CLONAL STRUCTURE OF ELYTRIGIA ATHERICA

A STUDY TO REVEAL THE GROWTH STRATEGY
OF A NATIVE INVASIVE SPECIES

MASTER'S THESIS

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Abstract

E. atherica is a grass species (Triticeae: Poaceae) which can reproduce sexually as well as clonally. It occurs on the higher elevations of European salt marshes. However, during the last decades it has started to invade the lower salt marshes. Seedling recruitment is important during initial colonisation, but over time sexual reproduction may decrease due to intraspecific competition. Competition and stochastic mortality could lead to a net decrease in clonal diversity, thereby increasing the portion of vegetative reproduction. Therefore, it is hypothesised that the ratio of sexual and vegetative reproduction changes towards more vegetative reproduction during colonisation of *E. atherica*. In this study, clonal diversities in differently sized patches of *E. atherica* within two populations on lower salt-marsh sites of different age (25 and 35 yrs) were investigated on the island of Schiermonnikoog, The Netherlands. Microsatellites were used to distinguish between clones.

The clonal diversity in small (< 5 m^2) and intermediate (5-20 m^2) sized patches was variable in both populations, ranging from 0.077 to 0.444 and from 0.200 to 0.750 on the younger and older site respectively. Furthermore, a trend of decreasing clonal diversity with increasing patch size was found for both sites. Finally, a higher clonal diversity on the older compared to the younger site was found.

These results are consistent with the hypothesis. Three developmental phases are distinguished during colonisation of *E. atherica*: (1) seedling recruitment increases the clonal diversity; (2) vegetative reproduction creates patches, decreasing clonal diversity. However, patches trap water dispersed seeds, increasing chances for seedling recruitment in the population. This sexual reproduction outweighs vegetative reproduction, thus increasing clonal diversity; (3) as the ramet density increases in patches over time, seedling recruitment decreases due to shading; vegetative reproduction increases. Together with intraspecific competition and/or stochastic mortality, this will decrease the clonal diversity over time.

Introduction

Sexual and vegetative reproduction

Clonality is a common phenomenon in the plant world. Vegetative (clonal) reproduction (see Table 1 for a glossary) means that a plant reproduces vegetatively or, in other words, without meiosis and fertilisation but with normal mitosis. The plant that is reproducing clonally is therefore genetically identical to its clonal offspring. Many plant species reproduce clonally by means of stolons or rhizomes, which are respectively above- and below-ground lateral roots, but other means of vegetative reproduction are also known to occur, for example via seed-shaped vegetative parts. An individual, also called a genet or clone, is thus composed of one or more potentially or actually independent plant subunits, called ramets, which all share an identical genome.

Vegetative reproduction may have (short-term) advantages over sexual reproduction. For example, physiological aid by the mother plant may increase the survival probability of clonal offspring (D'Hertefeldt & Jónsdóttir 1999) and the propagation of clonal plants is favoured by the absence of costs of sexual reproduction (e.g. flowering) (Ronsheim & Bever 2000; Lovett Doust & Laporte 1991; Rydgren & Økland 2003). However, sexual reproduction offers the (long-term) advantage of mixing genomes to get rid of deleterious mutations which otherwise accumulate over time, thereby decreasing the fitness of an individual. Furthermore, the mixing of genomes creates novel allelic combinations, which is favourable for the adaptation of a population to changed environmental factors (Barton & Charlesworth 1998). In addition, seed dispersal is a major advantage for survival while vegetative reproduction is limited to the immediate surroundings of the mother plant. Seed dispersal increases the chance to establish at new sites and it offers the possibility to disperse in time (Howe & Miriti 2004).

Many clonal plant species are able to reproduce sexually as well as vegetatively (Cook 1983) and, consequently, most of these species were found to exhibit a considerable amount of clonal diversity (Ellstrand and Roose 1987; Widen *et al.* 1994).

The balance between sexual and vegetative reproduction is influencing the clonal diversity of populations of clonal plants (Eriksson 1997). In general, sexual reproduction raises the clonal diversity per area since more genets are added to the population. Vegetative reproduction, on the other hand, does not add clonal diversity directly. However, vegetative reproduction might indirectly affect the clonal diversity as more ramets are produced that potentially contribute to sexual reproduction. Processes such as seed production, seedling establishment, stochastic mortality, spatial heterogeneity and inter- and intraspecific competition are important factors influencing the sexual and vegetative reproduction and consequently the clonal diversity of populations (Harada *et al.* 1997). These factors could change in influence over time, thereby changing the amount of sexual and/or vegetative reproduction present in the population. For example, when the chance of seedling establishment decreases due to changed environmental circumstances, the amount of sexual reproduction decreases as well. This has influence on the clonal diversity of the population. It may be interesting to study the change in clonal diversity through time under influence of changing environmental conditions. Experiments could be done to study the effects of controlled factors on the clonal diversity in populations. However, the study of natural populations is favoured over experiments since a large time-scale is needed before effects are visible. The colonisation

phase in clonal plant species may be a suitable time-frame to study clonal diversity under natural circumstances. The increasing abundance of the species itself is likely to be one of the major factors that change the environmental conditions and hence the clonal diversity.

Table 1. Glossary

Term	Description
Ramet	Functional plant unit potentially able to live on its own (Scrosati 2002).
Genet	Sexually produced individual consisting of one or more potentially or actually independent ramets all sharing the same genome.
Clone	Genet used in a spatially explicit way.
Sexual reproduction	Recruitment of a new genet.
Vegetative reproduction	Growth of genetically identical ramets from a parent plant.
Clonal diversity	Number of genets found (G) divided by the number of ramets sampled (N); (G/N) (Ellstrand & Roose 1987).
Clonal probability	Chance that two ramets share the same genotype and can be assigned to the same clone.
Seedling establishment	Germination and rooting of seeds.
Seedling recruitment	Establishment of seedlings plus their survival.

Eriksson (1989) proposed two seedling recruitment patterns: ISR, initial seedling recruitment, in which there is predominantly vegetative reproduction after an initial population foundation by seedling recruitment, and RSR, repeated seedling recruitment, in which there is ongoing seedling recruitment within the population over time.

Several studies have shown that in ISR as well as in RSR seedling recruitment is high during colonisation. But subsequently, RSR predicts a low but constant seedling recruitment retaining a high clonal diversity because genet loss due to competition and stochastic mortality is balanced by genet gain. Contrasting, ISR predicts that seedling recruitment ceases or takes place only during so-called 'windows of opportunity' which are related to disturbances in the environment (Kanno & Seiwa 2004; Watkinson & Powell 1993; Li & Baskin 1999; Auge & Brandl 1997; Travis & Hester 2005). ISR will therefore lead to a decrease in clonal diversity since genet loss will outweigh genet gain. Ultimately, only a few genets will be left in a population. Large-scale environmental disturbances will reset the conditions of a site to those prior to colonisation, after which a new loop will start. Contrary, seedling recruitment in species exhibiting RSR may be dependent on small-scale disturbances.

A species adopting the ISR strategy will show an initial increase in ramets as well as genets since many seedlings will recruit in the area. Over time, all space will be inhabited by ramets. As the amount of genets decreases due to factors such as competition or stochastic mortality, vegetative reproduction will fill up the gaps (Fig. 1a). A species adopting the RSR strategy will also show an initial increase in ramets as well as genets,

but over time the amount of genets will remain constant since seedling recruitment outweighs genet loss (Fig. 1b).

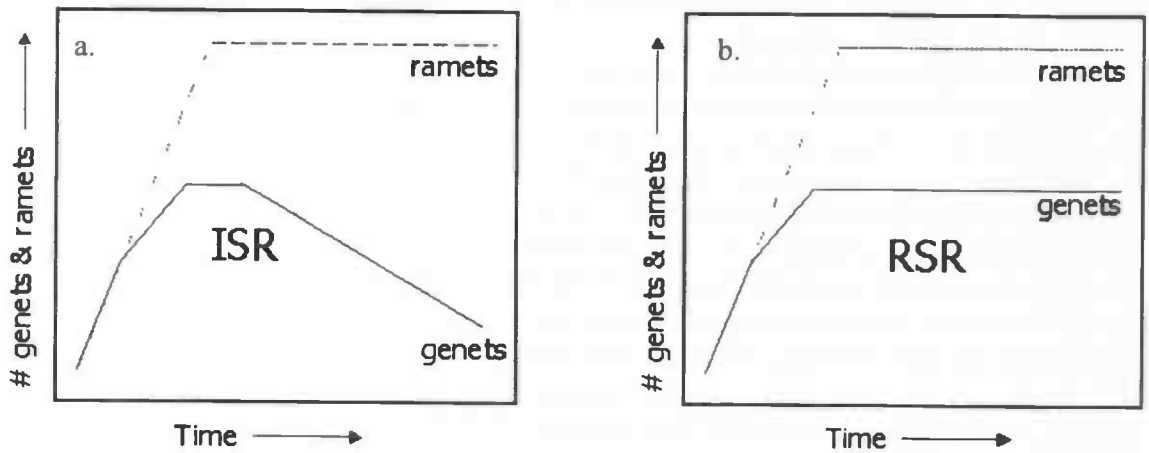

Figure 1. Number of genets and ramets through time in a fixed area in colonising populations of an ISR (a) and a RSR (b) species.

Although the clonal diversity in a population may depend on the clonal life history of the plant (Eriksson 1989), it may also depend on the spatial scale at which the population is investigated (Fischer & Van Kleunen 2002). The population of a clonal plant as a whole may have a high clonal diversity, but when looked at a small scale the clonal diversity found may be dependent on the type of vegetative spread. The phalanx type of clonal spread results in extensive mats of individual clones bordering each other, while the guerrilla strategy results in an intermingledness of clones (Eriksson 1997) (Fig. 2). If the phalanx type is present, sampling at a small scale could easily result in an underestimation of the clonal diversity. To illustrate this with Figure 2, sampling four neighbouring ramets in a square in phalanx will probably capture one genet, while at least two but probably all three genets will be captured in guerrilla. The chosen scale of sampling is therefore very important for the outcome of the study.

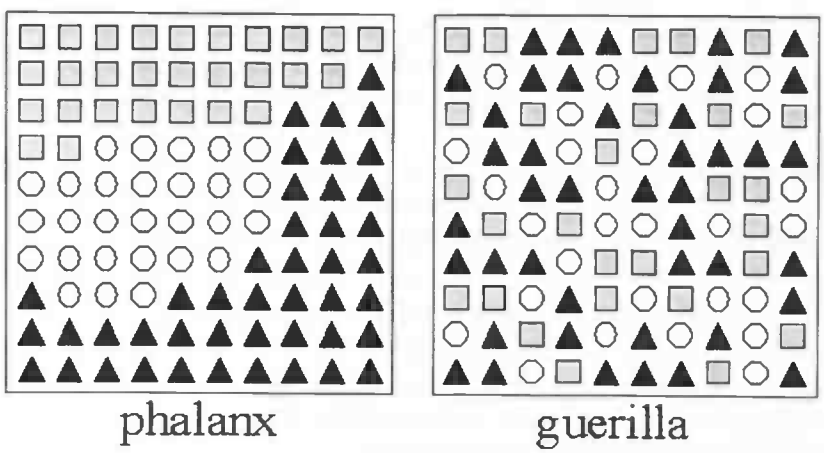

Figure 2. Two types of spatial distribution of ramets. The phalanx strategy results in a clumped distribution of ramets while the guerrilla strategy results in an intermingledness of genets. The number of ramets is the same for each genet in the two distributions: (▲) 44; (■) 28; (○) 28. Modified from Charpentier (2002).

Elytrigia atherica

Invasions by exotic (i.e. alien or non-native) plant species have been studied thoroughly during the last decades (Greimler *et al.* 2002; Stohlgren *et al.* 1998; Gorchov & Trisel 2003). Native plant species have only recently been recognized to be able to start dominating their home habitat and/or invade and dominate new habitats. These plants often happen to be clonal grass species, such as *Brachypodium pinnatum* (Bobbink 1991), *Calamagrostis spp.* (Soukupova 1992) and *Elytrigia atherica* (*Elymus athericus*) (Bakker 1989; Van Wijnen *et al.* 1997).

Elytrigia atherica, the subject species of this study, is a clonal grass species, occurring along the coast from northern Portugal to southern Denmark and on the coasts of the British Isles. *E. atherica* occurs mainly on higher elevations (annual inundation frequency ~53) (Bockelmann 2002) of salt marshes. However, since the last decades *E. atherica* has started to invade the lower salt marshes (annual inundation frequency ~166) (Bakker *et al.* 1993; Bockelmann 2002; Bakker *et al.* 2003). During young successional stages of both higher and lower salt marshes, *E. atherica* can be found sparsely dispersed in groups of ramets called patches. The patches grow over time ultimately forming extensive swards with patches joined together. On older salt-marsh sites, *E. atherica* will dominate most other species, representing the climax state of the salt marsh and reducing salt marsh biodiversity (Van Wijnen *et al.* 1997).

There may be several causes for the increase in cover by *E. atherica* on the higher salt marshes and colonisation by *E. atherica* on the lower salt marshes. It is known that nitrogen addition favours growth of *E. atherica* (Leendertse *et al.* 1997). An increase in nitrogen availability in the soil due to increased nitrogen contents in atmospheric deposition and water runoff from the mainland could explain the sudden spread of *E. atherica* (Van Wijnen & Bakker 1999). Stewart and Lee (1974) found that some plant species in saline environments increase their salt tolerance by producing nitrogen-based, osmotically active compounds. If this holds for *E. atherica*, an increased concentration of nitrogen in the Wadden Sea could then favour the spread of *E. atherica* to lower marshes where it faces a more saline environment.

E. atherica can reproduce clonally as well as sexually. Colonisation of new sites by *E. atherica* happens mainly through seedling recruitment (Bockelmann 2002), but over time the favourable conditions for seedling recruitment are likely to disappear. Vegetation density is positively correlated with seed trapping (Buitenwerf and Veeneklaas pers. comm.), but at the same time vegetation density and, more important, canopy height can also be expected to be negatively correlated with seedling recruitment due to competition for light (Tilman 1988). Young patches of *E. atherica* are expected to have a low ramet density and thus should have more favourable conditions for seedling recruitment than older patches which are expected to have a high ramet density. This results in rapidly increasing clonal diversities in small patches, but as the patches grow over time, the ramet densities will increase and the favourable conditions for seedling recruitment are expected to disappear. In addition, intraspecific competition between genets and stochastic mortality are expected to decrease the amount of genets in a patch over time, slowly decreasing the clonal diversity.

Based on this reasoning, the hypothesis is that the ratio of sexual and vegetative reproduction shifts from an initially high sexual reproduction towards a more vegetative reproduction over time within patches and whole populations of *E. atherica*.

H_0: No change in the ratio of sexual and vegetative reproduction over time
H_a: Change in ratio of sexual and vegetative reproduction towards more vegetative reproduction over time

ISR is the strategy that fits this hypothesis. Investigating the ratio of sexual and vegetative reproduction in patches and populations of *E. atherica* on the lower salt marsh during colonisation may give insight in its growth strategy. The time factor is approached in two different ways: (1) two salt-marsh sites of different age (25 and 35 years old) are compared and (2) patches of different size (0.3-85.5 m^2) within these sites are compared. Patch size is assumed to be positively correlated with patch age.
The patch is regarded as the scale at which the hypothesis is best applicable. That is because patches within a population generally have different sizes and therefore, as assumed, have different ages. Patches may therefore be in different stages of succession. However, comparing patches of different ages within one site may give a wrong picture since environmental factors that affect the clonal diversity may change over time as overall salt marsh succession progresses. Therefore, patches with different sizes in two sites are compared.

