Intraspecific variation in Rhinanthus angustifolius. Local adaptation, phenotypic plasticity and geographical structure.

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Introduction

During the last glacial period Scandinavia was covered by a thick ice sheet, making it impossible for plants to survive. Most plants living in Scandinavia today must therefore have colonized the region during the approximately 15 000 years that have passed since the ice retreated (Jonsell, 2004). The only exceptions are a few species that probably survived in northern ice-free areas (Stewart & Lister, 2001; Stewart *et al.*, 2010). After the ice had disappeared, plants started to colonize from several directions and since then, new species have continued to fill up the newly available areas (Sjörs, 1956; Gjærevoll, 1992). However, Scandinavia has still relatively few species compared to other temperate regions which were not so severely affected by the Pleistocene glaciations (Gjærevoll, 1992)

The time that has passed since the last ice age has also been too short for the emergence of any conceivable number of endemic species (Jonsell, 2004). Speciation is generally regarded as a slow process, especially if it is driven by divergent selection in allopatric populations (Rieseberg, 2007). However, speciation in plants may be rapid if it involves saltatorial genetic processes such as hybridization and polyploidization (Rieseberg & Willis, 2007; Soltis & Soltis, 2009; Madlung, 2013); thus, endemic plant species in Scandinavia almost exclusively represent taxa that have evolved recently through rapid and sympatric speciation events (Borgen, 1987; Jonsell, 2004).

Many plant species in Scandinavia have adapted to local ecological conditions, resulting in phenotypically and genetically distinguishable entities, such as ecotypes (Turesson, 1922; Jonsell, 2004). Some of these are unique to Scandinavia and therefore constitute an important part of the biological diversity of the region, together with other locally evolved lineages of plants and animals (Lundquist *et al.*, 2007).

Ecotypic differentiation

When plant population establish in environments differing from the original habitats of the parental populations, natural selection may result in ecotypic differentiation, i.e. the evolution of phenotypically and genetically divergent populations bound to specific habitats (Turesson, 1922; Silvertown & Charlesworth, 2001). The logic behind this argument is that fitness is related to the local environmental conditions, i.e. phenotypic traits that are favourable under certain conditions may be less favourable under other conditions (Linhart & Grant, 1996). If gene flow between ecotypes is limited – for example, through differences in flowering time – divergent selection may ultimately result in the evolution of separate biological species (Abbott & Comes, 2007; Foster *et al.*, 2007).

Ecotypic differentiation can take a variety of forms, from continuous or stepped clines (Gregor *et al.*, 1938) to the existence of distinct ecotypes (*sensu* Turesson, 1922). The resulting pattern depends on several factors, such as the amount of gene flow between populations, whether the underlying selection pressure is continuous or discontinuous, and whether the characters under selection are encoded by single or multiple genes (Linhart & Grant, 1996). If gene flow is limited and selection is both strong and discontinuous, the most likely outcome is the formation of ecotypes. In many cases, one might expect the same ecotype to evolve in a repeatable (parallel) manner (Turesson, 1922; Rolán-Alvarez, *et al.*, 2004; Foster *et al.*, 2007), especially if adaptation occurs primarily from standing genetic variation (Barrett & Schluter, 2007; Colosimo *et al.*, 2005).

Ecotypes – taxonomy and conservation

Scandinavia – and Europe as a whole – has gone through considerable changes in land-use practice during the past century. As an effect of modernized farming, traditionally-managed hay meadows and seminatural grasslands (e.g. pastures) have declined and been replaced by intensively managed agricultural fields and forests (Bernes, 1994). Since hay meadows and pastures are among the most species-rich habitats in Scandinavia (Ekstam *et al.*, 1988; Bernes, 1994), the decline and fragmentation of these habitats has urged the need to consider how to best preserve the species inhabiting them – and the genetic diversity and evolutionary potential possessed by the species. In this regard, it becomes meaningful to ask questions like: How is the genetic diversity of the species structured?, and, Are there any specialized ecotypes that should be considered?

To ensure that the genetic diversity of species is conserved – including diversity that might be unique to Scandinavia – conservation authorities need to consider variation within species (Jonsell, 2004; Lundquist *et al.*, 2007). In particular, it seems reasonable to include ecotypes and other locally-adapted populations in conservation plans. However, this work may be hampered by the lack of knowledge regarding the distinctness and conservation value of many ecotypes, as well as the evolutionary processes underlying them. The distinctness of ecotypes can, for example, be blurred or exaggerated by plastic responses to environmental variation (phenotypic plasticity; Pigliucci, 2001). One factor that may influence the conservation value of ecotypes is whether or not they correspond to coherent evolutionary units (Moritz, 1994).

