

LISANNA SCHMIDT

Phenotypic and genetic differentiation in
the hybridizing species pair *Carex flava*
and *C. viridula* in geographically
different regions



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Department of Botany, Institute of Ecology and Earth Sciences,
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LIST OF ORIGINAL PUBLICATIONS

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

- I.** Schmidt L, Fischer M, Schmid B, Oja T. (2017) Despite admixing two closely related *Carex* species differ in their regional morphological differentiation. *Plant Systematics and Evolution*, 7:901–914.
- II.** Schmidt L, Fischer M, Oja T. (2018) Two closely related species differ in their regional genetic differentiation despite admixing. *AoB plants*, 10:1–17.
- III.** Schmidt L, Schmid B, Oja T, Fischer M. (2018) Genetic differentiation, phenotypic plasticity and adaptation in a hybridising pair of a more common and a less common *Carex* species. *Alpine botany*, 128: 149–167.

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The author of the dissertation substantially contributed to developing the idea and study design of all the papers, and was responsible for sample selection and collection, field and lab measurements (**I**), molecular laboratory work (**II**), experimental work (**III**), data analysis, interpretation of the results and writing of the publications (**I–III**).

INTRODUCTION

This thesis examines phenotypic and genetic differentiation and diversity in three taxa, a more common and a less common species of the *Carex flava* complex, and their hybrid. It addresses whether the less common species is more differentiated between regions than the more common one, and whether the more common species is rather adapted to regional conditions than the less common one. The thesis also addresses the alternative hypothesis that both species show similar levels of differentiation and regional adaptation, which would be expected if backcrossing and introgression from hybrids to parental species are very important.

Plant species show remarkable morphological and genetic variation across their distribution areas. Also differential adaptation of plant populations within species along environmental gradients is common. Geographic distance between populations imposes physical limits to dispersal and different populations experience different environmental and ecological factors. Such geographic and environmental variability can lead to morphological and genetic differentiation of populations within species. The larger a species areal is, the more likely populations of the same species vary morphologically and genetically among regions. The extent of regional differentiation depends on various factors, among them the reproductive system, dispersal mechanisms and population characteristics. A further factor potentially affecting regional differentiation is hybridization. Hybridization and backcrossing between closely related co-occurring species may affect fitness and adaptation of parental species. Moreover, new varieties or species might arise from hybridization. Studying the morphological and genetic variability of hybridizing species provides knowledge of how hybridization influences evolution and provides taxonomic information valuable for other disciplines, including conservation biology.

Global change has profound effects on biodiversity (Vallejo-Marín and Hiscock, 2016). Climate change, intensified land use, pollution and the spread of invasive species are among the drivers of global change, of which land use change and climate change are expected to have the largest impact on biological diversity (Sala *et al.*, 2000). Rapid environmental change, such as habitat conversion or climate change, can induce local maladaptation of formerly adapted populations (Wilczek *et al.*, 2014; Anderson *et al.*, 2015) and cause the movement of species toward higher latitudes and elevations to track the climate optimal for growth (Kelly and Goulden, 2008). Between-species gene flow may also increase under global change, if human activities, such as disturbance, lead to more frequent co-occurrence of related species (e.g. Kane *et al.*, 2009). One of the major conservation goals is to maintain biodiversity and conditions that support it, which is a challenging task in the face of ongoing global change (Brennan *et al.*, 2015). Thus, with continuous climate change and increasing anthropogenic disturbance the importance of understanding ecological and evolutionary species responses to environmental changes is increasing.

Organisms can respond to global changes and variability in their habitat by acclimation, adaptation or migration (Chunco, 2014; Franks *et al.*, 2014).

Plants, due to their sessile habit, are especially exposed to changes in their environment. Their ability to migrate depends on pollen and seed dispersal, which differs between species (Govindaraju, 1988). Populations and species not able to respond to the climate change are under extinction risk (Hoffmann and Sgro, 2011; Wilczek *et al.*, 2014). Acclimation via non-genetic phenotypically plastic response has costs and constraints (Gienapp *et al.*, 2007; Nicotra *et al.*, 2010). Mutations may increase genetic variability, albeit at a very slow pace. Ways to compensate for the lack of genetic material could be intraspecific gene flow or interspecific gene flow via hybridization (Hamilton and Miller, 2016; Goulet *et al.*, 2017). Spatial and temporal isolation between species is on the one hand increased by habitat destruction and fragmentation. On the other hand it is reduced due to anthropogenic dispersal of invasive species and due to shift in geographic distributions under global warming (Chunco, 2014; Vallejo-Marín and Hiscock, 2016). Accidental, facilitated or intentional anthropogenic dispersal could lead to hybridization of native species with exotic congeners (Brennan *et al.*, 2015; Mesgaran *et al.*, 2016). Besides, habitat modification by increased disturbance could result in breakdown of ecological isolation barriers, meaning that species that were isolated by having different flowering times or ecological preferences come to contact and hybridize (Bardy *et al.*, 2011; Guo, 2014). With increasing anthropogenic activity hybridization is possibly an increasingly important way for species to cope with global changes (Donovan *et al.*, 2010; Krebs *et al.*, 2010; Hamilton and Miller, 2016).

According to the widely accepted Biological Species Concept, species are reproductively separated from each other (Mayr, 1970). However, hybridization and introgression (i.e. backcrossing of hybrid individuals with one of its parent species) is a common phenomenon and a natural feature in species evolution (Rieseberg and Carney, 1998; Guo, 2014). About 10% of animals and a quarter of plants are estimated to hybridize (Arnold, 1997; Seehausen, 2004; Mallet, 2005). Even *Homo sapiens* may have a hybrid genomic ancestry (Evans *et al.*, 2006), and also taxa from different genera, for example crossings between chokeberry (*Aronia*) and rowan (*Sorbus*), have resulted in widely cultivated intergeneric hybrids (Jeppsson, 2000). These observations contradict Mayr's criterion of complete reproductive isolation. However, hybridization is not similarly likely in all plant species. Taxa in closely related, sympatric species groups cross more readily and form hybrid zones (Mallet, 2005; Abbott *et al.*, 2013; Abbott, 2017). Such taxonomically complex groups (TCG) are found within various genera, including *Carex* (Naczi and Ford, 2008; Smith and Waterway, 2008; Escudero *et al.*, 2010a), *Euphrasia* (French *et al.*, 2005), *Hieracium* (Shi *et al.*, 1996), *Thymus* (Federici *et al.*, 2013), *Gentiana* (Christe *et al.*, 2014), *Solanum* (Jacobs *et al.*, 2011), *Veronica* (Bardy *et al.*, 2011) and many others. Often species are identified solely on the basis of morphological differences (Ennos *et al.*, 2005). In other cases morphology is combined with molecular study (Bardy *et al.*, 2011; Jacobs *et al.*, 2011; Federici *et al.*, 2013). In some cases though, data on morphology and molecular variation show contrasting results (Christe *et al.*, 2014). As neutral molecular markers are not

subject to selection, in contrast to most morphological characteristics, both approaches are required to obtain a fuller picture of the identity of populations. Moreover, the use of reciprocal transplant and common garden experiments allows more objective taxonomical conclusions by enabling to distinguish environmentally and genetically determined differences (Kaplan, 2002).

Hybridization has contrasting evolutionary consequences, as it is seen to provide solutions for species adaptation to global changes as well as causing challenges to conservation (Brennan *et al.*, 2015; Hamilton and Miller, 2016). Hybridization contributes to diversification with the formation of varieties, subspecies or new species (Rieseberg and Willis, 2007; Abbott *et al.*, 2013). The fitness of those novel types may differ from the one of parental species (Rieseberg, 1995; Campbell and Waser, 2001; Donovan *et al.*, 2010; Brennan *et al.*, 2015). Variation acquired by mutations takes time, but hybridization results in novel variation through recombination and backcrossing within a single generation and could increase genetic variability (Abbott *et al.*, 2013; Goulet *et al.*, 2017). Controversially, crossing between species could lead to negative impacts on fitness and adaptation of parental species (Allendorf *et al.*, 2001), result in extinction through outbreeding depression (Rhymer and Simberloff, 1996), or loss of the less common parental species (Ducarme and Wesselingh, 2005; Lepais *et al.*, 2009; Beatty *et al.*, 2010; Mesgaran *et al.*, 2016). Studies examining the genetic consequences of hybridization for hybrids and parental species in different parts of their distribution ranges, although rarely done, are important contributions to evolutionary ecology and conservation biology (Allendorf *et al.*, 2001; Chunco, 2014).

Genetic diversity is considered essential for population fitness and evolutionary potential of species, with higher diversity indicating better adaptive ability (Amos and Harwood, 1998). Genetic diversity directly affects biological diversity and also has ecological consequences at the population, community and ecosystem levels (Pertoldi *et al.*, 2007; Hughes *et al.*, 2008). Loss of genetic variability reduces the ability to adapt to future changes (Amos and Harwood, 1998; Hoffmann and Sgro, 2011). Levels and distribution of morphological and genetic variation differ within and between species, resulting in regional differentiation (Linhart and Grant, 1996). Regional differentiation is driven by differences in natural environments across the distribution as several evolutionary forces, genetic drift, gene flow, migration and mutation, shape populations differently (Slatkin, 1987). The relative importance of these forces depends on selection strength, population characteristics and plant life history traits, especially migration and dispersal (Loveless and Hamrick, 1984). Higher differentiation is typically found for clonally reproducing, selfing species and for species with small fragmented populations, and lower regional structuring is expected for outcrossing or wind pollinated species (Govindaraju, 1988; Ellstrand and Elam, 1993; Gitzendanner and Soltis, 2000).

Genetic variation and structure of natural populations from different parts of distribution area have been studied for several plant species (e.g. Schönswetter *et al.*, 2006; Kull and Oja, 2010), and varying relationships between species

characteristics and genetic diversity found (Leimu *et al.*, 2006 and references therein). Additional attention has been paid to genetic differentiation and changes of intra-population diversity along elevation gradients (Reisch *et al.*, 2005; Ohsawa *et al.*, 2007; Ohsawa and Ide, 2008; Frei *et al.*, 2012). Most of these studies concerned regional differentiation of single or several species without considering the possibility of hybridization (but see Friedman *et al.*, 2008; Korpelainen *et al.*, 2010; Krebs *et al.*, 2010). However, if there are close relatives, the distribution of genetic diversity and differentiation pattern may be affected by hybridization. As the expectation of higher regional differentiation in the rare species might be counter-balanced by introgression from the more common species, the study of regional differentiation of closely related hybridising species would be especially interesting for pairs of common and rare species co-occurring over large stretches of their ranges.

Different responses of genotypes to different environments are known as genotype-by-environment interactions (GxE; Schlichting, 1986), which indicate genetic variation in plasticity, and possibly adaptation to local environments. Phenotypic plasticity is the ability of an individual organism to produce different phenotypes in different environments (Schlichting, 1986; Schmid, 1992; West-Eberhard, 2005). The ability to respond to variable and unpredictable environments with physiological and morphological changes, i.e. phenotypic plasticity, is especially important for plants, which are constrained in their ability to move to more favourable sites. The degree of plasticity differs both within and between species (Schmid, 1992; West-Eberhard, 2005). But plastic responses have limitations, due to lack of extrinsic resources or architectural constraints (DeWitt *et al.*, 1998). On the other hand, developmental instability could affect plasticity positively (Schlichting, 1986). The evolution of plasticity may be affected by disruption or change in the genetic system through hybridization or inbreeding (Schlichting, 1986). With continuous climate change and rising anthropogenic influence on the environment of plant communities it is important to improve our understanding of plant genetic and plastic responses in a warming climate (Gienapp *et al.*, 2007; Nicotra *et al.*, 2010). However, plasticity tends to be underestimated in taxonomic and evolutionary work, and in many cases taxa differentiated solely by morphological characters were in later studies proven to be genetically identical (e.g. Kaplan, 2002).

