Spatial genetic structure in the clonal marine angiosperm *Cymodocea nodosa*: the influence of dispersal potential, mating system and species interactions

Thesis

Spatial Genetic Structure in the clonal marine angiosperm *Cymodocea nodosa*: the influence of dispersal potential, mating system and species interactions.

Maria Valeria Ruggiero

Laurea in Scienze Biologiche

Università degli Studi di Napoli "Federico II"

Italia

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Thus have I concisely given the result of my thoughts; and my verdict is that Being and Bpace and Beneration, these three, existed in their three ways before the heaven.

Plato - Timaeus

ABSTRACT

In the present thesis, the factors influencing population's genetic structure in the clonal marine angiosperm *Cymodocea nodosa* have been investigated.

C. nodosa is a dioecious seagrass, exhibiting both vegetative propagation and sexual reproduction. Seed dispersal is expected to be extremely restricted.

I selected seven microsatellite loci through genomic library screening to investigate the relative effects of sexual and clonal reproduction on the genetic diversity and structure in a *Cymodocea nodosa* population from the Gulf of Naples (Italy). High clonal diversity and genet density were found. Autocorrelation analyses confirmed the expectations of very restricted seed dispersal (observed dispersal range 1-21m) in this species.

The effect of mating system on genetic structure were investigated comparing the clonal architectures of the dioecious *Cymodocea nodosa* and monoecious *Zostera noltii.* An intermingled configuration of genets has been found in the dioecious *Cymodocea nodosa* while a clumped distribution of clones in the hermaphroditic *Zostera noltii* has been observed. I hypothesise that the possibility of reduction in the seed-set would drive genet distribution.

On a phylogeographic spatial scale, the existence of population differentiation and of genetic disjunction within the Mediterranean Sea was investigated in *Cymodocea nodosa*. Populations displayed a wide variability in clonal diversity. A Bayesian analysis revealed that "supra-population" panmictic units are present in the Mediterranean basin. Genetic substructure from a phylogeographic tree coincided with major geographical boundaries within the Mediterranean basin.

In general, in *Cymodocea nodosa*, seed dispersal is poor at the within-population level, but long-range dispersal events can occur, allowing high levels of gene flow at a phylogeographic scale. The observed "guerrilla" clonal architecture allows to reduce the effect of genetic identity on the genetic structure of the population, but it is also

advantageous by allowing pollen availability and therefore a sufficient seed-set in this dioecious species.

SUMMARY

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CHAPTER I – General Introduction

1.1 - Genetic structure and diversity in clonal plants

Genetic diversity is spatially structured. This spatial distribution is a product of environmental influences, including life history traits and demographic past history of the plant species (Loveless and Hamrick 1984; Slatkin 1985). Knowledge of how genetic diversity is spatially structured is therefore of primary importance to infer these causal factors and also the underlying genetic processes such as differential selective pressures, gene flow and drift (Barbujani 1987; Epperson 1993).

The assumption that less genetic diversity should be found in clonal as opposed to non-clonal plants, due to the rarity of sexual reproduction in the former has been prevailing for long time (Widén *et al.* 1994). However, an increasing number of studies based on molecular markers questioned this view, showing similar levels of genetic diversity among clonal and non-clonal plants (Ellstrand & Rose 1987; Hamrick & Godt; 1989; Widén *et al.* 1994).

In the following paragraphs, I will outline how the differential contribution of sexual and vegetative reproduction affects the genetic diversity and structure in clonal plant populations and how the two modes of reproduction interact and affect each other.

1.1.1 - Genetic and genotypic diversity

Sexual reproduction features can affect genetic diversity of populations in different ways: i) the mating system - which describes how male and female gametes unite and transmit hereditary information to the progeny - affects the levels of intra-individual variability (heterozygosity). If predominantly related individuals mate, then

the relative levels of outcrossing/inbreeding are affected; ii) the success rate of sexual reproduction, predominantly controlled by sexual investment, seed production and seedling recruitment rates, will affect the genotypic variation of the populations.

(i) Plant mating systems are diverse, ranging from self-incompatibility in obligate outcrossing, dioecious plant species to self-compatibility in hermaphroditic plants. These differences result in different levels of mating among genetically related individuals, or inbreeding, with profound effects on the genetic variability of populations (Fig. 1.1). Increasing levels of inbreeding lead to lower levels of heterozygosity, so that in the extreme case of self-compatible hermaphroditic species and in the absence of mechanisms to avoid self-fertilization, inbreeding can lead to severe losses of genetic diversity. On the other hand, the costs of inbreeding are generally reduced in populations with previous history of related mating because the genetic load has already been purged (Charlesworth 2003).

In self-compatible clonal plants, a special case of inbreeding arises from fertilization between different flowers belonging to same genetic individuals. Such phenomenon is generally called "geitonogamy" (Handel 1985; Eckert 2000; Reusch 2001a). In dioecious species, only biparental inbreeding is possible, but also in this case, the genetic diversity of the populations can be severely affected by high rates of reproduction between related individuals.

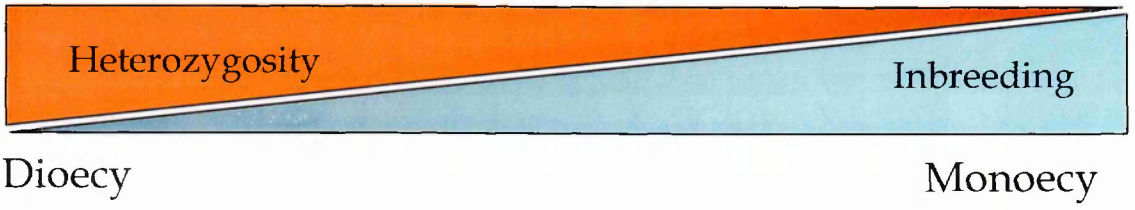

Fig. 1.1: Effect of the mating system on the heterozygosity of the population. From dioecy to self-compatible monoecy, an increase in inbreeding levels with a consequent decrease in heterozygosity occurs.

It is also known from many studies on plant populations (reviewed in Charlesworth 2003) that inbreeders are generally characterised by lower genetic diversity and larger between-populations differentiation than outcrossing species.

(ii) Watkinson & Powell (1993) showed in an influential paper using mathematical models, that the ratio of seedling (sexual) to ramet (vegetative) recruitment is fundamental for the clonal structure of populations. Interestingly, even low levels of sexual recruitment are capable to maintain high genetic diversity in plant populations. Eriksson (1993) conceptualized sexual recruitment strategies into two extreme cases at both ends of a continuum. Under Initial Seedling Recruitment (ISR), seeds disperse far from the original population, and new populations, resulting from seed dispersal, are expected to reflect the initial colonizing cohort. Such a strategy will avoid strong intra-specific competition that is often responsible for marked suppression of recruitment after the initial colonization (Eriksson 1993; Cheplick 1992). Under Repeated Seedling Recruitment (RSR) seedlings are recruited within the original populations. Consequently populations consist of a mixture of genotypes that represent different cohorts; it is believed that small-scale disturbances promote this kind of within-population recruitment (Eriksson 1993). The two different strategies are expected to result in different spatial patterns in the population. In the case of ISR, populations should consist of few large clones, while in the RSR a pattern with many small clones should be produced. The clonal architecture of a population can thus provide hints on the type of sexual recruitment, when ecological/demographic data are lacking. One has to take nonetheless into consideration that, as every classification in biology, differentiation between ISR and RSR is not discrete, but must be considered as a gradient of different states.

1.1.2 - Genetic structure in clonal plants

Spatial genetic structure is classically defined as "the non-random distribution of alleles in space or time" (Loveless & Hamrick 1984; Epperson 2000; Vekemans & Hardy 2004). Structure can arise at both the within-population and the between-population level. Although clines or patchiness of selectively relevant genes or markers may result from selective pressures in heterogeneous environments (Hedrick 1986; Linhart & Grant 1996; Bockelmann *et al.* 2003), in clonal plant populations it mainly derives from two different sources: i) a restricted dispersal of sexual products, leading to the formation of local family structures and ii) the asexual replication of genotypes through vegetative growth, so that genetic structure is driven by genetic identity.

(i) Highly leptokurtic seed dispersal curves (Ouborg *et al.* 1999; Cain *et al.* 2000) are typical of most plant species. Plants stand still – but their genes do not: given their limited mobility, spatial genetic structure is expected to occur frequently within plant populations. In this context, pairwise genetic relatedness among individuals decreases with increasing geographic distance, a process that was dubbed 'isolation-by-distance' (Wright 1943). The relative contribution of seed and pollen movement to overall within-population gene flow influences kinship structure and inbreeding levels. Various scenarios arise from the different combinations of the two modes of dispersal (Fig. 1.2). When seed and pollen dispersals are poor, mating by proximity generates genetic structure (Epperson 2000), increasing local levels of inbreeding. In the case of a higher dispersal of pollen than seed, genetic structure can be present but inbreeding is avoided. Pollen dispersal has thus the general function of

flattening the negative effects of genetic structure on inbreeding levels (Loveless & Hamrick 1984; Kalisz *et al.* 2001).

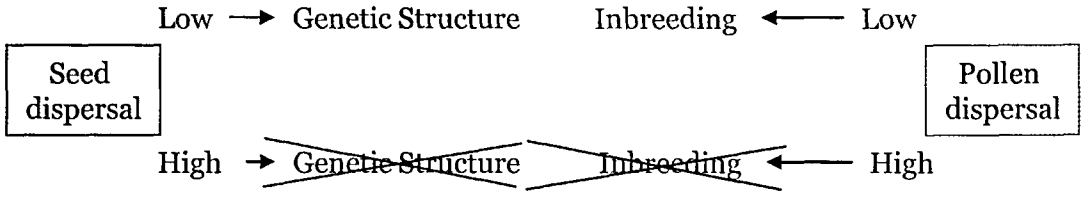

Fig. 1.2: Relative effects of pollen and seed dispersal on genetic structure and inbreeding of populations.

(ii) The clonal growth in plants results in different organizational levels: the ramet (*sensu* Harper 1977), represents the potentially independent physiological unit; depending on the longevity of rhizome connections between ramets, several ramets can form physiologically integrated clusters. The sexual individual, or genet, is formed by all ramets or ramet clusters which originated from the same zygote. From an evolutionary point of view, it is only the genet that matters and that eventually transmits genes to the next generation.

The main effect of clonal growth on the genetic structure is an "identity effect". In the case of vegetative replication of a genotype, alleles will not be distributed at random, due to allele identity at the spatial scale determined by the extent of clonal expansion and form. Lovett Doust (1981) described two clonal growth forms with markedly different genet architecture (Fig 1.3): in the 'phalanx' type of growth, ramets are connected by short internodes and are closely spaced. Such strategy leads to a mosaic structure in which clones are recognizable as discrete units and most neighbour interactions are intra-clonal; in the 'guerrilla' strategy, internodes are long and ramets are widely dispersed, leading to an intermingled make-up of genets, in which most neighbour interactions are inter-clonal.

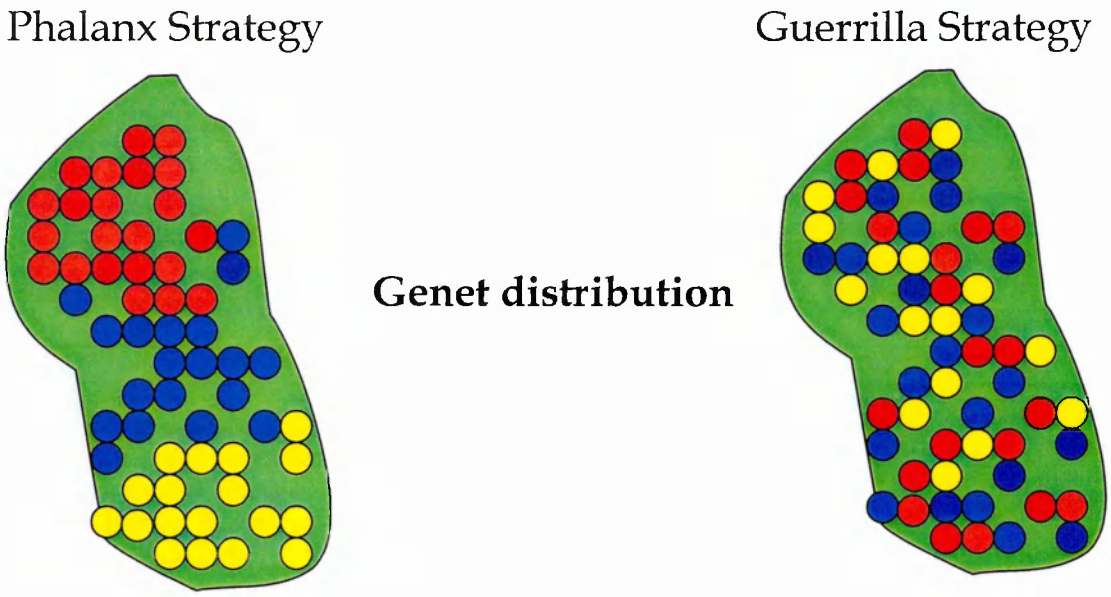

Fig. 1.3: Genet distribution resulting from different clonal growth forms: a phalanx strategy leads to a clumped distribution, a guerrilla strategy leads to an intermingled configuration of clones.

The two different growth forms will result in different genetic structures. When a guerrilla strategy is adopted, genets are highly intermingled and, at a given spatial scale, high genet density can be found; in such a case, the "identity effect" is reduced, accordingly to the level of genets intermingling. On the contrary, in the presence of a phalanx clonal growth, such effect is maximized and the maximal distance at which genetic structure can be found depends on the size of the clones.

The effects of genotype replication on population's genetic structure have been addressed in a number of studies (Montalvo *et al.* 1997; Reusch *et al.* 1998; Chung & Epperson 2000; Hämmerli & Reusch 2003c). Most of these studies approached this topic through autocorrelation analysis (Sokal & Wartenberg 1983). In brief, autocorrelation techniques describe the similarity of a variable, (e.g. alleles or genotypes), as a function of the distance of measurements of these variables in space or time. The autocorrelation coefficient is usually evaluated under the assumption that

the observations are random, independent samples of a population with an unknown distribution function: values which are significantly greater than expected occur when pairs within a distance class have scores more similar than it would be expected if the variable was randomly distributed. Conversely, values significantly lower than expected indicate that scores of the variable are more dissimilar than expected by chance. Results are usually shown as correlograms, graphic displays in which the values of the autocorrelation coefficients are plotted against distance classes, allowing the researcher to exactly identify the scale at which, for example, significant correlation occur. The most used autocorrelation indexes are the joint-count statistics (Congalton 1988), the Moran's I (Moran 1948), the Geary's c (Geary 1954) and the kinship coefficient f_{ij} (Loiselle *et al.* 1995).

1.1.3 - Evolutionary relationships between Mating system and Clonal growth

The interactions between clonal architecture and mating patterns remain largely unexplored, and studies on clonal growth and sexual reproduction have been rarely associated. Clonal architecture has been determined in various clonal plants and algae (Maddox *et al.* 1989; Montalvo *et al.* 1997; Kudoh *et al.* 1999; Pornon & Escaravage 1999; Ivey & Richards 2001; Xie *et al.* 2001; Hangelbroek *et al.* 2002; van der Strate *et al.* 2002; Albert *et al.* 2003). None of these studies, however, has taken into consideration the effects of clonal architecture on the reproductive potential of the population. Clonal growth forms, in fact, can affect size and spatial distribution of genets, interfering with patterns of pollen dispersal and with mating opportunities (Handel 1985; Charpentier 2002). As already cited, Lovett Doust (1981) identified

two main clonal growth strategies: the phalanx type of recruitment leads to a mosaic structure in which genetically identical ramets are clustered together and clones are recognisable as discrete units; the guerrilla type leads to an intermingled make-up of genets. In self-compatible species, a phalanx growth strategy is expected to be advantageous because, although large clonal patches have been predicted to increase selfing through geitonogamy (Eckert 2000), the cost of inbreeding is less important than the cost of a reduced seed set through limitation of compatible pollen. In dioecious species, monoclonal patches are also monosexual and fertilization can only take place from outside, hence outcrossing is obligate. Any possible reduction in the seed set, due to deficit of pollen in the immediate neighbourhood, can be lowered by an intermingled composition of genets (Charpentier 2002). Obligate outbreeding associated with a dioecious mating system should therefore be favoured by a guerrilla growth strategy. These hypotheses are supported by data on clone distribution in species with different levels of outcrossing (Stebbins 1950): on 71 perennial grasses, 93% of guerrilla-growing species were found to be self-incompatible and 77% of phalanx-growing species were found to be self-compatible.

Evolutionary interactions between clonal growth strategies and mating system evolution are still to be clarified. Clonal growth is a complex multi-trait feature affecting survival of individuals and, by determining the spatial distribution of flowering units, may impose selective pressures on traits which regulate mating system. Inversely, clonal growth traits (e.g. rhizomatous growth length) could be driven by their mating system costs (Charpentier 2002).

1.1.4 - Dispersal potential and Phylogeography at Sea

Natural populations are spread across large areas and are connected through occasional migration or gene flow between them. The similarity of these populations depends therefore on the number of migrants exchanged per generation. If there is high migration, the populations approach panmixia; if populations are not exchanging migrants, genetic differentiation increases with time due to mutation, genetic drift and also selection. On long temporal scales, speciation could occur.

The term "phylogeography", originally introduced by Avise *et al.* (1987), can be defined as the relationship between gene genealogies (phylogenetics) and geography. Molecular markers are typically used to infer a phylogeny, or gene tree, which reflects the evolutionary relationships of the individuals and populations sampled. By combining the resulting gene trees with the geographical location from which each individual was sampled, the geographical distributions of major gene lineages that comprise the gene tree can be elucidated. The resulting phylogeographic patterns are then interpreted within the context of evolutionary and biogeographic models. Phylogeography is therefore a powerful approach for investigating a wide range of issues, including the relative roles of gene flow, bottlenecks, population expansion, and vicariant events in shaping geographical patterns of genetic variation.

Several models of gene flow are used to describe the way in which dispersal takes place (Ouborg *et al.* 1999). The most frequently used is the infinite island model (Wright 1978; Slatkin 1985) in which an infinitely large source population sends migrants to a finite set of subpopulations at a constant rate. In the finite island model, migration is equally likely among a set of populations with effective population sizes (Wright 1931). The stepping-stone model (Kimura & Weiss 1964) describes island

migration along a linear set of populations, where each population receives migrants only from neighbouring populations. In continuum models (Wright 1940), the migration rate is a fixed function of distance and, finally, in the migration matrix model, migration rates may be different and are defined for each pair of populations in a migration matrix (Bodmer & Cavalli-Sforza 1968).

At sea, the spatial scale at which phylogeographic structure occurs is often greater than what observed in terrestrial habitats. The continuity and uniformity of marine environment make the potential for genetic exchange theoretically unlimited (Palumbi 1992; 1994). However, various exceptions to this rule have been identified. Genetic pools of the majority of widely distributed species are rarely homogenous from one end to the other of their distribution area (Féral 2002). This evidence could be related to two factors: from the one hand, dispersal of marine organisms is often restricted, limiting the potential for genetic exchange among populations; on the other hand, local adaptation to complex ecosystem zonations could lead to the build up of genetic divergence between populations.

The effect of the dispersal potential can be observed in marine invertebrates, where migration is mediated by the planktonic, vagile larvae and the dispersal potential depends mainly on the duration of the planktonic period (reviewed in Féral 2002). Marine macrophytes are expected to display high levels of population subdivision, due to their very limited seed or spore dispersal potential (Denny & Shibata 1989; Orth 1994). Seed density is known, in fact, to decline leptokurtically with distance, with an extended tail of long-distance dispersal events (Orth *et al.* 1994; Ouborg *et al.* 1999; Cain *et al.* 2000; Nathan *et al.* 2000). While short range dispersal has crucial effects at the within-population level, influencing dynamics and persistence of populations (as

discussed above), long-distance dispersal events act at the between-population level, affecting levels of gene flow, the colonisation of new sites and/or the extinction of local populations (Ouborg *et al.* 1999; Cain et al 2000). Gene flow, however, is not the same as dispersal. Gene flow refers to the movement of genes, which involves both seeds and pollen, whereas dispersal refers to seeds (and propagules) able to establish themselves (Ouborg *et al.*, 1999). It requires therefore also survival to reproduction and contribution of progeny to next generation (Avise 1998; Féral 2002).

Local adaptation could in fact occur, in the presence of complex zonations, caused by marked gradients (temperature, salinity, light, trophic abundance...) which provide a mosaic of different habitats. Local adaptation could therefore have an important role in building up genetic divergence among marine organisms' populations (Neigel 1997), as shown, for example, in transplant studies in seagrasses (Orsini *et al.* 2001; Hämmerli & Reusch 2002).

1.2 - Seagrasses: the plants that discovered the Sea

Seagrasses inhabit coastal shallow ecosystems with sedimentary bottoms worldwide, except for Antarctica (den Hartog 1970; Kuo & McComb 1989; Larkum & den Hartog 1989). Their positive influence on productivity of coastal ecosystems has made this group one of the main focuses of marine biologists worldwide. Seagrasses have the function of providing habitat for a wide variety of economically important species, stabilizing sediments, filtering seawater and removing excess of nutrients. Their ecologic and economic values are among the highest of all ecosystems, even with respect to the terrestrial ones (Costanza *et al.* 1997). In the last years, great concern has developed about the conservation of seagrass ecosystems because of ever growing disturbance levels due to natural and –mainly- anthropic impact (Short & Wyllie-Escheverria 1996; Meffe & Carroll 1997; Green & Short 2003), which are leading to increased meadows regression rates. For example, in France, a disappearance of *Posidonia oceanica* beds between 0 and 20 m has been observed in the last 30 years; in Spain, a comparison of old marine charts with present distribution data in Catalonia indicates that the meadow area is now about 75 percent of that at the beginning of the 20th century (Procaccini *et al.* 2003). The regression of *Posidonia oceanica* in the Mediterranean Sea has been related to a poor adaptive potential of the species, so that the ecological equilibrium of the species is "as fragile as for a relictual species" (Pérès 1985b). Pérès says that "... [The species] resisted to the physical, climatic and ecological catastrophes that marked the history of that Sea. [...]. It will not resist to the growing human populations around the basin and to the growing of their diverse activities".

1.2.1 - Definition and systematics.

In the late Cretaceous (160-45 mya), angiosperms colonized the marine environment (den Hartog 1970), but the passage to the aquatic life occurred in only 2% of the about 350000 angiosperm species, in 50-100 independent events (Cook 1990). The first working definition of "seagrasses" by Arber (1920) was based on the four main features allowing angiosperm to survive in the marine environment:

1. Toleration towards a saline medium
2. The power of vegetating while wholly submerged
3. A sufficiently developed anchoring root system to withstand the wash of waves and tides
4. The capacity for hydrophilous pollination.

This definition reveals its limits when dealing with genera that comprise truly marine species together with brackish waters ones. If "seagrasses" are grouped according to their ability to survive in steno-aline environments, some members of the genera *Ruppia* L., *Lepilaena* Drumm ex Harvey and *Potamogeton* L. should be comprised in the category, while other species of the genera should not. In 1970, den Hartog excluded *Ruppia* and *Lepilaena* from seagrasses.

The number of recognised seagrass species has therefore changed several times during the last 30 years. Kuo & McComb (1989) identified 58 species, excluding brackish water taxa, while more recently, Waycott (1996), defined seagrasses as "those marine tolerant angiosperms that are inhabitants of marine-influenced rather than freshwater environments". Today, about 60-70 species are recognised, since new species (e.g. in the genus *Halophila*, Larkum 1995) have been described after the Kuo & McComb paper.

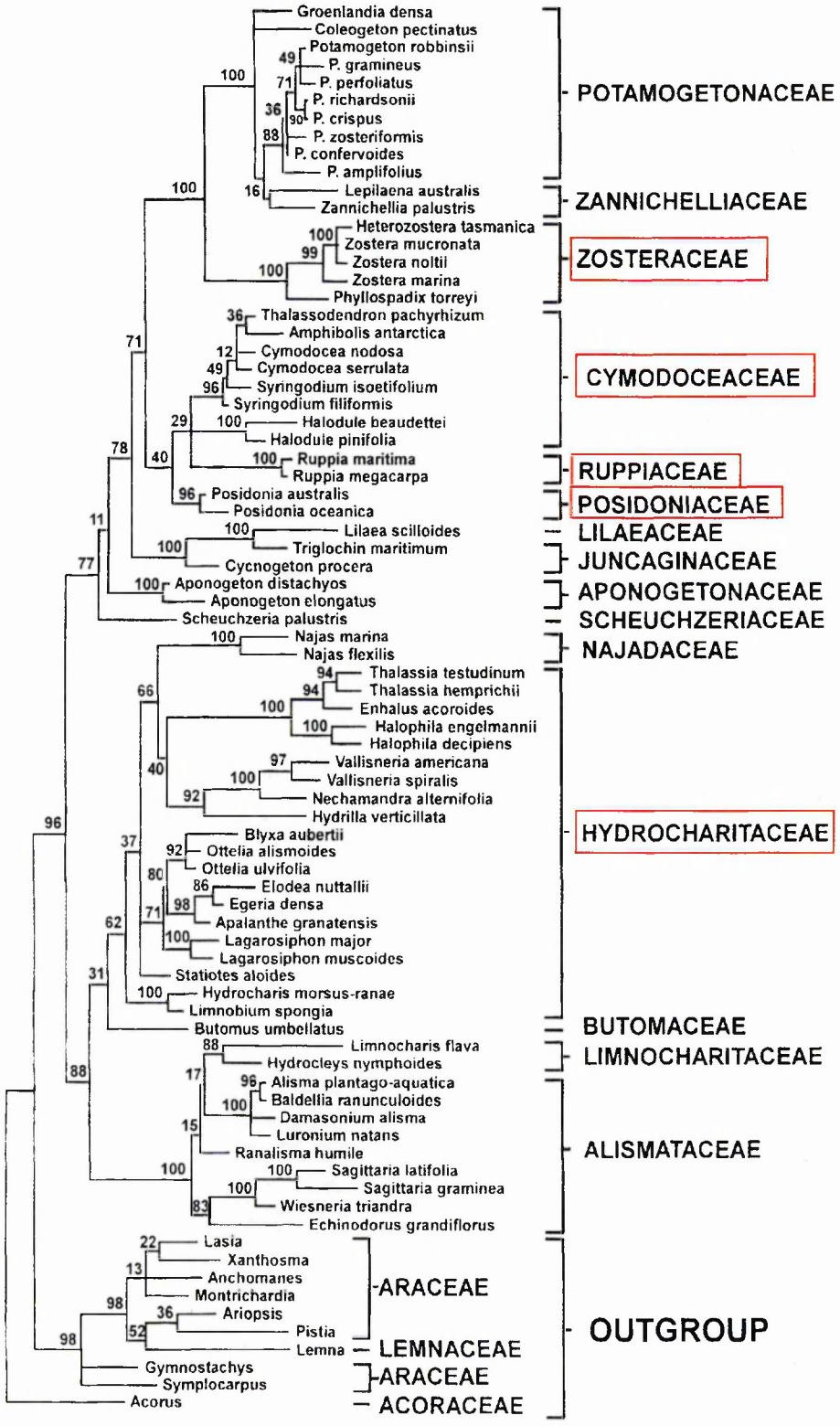

Fig. 1.4: Phylogenetic tree based on *rbcL* sequence, showing genealogical relationships within aquatic angiosperms. Seagrass families are highlighted (modified from Les *et al.* 1997).

