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REVIEW OF SOME USEFUL METHODS IN TAXONOMICAL INTERPRETATION OF DIFFICULT TAXA OF MEDICINAL AND AROMATIC PLANTS. CASE: THYMUS L.

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INTRODUCTION

It is known that there is a problem with many taxa (genera, species, and especially intra/species categories) regarding both their botanical classification and determining in the nature. Usually, such genera include a great number of species, whereas in some cases they are not obligatory diversified. The botanically complicated from the aspect of their classification and identification are genera such as *Hieracium* L., *Viola* L., *Verbascum* L., *Ranunculus* L., *Orchis* L., *Dactylorhiza* Necker ex Nevski, *Atriplex* L., *Campanula* L., *Orobanche* L., *Festuca* L., etc. Similar situation refers also to the medicinal and aromatic plants and may cause complications in wild collection and thus in further evaluation of their drug quality, as well as in collecting and descriptors development of accessions for gene bank needs. The following taxa of medicinal and aromatic plants may be stressed in regard of suspicious taxonomical interpretation of their species: *Mentha* L., *Achillea* L., *Hypericum* L., *Satureja* L., *Micromeria* Benth., *Allium* L., *Geranium* L., *Rosa* L., *Thymus* and some others. Difficulties in botanical determination of populations of these taxa are related to high morphological plasticity and variability in morphological features, occurrence of many similar (overlapping) characters, breeding between the genetically related species leading to numerous hybrids and frequent apomixis. On the other hand, the problems might be linked with insufficiently developed and/or often confused dichotomous keys, as well as too sophisticated tools and expensive methodologies in survey of biochemical, molecular and genetic traits. Although the structural (morphological) characters are usually only criterion for taxonomic evidence (Judd et al., 1999), there is a set of useful methods in specimen identification, including histological features, such as the structure of sieve-element plastids (Behnke 1994), general leaf anatomy (Carlquist, 1961, Barthlott, 1990, Rathnam, 1976), and secretory structures (Metcalfe, 1966), followed by embriology, chromosome number and structure (Nogueira et al., 1995), palynology, determining of secondary compounds (Adams, 1977, Dalgren, 1983, Stuessy, 1990), and recent approaches in molecular systematic (Sytsma and Hahn, 1997, Rice et al., 1997, etc.)

1.1. Why *Thymus* L. is complicated taxa for taxonomic evidence of its species?

Genus *Thymus* is one of most important genera as regards number of species within the Lamiaceae family. This family comprises more or less 220 genera. *Thymus* belongs to tribe Mentheae, subfamily Nepetoideae. Although the number of species within this genus varies depending on taxonomical viewpoint, there is more than 200 species.

Thymus is distributed throughout the arid, temperate and cold regions of the Old World north of the Equator and on the coasts of Greenland (Morales, 1989). However the central area of this genus surrounds the Mediterranean Sea. According to Jalas (1971), *Thymus* is divided into eight sections: *Micantes*, *Mastichina*, *Piperella*, *Teucrioides*, *Pseudothymbra*, *Thymus*, *Hyphodromi* and *Serpyllum*. The first five are only found on the Iberian Peninsula, in the northwest of Africa and the Macronesian Region. Section *Hyphodromi* extends through Mediterranean area and comprises around 60 species. Three subsections can be distinguished in this section: *Subbracteati*, *Serpyllastrum* and *Thymbropsis*. Section *Serpyllum* appears to be the oldest in the genus. Around 150 species belongs to this section. They occur throughout the area of the genus. This group is taxonomicaly difficult, and Jalas (1971) divided it into 7 subsections (*Insulares, Pseudopiperellae Isolepides, Isolepides,Kotschyani, Pseudomarginati, Alternantes, Serpyllu*).

In Flora of Serbia the total of 31 species of genus *Thymus* is listed with more than 60 varieties, which mostly grow in various meadow and pasture communities and dry, sunny rocky habitats both on limestone and serpentine.

