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Morphological, cytological, and genetic diversity of the *ex situ* and *in situ* populations of the giant plantain, yellow flax, late-flowering carnation, and sand carnation

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1. INTRODUCTION AND OBJECTIVES

The loss of plant diversity in the world is becoming increasingly serious problem. According to a report by the Royal Botanic Gardens, Kew (2020), nearly 40% of the 350,000 known vascular plant species are threatened with extinction. *In situ* conservation is the preferred conservation method, but some biotic and abiotic factors can impede the long-term survival of the populations. *Ex situ* means the conservation of the species outside their natural habitats which is an important complement to the *in situ* method. The Global Strategy for Plant Conservation (GSPC) has set the target of conserving at least 75% of protected species in *ex situ* collections by 2020.

The aim of *ex situ* conservation is to ensure high genetic representativeness of the protected species. This objective includes both morpho-phenological variability and the provision of taxon-specific collections with high genetic variability. In many cases, there is a lack of literature on the morpho-phenological and population genetic specificity of protected species, which also hinders the most accurate and specific sampling. In addition, limited data is available on *ex situ* populations established as living collections in botanical gardens. Adverse processes can occur in these living collections, such as a significant loss of genetic variability due to genetic drift and inbreeding depression. Genetic drift is particularly significant in small and isolated populations. These protected species in the living collections are planted in a different habitat condition far from their original location. Adaptation to new environmental conditions may cause morpho-phenological changes, which may inhibit subsequent reintroduction (repatriation) processes. At the same time, regular horticultural maintenance may have a negative impact on the genetic material conserved, leading to reduced selection pressure or even unwanted selection.

The giant plantain (*Plantago maxima* Juss. ex Jacq.) is a highly protected member of the native flora. Its Hungarian populations represent the westernmost populations along species' range. Until now, the morpho-phenological, cytological and population genetic knowledge of natural populations is incomplete. The populations are threatened by the gradual conversion of the habitats due to excessive river regulation, the spread of invasive species and the increased risk of fire due to military activities. An *ex situ* population of the species was established in 2016 in the Soroksár Botanical Garden, and conservation biological assessment of its populations is therefore very timely.

Yellow flax (*Linum flavum* L.) is a protected species in Hungary, and its populations are threatened by the gradual disappearance of its habitat. The *ex situ* stock was established in the Soroksár Botanical Garden between 1987-1993, collected from Alsószentiván. The research will not only provide an opportunity to explore the background diversity of the *in situ* populations, but also to evaluate the impact of the long-term conservation.

The late-flowering carnation (*Dianthus serotinus* W. et K.) from the section *Plumaria* is an endemic and protected species in Hungary. The *ex situ*

population was planted in the Soroksár Botanical Garden in 1975-1976 from Kápolnadomb, on the outskirts of Dunaharaszti. The original habitat was destroyed, so the population in the botanical garden preserves the last trace of this population. The other protected member of the section, which occurs in sandy areas is the sand carnation (*Dianthus arenarius* L.). Based on recent phylogenetic studies, the only proven occurrence of this species in Hungary was confirmed from the Scots pine forest area near Fenyőfő (Somogyi 2014). The morphological, chromosomal and population genetic evaluation of the two taxa is an important prerequisite for the development of *ex situ* conservation.

Objectives of this study were as follows:

1. Assessment of the phenotypic traits in the *in situ* and *ex situ* populations of the plant taxa studied. Determine the morphological variability of natural populations. Evaluation and comparison of morphological variability in *ex situ* versus *in situ* populations.
2. Assess the accompanying species in the habitat to compare *in situ* and *ex situ* populations of the giant plantain and yellow flax taxa.
3. Determination of species' chromosome numbers and fluorescent *in situ* hybridisation of the chromosomes of the giant plantain and late-flowering carnation and sand carnation populations to reveal the natural variability of the *in situ* populations.
4. ISSR and cpDNA marker analysis to explore the genetic diversity of the giant plantain populations. Comparison of chloroplast DNA haplotypes with samples from a Kazakhstan population to map potentially unique native haplotypes relative to the central population.
5. Application of universal and newly developed taxon specific cpDNA markers to map haplotypes in late-flowering carnation and sand carnation populations.

2. MATERIALS AND METHODS

The studied species, localities and the morphological traits examined

The locations and *in situ/ex situ* status of populations related to the taxa under study are listed in Table 1. The morphometric analyses of the four taxa were carried out to identify the vegetative and generative traits that characterise the species and could separate the populations from each other.

Table 1. Geographic location and *in situ/ex situ* status of the populations of studied taxa.

Taxon	Populations (status)
<i>Plantago maxima</i> Juss. ex Jacq.	Kakucs (<i>in situ</i>), Táborfalvai Katonai Lőtér (<i>in situ</i>) Tatárszentgyörgy (<i>in situ</i>) Kunpeszéri Szalag-erdő (<i>in situ</i>) Soroksári Botanikus Kert (3 subpop. moist, mesophilic, steppic) (<i>ex situ</i>) Kazahsztán (<i>in situ</i>)
<i>Linum flavum</i> L.	Pócalja (<i>in situ</i>) Érd-Kakukk-hegy (<i>in situ</i>) Érd-mészkö plató (<i>in situ</i>) Soroksári Botanikus Kert (<i>ex situ</i>)
<i>Dianthus serotinus</i> W. et K.	Szigetmonostor (<i>in situ</i>) Farmos (<i>in situ</i>) Tatárszentgyörgy (<i>in situ</i>) Soroksári Botanikus Kert (<i>ex situ</i>)
<i>Dianthus arenarius</i> L.	Fenyőfő (subpop. Anyafás újulat, Kurucerdő, Hangosrét) (<i>in situ</i>) Latvia (Jaunupe, Tārgale Parish) (<i>in situ</i>)
<i>Dianthus plumarius</i> L.	Balatongyörök (<i>in situ</i>)

In the case of the giant plantain, we evaluated and compared all the native populations with each other and with the *ex situ* collection we established in 2016. In addition to the native peripheral populations, a central sample from Kazakhstan was included in the chloroplast study.

When selecting the yellow flax populations for study, we selected populations occurring in protected areas, but also chose areas where human impact is already more pronounced, to assess the potential plasticity among the studied populations. Within the *Plumaria* section of the genus *Dianthus*, due to the difficulty of the species delimitation and the lack of distinguishing morphological traits, the selection of the studied populations was based on the Vascular plants of Hungary online database and on previous molecular studies by Somogyi (2014). The occurrence of sand carnation in Hungary was first proposed by Baksay (1972) based on a morphological and cytological study. Somogyi (2014) confirmed the presence of the species in the pine forest near Fenyőfő by molecular studies. As

sand carnation is possibly a relict species in our country, we considered it appropriate to include this species in our studies.

