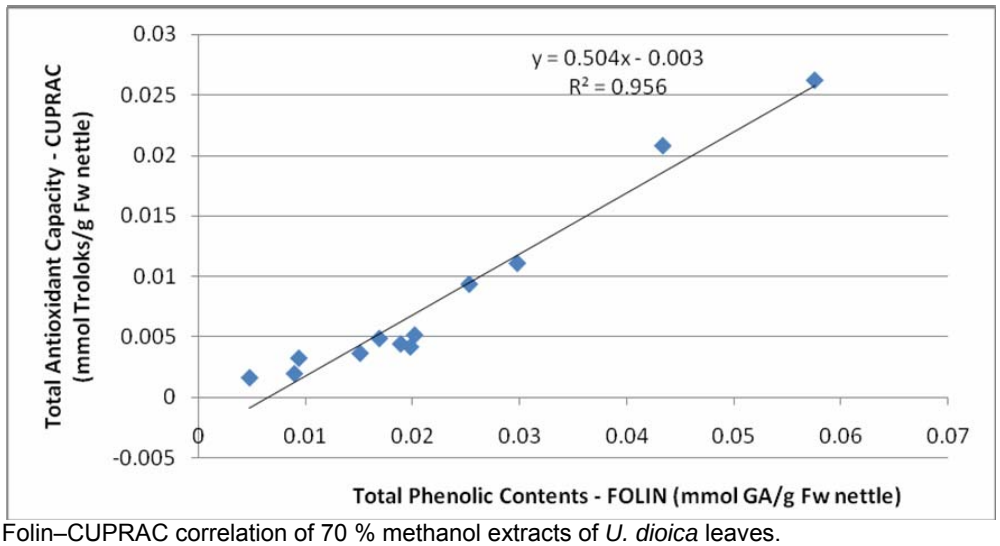

Figure 4: Folin–CUPRAC correlation of 70 % methanol extracts of *U. dioica* leaves.

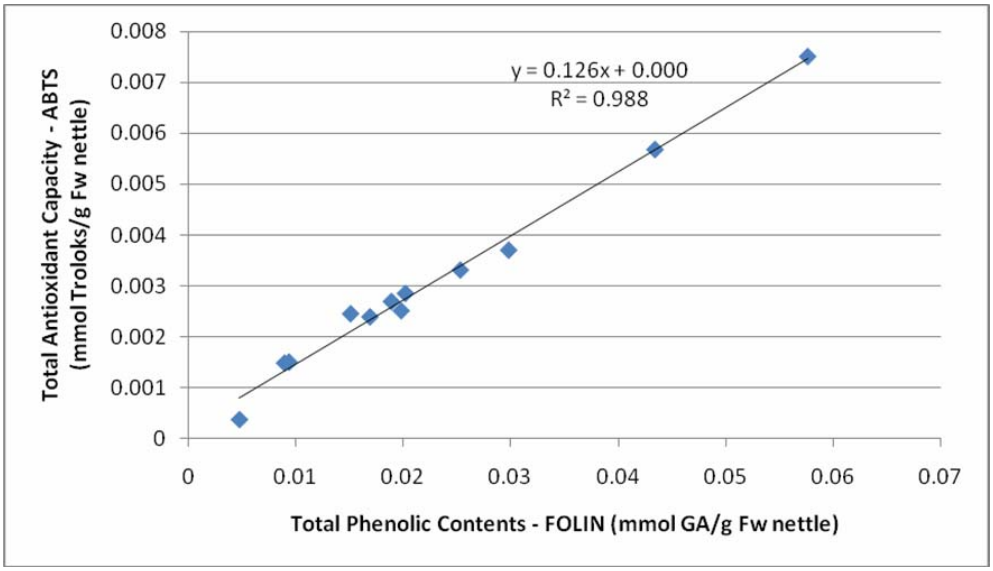

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Figure 5: Folin–ABTS correlation of 70 % methanol extracts of *U. dioica* leaves.