Local adaptation denotes better performance of local plants in local environments, and regional adaptation indicates adaptation to environmental, e.g. climatic, conditions over a larger region, where organisms experience similar selection pressures. Plants often show local adaptation along environmental gradients (Kawecki and Ebert, 2004; Leimu and Fischer, 2008). Genetic variability, depending on mutations, gene flow and drift, is necessary for an adaptive response (Hereford, 2009). Gene flow has contrasting effects on the development of adaptations, it can stimulate adaptive evolution by allowing the spread of advantageous alleles and increasing genetic diversity in the population, or it can prevent local adaptation and rather cause maladaptation by

demolishing existing advantageous allele combinations (Lenormand, 2002; Hereford, 2009; Blanquart *et al.*, 2012). Adaptation of species may also depend on their commonness and population sizes, in a way that populations of common species are in general more connected and have thus higher levels of gene flow among them, compared with species with sparser distributions (Loveless and Hamrick, 1984; Slatkin, 1987). Small size of populations could prevent local adaptation due to effects of genetic drift (Hamrick and Godt, 1996; Leimu and Fischer, 2008). Hybridization may also affect the ability to adapt by influencing genetic diversity in populations (e.g. Chunco, 2014; Brennan *et al.* 2015; Hamilton and Miller, 2016).

The relative importance of phenotypic plasticity, genetic differentiation, genotype by environment interactions and local adaptation can be studied with transplantation experiments (Kawecki and Ebert, 2004). Reciprocal transplant experiments have been used to explore the response of different genotypes across various environments since Turesson (1922), but mostly these experiments concerned single species over restricted geographic areas (e.g. Joshi *et al.*, 2001; McKay *et al.*, 2001; Becker *et al.*, 2006; Gonzalo-Turpin and Hazard, 2009; Anderson *et al.*, 2015; Ensslin and Fischer, 2015; Samis *et al.*, 2016; Hamann *et al.*, 2017) and effect of hybridization is understudied.

This thesis focuses on the *Carex flava* L. species complex, belonging to genus *Carex* (*Cyperaceae*). The large cosmopolite genus consists of approximately 2100 species that grow mostly on moist open habitats, including marshes, bogs, fens, shore-lines, and wet meadows in cold and temperate Northern Hemisphere, but are also found in deserts and rain forests (Reznicek, 1990; Naczi and Ford, 2008; Mohlenbrock, 2011; Spalink *et al.*, 2016). Many of the sedges are good colonizers and weedy species (Bryson and Carter, 2008). The high ecological diversity of occurrences of *Carex* plants is a result of post-glacial colonization and active radiation in newly colonized temperate habitats (Escudero *et al.*, 2012; Lévillé-Bourret *et al.*, 2018). Other important factors influencing the evolution of the genus are hybridization and the mode of chromosome evolution. Features like perennial habit, vegetative reproduction, cross-compatibility and incomplete reproductive isolation make hybridization common in sedges (Schmid, 1982; Cayouette and Catling, 1992). Sedges have holocentric chromosomes with diffuse centromeres, which enable rapid chromosome number evolution (Hipp *et al.*, 2010; Escudero *et al.*, 2012). Possible links between chromosome evolution and diversification were found in some studies where genetic differentiation was reported to follow chromosome rearrangements at the species and genus levels (Hipp, 2007; Escudero *et al.*, 2010a; Hipp *et al.*, 2010). Aneuploidy leading to taxa with variable eu-diploid chromosome numbers is a major process accompanying speciation in *Carex* (Davies, 1956a).

The research objects of this thesis are *C. flava*, *C. viridula* and their putative hybrid *C. x subviridula*. *Carex flava* populations are more widely distributed across Europe than the ones of *C. viridula* (Davies, 1956b; Schmid, 1981; Koopman, 2011), though Crins and Ball (1989b) considered *C. viridula* as most

widespread in the section *Ceratocystis*. The occurrence of *C. viridula* has decreased over time and populations have become more fragmented due to human influence, such as eutrophication of lakes, drainage of mires and regulation of water levels (Davies, 1953b; Pykälä and Toivonen, 1994). Its decrease in southern parts of distribution, e.g. Switzerland (Bornand *et al.*, 2016) may be explained by climate change (Kelly and Goulden, 2008). *Carex flava* plants are considered to be more competitive and the populations are larger than *C. viridula*'s (Davies, 1956b; Schmid, 1984b, 1986b; Crins and Ball, 1989b). Rabinowitz (1981) suggested three possible attributes of rarity, limited geographic distribution, small local population size and high habitat specificity. Based on this criteria, *C. viridula* can be considered "rare" due to its small and fluctuating population sizes. In both taxa, most seeds are produced via selfing, though they are able to outcross (Vonk, 1979; Schmid, 1984a). At sites where the taxa co-occur they hybridize (Schmid, 1983). These are often sites under anthropogenic influence (Cayouette and Catling, 1992; Pykälä and Toivonen, 1994). The hybrids are partially male fertile and thus can potentially backcross to parental lines adding to morphological variation in the hybrid swarm (Schmid, 1982).

The main focus of previous studies with *C. flava* agg. has been on solving their systematic relations, chromosome evolution (Davies, 1955; Schmid, 1982), phenology (Vonk, 1979), and ecological (Schmid, 1984a, 1984b, 1986a), morphological (Stoeva and Stepankova, 1990; Salo *et al.*, 1994; Blackstock and Ashton, 2001; Więclaw, 2014) or genetic variation (Bruederle and Jensen, 1991; Kuchel and Bruederle, 2000; Hedrén, 2002; Jiménez-Mejías *et al.*, 2012). Different opinions of their taxonomic division have prevailed over time, most widely accepted is currently the presence of four species and several interspecific units (Koopman, 2011; Jiménez-Mejías *et al.*, 2014). High morphological variation within species has been found and explained by phenotypic plasticity and hybridization (Blackstock and Ashton, 2010; Jiménez-Mejías *et al.*, 2012). In genetic studies allozyme markers have been mostly applied (except nuclear ITS by Jiménez-Mejías *et al.*, 2012). Although taxonomic differences between *C. flava* and *C. viridula* have been recognized, and population structure and genetic differences in the *C. flava* group have been studied, the effect of hybridization on regional differentiation has not been addressed. Moreover, in many taxonomic studies of morphological variation in *C. flava* agg. no clear distinction has been made between plastic and genetic sources of variation. The taxa merit further attention because the evolutionary role and potential of hybrids is possibly underestimated and the group could provide improved knowledge about the evolution of hybridizing species.

For studying variation and differentiation of *C. flava*, *C. viridula* and their hybrid, we combined several independent methods and examined 572 plants from 43 populations in three regions, Estonia, Lowland Switzerland and Highland Switzerland. The methods included morphological and molecular genetic variation analysis and reciprocal transplantation. For a subsample of plants, we also explored the chromosome numbers. We started with the analysis

of morphological variation and continued with a molecular analysis addressing regional differentiation in natural populations. Molecular techniques are a good choice when assessing the impact of hybridization and the role of genetic introgression in plant populations (Nolte *et al.*, 2009; Jiménez-Mejías *et al.*, 2012; Derieg *et al.*, 2013). Also, there are examples of morphologically identified hybridizing taxa turning out not to differ genetically from each other (Jacobs *et al.*, 2011). Ducarme and Wesselingh (2005) emphasized that hybrids are very variable and many of them are so close to parental species that only genetic markers are able to reveal hybridization. Microsatellites, as highly polymorphic codominant markers, are successfully used in population genetic studies (Frei *et al.*, 2012; Volis *et al.*, 2016) and studies of closely related species (Korpelainen *et al.*, 2010).

To resolve whether morphological variation has a genetic basis or is environmentally induced, we transplanted shoots from genets of all studied populations in the three regions to three common gardens, one in each region. Including hybrid genotypes to the reciprocal transplant experiment enabled us to assess the effect of hybridization on species diversification and to test the performance of hybrids and parents in contrasting natural environments (Emms and Arnold, 1997; Campbell and Waser, 2001; Miglia *et al.*, 2005; Donovan *et al.*, 2010). Furthermore, growing experimental plants in local and foreign regions enabled direct tests of regional adaptation. We observed variation in morphology and phenotype of the hybridizing taxa in different altitudes in Switzerland and in Estonia. We hypothesized that either, due to hybridization, the taxa may behave similarly and show similar levels of regional differentiation in all common gardens, or alternatively that regional differentiation of the less common *C. viridula* may be more pronounced than of the more common *C. flava*. In this study, for the first time morphological and genetic data are combined with a large reciprocal transplant experiment in order to test inter- and intraspecific variation of hybridizing species.

The main aims of this doctoral thesis were:

- to address whether hybridization plays a significant role in the evolution of *C. flava* group (I, II, III)
- to assess the genetic variability of *C. flava*, *C. viridula* and their hybrid *C. x subviridula* in geographically distant and climatically different regions (II)
- to study whether, despite hybridization, the more common taxon *C. flava* is morphologically and genetically less differentiated between regions than the less common *C. viridula* (I, II, III)
- to assess whether phenotypic differences within and between taxa are more determined by the genotype or more by the environment (III)
- to evaluate, whether *C. flava*, *C. viridula* and their hybrids are adapted to different regions, and whether this is related to the regional climates (III)

1. MATERIALS AND METHODS

1.1. Study species and sampling

We investigated the two sedges *Carex flava* L. (Fig. 1) and *C. viridula* Michaux., and their hybrid *C. x subviridula* Fernald. (*C. flava* x *C. viridula*), all belonging to the *C. flava* species aggregate, section *Ceratocystis* Dumort (**I**, **II**, **III**). Throughout the work we follow Koopman (2011) in terms of nomenclature. Two other taxa *C. lepidocarpa* and *C. demissa*, also occurring in the study regions, were not included in the analyses due to the small number of populations found.



Figure 1. The studied taxa (a) *Carex flava* L., (b) *Carex viridula* Michaux. and (c) *C. x subviridula* Fernald.

The field work was conducted in Switzerland in 2012 and in Estonia in 2013. Switzerland was divided into Low- and Highland based on elevational differences. Temperate climate in Lowland Switzerland (below 1000 m.a.s.l.) is mild, warm, and moist and the vegetation season is long. Estonia is situated in a transition zone between maritime and continental temperate climates. There is less precipitation, temperatures are lower and vegetation season shorter than in Lowland Switzerland. In Highland Switzerland (above 1000 m.a.s.l.), where climate is alpine, it is colder, wetter and vegetation season is shorter compared to the two other study regions.

From 43 populations of the three taxa we collected a total of 572 genetic individuals (genets). For microsatellite analysis, leaves were collected from the same 572 genets in the field and stored cool in plastic bags with silica gel until DNA extraction (**II**), of which 380 were used. Of each genet we collected three shoots (ramets) for the reciprocal transplantation experiment (**III**). The plants collected in Switzerland were planted to pots in the experimental garden of the University of Zurich, Switzerland, in 2012. In the summer of 2013, after completing field work in Estonia, three common gardens were established, one in each of the three study regions (Fig. 2). Morphological measurements were

made in total three times, first on freshly collected plants (I), then in two subsequent years (2014 and 2015) plants were measured in transplant gardens (III). Vouchers of genets from studied populations are kept in the herbarium of the Institute of Ecology and Earth Sciences of the University of Tartu.



Figure 2. Transplant gardens in (a) Tartu (36m), Estonia, (b) Zurich (503m), Lowland Switzerland and (c) Schynige Platte (1976m), Highland Switzerland.