At the end of the XVIII century, seagrass classification reflected superficial morphological similarities with algae (in 1792, Cavolini assigned the generic name of *Phucagrostis* -namely "seaweed grass"- to the actual genus *Cymodocea*) and marine angiosperms remained poorly known until the early XX century, when seagrasses were placed within the monocotyledonous sub-class Alismatidae. Initially Ascherson and Graebner placed seagrasses in two families (Hydrocharitaceae and Potamogetonaceae) and this partition persisted until first phylogenetic studies arose in the late XX century. Molecular phylogenetic studies, using *rbcL* and *trnL* intron sequences (Les et al 1997, Procaccini *et al.* 1999) revealed that marine angiosperms evolved at least three times (Fig. 1.4). Monophyletic lineages occur within Hydrocharitaceae, Zosteraceae and the Cymodoceaceae "complex", the latter consisting of genera traditionally classified within the Cymodoceaceae, Posidoniaceae and Ruppiaceae (Les *et al.* 1993; Les & Haynes 1995; Les *et al.* 1997).

1.2.2 - The trade-off between Clonality and Sexual reproduction in Seagrasses

Seagrasses, as their terrestrial counterparts, are characterized by a root system, a vascular system and sexual reproduction through flowering and seed set. Although their common organisation in roots, rhizomes (modified stems) and leaves, the adaptation to different habitats and life-styles has led to different *habitus* (Fig. 1.5).

Seagrasses are capable of vegetative propagation, mainly through horizontal rhizome elongation. Vegetative growth has always been considered the primary source of expansion and persistence of seagrass populations (Tomlinson 1974; Duarte & Sand-Jensen 1990). Clonal spread depends on rhizome elongation (i.e. rate of addition and

size of rhizome internodes) and branching pattern (i.e. branching frequency and angles, Marbà & Duarte 1998).

Variability in clonal spread seems to be species-specific, although some intra-specific variation can be observed in response to different environmental constraints, such as burial avoidance (Marbà & Duarte 1994), nutrient availability (Pérèz *et al.* 1994), changes in light and/or temperature (Terrados 1997a,b). Horizontal spread rates are variable, and range from an average of 2 cm y^{-1} in the Mediterranean endemic *Posidonia oceanica* to up to an average of 360 cm y^{-1} for the tropical species *Halophila ovalis* (Marbà & Duarte 1998).

Large, long-lived individuals can therefore form, within which clonal integration, i.e. the capacity to mobilize energy and nutrients among ramets, has been described (Tomasko & Dawes 1989; Terrados *et al.* 1997a,b; Nielsen & Petersen 2000).

Large clones have been, in fact, described by means of molecular markers in various seagrass species, such as in *Posidonia oceanica*, where an ancient, post-glacial clone was found in the North-Adriatic Sea (Ruggiero *et al.* 2002) and in *Zostera marina* (Reusch *et al.* 1999a). Clonality in seagrasses has been considered as a mean to preserve adaptive gene complexes in stable aquatic environments (Waycott *et al.* 1996).

Seagrasses have maintained the capacity of flowering and producing seeds, although sexual reproduction in seagrasses is generally thought to be rare (Sculthorpe 1967; den Hartog 1970; Les 1988). A peculiar feature of their sexual reproduction system is the hydrophilous pollination: hydrophilous pollen is only functional under water, in contrast to what happens in terrestrial angiosperms (Corbet 1990).

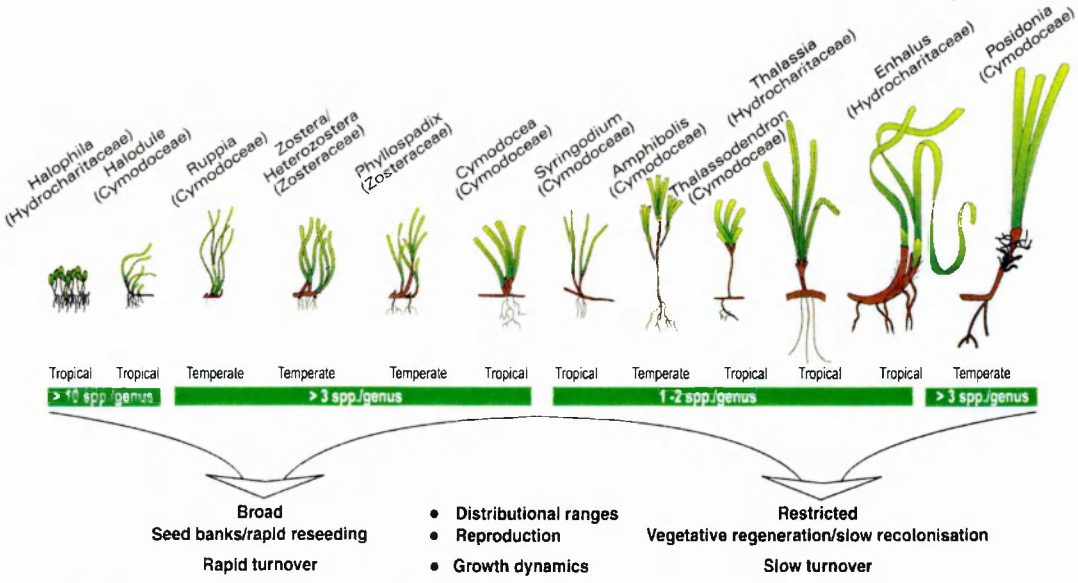

Fig. 1.5: Generic Seagrass model illustrating the different *habitus* of several genera in relation to their main ecological features.

Hydrophilous pollination arose several times during seagrass evolution and derives, at least in Potamogetonaceae and in part of Hydrocharitaceae, from anemophilous pollination (Les & Haynes 1995; Philbrick & Les 1996). The polyphyletic origin is reflected in the various kinds of hydrophilous pollination known in seagrasses. Two main models have been described: a two-dimensional one, in which the pollen is transported at the water surface, and a three-dimensional one, in which pollen is transported below water surface. Both systems required modifications of floral apparatus and of pollen grains, which in marine plants are filamentous. In some species, filamentous pollen can reach lengths of several millimetres and it can lack the exin layer (Philbrick 1991; Cox & Humphries 1993; Ackerman 2002).

A striking feature of seagrasses is that 69% of them are dioecious, against the 4% of terrestrial plants (Waycott *et al.* 1996). Dioecy has been hypothesized to represent an adaptation to hydrophilous pollination, to promote sufficient levels of outcrossing (Cox 1993); this view has been questioned by Les (1997), who pointed out that dioecy should be an ancestral condition, instead of a derived adaptive character. He

hypothesized that consequences of hydrophilous pollination were mainly a reduced rate of sexual reproduction due to inefficient pollen transfer and a widespread clonal growth.

1.2.3 - Genetic variability and population structure in Seagrasses

Until recently, seagrass populations have been considered to have low levels of genetic variability. This belief could have resulted from the lack of molecular markers polymorphic enough to unravel effective levels of variability. Most of the studies in the 80's and 90's revealing low polymorphism of seagrass populations were, in fact, conducted by means of allozymes (reviewed in Reusch 2001b, Waycott *et al. in press*). With the advent of DNA-based molecular markers, from RAPD to microsatellite loci, the general concept of widespread clonality and rare sexual reproduction in these plants has been questioned (Reusch 2001b). For example, the three species *Thalassia testudinum* (Kirsten *et al.* 1998), *Posidonia australis* (Waycott 1998) and *Zostera marina* (Olsen et al 2004, Reusch *et al.* 1999b; 2000) were thought to comprise only a few clones based on allozyme markers, while DNA based markers revealed several distinct genotypes at the m-scale. Moreover, new molecular markers for seagrasses are in continuous development, possibly revealing different levels of resolution, even within the same class of markers (e.g. new microsatellite loci for *Posidonia oceanica*, Alberto *et al.* 2003a)

Clonal diversity was found to be widely variable among locations within species. For example, in *Posidonia oceanica*, there are sites in the Adriatic Sea with only a single detectable genotype (Ruggiero *et al.* 2002); whereas, other populations in more central areas of the Mediterranean are multi-clonal (Procaccini *et al.* 2001; Arnaud-Haond *et al.* in press). A similar range in clonal diversity has been observed in

the Australian species *Posidonia australis* (Waycott 1998), in the northern-temperate species *Zostera marina* (Reusch *et al.* 2000; Olsen 2004) and in *Zostera noltii* (Coyer *et al. in press*).

This variability is probably due to the differential contribution of sexual versus vegetative reproduction, but nearly nothing is known regarding how successful recruitment varies in seagrass populations. Levels of clonal diversity can be affected by several factors, such as i) physical disturbance, as shown in *Zostera marina*, where clonal diversity was higher at sites with greater disturbance (Hämmerli & Reusch 2002) and ii) marginal position respect to the distribution range of the species ('geographic parthenogenesis' of Bierzychudek, 1985), as seemed to be the case for edge populations in *Cymodocea nodosa* (Alberto *et al.* 2001); *Posidonia oceanica* (Ruggiero *et al.* 2002); *Zostera marina* (Reusch *et al.* 1999a; Billingham *et al.* 2003); *Posidonia australis* (Waycott *et al.* 1997).

In general, it is not possible to easily identify common trends among congeneric species, or among species having similar or identical mating system features. Knowledge of local environmental factors, current regimes, human impact and historical colonization events of the area are of major importance to understand and predict population structure.

1.2.4 - Mediterranean Seagrasses

Only five species of seagrasses can be found in the Mediterranean Sea. The most common are the Mediterranean endemic *Posidonia oceanica* (L.) Delile, *Cymodocea nodosa* (Ucria) Ascherson and, in restricted shallow areas, *Zostera noltii* (Hornem.). *C. nodosa* is considered the pioneer species of *P. oceanica* beds, the latter representing the

"climax" stage. When *P. oceanica* beds regress and gaps are opened in the canopy, *C. nodosa* often replaces *P. oceanica* (den Hartog 1977) and mixed meadows can be observed at this stage. *Z. noltii* can be often found in mixed stands with *C. nodosa* in shallow waters. Mixed stands (Fig. 1.6) are, however, not persistent (Buia & Mazzella 1991).

The other two species, *Zostera marina* L. and *Halophila stipulacea* (Forssk.) Ascherson are less abundant in the Mediterranean basin. *Zostera marina* is restricted to river deltas and lagoons characterized by low salinity and brackish waters. *H. stipulacea* was first recorded in the Eastern Mediterranean Sea in 1895. The current opinion, supported by recent DNA-based studies (Ruggiero & Procaccini 2004) is that *H. stipulacea* entered the Mediterranean Sea from the Red Sea after the opening of the Suez Canal in 1869 (the "Lessepsian" hypothesis).

Fig. 1.6: *Cymodocea nodosa* and *Posidonia oceanica*.

In the following paragraphs, some ecological and reproductive features of the two species object of this thesis are presented.

Cymodocea nodosa

Cymodocea nodosa (Ucria) Ascherson is widely distributed along Mediterranean coastlines and extends on the Atlantic coasts from Southern Portugal to Northern coasts of Africa up to Senegal (den Hartog 1970, Fig. 1.7). It grows in dense meadows on fine sand bottoms up to a depth of 20m. Vegetative reproduction has been considered predominant in this species but seeds and seedlings are often recorded *in situ*, especially in the south-western part of the Mediterranean basin (Pirc *et al.* 1983; Cancemi *et al.* 2002), suggesting here high levels of sexual recruitment.

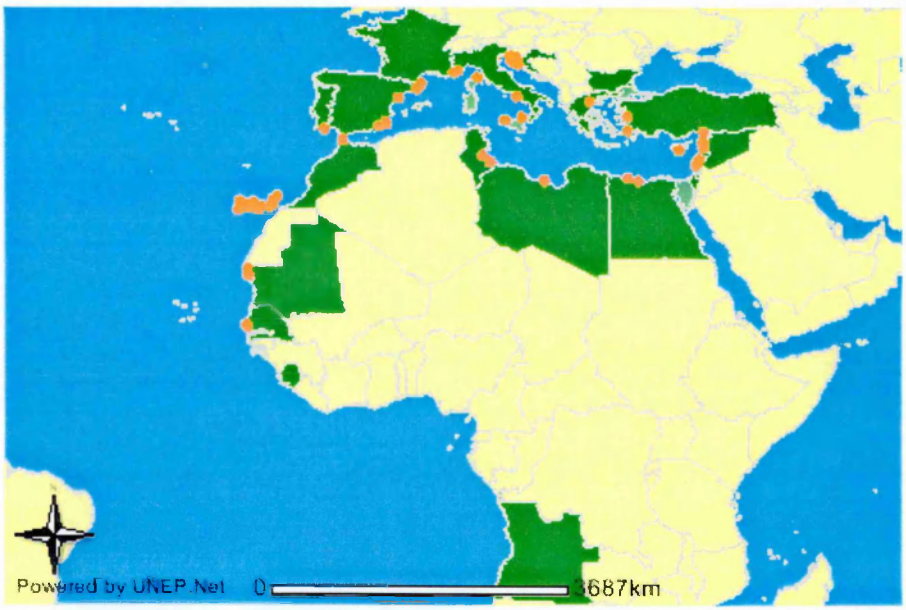

Fig. 1.7: Geographic distribution of *Cymodocea nodosa* (from UNEP.net). Dots represent point observations.

C. nodosa is a diploid (Koce *et al.* 2003), dioecious species, with male flowers reduced to two anthers borne on a stalk and sessile female flowers bearing two ovaries (Fig. 1.8, Caye & Meinesz 1985). Flowering occurs in spring (April-May) and fruits can be found attached to the mother plant until August (Fig. 1.9). Mature fruits are drupes with a fleshy pericarp that remain buried in the sediment in a dormant stage for about 8

months, until germination (Buia & Mazzella 1991). The potential for seed dispersal in this species is therefore quite limited.

C. nodosa also presents high potential for space colonization through elongation of horizontal (plagiotropic) rhizomes (Fig. 1.10) (Duarte & Sand-Jensen 1990). Average horizontal elongation rate for a population in the Island of Ischia (Gulf of Naples – Italy) was estimated in 30 cm/y (Cancemi *et al.* 2002). It has a perennial life-form, with the maximum rate of ramet recruitment in spring (Buia & Mazzella 1991; Cancemi *et al.* 2002).

Previous studies have reported very different levels of genetic variability in this species: a population in Ischia (Gulf of Naples) showed very high polymorphism of RAPD markers (Procaccini *et al.* 1996), while a population at the northern range limit of the species in the Atlantic (Ria Formosa lagoon, Portugal), using the same type of markers, has shown a very low clonal diversity (Alberto *et al.* 2001). None of the two studies has taken into account the spatial genetic structure of the studied populations.

Fig. 1.8: *Cymodocea nodo.* and female flowers borne on different plants (modified from den Hartog 1970).

a)

b)

Fig. 1.9: *Cymodocea nodosa*: Fruits (a) and seeds (b) attached to the mother plant.

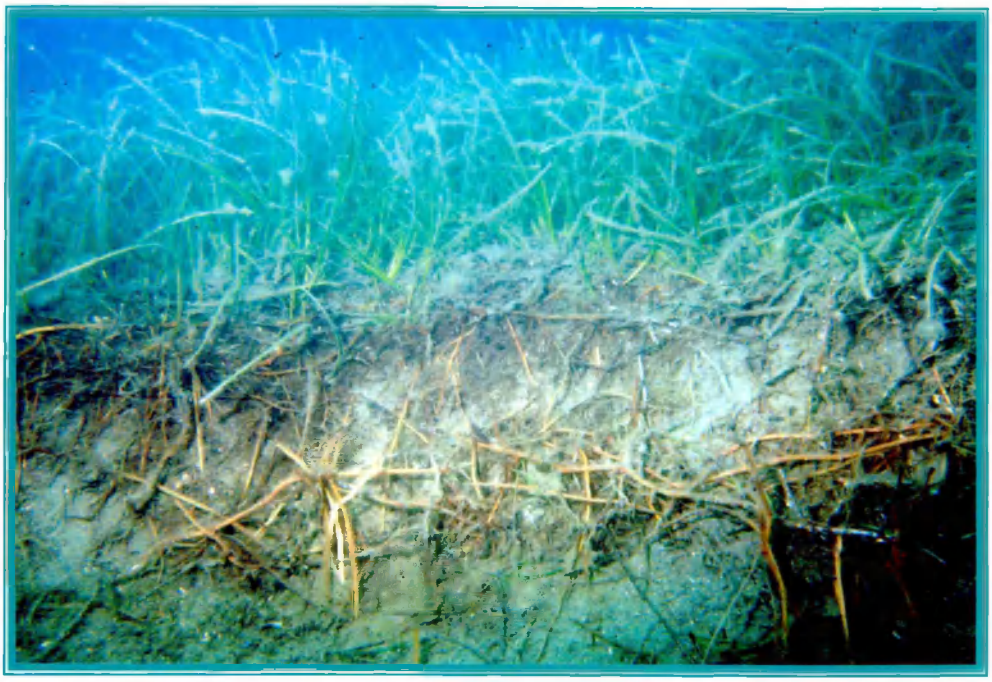

Fig. 1.10: *Cymodocea nodosa:* intermingled horizontal (plagiotropic) and vertical (orthotropic) rhizomes. Sediment is entrapped within the rhizome net, forming a typical "turf" structure (Buia & Mazzella 1991).

Zostera noltii

A recent debate has arisen about the systematic location of *Zostera noltii* Hornem (Fig. 1.11) within the genus. The genus *Zostera* has been subdivided in three sub-genera: *Zostera, Zosterella* and *Heterozostera. Z. noltii* has traditionally been classified within the sub-genus *Zosterella*, but in 2001 Tomlinson & Posluszny have proposed to elevate this subgenus to a new genus *Nanozostera* based on morphological characters. This view has been questioned by Les *et al.* (2002), through both morphological and molecular markers. I accept here *Z. noltii* as part of the sub-genus *Zosterella.*

Zostera noltii is widely distributed on Atlantic coasts along Europe, reaching its northern limit on the southern coasts of Norway (Fig. 1.12). Southward it extends until Mauritania (den Hartog 1970). In the Mediterranean Sea, *Z. noltii* is found in shallow waters, up to 6-7 m and it is rarely emerged in the intertidal.

Fig. 1.11: *Zostera noltii*

It can be found in monospecific stands, although more often it is in association with *C. nodosa*. *Z. noltii* is hermaphroditic, with male and female flowers on the same floral axis. Flowering shoots (Fig. 1.13) bear several inflorescences (spadices), containing 4-5 flowers of each sex (den Hartog 1970).

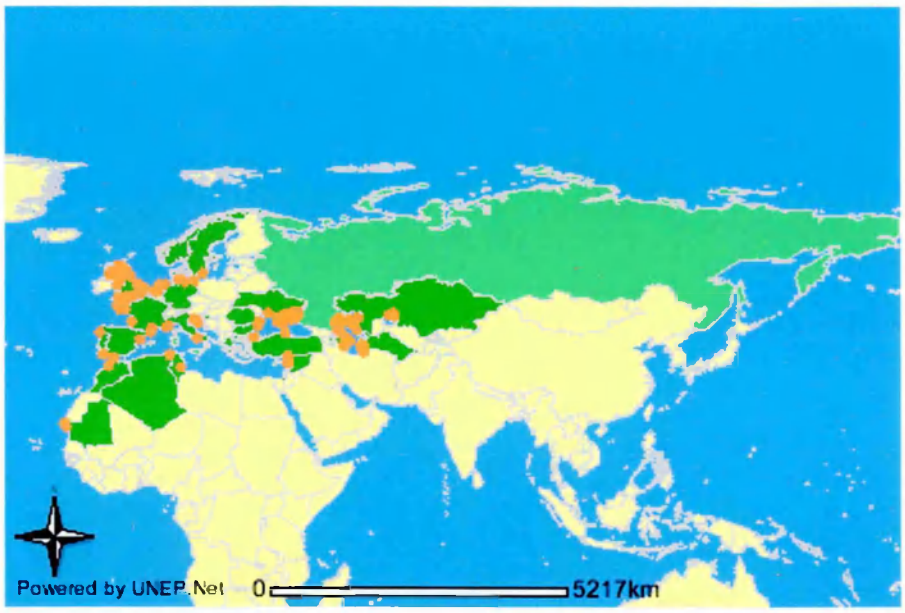

Fig. 1.12: Geographic distribution of *Zostera noltii* (from UNEP.net). Dots represent point observations.

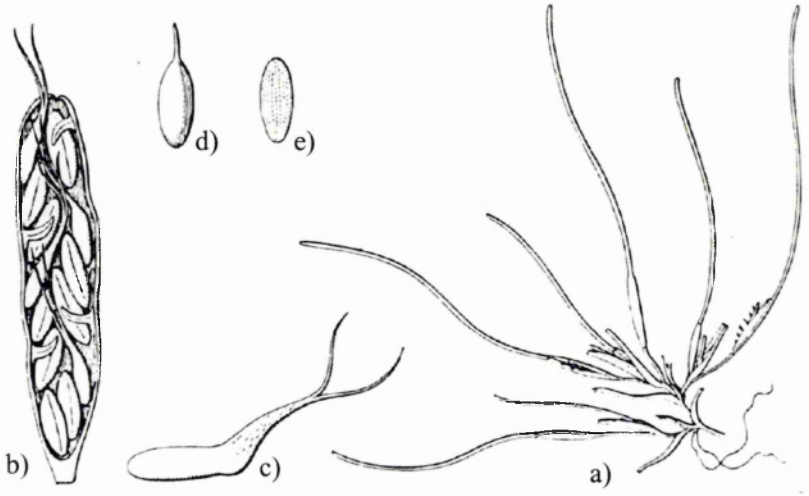

Fig. 1.13: *Zostera noltii*: a) flowering shoot; b) spadix; c) female flower; d) fruit; e) seed (modified from den Hartog 1970).

A recent study on the phylogeography of *Z. noltii* along its whole distribution area, showed large variability in genetic diversity among populations, with populations ranging from mono-clonal to complete clonality (Coyer *et al. in press*).

1.3 – Microsatellites as molecular markers

Molecular markers are essential tools in addressing ecological and evolutionary questions in conservation biology, evolutionary and population studies (Queller *et al.* 1993; Jarne & Lagoda 1996). Microsatellites have been shown to be the ideal class of genetic marker, having highly variable loci (Powell *et al.* 1996; Hancock 1999) with codominant alleles that allow distinguishing between homozygous and heterozygous genotypes. Microsatellite loci are often species specific, necessitating labour-intensive development for each target species separately; because of their advantages compared to other genetic markers, however, their development and utilization is in continuous progress in many taxa.

Microsatellites are single-sequence repeats on the DNA, which have been found in every organism examined so far. They are made up of tandemly repeated short sequence stretches with a maximum length of six bases. Estimates of microsatellite mutation rates range from 10^{-2} events per locus per replication in *Escherichia coli* (Levinson & Gutman 1987a), to $6x10^{-6}$ in *Drosophila* (Schug *et al.* 1997). Mutational process of microsatellites leads to the birth of new alleles through addition/deletion of one (or possibly more) repeat units (Fig. 1.14) and it seems to be very complex.

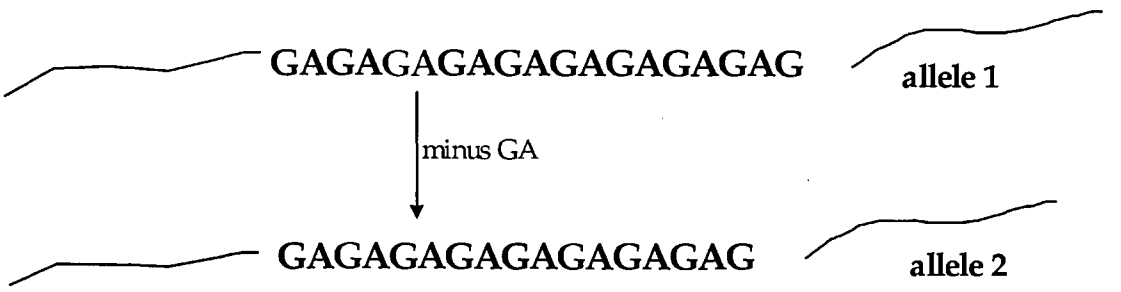

Fig. 1.14: Example of a microsatellite locus illustrating one possible mutation event (the deletion of one repeat unit), leading to a new allele.

There are two potential mechanisms which can explain their high mutation rates. The first is recombination between DNA molecules by unequal crossing-over or by gene conversion (Smith 1976; Jeffreys *et al.* 1994). The second mechanism involves slipped-strand mispairing during DNA replication (Levinson & Gutman 1987b). The length of the microsatellite repeats may have an effect on the mutation rate such that longer repeats are more polymorphic than shorter ones (Chakraborty *et al.* 1997). This is probably because the risk for a misalignment is greater for longer repeat arrays. Most microsatellite arrays are shorter than a few tens of repeat units, strongly suggesting that there must be size constraints on the expansion of repeat arrays. However, there is no direct evidence for selective constraints acting on allele length at microsatellite loci.

To estimate population differentiation and genetic distances from microsatellite data, several theoretical mutation models for microsatellites evolution have been proposed. The first and best known model described is the stepwise mutation model, (SMM, Kimura & Ohta 1978), in which single step mutations, either additions or deletions, are assumed to occur with equal probabilities. This model implies the potential for homoplasy, because alleles may also mutate towards allele states that are already present in the population. In the infinite allele model (IAM, Kimura & Crow 1964) mutation can involve any number of tandem repeats and always results in a new allele state not previously present in the population. In addition to these models, Di Rienzo *et al.* (1994) described the two phase model (TPM), where a limited proportion of mutations involve several repeats. Although rarely cited in microsatellite literature, a K-allele model (KAM) could also be considered for microsatellites. Under this model, there are K possible allelic states, and any allele has a constant probability of mutating towards any of the other K–1 allelic states (Crow & Kimura 1970). Microsatellite

regions have been characterized in few seagrass species; up to now, primers are available for *Posidonia oceanica* (Procaccini & Waycott 1998; Alberto *et al.* 2003a), *Zostera marina* (Reusch *et al.* 1999b; 2000), *Zostera noltii* (Coyer *et al.* 2004) and *Cymodocea nodosa* (Alberto *et al.* 2003b; Ruggiero MV, Chapter II of present thesis).

1.4 - Thesis outline

The thesis investigates the relative effects of sexual and clonal reproduction on the genetic diversity and structure in *Cymodocea nodosa* populations from the Mediterranean basin. In this outline I will depict the approaches underlying the studies described in each chapter. Details of methodologies and data analyses are described in each chapter. All studies are based on the use of microsatellite loci as molecular markers for clonal identification and assessment of genetic diversity parameters. Except for Chapter 1, for which more detailed informations are given in the thesis, all chapters are presented as the final version submitted to international journals.

