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Genetic diversity and evolution of *Pulmonaria angustifolia* L. and *Myosotis laxa sensu lato* (Boraginaceae)

UNIVERSITY OF TARTU
Press

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers that are referred to in the text by Roman numerals:

I **Kook E**, Vedler E, Püssa K, Kalamees R, Reier Ü. Pihu S. 2015. Intra-individual ITS polymorphism and hybridization in *Pulmonaria obscura* Dumort. and *Pulmonaria angustifolia* L. (Boraginaceae). Plant Systematics and Evolution 301 (3): 893–910.

II Pihu S, Öpik M, **Kook E**, Reier Ü. 2009. Morphological and genetic relationships of *Myosotis laxa* ssp. *baltica* and ssp. *caespitosa*, and typification of *M. laxa* ssp. *baltica*. Acta Societatis Botanicorum Poloniae, 78 (1): 37–49.

III **Kook E**, Pihu S, Reier Ü, Thetloff M, Aavik T, Helm A. 2016. Do landscape and environmental factors affect genetic and phenotypic variability within *Myosotis laxa s. lato* (Boraginaceae)? Annales Botanici Fennici 53: 56–66.

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I was mainly responsible for the idea, design, sampling, analysis of data and writing the text of paper **I**. For paper **II** I did the sampling and participated in data analysis and writing. For paper **III**, I was mainly responsible for sampling and writing the text and contributed to data analysis.

1. INTRODUCTION

Genetic diversity is the basis for organisms to survive in, and adapt to, changing environment. High genetic diversity ensures high species diversity and, therefore, the functionality of communities and ecosystems. Different evolutionary processes, like gene flow within species, hybridization, polyploidization, inbreeding and genetic drift can change genetic diversity within species. On the greater scale, these evolutionary processes are affected by environmental conditions and the landscape structure where the species exist. As such, the genetic pattern in a species can reveal the main evolutionary processes which are shaping that species genetically, and give us the opportunity to understand species dynamics and to target activities of conservation more precisely.

Interspecific hybridization can cause strong changes in the plant genetic variance. Extent of interspecific natural hybridization in plants, and the role of hybridization as significant evolutionary force have been reviewed by different authors (Yakimowski & Rieseberg 2014; López-Caamal & Tovar-Sánchez 2014; Whitney et al. 2010; Soltis & Soltis 2009). Natural hybridization is considered to be the source of genetic novelty which increases adaptive potential, promotes speciation, and therefore is worth conserving (Hochkirch 2013; Ennos et al. 2012). In some cases, hybridization can increase genetic variability in rare species, adding adaptive potential and counteracting inbreeding depression (López-Pujol et al. 2012; Thompson et al. 2010). At the same time, hybridization followed by outbreeding depression, genetic swamping and extinction is considered to be a serious threat to rare species (Gómez et al. 2015; Ruhsam et al. 2014; Gomez-Mestre & Jovani 2013). Thus, plant hybridization can be followed by opposite outcomes that are rather difficult to predict (Frankham et al. 2011; Wolf et al. 2001). Despite evidence that hybridization is promoted by anthropogenic impacts directly or indirectly (Gómez et al. 2015; Ayres et al. 2008), Whitney et al. (2010) have shown that an ability to hybridize is rather the characteristic of certain taxon, not the response to the conditions. They found that 40% of plant families contain hybrids and that pattern was consistent among regions. In addition, hybridization propensity of the plant orders showed a strong phylogenetic signal (Whitney et al. 2010).

A plant species exists in nature as a set of populations that can vary phenotypically and genetically. Population differentiation is promoted by isolation: weak or absent gene flow due to long geographic distances (van Strien et al. 2015; Wang & Bradburd 2014; Jenkins et al. 2010) or differences in environmental conditions of populations (Wang & Bradburd 2014; Sexton et al. 2014). In plants, the most common isolation pattern is isolation by distance (Sexton et al. 2014). In addition to the pure geographic distance, the landscape structure can promote or impede plant pollination and dispersal, and so increase or decrease genetic differentiation (Ferreira et al. 2013; Holderegger et al. 2010; Sork & Smouse 2006; Goverde et al. 2002; Bullock et al. 2001). Genetic differentiation and isolation of populations can be caused by adaptation to local

environmental conditions (Baranzelli et al. 2014; Gray et al. 2014; Wang & Bradburd 2014; Temunović et al. 2012). Adaptive differences between populations inhabiting different environments can persist independently of gene flow – genotypes from other environments have smaller fitness (Wang & Bradburd 2014; Räsänen & Hendry 2008; Nosil et al. 2005). Environmental differences and adaptive genetic differentiation in populations can led to the formation of independently evolving groups within species and induce ecological speciation (Givnish 2010; Rundle & Nosil 2005).

Plants with the same genotype can exhibit different phenotypes due to phenotypic plasticity (Valladares et al. 2007; Schlichting & Smith 2002) or epigenetic responses to environmental impacts (Klironomos et al. 2013; Zhang et al. 2013; Angers et al. 2010). Both phenotypic plasticity and epigenetic regulation are considered evolutionary forces which can impact dynamics of adaptive genetic change (reviewed by Schlichting & Wund 2014). In fluctuating, dynamic environments, where adaptive genetic changes cannot follow the environmental change, plasticity is favoured by selection (Palacio-López et al. 2015; Gomez-Mestre & Jovani 2013; Hallsson & Björklund 2012; Scheiner & Holt 2012). In addition, in some environments, epigenetic responses can be favoured in comparison to adaptive genetic changes (Wund 2012; Chevin & Lande 2011; Sultan & Spencer 2002). For example, differences in the expression of the regulator genes between annual and perennial monocarpic life cycles are quite small, and prolonged cold periods in spring can lead to annual life cycle (Satake 2010).

Prolonged cold periods are common on the Baltic Sea coast: as the sea freezes during winter, spring is longer and occurs later in coastal areas than inland (Jaagus & Ahas 2000). Moreover, the coastal vegetation of the Baltic Sea is influenced by post-glacial rebound, wave and ice erosion, annual water level fluctuations (low level in spring, high level in autumn) and temporal dynamics in soil salinity and pH (Jonsell 1988; Ericson 1980). After the dispersal of non-adapted species into these dynamic conditions, adaptation at the genetic level is likely too slow to enable that species to persist. Thus, the revealing of the effects of epigenetic regulation and plasticity is rather expected in these conditions. The post-glacial period in the Baltic Sea region is considered to be too short for speciation (Ingelög et al. 1993), but dynamic environmental conditions on the Baltic coast can lead to rapid population differentiation and the development of coastal microendemic taxa (Jonsell 1988).