Genus *Thymus* is famous of high diversity of its species, including intra-species (population) variability and a number of described subspecies, varieties and forms. Difficulties in their taxonomical

interpretation are related to high morphological variability of populations in many morphological, micromorphological traits and secondary compounds (chemical polymorphism), caused by influence of both environmental factors and genetic variation due to the frequent hybridization leading to variable chromosome number and expressed gynodioecy, a sexual polymorphism in which natural populations contain two type of plants – females and hermaphrodites (Thompson, 2002). It was shown that high female frequency causes increased heterozygosity (Gouyon and Couvet, 1987), thus increasing the population variability. Moreover, expressed variability in many morphological features even within a single plant does not allow simple and proper determination of a population. Additional problems are caused by often confuse botanical keys comprising a plenty of characters (leaf and calyx shape and size, indumentum, inflorescences, branching, etc.) and out of some are overlapping and/or insufficiently obvious both in native (fresh) and herbarium accessions. Taxonomic evidence for lower taxonomic categories (varieties, forms) is even more suspicious, insecure and difficult. It is clear that improper botanical determination of *Thymus* species might cause confusion in evaluation of a drug quality, especially because a majority of raw material at the markets of medicinal and aromatic plants originates from wild collecting.

The aim of this study was to highlight the significance of some frequently used methods in taxonomic interpretation of complicated taxa of medicinal and aromatic plants, focused on genus *Thymus* L.

MATERIAL AND METHODS

Plant material of several populations and species of the genus *Thymus* L. was collected from their natural habitats from Serbia during the vegetation season in 2002-2003, and from the living collection of the Royal Botanical Gardens Kew for DNA sequencing.

In order to evaluate the various methodological approaches in taxonomic evidence of *Thymus* populations/species, accessions were studied through plant morphology, anatomy, histochemisry, essential oil composition, and DNA sequencing.

For morphological studies, herbarium specimens were used for measurements of leaf length and width (n=70). Within the survey of leaf and stem anatomical traits (n=50), the plant material was fixed in FAA, followed by the standard paraffin procedure, microtome sectioned on 7-10 μm and stained with safranine and aniline blue, while the image analysis was done with software LEICA IM1000. Micromorphological research of leaf and calyx glands was conducted by SEM. Essential oils were obtained by hydro-distillation of dried aerial parts of plants collected in blossoming stage (June 2004), with yields covering a range between 0.1 and 0.8 ml/100 g of plant material. Chemical analysis of essential oil samples was performed by GC-FID (Gas Chromatography - Flame Ionization Detector) and GC-MSD (Gas Chromatography/Mass Selective Detector). Histochemical investigations were performed on fresh plant material by use of standard procedures for different chemical compounds, including sesquiterpene lactones (conc. sulphuric acid) (Geissmann et al. 1971), total lipids (Nile blue and Sudan) (Cain, 1947 and Jensen 1962), carbohydrates (PAS reaction) (O'Brian et al. 1981) and proteins (Ninhidrin-Schif reagens)(Ruzin 1999). In molecular analysis fresh plant material of each specimen was dried in 5g Silica gel. Total DNA was extracted and purified according to standard protocols. The internal transcribed spacer (ITS) regions of 18S- 26S nuclear rDNA was amplified as described in Baldwin (1992). Amplified region was then sequenced and analyzed with PAUP software.

Obtained results were statistically processed by descriptive statistics, analysis of variance (ANOVA, LSD test) and cluster analyses based upon the Euclidean distances using program package Statistica 5.0.

RESULTS AND DISCUSSION

Degree of variability and corresponding clustering of surveyed accessions of the genus *Thymus* L. was separately analyzed and discussed for each applied method.

1. Morphology

Results of variability in leaf length, width and length/width ratio (ANOVA) have shown that all used characters were statistically significant ($p < 0.001$), i.e. might be important in discrimination of *Thymus* populations. According to the cluster analysis (Fig. 1), the *Th. pannonicus*, was separated from the other analyzed populations, and in the less extent the endemic species *Th. dacicus*. This corresponds with the limited and geographically isolated habitats of these species in the studied region of the northern Serbia (Vojvodina).

