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Which factors limit the establishment of brachypodium retusum : a key species in ecological restoration of Mediterranean steppes ?

Christel Vidaller

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THÈSE

Présentée pour l'obtention du grade de
Docteur de l'Université d'Avignon et des Pays de Vaucluse

Quels facteurs limitent l'installation de *Brachypodium retusum* – une espèce clé dans la restauration écologique des steppes Méditerranéennes ?

Which factors limit the establishment of *Brachypodium retusum* – a key species in ecological restoration of Mediterranean steppes?

Christel VIDALLER

Sous la direction d'Armin BISCHOFF & Thierry DUTOIT

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Résumé

Le brachypode rameux (*Brachypodium retusum*) est une espèce herbacée pérenne qui domine les pelouses sèches de Méditerranée occidentale. Dans notre zone d'étude du Sud de la France, la recolonisation spontanée est très faible après perturbation du sol. Cette observation ne correspond pas aux résultats d'études menées dans d'autres régions montrant une capacité de colonisation élevée. L'objectif principal de cette thèse était donc de tester les différentes hypothèses pouvant expliquer les différents patrons de colonisation.

Dans un premier chapitre, nous avons testé si ces différents patrons de colonisation résultent d'une différenciation génétique entre les populations. Des marqueurs AFLP ont été utilisés pour analyser la structure génétique de 17 populations de Méditerranée occidentale dont la différenciation neutre entre populations. Dans un sous-échantillon de 13 populations françaises, la différenciation de traits phénotypiques sous sélection a été testée dans un jardin commun et comparée à la différenciation neutre. Dans le deuxième chapitre, nous présentons une étude sur la différenciation adaptative des traits phénotypiques et avons testé une potentielle réponse différentielle à la manipulation de facteurs environnementaux clés. Les deux derniers chapitres de la thèse ont analysé les facteurs environnementaux qui limitent la recolonisation *in situ*, indépendamment du génotype ou de la population. Dans le troisième chapitre, nous avons testé l'effet du pâturage et du feu sur le recouvrement végétatif ainsi que sur la reproduction sexuée de *B. retusum* installé. Enfin, dans le quatrième chapitre, nous avons mesuré l'effet de l'arrosage initial et du pâturage sur l'installation de plantules transplantées initialement germées en serre et de plantules issues de semis *in situ*. Nos résultats ont montré que les populations de *B. retusum* sont génétiquement différenciées pour les marqueurs neutres mais également pour les traits phénotypiques. Cette différenciation est supérieure à la dérive seule et suggère une adaptation aux conditions environnementales, en particulier aux températures estivales et à la fréquence du gel en hiver. Une réponse différentielle à la manipulation expérimentale de facteurs environnementaux (sol, pâturage, humidité du sol) a confirmé le caractère adaptatif de la différenciation génétique. Les expériences *in situ* ont montré que le feu a un effet positif sur la reproduction de *B. retusum* et sur la communauté végétale associée, alors que son recouvrement végétatif n'était pas supérieur à la moyenne de la communauté. L'exclusion du pâturage pendant deux saisons n'a eu d'incidence sur aucun des paramètres mesurés chez les populations adultes. L'arrosage initial a affecté l'installation des plantules au cours de la première saison. Au cours de la deuxième saison, l'effet seul de l'arrosage n'était pas significatif, cependant son effet est resté positif sur la survie uniquement dans les parcelles pâturées. Le pâturage, au début du cycle de vie de *B. retusum*, a eu un effet négatif sur le recrutement et la croissance des plantules. En conclusion, la différenciation adaptative entre les populations peut avoir contribué aux différences régionales en termes de capacité de colonisation et doit être prise en compte lors du ciblage des populations sources pour l'introduction de plantes en restauration écologique. La réponse positive de *B. retusum* a indiqué que le feu était une force sélective importante dans le passé qui pourrait être utilisée pour favoriser l'espèce et la communauté végétale associée dans des opérations de conservation et de restauration. L'exclusion du pâturage à court terme est tolérée par les populations adultes de *B. retusum*, mais l'abandon à long terme entraîne une diminution de son recouvrement par rapport aux graminées pérennes à croissance rapide. Enfin, lors des premiers stades de l'installation des plantules, il est nécessaire d'éviter le pâturage pour garantir le succès de l'introduction, sinon le stress dû au pâturage doit être compensé par un arrosage.

Mots-clés: AFLP; jardin commun; pelouse sèche; exclos; adaptation locale; pelouse méditerranéenne; marqueurs neutres; origine des plantes; poaceae; différenciation entre populations; brûlage dirigé; θ_{ST} ; P_{ST} ; traits phénotypiques; recrutement des plantules; Thero-Brachypodietea

Abstract

Ramosé false brome (*Brachypodium retusum*) is a perennial herbaceous species that dominates dry grasslands of the Western Mediterranean. In our southern French study area, spontaneous re-colonisation is very low after soil disturbance. This observation does not correspond to the results of studies from other regions showing a high colonisation capacity. The major objective of this PhD thesis was to test different hypotheses potentially explaining the different colonisation patterns.

In the first chapter, we tested whether these different colonisation patterns are the result of genetic differentiation among populations. AFLP markers were used to analyse genetic structure including neutral population differentiation in 17 Western Mediterranean populations. In a sub-sample of 13 French populations, differentiation in phenotypic traits under selection was tested in a common garden and compared to neutral differentiation. In the second chapter, we present a study on adaptive differentiation in phenotypic traits testing a potentially differential response to the manipulation of key environmental factors. The last two chapters of the PhD analysed environmental factors that limit re-colonisation in the field independent of genotype or population. In the third chapter, we tested the effect of grazing and fire on vegetative recovery as well as on sexual reproduction of established *B. retusum* and in the fourth chapter we measured the effect of initial watering and grazing on the establishment of transplanted seedlings pre-grown in a greenhouse and of field-sown seedlings.

Our results showed that populations of *B. retusum* are genetically differentiated in neutral markers but also in phenotypic traits. This differentiation is superior to drift alone and suggests adaptation to environmental conditions, particularly to summer temperature and winter frost frequency. A differential response to experimental manipulation of environmental factors (soil, pasture, soil moisture) confirmed the adaptive character of genetic differentiation. The field experiments showed that fire has a positive effect on *B. retusum* reproduction and on the associated community whereas vegetative recovery was not higher than community average. Two seasons of grazing enclosure did not affect any of the measured parameters in adult populations. Initial watering affected seedling establishment in the first season. In the second season, the watering main effect was not significant but interestingly the effect remained positive on survival in grazed plots whereas no such effect was observed in enclosures. Grazing in early life cycle stages of *B. retusum* had a negative effect on seedling recruitment and growth. In conclusion, adaptive differentiation between populations may have contributed to regional differences in colonisation capacity and needs to be taken into account in targeting source populations for plant introduction in ecological restoration. The positive response of *B. retusum* indicated that fire was an important selective force in the past which may be used to favour the species and its associated plant community in current conservation and restoration management. Short-term grazing enclosure is tolerated by mature *B. retusum* populations but long-term abandonment results in a decrease of cover relative to high-growing perennial grasses. In early stages of seedling establishment grazing should be avoided to guarantee introduction success - or grazing stress needs to be compensated by watering.

Key-words: AFLP; common garden; dry grassland; enclosures; local adaptation; Mediterranean grassland; neutral markers; plant origin; poaceae; population differentiation; prescribed burning; θ_{ST} ; P_{ST} ; phenotypic traits; seedling recruitment; Thero-Brachypodieta

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Introduction

I. Theoretical background

Global biodiversity is often described as species richness (May 1992). However, more recent approaches to quantify biodiversity loss and protection use a broader definition including all complexity levels from genes to ecosystems (Sutherland et al. 2013). Biodiversity related to genes includes intra-species variation in genetic information such as intra- and inter-population differentiation and as species diversity, it is influenced by evolutionary forces. The genetic structure of populations is the result of adaptive (selection) and neutral processes (genetic drift, mutations) but their relative contribution is largely unknown (Brousseau et al. 2015) (Figure I). Selection, genetic drift and gene flow shape diversity, structure and genetic variation of plant populations (Loveless & Hamrick 1984; Lenormand 2002; Leimu & Fischer 2008).

Natural selection may result in adaptive population differentiation due to genetically based phenotypic differences that improve local fitness in different environments. Adaptive differentiation occurs when differences among individual heritable traits cause differences in survival and reproduction. Adaptation is an evolutionary process through which traits are modified by natural selection. However, selection is not the only force resulting in population differentiation and may be counterbalanced by other evolutionary forces such as drift, gene flow and mutation (see Appendix I for definitions).

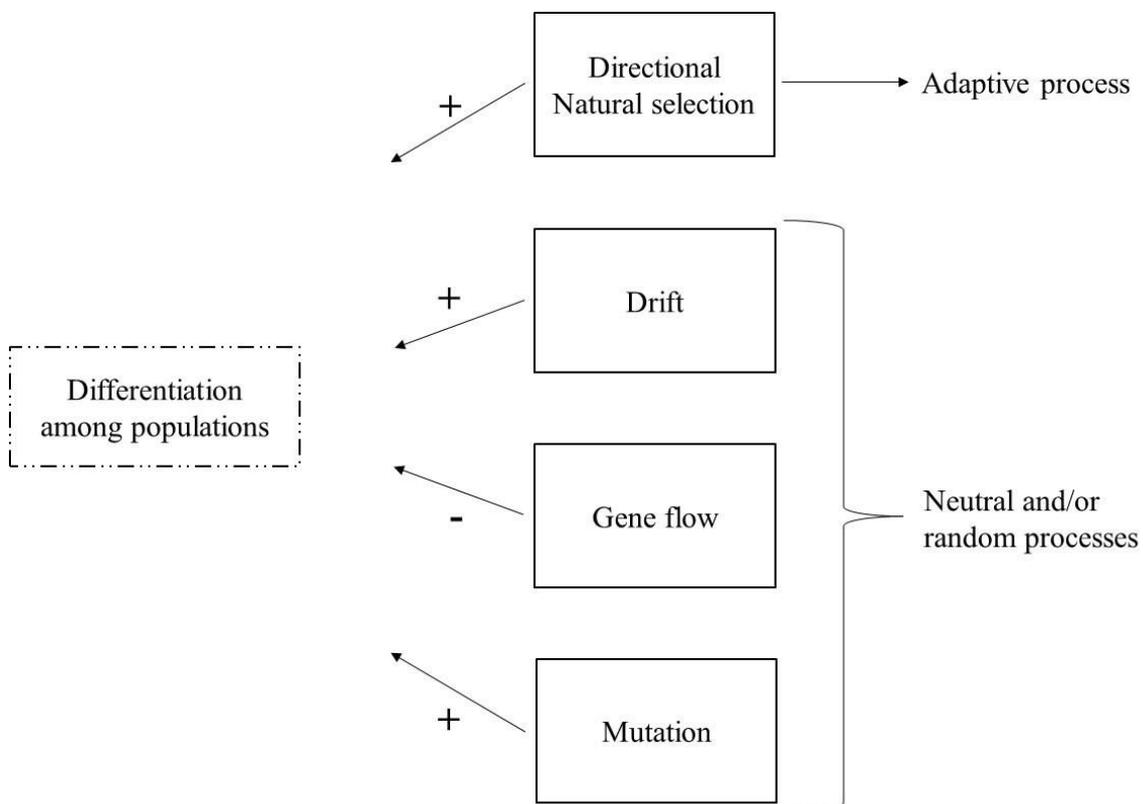


Figure I: Evolutionary forces and their effect on population differentiation.

Main ecological factors driving biodiversity are abiotic (resource availability, water availability, climate) or biotic (intra species or inter species competition). Plant distribution and biomass are largely determined by climate and locally modified by soil conditions (Polis 1999). Local adaptation is often the result of natural selection by abiotic factors such as climate and soil (Macel et al. 2007; Hancock et al. 2011; Manel et al. 2012). In addition to these major selective factors, grazing, water availability and fire shape populations and communities (Naveh 1975; O'Connor 1991; Blondel 2006) (Figure II).

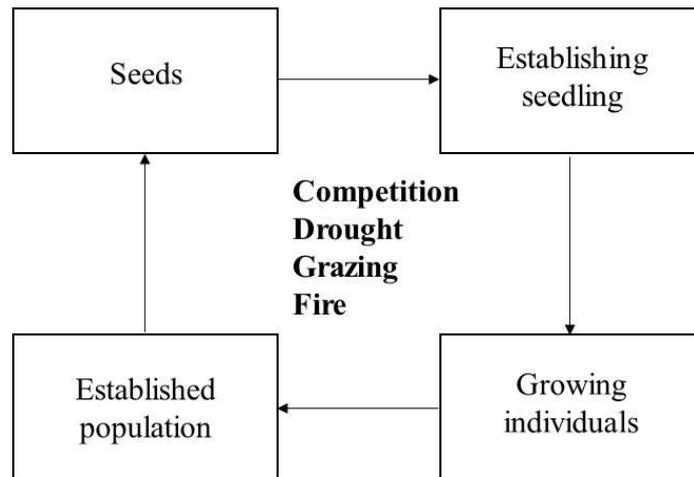


Figure II: Main factors driving population dynamics at several life cycle stages of a perennial grass after seed input. Modified from O'Connor (1991). Fire factor added according to Naveh (1975).

Adaptive differentiation is widespread in grassland species, and plants often show adaptation at regional scale (Becker et al. 2008; Bucharova et al. 2016). In grassland populations, climate is the major large-scale environmental factor driving genetic differentiation, in particular temperature, precipitation and their seasonal distribution (Manel et al. 2012; Bucharova et al. 2016). Soil conditions often vary at smaller scales resulting in small-scale adaptive differentiation (Van der Putten et al. 2004; Macel et al. 2007). Soil chemistry, but also soil structure and organisms are potential selective forces (Wardle 2002). Several studies showed grass species adaptation to soil type including serpentine soils (e.g Freitas & Mooney 1996) for *Bromus hordeaceus* and Meimberg et al. 2010 for *Aegilops triuncialis*) or fertility gradients (Sherrard & Maherali 2012). PH is, for example, one key soil chemistry factor to which plant populations may be adapted (Macel et al. 2007; Raabová et al. 2011). Soil characteristics and climate also influence land management which is a third important driver of adaptive

differentiation in grassland systems. In particular, grazing intensity depending on soil quality is known to affect plant performance and may select for genotypes that tolerate aboveground plant damage (Bullock et al. 2001; Hufford & Mazer 2012).

Apart from genotype-environment interactions, in particular affecting the outcome of plant introduction measures, environmental factors may limit colonisation and seedling recruitment independent of plant genotype or population. Mediterranean dry grasslands are semi-natural habitats that depend on human land management. Sheep grazing is one of the key management factors driving plant species composition and diversity (reviewed by Milchunas & Lauenroth 1993). Several studies have tried to identify plant traits linked to grazing but few general patterns have been found. In arid and semi-arid environments such as Mediterranean dry grasslands, water availability is known to be a strongly limiting factor for plant species establishment (Noy-Meir 1973; Bochet et al. 2007). However, an increase in soil humidity may also increase competition to ruderal plant species better adapted to more humid mesophilic conditions. Wildfires and fire management by shepherds were important drivers in the past but their role in maintaining the ecosystem is nowadays less clear because fire prevention has largely reduced the role of fire in the last decades (Naveh 1975; Parr & Andersen 2006). All these factors are acting on critical stages of the perennial grass life cycle (Figure II, O'Connor 1991). Competition and drought affect populations mainly during early stages whereas grazing effects are equally strong during the whole life cycle. Additionally, complex interactions between these major factors complicate predictions on plant responses such as plant establishment and colonisation processes.

II. Applied background

Since the Neolithic age, humans have strongly modified ecosystems and biodiversity. Sedentism and the development of agriculture resulted in the domestication of animals, selection of plant varieties, landscape opening and fragmentation (Diamond 2002; Skoglund et al. 2012). The beginning of the XIXth century with the beginning Industrial Age marked an increase in ecosystem disruption. Over-exploitation, fossil fuel use, urbanization and agricultural intensification led to a greater human impact on several inter-dependent ecological scales such as populations, communities, ecosystems and landscapes (Lewis & Maslin 2015). Mediterranean ecosystems underwent already great changes in biota and ecosystems during the Roman period (Pausas & Vallejo 1999; Blondel et al. 2010). They are thus the result of a complex “coevolution” of humans and other organisms (Di Castri 1981). This coevolution

involving a diversification of habitats has rather increased biodiversity. The Mediterranean region is today considered as a global biodiversity hotspot including a high number of endemic species (Medail & Quezel 1997; Myers et al. 2000).

Dry grasslands of temperate Europe are well studied since many decades (Tansley 1939; Smith 1980; Hillier et al. 1990; Poschlod & WallisDeVries 2002). Mediterranean dry grasslands are less well studied but the number of publications is currently increasing (Puerto et al. 1990; Lahav & Steinberger 2001; Peco et al. 2005; Buisson et al. 2006; Buisson & Dutoit 2006; Fadda et al. 2008; Henry et al. 2010; Henry et al. 2010; Peco et al. 2012; Robin et al. 2018). Due to the recent decline of these grasslands, an increasing number of studies focuses on ecological restoration (Buisson et al. 2006; Buisson & Dutoit 2006; Fadda et al. 2008; Henry et al. 2010; Robin et al. 2018). The ecological restoration of Mediterranean dry grasslands (“steppes”) of the Thero-Brachypodietea type (San Miguel 2008) based on spontaneous succession is a very slow process (Römermann et al. 2005). Even after several decades of restoration, the species composition is often different from that of the reference community. In particular, key species hardly establish (Blondel & Aronson 1999).

Brachypodium retusum is a dominant widespread core species of dry Mediterranean grasslands managed by grazing. The species is called key species in this thesis due to its dominance and its number of interactions with other species (Saiz & Alados 2011). Approaches to restore such grasslands and to re-establish *B. retusum* have failed in the greatest French Mediterranean steppe area of the Crau plain. Several previous studies have showed that the restoration of initial environmental conditions is not sufficient to restore the plant community with its dominant species. Even the transfer of *B. retusum* rich hay has not been successful in re-establishing populations suggesting a low seedling recruitment (Coiffait-Gombault et al. 2012a, b; Dutoit et al., 2013). The absence of the dominant species has a strong effect on the community structure and functioning (Raventós et al. 2012).

Surprisingly, studies in other Mediterranean systems have not confirmed the slow re-colonisation found in the Crau plain. The species rapidly re-occur after wildfires and is generally frequent in habitats of moderate disturbance (Caturla et al. 2000; De Luis et al. 2004; Cassagne et al. 2011). This may be explained by the strong resprouting capacity from belowground organs representing a high resilience to disturbances and a competitive advantage over annual species. However, a high re-colonisation was even found after soil disturbance by ploughing (Caturla et al., 2000).

III. Main thesis topics

The PhD project aims to identify the causes for the low establishment of *Brachypodium retusum* in Mediterranean steppes and in particular in the Crau area. Two main hypotheses are tested (i) The genetic differentiation explains differences in colonisation capacity and (ii) colonisation depends on environmental conditions such as humidity, grazing or fire that may limit or favour seedling recruitment. We expect that this project will allow us to better understand the ecology of this key species in order to improve the efficiency of restoration measures.

Basic ecology provides useful fundamental knowledge for restoration ecology which consequently provides guidance for ecological restoration measures (Palmer et al. 2006). Conversely, ecological restoration provides opportunities to set up ecological experiments, which contribute to fundamental knowledge in basic ecology (Bradshaw 1987) (Figure III).

Human impact affected and still affects several inter-dependent ecological scales varying through space and time (Lewis & Maslin 2015). Understanding the colonisation capacity of a species and the restoration of an ecosystem requires a better understanding of limiting factors at several ecological scales.

This thesis uses the concepts of four main thematic fields (Appendix I), population genetics, population ecology, community ecology and restoration ecology to identify potential filters and limits of *B. retusum* establishment and their consequences for plant introduction. This transdisciplinary approach allows evaluating the limiting factors at several scales and improving restoration success.

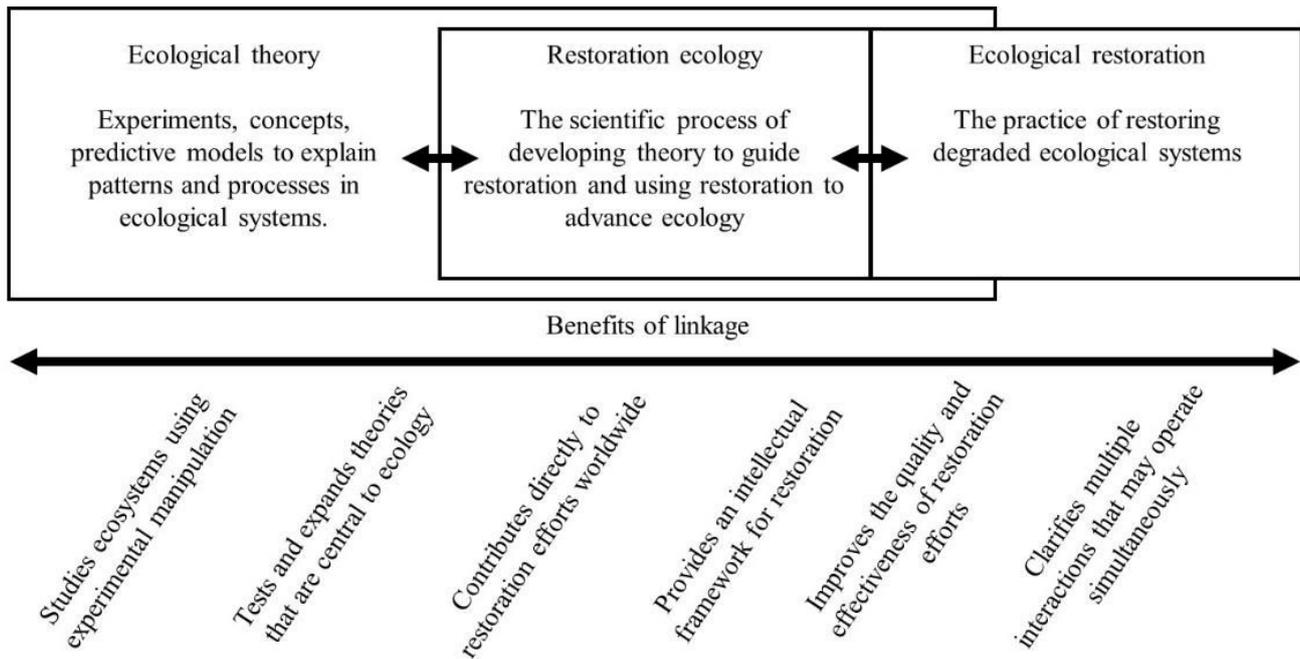


Figure III: The relationship between ecological theory, restoration ecology, and ecological restoration. Modified from Palmer et al., (2006).

Species genetics determines plant performance and environmental interactions may result in adaptive differentiation. In ecological restoration, plant provenance and related genetic differentiation are important issues for targeting source populations. Furthermore, environmental factors limiting plant establishment need to be analysed. Low establishment rates after disturbance and low seed production (Appendix II) of resident populations may limit establishment. Seedling recruitment after arrival of seeds may represent another bottleneck depending on environmental factors. The PhD focuses on two major research topics: (i) genetic differentiation and adaptation and (ii) environmental conditions limiting seedling recruitment and adult plant performance in *B. retusum* populations and its associated plant community. The results are used to discuss and evaluate consequences for plant introduction in ecological restoration.

a. Genetic differentiation and adaptation

Genetic and phenotypic variation as a function of environmental variation and adaptation are common at geographically large scales often representing strong environmental gradients (Etterson 2004; Brouillette et al. 2014; Stojanova et al. 2018). Spatial variation in abiotic and

biotic factors along environmental gradients select for phenotypic traits and thus contribute to genetic structuring and intraspecific differentiation among populations (Manel et al. 2012; Hsiung et al. 2017). Such genetically based phenotypic differences improve the fitness of local populations in different environments resulting in local adaptation. However, genetic structure is also the result of neutral processes (genetic drift, mutations) and the relative contribution of adaptive and neutral processes is still not well understood (Brousseau et al. 2015).

Adaptive differentiation is widespread in grassland species and plants often show adaptation at regional scale (Becker et al. 2008; Bucharova et al. 2017). Climatic and edaphic conditions (Nevo et al. 1988) are the main drivers of adaptive population differentiation that may also occur at small spatial scales, as reported for many grass species (Hamrick & Allard 1972; Nevo et al. 1981; Gutterman & Nevo 1994; Hsiao & Rieseberg 1994; Owuor et al. 1997). Phenotypic traits are typically under directional natural selection related to climate, and the direction and magnitude of this selection varies among local populations (Merilä & Crnokrak 2001). Strong directional selection is common in the wild (Endler 1986; Kingsolver et al. 2001) and most traits are moderately to highly heritable (Mousseau & Roff 1987; El-Lithy 2004).

The adaptation to soil conditions was first detected on soils contaminated with heavy metals indicating that adaptive differentiation may occur quite rapidly (Schat et al. 1996; Antonovics 2006). Furthermore, the pH value and related chemical and biotic properties may represent a strong selective force (Macel et al. 2007; Raabová et al. 2011). Soil humidity is a climate driven soil parameter and a strong limiting factor for plant fitness in arid and semi-arid ecosystem resulting in drought adaptation (Shah & Paulsen 2003; Suriyagoda et al. 2010). However, an increase in soil moisture often results in higher competition for other resources representing a counteracting selective force (Kadmon 1995).

Ecosystem management is a third potential driver of adaptive differentiation in semi-natural systems such as grasslands. Grazing changes site conditions by biomass removal, plant damage, reduced competition and creates heterogeneity in grassland vegetation (Bullock et al. 2001; Hufford & Mazer 2012). It seems to be likely that long-term grazing favours genotypes adapted to grazing (Hufford & Mazer 2012). However, grazing may also obscure adaptation to other environmental factors if this selection pressure has not been consistent in the past (Hufford et al. 2008). Isolation-by-environment (Orsini et al. 2013) is an adaptive evolutionary process increasing population differentiation. Thus, reduced establishment success of introduced or immigrating populations may occur due to maladaptation and should be carefully taken into account in restoration ecology.

Gene flow between different environments may, however, homogenise the gene pool and thus counterbalance adaptive differentiation (reviewed in Räsänen & Hendry 2008). Gene flow may be reduced due to isolation-by-environment but also due to isolation-by-distance (Wright 1931). Isolation-by-distance is a neutral evolutionary process depending on geographic distances and physical barriers (i.e. isolation by resistance; Orsini et al. 2013) between populations.

b. Environmental drivers of early establishment, vegetative recovery and reproduction following disturbance

Seedling recruitment, establishment, adult resistance to disturbance and seed production are crucial for spontaneous re-colonisation and/or plant introduction. Previous work has shown that seedling recruitment of *B. retusum* is very low on restoration sites of the Crau plain (Buisson et al. 2006; Coiffait-Gombault et al. 2012a). Drought stress and competition by the established plant community have been identified as key factors limiting seedling recruitment (Pywell et al. 2007; Nuttle 2007). It is therefore necessary to study the response of *B. retusum* to these factors and their variation to better understand population dynamics and colonisation processes. Such knowledge is indispensable to improve the success of re-introduction in ecological restoration or re-vegetation approaches. Studies in Spain and France have demonstrated that *B. retusum* benefits from wildfires (Caturla et al. 2000; De Luis et al. 2004; Cassagne et al. 2011). The dense belowground rhizome system allows a rapid post-fire recovery representing a competitive advantage compared to other species of the plant community. Additionally, controlled burning was used in the past to improve forage quality of Mediterranean grasslands (Stouff, 1997; Stouff, 1986). Thus, the absence of fire may explain the poor establishment of the species currently observed in the Crau area following arable use. Post-fire resprouting from belowground organs would not explain a poor seedling recruitment (Caturla et al. 2000; De Luis et al. 2004). However, favouring adult, already established plants increases seed production and potentially seed rain arriving at restoration sites.

Grazing was found to positively affect the growth of *B. retusum* (Coiffait-Gombault et al. 2012a, b). Similarly to fire, grazing indirectly favours the species since the resprouting capacity represent a competitive advantage over other species of the plant community such as annuals.

Humidity is another key factor in seedling recruitment. Soil moisture limits germination of most plant species. The high germination of *B. retusum* under optimum humidity in the laboratory (Coiffait-Gombault et al. 2012b) suggests that soil moisture may increase seedling recruitment

in Mediterranean grasslands. However, an increase in soil humidity may also increase competition to ruderal plant species better adapted to more humid mesophilic conditions. Wetter parts of the Crau plain are occupied by such mesophilic species and *B. retusum* is absent there (Molinier & Talon 1950; Masson et al. 2015). Nevertheless, higher water availability may still promote the early stages of seedling establishment (germination, seedling survival and growth).

c. Plant introduction and seed provenance question

Adaptive and neutral population differentiation are important issues in ecological restoration involving plant introduction. Whereas almost all plants show phenotypic and genetic differentiation among populations and regions (Jay et al. 2012) it is less clear whether and how local adaptation contributes to this differentiation (Leimu & Fischer 2008). The current debate on where to select seed material for ecological restoration reflects this ambiguity. Many authors advocate the use of local or regional seed sources which are increasingly adopted by practitioners (McKay et al. 2005; Vander Mijnsbrugge et al. 2010; Bucharova et al. 2017). This approach, however, has been questioned because local adaptation may not be common enough (Bischoff 2014) and local seed sources may frequently be inbred and genetically impoverished (Broadhurst et al. 2008). Hence, knowledge on genetic diversity and differentiation patterns and on the relation to phenotypic variation and local adaptation is increasingly required in order to improve strategies for revegetation. In spite of the known value for monitoring, evaluation and decision-making, genetic aspects are a frequently overlooked issue in restoration approaches (Mijangos et al. 2015).

Observed phenotypic trait variability in the field can be the result of environmental variation, phenotypic plasticity and genetic variation (Falconer & Mackay 1996). Disentangling these ultimate reasons behind trait differentiation among populations and thus gaining insights into mechanisms behind trait differentiation requires integrated approaches that are able to partition the observed trait variability into its respective components. The combination of quantitative and molecular population genetic methods allows such a partitioning even in natural plant communities (Stinchcombe 2014). It further allows quantifying the contribution of adaptive differentiation and random demographic processes to observed genetic differentiation patterns (Leinonen et al. 2013). However, adaptive responses of plant populations to local environmental conditions require the existence of genetically based phenotypic variability. The distribution of such genetic variability within and among populations depends on factors

that are also major drivers of micro-evolutionary patterns. A plant species balance between clonal and sexual reproduction is such a factor potentially influencing both the within-population genetic structure and gene dispersal (Silvertown 2008). Clonality is advantageous in habitats facing strong aboveground but low belowground disturbance such as grasslands. It may facilitate resource uptake in heterogeneous environments and is increasing with population age and successional stage (Silvertown 2008). It may, however, be associated with severe fitness costs such as increased selfing, inbreeding depression or reduced mate availability that in turn may reduce within-population genetic diversity (Honday & Jacquemyn 2008).

IV. The study species: *Brachypodium retusum*

Brachypodium genus belong to the Poaceae family and comprises about 20 worldwide distributed taxa including 18 species (Catalán & Olmstead 2000; Catalán et al. 2012). Three of them are annual and 15 are perennial species (Catalán et al. 2012). *Brachypodium* shows an intermediate evolutionary placement within the grass temperate pooid clade, being closer to the basal than to the recent Pooideae lineages (Catalán et al. 2015).

Ramose false brome (*Brachypodium retusum* P. Beauv.) is a perennial and allopolyploid (presumed hexaploid) species with a chromosomal base number of $2n=38$ (Wolny & Hasterok 2009; Wolny et al. 2011; Betekhtin et al. 2014). It is a rhizomatous C3 grass species adapted to the Mediterranean summer drought (Contu 2013). The species is widespread in the Western Mediterranean (Spain, Southern France, and Italy) but also occurs in the Eastern parts (Catalán et al. 2015). At regional Southern French level, it occurs on calcareous soils of lower mountain ranges but also on decarbonated Red Mediterranean soils of former river valleys whereas *B. retusum* is absent from lowlands with deep clay and loam soils (Vidaller et al. 2018). *B. retusum* is an outcrossing wind-pollinated species with low self-compatibility ranging from 0 to 30.6% (Catalán et al. 2015). It flowers from April to July and shows high clonal growth but a low clonal spread. Its rhizomes form a dense network close to the soil surface resulting in a high tolerance to above-ground disturbance such as wildfires or grazing (Caturla et al. 2000). *B. retusum* seeds do not require vernalisation (Khan & Stace 1999).

Brachypodium retusum is among the first species resprouting after fires and its regeneration is not affected by the frequency or severity of fire (Caturla et al. 2000, De Luis et al. 2004). Due to its dense rhizome-root system, the species stabilizes the soil (Martinez-Fernandez et al. 1995;

Cerdà 1998; Baets et al. 2008). *B. retusum* may facilitate seedling recruitment of other species by reducing post-fire mortality (Raventós et al. 2012). It may further reduce negative density-dependent effects among neighbouring seeder plants during the first post-fire year by reducing mortality (Raventós et al. 2012). Saiz & Alados (2011) found in a dwarf shrub community of south-eastern Spain ecological community a high number of ecological network links for the species. This number of links is an indicator for interactions with other species suggesting that *B. retusum* is a highly interacting species (Saiz & Alados 2011).

Few studies have tested the spatial association between *B. retusum* and other species. Maestre et al. (2005) found a negative small-scale correlation between *B. retusum* and *Stipa tenacissima* in a semi-arid steppe and a positive correlation between *B. retusum* and *Anthyllis cytisoides*.

The species was found to be highly competitive in Spanish pine woodlands and shrublands (Pausas et al. 2002; De Luis et al. 2004; Maestre et al. 2004) reducing seedling survival of pine species by strong competition for water (Clary et al. 2004) and for soil nitrogen (Bonet 2004; Casals et al. 2005).

Although *B. retusum* showed high regeneration and competitive ability in Spanish studies, seedling recruitment and colonisation was very low in our southern French study area (see III, (Buisson et al. 2006; Coiffait-Gombault et al. 2012a, b). Moreover, *B. retusum* represents a main nutritional resource for sheep although many annuals show a higher nutritive value (Tatin et al. 2013), thus its re-establishment in degraded areas would represent an agro-economic benefits for stockbreeders.

V. The study system: Dry Mediterranean grasslands

Grasslands are herbaceous communities dominated by grasses (Poaceae) or other graminoids (Cyperaceae, Juncaceae). Species-rich grasslands represent 27 % of the Earth's ecosystems (Henwood 1998). The total area of Mediterranean basin dry grasslands has considerably declined in the last decades but they still cover 424,371 km² (Dixon et al. 2014). Increased land use intensity, in particular transformation to arable use and land abandonment followed by shrub encroachment are threatening these highly diverse ecosystems. Mediterranean dry grasslands are under decline and protected by the EU habitats directive. *B. retusum* is still widespread in the Mediterranean but also suffers from the loss of this habitat (San Miguel 2008).

Grasslands of the Mediterranean basin depend on three major environmental factors: climate,

edaphic conditions and human impact, in particular livestock grazing (Blondel 2006). Fire and grazing are common disturbances in Mediterranean grasslands and shape populations and community (Naveh 1975). Their strong decrease (fire protection, land abandonment) may partly explain the decline of Mediterranean steppes (San Miguel 2008).

a. Study area

The study and sampling area to analyse genetic differentiation comprises Spain (Andalucía and Catalunya), Italy (Liguria and Sicilia) and southern France (departments Vaucluse, Bouches-du-Rhône, Gard, Hérault and Aude). The core study area in south-eastern France is characterised by a typical Mediterranean climate with a high amount of sunshine (over 3000 hours/year), a hot and dry summer and a mild winter (mean temperature of 7°C) The annual temperature average is 14 ° C and the maximum rainfall from spring to autumn (500 mm/year).

Within the core study area *B. retusum* occurs on base-rich calcareous soils (Rendzina) of lower mountain ranges with pH values of 7.5 to 8.0 and on Red Mediterranean Soils (Haplic Cambisol, see below) with pH values of 6.5 to 7.5. The latter soils originate from former river beds of large streams (Rhône, Durance) and are characterised by a high density of stones shaped by these streams (pebbles, cobbles). Many areas have been grazed by sheep since the Neolithic age but recent grazing history is not known for all sites (Badan et al. 1995; Leveau 2004).

b. In situ experimental field : the Crau plain

The Crau plain with an area of about 600 km² is located in south-eastern France (Bouches-du-Rhône department; 43°33'N 4°52'E; 10 m above sea level). The study site was used as a model system for tests on the influence of environmental factors in the field. The plant diversity is high and representative of Mediterranean “steppe” ecosystems (Wolff et al. 2013). The stony surface limits plant cover. Fifty percent of the soil surface is covered by coarse pebbles or cobbles (Bourrelly et al. 1983).

The plain is part of the paleo-delta of the Durance River which formerly flowed into the Mediterranean sea and had successively covered the plain with sediments from the prealps 650 000 to 120 000 years ago (Devaux et al. 1983; Colomb & Roux 1986). The geological history and Mediterranean climate have formed the Red Mediterranean Soil (Haplic Cambisol) with low fertility levels, in particular of phosphorus and potassium (Römermann et al. 2005). The Crau soil is particularly shallow (40-60 cm) with an impermeable layer of “puddingstone” (5 to 40 m deep) which is not the case at other sites of the same soil type (Devaux et al. 1983).

The absence of access to groundwater increases drought stress limiting plant growth at the Crau sites.

Sheep grazing in the Crau plain began 4000 years ago during the Roman period and continued during the Middle ages until nowadays (Badan et al. 1995; Leveau 2004). Most of the Crau plain is intensively grazed during spring. A large part of the steppe vegetation was lost due to irrigation (since the middle of the XVIth century) transforming the dry into mesophilic grasslands used for hay making (“La Crau verte”). The remaining steppe vegetation “La Crau sèche” is classified as a nature reserve (since 2001) and a Natura 2000 habitat. In the XXth century, even a part of the dry Crau was transformed into arable fields and orchards. Today only 20% of the initial steppe area is still intact representing 8600 ha of which 5811 ha are belonging to the nature reserve (Wolff et al. 2013).

VI. Research questions and thesis structure

My PhD thesis aims to answer to the following major questions:

- (1) What are the spatial and environmental scales of genetic differentiation? Is differentiation adaptive? What are the major drivers of differentiation and adaptation? What are the implications for provenance choice in ecological restoration?
- (2) Which ecological factors limit seedling recruitment and establishment? In particular, do fire, grazing, water supply affect germination, survival, growth and reproduction and are interactions between these factors significant? How can we use this information to improve the success of introduction and restoration measures?

Genetic differentiation in plant species may result from adaptation to environmental conditions but also from stochastic processes. The drivers selecting for local adaptation and the contribution of adaptation to genetic differentiation are often unknown. Previous restoration and succession studies have revealed different colonisation patterns for *Brachypodium retusum*, a common perennial Mediterranean grass. In order to understand these patterns, we measured AFLP marker differentiation in 17 Mediterranean populations and this neutral differentiation was compared to differentiation in phenotypic traits (**Chapter 1**). We further tested adaptation to different environmental factors in a common garden experiment (**Chapter 2**).

Colonisation processes are often limited by the availability of a sufficient amount of seeds (low seed production and dispersal) and by seedling recruitment as a critical life cycle stage. In Mediterranean dry grasslands, three main environmental factors influence seed production and establishment. Sheep grazing is the dominant type of management largely affecting plant populations. Wildfires and fire management were important drivers in the past but their role in maintaining the ecosystem is nowadays less evident because fire prevention and abandonment of traditional prescribed burning have largely reduced their impact in the last decades. Drought stress may be a third factor limiting seedling recruitment being particularly strong during the dry and hot Mediterranean summer but in our study region also occurring in autumn and spring. The last two parts of the thesis analyse environmental factors that may limit seedling recruitment, growth and reproduction: the effect of grazing and fire on vegetative regeneration as well as on the sexual reproduction of adult *B. retusum* and its associated plant community (**Chapter 3**) and the effect of watering and grazing on early establishment (**Chapter 4**).

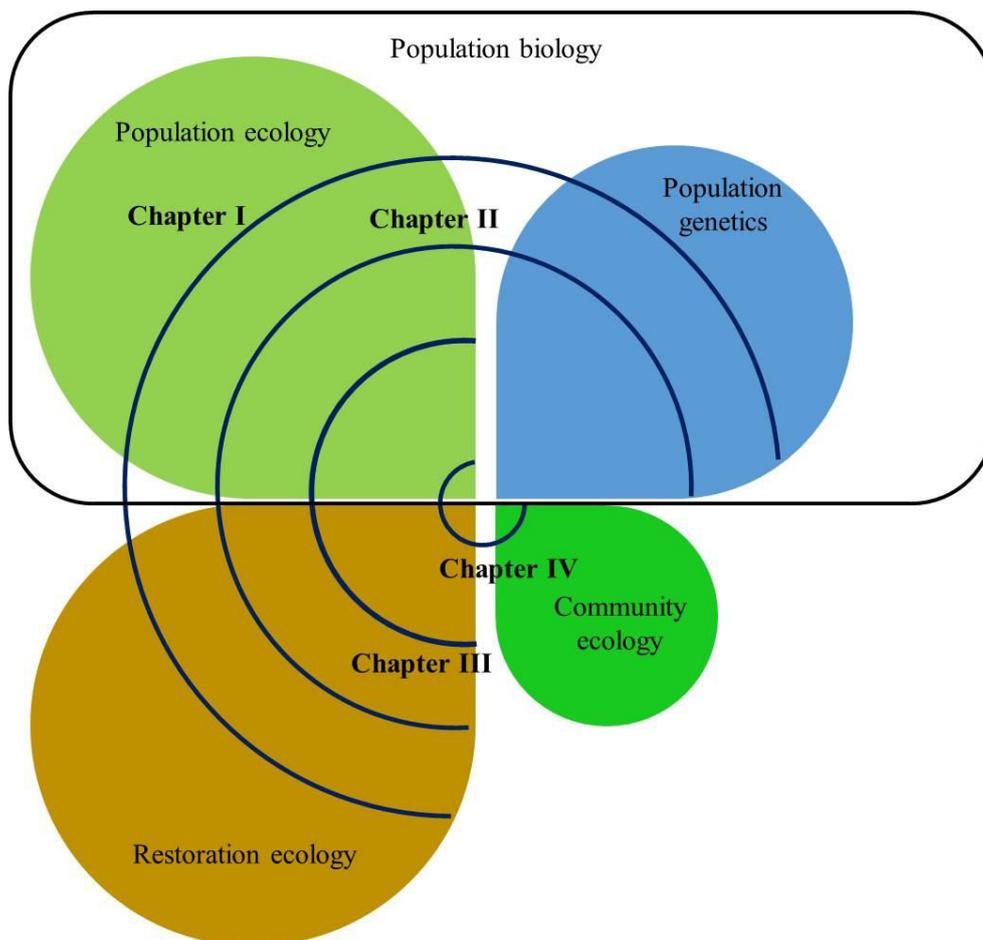


Figure IV: Ecological disciplines of the different chapters illustrating the transversal approach.

Chapter I: Comparison of neutral and adaptive differentiation in the Mediterranean grass species *Brachypodium retusum*. (Submitted to *Molecular Ecology*)

Chapter II: Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum* - the role of soil conditions, grazing and humidity. (Published in *American Journal of Botany*, 2018 (105) 1123-1132)

Chapter III: Fire increases the reproduction of the dominant grass *Brachypodium retusum* and Mediterranean steppe diversity in a combined burning and grazing experiment. (Accepted with minor revision in *Applied Vegetation Science*)

Chapter IV: Which factors limit early establishment of *Brachypodium retusum* a dominant species of Mediterranean grasslands? (Submission to *Basic and Applied Ecology* by the end of October 2018)

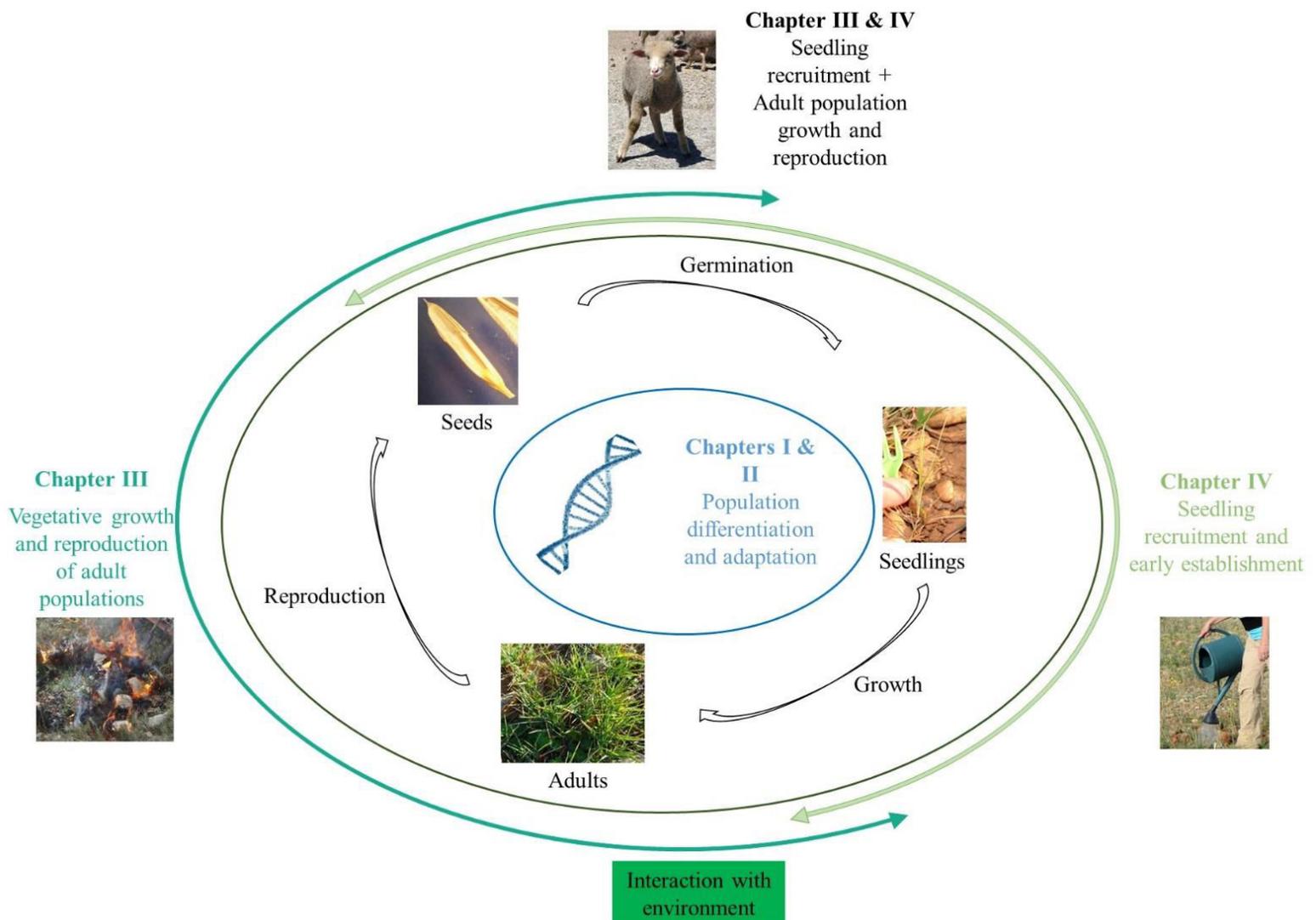


Figure V: Thesis structure.

Transition to Chapter I

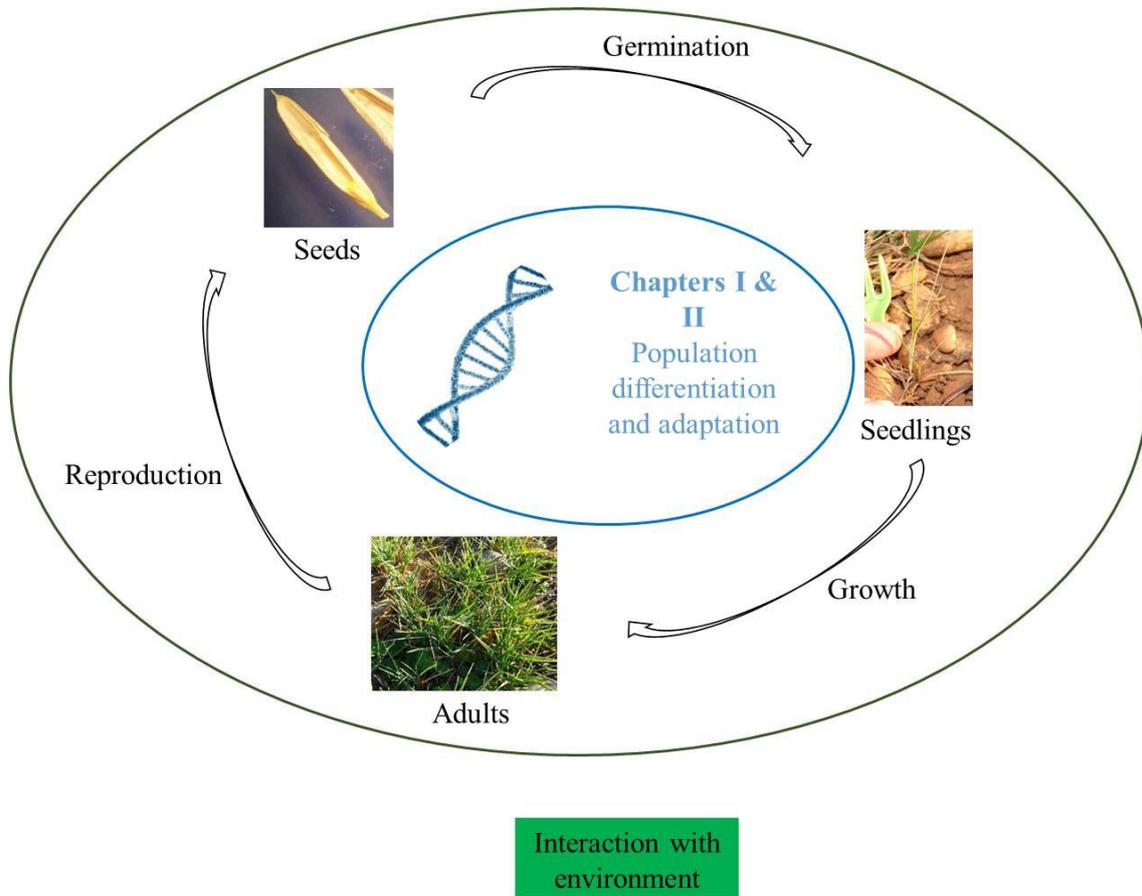


Figure VI: Chapter I structure.

In Chapter I, we focused on neutral and quantitative differentiation of *B. retusum* populations which to my knowledge has never been studied before. We analysed genetic diversity, structure and differentiation in Western Mediterranean populations using AFLP markers. We further compared this neutral variation with variation in phenotypic traits testing a subset of Southern French populations in order to evaluate the relative contribution of drift, gene flow and selection. We hypothesized that small-scale environmental gradients influence adaptive differentiation in phenotypic traits but not or to a much lesser degree differentiation in neutral genetic markers.

Contribution of the thesis:

- Genetic diversity and structure at Western Mediterranean scale.
- Effect of drift and environmental conditions on genetic differentiation.
- Recommendations for targeting source populations in ecological restoration.

Chapter I

Comparison of neutral and adaptive differentiation in the Mediterranean grass species *Brachypodium retusum*.

Submitted to *Molecular Ecology* in October 2018

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Abstract**➤ Background and Aims**

Evolution of phenotypic differentiation between populations is the result of simultaneously acting natural selection and genetic drift. Considerable differentiation in phenotypic traits has been found in the Mediterranean grass *Brachypodium retusum* (Pers.) P.Beauv., the dominant species of a vulnerable steppe habitat. However, scales and drivers of population differentiation are largely unknown. In our study, we compared molecular and phenotypic variation in order to (1) analyse spatial patterns of neutral genetic variation, (2) test for effects of selection on differentiation and (3) identify major drivers of adaptive differentiation.

➤ Methods

We collected plant material of seventeen populations in the Western Mediterranean (France, Spain, Italy) covering a large part of the species range. Neutral population differentiation was estimated using AFLP markers. A regional-scale subset of French populations was sampled in close-by pairs from calcareous and red Mediterranean soils as major habitat types. These populations were grown for two years in a common garden to measure phenotypic traits of vegetative growth and reproduction. We calculated pairwise θ_{ST} values based on AFLP markers and P_{ST} values using phenotypic traits.