To investigate the phylogeny of closely related species including possible reticulate evolution, and population differentiation under variable environmental conditions, the marker must have enough variation both at the intraspecific and interspecific level. Internal transcribed spacer, nrDNA ITS is a valuable and informative marker in plant phylogenetics (reviewed by Poczai & Hyvönen 2010; Calonje et al. 2009). Mostly exploited at the intraspecies and intrageneric level (Cecchi et al. 2011; Mader et al. 2010; Conesa et al. 2008; Hilger et al. 2004), it provides additional support for phylogenetic hypotheses with other nuclear and chloroplast markers at the family level as well (for the example in Boraginaceae,

see Cohen 2014). However, intra-individual variation of ITS and the occurrence of pseudogenes may create confusion and led to erroneous inferences in phylogenetics (Alvarez & Wendel 2003; Bailey et al. 2003). Nevertheless, if taken into account properly (Feliner & Rossello 2007), intra-individual ITS variation can give valuable information about hybridization, polyploidization and phylogeography (Hřibová et al. 2011; Xiao et al. 2010; Záveská Drábková et al. 2009; Kovarik et al. 2005; Koch 2003). Additionally, the ability of two taxa to hybridize is predictable on the basis of the identity of the conserved paired area (helix III) of secondary structure of ITS2 (Muller et al. 2007). Two taxa differing in at least one base-pair change (compensatory base change, CBC) in this area exhibit a reproduction barrier (Coleman 2009; Muller et al. 2007; Coleman 2007). Besides the use in phylogenetic research (reviewed by Poczai & Hyvönen 2010; and Calonje et al. 2009), nrDNA ITS is used also in phylogeographic study as a molecular marker (Koch 2003).

Phylogenetic relationships in the Boraginaceae have been investigated by different methods using morphological traits and molecular markers of plastid and nuclear DNA (Weigend et al. 2010; Mengoni et al. 2006; Selvi, Bigazzi, et al. 2006; Hilger et al. 2004; Langstrom & Chase 2002). Some studies are limited to the ITS1 sequence and not the whole ITS1–5.8S–ITS2 (Selvi, Coppi, et al. 2006; Hilger et al. 2004) due to difficulties in amplification of ITS2 or the unavailability of ITS2 sequences in GenBank (Cecchi et al. 2011; Hilger et al. 2004; Kirchner 2004). Polyploidy and variation in chromosome numbers are common in Boraginaceae (Coppi et al. 2006; Vosa & Pistolesi 2004; Selvi & Bigazzi 2002), for example in *Pulmonaria* the chromosome number ranges from 8 to 30+2B (Sauer 1975). Variation of ploidy level within species, hybridization between different taxa and difficulties in amplification may be circumstantial evidence of relatively high intra-individual ITS polymorphism in Boraginaceae. Hybridization and hybrid speciation in the genus *Pulmonaria* has recently been revealed for nine *Pulmonaria* species from seven European countries (Meeus et al. 2015).

The current study examines some regionally interesting taxa from Boraginaceae: rare and endangered *Pulmonaria angustifolia* L. with its common congener *Pulmonaria obscura* Dumort. (Fig.1) and morphologically highly variable *Myosotis laxa s. lato* (Fig.2). Beyond the possible genetic impoverishment of *P. angustifolia*, this pair of *Pulmonaria* species allows study of the evolutionary processes within and between closely related rare and abundant taxa. *Myosotis laxa s. lato*, considered to be one of the microendemic groups characteristic of the coastal areas of the Baltic Sea (Jonsell 1988), is the good system to investigate possible endemic speciation. Considering the occurrence of *M. laxa s. lato* both in coastal and mainland areas, analysis of genetic and phenotypic differentiation in the context of landscape structure and environmental conditions enables us to shed light on the origin of the coastal form of *M. laxa s. lato*.

Fig. 1. Studied species. **A**. *Pulmonaria angustifolia* L. **B.** *P. angustifolia*, juvenile. **C**. *Pulmonaria obscura* Dumort.

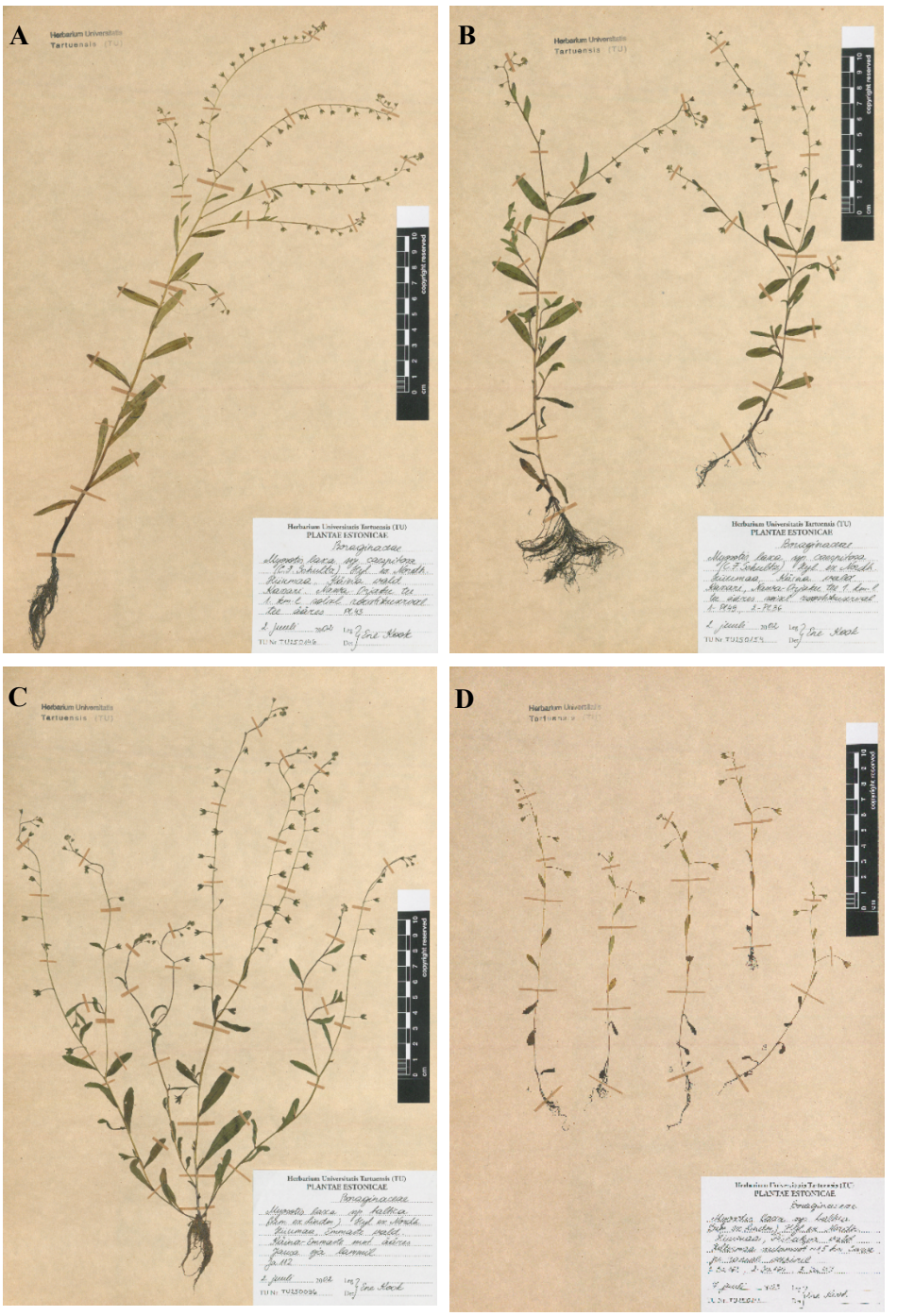

Fig. 2. Studied species. Morphological variation in *Myosotis laxa s. lato*. **A** – mainland form, **B,C** – intermediate, **D** – coastal form.