RAPD-PCR results show various degrees of variation for each accession when comparisons of patterns are done on individual basis as comparisons of the patterns for the DNA of the leaf, stem and root of the same accession (Figure 6). In Figure 6 (a) root DNA RAPD profiles of all three accessions have a high degree of observable variation that is controversial for the reasoning without further assessing and confirming the results with various other accessions from the same neighbourhood. At this point the important thing is to show that there exists innate or acquired type of differences intraindividually as a result of either somatic mutations caused by environmental stresses or germinal mutations that are heritable and for the next round of evaluations should be discovered. The mosaic level of DNA can be assessed with our replicative PCR approach, even with three replicates sometimes it is possible to catch the differences in DNA (Figure 6 (a) part root tissue DNA seems to be highly mosaic for all accessions with many gain/loss of bands and changes in band intensities (increase/decrease) and in (b) part for Tokat sample leaf, stem and root profiles exhibit increase and decrease in band intensities in three replicate PCRs. The homogenous band profiles for other tissue DNAs can be an indication for less variation in DNA of those accessions and parts. This part is especially important when the plant considered to be used as an effective herbal CAM agent for curing diseases, there is really a need for further validations and evaluations in this respect to show the cause for such different levels of variation that might be even because of the accumulation of toxicants in the vicinity of the plant causing a target organ toxicity, genotoxicity. For primer OPB 12 the high variation for all three accessions' root results can be due to such a target organ toxicity event and can be a biomarker of exposure in this respect to be further evaluated as a SCAR marker. Each accession is highly different in RAPD-PCR patterns for the primers OPB 12 and 5 (Figure 6) for all parts of the plants (other informative primer-that are listed in Table 1- amplified results are not shown in this work).

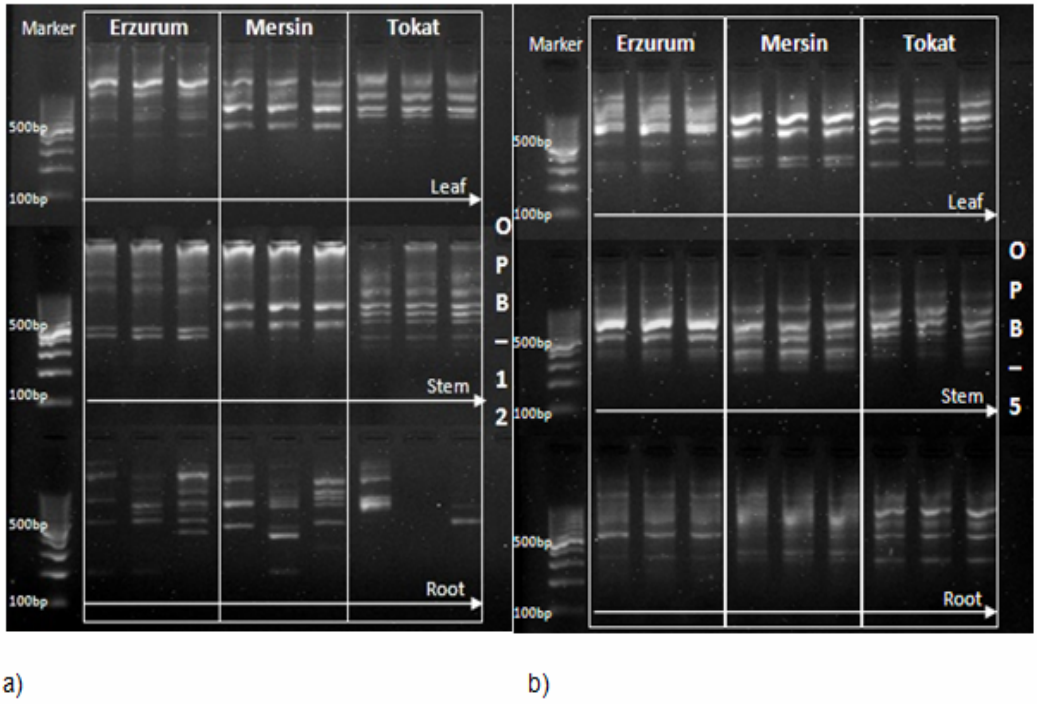

Figure 6: RAPD-PCR results for selected primers OPB12 (a) and OPB5 (b) for leaf, stem and root of three city accessions: Erzurum, Mersin and Tokat to show the various degrees of genomic variation; intraindividual (three replicates for each DNA sample) and interindividual for each city accessions different tissue DNAs.