Germination study

Germination biological studies were conducted with yellow flax and late-flowering carnation. Germination was carried out in the greenhouse of Soroksár Botanical Garden (47°24' - 19°09') under natural light conditions in spring 2019. The seeds were sown in Petri dishes lined with absorbent paper. Germination was evaluated once a week from 24.04.2019 to 06.11.2019. Germination percentage was calculated using the following formula: Germination % = Number of seeds germinated/Total seeds sown x 100

Assessment of the vegetation accompanying the studied species

A complete survey of the species inventory was also performed in the habitat of the *in situ* and *ex situ* populations of the giant plantain and yellow flax. For giant plantain, the survey was conducted in 2020, and species present in each population were recorded in 5 1x1 m quadrats. For the yellow flax one species list per population was recorded. Based on the species lists, the indicator values for the species were extracted from the FLÓRA database (Horváth *et al.* 1995). The attributes examined were Borhidi's relative temperature requirement - TB, relative indicators of groundwater and soil moisture - WB, and relative soil reaction – RB. The floristic similarity of the habitats was measured using the Jaccard index (Jaccard 1908).

Metaphase chromosome preparation and FISH

Chromosomal analyses were performed on giant plantain, late-flowering carnation and sand carnation species, using 5-20 seed samples collected randomly from each population. Chromosome preparation was performed following the work of Mlinarec *et al.* (2006). FISH (fluorescence in situ hybridization) analysis was performed on *Plantago maxima*, *Dianthus serotinus* (Szigetmonostor) and *Dianthus arenarius* (Fenyőfő/Hangosrét) according to the work of Mlinarec *et al.* (2012) with minor modifications. Clone pTa794, used as a 5S rDNA probe, contained the complete 410-bp BamHI fragment from the 5S rRNA gene and spacer region of wheat. The 2.4 kb HindIII fragment from the partial 18S rDNA and ITS1 of *Cucurbita pepo* was cloned in vector pUC19 and used as a 35S rDNA probe.

ISSR study of *in situ* and *ex situ* populations of the giant plantain

A total of 100 specimens from all Hungarian *Plantago maxima* populations were sampled and subjected to ISSR analysis. Eight primer pairs (UBC 807, 808, 809, 811, 816, 818, 835) were selected for the analysis. Gel images were evaluated

using GelAnalyzer 19.1 software. Binary data of ISSR markers were analysed as dominant markers using GenAlEx 6.51b2.

CpDNA analysis in *Plantago maxima*, *Dianthus serotinus* and *Dianthus arenarius*

Among the chloroplast markers successfully used in the genus *Plantago*, we used the marker *trnL-trnF* to compare domestic and Kazakh populations (Ronsted *et al.* 2002). Beside our samples we added samples from the GenBank. For the *Dianthus* taxa, we assessed universal chloroplast markers (*5' rps12-rpL20*, *rpoB-trnCGCAR*, *psbM-trnDGUC*, and *ycf1b*) and six new primer pairs designed for the two taxa. We included specimens of *Dianthus plumarius* species to test the within-section variability of the primers. The six new primer pairs were developed on the variable regions of the chloroplast genome of *Dianthus caryophyllus* (GenBank KU904222.1) and *Dianthus superbus* var. *longicalycinus* (GenBank KM668208.1).

Statistical evaluation of the morphometric traits and germination percentage

For statistical evaluation of vegetative and generative parameters, multivariate analysis of variance (MANOVA) was used using IBM SPSS 27 software, and discriminant analysis was also run. Analysis of variance (ANOVA) was used to evaluate germination power.

Evaluation of molecular data

The ISSR binary data matrix was evaluated using GenAlEx 6. software. Spatial genetic structuring of populations was assessed using STRUCTURE 2.3.4 software based on the Bayesian clustering. BioEdit Sequence Alignment Editor 7.2.5 software was used to edit and visualize chloroplast sequences and to analyse SNPs and indels. Maximum likelihood (ML) and Bayesian analyses were performed to evaluate phylogenetic relationships for *Dianthus* species by testing 10 primers, and ML analyses were run for the selected 5 primers. Haplotype network analysis was used to assess lineage relationships between the *Plantago* and for the *Dianthus* sequences. Haplotype networks were generated using PopART software with Templeton-Crandall-Singh (TCS) analysis.

3. RESULTS

Results with *Plantago maxima*

Morphometric studies

Our morphometric studies performed between 2018 and 2020, have shown that leaf number is a stable parameter in both *in situ* and *ex situ* stands, and therefore can be evaluated this as a taxon-specific marker. Leaf number also showed greater stability among habitats. It is important to highlight that along the measured morphological parameters, the *ex situ* population matched the morphological variability observed in the *in situ* populations. This result may indicate a stabilisation of the *ex situ* stands, which is favourable in case of long-term conservation.

In the study period between 2016-2020, a stabilisation process was observed in both leaf size and number of leaves among the three subpopulations (moist, mesophilic and steppic) of the *ex situ* stand compared to the year of establishment in 2016. In 2017, the number of leaves decreased significantly, which can be explained by the stress caused by the introduction into a new environment. The number of leaves has gradually increased over the years, which may show a stabilisation of the subpopulations. In the year 2019-2020, a decrease in leaf length was observed, coupled with an increase of the leaf number. Individuals from the steppe and moist subpopulations had higher leaf parameters compared to individuals from the mesophilic subpopulation. This difference in size can be explained by possible competition pressure, as species from both subpopulations exerted an increased shading effect on the giant plantain individuals. Plants from the moist subpopulation developed significantly more leaves compared to the other two subpopulations. This result may be related to the environmental adaptation of the species and the start of the generative phase. In addition to the morphometric studies, it is important to highlight the result that in the *ex situ* moist subpopulation, three individuals developed inflorescences in 2019 and four individuals in 2020, which is an important indicator of the stabilization of the subpopulation to the new environment.

Assessment of the surrounding vegetation of the studied species

The species lists of the habitats were classified according to the Jaccard index using a multivariate hierarchical classification (UPGMA - Unweighted pair-group average) based on the presence-absence data. Two major groups were formed: one group consisting of the 4 *in situ* stand while the other of the 3 *ex situ* stands. The separation of the *ex situ* stands from the *in situ* stands and the different association relationships behind this may explain the lack of flowering in the two *ex situ* stands and the low number of flowering individuals in the moist *ex situ*

stand. However, it is important to point out that many of the species that occur in the *in situ* populations are also found on the *Molinia* fen meadow of the Soroksár Botanical Garden, but not in the vicinity of the *ex situ* stands. This result may provide important information for the designation of new *ex situ* habitat for the future. *In situ* populations are further subdivided according to geographical distance. The only exception is the Táborfalva population, which is significantly less poor in species due to strong competitive pressure (*Solidago* spp., *Phragmites australis*), trampling from grazing and military activity.

Chromosome study

We confirmed the chromosome number $2n=2x=12$ in all Hungarian giant plantain populations and we reported for the first time the FISH pattern for the 35S and 5S rDNA regions of the species (Figure 1.). The species has five metacentric and one acrocentric chromosome pairs with a karyotype formula of $5m+1a$. FISH mapping revealed that the 35S and 5S rDNA regions are located on the acrocentric chromosome number 6. The chromosome pair is heteromorphic. 35S rDNA probe is located in one, subterminal position. The 5S rDNA region is located in two positions, proximal and subterminal to the centromeric region on the long arm. The 5S rDNA locus located proximal to the centromere region is smaller in size.

