PHD THESIS

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Taxonomical evaluation of Central-European *Diantus* species of sect. *Plumaria* (Opiz) Asch. et Graebn based on molecular evidences.

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PRELIMINARY WORKS AND MAJOR AIMS

Dianthus section *Plumaria* (Opiz) Asch. et Graebn. (syn. sect. *Fimbriatum* Boiss.) is the largest group within carnations. In some cases it was treated as a genus on its own (e.g. Opiz, 1852). The representatives of sect. *Plumaria* are widespread throughout Eurasia, but Williams (1893) and Meusel & Mühlberg (1979) mention species of this section also from the tropical African region and South-Africa. This latter statement however, appears to be contradicted by recent molecular evidence (Valente et al. 2010).

Williams (1893) listed 49 species from the sect. *Fimbriatum,* that. were enrolled into 5 subsections (*Plumarioides*, *Schistostolon*, *Cycaxostolon*, *Gonaxostolon* és *Monerestolon*). However, based on the morphological traits that Williams used in his classification, species with nearly identical morphology were classified into different infrageneric groups. Thus this division of *Dianthus* species is certainly not meaningful.

Kovanda (1982) reported about 30 species worldwide belonging to the sect. *Plumaria,* with a distribution area ranging from Western Europe up to North Africa, as well as to the Himalayas.

According to Vierhapper (1901) and Ascherson & Graebner (1929) the Central- and South-Eastern European region can be considered an important diversification center of sect. *Plumaria* justified also by the large number of feather carnation taxa occurring in this region.

In Europe sect. *Plumaria* is represented by approximately 10–20 species, the precise number of taxa depends on the species concept of the different authors. The most detailed taxonomic review of the European sect. *Plumaria* taxa was carried out by Novák (1928). In his work 9

European species are listed with altogether 70 infraspecific feather carnation taxa, including subspecies, varieties and forms as well.

Molecular investigation of Central- and South-eastern European species of *Dianthus* sect. *Plumaria* has recently become topical in several aspects. The section is morphologically uniform and no such clear character is available that enables the unambiguous separation of the species from each other. There is no widely accepted consensus about the exact number of taxa belonging to the section. Species concept of the authors was sometimes very different: some taxa were ranked as separate species by certain authors, while others treated them only as subspecies. For the above mentioned reasons the nomenclature and the chorology of the species belonging to the sect. *Plumaria* raises several unresolved questions.

Main goals

By performing morphological, molecular and pollination-ecological investigations on *Dianthus* species of sect. *Plumaria* from Central- and South-East Europe our major objectives were:

1. to confirm or reject the monophyletic origin of sect. *Plumaria*.
2. to clarify the relationship among the species of the section.
3. to evaluate the taxonomic position of the Central European species within sect. *Plumaria* .
4. to reveal nomenclatural problems, phylogenetic relationships within the Central European range and to outline species' borders.
5. to review those ecological factors that are involved in the formation of the white, feather-like petals.
6. to clarify the association between flower morphology and pollination type of *Dianthus plumarius* L.

MATERIAL AND METHODS

Morphometric analyses

Based on the related literature (respective publications?) we summed up and evaluated the morphological characters mentioned in connection with the European taxa belonging to the sect. *Plumaria*. For morphometric analyses we selected those morphological traits that are easily measured, assumed to be (more-or-less) free from the influence of environmental factors and thus seem to be proper for delimiting different taxa. The selected 14 morphological traits were tested on live- and herbarium material. The results were analysed by non-metric multidimensional scaling (NMDS).

Differences among the seed length values of particular taxa and micro-morphological characters (the texture of the seed surfaces, texture of the pollen grain surfaces, pollen size) were also investigated.

Sequence analyses

We used nuclear ribosomal ITS sequences being very popular in and suitable for taxonomic studies, together with 3 chloroplast sequences: *trn*K-*mat*K intron, *psb*A-*trn*K spacer and *trn*H-*psb*A intergenic spacer. Altogether we investigated 112 sequences including 106 *Dianthus* samples (20 are newly sequenced and 92 published previously by Valente et al. 2010). The sequence alignment was carried out by ClustalX program. Phylogenies of combined ITS and chloroplast data were inferred with Maximum Parsimony (MP) using MEGA 4.0 and with a Bayesian analysis using MrBayes v. 3.1.1., the phylogenetic trees were visualized and edited with Dendroscope v3.1.0 and the Tree Explorer of MEGA3.1.

AFLP analyses

In total we investigated 187 specimens from 83 localities using AFLP method. The AFLP protocol was carried out according to Teege et al. (2011), with some minor modifications. The AFLP chromatograms were analyzed using the software Gene Marker®. Fragments between 75 and 300 bp length were manually scored in a 0/1 data matrix. A Neighbor-Joining (NJ) tree using Nei–Li distances (NEI and LI 1979) was constructed by PHYLIP software.

