Evolutionary potential in functional traits of a wetland macrophyte (*Juncus effusus*) relevant for natural degradation of contaminants

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SUMMARY

Nutrients such as nitrogen and phosphorus are natural components of aquatic ecosystems and essential for plant growth. However, if progressive intensification of agriculture and urbanization cause too many nutrients to enter the ecosystem, then air and water can be polluted. Wetland ecosystems provide the important ecosystem service of natural degradation and retention of such excessive substances. The remediation potential of wetland plants and associated microorganisms has increased interest in the rehabilitation of lost and degraded wetlands. In addition, wetland constructions have frequently been used as cost-effective and sustainable biotechnologies to compensate for the loss of wetlands in the last century. Plant traits can have a direct or indirect impact on wetland ecosystems through accumulation, metabolism and microbial transformation processes in the soil. The influence of plants' functional traits and their interaction with the soil microbiome in the reduction of wetland pollutants have been well investigated with respect to the among-species variability. However, the extent of the intraspecific variability of these functional traits may be as important as interspecific variability in relation to ecosystem functions. Understanding the causes and consequences of intraspecific genetic variation related to nutrient degradation processes is fundamental for predicting evolutionary responses, but it can also aid applied approaches in wetland restoration and construction.

Applying a combination of quantitative and molecular genetics, the aim of the thesis is to quantify intraspecific variability in functional traits that are potentially relevant for the degradation of nitrogen compounds, and to investigate its genetic basis and response to selection. In our research, we used the wetland plant *Juncus effusus*, which has been established as a model plant in basic and applied research on wetland ecosystems. To understand whether and how quantitative genetic variation is expressed depending on nitrogen availability and how it is structured within and among natural European populations, we used common garden studies to evaluate and compare trait variation across multiple provenances, lineages and treatments. Microsatellites were used to study genetic diversity and population

structure to investigate the different environmental forces and their relative contributions to adaptation. Then we investigated possible relationships between genetic diversity and environmental factors such as climate and soil conditions. Furthermore, we asked whether trait expression shows signatures of local adaptation by comparing quantitative genetic trait divergence and neutral molecular markers (Q_{ST} – F_{ST} comparisons) and relating trait variation to soil conditions of the plant's origin.

We quantified a strong plastic behavior in response to nitrogen availability in all measured traits. The genetic variation of functional mean traits was strongly pronounced among populations, but very low at population level. We could not find a significant link between nitrogen availability and genetic trait expression. Differentiation of quantitative traits was substantial but did not exceed neutral expectations, as detected in Q_{ST} – F_{ST} comparisons. However, adaptive trait divergence in response to environmental soil conditions might be present, which is indicated by significant trait clines. Molecular analyses showed moderate genetic diversity and very strong genetic differentiation among populations. These findings confirmed our hypothesis, which is based on the life history of species *Juncus effusus*, as a successful colonizer with a predominantly selfing mating system. A Bayesian analysis of population structure across the European range showed that *J. effusus* comprises three genetically well-differentiated lineages that partly separate longitudinally but also co-occur sympatrically, probably as a result of glacial lineage diversification and secondary contact. The lineage Eff3 differed substantially from the other lineages by clines in population genetic diversity with environmental conditions and by a dominant occurrence at higher altitudes suggesting more particular ecological responses compared to lineages Eff1 and Eff2. In summary, however, we found only little and inconsistent evidence of selective mechanisms varying between the lineages, which supports the idea that lineages within *J. effusus* are the primary results of neutral divergence.

ZUSAMMENFASSUNG

Nährstoffe wie Stickstoff und Phosphor sind natürliche Bestandteile aquatischer Ökosysteme und für das Pflanzenwachstum unerlässlich. Wenn jedoch zu viele Nährstoffe in das Ökosystem gelangen, können Luft und Wasser verschmutzt werden. Diese Verschmutzung wird hauptsächlich durch die fortschreitende Intensivierung der Landwirtschaft und Urbanisierung verursacht. Feuchtgebietsökosysteme leisten einen wichtigen Beitrag durch natürlichen Abbau und Rückhaltung solch überschüssiger Nährstoffe. Das Sanierungspotential von Feuchtgebietspflanzen und den im Boden lebenden Mikroorganismen hat das Interesse an der Sanierung von verloren gegangenen und degradierten Feuchtgebieten erhöht. Darüber hinaus werden künstlich angelegte Feuchtgebietsanlagen häufig als kostengünstige und nachhaltige Biotechnologie eingesetzt, um den Verlust von natürlichen Feuchtgebieten auszugleichen. Pflanzeneigenschaften können die Ökosystemprozesse von Feuchtgebieten direkt oder indirekt durch Anreicherung, Metabolisierung und durch Beeinflussung mikrobieller Umwandlungsprozesse im Boden beeinflussen. In der Vergangenheit wurde die Variabilität von funktionellen Merkmalen, die für den Abbau von Schadstoffen in Feuchtgebietsökosystemen relevant sind, vor allem auf Ebene der gesamten Pflanzengesellschaft betrachtet. Allerdings kann das Ausmaß der intraspezifischen Variabilität für diese funktionellen Merkmale ebenso wichtig sein wie die interspezifische Variabilität in Bezug auf die Ökosystemfunktion. Das Verständnis für die Ursachen und Folgen intraspezifischer genetischer Variationen im Zusammenhang mit Abbauprozessen von Nährstoffen ist grundlegend für die Vorhersage evolutionärer Reaktionen, kann aber auch bei der Wiederherstellung und Konstruktion von Feuchtgebieten nützlich sein.

Ziel des Projektes ist die Quantifizierung der intraspezifischen Variabilität von funktionellen Merkmalen, die für den Abbau von Stickstoffverbindungen relevant sind, um deren genetische Basis und die Antwort auf die Selektion zu untersuchen. In unserer Studie haben wir die Feuchtgebietspflanze *Juncus effusus* verwendet, die sich als Modellpflanze in der Grundlagen-

und angewandten Forschung zu Feuchtgebietsökosystemen etabliert hat. Um zu verstehen, ob und wie quantitative genetische Variation in Abhängigkeit von der Stickstoffverfügbarkeit ausgeprägt wird und wie sie innerhalb und zwischen natürlichen europäischen Populationen strukturiert ist, haben wir ein *common garden* Experiment durchgeführt, um die Merkmalsvariation über mehrere Herkünfte, Linien und Behandlungsmethoden hinweg zu bewerten und zu vergleichen. Mit Hilfe von Mikrosatelliten wurden die genetische Vielfalt und die Populationsstruktur untersucht, um die verschiedenen evolutionären Kräfte und ihre relativen Auswirkungen auf die Adaptation zu bestimmen. Zusätzlich untersuchten wir mögliche Zusammenhänge zwischen genetischer Vielfalt und Umweltfaktoren wie Klima- und Bodenbedingungen. Darüber hinaus haben wir, durch den Vergleich der genetischen Differenzierung in quantitativen Merkmalen und neutralen molekularen Markern (Q_{ST} – F_{ST} Vergleiche) überprüft, ob die Merkmalsausprägung Signaturen der lokalen Anpassung aufweist und ob die Merkmalsvariation mit den Bodenverhältnissen der Pflanzenherkunft in Beziehung steht.

Wir quantifizierten ein starkes plastisches Verhalten als Reaktion auf die Stickstoffverfügbarkeit in allen gemessenen Merkmalen. Die genetische Variation der funktionellen Merkmale war zwischen den Populationen stark ausgeprägt, aber auf Populationsebene sehr gering. Es konnte kein signifikanter Zusammenhang zwischen der Verfügbarkeit von Stickstoff und der Expression genetischer Merkmale gefunden werden. Die Differenzierung der quantitativen Merkmale war beträchtlich, übertraf aber nicht die neutralen Erwartungen, wie mittels Q_{ST} – F_{ST} Vergleichen festgestellt wurde. Allerdings kann eine adaptive Merkmalsdivergenz als Antwort auf die Umweltbedingungen im Boden nicht ausgeschlossen werden, was durch signifikante Merkmalsgefälle in Abhängigkeit von Umweltfaktoren bestätigt wurde. Molekulare Analysen zeigten eine moderate genetische Vielfalt und eine sehr starke genetische Differenzierung zwischen den Populationen. Diese Ergebnisse bestätigten unsere Hypothese, die auf der Lebensgeschichte (*life history*) der Art *Juncus effusus*, als erfolgreicher Kolonisator mit einer überwiegenden Selbstbefruchtung, beruht. Eine Bayes'sche Analyse der Populationsstrukturierung im europäischen Raum zeigte,

dass *J. effusus* aus drei genetisch gut differenzierten Linien besteht, die sich teilweise entlang der geografischen Länge trennen, aber auch sympatrisch auftreten. Dies lässt eine genetische Diversifikation der Linien als Folge glazialer Isolationsmechanismen im quartären Eiszeitalter mit einem anschließenden sekundären Kontakt vermuten. Die Linie Eff3 unterschied sich wesentlich von den anderen Linien durch einen signifikanten Zusammenhang zwischen genetischer Diversität und Umweltbedingungen. Kombiniert mit einem dominanten Vorkommen in größeren Höhenlagen legt das eine spezifischere ökologische Reaktion der Linie Eff3 im Vergleich zu Eff1 und Eff2 nahe. Zusammenfassend haben wir jedoch nur wenige und inkonsistente Beweise auf selektive Mechanismen gefunden, die zwischen den Linien variieren. Dies unterstützt die Idee, dass die Linien innerhalb von *J. effusus* die primären Ergebnisse einer neutralen Divergenz sind.

CHAPTER 1

General Introduction

CHAPTER 1: General Introduction

Wetlands and their ecological relevance

Wetland ecosystems, including rivers, lakes, marshes and coastal areas, provide ecosystem services that are indispensable for human well-being. Besides the obvious benefit of strong cultural and spiritual aspects, wetlands contribute to economic production, flood control, sediment retention, carbon sequestration and water purification (Millennium Ecosystem Assessment 2005). Despite the significant importance of wetlands, they are one of the most threatened ecosystems worldwide (Daryadel & Talaei 2014). More than 50% of the world's wetlands have been lost over the last 200 years, and up to 90% of wetlands in parts of Europe have disappeared (Clarkson *et al.* 2014). The main drivers of wetland degradation and loss are ongoing urbanization, progressive agricultural intensification and industrial activities resulting in land conversion, water withdrawal, overexploitation, invasion of exotic species and the immense enrichment of nutrients and pollutions (Maltby 2009; Asselen *et al.* 2013).

The water purification capacity of wetlands, in particular, has for long been recognized as a valuable ecosystem service. Many studies have reported that wetlands play an important long-term role in removing nutrients and other pollutions from the environment (Woltemade 2000; Fisher & Acreman 2004; Rendón-López *et al.* 2016). They significantly improve water quality, enhancing the sustainability of food supplies, biodiversity and human well-being. These findings have increased the interest in rehabilitating lost and degraded wetlands and returning them to their 'near-natural' state (van der Valk 2009). Apart from this, constructed wetlands as artificial systems are created to imitate natural wetland conditions (Gokalp & Karaman 2017). Most of them are designed to utilize the natural processes of wetland vegetation and associated microorganisms for wastewater treatment, but also to provide habitat and aesthetic values (Kadlec *et al.* 2000). Compared to conventional wastewater treatments, constructed wetlands are more cost-effective, simple to construct and operate, and tolerate fluctuations in flow (Haarstad *et al.* 2011). To summarize, constructed wetlands provide sustainable restoration approaches linking social and economic development with environmental

protection (Millennium Ecosystem Assessment 2005). However, whereas the understanding of remediation and purification processes has been advanced by numerous studies on whole wetland plant communities, within-species variability, with respect to this ecosystem function, is largely neglected by fundamental research. This study helps to understand how intraspecific genetic variability affects community-based regulating ecosystem services by analysing the genetics of functional traits related to nutrient degradation processes in the representative wetland plant *Juncus effusus*. This plant has been established as a model plant in basic and applied research on wetland ecosystems and is ecologically well characterized by numerous studies, but potentially related intraspecific trait variability has not yet been described.

The nitrogen cycle: processes, agents and human influence

One of the most human-induced sources of nutrients in the environment are point sources that enter via industrial wastewater effluent and diffuse sources from agricultural run-off (García-García *et al.* 2009). Nutrients like nitrogen (N) are essential for plant growth as they are an important component of chlorophyll, nucleic acids, enzymes, amino acids and proteins. An increased application of nitrogen in agriculture maximises crop production, but the cheap production of fertilizers, thanks to the Haber-Bosch process, have led to an excessive use of nitrogen in agriculture (Erisman *et al.* 2011). Excessive nitrogen concentrations can result in eutrophication characterized by algae blooms, light limitation and oxygen depletion (Smith *et al.* 1999) which can entail considerable risks for biodiversity (Stevens *et al.* 2004). Nitrogen is a very mobile element and can enter surface waters and groundwater through the soil. In landscapes with riparian buffer zones, wetland plants and associated microorganisms in their rhizosphere have an immense potential for regulating nitrogen concentrations via accumulation and metabolization (Mander *et al.* 2005; Dhir *et al.* 2009). They, therefore, have the ability to remove nitrogen from the environment before the contaminated water enters a stream (Verhoeven *et al.* 2006). If wetland vegetation is herbaceous, then assimilated nitrogen by plants can be completely eliminated from the environment by uprooting the plants (Mander *et al.* 2005; Verhoeven *et al.* 2006). Two ecosystem processes are described as primarily

relevant for nitrogen removal in wetlands – nitrogen uptake and denitrification (Sutton-Grier *et al.* 2013). Plants take up nitrogen as ammonium (NH_4^+) and nitrate (NO_3^-), which lead to an extensive production of organic matter and incorporation into biomass (White & Reddy 2009). The organic N in wetland soil accounts for more than 95% of the total soil N and can be converted to NH_4^+ by microorganism mineralization (White & Reddy 2009). Products of microbial activity can in turn be absorbed and utilized by plants. The forms of inorganic N are affected by redox potential and the pH of the soil and are controlled by processes of the microbial community (Rydin & Jeglum 2006; Husson 2013). Under oxygen conditions, NH_4^+ can be converted to nitrite (NO_2^-), mediated by the bacteria of genus *Nitrosomonas*, and then to NO_3^-, mediated by *Nitrobacter*. When nitrate diffuses in anaerobic zones, it can be reduced stepwise to nitrogen gas (N_2) or nitrous oxide (N_2O) (van der Valk 2006). This denitrification process is the main process of permanent N removal in wetlands (Mayo & Bigambo 2005), resulting from the low redox potential and high carbon (C) content found in most wetlands (Rydin & Jeglum 2006). These processes require a close interaction between plants and their rhizosphere inhabiting microorganisms. Plant species can differ in important traits, which may affect belowground organisms; for example, wetland plants can differ in their oxygen release in the rhizosphere via aerenchyma (Armstrong 1967), which in turn can affect the redox potential of the soil (Blossfeld *et al.* 2011) and microbial communities both supporting mineralization and nitrification processes (Lai *et al.* 2011). Thus, an understanding of these interactions is essential for defining plant functional traits in relation to the degradation of contaminants.

Plant functional traits and trait variability

Understanding the role of biodiversity in ecosystem functioning is of great interest for human well-being and in the face of global change it is linked to the supply of goods and services (Díaz *et al.* 2006; Cardinale *et al.* 2012). Biodiversity is mainly explored at three levels, from diversity of genes to variety of species and the ecosystems they form. Evidence over the last two decades shows that biodiversity at any biological level affects ecological processes and

thus ecosystem functioning (Cardinale *et al.* 2012). In this sense, the focus should not only be on taxonomic identify of species, but also on the diversity and abundance of functional traits within communities to predict ecosystem functioning (Gagic *et al.* 2015; Laureto *et al.* 2015; Gross *et al.* 2017). In general, plant communities with a high functional trait variability show greater productivity than communities with reduced trait variation because of less niche overlap and a maximum, efficient use of resources (Cadotte *et al.* 2009). Such effects of species diversity are mainly mediated via complementarity and sampling effects, promoting community stability and productivity (Hooper *et al.* 2005). Firstly, complementarity is the result of niche partitioning and differences in resource requirements, leading to an increased efficiency of resource use by the community and therefore more productivity (Tilman *et al.* 1997; Roscher *et al.* 2008). For example, efficient nitrogen uptake was observed in species communities with different timing and depths of resource uptake, which promote coexistence through reduced competition (McKane *et al.* 1990). Complementarity effects also comprise the facilitation effects mediated by species that modify the environment and favour the co-occurrence of other species (e.g. nitrogen enrichment by nitrogen-fixing legumes) (Spehn *et al.* 2002). Secondly, the sampling effect states that highly competitive species with inherently high productivity are more likely to be present in species-rich communities (Huston 1997).

Wetland plants are directly involved in the natural degradation of contaminants and their interaction with microorganisms is fundamental for all processes involved. Evidence of significant differences among species in terms of functional traits, relevant for the degradation of contaminants, is comprehensive and has been described e.g. for efficiency of nutrient remediation (Smialek *et al.* 2006; Borin & Salvato 2012; Menon & Holland 2013), root traits such as length, longevity and porosity (Lai *et al.* 2011) and organic matter decomposition (Gingerich & Anderson 2011). However, an often-overlooked part of plant diversity, functional traits can also be highly variable within species that affect community structures and ecosystem processes (Siefert *et al.* 2015). This is surprising as intraspecific variability can be as important as interspecific variability. For example, genetic diversity has a positive effect on plant productivity (Crutsinger *et al.* 2006), decomposition rate (Silfver *et al.* 2007), nitrogen

cycling (Schweitzer *et al.* 2005) and ecosystem resilience (Reusch *et al.* 2005). As in the case of studies of interspecific variability, effects of within-species diversity might be caused by the presence of highly productive genotypes in polycultures (i.e. sampling effect) or by a more efficient use of resources in mixed genotypes or facilitation among interacting genotypes (i.e. complementarity effect) (e.g. Crutsinger *et al.* 2006; Cook-Patton *et al.* 2011; Ellers *et al.* 2011; Castagneyrol *et al.* 2012; Schöb *et al.* 2015). However, there are very few studies that investigate intraspecific variability in traits relevant to the degradation of contaminants in natural populations. Further evidence for the importance of intraspecific variability can be found in crop science. For some crop species it has been shown that nitrogen use efficiency, i.e. grain yield per unit of available nitrogen in the soil, and related traits like root architecture are indeed genetically variable (Hirel *et al.* 2007). It can therefore be assumed that different provenances, populations and genotypes also differ within non-crop species in terms of their genetic remediation potential. Genetic variation in the phenotypic expression of quantitative traits is the premise for adaptive differentiation in response to selection imposed by environmental conditions. Thus, if we want to understand whether and how traits evolve, we need to know whether trait variation is heritable and whether is selection on trait states.

Evolutionary and ecological genetics

The two cornerstones of modern biology are Darwinian evolution and Mendelian genetics, which were combined into a unified theory of evolution in the 1930s ("Modern Synthesis," Huxley 1943). The modern synthesis entailed some changes in the concept of evolution and evolutionary processes. It defined evolution as the process of “change in allele frequency within populations,” emphasizing the genetic basis of evolutionary change. Four fundamental forces of evolution are known -selection, mutation, genetic drift and gene flow- allowing for changes in allele frequency. A main source of novel genetic variants is mutation via nucleotide alterations in chromosomes. A large part of mutations is deleterious and will be eliminated from a population by selection or kept at very low frequencies in populations (Silvertown & Charlesworth 2001; Charlesworth 2012). However, if the environment changes, a new mutant

allele may be favoured and becomes more abundant in a population over time (Barton 2010). In general, the formation of new species due to chromosomal mutations is rare, but fixation can be increased by the reproduction system, e.g. in selfing species due to small effective population size (Nei & Nozawa 2011). Natural selection is a key mechanism of evolution and favours organisms that contain certain genotypes that allow them to adapt by successfully reproducing under specific environmental conditions (Frankham *et al.* 2002). Consequently, selection occurs if the fitness of genotypes differs, which leads to a phenotypic change, depending on which phenotype is favoured by selection. There are three basic forms of selection: (1) directional selection, which favours one extreme of the phenotype distribution initiated by selection against one extreme of trait expression, (2) disruptive selection, which favours both extreme phenotypes by selection against the population mean and (3) stabilizing selection, which favours phenotypic intermediates caused by selective pressure against two extreme types of a phenotypic trait (Nielsen 2005). Consequently, selection can increase or decrease genetic diversity depending on the direction of the selective pressure. However, most changes in allele frequency do not provide fitness advantages to organisms carrying them (Nielsen 2005). The processes by which allele frequencies fluctuate randomly over the generations are known as genetic drift and gene flow. Genetic drift causes shifts in allele frequency by randomly transmitting alleles from generation to generation (Frankham *et al.* 2002). Over time, populations tend to differ in their allele frequency because of an independent occurrence of genetic drift in different isolated populations, and within populations alleles can become fixed and thus reduce genetic diversity particularly within small populations (Silvertown & Charlesworth 2001). However, new alleles can be introduced into a population by mutation or, probably more often, by gene flow. This introduction is caused by gene migration between populations (Slatkin 1985). Gene flow between populations causes homogenization of genetic material and is therefore an antagonist of genetic drift. However, population divergence can occur despite gene flow if selection is strong enough (Latta 2004). Conversely, if gene flow overcomes the strength of selection, the evolution of local adaptation can be prevented by the movement of genes between populations (Ellstrand 2014). These

interactions show that population structuring is very complex in its development. It is therefore important to distinguish between neutral and adaptive processes (Holderegger *et al.* 2006). Adaptive genetic variation has an effect on fitness and thus allows for the assessment of a population or species' evolutionary potential. In contrast, neutral genetic variation has almost no direct effects on fitness and is therefore considered to be selectively neutral (Holderegger *et al.* 2006). However, selection can also affect neutral genetic diversity by a "selective sweep" or "background selection," if a neutral locus is physically located near the target of selection (Lohmueller *et al.* 2011). Consequently, there are several main mechanisms that can influence evolutionary change within populations and affect the level of genetic diversity. The modern synthesis of evolutionary theory provides empirical and theoretical foundations for studying genetic diversity, including discrete allelic states and derived quantitative characters, which lead to different metrics of genetic diversity such as genetic variance and heritability (Fisher 1930; Wright 1968; Hughes *et al.* 2008).

Quantitative genetic diversity in functional traits

Quantitative genetics is the study of the inheritance of quantitative phenotypic traits which typically show a continuous distribution that is influenced by many genetic loci with very small allelic effects, but with strong environmental effects on a phenotype (Frankham *et al.* 2002). These phenotypes are more commonly found in nature than phenotypic differences studied by Mendel, which fall into a number of discrete phenotypic classes. Inherited variation provides the raw material of evolution but the expressed phenotype of a species depends on both genetics and the environment (Falconer 1989). Thus, the total phenotypic variation (V_P) is the result of environmental (V_E) and genetic causes (V_G) and can be summarized as follows (Falconer 1989):

$$V_P = V_G + V_E + 2V_{GE}\,,$$

where V_{GE} is the covariance among genetic and environmental effects, which means that different genotypes may differ in the same environment.

The genetic variance itself can be portioned as follows:

$$V_G = V_A + V_D + V_I \,,$$

where V_A is the additive genetic variance or breeding value, V_D is the dominance variance and V_I is the interaction variance (Falconer 1989). The latter two are jointly described as non-additive genetic variances because particular traits are produced by the interaction of alleles at the same locus and different loci, respectively (Lynch & Walsh 1998). The additive effect that genes have on a phenotype is particularly important because it causes the resemblance between parents and offspring, and thus determines responses to selection (Silvertown & Charlesworth 2001). To determine how much of phenotypic variation is under genetic control, reflecting the adaptive evolutionary potential of the population, the broad-sense heritability of a trait can be measured as a ratio of genetic variance to phenotypic variance (Lynch & Walsh 1998).

$$H^2 = \frac{V_G}{V_P}$$

Here, the V_G can be estimated in clonal or selfing species, assuring that progeny will have the same genotype as their parent. However, it is also a useful tool for sexual species because highly inbred lines can grow by inbreeding several generations (Silvertown & Charlesworth 2001). The heritability of quantitative traits can be quantified in uniform common garden studies by comparing trait variation in individuals from different populations and genotypes under similar environmental conditions, the purpose of which is to minimize environmental effects (Frankham *et al.* 2002). However, the expressed genetic contributions to trait variance may vary across different environments (Pamilo 1988), limiting the conclusions that can be drawn from a single common garden environment alone. Simulating different environmental conditions in a common garden experiment allows for studying phenotypic plasticity and genotype-by-environment interactions and hence provides a general understanding of the observed trait variation and differentiation. Phenotypic traits can be influenced by environmental conditions, such that the same genotype can express different phenotypes in different environments. These responses are known as phenotypic plasticity and are often

quantified and illustrated as reaction norms (Pigliucci *et al.* 2006). If this plasticity varies between genotypes in direction or magnitude in different environments, it is classified as genotype-by-environment interaction (Falconer 1989). Thus, plants are able to adapt to environmental changes due to their genetic architecture and/or phenotypic plastic responses.

