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Interspecific in vitro hybridization in genus *Centaurium* and identification of hybrids via flow cytometry, RAPD, and secondary metabolite profiles

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Abstract: Most of the species from the genus *Centaurium* Hill readily hybridize between themselves in natural habitats. Artificial interspecific hybrid plants were obtained under in vitro conditions using reciprocal crosses between 4 species: *Centaurium erythraea*, *C. littorale*, *C. maritimum*, and *C. pulchellum*. Among 76 crossing experiments performed, 3 types of crosses produced seeds and gave 20 interspecific hybrid plants. Nuclear DNA quantification, fingerprinting of molecular markers, and determination of variation in main secondary metabolite content were employed to confirm their hybrid nature. All 3 approaches were capable of detecting hybrid plants. RAPD molecular markers proved to be the most reliable, always placing the hybrids between their parents in dendrograms, while secoiridoid glycoside content also showed intermediate profiles in the hybrid plants in relation to the parents. Hence, each of the techniques employed can be recommended for elucidating the mechanisms of interspecific hybridization in natural populations of *Centaurium* species in further studies. This could help to comprehend their breeding system and recognize interspecific hybridization as one of the crucial steps in polyploid formation and speciation in this genus.

Key words: *Centaurium*, interspecific hybridization, molecular markers, ploidy level, secoiridoid glycosides

1. Introduction

Interspecific hybridization is a process with a fundamental and critical role in the evolution and ecological adaptation of plant species, and it has become increasingly clear that hybridization among them sometimes results in the formation of entirely new species (Rieseberg and Carney, 1998; Rieseberg et al., 2003; Chapman and Burke, 2007; Soltis and Soltis, 2009). It greatly assists interspecific gene flow and can create new gene combinations that are not available within the limits of a species (Sikka and Joshi, 1960). Recent estimates indicate that at least 25% of plant species, evolutionarily the youngest ones, participate in hybridization and introgression with other species (Mallet, 2005).

The genus *Centaurium* Hill (Gentianaceae) comprises approximately 20 species, mainly annual or biennial, which primarily inhabit wet and disturbed terrains of the Mediterranean region (Mansion, 2004). Interspecific hybridization among natural populations was previously described in the genus *Centaurium* (Melderis, 1931; Zeltner, 1970, 1978; Ubsdell, 1976a, 1976b, 1979; Mansion and Struwe, 2004; Mansion et al., 2005; Guggisberg et al., 2006). It is one of the major causes of the reticulate pattern of morphological variation, which results in insufficiently clear taxonomical and phylogenetic relationships in the genus. Morphological variation among hybrid plants includes a whole range of intermediate forms, which are further affected by diverse environmental factors. Thus, the following interspecific hybrids were recorded: *Centaurium* × *tenuiflorum* (*C. erythraea* × *C. tenuiflorum*), *C. bianoris* (*C. maritimum* × *C. tenuiflorum*), *C. intermedium* (*C. erythraea* × *C. littorale*), *C. malzacianum* (*C. maritimum* × *C. pulchellum*), and *C. centaurioides* (*C. tenuiflorum* × *C. pulchellum*) (Ubsdell, 1979; Mansion et al., 2005; Guggisberg et al., 2006). Therefore, the genus *Centaurium* represents an ideal model for investigating interspecific hybridization as a powerful step in polyploid formation and speciation (Guggisberg et al., 2006). The studied species, *C. erythraea* Rafn, *C. littorale* (Turner) Gilmour, and *C. pulchellum* (Sw.) Druce, are characterized by showy pink flowers that are hermaphrodite and insect-pollinated, but also have a high capacity for autonomous selfing (Ubsdell, 1979; Brys and Jacquemyn, 2011), while *C. maritimum* (L.) Fritsch has yellow, self-compatible flowers (Mišić et al., 2009; personal observation).

In the past, verification of natural hybridization among some *Centaurium* species was done only by means of morphological and cytological data (Ubsdell,

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1976a, 1976b), which could identify the existence of interspecific hybrids but were not sufficient for definite confirmation. In this field, DNA markers are considered the most reliable tool (Mallet, 2005). Among the variety of molecular marker techniques available, RAPD (Welsh and McClelland, 1990; Williams et al., 1990) has been extensively used for DNA fingerprinting of genotypes because of its technical simplicity, the low cost and no requirement for sequence information, a demand for small quantities of DNA, and because it can produce abundant polymorphic markers (Wolfe and Liston, 1998). Despite criticism, RAPD analyses are now extensively used for estimating genetic relationships among closely related populations or species and providing molecular evidence concerning the hybrid origin of plants (Smith et al., 1996; Baumel et al., 2003; González-Rodríguez et al., 2004; Mehetre et al., 2004a, 2004b; Divakaran et al., 2006; Singh et al., 2007; Ali et al., 2008; Srivastava et al., 2010; Matoba et al., 2011; Mukherjee et al., 2013).

Chemical markers are another useful tool for the identification and confirmation of the hybrid nature of plants since qualitative and quantitative differences in the secondary chemistry of progeny can arise due to hybridization (Orians, 2000). *Centaurium* species are a rich source of secoiridoid glycosides (SGs), among which the most abundant are gentiopicrin (GP), sweroside (SW), and swertiamarin (SM) (van der Sluis, 1985; Jensen and Schripsema, 2002; Mišić et al., 2009; Šiler et al., 2010, 2012). Since the qualitative content and mutual quantitative ratios of these compounds are species-specific, they have been widely used as markers in systematic classification for taxonomical purposes (Hostettmann-Kaldas et al., 1981; van der Sluis, 1985; Jensen and Schripsema, 2002).

