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Numerical Taxonomic Studies on Some Species of the Genus *Thymus* L. (Labiatae) in Turkey

Tülay Aytaş Akçin
Department of Biology, Faculty of Arts and Science,
University of Ondokuz Mayıs, Samsun, Turkey

Abstract: The similarities of some species of the genus *Thymus* L. (Labiatae) from Turkey, were studied using the morphological and chemical characters. Chemical analyses were carried out by gas chromatography/mass spectrometers. For each taxa, 20 quantitative characters and 130 volatile oil substituents were determined and the data subjected to numerical taxonomic analyses. The results exhibited that the dendrograms obtained from morphological and chemical data were similar. To the results, the closest taxa were *T. sipyleus* Boiss. and *T. leucotrichus* Hal. Likewise, *T. praecox* Opiz., *T. longicaulis* C. Presl. and *T. thracicus* Velen. were closely related species. However, *T. pseudopulegioides* Klokov and Des. Shost was distinct from the other taxa.

Key words: Labiatae, *Thymus* L., numerical taxonomy, Turkey

INTRODUCTION

The genus *Thymus* L. contains some hundreds of species which distributed over nearly the whole of the world (Klokov *et al.*, 1954).The Mediterranean region can be described as the center of the genus (Stahl-Biskup and Saez, 2002). Jalas (1980) reported that the genus *Thymus* L. includes 37 species in Turkey and 14 of which are endemics. First description of the genus was by Linneaus (1753) in his book Species Plantarum. In the revision by Jalas (1971b) the genus were divided into two subgenera: Coridothymus and Thymus. In this revision, some of the species found in the sect. Pseudotymbra or Serphyllum were scattered in sect. Hypodromi (Jalas, 1971b).

Thymus L. is an aromatic plant with increasing importance in food processing (Letchamo *et al.*, 1994). *Thymus* species have been used for more than 2000 years as medicinal herbs and many of them are still in use (Tzakou and Constantinidis, 2005). The genus *Thymus* L. called "thyme" is one of the plants used as a folk medicine in Turkey (Başer *et al.*, 1991a). Volatile oil constituents of thyme are used as anticeptics, antispazmodics and fungusidal (Meriçli, 1986a; Çingi *et al.*, 1991).The anticeptic, antioxidative, insectecidal, preservative and anaesthetic properties of thyme are due to their biologically active substances, such as thymol, carvacrol, linalool, geraniol and other volatiles in the essential oil (Van-Den Broucke and Lemli, 1983). Recent studies have showed that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities (Stahl-Biskup and Saez, 2002). The aromatic and medicinal properties of the genus *Thymus* have made it one of the most popular plants throughout all of the world (Nickavar *et al.*, 2005).Although thyme is useful in the food and aroma industries and nowadays a serious drug in phytotherapy, both the taxonomy and the chemistry of the genus appear quite complex (Zarzuelo and Crespo, 2002).

There are many works on thyme flavonoid aglycones and glycosides (Hernandez *et al.*, 1987; Tomas-Barberan and Wollenweber, 1990; Rustaiyan *et al.*, 2000; Stahl-Biskup and Saez, 2002).Previous studies suggested that the excreted flavonoids were very useful taxonomic markers within the genus, since different flavonoid patterns were found among various species of the genus (Hernandez *et al.*, 1987).It was also determined that the thyme species collected from similar geographical localities exhibited slight differences in the relative amounts of each compound (Tomas-Barberan and Wollenweber, 1990). Meriçli (1986 a) reported that some species of *Thymus* L. contained oil dots at different quantity varying from one sampling locality to other. Yamaura *et al.* (1989) noted that the synthesis of essential oils in aromatic plants might be influenced by environmental factors, such as temperature, photoperiods and water stress. It was reported that the thyme species showed chemical polymorphism (Schmidt *et al.*, 2004). Tzakou and Constantinidis (2005) suggested that further studies are needed, which should cover the essential oils from different individual plants or specimens from various geophraphical regions.