2. Plant anatomy

Regarding stem and leaf anatomy the following traits were analyzed: stem diameter, the width of stem ribs and diameter of the central cylinder, and the length of upper and lower epidermis, the width of palisade tissue and leaf thickness, respectively.

2.1 Stem anatomy

According to the features in stem anatomy significant differences were obtained in all studied characters, particularly in stem diameter (the highest in population of the *Th. pannonicus* - 1640.75μm, and the lowest in population of the *Th. dacicus* -933.35μm). The highest diameter of the central cylinder was found in population of *Th. marschallianus* (954.85μm).

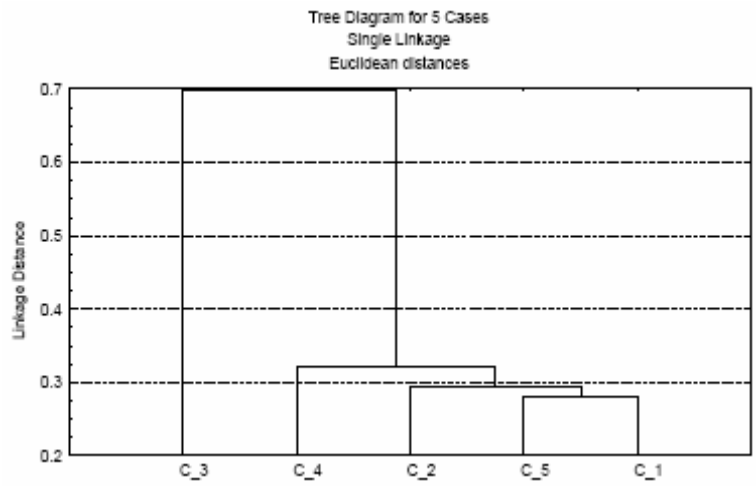

Fig. 1. Cluster for leaf size character of different *Thymus* populations
C1- *Thymus glabrescens* C2 – *Th. glabrescens* C3 – *Th. pannonicus* C4 – *Th. dacicus* C5 – *Th. marschallianus*

All studied characters of stem anatomy were statistically significant ($p < 0.001$), and thus might be relevant for species discriminating.

1.1. Leaf anatomy

It was shown that investigated populations differ in all studied characters of leaf anatomy which all were statistically significant ($p < 0.001$). The greatest variability was found in leaf thickness ranging from 154.14 ± 15.75 to 204.83 ± 14.45 in *Th. marschallianus* and *Th. glabrescens*, respectively.

Considering relation between all surveyed anatomy traits (stem and leaf anatomy) and population discrimination (Fig. 2), population of *Th. pannonicus* exhibited much different anatomy traits, whereas populations of the same species, the *Th. glabrescens* were grouped together.

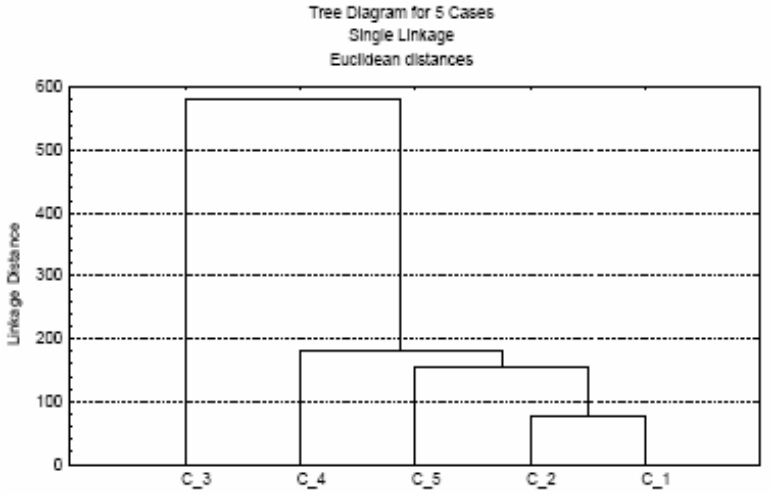

Fig. 2 Clustering of thyme populations according to stem and leaf anatomy features
C1- *Thymus glabrescens* C2 – *Th. glabrescens* C3 – *Th. pannonicus* C4 – *Th. dacicus* C5 – *Th. marschallianus*;