1.2. Chromosome counts

The immature male spikes collected in the common garden in Zurich in spring 2013 and in Estonia in spring 2014 were pale whitish, indicating the propitious stage for chromosome counts, i.e. the metaphase of meiosis, where chromosomes are tightly packed and most visible. For chromosome fixation we used aceto-carmin squash preparations of pollen mother-cells (Belling, 1926; Schmid, 1982). Samples were preserved in Carnoy's solution (ethanol: acetic acid = 3:1) and stored them at 4 °C. For the preparation of meiotic plates, we fixed anthers, separated from spikes, in an aceto-carmin solution with a drop of iron ions. After two days in a freezer, anthers were washed with acetic acid, macerated with a scalpel and again stained with aceto-carmin. After a few minutes a cover glass was carefully placed and excess fluid absorbed with blotting paper. Slides were heated over a lighter or spirit flame for a few seconds, which flattened the cells and made them stick to the slide and coverslip. Next, the cover slide was pressed to squash the cells and spread the chromosomes. Samples were observed with a phase-contrast microscope (Leica DMLB) to improve resolution with Köhler illumination and photographed with a digital camera (Nikon, D5200). Of approximately 150 samples, the chromosomes were counted in 26 cases, representing several populations of *C. flava* and *C. viridula*.

1.3. Morphological variation analysis

For analysing morphological variation within and between species (I), 14 morphological characters were measured in fruiting plants. In total, five vegetative (plant height, leaf length, leaf width, bract length, stem curvature) and nine floral reproductive traits (length and width of female spikes, deflexion

of male spike, length of male spike and its peduncle length, utricle beak length, utricle width and utricle length) were measured (I). The macro-morphological traits were measured with the use of a standard ruler. Traits related to utricle dimensions were measured with the Quick Photo Micro 2.2 software (Promicra) on photographs taken with an Olympus SP-500UZ digital camera mounted on an Olympus SZX7 binocular stereo microscope system.

1.4. Microsatellite genetic analysis

For estimating molecular genetic diversity and differentiation of 380 genets within and between taxa, microsatellites were used (II). We isolated total genomic DNA from silica-dried leaves using the CTAB method (Doyle, 1987). Cross-species amplification was tested with nine primers developed for *C. scoparia* (Hipp *et al.*, 2009), two primers developed for *C. rugulosa* (Ohbayashi *et al.*, 2008), four primers developed for *C. kobomugi* (Ohsako and Yamane, 2007) and two primers developed for *C. extensa* (Escudero *et al.*, 2010b). Of the 17 primer pairs tested, 10 aligned successfully with recipient DNA, cross-amplified in studied taxa, showed polymorphism and identifiable peaks in fragment analysis (II). Primers were optimized for a range of temperatures and MgCl₂ or MgSO₄ concentrations. PCR were performed using a Techne TC-5000 thermocycler. PCR products were multiplexed, fragment analysis was performed on a 3730xl DNA Analyzer. Microsatellite alleles were sized against LIZ 500 size standard (Applied Biosystems) and determined using the Peak Scanner Software v1.0 (Applied Biosystems).

1.5. Reciprocal transplant experiment

In 2013 three shoots of 572 genets representing all study taxa and 43 sampling populations were transplanted to three common gardens in Schynige Platte, Switzerland (46.65° N, 7.91° E, 1976m), Zurich, Switzerland (47.40° N, 8.55° E, 503 m) and Tartu, Estonia (58.39° N, 26.72° E, 36m; Fig. 2, Fig. 3) (III). The climatic conditions in the common gardens were similar to the respective region where the populations had been sampled (Fig. 1, III). The number of individuals differed between the gardens, because few individuals did not survive transportation between countries. Pots filled with one litre of commercial potting soil mixture and containing a single individual were arranged randomly at the transplant sites. Plants were weeded, watered when dry and covered during the period of snow cover with fleece in Schynige Platte and Estonia, whereas there was no snow cover in Zurich.

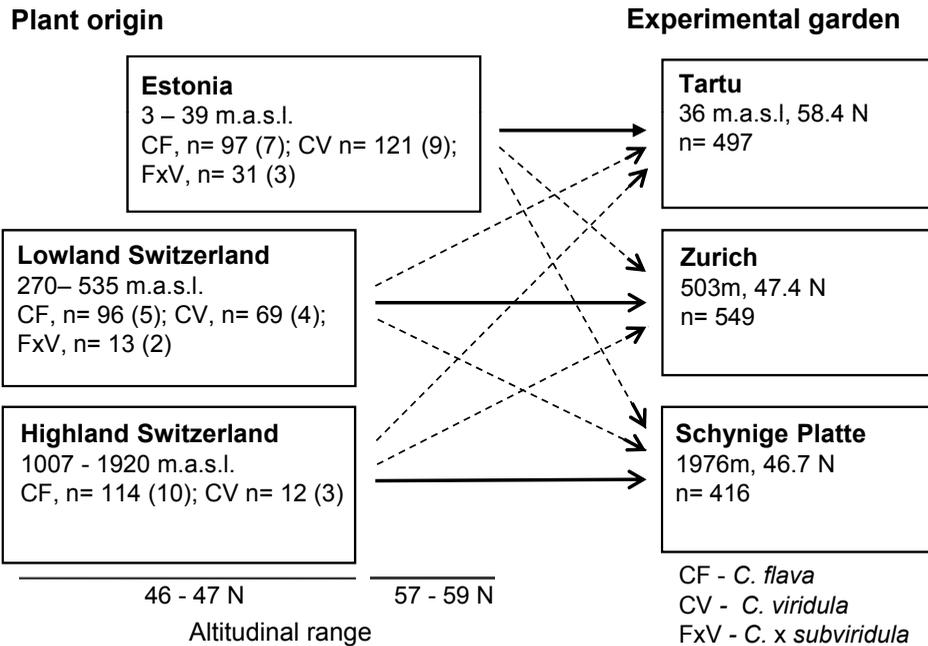


Figure 3. Design of the transplantation experiment. Plants of the three taxa (*C. flava*, *C. viridula* and *C. x subviridula*) were collected from three regions of origin and planted reciprocally to three gardens, one in each of the three regions. For plant origin region number of individuals and in brackets number of populations is presented. Black lines indicate transplanting to the respective regional garden and dashed lines indicate transplanting to a garden in a different region.

In the second year of the experiment (2014) eleven morphological and in the third year (2015) seven performance-related (fitness) traits were measured for all plants in each transplant garden. As morphological traits, we measured both vegetative and reproductive traits: plant height, stem curvature, bract length, leaf length and – width, length of female spikes, deflexion of male spike, length of male spike and its peduncle length, and utricle width and length. Traits associated with performance were number of vegetative and reproductive shoots, total number of shoots, biomass, survival, number of seeds and number of seeds with nut. Plant height is considered both morphological and performance trait and was measured in both year. Utricles were scanned using an Epson perfection V700 photo scanner and measured with the image analysis system WINSEEDLE. Aboveground biomass was collected by cutting shoots with scissors 2 cm above the soil surface, and biomass was dried at 70 °C for 24 h and weighed. Stem curvature and male spike curvature were assessed qualitatively. All other traits were quantitative and either counted or measured with the ruler.

1.6. Data analysis

Morphological traits (**I**, **III**), performance related traits (**III**), principal component scores (**II**) and primary genetic statistics (**II**) were used as univariate response variables. As explanatory categorical variables, we used taxa, region of origin and their interaction (**I–II**). In study **III**, additional predictor variables and selected interaction terms were used to assess the effect of plant origin, transplant garden and difference in mean annual temperature and -precipitation on plant morphology and performance. A contrast regional vs. foreign origin was used to test whether “regional” plants replanted to their region of origin showed better performance than “foreign” plants transplanted from other regions (i.e. regional vs. foreign contrast sensu Kawecki and Ebert, 2004). Response variables were log-transformed prior to analysis, when necessary to obtain normally distributed residuals and homoscedasticity. We used mixed-effect ANOVA to analyse the effects of random factors and fixed categorical predictors and their interactions on the variance of single response variable (**I–III**) and for the binomial variable survival we used General Linear Models GLM (**III**). Population (**I–II**), genet and population in interaction with garden (**III**) were the random factors in the models. Satterthwaite approximation was used to adjust degrees of freedom and p-values (**III**). For more detailed overviews of significant differences within and between species and regions we also conducted pairwise comparisons with a post-hoc test (Tukey’s HSD) (**I–III**). Pearson correlation coefficients were used when assessing the correlation of continuous variables (**I**, **III**). Principal Component Analysis (PCA) with morphological traits (**I**, **III**) and Multidimensional scaling analysis (MDS) based on Reynolds genetic distances (**II**) were used to visualize inter- and intraspecific dissimilarities within and among regions.

Microsatellite data was used to evaluate molecular genetic diversity, population genetic structure and genetic differentiation (**II**). Firstly, primary genetic statistics- the effective number of alleles (N_e), percentage of polymorphic loci (PL), expected heterozygosity (H_e), observed heterozygosity (H_o) and allelic richness (A_r) were calculated for each population across all loci. The inbreeding coefficient (F_{IS}) was calculated as $(H_{exp} - H_{obs}) / H_{exp}$ and allowed us to estimate the prevailing mating systems by region (i.e. Estonia, Low- and Highland Switzerland) and taxa. Then, the Bayesian model-based clustering approach was used to assign individuals to genetic clusters (K) without a priori knowledge of taxonomy or population, and to identify the hybrid individuals with admixture analysis. To compare the degree of differentiation among groups of populations categorized by taxa and region of origin, between-group F_{ST} values were calculated. Finally, analysis of molecular variance (AMOVA) enabled to determine the distribution of microsatellite variation among groups of populations, among populations within groups, and among individuals within populations.

2. RESULTS

2.1. Differentiation between *C. flava* and *C. viridula*

Differences between *C. flava* and *C. viridula* in natural populations were assessed using 14 morphological traits (Table 4, **I**), chromosome counts and ten microsatellite primer pairs (Table S1, **II**). Both univariate and multivariate analyses of 14 morphological traits supported the overall differentiation of *C. flava* and *C. viridula* growing in natural populations, with reproductive traits attaining the highest discriminative power (Table 5–6, Fig. 3, **I**). The highest genetic differentiation between these two taxa was found in the northern study region in Estonia ($F_{ST} = 0.47$) and the lowest one in Highland Switzerland ($F_{ST} = 0.19$, **II**). These findings were further supported by Bayesian cluster analysis (**III**). Overall, the STRUCTURE results indicated a clear differentiation between the taxa and in addition further variation between regions. The ΔK method in the Bayesian program STRUCTURE classified all individuals into two clusters, illustrating a clear distinction between the taxa ($K = 2$; Fig 2, **II**). The log-likelihood distribution method detected three clusters. $K = 3$ yielded a higher resolution than $K = 2$, also revealing differences between the regions, namely admixture between plants of *C. flava* and *C. viridula* in Highland and Lowland Switzerland ($K = 3$; Fig. 2, **II**). The results of the clustering analysis are in line with the results on genetic differentiation and diversity. ANOVAs testing the effects of taxon, region of origin and the interaction between taxon and region of origin on genetic diversity statistics revealed significant differences between the regions (for the percentage of polymorphic loci) and significant taxon-by-region interactions (for three measures of genetic diversity; Table 3a, **II**).

The maintenance of the species differences in different environments was further explored in reciprocal transplantation experiment (**III**). In the three experimental gardens in climatically different regions, taxa remained different from each other in nine of 15 studied vegetative, reproductive and fitness traits (Table 1, **III**). It is noteworthy, that leaf width and utricle length were important traits discriminating the taxa in the natural populations as well as in the transplant gardens. Difference between the taxa was further supported by multivariate principal component analysis of 11 morphological traits measured for all plants in each common garden (**III**). Tukey multiple-comparison tests and ANOVA performed with principal components PC1 and PC 2 clearly separated *C. flava* and *C. viridula* also in the experimental gardens, except the ones originating from Highland Switzerland (Table 2, Fig. 2, **III**), which were all characterised by smaller stature.