Table 1: Arithmetic averages of 20 quantitative characters in investigated *Thymus* L. taxa

Quantitative characters	Taxa					
	T. leucotrichus	*T. sipyleus*	*T. praecox*	*T. thracicus*	*T. longicaulis*	*T. pseudopulegioides*
Stem length (cm)	12.89	17.02	15.95	14.84	20.00	17.96
Flowering branches length (cm)	3.54	5.70	6.23	7.91	12.84	16.30
Cauline leaves width (mm)	0.98	1.27	4.11	2.31	2.45	8.75
Cauline leaves length (mm)	6.42	6.31	7.05	10.97	9.66	14.12
Axillary fascisles width (mm)	0.90	1.05	0.89	0.71	0.85	1.65
Axillary fascisles length (mm)	2.11	2.20	2.09	2.00	2.44	3.04
Petiole length (mm)	1.30	1.16	1.51	1.07	1.44	2.29
Vertisillasters length (mm)	1.92	1.30	2.02	1.77	2.43	1.90
Flower numbers at Vertisillasters	19.52	20.42	25.80	28.44	32.00	31.12
Pedicel length (mm)	1.75	1.42	2.40	2.60	2.37	2.91
Bract width (mm)	2.51	1.86	2.49	1.41	1.72	5.61
Bract length (mm)	6.13	5.09	5.60	5.78	5.52	8.56
Bracteole width (mm)	0.40	0.50	2.50	0.50	0.40	0.60
Bracteole length (mm)	1.70	1.74	1.54	2.00	1.61	1.61
Calyx width (mm)	1.53	1.36	1.45	1.30	1.34	1.44
Calyx length (mm)	4.76	4.10	4.02	4.92	3.92	4.46
Upper calyx teeth length (mm)	1.18	0.99	1.03	1.50	0.89	1.07
Lower calyx teeth length (mm)	2.01	2.35	2.20	1.94	1.93	2.55
Corolla length (mm)	6.78	4.45	6.05	5.86	4.52	7.64
Style length (cm)	2.30	2.50	2.80	2.20	2.50	3.30

The main objective of this study was to classify some Turkish *Thymus* L. species by using numerical taxonomic methods and to compare the results obtained from the morphological and chemical data.

MATERIALS AND METHODS

Specimens belonging to different taxa of *Thymus* L. were collected from Black Sea Region, Turkey during the period between May and August of 1995-1997.The specimens were identified by referring to the information in the Flora of Turkey and the East Aegean Islands, Vol.7 (Davis, 1982). Descriptions of all species were made using both fresh material and herbarium specimens. For each specimen, forty-nine distinctive morphological characters were determined. The maximum and minimum boundaries of twenty quantitative characteristics were measured and their arithmetic averages were calculated (Table 1).

The essential oil content was analysed by a Hewlett-Packard Gas Chromatography (GC) and Gas Chromatography/mass Spectrometers (GC/MS) as described by Tanker (1973) and Başer *et al.* (1991b). The operation conditions were as follows: initial column temperature was programmed to 220°C at 4°C min^{-1} and 240°C at 1°C min^{-1}. Carrier gas was helium with a flow rate of 1 mL min^{-1}. Individual components were identified by comparing with mass spectra of the authentic samples and with the Wiley/NBS Registry of mass spectral data library.

For numerical analyses, the SPSS program (version 8.0) was applied (Wolf and Whitkus, 1987; Reiseberg *et al.*, 1987). The average taxonomic distances between pairs of OTUs were computed using Euclidean distance method (Sneath and Sokal, 1973; Doğan *et al.*, 1992). Arithmetical averages of the twenty morphological characters were used. The volatile oil components of each taxa was assumed as present or absent to produce data matrix showing the similarities among the taxa. UPGMA (Unweighted Pair Group Method using Arithmetic averages) trees obtained from the two analyses (i.e., morphological and chemical) were compared with each other.