The main objectives of this study were to examine interspecific crossability of *Centaurium* species under precisely controlled in vitro conditions; to verify the hybrid nature of progeny plants that originated from these crosses by means of cytogenetic, phytochemical, and molecular methods; and to identify markers for rapid screening and detection of interspecific hybrids. A better understanding of hybridization between species of the genus *Centaurium* should lead to determination of the phenetic relationships within this genus, appreciation of their breeding system, and recognition of hybridization as a valuable step in polyploid formation and speciation.

2. Materials and methods

2.1. Plant material

Seeds of 4 *Centaurium* species, namely *Centaurium maritimum* (L.) Fritsch, *C. erythraea* Rafn, *C. pulchellum* (Sw.) Druce, and *C. littorale* (Turner) Gilmour, were used for in vitro culture establishment. Yellow centaury (*C. maritimum*) seeds were collected in July 2002 in the area of Podgorica (Montenegro), and seeds of common centaury (*C. erythraea*) were collected in August 2001 in the area of Lake Vlasina (Serbia). Seeds of lesser centaury (*C. pulchellum*) were collected in July 1999 near Rakovac (Vojvodina, Serbia), while seeds of seaside centaury (*C. littorale*) were obtained from a botanical garden in Nancy, France (Conservatoire et Jardins Botaniques de Nancy) and were gathered in 1999. All the seeds were stored at –20 °C in the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" Belgrade, Serbia, until use.

2.2. In vitro experimental design studies

2.2.1. In vitro culture establishment

Seeds were surface-sterilized in 20% commercial bleach with 2 drops of liquid detergent for 10 min and then rinsed 5 times with sterile distilled water. They were aseptically transferred to solid half-strength MS medium (Murashige and Skoog, 1962) supplemented with 100 mg L^{-1} *myo*-inositol, 20 g L^{-1} sucrose, and 7 g L^{-1} agar. The pH of the medium was adjusted to 5.8 before sterilizing at 114 °C for 25 min. All cultures were grown under long-day conditions (16 h light/8 h dark cycle) at 25 ± 2 °C and relative air humidity of 60%–70%. White fluorescent tubes provided a photon flux rate of 32.5 μmol m^{-2} s^{-1} at the level of the plant cultures. The cultures were maintained by regular subculturing at 4-week intervals on the same medium, until flowering.

2.2.2. In vitro flowering and pollination

Plants of *Centaurium maritimum* and *C. pulchellum* flowered spontaneously. Plants of *C. erythraea* were stimulated to flower by mechanical stress (cutting off the center of the basal rosette). Plants of *C. littorale* were treated with 0.01 mM GA_3 (gibberellic acid, Serva, Heidelberg, Germany), as proposed by Cvetić et al. (2004), to provoke flower stalk elongation, by applying the solution in the center of the rosettes 3 times at 2-day intervals.

Emasculation prior to flowering (a few days before opening of the flowers) was conducted under aseptic laboratory conditions to prevent self-pollination. To confirm the success of the procedure, 38 emasculated flowers were not pollinated and were used as the control group. Eight combinations of interspecies cross-pollination were performed (Table 1). In each attempt, a single emasculated flower was pollinated with one fully matured anther from a male parent plant. After maturation, each developed capsule was collected and examined for seed production. Spherical and fully filled seeds with no morphological abnormalities were transferred to fresh half-strength MS medium. Seedlings were grown under in vitro conditions until enough plant material was obtained for further analysis.

2.3. Flow cytometry analysis

The relative nuclear DNA (nDNA) amount of each plant (of both parental and putative hybrid plants) was estimated

Table 1. Number of capsules with seeds and plants obtained from 8 interspecific cross experiments performed in genus *Centaurium* under in vitro conditions. Successful crosses are presented in bold.

Crossing experiments		Attempts made	No. of capsules with viable seeds	No. of plants obtained
Female parent	Male parent			
C. maritimum	*C. erythraea*	20	**1**	**1**
C. erythraea	*C. maritimum*	6	0	0
C. maritimum	*C. pulchellum*	10	0	0
C. maritimum	*C. littorale*	6	0	0
C. littorale	*C. erythraea*	6	**5**	**11**
C. erythraea	*C. littorale*	3	0	0
C. pulchellum	*C. erythraea*	10	0	0
C. littorale	*C. pulchellum*	15	**2**	**8**

by flow cytometry analysis. Approximately 1 cm^2 of fully developed leaves were finely chopped with a razor blade in a petri dish containing 700 µL of ice-cold DNA buffer solution, as proposed by Arumuganathan and Earle (1991). A piece of *Lactuca sativa* L. leaf was added to each sample as an internal standard. The volume of the solutions was adjusted to 2 mL using the same buffer, and subsequently, fluorescent dye (DAPI, 2 µg mL^{-1}) was added. Each sample solution was filtered using a 50 µm mesh nylon filter to remove residual tissue. After 15–30 min of incubation at room temperature the relative fluorescence of stained nuclei was measured with a CyFlow ML flow cytometer (Partec GmbH, Münster, Germany), which is presented by a histogram of fluorescence values of 2000 nuclei per sample. Software Flomax (version 2.4 d, Partec GmbH, Münster, Germany) was used for detecting and processing the signals. The relative nDNA amount was estimated by directly comparing the mean position of the nuclear peak of analyzed plants to the internal standard (Arumuganathan and Earle, 1991). The H2–H10 and H13–H19 hybrids, as well as the control plants of each species with previously determined chromosome numbers, were subjected to flow cytometry analysis.

