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Morphological, phytochemical and molecular characterization of intraspecific variability of wormwood (*Artemisia absinthium* L.)

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Summary

A trial with nine wormwood accessions was installed to carry out a systematic evaluation of intraspecific diversity. Six morphological features, essential oil (EO) yield and thujone content were measured. Besides, 11 RAPD and 15 ISSR molecular markers were tested to determine the genetic diversity of the accessions. The experiment was carried out in open field in 2016.

Accession "Pákozd" exhibited largest growth (64.9 cm) and genotype "Norwegen" was the smallest (29.9 cm). This latter accession had also the smallest but thickest leaves. Concerning morphological features, the Norwegian population was the most homogenous one (CV%: 10.6-20.1) while "Belgin" brought about largest variability (CV%: 18.4-45.3).

Based on EO yield, the studied accessions were divided into three significantly diverse groups. The highest yield was produced by "Spanish" accession (3.215 ml/100 g), "Norwegen" and "Belgien" produced medium values (1.569-1.892 ml/100 g) and six accessions showed EO yields below 1% (0.349-0.832 ml/100 g). Three accessions ("Leipzig", "Belgien" and "Norwegen") had high amount of thujone in the oil (50-89%) while in all other accessions thujones were absent or present only below 1%. "Belgien" accession had balanced ratio of α- and β-thujones while in the other ones β-thujone was the absolute main component.

High polymorphism was found among the wormwood accessions also by molecular markers: 81.15% for RAPD and 73.10% for ISSR primers. Based on the Nei's genetic distances the three groups of genotypes were identical to those in the case of EO yield.

The study confirmed the large intraspecific variability of wormwood but revealed that it is not definitely connected to geographical origin of the populations.

Abbreviations: EMA-HMPC: European Medicines Agency – Committee on Herbal Medicinal Products; **CV**: Coefficient of Variation; **DNA**: Deoxyribonucleic Acid; **ISSR**: Inter simple Sequence Repeat; **RAPD**: Random Amplified Polymorphic DNA; **EO**: Essential oil

Keywords: essential oil, leaf shape, thujone; RADP; ISSR; DNA; morphology.

Introduction

Artemisia absinthium (Compositae) is a perennial herb, growing to 40-150 cm in height (Maw, Thomas and Stahevitch, 1985) and developing abundantly branching shoots. Wormwood oil has been used for centuries as anthelmintic, anticold, anti-inflammatoy, antimicrobial, antidepressant, digestive, carminative, choleretic drug, curing insect and spider bites, herpes and parasitic worm infections (Guarrera, 2005; Goud and Swamy, 2015). The indications of EMA-HMPC (2009) monograph include temporary loss of appetite and gastro-intestinal disorders.

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The species exhibit a very large intraspecific variability concerning its morphological traits and active ingredients. Systematic research is, however, rather scarce on this aspect, except references on essential oil composition. Studies on morphological and anatomical features of the genus detected significant marker characteristics of selected species within the genus. Thus, Haghighi and co-workers (2014) used seven morphological characters such as leaf type, leaf colour on adaxial and abaxial surfaces, attachment of capitalism, etc. to differenciate 15 species including *A. absinthium*. Other investigations focused on leaf anatomy as important qualitative feature in classification of this species (Mehrotra et al., 1990; Nazar and Mahmood, 2011). Noorbakhsh and co-workers (2008) described 13 leaf characteristics in 28 species of Artemisia but they had no stable relationship with taxonomy.

The most important active constituents of the herb are volatile compounds and bitter substances. The major volatile components of wormwood are thujone, myrcene, sabinene, trans- and cis-epoxyocimene, trans-verbenol, carvone, sabinyl acetate and chrysanthenyl acetate. However, numerous studies have shown that *Artemisia absinthium* displays significant intraspecific variation in the terpenic constituents and the spectrum is varying from region to region (Basta et al 2007; Mohammadi et al., 2015). Quantitative evaluation of phytochemical diversity of wormwood populations from different natural geographic areas supports the existence of distinct natural chemotypes within the species. Wormwood is usually known and reported to be rich in bicyclic monoterpene thujone (Juteau et al., 2003; Meschler and Howlett, 1999). The presence and concentration of both isomeric forms α- and β-thujones in the oil were described by several authors. Nin et al. (1995) and Juteau et al. (2003) defined characteristic "pure" thujone chemotype while on the other side, absence of thujones in wormwood oil was reported also several times by Orav and co-workers (2006), Carnat et al. (1992), Ariño et al. (1999c) etc. Because of restricted concentration of thujones in food products (EU Council Directive, 1988), these thujone-free chemotypes might raise considerable attention.