DNA-based marker systems to assess genetic diversity

The evolutionary potential of a trait depends on how much it varies genetically (Munday *et al.* 2013). Probably the most common way to assess genetic diversity in natural populations is the use of molecular markers. Of the different kinds of molecular markers (Mondini *et al.* 2009), microsatellites have become the preferred marker for population genetics and genome mapping (Schlötterer 2000). Microsatellite sequences are short, tandem repeats of two to six base pairs that are highly polymorphic and randomly distributed throughout the genome (Gupta *et al.* 1996). Variations in the number of base repeats is mainly due to intra-allelic polymerase slippage during DNA replication (Ellegren 2004). These variations can be amplified by applying the standard polymerase chain reaction (PCR) technique using fluorescent primer sequences from the flanking regions, and products are resolved in an automatic sequencer (Schlötterer 2000). Microsatellites mutate very rapidly at a mutation rate of 10^{-2} to 10^{-6} per locus, per replication (Schlötterer 2000). This high level of polymorphism makes them suitable for identifying individuals or particular strains of plants (Silvertown & Charlesworth 2001). They are ideal markers to disentangle fine-scale population structuring, which probably reflects relatively recent population genetic events (but see Karl *et al.* 2012). Microsatellites are generally considered to be neutral, but some recent studies have found a close link between them and genes which are under selection (Ellegren 2004; Nielsen *et al.* 2006). The next important feature of microsatellite markers is that they are codominant, which means that heterozygotes can be distinguished from homozygotes, enabling the calculation of allele frequencies (Freeland *et al.* 2011). The calculation of allele frequencies in turn serves as the basis for further, numerous analytical methods in population genetics (Avise 1994). Based on all these facts, microsatellites are ideal for studying population structuring, as they allow us to

understand the role of different evolutionary forces that determine the genetic structure of populations.

Evolutionary potential, genetic diversity and local adaptation

In order to predict the evolutionary potential of species under environmental change, it is necessary to consider the basic principles of selection. Natural selection cannot evoke evolutionary responses without genetic variation. Ultimately, if traits have variation that is heritable, genetic variation presents the adaptive potential of populations (Fisher 1930). Thus, to understand the expression of traits we need to know both genetics and selection. As discussed earlier, population structure can be the result of random, neutral processes and of selection, whose interaction provides important insights into the level of local adaptation and is therefore of fundamental importance in evolutionary and conservation biology (Latta 2008). Local adaptation is expected if selective forces affect phenotypes in locally distinct environmental conditions and overcome the action of other evolutionary forces like gene flow (Blanquart *et al.* 2012). Local adaptation in response to environmental gradients seems to be common in plant species (Leimu & Fischer 2008); it can be found on small scales (Lenssen *et al.* 2004) and even within species (Knight & Miller 2004). Several approaches have been developed to assess the adaptive divergence of populations. Early indications of local adaptation as a driving force for phenotypic variation were obtained by correlating mean phenotypic trait expression with ecologically relevant environmental variables (e.g. Huxley 1938). This qualitative approach is still used to analyse trait variation among and within species along environmental gradients (Hulshof *et al.* 2013; Cochrane *et al.* 2016). Such a correlation between phenotype and environment can be reflected in the genetic structure of populations. Allele frequencies can differ among populations from different habitats as a result of natural selection's producing locally adapted genotypes across environmental gradients (Schoville *et al.* 2012). If natural selection against maladapted immigrants from different environments impedes gene flow among populations, populations become more and more isolated with increasing ecological distance, leading to an isolation-by-environment pattern (IBE; Wang &

Bradburd 2014). The gradients driving local adaptation in plants are primarily climate (Hancock *et al.* 2011; Yoder *et al.* 2014) and altitudinal differences (Shi *et al.* 2011a; Manel *et al.* 2012), and soil properties, which have rarely been investigated to date (Smith *et al.* 2012). However, gene flow can also be limited by physical barriers and geographical distances, causing a similar population structure as that under IBE (Sexton *et al.* 2014). The combination of genetic drift and limited gene flow can lead to a pattern in which the genetic differentiation among populations rises with increasing spatial distance, a pattern described by Wright (1943) as isolation-by-distance (IBD). Consequently, it is important to disentangle IBE and IBD patterns to provide evidence for genetic differentiation driven by environmental conditions that are independent from neutral patterns of genetic drift (Nadeau *et al.* 2016). The interpretation of putative adaptive mechanisms is also complicated by phenotypic plasticity, which can accelerate environmental responses (Fitzpatrick 2012). The next most powerful approach to disentangle adaptive from neutral phenotypic divergence is the comparison of quantitative genetic (Q_{ST}) and neutral marker (F_{ST}) differentiation among natural populations. Genetic differentiation in quantitative traits among populations was estimated as Q_{ST}, a quantitative genetic analogue of Wright's F_{ST}, giving a standardized measure of population structure in a genetic locus (Whitlock & Guillaume 2009). Lande (1992) showed that the value of Q_{ST} for a neutral quantitative trait is the same as the F_{ST} value for neutral genetic loci. In principle, the Q_{ST} of a trait can be compared with the F_{ST} calculated from a set of selectively neutral loci in expressing the impact of adaptive processes on genetically based phenotypic trait variation among populations (Miller *et al.* 2008). If genetic differentiation in quantitative traits (Q_{ST}) is greater than neutral genetic differentiation (F_{ST}), trait divergence is interpreted as a signature of directional selection, whereas in the opposite case, i.e. $Q_{ST} < F_{ST}$ indicates stabilizing selection. If Q_{ST} are not significantly different to the F_{ST} values, then trait divergence among populations could have been achieved by neutral processes alone (Whitlock 2008). A growing number of studies followed this approach to find evidence of adaptation to local environments in a wide range of organisms (meta-analysis: Leinonen *et al.* 2008).

In summary, quantitative genetics is a tool to estimate the heritable variation of phenotypic traits that are most relevant for adaptive evolution. However, quantitative genetics cannot provide information about underlying genetic structures in certain traits of interest as molecular genetic methods can (Munday *et al.* 2013). Consequently, a combination of molecular and quantitative data allows for a deeper understanding of processes controlling adaptation to changing environmental conditions (Munday *et al.* 2013).

Juncus effusus – a representative wetland plant

Juncus L. is the largest genus in monocotyledonous family *Juncaceae*, which contains more than 300 species. Most *Juncus* species prefer wet soil conditions and are dominant in temperate wetland ecosystems worldwide (Buchenau 1892; Kirschner *et al.* 2002). One of the best studied species of the genus is *Juncus effusus*. *Juncus effusus* commonly known as “soft rush” or “common rush” has a broad native range, occurring in Europe and in large parts of Asia, Africa and America. It is known to vary greatly in morphological traits across its worldwide distributional range, leading to a description of many subspecies (Wu & Clemants 2000; Kirschner *et al.* 2002; USDA-NRCS 2017) with controversial intraspecific taxa (Zika 2003). It is often found growing as dense tufts in ditches, marshes, bogs, pastures and along margins of lakes and rivers and exhibit a wide ecological tolerance of wet, acidic and nutrient-poor soil conditions (McCorry & Renou 2003). It is a long-lived perennial herb which is able to reproduce vegetatively by short rhizomes. Like many other species in this genus, sexual seed reproduction is predominantly performed by selfing (Buchenau 1892; Richards & Clapham 1941) accompanied by an extreme seed production with a high potential for long-distance dispersal via wind, animals and water (Richards & Clapham 1941; Ervin & Wetzel 2001). However, molecular marker studies in *Juncus effusus* are scarce. Two studies demonstrated that *Juncus effusus* showed strong inbreeding coefficients and a strong genetic structure within species (Michalski & Durka 2012; Michalski & Durka 2015). This species is an ideal model for research on the functioning of wetland ecosystems and is ecologically well characterized by a number of studies, e.g. on seed production (Ervin & Wetzel 2001), germination and

establishment (Lazenby 1955), growth rates and production of biomass (Wetzel & Howe 1999), litter decomposition (Gingerich & Anderson 2011) or the interaction between co-occurring plant species (Ervin 2005). With respect of its high potential for environmental remediation, it has been studied in relation to metal accumulation (Mays & Edwards 2001; Deng *et al.* 2004), the uptake and metabolization of nitrogen (Smialek *et al.* 2006) and phosphorus (DeBusk *et al.* 1995), wastewater remediation (Wiessner *et al.* 2005; Borin & Salvato 2012) and microbial activity in the rhizosphere (Moran & Hodson 1989; Nikolausz *et al.* 2008). Many studies on the impact of plant functional traits on ecosystem processes in wetlands focused on variability among species; however, potentially related intraspecific trait variations within and among natural *J. effusus* populations has not been described so far.

Juncus effusus shows additional characteristics which make the species an ideal model for future evolutionary and ecological investigations regarding the impact of functional traits on ecosystem functioning. First, the diploid chromosome set (2n = 42) of *Juncus effusus* fits assumptions of many theoretical models on molecular and quantitative genetics. Second, *Juncus effusus* has a relatively small genome size (c-value of 1C = 0.30 pg; Bennett & Leitch 2012), supporting marker-assisted studies. Third, the habit of *Juncus effusus* is small in contrast to other wetland species like *Phragmites australis*, which benefits greenhouse work with large sample sizes.

Objectives and thesis outline

To summarize, many studies have investigated the impact of functional traits on ecosystem processes in wetlands. However, species are usually treated as constant entities, neglecting intraspecific genetic variation. This study deals with various methods for evaluating the evolutionary potential of *Juncus effusus*, including quantitative and molecular genetics. The aim is it to quantify the intraspecific variability in functional traits relevant to the degradation of nitrogen compounds and to study their genetic basis and response to selection. In particular, genetic diversity, heritability and genetic correlations are studied, allowing for hypotheses about the adaptive potential of populations and phenotypic evolution. The content and objectives of each chapter are briefly presented below.

Chapter 2 presents the results from the common garden experiment, in which seed families of *Juncus effusus* from seven European origins were grown under low and high nitrogen availability in the soil. It assesses the intraspecific genetically based variability of functional traits, potentially relevant to the degradation of nitrogen compounds. It asks whether and how quantitative genetic variation is expressed differently depending on nitrogen availability and how it is structured within and among natural European populations of *Juncus effusus*.

The study presented in **chapter 3** is based on the results of a further common garden experiment. It investigates molecular and phenotypic variation in response to different nitrogen availabilities for a regional set of populations of *Juncus effusus*. We asked whether trait expression shows signatures of adaptive differentiation by using $Q_{ST} - F_{ST}$ comparison and relating trait variation to soil conditions of population origin.

Chapter 4 investigates the large-scale genetic population structure of *Juncus effusus* using microsatellite markers. Here, we attempt to discover how genetic diversity is structured across Europe and how local adaptation to soil and climatic conditions contributes to observed population structures.

CHAPTER 2

Strong Divergence in Quantitative Traits and Plastic Behavior in Response to Nitrogen Availability among Provenances of a Common Wetland Plant

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Abstract

Wetland ecosystems provide important ecosystem services such as the degradation and retention of excessive nutrient loadings. Plants may affect these processes directly or indirectly via the interaction with the rhizosphere community. Many studies on the impact of plant functional traits on ecosystem processes in wetlands focused on variability among species, neglecting the importance of intraspecific variability. Here we assessed the intraspecific genetically based variability of a common wetland plant for traits, potentially relevant for the removal of nitrogen compounds from the soil. We asked whether and how quantitative genetic variation is expressed differently depending on nitrogen availability and how it is structured within and among natural European populations of *Juncus effusus*. We partitioned the observed genetic variation into within- (broad-sense heritability) and among (Q_{ST}) population components for mean traits and plasticities. We found a strong plastic behavior for all measured traits in response to nitrogen availability. Genetic variation for mean traits differed strongly among populations but was very low at population level. We could not demonstrate a general effect of nitrogen availability on genetic trait expression. Our results suggest that basic and applied studies on wetland ecosystem processes will benefit from a deeper understanding of intraspecific genetic variation for traits and plastic behavior which is also fundamental for breeding approaches or to predict evolutionary responses.

Introduction

Progressive agricultural intensification as well as ongoing urbanization result in immense nutrient loading in both freshwater and coastal ecosystems (Tilman *et al.* 2002). The eutrophication of natural communities by an increased input of nitrogen via fertilizers and wastewaters can entail substantial risks for biodiversity (Smith *et al.* 1999; Stevens *et al.* 2004). Wetland ecosystems provide an important service by the natural decomposition and retention of such excessive substances (Millennium Ecosystem Assessment 2005). Two ecosystem processes have primarily been described to be relevant for nitrogen removal in wetlands. Firstly, plant uptake associated with incorporation into biomass and secondly, denitrification by anaerobic microorganisms in the soil (Sutton-Grier *et al.* 2013). These processes are not independent from each other because of the close interaction between plants and the rhizosphere community. For example, among other exudates, roots of wetland plants release oxygen into the rhizosphere via aerenchymatous tissues (Armstrong 1967), which can affect the redox potential of the soil (Blossfeld *et al.* 2011) and microbial communities both supporting mineralization and nitrification processes. Products of the microbial activity can in turn be resorbed by the plants (Rydin & Jeglum 2006; White & Reddy 2009). In general, this interaction between plant and rhizosphere has an immense potential to affect the accumulation, metabolization and hence, the removal of contaminants from the environment (Dhir *et al.* 2009). Although these processes and involved functional plant traits still remain poorly understood, there is a strong scientific and practical interest in the utilization of wetland plants and associated microorganisms to remediate contaminated waters, soils and sediments.

Understanding patterns of intraspecific trait diversity within and among populations is fundamental for both the prediction of evolutionary pathways under changing environmental conditions, and targeted selection of lines with particular properties. In the past, the variability of functional traits relevant for removal of contaminants in wetland ecosystems has mostly been assessed among species. For example, significant differences among species have been described for nitrogen uptake efficiency (Borin & Salvato 2012), root traits such as length, longevity or porosity (Lai *et al.* 2011), and organic matter decomposition (Gingerich & Anderson

2011) all of which are linked to many wetland processes. However, the extent of intraspecific variability for these functional traits is less well known. This is surprising, since intraspecific variability may be as important as interspecific variability with respect to ecosystem function (Silfver *et al.* 2007; Lecerf & Chauvet 2008).

Observed intraspecific phenotypic variation is the result of heritable differences in genetic architecture (Linhart & Grant 1996) and the ability of genotypes to express different phenotypes (trait means) in different environments, known as phenotypic plasticity (Miner *et al.* 2005). At the population-level both, genetic diversity and plasticity, are subject to neutral evolution and adaptive evolutionary changes in response to the local environment that both can lead to a diversification in traits and reaction norms across populations. Assessing genetic diversity in quantitative traits is a challenging task. Often, this is realized in common garden studies using a seed family design with individuals of known relatedness. Trait heritability is then defined as the proportion of phenotypic variance that is attributable to genetic variation, i.e. explained by relatedness (Falconer 1989). However, heritability may differ depending on the environment in which it is expressed (Hoffmann & Merilä 1999; Sgrò & Hoffmann 2004). Various hypotheses with contrasting expectations have been formulated for the relation between environment and genetic variation expressed (see Hoffmann & Merilä 1999 for details). On the one hand, heritability is predicted to increase under rare, stressful conditions due to the expression of genes behaving neutral in normal environments and which hence are not affected by natural selection (Holloway *et al.* 1990; Pigliucci *et al.* 1995). In contrast, decreased values for heritability under stressful conditions could be expected if environmental and phenotypic variation increase while genetic variation remains constant (Charmantier & Garant 2005). Assessing trait variability under a range of experimental treatments will hence allow a more general assessment of expressed genetic variability and plastic behavior.

Juncus effusus, the soft rush, has been established as a model plant in basic and applied research on wetland ecosystems. It is well characterized ecologically by a number of studies, e.g. on seed production (Ervin & Wetzel 2001), germination and establishment (Lazenby 1955), growth rates and production of biomass (Wetzel & Howe 1999), litter decomposition

(Gingerich & Anderson 2011) or the interaction with co-occurring plant species (Ervin 2005). In respect of contaminant removal it has been studied, e.g. on metal accumulation (Mays & Edwards 2001), wastewater remediation (Borin & Salvato 2012) and microbial activity in the rhizosphere (Nikolausz *et al.* 2008). However, potentially related intraspecific trait variation within and among natural populations has not been described so far.

Like many other species in this genus, *J. effusus* has been described as predominantly selfing (Buchenau 1892) and its high seed production (Stockey & Hunt 1994) makes the species an efficient colonizer. Consequently, molecular marker studies found large inbreeding coefficients and a strong degree of genetic structuring within the species (Michalski & Durka 2012; Michalski & Durka 2015) which is expected for selfing species (Hamrick & Godt 1996).

Here we assess intraspecific variability for functional traits of *Juncus effusus* putatively related to the removal of excessive nitrogen in the soil, and ask how this variability changes depending on nitrogen availability, how it is genetically structured within and among populations and whether it relates to soil nitrogen availability of the site of origin as a signature of adaptation. We hypothesize that, (1) as a consequence of the mating system and other life history traits of *J. effusus*, genetic variation for quantitative traits will be more strongly expressed among than within populations; (2) under nitrogen deficiency, genetically based variation of traits will differ from that expressed under higher nitrogen availability, and (3) expressed trait variability related to nitrogen acquisition varies with soil nitrogen of sample origin revealing signatures of adaptation.

Material and Methods

Study species and experimental design

Juncus effusus L. (Juncaceae) is a perennial herb and widespread in temperate wetland ecosystems. It has been described to vary substantially in morphological traits across its worldwide distributional range (Kirschner *et al.* 2002). In Europe only *J. effusus* ssp. *effusus* has been described (Kirschner *et al.* 2002). Between 2010 and 2012, we collected seed families, i.e. offspring from a single maternal individual, from seven locations in Europe (Table 1). From each location (all with population sizes N > 100), individuals were selected arbitrarily across the whole site. As the species is highly selfing (Michalski & Durka 2012; Michalski & Durka 2015) seed families were assumed to represent the maternal genotype. Before the start of the experiment seeds from all families were stored in paper bags at room temperature. In December 2012, we germinated seeds in quickpots (96 cells, 4 cm diameter x 8 cm deep; Hermann Meyer KG, Rellingen, Germany) filled with a mixture of sand and commercial soil (1:2, vol/vol; Fruhstorfer soil type P) in a climatic chamber with a 12 h photoperiod and mean day and night temperature of 30 °C and 20 °C, respectively. Germination success was high and did not vary with the year of collection (J. Born; pers. obs.). Eight weeks after sowing, trays with seedlings were placed in a greenhouse under frost-free conditions. In May 2013, we planted individual seedlings, all approximately of the same size, in 3 l pots (17 cm diameter x 18.5 cm deep) filled with a mixture of sand and unfertilized commercial soil (1:2, vol/vol; Fruhstorfer soil type 0). Sample extracts (N = 10) from 10 g air-dried soil mixture suspended in 40 ml 1 M potassium chloride gave average concentrations of 2.37 NO^{3-} - N mg / kg and 3.08 NH^{4+} - N mg / kg detected by flow-rate injection analyzer FIAstar^{TM} 5000 Analyzer (FOSS Analytical, Denmark).

Each of the seven populations was represented by three to eight seed families (mean: 7.1 seed families per population) with up to 20 individuals per seed family (Table 1). Because of a low initial seed number and insufficient establishment rate, not enough offspring was available to ensure a complete balanced design. In total, 946 individuals were randomly arranged and

kept at ambient temperature in a greenhouse (51°23'N, 11°52'E). All individuals were constantly watered until the end of the experiment in August 2013. One randomly chosen half of the individuals from each seed family received dissolved ammonium nitrate (NH_4NO_3) once a week in a dose of 0.05 g for 3 months (high-nitrogen treatment, HNT), equivalent to a total nitrogen input of 80 kg N / ha * yr, representative for agriculturally managed ecosystems. The other half received a water equivalent without additional nitrogen supply (low-nitrogen treatment, LNT).

Table 1 Location of studied *Juncus effusus* populations, sample sizes used in the common garden experiment and soil nitrogen content at population origin

ID	country	population	latitude (°N)	longitude (°E)	nr. of seed families/ total nr. of plants	Soil N content (g / kg)
KP	Estonia	Kõpu	58.3367	25.2650	8 / 160	1.95
GR	Germany	Groß Rosenburg	51.9124	11.9178	8 / 160	1.25
OB	Germany	Offenbach	49.2029	8.2123	8 / 160	1.05
PS	Germany	Pressel	51.5655	12.7322	8 / 160	2.50
SF	Germany	Siptenfelde	51.6605	11.0484	8 / 160	1.80
DL	United Kingdom	Dunfermline	56.0598	-3.3866	3 / 35	2.05
FD	United Kingdom	Findochty	57.6989	-2.8902	7 / 111	2.90

Trait selection and measurements

We assessed plant functional traits putatively linked to nitrogen removal in wetland ecosystems, i.e. related to first the accumulation of nitrogen in plant biomass which is a function of both plant productivity and the C:N ratio of the biomass produced (Sutton-Grier *et al.* 2013) and second to denitrification rates of the soil which can potentially be influenced via carbon input (quantity and quality) and modification of redox conditions, e.g. via radial oxygen loss.

More in particular, we quantified plant productivity as above- (AGBM) and belowground biomass (BGBM) at the end of the experiment, plant height (H), i.e. mean of the two longest leaves, and number of stems produced (S). The latter two measurements were taken twice, at the start and end of the experiment to estimate a relative growth rate ($RGR = (H*S)_{end}$ -

$(H^*S)_{start}$) / $(H^*S)_{start}$). Harvested biomass was dried at 60 °C for 48 h and weighed. As a component of the leave economic spectrum (c.f. Wright *et al.* 2004) an approximation for leaf dry matter content (LDMC) was calculated as the ratio of dry to fresh weight of aboveground biomass. Additionally, for each sample a fraction (~ 10 mg) of above- and belowground biomass was milled (Retsch MM200, Retsch GmbH, Haan, Germany) and analyzed for carbon and nitrogen content using a CHNS/O elemental analyzer (Vario EL III, Element Analyzer, Elementar, Hanau, Germany). From these data, we calculated C:N ratios (C% : N%) for above- (AG-C:N) and belowground biomass (BG-C:N) as well as total aboveground N accumulation (AG-N) as N content per g leaf tissue multiplied by the dry weight. Root porosity, which is likely to be related to the radial oxygen loss of the roots (Lai *et al.* 2012), was measured using the microbalance method described in Visser and Bögemann (2003). In short, for each individual three root tips were extracted and 10 mm behind the root apex a 30 mm long root segment was resected. After carefully removing surface water from the root sample it was weighted. Then, the sample was placed in a water-filled 1.5 ml Eppendorf tube and put under vacuum conditions for 5 min and the now infiltrated root segment was weighed again. Root porosity for each sample was calculated as the weight difference relative to the weight after infiltration and multiplied by the specific weight for *J. effusus* roots given in Visser and Bögemann (2003). Porosity values for the three samples were averaged to obtain an individual estimate in percent (POR). In addition to these plant functional traits we also measured the pH value of the soil at the end of the experiment by analyzing a representative sample using the method described by Hoffmann (1991). Soil samples were air-dried, sieved at 2 mm and 10 g were suspended in 25 ml of a 0.01 M calcium chloride solution and analyzed using a pH Meter (Knick pH-Meter 766 Calimatic, Berlin, Germany). As we used standardized soil for growing the plants, variation in the pH value should be related directly or indirectly to the plant. For example, variation in the pH value may reflect differences in transformation rates of inorganic nitrogen regulated by microbial processes (Rydin & Jeglum 2006). Sampling of belowground biomass, C:N analyses of above- as well as belowground biomass and pH measurements were done on a randomly chosen half of the individuals grown per seed family and treatment.

Statistical analyses

All statistical analyses were performed in R (R Development Core Team 2014) with the full data set including both treatments and two subsets including the LNT and HNT data separately. For the following analyses we used generalized linear mixed models implemented in a Bayesian framework in the package 'MCMCglmm' (Hadfield 2010) allowing to deal with an unbalanced experimental design (Ogle & Barber 2012). Visual inspection of diagnostic plots showed normal distribution of model residuals except for number of stems and relative growth rate. To meet standard assumptions of linear models data on these traits were log transformed.

To assess the effect of nitrogen addition on observed trait variation a model was run allowing for different residual variances for the two treatment factors (fixed effect) and additionally included sample origin, seed family nested in origin and their interactions with treatment (as a measure of plasticity) as random effects.

In order to assess the impact of genotypic effects on trait means and plasticity, we compared the quality of different models with and without varying sets of random effects reflecting these genotypic effects. We started with a model without random effects and added stepwise a random term, i.e. population, followed by seed family within population and the interactions of sample origin and seed family with treatment (as estimates for plasticity), and compared the deviance information criterion (DIC) values between models. A decrease of DIC values by more than 10 for the more complex model was considered to indicate a substantial improvement.

Genetic diversity at population level was quantified as broad-sense heritability (H^2) derived from the partitioning of the total phenotypic variance into within (V_{WF}) and among family components (V_{AF}) as $H^2 = V_{AF} / (V_{AF} + V_{WF})$ (Lynch & Walsh 1998). Quantitative genetic trait divergence (Q_{ST}) among populations was estimated as $Q_{ST} = V_{AP} / (V_{AP} + 2V_{WP})$, where V_{AP} is among population variance and V_{WP} the within population variance given by the family variance (Spitze 1993). In selfing species such as *Juncus effusus*, the total genetic variation, here given

by the family variance, is the variation important to natural selection at local level. Hence, it is not informative to separate into additive and non-additive components (Banta *et al.* 2007).

