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Identification of *Conocephalum conicum* and *C. salebrosum* (*Hepaticophyta*) based on DNA markers

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Abstract: *Conocephalum conicum* (L.) Dumort. is one of the most common thallose liverworts in Poland. Enzymatic protein electrophoresis allowed to identify sibling species S and L within *Conocephalum conicum* at the end of the 1970s. Morphological and anatomical analyses resulted in determining the features permitting the identification of the species S and L and enabled to describe the species S as *Conocephalum salebrosum* Szweykowski, Buczkowska & Odrzykoski. Despite long-term investigations, *Conocephalum* species have not been analyzed with DNA markers so far. The aim of the present study was to find species-specific DNA markers, and to determine the degree of genetic similarity by analyzing several types of markers based on the PCR reaction. The following markers were used: RAPD, ISJ and sequences of the bacterial *katG* gene. Each of the DNA marker categories revealed differences between *Conocephalum conicum* and *C. salebrosum*. The Nei's genetic similarity coefficient calculated on the basis of the above DNA markers was 0.49 and confirmed the distinct taxonomic status of both species.

Keywords: *Conocephalum conicum, Conocephalum salebrosum,* DNA markers, genetic differences

Introduction

Conocephalum conicum (L.) Dumort. is one of the most common thallose liverworts in Poland. It can be found in wet, shaded sites in highlands and lowlands. Due to the considerable size of its thallus and a great number of localities, this species has became the object of interest of taxonomists and evolutionists. An analysis of flavonoids enabled to distinguish two geographical forms of this taxon, German and North-American, and gave rise to thorough research (Markham et al. 1976). Enzymatic protein electrophoresis allowed to identify in Poland genetic races S and L within *Conocephalum conicum* at the end of the 1970s (Szweykowski and Krzakowa 1978, Szweykowski et al. 1981). Their identification was based on the following isoenzymes: AAT, GDH and PX. The existence of a reproductive barrier between the European genetic races of *Conocephalum conicum* (Odrzykoski 1987) was also confirmed. The sibling species differed also in the sequence of the chloroplast *psbA* gene (Kim et al. 2001). However, both taxa have not been compared based on their nuclear DNA so far.

Morphological and anatomical analyses resulted in determining numerous diagnostic features permitting the identification of the sibling species S and L without the need to apply molecular methods. Morphological markers enabled to describe the species S as *Conocephalum salebrosum* Szweykowski, Buczkowska & Odrzykoski (Szweykowski et al. 2005).

The aim of the present study was to determine genetic similarity for *Conocephalum conicum* and *C. salebrosum* on the base of nuclear DNA analysis, and to find species-specific DNA markers as well.

Fig. 1. Distribution of analyzed *Conocephalum sp*. populations (square – *C. conicum*, round *C. salebrosum*).

Materials and Methods

The experimental materials comprised four population samples of *Conocephalum conicum* and four population samples of *C. salebrosum*. The distribution of the examined populations is presented in Fig. 1 (dots - *C. conicum*, squares - *C. salebrosum*). The materials were first identified on the basis of morphological characters. Isoenzymatic identification was performed to verify the determinations. AAT and GDH phenotypes were used as species-specific markers. Electrophoresis was performed in starch gel, in 0.1 M lithium-borate buffer, at pH 8.1. DNA was isolated by the modified CTAB procedure (Doyle and Doyle 1990).

RAPD, ISJ and sequences complementary to the bacterial *katG* gene were used to determine genetic similarity. Genome-scanning RAPD (Random Amplified Polymorphic DNA) markers are widely used in studies on genetic diversity of the bryophytes. They amplify DNA fragments with unknown functions and locations. The ISJ (Intron Splice Junction) markers have been designed on the basis of the exon-intron boundary sequences, so they amplify transcription-active sites (Weining and Landridge 1991). The PCR reaction for ISJ and RAPD markers was carried out in 20 μl of a reaction mixture containing 80 ng genomic DNA, 20 ng primer, 2 mM $MgCl_2$, 200 μM dNTP (dATP, dGTP, dCTP, dTTP), 1 μl 10x concentrated PCR buffer and 1 U Tfl polymerase (Epicentre Technology). The last group of markers, i.e. sequences complementary to the bacterial *katG* gene, have already proved to be perfect species-specific markers in many plant taxa (Zieliński and Polok 2005, Szczecińska et al. 2006, Bączkiewicz et al. 2008, Sawicki and Zieliński 2008). An analysis with the use of these markers was performed in 20 μl of a reaction mixture composed of 100 ng genomic DNA, 20 ng primer, 2 mM $MgCl_2$, 200 μM dNTP (dATP, dGTP, dCTP, dTTP), 1 μl 10x concentrated PCR buffer and 1 U Tfl polymerase (Epicentre Technology). Primer sequences are shown in Table 1. Electrophoresis of PCR products was performed in 1.2% (RAPD) or 1.5% (ISJ, *katG*) agarose gel.