➤ Key Results

Global dataset θ_{ST} indicates low but significant differentiation between populations of the Western Mediterranean. In the French populations, P_{ST} of vegetative and reproductive traits were higher than θ_{ST} suggesting that directional selection contributed to population differentiation. We also found significant local-scale differentiation between soil types but differentiation magnitude was much higher at the regional scale. July temperature and winter frost frequency were identified as major drivers of adaptive differentiation.

➤ Conclusions

The study demonstrated the importance of combining neutral marker and phenotypic trait analysis to evaluate genetic structure. Despite low differentiation in AFLP markers, environmental pressure was sufficient to maintain phenotypic differentiation at regional scale. Such information is crucial for targeting source populations in ecological restoration.

Key words: AFLP, common garden, neutral markers, phenotypic traits, dry grassland, θ_{ST} , P_{ST}

INTRODUCTION

Selection, genetic drift and gene flow shape diversity, structure and genetic variation of plant populations (Lenormand 2002; Leimu and Fischer 2008). Adaptive differentiation is widespread in grassland species, and plants often show adaptation at regional scale (Becker *et al.* 2008; Bucharova *et al.* 2016). In grassland populations, climate is the major large-scale environmental factor driving genetic divergence, in particular temperature, precipitation and their seasonal distribution (Manel *et al.* 2012; Bucharova *et al.* 2016). Soil conditions often vary at smaller scales resulting in small-scale adaptive differentiation (Van der Putten *et al.* 2004; Macel *et al.* 2007). Soil chemistry, but also soil structure and organisms are potential selective forces (Wardle 2002). Several studies showed grass species adaptation to soil type including serpentine soils (e.g. Freitas and Mooney 1996 for *Bromus hordeaceus* and Meimberg *et al.* 2010 for *Aegilops triuncialis*) or fertility gradients (Sherrard and Maherali 2012). PH is, for example, one key soil chemistry factor to which plant populations may be adapted (Macel *et al.* 2007; Raabová *et al.* 2011). Soil characteristics and climate also influence land management which is a third important driver of adaptive differentiation in grassland systems. In particular, grazing intensity depending on soil quality is known to affect plant performance and may select for genotypes that tolerate aboveground plant damage (Bullock *et al.* 2001; Hufford and Mazer 2012).

Nevertheless, genetic differentiation is not only the result of adaptation to different environments. Stochastic processes such as genetic drift considerably contribute to population differentiation and may even overwhelm local adaptation (Hereford and Winn 2008; Hereford 2009). Genetic drift and limited dispersal favour isolation-by-distance effects not related to environmental gradients (Sexton *et al.* 2014).

Our model species, *Brachypodium retusum*, is a common perennial grass that grows in dry grasslands, shrublands and open woodlands of the Western Mediterranean basin. It is the characteristic and dominant species of the Natura 2000 habitat “pseudo-steppe with grasses and annuals” protected by the EU habitats directive (San Miguel 2008). This dry grassland community is still widespread in the Western Mediterranean but threatened by overgrazing, conversion to arable land or on the opposite, by land abandonment (San Miguel 2008). Restoration approaches using *B. retusum* seed have often failed due to poor recruitment (Coiffait-Gombault *et al.* 2012) and there is an urgent need for a better understanding of relations between genetic differentiation and local environment to improve restoration success by better targeting source populations (Breed *et al.* 2018). In the French part of the study region,

two major environmental gradients have been identified. First, the species occurs on calcareous soils of lower mountain ranges but also on decarbonated Red Mediterranean soils of former river valleys whereas *B. retusum* is absent from lowlands with deep clay and loam soils (Vidaller *et al.* 2018). Second, the major climatic factor varying in the study region is rainfall increasing from the east to the west. In a previous study on a subset of populations, significant differentiation was found between soil types within sites representing the same climate although differentiation between sites of different climate was much stronger (Vidaller *et al.* 2018).

Classical common garden experiments to test for differentiation do not allow distinguishing drift and adaptation as driving forces (Vidaller *et al.* 2018). Molecular markers provide a powerful tool to study neutral genetic variation and to compare it with potentially adaptive quantitative traits (Brommer 2011; Leinonen *et al.* 2013; Durka *et al.* 2017). Meta-analyses have shown that *differentiation in quantitative traits* is often higher than *differentiation in neutral markers* since gene flow reduces neutral variation but differentiation in traits that are under strong selection is maintained (Leinonen *et al.* 2008; Kort *et al.* 2013). Thus, comparisons of *quantitative traits and neutral markers* provide valuable insights into the causes of spatial genetic divergence among populations (Leinonen *et al.* 2008). They may in particular help to disentangle the contribution of climate and genetic drift to population differentiation often occurring at similar spatial scales (Durka *et al.* 2017).

Using AFLP markers, we aimed at a better understanding of spatial genetic structure in the Western Mediterranean. Using a subset of Southern French populations (regional scale), we tested whether neutral genetic variation correspond to variation in phenotypic traits measured in a common garden. We correlated differentiation at regional level to climate and soil factors to identify the major environmental drivers.

More specifically, we address the following research questions:

- (1) What is the structure of genetic diversity of *B. retusum* at Western Mediterranean scale?
- 2) Does the comparison of phenotypic differentiation and neutral genetic differentiation allow the detection of environmental selection pressure at regional scale in Southern France?
- (3) What are the major drivers of adaptive differentiation; in particular, what is the role of local-scale soil type compared with large-scale climate?

MATERIALS AND METHODS

Study species and sampling

Ramose false brome (Poaceae, *Brachypodium retusum* P. Beauv.) is a rhizomatous perennial C3 grass species. It is a predominantly outcrossing wind-pollinated species that shows high clonal growth but also sexual reproduction. Its rhizomes form a dense network close to the soil surface resulting in a high resilience to above-ground disturbance such as wildfires or grazing (Caturla *et al.* 2000). *B. retusum* is a perennial and allopolyploid (presumed hexaploid) species with a chromosomal base number of $2n=38$ (Wolny *et al.* 2011).

Plant material of 17 populations was collected in the Western Mediterranean basin from Northern Italy to Southern Spain focusing on Southern French populations (Table S1). For 15 populations, we collected plant material (seeds or leaves) from twenty patches with a minimum distance of 10 m between patches to avoid that patches belong to the same individual. In each patch, we used only one seed or leaf for further analyses resulting in 20 different mother plants per population. Two populations were bulk samples with unknown number of mother plants (SES, ROQ, Table S1). Southern French populations were collected in pairs from base-rich calcareous soils of lower mountain ranges and from close-by decalcified red Mediterranean soils (Haplic cambisols). Calcareous soils are shallow on calcareous bedrock with an average soil pH of 8.4. Red Mediterranean soils show a higher clay content resulting in a better nutrient and water retention (pH 7.3). Because of the higher soil fertility and accessibility, grasslands on red Mediterranean soils were more intensively used in the past (in particular grazing) than those on calcareous soils. In sixteen populations, seeds were sampled at natural sites and later on grown in a common garden (see below) or in a growth chamber. Leaves for AFLP analysis were then sampled from these seed-grown plants. In one population, leaves were directly sampled in the field.

Sample preparation and DNA extraction

Leaves of 20 plants per population (ten for ROQ population) were collected for AFLP analysis resulting in a total of 366 plants were analyzed. Between 10-15 mg of silica-dried and further frozen leaves from the field, greenhouse-cultivated samples were ground in a mixer mill “TissueLyser” (Quiagen-Retsch). Total DNA was extracted using NucleoSpin Plant II Kit (Macherey & Nagel, Germany) (Detailed method description 1). DNA concentrations were measured using a photometer (Biophotometer, Eppendorf, Germany).

AFLP genotyping

According to Vos *et al.* (1995), 100 ng of DNA was digested using the restriction enzymes *EcoRI* and *Tru 9I* (Fisher Scientific, France) for 3 h at 37°C and then for 3h at 65°C in a total volume of 25 µl (15 µL + 10µl of DNA). Digestion products were immediately ligated to 0.5 µL Eco and 25 µL Mse adaptors for 3 h at 37°C and treated with T4 DNA Ligase and 0.1 µL of 100 mM ATP to a final volume of 25 µL (5 µL + 20 µl of restriction products). Ligation products were diluted eight times and pre-selective PCR amplification was performed using *EcoRI*+A, Mse+C primers and *Taq* DNA polymerase in a 44.5 µL volume. The pre-amplification thermocycle profile was 94°C for 2 min, followed by 20 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 1 min and 72°C for 10 min. Four primer combinations were chosen for the selective amplification PCR: ASI: *EcoRI*-AAC/*MseI*-CAA, ASII: *EcoRI*-AGG/ *MseI* -CGG, ASIII: *EcoRI*-AGC/ *MseI* -CAG, ASIV: *EcoRI*-ATG/ *MseI* -CTA dyed with 6-FAM fluorescence at 5' Eco end (Eurofins Genomics, Ebersberg, Germany). Hundred times diluted pre-amplification products were used to perform selective amplification in a final volume of 20 µL (15 µL + 5 µL of diluted pre-amplification products). As selective amplification thermocycle profile, we used 94°C for 2 min, 10 cycles of 94°C for 30 s, 65°C for 30 s (step -1°C per cycle), 72°C for 1 min, followed by 22 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, 72°C for 5 min and 4°C for 2h. The fragment length produced by the amplification was separated and quantified by capillary electrophoresis using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, U.S.A.) with GS600 LIZ size marker.

Analysis of phenotypic traits in common garden plants

In order to understand genetic differentiation based on quantitative traits, individuals from 13 French populations were grown for two years in a common garden. In December 2015, collected seeds were germinated in peat plugs (4 cm x 4 cm), placed in a growth chamber (temperature 15/20 °C, 12/12h, night/day) and watered regularly. In March 2016, 24 plugs per population (corresponding to 24 patches) were randomly selected and transplanted to a previously ploughed experimental site in Avignon-Montfavet. Two plugs per population were planted to random positions within each of 12 blocks (3 * 3m²). During the first four months, seedlings were moderately watered. Plots were hand-weeded to limit competition. Plant height, diameter, tiller number, leaf number and the length of the longest leaf were measured for two growing seasons.

Data analysis

Large-scale genetic structure using AFLP

Peaks were scored in Peak Scanner V 1.0 (Applied Biosystems) as present (1), absent (0), or “no data” (NA) if they were not unambiguously identified. In Raw Geno 2.0 (Arrigo *et al.* 2009), only fragments longer than 100 bp and smaller than 500 bp were considered. Maximum binning between peaks was set at 1.75 and minimum at 1.5. The reliability of AFLP markers was checked by repeating the complete analysis from DNA amplification to AFLP screening on 16 samples for each pair of primers. A Bayesian analysis using BAYESCAN (Foll and Gaggiotti 2008) was run to detect loci potentially under selection (outliers) and remove them for further analysis to focus on neutral genetic diversity. Two datasets were used, a global one to understand large-scale genetic structure and a subsample to analyse genetic structure at regional scale using AFLP and quantitative traits.

Diversity indices were estimated using R package poppr. Parameters of genetic diversity (percentage of polymorphic loci, number of multilocus genotypes, Shannon-Wiener Index of MLG diversity, Nei’s diversity index of expected heterozygosity and standardized index of association) were calculated for each population. Genetic differentiation among populations was analysed using divergence at neutral marker θ_{ST} , an analogue of F_{ST} for binary data using GenAIEx 6.5 (Peakall and Smouse 2012). The significance test of θ_{ST} was based on 9999 permutations. An UPGMA tree was constructed from the matrix of pairwise population differences (R package phangorn).

Regional scale genetic structure: phenotypic and neutral marker differentiation

For a subset of thirteen Southern French populations, the mean θ_{ST} was compared to the mean P_{ST} based on phenotypic traits. A Bayesian analysis using the software STRUCTURE 2.2 (Pritchard *et al.* 2007) was applied to assess genetic structure among AFLP samples. STRUCTURE uses an iterative approach to cluster genotypes into K populations without *a priori* knowledge to which population individuals belong. From $K=1$ to $K=13$ populations, and for each K solution, ten replicates were run with 100,000 burn-in simulations followed by 2 million simulations per replicate. Using STRUCTURE, direct posterior probabilities for K (log-likelihood) as well as the *ad hoc* statistic ΔK (Evanno *et al.* 2005) were estimated using uniform *a priori* values of K between one and thirteen (total number of populations).

Separate PCA were run on vegetative (plant height, diameter, tiller number, leaf number and length) and reproductive phenotypic traits (inflorescence and spikelet number). We used first axis PCA scores as a synthetic response variable considering several phenotypic traits at the same time to calculate P_{ST} values (divergence in quantitative traits). Calculations were run using R package Pstat with 9999 bootstraps (Da Silva and Da Silva 2018) but sum of squares (SS) were used as variance estimates instead of mean sum of squares in order to compare P_{ST} to SS based θ_{ST} . P_{ST} is analogous to Q_{ST} and is used if heritability estimates cannot be measured (Brommer 2011)

$$P_{st} = \frac{c\sigma_B^2}{c\sigma_B^2 + 2h^2\sigma_W^2} = \frac{\frac{c}{h^2}\sigma_B^2}{\frac{c}{h^2}\sigma_B^2 + 2\sigma_W^2}$$

with σ_B^2 being the phenotypic variance between populations, σ_W^2 the phenotypic variance within populations, c the proportion of the total variance explained by additive genetic effects and h^2 the heritability, proportion of phenotypic variance explained by additive genetic effects. The null assumptions were chosen for $\frac{c}{h^2}$ parameters with $c=h^2=1$ (Brommer 2011, Detailed method description 2).

In order to evaluate local soil type versus regional-scale differentiation (climate, drift), the two adjacent populations from red Mediterranean and calcareous soils were considered as originating from the same site resulting in a total of six sites and two soil types per site. Thus, each population represented a particular combination of site and soil. For AFLP markers, we applied an analysis of molecular variance (AMOVA) based on θ_{ST} statistics using GenAlEx 6.0. An ANOVA was calculated to analyse the effect of site, soil and the soil \times site interaction on first PCA axis. A multiple regression on distance matrices (MRM) was used to identify variables (climate distance, geographic distance, genetic distance and pH distance) that best predicted P_{st} distance among populations. Additionally, partial distance-based redundancy analyses (dbRDA) was applied to evaluate the relationship between divergence in phenotypic traits and environmental variables (Rpackage Vegan). Partial dbRDA were fitted separately for P_{st} of vegetative and reproductive traits using permutation testing (Legendre and Anderson 1999). First, a marginal test was performed using only environmental variables as predictors. Second, a conditional test was run using genetic distance and geographical coordinates as covariates to take into account the potential confounding effects of isolation by neutral genetic

drift. The genetic distance matrix was transformed into continuous rectangular vectors via principal coordinates analysis. The significance of environmental factors was evaluated using a dbRDA permutation test (9999 permutations). All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)) except for genetic structure.

RESULTS

AFLP analysis allowed genotyping of 366 individuals on 587 loci. After filtering for reliable non-redundant loci (error rate <4.39 %), suppressing unscored individuals and non-polymorphic loci, we created two datasets. A global dataset (98.48% of polymorphic loci) with 322 individuals was genotyped on 323 loci and a Southern French dataset (97.27% of polymorphic loci) with 258 individuals was genotyped on 320 loci. Only two outliers under selection were found by BAYESCAN analysis and removed from the datasets. No clone was found in any of the datasets.

Large-scale genetic structure using AFLP (global dataset)

Average Shannon diversity (H) of tested populations ranged from 2.302 to 3.044, and expected heterozygosity, H_e , from 0.134 to 0.205 (Table 1). In twelve populations, the standardized index of association was significantly different from zero rejecting the null hypothesis of no linkage between markers of twelve populations. In five populations, no linkage between markers was detected. The significant r_{barD} values ranging from 0.001 to 0.101, suggest asexual reproduction but occurring at different rate in these twelve populations. In the global data set, the Italian NIT was the only population showing private alleles.

Table1: Genetic diversity estimates in total dataset populations based on AFLP data. Populations are ranked according to longitude from east (top of the table) to west. Pop: Population name, N: Sample size, H: Shannon-Wiener Index of MLG diversity (Shannon, 2001), He: Nei's diversity index of expected heterozygosity, rbarD: standardized index of association, p.rbarD: p.value of rbarD.

| Country | Pop | N | H | He | rbarD | p. rbarD |
|---------|-------|--------|-------|-------|-------|----------|
| Italy | NIT | 19 | 2.944 | 0.205 | 0.101 | 0.001 |
| France | ME_C | 20 | 3.044 | 0.166 | 0.001 | 0.038 |
| France | ME_R | 19 | 2.944 | 0.172 | 0.003 | 0.003 |
| France | CA_C | 20 | 2.995 | 0.164 | 0.004 | 0.001 |
| France | CA_R | 20 | 3.044 | 0.165 | 0.002 | 0.021 |
| France | SR_C | 18 | 2.890 | 0.159 | 0.000 | 0.180 |
| France | SR_R | 20 | 2.995 | 0.166 | 0.007 | 0.001 |
| France | SM_C | 20 | 2.995 | 0.158 | 0.004 | 0.001 |
| France | SM1_R | 20 | 2.995 | 0.165 | 0.006 | 0.001 |
| France | SM2_R | 20 | 2.995 | 0.147 | 0.008 | 0.001 |
| France | NI_C | 20 | 2.995 | 0.168 | 0.002 | 0.009 |
| France | NI_R | 20 | 2.995 | 0.159 | 0.000 | 0.433 |
| France | MO_C | 19 | 2.944 | 0.150 | 0.000 | 0.634 |
| France | MO_R | 20 | 2.995 | 0.176 | 0.002 | 0.019 |
| France | ROQ | 10 | 2.302 | 0.165 | 0.003 | 0.059 |
| Spain | NES | 18 | 2.890 | 0.142 | 0.001 | 0.152 |
| Spain | SES | 17 | 2.833 | 0.134 | 0.007 | 0.001 |
| Average | | 18.941 | 2.929 | 0.162 | 0.009 | 0.091 |

In the global dataset, the average θ_{ST} (0.102; $P < 0.001$) revealed moderate genetic differentiation among the 17 populations. Pairwise θ_{ST} between populations ranged from 0.006 to 0.224 and were significantly different from zero except for one population pair (Table S2). The UPGMA tree calculated on the pairwise θ_{ST} matrix revealed differentiation of non-French populations but no clear geographical pattern for French populations (Fig. 1).

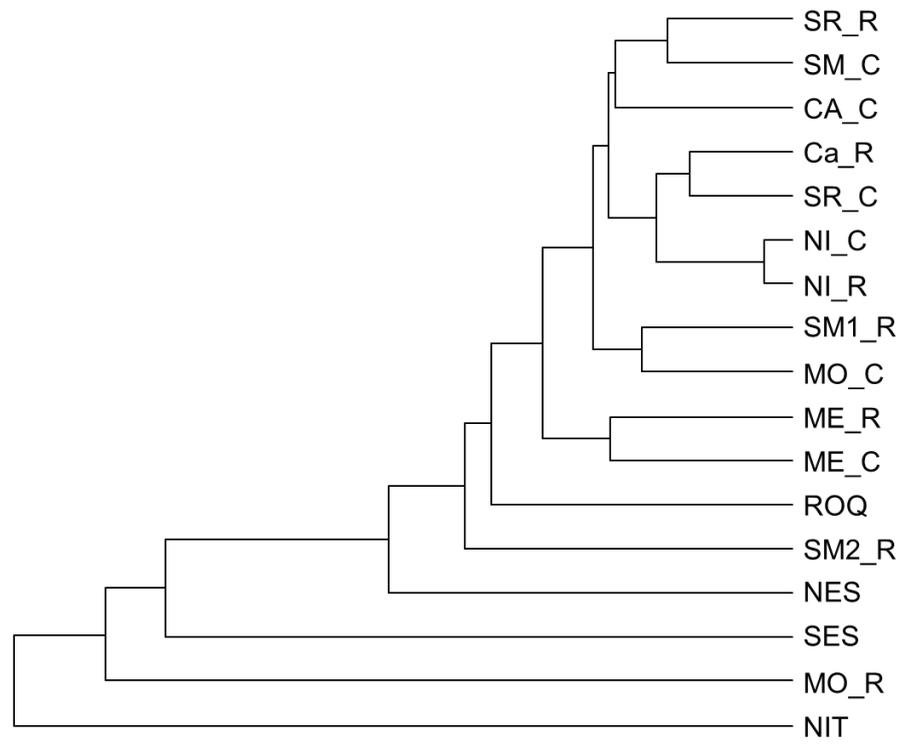


Figure 1: UPGMA dendrogram showing the relationship between global dataset populations based on θ_{ST} .

Regional scale genetic structure: phenotypic and neutral marker differentiation

In the Southern French dataset, the average θ_{ST} (0.072; $P < 0.001$) was lower than in the global data set and pairwise θ_{ST} between populations ranged from 0.006 to 0.197 (Table S2). The STRUCTURE analysis provided the strongest support when samples were clustered into two groups (K=2) and five groups (K=5) based on ΔK method of Evanno (Fig. S1). At K=5, both assignment ratio by individual and geographical distribution of genetic cluster are indicating a high level of admixture (Fig. 2 and 3).

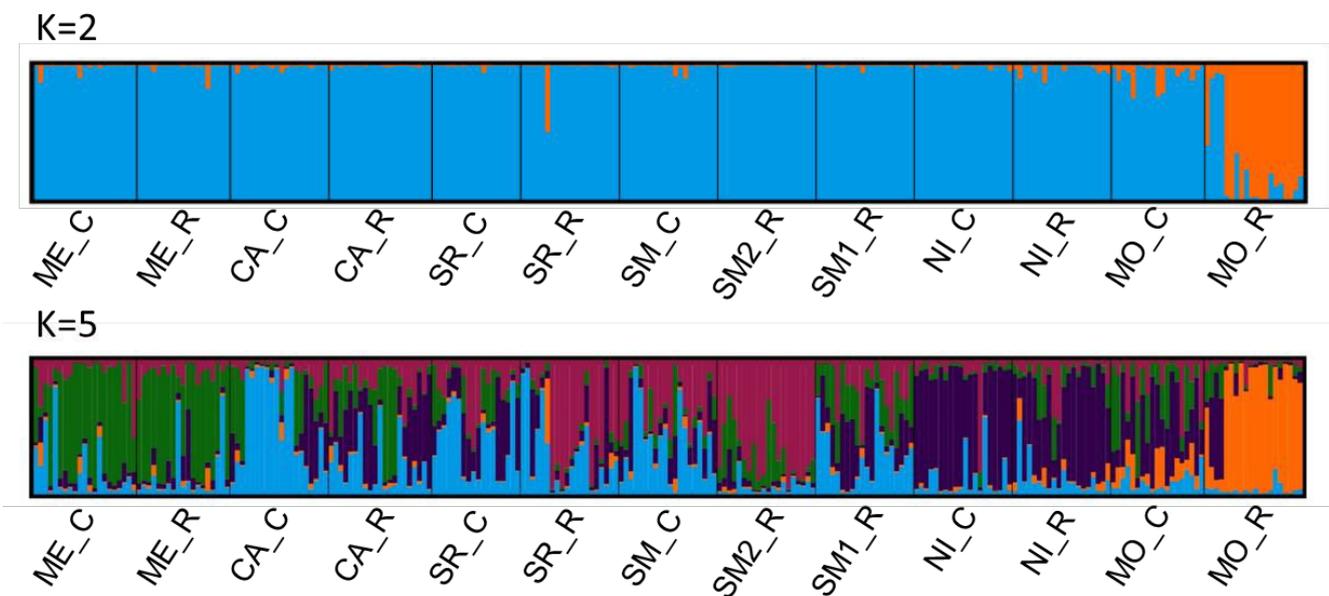


Figure 2: Genetic structure according to STRUCTURE 2.2 for K=2 and K=5 (optimum values according to Evanno method). The y-axis indicates the assignment ratio of each individual and individuals are assorted by population ranked from east (left) to west (right). Different colours correspond to clusters.

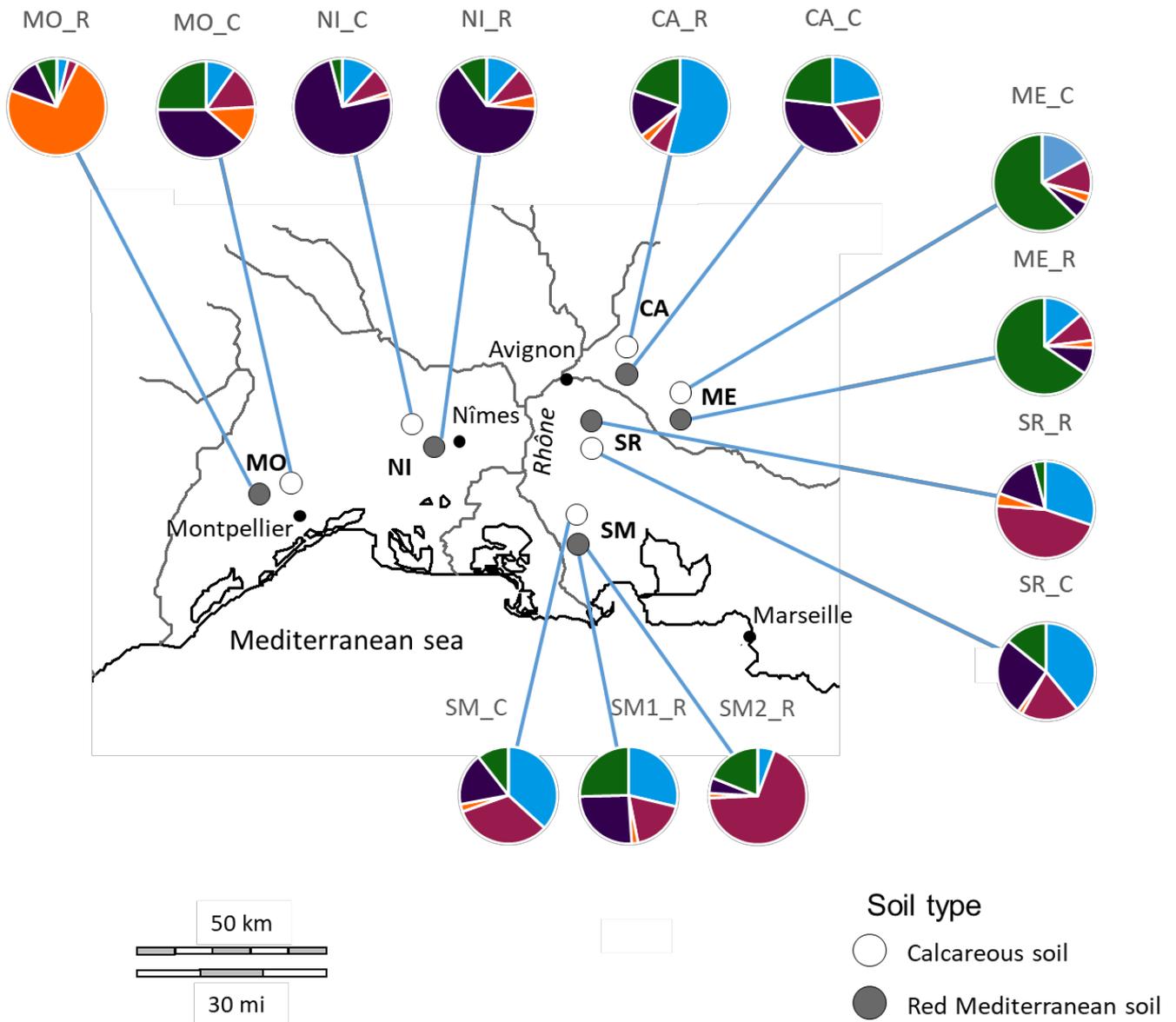


Figure 3: Geographical distribution of the genetic clusters identified by STRUCTURE 2.2 (pie charts) in Southern French *Brachypodium retusum* populations on red Mediterranean and calcareous soil.

Compared to differentiation in neutral AFLP markers, the phenotypic differentiation was higher. The average P_{st} for vegetative (0.171; $P < 0.001$) and reproductive traits (0.138; $P < 0.001$) revealed significant phenotypic differentiation among the 13 populations. Pairwise P_{st} between populations were 0.062 to 0.432 for vegetative and 0.066 to 0.355 for reproductive traits, respectively (Table S3). Average population differentiation was at least two times greater

for quantitative traits than for neutral markers. Confidence intervals do not overlap. Moreover, critical $\frac{c}{h^2}$ values for vegetative and reproductive traits are low: 0.3 and 0.4 (Fig 4A and 4B).

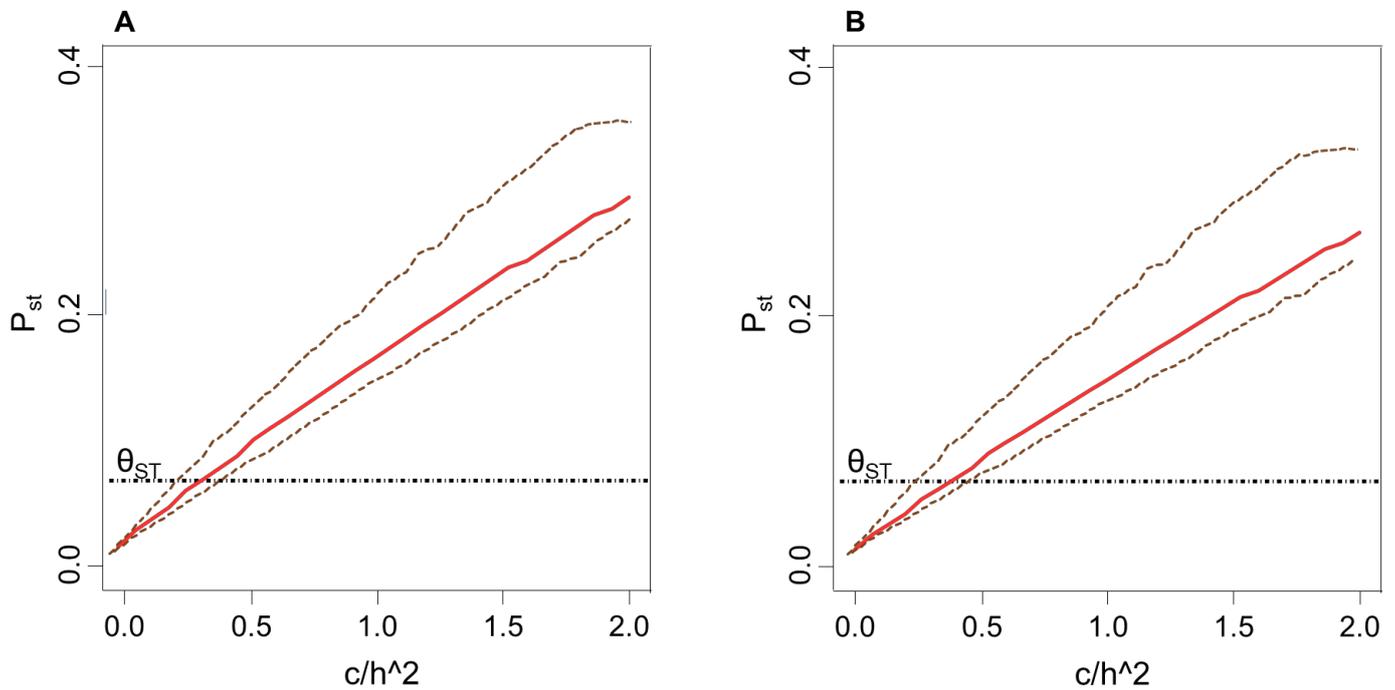


Figure 4: P_{st} simulation (\pm c.i.) for continuous varying $\frac{c}{h^2}$ and upper θ_{ST} comparison. A: Vegetative traits, B: Reproductive traits.

Site of origin contributed more to the differentiation than soil of origin. The site of origin accounted for 2.33 % of total variation for AFLP markers, 12.85 % for vegetative traits and 8.37 for reproductive traits whereas the soil of origin accounted for 0.62, 1.92 and 0.05 %, respectively (Table 2). Variation between soil types was still significant in AFLP markers (highly significant) and vegetative traits but not in reproductive traits. The significant soil x site interaction in molecular and phenotypic traits indicated that soil type variation depend on site of origin.

Table 2: Analysis of variance on AFLP (based on AMOVA) and phenotypic vegetative and reproductive traits (based on ANOVA performed on first axis PCA scores). MSD: Mean squared deviation and percentage of variation explained by collection site, soil type within collection site and site x soil interaction.

| | df | MSD | % of total variance |
|----------------------------|-----|--------|---------------------|
| AFLP | | | |
| Among sites | 5 | 85.370 | 2.33 *** |
| Among soils | 1 | 52.063 | 0.62 *** |
| Among soil*site | 5 | 56.310 | 5.00 *** |
| Within populations | 245 | 26.927 | 93.06 |
| Vegetative traits | | | |
| Among sites | 5 | 29.534 | 12.85 *** |
| Among soils | 1 | 19.676 | 1.92 * |
| Among soil*site | 5 | 14.531 | 6.76 ** |
| Within populations | 242 | 4.138 | 65.25 |
| Reproductive traits | | | |
| Among sites | 5 | 26.671 | 8.37 *** |
| Among soils | 1 | 0.775 | 0.05 NS |
| Among soil*site | 5 | 16.122 | 5.23 ** |
| Within populations | 242 | 6.028 | 80.38 |

Multiple regression analysis showed that phenotypic differentiation in vegetative and reproductive traits was correlated to climatic distance between sites but also provided evidence that differentiation in vegetative traits was partly explained by neutral genetic distances (Table 3).

Table 3: Multiple regression analysis (MRM). Standardized regression coefficients of multiple regression on distance matrices, with climatic distance, geographic distance and genetic distance in AFLP markers (θ_{ST}) as explanatory and pairwise P_{st} distances as response variables. R^2 : multiple correlation coefficient.

| Variables | P_{st} vegetative traits | | P_{st} reproductive traits | |
|---------------------|----------------------------|---------|------------------------------|---------|
| | Coefficient | P-value | Coefficient | P-value |
| Climate | 0.18 | 0.04 * | 0.07 | 0.05 * |
| Geographic distance | 0.10 | 0.20 NS | 0.03 | 0.06 . |
| θ_{ST} | 1.65 | 0.04 * | 1.69 | 0.13 NS |
| pH | 0.03 | 0.60 NS | 0.01 | 0.70 NS |
| R^2 | 0.57 | 0.02 * | 0.52 | 0.04 * |

The dbRDA results showed a significant correlation between phenotypic trait P_{st} and climate factors when not corrected for differentiation in neutral markers confirming MRM results (Table 4A). When neutral variation in AFLP markers was taken into account a part of these significant correlations disappeared (Table 4B). However, correlations between phenotypic traits and mean July temperature and number of frost days remained significant (marginally significant for reproductive traits, Table 4B, Fig. S2). Additionally, the correlation between corrected vegetative traits and mean annual temperature was marginally significant.

Table 4: Partial distance-based redundancy analyses (dbRDA) testing for effects of environmental factors on divergence in vegetative and reproductive traits (P_{st}). A: marginal test of individual sets of predictor variables and B: partial (conditional) test including geographic distance (latitude, longitude) and genetic distance as covariates. F -values, significance levels of ANOVA-like permutation tests and percentage of variation explained by each environmental factor. . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

| | P_{st} vegetative traits | | P_{st} reproductive traits | |
|-----------------------------|----------------------------|-------------------------|------------------------------|-------------------------|
| | F-values | Explained variation (%) | F-values | Explained variation (%) |
| A- Marginal tests | | | | |
| Precipitation | 6.606 * | 18.12 | 3.883 * | 15.48 |
| Mean annual temperature | 4.007 * | 8.29 | 2.072 NS | 8.27 |
| Mean January temperature | 5.336 * | 13.84 | 3.651 * | 13.61 |
| Mean July temperature | 5.048 * | 13.04 | 3.669 * | 14.20 |
| Days T<0 | 6.181 * | 16.95 | 3.796 * | 15.15 |
| pH | 4.001 * | 13.20 | 2.507 NS | 9.94 |
| B- Conditional tests | | | | |
| Precipitation | 0.803 NS | 3.30 | 0.905 NS | 7.21 |
| Mean annual temperature | 3.000 NS | 10.20 | 1.318 NS | 11.50 |
| Mean January temperature | 2.142 NS | 8.21 | 1.120 NS | 10.81 |
| Mean July temperature | 5.362 * | 22.60 | 2.961 . | 26.20 |
| Days T<0 | 6.345 * | 25.80 | 3.488 . | 31.00 |
| pH | 2.839 NS | 11.31 | 2.124 NS | 19.42 |

DISCUSSION

Structure of genetic diversity revealed by neutral molecular markers

In this study, we significantly extend the knowledge on the genetic diversity structure of *Brachypodium retusum*, the key grass species of a vulnerable grassland habitat in the Western Mediterranean of Europe. First, *B. retusum* showed a high level of genetic variability and diversity within populations. The number of polymorphic loci was higher than in other

perennial *Brachypodium* species (*B. pinnatum*, *B. sylvaticum*) but its genetic diversity was lower (Bařa *et al.* 2012; Mo *et al.* 2013). *B. retusum* is a widespread outcrossing wind-pollinated species with high self-incompatibility (Catalán *et al.* 2015). These reproductive characteristics favor high levels of genetic variability (Ge *et al.* 1999; Nybom and Bartish 2000). However, longevity and in particular clonal growth may have reduced diversity compared with other perennial *Brachypodium* species (Young *et al.* 1996). Indeed, most populations showed linkage disequilibrium, associations between alleles at different loci (r_{barD} , table 1), indicating a potential effect of asexual reproduction on genetic diversity.

Second, the cluster analysis of genetic differentiation (UPGMA, Fig. 1) revealed that Spanish and Italian populations are well separated from Southern French ones. At regional Southern French scale, analyses of AFLP data showed a low differentiation between populations (θ_{ST} of 7.2%) associated to strong admixture and weak geographical pattern (Structure, Fig. 2 and 3). Such a low genetic structuring at regional scale is typical for outcrossing species with long-distance gene flow and was also found for the outcrossing *B. pinnatum* (Bařa *et al.* 2012). The self-compatible *B. sylvaticum* showed, however, a higher structuration and differentiation of populations since selfing reduces gene flow between populations (Rosenthal *et al.* 2008).

Regional scale genetic structure: phenotypic and neutral marker differentiation

In Southern French populations, phenotypic differentiation (P_{ST}) was higher than neutral molecular differentiation (θ_{ST}) supporting divergent natural selection and adaptation to local environments (Volis *et al.* 2005; Leinonen *et al.* 2013). Critical $\frac{c}{h^2}$ was low confirming that the difference between neutral molecular and phenotypic differentiation is significant and robust (Brommer 2011).

No isolation-by-distance was found in phenotypic traits but a significant effect of θ_{ST} on vegetative phenotypic traits still showed an influence of neutral processes on phenotypic differentiation. Genetic drift and gene flow are major drivers of population differentiation even in phenotypic traits (e.g. Lande 1976; Orsini *et al.* 2013). However, effects of environmental conditions on phenotypic differentiation measured in a common garden experiment remained significant after controlling geographical-distance and genetic distance based on AFLP in the dbRDA analysis (Table 4). This finding supports that divergent directional selection led to adaptive differentiation. The major environmental factors associated to phenotypic differentiation among Southern French populations were summer temperature and number of

frost days. Summer temperatures are closely related to drought stress strongly affecting plant fitness and survival in Mediterranean systems and in particular limiting seedling recruitment (Bochet *et al.* 2007; Thomas *et al.* 2010; Vidaller *et al.* 2018). Southern French populations are relatively close to the northern range limit of *B. retusum* (San Miguel 2008; Catalán *et al.* 2015). Thus, it is likely that frost is a strong selective force contributing to population differentiation (e.g. Kreyling *et al.* 2012a; b). Frost has been identified as a major driver of population differentiation in trees (Savolainen *et al.* 2004; J. Kreyling *et al.* 2012a) but also in some herbaceous species (Agrawal *et al.* 2004; Kreyling *et al.* 2012b).

Soil type within sampling sites explained a significant part of variation among populations in neutral markers and in vegetative phenotypic traits but explained variation was much smaller between soils within sites than between sites suggesting that soil was not a major driver of genetic structure. The soil pH as the principal difference between the two tested soil types had no effect on phenotypic differentiation (P_{st}) when corrected for the influence of genetic drift or geographical distance. Small-scale adaptive differentiation to soil conditions has been shown for sites contaminated with heavy metals (Ernst 1987) but environmental gradients were very strong in the cited studies. If gradients are less strong gene flow may prevent adaptive differentiation at smaller scales (Leimu and Fischer 2008; Hereford 2009). Thus, adaptation to soil conditions often showing small-scale variation was not always detected or its magnitude was smaller than that of climate acting on larger scales and thus being less sensitive to homogenizing gene flow (Macel *et al.* 2007; Raabová *et al.* 2007).

CONCLUSIONS

Brachypodium retusum populations of the Western Mediterranean basin exhibited a high genetic diversity and weak to moderate differentiation between populations. Patterns of differentiation in neutral markers suggest a predominant influence of genetic drift. At regional scale in Southern France, both divergent selection and drift were detected by comparing phenotypic and neutral molecular differentiation. The major driver of adaptive differentiation was climate, in particular summer temperature and winter frost frequency. The two main soil types of the region contributed significantly to population differentiation but explained much less variation than climate and genetic drift at larger scales. Local adaptation is therefore highly probable in *B. retusum*. Accordingly, plant provenance targeting in ecological restoration needs to consider this spatial and environmental pattern of population differentiation. Local seed

collected on the same soil type should be preferred but proximity of source populations seems to be more important than corresponding soil type.

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SUPPORTING INFORMATION

Table S1: Soil characteristics, annual precipitation, mean annual air temperature (including January and July mean in brackets), and the number of frost days ($T < 0$) at collection sites. Sites are ranked from east to west. Climate data are based on daily averages from the nearest meteorological stations (1980-2010). Soil: C = calcareous soil, R = red Mediterranean soil, * Soil on granite bedrock, not red Mediterranean soil but chemical properties closer to F than to C.

| Country | Site | Soil | pH | x | y | Precipitation (mm*a ⁻¹) | Temperature (°C) [Jan/Jul] | Days T<0 |
|---------|------|------|-----|-----------|-----------|--|-------------------------------|-------------|
| IT | NIT | C | 8.1 | 43.852352 | 7.955755 | 469 | 16.6 [10.1/23.4] | 1 |
| FR | ME | C | 8.0 | 43.756456 | 5.199526 | 583 | 14.5 [6 /23.7] | 49 |
| FR | ME | R | 8.0 | 43.750425 | 5.210495 | 583 | 14.5 [6 /23.7] | 49 |
| FR | CA | C | 8.2 | 43.898827 | 4.941070 | 676 | 14.6 [5.9/24.2] | 33 |
| FR | CA | R | 6.9 | 43.898319 | 4.932395 | 676 | 14.6 [5.9/24.2] | 33 |
| FR | SR | C | 7.8 | 43.768047 | 4.831898 | 625 | 15.1 [6.6/24.3] | 30 |
| FR | SR | R | 6.8 | 43.798952 | 4.841659 | 625 | 15.1 [6.6/24.3] | 30 |
| FR | SM | C | 8.1 | 43.705354 | 4.797561 | 517 | 15.3 [7.4/24.4] | 24 |
| FR | SM | R | 6.8 | 43.523843 | 4.832804 | 517 | 15.3 [7.4/24.4] | 24 |
| FR | SM | R | 6.8 | 43.633055 | 5.019083 | 517 | 15.3 [7.4/24.4] | 24 |
| FR | NI | C | 8.0 | 43.784700 | 4.259389 | 701 | 14.9 [6.7/24.1] | 34 |
| FR | NI | R | 7.8 | 43.722868 | 4.339264 | 701 | 14.9 [6.7/24.1] | 34 |
| FR | MO | C | 8.9 | 43.645416 | 3.691638 | 870 | 14.1 [6.4/23.2] | 51 |
| FR | MO | R | 6.7 | 43.607580 | 3.449269 | 870 | 14.1 [6.4/23.2] | 51 |
| FR | ROQ | C | 8.1 | 42.981988 | 2.763333 | 652 | 15.1 [7.7/23.8] | 11 |
| SP | NES | R* | 7.4 | 41.706568 | 2.863655 | 666 | 16.1 [9 /24.2] | 13 |
| SP | SES | C | - | 37.563303 | -4.481613 | 493 | 17.1 [8.6/27.6] | 3 |

Detailed method description 1: DNA extraction

1- Sample homogenization by centrifugation

2- Cell lysis

400 μL PL1 + 10 μL RNase A \rightarrow 30 ' / 65°C in oven

Centrifugation 5 ' / 8000 rpm

3- Filtration / clarification of lysate

Transfer the supernatant to a new tube on a NucleoSpin® Filter (violet ring)

Centrifugation 2 ' / 8000 rpm

Transfer lysate in a 2mL tube avoiding the pellet

4- Adjust DNA binding conditions

Add 450 μL PC and mix pipetting up and down – 5 times

5- Bind DNA

Place a NucleoSpin® Plant II Column (green ring) into a new Collection Tube (2 mL) and load a maximum of 700 μL of the sample.

Centrifugation 1 ' / 8000 rpm

Repeat this step once

6- Wash and dry silica membrane

1st wash: 400 μL PW1 Centrifugation 1 ' / 8000 rpm

2nd wash: 600 μL PW2 Centrifugation 1 ' / 8000 rpm

3rd wash: 200 μL PW2 Centrifugation 2 ' / 8000 rpm

7- Elute DNA

Pipette 100 μL of PE buffer (65°C)

Incubate 5' at 65°C

Centrifugation 1 ' / 8000 rpm

Detailed method description 2: Critical $\frac{c}{h^2}$ formula

$$\frac{c}{h^2} (\text{critical}) = \frac{2\sigma_W^2(\text{lower})F_{st}(\text{upper})}{1-F_{st}(\text{upper})} \quad \text{with} \quad \sigma_W^2(\text{lower}) = \frac{[1-P_{st}(\text{lower})]\frac{c}{h^2}}{2P_{st}(\text{lower})}$$

Table S2: Pairwise θ_{ST} among populations. . P < 0.1, * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

| | NIT | ME_C | ME_R | CA_C | CA_R | SR_C | SR_R | SM_C | SM1_R | SM2_R | NI_C | NI_R | MO_C | MO_R | ROQ | NES |
|-------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| ME_C | 0.183 *** | | | | | | | | | | | | | | | |
| ME_R | 0.190 *** | 0.044 *** | | | | | | | | | | | | | | |
| CA_C | 0.180 *** | 0.060 *** | 0.047 *** | | | | | | | | | | | | | |
| CA_R | 0.164 *** | 0.058 *** | 0.056 *** | 0.038 *** | | | | | | | | | | | | |
| SR_C | 0.187 *** | 0.059 *** | 0.063 *** | 0.038 *** | 0.024 *** | | | | | | | | | | | |
| SR_R | 0.200 *** | 0.062 *** | 0.059 *** | 0.045 *** | 0.043 *** | 0.035 *** | | | | | | | | | | |
| SM_C | 0.203 *** | 0.065 *** | 0.075 *** | 0.040 *** | 0.042 *** | 0.040 *** | 0.030 *** | | | | | | | | | |
| SM1_R | 0.191 *** | 0.057 *** | 0.053 *** | 0.049 *** | 0.046 *** | 0.030 *** | 0.041 *** | 0.038 *** | | | | | | | | |
| SM2_R | 0.227 *** | 0.079 *** | 0.071 *** | 0.089 *** | 0.076 *** | 0.068 *** | 0.055 *** | 0.079 *** | 0.068 *** | | | | | | | |
| NI_C | 0.182 *** | 0.069 *** | 0.065 *** | 0.054 *** | 0.035 *** | 0.038 *** | 0.041 *** | 0.055 *** | 0.049 *** | 0.086 *** | | | | | | |
| NI_R | 0.172 *** | 0.060 *** | 0.059 *** | 0.051 *** | 0.025 *** | 0.033 *** | 0.046 *** | 0.050 *** | 0.045 *** | 0.082 *** | 0.006 *** | | | | | |
| MO_C | 0.174 *** | 0.067 *** | 0.058 *** | 0.060 *** | 0.045 *** | 0.063 *** | 0.063 *** | 0.054 *** | 0.036 *** | 0.090 *** | 0.049 NS | 0.044 *** | | | | |
| MO_R | 0.224 *** | 0.162 *** | 0.159 *** | 0.149 *** | 0.153 *** | 0.173 *** | 0.153 *** | 0.156 *** | 0.161 *** | 0.197 *** | 0.156 *** | 0.147 *** | 0.113 *** | | | |
| ROQ | 0.180 *** | 0.083 *** | 0.071 *** | 0.068 *** | 0.069 *** | 0.070 *** | 0.089 *** | 0.077 *** | 0.078 *** | 0.114 *** | 0.065 *** | 0.058 *** | 0.073 *** | 0.180 *** | | |
| NES | 0.205 *** | 0.089 *** | 0.102 *** | 0.093 *** | 0.084 *** | 0.101 *** | 0.106 *** | 0.087 *** | 0.104 *** | 0.136 *** | 0.105 *** | 0.080 *** | 0.099 *** | 0.214 *** | 0.091 *** | |
| SES | 0.177 *** | 0.150 *** | 0.151 *** | 0.151 *** | 0.132 *** | 0.152 *** | 0.157 *** | 0.153 *** | 0.167 *** | 0.207 *** | 0.140 *** | 0.122 *** | 0.131 *** | 0.239 *** | 0.176 *** | 0.150 *** |

Figure S1: Delta K, calculated according to Evanno et al. (2005) is plotted against the number of modeled gene pools (K).

