ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 17(1) 2018, 159–164

www.acta.media.pl ISSN 1644-0692 DOI: 10.24326/asphc.2018.1.15

ORIGINAL PAPER

Accepted: 8.11.2017

SIMPLE VERIFICATION OF *in vitro* – GROWN CLONES OF THE GENUS *Drosera* L. USING ITS MOLECULAR MARKERS

Monika Fratrikova[1], Miroslav Bauer[2,3], Martin Jopcik[1], Jana Libantova[1]✉

[1] Institute of Plant Genetics and Biotechnology, PSBC, SAS, Akademicka 2, P.O.Box 39A, 950 07 Nitra, Slovak Republic

[2] NAFC Research Institute for Animal Production, Nitra, Hlohovska 2, 951 41 Lužianky, Slovak Republic

[3] Department of Botany and Genetics, Faculty of Natural Sciences, Constantine the Philosopher University, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic

ABSTRACT

Drosera L. is a genus of carnivorous plants that comprises approximately 250 species, although this number is probably not complete. Some of these taxa exhibit only small differences in morphological traits that can be partly influenced if the taxa are propagated *in vitro*. Here, we focus on the verification of putative clones of *Drosera spathulata* Labill., *Drosera rotundifolia* L. and *Drosera binata* var. *Dichotoma* species cultivated *in vitro* using molecular markers covering the internal transcribed spacer (ITS) region of 45S ribosomal DNA (rDNA). Following the polymerase chain reaction (PCR) amplification of ~360-bp DNA fragments and sequencing, the sequences were aligned with corresponding sequences in the National Center for Biotechnology Information (NCBI) database. In addition, each of tested PCR amplicons had a specific restriction profile that predominantly enables the differentiation of *D. rotundifolia* and *D. spathulata*; the shape of the leaves does not have to be a clear morphologically distinguishable trait.

Key words: *Drosera binata*, *Drosera rotundifolia*, *Drosera spathulata*, ITS nucleotide sequences, molecular identification

INTRODUCTION

Currently, chloroplast (*rbc*L) and ribosomal internal transcribed spacer (ITS) DNA sequences are extensively employed in molecular studies aimed at determining inter- and intrageneric relationships in evolving taxa, as these sequences have high mutation rates [Williams et al. 1994, Baldwin et al. 1995, Fineschi et al. 2002, Rosato et al. 2016]. The *rbc*L analysis of ~100 plant species including families of the subclasses Rosidae, Hamamelidae, Nepenthaceae and Caryophyllidae placed monophyletic Droseraceae in the same clade as Caryophyllidae and Nepenthaceae. Combined *rbc*L and 18S ribosomal DNA (rDNA) sequence data were then used to infer phylogenetic relationships among the genera of the Droseraceae family, in which three of four genera, *Drosera*, *Dionaea* and *Aldrovanda*, exerted monophyletic character [Rivadavia et al. 2003]. Moreover, molecular analyses clustered most of the taxa of the *Drosera* L. genus into the *regia* clade, which contains *Drosera regia*; the *capensis* clade, which involves the South African and temperate species outside of Australia; and the *peltata* clade, which contains Australian endemics [Williams et al. 1994].

The majority of *Drosera* species have active fly traps and capture their prey with mobile glandular hairs that are present on the adaxial leaf surface

✉ jana.libantova@savba.sk

[Rivadavia et al. 2003]. Obovate- or spathulate-shaped leaves constitute a morphologically distinct trait of most of these taxa. Therefore, other characteristics such as the number of styles and the presence/absence of both stipules and specialised organs (tubers and gemmae) have been used for taxonomic classification. Later, chromosome number [Kondo et al. 1976], pollen morphology [Takahashi and Sohma 1982] and seed germination type [Conran et al. 1997] were also used to indicate the specificity of individual species. Most recently, DNA analyses including randomly amplified polymorphic DNA and the ITS region of the 18S-5.8S-26S nuclear ribosomal cistron have been used to identify nuclear genetic variation of individual species. In the case of 45S rDNA, the genetic regions are highly conserved, but ITS divergence is sufficient for resolving individual species within most genera [Matyasek et al. 2012].

In this work, we used ITS molecular markers for the verification of putative *in vitro* clones of *Drosera spathulata*, *Drosera rotundifolia* and *Drosera binata* species. In addition, for each of tested taxa, we proposed a specific restriction profile of amplified ITS polymerase chain reaction (PCR) products.

