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Evaluation of some *Achillea* L. accessions based on morphological, cytological and chemical characteristics

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Summary: Taxonomical evaluation of six taxa of the *Achillea* aggregate was carried out to determine their potential for breeding and cultivation. We used complex morphological, cytological and chemical aspects of characterisation. Three taxa of tetraploid genom and high chamazulene contents (33–40% ess.oil) proved to be *A. collina* Becker. A strain of wild origin had also high -bisabolol content and high oil level, comparable with the selected cultivars. A population was identified as new chemotype of *A. pannonica* Scheele with typical morphological and cytological traits, a wide sesquiterpene spectrum but lacking of 1.8 cineole and -pinene. We found a mixed population which consisted of both *A. collina* and *A. pannonica* plants. A tetraploid, azuleneless taxon could be concluded as *A. pratensis* Saukel and Länger not described before in Hungary. The diverse performance of the populations calls the attention to the significance of controlled plant raw in phytopharmaceutical products.

Key words: taxonomy, chromosome, flow cytometry, karyotype, essential oil, chamazulene

Introduction

Yarrow (*Achillea*) species around the world are utilized for their anti-inflammatory, spasmolytic, digestive, antimicrobial, and recently, antioxidant properties. The whole aerial parts and primarily the inflorescences contain essential oil, guajanolide sesquiterpenes, and fenolics, flavonoids, as main active ingredients. About 140 species of the genus had already been described, among which the taxa of *A. millefolium* aggregate seem to have the highest importance in Europe. The species especially of this group are however, often very hard to distinguish from each other. Intraspecific differentiation, appearance of autopolyploids and spontaneous hydridisation in overlapping distribution areas may increase the genetical, morphological and chemical diversity even today (*Ehrendorfer*, 1998). Morphological characteristics may change according to habitat or weather conditions, show phenocopies (*Dabrowska*, 1977a; *Németh* et al., 1993; *Saukel & Länger*, 1992a). Chromosome numbers has been used for decades as additional marker for description and determination. In this genus, basic chromosome number is X=9. Polyploids are known up to octaploid level (*Tutin*, 1976). The spectrum of other essential oil components may also vary considerably both qualitatively and quantitatively. In chemotaxonomical characterisation mainly chamazulene (mentioned also as azulenes, proazulenes) has been the main marker. However, as the chemosyndroms may include ecological, ontogenetical, organic and artificial effects (*Németh*, 2005), the terpenoid spectrum alone seems to be hardly enough for exact taxonomical identification. In the last period, molecular markers started to be increasingly used in taxonomical investigations. *Guo* et al. (2005) demonstrated the hybrid origin of several European and Asian species and also their relationships to North American yarrows by AFLP analysis showing also a continuous evolution of the genus.

Only some recent investigations use a complex approach for identification of yarrow species. *Rauchensteiner* et al. (2002) proved, that an appropriate diversification may only be possible by using detailed macro- and micro-morphological determination and phytochemical analysis (mono- and sesquiterpenes).

In a former gene reservation project (national project nr. NKFP 036) we collected 47 wild growing taxa and evaluated them in 2002-2005 according to biomass and essential oil accumulation (*Németh* et al., 2007). The best strains had been

choosen for further taxonomical and chemical evaluation, in order to determine their potential for introduction and breeding in Hungary. In this work we intended to use the complex approach of characterisation.

Materials and methods

Plant material. Six accessions of an *Achillea* collection of the Department of Medicinal and Aromatic Plants of the Corvinus University had been investigated. The material included two varieties: 'Proa' (selected at Pharmaplant, Germany in 1973) and 'Alba' (selected at Safarik University, Slovakia in 1992). The seeds were provided by the breeders. Four other *Achillea strains* originated from wild growing populations, the seeds of which were collected by us in 2002 and preserved in gene-bank. Numbering of these latter ones according to their origin is the following: nr.14 (Solymár), nr.27 (Csomortan), nr.39 (Csikszentkirály), nr.41 (Cseretnek).

The plantations were developed by seed sowing in greenhouse and planting the seedlings in open field in April 2005 at the research station of the university in Budapest. The measurements had been carried out in the second year old populations in 2006.