In Chapter II, I will illustrate the methodologies applied in order to identify microsatellite regions in the *Cymodocea nodosa* genome. The subsequent steps to optimize the genotyping system for use with hundreds of samples are also described. The seven microsatellite loci selected have been used in all of the following chapters.

In Chapter III, the fine-scale genetic structure and clonal architecture in a continuous population of *Cymodocea nodosa* were assessed. The resulting picture allowed to discriminate between the contribution of gene dispersal and seedling recruitment through sexual reproduction on the one hand; and growth form and rate of clonal reproduction on the other hand. To this end, spatial autocorrelation analyses have been performed both at the ramet level and at the genet level, allowing to estimate average clone size and gene dispersal within the population. The spatial distribution of genets has been depicted in a detailed clonal map.

In Chapter IV, the interactions between mating system and clonal growth in seagrasses have been investigated through the comparison of clonal architecture in the dioecious *Cymodocea nodosa* and the hermaphroditic *Zostera noltii*. Theory predicts

that different mating systems should be associated with different clonal architectures. I determined clonal growth patterns of the two species through overlapping sampling schemes to even out any environmental heterogeneity, in order to test the hypothesis that dioecy is related to a "guerrilla" growth strategy, while a phalanx-growth form is typical of hermaphroditic species.

In Chapter V, the large-scale genetic structure was determined among *Cymodocea nodosa* populations within the Mediterranean basin. In this chapter I investigated: i) geographic patterns of genetic diversity within the distribution area of the species; ii) levels and directionality of gene flow between populations and iii) the correspondence between panmictic units and geographically defined populations. To these aims, classical estimators of genetic diversity and structure were assessed for the sampled populations. An assignment test was used to determine directionality of the gene flow, while through a bayesian approach the existence of "supra-population" panmictic units was investigated.

CHAPTER II – Polymorphic microsatellite loci for the marine angiosperm *Cymodocea nodosa*[♦]

Introduction

Cymodocea nodosa (Ucria) Ascherson is a dioecious marine angiosperm, widely distributed in the Mediterranean Sea, and extending in the Atlantic Ocean from Southern Portugal to the Northern coasts of Africa (den Hartog 1970). It grows in dense meadows, often in association with other seagrasses (Buia & Mazzella 1991).

C. nodosa is characterized by high rates of both sexual reproduction and clonal propagation through rhizome elongation (Caye & Meinesz 1985; Duarte & Sand-Jensen 1990). It could represent a good model to study how the two modes of reproduction affect the population's genetic structure and to assess genet dynamics and gene flow at different spatial scales.

Only two published studies up to now have dealt with the genetic variability in *C. nodosa*, in which RAPD molecular markers revealed very different levels of polymorphism in two distinct populations (Procaccini & Mazzella 1996; Alberto *et al.* 2001). The development of more appropriate molecular markers for this species is thus becoming essential. Microsatellite loci can be considered markers of choice in population genetic studies due to their high polymorphism and codominant mode of inheritance; they are, however, species-specific, necessitating labour-intensive development for each target species separately. Microsatellite loci have been already

[♦] A shorter version of this chapter has been published as: Ruggiero MV, Reusch TBH, Procaccini G (2004) Polymorphic microsatellite loci for the marine angiosperm *Cymodocea nodosa*. *Molecular Ecology Notes*, **4**, 512-514. See Appendix III for a reprint of the cited paper.

selected in the *Cymodocea nodosa* genome (Alberto *et al.* 2003), although they are characterized by only one kind of repeat (CT). Here, the development of seven new polymorphic microsatellite loci characterized by different types of repeat units is described.

Materials & Methods

DNA Extraction

About 1 g of fresh or frozen leaf tissue has been cleaned from epiphytes and ground in liquid N_2. DNA was extracted in 8 ml of CTAB buffer, according to Doyle & Doyle (1987), modified as in Procaccini & Mazzella (1996) after addition of 0.5% SDS (Sodium Dodecyl Sulphate), 67 mM β-mercaptoethanol and 0.4% PVP (Polyvinilpirrolidone). After incubation for 1^h at 60°C, samples were purified twice in Chlorophorm/ Isoamylic Alcohol (24:1) and centrifuged for 15' at 3000rpm (rotations per minute). Supernatant was then precipitated with 1 vol of cold Propylic Alcohol by incubation for 1^h at room temperature and centrifuged at 4°C for 30' at 13000 rpm. Resulting nucleic acids were then resuspended in 200μl of TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) and processed to eliminate RNA from the solution. RNAase A was added to a final concentration of 10μg ml^{-1} and the sample was incubated at 37°C for 1^h. The enzyme and remaining proteins were eliminated through an extraction with Phenol/Chlorophorm/Isoamylic Alcohol (25:24:1) and one or two extractions in Chlorophorm/Isoamylic Alcohol (24:1). After each wash, samples were centrifuged for 15' at 3000rpm. The DNA solution was then precipitated by addition of 1/10 vol 3M NaAc, pH 5.2 and 2 vol absolute Ethylic Alcohol and incubation at -20°C

overnight. After eliminating the supernatant, the pellet was resuspended in 100μl TE. Yield in high-quality DNA was about 10-50μg /g fresh tissue.

DNA GENOMIC LIBRARY CONSTRUCTION

A schematic diagram of the necessary steps to obtain microsatellite sequences from the genome of the studied species is shown in Fig. 2.1.

High-quality genomic CTAB-extracted DNA was digested overnight at 37°C by blunt-end restriction enzymes (*Alu I, Hae III* and *RsaI*, Amersham). Digestion products were then separated through electrophoresis on 2% agarose gel (Biorad) and fragments from 300 to 600 bp were excised from the gel and purified (QIAquick Gel Extraction Kit- QIAGEN). 300 to 600 bp fragments were then ligated into a previously blunt-end restricted p-BlueScript plasmid vector (Stratagene), through the T4 ligase (Amersham) at room temperature overnight. Ligation products were then transformed into *Escherichia coli* electrocompetent cells. Cells were then plated on LB (Luria-Bertani) Ampicillin selective 25cm Ø plates and incubated over-night at 37°C. The amount of cells to plate (in μl) was calculated so to obtain ~1500-2000 colonies per plate. Fifteen plates allowed therefore a total of ~ 30000 colonies.

COLONY-HYBRIDISATION

A colony-hybridisation protocol was followed in order to identify bacterial clones carrying plasmids containing the microsatellite motifs as insert.

Colonies from each plate were transferred by lifting on Hybond N+ Nylon membranes (Amersham). Membranes were labelled in order to identify the position of colonies relative to the plates. Colonies were allowed to re-grow at 37°C for four to six

hours and preserved at 4°C. DNA was then fixed on membranes by autoclaving and UV linking (Stratalinker, Amersham).

Hybridisation and pre-hybridisation of membranes were carried out in Denhart's buffer (as in Sambrook *et al.* 1989) with the addition of Salmon sperm DNA to avoid high background signal.

The ^{32}P-labelled probes used for the hybridisation consisted in five repeated di- or trinucleotide motifs: $(ATT)_8$, $(ACT)_8$, $(AT)_{12}$, $(GA)_{12}$ and $(CA)_{12}$. Membranes were pre-hybridised at the hybridisation temperature for 4^h. Hybridisation temperatures were as follows: 58°C for $(ACT)_8$, $(GA)_{12}$ and $(CA)_{12}$; 50°C for $(ATT)_8$ and $(AT)_{12}$. Hybridisation was carried out overnight. After several washes at decreasing temperatures, membranes were exposed to autoradiographic films (X-OMAT AR, Kodak) at -80 °C and developed and fixed after 8-10^h.

Positive colonies (containing a microsatellite repeat into their plasmids) were identified through comparison between the autoradiographic film and the plate. Colonies were picked up and transferred to LB+Ampicillin growth medium and incubated at 30°C overnight. Plasmids were then purified (QIAprep Spin Miniprep Kit, Qiagen) from the selected colonies and fragments were sequenced using universal M13 primers through automated sequencing (CEQ 2000XL DNA Analysis System, Beckman Coulter).

GENOMIC LIBRARY ENRICHMENT

The "enrichment" of a genomic library implies that fragments are pre-selected for microsatellite motifs before ligating them in plasmid vectors. The enrichment method is based on the capability of streptavidin-coated magnetic beads to strongly bind

biotinilated oligonucleotides represented by a microsatellite repeat. If a DNA fragment contains a microsatellite, it will hybridise to the complementary biotinilated oligo and will form a complex together with the streptavidin-coated magnetic beads. The complex will be sequestered from unbound fragments applying a magnetic force to the beads. See Fig. 2.2 for a diagram of the process.

After restriction of genomic DNA, as described above, size-selected and purified blunt-end fragments were ligated into p-BlueScript plasmid vector. Plasmids underwent to an asymmetric PCR reaction (the forward universal primer M13 was in excess respect to the reverse primer) in order to obtain single-strand copies of the fragment population. Single-strand fragments were then purified and hybridised in SSC 6X (Sambrook *et al.* 1989) to biotinilated microsatellite oligos. The probes used for the hybridisation consisted in five repeated di- or trinucleotide motifs: $(ATT)_8$, $(ACT)_8$, $(AT)_{12}$, $(GA)_{12}$ and $(CA)_{12}$. Temperature was chosen according to the probe sequence and reaction was carried out for 30' in a shaking bath. After hybridisation, the biotinilated oligos in duplex with the single-strand fragments, putatively containing microsatellite sequences, were bound to the streptavidin-coated beads at room temperature for 15'. The complex was then washed several times in increasing stringency conditions. The last step was the elution of the "positive" fragments from the biotinilated oligos.

From the enriched single-strand fragment population, double-strand fragments were i) reconstituted through PCR using universal primers M13, ii) double-digested with HindIII + EcoRV and iii) ligated into p-BlueScript, double-digested with the same restriction enzymes. After this last step, the classical colony-hybridisation protocol was followed as described above.

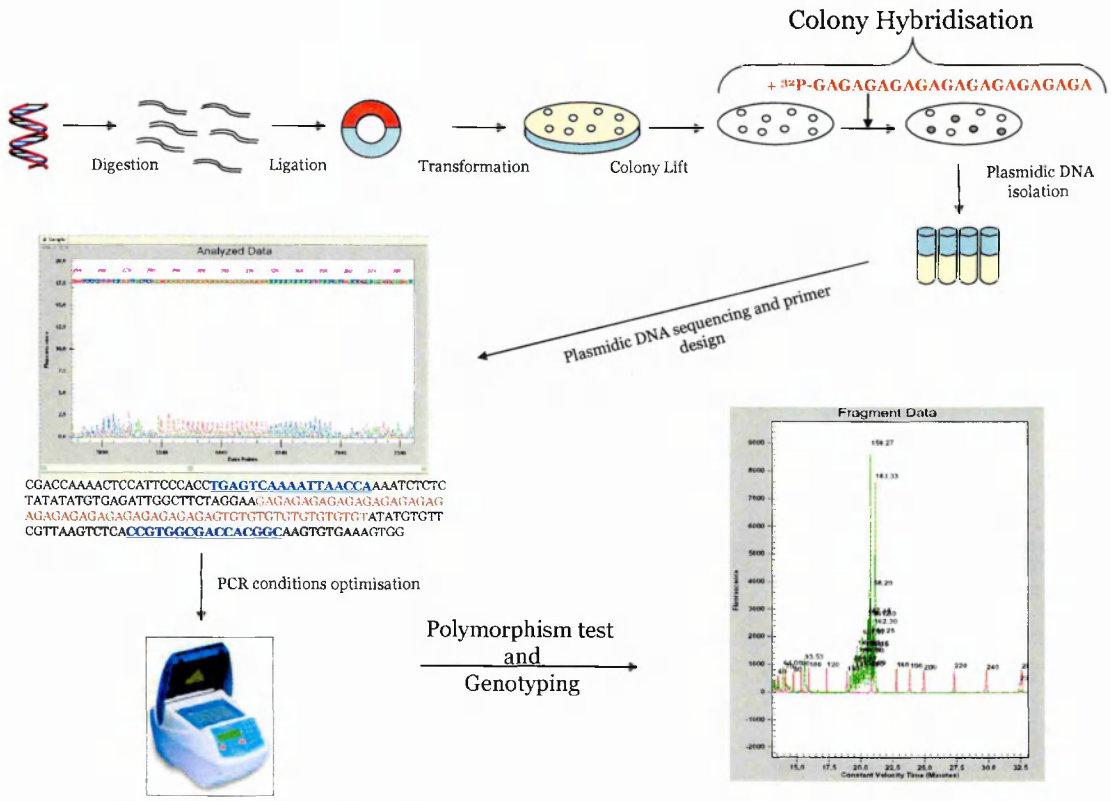

Fig. 2.1: Schematic diagram illustrating the main steps in a genome library screening.

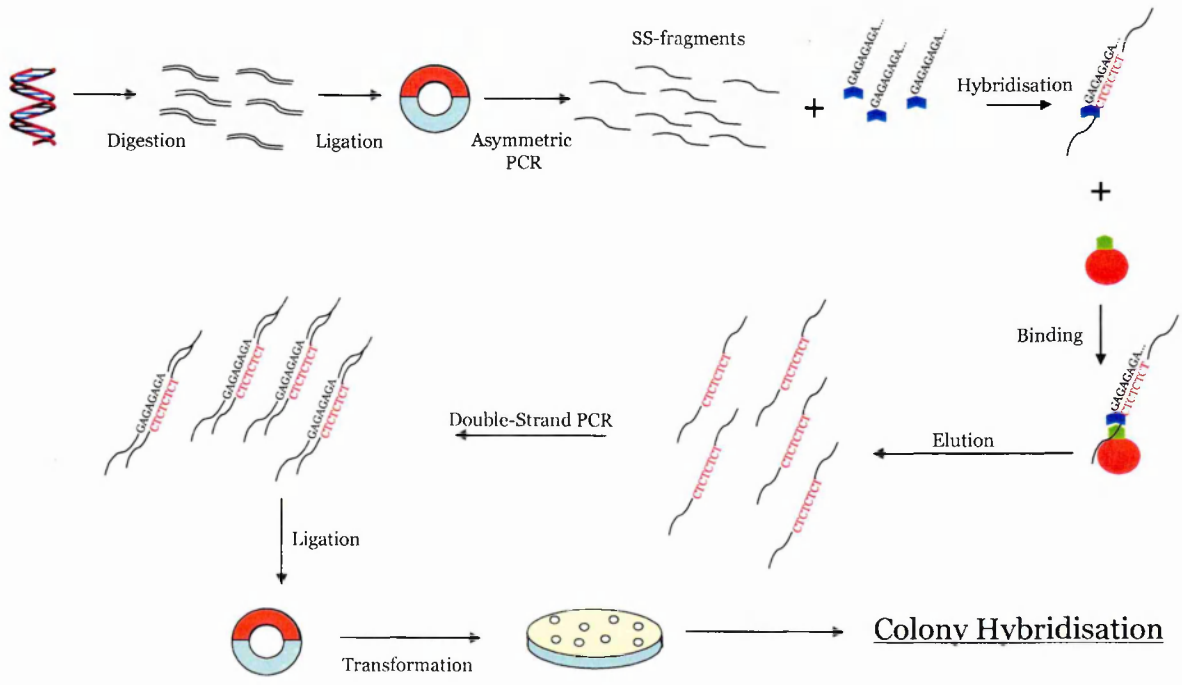

Fig. 2.2: Schematic representation of the enrichment procedure. From Colony Hybridisation on, steps are the same as in the "classical" library screening (Fig. 2.1).

TEST FOR POLYMORPHIC LOCI

In order to test for polymorphism of loci, 50 individual *C. nodosa* shoots from a population in the Island of Ischia (Gulf of Naples, Italy) and 60 individuals from 9 other geographically distinct populations from the Mediterranean Sea were genotyped. ^{32}P-labelled primers were used in the PCR reactions and products were run on a denaturing 6% acrylamide-bisacrylamide gel and visualized by autoradiography.

PCR conditions were as follows: 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM each primer, 0.5 u Taq (Roche) in a total volume of 10 μl. Template DNA can be as low as 2 ng. PCR cycles were as follows: an initial denaturation step of 4' at 94°C; 35 cycles consisting in 1' at 94 °C, 1' at 58 °C and 1' at 72 °C, followed by a final extension step of 7' at 72 °C. All PCR reactions were conducted in a GeneAmp 9700 Thermocycler (PE Applied Biosystems).

OPTIMISATION OF THE GENOTYPING SYSTEM: MULTIPLEX PCR AND AUTOMATED DETECTION.

For the polymorphic loci, PCR conditions were optimised for genotyping through automated fragment analysis (CEQ 2000XL DNA analysis system, Beckman Coulter). Automated capillary fragment analysis is based on the same principle as the polyacrylamide gels electrophoresis. One of each couple of primers for each microsatellite locus is labelled with a fluorescent dye (CY5 or IRD700 in this work) before they are used in PCR. A few µl of PCR product are applied to the capillaries, filled with polyacrylamide gel, through anode-directed electroosmosis, and fragments are separated through electrophoresis in buffer solution with high voltage (~ 3000 V). A laser (optical sequencer) detects the fluorescent dye from the primers and fluorescent

signals are transformed into peaks (alleles) at different locations (size of allele) and height (different intensity of amplification). An internal size standard facilitates the determination of the exact size of each allele. These peaks are then scored according to their size individually for each sample. Finally, the genotype of each sample is characterized for each analysed locus. Samples can present either two identical (homozygosis) or different (heterozygosis) alleles. A matrix listing multilocus genotypes of all samples is eventually will represent the basis of subsequent data analyses (See Appendix I).

PCR conditions were optimised for multiplex reactions, allowing to score more than one locus per sample per PCR. Optimisation for multiplexing implies to i) identify loci that differ in alleles size or in fluorescent dye colour that could thus be univocally scored; ii) identify the annealing temperature at which all multiplexing loci can be reliably amplified; iii) identify the amount of template DNA, primer and salt concentration and units of Taq polymerase to be used in the PCR reaction.

GENETIC POLYMORPHISM DISPLAYED BY THE MICROSATELLITE LOCI

Number of alleles and heterozygosity values were tested using the software GENETIX (Belkhir *et al.* 1996-2002; website: www.univ-montp2.fr/~genetix/ genetix/ intro.htm), in order to assess levels of polymorphism of the microsatellite loci detected and, consequently, their power of resolution in population genetic studies. Linkage disequilibrium was estimated through the software GENETIX on the Ischia population after eliminating replicated genotypes.

Results and Discussion

From the enrichment trials, ~40000 colonies were screened on the total of several trials, allowing 26 positive clones. None of the obtained microsatellite fragments was suitable, due to the presence of the microsatellite repeat in a terminal position, not allowing the design of suitable primers. Artefacts in the enrichment procedure could account for that. Few repeats within the positive fragments were too short to be considered as putatively polymorphic loci.

On the contrary, from the total genomic library 15 suitable positive clones were obtained from ~60000 screened colonies. Primers were designed on each positive clone using the web-based software Primer3 (website: www-genome.wi.mit.edu). In Appendix I, nucleotidic sequences of the complete microsatellite regions are shown.

The test for polymorphism showed that more than two alleles were found in 7 out of the 15 microsatellite loci. Only the probe $(ACT)_8$ did not allow any positive clone.

Features of loci and primer sequences are shown in Table 2.1. Loci consisted in one tri-nucleotide, four simple di-nucleotides and 2 complex di-nucleotides. The seven loci were characterized by a total of 57 alleles among all analyzed populations, of which 34 were found in Ischia. In Table 2.2, PCR conditions for multiplexing are shown; all PCR conditions are as described in Materials & Methods, except for primers concentrations.

Heterozygosity values and number of alleles for each locus are shown in Table 2.1. Observed heterozygosity ranged from 0.240 for *Cy 1* to 0.860 for *Cy 16*. Number of alleles ranged from 5 to 13, considering all populations analyzed. No significant linkage disequilibrium was found in the Ischia population, after Bonferroni correction.

Polymorphism of loci allows thus a reliable assessment of genetic diversity in population genetic studies.

Alberto *et al.* (2003b) found a deficit in heterozygosity of microsatellite loci among seedlings of *C. nodosa*, possibly due to a Wahlund effect in the tested population. In contrast, our newly described loci show a significant excess of heterozygosity in the Ischia population (Table 2.1), possibly due to a selective heterozygote advantage.

Table 2.1: Primer sequences, number of alleles found in Ischia and other 9 tested populations, size of alleles, observed and expected heterozygosity in the Ischia population and GenBank accession numbers for seven *Cymodocea nodosa* microsatellite loci. * = labelled primer.

	Primer Sequence (5'-3')	Microsatellite Repeat	Number of Alleles: Ischia (N=50)	Number of Alleles: Nine populations (N=60)	Size range of Alleles	H_o	H_{exp}	GenBank Accession
Cy 1	**F** GGAGCAAGTCCGAAGAAGAG **R** GAGGAGGAAGGAATGGCTG *	$(CT)_{16}$	3	6	119-133	0.240	0.250	AY559051
Cy 3	**F** CGTGGCTCTTTCCGTAAATC * **R** CACGCACCCAACAGAAAAG	$(GA)_{12}$	3	6	140-152	0.600	0.562	AY559052
Cy 4	**F** GGCTTCAATAATGATGCGGT * **R** CACAAGAACCATTCACCCCT	$(TAA)_9$	4	8	154-169	0.720	0.648	AY559053
Cy 16	**F** ACTTTCACACTTGCCGTGGT * **R** CACCTCGACCAAAACTCCAT	$(CA)_8(CT)_{22}$	11	13	175-207	0.860	0.766	AY559054
Cy 17	**F** CTGCTGGCAGGTGAAGAAAT * **R** CCGAAGTTGTGCTTTGATCC	$(CT)_{17}CG(AT)_{10}$	4	5	228-260	0.660	0.623	AY559055
Cy 18	**F** CGCTCCTTCTTCTACCAGCA * **R** CTGCGGGTGCGTCTCT	$(CA)_{16}$	4	9	141-163	0.600	0.641	AY559056
Cy 20	**F** ACATGCTTTGGTTGCACAGA * **R** ACTCCCACATCTCCCTCAAA	$(TC)_{19}$	5	10	179-211	0.820	0.710	AY559057

Table 2.2: PCR conditions for multiplexing. One quadruplex (a) and one triplex (b) are described. Primer concentrations are provided. All other conditions and annealing temperature are as in the text. Starting template DNA can be as low as 2 ng.

Locus	Dye	Multiplex	Primer Concentration (μM)
Cy 1	IRD700	a	0.4
Cy 3	IRD700	a	0.2
Cy 4	CY5	a	0.05
Cy 16	CY5	a	0.05
Cy 17	CY5	b	0.4
Cy 18	CY5	b	0.4
Cy 20	IRD700	b	1

CHAPTER III – Local genetic structure in a clonal dioecious angiosperm♦

Abstract

We used seven microsatellite loci to characterize genetic structure and clonal architecture at three different spatial scales (from meters to centimetres) of a *Cymodocea nodosa* population in the Island of Ischia (Gulf of Naples – Italy). *Cymodocea nodosa* exhibits both vegetative propagation by stolonization and sexual reproduction. Seeds remain buried in the sediment nearby the mother plant in a dormant stage until germination. Seed dispersal potential is thus expected to be extremely restricted. High clonal diversity (up to 67% of distinct genotypes) and a highly intermingled configuration of genets at different spatial scales were found. No significant differences in genetic structure were found among the three spatial scales, indicating that genetic diversity is evenly distributed along the meadow. Autocorrelation analyses of kinship estimates confirmed the absence of spatial clumping of genets at small spatial scale and the presence of very restricted seed dispersal (observed dispersal range 1-21m) in this species.

♦ The present study has been submitted for publication to *Molecular Ecology* as:
Ruggiero MV, Reusch TBH and Procaccini G *Local genetic structure in a clonal dioecious angiosperm.*

Introduction

Spatial genetic structure in plant populations, which is the non-random distribution of alleles, results from local genetic drift in combination with restricted dispersal of sexual products. In addition, clines or patchiness of selectively relevant genes or markers may result from selective pressures in heterogeneous environments (Heywood 1991). In the absence of differential selection and under restricted gene flow, pairwise genetic relatedness among individuals decreases with increasing geographic distance, a process that was dubbed 'isolation -by-distance' (Wright 1943).

Many plant species exhibit a mixed-mating system, relying both on sexual recombination and asexual replication of genotypes. While many plants reveal highly leptokurtic seed dispersal (Ouborg *et al.* 1999; Cain *et al.* 2000), in clonal plants an additional source of genetic structure is due to asexual replication of genotypes through vegetative growth, whose effects on population's genetic structure need to be considered in clonal plant studies (Montalvo *et al.* 1997; Reusch *et al.* 1998; Chung & Epperson 2000; Hämmerli & Reusch 2003c).

To assess genetic structure in clonal plants, several factors should therefore be considered. First, the relative contribution of seed and pollen dispersal on overall within-population gene flow, influences kinship structure and inbreeding levels: when seed and pollen dispersals are poor, mating by proximity generates genetic structure (Epperson 2000), increasing local levels of inbreeding. In the case of a higher dispersal of pollen than seed, genetic structure can be present but inbreeding is avoided. Pollen

dispersal has thus the general function of flattening the negative effects of genetic structure on inbreeding levels (Loveless & Hamrick 1984; Kalisz *et al.* 2001).

Secondly, seedling recruitment strategies can influence genotypic diversity of populations. Eriksson (1989; 1993) described two different strategies: in the Initial Seedling Recruitment (ISR), seeds disperse far from the original population, and populations are expected to consist of few large clones; in the Repeated Seedling Recruitment (RSR) seedling are recruited within the original populations, thus increasing local genetic diversity and producing a pattern with many small clones.

Thirdly, vegetative recruitment strategies can affect clonal architecture and, consequently, genetic structure: the phalanx type of recruitment leads to a mosaic structure in which genetically identical ramets are clustered together and clones are recognisable as discrete units; the guerrilla type leads to an intermingled make-up of genets (Lovett Doust 1981). Clonal architecture affects sexual recruitment both in self-compatible species in which geitonogamy (pollination within the same genet) can be enhanced by clustering of genets and in dioecious species, favouring or preventing sexual products to encounter (Charpentier 2002).

Autocorrelation analysis (Sokal & Wartenberg 1983; Smouse & Peakall 1999) of neutral molecular markers can provide insights into plant populations' spatial genetic structure, when it results mainly from seed and pollen dispersal in equilibrium populations. It has been widely used in many terrestrial plants (Epperson 2000), but rarely taking clonality into consideration (but see Montalvo *et al.* 1997; Reusch *et al.* 1998; Chung & Epperson 2000; Hämmerli & Reusch 2003c; van der Strate *et al.* 2002).

Marine clonal angiosperms (seagrasses) constitute a polyphyletic assemblage of about 60 species (Les *et al.* 1997), characterized by both sexual and clonal reproduction.