Excavation can immediately reveal clonal structures. However, using this technique is often impossible regarding physical effort and time needed. In addition, the clonal structure is often literally too complex, especially in clonal species where genets grow mixed. Furthermore, root connections within a genet may disappear over time (Barnes 1966; Piqueras & Klimes 1998), which will result in an overestimation of identified genets. A single genet broken in two will be counted twice although both fragments belong to the same genet.
Genetic markers can be used to rapidly identify genets among a large number of samples. Molecular techniques that reveal genetic markers such as allozyme, RAPD, AFLP and microsatellite analysis are often used in clonal plant research (Suyama *et al.* 2000; Pluess & Stöcklin 2004; Stehlik & Holderegger 2000; Schläpfer & fischer 1998; Albert *et al.* 2005; Kreher *et al.* 2000). These techniques (except allozyme analysis) compare small (presumed selectively neutral) parts of the genome that may differ in size between individuals. These parts are revealed as bands on electrophoresis gels. The faster the band migrates through the electrophoresis gel, the smaller its length. Bands with different size are interpreted as different alleles. Contrary to sexual reproduction, vegetative reproduction does not involve recombination of chromosomes. Therefore, all ramets derived from a common seedling share an identical genome (with the exception of accumulated somatic mutations) and consequently will reveal identical bands. In this study, microsatellites were used, but the hexaploidy of *E. atherica* made allelic interpretation impossible in this study. Therefore, standard calculations based on allelic distributions could not be carried out. The absence or presence of bands was interpreted as a character enabling genet identification (Jarne & Lagoda 1996). Characters are composed of 0 and 1 indicating absence and presence of known bands respectively.

Because allelic distributions are not known, two individuals which share all bands do not necessarily need to be clones since the relative proportions of alleles can be different. However, the higher the amount of bands checked, the lower the chance for this error.
The characters produced with the microsatellite analysis are not only useful to distinguish between genets, but can also help answering questions regarding relatedness between individuals. Are individuals within patches more related than individuals from different patches? In other words, does a patch provide its own related offspring or is seed dispersal mixed at the sampled scale? Related offspring within a patch is likely to contain alleles already present in genets in that patch, since only pollen dispersal from genets outside the patch can add novel alleles. Genets that were dispersed as seeds from outside the focal patch are consequently likely to contain alleles not present in genets already present in that patch. Thus if genets in a patch are related to each other, they will show absences and presences of bands that resemble each other more closely than if genets are not related to each other.

Materials and methods

Study species

Elytrigia atherica (Link) Carreras Mart. (Triticeae: Poaceae; species names *sensu* Van der Meijden (1996)) is a hexaploid (n=7), clonal grass species closely related to wheat. *E. atherica* occurs along the coasts of the British Isles and from northern Portugal to southern Denmark. Salt marshes are its main habitat. The seaward spread of *E. atherica* is limited by high concentrations of NaCl in the soil. *E. atherica* therefore generally occurs on higher elevations of salt marshes which consequently have a lower inundation frequency leading to a lower concentration of NaCl compared to lower marshes. *E. atherica* produces rhizomes for vegetative reproduction but also seeds in spikelets. Above ground vegetation decays in the autumn, but the rhizomes stay alive and form new shoots in spring. Concerning fertilisation, *E. atherica* has been found a highly outcrossing species (Bockelmann 2002).

Study site

The study site is situated on the Oosterkwelder on the Dutch back-barrier island of Schiermonnikoog (53°30'N, 6°10'E) (Fig. 3). Schiermonnikoog spreads eastwards due to tidal sand deposition on the eastside of the island as an effect of the dominant sea currents. Over at least 140 years, this has resulted in a chronosequence of salt-marsh sites in the shelter of the island (Bakker 1989; Olff *et al.* 1997). Pioneer salt marsh species facilitate silt deposition, raising nutrient levels and elevation, and thereby promoting succession towards a productive salt marsh dominated by tall-growing species such as *Atriplex portulacoides* on the low salt marsh and *E. atherica* on the high salt marsh (Leendertse *et al.* 1997; Olff *et al.* 1997).

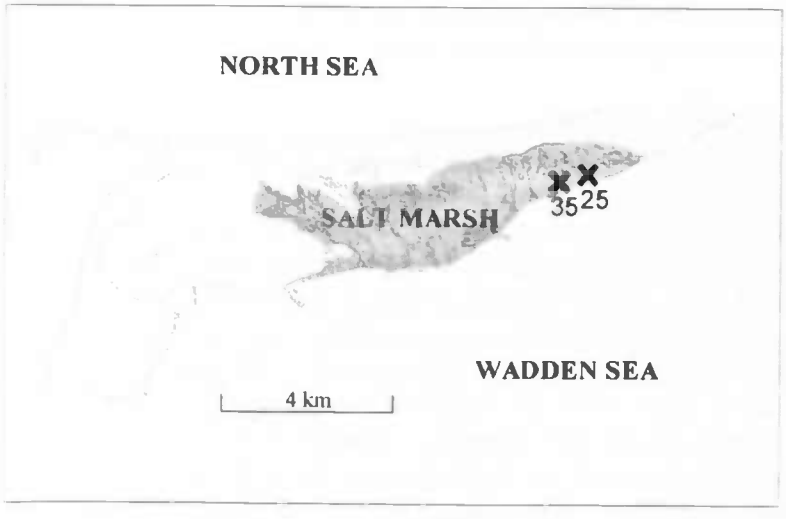

Figure 3. The Dutch barrier island of Schiermonnikoog

This linear chronosequence offers an ideal situation to study the colonisation and succession of *E. atherica* by comparing salt-marsh sites of different age. Since cattle-grazing and cutting took place on all salt marshes emerging before 1958 (Bakker 1989), the study sites were chosen to be situated on younger sites as to guarantee natural habitats.

The age of a site is determined by the time passed since the first terrestrial plants settled there (Olff *et al.* 1997).
Salt marshes in the Wadden Sea area have a characteristic structure. The pioneer zone is predominantly inhabited by *Salicornia spp.* and *Spartina anglica*. The lower salt marsh has an annual inundation frequency of 166 and is characterised by *Puccinellia maritima* and *Limonium vulgare*. The lower salt marsh evolves gradually into the higher salt marsh, which has an annual inundation frequency of 53. The lower salt marsh is defined as where *E. atherica* occurs but never reaches a cover of more than 50% (Bockelmann 2002). The higher salt marsh is characterised by the dominance of *E. atherica* and *Festuca rubra*. (Bakker 1989; Olff *et al* 1997)

Two salt-marsh sites of different age, 25 and 35 years old, were chosen for this study. Note that the age of the site does not indicate the age of the present *E. atherica* population, but the time-span over which the area has been vegetated. However, according to the ages of the sites, the two populations of *E. atherica* differ approximately 10 years in age. On the younger site, *E. atherica* is occurring in many, predominantly small patches (< 5 m^2). On the older site, *E. atherica* occurs in patches ranging in size from less than 1 m^2 up to patches larger than 80 m^2, without a bias towards any patch size. (See the Results section for detailed information on patch size distribution and Figure 4 for *E. atherica* mappings of the two sites). Eastwards of the younger site, *E. atherica* is in the earliest phase of colonisation in which seedlings just started to establish and patches are scarce. Westwards of the older site, *E. atherica* becomes very abundant, eventually covering the lower salt marsh completely.
Three blocks per site were established, each 50m x 50m in the younger site and 25m x 100m in the older site, perpendicular to the coastal border towards 330°N. In spring 2003, the abundance of *E. atherica* was mapped in each block by R.M. Veeneklaas and B. de Ridder (unpublished data). Three degrees of cover by *E. atherica* (<20%; 20-50%; >50%) were used. The clone maps created in this study were put on top of these maps.

Vegetation description
The abundances of the present species in 60 2m x 2m plots were estimated, 30 in blocks on the 25 year old salt-marsh site and 30 in blocks on the 35 year old salt-marsh site. The plots were randomly chosen from areas with an elevation high enough for *E. atherica* to occur and in each site half of the plots contained *E. atherica* while the other half did not.
The abundance of each species in each plot was estimated according to the method of Braun-Blanquet modified by Barkman *et al.* (1964) and transformed to an ordinal scale from 0 to 9, where 0 means absence and 9 means 75-100% cover (Table 2). This ordinal scale is used as a ratio scale in statistical analysis, the assumption being that all transitions are equally likely. For example, a change from 3 to 4 is as likely as a change from 7 to 8. This assumption is not unlikely since the scale roughly shows an exponential increase of cover, which in a way reflects the vegetative growth. It may be considered equally likely to expand from 1-5 individuals to >5 individuals as from 50% cover to 75% cover.

Table 2. Braun-Blanquet abundances descriptions and the ordinal transformation.

Braun-Blanquet	Ord. transf.	Percentage cover or description
-	0	Absent
r	1	1-5 individuals, very low cover
+	2	>5 individuals, very low cover
1	3	Frequent occurrence, cover <5%
2m	4	Very abundant, cover <5%
2a	5	5-12.5% cover
2b	6	12.5-25% cover
3	7	25-50% cover
4	8	50-75% cover
5	9	75-100% cover

Mean and standard error of abundances of present species in the two sites were derived from the data and a 2-tailed *t*-test was used to calculate whether there was a significant difference between the two sites. The difference between the number of species present per site was also checked for differences. Mean and standard error were also calculated for the data clustered in plots with and without *E. atherica* present both for the younger and the older site. A *t*-test was performed on these data as well to check for significant differences in abundances between species. The significance levels were adjusted with the Bonferroni procedure to control Type I error rates.

The abundance of *E. atherica* at the scale of the site was also calculated for all blocks on the younger and the older site by dividing the area where *E. atherica* was present by the total area of the site. The mapping data of *E. atherica* has been used to calculate the overall abundances.

With the same mapping data, the patch size distribution was calculated with patches divided in three patch size classes: small (<5 m^2) intermediate (5-20 m^2) and large (>20 m^2). A cover index was also included in the mapping which enabled calculation of an average cover index per patch size class per site.

Sample design

An exponential grid sample design was chosen (modified from Van der Velde 2000) meaning that sampling took place at different spatial scales. In this way, the sampling intensity and the information gained from the samples were optimised for several purposes. First of all, this design enables determination of the scale that captures most or all genets. Secondly, this design results in an even distribution of sample pairs in several distance classes which is beneficial in clonal probability calculations. Furthermore, this design enables comparison between differently sized patches since all patches have a comparable minimal sampling area. Finally, this design gives a clear overall picture of clonal diversity in the two populations.

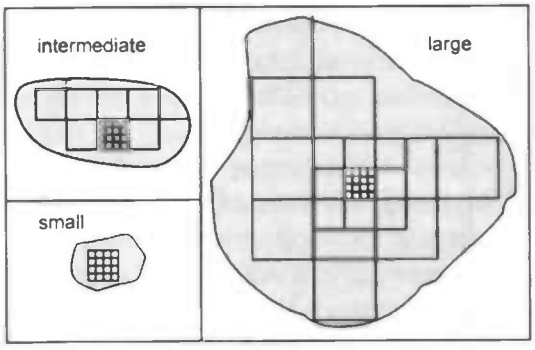

Figure 5. Patches of different sizes with sampling grids at different scales.

A small (<5 m^2), an intermediate (5-20 m^2) and a large (>20 m^2) patch were selected in every block if possible. These patches are denoted as S, M and L, respectively. Within all patches sampling took place within 1 m^2 on a 25cm x 25cm grid. Outside this 1 m^2, sampling took place on a 1-m^2 grid. In large patches, sampling took place in a 4 m^2 grid (Fig. 5) outside the central 9 m^2. Finally, several samples were taken from the edges of adjacent patches. The coordinates of the leaf samples were noted.

Leaf tissue (8-10 cm) was sampled and put in 2 mL air-tight tubes containing dried silica-gel. Preferably young leaves were collected as they contain more cells per area so yield more DNA than older leaves for the same amount taken. In addition, it is easier to destruct the cell walls of young tissue since these are not fully developed to their final rigid stage. It also contains less secondary metabolites (e.g. anti-herbivory proteins) and therefore yields cleaner DNA.

Microsatellite analysis

The first step of the microsatellite analysis involves total DNA extraction. In the laboratory, approximately 0.5 cm^2 leaf tissue was crushed in a 1.5 mL eppendorf tube with a metal mortar after being frozen in liquid nitrogen. The rest of the sample material was stored as a reserve. The total DNA of dried leaves was obtained using the CTAB method (Doyle & Doyle 1987; Appendix 1). It involves dissolving the cell membranes and extracting the DNA for 20 minutes at 60°C and 45 minutes at room temperature by extraction buffer containing CTAB. The extracts were purified with two chloroform-isoamylalcohol (24:1) extractions denaturing proteins, an isopropanol precipitation to remove polysaccharides and an ethanol wash step to remove salt and RNA. The total DNA extract, dissolved in 20 µL TE, was stored at -20°C.

Microsatellites are short (20-200 bp) stretches of DNA containing a repetitive sequence such as $(AG)_n$ or $(TAT)_n$. The repetitive sequence is often composed of 2, 3 or 4 base-pairs. The length of these microsatellites is hypervariable in the number of sequence repetitions (mutation rate ~10^{-5}). That is because replication slippage or unequal crossing-over during meiosis adds or deletes one or more copies of the repetitive sequence in the microsatellite. These kinds of mutations happen much more often than normal point mutations. Many alleles per microsatellite can therefore often be found in a single population since the microsatellite may differ in length among individuals. Microsatellites are assumed to be selectively neutral, thus patterns in microsatellite distribution are brought about by genetic drift, mutation and migration (dispersal in plants) and so reflect population demographic processes (Jarne & Lagoda 1996). In this study, 5 microsatellites were visualised with five primer pairs originally designed for *Elymus caninus* (Sun *et al.* 1998) and *Triticum aestivum* (Röder *et al.* 1998). Each primer is an oligonucleotide containing base-pairs that complement one of the two flanking sequences

of the microsatellite (Table 3). The primers attach to their complementary DNA and act as starting points for *Taq*-polymerase, the enzyme that can be used to artificially replicate the microsatellite.

Table 3. Cross-species microsatellite markers. The upper sequence is the labelled forward primer, the lower sequence the reverse primer.

Locus	Label	Primers (5'→3')	Species	Assessment
ECGA22	HEX green	GAA GGT GAC TAG GTC CAA C ATA GTC TCG GTC AGG CTC	*Elymus caninus*	Sun *et al.* 1998
ECGA89	HEX green	TTA GCT CTT TAC TTA TTC AAA C TCC TAT GAT CAA GCA CAA G	*Elymus caninus*	Sun *et al.* 1998
WMS2	FAM blue	CTG CAA GCC TGT GAT CAA CT CAT TCT CAA ATG ATC GAA CA	*Triticum aestivum*	Röder *et al.* 1998
WMS6	NED yellow	CGT ATC ACC TCC TAG CTA AAC TAG AGC CTT ATC ATG ACC CTA CCT T	*Triticum aestivum*	Röder *et al.* 1998
WMS44	FAM blue	GTT GAG CTT TTC AGT TCG GC ACT GGC ATC CAC TGA GCT G	*Triticum aestivum*	Röder *et al.* 1998

Each 10 µL polymerase chain reaction (PCR) solution contained 1 µL of total DNA (~5-10 ng), 1 µL of forward and 1 µL of reverse primer (0.5 µM each; final concentrations noted everywhere) (Biolegio, Malden, NL; Applied Biosystems, Warrington, UK), 0.1 µL *Taq*-polymerase (1 U/µL), 1 µL 10x PCR-buffer, 1 µL containing a solution of dNTPs (200 µM each) (Roche Diagnostics, Almere, NL), 1 µL BSA (1% or 0.2 µg/µL) (Roche Diagnostics, Almere, NL) and 3.9 µL MilliQ (Appendix 2). The forward primer was fluorescently labelled to be able to visualize PCR-products later on. All PCR reactions were performed on the same PCR machine (PE 9700 PCR, Perkin-Elmer) to standardize the procedure. See Appendix 2 for the PCR program descriptions.
The PCR products were electrophoresed on an ABI PRISM™ 377 automated fragment analyser (Perkin-Elmer; Appendix 3) together with a size marker (Rox 350). Per gel-scan 48 samples were analysed and for each sample either two or three PCR products of different microsatellites were pooled (Table 4). All electrophoreses were performed on the same machine to standardize the procedure.

