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Evaluation of yarrow (*Achillea*) accessions by phytochemical and molecular genetic tools

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Summary

Yarrow (*Achillea*) species are known and utilized worldwide. In the recent study our primarily goal was to get information about the intraspecific diversity of *A. collina* in the Carpathian Basin. Five cultivated genotypes and six populations of wild origin were compared involving seven other species as control. Essential oil (EO) and proazulene (PA) contents were determined and the DNA samples were evaluated by RAPD (11 primers) and ISSR (12 primers) methods.

The EO content varied between 0.010 (*A. distans*) and 0.365 (*A. collina*) ml/100 g DW, the PA content was found between 0.021 and 0.173 % DW. The used RAPD markers provided 140 bands (97.14 % polymorphic). They distinguished primarily among species and less characteristically among the *A. collina* populations. With ISSR primers we detected 188 bands (97.34 % polymorphic). ISSR markers and combined RAPD and ISSR method enabled an informative intraspecific evaluation of *A. collina* accessions. The largest genetic distances were found between *A. ptarmica* and the members of sect. *Achillea* (genetic distances 0.52 - 0.72). Similarity is highest (genetic distance 0.27) among the populations of lower geographical distances. Nei's genetic distances of cultivated populations are also relatively low (0.23 - 0.36). Some wild accessions may represent valuable biological resources for breeding.

Abbreviations

AFLP Amplified Fragment Length Polymorphism; **DNA** Deoxyribonucleic Acid; **DW** Dry Weight; **EO** Essential OIl; **ISSR** Inter-Simple Sequence Repeat; **PA** Proazulene; **PCA** Principal Coordinate Analysis; **RAPD** Random Amplified Polymorphic DNA; **UPGMA** Unweighted Pair Group Method with Arithmetic averages

Introduction

*Achille*a species are well known medicinal plants having an important role both in folk medicine and in the modern phytotherapy. Main indications include loss of appetite, bloating, flatulence, minor menstrual spasms and wound healing (Final community herbal monograph, 2011). The majority of the drug is still obtained from wild collection.

The genus *Achillea* consists of six sections and appr. 140 species (Guo et al., 2005), which are allogamous, herbaceous perennials in the Northern hemisphere. The presence of spontaneous hybrids, allo- and autopolyploids, aneuploids, and phenocopies results in a big cytological, morphological and chemical variability not only at species but also at intraspecific level. Therefore, taxonomic evaluation, identification of species and systematic classification has been a difficult task for decades. With regard to species identification earlier studies were obviously focusing on morphological traits. Although the majority of characteristics proved to be extremely variable, some of them like fruit size (Dabrowska, 1977), shape of leaflets and rayflorets (Rauchensteiner et al., 2002), and pollen morphology (Akyalcin et al., 2011) have been defined as stable ones at least in the investigated taxa. Cytological studies ascertained that in some species different caryotypes are present which, however, do not necessarily show connection with morphological traits (Dabrowska, 1977; Daniheka and Rotreklová, 2001).

Phytochemical parameters represent a valuable part of taxonomic evaluation of yarrow species. Based on the pharmacopoeial requirements, most frequently, the presence of chamazulene in the essential oil has been in the focus of investigations (Tétényi et al., 1962; Oswiecimska, 1962; Michler et al., 1992) although flavonoids may have taxonomic importance, as well (Valant, 1978). It is widely accepted now that chamazulene is a characteristic of the members of *Millefolium* group; however, the presence of azulenes is not a universal phenomenon for all of these species. Earlier, a firm connection between chromosome number of the species and the potential for accumulation of chamazulene was supposed. Recently, it has been accepted that the production of proazulenes in polyploids may depend on the chemism of the parent/original diploid species (Kästner et al., 1992; Ma et al., 2010*)*. By the development of analytical methods evaluation of a wider range of oil components and their enantiomers provided a more complex approach in identification and systematics (Orth, 2000; Rauchensteiner at al., 2002; Radulovic et al., 2007).

The use of molecular markers in the systematic studies of yarrow was introduced from the 1990th. Wallner et al. (1996) proved the applicability of RFLP and PCR based fingerprinting methods for characterisation of micropropagated *Achillea* clones. Investigations by nrITS and plastid trnL-F DNA sequences revealed phylogenetic connections: differentiation patterns of *Achillea* s.l. in time and space (Guo et al., 2004), although this method could not assure a well established separation of *A. millefolium* aggr. In the work of Guo et al. (2005) characterization was realized by AFLP markers and more recently, Ma et al. (2010) demonstrated the ongoing introgression of diploid progenitor and tetraploid progenies in the *A. millefolium* complex by analysing single copy nuclear genes and AFLP markers.

Besides taxonomic studies, several publications appeared in the last years about RAPD, ISSR (Ebrahimi et al., 2012; Farajpour et al., 2011; Gharibi et al., 2011) and AFLP (Rahimmalek et al., 2009) assessment of yarrow species. Characteristically, the authors compared populations from different geographical locations for conservation purposes without describing the phytochemical values of the plant material. These studies are focusing on indigenous species (*A. santolina, A. tenuifolia, A. eriophora*, etc.), not found at the international market and official therapies.

It can be established that practice-oriented investigations on economically important taxa of yarrow are scarce up to now. The goal of our investigations was to look for reliable, relatively simple methods for differentiation/determination of intraspecific taxa of *A. collina*, the most frequently collected and cultivated species. According to our knowledge, molecular genetic study of intraspecific diversity of this species has not been published till now.