Many ecotypes are recognized as taxa, often at the infraspecific level (Jonsell, 2014), but in some cases as separate species. The choice of level depends not only on the distinctness of the ecotypes but also on local taxonomic traditions (Hamilton & Reichard, 1992; Stuessy *et al.*, 2014). Naming of ecotypes as taxa facilitates communication and conservation management, for example by enabling their inclusion in regional or national redlists (ArtDatabanken, 2015). Conservation of infraspecific taxa is, however, only meaningful if they are circumscribed in such a way that they correspond to biologically meaningful entities (Mace, 2004). To enable the proper identification of such units, more detailed research is needed, especially on species showing evidence of ecotype formation (Moritz, 1994).

The annual hemiparasitic rhinanthoids

Rhinanthus angustifolius, the species of focus in this thesis, belongs to the rhinanthoid clade (subfamily Rhinanthoideae) of the family Orobanchaceae – a family that is almost exclusively composed of parasitic and hemiparasitic species (Judd *et al.*, 2008). *Rhinanthus angustifolius* and several of the other annual hemiparasitic species in the clade (hereafter called 'annual rhinanthoids') have undergone extensive ecotypic differentiation (Turesson, 1922; Karlsson, 1974). Many seasonal ecotypes, differing in flowering time and associated characters, have been described (*Euphrasia*: de Soó, 1929; Karlsson, 1974, 1976; *Odontites*: Snogerup, 1983; *Rhinanthus*: ter Borg, 1972; Zopfi, 1993a-b, 1995), but differentiation in characters unrelated to flowering time, such as leaf shape, hairiness and flower morphology, is also common (de Soó, 1929; de Soó & Webb, 1972).

The annual rhinanthoids share the same basic plant architecture and therefore undergo the same phenotypic adaptations to achieve differences in flowering time (Karlsson, 1974). Plants of these species consist of a main stem with a well-defined, and largely genetically determined, number of nodes. At each node the plants produce either branches or flowers – branches are produced at the lower nodes and flowers at the higher nodes. To achieve earlier or later flowering start, the number of nodes below the lowermost flower is decreased or increased, respectively. As a result, early-flowering individuals usually have fewer branches and fewer nodes than those with later flowering times (Karlsson, 1982; Wesselingh, 2016).

The annual rhinanthoids described here are root hemiparasites, which means that they are able to photosynthesize on their own but rely on attachment to the roots of other plants for efficient water and nutrient uptake (Irving & Cameron, 2009). The annual rhinanthoids can parasitize on a wide variety of hosts; *R. minor* has, for example, been recorded to parasitize on more than 50 different plant species (Gibson & Watkinson, 1989).

The annual rhinanthoids often have specific effects on their host. Forbs, for example, have a more well-developed defence against root parasites than grasses, and are therefore less affected by the presence of such parasites (Cameron *et al.*, 2006). Consequently, annual rhinanthoids can have an indirect effect on the composition of the plant community in which they occur, increasing the abundance of forbs, decreasing the abundance of more competitive grasses, and as a result having a positive effect on total species richness (Bardgett *et al.*, 2006; Cameron *et al.*, 2009). For this reason, *R. minor* is often sown in seminatural grasslands to restore and increase their biodiversity (Pywell *et al.*, 2004; Westbury *et al.*, 2006).

The annual rhinanthoids may in turn be influenced by the identity of the host parasitized (e.g. Snogerup, 1982; Svensson & Carlsson, 2004; Cameron & Seel, 2007; Fig. 1). Both the performance and phenotype of the parasite are known to be affected by the growth rate of the host and by the strength of its defence against root parasites (Cameron *et al.*, 2006; Hautier *et al.*, 2010). Such host effects could potentially blur or exaggerate the distinction between ecotypes sampled in habitats differing in plant community composition.