Macroscopic morphological observations. They were carried out at the living plants during flowering and on the herbarium samples which were collected during this period. Determination of morphological traits was made according to *Simon* (1992), *Tutin* (1976), *Saukel and Länger* (1992/b) and *Rauchensteiner* et al. (2002). Voucher specimens had been deposited at the Department of Medicinal and Aromatic Plants, BCU, Budapest.

Cytological studies. For observation and counting of the chromosomes in metaphase, meristematic cells of primary roots 48 hours after germination in Petri-dishes were used in 10–15 replications. Squash preparations were made from tissues (pre-treated with colchicines 0.1% at 20 °C for 2,5 hours). It was followed by fixation in acetic acid (45%) 10 °C for 20 minutes and hydrolysis with 1 mol/l HCl solution at 60 °C for 5 minutes. The preparations were stained by 2% aceto-carmine at room temperature for 24 hours.

For determination of the ploidy level, the microscopic observations were completed by flow cytometrical analysis in Beckton-Dickinson FACScan equipment. After the development of the second leaves, the seedlings were lysed at 0 °C in 500 l NBI isolation fluid (*Gailbraith* et al., 1983). The nuclei had been separated from the lysed cells by a sieve of 30 m and for their staining propidium-iodide fluorochrome (25g/ml) was used for 3 minutes. After 1500 measurements characteristic for the DNA content of samples, the results had been evaluated by Beckton Dickinson CellQest program. As diploid control *A. crithmifolia* (2n=18) plants were used from our collection.

The parametric characterisation of the chromosomes of taxon nr. 41 had been carried out according to *Sahin* et al. (2006).

Essential oil analysis. Representative samples for the populations were taken at full flowering and dryed at room temperature. 6x100 g samples were distilled in Clevenger type apparatus according to the PhEu IV. The components of the essential oils had been determined by the following methods:

Gas-chromatograph GC 6890N, detector MS 5975, Agilent Technologies equiped with a capillary column HP-5MS, (30m x 250 µm, film thickness 0.25 µm). The injector and detector temperatures were 250°C. Carrier gas was Helium, at a speed of 0.5 ml/min. constant. The column temperature program was: 50 °C (0.5min); 4°C/min. 150 °C (5 min) 12 °C/min. 220 °C (10 min.). The identification of the oil components was performed by MS spectra library (NIST), by own compound library and by retention times.

Results

Morphology. During the vegetation period, populations no.27 and no.39 developed the first flowers at the end of June, which were followed by variety 'Proa', strain no. 41, variety 'Alba' and no.14. The last one flowered about 3 weeks later than the first material.

The investigated accessions performed also different morphological characteristics and homogeneity (*Table 1*). The largest bushes were observed in variety 'Alba' and largest leaves in strain nr.39. Flowers were uniformly white in all of the accessions. The thousand seed mass varied 0.096–0.160 g, which includes a twice fold difference.

Table 1. Morphological parameters of the investigated *Achillea* populations (Budapest, 2006)

	Height	Bush width	Length	Width of leaves	Thousand seed mass (g)
'Alba'	77	66	6.2	1.0	0.103
'Proa'	69	52	5.6	0.9	0.136
No.14	72	48	5.3	1.0	0.096
No.27	54	50	6.1	1.0	0.160
No.39	71	50	8.0	1.3	0.142
No.41	57	47	5.7	1.2	0.096

The populations exhibited appropriate homogenous appearance in case of no.27, variety 'Proa' and no.14. However, the stands were heterogeneous with considerable individual differences especially in case of no.39, no.41 and also in variety 'Alba'.

Chromosomes. Our method for determination, counting and evaluation of chromosomes in root tip meristematic cells proved to be superior to several other ones in former literature (*Hofmann* et al., 1992; *Danihelka & Rotreklová*, 2001/a). We established, that four accessions (varieties 'Alba' and 'Proa' and strains no.14 and no.41) had the chromosome number 2n=36, which shows a tetraploid genom (*Figure 1*). In population no.27 the investigated cells contained 36 pairs of chromosomes (2n=72), (*Figure 2*). The strain no.39 proved to be a mixed material with the majority of cells containing 2n=72 chromosomes but in some samples cells with 2n=36 chromosomes were also found (*Figures 3* and *4*).