Pulmonaria angustifolia and *P. obscura* are insect-pollinated forest herbs. *P. obscura* grows in deciduous forests preferring shade, whereas *P. angustifolia* occurs in more open habitats (Merxmueller & Sauer, W. 1972). Both species are distylous, allogamous and self-incompatible (Kühn et al. 2004). *Pulmonaria obscura* is a widely distributed and common species in Estonia and Latvia, whereas *P. angustifolia* is on the northern border of its distribution and has only a few small populations in both countries (Kukk & Kull 2005; Lazdauskaite et al. 1996; Hultén & Fries 1986). *P. angustifolia* needs moderate forest disturbance for regeneration (Reier et al. 2005), and thus it has been greatly affected by changes in forest management in Estonia during the last half-century. *Pulmonaria obscura* and *P. angustifolia* are closely related species: based on nuclear and chloroplast markers they are sister groups on the phylogenetic tree of the family Boraginaceae (Cohen 2014; Weigend et al. 2010). Similar chromosome number (2n=14 in *P. angustifolia*, 2n=14 or 2n=28 in *P. obscura*) and the occurrence of hybrids between *P. obscura* and *P. angustifolia* also confirm their close relationship (Góralski et al. n.d.; Lazdauskaite et al. 1996b; Sauer 1975).

M. laxa s. lato is an octoploid herb in the family Boraginaceae, subfamily Cynoglosseae (Weigend et al. 2010). This taxon is characterised by significant morphological variation (Apelgren 1990a; Apelgren 1990b; Grau & Merxmüller 1972), and annual, biennial or perennial life cycles (Koutecká & Lepš 2013; Koutecká & Lepš 2011; Kühn et al. 2004; Ulvinen 1998; Grau & Merxmüller 1972). *Myosotis baltica* Sam. as a separate taxon was described by G. Samuelsson (1926), based on material from Sweden (stored at the herbarium of the Swedish Museum of Natural History, S). However, that description was questioned by H. Lindberg (1934), because he had already described the same species as *M. laxa* Lehm. (Lindberg 1915) based on material from Åland (stored at the Herbarium of Finnish Museum on Natural History, H). Later, *M. caespitosa* and *M. baltica* were considered subspecies of *M. laxa* (Nordhagen 1940). Investigations of morphological traits of *M. laxa* ssp. *caespitosa* and ssp. *baltica* showed that there are no clear distinctions between "*Myosotis baltica* type" and "*Myosotis caespitosa* type" and no clear grouping of the material can be made based on the morphological variation (Apelgre n 1990b, p.297). Therefore, in this work, the taxon is considered *M. laxa s. lato* (includes var. *caespitosa*, var. *baltica* and var. *laxa sensu* Apelgren), var. *caespitosa* is considered to be the mainland form and var. *baltica* is considered to be the coastal form of *Myosotis laxa s. lato.*

M. laxa s. lato is broadly distributed in Europe, Asia and North America (Hultén & Fries 1986; Grau & Merxmüller 1972). The area of the coastal form is considered to be located in the Baltic Sea region, where it is most common in south-western Finland and Åland (Ulvinen 1998; Apelgren 1990b; Hultén & Fries 1986). However, outside the Baltic Sea region, the coastal form is found in north-western Russia on the coast of Lake Ladoga (Budantsev 2006; Tzvelev 2000) and in the Caspian Basin, in the northern part of Central Asia, Altai (Viljasoo 1969; Popov 1953) and Mongolia (Byazrov et al. 1983), where it

grows on the floodplains of rivers. The coastal form of *M. laxa* is annual, has leaves in inflorescence, long pedicels and calyces, smaller flowers and is shorter than *M. laxa s. lato* (Tzvelev 2000; Lazdauskaite et al. 1996a; Krok & Almquist 1994; Grau & Merxmüller 1972; Samuelsson 1926). The mainland form of *M. laxa s. lato* grows in different moist habitats like swamps and moist grasslands, but also on river banks and sea coasts, sometimes in the same location with the coastal form (Tzvelev 2000; Lazdauskaite et al. 1996a; Krok & Almquist 1994; Grau & Merxmüller 1972). The largest morphological variation within *M. laxa s. lato* occurs in the SW Archipelago of Finland, thus it is speculated that the coastal form evolved in the Finnish archipelago and later dispersed to other coastal regions around the Baltic Sea (Apelgren 1990b). However, the existence of *M. laxa* plants similar to the coastal form of *M. laxa* in continental areas (Byazrov et al. 1983; Viljasoo 1969; Popov 1953) casts doubt on a Finnish origin.

In this thesis, we are to answer following questions:

- Is the intra-individual polymorphism of ITS1-5.8S-ITS2 present in *P. angustifolia*, *P. obscura* and in *M. laxa s. lato*?
- What kind of phylogenetic relationship is indicated by intra-individual polymorphism of ITS1-5.8S-ITS2 between *P. angustifolia* and *P. obscura*?
- Does the morphological and molecular variation support the ongoing speciation in *M. laxa s. lato*?
- Which factors are the most significant in determining of morphological and molecular variation in the *M. laxa s. lato* – geographic distance, landscape structure or environmental conditions?
- Does the morphologically typical coastal form of *M. laxa* occur in Estonia?

2. MATERIALS AND METHODS

2.1 Sampling

Leaf samples of *P. angustifolia* were collected from two populations in Estonia and four populations in Latvia and from one population in Poland (Table 1 in paper **I**). Sampling was carried out such that individuals sampled were separated by at least 1 m. All populations of *P. angustifolia* and *P. obscura* were isolated from each other. From the Krustkalni Nature Reserve mixed population (Latvia), *P. angustifolia*, *P. obscura*, and intermediate specimens were sampled. The sampled populations include most of the populations of *P. angustifolia* in Estonia and Latvia.

Leaf samples were collected and morphological traits were measured for *Myosotis laxa s. lato*, *M. scorpioides* (L.) Hill and *M. arvensis* (L.) Hill from 11 geographically separated locations (Fig. 2 in paper **II** and Fig. 1 in paper **III**) on the Estonian islands of Saaremaa and Hiiumaa, the Estonian mainland and in Hjortö and Björkö (Åland Islands, Finland). To map morphological variation of *M. laxa s. lato*, 16 morphological traits were measured for every specimen (Text p. 40 and Table 2 in paper **II**). Eleven of these morphological traits were used to create a distance matrix of morphological data for the studied populations (Table 1 in paper **III**).

To characterize spatial and environmental impact on phenotypic and genetic variability of *M. laxa s. lato*, for every population geographical coordinates were fixed and landscape structure and abiotic environment were described (**III**). Because the species dispersal occurs mainly via water, the sea/land ratio was used as the proxy of landscape structure around the populations. Environmental factors, known to be important in determining plant performance were described for each sampling site (including soil moisture, soil type, precipitation, minimum temperature in February and the effect of seawater, i.e. wether or not the sampled sites were periodically inundated) (**III**).

Voucher specimens are deposited in the herbarium (TU) of the Natural History Museum, University of Tartu.

2.2 DNA extraction and cloning

For DNA extraction, leaf samples were collected, frozen at –20 °C and lyophilised with cooling trap Hetotrap CT60 at –60 °C (**II**) or dried with silicagel (**I, III**). DNA was extracted by standard protocol (Doyle & Doyle 1987). ITS1-5.8S-ITS2 was amplified with primers ITS4 and ITS5 (**II**) or ITSLeu1 and ITS4 (**I, III**) (White et al. 1990). The PCR bands of *Pulmonaria* that resulted in ambiguous bases were cloned into *Escherichia coli* and 15–25 clones per band were sequenced (**I**).