Rhinanthus angustifolius

Rhinanthus angustifolius grows in open grassland habitats in Western Eurasia (de Soó & Webb, 1972). The species is widespread in Europe and has its northern

Figure 1. Two different host species affecting the phenotype of an annual rhinanthoid.

distribution range in Scandinavia (Hultén, 1971). Studies of molecular variation on a European scale indicate that the species most likely had Pleistocene refugia in both the south-western and south-eastern parts of Europe, and that the populations present in Northern Europe today were mainly recruited from the southeast (Vrancken *et al.*, 2009). Present-day populations in Sweden occur in e.g. pastures, coastal meadows and along roads (Krok & Almquist, 1984; Mossberg & Stenberg, 2003). It is also a characteristic species of traditionally-managed hay meadows (Ekstam *et al.*, 1988).

Rhinanthus angustifolius has an erect stem and opposite, simple leaves. The plant can become up to c. 50 cm tall. The yellow two-lipped flowers are arranged in terminal racemes and pollinated by bumblebees (Kwak, 1978; Natalis & Wesselingh, 2012). The seeds are contained within capsules and stay in the capsule until they are shaken out by the wind or by a passing animal.

The seeds of *R. angustifolius* are heavy and have no obvious adaptation for dispersal. They are therefore assumed to disperse mainly within the population. However, long-distance dispersal with machinery and hay transport happens occasionally and has probably been of major importance for the dispersal of *R. angustifolius* in the pre-industrial European landscape, when traditionally-managed hay meadows were still common (Vrancken *et al.*, 2012).

Rhinanthus angustifolius is highly polymorphic and several ecotypes connected to different – often human-created – habitats have been described (de Soó & Webb, 1972; Mossberg & Stenberg, 2003). The patterns of variation are, however, complex and reticulate, and there is a lack of consensus regarding the number and taxonomic levels of described taxa. The pattern of phenotypic variation is further complicated by extensive hybridization with closely related species such as *R. minor* (Kwak, 1980; Vrancken *et al.*, 2012) and possibly by plastic responses to different hosts and other environmental factors (Houston & Wolff, 2012).

In Swedish floras, *R. angustifolius* is divided into two major subspecies: ssp. *angustifolius* and ssp. *grandiflorus* (Krok & Almquist, 1984; Mossberg & Stenberg, 2003). The subspecies are considered to be ecotypes adapted to different land-use practice (ter Borg, 1972; de Soó & Webb, 1972). *Rhinanthus angustifolius* ssp. *angustifolius* is the most widespread subspecies, occupying a variety of open grasslands, such as pastures, road verges and coastal meadows. It produces 5–20 nodes below the lowermost flower, and the stem is often branched with long and flower-bearing side branches. Subspecies *angustifolius* is reported to flower during the latter part of the summer – normally between July and September. Subspecies *grandiflorus*, on the other hand, inhabits traditionally-managed hay meadows and has fewer nodes below the lowermost flower – normally between 2 and 6. The stem is unbranched or has a few short branches, normally without flowers. Subspecies *grandiflorus* is described as having an earlier flowering time than ssp. *angustifolius*: from May to June. The early flowering time is considered as an adaptation to hay making practice, allowing seed set before mowing (ter Borg, 1972).

In addition to the two major subspecies of *R. angustifolius*, there are at least two other putative ecotypes occurring in Sweden today. One of them occupies extensively farmed cropland and has seeds with smaller wing size than normally observed in *R. angustifolius*. This putative ecotype is currently considered as a subspecies, *R. angustifolius* ssp. *apterus*, and the small wing size has been interpreted as an adaptation to the traditional way of seed rinsing, where the largest-winged seeds are more easily separated from crop seeds (Carlsson, 1995; ArtDatabanken, 2015). Subspecies *apterus* is almost extinct and included in the Swedish redlist (ArtDatabanken, 2015).

Another putative ecotype of *R. angustifolius* occurs in calcareous spring fens on the Baltic island of Gotland. It differs from the widespread form of *R. angustifolius* by its red coloration and greater number of nodes and later flowering time (Lindell, 2006). The putative fen ecotype has phenotypic similarities with another putative fen ecotype occurring in similar spring fens on the Baltic island of Saaremaa, Estonia (Lindell, 2006). The fen ecotype on Saaremaa has for long been considered as a separate taxon, and is currently regarded as a locally endemic species, *R. osiliensis*. The Gotlandic fen ecotype has provisionally been treated as *R. osiliensis* by Swedish authorities (ArtDatabanken, 2015); however, results from a recent molecular-genetic study do not support this treatment (Talve *et al.*, 2014).