Seagrasses are structuring species along coastal ecosystems worldwide. Their recognized ecological and economic importance (Costanza *et al.* 1997) justifies the growing concern about their worldwide documented regression (Short & Wyllie-Escheverria 1996) and has encouraged an increasing effort in population genetics studies (reviewed in Reusch 2001b). Few published studies have dealt up to now with fine-scale genetic structure in seagrasses; in the monoecious, self-compatible *Zostera marina*, the dominant seagrass species in the northern hemisphere, three studies investigated the contribution of clonal growth to genetic structure through autocorrelation analyses of microsatellite loci (Reusch *et al.* 1998; Hämmerli & Reusch 2003c; Olsen *et al.* 2004). Recently, spatial autocorrelation has been assessed in the seagrass species *Zostera noltii* (Coyer *et al.* 2004 *in press*) and in *Posidonia oceanica* (Procaccini G *et al.*, unpublished).

Cymodocea nodosa is widely distributed in the Mediterranean Sea, and extends on the Atlantic coasts from Southern Portugal to Northern coasts of Africa (den Hartog 1970). It grows in dense meadows, often in association with other seagrasses, as the Mediterranean endemic *Posidonia oceanica*, of which it represents the preceding species in the ecological succession and with *Zostera noltii* (Buia & Mazzella 1991). Vegetative reproduction has been considered predominant in this species but seeds and seedlings are often recorded *in situ*, especially in the south-western part of the Mediterranean basin (Pirc *et al.* 1983; Cancemi *et al.* 2002), suggesting a high level of sexual recruitment. A previous study on the genetic variability of a *C. nodosa* population in Ischia (Naples- South-Western Mediterranean), reported, in fact, a very high polymorphism of RAPD markers (Procaccini & Mazzella 1996). In contrast, a population at the northern range limit of the species in the Atlantic (Ria Formosa

lagoon, Portugal), using the same type of markers, has shown a very low clonal diversity (Alberto *et al.* 2001). None of the two studies has taken into account the spatial genetic structure of the studied populations.

In the present study, we describe the clonal architecture and the genetic structure in a continuous Mediterranean meadow of *Cymodocea nodosa* (Ucria) Ascherson in order to test how the combined effects of seed dispersal and dioecious habit can affect genetic structure in a marine clonal plant. The potential for seed dispersal in this species is extremely limited: detached seeds remain buried in the sediment nearby the mother plant in a dormant stage for about 8 months, until germination (Buia & Mazzella 1991). We expect that the restricted dispersal potential of seeds leads to a significant kinship structure at small spatial scales. In comparison with the monoecious seagrass species *Zostera marina* (Hämmerli A 2002), where a clumped distribution of clones was recorded, the dioecious habit of *C. nodosa* should lead to an intermingled configuration of clones. Due to the obligate outcrossing of the species, levels of biparental inbreeding should only be related to pollen dispersal potential. Seven species-specific microsatellite loci (Ruggiero *et al.* 2004, Chapter II of present thesis) have been used to identify multi-locus genotypes and their spatial distribution within the studied population. The differential contribution of sexual and vegetative recruitment to kinship structure was assessed through spatial autocorrelation analyses. A hierarchical sampling scheme has been adopted, in order to assess the minimum spatial scale at which a genetic structure could be revealed.

Materials & Methods

SPECIES: *C. nodosa* is a diploid (Koce *et al.* 2003), marine macrophyte, presenting both vegetative propagation by stolonization and sexual reproduction by germination of seeds. It grows both on sandy and rocky bottoms at a depth that rarely exceeds 18-20m (den Hartog 1970).

C. nodosa is a dioecious species, with sessile female flowers bearing two ovaries (den Hartog 1970; Caye & Meinesz 1985). Flowering occurs in spring (April-May) and mature fruits can be found attached to the mother plant until August. Its filamentous pollen is thought to be an adaptation to hydrophilous pollination (Cox & Humphrey 1993).

C. nodosa has a perennial life-form, with the maximum rate of ramet recruitment in spring (Buia & Mazzella 1991; Cancemi *et al.* 2002). It presents a high potential for space colonization through elongation of horizontal (plagiotropic) rhizomes (Duarte & Sand-Jensen 1990). Average horizontal elongation rate for a population in the island of Ischia (Naples – Italy) was estimated in 30 cm/y (Cancemi *et al.* 2002).

SAMPLING SCHEME: The studied meadow is located near the Castello Aragonese in Ischia (40° 44' N; 13° 58' E; Naples, Italy) in a relatively sheltered area. It is a continuous meadow at a depth of 4-6m on sandy bottom covering an area of about 1800m^2. All sampling was conducted with SCUBA diving (Fig. 3.1). Sampling covers the whole area of the meadow and has been conducted on three different spatial scales (Fig. 3.2). 1) A 30x60m area was sampled according to a grid with square meshes of 2m size. A total of 304 samples has been collected; 2) Five 80x80cm quadrats were chosen in randomly generated positions within the settled grid and shoots were collected at a

reciprocal distance of 20cm. A total of 25 shoots from each quadrat were sampled. 3) Rhizome fragments from first sediment layer (20cm depth) were collected in five locations within the grid, using 20cm diameter metallic corers. Fourteen to 36 rhizome fragments were found in each sediment core. A total of 122 fragments were collected. Tissue was brought to the laboratory, accurately cleaned from epiphytes in order to reduce contamination, and silica-gel dried.

Fig. 3.1: A SCUBA diver, sampling in the studied *Cymodocea nodosa* meadow.

DNA EXTRACTION AND MICROSATELLITE MULTI-LOCUS GENOTYPE DETECTION:

Five mg of silica-gel dried tissue from each individual sample have been ground through Mixer Mill MM300 (QIAGEN). Subsequent DNA extraction has been carried out using the Qiagen DNAeasy Plant Mini Kit (QIAGEN). Seven polymorphic microsatellite loci (Ruggiero *et al.* 2004, Chapter II of present thesis) were used to obtain multilocus individual genotypes. PCR conditions are as in Ruggiero *et al.* (2004, Chapter II of present thesis). Allele detection was conducted through automated sequencing (CEQ 2000XL DNA Analysis system, Beckman Coulter) for fragment analysis.

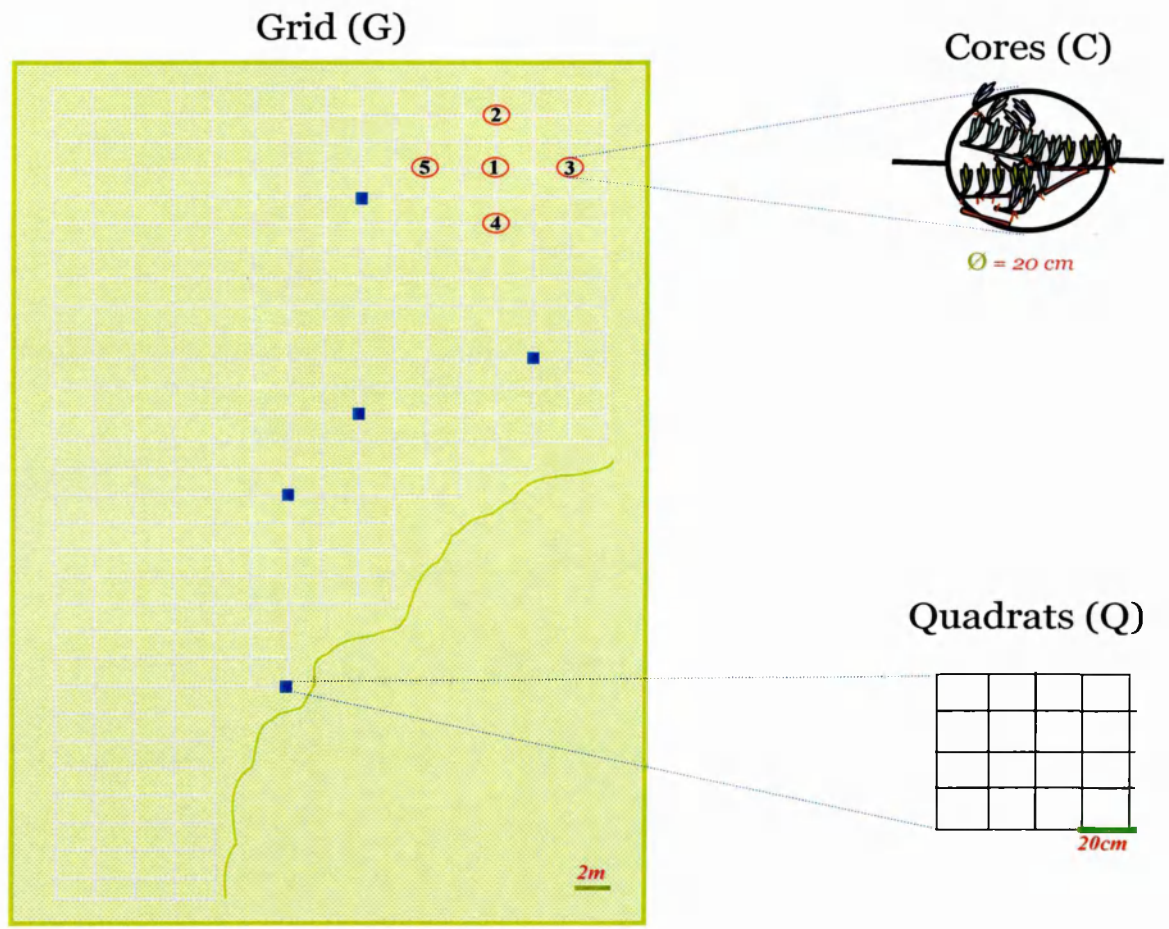

Fig. 3.2: Sampling design: for the Grid and the Quadrats, shoots were collected at the grid nodes; for the Cores, all shoots and rhizome fragments within a diameter of 20cm were collected. In brackets, the codes used to identify each sampling scale in the text are shown.

DATA ANALYSIS: Data analysis was undertaken both on the whole data-set and on separate data-sets from each sampling scale (from now on, referred to as Grid, Quadrats and Cores). Number of genotypes was calculated with the software GIMLET (Valière 2002) and overall genetic diversity was calculated as the percent of different genotypes over the total number of sampled ramets (G/N, Pleasant & Wendel 1989). The number of theoretical genotypes was calculated according to the formula:

$$N_g = \prod_{i=1}^{L} [a_i(a_i + 1)]/2$$

(Parks & Werth 1993)

The probability of identity (*Pi*) according to Waits *et al.* (2001) was also estimated through GIMLET software.

The minimum number of individuals to sample in order to get a reliable estimate of clonal diversity was assessed resampling at random an increasing number of individuals (10, 30, 50, 100, 200, 300, 400, 500, 546) from the data-set and calculating the relative G/N values. The final point (546 samples) represents the complete data-set.

Number of alleles/locus, observed and expected heterozygosity and the estimator *f* of inbreeding according to Weir & Cockerham (1984) have been estimated through the software GENETIX (Belkhir *et al.* 1996-2002; website: www.univ-montp2.fr/~genetix/genetix/intro.htm), after removal of replicated genotypes. Significance of *f* was assessed through estimation of the 95% CI after 1000 bootstraps. Deviation from Hardy-Weinberg proportions was tested at the genet level using a Markov-chain algorithm (Guo & Thompson 1992) implemented in the GENEPOP 3.3 software (Raymond & Rousset 1995).

In order to determine if clonal diversity (G/N), inbreeding coefficient (*f*) and observed heterozygosity (H_o) were significantly different among the three sampling spatial scales, an analysis of variance (ANOVA) was conducted, considering the five quadrats, the five cores and five sub-plots of the whole grid. The sub-plots size was 8x8m in order to obtain a comparable sample size among the three spatial scales. Position of sub-plots was chosen such as to avoid the resampling of quadrats and/or cores and to preserve independence of samples.

Size-class distribution was determined for the three spatial scales considering the number of clonemates as an estimate of clone size. The spatial spread of clones was also estimated as the linear distance between the most distant clonemate pair.

Autocorrelation analyses using the kinship coefficient f_{ij} (Loiselle *et al.* 1995) were conducted through the software SPAGEDI (Hardy & Vekemans 2002) on the grid and on the quadrats, averaging over all loci. For the "grid" scale, two different data sets were considered in the analyses, in order to assess the differential contribution of isolation by distance and clonal growth to genetic structure: a) for the ramet level all sampled individuals have been included; b) for the genet level distinct genotypes were included only once. In the latter case, a randomly chosen data point from each clone was taken into the data-set. 16 distance classes were fixed. The size of the smallest distance class was calculated according to Epperson & Chung (2001) and resulted in 2.8m. Size of each distance class was thus approximated to 3m. 95% confidence envelopes were defined through 1000 permutations of genes and spatial locations. A two tailed t-test was conducted in order to test for significant differences in kinship values between ramets and genets.

For the quadrat scale, the analysis was conducted only considering the ramet level (i.e. all individuals were included) because sample size for genets was too small to allow any significance of the analysis. For this scale, size of distance classes was set to 20cm.

An interesting application of autocorrelation analyses is the estimate of Sp statistics (Vekemans & Hardy 2004). Because it is independent on the sampling scheme, it allows a quantitative estimate of genetic structure. The rationale is based on the expectation that in the presence of isolation by distance, correlation parameters decrease linearly with the logarithm of the distance at least in a spatial range depending on gene dispersal and effective density of the population. The Sp value results from the slope of the regression of a kinship coefficient (f_{ij} of Loiselle *et al.* 1995) against the logarithm of the distance:

$$Sp = -b_F / (1-F_I)$$

where $-b_F$ is the regression slope and F_I is an estimate of inbreeding coefficient of the population. The b_F and F_I values were provided by the software SPAGEDI.

The regression slope also allows an estimate of gene dispersal (σ_g) through an estimate of neighbourhood size (Nb). The σ_g was calculated as:

$$\sigma_g = (Nb/4\pi D_e)^{0.5}$$

where D_e is the effective density of the population and $Nb = -1(1- F_I)/b_F$. gene dispersal can only be estimated within the range of linearity of the regression (Rousset

1997). This range goes from σ_g to approximately 20 σ_g for microsatellite markers (Heuertz *et al.* 2003).

These estimates were calculated for the genet level only, being gene dispersal within clonal neighbourhood uninfluent for a dioecious species. Actual sampling density was 0.245 shoots/m^2 (304 samples over an approximate area of 1240m^2). Considering that D_e can be estimated as from one half to one tenth of the actual density in natural populations, we have chosen the lower value ($0.1D_e$) because in a clonal plant effective population size and density are additionally reduced by replication of genets.

The estimated value of σ_g was used to calculate the theoretical range of linearity of the relationship and the process was reiterated within the new distance ranges until the value of σg was stabilized.

Results

GENETIC DIVERSITY: Seven microsatellite loci displayed 35 alleles in total (Table 3.1). Observed heterozygosity ranged from 0.26 for locus *Cy 1* to 0.79 for locus *Cy 20*. Inbreeding coefficients (*f*) of single loci ranged from -0.123 for *Cy 20* to 0.099 for *Cy 17*. Values were significantly negative for all loci except for *Cy 17*.

The theoretical number of possible genotypes with the seven loci used (Ng) was $3.42 \cdot 10^9$. Probability of identity (P_i) values for each multilocus genotype were always lower than the threshold of 0.001 recommended for the rejection of identity by chance of genotypes (Waits *et al.* 2001) and ranged from $5.14 \cdot 10^{-10}$ to $6.95 \cdot 10^{-4}$. The microsatellite loci used in this study allowed thus the unequivocal assignment of ramets to clones.

Table 3.1: The seven *Cymodocea nodosa* microsatellite loci used in the present study. Number of alleles, observed and expected heterozygosity and inbreeding coefficient (*f*, Weir & Cockerham, 1984), calculated on the whole dataset after removal of replicated genotypes are shown.

Locus name	N. alleles	H_o	H_{exp}	*f*
Cy 1	3	0.2632	0.2462	-0.066*
Cy 3	3	0.5944	0.5462	-0.087*
Cy 4	4	0.6957	0.6537	-0.046*
Cy 16	11	0.7647	0.7516	-0.016*
Cy 17	4	0.5418	0.6002	0.099*
Cy 18	5	0.6037	0.5910	-0.020*
Cy 20	5	0.7864	0.6993	-0.123*

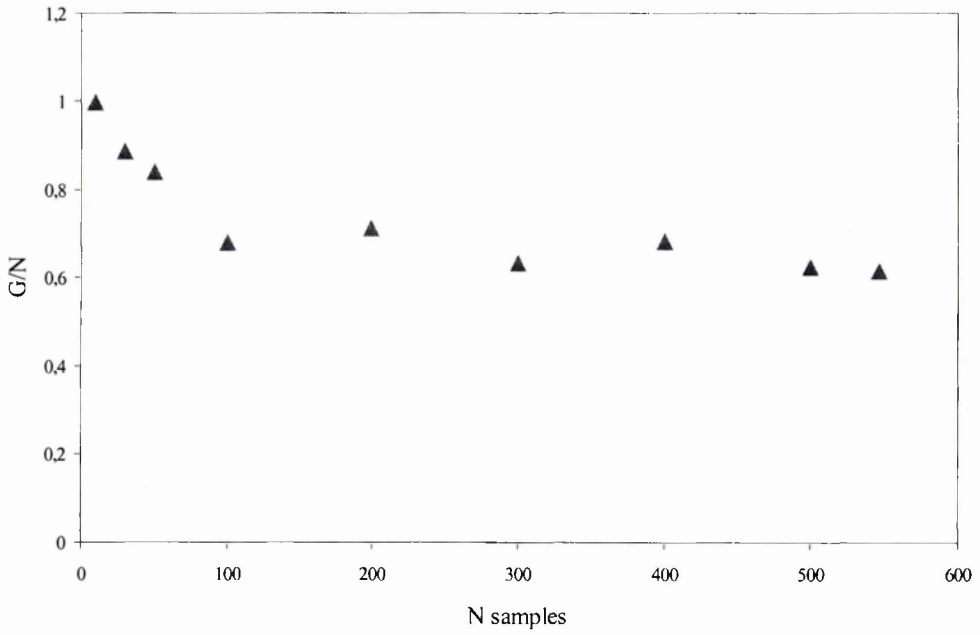

Fig. 3.3: Number of recorded genotypes in relation to the number of sampled individuals (G/N) in the seagrass *Cymodocea nodosa*. The last point represents the complete data-set (546 samples). 100 individuals are needed to obtain a reliable estimate of clonal diversity.

Determination of clonal diversity (G/N) on random subsets of the dataset revealed that 100 shoots is the minimum number of samples needed to get a reliable estimate of clonal diversity (Fig. 3.3). Higher sample sizes did not improve the estimates, while replicates of smaller size revealed higher values of G/N. For that reason, clonal diversity values for quadrats and cores was considered over the whole data-set within each of the two sampling scales. Clonal diversity (G/N) was 0.67 for the grid, 0.62 for quadrats and 0.55 for cores (Table 3.2).

Observed heterozygosity values were 0.63 for the grid, 0.61 for the quadrats (ranging from 0.54 to 0.70) and 0.56 for the cores (ranging from 0.52 to 0.74). None of the data sets was in Hardy-Weinberg proportions. The inbreeding coefficient (*f*) was significantly negative (95% CI after 1000 bootstraps) in all three spatial scales (Table 3.2), ranging from -0.07 for the grid to -0.02 for the cores. Values calculated on the whole data-set were similar to the ones calculated on the sub-sets (Table 3.2).

Table 3.2: Diversity values for the three sampling scales and overall. G/N, observed and expected heterozygosity, accordance to Hardy-Weinberg proportions (HW, P-value) and inbreeding coefficient (*f*, Weir & Cockerham, 1984), calculated after removal of replicated genotypes are shown. N=Sample size, G=number of genotypes, U=number of genotypes found only once within each sampling scale.

	N	G	U	G/N	U/G	Mean n. all./Locus	Hexp (Std. Dev)	Hobs (Std. Dev.)	HW	*f*
Grid	304	204	173	0.67	0.85	5.14	0.5905 (0.1572)	0.6331 (0.1730)	**	-0.0696
Quadrats (all)	123	76	61	0.62	0.80	4.43	0.5769 (0.1851)	0.6109 (0.2025)	**	-0.0524
Cores (all)	119	65	52	0.55	0.80	4.43	0.5290 (0.1715)	0.5429 (0.2261)	**	-0.0184
Overall	546	323	266	0.59	0.82	5.43	0.5838 (0.1646)	0.6059 (0.1761)	**	-0.0363

**=P-value <0.01

Analysis of variance at the genet level revealed no significant differences in clonal diversity (G/N; P=0.36), observed heterozygosity (P=0.44) and *f* (P=0.78) among the three spatial scales (Table 3.3).

Table 3.3: One-way ANOVA for inbreeding coefficient (*f*), clonal diversity (G/N) and observed heterozygosity (H_{obs}) among the five quadrats, the five cores and five sub-plots of the whole grid. The sub-plots were chosen in order to preserve independence of samples.

		f			G/N			H_{obs}		
Groups	*df*	*MS*	*F*	*P-value*	*MS*	*F*	*P-value*	*MS*	*F*	*P-value*
Sampling scale	2	0.0019	0.2479	0.7843	0.0385	1.1047	0.3628	0.0051	0.8818	0.4392
Residual	12	0.0079			0.0348			0.0057		

CLONE DISTRIBUTION AND SIZE: Size-class distribution of genotypes (Fig. 3.4 and Table 3.4) was highly skewed for all sampling scales. In general, few big clones were present, together with many small clones and many individual genotypes. At the "grid" scale, 173 genotypes (~85% of genotypes) were sampled only once and 31 genotypes were represented by at least two individuals (~15%). The two largest clones were represented by 16 and 17 clonemates, spreading over a distance of 43-48 m. In Fig. 3.4, a map of the spatial position of genotypes is shown. Most of the clones were not recognisable as discrete units, except for few, very small groups (e.g., 38, 39, 106, 108).

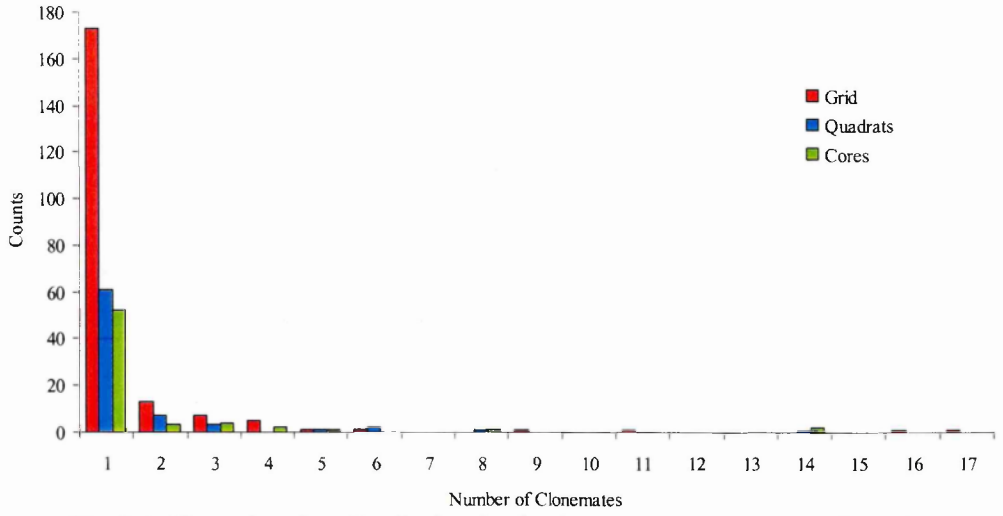

Fig. 3.4: Clone size-class distribution for the three sampling scales: number of genotypes (y-axis) consisting of the same number of clonemates (x-axis). The number of genotypes found only once was 173 for the grid, 61 for the quadrats and 52 for the cores, respectively.

Table 3.4: List of clones. Genotypes found only once are not listed. Number of clonemates and maximum linear spread are shown. Sharing of clones among the three sampling scales is also shown. G= Grid; Q= Quadrats; C=Cores.

Genotype #	N clonemates	Belongs to	Max linear Distance (m)	Genotype #	N clonemates	Belongs to	Max linear Distance (m)
1	4	G/Q	60.53	106	2	G	2.00
7	7	G/Q	21.54	108	2	G	2.00
8	2	G	14.00	116	3	G	8.25
10	4	G/Q	24.74	121	2	G	7.21
11	31	G/Q	43.27	124	2	G	16.50
13	9	G	32.00	128	5	G/C	8.24
15	10	G/Q	17.89	168	2	G	4.47
18	5	G	10.20	173	3	G/Q	16.00
26	15	G/C	28.28	182	2	G/Q	10.20
27	12	G/Q	11.18	200	2	G/Q	2.00
38	2	G	2.00	201	4	G/Q	4.47
39	4	G	4.47	206	6	Q	0.85
43	25	G/Q/C	48.37	211	15	Q/C	7.21
46	2	G	18.11	233	3	Q	18.44
47	3	G	38.47	234	2	Q	7.21
48	2	G	13.42	236	2	Q	18.44
50	2	G	60.00	238	3	Q	0.28
59	2	G/Q	17.00	243	2	Q	14.00
61	2	G	8.25	252	2	Q	0.28
64	4	G	22.80	256	2	Q	0.28
67	6	G	27.20	260	5	Q/C	39.29
68	2	G/Q	12.60	271	2	C	4.00
69	2	G	11.31	285	3	C	8.00
73	4	G	13.42	288	2	C	-
76	3	G	16.12	303	3	C	-
78	2	G/C	21.60	304	2	C	-
93	16	G/Q/C	20.00	308	3	C	-
95	4	G	22.09	309	5	C	-
102	3	G	12.65				

Genotype #	N. clonemates
Core #1	
26	2
271	1
Core #2	
26	1
93	2
271	1
285	2
Core #3	
26	3
43	1
78	1
93	2
303	3
304	2
Core #4	
26	4
93	2
128	1
285	1
308	3
309	5
Core #5	
26	4
43	2
93	2
128	3
211	14
260	4

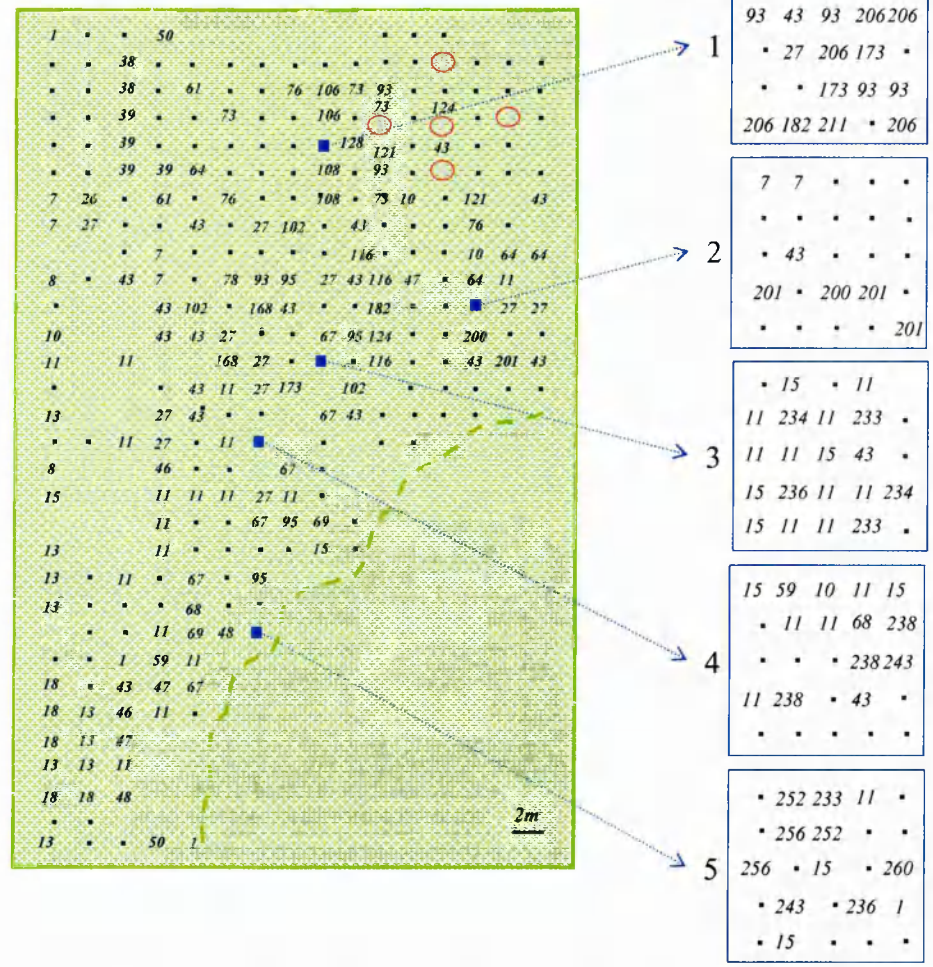

Fig. 3.5: Genotype distribution among grid, quadrats and cores. Position of quadrats and cores within the grid is also shown. Points indicate unique genotypes (genotypes found only once). The absence of points or numbers indicates gaps in the meadow. Unique genotypes are not listed for the cores. Genotype reference numbers are as in Table 3.4.