2.3 Herbarium specimens

To investigate occurrence and distribution of coastal form of *M. laxa s. lato* in Estonia, herbarium specimens of *M. laxa s. lato* were examined in six herbaria in Estonia (Tartu University, TU; Estonian Univesity of Life Sciences, TAA), Sweden (Swedish museum of Natural History, S), Finland (Finnish Museum of Natural History, H), Germany (Berlin-Dahlem Botanical Garden and Botanical Museum, B) and UK (Royal Botanic Gardens Kew, K). Special attention was paid to morphologically typical specimens of the coastal form of *M. laxa*. Material collected by Samuelsson and identified according to the type description (Samuelsson 1926) was investigated from S and B (Samuelsson did not fix any type specimen). Material identified by Lindberg as *M. laxa* was investigated at H (including specimens from Exsiccatae Fennicae).

2.4 Analysis of intra-individual polymorphism

Pulmonaria angustifolia and *P. obscura* sequences of ITS1-5.8S-ITS2 were aligned with Clustal X (Larkin et al. 2007). The ITS1, 5.8S and ITS2 were analysed separately (**I**). To check functionality of nrDNA ITS variants, completeness of the conserved motifs of 5.8S gene and ITS1 was controlled and 5.8S secondary structures were constructed in the mFold (Zuker 2003). ITS2 secondary structures for sequence-structure phylogenetic analysis were constructed in ITS2 Database (Koetschan et al. 2012). Sequences with substitutions in the conserved motifs and/or lacking the proper secondary structure were eliminated from phylogenetic analyses. To detect the rate of intra-individual polymorphism, nucleotide sequence divergence was calculated between and within specimens separately for ITS1, 5.8S and ITS2 as well as number of haplotypes and haplotype diversity, and sequence identity matrix (**I**)

2.5 Phylogenetic analysis

Phylogenetic analyses for *P. angustifolia* and *P. obscura* were done with sequence information of the whole ITS1-5.8S-ITS2 and with sequence information of both spacers (ITS1, ITS2) and 5.8S gene separately, with MEGA 5.1 (Tamura et al. 2011). Phylogenetic analysis of sequence-structure information of ITS2 was done with ProfDistS 0.9.9 (Wolf et al. 2008) (**I**).

M. laxa s. lato sequences of ITS1-5.8S-ITS2 were aligned with Clustal W (**II**) in BioEdit (Hall 1999) or Clustal X (**III**) (Larkin et al. 2007). Phylogenetic analysis (parsimony analysis and neighbour-joining) was performed with PAUP 4.0b10 (Swofford 2002) (**II**) and with MEGA 5.1 (Tamura et al. 2011) (**III**). For every population with more than one specimen, consensus sequence of ITS1-8.8S-ITS2 was created with BioEdit to calculate genetic distance matrix for populations (Hall 1999) (**III**).

2.6 Morphological differentiation of coastal and mainland form of *M. laxa s. lato*

To shed light on the differentiation of the two forms on the basis of morphological dataset, principal component analysis, stepwise discriminant analysis, analysis of variance (ANOVA) and cluster analysis (UPGMA) were conducted (StatSoft Inc. 2001) (**II**). For comparison of means of morphological traits, t-test and Tukey test, and for part of traits, nonparametric Kruskal-Wallis test were performed. Morphological trait values of individuals were averaged for calculation of the morphological distance matrix (**III**).

2.7 Analysis of correlation between plant traits and spatial and environmental factors

To investigate the correlation between plant traits (genetic and morphological distances between populations) and environment, landscape structure and geographical distance, five distance matrices were calculated (**III**). Partial Mantel tests were conducted in R (R Development Core Team 2013) using the *mantel.partial* function in the *vegan* package (Pearson method, 10 000 permutations). Tests were conducted for the following pairs of matrices, all controlled for geographic distance as a third (z) parameter: (1) landscape dissimilarity and genetic distances, (2) landscape dissimilarity and phenotypic distances, (3) environmental dissimilarity and genetic distances and (4) environmental dissimilarity and phenotypic distances. The relationship between genetic and phenotypic distances, and their relationship with geographic distance was tested with the "mantel" function in the vegan package (Pearson method, 10000 permutations) (**III**).

3. RESULTS

3.1 Analysis of intra-individual polymorphism

Intra-individual polymorphism was detectable in *P. angustifolia* and *P. obscura*, revealed in base insertions/deletions, substitutions and presence of non-functional sequences (in paper **I**). *P. angustifolia* had a higher level of intra-individual polymorphism than *P. obscura* (Fig. 1, Table 2, Table 3 in paper **I**). In ten sites, cloned sequences of *P. angustifolia* showed intra-individual polymorphism: all *P. angustifolia* individuals had nucleotides characteristic of both *P. angustifolia* and *P. obscura* in these sites. In five sites, all *P. angustifolia* individuals carried nucleotides characteristic of *P. obscura* (Table 3, **I**; GenBank sequences of *P. angustifolia* and *P. obscura* were used as reference sequences). *P. obscura* specimens showed intra-individual polymorphism in 1–5 sites, whereas *P. angustifolia* specimens had intra- individual polymorphism in 10–14 sites and were identical to *P. obscura* from GenBank in the five sites (Table 3, **I**). Haplotypes characteristic to *P. obscura* were found in *P. angustifolia*, but not vice versa (Appendix 1, **I**). Of 256 ITS1-5.8S-ITS2 sequences, 61 lack the stable secondary structure; these sequences were considered non-functional (pseudogenes) and excluded from phylogenetic analyses.

3.2 Phylogenetic analysis

Phylogenetic analyses in *P. angustifolia* and *P. obscura* based on sequence information of spacers ITS1 and ITS2 and the whole ITS1-5.8S-ITS2 resulted in highly congruent neighbour-joining trees. *Pulmonaria* cloned sequences were connected in a clade with two subclades. One of subclades, *P. angustifolia* clade did not contain any of the *P. obscura* sequence. The other clade consisted of *P. angustifolia* and *P. obscura* sequences (Figure 2, **I**).

Phylogenetic analysis on the basis of ITS2 sequence-structure resulted in similar tree topography, having slightly higher bootstrap support for the subclades of *Pulmonaria* clade than sequence-based trees (Figure 3, **I**). There were no compensatory base changes (CBC) in the conserved area of ITS2 secondary structure (**I**).

In *M. laxa s. lato*, neighbour-joining analysis of ITS1-5.8S-ITS2 resulted in a monophyletic group consisting of *M. laxa s. lato*, *M. scorpioides* and *M. rehsteineri* (bootstrap support 94, Figure 7, **II**). In the subcluster of *M. laxa s. lato* specimens from Sarve population were clustered together but there was no grouping of other specimens neither according to subspecies nor populations (Figure 7, **II**)

On the neighbour-joining tree of consensus sequences of 14 populations (including populations from Åland), *M. laxa s. lato* and *M. scorpioides* were connected in a clade (bootstrap support 100, Figure 2, **III**) and *M. laxa s. lato* was monophyletic. Clustering into subclades of the *M. laxa s. lato* clade was not

concordant with geographic origin or subspecies. Populations from Åland did not hold basal position in relation to *M. laxa s. lato* clade but were scattered in this clade. (Figure 2, **III**).