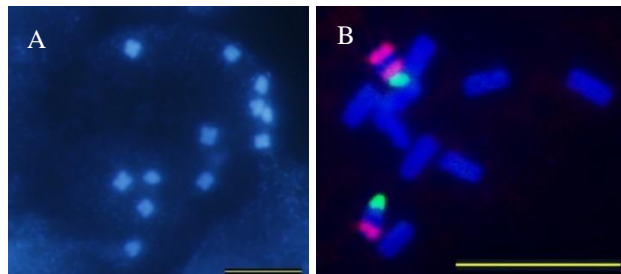


Figure 1. A) Metaphase chromosome of the giant plantain ($2n=2x=12$). B) FISH mapping: 35S rDNA probe (green) and (B) 5S rDNA probe (red). Scale = 10 μ m (Photos: Jelena Mlinarec).

ISSR study

The 8 ISSR primers yielded 100 distinct and reproducible electrophoretic bands in the range 280–2000 bp for the 100 individuals tested, with a polymorphism rate of 87.0%. The highest polymorphism was in the Kunpezsér population, where two private bands were detected. The lowest Nei genetic diversity was found in the Kakucs and the *ex situ* populations ($He = 0.206$) and the highest in the Kunpezsér population ($He = 0.257$). The Nei genetic diversity among the *ex situ* population and the Kakucs population, is completely identical, which supports the conclusion that the *ex situ* population is originating from and closely related to the Kakucs population. The AMOVA analysis suggests that 8% of the total genetic diversity can be attributed to inter-population relationships and the remaining 92% to intra-population relationships. The STRUCTURE analysis

indicates admixture between individuals within clusters. Population genetic structure based on STRUCTURE analysis shows the separation of the Kunpeszér population and its location in separate clusters, as illustrated in Figure 2.

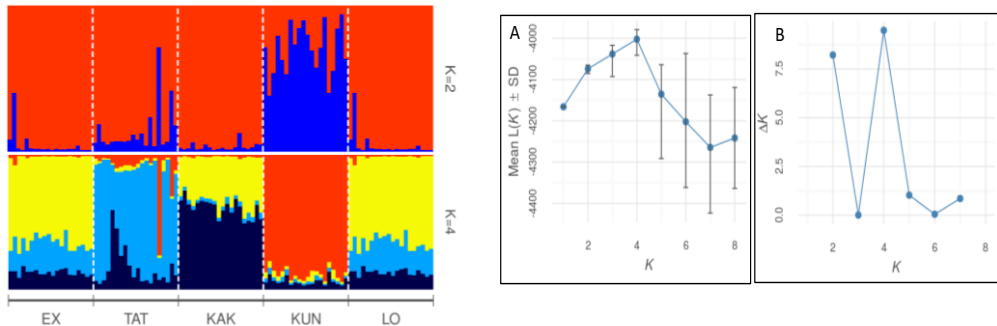


Figure 2. Estimated genetic population structure of *Plantago maxima* populations detected by STRUCTURE analysis based on ISSR markers for K = 2 and K = 4. STRUCTURE HARVESTER result. (A) probability mean $\ln K$ and (B) delta K. EX - Soroksár Botanical Garden – *ex situ*, TAT - Tatárszentgyörgy, KAK - Kakucs, KUN - Kunpeszér, LO - Táborfalva Katonai Lő- és Gyakorlótér.

CpDNA analysis of the *in situ* and *ex situ* *Plantago maxima* populations

The total length of the *trnL-trnF* sequence is 837 bp. The haplotype network contains four *Plantago maxima* haplotypes (H1-H4), haplotypes H5-H8 belong to different *Plantago* species (Figure 3.). No polymorphism was found among the sequences of the individuals from the native populations, which share a common haplotype (H1). There are two polymorphic positions among Hungarian and Kazakh samples: mononucleotide microsatellite (A) at position 226-236 bp, mononucleotide microsatellite (T) at position 589-597 bp. The Kazakh population has two haplotypes (H2-H3) based on the length of the two microsatellite regions. The *Plantago maxima* sample downloaded from NCBI represents the fourth haplotype (H4) with two SNPs at positions 38 and 247 bp.

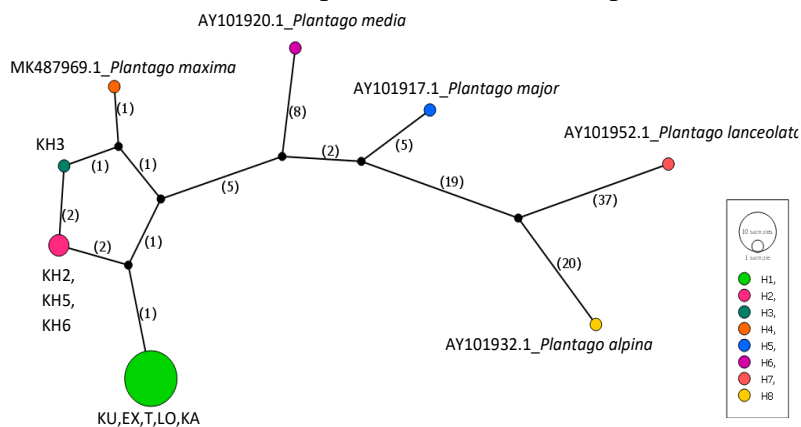


Figure 3. Haplotype network of the of *trnL-trnF* cpDNA region of *Plantago* samples performed with Templeton-Crandall-Singh analysis. Black dots indicate missing intermediate haplotypes not observed in the analysed sample set. The numbers on the branches indicate the mutational steps (number of base pair changes) between haplotypes.

Results with *Linum flavum*

Morphometric studies

Between 2018-2020, comparative evaluations were carried out in three *in situ* and one *ex situ* yellow flax populations. MANOVA revealed that individual height, petal width and number of flowers were strongly influenced by the year. The leaf length parameter was not affected by the year, but significant variation was observed among populations, making this trait suitable for exploring diversity among populations. The *ex situ* population from the botanical garden diverged from the *in situ* population along most of the studied morphological traits. Individuals of the garden stand were taller and developed larger leaves than individuals of the *in situ* populations, which can be explained by the shading due to the tree canopy. This split from natural variability may indicate a process of adaptation to garden conditions, which may adversely affect subsequent reintroduction efforts. Based on the discriminant analysis according to the first axis (which explained 89.5% of the variance), two major groups emerged. This shows the separation of *in situ* and *ex situ* populations, confirming the results obtained by MANOVA.

Germination study

ANOVA revealed a significant difference in final germination percentages among the populations studied ($F(3, 16) = 23.065, p < 0.001$). The highest germination percentage was in the botanical garden population (45.6%) and the lowest in the population of Pócalja (3.6%). The botanical garden population differed from the other populations during germination. Already at the first time of data collection, it reached a high germination percentage (42.40%), which was significantly different from the germination of *in situ* populations with slower germination ($F(3, 16) = 55.711, p < 0.001$).

