

TIINA TALVE

Genetic diversity and
taxonomy within the genus *Rhinanthus*



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Department of Botany, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

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

- I **Talve T**, Orav K, Angelov G, Pihu S, Reier S, Oja T. 2012. Comparative study of seed germination and genetic variation of rare endemic *Rhinanthus osiliensis* and related widespread congener *R. rumelicus* (Orobanchaceae). *Folia Geobotanica*, 47, 1–15.
- II Oja T, **Talve T**. 2012. Genetic diversity and differentiation in six species of the genus *Rhinanthus* (Orobanchaceae). *Plant Systematics and Evolution*, 298, 901–911.
- III **Talve T**, McGlaughlin ME, Helenurm K, Wallace LE, Oja T. 2013. Population genetic diversity and species relationships in the genus *Rhinanthus* L. based on microsatellite markers. *Plant Biology*, 16, 495–502.
- IV **Talve T**, Mürk M, Lindell T, Oja T. 2014. *Rhinanthus* plants found in calcareous fens on Gotland (Sweden): are they related to *R. osiliensis* form Saaremaa (Estonia)? *Biochemical Systematics and Ecology*, 54, 113–122.

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The author's contribution to the papers:

Paper I – collecting (80%) and analysing data (80%), writing the text (80%);

Paper II – collecting (90%) and analysing data (100%), writing the text (40%);

Paper III – collecting (100%) and analysing data (80%), writing the text (50%);

Paper IV – collecting (80%) and analysing data (100%), writing the text (50%).

I. INTRODUCTION

Rapidly decreasing biological diversity, including plant diversity, constitutes a very real scientific concern at present time. Biological diversity consists not only of the range of ecosystems, species or populations, but also includes different aspects of genetic variation. Evaluation of genetic diversity and assessment of genetic differentiation are essential for unraveling the common problem in plant taxonomy, namely – complex patterns of morphological variation within and between species, which obscure boundaries between species and make it difficult to estimate species diversity.

There are many plant genera (i.e. grass genus *Bromus*, orchid genus *Dactylorhiza*, *Rhinanthus* and many others) that are well known for their complicated species relationships and taxonomy. Therefore, without thorough genetic evaluations, it is possible to overrate or underestimate the species abundance in a particular taxon and levels of plant diversity respectively. Genetic studies of related species, especially rare and common congeners, provide important information not only about the present day species diversity but also about the evolutionary history and the processes that have shaped this diversity (Charlesworth and Wright 2001). At the same time, such genetic studies help to identify the evolutionary lineages, which sometimes differ from the delimitation of traditional taxonomic units (Karron 1991; Gitzendanner and Soltis 2000; Cole 2003; Vrancken et al. 2012). Moreover, genetic diversity is a main factor for viability and is fundamental for conservation purposes (Hamrick and Godt 1989; Barrett and Kohn 1991; Frankham 1997; Frankham 1998).

For an adequate understanding of genetic variation and gene pool in a population, evaluation of the breeding mode of a species is needed. Generally, outbreeding species are genetically more diverse compared to inbreeding species (Hamrick and Godt 1989; Frankham 1997). Mating between relatives could have a negative effect, called inbreeding depression, on offspring fitness (Charlesworth and Charlesworth 1987; Keller and Waller 2002). The size and fragmentation of a population may also affect the level of genetic diversity. Rare, endangered and endemic species often have small population sizes and are expected to have low genetic diversity due to bottlenecks, genetic drift and inbreeding (Barrett and Kohn 1991; Frankham 1998).

This thesis focusses on a taxonomically difficult genus *Rhinanthus* L. (Orobanchaceae, previously Scrophulariaceae) which embraces annual root-hemiparasitic plants, distributed mainly in Europe (Soó and Webb 1972). The main habitats of *Rhinanthus* species are grasslands and meadows. Species of genus *Rhinanthus* is taxonomically intricate due to great morphological polymorphism, ecotypic variation and seasonality (Soó and Webb 1972; Westbury 2004). According to *Flora Europaea* (Soó and Webb 1972) the genus comprises approximately 30 taxa including ten endemic species and over ten taxa with unclear status. In nature, we often meet individuals with intermediate characters. This remarkable morphological variation has been explained by

phenotypic plasticity depending on the environmental growth conditions (Soó and Webb 1972; Westbury 2004) or by hybridization, which is often detected even between species from different sections (Kwak 1978; Kwak 1980; Westbury 2004; Ducarme and Wesselingh 2005; Ducarme et al. 2010; Natalis and Wesselingh 2012; Ducarme and Wesselingh 2013). In addition, morphological variation of hemiparasitic *Rhinanthus* may be directly affected by hosts and their quality (Seel and Press 1993). Genus is divided into five sections according to flower characteristics (Soó and Webb 1972): *Rhinanthus* L., *Breviostres* (Sterneck) Soó, *Anomali* (Sterneck) Soó, *Anoectolemi* Chab. and *Cleistolemus* Chab. Short rounded teeth of upper lip of corolla are typical of section *Rhinanthus* and short square teeth are characteristic of section *Breviostres*. Small section *Anomali* consists of only one endemic species and is described by convex margins of upper lip of corolla, long teeth and an opened throat. Last two sections have both long teeth and concave margins of upper lip of corolla. Species of section *Anoectolemi* have an opened throat, while in the biggest section *Cleistolemus* the throat of the flowers is closed. This genus has garnered special interest in Estonia, because of our most famous endemic species *Rhinanthus osiliensis*.

During the last decade genetic relationships in genus *Rhinanthus* have been studied several times with different molecular markers. For example, ITS sequences, RAPD, ISSR, AFLP and microsatellites markers have been used to investigate the taxonomy and phylogeny within the genus *Rhinanthus* (Böhme 2001; Ducarme and Wesselingh 2005, 2013; Houston and Wolff 2012; Vrancken et al. 2009, 2012; Pleines et al. 2013). Each of these studies has improved our understanding about the relationships between species in the genus and, altogether, they have provided a more sophisticated picture about *Rhinanthus* as a whole. At the same time, new level of knowledge clearly demonstrated that taxonomic and phylogenetic relationships in *Rhinanthus* are still arguable.