Additionally, we quantified the heritability of plasticity and respective Q_{ST}'s as described above but with variances explained by the seed family × treatment and origin × treatment interaction, respectively. All variance components were extracted from models fitted for each population separately containing treatment as fixed and seed family and seed family × treatment as random effects. Models for Q_{ST} estimation included only origin and seed family as random terms and for Q_{ST} of plasticity also the interaction terms origin × treatment and seed family × treatment were included. We accounted for potential heteroscedasticity introduced by the two treatment conditions by standardizing the data for each condition to a mean of zero and a standard deviation of one hence, effectively removing the treatment effect for this analysis. Credibility intervals for all estimates were directly taken from the posterior distribution. Estimates were deemed significantly different from zero, if the respective term increased model fit by Δ DIC > 10 compared to a model without the respective term.

In order to test the hypothesis that genetic variation differed depending on N availability, we used models explaining treatment specific heritability or Q_{ST} values by treatment as fixed and sample origin (in case of heritabilities) and functional traits as random effects.

To assess whether the genetic basis for trait expression differed between the two treatment conditions we did cross-environment genetic correlations as simple Pearson's correlations between seed family means from each treatment. This method may result in downwardly biased estimates but provides a conservative test of whether a genetic correlation is different from zero (Astles *et al.* 2006).

We tested for potential adaptive patterns in trait expression by correlating treatment specific population mean traits (X^{pop}_{LNT} and X^{pop}_{HNT}, for the low- and the high-N treatment respectively) and absolute plasticity ($X^{pop}_{LNT} - X^{pop}_{HNT}$) against soil nitrogen content in upper layer (0-20 cm depth) of the population origin (Table 1). Data was extracted from the LUCAS topsoil database (Tóth *et al.* 2013) using the average between the two nearest available data points (min distance: 4.1 km, max distance: 17.0 km, mean: 9.2 km). Soil N availability obtained by this

approach might not necessarily reflect local soil conditions at the population origin rather it represents a regional estimate and hence, correlations with trait means will reflect more regional adaptive patterns. Soil nitrogen content did not show an obvious spatial pattern, i.e. there was no correlation with latitude and longitude of population origin ($P > 0.5$).

Results

All investigated quantitative traits showed a significant plastic response to the two treatment conditions. As expected, mean values for above- and belowground biomass and aboveground N accumulation increased strongly by more than 100% when nitrogen was added. Traits such as LDMC (-32.3%), above- and belowground C:N (-16.5%, -9.8%) decreased under the high-nitrogen treatment (Table 2) as did root porosity (-6.7%; Fig. 1). The complete dataset is available in Appendix S1.

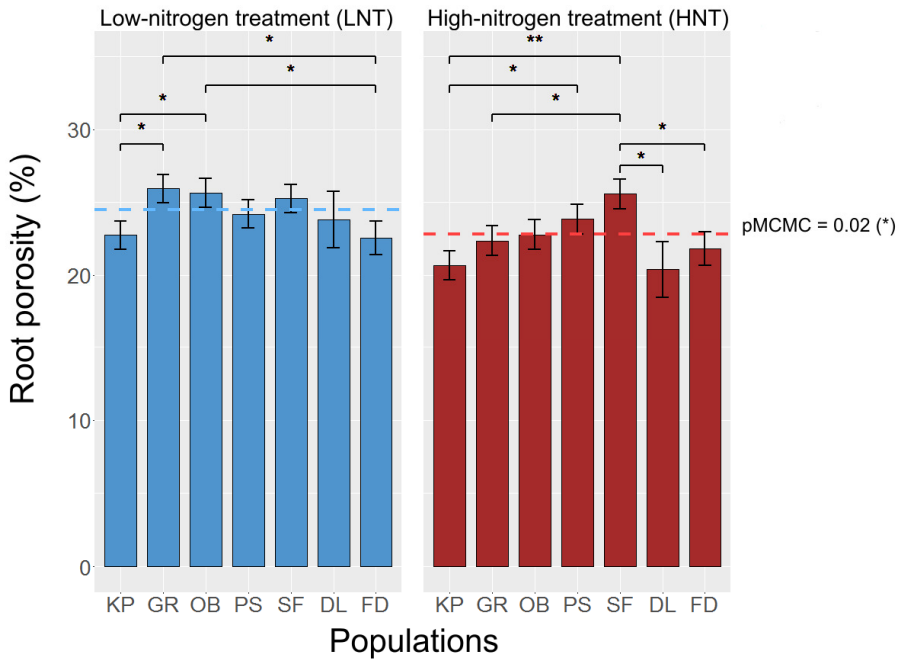

Fig. 1 Effect of treatment and populations on root porosity in *Juncus effusus*. Bars represent estimated trait means and standard errors for each population (for acronyms see Table 1) grouped by treatment. Dashed lines indicate treatment means across populations.

All measured traits differed significantly among population origin and seed families within populations except for belowground biomass and belowground C:N (Table S1). The plastic response to the treatment significantly differed among populations for plant height, belowground biomass and LDMC. Also, seed families differed in their response to the treatment in traits such as plant height, relative growth rate, aboveground C:N and -biomass as well as pH value and root porosity (Table S1).

Broad-sense heritability estimates (H^2) across treatments were low for most traits and populations investigated (Table S2). Estimates obtained for each treatment separately differed

substantially among populations and traits even for the same trait and population, e.g. for aboveground biomass in population FD (LNT: H^2 = 0.618, HNT: H^2 = 0.011). Broad-sense H^2 of plasticity was generally low with few exceptions, i.e. plant height ($H^2_{plasticity}$ = 0.541) in population FD and aboveground biomass ($H^2_{plasticity}$ = 0.219) in population KP (Table S3).

Overall genetic differentiation in mean traits among populations, quantified as Q_{ST}, varied substantially between Q_{ST} = 0.163 to 0.737 for root porosity and plant height, respectively (Fig. 2, Table S4). Estimating Q_{ST} values for treatments separately revealed a strongly differently expressed genetic differentiation for belowground biomass and belowground C:N, whereas in most other traits the level of genetic differentiation was similar between treatments (Fig. 2, Table S4). Estimates for Q_{ST} in the plastic response was generally lower than for mean traits with the strongest genetic differentiation for plasticity in belowground biomass (Q_{ST} = 0.405; Fig. 2, Table S4). On average, the expression of genetic diversity at within- and among population level did not vary consistently with N availability.

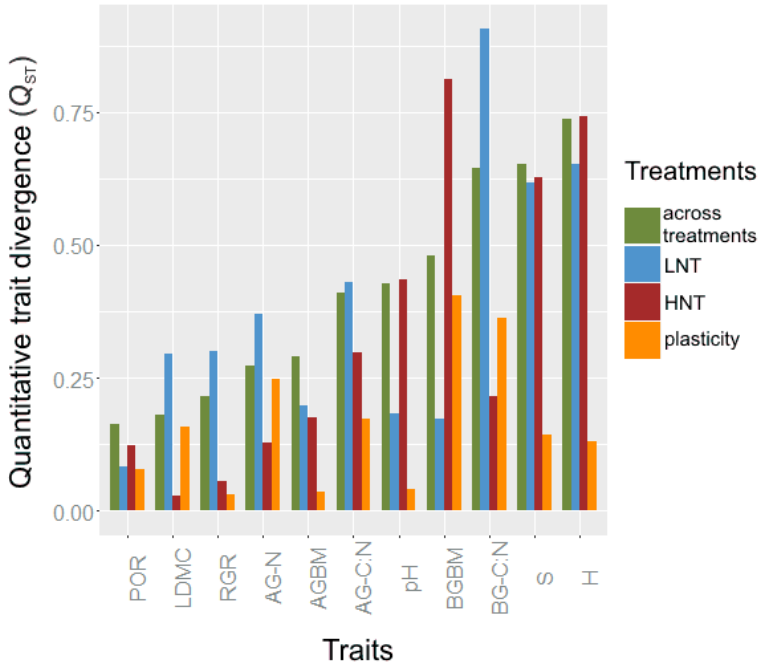

Fig. 2 Quantitative trait divergence (Q_{ST}) among studied populations of *Juncus effusus* across treatments, for the low- (LNT) and high-nitrogen treatment (HNT) separately and for trait plasticities. POR, root porosity; LDMC, leaf dry matter content; RGR, relative growth rate; AG-N, total aboveground N accumulation; AGBM, aboveground biomass; AG-C:N, carbon to nitrogen ratio of aboveground biomass; pH, soil pH; BGBM, belowground biomass; BG-C:N, carbon to nitrogen ratio of belowground biomass; S, number of stems; H, plant height.

Cross-environment genetic correlations significantly differed from zero for most traits except for belowground biomass, aboveground N accumulation and root porosity (Table S4).

Expressed population mean traits were not related to soil N content of population origin except for root porosity, which significantly decreased with soil N content in the low-N treatment (Fig. 3 A) as well as aboveground N accumulation in high-N treatment (Table S5). Absolute trait plasticity was related to soil N for aboveground N accumulation only, which significantly decreased with more N availability (Fig. 3 B). Also, variability in some trait means showed significant spatial patterns (Table S5), i.e. correlations with latitude: root porosity (LNT), aboveground N accumulation (HNT), and with longitude: aboveground C:N (LNT) and relative growth rate (HNT).

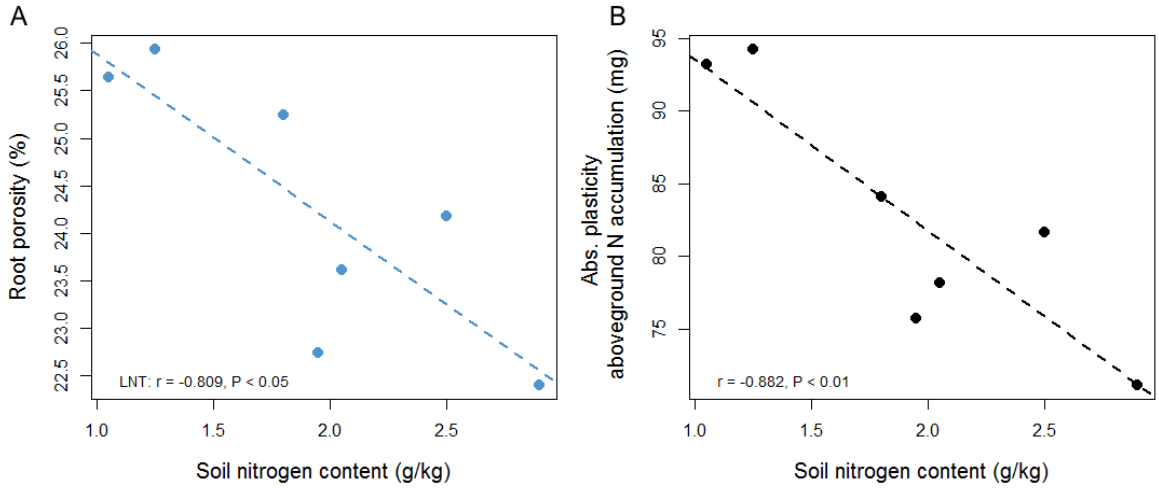

Fig. 3 Correlation of soil nitrogen content of site of origin with populations means of (A) root porosity in the low-N treatment (LNT) and (B) absolute plasticity of aboveground nitrogen accumulation (mg) for *Juncus effusus*. For visualization, regression lines are drawn.

Table 2 Estimated overall trait means for the low- (LNT) and the high-nitrogen treatment (HNT), the treatment effect expressed as percentage difference and the proportion of individual genotypic effects relative to the variance explained by all genotypic effects for representatives of seven populations of *Juncus effusus*.

		Fixed effects				Difference in % and significance level		Random effects			
Functional traits (unit)		mean LNT (95% CI)		mean HNT (95% CI)				Population (%)	seed family (%)	population × treatment (%)	seed family × treatment (%)
H	(cm)	28.06	(23.34 - 33.02)	41.62	(36.80 – 46.62)	48.3	(***)	78.49	9.88	8.87	2.75
S (log+10)		3.77	(3.62 - 3.92)	4.37	(4.21 – 4.52)	15.9	(***)	75.44	14.04	7.02	3.51
RGR (log+10)		3.04	(2.95 - 3.13)	3.81	(3.72 - 3.90)	25.3	(***)	36.00	28.00	8.00	28.00
AGBM	(g)	1.93	(1.53 – 2.35)	8.52	(8.14 - 8.95)	341.5	(***)	37.13	15.61	15.61	31.65
BGBM	(g)	2.46	(2.03 – 2.91)	7.11	(6.55 - 7.65)	189.0	(***)	19.91	10.63	53.62	15.84
LDMC	(mg g^{-1})	482.10	(444.80 – 524.50)	326.60	(294.40 - 368.80)	-32.3	(***)	11.16	18.61	54.93	15.30
AG-C:N		44.41	(41.26 – 47.58)	37.08	(34.03 - 39.93)	-16.5	(***)	49.10	19.33	11.26	20.30
BG-C:N		88.73	(85.16 – 92.34)	80.01	(76.45 - 83.50)	-9.8	(***)	64.96	16.58	10.70	7.76
AG-N	(mg)	19.72	(15.87 – 23.60)	102.96	(98.33 – 108.58)	422.1	(***)	17.34	15.24	56.98	10.44
pH		6.36	(6.27 – 6.44)	6.43	(6.35 - 6.50)	1.1	(*)	44.44	11.11	16.67	27.78
POR	(%)	25.23	(23.76 – 26.56)	23.54	(22.25 - 24.87)	-6.7	(*)	17.36	16.85	9.45	56.34

H, plant height; S, number of stems; RGR, relative growth rate; AGBM, aboveground biomass; BGBM, belowground biomass; LDMC, leaf dry matter content; AG-C:N, carbon to nitrogen ratio of aboveground biomass, BG-C:N, carbon to nitrogen ratio of belowground biomass; AG-N, total aboveground N accumulation; pH, soil pH; POR, root porosity. *, pMCMC < 0.05; **, pMCMC < 0.01; ***, pMCMC < 0.001.

Discussion

The role of intraspecific variation for ecosystem functioning has increasingly been recognized recently (Hajjar *et al.* 2008; Hughes *et al.* 2008). However, the evidence for an impact of plant genetic diversity on wetland ecosystem processes is scarce. In one of the rare examples, Tomimatsu *et al.* (2014) demonstrated that polycultures of different *Phragmites australis* genotypes showed higher primary production and nitrogen removal than monocultures. They argued that the genetic diversity effect was driven by both competitive interaction and complementarity effects. Niche differentiation among genotypes can contribute to complementarity effects provided that differences in functional traits are genetically based, which however, is generally unknown. Here we described intraspecific genetic variability in functional traits of *Juncus effusus*, putatively linked to the natural removal of nitrogen in wetland ecosystems. In response to varying availability of nitrogen we found a strong divergence in the expression of traits and in the plastic behavior among populations. Variation in trait expression and plasticity could partly be related to soil N availability of population origin suggesting possible signatures of adaptation, which in summary may indicate a genetically based impact on plant associated ecosystem functions.

Treatment effects

Under higher nutrient supply *J. effusus* showed a higher productivity, indicated by a number of growth related parameters (cf. Lazenby 1955). Consequently, the C:N ratio of plant biomass decreased while accumulation of nitrogen increased, reaching values similar to what has been reported previously for this species (Boyd 1971; Kao *et al.* 2003). Only ammonia levels much higher than in our study (> 200 mg / l) have been found to inhibit growth in *J. effusus* (Clarke & Baldwin 2002). The addition of ammonium nitrate has a well-known acidifying effect also on wetland soils (Min *et al.* 2011). Unexpectedly, in our experiment under the high-N treatment the soil pH value increased significantly, compared to the low-N treatment. As we used standardized soil for growing the plants, variation in the pH value should be related directly or

indirectly to the plant and soil pH can hence interpreted as extended phenotype (cf. Schweitzer *et al.* 2004; Phillips 2009). A number of plant specific effects are known to influence soil pH and hence, may contribute in shaping this particular response such as the ratio between cation and anion uptake and the release of CO_2, oxygen and different organic compounds. Firstly, different *Juncus* sp. have been shown to alter rhizospheric pH in contrasting ways (Blossfeld *et al.* 2011). For *J. effusus* the authors observed a strong oxidative acidification caused by oxygen release from the roots (Blossfeld *et al.* 2011). On the other hand, in an oxygenated rhizosphere, nitrification of ammonia to nitrate can occur and the uptake of nitrate is in many plants accompanied by an increase in soil pH (Sorrell & Orr 1993; Schubert & Yan 1997). The preferential uptake of either ammonia or nitrate over the other could hence also affect soil pH. For example, the wetland plant *Typha latifolia* prefers ammonia over nitrate indicated by strongly differing uptake kinetics for these N sources (Brix *et al.* 2002). However, for *J. effusus*, uptake kinetics for ammonium and nitrate reported are very similar (Yao *et al.* 2011). In general, plant residues and root exudates can increase soil pH also with impact on soil bacterial communities (Butterly *et al.* 2011; Shi *et al.* 2011b). Consequently, more specific experiments are needed to explain this treatment effect in detail.

Mean values for root porosity in *J. effusus* from our study are similar to previously reported results (Visser & Bögemann 2003; Visser & Bögemann 2006). However, under the low-N treatment we found significantly higher root porosity than under less nitrogen limited conditions suggesting a functional role of root porosity for nitrogen cycling. Only recently, a hydroponic experiment on rice, which, similarly to *J. effusus* (Visser & Bögemann 2006) forms aerenchyma in the roots constitutively, revealed similar results (Abiko & Obara 2014). The increase in aerenchyma formation has been demonstrated to reduce metabolic costs of soil exploration, i.e. by a reduction of root respiration by replacing living cortical cells with aerenchyma (Zhu *et al.* 2010). Thus it can increase the remobilization of nutrients (Postma & Lynch 2011) which is both beneficial for N acquisition and plant growth in nitrogen deficit soils (Saengwilai *et al.* 2014).

Genotypic effects

In our study, genetic variation was present for almost all investigated trait means and trait plasticities but its expression differed among the traits and whether it was assessed at within or among-population level. In general, within-population genetic variation for quantitative traits (H^2) was relatively low. A review by Geber and Griffen (2003) found on average larger heritabilities in species with outcrossing and mixed mating systems ($h^2 = 0.29$) compared to inbreeding species ($h^2 = 0.15$). Indeed, the overall level of heritability estimated for *J. effusus* in our study is even lower (H^2 averaged across traits, populations and treatments = 0.09) confirming our hypothesis based on the species' life history traits. Both, frequent founder events and a predominantly selfing mating system are expected to reduce effective population sizes. As a consequence, genetic drift, i.e. random change in number of gene variants in a population, will be especially pronounced and may lead to a loss of genetic diversity and to a fixation of alleles within populations (Frankham *et al.* 2002). Heritabilities have also been reported to differ among trait types with values for ecophysiological traits (without secondary chemistry) often slightly smaller than for morphology or vegetative performance (cf. Geber & Griffen 2003; Aspinwall *et al.* 2013). Also in our study, heritability estimates in ecophysiological traits such as root porosity, above- and belowground C:N tended to be lower than for vegetative traits such as plant height and number of stems, putatively indicating a stronger selection on the latter (Mousseau & Roff 1987). It has been suggested that the presence of genetic variation for the degree of aerenchyma formation in response to N stress would enable breeders to select for genotypes with a consistently high, low, or plastic trait expression (Saengwilai *et al.* 2014) with direct and indirect impact on N removal from wetland soils. Indeed, aerenchyma formation or root porosity has been found to vary genetically in a number of crop species (e.g. Colmer 2003). Here we found some genetic differentiation among populations, but only very little heritable trait variation within populations. However, low heritabilities can also reflect large environmental or error variance obscuring genetic variability among individuals (Schwaegerle & Levin 1991).

Genetically based trait variation residing among populations was found to be substantial and significant for all traits. Strong quantitative genetic differentiation as a result of genetic drift is not unexpected for selfing species and has been demonstrated for a range of non-crop plant species and traits, e.g. *Medicago truncatula*, *Senecio vulgaris, Arabidopsis thaliana, Amphicarpaea edgeworthii* or *Hordeum spontaneum* (Bonnin *et al.* 1996; Steinger *et al.* 2002; Stenøien *et al.* 2005; Liang *et al.* 2009; Volis 2011). In addition to genetic drift, adaptive divergence can also contribute to genetic differentiation among populations. In order to assess the relative contribution of selective processes to observed quantitative differentiation patterns it has been suggested to compare quantitative trait divergence with neutral expectations derived from molecular markers (Leinonen *et al.* 2013). However, in selfing species such as *Juncus effusus* with strong quantitative trait and genetic marker differentiation diversifying selection might well contribute to trait variation but might be hard to detect by this approach. Indeed, we found population mean traits for root porosity (LNT) and aboveground N (HNT) significantly negative correlated to soil N availability of the region of population origin suggesting that variation in the expression of these traits is at least partly adaptive, despite being little differentiated among studied population compared to other investigated traits such as plant height. From these correlations it seems plausible that a lower soil N availability selects for an increased efficiency in N acquisition in *J. effusus*. Our results provide hence a promising fundament for future studies investigating adaptive trait variation based on a larger sample.

In contrast to our hypothesis of differently expressed genetic variation in functional traits depending on N availability, neither within-population nor among-population genetic variation was more strongly expressed in one of two N treatments. However, it has also been argued that unfavorable conditions may have rather unpredictable effects on the expression of genetic variation. More in particular, large changes in heritability of mean traits in either direction as a response to environmental changes could be explained by a high heritability of plasticity (Gavrilets & Scheiner 1993; Hoffmann & Merilä 1999). Indeed, the large differences in treatment specific Q_{ST} values for belowground C:N (LNT: Q_{ST} = 0.906; HNT: Q_{ST} = 0.216) and

belowground biomass (LNT: Q_{ST} = 0.173; HNT: Q_{ST} = 0.811) were accompanied by the largest Q_{ST} values for plasticity (Q_{ST} = 0.363 and Q_{ST} = 0.405, for belowground C:N and belowground biomass, respectively), supporting this explanation.

There is an ongoing debate on whether plants can affect nitrogen-cycling and hence soil N availability and in turn their own fitness rather indirectly via interactions with the soil microbial community (Knops *et al.* 2002) or actively via litter chemistry (Chapman *et al.* 2006). Regardless of the underlying mechanisms, genetically determined intraspecific differences in functional traits – as demonstrated here for *Juncus effusus* – eventually place ecosystem processes in an evolutionary framework.

Supplementary data and acknowledgements

Appendix S1 Dataset of all studied individuals and their quantitative traits in response to nitrogen availability.

Table S1 Model comparisons to evaluate different genotypic effects on observed trait variance in *Juncus effusus* across treatments.

Table S2 Broad-sense heritability (H^2) for quantitative traits of seven *Juncus effusus* populations across treatments and for the low- (LNT) and high-nitrogen treatment (HNT) separately.

Table S3 Broad-sense heritability (H^2) of trait plasticity for seven *Juncus effusus* populations.

Table S4 Estimates of quantitative genetic differentiation (Q_{ST}) among studied populations of *Juncus effusus* across treatments and for the low- (LNT) and high-nitrogen treatment (HNT) separately, for trait plasticities as well as cross-environment genetic correlations given as simple Pearson's correlation *r* between seed family means.

Table S5 Correlations of population means of *Juncus effusus* with environmental variables of site of origin.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2016.10.002.

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CHAPTER 3

Trait Expression and Signatures of Adaptation in Response to Nitrogen Addition in the Common Wetland Plant *Juncus effusus*

PlosOne (submitted)

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Abstract

Wetland ecosystems are known to mitigate high nutrient loadings and thus can improve water quality and prevent potential biodiversity loss caused by eutrophication. Plant traits affect wetland processes directly through effects on accumulation or metabolization of substances, and indirectly by affecting microbial transformation processes in the soil. Understanding the causes and consequences of intraspecific variation in plant functional traits and associated ecosystem processes can aid applied ecological approaches such as wetland restoration and construction. Here we investigated molecular variation and phenotypic variation in response to three levels of nitrogen availability for a regional set of populations of the common wetland plant *Juncus effusus*. We asked whether trait expression reveals signatures of adaptive differentiation by comparing genetic differentiation in quantitative traits and neutral molecular markers (Q_{ST} - F_{ST} comparisons) and relating trait variation to soil conditions of the plant's origin. Molecular analyses showed that samples clustered into three very distinct genetic lineages with strong population differentiation within and among lineages. Differentiation for quantitative traits was substantial but did not exceed neutral expectations when compared across treatments or for each treatment and lineage separately. However, variation in trait expression could be explained by local soil environmental conditions of sample origin, e.g. for aboveground carbon-to-nitrogen (C:N) ratios, suggesting adaptive differentiation to contribute to trait expression even at regional level.

Introduction

Wetland ecosystems are known to mitigate high nutrient loadings and thus can improve water quality and prevent potential biodiversity loss caused by eutrophication (Millennium Ecosystem Assessment 2005). The potential of wetlands to limit environmental damages is more and more recognized also by applied approaches using managed and constructed wetlands (Kuschk *et al.* 2012). Two processes are primarily driving the reduction of high nutrient loads by wetlands: First, a direct accumulation of substances by the vegetation, and second microbial transformation processes (Rydin & Jeglum 2006; White & Reddy 2009). For example, high inorganic nitrogen loads can be reduced by denitrification processes with anaerobic soil microorganisms converting nitrate to nitrogen gas that is lost to atmosphere (Sutton-Grier *et al.* 2013). Plant traits can directly and indirectly affect the soil microbiome and its activity and hence microbial N processing (terHorst & Zee 2016). For example, the quantity and quality of leaf litter as well as root exudates can influence soil microclimate thus affecting soil microorganisms (Chapin 2003; Orwin *et al.* 2010). The effect of plant functional traits and their interaction with the soil microbiome on the reduction of contaminants in wetland ecosystems is well studied with respect to among-species variability, e.g. for the efficiency of nitrogen and phosphorus retention (Smialek *et al.* 2006; Menon & Holland 2013), root traits (Lai *et al.* 2011) and for the degradability of plant tissue (Moran & Hodson 1989; Gingerich & Anderson 2011). However, recently an increased number of studies demonstrate that also intraspecific variation in functional traits can be highly variable even at regional and locale scale (Jung *et al.* 2010; Moreira *et al.* 2012).