Both, *C. flava* and *C. viridula* turned out to be aneuploid with more than one eu-diploid number in Switzerland and in Estonia (Fig. 4). Nevertheless, chromosome numbers differed between the taxa. Among our samples, *Carex flava* had a haploid chromosome number of $n=30$ in seven populations (Kerns,

Mels, Saignelegier, Rehetobel, Kaltbrunn, Kreuzlingen and Leie), n=29 in two populations (Cadagno, Kreuzlingen) and n=31 in two populations (Kreuzlingen, Leie; locations of populations in Table 1–3, **I**). For *C. viridula* n=35 was counted in four populations (Disentis, Wetzikon, Amriswil and Paatsa) and n=34 in one population (Paatsa). In some populations several samples per populations could be used, but many samples were not in a propitious state. For obvious reasons we could not study pollen meiosis in hybrid individuals.

Based on the results on the morphology in the natural populations (**I**) and in common gardens (**III**), molecular genetic variation (**II**) and karyology we confirm that *C. flava* and *C. viridula* are, despite admixing, distinct species.

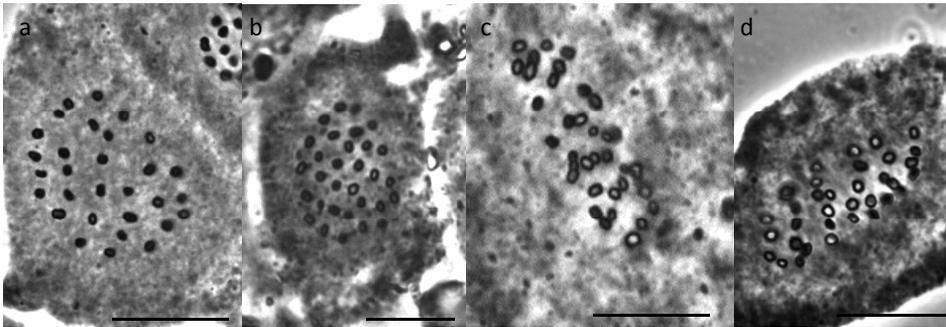


Figure 4. Meiosis in pollen mother cells in (a) *Carex flava*, Kaltbrunn, n=30, (b) *Carex flava*, Hudelmoos, n=31, (c) *C. viridula*, Kreuzlingen, n=35 and (d) *C. viridula*, Hudelmoos, n=35. Scale bar = 10 μ m.

2.2. Regional divergence of *C. flava*, *C. viridula* and *C. x subviridula*

The analyses of ten microsatellite primer pairs showed, for both parental taxa and for their hybrid, similar levels of genetic diversity in number of effective alleles (N_e), percentage of polymorphic loci (PL%), allelic richness (Ar) (Table 2, **II**), with significant differences in expected and or observed heterozygosities (H_o , H_e) (Table 3, **II**). Both parental taxa showed variable inbreeding coefficients in different populations, but on average F_{IS} was higher for *C. flava* than for *C. viridula*, 0.18 and 0.05 respectively. *Carex flava* and *C. viridula* differed in the regional distribution of their genetic variation. The genetic diversity in *C. viridula* populations was highest in Lowland Switzerland, followed by Estonia and lowest in Highland Switzerland, where population density of *C. viridula* is low. The genetic diversity in *C. flava* populations was highest in Highland Switzerland, intermediate in Lowland Switzerland and lowest in Estonia. Private alleles were detected in seven loci of *C. flava*, four of which were found in Highland Switzerland (Table S2, **II**).

Univariate analysis showed that both taxa differed among regions in five of 14 traits, with *C. flava* differing in height of tallest culm, utricle width and

utricle beak length; and *C. viridula* in five reproductive traits (Table 5, **I**). In transplant gardens *C. flava* plants showed strong plasticity in vegetative traits (Table 3, **III**). We detected also genetically determined variation, for example, *C. flava* plants from Highland Switzerland were of smaller size and this remained the case in the Lowland Switzerland and Estonian gardens, as also illustrated by multivariate analysis (Table 2, Fig 2, Table S3, **III**). *Carex viridula* was found both morphologically and genetically more differentiated between regions than *C. flava* (**I, II**). Based on multivariate principal component analysis using 14 morphological traits, *C. viridula* populations showed higher regional differentiation than *C. flava*, with the exception of *C. flava* populations from Highland Switzerland, which were mostly smaller in their vegetative traits (Fig. 2–3, **I**).

Higher genetic differentiation of *C. viridula* among regions than of *C. flava*, based on ten microsatellite primer pairs, was supported by three different analyses: AMOVA (Table 4, **II**), pairwise F_{ST} values (Fig. 3, **II**), Bayesian clustering (Fig. 2, **II**). Within and between regions differences among populations were also illustrated with ordination of multidimensional scaling analysis (Fig. 4, **II**). According to the hierarchical AMOVA, the proportion of genetic variance between the regions was 20.77% for *C. viridula* and 6.84 % for *C. flava*. Nevertheless, based on pairwise F_{ST} values, *C. flava* populations from Estonia showed genetic differentiation from conspecific populations from two other study regions (Fig. 3, **II**). Overall, the morphology and performance of *C. viridula* depended more on the region of origin than that of *C. flava* and the hybrids, leading to significant origin-by-site interactions (Table 3, **III**).

When the three taxa grew in the three experimental sites, the intraspecific differentiation, as observed in nature, was generally maintained, but differed between the gardens (Table 2, Fig. 2, **III**). This indicates the importance of taking environmental variation into account when measuring phenotypic differentiation, and the importance of using more than one garden site.

Hybrids were found in the two study regions Lowland Switzerland and Estonia in several sites, where *C. flava* and *C. viridula* grew in the vicinity. Hybrids from these two regions differed remarkably from each other, both morphologically (Fig. 3, **I**) and genetically (Fig. 3–4, **II**). Moreover, in nature the hybrids from Lowland Switzerland were morphologically more similar to *C. flava*, suggesting that they were mostly backcrosses with *C. flava*, whereas the hybrids from Estonia were intermediate between the parental species (Table 7, Fig. 3, **I**). Multivariate analysis of all individuals in the transplant gardens revealed that the hybrids from Lowland Switzerland retained their similarity to *C. flava*, except in the Schynige Platte garden, whereas hybrids originating from Estonia showed different patterns depending on the garden (Table 2, Fig. 2, **III**). Of the several transgressive traits found in hybrid individuals in natural populations, the extreme values of leaf length and male spike length remained in some transplant gardens (comparison of Table 7, **I** and Table S2, **III**). However, in the transplant gardens hybrids from Estonia showed more extreme characters than hybrids from Lowland Switzerland. Estonian

hybrids had more reproductive and vegetative shoots in the Estonian and Lowland Switzerland gardens and they survived better than either parental taxa. The genetic diversity (H_e) of hybrids was as high as or higher than those of the parental taxa (Table 2, **II**). These results offer compelling evidence for high plasticity and relatively high genetic variation of hybrids.

2.3. Genetically and environmentally determined variation in transplants

One of the goals of the reciprocal transplant experiment was to estimate whether phenotypic differences within species are rather determined by the genotype or by the environment. The two-year experiment showed strong environmentally induced variation for all three taxa in most of the morphological and performance traits examined, except for measures of fecundity and survival (garden effect, Table 1, **III**). Slightly more variation, on average, was explained by the transplant gardens in *C. flava* and the hybrid *C. x subviridula* than in *C. viridula* (Table S6, **III**).

Morphology and performance were more determined by genetic variation between regions of origin in *C. viridula* than in *C. flava* or hybrid. However, genetically determined variation, due to the region of origin, was found for fewer traits than the environmental garden effects were (Table 1, Table S5, **III**). Significant effects of the region of plant origin detected in morphological and fitness traits indicated that *C. flava*, *C. viridula* and *C. x subviridula* have evolved some regional differences in parallel, such as shorter heights of plants originating from Highland Switzerland (Table S2, **III**).

Independent of the region of origin, all three taxa showed decreased vegetative and reproductive growth in the alpine garden at 1976m and increased growth in the northernmost garden at 36m in Tartu (Table S2, **III**). This finding reflects a general increase in performance with increasing latitude and decreasing elevation of the test environment. However, our results also indicated ample genetic variation in plasticity, i.e. taxon by environment (i.e. transplant garden), population by environment, or genotype-by-environment interactions. We found that phenotypic differences between *C. flava* and *C. viridula* were determined more by the genotype within taxa than by the environment, as revealed by seven of 15 significant taxon \times origin interactions compared with only two of 15 significant taxon \times garden interactions (Table 1a, **III**). The genotype-by-environment effect was also evident in origin by garden interactions, which were found significant in nine of 15 traits, showing that not only taxa as a whole showed different responses to the gardens, but that the response to the gardens also depended on the region of plant origin (Table 1, **III**). Three-way taxa \times origin \times garden interactions were found significant for nine of 15 traits (Table 1a, **III**), respective for five of 15 traits with hybrids in the model (Table 1b, **III**), indicating that differences in the plastic response of plants from different origins differed between taxa.

2.4. Regional adaptation of *C. flava*, *C. viridula* and *C. x subviridula*

Regional adaptation of the three taxa was tested with regional vs. foreign contrasts. Overall significant differences between regional plants and non-regional (foreign) ones were detected in two of 15 traits for *C. flava*, in four of 15 traits for *C. viridula* and in five of 15 traits for *C. x subviridula* (regional effect, Table 4, **III**). In all significant cases the regional plants performed better than the foreign ones, indicating general adaptation to regional conditions. For example, both *C. flava* and *C. viridula* plants produced more seeds when grown in local gardens and hybrids were in local gardens taller and had longer leaves (Table S2, **III**).

The response of regional plants in specific gardens was examined with “regional vs. foreign” × garden interactions. Significant interaction terms were found for *C. flava* in three of 15 traits, for *C. viridula* in seven of 15 traits and for hybrids in two of 15 traits (Table 4, **III**), indicating that effects of the match of regional origin with region of the garden on the morphology and performance also differed among gardens. The direction of significant “regional vs. foreign” × garden interactions indicated both cases of adaptation (*C. flava*: 5 cases, *C. viridula*: 9 cases, hybrid: 3 cases) and of maladaptation (*C. flava*: 3 cases, *C. viridula*: 12 cases, hybrid: 1 case).

We also expected to find evidence for local adaptation when examining the relationship between plant performance and climatic differences (in mean annual temperature and precipitation) between experimental garden and population of plant origin. The effect was significant for trait “seeds with nut” for *C. flava* (Table S4, **III**).

Overall, these results suggest some regional adaptation for all taxa. However, at closer examination in each garden, *C. viridula* plants turned out to show more cases of maladaptation than of adaptation, whereas the two other taxa showed more cases of adaptation than of maladaptation. The climatic factors mean annual temperature and precipitation were not the main reason, however, for the observed cases of adaptation.

3. DISCUSSION

3.1. Difference between *C. flava* and *C. viridula*

According to the “semipermeable species boundaries” theory, alleles at some loci can be exchanged between species, and species boundaries are expected to vary geographically, especially between recently diverged species (Arnold, 1992; Rieseberg and Burke, 2001; Abbott *et al.*, 2013). Thus, hybridization coupled with backcrossing is expected to affect population differentiation on a regional scale. In case of gene flow between sympatric species, the genome may become admixed and species may evolve some traits in parallel, which can lead to regional differences. We detected hybrid individuals at sites where *C. flava* and *C. viridula* grew together, indicating hybridization. To study whether *C. flava* and *C. viridula* are affected by the hybridization, we examined their morphology, performed molecular genetic analysis and planted the plants to common gardens. In the natural populations, the two taxa differed significantly in 71.4% of the studied morphological traits, with reproductive traits attaining the greatest discriminative power (I). We further observed the taxa in three transplant gardens and found that in 60% of morphological and performance traits the differences between the two taxa were maintained (III). With the use of neutral molecular markers we detected admixture between *C. flava* and *C. viridula* (II). Our results of chromosome counts support the difference between taxa, but also indicate aneuploidy. However, the morphological and genetic differences between species were more prominent than a harmonising effect of admixture, both in within-region and between-region analyses. The transplant experiment also revealed that the two taxa shared similar patterns of within-taxa differentiation between regions of origin, indicating parallel evolution and regional connectedness.