The main research object of this study is the glandular-hairy Estonian neoendem *R. osiliensis* (Ronniger et Saarsoo) Vassilcz. (\equiv *R. rumelicus* Velen. subsp. *osiliensis* Ronniger et Saarsoo), the taxonomic position of which has been unclear from the time of its discovery in 1933 (Saarsoo 1933). Initially, this taxon was described as *R. rumelicus* subsp. *osiliensis* Ronniger et Saarsoo (Ronniger 1934). Its presumable progenitor – glandular-hairy *R. rumelicus* Velen. has a clearly distinct distribution and morphology. Habitat of *R. osiliensis* is calcareous spring fens located in the western part of the island Saaremaa. *Rhinanthus rumelicus* grows in grasslands distributed mainly across Eastern and Central Europe, including Balkan Peninsula (Soó and Webb 1972). The leaves of *R. rumelicus* are twice as wide as *R. osiliensis* and the flowering times of the two never overlap. *Rhinanthus rumelicus* flowers in May and June but *R. osiliensis* at the end of July or in August. It has been suggested that *R. osiliensis* derived from *R. rumelicus* after the postglacial colonization by the Balkan refugia (Eichwald 1960). The species name *R. osiliensis* was for the first

time used in the Flora of the USSR (Vasilchenko 1955). Further, Eichwald (1960) also suggested its recognition at species level based on its distinct morphology, ecological preference and non-overlapping distribution. Nonetheless, the discussion around the taxonomical status of *R. osiliensis* still persists. Another glandular-hairy species *R. wagneri* Degen, which is morphologically very similar to *R. rumelicus* and share the same distribution range, was also included in this study. Main differentiation is that *R. wagneri* have fewer glandular hairs in the upper part of the plant. In addition, plants very similar to *R. osiliensis* have been found in Sweden on the island of Gotland (Lindell 2006). The habitats of these unidentified *Rhinanthus* sp. and *R. osiliensis* are spring fens and their flowering period overlaps. The geographic distances between the two growth locations are approximately 200 km in beeline. Hairiness characters are the only morphological difference between them. *Rhinanthus osiliensis* has longer glandular hairs on the sepals and calyx. Accordingly, an important part of this study was dedicated to the genetic comparison between Estonian *R. osiliensis* and other glandular-hairy species, including the unnamed *Rhinanthus* sp. from Gotland.

Furthermore, for a better overview of genetic variation within the genus, three common species: *R. alectorolophus* (Scop.) Pollich with long white simple hairs, two glabrous *R. minor* L. and *R. angustifolius* C.C. Gmelin (syn. *R. serotinus*) and one Bulgarian endemic *R. javorkae* Soó were included in this study. *Rhinanthus angustifolius*, *R. alectorolophus* and *R. minor* are widely distributed throughout Europe (Soó and Webb 1972), whereas endemic *R. javorkae* is distributed only in one location in South-West Bulgaria (Asenov 1995).

For studying this intriguing genus we used two different molecular markers. Isozymes as codominant genetic markers were used for preliminary estimates of genetic diversity and breeding mode in populations and species. The important advantage of this method is the great amount of available data for many species, which is irreplaceable information for making general conclusions (Hamrick and Godt 1989; Hamrick et al. 1991). Regardless of criticisms leveled against isozymes, they are still widely used for evaluating genetic diversity and species boundaries (e.g., Lipsen et al. 2013, Dostálek et al. 2014).

Microsatellites, or simple sequence repeats (SSRs) as polymorphic, codominant and rapidly mutating (Morgante and Olivieri 1993) markers are very popular and useful for investigating genetic diversity of populations of rare species (e.g., Furches et al. 2009; Riley et al. 2010) and studying taxonomically intricate species (e.g., Dunbar-Co and Wieczorek 2011; Mitsui and Setoguchi 2012; Ferris et al. 2014). Therefore, we chose SSR markers for a more detailed investigation of the *Rhinanthus* species.

In sum, the aims of this doctoral thesis were:

- to develop new microsatellite primers for rare endemic *R. osiliensis* (III)
- to estimate the breeding mode in *R. osiliensis*, *R. rumelicus*, *R. wagneri*, *R. angustifolius*, *R. alectorolophus*, *R. minor* and *R. javorkae* (I, II, III, IV)
- to estimate and compare genetic diversity of Estonian endemic *R. osiliensis*, Bulgarian endemic *R. javorkae* and common species *R. rumelicus*, *R. wagneri*, *R. angustifolius*, *R. alectorolophus* and *R. minor* (I, II, III, IV)
- to assess species boundaries in genus *Rhinanthus* (I, II, III, IV)
- to characterize genetic variation of the unidentified *Rhinanthus* taxon from Gotland, Sweden and determine its relation to the *R. osiliensis* (IV)

2. MATERIALS AND METHODS

2.1. Study species and sampling

We investigated seven *Rhinanthus* species from three different sections. *Rhinanthus osiliensis* (I, II, III, IV) (Fig. 1A), *R. rumelicus* (I, II, III) (Fig. 2B), *R. wagneri* (II, III), *R. angustifolius* (II, III, IV) and *R. alectorolophus* (II) (Fig. 3C) belonging to section *Cleistolemus*, *R. minor* (II, III, IV) to section *Rhinanthus* and *R. javorkae* (III) (Fig. 4D) to section *Anoectolemi*. In addition, taxonomically unclear individuals found from Sweden were studied and compared with *R. osiliensis*, *R. angustifolius* and *R. minor* from Estonia (IV).



Fig. 1. Studied species **A.** *Rhinanthus osiliensis* (Ronniger et Saarsoo) Vassilcz. **B.** *Rhinanthus rumelicus* Velen. **C.** *Rhinanthus alectorolophus* (Scop.) Pollich **D.** *Rhinanthus javorkae* Soó.