Real-time PCR HRM results

Real-time PCR results are evaluated in two parts as: 1. Further evaluation of specific two amplicons; 297 bp chloroplast DNA sequence 16S rRNA gene (5' end) with Val-tRNA gene region and 463 bp Agglutinin isolectin VII precursor (chia5.7.2) gene exon 3 of *U. dioica* accessions using HRM analysis and 2. Using the genotype auto-calling of HRM sequence matching function to determine the mighty phytogeographical ecotypes which are above a confidence threshold and confirms with the selected representative controls in Tables 7 and 8 for the cpDNA specific and Agglutinin isolectin VII precursor (chia5.7.2) gene exon 3 specific amplicons respectively. Changes in the melting profiles of amplicon(s) can be in the form of a shift in the melting temperature or an obvious difference in the shape of the melt curve where both of these parameters are a function of the amplicon sequence. This part of experimental results confirms further dissimilarities and similarities of specific amplicons

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that can not be shown by RAPD-PCR analysis, but still consistent with both RAPD-PCR variation results and HRM ecotype determination results for both specific amplicons (Tables 7-8 and Figures 7-8). For the Irano-Turanian samples there is a need for further assessment of the samples so those samples below the threshold confidence value which is an integrity check for auto-called genotypes and do not confirm with the auto-called genotypes of control samples are excluded to give the results in Figures 9 a, b columns. Genotypes automatically called by comparing samples and controls in difference plot that magnify curve differences by subtracting each curve from the most abundant type or a user defined reference. Rotor-Gene Q software conveniently identifies known genotypes or variations, clusters similar curve shapes automatically into groups representing different genotypes: genotype auto-calling.

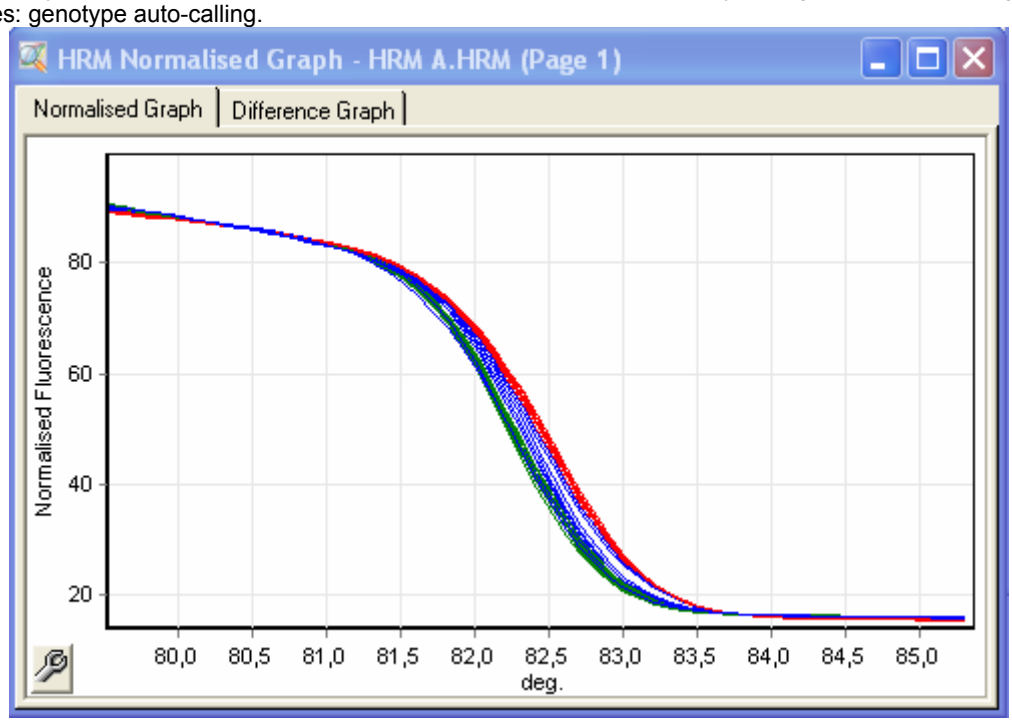

Figure 7: Normalized graph of all samples in Table 7 after HRM analysis. The color codes are given for the cities that fall in the borders of phytogeographic regions (Figure 1). Blue samples for Irano-Turanian, red for Mediterranean and green for Euro-Siberian city accessions of *U. dioica* HRM analysis using cp primers. Blue samples can not be differentiated from the other two, red and green.