Morphological variation due to phenotypic plasticity is common among plants. Experimental transplantations between regions of origin reveal how much of the variation is due to genetic differences among populations and how much due to phenotypically plastic responses (Kawecki and Ebert, 2004). Our transplant experiment revealed stronger environmental garden effects than effects of regional genetic origin (III). Taxa in the *C. flava* group are known to have high morphological variability (Crins and Ball, 1989a; Stoeva and Stepankova, 1990; Hedrén, 2002). In our study III all taxa showed high plastic responses to the three common garden environments. Transplant garden explained on average slightly more variation in *C. flava* and the hybrid *C. x subviridula* than in *C. viridula*. A profound effect of the environment on the phenotype is consistent with earlier smaller-scale experiments with taxa of the *C. flava* group (Davies, 1953a; Schmid, 1986b), where Schmid (1992) was even speaking of general-purpose genotypes.

When we assessed whether phenotypic differences within and between taxa were determined rather by the genotype or by the environment, and found

strong environmental effects, the differences between taxa were nevertheless maintained in the transplant gardens (III). Thus we detected consistent quantitative-genetic variation among taxa, which indicates that the differences between the taxa had indeed a genetic basis (III). This finding is different from Schmid's (1980) small scale common garden experiment, who compared field-collected and cultivated material of *C. flava* and *C. viridula* and found stronger morphological differences in the field compared to differences measured in the common garden. Hedrén (2004) deduced from this finding that *C. flava* and *C. viridula* may differ in their preference for optimal conditions, and that the separating morphological characters may be developed under the conditions optimal for both species. Kaplan (2002) found in a common garden experiment with several *Potamogeton* taxa that most of the morphological characters considered as suitable for distinguishing taxa were under strong environmental influence, and that the taxa identified earlier did not reflect genetically distinct lineages. Our transplant experiment clearly confirmed that the differences in growth and performance between the studied *Carex* taxa have a genetic basis, as these differences were maintained when the plants were raised in common gardens.

Most phenotypically plastic characters in plants are generally related to plant growth and leaf morphology. Least plasticity is exhibited by reproductive structures, in particular flowers (Schmid, 1992), though direct fitness measures like seed size and number may still be plastic to some extent (Nicotra *et al.*, 2010). But several characters of the anatomy of leaves are found to be weakly affected by the environment and to be taxonomically useful (Bugg *et al.*, 2013). Although a large part of the environmentally induced morphological variability in plants of the *C. flava* group is found in vegetative traits, and reproductive traits are considered more constant (Davies, 1953a; Schmid, 1983; Crins and Ball, 1989a), we detected strong effects of environment also on reproductive traits (spike and utricle dimensions) (III). This is consistent with Gonzalo-Turpin and Hazard (2009) who found plasticity in floral traits and seed size of the grass *Festuca eskia* Ramond ex DC. They suggested this plastic response to be adaptive and to provide the potential to successfully adapt to climate change. In earlier studies with herbarium material collected in natural populations, utricle beak length, but also the width and length of utricles were traits attaining most power in discriminating taxa of the *Carex flava* group (e.g. Schmid, 1984b; Hedrén, 2002). In addition to the aforementioned characters, we also found that the width of leaves discriminated the taxa very well, both in the natural populations and in the common gardens.

Against the biological species concept, Hedrén (2002) recommends to treat hybridizing taxa as distinct species if they function as evolutionarily independent units, and if introgression between them does not obscure differentiation. This would be in agreement with earlier studies finding significant differences between *C. flava* and *C. viridula* based on morphology (Davies, 1953a; Schmid, 1983; Crins and Ball, 1989a; Stoeva and Stepankova, 1990; Pykälä and Toivonen, 1994; Więclaw, 2014), allozyme data (Bruederle and

Jensen, 1991; Hedrén, 2002) and ITS sequences (Jiménez-Mejías *et al.*, 2014). Hedrén (2002) also concluded that there is no notion of effects of introgression for *C. flava* and *C. viridula*, as plants from mixed populations are as different from each other as are plants from pure populations. However, Schmid (1982) found considerable genetic and karyological variability in the *C. flava* group due to introgression. He suggested that the taxa remain morphologically distinct as selection favours individuals with parental character combinations and as taxa cross quite rarely due to their scattered distributions. The maintenance of between-species differences despite of interspecific gene flow was also found for sympatric *Senecio* and *Helianthus* taxa. The *Senecio* taxa were suggested to remain different due to general selection against hybrids and locally maladapted hybrid individuals (Brennan *et al.*, 2016). Kane *et al.* (2009) detected high levels of gene flow between *Helianthus annuus* L. and *H. petiolaris* Nutt., but found that the two taxa remain morphologically and ecologically distinct. The individuals with mixed ancestry were morphologically similar to either parent, which suggested that the genes involved in species-specific traits did not cross the species boundaries. The *Carex flava* group has received quite some research attention, but in this thesis morphological, molecular and translocation evidence has been combined for the first time, and the taxa were studied in geographically distant regions. We conclude that *C. flava* and *C. viridula* are indeed separate taxa, but that hybridization between them leads to some admixture and to a certain morphological similarity. As our results are comparable with most earlier studies concerning other parts of distribution, it is probable that the results of between species differences are not restricted to Estonia and Switzerland, but are applicable to other unstudied parts of their distribution.

A potential explanation why parental species can stay distinct in the face of interspecific gene flow is ecological differentiation (Harrison, 1993; Goulet *et al.*, 2017). Small ecological differences in habitat requirements were found important in the maintenance of *Helianthus* taxa, where the introduction of the more common *H. annuus* to the more narrowly distributed *H. bolanderi* A. Gray resulted in the loss of the latter taxon due to hybridization (Carney *et al.*, 2000). While there are some ecological differences between the habitats of the species of the *C. flava* group in terms of soil moisture and productivity, there are still many localities where *C. viridula* and *C. flava* occur together (Schmid, 1982). The question, which separation mechanisms prevail in the *C. flava* – *C. viridula* system, can therefore not be answered based on this study, but would require further research, e.g. on flowering times of the different taxa in mixed populations.

3.2. Genetic diversity and regional differentiation in the different taxa

Against our expectation, we observed similar levels of genetic diversity for *C. flava* and *C. viridula*, and on average higher inbreeding coefficient for *C. flava* (II). Surprisingly low genetic diversity for *C. flava* was detected with the use of allozymes in studies with populations from Fennoscandia (Hedré, 2004), and in Central Europe and Scandinavia (Bruederle and Jensen, 1991). Hedré (2004) found *C. flava* mostly in mixed populations and suggested that its low genetic diversity is a result of strong inbreeding. The proportion of fruits formed by selfing depends on the distance between male and female flowers (Hedré, 2004). Interestingly, in the high elevation garden the male spike peduncles were shorter than in the two other gardens, indicating a possible tendency for higher self-fertilization in the more extreme weather conditions during alpine summers. However, based on the microsatellite data the inbreeding coefficient F_{IS} was not significantly higher in populations originating from Highland Switzerland (II). Our results on inbreeding obtained with microsatellite markers, which have higher mutation rates and resolution than allozymes (Sunnucks, 2000), are similar to the earlier findings with the use of allozymes.

Based on Rabinowitz (1981) rarity criteria, we considered *C. viridula* “rare”, as despite wide geographic distribution it is neither very frequent nor very abundant (Pykälä and Toivonen, 1994). In the literature there are a number of examples of rare species with low levels of genetic diversity (Ellstrand and Elam, 1993; Leimu *et al.*, 2006; Talve *et al.*, 2013). On the other hand, different rare species may have different population sizes, habitat specificities and geographic ranges, and thus their levels of genetic diversity may be very variable (Gitzendanner and Soltis, 2000). Moreover, Rabinowitz (1981) suggested that rare species may evolve means of maintaining high levels of genetic variability despite their rarity, such as increased outcrossing. Moreover, increased outcrossing is especially evident in groups such as *Carex flava*, where all species appear to hybridize freely (Volkova *et al.*, 2008). A mixed breeding system of selfing and outcrossing was suggested for the *C. flava* group members (Hedré, 2004), which is in agreement with our results (II). Accordingly, interspecific gene flow was given as the main reason for high levels of genetic variation in *C. hirsutella* Mackenzie in the *Carex complanata* complex (Smith and Waterway, 2008). Another explanation for the presence of relatively high levels of genetic diversity in isolated small populations could be a delayed loss of genetic diversity, as described for *Carex magellanica* Lam. in populations in Estonia due to recent loss of habitats (Kull and Oja, 2010). Similarly to extinction dept, loss of genetic diversity may be delayed (Helm *et al.* 2009). The distribution of *C. viridula* is characterised by fragmented habitats and declines or fluctuations in habitat quality. In Switzerland it is considered as potentially endangered (Bornand *et al.*, 2016).

We suggest that the detected similar levels of genetic diversity for the rare taxon *C. viridula*, with its generally small populations, and the more common taxon *C. flava*, with generally larger populations, reflects higher inbreeding in *C. flava* than in *C. viridula*, and a possible delay of genetic diversity losses in *C. viridula*.

Genetic variation is not distributed equally among populations of the same species across an entire distribution areal, which becomes more apparent as genetic differentiation. Regional differentiation of populations of the same species is a common phenomenon, as geographic distance between populations sets physical limits to dispersal and reproduction (Loveless and Hamrick, 1984; Slatkin, 1987; Balloux and Lugon-Moulin, 2002). Genetic differentiation among plant populations over small and large scales may also result from different adaptive responses to ecological gradients (Linhart and Grant, 1996; Ohsawa and Ide, 2008). In our study, we observed higher genetic and morphological differentiation of the less common species *C. viridula* than of the more widespread *C. flava* (I, II). This is in line with our prediction that the species with the smaller and more scattered populations should show stronger regional differentiation. On the other hand, for wind pollinated species, such as the *C. flava* group members, in general higher levels of gene flow are expected than for selfing or animal-pollinated species (Govindaraju, 1988), which would speak against high differentiation in *C. viridula* populations. Kull and Oja (2010), however, found that wind pollination is not very effective for small herbs in closed habitats. Compared with *C. flava*, *C. viridula* plants are shorter and their male spikes are closer to the ground, which most likely makes pollen dispersal by wind less efficient. However, seeds of *C. viridula* have a special feature enhancing long-distance dispersal, namely air-filled in the utricle (Crins and Ball, 1989b). While this feature was suggested to enable *C. viridula* to colonize and survive in fluctuating wet habitats, like lake shores (Crins and Ball, 1989b), it is unlikely that long-distance dispersal is very frequent. A further possible explanation for higher differentiation of *C. viridula* than of *C. flava* is the loss of suitable habitats and subsequent fragmentation of the previously larger populations (Pykälä and Toivonen, 1994). Similarly, Reisch *et al.* (2005) found high differentiation along an altitudinal gradient for *Primula farinosa* L., and suggested that isolation by distance is the most likely reason, because the distribution of *P. farinosa* has decreased in the 20th century due to land development and loss of suitable habitats. Similar findings were also reported by Schönswetter *et al.* (2006) for rare and disjunct *Carex atrofusca* Schkuhr populations. They explained this strong differentiation with genetic drift, which is also possible for *C. viridula*. This is perhaps surprising, given that we detected similar levels of neutral genetic diversity for *C. viridula* compared to *C. flava*, but ten markers is by far not explaining the whole within-species variability (II). We suggest that the higher morphological and genetic differentiation observed between *C. viridula* populations than between *C. flava* populations is most likely due to stronger genetic drift and fragmentation of populations.