1.3 Micromorphology – studies on gland number and size

The presence of leaf glands and their size at lower and upper leaf epidermis and calyx varied among the studied *Thymus* populations, whereas the counting of the leaf glands of the upper and lower epidermis per mm^2 of three populations collected from three habitats of Stara mountain on the southeast of Serbia, of the endemic species *Thymus vandasii* pointed out the existence in intra-specific variability in this character, ranging from 1.15 ± 0.72 to 13.51 ± 0.86 and from 5.88 ± 1.35 to 5.88 ± 1.35 in upper and lower epidermis, respectively. According to the size of glands present at leaves and calyx studied in 10 different populations/species of the genus *Thymus* (Fig. 3.) grouping in two clades was obtained, where different populations of the same species such as *Th. marschallianus* and *Th. glabrescens* occurred in both groups, suggesting that gland size varies not only among different species, yet also among populations of the single species. Therefore the characters such as gland number and size could not be recommended in discrimination of *Thymus* species.

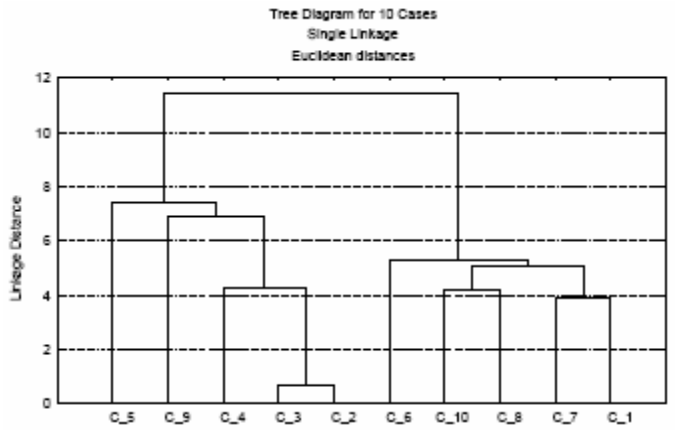

Fig. 3. Cluster according the size of leaf and calyx glands
C1- *Thymus pannonicus*; C2- *Th. glabrescens* C3- *Th. glabrescens* C4- *Th. glabrescens* C5- *Th. marschallianus* C6- *Th. pannonicus* C7- *Th. glabrescens* C8- *Th. marschallianus* C9- *Th. dacicus* C10- *Th. vandasii*

4. Histochemistry

Histochemical analysis of the target components has not revealed any differences among studied species (Tab. 1).

Table 1. Histochemical determining of some compunds in *Thymus* populations

Population	Lipids	Carbohydrates	Proteins	Sesquiterpene lactones
Th. glabrescens	+	+	+	+
Th. marschallianus	+	+	+	+
Th. pannonicus	+	+	+	+
Th. dacicus	+	+	+	+
Th. glabrescens	+	+	+	+

Studies on sesquiterpene lactones by use of conc. sulfuric acid have only confirmed that leaf glands are the structures responsible for secondary compounds (essential oil) accumulation It is possible that some more subtle methods in histochemistry should be performed, targeting the specific secondary compounds to evaluate eventual differences among different *Thymus* populations. This could be especially important for identifying of phenolic components knowing that *Thymus* taxa are characterized as either phenolic or non-phenolic (Stahl-Biskup, 2002).