At the "quadrats" scale (Fig. 3.4, and Table 3.4), 61 genotypes were sampled only once (~80%) and 15 genotypes were represented by at least two individuals (~20%). Some genotypes are present in more than one quadrat (Table 3.4): within each quadrat, largest clones spread over a distance of 80-100 cm. In Fig. 3.5, the spatial position of genotypes within the quadrats is shown. Although clumping is more pronounced at the "quadrat" scale, intermingling is still evident.

At the "core" scale, (Fig. 3.4, Table 3.4), 52 genotypes were sampled only once (~80%) and 13 genotypes were represented by at least two individuals (~20%). Considering single cores, the largest clone was represented by 14 ramets. Six of the 13 clones were shared among the cores (Table 3.4).

AUTOCORRELATION ANALYSIS: For the "grid" sampling scale, a moderate genetic structure was observed, with kinship values of 0.0456 and 0.0436 at the smallest spatial class (3m) for the ramet level and for the genet level respectively (Table 3.5). Values were significantly positive until a distance of about 10m (Fig. 3.6) for the genet level and about 16 m for the ramet level.

Table 3.5: Sp statistics for genets and ramets. Gene dispersal estimates are for the genet level only. f_{ij}= Kinship coefficient at the minimum distance class (3m); Nb = Neighbourhood size; σ_g = lower limit of gene dispersal range; $20\sigma_g$ = upper limit of gene dispersal range.

	Ramets	**Genets**
f_{ij} (3m)	0.0456	0.0436
Sp value	0.0185	0.0156
Nb	---	54.08
σ_g	---	1.081
$20\sigma_g$	---	21.63

There were no significant differences in means between ramets and genets (t-test P value 0.86), indicating a low contribution of genotype identity to kinship values. The threshold of 10m can be considered as the diameter of the minimum panmictic unit (i.e. the neighbourhood size, *sensu* Wright 1943) and provides an estimate of the local gene dispersal. No significant kinship structure could be revealed for the fine-scale quadrats (data not shown) for any distance class.

SP STATISTICS: Sp values were, being 0.0185 for the ramet level and 0.0156 for the genet level (Table 3.5), indicating moderate levels of genetic structure. Value for the ramet level was slightly higher than for the genet level. Gene dispersal was also low and ranged from about 1 to 22m, with an average value of 11.35m. Nb value for the genet level was 54.

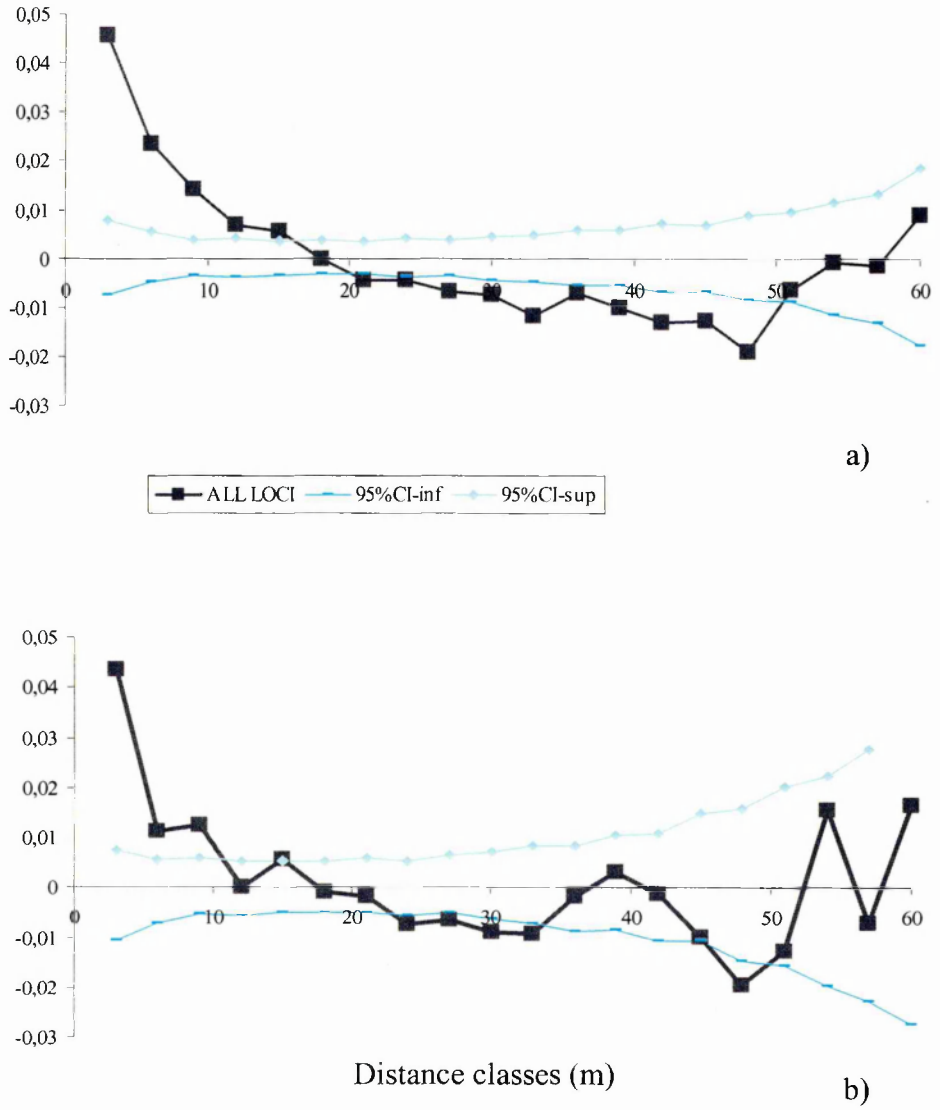

Fig. 3.6: Spatial autocorrelation of kinship (f_{ij}) over all loci at the grid scale (30x60m). Two data-sets were analyzed: including all samples and b) including a random sample for each clone. 95% confidence intervals derived by permutation in SPAGEDI are also shown.

Discussion

High clonal diversity was found in the studied population, reaching values as high as 67% of distinct genotypes. The clonal map showed that genets were highly intermingled and not recognizable as discrete, clumped units. These findings can be related to three interacting processes: i) The balance between the persistence of old, founder genets and the rate and success of sexual reproduction in the population; ii) A poor seed dispersal potential and a Repeated Seedling Recruitment strategy (RSR, Eriksson 1989; 1993); iii) A "guerrilla" clonal growth form that leads to intermixing of clones.

i) Watkinson & Powell (1993) predict that the ratio of seedling to ramet recruitment is fundamental in the make-up of clonal structure and that even low levels of sexual recruitment are capable to maintain high genetic diversity in plant populations. They also predict that inequality in size of genets increases with time. The size-class distribution of genets found in the population is consistent with such a scenario, revealing the presence of few very large clones, together with many small clones and a high percentage of unique genotypes. Smaller genets (as small as 30cm) and unique genotypes could be the result of recent events of seedling recruitment. Bigger clones can reach considerable sizes, with linear spread between the most distant clonemates as high as 60m. These large clones could represent founder genets of the population. In fact, given the described horizontal rhizome elongation rate of 30cm y^{-1} for a *C. nodosa* population in Ischia (Cancemi *et al.* 2002) and assuming physical connection of ramets, small clones and unique genotypes should date up to 1 year before, while a 60 m clone should be at least 200 years old.

This estimate is not unrealistic for seagrasses. Ancient clones were discovered in two other seagrass species: in the Mediterranean endemic *Posidonia oceanica*

(Ruggiero *et al.* 2002), and in *Zostera marina* (Reusch *et al.* 1999a), the most important seagrass in the northern temperate hemisphere, clones dating up to thousands years ago have been found. The presence of large clones could also account for the observed excess of heterozygosity found in the studied population. Hämmerli & Reusch (2003b) showed a significant positive correlation between heterozygosity and clone size, which can be considered as a measure of genet fitness. The higher frequency of multilocus heterozygote genotypes could thus be the result of a selective heterozygote advantage due to local adaptation. Nonetheless, an overestimation of clone age is possible, due to rafting of shoots or rhizome fragments that reattach and grow in distant locations within the meadow.

ii) The coexistence of older genets together with newly recruited ones suggests a low dispersal of seeds together with a RSR; seeds seem to be, in fact, maintained within the population, so that derived genotypes intermingle with the pre-existing ones. Eriksson & Fröborg (1996) argue that the potential for seed germination and seedling recruitment and the subsequent emergence of new genotypes is enhanced by the opening of gaps within the canopy. This is likely to occur in the studied population. The study site is located in a relatively sheltered area, but in the summer season the meadow suffers of anthropic impact through boat anchoring, that can induce detachment of discrete portions of the meadow, leaving wide gaps within the canopy.

iii) Intermingling of genets is also likely to derive from a "guerrilla" growth strategy. In the monoecious seagrass *Zostera marina* (Hämmerli A 2002) a clumped distribution of clones has been described, attributable to a "phalanx" growth strategy. In the two species, tight proximity of clonemates seems not to negatively influence reproductive success, probably because mechanisms of cryptic self-incompatibility

(Hämmerli & Reusch 2003a) have evolved in order to reduce levels of geitonogamy. In *Cymodocea nodosa*, in contrast, a highly intermingled arrangement of clones has been observed. This is expected in a dioecious species, allowing the population to avoid drawbacks of a clumped distribution of genets. The formation of monoclonal-unisexual discrete patches could in fact result in a lack of gametes of the opposite sex in the immediate proximity, leading to low sexual reproduction rates (Charpentier 2002).

Correlograms obtained separately for genets and ramets show minor differences, both in kinship values at the smallest distance class and in the estimated neighbourhood size. Although values for the genet level are somewhat higher than for the ramet level, nearly all of the nonrandom, spatial genetic structure is caused by sexually reproduced individuals, not by clones. This is probably due to the absence of spatial clumping of identical genotypes even at small distance classes. With nearly random distributions of clones in local populations, it is expected that clonal reproduction should not substantially increase the degree of local consanguineous mating (Chung & Epperson 2000).

Correlation decreases quite rapidly with distance, giving an estimate of the neighbourhood size diameter of about 10 m for the genet levels. This value is nevertheless higher than in other seagrass species, for example in the eelgrass *Zostera marina* (Hämmerli & Reusch 2003c). Genetic structure as from the Sp value was once again slightly higher for the ramet level than for the genets. A comparison with terrestrial plants from the exhaustive review of Sp values in Vekemans & Hardy (2004), shows that Sp value for *C. nodosa* is similar to that of predominantly outcrossing, herbaceous species, corresponding to the herbaceous habit and dioecy of the species. Gene dispersal as estimated from the regression analysis is coherent with what was

found from the correlograms, giving a range from 1 to 22m, with an average of about 11m. This estimate is comprehensive of both seed and pollen dispersal, indicating that male gametes could also poorly disperse and that gene flow could not be extended enough to avoid biparental inbreeding. However, the small values of kinship and the high heterozygosity observed suggest that biparental inbreeding is negligible in the studied *C. nodosa* population. At a smaller scale, autocorrelation within each quadrat was not significant, indicating the absence of family structure within a neighbourhood of about 1m. This is in accordance to the estimates obtained for gene dispersal (σ_g) at the grid level, with the lower range boundary of about 1m.

C. nodosa is thought to be characterized by a poor potential for seed dispersion, because seeds are released below the superficial sediment layer nearby the mother plant. Values of gene dispersal from the present study suggest that physical factors such as sediment resuspension following storms and "sweeping" by waves in the presence of underwater currents especially in shallow waters, can enhance seed dispersal, although only at a meters scale.

Plant populations have a spatial component that needs to be thoroughly considered in order to assess the correct sampling scale at which genetic structure and diversity can be revealed (Widén *et al.* 1994). In this study, three different sampling scales have been analysed, from meters (grid) to millimetres (cores). The absence of significant differences in the genetic estimates among the three spatial scales could be explained with the observation that genotypes are highly intermingled. The high density of different genets in the studied population is evident even when considering the smaller spatial scales. In a quadrat of 80x80cm up to 22 different genotypes were found, and sampling in a core of 20cm in diameter allowed up to 19 genotypes. This finding

leads to the conclusion that genotypic and genetic diversity must be evenly distributed among the population, regardless of the spatial scale.

According to the expectations, *C. nodosa* in the studied population exhibits high rates of sexual recruitment and poor dispersal potential, two attributes that are characteristic for a RSR strategy. The success of sexual reproduction in this population can be related to a "guerrilla" growth strategy that leads to a highly intermingled distribution of genets, thus allowing the presence of gametes of the opposite sex in the immediate proximity (Charpentier 2002).

In order to assess implications of reproductive features on demography and spatial layout of plant populations, further studies comparing genetic structure in species with similar ecological requisites but different mating systems are desirable.

CHAPTER IV – The effects of mating system on clonal architecture: a comparative study in two marine angiosperms*♦

Abstract

In this paper we present a comparative study of the clonal architectures of *Cymodocea nodosa* and *Zostera noltii* at the same location in a mixed stand, in order to verify the hypothesis that clonal growth strategies and the resulting genet architecture are driven by mating system costs. Microsatellite loci have been used to identify clones and assess their spatial distribution in both species. An intermingled configuration of genet have been found in the dioecious, obligate outcrossing *Cymodocea nodosa* and, on the contrary, a clumped, "phalanx-type" distribution of clones in the hermaphroditic, self-compatible *Zostera noltii* has been observed. We hypothesise that the possibility of reduction in the seed-set would drive genet distribution, rather than inbreeding avoidance.

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* Patrizia Pirozzi and Stefano Capone contributed to this study, principally in genotyping *Zostera noltii* samples.

♦ The present study has been submitted to *Evolutionary Ecology* as:
Ruggiero MV, Capone S, Pirozzi P, Reusch TBH and Procaccini G *The effects of mating system on clonal architecture: a comparative study in two marine angiosperms.*

Introduction

Plant mating systems are varied, ranging from self-compatibility in hermaphroditic plants to self-incompatibility and obligate outcrossing in dioecious plants. These differences result in different levels of inbreeding with profound effects on the genetic variability of populations. In many plant species inbreeders are generally characterised by lower genetic diversity and larger between-populations differentiation than outcrossing species (reviewed in Charlesworth 2003).

For the study of mating system evolution, clonal plants need special attention because their within-population genetic diversity is hierarchically organized. Their genetic diversity and structure rely both on sexual recombination and asexual replication of identical genotypes. On the one hand, the balance between the two kinds of reproduction influences genetic structure, the effective population size and the number of genets per area (Ashton & Mitchell 1989; Eckert & Barrett 1992; Eriksson 1996; Reusch 2001a). Watkinson & Powell (1993) predict that the ratio of seedling to ramet recruitment is fundamental in the make-up of clonal structure and that even low levels of sexual recruitment are capable to maintain high genetic diversity in plant populations. On the other hand, clonal growth forms affects the size and spatial distribution of genets, interfering with patterns of pollen dispersal and thus with mating opportunities (Handel 1985; Charpentier 2002).

Lovett-Doust (1981) described two extremes along a continuum that are useful to conceptualize clonal growth forms in plants: in the phalanx type of growth, ramets are connected by short internodes and are closely spaced. Such strategy leads to a mosaic genotypic structure in which clones are recognisable as discrete units and most neighbour interactions are intra-clonal; in the guerrilla strategy, internodes are long and

ramets are widely dispersed, leading to an intermingled make-up of genets, in which most neighbour interactions are inter-clonal.

In self-compatible species, a phalanx growth strategy is expected to be advantageous because, although large clonal patches have been predicted to increase selfing through geitonogamy (Eckert 2000), the cost of inbreeding is balanced by the cost of a reduced seed set through limitation of compatible pollen. In dioecious species, monoclonal patches are all together monosexual and outcrossing is obligate. Avoidance of inbreeding should not thus drive growth form, while reduction in the seed set, due to deficit of pollen in the immediate neighbourhood, can be limited through an intermingled composition of genets (Charpentier 2002). Dioecy and concomitant obligate outbreeding should therefore be favoured by a guerrilla growth strategy. These hypotheses are supported by data on clone distribution in species with different levels of outcrossing (Stebbins 1950, cited in Silander 1985): of 71 perennial grasses, 93% of guerrilla-growing species were found to be self-incompatible while only 77% of phalanx-growing species were found to be self-compatible.

The interactions between clonal architecture and mating patterns remain largely unexplored, and studies on clonal growth and mating have rarely been associated. Clonal architecture has been determined in various clonal plants and algae (Maddox *et al.* 1989; Montalvo *et al.* 1997; Kudoh *et al.* 1999; Pornon & Escaravage 1999; Ivey & Richards 2001; Hangelbroek *et al.* 2002; van der Strate *et al.* 2002; Xie *et al.* 2001; Albert *et al.* 2003). None of these studies, however, has taken into consideration the effects of clonal architecture on the reproductive potential of the population. In the marine plant *Zostera marina*, however, the effect of clonal distribution on the seed-set has been investigated (Hämmerli & Reusch 2003a).

Marine angiosperms (seagrasses) constitute a polyphyletic group of about 60 monocotyledon species, belonging to the sub-class Alismatidae, and evolved independently along at least three phylogenetic lineages (Les *et al.* 1997). Most seagrass species exhibit a mixture of clonal growth along with sexual reproduction. Within sexual reproduction, a multitude of breeding system can be found comprising hermaphroditism, monoecy and dioecy (Les 1988). In sharp contrast to terrestrial plants, however, 78% of seagrass species are dioecious. This high representation of dioecious species at sea has been regarded as an adaptation to hydrophilous pollination, in order to promote sufficient levels of outcrossing (Cox 1993). The wide array of mating systems found in marine angiosperms makes this group a useful model to investigate the influence of breeding on clonal architecture and thus to population's genetic structure.

The dioecious *Cymodocea nodosa* (Ucria) Ascherson and the monoecious *Zostera noltii* Hornem are widely distributed in the Mediterranean Sea and are often in association in mixed stands on subtidal sandy bottoms. Recent studies have investigated genetic structure in *Zostera noltii* and *Cymodocea nodosa.* In *Z. noltii*, small, contiguous clones were observed in two populations (Coyer et al, *in press*). In *C. nodosa*, in contrast, a highly intermingled configuration of clones has been observed at a cm scale (Ruggiero MV, Chapter III of present thesis).

In this paper we present a comparative study of the clonal architectures of *Cymodocea nodosa* and *Zostera noltii* at the same location in a mixed stand, in order to verify the hypothesis that clonal growth strategies and the resulting genet architecture are driven by mating system costs. When in mixed stands, species undergo to the same environmental constraints, so that a comparison of their genetic structure can only be based on their different reproductive features and thus environmental heterogeneity can

be disregarded. In mixed stands, however, interactions between the species such as competition or facilitation could occur, influencing local genetic structure of one or both species. In order to test for this hypothesis, we compared clonal structure and genetic diversity between monospecific and mixed stands for each species. Clones were identified by means of polymorphic species-specific microsatellite *loci* as molecular markers in both species.

Materials & Methods

SAMPLING SCHEME: The study site is located at the embayment of the Castello Aragonese in Ischia (40° 44' N; 13° 58' E; Naples, Italy). Here *Cymodocea nodosa* forms a continuous meadow at 4m depth on a sandy bottom on an area of approximately 1800 m^2. At the borders of the meadow, *C. nodosa* can be found in association with *Z. noltii.* The latter forms monospecific patches outside the *C. nodosa* meadow. At the studied site, thus, both species can be found in monospecific and mixed stands. In 2002, shoots of *C. nodosa* and *Z. noltii* have been collected in a plot of 6x30 m; *C. nodosa* was collected according to an imaginary grid of 2 m size meshes; *Z. noltii* was collected within the same grid in three of the four transects parallel to the long side of the plot (30m). In 2003, five plots of 1 x 3 m with an imaginary grid with meshes of 50 cm side have been randomly located within the mixed stand. At each point of the grid, one shoot for each species has been collected, for a maximum of 21 shoots per species per plot.

In order to verify if ecological interactions between the two species affected genetic structure, masking the effect of mating system alone, the five plots were replicated in monospecific patches for each species and results were compared between monospecific and mixed stands. All sampling was conducted by SCUBA diving. From now on, we will refer to the larger plot as to "grid".

DNA EXTRACTION AND MICROSATELLITE MULTI-LOCUS GENOTYPE DETECTION: 5 mg of silica-gel dried tissue from each individual sample have been ground through Mixer Mill MM300 (QIAGEN). Subsequent DNA extraction has been carried out using the Qiagen DNAeasy Plant Mini Kit (QIAGEN).

Six polymorphic microsatellite loci were used for *Z. noltii* (Coyer *et al.* 2004) and 6 microsatellite loci were used for *C. nodosa* (Ruggiero *et al.* 2004, Chapter II of present thesis), in order to obtain multilocus individual genotypes (Table 1a, b). In Table 1c multiplex reactions for *Z. noltii* are shown and all other conditions were as follows: 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 u Taq (Roche) in a total volume of 10 μl. PCR cycles were as follows: an initial denaturation step of 5' at 94°C; 40 cycles consisting in 40'' at 94 °C, 40'' at T ann. and 40'' at 72 °C, followed by a final extension step of 10' at 72 °C. All PCR reactions were conducted in a PCR-Express thermocycler (Hybaid).

PCR conditions for *C. nodosa* are as in Ruggiero *et al.* (2004, Chapter II of present thesis). Allele detection was conducted through automated sequencing (CEQ 2000XL DNA Analysis system, Beckman Coulter) for fragment analysis.

DATA ANALYSIS: For each species, number of genotypes, identification of clones and probability of identity of genotypes (Pi, Waits *et al.* 2001) were calculated with the help of the software GIMLET (Valière 2002). The probability of identity (Pi) was estimated in order to test identity by chance of genotypes. A threshold of Pi values of 0.001 is recommended for the rejection of the hypothesis (Waits *et al.* 2001). Clonal diversity was calculated as the percent of different genotypes over the total number of sampled ramets (G/N, Pleasant & Wendel 1989).

Mean number of alleles/locus, observed and expected heterozygosity and the estimator *f* of inbreeding according to Weir & Cockerham (1984) have been estimated through the software GENETIX (Belkhir *et al.* 1996-2002; website: www.univ-montp2.fr/~genetix/genetix/intro.htm), after removal of replicated genotypes.

Significance of f was assessed through estimation of the 95% CI after 1000 bootstraps. All analyses were conducted both on single plots and considering overall samples. In particular, overall N, G, S and mean number of alleles/locus were calculated pooling the five plots, while G/N, H_{exp}, H_{obs} and f were averaged over the five plots.

Differences in G/N, observed heterozygosity, inbreeding coefficient and mean number of alleles per locus between the two species were statistically compared through t-tests. T-tests were also conducted to compare differences in above variables between monospecific stands of *Z. noltii* and *C. nodosa* alone with mixed stands of *Z. noltii/C. nodosa.*

Table 4.1: Microsatellite loci used in the present study. Repeat motif, Number of Alleles and Size range of alleles for each locus are shown. a) loci for *Cymodocea nodosa*; b) loci for *Zostera noltii*; c) PCR conditions for multiplexing for *Zostera noltii* loci. Three triplex are described (a, b, c). Primer concentrations and annealing temperatures are provided. All other conditions are as in the text.

	Microsatellite Repeat	N Alleles	Size range of Alleles
Cy 3	$(GA)_{12}$	4	140-150
Cy 4	$(TAA)_9$	3	154-169
Cy 16	$(CA)_8(CT)_{22}$	7	175-201
Cy 17	$(CT)_{17}CG(AT)_{10}$	6	230-260
Cy 18	$(CA)_{16}$	3	143-155
Cy 20	$(TC)_{19}$	6	179-211

a)

	Microsatellite Repeat	N Alleles	Size range of Alleles
3f-8	$(TC)_{17}$	4	197-216
3f-11	$(CT)C(CT)_{15}$	4	277-297
B3	$(GA)_{21}$	5	180-223
3b-8	$(GA)_{14}$	3	139-149
3b-1	$(GA)_4G(GA)_{20}$	4	91-116
3d-6	$(TC)_{13}(TGTC)_3(TC)_9$	5	185-225

b)

Locus	Dye	Multiplex	Primer Concentration (μM)	T ann. (°C)
3f-8	CY5	a	0.25	58
3f-11	IRD700	a	0.5	58
B3	CY5	b	0.25	56
3b-8	IRD700	b	0.5	56
3b-1	CY5	c	0.5	60
3d-6	IRD700	c	0.5	60

c)

Results

The microsatellite loci used displayed a comparable number of alleles for the two species (Table 4.1). In total, 29 alleles were found for *C. nodosa* and 24 alleles for *Z. noltii*. Mean number of alleles was higher for *C. nodosa* than for *Z. noltii*, with respective overall values of 5.7 and 4.2 (Table 4.2). All Pi values ranged from $1.04 \cdot 10^{-8}$ to $2.41 \cdot 10^{-4}$ in *C. nodosa* and from $1.24 \cdot 10^{-9}$ to $1.70 \cdot 10^{-4}$ in *Z. noltii*, indicating that the risk for falsely inferring clonal identity by chance of genotypes is very low (i.e. smaller than P=0.001, Waits *et al.* 2001). Overall clonal diversity values were very different among the two species (Table 4.2); G/N value was 0.628 (±0.227) for *C. nodosa* while it was much lower for *Z. noltii* (0.125 ± 0.034). Number of single genotypes (i.e. found only once) was higher for *C. nodosa* (overall 73) respect to *Z. noltii* (overall 5). Overall number of observed genotypes was generally less than the sum of genotypes for each plot because of the presence of shared genotypes. Heterozygosity values were higher in *Zostera noltii* (overall value 0.903 ± 0.082) than in *Cymodocea nodosa* (overall value 0.673 ± 0.077). Both species presented an excess of heterozygosity, as shown by negative overall *f* values, although *C. nodosa* value approached 0 (-0.053 ± 0.105 for *C. nodosa*, -0.254 ± 0.167 for *Z. noltii*). All differences in the calculated parameters between the two species were significant (Table 4.3).