Understanding the causes and consequences of this intraspecific variation is not only a fundamental challenge for ecological research but it can also aid applied approaches in wetland restoration and construction because it is increasingly recognized as important driver of ecosystem functioning (Silfver *et al.* 2007; Lecerf & Chauvet 2008). Across a species' distribution range substantial trait variation can arise because of two fundamental drivers. First, differences in

trait expression among accessions within a single species can cause by following neutral processes such as drift, migration, or lineage diversification. Second, trait diversification can be driven by selective processes in response to local biotic and abiotic environmental conditions, such as nutrient-, light- and water availability (Guo *et al.* 2011; Wang *et al.* 2012; Zhou *et al.* 2012).

Detecting the signature of adaptive processes in trait variation and differentiation is a challenging task because it requires the partitioning of the observed phenotypic trait variance into genetically and environmentally based variance. In order to minimize the latter common garden studies are often used to assess and compare trait variation across multiple provenances or genotypes. However, the expressed genetic contributions to trait variance may vary across different environments (Pamilo 1988) limiting conclusions that can be drawn from a single common garden environment alone. Simulating different environmental conditions in a common garden experiment would hence allow a more general understanding of the observed trait variation and differentiation.

The impact of adaptive processes to genetically based phenotypic trait variation expressed among populations can be assessed by the comparison between such differentiation patterns and neutral expectations based on neutral molecular loci (Whitlock 2008; Leinonen *et al.* 2013). Larger genetic differentiation in quantitative traits (Q_{ST}) compared to neutral genetic differentiation (F_{ST}) is interpreted as a signature of directional selection whereas the opposite case, i.e. $Q_{ST} < F_{ST}$, indicates stabilizing selection. This approach has been employed by an increasing number of studies finding evidences for adaptation to local environments in a wide range of organisms (Leinonen *et al.* 2008). The Q_{ST} - F_{ST} approach can detect signatures of adaptation but not the environmental regimes causing trait divergence and the observed differentiation patterns. Consequently, another more qualitative approach to detect adaptive differentiation is to test for clines in mean phenotypic trait expression along environmental variables (Alexander *et al.* 2012; Konarzewski *et al.* 2012).

Here we study quantitative trait expression and molecular genetic differentiation in response to differing nitrogen availability among a regional set of populations of the common wetland plant *J. effusus* looking for signatures of adaptation. *Juncus effusus*, the 'Common Rush' is a perennial, self-compatible herb widely distributed in temperate wetland ecosystems (Richards & Clapham 1941; Kirschner *et al.* 2002). It is a model species for research on wetland ecosystem functioning and well characterized by a number of studies in respect to contaminant removal, e.g. on metal accumulation (Mays & Edwards 2001), nitrogen remediation (Borin & Salvato 2012; White & Cousins 2013) and microbial activity in rhizosphere (Nikolausz *et al.* 2008). A previous study has shown that *J. effusus* in Germany consists of sympatrically occurring, morphologically rather similar, but genetically highly differentiated lineages (Michalski & Durka 2015). A quantitative genetic experiment revealed strong differentiation in the expression of functional traits among few European *Juncus effusus* populations partially explained by environmental conditions of the source location suggesting a contribution of adaptive divergence (Born & Michalski 2017). To further understand functional trait expression within *J. effusus* and considering possible within-species lineage differentiation we ask:

1) What is the relative contribution of neutral and selective processes in quantitative trait differentiation of *J. effusus* and do observed patterns differ among lineages?
2) Is there a clinal differentiation of trait expression along soil environmental conditions at regional scale indicating adaptive responses?

Material and Methods

Between 2013 and 2014, seed families, i.e. offspring from a single maternal individual, were collected at 21 locations across central Germany and covering a wide range of habitats. *Juncus effusus* is not endangered or a protected species in Germany and no specific permissions for leaf and seed sampling were required. Spatial distance among populations ranged from 1.1 km (PWA - PWI) to 222.5 km (ZM – JEM). At each location (with population sizes varying between 20 and > 100 adult individuals), seed material was sampled from 4 - 6 individuals arbitrarily selected across the whole site, resulting in a total of 111 maternal individuals. Leaf material was sampled from the maternal and additional individuals resulting in a larger sample per location (N = 7 - 12, mean: 8.5) for molecular genetic analysis. Sampled leaf and seed material was dried with silica gel and stored in paper bags at room temperature. For each population and site a mean seed mass was estimated by measuring length and width of seeds from 3 - 5 seed families by optical scanning with high resolution and applying image analysis implemented in WinSeedle (Regent Instruments Inc., Québec, Canada) and calculating a volume assuming an ellipsoid shape of the seeds. Topsoil data for each location was taken from the LUCAS topsoil dataset (Tóth *et al.* 2013). In order to obtain population-specific data soil environmental variables (particle size distribution: clay-, silt- and sand content, coarse fragments, soil pH, organic- and inorganic compounds: organic carbon and carbonate, phosphorus-, nitrogen- and potassium content, and cation exchange capacity) were averaged across all data points available within a radius of 15 km of each population (1 - 7 per population, mean 4.8). Soil parameters obtained by this approach might not necessarily represent exact local conditions at the population origin rather small-scale regional patterns.

Molecular genetic data

Genomic DNA was extracted from dried leaves using the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol and quality was checked by gel electrophoresis and NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA). Genotyping was done using 14 microsatellite loci (Jeff04, Jeff06, Jeff10, Jeff11, Jeff29, Jeff36, Jeff52, AY493568, AY493569, Jeff058, Jeff059, Jeff067, Jeff069, Jeff074) previously described by Michalski and Durka (2012); Michalski and Durka (2015) and two additional loci (Jeff111 and Jeff115, Table S1). PCR fragments were obtained using directly fluorescent-labeled primers or primers with universal fluorescent-labeled M13R and CAG tails (Schuelke 2000). Amplification reactions with directly fluorescent-labeled primers were carried out in a volume of 8 µl containing 1 µl genomic DNA (~ 20 ng / µl), 4 µl QIAGEN Multiplex Mastermix, 1.2 µl H_2O, 1.0 µl primer mix and 0.8 µl QIAGEN Q-Solution. PCR conditions were as follows: 95 °C for 15 min; followed by 35 cycles of 95 °C for 30 s; 58 °C for 40 s and 72 °C for 1 min, followed by 72 °C for 15 min. The amplification with M13R or CAG tailed primers was performed in a 5 µl reaction mixture that contained 1.0 µl genomic DNA (~ 20 ng / µl), 2.5 µl QIAGEN Multiplex Mastermix, 0.25 µM of forward and reverse primer mix and 0.25 µM of fluorescently labelled M13R or CAG primer. A touchdown PCR was run as follows: 95 °C for 15 min; followed by 20 cycles (94 °C for 30 s, 60 °C for 30 s with decrease of 0.5 °C per cycle, 72 °C for 90 s), followed by 20 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s) and close with 72 °C for 10 min. Fragments were run on an ABI 3130 (Applied Biosystems, Foster City, California, USA) with size standard LIZ 500 (Applied Biosystems) and scoring was done using GeneMapper 5 (Applied Biosystems).

Quantitative genetic data

Trait expression in response to differing nitrogen availability was quantified in a common garden experiment in the experimental field station Bad Lauchstädt (51°23'N, 11°52'E). In December 2014, seeds were germinated in quickpots (96 cells, 4 cm diameter x 8 cm deep; Hermann Meyer KG, Rellingen, Germany) filled with a 2:1 (vol/vol) mixture of commercial soil (Fruhstorfer soil type P) and sand using a climate chamber with a 12 h photoperiod and mean day and night temperature of 30 °C and 20 °C, respectively. In April 2015, seedlings of approximately the same size were transplanted into 3 l pots (17 cm diameter x 18.5 cm deep) filled with a mixture of commercial soil and sand (2:1, vol/vol; Fruhstorfer soil type P). Sample extracts (N = 10) from 10 g air-dried soil mixture suspended in 40 ml 1 M potassium chloride gave average concentrations of 49.3 mg / kg NO^{3-} - N and 0.45 NH^{4+} - N mg / kg detected by flow-rate injection analyzer FIAstar™ 5000 Analyzer (FOSS Analytical, Denmark). All individuals were constantly watered and after one month of transplanting plants received either no or additional fertilizer of weekly doses of dissolved ammonium nitrate (NH_4NO_3), equivalent to a total of 72 kg N ha^{-1} yr^{-1} and 153 kg N ha^{-1} yr^{-1}, respectively. Thus, the fertilization treatment consistent of three levels: (1) a low nitrogen load with no additional nitrogen (T0), (2) a moderate nitrogen load (T70) and (3) a high nitrogen load (T150). Because of a low initial seed number and insufficient germination- and establishment rate, for a number of populations not enough offspring could be raised to ensure a fully balanced design (Table 1). Pots were arranged randomly in the greenhouse.

Table 1 Location of studied *Juncus effusus* populations, sample sizes used in the common garden experiment, lineage membership according to STRUCTURE and expected heterozygosity (H_e).

ID	Population	Latitude (°N)	Longitude (°E)	No. of seed families / total no. of individuals			Population structure Q values			H_e
				T0	T70	T150	Eff1	Eff2	Eff3	
BB	Brandberge	51.5117	11.9263	4 / 16	-	4 / 12	**0.975**	0.023	0.003	0.359
DB	Drübeck	51.8531	10.7057	5 / 14	-	-	0.116	**0.877**	0.007	0.356
EB	Ettersberg	51.0320	11.2638	5 / 20	4 / 11	4 / 12	0.001	**0.996**	0.003	0.181
ES	Esperstedt	51.4192	11.6676	6 / 24	4 / 12	4 / 12	0.001	**0.998**	0.001	0.042
ET	Elendstal	51.7475	10.6810	6 / 24	4 / 12	4 / 12	0.001	0.002	**0.997**	0.133
GF	Gräfenroda	50.7438	10.7914	6 / 24	4 / 12	4 / 12	**0.997**	0.002	0.001	0.256
GR	Groß Rosenburg	51.9124	11.9178	5 / 14	-	-	0.002	**0.994**	0.004	0.144
JAM	Jävenitzer Moor	52.5029	11.4720	5 / 19	4 / 12	4 / 12	**0.986**	0.003	0.011	0.275
JEM	Jemmeritzer Moor	52.6365	11.2620	6 / 24	4 / 12	4 / 11	**0.989**	0.009	0.002	0.302
MS	Massanei	51.0587	13.0458	5 / 20	-	4 / 12	0.005	**0.989**	0.006	0.075
OH	Oberhof	50.7150	10.7769	5 / 17	-	4 / 12	**0.993**	0.005	0.002	0.197
PWA	Pressel Wald	51.5739	12.7381	5 / 20	4 / 12	4 / 12	**0.924**	0.022	0.055	0.341
PWI	Pressel Wiese	51.5655	12.7322	5 / 20	4 / 12	4 / 12	0.021	**0.941**	0.038	0.194
RB	Rappbodetalsperre	51.7407	10.8875	5 / 16	-	-	0.002	0.002	**0.996**	0.027
RO	Rösa	51.6114	12.4493	5 / 20	4 / 12	4 /12	0.003	**0.981**	0.017	0.203
SC	Schierke	51.7732	10.6388	6 / 22	-	-	0.002	0.002	**0.996**	0.250
SF1	Siptenfelde	51.6605	11.0484	4 / 16	-	-	**0.996**	0.002	0.002	0.188
SF2				5 / 20	-	-	0.002	**0.997**	0.001	0.050
WD	Wermsdorf	51.3017	12.9030	4 / 15	-	4 / 12	0.001	**0.988**	0.011	0.144
WL	Wörlitz	51.8336	12.4348	5 / 20	4 / 12	4 / 12	0.024	**0.967**	0.010	0.181
ZM	Zella Mehlis	50.6722	10.6739	5 / 20	4 / 12	4 / 12	**0.997**	0.001	0.002	0.000
ZR	Ziegelroda	51.3455	11.4931	4 / 14	4 / 12	4 / 12	0.002	**0.996**	0.002	0.198

At the start of the experiment, initial plant biomass was indirectly assessed as plant height (H), i.e. the mean height of the two longest leaves, times the number of stems (S). We repeated this measurement at the end of experiment to estimate a relative growth rate ($RGR = (H*S)_{end} - (H*S)_{start}) / (H*S)_{start}$). Ten weeks after initiating the treatment, all plants were harvested. For each individual, the weight of the above- (AGBM) and belowground biomass (BGBM) was determined after drying at 60 °C for 48 h. A shoot to root ratio was calculated as the quotient between AGBM and BGBM. To assess the biomass quality, we calculated leaf dry matter content (LDMC) as dry divided by fresh mass. Two individuals per seed family and treatment were randomly selected for chemical analyses. A representative fraction (~ 10 mg) of above- and belowground biomass was milled and C and N concentrations were *measured using* a CHNS/O elemental analyzer (Vario EL III, Element Analyzer, Elementar, Hanau, Germany). From these data, we calculated C:N ratios for above- (AG-C:N) and belowground biomass (BG-C:N) as well as total aboveground N accumulation (AG-N) as N content per g leaf tissue multiplied by the dry weight. The same subset of individuals was also used for pH measurement of the pot soil using the method described by Hoffmann (1991). Air-dried and sieved (2 mm) soil samples were suspended in a ratio 1:2.5 with 0.01 M $CaCl_2$ solution and analyzed using a pH Meter (Knick pH-Meter 766 Calimatic, Berlin, Germany). Root porosity was estimated using the microbalance method described in Visser and Bögemann (2003). Eight weeks after the start of the experiment, we extracted for each individual three root tips. Ten mm behind the root apex, a 30 mm long root segment was excised and surface water was carefully dried with tissue paper and the weight of the root sample was determined. Samples were then placed in a water-filled 1.5 ml Eppendorf tube and put under vacuum conditions for 5 min and infiltrated root segments were weighed again. Root porosity was calculated as the weight difference relative to the weight after infiltration and multiplied by the specific weight for *J. effusus* roots given in Visser and Bögemann (2003). The three values obtained were averaged to obtain an individual estimate.

Genetic diversity and population structure

For the microsatellite data, we used a principal coordinate analysis (PCoA) computed in GenAlEx (Peakall & Smouse 2012) to visualize genetic similarities among *J. effusus* individuals (Fig. S1). Population structure was determined using a Bayesian model-based clustering approach implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003; Falush *et al.* 2007). An admixture model with correlated allelic frequencies was used to determine the most likely number of clusters (K). We used 10 independent runs each with 150,000 iterations of which 50,000 were discarded as burn-in. The optimum K was identified using the approach of Evanno *et al.* (2005) implemented in the web program STRUCTURE HARVESTER 0.9.94 (Earl & vonHoldt 2012). Individuals were assigned to lineages based on individual assignment probabilities (Q values > 0.7). For each population expected heterozygosity H_e (Nei 1987) and global genetic differentiation (F_{ST}, (Nei 1987)) among populations and lineages identified above was calculated using FSTAT (Goudet 1995, Version 2.9.3.2).

Data analyses

To assess the quantitative trait expression in response to treatment and lineage, we used a linear mixed effect model implemented in package 'lme4' (Bates *et al.* 2014) for R (R Development Core Team 2014) explaining the observed variation in each trait separately by N supply, lineage identity and the lineage × treatment interaction as fixed effects, and seed family and their interaction with treatment as random effect. Subsequently we did post hoc analyses to test for pairwise differences among treatments and lineages within treatment.

For each quantitative trait genetic differentiation among populations was estimated as $Q_{ST} = V_{AP} / (V_{AP} + 2 V_{WP})$ (Spitze 1993) by partitioning the total phenotypic variance into variance among- (V_{AP}) and within (V_{WP}) populations, with the latter given by the seed family component. Variance components were derived from generalized linear mixed models using a Bayesian framework implemented in the package 'MCMCglmm' (Hadfield 2010) for R (R Development

Core Team 2014). Bayesian approaches have been recommended for Q_{ST} estimation as they allow for flexible experimental designs and return mostly unbiased precision estimates (O'Hara & Merilä 2005; Ogle & Barber 2012). For each trait we calculated (1) an across-treatment Q_{ST} for all populations using models including treatment as fixed, and population and seed family as random effects, and (2) treatment-specific Q_{ST} values with only population and seed family as random effects. And (3) we calculated lineage-specific across-treatment Q_{ST} values. For (3) Q_{ST} values were calculated for only two out of three genetic lineages found (see below), as sample size for the third lineage was very low (see results). Credibility intervals for all estimates were directly taken from the posterior distribution.

To test whether quantitative genetic differentiation in mean traits (Q_{ST}) differed from neutral expectations we followed the approach of Whitlock and Guillaume (2009) by reporting the difference between the observed Q_{ST} and a simulation expected under neutrality (Q^n_{ST}). A significant positive or negative deviation from zero directly indicates directional or stabilizing selection, respectively. The distribution of Q^n_{ST} values was calculated by simulating a neutral among-population variance 1000 times as $V^n_P = F_{ST} * (2 * V_A / (1 - F_{ST}))$ and then multiplying by a factor $r / (n_{pop} - 1)$ with n_{pop} the number of populations considered and r being a random number drawn from a chi² - distribution with $n_{pop} - 1$ degrees of freedom, to simulate the sampling distribution around this expectation. For each simulation, the F_{ST} value for the respective set of populations was used and a V_A value was sampled from the posterior distribution of the models described above. Q^n_{ST} values were then computed using the observed within-population variance. The test statistic was calculated as the difference between 1000 Q_{ST} values drawn from the posterior distribution of the model and 1000 simulated Q^n_{ST} values and considered to be significant if the 95% credible interval did not include zero.

In order to assess whether variability and differentiation in trait expression relates to soil environmental characteristics of population origin we performed several analyses. (A) Soil environmental parameters were reduced by applying a principal component analysis (PCA) on

standardized soil parameters using function prcomp() from 'stats' package for R (R Development Core Team 2014). Then, the first two axes scores accounting for the majority of variation (43% and 30% of the total variation, respectively; Fig. S2) were used separately to explain trait expression in response to (1) soil environment of the region of origin in dependence of experimental N supply and (2) soil environment in dependence of intraspecific lineage affiliation for each treatment conditions separately to test for potential lineage-specific patterns. For (1) we used linear mixed effect models with PCA axis scores and experimental treatment as fixed and lineage, population and seed family as random effects to account for repeated measurements. For (2) we calculated models for each treatment condition separately explaining individual trait variation by PCA axis scores, lineage and their interaction as fixed and population and seed family as random effects. To account for possible maternal effects mediated by seed size (Roach & Wulff 1987), we repeated all analyses using mean seed mass per population as covariate. Additionally, we tested for relations between expected heterozygosity (H_e) and first axis scores of soil parameters. (B) Variation in pairwise trait differentiation (Q^{ij}_{ST}) between populations was explained by pairwise environmental distances jointly considering neutral genetic differentiation (F^{ij}_{ST}) using a multiple matrix regression approach (Wang 2013). Environmental distances were calculated as Euclidean distances using standardized soil parameters. Trait and molecular genetic differentiation matrices were standardized prior to the analyses in order to allow comparisons of coefficients. Significances for individual regression coefficients were assessed by comparing observations against a null distribution obtained by permuting the dependent matrix 9999 times. This approach was applied to explain (1) across-treatment trait differentiation with the subset of populations present in all treatment conditions (N = 12), and (2) for each treatment condition separately and (3) for each lineage and treatment condition separately.

Results

Genetic diversity and population structure

In a total of 111 individuals, we found 74 different alleles at 16 different microsatellite loci. The expected heterozygosity varied substantially among populations (mean H_e = 0.187, SD = 0.089; Table 1). Genetic differentiation among populations was very pronounced (global F_{ST} = 0.66; SD = 0.120). Bayesian cluster analysis revealed a single most likely solution with samples forming three distinct genetic lineages (K = 3, ΔK = 273.4) subsequently described as lineages Eff1, Eff2 and Eff3, with the latter represented by individuals of only three populations (Fig. 1). All individuals could be unambiguously assigned to either lineage (Q > 0.7). Only one location showed a strong admixture (SF) and samples from this location were assigned to either Eff1 or Eff2 and thus, samples were considered as two populations in the following analyses. Between lineage differentiation was substantial with $F_{ST}^{\text{Eff1-Eff2}}$ = 0.405, $F_{ST}^{\text{Eff1-Eff3}}$ = 0.372 and $F_{ST}^{\text{Eff2-Eff3}}$ = 0.412. Within lineages, genetic differentiation among populations was still very pronounced with F_{ST} = 0.289, 0.385 and 0.614 for Eff1, Eff2 and Eff3, respectively.

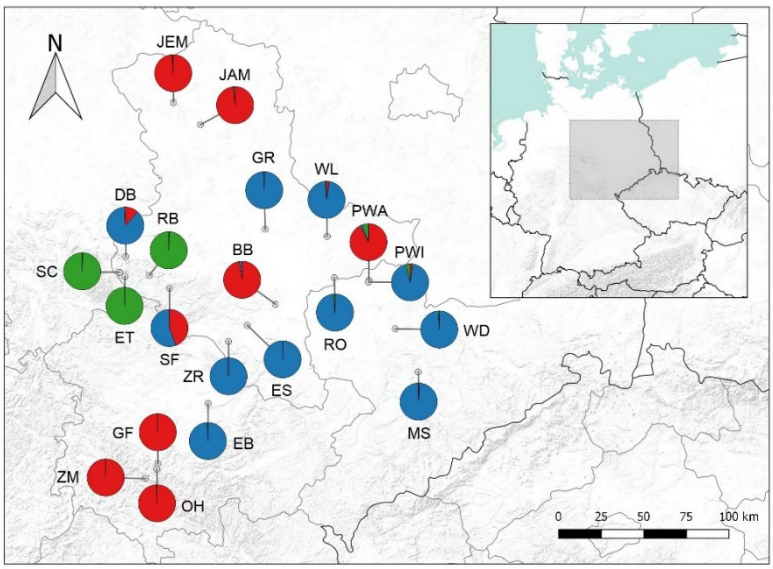

Fig. 1 Locations of the 21 sampled *Juncus effusus* populations and, in colour, lineages membership (red: Eff1, blue: Eff2, green: Eff3). For population acronyms see Table 1.

Effect of N addition on plant trait expression

The overall effect of N addition and lineage on trait expression was assessed for individuals from lineages Eff1 and Eff2 only as sample size for Eff3 was too low. In general, all investigated quantitative traits responded significantly to the N addition treatment, except for root porosity (Fig. 2). As expected traits such as plant height, number of stems, relative growth rate, above- and belowground biomass as well as aboveground N increased under N supply, whereas above- and belowground C:N and Root:Shoot decreased (Fig. 2). Differences between lineages were inconsistent across treatment conditions and mostly expressed in vegetative characters such as plant height and number of stems (Fig. 2).

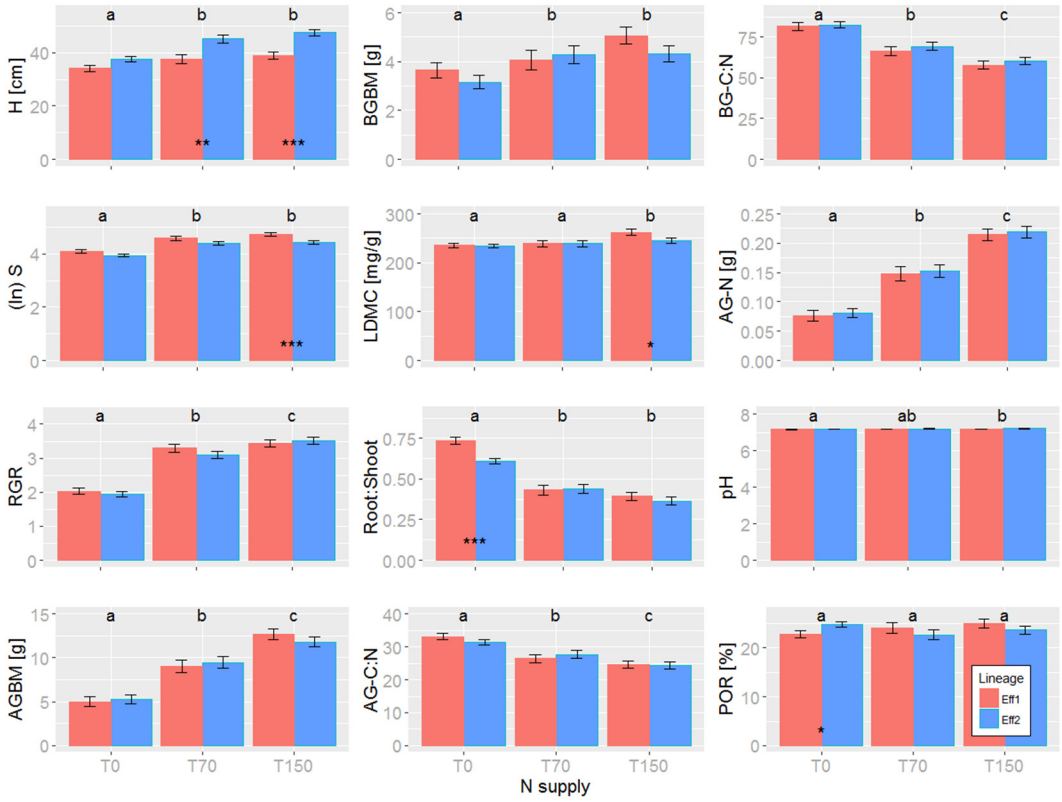

Fig. 2 Quantitative trait expression in response to treatment and lineage membership in *Juncus effusus*. Different letters indicate significant differences among N supplies and asterisks indicate significantly differences ($\alpha < 0.05$) among lineages (red: Eff1, blue: Eff2) within treatments. H: plant height; S: number of stems; RGR: relative growth rate; AGBM: aboveground biomass; BGBM: belowground biomass; LDMC: leaf dry matter content; Root:Shoot: ratio root to shoot; AG-C:N: carbon to nitrogen ratio of aboveground biomass; BG-C:N: carbon to nitrogen ratio of belowground biomass; AG-N: total aboveground N accumulation; pH: soil pH; POR: root porosity.

