Restoration genetics of north-west European saltmarshes: A multi-scale analysis of population genetic structure in *Puccinellia maritima* and *Triglochin maritima*

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Statement of originality

I hereby confirm that this PhD thesis is an original piece of work conducted independently by the undersigned and all work contained herein has not been submitted for any other degree. All research material has been duly acknowledged and cited

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Date: 22/09/2014

General abstract

Increasing human pressure combined with sea level rise and increased storminess is threatening coastal ecosystems around the world. Among these ecosystems, saltmarshes are particularly endangered due to their position in temperate areas with low wave action where human density is often high (e.g. estuaries). Around the UK, centuries of land reclamation have led to a substantial decrease of the area of saltmarsh. Over the past decades, restoration schemes have been implemented in numerous coastal locations in an attempt to counteract this loss. Such schemes involve allowing sea water to inundate a previously embanked area and letting the vegetation develop naturally, thereby reverting to saltmarsh through natural colonisation. However, surveys of restored areas that have looked at the recovery of plant species diversity or functional characteristics often show that restored saltmarshes do not reach the state of a natural saltmarsh ecosystem. While there is much data at the species level, recovery of plant intra-specific diversity (genetic diversity) has not been assessed in restored saltmarsh although this component of biodiversity is receiving increasing attention for its effect on ecosystem function.

This thesis represents the first attempt to (1) characterize the nation-wide genetic structure of two important north-west European saltmarsh plant species, the common saltmarsh grass (*Puccinellia maritima)* and the sea arrowgrass (*Triglochin maritima)* and (2) compare levels of genetic diversity and structure between restored and natural ecosystems. Microsatellite molecular markers were developed for both species. Using innovative methods to analyse the genetic data obtained for these two polyploid species, this thesis highlights that genetic diversity at the national scale is organised regionally for both species, although gene-flow is still restricted between populations within the same region. Gene-flow between populations is determined by different processes depending on the species. While coastal processes mainly influence gene dispersal in *P. maritima*, overland routes of dispersal are involved for *T.*

maritima. These differences are believed to be due to differences in dispersal ecology between the two species. Although gene-flow exists between distant saltmarshes, the genetic analysis of *P. maritima* and *T. maritima* colonists arriving on restored sites highlighted their local origin and reaffirmed that it is preferable to restore saltmarsh where a nearby natural saltmarsh can act as a source of colonists. A multiple paired-site comparison identified similar genetic diversity between restored and natural saltmarshes indicating that restoration of local genetic diversity is rapid for both species. A single site comparison at Skinflats in the Forth estuary compared fine-scale spatial genetic structure between the restored and natural saltmarsh. Interestingly, no structure was detected for *T. maritima* either in restored or natural saltmarsh. In contrast, a strong genetic structure organised along the elevation gradient was observed in the natural saltmarsh for *P. maritima* but was absent in the restored saltmarsh. The origin of this structure is not clear but could be due to restricted gene-flow between individuals from different elevations due to strong post-zygotic selection, as suggested in previous work. In any case, this lack of structure in the restored saltmarsh indicates that genetic recovery is incomplete in this respect for *P. maritima*. This thesis introduces the growing field of restoration genetics to saltmarsh ecology and identifies the principal population genetic trends in two of the species dominating the vegetation of north-west European saltmarshes community. The information given here will be useful for restoration practitioners and provides a strong foundation for future work characterizing the importance of genetic diversity for saltmarsh function.

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Chapter I: General Introduction

1. Saltmarsh presentation

a. Description

Saltmarsh is very often referred as the ecotone between land and sea being located in temperate areas around the world with relatively low wave action such as creeks, embayments or estuaries (Adam 2002). The influence of regular tidal flooding creates a range of abiotic conditions along the elevation gradient of the saltmarsh including submersion time (Boorman *et al.* 2001), salinity (de Leeuw *et al.* 1991; Wang *et al.* 2007), nutrient levels (Levine *et al.* 1998; Rozema *et al.* 2000) or redox potential (Armstrong *et al.* 1985).

Under this environmental stress, the vegetation is species-poor and composed of well-adapted halophytes (Nottage & Robertson 2005). The species composition of saltmarshes differs between regions across the world (Adam 2002). For example, the vegetation community is largely dominated by *Spartina alterniflora* in the United States whereas more species co-occur in north-west European saltmarshes. This thesis focuses on the latter but some US studies have inspired the work presented here.

The vegetation community of north-west European saltmarsh is a great example of community switch following a gradual change in environmental conditions. The distribution of plant species in saltmarsh depends primarily upon two factors, (i) the gradient in abiotic conditions described earlier but also (ii) interspecific interactions such as competition (Pennings *et al.* 2005). It has been demonstrated that species able to cope with an important abiotic stress make poor interspecific competitors when stress decreases (Engels & Jensen, 2010). Plants are therefore limited on their lower limit of distribution within the saltmarsh by abiotic stress and on their upper limit by competition with less stress tolerant species (Engels & Jensen 2010). These different effects result in a very characteristic zonation of vegetation along the elevation gradient which is thought to be a good representation of the different successional stages of a

saltmarsh, at least in estuarine marshes (De Leeuw *et al.* 1993). The lower salt-marsh is composed of colonist species settling on the bare mudflat, the characteristic species are, among others, *Salicornia spp.*, *Puccinellia maritima* or *Spartina spp.* (Rodwell *et al.* 2000). These early colonists accrete and stabilize sediments which reduce the stress due to wave action and/or immersion. Mid and high salt-marsh communities can then develop and species such as *Triglochin maritima*, *Plantago maritima*, *Limonium vulgare*, *Armeria maritima* or *Glaux maritima* become established, progressively replacing the low marsh species. The highest part of the saltmarsh is dominated by plants such as *Festuca rubra*, *Elymus pycnanthus or Elymus repens* marking the transition with terrestrial vegetation. This simple representation of a gradual change in vegetation along the elevation gradient is of course rendered more complex by the creek network which is commonly found in saltmarshes. These structures create locally the environmental conditions needed for some communities to persist or develop nested inside another community type. Moreover, the location of the different community types within the tidal frame is not constant and regional variation has been observed (Rodwell *et al.* 2000). This was commonly attributed to differences in abiotic parameters between locations (salinity, wave energy, tidal magnitude).

For a long time considered as a space without real value, recent studies have demonstrated the ecosystem services that the saltmarsh ecosystem is able to fulfil. Saltmarshes were demonstrated to be among the most productive ecosystems of the planet (Lefeuvre *et al.* 2003). Apart from the obvious value that this characteristic may have on agricultural activities such as livestock grazing (Jensen 1985; Bos *et al.* 2005), an important part of the carbon captured via this biomass production is not subsequently liberated to the atmosphere, thus making saltmarshes an important carbon sink (Chmura *et al.* 2003; Chmura 2013).

The vegetation on the saltmarsh also permits the stabilisation of the coastline by the combination of different action. Firstly, root systems are thought to stabilise sediments,

therefore reducing erosion of the seaward edge of the saltmarsh due to gradual and smooth phenomena such as sea level rise or tidal cycle (Van Eerdt 1985; Feagin *et al.* 2009). Secondly, it was demonstrated that saltmarsh vegetation decreases wind wave velocity, thus facilitating sedimentation when compared to bare mudflats (Möller *et al.* 1999; Neumeier & Amos 2006). This attenuation of wave velocity also has the effect of attenuating the damage made to sea walls during storm events, thus reducing repair costs (King & Lester 1995).

Saltmarsh plants are also known to be good bioremediators due to their capacity for absorbing and storing heavy metals (Williams *et al.* 1994a; Reboreda & Caçador 2007). Saltmarshes are thus useful heavy metal filters intercepting polluted river flows before they reach the sea.

Finally, this ecosystem is also an important refuge for birds (Hughes 2004) or economically important species of fish and crustaceans (Boesch & Turner 1984; Laffaille *et al.* 2000). The amount of wildlife found within saltmarshes also makes them interesting from a touristic and recreational point of view.

The distribution of saltmarshes is quite widespread around Great Britain (Fig. I-1) but their sizes vary across regions due to differing geomorphology. Saltmarshes spanning over several hundred hectares are common in England. They are notably found around large estuarine systems or sheltered areas such as North Kent, the Wash, Essex, Severn estuary or the Solent (Boorman 2003). Further north, in Scotland, the geomorphology precludes the development of such large systems; saltmarshes are therefore more fragmented being most of the time restricted to small estuaries or sea loch heads (Boorman 2003).

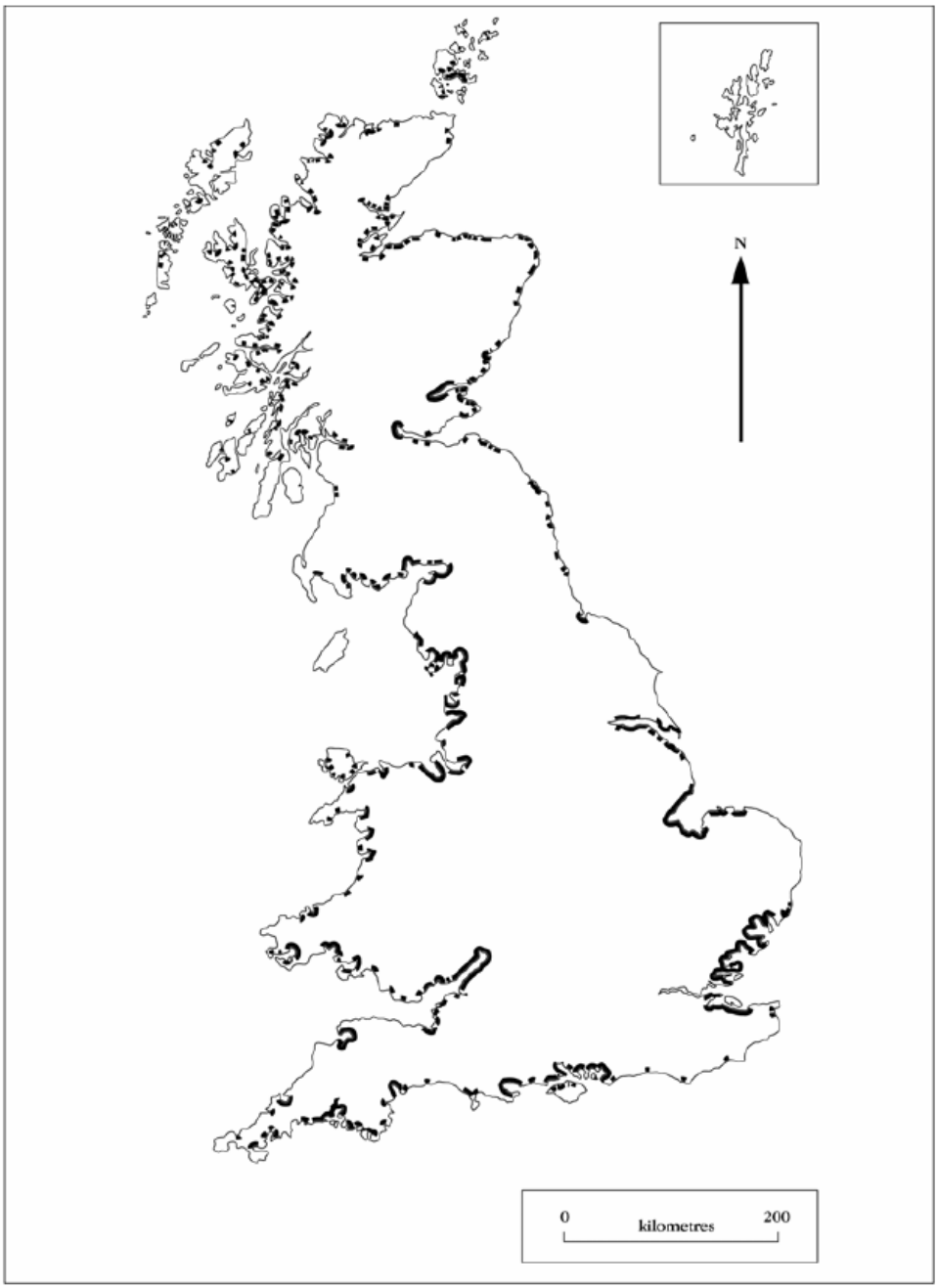

Fig. I-1 Map showing saltmarsh distribution around the UK (from Boorman, 2003; after Burd, 1989)

b. Threats

Despite the recent demonstration of the ecological and, therefore, economical value of this environment, its global extent has decreased dramatically during past centuries notably because of the human pressure concentrated on coastal areas (Lotze *et al.* 2006, Gedan *et al.* 2009). The principal cause of this decline is land reclamation and conversion of saltmarsh into agricultural, industrial or residential areas. This land reclamation was very often obtained

through diking and drainage of the embanked area (Fig. I-2). Where saltmarshes were not embanked, they were often used as dumping grounds; this had the detrimental effects of increasing the concentration of toxic materials such as heavy metals in the sediments, although the effects of their toxicity on halophytes are poorly known (Williams *et al.* 1994a; b).

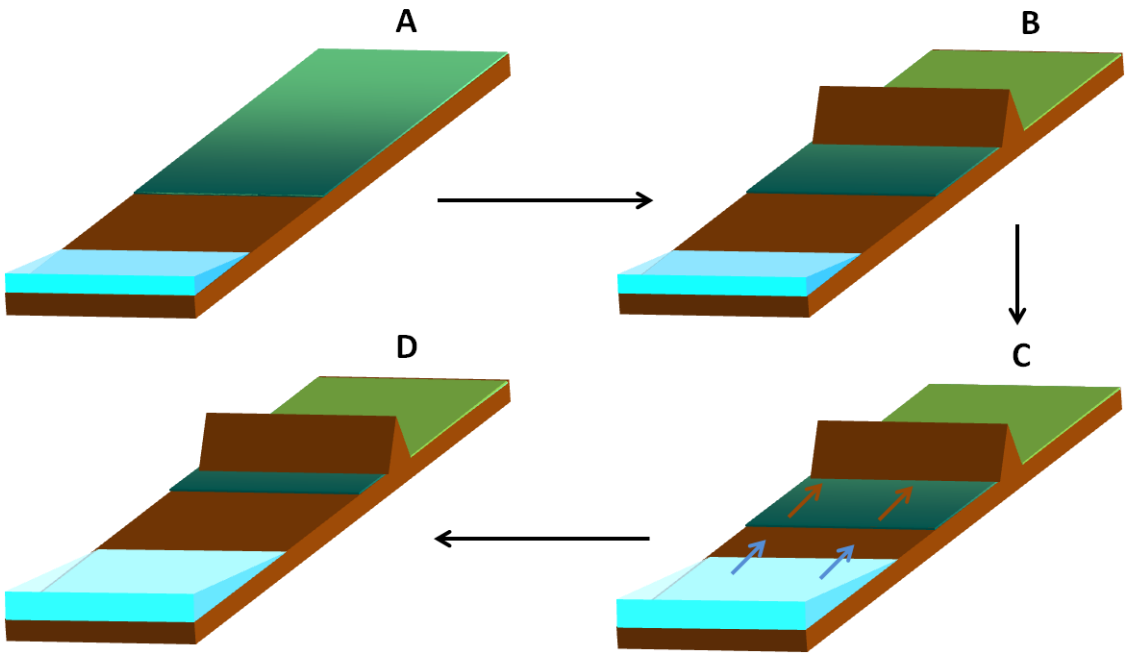

Fig. I-2 Illustration of the two main impacts causing saltmarsh destruction, land reclamation and coastal squeeze. A: Representation of an undisturbed saltmarsh; B: The building of a sea wall excludes sea water, the land reclaimed behind the sea wall can then be converted for agricultural, industrial or urban use; C: Increased tidal range and flow velocities due to land claim coupled to sea level rise causes erosion of the front edge of the saltmarsh. This saltmarsh, limited on its upper zone by the sea wall, cannot migrate landward, causing the phenomenon of "coastal squeeze"; D: Area of saltmarsh left after the effects of both land reclamation and coastal squeeze.

Levels of pollution within saltmarshes are also influenced by their geographical position. Being frequently located within estuaries, they are the last filter of runoff water at the outlet of entire catchments (Gedan *et al.* 2009). During the past decades, for example, the extensive use of fertilizers further upstream in the catchment has led to eutrophication of saltmarshes due to an increased concentration in nutrients such as phosphorus and nitrogen. Halophytes commonly grown under nutrient limiting conditions, their competitiveness is affected which has an impact on zonation within the saltmarsh (Gedan *et al.* 2009). Moreover, increased

levels of nutrients increase above-ground biomass while decreasing below-ground biomass, thus weakening the stability of saltmarsh sediments and increasing erosion (Deegan *et al.* 2012).

This erosion of the seaward edge is also one of the main reasons for saltmarsh loss. There is however, still debate about the main causes of this erosion. On one hand, physical factors such as land reclamation increasing tidal range and current velocities, increased wind and wave activity together with sea level rise have been invoked as the main reasons for saltmarsh erosion (van der Wal & Pye 2004) causing the well-known effect of "coastal squeeze" (Fig. I-2). On the other hand, biological processes such as bioturbation and herbivory by *Nereis diversicolor* have been proposed as the main factors destabilising saltmarshes in South-East England (Hughes & Paramor 2004). However, this last hypothesis has been questioned and further research is needed to confirm its effect on saltmarsh erosion (Wolters *et al.* 2005a).

Finally, British saltmarshes have also been subject to biological invasion by *Spartina anglica* which is considered as one of the greatest examples of invasion following hybridization (Baumel *et al.* 2001). This species results from a chromosome doubling of the sterile *Spartina x townsendii* itself the result of the hybridization between the native European species *Spartina maritima* and the introduced North-American species *Spartina alterniflora* (Baumel *et al.* 2001). Although considered from some aspects as an ally in ecosystem restoration (e.g. mudflats stabilisation, prevention of erosion), its detrimental effects on the community have also been underlined (monospecific stands replacing diverse communities, helping reclamation of land for agriculture) (Doody 1990).

The combination of all these causes has resulted in a continuous reduction of the area occupied by saltmarshes in the UK over the last centuries. Although the general consensus is that the overall area of saltmarsh is still currently decreasing (Phelan *et al.* 2011), the rate of this decrease is hard to quantify. This is firstly due to the lack of a nationwide baseline survey

of the saltmarsh extent (but see Burd 1989). Secondly, where local censuses of saltmarsh exist, differences concerning the methodology used to infer saltmarsh area preclude their combination into a single inventory (Phelan *et al.* 2011). Finally, regional variation exists at the scale of the UK making generalization from local examples impossible at the scale of the UK. For example, while a net loss of saltmarsh was observed in south-east England, saltmarsh accretion was noticed in north-west England (Adam 2002).

c. Restoration

Several international Conventions have been translated into UK policies in order to protect wetlands and intertidal areas (Foster *et al.* 2013). Among these, we find the Ramsar Convention especially designed for protecting wetlands, or the Bonn and Bern Conventions which gave birth respectively to the Habitats and Birds European Directive. The Convention on Biological Diversity (CBD) signed by the UK in Rio in 1992 also lead to the UK Biodiversity Action Plan (UK BAP) which clearly set the target for preserving the total area of saltmarshes around the country at the same level as the 1992 baseline (UK Biodiversity Group 1999). In order to reach these targets, conservation areas such as Sites of Special Scientific Interest (SSSIs), Special Protection Areas (SPAs) or Special Areas of Conservation (SACs), were designated around the country (Foster *et al.* 2013). However, in order to counteract the yearly loss of saltmarsh, restoration schemes have also had to be implemented (UK Biodiversity Group 1999).

Different restoration methods exist in order to recreate a saltmarsh (Nottage & Robertson 2005). Around the UK, two main options are most often adopted. The first method, called de-enbankment, consists in removing the existing sea-wall in order to let the sea re-enter in the previously embanked area (Esteves 2014). Although the totality of the sea-wall may be removed, the option the most often used in the UK is breaching the existing sea wall at one or several locations (Fig. I-3). This method has the advantage of providing relatively sheltered

conditions for sedimentation to occur and vegetation to develop (Esteves 2014). Engineering work may be needed in order to either re-level the surface of the saltmarsh permitting the tide to inundate the entire site where the embanked area has been artificially raised, or to recreate a creek network typical of a natural saltmarsh (Garbutt & Boorman 2009). Whereas transplants are often used to restore the vegetation of the saltmarsh in the US (Travis & Grace 2010), in the UK, natural processes of recolonisation are expected to take place with a progressive substitution of the pre-existing terrestrial vegetation by saltmarsh species.

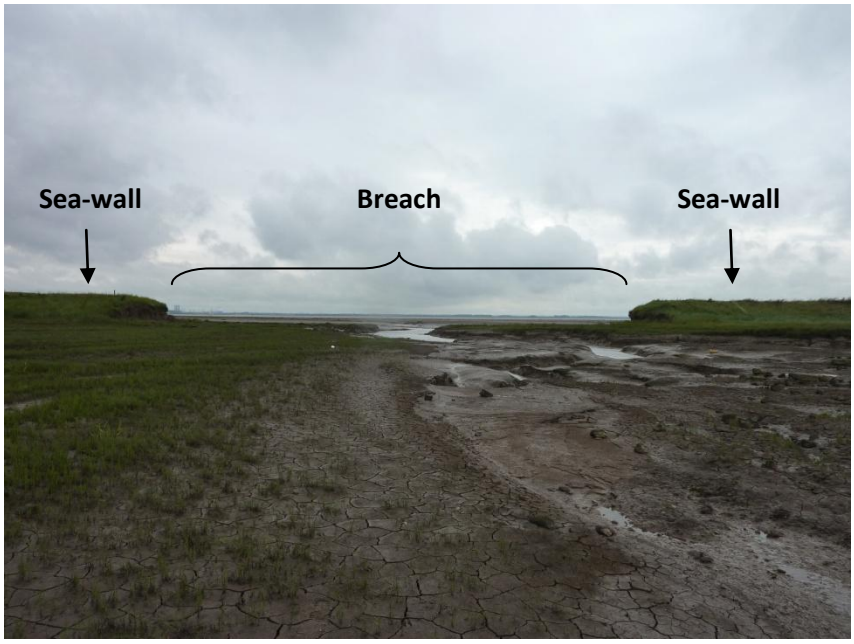

Fig. I-3 Breach into the sea wall permitting the sea water to enter in the restored site of Paull Holme Strays (Hull estuary, UK). Photo: Romuald Rouger

The second method called Regulated Tidal Exchange (RTE) consists in allowing the sea water to enter into the site via one or a few pipes fitted through the sea wall (Nottage & Robertson 2005) (Fig. I-4). This method has multiple advantages. Firstly, the site can be completely isolated from wind wave action allowing the vegetation to develop under undisturbed conditions. Secondly, this technique permits habitats to be recreated together with providing the water reservoir needed to cope with extreme events. The sea-wall being conserved, control of the pipe flow can be used in order to either exclude or retain sea water within the site (Esteves 2014). However, this method modifies the tidal regime inside the restoration

scheme. This may lead to slight differences concerning vegetation communities and their position within the tidal frame between restored and neighbouring natural sites.

Fig. I-4 Pipe fitted through the sea wall permitting the sea water to enter in the restored site of Skinflats (Forth estuary, UK). Photo: Romuald Rouger

A recent census reported 54 saltmarsh restoration schemes implemented since 1991 in the UK which corresponds to a total recreated area of 2276 ha (Esteves 2014). This figure is slightly above the target given by the UK Biodiversity Action Plan specifying the recreation of 100ha/yr of saltmarsh over a period of 15 years between 1998 and 2013, plus an additional 600 ha to counteract the loss which is thought to have occurred between 1992 and 1998 (UK Biodiversity Group 1999). Although the number of restoration schemes implemented in the UK is bigger when compared to other north-west European countries such as the Netherlands or Germany, the size of the UK restoration sites is on average smaller and rarely exceeds 100 ha (Wolters *et al.* 2005b).

Following saltmarsh recreation, a scientific survey is often undertaken in order to assess restoration success. Aspects surveyed encompass, among others, recovery of bird populations (Curado *et al.* 2013) or of the invertebrate community (Garbutt *et al.* 2006), functional recovery such as carbon and nutrient cycling (Burden *et al.* 2013) or sedimentation patterns

(Garbutt *et al.* 2006). However, the most commonly surveyed aspect concerns plant population recovery. A comparison between restored communities and neighbouring reference saltmarshes showed that the plant species richness in restored saltmarshes is very often lower than what is observed in natural saltmarshes (Wolters *et al.* 2005b). Another study argued that dispersal limitation was the main reason for this deficit in species richness within recently recreated saltmarsh (Erfanzadeh *et al.* 2010a).

2. Restoration genetics in saltmarshes

a. Why is restoration genetics important?

Ecological restoration is defined by the Society for Ecological Restoration as "the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed" (SER 2004). In order to achieve this goal, population genetic parameters were designated as an important feature to take into account, thus giving birth to the field of "restoration genetics" (Falk *et al.* 2001, 2006). Unfortunately, this aspect is very often ignored during ecosystem restoration although it is estimated that "[overlooking] genetic variation is to ignore a fundamental force that shapes the ecology of living organisms"(Falk *et al.* 2006). The importance of the information given by population genetics studies to restoration project can be located at various levels that are briefly reviewed here.

At a macro-geographical scale, the measures of inter-population differentiation obtained using molecular markers allow the limits between coherent phylogeographical units to be identified (e.g. Tan *et al.* 2005; Neel 2008; Gonzalez-Perez *et al.* 2009). Landscape features acting as barriers to gene-flow are then easily highlighted and represent valuable information concerning the connectivity of a restored site to similar environments across the landscape. This is most important when natural processes of recolonisation are supposed to occur, in which case connectivity may speed up the restoration process. In the case where

transplantation of individuals to the recreated site is involved, care must be given to potential patterns of local adaptation in which case the use of non-local transplants may be detrimental to the long-term sustainability of the restored community (McKay *et al.* 2010). Quantitative trait analyses using common genecological protocols, such as reciprocal transplant or common garden experiments, may then be very useful in order to, first, determine that part of the variation which is explained either by phenotypic plasticity or by genetic control and, secondly, to designate the best suited source population given the physical characteristics of the restored site (McKay *et al.* 2010).

At the intra-population level, genetic diversity is now widely recognised as an insurance of the community against environmental uncertainty. The more diverse the population, the greater is the probability of finding individuals able to cope with new random environmental stress (Jump *et al.* 2009a). Moreover, recent studies have also shown that genetic diversity has positive effects on ecosystem functioning, such as productivity, resilience to disturbance or litter decomposition, through facilitation or complementarity between genotypes (Reusch *et al.* 2005; Schweitzer *et al.* 2005; Hughes *et al.* 2008; Hughes & Stachowicz 2009). These effects have also been shown to scale up to higher trophic levels by, for example, positively influencing the species richness and abundance of invertebrates (Reusch *et al.* 2005; Johnson *et al.* 2006).

Intra-population genetic parameters can also reveal important influences on individual performance. Size of effective population, for example, is an important aspect to consider. When natural processes of recolonisation from neighbouring source populations are involved in the restoration process, founder events can lead to a low effective population size, at least in the early stage of recolonisation. The effect of genetic drift on the population can then lead to the fixation of deleterious alleles in the population (Ellstrand & Ellam 1993). Inbreeding coefficient is also an important aspect to consider. Indeed, high levels of inbreeding may

produce the well-known detrimental effects of inbreeding depression (Keller & Waller 2002). Conversely, the use and subsequent breeding of transplants with the local pre-existing population may give rise to the phenomenon of outbreeding depression (Falk *et al.* 2006).

Knowing whether inbreeding or outbreeding has an effect on individual performance and which level of genetic diversity should be targeted to avoid these effects in the restored population generally involves heavy experimental work. Therefore, the simplest way to control the genetic state of a restored population has been to compare indices of intra-population genetic diversity with those observed in a functionally similar ecosystem nearby (Lloyd *et al.* 2012; Oudot-Canaff *et al.* 2013).

Although genetic diversity is considered as an important aspect to consider in restoration ecology, it has rarely been within the scope of any study looking at saltmarsh restoration schemes across North-West Europe. However, some of the saltmarsh restoration work undertaken in the US has involved population genetic studies.

b. The case of *Spartina alterniflora* in US saltmarshes

Over the past 15 years, several population genetic studies have investigated the genetic diversity of *Spartina alterniflora* at multiple scales within its native range along the Atlantic and Gulf coast of the US. The applicability of some of these results for restoration purposes were assessed by Travis *et al.* (2006) but can now be complemented by recent studies that are interesting to briefly review here.

The first observation about this species is that inbreeding, when it occurs, has strong negative effects on individuals performance (Daehler 1999; Travis *et al.* 2004). Secondly, genotypes are pre-adapted to the local conditions they encounter. This pre-adaptation is both geographically based, since individuals coming from locations distant from the restored sites had poorer performance than local individuals (Travis & Grace 2010), but also based on the position of

these genotypes within the saltmarsh, tall and short forms of *S. alterniflora* being found at different locations within the same saltmarsh (Gallagher *et al.* 1988). The amount of genetic diversity to be found within a restored *S. alterniflora* saltmarsh must therefore be high enough to avoid inbreeding depression but limited in order to avoid maladaptation of geographically distant genotypes.

Surveys comparing the level of genetic diversity in reference and restored sites where natural processes of recolonisation are occurring found that the totality of the genetic diversity was recovered within only two years (Travis *et al.* 2002). This ensures that inbreeding depression is as likely to occur within the restored as in the reference saltmarsh. Moreover, the lack of genetic differentiation detected between saltmarshes a few kilometres distant (Novy *et al.* 2010) combined with the observation that a strong biogeographical structuring occurs at greater scale (O'Brien & Freshwater 1999; Blum *et al.* 2007) suggests that exchange of propagules must occur regularly between saltmarshes in close vicinity but very rarely with more distant saltmarshes. Colonists arriving on a restored site are thus very likely of local origin and therefore pre-adapted to site conditions.

However, natural recolonisation is not always the option adopted by restoration practitioners. *S. alterniflora* transplants are often used in order to speed up the recovery process, notably when sites are isolated. In order to define the maximum distance at which transplants need to be collected to avoid any risk of maladaptation, a common garden experiment combined with a population genetic study was undertaken. This work highlighted that transplants must be collected inside a radius of 300 km around the restored marsh (Travis & Grace 2010). A sufficient number of different genotypes must be collected within this radius in order to avoid the occurrence of inbreeding depression described earlier, but also because recent evidence shows that clone performance is increased in genotypically diverse plots compared to monoculture (Wang *et al.* 2012; Hughes 2014). Moreover, genotypic diversity shows a long-

term steady decline over time once the ecosystem is saturated, so selecting enough clones in the early stage of the restoration ensures long-term sustainability of the population (Travis & Hester 2005).

Although this corpus of evidence is of great value for restoration practitioner in the US, it is difficult to directly extrapolate this work to the very different vegetation communities found in North-West European saltmarshes. However, it provides an inspirational basis for designing studies on restoration genetics in European saltmarshes.

3. PhD aims

The objective of this PhD is to use population genetics techniques to answer questions which are relevant from a restoration point of view in North-West European saltmarsh communities. Following this goal, four research chapters are presented in this thesis. They are all written under the form of publishable papers in peer-reviewed journals. Some of them have already been published (Chapter II and III); others need further revision before submission to peer-reviewed journal (Chapter IV and V). The four research points addressed in this thesis are listed below:

- **Chapter II: Development of microsatellites markers for the two saltmarsh species *Puccinellia maritima* and *Triglochin maritima*.**

  No molecular markers readily existed by the start of this PhD for the two species I decided to focus on. This methodological chapter encompasses two technical notes which have been published in "Conservation Genetics Resources" and "Genetics and Molecular Research" for *T. maritima* and *P. maritima* respectively.

- **Chapter III: UK-wide population genetic study of the two saltmarsh species *P. maritima* and *T. maritima*.**

The objective of this chapter is to investigate the genetic structure of the two species at a macro-geographical scale in order to estimate the amount of genetic exchange between regions and identify which patterns govern the differentiation between populations. This chapter gives an overview of the phylogeographical unit and the large scale barrier to dispersion between these units.

This chapter mainly comprises a paper recently published in “Molecular Ecology”.

- **Chapter IV: Origin of colonists and subsequent development of genetic diversity within restored saltmarshes.**

  The objective of this chapter is two-fold. Firstly, to identify and compare what are the likeliest colonisation source for the two species investigated. Secondly, to investigate how levels of genetic diversity compare with a neighbouring reference saltmarsh.

- **Chapter V: Fine-scale spatial genetic structure of saltmarsh plants in both restored and natural environments.**

  When they are made, comparisons of levels of genetic diversity between restored and reference ecosystems often overlook how this genetic diversity is distributed in both ecosystems. The goal of this chapter is, firstly, to discuss the importance of this aspect in ecosystem restoration and, secondly, to investigate and compare levels of fine-scale spatial genetic structure between a restored and a natural ecosystem for the two species.