Aims of the thesis

The overall aim of this thesis is to increase our understanding of the intraspecific variation of *R. angustifolius*, and in particular how factors such as local adaptation, geography and phenotypic plasticity affect the variation and diversity within the species. Moreover, we aim to evaluate the distinctness of putative ecotypes in order to determine their taxonomic value and usefulness as conservation units.

In **Paper I** we focus on the two major subspecies of *R. angustifolius*, occurring in Sweden today – ssp. *angustifolius* and ssp. *grandiflorus*. The aim of the study is to evaluate whether the subspecies constitute phenotypically distinct ecotypes – as is commonly assumed in the floristic literature – and furthermore, we investigate to what extent phenotypic plasticity affects the distinctness of the two subspecies, especially in regard to the identity of the host species.

In **Paper II** we examine whether the same subspecies constitute separate evolutionary lineages, i.e. whether there is molecular-genetic support for the current division of Swedish *R. angustifolius* into two major subspecies, or whether the intraspecific genetic structure is more strongly determined by geographical distance.

In **Paper III** we move the focus to the putative ecotype occurring in a few calcareous spring fens on the Baltic island of Gotland. We evaluate whether the fen populations constitute a phenotypically and genetically distinct ecotype worthy of taxonomic recognition. We also use molecular-genetic markers to assess the evolutionary relationship between the Gotlandic fen ecotype, the common form of *R. angustifolius* and another putative spring fen ecotype occurring on the Baltic island of Saaremaa – currently regarded as a separate species, *R. osiliensis*.

In **Paper IV** we use another set of molecular markers to get a better understanding of the evolutionary history of the spring fen ecotypes on Gotland and Saaremaa. In particular, we are interested in whether the phenotypically similar ecotypes have evolved at repeated occasions as a response to similar environmental conditions, or whether they have a common origin.

Methods

In the following part, I give a general overview of the methods used in this thesis. Detailed descriptions of materials and methods are given in each of the four papers.

We use a combination of common-garden experiments and molecular-genetic methods to study intraspecific variation in *R. angustifolius*. Common-garden experiments are commonly used to quantify genetic and environmental sources of phenotypic variation in one, or preferably, several standardized growth environments (Silvertown & Charlesworth, 2001; Pigliucci, 2001). Phenotypic characters are often influenced by selection, which on the one hand underlies many of the patterns that are of interest in a common-garden experiment (e.g. the existence of ecotypes, Turesson, 1922), but on the other hand can mask or obscure evolutionary relationships among populations. Molecular-genetic methods are therefore useful as complementary tools for studying genetic structure and delimiting taxa at the infraspecific level (Stuessy *et al.*, 2014).

Common-garden experiments

To evaluate patterns of plasticity and the phenotypic distinctness of the putative ecotypes within *R. angustifolius*, we perform two separate common-garden experiments in which plants of putative ecotypes are grown with several host species under seminatural garden conditions (Fig. 2). In the first experiment (Paper I) we compare plants of ssp. *angustifolius* and ssp. *grandiflorus*, and in the second experiment (Paper III) we compare the putative fen ecotype on Gotland with the common grassland form of *R. angustifolius*.

We collect *Rhinanthus* seeds for the common-garden experiments in the natural habitats of the putative ecotypes (Fig. 3). Seeds from each seed plant are sown in a separate pot – each containing one of three (Paper III) or four (Paper I) host species – and the pots are randomly placed in a sunny part of an outdoor garden at Lund University (Fig. 4). All the host species used in the experiments are common in Sweden and variously abundant in grassland habitats occupied by *R. angustifolius.*

The *Rhinanthus*-plants are scored for a number of phenotypic characters, with focus on features that are deemed important for division into ecotypes or infraspecific taxa