Microsatellite analyses

The studied material comprises 11 populations belonging to 5 taxa of sect. *Plumaria.*, These originated from different localities of the Carpathian Basin and the Carpathian Mountains. Additionally, *D. giganteus* D'Urv samples were also collected from several localities of the South Carpathians which served as outgroup.

We used 3 nuclear microsatellite markers: MS-DINCARACC (Smulders et al. 2000); DCA221; DCD010 (Smulders et al. 2003). The PCRs were performed according to the amplification protocol by Smulders et al. (2000, 2003). In our analyses we considered the presence (1) and absence (0) of the particular alleles, and the allele frequencies. Results were evaluated with PAST Program v. 2.13. We tested several multivariate data analysis methods operating with different principles, furthermore we performed with BAPS software a model-based clustering method processing genotypic data by Bayesian approach.

Pollination observations

Only a few pollination observation data were available from the literature in connection with feather carnation taxa (Erhardt 1990, 1991; Balao et al. 2011). Therefore field observations were carried out in a natural population of *D. plumarius* at Sas-hegy (Budapest, Hungary). The pollination studies were carried out eight times during the peak flowering period of the species in 2009.

RESULTS

Morphometric analyses

The results of our morphometric investigations highlight the fact that in the related literature the listed morphological traits linked to each taxa are not proper to delimite specimens of the different taxa when considering a larger sample size. Our studies revealed that flower morphological characters were most useful to separate the particular species complexes.

Sequence analyses

The combined ITS and chloroplast dataset of 112 samples investigated in the MP-analysis contained 2,254 nucleotide positions (as characters), of which 123 were parsimony informative. The proportion of well supported groups (BS above 65% and PP above 95%) were relatively low on both the MP and the Bayesian trees.

On the Bayesian tree, the members of sect. *Plumaria* fall into 19 different clades, either as individual species/accessions or as species groups. From these, 16 comprise a large polytomy. Only 5 clades have high statistical support (PP above 95%). Some clades include both the members of sect. *Plumaria* and species from other *Dianthus* sections.

AFLP analyses

The selective amplification of the chosen primer pairs resulted 552 fragments of lengths between 75 and 300 bp. On the neighbor-joining AFLP tree 4 major groups are present: (i) „Plumarius group" including all subspecies of the species *D. plumarius*, and *D. serotinus*; (ii) „Petraeus group" including the East-Carpathian taxa, clustering with all the *D. arenarius* samples; (iii) "Superbus group" composing of all the analysed samples of *D. superbus*; (iv) "Monspessulanus group" containing the *D. monspessulanus* and *D. hyssopifolius* samples.

Microsatellite analyses

In total, 127 different alleles were detected across the 3 microsatellites genotyped on 239 individuals. The multivariate data analyses of the 3 microsatellite loci resulted three major groups: „Giganteus", „Plumarius" and „Petraeus". Within the clades, no further subdivision, either on any taxonomic or on the population level can be recognised

Pollination observations

The flowers of *D. plumarius* were mainly visited by small- and medium-sized owlet moths and hawkmoths. The data support our conception that the flowers of *D. plumarius* fit well a nocturnal pollination syndrome. Although different hoverfly species were constant visitors of the flowers at any time of the day, these can be regarded as pollen rubbers, or they might be important pollen transporters only in small geographical ranges: within populations or at individual level.

SUMMARY

Based on the analysed morphological characters the Central-European species of sect. *Plumaria* cannot be unambiguously separated. Only species complexes are outlined (*Plumarius* group and *Petraeus* group). The existence of an additional group is depending also on the clustering method used. This third group comprises the samples of *D. superbus*, *D. monspessulanus* and *D. gratianopolitanus*.

The taxa cannot be separated reliably either by taking the micromorphological characters of seed- and pollen surface, pollen size into consideration. This result confirmed the recent origin, and the rapid diversification of *Dianthus* species, as the time frame was possibly not long enough for the accumulation of morphological differences.

On the phylogenetic trees of the combined sequence dataset the specimens belonging to the sect. *Plumaria* are located mainly in clades with low support, nevertheless there are several evidences proving the polyphyly of sect. *Plumaria*. Our study evidenced that the morphological traits (light colour of the corolla, deeply divided, featherlike petals, long calyx tube, fragrantflowers based on feather carnations species are classified are not the signs of common origin.

Literature data together with our own pollination observations confirmed that the above described flower morphological traits are characteristic features of the nocturnal pollination syndrome. We provided pollination observation data for the first time on the potential pollinators of *D. plumarius*.

The clades of feather carnation species for which pollinator observations were documented are well separated from each other. This evidence supports our presumption about the parallel evolution of the nocturnal pollination syndrome within the genus *Dianthus*.

Thus the characteristic flower morphology of feather carnations is the consequence of adaptation to nocturnal pollinators (moth and hawkmoths).