4. Study system

The two species that I decided to focus on during this thesis are *Puccinellia maritima* and *Triglochin maritima*. These plants are two important constituent of saltmarsh communities, generally occurring at different levels within the tidal frame (Rodwell *et al.* 2000). I give, here, a brief description of both species and the localisation of the sites where samples were collected.

a. Puccinellia maritima

P. maritima, or common saltmarsh-grass, is a perennial grass generally occuring from the pioneer stage up to the intermediate communities of the saltmarsh. It is widely distributed around the UK but also across north-west Europe (Gray & Scott 1977). Producing large clones through stoloniferous extension, this plant is considered as an engineer species due to its ability to accrete and stabilize sediments (Langlois *et al.* 2001). This sediment accretion results in the formation of "hummocks" (Langlois *et al.* 2003) permitting a diminution of the environmental stress and therefore allowing other species to establish.

Wind-pollinated, sexual reproduction in this species is mostly by outcrossing (Gray & Scott 1977) while important asexual reproduction also occurs by the breaking of stolons or detachment of vegetative propagules that are subsequently dispersed by tides (Festoc 1999). The allocation between asexual and sexual reproduction in the species varies broadly between populations and individuals (Gray 1985, 1987).

Morphology is also a varying parameter between populations of this species (Fig. I-5). Using a common garden experiment on multiple accessions collected across the UK and measuring each of them for 19 morphological traits, Gray & Scott (1980) observed a slight grouping of the individuals according to their geographic region of origin. They also found a morphological grouping of the individuals depending on whether they were coming from grazed or ungrazed

saltmarshes. The fact that these morphological differences between accessions were conserved even within the common garden allowed the authors to suggest a genetic control of this morphological variation (Gray & Scott 1980).

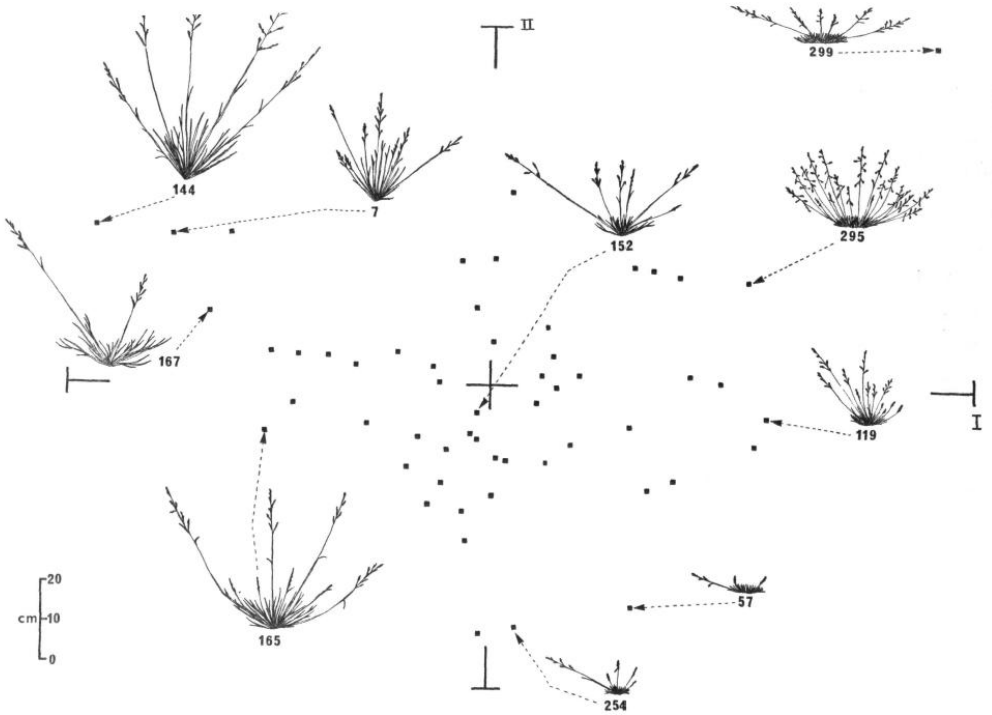

Fig. I-5 First two axis of the PCA obtained after the measurement of 19 morphological parameters on 56 accessions collected across the UK. Plant representations serve to visualize the extent of this morphological variation (from Gray & Scott 1980)

Chromosome counts were made in order to see whether ploidy types could be related to these different growth forms. Although, previous counts found variability in ploidy across the geographic range of *P. maritima*, all accessions collected in the UK were found to be octoploid (x=7, 2n=56) (Scott & Gray 1976). Unfortunately, the origin of this level of ploidy is unknown (allo vs auto-polyploidy).

Interestingly, morphological differentiation was also detected between individuals located at different stages of succession within the same saltmarsh. Plants from mature populations were found to be on average larger, with longer leaves, producing more vegetative and flowering tillers, more inflorescences and panicles, and having a better seed production (Gray 1987) than

plants from pioneer populations. Moreover, they had better resistance against intra-specific competition (Festoc, 1999). Again, these differences were conserved in a common garden experiment suggesting the genetic control of these characteristics.

Genecological studies also observed that more morphological variation was found to exist between individuals coming from the pioneer stage of the vegetation than between individuals from the mature population (Gray *et al.* 1979; Gray 1985, 1987). This suggested that the pioneer community comprises a mix of random colonists exhibiting a wide range of phenotypic variation. The progressive closure of the vegetation favours phenotypes which are most adapted to these conditions (Gray 1985). This has the effect of decreasing the morphological variation found within mature communities. This hypothesis was further confirmed when growing seeds collected from the mature community which exhibited greater morphological variation than their parental cohorts, also suggesting the phenomenon of post-zygotic selection (Gray 1987).

Nevertheless, Festoc (1999) failed to find more morphological variation within the pioneer community than within the mature community. This author therefore suggested a gradual substitution of the genotypes as the population matures rather than a gradual selection for a particular genotype from the pioneer morphological pool.

In addition, molecular investigations using RAPD markers detected genetic differentiation between the populations from pioneer and mature communities. Festoc (1999) argued that this differentiation was due to post-zygotic selection acting at both stages of succession and not only at the mature stage as proposed by Gray (1987). However, this assertion must be taken cautiously. Firstly RAPD markers are neutral and, therefore, not the best adapted to detect the action of diverging selection pressures. Secondly the differentiation observed here may well be only due to the isolation by distance phenomenon although this was not tested by the author.

b. Triglochin maritima

T. maritima, or sea arrowgrass, is a Juncaginaceae generally occurring in mature stages of vegetation within the saltmarsh (Rodwell *et al.* 2000). Widely distributed around the globe, its distribution is mainly around the coast in the UK. It is also found inland at some locations where environmental conditions are suitable (Davy & Bishop 1991). Perennial, *T. maritima* clones elongate through centrifugal rhizomatic expansion. When the shoots in the centre of the clone begin to die, it creates characteristic rings of vegetation (Fig. I-6) (Heslop-Harrison & Heslop-Harrison 1958). These rings were found to have a strong positive influence on the vegetation because they increase substrate elevation which reduces stress due to waterlogging and improves seed supply of other species (Fogel *et al.* 2004).

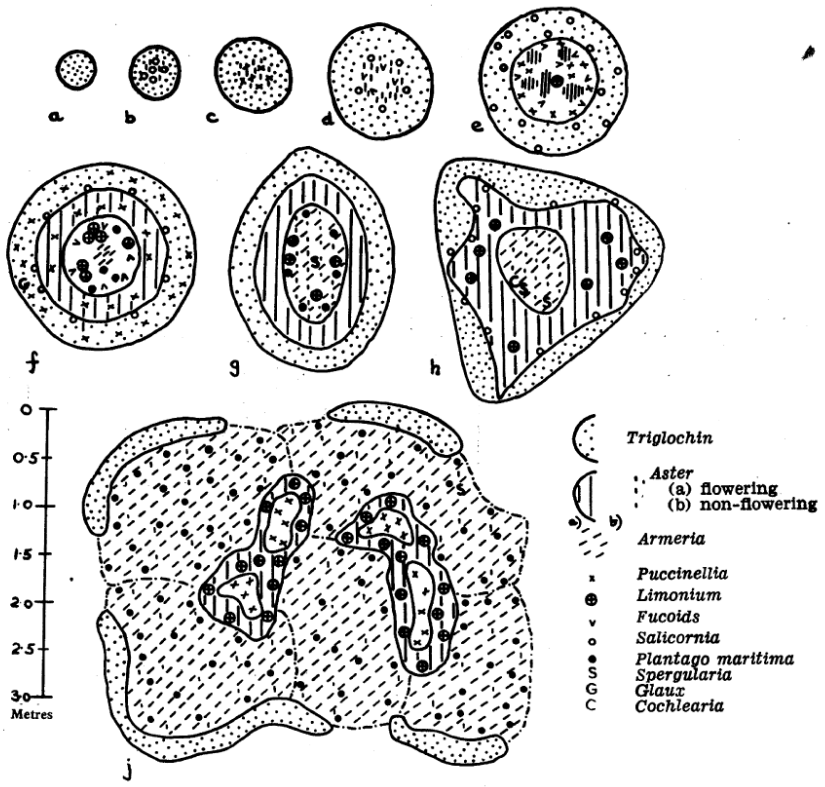

Fig. I-6 Examples of *T. maritima* rings layout as observed on the field (from Heslop-Harrison & Heslop-Harrison 1958)

Similarly to *P. maritima*, sexual reproduction in *T. maritima* is mostly by outcrossing. Wind-pollinated, flowers are strongly protogynous which restricts autopollination (Davy & Bishop

1991). Seeds are dispersed by tides and retain good viability even after a prolonged stay in sea water (Koutstaal *et al.* 1987) suggesting potential for long-distance dispersal. It is also thought that dispersion may occur via ducks feeding on this species (Davy & Bishop 1991).

Less genecological studies were made looking at the morphological variation of this species when compared to *P. maritima*. However, a difference in growth rate difference was observed between individuals collected from low and high marsh. Differences were conserved when grown under the uniform conditions of a common garden experiment suggesting a genetic basis for this difference (Jefferies 1977). Phenotypic plasticity providing an ability to cope with changing environmental conditions was also observed within this species (Jefferies & Rudmik 1991).

The phylogeography of this species across Europe was investigated recently using AFLP markers (Lambracht *et al.* 2007). Two genetic groups were detected, one gathering populations from the Portuguese, Spanish and French Atlantic coast, the other grouping populations from the North and Baltic Seas, central Europe inland populations and Adriatic sea. It was suggested that this clustering originated from the range expansion following the last glacial maximum (LGM) from two glacial refugia, one located along the South-West Atlantic coast of Europe and the other located inland in Central Europe and along the Mediterranean coast. Interestingly, the UK is located at the limit between these two geographical areas thus gathering individuals belonging to the two genetic groups (Lambracht *et al.* 2007).

Finally, cytological investigations of this species indicate that *T. maritima* is mostly octoploid across northern Europe (2n=48, x=6), although a large variation of ploidy was found to exist across its range, with diploid individuals detected in Romania while 20-ploid individuals were found in Japan (Davy & Bishop 1991).

c. Sites presentation

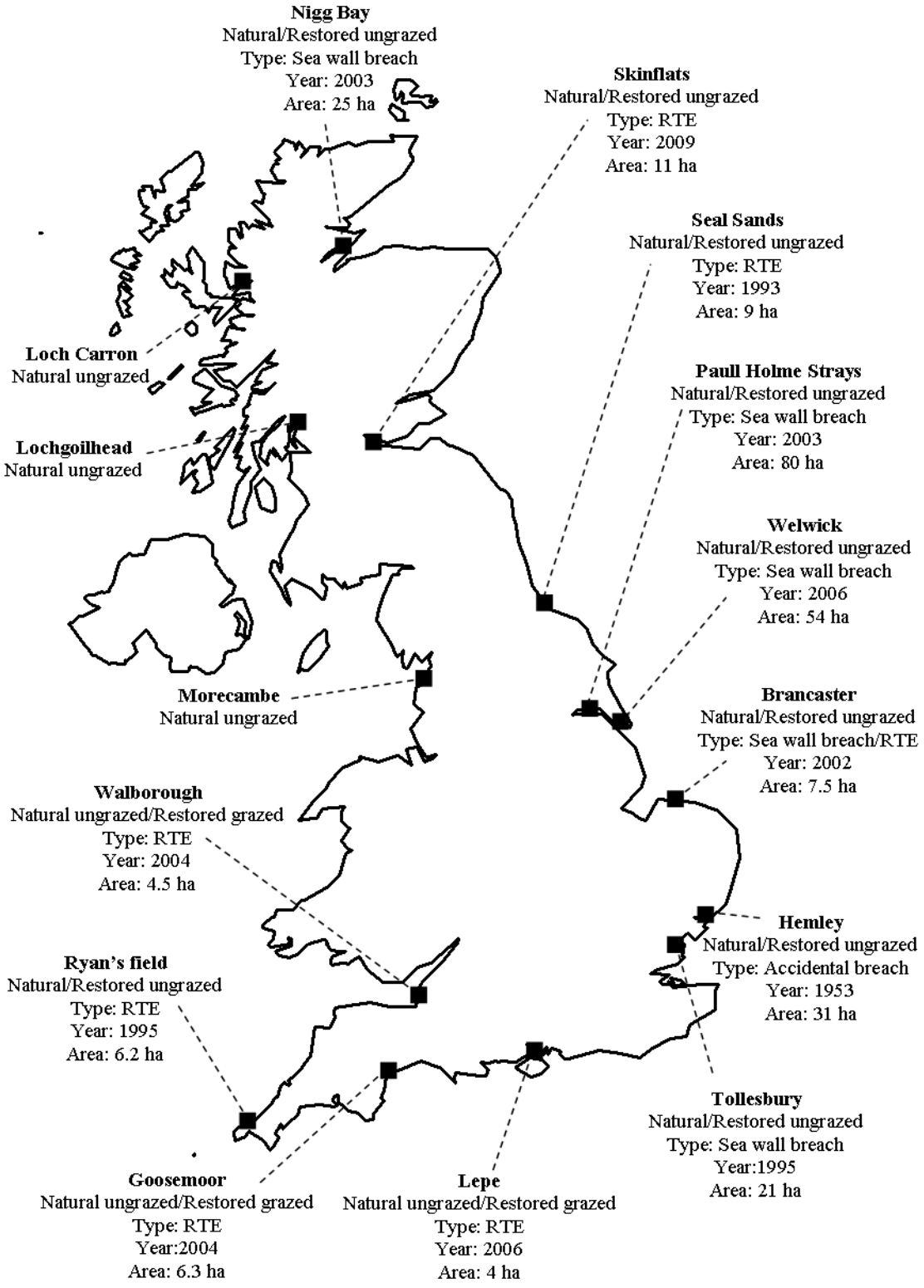

Fig. I-7 Presentation of the study sites visited across the UK. Most sites encompass both a restored and a natural saltmarsh (save Morecambe, Lochgoilhead and Loch Carron). Characteristics of each restored site are given. RTE stands for "Regulated Tidal Exchange".

Overall 15 sites were visited across the UK (Fig. I-7). All paired restored/natural saltmarshes were selected based on the database made publicly available on the internet by the Associated British Ports Marine Environmental Research Ltd (ABPmer 2014) and the census of the restored schemes made by Wolters *et al.* (2005b). The selection criteria were as follow: (1) presence of at least one target species on both restored and natural saltmarshes; (2) age of the restored saltmarshes ranging evenly; (3) good spread of the saltmarshes along the UK coastline. A few natural sites (Loch Carron, Lochgoilhead, Morecambe) were selected in areas where no restoration schemes have been implemented so far in order to have a regular coverage of the British coastline. Four additional saltmarshes were also visited within the Forth estuary in close proximity of Skinflats.

Chapter II: Development of microsatellite markers for the two saltmarsh species *Puccinellia maritima* and *Triglochin maritima*

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Part A: Development and cross-species amplification of twelve microsatellite loci for *Puccinellia maritima*, an important engineer saltmarsh species.

1. Abstract

The grass *Puccinellia maritima* is an important saltmarsh ecosystem engineer exhibiting wide morphological variation, which is partly genetically determined. Nevertheless, nothing is known about its population genetics or how neutral genetic variation is distributed throughout its geographical range. Here we describe 12 polymorphic microsatellites pooled into two multiplexes for this octoploid species. Assessment of 24 samples from 3 populations revealed 4 to 29 alleles per locus, with variation in allele presence and abundance between populations. The transferability of these markers is reported based on their cross amplification in 6 other *Puccinellia* species of different ploidy level.

Key words: Microsatellite; Multiplex; Polyploidy; *Puccinellia maritima*; Saltmarsh.

2. Introduction

Saltmarshes are coastal ecosystems characterized by the presence of salt-tolerant vegetation that is frequently flooded by sea tides (Nottage & Robertson 2005). During past centuries, the extent of these coastal ecosystems has been substantially reduced for reasons including land reclamation for agriculture or urbanisation and coastal squeeze between sea defences and rising sea level (Adam 2002). Restoration programmes have been implemented throughout Europe to counteract this loss.

Puccinellia maritima is a perennial and octoploid saltmarsh plant species (Scott & Gray 1976). This species is often the dominant plant of the early successional stages of European saltmarsh. *P. maritima* is considered as an engineer species due to its ability to accumulate and stabilise sediment thereby creating suitable habitats for the establishment of other saltmarsh plants (Langlois *et al.* 2001, 2003). This species shows great morphological variation between populations either along the coastline or along the tidal gradient. This variation has been shown to be partially genetically determined through classical genetic studies (Gray & Scott 1977, 1980; Gray *et al.* 1979; Gray 1985). However, the distribution of genetic variation within and between populations of *P. maritima* is poorly understood whilst gene flow and genetic structuring at regional scales remains unknown, largely due to the lack of variable genetic markers in this species.

Here we describe 12 newly developed microsatellite markers that will enable the study of the genetic diversity and structure of this species at both local and regional geographic scales, and allow us to develop a greater evidence base for saltmarsh restoration and management.

3. Material and methods

Ten *P. maritima* individuals from 5 populations were sampled across the UK (Walborough: 51°19'N, 2°59'W; Chalkdock: 50°48'N, 0°52'W; Lepe: 50°47'N, 1°21'W; Abbotts Hall: 51°47'N,

0°51’E; Goosemoor: 50’40’N, 3°27’W). Genomic DNA was extracted from silica dried leaves using ISOLATE Plant DNA Mini Kit (Bioline, London, United Kingdom). An equimolar DNA solution was prepared from the 10 samples and sent to Genoscreen (Lille, France) for development of a microsatellite-enriched library using 8 different probes (TG, AAC, AGG, ACAT, TC, AAG, ACG, ACTC), and sequencing by 454 GS FLX Titanium (Roche Applied Science, Meylan, France) according to Malausa *et al.* (2011).

The resulting reads were analysed with the software QDD2 (Meglécz *et al.* 2010) to detect microsatellite loci and design primers for each of them. Default parameters of the software were used apart from the maximum length of PCR product for primer design which was set to 400 bp. The selection criteria used for choosing candidate primers to test is adapted from Lepais & Bacles (2011). First, microsatellites containing the AT motif were discarded due to the difficulty of their amplification (Temnykh *et al.* 2001) and only di- or tri-nucleotide repeats were selected. Candidate primers were grouped in six classes of 50 bp according to the expected size of their PCR products (90-140 bp, 140-190 bp, 190-240 bp, 240-290 bp, 290-340 bp, 340 bp and above). Four primers out of each size class were selected for a first screening of 24 loci. This selection was based on the statistics given by QDD2, “A” design with the lowest penalty score being selected when possible. Two additional screenings of 24 loci each were conducted by preferentially selecting candidates in the size classes for which less reliable loci were found in the previous screening. Our objective was to obtain a sample of primer pairs spread across size classes that would allow us to maximize the number of loci that could be included in each multiplex PCR (Lepais & Bacles 2011). Sequence data for each tested locus was submitted to Genbank (http://www.ncbi.nlm.nih.gov/genbank/), accession numbers KC588847 to KC588900. Each primer pair was run in simplex PCR format using a M13 tailed primer protocol (Schuelke 2000). PCR reactions were carried out in a final volume of 10µl using 1X Type-it Multiplex PCR Master Mix (Qiagen, Manchester, U.K.), 0.05µM of M13-forward primer, 1µM of reverse primer, 1X M13-fluorescent dye using four different dyes (6-FAM, HEX,

TAMRA, ATTO 565; MWG Operon, Ebersberg, Germany) and approximately 20ng of genomic DNA. PCR cycles were performed on DNA engine Tetrad® 2 (Peltier Thermal cycler) with a starting step of 5 min at 95°C followed by 32 cycles of 30s at 95°C, 90s at 60°C, 30s at 72°C, then 8 cycles of 30s at 94°C, 45s at 53°C, 45s at 72°C, and finishing with a final elongation step of 10 mins at 72°C. PCR products labelled with different fluorescent dyes were pooled before and analysed in an ABI 3730xl sequencer (Applied Biosystems, Warrington, U.K.). Fragment data were analysed with Peak Scanner (Applied Biosystems). The clarity and exploitability of the signal was assessed by running PCR on seven samples from three populations. Primer pairs showing no amplification, too much stutter, or amplifying monomorphic loci were discarded.

The selected primers were checked for multiplex compatibility using Multiplex manager (Holleley & Geerts 2009), and used to design multiplex assays. Multiplex PCR reactions were carried in a final volume of 10µl using 1X Type-it Multiplex PCR Master Mix (Qiagen), variable concentrations of each fluorescently labelled primer (see Table II-1), 0.5X Q-solution (Qiagen) and 20 ng of genomic DNA. The PCR cycle was 5 min at 95°C followed by 32 cycles of 30s at 95°C, 3mins at 62°C, 30s at 72°C and then a final elongating step of 30 mins at 60°C.

DNA of 8 individuals from each of three populations (Walborough, Goosemoor and Nigg Bay: 57°44'N 4°2'W) was amplified with the two designed multiplexes. Alleles were scored using the software STRand (Veterinary Genetics Laboratory, University of California, http://www.vgl.ucdavis.edu/informatics/strand.php/) with allele binning performed using MsatAllele (Alberto 2009) modified by M. Vallejo-Marin to allow binning of more than two alleles per locus. Population genetic studies for polyploid species are still challenging since some common statistics such as expected heterozygosity or deviation from Hardy Weinberg equilibrium cannot be computed for polyploids. Consequently, since *P. maritima* is octoploid (Scott & Gray 1976), SPAGeDi (Hardy & Vekemans 2002) was used to determine number of alleles per locus and number of private alleles per locus by specifying ploidy level.

Cross species amplification was tested on 6 *Puccinellia* species differing in ploidy level. Genomic DNA from 10 individuals of *P. vahliana* (2x, five from Ny-Ålesund, Svalbard, Norway; five from Ringhorndalen, Svalbard, Norway), 8 individuals of *P. angustata* (6x, three from Innerholmen, Svalbard, Norway; five from Björndalen, Svalbard, Norway) and 2 individuals of *P. svalbardensis* (6x, Innerholmen, Svalbard, Norway) was extracted from silica dried material using CTAB protocol (Murray & Thompson 1980). Genomic DNA from 2 individuals of *P. convoluta* (2x or 4x, one from Tavira, Algarve, Portugal, one from Alvor, Algarve, Portugal), 1 individual of *P. festuciformis* (6x, Quinta do Lago, Algarve, Portugal) and 1 individual of *P. stenophylla* (10x, Alvor, Algarve, Portugal) was extracted from dry herbarium samples using ISOLATE Plant DNA Mini Kit (Bioline).

4. Results and discussion

The sequencing strategy yielded 13 253 reads (average length = 284.73 bp, SD = 117.67 bp) and 936 candidate microsatellite loci. From the 72 screened primers pairs, 60 were monomorphic or yielded unreliable bands. Twelve primer pairs showed a clear, repeatable and polymorphic signal across different samples, and were compatible with multiplex PCR amplification. Two multiplex assays of six primers each were constructed (Table II-1).

The number of alleles per locus spanned from 4 to 29. The maximum number of alleles for an individual at a single locus was eight, which is in accordance with the ploidy level of this species. The number of private alleles varied from 16 to 22 across all loci on 8 individuals per population across the three sampled populations (Table II-2). The high level of polymorphism and the frequent private alleles show the efficiency of these loci to study relatedness between individuals and differentiation between populations.

Table II-1 Characteristics of the 12 microsatellites primers developed for *Puccinellia maritima*.

Multi-plex	Locus	Repeat motif	Allele size range (bp)	Primer sequences (5'->3')	[Primer] (nM)	Dye	Genbank accession no.
1	Pm29	$(AAC)_6$	132-165	F: CATCCTCGAGAGGGAGAAA R: ACACATATCAGCCCTCGGT	200	FAM	KC588874
	Pm61	$(AAC)_6$	252-336	F: GAATCATGTGCGAACCTGTG R: ATCTTCAGCAATGCCTGGAT	200	FAM	KC588894
	Pm26	$(AC)_7$	109-115	F: TGGGGACATCGAAATGGTAT R: TCAAATAGCTGCTGGGAACC	100	HEX	KC588871
	Pm65	$(AC)_7$	226-314	F: ATCGTAGGAGATGCACGCTT R: CGCCAGGAGCTGTTAAATGT	200	HEX	KC588896
	Pm10	$(AAC)_8$	222-234	F: TCAGCTCAAACTCTCAGGCA R: ACCAAGCTCACCAATCAACC	400	TAMRA	KC588856
	Pm19	$(AG)_9$	312-352	F: GCAGGTTTGATAGAGGCAGG R: TGGTAACCTAGCGAGCAGTG	400	TAMRA	KC588865
2	Pm27	$(AAG)_{14}$	91-208	F: ATCATTGGCCTCTCGTTGTC R: AGTGTTGGGCGTATAGGCTG	400	FAM	KC588872
	Pm25	$(AGG)_6$	99-117	F: CTAGTTGCAGCCATGGGATT R: CCGGAACCATTAGAAGACGA	100	HEX	KC588870
	Pm34	$(AAC)_9$	171-309	F: TGGCAAATTTACACCACGAA R: GCAAGCAATGAAAACACGAA	100	HEX	KC588877
	Pm23	$(AAC)_{10}$	337-352	F: CTTGTTTGGGACTGAAAGGC R: GACCAGCACGGCATATGTTA	100	HEX	KC588869
	Pm39	$(AG)_9$	243-299	F: TTTCGGTCATTAGGATTCGC R: AAGGCCTGGCTAGATGTGAA	400	TAMRA	KC588880
	Pm12	$(AGG)_6$	213-225	F: GGGTGACTGGGGTGATAAGA R: AATCCACGAATTTCCACCAA	200	ATTO-565	KC588858

Annealing temperature of each multiplex, Ta=62°C.

Table II-2 Characteristics of the 12 microsatellite loci described for *Puccinellia maritima* across 3 populations.

Locus	Nigg Bay (n=8)			Walborough (n=8)			Goosemoor (n=8)			Overall (n=24)	
	N_A	Ni	PA	N_A	Ni	PA	N_A	Ni	PA	N_A	Ni
Pm10	3	2-3	0	5	3-5	0	5	2-5	0	5	2-5
Pm12	3	1-3	1	3	1-3	0	3	1-3	0	4	1-3
Pm19	9	1-6	2	10	4-5	5	9	2-4	3	17	1-6
Pm23	4	1-4	2	2	1-2	1	3	1-3	0	6	1-4
Pm25	4	1-4	1	3	1-3	0	5	1-3	1	6	1-4
Pm26	4	2-4	0	3	1-3	0	4	1-3	0	4	1-4
Pm27	15	4-7	7	10	1-3	2	19	2-5	7	29	1-7
Pm29	6	2-3	1	8	2-5	3	4	1-3	0	9	1-5
Pm34	13	3-8	3	12	3-6	2	10	3-6	2	18	3-8
Pm39	8	2-5	2	9	1-6	2	16	1-5	8	21	1-6
Pm61	5	1-3	0	6	2-4	1	7	3-5	1	8	1-5
Pm65	5	2-5	0	6	2-4	0	6	3-5	0	6	2-5
Overall	79	-	19	77	-	16	91	-	22	133	-

Number of individuals (n), Number of alleles (N_A), Number of alleles per individual (Ni), Number of private alleles (PA).

The results of cross-species amplification differed between species (Table II-3). Apart from Pm23, most of the loci amplified in at least one other species. Cross amplification was most successful in *P. vahliana*, *P. angustata* and *P. svalbardensis*. However, it should be noted that DNA from *P. convoluta*, *P. festuciformis* and *P. stenophylla* was extracted from dry herbarium samples and that DNA amplification from fresh samples may give superior results.

Table II-3 Cross-species amplification of the 12 microsatellite loci designed for *P. maritima* in other *Puccinellia* species. The amplification was considered as successful when half or more of the samples showed amplification.

Locus	*P. vahliana* N=10			*P. angustata* N=8			*P. svalbardensis* N=2			*P. convoluta* N=2			*P. festuciformis* N=1			*P.stenophylla* N=1		
	S	N_A	np	S	N_A	np	S	N_A	np	S	N_A	np	S	N_A	np	S	N_A	np
Pm10	+	2	10	+	2	8	+	3	2	+	7	2	-	0	0	+	1	1
Pm12	+	1	5	+	1	4	+	2	2	-	0	0	-	0	0	-	0	0
Pm19	+	4	5	-	1	3	+	3	2	-	0	0	-	0	0	-	0	0
Pm23	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0
Pm25	+	1	5	+	2	4	+	2	2	+	3	2	-	0	0	-	0	0
Pm26	+	2	10	+	2	8	+	2	2	+	1	1	-	0	0	-	0	0
Pm27	+	1	9	+	2	7	+	1	2	-	0	0	-	0	0	-	0	0
Pm29	+	2	10	+	2	8	+	4	2	+	4	2	-	0	0	-	0	0
Pm34	+	1	5	-	1	1	+	1	2	+	2	2	-	0	0	-	0	0
Pm39	-	0	0	-	0	0	+	2	2	-	0	0	-	0	0	-	0	0
Pm61	+	1	5	+	3	4	+	1	2	+	1	1	-	0	0	-	0	0
Pm65	+	1	5	-	1	3	+	2	2	+	1	1	-	0	0	-	0	0

Number of individuals (N), Amplification success (S), Number of alleles across all individuals amplified (N_A), Number of samples positively amplified (np).

5. Conclusion

The twelve polymorphic microsatellite loci described here will be highly useful to study the population genetics of *P. maritima* across a broad range of applications in molecular ecology and habitat restoration. Moreover, cross amplification of these markers demonstrates their utility for research in congeneric species such as *P. svalbardensis*, a rare endemic plant from the island Svalbard, Norway.

6. Acknowledgements

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sequencing, and DNA Sequencing and Services (Dundee, U.K.) for fragment analysis. We are most grateful to Pernille Bronken Eidesen from the University Centre in Svalbard, Longyearbyen, Norway, and Maria Manuela David from the Herbarium of the University of Algarve, Faro, Portugal for sample collections of related *Puccinellia* species.

Part B: Isolation and characterization of 20 microsatellite loci for the saltmarsh plant *Triglochin maritima* L.

1. Abstract

Twenty microsatellite markers were developed for the polyploid plant *Triglochin maritima* L., an important component of declining saltmarsh ecosystems that are now subject to much restoration effort. All loci were polymorphic when tested across 24 individuals from three populations. The average number of alleles per population was 6, ranging from 2 to 12. Private alleles were identified in each population, demonstrating the utility of these markers for the investigation of the population genetic structure and diversity of this species.

Keywords: Microsatellite; 454 sequencing; Polyploidy; *Triglochin maritima L.*; Saltmarsh.

2. Introduction

The halophytic *Triglochin maritima* L. (Davy & Bishop 1991) is a major component of European saltmarsh communities, which are under significant threat from changes in sea level and land use. In Europe, AFLP molecular marker data have shown that individuals of *T. maritima* are derived from two principal lineages originating from past glacial refugia which have not extensively intermixed until the present day (Lambracht *et al.* 2007). However, the process by which this phylogeographic structure has been maintained is not well understood, especially since this species is wind pollinated and water dispersed, which should enable extensive gene flow. Given the increasing implementation of saltmarsh restoration programs, the determination of the population structure and diversity of this species will provide important information for conservation and restoration management. Here we describe 20 new microsatellite loci for *T. maritima,* which will enable investigation of the population genetic characteristics of this species throughout its distribution.