MATERIALS AND METHODS

Plant material and DNA isolation. Plants of *D. binata* var. *Dichotoma*, *D. rotundifolia* L. and *D. spathulata* Labill. were provided as gifts from the Institute of Experimental Botany, Czech Academy of Sciences and cultivated aseptically on agar media as described previously [Bobak et al. 1995]. Total genomic DNA was isolated from the fresh leaf material (1 g) of investigated taxa in accordance with the methods of Bekesiova et al. [1999].

PCR amplification and sequencing. PCR fragments were amplified using the primers DribFOR (5´-AACCCCGGCGCAAGTCGCGCCAAGGA-3´) and DribREV (5´-AGCTTCCCATTTGGGCCAACCGCG-3´) designed for the sequences covering the ITS1, 5.8S rRNA and ITS2, accounting for the availability of overlapping sequences of analysed species stated in National Center for Biotechnology Information (NCBI) database (AB355664.1, HM204879.1 and AB685414.1). The PCR conditions were: one cycle at 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s; and a final elongation step at 72°C for 10 min. The DNA amplified by PCR was cloned into a pGEM-Teasy (Promega, USA) vector and sequenced using an ABI Prism 310 genetic analyser with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA).

Bioinformatic analysis of sequencing data. Nucleotide sequences of the 18S-5.8S-26S rRNA genes of *D. rotundifolia* L. (AB355664.1), *D. binata* (HM204879.1) and *D. spathulata* (AB685414.1) were collected from the NCBI GenBank database.

The nucleotide sequences of the PCR amplicons of the analysed species cultivated *in vitro* were aligned with the rDNA sequences of the corresponding species using the CLUSTAL-W v1.2.4 program [Thompson et al. 1994]. PCR fragment restriction analysis was then performed using the NEBcutter v2.0 program (http://nc2.neb.com/NEBcutter2/). The identities of the studied taxa were also confirmed using the *Eco*RV, *Bgl*I and *Sph*I restriction enzymes.

RESULTS AND DISCUSSION

The verification of the identity of individual species using DNA molecular analysis is a crucial step for the genetically related individual *Drosera* species that have similar morphological characters [Tungkajiwangkoon et al. 2016] that may be slightly altered from *in vitro* cultivation [Batagin et al. 2009, Trejgell et al. 2012]. In our case, this type of molecular verification of individual carnivorous species precedes their further detailed characterisation in respect of molecular processes involving the insect prey digestion.

The 364-bp- and 368-bp-long fragments covering the sequences of ITS1 of the 5.8S and ITS2 of 45S rDNA, respectively, of *D. spathulata*, *D. binata* and *D. rotundifolia* were amplified using the DribFOR and DribREV primers. The primers were designed in relatively conserved border regions of ITS1 and ITS2 within the ~ 680 bp partial 45S rDNA sequence of tested plant species available in NCBI database (fig. 1). After PCR, the DNA amplicons of the putative *D. rotundifolia*, *D. spathulata* and *D. binata* clones were TA cloned and sequenced. The alignment of obtained primer flanking