2.4. DNA extraction

Freshly harvested young leaves (0.15–0.2 g) of in vitro grown plants were ground in liquid nitrogen. DNA was extracted using a CTAB-based procedure described earlier by Doyle and Doyle (1990). The DNA purity and concentration of samples were determined by comparing the absorbance ratios at 260 nm to those at 280 nm or 230 nm in a UV visible spectroscopy system (Agilent 8453, Agilent Technologies, Waldbronn, Germany).

2.5. RAPD-PCR reactions

RAPD-PCR amplifications were performed, as optimized earlier for the genus *Centaurium* by Skorić et al. (2012), in 25 µL of reaction volume containing 50 ng of template DNA, 2.5 mM $MgCl_2$, 2 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 200 µM dNTPs (of each dATP, dCTP, dGTP, and dTTP), and 7.5 pmol of primer in 1X $(NH_4)_2SO_4$ reaction buffer supplied with the polymerase. Among 50 random decamer primers tested (Metabion, Martinsried, Germany), 10 (Table 2) were selected for the analyses based on reproducibility and production of distinguishing banding patterns. All amplification reactions were repeated at least twice. Reactions without DNA were used to check possible contamination of PCR reactions. Hybrids H5, H13, and H19 were omitted from the analysis due to contamination of the DNA samples.

PCR amplifications were performed in a peqSTAR 96 Universal Gradient thermocycler (Peqlab, Biotechnologie GmbH, Erlangen, Germany), with the following amplification profile: initial denaturation step at 95 °C for 5 min, followed by 45 cycles consisting of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. The final extension step lasted 10 min at 72 °C, and a hold temperature was set to 4 °C at the end. The amplification products were separated by electrophoresis on 1% (w/v) agarose gels in 1X Tris/borate/EDTA (TBE) buffer at 1.5 V cm^{-1} for 1.5 h. The PCR products were stained with ethidium bromide (0.5 µg mL^{-1}) and visualized by an UV transilluminator (ST4 3026-WL/26M, Vilber Lourmat, Torcy, France). The 100 bp DNA Ladder Plus (Fermentas, Vilnius, Lithuania) was used as fragment size marker. To produce more accurate identification of bands of hybrid plants and their parents, RAPD-PCR products for all samples obtained with the 3 most efficient primers (OPB 18, OPF 14, and OPT 14) were separated and analyzed by Lab-on-a-Chip (DNA 7500 LabChip) using an Agilent Bioanalyzer 2100 system with Agilent 2100 Expert software (Agilent Technologies, Santa Clara, CA, USA).

Table 2. Sequence of selected RAPD decamer primers used for evaluation of the efficiency of 3 types of crossing experiments, total number, and number of polymorphic bands.

Crossing experiment	Primer name	Sequence (5'→3')	Total no. of bands	No. of polymorphic bands	Polymorphism (%)
C. maritimum × *C. erythraea*	OPB 11	GTAGACCCGT	9	8	88.9
	OPB 15	GGAGGGTGTT	7	7	100.0
	OPB 17	AGGGAACGAG	8	8	100.0
	OPB 18	CCACAGCAGT	4	4	100.0
	OPF 05	CCGAATTCCC	8	8	100.0
	OPF 14	TGCTGCAGGT	8	5	62.5
	OPH 14	ACCAGGTTGG	6	6	100.0
	OPO 07	CCGCAGCCAA	11	7	63.6
	OPO 16	TCGGCGGTTC	8	7	87.5
	OPT 14	AATGCCGCAG	8	7	87.5
Total			77	67	
C. littorale × *C. erythraea*	OPB 11	GTAGACCCGT	8	6	75.0
	OPB 15	GGAGGGTGTT	14	9	64.3
	OPB 17	AGGGAACGAG	3	2	66.7
	OPB 18	CCACAGCAGT	12	4	33.3
	OPF 14	TGCTGCAGGT	10	3	30.0
	OPT 14	ACCAGGTTGG	8	6	75.0
Total			55	30	
C. littorale × *C. pulchellum*	OPB 11	GTAGACCCGT	10	9	90.0
	OPB 18	CCACAGCAGT	9	4	44.4
	OPF 14	TGCTGCAGGT	7	4	57.1
	OPT 14	AATGCCGCAG	13	9	69.2
Total			39	26	

Banding pattern analysis and binary matrix construction for RAPD data were performed by TotalLab TL120 1D v. 2009 software (Nonlinear Dynamics Ltd., Newcastle, UK). Presence of bands was scored as "1" and absence of bands as "0". Only consistently reproducible, well-resolved fragments were included. From the resulting 1/0 data matrix, a similarity matrix was calculated using Jaccard's coefficient of similarity.