Molecular markers have only exceptionally been used for studying intraspecific variability and relationship of accessions of this genus and especially of this species. RAPD investigations in *Artemisia annua* were performed in India already 17 years before (Sangwan et al., 1999). Nazar and Mahmood (2011) established the genetic diversity of some collected *Artemisia* species including *A. absinthium* in Pakistan, using RAPD markers as well. Application of ISSR analysis has been used for *A. herba-alba* in Tunisia to detect genetic polymorphism within this species. According to our knowledge, the combination of both RAPD and ISSR markers for revealing genetic variability of *A. absinthium* has not been published yet.

Wild growing plants assure rich resources for optimising the botanical raw material for diverse utilization areas. Optimisation of the raw material, however, presumes the thorough knowledge on the genotypes used in breeding programs and production. The objective of our study was the comparison and better recognition of 9 genetic stock accessions originating from different European regions. The populations were grown under uniform environmental conditions in Hungary and evaluated by morphological, chemical and genetic tools.

Materials and methods

Plant material and cultivation

Plant material for the study included gene bank collections, market items and seeds collected from wild habitats (Tab. 1). Propagation was carried out by seed sowing in March 2016 and seedling raising in greenhouse for 2 months. The seedling with 5-6 leaves were planted into open field plots in June, installing three replicates at the Experimental Station of Szent István University in Budapest. The soil is sandy-loam, pH 7.8, humus content is low. The plants were grown without additional fertilization; mechanical weed control and irrigation were assured in dry periods. Measurements and sampling were carried out at the beginning of August at vegetative stage. 10 individuals from each accession were randomly chosen to evaluate harvest. Details of the measurements are described below.

Tab. 1: List of the investigated *A. absinthium* accessions

Accession		Origin of population
No.	Sign	
1	Belgien	Accession of Gatersleben genebank (collected in Belgium)
2	Csór	Wild collected population: Csór, Hungary
3	English	Market item from England ('Premier seeds direct' company)
4	Hungarian	Market item from Hungary ('Herbaria' company)
5	Leipzig	Accession of Gatersleben genebank (collected in Germany)
6	Norwegen	Accession of Gatersleben genebank (collected in Norway)
7	Pákozd	Wild collected population: Pákozd, Hungary
8	Spanish	Wild collected population: Teruel, Spain
9	Wild Soroksár	Wild collected population: Soroksár, Hungary

Morphological measurements

Six randomly selected individuals were chosen to collect seven leaves at the central part of the shoots from each of them. In total, 378 leaves of nine accessions were investigated at leaf rosette period. After collecting, the leaves were pressed and a herbarium was prepared following the techniques of DeWolf (1968). The thickness (as mean of five target points on midway between the margins and midrib at the widest part of the leaf) was measured using a Digimatic Thickness Gauge equipment. The total length of the leaf and that of the blade were measured by vernier caliper at the herbarium specimens and the ratio between them has been calculated (Fig. 1).

Plant height (from ground level to tallest shoot) and width (mean of the widest diameters) were measured immediately before start of harvesting.

Essential oil extraction

After the morphological measurements, the shoots of each individual were cut at about five cm height above the soil surface. The plant material was dried at room temperature (20-25 °C) in shade for two weeks. Leaves were separated from stem parts and only the former ones were used for essential oil distillation.

Plant samples (g) were distilled in a Clevenger-type apparatus according to the method recommended by the VII. Hungarian Pharmacopoeia. Essential oils were extracted from 50 g dried leaves of each accession by hydro-distillation (500 ml water) for 2.5 hours.