Table 7: Chloroplast primer specified HRM analysis sequence matching with genotype autocalling function worked with the appropriate Tm peaks and confidence % with the defined controls for Mediterranean, Euro-Siberian and Irano-Turanian phytogeographic accessions (personal expertise is very important in this part). The numbers indicate different plants from the same location.

Name	Tm Peak (°C)	Genotype	Confidence (%)
Antalya-1	82,55	Mediterranean	100,00
Antalya-2	82,55	Mediterranean	99,98
Muğla-1	82,58	Mediterranean	99,99
Mugla-2	82,60	Mediterranean	99,99
Mugla-3	82,60	Mediterranean	99,83
Mersin-1	82,55	Mediterranean	99,98
Mersin-2	82,60	Mediterranean	99,89
Mersin-3	82,63	Mediterranean	99,90
Izmir-1	82,57	Mediterranean	99,96
Izmir-2	82,60	Mediterranean	99,94
Izmir-3	82,67	Mediterranean	100,00
Tekirdag-1	82,33	Euro-Siberian	99,79

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Tekirdag-2	82,30	Euro-Siberian	99,98
Tekirdag-3	82,30	Euro-Siberian	99,98
Istanbul (Ant.)-1	82,33	Euro-Siberian	99,83
Istanbul (Ant.)-2	82,35	Euro-Siberian	99,60
Istanbul (Eur)-1	82,35	Euro-Siberian	99,79
Istanbul (Eur)-2	82,35	Euro-Siberian	99,41
Ordu-1	82,30	Euro-Siberian	100,00
Ordu-2	82,32	Euro-Siberian	99,85
Ordu-3	82,30	Euro-Siberian	99,97
Van-1	82,45	Irano-Turanian	100,00
Van-2	82,38	Irano-Turanian	99,29
Tokat-1	82,48	Irano-Turanian	99,96
Tokat-2	82,37	Irano-Turanian	99,32
Tokat-3	82,52	Mediterranean	99,92
Konya-1	82,32	Euro-Siberian	99,97
Konya-2	82,40	Irano-Turanian	99,81
Konya-3	82,45	Irano-Turanian	99,99

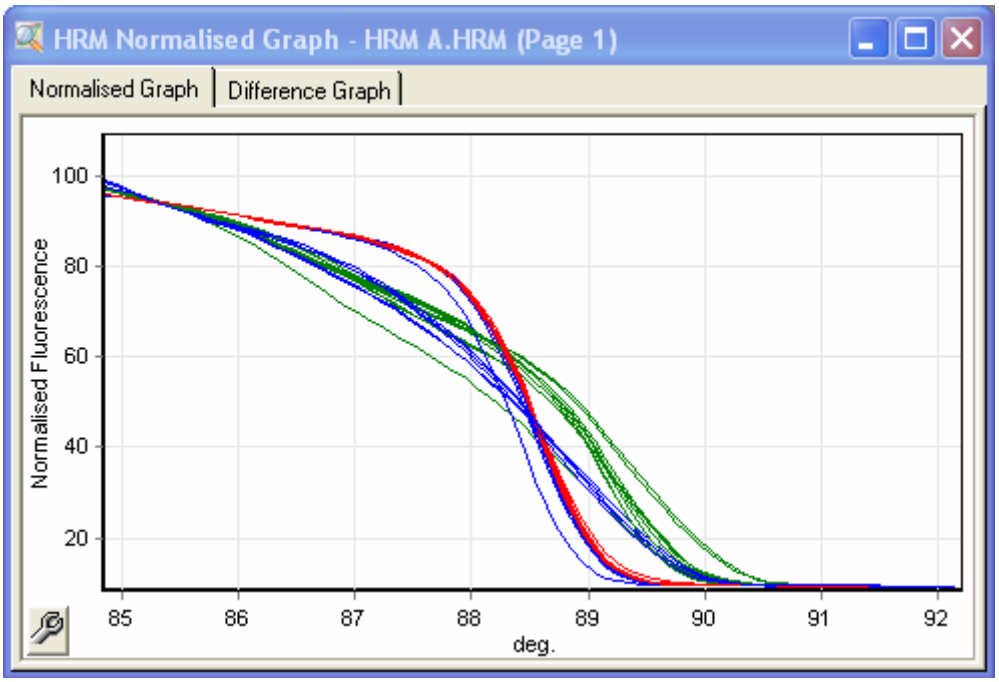

Figure 8: Normalized graph of all samples in Table 8 after HRM analysis. The color codes are given for the cities that fall in the borders of phytogeographic regions (Figure 1). Blue samples for Irano-Turanian, red for Mediterranean and green for Euro-Siberian city accessions of *U. dioica* HRM analysis using UDA primers. Blue samples can not be differentiated from the other two.