2.6. Determination of SGs by HPLC

Plants for high pressure liquid chromatography (HPLC) analysis were harvested in the flowering stage, detached into aerial parts and roots, and air-dried at 30 °C. Each sample (300 mg, powdered) was extracted with 10 mL of methanol overnight. All samples were filtered through 0.2 µm cellulose filters (Agilent Technologies, Santa Clara, CA, USA) and stored at 4 °C until use. All extractions were performed in triplicate for each plant species or hybrid. The following hybrid plants were subjected to the analysis: H2, H5-H10, and H13-H18.

Quantification of GP, SW, and SM in aerial parts and roots of parental plants and their hybrid progeny was performed on a HPLC-DAD system, model HP1100 (Hewlett Packard, Santa Clara, CA, USA), as described earlier by Šiler et al. (2012). Concentrations of GP, SW, and SM in both aerial parts and roots are presented as percentages of dry weight (DW).

2.7. Statistical analyses

A dendrogram based on the RAPD binary matrix was constructed by unweighted paired group method using arithmetic averages (UPGMA) clustering analysis with Jaccard's coefficient of dissimilarity. The robustness of clusters was calculated by bootstrap test with 1000 replications. Similarity calculations and UPGMA analysis were performed using PAST software (PAlaeontological STatistics, version 1.89) (Hammer et al., 2009).

Statistical analyses for SG concentration data were performed using Statgraphics Centurion XV software (version 15.1.02 StatPoint, Inc., 1982–2006, USA). The

data were subjected to analysis of variance (ANOVA), and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at a confidence level of $P \leq 0.05$.

Conjoint cluster analysis based on GP, SW, and SM content in aerial parts of all 4 *Centaurium* species and all hybrid plants was employed to create a dendrogram describing dissimilarities between studied individuals. Ward's method, using Euclidean distance as a dissimilarity measure, was performed using PAST software.

3. Results

3.1. In vitro flowering and cross experiments

Plants of *Centaurium maritimum* and *C. pulchellum*, used as parental species in the present study, spontaneously flowered 6–9 weeks after seed germination. Parental plants of *C. erythraea* and *C. littorale* needed additional treatments to obtain the whole ontogenetic cycle. Flowering of both species was recorded 8–10 weeks after the treatments. The period of anthesis was 2–3 days for *C. pulchellum* and 4–5 days for *C. littorale*, *C. erythraea*, and *C. maritimum*.

In sum, 76 hybridization attempts were made (Table 1). The crossing experiment between *Centaurium maritimum* as a female parent (Figure 1a) and *C. erythraea* as a male parent (Figure 1b), after seed formation (Figure 1c), gave only one hybrid plant, H2 (Figure 1d). Two types of crosses, *C. littorale* × *C. erythraea* and *C. littorale* × *C. pulchellum* (with *C. littorale* as female parent in both assays), produced 11 (H13–H23) and 8 (H3–H10) hybrid plants, respectively. Reciprocal crosses *C. erythraea* (female parent) × *C. maritimum* (male parent) and *C. erythraea* (female parent) × *C. littorale* (male parent) did not result in seed formation (Table 1). Control flowers, which were emasculated but not pollinated, did not produce any seeds either.

3.2. Relative ploidy level of hybrids

The relative nDNA amount per cell of each analyzed plant was compared with control plants with known chromosome numbers and ploidy levels. Parental individuals of *Centaurium erythraea*, *C. littorale*, and *C. pulchellum* used in our experiment were tetraploids, whereas the specimen of *C. maritimum* was diploid (Table 3).

The relative nDNA amount per cell of all hybrid plants expressed values approximately intermediate to those observed in parental species (Table 3). The assumed ploidy level for hybrid plants derived from crosses *Centaurium maritimum* × *C. erythraea* and *C. littorale* × *C. pulchellum* was triploid, while *C. littorale* × *C. erythraea* gave hybrid plants, which appear to be tetraploids.

3.3. Molecular marker data

The primers used for the evaluation of hybridization of each cross type, total number of bands gained, and percent of polymorphic bands are presented in Table 2. Results of RAPD-PCR banding patterns confirmed hybridization on the basis of presence/absence of species-specific bands belonging to parental individuals. In cross *C. maritimum* × *C. erythraea*, 30% of bands were shared between the male parent and the offspring plant H2, thus confirming its hybrid nature (Figure 2). For other crosses (*C. littorale* × *C. erythraea* and *C. littorale* × *C. pulchellum*) the percentages of markers shared between the male parent and offspring were 13.33%–20% and 10.8%–13.5%, respectively. We also detected one band obtained with OPH 14 primer in the H2 plant and one band obtained with OPF 14 primer in hybrid plants H3, H4, H7, and H8 (from cross *C. littorale* × *C. pulchellum*), which were present only in hybrid offspring but not in parental plants.

All 3 dendrograms (Figure 3) clearly placed hybrid plants between their parents. In 2 crossing experiments (*C. littorale* × *C. erythraea* and *C. littorale* × *C. pulchellum*) all obtained hybrid plants are clustered closer to the female parent (in both cases, *C. littorale*). The third type of cross (*C. maritimum* × *C. erythraea*) resulted in only one hybrid plant, which is closer to the pollen-donor parent in the dendrogram.

Electropherograms obtained by Agilent 2100 Bioanalyzer using selected primers exhibited higher resolution and band identification (Figure 4). In this manner, determination of characteristic bands shared between parental and progeny hybrid plants proved more accurate.