3.3 Morphological differentiation in the *M. laxa s. lato*

Nine morphological characters were statistically significantly different between the coastal form and mainland form of *M. laxa* (Table 5, Figure 5, **II**). When the analysis was done by populations, the variation of several characters overlapped largely for different taxa from some populations, while in other populations the differences were clear (data not shown).

Principal component analysis of morphological characters of *M. laxa* coastal form, *M. laxa* mainland form and *M. scorpioides* showed that *M. scorpioides* can be well discriminated from both coastal and mainland form of *M. laxa* (Figure 3, **II**). The majority of specimens of *M. laxa s. lato* forms a continuum being indistinguishable on the base of morphological characters. However, a group of coastal specimens separates clearly from the remaining set. The UPGMA analysis resulted in the similar distribution of specimens (Figure 4, **II**). The discriminant analysis showed that *M. scorpioides* was 95% correctly classified, but the coastal form and mainland form of *M. laxa* were not well discriminated (Table 4, **II**). As the fruits were not available for all specimens, the fruit size was analysed separately. Fruits of typical coastal form were significantly longer than those of mainland form (Table 6, **II**). The difference in width of fruits was not significant, but fruits of typical coastal form were significantly bigger than these of the same form from the other populations ($t=-4.24$, $p=0.0007$).

3.4 Analysis of correlation between plant traits and spatial and environmental factors

Partial Mantel test revealed that genotypic distances between *M. laxa s. lato* populations are influenced by landscape dissimilarity (controlled for geographic distance), expressed as the proportion of sea and mainland in the surrounding landscape (Table 3, **III**). Geographic distance alone had no significant effect on genotypic or phenotypic variation (Table 3, **III**). There was also a significant correlation between genetic and phenotypic distances ($r=0.2$, $p=0.03$) (**III**). Phenotypic distance itself was related to neither landscape dissimilarity nor environmental dissimilarity in populations (Table 3, **III**).

3.5 Updating of distribution data of the coastal form of *M. laxa* and typification of *M. laxa* Lehm. ssp *baltica* (Sam) Hyl. ex Nordh.

Twenty morphologically typical specimens of the coastal form collected in Estonia were found in local herbaria (TAA, TU). During the fieldwork in Estonia 2002–2003, 21 specimens were collected that were typical according to Samuelsson's (1926) description (**II**). Thus the presence of typical coastal form of *M. laxa s. lato* in Estonia is supported both by herbarium specimens and fresh material. *M. laxa* Lehm. ssp *baltica* (Sam) Hyl. ex Nordh. has been typified and *Myosotis baltica* type specimens can be found in the Swedish Museum of Natural History (lectotype – S HS-6990 and isolectotype – S HS-6991).

4. DISCUSSION

4.1 Intra-individual polymorphism in study taxa

Intra-individual polymorphism of ITS1-5.8S-ITS2 is not rare in plants (reviewed by Poczai & Hyvönen 2010). In our study, intra-individual polymorphism was detected both in *P. angustifolia* and *P. obscura* (**I**). In both species, intra-individual evolutionary divergence of 5.8S gene and ITS1 and ITS2 spacers was higher than divergence between individuals. *P. angustifolia* and mixed populations showed higher intra-individual polymorphism than in *P. obscura* populations: more polymorphic sites, higher evolutionary divergence and higher haplotype diversity than *P. obscura*.

In general, intra-individual polymorphism of ITS1-5.8S-ITS2 is especially characteristic of hybrids and allopolyploids (Poczai & Hyvönen 2010, Coleman 2009). *P. obscura*, known as both diploid and tetraploid, was therefore expected to exhibit similar or higher intra-individual polymorphism than diploid *P. angustifolia*. Analysis of intra-individual polymorphic sites revealed that *P. angustifolia* and mixed population specimens had nucleotides characteristic both of *P. angustifolia* and *P. obscura* in polymorphic sites. In addition, identical or nearly identical sequences of 5.8S, ITS1 and ITS2 were found between *P. angustifolia*, *P. obscura* and mixed population specimens (**I**). This pattern of intra-individual polymorphism allows us to conclude hybridization with *P. obscura* in all studied *P. angustifolia* populations, not only in the mixed population. Analysis of secondary structure of ITS2 confirms the hybridizing ability of *P. angustifolia* and *P. obscura* because no compensatory base change between these species were detected (**I**). Despite being isolated, all *P. angustifolia* populations and mixed population showed a similar pattern of intra- individual polymorphism.

So our results suggest hybridization between *P. angustifolia* and *P. obscura* and a hybrid origin of all *P. angustifolia* populations and mixed population. Greater evolutionary divergence of ITS1 and ITS2 between individuals of the mixed population than between the *P. angustifolia* and *P. obscura* pure populations indicates that hybridization is more or less continual in the mixed population and relatively infrequent in the pure *P. angustifolia* populations.

In case of intra-individual polymorphism of ITS1-5.8S-ITS2, non-functional sequences (pseudogenes) can be numerous (Harpke & Peterson 2008; Xiao et al. 2010) or missing (Záveská Drábková et al. 2009). Thus, the percentage of pseudogenes of ITS1-5.8S-ITS2 in *P. angustifolia* (23%) and *P. obscura* (31%) can be considered moderate. In addition to substitutions in the conserved motifs of 5.8S gene, one functionality-breaking substitution was found in the non-conserved area of 5.8S in the *P. obscura*. So the check for functionality of 5.8S is more substantial by the secondary structures rather than by conserved motifs only.

In the *M. laxa s. lato*, ITS1-5.8S-ITS2 sequences were well-readable and had very few ambiguous sites (data unpublished). *M. laxa s. lato* is an octoploid and therefore is rather expected to have different variants of ITS1-5.8S-ITS2 in the

same specimen. The low rate of intra-individual polymorphism of *M. laxa s. lato* can be explained by a high rate of selfing, because selfers are generally less polymorphic than outcrossers (Glemin et al. 2006). Another explanation for the low rate of intra-individual polymorphism can be autopolyploidy of *M. laxa s. lato*.

4.2 Phylogenetic relationships of *Pulmonaria angustifolia* and *Pulmonaria obscura*

Adding pseudogenes into the phylogenetic analysis can lead to erroneous results and incorrect conclusions about the phylogeny of the investigated taxa (see Poczai & Hyvönen 2010; Feliner & Rossello 2007). In our dataset, 24% of sequences lack a stable secondary structure and so these sequences were excluded from phylogenetic analyses.

On the neighbour-joining trees of ITS1 (sequence-based) and ITS2 (based on sequence-structure information) part of the *P. obscura* sequences clustered together with *P. angustifolia* and mixed population sequences, but not vice versa (**I**). That pattern was consistent through the trees based on the whole ITS1-5.8S-ITS2, on the ITS1 and ITS2 spacers separately and on the ITS2 sequence-structure information (**I**). It can conclude that the genetic material of *P. obscura* occurs in *P. angustifolia* and mixed population specimens showing a hybrid origin of all studied *P. angustifolia* populations and mixed population. Subclades of the ITS2 sequence–structure tree had significant bootstrap support while subclades of the ITS1 had not – so the structure information increases the precision of phylogenetic analysis.