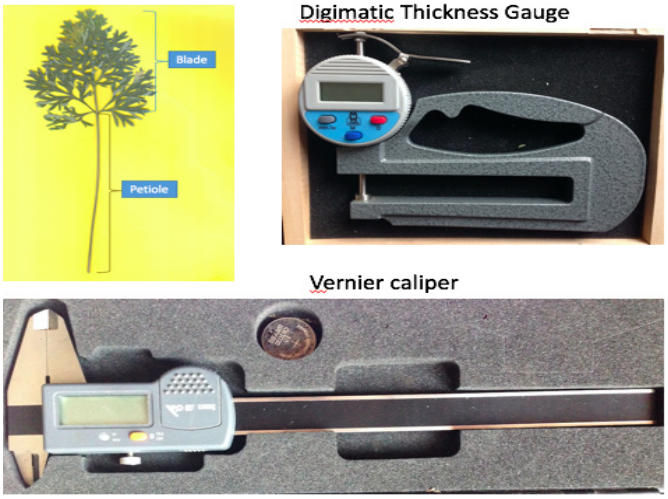

Fig. 1: Herbarium leaf of *A. absinthium* and devices for leaf measurements

The oils were collected, stored in an airtight vial and kept in a refrigerator at 4 °C until the analysis took place.

Gas chromatography

The GC analyses were carried out using an Agilent Technologies 6890N instrument equipped with HP-5 and HP-5MS capillary columns (5% phenyl, 95% dimethyl polysiloxane, length: 30 m, film thickness: 0.25 mm, id. 0.25 mm), programed as follows: initial temperature 60 °C, then by a rate of 3 °C/min up to 240 °C; the final temperature was kept for 5 min; injector and detector temperature: 250 °C; carrier gas: helium (constant flow rate: 1 mL/min); split ratio: 30:1.

Mass spectrometric analysis

GC-MS analyses were carried out using an Agilent Technologies 6890N GC equipped with an Agilent Technologies MS 5975 inert mass selective detector and an ionization energy of 70 eV. The MS were recorded in full scan mode that revealed the total ion current (TIC) chromatograms. α- and β-Thujone content were identified by linear retention indices that were calculated using the generalized equation of Van Den Dool and Dec. Kratz (1963) by using NIST MS Search 2.0 library and Adams mass spectra library (P. Adams, 2007).

DNA isolation

Young leaves from 10-15 plants of each accession were collected and ground in liquid nitrogen to a fine powder. Genomic DNA was extracted from fresh leaves by a DN easy Plant Mini Kit (Qiagen, BioScience, Hungary). The concentration and quality of extracted DNA from each accession were assessed using NanoDrop (BioScience, Hungary) and visually checked on 1.5% agarose gel.

Out of the primarily screened 13 RAPD and 15 ISSR primers only 11 RAPD and all the 15 ISSR primers produced clear, reproducible and scorable bands, thus the investigations have been carried out by these ones. Their sequences are presented in Tab. 2.

RAPD and ISSR amplification

The PCR reaction was performed in a 12 ml volume containing the reaction buffer (approximately 15-25 ng genomic DNA, 1 mM primer (for RAPD) or 2mM primer (for ISSR), 6 ml of 2× GoTaq Hot Start Green Master Mix (Promega), 3 mM $MgCl_2$ and nuclease free

Tab. 2: The evaluated RAPD and ISSR primers

RAPD primer name	**Sequence**	**Size of fragment (bp)**	**Number of fragments**
A10	5'-GTGATCGCAG-3'	1450-250	10
B10	5'-CTGCTGGGAC-3'	1500-300	16
E3	5'-CCAGATGCAC-3'	1000-250	11
M2	5'-ACAACGCCTC-3'	1300-300	15
M3	5'-GGGGGATGAG-3'	1300-300	14
OPA-02	5'-TGCCGAGCTG-3'	1100-300	11
OP A20	5'-GTTGCGATCC-3'	1500-400	6
OPB-11	5'-GTAGACCCGT-3'	1200-300	10
OP G18	5'-GGCTCATGTG-3'	1300-350	10
OP G13	5'-CTCTCCGCCA-3'	1300-300	11
Seq-2	5'-GGGTTTAGGG-3'	1300-400	8
Total no. of bands (RADP)			122
Number of polymorphic loci			99
Percentage of polymorphic loci			81.15%
ISSR primer name	**Sequence**	**Size of fragment (bp)**	**Number of fragments**
443	5'-ACACACACACACACACACT-3'	1200-200	14
818	5'-CACACACACACACAG-3'	1100-200	13
825	5'-ACACACACACACACACT-3'	1300-200	16
849	5'-GTGTGTGTGTGTGTGTCG-3'	1100-240	13
A7	5'-AGAGAGAGAGAGAGAGAGAGT-3'	950-200	14
Caa5	5'- CAACAACAACAACAA-3'	1100-200	11
CAg5	5'- CAGCAGCAGCAGCAG-3'	1000-180	17
Ctc4rc	5'-CTCCTCCTCCTCRC-3'	1000-230	16
Issr1	5'-CACACACACACACACAGT-3'	1500-150	14
Issr2	5'-GAGAGAGAGAGAGAGAG-3'	900-220	12
Issr3	5'-GTGTGTGTGTGTGTGTC-3'	850-300	12
Issr4	5'-ACACACACACACACACTG-3'	1500-250	12
Issr5	5'-AGTGAGTGAGTGAGTG-3'	1300-120	17
Issr6	5'-GATAGATAGATAGATAGATA-3'	1100-250	8
Issr7	5'-TCTTCTTCTTCTTCTTCT-3'	1500-530	7
Total no. of bands (ISSR)			196
Number of polymorphic loci			144
Percentage of polymorphic loci			73.10%
Total no. of bands (RADP+ISSR)			318
Number of polymorphic loci			243
Percentage of polymorphic loci			76.18%