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Table 8: UDA primer specified HRM analysis sequence matching with genotype autocalling function worked with the appropriate Tm peaks and confidence % with the defined controls for Mediterranean, Euro-Siberian and Irano-Turanian phytogeographic accessions (personal expertise is very important in this part). The numbers indicate different plants from the same location.

Name	Tm Peak 1 (°C)	Tm Peak 2 (°C)	Genotype	Confidence (%)
Antalya-1	82,75		Mediterranean	100,00
Antalya-2	82,75		Mediterranean	99,73
Muğla-1	82,75		Mediterranean	99,61
Mugla-2	82,73		Mediterranean	99,18
Mugla-3	82,75		Mediterranean	99,91
Mersin-1	82,70		Mediterranean	96,94
Mersin-2	82,73		Mediterranean	98,54
Mersin-3	82,70		Mediterranean	98,11
Izmir-1	82,77		Mediterranean	99,25
Izmir-2	82,75		Mediterranean	99,84
Izmir-3	82,77		Mediterranean	99,84
Tekirdag-1	80,65	83,40	Euro-Siberian	97,92
Tekirdag-2	80,58	83,33	Euro-Siberian	100,00
Tekirdag-3	80,63	83,33	Euro-Siberian	99,62
Istanbul (Ana.)-1	81,15	83,27	Euro-Siberian	85,09
Istanbul (Ana.)-2	81,10	83,40	Euro-Siberian	83,91
Istanbul (Eur.)-1	80,70	83,38	Euro-Siberian	96,90
Istanbul (Eur.)-2	80,75	83,42	Euro-Siberian	96,02
Ordu-1	81,20	83,40	Euro-Siberian	96,67
Ordu-2	80,70	83,45	Euro-Siberian	98,36
Ordu-3	81,05	83,25	Euro-Siberian	99,08
Van-1		83,38	Irano-Turanian	100,00
Van-2	82,83	83,35	Irano-Turanian	99,02
Tokat-1		82,70	Mediterranean	95,54
Tokat-2		82,55	Mediterranean	50,38
Tokat-3		82,68	Mediterranean	93,84
Konya-1	79,15	82,78	Irano-Turanian	93,88
Konya-2	78,97	82,82	Irano-Turanian	95,19
Konya-3	79,17	82,85	Irano-Turanian	93,75