Assessment of the surrounding vegetation co-occurring with the studied species

The numbers of species per stand were as follows: Soroksár Botanical Garden: 31 species, Érd, Szarmata limestone plateau: 58 species, Érd, Kakukk-hegy: 40 species, Pócalja: 66 species. Based on the presence-absence data of the species lists of the habitats, a multivariate hierarchical (UPGMA - Unweighted pair-group average) classification according to the Jaccard index was performed, as illustrated in Figure 4. Based on the clustering, the Érd, Kakukk-hegy population was separated as the least disturbed habitat and several protected species such as *Himantoglossum jankae* and *Dactylorhiza viridis* were found in the area. The three more anthropogenically exposed populations, including the *ex situ* population, formed a common group.

Results with *Dianthus serotinus* and *Dianthus arenarius*

Morphometric studies

Over the three years (2018-2020), the MANOVA for year as a block effect among the *in situ* populations yielded significant differences for all parameters tested, except for the petal length parameter ($F(2, 163) = 0.467, p=0.628$). Significant differences were found among habitats except for two traits (calyx width ($F(2, 163) = 0.479, p=0.620$) and petal width ($F(2, 163) = 0.195, p=0.823$).

In the two-year study among the *ex situ* and *in situ* populations, the MANOVA test revealed significant effects in the years for rosette diameter ($F(1, 138) = 16.664, p<0.001$), leaf length ($F(1, 138) = 4.825, p<0.05$) and calyx length ($F(1, 138) = 27.852, p<0.001$). There was no significant difference among habitats only in the case of the calyx length ($F(3, 138) = 1.460, p=0.228$).

A morphology-based comparison of *Dianthus serotinus* and *Dianthus arenarius* species populations was carried out in 2018. Individuals from the *ex situ* population were not surveyed in 2018 due to statistically insignificant number of individuals. MANOVA revealed significant differences among populations in rosette diameter ($F(3, 74) = 5.775, p<0.01$), calyx width ($F(3, 74) = 5.936, p<0.01$), leaf length ($F(3, 74) = 4.127, p<0.01$), petal length ($F(3, 74) = 7.530, p<0.001$) and number of flowers ($F(3, 74) = 8.508, p<0.001$). In 2020, we compared populations based on node size. We found significantly larger node size ($F(6, 108) = 41.466, p<0.001$) in all *Dianthus serotinus* populations we examined compared to *Dianthus arenarius* Fenyőfő subpopulations. While node size may be a taxonomically stable morphological trait, it is also important to note the possibility that this difference is simply caused by habitat differences.

Germination study

In the germination study with late-flowering carnation, we conducted the studies with batches from *ex situ* collections in Debrecen and Bern, due to the low seed production and damage caused by insects in the Soroksár Botanical Garden specimens in 2018. There was no clear separation in germination power among *in situ* and *ex situ*, however ANOVA revealed significant differences among collections based on final germination percentages ($F(4, 16) = 21.401, p<0.001$). The population from Bern exhibited the highest value (94.71%). In the germination test, populations with high germination power produced significantly higher germination values already at the first time of data collection.

Chromosome study

All three *Dianthus arenarius* subpopulations within Fenyőfő were exclusively hexaploids, with the chromosome number $2n=6x=90$. All *Dianthus serotinus* populations included in the study were also hexaploids ($2n=6x=90$), no mixed cytotype populations were detected. The *Dianthus arenarius* population from Latvia showed to be tetraploid with the chromosome number $2n=4x=60$. The metaphase chromosome preparations are illustrated in Figure 4.

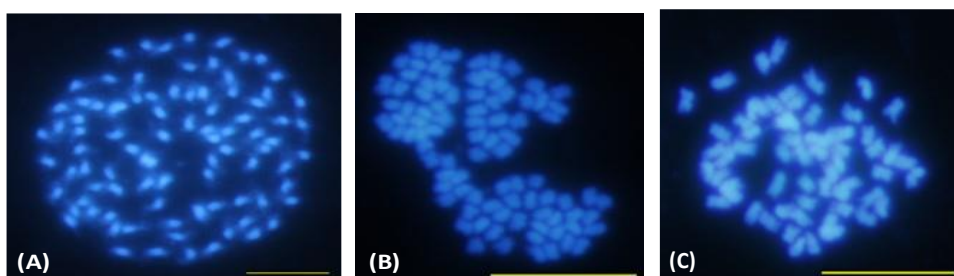


Figure 4: Metaphase chromosome preparations of (A) *Dianthus arenarius* Fenyőfő (Hangosrét) $2n=6x=90$, (B) *Dianthus serotinus* Szigetmonostor $2n=6x=90$, and (C) *Dianthus arenarius*, Latvia $2n=4x=60$. Scale bar = 10 μ m.

The FISH analysis showed that the Hungarian samples of *Dianthus serotinus* and *Dianthus arenarius* were similar both karyologically and cytogenetically. A slight difference in the number of FISH signals was found among the samples from Fenyőfő and Szigetmonostor, but no difference in the number and position of loci. In both samples, four 35S rDNA and one 5S rDNA loci were detectable. The 35S rDNA locus was located terminally, and the 5S rDNA locus had two 5S rDNA regions terminally and four interstitially.

CpDNA analysis of the *Dianthus* taxa

Based on preliminary studies, we selected five of the 10 primers (P7, P15, P16, P19, P20) that were considered polymorphic to include and test on larger sample size and additional taxa. Based on the resulting phylogenetic tree, no clear taxonomic segregation among the tested species could be detected. Specimens of several species were observed in different clades of the phylogenetic tree. For example, *Dianthus gratianopolitanus* individuals were grouped into separate clades. In the case of *Dianthus serotinus*, clustering by population was observed, where Szigetmonostor and Farnos samples clustered to separate clades. *Dianthus arenarius* samples from Latvia fall into a separate clade. However Soroksár and Fenyőfő samples, did not show clear clustering, as individuals on the phylogenetic tree grouped into different clades. In the clade marked in grey, all *Dianthus plumarius* samples grouped in a large polytomous branch, together with the

Soroksár and Fenyőfő samples, and the *Dianthus plumarius* subsp. *lumnitzeri* (syn. *Dianthus moravicus*) sample (Figure 5.).