Table 4. Pooling of PCR products with mixed amounts in µL

ECGA22 : WMS6 : WMS44	7 (plate) : 7 : 3
WMS2 : ECGA89	7 (plate) : 5

The computer program Genescan (Perkin-Elmer) was used to prepare the digitalized results before analysing in the computer program Genotyper (Perkin-Elmer). Genotyper scored the presence of fragments with predetermined sizes. The sizes of the fragments were determined by checking by hand a subset of all samples which comprises the common fragments. In addition to this automatic procedure, all results produced by Genotyper were compared with the digital gels to check for errors. Appendix 4 shows the criteria for peak scoring by Genotyper.

Genotyping
Fragment information was put into the computer program Access (Microsoft) where all samples were assigned identities based on their genotypes. Unfortunately, codominant analysis was not possible in this study, because *E. atherica*'s hexaploidy generates allele

configurations which are not identifiable in a reliable way. To illustrate this, it is difficult to distinguish between the allelic configurations 1:5 and 2:4 if only two alleles are known to be involved. In addition, each locus could generate six alleles, but often more than six bands are found per microsatellite. This indicates that more loci could be involved which complicates the identification even more since it is unknown which alleles belong to which locus. Because of these difficulties, genotypes were treated as characters comprising information on absence and presence of known bands. Each different character was assumed to indicate a different genet. For clarity reasons the term genotype will be used instead of the term character.

To gain insight in the power of each investigated microsatellite to distinguish between genets, the number of genotypes revealed by each combination of primer pairs (ranging from 1 primer pair to all 5 primer pairs) was calculated. Part of the left half of a quadratic polynomial regression was fitted through the data instead of a linear regression, because with an increasing number of microsatellites investigated the curve will level off and less or no new genotypes will be revealed with addition of microsatellites. Using a quadratic polynomial will increase the estimated number of genets needed to capture all genets compared to linear regression, but it resembles reality more than with linear regression.

It is possible that two different genets could not be distinguished based on their genotype revealed by the 5 microsatellites. The probability that a certain genotype (i.e. a certain occurrence of bands) that was sampled twice would in fact belong to different genets (i.e. not identical by descent), which would underestimate the amount of genets found, was calculated using the formula

$$P_{gen} = 2^h \prod^{k} p_i$$

in which P_{gen} is the probability of a certain combination of bands to occur by chance, h is the amount of polymorphic microsatellite genotypes, k is the number of microsatellites investigated and p_i is the frequency of the ith microsatellite genotype. Every genet was taken into account only once. This formula is modified after Reusch *et al.* (1999) and Parks & Worth (1993). They used p_i as the frequency of the ith allele. Since it is not sure which bands are produced by a certain locus, h cannot be determined in this way. The analysis is therefore performed with frequencies of genotypes derived from separate microsatellites. Hence, h is the amount of microsatellites that show polymorphy in the genotypes. However, this could introduce a bias in the results since a lot of information about the band patterns is lost. Anyway, the probabilities found are likely to be overestimated, because there is a chance that other microsatellite genotypes will show up which decreases the frequencies of occurrence of other microsatellite genotypes. Therefore, this bias is considered not dangerous to the conclusion that, if the results indicate this, individuals with an identical genotype are belonging to the same genet.

Data analysis

After assigning identities to the samples from the obtained fingerprints, these data were linked to the sample locations and maps were made to visualise the results. Clonal diversity was estimated as G/N (Ellstrand & Roose 1987) in which G is the number of different genotypes found divided by N which is the number of samples analysed. G/N equals 1 when each ramet represents a different genet. Accordingly, sexual reproduction

may be assumed to prevail in the population. The clonal diversity G/N approximates 0 when all ramets belong to the same clone, indicating that sexual reproduction is absent in the population. The clonal diversity measured is highly dependent on the number of samples taken. It is therefore important that sample numbers (as well as areas) are comparable. The clonal diversity per core area of each patch was calculated. The clonal diversity of the core area of patches has the advantage that it is comparable among all patches irrespective of their size, because the investigated core area is equal in size and the number of samples is approximately the same. Measurements taken at different spatial scales and with different sample sizes could induce a bias when compared with each other.

By using spatial statistics, spatial autocorrelation of clones was determined giving the clonal probability which is described as the chance that at a certain distance interval two ramets belong to the same genet. The clonal probability thus indicates the extent and the interminglednes of clones. The spatial autocorrelation of clones in patches for the two sites was estimated by values of Moran's *I* statistic for different distance classes. In order to facilitate the calculation, the computer program SPAGeDi (Hardy & Vekemans 2002) was used. Relatedness coefficients (called 'relationship coefficients' in SPAGeDi) are defined by the program as the proportion of genes in one individual with alleles identical to these of a reference individual. When assigning a number to clonal identities and using these as distinct alleles belonging to only one locus of a haploid organism, the spatial autocorrelation of clones will be calculated.

To calculate Moran's *I*, SPAGeDi uses the function

$$r_{ij} = \sum_l \sum_a (p_{ila} - p_{la})(p_{jla} - p_{la}) \,/\, \sum_l \sum_a \mathrm{Var}(p_{ila}) + 1/(n_l - 1)$$

where p_{ila} stands for the frequency of allele a at locus l in individual i; r is the relatedness coefficient of Wright (1946) of which Hardy & Vekemans (1999) showed that it estimates Moran's *I* statistic; i and j are distinct individuals; $\mathrm{Var}(p_{ila})$ is the variance of individual allele frequencies for all loci. By calculating r_{ij} for all possible pairs and averaging r_{ij} over all distance classes, the mean values of Moran's *I* are computed as proposed by Dewey and Heywood (1988). Each genotype, or character, was assigned a specific number. The calculation involved one 'locus' with many 'alleles', each allele being a different genet.

The obtained correlogram shows for each distance class the fraction of pairs that share the same identity. Values of Moran's *I* statistic lie between -1 and +1, where a value of -1 indicates complete negative autocorrelation (all ramets picked belong to separate genets), 0 indicates no autocorrelation in clones (complete randomness) and +1 indicates total positive autocorrelation.

To maximize the confidence of the calculated Moran's *I* values and to raise their significance, the proportion (%) of all individuals represented at least once in the distance class was tried to set to >50% and the coefficient of variation of the number of times each individual is represented was tried to set to <1.

Relatedness within patches was estimated by calculating the average amount of marker differences between pairs. A marker difference is when a certain band is present in one

and absent in another individual. Only different genets were taken into account. Assumed was that close relatives have less marker differences than unrelated individuals.
Furthermore, an UPGMA tree was created using the computer program Mix from the Phylip package (Felsenstein 1989). The calculation was based on Wagner parsimony which states that a transition from absence to presence of a band is as likely as a transition from presence to absence of a band. The tree shows the simplest parsimony of all genets and any clustering of genets that reflects spatial clustering indicates some extent of relatedness.

Prior to every ANOVA, homogeneity of variances was estimated with Levene's test. The clonal diversities were tested with a two-way analysis of variance (ANOVA) with clonal diversity in the core area of patches as the dependent factor and site and patch size class as the independent factors. The relatedness within patches was tested with a two-way ANOVA with mean number of band differences as the dependent factor, site and patch size class as independent factors and patch size in m^2 as a covariate.

Results

Vegetation description

All 16 species except *Armeria maritima*, *Atriplex portucaloides*, *Puccinellia maritima*, *Suaeda maritima* and *Triglochin maritima* showed significant differences in abundance between the younger and the older salt-marsh site (data from plots with *E. atherica* and without *E. atherica* combined; *t*-test; Bonferroni corrected $\alpha = 0.003$). These species declined in abundance in contrast to *Atriplex prostrata* and *Elytrigia atherica* which increased in abundance. The number of species present decreased as well, indicating lower species diversity on the older site than on the younger site.

The same data were clustered in either presence of *E. atherica* or absence of *E. atherica* within either the younger or the older salt-marsh site. On the older site, *Artemisia maritima*, *Festuca rubra*, *Limonium vulgare* and *Suaeda maritima* declined in abundance in plots with *E. atherica* compared to plots without *E. atherica* (*t*-test; $\alpha = 0.003$; Table 5). The number of species also declined on the older site in plots with *E. atherica*, but this effect was marginally significant ($P = 0.04$). On the younger site presence of *E. atherica* did not alter the composition of the plots.

Table 5. Mean and standard deviation of abundances (Braun-Blanquet transformed to ordinal scale) of the vegetation in the 25 year old and the 35 year old salt-marsh site measured in 60 2x2 m plots with (*Ea*) and without (Non-*Ea*) *E. atherica* for different species (n=15 in each group). Bold figures indicate significant differences (Bonferroni corrected $\alpha = 0.003$) between Non-*Ea* and *Ea* within a site (*t*-test).

	25 year old site				35 year old site			
	Non-*Ea*		*Ea*		Non-*Ea*		*Ea*	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Armeria maritima	0.133	0.091	0.200	0.200	0.000	0.000	0.000	0.000
Artemisia maritima	6.200	0.327	6.533	0.291	**6.267**	0.300	**3.600**	0.576
Aster tripolium	1.800	0.279	1.733	0.284	0.667	0.252	0.333	0.126
Atriplex portucaloides	1.400	0.214	1.400	0.289	0.867	0.256	0.533	0.192
Atriplex prostrata	0.667	0.252	0.733	0.153	2.800	0.200	3.400	0.273
Elytrigia atherica	**0.000**	0.000	**1.867**	0.256	**0.000**	0.000	**7.867**	0.506
Festuca rubra	8.733	0.118	8.933	0.067	**8.733**	0.118	**4.533**	0.716
Glaux maritima	1.667	0.374	2.267	0.483	0.000	0.000	0.267	0.182
Limonium vulgare	4.933	0.248	4.400	0.321	**1.733**	0.384	**0.200**	0.145
Plantago maritima	1.267	0.358	1.067	0.358	0.133	0.091	0.000	0.000
Puccinellia maritima	1.733	0.248	1.533	0.307	2.000	0.000	0.000	0.000
Salicornia europaea	2.000	0.258	2.267	0.118	0.400	0.163	0.067	0.067
Spartina anglica	0.467	0.133	0.467	0.236	0.000	0.000	0.000	0.000
Spergularia maritima	1.333	0.187	1.333	0.232	0.000	0.000	0.000	0.000
Suaeda maritima	2.600	0.190	2.467	0.215	**2.667**	0.232	**1.200**	0.243
Triglochin maritima	0.600	0.190	0.600	0.214	0.467	0.165	0.133	0.133
Number of species	11.133	0.389	11.867	0.467	7.467	0.446	5.667	0.361

The overall abundance of *E. atherica* at the scale of the site, calculated with the mapping data, also differed between the younger and the older salt-marsh site. An abundance of 1.15% on the younger site and 20.8% on the older site was found, which means an 18-fold increase in abundance in 10 years (Fig. 6). The abundances found are not consistent

with the data in Table 5. This is because in Table 5 plots have explicitly been chosen for their absence or presence of *E. atherica* and, furthermore, are revealing abundacies at a different scale.

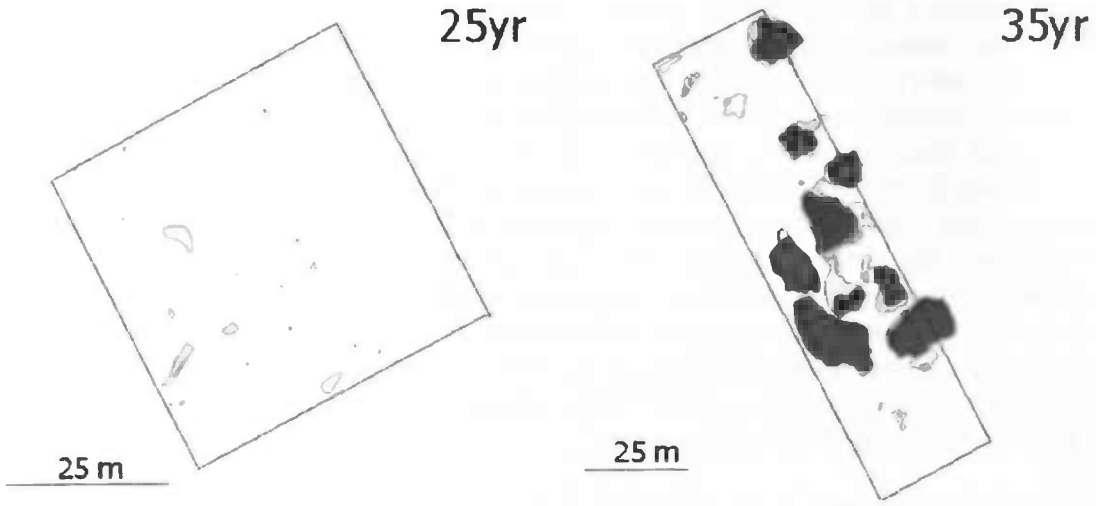

Figure 6. Two blocks showing the borders in which *E. atherica* was mapped. The young salt-marsh site (25yr) and the old salt-marsh site (35yr). Light grey indicates less than 20% cover by *E. atherica*, medium grey 20-50% and dark grey more than 50%.

The patch size distribution was clearly different between the younger and the older salt-marsh site (Fig. 7). On the younger site, small patches were far more abundant than on the older site, whereas on the older site, intermediate and large patches were more abundant than on the younger site.

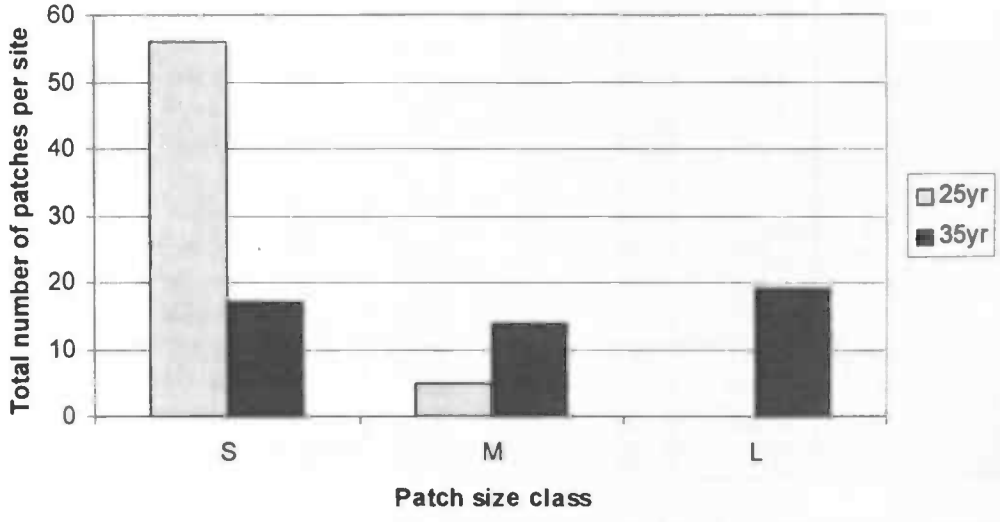

Figure 7. Patch size class distribution as number of small (S), intermediate (M) and large (L) patches for the three blocks (7500 m^2) on the 25 year old salt-marsh site (grey bars) and the three blocks (7500 m^2) on the 35 year old salt-marsh site (black bars).

The cover of *E. atherica* in patches of equal sizes did not differ between the sites, but cover increased with patch size (Fig. 8). This indicates that when patches get older, the cover of *E. atherica* increases.