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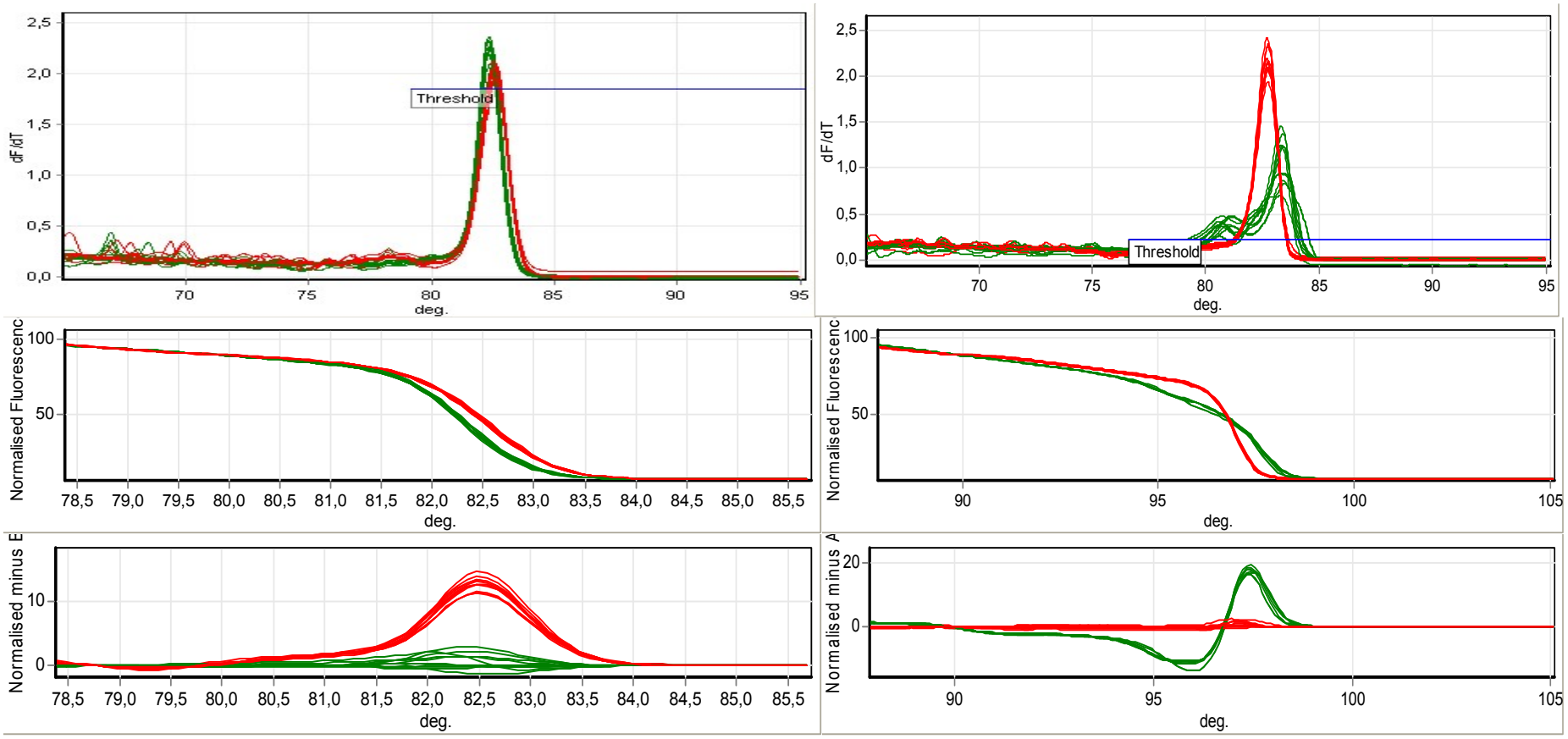

a) b)

Figure 9: (a) column is for the 16S rRNA gene (5' end) with Val-tRNA gene region and (b) for Agglutinin isolectin VII precursor (chia5.7.2) gene exon 3 melt curves (1st lane), normalised curves (2nd lane) and difference plots (3rd lane). Green color for Mediterranean, red color for Euro-Siberian accessions (2-3 accessions from each locality).

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Conclusion

U. dioica is a very important CAM plant widely used in Turkey and worldwide. The details in this respect are important especially related with its genetic background and the factors having any kind of effect on the genetic profile of the plant. Ecotypes have no main taxonomic rank in modern biological classification. However some scientists consider them "taxonomically equivalent to subspecies". In the context of work with CAM plant species and especially *U. dioica* ecotype determination can have a critical value for many further evaluations. In this respect it will be very beneficial to remind about the definition of ecotype, sometimes called ecospecies: ecotype describes a genetically distinct geographic variety, population or race within species (or among closely related), which is adapted to specific environmental conditions. Typically, ecotypes exhibit phenotypic differences (such as in morphology or physiology) stemming from environmental heterogeneity and are capable of interbreeding with other geographically adjacent ecotypes without loss of fertility or vigor. Our work is important and a novel contribution in this respect to fill the missing parts related with morphologic complications of a CAM plant with heterogeneous physiology, genetics and environmental impacts. Evaluation of morphologic parameters related with efficacy, genetic background of the species due to innate and acquired changes in the genome and the geography of the plant all should be evaluated with sound expertise in the field and HRM sequence matching analysis with genotype auto calling function introduced in this research enables the defining of ecotypes which will help a lot to solve many undetermined and controversial issues related in the field.

Acknowledgment

This work is supported by the Scientific Research Fund of Fatih University under the project numbers P50031103_B and P50031104_G.

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