water). PCR amplification was conducted in a SuperCycler SC-200 thermocycler (Kyratec). The thermal cycle used for RAPD primers was 2 min at 95 °C followed by 35 cycles at 94 °C for 30 s; 1 min at specific annealing temperature (the optimal annealing temperature was determined for each individual primer); 1 min at 72 °C and final extension at 72 °C for 5 min. The program used for ISSR primers was 3 min at 95 °C followed by 35 cycles at 94 °C for 30 s; 45 s at specific annealing temperature; 45 s at 72 °C and final extension at 72 °C for 5 min.

Amplified DNA fragments were separated in a 1.5% agarose gel (SeaKem LE Agarose, Lonza) at 100 V for 90-120 min in 1× Tris-Acetate EDTA (TAE) buffer (pH 8.0) and stained by 1% (w/v) ethidium bromide. The PCR products were visualized under UV light by AlphaImager EP Imaging System (Cell Bioscience). The 100 bp ladder (Promega) was used as a molecular weight size marker.

Data analysis

SPSS version 22 was used to analyze the data. MANOVA test has been conducted for evaluating the measured morphological characters of

the leaves. One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test were carried out to analyze the significant differences of EO content of the nine accessions. Homogeneity of variances was checked by Levene's test and the normality of variances was checked by Kolmogorov-Smirnov method. In case of violated homogeneity of variances, Games-Howell was used in post hoc test to determine the significant difference between accessions.

Amplified DNA fragments with reproducible bands of each locus were scrored as binary present (1) or absent (0) and data matrices of RADP and ISSR loci were assembled for further analysis. The results were summarized in Microsoft Excel table. Popgene version 1.32 (YEH et al., 1997) was used to estimate number of polymorphic bands, percentage of polymorphic bands, Nei's (1972) gene diversity (h) and Shannon's Information Index (I) (LEWONTIN, 1972) for dominant marker data or all loci and also for each population separately. Genetic relationship among genotypes was studied by UPGMA (Unweighted Pair Group Method with Arithmetic averages) cluster analysis and principal component (PCA) analysis using PAST software (HAMMER et al., 2001).

Results and discussion

Morphological measurements

According to MANOVA Tests of between-subjects effects, there have been significant differences detected in the case of each of the four leaf morphological characters: F. thickness$_{(8;363)}$=13.77 ($p<0.001$); F. lenght leaf$_{(8;363)}$=18.17 ($p<0.001$); F. length petiole$_{(8;363)}$=15.03 ($p<0.001$); F. ratio blade petiole$_{(8;363)}$=9.84 ($p<0.001$). The results are summarized in Tab. 3.

The thickness of blades of the investigated accessions ranged from 0.31 mm to 0.49 mm. The Games-Howell test distinguished 4 subsets at $p = 0.05$ significance level. Accessions from "Csór" and "Norwegen" have the smallest thickness of leaf while "Belgien" was characterised by the thickest leaves. The length of leaf and the length of petiole seem to be in tight connection, as highest values for both of them were found in accessions "Hungarian" and "Leipzig" while the length of leaf and petiole were shortest in the accession "Norwegen". The marginal values for the leaf length were 209 mm ("Leipzig") and 124 mm ("Norwegen").

The ratio between blade and petiole was influenced by the origin, too. It varied from 0.76 ("Spanish") to 1.12 ("Belgien"). These accessions proved to be significantly different from all the other ones and also from each other. For these values, the investigated four accessions of Hungarian origin revealed similar ratios between 0.8-0.9.