For isozyme analysis (**I**, **II**), the seeds of studied species were collected from five to ten randomly selected plants at least 3 m apart, air-dried at room temperature and stored in paper bags at 4°C until the germination. The seed accessions from each population were pooled to obtain representative samples. In addition, seeds of *R. alectorolophus* from one natural population from Germany and of *R. minor* from France were provided by the Muséum d' Histoire Naturelle and Rennsteiggarten Oberhof, respectively. Seeds were placed on wetted filter paper in Petri dishes at the end of October and stratified in darkness at 4 °C for six months. Germinated seeds were planted and cultivated in the greenhouse until the isozyme analysis. For microsatellite analysis (**III**, **IV**), healthy leaf samples were collected from randomly selected plants at least 3 m apart and stored in silica gel until DNA extraction. All vouchers are kept in the herbarium of The Natural History Museum, University of Tartu (TU).

2.2. Isozyme analysis

Enzyme analyses were used in papers **I** and **II**. The samples were prepared from fresh leaves of previously grown seedlings in the extraction buffer. In total, five enzyme systems with readable resolution were used for further analysis: shikimate dehydrogenase (SKD, EC 1.1.1.25), aspartate aminotransferase (AAT, EC 2.6.1.1), 6-phosphogluconate dehydrogenase, (6PGD, EC 1.1.1.44), esterase (EST, EC 3.1.1.2) and phosphoglucomutase (PGM, EC 2.7.5.1). The enzymes were separated on 20% acrylamide gels with modified gel-buffer systems and were stained with standard histochemical methods (Wendel and Weeden 1989). The isozymes phenotypes correspond to respective genotypes and the interpretation on enzyme banding patterns is based on the subunit structure (Wendel and Weeden 1989). Different loci of isozymes are labeled by capital letters followed by a number reflecting allozymes in the order of decreasing mobility, e.g. *AAT-B2*.

2.3. Microsatellite analysis

The second tool for estimating genetic variation among and within our studied species was microsatellites (**III**, **IV**). Total genomic DNA was isolated from silica-dried leaves using the CTAB method (Doyle and Doyle 1990). In paper **III**, six new microsatellite primers were developed for *R. osiliensis* and analysis were performed to compare genetically two endemics *R. osiliensis* and *R. javorkae* with widespread species *R. rumelicus*, *R. wagneri*, *R. angustifolius* and *R. minor*. In paper **IV**, 15 primers developed for *R. angustifolius* (Ducarme et al., 2008) eight primers developed for *R. minor* (Houston and Wolff, 2009) and six primers previously developed for *R. osiliensis* (**III**) were tested for cross-species amplification and finally fifteen successfully amplified microsatellite

primers were used for further analysis (IV). Primers were optimized for a range of temperatures and $MgCl_2$ or $MgSO_4$ concentrations. PCR was performed using a Techne TC-5000. Microsatellite alleles were detected on an Avant 3100 Genetic Analyser (Applied Biosystems, Foster City, USA) and were sized against LIZ 500 size standard (Applied Biosystems) using Peak Scanner Software v1.0 (Applied Biosystems).

2.4. Data analysis

In paper I, the mean population germination percentages and one-way ANOVA based on germination rates were calculated. We also calculated the Spearman rank correlation r_s between germination percentage and the genetic diversity measures. The t-test was used to assess the differences of germination rates among species.

For genetic diversity evaluation, the percentage of polymorphic loci (P), the mean number of alleles per locus (A), observed (H_O) and expected heterozygosity (H_E) were calculated for each population (I, II, III, IV). Received values were averaged for each species to express mean genetic variation at the population level. The species-level values are proper for comparing genetic diversity among species using isozymes (Hamrick and Godt 1989). Thus, genotypes of species were pooled and genetic diversity parameters were calculated (I, II). Significant differences of genetic diversity values (P , A , H_O , H_E), inbreeding coefficient (F_{IS}) and genetic differentiation (F_{ST}) between *R. osiliensis* and *R. rumelicus* were evaluated with two-side test (I). Inbreeding coefficient and its significant departure from zero were estimated through the chi-square test in each locus and overall in every studied population (I, II, III).

To estimate intra- and intergenetic differentiations, pairwise F_{ST} values between populations were calculated (I, IV). The partitioning of genetic diversity among studied species, populations within species and within populations was estimated with an analysis of molecular variance (AMOVA) (I, III). In addition, a principle coordinates analysis using the covariance standardized method of Nei's genetic distances among the populations to determine the genetic grouping of populations and species (III, IV). Finally, the Bayesian model-based clustering approach was used to assign individuals to genetic clusters (K) without *a priori* knowledge of taxonomy or population locations (III, IV). For illustrate the species relationships, a cluster analysis on the basis of the Nei's genetic distances was performed, using the unweighted pair group method with arithmetic mean (UPGMA) (I, II, III) and Neighbor-Joining (IV) methods.

3. RESULTS

3.1. Breeding mode

Inbreeding coefficient (F_{IS}) was calculated by isozymes (**I**, **II**) and microsatellites (**III**). We also could estimate inbreeding in paper **IV**, comparing observed and expected heterozygosity what reflects deviation from Hardy-Weinberg equilibrium. Inbreeding coefficient values in *R. osiliensis* and *R. javorkae*, indicated predominant inbreeding in these endemics (**I**, **II**, **III**). Germination rate was in correlation with inbreeding coefficient whereas rare *R. osiliensis* had significantly lower germination rates and longer dormancy of seeds compared to closely related widespread *R. rumelicus* (Fig. 1, in paper **I**). Our results confirm the prevalence of autogamy in *R. minor* in contrary to other common species, but in consistency with its flower morphology (**III**, **IV**). Expected heterozygosity was higher than observed heterozygosity in almost every population of all studied species, except one population of *R. osiliensis* and one population of unnamed *Rhinanthus* species from Gotland (**IV**). This result reveals non-random mating mode in most of the studied populations.

3.2. Genetic diversity within genus *Rhinanthus*

In total, seven *Rhinanthus* species from three sections were genetically studied using isozymes and microsatellites. We used five enzymes and seven polymorphic loci to evaluate genetic diversity within species (**I**, **II**). Six new microsatellite markers were developed for endemic *R. osiliensis* and were successfully amplified in *R. rumelicus*, *R. wagneri*, *R. angustifolius*, *R. minor*, *R. javorkae* and unidentified *Rhinanthus* sp. (**III**, **IV**).