In Table 4.3, t-test P-values between monospecific and mixed stands for each species are also shown for clonal diversity (G/N), average expected and observed heterozygosity, inbreeding coefficients and mean number of alleles per locus. No significant differences were found between monospecific and mixed stands for the above parameters, indicating no effects of interspecific interactions on the genetic diversity of the two species.

Table 4.2: Number of samples (N), number of genotypes (G), number of unique genotypes (i.e. found only once, U) expected and observed heterozygosity, inbreeding coefficient (*f*) and mean number of alleles per locus for each plot (G=grid 30x60m; M=plot 1x3m; C= *Cymodocea nodosa*; Z=*Zostera noltii*) and overall. Overall N, G, U and mean number of alleles/locus were calculated pooling the five plots; G/N, Hexp, Hobs and *f* were averaged over the five plots (in brackets, standard deviations are shown). a) *Cymodocea nodosa*; b) *Zostera noltii*.

	N	G	U	G/N	Hexp	Hobs.	*f*	Mean N All./locus
GC	56	41	34	0.732	0.644	0.683	-0.049	4.833
MC1	20	7	5	0.350	0.599	0.786	-0.241	3.167
MC2	21	8	4	0.381	0.639	0.708	-0.042	3.333
MC3	20	18	17	0.900	0.587	0.556	0.082	3.833
MC4	20	12	9	0.600	0.620	0.681	-0.054	4.167
MC5	21	17	14	0.810	0.600	0.628	-0.016	4.167
All MC	102	60	47	0.608 (±0.247)	0.609 (±0.208)	0.671 (±0.086)	-0.054 (±0.117)	4.833
Overall	158	95	73	0.628 (±0.227)	0.615 (±0.023)	0.673 (±0.077)	-0.053 (±0.105)	5.667

a)

	N	G	U	G/N	H_{exp}	$H_{obs.}$	*f*	Mean N All./locus
GZ	41	7	4	0.170	0.614	0.833	-0.288	3.000
MZ1	12	2	1	0.167	0.625	1.000	-0.333	3.000
MZ2	21	2	-	0.095	0.604	0.833	-0.053	3.000
MZ3	20	2	-	0.100	0.625	1.000	-0.333	3.000
MZ4	20	2	-	0.100	0.604	0.833	-0.053	3.000
MZ5	17	2	-	0.118	0.542	0.917	-0.467	2.500
All MZ	90	5	1	0.116 (±0.070)	0.600 (±0.034)	0.917 (±0.083)	-0.248 (±0.186)	4.167
Overall	131	12	5	0.125 (±0.034)	0.602 (±0.031)	0.903 (±0.082)	-0.254 (±0.167)	4.167

b)

In Fig. 4.1, the distribution of *Zostera noltii* genotypes in the grid plot (a) and the five plots (b) is shown. Few clones were present at both spatial scales, with all ramets of a clone shortly spaced. At the grid scale, large clones were found, spreading up to 10 m. At the plots level, clumping of clones was even more evident, ramets being regrouped at a cm scale. Most of the plots were formed by two genotypes. Four single genotypes in the grid and only one in all the plots were found. None of the genotypes from the grid were found in the five plots while most of the genets from the plots were shared among them, except for genet #12 (Fig. 4.1a).

Table 4.3: t-test P-values for differences in observed heterozygosity, mean number of alleles per locus, inbreeding coefficient and clonal diversity between *Cymodocea nodosa* and *Zostera noltii* (a) and within each species between monospecific and mixed stands (b). In each comparison, groups include five replicates (N=5). Significant differences were found between *C. nodosa* and *Z. noltii* for all parameters, while no significant differences were found between stands within each species.

	G/N	H_{obs}	*f*	Mean N All./Locus
C. nodosa/Z. noltii (a)	0.000	0.001	0.032	0.004
C. nodosa (Mono/Mixed) (b)	0.950	0.644	0.690	0.390
Z. noltii (Mono/Mixed) (b)	0.617	0.897	0.739	0.398

In *C. nodosa* (Fig. 4.2), many more clones were present at both spatial scales with respect to *Zostera noltii*. At the grid scale, large clones can be identified, spreading up to 18 m. Clonemates were widely spaced and no grouping was evident. At the plot scale, genets were more clumped than at the grid scale, but still high density of different genotypes was evident. A high number of single genotypes were found, both at the grid scale and at the plot scale (see plots #3, 4 and 5 in Fig. 4.2). Some of the genets present at the grid scale were found also in the plots (genets # 2, 4, 37, 38 and 41), while only two genets (#2 and #47) were shared among the plots. No significant differences were

found among monospecific and mixed stand with regard to the clonal architecture (data not shown), indicating that interspecific interactions had no effects on the genetic structure of the two species.

Discussion

Up to our knowledge, this is the first study in which genotypic and genetic diversity of two clonal plant species with contrasting mating systems are described. Significant differences in genotypic diversity and clonal architecture between a monoecious and a dioecious marine plant were observed that may correlate with the predicted fitness costs through clumped or intermingled growth forms. The clonal map showed a marked clumping of clonemates for the monoecious *Z. noltii*, resulting in a mosaic structure in which clones were recognisable as discrete units, as expected for a phalanx growing plant. In the dioecious *Cymodocea nodosa*, genet distribution was, in contrast, typical of a guerrilla plant, with highly intermingled genets and widely spaced clonemates even at small scales. Both *C. nodosa* and *Z. noltii* presented extensive vegetative propagation at different spatial scales, with genets spreading from centimetres up to tens of meters. Density of clonemates, however, was higher in *Zostera noltii*, whereas *Cymodocea nodosa* genets were represented by few ramets covering large areas (see genet distribution in the grid plot in Figs. 4.1, 4.2).

Already in 1950 Stebbins showed that a phalanx growth strategy is associated with self-compatibility while a guerrilla strategy is typical of self-incompatible species, in which inbreeding is only biparental and the reproductive potential can be severely affected by a clumped distribution of genets. More recently, clumping of clonemates has been described in various self-compatible clonal plants.

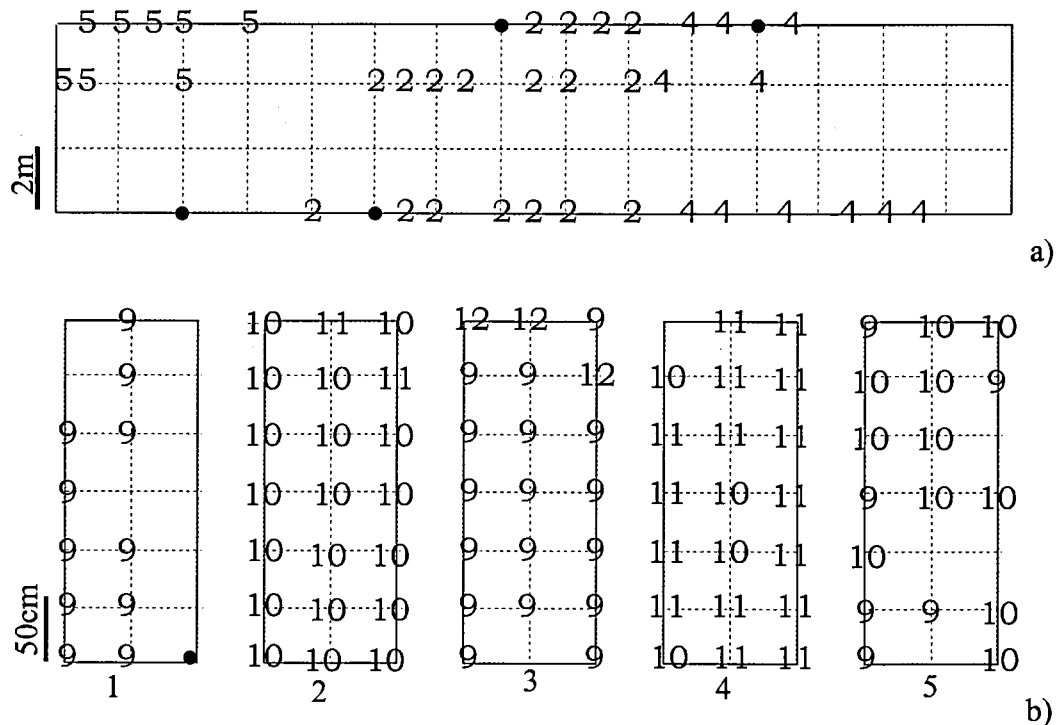


Fig. 4.1: Genotype distribution in a) grid and b) plots for *Zostera noltii*. Each number represents a different genotype. Dots represent single genotypes.

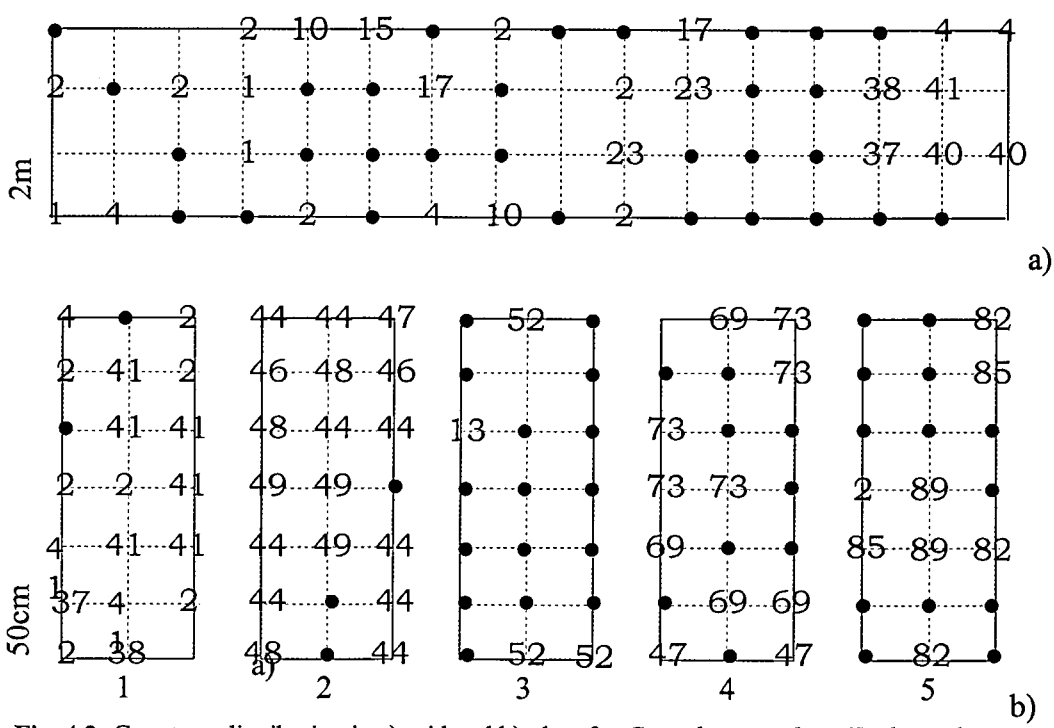


Fig. 4.2: Genotype distribution in a) grid and b) plots for *Cymodocea nodosa*. Each number represents a different genotype. Dots represent single genotypes.

In the well studied monoecious seagrasses *Zostera marina*, where clones were found to be clustered at small spatial scale of 1-5 m (Hämmerli & Reusch 2003c), pollen limitation seems to severely affect the size of seed-set, independently on the genetic composition of the neighbourhood (Reusch 2003), although some levels of geitonogamy are present (Reusch 2001a). In the pondweed *Potamogeton pectinatus*, most ramets from a genet tended to be in each other's vicinity (Hangelbroek *et al.* 2002). Interestingly, at least in *Z. marina*, mechanisms of cryptic self-incompatibility seem to have evolved in order to reduce levels of geitonogamy (Hämmerli & Reusch 2003a). This may represent an alternative strategy to altering the growth form together with the evolution of dioecy as observed here for *C. nodosa.* In dioecious plants, a dispersed distribution of clonemates was observed in *Eurya emarginata* (Chung & Epperson 2000) and seed-set limitation in large clonal patches has been reported in the self-incompatible *Linnea borealis* (Wilcock & Jennings 1999). These studies support the idea that dioecious/self-incompatible species tend to a guerrilla growth strategy and that large clones may suffer from reduced fecundity due to deficit in compatible pollen. Reproductive output seems thus to be more critical in shaping clonal structure than inbreeding avoidance, in both monoecious and dioecious species.

It is generally recognized that self-compatible plants are characterized by a lower genetic diversity compared to self-incompatibles (Charlesworth 2003). In the present study, the dioecious *Cymodocea nodosa* presented a high genotypic diversity, with many clones of different sizes and a high proportion of single genets, possibly indicating a high level of seedling recruitment. This is in accordance with what observed *in situ* in the southern Mediterranean Sea (Pirc *et al.* 1983; Cancemi *et al.* 2002) and what described in a recent study on the genetic structure of the species

(Ruggiero MV, Chapter III of present thesis). In the monoecious *Zostera noltii*, instead, a very low genotypic diversity was observed, with few large clones and few single genotypes, indicating that vegetative propagation exceeds sexual reproduction in the studied population.

Dorken *et al.* (2002) found similar levels of heterozygosity between monoecious and dioecious populations of the clonal aquatic plant *Sagittaria latifolia.* Authors suggest that inbreeding depression, favouring survival of outcrossed offsprings, could be responsible for the maintenance of high levels of genetic polymorphism in the selfing populations. A similar explanation could be applied to the observed excess of heterozygosity in the present study for both species. Inbreeding depression could also account for the higher heterozygosity observed in *Z. noltii* compared to *C. nodosa*. On the other hand, the presence of few large heterozygote clones in the former species could be possibly resulting from a selective heterozygote advantage due to local adaptation: in the monoecious *Zostera marina*, Hämmerli & Reusch (2003b) show a significant positive correlation between heterozygosity and clone size.

Sampling on the two different spatial scales was conducted at distance of one year. The grid was sampled in 2002, while the five plots were sampled in 2003. In *C. nodosa*, some genotypes were present in both the grid and the plots, indicating that these clones were persistent from one year to the other, while for *Z. noltii* none of the genotypes from 2002 were present in 2003. Most of the genotypes from 2003 plots didn't share any genotype in *C. nodosa*, indicating that these possibly younger clones are slow growing. In *Z. noltii*, the genotypes from 2003 are large and shared among the five plots. The comparison between the two groups of genotypes for each species reveals thus that while *C. nodosa* is characterized by persistent, slow growing clones, in

Z. noltii clones suffer of high mortality, but grow faster. This was expected since *C. nodosa* represents a structuring species, compared to *Z. noltii* and it is characterized by a longer life-span than the latter (Buia & Mazzella 1991). *Z. noltii* presents higher horizontal rhizomes growth rate (68 cm/y) compared to *C. nodosa* (40 cm/y, Marbà & Duarte 1998) and it occupies easily the gaps in the canopy left by *C. nodosa* through fast vegetative spreading.

Interactions in mixed meadows could affect genotypic diversity and genetic structure of the two species, due to either facilitation or competition. As a control for interspecific interactions, sampling was replicated in nearby monospecific stands. No significant differences were found between monospecific and mixed stands, strongly suggesting that all differences observed in genetic diversity and structure could only be attributable to differences in reproductive features between the two species.

Evolutionary interactions between clonal growth strategies and mating system evolution are still to be clarified. Clonal growth is a complex multi-trait feature affecting survival of individuals and, by determining the spatial distribution of flowering units, may impose selective pressures on traits which regulate mating system. Our findings here are consistent with the idea that clonal growth traits (e.g. rhizomatous growth length, branching angle) could be driven by their associated mating system costs (Charpentier 2002). More experimental studies, in particular with co-occurring monoecious and dioecious species, are necessary and possible in seagrasses, in order to assess evolutionary relationships between mating pattern and clonal growth.

CHAPTER V – Geographic patterns of populations structure in the marine angiosperm *Cymodocea nodosa* in the Mediterranean Sea.♦

Abstract

Cymodocea nodosa is a clonal marine angiosperm (seagrass), widely distributed in the Mediterranean Sea and extending also on the Atlantic coasts, from Southern Portugal to Northern coasts of Africa. In the present study, we determine levels of gene flow and patterns of genetic variability by means of microsatellite loci, in order to investigate the extent of population differentiation and the existence of genetic differentiation within the Mediterranean Sea. Populations displayed wide variability in clonal diversity (G/N), with values ranging from 0.05 to 1.00. A Bayesian analysis of population structure revealed that all populations from the Adriatic Sea formed a single panmictic unit, as do two populations from Ischia (Gulf of Naples) and the two populations of Malta and Messina. The neighbour-joining tree on Cavalli Sforza chord distance and the Assignment test showed that patterns of genetic diversity were coincident with geographical boundaries within the basin. Results are discussed in the light of oceanographic features of the Mediterranean Sea.

♦ The present study will be submitted to *Marine Biology* as:
Ruggiero MV, Reusch TBH and Procaccini G *Geographic patterns of population structure in the marine angiosperm* Cymodocea nodosa *(Ucria) Ascherson in the Mediterranean Sea.*

Introduction

At sea, the spatial scale of significant genetic structure within species is often greater than in terrestrial habitats. The continuity of the marine environment make the potential for genetic exchange theoretically unlimited (Palumbi 1992; 1994). However, in contrast to the notion of modest phylogeographic divergence over large areas, marine macrophytes are expected to display high levels of population subdivision, due to their very limited seed or spores dispersal potential (Denny & Shibata 1989; Orth 1994). Determining the extent and patterns of gene flow across populations' ranges of geographical distribution is of primary importance in order to shed light on biological and physical factors driving population differentiation and eventually speciation (Slatkin 1993; Palumbi 1994). In the marine environment, currents within and among basins can affect species distribution ranges, driving dispersal of propagules through water circulation patterns (Féral 2002). In contrast, the survival of local populations is under the influence of both ecological plasticity and the genetic make-up of founding individuals (Avise 1998).

The Mediterranean Sea can be divided into ten different biogeographical regions, due to its extension, geological history, and its varied hydrological and climatic conditions (Pérès 1985a). The main physical subdivision of the Mediterranean Sea is between the Western and Eastern basin that are separated by a "terrace" between Tunisia and Sicily (the Siculo-African sill, Pickard & Emery 1990). From an ecological point of view, each biogeographical region is characterized by peculiar hydrographical properties, presenting different surface temperatures and salinities and providing a mosaic of different environments (Pérès 1985a; Sarà 1985).

Marine angiosperms (seagrasses) are structuring species along shallow coastal shorelines worldwide (den Hartog 1970), exerting valuable functions as important primary producers, and providing habitat for a wide variety of economically important species, stabilizing sediment and removing excess nutrient. Their recognized ecological and economic importance (Costanza *et al.* 1997) justifies the growing concern about their world-wide documented regression (Short & Wyllie-Escheverria 1996; Green & Short 2003) and has encouraged an increasing effort in population genetics studies (reviewed in Reusch 2001b).

Recently, a number of studies have approached phylogeographic patterns and genetic variability in seagrasses by means of microsatellite markers. In both *Zostera marina* (Olsen *et al.* 2004), the most extensively distributed marine angiosperm in the northern temperate hemisphere, and in the congeneric *Zostera noltii* (Coyer *et al.* in press), a wide variation in clonal diversity among populations and high values of genetic differentiation between populations were found at large phylogeographic scales. In the endemic Mediterranean marine angiosperm *Posidonia oceanica*, low genetic diversity and low levels of gene flow were detected, and populations clustered in three main groups, corresponding to the main biogeographical sectors of the Mediterranean Sea (Procaccini *et al.* 2001; 2002).

In the present study, levels of gene flow and patterns of genetic variability have been investigated in the marine angiosperm *Cymodocea nodosa* by means of microsatellite loci, in order to investigate the extent of population differentiation and the existence of genetic divergence within the Mediterranean Sea. *Cymodocea nodosa* is a dioecious seagrass species, presenting both vegetative propagation by stolonization and sexual reproduction by germination of seeds. Its distribution area extends mainly in the

Mediterranean Sea but it also expands on the Atlantic coasts, from Southern Portugal to Northern coasts of Africa (den Hartog 1970). It grows in dense meadows, often in association with other seagrasses as the Mediterranean endemic *Posidonia oceanica*, of which it represents the preceding species in the ecological succession, and with *Zostera noltii* (Buia & Mazzella 1991). Although vegetative reproduction has been considered predominant in this species, seeds and seedlings are often recorded *in situ*, especially in the south-western part of the Mediterranean basin (Pirc *et al.*, 1983; Cancemi *et al.*, 2002), suggesting high levels of sexual recruitment in this region. Mature fruits remain buried in the sediment nearby the mother plant in a dormant stage for about 8 months, until germination (Buia & Mazzella 1991). The potential for seed dispersal in this species is then quite limited. A recent study on the spatial genetic structure of a *C. nodosa* population from the Island of Ischia (Gulf of Naples, Ruggiero MV, Chapter III of present thesis) confirmed the limited dispersal potential of the species, showing that seeds can travel few meters far from the mother plant before germination, but that vegetative shoots dispersal could be an important mean of long-range dispersal even within the same locality (>100 m, *sensu* Cain *et al.* 2000).

Our aim was to verify whether genetic markers confirm the hypothesis of a limited dispersal potential in *Cymodocea nodosa* which could lead to clear patterns of population differentiation within the Mediterranean Sea.

Materials & Methods

SAMPLING: Nine to 50 individual *C. nodosa* shoots were randomly collected by SCUBA diving in 17 localities along the Mediterranean coasts (Fig. 5.1, Table 5.1) at a distance of at least 2m from each other within each locality, in order to minimize the possibility to sample clonemates (genetically identical shoots). Fresh tissue was accurately cleaned from epiphytes and preserved by drying on silica-gel or fixed in ethanol. Ethanol preserved tissue was rehydrated, cleaned from epiphytes and processed immediately after rehydratation.

DNA EXTRACTION AND MICROSATELLITE MULTI-LOCUS GENOTYPE DETECTION: 5 mg of silica-gel dried tissue from each individual were ground through Mixer Mill MM300 (QIAGEN). Ethanol preserved tissue was ground in liquid N_2. Subsequent DNA extraction was carried out using the Qiagen DNAeasy Plant Mini Kit (QIAGEN).

Seven polymorphic microsatellite loci (Ruggiero *et al.* 2004, Chapter II of present thesis) were used to obtain multilocus individual genotypes. PCR conditions are as in Ruggiero et al. (2004). Allele detection was conducted through in automated sequencing (CEQ 2000XL DNA Analysis system, Beckman Coulter) for fragment analysis.

DATA ANALYSIS: In order to discriminate genets (the products of one zygote *sensu* Harper 1977) from ramets (the physiological units forming a clone), the percent of different genotypes over the total number of sampled ramets (G/N, Pleasant & Wendel 1989) was calculated. Distinct genotypes were identified with the help of the software GIMLET (Valière 2002). The probability of identity (Pi) by chance of genotypes according to Waits *et al.* (2001) was also estimated through GIMLET software.

Number of alleles/locus, non-biased heterozygosity (Nei 1987) and observed heterozygosity have been estimated through the software GENETIX (Belkhir *et al.* 1996-2002), after removal of replicated genotypes. The estimator f of F_{IS} according Weir & Cockerham (1984) was calculated through the software FSTAT v. 2.9.1 (Goudet 2000.). Significance of f was assessed after 119000 randomisations of alleles among individuals within samples. Total gene diversity (H_T, Nei 1987) for each locus was also calculated by FSTAT v. 2.9.1.

The estimator θ of F_{ST} according to Weir & Cockerham (1984) was determined for each locus and overall populations through the software FSTAT v. 2.9.1 after eliminating replicated genotypes; standard errors for θ were determined through jackknifing over loci.

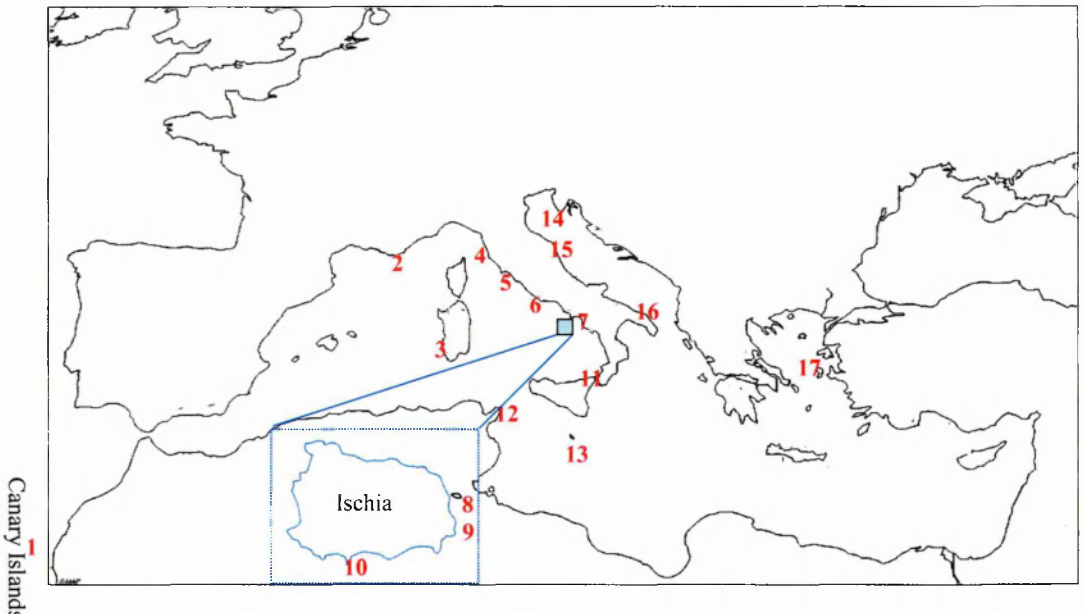


Fig. 5.1: Geographic distribution of sampled populations. Refer to Table 5.1 for population codes.

Table 5.1: Populations codes and coordinates for the 17 sampled populations.