RESULTS AND DISCUSSION

The phenogram resulting from UPGMA using the averages of twenty morphological quantitative characters is presented in Fig. 1. From the morphological data it appears that two species, *T. sipyleus* and *T. leucotrichus* are the most similar taxa at 96.0% similarity level. SPSS program clearly suggested that two main clusters were apparent, one comprising *T. sipyleus* and *T. leucotrichus*; the other grouping *T. praecox*, *T. thracicus*, *T. longicaulis* and *T. pseudopulegioides*. In the second grouping, *T. pseudopulegioides* formed basal node to this grouping.

Actually, these results are basically similar to phylogenetical data in Flora of Turkey (Davis, 1982). However, *T. leucotrichus* differs from *T. sipyleus* in that

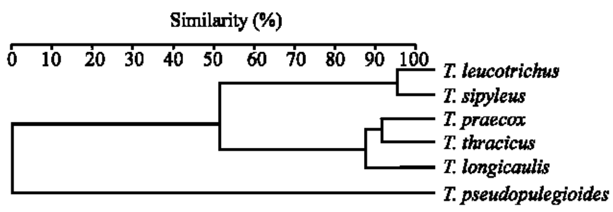

Fig. 1: Phenogram based on morphological characters

Table 2: The compounds detected in the volatile oils of five *Thymus* L. taxa

	Taxa				
Compounds	*T. sipyleus* (%)	*T. præcox* (%)	*T. thracicus* (%)	*T. longicaulis* (%)	*T. pseudopulegioides* (%)
α- pinene	0.37	0.08	0.46	0.39	0.42
α-thujene	-	0.04	0.38	0.31	0.13
Camphene	0.43	0.15	0.72	0.72	0.03
β-pinene	1.31	0.08	0.18	0.12	0.04
Sabinene	0.59	0.09	0.01	0.36	0.04
g-3-carene	-	0.01	0.06	-	0.02
Myrcene	2.11	0.52	1.05	0.42	0.44
α-terpinene	-	0.19	1.98	0.50	0.61
Dehydro-1,8-cineole	-	0.01	-	0.03	-
Limonene	0.73	0.34	0.42	0.98	0.34
1,8-cineole	7.46	1.69	1.54	0.29	0.02
β-phellandrene	-	0.02	-	0.05	0.09
(z)-b-ocimene	0.03	0.03	0.03	-	0.07
γ-terpinene	0.19	0.69	11.08	1.82	1.33
(E)-β -ocimene	0.10	0.26	-	-	-
5-methyl-3-heptanone	-	0.20	0.15	0.23	0.43
p-cymene	0.47	2.44	25.44	15.01	13.69
Torpinolene	0.02	0.07	0.12	0.11	0.09
3-octyl acetate	-	0.06	-	0.19	0.09
Octenyl acetate	-	0.14	-	0.61	0.56
3-octanol	0.06	0.22	0.17	0.48	0.26
Nonanol	0.02	0.01	-	0.14	-
α-p-dimethyl styrene	-	0.01	0.11	0.02	0.04
1-octen-3-ol	0.28	0.49	0.58	1.16	1.07
Trans-sabinene hydrate	1.16	0.36	0.46	0.45	0.27
3-nonenyl acetate	-	0.01	-	0.05	-
Bicycloelemene	0.05	0.02	-	-	-
α-copaene	0.08	0.06	-	-	0.05
Dihydroedulane II	-	-	0.05	0.02	-
Camphor	2.40	0.33	0.38	0.03	-
Dihydroedulane I	-	-	-	0.02	0.20
β-bourbonene	0.48	0.42	0.09	0.02	0.20
Linalool	9.14	4.94	0.40	2.25	5.25
Cis-sabinene hydrate	0.18	0.15	0.45	0.44	0.26
Octanol	0.01	0.02	0.04	0.02	-
Linalyl acetate	0.23	0.45	-	1.02	14.79
Trans-p-menth-2-en-ol	0.03	0.01	0.04	0.01	-
Bornyl acetate	0.12	0.48	0.14	1.18	-
Thymol methyl ether	-	-	0.77	0.28	2.17
β-elemene	0.15	0.35	-	-	-
Terpinen-4-ol	0.77	0.23	0.64	0.39	0.40
β-caryophyllene	11.66	11.75	0.70	2.10	2.00
Carvacrol methyl ether	-	-	-	-	4.57
Cis-dihydrocarvone	-	0.04	-	0.08	0.11
Aromadendrene	-	-	-	-	0.44
Trans-dihydrocarvone	-	0.03	0.04	0.01	0.03
Nonanol	0.01	-	0.06	0.02	-
Trans-pinocarveol	-	0.01	0.06	0.04	-
Alloaromadendrene	0.71	0.80	-	-	-
(Z)-β-farnesene	2.31	2.79	-	0.01	-
δ-terpineol	0.56	0.06	0.10	-	-
Trans-verbenol	-	0.06	0.06	0.04	0.13
α-humulene	0.73	2.10	0.05	0.08	0.14
Neral(sitral a)	0.75	-	-	-	-
α-terpineol	3.08	0.95	0.22	1.67	2.17
Borneol	2.26	0.50	3.21	-	-
α-terpnyl acetate	-	11.96	-	37.98	9.06
Germacrene D	3.38	5.33	0.09	0.24	0.47
Neryl acetate	-	-	-	0.04	0.57
Geranial(sitral b)	1.42	-	-	-	-
α-cadinene	0.20	0.44	-	-	-
β-bisabolene	-	2.83	4.71	3.54	1.54
Bicyclogermacrene	1.84	1.17	-	-	0.23
Geranyl acetate	0.04	0.06	-	1.12	3.61
Citronellol	0.18	-	-	-	-