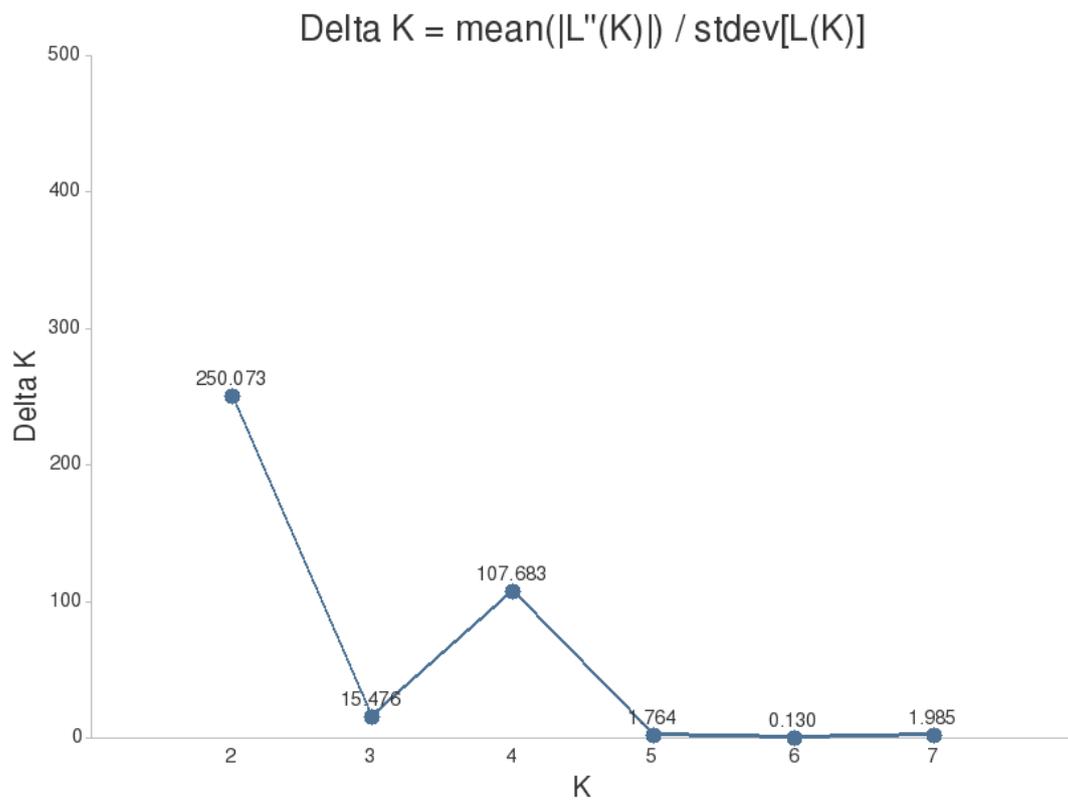
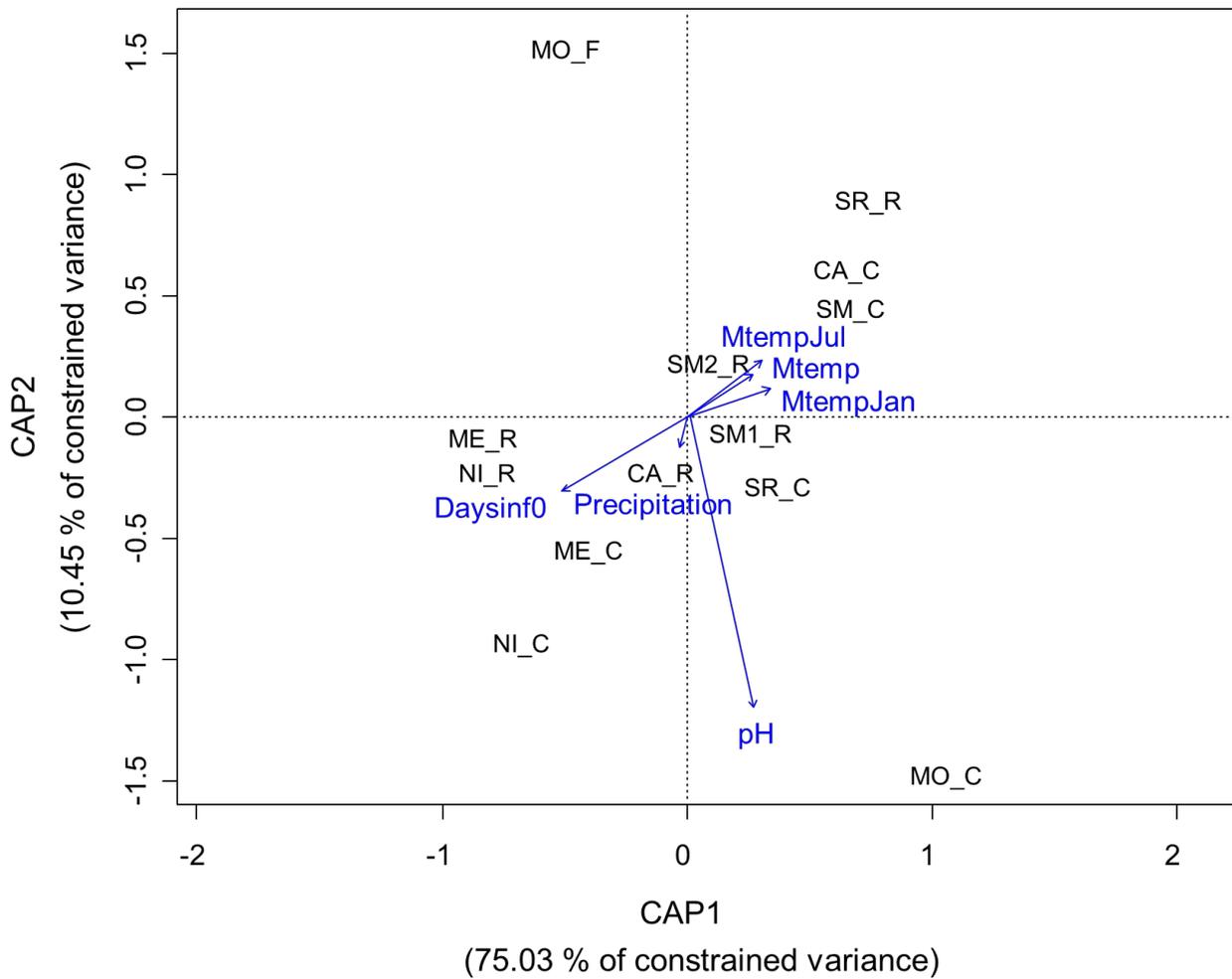


Table S3: Pairwise P_{st} between Southern French populations for vegetative (A) and reproductive (B) traits. All pairwise comparisons were significant ($P < 0.001$).

| A | ME_C | ME_R | CA_C | CA_R | SR_C | SR_R | SM_C | SM1_R | SM2_R | NI_C | NI_R | MO_C |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| ME_R | 0.079 | | | | | | | | | | | |
| CA_C | 0.131 | 0.160 | | | | | | | | | | |
| CA_R | 0.083 | 0.088 | 0.146 | | | | | | | | | |
| SR_C | 0.089 | 0.121 | 0.112 | 0.100 | | | | | | | | |
| SR_R | 0.160 | 0.164 | 0.075 | 0.176 | 0.112 | | | | | | | |
| SM_C | 0.128 | 0.146 | 0.071 | 0.141 | 0.095 | 0.078 | | | | | | |
| SM1_R | 0.116 | 0.129 | 0.071 | 0.101 | 0.062 | 0.095 | 0.081 | | | | | |
| SM2_R | 0.139 | 0.145 | 0.099 | 0.143 | 0.096 | 0.078 | 0.089 | 0.089 | | | | |
| NI_C | 0.130 | 0.098 | 0.271 | 0.148 | 0.200 | 0.267 | 0.283 | 0.223 | 0.242 | | | |
| NI_R | 0.127 | 0.091 | 0.218 | 0.125 | 0.176 | 0.237 | 0.221 | 0.175 | 0.200 | 0.062 | | |
| MO_C | 0.098 | 0.119 | 0.085 | 0.099 | 0.089 | 0.088 | 0.083 | 0.074 | 0.125 | 0.225 | 0.198 | |
| MO_R | 0.288 | 0.245 | 0.412 | 0.297 | 0.351 | 0.432 | 0.415 | 0.362 | 0.400 | 0.221 | 0.189 | 0.376 |

| B | ME_C | ME_R | CA_C | CA_R | SR_C | SR_R | SM_C | SM1_R | SM2_R | NI_C | NI_R | MO_C |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| ME_R | 0.075 | | | | | | | | | | | |
| CA_C | 0.098 | 0.097 | | | | | | | | | | |
| CA_R | 0.150 | 0.113 | 0.087 | | | | | | | | | |
| SR_C | 0.082 | 0.079 | 0.072 | 0.080 | | | | | | | | |
| SR_R | 0.155 | 0.138 | 0.121 | 0.082 | 0.115 | | | | | | | |
| SM_C | 0.091 | 0.087 | 0.101 | 0.160 | 0.074 | 0.167 | | | | | | |
| SM1_R | 0.170 | 0.089 | 0.084 | 0.098 | 0.080 | 0.114 | 0.091 | | | | | |
| SM2_R | 0.096 | 0.096 | 0.124 | 0.098 | 0.072 | 0.098 | 0.095 | 0.087 | | | | |
| NI_C | 0.135 | 0.134 | 0.178 | 0.198 | 0.149 | 0.235 | 0.165 | 0.199 | 0.178 | | | |
| NI_R | 0.136 | 0.132 | 0.187 | 0.211 | 0.142 | 0.239 | 0.138 | 0.193 | 0.164 | 0.073 | | |
| MO_C | 0.116 | 0.093 | 0.089 | 0.085 | 0.084 | 0.098 | 0.098 | 0.066 | 0.123 | 0.192 | 0.177 | |
| MO_R | 0.219 | 0.236 | 0.298 | 0.339 | 0.333 | 0.355 | 0.285 | 0.318 | 0.268 | 0.198 | 0.201 | 0.331 |

Figure S2: Distance-based redundancy analysis (dbRDA) showing the influence of environmental variables on pairwise vegetative traits P_{st} among populations. The vectors illustrate correlations between the original environmental variables and the dbRDA-axes.



Transition to Chapter II

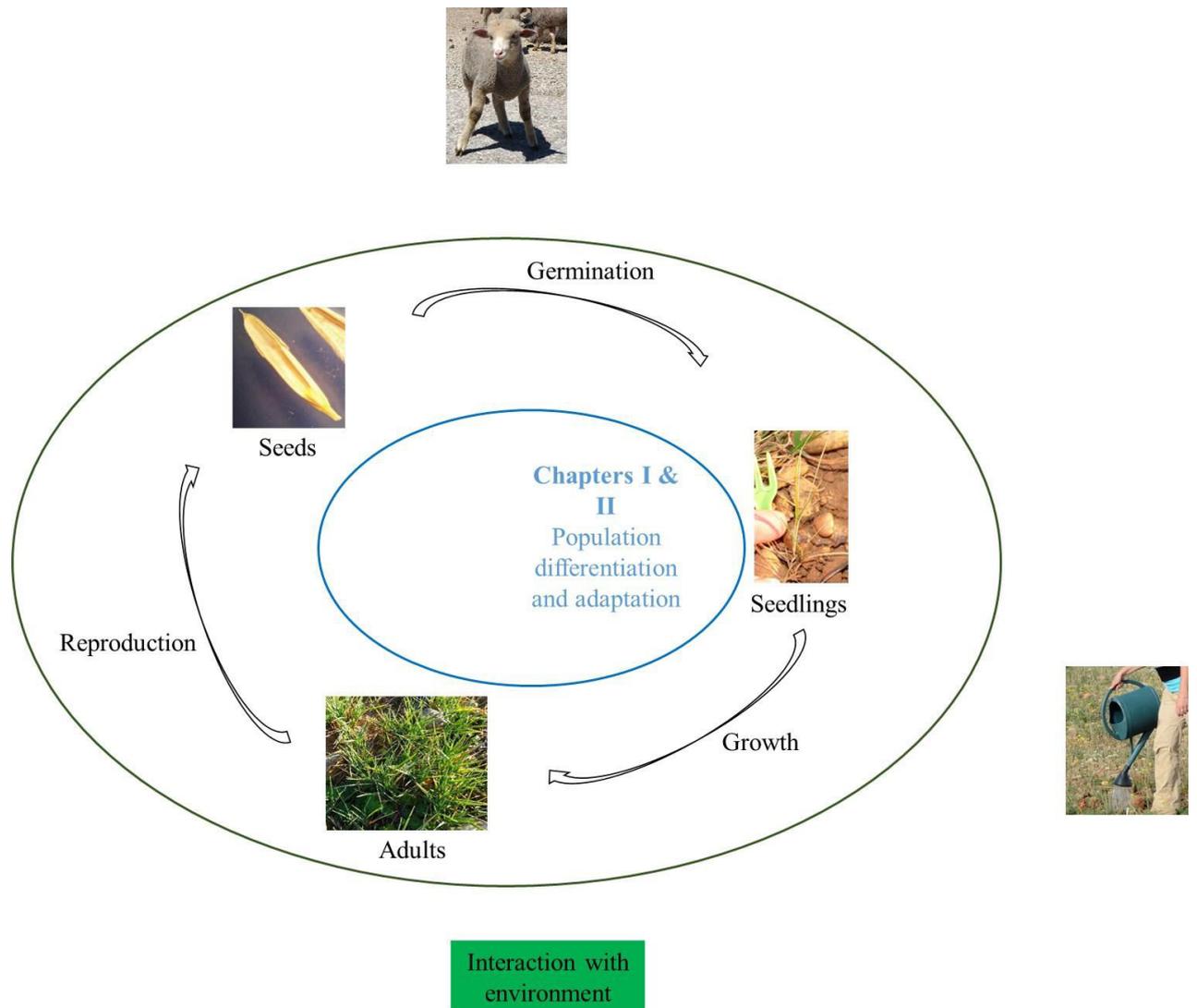


Figure VII: Chapter II structure.

In Chapter I, we showed an adaptive differentiation of French populations related to climate, in particular to mean July temperature and frost day number. Adaptive differentiation was tested regarding principal component axis of vegetative or reproductive traits.

In Chapter II, we tested population differentiation using different subsets in a common garden and in a growth chamber experiment. The major common garden experiment is the same used for the comparison of phenotypic traits and molecular markers in Chapter I. However, in Chapter II only first-year measurements were included since all data were collected one year earlier than in Chapter I studies. We further included a stone cover treatment not detailed in

Chapter I and set up additional experiments to test for grazing (simulated by clipping) and humidity (soil moisture) effects on Southern French *B. retusum* populations. The major aim was to test differential population responses related to environmental conditions of collection sites. We hypothesised that *B. retusum* populations are adapted to the main gradients varying among collected populations: such as humidity (soil moisture), stone cover and grazing.

Contribution of the thesis:

- Differentiation of *B. retusum* populations in phenotypic traits at regional scale in Southern France and between major soil types.
- Adaptation to humidity, stone cover and grazing.
- Recommendations for targeting source populations in ecological restoration.
- Environmental requirements for successful establishment.

Chapter II

Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum* - the role of soil conditions, grazing and humidity.

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Abstract

➤ Background and Aims

Genetic differentiation in plant species may result from adaptation to environmental conditions but also from stochastic processes. The drivers selecting for local adaptation and the contribution of adaptation to genetic differentiation are often unknown. Restoration and succession studies have revealed different colonization patterns for *Brachypodium retusum*, a common Mediterranean grass. In order to understand these patterns, we tested population differentiation and adaptation to different environmental factors.

➤ Methods

Structured sampling of twelve populations from six sites and two soil types within site was used to analyze the spatial and environmental structure of population differentiation. Sampling sites differ in grazing intensity and climate. We tested germination and growth in a common garden. In subsets, we analyzed the differential response to stone cover, grazing and soil moisture.

➤ Key Results

We found significant differences among populations. Site explained population differentiation better than soil suggesting a dominant influence of climate and/or genetic drift. Stone cover had a positive influence on seedling establishment and populations showed a differential response. However, this response was not related to environmental differences between collection sites. Regrowth after clipping was higher in populations from the more intensively grazed Red Mediterranean soils suggesting an adaptation to grazing. Final germination was generally high even under drought but germination response to differences in soil moisture was similar across populations.

➤ Conclusions

Adaptive population differentiation in germination and early growth may have contributed to different colonization patterns. Thus, the provenance of *B. retusum* needs to be carefully considered in ecological restoration.

Key words: Common garden; local adaptation; plant origin; Poaceae; population differentiation; seedling recruitment

INTRODUCTION

Genetic differentiation among populations is an important strategy of plant species to survive under different environmental conditions (Linhart and Grant, 1996; Bischoff and Hurault, 2013). Adaptive differentiation occurs if environmental gradients are strong and gene flow counteracting differentiation is low (Kawecki and Ebert, 2004). Climate is a major driver of local adaptation and genetic differentiation in plant populations, in particular on large geographic scales (Etterson, 2004; Leinonen et al., 2009; Bucharova et al., 2016). However, microclimatic gradients may also act on much smaller scales resulting in small-scale adaptation and differentiation (Knight and Miller, 2004; Lenssen et al., 2004; Bischoff et al., 2006).

The adaptation to soil conditions was first detected on soils contaminated with heavy metals indicating that adaptive differentiation may occur quite rapidly (Schat et al., 1996; Antonovics, 2006). Furthermore, the pH value and related chemical and biotic properties may represent a strong selective force (Macel et al., 2007; Raabová et al., 2011). Humidity is also a limiting factor for plant growth and reproduction leading to drought adaptation (Shah and Paulsen, 2003; Suriyagoda et al., 2010). However, an increase in soil moisture often results in higher competition for other resources representing a counteracting selective force (Kadmon, 1995).

Ecosystem management is a third potential driver of adaptive differentiation in semi-natural systems such as grasslands. Grazing changes site conditions by biomass removal, plant damage, reduced competition and creates heterogeneity in grassland vegetation (Bullock et al., 2001; Hufford and Mazer, 2012). It seems to be likely that long-term grazing favors genotypes adapted to grazing (Hufford and Mazer, 2012). However, grazing may also obscure adaptation to other environmental factors if this selection pressure has not been consistent in the past (Hufford et al., 2008).

Genetic differentiation is not only the result of adaptation to different environments. Stochastic processes such as genetic drift considerably contribute to population differentiation and may even overwhelm local adaptation (Hereford and Winn, 2008; Hereford, 2009). Genetic drift and limited dispersal favor isolation by distance effects not related to environmental gradients (Sexton et al., 2013). Durka et al. (2017) found both isolation by distance due to stochastic processes, and isolation by environment in seven tested plant species. The contribution of adaptive differentiation to genetic structure may even be low compared with genetic drift (Michalski and Durka, 2012, Sexton et al. 2013).

Genetic differentiation in quantitative and thus potentially adaptive traits is typically tested in common garden experiments (Petit et al., 2001; Leinonen et al., 2009; Bischoff and Müller-Schärer, 2010). Tests for local adaptation require reciprocal transplantation to original collection sites (Blanquart et al., 2013). However, common garden experiments comparing the response to the manipulation of key environmental factors allow conclusions on adaptive differentiation and may reveal more specific information on drivers of local adaptation than reciprocal transplantations in the field (Lenssen et al., 2004; Pahl et al., 2013).

We used the perennial grass *Brachypodium retusum* (Pers.) P.Beauv. as a model species to test for neutral and adaptive population differentiation at a regional scale in Southern France. The species is dominant in dry grasslands of the Western Mediterranean basin but also occurs in matorral and open woodlands (Caturla et al., 2000; Catalán et al., 2015). In contrast to studies showing a high colonization capacity of the species (Caturla et al., 2000; Luis et al., 2004; Cassagne et al., 2011), spontaneous natural regeneration of *B. retusum* was poor in the La Crau nature reserve (Southern France) thirty years after conversion from arable use to traditional sheep grazing complicating ecological restoration operations (Coiffait-Gombault et al., 2012). We hypothesize that genetic differentiation contributes to the different colonization patterns and that this differentiation results from adaptation to major environmental factors distinguishing habitats. In the study region, *B. retusum* occurs in lower calcareous mountain ranges and on more or less decalcified red Mediterranean soils of former riverbeds such as the Crau area. The latter habitat is also characterized by stones (pebbles, cobbles) shaped by the former rivers. These stones were found to favor growth and survival of *B. retusum* because rhizomes and roots can grow underneath benefitting from higher soil moisture during the hot and dry Mediterranean summer (Buisson et al., 2015). Apart from soil characteristics and stone cover, these two habitats show also large differences in soil moisture and grazing intensity. The information on population differentiation and adaptation to environmental factors is crucial to predict colonization processes but also to improve seed sourcing in ecological restoration (McKay et al., 2005; Vander Mijnsbrugge et al., 2010).

In our study, we tested *B. retusum* populations from calcareous and closest red Mediterranean soils replicated at different geographical distances in order to evaluate whether local-scale differentiation along strong environmental gradients can be as important as large-scale differentiation driven by macro-climate or genetic drift (Bischoff et al., 2006; Hereford and Winn, 2008; Durka et al., 2017). We further analyzed differential responses of these populations

to grazing simulated by clipping, to stone cover and to soil moisture. We specifically wanted to answer the following research questions:

(1) At which scales does population differentiation occur: what is the role of local-scale soil type compared with large-scale variation? (2) Is this differentiation adaptive in terms of soil conditions (stone cover), management (grazing simulated by clipping) and humidity (soil moisture, germination)?

MATERIALS AND METHODS

Study species

Ramose false brome (Poaceae, *Brachypodium retusum* P. Beauv.) is a rhizomatous perennial C3 grass species. Its rhizomes form a dense network under the soil surface, resulting in a high resilience to above-ground disturbance such as wildfires or grazing (Caturla et al., 2000). Vegetative growth is characterized by tillering at the stem base but also by lateral branching at upper stem parts. *B. retusum* is an outcrossing wind-pollinated species. It flowers from April to July and shows high clonal growth and reproduction when rhizome connections disintegrate. However, rhizomes are short and just allow a slow clonal spread (<5 cm *a⁻¹ in our experiments).

Collection sites and sampling

The study and sampling sites were situated in the central part of the French Mediterranean climate zone from Montpellier to the lower Durance valley. Seeds were collected from six populations on base-rich calcareous soils (Calcaric cambisol) of lower mountain ranges with pH values of 7.8 to 8.9 (Table 1, Fig. 1). Close to each of these six populations on calcareous grasslands, six populations were also collected from usually decalcified red Mediterranean soils (Haplic cambisols) with pH values of 6.7 to 8.0. Three additional populations were collected in the Crau nature reserve as the focal site of Mediterranean dry grassland restoration (Buisson and Dutoit, 2006) in order to obtain a better representation of this largest red Mediterranean soil area of the study region. For further analysis, the four Crau populations (distance: 4-11 km) were pooled to the red Mediterranean soil population of Saint-Martin-de-Crau (SM, Table 1).

Red Mediterranean soils show a higher clay content than calcareous soils resulting in a better nutrient and water retention. Due to the higher soil fertility, grasslands on red Mediterranean

soils were more intensively used (in particular grazing, less frequently mowing) than those on calcareous soils (Table 1). They are also rich in hydrated oxides of iron and aluminum and in silica whereas calcareous soils are poorer in these elements. Up to 50% of red Mediterranean soil surface is covered by cobbles and pebbles deposited by former rivers. Calcareous soils are less deep on calcareous bedrock and contain numerous angular brittle stones.

The study area is characterized by a typical Mediterranean climate with hot and dry summers and mild and humid winters. The six sampling sites differ in climatic conditions (Table 1) whereas differences between soil types within sites are small. Annual precipitation depends on the proximity to mountain ranges and is generally higher in the western than in the eastern part of the study area. Close to the Mediterranean Sea, winter temperatures are higher resulting in a lower frost frequency.

Table 1: Soil characteristics, grazing intensity, annual precipitation, mean annual air temperature (including January and July mean in brackets), and the number of frost days ($T < 0$) at collection sites of the twelve populations. Sites are ordered from east to west. Climate data are based on daily averages from the nearest meteorological stations (1980-2010). *irregular mowing

| Site code | Site name | Soil | Coordinates | pH | Grazing | Precipitation (mm*a ⁻¹) | Temperature (°C) Mean [Jan/Jul] | Days T<0 |
|-----------|------------------------|-------------------|-------------------------|-----|---------|-------------------------------------|---------------------------------|----------|
| Me | Mérindol | Calcareous | 43.756456 N, 5.199526 E | 8.0 | No* | 583 | 14.5 [6.0/23.7] | 49 |
| | | Red Mediterranean | 43.750425 N, 5.210495 E | 8.0 | Low | | | |
| Ca | Caumont | Calcareous | 43.898827 N, 4.941070 E | 8.2 | No | 676 | 14.6 [5.9/24.2] | 33 |
| | | Red Mediterranean | 43.898319 N, 4.932395 E | 6.9 | No | | | |
| SR | Saint-Rémy-de-Provence | Calcareous | 43.768047 N, 4.831898 E | 7.8 | No | 625 | 15.1 [6.6/24.3] | 30 |
| | | Red Mediterranean | 43.798952 N, 4.841659 E | 6.8 | High | | | |
| SM | Saint-Martin-de Crau | Calcareous | 43.705354 N, 4.797561 E | 8.1 | No | 517 | 15.3 [7.4/24.4] | 24 |
| | | Red Mediterranean | 43.567465 N, 4.834645 E | 6.8 | High | | | |
| Ni | Nîmes | Calcareous | 43.784700 N, 4.259389 E | 8.0 | No | 701 | 14.9 [6.7/24.1] | 34 |
| | | Red Mediterranean | 43.722868 N, 4.339264 E | 7.8 | No* | | | |
| Mon | Montpellier | Calcareous | 43.645416 N, 3.691638 E | 8.9 | No* | 870 | 14.1 [6.4/23.2] | 51 |
| | | Red Mediterranean | 43.607580 N, 3.449269 E | 6.7 | No* | | | |

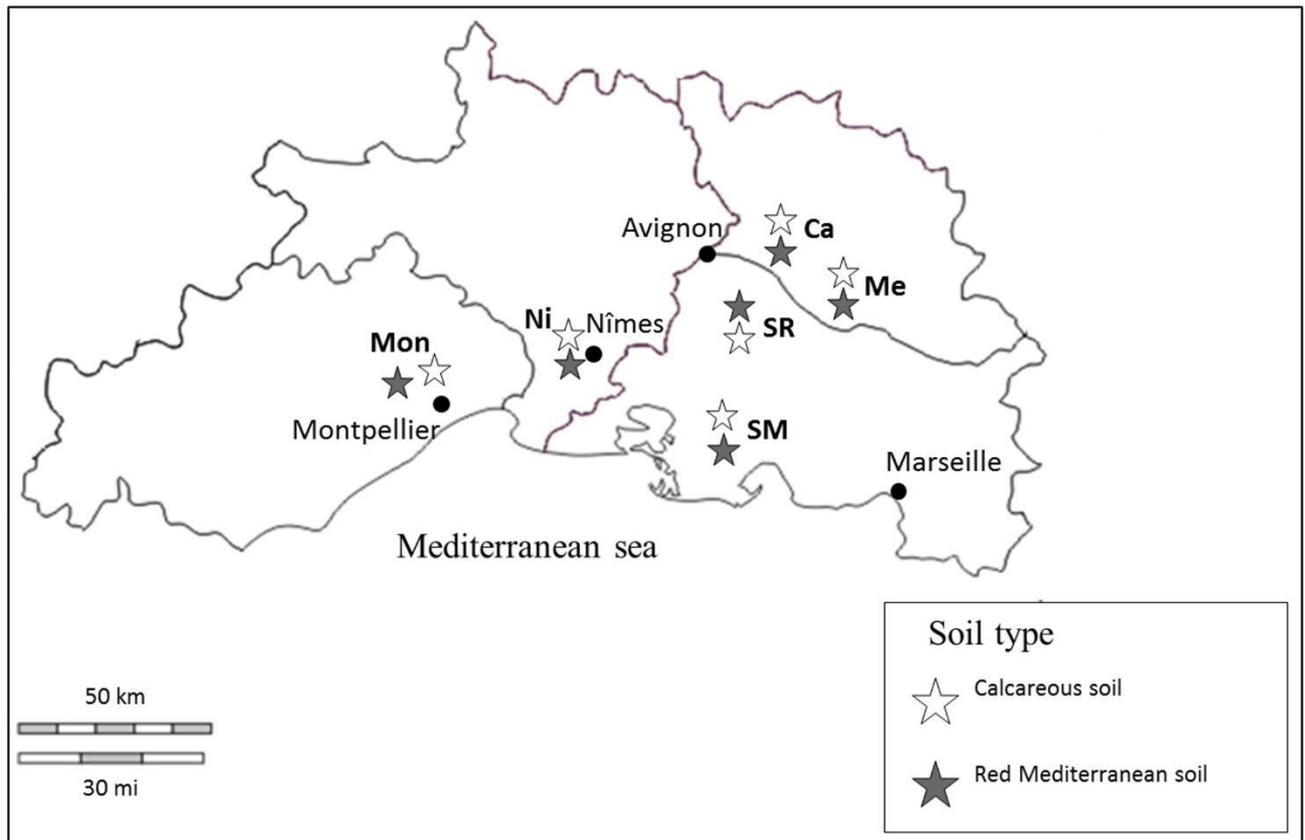


Figure 1: Position of six sampling sites each comprising two populations from different soil types.

Seed collection and preparation

In each population, all inflorescences of each of fifty 1 m² patches were collected. The minimum distance between patches was 10 m and sampling was done in one or several parallel lines depending on population shape (linear versus polygon). Due to clonal growth of the species, the real patch size of individuals is unknown. A distance of 10 m was considered as sufficient to avoid that different patches belong to the same individual (Montalvo and Ellstrand, 2000). On average, one patch comprised forty inflorescences resulting in a total of 2000 collected inflorescences per population. In germination experiments, the number of seed families may be higher than the number of patches because one patch may comprise more than one individual. This pooling at patch level reduces intra-population variation compared to half-sib designs based on individual seed families. In all other experiments, only one seedling per patch was kept for planting corresponding to classical half-sib designs.

Population differentiation under common conditions

Population differentiation was tested in a growth chamber germination experiment and in a common garden study. Inflorescences were shaken in tubes with glass beads using a vortex mixer to separate seeds from glumes. Transparency under transmitted overhead projector light was used as a criterion to remove empty seeds without embryo. In December 2016, seeds were germinated in peat plugs (4 cm x 4 cm). Four seeds of each sampled patch were sown per plug and altogether forty patches of each population were randomly selected for a total of 160 seeds and 40 plugs per population. The plug trays were placed in a growth chamber (temperature 15/20 °C, 12/12h, night/day) and watered regularly. Coleoptile emergence used as a proxy of germination was recorded every 2-3 days for seven weeks. In March 2016, 24 plugs per population (corresponding to 24 patches or seed families) were randomly selected and transplanted to a previously ploughed experimental site in Avignon-Montfavet. Thus 48% of the initially collected patches were represented in the field. Seedling number was reduced to one (average size) per plug prior to transplantation. Two plugs per population were planted to random positions within each of 12 blocks (3 * 3m²). During the first four months, seedlings were moderately watered. Plots were hand-weeded to limit competition. Plant height, diameter, tiller number, leaf number and the length of the longest leaf were measured once a month. Young *B. retusum* plants allocate resources to tillering and leaf growth. Later, tillers elongate and older leaves die. Therefore, we calculated cumulative leaf length as an aboveground biomass estimate for young seedlings (leaf length * number of leaves) and cumulative tiller length for older plants (plant height * number of tillers).

Adaptation to differences in soil conditions, management and soil moisture

In May 2016, stones of 10 – 20 cm length and 7 to 15 cm width were placed in a circle around half of the seedlings of the common garden experiment to test their effect on plant growth. The stones were taken from the red Mediterranean soil of the Caumont population (Ca, Table 1, Fig. 1). Measurements were the same as in the population differentiation study under common conditions detailed above.

A subset of four population pairs (red Mediterranean and calcareous soils x Me, Ca, SR, SM sites) was grown one year earlier in a similar common garden experiment to simulate grazing by clipping established plants. Clipping of second year plants prevents high mortality compared to clipping of young seedlings. The design was similar to the differentiation study. In November

2014, seeds were sown to peat plugs and placed in a greenhouse. In February 2015, two seedlings of each population were planted to each of 12 blocks (24 seedlings in total). Due to low germination, seedling number was only one per block for the Me population from calcareous soil (12 seedlings in total). Plants were moderately watered during the first year but not in the second year. In April 2016, half of the seedlings were clipped 2 cm above the soil. We measured the same traits as in the differentiation study. Additionally, the number of inflorescences per plant was counted at the end of the season. This was not possible in the population differentiation study under common conditions since plants rarely (<10%) flower in the first year.

The response to soil moisture during germination was analyzed in a controlled experiment manipulating moisture levels. The major objective was to test adaptive differentiation in germination response under dry conditions. Five moisture levels from 0.3 to 20% soil moisture were adjusted by adding water to plastic boxes filled with 1.15 kg of sand and using a Theta Probe ML2x sensor (Delta-T Devices Ltd, Cambridge, United Kingdom). A subset of ten populations was chosen for this experiment. Six seeds of each population (four for calcareous population of SM due to limited seed number) were sown to each of 12 boxes of each of the five soil moisture levels resulting in a total of 360 seeds per population assigned to 60 boxes. Boxes were closed by a translucent lid to keep initial moisture levels. The boxes were placed in a growth chamber with a 12-hours cycle of light and temperature (day: 25°C; night: 12°C). Germination and moisture were measured every three days for a period of six weeks. Germinated seeds were removed. At the end of the experiment, the water potential of the sand was measured using WP4C Dewpoint Water Potentiometer (Decagon Devices, Pullman, Washington, United States of America). Water potentials were close to 0 (saturation) for the highest and -0.18 MPa for the lowest moisture level (Appendix S1, see Supplemental Data with this article).

Statistical analysis

Linear models were used to analyze population and treatment effects on germination and growth traits. Population was divided in a soil and site component. The two adjacent populations from red Mediterranean and calcareous soils were considered as originating from the same site resulting in a total of six sites and two soil types per site. Thus, each population represented a particular combination of site and soil. Site, soil and their interaction were

included as fixed effects in the models. The effect size of site of origin, soil of origin and the site x soil interaction was calculated as $100 \times \text{factor sum of square} / \text{total sum of squares}$.

The common garden studies were designed as completely randomized block experiments. Thus, block was included as an additional random factor. The adaptation treatments (stone cover, clipping) were included as fixed factor for measurements taken after treatment start. In some cases $\log_{10}(x+1)$ transformation was required to comply with assumptions of linear models (normality, homoscedasticity).

In the germination experiments, final germination percentage in plugs (population differentiation study) and in boxes (soil moisture test) was calculated as response variable. Germination percentage was $\arcsin(\sqrt{x/100})$ transformed prior to analysis (Quinn and Keough, 2002). Since position of plugs was completely randomized before transplantation to the field, no block effect was considered (germination in the population differentiation study). The soil moisture test on germination was analyzed in a split-plot model. The box effect was included as a random factor nested in moisture level. Accordingly, moisture was tested against box whereas soil, site and all interactions were tested against the model error.

A Tukey HSD posthoc test was calculated to analyze differences between treatment levels more in detail if the treatment main effect or interactions were significant. Posthoc tests were preferred over a priori linear contrasts because we had no clear a priori hypothesis on specific adaptation to tested environmental factors. All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)).

RESULTS

Population differentiation under common conditions

A high variation in germination capacity was found between populations ranging from 10% (Ca, red Mediterranean soil) to 75% (Ni, calcareous soil, (Fig. 2). Site and soil of origin as well as their interaction had a significant influence on germination (Table 2). Populations from calcareous soils and from the westernmost sites germinated significantly better than those from red Mediterranean soils and eastern parts of the study area, respectively. Differences between soil types depended on site of origin, resulting in a significant interaction.

Population differentiation in plant growth was smaller than in germination but still significant (Table 2, Fig. 2). Differentiation in growth traits did not decrease during the growing season (Appendix S2). Site of origin contributed more to the differentiation than soil of origin. Effect size of site of origin was between 3% of total variation for leaf length and 14% for leaf number whereas effect size of soil of origin was always lower than 1% (Fig.3). The site x soil interaction explained 1% and 7.7 % of the model variation. In agreement with the germination results, the westernmost populations from Ni and Mon showed a higher performance in early (leaf length, cumulative leaf length) and late growth traits (tiller length and number, cumulative tiller length, tussock diameter). The soil type effect was only significant for leaf length being higher in populations from red Mediterranean soils than in those from calcareous soils. In several traits, in particular in leaf length, tiller length and plant diameter, a significant soil x site of origin interaction was detected. For the westernmost sites of origin, growth was higher in populations from red Mediterranean soils whereas no differences or even a higher performance on calcareous soils were found for populations from the eastern and central parts of the study area.

Table 2: Differentiation in *Brachypodium retusum* populations from different sites and soil types, and differential population response to stone cover treatment. *F*-values and significance levels from two-way ANOVA before stone treatment (growth traits: May) and three-way ANOVA afterwards (July) including the stone effect. . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

| | | df | Germination | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter |
|------------------------|----------------------|-------|-------------|-------------|-------------|--------------|---------------|------------------------|--------------------------|----------------|
| Before treatment (May) | Site | 5,338 | 31.41 *** | 1.82 NS | 11.81 *** | 6.62 *** | 4.87 *** | 8.12 *** | 6.98 *** | |
| | Soil | 1,338 | 36.83 *** | 0.00 NS | 5.30 * | 0.00 NS | 0.02 NS | 1.05 NS | 0.00 NS | |
| | Soil × Site | 5,338 | 3.08 ** | 0.64 NS | 6.60 ** | 2.06 . | 1.76 NS | 2.00 . | 2.13 . | |
| After treatment (July) | Site | 5,315 | | 1.73 NS | 3.99 ** | 7.09 *** | 6.93 *** | 4.89 *** | 8.48 *** | 4.01 ** |
| | Soil | 1,315 | | 0.00 NS | 5.07 * | 0.37 NS | 0.34 NS | 1.32 NS | 0.38 NS | 1.61 NS |
| | Stones | 1,315 | | 1.58 NS | 1.09 NS | 4.24 * | 5.48 * | 0.03 NS | 5.67 * | 4.16 * |
| | Soil × Site | 5,315 | | 0.97 NS | 3.02 * | 2.18 * | 1.33 NS | 2.04 . | 1.76 NS | 2.52 * |
| | Stones × Site | 5,315 | | 2.02 . | 0.54 NS | 1.56 NS | 2.87 * | 0.18 NS | 2.24 * | 0.38 NS |
| | Stones × Soil | 1,315 | | 0.10 NS | 1.90 NS | 0.59 NS | 0.02 NS | 1.14 NS | 0.05 NS | 0.44 NS |
| | Stones × Soil × Site | 5,315 | | 0.49 NS | 0.18 NS | 2.26 * | 0.21 NS | 1.18 NS | 0.79 NS | 0.26 NS |

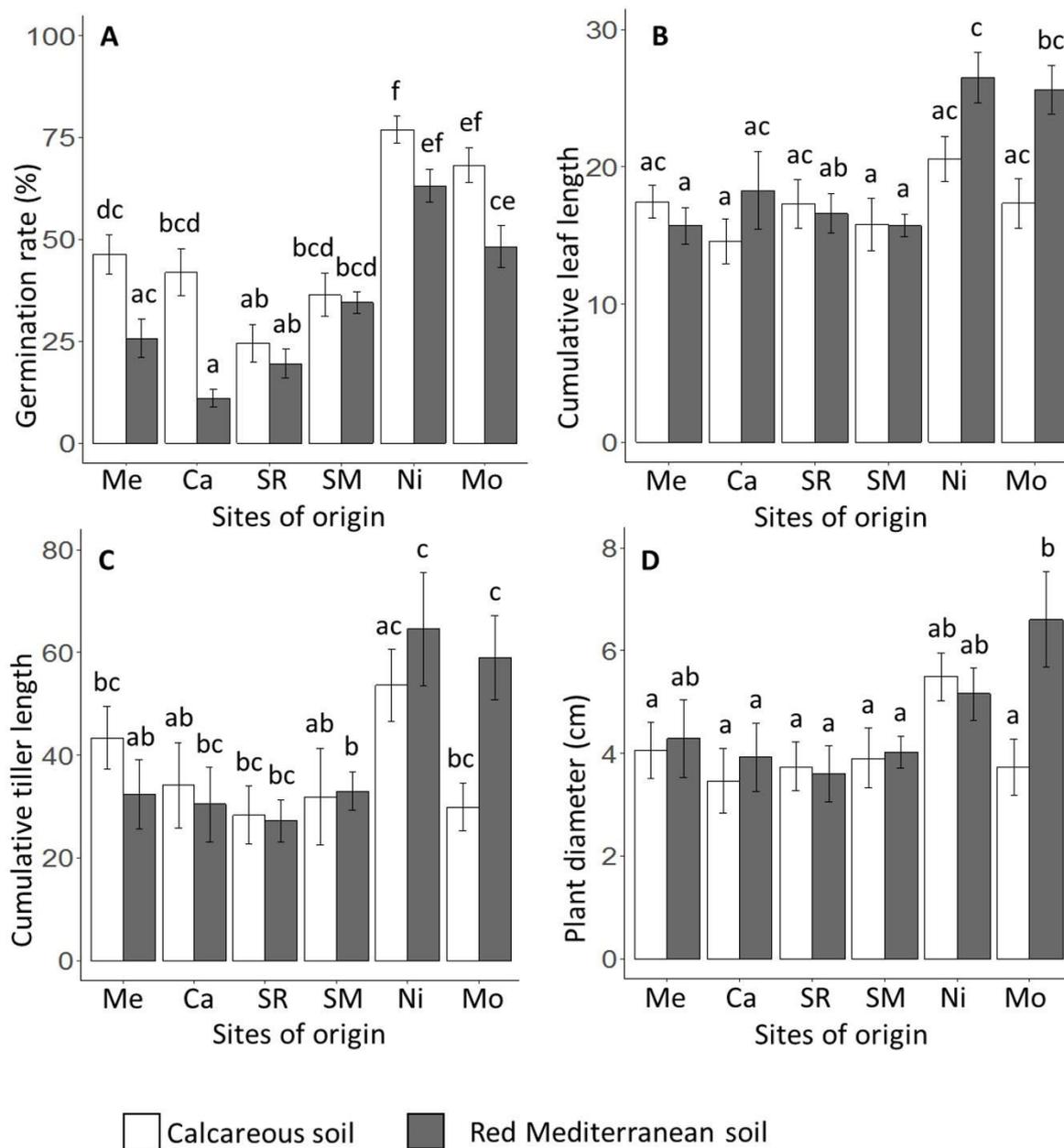


Figure 2: Population differentiation decomposed in collection site and soil type within collection site (mean values \pm SE, see Table 2 for statistics). A: Final germination, B: Cumulative leaf length, C: Cumulative tiller length and D: Plant diameter. Different lower case letters indicate significant differences ($P < 0.05$).

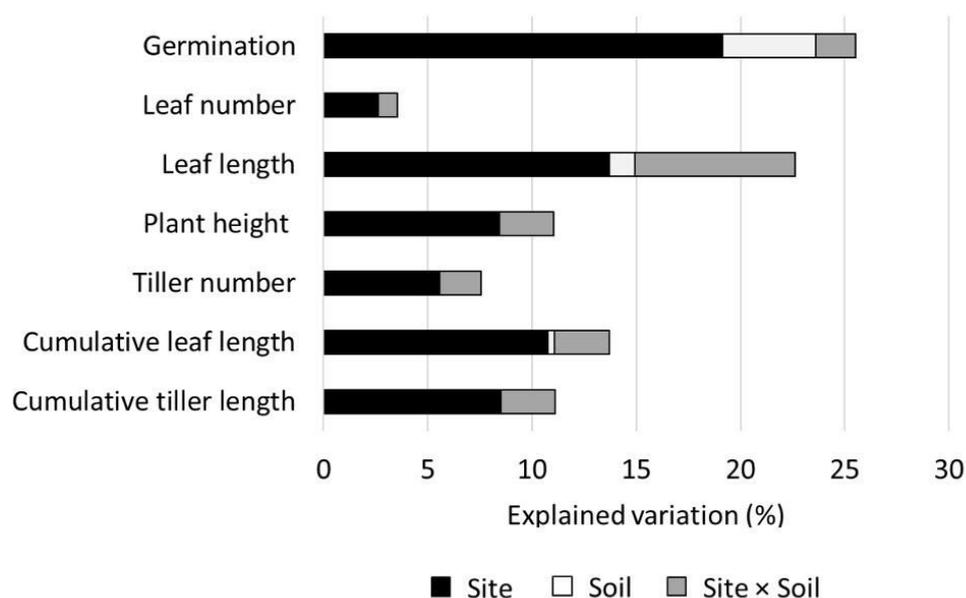


Figure 3: Percentage of variation explained by collection site, soil type within collection site and site x soil interaction for growth traits (May).

Adaptation to differences in soil conditions, management and soil moisture

Stone cover around the seedlings significantly increased plant height, tiller number and thus cumulative tiller length but not cumulative leaf length (Table 2, Fig. 4). The effect of stone cover was the same for plants from the two soil types, while stone cover had contrasting effects on leaf number and cumulative leaf length as well as on tiller number and cumulative tiller length of plants from the different sites. The populations from SR including the SM population showed a consistent positive response to stone cover whereas the response of the Me populations was negative or neutral.

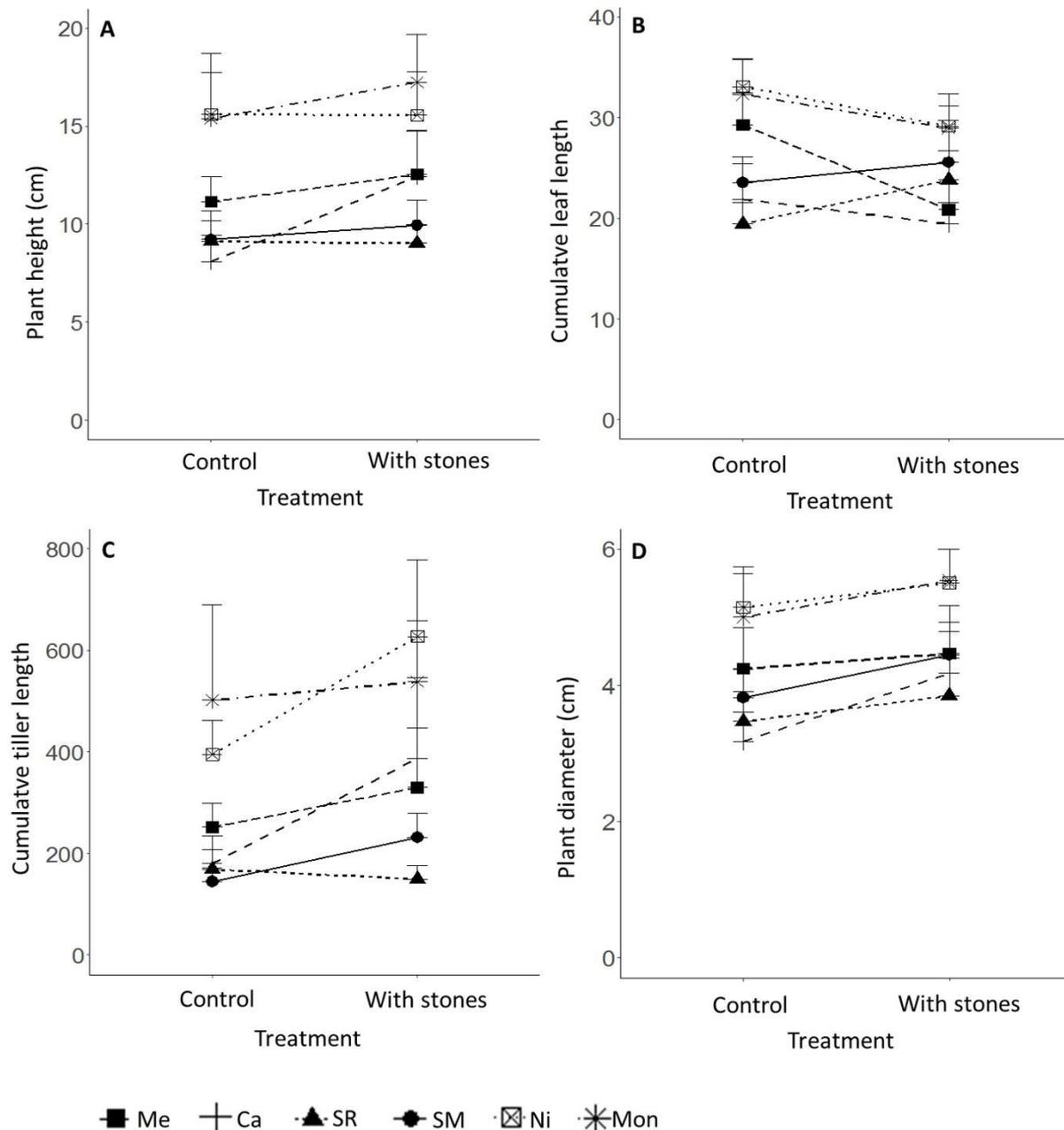


Figure 4: Differential response to stone cover (mean values \pm SE) between *Brachypodium retusum* populations in southern France. A: Plant height, B: Cumulative leaf length, C: Cumulative tiller length, D: Plant diameter.

Simulated grazing (clipping) significantly reduced plant height, leaf number, tiller number, cumulative tiller length, cumulative leaf length, and inflorescence number (Table 3, Fig. 5). The negative effect on tiller number was the result of cutting underneath lateral branches that were also counted as tillers. While site \times treatment interactions were not significant, several significant soil \times treatment interactions were detected. Populations from red Mediterranean soils showed significantly higher compensatory growth resulting in smaller cumulative tiller length, and inflorescences number between clipped and non-clipped plants.

Table 3: Differential response in a subset of populations to simulated grazing. *F*-values and significance levels from three-way ANOVA on site, soil (within site) and clipping effects. Vegetative traits measured in spring (May), inflorescence number in June of the second growing season. . P < 0.1, * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

| | df | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter | Inflorescence number |
|------------------------|-------|-------------|-------------|--------------|---------------|------------------------|--------------------------|----------------|----------------------|
| Site | 3,132 | 1.20 NS | 1.13 NS | 0.27 NS | 0.63 NS | 1.47 NS | 0.64 NS | 1.30 NS | 2.48 . |
| Soil | 1,132 | 0.02 NS | 9.43 ** | 3.16 . | 0.05 NS | 4.13 * | 2.87 . | 1.54 NS | 3.91 * |
| Clipping | 1,132 | 12.31 *** | 3.19 . | 220.98 *** | 10.48 ** | 11.46 *** | 211.72 *** | 7.23 ** | 58.39 *** |
| Soil × Site | 3,132 | 0.67 NS | 2.12 NS | 0.81 NS | 0.16 NS | 2.05 NS | 1.18 NS | 0.67 NS | 1.98 NS |
| Clipping × Site | 3,132 | 0.66 NS | 0.49 NS | 0.81 NS | 0.02 NS | 0.99 NS | 1.49 NS | 0.94 NS | 0.23 NS |
| Clipping × Soil | 1,132 | 0.08 NS | 0.09 NS | 3.93 * | 2.17 NS | 0.12 NS | 3.07 . | 0.48 NS | 3.37 . |
| Clipping × Soil × Site | 3,132 | 1.12 NS | 0.61 NS | 1.65 NS | 0.76 NS | 0.43 NS | 1.08 NS | 0.40 NS | 0.29 NS |

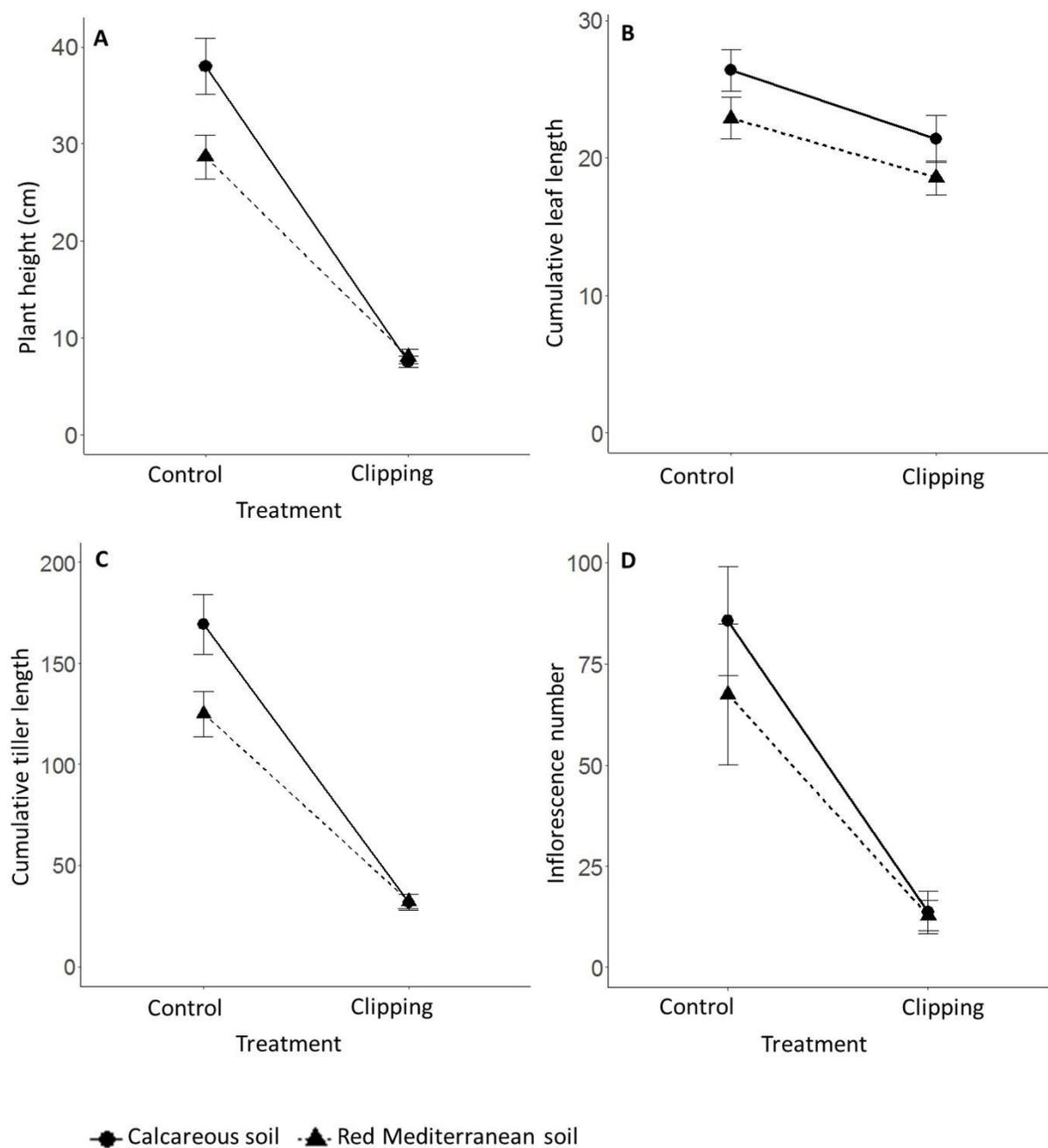


Figure 5: Differential response of a subset of populations to simulated grazing (mean values \pm SE). Since site \times clipping interaction was never significant only soil type \times clipping combinations are presented. A: Plant height, B: Cumulative leaf length, C: Cumulative tiller length, D: Inflorescence number by soil type.