Results

An analysis of DNA markers enabled to identify 113 bands (53 *katG*, 38 ISJ and 22 RAPD), including 99 for *Conocephalum salebrosum* and 53 for *C. conicum*. *C. salebrosum* populations showed higher polymorphism than *C. conicum* populations- 75% vs. 70% polymorphic bands. The DNA markers used in the study permitted molecular identification of these species. Among 19 species-specific bands, 14 were revealed by primers complementary to the bacterial *katG* gene. Among *katG* primers, the most species-specific markers were revealed by *katG8* - 4 and *katG9* – 5 (Fig. 2), which constituted respectively 36% and 35% of all bands obtained with these primers. RAPD and ISJ markers revealed 3 and 2 species-specific bands, respectively. The genetic simila-

Tab. 1. Primers used in the analysis of *Conocephalum* species.

Type of marker	Abbreviation	Sequence
RAPD	OPB-13	5‘ TTCCCCCGCT 3’
	OPD-03	5’ GTCGCCGTCA 3’
ISJ	ISJ-06	5’ ACTTACCTGAGCCAGCGA 3’
	ISJ-08	5’ GACCGCTTGCAGGTAAGT 3’
	ISJ-10	5’ ACTTACCTGCATCCCCCCT 3’
katG	katG-5-1	5’ CGACAACGCCAGCTTGGAC 3’
	katG-5-2	5’GGTTACCGTAGATACGCCCC 3’
	katG-6-1	5’GCAGATGGGGCTGATCTACG 3’
	katG-6-2	5’ ACCTCAATGCCGCTGGTG 3’
	katG-7-1	5’ GCTGGAGCAGATGGGGTTG 3’
	katG-7-2	5’ATCCACCGGCAGCGAGAG 3’
	katG-8-1	5’ GTCACTGACCTCTCGCTG 3’
	katG-8-2	5’ CGCCCATGCGGTCGAAAC 3’
	katG-9-1	5’GCGAAGCAGATTGCCAGCC 3’
	katG-9-2	5’ACAGCCACCGAGCACGAC 3’

rity coefficient based on DNA markers was 0.49 (Fig. 3), including 0.65 for RAPD, 0.47 for ISJ and 0.46 for *katG* markers.

Discussion

The Nei’s genetic similarity coefficient based on analysis of isoenzymes data is usually lower than 0.65 for good biological and taxonomical species (Crawford 1983). This value seems to be correct also for data obtained using DNA markers (Zieliński and Polok 2005). The analysis of nuclear DNA shows that *Conocephalum conicum* and *C. salebrosum* are good biological species (I=0.49). The same classes of markers have been already used to analyze taxa of the *Polygonatum* genus (Szczecińska et al. 2006). For two closely related species, *P. odoratum* and *P. multiflorum,* the genetic similarity coefficient amounted to 0.54, i.e. was even higher than that obtained in the case of *Conocephalum*.

The taxonomic distinctness is also confirmed by a high number of species-specific bands. The existence of species-specific markers in each of the classes of primers in nuclear DNA, specific *psb*A organelle DNA sequences (Kim et al. 2001), as well as isoenzymatic (Szweykowski and Krzakowa 1978, Odrzykoski and Szweykowski 1991) and biochemical (Markham et al. 1976, Toyota et al. 1997) markers, proves the taxonomic distinctness of *C. conicum* and *C. salebrosum*. Szweykowski et al. (2005) found morphological markers that enabled to identify these taxa without the need to apply molecular methods, which in turn made it possible to describe *C. salebrosum*.

Sequences complementary to the bacterial *katG* gene proved to be good species-specific markers in previous research on members of the genus *Polygonatum* (Szczecińska et al. 2006), *Pinus* (Zieliński and Polok 2005) and the genus *Sphagnum* (Sawicki and Zieliński 2008). Also in this study *katG* primers revealed the greatest number of species-specific bands. Among 53 identified *katG* bands, 14 enabled to distinguish between *C. conicum* and *C. salebrosum*, not indicating intraspecific polymorphism. The commonly used genome-scanning RAPD and ISJ markers were less effective in terms of species identification.

High levels of intraspecific polymorphism were recorded in both *Conocephalum* taxa, i.e. 75% and 70% for *C. salebrosum* and *C. conicum* respectively. Such high variation may be a consequence of a high proportion of vegetative reproduction, and limited gene flow or the lack of gene flow between populations.

