- Short communication
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RAPD ANALYSIS OF *ASPLENIUM ADULTERINUM* MILDE FROM *IN VITRO* CULTURE

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Abstract.

Asplenium adulterinum Milde represents one of the natural heritages which have to be protected due to their scientific importance. In the actual context of global warming with myriad effects on biodiversity, the *ex-situ* conservation of *A. adulterinum* should be a priority. The possibility to obtain genetically stable individuals through *in vitro* techniques is a requirement of *ex situ* conservation. The genetic similarity of *A. adulterinum* gametophytes maintained 8 years *in vitro* culture was assessed. Explants represented by gametophyte fragments were analyzed for genetic stability through RAPD. Close similarity (88.88%) was found between gametophytes maintained in the same conditions (medium, illumination, temperature, passages number).

Asplenium adulterinum Milde known as Adulterated Spleenwort or Ladder Spleenwort is a vulnerable European fern species, mentioned in the IUCN Red List, with stable populations, but in a continuing decline of subpopulations (Christenhusz *et al*. 2017). It is a protected species through international laws, restricted to serpentine rocks, a critically endangered in the Red Data Book of the Vascular Plants in Romania (Dihoru and Negrean 2009). This species is restricted to serpentine rocks in Europe (Tutin *et al.* 1980). The international laws which protect the interest species are represented by Habitats Directive Annex II and Annex IV (Directive 92/43/EEC) (plant species demanding strict protection in all European Union countries) and in the Bern Convention. In this concern, the *ex situ* conservation studies of *A. adulterinum* are needed.

Climate change, habitat fragmentation, and invasive species are the main factors affecting biodiversity and the cause of the extinction of species (Mantyka-Pringle *et al*. 2012). In the case of *A. adulterinum*, the limiting factors are represented by deforestation (leading to modification of the wind speed, humidity, and microclimate), the competition with other species, and the quarry exploitation of serpentine rocks (Mihailescu *et al*. 2015). Including the species in Red lists and international laws, its *in situ* conservation in the natural habitats is to be ensured. The *ex-situ* techniques are applied as an additional conservation method for the *in-situ* methods, being a backup collection for the most vulnerable material (Hawkes *et al.* 2000).

All over the world, fern conservation is realized by combing the *in situ* (habitat protection, Red lists) with *ex situ* methods (artificial spore banks, wet storage, dry storage, cryopreservation, *in vitro* techniques, quasi *in situ*) (Ibars and Estrelles 2012). The plant tissue culture techniques represent a useful tool to conserve genes and guarantee the survival of the endangered species, without affecting the effectiveness of natural populations.

Genetic integrity of the plant material induced and maintained *in vitro* represents a base criterion of *in vitro* conservation. In recent years, different tools like RAPD, ISSR, REMAP, IRAP, AFLP, and MSAP were used for genetic analysis in fern conservation (Peredo *et al*. 2011).

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So far, no studies concerning the genetic similarity of *A. adulterinum* regenerated through *in vitro* culture and maintained in medium-term conservation were identified. Thus, in the present study, the genetic similarity of *A. adulterinum* gametophytes maintained *in vitro* culture was rated with a view to certify that the medium-term conservation represents a proper conservation strategy for this species.

Asplenium adulterinum gametophytes were obtained through *in vitro* spore germination (Aldea *et al*. 2016). The spores were collected from one single plant. The medium variant was represented by the agarised MS (Murashige & Skoog 1962) added with 3% sucrose, IAA (1.8 mg/l), Kn (0.02 mg/l), and activated charcoal (1g/l), at 24 ± 2°C and 16 hrs light/8 hrs dark with the light intensity of 27µmol m^{-2} sec^{-1}. The *in vitro* cultures were maintained in the same conditions (medium variant, temperature, light) for 8 years with subculture passages at 3 months.

Eleven samples (10 - *A. adulterinum* and 1 - *A. trichomanes*) were used for total genomic DNA extraction to analyze the genetic stability. *A. trichomanes* was used as an outlier because the interest species is considered a natural hybrid between *A. trichomanes* and *A. viride* (Lovis 1955).

DNA was extracted from 0.5-1g/ sample using the DNA extraction method of Doyle and Doyle (1987). DNA concentration and quality were evaluated by NanoDrop 2000.

Between 1- 4 µl of DNA template was enough for successful amplification. PCR were performed in a 25 µl volume containing 5 µl MangoTaq Colored Reaction Buffer, 2.5 mM $MgCl_2$, 0.5 mg/ml BSA, 0.2 mM of each dNTP, 1 µM of primer, 1.25 U of MangoTaq™.

The amplification was realized with Kit A Roth RAPD primer. Amplification was done with an Eppendorf Mastercycler following the program: initial denaturation 95°C 5 min, followed by 35 repeats: 95°C 30s, 40°C 30s, 72°C 1 min, and final elongation at 72°C for 5 min.

A total of 7 random primers were utilized for RAPD analysis, out of which 5 random decamer primers were selected based on the clarity of banding patterns. The primer sequences are presented in Table 1.