Concerning the evaluated leaf morphological traits, it was established that the Norwegian population can be considered as the most homogenous one (CV%: 10.6-20.1) while the greatest variability was shown by the accession "Belgien" (CV%: 18.4-45.3).

The four Hungarian accessions had taller plants than all the accessions of other origin except the accession "English" (Tab. 4.). In the whole population, genotype "Pákozd" exhibited the highest growth (46.2 cm) and genotype "Norwegen" had the shortest shoots (18.7 cm). This latter accession was determined as the most homogenous one (CV%=11%). On the other side, the largest variability was detected in population "English" (CV%=31.17%).

Similarly to the discussed other characteristics, plant width brought about considerable differences among accessions (Tab. 4). "English" and "Pákozd" were the biggest with largest values for plant width (59.4-64.9 cm) while "Norwegen" was separated from all the others presenting the smallest bushes (29.9 cm). It should be mentioned, that the thinnest leaf blade and smallest leaves were also detected in this latter one.

Tab. 4: Height and width of different wormwood accessions

Accession	Height (cm)		Width (cm)	
	Mean	St. dev.	Mean	St. dev.
Belgien	$41.1^{b,c}$	9.11	$42.9^{b,c}$	10.16
Csor	$42.0^{b,c}$	9.16	$54.0^{b,c,d}$	11.08
English	47.5^{c}	14.80	$59.4^{c,d}$	11.78
Hungarian	$40.2^{b,c}$	9.37	$50.3^{b,c,d}$	4.55
Leipzig	$40.8^{b,c}$	13.46	$50.8^{b,c,d}$	9.85
Norwegen	18.7^{a}	2.06	29.9^{a}	4.25
Pakozd	46.2^{c}	8.84	64.9^{d}	16.74
Spanish	32.7^{b}	7.53	$47.4^{b,c,d}$	5.72
Wild Soroksar	$42.7^{b,c}$	12.60	56.3^{d}	8.25
Mean	39.1		50.7	

Different letters at column represent statistical significant difference between accessions according to Games-Howell test at p=0,05

Tab. 3: Leaf characteristics of nine wormwood accessions

Accession	Thickness of blade (mm)		Length of leaf (mm)		Length of petiole (mm)		Ratio of blade/petiole	
	Mean	St. dev.	Mean	St. dev.	Mean	St. dev.	Mean	St. dev.
Belgien	0.49^{d}	0.089	$167^{b,c,d}$	50.5	$83^{b,c}$	37.8	1.12^{e}	0.306
Csór	0.31^{a}	0.075	$181^{c,d,e}$	32.1	$97^{c,d}$	21.9	$0.89^{b,c}$	0.164
English	$0.37^{a,b}$	0.098	$185^{d,e}$	42.9	$100^{c,d}$	33.1	$0.91^{a,b,c,d}$	0.278
Hungarian	0.40^{b}	0.095	206^{e}	55.2	115^{d}	35.3	$0.81^{a,b}$	0.218
Leipzig	$0.42^{b,c}$	0.075	209^{e}	56.2	111^{d}	33.2	$0.91^{b,c,d}$	0.181
Norwegen	0.32^{a}	0.056	124^{a}	13.0	61^{a}	8.6	$1.03^{d,e}$	0.207
Pákozd	$0.37^{a,b}$	0.161	148^{b}	42.0	78^{b}	27.4	$0.93^{b,c,d}$	0.220
Spanish	$0.47^{c,d}$	0.097	155^{b}	36.5	$90^{b,c}$	24.8	0.76^{a}	0.172
Wild Soroksár	$0.41^{b,c,d}$	0.131	151^{b}	34.7	79^{b}	21.3	$0.94^{b,c,d}$	0.195
Mean	0.39		170		91		0.92	

Different letters at column represent statistical significant difference between accessions according to Games-Howell test at p=0,05

Characteristics of the essential oil

Essential oil yield

The essential oil yield of the investigated accessions was 1.107 ml/100 g as a mean, however, it varied on a large scale: between 0.349 ml/100 g ("Wild Soroksár") and 3.215 ml/100 g ("Spanish"). According to ANOVA test of between-subjects effects for this trait, significant differences could be detected among accessions: $F_{(8;81)}$=58.707 ($p<0.001$). The Tukey test provided 3 subsets at p=0.05 significance level (Tab. 5). Based on this, all accessions from Hungary together with "English" and "Leipzig" are statistically equal, practically below 1%. Among them, highest mean value was reached by "Leipzig" and lowest level by "Wild Soroksár". In general, these values correspond to former data of Orav and co-workers (2006) who obtained 0.1-1.1% essential oil from plant material coming from different European regions or to the reference of Basta et al. (2007) about Greek wild plants (0.31%).