3. Material and methods

Leaves were collected from four different individuals from three populations (Lepe: 50°47'N, 1°21'W; Nigg Bay: 57°44'N, 4°2'W; Brancaster: 52°58'N, 0°37'E). Genomic DNA was extracted using ISOLATE Plant DNA Mini Kit (Bioline) following the manufacturer's instructions. An equimolar DNA solution (total DNA 2 µg) combining DNA from all individuals was used in the preparation of the microsatellite enriched library and sequencing using commercial services provided by Genoscreen (Lille, France) using 454 GS FLX Titanium (Roche Applied Science). 30817 sequences were obtained and analysed with QDD2, using default parameters to detect microsatellite loci (Meglécz *et al.* 2010). The obtained candidate loci were then selected following the method presented by Lepais & Bacles (2011), selecting "A" or "B" calibre primers with uninterrupted microsatellite repeats and the lowest penalty score. The sequence data of

48 loci tested for this publication were submitted to Genbank, accession numbers KF147933 to KF147980.

Simplex PCR reactions for each selected primer pair were conducted using the "M13 tail" protocol designed by Schuelke (2000). Reactions were carried out in a final volume of 10µl with 1X of Type-it Multiplex PCR Master Mix (Qiagen), 0.05µM of M13-forward primer, 1µM of reverse primer and between 10 and 40 ng of template DNA. The PCR cycle proceeded according to 5 mins at 95°C followed by 32 cycles of (30 s at 95°C, 90 s at 58°C, 30 s at 72°C), 1µM of M13-sequence oligonucleotide tagged with either FAM, HEX, TAMRA or ATTO565 was then added and the PCR continued with 8 cycles of (30 s at 94°C, 45 s at 53°C, 45s at 72°C), and a final extension step of 10 mins at 72°C. PCR products were analysed by DNA Sequencing and Services (Dundee, UK) using a 3730 DNA Sequencer (Applied Biosystems) with reference to a LIZ 500 size standard. Only primers showing clear and replicable patterns were selected to be included into multiplexes. 20 loci were then combined into 3 different multiplex PCR combinations (Table II-4).

Multiplex PCR was performed in a final volume of 10µl with 1X Type-it Multiplex PCR Master Mix (Qiagen), 0.5X Q solution (Qiagen) for multiplex 1 and 3, variable concentration of fluorescently labelled forward and reverse primer (Table II-4) and between 10 and 40 ng of template DNA. The PCR conditions were 5 mins at 95°C followed by 32 cycles of (30 s at 95°C, 90 s at 62°C, 30 s at 72°C) and a final extension of 60°C for 30 mins.

T. maritima is a polyploid species having variable ploidy level but being typically octoploid (Davy & Bishop 1991). Therefore, classic statistics such as deviation from Hardy-Weinberg equilibrium could not be calculated due to uncertainty concerning allelic dosage. Number of alleles per locus, and number of alleles per individual were calculated manually. Number of private alleles was calculated using a custom script in the R programming language and is available from the authors on request.

Table II-4 Characteristics of 20 new microsatellite loci for *Triglochin maritima L.*

Multi-plex	Marker	Motif	Dye	Primer sequence (5'-3')	[Primer] (nM)	Fragment size	GenBank accession number
1	Tm26	$(AAG)_5$	FAM	F: GGGAACACCTGAGAAGGACA R: CGAGGTTCCTCTTCCATTCA	200	88-121	KF147958
	Tm22	$(AC)_9$	FAM	F: AGTGAAATCATGGCCTGGAG R: ACCTCGTCACTGCACATCAG	200	327-339	KF147954
	Tm33	$(AAC)_5$	HEX	F: CCAACGAGGTGTAGGTTTGG R: TGATGTGGTGGGGTTTGTTA	200	201-207	KF147965
	Tm45	$(AAC)_9$	HEX	F: TGGGTATGTTGGATTTGGTGT R: CCAAGATTGCATGTGCACTAA	400	288-312	KF147977
	Tm14	$(AAG)_{13}$	TAMRA	F: GGGTGACCCAGAGTCTCAAA R: ATGCAACCTTCTGCTTACGG	400	243-276	KF147946
	Tm01	$(AAC)_7$	ATTO-565	F: TGCATGAGTCCATCACCTTC R: TCTTCCATTCGCTAGGCAGT	100	128-134	KF147933
	Tm17	$(AAG)_5$	ATTO-565	F: CAACTGACACATGCACCTCC R: GAGCTTGTCTGGGTCTCACC	100	289-322	KF147949
2	Tm28	$(AG)_6$	FAM	F: AGAAGGCTGAGACGAAAACG R: TGGCGAGTACTGTGGATGAG	200	115-127	KF147960
	Tm18	$(AG)_8$	FAM	F: TGCTGGAAGGATGAAAAGAC R: CTACACGCGTTTTATGTGCG	200	293-309	KF147950
	Tm09	$(AAG)_7$	HEX	F: AGATATGGTTGCGAATTGGG R: ACATTACACCTTCCATCCGC	100	203-209	KF147941
	Tm46	$(AAC)_5$	HEX	F: CGGTATTCGCAAGCTTGATT R: GTGCACGCCCACTAACATT	400	346-391	KF147978
	Tm15	$(AAG)_8$	TAMRA	F: GGATCTGGCTTGGAAACAAA R: TGGTCTTCTCCTCTCCTCCA	200	244-283	KF147947
	Tm41	$(AGG)_6$	ATTO-565	F: TTGCATTCCATCTCCAATGA R: GGACGGCCTTGAAGTAACAA	200	303-324	KF147973
3	Tm36	$(AAG)_{11}$	FAM	F: TTTGCCTGTTTTATTTCGTCG R: CGAGGATTTCTACCGCACTC	200	221-251	KF147968
	Tm06	$(AG)_{10}$	HEX	F: ACGACCTCCAACGAAACAAC R: GCTTACACCTCCGCTATGGA	200	131-145	KF147938
	Tm10	$(AAG)_6$	HEX	F: GATCCACAAACGGATCCAAC R: GGGGAAATTAGGGCAAAGAG	200	230-251	KF147942
	Tm44	$(AAC)_7$	HEX	F: TTCTCCACTTCGCAGGACTT R: CATCTGTCGTTATTTCCATTGC	400	328-352	KF147976
	Tm04	$(AG)_7$	TAMRA	F: ATCTTGGGGAGCTAAACGGT R: CAGCAACAAACCTCCCAAAT	400	110-118	KF147936
	Tm42	$(AG)_6$	TAMRA	F: CTCTTTGCCTTCGTCGATTC R: GTGCTGAGGTGGAGGCTTAG	400	293-299	KF147974
	Tm07	$(AG)_9$	ATTO-565	F: CCAGAAAATCTAGCAACGGC R: AGGCCGATCTTGACAATCAC	100	140-144	KF147939

4. Results and discussion

The average number of alleles per locus was 6, ranging from 2 to 12. For all loci, the maximum number of alleles per locus per individual was never more than 4. The number of private alleles within populations across all loci ranged from 10 to 13 indicating that these markers will be useful to discriminate populations in further genetic studies (Table II-5). These 20

microsatellite markers will, therefore, provide a valuable tool to study the population genetics of this species throughout its range.

Table II-5 Genetic characterization of three *T. maritima* populations by the 20 described microsatellite loci.

Loci	Brancaster (n=8)			Lepe (n=8)			Nigg Bay (n=8)			Total	
	Na	Ni	PA	Na	Ni	PA	Na	Ni	PA	Na	Ni
Tm26	4	1-2	0	9	1-2	1	8	1-2	0	10	1-2
Tm22	4	1-3	0	5	2-4	1	4	2-3	1	6	1-4
Tm33	2	1-2	0	1	1	0	2	1-2	0	2	1-2
Tm45	3	1-2	0	4	1-2	0	5	1-3	3	7	1-3
Tm14	6	1-2	0	6	1-2	0	9	1-4	3	10	1-4
Tm01	2	1-2	0	3	1	1	2	1-2	0	3	1-2
Tm17	3	1-2	0	3	1-2	0	3	1-3	0	3	1-3
Tm28	6	2-4	1	6	2-4	0	4	1-3	0	7	1-4
Tm18	5	1-2	1	5	1-2	1	4	1-2	0	6	1-2
Tm09	3	1-2	0	3	1-2	0	2	1-2	0	3	1-2
Tm46	5	1-4	0	3	2-3	0	5	1-3	0	5	1-4
Tm15	8	1-2	2	5	1-2	0	9	2-3	3	12	1-3
Tm41	7	2-4	1	6	2-3	1	5	2-4	0	8	2-4
Tm36	6	2-3	1	8	3	2	5	2-3	0	9	2-3
Tm06	4	2	0	6	1-2	2	3	1-2	0	6	1-2
Tm10	4	1-2	1	5	1-2	1	5	1-2	1	7	1-2
Tm44	5	1-2	2	2	1-2	0	4	1-2	1	6	1-2
Tm04	4	1-2	0	4	1-2	0	3	1-2	0	4	1-2
Tm42	2	1-2	2	3	1-2	0	2	1-2	1	3	1-2
Tm07	3	1-2	0	2	1-2	0	3	1-2	0	3	1-2
Total	86	-	11	89	-	10	87	-	13	120	-

Number of individuals (n), Number of alleles (NA), Number of alleles per individual (Ni), Number of private alleles (PA).

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Chapter III: UK-wide population genetic study of the two saltmarsh species *Puccinellia maritima* and *Triglochin maritima*.

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1. Abstract

Little is known about the processes shaping population structure in saltmarshes. It is expected that the sea should act as a powerful agent of dispersal. Yet, in contrast, import of external propagules into a saltmarsh is thought to be small. To determine the level of connectivity between saltmarsh ecosystems at a macro-geographical scale, we characterised and compared the population structure of two polyploid saltmarsh species, *Puccinellia maritima* and *Triglochin maritima* based on a seascape genetics approach. A discriminant analysis of principal components highlighted a genetic structure for both species arranged according to a regional pattern. Subsequent analysis based on isolation by distance and isolation by resistance frameworks indicated a strong role of coastal sediment transport processes in delimiting regional structure in *P. maritima* while additional overland propagule dispersal was indicated for *T. maritima.* The identification and comparison of regional genetic structure and likely determining factors presented here allows us to understand the biogeographical units along the UK coast, between which barriers to connectivity occur not only at the species level but at the ecosystem scale. This information is valuable in plant conservation and community ecology and in the management and restoration of saltmarsh ecosystems.

Keywords: *Puccinellia maritima*, *Triglochin maritima*, Saltmarsh, DAPC, Isolation by resistance, Polyploidy.

2. Introduction

Saltmarshes are an excellent example of ecotones since they lie at the transition between terrestrial and marine ecosystems. The influence of the tides on this environment creates a range of extreme conditions (e.g. disturbance, salinity, inundation) leading to a species poor ecosystem, typically of salt tolerant plants that form vegetation communities strongly stratified along an elevation gradient. Saltmarshes provide a broad range of ecosystem services, such as preventing land erosion, providing nitrogen and carbon storage, and forming a refuge for economically important species of fish or crustacean (Gedan *et al.* 2009). However, the extent of this ecosystem has been dramatically reduced by historical land reclamation and is under further threat, both due to continuing anthropogenic pressures and saltmarshes being trapped between rising sea levels and fixed sea walls, the phenomenon dubbed "coastal squeeze" (Gedan *et al.* 2009). To counteract the loss, restoration programmes relying on natural recolonisation of the plant community have been implemented in the UK (Wolters *et al.* 2005b). Understanding the mechanisms shaping the connectivity between saltmarsh ecosystems is therefore of primary importance to design efficient management and restoration policies.

The halophytes composing the north-western Europe saltmarsh vegetation are known to be morphologically variable across their range. They were, therefore, extensively used in classical morphogenetic studies. For example the early work done on morphological variation in *Plantago maritima* was highly important in the identification and definition of plant 'ecotypes' (Gregor 1938). While, in *Aster tripolium*, the production of ray-florets varies widely both within and between populations and this characteristic was shown to be at least partially genetically inherited (Clapham *et al.* 1942; Duvigneaud & Jacobs 1971; Gray 1987; Huiskes *et al.* 2000). *Suaeda maritima* is also a species displaying a noticeable ecotypic variation (Gray 1974). All

these examples suggested that the genetic diversity within saltmarsh species is strongly structured across a macro-geographical scale.

Studies using modern molecular markers to investigate the structure of the genetic diversity within these species showed that genetic differentiation existed between saltmarsh plant populations. (*Aster tripolium*: Krüger, Hellwig, & Oberprieler, 2002; Brock *et al.*, 2007; *Armeria maritima*: Baumbach & Hellwig 2007; *Suaeda maritima*: Prinz, Weising, & Hensen, 2009; *Spergularia media*: Prinz, Weising, & Hensen, 2010; *Triglochin maritima*: Lambracht, Westberg, & Kadereit, 2007). However, these studies largely focused on populations collected in inland saline habitat. Because these populations are highly fragmented and sometimes small, they are particularly sensitive to founder effects or genetic drift. Consequently, they give little information about the genetic structure of these species along a coastal system and the putative mechanisms shaping it.

In their population genetic study of *Spergularia media*, Prinz *et al.* (2010) noticed that coastal populations showed an overall lower ϕ_{ST} than inland populations. In *Elytrigia atherica*, populations separated by only few hundred meters but experiencing contrasting selective pressure were less genetically related than distant populations sharing the same environmental conditions (Bockelmann *et al.* 2003). Moreover, other studies looking at the genetic structure around Europe of multiple coastal plant species closely associated with sandy or rocky habitats highlighted extensive geographical clustering (Kadereit *et al.* 2005; Weising & Freitag 2007) and that genetic distance between populations was correlated with the coastal geographic distance separating them (Clausing *et al.* 2000). Altogether, these results suggest that direction and levels of gene flow along the coast is likely to be of critical importance in shaping the population genetic structure of saltmarsh species.

During the past decade, the emerging field of landscape genetics aimed to investigate the impact of landscape features on structuring the genetic diversity of a species (Manel &

Holderegger 2013). In marine or coastal species, specific features will have an impact on the genetic structure (e.g. ocean circulation, tidal regime, wind direction, salinity gradient). The integration of these parameters into the analysis of genetic diversity within a species was named "seascape genetics" (Galindo *et al.* 2006; Selkoe *et al.* 2008). This approach has rarely been used to study the genetic structure of western-European saltmarsh species, although research into the genetic structure of sea beet (*Beta vulgaris* ssp. *maritima*) by Fievet *et al.* (2007) provides a notable example.

We sought to identify the effect of coastal environmental processes on the genetic connectivity among UK saltmarshes. The overall aim of such work is to inform saltmarsh restoration practice through developing a greater understanding of dispersal and colonisation dynamics around the UK coasts. Here, we used a seascape genetics approach in two common, but ecologically contrasting, saltmarsh species: *Puccinellia maritima* and *Triglochin maritima*. Using microsatellite markers designed for these species, we asked (1) What is the level of genetic connectivity between populations? (2) Is the genetic structure comparable between species? (3) What factors best explain the population genetic structure of both species (e.g. isolation by distance, tidal currents, etc.)?

3. Material and Methods

a. Study species

Puccinellia maritima is a perennial grass naturally occurring from the early stages of saltmarsh succession (Gray & Scott 1977). This colonist species is considered to be an engineer species that permits sediment accretion, which in turn facilitates plant community development (Langlois *et al.* 2003). Colonisation by *P. maritima* is, therefore, of primary importance for the development of the biotic and abiotic environment of a saltmarsh. The sexual reproduction of this species is predominantly by outcrossing with caryopses being dispersed by the tides.

Asexual reproduction also occurs through dispersion of uprooted tillers (Brereton 1971; Gray & Scott 1977). Morphogenetic analysis of this species showed that this plant is morphologically variable across its range and that much of this variation is under genetic control (Gray & Scott 1980; Gray 1985, 1987).

Triglochin maritima is also a perennial species but typically occurs once sediments are stabilized. Although self-compatible (Lambracht *et al.* 2007), its flowers are strongly protogynous, preventing auto-pollination. Dispersal mainly occurs by seeds which show good viability after a floatation time of several months in sea water (Davy & Bishop 1991). Asexual propagation occurs only via centrifugal expansion of individuals producing characteristic rings (Davy & Bishop 1991). Under uniform glasshouse conditions, differences in growth between populations collected at different elevations on the same saltmarsh have been hypothesized to be genetically based (Jefferies 1977).

b. Sample collection and molecular work

Table III-1 Location and number of samples collected in each population of *T. maritima* and *P. maritima*. The number of samples successfully amplified is given in parentheses.

Population	Code	Sampling year	*T. maritima*	*P. maritima*	Longitude	Latitude
Brancaster	B	2011	30 (29)	30	0.6230°E	52.9721°N
Goosemoor	G	2011	30	30	3.4541°W	50.6836°N
Lepe	L	2011	22	30	1.3860°W	50.8621°N
Nigg Bay	N	2011	30 (29)	30 (29)	4.0166°W	57.7374°N
Ryan's field	R	2011	30	30 (28)	5.4328°W	50.1768°N
Seal Sands	SE	2011	30	30	1.2140°W	54.6233°N
Skinflats	SK	2011	30	30	3.7320°W	56.0553°N
Walborough	W	2011	30	30	2.9847°W	51.3140°N
Hemley	H	2012	30	30 (29)	1.3389°E	52.0324°N
Lochcarron	LC	2012	30 (29)	30	5.4517°W	57.4179°N
Lochgoilhead	LG	2012	20 (19)	30	4.9142°W	56.1601°N
Morecambe	M	2012	0	30(29)	2.8082°W	54.1412°N
Paull Holme Strays	P	2012	30 (29)	30	0.1749°W	53.6821°N
Tollesbury	T	2012	30	30	0.8333°E	51.7699°N
Welwick	WE	2012	30 (29)	30	0.0203°E	53.6477°N

Samples of *P. maritima* and *T. maritima* were collected from 15 and 14 populations respectively across the UK over two successive field seasons in summer 2011 and 2012 (Table III-1). Samples were collected randomly on each saltmarsh where the species occurred

allowing a minimum distance of at least 5 m between samples. The average distance between adjacent sampled individuals per site was 16 - 36 m for *T. maritima* and 15 - 43 m for *P. maritima*. In one exception to this sampling regime, the Lochgoilhead site, the minimum distance between two adjacent individuals was reduced to one 1 m, with average distance between individuals sampled for *T. maritima* and *P. maritima* of 5 and 6 m respectively due to the small size of this population. Care was taken to avoid collection of physically linked individuals within any site. Samples were dried immediately in fine-grained silica gel and stored in a dry and dark place until analysed.

Genomic DNA was extracted from approximately 10 mg of dried leaf tissue using the DNeasy 96 plant kit (Qiagen) following the manufacturer's instructions. DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and adjusted to 5-20ng/µl using ultra-pure PCR water (Bioline). PCR protocols are detailed in Rouger *et al.* (2014) for *P. maritima* and Rouger & Jump (2013) for *T. maritima*. Fragment analysis was conducted by DNA Sequencing and Services (University of Dundee, UK) using an ABI 3730 DNA Sequencer (Applied Biosystems). Fragment sizes were scored using the software STRand (Toonen & Hughes 2001) and alleles allocated to their respective size classes using the package MsatAllele (Alberto 2009) in R (R Core Team 2013). Loci Pm27 (*P. maritima)* and Tm07 (*T. maritima*) were not included in this analysis due to high amplification failure rates. The full analysis was, therefore, based on 11 loci in *P. maritima* and 19 loci in *T. maritima*.

c. Data analysis

P. maritima and *T. maritima* are both polyploid species. Although variable in ploidy across their range, the two species are reported to be octoploid in the UK (Scott & Gray 1976; Davy & Bishop 1991). Population genetics analysis of polyploids is still challenging due to diverse technical and statistical issues among which the difficulty to characterize allelic dosage of each individual or differing inheritance pattern between loci (Dufresne *et al.* 2014). To circumvent

these problems, each allele was scored as present or absent in each individual. Each individual was then characterized by a binary vector as long as the total number of alleles detected across all individuals. The presence/absence matrix obtained was then comparable to a dataset obtained with classic genetic fingerprinting method such as AFLP. Although part of the genetic information is lost (e.g. it is not possible to calculate allelic frequency), this method is known to give satisfactory results in recent population genetic work in polyploids (Sampson & Byrne 2012; Vallejo-Marin & Lye 2012).

i. Genetic parameters

GenAlEx 6.5 (Peakall & Smouse 2012) was used to calculate the number of alleles detected per population. The number of genotypes within each population was calculated by detecting individuals sharing the same genotype using the software GenoType (Meirmans & Van Tienderen 2004). Occurrence of identical multilocus genotypes (clones) in our dataset was variable between populations in *P. maritima* and absent in *T. maritima* (Table III-2 in the Results section). Consequently, all sampled individuals were included in subsequent analysis and measures of diversity within or between populations were chosen accordingly. Genetic diversity in each population was measured using the Kosman index of diversity within populations following the equation:

$$KW_{\rho}(P) = \frac{1}{n} Ass^{\rho}_{max}(P, P)$$ (Equation 5 from Kosman & Leonard, 2007)

where each individual of the population P of size n is paired to another individual from the same population as to maximize the sum of distance between pairs ($Ass^{\rho}_{max}(P, P)$). The distance between individuals (ρ) was calculated from the presence/absence matrix using Dice dissimilarity index commonly used to calculate genetic distance in polyploids (Vallejo-Marin & Lye 2012; Cidade *et al.* 2013). Dice dissimilarity coefficient between individuals was calculated in R using the package ade4 (Dray & Dufour 2007). KW was calculated with a custom R script

(Appendix III-1) using the assignment problem algorithm implemented in the function *solve_LSAP* of the package clue (Hornik 2005).

ii. Genetic structure

A discriminant analysis of principal components (DAPC) was used to assign individuals to a predefined number of genetic clusters with the R package adegenet (Jombart 2008; Jombart *et al.* 2010). This method offers a good alternative to Bayesian analysis of assignment such as STRUCTURE (Pritchard *et al.* 2000). This multivariate approach is particularly suitable for polyploids as it does not assume populations to be at Hardy-Weinberg equilibrium and does not require assumptions about the inheritance pattern of each locus (Dufresne *et al.* 2014). We used sequential K-means clustering (all PCs retained, 100 starts of 10^6 iterations each) on our dataset to characterize the most likely number of clusters detected in each species based on the Bayesian information criterion. The analysis was run for K spanning from 1 to 25. DAPC was then run using values of K around the most likely number of clusters as a priori clusters. The posterior probabilities of assignment for each individual were then input into distruct (Rosenberg 2003) to help visualise results at different values of K.

The partitioning of genetic variation between groups was assessed based on a two-level hierarchical AMOVA (Excoffier *et al.* 1992). The first level was defined by the clusters discriminated with the DAPC while the second level was defined according to the populations sampled. The AMOVA was constructed for different values of K and significance levels were tested using 999 permutations following the procedure given by Excoffier *et al.* (1992) and implemented in the package ade4 in R (Dray & Dufour 2007). The matrix of genetic distance between individuals used for the AMOVA was the Dice dissimilarity matrix calculated previously.

Even though the AMOVA framework is very flexible to test divergence between populations under different evolutionary scenarios, it makes assumptions that are very likely to be violated

for the two species studied (no-inbreeding, no migration, pure drift, random sampling at each level) (Excoffier *et al.* 1992). Therefore, the Kosman distance between population (KB) was preferred over pairwise ϕ_{ST} to measure distance between populations based on the matrix of genetic distance between individuals (Kosman & Leonard 2007). This dissimilarity index is particularly well suited for the study of organisms using clonal reproduction and/or which are likely to depart from Hardy-Weinberg equilibrium. This time, each individual of a population P_1 is paired to an individual of a population P_2 as to minimize the sum of distance between pairs (noted $Ass^{\rho}_{min}(P_1, P_2)$, where ρ is the between individuals dissimilarity coefficient used). This sum is then divided by the number of pairs:

$$KB_{\rho}(P_1, P_2) = \frac{1}{n} Ass^{\rho}_{min}(P_1, P_2)$$ (Equation 5 from Kosman & Leonard, 2007)

This requires population size to be the same between populations. As this is not always the case, KB was calculated from the average of 1000 bootstrap replicates of 30 individuals. Again, the matching of individuals giving the minimum sum was found using the assignment problem algorithm implemented in the function *solve_LSAP* of the R package clue (Hornik 2005) using a custom script (Appendix III-1).

This measure of dissimilarity between populations was used to conduct a Nonmetric Multidimensional Scaling (NMDS) ordination of populations with the R package vegan (Dixon 2003). We examined the solution along three axes using a maximum number of random starts of 100. To assist the pattern of genetic structure observed both on the DAPC and on the NMDS ordination, a Mantel test of correlation between the matrixes of population dissimilarity of the two species was made using the package ade4 in R.

iii. Factors shaping the genetic structure

Isolation by distance between populations was tested using Mantel test in the R package vegan using 999 permutations. The KB dissimilarity between populations was tested against

great circle geographical distance (d_{GC}) calculated using the function *distMeeus* implemented within the R package geosphere and against coastal distance between saltmarshes (d_C) calculated manually from a 1:250 000 map of the UK. Unmodified and log transformed distance were used.

The correlation between latitude and population dissimilarity was also tested. Latitudinal distance (d_{lat}) between populations was calculated as the great circle distance between the projections of the population coordinates onto the prime meridian. Unmodified and log transformed latitudinal distance were tested.

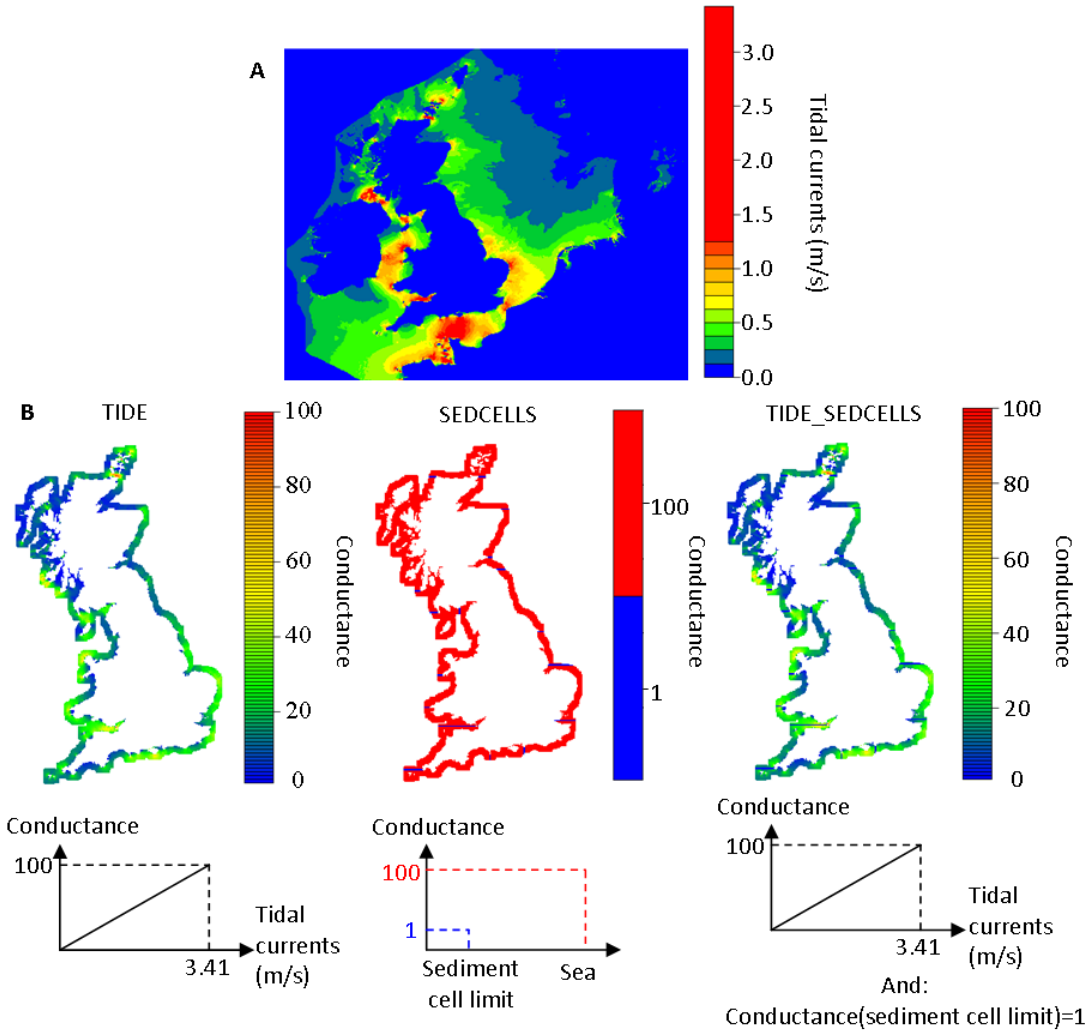

Fig. III-1 Map of (A) tidal currents velocities around the UK and of (B) the three isolation by resistance models built in this analysis (TIDE, SEDCELLS, TIDE_SEDCELLS)

Aquatic dispersal in saltmarsh plants occurs primarily through sea currents around the coast. Current dynamics around the UK must, therefore, play a key role in gene flow between saltmarshes. This hypothesis was tested using the isolation by resistance framework implemented in CIRCUITSCAPE (McRae 2006). This method uses circuit theory seeing the landscape as a conductive surface. The landscape is divided into cells of equal dimensions and characterized by a resistance (or conductance) value, the most permeable cells to movement or gene flow having the least resistance (or highest conductance). Based on this landscape grid, the program calculates pairwise resistance between each pair of populations. This matrix of pairwise resistance can then be tested for correlation with population dissimilarity using a classic Mantel test. Three models were built and tested following this method (Fig. III-1.B). Instead of using coastal currents which are generally weak around the UK, the first model (TIDE) used tidal current velocities around the UK to determine landscape conductance. Tidal current data were provided on a 0.025° longitude by 0.0167° latitude grid (J. Polton, National Oceanography Center) (Fig. III-1.A). We restricted our analysis to a 10 cell wide band around the UK coastline. Values of tidal currents within this band were divided into centiles and a value of cell conductance was allocated to each centile on a scale from 1 to 100. The second model (SEDCELLS) was based on the sediment units defined in May & Hansom (2003). The exchange of sediments between these units is understood to be very limited, dividing points between sediment cells being headlands around which almost no sediments can pass or embayments which act as sediments sinks due to converging longshore currents (May & Hansom 2003). The model SEDCELLS hypothesizes that such limited exchange occurs also for plant propagules, thus restricting gene flow between populations belonging to different sediment units. In this second model, the same geographical grid as for the 'TIDE' model was used. Each cell was given a conductance value of 100 apart from the sediment cell boundaries where a three-cell wide band was given a lower conductance value of 1. The third model (TIDE_SEDCELLS) aggregates the two first models. Each cell had the conductance value

allocated in the 'TIDE' model except at the sediment unit boundaries where a three cell wide band was given a conductance value of 1.

Grids of landscape conductance for each model were produced out of the tidal current data using a custom script in R. They were then imported into the software CIRCUITSCAPE and pairwise landscape resistance between populations were inferred using a cell connection scheme of eight neighbours (McRae 2006). Similarly to isolation by distance models, unmodified and log transformed resistance were used to test correlation with genetic dissimilarity between populations.

When comparing models, the best model should not only show the best correlation to genetic distance but also a significant partial correlation when controlling for the other competing models (McRae & Beier 2007). Therefore and in order to compare the different models investigated in this study, we used partial Mantel tests implemented in the package vegan in R.

4. Results

a. Genetic parameters

The 19 microsatellite loci used in *T. maritima* yielded 182 alleles overall with the number of alleles detected in each population varying from 87 to 117 (only 19 individuals were successfully amplified in the population of Lochgoilhead where 87 alleles were detected). In *P. maritima*, the 11 microsatellite loci used yielded 175 alleles. The number of alleles per population spanned from 65 to 103 (Table III-2). The average genetic diversity within populations measured with KW was 0.7143 and 0.7100 for *T. maritima* and *P. maritima* respectively. The number of different multilocus genotypes detected within *P. maritima* was lower than the number of samples amplified in 9 out of 15 populations. Furthermore, the populations of Brancaster, Welwick and Paull Holme Strays were shown to share common genotypes (Brancaster-Welwick: 1 shared genotype, Brancaster-Paull Holme Strays: 1 shared

genotype, Welwick-Paull Holme Strays: 2 shared genotypes). In *T. maritima*, each multilocus genotype was represented by a unique sample (Table 2).

Table III-2 Genetic diversity parameters calculated for sampled populations of *T. maritima* and *P. maritima*.