3.4. Secoiridoid glycosides profiles

Species involved in hybridization experiments showed variations in quantitative content of SGs in aerial parts (Figure 5a), while GP was generally the dominant SG in roots of all investigated species (Figure 5b). GP and SM were the most abundant compounds in the aerial parts of *Centaurium maritimum,* while *C. pulchellum* and *C. erythraea* accumulated SM as the major SG. *C. littorale* primarily contained GP in aerial parts. Hybrid plants generally displayed SG concentration patterns in aerial parts intermediate to those of the parental specimens in all types of crosses. In contrast, SG concentration profiles of roots were more uniform than those of aerial parts where the quantitative content of SM, GP, and SW varied among hybrid specimens of the same type of cross (Figures 5a and 5b).

Cluster analysis of SG profiles gave 2 clearly segregate clusters (Figure 6). The first cluster (A) is composed of accessions *Centaurium littorale* × *C. erythraea* cross and hybrid progeny derived from this cross. Hybrids are placed in between their parents, albeit closer to the female parent (*C. littorale*). The second cluster (B) is much more heterogeneous; the B1 subcluster is composed of all hybrid progenies derived from cross *C. littorale* × *C. pulchellum,* which are much closer to the male parent. The B2 subcluster indicates the genetical proximity of 2 parental

Figure 1. In vitro flowering. a- *Centaurium maritimum* (yellow flower, ovule parent), b- *C. erythraea* (pink flower, pollen parent), c- after pollination and formation of seeds, d- they bring out H2 hybrid plant with intermediate orange colored flowers.

species, *C. maritimum* and *C. pulchellum*, by placing the hybrid plant a bit further from their parents.

4. Discussion

4.1. In vitro flowering and visual verification of interspecies hybridization

There are only a few published papers about in vitro interspecific pollination (Van Tuyl et al., 1991; Janson, 1993; Chi, 2000). This work is the first successful attempt to produce *Centaurium* interspecies hybrid plants via sexual reproduction, and it was done under in vitro conditions. Conducting hybridization experiments under in vitro conditions has a few advantages: growth under strictly regulated aseptic environmental conditions, year-round availability of plant material, and an exceptionally high rate of propagation and growth, which was particularly useful in propagation of sterile hybrids such as H2, in this study. Additionally, micropropagation ensures sufficient

Table 3. Relative nuclear DNA amount per cell and ploidy levels of investigated parental and interspecific hybrid plants.

Accession	Relative nDNA amount per cell	Ploidy level
C. maritimum (female parent)	0.26	2X
C. erythraea (male parent)	0.38	4X
Hybrid H2	0.32	3X
C. littorale (female parent)	0.40	4X
C. erythraea (male parent)	0.39	4X
Hybrid H13	0.39	4X
Hybrid H14	0.40	4X
Hybrid H15	0.39	4X
Hybrid H16	0.39	4X
Hybrid H17	0.41	4X
Hybrid H18	0.39	4X
Hybrid H19	0.40	4X
C. littorale (female parent)	0.39	4X
C. pulchellum (male parent)	0.44	4X
Hybrid H3	0.30	3X
Hybrid H4	0.30	3X
Hybrid H5	0.28	3X
Hybrid H6	0.30	3X
Hybrid H7	0.29	3X
Hybrid H8	0.30	3X
Hybrid H9	0.30	3X
Hybrid H10	0.30	3X

plant material for chemical and molecular analyses. In vitro plant material is free of foreign DNA which is of great importance, especially for RAPD analysis, in which small unspecific primers are used.

Various species from the genus *Centaurium* are capable of in vitro flowering and seed production (Cvetić et al., 2004; Todorović et al., 2006; Mišić et al., 2009; Todorović et al., 2009). The species used in this study were able to complete the full ontogenetic cycle under in vitro conditions. Flowers that were emasculated just before opening did not produce any seeds, which confirmed the results reported by Brys and Jacquemyn (2011) that early emasculation successfully disabled self-pollination. The visible indicator of the hybrid origin of H2 offspring from cross *C. maritimum* (yellow flowers) × *C. erythraea* (pink flowers) was the intermediate orange flower color (Figure 1d). Unfortunately, visual hybrid verification could not be applied in other experiments where crosses between pink-flower species were performed.

4.2. Hybrid verification by nDNA estimation

The relative nDNA content of the individuals with known chromosome numbers and ploidy levels, and comparison with the literature, can give information on genome size and genome constitution of the parental and hybrid plants (Ubsdell, 1976a; Morgan-Richards et al., 2004; Mansion et al., 2005). The results of assumed ploidy level for all 4 *Centaurium* species included in hybridization experiments correspond with results published earlier (Zeltner, 1978; Mansion et al., 2005; Guggisberg et al., 2006). The interspecific hybrid plants would be expected to express an average nuclear DNA content of parental species after flow cytometry analysis (Meiners et al., 2011). In the case of the *C. maritimum* (2x) × *C. erythraea* (4x) cross, the H2 hybrid appears to be triploid, and its genome size is equivalent to 1 *C. maritimum* and 2 *C. erythraea* chromosome sets, suggesting that fertilization of 2 normally reduced gametes occurred (Table 3). Unfortunately, this technique did not allow us to distinguish between 2 parental species in the