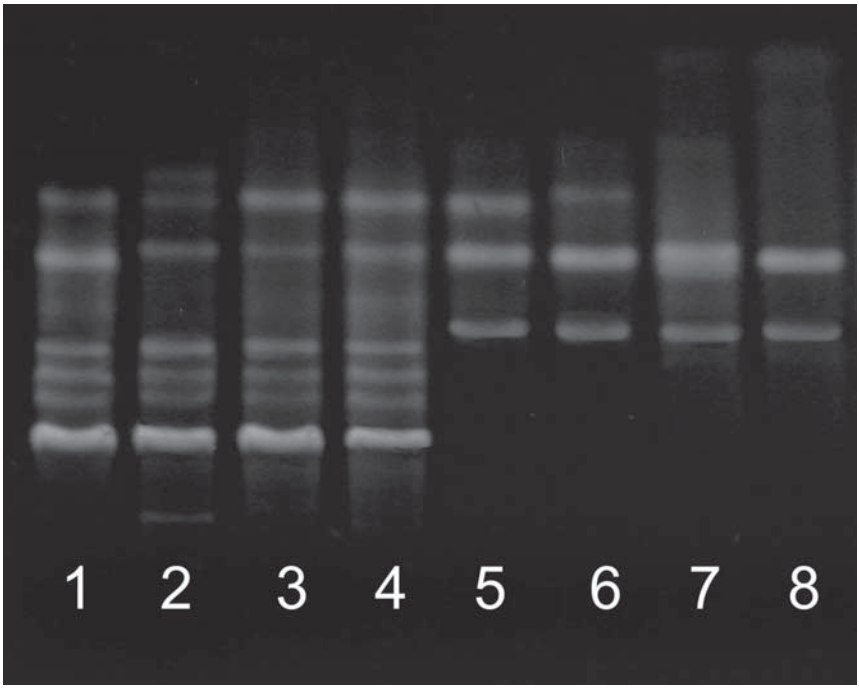

Fig. 2. DNA amplification pattern for *C. conicum* (1-4) and *C. salebrosum* (5-8) with *katG*-5 primers.

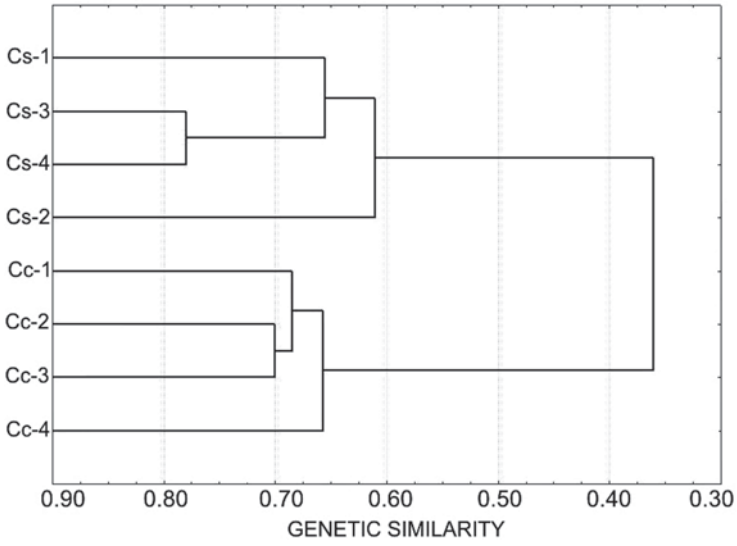

Fig. 3. UPGMA dendrogram of 10 populations of the genus *Conocephalum* based on Nei's genetic similarity.

Conclusions:

- *Conocephalum conicum* and *C. salebrosum* can be considered as a good biological species, for which numerous species-specific, nuclear DNA amplified markers were found.
- The genetic similarity coefficient determined n the base of nuclear DNA markers confirms the taxonomic distinctness of the species examined.
- The molecular markers applied in the study revealed high interpopulation polymorphism of *Conocephalum sp*. populations, characterized by a high proportion of vegetative reproduction.
- Sequences complementary to the bacterial *katG* gene proved to be the most suitable for the identification of *C. conicum* and *C. salebrosum*.

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Odlišení játrovek *Conocephalum conicum* a *C. salebrosum* (*Hepaticophyta*) na základě DNA markerů

Conocephalum conicum (L.) Dumort. je jednou z nejrozšířenějších frondózních játrovek v Polsku i ve střední Evropě. Elektroforéza enzymatických proteinů pomohla již koncem 70-tých let 20. století rozlišit tzv. sibling species S and L uvnitř druhu *Conocephalum conicum*. Posléze byl i na základě morfologických rozdílů "druh S" popsán jako nový druh *Conocephalum salebrosum*. Moderní metody dovolují oprávněnost existence nového druhu potvrdit i na základě studia markerů DNA.

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