Carex viridula plants of different regions of origin differed in their overall growth, seed production, and survival in the common gardens, indicating a genetic basis for trait differences between regions. This might suggest the presence of ecotypes with genetically fixed traits, as also suggested for *Carex spicata* Huds. (Janyszek *et al.*, 2008). According to Pykälä and Toivonen (1994), the presence of interspecific taxa of *C. viridula* in Fennoscandia indicates its high evolutionary activity compared with other parts of the distribution range. This could be explained by a central-marginal hypothesis, as Fennoscandia is located in the periphery of the distribution area. Schmid (1986b) suggested that the evolution of the different subspecies among the *C. flava* group, particularly in *C. viridula*, may be explained by the combination of local selection and random genetic drift in temporally and spatially small and isolated populations with quite short individual generation times. Our transplant experiment revealed a genetic basis for regional differentiation in *C. viridula*. Possibly such genetically different regional phenotypes may be evolving towards more pronounced ecological preferences and finally reproductive barriers.

Greater dispersal is expected for wind-pollinated species whose populations are large and continuous in space and time, such as the ones of *C. flava*. Such high gene flow between populations generally leads to low genetic structuring of metapopulations (Slatkin, 1987; Gitzendanner and Soltis, 2000). Our results of morphological variability and genetic structuring of *C. flava* populations (**I**, **II**) are in accordance with theoretical studies, which have shown that already quite small amounts of long-distance pollen dispersal can prevent population differentiation for neutral alleles (Loveless and Hamrick, 1984).

At high altitudes, plants endure several constraints for growth and reproduction – the shortness of the growing season, low temperatures, and long persistence of snow cover. This could lead to differentiation from populations at other altitudes (Premoli, 2003; Ohsawa *et al.*, 2007). According to Schmid (1983) *C. flava* is replaced by *C. flava* var. *alpina* at high altitudes (over 1000 m) in central Europe. The var. *alpina* is characterised by shorter stems (less than 30 cm) and leaves (less than 10 cm), which is similar to the size of *C. flava* in natural populations in Highland Switzerland, as described in the study **I**. This supports the recognition of the specimens of *C. flava* in Highland Switzerland as var. *alpina*, as recommended by Schmid (1983). This is further supported by our finding that the smaller stature of plants from *C. flava* populations from Highland Switzerland than of the other regions was maintained in the common garden.

3.3. Regional adaptation

Plants are locally or regionally adapted if they have higher fitness in their local or regional habitat than plants from foreign habitats (Kawecki and Ebert, 2004). While adaptation to local conditions is quite common (Leimu and Fischer, 2008), plants of many local populations were also found to be less fit than

plants from foreign populations, indicating maladaptation to local conditions (Hereford, 2009; Anderson *et al.*, 2015). Our transplant study showed various genotype-by-environment interactions, which encouraged us to examine the patterns for regional adaptation of our study taxa (III). We found generally better performance of local *C. flava*, *C. viridula* and hybrid *C. × subviridula* plants than of foreign ones for several performance traits, whereas other traits were indifferent in this respect. We examined the regional vs. foreign response in each of the different gardens to test for changes in fitness and morphology of regional plants in their own habitat. In several cases the regional plants performed better than foreign ones, but we also detected maladaptation in *C. viridula*, and fewer such cases for *C. flava* and the hybrid. Maladaptation is supposed to be more likely in small and isolated populations due to higher impacts of genetic drift and deleterious mutations (Ellstrand and Elam, 1993; Pertoldi *et al.*, 2007; Leimu and Fischer, 2008; Farkas *et al.*, 2015). Therefore, we had predicted limited local adaptation for the more rare *C. viridula*, due to effects of genetic drift in small populations. While our study II showed that the neutral molecular microsatellite diversity of *C. viridula* was not lower than in *C. flava*, populations of *C. viridula* were more differentiated between regions indicating stronger effects of genetic drift. While this is in line with the idea that plants from species with smaller populations are less likely to be locally adapted (Leimu and Fischer, 2008), there are also case studies showing local adaptation in plants that are by some means rare. Becker *et al.* (2006) detected local adaptation in *Carlina vulgaris* L. despite small, isolated and fragmented populations; and McKay *et al.* (2001) found adaptation in *Boecheera fecunda* (Rollins) Dorn (syn. *Arabis fecunda*), despite its small populations and low neutral genetic variation.

Maladaptation may also result from sudden changes in local or regional environmental conditions, which renders organisms adapted to past rather than current conditions. Although *C. viridula* is common in Finland it has declined or disappeared from many localities where environmental conditions changed due to the drainage of mires, regulation of water levels and eutrophication of shores (Pykälä and Toivonen, 1994). Current rapid climatic change may similarly affect adaptation, as already detected for the model organism *Arabidopsis thaliana* (L.) Heynh. (Wilczek *et al.*, 2014). Maladaptation may become more and more apparent with proceeding climate change, where genotypes from warmer, lower-elevation populations gain a fitness advantage over local genotypes at high elevation or high latitudes, where plants are adapted to a colder climate (Anderson *et al.*, 2015). Hereford (2009) adds environmental maternal effects as a potential cause for local maladaptation, which may also have played a role in our clonal study organism. These results suggest that the higher number of maladaptation cases for *C. viridula* was mainly due to increased genetic drift in its generally smaller populations or to more pronounced environmental change in its populations.

In widely spread species with mixed mating systems, like *C. flava*, high gene flow among populations is expected (Rieseberg and Burke, 2001). Based

on neutral genetic markers we found low regional genetic differentiation between populations of *C. flava* (III), indicating high gene flow, which could potentially prevent adaptation to regional conditions, as it tends to homogenize allele frequencies (Lenormand, 2002; Kawecki and Ebert, 2004; Hereford, 2009). On the other hand, high gene flow increases genetic diversity within populations, which is required for adaptations to evolve, and there are numerous studies showing that local selection was strong enough to override gene flow (e.g. Leimu and Fischer, 2008 and references therein).

The presence or absence of local or regional adaptation was shown to depend on the geographic scale of the study (Joshi *et al.*, 2001; Macel *et al.*, 2007). Becker *et al.* (2006) found regional adaptation in fragmented *Carlina vulgaris* populations and explained it by large climatic differences between the study sites, which would have not been detected in a small scale study (< 200 km distance). Similarly, Galloway and Fenster (2000) studied intraspecific differentiation in self-compatible, predominantly outcrossing *Chamaecrista fasciculata* with limited gene flow, and detected local adaptation only when transplanting at the furthest spatial scales of 2000 km, which is similar to the distance between Estonia and Switzerland. Besides, a narrow habitat niche was shown to limit divergent selection and thereafter adaptation of *Geum reptans* L. in Alpine landscape (Hamann *et al.*, 2017). *Carex flava* occurs in a wide variety of moist to wet habitats, open meadows and fens, as well as in partially shaded shrub-carrs and swamps, and at different elevations (Crins and Ball, 1989b; Pykälä and Toivonen, 1994). Therefore it is not very likely that environmental conditions are very similar in these habitats, and a narrow habitat niche is not likely the cause for observed pattern of regional adaptation. Rather, it supports that regional adaptation is supported by environmental differences and thus selection pressures.

Another important factor to consider is the temporal scale of a study and the specific climatic conditions in the study years. Evidence for local adaptation has been found to differ between subsequent experimental years (e.g., Rice and Mack, 1991). Moreover, local adaptation may only become apparent with time, depending on plant longevity (Bennington *et al.*, 2012). In our transplant experiment the two years of the measurements, 2014 and 2015, differed in their weather conditions, especially in Switzerland. In 2014, there was a short heatwave in June, July saw frequent rains and August was relatively cold. In contrast, in 2015 the summer was quite hot, 2–2.5 °C above the norm (annual weather report, MeteoSwiss, 2015). In Estonia, the annual temperature was 1.5 °C above the norm in both years and rainfall was below the usual (annual weather report, Estonian Weather Service, 2015). Fluctuating environmental conditions at study locations are considered as methodological challenge and suggest that experiments should last several years (Volis *et al.*, 2016). In short term experiments local adaptation may be underestimated, as revealed, for example, in a two-species transplant experiment that was revisited every other year from 1981–1993 and then again after 30-years of establishing. After three decades *Dryas octopetala* L. plants showed stronger pattern of local adaptation

than in the beginning of the experiment, and the second study species *Eriophorum vaginatum* L. showed differences in survival after three decades, which had not been detected 17 years earlier (Bennington *et al.*, 2012). To address these issues, we did our experiment for two years involving three gardens and plants from many field populations. This allowed us to test effects of climatic differences (see next paragraph) on plant performance between the actual climate in each garden and the long-term local climatic conditions in each of the 43 populations. Thereby testing general adaptation to local climates and enabling to make conclusions which do not depend on the very climate of the study years, but only uses it as a tool.

Different abiotic (e.g. climate, abiotic soil components) and biotic (e.g. mutualists, herbivores, pathogens) selection pressures can influence the evolution and expression of local and regional adaptation (Macel *et al.*, 2007; Raabová *et al.*, 2007; Münzbergová *et al.*, 2017). Precipitation and temperature are considered the main climatic variables causing large environmental variation (Volis *et al.*, 2016), whereas other factors have a more local effect (Macel *et al.*, 2007). We related differences between temperature and precipitation at the populations of origin and at the transplant gardens to plant fitness traits (III). When precipitation in the garden was similar to precipitation at the populations of origin the number of seeds with nuts was significantly higher for *C. flava* than when precipitation was different, suggesting that adaptation is at least partly due to local climatic conditions. For *C. viridula* we did not detect such effects of climatic differences, suggesting that the regional adaptation detected for some traits (indicated by a regional versus foreign contrast for plant performance in the gardens) was due to selection pressures exerted by other biotic or abiotic factors.

It is important to note that the degree of regional adaptation detected in our study is a conservative estimate, as it was largely designed to detect adaptation to climate, which was characterized by mean annual temperature and precipitation. However, adaptation can also be affected by other factors not considered in our study. For example, adaptation has been found to depend on specific mutualists, pathogens or herbivore populations (Joshi *et al.*, 2001; Anderson *et al.*, 2015). While in our experiment, we did not observe notable damages by herbivores and therefore refrained from considering it more closely, we cannot rule out that selection by herbivory differs between natural populations. Therefore we cannot draw conclusions on effects of adaptation to other biotic interactions or to soil conditions. In a multi species study Bucharova *et al.* (2016) explained better performance of regional transplants than of non-regional ones, with stronger selection pressures originating from regional microbial communities and differences in photoperiod rather than temperature and precipitation. In our experiment, we did not study the effect of microbial communities in the soil. Day length, however, differed between Estonia and Switzerland, and it is possible that part of the regional adaptation was due to differences in day length.

3.4. Role of hybridization

Interspecific hybridisation in flowering plants is a major evolutionary pathway (Arnold, 1992; Rieseberg and Willis, 2007; Abbott *et al.*, 2013). Understanding the impact of hybridization on evolution requires studying morphological and molecular variation. We detected and sampled putative hybrid individuals in two locations in Estonia and in another two locations in Lowland Switzerland where *C. flava* and *C. viridula* grew in sympatry. As putative hybrids we considered individuals with empty utricles (without seed) and with a pale green and somewhat sturdy habitus. Hybrids were less abundant than plants of *C. flava* and *C. viridula*. Similarly, Więclaw and Wilhelm (2014), who studied hybridization in natural *C. flava* populations in Poland, found hybrids only in few of their many study locations (in 8 of 90), where two or more taxa of the group co-occurred. However, in contrast to us, they found numerous hybrid individuals in all eight sites. Derieg *et al.* (2013) also reported that there were only small sets of individuals of hybrids between *Carex cryptolepis* Mack. and *C. viridistellata* Derieg. These observations are in line with the idea that hybrids are often found to be maladapted in their parental environments, which is considered as an important reason for the maintenance of phenotypic differences between hybridising species (e.g. Brennan *et al.*, 2016).