The measurements had been ascertained by flow cytometric analysis. The histograms of the fluorescent

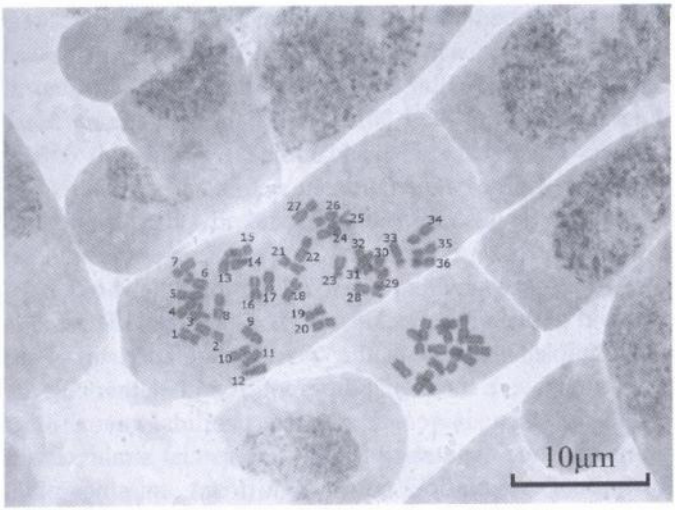

Figure 1. Chromosomes of tetraploid taxon no. 41

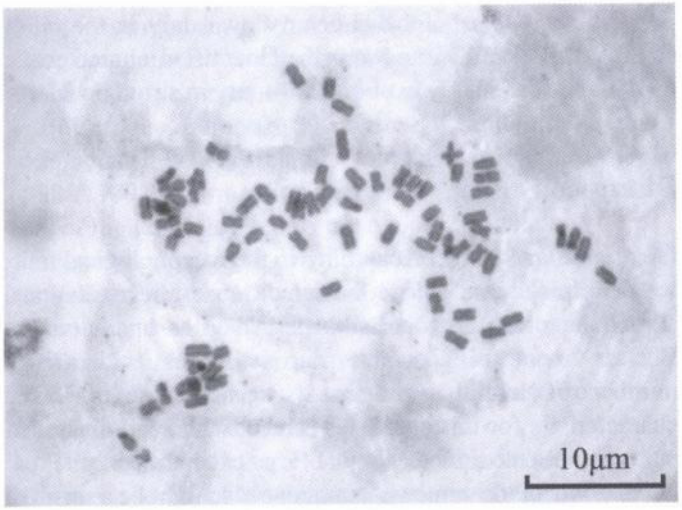

Figure 2. Chromosomes of octoploid taxon no. 27

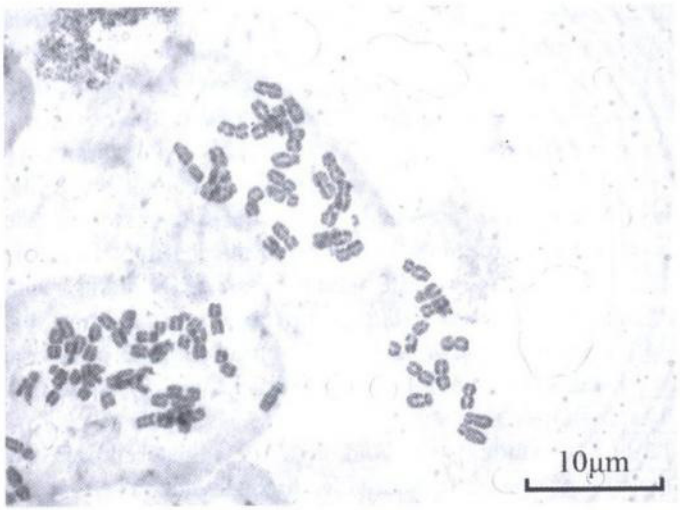

Figure 3. Chromosomes of tetraploid individual in taxon no. 39

signals clearly show the different amounts of DNA in meiotic cells of the samples (*Figures 5* and *6*).