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Materials and methods

Plant material

Eleven *Achillea collina* Becker accessions have been included into the investigation. Three of them are officially registered cultivars, two ones are cultivated commercial materials without special denomination and six accessions originate from wild populations (Tab. 1). For comparative studies, seven other species of different ploidy level were used as control. Among them *A. ptarmica* belongs to Section *Ptarmica* (DC.) W. Koch. All other species are members of the Section *Achillea* and except *A. crithmifolia* and *A. filipendulina* of the *A. Millefolium* agg. (GUO et al., 2004).

Seeds of accessions nr. 1-2 were obtained from the maintainers of cultivars, nr. 3 derived from own maintenance breeding, nr. 4-5 were obtained from farmers, 6-11 were collected from wild populations of Hungary and accessions nr. 12-18 were obtained from the genebank of the National Botanical Garden, Vácrátót. The species identity has been controlled according to the morphological traits and through checking the ploidy level by flow cytometry.

Plants were grown from seeds in climatic chambers in 2014. In 5-6 weeks they reached a 4-5 leafy stadium when bulk sampling (KRAFT and SÄLL, 1999) from 10-15 plants/accession was carried out for PCR trials as well as checking of chromosome numbers by flow cytometry (Fig. 1) according to the modified method of GALBRAITH et al. (1983).

After 2 months, the seedlings were planted into open field plots in three replicates at our experimental station in Budapest. At flowering stage, representative bulk samples with max. 20 cm of shoots were taken from each plot for essential oil extraction. The plant material was dried at room temperature and stored at +4 °C until distillation.

The accessions are maintained as living genebank collection at the Research Station of the Faculty of Horticulture, Corvinus University, Budapest.

Essential oil extraction and analysis

The essential oil content was measured from the dried drug in triplicate, by hydrodistillation in a Clevenger-type apparatus according to the Hungarian Pharmacopoeia VII *(Millefolii herba*). After 3 hours of distillation, n-hexane was added to take up the essential oil. After evaporation of the hexane, the collected extract was stored in a cool place. The essential oil content was calculated as ml/100 g dried plant material. Water content of the drug was determined by heating 4 g of the drug at 105 °C for 3 hours.

The proazulene content in the essential oil samples was determined by spectrophotometric method at 608 nm as described in the European Pharmacopoeia VII *(Millefolii herba*) in triplicate and calculated as a percentage of the dry weight expressed as chamazulene.

DNA isolation

One young leaf from each of 10-15 plants of each accessions was collected and ground together with liquid nitrogen. Genomic DNA was extracted from these bulk samples of fresh young leaves by DNeasy Plant Mini Kit (Qiagen, BioScience, Hungary). DNA concentration and quality was assessed using NanoDrop (BioScience, Hungary) and visually checked on 1% agarose gel.

Out of the primarily screened 17 RAPD and 13 ISSR primers only 11 RAPD and 12 ISSR primers produced clear, reproducible and scorable bands, thus, the investigations have been carried out by these ones.

Tab. 1: List of the investigated *Achillea* accessions

Accession		Species	Origin of population
Nr.	sign	/chromosome nr.*/	
Sect. *Achillea*			
Agg. *A. millefolium* s.l.			
1	C1	*A. collina* Becker (4x)	German variety 'Proa'
2	C2	*A. collina* Becker (4x)	Slovakian variety 'Alba'
3	C3	*A. collina* Becker (4x)	Hungarian variety 'Azulenka'
4	C4	*A. collina* Becker (4x)	Cultivated commercial plant material, Gyula 46° 38' 50.2" N/ 21° 16' 42.3" E
5	C5	*A. collina* Becker (4x)	Cultivated commercial plant material, Földes 47° 17' 22.8" N/ 21° 21' 47.8" E
6	CW1	*A. collina* Becker (4x)	Wild collected population, Aszód 47° 39' 12.1" N/ 19° 29' 3.6" E
7	CW2	*A. collina* Becker (4x)	Wild collected population, Csörötnek 46° 56' 59.3" N/ 16° 22' 14.7" E
8	CW3	*A. collina* Becker (4x)	Wild collected population, Diósd 47° 24' 29.8" N/ 18° 56' 36.5" E
9	CW4	*A. collina* Becker (4x)	Wild collected population, Mikóújfalu 46° 3' 13.5" N/ 25° 50' 6.7" E
10	CW5	*A. collina* Becker (4x)	Wild collected population, Nagymaros 47° 47' 16.9" N/ 18° 57' 14.9" E
11	CW6	*A. collina* Becker (4x)	Wild collected population, Remeteszőlős 47° 33' 23" N/ 18° 55' 44.6" E
12	ASP	*A. asplenifolia* Vent. (2x)	Genebank of Vácrátót Bot.Garden
14	DIS	*A. distans* Walds. et Kit. (6x)	Genebank of Vácrátót Bot.Garden
16	MIL	*A. millefolium* L. s.s. (6x)	Genebank of Vácrátót Bot.Garden
17	PAN	*A. pannonica* Scheele (8x)	Genebank of Vácrátót Bot.Garden
Excl. agg. *A. millefolium*			
13	CRI	*A. crithmifolia* Walds. et Kit. (2x)	Genebank of Corvinus University
15	FIL	*A. filipendulina* Lam. (2x)	Genebank of Vácrátót Bot.Garden
Section *Ptarmica*			
18	PTR	*A. ptarmica* L. (2x)	Genebank of Vácrátót Bot.Garden