The test of soil moisture effects on germination in different *B. retusum* populations revealed high germination rates even under dry conditions (0.3% volumetric water content) and a significant decrease of germination under maximum soil moisture (at saturation level: 19.5%, Fig. 6). The highest germination was obtained for intermediate soil moisture levels. With 50% to 80% on average, germination rates were higher than in peat plugs used for the germination test on population differentiation. In agreement with the peat plug germination test, site and soil type effects were significant with a higher germination rate in populations from calcareous soils and from the westernmost sites, respectively (Fig. 6).

The interaction between soil type of origin and soil moisture was not significant and the one between site and soil moisture was only marginally significant suggesting similar adaptation to dry conditions. The marginally significant interaction was explained by a stronger negative response of the Ni populations to drought stress compared with populations from the three other sites.

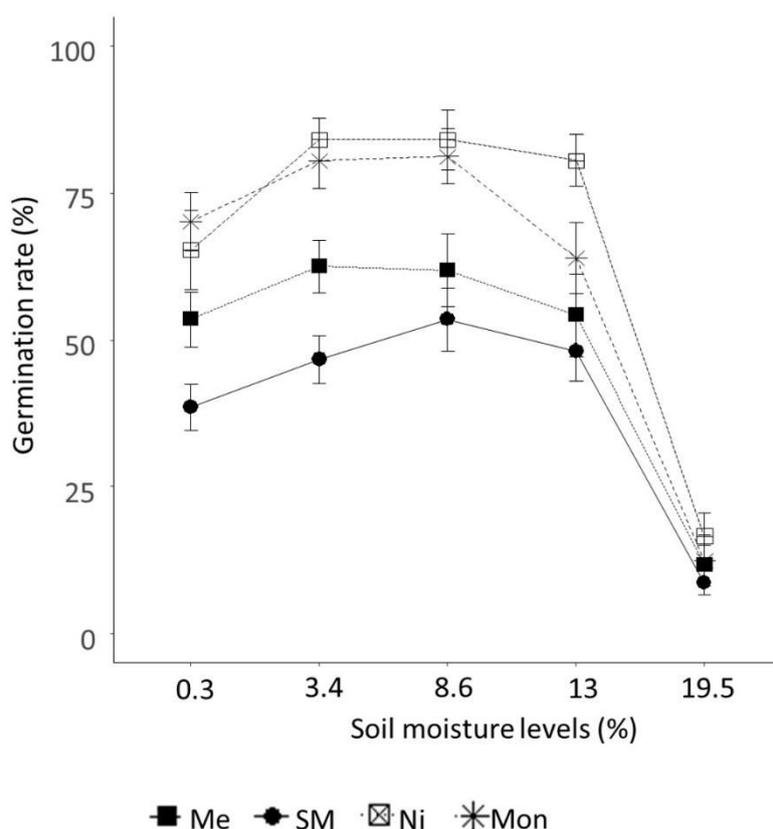


Figure 6: Differential germination response of a subset of populations to soil moisture (mean values \pm SE). Since the soil of origin \times soil moisture interaction was never significant only site of origin effects are presented. Soil moisture ($F_{4,55} = 53.71$, $P < 0.001$), site of origin ($F_{3,505} = 27.96$, $P < 0.001$), soil of origin ($F_{1,505} = 33.86$, $P < 0.001$), site of origin \times soil of origin \times moisture interaction ($F_{12,505} = 1.726$).

DISCUSSION

Population differentiation under common conditions

Our common garden study demonstrated genetic differentiation among populations at a regional scale. We found significant differences between populations for germination capacity and several morphological traits such as leaf length, tiller number and height whereas variation in leaf number per tiller was low. In particular, germination and tiller number are closely linked to seedling recruitment and establishment supporting our hypothesis that different colonization patterns observed at different Mediterranean sites (high: Caturla et al. 2000, Luis et al. 2004; low: Coiffait-Gombault et al., 2012) may result from genetic differentiation.

Site of origin (climatic differences) better explained this differentiation than soil type. Independent of soil type, the two eastern populations showed significantly lower germination rates and growth than western populations. The significant effect of site is likely the result of climatic differences between eastern and western parts of the study area although our design does not allow to distinguish random genetic drift from climatic differences as drivers of differentiation. The sites of the western populations (Ni, Mon) are closer to the Cévennes Mountains and receive more rainfall than those of the Eastern populations (Ceresetti, 2011).

Climate is known to be a major driver of population differentiation and adaptation (Linhart and Grant, 1996; Etterson, 2004; Ravenscroft et al., 2014) Water availability and temperature have a particularly strong effect on plant fitness leading to population differentiation in the case of spatial variation (Shah and Paulsen, 2003; Xu and Zhou, 2006). Yuan et al. (2016) found for example that population differentiation in the grass species *Leymus chinensis* was driven by precipitation, temperature and soil water content. A mainly soil moisture driven differentiation was also shown for *Boechera stricta* (Lee and Mitchell-Olds, 2011). However, the authors also found that isolation by distance due to stochastic processes such as genetic drift considerably contribute to population differentiation. Adaptive differentiation and isolation-by-distance effects often act at the same time on genetic structure of populations and their contribution to differentiation may be difficult to distinguish (Michalski and Durka, 2012; Durka et al., 2017). Nevertheless, shoot and leaf growth (number and length) were lower in populations from dryer Eastern sites rather selecting for growth forms with smaller and less numerous leaves and shoots that are better protected against drought stress (Liancourt and Tielbörger, 2009; Pérez-Ramos et al., 2013). The significant site effects on the cited growth traits were stable throughout the

whole growing season and did not decrease with time suggesting a minor influence of environmental maternal effects on our results. Environmental maternal effects may contribute to population differentiation but particularly affect early traits and vanish during plant development (Roach and Wulff, 1987; Donohue, 2009).

Soil type is also known to select for population differentiation in plants (Linhart and Grant, 1996; Raabova et al., 2011). In our study, we detected a significant soil type of origin effect on differentiation at a small scale of 10 to 20 km but only for germination capacity and leaf length. Germination is under stronger control of environmental maternal effects than growth traits affecting population differentiation in such early traits (Roach and Wulff, 1987; Bischoff and Müller-Schärer, 2010). However, it is also possible that selection for clonal growth under higher grazing pressure of red Mediterranean soils has reduced allocation of resources to seeds compared with less grazed calcareous sites. Although providing some evidence for soil of origin effects, our results do not confirm earlier studies showing a high small-scale differentiation along strong environmental gradients (Lenssen et al., 2004; Bischoff et al., 2006; Raabová et al., 2007; Bischoff and Hurault, 2013). Since no specific barriers prevented pollen dispersal at this scale, gene flow may have counteracted local adaptation to soil type (Kawecki and Ebert, 2004).

Adaptation to differences in soil conditions, management and soil moisture

Brachypodium retusum populations showed a differential response to stone cover and clipping, and to a lesser degree to soil moisture in the germination phase.

Our study demonstrated a clearly positive effect of stone cover on the growth of *B. retusum*, in particular on plant height, tiller number and plant diameter. Stone cover increases soil moisture by shading and water condensation and protects lower stem parts and roots from strong solar radiation and heat (Peters et al., 2008; Buisson et al., 2015). A differential response of Aizoaceae species demonstrated that plants might be locally adapted to type and density of stone cover (Ellis and Weis, 2006). In our study, however, the differential response was not related to the dominant soil types but to site of origin. Although the texture and depth of calcareous soils and red Mediterranean soils are different, limestone layers and pieces close to the soil surface may have similar effects on microclimate. Thus, the differential response to stone cover cannot be related to differences to local environmental conditions at collection sites.

As expected, clipping largely reduced plant height, tiller number and leaf number throughout the whole season resulting in a highly reduced reproduction. The effect was less strong in populations from red Mediterranean soils than in populations from calcareous sites suggesting an adaptation to higher grazing intensity. Grazing exerts strong directional selection and may result in genetic differentiation and local adaptation to long-term differences in grazing intensity (Linhart and Grant, 1996; Hufford and Mazer, 2012). Higher compensatory growth may be one important adaptation of grasses to higher grazing intensity (Sarmiento, 1992). Although we do not exactly know whether our collection sites show such long-term differences, red Mediterranean soils were more grazed in the past than calcareous soils because slopes are less steep and rocky, and soils are more fertile both favoring sheep grazing (Verheye and De la Rosa, 2009).

Drought stress reduces germination and usually plant species show a positive correlation between soil moisture and germination rates (Bochet et al., 2007; Thomas et al., 2010). The ability to germinate fast under moderate to low soil moisture may be crucial for species colonizing dry environments (Schütz et al., 2002; Bochet et al., 2007). All tested *B. retusum* populations showed a high germination capacity even under the lowest soil moisture level (-0.18 MPa=0.3 % volumetric water content), but a considerable drop under saturated conditions. The observed germination pattern differs from the results of Bochet et al. (2007) who found a strong decrease in *B. retusum* germination already at water potentials of -0.01 and -0.05 MPa. Although the results may depend on age of seeds and specific experimental conditions this difference suggests genetic variability in germination response to soil moisture. Environmental maternal effects may contribute to population differentiation in germination but there is little evidence for a contribution to adaptive responses through transgenerational plasticity (Galloway and Etterson 2007, Bischoff and Müller-Schärer 2010). In our study, the response to soil water potential was not much different among populations. Only the population of one of the two sites with the most humid climate (Ni) showed a stronger decline under dry conditions suggesting weak adaptation to climate-mediated soil moisture.

CONCLUSIONS

Significant population differentiation was found at a regional scale of 30 to 200 km. Differentiation between major soil types representing strong environmental differences were found at even smaller scales (10-20 km). However, differentiation between soil types was weaker than differentiation among sites suggesting a major influence of climate and/or genetic drift on population differentiation.

Stone cover increased *B. retusum* growth and the differential response of populations suggests adaptation to this environmental factor. However, we could not relate this response to the home environment of tested populations. Further experiments are required to identify potential drivers of adaptation to stone cover (density at the surface or in different soil layers). The response to clipping was also clearly different among populations and the differential response could be linked to the major soil type suggesting that different grazing intensity in the past has selected for different ecotypes. *B. retusum* showed a high germination under dry soil conditions but there was only weak evidence for a differential response of tested populations. The study demonstrated that population differentiation may well explain different colonization patterns observed in different Mediterranean regions (Caturla et al., 2000; Luis et al., 2004; Coiffait-Gombault et al., 2012). Since observed differences may largely influence seedling establishment in Mediterranean dry grasslands, plant origin needs to be carefully considered in ecological restoration involving planting or sowing of *B. retusum*.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

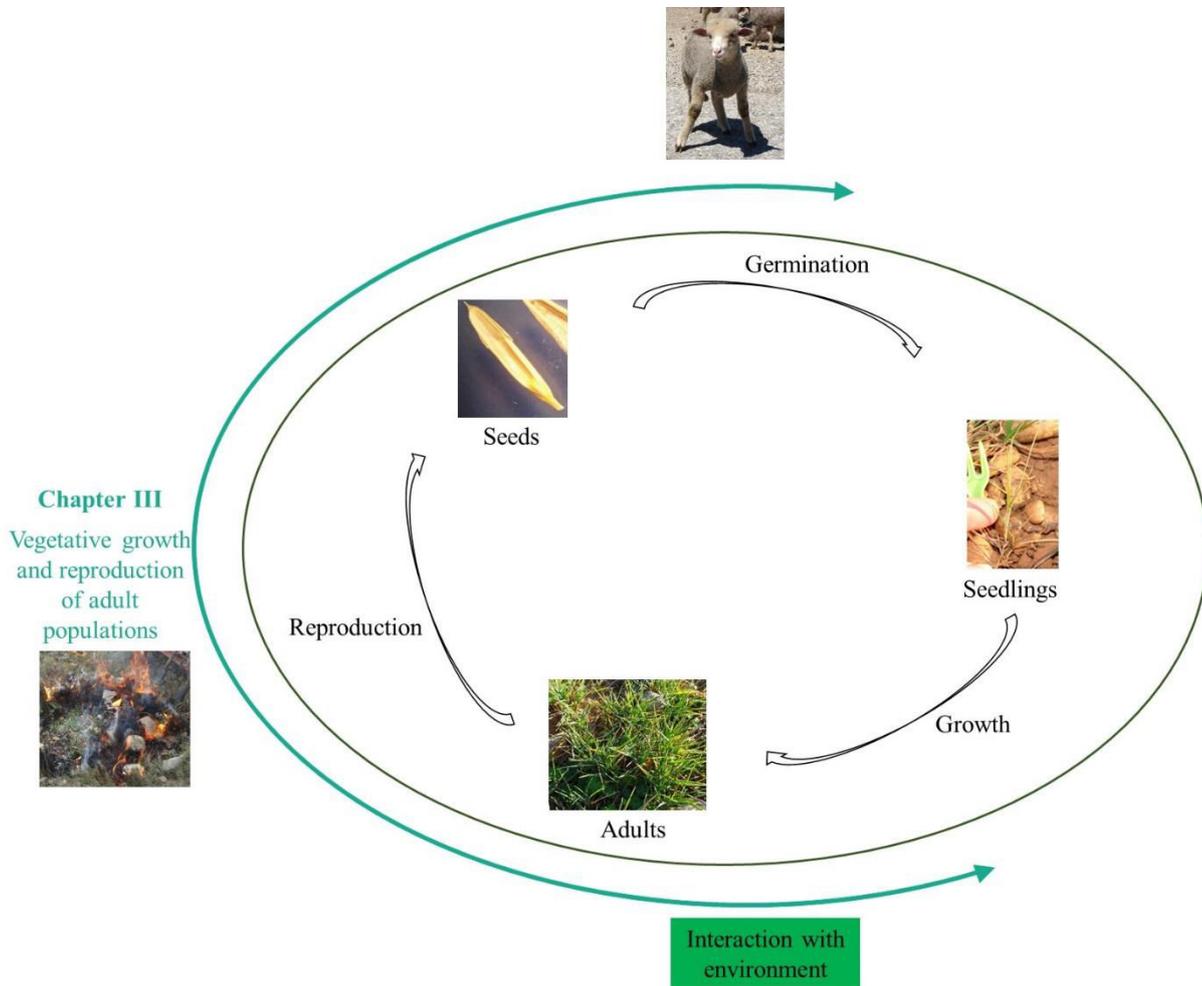
Appendix S1: Comparison of soil moisture levels measured by Theta Probe ML2x sensor (mV and %) and of water potential measured by WP4C Dewpoint Water Potentiometer.

| Soil moisture levels (mV) | Soil moisture (%) | Water content (mL) | Water potential (Mpa) |
|---------------------------|-------------------|--------------------|-----------------------|
| 500 | 19.5 | 250 | Saturation |
| 350 | 13 | 175 | Saturation |
| 250 | 8.6 | 150 | -0.01 |
| 150 | 3.4 | 100 | -0.12 |
| 100 | 0.3 | 30 | -0.18 |

Appendix S2: Differentiation in *Brachypodium retusum* populations from different sites and soil types. *F*-values and significance levels from two-way ANOVA for measurement dates not presented in Table 2. . P < 0.1, * P < 0.05, ** P < 0.01, *** P < 0.001, NS not significant.

| Growth | | df | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter |
|---------|-------------|-------|-------------|-------------|--------------|---------------|------------------------|--------------------------|----------------|
| March | Site | 5,338 | 1.17 NS | 18.20 *** | 12.44 *** | 0.43 NS | 7.02 *** | 5.37 *** | |
| | Soil | 1,338 | 3.39 . | 0.13 NS | 0.01 NS | 2.60 NS | 1.12 NS | 0.66 NS | |
| | Soil × Site | 5,338 | 0.93 NS | 2.78 * | 3.70 ** | 0.36 NS | 0.97 NS | 2.15 * | |
| April | Site | 5,338 | 4.99 *** | 16.95 *** | 12.18 *** | 5.58 *** | 9.89 *** | 10.36 *** | |
| | Soil | 1,338 | 6.67 * | 1.06 NS | 1.23 NS | 0.09 NS | 4.49 * | 0.15 NS | |
| | Soil × Site | 5,338 | 1.50 NS | 7.59 *** | 2.27 * | 1.60 NS | 2.41 * | 1.92 . | |
| October | Site | 5,338 | 1.56 NS | 15.03 *** | 5.69 *** | 5.40 *** | 5.58 *** | 3.39 ** | 9.24 *** |
| | Soil | 1,338 | 0.40 NS | 4.98 * | 0.04 NS | 0.08 NS | 0.53 NS | 0.80 NS | 1.21 NS |
| | Soil × Site | 5,338 | 1.83 NS | 10.90 *** | 1.10 NS | 2.81 * | 2.73 * | 1.38 NS | 5.31 *** |

Transition to Chapter III



In Chapters I and II, we showed a contribution of drift and adaptation to major environmental factors to *B. retusum* population differentiation. Adaptive population differentiation in germination and early growth may have contributed to different colonisation patterns observed in previous studies. We further demonstrated under favourable conditions in a common garden and in growth chambers that germination seedling recruitment and sexual reproduction are quite high suggesting a potentially high colonisation capacity (for seed production see Appendix 2).

In Chapter III, we analyse *in situ* growth and reproduction in adult *B. retusum* populations to evaluate environmental factors limiting the availability of propagules in the study area. Although decreased in importance due to fire protection measures, wildfires and pastoral fires used by shepherd to improve nutritional value have shaped plant populations and communities.

Grazing is required to maintain species composition of the ecosystem by preventing shrub encroachment. However, grazing also represents a major stress limiting growth and reproduction. We hypothesize that *B. retusum* benefits from fire and grazing and that sexual reproduction increases after burning. The study focuses on *B. retusum* but also include the surrounding plant community and its biodiversity.

Contribution of the thesis:

- Knowledge on grazing and fire as major factors influencing vegetative growth and reproduction compared to the associated plant community.
- Effect of fire and grazing on the associated plant community.
- Recommendations for conservation management and ecological restoration.

Chapter III

Fire increases the reproduction of the dominant grass *Brachypodium retusum* and Mediterranean steppe diversity in a combined burning and grazing experiment.

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Abstract

➤ Questions

What is the effect of fire and grazing on vegetative growth and sexual reproduction of *Brachypodium retusum*, the dominant species of Mediterranean Thero-Brachypodieta grasslands (“steppes”) and on species composition of the associated plant community?

➤ Location

“La Crau”, South-Eastern France.

➤ Methods

We set up a split-plot experiment testing the effects of fire season (winter, summer) and grazing on plant cover, inflorescence number, seed set and germination of *B. retusum* in traditionally managed grasslands. We further analyzed plant species composition and diversity in vegetation relevés. The same fire treatments were tested in a second experiment in long-term exclosures abandoned 17 years ago.

➤ Key Results

B. retusum showed a significant rapid post-fire recovery but increase in plant cover was not faster than in other species of the plant community. Fire increased *B. retusum* inflorescence production and seed set per inflorescence and this effect was stronger in the summer fire treatment. At community level, fire significantly increased species richness, evenness and Bray-Curtiss dissimilarity in the second post-fire season and again, the summer fire effect was stronger. Grazing exclusion for two seasons had only a small effect on *B. retusum* and plant community. The effect of both fire treatments on *B. retusum* was similar in long-term exclosures. In these exclosures, fire resulted in a community shift towards traditionally managed steppes.

➤ Conclusions

The concomitant positive effects of experimental burning on *B. retusum* reproduction and on plant diversity of Mediterranean steppe vegetation suggest that the system is adapted to fire as an important driver of community dynamics. Annual species surviving as seeds are as successful in post-fire recovery as perennial resprouters. Prescribed burning may be an alternative strategy to restore community structure in abandoned steppes showing a decline in typical grassland species after several years of grazing abandonment.

Keywords: exclosures; Mediterranean grassland; plant community; plant-environment interactions; population dynamics; prescribed burning; Thero-Brachypodieta.

INTRODUCTION

Mediterranean dry grasslands are semi-natural habitats that depend on human land use such as grazing and related fire management shaping plant populations and communities (Naveh 1975; Bond & Van Wilgen 1996). Both increased land use intensity, in particular transformation to arable use, and land abandonment followed by shrub encroachment are threatening these highly diverse ecosystems (San Miguel, 2008). As in other European grassland ecosystems, diversity is linked to traditional extensive land use (Poschlod & WallisDeVries 2002; Pywell et al. 2007; Gazol et al. 2012). Sheep grazing is one of the key land use factors driving plant species composition and diversity (reviewed by Cingolani et al. 2005). Wildfires and fire management by shepherds in the Mediterranean region were also important drivers until the industrial revolution but their role in maintaining the ecosystem is nowadays less clear because fire suppression and abandonment of traditional pastoral fires have largely reduced their impact in the last decades (Naveh 1975; Parr & Andersen 2006).

Many plant species and communities are adapted to fire and depend on specific fire regime (Bond & Van Wilgen 1996). Changes in such disturbance regime often lead to shifts in plant community composition (Bond & Keeley 2005). In Mediterranean “Matorral” (shrublands), an increase in fire frequency favors seeder shrubs and resprouter grasses such as *Brachypodium retusum* (Vilà-Cabrera et al. 2008). Accordingly, fire suppression may change community structure and diversity of such ecosystems (Bond et al. 2005). At species level, plants with different adaptive strategies to survive fires have been selected over centuries (Buhk et al. 2007). Resprouting from protected tissues and/or from undamaged below-ground organs are common strategies but also increased seed production, fire-resistant seeds, fire-stimulated germination or flowering (Clarke et al. 2013; Lamont & He 2017; Pyke 2017). For example, some fynbos and chaparral geophyte species only start flowering after fire and disperse seeds on bare soil to benefit from reduced competition and higher nutrient availability (Keeley 1993; Tyler & Borchert 2003).

As fire, grazing favors resprouting either from belowground organs or from the stem base. This may be combined with other avoidance strategies such as small growth, early germination and flowering, and a short flowering period (Landsberg et al. 1999; Díaz et al. 2001). In grasslands, seedling recruitment is often quite low because grazing and dense grassland swards may hamper seed production, germination and seedling recruitment (Drobnik et al. 2011). However, Mediterranean steppe vegetation is a quite open grassland type and the grazing period is short allowing the survival of many annual species (Briske & Noy-Meir 1998). Similar to fire,

grazing increases mineralization of organic matter and nutrient availability during the seedling recruitment period (Bond & Keeley 2005). Although several studies have shown that grazing is needed to maintain the unique species composition of Mediterranean steppe communities, the consequences of short-term and long-term grazing abandonment are not precisely known (Saatkamp et al. 2018). There is also a lack in studies analyzing the interaction of fire and grazing in Mediterranean grasslands. Annual fire may increase the productivity of ungrazed sites but not of recently grazed sites (Buis et al. 2009). The fire-by-grazing interaction was also found to depend on the annuals–perennials ratio of plant communities (Noy-Meir 1995).

In our study, we analyzed fire and grazing effects in a Mediterranean steppe (Thero-Brachypodietea) dominated by the resprouter grass *Brachypodium retusum*. This steppe community is under decline and protected by the EU habitats directive (San Miguel 2008). *B. retusum* is still widespread in the Mediterranean but also suffers from the loss of this habitat. In ecological restoration, *B. retusum* shows poor re-establishment (Coiffait-Gombault et al. 2012; Vidaller et al. 2018) and it is obvious that restoration remains incomplete if the dominant species of pristine steppes is lacking. Thus, a better knowledge on the ecology of the species and effects of major management factors is needed to better conserve this plant community. Fire and grazing are two key environmental/management factors that have largely decreased (fire protection, land abandonment) potentially explaining the decline of Mediterranean steppes (San Miguel 2008).

Previous studies have demonstrated that *B. retusum* benefits from fire (Caturla et al. 2000; De Luis et al. 2004; Cassagne et al. 2011). Wildfires particularly occur in undergrazed or abandoned areas but also prescribed burning was used in the past to improve forage quality (Stouff 1986; San Miguel 2008). The dense below-ground rhizome network of *B. retusum* allows a fast post-fire regrowth (Caturla et al. 2000; De Luis et al. 2004). Fire may increase reproductive stalk number (Caturla et al. 2000) but it is unknown whether fire also promotes seed set and viability. We further do not know whether fire season influences population and community dynamics of Mediterranean steppes and their dominant species. Wildfires mainly occur during the dry and hot summer period whereas shepherds burned grasslands at the end of winter (pastoral fires) to remove standing litter and favor resprouting (Stouff 1986). Both fire types may also promote annual species by providing favorable conditions for autumn and spring germination.

Similarly, grazing was found to have a positive effect on the growth of *B. retusum* (Coiffait-Gombault et al. 2012) and on the diversity of its associated plant community (Saatkamp et al.

2018). However, grazing may reduce sexual reproduction due to vegetative biomass and/or inflorescence removal (Vidaller et al. 2018). We were particularly interested in combined grazing and fire effects on the dominant species *Brachypodium retusum* and on its associated plant community. We hypothesize that both fire and grazing favor *B. retusum* relative to other species of the plant community. We further hypothesize that other resprouting species but also annual species benefit from fire and grazing increasing biodiversity and changing plant species composition. Our last hypothesis is that fire effects are stronger in ungrazed situations and that prescribed burning may be an efficient strategy to restore steppe communities in undergrazed or abandoned grasslands. We set up two experiments to specifically analyze the following research questions:

- (1) What is the effect of fire and grazing on the vegetative growth and sexual reproduction of *B. retusum* compared with co-occurring species cover of the Mediterranean steppe?**
- (2) How do fire and grazing affect plant species composition and diversity?**
- (3) Do fire effects depend on season and can fire help to maintain diversity of grazed or abandoned grasslands?**

MATERIALS AND METHODS

Study area

The study was set up in the “Crau” Mediterranean grassland (“steppe”, EUNIS habitat E1.311, Natura 2000 habitat type 6220) in South-Eastern France. The study region is characterized by hot/dry summers and mild/humid winters with an annual precipitation of 517 mm (1980-2010) and a mean temperature of 15.3 °C (Meteo-France, meteorological station of Saint-Martin-de-Crau, Appendix S1). Summer drought is common but the summer 2016 was even drier than average (55 mm instead of 100 mm on average). The soils of the study area are decalcified Red Mediterranean Soils (Haplic cambisol) with a mean pH of 6.8. The vegetation has been shaped by a long history of grazing back to the Neolithic (Henry et al. 2010) and by former winter fires (Stouff 1986; Chalvet 2016). The steppe is still annually grazed by itinerant sheep during spring. Due to risks for inhabitants and land users, winter fires are no longer used as a management strategy since the end of the 19th century (Chalvet 2016). However, wildfires still occur accidentally during summer, in particular close to roads and field tracks. No fire was observed at the study site since 1973 (Prométhée fire database).

The grassland is species-rich, dominated by annual plants and the perennial grass *Brachypodium retusum* (Buisson & Dutoit 2006, Appendix S2). In long-term exclosures (since 2001), 40% of initially occurring grassland species disappeared and have been replaced by high-growing herbaceous ruderal species (Saatkamp et al. 2018).

The study species, *Brachypodium retusum* (Pers.) P. Beauv., (ramose false brome, Poaceae), is a perennial grass species. Its rhizomes form a dense network close to the soil surface allowing resprouting after destruction of aboveground organs by fire or grazing (Caturla et al 2000). The species shows tillering at the stem base but also lateral branching at upper stem parts. Clonal growth is common but slow. *B. retusum* usually flowers from April to July but grazed or cut plants may also flower later in the season. At grazed sites, the species is highly dominant.

Long-term grazing exclosure results in an increase of *B. retusum* height and the development of other competitive grass perennial species such as *Dactylis glomerata* (Saatkamp et al., 2018).

Design

Two experiments testing the effect of late-winter fires (= winter fires) simulating pastoral fires, summer wildfires (= summer fires) and sheep grazing were set up in March 2016 and run for two seasons. The winter fire treatment was applied once in March 2016 and the summer fire treatment once in July 2016. In the first experiment, fire treatments were applied to the traditionally grazed steppe (Appendix S3, A). In the second experiment, fire treatments were applied to long-term exclosures (abandoned grassland, Appendix S3, B).

Effect of fire and grazing in a traditionally managed steppe area

The experiment was set up at two different sites comprising three blocks each (Negreiron 43°32'18.28"N / 4°49'0.75"E, Collongue 43°31'37.55"N / 4°49'55.82"E) to reflect heterogeneity in grazing but the site effect was finally pooled into block for statistical analysis in order to increase parsimony.

Blocks were split into two parts (half-blocks) of which one was fenced to prevent grazing. The three fire treatments (summer fire, winter fire, no fire) were applied to three plots of 4 m² within each of the twelve half-blocks (split-plot design) in order to test combined effects of fire and grazing (36 plots in total). The distance between plots within half-blocks was 1 m, between half-blocks within blocks 50 m and between blocks at least 200 m. The position of fire

treatments was randomized within half-blocks and that of grazing treatment was randomized within blocks.

A thermal weeder was used to burn plots to simulate winter fire and summer wildfire. Thermal weeding was achieved using a portable device with a gas cylinder and a ramp comprising four nozzles for flame weeding (Appendix S4). Due to fast fire spread during summer, we removed the device once the vegetation was burning. We stopped the fire manually when flames arrived at the plot border. To prevent grazing, the control half-blocks were fenced with stretched, non-electrified nets (1 m high). The other half-blocks allowed free sheep grazing during the grazing period from April to June. The mean grazing intensity was of 646 sheep*ha⁻¹*season⁻¹ at Négreiron site and 328 sheep* ha⁻¹*season⁻¹ at Collongue site.

In July 2016, the aboveground vegetation was harvested in two plots of 50*50 cm in each grazed and ungrazed half-block to measure biomass removal by sheep. Samples were dried at 80°C to a constant biomass and weighed afterwards. Grazing reduced aboveground biomass from 70 ± 11g/m² in ungrazed plots to 50 ± 9g/m² at the end of the grazing period.

Effect of fire in long-term exclosures

The same fire treatments were established in long-term grazing exclosures. The aboveground vegetation was harvested as described above. Standing biomass (110 g/m² ± 16) was twice as high as in traditionally grazed steppe area. The design included three plots per block comprising the three fire treatments and six replicate blocks (18 plots in total). Corresponding to the first experiment, three blocks were set up at the Négreiron site fenced in August 2001 (3 ha) and three blocks at the Collongue site fenced in June 1999 (2.9 ha). The distance between blocks and to exclosure fences was at least 50 m.

Data collection

B. retusum survey

The cover percentage of the species was estimated at six dates (May, July, October 2016 and February, May, July 2017) including two growing seasons. At the same time, total vegetation cover was estimated. Cover was measured as the vertical projection of all aboveground organs. Visual cover estimation is as reliable as the more objective pin-point or point-intercept method and allows an analysis of larger areas (Damgaard 2014). Cover values were always estimated by the same person to avoid bias of different observer estimations. *B. retusum* cover was divided

by total vegetation cover to obtain weighted cover. The cover of May 2016 and 2017 was finally used for analyses representing maximum vegetative development before aboveground organs partially die due to summer drought. The regeneration of *B. retusum* was measured by comparing May 2017 and October 2016 cover. In order to evaluate sexual reproduction, the number of inflorescences was counted in each plot during flowering peak in July 2016 and 2017. At the same time, twenty inflorescences per plot were harvested to count seeds. If number of inflorescences per plot was lower than twenty all inflorescences were sampled. Seeds were prepared by crushing the inflorescences in a plastic tube with glass beads using a vortex, then spreading the separated spikelets on an overhead projector in order to check whether spikelets are filled. Empty seeds or spikelets were removed from seed counts. Seeds were stored at room temperature and germinated in peat plugs (4 x 4 cm). Four seeds of each experimental plot (block x treatment combination) were sown to one plug. The plug trays were placed in a growth chamber under optimum germination conditions (temperature 15/20 °C, 12/12h, night/day, Vidaller et al. (2018)) and watered regularly. Coleoptile emergence was used as a proxy of germination and was recorded every 2-3 days for seven weeks.

Vegetation survey

Species composition of the associated plant community was analyzed in May 2016 and May 2017 during the peak of flowering. The cover of all vascular plant species was estimated using a modified Braun-Blanquet scale (Braun-Blanquet 1932: + = one individual per plot, 1 < 5% cover, 2: 5 - 25 %, 3: 26 - 50 %, 4: 51 - 75 %, 5:> 75%). Contrary to the *B. retusum* survey, the sum of individual species cover may be higher than 100%.

Data analysis

In the combined fire and grazing experiment, a split-plot ANOVA was performed to analyze fire treatment and grazing effects on individual response variables (individual species cover, *B. retusum* inflorescence, seed number and germination rate, species richness, diversity indices). Grazing (whole-plot factor) was tested against the block x grazing interaction. Fire (split-plot factor), the fire x grazing interaction and block were tested against the model residuals. In the long-term enclosure experiment, a reduced model was applied only including the fire treatment and block. When necessary log₁₀(x+1) and square root transformations were used to comply with assumptions of linear models (normality, homoscedasticity). A Tukey HSD posthoc test

was calculated to analyze differences between treatment levels if treatment main effects or interactions were significant.

In order to analyze plant community data, Braun-Blanquet scores were transformed into percentage cover: + = 0.5%; 1 = 2.5%; 2 = 15.0%; 3 = 37.5%; 4 = 62.5%; 5 = 87.5%. Species richness (S), evenness (J') and Bray-Curtis index (BC) were calculated using vegan v2.4-2 package. J' evenness was calculated as $H'/\ln(S)$, with H' being the Shannon diversity index (Pielou 1969). Species composition was compared using NMDS (Non-Metric Multidimensional Scaling) based on the similarity index of Bray-Curtis (Borcard et al. 2011) in order to illustrate changes in plant species composition as well as the species most correlated with each treatment. NMDS analyses were run using 30 random starting configurations in 1 to 15 dimensions. The run with the lowest stress value was finally applied. A permutation multivariate analysis of variance (PERMANOVA, Anderson 2001) with 9999 permutations was used to test whether the community composition was significantly different between the fire and grazing treatments. A pairwise posthoc test with Bonferroni adjustment was calculated to analyze differences between levels of the fire treatment. All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)) including multivariate analyses using the vegan package.

RESULTS

B. retusum survey

In the combined fire and grazing experiment, fire significantly reduced *B. retusum* cover in the following two years (Tab. 1, Appendix S5). Several months after fire treatments (October 2016), even cover relative to neighboring plants (weighted cover) was lower (Tab.1). Accordingly, inflorescence and seed production were significantly reduced but a higher germination rate was found in seeds produced in winter fire plots compared with control plots (Tab.1). In the second post-fire year (2017), the fire main effect on weighted cover of *B. retusum* was not significant any more (Fig. 1A, Tab.1). *B. retusum* showed a rapid regeneration of its vegetative cover in the burned plots (Fig. 1C, Tab.1) compared with control plots in which its cover decreased from 2016 to 2017. At the end of the survey period in July 2017, inflorescence number was higher in burned plots but differences were only significant between summer fire treatment and control (Fig. 2A, Tab.1). We also found a significantly higher seed number per

inflorescence in summer and winter fire plots (Fig. 2C, Tab.1) whereas germination rate was no longer different between seeds from the different treatments.

The effect of grazing was not significant for any of these variables (Tab. 1). Only a significant fire x grazing interaction occurred in 2017 for *B. retusum* cover. Grazing reduced absolute and weighted cover in unburned controls.

In the long-term exclosures, fire effects were similar to those in the combined grazing and fire experiment (Tab. 1). Cover (weighted and non-weighted), inflorescence number and seed number per inflorescence decreased in the beginning of the first year, but recovered rapidly in the second post-fire year (Tab. 1, Fig. 1B, 1D). Although absolute *B. retusum* cover was still lower in 2017, relative (weighted) cover was not different any more to the control whereas inflorescence number was even higher in the summer fire treatment compared with the unburned control (marginally significant). Due to the low seed set in all treatments, differences in seed number per inflorescence were not significant.

Table 1: ANOVA F-values and significance level for effects of fire and grazing on *Brachypodium retusum* in the two experiments: cover and weighted cover adjusted for total vegetation cover (both May), post-fire regeneration (cover difference between October 2016 and May 2017), inflorescence number, seed number per inflorescence (both July) and germination rate. F×G= fire x grazing interaction. . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant. Factor effect: ↓:decrease; ↑: increase.

| | <i>df</i> | Cover (%) | Weighted cover (%) | Regeneration (%) | Inflorescences (number.4m ⁻²) | Seeds (number.inflo ⁻¹) | Germination (%) |
|---|-----------|-----------|--------------------|------------------|---|-------------------------------------|-----------------|
| A. Combined fire and grazing experiment | | | | | | | |
| 2016 | Fire | 1.10 | 469.29 *** ↓ | 365.43 *** ↓ | 18.99 *** ↓ | 42.44 *** ↓ | 4.06 * ↑ |
| | Grazing | 1.10 | 0.39 NS | 1.43 NS | 0.18 NS | 0.56 NS | 0.01 NS |
| | F×G | 1.10 | 0.13 NS | 0.13 NS | 0.09 NS | 1.59 NS | 0.03 NS |
| 2017 | Fire | 2.20 | 12.35 *** ↓ | 2.21 NS | 143.04 *** ↑ | 11.48 *** ↑ | 5.86 ** ↑ |
| | Grazing | 1.10 | 1.62 NS | 0.58 NS | 1.12 NS | 0.01 NS | 0.24 NS |
| | F×G | 2.20 | 3.64 * | 3.62 * | 0.65 NS | 0.90S | 0.18 NS |
| B. Fire experiment in long-term exclosures | | | | | | | |
| 2016 | Fire | 1.10 | 311.00 *** ↓ | 115.60 *** ↓ | 1.10 NS | 1.08 NS | 0.87 NS |
| 2017 | Fire | 2.20 | 0.66 NS | 1.85 NS | 72.94 *** ↑ | 3.76 . ↑ | 2.27 NS |

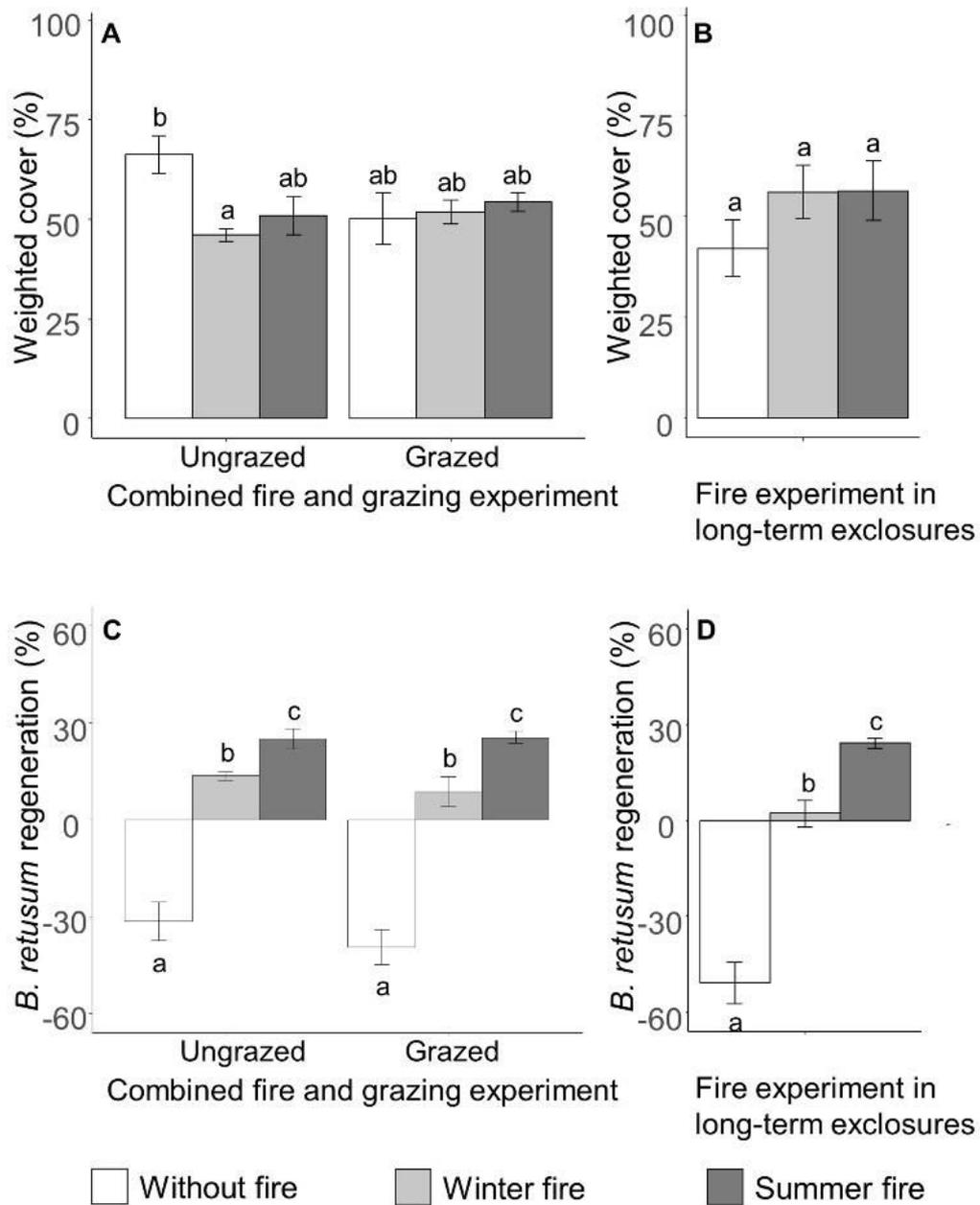


Figure 1: Effects of fire and grazing on vegetative growth of *B. retusum* in the combined fire and grazing experiment (left) and the fire experiment in long-term enclosures (right). A, B: weighted cover one year after fire treatments (May 2017, mean \pm SE); C, D: Post-fire regeneration of cover (mean difference between October 2016 and May 2017, \pm SE).

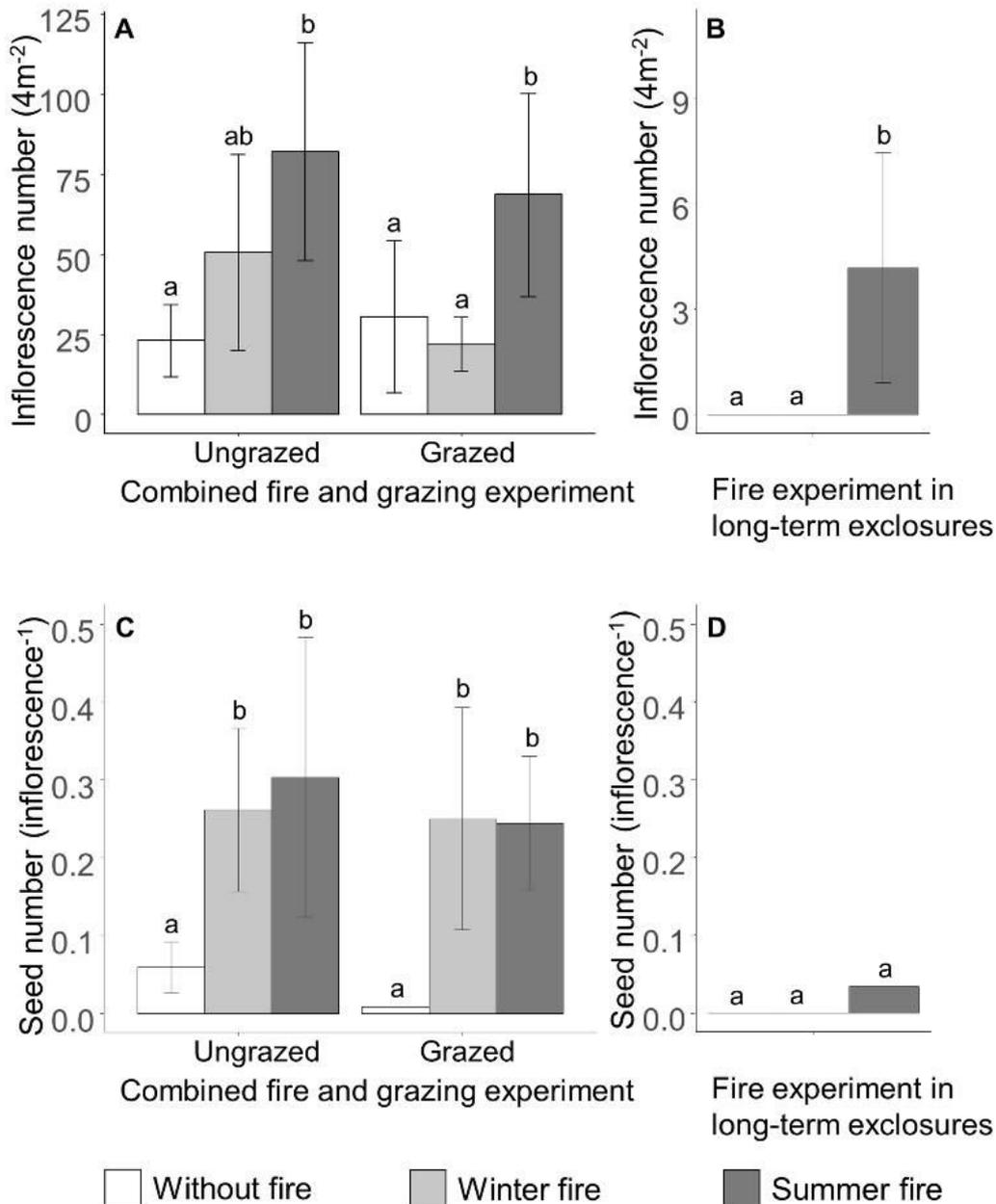


Figure 2: Effects of fire and grazing on the reproduction of *B. retusum* (mean \pm SE) in the combined fire and grazing experiment (left) and the fire experiment in long-term exclosures (right) in the second post-fire year (July 2017). A, B: Inflorescence number; C, D: Seed number per inflorescence.

Plant community

In the combined fire and grazing experiment, fire significantly reduced species richness three months after treatments in 2016 but one year later, in 2017, species richness was significantly higher in burned plots than in unburned controls (Tab. 2, Fig. 4). The second year differences

in species richness were significant between summer fire and control plots whereas winter fire species richness was not different from the other treatments. In both years, evenness and Bray-Curtis dissimilarity were significantly higher in the fire treatments. Regardless of the year, grazing effect and the fire x grazing interaction were not significant.

In the long-term exclosures, species richness was not significantly affected three months after fire treatment (May 2016) but in the second year, a significant increase was measured between summer fire and control. As in the first experiment, evenness (marginally) and Bray-Curtis dissimilarity were significantly higher in burned plots compared to the controls. However, the fire effect on Bray-Curtis dissimilarity was not significant any more in the second post-fire year.

Table 2: ANOVA *F*-values, significance levels for effects of fire and grazing on species richness (4m²), evenness and Bray-Curtis dissimilarity in the two experiments. F×G= fire x grazing interaction. . *P* < 0.1, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, NS not significant. Factor effect: ↓: decrease; ↑: increase.

| | | <i>df</i> | Species-Richness | Evenness | Bray-Curtis |
|---|---------|-----------|------------------|-------------|-------------|
| A. Combined fire and grazing experiment | | | | | |
| 2016 | Fire | 1.10 | 4.97 * ↓ | 12.26 ** ↑ | 24.14 *** ↑ |
| | Grazing | 1.10 | 0.45 NS | 0.01 NS | 0.45 NS |
| | F×G | 1.10 | 0.01 NS | 3.12 NS | 0.16 NS |
| 2017 | Fire | 2.20 | 4.18 * ↑ | 14.00 *** ↑ | 3.32 * ↑ |
| | Grazing | 2.20 | 0.03 NS | 0.00 NS | 0.04 NS |
| | F×G | 2.20 | 0.42 NS | 0.31 NS | 0.40 NS |
| B. Fire experiment in long-term exclosures | | | | | |
| 2016 | Fire | 1.10 | 3.05 NS | 4.82 . ↑ | 32.06 ** ↑ |
| 2017 | Fire | 2.10 | 5.03 * ↑ | 3.95 * ↑ | 1.45 NS |

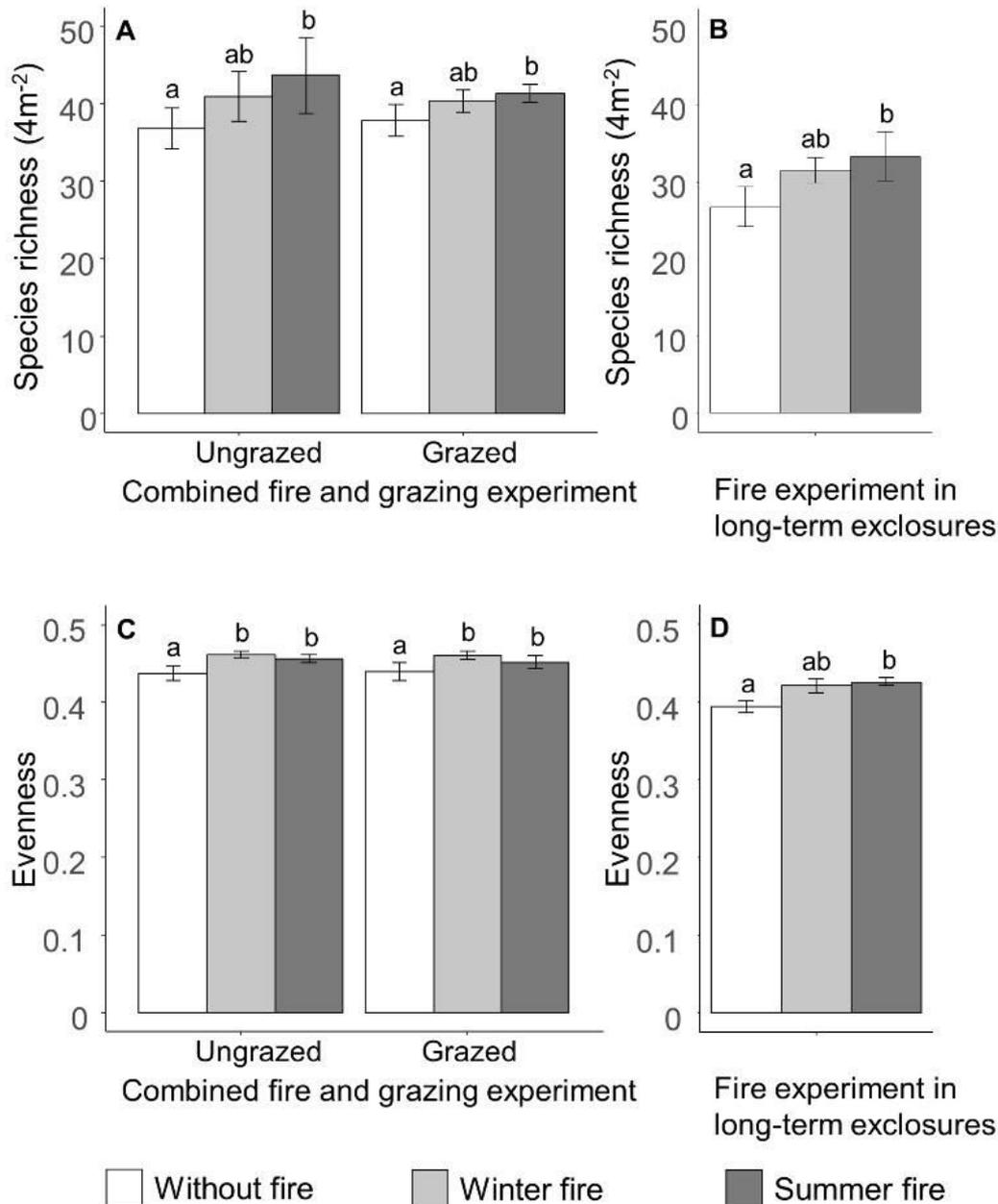


Figure 3: Effects of fire and grazing on the plant community (mean \pm SE) one year after fire treatments (May 2017) in the combined fire and grazing experiment (left) and the fire experiment in long-term exclosures (right). A, B species richness; C, D: evenness.