The karyotype of taxon no. 41 is presented in *Figure 7*. It was established, that the nuclei of these cells contained eleven pairs of metacentric and seven pairs of submetacentric chromosomes. It includes at least a single pair of

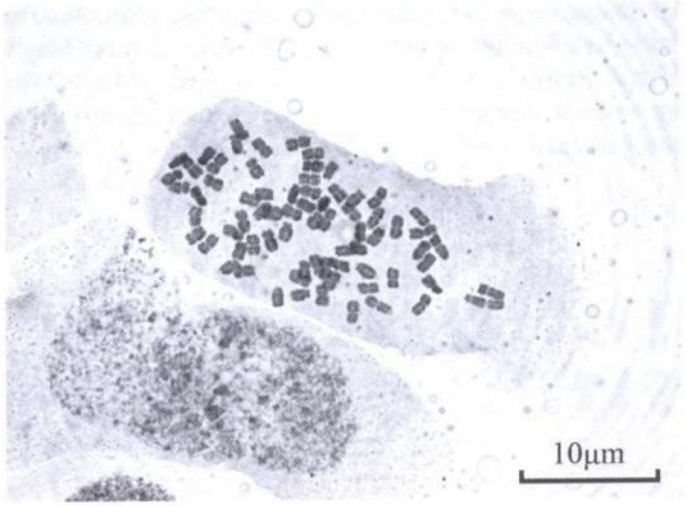

Figure 4. Chromosomes of octoploid individual in taxon no. 39

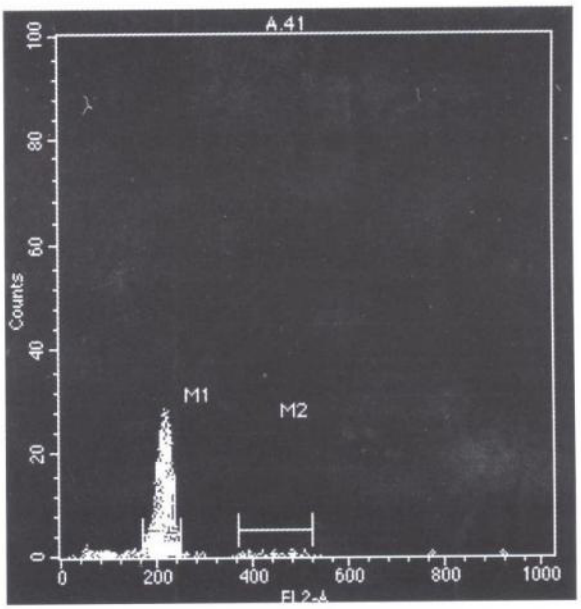

Figure 5. Flow cytometrical histogram of tetraploid taxon no. 41

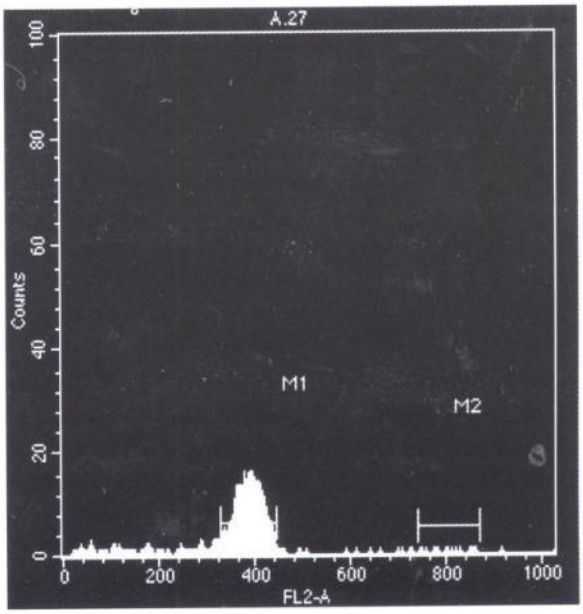

Figure 6. Flow cytometrical histogram of octoploid taxon no. 27

chromosomes with satellite. It has the ploidy level: 4x. The karyotype formula is 11m+ 7 sm. The chromosome length range (μm): 1,38–2,40 and TKL (μm): 68,88. The chromosome length ranges and arm ratios are lower, than in the species mentioned by *Sahin* et al. (2006).