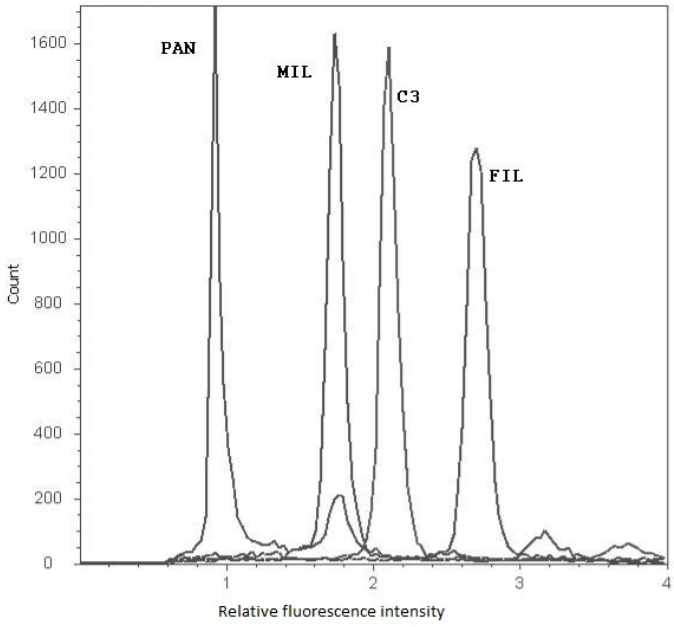

Fig. 1: Histograms of relative fluorescence intensity of octoploid (*A. pannonica*), hexaploid (*A. millefolium*), tetraploid (*A. collina*) and diploid (*A. filipendulina*) *Achillea* species (from left to right), (codes of accessions as in Tab. 1).

RAPD Analysis

11 RAPD primers have been used, the optimum annealing temperature was determined for each one individually (Tab. 2). Amplification reactions were performed in 12 µl volume containing 15-25 ng genomic DNA, 1 µM primer, 6 µl of 2× GoTaq Hot Start Green Master Mix (Promega), 3 mM $MgCl_2$ and nuclease free water. PCR amplification was performed in a SuperCycler SC-200 thermocycler (Kyratec) under the following conditions: 2 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at specific annealing temperature, 1 min at 72 °C and a final extension for 7 min at 72 °C. 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.

ISSR Analysis

ISSR analysis has been performed with 12 primers (Tab. 2), the optimum annealing temperature was determined for each one individually. PCR reactions were carried out in a volume of 12 µl containing 15-25 ng genomic DNA, 2 µM of primer, 6 µl of 2× GoTaq Hot Start Green Master Mix (Promega), 3 mM $MgCl_2$ and nuclease free water. The SuperCycler SC-200 (Kyratec) was programmed as follows: an initial cycle of 3 min at 95 °C, followed by 35 cycles each consisting of 30 s at 94 °C, 45 s at specific annealing temperature, 45 s at 72 °C and final extension of 7 min at 72 °C. Fragment separation and visualization was performed as above.

Statistical analysis

The results of essential oil and proazulene analysis were evaluated by one-way ANOVA using the IBM SPSS Statistics 22 program. The pairwise comparisons of the variances were made by the Tukey Post Hoc test.

Amplified DNA fragments were scored visually for presence (1) or absence (0) of homologous bands and the results were summarized in Microsoft Excel table. Popgene version 1.32 (Yeh and Boyle, 1997) was used to estimate number of polymorphic bands, percentage of polymorphic bands, Nei's (1973) gene diversity (h) and Shannon's Information Index (I) (Lewontin, 1972) for dominant marker data. Genetic relatedness among genotypes was studied by UPGMA (Unweighted Pair Group Method with Arithmetic averages) cluster analysis and principal coordinate analysis (PCA) using Past software (Hammer et al., 2001).

Tab. 2: The tested RAPD and ISSR primers

RAPD primer name	Sequence	Annealing temp. (°C)	No. of bands
OPA-20	5'-GTTGCGATCC-3'	39	10
OPG-18	5'-GGCTCATGTG-3'	38	14
OPB-11	5'-GTAGACCCGT-3'	38	14
OPA-02	5'-TGCCGAGCTG-3'	35	13
OPG-13	5'-CTCTCCGCCA-3'	43	13
m2	5'-ACAACGCCTC-3'	41	13
g11	5'-TGCCCGTCGT-3'	48	14
seg1	5'-AGGGGTCTTG-3'	35	15
seq2	5'-GGGTTTAGGG-3'	35	9
seq3	5'-GACAGACAGG-3'	35	15
seq4	5'-CGAAGCTACC-3'	35	10
Total no. of bands (RAPD)			**140**
Number of polymorphic loci			136
Percentage of polymorphic loci			97.14%
ISSR primer name	Sequence	Annealing temp. (°C)	No. of bands
818	5'-CCCCCCCAAAAAAAG-3'	47	10
825	5'-AAAAAAAACCCCCCCCT-3'	49	14
849	5'-GGGGGGGGTTTTTTTTC-3'	49	14
CAg5	5'-CCCCCAAAAAGGGGG-3'	49	15
ctc4rc	5'-CCCCCCCCCTTTTR-3' *	50	18
issr1	5'-CACACACACACACACAGT-3'	51	21
issr2	5'-GAGAGAGAGAGAGAGAG-3'	49	10
issr3	5'-GTGTGTGTGTGTGTGTC-3'	49	12
issr4	5'-ACACACACACACACACTG-3'	51	21
issr5	5'-AGTGAGTGAGTGAGTG-3'	45	18
issr6	5'-GATAGATAGATAGATAGATA-3'	47	14
issr7	5'-TCTTCTTCTTCTTCTTCT-3'	45	15
Total no. of bands (ISSR)			**188**
Number of polymorphic loci			183
Percentage of polymorphic loci			97.34%
Total no. of bands (RAPD+ISSR)			**328**
Number of polymorphic loci			319
Percentage of polymorphic loci			97.26%