Based on our AFLP results, four lineages are outlined within the Central-European region: "*Plumarius* group" (incl. *D. serotinus* and *D. gratianopolitanus*), "*Petraeus* group" (incl. *D. arenarius*), "*Superbus* group" and finally "*Monspessulanus* group" (incl. *D. monspessulanus* and *D. hyssopifolius*). The existence of these 4 groups was not contradicted by the phylogenies inferred on the grounds of gene sequence. Moreover, *D. superbus* was the only species that was represented by several samples composing a monophyletic group on the phylogram. The existence of different lineages can be evidenced partly by morphology (*Plumarius* – *Petraeus* groups), partly by differences in scent profile found by previous authors (*D. arenarius* – *D. superbus* – *D. monspessulanus*; Jürgens és mtsai. 2003).

For the Hungarian flora, the major outcome of our AFLP analyses is the molecular confirmation of the presence of *D. arenarius* subsp. *borussicus* Vierh. in Hungary. A further important result is that we found *D. serotinus*, the other native sand dwelling species, to be more widespread compared to *D. arenarius*, not only along the interfluves between Danube and Tisza rivers. Moreover, its presence was also evidenced e.g. from East Hungary (Nyírség). We proved that the two sand dwelling species native in

Hungary are not closely related, furthermore, we demonstrated that *D. serotinus* is more closely related to the members of the *D. plumarius* species complex, while *D. arenarius* is closely related to Dacic and Balkan feather carnations, so it is included in the *D. petraeus* species complex.

Based on the 3 nuclear microsatellite markers the taxonomic separation of feather carnation groups are poorly resolved and they show a geographical separation either at a larger regional level, regardless of the clustering method used. The specimens collected from Romania (*D. petraeus* subsp. *orbelicus* (Velen.) Greuter et Burdet, *D. petraeus* subsp. *petraeus* W. et K., *D. superbus* subsp. *alpestris* Kablík. ex Čelak.) are located in one clade while the other clade contains the *D. plumarius* specimens collected from the Transdanubian Middle Mountains (Hungary).

The delimitation of different *D. plumarius* subspecies was not possible based on either sequences-, AFLP-, or microsatellite results.

Based on our molecular genetic as well as nomenclatural studies we concluded that in Hungary only 3 feather carnations can be distinguished along the dry grasslands, instead of the 5 previously accepted taxa. These are as following. *Dianthus plumarius*, *D. serotinus* and *D. arenarius*.

Up to now, the following taxa were listed from the Hungarian Middle Mountains: *D. plumarius* subsp. *lumnitzeri* (Wiesb.) Dom., *D. plumarius* subsp. *praecox* (Kit. ex Schult.) Dom. and *D. plumarius* subsp. *regis-stephani* (Rapcs.) Baksay. Since there are neither clear morphological characters nor molecular differences, these *D. plumarius* subspecies cannot be treated as separate taxa.

NEW SCIENTIFIC RESULTS

1. The polyphyletic origin of sect. *Plumaria* in worldwide context was revealed by molecular genetic tools.

2. The AFLP results imply that the Central European species of sect. *Plumaria* belong to at least 4 lineages. These are: *Plumarius* group, *Petraeus* group, *Monspessulanus* group and *Superbus* group. The existence of the above mentioned 4 lineages can be justified by our morphological investigations and by differences in the scent profile found by previous authors.

3. By our field observations we could confirmed that *Dianthus plumarius* population in Sas-hegy (Budapest) fit well into the nocturnal pollination syndrome. The pollination observation data support the presumption that the flower morphological traits used for the classification of sect. *Plumaria* are most likely the results of parallel evolution. The feather-like petals, the long calyx tube, the light petal colour and the strongly scented flowers evolved as a result of convergent adaptation to pollination by nocturnal insects (moths and hawkmoths).

4. We demonstrated that the feather carnation species analysed cannot be clearly separated from each other by morphological characters, only larger species complexes can be delineated.

5. AFLP results confirmed that *D. arenarius* growing in the herb layer of the Scots pine forest near Fenyőfő is native in Hungary. Its Hungarian area was previously considerably overestimated. Most

probably *D. arenarius* can be considered a relict species in the Carpathian basin.

6. AFLP data debated the assumption that *D. serotinus* is the nearest relative of *D. arenarius*. In contrast, *D. serotinus* is more closely related to those taxa that grow in the rocky grasslands of the Hungarian Middle Mountain region (*D. plumarius* complex).

7. By population genetic investigations we have proved that in rocky outcrops of the Transdanubian Middle Mountain one single feather carnation taxon (species?) occurs and all the populations have to be included under the name *Dianthus plumarius* L.

8. Neither morphological nor molecular evidences support the fact that the endemic and the strictly protected *D. plumarius* subsp. *regis-stephani.* is a separate taxa. Its morphological diversity fits into the variability of *D. plumarius*.

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