Altogether, isozymes and microsatellites disclosed similar genetic diversity results. Genetic diversity parameters were highly variable between populations within species studied (**I**, **II**, **III**, **IV**). Endemics *R. javorkae* and *R. osiliensis* showed low genetic diversity parameters compared to widespread species. For example, observed number of alleles (A), observed (H_O) and expected heterozygosity (H_E) in endemic *R. osiliensis* were lower in all studied papers compared to presumable congener *R. rumelicus* (Table 3 in **I**, Table 3 in **II** and Table 4 in **III**). The same results were identified when comparing *R. osiliensis* to common species *R. angustifolius* (**II**, **III**, **IV**) and *R. wagneri* (**II**, **III**). Bulgarian endemic *R. javorkae* was studied only with microsatellites in paper **III** but genetic diversity was the lowest compared to the other five species including Estonian endemic *R. osiliensis*. Surprisingly, low genetic variation was also identified in common *R. alectorolophus* using isozymes (**II**) and in *R. minor* using microsatellites (**III**, **IV**).

3.3. Species boundaries in genus *Rhinanthus*

Genetic differentiation and species relationships within genus were inferred from data obtained by isozymes and SSR analyses. Results based on isozymes and microsatellites data showed similar differentiation patterns. The UPGMA and Neighbor-Joining dendrograms, based on Nei's genetic distances, illustrate the genetic relationships between studied populations and species. Four studied species: *R. alectorolophus* from section *Cleistolemus* (II), *R. minor* from section *Rhinanthus* (II, III, IV), Bulgarian endemic *R. javorkae* from section *Anoectolemi* (III) and unspecified species from Gotland (IV) are genetically most distinct from the other studied species.

All studied species of section *Cleistolemus* (*R. osiliensis*, *R. rumelicus*, *R. wagneri*, *R. angustifolius*), except *R. alectorolophus*, were close to each other with slight differentiation (I, II, III, IV). Our results demonstrate the genetic distinctness of *R. alectorolophus* and argue against its placement in section *Cleistolemus*. In agreement with current taxonomy, *R. minor*, which belongs to section *Rhinanthus*, occupies a distinct position in an UPGMA dendrogram (II, III and IV). Strong genetic differentiation of rare endemic *R. javorkae* was also in accordance with its position in a different section *Anoectolemi* (III). We found that individuals of unnamed *Rhinanthus* from Gotland comprised a clearly differentiated genetic unit (IV). These findings are also supported by the Bayesian clustering analysis (Fig. 3 in paper III and IV).

3.4. Species relationships between *R. osiliensis* and unspecified taxon from Gotland

Data, based on fifteen microsatellite primer pairs, clearly separated unassigned *Rhinanthus* sp. from narrow endemic *R. osiliensis* (IV). All genetic diversity parameters (P , A , H_O , H_E) were remarkably lower in *Rhinanthus* sp. (Table 4, IV). Comparing those two taxa, 95 alleles were detected (Appendix A, IV). All 15 loci were polymorphic in *R. osiliensis* and 54 private alleles were detected. In contrast, only 4 unique alleles were found in *Rhinanthus* sp. and 4 loci were totally monomorphic. Genetic differentiation of populations (F_{ST}) within species was high in both studied species (Table 4, IV). *Rhinanthus* sp. genetic differentiation from *R. osiliensis* was supported by three different analyses: Neighbor-Joining dendrogram (Fig. 1, IV), principal component analysis (Fig. 2, IV) and Bayesian clustering (Fig. 3, IV). In addition, results showed that *R. osiliensis* and *Rhinanthus* sp. are both more closely related to *R. angustifolius* than to each other.

4. DISCUSSION

4.1. Breeding mode

Breeding mode is one of the factors that significantly influence genetic diversity of populations and species. Generally, inbred species with restricted geographic distribution have lower genetic variation compared to common outbreeding plant species (Hamrick and Godt 1989; Frankham 1997). Rare and endemic species with small population sizes usually have reduced genetic diversity possibly due to bottlenecks, genetic drift and inbreeding (Barrett and Kohn 1991; Frankham 1998). However, several studies have found that the present generalizations may be too simplified and some restricted rare species also have high genetic diversity.

High breeding mode variation has been detected in plants even in closely related taxa (Jain 1976; Schemske and Lande 1985). Therefore it is informative to evaluate the breeding mode using genetic markers. Inbreeding coefficient F_{IS} shows heterozygote deficiency or excess, indicating breeding mode of the population (Weir and Cockerham 1984).

Our results showed a positive inbreeding coefficient as well as homozygote excess in most of the studied populations regardless of species, indicating non-random mating (**I, II, III, IV**). Non-random mating is common in plants and could be explained by several factors. Studied *Rhinanthus* species are mainly pollinated by bumblebees (Kwak 1978; Ducarme and Wesselingh 2005; Natalis and Wesselingh 2012) with flying distances less than 50 m (Saville et al. 1997), which rarely lead to pollination between populations. Moreover, bumblebees usually visit several flowers on the same plant causing geitonogamous pollination (Utelli and Roy 2000; Matsuki et al. 2008). In addition, the quite large seeds of *Rhinanthus*, distributed only by gravitation (Eichwald 1960; Reitalu 2003; Westbury 2004), usually germinated in close proximity to the mother plant, boosting the mating between relatives. Sometimes, selfing is likely to be an adaptive breeding mode under conditions of population bottlenecks or decreased pollinator visitations (Jain 1976; Schemske and Lande 1985). Therefore, *Rhinanthus* species supposedly should be predominantly autogamous.

Among the studied species, widespread *R. minor* showed significantly high fixation index, indicating selfing as a mating mode in contrary to other common species (**II, III, IV**). It has been previously suggested that *R. minor* can self-pollinate (Westbury 2004). In addition, Ducarme and Wesselingh (2013) also showed high inbreeding coefficient for this species. The morphology of the flower of *R. minor* promotes self-fertilisation as well (Kwak 1979; Ducarme and Wesselingh 2013).

In contrast, *R. angustifolius* needs bumblebee visitation for pollination (Kwak 1979, 1980) which results in higher outcrossing rate (Ducarme and Wesselingh 2013). We found considerable variation in levels of genetic

diversity and inbreeding coefficient in populations of common *R. angustifolius*: some of them had a mixed mating mode, some were more inbred. These findings are not surprising for such a polymorphic and widely distributed species as *R. angustifolius*. Another widespread species *R. rumelicus* revealed a similar pattern of genetic variation and mating mechanisms to *R. angustifolius*.