In 2017, one year after fire treatments, NMDS ordination showed a clear separation between the plant community of the combined fire and grazing experiment in the steppe area and the fire experiment in long-term exclosures (Fig. 4). Along axis 1 of the NMDS, *B. retusum* and ruderal species such as *Sonchus oleraceus*, *Avena sterilis* and *Avena barbata* characterize the vegetation of the long-term exclosures whereas short annual and stress-tolerant species such as

Polycarpon tetraphyllum, *Evax pygmaea*, *Euphorbia falcata*, *Galium murale* are more abundant in the traditionally managed steppe. In the long-term exclosure, fire changed the plant community more than in the steppe (Fig. 4). The burned long-term exclosures were closer to the traditionally managed steppe than the unburned controls. The PERMANOVA testing differences in species composition between treatments showed a significant effect of fire in the combined fire and grazing experiment ($F_{2,35}=2.865^*$) and in long-term exclosures (marginally significant, $F_{2,17}=1.78^{(*)}$). In the combined fire and grazing experiment, the effect of summer fire was stronger than that of winter fire whereas no such differences occurred in the long-term exclosures (Appendix S6). The effect of grazing and the grazing x fire interaction were not significant (grazing: $F_{1,35}=1.512^{NS}$, fire x grazing: $F_{2,35}=0.212^{NS}$).

In both experiments, the cover of annual species such as *Medicago monspeliaca*, *Euphorbia falcata*, and *Bromus rubens* as well as the perennial grass *Poa bulbosa* particularly benefitted from fire treatments (Appendix S7). In particular, summer fire had a strong positive effect on the cover of *M. monspeliaca* and *B. rubens* whereas *E. falcata* and *P. bulbosa* also benefitted from winter fire. The grazing effect was not significant for these species.

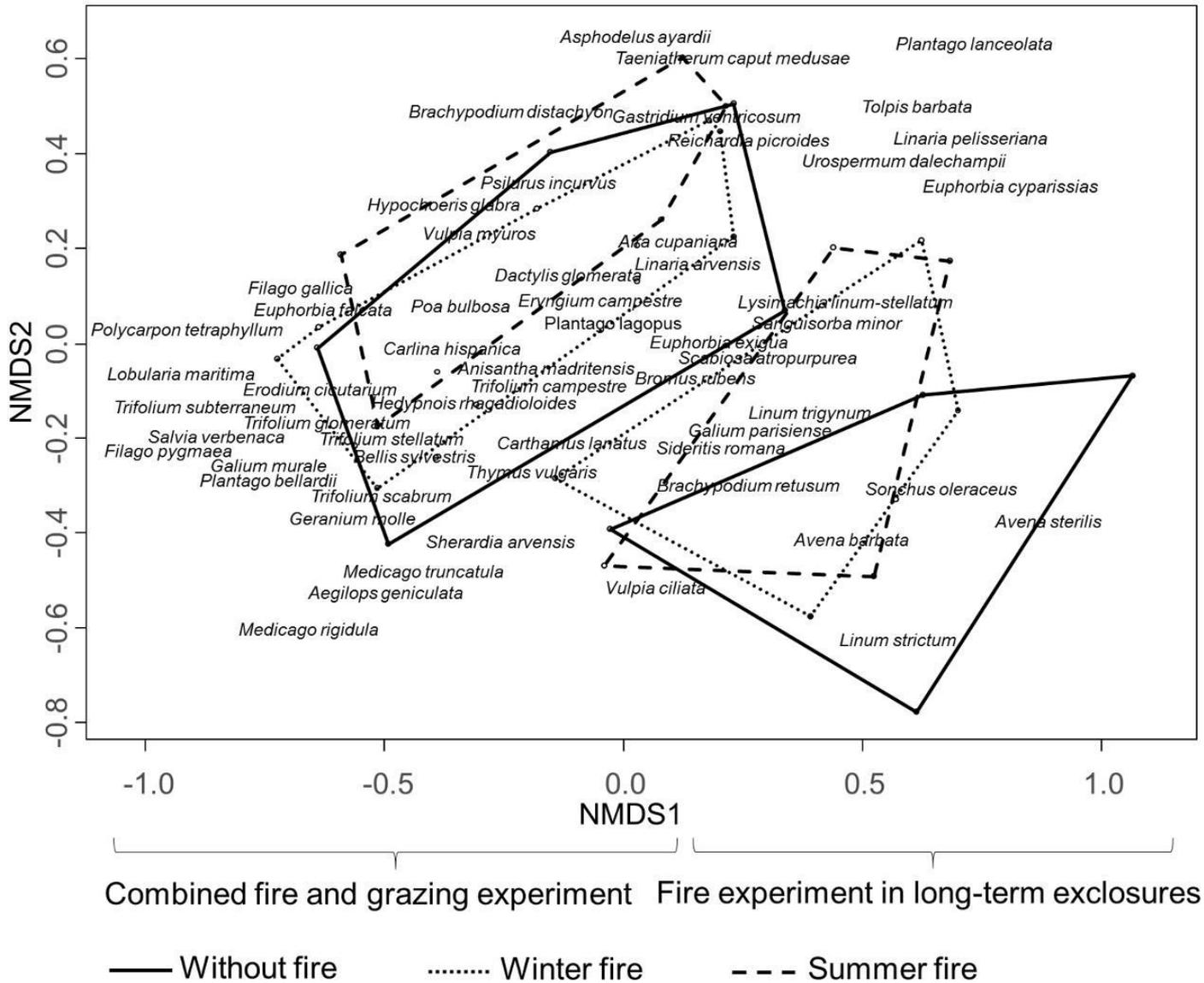


Figure 4: Effect of fire on plant species composition in the combined fire and grazing experiment set up in traditionally managed steppe and in the fire experiment set up in long-term exclosures using NMDS on May 2017 data. Polygons indicate the position of the outmost plots in each treatment (NMDS stress: 0.1471418).

DISCUSSION

In the combined fire and grazing experiment, the fire effect was much stronger than the grazing effect on both the performance of the dominant species *B. retusum* and plant species composition. In our case, grazing reduced aboveground biomass by 40% but did not significantly influence the majority of measured *B. retusum* traits and community parameters. Fire had a similar effect in the combined fire and grazing experiment at traditionally managed sites and in the long-term exclosures allowing a fast recovery of typical steppe species such as *B. retusum*.

Effects of fire and grazing on *B. retusum*

B. retusum can survive fire by resprouting from rhizomes favored by axillary buds (Clarke et al. 2013). Adapted plant species such as rhizomatous resprouters may even benefit, since fire increases resource availability (Neary et al. 1999), stimulates re-growth from below-ground buds (Fidelis et al. 2014) and opens the canopy reducing competition for light, water and nutrients (Everson & Everson 1987). In our study, *B. retusum* cover increased rapidly after fire whereas controls showed a decrease in cover due to the summer drought in 2016. In particular, reduced competition for water resulting from biomass removal was likely the reason for the better post-fire regeneration (Everson & Everson 1987). However, weighted cover of *Brachypodium retusum* relative to total vegetation cover was in the beginning significantly lower than in fire treatments indicating that post-fire regeneration was slower than in other species of the plant community. Thus, our results do not confirm studies in Eastern Spain showing a faster recovery of *B. retusum* compared to accompanying species resulting in a rapid increase of weighted *B. retusum* cover (Caturla et al. 2000, De Luis et al. 2004).

Regeneration was faster in the summer than in the winter fire treatment suggesting that *B. retusum* is better adapted to natural summer fire. The period of fire management by shepherd may not have been long enough for an evolutionary response whereas summer fire disturbance is much older allowing adaptive processes. The stronger summer fire effect on *B. retusum* regeneration may also be the result of a higher standing biomass and a lower water content (dry leaves and stems) corresponding to higher fire temperatures and biomass loss compared with winter fire (Hobbs 1991; Trabaud 1979). Nevertheless, the traditional winter fire management is already well-known to improve pasture quality and vigor by allowing the regeneration of *B. retusum* aboveground biomass (Stouff 1986).

In the long-term exclosures, fire effects on *B. retusum* cover were similar to those of the combined grazing and fire experiment in the traditionally managed steppe. However, the species reached cover values of the unburned control already in the second post-fire year indicating a faster recovery. Long-term exclosure from grazing results in a strong biomass increase mainly due to the growth of perennial grass species (Bond & Parr 2010). Abandonment of grazing also increases litter biomass, C:N ratios and decreases soil nutrient content (Peco et al. 2012; Saatkamp et al. 2018). Thus, fire temperatures are higher and therefore fire has a stronger effect on plant species than in grazed systems with lower living and dead biomass (Hobbs et al. 1991). The high recovery of *B. retusum* in our exclosures suggests that wildfire or prescribed burning may help to maintain the typical species composition and community structure in long-term absence of grazing.

In addition to vegetative resprouting, fire increased allocation of resources to reproductive organs in the second post-fire year favoring seed production and quality. The production of inflorescences was significantly higher in both fire treatments, but in particular after summer fire. Fire-stimulated flowering is already known for several species in the Mediterranean regions of Australasia and South Africa with peak flowering occurring five to eighteen months after fire (Lamont & Downes 2011, Pyke 2017). Flowering may be triggered by fire-specific cues such as chemical compounds of smoke and ash or scorching of below-ground storage organs (Keeley 1993; Tyler & Borchert 2003) but also by a fire-induced increase in light and temperature (Fidelis & Blanco 2014). As resprouting, inflorescence production may be favored by a higher resource availability due to reduced competition and higher nutrient or water availability (Coates & Duncan 2009).

Fire also increased the seed set measured as seed production per inflorescence in the second post-fire year whereas high fire damage to photosynthetically active tissues (leaves, stems) reduced seed set in the first year. A higher post-fire seed set in the following years was also measured for two Australian perennials (Lamont & Runciman 1993). The effect on ex-situ germination was less strong being only positive in the same year of the fire treatments. In the second season, when the increase in inflorescence production and seed set occurred, differences in germination were not significant any more. Germination is an important trait under selection for adaptation to fire (Keeley et al. 2012). Many species from a wide range of taxa show a rapid and increased germination after fire (Naveh 1975; González-Rabanal et al. 1994). However, *B. retusum* seeds were produced after fire excluding a direct exposure to fire cues. Our germination

experiment was set up in a growth chamber not taking into account modified conditions in burned plots (nutrient availability, destruction of organic matter, presence of ashes, etc.). Moreover, in some species of fire-prone ecosystems, there is no evidence that fire triggers germination (Le Stradic et al. 2015).

The grazing main effect on vegetative growth and on reproduction was not significant neither in the first nor in the second season. Although statistical power to test grazing as the whole-plot factor of the split-plot design was lower than for fire as split-plot factor the small differences between grazing treatment means confirm that grazing impact is much less strong than fire impact. *B. retusum* tolerates the short-term absence of grazing even though grazing is an important factor in maintaining population cover (Coiffait-Gombault et al. 2012). However, a significant fire x grazing interaction was measured during the second season with plant cover being lower only in unburned plots. The result confirmed our hypothesis that fire impact is stronger in ungrazed situations. Higher fire temperatures due to higher litter biomass already cited for abandoned grasslands (Peco et al. 2012, Hobbs 1991) may have increased the impact of fire in ungrazed control plots. However, this significant interaction was also visible in winter fire plots that were burned before grazing. This effect could be explained by the behavior of sheep avoiding recently burned *B. retusum* plants, thus increasing the positive effect of fire by reducing post-fire biomass consumption.

Effects of fire and grazing on the plant community

In the combined fire and grazing experiment, fire reduced species richness three months after treatments but in the second year, the fire effect was already strongly positive. Fire also increased evenness by removing biomass of the dominant *B. retusum* and community dissimilarity. Infrequent and/or irregular fire disturbance may result in species loss and community composition changes (Laurance 2003). However, in fire-prone ecosystems, such disturbance maintains species richness and community structure (Bond et al. 2005, Fidelis et al. 2012). Accordingly, fire suppression may considerably reduce plant diversity of fire-prone systems (Sparks et al. 1998, Leach & Givnish 1996).

Again, the effect of summer fire was stronger than that of winter fire. Fire season was found to affect plant communities. Fire during the growing season may, for example, increase plant diversity, in particular the number of annuals and cool-season perennials whereas no such effect was observed for dormant season (fall, spring) fire (Biondini et al. 1989; Howe 1994). The

growing season of *B. retusum* and most other species of the community starts in late winter and lasts until July corresponding to the two fire dates. The stronger summer fire effect may be the result of a higher standing biomass and a lower water content (dry leaves and stems) corresponding to higher fire temperatures and biomass loss (Hobbs 1991; Trabaud 1979).

The fire effect on long-term exclosures was similar to that on traditionally managed steppe plots. Fire generally increased diversity in the second season. Contrary to the traditionally managed steppe, there was no decrease in species richness confirming the particularly beneficial effect of fire in abandoned grassland for conserving species-rich dry grasslands (Bond & Parr 2010). Plant species composition of burned long-term exclosures was closer to the traditionally managed steppe than that of the unburned control.

In both experiments and systems, differences in species composition were mainly explained by an increase in native annual seeder species whereas fire reduced the cover of resprouter grasses in the first post-fire year. Similar to the results on the dominant species *B. retusum*, fire-induced community changes do not correspond to those of earlier studies in Spanish *B. retusum* grasslands (Caturla et al. 2000, De Luis et al. 2004). The La Crau steppe is a more open and less nutrient-rich grassland type than the Spanish field sites allowing the development of a higher diversity of annual species (Buisson & Dutoit 2006). Apart from resprouting, seedling recruitment is therefore a second successful strategy of post-fire regeneration as it is already known for other dry and nutrient-poor grasslands (Valone & Kelt 1999). Although the short study period does not allow conclusions on the duration of facilitative effects, we were able to demonstrate that fire helps annual species to develop. Further research is needed to identify the optimum frequency of fire disturbance to maintain such positive effects on plant diversity.

Grazing was expected to have positive but less strong effects on plant diversity of the steppe community. Such positive effects are mediated by sward opening, biomass reduction and soil surface disturbance (Coiffait-Gombault et al. 2012; Saatkamp et al. 2018). In our study, the effects of grazing and the interaction between fire and grazing were not significant. Again, the small differences between treatment means confirmed that grazing has a much smaller effect than fire in spite of lower statistical power for testing whole-plot effects. The hypothesis that fire effects are stronger in ungrazed situation was not supported at community level. As already demonstrated for the dominant species *B. retusum*, the plant community tolerated the short-term absence of grazing without species loss or differences in diversity indices. These findings confirmed results of a previous study in the same system showing only small changes in the first five years after grazing abandonment (Saatkamp et al. 2018).

CONCLUSIONS

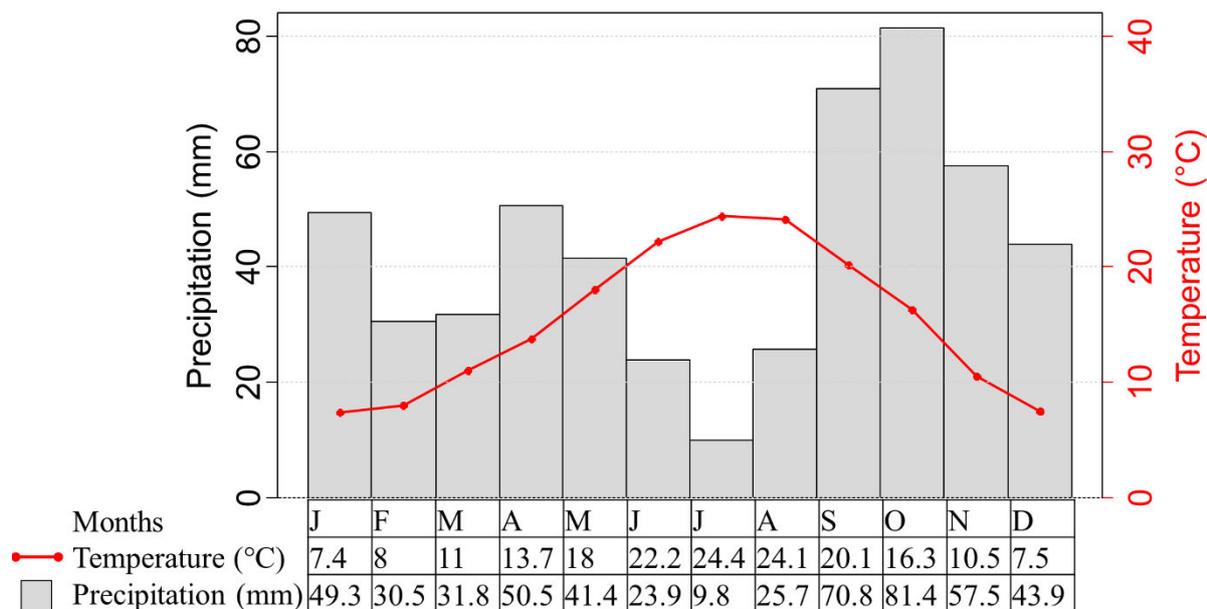
We showed a concomitant positive effect of fire on the reproduction of the dominant grass *B. retusum* and the species diversity of its associated plant community. Contrary to our expectations, fire did not favor perennial resprouters such as *B. retusum* over annuals confirming that both strategies resprouting from below-ground organs and seedling recruitment are successful in short term post-fire vegetation recovery. Short-term grazing exclusion had only small effects on *B. retusum* and associated plant community. However, the long-term enclosures abandoned 17 years before our experiments showed large differences to the traditionally managed steppe such as a lower *B. retusum* reproduction and a lower plant diversity. In such abandoned or undergrazed systems, fire has a particularly positive effect on *B. retusum* performance and plant diversity and may thus compensate for long-term grazing absence to conserve this unique Mediterranean grassland ecosystem.

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SUPPORTING INFORMATION

Appendix S1: Climate chart of Saint-Martin de Crau (nearest meteorological station, 10 km from our study site.) showing long-term means of air temperature and precipitation (source: Meteo-France).



Appendix S2: Vascular plant species found at the study sites.

Species

Aegilops geniculata Roth

Aegilops triuncialis L.

Aira cupaniana Guss.

Ajuga iva (L.) Schreb.

Allium sphaerocephalon L.

Andryala integrifolia L.

Anisantha madritensis (L.) Nevski,

Anisantha rubens (L.) Nevski

Arenaria serpyllifolia L.

Asperula cynanchica L.

Asphodelus ayardii Jahand. & Maire

Avena barbata Pott ex Link

Avena sterilis L.

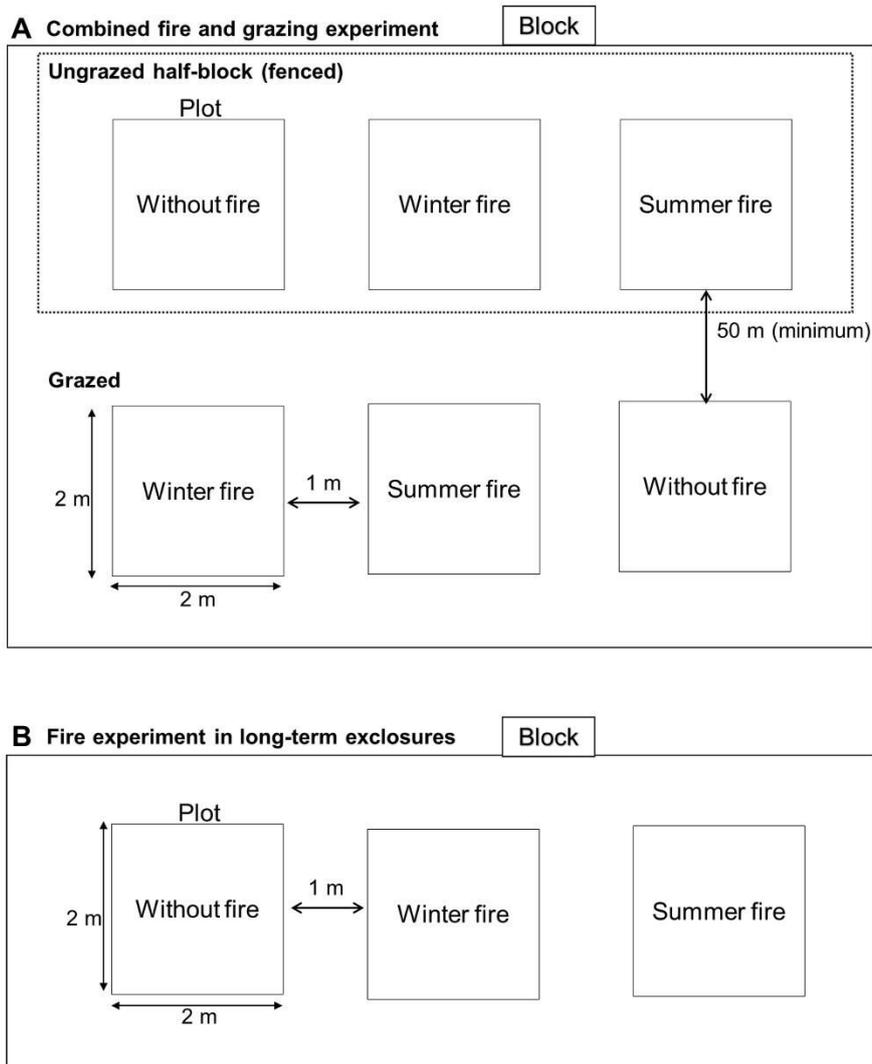
Bellis sylvestris Cirillo
Biscutella laevigata L.
Bothriochloa ischaemum (L.) Keng
Brachypodium distachyon (L.) P.Beauv.
Brachypodium retusum (Pers.) P.Beauv.
Bromus hordeaceus L.
Carduus nigrescens Vill.
Carduus pycnocephalus L.
Carlina hispanica Lam.
Carlina lanata L.
Carthamus lanatus L.
Catapodium rigidum (L.) C.E.Hubb.
Centaurea melitensis L.
Centranthus calcitrapa (L.) Dufr.
Cerastium glomeratum Thuill.
Clinopodium nepeta (L.) Kuntze.
Convolvulus cantabrica L.
Crassula tillaea Lest.-Garl.
Crepis foetida L.
Crepis sancta (L.) Bornm.
Crepis vesicaria L.
Crucianella angustifolia L.
Cynodon dactylon (L.) Pers.
Cynosurus echinatus L.
Dactylis glomerata L. ssp. *hispanica* (Roth) Nyman
Daucus carota L.
Draba verna L.
Echium asperrimum Lam.
Erigeron sp.
Erodium cicutarium (L.) L'Hér.
Eryngium campestre L.
Euphorbia cyparissias L.
Euphorbia exigua L.
Euphorbia falcata L.
Euphorbia segetalis L.
Euphorbia seguieriana Neck.

Filago germanica (L.) Huds.
Filago pygmaea L.
Filago pyramidata L.
Foeniculum vulgare Mill.
Galactites tomentosa Moench
Galium murale (L.) All.
Galium parisiense L.
Gastridium ventricosum (Gouan) Schinz & Thell.
Geranium molle L.
Hedypnois rhagadioloides (L.) F.W.Schmidt
Helianthemum salicifolium (L.) Mill.
Helictochloa bromoides (Gouan) Romero Zarco
Hippocrepis ciliata Willd.
Hirschfeldia incana (L.) Lagr.-Foss.
Hordeum murinum L.
Hypochoeris glabra L.
Lactuca serriola L.
Lagurus ovatus L.
Lamium amplexicaule L.
Leontodon tuberosus L.
Linaria arvensis (L.) Desf.
Linaria pelisseriana (L.) Mill.
Linum strictum L.
Linum trigynum L.
Lobularia maritima (L.) Desv.
Logfia gallica (L.) Coss. & Germ.
Logfia minima (Sm.) Dumort.
Lysimachia linum-stellatum L.
Medicago minima (L.) L.
Medicago monspeliaca (L.) Trautv.
Medicago rigidula (L.) All.
Medicago truncatula Gaertn.
Melica ciliata L.
Misopates orontium (L.) Raf.
Neatostema apulum (L.) I.M.Johnst.
Onopordum illyricum L.

Petrorrhagia prolifera (L.) P.W.Ball & Heywood
Phleum pratense L. ssp *nodosum* (L.) Dumort.
Plantago bellardii All.
Plantago coronopus L.
Plantago lagopus L.
Plantago lanceolata L.
Poa annua L.
Poa bulbosa L.
Polycarpon tetraphyllum (L.) L.
Psilurus incurvus (Gouan) Schinz & Thell.
Ranunculus paludosus Poir.
Reichardia picroides (L.) Roth
Rostraria cristata (L.) Tzvelev
Ruta montana (L.) L.
Sagina apetala Ard.
Salvia verbenaca L.
Sanguisorba minor Scop.
Scabiosa atropurpurea L.
Schedonorus arundinaceus (Schreb.) Dumort.
Scorzonera laciniata Jacq.
Senecio vulgaris L.
Seseli tortuosum L.
Sherardia arvensis L.
Sideritis romana L.
Silene gallica L.
Silene nocturna L.
Silene otites (L.) Wibel
Sonchus asper (L.) Hill
Sonchus oleraceus (L.) L.
Stipa capillata L.
Taeniatherum caput-medusae (L.) Nevski
Teucrium chamaedrys L.
Teucrium polium L.
Thymus vulgaris L.
Tolpis barbata (L.) Gaertn. ssp *umbellata* (Bertol.) Jahand. & Maire
Torilis nodosa (L.) Gaertn.

Tragopogon porrifolius L.
Trifolium campestre Schreb.
Trifolium cherleri L.
Trifolium glomeratum L.
Trifolium scabrum L.
Trifolium stellatum L.
Trifolium subterraneum L.
Trifolium suffocatum L.
Trifolium tomentosum L.
Tyrimnus leucographus (L.) Cass.
Urospermum dalechampii (L.) Scop. ex F.W.Schmidt
Urospermum picroides (L.) Scop. ex F.W.Schmidt
Valerianella discoidea (L.) Loisel.
Verbascum sinuatum L.
Veronica arvensis L.
Vulpia ciliata Dumort.
Vulpia myuros (L.) C.C.Gmel.

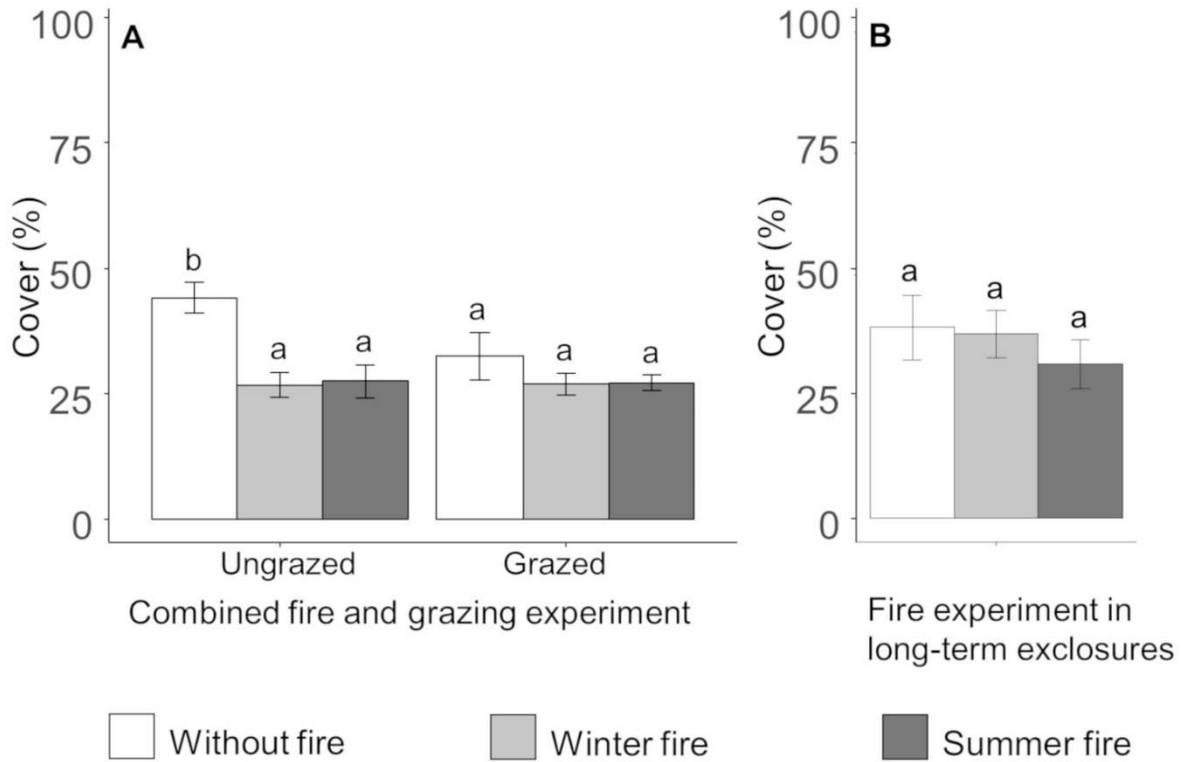
Appendix S3: Design of the combined fire and grazing experiment (A) and the fire experiment in long-term enclosures (B). The figure shows one out of six experimental blocks in each experiment.



Appendix S4: Thermal weeder device.



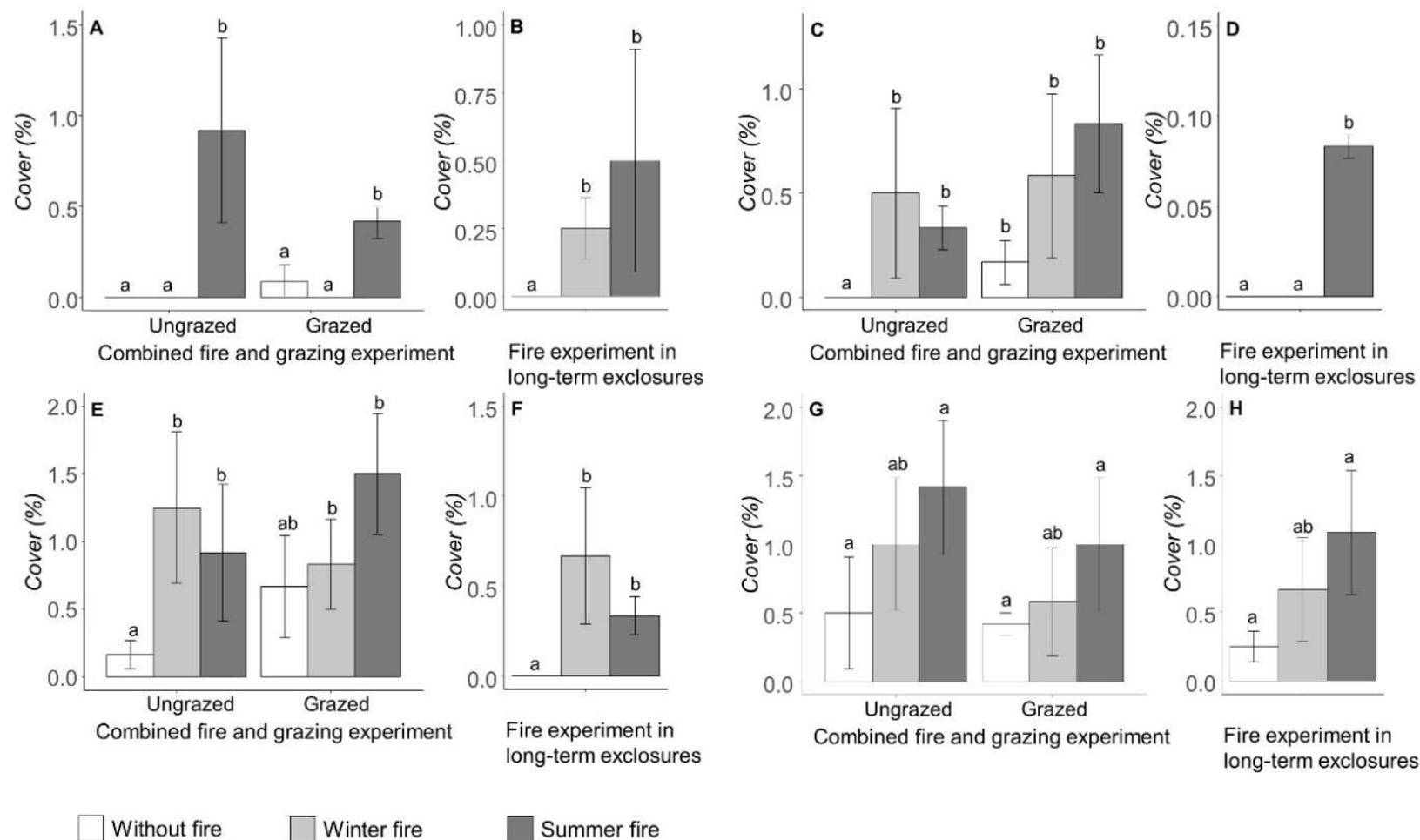
Appendix S5: Effects of fire and grazing treatments on *B. retusum* cover (mean \pm SE) in the combined fire and grazing experiment (A) and the fire experiment in long-term exclosures (B) one year after fire treatments (May 2017).



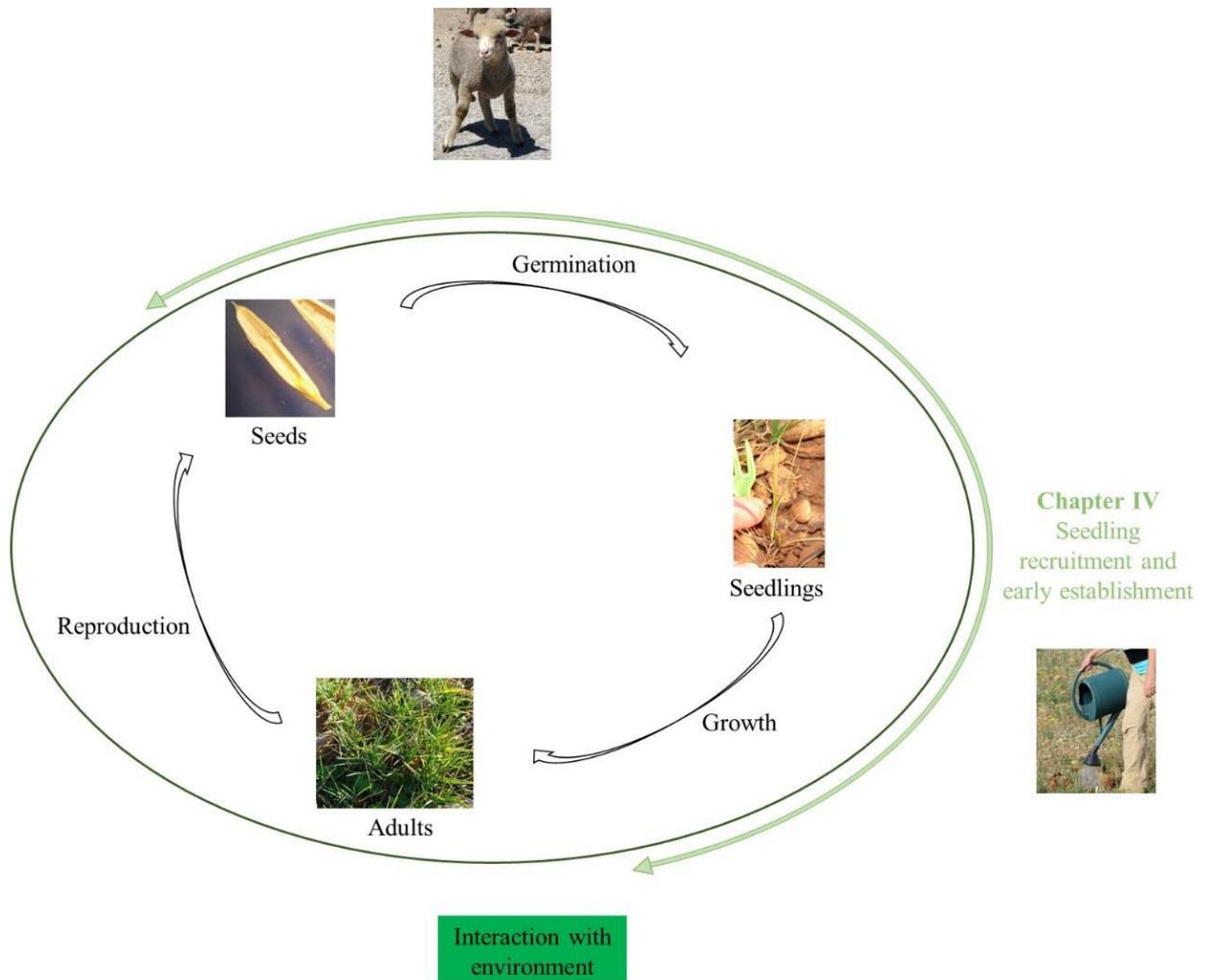
Appendix S6: Pairwise contrasts of Permutational Multivariate Analysis of Variance (PERMANOVA) with Bonferonni adjustment to test differences between different levels of the fire treatment. A: Fire treatments in combined fire and grazing experiment B: Fire treatments in long-term exclosures experiment.

| Spring | pairs | | | F.Model | R2 | p.value | p.adjusted |
|---|-------------|----|-------------|---------|-------|---------|------------|
| A. Fire treatments in combined fire and grazing experiment | | | | | | | |
| 2016 | Control | vs | Winter fire | 3.777 | 0.100 | 0.001 | 0.003 |
| 2017 | Summer fire | vs | Control | 2.992 | 0.089 | 0.005 | 0.021 |
| | Summer fire | vs | Winter fire | 0.183 | 0.005 | 0.993 | 1.000 |
| | Control | vs | Winter fire | 2.633 | 0.071 | 0.022 | 0.066 |
| B. Fire treatments in long-term exclosures experiment | | | | | | | |
| 2016 | Control | vs | Winter fire | 2.956 | 0.118 | 0.005 | 0.017 |
| 2017 | Summer fire | vs | Control | 1.998 | 0.129 | 0.022 | 0.067 |
| | Summer fire | vs | Winter fire | 0.193 | 0.009 | 0.998 | 1.000 NS |
| | Control | vs | Winter fire | 2.431 | 0.066 | 0.026 | 0.071 NS |

Appendix S7: Effects of fire and grazing treatments on plant species most benefitting from fire (mean \pm SE) in the combined fire and grazing experiment (left) and the fire experiment in long-term exclosures (right) one year after fire treatments. A, B: *Medicago monspeliaca*, C, D: *Euphorbia falcata*, E, F: *Poa bulbosa*, G, H: *Bromus rubens*.



Transition to Chapter IV



In Chapter III, we showed that fire had a positive effect on vegetative regeneration and reproduction of *B. retusum* even though its recovery is lower than community average. Moreover, it has a positive effect on species richness and diversity of the associated community. Short-term grazing exclosure (2 years) had no effect on *B. retusum* but long-term abandonment (17 years) reduced relative *B. retusum* cover and resulted in a community shift towards ruderal perennials.

In Chapter IV, we tested *in situ* germination and seedling establishment to evaluate colonisation capacity in the field. In Mediterranean dry grasslands, the limiting factors may be water availability and grazing although grazing is required to maintain the system with its dominant plant species and a higher soil moisture favour other species not adapted to summer drought. We hypothesize that initial watering favours germination and seedling recruitment whereas early grazing has a negative effect.

Contribution of the thesis

- Knowledge on environmental factors limiting germination and early establishment: water availability and grazing.
- Information on general *in situ* colonisation capacity
- Recommendations for conservation management and plant re-introduction.

Chapter IV

Which factors limit early establishment of *Brachypodium retusum* a dominant species of Mediterranean grasslands?

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Abstract

In Mediterranean dry grasslands, water availability and grazing regimes are known to be limiting factors for plant species establishment. However, their joint effect on species establishment have received little attention especially on *Brachypodium retusum*, the dominant perennial grass species of Mediterranean dry grasslands which populations are particularly impacted by land use changes.

In our study, we aimed to analyse the effects of grazing and watering on seedling recruitment and early establishment of *B. retusum* in a former cultivated field located in “La Crau”, a vast steppe vegetation area in Southern France, for improving the re-establishment of this species in future ecological restoration projects.

A split-plot experiment was set up in order to test the effects of grazing and initial watering on *in situ* seeds germination. Grazing and initial watering effects have also been tested on seedling survival, seedling height, leaf length, tiller number, leaf number and seedling diameter for (i) transplanted seedlings and (ii) sown seedlings.

Grazing negatively affected germination rates whereas watering effect was not significant. At the end of the survey period, survival was 49 % for transplanted seedlings and 36 % for sown seedlings after two grazing seasons. One original significant interaction occurred between grazing and watering. Initial watering had a positive effect in grazed plots compensating for recurrent grazing “perturbations”. Grazing also significantly reduced plant height, leaf number and length but these effects disappeared six months after the end of the grazing period. Nevertheless, grazing also significantly increased tillering while watering had an expected significant positive effect on plant growth. These results are then discussed for restoring *B. retusum* populations in former arable fields. Sowing in ungrazed fenced areas instead of watering and transplanting seedlings in grazed areas could be then advised and would allow to reduce the economic cost and environmental impacts of such operations.

Keywords: Dry grassland; Plant-environment interactions; Poaceae; Population dynamics; Thero-Brachypodietea

INTRODUCTION

European semi-natural grasslands are under constant decline and belong to the most threatened habitats today (Poschlod & WallisDeVries, 2002; Pywell et al., 2007). Grasslands have been frequently converted to arable fields because improved drainage and irrigation techniques allow nowadays crop production at sites previously limited to extensive grassland use (Bischoff, Warthemann, & Klotz, 2009; Tatin, Wolff, Boutin, Colliot, & Dutoit, 2013). Under Mediterranean climate, strong summer drought limits standing biomass, and the combination of grazing and climate has resulted in particularly species-rich grasslands also called pseudo-steppes (Blondel, Aronson, Bodiou, & Boeuf, 2010; San Miguel, 2008). Ecological restoration of Mediterranean grasslands based on spontaneous succession is a slow process. In particular, characteristic species that are limited to this type of grasslands slowly establish or even remain absent (Blondel & Aronson, 1999; Coiffait-Gombault, Buisson, & Dutoit, 2012; Römermann, Dutoit, Poschlod, & Buisson, 2005).

Water availability is well-known to be a limiting factor of plant establishment (Noy-Meir, 1973; Ronnenberg, Hensen, & Wesche, 2011). Drought stress reduces germination and increases seedling mortality (Bochet, García-Fayos, Alborch, & Tormo, 2007; Thomas, Morris, Auld, & Haigh, 2010). Our test species *Brachypodium retusum* showed relative high germination under drought but still germination increased with higher soil moisture (Vidaller, Dutoit, Ibrahim, Hanslin, & Bischoff, 2018). However, an increase in water availability also increases competition for light and other resources (Kadmon, 1995). In our study area, higher soil moisture favoured undesired ruderal species such as the commonly high-growing grass *Brachypodium phoenicoides* that outcompete typical pseudo-steppe species (Masson, Mesléard, & Dutoit, 2014).

Mediterranean dry grasslands depend on human land management, in particular on grazing (San Miguel, 2008; Apostolova, Dengler, Di Pietro, Gavilán, & Tsiripidis, 2014). Land abandonment results in shrub encroachment which is apart from land use intensification the major threat to this type of ecosystem (San Miguel, 2008). Grazing was expected to have positive but less strong effects on plant diversity of the steppe community. Grazing animals remove biomass reducing above-ground competition and create gaps by trampling both shaping plant communities and favouring the co-existence of different species (Bullock, Pywell, Burke, & Walker, 2001; Coiffait-Gombault et al., 2012; Drobnik, Römermann, Bernhardt-Römermann, & Poschlod, 2011). Thus, grazing may have positive effects on seedling establishment (Bullock et al., 2001). However, grazing is also known to increase seedling mortality hampering

establishment (Buisson, Corcket, & Dutoit, 2015). Although showing a relatively low palatability, young seedlings of our study species *B. retusum* are negatively affected by grazing (Buisson et al., 2015; Vidaller et al., 2018).

Brachypodium retusum is the dominant perennial grass species of dry grasslands in the Western Mediterranean basin but also occurs in shrublands (matorral, garrigue) and open woodlands (Catalán, López-Álvarez, Díaz-Pérez, Sancho, & López-Herránz, 2015; Caturla, Raventós, Guàrdia, & Vallejo, 2000). The species is still abundant in the Mediterranean but *B. retusum* grasslands (Thero-Brachypodietea) are under decline and protected by the EU habitats directive (San Miguel, 2008). Due to its high cover and numerous interactions with other species of the community (Saiz & Alados, 2011), successful restoration of plant community structure of such grasslands depends on the re-establishment of *B. retusum*. In our study area, the “La Crau” nature reserve in South-Eastern France, *B. retusum* was not able to re-colonise restoration sites within thirty years although pristine dry grasslands with a high population density of the species occur adjacent to these sites (Coiffait-Gombault et al., 2012). Apart from low seed production (Vidaller et al., 2018) and dispersal limitation (Bischoff, 2000), conditions of establishment after arrival of seeds may represent a strong environmental filter (Nuttall, 2007; O’Connor, 1991; Pywell et al., 2007). We hypothesize that water availability and grazing are key factors limiting seedling establishment. We further hypothesize that initial watering has a positive effect whereas initial grazing has a negative effect on seedling recruitment. We were particularly interested in the interaction of both factors since the joint effect of water and grazing stress on seedling establishment has rarely been tested so far (Ibáñez & Schupp, 2001; Soliveres et al., 2011).

We present results of a full-factorial field experiment manipulating water availability and grazing on an ex-arable field converted to traditional dry grassland management in 1985 (Römermann et al., 2005; Buisson, Dutoit, Torre, Römermann, & Poschlod, 2006). Since germination and initial seedling growth are particularly critical steps in plant establishment (Bochet et al., 2007; Thomas et al., 2010), we tested effects on seedlings grown from field-sown seeds and on seedlings pre-grown under greenhouse conditions. We specifically asked the following questions: (1) What is the effect of initial watering and grazing on *B. retusum* field germination? (2) What is the effect of these factors on survival and growth of sown and transplanted *B. retusum* seedlings? (3) Does the effect of watering depend on grazing?

MATERIALS AND METHODS

Study area

The study site is part of the “La Crau” Nature reserve in South-Eastern France. The area is characterized by hot/dry summers and mild/humid winters with an annual precipitation of 517 mm (1980-2010) and a mean temperature of 15.3 °C (Figure S1). The soils of the study area are decalcified Red Mediterranean Soils (Haplic cambisol) with a mean pH of 6.8. The sites are grazed by itinerant sheep from February to June.

The experiment was set up on a restoration site in “Peau de Meau” (43°34'09N / 4°49'55E; Figure S2). The site was managed as an arable field between 1960 and 1985 to grow melon and cereal crops (Buisson et al., 2006). Afterwards, the site has been traditionally grazed as the surrounding pristine grasslands. These surrounding reference sites belong to the Mediterranean Thero-Brachypodietea habitat “Pseudo-steppe with grasses and annuals” (EUNIS habitat E1.311, Natura 2000 habitat type 6220, San Miguel, 2008). Although management was the same as on reference site *B. retusum* has not re-occurred on the restoration site since 1985.

Brachypodium retusum (Pers.) P. Beauv., (ramose false brome, Poaceae), is the dominant species of this habitat in our study area. The rhizomes of this perennial grass form a dense network close to the soil surface allowing resprouting after destruction of above-ground organs (Caturla et al., 2000). The species shows tillering at the stem base but also lateral branching at upper stem parts. Clonal growth is common but slow. *B. retusum* usually flowers from April to July and seeds germinate after autumn rainfalls or during early spring when humidity is sufficient (Vidaller et al., 2018).

Experimental design

In autumn 2016, we set up a split-plot experiment testing the effects of grazing, initial watering and its interaction on germination rate, survival, seedling height, leaf length, tiller number, leaf number and seedling diameter for (i) transplanted seedlings and (ii) sown seedlings (Figure 1). Blocks were split into two parts (half-blocks) of which one was fenced to prevent sheep grazing. The two watering treatments (watering, no watering), were applied to two plots of 9 m² within each half-block (split-plot design) in order to test combined effects of watering and grazing (20 plots in total). A distance of 40 m between half-blocks within blocks was chosen to avoid any influence of enclosures on grazed plots since grazing is often much stronger close to enclosures (Saatkamp,

Henry, & Dutoit, 2018). The position of water treatments was randomized within half-blocks and that of grazing treatment was randomized within blocks.

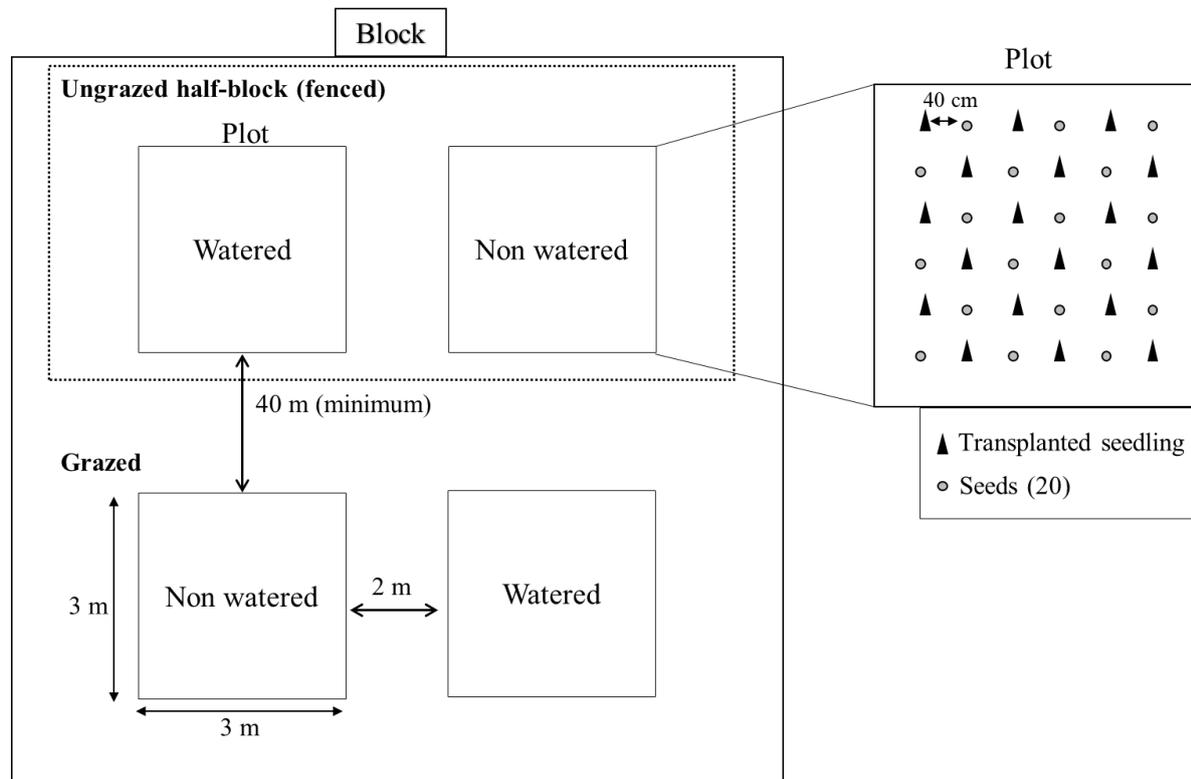


Figure 1: Experimental design of the experiment. The figure shows one out of five experimental blocks.

Local seeds collected in the previous year were used in the experiment. In each plot, 18 seedlings previously germinated and grown for 2 months in peat plugs (4 cm x 4 cm) in a growth chamber (temperature 15/20 °C, 12/12h, night/day) were transplanted in October 2016. At the same time, 20 seeds were sown to each of 18 pockets between transplanted seedlings. Transplanted seedlings and one emerging seedling per pocket were marked for continuous survival and growth measurements.

In autumn 2016 (late October to early December) and in spring 2017 (early April to early July), plots of the initial watering treatment were irrigated every seven to fourteen days in order to add 60 l/m² of water per month. Thus irrigation doubled the average monthly precipitation during this period. The irrigation treatment was interrupted in winter and early spring since soils were water saturated. The grazing enclosures was realised using fences with stretched, non-electrified nets (1 m high).

Data collection

Field final germination was measured by counting the number of emerging coleoptiles in May 2017. Growth and survival were measured at the end of autumn, after the summer drought and the autumnal rainy season to be sure to identify the living individuals from the dead ones (December 2016 and 2017), during the grazing period (May 2017 and 2018) and after the end of the grazing period (July 2017). The growth measurements included plant height, diameter, tiller number, leaf number and the length of the longest leaf to characterise plant performance (Vidaller et al., 2018).