Quantitative genetic divergence

Quantitative genetic differentiation in mean traits across treatment conditions ranged from Q_{ST} = 0.021 to 0.548 and was substantial for most traits except for relative growth rate, AG-C:N and root porosity (Table S2). Genetic differentiation was inconsistently expressed when treatment conditions were treated separately, most notably for AG-C:N, BG-C:N, above- and belowground biomass, but without a clear trend towards less or more differentiation at a specific N addition level. (Table S2). Genetic differentiation differed between lineages strongly for plant height (Eff1: Q_{ST} = 0.699, Eff2: Q_{ST} = 0.221) and LDMC (Eff1: Q_{ST} = 0.992, Eff2: Q_{ST} = 0.067), whereas for most other traits differentiation patterns were more similar (Table S2). The observed genetic differentiation for the measured traits did not significantly exceed neutral expectations when compared across treatments or for each treatment and lineage separately (Fig. 3, Fig. S3, Fig. S4). Differentiation lower than expected under neutrality was found in several comparisons, most often for relative growth rate but also for soil pH, root to shoot ratio or aboveground N accumulation.

Expected heterozygosity at population level significantly increased with PCA scores of the first axis only (r = 0.654, P < 0.05), which mainly represented sand and clay content and cation exchange capacity.

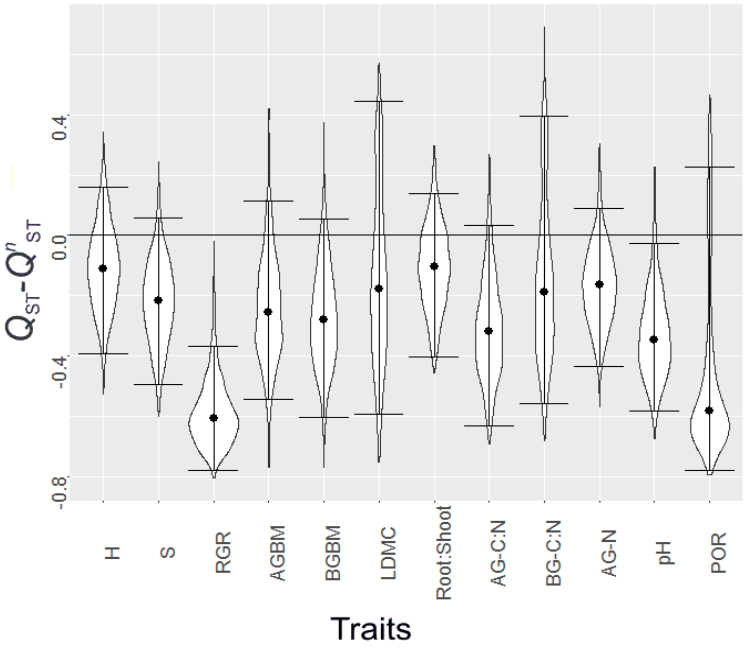

Fig. 3 Violin plots showing the comparison between quantitative genetic differentiation among populations (Q_{ST}) across treatment conditions and a neutral expectation (Q^n_{ST}) for all measured plant traits. Dots indicate the posterior median of the difference and bars the 95% credibility interval. A signature of directional or balancing selection is indicated by a significant deviation from the zero expectation. For trait explanations see Fig. 2.

Trait clines with soil environments

Overall mean trait expression at population level varied with soil environmental variation expressed as PCA axis 1 for above- and belowground C:N ratio (Fig. 4), number of stems as well as LDMC and belowground biomass (Table S3). Overall, trait expression did not co-vary with PCA axis 2. These results were not altered by the inclusion of seed mass as covariate (data not shown). Significant differences between lineages Eff1 and Eff2 were present for some relationships, e.g. slopes of the clines along PCA axis 1 differed for plant height, aboveground N and pH in the pot soil, but were not consistently expressed across the different N addition levels (Table S4, S5).

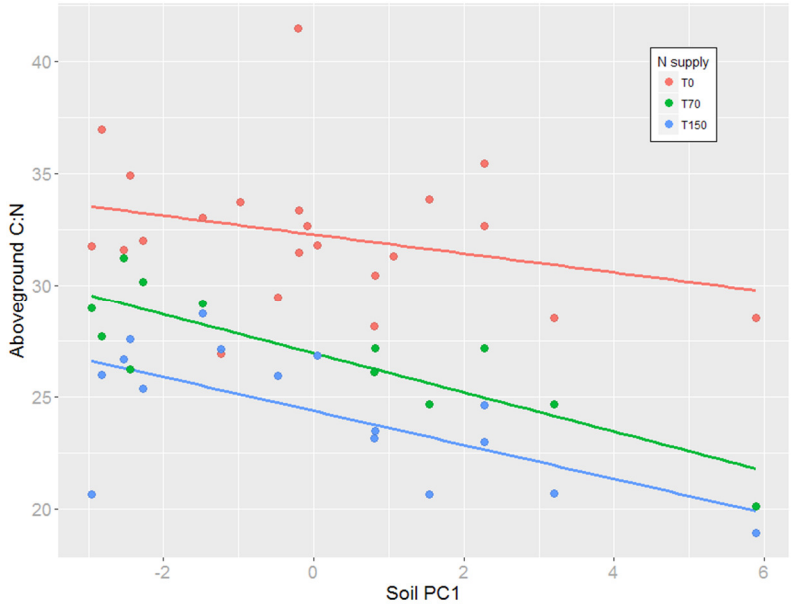

Fig. 4 Correlation between aboveground C:N ratio and soil environmental parameters of population origin (factor scores for the first axis of a PCA on all soil parameters) in dependence of experimental N supply. Note that for visualization only population mean values are plotted. Colours represent the different nitrogen addition levels applied.

Pairwise genetic trait differentiation between treatments increased significantly with distance in soil environment for aboveground C:N, whereas differentiation in plant height and number of stems mirrored neutral genetic differentiation (Table S6). A similar pattern was found when treatment conditions were analysed separately. Treating each lineage separately within treatment conditions, only few significant correlations could be found which were also inconsistently expressed among treatment conditions and the two lineages considered (Table S7).

Discussion

Supporting recent findings by Michalski and Durka (2015), our results show that in Central Europe *Juncus effusus* consists of multiple, genetically well separated lineages that partly co-occur at the same location but show only limited differences in trait expression. It has been suggested, that the co-existence of these genotypic lineages is a result of an allopatric origin with secondary contact and a divergence in flowering phenology could contribute to the maintenance of sympatric lineages (Michalski & Durka 2015).

Effect of N addition on plant trait expression

Nitrogen addition increased plant height, number of stems, relative growth rate as well as above- and belowground biomass in *J. effusus*, which can be expected from previous studies investigating the effect of N fertilization (Xu *et al.* 2006; Macek & Rejmánková 2007). Furthermore, our results showed that N addition increased total aboveground N accumulation and decreased the C:N ratio in leaves and roots, as was also observed in several other studies (Esmeijer-Liu *et al.* 2009; Mao *et al.* 2014). Soil N availability stimulates plant growth and N incorporation into biomass which is well known for terrestrial plant species (Xia & Wan 2008). Surprisingly, in our study *J. effusus* showed a weak but significantly higher LDMC under the highest N availability compared to the other treatment conditions. In general, LDMC correlates negatively with relative growth rate (Cornelissen *et al.* 2003) which in turn is positively affected by increased N availability as shown by our results (but see e.g. (Mao *et al.* 2014)). Often, light competition induced by N enrichment will decrease LDMC and in turn may increase the SLA ('specific leaf area'). However, all individuals experienced similar sunlight conditions and light competition because treatments were applied arbitrarily across pots. Furthermore, we found a greater above- than belowground growth under N addition indicated by a significantly lower root to shoot ratio in N treatments. Changes in allocation in response to N concentration expected and consistent with other studies (Poorter *et al.* 2012). Whereas N limitation may stimulate root growth to increase nutrient uptake and N addition may lead to a shift of N from

belowground biomass to leaf biomass because of a N demand for physiological activities in leaves, e.g. for competition for light (Xia & Wan 2008). Higher root porosity under low N conditions can increase the remobilization of nutrients (Lai *et al.* 2011) and thus, improve N acquisition and plant growth under N limitations (Postma & Lynch 2011; Saengwilai *et al.* 2014). However, compared to similar experiments on wetland macrophytes, in our study root porosity of *J. effusus* did not significantly decrease with N addition (cf. Born and Michalski (2017)).

Quantitative genetic divergence

We found high differentiation among populations and lineages at molecular and quantitative trait level confirming earlier results (Michalski & Durka 2015; Born & Michalski 2017), which are probably related to the life history of *J. effusus*. The species is an efficient pioneer species and colonizer due to a fast growth rate (Ervin & Wetzel 2002), high seed production (Stockey & Hunt 1994) and a predominantly selfing mating system (Buchenau 1892; Michalski & Durka 2015). Both frequent founder events and selfing mating system are expected to reduce effective population size and increase genetic drift effects. Consequently, at population-level, genetic variation can be reduced associated with strong genetic differentiation among populations (Frankham *et al.* 2002).

Indeed, Q_{ST}-estimates for *J. effusus* under N addition were exceptionally high for some traits such as aboveground C:N (T70: Q_{ST} = 0.886) and belowground C:N (T70: Q_{ST} = 0.860, T150: Q_{ST} = 0.941). Similarly, populations of the predominantly selfing *Senecio vulgaris* showed a strong degree of quantitative trait divergence for growth and life history traits (Q_{ST} = 0.26 – 0.77, Steinger *et al.* (2002)). The partially self-fertilizing species *Arabis fecunda* even showed an average Q_{ST} of 0.94 for morphological traits (McKay *et al.* 2001). A meta-analysis of quantitative trait divergence revealed that vast majority of the Q_{ST} values typically exceeds neutral expectations based on molecular markers indicating a predominant role of divergent selection in shaping quantitative trait differentiation (Leinonen *et al.* 2008). In our study, the

average Q_{ST} of 0.36 across all populations and treatments was comparable to the average of Q_{ST} = 0.35 reported by Leinonen *et al.* (2008). However, when compared to neutral expectations, signatures of adaptive differentiation could not be found for any of the traits assessed in our study. For most traits differentiation did not differ from neutral expectations and for some traits (e.g. soil pH or relative growth rate) differentiation patterns rather showed evidence for stabilizing selection ($Q_{ST} < Q^n_{ST}$). Neutral differentiation and stabilizing selection as causes for quantitative trait differentiation is often found for rare species with small population sizes and high level of habitat fragmentation and isolation e.g. *Liatris scariosa* (Gravuer *et al.* 2005), *Primula sieboldii* (Yoshida *et al.* 2008) and *Psilopeganum sinense* (Ye *et al.* 2014). It has been further argued that the ecological niche of rare species is restricted causing a homogenous selection pressure, resulting in a relatively low quantitative trait divergence (Petit *et al.* 2001). However, the ecological niche and distributional range of *J. effusus* is rather broad questioning the importance of stabilizing selection for trait expression in this species.

Quantitative trait differentiation estimated by Q_{ST} can be biased, possibly limiting the conclusions that can be drawn from Q_{ST} - F_{ST} comparisons. First, maternal effects may affect trait expression in general and can bias Q_{ST} estimates downwards (De Kort *et al.* 2013) which has to be considered particularly for early life traits like initial growth and survival (Hernández-Serrano *et al.* 2014). In our study, the majority of traits were measured at the end of the growing season on adult individuals reducing the probability of such a bias. Still, maternal effects cannot be ruled out completely as shown by the effects of seed size on treatment specific trait clines (Table S4, S5). Second, non-additive genetic effects may also decrease Q_{ST} estimates possibly resulting in $Q_{ST} < F_{ST}$ outcomes without stabilizing selection. Whereas dominance effects for inbred species such as *J. effusus* might be of less importance (Goudet & Büchi 2006), epistatic and pleiotropic effects may introduce a bias which is often neglected in the Q_{ST} - F_{ST} approach. However, for our study this bias is not very likely as it would lead to false positive signatures of adaptive divergence only. The use of microsatellite data for Q_{ST} - F_{ST}

comparisons has been criticized because the potentially large number of alleles may result in downwardly biased F_{ST} estimates (Hedrick 2005). Instead, the use of SNP data has been recommended. Indeed, preliminary results for SNP genotyping using same set of populations and individuals and a small set of loci (N = 32) resulted in a higher overall F_{ST} estimate (F_{ST} = 0.823 (SNP) vs. F_{ST}= 0.660 (Microsatellite); Born, unpublished data). But this would only query positive signatures of adaptation that were not found in this study.

Trait clines with soil environments

Genetically based phenotypic trait variation along environmental or geographical clines has been reported for many plant species (Carlson *et al.* 2011; Liu *et al.* 2016; Michalski *et al.* 2017) and is considered to be a signature of adaptation (but see Colautti & Lau 2015). Whereas a plant's response to climatic conditions, latitude or elevation of origin is frequently studied, the impact of soil properties on adaptive trait expression has been much less investigated (Macel *et al.* 2007). Here, we found that at population-level mean trait expression as well as pairwise trait differentiation for several traits (e.g. AG-C:N, Root:Shoot ratio or plant height) correlated significantly with soil environmental data of the site of origin and distances, respectively, suggesting adaptive trait variation in response to soil characteristics.

However, these correlations were not consistently found for these traits when data of the different N concentrations applied was analyzed separately, suggesting that in different environments the (genetic) basis for trait expression can differ substantially (Hoffmann & Merilä 1999). In the two N addition treatments, most consistently aboveground C:N ratio showed patterns of adaptive trait variation and differentiation. Plants originating from poorer, sandier soils with less capacity to hold exchangeable cations (CEC) expressed significantly higher C:N ratios as compared to plants from more fertile soils. Indeed, it is known from field observations that the aboveground C:N ratio of herbaceous plants increases when nutrient availability in the soil becomes more limited (Di Palo & Fornara 2015). Such responses have been explained by ecophysiological mechanisms of carbon (re-)allocation (Hermans *et al.* 2006). Our findings

suggest that these mechanisms are not purely plastic for *J. effusus* (cf. Liu *et al.* 2016), but are selectively modified by local soil conditions.

We found only little and inconsistent evidence for selective mechanisms to vary between the lineages within *J. effusus* which would be indicated by significant interactions between lineage and soil environmental conditions of origin when explaining population mean traits. This would support the idea that the lineages within *J. effusus* are the result of neutral divergence following e.g. variance (Michalski & Durka 2015).

In summary, increased effects of genetic drift and limited gene flow for the selfing colonizer *J. effusus* resulted in a very pronounced neutral genetic differentiation with few differences between lineages within the species. Adaptive trait differentiation in response to soil environmental conditions might still be present as indicated by significant trait clines but could not be detected by Q_{ST} - F_{ST} comparisons.

Supplementary data and acknowledgements

Table S1 Characteristics of two newly developed microsatellite markers in *Juncus effusus* including repeat motif, primer sequence for forward- and reverse primer, reaction mixture using universal fluorescent-labeled tailed primers, allelic size range and accession number in gene bank.

Table S2 Quantitative trait divergence (Q_{ST}) among studied *Juncus effusus* populations across treatments, for the different N addition level (T0, T70 and T150) and for lineages Eff1 and Eff2 across treatments separately. Estimates in bold are deemed significantly different from zero (lower CI 95% > 0.1).

Table S3 Effects of soil environment of the source location (measured as the first and second axis of a principal component analysis of all soil parameters, PC1 and PC2) and experimental nitrogen addition and their interaction on mean quantitative trait expression in *Juncus effusus*.

Table S4 Effects of soil environment of the source location (measured as the first axis of a principal component analysis of all soil parameters, PC1) and lineage membership and their interaction on mean quantitative trait expression in *Juncus effusus*.

Table S5 Effects of soil environment of the source location (measured as the second axis of a principal component analysis of all soil parameters, PC2) and lineage membership and their interaction on mean quantitative trait expression in *Juncus effusus*.

Table S6 Multiple matrix regression with randomization analysis (MMRR) explaining pairwise trait differentiation between populations (Q^{ij}_{ST}) jointly by (A) pairwise soil environmental distance and (B) pairwise neutral genetic differentiation (F^{ij}_{ST}) for the subset of 12 *Juncus effusus* populations across treatment conditions and for each treatment T0 (N = 22), T70 (N = 12) and T150 (N = 16) separately.

Table S7 Multiple matrix regression with randomization analysis (MMRR) explaining pairwise trait differentiation between populations (Q^{ij}_{ST}) jointly by (A) pairwise soil environmental distance and (B) pairwise neutral genetic differentiation (F^{ij}_{ST}) for the lineages Eff1 and Eff2 and treatment conditions (T0, T70 and T150) separately.

Fig. S1 Principal coordinate analysis (PCoA) plot based on pairwise genotypic distances among individuals. Circles represent individuals grouped in three distinct clusters indicated by colors (red: Eff1, blue: Eff2, green: Eff3).

Fig. S2 The Principal component analysis (PCA) of 11 soil variables for 22 *Juncus effusus* populations. Grey arrows indicate loadings of each soil variable on the two axes (particle size distribution: clay-, silt- and sand content, coarse fragments, pH (CaCl2), organic carbon (OC), potassium- (K), phosphorus- (P), carbonate- (CACO3) and total nitrogen (N) content and cation exchange capacity (CEC)), while colored symbols represent populations and their underlying genotype (red: Eff1, blue: Eff2, green: Eff3) in the environmental space.

Fig. S3 Violin plot shows the comparison of quantitative genetic divergence (Q_{ST}) and with the expected distribution under neutrality (Q^{n}_{ST}) for each treatment separately (A: T0, N = 22; B: T70, N = 12 and C: T150, N = 16).

Fig. S4 Violin plot shows the comparison of quantitative genetic divergence (Q_{ST}) and with the expected distribution under neutrality (Q^{n}_{ST}) within detected lineages Eff1 (A, N = 8) and Eff2 (B, N = 11).

Supplementary data available online after publication.

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CHAPTER 4

Genetic Analyses Reveal That Multi-Facetted Processes are Shaping the Intraspecific Differentiation Patterns of the Widespread Wetland Plant *Juncus effusus* in Europe

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Manuscript

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Abstract

Understanding natural processes driving spatial genetic variation and population structure is a fundamental issue in ecology and evolutionary biology. One effective method is to assess the relative importance of geographic and environmental variables on the genetic structure of populations. Here we studied the genetic differentiation of 128 *Juncus effusus* populations across Europe using 16 microsatellite loci as well as environmental and geographical variables. To test the patterns of isolation-by-distance (IBD) and isolation-by-environment (IBE), we used a multiple matrix regression approach (MMRR) that explains the genetic differentiation between populations by spatial, soil and climatic distances. Additionally, linear and curvilinear relationships of unbiased expected heterozygosity with local environmental conditions and spatial coordinates were tested. Our results revealed the presence of three genetically well-differentiated lineages within *Juncus effusus*. The lineage Eff3 differed notably from the other lineages by clines in population genetic diversity with environmental conditions and by a dominant occurrence at higher altitudes suggesting more particular ecological responses compared to lineages Eff1 and Eff2. A strong correlation between genetic structure and spatial distance was found within each lineage, whereas all lineages showed differing relative contributions of climate and soil conditions to pairwise molecular differentiation. These findings suggest that geography plays a dominant role in explaining genetic variation among populations, but environmental factors may also contribute to spatial genetic structure in *Juncus effusus*.

Introduction

Understanding processes driving the distribution of genetic diversity and population divergence and hence the genetic structure of species is fundamental to ecology and evolutionary biology. Physical barriers and geographical distances limiting gene flow are the most common explanations for genetic structure and hence, functional and ecological divergence among populations (Rieseberg & Willis 2007). If gene flow and the effects of genetic drift because of dispersal limitation are in equilibrium, genetic differentiation between populations will increase with spatial distance, a pattern firstly described by Wright (1943) as 'isolation-by-distance' (IBD). However, the distribution of genetic diversity across a species' range can be affected additionally by heterogeneous environmental conditions throughout this range via several non-mutual exclusive processes (Lira-Noriega & Manthey 2013). For example, populations in more extreme environments are expected to experience more stochastic reduction of genetic diversity because of more rapid cycles of extinction and recolonization and associated bottlenecks than in more favourable conditions (Eckert *et al.* 2008). Although formulated mostly in the context of species geographic ranges, this hypothesis should hold true for environmental conditions in general leading to a decline of genetic diversity at the limits of a species' environmental range. In addition, local adaptation, the increase of locally beneficial genes in a specific environment may shape genetic structuring, as it reduces effective gene flow by natural selection against immigrants from different environments (Andrew *et al.* 2012; Sexton *et al.* 2014) leading to 'isolation-by-environment' (IBE). Disentangling neutral and putatively non-neutral environmentally driven effects on genetic structuring is often challenged by the correlation of spatial distances with environmental parameters which in turn can interrelate among each other. For example, habitat and soil type can be linked with gradients of temperature, precipitation, soil characteristics and vegetation density (Lee & Mitchell-Olds 2011; Andrew *et al.* 2012; Gray *et al.* 2014). To add even more complexity, also postglacial recolonization patterns can imprint on observed genetic population structure (Nadeau *et al.* 2016). Repeated founder events and secondary contact from previously separated lineages

during postglacial recolonization can develop genetic structure in gene frequencies similar to IBD and IBE patterns (de Lafontaine *et al.* 2013). Hence, the distribution of genetic diversity within a species is shaped by many processes whose interactions are not always clear or easy to identify.

Wetland ecosystem can be found in an incredible variety of types shaped by different hydrological and geomorphological factors (van der Valk 2006). Minor changes in these factors affect the diversity of vegetation types and may require adaptation of species and populations to survive in wetlands. Particularly one feature makes wetland soils unique and differing from terrestrial systems – anaerobic soils. Not all wetland soils are anaerobic, but oxygen is often absent or only available in low concentrations due to permanently or seasonally saturation by water, which required specific morphological adaptations like formation of interconnected gas spaces from leaves to roots to ensure gas exchange within plants (Armstrong 1980). Thus, soil heterogeneity promotes a variety of morphological physiological plant responses and can act as important agent of selection (Pregitzer *et al.* 2010).

However, evidence for adaptive genetic differentiation driven by environmental conditions has been most often demonstrated for climatic conditions (e.g. Durka *et al.* 2017). Whether and how other environmental factors such as soil properties affect differentiation patterns is much less investigated. In one of rare example, Misiewicz and Fine (2014) found for the Amazonian tropical tree *Protium subserratum* a higher level of genetic differentiation between adjacent populations in different soil types than between geographically more distant populations of same soil type. For several wetland plants, a pronounced genetic differentiation among populations was documented indicating limited gene flow among often unconnected wetland habitats in terrestrial landscapes (Barrett *et al.* 1993; Santamaría 2002; Nies & Reusch 2005). Hence, a strong increase in genetic differentiation with increasing by geographic distance between populations may be common for wetland plants. When ecological characteristics in wetland ecosystems differ, gene flow may also be reduced between

populations that are differently adapted (Nosil *et al.* 2005), but only few studies examined the role of environmental variation on genetic structure in wetland plants (but see Zhou *et al.* 2013).

Here we investigate the large-scale genetic population structure of the wetland plant *Juncus effusus* ssp. *effusus* which is native in Europe and large parts of Asia and Northern Africa (Kirschner *et al.* 2002). It occurs in a wide range of wetlands exhibiting a broad ecological tolerance of wet, acidic and nutrient-poor soil conditions (McCorry & Renou 2003). *Juncus effusus* is a model species for research on wetland ecosystems functioning and well characterized by a number of studies focussing on ecological aspects like production and germination of seeds (Lazenby 1955; Ervin & Wetzel 2001), growth rate and biomass production (Wetzel & Howe 1999) and litter decomposition (Gingerich & Anderson 2011) as well as on functional aspects like uptake and metabolization of nitrogen (Smialek *et al.* 2006), phosphorus (DeBusk *et al.* 1995) and heavy metal (Mays & Edwards 2001) or associated soil microbial activity (Moran & Hodson 1989; Nikolausz *et al.* 2008). Like many other species in this genus, *Juncus effusus* is a predominantly selfing species (Buchenau 1892; Michalski & Durka 2015) with a high seed production (Stockey & Hunt 1994) rendering the species an efficient colonizer. These properties are known to reduce effective populations sizes, increase genetic drift and population differentiation and may even cause a fixation of alleles within populations (Frankham *et al.* 2002; Ingvarsson 2002). Indeed, molecular data –though on few populations only- is indicating the existence of several genetically well separated but partly sympatrically co-occurring lineages within the species (Michalski & Durka 2015). However, potential lineage-specific patterns of trait diversity have not been considered so far, except for a recent study revealing strong genetic differentiation among *Juncus effusus* populations in quantitative trait expression (Born & Michalski 2017).