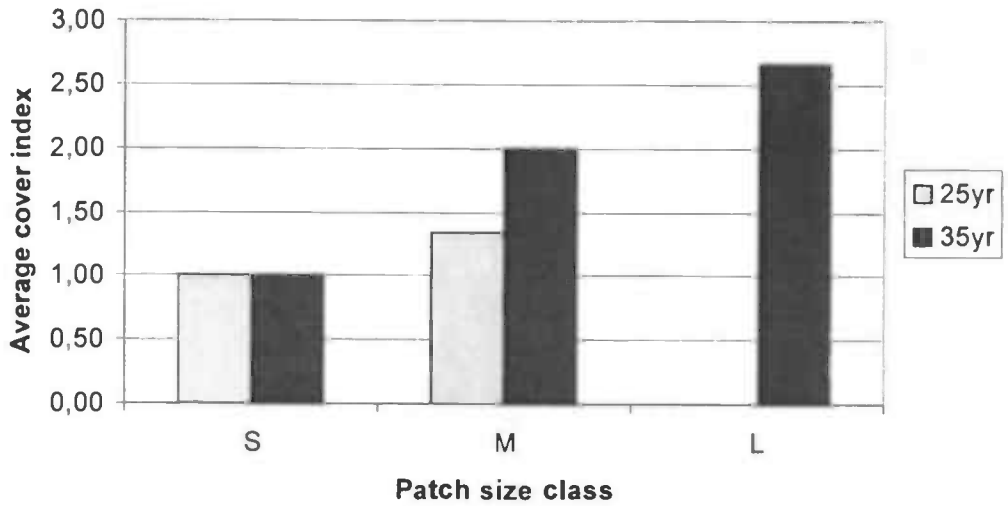

Figure 8. Average cover index per patch size class per site. The cover index was estimated in three classes: 1 < 20%; 2 = 20-50%; 3 > 50%. S means small, M means intermediate and L means large patch size. The grey bars represent data from the 25 year old salt-marsh site, the black bars represent data from the 35 year old salt-marsh site.

Genotyping

A total of 42 different bands were found that were reliable and polymorphic. Table 6 shows the number of bands detected per primer.

Table 6. Bands detected per microsatellite primer.

Microsatellite	ECGA22	ECGA89	WMS2	WMS44	WMS6	Total
Bands detected	7	7	9	10	9	42

All samples with identical multilocus genotypes were assigned to one genet assuming that they belong to the same genet. In order to test whether this assumption could be made, the expected frequency P_{gen} was calculated, which gives the probability that a certain multilocus genotype possibly generated by these 5 primers could occur more than once in the sample pool based on chance events, thus without being identical by descent. P_{gen} ranged from less than 0.0000 to 0.0773. In 48% of the cases the probability was below 0.001. Only in 1.6% of the cases, the probability was above 0.05. These results indicate that the chance for this error is negligible and that all ramets with an identical genotype belong to the same genet.

Detected genotypes increase with the number of primers (Fig. 9). On average, 4.58 primers are needed to distinguish all genotypes found with 5 primers. The least informative primers were ECGA89 and ECGA22; the most informative was WMS6.

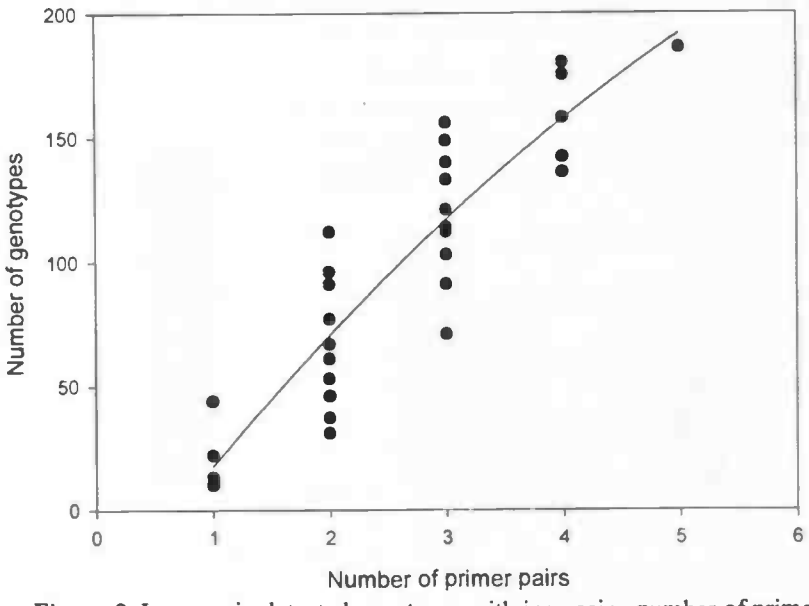

Figure 9. Increase in detected genotypes with increasing number of primer pairs. There are 5 combinations of one and four primers, 10 combinations of two and three primers and 1 combination of all five primers. A quadratic polynomial regresses the values with $R^2 = 0.82$.

Clonal diversity

410 out of 495 samples taken could be scored for all 5 primers (83%). A total of 186 different genotypes was found among these 410 samples. This gives a grand clonal diversity of 0.45. (See Appendix 5 for general information on sample sizes and clonal diversities.)

Patches were generally found to be multiclonal, since multiclonality was found in 14 out of 15 patches (93%). All clones were delimited by their patch. This indicates that long-distance clonal spreading or dispersal of broken off vegetative parts to a nearby, suitable site is not a general dispersal strategy of *E. atherica*. Growth of clones within patches was mixed or, in other words, there were no sharp boundaries between different clones. Figure 10 shows parts of clone maps from this study. (See Appendix 6 for the clone maps.) The large and intermediate patch of 35-1, the first block on the older salt-marsh site, are representative for most patches as they contain some extensive clones with many small clones in between them; it seems all clone sizes are present in these patches. On 25-1, the first block on the younger salt-marsh site, the same situation is present in the intermediate and small patches. However, the large and intermediate patch of 35-3, the third block on the older salt-marsh site, each show only one dominant clone with many small clones in between.

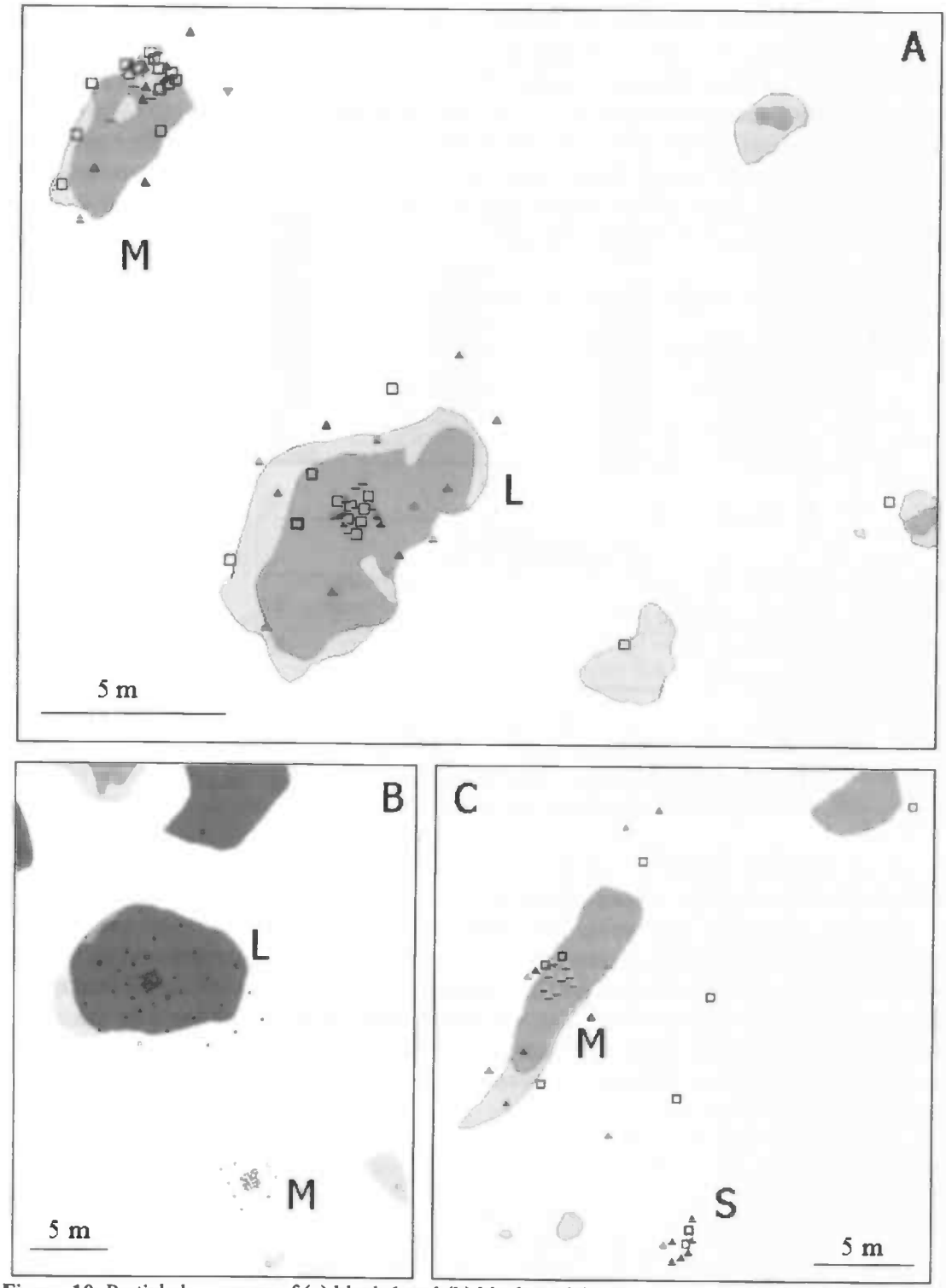

Figure 10. Partial clone maps of (a) block 1 and (b) block 3 of the older salt-marsh site and (3) block 1 of the younger salt-marsh site. Small, intermediate and large patches are denoted as S, M and L, respectively. Open squares indicate genets consisting of only one sampled ramet, while coloured triangles represent genets that occur more than once. The green polygons are patches. Light grey indicates <20% cover, medium grey 20-50% cover and dark grey >50% cover by *E. atherica*.

Since clones have been found to be limited to their patch, the chance is high that the clonal probability (i.e. the chance to pick two ramets from the same genet) is zero if two samples are picked from different patches. The average clonal probability in patches on the younger salt-marsh site starts at 0.47 at 0.25m but immediately drops to values around 0 (Fig. 11). Contrasting, on the older salt-marsh site the clonal probability has values around 0 at all distance classes. Thus, clonal probability is high at distances up to 0.25 m on the younger site after which spatial autocorrelation is absent. The older site lacks spatial autocorrelation at all distance classes.

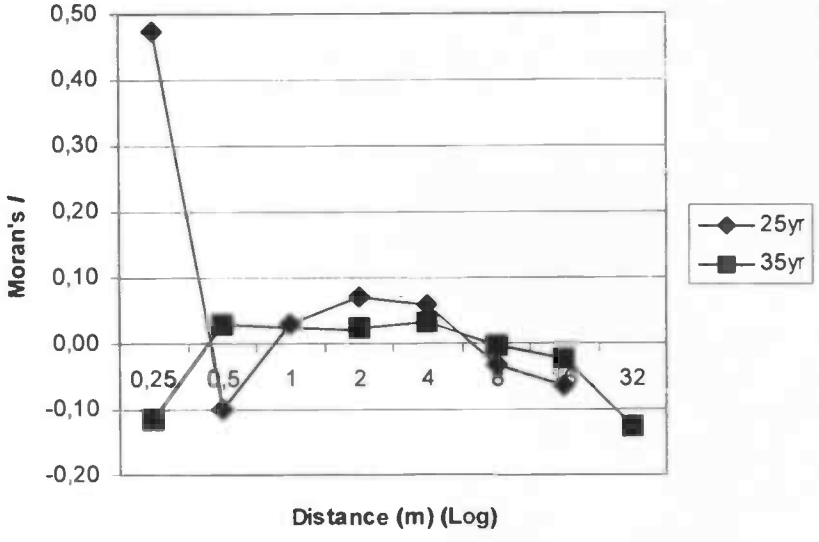

Figure 11. Correlogram of clonal probability using average values of Moran's *I* for 8 logarithmic distance intervals.

The clonal diversity shows a negative trend among patches of different size classes within a site, meaning that when patches grow larger over time, they contain less genets per unit area (Fig. 12). However, this trend was not significant. Furthermore, there appears to be a lower clonal diversity in the younger marsh in comparison to the older marsh for the two comparable patch size classes, although this was not significant either. The clonal diversity on the older site was significantly higher compared to the clonal diversity of the lower site when all patch size classes were combined (two-way ANOVA; $F_{1;8} = 7.007$; $P = 0.029$). The sample sizes may be too low for other relationships to be significantly different.

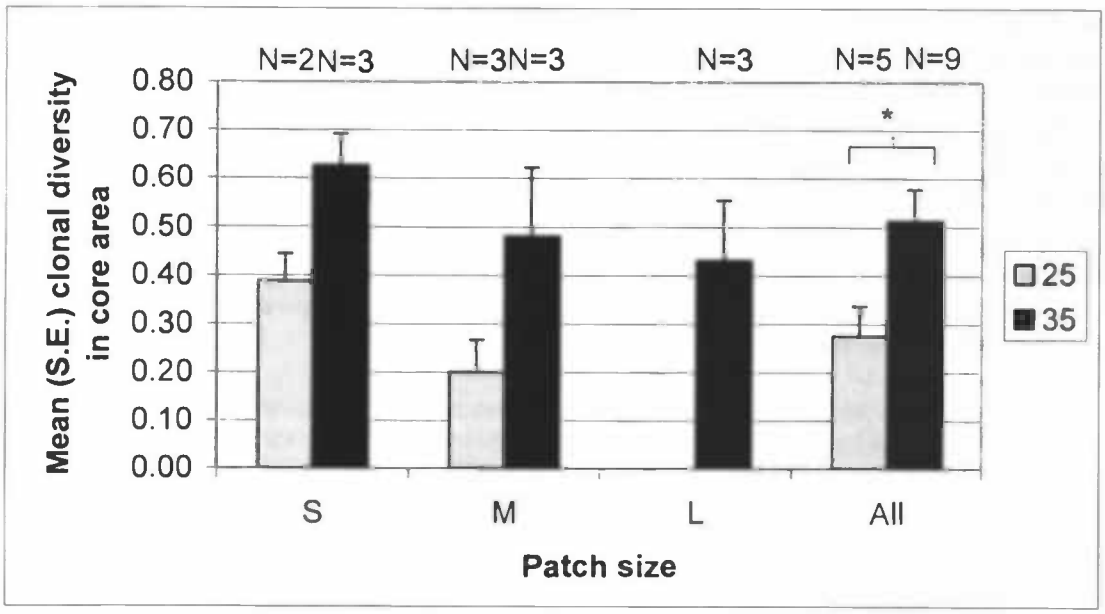

Figure 12. Mean (S.E.) clonal diversity in the core area of patches for the younger (light grey) and the older (dark grey) site and for different size classes. S means small, M means intermediate and L means large patch size. N gives the sample size of patches. * is significant at $\alpha = 0.05$.

Patches with an area less than 10 m^2 have variable clonal diversities, albeit higher for the older salt-marsh site compared to the younger salt-marsh site (Fig. 13). This difference between the two sites has also been shown in Figure 12. For patches larger than 10 m^2, clonal diversity decreases with patch size.

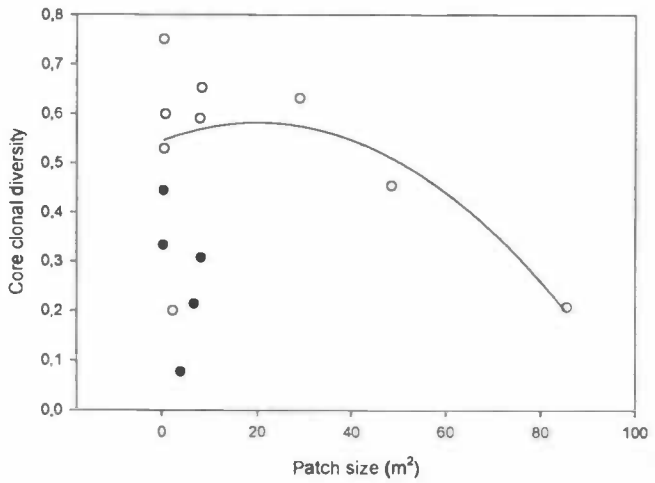

Figure 13. Relationship between core clonal diversity and patch size in m^2. Open circles represent values for patches from the older salt-marsh site and filled circles represent values for patches of the younger salt-marsh site. The fit through the older site values is a quadratic polynomial. $R^2 = 0.386$.