	Locality	Label	Coordinates	
1	Canary Islands (Spain)	Can	28°14'N	014°16'W
2	Le Brusque (France)	Lbr	43°05'N	005°48'E
3	Oristano (Italy)	Ors	39°52'N	008°26'E
4	Livorno (Italy)	Liv	43°30'N	010°19'E
5	Civitavecchia (Italy)	Civ	42°06'N	011°46'E
6	Caprolace (Italy)	Cap	41°21'N	012°58'E
7	Capo Miseno (Italy)	Cms	40°47'N	014°05'E
8	Ischia – Castello (Italy)	ICT	40°44'N	013°58'E
9	Ischia – Carta Romana (Italy)	ICR	40°44'N	013°58'E
10	Ischia – Maronti (Italy)	IMR	40°42'N	013°54'E
11	Messina (Italy)	Mes	38°12'N	015°34'E
12	Tunis (Tunisia)	Tun	36°55'N	010°45'E
13	Malta	Mlt	35°56'N	014°20'E
14	Koper (Slovenia)	Kop	45°33'N	013°43'E
15	Ancona (Italy)	Anc	43°37'N	013°31'E
16	Lecce (Italy)	Lec	40°22'N	018°20'E
17	Edremit (Turkey)	Edr	39°33'N	026°37'E

Clonal diversity (G/N) and θ were also calculated on the subsets corresponding to the Western and Eastern Mediterranean populations, considering as Western group populations from 2 to 13 and Eastern group populations 14 to 17 as from Fig. 5.1. Pairwise Cavalli-Sforza & Edwards chord distance (1967) was calculated after 1000 bootstraps of allelic frequencies and a cladogram was constructed through Neighbour-joining analysis, using the softwares SEQBOOT, GENDIST, NEIGHBOUR and CONSENSE in the program package PHYLIP ver. 3.57c (Felsenstein 1986-1995).

The software BAPS (Bayesian Analysis of Population Structure, Corander *et al.* 2003, available at http://www.rni.helsinki.fi/~mjs) was used to verify if populations could be clustered into higher level panmictic units. The program was run for 10^6 iterations after a burn-in period of 50000 after eliminating replicated genotypes. A Neighbour-joining tree was then constructed on Cavalli-Sforza chord distance clustering populations belonging to panmictic units identified by the BAPS analysis.

Values of θ were also calculated on the subsets corresponding to the western and eastern Mediterranean demes (Canary Islands population was thus excluded from the analysis), in order to verify levels of gene flow within each basin.

To assess directionality of gene flow, an Assignment Test was conducted through the GENECLASS software (Cornuet *et al.* 1999) using the Bayesian method and 10000 simulation steps, with a probability threshold of 0.5. The test was done considering the panmictic units identified through the BAPS analysis.

Results

POLYMORPHISM OF MICROSATELLITE LOCI: The seven microsatellite loci used allowed 75 alleles in total in for the 17 populations analysed, ranging from 9 alleles at the Canary Islands (average number of alleles: 1.28) to 39 alleles in at Tunis (average number of alleles: 5.57). Twenty private alleles were found in total (percentage of private alleles: 27%). The highest number of private alleles (4) was found in at Edremit and Tunis. In Table 5.2 allelic diversity for the microsatellite loci used is shown. The most polymorphic loci were *Cy 20* and *Cy 16* (H_T values 0.834 and 0.831 respectively); the less polymorphic were *Cy 17* and *Cy 1* (H_T values 0.567 and 0.595 respectively). All loci showed moderate levels of genetic differentiation among populations, with *Cy 1* displaying the highest θ value (0.596), according to the fact assumption that least polymorphic loci are expected to have higher F_{ST} values (Hedrick 1999). *Cy 1* showed the higher inbreeding coefficient (f=0.276) while loci *Cy 18* and *Cy 20* showed an excess of heterozygosity (f values <0).

Multi-locus genotypes obtained from the seven microsatellite loci used displayed values of probability of identity (P_i) ranging from $5.33 \cdot 10^{-15}$ to $6.36 \cdot 10^{-06}$; all values are lower than the threshold of 0.001 recommended for the rejection of identity by chance of genotypes (Waits et al 2001).

Table 5.2: Allele size range, number of alleles (α), total gene diversity (H_T, Nei 1987), Weir & Cockerham (1984) estimators of F_{ST} (θ) and F_{IS} (f) for each locus.

Locus	Allele size range (bp)	A	H_T	θ	f
Cy 1	119-131	5	0.595	0.596	0.276
Cy 3	142-154	7	0.648	0.150	0.164
Cy 4	154-176	8	0.735	0.145	0.018
Cy 16	171-209	21	0.831	0.228	0.042
Cy 17	228-260	6	0.567	0.124	0.032
Cy 18	130-163	11	0.767	0.227	-0.174
Cy 20	173-213	17	0.834	0.232	-0.018

GENETIC VARIABILITY OF POPULATIONS: Populations varied widely in terms of genetic variability, with G/N values ranging from 1 for Tunis and Oristano to 0.05 for Messina. Four populations (Ancona, Lecce, Canary Islands and Messina) showed only two distinct genotypes and therefore were not considered in the following analyses. Observed heterozygosity ranged from 0.29 for Koper to 0.69 for Tunis (Table 5.3). A significant excess of heterozygosity was detected in the south-Tyrrhenian populations Capo Miseno and Ischia Castello, while a significant deficit of heterozygosity was found in the North-Tyrrhenian populations Livorno, Civitavecchia and Le Brusque. In general, higher clonal diversity was found in populations from the Western part of the Mediterranean, respect to the Eastern basin (total G/N=0.71 and 0.36, respectively).

Table 5.3: Number of samples (N), number of genotypes (G), genotypic diversity (G/N), non-biased and observed heterozygosity (Hn.b. and Hobs respectively), inbreeding coefficient (f) according to Weir & Cockerham (1984) and mean number of alleles/locus for each population, calculated after removal of replicated genotypes. Heterozygosity and inbreeding coefficient were not calculated for four populations (Ancona, Lecce, Canary Islands and Messina), in which only 2 genotypes were recorded. *= f significant at the 5% nominal level, after 119000 permutations.

Pop code	Pop label	N	G	G/N	$H_{n.b.}$	H_{obs}	f	Mean. N. Alleles/Locus
1	Can	20	2	0.10	--	--	--	1.286
2	Lbr	47	30	0.64	0.501	0.424	0.156*	3.143
3	Ors	25	25	1.00	0.625	0.629	-0.006	4.286
4	Liv	34	33	0.97	0.632	0.519	0.180*	5.000
5	Civ	42	40	0.95	0.544	0.471	0.135*	4.000
6	Cap	38	13	0.34	0.494	0.516	-0.047	3.429
7	Cms	18	6	0.33	0.509	0.619	-0.244*	2.714
8	ICT	40	29	0.73	0.561	0.616	-0.099*	4.286
9	ICR	9	8	0.89	0.599	0.607	-0.015	3.714
10	IMR	50	34	0.68	0.479	0.513	-0.071	3.429
11	Mes	40	2	0.05	--	--	--	2.286
12	Tun	33	33	1.00	0.657	0.649	0.012	5.571
13	Mlt	40	38	0.95	0.602	0.613	-0.018	4.000
14	Kop	11	9	0.82	0.305	0.286	0.068	2.286
15	Anc	15	2	0.13	--	--	--	2.000
16	Lec	19	2	0.11	--	--	--	2.143
17	Edr	24	9	0.38	0.389	0.397	-0.020	2.429

PATTERNS OF POPULATIONS GENETIC STRUCTURE AND GENE FLOW: Average value of θ for the 17 populations was 0.241 ± 0.054, indicating marked genetic differentiation between populations. Pairwise values of θ are shown in Table 5.4. The higher genetic differentiation (θ = 0.680) was between Canary Islands and Koper, the lower (θ = 0.000) between Messina and Lecce. When differentiating between western and eastern populations, the average values of θ obtained were respectively 0.220 ± 0.054 and 0.391 ± 0.122, indicating that gene flow is more effective within the Western than within the Eastern basin.

Table 5.4: Pairwise θ values between populations.

	Can	Cap	Cms	ICR	Mes	ICT	IMR	Mlt	Ors	Kop	Lec	Tun	Edr	Liv	Civ	Lbr
Anc	0.625	0.320	0.363	0.306	0.243	0.341	0.386	0.244	0.167	0.163	0.250	0.196	0.383	0.208	0.302	0.277
Can		0.513	0.508	0.366	0.511	0.398	0.471	0.358	0.397	0.680	0.563	0.294	0.605	0.338	0.459	0.473
Cap			0.229	0.137	0.211	0.146	0.183	0.141	0.188	0.392	0.228	0.229	0.339	0.164	0.303	0.355
Cms				0.120	0.264	0.170	0.212	0.175	0.277	0.481	0.259	0.237	0.439	0.181	0.308	0.379
ICR					0.130	0.036	0.074	0.096	0.222	0.415	0.142	0.157	0.340	0.128	0.282	0.357
Mes						0.135	0.152	0.172	0.088	0.208	0.000	0.049	0.393	0.046	0.145	0.269
ICT							0.095	0.145	0.233	0.395	0.182	0.188	0.300	0.147	0.287	0.364
IMR								0.189	0.272	0.424	0.197	0.233	0.364	0.218	0.330	0.417
Mlt									0.203	0.324	0.136	0.135	0.302	0.130	0.295	0.284
Ors										0.253	0.140	0.152	0.347	0.145	0.252	0.280
Kop											0.180	0.247	0.466	0.265	0.310	0.368
Lec												0.074	0.396	0.029	0.189	0.286
Tun													0.317	0.130	0.228	0.279
Edr														0.283	0.390	0.413
Liv															0.158	0.188
Civ																0.165

The NJ tree on Cavalli-Sforza chord distance for the 17 populations is shown in Fig 5.2. Although supported by low bootstrap values, 5 clusters were recognizable: a) Caprolace grouped with Oristano; b) Messina with Lecce, Ancona and Koper; c) Livorno with Le Brusque and Civitavecchia; d) Capo Miseno with the three populations from Ischia (Castello, Cartaromana and Maronti); e) Malta with Tunis. Canary Islands and Edremit were isolated from all other populations. The divergence of Edremit from all other populations was highly supported (98.2% boostrap).

The best partition after the bayesian analysis of population structure (BAPS) revealed that 13 panmictic units over 17 geographic localities were recognisable (Fig. 5.3a). Three demes represented by more than one population were found: a cluster comprising the eastern populations Ancona, Koper and Lecce (from now on coded as AKL); a cluster grouping the very close populations from Ischia-Cartaromana and Ischia-Castello (coded as IS1) and a cluster grouping the two populations from Messina and Tunis (coded as MET). All other populations could be considered genetically distinct from each other. When grouping populations into demes, the resulting Neighbour-joining tree on pairwise Cavalli-Sforza chord distance was somewhat different than the formerly described one, though bootstrap values remained low (Fig. 5.3b). Edremit clustered within the eastern deme (Ancona, Koper, Lecce), while it was isolated in the previous tree; the Sicily Channel deme (Messina and Tunis) grouped with Malta, while Messina was linked to the eastern populations in the former analysis; Caprolace clustered with the Ischia populations and Capo Miseno, while it was previously grouped with Oristano. The northern Tyrrhenian group (Livorno, Civitavecchia and Le Brusque) was maintained. Canary Island populations remained isolated and basal to all other populations. Divergence of the north-Tyrrhenian

populations was highly supported by bootstrap values (95.4%). In general, the phylogenetic pattern resulting from grouping populations into panmictic units was more coherent with geographic features of the basin.

Results from the assignment test are shown in Table 5.4 and Fig. 5.4. Most of the genotype exchanges were among the central Mediterranean populations (Fig. 5.4), namely the Ischia populations (IMR and the Ischia deme IS1), Livorno, the Messina-Tunis deme (MET) and Civitavecchia. IMR exchanged migrants with the IS1 and with the MET demes; IS1 exchanged migrants with IMR, Capo Miseno, MET and Livorno, while only one with Caprolace; Livorno exchanged migrants mainly with Civitavecchia and Le Brusque, and, although less extensively, with almost all populations, except for Canary Islands, Edremit and Ischia-Maronti; the MET deme exchanged with the central Mediterranean populations IMR, Caprolace, Malta, IS1 and Livorno and also with the eastern deme AKL; Civitavecchia exchanged only but extensively with Livorno; Oristano only with Caprolace and Livorno. The eastern populations (deme AKL) were exchanging genotypes with the Messinian-Tunisian deme (MET) and only one with Livorno.

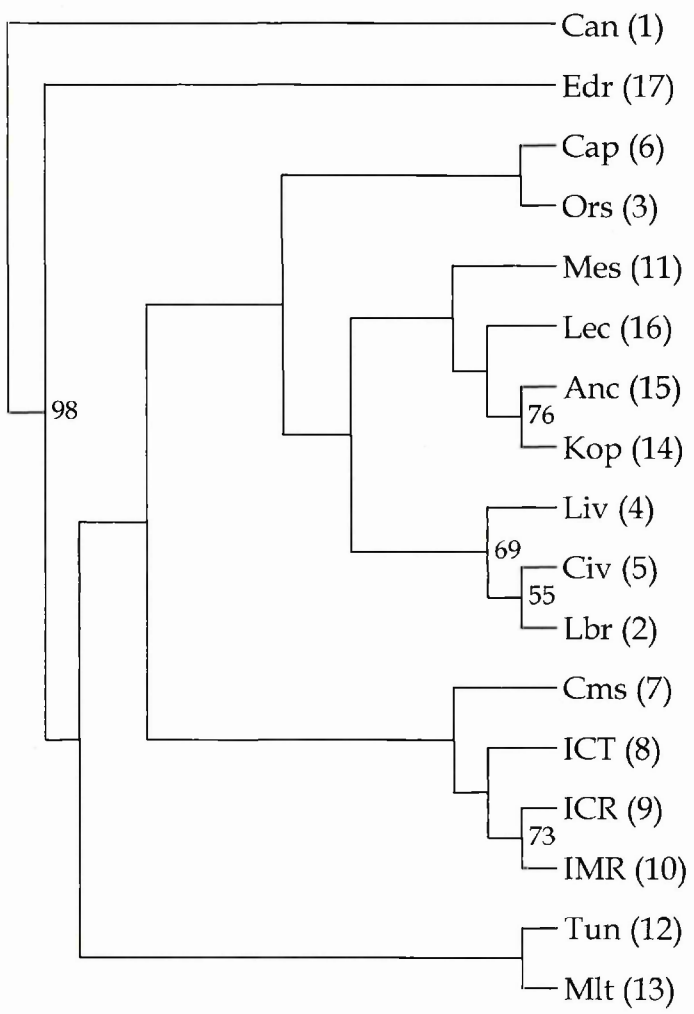
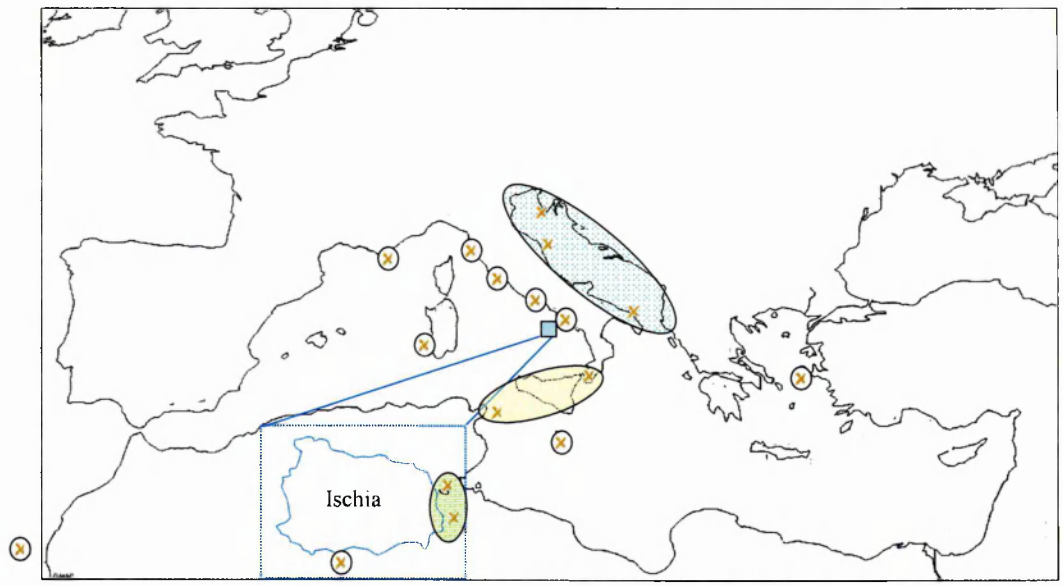
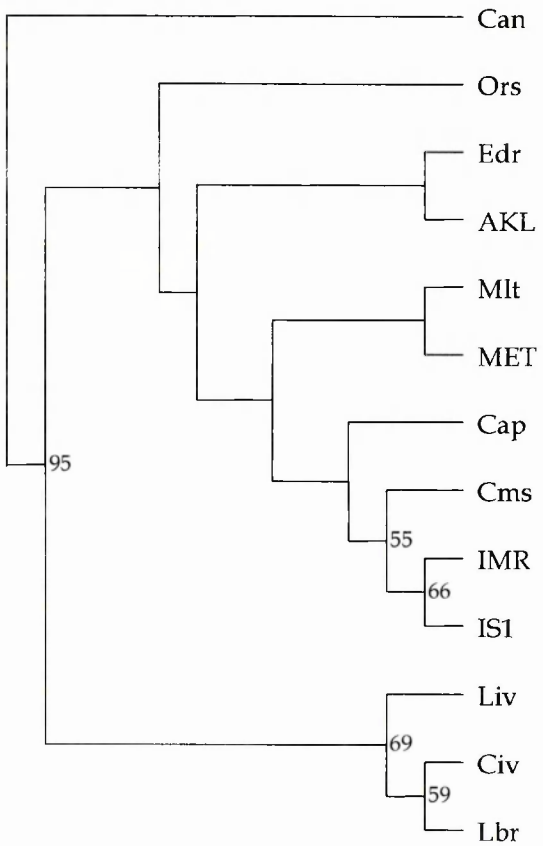


Fig. 5.2: Neighbour-joining tree on Cavalli-Sforza chord distance. Bootstrap values higher than 50% are shown. In brackets, the population codes are also given.



a)



b)

Fig. 5.3: Population structure according to a) Bayesian analysis and b) Neighbour-joining tree on Cavalli-Sforza chord distance, after identification of demes. AKL (Blu circle) = Ancona, Koper, Lecce; IS1 (Green circle) = Ischia-Castello, Ischia-Cartaromana; MET (Yellow circle) = Messina, Tunis. All other populations (White circles) represent single- panmictic units.

Table 5.5: Assignment test based on Bayesian method. The test was done considering the panmictic units identified through the BAPS analysis. Threshold probability for rejection of assignment was 0.05. The number of genotypes from populations on the left that can be assigned to populations on the top (donors) is shown. -- = no exchanging genotypes.

	AKL	Can	Cap	Cms	IS1	IMR	Mlt	Ors	MET	Edr	Liv	Civ	Lbr
AKL	x	--	--	--	--	--	--	--	4	--	1	--	--
Can	--	x	--	--	--	--	--	--	--	--	--	--	--
Cap	--	--	x	--	1	--	--	1	2	--	2	--	--
Cms	--	--	--	x	4	--	--	--	--	--	1	--	--
IS1	--	--	--	--	x	3	--	--	1	--	1	--	--
IMR	--	--	--	--	23	x	--	--	7	--	--	--	--
Mlt	--	--	--	--	--	--	x	--	3	--	1	--	--
Ors	--	--	--	--	--	--	--	x	--	--	--	--	--
MET	--	--	--	--	--	--	--	--	x	--	1	--	--
Edr	--	--	--	--	--	--	--	--	--	x	--	--	--
Liv	--	--	1	--	--	--	1	1	1	--	x	--	--
Civ	--	--	--	--	--	--	--	--	--	--	1--	x	--
Lbr	--	--	--	--	--	--	--	--	--	--	8	--	x

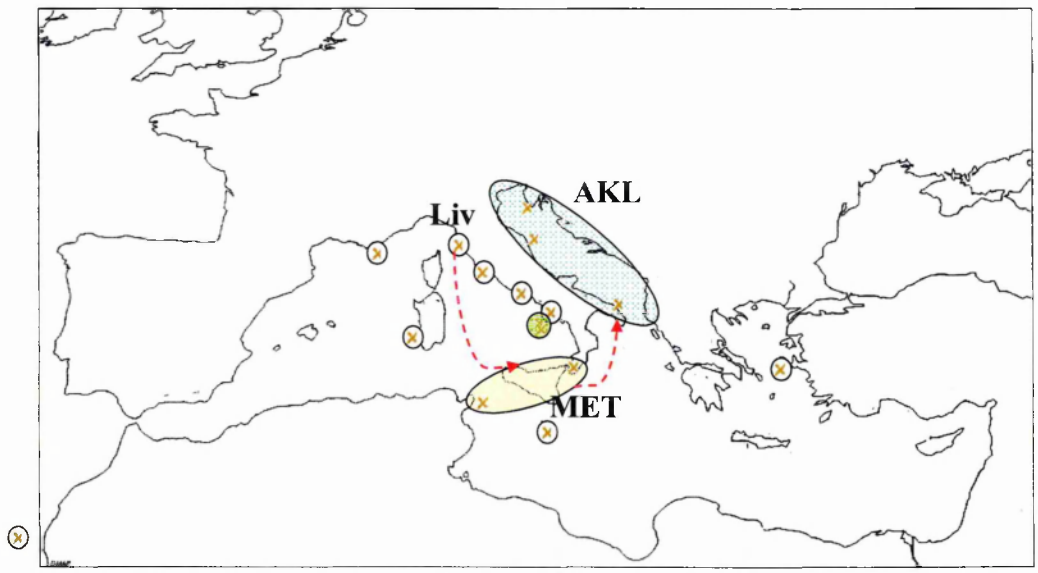


Fig. 5.4: Main migrant exchanging pathways between the Western and the Eastern basin as from the assignment test. Arrows connect exchanging demes. Circles indicate panmictic units as from the BAPS analysis (see Fig. 5.3). Assignment was conducted using the bayesian method with a threshold probability of 0.5.

Discussion

Although *Cymodocea nodosa* is characterized by a poor seed dispersal potential (Ruggiero MV, Chapter III of present thesis), in the present study microsatellite loci showed that long-distance dispersal events can occur in this species. Although a moderate genetic divergence is present, in fact, the phylogeographic tree failed to support strong population differentiation and the Bayesian analysis showed that supra-population panmictic units are present, two of which covering hundreds of kilometres. Although surprising, this result is in accordance with the high between-population connectivity observed in the seagrasses *Zostera marina* (Reusch 2002; Olsen *et al.* 2004), and in *Zostera noltii* (Coyer *et al*, in press), in which high levels of gene flow were observed up to 150 km. The possibility of drifting of vegetative or reproductive shoots has been taken into consideration in the cited studies and could also account for the long-distance gene flow observed in the present work.

Populations of *Cymodocea nodosa* throughout the Mediterranean Sea showed a moderate genetic structure, which seems to be mainly linked to geographical factors and surface currents, probably acting as genetic exchange avenues. Results from the Bayesian analysis of population structure showed that most populations can be considered as distinct genetic units, confirming the observed high value of θ. However, three "supra-population" demes were recognisable, where high levels of gene flow or a recent common origin could account for panmixia of the grouped populations. Among the sampled populations, different levels of clonal diversity (G/N) were found, with Eastern populations less diverse than the Western ones. This observation is in line with what observed in the endemic seagrass species *Posidonia oceanica* (Procaccini *et al.* 2002), although in this hermaphroditic species values of genetic diversity were lower.

Although a clear difference can be detected between the two portions of the basin, significance of the differences cannot be determined, due to the different number of populations within each group.

Interestingly, subdivision between Western and Eastern populations is not supported by the phylogeographic tree. The main cluster comprises, in fact, both South-Tyrrhenian and Eastern populations together with a Siculo-Tunisian group. The Siculo-Tunisian sill is considered to be the boundary between the Eastern and the Western basins of the Mediterranean Sea, and could probably act as a connection area between these sectors.

This finding is also confirmed by the assignment test, showing that gene flow is mainly directed from the genetically variable western basin toward the genetically impoverished eastern basin through the intermediate Siculo-Tunisian deme. The main westerly surface currents associated to the inflow of Atlantic Water through the Strait of Gibraltar (Pinardi 2000) could account for that.

In the Western basin, most of the populations remain as isolated demes, indicating that genetic differentiation within this basin is pronounced. Only two supra-population demes (Ischia-Castello with Ischia-Cartaromana and Messina with Tunis) were in fact observed. Here, grouping of the two Ischia sampling sites into one deme is intuitive, due to their close proximity. The two sites are, in fact, located on the opposite coastlines of a small bay and are separated by about 0.5km distance. Superficial Atlantic Waters (AW) incoming through the Gibraltar Strait and branching northwards at the Tunisian Channel could instead provide avenues for an extensive migrant exchange between the Messina and Tunis populations (Pinardi 2000). The Messina population showed an extremely low clonal diversity, with only two large clones. This high

clonality could be explained by local adaptation to the peculiar features of the sampling site (Olsen *et al.* 2004). Local adaptation could have led to the preferential growth of few large genotypes through two different factors: on the one side, the physical isolation of the sampled site with consequent lack of allochtonous recruitment, and, on the other side, the hydrodynamic features of the highly disturbed Messina Strait, characterized by frequent whirls (Brandt *et al.* 1997) due to the contrasting currents along the Strait.

The phylogeographic tree obtained considering panmictic units allowed a geographically coherent pattern for the Western Mediterranean Sea. The disjunction of the North-Tyrrhenian from all other populations is supported by high bootstrap values in the phylogeographic tree. A distinct South-Tyrrhenian group, comprising the Ischia populations with Capo Miseno and Caprolace is nested within the main cluster. A differentiation between Northern and Southern Tyrrhenian populations has already been observed in the seagrass *Posidonia oceanica* (Procaccini *et al.* 2001; 2002) and could be due to seasonal patterns of superficial closed circulation cells in the north, centre and South-Tyrrhenian Sea (Tait 1984; Astraldi & Gasparini 1994) that could act as physical barriers to the dispersal of seeds and/or vegetative propagules between the different sections of this basin. Moreover, the three North-Tyrrhenian populations (Livorno, Civitavecchia and Le Brusque) were affected by significant levels of inbreeding, while two populations from the Gulf of Naples (Capo Miseno and Ischia-Castello) had more heterozygous genotypes than expected. An excess of heterozygosity in Ischia populations has also been shown in the other two seagrass species *Zostera noltii* (Ruggiero MV, Chapter IV of present thesis) and *Posidonia oceanica* (Procaccini G, unpublished data). It seems thus that in this region selective heterozygote advantage

and/or strong inbreeding depression are present, probably in response to stressful environmental conditions (Hämmerli & Reusch 2002).

Oristano population remains isolated, although within the main cluster. Its geographical isolations from the Tyrrhenian Sea could account for its genetic differentiation. The assignment test shows coherently that the Oristano population has only minor genetic exchanges with Livorno northwards and with Caprolace southwards. The population from outside the Mediterranean basin (Canary Islands) is clearly isolated, as expected from its geographic position. Its marginal distribution could also explain the observed high rate of clonal reproduction (Eckert 2002). Similarly, RAPD markers revealed almost complete uniclonality in a *Cymodocea nodosa* population at the northern limit of the species in the Atlantic Ocean (Alberto *et al.* 2001). At the same site, low clonal diversity was also found in the seagrass *Zostera marina* (Billingham *et al.* 2003). The studied site, however, represents the southernmost range in this northern temperate species.