Here the following questions are addressed with the use of a molecular approach and the modelling of species distribution:

1. How is genetic diversity of *J. effusus* structured across Europe?
2. How is diversity and structure affected by environmental drivers such as climate and soil conditions?
3. Does local adaptation to soil or climatic conditions contribute to observed population structure?

Material and Methods

Study area and populations

Between 2010 and 2015, leaf material was collected from 128 locations representing the distributional range of *Juncus effusus* in Europe (Table S1). From each location, individuals were selected arbitrarily across the whole site. Sampled leaf material was dried with silica gel and stored in paper bags for future DNA extraction.

For each location, soil environmental variables (particle size distribution: clay-, silt- and sand content, coarse fragments, soil pH, organic- and inorganic compounds: organic carbon and carbonate, phosphorus-, nitrogen- and potassium content, and cation exchange capacity) were extracted from the LUCAS topsoil dataset (Tóth *et al.* 2013) as averaged values across all data points available within a radius of 15 km of each location (1 - 9 data points per location, mean 3.6). Hence, soil parameters reflect conditions at the respective regional scale rather than exact local conditions. The LUCAS topsoil database did not cover soil conditions for all locations (e.g. Serbia, Ukraine). Hence, these soil properties were recorded for 112 populations. Additionally, for each location bulk density and available water capacity of the top soil layer were extracted from the world soil database (Wieder 2014). Bioclimatic variables (BIO1-BIO19) for each location were extracted from the WordClim database at a raster of 2.5 min (Fick & Hijmans 2017).

Genotyping

Leaf material from 1 – 10 individuals per location (in total N = 1048) was used to extract genomic DNA as described in manufacturer's protocol using the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany). DNA concentration was checked by gel electrophoresis and NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA). A suitable set of microsatellite loci was used for genotyping (Table S2).

Amplification reactions were done using directly fluorescent-labeled primers or primers with universal fluorescent-labeled M13R and CAG tails (Schuelke 2000). For amplification with directly fluorescent-labeled primers reactions contained in a total volume of 8 µl: 1 µl genomic DNA (~ 20 ng / µl), 4 µl QIAGEN Multiplex Mastermix, 1.2 µl RNase-free H_2O, 0.8 µl QIAGEN 5 × Q-Solution and 0.2 µM of each forward and reverse primer. The PCR was run on an Eppendorf Thermal cycler with the following PCR program: 95 °C for 15 min; followed by 35 cycles of 95 °C for 30 s; 58 °C for 40 s and 72 °C for 1 min, followed by 72 °C for 15 min. The PCR mix for M13R or CAG tailed primers contained of 1.0 µl genomic DNA (~ 20 ng / µl), 2.5 µl QIAGEN Multiplex Mastermix, 0.05 µM of the M13R or CAG tagged primer, and 0.25 µM of each untagged and fluorescently labelled M13R or CAG primer in in final 5 µl reaction volume. A touchdown PCR was run as follows: 95 °C for 15 min; followed by 20 cycles (94 °C for 30 s, 60 °C for 30 s with decrease of 0.5 °C per cycle, 72 °C for 90 s), followed by 20 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s) and final step with 72 °C for 10 min. Fragment analysis was performed on an ABI 3130 (Applied Biosystems, Foster City, California, USA) with a formamide and size standard LIZ 500 (Applied Biosystems) mixture and using GeneMapper v.5 (Applied Biosystems) software for genotyping.

Statistical analyses

Population structure and genetic diversity

The Bayesian model-based clustering program InStruct (Gao *et al.* 2007) was used to assess population structure. InStruct is an alternative implementation of commonly used Bayesian clustering methods allowing partial self-fertilization or inbreeding. To determine the most likely number of clusters (K), ten independent runs were performed for each K (K = 1 – 10) by sampling every 100th iteration out of a total of 500.000 and a burn-in of 150.000 iterations under the admixture model. The optimum number of clusters was determined by the ΔK-method described by Evanno *et al.* (2005). Optimal alignment of the independent runs for the best K was achieved by running CLUMPP (Jakobsson & Rosenberg 2007, version 1.1.2). using the 'Greedy' algorithm testing 1000 random input orders of runs. Individuals were assigned to clusters or referred to lineages hereafter if the individual assignment probability Q was larger than 0.7. As most individuals could be assigned to a lineage unambiguously (see Results), all following analyses were done at the lineage level and a local population was defined as individuals at a certain location belonging to the same lineage. Consequently, some locations harboured more than one population. For each population unbiased expected heterozygosity (uH_E) (corrected for sample size) and lineage specific genetic differentiation estimates were calculated using GenAlEx (Peakall & Smouse 2012).

Testing for differentiation patterns

In order to test for patterns of isolation-by-distance (IBD) and isolation-by-environment (IBE), we used a multiple matrix regression approach (MMRR) as described by (Wang 2013) explaining between population genetic differentiation jointly by spatial, soil and climatic distances. Soil and climatic distances between pairs of populations were calculated as simple Euclidian distances between scaled environmental parameters and analyses were done with 9999 permutations to assess significances. Additionally, we tested for linear and curvilinear relations of unbiased expected heterozygosity (uH_E) with local environmental conditions and spatial coordinates. In order to reduce the number of variables to be tested we performed principal component analysis (PCA) on standardized variables. Then, for each climatic and soil parameter, only the first two PCA axis scores, explaining 71.0% and 46.8% of the total variation, respectively (Fig. 3, Fig. S1), were used to explain unbiased expected heterozygosity for populations with sample size N > 2.

Climatic envelope model

Climatic envelope models of *Juncus effusus* were parameterized A) for the whole European distribution of the species by using occurrence information retrieved from GBIF (gbif.org 20th Nov 2017; doi:10.15468/dl.mciu4b) and B) for each lineage separately by using our own lineage specific occurrence data based on individuals with InStruct Q values > 0.7. For A) from all 465,545 data points retrieved only those with a complete and a coordinate uncertainty of less than 5000 m were retained (N = 344,515). To further reduce this data set we randomly sampled 25 points in every 4° longitude intervals across the whole west to east distribution range in Europe. After removing duplicate entries and those with a minimum nearest-neighbour distance of < 15 km to avoid modelling bias based on spatial autocorrelation issues, and adding our own observations, a total of 350 data points was used for modelling.

Climatic attributes at these occurrence locations were obtained for 19 bioclimatic variables from the WorldClim 1.4 database at a spatial resolution of 2.5 arc-minutes (Hijmans *et al.* 2005 ;available at: http:// www.worldclim.org/). To reduce the number of climatic predictors and to

minimize collinearity, a principal components analysis on the extracted variables was carried out. The cumulative total variance explained by the first 4 principal components analysis (PCA) axes was 91.3% (48.4%, 25.1%, 9.9% and 7.8%, respectively). The climatic predictors with the highest loadings on components 1 – 4 were BIO19 (precipitation of the coldest quarter), BIO1 (annual mean temperature), BIO2 (mean diurnal temperature range) and BIO15 (precipitation seasonality), respectively. Variables having Pearson correlation moments with these predictors above 0.6 were excluded, leaving BIO1, BIO2, BIO10 (mean temperature of warmest quarter), BIO15, BIO18 (precipitation of warmest quarter) and BIO19 as predictors for further analyses. We used MAXENT v. 3.4.1 (Phillips *et al.* 2017) with the default values of the convergence threshold and the maximum number of iterations (500), using repeatedly randomized samples of 50% of the localities for cross-validation model training and testing, respectively ('random seed'). The functions of environmental variables were selected automatically based on considerations of sample size ('auto features'). The beta ('regularization') multiplier was set to 5 to fit smoother curves. The results of 25 cross-validated model runs were averaged and the resulting probabilities projected onto the current climate. The predictive ability of the models was evaluated using the area under the curve of the receiver-operating characteristic (AUC).

Results

Genetic diversity and population structure

Across 1048 samples and using 16 microsatellite loci, in total we detected 121 different alleles with 2 – 14 (mean = 7.6) alleles among loci. Bayesian cluster analysis with InStruct revealed highest DeltaK values (ΔK = 426.3, Fig. S2) for three distinct genetic groups which we refer to as lineages in the following (Eff1, Eff2 and Eff3, Fig. 1A, Table S1). Most individuals (993 out of 1048; 95%) and populations (107 out of 128; 84%) showed only low levels of admixture (assignment probability Q > 0.7). A total of 28%, 30% and 37% of the 1048 individuals were assigned to lineages Eff1, Eff2 and Eff3, respectively. A plausible solution based on the InStruct analysis is also the structuring into four larger groups (ΔK = 104.0), with lineage Eff3 splitting into two subgroups (Fig. 1B, Fig. S3). No clear spatial pattern of lineage membership was evident, except for Western Europe in which lineage Eff1 dominated (Fig. 1A). Within lineages populations exhibited low levels of genetic diversity (Eff1: uH_E = 0.167 (SE = 0.009); Eff2: uH_E = 0.132 (SE = 0.007) and Eff3: uH_E = 0.148 (SE = 0.008), but very strong genetic differentiation among populations with F_{ST} = 0.644 (SE = 0.033); 0.655 (SE = 0.039) and 0.793 (SE = 0.020) for Eff1, Eff2 and Eff3, respectively. Between lineage differentiation revealed a similar moderate level for all comparisons (F_{ST} = 0.317 (SE = 0.056); F_{ST} = 0.224 (SE = 0.045) and F_{ST} = 0.239 (SE = 0.033); for Eff1 - Eff2, Eff1 - Eff3 and Eff2 - Eff3, respectively).

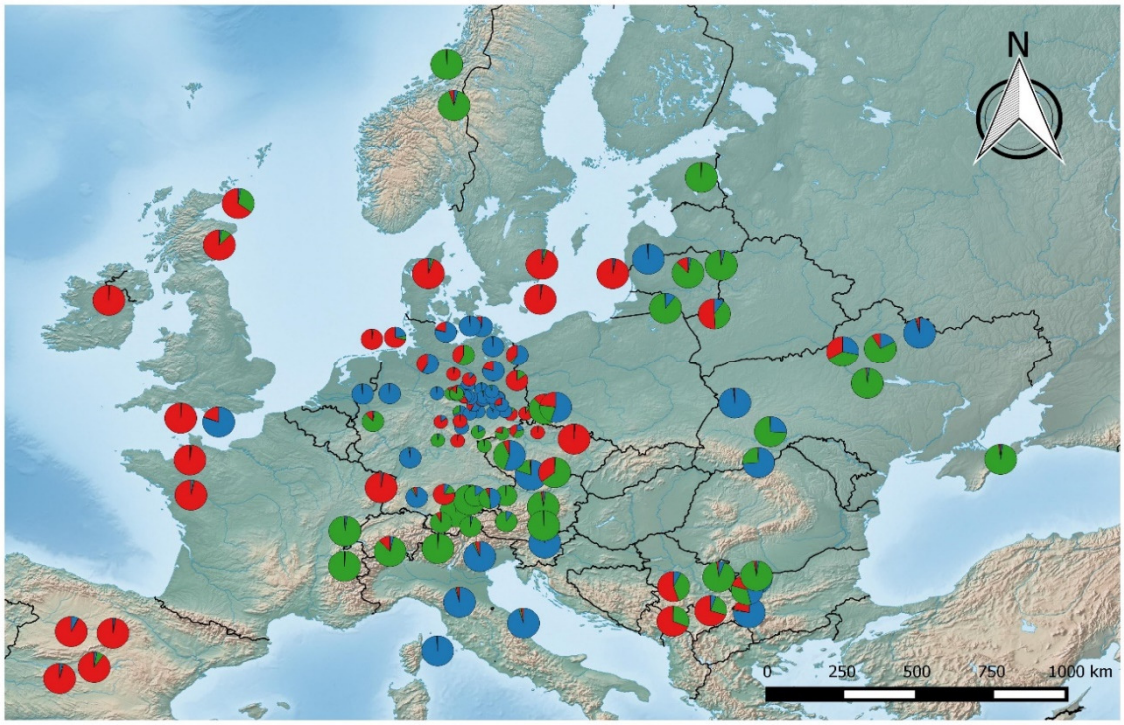

Fig. 1A Spatial distribution of 128 sampled *J. effusus* occurrences and in colour, membership probabilities to lineages identified by InStruct (K = 3; red: Eff1, blue: Eff2, green: Eff3).

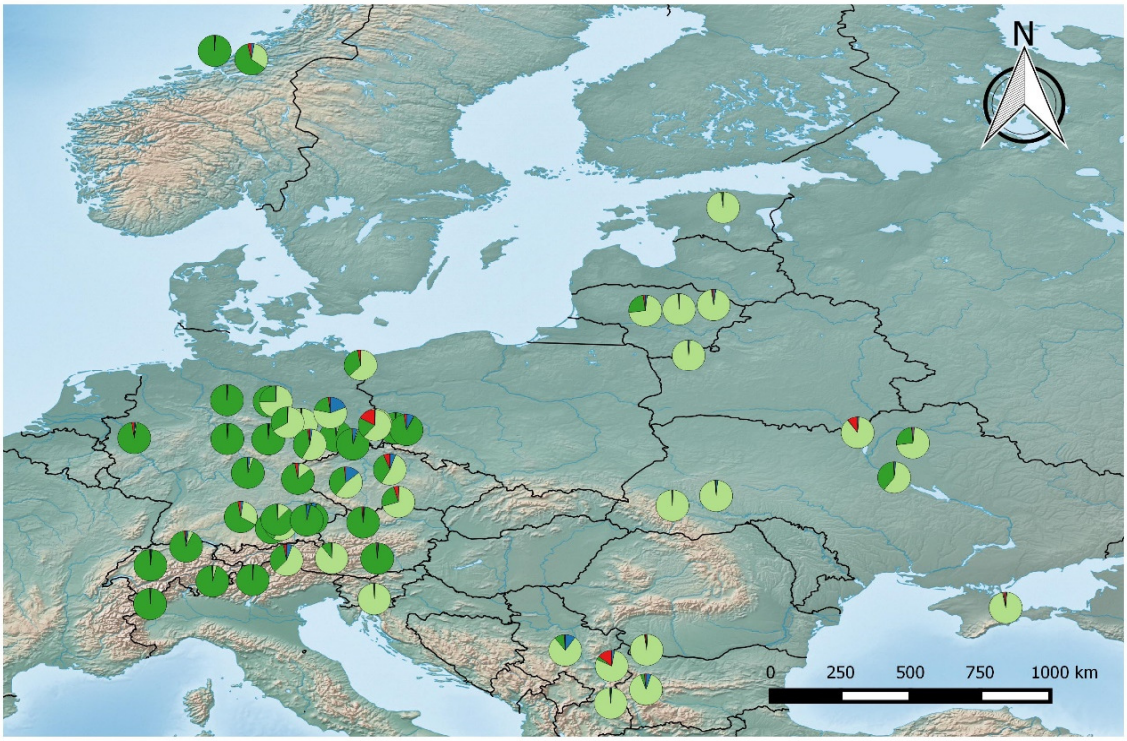

Fig. 1B Spatial distribution of subgroups (dark and bright green) within *Juncus effusus* lineage Eff3 as identified by InStruct with K = 4.

Climatic envelope model

The cross-validation of the climate envelope models revealed a high mean model fit for A) the whole species as well as for B) each lineage separately (AUC =0.970 (SD 0.008), 0.980 (0.016), 0.983 (0.009), and 0.980 (0.006), for the whole species, lineages Eff1, Eff2 and Eff3, respectively. Estimations of the relative contributions of the environmental variables to the Maxent model suggests that annual mean temperature (BIO1), mean diurnal temperature range (BIO2) and precipitation of the coldest quarter (BIO19) are the most important in all cases. Predicted climatic suitability for lineages Eff1 and Eff2 was very similar to each other and to the whole species. Lineage Eff3 differed notably from the other lineages by preferring harsher conditions in more montane, alpine areas and reaching higher latitudes (Fig. 2).

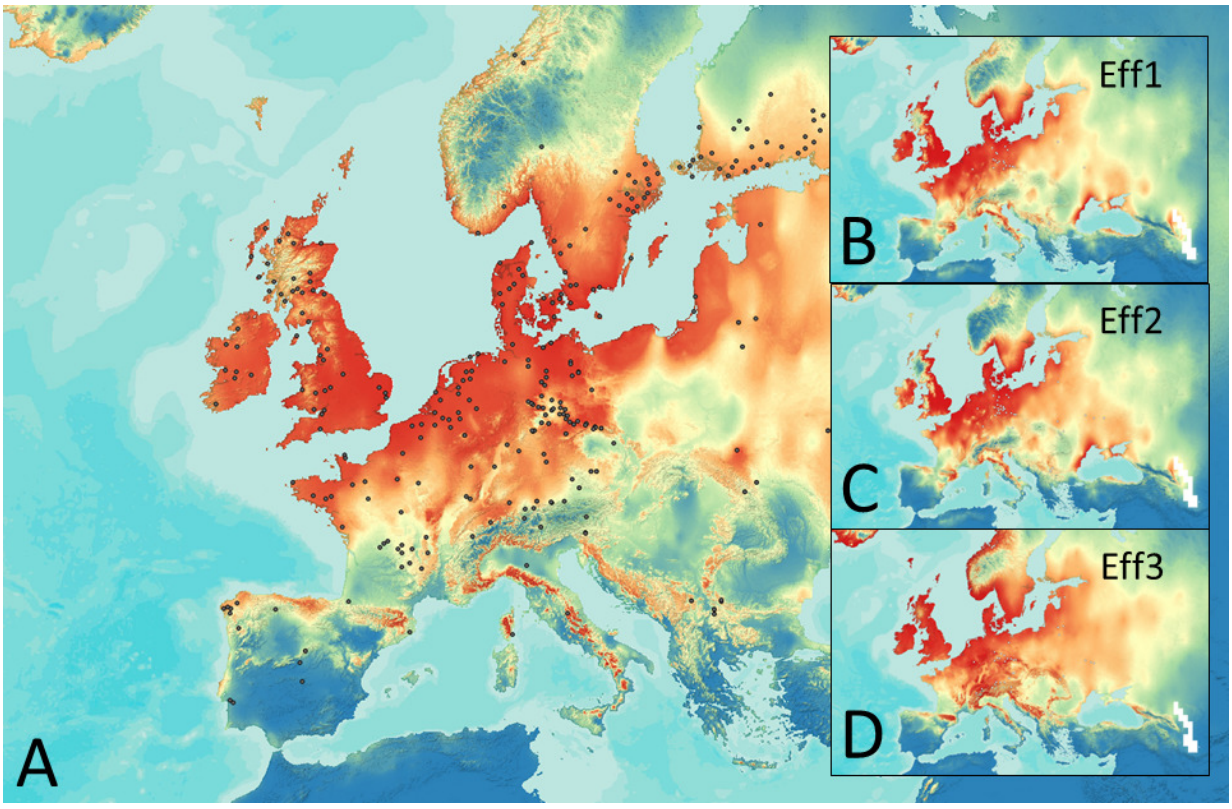

Fig. 2 Current distribution and mean predicted climatic suitability for *Juncus effusus* across Europe based on Maxent modelling. Climatic suitability is indicated in a gradient from red (high suitability) to blue (no suitability). A: Modelling for the whole species, B, C and D: Modelling for samples of lineages Eff1, Eff2 and Eff3, respectively.

Ecological differentiation

Lineage-specific unbiased heterozygosities (uH_E) at population level did not covary with climatic or soil conditions, latitude or longitude for lineages Eff1 and Eff2 ($P > 0.27$). Lineage Eff3 however, showed higher genetic diversity at intermediate longitudes ($\beta^2 = -0.0005$, $P < 0.01$), at intermediate values along climate PC axis 2 ($\beta^2 = -0.004$, $P < 0.01$, loaded mainly by temperature seasonality and annual range, BIO4 and BIO7, respectively; Fig. 3), and higher genetic diversity with increasing values along soil PC axis 2 ($\beta^2 = 0.02$, $P < 0.01$, which was mainly loaded positively by available water capacity and organic carbon content; Fig. S1).

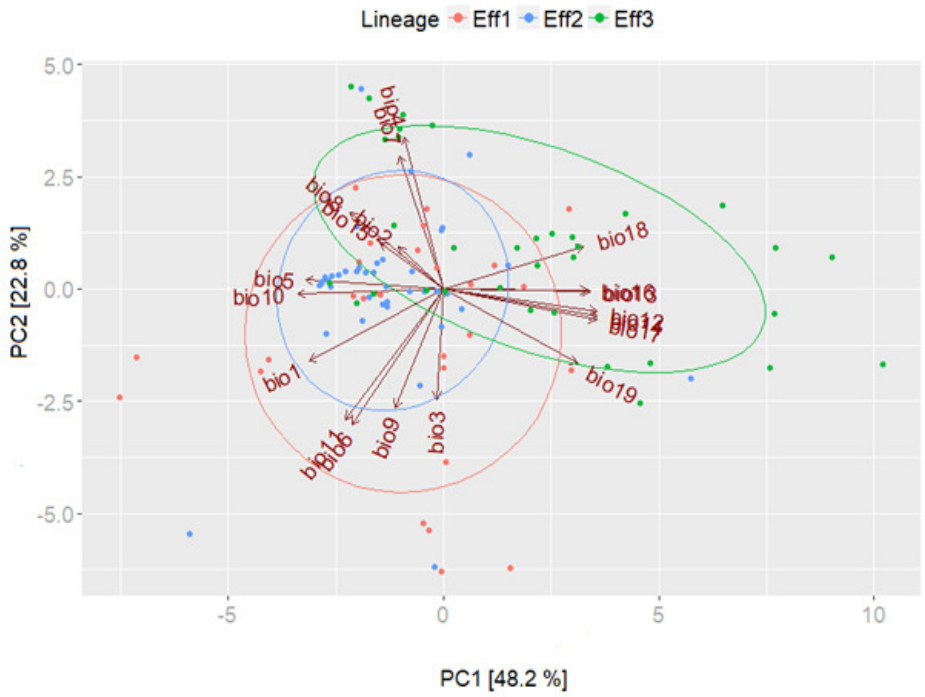

Fig. 3 Distribution of intra-specific diversity of *Juncus effusus* in depends of climatic conditions represented by the first two axes of a principal component analysis (PCA) performed on 19 bioclimatic variables. Red arrows indicate loadings of each variable on the two axes, while colours represent lineage (red: Eff1, blue: Eff2, green: Eff3) in the environmental space. For illustrative purposes only non-admixed populations (84%) are depicted.

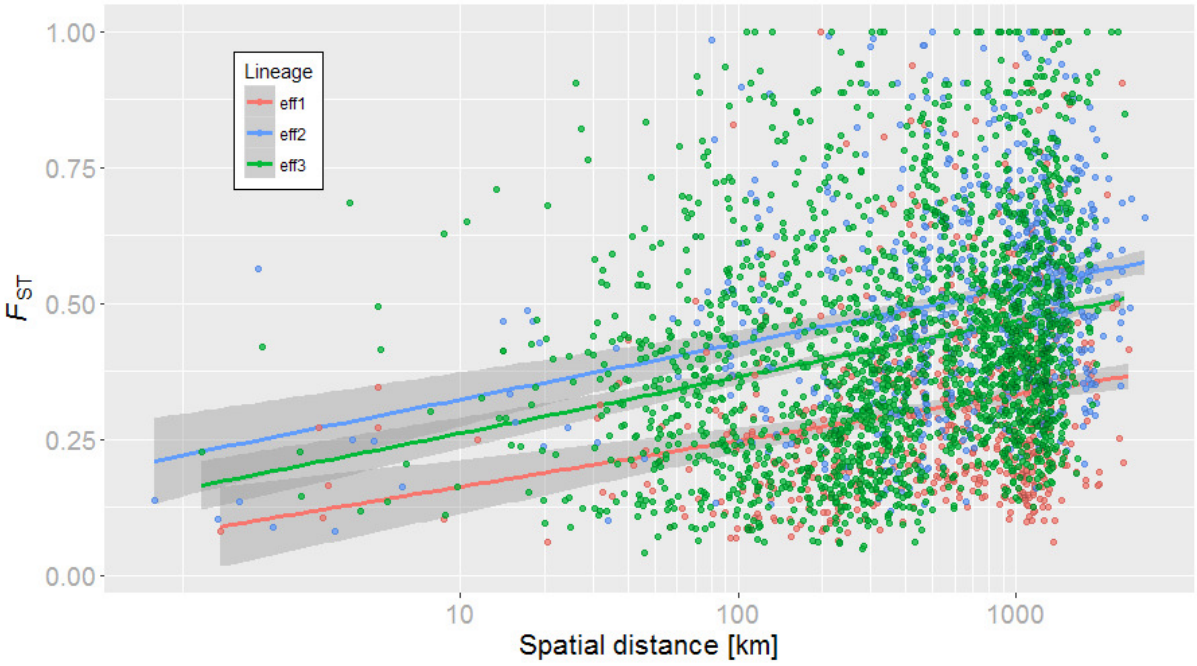

Fig. 4 Scatterplot of pairwise F_{ST} vs. spatial distance of sampled *Juncus effusus* populations separated by lineage membership (red: Eff1, blue: Eff2, green: Eff3). Note that the slopes of the relationship did not differ significantly among lineages (P > 0.65).