Relatedness

The mean number of band differences increased with the patch size class (two-way ANOVA $F_{2;7} = 4.726$ $P = 0.050$; Table 7), meaning that genets in larger patches are less related than genets in small patches. A contrast test showed that large patches had significantly more band differences among genet pairs than small and intermediate patches ($P = 0.030$) whereas small and intermediate patches did not differ. The mean number of band differences within patches did not differ between the younger and the older salt-marsh site (two-way ANOVA; $F_{5;8} = 1.838$ $P = 0.212$), which means that the relatedness in patches is equal on the younger and the older site. Patch 25-2T (Table 7) is in fact not a patch but an area with many seedlings; all ramets were sampled in this area and every ramet was found a separate genet.

Table 7. Patch area, number of genets and mean number of band differences between all pairs of genets within a patch. The younger and the older salt-marsh site are denoted as 25 and 35, respectively. The block number is denoted 1, 2 or 3 and S, M and L indicate the patch size classes small, intermediate and large, respectively. T is explained in the main text.

Patch	Patch area (m^2)	Number of genets	Mean number of band differences	Range
25-1S	0.36	4	1.5	1-2
25-1M	8.16	11	5.58	1-9
25-2M	6.67	11	4.27	1-9
25-2T	110.50	15	6.14	1-12
25-3S	0.30	3	1.33	1-2
25-3M	3.98	1	-	-
35-1S	0.38	3	2.00	1-3
35-1M	8.30	20	3.41	1-8
35-1L	29.13	16	6.12	1-13
35-2S	0.48	11	3.60	1-11
35-2M	7.94	19	3.93	1-10
35-2L	48.44	14	8.02	1-16
35-3S	0.69	6	4.93	1-11
35-3M	2.29	6	1.87	1-3
35-3L	85.49	11	2.44	1-4

Contrary to the results from the mean number of marker differences, the consensus UPGMA tree from all genets occurring in patches showed much structure (Fig. 14). The first 7 clusters from the top are mainly composed of genets from the younger marsh, while the other 29 clusters are predominantly composed of genets from the older salt-marsh site. This indicates that there is no complete mixing of alleles present between the two sites but that pollen and/or seed dispersal is limited to a high degree. In addition, since the branches of most young site clusters are longer after the first bifurcation than of old site clusters, the relatedness between clusters may be lower than among old site clusters. Appendix 7 shows the same UPGMA tree but with patch information for each genet. In many cases, genets from one patch are clustered together indicating a high relatedness within patches. Other bifurcations like those indicated with A, B and C show bigger differences between clusters because the lengths of the branches that divide them

Figure 14. UPGMA tree of all genets occurring in patches using Wagner pa
All bifurcations have likelihood values of 0.9 or higher. The thick vertical li
demarcates the genotypic difference below which genets were clustered. Clu
grouped in a box with white indicating genets from the younger salt-marsh s
black indicating genets from the older salt-marsh site. A, B and C are import

Discussion and conclusions

Site characteristics

Most species declined in abundance on the older site compared to the younger site except for *Atriplex prostrata* and *E. atherica*, which increased in abundance. The conclusion is thus that *E. atherica* is a strong interspecific competitor. In agreement with this, the biodiversity was found to be lower on the older salt-marsh site compared to the younger salt-marsh site, which has also been found by Van Wijnen *et al.* (1997). The increase in abundance of the annual *A. prostrata* may be explained by the fact that it occurs abundantly where floodmark is catched. Tall-growing *E. atherica* patches may trap floodmark very well (Veeneklaas pers. comm.).

The equality in species presence and abundance in *Elytrigia* and non-*Elytrigia* plots on the younger site (Table 5) could indicate that presence of *Elytrigia* is a chance process. *Elytrigia* simply did not yet arrive in these plots. However, absence of differences on the factors that were investigated is, of course, no reason to conclude that chance events determine the presence of *E. atherica*. Kuijper (2004) showed that seedling recruitment of *E. atherica* can be strongly reduced by herbivory, especially at unproductive sites as is the younger site in this study. Although hares and geese prefer other salt marsh species than *E. ahterica*, herbivory on seedlings on the younger site can prevent *E. atherica* from colonisation. In addition, Kuijper (2004) showed that, although *E. atherica* is able to outcompete small-statured vegetation on the long term, it has difficulty to invade short vegetation in the early phases of colonisation. This may explain the absence of differences in vegetation between plots with and without *E. atherica*. The colonisation was not far enough to bring about differences in the vegetation.

On the older salt-marsh site, the four species which abundance declined in plots with *E. atherica* compared with plots without *E. atherica* (*Artemisia maritima*, *Festuca rubra*, *Limonium vulgare* and *Suaeda maritima*) belong to the top 5 of most abundant species. Here, apparently, *Elytrigia* does compete with these 4 species. Other species, which are able to live among these abundant ones, did not decrease in abundance significantly, probably because they are able to live in *Elytrigia*-dominated stands as well, as long as the density of *Elytrigia* ramets is not too high thereby shading these species.

The patch size class distribution is very straightforward regarding differences between the younger and the older salt-marsh site (Fig. 7). More small patches on the younger site and more intermediate and small patches on the older site indicates a fast growth of patches. However, assuming that small patches on a 25 year old site will become intermediate and large patches on a 35 year old site, one would expect the amount of small patches on the younger site to be the same as the amount of intermediate and large patches on the older site. This is not the case, since around 15 intermediate and 20 large patches on the older site do not add up to 55 small patches on the younger site. A reason for this could be that small patches lying close to each other will merge over time forming bigger patches. The merging of small patches together would raise the clonal diversity within patches without sexual reproduction needing to take place. However, the merging of patches itself will not increase the clonal diversity per standard area.

The cover estimate of patches of different size classes increases with the size of the patch (Fig. 8). This means that when patches grow in size, the ramet density within a patch also

increases. This indicates that patches of *E. atherica* do not only grow at the border, but rather grow in all directions and at all locations. This could be linked to the fact that clones of *E. atherica* were found to grow in a mixed fashion.

Identification of genets

The sample design and the resolution of genetic analysis are important factors that influence the amount of genets found (Ellstrand & Roose 1987). When the sampling intensity is raised, more genets are likely to be found. There is an optimal intensity, depending on the clonal diversity in the population, where all genets will be found with the least samples. One of the reasons why the exponential grid sample design was chosen, was to determine the scale that captures most or all genets with the least samples. Unfortunately, the results show that it is very unlikely that all genets present were sampled, even at the smallest scale. When comparing the amount of genets found at a certain scale with the amount of genets found at a smaller scale within the same area, new genets were usually found. This indicates that saturation has not yet been reached, at least on all but the smallest scale at which has been sampled. Furthermore, in most core areas, many genets were sampled only once, indicating that many small clones can be present even in patches dominated by a single large clone. Therefore, making predictions about the actual amount of genets per standard area is difficult. However, comparisons between patches and sites can still be made.

Another aspect of identification of genets is the resolution of the genetic analysis. Ellstrand and Roose (1987) found that the clonal diversity was positively correlated with the amount of molecular markers used to determine the clonal diversity. When using a low resolution, so with a small amount of scorable bands, the chances are high that individuals are grouped together as being clonally related when in fact they are not. As was shown in Figure 9 in two specific combinations of 4 out of 5 microsatellites, almost all the genotypes that were distinguished by all 5 microsatellites could be revealed. This indicates that the two microsatellites that were absent in the two combinations, although they have substantial discriminative power, did not alter the genotyping results very much. Therefore, it can be assumed that samples scored as belonging to a single genet, in fact are so.

In this study, 5 microsatellites were investigated that together were scored for presence or absence of 42 bands. A subset of individuals from the sampled large patch in the third block on the older salt-marsh site was checked for differences in band presences. Most often a difference of one band in primer WMS6 was found between the dominant genet and different genets in this patch. This can be explained logically by the high polymorphism found in this primer, but this could also be due to false scoring because of the high complexity of WMS6. However, 3 genets were differentiated from the dominant genet by differences in primers other than WMS6. In addition, the checked individuals belonging to the dominant genet had an identical banding pattern, even in regions and at complexity levels (e.g. relative peak sizes) not taken into account in the analysis. These facts make the genotypic analysis of this patch, as well as all other samples, reliable.

Clonal diversity

The grand clonal diversity of 0.45 is substantially higher than 0.17, the mean from 26 studies found by Ellstrand and Roose (1987). This could mean that sexual reproduction is

more abundant in *E. atherica* compared to other clonal species, but this result could also be an effect of sampling. However, although the sampling scale was very small, many clones were found, which indicates much sexual reproduction.
In studies on the spatial autocorrelation of clones, a high positive autocorrelation decreasing towards or below zero has often been found (Epperson & Clegg 1986; Harada *et al.* 1997; Stehlik & Holderegger 2000; Schläpfer & Fischer 1998; Kudoh *et al.* 1999; Chung & Epperson 2000), as a result of the spatial limit of clonal dispersal (isolation by distance (Wright 1943). In the present study, however, the clonal probability in patches is only high on the younger salt-marsh site at distances up to 0.25m. At larger distances on the younger site and on the higher salt marsh as a whole, clonal probability is around 0, which means that clones lack spatial autocorrelation. This indicates that, within multiclonal patches, clones have no sharp boundaries within the patch. In other words, the interminglednes reduces the clonal probability to such an extent that spatial autocorrelation is absent. A reason for this absence of clonal probability in *E. atherica*, in contrast to the findings in other species, could be that it is advantageous for *E. atherica* to initially spread over the area; the cover will be increased at a later stage. In addition, the thin growth form of *E. atherica* is not very useful in physical inter- or intraspecific competition. Accordingly, guerrilla strategists are often herb species, while phalanx strategists are often woody species (Chung *et al.* 2000 and references therein).
Two types of growth strategies for clonal plants are recognized: the phalanx and the guerrilla strategy (Eriksson 1997). The present study showed that clones were intermingled meaning that, at least during colonisation, the guerrilla strategy prevailed. The results from the clonal probability calculations also point into that direction, since a mixed growth form would give neither a high nor a low clonal probability. However, it should be noted that the strategy adopted by a certain species does not necessarily has to be fixed, but can change according to the prevailing conditions (Kik 1987). The interminglednes of clones as well as the occurrence of small genets in patches dominated by a few large genets has also been found in *Uvularia perfoliata* (Kudoh *et al.* 1999).

The finding that the clonal diversity is lower in large patches compared to intermediate and small patches indicates that the ratio of sexual and vegetative growth changes towards more vegetative reproduction and/or less sexual reproduction. It is interesting to understand whether vegetative reproduction increased, sexual reproduction decreased or both were at hand and in what magnitude.
An important fact is that, whatever the change in vegetative reproduction may be, there will be no effect on the clonal diversity found, since the chance for a genet to be sampled will not change. However, this holds only when the change in the amount of vegetative reproduction is the same for every genet. For example, the chance to pick two certain genets will be equal at any time whatever the rate of vegetative growth as long as both genets were growing at the same rate. A change in sexual reproduction, however, will result (1) in a constant clonal diversity when sexual reproduction ceases or (2) in an increasing clonal diversity when sexual reproduction remains constant or increases, whatever the change in vegetative reproduction. Table 8 summarizes these effects on clonal diversity with changes in sexual and vegetative reproduction.

Table 8. The effects of change in sexual and vegetative reproduction on clonal diversity.

Clonal diversity without competition or stochastic mortality		Vegetative reproduction		
		Decreasing	Constant	Increasing
Sexual reproduction	Decreasing	> or =	> or =	> or =
	Constant	>	>	>
	Increasing	>	>	>

As a consequence of the results of this thought experiment described above, a decreasing clonal diversity can only be explained by genet loss due to intra- or interspecific competition or stochastic mortality. The amount of genet loss should then outweigh the sexual reproduction to arrive at a net decrease in clonal diversity. Figure 15 shows different possibilities to arrive at a net genet loss (i.e. genet loss > genet gain) and a subsequent decrease in clonal diversity. Three scenarios are possible: (1) genet gain remains constant and the genet loss increases; (2) genet loss remains constant and genet gain decreases; (3) genet loss increases and genet gain decreases. The last scenario will result in a net genet loss more rapidly than the other two scenarios.

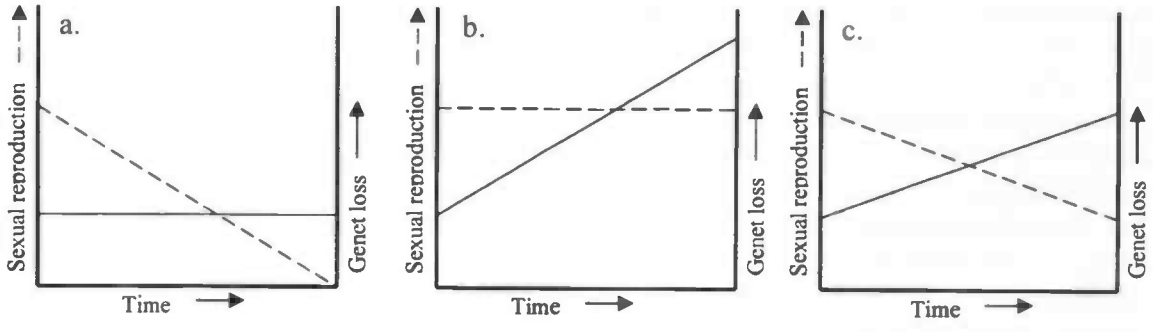

Figure 15. Three scenarios for sexual reproduction (dashed line) and genet loss (solid line) over time. In each graph sexual reproduction starts at a higher rate than genet loss which results in an increasing clonal diversity. In a, the sexual reproduction decreases over time ultimately crossing the constant genet loss after which clonal diversity will decrease. Vice versa for b. In c sexual reproduction decreases and genet loss increases.

As the probability for seedling establishment is likely to decrease with increasing ramet density in the patch over time (Tilman 1988), the intraspecific competition is likely to be increased with increasing ramet density. This points to the third scenario in which both the genet gain decreases and the genet loss increases ultimately resulting in a decreasing clonal diversity.

The trend of decreasing clonal diversity with increasing patch size is based on very low sample sizes. That is possibly the reason that no significant differences were found between the size groups. Another option for this result could be that the variance in clonal diversity found in small and intermediate patches is comparable to the variance in the three large patches which by chance showed a negative linear relationship. This would mean that in reality there is no difference in clonal diversity between patches of different sizes. More replicates are needed to decide upon that. This could be investigated by comparing clonal diversities in core areas only, reducing time and costs.

Relatedness and seed dispersal

The mean number of band differences increased significantly with patch size class. This would mean that genets in bigger patches are less related than genets in small patches. However, this result has not been found when, instead of patch size class, patch size in m^2 was used. Patch size in m^2 is a variable that represents the real conditions better than patch size classes. Therefore, the conclusion that genets in bigger patches are less related than genets in small patches may be wrong.

A reason for the absence of differences in relatedness could be that seeds disperse very well at the scale used in this study. This seems plausible regarding the hydrodynamic conditions in salt marshes. Studies on *E. atherica* conducted on a larger scale showed clear differences in relatedness (Bockelmann 2002), whereas other studies at the same scale but with different salt-marsh plant species also reported absence of differences in relatedness (Travis & Hester 2005).

Another study by Bockelmann (2002) showed that the population on the lower salt marsh is slightly genetically differentiated from the higher salt-marsh population. She explained this with adaptation to specific environmental conditions. Apparently, the environmental conditions do not differ that much between the two lower salt-marsh sites in this study as to result in genetic differentiation or in differences in relatedness.