	T. maritima				*P. maritima*			
Population	N	N_A	N_G	KW	N	N_A	N_G	KW
Brancaster	29	107	29	0.7375	30	89	23	0.7289
Goosemoor	30	115	30	0.7321	30	85	27	0.7309
Lepe	22	107	22	0.7088	30	92	30	0.7545
Nigg Bay	29	114	29	0.7375	29	91	19	0.6986
Ryan's field	30	95	30	0.7046	28	65	26	0.6271
Seal Sands	30	117	30	0.7409	30	81	21	0.7408
Skinflats	30	104	30	0.7123	30	66	18	0.6602
Walborough	30	116	30	0.7380	30	103	30	0.7627
Hemley	30	111	30	0.7277	29	101	29	0.7142
Loch Carron	29	91	29	0.6808	30	89	30	0.7584
Lochgoilhead	19	87	19	0.6440	30	88	30	0.7213
Morecambe	-	-	-	-	29	87	19	0.6601
Paull Holme Strays	29	106	29	0.6993	30	79	18	0.6196
Tollesbury	30	112	30	0.7201	30	96	30	0.7510
Welwick	29	110	29	0.7167	30	82	22	0.6805
Total	396	182	396		445	175	369	

N: Number of individuals successfully amplified; N_A: Number of alleles detected; N_G: Number of multilocus genotypes identified

b. Genetic structure

The sequential K-means clustering showed that the most likely number of clusters for both species was around K=5. Although, based on the BIC score, this value was clear for *T. maritima*, it was more ambiguous for *P. maritima* (Fig. III-2). This pattern was further confirmed with *T. maritima* showing a clear separation between segregated clusters while the limits between groups in *P. maritima* were not as distinct.

Two particularly important observations can be made from the DAPC analysis. Firstly, a regional clustering of the genetic structure was observable along the coast for both species. This was confirmed by the two level hierarchical AMOVA using regions indicated by the DAPC. The amount of genetic variation explained among regions was significant in both species for all values of K considered (Table III-3). However, at any value of K, the genetic variation explained between populations within regions remained significant, indicating that differentiation between populations remains for both species.

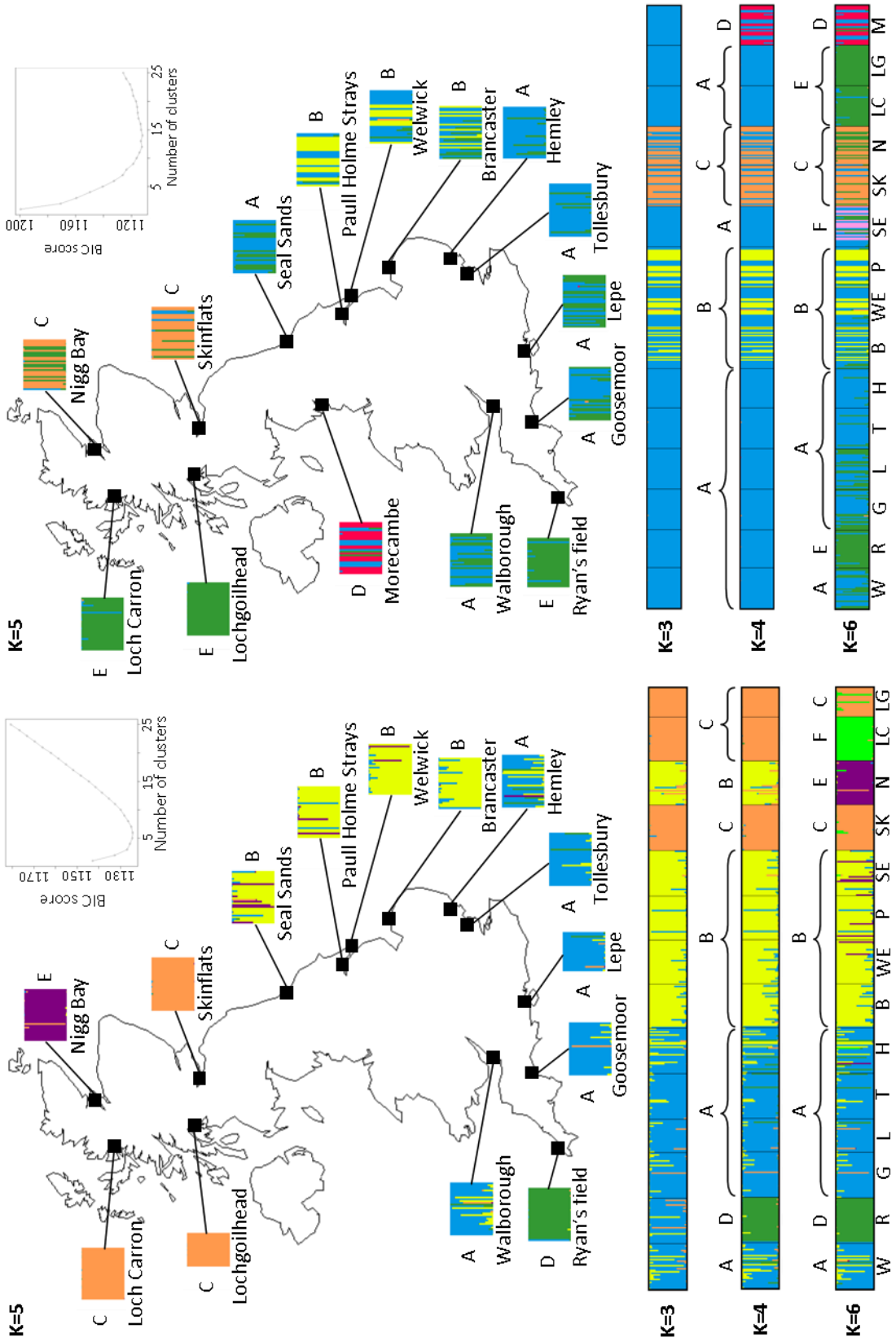

Fig. III-2 Discriminant Analysis of Principal Components showing the genetic clustering of populations of *T. maritima* (bottom) and *P. maritima* (top) at successive values of K. The letters define the regions used as input into the subsequent AMOVA. Population codes used here are given in Table III-1.

Table III-3 Two-level AMOVA for *P. maritima* and *T. maritima.* Regions were segregated based on Discriminant Analysis of Principal Components at successive values of K.

	Triglochin maritima						*Puccinellia maritima*					
	df	SS	MS	Est. Var.	%	p	df	SS	MS	Est. Var.	%	p
K=4												
Among regions	3	6.37	2.12	0.015	4.17	***	3	10.19	3.40	0.025	6.81	***
Among populations within region	10	7.57	0.75	0.015	4.28	***	11	14.07	1.28	0.033	8.98	***
Within populations	382	124.83	0.33	0.327	91.54	***	430	131.95	0.31	0.307	84.21	***
K=5												
Among regions	4	7.65	1.91	0.017	4.65	***	4	12.19	3.05	0.023	6.27	***
Among populations within regions	9	6.29	0.70	0.013	3.72	***	10	12.07	1.21	0.030	8.42	***
Within populations	382	124.83	0.33	0.327	91.63	***	430	131.95	0.31	0.307	85.31	***
K=6												
Among regions	5	8.91	1.78	0.019	5.39	***	5	13.94	2.79	0.024	6.59	***
Among populations within regions	8	5.03	0.63	0.011	3.02	***	9	10.32	1.15	0.028	7.88	***
Within populations	382	124.83	0.33	0.327	91.59	***	430	131.95	0.31	0.307	85.53	***

*** statistically significant at p<0.001

Secondly, the regional organisation of the genetic structure showed similarities between species, as confirmed by the Mantel test comparing the matrices of population dissimilarity between the two species (r=0.608, p<0.001). These similarities in genetic structure between species were further developed looking at the NMDS ordination where the groups segregated previously for both species on the DAPC were coherent with the results of this analysis. (Fig. III-3). In *T. maritima*, the populations grouped by the DAPC within an eastern group (Paull Holme Strays, Welwick, Brancaster and Seal Sands) were shown to be close to a cluster incorporating most southern populations (Walborough, Lepe, Hemley, Tollesbury and Goosemoor) (Fig. III-3). A similar pattern was found in *P. maritima*. Interestingly, the NMDS permitted us to explain some of the differences observed between species on the DAPC. For example, the DAPC allocated the *P. maritima* population of Seal Sands within the southern group although it is part of the eastern cluster for *T. maritima*. However, the NMDS indicated that the *P. maritima* population of Seal Sands is one of the closest populations to the eastern group, being only differentiated from Welwick along the dimension 3 of the NMDS (Fig. III-3). Similarly, the DAPC

indicated that populations of Ryan's field were either segregating out of the southern group for *T. maritima* or related to the populations of Loch Carron and Lochgoilhead for *P. maritima*. However, the NMDS indicates that for both species, the population of Ryan's field is more or less equidistant to these two options confirming the similarity of genetic structure between these two species.

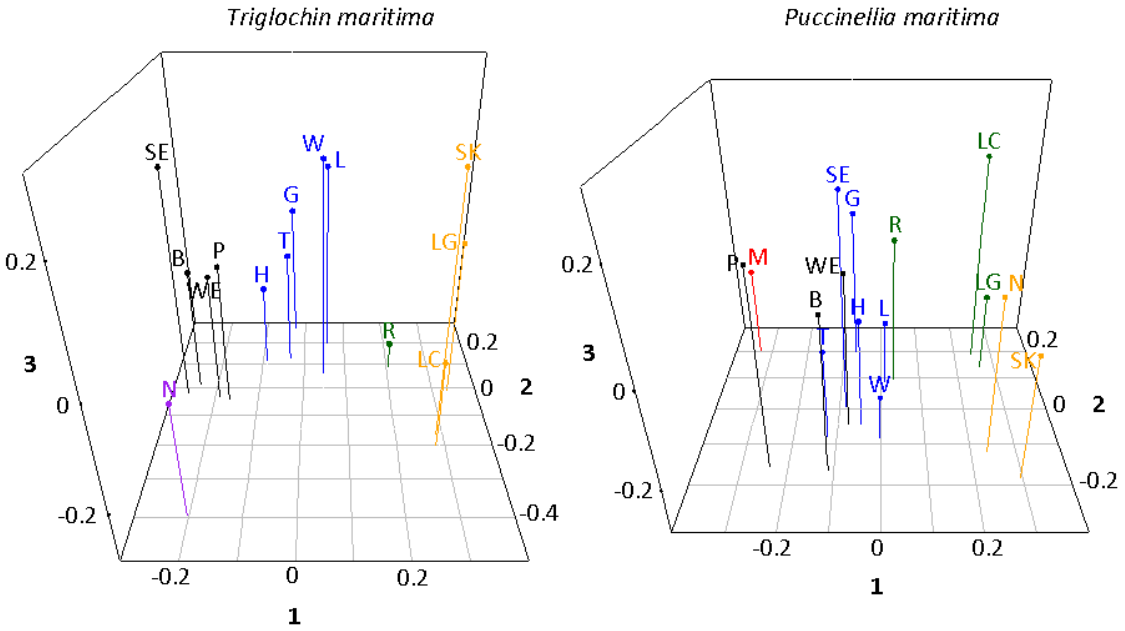

Fig. III-3 Nonmetric Multidimensional Scaling (NMDS) ordination of *T. maritima* and *P. maritima* based on Kosman genetic distance between populations. The solution using three dimensions gave a stress value of 10% and 7% for *P. maritima* and *T. maritima* respectively. Colour of each population is based on the colours obtained with the DAPC at K=5, with yellow replaced by black for clarity. Population codes used here are given in Table III-1.

Although the genetic structure of these species is globally similar, one incongruence could still be identified. The *P. maritima* population of Skinflats showed high similarity with the northern Scottish population of Nigg Bay. In *T. maritima* on the contrary, Skinflats and Nigg Bay were well separated both on the DAPC and on the NMDS.

c. Factors shaping genetic structure

i. Isolation by distance models

For any geographic distance investigated, correlation with genetic dissimilarity obtained with log transformed and untransformed distances were compared using partial Mantel tests. Log transformed distance always showed the better correlation of the two (Table III-4). Subsequent results are therefore only given considering log transformed distances.

Table III-4 Mantel test of isolation by resistance and partial Mantel test comparing competing models. KB: Kosman genetic distance between populations, d_{GC}: Great circle geographical distance, d_C: Coastal distance between saltmarshes, d_{lat}: Latitudinal distance, TIDE: Pairwise landscape resistance using tidal current velocities, SEDCELLS: Pairwise landscape resistance using sediment cells, TIDE_SEDCELLS: Pairwise landscape resistance using both sediment cells and tidal current velocities.

	T. maritima		*P. maritima*	
	r	p	r	p
Mantel test				
KB~log(TIDE)	0.5611	***	0.5744	***
KB~log(SEDCELLS)	0.6076	***	0.7017	***
KB~log(TIDE_SEDCELLS)	0.5877	***	0.6243	***
Partial Mantel test				
Comparison log vs unmodified				
KB~log(d_{GC}),d_{GC}	0.336	**	0.5964	***
KB~log(d_C),d_C	0.3938	***	0.6099	**
KB~log(d_{lat}),d_{lat}	0.3209	***	0.4538	***
KB~log(TIDE),TIDE	0.4232	**	0.3471	*
KB~log(SEDCELLS),SEDCELLS	0.4264	***	0.6288	***
KB~log(TIDE_SEDCELLS),TIDE_SEDCELLS	0.4205	**	0.4106	**
Comparison between competing models				
KB~log(d_{GC}),log(d_C)	0.4118	**	0.1707	0.071
KB~log(d_{GC}),log(SEDCELLS),	0.4395	**	0.137	0.121
KB~log(SEDCELLS),log(d_C)	0.1272	0.192	0.2909	**

statistically significant at *p<0.05; **p<0.01; ***p<0.001

Mantel tests assessing correlation between either coastal (d_C) or great circle distance (d_{GC}) to genetic dissimilarity between populations (KB) showed a strong correlation in both species (Fig. III-4). Interestingly, the correlation between d_{GC} and KB showed a higher Mantel's r than the correlation between d_C and KB in *T. maritima*. A partial mantel test showed that this difference was significant (Table III-4). Latitudinal distance (d_{lat}) was also significantly

correlated to genetic dissimilarity between populations within both species (using log transformed distance; *T. maritima*: r=0.6430, p<0.001; *P. maritima*: r=0.5073, p<0.001).

ii. Isolation by resistance models

Similar to isolation by distance models, partial Mantel tests indicated that log-transformed resistance showed the best correlation with genetic dissimilarity between populations for any isolation by resistance model tested. Therefore, results are only given using log transformed resistance.

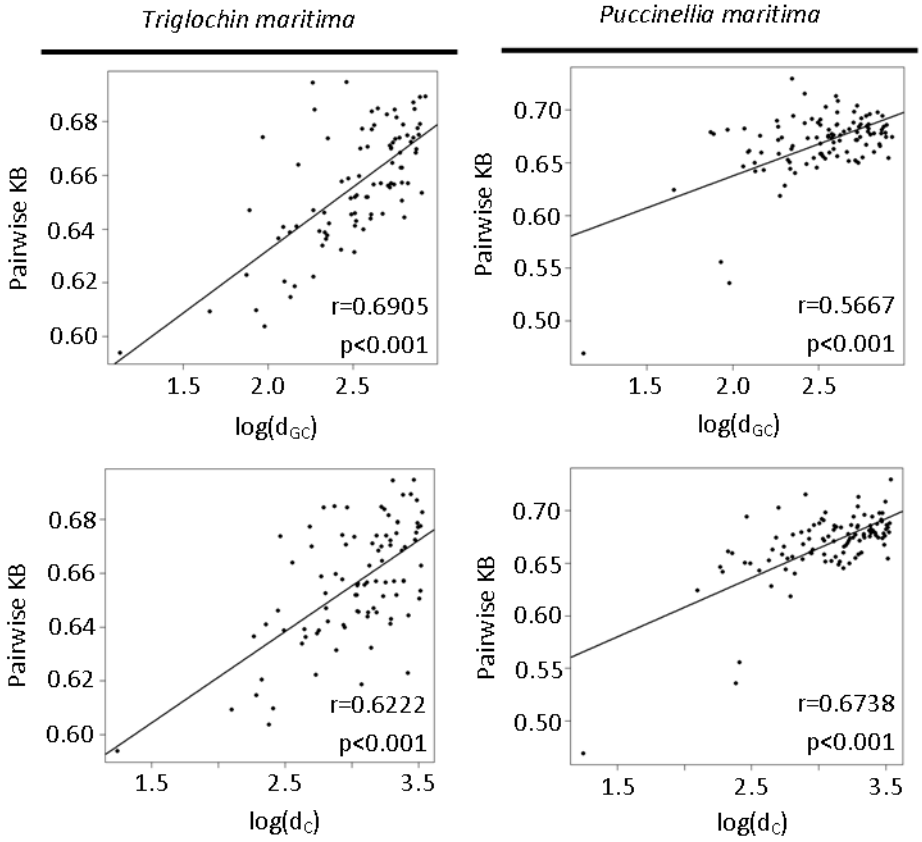

Fig. III-4 Mantel test of isolation by distance between populations of *T. maritima* and populations *P. maritima*. KB: Kosman distance between populations, d_{GC}: great circle distance, d_C: coastal distance. Linear regression lines were added for clarity.

The three models of isolation by resistance used in this study (TIDE, SEDCELLS, TIDE_SEDCELLS) were all shown to be significantly correlated with genetic dissimilarity between populations (Table III-4). For each species, the model taking into account only sediment cells (SEDCELLS)

showed the best correlation with genetic dissimilarity between populations. The best models of isolation by distance and isolation by resistance were compared for both species using a partial Mantel test (Table III-4). For *T. maritima*, the model of isolation by distance using the log transformed great circle distance had a significantly better correlation to genetic dissimilarity between population than the isolation by resistance model SEDCELLS. Contrastingly for *P. maritima*, the isolation by resistance model SEDCELLS showed a significantly better correlation than the isolation by distance model using the log transformed coastal distance between populations.

5. Discussion

Instinctively, we might expect the action of the sea on saltmarshes to act as a powerful agent of dispersal leading to genetic homogenisation of populations across a broad geographical scale. Strengthening this hypothesis, previous studies have suggested that the exchange of genetic material between isolated saltmarshes was possible due to the action of tidal currents dispersing seeds that retain good viability even after a prolonged floatation or even immersion in sea water (Koutstaal *et al.* 1987). Nevertheless, Huiskes *et al.* (1995) showed that more propagules were exported out of the saltmarsh than imported within. The strength of this source-sink asymmetry of propagule exchange between saltmarshes is an important parameter structuring plant genetic diversity. Therefore, knowing how this genetic diversity is organized should enable us to make useful inferences about connectivity between saltmarshes.

a. Genetic structure

In the case of our two species, the greatest part of the genetic variation was shown to be nested within populations, indicating that gene flow occurs at such a rate that genetic divergence between populations is limited around the UK. However, significant patterns of

genetic structure were highlighted. The AMOVA and the DAPC both converged toward a geographical organization of the genetic diversity within these two species indicating a stronger gene flow between populations located within the same geographical region. The strength of this genetic exchange was nonetheless still limited as suggested by the small but still significant differentiation between populations belonging to the same regions. Previous investigation showed a similar pattern in the perennial and sea-dispersed species *Spartina alterniflora*, a species dominating North American saltmarsh communities. The genetic diversity of this species was shown to be regionally structured along the Atlantic coast although genetic differences between populations were still maintained within each region (O'Brien & Freshwater 1999; Blum *et al.* 2007).

In order to estimate the strength of gene flow between populations, migration rates between populations are classically calculated from F_{ST} estimates (Wright 1949). Unfortunately, this method relies on the measurement of allelic frequencies within populations, which is not possible here because of the ploidy level of the two species. However, the *P. maritima* genotypes found in common between the populations of Brancaster, Paull Holme Strays and Welwick are a good indication of effective exchange of propagules between populations within the same region. Detailed work aiming to estimate more precisely the strength of this propagule exchange is necessary to allow us to better understand dispersal dynamics at this most local scale.

b. Comparison of genetic structure between species

In a study comparing the phylogeography of five coastal plant species, Kadereit *et al.* (2005) found that genetic structure was conserved between species, suggesting that all these species were under similar processes of coastal dispersal. A strong correlation between the genetic structures of the two species was shown here by a Mantel test and graphically confirmed by the NMDS ordination of the populations. This similarity indicates that both species we

investigated are likely to share similar dispersal vectors, although our data confirm that asexual propagation occurs more frequently in *P. maritima* than *T. maritima* (Table III-2).

c. Factors shaping the genetic structure

i. Isolation by distance

A strong correlation was found between genetic and geographic distance for both *T. maritima* and *P. maritima* confirming the regional organization of the genetic diversity found earlier with the DAPC and the AMOVA. A similar pattern was reported for *Spartina alterniflora* along the Atlantic coast of North America (O'Brien & Freshwater 1999; Blum *et al.* 2007; Travis & Grace 2010) and, more weakly, in the invasive European species *Elytrigia atherica* (Bockelmann *et al.* 2003).

Here, although coastal distance explains genetic dissimilarity between *P. maritima* populations significantly better than great circle distance, this is not the case for *T. maritima,* where great circle distance explains genetic dissimilarity significantly better. This pattern typified in both the DAPC and the NMDS ordination by the *T. maritima* population of Skinflats located on the east coast of Scotland (Fig. III-2 and III-3). This population is genetically closer to Lochgoilhead and Loch Carron (both located on the west coast) than to its neighbouring east-coast populations of Nigg Bay and Seal Sands.

In the UK, the retreat of the ice sheet following the last glacial maximum followed a latitudinal gradient (Siegert 2001). The strong correlation between genetic and latitudinal distance for both *T. maritima* and *P. maritima* might, therefore, be a signature of the sequential UK colonisation by these two species following a latitudinal gradient after the last glacial maximum (LGM). However, this result must be taken cautiously due to the strong correlation between latitudinal and coastal distance in our study (Mantel test: $r=0.518$, $p<0.001$).

ii. Isolation by resistance

One issue with the isolation by distance model is that it ignores landscape heterogeneity when used to predict expected gene flow between two populations (McRae 2006). The isolation by resistance framework was developed to overcome this issue and its application has permitted the testing of more precise scenarios to explore the importance of landscape features acting as barriers to gene flow (e.g. Goulson *et al.*, 2011). In our study, the model SEDCELLS, only considering sediment cells around the UK, gave the best results among the three tested here suggesting that the same processes that shape the geomorphology of the UK coastline are also important in shaping its biodiversity. Tidal currents do not seem to play the most important role at the scale investigated. However, the effect of tidal currents was only tested at a large geographical scale; its effect on mixing the genetic pool and therefore having an impact on a finer scale spatial genetic structure (i.e. within an estuary) needs further investigation.

In *T. maritima*, although the resistance matrix obtained with the SEDCELLS model was significantly correlated with genetic distance between populations, the correlation coefficient was significantly higher when using great circle distance between populations. This confirmed the impact of other than strictly coastal processes on shaping the genetic structure of this species. In their phylogeographic analysis of *T. maritima,* Lambracht *et al.* (2007) suggested that this species colonised the Baltic sea after the LGM from an inland refuge habitat. It is, therefore possible that the overland pattern of dispersal highlighted here is due to a stepping-stone dispersal process through inland habitat connecting apparently distant populations. However, these inland populations are rare in the UK (Davy & Bishop 1991) and their effectiveness in connecting distant populations may be questionable. Similarly or in conjunction with this last hypothesis, zoochory may connect distant populations of *T. maritima*. Indeed, migrating geese or ducks have been reported to feed on this species (Charman & Macey 1978; Davy & Bishop 1991).

In contrast, the resistance matrix obtained with the SEDCELLS model in *P. maritima* was significantly better correlated overall to genetic distance between populations. Sediment cell boundaries may, therefore, act as a strong barrier to dispersal of this species. This finding confirms that dispersal in *P. maritima* is primarily through a coastal process as already suggested for other coastal species around Europe (Kadereit *et al.* 2005). Moreover, our findings in *P. maritima* are comparable with work exploring the influence of marine currents on the genetic structure of sea beet (*Beta vulgaris ssp. maritima*) along the north-western coast of France (Fievet *et al.* 2007), where the separation between the two genetic groups discriminated follows the direction of a marine current similar to the ones delineating sediment cells around the UK.

The differential dispersal strategy between our two species may also be explained by their ecology. Recourse to sexual reproduction is known to vary between populations of *P. maritima* (Gray & Scott 1977; Erfanzadeh *et al.* 2010a) whereas it is the principal means of propagation in *T. maritima.* Interspecific differences in the prevalence of sexual reproduction may, therefore, partially explain the association of *P. maritima* with coastal processes via the long-shore dispersal of uprooted fragments while more abundant gene dispersal through the smaller units of seed and pollen may connect more distant populations of *T. maritima*.

6. Conclusion

Genetic diversity around the UK for *Puccinellia maritima* and *Triglochin maritima* is organized regionally, however, different parameters are at the origin of this structure. While the genetic organisation of *P. maritima* is shaped by a coastal process, our data indicate a stronger ability of *T. maritima* to disperse overland. Multispecies seascape genetic analysis such as that presented here is highly valuable for ecosystem management since it helps to designate coherent units of conservation and barriers to ecosystem connectivity (e.g. Kelly & Palumbi 2010; Coleman *et al.* 2011). Furthermore, it can inform saltmarsh restoration strategy by

demonstrating the likely extent of and barriers to dispersal processes underpinning colonisation of target restoration sites.

Saltmarsh is a species poor ecosystem and molecular tools are now rapidly available for non-model species. Future research should exploit the opportunity to take a community genetics approach to understanding genetic diversity and structure in this ecosystem, and thereby provide valuable information on habitat connectivity and the development of plant communities in newly restored saltmarsh sites.

7. Acknowledgements

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Chapter IV: Origin of colonists and subsequent development of genetic diversity within restored saltmarshes

To be submitted in *Applied Vegetation Science*

1. Abstract

It is now well recognised that genetic diversity has an impact on the performance and functioning of plant communities, but this aspect of biodiversity is rarely investigated when restoring plant populations. This is the case for north-west European saltmarsh communities where no study has yet been undertaken in order to characterize the impact of ecosystem restoration on genetic diversity. Consequently, in this study we investigate the level of genetic diversity in restored and natural populations of two saltmarsh plants with distinct dispersal ecology, *Puccinellia maritima* and *Triglochin maritima*. We sought to determine: (1) Where do colonists arriving on a restored saltmarsh come from? (2) How does the level of genetic diversity in restored schemes compare to what is observed in natural saltmarshes? (3) How does the level of genetic diversity in restored saltmarshes change through time? Plants were collected in both restored and natural sites across the United Kingdom and analysed at 11 microsatellite loci for *P. maritima* and 19 for *T. maritima*. Focusing on the regulated tidal exchange (RTE) restoration scheme of Skinflats located within the Forth estuary in Scotland, a discriminant analysis of principal components confirmed the regional origin of colonists. The strong genetic segregation between *P. maritima* populations within the estuary even pinpointed the immediately neighbouring natural saltmarsh as the main colonisation source of the restored saltmarsh for this species. Wilcoxon tests carried out on measures of genetic diversity failed to show any significant differences in genetic diversity between restored and natural sites. Similarly, linear regression failed to show any significant relation between genetic diversity in restored sites and age since restoration. Interestingly, for *P. maritima*, some values of genetic diversity in the restored population were significantly related to levels of genetic diversity in the neighbouring natural population. Overall our results suggest that, although restoration of genetic diversity is quick, it is constrained by the pre-existing regional level of genetic diversity. Restoring high levels of genetic diversity therefore involves recreating

saltmarshes in close proximity to pre-existing saltmarshes and ensuring that sufficient genetic diversity exists in these natural sites by allowing good connectivity and sufficient population size.

Keywords: Genetic diversity; Restoration; Saltmarsh; *Puccinellia maritima*; *Triglochin maritima* Polyploidy

2. Introduction

During the past centuries, an ever increasing human population has settled on the coast to the extent that the coastal human population density is now three times higher than the average global density (Small & Nicholls 2003). If this trend continues, it is estimated that coastal populations could reach 6 billion by 2025 (Kennish 2002). As a consequence, the pressure on coastal ecosystem is considerable and leads to ongoing degradation of these environments worldwide (Lotze *et al.* 2006). Represented all around the world in temperate area with low-energy wave action such as estuaries, saltmarshes are a prime example of coastal habitats globally impacted by human pressures (Adam 2002).

In the UK, sea defences have commonly been built to protect urban areas and convert saltmarshes into agricultural land, leading to a decrease of the area occupied by this environment over the past centuries (Foster *et al.* 2013). Impacting the long list of ecosystem services provided by saltmarshes (Gedan *et al.* 2009), the sustainability and efficiency of such hard defences against the sea have been criticised over the past decades. Besides, flood defences have become a major concern due to the recent dramatic flooding that has affected parts of the country (Carrington 2014).

The historic reduction in saltmarsh area in combination with increased recognition of their value has resulted in the implementation during the past decades of a saltmarsh restoration programme around the UK. Qualified as “managed realignment”, schemes usually consist of breaching the sea wall to let the sea water inundate an area previously embanked (Esteves 2013). Natural processes of recolonisation are then expected to take place, the terrestrial vegetation being progressively replaced by the characteristic halophytic communities of saltmarshes. Assessment of the success of such schemes typically focus on various aspects including the development of the plant community (Wolters *et al.* 2005b), bird use (Curado *et al.* 2013) or other functional parameters such as carbon sequestration (Burden *et al.* 2013).

Genetic diversity within and between saltmarshes is a frequently overlooked aspect of saltmarsh restoration (Friess *et al.* 2012). However, an ever increasing body of evidence shows the importance and impacts of genetic diversity at a broad range of ecosystem levels.

Poor performance of the individuals composing the restored population is a frequent example of such genetic effect. Local adaptation of genotypes to their environment may lead to their maladaptation when colonising distant habitats (e.g. Noël *et al.* 2011; Raabova *et al.* 2011). Studying *Spartina alterniflora*, a plant dominating North America saltmarsh communities, Travis & Grace (2010) showed that transplant performance within restoration sites was predicted by its genetic distance to this site. They therefore suggested the use of locally adapted genotypes for saltmarsh restoration. Individual performance may also be impacted several generations after restoration by the combination of founder effect and poor genetic connectivity with other populations leading to the prejudicial effects of inbreeding depression (Reed & Frankham 2003).

Interestingly, recent works have shown that the detrimental effects of reduced genetic diversity are not restricted to the individual level. At the population scale, genetic diversity also provides the adaptive reservoir needed to cope against future environmental uncertainty, otherwise described as the "option value" of genetic diversity (Jump *et al.* 2009a). Besides, experiments in monospecific plots have shown that genotypic diversity have a positive impact on productivity and resistance to disturbance (Hughes & Stachowicz 2004, 2009; Drummond & Vellend 2012). At the community level, a correlation was described between genotypic and species diversity (Vellend & Geber 2005; Vellend 2006). Moreover, these beneficial effects of genotypic diversity were shown to impact higher trophic levels by increasing the abundance (Reusch *et al.* 2005) and species richness of invertebrates in the community (Crutsinger *et al.* 2006).

This positive relation of plant genotypic diversity with community richness and productivity is thought to be particularly acute when only one or a few species dominate (Reusch and Hughes, 2006). This is exactly the case in saltmarshes where a positive effect of intraspecific genetic and trait diversity affected plant performance in *Spartina alterniflora* (Hughes 2014). In the case of North-Western European saltmarsh, communities are classically composed of few species dominated by one or two species. Such effects of genetic diversity are therefore likely to occur.

The level of genetic diversity within a restored saltmarsh depends on two components related to the dispersal ecology of the species composing the community. First, the origin and abundance of the individuals colonising the restored site, which relies on the strength of propagule exchange with the existing neighbour habitats via seed and plant fragments. Secondly, the level of genetic connectivity of this habitat with other saltmarshes which depends both on propagule exchange but also on pollen-flow between populations. Therefore, the goal of our study is to give an overview of the current genetic state of recently restored saltmarshes in the UK using two species having distinct dispersal ecology, *Puccinellia maritima* and *Triglochin maritima* (Gray & Scott, 1977; Davy & Bishop, 1991).

To achieve this goal, we use microsatellite markers (1) to assess directly the origin of colonists arriving on the saltmarsh restoration site of Skinflats located in the Forth estuary in Scotland. Secondly, using multiple pairs of restored and natural sites across the country, we assessed (2) how genetic diversity compares between restored and natural sites and (3) how genetic diversity develops through time in restored habitats.