Lineage-specific genetic differentiation among populations followed a strong isolation-by-distance pattern with a similar slope of the relationship among lineages (Eff1: β_D = 0.04, P = 0.04; Eff2: β_D = 0.04, P < 0.001; Eff3: β_D = 0.04, P = 0.01 Fig. 4; Table 1). Testing for the relative contributions of environmental distances (climate, soil) on pairwise genetic differentiation and jointly considering spatial distances using the MMRR approach we found a significant contribution of soil distances in lineage Eff1 (β_{soil} = 0.043, P = 0.027; Table 2) and climatic distances in lineage Eff2 ($\beta_{climate}$ = 0.041, P = 0.014; Table 2).

Table 1 Summary of multiple matrix regression with randomization (MMRR) comparing effects of genetic differentiation estimated by pairwise F_{ST} with (log-transformed) geographic distances (isolation-by-distance, IBD).

Lineage	# of sites	β_D	P_D	R^2
Eff1	36	**0.04**	**0.04**	0.03
Eff2	39	**0.04**	**<0.001**	0.05
Eff3	65	**0.04**	**0.01**	0.04

Results include regression coefficients (β) and their P values as well as coefficients of determination (R^2). Significant P values ($\alpha < 0.05$) in bold.

Table 2 Summary of multiple matrix regression analyses with randomization (MMRR) comparing the relative effects of spatial distances, difference in soil and climate conditions on genetic differentiation measured as pairwise F_{ST} (isolation-by-environment, IBE). Note that for this analysis all matrices were scaled before analysis.

Lineage	# of sites	$\beta_{spatial}$	$P_{spatial}$	β_{soil}	P_{soil}	$\beta_{climate}$	$P_{climate}$	R^2
Eff1	34	0.021	0.460	**0.043**	**0.027**	0.013	0.559	0.08
Eff2	33	0.022	0.340	0.026	0.187	**0.041**	**0.014**	0.07
Eff3	57	0.029	0.274	-0.018	0.300	0.032	0.202	0.05

Results include regression coefficients (β) and their P values as well as coefficients of determination (R^2). Significant P values ($\alpha < 0.05$) in bold.

Discussion

A Bayesian analysis of genetic population structuring of *Juncus effusus* across Europe showed the presence of three genetically well-differentiated lineages within the species (Eff1, Eff2, Eff3) with one lineage Eff3 showing further sub-differentiation. The existence of multiple genetically separated lineages within European *J. effusus* has been suggested earlier based on molecular studies on only few populations (Born & Michalski, submitted; Michalski & Durka 2015), however, the quantitative and spatial extent of this differentiation across Europe was not known before. The distribution of these lineages across Europe was not arbitrarily on spatial and climatic scale (Fig. 1A, Fig. 2). Lineage Eff1 was more common in Western Europe, whereas Eff2 and Eff3 were spread across the rest of Europe; however, Eff3 dominated in higher altitudes and more alpine regions indicating a preference for harsher environmental conditions including low temperature, dryness and shorter growing season. The two subgroups within Eff3 separated spatially with one group mainly occurring in Germany, Austria and Switzerland, whereas the other was more prevalent in Eastern Europe (Fig. 1B). The northern hemispheric distributed *J. effusus* s.l. has been described to harbour a great amount of local and regional phenotypic variation associated with flowers and inflorescences (Kirschner *et al.* 2002). In particular for North America a number of subtaxa have been described (Fernald & Wiegand 1910). However, for European *J. effusus*, only a single subtaxon with compact inflorescences similar to that of *J. conglomeratus* has been distinguish by some authors (*J. effusus* var. *subglomeratus* DC or *var. compactus* Lej. & Courtois) (Buchenau 1890; Fernald & Wiegand 1910; Tweed & Woodhead 1946; Fernández-Carvajal 1982). Indeed, data on *J. effusus* by Michalski and Durka (2015) suggests that probably one of the lineages identified here might correspond to this earlier described subtaxon. Still, the strong genetic differentiation pattern found here has not been recognized in the taxonomic literature yet probably caused to some extent by the fact that the lineages do occur partly in sympatry with some locations in mid- and Eastern Europe even supporting all three lineages and increasing local morphological variability. Such ‘hidden’ or cryptic intraspecific diversity has been found predominantly in

animals and fungi but less commonly in higher plants (Bickford *et al.* 2007). Cryptic diversity may arise in response to selection processes resulting in morphological stasis or due to reproductive isolation, for instance, when lineages differ in their flowering time (Baack *et al.* 2015). However, the data gathered so far do not allow speculating about the causes and the time-scale of diversification in *J. effusus.* The long-term persistence of lineages within *J. effusus* is probably supported by the selfing mating system and flowering time differentiation maintaining reproductive isolation among the lineages even in sympatry (Michalski & Durka 2015). Clearly, for *J. effusus* further work on morphological and ecological differentiation preferentially on global scale is required as already suggested by Kirschner *et al.* (2002).

At lineage-level our results showed low amounts of population-level genetic diversity, but strong between-site differentiation confirming expectations for predominantly selfing species (Frankham *et al.* 2002). Across lineages, however, some sites harboured a substantial amount of genetic diversity as a result of admixture between lineages (e.g. Zitt: uH_e= 0.64, all three lineages present). Postglacial colonization at the Northern hemisphere is often associated with a decline of genetic diversity towards higher latitudes likely because of founder effects during northwards migration from refugia after the last glaciation (Hewitt 2000). For individual lineages within *J. effusus*, variability in genetic diversity did not covary consistently with latitude or longitude and thus did not allow general conclusions about possible lineage specific migration routes after the last glacial maximum (Voss *et al.* 2012; Leipold *et al.* 2017; Michalski *et al.* 2017). The distribution of genetic diversity along ecological clines is a key question. For instance, Huang *et al.* (2016), found a strong correlation between genetic diversity in the shrub *Caragana microphylla* for many environmental factors such as cold index and mean annual rainfall. Significant relationships between temperature factors and genetic diversity were also observed in a perennial, densely tufted grass, *Stipa grandis* (Zhao *et al.* 2006). These significant correlations, together with the missing IBD pattern, suggest that natural selection driven by temperature and precipitation fluctuations was responsible for the adaptive eco-geographic differentiation of the *S. grandis* populations (Zhao *et al.* 2006). Genetic variation

often varies along climatic gradients, particularly temperature and precipitation are important abiotic factors which are also well known to shape species distribution across broad scales. In many latitudinal and elevational diversity studies, climatic conditions determine the selective regime (Shi *et al.* 2011a; Hahn *et al.* 2017), but can also affect phenological traits such as time and duration of flowering resulting in reproductive isolation (Blionis *et al.* 2001; Gómez-García *et al.* 2009). But also soil as extremely heterogeneous collection of factors and microhabitats can influence the fitness of plants directly or indirectly via plant-soil (biota) interactions (Wall *et al.* 2012; Bergmann *et al.* 2016). These fitness consequences of environmental conditions on plant populations have the potential to influence range responses of species including migration and fragmentation processes (Van Nuland *et al.* 2017). However, in our study only for lineage Eff3 significant clines in population genetic diversity with environmental conditions could be found suggesting more particular ecological responses as compared to lineages Eff1 and Eff2 further substantiating the results of the distribution modelling (Fig. 1A; Fig. 2). However, this potential ecological niche differentiation of lineage Eff3 was not obviously reflected in a particular isolation-by-environment pattern (Table 2). At least, compared to lineages Eff1 and Eff2, adding environmental distances to explain genetic differentiation in Eff3 did not substantially increase explained variation substantially when compared to the variation explained by spatial distances alone. *Juncus effusus* is characterized by an extremely high seed production (Ervin & Wetzel 2001), a very efficient seed distribution via wind, animals and water and a dominant long-lasting soil seed bank (Richards & Clapham 1941). On the other hand, the species is highly selfing and gene flow by pollen between established populations is probably not very pronounced. Hence, *J. effusus* is likely to exhibit a strong meta-population dynamic with strong drift effects which might obscure large-scale spatial patterns in the distribution of genetic diversity associated either with earlier migration dynamics or potential adaptation processes in response to local environmental conditions.

Supplementary data

Table S1 Location of studied *Juncus effusus* populations and number of individuals with assignment probabilty (Q) to the three lineages identified by InStruct.

Table S2 Characteristic of sixteen microsatellite markers in *Juncus effusus* including repeat motif, primer sequence for forward- and reverse primer, type of reaction mixture using directly fluorescent-labeled primers (A) and universal fluorescent-labeled tailed primers (B), allelic size range and accession number in gene bank.

Fig. S1 The Principal component analysis (PCA) of 13 soil variables extracted from the LUCAS topsoil database and the world harmonized soil database for 74 *Juncus effusus* populations.

Fig. S2 Graph of DeltaK values obtained from InStruct to determine the optimal number of clusters in *Juncus effusus*, based on genotyping 1048 individuals using 16 microsatellite loci (Table S2).

Fig. S3 Population structure based on Bayesian clustering approach for K = 3 (A) and K = 4 (B) using InStruct analysis. (A) All sampled *Juncus effusus* individuals (N = 1048) are partitioned in three coloured clusters (red = Eff1, blue = Eff2 and green = Eff3) and (B) demonstrating a splitting of Eff3 into two subgroups. Each individual is represented by a single vertical line.

Supplementary data available online after publication.

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CHAPTER 5

Synthesis

CHAPTER 5: Synthesis

The objective of this thesis is to assess molecular variation as well as intraspecific variation in functional traits of the common wetland plant *Juncus effusus* to understand how intraspecific variability responds to different habitat conditions related to soil conditions and thus affects important ecosystem processes in wetlands. The importance of intraspecific variability for ecosystem functioning has been underappreciated hitherto. However, understanding the causes and consequences of intraspecific trait variability is fundamental for both ecological research and for the use of genetic resources by humans. This chapter summarizes the study's findings and conclusions and discusses possible evolutionary responses and implications for applied ecological approaches, after briefly summarizing the main results of each chapter.

(I) **Chapter 2** focuses on the expression of intraspecific variability in functional traits in response to nitrogen availability in the soil and its structure within and among natural populations. A common garden study provided a strong plastic behavior for all measured traits in response to nitrogen availability. The genetic diversity of functional traits differed greatly among populations (Q_{ST}) but was less pronounced within populations (H^2). However, a significant pattern of differentiated genetic trait expression in response to nitrogen availability could not be observed.

(II) The objective of **chapter 3** is to study molecular diversity and phenotypic variation in response to differing nitrogen availability and looks for signatures of adaptation. Differentiation of quantitative traits (Q_{ST}) was strongly pronounced but did not exceed neutral expectations (F_{ST}) in response to nitrogen availability, indicating the lack of directional selection. However, quantitative genetic differentiation could be explained by environmental conditions of sample origin, suggesting that differential trait expression may by the consequence of local adaptation.

(III) **Chapter 4** asks whether and how molecular genetic variation is partitioned within and among European populations. In addition, we used a multiple matrix regression approach (MMRR) that explains population genetic differentiation by spatial, soil and climatic distances. The genetic structure among populations was strongly pronounced, resulting in three genetically well-differentiated lineages. The distribution of these lineages across Europe was not randomly on spatial and climatic scale. A strong correlation between genetic structure and spatial distance was found within each lineage, whereas all lineages showed differing relative contributions of climate and soil conditions to pairwise molecular differentiation.

Assessing the importance of intraspecific variability

Understanding patterns of intraspecific variability within and among natural populations is of fundamental importance if we want to predict the potential of natural adaptation to changing environmental conditions and their effects on the environment. As outlined in **chapter 4**, the genetic diversity and genetic structure of *Juncus effusus,* as evaluated with microsatellite markers, were mainly influenced by neutral processes like genetic drift and gene flow. *Juncus effusus* exhibited a moderate genetic diversity at the species level and a strong neutral genetic differentiation among populations. These results are consistent with **chapter 3 and 4** and can be explained by the life history of the species. *Juncus effusus* is an efficient pioneer species and colonizer due to its fast growth rate (Ervin & Wetzel 2002), high seed production (Stockey & Hunt 1994) and is characterized by a predominantly selfing mating system (Buchenau 1892; Michalski & Durka 2015). Both frequent founder events and the predominantly selfing breeding system increase genetic drift and reduce effective population size. These processes in turn can reduce genetic diversity and simultaneously increase genetic structure by reducing the gene flow between populations (Frankham *et al.* 2002).

In addition to the analysis of genetic variation based on neutral markers described in **chapters 3 and 4**, I assessed the molecular basis for genetic differentiation in candidate genes that are putatively involved in the transport and metabolization of nitrogen and are thus important for the potential of degradation. A promising approach for identifying relevant genes in a non-model species is the de-novo sequencing of the transcriptome by a next-generation sequencing approach, which has not yet been undertaken to study this species. For this purpose, an RNA-library of 19 genotypes' pooled extracts of leaf, root and inflorescence material was constructed, all of which had originated from all over Europe. After correcting the quality of sequence reads obtained by both Roche 454 and Illumina HiSeq 2000 in the sequencing of this library, a transcriptome was assembled de-novo. Raw sequence reads and the transcriptome assembly were stored at the NCBI and the SRA and TSA archives (https://www.ncbi.nlm.nih.gov/) under the accessions *SRR4450626*, *SRR4450627* and

GFBP00000000, respectively (BioProject *PRJNA345287*). Additionally, contigs were blasted against NCBIs non-redundant amino acid database and were further assigned to their respective Gene Ontology (GO) categories. Single nucleotide polymorphisms (SNP) were detected by mapping Illumina reads against the assembly and further processing. For 20,557 out of 158,591 contigs in the assembly, we identified a total of 124,665 biallelic SNP markers. The SNP position was determined in the consensus sequence to check for synonymous and non-synonymous mutations. A synonymous SNP does not affect the amino acid, while a non-synonymous SNP changes the amino acid of a protein. The proportion of non-synonymous to synonymous mutations (K_a/K_s) is indicative of a possible underlying neutral, balancing or diversifying selection (Nei & Gojobori 1986; Nei 2005). The K_a/K_s ratios of the annotated contigs varied for different biological processes but did not indicate a strong impact of positive selection on genes related to nitrogen compound metabolic processes (Fig. S1). Using the de-novo transcriptome assembly, 16 candidate genes were identified to be putatively linked to the transport and metabolization of nitrogen, as well as associated SNP loci using GO terms from the annotation results. Subsequently, a subset of 32 SNP loci consisting of 20 non-synonymous and 12 synonymous SNPs (Table S1) was genotyped for 758 individuals from 105 populations across Europe, using a KASP genotyping array (CD Genomics, Germany). For all SNP loci, we calculated a low level of genetic diversity (uH_E = 0.132, SE = 0.003) and a very strongly pronounced genetic differentiation (F_{ST} = 0.682, SE = 0.017). However, genetic diversity indices did not differ between synonymous (N = 12; uH_E = 0.126, SE = 0.005; F_{ST} = 0.644, SE = 0.037) and non-synonymous SNPs (N = 20; uH_E = 0.136, SE = 0.004; F_{ST} = 0.705, SE = 0.014). A similar proportion of SNPs presenting low and high F_{ST} values in synonymous and non-synonymous SNPs supports the assumption of neutrality of genes of N uptake and metabolization in which genetic differentiation among populations is determined by neutral processes alone as affecting all loci equally (Barreiro *et al.* 2008). An assessment of expected heterozygosity and fixation indices based on the genotyping of 32 SNP loci (uH_E = 0.104, SE = 0.003; F_{ST} = 0.839, SE = 0.006) exceeded those observed by 16 microsatellite loci (uH_E = 0.233, SE = 0.006; F_{ST} = 0.658, SE = 0.017) using the same set of populations and individuals.

The lower global F_{ST} based on microsatellite markers was likely the result of higher allele numbers compared to biallelic SNPs (Whitlock 2011).

In **chapter 4**, a Bayesian analysis of population structuring provided evidence of three genetically highly differentiated *Juncus effusus* lineages across Europe. However, with the SNP data set, we received contradictory results, as the Bayesian population structure analysis showed a subdivision of two genetic groups based on 106 *Juncus effusus* populations. This result was evident for both synonymous and non-synonymous SNPs. In addition, the principle coordinate analysis (PCoA) of genotypic pairwise distances was used for the SNP data set to visualize the similarity among individuals. The PCoA revealed a triangular pattern formed by three genetic groups, whereby the first two axes are explained by 81% of the total variance. This result seems more plausible because of the division into three genetic groups with fewer admixed individuals and the allocation of individuals to the genetic groups -as seen in the use of microsatellite markers. Previous studies have reported that randomly selected microsatellites, in particular dinucleotide repeats, have on average a greater informativeness for population structure inference than random selected SNPs (Rosenberg *et al.* 2003; Liu *et al.* 2005), indicating that the use of microsatellites in **chapter 4** leads to a better inference of population structure in *Juncus effusus* compared to the 32 randomly selected SNPs. However, in studies with a large number of SNPs (~3000), the relative performance of SNPs is significantly improved and SNPs perform better than microsatellites (Glover *et al.* 2010; Gärke *et al.* 2012; Fischer *et al.* 2017). Thus, the potential of SNPs for a better inference of the population structure strongly depends on their number (Rosenberg *et al.* 2003).

In future research, the use of the candidate genes and SNPs identified in this study can be used as the basis for studies on differential expression using qRT-PCR or for association mapping of quantitative traits (i.e. quantitative trait loci, QTL (Hall *et al.* 2010; Yang *et al.* 2017)). In addition to the candidate gene approach, genotyping by sequencing approaches can be used to identify thousands of SNPs in a single procedure to predict genome-wide allele frequency differences and perform genome-wide association studies, in order to discover genetic structures that relate to complex quantitative traits (McCarthy *et al.* 2008). A suitable

method for a genome-wide SNP discovery and genotyping in *Juncus effusus* is the double-digest restriction, site-associated DNA sequencing (ddRAD-seq) method which requires no previous genomic knowledge (Peterson *et al.* 2012).

Detecting signatures of adaptive processes in trait variation and differentiation is a challenging task and often realized in common garden experiments with a simulated range of experimental treatments that allow for a more general understanding of expressed genetic variability and plastic behavior. In our common garden studies, genetic variation of quantitative traits was low at population-level, whereas genetically-based trait variation residing among populations was substantial and significant for all studied traits. These results are not unexpected for a selfing species and have been demonstrated for a range of plant species and traits (discussed in **chapters 2 and 3**). We found a strong divergence in the expression of functional traits and in the plastic behavior among populations in response to the varying availability of nitrogen. However, in contrast to our hypothesis of differently expressed genetic variation in functional traits that depend on the availability of N, we could not demonstrate a general effect of nitrogen availability on genetic variation in functional traits. Variation in trait expression could be partly related to local soil, environmental conditions of sample origin, suggesting possible signatures of adaptation, which may indicate a genetic influence on plant-associated ecosystem functions. However, these trait correlations were not found to be consistent in different nitrogen availabilities, suggesting a different genetic basis for trait expression in diverse environments (**chapter 3**).

Assessing evolutionary potential across large environmental scales, using estimates of heritability and genetic correlation from common garden experiments, may be subject to uncertainties (Mitchell-Olds & Rutledge 1986). First, studies have reported a higher heritability under greenhouse conditions (Geber & Griffen 2003; Gardner & Latta 2008), which can be caused either by reduced environmental variation in greenhouse in comparison to wild plant populations with a potentially higher impact of biotic interactions on trait expression. Greater heritability under greenhouse conditions may also be a consequence of greater genetic

variance due to the expression of genes under novel greenhouse conditions (Hoffmann & Merilä 1999). Second, genetic correlations and heritabilities can shift when populations are subject to other environmental conditions (Sgrò & Hoffmann 2004). Thus, to understand and predict the evolutionary potential of a population, we need measurements in natural populations, which are much more difficult to attain in the field; therefore, such measurements are still limited due to necessary determination of the genetic relationship of a large number of individuals (Wilson & Poissant 2016).

Evidence of selection

The impact of local adaptation on genetically-based population divergence in trait expression requires knowledge about the strength and distribution of selection acting on quantitative traits and calls for insight into neutral processes that affect the whole genome, including genetic drift and gene flow. One of the commonly applied approaches for distinguishing between different evolutionary processes is to compare differentiation patterns in functional traits (Q_{ST}) and neutral expectations based on neutral markers (F_{ST}) (Whitlock 2008; Leinonen *et al.* 2013). In **chapter 3** most trait-specific Q_{ST} are equal to or lower than the estimated F_{ST}, indicating that trait differentiations could be reached by genetic drift alone or are caused by stabilizing selection. The results contrast those of most previous studies, where Q_{ST} exceeds the F_{ST}, suggesting that populations might be under selective pressure for a specific trait (Leinonen *et al.* 2008). The mainly dominant pattern of $Q_{ST} > F_{ST}$ can be explained partly with publication bias (Lamy *et al.* 2012), which increases the importance of this study by attention on evolutionary bases of phenotypic stasis.

Although the Q_{ST}-F_{ST} comparison is an effective method for testing the relative importance of neutral and adaptive processes, there are several limitations (discussed in **chapter 3**). The Q_{ST}-F_{ST} comparison requires a large number of populations to increase statistical power and cannot differentiate between selection and genetic drift, if Q_{ST} values are the same as F_{ST}. An alternative is the multivariate method proposed by Martin *et al.* (2008), which used the genetic covariance matrix within- and among-populations to test for proportionality as well as

Ovaskainen *et al.* (2011) method, which used the population-level coancestry matrix, based on neutral marker data. Using a Bayesian framework, the genetic coancestry matrix, which is based on neutral markers, is used as a neutral expectation (analogous to F_{ST}) and can compared with the genetic covariance matrix, which is based on quantitative traits (analogous to Q_{ST}) for each population to reject the null hypothesis of random genetic drift even in the case where F_{ST} and Q_{ST} are equal (Ovaskainen *et al.* 2011; McKinney *et al.* 2014). Consequently, the use of this new method could help to uncover signatures of divergent and stabilizing selection for my data, where Q_{ST} is mainly equal to the estimated F_{ST}.

To infer past selection processes or their absence, we evaluated population divergence by comparing quantitative traits and neutral genetic loci (**chapter 3**). However, an alternative approach would have been to conduct a reciprocal transplant experiment to assess local adaptations to current selective pressures in populations that differ across sites and to assess how the fitness rate differs at each of the several common garden sites. For this experimental approach, populations differing in nitrogen bioavailability and alleles of candidate genes would have to be collected to assess fitness traits such as above- and belowground biomass, seed and flower production and the interaction between destination and origin of populations, which may indicate local adaptation in response to different habitat conditions. As proposed in Bubier *et al.* (2011) and Mao *et al.* (2014), the effects of nitrogen addition on functional traits, as important drivers of ecosystem functioning, may be undetectable in short-term experiments; ideally, experiments conducted over multiple growing seasons or generations are necessary. The next factor that complicates the application of the Q_{ST}-F_{ST} comparison in testing for adaptive evolution is the missing genotype-by-environment interaction in lab-based studies. This complication is due to the use of collected individuals in the field. The obtained pattern of genetic variance among traits in the lab are not completely consistent with those in natural populations (Wilson & Poissant 2016); thus we also need measurements in wild populations to help understand phenotypic evolution. The common garden study and the transplant experiment can be seen as complementary approaches, rather than competing approaches,

for assessing quantitative genetic variation and potential adaptive differentiation in the phenotypic traits of *Juncus effusus*.

Implications for applied approaches

Wetland ecosystems provide a wide range of ecosystem services which are indispensable for human well-being and for the conservation of biodiversity (Millennium Ecosystem Assessment 2005). The common wetland plant *Juncus effusus* is often used in constructed wetlands to treat domestic wastewater. It is an ideal candidate for the use of phytoremediation due to its fast growth rates (Ervin & Wetzel 2002) and potential to tolerate wide ranges of nutrients and metals (Gruber *et al.* 2008).

Recent studies demonstrated that intraspecific variability can have large effects on ecosystem functioning and similar ecological consequences, such as species diversity (Reusch *et al.* 2005; Crutsinger *et al.* 2006; Cook-Patton *et al.* 2011). Thus, knowledge of genetic diversity and differentiation related to phenotypic variation and local adaptation is essential for aiding applied approaches in wetland restoration and construction (Kettenring *et al.* 2013). This study has shown that increased effects of genetic drift and limited gene flow for the selfing species *J. effusus* resulted in a very pronounced neutral genetic differentiation and a strong divergence in the expression of traits among populations. The impact of genetic variation on nutrient cycling has the potential to be substantial due to genes, affecting the amount and chemical quality of plant litter. In turn, leaf litter quality and quantity can affect plant growth by influencing the community of soil microorganisms supporting, for example, nitrogen mineralization and nutrient immobilization (Schweitzer *et al.* 2004; Schweitzer *et al.* 2005). I performed a one-year mesocosm experiment in which genotypic richness varied to assess the effect of genotypic interactions in regulating nitrogen removal in the wetland plant *Juncus effusus* (unpublished data). The study found a different reaction among genotypes in their biomass production in response to diversity (Fig. 1A). In addition to finding an obtained genotypic effect, we also detected a tendency for a diversity effect in which plant productivity increased in polycultures rather than monocultures (Fig. 1B). Our findings emphasize the

importance of quantifying the genetic basis of ecological relevant traits in N cycling to improve the functioning of constructed wetlands. However, a further study with a higher number of genotypes and replications will need to be undertaken to confirm a clear diversity effect and to obtain a more precise assessment of the fate of nitrogen.