Data analysis

Linear models were run to analyse the effects of watering (yes/no), grazing (yes/no) and planting type (field sown/greenhouse sown). The full model included the three factors, all two-way interactions, the three-way interaction watering x grazing x planting type and block. In order to consider the split-plot design of the experiment grazing was tested against the grazing x block interaction. Watering and the watering x grazing interaction were tested against the grazing x watering x block interaction. Finally, planting type, block and all interactions with planting type were tested against the model residuals. A repeated measures analysis based on this model was calculated for survival since more data points were available than for growth traits. A reduced model only including grazing, watering, their interaction and block was applied to germination data. As in the full model grazing was tested against the grazing x block interaction. Watering, the watering x grazing interaction and block were, however, tested against the model residuals. When necessary $\log_{10}(x+1)$ and square root transformations were used for count and proportion data, respectively, to comply with assumptions of linear models (normality, homoscedasticity). A Tukey HSD posthoc test was calculated to analyze differences between treatment levels if treatment main effects or interactions were significant.

For growth traits, the effect size of grazing, watering, planting type, and of their interactions was calculated as $100 * \text{sum of square} / \text{total sum of squares}$. All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)).

RESULTS

Field germination ranged from 7.1 % (unwatered and grazed plots) to 12.9 % (watered and ungrazed plots). Grazing had a significant negative impact on germination (Table 1, Fig. 2) as *B. retusum* seeds from ungrazed plots germinated significantly better than those from grazed plots. The effect of watering and the grazing × watering interaction were not significant.

Table 1: Effects of initial watering and grazing on *B. retusum* germination and growth (July 2017 and May 2018) for transplanted seedlings and sown seedlings. T×W= type x watering interaction, T×G= type x grazing interaction, W×G= watering × grazing interaction, T×W×G= type × watering × grazing interaction. ANOVA *F*-values, significance levels: . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant. Factor effect: ↓ (decrease) ↑ (increase).

| Date | Factors | df | Germination (semis) | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter |
|------|----------|----|------------------------|-------------|--------------|--------------|------------------|---------------------------|-----------------------------|-------------------|
| 2017 | Type | 1 | - | 4.650 . | 10.650 ** | 33.326 *** | 34.278 *** | 7.417 * | 39.259 *** | 126.101 *** |
| | Watering | 1 | 1.106 NS | 0.607 NS | 0.020 NS | 1.498 NS | 4.649 * ↑ | 1.345 NS | 0.681 NS | 7.098 ** ↑ |
| | Grazing | 1 | 10.540 * ↓ | 0.313 NS | 3.821 . ↓ | 10.557 ** ↓ | 16.291 ** ↑ | 0.573 NS | 0.465 NS | 13.222 ** ↑ |
| | T×W | 1 | - | 0.257 NS | 0.005 NS | 0.065 NS | 2.381 NS | 0.051 NS | 0.959 NS | 0.246 NS |
| | T×G | 1 | - | 0.977 NS | 1.574 NS | 1.278 NS | 0.023 NS | 1.141 NS | 0.484 NS | 2.181 NS |
| | W×G | 1 | 0.478 NS | 0.006 NS | 0.542 NS | 0.713 NS | 0.207 NS | 0.588 NS | 0.032 NS | 1.062 NS |
| | T×W×G | 1 | - | 5.523 * | 3.804 . | 11.354 ** | 7.116 * | 5.733 * | 10.157 ** | 0.153 NS |
| 2018 | Type | 1 | - | 1.605 NS | 12.836 * | 24.480 *** | 7.387 * | 7.183 * | 18.079 ** | 20.008 *** |
| | Watering | 1 | - | 0.430 NS | 0.584 NS | 0.002 NS | 0.289 NS | 1.293 NS | 0.273 NS | 0.887 NS |
| | Grazing | 1 | - | 2.577 NS | 19.594 *** ↓ | 28.501 *** ↓ | 1.506 NS | 11.100 ** ↓ | 12.621 *** ↓ | 1.482 NS |
| | T×W | 1 | - | 0.175 NS | 0.813 NS | 0.063 NS | 0.002 NS | 0.687 NS | 0.002 NS | 0.303 NS |
| | T×G | 1 | - | 1.157 NS | 0.298 NS | 0.041 NS | 1.034 NS | 1.233 NS | 0.696 NS | 0.789 NS |
| | W×G | 1 | - | 0.076 NS | 0.864 NS | 1.068 NS | 1.103 NS | 0.207 NS | 1.611 NS | 0.014 NS |
| | T×W×G | 1 | - | 2.241 NS | 0.537 NS | 0.144 NS | 0.180 NS | 1.308 NS | 0.042 NS | 0.291 NS |

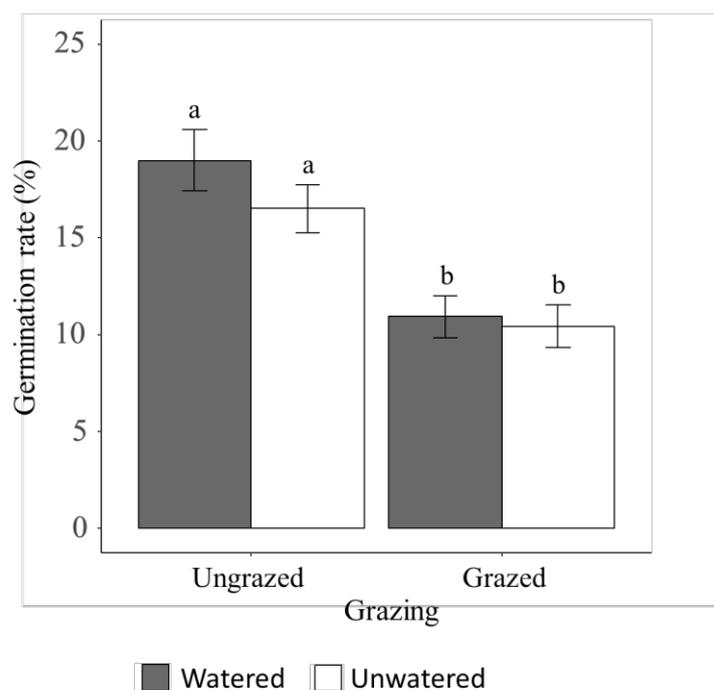


Figure 2: Effects of initial watering and grazing on final germination of *Brachypodium retusum* (mean values \pm SE).

Sown seedlings showed significantly lower survival rates than planted seedlings, an effect that became stronger during the study period (Table A.1, Fig. 3). Watering had no significant effect on survival rates whereas grazing had a significantly negative effect. A significant grazing x watering interaction indicated that the watering effect depended on grazing. A positive effect of watering was only apparent in grazed plots. The significant grazing x watering x date interaction showed that this grazing-dependent watering effect also changed with time (Table A.1). In December 2017 and May 2018, watering had a significantly negative effect on survival in ungrazed plots.

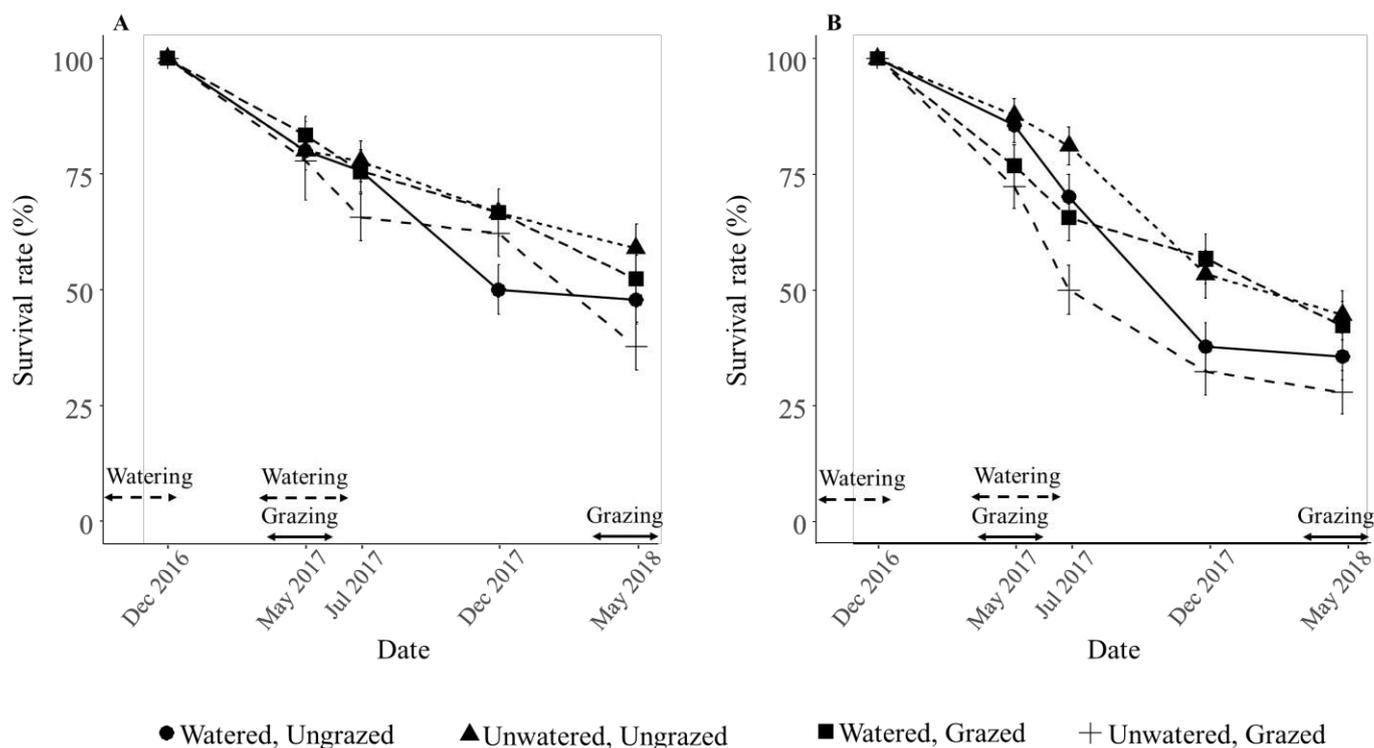
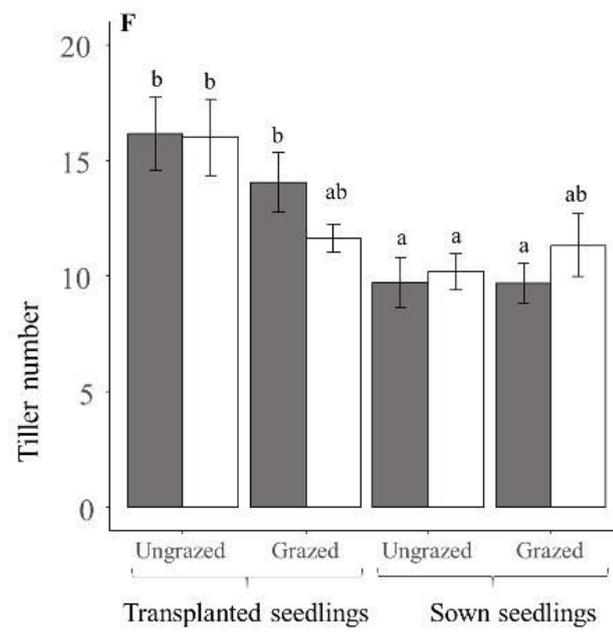
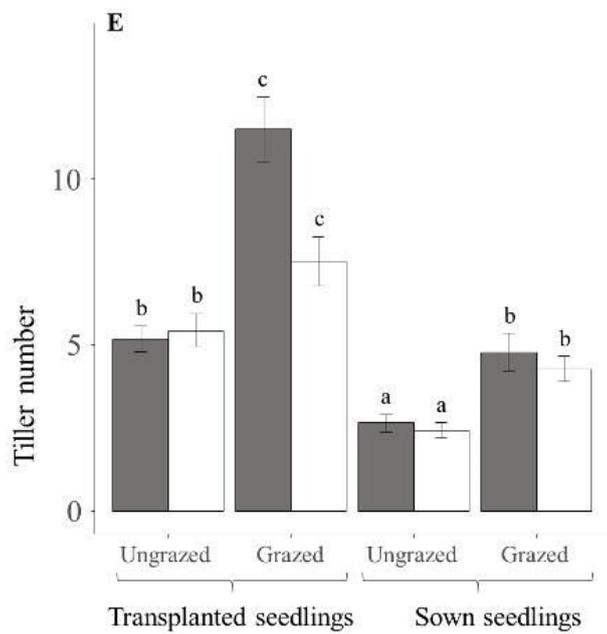
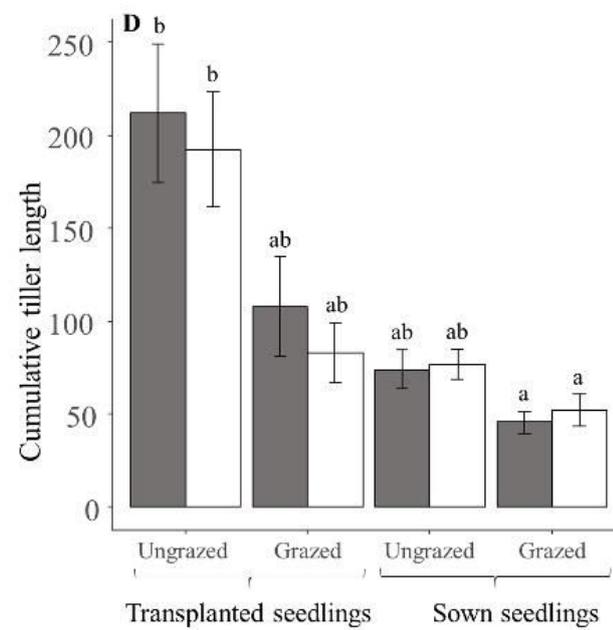
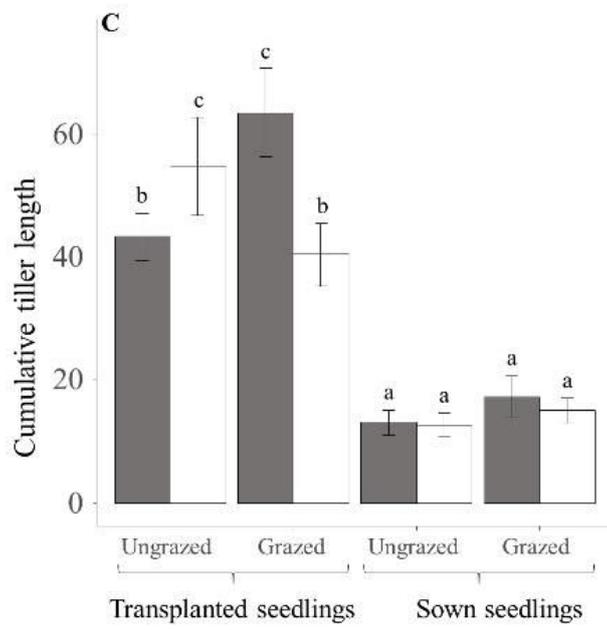
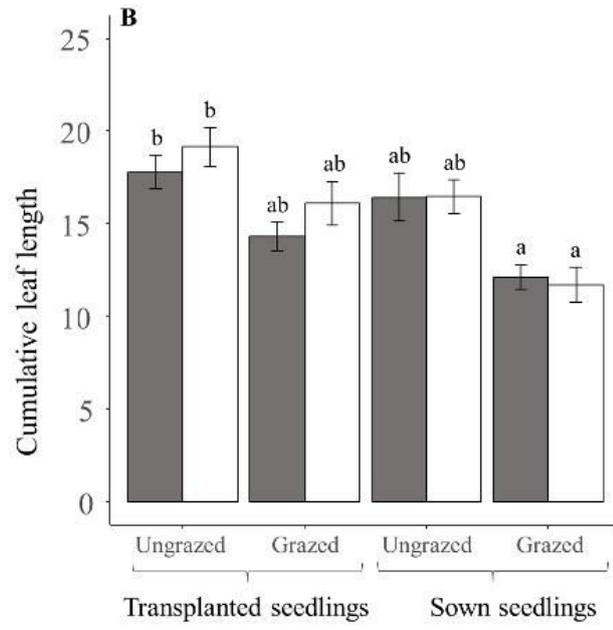
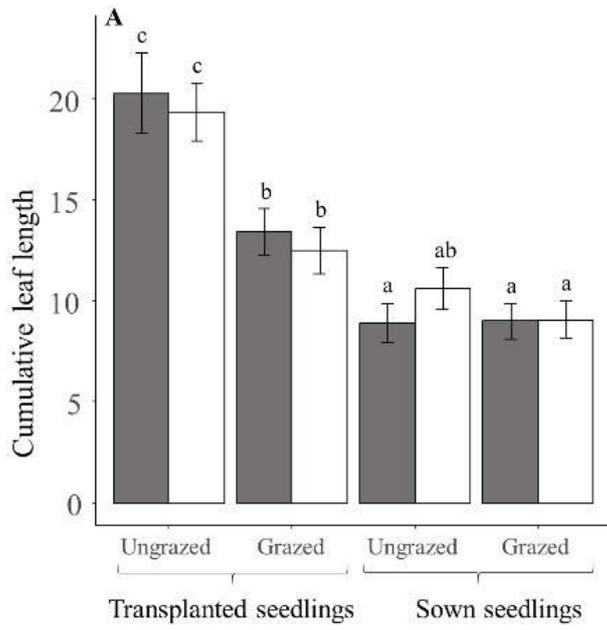


Figure 3: Effects of initial watering and grazing on survival rate of *B. retusum* transplanted seedlings (A) and sown seedlings (B) (mean values \pm SE).

The watering effect was not significant at the end of the study period (Table 1, Fig. 1). However, watering significantly increased tiller number, cumulative tiller length and plant diameter in at least two of the previous three observation dates (Table 1; Table A.2). Grazing significantly reduced leaf length, plant height, cumulative leaf length and cumulative tiller length (Table 1, Fig. 4). Nevertheless, it increased significantly tiller number and plant diameter (Table S1, Fig. 4). However, a high recovery was measured after the grazing period 2017 resulting in non-significant grazing effects on all measured variables in December 2017 (Table A.2). Significant interactions (watering \times grazing and watering \times grazing \times seedling type) were only found at the earliest dates in May and July 2017 and disappeared later on. Thus the growth measurements do not confirm the grazing-dependent increase in survival rates



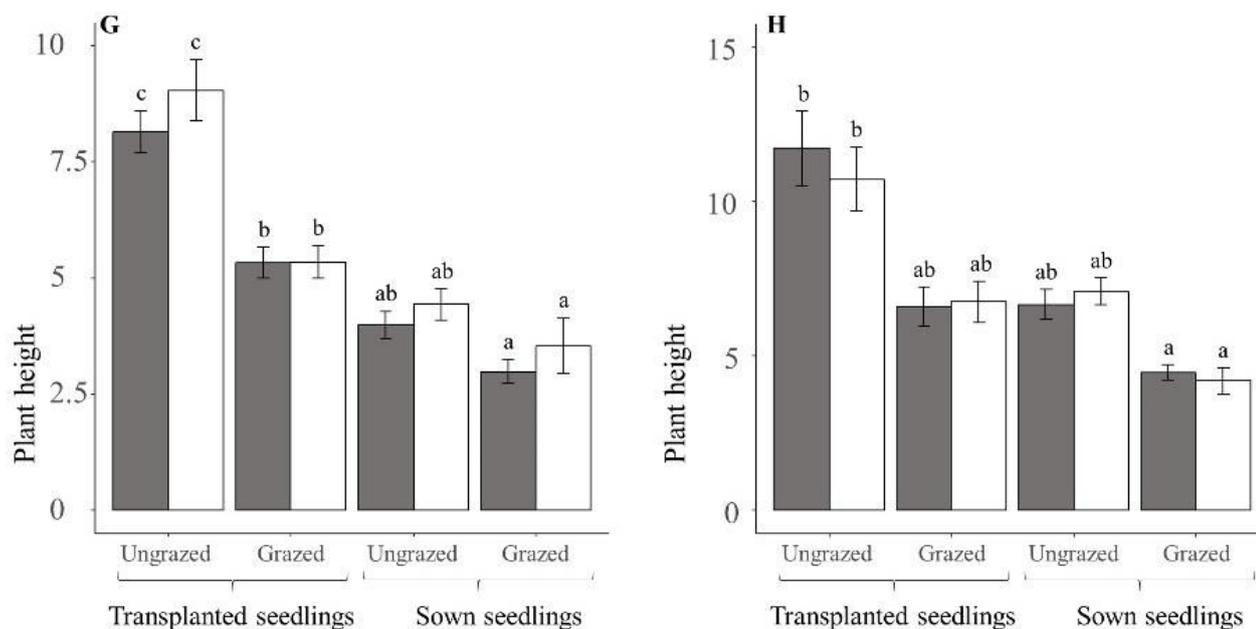


Figure 4: Effects of initial watering and grazing on sown and planted *B. retusum* seedlings growth (mean values \pm SE) at the end of the first grazing period (left side of the panel, July 2017) and at the end of the study period (right side of the panel, May 2018). A, B: Cumulative leaf length; C, D: Cumulative tiller length; E, F: Tiller number; G, H: Plant height. Different lower case letters indicate significant differences ($P < 0.05$).

At the end of the study period, in May 2018, only a seedling type and grazing factors were significant (Table 1, Fig. 4). Transplanted seedling still showed a higher growth than sown seedlings except for leaf number. *B. retusum* for grazing plots show lower leaf length, plant height, cumulative leaf length and cumulative tiller length (Table 1, Fig. 4).

Effect size of watering was always lower than that of grazing (Figure 6). In July 2017, watering explained between 0.004 % for leaf length and 1.43 % for plant diameter. Grazing explained from 0.73 % for cumulative tiller length to 21.78% for tiller number. The site x soil interaction explained 0.02 % for leaf number and 2.13 % of the model variation for cumulative leaf length. In May 2018, watering explained between 0.001 % for plant height and 1.43 % for cumulative leaf length. Grazing explained from 4.23 % for plant diameter to 40.85 % for plant height. The site x soil interaction explained 0.04 % for plant diameter and 6.94 % of the model variation for tiller number.

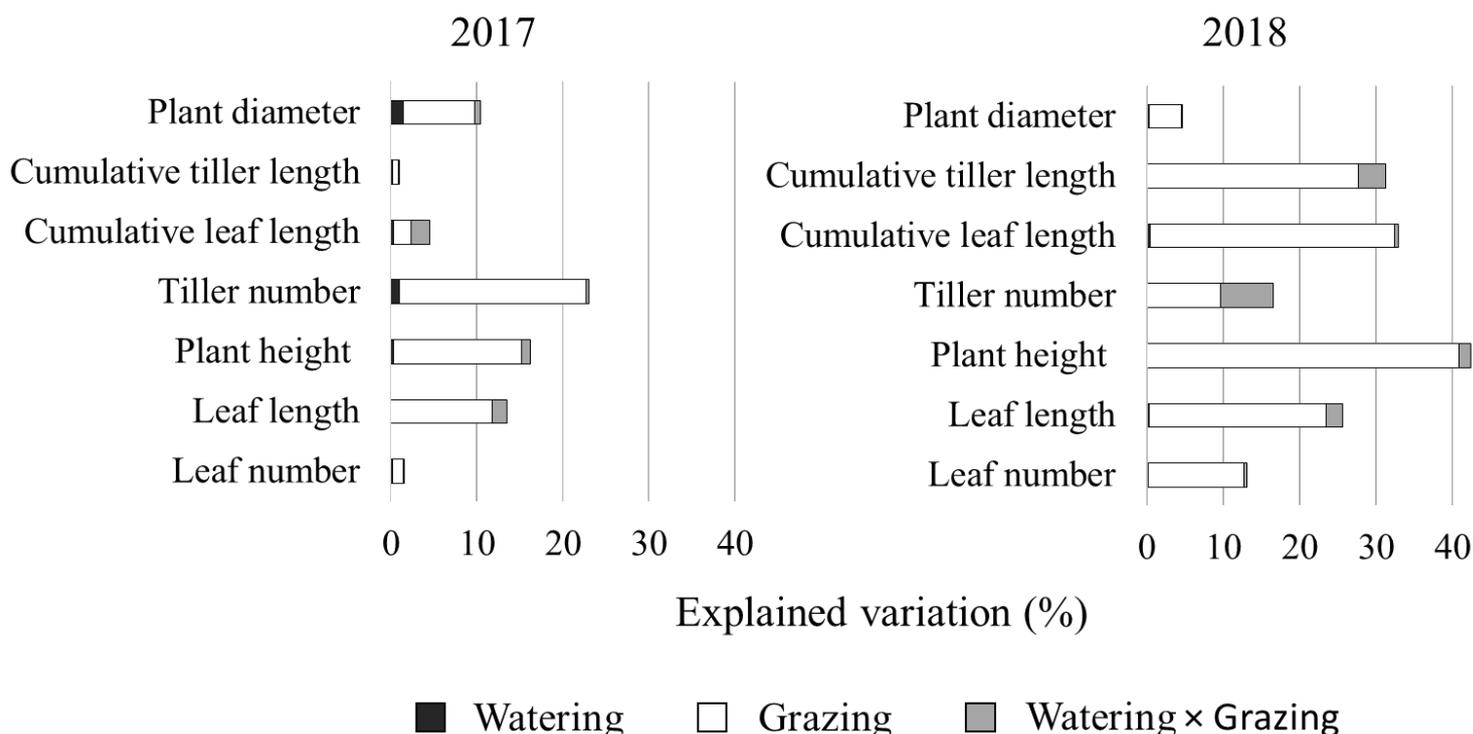


Figure 6: Percentage of variation in plant growth explained by watering, grazing and their interaction (July 2017 and May 2018).

DISCUSSION

Field germination

In our study, watering had no significant effect on the germination of *Brachypodium retusum*. The result confirmed a previous growth chamber experiment showing that already at low to moderate moisture levels the response to water addition is not significant (Vidaller et al., 2018). However, it does not correspond to other growth chamber studies in dry or Mediterranean ecosystems showing a very sensitive germination response to soil moisture (Bochet et al. 2007; Thomas et al. 2010). Additionally, pre-emergence mortality increases with drought resulting in a stronger drought effect on seedling emergence than on germination (Tobe et al. 2005). Our field germination data correspond to seedling emergence since we did not analyse germination in the soil. The typical Mediterranean autumn and early-spring rainfall may have been sufficient to avoid seedling mortality in the soil explaining the absence of watering effects on field germination in our study.

Grazing had a negative effect on field germination. Gap creation by trampling livestock increases field germination (Bullock et al. 2001) but trampling, urine and faeces excretion may

have the opposite effect (Bilotta, Brazier, & Haygarth, 2007). Trampling is known to damage seeds or emerging coleoptiles (James, Svejcar, & Rinella, 2011; Rother, Jordano, Rodrigues, & Pizo, 2013). Dung pads may reduce light required by many seeds to germinate (Milotić & Hoffmann, 2016). Furthermore, grazing sheep may have damaged seedlings before recording germination in our study.

Survival and growth

The main watering effect on seedling survival was not significant neither in field sown seedlings nor in seedling pregrown in a greenhouse confirming that the species is well adapted to drought (Vidaller et al., 2018). *Brachypodium retusum* is able to store water in rhizomes and respond fast to rainfall (Clary, Savé, Biel, & Herralde, 2004). However, the watering effect depended on grazing and the watering \times grazing interaction was also significantly affected by observation date. Watering had a positive effect in grazed but not in ungrazed plots. This effect was stronger during and after the grazing season representing a particularly stressful period for seedlings. Watering allowed a higher resilience to grazing whereas watering may increase competition to ruderal species when not stressed by grazing. In similar Mediterranean dry grasslands, higher soil moisture favoured ruderal species such as the high-growing grass *Brachypodium phoenicoides* outcompeting typical pseudo-steppe species (Masson et al., 2014). Water addition to ungrazed plots may also increase soil pathogen infestation reducing plant performance and influencing interspecific competition (Van der Putten & Peters, 1997; Van der Putten, Vet, Harvey, & Wäckers, 2001).

Contrary to germination and survival, watering treatment had a significantly positive effect on growth and the effect varied among seasons. In ecosystems with seasonal aridity, water is a limiting factor for growth (Noy-Meir, 1973) mainly due to a trade-off between drought tolerance and growth capacity (Pérez-Ramos, Volaire, Fattet, Blanchard, & Roumet, 2013). Very young *B. retusum* plants allocate resources to tillering and leaf growth. Later, tillers elongate and older leaves die (Vidaller et al., 2018). Watering increased leaf growth and tillering in the beginning and tiller growth up to seven months after treatment stop.

Grazing had a negative effect on *B. retusum* survival, in particular in the beginning of the study period. Grazing is known to increase early seedling mortality hampering establishment of the species (Buisson et al., 2015). Although palatability is low compared with dicots and several other grasses, young seedlings are still attractive to sheep (Buisson et al., 2015) and play an

important role in sheep nutrition (Meuret et al. 2013). Apart from biomass removal by grazing sheep, trampling, urine and faeces excretion may also reduce survival as already detailed in the germination section (Bilotta, Brazier, & Haygarth, 2007). Negative grazing effects on survival disappeared six months after the end of the grazing season confirming a high resilience of *B. retusum* to above-ground disturbance (Caturla et al., 2000; De Luis, Raventós, Cortina, González-Hidalgo, & Sánchez, 2004). Additionally, negative grazing effects were mitigated by watering resulting in a significant grazing x watering interaction as discussed above.

Grazing significantly reduced leaf growth and plant height but increased tillering and plant diameter after the first grazing period. These significantly positive grazing effects disappeared at the end of the study period. Reduced growth of defoliated grasses caused by the loss of photosynthetic active tissues has been widely observed (Ferraro & Oesterheld, 2002; Van Staalduinen & Anten, 2005). However, different compensatory growth mechanisms may limit the damage such as stimulating photosynthesis, removing old and dead tissues, changed resource allocation and tiller production (McNaughton, 1983; Oesterheld & McNaughton, 1988, 1988; Zhao et al., 2008). Six months after the end of the first grazing period, grazing effects were not significant any more confirming again a high resilience to above ground perturbations (Caturla et al., 2000).

As expected, seedlings pre-grown in a greenhouse established better than field-germinated seedlings as it was demonstrated by other studies (Godefroid et al., 2011; Menges, 2008). Greenhouse grown seedlings showed already a well-developed root and rhizome network that was transplanted in peat plugs. Rhizome establishment in *B. retusum* is a key factor in resistance and tolerance to environmental stress (Caturla et al., 2000). A lower mortality of established seedling is, however, also apparent in other species resulting in a mortality decrease with size and age of seedlings (Gilbert, Harms, Hamill, & Hubbell, 2001; Quintana-Ascencio, Ramírez-Marcial, González-Espinosa, & Martínez-Icó, 2004). Although significant, the magnitude of the seedling type effect on survival was relatively low (10%) thus not strongly advocating expensive pre-growth and transplanting. Differences in growth were much higher than differences in survival. In particular, plant height and diameter were greater in pre-grown seedlings even two seasons after planting. The large differences in tiller number and cumulative tiller length in the beginning became, however, much smaller at the end of the experiment suggesting a catch-up in these growth traits. The response of pre-grown and field-germinated seeds to the water and grazing treatments was similar resulting in non-significant interactions.

CONCLUSIONS

Initial watering had a positive effect on early seedling establishment but not on germination indicating that in Mediterranean dry grasslands, the second step of seedling recruitment is limited by water availability. However, the watering effect disappeared after two growing seasons except for a significantly higher survival in grazed and watered plots. Grazing had a generally negative effect on field germination and seedling establishment during the first two years. In ecological restoration, initial fencing (at least two years) increases thus germination, survival and growth of introduced populations (Buisson et al., 2015). Fencing may also be sufficient to avoid water limitation and the need to irrigate grazed plots. Alternatively, watering may help to mitigate grazing stress if fencing is not possible. Seedlings pre-grown under optimum conditions established better than field-sown seedlings. However, the effect magnitude was relatively low for seedling survival, and seedling recruitment of field-sown seed was higher than expected from previous studies showing a very low re-colonisation of restored sites with adjacent source populations (Coiffait-Gombault 2012, Helm et al. in press). Further observation is required to evaluate whether a long-term fitness advantage justifies the higher costs of pre-growth and planting compared with simple sowing.

ACKNOWLEDGEMENTS

We thank Olivier Blight, Lucie-Valentine Dupont, Perrine Lagarde and Lorenzo Ottaviani for field assistance. We also thank Fanny Sauguet for identifying field plots and the sheep farmers Mr and Ms Groulet, the Federal State Nature Conservation Authority (CEN-PACA) and Crau Nature Reserve for the permission to set up the experiment. The study was funded by the French Federal State PACA (PhD grant).

SUPPORTING INFORMATION

Figure S1: Monthly rainfall and average temperatures for the study period (2016-2018). Data from the nearest meteorological station in Saint-Martin de Crau). Treatment periods are indicated underneath.

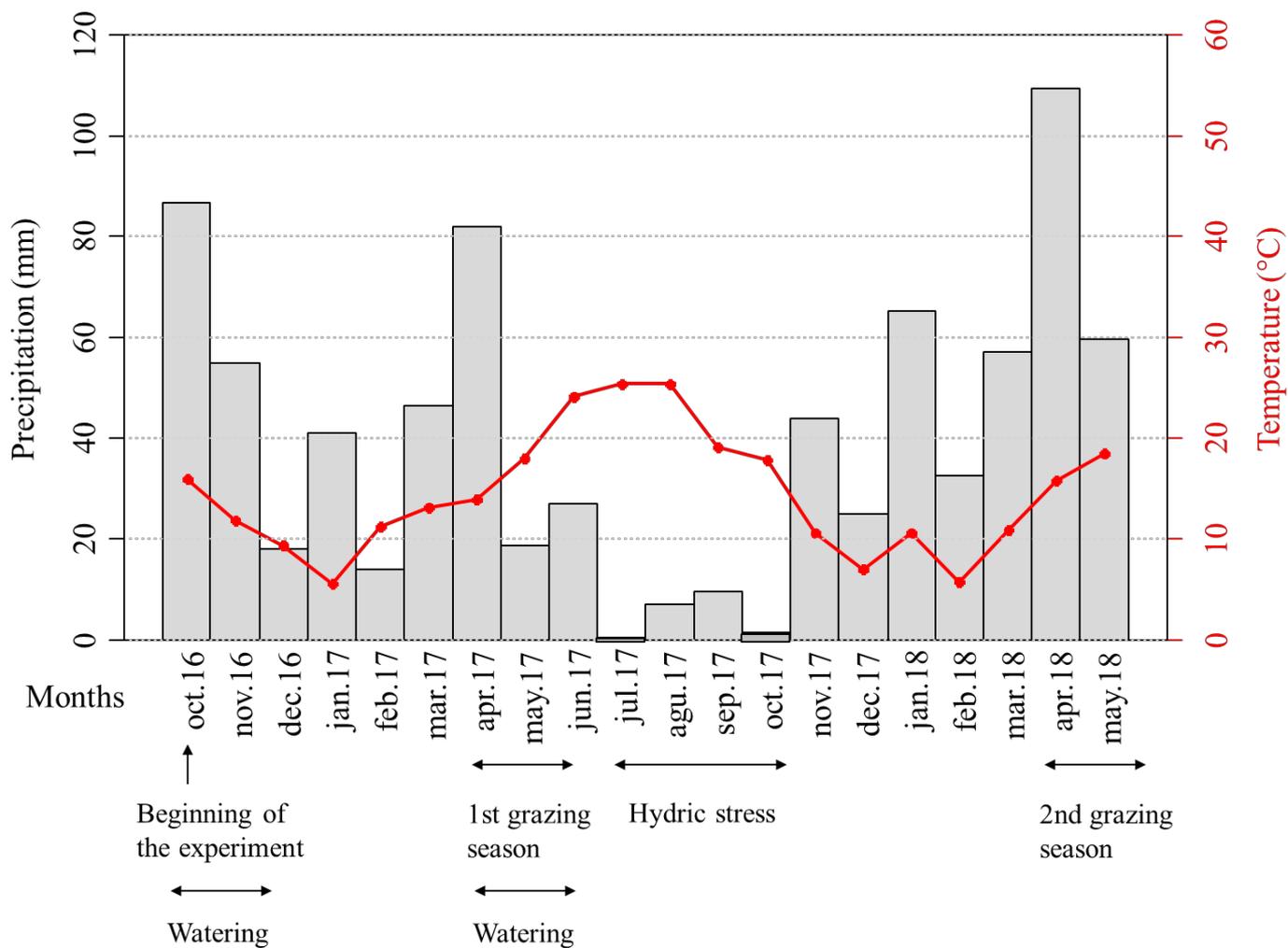


Figure S2: Satellite image of the study area (May 2017). Darker spots correspond to the grazing exlosures.



Table S1: Effects of initial watering and grazing on *B. retusum* germination and growth for transplanted seedlings and sown seedlings. T×W= seedling type x watering interaction, T×G= seedling type x grazing interaction, W×G= watering × grazing interaction, T×W×G= seedling type × watering × grazing interaction. ANOVA *F*-values, significance levels: . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant. Factor effect: ↓ (decrease) ↑ (increase).

| Date | Factors | df | Germination (semis) | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter |
|---------|----------|----|------------------------|--------------|-------------|--------------|------------------|---------------------------|-----------------------------|-------------------|
| May2017 | Type | 1 | - | 101.467 *** | 35.337 *** | 38.501 *** | 86.244 *** | 63.465 *** | 66.731 *** | 134.109 *** |
| | Watering | 1 | 1.106 NS | 2.871 . ↑ | 0.075 NS | 0.990 NS | 3.188 . ↑ | 0.715 NS | 3.548 * ↑ | 2.368 NS |
| | Grazing | 1 | 10.540 * ↓ | 41.998 *** ↓ | 3.784 . ↓ | 4.204 . ↓ | 0.201 NS | 10.629 ** ↓ | 1.151 NS | 0.281 NS |
| | T×W | 1 | - | 2.532 NS | 0.205 NS | 1.203 NS | 0.279 NS | 1.252 NS | 0.784 NS | 0.357 NS |
| | T×G | 1 | - | 4.397 * | 0.841 NS | 0.424 NS | 0.001 NS | 0.635 NS | 0.179 NS | 0.012 NS |
| | W×G | 1 | 0.478 NS | 0.743 NS | 0.337 NS | 0.063 NS | 0.648 NS | 0.084 NS | 0.314 NS | 0.082 NS |
| | T×W×G | 1 | - | 1.568 NS | 2.707 NS | 0.248 NS | 7.236 * | 2.920 NS | 2.316 NS | 10.628 ** |
| July17 | Type | 1 | - | 4.650 . | 10.650 ** | 33.326 *** | 34.278 *** | 7.417 * | 39.259 *** | 126.101 *** |
| | Watering | 1 | - | 0.607 NS | 0.020 NS | 1.498 NS | 4.649 * ↑ | 1.345 NS | 0.681 NS | 7.098 ** ↑ |
| | Grazing | 1 | - | 0.313 NS | 3.821 . ↓ | 10.557 ** ↓ | 16.291 ** ↑ | 0.573 NS | 0.465 NS | 13.222 ** ↑ |
| | T×W | 1 | - | 0.257 NS | 0.005 NS | 0.065 NS | 2.381 NS | 0.051 NS | 0.959 NS | 0.246 NS |
| | T×G | 1 | - | 0.977 NS | 1.574 NS | 1.278 NS | 0.023 NS | 1.141 NS | 0.484 NS | 2.181 NS |
| | W×G | 1 | - | 0.006 NS | 0.542 NS | 0.713 NS | 0.207 NS | 0.588 NS | 0.032 NS | 1.062 NS |
| | T×W×G | 1 | - | 5.523 * | 3.804 . | 11.354 ** | 7.116 * | 5.733 * | 10.157 ** | 0.153 NS |

| Date | Factors | df | Germination (semis) | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter |
|---------|----------|----|------------------------|-------------|--------------|--------------|------------------|---------------------------|-----------------------------|-------------------|
| Dec2017 | Type | 1 | - | 8.247 * | 1.937 NS | 18.202 ** | 22.778 *** | 12.335 ** | 54.114 *** | 45.803 *** |
| | Watering | 1 | - | 0.979 NS | 0.057 NS | 0.159 NS | 5.512 * ↑ | 0.816 NS | 4.573 * ↑ | 4.655 * ↑ |
| | Grazing | 1 | - | 0.001 NS | 0.191 NS | 1.748 NS | 1.523 NS | 0.003 NS | 0.062 NS | 0.871 NS |
| | T×W | 1 | - | 0.038 NS | 0.115 NS | 1.425 NS | 0.023 NS | 0.676 NS | 1.255 NS | 0.746 NS |
| | T×G | 1 | - | 2.771 NS | 0.698 NS | 0.404 NS | 0.498 NS | 2.116 NS | 0.001 NS | 0.282 NS |
| | W×G | 1 | - | 1.997 NS | 0.676 NS | 2.302 NS | 4.305 * | 0.311 NS | 1.977 NS | 2.728 . |
| | T×W×G | 1 | - | 1.850 NS | 0.086 NS | 0.194 NS | 0.612 NS | 0.221 NS | 0.037 NS | 0.271 NS |
| May2018 | Type | 1 | - | 1.605 NS | 12.836 * | 24.480 *** | 7.387 * | 7.183 * | 18.079 ** | 20.008 *** |
| | Watering | 1 | - | 0.430 NS | 0.584 NS | 0.002 NS | 0.289 NS | 1.293 NS | 0.273 NS | 0.887 NS |
| | Grazing | 1 | - | 2.577 NS | 19.594 *** ↓ | 28.501 *** ↓ | 1.506 NS | 11.100 ** ↓ | 12.621 *** ↓ | 1.482 NS |
| | T×W | 1 | - | 0.175 NS | 0.813 NS | 0.063 NS | 0.002 NS | 0.687 NS | 0.002 NS | 0.303 NS |
| | T×G | 1 | - | 1.157 NS | 0.298 NS | 0.041 NS | 1.034 NS | 1.233 NS | 0.696 NS | 0.789 NS |
| | W×G | 1 | - | 0.076 NS | 0.864 NS | 1.068 NS | 1.103 NS | 0.207 NS | 1.611 NS | 0.014 NS |
| | T×W×G | 1 | - | 2.241 NS | 0.537 NS | 0.144 NS | 0.180 NS | 1.308 NS | 0.042 NS | 0.291 NS |

Table S2: Effects of initial watering, grazing, seedling type and observation date on *B. retusum* survival. ANOVA *F*-values, significance levels: . $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

| Factors | df | Survival rate |
|----------------------------------|----|---------------|
| Type | 1 | 7.889 ** |
| Watering | 1 | 0.850 NS |
| Grazing | 1 | 3.151 . |
| Date | 4 | 65.450 *** |
| Type × watering | 1 | 0.366 NS |
| Type × grazing | 1 | 1.313 NS |
| Watering × grazing | 1 | 31.211 *** |
| Type × date | 4 | 1.733 NS |
| Watering × date | 4 | 0.279 NS |
| Grazing × date | 4 | 1.072 NS |
| Type × watering × grazing | 1 | 1.368 NS |
| Type × watering × date | 4 | 0.679 NS |
| Type × grazing × date | 4 | 1.315 NS |
| Watering × grazing × date | 4 | 4.275 ** |
| Type × watering × grazing × date | 4 | 0.600 NS |

Chapter synthesis_ Transition to the general discussion

The thesis has focused on intrinsic, extrinsic factors and their interaction limiting the re-establishment of the common Mediterranean species *Brachypodium retusum*. In Chapter I, we showed that *B. retusum* Western Mediterranean populations exhibit a high neutral genetic diversity despite asexual reproduction indicating a high gene flow between populations. French populations showed a significant differentiation due to conjoint action of drift and selective adaptation related to mean July temperature and frost day number. Differentiation was also shown at local scale between populations originating from different soil types but its magnitude was much smaller than at regional scale between populations from the same soil type. In Chapter II, we focused on quantitative traits and confirmed this finding on the magnitude of local soil and regional climate/drift effects on differentiation. We further found differential population responses to stone cover and grazing manipulated in common garden experiments and to a much lesser degree to soil moisture tested in a growth chamber. In Chapter III, we studied environmental factors limiting adult *B. retusum* regeneration and reproduction. Grazing as the major management factor and fire occurring spontaneously but also used by shepherds in the past were tested in a full factorial experiment. *B. retusum* regenerated quickly after fire but surprisingly regeneration was not faster as community average. Accompanying annual species regenerated faster than *B. retusum*. Fire enhanced *B. retusum* reproduction. Fire had also a positive effect on the species-richness and diversity of the associated community. Short-term grazing enclosure was tolerated (2 years) but long-term abandonment (17 years) had a negative effect on *B. retusum* reproduction and community. The experiment on *in situ* germination and seedling establishment (Chapter IV) showed that colonisation capacity starting from seeds is also relatively high in the field. However, a longer observation period is required to finally evaluate the level of *in situ* colonisation. Watering had a positive effect on early growth and on final survival. The latter effect was, however, only observed in grazed plots indicating a beneficial watering effect in plants suffering from grazing stress. As hypothesized, early grazing had a negative effect on growth and survival.

General discussion

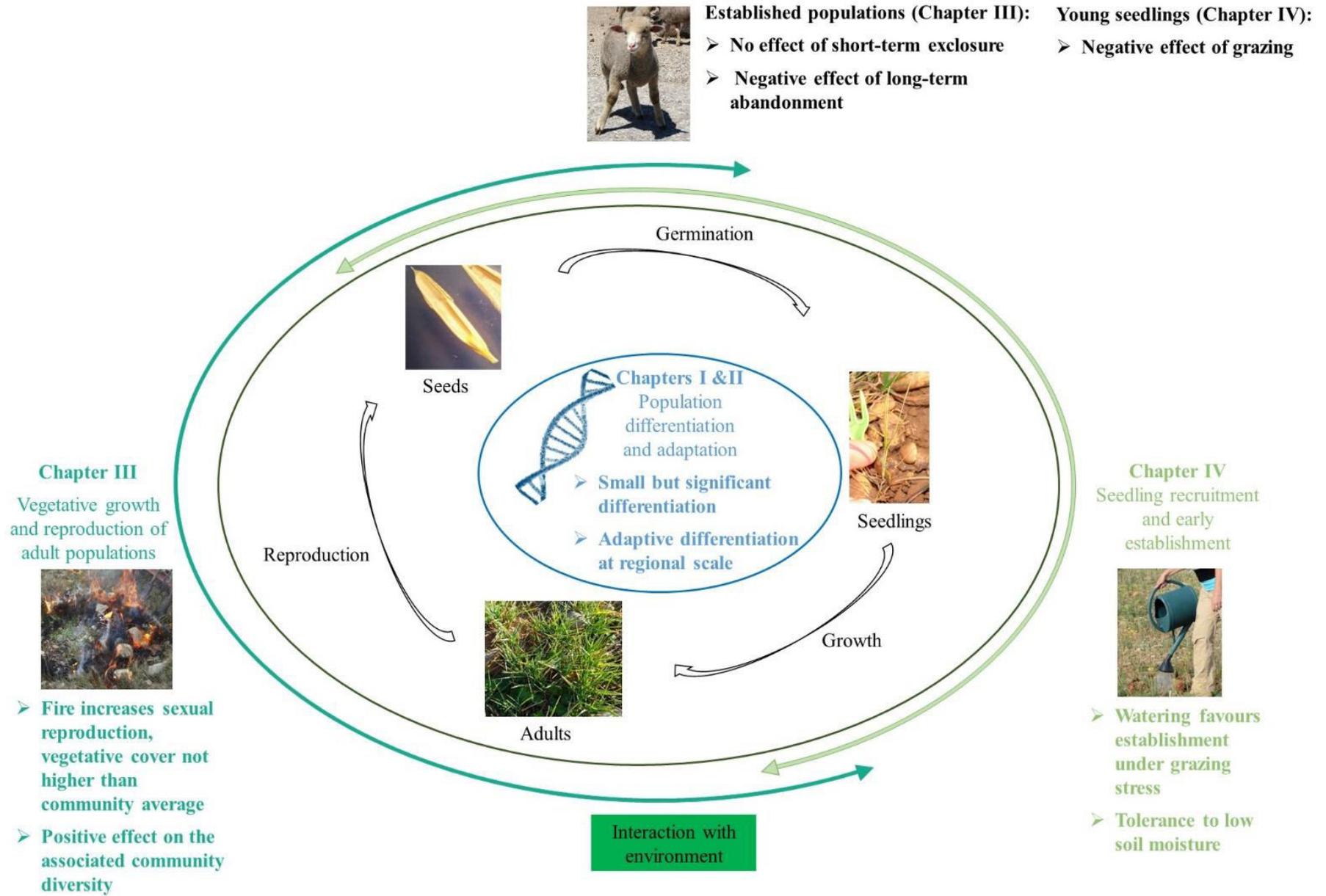


Figure VIII: Thesis structure and major results.

I. Genetic differentiation and adaptation in *B. retusum* populations

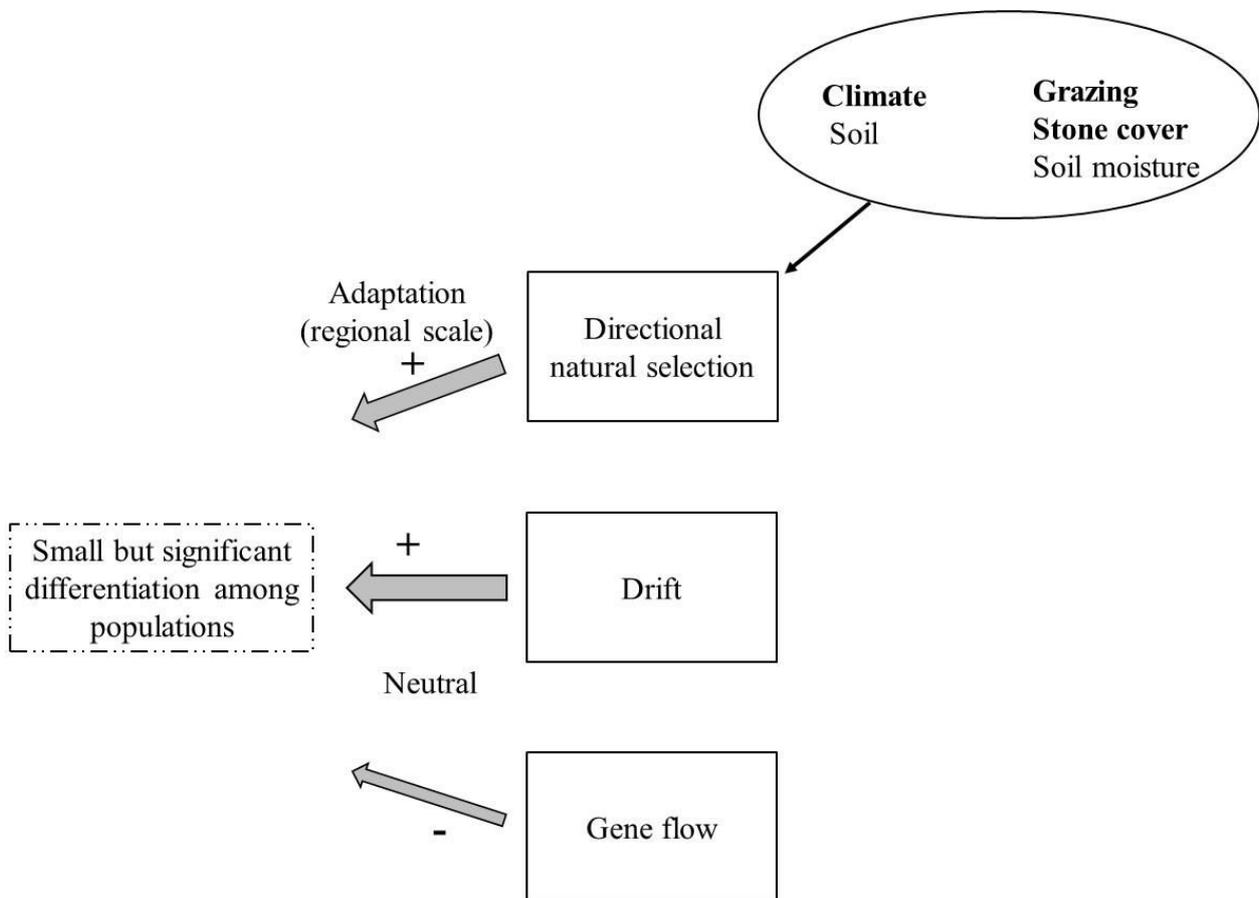


Figure IX: Evolutionary forces and their effect on population differentiation of *B. retusum*. + increase; - decrease; arrow width represents the relative contribution of each evolutionary force.

a. Genetic structure of *B. retusum* is driven by gene flow, drift and adaptive differentiation

Gene flow is a random process that can constrain evolution by preventing adaptation to local conditions but it may also increase evolutionary potential by spreading new genes (Kawecki & Ebert 2004). Its effects depend on the distance between populations, breeding system, pollination agents, seed dispersal mechanisms and physical boundaries. Previous studies have shown that gene flow is common in perennial outcrossing species (Hamrick & Godt 1996). In Chapter I, we found that gene flow is quite high and limit population differentiation in the Western Mediterranean basin.