Relatively high concentrations of 1.569 ml/100 g and 1.892 ml/100 g were determined from accessions "Norwegen" and "Belgien", respectively. These results are similar to the findings of Msaada and co-workers (2015) in Tunisia who described an essential oil content of 1.1-1.46% in wild growing plants. Also plant material from Cuba produced similar yield (1.25%), (Pino et al., 1997). The "Spanish" accession in our study presented an exceptionally high level of volatile compounds which is significantly different from each of the other ones (3.215 ml/100 g). This high level could hardly be found in the literature.

The "Spanish" accession was the most homogenous one in term of essential oil yield (CV%=12%) while the largest variability was determined in the population "Leipzig" from Germany (CV%=64.4%).

Our results ascertain that the yield of essential oil of wormwood may differ considerably depending on the investigated plant material (Nguyen and Németh, 2016). At the same time, it can be concluded that the differences are based on genetic variability instead of being the result of diverse ecological conditions as they have manifested themselves in the same environment at our experimental site.

Tab. 5: Essential oil yield (ml/100 g DM) of dried leaves of the studied *Artemisia absinthium* accessions grouped according to the Tukey test

Wormwood accessions	N	Subset		
		1	2	3
Wild Soroksar	10	0.3490		
Hungarian	10	0.3620		
Csor	10	0.5210		
Pakozd	10	0.5530		
English	10	0.6750		
Leipzig	10	0.8320		
Norwegen	10		1.5690	
Belgien	10		1.8920	
Spanish	10			3.2150
Sig.		.152	.662	1.000

Means for groups in homogeneous subsets are displayed. The error term is: Mean Square(Error) = 0.156.
a. Uses Harmonic Mean Sample Size = 10.000. b. Alpha = 0.05

Tab. 6: Thujone contents of the studied *A. absinthium* accessions (GC area %)

Accession	α-thujone			β-thujone		
	Min.	Max.	St.dev.	Min.	Max.	St.dev.
Belgien	0.00	51.68	33.24	0.00	89.89	30.09
Csor	0.00	0.27	0.18	0.00	0.30	0.12
English	0.00	0.25	0.63	0.00	1.92	0.63
Hungarian	0.00	0.27	0.10	0.00	0.70	0.24
Leipzig	0.00	0.75	0.72	0.00	84.44	32.51
Norwegen	0.00	24.83	10.60	0.00	26.77	9.72
Pakozd	0.00	0.12	0.04	0.00	0.19	0.08
Spanish	0.00	0.00	0.00	0.00	0.06	0.02
Wild Soroksar	0.00	0.13	0.05	0.00	2.12	0.67
Mean	0.00	3.33		0.00	14.56	

Thujone content

Both isomeric forms of thujone, α- and β-thujone were determined and quantified in all studied wormwood oils (Tab. 6). There are well established differences among the oils. Three accessions ("Leipzig", "Belgien" and "Norwegen") have high amounts of thujonesin the oil while in the case of all other accessions thujones were absent or present only in trace amounts (below than 1%). Thujone content has been shown the variable distribution within individuals of these accessions (Fig. 2). The highest proportions (up to 91.55%) were found in an individual of "Belgien". It seems to be a unique chemical character which may be detected only by individual sampling like in our study.

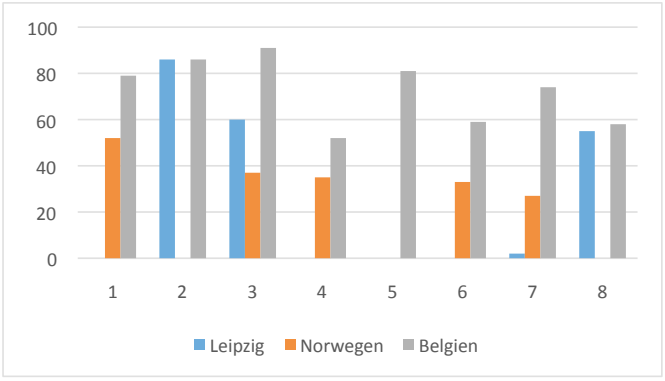

Fig. 2: Distribution of individual values in the thujon containing accessions (GC area %)

Among the thujone chemotypes, in the oils of accessions "Leipzig" and "Belgien" β-thujone content was much higher than that of the a isomer. In these spectra β-thujone varied from 50% to 89% of total oil while α-thujone was observed in less than 2% of total area percentage of the investigated oils. The majority of the references on wormwood indicate similar tendency. Blagojević et al. (2006) investigated Serbian natural populations and brought about 63.4% concentration for β-thujone with 0.4% for α-thujone.