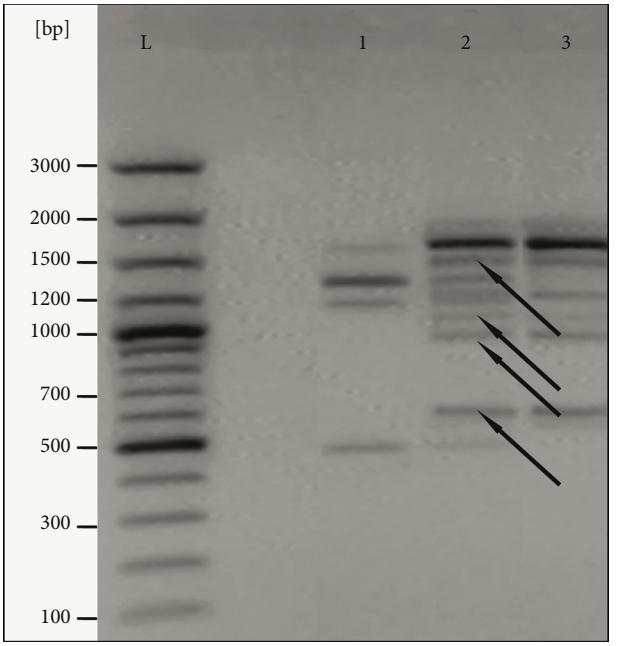

Figure 2. RAPD-PCR banding profiles of *Centaurium maritimum* (female parent, lane 1), hybrid H2 (lane 2), and *C. erythraea* (male parent, lane 3) generated with OPF 14 primer, separated in 1% (w/v) agarose gel. L- 100 bp DNA ladder. Arrows indicate the male-parent–derived bands, which confirm the interspecific hybridization.

case of the *C. erythraea* (4x) × *C. littorale* (4x) cross, since they have a similar relative nDNA amount per cell. Hybrids from this cross are certainly tetraploid, just as the parent plants, and had a mean relative nDNA amount equivalent to the sum of 2 *C. littorale* and 2 *C. erythraea* chromosome sets. This indicates that the fertilization of 2 normally reduced gametes occurred. Hybrid plants obtained from this cross flowered and produced some amount of seed. The confirmation of the hybrid nature of these plants was performed using molecular and chemical markers.

4.3. Molecular data undoubtedly confirm interspecific hybridization

The hybrid origin of all progeny plants was confirmed by the RAPD banding profiles. The presence of RAPD bands specific to the male parent in the hybrid RAPD profile (Figures 2 and 3) undoubtedly confirms the hybrid origin of progeny (Yin et al., 2001; Mehetre et al., 2004a, 2004b; Liu et al., 2007; Ali et al., 2008). Bootstrap values at nodes between the hybrids arising from the same type of crossing rarely exceed 75 (Figure 3), suggesting that there is no significant genetic difference between them. Nevertheless, since RAPD markers are biparentally inherited (Williams et al., 1990), one would expect a complete additive banding pattern in all F1 hybrids from the same cross. However, there is an evident deviation from complete additivity in RAPD fingerprints among different F1 hybrid plants (from

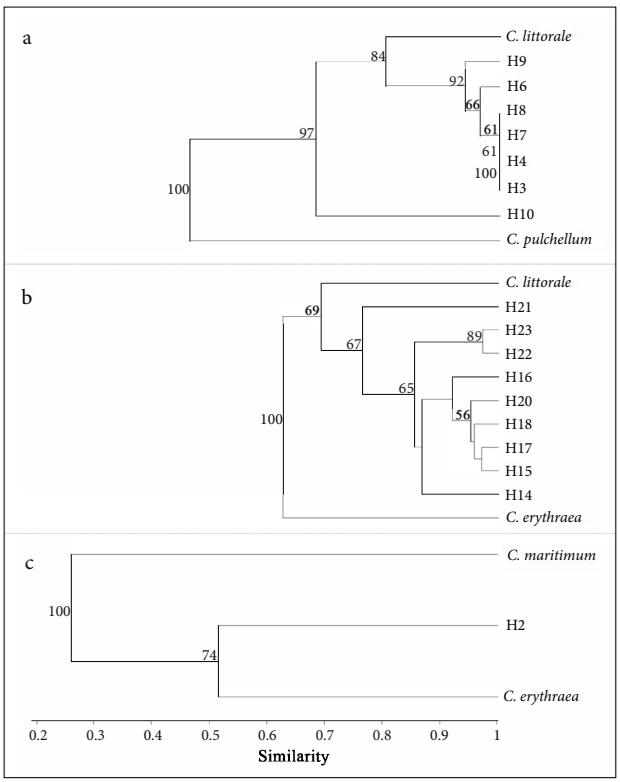

Figure 3. UPGMA dendrograms based on Jaccard's similarity coefficient, calculated from RAPD data, for 3 crossing experiments: a- *Centaurium littorale* × *C. pulchellum,* b- *C. littorale* × *C. erythraea*, and c- *C. maritimum* × *C. erythraea*; hybrid accessions are placed between their parents. Bootstrap values >50 are displayed at each node.

17.78% to 29% per hybrid[1]). Additionally, 2 nonparental bands in hybrid progeny were detected. An explanation for this phenomenon may be that recessive alleles of heterozygous parental plants remained undetected due to the dominant nature of RAPD markers (Williams et al., 1990). Recombination, mutation, or random segregation of chromosomes during hybrid formation often occurs as well. Similar observations were previously reported for *Cyrtandra* Forster & Forster, *Gossypium* L., and *Primula* L. hybrids (Smith et al., 1996; Mehetre et al., 2004a, 2004b; Terzioğlu et al., 2012).