Many hybrid individuals are intermediates or mosaics of parental characters, but others also show transgressive traits, i.e. traits more extreme than those of either parent (Rieseberg and Carney, 1998). While it is true that many hybrids have lower fitness than their parents, there is also large variation in the fitness of hybrids; and some even exceed the parents in terms of fitness (Rieseberg, 1995; Abbott, 2017). In our study, hybrids differed phenotypically from the parental species, and these differences depended on the region of plant origin and on the test environment (**I**, **III**). While hybrids from Estonia were generally intermediate between their parent species in 71.4% of the morphological characters, in Lowland Switzerland only 7% of the characters were intermediate (**I**). Hybrid intermediacy would be in accordance with previous studies with *C. × subviridula* (Stoeva and Stepankova, 1990; Więclaw, 2014). However, in the field we also detected traits with positively extreme values in hybrids (higher than that of either parent), and some of these traits, including male spike length, remained positively extreme in the transplant gardens (**III**). Such transgressive traits indicate novel evolutionary potential of hybrids, which may increase the ability of hybrid populations to cope with changes in environmental conditions. Compared with their parental taxa, hybrids had also similar levels of neutral genetic diversity and performance (**II**, **III**). These results are in line with several studies showing good performance of plant hybrids in different environments (Emms and Arnold, 1997; Campbell and Waser, 2001; Miglia *et al.*, 2005). Eroukhanoff *et al.* (2013) studied local adaptation of hybrid species (using sparrows as models) and noted that despite admixed genetic backgrounds the hybrids showed adaptation to environmental variation. We found indication for better performance of regional hybrids, but weak signal in specific sites, and

suggest that this may indicate that the observed hybrid lineages are young or that hybrid adaptation is constantly disrupted by backcrosses.

Carex flava and *C. viridula* differ in their chromosome numbers, with $n = 28-30$ and $n = 34-36$ respectively (Davies, 1955; Schmid, 1982; Stoeva and Stepankova, 1990; Jiménez-Mejías *et al.*, 2012), suggesting that most hybrids resulting from crosses between them have severely disturbed meiosis. This could be one reason why we were not able to count chromosomes in samples of hybrid individuals. Interestingly, in some putative hybrid individuals, which were also confirmed to be of hybrid origin by our microsatellite analysis, we found some 5% of utricles with partially developed seeds (own observation). This suggests some fertility, which in sedge hybrids could have been restored in backcrosses (Cayouette and Catling, 1992). Moreover, microsatellite analysis indicated that admixture proportions per hybrid individual were very variable, indicating that these comprised F_1 to F_n hybrids and backcrosses (II). The germination percentage of the seeds of our hybrids was zero, suggesting that they were indeed infertile. In other studies hybrids between *C. flava* and *C. viridula* have been found to be sterile, whereas hybrids between *C. viridula* and *C. lepidocarpa* or *C. demissa* have been partially fertile, reflecting their closer relationship than of the former ones (Schmid, 1982; Crins and Ball, 1989b). In the microsatellite analysis we did not detect more private alleles in the hybrids than only one in Estonia (II). Nevertheless, we cannot completely rule out the possibility that some hybrid individuals in our studied populations carried alleles deriving from other *Carex* taxa, which might have contributed to hybridization but were no longer extant at the site.

In our study the hybrids from different regions of origin showed morphological and genetic differentiation from each other and were differently differentiated from the parental taxa (I, II). The hybrids from Lowland Switzerland were morphologically and genetically similar to *C. flava*, whereas the ones from Estonia were more similar to *C. viridula* (I–III). These differences between hybrid origins could have arisen from genetic drift in hybrid populations or due to different introgression patterns in the two regions with hybrids. The direction of hybridization, i.e. the species serving as maternal and as paternal parent, is found to depend on several factors, among them on species abundance, phenology and floral morphology. Nolte *et al.* (2009) found genetic differences between two hybrid zones of fish (*Cottus*), and explained it by different selection pressures on the admixed populations, as local ecological conditions differed between the hybrid zones. The direction of hybridization may also be affected by the length of the style (Field *et al.*, 2011), and hybridization could occur more commonly from sedges with long-beaked utricles to short-beaked ones due to mismatch between pollen tube and style length (Derieg *et al.*, 2013). This would suggest that *C. flava* should serve as paternal parent and *C. viridula* as maternal parent in our system. *Carex viridula* has indeed been found to generally act as a female parent, while *C. flava* can occasionally also be pollinated by F_1 hybrids (Schmid, 1982).

The detected similarity of hybrids to *C. flava* in Lowland Switzerland lets us assume backcrossing with *C. flava*, which according to Schmid (1982) would lead to increasingly more fertile and *C. flava*-like swarms of introgressed forms. This matches well with the results of a 50-year-long observation of hybrid sunflowers (*Helianthus*), where Carney *et al.* (2000) found a shift to higher similarity with the more common taxon, which was more pronounced in morphology than in neutral genetic markers. Ducarme and Wesselingh (2005) studied *Rhinanthus* hybrid zones and found in one study population, probably due to genetic assimilation, backcrossing with the more abundant parent and loss of the less common species *R. minor* L. Another explanation for preferential backcrossing with *C. flava* could be its phenology, as it flowers earlier than *C. viridula* and backcrossing is expected to occur towards the taxon flowering earlier (Vonk, 1979). These considerations are in line with our finding of increased similarity of hybrids with *C. flava* in Lowland Switzerland, while they do not explain the higher similarity to *C. viridula* in Estonia. Possibly, the latter was due to different selection pressures in Estonia.

It would be very interesting to conduct studies similar to ours with many pairs of hybridizing and non-hybridizing related species in order to find out whether the differences between taxa and between regions of origin would be more pronounced for non-hybridizing species or not. Abbott (2017) suggested that combined morphological and population-genetic approaches are likely to lead to the discovery of many more plant hybrid zones in the future. Moreover, according to Cayouette and Catling (1992), a taxonomist should know the hybrids in order to know the species well enough to provide a comprehensive classification.

3.5. Implications for conservation and global change biology

In conservation biology it is often important to understand whether and if so, to what extent populations are genetically isolated from each other. Small isolated populations are subject to stronger effects of genetic drift, which will affect their evolutionary potential through the fixation of deleterious mutations and reduced genetic diversity (Ellstrand and Elam, 1993; Frankham, 1996). The knowledge of population structuring can therefore inform conservation strategies and management (Balloux & Lugon-Moulin 2002). Ennos (2005) suggested that for the conservation of taxonomically complex groups new strategies are needed. Conservation should concentrate on facilitating the evolutionary interactions among members of such groups that generate and maintain their taxonomic biodiversity (i.e. hybridization and introgression), rather than trying to preserve the individual taxonomic entities that such evolution produces.

Hybridization can add additional challenges for conservation, as due to backcrossing the rarer parental taxa could be threatened (Levin *et al.*, 1996; Rhymer and Simberloff, 1996; Allendorf *et al.*, 2001; Bardy *et al.*, 2011). *Pyrola minor* L. is an example, where due to hybridization with more abundant taxa fitness has decreased and the rarer taxon is facing extinction in some parts of its distribution (Beatty *et al.*, 2010). Another example is *Cakile maritima* Scop., which after introduction to Australia had a reproductive advantage over earlier introduced *C. edentula* (Bigelow) Hook. (Mesgaran *et al.*, 2016). *Cakile maritima* hybridized with the existing *C. edentula*, which led to the loss of the latter taxon in Australia, while the former still carries some of its genes. Assessing the outcome of hybridization is essential for conservation of many species (Chunco, 2014).

Hybridisation may not only pose challenges, but may also provide new opportunities to conserve evolutionary potential in changing environments by adding new phenotypic and genetic variation to populations (Chunco, 2014; Brennan *et al.* 2015; Hamilton and Miller, 2016). In our study, we found hybrids not to be very common, but those found performed well. We also found molecular and phenotypic patterns suggesting backcrossing between hybrids and parental species. While these patterns differed between the two study regions with hybrids, hybridisation did not pose a threat to any of the parental taxa, but may have contributed to genetic diversity.

Global change may affect hybridization in multiple ways. The opportunities for interspecific crosses could increase either directly by distributing species around the world or indirectly by eroding phenological and ecological barriers between species (Brennan *et al.* 2015, Chunco, 2014). Many altered and disturbed habitats are providing new environments available for hybrids (Vallejo-Marin and Hiscock, 2016). In our study regions, we detected hybrids at sites with various disturbances, including drainage, establishment of trails or artificial lakes. While it has been suggested that hybrids may even be more fit than their parental taxa, and especially so in disturbed habitats (Rieseberg, 1995; Campbell and Waser, 2001; Donovan *et al.*, 2010), in our study, hybrid fitness did not exceed the one of the parental taxa.

We observed considerable genetic variation in all taxa, although our evidence for local adaptation was not very pronounced. This suggests that our study taxa should in the long term be able to respond to future selection imposed by changed climates. Phenotypic plasticity has been suggested as an important means to persist under global change (Gienapp *et al.*, 2007; Nicotra *et al.*, 2010). However, the evolution of phenotypic plasticity is constrained by several costs, namely production, maintenance and developmental costs of plasticity (DeWitt *et al.*, 1998). In our study, all taxa survived and generally grew well in the three experimental gardens, suggesting that they might be generally quite robust to climate change. The strong effects of the garden on the phenotypic variation indicate that this was due to pronounced phenotypic plasticity, implying that the plasticity required to perform well in a different region, was not limited by such costs.

4. CONCLUSIONS

The combination of morphological field data, molecular genetic data and transplantation between regions in the study of two closely related hybridizing species allowed us to draw several important conclusions on the factors underlying phenotypic variation in these taxa. These concern the relative importance of phenotypic plasticity, genetic diversity, genetic differentiation, and adaptation, differences between the more rare and the more common taxon, and the role of hybridization.

- In this thesis, we showed that *C. flava* and *C. viridula* are indeed separate taxa, but that hybridization between them leads to some admixture and morphological similarity. Therefore, we suggest in future studies to take into account whether studied taxa are hybridizing and if they are then to include hybrids to the study.
- *Carex flava* with more continuous distribution and larger populations, *C. viridula* with more scattered distribution and smaller populations and hybrid *C. x subviridula* had on average similar levels of genetic diversity, with the exception of very high genetic diversity detected in the alpine environment. The detected similar levels of genetic diversity for rare taxa with small populations and more common taxa with larger populations, are suggesting outcrossing, but also possible delayed loss of genetic diversity.
- Based on morphological and neutral genetic data, *C. flava* was less differentiated among regions than *C. viridula*. We suggest that higher morphological and genetic differentiation observed in *C. viridula* populations is most probably due to effect of genetic drift and fragmentation of populations. Besides, the transplant experiment revealed genetic basis for regional differentiation of *C. viridula*. Hence, we suggest that these genetically different morphs indicate evolutionary activity and development of ecotypes of *C. viridula*. Moreover, our results support the recognition of the specimens of *C. flava* in Highland Switzerland as var. *alpina*.
- Our results of transplant experiment showed significant plasticity, genetic variation and genotype-by-environment interaction on the morphology and performance of the studied taxa. The environment had strongest influence on the phenotypic differences within all three studied taxa. Plant systematic studies need to take all three sources of variation into account. We recommend to work with plant populations from more than one region, as the vast variability plants are able to exhibit is most likely not revealed in small scale studies.
- For all three taxa significantly higher performance of regional plants over foreign ones was found. When we detected the effect in the gardens, we found several indications of maladaptation, especially for *C. viridula*. We can conclude that in our study system, the maladaptation was mainly due to increased genetic drift in its generally smaller populations or to more

pronounced environmental change in its populations. We suggest that the weak signal of local adaptation found in *C. flava* was due to gene flow between populations. We recommend to observe plants in transplantation experiment for several years, as years may be abnormal and the response of plants can change over years. Moreover, in adaptation studies we recommend to detect more factors, which could possibly influence plant adaptations, soil biota, competitors and herbivores.

We hope that this study inspires further studies of regional differentiation, adaptation and phenotypic plasticity of hybridizing pairs of species, ideally of more rare and more common species. This would allow a more general understanding of the role of hybridization for microevolution under global change.