Genetic diversity is affected by gene flow, drift and selection but also strongly depends on breeding system (Hamrick & Godt 1996). In Chapter I, a high genetic diversity was found and only one population showed a small number of private alleles. *B. retusum* is capable of sexual (cross-pollination) and vegetative reproduction (through ramet-forming rhizomes). However, the relative contribution of sexual and vegetative reproduction is not known. In Chapter I, we demonstrated that in spite of vegetative reproduction, the species maintain a high diversity suggesting that gene flow and sexual reproduction are high in the Western Mediterranean.

The ecological consequences of genetic diversity are numerous as it affects all higher organisation levels such as individuals, populations and even communities, with, in some cases, a similar magnitude to the effects of species diversity (Hughes et al. 2008). Genetic diversity may affect several ecological processes such as primary productivity, population recovery from disturbance, interspecific competition, community structure, and fluxes of energy and nutrients. Genetic diversity and variation enables species and populations to adapt to environmental changes and allows organisms to establish in different environments (Reed & Frankham 2003; Frankham 2005).

Adaptive differentiation is common in plants and often occurs conjointly with drift. A main current question in ecology and evolution is how local is adaptation (McKay et al. 2005; Sutherland et al. 2013). In Chapters I and II, we found a regional pattern of adaptive differentiation but also evidence for drift. Genetic drift can contribute to population differentiation and reduce the response to selection pressures (Wright 1931; Loveless & Hamrick 1984). It was found to be major force causing population differentiation in quantitative traits (e.g. Lande 1976; Linhart & Grant 1996). Moreover, geographic distance between populations may also contribute to the restricted gene flow (Slatkin 1987) and thus to differentiation. Sexton et al. (2014) found that concomitant isolation-by-distance and isolation-by-environment is the major pattern found in plant species. However, in Chapter I, isolation-by-environment remained significant even after controlling for isolation-by-distance confirming local adaptation.

The magnitude of local adaptation depends on several factors such as the environmental heterogeneity, the strength of selection pressure, among-population connectivity and genetic variation within populations (Leimu & Fischer 2008; Bolnick & Otto 2013). Local adaptation is considered to be stronger in populations that occur in heterogeneous environments, that experience high selection pressure, and that show restricted gene flow and sufficient genetic variation (Pigliucci & Marlow 2001; Conner & Hartl 2004). Strong directional selection is

common under near-natural conditions (e.g. Endler 1986; Kingsolver et al. 2001) and most traits appear to be moderately or highly heritable (e.g. (Mousseau & Roff 1987; El-Lithy 2004).

b. Main drivers of adaptive adaptation

Genetic variation within and among populations is strongly affected by ecological factors (Nevo et al. 1988; Macel et al. 2007; Manel et al. 2012). Climatic and edaphic conditions are the main causes for differences among populations and can result in small-scale differentiation as reported for many grass species (Hamrick & Allard 1972; Nevo et al. 1981; Gutterman & Nevo 1994; Hsiao & Rieseberg 1994; Owuor et al. 1997). In Chapters I and II, we showed that adaptive differentiation is more related to regional-scale climatic conditions than to local-scale soil type. Mean July temperature and winter frost frequency were identified as major climatic drivers of differentiation. The significant effect of site was likely the result of climatic differences between eastern and western parts of the study area. The sites of the western populations (Ni, Mon) are closer to the Cévennes Mountains and receive more rainfall than those of the Eastern populations (Ceresetti 2011). In Chapter II, we specified which phenotypic traits are the most differentiated and tested environmental factors to which populations may be adapted. We found significant differences between populations for germination capacity and several morphological traits such as leaf length, tiller number and height whereas variation in leaf number per tiller was low. In particular, germination and tiller number are closely linked to seedling recruitment and establishment supporting our hypothesis that different colonisation patterns observed at different Mediterranean sites (high: Caturla et al. 2000; Luis et al. 2004; low: Coiffait-Gombault et al. 2012a) may result from genetic differentiation.

Soil type within sampling sites explained a significant part of variation among populations in neutral markers and in vegetative phenotypic traits but explained variation is much smaller between soils within sites than between sites suggesting that soil was not a major driver of genetic differentiation. Small-scale adaptive differentiation to soil conditions has been found for grasses to heavy metal soil contamination (Rozema et al. 1985; Ernst 1987). However, at a small scale, gene flow may be stronger than selective pressure and thus counteract selection (Kawecki & Ebert 2004; Leimu & Fischer 2008; Hereford 2009). Gene flow is high for epizoochorous species (Fischer et al. 1996). Southern French population were connected in the past due to transhumance involving spring grazing in the lowlands and summer grazing in the mountains. This is particularly well documented for the exchange between our focal study site, the Crau plain, and the French Alps lasting from Roman period to 1962 (date of the last

pedestrian transhumance, (Gardelle 1965; Badan et al. 1995; Congés 1997)). However, the grazing period of our Mediterranean *B. retusum* collection sites is early spring which is far before the seed production and maturation period limiting the effect of epizoochorous dispersal by sheep.

We further were able to demonstrate in Chapter II that *Brachypodium retusum* populations respond differently to stone cover and clipping - whereas the differential germination response to soil moisture was relatively small. Stone cover had a generally positive effect on the growth of *B. retusum*, in particular on plant height, tiller number and plant diameter probably related to the more favourable microclimate. Stones were found to favour growth and survival of *B. retusum* because rhizomes and roots can grow underneath and thus benefitting from higher soil moisture during the Mediterranean summer (Lahav & Steinberger 2001; Peters et al. 2008; Buisson et al. 2015). We were not able to relate the differential response to stone cover to major soil type. Thus, populations from Red Mediterranean soils were not specifically adapted to the presence of pebbles and cobbles of such soils used in the experiment. A more detailed analysis of stone density, shape and sites combined with an experiment combining stones from red Mediterranean and calcareous soils may help to better explain the differential response.

Clipping largely reduced plant height, tiller number and leaf number resulting in a highly reduced reproduction. We also found a differential response of populations that could be linked to grazing intensity at collection sites. The grazing effect was less strong in populations from red Mediterranean soils which are under higher grazing intensity than in populations from calcareous sites. Grazing is known to exert strong directional selection and may result in genetic differentiation and local adaptation to long-term differences in grazing intensity (Linhart & Grant 1996; Hufford & Mazer 2012). Higher compensatory growth may be one important tolerance mechanism as an adaptation of grasses to higher grazing intensity (Sarmiento 1992).

Drought stress reduces germination, and usually plant species show a positive correlation between soil moisture and germination rates (Bochet et al. 2007; Thomas et al. 2010; Walder & Erschbamer 2015)). The ability to germinate fast under moderate to low soil moisture confer to species an advantage to colonize dry environments (Schütz et al. 2002; Bochet et al. 2007). All tested *B. retusum* populations showed a high germination capacity even under the lowest soil moisture level but a considerable drop under saturated conditions. The response to variation in soil moisture was similar among populations. Only one population (Ni) showed a strong decline under dry conditions which may be due to the lower drought tolerance of the relatively

humid collection site. These results suggest a weak adaptation to climate-mediated soil moisture. A strong decrease in germination under drought was, however, found in another study including *B. retusum* populations from Spain (Bochet et al. 2007). Although other factors such as seed age and quality or experimental conditions may influence germination results, such a strong difference to our results still suggests genetic differentiation in the response to soil moisture. In my thesis, the high stability of germination under drought was also confirmed *in situ* (Chapter IV) as the watering treatment did not increase germination.

II. Plant regeneration, reproduction and early establishment – effects and interactions with environmental factors

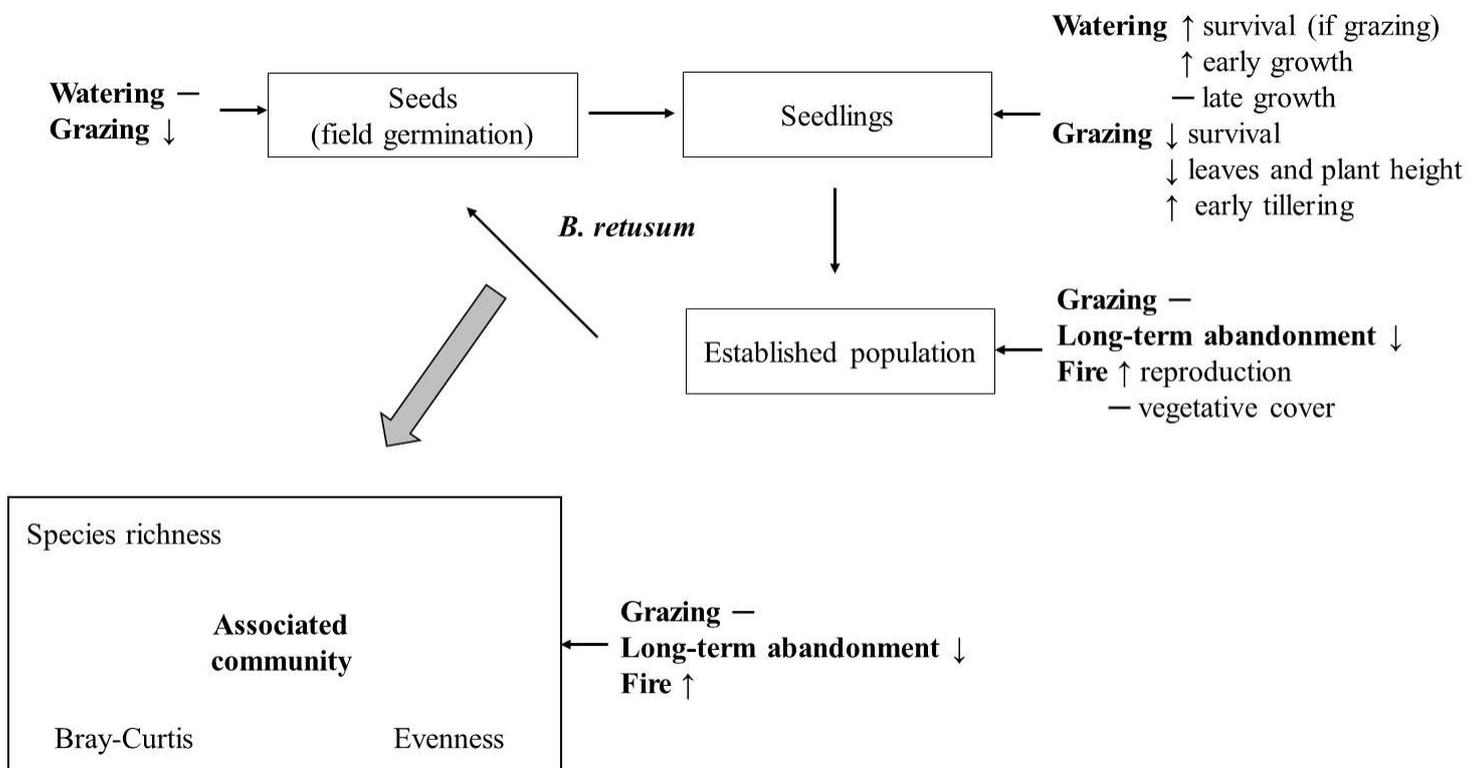


Figure X: Main factors driving *B. retusum* populations at different life cycle stages in field-sown seedlings, established adults and the associated community. ↑ increase; ↓ decrease; — no effect

Plant productivity and distribution are driven by climate and locally modified by soil conditions (Polis 1999). Additionally, biotic interactions such as herbivory regulate plant performance.

Interacting organisms are also directly affected by climate and soil increasing the complexity of interactions. Domestic grazers such as sheep are the major herbivores of Mediterranean grasslands and are indispensable to protect these systems against shrub encroachment (San Miguel 2008). Fire is closely related to climate but has specific effects on the vegetation (Bond & Keeley 2005a). In Mediterranean grasslands, characterized by summer drought, fire is common and thus known to be one of the major factors shaping plant populations and communities (Naveh 1975).

a. Fire does not favour vegetative recovery compared to other dry grassland species but increases sexual reproduction

Fire is an important ecological and evolutionary factor in several worldwide ecosystems such as boreal forests, eucalypt woodlands, shrublands, grasslands and savannahs. Although fire disturbance may reduce species richness in non-adapted systems with low fire frequency (Laurance 2003), fire effects on plant communities of fire-prone systems are positive (Bond & Keeley 2005b). In fire-prone grasslands, anthropogenic fire suppression has reduced species richness (Leach & Givnish 1996). Fire suppression may even result in a complete biome switch to non-adapted forest and a subsequent loss of animal and plant species (Bond & Van Wilgen 1996).

Fire has an effect on both plant species traits and community but effects are rarely analysed together. The fire impact on species and the associated community is, however, well documented for *Pinus* species (Richardson 2000). Similarly, we analysed the effect of fire on the population dynamics of one species (vegetative recovery, reproduction of *B. retusum*) and on the associated plant community.

In Chapter III, we showed a positive effect of winter and in particular of summer fire on *B. retusum* on reproduction and to a lesser degree also on regeneration of vegetative cover. However, vegetative regrowth of the species was not higher than community average and in the beginning, other species showed a faster regeneration. In particular, annual seeder species benefitted from fire, increasing the diversity of the associated community. *B. retusum* is a resprouter showing increased vegetative growth after injury or a strong change in growing conditions (Del Tredici 2001). Resprouting is a well-known tolerance strategy allowing regeneration after biomass removal (Clarke et al. 2013). In several perennial plants, fire can also trigger flowering (Lamont & Downes 2011). For example, in several fynbos and chaparral

geophyte species, flowering occurs soon after fire and seeds are dispersed on bare soil providing a fitness advantage over species not responding to fire (Keeley 1977; Keeley 1993; Borchert & Tyler 2009).

At the community level, species richness and diversity increased. Such positive effects of fire on community structure have also been reported for pine-grassland communities (Sparks et al. 1998). Fire effects at species and community scales may vary depending on fire regime (Bond & Van Wilgen 1996; Davies et al. 2016). Changes in such regimes with specific recurrence and period often result in plant community shifts (Bond & Keeley 2005). Furthermore, an increase in fire frequency may change the proportions of functional groups. In another Mediterranean grassland, fire increased the abundance of herbaceous species and reduced the abundance of both seeder and resprouter shrubs. *Brachypodium retusum* was dominant in all fire frequencies (Vilà et al. 2001).

In our study, fire increased herbaceous annual seeder species and reduced the cover of resprouter grasses in the first post-fire year. This result does not correspond to studies in Spanish *B. retusum* grasslands showing a faster recovery of resprouter species (Caturla et al. 2000, De Luis et al. 2004). This difference may be explained by site and soil differences as the Crau steppe is a more open and less nutrient-rich grassland type than the Spanish field sites allowing the development of a higher diversity of annual species (Buisson & Dutoit 2006). Fire may favour annual species through a higher post-fire seedling recruitment which was demonstrated in other dry and nutrient-poor grasslands (Valone & Kelt 1999). However, longer-term studies are necessary to identify the optimum fire regime to maintain such positive effects on plant diversity.

b. Grazing reduces early growth and establishment but is well tolerated in adult plants

Contrary to fire that has decreased in importance, grazing is still a strong driver of population and community dynamics.

In Chapter IV, we showed that grazing had a negative effect on early stages of establishment including reduced germination, survival and growth. However, the effect of grazing varies depending on developmental stage with high compensatory growth of *B. retusum* after grazing periods. Trampling, urine and faeces excretion may change the soil micro-environment (i.e., soil loss, reduced shade, (Bilotta et al. 2007) and therefore modify conditions that influence

germination, survival and growth. Defoliation and the related loss of photosynthetic have been identified as the most important grazing effect (Ferraro & Oesterheld 2002; Van Staalduinen & Anten 2005; Zhao et al. 2008). Compensatory growth following defoliation may compensate for such losses by stimulating photosynthesis, removing old and dead tissue, altering mass allocation, increasing growth rate, and producing more tillers in defoliated plants (McNaughton 1983; Oesterheld & McNaughton 1988; Oesterheld & McNaughton 1988; Zhao et al. 2008).

In Chapter III, we showed that grazing did not negatively affect adult *B. retusum* populations and the associated community. However, long-term abandonment of grazing may have negative effects on *B. retusum* since sexual reproduction decreases and other perennial species may outcompete *B. retusum* and annual species (Saatkamp et al. 2018).

At community level, grazing effects are contrasting including positive, negative and non-significant effects on plant diversity (Huston 1994; Proulx & Mazumder 1998). Grazing tends to favour small and prostrate plants over tall erect types and annuals over perennials (Noy-Meir et al. 1989; McIntyre et al. 1999; Díaz et al. 2007). Grazing favours the co-existence of different species by biomass removal reducing above-ground competition and creating gaps (Bullock et al. 2001; Drobnik et al. 2011; Coiffait-Gombault et al. 2012a).

c. Initial watering has a positive effect in the beginning largely disappearing after one year

In arid and semi-arid environments such as dry grasslands, water availability is known to be a limiting factor for plant establishment, productivity and performance (Noy-Meir 1973; Bochet et al. 2007). Drought stress has been identified as a key factor limiting seedling recruitment (Pywell et al. 2007; Nuttle 2007). In Chapter II, we showed a high germination even under low moisture. In Chapter IV, initial watering had no effect on seed germination and seedling survival confirming that the species is well adapted to low soil moisture (Vidaller et al. 2018). *B. retusum* rhizome has a high capacity to retain water and respond quicker to water additions after drought than shrub species (Clary et al. 2004). The watering effect was only positive (increased survival) in grazed plots suggesting that watering may compensate for additional grazing stress.

Watering increased growth at early stages of establishment but the effect disappeared in the following season. In ecosystems with periodic drought, water is a limiting factor for growth (Noy-Meir 1973) and there is a trade-off between the capacity to tolerate drought and growth

(Pérez-Ramos et al. 2013). Our study showed that initial watering had no long-term effects on growth of surviving plants but the higher survival in traditionally grazed grasslands still demonstrated a long-term positive effect on establishment of *B. retusum*.

III. Implications for ecological restoration - plant introduction and population management

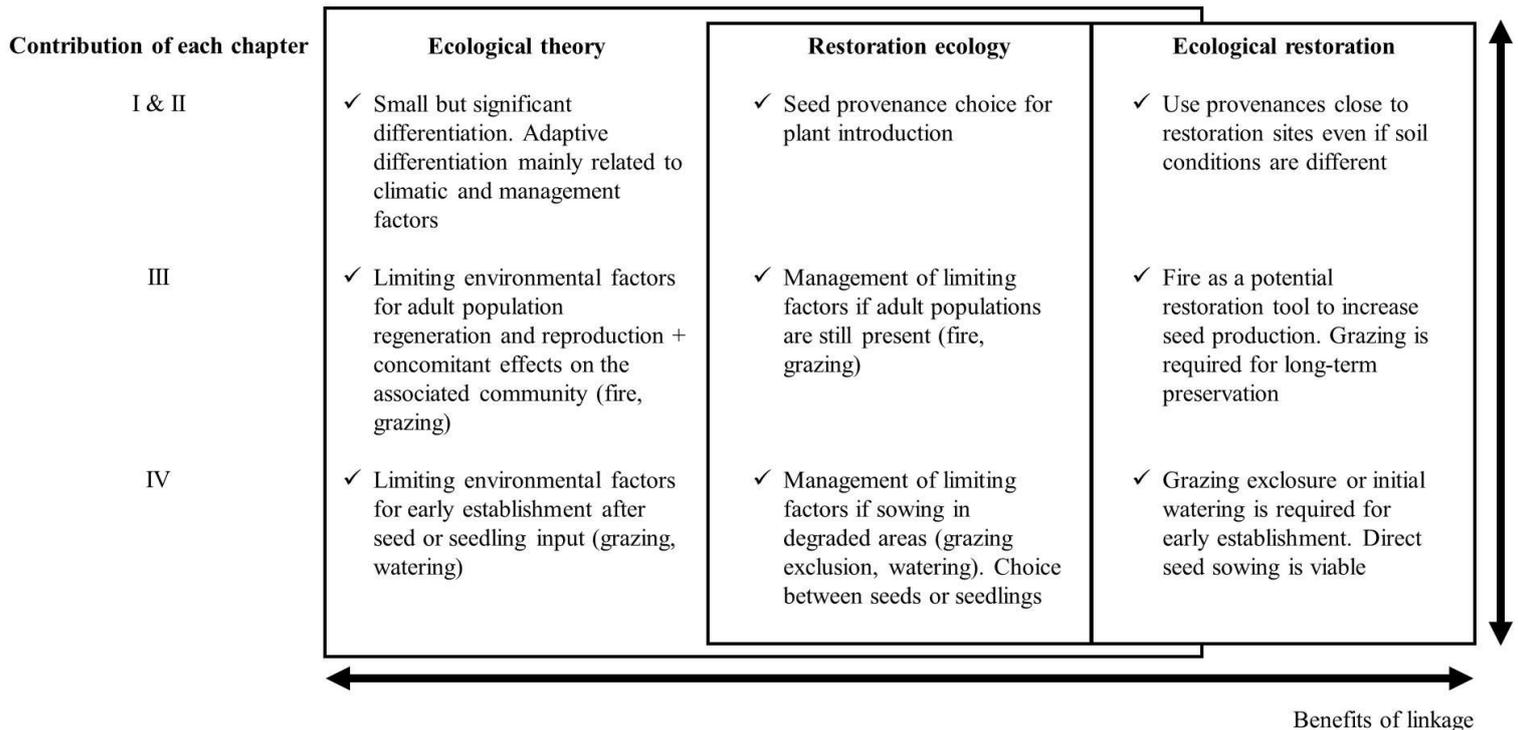


Figure XI: Contribution of the thesis to basic and applied ecology.

Ecological restoration involves different organisation levels from genes to communities. One restoration action is the translocation of dominant strongly interacting key species (Palmer et al. 2016) when restoration of abiotic conditions is not sufficient for re-establishment. The genetic structure of the introduced populations influences restoration success. Knowledge on inbreeding, reproductive viability, local adaptation, and genetic diversity is crucial in restoration (Hufford & Mazer 2003; Hufford & Mazer 2012). Moreover, a better understanding of key environmental drivers of establishment and their specific effects on population dynamics is required to evaluate and improve restoration success.

a. Adaptive differentiation and the seed provenance question

Recent studies illustrate the growing importance of taking into account genetic differentiation in ecological restoration by planting or sowing. Local origin of populations is usually recommended to establish locally adapted populations (Hufford & Mazer 2003; Vander Mijnsbrugge et al. 2010; Hufford & Mazer 2012; Bucharova et al. 2017). In Chapters I and II, we demonstrated adaptive differentiation advocating the importance of plant provenance in ecological restoration. Introducing non-local populations may thus compromise the success of plant introductions (Hufford & Mazer 2003; Hufford & Mazer 2012). We demonstrated that climate and genetic drift at regional scale are more important drivers than local soil type. We concluded that the close-by source populations facing similar climatic conditions should be preferred over populations from the same soil type.

Genetic diversity may also affect the performance of founder populations. If genetic diversity is too low, genetic bottlenecks may result in inbreeding depression and decrease the capacity to adapt to environmental factors. In our study, we showed a high neutral genetic diversity and gene flow which reduces the probability of founder effects. Moreover, hybridization of introduced non-local populations with remnant local populations may lead to outbreeding depression. The hybrids show a lower fitness than their parents which was in particular observed in F2 and further hybrid generations whereas F1 hybrids still show a heterosis effect (Hufford & Mazer 2003; Hufford & Mazer 2012; Zavodna et al. 2015).

Fragmentation and reduced population size also need to be considered since they directly affect genetic characteristic structure of populations. Habitat fragmentation may reduce population size and increases spatial isolation between populations. Although populations may respond differently to fragmentation, often a decrease in genetic diversity and an increase in population differentiation is observed due to increased random genetic drift, inbreeding and reduced gene flow (Young et al. 1996; Cheptou et al. 2017).

b. Fire increases sexual reproduction and has positive effect on plant community species-richness

Fire is already used as a restoration tool in Europe and particularly in North America, South Africa and Australia (Heinlein et al. 2000; Holmes et al. 2000; Carrick et al. 2015). Several studies showed that restoration of such fire-prone ecosystems needs a specific knowledge of the appropriate frequency and period (Bond & Van Wilgen 1996; Bond & Keeley 2005).

In Mediterranean ecosystems, the use of fire is more controversial mainly due to the higher human population density. Consequently, there is a knowledge gap on the effect of different fire regimes (Stouff 1997; Stouff 1986). Fire management has been abandoned since more than a century in our study region (Stouff 1997; Stouff 1986). In Chapter III, we demonstrated that *B. retusum* recovers quickly after fire and that fire even increases sexual reproduction. Additionally, species-richness and diversity of the associated plant community increase. These results suggest that fire is a key and currently missing ecological factor in preserving and restoring steppe ecosystems. Although *B. retusum* does not recover faster than other species of the plant community, the increase in seed production helps to increase seedling recruitment in burned areas and thus genetic diversity of the populations usually showing clonal growth. Our results also showed that fire may replace grazing as a conservation measure since the species composition of abandoned grasslands showed a post-fire community shift towards typical steppe communities. Since fire-specific effects on ecosystems are not well known, the use of fire for restoration and conservation should be carefully surveyed. In particular, potential negative effects on organisms of higher trophic levels such as animals and microorganisms need to be considered.

c. Young seedlings need grazing protection or additional watering whereas adult plants benefit from grazing.

Mediterranean dry grasslands depend on human land management, in particular on grazing (San Miguel 2008; Apostolova et al. 2014). In Chapter IV, we showed that grazing reduced survival if plants are not watered in the beginning. Thus grazing should be excluded in the beginning. Alternatively, irrigation may be used to increase establishment if grazing exclusion is not possible.

Grazing may have positive effects on seedling establishment if competition is strong (Bullock et al. 2001). However, grazing is also known to increase seedling mortality hampering establishment in less competitive systems (Buisson et al. 2015). Although showing a relatively low palatability, young seedlings of our study species *B. retusum* are negatively affected by grazing (Buisson et al. 2015; Vidaller et al. 2018). In Chapter III, we showed that short-term grazing enclosure is tolerated. However, long-term grazing enclosure results in an increase of more competitive grass species such as *Dactylis glomerata* and a decrease of *B. retusum* cover relative to other species (Saatkamp et al., 2018). In conclusion, *B. retusum* benefits from grazing in the long run. Moreover, grazing may promote the coexistence of different functional groups

and favour the annual species, thus increasing species richness (Bullock et al. 2001; Drobniak et al. 2011; Coiffait-Gombault et al. 2012a, b). Grazing management needs therefore to be considered in restoration (Papanastasis 2009) but it is necessary to determine the responses of different functional plant groups to grazing (Hellström et al. 2003).

d. *B. retusum* establishes well from seed

Greenhouse-grown transplants usually provide greater initial success than sown seeds (Maschinski & Whitham 1989; Godefroid et al. 2011). Seeds need to survive, germinate and emerge before reaching seedling stage resulting in a lower establishment compared with transplanting of seedlings. Thus, seedling mortality often decreases with time (Gilbert et al. 2001; Quintana-Ascencio et al. 2004) resulting in a decreasing slope of survivorship curves (Li et al. 1996).

In Chapter IV, we showed that survival and growth were lower in field-sown seedlings than in transplanted seedlings. However, mean survival was still 38% for sown seedlings at the end of the experiment indicating a successful establishment from seeds. The difference between sown and transplanted seedlings (48 % survival) was significant but small. The differences in growth traits diminished during the study period. Thus, we believe that the small fitness advantage of transplanting pre-grown seedlings to restoration sites does not compensate for higher effort and costs of this method. Tests on the effect of seed density are required to improve information on optimum conditions in the field and to provide practical guidance on seed-number limitation (Kirchner et al. 2006).

Conclusions – answers to hypotheses

The AFLP marker analysis demonstrated a relatively low but still significant population differentiation at Western Mediterranean and regional Southern French scale. Genetic diversity is high suggesting a high gene flow among populations. A part of this differentiation was found to be adaptive demonstrated by the comparison of neutral AFLP markers and phenotypic traits. Factors driving differentiation were climate, soil type, stone cover, grazing (simulated by clipping) and to a lesser degree soil moisture. Adaptive differentiation was particularly found in phenotypic traits that are crucial for early establishment such as germination and tiller number. The results support our hypothesis that different colonisation patterns observed at different Mediterranean sites (high: Caturla et al. 2000, Luis et al. 2004; low: Coiffait-Gombault

et al. 2012a) result at least partially from genetic differentiation among populations. Plant introduction measures in ecological restoration should therefore take into account plant provenance including geographical and environmental distance to restoration sites.

We confirmed the strong and predominantly positive effect of fire on *B. retusum* populations. Although vegetative recovery was not faster than community average, the species reached pre-fire cover already after two seasons and in particular increased seed production. Fire may thus favour refreshment of older populations by resprouting but also by higher seedling recruitment on the more open post-fire sites. When sites are undergrazed or abandoned, fire may replace grazing as a conservation measure to maintain dry grassland community structure and species richness. In ecological restoration, fire may support natural colonisation from still existing local populations through increased seed production and corresponding higher seed rain at restoration sites.

The effect of grazing was less positive than expected. Young seedlings showed a lower survival and growth at least if grazing stress was not compensated by initial watering. Two years of grazing exclusion did not significantly affect vegetative cover and reproduction of adult populations. However, long-term abandonment seems to have a negative effect on *B. retusum* since 17-year old exclosures showed a lower relative cover and a lower seed production than traditionally grazed plots. Initial watering has a positive effect on early growth traits whereas differences were not visible any more in the second growing season. However, in grazed plots a positive watering effect on survival was still significant at the end of the study period indicating that watering can compensate for grazing stress. Thus watering may improve seedling recruitment and establishment under strong grazing pressure and drought. If drought stress is not strong during the establishment period – which was the case during our experiment – one or two-year grazing exclusion may be the more cost-effective strategy to improve establishment success.

Perspectives

I. Genetic diversity and clonality

With our sampling design, we tried to guarantee that different individuals do not belong to the same clone. Accordingly, we cannot directly measure clone size and *in situ* clonal growth. Such information would be complementary in terms of colonisation capacity and genetic diversity of founder populations. In order to obtain more detailed information on clonality, small-scale

sampling at distances of 0.5 to 5 m is required. Genetic marker analysis applied separately to all small-scale samples allows an identification of clone size, clonal diversity and architecture (Duhovnikoff & Hazelton 2014). Moreover, clone age can be estimated based on maximum clone diameter since clonal spread by rhizomes is slow and regular ($<5 \text{ cm} \cdot \text{a}^{-1}$ measured in Chapter II common garden experiments, Steinger et al. 1996).

Sampling of different ramets in founder populations and tests in common garden experiments would help to better understand the relationship between population fitness and genetic diversity. Ramet senescence and its effect on survival and sexual reproduction can be studied using this approach. Clonal growth may not reduce intrinsic senescence. In iteroparous perennial plants, senescence was found to be positively correlated to time since first reproduction and mean reproductive lifespan (Silvertown et al. 2001). However, fragmented clones (clonal reproduction) may more likely escape from senescence at the genet level than clones that remain physiologically integrated (Silvertown et al. 2001).

II. Fire effects on seedling recruitment and restoration management

Reproduction is a key factor in *B. retusum* re-establishment and is enhanced by summer wildfire. However, we do not know how long such positive fire effects last. A medium or long-term experiment would be needed to understand post-fired reproduction dynamics, in particular the return period to a steady state without fire. We also did not measure fire effects on seedling recruitment. Several species better germinate and establish after fire due to favourable site modifications such as reduced competition, chemical compounds released from smoke and ash and a higher nutrient availability (Bond & Van Wilgen 1996). Experiments on such fire effects may be set up in traditionally managed grasslands but also on degraded sites in order to obtain information on fire as a restoration tool.

I am aware that fire may have negative effects on the epigeic and hypogeic fauna and soil microorganisms. The response of the fauna depends on fire characteristics such as intensity or propagation and animal species exhibit a large range of responses from negatively affected to fire-tolerant or even dependant (Swengel 2001). Before recommending fire as a restoration tool to maintain or restore Mediterranean dry grassland communities, potential negative effects on other organisms need to be evaluated.

III. *B. retusum* migration and dispersal

Seed dispersal is a key process that often limits the re-establishment of plant species. A better understanding of dispersal is needed to evaluate colonisation capacity based on seedling recruitment. Several studies on the ecological restoration of grassland ecosystems have shown that low dispersal represents a very strong environmental filter (Bakker et al. 1996; Bischoff 2000; Donath et al. 2003; Bischoff et al. 2009). Species without specific adaptation to wind dispersal are rarely dispersed more than 5 m per year (Jongejans & Telenius 2001; Coulson et al. 2001). So far, there is no accurate analysis of the dispersal of *B. retusum*. The fact that this very abundant species of intact steppe communities was not able to colonise adjacent areas of restoration sites within 30 years (Coiffait-Gombault et al. 2012a) suggests a low dispersal capacity. More knowledge on dispersal is crucial to better understand the role of this filter in steppe ecosystem restoration. Two main dispersal vectors have to be considered in our study region. Wind dispersal probably plays a major role (anemochory) because most of the steppe is exposed to strong winds from the north (“mistral”). Animal dispersal is probably the second important vector. Grassland species seeds can be transported by livestock (zoochory). Fischer et al. (1996) found the seeds of many species on grazing sheep (wool, hooves). However, the Crau plain is mostly grazed in spring and never during the *Brachypodium* seed production period in summer (Débit & Garde, 2013). It is therefore unlikely that sheep contribute to the dispersal of the species. As the second group of animals that disperse seeds in grassland ecosystems, ants are very abundant in the Crau (myrmecochory). The seed-eating ant *Messor barbarus* disperses over 60% of plant species (Cerdan et al. 1986). Dispersal by ants is generally limited to a few meters (Andersen 1988; Retana et al. 2004) but some seeds may also be transported much further (77 m, (Gomez & Espadaler 1998). Thus, it would be of high interest to test the efficiency of wind and ants as dispersal vectors. The wind speed can be manipulated in the laboratory to test the distance of dispersal by wind. *In situ* tests using platforms corresponding to the inflorescence position in the natural habitat may be used as an additional method to test for wind dispersal. Then the position of the seeds after wind transport indicates *in situ* dispersal distances. Myrmecochoric dispersal can be analysed by direct observation of seed transport by dominant granivorous ant species (*Messor barbarus*). Alternatively, seed deposits may be installed at different distances from the nest allowing a direct measurement of transport distances.

IV. Links between *B. retusum* and associated plant community

B. retusum is highly interacting with other species as shown for dense dwarf matorral communities in Spain (Saiz & Alados 2011). In general, it is the dominant species of the dry Mediterranean Thero-Brachypodietea grasslands (San Miguel 2008). Therefore, it seems to be likely that the species strongly affects its associated plant community but such plant-plant interaction have not been tested so far (Coiffait-Gombault 2011).

Plant-plant interactions may strongly influence the establishment of other species during plant succession and ecological restoration through mutualistic (facilitation) and competitive effects (Pywell et al. 2007; Brooker et al. 2008). In particular, facilitation effects are currently discussed as a restoration tool improving seedling recruitment and establishment of target species (Padilla & Pugnaire 2006). Thus, it would be interesting to compare the establishment of characteristic steppe species under *B. retusum* cover and at non-occupied control sites to evaluate facilitative effects.

V. Global change perspective

Current climate models predict a decrease in spring and summer precipitations in the Mediterranean climate zone resulting in stronger summer drought stress (Gibelin & Déqué 2003). Our experiments demonstrated a high drought tolerance of the species but stronger summer drought may still limit *B. retusum* populations and result in a retreat of corresponding dry grassland communities resulting in a further decline. Experimental tests simulating summer drought in the field or specifically manipulating soil moisture and temperature in growth chambers may help to better understand the climatic limits of the species and its associated plant community (e.g. Jentsch et al. 2007; Martin-StPaul et al. 2013).

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Appendices

Appendix I: Brief description of thematic fields used in the thesis and brief glossary.

Population biology

A **population** is a group of individuals belonging to the same species (interbreeding individuals) and living in the same geographic area (Krebs 1972). **Population biology** is the study of patterns of abundance, distribution and variation in space and time of populations and the interpretation of these patterns in terms of genetic and environmental influences. In the late 1950's and in during the 1960's population ecologists and evolutionists have tried to create a conceptual synthesis of these two research fields in order to define a population biology science (e.g., MacArthur & Connell 1966; Lewontin 1968). However, **population genetics** and **population ecology** studies have been done independently and population biology still lack a synthesis between population ecology and genetics concepts until the beginning of the XIXth century (Singh & Uyenoyama 2004). Since then, several authors began to synthetize both fields mainly in text books (e.g., Neal 2004).

Population genetics and **population ecology** are obviously linked since population characteristics and environmentally linked performance could induce changes in population genetics. Conversely, genetics composition of a population could affect population responses to environmental factors.

- **Population genetics**

Population genetics is the study of population genetic composition, the genotype and phenotype frequency distributions and changes in response to the processes of natural selection, genetic drift, mutation and gene flow. Selection, drift, gene flow and mutation are the main evolutionary forces. The relative impact of each force depends on species-specific intrinsic characteristics as well as the landscape's composition and history (e.g. Aguilée et al. 2011; Husemann et al. 2012).

Selection

Natural selection was considered by Darwin (1859) as the most powerful of the evolutionary forces. It occurs when differences among individual heritable traits cause differences in survival and reproduction. Depending of trait evolution direction, three types of natural selection can be distinguished. Directional selection drives a phenotype (observable trait) to one extreme rather the intermediate phenotype or the opposite extreme phenotype. Stabilizing selection occurs when an intermediate phenotype is favoured. Disruptive selection occurs when extreme phenotypes have an advantage over more intermediate phenotypes. Moreover, sexual selection is a part of natural selection and could be distinguished from it when selection acts on traits involved in reproductive success.

Adaptation is both an evolutionary process and a product of natural selection. Adaptation is a process of evolution in which traits are modified by natural selection and is a phenotypic trait shaped by natural selection.

However, it is currently known that selection is not the only force leading to evolution and could be counterbalanced by other evolutionary forces.

Drift

Genetic drift is another evolutionary force that could lead to population differentiation. Genetic drift is a change in the gene pool strictly due to chance fixation of alleles and is considered as the null model of population differentiation. It is the result of random processes in finite populations and its strength strongly depends on the effective size of a population, gene flow from other populations and the time scale of observation. In populations with an effective size larger than 500 individuals and over ecological timescale, drift is considered to be of limited importance (Hartl & Clark 2007). Nevertheless, in small and isolated populations with limited gene flow, drift can lead to significant variation of allele frequencies, the fixation of alleles even over short time scales and thus to rapid differentiation (Ellstrand & Elam 1993).

Gene flow

Gene flow, also called **gene migration**, is the transfer of genetic material from one population to another. It can counterbalance the effects of selection and drift by introducing new alleles into a population. Gene flow generally depend on the landscape and environment barriers (Manel & Holderegger 2013). However, differentiation can occur even in the presence of gene flow depending on the relative strength of the evolutionary forces.

- **Population ecology**

Population ecology study the environmental factors that influence abundance, distribution and variation in space and time of populations. Several factors impact size, birth, growth, reproduction, death of a population and their variations. Main factors could be separated in abiotic (resource availability, water availability, climate) and biotic factors (intra species or inter species competition) (Neal 2004).

Community ecology

Community ecology began to develop in the beginning of the XXth century with a debate on community definition (Clements 1916; Gleason 1926). Later, an ongoing debate arose, MacArthur (1972) suggested that community ecology main goal is to find general rules whereas Lawton (1999) suggested that “community ecology is a mess”. More recently, Simberloff (2004) added that general rules cannot be achieved due to the complex nature of communities. McGill et al. (2006) responded that general laws can be found focusing on four themes which are traits, environmental gradients, interaction milieu and performance currencies.

For this thesis, we retained that a **community** is a group of populations of different species living in the same area (Krebs 1972). Community ecology is the study of patterns in the diversity, abundance, and composition of species in communities, and the processes underlying these patterns (Vellend 2010).

Restoration ecology

Ecological restoration preceded **restoration ecology** and is practised since centuries by indigenous communities (Stevens 1997). **Ecological restoration** is “The process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed.” (Society for Ecological Restoration International Science and Working Policy Group, 2004).

It is only in the later XXth century, that emerged **restoration ecology** which is the science that develops and tests a body of theory focused on repairing damaged ecosystems (Palmer et al. 1997). Restoration ecology first focused on plant community restoration (Young 2000). Indeed, restoration operations often involve a focus on multi-species assemblages. Later on, population biologists working in a restoration framework focused on population restoration to a level that will allow them to persist over the long term within a dynamic landscape and include the ability to undergo adaptive evolutionary change. Indeed, common or dominant species removal, one whose total biomass (the sum of the biomass of all individuals) is greater than the average total

biomass of other species, is likely to have a significant impact on ecosystem functioning (Naeem 2006).

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Appendix II: *In situ* and common garden seed set.

Observations in the field suggested a low *in situ* seed production. Seed production and seed set were therefore measured *in situ* and in common garden. In order to measure population differentiation in the common garden studies of the Chapters I and II, in one inflorescence per sampled patch, the number of seeds and flowers was counted to calculate the seed set. Remaining inflorescences were shaken in tubes with glass beads using a vortex mixer to separate seeds from glumes. Transparency under transmitted overhead projector light was used as a criterion to remove empty seeds without embryo. *In situ* seed set could thus be measured. This experiment was also used to compare the seed set in a common garden with the seed set at the collection sites (see above).

Separate models were run for seed set at collection (parental generation, influenced by maternal environment) and common garden sites (F1). The parental seed set model included site, soil and their interaction. Soil and site effects combine seed set differences resulting from genetic differentiation but also from phenotypic plasticity according to different environmental conditions. The F1 seed set model additionally included block as all other common garden study models. The main objective of this approach was to test whether the general seed set level, but also differences between populations, are similar at original collection sites and under standard common garden conditions. As for the germination tests, seed set percentage was arcsin ($\sqrt{x/100}$) transformed prior to analysis.

A Tuckey HSD posthoc test was calculated to analyse differences between treatment levels more in detail if the treatment main effect or interactions were significant. Posthoc tests were preferred over a priori linear contrasts because we had no clear a priori hypothesis on specific adaptation to tested environmental factors. All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)).

Seed set data were obtained from a subset of populations planted in the common garden and compared with seed set of parental plants at the original collection sites. Mean seed set was lower than 10% in parental plants *in situ*, but between 20 and 25% in the common garden (Figure A). Seed set in parental plants differed between sites ($F_{5, 663}: 56.346$ ***), but the site effect depended on soil type (site x soil, $F_{5, 663}: 3.28$ **) whereas the soil of origin had no overall effect ($F_{1, 663}: 0.95$). Seed set was higher on Red Mediterranean soils than on calcareous soils at three sites and higher on Calcareous soils at one site (Saint-Rémy-de-Provence). In the common garden, these differences in seed set between original sites and soils disappeared.

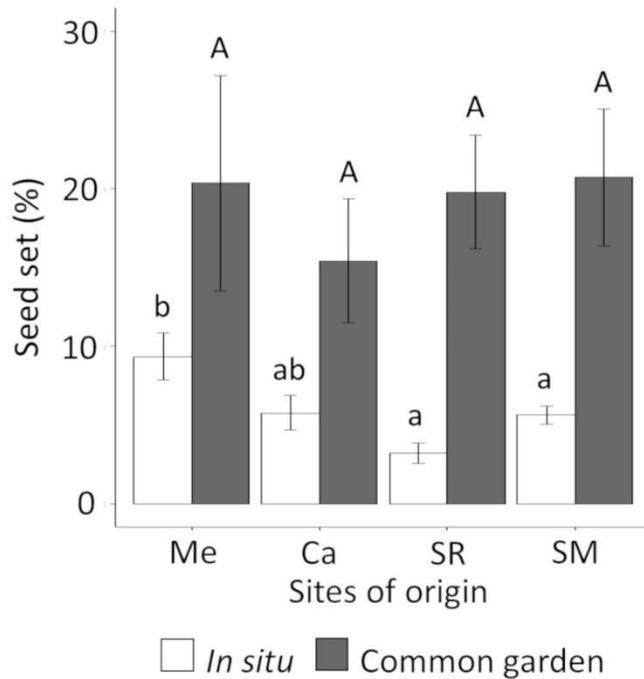


Figure A: Differences in seed set of parental plants (*in situ*) and F1 plants grown in a common garden (mean values \pm SE). Sites of origin: SM: Saint Martin-de-Crau, SR: Saint Rémy-de-Provence, Me: Mérindol, Ca: Caumont, Ni: Nîmes, Mo: Montpellier. Different lower case letters indicate significant differences ($P < 0.05$).

A strong influence of the maternal environment and a high phenotypic plasticity were demonstrated for seed set. Most populations showed a very low *in situ* seed set at natural sites and there was considerable differentiation among populations which may be a major constraint in ecological restoration based on seedling recruitment (Coiffait-Gombault et al. 2012a, b). However, in the common garden, differences completely disappeared due to more favourable growing conditions, and seed set was much higher. *B. retusum* produces flowers but allocates only resources to seeds if conditions for seed production are favourable. Such a reduction in seed production may be explained by abortion which is a widespread bet hedging strategy to concentrate resources to remaining seeds (Lovett Doust & Lovett Doust 1988). Furthermore, sterile florets may still have a positive function since they increase the dispersal of seeds with spikelets as dispersal units in grass (e.g. Monty et al. 2016 for *Bromus tectorum*).

Appendix III: Vidaller C., Dutoit T., Ibrahim Y., Hanslin H.M, Bischoff A. (published in *American Journal of Botany* (2018), 105 : 1123-1132). Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum* - the role of soil conditions, grazing and humidity.

Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum*: The role of soil conditions, grazing, and humidity

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PREMISE OF THE STUDY: Genetic differentiation in plant species may result from adaptation to environmental conditions, but also from stochastic processes. The drivers selecting for local adaptation and the contribution of adaptation to genetic differentiation are often unknown. Restoration and succession studies have revealed different colonization patterns for *Brachypodium retusum*, a common Mediterranean grass. In order to understand these patterns, we tested population differentiation and adaptation to different environmental factors.

METHODS: Structured sampling of 12 populations from six sites and two soil types within site was used to analyze the spatial and environmental structure of population differentiation. Sampling sites differ in grazing intensity and climate. We tested germination and growth in a common garden. In subsets, we analyzed the differential response to stone cover, grazing and soil moisture.

KEY RESULTS: We found significant differences among populations. The site explained population differentiation better than soil, suggesting a dominant influence of climate and/or genetic drift. Stone cover had a positive influence on seedling establishment, and populations showed a differential response. However, this response was not related to environmental differences between collection sites. Regrowth after clipping was higher in populations from the more intensively grazed Red Mediterranean soils suggesting an adaptation to grazing. Final germination was generally high even under drought, but germination response to differences in soil moisture was similar across populations.

CONCLUSIONS: Adaptive population differentiation in germination and early growth may have contributed to different colonization patterns. Thus, the provenance of *B. retusum* needs to be carefully considered in ecological restoration.

KEY WORDS Common garden; local adaptation; plant origin; Poaceae; population differentiation; seedling recruitment.

Genetic differentiation among populations is an important strategy of plant species to survive under different environmental conditions (Linhart and Grant, 1996; Bischoff and Hurault, 2013). Adaptive differentiation occurs if environmental gradients are strong and gene flow counteracting differentiation is low (Kawecki and Ebert, 2004). Climate is a major driver of local adaptation and genetic differentiation in plant populations, in particular on large geographic scales (Etterson, 2004; Leinonen et al., 2009; Bucharova et al., 2016). However, microclimatic gradients may also act on much smaller scales resulting in small-scale adaptation

and differentiation (Knight and Miller, 2004; Lenssen et al., 2004; Bischoff et al., 2006).

The adaptation to soil conditions was first detected on soils contaminated with heavy metals indicating that adaptive differentiation may occur quite rapidly (Schat et al., 1996; Antonovics, 2006). Furthermore, the pH value and related chemical and biotic properties may represent a strong selective force (Macel et al., 2007; Raabová et al., 2011). Humidity is also a limiting factor for plant growth and reproduction leading to drought adaptation (Shah and Paulsen, 2003; Suriyagoda et al., 2010). However, an increase in soil

moisture often results in higher competition for other resources that represent a counteracting selective force (Kadmon, 1995).

Ecosystem management is a third potential driver of adaptive differentiation in semi-natural systems such as grasslands. Grazing changes site conditions by biomass removal, plant damage, reduced competition, and creates heterogeneity in grassland vegetation (Bullock et al., 2001; Hufford and Mazer, 2012). It seems to be likely that long-term grazing favors genotypes adapted to grazing (Hufford and Mazer, 2012). However, grazing may also obscure adaptation to other environmental factors if this selection pressure has not been consistent in the past (Hufford et al., 2008).

Genetic differentiation is not only the result of adaptation to different environments. Stochastic processes such as genetic drift considerably contribute to population differentiation and may even overwhelm local adaptation (Hereford and Winn, 2008; Hereford, 2009). Genetic drift and limited dispersal favor isolation by distance effects that are not related to environmental gradients (Sexton et al., 2013). Durka et al. (2017) found both isolation by distance caused by stochastic processes, and isolation by environment in seven tested plant species. The contribution of adaptive differentiation to genetic structure may even be low compared with genetic drift (Michalski and Durka, 2012; Sexton et al. 2013).

Genetic differentiation in quantitative, and thus potentially adaptive traits, is typically tested in common garden experiments (Petit et al., 2001; Leinonen et al., 2009; Bischoff and Müller-Schärer, 2010). Tests for local adaptation require reciprocal transplantation to original collection sites (Blanquart et al., 2013). However, common garden experiments comparing the response to the manipulation of key environmental factors allow conclusions on adaptive differentiation and may reveal more specific information on drivers of local adaptation than reciprocal transplantations in the field (Lenssen et al., 2004; Pahl et al., 2013).

We used the perennial grass *Brachypodium retusum* (Pers.) P. Beauv. as a model species to test for neutral and adaptive population differentiation at a regional scale in Southern France. The species is dominant in dry grasslands of the Western Mediterranean basin, but also occurs in matorral and open woodlands (Caturla et al., 2000; Catalan et al., 2015). In contrast to studies showing a high colonization capacity of the species (Caturla et al., 2000; Luis et al., 2004; Cassagne et al., 2011), spontaneous natural regeneration of *B. retusum* was poor in the La Crau nature reserve (Southern France) 30 years after conversion from arable use to traditional sheep grazing, complicating ecological restoration operations (Coiffait-Gombault et al., 2012). We hypothesize that genetic differentiation contributes to the different colonization patterns, and that this differentiation results from adaptation to major environmental factors distinguishing habitats. In the study region, *B. retusum* occurs in lower calcareous mountain ranges and on more or less decalcified red Mediterranean soils of former riverbeds such as the Crau area. The latter habitat is also characterized by stones (pebbles and cobbles) shaped by the former rivers. These stones were found to favor growth and survival of *B. retusum* because rhizomes and roots can grow underneath and thus benefit from higher soil moisture during the hot and dry Mediterranean summer (Buisson et al., 2015). Apart from soil characteristics and stone cover, these two habitats show also large differences in soil moisture and grazing intensity. The information on population differentiation and adaptation to environmental factors is crucial to predict colonization processes, but also to improve seed sourcing in ecological restoration (McKay et al., 2005; Vander Mijnsbrugge et al., 2010).

In our study, we tested *B. retusum* populations from calcareous and closest red Mediterranean soils replicated at different geographical distances to evaluate whether local-scale differentiation along strong environmental gradients can be as important as large-scale differentiation driven by macroclimate or genetic drift (Bischoff et al., 2006; Hereford and Winn, 2008; Durka et al., 2017). We further analyzed differential responses of these populations to grazing simulated by clipping, to stone cover and to soil moisture. We specifically wanted to answer the following research questions:

(1) At which scales does population differentiation occur: what is the role of local-scale soil type compared with large-scale variation? (2) Is this differentiation adaptive in terms of soil conditions (stone cover), management (grazing simulated by clipping) and humidity (soil moisture, germination)?