There are few reports regarding the use of Lab-on-a-Chip technology for the separation of RAPD-PCR products (Tedeschi et al., 2011). This approach allowed us to improve the objective detection of PCR products characterized by a relatively low yield of amplification, thus making RAPD fingerprints more informative. This certainly nominates Lab-on-a-Chip technology for further extensive application in studies of interspecies hybridization.

[1] Data not presented and are available upon request.

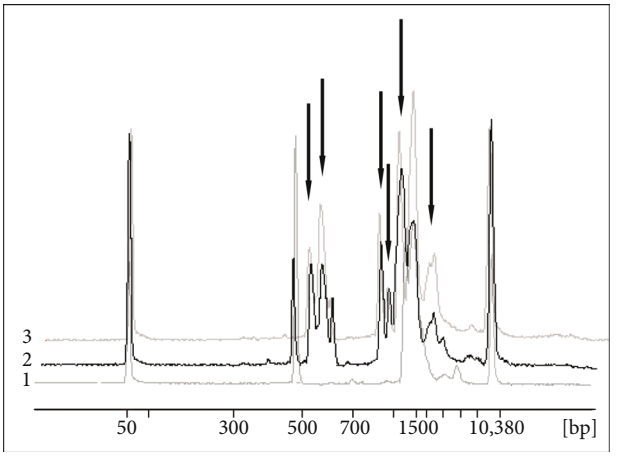

Figure 4. Electropherograms of RAPD fingerprints: 1- *C. maritimum* (female parent); 2- H2 (hybrid plant); and 3- *C. erythraea* (male parent), generated with OPF 14 primer. Arrows indicate the male-parent–derived informative bands.

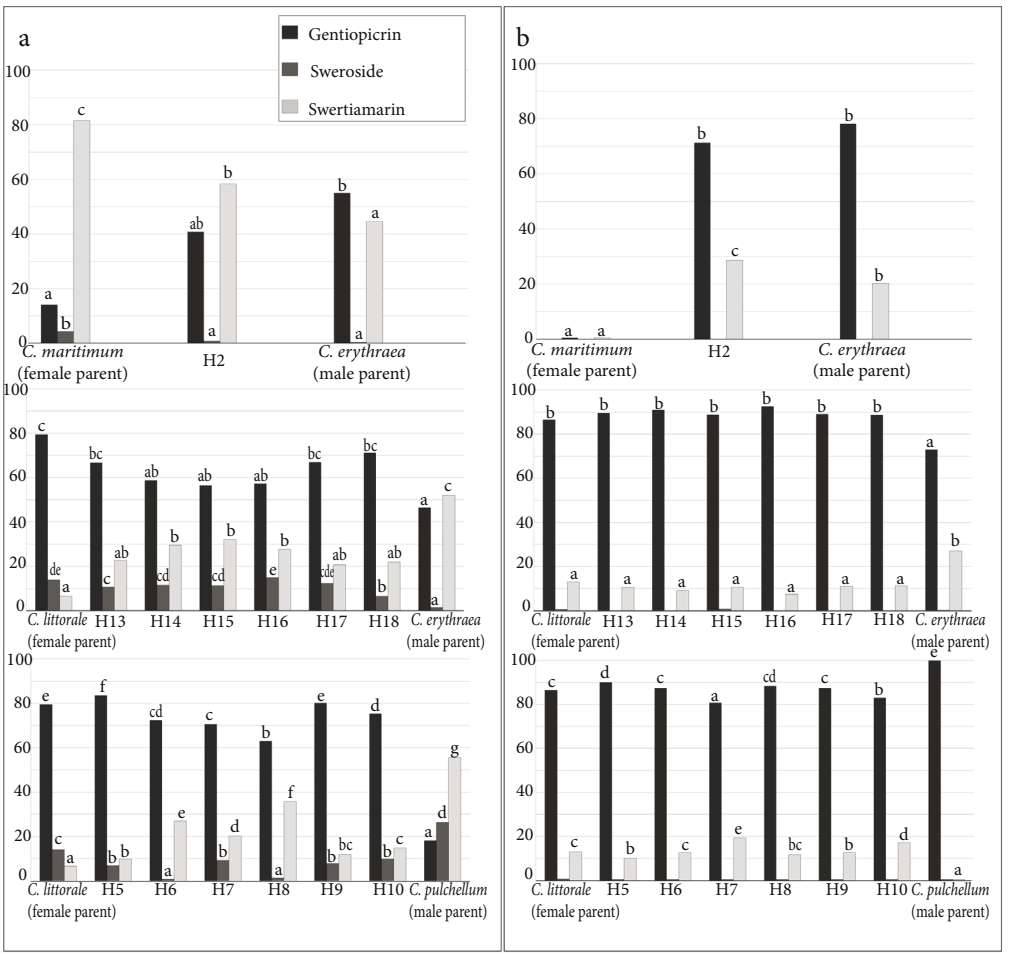

Figure 5. Content (% DW) of 3 secoiridoid glycosides (gentiopicrin, sweroside, and swertiamarin) in methanol extracts of: a- aerial parts and b- roots of parental and hybrid plants. Values are presented as means of 3 independent extractions. Within each parameter, values marked with the same letter are not significantly different at $P \leq 0.05$, according to LSD test.