Results and discussion

Essential oil and proazulene content

The essential oil content of the examined accessions varied between 0.002 (*A. distans*) and 0.365 (*A. collina* CW4) ml/100 g (Tab. 3). The

Tab. 3: Essential oil and proazulene content of the examined accessions (codes of accessions as in Tab. 1)

Accession code	Essential oil content (ml/100 g DW)		Proazulene content (% D.W.)	
	Mean	Standard deviation	Mean	Standard deviation
C1	0.248 c,d	0.082	0.105 b,c	0.029
C2	0.273 c,d	0.097	0.075 b	0.035
C3	0.290 c,d	0.051	0.173 b,c	0.052
C4	0.236 c,d	0.940	0.078 b	0.039
C5	0.248 c,d	0.108	0.074 b	0.043
CW1	0.202 b,c	0.145	0.061 a,b	0.018
CW2	0.135 a,b,c	0.088	0.021 a	0.005
CW3	0.235 c,d	0.141	0.135 c	0.057
CW4	0.365 d,e	0.183	0.106 b,c	0.038
CW5	0.317 c,d	0.212	0.148 c	0.086
CW6	0.199 c,d	0.103	0.079 b	0.075
ASP	0.249 c,d	0,038	0.136 c	0.014
DIS	0.485 e	0,044		
MIL	0.005 a	0,001		
PAN	0.198 b,c,d	0,026		
CRI	0.159 a,b,c	0,062		
FIL	0.189 a,b,c	0,009		
PTR	0.059 a,b	0,011		

Different letters represent statistically different subsets according to the Tukey test at p=0.05

accumulation levels of each species are in the range of data mentioned in other investigations (NÉMETH, 2005).

Evaluating the studied populations with giving consideration to the accumulation level of the essential oil, the Tukey test distinguished 5 subsets at p=0.05 significance level. Among them both *A. distans* having the lowest content (0.01 ml/100 g) and *A. crithmifolia* having the highest one (0.42 ml/100 g) represent distinct subsets. On the other hand, the largest homogenous subset includes all of the *A. collina* accessions, besides *A. crithmifolia*, *A. pannonica* and *A. filipendulina*. Taking into account only the *A. collina* accessions, the differences are much lower; significant differences were proven only for *A. collina* wild growing population CW2 and another wild growing population CW4 compared to all of the other ones. These accessions show the two extreme values of the essential oil content: CW4 (Mikóújfalu, Transylvania) produced the highest content (0.327 ml/100 g) and the genotype CW2 (Csörötnek, West-Hungary) the lowest one (0.135 ml/100 g). In other wild collected accessions (in Central Hungary) concentrations between these extreme values were detected. However, each *A. collina* sample could surpass the requirements of the European Pharmacopoeia. In our previous study on 23 Hungarian wild populations of this species we detected also significant (up to four-fold) differences among the samples, however, no connection between the geographical location and the level of the essential oil content could be ascertained (NÉMETH et al., 2007). The essential oil content of the selected cultivars and the cultivated genotypes was much more similar to each other, within a range of 0.2 and 0.3 ml/100 g without significant difference among them.

According to the recent chemotaxonomic conception, among the investigated species, proazulenes are only accumulating in *A. collina* and *A. asplenifolia* (KASTNER et al., 1992; RAUCHENSTEINER et al., 2002). This has been ascertained by our results. The distilled oil of the other species had a yellowish colour indicating the lack of azulenes, while the samples of the mentioned two species each showed a blue colour of different intensity. The concentration of proazulenes prescribed by the European Pharmacopoeia VIII is 0.1% which, however, was exceeded only by half of the samples. Besides the sample of *A. asplenifolia* it was the case in three wild growing accessions and in two selected cultivars of *A. collina* (Tab. 3).

The lowest proazulene content (0.020%) was detected in a wild growing *A. collina* population (CW2), while significantly the highest values (0.135% and 0.148%) were also measured in accessions of wild origin (CW3 and CW5, respectively). Among the cultivated genotypes, the registered cultivars 'Proa' (C1) and 'Azulenka' (C3) showed significantly the highest proazulene contents (0,110% and 0.133%, respectively). Both cultivated populations (C4 and C5), furthermore the cultivar 'Alba' (C2) and the sample from Remeteszőlős (CW6) have each statistically similar proazulene contents (0.074-0.079%). In previous Hungarian investigations the proazulene content of the wild growing populations was between 30% and 67% (NÉMETH et al., 2007). Although these are area percentages detected by GC method, therefore difficult to compare with the present data, significant differences among accessions were detected in both studies. Other trials on European wild yarrow populations *A. collina* has been rarely evaluated. In Germany, MICHLER et al. (1992) described large differences among populations concerning the presence of proazulenes.