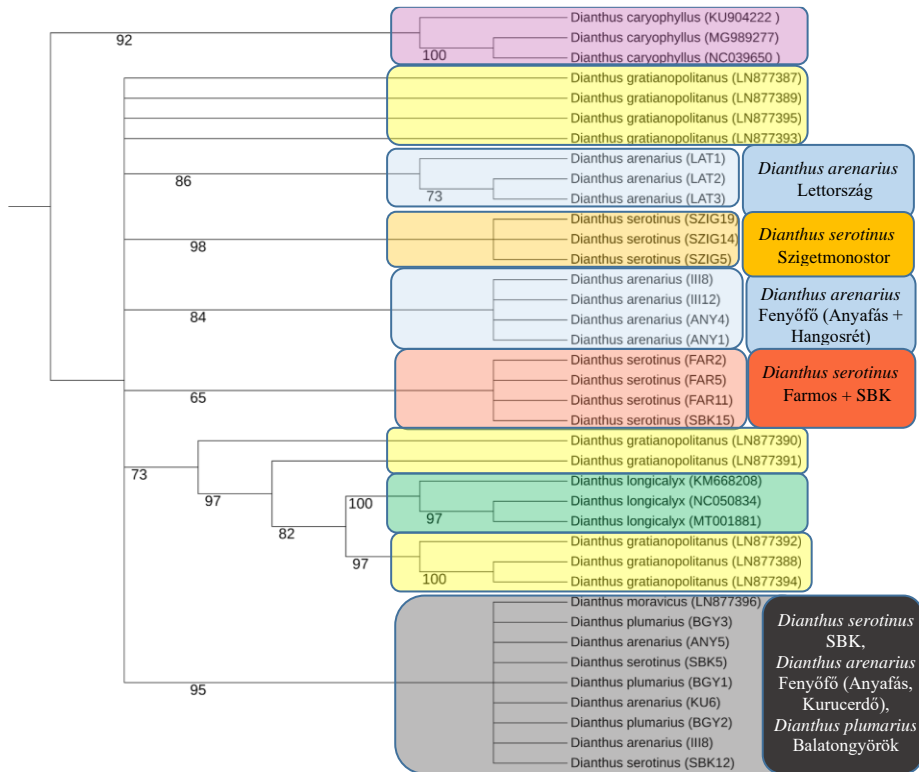


Figure 5: Maximum likelihood (ML) phylogenetic tree based on the five selected cpDNA regions. The species *Dianthus caryophyllus* was used as outgroup.

TCS analysis of 22 sequences resulted in 9 haplotypes. The Latvian samples were separated from the other groups by two haplotypes (H1 and H2). Fenyőfő samples were characterized by two haplotypes. The subpopulations of the Anyafás and the Hangosrét subpopulations were grouped into haplotype H3. However, the H8 haplotype also included samples from both the Anyafás and the Hangosrét subpopulations. For better clarity, the figure shows only the Fenyőfő sample, and the subpopulations are not shown. The samples from Farmos are of two haplotypes (H5 and H6). The samples from Szigetmonostor are of one haplotype (H3). The *ex situ* samples from Soroksár are classified into three haplotypes (H7, H8 and H9). The *Dianthus plumarius* samples from Balatongyörök are classified into haplotype H8.

4. CONCLUSIONS, RECOMMENDATIONS

Recommendations based on the giant plantain study

For morphological-based studies, it is beneficial to compare *ex situ* populations with the source *in situ* populations. Such kind of researches, however, are still very scarce today. Ensslin and Godefroid (2020) conducted morphology-based comparative studies with 12 species (*in situ* and *ex situ* stocks). In their studies, they found a significant change in phenotypic traits and variability in morphological traits decreased in *ex situ* collections in direct proportion with the increase in cultivation time. Sandner *et al.* (2022) compared a 30-year-old *ex situ* collection of *Digitalis lutea* in a study with two *in situ* populations, one of which was the original habitat of the *ex situ* collection. The garden individuals flowered earlier and differed from the natural population in generative traits, as they were adapted to self-pollination and bumblebees visited flowers less frequently. Greater morphological separation may therefore indicate unfavourable adaptive and genetic processes. In our morphological analyses with the giant plantain, we observed significant differences among *in situ* and *ex situ* stands along the parameters regarding leaf length and leaf width. It is important to highlight that in the *ex situ* stand, which was considered relatively young, we observed morphological variability similar as in the natural populations. This result is favourable, as the evolutionary potential is inherent in the genetic material of the collection. At the same time, it is important to highlight that the subpopulations have already diverged into distinct groups regarding leaf length and leaf width parameters, which can be evaluated as a local acclimatization. The emergence of a generative phase is an important indicator of the stabilisation of *ex situ* populations. For example, in a study with *Plantago lanceolata* only individuals with more than five leaves developed a generative phase with inflorescence (Antonovics and Primack 1982). Also, in the case of the giant plantain, we found that only the moist subpopulation with significantly higher leaf number had flowering individuals during the whole study period.

With the assessment of the surrounding vegetation of the studied species, we can get a better understanding of the species' environmental conditions and requirements. To our current knowledge, there is no detailed study comparing the surrounding vegetation of *in situ* and *ex situ* populations of the protected species. To ensure the success of repatriation, environmental parameter and species list surveys have been carried out in the *in situ* populations of *Salix lapponum*. The repatriation to the selected site was successful and a more detailed understanding of the habitats widen the knowledge on the biology and ecology of the species (Pogorzelec *et al.* 2020). During the habitat survey, species lists of *in situ* and *ex situ* populations of the giant plantain were classified into two separate groups based on UPGMA, which may explain the lack of flowering in the two subpopulations. This difference can be explained by differences in species composition rather than species number. However, comparing it with the morphological results, this difference did not result in a detectable morphological

adaptation, which is favourable for the long-term conservation of the species. When establishing new *ex situ* populations, it is recommended to plant the species in association conditions similar to those of the *in situ* populations, and the species lists recorded may be of help in the future.

Within the genus *Plantago*, intraspecific cytotype variability occurs. Rezsó Soó previously reported chromosome number of the native giant plantain and described the species as diploid ($2n=12$) (Soó 1970). Magulaev (1982), on the other hand, reported data from the central population with $2n=24$. During my research I assessed the chromosome number in all native populations and my results agreed with those reported by Soó (1970), with no cytotype differences among populations. FISH mapping has shown a species-specific pattern within the genus *Plantago*. To the best of our knowledge, is the first time that we have reported data on the FISH mapping of the giant plantain, showing a species-specific pattern.

Based on our analyses, native populations of the giant plantain show similar genetic diversity (mean value $He = 0.220$) compared to other protected and endemic *Plantago* species. This result is likely to be explained by gene flow among populations and recent isolation. AMOVA analysis suggests that 92% of the diversity is within populations. The low differentiation among populations can be explained by the small geographic distance, which was also observed for *Adonis vernalis* in the German exclave (Kropf *et al.* 2020).

The *ex situ* population of the giant plantain shows almost the same genetic diversity as the *in situ* populations ($He = 0.206$) and the same Nei genetic diversity as the Kakucs population. This result is favourable in terms of genetic representativeness. However, it is important to underline that due to the difference in genetic variability among populations (8%), it is recommended to establish further *ex situ* collections by including genetic material from other native populations in addition to the Kakucs population.

In the Hungarian populations of the giant plantain, we found one haplotype based on the *trnL-trnF* cpDNA region. However, in the Kazakhstan population, we found two haplotypes different from the one from Hungary. Haplotype differences among central versus marginal populations were also detected in *Poa badensis* (Plenk *et al.* 2019).

With regard to the *ex situ* conservation of the giant plantain, in the future, it is important to collect seeds from all the native populations and to establish new *ex situ* populations to conserve as much genetic diversity as possible. The seed collection, germination protocol used to establish the *ex situ* population seems to be suitable for further populations in the future. However, direct sowing the seeds in a designated patch of the *Molinia* meadow would be worth testing in the future. Based on my studies, the Soroksár Botanical Garden *Molinia* meadow may be suitable for the establishment of new populations, but when selecting sites, I recommend planting in moister areas where we have already experienced a generative phase in the existing *ex situ* subpopulation.