The UPGMA tree of genets from patches showed a division between genets from the younger and genets from the older salt-marsh site, indicating that pollen and/or seed dispersal is limited to a certain extent. However, the division is not sharp and thus pollen and/or seeds may disperse between the sites now and then.

The clustering of genets from the same patch indicates that genets are related within patches. This supports the hypothesis that seeds produced by a patch get trapped in that patch and, at least in early phases of patch development, have a chance to establish there.

Another explanation for the high relatedness between genets within patches is that, if minor differences in band patterns are present, somatic mutations are the cause for these differences. In *Prunus ssiori* somatic mutations accounted for 5 of the 44 genotypes scored (Nagamitsu *et al.* 2004) which shows that somatic mutations could introduce a bias in the genet identification. However, in this study most often the differences involve more than one band which makes it very unlikely that these were the result of somatic mutations.

The longer branch lengths for the clusters with predominantly younger site genets compared to those of the clusters with predominantly older site genets indicate that genets from the younger site are less related than genets from the older site. However, any reference is missing thus any conclusions cannot be made.

It is impossible to determine the extent of the differentiation and relatedness described above and thus to determine the reliability of the relatedness estimates due to the absence of a reference. Since almost no significant differences have been found in calculations using the mean number of marker differences, this indicates that the structure found in the UPGMA tree is not very reliable. However, it is interesting to see so much explainable structure, although the results of the UPGMA tree must be interpreted carefully.

Growth strategy

Summarizing the results, much variation in clonal diversity exists in the small and intermediate sized patches. Furthermore, a negative trend in clonal diversity was found from small to large patches. Finally, a significant difference in clonal diversity between the younger and the older salt-marsh site was found, with the older site revealing an overall higher clonal diversity.

Regarding these results, it could be that the variety in clonal diversity in small and intermediate patches represents stages toward patches with a high clonal diversity. The clonal diversity will then decrease slowly over time when patches grow larger than ~10 m^2 and consequently have reached a certain ramet density.

This pattern of clonal diversity over time is more consistent with the ISR than the RSR strategy. This conclusion is based on the results of clonal diversity in patches of different sizes on the older site. Comparing the clonal diversities of patches in differently aged sites is more difficult, since it is uncertain how fast patches grow. It is not known which patch size on the younger site should be compared with which patch size on the older site if the clonal diversity over time is to be investigated. However, simple predictions can be made. Recapitulate that patches grow in size over time and that the older site exhibits a higher clonal diversity than the younger site in general. The difference between clonal diversities over time should then be larger than the difference found when comparing differently sized patches within a site. Since smaller sized patches have less clonal diversity on the younger than on the older site, comparing these to larger sized patches on the older site will result in larger differences in clonal diversity. The result would therefore be even more pronounced, showing a rapid increase in clonal diversity followed by a slow decline. A rapid increase in clonal diversity followed by a steady decline, has also been found by Travis *et al.* (2002; 2004) and Travis and Hester (2005) and is typical for an ISR strategist. Unfortunately, the younger site lacked large patches, disabling the comparison of clonal diversities in patches that are in the decreasing phase on the younger site. Since ISR and RSR are particularly distinguished by the fate of the clonal diversity directly after the initial establishment phase, the conclusion that *E. atherica* adopted the ISR strategy can only be based on the results from the older site.

The question can be raised what will happen with the clonal diversity over longer time spans, since the decrease in clonal diversity is sharp in respect to the time frame in this study. The chance that a large clone dies due to competition or stochastic mortality can be assumed to be small in comparison to a small clone, because the amount of ramets is likely to be positively correlated to the chance for survival of the genet. Therefore, it seems likely that over time the decrease in clonal diversity will level off. Ultimately, a few, but very extensive genets will cover the area that has initially been covered by many smaller genets. This has also been found in *Spartina alterniflora* (Travis & Hester 2005) and *Prunus ssiori* (Nagamitsu *et al. 2004*).

In order to understand the growth strategy of *E. atherica*, patch size, clonal diversity and possibly influencing abiotic and biotic factors should be synthesised into a concept life history of the species.

Three developmental phases can be distinguished in growing patches:

1) During the first phase, initial seedling recruitment takes place at an uncolonised site. The clonal diversity increases sharply since every new

ramet is a separate genet. New seedlings or regions where ramets are not clonally related are in the first developmental phase.

2) During the second phase, some clonal growth takes place, thereby reducing the clonal diversity. Young genets do not flower during the first years, but do form rhizomes already during the first year (Bockelmann 2002). However, patches trap seeds. Since these small patches have a very low ramet density, the probability that a seedling establishes in the patch is very high. This increases the clonal diversity. The balance between vegetative and sexual reproduction will result in an increasing clonal diversity, albeit that this increase decreases as patches grow older. Patches with sizes up to approximately 10 m^2 and with low to intermediate densities of ramets are in the second phase of patch development.

3) During the third phase, the density of patches has increased, thereby decreasing the probability for trapped seeds to establish in the patch; the present ramets possibly shade the soil too much to allow seedling establishment. At the same time, intraspecific competition and stochastic mortality result in genet loss which reduces the clonal diversity. Patches larger than approximately 10 m^2 and with high ramet densities are in the third phase of patch development.

Figure 16 illustrates these phases on the basis of the clonal diversity data. Hypothetical lines have been drawn that show the initial seedling recruitment and the steady decrease in clonal diversity over time. This development in clonal diversity is consistent with the ISR strategy.

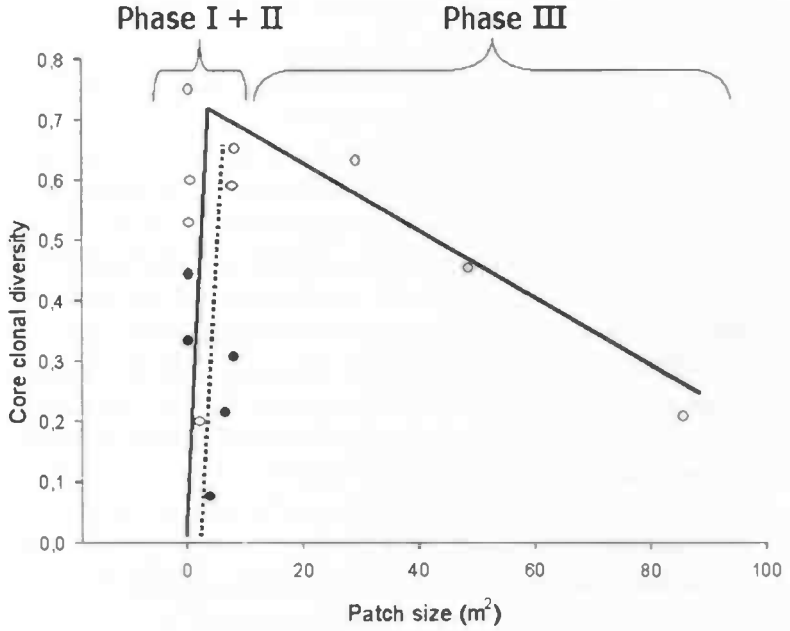

Figure 16. Relationship between core clonal diversity and patch size. Patch size is assumed to correlate with patch age. Open circles represent values for patches from the older salt-marsh site; filled circles represent values for patches of the younger salt-marsh site. The dashed line indicates the clonal diversity over time on the younger site; the solid line indicates the clonal diversity over time on the older site.

Biotic and abiotic factors influencing seedling establishment

The older salt-marsh site showed an overall higher clonal diversity than the younger salt-marsh site. This may be caused by the fact that herbivory is lower on the older site than on the younger site, as a significantly higher grazing pressure on *E. atherica* seedlings on a 25 year old site compared to a 35 year old site has been measured (Kuijper 2004). However, herbivory explained only 1.8% of the variance he found in his study which also involved competition by other plant species. Nevertheless, herbivory can be the major factor giving rise to the differences in clonal diversity found in the present study.

Another reason that the clonal diversity is higher on the older than on the younger site could be that elevation is higher and the clay layer thicker on the older than on the younger site (Van Wijnen & Bakker 1997; Olff *et al.* 1997). A higher elevation causes a decrease in the amount of inundations which is beneficial for establishing seedlings. A thicker clay layer is correlated with a higher amount of nitrogen in the soil, which is beneficial for *E. atherica* as well as for many other salt-marsh species (Leendertse *et al.* 1997 and references therein). Both the elevation and the nitrogen content of the soil are limiting factors in succession of salt marshes.

Another explanation for the change in the ratio of sexual and vegetative reproduction towards more vegetative reproduction over time could be that clonal plants itself changes the contribution to both modes of reproduction (Kanno & Seiwa 2004; Kudoh *et al.* 1999). However, this seems unlikely in *E. atherica* since young seedlings do not set seed during the first years and because the first developmental phase of colonisation is highly dependent on seed dispersal from other sites (Bockelmann 2002). This is the opposite of what would be expected if *E. atherica* influences its own seed production.

Concluding, *E. atherica* grows by means of both sexual and vegetative recruitment. It relies on sexual recruitment during the initial phases of colonisation but over time vegetative recruitment will take over and clonal diversity will decrease due to competition and/or stochastic mortality. Sexual reproduction involves pollen which can disperse very far as to result in absence or very weak relatedness.

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Appendix 1.

DNA extraction from dried plant tissue using CTAB

Materials:

Liquid nitrogen
CTAB extraction buffer
Chloroform-isoamylalcohol (24:1 vol:vol)
Isopropanol (-20°C)
Ethanol 70% (4°C)
0.1xTE-buffer (1 mM Tris-HCl pH 8.0; 0.1 mM EDTA pH 8.0)

CTAB extraction buffer contains per 100 mL:

CTAB	2 gr (2%)
NaCl	8.2 gr (0.14 M)
EDTA	4 mL of 0.5 M solution (pH 8.0)
Tris-HCl	10 mL of 1 M solution (pH 8.0)
Polyvinylpyrrolidon (PVP)	0.4 gr (1%)
MilliQ	Add up to 100 mL

Solution does not need to be autoclaved. Can be stored at RT for a few weeks.

DNA extraction:

- Put 0.5 cm^2 dried plant tissue, clipped into little pieces, into a 1.5 mL Eppendorf tube. Close the tube and put it into liquid nitrogen for at least 2 minutes. Open the tube carefully and crush the pieces immediately with a metal mortar until the material is powderized. The mortar should be put in 70% ethanol and dried with a Kimwipe tissue.
- Add 1 µL β-mercaptoethanol per 1 mL CTAB extraction buffer.
- Add 0.75 mL CTAB extraction buffer per tube and mix the tubes briefly on the vortex.
- Place the tubes in a 60°C shaker for 20 minutes.
- Place the tubes on a rocking table for 45 minutes at room temperature.
- Add per tube 0.75 mL chloroform-isoamylalcohol and mix 1 minute by hand.
- Centrifuge the tubes at 10000 rpm for 5 minutes.
- Bring the waterphase (upper layer) to a new 1.5 mL tube using a pipette.
- Repeat steps 6-8 once more.
- Add two-third of the waterphase volume (400 µL) of ice-cold isopropanol and keep the tubes at 4°C for 30 minutes.
- Centrifuge the tubes at 10000 rpm for 12 minutes. A pellet will possibly form.
- Remove the isopropanol carefully; keep the pellet in sight. Dry the tubes upside down on tissue.
- Add per tube 500 µL cold ethanol 70% and keep the tubes at 4°C for 10 minutes.
- Centrifuge the pellet at 10000 rpm for 3 minutes and remove the ethanol.
- Dry the pellet using a vacuum dryer.
- Dissolve the pellet in 50 µL 0.1TE-buffer for 1 hour at room temperature.
- Store the tubes at -20°C.

Appendix 2.

PCR protocol

1 µL DNA is mixed with 9 µL mastermix

Mastermix (per sample)

	Concentration added	Volume (L)
PCR buffer	10x	1.00
dNTP	2 mM	1.00
Forward primer	5 µM	1.00
Reverse primer	5 µM	1.00
BSA	10% (2 g/L)	1.00
Taq-polymerase	10 U/µL	0.10
MilliQ	-	3.90

PCR programs

	ECGA22 / ECGA89		WMS2 / WMS6		WMS44	
	T	time	T	time	T	time
Hot start	94°C	3:00	94°C	3:00	94°C	3:00
Denaturation	94°C	1:00	94°C	1:00	94°C	1:00
Annealing	55°C	1:00	50°C	1:00	65°C	1:00
Extension	72°C	1:00	72°C	1:00	72°C	1:00
Cycles	10 cycles		10 cycles		10 cycles	
Denaturation	94°C	0:30	94°C	0:30	94°C	0:30
Annealing	55°C	1:00	50°C	1:00	65°C	1:00
Extension	72°C	1:00	72°C	1:00	72°C	1:00
Cycles	28 cycles		28 cycles		16 cycles	
Final extension	72°C	20:00	72°C	20:00	72°C	20:00
Stop	4°C		4°C		4°C	

Appendix 3.

Electrophoresis on ABI PRISM™ 377

Preparation of genescan gel:

- Always wear gloves.
- Get a tube with 250 µL APS out of the freezer to thaw.
- Pull frame in holder towards you and place the first glassplate (36 cm).
- Wet spacers on one side with MilliQ and place them on the edges of the glassplate with the wetted side down.
- Place the second glassplate on top, close the clamps and put the overflow-catcher under the glassplates.
- Put 9.0 g urea in a 25 mL cylinder.
- Add 2.81 mL accugek (29:1).
- Add MilliQ up to 22.5 mL, seal the cylinder with parafilm and shake while holding the cylinder firmly to take up heat until all urea is dissolved. MilliQ can be added if the level dropped below 22.5 mL.
- Transport solution to a beaker. In case two gelruns are performed on one gel, add 0.25 g resin to the solution, shake and filter it.
- Add 2.5 mL 10x TBE.
- Add 250 µL 10% APS and homogenize.
- Add 5 µL TEMED and homogenize again.
- Immediately take up the solution into a syringe without a needle.
- Wet the upper part of the glassplates with the solution and slowly inject the rest of the solution in the middle between the glassplates while knocking on the frontline.
- Insert the comb (48 slots) with its teeth outwards and install the upper clamp.
- Allow the gel to polymerize for 2 hours.
- Remove the upper clamp and the comb.
- Clean the comb with demi and dry it with a tissue.
- Remove all gel material that is present outside the glassplates by using tissues wetted with demi and dry tissues.
- Put the comb with its teeth into the gel with approximately 1 mm of the teeth above the glassplates and 1 mm of the teeth into the gel.
- Put the upper buffer chamber on top and close all clamps again.

Platecheck and PreRun:

- Turn on the ABI-377 and the computer. Open the door, install the lower buffer chamber, install the gelsystem and close the door.
- Open ABI Prism™ 377 Collection, go to File/New/GenScan™ Run and start platecheck D.
- In the meantime, prepare 1.4 L of 1x TBE buffer by diluting 140 mL of 10x TBE buffer with demi.
- Check whether the signal of the platecheck is horizontal and whether there are any big peaks visible. Non-horizontal signals may be caused by a wrongly inserted gelsystem. Small peaks normally disappear rapidly, bigger distortions

may be caused by a dirty glassplate or air bubbles in the gel. Terminate the platecheck.

- Open the door and pour the prepared buffer in the buffer chambers.
- Install the heat transfer plate and go to Window/Manual Control and turn on the pump to see if there is any leakage in the pump system.
- Rinse the lanes and put the lid on the upper buffer chamber.
- Close the door and start PreRun GS PR 36D 1200.
- Go to Mual Control again and put the laser standby. Check the Status window for sufficient time and proper functions. PreRun at least for 15 minutes.
- Go to File/New/Genescan™ Sample and fill in all sample names for the right lane numbers. Activate the dyes used and close and save the file.

Preparing the samples:

- Mix the PCR products.
- Rox-mix = Rox 350 : loadingbuffer : formamid = 1 : 1.5 : 5
- Add 1.5 µL Rox-mix per well in 48 wells on a 96-well plate.
- Add 1 µL of PCR product mix per well.
- Spin the plate for 15 seconds at 2000 rpm.
- Denaturise the samples for 2 minutes at 95°C. Put the samples on ice in the dark immediately after denaturisation.