Rare and endemic species quite often have small population sizes with low genetic diversity due to bottlenecks, genetic drift and inbreeding (Barrett and Kohn 1991; Frankham 1998). In our study, taxa with extremely small distribution range (endemics *R. osiliensis*, *R. javorkae* and unnamed *Rhinanthus* sp.) showed remarkably high inbreeding levels (**I**, **II**, **III** and **IV**). In accordance with previous studies (Schemske and Lande 1985), our results also showed that mating modes vary widely even in closely related taxa (e.g. *R. rumelicus* and *R. osiliensis*).

Germination rate and duration of dormancy can also be related to inbreeding. Germination rate was in correlation with inbreeding coefficient, where *R. osiliensis* had significantly lower germination rates and longer dormancy of seeds compared to *R. rumelicus* (Fig. 1, **I**). *Rhinanthus minor* is a widespread species but its dormancy period is longer than expected compared to another common species *R. angustifolius* (ter Borg 2005; Mudrak et al. 2014). Our results coincide with previous study of germination in genus *Rhinanthus*, where two different types of dormancy were detected (ter Borg 2005). Duration of dormancy could be an adaptation to different environmental conditions (Meyer and Carlson 2004).

4.2. Genetic diversity within genus *Rhinanthus*

Genetic diversity is fundamental for species viability but also influences community structure. Species of *Rhinanthus* are especially important to study because they have a strong effect on ecosystems through host-parasite interactions (Rowntree et al. 2011). Isozymes and microsatellites revealed similar results. In general, endemic *R. osiliensis* and *R. javorkae* showed lower level of genetic diversity compared to the common *R. rumelicus*, *R. wagneri* and *R. angustifolius* (**I**, **II**, **III**). In addition, low genetic diversity was found in unidentified taxon found on Gotland, Sweden (**IV**). These results are supported by the common idea that narrowly distributed species have lower level of genetic diversity compared to widespread ones (Hirai et al. 2012; Yamada and Maki 2012). Decreased genetic diversity occurs due to bottlenecks, genetic drift and inbreeding (Barrett and Kohn 1991; Frankham 1998).

We found considerable genetic variation in populations of widespread *R. angustifolius*, *R. rumelicus* and *R. wagneri* with unique private alleles in latter two species (**II**, **III**). Our findings are supported by many others studies of widely distributed species (Frankham 1997; Dodd and Helenurm 2002; Gonzales and Hamrick 2005). Some species-specific alleles have been

previously found in *R. angustifolius* using RAPD and ISSR markers, probably indicating the ongoing speciation process (Ducarme and Wesselingh 2005).

Unexpected results were found in common species *R. minor* and *R. alectorolophus* where species showed lower genetic diversity compared to the other common species (*R. angustifolius*, *R. rumelicus*, *R. wagneri*) (II, III, IV). Low genetic variation in *R. minor* could derive from its flower morphology and breeding mode as discussed before.

Great variation of genetic diversity between populations within studied species is an expected result for genus *Rhinanthus*. There are many factors that influence the levels of genetic diversity among populations of *Rhinanthus* species. Generally, genetic diversity of a population is strongly affected by management, local history and climate fluctuations. Species of *Rhinanthus* do not form persistent seed banks and most of the seeds germinate in their first year. In addition, seeds remain dormant and a cold stratification period is needed for germination (ter Borg 2005). Therefore, a poor vegetation year or an unusual winter could have a dramatic influence on the population's genetic diversity. For example, a short and warm winter could be insufficient for breaking dormancy, resulting in most of the seeds not germinating in the following spring (ter Borg 2005).

Main habitats of *Rhinanthus* are grasslands. Seasonal management, such as mowing and litter removal is usual in those habitats and directly influences the diversity of plant community. Several papers have shown that management could have negative or positive influences to viability and also genetic diversity of populations. Mowing or grazing before seed production have strong negative effects due to annual life trait and no persistent seed bank of *Rhinanthus* (Magda et al. 2004; Mudrak et al. 2014). But, effective management after flowering and before seed release helps with seed dispersion (Strykstra et al. 1997; Bullock et al. 2003). Litter removal also has a positive effect on populations, especially on the survival of seeds (Mudrak et al. 2014). Moderate disruption of habitat could have positive effect on a population through formation of new micropatches and a better environment for *Rhinanthus*.

4.3. Species boundaries in genus *Rhinanthus*

The studied genus *Rhinanthus* includes many species with uncertain taxonomic rank and position. Our results demonstrated that assessment of genetic diversity may help to elucidate the species differentiation and taxonomic relationships within this genus.

Based on our SSR and isozyme data we can clearly distinguish *R. minor* (II, III, IV), Bulgarian endemic *R. javorkae* (III), *R. alectorolophus* (II) and unnamed *Rhinanthus* species (IV) from other studied species. The strong differentiation of *R. minor* and *R. javorkae* is coincident with morphological delimitation and existing taxonomy, where these two species belong to separate

sections in genus *Rhinanthus*. Previous study (Houston and Wolff 2012), based on microsatellites markers showed intraspecific differentiation of populations of *R. minor* according to geographic location but not according to subspecies. Recently, AFLP data revealed clear-cut genetic split into two strongly differentiated geographic groups within *R. minor* (Vrancken et al. 2012).

Isozyme results demonstrated that *R. alectorolophus* is distinct from other species in section *Cleistolemus* (II). This result is inconsistent with contemporary taxonomy but in accordance with study of Böhme (2001), where ITS sequence data placed *R. alectorolophus* also apart from other species of section *Cleistolemus*. The present delimitation and composition of sections is based purely on flower morphology and even from this point of view *R. alectorolophus* differs from others, having calyxes densely covered with long white hairs (Soó and Webb 1972). Investigation of *R. alectorolophus* with AFLP markers did not find any genetic differentiation between ecotypes of this species (Pleines et al. 2013). Thus, our results strongly substantiate the distinct position of *R. alectorolophus* and provide additional evidence for paraphyly of section *Cleistolemus*.