3. Material and methods

a. Study species

Puccinellia maritima and *Triglochin maritima* are the two species used to carry this study. These two halophytes are commonly found in saltmarshes but frequently in different vegetation communities (Rodwell *et al.* 2000). Because saltmarsh communities are arranged along an elevation gradient, these two species are typically found at different positions on the saltmarsh. *Puccinellia maritima* is a grass occurring at low elevation and often dominating the community (Gray & Scott 1977). Small individuals settling in the pioneer zone of the saltmarsh expand horizontally via creeping stolons leading to a better stabilization of the sediment and the creation of hummocks on which the colonisation of other species is facilitated (Langlois *et al.* 2003). Conversely, *Triglochin maritima* occurs in communities higher up the saltmarsh where individuals grow and expand centrifugally, sometimes forming characteristic rings of vegetation (Davy & Bishop 1991). The engineering role of these rings has been detailed in previous studies showing the facilitating effects on establishment of other species by increasing the elevation and reducing waterlogging (Fogel *et al.* 2004). Both species are wind-pollinated and predominantly outbreeding (Gray & Scott 1977; Davy & Bishop 1991). Although sexual reproduction is thought to be the main agent of gene dispersal for *T. maritima* (Davy & Bishop 1991), recourse to this mode of propagation was shown to be variable between *P. maritima* populations (Gray & Scott 1977). Thus, asexual reproduction occurs extensively in *Puccinellia maritima* by the separation and dispersal of uprooted tillers (Brereton 1971), while it is less common in *T. maritima*. Finally, both species were reported to be octoploid with *Puccinellia maritima* being 2n=56 (x=7) and *Triglochin maritima* being 2n=48 (x=6).

b. Collecting sites

In total, samples were collected from 19 locations around the UK over three successive summers (Table IV-1, Fig. IV-1). On every site, care was taken to not collect physically linked individuals. Plant material was placed into zip-lock bags pre-filled with silica gel and stored until analysis.

Table IV-1 Sampling locations for the two species, *Triglochin maritima* (*Tm*) and *Puccinellia maritima* (*Pm*). The number of years since restoration is given in parentheses. The number of samples successfully amplified is given in square brackets.

Location	Code	Site status	Sampling year	*Tm*	*Pm*	Long.	Lat.
Nigg Bay	N	Natural	2011	30 [29]	30[29]	4.017°W	57.737°N
		Restored	2011 (8)	30[29]	30	4.035°W	57.739°N
Skinflats	SK	Natural	2011	100	100	3.732°W	56.055°N
		Restored	P. maritima: 2010 (1) T.maritima: 2011 (2)	100[98]	100[99]	3.734°W	56.055°N
Forth I	FI	Natural	2011	30	30	3.737°W	56.080°N
Forth II	FII	Natural	2011	30	30[29]	3.653°W	56.013°N
Forth III	FIII	Natural	2011	30[29]	30	2.851°W	56.014°N
Forth IV	FIV	Natural	2011	30	30	3.795°W	56.099°N
Seal Sands	SE	Natural	2011	30	30	1.214°W	54.623°N
		Restored	2011 (18)	30[29]	30	1.200°W	54.617°N
Paull Holme Strays	P	Natural	2012	30[29]	30	0.175°W	53.682°N
		Restored	2012 (9)	30[28]	30	0.200°W	53.698°N
Welwick	WE	Natural	2012	30[29]	30	0.020°E	53.648°N
		Restored	2012 (6)	30	30[29]	0.007°E	53.648°N
Brancaster	B	Natural	2011	30[29]	30	0.623°E	52.972°N
		Restored	2011 (9)	30	30	0.632°E	52.972°N
Hemley	H	Natural	2012	30	30[29]	1.339°E	52.032°N
		Restored	2012 (58)	30	30	1.337°E	52.035°N
Tollesbury	T	Natural	2012	30	30	0.833°E	51.770°N
		Restored	2012 (17)	-	30	0.840°E	51.767°N
Lepe	L	Natural	2011	22	30	1.386°W	50.862°N
		Restored	2011 (5*)	30	30[29]	1.358°E	50.786°N
Goosemoor	G	Natural	2011	30	30	3.454°W	50.684°N
		Restored	2011 (7)	30[29]	30	3.453°W	50.682°N
Ryan's field	R	Natural	2011	-	30[28]	5.411°W	50.192°N
		Restored	2011 (16)	30	30	5.433°W	50.177°N
Walborough	W	Natural	2011	30	30	2.985°W	51.314°N
		Restored	2011 (7)	30	30	2.985°W	51.317°N
Morecambe	M	Natural	2012	-	30[29]	2.808°W	54.141°N
Lochgoilhead	LG	Natural	2012	20[19]	30	4.914°W	56.160°N
Loch Carron	LC	Natural	2012	30[29]	30	5.452°W	57.418°N

i. Origin of colonists

The saltmarsh system of Skinflats was used to assess the origin of the plants having colonized the restored site. Skinflats is a "regulated tidal exchange" (RTE) scheme located within the Forth estuary and having started in autumn 2009 (Fig. IV-1). Characteristic saltmarsh plants

started to settle on site from the first growing season in 2010. For *P. maritima,* one hundred colonists were collected inside the restored site in 2010 with an average distance between adjacent samples (AD) of 3 m. Although *T. maritima* was represented on site in 2010, the size of its population was too small to be sampled. One hundred samples of *T. maritima* were therefore collected over the second growing season in 2011 (AD = 12m). To assess whether the origin of these colonists is local, regional, or global; populations belonging to these three levels were also sampled. First, one hundred samples of each species were collected on the neighbouring natural saltmarsh of Skinflats (*T. maritima* AD: 14m; *P. maritima* AD: 6m). Secondly, thirty samples of both species were collected on each of four pre-existing saltmarshes belonging to the Forth estuary (*T. maritima* AD: 14-27m; *P. maritima* AD: 12-24m). Thirdly, thirty individuals on each of 14 natural saltmarshes for *P. maritima* and 12 natural saltmarshes for *T. maritima* were also collected around the UK (*T. maritima* AD: 5-36m; *P. maritima* AD: 6-44m) (Fig. IV-1, Table IV-1).

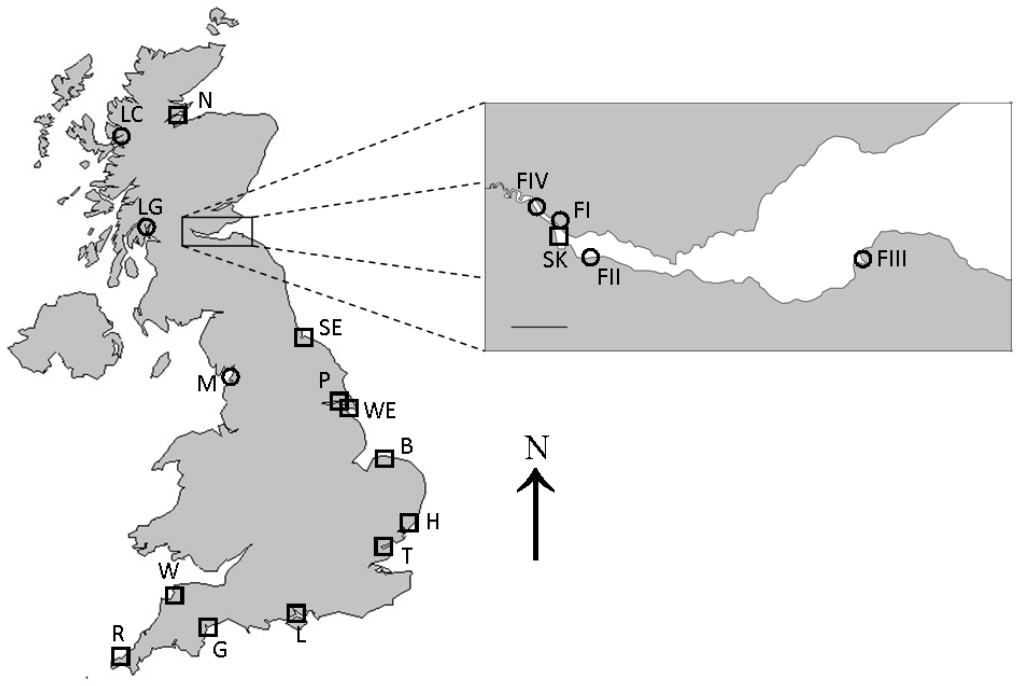

Fig. IV-1 Map of the sampling sites. Population codes are given in Table IV-1. Squares: Locations where both a restored and natural site were sampled for at least one species; Circles: Locations where only a natural site was sampled. Although FII is a restored saltmarsh, this scheme was implemented before the restored site of Skinflats and is therefore considered as a potential colonisation source.

ii. Comparison of restored vs. natural saltmarshes

Twelve pairs of sites combining a restored and a natural saltmarsh were visited and sampled around the UK (Table IV-1, Fig. IV-1). To standardize the sample size between locations, a random sub-sample of thirty individuals was selected in both the restored and natural sites of Skinflats. Individuals of *P. maritima* were available from both restored and natural saltmarsh at every location (Natural AD: 14-43m; Restored AD: 13-46m). For *T. maritima*, there were only 10 locations where samples could be collected both within natural and restored sites (Natural AD: 16-36m; Restored AD: 16-49m)

c. Molecular work

DNA was extracted from dried tissue using the DNeasy 96 plant kit (Qiagen) following the manufacturer's procedure. We genotyped all individuals using protocols and microsatellite markers developed recently for both species (*T. maritima*: Rouger & Jump 2013; *P. maritima*: Rouger *et al.* 2014). Fragment analysis was conducted using an ABI 3730 DNA Sequencer (Applied Biosystems) and resulting electropherograms were analysed using the software STRand (Toonen & Hughes 2001). Raw data were then imported into R (R Core Team 2013) and allele binning was made using the package MsatAllele (Alberto 2009).

Loci Pm27 for *P. maritima* and Tm07 for *T. maritima* were excluded from further analysis due to high amplification failure. Samples for which more than 3 loci were missing were discarded from the analysis. Overall, 11 loci were used for *P. maritima* and 19 loci for *T. maritima* giving 2.51% and 0.36% of missing data respectively.

d. Data analysis

Both *P. maritima* and *T. maritima* are polyploid species for which the inheritance pattern remains unknown. Analysis of molecular data in polyploid species is currently a challenge in population genetics (Dufresne *et al.* 2014). The main problem concerns the difficulty to infer

allelic dosage within each individual therefore preventing the calculation of classical statistics such as F statistics or deviations from Hardy-Weinberg equilibrium. Several methods aim to solve this issue. For example, the area under each peak on an electropherogram has been used to infer the copy number of copies of each allele within an individual (Esselink *et al.* 2004). Another approach uses a maximum likelihood method to calculate the allele frequencies at each locus within each population (Meirmans & Van Tienderen 2004; Teixeira *et al.* 2014). However, these methods make assumptions which are difficult to meet for species with a high ploidy level such as *P. maritima and T. maritima*. For each species, we therefore analysed our microsatellite data by coding each allele as present (1) or absent (0). The resulting presence/absence matrix was analysed using statistical tools which were designed for dominant markers such as AFLPs.

i. Origin of colonists

Saltmarshes were sampled at three different scales around the restored site of Skinflats. First at a local scale (natural saltmarsh of Skinflats), at a regional scale (natural saltmarshes belonging to the Forth estuary i.e. FI; FII; FIII; FIV) and at a global scale (natural saltmarshes sampled around the UK, see Fig. IV-1). In the first place, individuals coming from local and regional sites ("Inside estuary") were genetically discriminated from individuals coming from sites around the UK ("Outside estuary") using a discriminant analysis of principal components (DAPC) implemented in the package adegenet in R (Jombart 2008; Jombart *et al.* 2010). Selecting the number of principal components to retain in the DAPC was conducted using the cross-validation method function *xvalDapc* with a training set gathering 90% of the samples. The number of PCs giving the minimum "root mean squared error" was retained (Jombart 2014). Based on this DAPC, predictions about the origin of the colonists on the restored saltmarsh were made. Using the function *predict.dapc,* samples collected within the restored

saltmarsh were added as supplementary individuals and therefore assigned to either the cluster “Inside estuary” or “Outside estuary”.

Secondly, another DAPC was conducted using only individuals from inside the estuary. In this case, we tried to genetically discriminate individuals sampled in the regional pool of populations from individuals collected in the local natural saltmarsh of Skinflats. The same procedure as above was used to predict the origin of the colonists collected on the restored saltmarsh of Skinflats.

ii. Genetic diversity

We expect that colonisation after saltmarsh restoration will have a measureable effect on genetic diversity parameters through phenomena such as founder effects. Studies comparing restored and natural habitats classically look at parameters such as inbreeding coefficient (F_{IS}), expected heterozygosity (H_e) or deviation from Hardy-Weinberg equilibrium(Lloyd *et al.* 2012; Fant *et al.* 2013; Oudot-Canaff *et al.* 2013). Unfortunately, the ploidy level of our two species impedes the calculation of such statistics without making unreliable assumption about the behaviour of the microsatellite markers used. However, less complex but still informative parameters could be calculated.

Number of alleles (N_A), private alleles (PA), rare alleles (Na_{15}) and common alleles (Na_{50}) were calculated for each population. Rare and common alleles were counted based on band frequencies rather than allelic frequencies. Alleles with a band frequency lower than 15% of the population (allele present in 4 individuals or less in a population of 30 individuals) were considered as rare alleles; alleles with a band frequency higher than 50% in the population were considered as common alleles. Number of clones (G) was measured in each population in order to calculate clonal diversity (R). Individuals sharing the same multilocus genotype were determined using the software GenoType (Meirmans & Van Tienderen 2004). Clonal diversity was then calculated following the equation (G-1)/(N-1) (Dorken & Eckert 2001) where N is the

number of individuals within the population. Detection of clones was also used to check the number of shared genotypes between populations. Genetic diversity within populations was calculated using the Kosman index of diversity within population (KW) (Kosman & Leonard 2007) which is well suited to calculate genetic diversity in clonal species or when using markers likely to depart from Hardy-Weinberg.

All these parameters were computed using custom scripts in R (Appendix IV-1)

iii. Comparison of restored vs. natural saltmarsh

N_A, PA, N_{A15}, and N_{A50} are statistics which heavily depend on sample size. In order to compare these indices in populations with differing sample size, approaches such as rarefaction methods were developed for co-dominant markers in diploid species (Kalinowski 2004) but these are still inapplicable for polyploids. Comparison between restored and natural saltmarsh was therefore made only using populations with similar sample size. We therefore conserved the 12 locations where a restored and a natural population coexisted for *P. maritima* (SK, B, G, L, N, R, SE, W, H, P, T, WE) and restricted our analysis to 9 locations for *T. maritima* (B, G, N, SE, SK, W, H, P, WE). Sample size ranged from 28 to 30 individuals in all these populations. The *T. maritima* samples collected in Lepe (L) were excluded from the analysis since the population size in the restored site was 22 individuals only.

Comparison of all genetic measures (N_A, PA, N_{A15}, N_{A50}, R, and KW) between restored and natural saltmarshes were made using paired Wilcoxon signed-rank tests. The ratios of rare alleles (N_{A15}/N_A) and of common alleles (N_{A50}/N_A) were also compared between restored and natural saltmarshes using paired Wilcoxon signed-rank tests. Simple linear regressions on the ratio restored/natural of every measure of genetic diversity were used to test the effect of restored saltmarsh age. Relationships between genetic diversity measures in natural and restored saltmarsh were also tested using simple linear regressions. All tests were carried out using R.

4. Results

a. Origin of colonists

i. Outside vs. inside estuary

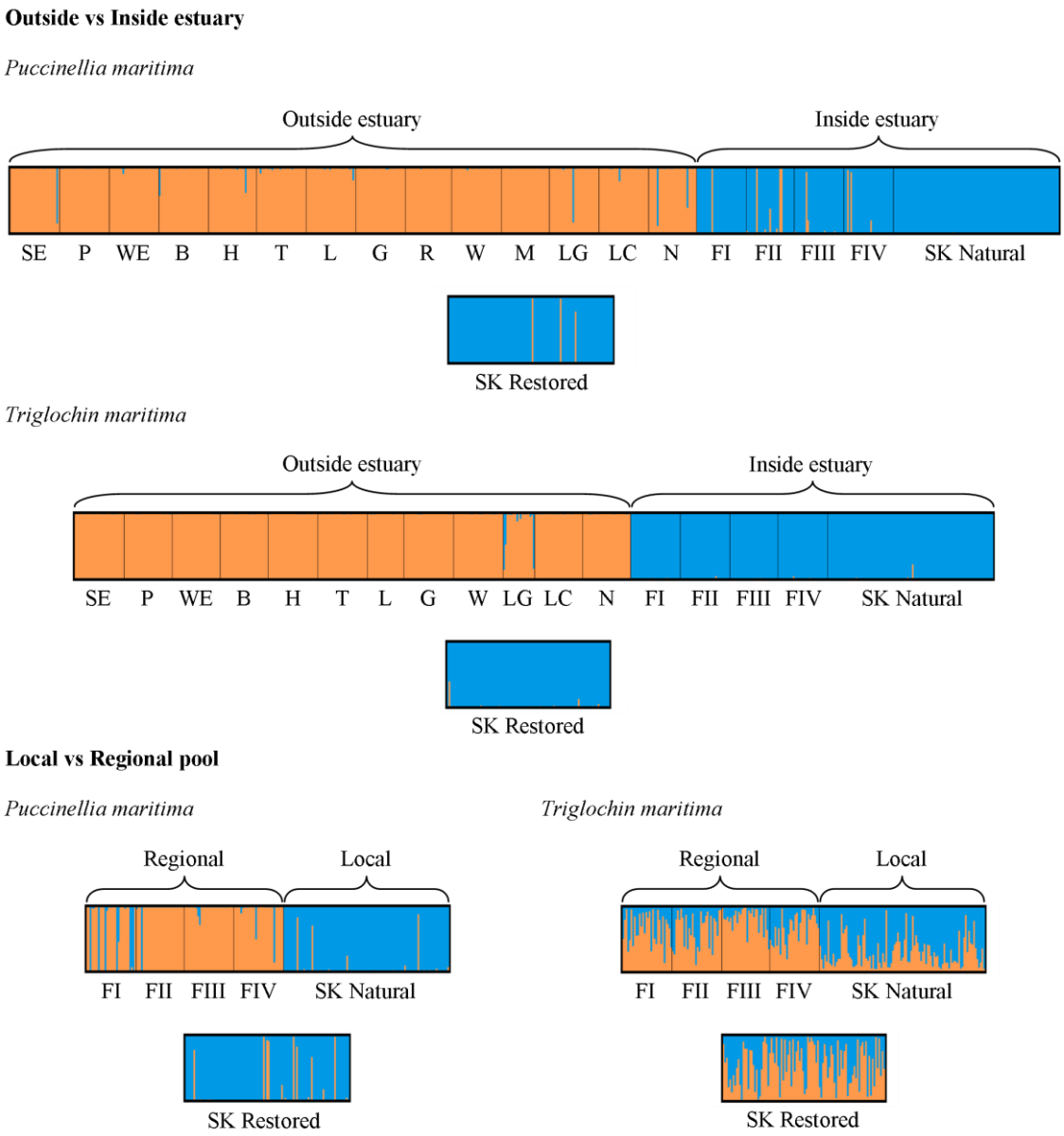

Fig. IV-2 DAPC results showing, first, the segregation of collected individuals into either a "global" (outside estuary) or a "regional" cluster (inside estuary) and the subsequent assignment of the individuals collected in the restored saltmarsh of Skinflats. Second, the segregation of individuals of the Forth estuary into either a "regional" or a "local" cluster and the subsequent assignment of the individuals collected in the restored saltmarsh of Skinflats.

Using cross-validation method, the optimal number of principal components to retain was 120 for *P. maritima* and 100 for *T. maritima* giving an assignment prediction success of 95.4% (5% Confidence interval: CI = 45.9-54%) and 99.0% (CI= 46.1-53.8%) respectively. Therefore, for both species, samples collected from natural saltmarshes inside the estuary could be clearly discriminated from samples collected in saltmarshes located outside the estuary (Fig. IV-2). Using these optimal numbers of principal components for the discriminant analysis permitted us to retain 98.7% and 95.9% of the genetic variance for *P. maritima* and *T. maritima* respectively.

In *P. maritima*, although the two clusters are well defined, one to two individuals in the populations of Nigg Bay (N), Lochgoilhead (LG) and Seal Sands (SE) showed similarity with the genetic cluster inside the estuary. In contrast, a few individuals collected within the Forth estuary showed relatedness with the genetic cluster defined by the individuals sampled around the UK (Fig. IV-2).

The segregation between the two genetic clusters was even clearer in *T. maritima,* where only three samples from Lochgoilhead showed similarity with samples collected from inside the estuary, while, no samples from inside the estuary were related to a population around the UK (Fig. IV-2).

The assignment of the Skinflats colonists to either cluster gave comparable results in both species. In *P. maritima*, apart from 3 individuals, all colonists were assigned to the “inside estuary” cluster. For *T. maritima*, all samples were assigned to the “inside estuary” cluster without exception (Fig. IV-2).

ii. Regional vs. Local

Cross validation gave an optimal number of principal components to retain of 40 for *P. maritima* and 60 for *T. maritima*. However, while the assignment prediction success is 91.5%

(CI = 42.9-56.3%) for *P. maritima*, it is only 59.8% for *T. maritima* and this is only marginally different from what is expected when assigning the individuals only by chance (CI = 42.9-56.8%). Using the optimal number of principal components in the subsequent discriminant analysis retained 94.7% and 90.5% of the variance for *P. maritima* and *T. maritima* respectively.

As expected by the cross validation, *P. maritima* individuals sampled in the local saltmarsh (Skinflats natural) were in general well discriminated from individuals collected in the other saltmarshes of the Forth estuary (FI, FII, FIII, FIV). On the contrary, the segregation between local and regional pools of *T. maritima* was unclear due to a considerable amount of admixture (Fig. IV-2).

Samples of *P. maritima* collected within the restored saltmarsh of Skinflats were generally assigned to the local genetic cluster composed of the samples collected on the natural saltmarsh of Skinflats. For *T. maritima*, the observed admixture between regional and local genetic cluster prevented reliable identification of the origin of the colonists.

b. Genetic diversity

The average number of alleles found in populations was 85 and 90 in *P. maritima* and *T. maritima* respectively but there was more variation between populations in *P. maritima* (SD= 12.3) than in *T. maritima* (SD= 9.8). Among all *P. maritima* populations visited, there were only seven populations where the number of genotypes detected was equal to the number of individuals sampled (R=1). In contrast, within *T. maritima* populations, all sampled individuals represented a unique multi-locus genotype (Table IV-2).

Table IV-2 Genetic diversity parameters for all locations samples for *P. maritima* and *T. maritima*. N: number of samples, N_A: number of alleles, PA: number of private alleles, NA_{15}: number of rare alleles, NA_{50}: number of common alleles, R: Genotypic diversity, KW: Kosman index of genetic diversity.

Pop.	Site status	*Puccinellia maritima*							*Triglochin maritima*						
		N	N_A	PA	N_{A15}	N_{A50}	R	KW	N	N_A	PA	N_{A15}	N_{A50}	R	KW
SE	Natural	30	81	1	26	29	0.690	0.741	30	71	0	24	26	1	0.706
	Restored	30	75	0	41	25	0.448	0.432	29	91	2	42	25	1	0.753
P	Natural	30	79	0	27	33	0.586	0.620	29	102	0	21	21	1	0.753
	Restored	30	83	0	33	29	0.690	0.687	28	95	1	19	19	1	0.785
WE	Natural	30	82	0	26	31	0.724	0.681	29	86	1	33	33	1	0.690
	Restored	30	68	0	33	34	0.5	0.340	30	84	1	28	28	1	0.708
B	Natural	30	89	0	32	27	0.759	0.729	29	94	1	43	26	1	0.722
	Restored	30	94	1	40	26	0.828	0.727	30	76	3	38	28	1	0.601
H	Natural	29	101	0	47	22	1	0.714	30	110	4	53	26	1	0.746
	Restored	30	101	0	46	23	0.931	0.704	30	104	1	42	30	1	0.751
T	Natural	30	96	0	36	23	1	0.751	30	93	1	37	26	1	0.722
	Restored	30	109	1	57	24	0.828	0.751	-	-	-	-	-	-	-
L	Natural	30	92	1	32	19	1	0.755	22	81	0	34	27	1	0.672
	Restored	29	74	0	30	26	0.679	0.639	30	86	1	24	27	1	0.768
G	Natural	30	85	1	30	24	0.897	0.731	30	99	4	45	28	1	0.718
	Restored	30	84	1	29	29	1	0.704	29	89	1	32	27	1	0.725
R	Natural	28	65	0	23	25	0.926	0.627	-	-	-	-	-	-	-
	Restored	30	60	0	15	30	0.862	0.685	30	87	2	38	30	1	0.681
W	Natural	30	103	0	36	25	1	0.763	30	109	1	51	27	1	0.754
	Restored	30	109	0	52	28	0.897	0.740	30	92	1	37	28	1	0.741
M	Natural	29	87	0	40	29	0.643	0.660	-	-	-	-	-	-	-
LG	Natural	30	88	0	37	23	1	0.721	19	94	0	45	20	1	0.761
LC	Natural	30	89	2	43	19	1	0.758	29	100	1	49	23	1	0.722
N	Natural	29	91	2	43	28	0.643	0.699	29	95	1	48	26	1	0.705
	Restored	30	70	0	25	28	0.621	0.635	29	92	1	32	18	1	0.754
SK	Natural	30	77	0	28	28	0.621	0.655	30	80	2	23	27	1	0.776
	Restored	30	76	0	38	28	0.586	0.601	30	77	0	34	25	1	0.651
FI	Natural	30	89	0	29	28	0.759	0.723	30	84	0	29	23	1	0.729
FII	Natural	29	70	1	32	27	0.750	0.621	30	90	0	30	26	1	0.720
FIII	Natural	30	89	0	33	22	0.897	0.710	29	76	2	33	26	1	0.650
FIV	Natural	30	83	3	20	27	0.828	0.769	30	93	1	37	24	1	0.722

A few *P. maritima* populations shared common genotypes with each other that we can distinguish in three different cases. First, all but four (L, R, H, T) natural saltmarshes shared at least one common genotype with their paired restored saltmarsh (Table IV-3). Secondly, some populations collected within the Forth estuary (SK Natural, SK Restored, FI, FII and FIV) also shared genotypes (Table IV-4). The outermost population FIII was the only one that did not have a genotype in common with the other populations of the estuary. Thirdly, populations from Brancaster, Paull Holme Strays and Welwick also shared clones with each other (Table IV-5).

Table IV-3 Number of shared *P. maritima* genotypes between restored and natural sites

	SK	B	G	L	N	R	SE	W	H	P	T	WE
Common genotypes	4	1	4	0	2	0	1	1	0	3	0	3

Table IV-4 Number of shared *P. maritima* genotypes between sites collected within the Forth estuary

	SK Nat.	SK Res.	FI	FII	FIII	FIV
SK Nat.						
SK Res.	4					
FI	3	3				
FII	1	1	1			
FIII	0	0	0	0		
FIV	0	0	1	0	0	

Table IV-5 Number of shared *P. maritima* genotypes between sites of Brancaster, Welwick and Paull Holme Strays

	B Nat.	B Res.	WE Nat.	WE Res.	P Nat.	P Res.
B Nat.						
B Res.	1					
WE Nat.	1	0				
WE Res.	2	0	3			
P Nat.	1	0	2	3		
P Res.	0	0	2	2	3	

In both species and for any genetic diversity measures considered, Wilcoxon tests detected no significant differences between restored and natural saltmarshes. In *P. maritima*, Kosman index of diversity within populations (KW) was lower in restored populations than in natural populations but marginally not significant (V=16 p=0.077). The proportion of rare alleles within populations (N_{A15}/N_A) was higher in restored saltmarsh than in natural saltmarshes although again this was marginally not significant (V=62 p=0.0772).

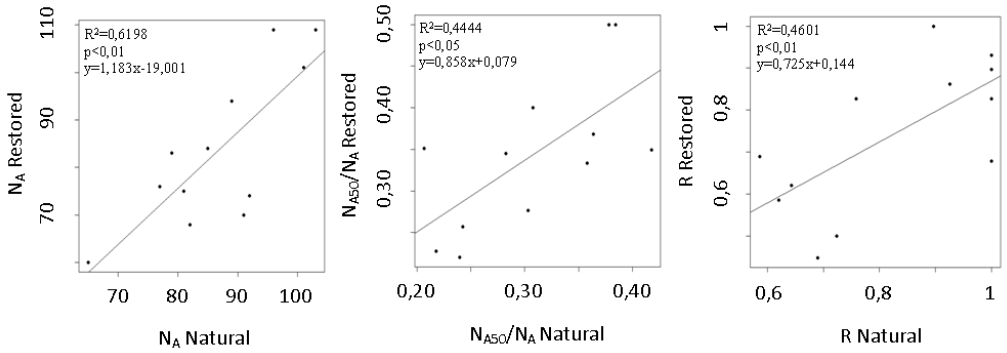

Fig. IV-3 Significant correlations between parameters of genetic diversity inferred in natural populations against restored populations of *P. maritima*. N_A: number of alleles; N_{A50}/N_A: proportion of common alleles; R: genotypic diversity.

Linear regressions did not detect a significant effect of restored saltmarsh age on any genetic diversity parameters. However linear regressions highlighted correlations between some genetic diversity (N_A, N_{A50}/N_A, R) measures found in natural populations of *P. maritima* and their paired restored populations (Fig. IV-3). Such correlations were absent between restored and natural populations of *T. maritima*.

5. Discussion

In the United States, "active" restoration is sometimes used in isolated saltmarshes to speed up the restoration process. It classically consists of transplanting or seeding *Spartina alterniflora*, a plant dominating US saltmarsh communities (Niedowski 2000; Travis *et al.* 2006). This technique allows control of the amount of genetic diversity available at the start of the restoration. In Europe where saltmarsh vegetation is more diverse than in North America, such a solution is difficult to apply. Saltmarsh restoration is, therefore, "passive" in Europe and relies only upon the natural colonisation of restored sites. In this context, physical characteristics of the site play a role (Erfanzadeh *et al.* 2010b; Pétillon *et al.* 2010) but propagule availability is the factor most affecting the colonisation of a restored saltmarsh (Erfanzadeh *et al.* 2010a).

For both species, our DAPC results clearly indicate that *P. maritima* and *T. maritima* individuals settling on the restored site of Skinflats are at least of a regional origin (i.e. inside the Forth estuary). These findings confirm other results suggesting that colonists on a restored saltmarsh are mostly of regional origin given the matching of the communities found between restored and neighbouring natural sites (Wolters *et al.* 2005b; Erfanzadeh *et al.* 2010a). From a management point of view, this has the advantage to reduce the probability of maladaptation of geographically distant individuals colonising the restored site. This probability was non negligible in the first place given the high and supposedly adaptive morphological variation of *P. maritima* notably (Gray 1974; Gray & Scott 1980). On the other hand, propagule exchange

with distant populations is likely to be too rare to permit the rapid restoration of a saltmarsh where natural communities are absent in close vicinity. It is therefore crucial to conserve a sufficiently dense network of saltmarsh in order to keep the potential for restoration high anywhere along the UK coastline where conditions allow.

At a finer scale, assignment inside the estuary gave contrasting results between species likely due to their different dispersal ecology. For *P. maritima*, most individuals from the Skinflats restored site were assigned into the local genetic cluster formed by the individuals collected on the Skinflats natural site. Only a few showed greater genetic similarity with samples from the regional pool. Colonisation is, therefore, very local for this species. Interestingly, the number of genotypes shared between populations inside the Forth estuary indicates a regular direct exchange of genetic material between populations within the estuary via exchange of vegetative fragments. However, it is clear that the rate of this genetic exchange is not strong enough to homogenize the *P. maritima* gene pool across the estuary. Although sexual reproduction varies between populations of *P. maritima* (Gray & Scott 1977), studies have found that seed production in this species can indeed be very limited or null (Erfanzadeh *et al.* 2010a). In the Forth estuary, vegetative fragments settling in a new population may therefore not breed with local genotypes, restricting the potential for homogenising the genepool across the estuary.

Genetic exchange between populations is stronger in *T. maritima,* as demonstrated by the DAPC being unable to segregate individuals into a local and a regional cluster. It is therefore impossible to tell whether the colonisation source for this species is local or regional. If seeds arriving on the saltmarsh are of local origin, extensive pollen-flow must exist between populations to generate a scenario close to panmixia in the estuary. On the contrary, if seeds arrive from all saltmarshes inside the Forth estuary, this seed exchange between populations may be enough to homogenise the gene pool inside the estuary. These scenarios are not

mutually exclusive and equally plausible given that *T. maritima* is protogynous, wind-pollinated and seeds are able to disperse and retain good viability over a long period in sea water (Davy & Bishop 1991). A study on *Beta vulgaris ssp. maritima*, another coastal species, comparing cytoplasmic and nuclear markers suggested that pollen-flow was more effective in genetically connecting populations (Fievet *et al.* 2007). Comparison between these two kinds of markers in *T. maritima* would permit more precise determination of the processes involved in shaping the genetic structure of this species.