Recommendations based on the yellow flax study

The *ex situ* collection of the yellow flax is a long-preserved, historic *ex situ* collection that was planted in the garden more than 35 years ago. The Soroksár Botanical Garden stand diverged from the three *in situ* stands along the morphological parameters studied. This split is presumably the result of a longer period of local adaptation. This result is in agreement with the findings of Rauschkolb *et al.* (2019), where they described a decrease in fitness parameters and rapid differentiation of individuals preserved in *ex situ* collections from the *in situ* populations. These results were explained by local adaptation to garden conditions and genetic changes (genetic drift and inbreeding depression). The more vigorous growth of the botanical garden individuals may be explained by the fact that while in natural populations low shading is typical, the *ex situ* population grows under a row of fruit trees in tall vegetation under considerable shading.

In our germination study, the germination speed of the seeds from *ex situ* populations differed from the seeds collected from the *in situ* populations, as seeds from *ex situ* populations reached significantly higher germination percentages already at the first evaluation date compared to the *in situ* populations. Later this difference disappeared compared to one *in situ* population, but still a remarkably high final germination percentage was observed. This result is also in line with the study by Ensslin *et al.* (2018). In a germination study with 72 herbaceous species, they found that seeds from *ex situ* populations achieved significantly higher germination power and also showed a loss of seed dormancy compared to wild populations. This loss of dormancy was particularly significant in short-lived species.

Competition causes individuals to adapt to stress and interspecific competition may lead to the acquisition of adaptive traits. Based on the species lists recorded in the yellow flax populations, the *ex situ* population was grouped with two *in situ* populations (Pócalja and Érd, Szarmata limestone plateau). The effect of disturbance on the species composition is also evident in these three populations, so our results are mostly attributed to it. The Kakukk-hegy in Érd is only accessible by permission, so the degree of disturbance in this stand is minimal. The competition pressure may also differ among habitats in *in situ* and *ex situ* stands, which may also induce trait-based variation. Morphological and germination differences were also observed in the *ex situ* stand, which may be explained by the disturbed habitat and stronger competition pressure.

Our results should be complemented by genetic studies in the future. Since the original *in situ* population was destroyed, we consider the maintenance of the *ex situ* population greatly important, as it carries the last genetic fingerprint of the original population. However, as we observed a more significant adaptation to the garden conditions, we consider this population less suitable for reintroduction. For this reason, we also suggest establishing a new *ex situ* population, which could be suitable for future repatriation activities.

Recommendations based on the late-flowering carnation and sand carnation study

The late-flowering carnation is a native and protected member of the section *Plumaria*, so the conservation of this species has a great importance. However, the morphological similarity of the species from this section and the low sequence variability and consequent low phylogenetic structure among species further complicates taxon identification. The presence of *Dianthus arenarius* was previously reported by Baksay (1972) from Fenyőfő and confirmed by AFLP analysis by Somogyi (2014). During our investigations we carried out a conservation assessment of the late-flowering carnation stand established in the Soroksár Botanical Garden. We planned to evaluate the population of *Dianthus arenarius* in Fenyőfő on morphological, cytological and molecular genetic basis and compare it with *Dianthus serotinus*, thus giving a basis for a conservation plan for the sand carnation population in Hungary.

In the study of late-flowering carnation, we also observed greater morphological variability among natural populations in 2018-2020. The SBK *ex situ* population was clustered with the populations of Farnos and Tatárszentgyörgy in the 2019-2020 study period. This can be considered as a positive result, as the *ex situ* population fitted into the morphological variability among natural populations. In the *in situ* morphological comparative study of late-flowering carnation and sand carnation, the populations of the two taxa did not differ along the parameters. This result agrees with the results of Somogyi (2014), who found that the differences between the studied macro- and micromorphological traits within the *Plumaria* section are rather due to environmental background factors. Node size was the only trait that could be considered as a perspective trait that separated the Fenyőfő population individuals (for two subpopulations) from the late carnation populations. However, this trait may also be influenced by shading by pines, as opposed to direct light exposure of late-flowering carnation populations.

In our germination study, we observed a significant difference in germination power among seeds from *ex situ* and *in situ* populations of late-flowering carnation. This difference was not *in situ/ex situ* status specific, as it was with the yellow flax. We found significantly lower germination power in the Farnos *in situ* and Debrecen *ex situ* populations than in the Bern *ex situ* and the Szigetmonostor, Tatárszentgyörgy *in situ* populations. This difference is in line with the results reported in the PhD thesis of Cevallos González (2021), where he found a positive correlation among collection site and seed weight and germination percentages.

The genus *Dianthus* is characterized by a large number of complex species groups and has one of the highest rates of species diversification (Valente *et al.* 2010, Fassou *et al.* 2022). Low genetic distances are observed within the genus, which limits the phylogenetic resolution at the species level, which could be explained by rapid radiation (Valente *et al.* 2010). The low mutation rate between

species (Fassou *et al.* 2022) also causes taxonomic uncertainties, which makes conservation activities harder to implement.

Within the genus *Dianthus*, in addition to the cytotype variability among populations, mixed cytotype populations also occur (Weiss *et al.* 2002). Chromosome analysis in both taxa revealed a chromosome number of $2n=6x=90$ in the Hungarian populations. This result agrees with the work of Rezső Soó, who draws attention to the uncertainty of the $2n=4x=60$ data in late-flowering carnation (Soó 1970). However, the hexaploid cytotype in the population of Fenyőfő is a surprising result, since previously Baksay (1972) described a chromosome number of $2n=4x=60$. In contrast, the Latvian *Dianthus arenarius* sample is indeed tetraploid $2n=4x=60$. There is no previous study with FISH within the genus *Dianthus*, however, it could be an important starting point for the elucidation of polyploidization processes within the genus. In *Dianthus serotinus* and *Dianthus arenarius* samples, no differences were found in the number and location of loci in the FISH pattern of hexaploid samples, which may also indicate recurrent evolution, rapid radiation and close relatedness within the section.

The genus *Dianthus* has a low mutation rate based on recent studies (Fassou *et al.* 2022). Species of *Dianthus* sect. *Plumaria* are presumed to be polyphyletic (Somogyi 2014). Previously used cpDNA regions (*3'trnK-matK*, *trnH-psbA* and *psbA-3'trnK*) showed no variability between the two species. In our studies, we wanted to develop markers that would help to identify the two species and detect haplotype diversity within populations. Overall, we observed higher sequence variability than with previously used markers, but this sequence variability was not suitable for phylogenetic resolution, which supports the idea of rapid radiation between taxa (Valente *et al.* 2010, Somogyi 2014, Fassou *et al.* 2022).