Among all the species studied, *R. osiliensis*, *R. angustifolius*, *R. rumelicus* and *R. wagneri* from section *Cleistolemus* showed some differentiation but are genetically connected to each other according to isozymes (I, II) and microsatellites (III, IV). The close relationships between them are in agreement with their overall morphology. *Rhinanthus osiliensis*, *R. rumelicus* and *R. wagneri* have quite similar features. The main difference between *R. osiliensis* and its closest congener *R. rumelicus* is that the leaves of the latter are remarkably wider. *Rhinanthus wagneri* is very similar to *R. rumelicus* but its stems and bracts are subglabrous (Soó and Webb 1972). The calyx is also subglabrous except margins and surface of the fruit (Soó and Webb 1972; Asenov 1995). Our results support the general view about low genetic differentiation that probably accompanies rapid and recent speciation, as indicated by several authors (Gottlieb 1973; Crawford 1985; Segarra-Moragues and Catalán 2002). It has been shown that annual species may have as low genetic differentiation as conspecific populations due to recent origin of these taxa and incompletely developed species boundaries (Gottlieb 1977). The origin from common ancestors with further adaptation to different ecological conditions (spring bogs for *R. osiliensis* and meadows for *R. rumelicus* and *R. wagneri*) could also be a possible explanation for these findings. Our data confirmed the previous ITS results (Böhme 2001) that showed low genetic differentiation between species and even between different sections.

The morphologically and ecologically diverse *R. angustifolius* is common everywhere in Europe and co-occurs with the three studied species (*R. wagneri*, *R. rumelicus* and *R. minor*). The flowering time of *R. angustifolius* completely overlaps with *R. wagneri* and partially with *R. rumelicus* (Asenov 1995) and *R. osiliensis* (Eichwald 1960; Reitalu 2003). Hybridization has been demonstrated between *R. minor* and *R. angustifolius* (Kwak 1980; Ducarme and

Wesselingh 2005; Ducarme et al. 2010). Unfortunately, hybrids are very polymorphic and often so close to their parental species that only genetic markers are able to identify hybridization and introgression (Ducarme and Wesselingh 2005). The hybridization and introgression of *R. minor* towards to *R. angustifolius* were detected in several studies (Ducarme et al. 2010; Natalis and Wesselingh 2013; Vrancken et al. 2009; Vrancken et al. 2012). Furthermore, a putative hybridization event between *R. javorkae* and *R. wagneri* could be suggested according to our results and personal observations (III).

Weak species boundaries could be explained by the recolonization routes after the last glaciation (Hewitt 2000). Two main Pleistocene refugia have been identified: in south-western Europe and in eastern Balkan/Caucasus, whereas other authors have suggested that *R. angustifolius* spread mainly from eastern refugium (Vrancken et al. 2009). Phylogeographic studies of *R. angustifolius* (Vrancken et al. 2009) and *R. minor* (Vrancken et al. 2012) detected differentiation of admixture between *R. minor* and *R. angustifolius*. The level of admixture was low in the west of Europe but higher in the east (Vrancken et al. 2012).

4.4. Species relationships between *R. osiliensis* and unspecified taxon from Gotland

Theoretical studies emphasize that speciation is a complex process of genetic and morphological differentiation. Empirical studies have shown the situation to be even more complicated. Our attempt to unravel the relationships between unspecified *Rhinanthus* sp. found on Gotland and rare endemic *R. osiliensis* from Estonian Saaremaa demonstrated their genetic distinctness, regardless of morphological and habitat similarity (IV). The populations of *Rhinanthus* sp. showed remarkably lower genetic diversity than *R. osiliensis*, despite the fact that they both have an extremely narrow distribution area (Hirai et al. 2012; Yamada and Maki 2012). Several private alleles were found when comparing genetic variation at the taxon level. The Neighbour-Joining, a principle coordinates analysis and Bayesian clustering clearly separated the *Rhinanthus* sp. from *R. osiliensis*.

Similar findings have been reported in studies of the population *R. minor* across Europe (Vrancken et al. 2012). Vrancken and colleagues (ibid.) discovered morphologically very similar but genetically surprisingly distinct populations of *R. minor* in different geographic regions. Moreover, those genetically distinct populations were genetically more similar to *R. angustifolius*. Our study also found that *R. osiliensis* is genetically more close to *R. angustifolius* (IV). Conversely, a few studies of *Rhinanthus* have found no genetic distinctness regardless of morphological differentiation (Houston and Wolff 2012; Pleines et al. 2013). Lack of genetic separation between six different subspecies of *R. minor* was found in the UK (Houston and Wolff

2012), but the studied individuals were distinct from *R. minor* and *R. angustifolius* found elsewhere in Europe. Another example of disarrangement within *Rhinanthus* is a study of *R. alectorolophus* (Pleines et al. 2013). Morphological characteristics distinguished three different subspecies of *R. alectorolophus* and three genetic clusters were also identified but they did not correspond to the taxonomic classification based on morphology. Such examples of discordance between levels of genetic and morphological diversity are abundant in plant taxonomy (e.g. Doyle 1997; Helsen et al. 2009). Recent speciation, cryptic species, hybridization or incomplete investigation may be behind the morphologically similar taxa (Bickford et al. 2007; Reeves and Richards 2011). Despite the proximity of islands Saaremaa and Gotland and their similar geological age and origin (Björck 1995; Tuuling et al. 2011) *Rhinanthus* sp. and *R. osiliensis* probably used different colonization routes after the last glaciation (Hewitt 2000). Thereafter, adaptation to spring fen habitats resulted in the convergence of morphologic characters of originally different species. Gene flow between two locations is effectively prevented by sea, which keeps the two species still dissimilar. This hypothesis is also supported by the presence of distinct march orchid's species on the islands (Ståhlberg and Hedrén 2008).

Our results showed that individuals of *Rhinanthus* sp. from Gotland and *R. osiliensis* form two different genetic entities and may not belong to the same species. In spite of their strong morphological resemblance, they possess remarkable genetic differentiation evaluated by microsatellite data (IV). Further genetic comparison between this unnamed *Rhinanthus* sp. and other species is crucial to determine the status and origin of this taxon. A first step in this investigation could be the comparison of this taxon with local populations of *R. angustifolius* and *R. minor* from Gotland.