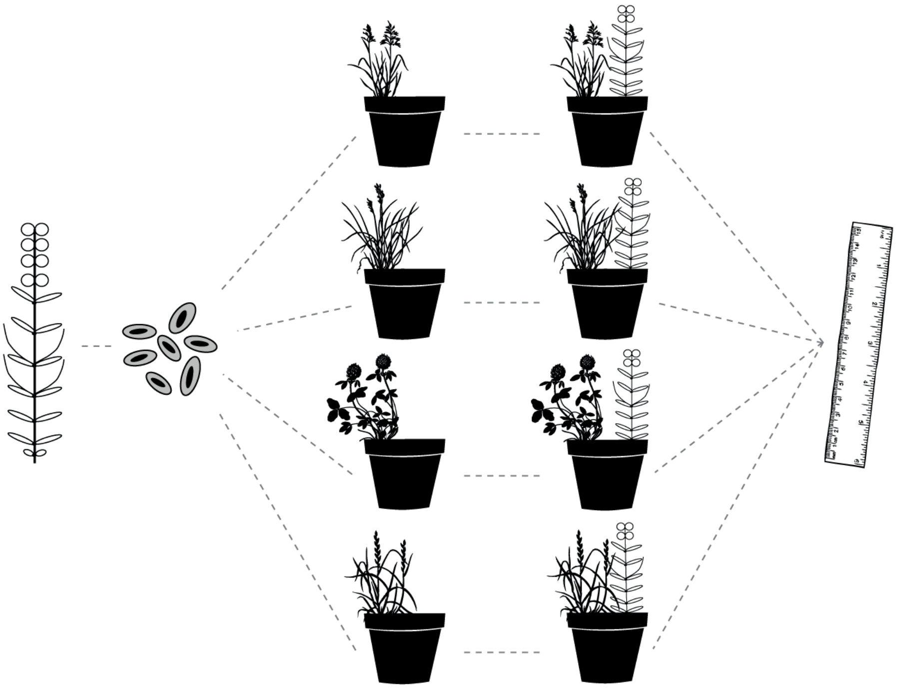

Figure 2. Set-up of the common-garden experiment.

based on previous literature. The common-garden data are subjected to a combination of univariate and multivariate statistical analyses to assess the specific questions of the different studies.

Molecular methods

During the last decades, a wealth of molecular-genetic methods for studying genetic diversity have been developed, and all have their specific pros and cons, depending on the type of organism and the question of interest (Sunnucks, 2000; Zhang & Hewitt, 2003; Stuessy *et al.*, 2014). In this thesis we use a combination of nuclear microsatellite markers and plastid sequence data to address questions regarding the genetic structure and the delimitation of taxa at the infraspecific level (Fig. 5).

Microsatellites are regions with tandem repeats of 2–6 nucleotides that mutate by adding or deleting repeat units, because of polymerase slippage or unequal crossing-over (Goldstein & Schlötterer 1999; Ellegren, 2004). Because of high mutation rates, considerable variation can often be observed within species, making nuclear microsatellites particularly useful for assessing patterns of population differentiation

Figure 3. Anneli collecting seeds of *R. angustifolius* in Ireviken, Gotland.

Figure 4. Anneli working in the outdoor garden at Lund University.

Figure 5. Anneli doing molecular work in the lab.

(Selkoe & Toonen, 2006). However, the high mutation rate and the way microsatellites mutate cause a high risk of homoplasy, i.e. that variants with the same number of repeats evolve repeatedly and independently in different lineages (Estoup *et al.*, 2002). Moreover, homologous microsatellite regions are not necessarily shared between related species, and flanking regions used for primer annealing may also differ (Selkoe & Toonen, 2006). For these reasons microsatellites are less suitable for studies above the species level.

To assess patterns of variation both within and between species, non-coding regions of the plastid genome may be sequenced and analysed (Shaw *et al.*, 2014). The risk of homoplasy is small if sequence variants differ at several positions. However, plastid genomes have in general much lower mutation rates than nuclear microsatellites (Wolfe *et al.*, 1997), and the mutation rates also differ between regions of the plastid genome (Shaw *et al.*, 2014). Therefore, it can be difficult to identify regions with the appropriate level of variation for the specific species and question considered (Shaw *et al.*, 2014).

Since we expect the putative ecotypes of *R. angustifolius* to have evolved recently, we use nuclear microsatellite markers with potentially high mutation rates to study the evolutionary relationship between ssp. *angustifolius* and ssp. *grandiflorus* (Paper II), and between the putative spring fen ecotype and other populations of *R. angustifolius* and *R. osiliensis* (Paper III). However, since the study of the fen ecotype (Paper III) includes populations in both Sweden and Estonia, which may have been separated from each other for a long period of time, we might expect some degree of homoplasy at the microsatellite loci in this analysis. Therefore we also obtain plastid sequence data (Paper IV) to evaluate some of the patterns observed in Paper III. We subject the molecular-genetic data to a combination of population-genetic analyses (Paper II and III) or phylogenetic analyses (Paper IV) to address the specific questions of each study.

Results and discussion

In the following part of the thesis I summarize and discuss the main findings of the four papers. Paper III and IV are presented together as they partly address the same questions, using different methods.