Table 2: Continued

	Taxa				
Compounds	*T. sipyleus* (%)	*T. præcox* (%)	*T. thracicus* (%)	*T. longicaulis* (%)	*T. pseudopulegioides* (%)
δ-cadinene	1.13	1.25	0.38	0.04	0.41
γ-cadinene	1.30	0.44	-	0.02	0.23
(E)-α -bisabolene	-	-	-	-	0.47
Cumin aldehyde	-	0.01	0.12	0.03	0.03
Nerol	0.14	0.01	-	0.02	0.23
3,7-giaiadiene	0.13	0.05	-	-	0.03
2,6-dimethyl-3-(E),5(E),7-octatrien-2-ol	0.14	0.02	-	-	-
Trans-carveol	-	0.04	0.03	0.05	0.01
Geraniol	0.06	0.03	-	0.30	1.26
Cis-calamenene	0.17	0.05	-	-	0.02
p-cymen-8-ol	-	0.01	0.42	0.12	0.02
Thymol acetate	-	-	0.02	0.02	0.03
(E)-geranyl acetone	0.03	0.02	-	0.01	-
Carvocrol acetate	-	0.02	0.04	0.01	-
Geranyl butyrate	0.07	-	-	-	-
Epicubebol	0.37	0.18	0.01	-	0.06
Geranyl-2-methyl butyrate	0.18	-	-	-	-
α-calacorene I	0.02	0.01	-	-	0.04
Shyobunol	0.06	0.01	-	-	0.04
Cubebol	0.86	0.11	-	-	0.02
Isocaryophyllene oxide	0.58	0.11	-	0.02	-
Caryophyllene oxide	5.20	2.01	0.77	0.91	0.92
(E)-nerolidol	1.27	9.47	-	-	-
Ledol	0.13	0.10	-	-	-
Humuilene epoxide II	0.20	-	0.05	0.03	-
Germacrene D-4-ol	3.04	4.98	-	-	-
Cubenol	0.23	-	-	-	-
Elemol	1.08	0.43	-	-	-
Globulol	-	-	-	-	0.09
Hedycaryol	3.09	-	-	-	-
Viridiflorol	0.27	0.16	-	-	-
Cumin alcohol	-	0.01	0.17	0.05	0.05
Spathulenol	3.83	0.81	0.09	0.03	0.83
Isothymol	-	0.02	0.20	0.11	0.03
Thymol	-	2.31	15.69	16.76	22.12
T-cadinol	2.36	0.45	-	-	-
T-muurolol	0.77	0.72	-	-	-
Isocarvacrol	-	0.01	0.33	0.10	0.10
δ-cadinol	0.16	0.14	-	-	-
carvacrol	0.15	9.59	18.21	1.97	2.35
α-eudesmol	1.64	0.08	-	-	-
α-cadinol	2.31	1.88	0.02	-	-
Cadelene	-	-	-	-	-
β-eudesmol	0.92	-	-	-	-
Intermedeol	1.11	1.81	-	-	-
Caryophylladienol I	0.13	-	-	-	-
Caryophylladienol II	0.40	0.11	-	0.05	0.04
Caryophyllenol I	0.05	0.03	-	-	-
(2E,6E)-farnesol	-	0.76	-	-	-
Caryophyllenol II	0.49	0.12	-	0.09	-
Hexadecanoic acid	0.54	0.26	0.40	-	0.33
Bornyl formate	0.19	-	-	0.18	-
Trans-α-hergamotol	0.19	-	-	-	0.09