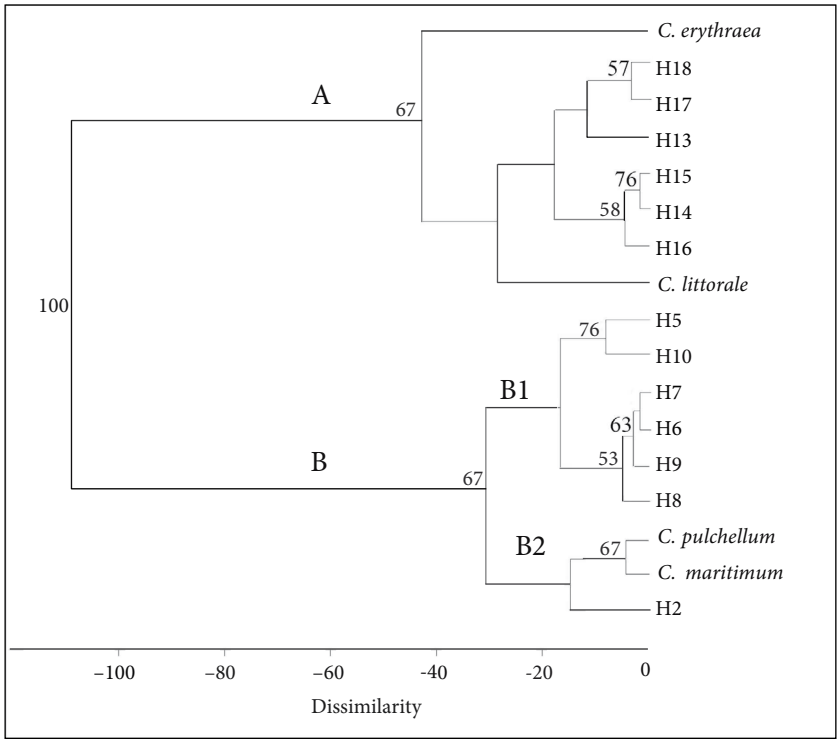

Figure 6. Dendrogram based on the content of secoiridoid glycosides in aerial parts of parental and hybrid plants from 3 crossing experiments.

4.4. Secoiridoid glycosides as a marker system in estimation of hybrid nature of progeny

SGs have been successfully utilized as phylogenetic and taxonomic markers of the genus *Centaurium* (van der Sluis, 1985; Jensen and Schripsema, 2002). Therefore, their accumulation was analyzed in the present study to aid in verification of interspecific hybridization. Quantification of all 3 traced SGs generally showed intermediate values of all hybrid plants in relation to their parents, which is not always the case (Rieseberg, 1995). Since aerial parts displayed more variability in SG profiles and yielded far more total SGs than roots, UPGMA analysis and construction of a dendrogram were conducted on the aerial parts alone. Observed chemical polymorphism clearly identified hybrid progeny, supporting molecular data and encouraging the use of SGs as chemical markers in the further identification of natural interspecific hybrids of the genus *Centaurium*.

4.5. Significance of the results for the elucidation of interspecies hybridization in nature

It has been suggested that many *Centaurium* species have a polyploid origin through natural hybridization, either via autopolyploidy or allopolyploidy (Zeltner, 1978; Ubsdell, 1979). On the basis of molecular data, Guggisberg et al. (2006) strongly support the allopolyploid origin for *C. bianoris* through natural hybridization between *C. maritimum* (as female parent) and *C. tenuiflorum* (as male parent) on Majorca Island. However, the authors do not give an explanation regarding the mechanism of formation of this allopolyploid species as there were no triploid plants found in natural populations. Although H2 triploid hybrid showed no fertility, we presume the possibility of contributions from such triploid plants in further polyploid formation during the evolution. A triploid bridge pathway could significantly assist autopolyploid formation regardless of the mating system as well as allopolyploid formation in outcrossing taxa (Ramsey and Shemske, 1998; Husband, 2004).

There is evidence of interspecific hybridization between *Centaurium littorale* (as female parent) and *C. erythraea* (as male parent) in nature (Ubsdell, 1976a, 1976b, 1979). As noticed by the author, extensive hybridization often occurs between *C. erythraea* subsp. *erythraea* and *C. littorale* subsp. *littorale* on the Lancashire coast (British Isles) and in similar areas in Germany and Denmark. Published data as well as our results indicate that there were no effective reproductive barriers to gene exchange between these 2 species, and interspecific hybridization readily occurs where their habitats are adjacent.

All successfully performed hybridization experiments in the present study suggest that efficient pre-zygotic reproductive barriers between *Centaurium* species, which act to prevent fertilization after flower pollination, do not exist. Postzygotic reproductive barriers, reflected in hybrid unviability, sterility, or hybrid breakdown (Rieseberg and Carney, 1998) certainly exist, but are in sufficiently effective.

Biosystematic studies that include experimental interspecific hybridization require a lot of time and effort, but can allow substantial insights into the genetic structure of a particular plant group and elucidate the nature of the mechanisms involved in evolutionary processes that led and can lead to speciation. Results of this study indicate that RAPD fingerprinting, flow cytometry analysis, and secondary metabolite analysis are useful tools for the identification and confirmation of the hybrid origin of plants. Furthermore, these techniques could be successfully applied in investigations conducted in order to elucidate the mechanisms of interspecific hybridization in natural populations of *Centaurium* species, which is the course of our further work.

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