CONCLUSIONS

Genetic diversity and differentiation levels of seven *Rhinanthus* species from three different sections and one unclear taxon were studied in this thesis. Two different molecular techniques – allozymes and microsatellites – were used for analyses. The results of this thesis demonstrate the importance of evaluating genetic variation for describing complex species taxonomy. Different aspects of genetic diversity provide valuable information about the species delimitation, relationships and species boundaries.

The following conclusions may be drawn from the obtained results:

- We developed six new microsatellite markers for endemic *R. osiliensis*. They were successfully amplified in *R. rumelicus*, *R. wagneri*, *R. angustifolius*, *R. minor* and *R. javorkae*. Allozymes and microsatellites showed similar results. However, microsatellites will be favored tools in the future for studying species of *Rhinanthus* due to their higher discriminating ability.
- Non-random mating and inbreeding were detected in most of the studied populations, probably due to particular behavior of pollinators, heavy seeds and flower morphology. Significantly different germination rate, duration of dormancy and inbreeding were found in *R. osiliensis* and *R. rumelicus*. Two different dormancy periods were detected, which could indicate adaptation to different climate conditions.
- Low genetic diversity was detected in endemics *R. osiliensis* and *R. javorkae*. Remarkably low genetic variation was found in *Rhinanthus* sp. from Gotland, Sweden. These results are in accordance with the theoretical hypothesis that species with restricted geographic range have low level of genetic diversity compared to species with widespread distribution. Unexpectedly, widespread *R. minor* showed also low level of genetic variation and high inbreeding mode. This finding could be explained by the exceptional flower morphology that induces autonomous self-pollination. This fact shows the complexity of patterns of genetic diversity among species in the genus *Rhinanthus*.
- *Rhinanthus minor* and *R. javorkae* were well differentiated from other studied species. Separation of *R. minor* and *R. javorkae* is congruent with previous taxonomy where those two species belong to two different sections. Surprisingly, *R. alectorolophus* that belongs to section *Cleistolemus* together with *R. osiliensis*, *R. rumelicus*, *R. wagneri* and *R. angustifolius* was strongly differentiated from those species. This result raises a question about the monophyly of this section. Low genetic differentiation of *R. osiliensis*, *R. rumelicus*, *R. wagneri* and *R. angustifolius* shows their close affinity and weak genetic structure of section *Cleistolemus*.

- We clearly showed that the unidentified *Rhinanthus* sp. found from Gotland, while morphologically similar to *R. osiliensis*, does not belong to the same taxon. Both *R. osiliensis* and *Rhinanthus* sp. are genetically more close to *R. angustifolius*. We suggest that these two cryptic species originated from two independent island colonization events and may be even from different refugia. Further genetic comparison between unspecified *Rhinanthus* sp. and local *Rhinanthus* species from Gotland is necessary to determine the species status and to check for possible alternative hypotheses about the origin of this unclear taxon.

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SUMMARY IN ESTONIAN

Perekond robirohu (*Rhinanthus*) geneetiline mitmekesisus ja taksonoomia

Meie planeedi kiiresti kahanev bioloogiline mitmekesisus on viimase aastakümne üks aktuaalsemaid uurimisteemasid. Bioloogiline diversiteet koosneb lisaks ökosüsteemide, liikide ja populatsioonide mitmekesisusele ka geneetilisest mitmekesisusest. Tänapäevaks on tõestatud, et liikide jätkuva ellujäämise üheks oluliseks faktoriks on piisav geneetiline varieeruvus. Seega aitab geneetilise mitmekesisuse uurimine lahendada nii keerukate liigikomplekside taksonoomilisi probleeme kui annab meile infot tänapäeva liigirikkuse, liikide mineviku ning evolutsiooni kohta. Nii ei ole tänapäeva looduskaitse mõeldav ilma haruldaste ning laialt levinud liikide võrdleva geneetilise analüüsita, mille põhjal saab koostada otstarbekad kaitsekavad. Geneetiline varieeruvus on otseselt seotud taimede paljunemisviisi ja populatsiooni suurusega. Isegi lähedastele taimeliikidele on iseloomulik suur paljunemisviiside varieeruvus ning risttolmlejatel on isetolmlejatega võrreldes reeglina kõrgem geneetiline mitmekesisus. Haruldastel ja endeemsetel liikidel on sageli väikesed populatsioonid, kus mitme protsessi (pudelikaela efekt, geneetiline triiv ning sugulusristumine) koosmõju viib madalama geneetilise varieeruvuseni. Niisiis on mitmekülgsed geneetilised uuringud liigirikkuse adekvaatseks hindamiseks ning kaitstmiseks asendamatud.

Perekond robirohi (*Rhinanthus* L.) koosneb ligikaudu 30 üheaastasest poolparasiitsest liigist, mis on peamiselt õite morfoloogia alusel jagatud viide sektsiooni: *Rhinanthus* L., *Breviostres* (Sterneck) Soó, *Anomali* (Sterneck) Soó, *Anoectolemi* Chab. ja *Cleistolemus* Chab. Perekonda iseloomustab taksonoomiline keerukus – kirjeldatud on palju ebaselge staatusega endeeme, alamliike ning ökotüüpe. Lisaks sellele esineb tunnustatud taksonite vahepealsete tunnustega indiviide, mida seletatakse nii fenotüübilise plastilisuse kui hübriidisatsiooniga. Kuigi viimastel aastatel on avaldatud mitmeid asjakohaseid teadustöid robirohu perekonna kohta, on endiselt palju vastamata küsimusi ning neid tekib aina juurde.