it has leaf with revolute margins and longer hairs. The numerical analyses of morphological characters showed that *T. praecox*, *T. thracicus* and *T. longicaulis* were interpreted as related species. Morphological evidences also supported these similarities. Particularly, *T. thracicus* and *T. longicaulis* are so similar in morphology that some members of *T. thracicus* are sometimes assigned as *T. longicaulis* (Jalas, 1971a). In the present study, it was found that some of the morphological characteristics, such as leaf shape and whether or not leaf margins are revolute were found to be useful to distinguish these *Thymus* L. species. *T. thracicus* differs from *T. longicaulis* in that it has flat leaf margin and oblanceolate leaf shape. In addition, Jalas (1971 a) suggested that characteristics, such as length of calyx and upper calyx teeth were also usable diagnostic characters for these species. Actually, in *T. longicaulis* the length of calyx teeth is 2.5-4.2 (-4.7) mm and the length of upper calyx teeth is 0.5-1.3 (-1.5) mm. In comparison in *T. thracicus* these characters are 4.2-5.5 mm and 1.2-1.8 mm, respectively. Therefore, it was found that these quantitative characters could be useful for distinguishing the two species in addition to other morphological characteristics mentioned above. The results of UPGMA analyses showed that *T. pseudopulegioides* was not similar to other taxa. These results agree with the fact that this taxon can be clearly distinguished from the other taxa based on morphological characters. *T. pseudopulegioides* is different from the other taxa despite the fact that the stem is hairy along the four edges and dimensions of cauline leaves (Table 1). It seems that UPGMA dendrogram using quantitative characters supports the morphological evidences.

Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometers (GC/MS) analyses of the volatile oils of five taxa of *Thymus* L. led to determination of one-hundred thirty different compounds (Table 2). Twelve of these compounds (i.e rosefuran, pentacosene, phytol, tricyclene, 3-nananone, perillene, myrtenal, cubebene, carvone, pinovarvone, nonyl acetate, cis carveol) were present in less than 0.02%. Thus, they were excluded from the data analyses. Thymol, which is characteristic essential oil of *Thymus* L. genus, was not found in *T. sipyleus* volatile oil. The chemical composition of the volatile oil from *T. sipyleus* showed that carvacrol was the lowest compounds of the oil with percentages of 0.15%. These results were also supported by Tanker (1973), as he pointed out that some of *Thymus* L. species with a lemon- like odour contains no thymol. Tanker (1973) and Meriçli (1986 a, b) suggested that the genus *Thymus* L.; except for *T. sipyleus* that has lemon-like odour, was characterized by its thymol odour due to thymol and carvacrol. The main components of the essential oil of *T. sipyleus* were β-caryophyllene (11.66%), linalool (9.14%), 1,8-cineole (7.46%), caryophyllene oxide (5.20%) and α-terpineole (3.08%). Citral-a (neral) and citral-b (geranial) were also determined in only volatile oil of *T. sipyleus* suggesting that it can be distinguished from the other species based upon the chemical characters.