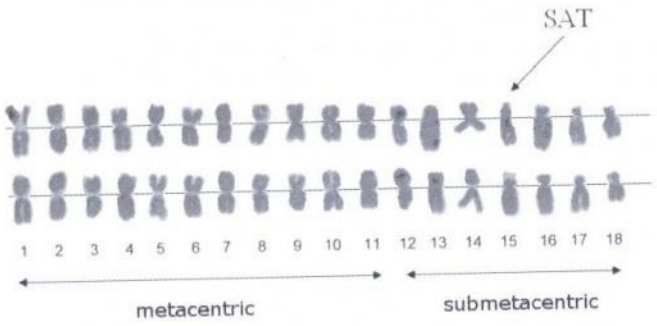

Figure 7. Karyotype of tetraploid taxon no. 41

Essential oil. The essential oil content of the flowering shoots proved to be the highest in 'Proa' and only this one reached the limit of the Pharmacopoea Europaea (PhEu), (0.2%). Population no.27 provided an oil level only under 0.1%, while in the other samples it lay between 0,1–0,2%.

Monoterpenes were hardly detected in the samples (*Table* 2). 1,8-cineole, camphor, borneol were present in some percentages in samples of 'Proa', no.39 and no.41. These compounds seem to be the most characteristic monoterpenes in the genus *Achillea* (*Németh*, 2005). Additionally, -thujone was detected in about 3% in samples of population no. 41.

Table 2. Essential oil content and main compounds (above 1% of the oil) of the investigated *Achillea* accessions (mean values)

	'Alba'	'Proa'	No.14	No.27	No.39	No.41
Essential oil content (%dw.)	0.130	0.220	0.168	0.068	0.109	0.114
Standard deviation	0.029	0.138	0.042	0.030	0.153	0.024
Main compounds (%ess.oil): 1,8 cineole	–	1.1	–	–	8.5	2.3
camphor	–	4.5	–	0.7	1.7	3.2
borneol	–	6.3	–	–	1.2	–
β-thujone	–	–	–	–	–	3.1
α-terpineol	0.5	1.1	3.2	2.3	1.2	–
β-caryophyllene	15.7	12.3	10.2	6.1	9.1	2.8
α-humulene	2.8	1.8	2.3	–	2.0	–
germacrene D	3.6	4.3	2.6	4.2	3.8	0.9
spathulenol	–	–	–	2.8	3.2	1.2
β-caryophyllene-oxide	4.8	3.7	3.1	8.8	6.7	6.1
γ-gurjunene	–	1.5	–	3.3	1.0	–
γ-muurolene	–	–	–	2.3	3.1	18.6
α-cadinol	1.2	1.1	–	3.0	4.8	–
longifolene	–	–	–	4.3	–	–
α-bisabolol	–	–	18.6	1.5	–	–
chamazulene	39.9	39.0	33.1	–	12.3	–
unknown Rf 31,77	–	–	–	20.5	–	–

The main compounds of the essential oil however, were sesquiterpenes in each sample (*Table 2*). Varieties 'Alba' and 'Proa' provided blue oil with high proportions of chamazulene (33–40% of ess. oil). The chamazulene contents of strains no.14 and no.39. reached only lightly lower levels, but no chamazulene or only traces were detected in the oils of strain no.27 and no.41 respectively.

Further characteristic sesquiterpene compounds in the oils were -caryophyllene (3-16%) caryophyllene-oxide (3-9%) and germacrene D (3-4%). They had been detected in each sample, however in the oils of population no.41 the proportions were somewhat different from the other ones.

Beside this universal sesquiterpenoids, some other compounds are characteristic only for special strains. In the flowers of population no.41 significant amounts of ã-muurolene (14-28%) were found, which, at the same time was missing or present only in very low proportions in other populations. – bisabolol had been shown only in the oil of strain no.14 (9-23%) and a still unidentified characteristic compound (Rf: 31.77) in about 20% only in strain no.27.

Discussion

***A. collina* Becker.** According to the morphological traits we determined that beside the selected varieties 'Alba' and 'Proa' the strain no.14 can be described as an *A. collina* Becker taxon. Its lanceolate, narrow leaves, with a high number of leaflets, and many branches seem to be characteristic for this species. The thousand seed masses of these taxa were about 0.1 g, except variety 'Proa' where the somewhat larger seeds might be a result of indirect selection during breeding.

The data on chromosome numbers of the taxa were in good coincidence with the morphological evaluation: both the selected cultivars, and taxon no.14 exhibited a tetraploid genom which is known for *A. collina* Becker (*Danihelka & Rotreklová*, 2001a).