Having demonstrated colonization for both species to be at least of regional origin, we expected a strong founder effect to act on newly created saltmarshes. However, none of our results supported this hypothesis. The level of genetic diversity in restored saltmarshes was at a similar level to what is commonly found in natural sites. Our results follow the same pattern that was suggested in other study systems where seed banks and relict populations (Oudot-Canaff *et al.* 2013) or artificial re-planting (Lloyd *et al.* 2012; Fant *et al.* 2013) are involved in recreating plant populations. In our study, only natural recolonisation of recreated saltmarshes took place; moreover, the impact of relict seed bank on vegetation development can be discarded given that all sites having been embanked for decades prior to restoration and *P. maritima* and *T. maritima* do not form persistent seed banks (Wolters & Bakker 2002). Interestingly, in a comparable environment, no differences in genetic diversity could be detected between naturally colonising *Spartina alterniflora* populations and reference sites in Louisiana saltmarshes (Travis *et al.* 2002). However, a recent example looking at restored populations of *Dactylorhiza incarnata*, a coastal dune orchid, indicated that recolonisation from a nearby population was causing severe impacts on genetic diversity in restored populations (Vandepitte *et al.* 2012) due to founder effect. For our two species, different parameters may have reduced founder effects in restored sites. First, even though no estimate was taken in any population, population size of both species was high in most restored saltmarshes (R. Rouger, pers. obs.), the local natural site being close enough to act as a regular

and strong source of colonists. Secondly, the high ploidy level of our two species dramatically increases the effective population size of *P. maritima* and *T. maritima* (Parisod *et al.* 2010) buffering the effects of genetic drift during the generation following restoration. Consequently, we predict that founder effect should be more acute in restored populations of diploid saltmarsh plants that are isolated from natural sites.

As expected from our findings with the DAPC, the level of genetic diversity in restored populations of *P. maritima* seemed to match the genetic diversity found in local reference saltmarshes. This observation is of particular importance from a management point of view as it demonstrates that in order to reach an adequate level of genetic diversity within a restored site; it is of crucial importance to conserve the level of genetic diversity in pre-existing local sites. A small and isolated natural population upon which deleterious effects of genetic drift or inbreeding depression are acting is unlikely to contribute a high level of genetic diversity to a restored population of *P. maritima*. We did not find such effects in *T. maritima*, potentially because there is less variation overall in the genetic measures for this species.

6. Conclusion

Besides confirming the need for a local natural saltmarsh in restoration schemes, our results demonstrate that genetic diversity is restored very quickly in restored populations of *P. maritima* and *T. maritima*. However, we highlight that genetic diversity within the restored saltmarsh depends on the existing local genetic diversity. From a management point of view, this implies that besides restoring saltmarshes, care must be given to conserve the existing genetic diversity in natural saltmarsh by ensuring connectivity and sufficiently high population size.

7. Acknowledgements

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Chapter V: Fine-scale spatial genetic structure of saltmarsh plants in both restored and natural environments.

To be submitted to *Evolutionary Ecology*

1. Abstract

An ever growing body of evidence shows that population genetic diversity is an important aspect to consider in ecosystem restoration due notably to the beneficial effects that genetic diversity may have on individual fitness and community performance. Nevertheless, most studies looking at restoration of plant genetic diversity in habitat restoration overlook how this genetic diversity is distributed within both restored and reference ecosystems although this may also be an important aspect to consider. Using a multiyear-point sampling within a restored and a natural saltmarsh, the aim of this study was to demonstrate the importance of considering fine-scale spatial genetic structure when restoring a habitat. To achieve this goal, we used as a model the two halophytes *Puccinellia maritima* and *Triglochin maritima*. Although similar levels of genetic diversity were observed in both restored and natural saltmarshes, the analysis of fine-scale spatial genetic structure led to different patterns depending on the species. For *T. maritima*, no fine-scale spatial genetic structure could be detected in either saltmarsh suggesting that genetic recovery is complete. For *P. maritima* on the contrary, a fine-scale genetic structure was detected arranged along the elevation gradient of the natural saltmarsh with individuals collected at low elevation being differentiated from individuals collected at higher elevation within the saltmarsh. This structure, probably driven by differential selective pressure between locations, could not be detected in the restored saltmarsh, thus suggesting incomplete genetic recovery. Even though the consequences of this difference in fine-scale spatial genetic structure need further investigation, this example illustrates the importance of looking not only at genetic diversity but also at its distribution to evaluate the genetic recovery of a community.

Keywords: Genetic structure, Saltmarsh, *Puccinellia maritima*, *Triglochin maritima*, Polyploids

2. Introduction

It is now broadly recognised that species and functional diversity plays a significant role in maintaining ecosystem functioning and stability (Hooper *et al.* 2005). The recovery of plant community assembly comparable to what is typically found in a natural environment has therefore been the focus of various studies looking at the success of restoration schemes (e.g. Seabloom 2003; Lindborg & Eriksson 2004; Galatowitsch & Richardson 2005). In species poor environments, recent studies have shown that genetic diversity in the dominant species may also have an influence on ecosystem performance. For example, genotypically diverse plots of *Zostera marina* were demonstrated to be more resistant to disturbance than monocultures (Hughes & Stachowicz 2004) but also more resilient and productive (Reusch *et al.* 2005; Hughes & Stachowicz 2009). In *Solidago altissima*, genotypic diversity was shown to have a positive effect on primary productivity and arthropod diversity (Crutsinger *et al.* 2006). The effect of genotypic diversity on community performance was even found in one instance to be similar in magnitude to the effect of species diversity (Cook-Patton *et al.* 2011). Furthermore, maintaining genetic diversity might be one way in which populations can be best prepared to survive environmental uncertainty (Jump *et al.* 2009a). In order to take this aspect into account when restoring a habitat, recent surveys of restoration projects have also looked at how neutral genetic diversity recovers to reach the level typically found in natural environments (Lloyd *et al.* 2012; Fant *et al.* 2013; Oudot-Canaff *et al.* 2013). However, comparisons of genetic diversity levels between restored and reference ecosystems generally overlook how this diversity is spatially distributed although this is a crucial aspect to assess.

Studies looking at how genetic diversity recovers in restoration schemes generally focus on areas of at least several hundred square meters while the effects of genetic diversity through facilitation or complementarities between genotypes have been validated on plots of much smaller sizes (e.g. Hughes & Stachowicz 2004, 2009; Reusch *et al.* 2005). Therefore, the effects

of genetic diversity within two environments showing similar levels of genetic diversity may depend heavily on their pattern of fine scale spatial genetic structure.

For example, if a strong genetic structure occurs, the genetic landscape can be reduced to a juxtaposition of small plots, each of them supporting a limited genetic diversity (Fig. V-1, Case 1). Conversely, if no fine scale spatial genetic structure is detected, it is reasonable to estimate that global level of genetic diversity is a good representation of the fine-scale level (Fig. V-1, Case 2).

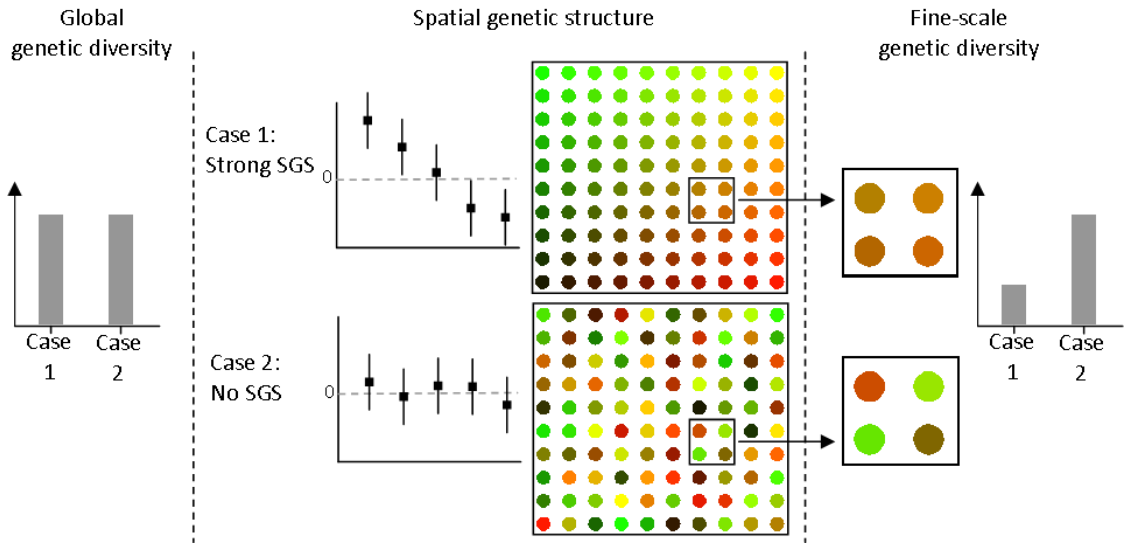

Fig. V-1 Influence of fine-scale spatial genetic structure (SGS) within two populations supporting the same global genetic diversity. Each coloured dot represents an individual, genetic proximity between two individuals is symbolised by their colour proximity. In case 1, genetically close individuals are spatially close to each other producing a strong fine-scale SGS; in case 2, individuals are arranged randomly across the landscape producing no SGS. Genetic diversity at a fine-scale in case 1 is, therefore, smaller than what is observed in case 2.

Fine-scale spatial genetic structure may originate from limited gene-dispersal across the landscape (Volis *et al.* 2010; Barluenga *et al.* 2011; Sebbenn *et al.* 2011) or specialisation of individuals to local variations in environmental conditions (Antonovics 2006; McLeod *et al.* 2012). In this latter case, effects of genetic diversity may also switch from beneficial to detrimental due to the presence of numerous individuals maladapted to local conditions therefore, weakening the stability of the community (Keller *et al.* 2000).

North-western Europe saltmarshes are species-poor environments where the vegetation is often dominated by one or two species (Rodwell *et al.* 2000). Since the area occupied by this environment has reduced over the past centuries, restoration schemes have been implemented to counteract the loss (Garbutt & Boorman 2009). A multiple comparison between restored and natural saltmarshes has recently shown that genetic diversity is very quickly restored in recreated saltmarsh (see Chapter IV); however, the distribution of this genetic diversity both in restored and natural saltmarsh remains uninvestigated.

Saltmarshes are typical environments were such fine-scale spatial genetic structure is likely to develop. Firstly because the perennial plants often dominating the vegetation community tend to spread asexually through rhizomatic or stoloniferous expansion leading to the spatial proximity of genetically identical individuals. Secondly, saltmarshes are at the transition between marine and terrestrial ecosystem and regularly inundated by tides. Environmental conditions such as soil-moisture, salinity or disturbance are, therefore, strongly spatially auto-correlated along an elevation gradient. Besides having a strong effect on the structuring of vegetation communities on the saltmarsh (Rodwell *et al.* 2000), this variation in environmental conditions was also demonstrated to be at the origin of intraspecific morphological variation (Davy & Smith 1985; Gray 1987). Although such variation in morphology should theoretically have no direct influence on neutral genetic variation, some studies have highlighted genetic differentiation between individuals collected at high and low elevation within the saltmarsh (Festoc 1999; Bockelmann *et al.* 2003). Lack of neutrality in the markers that both studies used being unlikely, authors attributed these observed patterns to a strong post-zygotic selection against seedlings coming from inter-habitat crosses thus restricting gene-flow between these two elevation zones (Festoc 1999; Bockelmann *et al.* 2003).

By using neutral microsatellite markers on two common north-western European saltmarsh species, the goal of this study was, firstly, to investigate and compare levels of genetic diversity

between a restored and a natural saltmarsh. Secondly, introducing an innovative method for the construction of spatial autocorrelograms of genetic variation, we aimed to assess how this genetic diversity is distributed within both restored and natural saltmarsh by looking at patterns of fine-scale spatial genetic structure.

3. Material and Methods

a. Study species

The two species used in this study are *Puccinellia maritima* and *Triglochin maritima*. They are both halophytes commonly found in saltmarshes and have an important role in the functionality of this ecosystem (Langlois *et al.* 2001, 2003; Fogel *et al.* 2004). Often dominating their respective vegetation communities, *P. maritima* is more prevalent at lower elevation on the saltmarsh while *T. maritima* is found in more mature community higher up within the tidal frame (Gray & Scott 1977; Davy & Bishop 1991). Both wind-pollinated, sexual reproduction is the principal mode of dissemination for *T. maritima* for which asexual reproduction only occurs through rhizomatic expansion. The utilization of sexual reproduction is reported to be more variable between *P. maritima* populations for which asexual reproduction via stoloniferous expansion or dissemination of viable fragments plays an important role in dispersal (Gray & Scott 1977). Both species are reported to be morphologically variable across their range. For *P. maritima*, morphological variation was found both within (Gray *et al.* 1979) and between populations (Gray & Scott 1980) and is thought to have a genetic basis. Molecular differentiation between plants from pioneer and mature stages of vegetation was also observed using RAPD markers (Festoc 1999) and attributed to increasing selection pressure along the elevation gradient. For *T. maritima*, growth differences were observed between individuals from high and low marsh (Jefferies 1977) and are possibly attributable to differences in selection pressure.

b. Study area

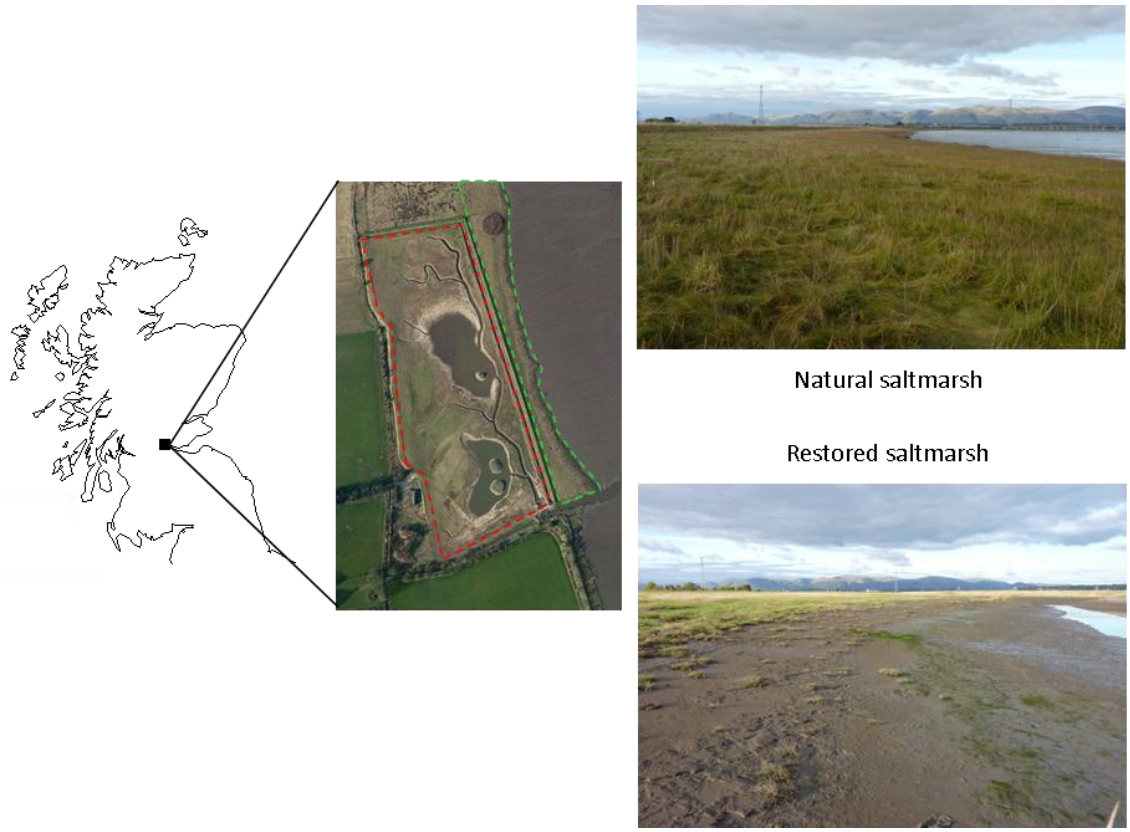

Fig. V-2 Location map of the restored and natural sites of Skinflats in the Forth estuary, Scotland. The restored and natural saltmarshes are respectively delineated in red and green on the aerial picture. Within the restored saltmarsh, the vegetation community is distributed around two pools excavated during restoration work. Exchange of seeds or vegetative propagules between them is thought to be limited due to the channel conformation visible on this aerial picture.

The site investigated is the saltmarsh of Skinflats located on the Forth estuary in Scotland. This site comprises both a restored and a natural saltmarsh (Fig. V-2). The natural saltmarsh is a good example of gradual community change along an elevation gradient. The seaward edge of the saltmarsh is occupied by the *Puccinellietum maritimae* communities (NVC type SM13) where a sub community dominated by *P. maritima* (NVC type SM13a) switches progressively to a sub community of *Plantago maritima-Armeria maritima* (NVC type SM13d) along the elevation gradient. The highest part of the saltmarsh is then occupied by the *Festuca rubra* community *Juncetum gerardi* (NVC type SM16) (Rodwell *et al.* 2000; Jump *et al.* 2009b). Lying behind the seawall, the restored part of the saltmarsh was originally a mesotrophic grassland.

Engineering work to restore this saltmarsh took place at a sufficiently low elevation to allow sea water entry via a pipe fitted through the sea wall. The first plant colonists were observed to develop on the bare mudflat after the first year of restoration in 2010.

c. Sampling regime

P. maritima individuals were among the first to colonise the recreated site. One hundred plants were collected each year in 2010, 2011 and 2012. Similarly, one hundred plants were sampled each year within the natural saltmarsh in 2011 and 2012. The position of each of these plants was recorded using a differential global positioning system (Leica Geosystems) allowing the acquisition of precise estimate of Northing, Easting and elevation (+/- 4cm).

Concerning the *T. maritima* population, the number of individuals observed within the restored site in 2010 was not large enough to be sampled. Therefore, one hundred plants were collected in 2011 and 2012 within the restored saltmarsh and one hundred plants were also collected within the natural saltmarsh in 2011 and 2012. Spatial coordinates of *T. maritima* samples were recorded using a handheld GPS (Garmin) in 2011 and a differential GPS in 2012. Data concerning the elevation of each *T. maritima* samples are therefore only available in 2012.

Using elevational data obtained for both species and data obtained from other sampling campaign (data not shown) we produced a three dimensional representation of both saltmarshes using the packages akima (Akima *et al.* 2009) and rgl (Adler & Murdoch 2012) in R (R Core Team 2013) (Fig.V-3).

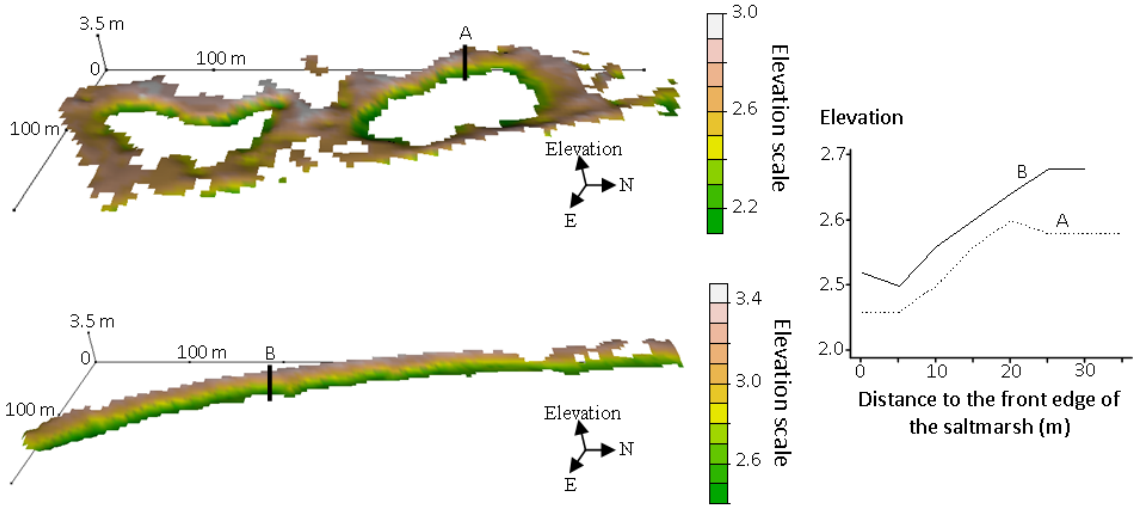

Fig. V-3 Three dimensional representation of the restored (top left) and natural saltmarsh (bottom left) and elevation profile (right). Elevation scale is given in meters. Profiles A and B were measured at the locations indicated on the three dimensional representations. As observed on the field, the community is on average higher in the natural saltmarsh.

d. DNA extraction and analysis

DNA from each sample was extracted using the DNeasy 96 plant kit (Qiagen) following the standard protocol provided by the manufacturer. Concentration of each DNA extract was controlled on NanoDrop 2000c (Thermo Fisher Scientific) and dilutions of 5-20 ng/µl were made using ultrapure PCR water (Bioline). Microsatellite markers were developed recently for both *P. maritima* (Rouger *et al.* 2014) and *T. maritima* (Rouger & Jump 2013). Each sample was therefore amplified with its respective set of markers following these published protocols. Allele scoring was made using the program STRand (Toonen & Hughes 2001). Allele binning was made using the package MsatAllele (Alberto 2009) in R. As observed in previous studies (Rouger & Jump 2014), loci Pm27 for *P. maritima* and Tm07 for *T. maritima* showed a high amplification failure rate and were therefore discarded from further analysis.

e. Data analysis

Both species were reported to be variable in ploidy across their range but thought to be octoploid in the UK (Scott & Gray 1976; Davy & Bishop 1991). Population genetics of polyploid species is still challenging due to their inherent characteristics such as the difficulty to infer allelic dosage in individuals or their greater probability to deviate from Hardy-Weinberg equilibrium (Dufresne *et al.* 2014). In order to consider this aspect, alleles within each locus were recorded as present or absent (0/1) which permits data analysis based upon methods which do not require the Hardy-Weinberg assumption. Such approach has already been used in the past for these species and gave reliable results (Rouger & Jump 2014).

i. Genetic diversity

Allele frequencies being difficult to infer using co-dominant markers on polyploid species (Dufresne *et al.* 2014), genetic diversity indices were calculated based on band frequencies instead. We calculated the number of alleles (Na), the number of rare alleles having a band frequency lower than 5% in the population (Na_5), the number of common alleles having a band frequency higher than 50% in the population (Na_{50}). Dice dissimilarity index was used to calculate inter-individual genetic distance using the package ade4 (Dray & Dufour 2007) in R. This matrix of inter-individual distance was then used to calculate Kosman index of genetic diversity within populations which is well suited to species likely to depart from Hardy-Weinberg such as polyploid or clonal species (Kosman & Leonard 2007). This index was computed using an R script designed previously (Rouger & Jump 2014). Matrix of inter-individual distances was also used as an input into the program GenoType (Meirmans & Van Tienderen 2004) in order to detect clones. Genotypic diversity was then calculated following the equation G-1/N-1 (Dorken & Eckert 2001) where G is the number of multilocus genotypes detected within the population and N, the number of individuals collected.

ii. Genetic autocorrelograms

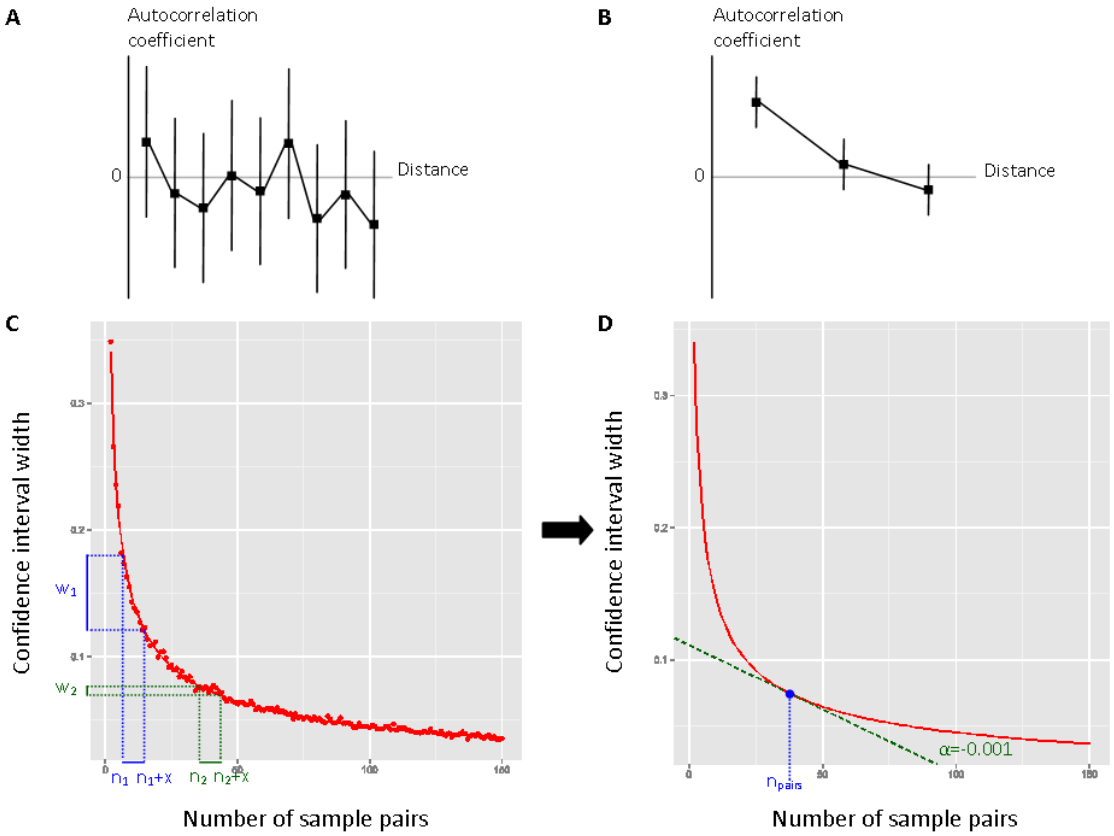

Fig. V-4 Description of the method used to designate the number of sample pairs to allocate in each size class of the spatial autocorrelogram. A: Illustration of the effect of having too few sample pairs in each size class, the width of the confidence interval is large and no reliable structure can be detected B: Illustration of the effect of having too many sample pairs, a genetic structure is detected but the number of size class is not enough to give a fine estimation of its extent. C: Relationship between the width of the confidence interval and number of sample pairs in each size class. If the number of sample pairs in each size class is small (n_1), adding x sample pairs per size class substantially decreases the width of the confidence interval (w_1). On the contrary, if the number of sample pairs in each size class is large already (n_2), adding x sample pairs per size class does not have a great impact on the confidence interval width (w_2) D: Inference of the number of sample pairs needed knowing α=-0.001, at that point adding one sample pair in each distance class (n_{pairs}+1) would only decrease the confidence interval width of 0.001.

Spatial autocorrelation of genetic variation has been commonly used to assess genetic structure across a landscape (Escudero *et al.* 2003). In this method, spatial distance between each pair of individuals is measured. Based on this, each pair of individuals is assigned to predefined distance classes. The selected spatial autocorrelation coefficient is calculated

within each distance class and significant structure is detected either through bootstrapping or permutation procedures. The statistical power of a spatial autocorrelogram therefore depends primarily upon the number of paired individuals used in each distance class to calculate the selected spatial autocorrelation coefficient. Indeed, too few sample pairs in each size class and the confidence interval, obtained by permutation or bootstrapping, will inflate giving unreliable results for each size class (Fig. V-4.A). On the other hand, too many sample pairs and the final number of size classes will be too small to precisely detect the extent of the genetic structure (Fig. V-4.B). In order to find the best trade-off between these two extremes, we developed a method that allowed us to designate the best number of paired samples to allocate in each size class.

First of all, the width of the confidence interval is calculated for each number of pairs per size class varying from a minimum (e.g. 2 sample pairs per size class) to a maximum (e.g. 200 sample pairs). The width of the confidence interval is calculated after 1000 random permutations across size classes or the average width across size classes of 1000 bootstraps in each of them. Graphically (Fig. V-4.C), the obtained relationship between the width of the confidence interval and the number of sample pairs in each size class is a log-log relationship:

$$\ln(width) = a + b \times \ln(pairs) \text{ (Equation 1),}$$

where *width* is the width of the confidence interval and *pairs* is the number of sample pairs in each size class. Parameters *a* and *b* are simply estimated using a linear regression. This log-log relationship implies that the gain obtained on the width of the confidence interval when adding one pair of samples to each size class is non-linear and higher when the number of pairs in each size class is small (Fig. V-4.C). We therefore defined arbitrarily a “diminution threshold”, noted α, below which we estimate that adding one sample pair to each distance class will not decrease substantially the width of the confidence interval. For example, if α=-0.001, adding only one sample pair to each size class would decrease the width of the

confidence interval by 0.001. Therefore, knowing how many pairs are needed to achieve this target is basically solving:

$$\frac{d(width)}{d(pairs)} = \alpha = -0.001 \text{ (Eq. 2)}$$

From equation 1:

$$\alpha = e^{a} \times b \times (pairs)^{b-1} \text{ (Eq. 3)}$$

And therefore,

$$pairs = \sqrt[-b+1]{\frac{e^{a} \times b}{\alpha}} \text{ (Eq. 4)}$$

This number of sample pairs needed is then rounded to the immediately higher integer (Fig. V-4.D).

This method was implemented using a custom R script (available on request). The correlation coefficient r computed in this script is closely related to Moran's I, and was developed by Smouse and Peakall (1999) and already implemented into GenAlEx (Peakall & Smouse 2012). In order to calculate this coefficient in each size class, the Dice dissimilarity matrix previously computed was used. Spatial autocorrelograms were built for each species and each year both in restored and natural saltmarshes. For each spatial autocorrelogram, the relationship between number of sample pairs and width of the confidence interval was estimated for both bootstrapping and permutation methods. The number of sample pairs to allocate in each size class was determined using α=-0.001 for both methods. Each method giving two comparable, but different, estimates of sample pairs to allocate in each size class, we kept the higher of the two values. Confidence intervals were inferred in each size class using 1000 permutations and 1000 bootstraps. The obtained autocorrelograms were validated by comparing those obtained using the software GenAlEx.

In order to investigate the effect of saltmarsh elevation on the genetic structure, elevation autocorrelograms were made based on differences in elevation between pairs of sample.

However, using the preceding method would create distance classes of only a few centimetres, which are not realistic given the precision obtained by the differential GPS (+/- 4 cm). We therefore allocated each pair of individuals into evenly spaced classes (increasing by 0.1m). Similarly, confidence intervals for each class were obtained using 1000 bootstraps and 1000 permutations.

iii. sPCA

In order to confirm and visualise directly on the saltmarsh the genetic structures detected by spatial autocorrelograms, we conducted a spatial Analysis of Principal Components (sPCA) (Jombart *et al.* 2008). Although a classic PCA summarizes much of the genetic variation present in a multivariate dataset into its first principal components, it does not take spatial information into account and may therefore miss a cryptic and spatially arranged genetic structure. sPCA, on the contrary, is a spatially explicit multivariate analysis permitting us to focus on the part of the genetic variation which is spatially structured by optimizing not only the genetic variance between samples but also their spatial autocorrelation.

Spatial information is entered in the sPCA in the form of a row standardized weighting matrix derived from a connection network between individuals. Two types of connection network were built and tested. The first connection network, called “Distance CN”, connects samples which are distant of no more than 30 m. The second connection network, called “Elevation CN”, connects samples between which difference in elevation is smaller than 0.1m. sPCA was then conducted using the package adegenet (Jombart 2008) in R for both species, both years, each site and using the two connection networks when this was possible (e.g. “Elevation CN” could not be used for *T. maritima* in 2011). Tests for *global* and *local* genetic structure according to the definition made by Thioulouse *et al.* (1995) were made using 999 permutations.

The visualisations of noticeable genetic structures were then made using an interpolation of the sample scores along the first sPCA component fitted on the previously constructed three dimensional representation of the saltmarsh.

4. Results

a. Genetic diversity

For *P. maritima*, 109 alleles were detected overall. The number of alleles detected each year in both restored and natural saltmarshes is similar and comprised between 80 and 86 (Table V-1). The only exception occurred in the restored saltmarsh in 2012 where this number of alleles increased to 98. It had the effect of increasing both the genetic (KW) and genotypic diversity (R) for that year in the restored saltmarsh (Table V-1). Genotypic diversity is relatively low overall meaning that multiple individuals are sharing the same multi-locus genotype. Interestingly, individuals sharing similar multilocus genotypes are not spatially clustered but spread inside the saltmarsh (Fig. V-5).