Genetic markers

In the RAPD analysis 140 bands were detected of which 97.14% was polymorphic. The numbers of obtained bands were between 9 and 15, the average was 12.36 polymorphic bands/primer. It is higher than obtained with *A. santolina*, *A. tenuifolia* (EBRAHIMI et al., 2012) and with *A. millefolium* (FARAJPOUR et al., 2011), (Tab. 2). Based on the PCA of RAPD markers, segregation of the taxonomically most distant *A. ptarmica* from the accessions of *A. collina* is obvious while samples of *A. crithmifolia* and *A. filipendulina* are between them (Fig. 2). These latter diploid species both belong to sect. *Achillea,* however, are less closely related to the polyploid members of the *A. millefolium agg*. (GUO et al., 2004). *A. collina* genotypes C3 (Hungarian cultivar 'Azulenka') and CW6 (wild collected genepool from Remeteszőlős, central Hungary) show the largest distances from the other ones while genotypes CW1, CW3 and C5 show the closest linkage to each other. The mentioned pattern, however, may not reflect geographical or genetic connection. In case of the studied genotypes, it could be established, that RAPD markers tend to distinguish primarily among species and less characteristically among intraspecific populations of *A. collina*.

Using the ISSR markers, the total number of detected bands was 188, about 30% more than in case of RAPD primers. The percentage of the polymorphic bands reached 97.34% exceeding the values of other related studies with *A. millefolium* (FARAJPOUR et al., 2012; GHARIBI et al., 2011). The mean number of polymorphic bands/primer was 15.25, ranging from 10 to 21, also higher than in RAPD analysis (Tab. 2).

Principal coefficient analysis of the studied samples shows a clear separation of *A. ptarmica* (Fig. 3) which reflects well the fact that it is a member of Sect. *Ptarmica* and taxonomically the less related species with all the other ones. This is a similar result as that in the analysis with RAPD markers. A well defined group is formed by four of the accessions of *A. collina* of wild origin and another one by the cultivated accessions of this species. CW2 which is a population of wild origin shows a larger separation from all of the other *A. collina* accessions and especially from the other wild growing ones. A single

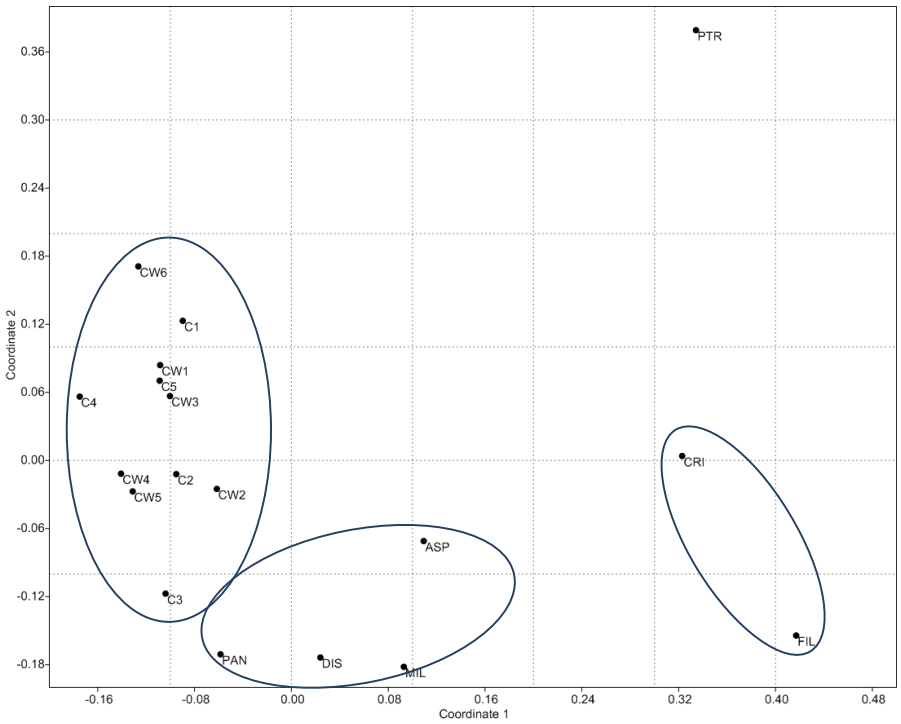

Fig. 2: Patterns of relationships among the investigated *Achillea* accessions revealed by principal component analysis based on RAPD data (codes of accessions as in Tab. 1)

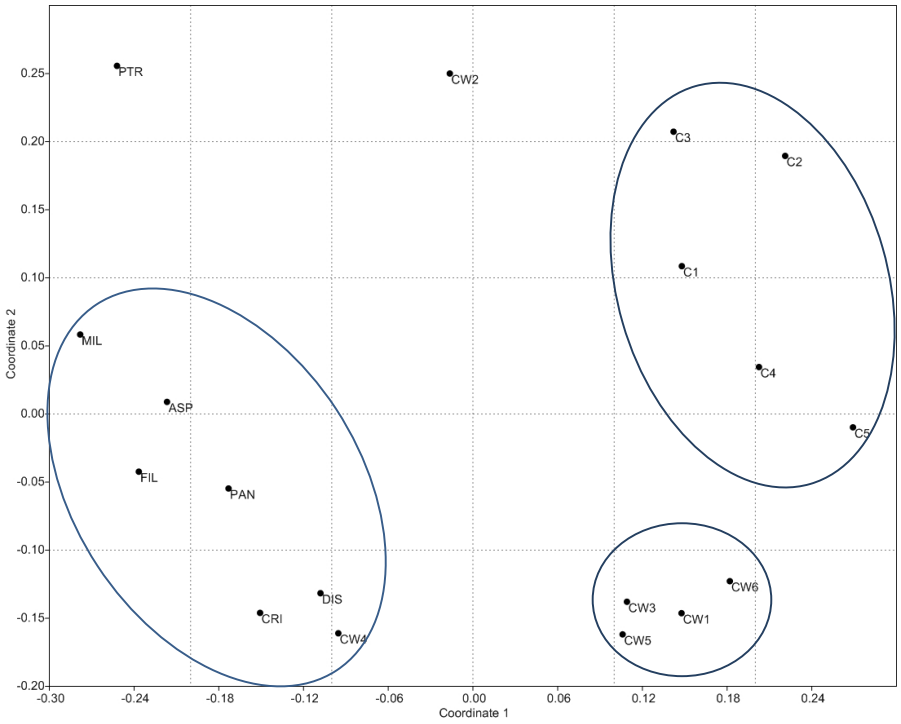

Fig. 3: Patterns of relationships among the investigated *Achillea* accessions revealed by principal component analysis based on ISSR data (codes of accessions as in Tab. 1)