Hybridization is more likely in the small and isolated populations of rare plant species where it is promoted by a lack of suitable reproducing partners (Thompson et al. 2010). *P. angustifolia* populations from Estonia and Latvia are small and isolated of each other and, growing on the northern border of the species area, thus they can be exceptional. But one studied population originated from the core area of *P. angustifolia* (Poland, Jelenia Góra) also consisted of *P. obscura* haplotypes and showed a similar pattern of polymorphism, thus suggesting a hybrid origin for the whole *P. angustifolia*.

4.3 Morphological and molecular variation in the *Myosotis laxa s. lato*

Analysis of morphological traits showed that nine traits were significantly different between coastal and mainland forms of *M. laxa* (**II**). At the population level, the variation of these characters overlapped largely for coastal and mainland form in some populations, while in the other populations the differences were clear. In addition, principal component analysis of morphological traits confirmed that only a small fraction of specimens of *M. laxa s. lato* was

morphologically different from most specimens of *M. laxa s. lat.* (**II**). So the wide morphological variation in the *M. laxa s. lato* did not support a clear differentiation of coastal and mainland forms. On the basis of material from Sweden and Finland, Apelgren (1990a; 1990b) showed that most of the studied specimens of *M. laxa s. lato* constitute a morphological continuum with more or less different coastal form at the one end. The current study shows rather similar morphological variation of *M. laxa s. lato* from the eastern coast of the Baltic Sea (Saaremaa, Hiiumaa and Estonian mainland). In Estonia, plants exhibiting morphologically typical coastal phenotype (sensu Samuelsson 1926) were found in the local herbaria (TU, TAA) and in the four populations sampled in the current study. Likely, *M. laxa s. lato* is shaped by similar ecological conditions and evolutionary processes in these areas.

In the neighbour-joining analysis of nrDNA ITS, all specimens of *Myosotis laxa s. lato* were clustered together with *M. scorpioides* as a sister group (**II, III**). As the grouping of *M. laxa* specimens into the subclades did not follow the locations or subspecies, it is clear that there is no special 'coastal' genotype. Moreover, on the basis of molecular variation of *M. laxa s. lato*, there is no sign of genetic differentiation of any subtaxa. Likely, there are slightly different genotypes in the *M. laxa s. lato*, which are all able to exhibit mainland, coastal and a variety of intermediate phenotypes. However, correlation between genetic and phenotypic distances (**III**) suggests that at least part of phenotypic variability should have a genetic background. Specimens from Åland Islands, the supposed centre of origin of coastal form of *M. laxa* did not hold basal position on the neighbour-joining tree, so they are not the ancestors of all other specimens of the coastal form. So the origin of the coastal form from Åland is doubtful. In addition, the lack of correlation between genetic distance and geographic distance (**III**) suggests that the coastal form has no single centre of origin.

Although the coastal form of *M. laxa* was initially described on the basis of morphological traits and growing in characteristic coastal habitats, the niche differentiation between the coastal and mainland form is not strict: Apelgren (1990a) documented specimens of wide morphological variety inhabiting the same coastal location. Mixed populations, where coastal and mainland forms of *M. laxa s. lato* were growing together, were found in the current study as well (**II**). Simultaneous occurrence of the two forms in coastal conditions can indicate that at least the first generations of the mainland form are able to grow on coasts without manifesting any response to environmental conditions.

Plant phenotype is under the direct impact of environmental conditions and acts as the target of natural selection. According to our results, environmental conditions had no impact on phenotypic distance (**III**). This is a bit controversial, because the coastal environmental conditions have been considered as the cause of population differentiation in *M. laxa s. lato*.

It is likely that morphological differences in the coastal form of *M. laxa* are not a direct response to the environmental conditions, but are induced by the epigenetically regulated shift in the life cycle from biennial/perennial to annual.

M. laxa s. lato can exhibit annual, biennial or perennial life cycles, but the coastal form is considered annual. This kind of variation in life cycle is epigenetically controlled by FLC (flowering locus C), in which prolonged cold periods can induce an annual life cycle (Aikawa et al. 2010; Satake 2010). The vegetation period starts later (the cold period is longer) in the coastal areas of Estonia than on the mainland due to the impact of the cold surface of the Baltic sea in spring (Jaagus & Ahas 2000), thereby allowing for cold-dependent epigenetic regulation of the life cycle of coastal plants. Outside of the Baltic Sea coastal area, *M. laxa* plants similar to coastal form have also been found in north-western Russia on the shore of Lake Ladoga (Budantsev 2006; Tzvelev 2000) and on river floodplains in Mongolia (Byazrov et al. 1983; FloraGREIF n.d.), where a similar effect caused by prolonged cold period and delayed spring can be observed. In addition, across the whole area, the coastal form is less abundant than mainland form and there are no examples of occurrence of the coastal form outside of the area of the mainland form of *M. laxa s. lato* (Kukk & Kull 2005; Ulvinen 1998; Lazdauskaite et al. 1996a; Krok & Almquist 1994; Hultén & Fries 1986; Jalas 1980; Grau & Merxmüller 1972). This pattern of distribution, against the background of a possible epigenetic basis of the characteristic morphology and life cycle, suggests an independent origin of the coastal form in different locations in the area of *M. laxa s. lato.*

4.4 Impact of spatial factors on genetic and phenotypic variability of *Myosotis laxa s. lato*

Whereas the genetic variability in the *Myosotis laxa s. lato* was significantly correlated to landscape dissimilarity, the structure of landscape must be considered the driver of evolutionary change at the genetic level. The composition of the surrounding landscape can affect pollination and seed dispersal, and therefore restrict, alternate or promote gene flow between populations (Sork & Smouse 2006; Bullock et al. 2001). It is likely that gene flow in the *M. laxa s. lato* occurs mostly from the mainland populations to the coast. Plants of the mainland form of *M. laxa s. lato* are larger, more branched, have more flowers and a longer flowering time, therefore produce more seeds than the coastal form. Seed dispersal of *M. laxa s. lato* by water likely occurs from higher mainland populations to the sea-level coastal populations. This scenario fits well into the model of counter-gradient gene flow, where gene exchange is strongest between dissimilar environments (Sexton et al. 2014). Possible consequences of this counter-gradient gene flow are inhibited genetic differentiation in coastal populations and selection for plasticity and epigenetic effects (Scheiner & Holt 2012; Sultan & Spencer 2002). Lack of correlation between genetic distance and environmental dissimilarity also supports the occurrence of directed gene flow in *M. laxa s. lato* populations (**III**).

Genetic distance and phenotypic distance of within *M. laxa s. lato* had no correlation with geographic distance. Plants commonly exhibit a genetic

correlation with geographic distance but this is not the rule (Sexton et al. 2014). The absence of correlation between genetic distance and geographic distance shows that phenotypically different populations of *M. laxa s. lato* belonging to the coastal form of *M. laxa* have no single centre of origin.

4.5 Updating of distribution data of the coastal form of *M. laxa*

Our study confirms the occurrence of the morphologically typical coastal form of *M. laxa* in Estonia, both in herbaria and in sampled sites. The oldest herbarium specimen of the typical coastal form of *M. laxa* of Estonian origin, collected in 1932, is stored in the Herbarium of the Natural History Museum of the University of Tartu (TU 257670) .