5. Essential oils analysis

To evaluate the importance of the chemical composition of essential oil of *Thymus* populations the reliable group of seven components (thymol, ρ-cimene, linalool, γ-terpinene, geraniol, nerolidol and α-terpineol) was chosen in eight different species, including of some previously studied (Kulevanova, 1996). Clustering of surveyed populations (Fig. 4) showed grouping of species in two distinct clades, one comprising the *Th. tosevii*, *Th. moesiacus*, *Th. jankae* (all classified into the sub-section Pseudomarginatii), and *Th. pannonicus* of the section Isolepides. The second group included species of the section Isolepides, such as: *Th. glabrescens*, *Th. marschallianus* and *Th. dacicus*. It might be concluded that there is a relation between taxonomic position and the determinants of essential oils in the *Thymus* species. Nevertheless, many studies underlined the strong impact of environmental conditions on the essential oil composition, as the monoterpene variation may represent an adaptive strategy in relation to environmental variation, contributing to the spatial and evolutionary dynamics of the chemotypes (Thompson, 2002). It was postulated that that in some cases the participation of particular essential oil component might be discriminator trait for *Thymus* species corresponding with their position within certain sections (Sáez and Stahl-Biskup, 2002), at least allowing the rough classification of *Thymus* taxa into the phenolic and non-phenolic group (Stahl-Biskup, 2002). This might explain the grouping of the *Th. pannonicus* within the "non-phenolic" group (absence of thymol) rather than group of genetically related species of the section Isolepides (Fig. 4). However, the critical approach of genetic determinacy in chemotype variation is needed, especially in the frame of adaptive responses of *Thymus* populations to specific habitat conditions, including soil and climate characteristics (Gouyon et al., 1986, Sáez, 1996), which was evident according to position of two different populations of the *Th. glabrescens* within the cluster (Fig. 4).

Fig. 4. Clustering of some Thyme populations and species according to main components of essential oils C1- *Thymus glabrescens* C2 – *Th. glabrescens* C3 – *Th. marschallians* C4 – *Th. pannonicu* C5 – *Th. dacicus* C6 – *Th. tosevii* C7 – *Th. moesiacus* C8- *Th. jankae*

6. The validity of chromosome number

In the genus *Thymus* following chromosome numbers are known: *2n*= 24, 26, 28, 30, 32, 42, 48, 50, 52, 54, 56, 58, 60, 84 and 90, corresponding to the diploid, tetraploid and hexaploid levels. The secondary basic numbers x= 14 and x= 15 probably originated from a basic number x= 7. The most frequent numbers are *2n*= 28, 30, 56 and 60. Aneuploidy has been an important phenomenon during the evolution of this genus and is responsible for the other numbers. There are a lot of interesting cases of different levels within the same species. This is true for *Th. mastichina* with *2n*= 30, 60; *Th. vulgaris 2n*= 28, 58; *Th. zygis*, *Th. leptophyllus*, *Th. glabrescens*, *Th. longicaulis*, *Th. praecox 2n*= 28,, 56; *Th. algeriensis 2n*= 30, 56; *Th. comptus 2n*= 26, 52; *Th. zygoides 2n*= 60, 90; *Th. longidentatus2n*= 30, 90; *Th. striatus* and *Th. herba-barona 2n*= 28, 56, 84. The latter is most remarkable because the chromosome numbers studied in the Western Mediterranean populations resulted to be *2n*= 28 in Mjorca, *2n*= 56 in Corsica and *2n*= 84 in Sardinia. Although the chromosome number has not been determined within this study, it should be considered as an important character in Thymus populations discrimination, but is it still unclear whether in some species chemical polymorphism and high ploidy levels are related (Sáez and Stahl-Biskup, 2002).

7. **Molecular analysis – DNA sequencing**

The internal transcribed spacer (ITS) regions of 18S-26S nuclear rDNA are considered to be rapidly evolving, but also have highly conserved areas that can be aligned over a broad taxonomic scale. Thus, it have become a major focus of comparative sequencing at the specific and generic levels in angiosperms. Having in mind that analysis of ITS gave good results in *Nepeta* (Lamiaceae) studies (Jamzad et al., 2003), we conducted screening survey and analysis of several *Thymus* species and populations from Serbia and RBG Kew living collection that are native to Iberian peninsula and British isles. Although thyme samples were from different geographical locations and members of different sections and subsections within the genus, the analysis showed that there is no significant difference in ITS region (Fig. 5), but it can be concluded, based on limited evidence, that the genus is monophyletic.