MATERIAL AND METHODS

Study species

Ramosé false brome (Poaceae, *Brachypodium retusum* P. Beauv.) is a rhizomatous perennial C3 grass species. Its rhizomes form a dense network under the soil surface, resulting in a high resilience to aboveground disturbance such as wildfires or grazing (Caturla et al., 2000). Vegetative growth is characterized by tillering at the stem base, but also by lateral branching at upper stem parts. *Brachypodium retusum* is an outcrossing, wind-pollinated species. It flowers from April to July and shows high clonal growth and reproduction when rhizome connections disintegrate. However, rhizomes are short and just allow a slow clonal spread ($< 5 \text{ cm} \times \text{year}^{-1}$ in our experiments).

Collection sites and sampling

The study and sampling sites were situated in the central part of the French Mediterranean climate zone from Montpellier to the lower Durance valley. Seeds were collected from six populations on base-rich calcareous soils (Calcaric cambisol) of lower mountain ranges with pH values of 7.8–8.9 (Table 1, Fig. 1). Close to each of these six populations on calcareous grasslands, six populations were also collected from usually decalcified red Mediterranean soils (Haplic cambisols) with pH values of 6.7–8.0. Three additional populations were collected in the Crau nature reserve as the focal site of Mediterranean dry grassland restoration (Buisson and Dutoit, 2006) in order to obtain a better representation of this largest red Mediterranean soil area of the study region. For further analysis, the four Crau populations (distance: 4–11 km) were pooled to the red Mediterranean soil population of Saint-Martin-de-Crau (SM, Table 1).

Red Mediterranean soils show a higher clay content than calcareous soils, resulting in a better nutrient and water retention. Because of the higher soil fertility, grasslands on red Mediterranean soils were more intensively used (in particular grazing, less frequently mowing) than those on calcareous soils (Table 1). They are also rich in hydrated oxides of iron and aluminum, and in silica whereas calcareous soils are poorer in these elements. Up to 50% of red Mediterranean soil surface is covered by cobbles and pebbles deposited by former rivers. Calcareous soils are less deep on calcareous bedrock and contain numerous angular brittle stones.

The study area is characterized by a typical Mediterranean climate with hot and dry summers, and mild and humid winters. The

TABLE 1. Soil characteristics, grazing intensity, annual precipitation, mean annual air temperature (including January and July mean in brackets), and the number of frost days ($T < 0$) at collection sites of the twelve populations. Sites are ordered from east to west. Climate data are based on daily averages from the nearest meteorological stations (1980-2010). *, Irregular mowing.

| Site code | Site name | Soil | Coordinates | pH | Grazing | Precipitation (mm*a ⁻¹) | Temperature (°C) Mean [Jan/Jul] | Days T<0 |
|-----------|------------------------|-------------------|-------------------------|-----|---------|-------------------------------------|---------------------------------|----------|
| Me | Mérindol | Calcareous | 43.756456 N, 5.199526 E | 8.0 | No* | 583 | 14.5 [6.0/23.7] | 49 |
| | | Red Mediterranean | 43.750425 N, 5.210495 E | 8.0 | Low | | | |
| Ca | Caumont | Calcareous | 43.898827 N, 4.941070 E | 8.2 | No | 676 | 14.6 [5.9/24.2] | 33 |
| | | Red Mediterranean | 43.898319 N, 4.932395 E | 6.9 | No | | | |
| SR | Saint-Rémy-de-Provence | Calcareous | 43.768047 N, 4.831898 E | 7.8 | No | 625 | 15.1 [6.6/24.3] | 30 |
| | | Red Mediterranean | 43.798952 N, 4.841659 E | 6.8 | High | | | |
| SM | Saint-Martin-de-Crau | Calcareous | 43.705354 N, 4.797561 E | 8.1 | No | 517 | 15.3 [7.4/24.4] | 24 |
| | | Red Mediterranean | 43.567465 N, 4.834645 E | 6.8 | High | | | |
| Ni | Nîmes | Calcareous | 43.784700 N, 4.259389 E | 8.0 | No | 701 | 14.9 [6.7/24.1] | 34 |
| | | Red Mediterranean | 43.722868 N, 4.339264 E | 7.8 | No* | | | |
| Mon | Montpellier | Calcareous | 43.645416 N, 3.691638 E | 8.9 | No* | 870 | 14.1 [6.4/23.2] | 51 |
| | | Red Mediterranean | 43.607580 N, 3.449269 E | 6.7 | No* | | | |

six sampling sites differ in climatic conditions (Table 1) whereas differences between soil types within sites are small. Annual precipitation depends on the proximity to mountain ranges and is generally higher in the western than in the eastern parts of the study area. Close to the Mediterranean Sea, winter temperatures are higher resulting in a lower frost frequency.

Seed collection and preparation

In each population, all inflorescences of each of 50 1-m² patches were collected. The minimum distance between patches was 10 m and sampling was done in one or several parallel lines depending on population shape (linear versus polygon). Because of clonal growth of the species, the real patch size of individuals is unknown. A distance of 10 m was considered sufficient to obtain samples from different individuals (Montalvo and Ellstrand, 2000). On average, one patch comprised 40 inflorescences resulting in a total of 2000 collected inflorescences per population. In germination experiments, the number of seed families may be higher than the number of patches because one patch may comprise more than one individual. This pooling at patch level reduces intrapopulation variation compared to half-sib designs based on individual seed families. In all other experiments, only one seedling per patch was kept for planting, corresponding to classical half-sib designs.

Population differentiation under common conditions

Population differentiation was tested in a growth chamber germination experiment and in a common garden study. Inflorescences were shaken in tubes with glass beads using a vortex mixer to separate seeds from glumes. Transparency under transmitted overhead projector light was used as a criterion to remove empty seeds without embryos. In December 2016, seeds were germinated in peat plugs (4 cm × 4 cm). Four seeds of each sampled patch were

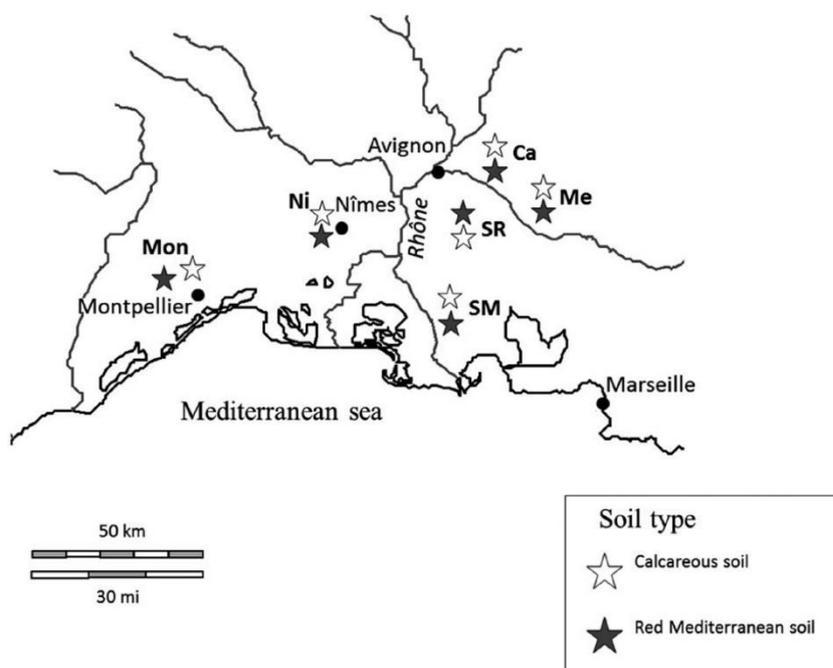


FIGURE 1. Position of six sampling sites each comprising two populations from different soil types.

sown per plug, and altogether, 40 patches of each population were randomly selected for a total of 160 seeds and 40 plugs per population. The plug trays were placed in a growth chamber (temperature 15/20°C, 12/12 h, night/day) and watered regularly. Coleoptile emergence used as a proxy of germination was recorded every 2–3 days for seven weeks. In March 2016, 24 plugs per population (corresponding to 24 patches or seed families) were randomly selected and transplanted to a previously ploughed experimental site in Avignon-Montfavet. Thus, 48% of the initially collected patches were represented in the field. Seedling number was reduced to one (average size) per plug prior to transplantation. Two plugs per population were planted to random positions within each of 12 blocks (3 m × 3 m each). During the first four months, seedlings were moderately watered. Plots were weeded by hand to limit competition.

Plant height, diameter, tiller number, leaf number, and the length of the longest leaf were measured once a month. Young *B. retusum* plants allocate resources to tillering and leaf growth. Later, tillers elongate and older leaves die. Therefore, we calculated cumulative leaf length as an aboveground biomass estimate for young seedlings (leaf length \times number of leaves) and cumulative tiller length for older plants (plant height \times number of tillers).

Adaptation to differences in soil conditions, management, and soil moisture

In May 2016, stones of 10–20 cm length and 7–15 cm width were placed in a circle around half of the seedlings of the common garden experiment to test their effect on plant growth. The stones were taken from the red Mediterranean soil of the Caumont population (Ca, Table 1, Fig. 1). Measurements were the same as in the population differentiation study under common conditions detailed above.

A subset of four population pairs (red Mediterranean and calcareous soils \times Me, Ca, SR, SM sites) was grown one year earlier in a similar common garden experiment to simulate grazing by clipping established plants. Clipping of second year plants prevents high mortality compared to clipping of young seedlings. The design was similar to the differentiation study. In November 2014, seeds were sown in peat plugs and placed in a greenhouse. In February 2015, two seedlings of each population were planted in each of 12 blocks (24 seedlings in total). Because of low germination, seedling number was only one per block for the Me population from calcareous soil (12 seedlings in total). Plants were moderately watered during the first year, but not in the second year. In April 2016, half of the seedlings were clipped 2 cm above the soil. We measured the same traits as in the differentiation study. Additionally, the number of inflorescences per plant was counted at the end of the season. This was not possible in the population differentiation study under common conditions because plants rarely (<10%) flower in the first year.

The response to soil moisture during germination was analyzed in a controlled experiment manipulating moisture levels. The major objective was to test adaptive differentiation in germination response under dry conditions. Five moisture levels from 0.3 to 20% were adjusted by adding water to plastic boxes filled with 1.15 kg of sand and using a Theta Probe ML2x sensor (Delta-T Devices Ltd, Cambridge, United Kingdom). A subset of 10 populations was chosen for this experiment. Six seeds of each population (four for calcareous population of SM due to limited seed number) were sown in each of 12 boxes and each of the five soil moisture levels; this resulted in a total of 360 seeds per population assigned to 60 boxes. Boxes were closed with a translucent lid to keep initial moisture levels. The boxes were placed in a growth chamber with a 12-h cycle of light and temperature (day: 25°C; night: 12°C). Germination and moisture were measured every three days for a period of six weeks. Germinated seeds were removed. At the end of the experiment, the water potential of the sand was measured using WP4C Dewpoint Water Potentiometer (Decagon Devices, Pullman, Washington, USA). Water potentials were close to 0 (saturation) for the highest and -0.18 MPa for the lowest moisture level (Appendix S1, see Supplemental Data with this article).

Statistical analysis

Linear models were used to analyze population and treatment effects on germination and growth traits. Population was divided into

a soil and site component. The two adjacent populations from red Mediterranean and calcareous soils were considered as originating from the same site resulting in a total of six sites and two soil types per site. Thus, each population represented a particular combination of site and soil. Site, soil, and their interaction were included as fixed effects in the models. The effect size of site of origin, soil of origin, and the site \times soil interaction was calculated as $100 \times$ factor sum of square/total sum of squares.

The common garden studies were designed as completely randomized block experiments; thus, block was included as an additional random factor. The adaptation treatments (stone cover, clipping) were included as a fixed factor for measurements taken after treatment start. In some cases $\log_{10}(x + 1)$ transformation was required to comply with assumptions of linear models (normality, homoscedasticity).

In the germination experiments, final germination percentage in plugs (population differentiation study) and in boxes (soil moisture test) was calculated as response variable. Germination percentage was $\arcsin(\sqrt{x/100})$ transformed prior to analysis (Quinn and Keough, 2002). Because the position of plugs was completely randomized before transplantation to the field, no block effect was considered (germination in the population differentiation study). The soil moisture test on germination was analyzed in a split-plot model. The box effect was included as a random factor nested in moisture level. Accordingly, moisture was tested against the box effect whereas soil, site, and all interactions were tested against the model error.

A Tukey honest significance test (HSD) posthoc test was calculated to analyze differences between treatment levels more in detail if the treatment main effect or interactions were significant. Posthoc tests were preferred over a priori linear contrasts because we had no clear a priori hypothesis on specific adaptations to tested environmental factors. All statistical analyses were run in R (R, version 3.3.1, R Development Core Team (2013)).

RESULTS

Population differentiation under common conditions

A high variation in germination capacity was found between populations ranging from 10% (Ca, red Mediterranean soil) to 75% (Ni, calcareous soil) (Fig. 2). Site and soil of origin as well as their interaction, had a significant influence on germination (Table 2). Populations from calcareous soils and from the westernmost sites germinated significantly better than those from red Mediterranean soils and eastern parts of the study area, respectively. Differences between soil types depended on site of origin, resulting in a significant interaction.

Population differentiation in plant growth was smaller than in germination, but still significant (Table 2, Fig. 2). Differentiation in growth traits did not decrease during the growing season (Appendix S2). The site of origin contributed more to the differentiation than soil of origin. Effect size of site of origin was between 3% of total variation for leaf length and 14% for leaf number whereas effect size of soil of origin was always lower than 1% (Fig. 3). The site \times soil interaction explained 1% and 7.7%, respectively, of the model variation. In agreement with the germination results, the westernmost populations from Ni and Mon showed a higher performance in early (leaf length, cumulative leaf length) and late growth traits (tiller length and number, cumulative tiller length, tussock

diameter). The soil type effect was only significant for leaf length being higher in populations from red Mediterranean soils than in those from calcareous soils. In several traits, in particular in leaf length, tiller length and plant diameter, a significant site × soil of origin interaction was detected. For the westernmost sites of origin, growth was higher in populations from red Mediterranean soils whereas no differences or even a higher performance on calcareous soils were found for populations from the eastern and central parts of the study area.

Adaptation to differences in soil conditions, management, and soil moisture

Stone cover around the seedlings significantly increased plant height, tiller number, and thus cumulative tiller length, but not cumulative leaf length (Table 2, Fig. 4). The effect of stone cover was the same for plants from the two soil types, while stone cover had contrasting effects on leaf number and cumulative leaf length as well as on tiller number and cumulative tiller length of plants from the different sites. The populations from SR including the SM populations showed a consistent positive response to stone cover, whereas the response of the Me populations was negative or neutral.

Simulated grazing (clipping) significantly reduced plant height, leaf number, tiller number, cumulative tiller length, cumulative leaf length, and inflorescence number (Table 3, Fig. 5). The negative effect on tiller number was the result of cutting underneath lateral branches that were also counted as tillers. While site × treatment interactions were not significant, several significant soil × treatment interactions were detected. Populations from red Mediterranean soils showed significantly higher compensatory growth resulting in smaller differences in

plant height, cumulative tiller length, and inflorescences number between clipped and nonclipped plants.

The test of soil moisture effects on germination in different *B. retusum* populations revealed high germination rates even under dry

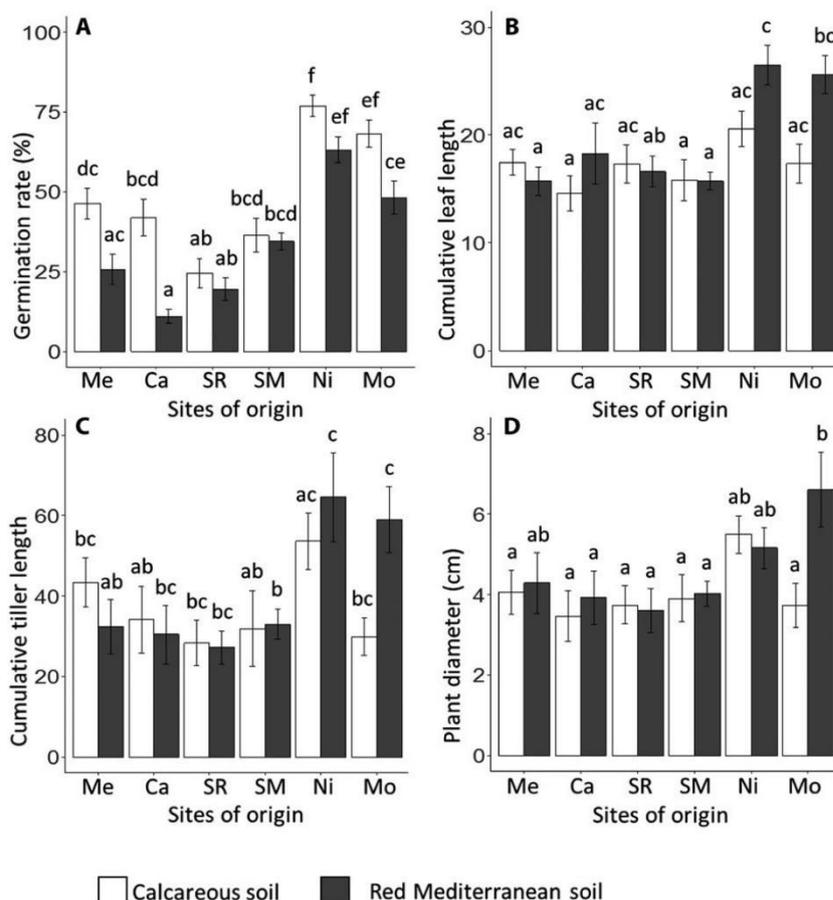


FIGURE 2. Population differentiation decomposed in collection site and soil type within collection site (mean values ±SE, see Table 2 for statistics). A, Final germination. B, Cumulative leaf length. C, Cumulative tiller length. D, Plant diameter. Different lower case letters indicate significant differences ($P < 0.05$).

TABLE 2. Differentiation in *Brachypodium retusum* populations from different sites and soil types, and differential population response to stone cover treatment. *F*-values and significance levels from two-way ANOVA before stone treatment (growth traits: May) and three-way ANOVA afterwards (July) including the stone effect. * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; NS, not significant.

| | | df | Germination | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter |
|------------------------|----------------------|-------|-------------|-------------|-------------|--------------|---------------|------------------------|--------------------------|----------------|
| Before treatment (May) | Site | 5,338 | 31.41 **** | 1.82 NS | 11.81 **** | 6.62 **** | 4.87 **** | 8.12 **** | 6.98 *** | |
| | Soil | 1,338 | 36.83 **** | 0.00 NS | 5.30 ** | 0.00 NS | 0.02 NS | 1.05 NS | 0.00 NS | |
| | Soil × Site | 5,338 | 3.08 *** | 0.64 NS | 6.60 *** | 2.06 * | 1.76 NS | 2.00 * | 2.13 * | |
| After treatment (July) | Site | 5,315 | | 1.73 NS | 3.99 *** | 7.09 **** | 6.93 **** | 4.89 **** | 8.48 *** | 4.01 ** |
| | Soil | 1,315 | | 0.00 NS | 5.07 ** | 0.37 NS | 0.34 NS | 1.32 NS | 0.38 NS | 1.61 NS |
| | Stones | 1,315 | | 1.58 NS | 1.09 NS | 4.24 ** | 5.48 ** | 0.03 NS | 5.67 * | 4.16 * |
| | Soil × Site | 5,315 | | 0.97 NS | 3.02 ** | 2.18 ** | 1.33 NS | 2.04 * | 1.76 NS | 2.52 * |
| | Stones × Site | 5,315 | | 2.02 * | 0.54 NS | 1.56 NS | 2.87 ** | 0.18 NS | 2.24 * | 0.38 NS |
| | Stones × Soil | 1,315 | | 0.10 NS | 1.90 NS | 0.59 NS | 0.02 NS | 1.14 NS | 0.05 NS | 0.44 NS |
| | Stones × Soil × Site | 5,315 | | 0.49 NS | 0.18 NS | 2.26 ** | 0.21 NS | 1.18 NS | 0.79 NS | 0.26 NS |

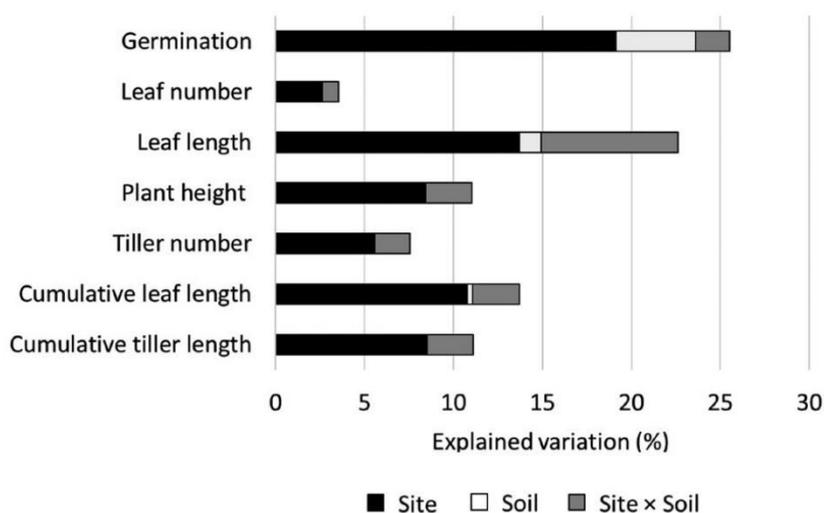


FIGURE 3. Percentage of variation explained by collection site, soil type within collection site and site x soil interaction for growth traits (May).

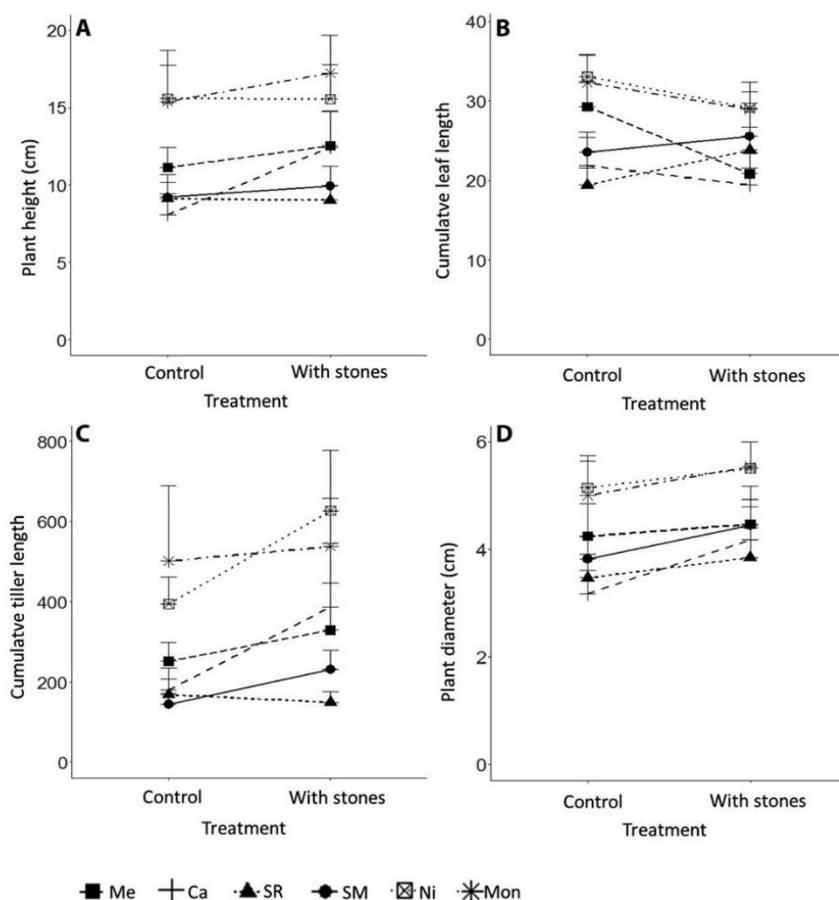


FIGURE 4. Differential response to stone cover (mean values +SE) between *Brachypodium retusum* populations in southern France. A, Plant height. B, Cumulative leaf length. C, Cumulative tiller length. D, Plant diameter.

conditions (0.3% volumetric water content) and a significant decrease of germination under maximum soil moisture (at saturation level: 19.5%, Fig. 6). The highest germination was obtained for intermediate soil moisture levels. With 50% to 80% on average, germination rates were higher than in peat plugs used for the germination test on population differentiation. In agreement with the peat plug germination test, site and soil type effects were significant with a higher germination rate in populations from calcareous soils and from the westernmost sites, respectively (Fig. 6).

The interaction between soil type of origin and soil moisture was not significant and the one between site and soil moisture was only marginally significant suggesting similar adaptation to dry conditions. The marginally significant interaction was explained by a stronger negative response of the Ni populations to drought stress compared with populations from the three other sites.

DISCUSSION

Population differentiation under common conditions

Our common garden study demonstrated genetic differentiation among populations at a regional scale. We found significant differences between populations for germination capacity and several morphological traits such as leaf length, tiller number, and height whereas variation in leaf number per tiller was low. In particular, germination and tiller number are closely linked to seedling recruitment and establishment, thus supporting our hypothesis that different colonization patterns observed at different Mediterranean sites (high: Caturla et al. 2000, Luis et al. 2004; low: Coiffait-Gombault et al., 2012) may result from genetic differentiation.

Site of origin better explained this differentiation than soil type. Independent of soil type, the two eastern populations showed significantly lower germination rates and growth than western populations. The significant effect of site is likely the result of climatic differences between eastern and western parts of the study area although our design does not allow us to distinguish random genetic drift from climatic differences as drivers of differentiation. The sites of the western populations (Ni, Mon) are closer to the Cévennes Mountains and receive more rainfall than those of the Eastern populations (Ceresetti, 2011).

TABLE 3. Differential response in a subset of populations to simulated grazing. *F*-values and significance levels from three-way ANOVA on site, soil (within site) and clipping effects. Vegetative traits measured in spring (May), inflorescence number in June of the second growing season. * $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; NS, not significant.

| | df | Leaf number | Leaf length | Plant height | Tiller number | Cumulative leaf length | Cumulative tiller length | Plant diameter | Inflorescence number |
|------------------------|-------|-------------|-------------|--------------|---------------|------------------------|--------------------------|----------------|----------------------|
| Site | 3,132 | 1.20 NS | 1.13 NS | 0.27 NS | 0.63 NS | 1.47 NS | 0.64 NS | 1.30 NS | 2.48 * |
| Soil | 1,132 | 0.02 NS | 9.43 *** | 3.16 * | 0.05 NS | 4.13 ** | 2.87 * | 1.54 NS | 3.91 * |
| Clipping | 1,132 | 12.31 **** | 3.19 * | 220.98 **** | 10.48 *** | 11.46 **** | 211.72 **** | 7.23 ** | 58.39 *** |
| Soil × Site | 3,132 | 0.67 NS | 2.12 NS | 0.81 NS | 0.16 NS | 2.05 NS | 1.18 NS | 0.67 NS | 1.98 NS |
| Clipping × Site | 3,132 | 0.66 NS | 0.49 NS | 0.81 NS | 0.02 NS | 0.99 NS | 1.49 NS | 0.94 NS | 0.23 NS |
| Clipping × Soil | 1,132 | 0.08 NS | 0.09 NS | 3.93 ** | 2.17 NS | 0.12 NS | 3.07 * | 0.48 NS | 3.37 * |
| Clipping × Soil × Site | 3,132 | 1.12 NS | 0.61 NS | 1.65 NS | 0.76 NS | 0.43 NS | 1.08 NS | 0.40 NS | 0.29 NS |

Climate is known to be a major driver of population differentiation and adaptation (Linhart and Grant, 1996; Etterson, 2004; Ravenscroft et al., 2014). Water availability and temperature have a particularly strong effect on plant fitness leading to population differentiation in the case of spatial variation (Shah and Paulsen, 2003; Xu and Zhou, 2006). Yuan et al. (2016) found for example that population differentiation in the grass species *Leymus chinensis* was driven by precipitation, temperature, and soil water content. A mainly soil moisture driven differentiation was also shown for *Boechera stricta* (Lee and Mitchell-Olds, 2011). However, the authors also found that isolation by distance due to stochastic processes such as genetic drift considerably contributes to population differentiation. Adaptive differentiation and isolation-by-distance effects often act at the same time on the genetic structure of populations and their contribution to differentiation may be difficult to distinguish (Michalski and Durka, 2012; Durka et al., 2017). Nevertheless, shoot and leaf growth (number and length) were lower in populations from dryer Eastern sites suggesting a selection for growth forms with smaller and less numerous leaves and shoots that are better protected against drought stress (Liancourt and Tielbörger, 2009; Pérez-Ramos et al., 2013). The significant site effects on the cited growth traits were stable throughout the whole growing season and did not decrease with time suggesting a minor influence of environmental maternal effects on our results. Environmental maternal effects may contribute to population differentiation, but particularly affect early traits and vanish during plant development (Roach and Wulff, 1987; Donohue, 2009).

Soil type is also known to select for population differentiation in plants (Linhart and Grant, 1996; Raabova et al., 2011). In our study, we detected a significant soil type of origin effect on differentiation at a small scale of 10–20 km, but only for germination capacity and leaf length. Germination is under stronger

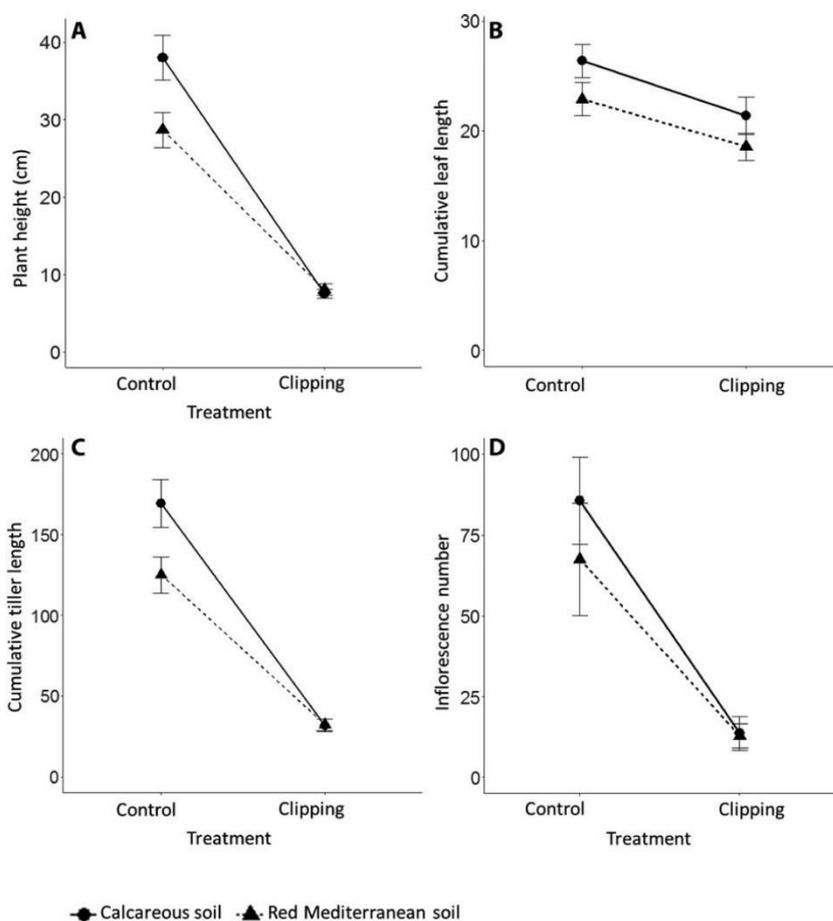


FIGURE 5. Differential response of a subset of populations to simulated grazing (mean values \pm SE). Since site \times clipping interaction was never significant only soil type \times clipping combinations are presented. A, Plant height. B, Cumulative leaf length. C, Cumulative tiller length. D, Inflorescence number.

control of environmental maternal effects than growth traits affecting population differentiation in such early traits (Roach and Wulff, 1987; Bischoff and Müller-Schärer, 2010). However, it is also possible that selection for clonal growth under higher grazing pressure of red Mediterranean soils has reduced allocation of resources to seeds compared with less grazed calcareous sites. Although providing some evidence for soil of origin effects, our

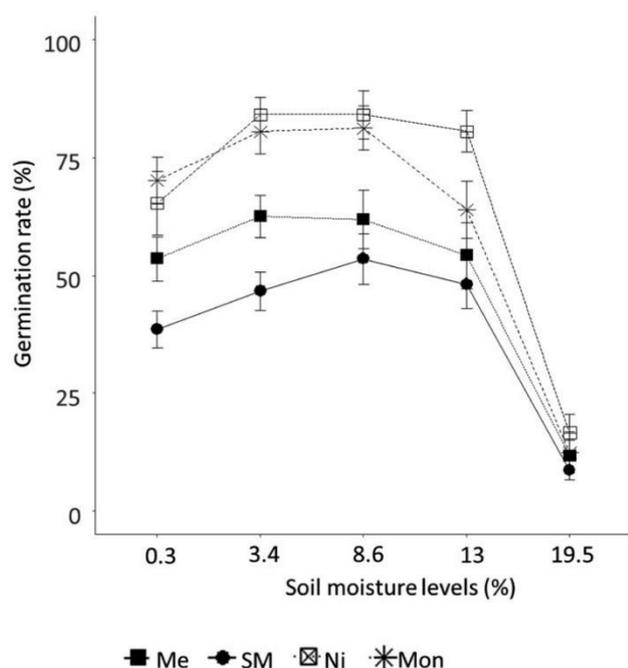


FIGURE 6. Differential germination response of a subset of populations to soil moisture (mean values \pm SE). Since the soil of origin \times soil moisture interaction was never significant only site of origin effects are presented. Soil moisture ($F_{4,55} = 53.71$, $P < 0.001$), site of origin ($F_{3,505} = 27.96$, $P < 0.001$), soil of origin ($F_{1,505} = 33.86$, $P < 0.001$), site of origin \times soil of origin \times moisture interaction ($F_{12,505} = 1.726$).

results do not confirm earlier studies showing a high small-scale differentiation along strong environmental gradients (Lenssen et al., 2004; Bischoff et al., 2006; Raabová et al., 2007; Bischoff and Hurault, 2013). Because no specific barriers prevented pollen dispersal at this scale, gene flow may have counteracted local adaptation to soil type (Kawecki and Ebert, 2004).

Adaptation to differences in soil conditions, management, and soil moisture

Brachypodium retusum populations showed a differential response to stone cover and clipping, and to a lesser degree to soil moisture in the germination phase.

Our study demonstrated a clearly positive effect of stone cover on the growth of *B. retusum*, in particular on plant height, tiller number, and plant diameter. Stone cover increases soil moisture by shading and water condensation, and protects lower stem parts and roots from strong solar radiation and heat (Peters et al., 2008; Buisson et al., 2015). A differential response of Aizoaceae species demonstrated that plants might be locally adapted to type and density of stone cover (Ellis and Weis, 2006). In our study, however, the differential response was not related to the dominant soil types, but to site of origin. Although the texture and depth of calcareous soils and red Mediterranean soils are different, limestone layers and pieces close to the soil surface may have similar effects on microclimate. Thus, the differential response to stone cover cannot be related to differences in local environmental conditions at collection sites.

As expected, clipping largely reduced plant height, tiller number, and leaf number throughout the whole season resulting in a highly reduced reproduction. The effect was less strong in populations from red Mediterranean soils than in populations from calcareous sites suggesting an adaptation to higher grazing intensity. Grazing exerts strong directional selection and may result in genetic differentiation and local adaptation to long-term differences in grazing intensity (Linhart and Grant, 1996; Hufford and Mazer, 2012).

Higher compensatory growth may be one important adaptation of grasses to higher grazing intensity (Sarmiento, 1992). Although we do not exactly know whether our collection sites show such long-term differences, red Mediterranean soils were more grazed in the past than calcareous soils because slopes are less steep and rocky, and soils are more fertile—both of these attributes favor sheep grazing (Verheye and De la Rosa, 2009).

Drought stress reduces germination, and usually plant species show a positive correlation between soil moisture and germination rates (Bochet et al., 2007; Thomas et al., 2010). The ability to germinate fast under moderate-to-low soil moisture may be crucial for species colonizing dry environments (Schütz et al., 2002; Bochet et al., 2007). All tested *B. retusum* populations showed a high germination capacity even under the lowest soil moisture level (-0.18 MPa = 0.3% volumetric water content), but a considerable drop under saturated conditions. The observed germination pattern differs from the results of Bochet et al. (2007) who found a strong decrease in *B. retusum* germination already at water potentials of -0.01 and -0.05 MPa. Although the results may depend on age of seeds and specific experimental conditions this difference suggests genetic variability in germination response to soil moisture. Environmental maternal effects may contribute to population differentiation in germination, but there is little evidence for a contribution to adaptive responses through transgenerational plasticity (Galloway and Etterson, 2007; Bischoff and Müller-Schärer, 2010). In our study, the response to soil water potential was not much different among populations. Only the population of one of the two sites with the most humid climate (Ni) showed a stronger decline under dry conditions suggesting weak adaptation to climate-mediated soil moisture.

CONCLUSIONS

Significant population differentiation was found at a regional scale of 30–200 km. Differentiation between major soil types representing strong environmental differences were found at even smaller scales (10–20 km). However, differentiation between soil types was weaker than differentiation among sites, suggesting a major influence of climate and/or genetic drift on population differentiation.

Stone cover increased *B. retusum* growth and the differential response of populations suggests adaptation to this environmental factor. However, we could not relate this response to the home environment of tested populations. Further experiments are required to identify potential drivers of adaptation to stone cover (density at the surface or in different soil layers). The response to clipping was also clearly different among populations, and the differential response could be linked to the major soil type suggesting that different grazing intensity in the past has selected for different ecotypes. *Brachypodium retusum* showed high germination under dry soil conditions, but there was only weak evidence for a differential response of tested populations. The study demonstrated that

population differentiation may well explain different colonization patterns observed in different Mediterranean regions (Caturla et al., 2000; Luis et al., 2004; Coiffait-Gombault et al., 2012). Because observed differences may largely influence seedling establishment in Mediterranean dry grasslands, plant origin needs to be carefully considered in ecological restoration involving planting or sowing of *B. retusum*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Extended abstract

The ecological restoration of Mediterranean Thero-Brachypodietea grasslands (“steppes”) based on spontaneous succession following soil disturbance is a very slow process. Even after several decades of restoration, the species composition is often different from that of the reference community. In particular, key species hardly establish. *Brachypodium retusum* is a dominant widespread core species of such dry Mediterranean grasslands managed by grazing. However, approaches to restore such grasslands and to re-establish *B. retusum* have failed until now in the greatest French Mediterranean steppe area of the Crau plain. Previous studies showed that the recreation of initial environmental conditions is not sufficient to restore the plant community with its dominant species. Even the transfer of *B. retusum* rich hay has not been successful to re-establish populations suggesting a low seedling recruitment. The absence of the dominant species has a strong effect on community structure and functioning. Furthermore, the species plays an important role in sheep nutrition and its re-establishment on degraded grassland systems would thus represent an agro-economic benefit.

Surprisingly, former studies in other Mediterranean systems have not confirmed the slow re-colonisation. The species rapidly re-occur after wildfires and is generally frequent in habitats of moderate disturbance. This may be explained by the strong resprouting capacity from belowground organs representing a competitive advantage over annual species. However, a high re-colonisation was even found after intensive soil disturbance by ploughing.

The PhD thesis aims to identify the causes for the low establishment of *Brachypodium retusum* in Mediterranean steppes and in particular in the Crau area. Two main hypotheses are tested (i) the genetic differentiation explains differences in colonisation capacity, (ii) colonisation depends on environmental conditions such as humidity, grazing or fire that may limit or favour seedling recruitment. This project allows us to better understand the ecology of this key species in order to improve the efficiency of restoration measures.

Specifically, this PhD thesis aims to answer to the following major questions:

- (1) What are the spatial and environmental scales of genetic differentiation? Is differentiation adaptive? What are the major drivers of differentiation and adaptation? What are the implications for provenance choice in ecological restoration?
- (2) Which ecological factors limit establishment? In particular, do fire, grazing, initial watering and their interactions improve or reduce the establishment of *B. retusum* acting on seed production, germination, survival and growth?

The thesis comprises a general introduction, four research chapters and a general discussion. Each research chapter is written as a stand-alone paper. One paper is currently published, one is in revision (minor), one is submitted and the last one in preparation for a submission by the end of October 2018.

Chapter I: Comparison of neutral and adaptive differentiation in the Mediterranean grass species *Brachypodium retusum*. (Submitted to *Molecular Ecology*)

Chapter II: Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum* - the role of soil conditions, grazing and humidity. (Published in *American Journal of Botany*, 2018 (105) 1123-1132)

Chapter III: Fire increases the reproduction of the dominant grass *Brachypodium retusum* and Mediterranean steppe diversity in a combined burning and grazing experiment. (Accepted with minor revision in *Applied Vegetation Science*)

Chapter IV: Which factors limit early establishment of *Brachypodium retusum* a dominant species of Mediterranean grasslands? (Submission to *Basic and Applied Ecology* by the end of October 2018)

Genetic differentiation in plant species may result from adaptation to environmental conditions but also from stochastic processes. The drivers selecting for local adaptation and the contribution of adaptation to genetic differentiation are often unknown. Previous restoration and succession studies have revealed different colonisation patterns for *Brachypodium retusum*, a common perennial Mediterranean grass. In order to understand these patterns, we analysed genetic structure including population differentiation in 17 Mediterranean populations using AFLP markers. In a subset of 13 populations this neutral differentiation was compared to differentiation in phenotypic traits (**Chapter I**). We further tested adaptation to different environmental factors in a common garden experiment (**Chapter II**).

In **Chapter I**, the mechanisms driving population differentiation and adaptation were analysed by comparing pairwise θ_{ST} and P_{ST} . Seeds or leaves from seventeen populations were collected in France (14 populations), Spain (2 populations) and Italy (1 population) for a total of 322 individuals genotyped with 323 AFLP markers. A subset of thirteen French populations was grown for two years in a common garden experiment to measure vegetative growth and reproduction. Based on AFLP marker analysis, diversity indices and their relations to climate and soil conditions were calculated. Population differentiation in neutral AFLP markers was estimated using pairwise θ_{ST} . First axis PCA scores of quantitative traits were fitted to determine

P_{ST} values (adaptive differentiation), and P_{ST} were compared to θ_{ST} . Global dataset θ_{ST} (0.102, $P < 0.001$) indicates significant differentiation between populations. P_{ST} (0.171; $P < 0.001$) was higher than θ_{ST} (0.072; $P < 0.001$) suggesting that phenotypic population differentiation is driven by directional selection leading to adaptive differentiation. This adaptive differentiation at a regional scale (Southern France) advocates a careful choice of plant origin in ecological restoration.

The aim of **Chapter II** was to focus on adaptive population differentiation by manipulating environmental factors in common garden and growth chamber experiments. Different subsamples of populations tested in the genetic analyses (Chapter 1) were used to analyse the spatial and environmental structure of population differentiation. These subsamples comprised twelve populations from six sites and two soil types (Red Mediterranean, calcareous) within site. Sampling sites differ in grazing intensity and climate. In these populations of different site and soil origin, we analysed the differential response to stone cover, grazing and soil moisture. We found significant differences among populations. Site explained population differentiation better than soil suggesting a dominant influence of climate and/or genetic drift. Stone cover had a positive influence on seedling establishment and populations showed a differential response. However, this response was not related to environmental differences between collection sites. Regrowth after clipping was higher in populations from the more intensively grazed Red Mediterranean soils suggesting an adaptation to grazing. Final germination was generally high even under drought but germination response to differences in soil moisture was similar across populations. Adaptive population differentiation in germination and early growth may have contributed to different colonisation patterns. The results confirm that the provenance of *B. retusum* needs to be carefully considered in ecological restoration.

Seed production and seedling establishment represent a strong filter for re-colonisation of plant species. In Mediterranean dry grasslands, three main factors influence population dynamics. Sheep grazing is one of the key management factors driving plant species composition and diversity. Wildfires and fire management were also important drivers in the past but their role in maintaining the ecosystem is nowadays less clear because fire prevention and abandonment of traditional prescribed burning have largely reduced their impact in the last decades. Drought stress may be a third factor limiting seedling recruitment.

The last two parts of the thesis analysed environmental factors that may limit establishment: the effect of grazing and fire on vegetative regeneration as well as on the sexual reproduction

of adult *B. retusum* (**Chapter III**) and the impact of watering and grazing on the growth of transplanted and sown seedlings of local origin (**Chapter IV**).

The aim of **Chapter III** was to understand the effect of fire and grazing on vegetative growth and sexual reproduction of adult *Brachypodium retusum* since seed production in potential source populations may largely influence seed availability and recruitment at adjacent restoration sites. These factors were also tested on species composition of the associated plant community in Mediterranean steppe of the Crau (South-Eastern France) because fire cannot be suggested as a restoration tool for increase *B. retusum* colonisation without considering its effects on the whole plant community.

We set up a split-plot experiment testing the effects of fire season (late winter, summer) and grazing on plant cover, inflorescence number, seed set and germination of *B. retusum* in traditionally managed grasslands. We further analysed plant species composition and diversity in vegetation relevés. The same fire treatments were tested in a second experiment in long-term exclosures abandoned 17 years ago using the same protocol of measurements.

B. retusum showed a significant rapid post-fire recovery but increase in plant cover was not faster than in other species of the plant community. Nevertheless, summer fire increased inflorescence production of the species and seed set per inflorescence. At community level, fire significantly increased species richness, evenness and Bray-Curtiss dissimilarity and again, the effect of summer fire was stronger. Grazing exclusion for two seasons had only a small effect on *B. retusum* and plant community. The effect of the two fire treatments on *B. retusum* was similar in long-term exclosures. In these exclosures, fire resulted in a shift in plant species composition towards the traditionally managed steppe.

The concomitant positive effects of experimental burning on *B. retusum* reproduction and on plant diversity of Mediterranean steppe vegetation suggest that the system is adapted to fire as an important driver of community dynamics. Seedling recruitment of annual species is as successful in post-fire vegetative recovery as resprouting of perennials such as *B. retusum*. Prescribed burning may be an alternative strategy to maintain community structure in abandoned Mediterranean grasslands. However, plant community and dominant species tolerate the absence of grazing in the short term (two years) showing no significant changes in tested response variables.

In **Chapter IV** we analysed the effects of grazing and watering on seedling recruitment and early establishment of *Brachypodium retusum*. Grazing is known to favour the species but the effect on seedling recruitment may still be negative. Inversely, soil moisture increases

competition and favours ruderal species in the long run but initial watering may still be beneficial to establish young seedlings.

We set up a split-plot experiment testing the effects of grazing and initial watering on seedling height, leaf length, tiller number, leaf number and seedling diameter for (i) transplanted seedlings and (ii) sown seedlings on ex-arable fields of the Crau.

Survival after one year was 40 % for transplanted seedlings and 27 % for sown seedlings. Grazing negatively affected field germination whereas the watering effect was not significant. Grazing reduced plant height but the effect disappeared six months after the grazing period. Watering had a significantly positive effect on early plant growth that disappeared in the second growing season. Main effects of both factors on survival were not significant but a significant interaction occurred: initial watering had a positive effect only in grazed plots compensating for recurrent “grazing stress”. In ecological restoration, grazing enclosure in the beginning may reduce negative drought effects on survival. If not possible, initial watering is then an alternative way to limit seedling mortality.

In conclusion, the adaptive differentiation between populations demonstrated in my studies may have contributed to regional differences in terms of colonising capacity. The combination of drought and grazing stress limits seedling recruitment and survival during the hot and dry Mediterranean summer. Fire generally increases seed production of potential source populations and may thus be an original tool for restoring degraded areas through indirectly favouring the arrival of seeds on restoration sites.

Keywords: AFLP; common garden; dry grassland; exclosures; local adaptation; Mediterranean grassland; neutral markers; plant origin; poaceae; population differentiation; prescribed burning; θ_{ST} ; P_{ST} ; phenotypic traits; seedling recruitment; Thero-Brachypodietea

Scientific communications and publications

Chapter I

Publication

- Vidaller C., Baumel A., Juin M., Dutoit T., Bischoff A. (submitted to *Molecular Ecology* in October 2018). Comparison of neutral and adaptive differentiation in the Mediterranean grass species *Brachypodium retusum*.

Communication

- Vidaller C., Baumel A., Juin M., Dutoit T., Bischoff A. (2018 – oral) Adaptive and neutral differentiation in the Mediterranean grass species *Brachypodium retusum* populations: consequences for ecological restoration. International Conference on Ecological Sciences, Sfecologie2018, Rennes, France, 22th - 25th October 2018.

Chapter II

Publication

- Vidaller C., Dutoit T., Ibrahim Y., Hanslin H.M, Bischoff A. (published in *American Journal of Botany* (2018), 105 : 1123-1132). Adaptive differentiation among populations of the Mediterranean dry grassland species *Brachypodium retusum* - the role of soil conditions, grazing and humidity.

Communication

- Vidaller C., Dutoit T., Bischoff A. (2016 – oral) La différenciation entre populations de *Brachypodium retusum*: Conséquences pour la restauration des steppes méditerranéennes. 7th symposium of the French Ecological Restoration Network – REVER 7, Bordeaux, France, 19th - 20th January 2016.
- Vidaller C., Dutoit T., Bischoff A. (2016 – oral) Differentiation between populations of *Brachypodium retusum*: Consequences for the restoration of the Mediterranean steppes. Conservation Sciences in the Mediterranean Region, Tour du Valat, Arles, France, 22th - 24th March 2016.
- Vidaller C., Dutoit T., Bischoff A. (2016 – oral) Differentiation among populations of *Brachypodium retusum*: Consequences for the restoration of Mediterranean steppes. 10th European Conference on Ecological Restoration, Freising, Germany, 22th - 26th August 2016.
- Vidaller C., Dutoit T., Bischoff A. (2016 – oral) Differentiation among populations of *Brachypodium retusum*: Consequences for the restoration of Mediterranean steppes. International Conference on Ecological Sciences, Sfecologie2016, Marseille, France, 24th - 28th October 2016.

Chapter III

Publication

- Vidaller C., Dutoit T., Ramone H., Bischoff A. (Accepted with minor revision in *Applied Vegetation Science* in October 2018). Fire increases the reproduction of the dominant grass *Brachypodium retusum* and Mediterranean steppe diversity in a combined burning and grazing experiment.

Communication

- Vidaller C., Dutoit T., Bischoff A. (2017 – oral) Influence du feu et du pâturage sur la croissance et la reproduction d'une espèce clé (*Brachypodium retusum*) d'une pelouse méditerranéenne (La Crau, Sud-est de la France). 13th Congrès international francophone en écologie végétale, ECOVEG13, Forêt Montmorency, Québec, Canada, 10th - 13th September 2017.
- Vidaller C., Dutoit T., Bischoff A. (2017 – poster) Le feu comme outil de restauration ? Résultats préliminaires sur une espèce clé (*Brachypodium retusum*) d'une pelouse méditerranéenne (La Crau). 8th symposium of the French Ecological Restoration Network – REVER 8, Arras, France, 8th - 9th March 2017.

Chapter IV

Publication

- Vidaller C., Dutoit T., Ramone H., Bischoff A. (planned to be submitted to *Basic and Applied Ecology* in 2018). Which factors limit early establishment of *Brachypodium retusum* a dominant species of Mediterranean grasslands?

Chapter III & IV

Communication

- Bischoff A., Vidaller C., Dutoit T. (2017 – oral) The effect of prescribed burning, initial watering and grazing on the restoration of the Mediterranean grass *Brachypodium retusum*. 7th World Conference on Ecological Restoration, Iguassu, Brazil, 27th August – 1st September 2017.
- Vidaller C., Dutoit T., Ramone H., Bischoff A. (2017 – oral) Influence du feu, de l'arrosage et du pâturage sur l'installation, la croissance et la reproduction d'une espèce clé (*Brachypodium retusum*) pour la restauration d'une pelouse méditerranéenne (La Crau, Sud-Est de la France). 9th symposium of the French Ecological Restoration Network – REVER 9, Tour du Valat, Arles, France, 4th - 6th April 2018.