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SUMMARY IN ESTONIAN

Kahe lähedase ja hübriidiseeruva tarnaliigi fenotüübiline ning geneetiline eristumine Eestis ja Šveitsis

Taimed varieeruvad silmapaistvalt morfoloogiliselt, geneetiliselt ning kohastumuste poolest üle terve oma levila. Geograafiline kaugus populatsioonide vahel takistab geenide vahetust. Teisalt mõjutavad erinevates piirkondades esinevaid populatsioone erisugused ökoloogilised tegurid ning keskkonnatingimused. Tulemuseks on liigisisene geograafiline eristumine, mille ulatus sõltub nende faktorite tasakaalust. Oluline roll on ka liigi levila suurusel, populatsioonide arvukusel ning taime elukäigutunnustel. Liigisisene morfoloogiline ja geneetiline eristumine on tugevam isesobivatel ja kлонаalselt paljunevatel liikidel kui ka liikidel kellel on väikesed populatsioonid ning katkendlik levila. Nõrgem eristumine seevastu on laialdase levikuga ja suurte populatsioonidega võrd- tolmlevatel liikidel. Samas kui sellise liigi elupaik on killustunud ja populatsioonid isoleeritud, siis suureneb geneetiline eristumine proportsionaalselt isolatsioonis oldud ajaga. Regionaalset eristumist mõjutab ka liikidevaheline hübriidiseerumine ja hübriidide tagasiristumine (introgression) vanemliikidega. Globaalsed muutused nagu kliimamuutused ja suurenenud inimtegevuse häiringud põhjustavad varasemalt eraldi kasvanud taimeliikide koosesinemist ja soodustavad hübriidiseerumist. Hübriidiseerumine võib põhjustada geenivahetust lähedaste liikide vahel ja seega suurendada nende kohasust lisades adaptiivset geneetilist mitmekesisust. Hübriidid võivad ületada oma kohasuselt vanemliike ja asustada uusi ökoloogilisi nišše, aga põhjustada ka ühe või mõlema vanemliigi väljasuremist. Hübriidiseeruvate liikide uurimine võimaldab mõista hübriidiseerumise mõju liikide evolutsioonile ja pakub mitmetes valdkondades, sealhulgas looduskaitstes vajalikku taksonoomilist informatsiooni.

Taksonoomilised järeldused põhinevad taimede varieeruvuse hinnangutel looduslikes populatsioonides, kuid genotüübi ja keskkonna koosmõju tõttu võivad ühel genotüübil sõltuvalt keskkonnast avalduda erinevad fenotüübid. Ajalooliselt on korduvalt taksonoomilist segadust tekitanud ühe taimeliigi regionaalsete ökotüüpide kirjeldamine eri liikidena.

Käesolev doktoritöö käsitleb kahe lähedalt suguluses oleva *C. flava* L. (kollane tarn), *C. viridula* Michx. var. *viridula* (ojatarn) ja nende oletatava hübriidi (*C. × subviridula* Fernald) fenotüübilist ja geneetilist regionaalset eristumist. Uuringute keskmes olevad kollane tarn ja ojatarn on levinud Euraasias ja Põhja-Ameerikas ning neid on leitud ka Põhja-Aafrikas. Mõlemad tarnad eelistavad niiskeid kasvukohti, kuid kollast tarna leiab sagedamini soisel ja niiskel niidul, kraavikaldal, harvemini leht- ja segametsas. Ojatarn seevastu eelistab regulaarsete häiringutega kasvukohti nagu järve kallas või tee serv. Samas leidub teda ka soos ja siirdesoos, kus konkurents on väiksem. Kollane tarn on üldiselt konkurentsivõimelisem ja võib kasvada ka tiheda taimkattega kasvukohtades, kust ojatarn välja tõrjutakse. Hoolimata vähesest konkurentsivõimest võib ojatarn mulla seemnepangas püsida aastaid ja häiringu korral jälle

kasvama hakata. Sobivates kasvukohtades on kollane tarn sagedasem ja tema populatsioonid on suuremad kui katkendliku levikuga ojatarnal. Inimtegevuse tagajärjel, nagu näiteks raba või soise niidu kuivendamine, võivad ennist eraldi kasvanud liigid kasvukoha häiringu tõttu hakata kasvama samas kasvukohas ja tekib võimalus hübriidiseerumiseks.

Antud doktoritöö peamiseks eesmärgiks on selgitada, kas katkendlikuma levila ja väiksemate populatsioonidega ojatarn on regioonide vahel rohkem eristunud kui laiemalt levinud, suuremate populatsioonidega kollane tarn ning kas laia levikuga liik on paremini kohastunud kui katkendliku levikuga liik. Samas, kui hübriidiseerumine ja tagasiristumine on toimunud ulatuslikult, on võimalik ka alternatiivne tulem, mille järgi mõlemad vanemliigid on regioonide vahel sarnaselt eristunud. Lisaks oli eesmärgiks hinnata hübriidsete isendite evolutsioonilist potentsiaali.

Täitmaks uuringu eesmäärke, korjasime kolmes regioonis: Eestis (kuni 100 m), Šveitsi lauskmaal (kuni 1000 m) ja Šveitsi Alpides (üle 2000 m) 43st looduslikust populatsioonist 572 isendit (genetit), igal genetil vähemalt kolm võsu. Regioonid erinevad teineteisest temperatuuri, sademete hulga ja vegetatsiooniperioodi poolest. Eesti asub mandrilise ja merelise kliima piirmail, kus on võrreldes Šveitsiga mõõdukas temperatuur ning aastane sademete hulk. Mandrilise kliimaga Šveitsi lauskmaal on soojem ja sajusem kui Eestis. Alpiinse kliimaga Šveitsi Alpides on vegetatsiooniperiood lühem, aastane temperatuur madalam ja sademeid rohkem kui kahes teises regioonis. Kõigil 572 isendil mõõdeti 14 morfoloogilist vegetatiivset ja reproduktiivset tunnust ning uuriti geneetilist varieeruvust (10 mikrosatelliitide nukleotiidsel järjestust). Hindamiseks, kas liigisiseseid ja liikidevahelised morfoloogilised erinevused on mõjutatud keskkonna või geenide poolt, teostasime vastastikuse (retsiprookse) ümberistutuskatse. Kolmest eelnevalt kirjeldatud regioonist kogutud 572 geneti kolm võsu istutasime kolme katseaeda (üks aed igas regioonis). Istutatud võsud kasvasid kaks aastat ja kahel järjestikusel suvel mõõdeti neil 11 morfoloogilist ja 7 kohasuse tunnust.

Hübriidiseerumine koos sellele järgneva tagasiristumisega võib viia teatavas ulatuses genoomi segunemiseni (*genome admixture*), kuid eri regioonides tekkinud samade vanemate vahelised hübriidid võivad areneda paralleelselt. Kõiki uuritud hübriide iseloomustas tugev kasv ning steriilsed kahvatukollased põisikud. Analüüside tulemused näitasid, et nii looduslikes populatsioonides kui ka ühisaedades erinesid kollane tarn ja ojatarn üksteisest, vastavalt 71.4% ja 60% morfoloogiliste tunnuste poolest. Taksonoomiliselt kõige olulisemad olid reproduktiivsed, õisiku mõõtmetega seotud tunnused. Morfoloogilised tunnused eristasid selgesti liike regiooni sees ja regioonide vahel. Loetletud kromosoomide arvud ja geneetilised markerid kinnitasid kahe taksoni eristumist, kuid näitasid ka aneuploidsust ja geneetilist segunemist, mis viitab tagasiristumisele aga ka sellele, et geneetiliselt ei ole kollane tarn ja ojatarn üksteisest täielikult eristunud. Lisaks viitasid ümberistutuskatse tulemused sellele, et mõlema uuritud taksoni morfoloogiline vastus sõltus päritolu regioonist, viidates paralleelsele evolutsioonile ja regionaalsele sidususele (*regional connectedness*).

Kuigi kolme uuritud taksoni, kollase tarna, ojatarna ja hübriidi, isendid olid pärit kolmest geograafiliselt ja kliimatiliselt erinevast regioonist, oli nende geneetiline varieeruvus küllaltki sarnane. Kõige suurema geneetilise mitmekesisuse polümorfsete lookuste protsent oli kollase tarna populatsioonides Šveitsi alpides, mis viitab võimalikule mutatsioonide kõrgele tasemele intensiivse kiirguse tingimustes. Kõige väiksema mitmekesisuse leidsime kollase tarna populatsioonides Eestis ja ojatarna populatsioonides Šveitsi alpides. Vaatamata sellele, et ojatarn on Šveitsis potentsiaalselt ohustatud liik ja tema elupaigad fragmenteerunud, on meie tulemuste kohaselt tema liigisisene geneetiline mitmekesisus kollase tarnaga samal tasemel. See annab alust oletada, et ojatarnal esineb geneetilise mitmekesisuse kadumise võlg: liigi levila ja arvukus on juba vähenenud ja geneetilise mitmekesisuse kahanemine seisab veel ees.

Liigisisese fenotüübilise ja geneetilise varieeruvuse ulatus ja jaotumus erinevates regioonides oli kollasel tarnal ja ojatarnal erinev. Võrreldes kollase tarnaga olid ojatarna populatsioonid regioonide vahel tugevamalt eristunud. Tulemus on kooskõlas teoreetilise hüpoteesiga, mille kohaselt katkendlikuma levikuga ja väiksemate populatsioonidega liigid näitavad suuremat regionaalset eristumist. Meie tulemused toetavad kollase tarna alpiinse vormi määratlemist omaette varieteedina *C. flava* var. *alpina*. Tuvastasime, et liikidevaheline hübriidiseerumine ei ole nii ulatuslik, et takistada ojatarna regionaalset eristumist.

Kolmes katseaias läbi viidud ümberistutuskatse näitas, et keskkond mõjutab tugevalt fenotüübi kujunemist nii kollasel tarnal, ojatarnal kui ka hübriidil. Tulemused näitasid ka regionaalsete keskkonnatingimustega kohastumust. Kõigis katseaedades tuvastasime kohasemaid samast regioonist pärinevad kohalikke populatsioone võrreldes võõrpopulatsioonidega: neil oli kas rohkem reproduktiivseid võsusid ja põisikuid või suurem üldine kasv ja ellujäämine. Erandina leidsime, mõndades aedades võõrpopulatsioonide kõrgemat kohasust, eriti ojatarnal, mis väljendus suuremas üldises kasvus ja paremas ellujäämises. Ümberistutuskatse tulemused näitasid, et kohastumuse uuringutes peaks ilmastikuolude kõrval arvestama ka bioloogiliste teguritega.

Meie kombineeritud uuring morfoloogilisest ja geneetilisest varieeruvusest looduslikes populatsioonides ning vastastikune ümberistutuskatse võimaldas teha uuritud taksonite morfoloogilise varieeruvuse kohta mitmeid olulisi järeldusi. Meie tulemused kinnitavad, et olla eraldi liigid on kollane tarn ja ojatarn on küllalt eristunud, kuid nendevaheline hübriidiseerumine ja tagasiristumine on põhjuseks teatud morfoloogilisele sarnasusele. Meie uuring näitas, et nii populatsioonigeneetilistes kui ökoloogilistes uuringutes lähedaste liikidega on oluline arvestada hübriidiseerumise võimalusega.

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Conference presentations:

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Schmidt, L., Oja, T. Allozyme diversity and morphological variation in the *Carex flava* L. complex (Cyperaceae). Plant Population Biology Conference, 16–18 May 2012, Zurich, Switzerland. *Poster presentation*

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„Scientific writing workshop“. 16–17 July, 1–2 September, 2016, University of Bern, Switzerland

„Data wrangling and plotting with R“. 4th march, 2016, University of Bern, Switzerland

„Career Compass Workshop: Design your career path by identifying and connecting to what matters to you“. 26th February and 1 April, 2016, University of Lausanne, Switzerland

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2017 Life Science career Day 2017. University of Lausanne, Switzerland

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