CONCLUSIONS

In this thesis different evolutionary processes were explored in the two genera of Boraginaceae using the pair of closely related species *Pulmonaria angustifolia* and *Pulmonaria obscura* and morphologically highly variable *Myosotis laxa s. lato.* Patterns of morphological characters and intra-individual polymorphism of nrDNA ITS provide valuable information on phylogeny and reticulation evolutionary events of these taxa. Linking spatial information to genetic and phenotypic differentiation reveals the impact of landscape and environmental factors in shaping plant genotype and phenotype.

- Intra- individual polymorphism of nrDNA is present in *P. angustifolia* and *P. obscura* revealing by intra-individual evolutionary divergence of ITS1 and ITS2 spacers and 5.8S gene, moderate to high haplotype diversity in each individual and the presence of the non-functional paralogs of nrDNA ITS. *M. laxa s. lato* showed a low rate of intra-individual polymorphism, likely due to self-fertilization or autopolyploidy.
- All studied populations of *P. angustifolia* were of hybrid origin, carrying genetic material from *P. obscura*. Analysis of ITS2 secondary structure showed no compensatory base change (CBC) between *P. angustifolia* and *P. obscura*, confirming their ability to hybridize. The occurrence of *P. obscura* genetic material in *P. angustifolia* was revealed by the nucleotide composition in the polymorphic sites, identical sequences between *P. angustifolia* and *P. obscura*, higher evolutionary divergence in the ITS1 and ITS2 spacers of *P. angustifolia* and by part of *P. obscura* sequences clustering together with *P. angustifolia* sequences on the neighbour-joining tree. Hybridization in most of the studied populations of *P. angustifolia* is likely caused by the small population size and isolation. However, both edge populations of *P. angustifolia* in Estonia and Latvia and the population from the core area in Poland showed similar genetic pattern, suggesting a hybrid origin for the whole *P. angustifolia*.
- Morphological and molecular variation in the *M. laxa s. lato* did not support species differentiation in this taxon. The morphologically typical coastal form, which slightly differs from most specimens of *M. laxa s. lato* was not monophyletic on the basis of neighbour-joining analysis. Whereas genetic variation in *M. laxa s. lato* was correlated with phenotypic variation, it can be concluded that different phenotypes have at least a partly genetic background. The coastal form is likely the result of an epigenetic shift in the regulation of the life cycle, induced by prolonged cold periods in spring in the typical coastal habitat. Additionally, an epigenetic background of coastal form of *M. laxa* is supported by its occurrence on lake shores and river floodplains outside of the Baltic sea region, but not outside of the area of *M. laxa* mainland form.
- The landscape structure around the populations was correlated to the genetic distance between populations, confirming a unidirectional dispersal of *M.*

laxa s. lato from mainland to the coast. The mainland form of *M. laxa* likely produces more seeds than the coastal form, because it has more flowers and a longer flowering time than the coastal form. Dispersal via water, which is characteristic of *M. laxa s. lato*, is probably directed from higher and more abundant mainland populations to the sea-level coastal populations and prevents genetic adaptation of the coastal populations. In the harsh coastal conditions and under the impact of directed gene flow, epigenetic effects and phenotypic plasticity can be favoured by selection.

- Similar morphological variation of *M. laxa s. lato* in Estonia to those in Finland and Sweden suggests that the evolutionary patterns in this taxon are mostly the same across the Baltic Sea coastal region. Therefore the occurrence of the morphologically typical coastal form of *M. laxa s. lato* in Estonia is rather expected. Specimens in the local herbaria and specimens from the populations sampled during the current work confirm the occurrence of the typical coastal form of *M. laxa* in Estonia.

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SUMMARY IN ESTONIAN

Kareleheliste *Pulmonaria angustifolia* L. ja *Myosotis laxa s. lato* (Boraginaceae) geneetiline mitmekesisus ja evolutsioon

Ökosüsteemide funktsioneerimine ja kohanemine muutuvate keskkonnatingimustega põhineb bioloogilisel mitmekesisusel. Bioloogiline mitmekesisus sõltub geneetilisest mitmekesisusest: alleelide ja genotüüpide paljusus liigi sees võimaldab kohastumist ja vähendab väljasuremise riski muutuvas keskkonnas. Geneetilist mitmekesisust loovad ja kujundavad evolutsiooniprotsessid – taimede puhul näiteks hübridiseerumine, polüploidiseerumine, geneetiline kohastumine, epigeneetiline regulatsioon. Need protsessid on mõjutatud keskkonnast ja maastikust, kus liik eksisteerib. Geneetilise mitmekesisuse muster liigi sees, erinevate alleelide ja genotüüpide hulk ning jaotus aga annab infot selle kohta, millised evolutsiooniprotsessid on antud liigi kujunemisel ja püsimisel olulised. Evolutsiooniprotsesside tundmine omakorda võimaldab mõista liigi dünaamikat ja ohustatuse määra.

Kaitset vajavad liigid on sageli aheneva areaaliga ning eksisteerivad väikeste isoleeritud populatsioonidena. Isolatsioonist tingitud vähene geenivool populatsioonide vahel ning geenitriivi tugev mõju toovad kaasa populatsioonide järkjärgulise geneetilise eristumise. Lisaks on sellistes populatsioonides enamasti kõrge sugulusristumise tase. Isolatsiooni, sugulusristumise ja geenitriivi tulemuseks on geneetiline vaesumine ja madal kohastumisvõime. Harvade sündmustena toimuv geenivool võib sellistes populatsioonides mõjuda isegi kahjulikult – geneetiliselt oluliselt erinevate vanemate järglaskond ei pruugi olla antud keskkonnas piisava kohasusega. Seega, et võtta vastu põhjendatud looduskaitselisi otsuseid, on oluline tunda väheneva liigi geneetilise mitmekesisuse taset ja teda mõjutavaid evolutsiooniprotsesse.

Käesolevas töös uurisin geneetilist mitmekesisust ja evolutsiooniprotsesse kareleheliste (Boraginaceae) sugukonna liikidel *Pulmonaria angustifolia* (sinine kopsurohi) ja *Pulmonaria obscura* (harilik kopsurohi) ning *Myosotis laxa s. lato* (muru-lõosilm). Markerina kasutasin nrDNA ITS-järjestust, mis on piisavalt varieeruv, et olla informatiivne lähedalt suguluses olevate liikide vahel ning võimaldab eristada ja iseloomustada ka liigisiseseid taksoneid. Lisaks, eriti polüploididel ja hübriididel, võib nrDNA ITS esineda ühe isendi sees mitmete erinevate variantidena. Selline isendisisene polümorfism annab samuti infot evolutsiooniprotsesside kohta uuritavas taksonis.