Paper I

Based on information in Swedish floras, we expect plants of *R. angustifolius* ssp. *angustifolius* and ssp. *grandiflorus* to conform to two distinct ecotypes differing in flowering time and related characters, when grown under uniform garden conditions. However, based on previous results from rhinanthoids, we also expect growth on different host species to have specific effects on the phenotype of the *Rhinanthus* plants (ter Borg, 1972; Zopfi 1993a, b, 1995). In concordance with the second prediction, the results of the common-garden study show consistent effects of host species on both the phenology and morphology of the *Rhinanthus* plants. The plants become '*angustifolius*-like' when grown on the most favourable host and '*grandiflorus*-like' when grown on the least favourable host. The major exception is the number of nodes, which, in concordance with previous results from other annual rhinanthoids (*Rhinanthus*: ter Borg, 1972; Zopfi, 1993b; *Odontites*: Snogerup, 1982), is unaffected by the identity of the host species. The specific effects of the host species most likely result from host-specific differences in growth rate (Hautier *et al.*, 2010) and the strength of defence against root parasitism (Cameron *et al.*, 2006). Interestingly, growth on the 'best' host (*Trifolium pratense*) causes a delay in flowering time, presumably as an effect of shading by the host early in the development.

In concordance with our first prediction we find significant differences between plants of the two subspecies. Plants of ssp. *angustifolius* are on average larger, have more nodes on the main stem and are more profusely branched than plants of ssp. *grandiflorus*. They also have a longer flowering period and a later flowering peak than plants of *grandiflorus*, most likely because the *angustifolius* plants produce more flower-bearing branches that flower later than the main stem. However, in contrast to the descriptions in Swedish floras, we find no difference in flowering start between plants of the two subspecies.

Even though we find some significant differences between the two subspecies, there is extensive between-population variation, resulting in a more-or-less continuous spectrum of populations – from those that are most '*angustifolius*-like' to those that are most '*grandiflorus*-like' – instead of two distinct groups as would be expected if the subspecies represent classical ecotypes (*sensu* Turesson, 1922).

A notable observation is that plants from coastal meadows generally are intermediate to plants conforming to the *angustifolius* and *grandiflorus* phenotypes. Accordingly, our results indicate the existence of two partly independent axes of adaptation, one contrasting plants from habitats with different mowing regimes (corresponding to the current division into two subspecies) and another contrasting plants from inland vs. coastal sites, irrespective of land-use history.

Altogether, the results of the common-garden study indicate a more complex and blurred pattern of variation than inferred from the current taxonomic treatment of the study species.

Paper II

It is possible for habitat-related groups of populations to constitute distinct evolutionary lineages, and thus be worthy of taxonomic recognition, even if there is extensive overlap at the phenotypic level (Kolseth *et al.*, 2005). To test whether this possibility holds true in *R. angustifolius*, we carry out a molecular-genetic study, using 11 nuclear microsatellite loci, to assess the evolutionary history and genetic distinctness of the two currently recognized subspecies (ssp. *angustifolius* and ssp. *grandiflorus*) as well as the three habitat-related groups of populations (hay meadows, road verges and coastal meadows) inferred from the common-garden study.

The results of the microsatellite analyses indicate that neither the two currently recognized subspecies nor the three habitat-related population groups inferred from common-garden data, constitute genetically coherent groups with separate evolutionary origins. Instead, we find a clear geographical structure, with adjacent populations being more similar to each other than populations separated by large geographical distances. As currently described taxa or population groups do not contain any unique variation, we recommend that, in the event that the habitats of *R. angustifolius* decline and become threatened, conservation should focus on multiple populations spread over a large geographical range, rather than on populations of the specific subspecies or habitat groups considered in the present study.

Papers III & IV

The results of the common-garden experiment in Paper III reveal that spring fen populations on Gotland conform to a phenotypically distinct ecotype (*sensu* Turesson, 1922), characterized by red coloration (the most distinctive and uniform feature) as well as a later flowering start, a larger node and branch number, and a denser cover of glandular hairs on bracts and calyces than normally found in *R. angustifolius*. Based on indirect evidence, the characters distinguishing the fen ecotype can be tentatively attributed to a history of local adaptation to the ecological conditions prevailing in the spring fen habitat.