Morphological similarities of *T. longicaulis* and *T. thracicus* have also been evident from the chemical features. *T. thracicus* oil was rich in p-cymene (25.44%), thymol (15.69%), carvacrol (18.21%) and γ-terpinene (11.08%). The main components of the essential oil of *T. longicaulis* were α-terpnyl acetate (37.98%), thymol (16.76%), p-cymene (15.01%) and β-bisabolene (3.54 %). Thymol, the main constituent of the essential oil, was found in high concentrations in *T. longicaulis* and *T. thracicus* volatile oil, whereas it was determined at lower amount in *T. praecox* volatile oil. On the other hand, *T. longicaulis* and *T. thracicus* are also similar to each other because of the higher thymol concentrations.

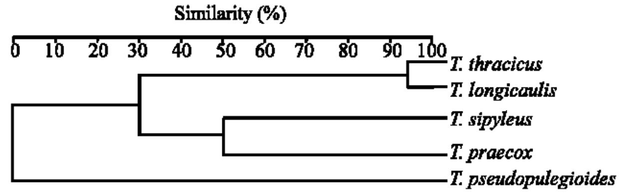

Fig. 2: Phenogram based on chemical characters

T. pseudopulegioides is distinguished from all of the other taxa by having carvacrol methyl ether at higher concentrations (4.57%). Similarly aromadendrane, neryl acetate, (E)-α-bisabolene and cadelene, were also only found in *T. pseudopulegioides* volatile oil. These results agree with the fact that *T. pseudopulegioides* is different from the other taxa with regard to chemical characters.

UPGMA dendrogram using chemical components for each taxon (Fig. 2) resulted in different from that obtained using quantitative characters (Fig. 1).It was unfortunate that *T. leucotrichus* could not be included in chemical based UPGMA analyses because it had essential oil very low concentration. Phenetic analyses based on chemical characters indicated that the most similar taxon were *T. thracicus* and *T. longicaulis* with respect to volatile oil components. In the phenogram, two main clustering were apparent, one formed by *T. thracicus* and *T. longicaulis* and the other formed by *T. sipyleus* and *T. praecox*. However, *T. pseudopulegioides* was placed at the base of four and was sister taxon to the two maining clusters.

T. sipyleus was closely related to *T. praecox* in UPGMA dendrogram based on chemical characters (Fig. 2) whereas it was the most similar to *T. leucotrichus* in morphological based UPGMA analyses (Fig. 1).This discrepancy is due to fact that *T. leucotrichus* could not be included in the present numerical classification based upon chemical characters because it had lower content of volatile oil.

The results suggested that the essential oil oils of *Thymus* L. taxa are not always correlated with the classification. On the other hand, morphologically related species may be share different compounds. For instance, α-terpnyl acetate was present in *T. longicaulis* at higher concentrations (37.98%) while it was absent from *T. thracicus*. This contention was also supported by Hernandez *et al.* (1987), as he pointed out that the volatile oils may be altered by environmental conditions. Previous studies have reported that the essential oil contents of thyme species were different depending on the locality (Adzet and Martinez, 1981; Tomas-Barberan *et al*., 1988; Tzakou and Constantinidis, 2005). The present study is also in accordance with this contention. The results supported an influence of ecological factors on the excretion of essential oils.

UPGMA dendrogram based on quantitative characters is more reliable and reflects the possible relationships of taxa. A comprehensive study covering all of the *Thymus* L. species seems to be necessary to construct a satisfactory classification.

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