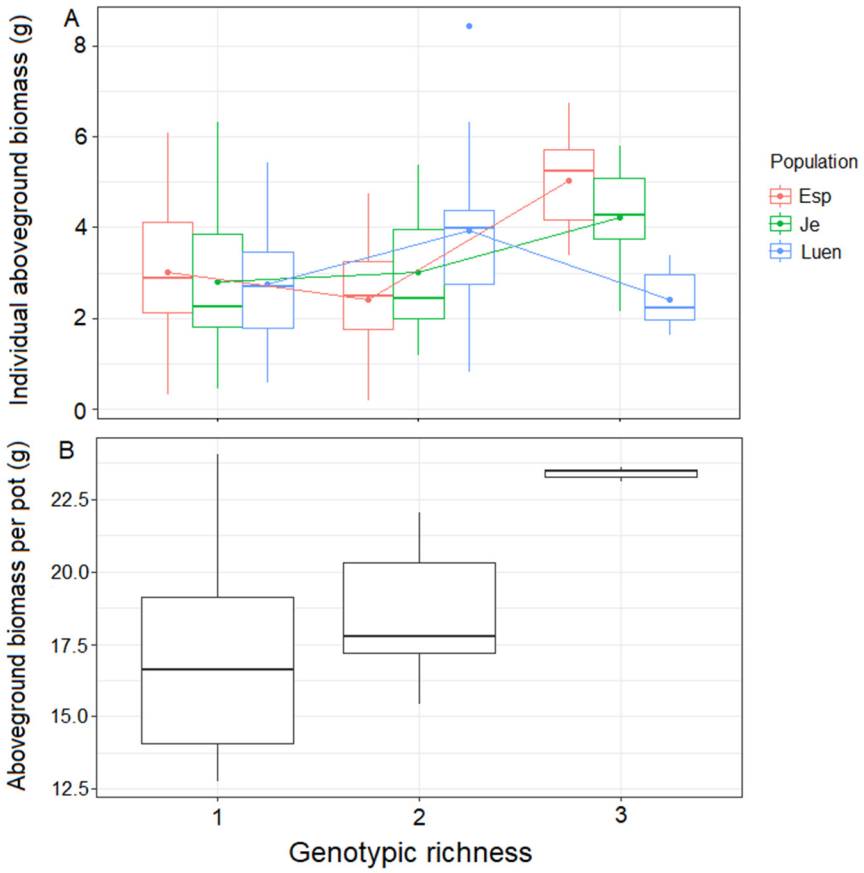

Fig. 1 Effect of genotypic richness on (A) individual biomass production of the respective genotypes of *Juncus effusus* and (B) total biomass production per pot. For population acronyms see chapter 4 (Table S1).

Lineage divergence, isolation and genetic drift in *Juncus effusus*

An important finding of the molecular genetic analysis was the occurrence of three genetically well-differentiated *Juncus effusus* lineages across Europe. This discovery of previously unrecognized lineages is fundamental for evolutionary biology. In **chapter 3**, we evaluated the phenotypic divergence among the observed lineages and found that they differ in few morphological traits; but all studied traits showed an overlap between lineages which makes it difficult to differentiate between them in *Juncus effusus*, despite considerable genetic variation (**chapter 4**). This lack of morphological differentiation justifies a description as “cryptic” lineage, which is mainly found in animals and fungi, and less commonly in higher plants (Bickford *et al.* 2007). Several factors support this interpretation of a cryptic nature of *Juncus effusus*, detailed in **chapter 4**. Firstly, both microsatellites and SNPs as nuclear markers provide a clear separation of genetic groups that are largely consistent for lineages Eff1 and Eff2 and associate individuals to similar genetic groups. Secondly, still considerable differences based on the between-lineage F_{ST} of microsatellite markers could be detected. Thirdly, in spite of the large distribution of *Juncus effusus* in Europe only 5.3% of studied individuals showed individual admixture portions lower than 0.7 classifying genotypes as hybrids. This represents the most extreme form of cryptic lineages occurring sympatrically with a low hybridization rate.

Molecular phylogeographic studies are helpful for understanding the evolutionary history of a species and the interactions between past changes in global climate and extensive vegetation shifts (Garcia *et al.* 2011). Past climate events, particularly the glacial and interglacial periods of the Quaternary Ice Age, have driven the evolution of divergent genetic lineages by isolating populations in three major European glacial refugia in the Iberian Peninsulas, Italy and the Balkan Peninsulas. Subsequently, postglacial colonization and range expansions from refugia allowed secondary contact from previously separated lineages (Hewitt 2004; Schönswetter *et al.* 2005). The Europe-wide spread of the cryptic *Juncus effusus* lineages and their sympatric occurrence makes it difficult to imagine a scenario in which these lineages arose within the current distribution range. We proposed, instead, an allopatric divergence from past changes in global climate and a secondary contact after glacial lineage

diversification. In **chapter 4**, we showed that Western Europe is dominated by the lineage Eff1, while lineages Eff2 and Eff3 were predominantly in Eastern Europe. This finding can be explained by different migration routes during the interglacial phases of the Quaternary Period, indicating that the Iberian Peninsula may serve as a refugial area for lineage Eff1, whereas lineages Eff2 and Eff3 survived in glacial refugia in the Italian and Balkan Peninsulas. Central Europe, particularly Germany, was highlighted as a very diverse area, possibly indicating sympatry as a result of glacial lineage diversification and the secondary contact of former isolated lineages. This had occurred after the last glacial period, proving the opportunity to form hybrid zones (Hewitt 1999). A common pattern seen in many European plants is a decline in genetic diversity from the south to north, which can be explained by multiple range contractions and expansions during the Quaternary Ice Age (Comes & Kadereit 1998; Feliner 2011). However, no significant differences between northern and southern populations of *Juncus effusus* were found ($P > 0.05$; U-Test). A clear large-scale spatial pattern in the distribution of genetic diversity is likely obscured by a predominantly selfing mating system of *Juncus effusus* which leads to strong meta-population dynamics with strong drift effects. In conclusion, *Juncus effusus* shows a deep genomic split into three morphological cryptic lineages, which is an indication of a complex evolutionary history. However, the data collected so far, do not allow a clear indication of causes and the time-scale of diversification in *J. effusus*. Thus, further phylogeographic studies are necessary to understand the evolutionary history of the cryptic *Juncus effusus* lineages. Ideally, a combination of different sequences - such as nuclear and neutral (as already used in this study), as well as cytoplasmic- should be used to provide a more comprehensive and reliable understanding of the global distribution of genetic diversity and its evolution.

In **chapter 3**, there was only little and inconsistent evidence of selective mechanisms varying between the lineages, which supports the idea that the lineages within *J. effusus* are the primary result of neutral divergence. As such cryptic lineages resulting from neutral genetic drift show, phenotypic and phylogenetic differences are proportional to time (Lynch 1990; Smith *et al.* 2011). However, the lack of taxonomically useful morphological characters in these

cryptic lineages supports stabilizing selection as an additional cause, resulting in morphological stasis in genetically well-differed groups. Thus, it is possible that a combination of adaptive and non-adaptive evolution underlies cryptic speciation in *Juncus effusus*. Some studies have proposed that stabilizing selection is often found in rare species that occupy similar ecological niches, leading to morphological stasis (Gravuer *et al.* 2005; Yoshida *et al.* 2008; Ye *et al.* 2014). However, *Juncus effusus* is a common and a widespread wetland plant with a broad ecological niche, which questions this explanatory approach. Furthermore, it has been also argued that species can track new habitats faster than adapting to temporally changing environments, resulting in morphological stasis (Smith *et al.* 2011). Aquatic systems in Europe have undergone dramatic expansion and contraction as a result of historical processes like Quaternary glaciation over the last 2.5 million years (Hewitt 2000); in the last century, it has also undergone heavy exploitation by humans for the provision of "goods and services (Millennium Ecosystem Assessment 2005)," which prevent adaptations to dynamically changing environments. This process was reported in some animals (Kozak *et al.* 2005; Lavoué *et al.* 2010; Smith *et al.* 2011) and proposed the evolution of long-term generalists that are able to survive throughout wide environmental fluctuations over geological timescale (Sheldon 1996).

Coexistence of lineages

A wide geographic range and the frequent sympatric co-occurrence – particularly in Central Europe - suggest that *Juncus effusus* lineages can coexist. It is generally assumed that a stable coexistence of species requires ecological differentiation or spatio-temporal niche differentiation in order to reduce interspecific competition (Schulze *et al.* 2005; White *et al.* 2010; Leys *et al.* 2016). In **chapter 4** isolation-by-environment (IBE) patterns based on climatic data and soil properties are observed along environmental clines. However, all lineages showed differing relative contributions of climate and soil conditions to pairwise molecular differentiation. In general, it is expected that these IBE patterns evolve for adaptive genetic variation (Blanquart *et al.* 2012; Aitken & Whitlock 2013), and under the assumption that the

IBE patterns result from selection, some studies have also interpreted it as the beginning of a speciation (Shafer & Wolf 2013). However, detection of these patterns alone is not evidence of local adaptation, as other mechanisms can progress this pattern (Wang & Bradburd 2014), e.g. genetic linkage or when environmental distance is associated with reproductive isolation. Therefore, it is helpful to identify ecological factors that drive adaptive divergence and isolation among populations by using environmental designs, as done in **chapter 3**. Our common garden experiment showed that adaptive trait differentiation in response to soil, environmental conditions might still be present, but no clear indication of an ecological and functional separation of the lineages was evident. **Chapter 4** provides evidence of differences between the lineages in terms of spatial variation in environmental factors. The lineage Eff3 showed a greater relative frequency at high elevations compared to the lineages Eff1 and Eff2 which occurred mainly in lowlands under similar climatic conditions. Elevational clines can include gradients of a series of environmental factors (Körner 2007), such as temperature and precipitation, which can be main drivers for genetic differentiation among populations along elevational gradients (Hirao & Kudo 2004; Shi *et al.* 2011a). Higher-altitude environments are characterized by severe limitations on survival and reproduction, including those induced by short growing seasons and low temperatures, and thus, the occurrence of populations at different altitudes might be due to local adaptation (Frei *et al.* 2014). Moreover, the mating system of *Juncus effusus* limits outcrossing events. It has been described as predominantly selfing (Buchenau 1892), evidenced by higher ovule production per flower than other wind-pollinated species (Michalski & Durka 2010) and by the high inbreeding coefficients of individual lineages documented in this study (F_{IS} = 0.527 – 0.694, unpublished data). Temporal niche segregation may also play an important role in *Juncus effusus*. The role of premating barriers by the non-identical timing of flowering between lineages was observed in our common garden study, where we documented strong differences between lineages from the date of the first flowering, with Eff1 flowering first (39% of individuals), followed by Eff2 (6% of individuals) and Eff3 did not show flowering while performing the common garden experiment (unpublished data). These phenological shifts can reduce pollen exchanges among populations. These

observations are consistent with the results of the study conducted by Michalski and Durka (2015) on *Juncus effusus*. Differences in flowering phenologies, which had evolved from isolation by time in sympatry, can be also found in other genera (Martin & Willis 2007; Ferriol *et al.* 2008). We also found in our study (**chapter 4**) that a low frequency of hybridization potentially reflects a limited opportunity for outcrossing as well as reduced seed and pollen dispersal among lineages. This is due to either historical processes or indicates a reduced fitness of hybrids. However, Michalski and Durka (2015) described a seed production and germination rate of *Juncus effusus* hybrids similar to parental lineages, suggesting that limited outcrossing events and flowering time differentiation seem to play a more important role in the maintenance of sympatric lineages than lack of hybrid fitness.

Concluding remarks

In this study, each chapter provides important insights into capacity for genetic adaptation of *Juncus effusus*, which is essential for predicting evolutionary potential. Quantitative genetics is still the most direct way to predict the evolutionary potential by measuring heritable variation in traits which are relevant to adapt to changing environmental conditions (Munday *et al.* 2013). On the other hand, modern molecular approaches are suitable for assessing the underlying genetic variation between populations (Mondini *et al.* 2009). Polygenic adaptation is common and even cryptic genetic diversity, which is hidden on phenotypic level, can be a powerful force in evolution (Masel 2006; Harrisson *et al.* 2014). Thus, ideally a combination of both quantitative and molecular genetics allows a deeper knowledge of the processes that control local adaptation to environmental conditions (Munday *et al.* 2013).

In the previous discussion, possible follow-up studies were proposed at several points. This last paragraph focuses on the importance of long-term studies to more accurately predict the evolutionary potential. A series of studies investigated the phenotypic responses of species to environmental change and concluded that microevolution has taken place. However, a review study by Gienapp *et al.* (2008) revealed that many responses described as adaptation to environmental change could be caused by phenotypic plasticity. Phenotypic plasticity provides

the potential for plant species to adjust to novel environmental conditions, but plastic responses are limited in the production of extreme phenotypes and cannot provide a long-term solution (Gienapp *et al.* 2008; Fitzpatrick 2012). In order to determine the extent of phenotypic plasticity, experiments over multiple generations are suitable, in particular to include the potential of developmental and transgenerational plasticity. Both mechanisms provide persisted longer responses to the environment, while developmental plasticity establishes during ontogeny, transgenerational plasticity results from environmental conditions experienced by previous generations through epigenetic inheritance or transmission of nutritional, somatic and cytoplasmic material (Munday *et al.* 2013). Thus, phenotypic plasticity and genetic adaptation can contribute to adaptation to altered environmental conditions, which increases the importance of distinguishing between true microevolutionary adaptations and environmentally induced plastic responses. The picture is complicated as previous studies have shown that phenotypic plasticity has a heritable component (Schlichting 1986; Scheiner & Lyman 1989) and is thus partially under genetic control (heritability of plasticity, e.g. Zeng *et al.* 2017), which makes the interaction between plastic and genetic change non-trivial. Future studies on the current topic are therefore recommended and essential to improve predictions of the future impacts of rapidly changing and unpredictable environmental conditions for a range of species and to develop recommendations for species conservation (Harrisson *et al.* 2014).

SUPPORTING INFORMATION

Table S1 Candidate genes putatively linked to transport and metabolization of nitrogen, and associated SNP loci were identified using the obtained GO annotation and other sources.

Contig ID	Gene family	Accession number	GO annotation / Reference	Length of total assembled sequences (bp)	Length of coding region (aa)	SNP locus in total assembled sequences (bp)	Allele	Amino acid changes	SNP type
Nitrogen transporter									
jeff_454_rep_c73586	Ammonium transporter	XM_013812793	GO:0015696	977	148	618	G / A	Valine / Isoleucine	Non-synonymous
comp195167_c0_seq2	Ammonium transporter	AF289477	GO:0072488	4573	503	1655	C / T	Alanine / Valine	Non-synonymous
						306	G / A	Alanine	Synonymous
jeff_454_rep_c95752	Nitrate transporter-like	XM_003573432	GO:0010167	1202	141	314	G / A	Leucine / Phenylalanine	Non-synonymous
						162	G / A	Tyrosine	Synonymous
jeff_454_rep_c3175	NRT1 / peptide transporter	XM_006643679	GO:0006807	2373	571	1199	C / T	Glycine / Serine	Non-synonymous
						563	A / C	Serine / Alanine	Non-synonymous
						390	C / T	Glycine	Synonymous
Nitrate reductase									
comp190758_c0_seq1	Nitrate reductase	XM_008680224	GO:0042128	3120	897	628	A / G	Glutamine	Synonymous
Amino acid synthetase, aminotransferase and dehydrogenase									
jeff_454_rep_c2162	Alanine aminotrans-ferase	KM051842	*(McAllister et al. 2012; Xu et al. 2012)*	1978	483	392	G / A	Valine / Isoleucine	Non-synonymous
comp198444_c0_seq1	Aspartate kinase-homoserine dehydrogenase	D78573	GO:0004072	3451	910	1640	A / G	Histidine / Arginine	Non-synonymous
						1405	A / C	Asparagine / Histidine	Non-synonymous
						1842	T / C	Aspartic acid	Synonymous

jeff_454_rep_c3396	Asparagine synthetase	KC140125	GO:0097164	2870	581	1595	T / A	Leucine / Glutamine	Non-synonymous
						1536	T / C	Alanine	Synonymous
Glutamine and Glutamate synthetase									
jeff_454_rep_c1573	Glutamine synthetase	XM_006647790	GO:0004356, GO:0009399	2025	372	1358	T / C	Asparagine / Serine	Non-synonymous
						414	C / T	Valine / Isoleucine	Non-synonymous
jeff_454_rep_c4534	Glutamine synthetase	XM_015776193	GO:0004356, GO:0009399	1717	356	1170	G / T	Alanine / Glutamic Acid	Non-synonymous
comp199208_c0_seq1	Glutamate synthase	XM_008676969	GO:0060359	7020	2173	1905	A / T	Valine / Aspartic acid	Non-synonymous
						1417	T / C	Lysine / Glutamic acid	Non-synonymous
						2624	C / T	Threonine	Synonymous
comp196768_c0_seq1	Glutamate decarboxylase	XM_006659422	GO:0015706	2212	503	718	T / C	Isoleucine / Valine	Non-synonymous
						1316	G / A	Valine	Synonymous
jeff_454_rep_c14893	Glutamate dehydrogenase	XM_013831963	*(McAllister et al. 2012)*	1021	260	973	A / G	Asparagine / Serine	Non-synonymous
						494	A / G	Leucine	Synonymous
Others									
jeff_454_rep_c37476	Vacuolar-resorting receptor	XM_006656279	GO:0015706	784	224	464	G / A	Valine / Methionine	Non-synonymous
						442	T / C	Glycine	Synonymous
jeff_454_rep_c894	Ferredoxin NAPD+ reductase	XM_006655630	GO:0004324	3399	360	1068	T / A	Leucine / Glutamine	Non-synonymous
						283	T / C	Alanine	Synonymous
comp192514_c0_seq1	Ferredoxin-nitrite reductase	D50556	GO:0048307	2051	586	775	A / G	Asparagine/ Aspartic acid	Non-synonymous
						1317	C / A	Phenylalanine / Leucine	Non-synonymous
						1014	G / T	Proline	Synonymous

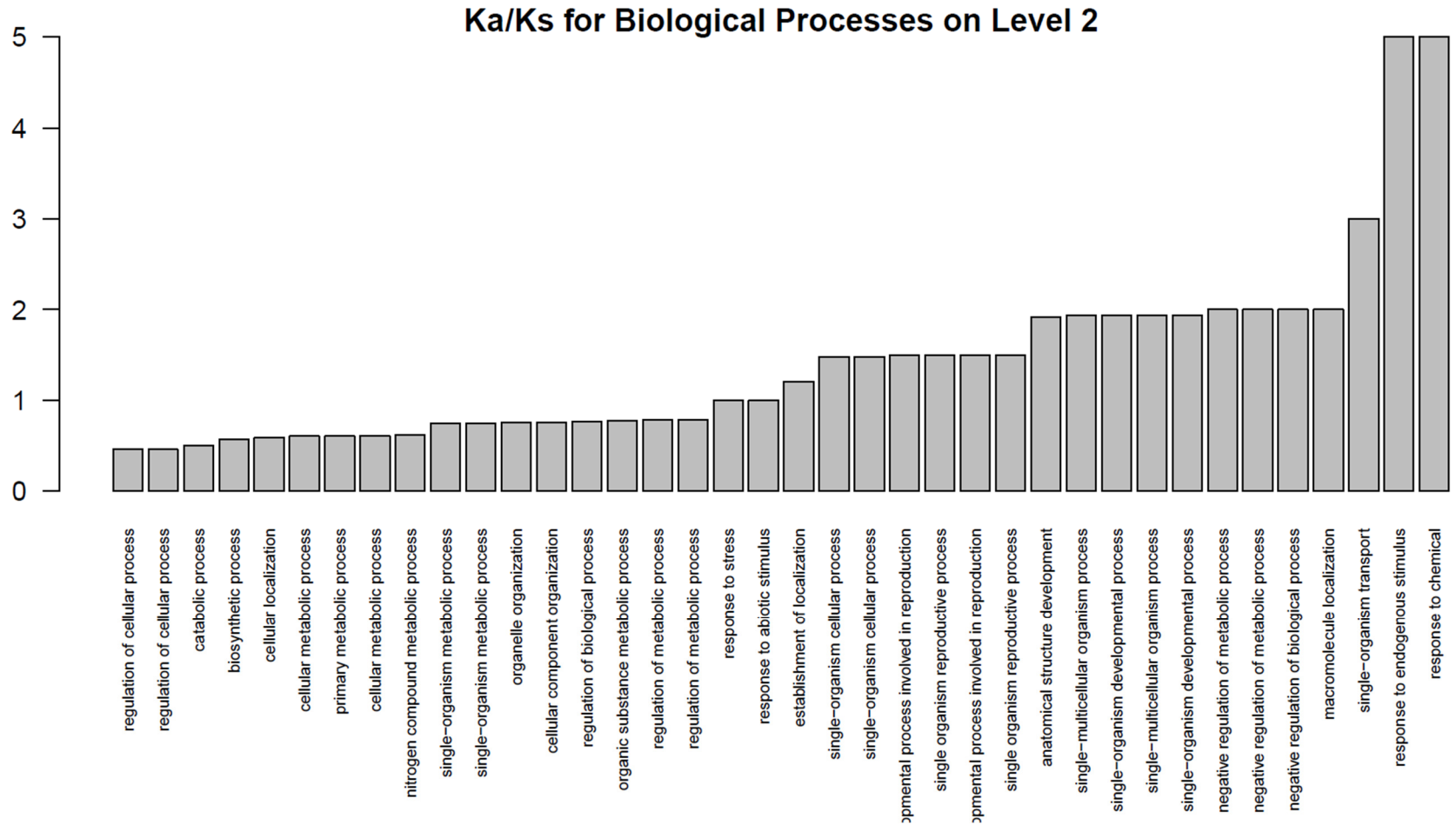

Fig. S1 Proportion of non-synonymous to synonymous mutations (Ka/Ks) for different classes of biological processes.

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APPENDIX

Lebenslauf

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Bildung und beruflicher Werdegang

ab Januar 2017	Wissenschaftliche Mitarbeiterin im Forschungsprojekt: FINAR - Managing Fungal Infections in the Era of Emerging Azole Resistance (gefördert vom BMBF) an der Martin-Luther-Universität Halle-Wittenberg
Mai 2013 - Gegenwart	PhD Student, Helmholtz-Zentrum für Umweltforschung in Halle, Department: Biozönoseforschung Förderung: Deutsche Forschungsgemeinschaft (DFG)
Oktober 2011 – April 2013	Masterabschluss in Biologie an der Martin-Luther-Universität Halle-Wittenberg, Note 'sehr gut' (1,3) Masterarbeit: “Epigenetische Variation bei der genetisch verarmten Pflanzenart *Ceratocapnos claviculata* (L.)“, Note 'sehr gut' (1,3)
Oktober 2008 – September 2011	Bachelorabschluss in Biologie an der Martin-Luther-Universität Halle-Wittenberg, Note 'gut' (2,0) Bachelorarbeit: “Genetische Diversität und Differenzierung invasiver *Prunus serotina* -Populationen entlang eines West-Ost-Gradienten in Europa“, Note 'sehr gut' (1,4)
September 2000 – Juli 2008	Abitur am Martin-Luther-Gymnasium Hartha

Publikationen

Born, J. & Michalski, S.G. (2017) Strong divergence in quantitative traits and plastic behavior in response to nitrogen availability among provenances of a common wetland plant. *Aquatic Botany,* **136,** 138-145.

Born, J. & Michalski, S.G. Trait expression and signatures of adaptation in response to nitrogen addition in the common wetland plant *Juncus effusus*. *PlosOne (submitted)*

Born, J. & Michalski, S.G. Genetic analyses reveal that multi-facetted processes are shaping the intraspecific differentiation patterns of the widespread wetland plant *Juncus effusus* in Europe. *Manuscript*

Konferenzbeiträge

Jennifer Born, R. Lutz Eckstein and Walter Durka (2013)**:** Epigenetic variation in a genetically depauperate range-expanding species, 43rd Annual Meeting of the Ecological Society of Germany, Austria and Switzerland. *Poster*

Jennifer Born, Stefan G. Michalski (2014): Adaptive divergence in functional traits of a widespread wetland plant, 44th Annual Meeting of the Ecological Society of Germany, Austria and Switzerland. *Talk*

Halle (Saale), den 12.07.2018

Jennifer Born

Eigenständigkeitserklärung

Hiermit erkläre ich, dass die vorliegende Arbeit mit dem Titel **„Evolutionary potential in functional traits of a wetland macrophyte (*Juncus effusus*) relevant for natural degradation of contaminants“** bisher weder bei der Naturwissenschaftlichen Fakultät I - Biowissenschaften - der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde.

Darüber hinaus erkläre ich, dass ich die vorliegende Arbeit eigenständig und ohne fremde Hilfe verfasst sowie keine anderen als die im Text angegebenen Quellen und Hilfsmittel verwendet habe. Textstellen, welche aus verwendeten Werken wörtlich oder inhaltlich übernommen wurden, wurden von mir als solche kenntlich gemacht.

Ich erkläre weiterhin, dass ich mich bisher noch nie um einen Doktorgrad beworben habe.

Halle (Saale), den 12.07.2018

Jennifer Born