Table V-1 Sampling details and genetic diversity parameters of *T. maritima* and *P. maritima* for each year in both restored and natural saltmarshes. Z: availability of elevation data; AD: average distance between adjacent samples; N: number of collected samples, in brackets is the number of successfully amplified samples; Na: number of alleles; Na_5: number of rare alleles (alleles present in less than 5% of individuals); Na_{50}: number of common alleles (alleles present in more than 50%); KW: Kosman index of diversity within population; R: Genotypic diversity.

Puccinellia maritima									
Site	Year	Z	AD (m)	N	Na	Na_5	Na_{50}	KW	R
Restored saltmarsh	2010	Yes	2.69	101(99)	85	29	25	0.595	0.408
	2011	Yes	17.41	100	86	21	27	0.671	0.374
	2012	Yes	5.31	100	98	30	28	0.728	0.737
Natural saltmarsh	2011	Yes	6.41	100	85	27	25	0.683	0.455
	2012	Yes	3.06	100	80	12	28	0.710	0.647
Triglochin maritima									
Site	Year	Z	AD	N	Na	Na_5	Na_{50}	KW	R
Restored saltmarsh	2011	No	11.94	100(98)	129	38	33	0.737	1
	2012	Yes	4.86	100(99)	129	39	32	0.733	1
Natural saltmarsh	2011	No	14.13	100	129	47	30	0.725	1
	2012	Yes	3.23	100(99)	123	27	27	0.744	0.949

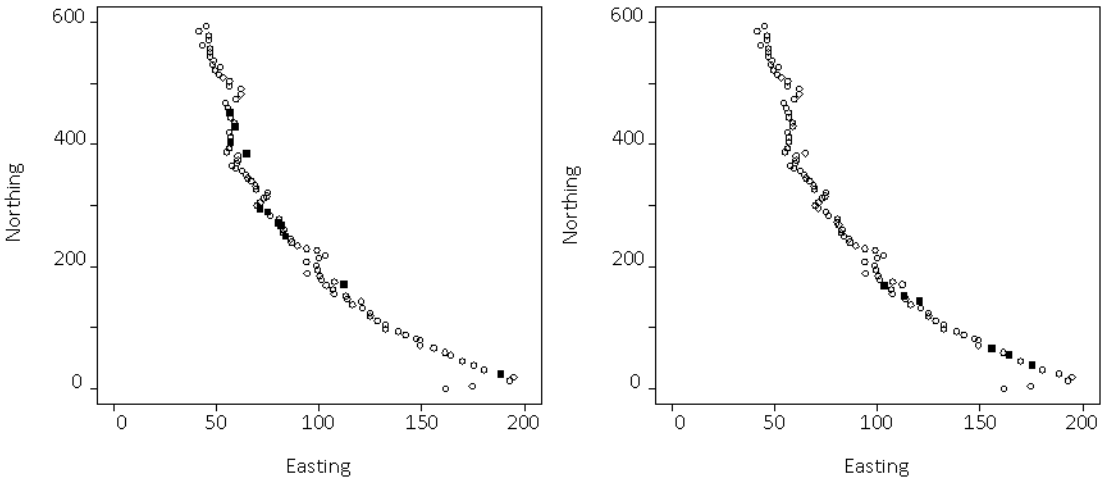

Fig. V-5 Example of spatial distribution of two multilocus genotypes of *P. maritima* within the natural saltmarsh in 2011. Identical multilocus genotypes are represented by filled squares. Left: genotype "8"; Right: genotype "49".

For *T. maritima*, 151 alleles were detected overall. The numbers of alleles detected each year in each saltmarsh were stable, only varying between 123 and 129 alleles. These comparable levels of allelic diversity also produced similar levels of genetic diversity (KW) between years and sites (Table V-1). Concerning the genotypic diversity, very few collected samples shared similar genotypes apart from 2012 in the natural saltmarsh where 3 individuals have been found carrying the exact same multilocus genotype. These three individuals were very close to each other, only separated by 0.80, 0.70 and 1.01m.

b. Genetic autocorrelograms

The spatial autocorrelograms of genetic variation in *T. maritima* did not identify any genetic structure in the restored saltmarsh either in 2011 or 2012 (Fig. V-6). Similarly, no genetic structure was observed in the natural saltmarsh in 2011. However, in 2012, a weak genetic structure was detected within the first distance class. The difference observed between the two years in the natural saltmarsh is due to the fact that the sampling regime was finer in 2012 than in 2011. Indeed, the average distance between adjacent samples in 2011 was of 14.13 m (Table V-1) and the first distance class of the spatial autocorrelogram spanned from 9.22 to 14.43 m. In comparison, in 2012, the average distance between adjacent samples was of 3.23

m (Table V-1) and the first distance class spanned from 0.39 and 1.37 m. However, the genetic structure detected in 2012 must be influenced by the fact that the first distance class of the spatial autocorrelogram comprises the three individuals sharing the same multilocus genotype detected earlier.

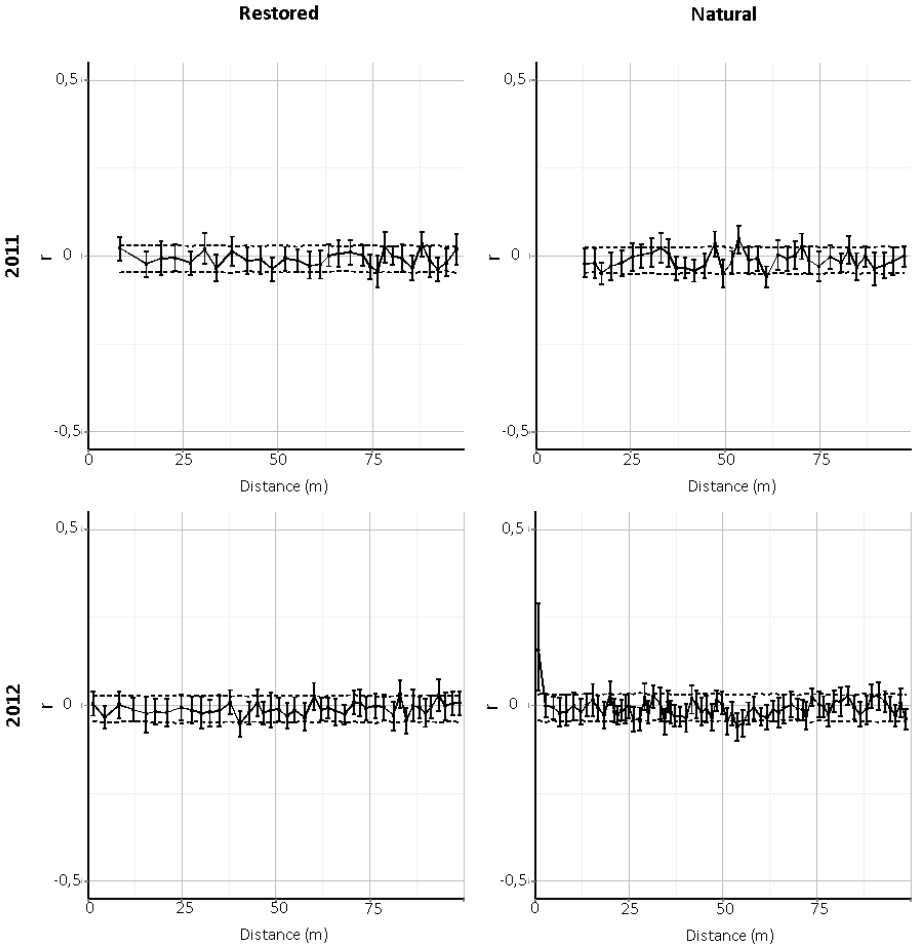

Fig. V-6 Spatial autocorrelograms of genetic diversity for *T. maritima*. r: Correlation coefficient of Smouse and Peakall (1999). Dotted lines: 95% confidence interval determined by permutation. Vertical bars: 95% confidence interval determined by bootstrapping.

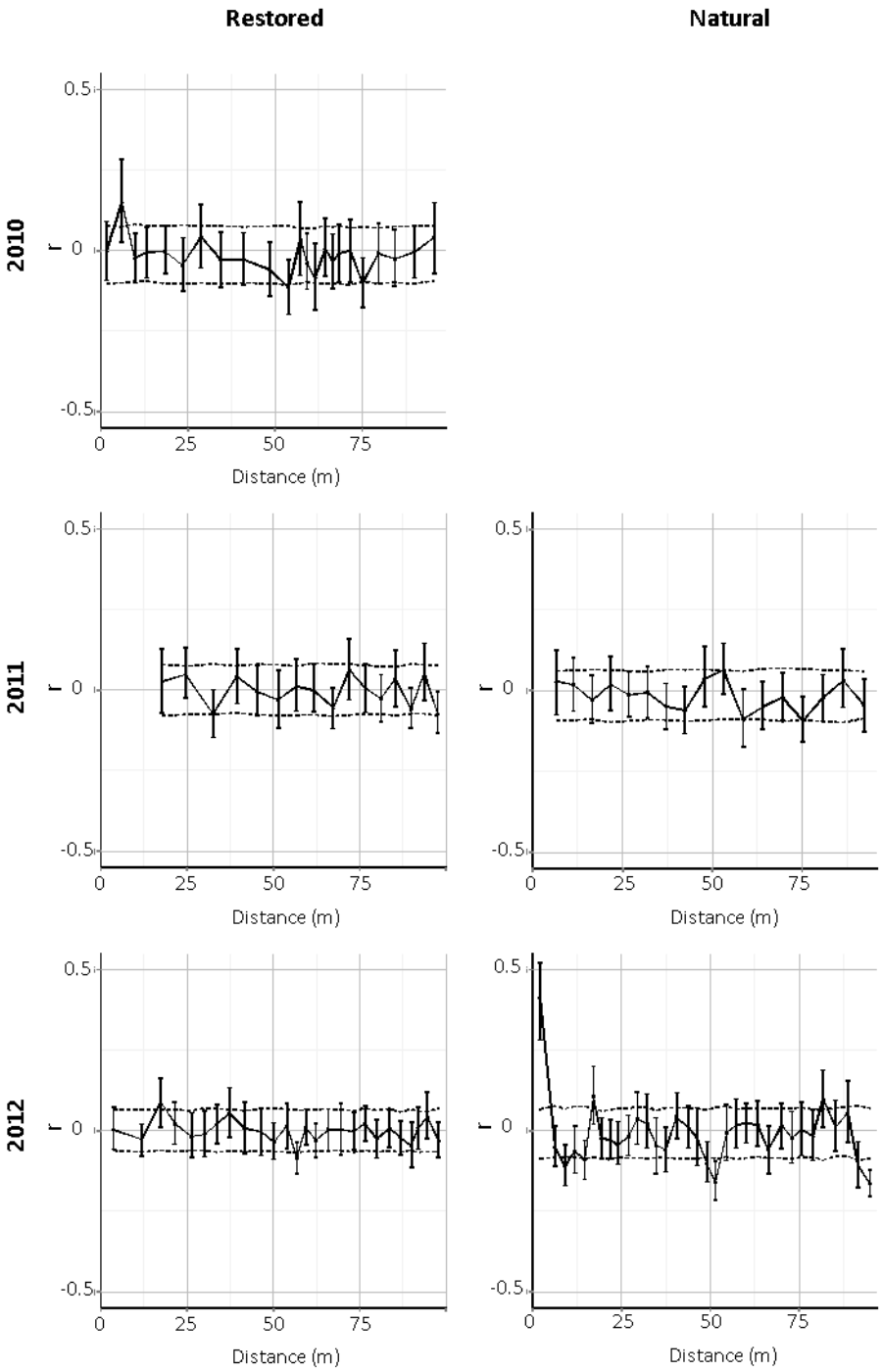

Fig. V-7 Spatial autocorrelograms of genetic diversity for *P. maritima*. r: Correlation coefficient of Smouse and Peakall (1999). Dotted lines: 95% confidence interval determined by permutation. Vertical bars: 95% confidence interval determined by bootstrapping.

Concerning *P. maritima*, and similarly to *T. maritima*, no genetic structure could be highlighted in the restored saltmarsh in 2010, 2011 or 2012 (Fig. V-7). Within the natural saltmarsh, no genetic structure was observed in 2011, whereas a weak spatial structure was detected in 2012. Again, the finer sampling regime adopted in 2012 (AD= 3.08m, first distance class span: 0.26 - 4.15 m) compared to 2011 (AD= 6.41 m, first distance class span: 2.97-8.50 m) permitted us to detect this spatial genetic structure in 2012.

Elevation autocorrelograms made for *T. maritima* in 2012 in both natural and restored saltmarshes did not identify any genetic structure arranged along the elevation gradient (Fig. V-8). For *P. maritima*, the elevation autocorrelograms could not detect any structure in the restored saltmarsh in 2010, 2011 or 2012 (Fig. V-9). In the natural saltmarsh, although no genetic structure is detected along the elevation gradient in 2011, a strong genetic structure was observed in 2012. It should, however, be observed that fewer distance classes were available in 2011 (Fig. V-9).

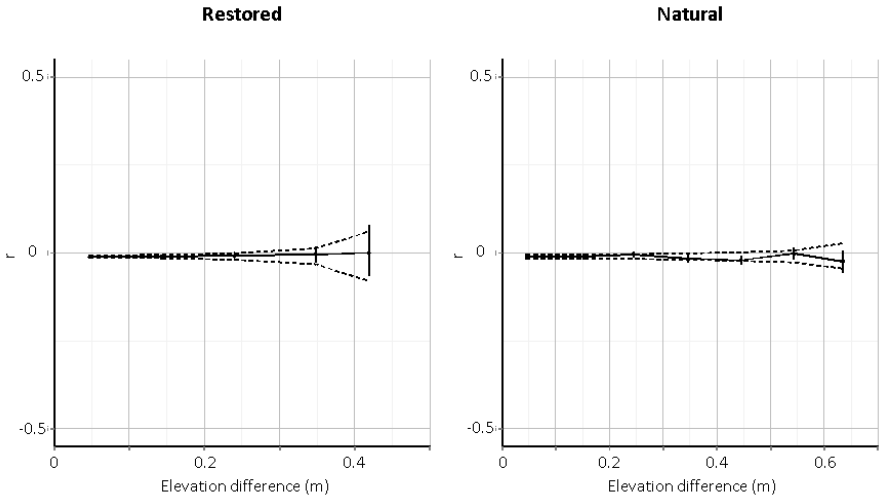

Fig. V-8 Elevation autocorrelograms of genetic diversity for *T. maritima*. r: Correlation coefficient of Smouse and Peakall (1999). Dotted lines: 95% confidence interval determined by permutation. Vertical bars: 95% confidence interval determined by bootstrapping.

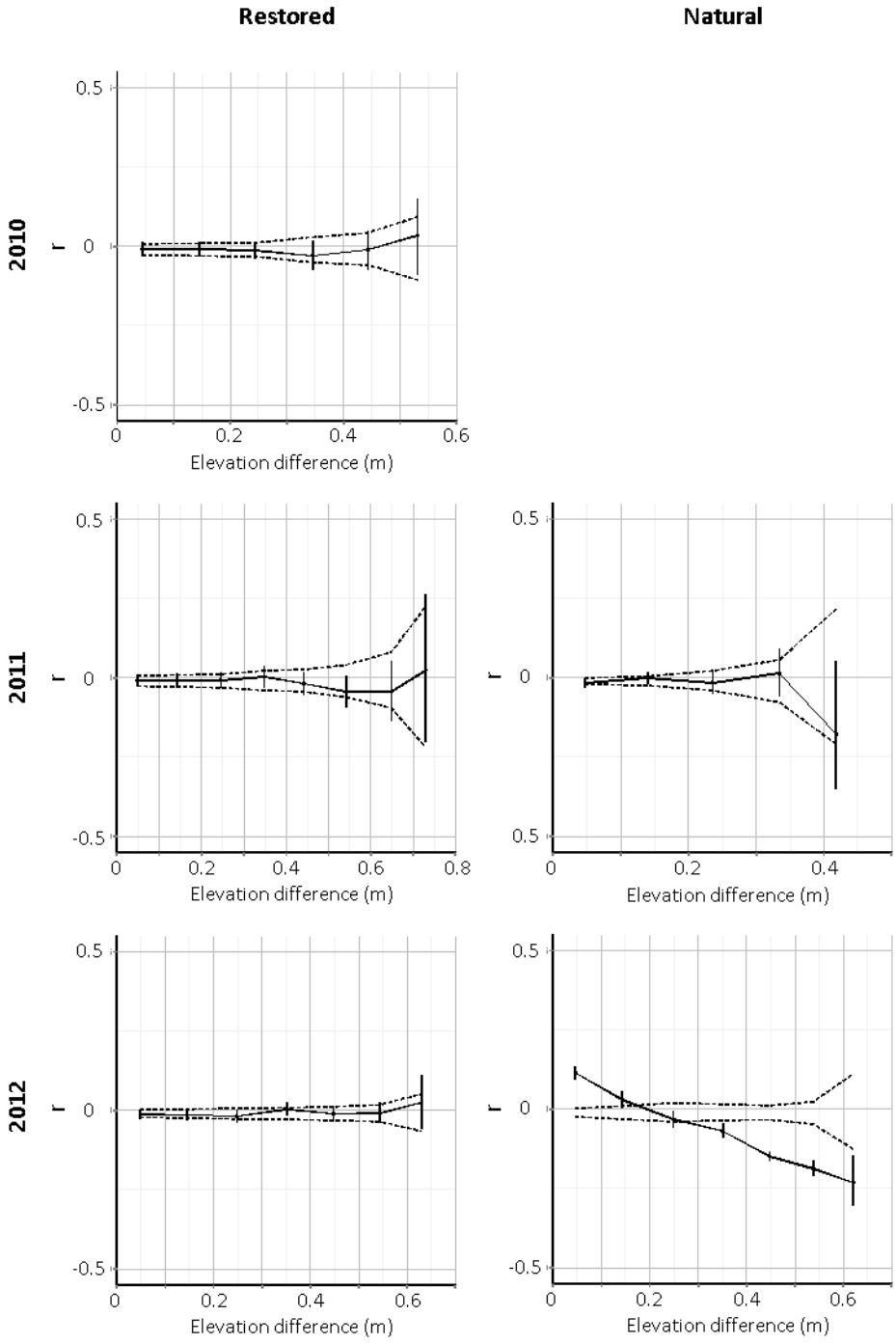

Fig. V-9 Elevation autocorrelograms of genetic diversity for *P. maritima.* r: Correlation coefficient of Smouse and Peakall (1999). Dotted lines: 95% confidence interval determined by permutation. Vertical bars: 95% confidence interval determined by bootstrapping.

c. Spatial Analysis of Principal Components (sPCA)

For *P. maritima,* the tests of significance for global and local genetic structures detected by sPCA produced a few discrepancies when compared to the results obtained by the genetic autocorrelograms (Table V-2).

Table V-2 Tests of global and local structures for the structure detected by sPCA with different connection network. Distance CN: connection network joining individuals no further apart than 30m; Elevation CN: connection network joining individuals with a difference in elevation of no more than 0.1 m.

			Distance CN		Elevation CN	
			Global structure	Local structure	Global structure	Local structure
P.maritima	Restored	2010	p=0.342 max(t)=0.0206	p=0.842 max(t)=0.0295	p=0.769 max(t)=0.0135	p=0.273 max(t)=0.0367
		2011	p<0.05 max(t)=0.03	p=0.467 max(t)=0.0192	p=0.667 max(t)=0.0136	p=0.065 max(t)=0.0282
		2012	p<0.01 max(t)=0.0241	p=0.073 max(t)=0.0247	p=0.547 max(t)=0.0144	p=0.447 max(t)=0.0217
	Natural	2011	p=0.491 max(t)=0.015	p=0.183 max(t)=0.0235	p<0.05 max(t)=0.0222	p=0.971 max(t)=0.022
		2012	p=0.096 max(t)=0.0215	p=0.536 max(t)=0.0237	p<0.001 max(t)=0.0789	p=0.569 max(t)=0.0183
T.maritima	Restored	2011	p=0.599 max(t)=0.0126	p<0.05 max(t)=0.0177	-	-
		2012	p=0.374 max(t)=0.0115	p=0.532 max(t)=0.0142	p=0.274 max(t)=0.0118	p=0.812 max(t)=0.0141
	Natural	2011	p=0.753 max(t)=0.0109	p=0.127 max(t)=0.0177	-	-
		2012	p<0.01 max(t)=0.0134	p=0.165 max(t)=0.0142	p<0.05 max(t)=0.013	p=0.195 max(t)=0.0143

Although no genetic structure could be observed for *P. maritima* in the restored saltmarsh with the spatial autocorrelograms (Fig. V-7), a significant global structure was detected both in 2011 and 2012 using the "Distance CN" (Table V-2). However, the visualisation, for 2012, of the original scores along the first component of the sPCA on the three dimensional representation of the saltmarsh (Fig. V-10) showed that this structure occurred at a larger scale than what was possible to be observed with the spatial autocorrelograms (greater than 100 m). Along this component, original sample scores seem to be differentially distributed around the two pools excavated during restoration (Fig. V-10). However, this pattern must be taken cautiously given the relative importance of the second component which still gives an "isolation by distance"

pattern of genetic structure but arranged in a different way (representation not shown). Interestingly, on the natural saltmarsh in 2012 where a weak spatial genetic structure was detected by the spatial autocorrelograms for *P. maritima* (Fig. V-7), no global structure was observed using the sPCA (Table V-2). The connection network used here, joining only individuals which are not further apart than 30m, is perhaps not adapted to detect the very fine structure observed on the spatial autocorrelogram.

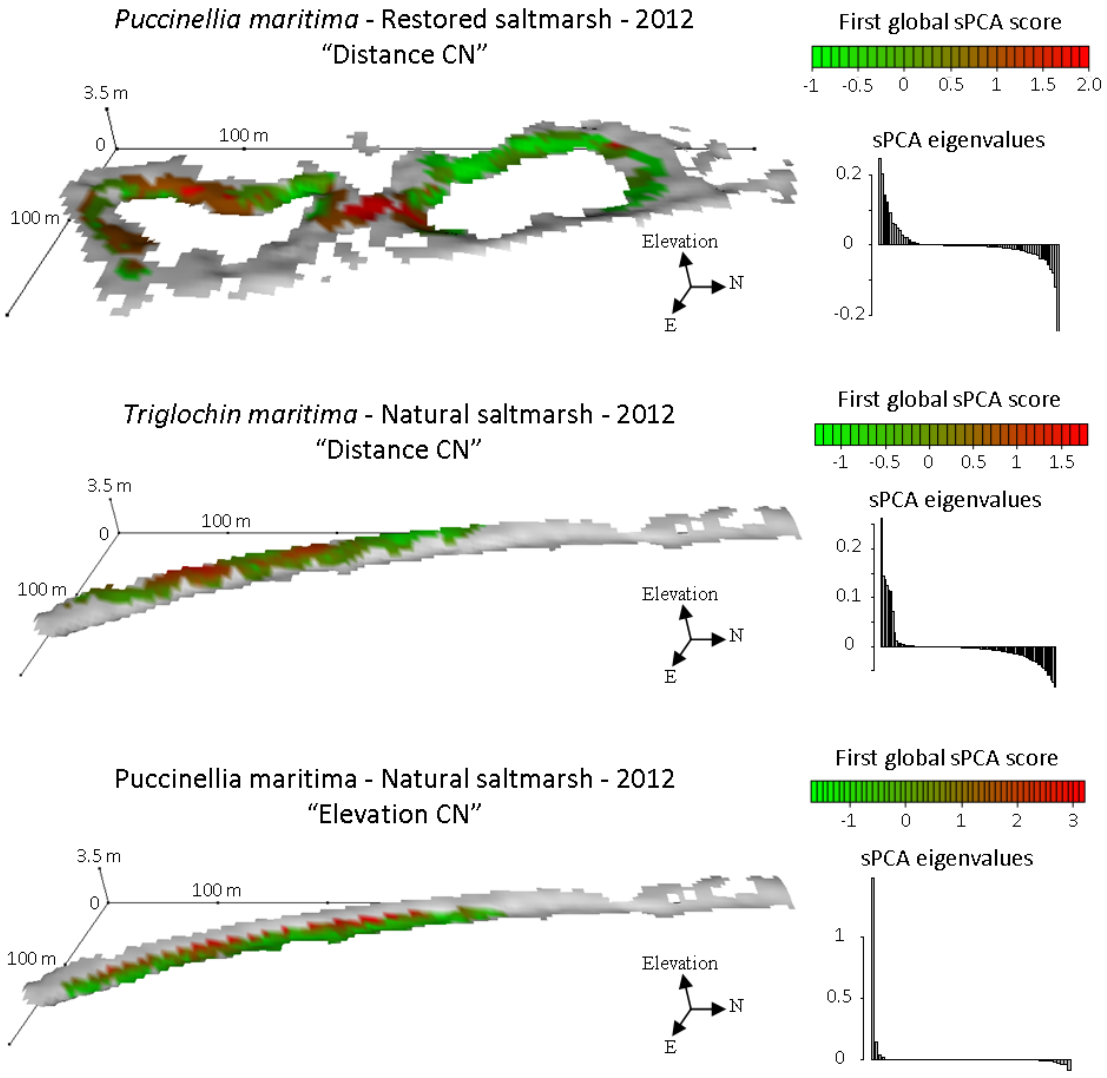

Fig. V-10 Significant spatial genetic structure detected by sPCA interpolated on the three dimensional representation of the saltmarsh. Each interpolated point is not further than 10m from a sampled individual. First global original scores of the sPCA were used. Plots of sPCA eigenvalues are shown on the right of each representation. Grey colour corresponds to areas which are further than 10 m from a sampled individual for that year.

Concerning the influence of the elevation on the distribution of genetic diversity of *P. maritima* within the natural saltmarsh, the sPCA using "Elevation CN" converged towards what was observed on the elevation autocorrelogram. The importance of the first component of the sPCA on the plot of eigenvalues indicates that an important part of the genetic variation is summarized by this component (Fig. V-10). The visualisation of this first component on the three dimensional representation of the saltmarsh illustrates the extent of this genetic structure with samples from the low and high part of the saltmarsh being well differentiated from each other although the spatial distance between the two zones is limited (around 20 m, Fig. V-10). Interestingly, the sPCA also indicated a genetic structure arranged along the elevation gradient in 2011 (Table V-2), although this genetic structure was not detected in the spatial autocorrelogram (Fig. V-9).

Concerning the sPCAs conducted on *T. maritima*, two global structures were detected. The first was detected using the "Distance CN" within the natural saltmarsh in 2012 (Table V-2). Although, a genetic structure was also detected on the spatial autocorrelogram for that year, the scale of the observed genetic structure differed between the two analyses. The spatial autocorrelogram detected a very fine spatial genetic structure (distance of around 1.5 m between genetically related individuals) where the sPCA detected an "isolation by distance" pattern spanning over several hundred meters (Fig. V-10). This structure was therefore expected to be observed in 2011 but was not detected. The second global structure on the natural saltmarsh in 2012 was detected using the "Elevation CN". However, the visualisation of this genetic structure on the natural saltmarsh is not as clear as the genetic structure obtained for *P. maritima* the same year using the same connection network (representation not shown). Moreover, this genetic structure was not confirmed by the corresponding elevation autocorrelogram. Interestingly, a local structure was also detected in the restored saltmarsh in 2011 using the "Distance CN". This suggests a significant negative spatial autocorrelation

between neighbouring samples which is difficult to explain assuming random arrival of colonists on site.

5. Discussion

Ecological restoration can be defined as "the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed" (SER 2004) and traditionally aims at restoring species diversity. Recent research developments have also pointed out the need of ensuring a good level of intra-specific genetic diversity because of the effects that genetic diversity may have on individual performance (i.e. less probability of inbreeding depression and/or maladaptation) (Falk *et al.* 2001; Hufford & Mazer 2003) but also on the whole community (option value of genetic diversity in buffering against environmental uncertainty, complementarity between genotypes increasing productivity, beneficial effects to higher trophic levels) (Hughes *et al.* 2008; Jump *et al.* 2009a). This is why a particular emphasis is now devoted to compare levels of genetic diversity between restored environments and reference ecosystems

a. Genetic diversity

Comparisons between the restored and natural populations of *P. maritima* and *T. maritima* in the Skinflats RTE scheme indicated that levels of genetic diversity were very similar between sites. This suggests that genetic diversity recovers very quickly after natural colonization of the recreated saltmarsh by these two species. This result is in accordance with a previous study that compared levels of genetic diversity within these two species between restored and natural saltmarshes at several different sites across the UK (see Chapter IV).

Numerous studies have identified that genetic diversity estimated with neutral markers such as microsatellites tells us little about the adaptive potential of populations (e.g. Holderegger *et al.* 2006) and that attention should be preferentially given to phenotypic traits experiencing

selection when assessing the adaptive potential of a population (Reed & Frankham 2001). However, a recent meta-analysis also highlighted a positive but weak correlation between neutral and quantitative genetic variation (Leinonen *et al.* 2008) suggesting that while analysis of neutral genetic diversity cannot be considered as a reliable indicator, it can still inform on the adaptive potential of populations (Jump *et al.* 2009a). In our case, we can therefore consider that the comparable levels of genetic diversity between restored and natural saltmarshes indicate that the adaptive potential to a change in environmental conditions is the same in both saltmarsh. Moreover, the reported beneficial effects of genetic diversity are generally based on neutral rather than quantitative genetic diversity (Reusch *et al.* 2005; Hughes *et al.* 2008; Hughes & Stachowicz 2009) which also suggests that beneficial effects of genetic diversity are as likely to occur in the restored as in the natural saltmarsh.

b. Genetic structure

As pointed out in the introduction (Fig. V-1), similar levels of genetic diversity are not enough to ensure that the fine-scale effects of genetic diversity via facilitation or complementarity can occur or that genotypes are distributed in accordance with their ecological requirements within the saltmarsh. Fine-scale spatial genetic structure is, therefore, also an important aspect to consider. The comparison of fine-scale spatial genetic structure between restored and natural saltmarsh highlighted differing results between *T. maritima* and *P. maritima*.

For *T. maritima*, asexual elongation producing large genotypes (Heslop-Harrison & Heslop-Harrison 1958) combined with specialization of genotypes to specific elevation zones (Jefferies 1977) suggested that a genetic structure would be found at least in the mature community of the natural saltmarsh. Surprisingly, the complementary analysis of fine-scale spatial genetic structure using spatial autocorrelograms and sPCA showed that genetic diversity is organized similarly in restored and natural saltmarshes with genotypes being distributed randomly within the saltmarsh. The only structures detected at a very-fine scale were either due to multiple

sampling of the same genotype (Fig. V-6, Natural 2012) or could not be confirmed by both sPCA and autocorrelograms (i.e elevation cline detected by the sPCA within the natural saltmarsh in 2012 but not observed on the elevation autocorrelogram). At a larger scale, although an isolation by distance pattern was observed on the sPCA within the natural saltmarsh in 2012 (Table V-2, Fig. V-10), it must be limited as such structure could not be detected in 2011 (Table V-2). This overall lack of fine-scale spatial genetic structure in *T. maritima* is consistent with previous results having highlighted a homogeneous gene-pool for this species at the scale of the Forth estuary (see Chapter IV). Seeds of *T. maritima* were reported to retain good viability and buoyancy even after a few months in sea water (Davy & Bishop 1991). Sexual reproduction of this species followed by dispersal by tidal currents is likely to hamper the formation of any fine-scale spatial structure for this species. Beneficial effects through complementarity and facilitation between genotypes, if they are to occur in this species, are as likely to happen in the restored as in the natural saltmarsh. We can consider that the genetic recovery of the restored *T. maritima* population is complete.

For *P. maritima* the type of fine-scale spatial genetic structure depended on the saltmarsh. Within the restored saltmarsh, the spatial genetic structure detected by the sPCA in 2011 and 2012 grouped genetically close individuals over a few hundred meters (Table V-2, Fig. V-10). Interestingly, this organisation was not observed in 2010 suggesting a scenario of progressive development of the genetic structure. The first colonists arriving on the saltmarsh in 2010 settled randomly on the bare mudflat leading to the observed lack of structure for that year. The limited dispersal due to the sheltered conditions inside the restored saltmarsh (R. Rouger, pers. obs.) in conjunction with the poor connectivity between the two pools excavated during the restoration (Fig. V-2) perhaps led to limited dispersal of seeds and vegetative propagules, producing the observed structure in 2011 and 2012. In the natural saltmarsh which is more exposed to tidal influence, such structure could not be observed. However, in this more mature community, a fine scale genetic structure organized along the elevation gradient was

clearly detected by both sPCA and spatial autocorrelogram in 2012 (Fig. V-9, Fig. V-10). Although, a similar structure was observed by the sPCA in 2011 (Table V-2), it was not found on the spatial autocorrelogram for that year (Fig. V-10). The elevation span of the samples collected in 2011 (2.38m-2.82m) compared to those collected in 2012 (2.44-3.1 m) explains the difference between these two years. Individuals located at higher elevation on the saltmarsh may have been missed during the sampling campaign in 2011.