Pulmonaria obscura ja *Pulmonaria angustifolia* on mitmeaastased rohttaimed, mis kasvavad viljaka mullaga metsakooslustes. Kui *P. obscura* on Eestis ja Lätis tavaline salumetsade rohttaim, siis *P. angustifolia* on samas piirkonnas väga haruldane, olles oma levila põhjapiiril ning esinedes vaid üksikute isoleeritud populatsioonidena. Mõlemad liigid on putuktolmlejad, neil esineb heterostüülia ning isesobimatus. Eestis ja Lätis kattub nende õitsemisaeg suures

osas. *P. angustifolia* vajab kasvamiseks häiringutega ja liigestatud reljeefiga metsaala, ta on soojalembene ja kasvab küngaste lõunapoolsetel nõlvadel. Seoses muutustega metsa majandamises on *P. angustifolia* arvukus Eestis viimase 50 aasta jooksul tugevalt langenud. *M. laxa s. lato* (muru-lõosilm) on rohttaim, mis kasvab niisketel niitudel, veekogude kallastel ja kraavides, eelistatult seal, kus konkurents pole väga suur. *M. laxa s. lato* võib olla ühe-, kahe-, või mitmeaastane ning on morfoloogiliselt väga varieeruv. Hoolimata suurest morfoloogilisest varieeruvusest pole liigisiseseid taksoneid kuigi arvukalt kirjeldatud. Tuntuim liigisisene takson on *M. laxa* rannikuvorm, algselt kirjeldatud eraldi liigina *Myosotis baltica* Sam. *M. laxa* rannikuvorm kasvab tüüpiliselt Läänemere rannakooslustes, sageli veepiiril, muudes *M. laxa s. lato* iseloomulikes kasvukohtades teda reeglina ei leidu. Siiski on rannikuvormiga sarnaseid taimi kirjeldatud ka Laadoga järve piirkonnast Venemaalt ja Mongooliast, kus ta kasvab järve- ja jõekallastel. Rannikuvorm on alati üheaastane, ta on võrreldes *M. laxa s. lato* maismaavormiga väiksem ja vähem harunenud, iseloomulikud on lehistunud õisik, väiksemad õied, suuremad viljad ning lühem õitsemisaeg. Kogu *M. laxa* areaali ulatuses esineb rannikuvorm väiksema arvu populatsioonidena ja sporaadilisemalt kui maismaavorm.

Käesoleva töö eesmärgiks on nrDNA ITS- järjestuse polümorfismi põhjal iseloomustada *P. angustifolia*, *P. obscura* ja *M. laxa s. lato* geneetilist mitmekesisust, neid liike mõjutavaid evolutsiooniprotsesse ning keskkonnatingimuste ja maastiku struktuuri võimalikku mõju fenotüüpilisele ja geneetilisele varieeruvusele.

P. angustifolia ja *P. obscura* uurimiseks kogusin taimi Eestist ja Lätist, kus kasvavad *P. angustifolia* kõige põhjapoolsemad populatsioonid ning Poolast, selliselt alalt, mis kuulub *P. angustifolia* areaali keskosasse. *M. laxa s. lato* taimed kogusin Saaremaalt, Hiiumaalt, Eesti mandriosast ning Hjortö ja Björkö saartelt Ahvenamaa saarestikus (Soome).

Liikidel *P. angustifolia* ja *P. obscura* esines nrDNA isendisisene polümorfsus, mis oli *P. angustifolia* puhul oluliselt suurem. Polümorfsus väljendus selles, et osa ITS-järjestustest olid mittefunktsionaalsed, isendisisene järjestuste keskmine evolutsiooniline divergents oli oluliselt kõrgem isendite vahelisest divergentsist ja igas isendis esines ITS- järjestus hulga erinevate haplotüüpidena.

P. angustifolia isenditel esines kümnes saidis nii *P. angustifolia* kui *P. obscura* iseloomulikke nukleotiide ja viies saidis ainult *P. obscura* nukleotiide (referentsidena kasutati kummagi liigi järjestust andmebaasist GenBank). Seega näitas nukleotiidide muster polümorfsetes saitides kõigi *P. angustifolia* isendite hübriidset päritolu. Fülogeneesianalüüs, kus osa *P. obscura* järjestusi moodustas monofüleetilise rühma koos *P. angustifolia* järjestustega, kinnitas samuti hübridiseerumist kahe liigi vahel. Lisaks muutustele metsa majandamises, võib lokaalne hübridiseerumine olla põhjuseks, miks *P. angustifolia* arvukus on Eestis viimase 50 tugevalt langenud. Kuna areaali keskosast pärinev *P. angustifolia* populatsioon osutus samuti hübriidseks, ei saa välistada, et liik *P. angustifolia* on tervikuna hübriidse päritoluga.

Osa *M. laxa s. lato* rannikuvormi isenditest eristus ülejäänud valimist morfoloogiliste tunnuste alusel, aga mitte geneetiliselt. NrDNA ITS-järjestuste põhjal konstrueeritud fülogeneesipuul olid *M. laxa* rannikuvormi morfoloogiliselt eristuvad isendid polüfüleetilised. *M. laxa s. lato* geneetiline varieeruvus populatsioonides oli positiivses korrelatsioonis populatsioone ümbritseva maastiku struktuuriga, mis näitab suunatud geenivoolu maastikul. Kuna *M. laxa s. lato* levib vee abil, siis geenivool on suure tõenäosusega suunatud sisemaa poolt ranniku poole ehk siis kõrgemalt madalamale. Selline pidev geenivool tõenäoliselt takistab rannikupopulatsioonide geneetilist eristumist.

Fenotüüpiline varieeruvus *M. laxa s. lato* populatsioonides ei olnud korrelatsioonis ümbritseva maastiku struktuuriga ega keskkonnatingimustega. Samas on rannikuvormi iseloomulikke morfoloogilisi tunnuseid on peetud just kohastumusteks iseloomulike keskkonnatingimustega Läänemere rannikul veepiiril ja selle vahetus läheduses. Tõenäoliselt ei reageeri *M. laxa s. lato* taimed keskkonnatingimustele mitte morfoloogiliste tunnuste plastilisusega, vaid kogu elutsükkel on epigeneetiliselt reguleeritud sõltuvalt keskkonnatingimustest. Läänemere rannikul, kus kevadel on kauem külm kui sisemaal ja vegetatsiooniperiood algab hiljem, toimib tõenäoliselt epigeneetiline elutsükli nihe, kus pikenenud külmaperiood kevadel indutseerib taimede üheaastase elutsükli. Selline elutsükli regulatsioon võib esineda mitte ainult Läänemere rannikualadel, vaid igal pool kus *M. laxa s. lato* kasvab talvel jäätuvate veekogude ääres.

Käesolevas töös uurisin evolutsiooniprotsesse ja geneetilist mitmekesisust kareleheliste (Boraginaceae) sugukonna liikidel *P. angustifolia* ja *M. laxa s. lato*, kasutades markerina nrDNA ITS. Leidsin, et nrDNA ITS on liikidel *Pulmonaria angustifolia* ja *Pulmonaria obscura* isendisiseselt polümorfne ning polümorfsus on liigil *P. angustifolia* oluliselt kõrgem. Selgus, et kõik uuritud *P. angustifolia* populatsioonid on hübriidsed, sisaldades *P. obscura* nrDNA ITS järjestusi. Hübridiseerumine võib olla üheks põhjuseks, miks *P. angustifolia* arvukus on Eestis tugevalt langenud, samas ei saa välistada, et liik *P. angustifolia* tervikuna on hübriidse päritoluga. Geneetiline varieeruvus *M. laxa* s. *lato* populatsioonides oli korrelatsioonis ümbritseva maastiku struktuuriga, mis näitab suunatud geenivoolu maastikul. Selline geenivool takistab geneetilist kohastumist rannikul asuvates populatsioonides ning *M. laxa* s. *lato* rannikuvormi iseloomulikke morfoloogilisi tunnuseid põhjustab tõenäoliselt taimede elutsükli epigeneetiline regulatsioon.

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