The patterns observed for microsatellite loci do not correspond to the existence of a genetically coherent fen ecotype: northern and southern populations of the fen ecotype are more differentiated from each other than from geographically adjacent populations of the common grassland form. A plausible explanation for the observed incongruence between the phenotypic and molecular patterns is that the Gotlandic fen ecotype has evolved repeatedly from *R. angustifolius* as a response to similar selection pressures in different spring fens on this island, a reasonable scenario for many plant ecotypes (Turesson, 1922; Karlsson, 1974, 1976; Levin, 2001). The hypothesis of repeated evolution of a spring fen ecotype on Gotland is also supported, or at least not refuted, by the plastid sequence data in Paper IV.

The molecular results of Paper III (microsatellite data) and Paper IV (plastid sequence data) support the hypothesis that fen ecotypes on Gotland and Saaremaa have separate evolutionary origins (Talve *et al.*, 2014) – extending the parallel-evolution scenario discussed above for different populations of the fen ecotype on Gotland. Based on all the molecular results and the phenotypic distinctness observed in the common-garden experiment, we conclude that the Gotlandic fen ecotype warrants distinction as a separate taxon and propose that southern and northern fen populations represent separate management units (Moritz, 1994), each with a high conservation value. We propose a varietal status for the Gotlandic fen ecotype, *R. angustifolius* var. *gotlandicus*, and give some recommendations for the conservation of this taxon.

Concluding remarks

The results of our studies indicate that *R. angustifolius* has undergone genetic adaptation to local environmental conditions in Scandinavia, but that its present-day populations generally differ in a continuous manner instead of forming distinct ecotypes. The major exception is the fen ecotype on Gotland (Fig. 6), which is phenotypically coherent (especially in coloration) and genetically different from populations occupying drier grassland habitats. Our studies are not designed to investigate the factors causing, or counteracting, local adaptation, but based on our results, it seems likely that the number of 'habitat axes' and the level of gene flow between populations could play major roles in determining the pattern of differentiation. As shown in Paper I, grassland habitats occupied by *R. angustifolius* cannot easily be categorized into distinct groups. Instead, they overlap in at least two factors that could cause local adaptation (e.g. management history and nearness to the coast) and thus explain the blurred and complex pattern seen in this common-garden experiment. Furthermore, our molecular results (Paper II and III) indicate that fen populations are more isolated than grassland populations, which could facilitate the evolution of a relatively distinct fen ecotype. The genetic basis of the characters under selection could also affect the distinctness of the ecotypes (Linhart & Grant, 1996), but this remains to be investigated.

In concordance with previous studies, results from the common-garden experiments show that characters related to size, branchiness and flowering time might be affected by environmental factors, such as the identity of the species serving as the host. Therefore, we urge caution in the use of these characters for delimitation of taxa within the annual rhinanthoids. By comparing the results of our two common-garden experiments, we also conclude that the choice of growth environments can have a major impact on the results and their interpretations: the host species used in Paper I caused greater plastic responses than the hosts used in Paper III. This demonstrates the importance of using several, carefully chosen environments in common-garden experiments (Pigliucci, 2001).

Altogether, our results highlight the need to critically evaluate infraspecific taxa that have been described solely on the basis of field observations and/or herbarium specimens. This is especially important if the characters used to delimit taxa are suspected to be plastic, as in the case of seasonal ecotypes in annual rhinanthoids. A combination of common-garden experiments and molecular-genetic analyses should be particularly informative in such evaluations.

The results in Paper III and IV support the common notion that ecotypes may evolve in a parallel manner. Ecotypes with parallel origins may be taxonomically recognized (Jonsell, 2004) but do not correspond to coherent evolutionary units and they should therefore not be automatically regarded as management units for conservation (Moritz, 1994). For example, translocation of individuals between populations with separate evolutionary origins should be avoided in order to preserve genetic integrity and potentially unique gene combinations (Moritz, 1999). This aspect emphasizes the advantage of performing molecular-genetic analyses to delimit management units in species showing evidence of ecotype formation.

Finally, I would like to point out that even though we found no strong support for the current taxonomic recognition of the 'meadow ecotype' (ssp. *grandiflorus*), it is still advisable to assign high conservation value to populations of *R. angustifolius* in traditionally-managed hay meadows, not only because of their cultural-historical value, but also because they have a potentially large effect on the maintenance of high species diversity in this habitat (Bardgett *et al.*, 2006; Cameron *et al.*, 2009)

Figure 6. Plant of the fen ecotype on Gotland.

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