wild growing accession of *A. collina* (CW4) and other species of the sect. *Achillea* form a further, larger group. In this pattern, species of the *A. millefolium* agg. do not reflect a close relationship with each other. The most characteristically separated wild growing population CW2 (Western Hungary) and CW4 (Transylvania) are located to larger distances (200-400 km) from the central populations (CW1, 3, 5 and 6). According to the results, ISSR markers proved to be appropriate first of all for the separation of *A. collina* accessions while the relationships of other species inside the section are less specific.

A joint evaluation of RAPD and ISSR analysis revealed a good separation of *Achillea* species (Fig. 4.). Similarly to the results of both RAPD and ISSR markers separately, *A. ptarmica* and the members

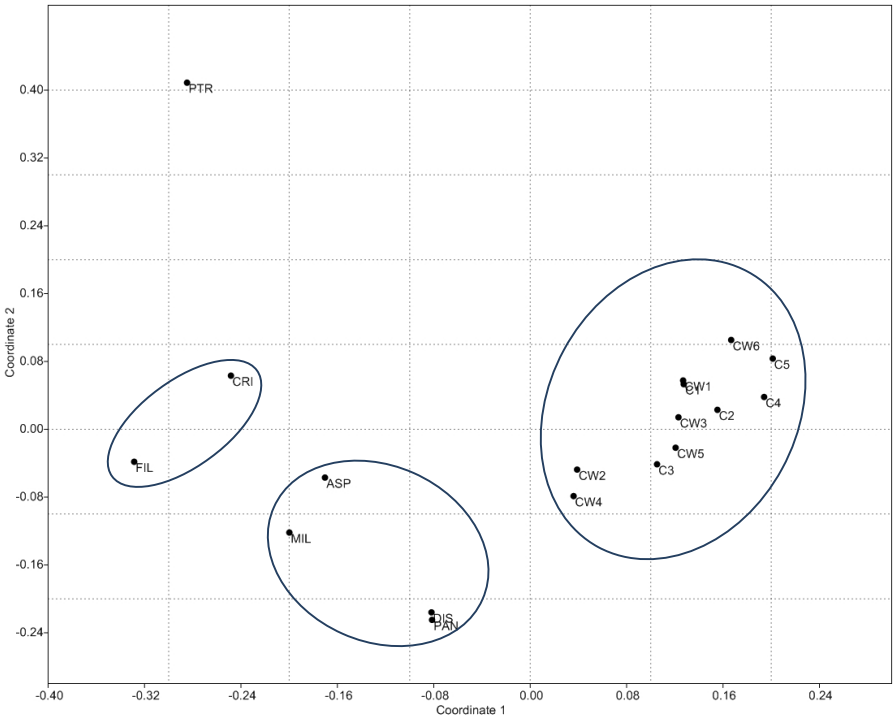

Fig. 4: Patterns of relationships among the investigated *Achillea* accessions revealed by principal component analysis based on RAPD and ISSR data (codes of accessions as in Tab. 1)

Tab. 4: Genetic distances matrix of the investigated *Achillea* accessions based on RAPD and ISSR data (codes of accessions as in Tab. 1)

	CW2	C3	C2	C1	C4	C5	CW6	CW1	CW5	CW3	CW4	DIS	PAN	FIL	CRI	ASP	MIL	PTR
CW2	1.00																	
C3	0.40	1.00																
C2	0.38	0.30	1.00															
C1	0.44	0.36	0.30	1.00														
C4	0.46	0.28	0.28	0.29	1.00													
C5	0.42	0.35	0.26	0.30	0.23	1.00												
CW6	0.45	0.44	0.36	0.36	0.34	0.26	1.00											
CW1	0.47	0.41	0.35	0.36	0.33	0.30	0.27	1.00										
CW5	0.47	0.45	0.38	0.43	0.32	0.33	0.36	0.30	1.00									
CW3	0.46	0.39	0.32	0.39	0.27	0.30	0.30	0.27	0.28	1.00								
CW4	0.44	0.42	0.45	0.44	0.38	0.38	0.38	0.36	0.39	0.32	1.00							
DIS	0.50	0.46	0.44	0.43	0.46	0.47	0.46	0.42	0.43	0.42	0.39	1.00						
PAN	0.48	0.46	0.45	0.46	0.49	0.49	0.48	0.48	0.50	0.50	0.40	0.25	1.00					
FIL	0.62	0.57	0.61	0.65	0.65	0.68	0.68	0.63	0.66	0.60	0.55	0.55	0.59	1.00				
CRI	0.65	0.64	0.64	0.62	0.69	0.64	0.57	0.54	0.58	0.62	0.64	0.56	0.61	0.54	1.00			
ASP	0.47	0.46	0.48	0.43	0.48	0.50	0.49	0.50	0.52	0.41	0.47	0.44	0.45	0.45	0.55	1.00		
MIL	0.50	0.47	0.49	0.53	0.51	0.59	0.54	0.49	0.50	0.46	0.45	0.38	0.46	0.53	0.51	0.37	1.00	
PTR	0.63	0.63	0.59	0.56	0.63	0.62	0.56	0.59	0.72	0.62	0.59	0.57	0.59	0.57	0.60	0.52	0.53	1.00