Run:

- Terminate the PreRun and open the door.
- Rinse the lanes and load the first row of samples using an 8-tip pipette and between 1 and 1.5 µL of sample.
- Open the sample sheet in the run folder, select Matrix D 36-2400, enter your name and the amount of lanes used.
- Press Run and save the Gel File.
- Check the Status window for sufficient time and proper functions. After 2 minutes, pause the run and load the second row of samples.
- Press Resume and wait 2 minutes.
- Press Pause and load the third row of samples.
- Press Resume and open the Gel Image window. When the marker larger than the bands of interest is revealed, terminate the run.
- When loading a second set of samples on the same gel, close all windows and choose File/New/GenScan™ Run. Prepare a new set of samples and make a new sample sheet. Restart the procedure as described after the PreRun has finished.

Cleaning:

- Shut down the machine and open the door.
- Remove the front heating plate and clean it with demi and tissue.
- Close the clamps again and remove the lid from the upper buffer chamber.
- Remove the gelsystem and pour the buffer in the sink.
- Remove the lower buffer chamber and pour the buffer in the sink.
- Clean the white heating plate on the ABI-377 with a wet and a dry tissue.

- Rinse the buffer chambers and the frame with demi.
- Separate the plates and remove and clean the spacers with demi.
- Remove the gel material by rolling it on tissue.
- Clean the glassplates with alconix and demi thoroughly and place them in a rack to dry.

Appendix 4.

Filter criteria for peak scoring in Genotyper

ECGA 22 - green

Peak name	Select	Within (bp)	Min. height
Unknown	All peaks	100.00 – 180.00	50
126	Highest peak	126.10 – 127.10	50
128	Highest peak	127.60 – 128.60	50
130	Highest peak	129.70 – 130.70	50
152	All peaks	152.00 – 152.70	50
154	Highest peak	153.60 – 154.60	50
156	Highest peak	155.65 – 156.65	50

ECGA 89 – green

Peak name	Select	Within (bp)	Min. height
Unknown	All peaks	180.00 – 250.00	50
196	All peaks	195.00 – 196.00	50
198	All peaks	197.00 – 198.00	50
200	All peaks	200.00 – 201.00	50
206	All peaks	206.00 – 207.00	50

WMS 2 - blue

Peak name	Select	Within (bp)	Min. height
Unknown	All peaks	170.00 – 300.00	50
185	Highest peak	183.50 – 186.25	50
266	Highest peak	262.25 – 268.25	50
283	Highest peak	279.50 – 285.50	50
296	Highest peak	293.00 – 298.00	25

WMS 44 - blue

Peak name	Select	Within (bp)	Min. height
Unknown	All peaks	85.00 – 180.00	50
116	Highest peak	115.00 – 116.00	50
138	Highest peak	138.00 – 139.00	50
140	Highest peak	139.25 – 140.25	50
142	Highest peak	141.60 – 142.60	50
144	Highest peak	143.70 – 144.70	50

WMS 6 – yellow

Peak name	Select	Within (bp)	Min. height
Unknown	All peaks	130.00 – 175.00	50
140	Highest peak	138.30 – 140.20	50
142	Highest peak	140.60 – 142.40	50
149	Highest peak	147.20 – 149.05	50

151	Highest peak	149.30 – 151.15	50
153	Highest peak	151.30 – 153.05	50
159	Highest peak	157.00 – 159.00	50
161	Highest peak	159.00 – 160.70	50
165	Highest peak	163.00 – 165.00	50

Unknown peaks were checked for uncommon alleles. A total of 15 additional alleles were scored.

Uncommon alleles

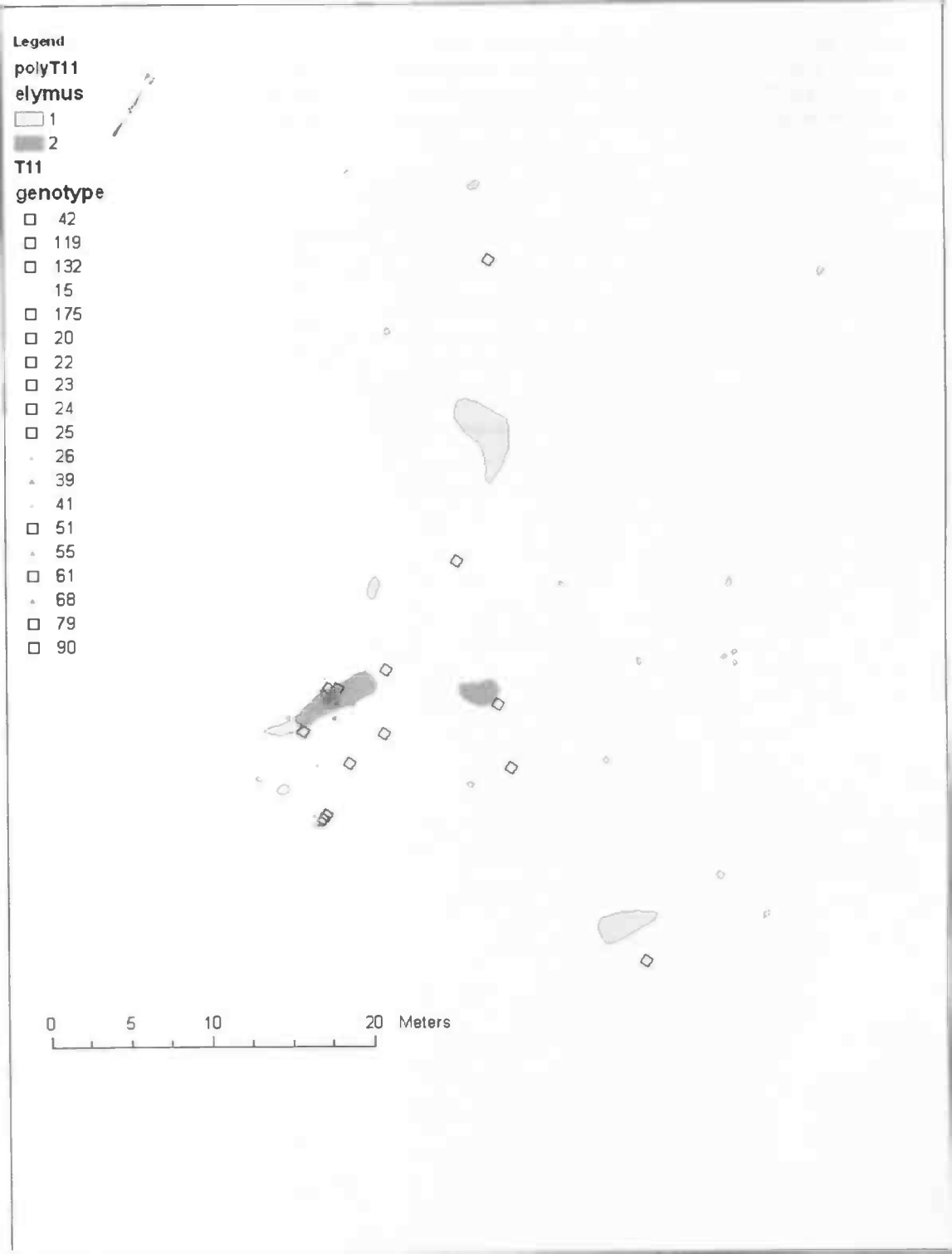
ECGA 22	ECGA 89	WMS 2	WMS 44	WMS 6
172	197	180	114	163
	199	200	115	
	207	248	127	
		262	145	
		285	149	

Appendix 5.

General information about sample sizes and clonal diversities

The younger and older salt-marsh sites are denoted as 25 and 35, respectively. The next digit indicates the number of the block. The letter denotes a small (S), intermediate (M) or large (L) patch. T is explained in the main text. The clonal diversity is calculated as the number of genets divided by the number of samples. Note that here all genets of each patch are used for the calculation instead of only the genets present in the core area.