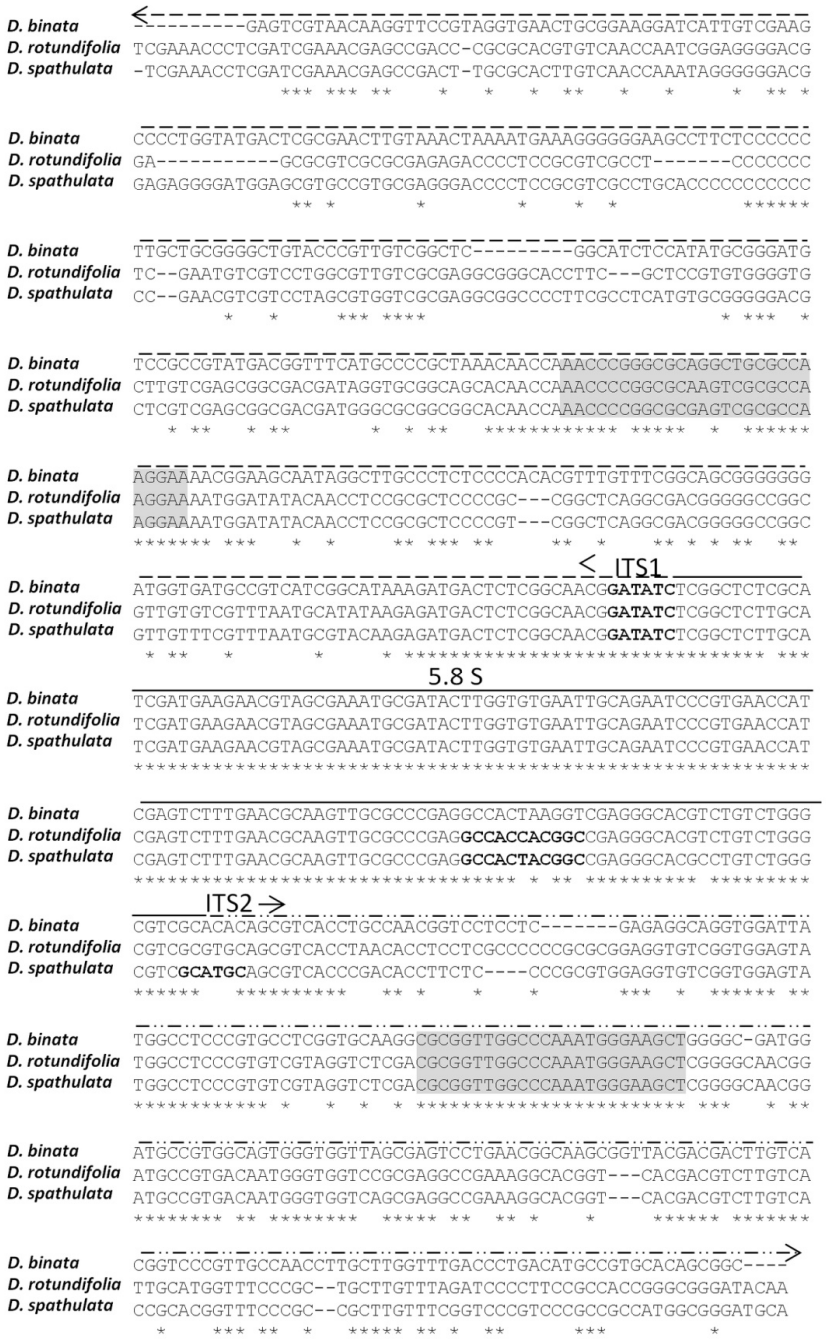

Fig. 1. Ribosomal DNA alignment of *Drosera binata* (HM204879.1), *Drosera rotundifolia* (AB355664.1) and *Drosera spathulata* (AB685414.1) involving the ITS1, 5.8S and ITS2 nucleotide sequence. The primers DribFOR and DribREV used in PCR amplification are shown in the grey *box*. In *bold* are shown the restriction sites GATATC, GCCNNNNNGGC and GCATGC, which correspond to *EcoR*V, *Bgl*I and *Sph*I restriction endonucleases, respectively

sequences with the corresponding sequences of *D. rotundifolia* (AB355664.1), *D. spathulata* (AB685414.1) and *D. binata* (HM204879.1) retrieved from the NCBI database confirmed the 99–100% sequence consistency of the analysed *in vitro* clones.

The occurrences of substitution mutations and indels within the analysed ITS sequences of these taxa were subsequently analysed. The regions of high homogenity occurred mostly in the regions belonging to the 5.8S rDNA (coding part of rDNA units), as in most angiosperms, indicating strong selection pressure imposed on functionality [Rosato et al. 2016]. In contrast, a higher level of variability was observed in the regions corresponding to ITS1 and ITS2 (fig. 1). Among the analysed species, *D. binata* and *D. spathulata* shared a higher genetic divergence (88% identity and 3% of indels) than did *D. spathulata* and *D. rotundifolia* (96% identity and 1% of indels). This is in agreement with the results of previous phylogenetic studies that involve morphological as well as *rbc*L and 18S rDNA data [Williams et al. 1994, Rivadavia et al. 2003, Renner and Specht 2011]. Their results showed that within one monophyletic *Drosera* group *D. spathulata* and *D. rotundifolia* both occurred in a *capensis* clade, and *D. binata* was grouped in a *peltata* clade.