Käesolev väitekiri keskendub perekonna *Rhinanthus* seitsme liigi: saaremaa robirohu *R. osiliensis* (I, II, III, IV), rumeelia robirohu *R. rumelicus* (I, II, III), *R. wagneri* (II, III), suure robirohu *R. angustifolius* (II, III, IV), *R. alectrolophus* (II) sektsioonist *Cleistolemus*, väikese robirohu *R. minor* (II, III, IV) sektsioonist *Rhinanthus* ning *R. javorkae* (III) sektsioonist *Anoectolemi* geneetilisele analüüsile. Lisaks uurisime ühte ebaselget robirohu taksonit Gotlandi saarelt Rootsist. Täitmaks uurimuse eesmärgi, töötasime välja kuus uut mikrosatelliitide praimerit Eesti endeemse liigi *R. osiliensis* jaoks. Need sobivad ka teiste robirohu liikide geneetilise mitmekesisuse analüüsimiseks. Uuritud liikide paljunemisviiside väljaselgitamiseks kasutasime nii iso-

ensüümide kui mikrosatelliitide meetodit. Mõlemad näitasid, et kõigis perekond *Rhinanthus* uuritud liikide populatsioonides esineb sugulusristumist ehk inbriidingut (**I, II, III, IV**). Robirohu liigid on putuktolmlejad, kuid tolmel-damine toimub sageli sama taime erinevate õite vahel ehk geitonogaamselt, mis vähendab suure tõenäosusega võõrtolmlemist ja geenivoogu erinevate populatsioonide vahel. Lisaks langevad rasked seemned emataime ümbrusesse, mis suurendab veelgi sugulusristumise tõenäosust. Haruldase endeemi *R. osiliensis*'e ja tema lähima laia levikuga sugulusliigi *R. rumelicus* seemnete idanevus ning puhkeperioodi kestvus osutusid väga erinevaks. Võib väita, et siin on tegemist adaptatsiooniga erinevate kasvukoha tingimuste ja regiooni kliimaga.

Kasutasime mõlemat mainitud molekulaarset meetodit ka liikide geneetilise mitmekesisuse hindamiseks ning võrdlemiseks perekonnas *Rhinanthus* (**I, II, III, IV**). Tulemused näitasid, et endeemsetel liikidel (*R. osiliensis* ja *R. javorkae*) on madalam geneetiline mitmekesisus kui laialt levinud liikidel (*R. rumelicus*, *R. wagneri*, *R. angustifolius*). Ka Gotlandi ebaselget taksonit iseloomustab väga madal geneetiline mitmekesisus. Need tulemused on kooskõlas teoreetiliste hüpoteesidega, mille kohaselt kitsa levikuga liigid ongi geneetiliselt vaesemad. Üllatusena leidsime, et ka laialt levinud liik *R. minor* on madala geneetilise varieeruvuse ja kõrge inbriidingu määraga. Lähimalt uurides ilmnes, et selle liigi õites on tolmukad ja emakas teineteisele väga lähedal ning see soodustab isetolmlemist. Meie tulemused näitasid, et üldlevinud arusaam, et putuktolmlejad liigid on valdavalt võõrtolmlejad ei pruugi vastata reaalsele pildile. Isegi ühe perekonna piires liikide paljunemisviisid märkimisväärselt varieerusid. Analüüsisime ka liikide omavahelist eristumist ja liigisisest varieeruvust (**I, II, III, IV**) uuritud liikide seas. Teistest uuritud liikidest eristusid enim *R. minor* seksioonist *Rhinanthus* (**II, III, IV**), Bulgaaria endeem *R. javorkae* seksioonist *Anoectolemi* (**III**) ning *R. alectorolophus* seksioonist *Cleistolemus* (**II**). *Rhinanthus minor* ja *R. javorkae* geneetiline eristumine on kehtiva klassifikatsiooniga vastavuses. Nad kuuluvad eraldi seksioonidesse. Ootamatu oli *R. alectorolophus* geneetiline erisus võrreldes teiste *Cleistomus* seksiooni kuuluvate liikidega. Siiski, on meie tulemused kooskõlas eelneva uuringuga, kus ka ITS järjestused eristasid *R. alectorolophus* teistest sama seksiooni liikidest. Väidame, et seksioon *Cleistolemus* ei pruugi olla monofüleetiline grupp ning vajab ümberklassifitseerimist. Teised seksiooni *Cleistolemus* liigid *R. osiliensis*, *R. angustifolius*, *R. rumelicus* ja *R. wagneri* olid geneetiliselt sarnased. Madal geneetiline mitmekesisus iseloomustab sageli hiljuti tekkinud noori liike. On tõenäone, et seksioonis *Cleistolemus* on liigitekkelised protsessid alles käimas ning liigid pole veel lõplikult geneetiliselt eristunud. Lisaks sellele toimub perekonna *Rhinanthus* liikide vahel sageli hübriidisatsioon, mis omakorda vähendab liikide geneetilist diferentseerumist.

Kuna hübriidid on morfoloogiliselt väga varieeruvad on neid võimalik kindlamalt tuvastada vaid geneetiliste meetoditega.

Hindasime ka Gotlandist leitud ebaselge robirohu populatsiooni geneetilist mitmekesisust ning võrdlesime saadud tulemusi morfoloogiliselt väga sarnase Saaremaa robirohuga *R. osiliensis* (IV). Tulemused näitasid, et Rootsist, Gotlandi saarelt leitud robirohu populatsioon ning Eesti endeem *R. osiliensis* on teineteisest geneetiliselt märkimisväärselt eristunud ning tegemist ei saa olla ühe liigiga. *Rhinanthus osiliensis* oli geneetiliselt lähedasem hoopis laialt levinud suurele robirohule *R. angustifolius*. Lõpliku taksonoomilise järelduse tegemiseks on vajalik ebaselge taksoni geneetiline võrdlus teiste Rootsis leiduvate robirohu liikidega. Käesolevas doktoritöös esitatud tulemused näitavad molekulaargeneetiliste uuringute tähtsust ja vajalikkust nii teoreetiliste kui ka praktiliste probleemide lahendamiseks. Geneetilise varieeruvuse andmete põhjal on võimalik selgitada keerulisi liikidevahelisi suhteid ja liigipiire, hinnata populatsioonide elujõulisust, kirjeldada geograafilist eristumist ning tuvastada hübriidisatsiooni. Alles peale eelnimetatud teoreetiliste probleemide lahendamist on võimalik pöörduda praktilise looduskaitse poole. Meie tulemused lubavad teostada haruldase saaremaa robirohu populatsioonide geneetilist inventuuri, ning koostada konkreetsetest geneetilisest andmetest lähtudes adekvaatsed ja efektiivsed kaitsestrateegiad ja kavad.

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