The PCR products were loaded on 1.5% agarose gel buffered with 1X TBE at a constant voltage of 4V/cm for 2 hrs. The size of the amplicons was determined by comparison with O'GeneRuler 1kb DNA Ladder (Thermo Scientific). PCR products were visualized under GENi Gel Documentation System from SynGene.

The presence/absence data matrix was used to generate a pairwise genetic distance matrix for binary data (using the GenAlEx 6.501 software) which was visualized using a cluster analysis (unweighted pair-group method with arithmetic averages, UPGMA) and illustrated in a dendrogram using MEGAX.

Despite its ecological role in biodiversity maintenance and the conservation interest underlined by its characteristics being a glacial relict (Holderegger 1994), a serpentine fern (Tutin *et al*. 1980), and the most threatened plant species in the Romanian flora (Bartók and Irimia 2015), few studies regarding *A. adulterinum ex situ* conservation were done. In this context, a study concerning the distribution, population rate, and conservation plant framework for this species was published by Zo£nierz *et al*. (2008), in Poland. Further studies highlight the usefulness of *in vitro* cultures method to create prothallia "gene bank" for serpentine fern species (Marszał-Jagacka and Kromer 2011, Aldea *et al*. 2016).

After any type of *ex situ* conservation protocol is mandatory to verify the genetic stability of *in vitro* conserved plant germplasm. The samples used were maintained on the MS added with plant growth factors for 8 years. In the present investigation, the amplification profiles of 11 samples from medium-term culture were from 150 to 980 bp that could be scored. Of these bands, 16 were specific to the species *A. adulterinum* compared with *A. trichomanes*. For 10 samples of *A. adulterinum* induced through spores' germination of the same individual, a total of 27 bands

were scored (Table 1). Cluster analysis was done based on the genetic distance matrix including the outlier (Fig. 1).

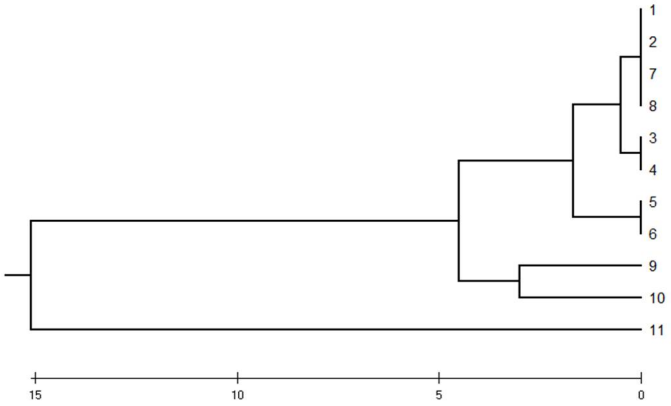

Fig. 1. UPGMA dendrogram based on genetic distances between *in vitro* maintained gametophytes of *A. adulterinum* inferred from on RAPD markers. 1-10: *A. adulterinum* gametophyte fragments from *in vitro* culture; 11- outlier represented by *A. trichomanes.*

Table 1. Description of random decamer primers used for the analysis of *in vitro* preserved *A. adulterinum*.

Primer code	Primer sequence	Fragment size (bp)	Polymorphic loci (%)
r-07	GAA ACG GGT G	300 - 750	75%
r-08	GTG ACG TAG G	320 - 930	85.71%
r-09	GGG TAA CGC C	260 - 500	0%
r-10	GTG ATC GCA G	420 - 1200	0%
r-17	GAC CGC TTG T	410 - 950	42.86%

The similarity indices ranged from 59. 25 to 100% among gametophytes maintained for 8 years in the same *in vitro* conditions. All the samples (1-8) display a minimum 88.88% similarity (have 24 out of 27 matching loci). The 3 subsets of samples display total identity (1, 2, 7, 8), (3, 4), and (5, 6). However, two of the samples (9 and 10) display significant differences compared to the rest of the samples (up to 11 out of 27 mismatching loci) and amongst each other (5 mismatching loci).

The RAPD technique, being a random method is prone to errors. In the present study the 3 different loci could be authentic or an artifact due to different DNA concentrations in the samples or the randomized annealing of the primers. Recent genetic studies showed that *in vitro* culture induced different mutation types in all species studied (Chandra and Thoyajaksha 2018). Variations may be attributed to *in vitro* stress induced by biochemicals (Devarumath *et al.* 2002) or subcultures number. In the case of long-term micropropagated *Olea europaea* shoots, different level of genetic variability was identified through RAPD after the seventh subculture on the same medium variant (Peyvandi *et al.* 2009). Taking into account that the cause of genetic variability cannot be accurately identified, more analyses or elimination of individuals with variability from *in vitro* culture are required. There is no technique that can completely guarantee the genetic

fidelity of the micropropagated plants (Mallón *et al*. 2010). Due to its attributes, the RAPD method is often used to verify genetic stability *in vitro* cultures.

Using the RAPD method an 88.88% similarity among the *A. adulterinum* gametophytes maintained in the same physical and chemical conditions for 8 years was observed. For a better analysis, the further molecular analysis should be performed.

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