The outranging oil content of the variety 'Proa' may show the result of the breeding. However, the other variety 'Alba' reached only hardly more than the half of it, while good results were found in one of the strains of wild origin (no.14). In addition, the standard deviation for this trait showed a relative inhomogeneity in 'Proa', while no.14 proved to be a more stable one.

Among tetraploid taxa the registered varieties and taxon no.14 provided high chamazulene content, which exceeds the PhEu requirement almost double fold. These seem to be in agreement with the morphological and cytological results, ascertaining the identification of the mentioned taxa as *A. collina* Becker. According to several references, this tetraploid species contains considerable proportions of proazulenes in its blue essential oil (*Németh*, 2005).

β-caryophyllene, caryophyllene-oxide, germacrene D and spathulenol had been found as major compounds in the oils of other Hungarian *A. collina* populations also in our former investigations (*Holm* et al., 2004). The population no.14 of wild origin seems to be a distinct chemotype based on its high -bisabolol content. The two selected varieties show only slight differences in composition.

***A. pannonica* Scheele.** Taxon no.27 with its large rosette leaves and flowers, greyish-green, pubescent stem proved to be most likely an *A. pannonica* Scheele population. It yielded the largest seeds, which is in coincidence with the findings of *Dabrowska* (1977/b).

The low content of essential oil and the lack of chamazulene seems to be in agreement with former results (*Héthelyi* et al., 1989; *Németh*, 2005). However, only traces of 1,8-cineole and -pinene were found in the oil which is in contradiction with several previous reports (*Hofmann* et al., 1992; *Kastner* et al., 1992; *Németh* et al., unpublished) where these compounds are characteristic ones for the oil of this species. At the same time, this taxon showed the richest sesquiterpene composition with an unidentified major compound. It is in correlation with the statement of *Kubelka* et al. (1999) who mention that the sesquiterpene pattern of taxa of higher ploidy seems to be more variable.

Population no.39. proved to be a mixed one which consists of different types of individuals. Beside erect, vigorous and hairy plants, abundantly branching, prostrate, ones with light- green, somewhat rigid leaves were also present. It was the most heterogenous one also concerning the seed mass and essential oil content.

The majority of the oil samples of this taxon were blue. However, some of them contained no chamazulene, the individual values ranged between 0% and 32%. The spectrum of the majority of the other sesquiterpenes showed similarity mainly with the oils of taxon no. 27. It could be concluded, that this population might be a mixed *A. collina* Becker and *A. pannonica* Scheele stand.

***A. pratensis* Saukel and Länger.** Taxon nr.41 possess smaller bush, broader, light greenish leaves with low number of leaflets wich are nearly planar and not similar to any of the formerly mentioned taxa. The anatomical data of the flowers (ligule width, corolla tube length) were not uniform inside the population.

According to the tetraploid chromosome numbers and the thousand seed mass it was identical with taxa of *A. collina*. The karyotype of this strain seemed to be also similar to the results of *Maffei* et al. (1993) in a taxon identified as *A. collina*.

However, the essential oil without chamazulene does not coincides with this establishment. Other qualitative (i.e. presence of -thujone, lack of germacrene D, humulene) and quantitative (i.e. low level of -caryophyllene, high proportions of ã-muurolene) differences in the oil composition were also obvious and supported the idea not identifying the taxon as *A. collina* Becker.

Saukel & Länger (1992/b), *Kastner* et al. (1992) and recently *Danihelka & Rotreklová* (2001/b) described the presence of a tetraploid, azulenless taxon, *A. pratensis* Saukel and Länger in Central European habitats, which might often have been mistaken to *A. collina* Becker. According to *Kubelka* et al (1999) *A. pratensis* Saukel and Länger is characterized by eudesmanolides and therefore separates clearly from proazulene containing species.

In our experiment, based of the complex morphological, cytological and chemical evaluation it was established, that the taxon no.41 of West-Hungarian wild origin might be *A. pratensis* Saukel and Länger. The collection site of this population was close to areas were already *A. pratensis* Saukel and Länger had been found (*Vetter*, 1995). However, this species had not yet been described in the territory of Hungary.

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