As stated above, *D. rotundifolia* and *D. spathulata* showed high genetic relatedness, as the analysed ITS sequences were 96% identical. *Drosera rotundifolia* has round or nearly round leaf blades that are wider than they are longer, whereas *D. spathulata*, which includes diploids, tetraploids and hexaploids, can have obovate to spathulate leaf shapes. Molecular analysis appears to be justified,

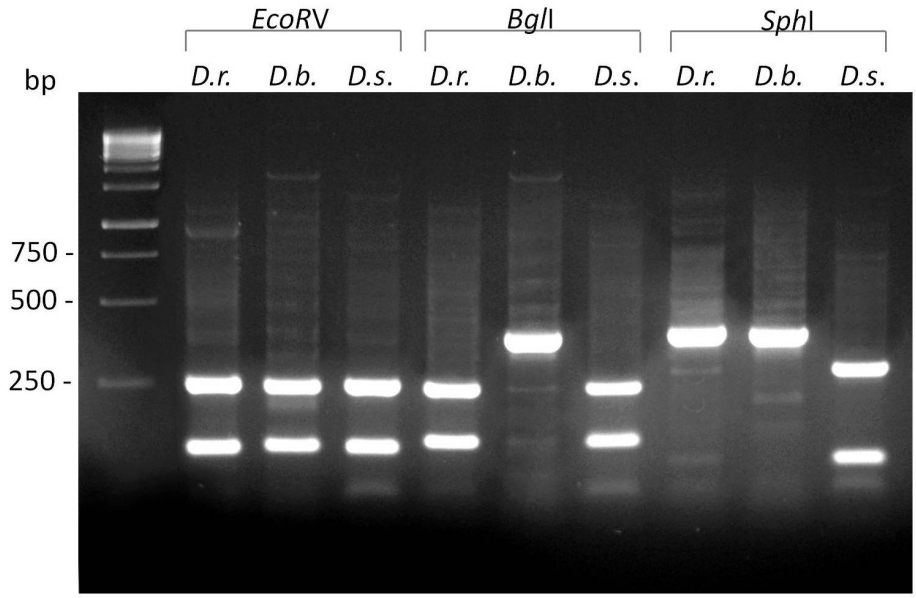

Fig. 2. Restriction analysis of the 18S ribosomal DNA (rDNA) polymerase chain reaction (PCR) fragment ~350 bp in length amplified with *Drosera rotundifolia*, *Drosera spathulata* and *Drosera binata* templates. Restriction fragments 110 bp and 240 bp long were identified in all tested samples following restriction by the *EcoR*V enzyme. Restriction analysis with *Bgl*I resulted in 135-bp and 215-bp products when the PCR products *of D. rotundifolia* and *D. spathulata* were digested. The restriction endonuclease *Sph*I specifically digested the PCR product of *D. spathulata*, and the lengths of restriction products were 100 bp and 250 bp

especially in the cases of vague morphological differences. To simplify the process of the molecular identification of *D. binata*, *D. rotundifolia* and *D. spathulata* maintained as clones under *in vitro* conditions, we chose suitable restriction enzymes conferring specific restriction profiles of ~350-bp ITS DNA sequences to corresponding taxa. As shown in Figure 2, the restriction endonuclease *Bgl*I is applicable to the differentiation of *D. binata* from *D. rotundifolia* and *D. spathulata*, and the restriction enzyme *Sph*I conferred a specific restriction profile to *D. spathulata*.

The latest studies report the identification of approximately 250 species within the genus *Drosera* L. [Gonella et al. 2016]. However, discoveries of new taxa [Gonella 2015] as well as the taxonomic revision of some *Drosera* species [Rivadavia et al. 2014] indicate that this number is not complete. In addition to morphological characterisation, molecular analysis appears to be a useful tool for phylogenetic studies, the identification of new taxa and the verification of individual species.

CONCLUSIONS

In addition of phylogenetic analysis, the variability within the ITS1 and ITS2 sub-regions of 45S rDNA might be used for rapid identification of individual taxa mainly in cases of ambiguous species assignment based on morphological characteristics. In our study this approach enabled us to confirm the identity of *D. rotundifolia*, *D. spathulata* and *D. binata* clones cultured *in vitro*. Moreover, the presence of specific restriction sites in 45S rDNA amplicons of *D. rotundifolia*, *D. spathulata* and *D. binata* allows simple and fast PCR-RFLP differentiation of these species during *in vitro* cultivation without DNA sequencing.

REFERENCES

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue, M.J. (1995). The ITS region of nuclear ribosomal DNA – a valuable source of evidence on angiosperm phylogeny. Ann. Mo. Bot. Gard., 82, 247–277.