The haplotype tree shows that shared haplotypes were also found between the studied species. This result has been described for *Dianthus gratianopolitanus* and *Dianthus orientalis*, where samples from different populations represented several distinct lineages within a large clade (Fassou *et al.* 2022). These results were explained by a combination of common ancestral variation and the existence of continuous secondary genetic contact zones (Fassou *et al.* 2022). The shared haplotypes in the Soroksár samples may be indicative of potential interspecific hybridization processes within the garden.

The *ex situ* population of late-flowering carnation is the last genetic fingerprint of the natural *in situ* population, however, the potential introgression detected in the genetic studies with *Dianthus* taxa in the garden indicates an unfavourable genetic process. In the future, it would be important to establish new *ex situ* populations of *Dianthus serotinus* and *Dianthus arenarius* taxa from Fenyőfő, but separate management of genetic material is of paramount importance due to the possible introgression that has occurred within the garden.

NEW SCIENTIFIC RESULTS

1. In my dissertation, comparative study was performed on four plant species protected in Hungary. I evaluated the biological and conservation value of the *in situ* and *ex situ* populations:

1.1. Based on the results of the morphological studies, the *ex situ* populations of the giant plantain and late-flowering carnation fit into the morphological variability experienced in natural populations. In the case of yellow flax separation was observed, which is most probably the consequence of long-term *ex situ* conservation.

1.2. In the case of late-flowering carnation and yellow flax, I demonstrated the impact of *ex situ* conservation on the long-term germination. In the case of yellow flax, I found the absence of seed dormancy of the *ex situ* stock. In late-flowering carnation, the different germination characteristics of the *ex situ* stocks was demonstrated.

1.3. In the case of the giant plantain, late-flowering carnation and sand carnation, I performed for the first time fluorescence *in situ* hybridization on the chromosomes, and I clarified the chromosome number of the investigated populations in order to reveal the background diversity.

2. For the first time, I reported the genetic diversity of the Hungarian giant plantain populations based on ISSR. I found that among the natural populations, the Kunpezér population has the highest genetic variability and that the *ex situ* populations have a similar genetic diversity compared to the *in situ* populations.

3. Based on the *trnL-trnF* cpDNA region, I detected a distinct haplotype in the Hungarian populations compared to the central, Kazakh population. This result highlights the conservation biological value of the Hungarian peripheral populations.

4. I did not find morphological traits that are possibly suitable for taxon identification between late-flowering carnation and sand carnation. In terms of chromosome number, the Hungarian populations did not differ from each other ($2n=90$), but the sand carnation samples from Latvia were different ($2n=60$). I got a similar result based on the cpDNA region test, where I found differentiation only among the Latvian and Hungarian populations. According to my results, it can be assumed that introgression is acting on the sand carnation population in Fenyőfő.

PUBLICATIONS CONNECTED TO THE DISSERTATION

Papers in impact factored journals

- Kovács, Zs.,** Csergő, A. M., Csontos, P., Höhn, M. (2021): Ex situ conservation in botanical gardens – challenges and scientific potential preserving plant biodiversity. NOTULAE BOTANICAE HORTI AGROBOTANICI CLUJ-NAPOCA 49 : 2 Paper: 12334 , 21 p. [Q2, IF 1,168]
- Csontos, P., Tamás, J., **Kovács, Zs.,** Schellenberger, J., Penksza, K., Szili-Kovács, T., Kalapos, T. (2022): Vegetation dynamics in a loess grassland: plant traits indicate stability based on species presence, but directional change when cover is considered. *PLANTS-BASEL* 11:6 , Paper: 763. [Q1, IF 4,658]
- Kovács, Zs.,** Mlinarec, J., Höhn, M. (2023): Living on the edge: morphological, karyological and genetic diversity studies of the Hungarian *Plantago maxima* populations and established ex situ collection. *BOTANICAL STUDIES* 64:2 pp. 1-15. [Q2, IF: 2,673]

In reviewed article (MTA listed)

- Kovács, Zs.,** Barabás, S., Höhn, M. (2018): Az óriás útifű (*Plantago maxima* Juss. ex Jacq.) csírázásbiológiai vizsgálata. *BOTANIKAI KÖZLEMÉNYEK* 105 : 2 pp. 243-252. [Q4]
- Kovács, Zs.,** Barabás, S., Csontos, P., Höhn, M., Honfi, P. (2019): Az óriás útifű (*Plantago maxima* Juss. ex Jacq.) ex situ védelemben vonása II.: Élőhelypreferencia-vizsgálat. *BOTANIKAI KÖZLEMÉNYEK* 106 : 2 pp. 157-172. [Q4]
- Kovács, Zs.,** Tillyné Mándy, A. (2019): In vitro steril magvetés és mikroszaporítás az ex situ konzerváció szolgálatában. *KERTGAZDASÁG* (1998) 51 : 3 pp. 19-30.

Book chapters

- Höhn, M., Papp, L. ifj., **Kovács, Zs.,** Németh, A., Pándi, I., Papp, L., Radvánszky, A., Zsigmond, V. (2020): Ex situ növénymegőrzés – Gyűjteményes kertek a növényvilág megőrzésért Budapest, Magyarország: Magyar Arborétumok és Botanikus Kertek Szövetsége. ISBN: 9786158175302

Conference papers (abstracts):

- Kovács, Zs.,** Barabás, S. (2016): Csírázásbiológiai vizsgálatok az óriás útifű (*Plantago maxima* Juss.) ex-situ védelemben vonásához. In: XI. Aktuális Flóra- és Vegetációkutatás a Kárpát-medencében nemzetközi konferencia pp. 180-182.