For better delimitation on lower taxonomical level (species/population level) analysis of amplified fragment length polymorphism (AFLP) should be conducted. AFLP technique has a potential for populational studies where fine scale monitoring of individual genotypes is important (Travis et al., 1996).

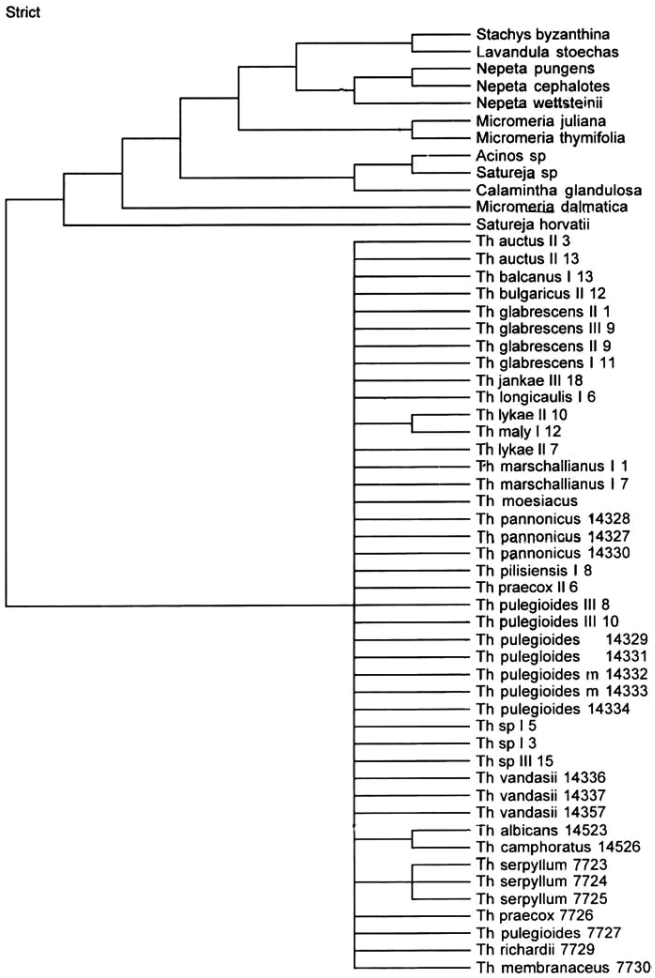

Fig. 5. Strict consensu tree for 41 *Thymus* sample and outgroups based on ITS nuclear region

SUMMARY

To evaluate the significance of some well accepted methods in taxonomic evidence of botanically complicated taxa of medicinal plants, a number of populations of thyme species was studied in their morphological and anatomical traits, as well as in analyses in histo-chemistry, the main components of essential oils and molecular markers. Results confirmed a high variability in all tested characters, suggesting existence of morpho- and chemotypes.

CONCLUSIONS

Results on infra-specific and population variability in *Thymus* L., obtained due to performing of different methods acceptable for taxonomic evidence, such as morphology, anatomy, histo- chemistry, essential oil analysis, and DNA sequencing, suggest that traditional methodological principles in taxa identification, in first morphological and anatomy traits should be combined and used as a set of characters. Chemical polymorphism based upon the content of particular components of essential oils should be estimated in relation to environment, and in further research should be correlated with molecular (genetic) studies. AFLP analysis should be conducted to establish more clear delimitation between different species, and to make clear correlation between different sections and subsections within the genus. At se same time, further anatomical and morphological investigation should back up molecular marker analysis, and result in congruent determination keys and descriptors for thyme species.

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