"Belgien" accession has balanced percentages of both forms of thujone which may be compared to the data of Rezaeinodehi and Khangholi (2008) on wild collected Iranian wormwood: 18.6% and 23.8%, for α- and β-thujones, respectively. The remaining genotypes of our study (all accessions from Hungary, "English" and "Spanish"), however, can be declared as "thujonless" ones because thujones could not be demonstrated among the main components of their oils. Several

authors reported oil composition without or with trace amounts of thujones. These samples included raw material from different regions of the world, e.g. Estonia (Orav et al., 2006), France (Carnat et al., 1992), Cuba (Pino et al., 1997), etc.

Molecular aspect

Using 11 RAPD primers, we detected 122 bands which means 11 bands per primer on average. The bands were in the range of 250-1500 bp. The primer B10 gave the highest number of bands (16 bands) while the primer OP-A20 gave the lowest number of bands (6 bands). Tab. 2 shows the number and the size of DNA fragments produced by each of the RAPD and ISSR primers.

A total of 196 scorable bands were generated from 15 ISSR primers. The average number of amplified bands per primer was 13. The Primer ISSR5 and Cag5 composed of trinucleotide repetitions produced as many as 17 bands while primer ISSR7 amplified the lowest number of bands (7 ones). The size of the bands amplified by the ISSR primers ranged from 120 bp (by primer ISSR5) to 1500 bp (by ISSR1, ISSR4 and ISSR7) (Tab. 2). The proportions of polymorphic bands among wormwood accessions were high, with 81.15% for RAPD and 73.10% for ISSR.

When comparing 15 samples of three Artemisia species (*A. vulgaris, A. absinthium, A. roxburghiana*), the proportion of polymorphic bands was 68% (Nazar and Mahmood, 2011). Sangwan et al. (1999) indicated 63.6% polymorphism for RAPD primers during the investigations on 8 plants of *Artemisia annua* L. in India. However, these and related studies are hardly comparable to the present one as they evaluated interspecific variability or intraspecific diversity of other *Artemisia* species.

The largest genetic distance coefficients were determined for accessions "Belgien" and "Spanish" (>0.4 value detected in 4 cases out of 8), (Tab. 7). Nei's genetic distances among the accessions from Hungary are relatively low, between 0.26 and 0.34. The closest similarity was observed between samples collected from wild growing regions in Hungary: "Pákozd" and "Wild Soroksar" (0.26) while the greatest dissimilarity was observed between the accessions "Belgien" and "Csór" (0.47).

Based on the genetic distance matrix of the 9 accessions, the UPGMA dendogram demonstrates the grouping of the investigated accessions in three main clusters (Fig. 3). "Spanish" accession seems to form alone a distinct group (group 1), accessions "Norwegen" and "Belgien" were classified into Group 2 while all the Hungarian accessions were located in the same Group 3 with two further accessions, one from market in England and the other from German seed bank exchange ("Leipzig").

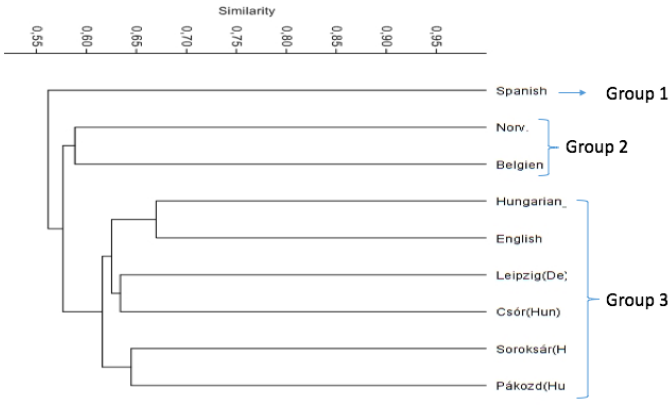

Fig. 3: Dendrogram showing the relationship among the studied *A. absinthium* accessions (Generated by combination of RAPD and ISSR primers using UPGMA method)