Batagin, K.D., de Almeida, C.V., Tanaka, F.A.O., de Almeida, M. (2009). Morphological alterations in leave of micropropagated pineapple plants cv. IAC "Gomo-de-mel" acclimatizated in different conditions of luminosity. Acta Bot. Bras., 23, 85–92.

Bekesiova, I., Nap, J.P., Mlynarova, L. (1999). Isolation of high quality DNA and RNA from leaves of the carnivorous plant *Drosera rotundifolia*. Plant Mol. Biol. Rep., 17, 269–277.

Bobak, M., Blehova, A., Kristin, J., Ovecka, M., Samaj, J. (1995). Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured *in vitro*. Plant Cell Tissue Organ Cult., 43, 43–49.

Conran, J.G., Jaudzems, V.G., Hallam, N.D. (1997). Droseraceae germination patterns and their taxonomic significance. Bot. J. Linn. Soc., 123, 211–223.

Fineschi, S., Anzidei, M., Cafasso, D., Cozzolino, S., Garfi, G., Pastorelli, R., Salvini, D., Taurchini, D., Vendramin, G.G. (2002). Molecular markers reveal a strong genetic differentiation between two European relic tree species: *Zelkova abelicea* (Lam.) Boissier and *Z. sicula* Di Pasquale, Garfi & Quezel (Ulmaceae). Conserv. Genet., 3, 145–153.

Gonella, P.M., Fleischmann, A., Rivadavia, F., Neill, D.A., Sano, P.T. (2016). A revision of *Drosera* (Droseraceae) from the central and northern Andes, including a new species from the Cordillera del Cóndor (Peru and Ecuador). Plant Syst. Evol., 302, 1419–1432.

Gonella, P.M., Rivadavia, F., Fleischmann, A. (2015). *Drosera magnifica* (Droseraceae): the largest New World sundew, discovered on Facebook. Phytotaxa, 220, 257–267.

Matyasek, R., Renny-Byfield, S., Fulnecek, J., Macas, J., Grandbastien, M.A., Nichols, R., Leitch, A., Kovarik, A. (2012). Next generation sequencing analysis reveals a relationship between rDNA unit diversity and locus number in *Nicotiana* diploids. BMC Genomics, 13, 722.

Kondo, K. (1976). A cytotaxonomic study in some species of *Drosera*. Rhodora, 78, 532–541.

Renner, T., Specht, C.D. (2011). A sticky situation: assessing adaptations for plant carnivory in the Caryophyllales by means of stochastic character mapping. Int. J. Plant Sci., 172, 889–901.

Rivadavia, F., Gonella, P.M., Sano, P.T., Fleischmann, A. (2014). Elucidating the controversial *Drosera montana* complex (Droseraceae): a taxonomic revision. Phytotaxa, 172, 141–175.

Rivadavia, F., Kondo, K., Kato, M., Hasebe, M. (2003). Phylogeny of the sundews, *Drosera* (Droseraceae), based on chloroplast *rbcL* and nuclear 18S ribosomal DNA sequences. Am. J. Bot., 90, 123–130.

Rosato, M., Kovarik, A., Garilleti, R., Rossello, J.A. (2016). Conserved organisation of 45S rDNA sites and rDNA gene copy number among major clades of early land plants. Plos One, 11, e0162544.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994). CLUSTAL-W – Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acid. Res., 22, 4673–4680.

Takahashi, H., Sohma, K., 1982. Pollen morphology of the Droseraceae and its related taxa. Science reports of the research Institutes Toboku University, 4th Series, Biology, 38, 81–156

Trejgell, A., Dąbrowska, A., Tretyn, A. (2012). Micropropagation and influence of *in vitro* culture on development of *Cirsium pannonicum* (L. f.) link regenerants. Acta Sci. Pol. Hortorum Cultus, 11, 81–90.

Tungkajiwangkoon S., Shirakawa, J., Azumatani, M., Hoshi, Y. (2016). Breeding and cytogenetic characterizations of new hexaploid *Drosera* strains colchicines induced from triploid hybrid of *D. rotundifolia* and *D. spatulata*. Cytologia, 81, 263–269.

Williams, S.E., Albert, V.A., Chase, M.W. (1994). Relationships of Droseraceae – a cladistic-analysis of *rbcL* sequence and morphological data. Am. J. Bot., 81, 1027–1037.