- Kovács, Zs.,** Barabás, S. (2017): Ex-situ conservation of the giant plantain (*Plantago maxima* Juss.) in Soroksár Botanical Garden In: 3rd Conference of Eastern and Central European Botanic Gardens : Botanic Gardens - delivering public goods and supporting society Budapest, Magyarország 40 p. pp. 31-32.
- Kovács, Zs.,** Barabás, S., Honfi, P., Höhn, M. (2018): Az óriás útifű (*Plantago maxima* Juss.) csírázásbiológiai vizsgálatai és ex situ védelembe vonása. BOTANIKAI KÖZLEMÉNYEK 105 : 2 pp. 288-289. , 2 p.
- Kovács, Zs.,** Höhn, M. (2018): *Dianthus arenarius* és *Dianthus serotinus* chorológiai jellemzői, morfológiai és diverzitásvizsgálata a Pannonicum területén. In: Bodor, Péter; Fodor, Marietta (szerk.) Ifjú Tehetségek Találkozója Budapest, Magyarország : Szent István Egyetem 169 p.
- Kovács, Zs.** (2019): Az óriás útifű (*Plantago maxima* Juss.) ex-situ fenntartásának első eredményei a Soroksári Botanikus Kertben In: Komlósi, István; Juhász, Csaba; Juhász, Lajos; Gyüre, Péter; Vári, Erzsébet (szerk.) XXXIV. Országos Tudományos Diákköri Konferencia. Agrártudományi Szekció. Előadáskivonatok. Debrecen, Magyarország : Debreceni Egyetem Agrár- és Gazdálkodástudományok Centruma Mezőgazdaság-, Élelmiszertudományi és Környezetgazdálkodási Kar pp. 309-309.
- Kovács, Zs.,** Csontos, P., Höhn, M. (2019). Ex situ conservation: problems and future perspectives with some examples from the Hungarian flora In: Benedek, Klára Vth Horticulture and Landscape Planning Conference from Transylvania pp. 12-12.
- Kovács, Zs.,** Höhn, M., Csontos, P. (2020): Az ex situ konzerváció lehetőségei és korlátai, nemzetközi és hazai példák bemutatásával: Magyar Biológiai Társaság, Botanikai Szakosztály, 1499. szakülésének kivonata BOTANIKAI KÖZLEMÉNYEK 107 : 2 p. 250
- Kovács, Zs.,** Mlinarec, J., Höhn, M. (2021): Chromosome-based comparison of *Dianthus arenarius* L. and *Dianthus serotinus* Waldst. & Kit. Populations In: Fodor, Marietta; Bodor-Pesti, Péter; Deák, Tamás (szerk.) SZIENTific Meeting for Young Researchers 2020 : ITT Ifjú Tehetségek Találkozója 2020 Budapest, Magyarország : Szent István University pp. 249-250.
- Kovács, Zs.,** Höhn, M. (2022): Experiences in the ex situ conservation of the giant plantain (*Plantago maxima* Juss. ex Jacq.) in Soroksár Botanical Garden. In: Höhn, Mária (szerk.) Abstract Booklet of the 9th European Botanic Gardens Congress (EuroGard IX) Budapest, Magyarország : MABOSZ 131 p.
- Höhn, M., Miklósi, Sz., Bottlik, G., **Kovács, Zs.** (2022): Conserving natural forest steppe within the territory of the Soroksár Botanical Garden. In: Höhn, Mária (szerk.) Abstract Booklet of the 9th European Botanic Gardens Congress (EuroGard IX) Budapest, Magyarország MABOSZ (2022) 131 p.

REFERENCES

1. Antonovics, J., Primack, R.B. (1982): Experimental ecological genetics in *Plantago*: VI. The demography of seedling transplants of *P. lanceolata*. *The Journal of Ecology*, pp. 55-75.
2. Baksay, L. (1972): Biosystematik der *Dianthus plumarius* L. (sensu lato) in Ungarn. In: *Symposia Biologica Hungarica* 12. 149-161.
3. Cevallos González, J. D. (2021): Delineation of seed transfer zones based on ecological knowledge and testing based on seed traits from different provenance for ecological restoration use. 113 p. Egyetemi doktori értekezés, ELTE.
4. Ensslin, A., Godefroid, S. (2020): Ex situ cultivation impacts on plant traits and drought stress response in a multi-species experiment. *Biological Conservation*, 248: 108630.
5. Ensslin, A., Van de VyVer, A., Vanderborcht, T., Godefroid, S. (2018): Ex situ cultivation entails high risk of seed dormancy loss on short-lived wild plant species. *Journal of Applied Ecology*, 55(3): 1145-1154.
6. Horváth, F., Dobolyi, Z. K., Morschhauser, T., Lőkös, L., Karas, L., Szerdahelyi, T. (1995): FLÓRA adatbázis 1.2: Taxonlista és attribútum-állomány. Flóra munkacsoport. Vácátót, Magyarország: MTA Ökológiai és Botanikai Kutatóintézete 267 p
7. Jaccard, P. (1908): Nouvelles recherches sur la distribution florae. *Bulletin de la Société Vaudoise des Sciences Naturelles*, 44, 223-270
8. Kropf, M., Bardy, K., Höhn, M., Plenck, K. (2020): Phylogeographical structure and genetic diversity of *Adonis vernalis* L. (Ranunculaceae) across and beyond the Pannonian region. *Flora*, 262, 151497.
9. Magulaev, A. Y. (1982): The number of chromosomes of the species of Asteraceae, Caryophyllaceae and Plantaginaceae of the North Caucasus. *Scientific Reports of Higher School for Biological Sciences*, 11(227), 74-79.
10. Mlinarec, J., Papeš, D. A., Besendorfer, V. (2006): Ribosomal, telomeric and heterochromatin sequences localization in the karyotype of *Anemone hortensis*. *Botanical Journal of the Linnean Society*, 150, 177–186.
11. Mlinarec, J., Šatović, Z., Mihelj, D., Malenica, N., & Besendorfer, V. (2012): Cytogenetic and phylogenetic studies of diploid and polyploid members of tribe Anemoninae (Ranunculaceae). *Plant Biology*, 14(3), 525-536.
12. Plenck, K., Bardy, K., Höhn, M. and Kropf, M. (2019): Long-term survival and successful conservation? Low genetic diversity but no evidence for reduced reproductive success at the north-westernmost range edge of *Poa badensis* (Poaceae) in Central Europe. *Biodiversity and conservation*, 28, 1245-1265.
13. Pogorzelec, M., Bronowicka-Mielniczuk, U., Serafin, A., Parzymies, M. (2020): The importance of habitat selection for the reintroduction of the endangered *Salix lapponum* L. in eastern Poland. *Journal for Nature Conservation*, 54, 125785.
14. Rauschkolb, R., Szczeparska, L., Kehl, A., Bossdorf, O., Scheepens, J. F. (2019): Plant populations of three threatened species experience rapid evolution under ex situ cultivation. *Biodiversity and Conservation*, 28, 3951-3969.
15. Rønsted, N., Chase, M.W., Albach, D. C., Bello, M. A. (2002): Phylogenetic relationships within *Plantago* (Plantaginaceae): evidence from nuclear ribosomal ITS and plastid trnL-F sequence data. *Botanical Journal of the Linnean Society*, 139(4), 323-338.
16. Sandner, T.M., Gemeinholzer, B., Lemmer, J., Matthies, D., Ensslin, A. (2022): Continuous inbreeding affects genetic variation, phenology, and reproductive strategy in ex situ cultivated *Digitalis lutea*. *American Journal of Botany*, 109(10), 1545-1559.
17. Somogyi, G. (2014). A *Dianthus* sect. *Plumaria* (Opiz) Asch. et Graebn. közép-európai fajainak komplex molekuláris taxonómiai értékelése= Taxonomical evaluation of Central-European

- Dianthus* species of sect. *Plumaria* (Opiz) Asch. et Graebn based on molecular evidences. (Egyetemi doktori értekezés, Budapesti Corvinus Egyetem).
18. Soó, R. (1970): A magyar flóra és vegetáció rendszertani-növényföldrajzi kézikönyve IV. Akadémiai Kiadó. Budapest. 594 pp.
 19. Valente, L. M., Savolainen, V., Vargas, P. (2010): Unparalleled rates of species diversification in Europe. *Proceedings of the Royal Society B: Biological Sciences*, 277, 1489–1497.
 20. Weiss, H., Dobeš, C., Schneeweiss, G. M., Greimler, J. (2002): Occurrence of tetraploid and hexaploid cytotypes between and within populations in *Dianthus* sect. *Plumaria* (Caryophyllaceae). *New Phytologist*, 85-94.