Patch	Size	Cover estimate	No. of samples	Number of clones	Clonal diversity
25-1S	0.364	<20%	9	4	0.44
25-1M	8.160	20-50%	25	11	0.44
25-2T	-	-	15	15	1.00
25-2M	6.670	<20%	31	11	0.35
25-3S	0.296	<20%	9	3	0.33
25-3M	3.977	<20%	19	1	0.05
35-1S	0.375	<20%	4	3	0.75
35-1M	8.299	20-50%	34	20	0.59
35-1L	29.130	20-50%	37	16	0.43
35-2S	0.482	<20%	20	11	0.55
35-2M	7.935	20-50%	35	19	0.54
35-2L	48.438	>50%	30	14	0.47
35-3S	0.688	<20%	10	6	0.60
35-3M	2.285	20-50%	33	6	0.18
35-3L	85.495	>50%	59	11	0.19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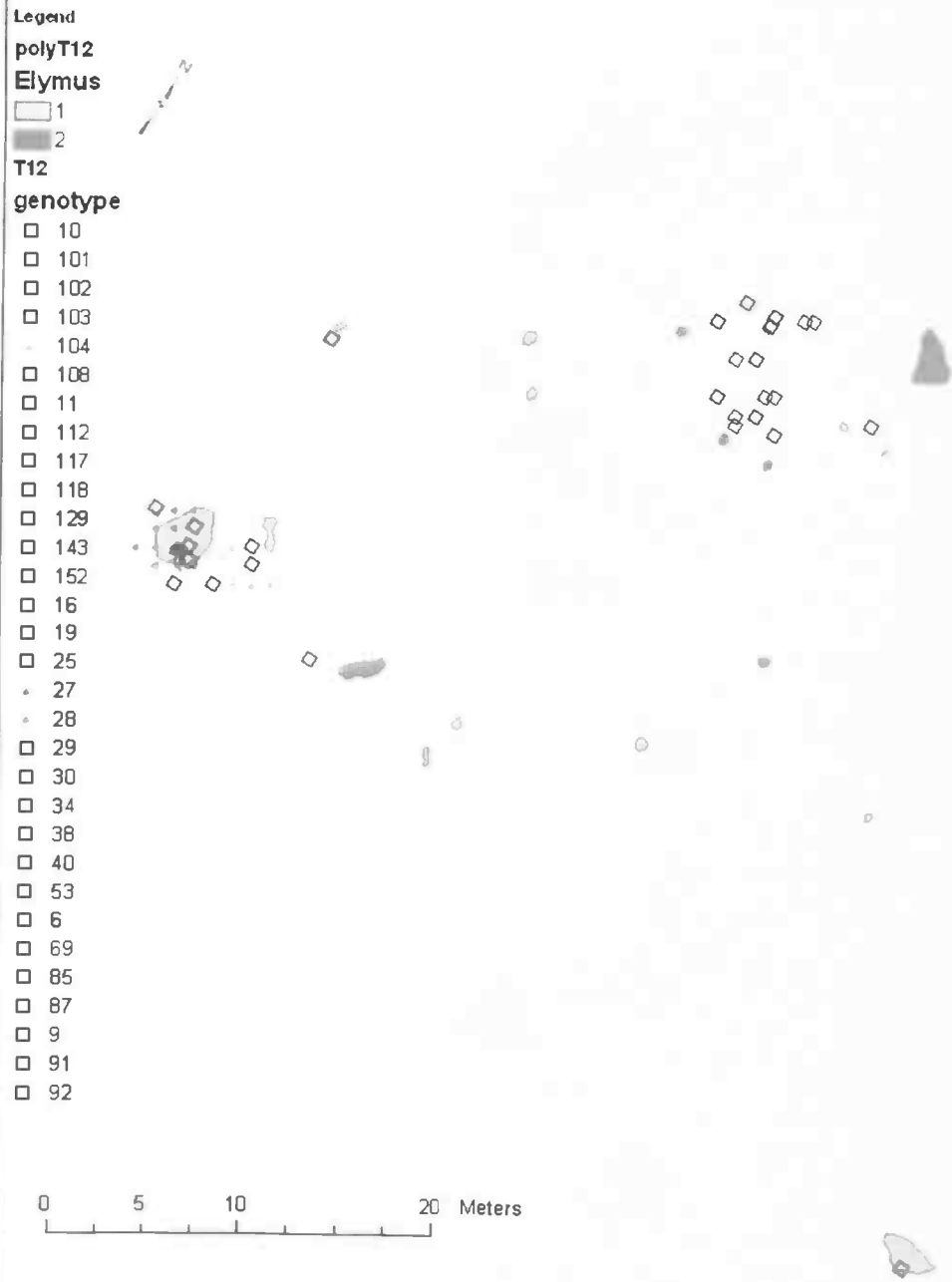
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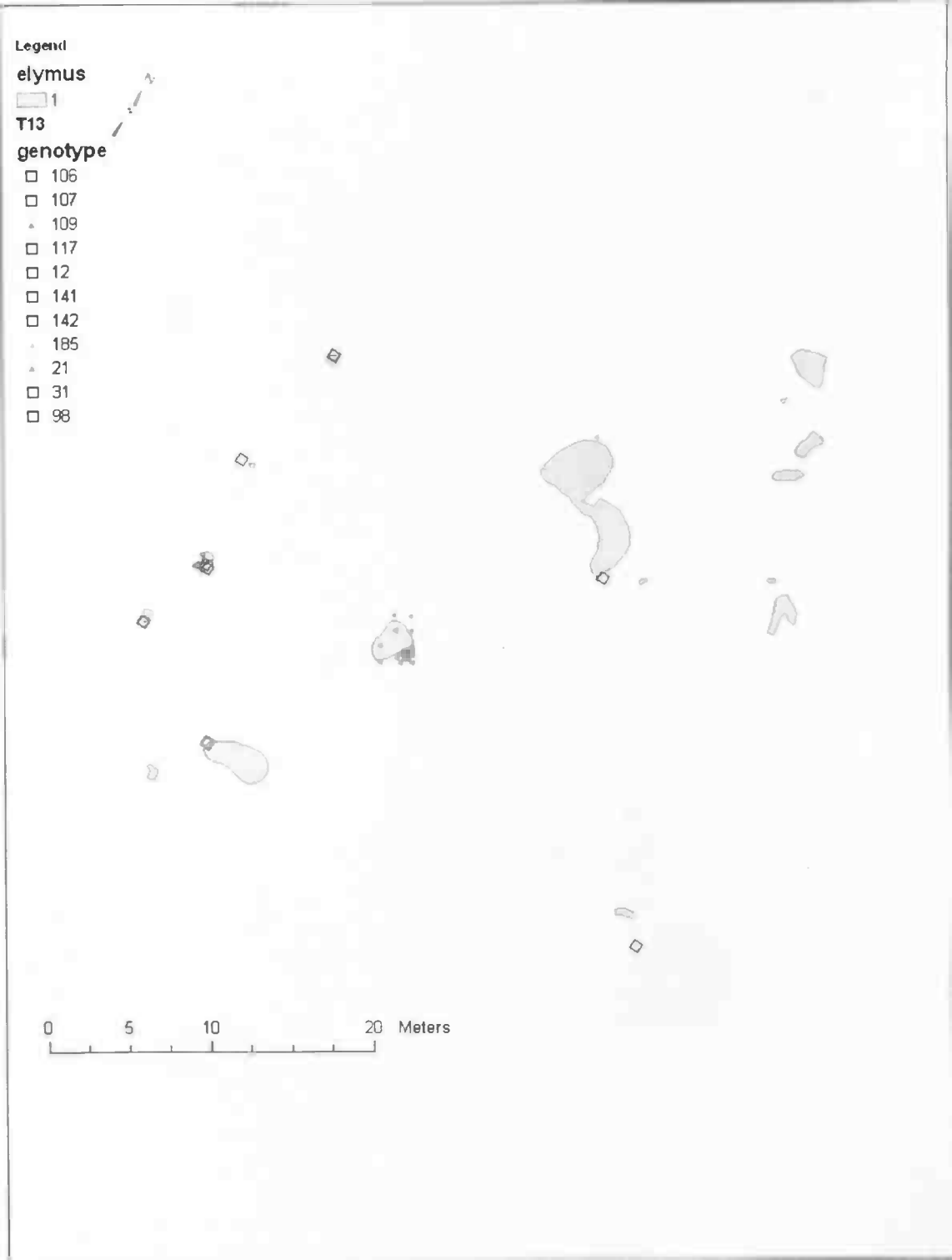
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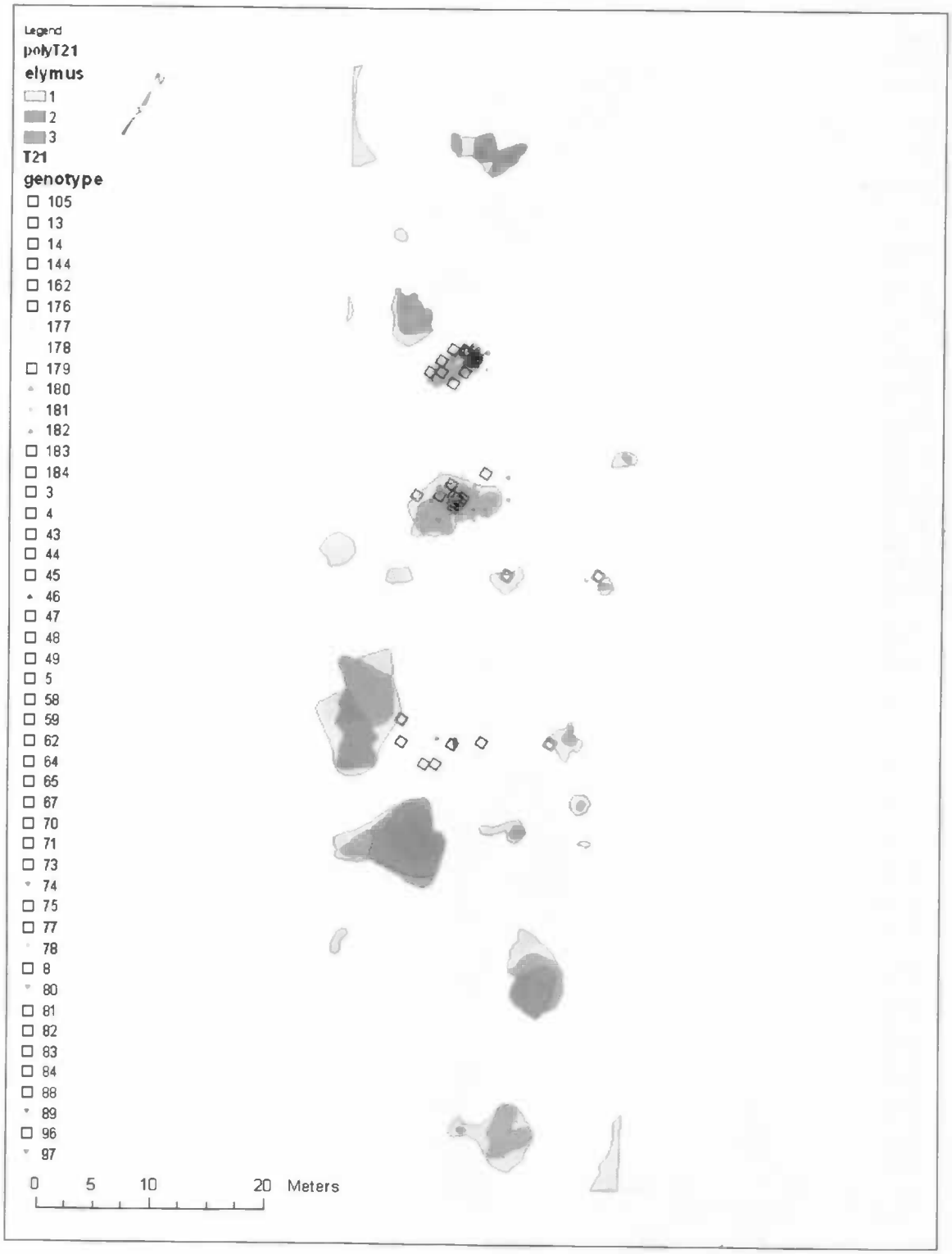
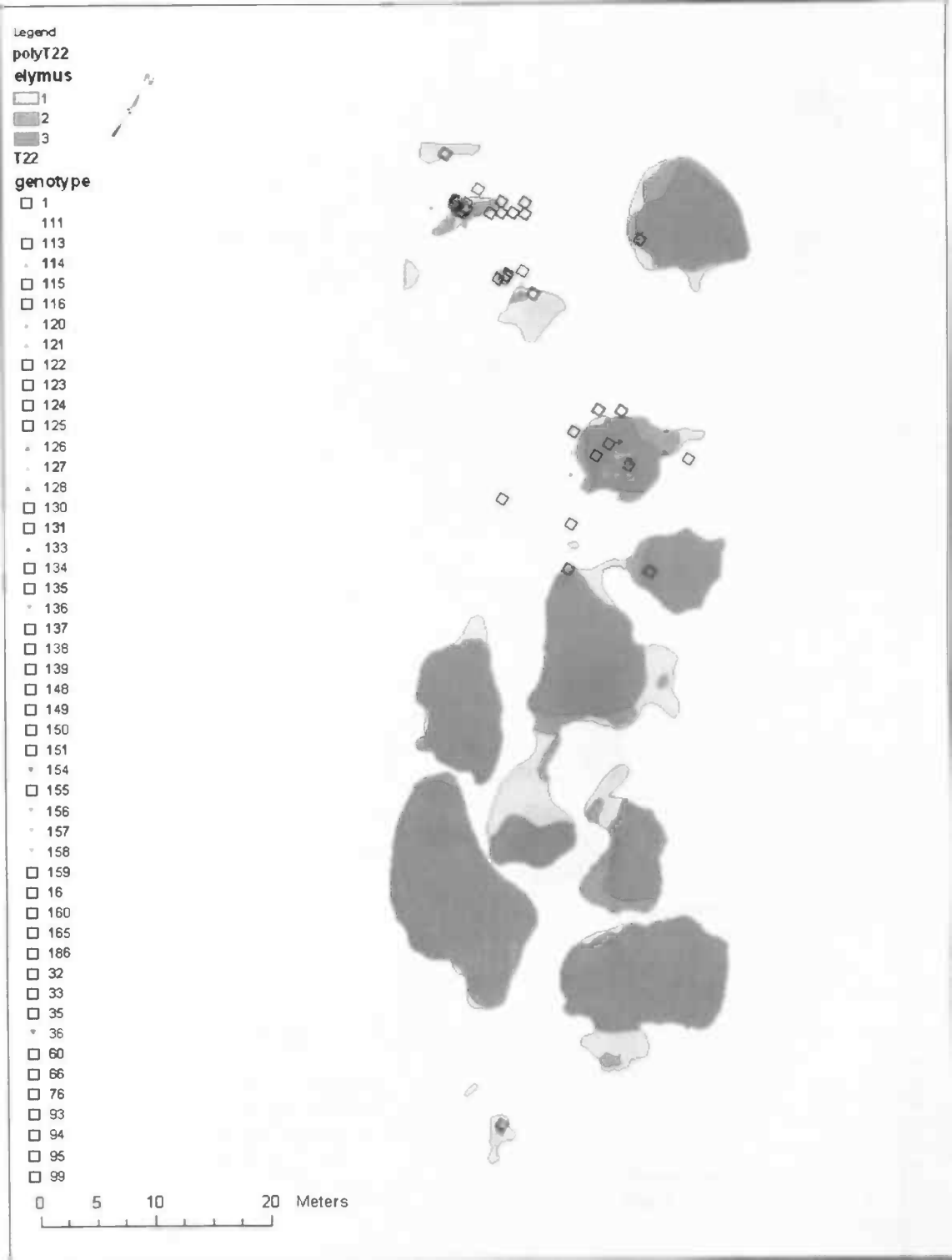
Meters

Appendix 6.d

Clone map of the first block on the older salt marsh site

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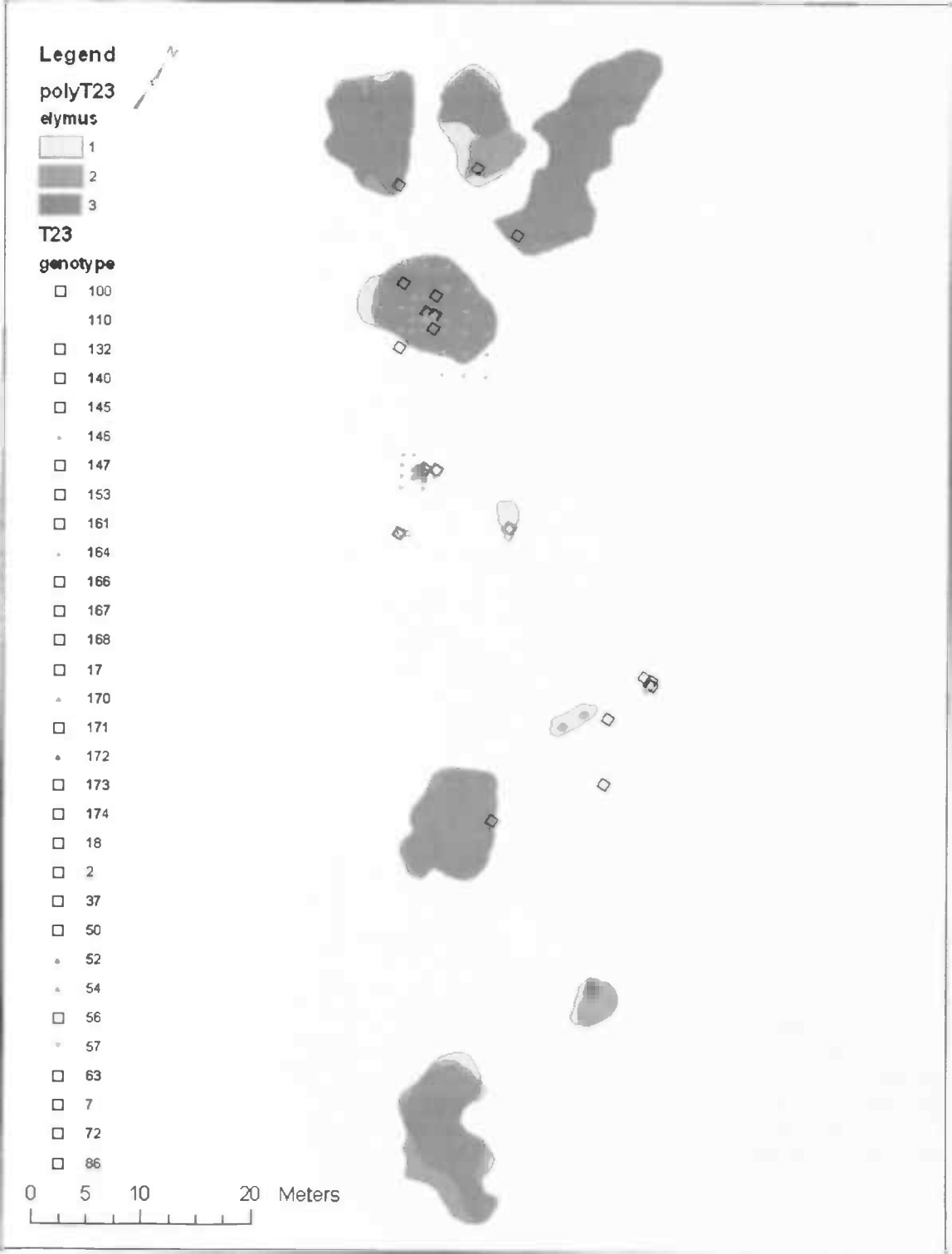
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167
168
17
170
171
172
173
174
18
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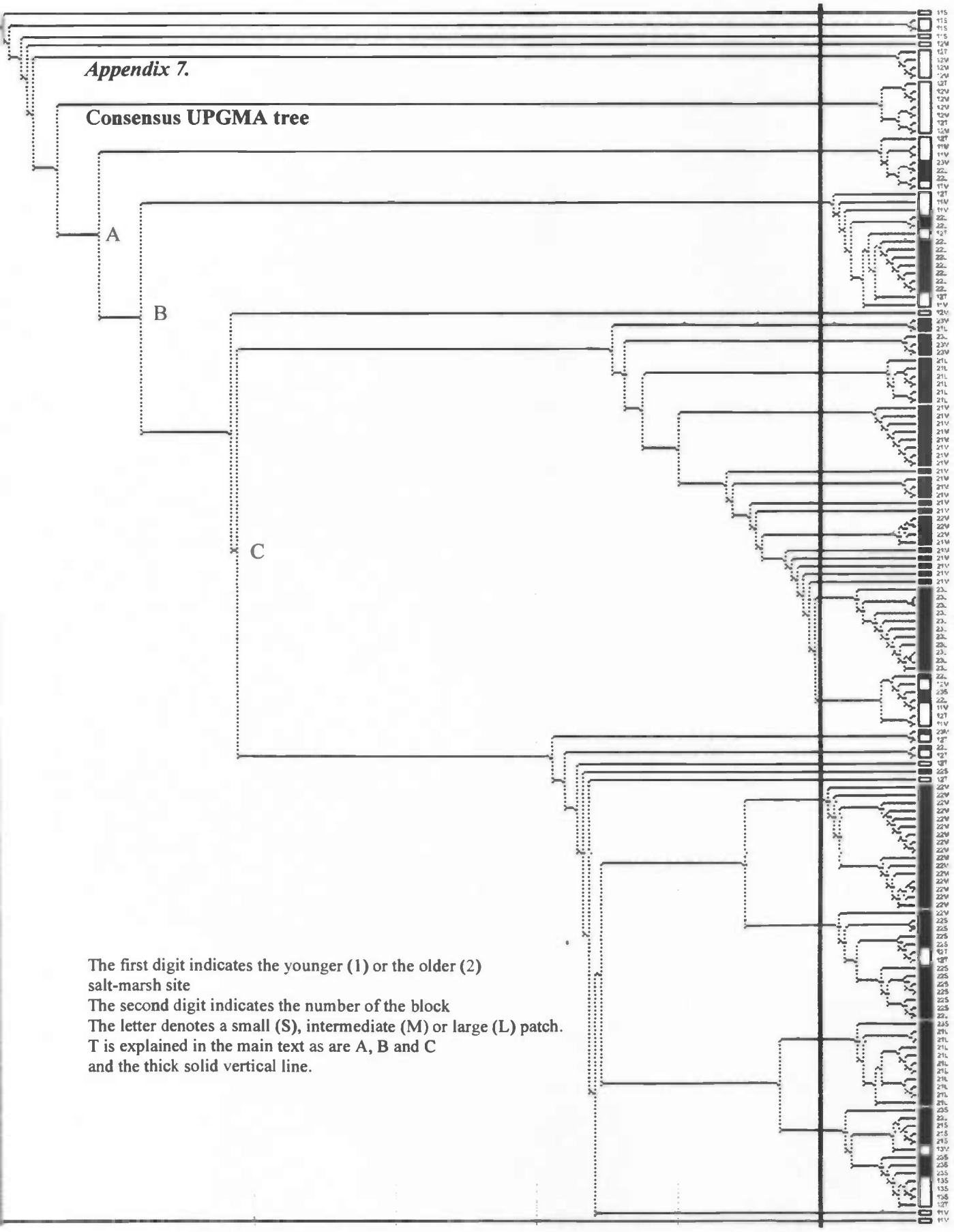
10
20
Meters

Appendix 7.

Consensus UPGMA tree



The first digit indicates the younger (1) or the older (2) salt-marsh site
The second digit indicates the number of the block
The letter denotes a small (S), intermediate (M) or large (L) patch.
T is explained in the main text as are A, B and C and the thick solid vertical line.