Classical genecological works conducted on *P. maritima* have demonstrated that individuals from the mature community were morphologically different and less variable than individuals found in juvenile communities of the saltmarsh and that this morphological variation was heritable (Gray 1985; Festoc 1999). Numerous similar examples of local adaptation to environmental conditions despite gene-flow have now been described (Gonzalo-Turpin & Hazard 2009; Andrew *et al.* 2012; Muir *et al.* 2014) but there is still debate whether this adaptive divergence can be observed using neutral genetic markers. Recently, some authors hypothesized that local adaptation of populations to their environmental conditions may reduce overall gene-flow between them and therefore increase their neutral differentiation by genetic drift (Nosil *et al.* 2009; Orsini *et al.* 2013). This mechanism, known under the concept of "isolation by adaptation", could explain the pattern we observed here. Moreover, previous studies using RAPD markers have also described genetic differences between *P. maritima* populations depending on their position within the saltmarsh (Festoc 1999). Genetic differentiation along a maritime elevation gradient was also demonstrated in *Elymus athericus* using microsatellites (Bockelmann *et al.* 2003) or in *Spartina alterniflora* based on morphological analysis (Gallagher *et al.* 1988). Although, this would need further research to be confirmed, our study suggests the existence of such a genetic cline due to "isolation by adaptation" over a very small spatial scale, the high and low communities in the natural saltmarsh of Skinflats being only separated by 10-20 meters.

The differences in fine-scale spatial genetic structure between restored and natural populations of *P. maritima* indicated that genetic recovery remains incomplete for this species even three years after restoration. Moreover, if the adaptive origin of this genetic cline was to be confirmed, its absence within the restored saltmarsh suggests that individuals are not yet distributed according to their environmental requirement. However, the development of the vegetation cover will inevitably increase competition between genotypes potentially leading to a progressive sorting of genotypes according to their environmental requirements. We can therefore predict that this genetic cline will progressively develop in the restored saltmarsh although over what time scale is unclear.

6. Conclusion

Levels of genetic diversity within the restored populations of *P. maritima* and *T. maritima* were found to be similar to those observed in the reference ecosystems. However, the analysis of their fine-scale spatial genetic structure demonstrated that, although the distribution of genetic diversity was the same in restored and natural saltmarshes for *T. maritima*, strong differences could be noticed for *P. maritima*. We therefore consider that genetic recovery is complete for *T. maritima* but incomplete for *P. maritima*. The consequences within the restored saltmarsh of this lack of genetic organisation are not known and need further investigation. However, this result highlights the importance of considering not only levels of genetic diversity but also the organization of genetic structure to assess the success of genetic recovery during habitat restoration.

7. Acknowledgements

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Chapter VI: General discussion

Coastal defence around the UK faces new challenges due to the effects of global change. Sea level rise in conjunction with the action of more frequent extreme events like storm surges dramatically increases the risk of coastal flooding around the UK (Leake *et al.* 2007). Given these risks, the traditional "hold-the-line" policy aiming to conserve already existing coastal defence at all costs has been criticized (Wolters *et al.* 2005b). Developing innovative "soft-engineering" techniques of coastal protection such as saltmarsh recreation is considered as a viable alternative and multiple restoration schemes have been implemented throughout the UK (Esteves 2013).

The ecological effectiveness of this strategy has recently been analysed by meta-analyses of existing data concerning restoration schemes around the UK. These meta-analyses highlighted that restored saltmarshes are often not reaching the ecological state of a natural saltmarsh (Wolters *et al.* 2005b; Esteves 2013). Furthermore, it was also pointed out that scientific surveys of restoration schemes are too often missing and that, when they are undertaken, data are rarely made publicly available (Esteves 2013). Amongst the most overlooked aspects of saltmarsh restoration are genetic parameters, although they are now acknowledged to be an important aspect to consider when restoring communities (see Chapter I).

Several studies have nonetheless used saltmarsh plants as models for classical population genetics work. Besides the work already made on *P. maritima* and *T. maritima* described in Chapter I, an important literature exists concerning the analysis of intra-specific morphological variation in saltmarsh plants. Some of the most striking examples concern the floral polymorphism in *Aster tripolium* (Duvigneaud & Jacobs 1971; Huiskes *et al.* 2000) or the ecotypic variation in *Plantago maritima* (Gregor 1938) and *Suaeda maritima* (Boucaud 1962; Ihm *et al.* 2004).

Following these morphological analyses, investigations of molecular variation in some of these plants were also undertaken. These studies had differing aims, unfortunately unrelated to

saltmarsh restoration. Some studies aimed at assessing and comparing levels of genetic diversity between coastal populations and inland populations occurring either naturally or having been artificially facilitated by anthropogenic salt pollution (*Aster tripolium*: Krüger *et al.* 2002; Brock *et al.* 2007; *Spergularia media*: Prinz *et al.* 2010; *Suaeda maritima*: Prinz *et al.* 2009; *Armeria maritima*: Baumbach & Hellwig 2003, 2007; *Salicornia ramosissima*: Krüger *et al.* 2002). Other studies have used molecular markers in order to disentangle taxonomy, most notably of the genus *Salicornia* (Jefferies & Gottlieb 1982; Noble *et al.* 1992; Murakeözy *et al.* 2007). A few studies have also employed genetic markers in order to understand the phylogeography of saltmarsh plant species (i.e. *Spartina anglica*: Baumel *et al.* 2001; *Elymus athericus*: Bockelmann *et al.* 2003; *Salicornia sp.* Kadereit *et al.* 2007; *Triglochin maritima*: Lambracht *et al.* 2007).

The goal of this PhD was therefore to investigate the population genetics of two species considered as important ecosystem engineers of European saltmarshes with the particular aim of giving useful guidance for restoration practices. In this general discussion, I give the principal outcomes of this work and ideas concerning future directions which could be investigated in order to improve our understanding of restoration genetics in saltmarshes.

1. Molecular markers and the issue of polyploidy in saltmarshes

The molecular markers used so far in most saltmarsh plant population genetics studies were dominant markers (e.g. RAPD, AFLP). To my knowledge, the only use of co-dominant markers in north-western European saltmarshes was made for *Elymus athericus* (Bockelmann *et al.* 2003; Refoufi & Esnault 2006; Scheepens *et al.* 2007) and *Salicornia* sp. (Vanderpoorten *et al.* 2011). Some microsatellite markers were also developed for *Spergularia media* (Prinz *et al.* 2009a) but never used so far in a population genetics study.

Elymus athericus and *Spartina anglica* are classically considered as invasive species in north-western European saltmarshes (Baumel *et al.* 2001; Refoufi & Esnault 2006) and the taxonomy of the genus *Salicornia* is still challenging due to the occurrence of numerous cryptic species (Vanderpoorten *et al.* 2011). These species were, consequently, not the best suited for the study of restoration genetics in north-west European saltmarsh communities. Chapter II therefore focuses on designing the first sets of microsatellite markers available for *Puccinellia maritima* and *Triglochin maritima*, which are two recognized important components of saltmarsh succession and ecosystem functioning (see Chapter I).

Although potentially highly informative, co-dominant markers lose part of their efficiency when they are used in polyploid species due to the uncertainty concerning the allelic dosage of each individual and the lack of information concerning their patterns of inheritance (Dufresne *et al.* 2014). These difficulties were typified during the analysis of the genetic data obtained for *Puccinellia maritima* (2n=8x=56; x=7) and *Triglochin maritima* (2n=8x=48; x=6) in Chapter III, IV and V. In these chapters, microsatellite alleles were scored as present or absent and most subsequent statistics were distance based statistics (i.e. AMOVA, Kosman index) rather than allelic frequency based (i.e, F statistics, H_o, H_e).

Further practical or theoretical developments are, therefore, needed in order to make a better use of the information given by co-dominant markers in polyploid species. For example, modern cytogenetic techniques of *in situ* hybridization (Devi *et al.* 2005) can give very useful information. “Genomic *in situ* hybridization” (GISH) can permit us to detect whether the species investigated is an allopolyploid or an autopolyploid (Chester *et al.* 2012) and consequently give valuable precisions about the possible inheritance patterns of the markers used in this species. Microsatellite markers used can also be mapped using “fluorescence *in situ* hybridization” (FISH) (Santos *et al.* 2010) which would give additional information about the inheritance pattern of each locus but also the maximum number of copies which are to be

detected for each locus. However, none of these two cytogenetic techniques are enough for detecting the allelic dosage of each individual. The analysis of microsatellite data under the form of presence/absence data must therefore stay the rule until further technical developments occur.

Treating microsatellite data as dominant markers gives reliable results and has been used in numerous other studies (e.g. Sampson & Byrne 2012; Vallejo-Marin & Lye 2012). However, since the way the genetic datasets are assembled differs between marker types, some bias may appear (Fig. VI-1). Unfortunately, no theoretical study has tried to look at the effect of these biases on the inferred statistics. Polyploidy being a common feature in saltmarsh plants (i.e. *Suaeda maritima*: 4x, *Halimione portulacoides*: 4x, *Elymus athericus*: 6x, *Spartina anglica*: 4x, *Salicornia* sp.: 2-4x) (Baumel *et al.* 2001; Scheepens *et al.* 2007; Koce *et al.* 2008; Vanderpoorten *et al.* 2011), these kinds of study are crucially needed in order to better handle the genetic data obtained for these species.

Dominant marker (e.g. AFLP)

Locus	A	B	C	D	E	F	G	H	I
n_1	1	0	1	1	0	1	1	0	1
n_2	0	0	1	1	0	0	1	1	1
n_3	1	1	0	1	0	1	1	0	0
n_4	0	1	0	1	1	1	0	0	0

Co-dominant marker (e.g. microsatellite)

Locus	A						B	
Allele	252	254	256	260	264	266	130	136
n_1	1	0	1	1	0	1	0	1
n_2	0	0	1	1	0	0	1	1
n_3	1	1	0	1	0	1	0	0
n_4	0	1	0	1	1	1	0	0

Fig. VI-1 Representation of two datasets obtained with either dominant or co-dominant markers. These datasets can subsequently be used to calculate Dice dissimilarity index between each pair of individuals which is given by the formula $Dice_{n1n2}=1-(2a/(2a+b+c))$, where "a" is the number of position where a band is detected in both samples n_1 and n_2, "b" the number of position where a band is detected in n_1 but not in n_2 and "c" the number of position where a band is detected in n_2 but not in n_1. Used on dominant markers, each locus has an equal "weight" in this equation. Used on co-dominant markers, however, the "weight" of each locus within the equation will depend upon the number of alleles being amplified and this may cause a bias towards the allelic "richest" loci. In this example, locus A will have a bigger influence on the index of dissimilarity than locus B.

Although their analysis is challenging, microsatellite data collected for *P. maritima* and *T. maritima* gave useful information for restoration practitioners to consider. The outcome of this work can be here divided within two sections. Firstly, a section helping to understand how, at a macro-geographical scale, saltmarshes interact with each other (connectivity, gene-flow, exchange of propagules). Secondly, at a micro-geographical scale, a part enabling us to understand how a restored saltmarsh recovers from the genetic perspective and whether this recovery is complete.

2. Dispersal and connectivity between UK saltmarshes

Chapter III permitted us to delineate the main biogeographical units of both species at the scale of the UK. The goal here was to determine whether the gene-pool at the scale of the country was homogeneous, meaning that extensive gene-flow between saltmarshes occurs, or structured, meaning some gene-flow limitation between saltmarshes. Interestingly, the structure detected for both *P. maritima* and *T. maritima* seemed comparable with biogeographical regions gathering relatively geographically close populations. Nevertheless, differentiation between populations within each region was still significant. Overall, this means for both species that, although gene-flow is occurring between distant saltmarshes, the strength of this flow is not strong enough to entirely homogenize the gene-pool across the country.

The analysis of the origin of colonists of the Skinflats site made in Chapter IV enabled us to discover how the amount of gene-flow detected between populations in Chapter III is translated practically in terms of colonist arrival on a restored site. This work highlighted that saltmarsh re-colonization was a very local process for both species. In this context, conserving a good network of saltmarsh across the country is imperative in order to ensure a good capacity of recovery for restored saltmarshes wherever these schemes are implemented along

the UK coastline. It also confirms the importance of restoring saltmarshes not too distant from potential colonization sources, as advised in previous studies (Wolters *et al.* 2005c).

Chapter III also permitted us to determine which features are shaping the genetic structure observed within *P. maritima* and *T. maritima*. Although the genetic structure observed for both of them was globally similar, the factors underlying this structure were surprisingly different. Genetic structure for *P. maritima* was mainly influenced by coastal processes and most especially coastal geomorphology, while an overland route of gene flow was also implicated in shaping the genetic structure of *T. maritima*. These differences are likely to be due to the differences in ecology between the two species, *P. maritima* dispersing asexually via tidal processes while long-distance dispersal of seeds and pollen is also occurring in *T. maritima* (Chapter I).

These observed differences between *P. maritima* and *T. maritima* therefore raise the question of how generalisable the results obtained here are for other saltmarsh species. However, while differences in ecology inevitably exist between all saltmarsh species, for most of them, sea tides are the main agent of dispersal (Huiskes *et al.* 1995) which allow us to hypothesize that although the nation-wide genetic structure may differ between saltmarsh species, a local source of colonization will still be important for restoring the population of any of them. In order to clarify this aspect, further work involving the population genetics study of *Aster tripolium* was planned to be undertaken during this thesis. This species differs from *P. maritima* and *T. maritima* by having a large seed production subsequently dispersed by both the action of wind and sea tides (Clapham *et al.* 1942). Unfortunately, the development of molecular markers within this species was not successful.

3. Genetic diversity and structure

The local origin of colonists implies that a single source population is often involved for restoring a saltmarsh community. In classical population genetics, colonization events from single source population can cause the well-known founder effects (Provine 2004) leading to a reduced level of genetic diversity within the newly colonized site. Moreover, levels of genetic diversity within restored communities have been shown to be an important aspect to consider (Chapter I). Indeed, a depleted genetic diversity when compared to a natural environment may negatively impact population performance and potentially ecosystem function (Hughes & Stachowicz 2004, 2009; Reusch *et al.* 2005; Reusch & Hughes 2006; Johnson *et al.* 2006; Hughes *et al.* 2008; Cook-Patton *et al.* 2011). The goal of Chapter IV was therefore to compare levels of genetic diversity between restored and natural populations of *P. maritima* and *T. maritima*.

This multi-site comparison highlights that levels of genetic diversity within restored saltmarshes are equivalent to neighbouring natural saltmarshes. This was true even for recently recreated saltmarshes, suggesting that the recovery of genetic diversity within restored saltmarsh is almost immediate (Chapter IV). High level of ploidy in *P. maritima* and *T. maritima* generally imply a larger effective population size (Parisod *et al.* 2010) and may therefore avoid the occurrence of a founder effect and associated genetic drift. However, this absence of founder effect was also observed on recently salt contaminated inland sites of Central Europe where neither populations of tetraploid species (*Suaeda maritima*) or diploid species such as *Aster tripolium*, *Salicornia ramosissima*, *Spergularia media* show a lower genetic diversity when compared to natural sites (Krüger *et al.* 2002; Brock *et al.* 2007; Prinz *et al.* 2009b, 2010). These previous studies indicate that the results highlighted within this thesis for *P. maritima* and *T. maritima* can be generalised to the other saltmarsh species, whatever their level of ploidy.

Because of the local origin of colonists, similar levels of genetic diversity between restored and neighbouring natural saltmarshes do not automatically imply that the restored saltmarsh is in good condition from a genetic point of view since this will indeed depend on the starting level of genetic diversity present within the already existing saltmarshes. Conserving genetic diversity within sites must therefore be an important aspect to consider (Chapter IV). Further studies also looking at the practical effect of genetic diversity within saltmarsh communities could also give useful information. Such work was undertaken in the US on saltmarsh dominated by *Spartina alterniflora* and highlighted that this species is subject to inbreeding depression when genetic diversity is low (Daehler 1999) and that genetic diversity improves plant and community performance (Wang *et al.* 2012; Hughes 2014). Very little is known yet concerning these aspects within north-west European native saltmarsh species.

An experiment trying to look at the effect of *T. maritima* genotypic diversity on decomposition rate was implemented during this PhD but did not yield analysable data due to the technical difficulties of installing litter bags within the heavily and frequently disturbed saltmarsh environment. Such studies within dominant saltmarsh species would allow us to gain a better understanding of the level of genetic diversity needed in saltmarsh species in order to avoid potential detrimental effects of low genetic diversity.

Assessing genetic recovery within an ecosystem is classically restricted to the comparison of genetic diversity between restored and natural environments (Gonzalez-Perez *et al.* 2009; Lloyd *et al.* 2012; Vandepitte *et al.* 2012; Fant *et al.* 2013; Oudot-Canaff *et al.* 2013). Chapter V went one step further by also looking at how this diversity is distributed in both environments. Interestingly, no differences in genetic structure between restored and natural environment could be detected for *T. maritima* indicating that the distribution of *T. maritima* individuals within both saltmarshes is random and independent of their genetic identity. On the contrary, for *P. maritima* a strong genetic structure was detected within the natural saltmarsh arranged

along the elevation gradient. Such structure could not be observed in the restored saltmarsh. This kind of genetic cline in *P. maritima* following the saltmarsh gradient in elevation was also detected in a previous study (Festoc 1999) and was suggested to be related to the morphological variation observed within the species along the same gradient (Gray 1987; Festoc 1999). Differential selective pressure was suggested to be at the origin of this cline (Gray 1987; Festoc 1999). In our case, however, the uses of microsatellites that are neutral genetic markers do not allow us to speculate on the origin of the observed cline. Reciprocal transplant experiments between the lower and the upper part of the saltmarsh could permit to determine whether clones are adapted to the conditions they are developing in (Seliskar 1985). Although heavier to implement, the development and population genetics analysis of quantitative trait loci (QTL) linked to morphological characteristics of the plant could also shed further light on the origin of this genetic cline (Ma *et al.* 2010).

Despite the uncertainty concerning the origin of this cline, its absence within the restored saltmarsh suggests that genetic recovery is not yet complete concerning *P. maritima*. Although, the potential consequences of an incomplete genetic diversity are well documented, it's hard to predict exactly what will be the consequences of a deficit in genetic structure at the community level (i.e. maladaptation, fragility of the community). Further studies are needed to study this aspect as it could be valuable for saltmarsh restoration practitioners but also generalisable to other ecosystems where environmental gradients occur (i.e. mountain communities, mangroves).

4. General conclusion

Coastal protection is becoming an increasingly important challenge because of both increasing human pressure and global change causing sea level rise and increased storminess. In response, soft engineering techniques such as saltmarsh restoration are slowly starting to be implemented around the world. The genetic recovery of vegetation communities in restored

environments has recently become the focus of a number of studies, which point to the importance of genetic parameters on community performance. In saltmarsh recreation, most of the surveys looking at restoration of genetic diversity were undertaken in the US. This PhD represented the first attempt to investigate this aspect in North-west European saltmarshes. The main points highlighted here were (1) that genetic diversity within saltmarsh plants is structured at a macro-geographical scale within geographically coherent regions; (2) that although gene-flow exists between these regions, colonists directly arriving on a restored site are of local origin, (3) that genetic diversity within recreated saltmarsh is quickly equivalent to the levels found in natural saltmarshes, (4) that differences in genetic structure are still observable between saltmarshes. Taken together, these results provide useful information for restoration practitioners to consider and lead the way to further work in the field of saltmarsh restoration genetics within north-west European saltmarshes.

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Appendices

a. Appendix III-1: R script used to calculate Kosman indices

This R code is constrained by the document margins. Each line of code starts with ">".

```
>library(ade4)
>library(clue)
>
>########################
>#Kosman within populations#
>########################
>
>Pdatabin<-read.table("Pglobalpopgenbin.txt",h=T,sep=",")#Uploading of the allele Presence/Absence matrix. NAs are amplification failure but were replaced by 0s beforehand
>Pdist<-dist.binary(Pdatabin,method = 5, diag = TRUE, upper = TRUE)#Dice distance calculation
>Pdistbis<-as.matrix(Pdist)
>
>Ppopnames<-c("B","G","L","N","R","SE","SK","W","H","LC","LG","M","P","T","WE")
>
>PNcumul<-
matrix(c(0,30,60,90,119,147,177,207,237,266,296,326,355,385,415,445),nrow=1,dimnames=list("N",c("Origin",Ppopnames)))
>
>Tdatabin<-read.table("Tglobalpopgenbin.txt",h=T,sep=",")#Uploading of the allele Presence/Absence matrix. NAs are amplification failure but were replaced by 0s beforehand
>Tdist<-dist.binary(Tdatabin,method = 5, diag = TRUE, upper = TRUE)#Dice distance calculation
>Tdistbis<-as.matrix(Tdist)
>
>Tpopnames<-c("B","G","H","L","LC","LG","N","P","R","SE","SK","T","W","WE")
>
>TNcumul<-
matrix(c(0,29,59,89,111,140,159,188,217,247,277,307,337,367,396),nrow=1,dimnames=list("N",c("Origin",Tpopnames)))
>
>#Puccinellia
>P_KW<-matrix(0,ncol=length(Ppopnames),dimnames=list("KW",Ppopnames))#matrix where the results are going to be stored for Puccinellia
>
>for(i in 1:(length(Ppopnames))){
>	mat<-Pdistbis[(PNcumul[1,i]+1):PNcumul[1,i+1],(PNcumul[1,i]+1):PNcumul[1,i+1]]#Subset of the distance matrix corresponding to one population (looped over all populations)
>	P_KW[1,i]<-
(sum(mat[cbind(seq_along(solve_LSAP(mat,maximum=TRUE)),solve_LSAP(mat,maximum=TRUE))]))/(PNcumul[1,i+1]-PNcumul[1,i])#Calculation of KW for this subset (looped over all populations)
>}
>
>#Triglochin
>T_KW<-matrix(0,ncol=length(Tpopnames),dimnames=list("KW",Tpopnames))#matrix where the results are going to be stored for Triglochin
>
>for(i in 1:(length(Tpopnames))){
>	mat<-Tdistbis[(TNcumul[1,i]+1):TNcumul[1,i+1],(TNcumul[1,i]+1):TNcumul[1,i+1]]#Subset of the distance matrix corresponding to one population (looped over all populations)
>	T_KW[1,i]<-
(sum(mat[cbind(seq_along(solve_LSAP(mat,maximum=TRUE)),solve_LSAP(mat,maximum=TRUE))]))/(TNcumul[1,i+1]- TNcumul[1,i])#Calculation of KW for this subset (looped over all populations)
>}
>
>#################################
>#Kosman distance between populations#
>#################################
>
```

```
>Pdatabin<-read.table("Pglobalpopgenbin.txt",h=T,sep=",")#Uploading of the allele Presence/Absence matrix. NAs are amplification failure but were replaced by 0s beforehand
>Tdatabin<-read.table("Tglobalpopgenbin.txt",h=T,sep=",")#Uploading of the allele Presence/Absence matrix. NAs are amplification failure but were replaced by 0s beforehand
>Pdist<-dist.binary(Pdatabin,method = 5, diag = FALSE, upper = FALSE)#Dice distance calculation
>Tdist<-dist.binary(Tdatabin,method = 5, diag = FALSE, upper = FALSE)#Dice distance calculation
>Pdistmatind<-as.matrix(Pdist)
>Tdistmatind<-as.matrix(Tdist)
>PPop<-c("B","G","L","N","R","SE","Sk","W","H","LC","LG","M","P","T","WE")
>TPop<-c("B","G","H","L","LC","LG","N","P","R","SE","Sk","T","W","WE")
>
>PNcumul<-
matrix(c(0,30,60,90,119,147,177,207,237,266,296,326,355,385,415,445),nrow=1,dimnames=list("N",c("Origin",PPop)))
>TNcumul<-
matrix(c(0,29,59,89,111,140,159,188,217,247,277,307,337,367,396),nrow=1,dimnames=list("N",c("Origin",TPop)))
>
>f<-function(x){
>	b<-Pdistmatind[x[1],x[2]]#function permitting to build a bootstrapped distance matrix in the next loop
>	return(b)
>}
>
>nbbootstrap<-1000
>
>Pdistmatpop<-matrix(0,ncol=length(PPop),nrow=length(PPop))#Matrix where the results are going to be stored for each bootstrap step
>
>Psuc<-array(dim=c(length(PPop),length(PPop),nbbootstrap))#Array storing the results of every bootstrap step
>
>for(k in 1:nbbootstrap){
>
>for(i in 1:(length(PPop))){
>	for(j in 1:i){
>		if(j==i){
>		}else{
>			row<-sample((PNcumul[1,i]+1):PNcumul[1,i+1],30,replace=T)#Bootstrap sampling of 30 individuals in the first population
>			col<-sample((PNcumul[1,j]+1):PNcumul[1,j+1],30,replace=T)#Bootstrap sampling of 30 individuals in the second population
>			a<-expand.grid(row,col)
>			mat<-matrix(apply(a,1,f),ncol=30,byrow=F)#Building of the bootstrapped distance matrix, with individuals bootsrapped from the population 1 in rows and individuals from the population 2 in columns
>			Pdistmatpop[i,j]<-
(sum(mat[cbind(seq_along(solve_LSAP(mat)),solve_LSAP(mat))]))/30 #Calculation of KB for this pair of population
>		}
>	}
>}
>
>Psuc[,,k]<-Pdistmatpop #Looped over the number of bootstrap
>print(k)
>}
>
>P_avdistmatpop<-apply(Psuc,c(1,2),mean) #Final result: Average value of the nbbootstrap values calculated
>SE<-function(x){
>	sd(x)/sqrt(length(x))#Standard error
>	}
>P_SEdistmatpop<-apply(Psuc,c(1,2),SE)
>
>Tdistmatpop<-matrix(0,ncol=length(TPop),nrow=length(TPop))#Matrix where the results are going to be stored for each bootstrap step
>
>Tsuc<-array(dim=c(length(TPop),length(TPop),nbbootstrap))#Array storing the results of every bootstrap step
```

```
>
>f<-function(x){
>	b<-Tdistmatind[x[1],x[2]]#function permitting to build a bootstrapped distance matrix in the next loop
>	return(b)
>}
>
>for(k in 1:nbbootstrap){
>
>for(i in 1:(length(TPop))){
>	for(j in 1:i){
>		if(j==i){
>		}else{
>			row<-sample((TNcumul[1,i]+1):TNcumul[1,i+1],30,replace=T)#Bootstrap sampling of
30 individuals in the first population
>			col<-sample((TNcumul[1,j]+1):TNcumul[1,j+1],30,replace=T)#Bootstrap sampling of 30
individuals in the second population
>			a<-expand.grid(row,col)
>			mat<-matrix(apply(a,1,f),ncol=30,byrow=F)#Building of the bootstrapped distance
matrix, with individuals bootsrapped from the population 1 in rows and individuals from the population 2 in
columns
>			Tdistmatpop[i,j]<-
(sum(mat[cbind(seq_along(solve_LSAP(mat)),solve_LSAP(mat))]))/30 #Calculation of KB for this pair of population
>		}
>	}
>}
>
>Tsuc[,,k]<-Tdistmatpop #Looped over the number of bootstrap
>print(k)
>}
>
>T_avdistmatpop<-apply(Tsuc,c(1,2),mean) #Final result: Average value of the nbbootstrap values calculated
>SE<-function(x){
>	sd(x)/sqrt(length(x)) #Standard error
>	}
>T_SEdistmatpop<-apply(Tsuc,c(1,2),SE)
```

b. Appendix IV-1: R script used to compute genetic diversity parameters

This R code is constrained by the document margins. Each line of code starts with ">". This code was designed for *Puccinellia maritima* but also works for *Triglochin maritima*.

```
>library(clue)
>library(ade4)
>
>#Import of the datasets
>Pbin_30SK<-read.table("Pbin_30SK.txt",sep=",",header=T) #Presence/absence data (each column is an allele, each row is a sample)
>
>P_geno<-read.table("P_30SKgeno0.txt",header=F) #Genotype information (first column is sample IDs, second column is the genotype number, two individuals sharing the same genotypes have the same genotype number)
>
>#Replacement of missing value (-9) by 0s
>Pbin_30SK_missing0<-Pbin_30SK
>Pbin_30SK_missing0[Pbin_30SK_missing0==-9]<-0
>
>Ppop<-
c("SKR","BR","BN","GR","GN","FI","FII","FIII","FIV","LR","LN","NR","NN","RR","RN","SER","SEN","SKN","WR","WN","HR","HN","LC","LG","M","PR","PN","TR","TN","WER","WEN")# Population names
>Peff<-c(30,30,30,30,30,30,29,30,30,29,30,30,29,30,28,30,30,30,30,30,30,29,30,30,29,30,30,30,30,29,30)# Population sizes
>
>PGendiv<-list(      KW=matrix(NA,nrow=1,ncol=length(Ppop),dimnames=list("KW",Ppop)),
>                           Na=matrix(NA,nrow=1,ncol=length(Ppop),dimnames=list("Na",Ppop)),
>                           Na15=matrix(NA,nrow=1,ncol=length(Ppop),dimnames=list("Na15",Ppop)),
>                           Na50=matrix(NA,nrow=1,ncol=length(Ppop),dimnames=list("Na50",Ppop)),
>                           PA=matrix(NA,nrow=1,ncol=length(Ppop),dimnames=list("PA",Ppop)),
>                           R=matrix(NA,nrow=1,ncol=length(Ppop),dimnames=list("R",Ppop))
>                     )#List where the results will be stored
>
>#Genetic diversity indices
>
>Peffcum<-c(0,cumsum(Peff))
>
>for(i in 2:length(Peffcum)){
>
>          #Calculating the number of alleles
>          mat<-Pbin_30SK_missing0[(Peffcum[i-1]+1):Peffcum[i],]
>          b<-apply(mat,2,sum)
>          PGendiv$Na[1,i-1]<-sum(table(factor(b,levels=c(1:length(mat[,1])))))
>
>          #Calculating the number of rare alleles (band frequency lower than 15%, 4 individuals or less in a population of 30 individuals)
>          PGendiv$Na15[1,i-1]<-sum(table(factor(b,levels=c(1:floor(length(mat[,1])*0.15)))))
>
>          #Calculating the number of alleles with a band frequency higher than 50%
>          PGendiv$Na50[1,i-1]<-sum(table(factor(b,levels=c(ceiling(length(mat[,1])*0.50):length(mat[,1])))))
>
>          #Calculating KW
>          Pdist<-dist.binary(mat,method = 5, diag = TRUE, upper = TRUE)
>          Pdist<-as.matrix(Pdist)
>          PGendiv$KW[1,i-1]<-
(sum(Pdist[cbind(seq_along(solve_LSAP(Pdist,maximum=TRUE)),solve_LSAP(Pdist,maximum=TRUE))]))/(length(Pdist[,1]))
>
```

```
>	#Calculating genotypic diversity (this code takes into account missing data, two MLGs are only compared based on the loci which amplified)
>	vec<-P_geno[(Peffcum[i-1]+1):Peffcum[i],2]
>	PGendiv$R[1,i-1]<-(length(unique(vec))-1)/(length(vec)-1)#The genotypic diversity is calculated according to the equation G-1/N-1
>
>	#Calculating number of private alleles
>	matcomp<-Pbin_30SK_missing0[-((Peffcum[i-1]+1):Peffcum[i]),]
>	veccomp<-apply(matcomp,2,sum)
>	a<-table(veccomp)
>	b<-a[names(a)==0]
>	if(length(b)==0){
>	PGendiv$PA[1,i-1]<-0
>	}else{
>	PGendiv$PA[1,i-1]<-b
>	}
>
>}
>
>#Inference of shared genotypes between populations
>
>Pvec<-P_geno[,2]
>
>PSharedgeno<-matrix(NA,nrow=length(Ppop),ncol=length(Ppop),dimnames=list(Ppop,Ppop))
>for (i in 1:(length(Peffcum)-2)){
>	Pvec1<-Pvec[(Peffcum[i]+1):Peffcum[i+1]]
>	for (j in (i+1):(length(Peffcum)-1)){
>		Pvec2<-Pvec[(Peffcum[j]+1):Peffcum[j+1]]
>		PSharedgeno[j,i]<-length(intersect(unique(Pvec1),unique(Pvec2)))
>	}
>}
```