Conclusions

Our results show that the data obtained by different methods such as morphological, chemical and molecular analysis may effectively demonstrate the wide intraspecific variability of wormwood. At the same growing habitat, under the conditions of our experiment, characteristic differences in growth, width of the bushes, size and form of the leaves were registered. Besides the actual mean values, the internal homogeneity of the studied accessions proved to be different. The coefficient of variation for the morphological traits ranged from 10,6% till 45.3% and was the highest in the case of the length of petiole. Differences in leaf shape of the same individual contribute to the heterogeneity of populations and present additional difficulties in evaluation.

It could be established that the accession "Norwegen" presented the smallest bushes, thinnest leaf blade and smallest leaves. However, on the other side "English" demonstrated the highest growth, "Spanish" developed the thickest leaves while accessions "Leipzig" and "Hungarian" provided the largest leaves. Thus, unfortunately, a well established connection among these morphological traits can be excluded.

The yield of the EO demonstrated similarly large differences among genotypes, it was determined between 0.349 and 3.215 ml/100 g. Besides, intra-accession differences were also considerable, even higher than in the case of the morphological traits: CV for EO yield ranged between 18.4% and 64.4%. The three groups representing significantly different levels of this yield do not seem to be in any

Tab. 7: Genetic distance matrix of the investigated wormwood accessions based on RAPD and ISSR data

Accession	Csór	Wild Sorok.	Pákozd	Hungarian	English	Norwegen	Belgien	Leipzig	Spanish
Csór	1								
Wild Soroksár	0.30	1							
Pákozd	0.34	0.26	1						
Hungarian	0.32	0.35	0.3	1					
English	0.33	0.27	0.34	0.27	1				
Norwegen	0.30	0.28	0.35	0.33	0.29	1			
Belgien	0.47	0.40	0.37	0.31	0.44	0.42	1		
Leipzig	0.43	0.34	0.34	0.34	0.39	0.35	0.38	1	
Spanish	0.44	0.39	0.37	0.46	0.40	0.40	0.43	0.38	1

connection with the origin of the accession. The genotypes showing significantly similar values such as "Norwegen" and "Belgien" are both genebank accessions of presumably wild origin, thus they have no obvious relationship with each other. In the largest group of relatively low EO yield can be found all the Hungarian wild growing populations. However, they are grouped together with geographically very distinct origins such as the English and the German one. Unfortunately, the former accession has been obtained from a market item, thus may not represent a natural habitat. Further systematic study would be necessary to establish any tendency for the yield of EO in connection with geographical directions.

Especially interesting is the variation concerning thujone content of the EO. Our results demonstrate that thujone is not necessarily the main compound of the essential oil of wormwood as it is frequently interpreted by LACHENMEIER et al. (2006). Among the 9 investigated accessions only three accumulated considerable concentrations of thujones in their essential oil. The variability for thujone content exceeded both that of the morphological traits and that of the accumulation level of volatile compounds: e.g. in accession "Belgien" no individuals were found without thujones while in accession "Leipzig" the individual deviations ranged from 0.0% to 86.2%. As for the isomers, β-thujone proved to be the major one, in some cases α-thujone reached comparable percentages, however, no sample was found where this latter one would have been the only isomer. Based on the accumulation level of thujone and its two isomers, it seems to be relevant defining the following chemotaxonomic groups of the species:

- high content of β-thujone (above 50%) + low content of α-thujone (below 10%),
- medium content of β-thujone (from 10 to 50%) + medium content of α-thujone (from 10 to 50%),
- low content of β-thujone (below 10%) + low content of α- thujone (below 10%).

As the minimum values depend to some extent on the sensitivity of the equipment and investigation parameters, it does not seem reasonable to identify an absolute zero variant in this respect.

Based on the study, it may be concluded that large variability of morphological traits such as growth and leaf shape cannot be necessarily connected to the essential oil accumulation potential of the accessions. Similarly, no characteristic marker trait has been found for essential oil composition and chemotype although it would be an advantageous phenomenon for breeding and cultivation. At the same time, the grouping of the accessions based on the EO yield coincides with the groups based on the applied RAPD and ISSR molecular markers.

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Conflict of interest

The authors declare that they have no conflict of interest.

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