of sect. *Achillea* are the most characteristically distinguishable from each other. The accessions of the two related species *A. crithmifolia* and *A. filipendulina* (Section *Achillea*, exc. *A. millefolium* agg.) are situated closer to each other in the PCA analysis than to any of the accessions in agg. *A. millefolium*. The studied genotypes *of A. distans* and *A. millefolium* seem to be closely linked with each other. AFLP profiles of these two species were also hardly distinguishable from each other (GUO et al., 2005) being two related polyploids of the *A. millefolium* agg. The species *A. pannonica*, *A. distans* and *A. millefolium* gave overlapping patterns and could not be clearly separated based on essential oil markers, either (RAUCHENSTEINER et al., 2002).

Accessions of *A. collina* represent another group. By the joint evaluation of RAPD and ISSR markers, cultivated and wild growing ac-

cessions do not seem to separate characteristically from each other, except the geographically more distant CW2 and CW4 which are situated in the PCA coordinate system characteristically far away.

In coincidence with the above mentioned, the largest genetic distance coefficients were proven between *A. ptarmica* and the members of sect. *Achillea* (genetic distances between 0.52 and 0.72), (Tab. 4). Distances of the species inside the Section Achillea are in most cases below 0.5. The genetic distances of CW2 originating from Western Hungary are the largest to any of the other wild originated accessions exceeding 0.4. On the other side, similarity is highest (genetic distance 0.27 both) between the populations CW1-CW3 and CW1-CW5 where geographical distances of the original locations are 52-55 km. Nei's genetic distances among the cultivars are also relatively low, between 0.23 and 0.36.

The values of the Shannon diversity index (H) (LEWONTIN, 1972) reflect similar results. This value calculated for all of the accessions in this study is 0.5209 while taking into account only the *A. collina* accessions it shows a lower value: 0.4098. The intraspecific diversity seems to be higher among the wild growing accessions (H=0.3793) than among the cultivars (H=0.3028).

Based on the investigated markers, it can be established, that the studied RAPD provided a more specific approach for distinction of species while the used ISSR and combined RAPD and ISSR primer evaluation enabled an informative evaluation among *A. collina* accessions, as well. Nevertheless, the separation of populations based on the investigated molecular markers does not reflect any connection with their essential oil and proazulene contents.

According to the results of this study, a common origin of the cultivated populations might be anticipated. 'Proa' is the first selected cultivar of *A. collina*. It is a German cultivar, which has been on the market since 1973. Compared to this, 'Alba', a Slovakian cultivar was registered almost twenty years later in 1992. As no relevant information on the original genetic background of these selections is available, a relationship cannot be excluded. Besides, even in case of their different origins, during the decades of their cultivation in Central Europe, a stepwise reduction of the genetic divergence might be hypothesized. It is supported by the fact that the two accessions from commercial cultivation seem to be closely related to them, as well. 'Azulenka', however, a recently (2013) registered Hungarian cultivar which has been selected from a wild population of central Hungary shows the lowest similarity with the formerly mentioned taxa.

Genetic relationships of the accessions collected from the wild show a connection with their original geographical habitats. This is similar to the findings of Iranian authors in case of some other yarrow species (FARAJPOUR et al., 2011; GHARIBI et al., 2011) although the geographic distances of the studied populations were much larger than in our study. In our case the most distant populations (200-600 km from Central Hungary) proved to have the smallest similarity with each other and with the central ones. The populations within a 50-60 km range of distance show much higher similarity. This finding may be in connection with the fact that *A. collina* is a common weed species in these area having the potential to be transported and distributed easily around resulting in a decreased genetic diversity inside the mentioned central region. As since the start of our work, SSR markers are also available, a continuation of this work is forseen inclusing further *A. collina* accessions from the region.

Conflict of interest

The authors declare that they have no conflict of interest.

References

AKYALCIN, H., ARABACI, T., YILDIZ, B., 2011: Pollen morphology of six *Achillea* L. sect. *Achillea (Asteraceae)* species in Turkey. Turk. J. Bot. 35, 183-201.

DABROWSKA, J., 1977: Observations of the fruit size of nine taxons from the genus *Achillea* L. representing natural di-, tetra-, hexa- and octaploids. Herba Pol. 28, 55-66.

DANIHELKA, J., ROTREKLOVÁ, O., 2001: Chromosome numbers within the *Achillea millefolium* and *A. distans* groups in the Czech republic and Slovakia. Folia Geobot. 36, 163-191.

EBRAHIMI, M., FARAJPOUR, M., RAHIMMALEK, M., 2012: Inter- and intra-specific genetic diversity of Iranian yarrow species *Achillea santolina* and *A. tenuifolia* based on ISSR and RAPD markers. Genet. Mol. Res. 11, 2855-2861.

FARAJPOUR, M., EBRAHIMI, M., AMIRI, R., NOORI, S.A.S., SANJARI, S., GOLZARI, R., 2011: Study of genetic variation in yarrow using inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers. Afr. J. Biotechnol. 10, 11137-11141.

Final community herbal monograph on *Achillea millefolium* L., herba, EMA/HMPC/290284/2009 http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_Community_herbal_monograph/2011/09/WC500115470.pdf (accessed: 23.02.2015)

GALBRAITH, D.W., HARKINS, K.R., MADDOX, J.M., AYRES, N.M., SHARMA, D.P., FIROOZABADY, E., 1983: Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220, 1049-1051.

GHARIBI, S., RAHIMMALEK, M., MIRLOHI, A., MAJIDI, M.M., TABATABAEI, B.E.S., 2011: Assessment of genetic diversity in *Achillea millefolium* subsp. *millefolium* and *Achillea millefolium* subsp. *elbursensis* using morphological and ISSR markers. J. Med. Plants. Res. 5, 2413-2423.

GUO, Y.P., EHRENDORFER, F., SAMUEL, R., 2004: Phylogeny and systematics of *Achillea* (Ateraceae-Anthelmideae) inferred from nrITS and plastid trnL-F DNA sequences. TAXON 53, 657-672.

GUO, Y.P., SAUKEL, J., MITTELMAYR, R., EHRENFORFER, F., 2005: AFLP analyses demonstrate genetic divergence, hybridization and multiple polyploidization in the evolution of *Achillea* (Asteraceae-Anthemideae). New Phytologist 166, 273-290.

HAMMER, Ř., HARPER, D.A.T., RYAN, N.P.D., 2001: PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 9.

KAFT, T., SÄLL, T., 1999: An evaluation of the use of pooled samples in studies of genetic variation. Heredity 82, 488-494.

KÄSTNER, U., SAUKEL, J., ZITTERL-EGLSEER, K., LANGER, R., REZNICEK, G., JURENITSCH, J., KUBELKA, W., 1992: Ätherisches Öl – ein zusätzliches Merkmal für Charakterisierung der mitteleuropaischen Taxa der *Achillea millefolium*-Gruppe. Sci. Pharm. 60, 87-99.

LEWONTIN, R., 1972: „The Apportionment of Human Diversity". Evol. Biol. 6, 391-398.

MA, J.X., LI, Y.N., VOGL, C., EHRENDORFER, F., GUO, Y.P., 2010: Allopolyploid speciation and ongoing backcrossing between diploid progenitor and tetraploid progeny lineages in the *Achillea millefolium* species complex: analyses of single-copy nuclear genes and genomic AFLP. BMC Evol. Biol. 10, 100. doi: 10.1186/1471-2148-10-100

MICHLER, B., PREITSCHOPF, A., ERHARD, P., ARNOLD, C., 1992: *Achillea millefolium*: Zusammenhänge zwischen Standortfaktoren, Ploidiegrad, Vorkommen von Proazulenen und Gehalt an Chamazulen im ätherischen Öl. PZ-Wissenschaft 137, 23-29.

NEI, M., 1972: Genetic distance between populations. Am. Nat. 106, 83-292.

NÉMETH, É., 2005: Essential oil composition of species in the genus *Achillea*. J. Essent. Oil. Res. 17, 501-512.

NÉMETH, É., BERNÁTH, J., TARJÁN, G., 2007: Quantitative and qualitative studies of essential oils of Hungarian *Achillea* populations. J. Herbs. Spices. Med. Plants, 13, 57-69.

ORTH, M., JUCHELKA, D., MOSANDL, A., CZYGAN, F., 2000: Enantiomere Monoterpene im ätherischem Öl von *Achillea millefolium s.l.* – eine zusatzliche taxonomische Bestimmungshilfe? Pharmazie 55, 456-459.

OSWIECIMSKA, M., 1962: Variable content of volatile oil and azulenes in

herbs milfoil. Part I. variability independent of habitat. Dissertationes Pharmaceuticae 14, 539-545.

RADULOVIC, N., ZLATKOVIC, Z., PALIC, R., STOJANOVIC, G., 2007: Chemotaxonomic significance of the Balkan *Achillea* volatiles. Nat. Prod. Commun. 2, 453-74.

RAHIMMALEK, M., TABATABAEI, B.E.S., ARZANI, A., ETEMADI, N., 2009: Assessment of genetic diversity among and within *Achillea* species using amplified fragment length polymorphism (AFLP), Biochem. Syst. Ecol. 37, 354-361. doi: 10.1016/j.bse.2009.06.2002

RAUCHENSTEINER, F., NEJATI, S., WERNER, I., GLASL, S., SAUKEL, J., JURENITS, J., KUBELKA, W., 2002: Determination of taxa of the *Achillea millefolium* group and *Achillea crithmifolia* by morphological and phytochemical methods I. Characterisation of Central European taxa. Sci. Pharm. 70, 199-230.

TÉTÉNYI, P., TYIHÁK, E., MÁTHÉ, I., SVÁB, J., 1962: Untersuchungen über die Azulenverbindungen der *Achillea*-Arten. 1. Mikrochemische Untersuchungsmethoden. Die Pharmazie 17, 463-466.

VALANT, K., 1978: Characteristische Flavonoidglykoside und verwandtschaftliche Gliederung der Gattung *Achillea*. Naturwissenschaften 65, 437-438.

WALLNER, E., WEISING, K., ROMPF, R., KAHL, G., KOPP, B., 1996: Oligonucleotide fingerprinting and RAPD analysis of *Achillea* species: Characterisation and long-term monitoring of micropropagated clones. Plant. Cell Rep. 15, 647-652.

YEH, F.C., YANG, R.C., BOYLE, T.B.J., YE, Z.H., MAO, J.X., 1997: POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.

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