The phylogeographic analysis shows genetic similarity between the Aegean population (Edremit) and the three Adriatic populations (Koper, Ancona and Lecce). Although the distance among Adriatic populations can be measured in hundreds of kilometres, the three Adriatic localities form a large panmictic unit, while the Edremit population remains as an isolated deme. The Adriatic Sea is characterized by surface currents that form a closed gyre in this basin (Poulain 1999), so that gene flow could be more effective within the basin than between Adriatic and outside populations. A rapid re-colonisation in the Adriatic Sea after the last glaciation event could also be considered as a possible cause for the lack of differentiation among Adriatic populations. Lower heterozygosity and lower allelic diversity were found in the

northernmost Adriatic population (Koper) respect to the Aegean population (Edremit), suggesting that the latter could represents a hypothetical source populations for Adriatic populations (Hewitt 1996; Johnson *et al.* 2000). A similar situation in the Adriatic Sea, related to a northward post-glacial recolonization avenue has also been observed for the seagrass *Posidonia oceanica*, which is present in the northern part of the basin with a single, highly inbred clone (Ruggiero *et al.* 2002), probably coming from the Aegean Sea. The presence of a single panmictic unit covering the whole Adriatic Sea is in contrast with the high θ value within the whole eastern group. Nonetheless, the high genetic differentiation of the Edremit population, as shown by the phylogeographic tree including single populations (Fig. 5.2), could bias upwards the estimate of genetic differentiation within the Eastern basin. These results need to be confirmed, because the present study suffers from a relatively poor representation of genotypes in two of the three populations. A more extensive sampling in the Levantine basin and Aegean Sea could also clarify on post-glacial recolonization events in this area. Moreover, because size homoplasy for microsatellite alleles could occur, errors in the estimation of between-populations differentiation are possible (Estoup *et al.* 2002). Two kinds of errors can occur (Adams *et al.* 2004): 1) alleles can be identical in state but not identical by descent. In this case, population differentiation could be underestimated. 2) Alleles can be not identical in state, but identical by descent for the microsatellite repeat. In this case, population differentiation could be overestimated. Further efforts are thus needed to reveal the possible presence of size homoplasy in our data-set, considering the DNA sequence of the observed alleles and/or their SSCP profiles (Single Strand Conformation Polymorphism).

A wide range of genetic diversity has been observed in populations of *Cymodocea nodosa* throughout the Mediterranean Sea. Most populations were in Hardy-Weinberg equilibrium, suggesting high effective population size and the absence of biparental inbreeding in this dioecious species. Populations ranged from almost complete monoclonality (only two genotypes were recorded in Ancona, Lecce, Messina and Canary Islands), to a complete lack of clonality (i.e. each sample was genetically distinct in Tunis and Oristano). Recent findings in seagrass species confirm the marked variability in genetic diversity and size of genets in distinct populations (*Posidonia australis*, Waycott *et al.* 1997, Waycott 1998; *Zostera marina*, Reusch *et al* 2000, Olsen et al. 2004; *Zostera noltii,* Coyer et al, in press; *Posidonia oceanica*, Procaccini *et al.* 2001, 2002; Arnaud-Haond *et al.*, *in press*). In clonal plants such as seagrasses, variations in genetic diversity can be related to the relative proportion of clonal versus sexual reproduction, the breeding system, and the effective population size (Ashton & Mitchell 1989; Eckert & Barrett 1992; Eriksson 1996; Reusch 2001a).

Although geographic distance and/or hydrographical pathways of gene flow seem to be responsible for population genetic structure in the Mediterranean basin, there is also evidence for population differentiation as a consequence of local adaptation to the extremely heterogeneous environmental conditions in the Mediterranean Sea (Sarà 1985). A more extensive sampling covering the whole Mediterranean basin would be desirable in order to better understand to what extent isolation of populations depends on physical barriers to dispersal and on the dispersal potential of the species itself and how much local adaptation contributes to the variation range in genetic diversity between populations.

OVERALL CONCLUSIONS

The main aim of the thesis was to unravel the reciprocal interactions of sexual reproduction and clonal spread in *Cymodocea nodosa* as a model species for marine clonal plants.

The different aspects of each of the two modes of reproduction have been investigated through the neutral genetic variation displayed by microsatellite loci, currently considered the markers of choice for population genetics and molecular ecology studies. The selection and optimisation of seven species-specific molecular markers has been described in detail in Chapter II. The seven loci displayed high number of alleles and high heterozygosity over all analyzed populations, allowing the correct identification of individual genotypes.

Sexual reproduction has been shown to be frequent and successful in a population in Ischia (Gulf of Naples, Italy). Seed dispersal is poor (1-21m), as expected based on the reproductive features of the species, and seedlings are recruited continuously within the population. A mixture of small clones, together with large ones has been, in fact, observed, indicating the coexistence of newly recruited genotypes with older clones, some of which possibly ancient (Chapter III). In contrast with the described poor dispersal potential, long-range dispersal events can occur sporadically at phylogeographic scales (Chapter V). Gene flow is moderated and it is mainly driven by surface currents and geographical barriers. Most populations consisted of distinct

panmictic units, but "supra-population" units, grouping geographically distant meadows, were observed.

The extent and shape of clones has been investigated in Chapter III. Clonal growth form was ascribed to the so-called "guerrilla strategy", which leads to an intermingled configuration of genets. Clones are not, in fact, recognisable as distinct, discrete units. Such clone configuration is advantageous in several ways: on the one hand, it reduces the effects of genetic identity on the genetic structure of the population, reducing therefore the possibility of biparental inbreeding at small spatial scales; on the other hand, the breakdown of clonal continuity also interrupts monosexual patches in dioecious species, by favouring opposite sexual products to encounter and thus allowing the maintenance of a sufficient seed-set (Chapter III). The latter hypothesis has been tested in Chapter IV, where the genet distribution has been compared between the dioecious *Cymodocea nodosa* and the monoecious *Zostera noltii*. The basic hypothesis was that the need to maintain a consistent seed-set would drive genet distribution, leading to an intermingled configuration of genets in a dioecious, obligate outcrossing species. On the contrary, a clumped, "phalanx-type" distribution of clones should be more advantageous in a hermaphroditic, self-compatible species. No clear effects of interspecific competition on the genetic diversity and clonal architecture were recognisable in the mixed stands.

As a final remark, the wide variety in genetic diversity recorded on 17 *Cymodocea nodosa* populations along the Mediterranean coast-lines (Chapter V) exhorts for cautions in the attempt to generalize conclusions on the genetic features and ecological behaviour of seagrasses. Such a variation has been found, in fact, to be

common in seagrasses and can be related to their plasticity in response to the widely differentiated environmental factors at sea.

Determining clonal distribution and seed dispersal solely through classical ecological methods, such as tracing rhizomes or trapping seeds, is an extremely labour-intensive matter in an underwater environment. Molecular markers have, once again, revealed their value in flanking and integrating ecological investigations in marine plants.

Many gaps remain in the knowledge of marine plants' evolutionary biology. A tight cooperation and integration of both ecophysiological and demographic methods on the one hand, and the application of molecular markers on the other hand (in one word: Molecular Ecology) could hopefully lead to unravel the consequences of environmental factors on the differential fitness of the two evolutionary subjects in clonal plants: the ramet and the genet.

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Appendix 1

Here are presented the complete sequences of the positive clones identified in the *Cymodocea nodosa* genome through genomic library screening.

Locus *Cy 1*
GenBank Accession number: AY559051
Lenght: 169 bp

```
  1 tgtaaggggg gaggcaccac cagcaatggt ggggtgggag caagtccgaa gaagagaaga
 61 gaagatagct agatagctct ccaacttctt tgcttacgtc tcgtctctct ctctctctct
121 ctctctctct ctctctcctt tgctgcccca gccattcctt cctcctcgg
```

Locus *Cy 3*
GenBank Accession number: AY559052
Lenght: 157 bp

```
  1 ccgtgtccgt ggctctttcc gtaaatctat cgaaacctcc gaccctgact tttttcttcc
 61 cccacccctt ttctgttggg atgagagaga gagagagaga gagagagaaa gggggagagt
121 gcggaagaga ccccttttct gttgggtgcg tgcggga
```

Locus *Cy 4*
GenBank Accession number: AY559053
Lenght: 173 bp

```
  1 ccgatggctt aacataatga tgcggtactg cagacaaata ataataataa taataataat
 61 aataatgacg accaagaagg tgtcatggta gaagcgtagc ggaggaccac accataatca
121 ccatgtgcgg taagaataat aaggggtgaa tggttcttgt gattccggag ggg
```

Locus *Cy 16*
GenBank Accession number: AY559054
Lenght: 313 bp

```
  1 ccactttcac acttgccgtg gtcgccacgg tgagacttaa cgaacacata tacacacaca
 61 cacacacact ctctctctct ctctctctct ctctctctct ctctctctct cttcctagaa
121 gccaatctca catatataga gagattttgg ttaattttga ctcaggtggg aatggagttt
181 tggtcgaggt gggtgcacag ggctctgtgg cacggctctc tgtggcacat gcacaagtcg
241 caccacaagc ccagagaagg accgttcgag ctcaacgacg tgttcgtctc ctcaacgccg
301 cccccgccac cgg
```

Locus *Cy 17*
GenBank Accession number: AY559055
Lenght: 375 bp

```
  1 ctacgtgcca tttttcattt cctatattta taccttcttc cgacatctga ataatatata
 61 tataagaagt cagttaccgt catatatata tgtatgtaga ataatgggac tgctggcagg
121 tgaagaaatt gatccgattg ttatatatat atctatacgt gccccatctc tactgccccc
181 catcatcatc ttcttcttcc tctgtttttc gcctacatct cgtccgacgt atataaacta
241 acctctctct ctctctctct ctctctctct ctctctcgat atatatatat atatatatga
301 atgatgactt ttaagcttcg atcggatcaa agcacaactt cggtataacc gaagttttta
361 aattttattt taagt
```

Locus *Cy 18*
GenBank Accession number: AY559056
Lenght: 328 bp

```
  1 ctcatgaacc gctgcagatg catttgctgt tgctccgagg ccggattctg cgtttgcatc
 61 ttacttcatt aaacaacaac aataaaaaca actactgctg ccgctgccgc cgccgctgct
121 actgctacta ccactgccgt tcaacggaaa ttcgtactat caatcgctcc ttcttctacc
181 agcagaacga cggcatttgc atccatgtat atacattaca ttacatatat agacacacac
241 acacacacac acacacacac acacagagga agaggaagag aaagagaggg agagagagag
301 agggagagac gcacccgcag cgcagcag
```

Locus *Cy 20*
GenBank Accession number: AY559057
Lenght: 366 bp

```
  1 ggtctctacc tarrataaga catgctttgg ttgcacagaa caggtggaca gttggattct
 61 aggacaacaa tttctctctt tacctctacc ctctctctct ctctctctct ctctctctct
121 ctctctctca ctagactctc tctttcctct aacttgtgga tcttaacaac ctctccacct
181 tttatttagg tttgagggag atgtgggagt tttggttgcc cttgtggtgc ccaattgagc
241 cccattttgc caggtggata gcccaaagcc accatatatg aaaatcccat ttttaaacac
301 taggcaatag atctctatga gggagatgtg ggagtcttgg ttgcccttgt ggcacccaat
361 tgagtt
```

APPENDIX 2a

Here the Data Matrix used for analyses in Chapter III is presented

GRID

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
A1	127127	142148	154154	175201	230230	144144	195211
A2	119127	142142	154169	175189	230260	144144	179179
A3	119127	142150	154163	179201	234234	144144	179179
A4	119127	142142	154163	175189	230234	144144	179205
A5	127127	142150	154169	179201	230234	144144	179179
A6	119127	142150	154169	175193	234260	144154	179179
A7	127127	142142	163169	175201	230234	144154	179195
A8	127127	142142	163169	175201	230234	144154	179195
A10	119127	142142	154163	175201	230234	144154	195205
A11	127127	142150	154163	175201	230230	144154	179211
A12	127127	142142	154163	175201	230234	144154	179195
A13	127127	142150	154163	175201	234236	144154	179211
A14	119127	142150	154163	175201	234236	144154	195205
A15	119127	142150	154163	175201	234236	144154	179211
A16	127127	142150	154163	201201	236260	154154	179211
A17	119127	142142	154163	175201	230234	144154	195205
A18	127127	142148	154163	175175	230230	144144	195211
A19	119127	142142	163163	175175	230236	144154	179211
A20	119127	142150	154163	175201	234236	144154	179211
A21	119127	142150	154163	175201	234236	144154	179211
A22	119127	142150	154163	175201	234236	144154	179211
A24	119127	148150	154163	175201	230230	144154	195209
A25	119127	142148	154154	175201	230230	144144	195211
A26	119127	142148	154154	175201	230230	144144	195211
A27	119127	142148	154154	175201	230230	144144	195211
A28	119127	142150	154163	175201	234236	144154	179211
A29	119127	142148	154154	175201	230230	144144	195211
A30	119127	142148	154154	175201	230230	144144	195205
A31	119127	142150	154163	175201	234236	144154	179211
B1	119127	142142	154154	175201	234236	144154	195205
B2	119127	142150	154169	179201	234234	144144	179179
B3	119127	142150	154163	175189	230234	144154	179205
B4	119127	142142	154154	175189	230234	144144	179205
B5	119127	142142	166169	189201	230260	144152	179195
B6	119127	142150	154163	175201	234260	144154	179195
B7	127127	142148	154154	189201	230230	144154	179195
B8	127127	142142	154154	189189	230234	144154	179195
B10	119127	142150	154163	175201	230236	144154	179179
B16	127127	142142	154169	175201	230230	144154	179205
B21	119127	142148	154169	175203	230260	154154	179179
B22	119127	142148	154163	175175	230230	144144	195211
B23	119127	148150	154169	175189	234236	152152	195211
B24	119127	142150	163169	189201	236260	144154	179211
B25	119127	148150	154163	175201	230236	144154	195211
B26	119127	142150	154163	175201	234236	144154	179211
B27	119127	142150	154163	175201	234236	144154	179211
B28	119127	142150	154163	175201	234236	144154	179211
B29	119127	142148	154154	175201	230230	144144	195211
B30	119127	142142	154169	179189	230260	144152	179179
B31	119127	142148	154169	201201	230230	144144	179205
C1	127127	142142	154163	177201	234234	144152	179179
C2	127127	142142	154169	175201	234260	144154	179195
C3	127127	142142	154169	175201	234260	144154	179195
C4	127127	142142	154154	175189	230234	144144	179195
C5	127127	142142	154154	175189	230234	144144	179195
C6	127127	142142	154154	175189	230234	144144	179195
C7	127127	142142	166169	189201	230260	144152	179195
C8	127127	142148	169169	177191	230236	154154	179209
C9	119127	142148	154163	193193	230236	152154	179179
C10	127127	142142	154163	175201	230234	144154	195205
C13	127127	142150	154163	175201	234236	144154	179211
C16	127127	142150	154163	175201	234236	144154	179211
C21	127127	142150	154163	175201	234236	144154	179211
C22	127127	142150	154163	177201	230230	152152	179179
C23	127127	142142	163169	175189	230260	144154	179211
C24	127127	142148	154154	175201	230230	144144	195211

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
C25	127127	142142	154163	175201	230234	144154	195205
C26	127127	142150	154163	175201	234236	144154	195211
C27	127127	142150	154163	175201	234236	144152	179211
C28	127127	142150	154163	175201	234236	144154	179211
C29	127127	142148	154163	175175	230230	154154	195211
C31	127127	142148	154169	189201	230234	144152	179179
D1	121127	142142	154169	179189	230260	144144	179179
D2	127127	142142	154154	175189	230234	142144	179195
D3	127127	142142	154169	175189	230260	144154	179179
D4	127127	142150	163169	175189	230234	144152	187211
D5	127127	142142	166169	189201	230260	144144	179195
D6	127127	142142	154154	175189	230234	144144	179195
D7	127127	142148	154163	175201	230234	144154	195211
D7c	127127	142142	154154	175189	230234	154154	179195
D8	127127	148150	154169	189201	230234	144154	195209
D9	127127	142142	163169	175201	230234	144154	179195
D10	127127	142142	163169	175201	230234	144154	179195
D11	127127	142142	154163	175201	230234	144154	195205
D12	127127	142142	154163	175201	230234	144154	195205
D14	127127	142150	163163	175201	230234	144154	195205
D15	127127	142142	154154	189189	230234	144154	179195
D16	127127	142142	154154	189189	230234	144154	179195
D17	127127	142150	154163	175201	234236	144154	195211
D18	127127	142150	154163	175201	234236	144154	179211
D19	127127	142150	154163	175201	234236	144154	179211
D20	127127	142150	154163	175201	234236	144154	179211
D21	127127	142142	154163	175201	236236	144154	179211
D22	127127	148148	154154	189189	234236	144154	179211
D23	127127	142150	154163	175201	234236	144154	179211
D24	121127	142148	154163	189207	230234	144154	205211
D25	127127	142150	154163	175201	234236	144152	179211
D26	127127	142150	154163	175201	234236	144154	179211
D31	121127	142142	154169	179189	230260	144144	179179
E2	119127	142150	163169	175189	230234	142144	179205
E3	127127	142142	154154	175189	230234	154154	179195
E4	127127	142142	154154	175189	230260	144144	179179
E5	127127	142150	154163	189201	234260	144144	179195
E6	127127	142142	154154	175189	230234	144154	179195
E7	127127	142142	163169	175201	230234	144154	179179
E8	127127	142142	154163	175201	230234	144154	195205
E9	127127	142142	163169	175201	230234	144154	195205
E10	121127	148150	154163	175201	230230	144154	195211
E11	127127	142142	154163	175201	230234	144144	195205
E12	127127	142142	154163	175201	230234	144154	195205
E14	127127	142142	154163	175201	230234	144154	195205
E15	127127	142142	154163	175201	230234	144154	195205
E16	127127	142148	154163	175175	230230	154154	195205
E17	127127	148150	154163	175201	230236	144154	179195
E18	127127	142150	154163	175201	234236	144154	179211
E19	127127	142150	154169	175193	230230	154162	179209
E20	127127	142142	163163	175189	234236	144154	179209
E21	127127	142150	154163	175201	234236	144144	179211
E22	121127	142150	154163	179191	230234	144154	179179
E23	127127	142150	154163	189201	234236	144154	179209
E24	127127	142150	154163	175201	234236	144154	179211
E25	127127	142150	154163	175201	234236	144144	179211
E26	127127	142150	154163	189191	234236	154154	179211
E31	127127	142148	154154	175201	230230	144144	195211
F2	127127	142150	163169	175201	230230	154154	179209
F3	127127	142150	163169	175189	230234	144144	179205
F4	127127	148150	154154	175193	260260	144154	179179
F5	127127	142142	154154	175189	230234	154154	195211
F6	119127	142142	154169	175201	234260	152152	179195
F7	127127	142150	154163	189201	236260	144154	179195
F8	121127	142150	169169	175193	230234	152152	179209
F9	127127	142150	169169	175193	230260	144154	179205
F10	121127	142142	163169	175175	230234	144154	179209
F11	127127	142142	154154	175175	230230	144154	195195
F12	127127	142142	154154	189189	230234	144154	179195
F13	127127	142142	154154	189189	230234	154154	179195
F14	127127	142150	154163	175201	234236	144154	179211
F15	127127	142150	163169	175189	230236	144154	179211
F16	127127	142150	154163	175201	234236	144154	179211
F17	127127	148150	154163	175175	230230	144154	179195
F18	127127	148150	154163	175201	230236	144154	195211
F19	127127	142150	154163	175201	234236	154154	179211

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
F20	127127	142148	154154	175189	234234	144152	179209
F21	127127	142142	154163	175175	230230	144144	179209
F22	119127	142142	163169	175179	230230	144154	179205
F23	127127	142148	154163	175175	230230	154154	195211
G2	127127	142150	154169	179201	230234	152152	179195
G3	127127	142150	163169	189191	230230	144154	179209
G4	127127	142142	163169	175189	230230	144144	179195
G5	127127	142142	154169	175201	234260	154154	179195
G6	127127	142142	154163	175175	234234	144154	195205
G7	127127	142142	163169	175201	230260	144154	179179
G8	127127	142142	154154	189189	230234	144154	179195
G9	127127	142142	154169	175201	230236	144154	179179
G10	121127	148150	154163	189201	230230	144154	195211
G11	127127	142142	154154	189189	230234	154154	179195
G12	127127	142148	154163	175201	234236	144154	179211
G13	127127	142142	154154	189189	230234	144154	179195
G14	127127	142142	154154	189189	230234	144154	179195
G15	127127	142150	163163	175201	230234	154162	195205
G18	127127	142142	154154	189189	230234	144154	179195
G18c	127127	142150	154163	175201	234236	144154	179211
G19	127127	142150	154163	175201	234236	144144	179211
G20	127127	142150	154163	175175	230230	144144	179195
G21	127127	142148	154163	175175	230230	144154	195211
G22	127127	142148	154154	177191	230230	154154	195211
H2	127127	142150	163169	175189	230234	144152	179205
H3	127127	142150	154163	189201	236260	144154	179195
H4	121127	142142	154166	179201	230234	144154	179195
H5	127127	142150	163169	175193	230260	144154	179211
H6	121127	142150	163169	175175	234260	144154	179195
H7	127127	142142	163169	175193	230230	144154	179211
H8	127127	142142	154163	175201	230234	144144	195205
H9	121127	148150	154163	189201	230230	144154	195205
H10	127127	142148	154163	175175	230230	144154	195211
H11	127127	142142	154163	175201	230234	144154	195205
H12	127127	142142	154154	189189	230234	144144	195205
H13	127127	148150	169169	189193	230260	144154	179179
H14	127127	148148	154163	189201	230230	144154	195211
H17	127127	142150	154163	175201	234236	144144	179211
H18	127127	142150	154163	175201	234236	144154	179211
H19	127127	142148	154163	175175	230230	144154	195211
H20	127127	142142	154169	175201	230230	144154	179179
I2	127127	148150	154169	189201	230236	144144	179179
I3	127127	142150	154169	179201	234234	144144	179179
I4	127127	142150	154169	179201	234234	144144	179179
I5	119127	148150	154163	189201	230230	144154	195205
I6	119127	148150	154163	189201	230230	144154	195211
I7	119127	148150	154163	189201	230230	144154	195211
I8	127127	142150	163169	175189	230234	144154	179205
I9	127127	142142	154163	175189	230230	144154	179195
I10	127127	142142	154154	189189	230234	144154	179195
I11	127127	142142	154154	189189	230234	154162	179195
I12	127127	142150	154163	175201	234236	144144	179211
I14	121127	148150	154163	189201	230230	144144	195211
I15	127127	142150	154163	175201	234236	144144	179211
I16	127127	148150	154163	175201	230234	144144	195211
I17	127127	142150	154169	179201	234236	144144	179205
I18	121127	142142	154154	179189	234260	154154	179205
I19	127127	142150	154163	189201	234236	144154	179209
I20	127127	142148	154163	175175	230230	144144	195211
L2	127127	142150	154169	179201	234234	154154	179179
L3	127127	148150	154154	175193	260260	144154	179179
L4	127127	142150	154163	175179	230260	144154	179179
L5	121127	142142	163169	179195	230234	154154	179209
L6	121127	142148	163169	179201	230234	144154	179195
L7	127127	142148	163169	175191	236260	152154	179195
L8	127127	142142	154163	175201	230234	144154	195205
L9	127127	148150	163169	175175	230230	154154	195195
L10	127127	142142	154163	175201	230234	144154	195205
L11	127127	142142	154154	189189	230234	152152	179195
L12	127127	142148	154163	175175	230230	144154	195211
L13	127127	150150	154163	175201	230234	144154	195211
L14	127127	142142	154163	175201	230234	144144	195205
L15	127127	142142	154163	175201	230234	144154	195205
L19	127127	142150	154163	189201	234236	144144	179209
L20	127127	142142	154163	175177	230230	154154	179209
M1	127127	142142	163163	189201	230234	144154	195205

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
M2	127127	142148	154163	175201	230234	154154	195211
M3	121127	148150	154163	189201	230230	144154	195211
M4	127127	148150	154154	175193	260260	144154	179179
M5	127127	142148	154154	189201	230230	154154	179195
M6	121127	148150	154163	189201	230230	144154	195211
M7	127127	148150	154154	175193	260260	144154	179179
M8	127127	142150	154163	175201	230230	154154	179211
M9	121127	150150	154154	175175	234260	144154	179179
M10	127127	148150	163169	175175	230230	154154	195195
M11	127127	148150	154163	189201	230230	144154	195211
M12	121127	148150	154163	189201	230230	152152	195211
M13	127127	148150	163169	175175	230230	154154	195195
M14	127127	142142	154169	175175	230230	152154	179195
M15	127127	148150	154163	175201	230234	144154	195211
M16	127127	142142	154154	179203	236236	144154	179205
N1	127127	142142	154154	175189	234234	144154	195209
N2	127127	142148	154169	175201	230230	144144	179195
N3	127127	142148	163163	175201	230230	144154	179211
N4	127127	142148	154163	175201	230234	144144	195211
N5	127127	142150	163169	175175	230234	144154	179179
N6	127127	142150	154169	175193	230260	144152	179205
N7	127127	142142	154163	175201	230234	144154	179195
N8	127127	142150	163169	175189	230234	144154	179195
N9	121127	150150	154163	175201	230236	154154	179195
N9c	127127	142142	154163	175201	230234	152152	195205
N10	127127	142150	154163	175201	234236	144152	179211
N11	121127	142148	154163	193193	230236	154154	179179
N12	127127	142142	163169	175201	230234	154162	179195
N13	119127	142150	154154	175175	230236	152154	179179
N14	127127	142142	154169	175175	230230	154154	179195
N15	127127	142150	154163	191193	230230	144154	179205
N16	119127	142142	154154	179203	236236	144154	179195
O1	121127	142150	163163	189201	234236	144144	195205
O2	127127	142142	154169	175193	230234	144154	195209
O3	121127	142150	154154	175201	234234	144154	195205
O4	121127	148150	154163	189201	230230	152152	195211
O5	127127	142142	154163	175201	230234	144154	195205
O6	127127	148150	154169	175175	230260	152154	179179
O7	121127	148150	154163	189201	230230	152162	195211
O8	127127	142150	154154	175201	234236	144154	195211
O9	127127	142150	154163	175201	234236	154154	195211
O10	127127	142150	163163	175175	230234	154154	179205
O11	127127	142142	154154	175179	230230	144154	179205
O12	121127	142148	154154	193193	230236	144144	179179
O13	127127	142150	154154	175179	230236	144144	179179
O14	127127	142142	163169	175179	230234	154154	179209
O15	127127	142148	154154	175201	230234	144144	195211
P2	127127	142142	154163	175189	230230	144154	179179
P3	121127	148150	154163	175201	234234	154154	179195
P4	121127	142148	154169	175175	234260	144154	179179
P5	127127	142142	163163	175175	234236	144154	179205
P6	121127	142150	163163	189201	230230	144154	195211
P7	127127	142148	154154	189201	230230	154154	179195
P8	127127	142150	154163	189201	236260	144154	179195
P9	127127	142142	154163	175201	230234	144154	179195
P10	127127	148150	163169	175175	230230	152152	195195
P10c	127127	142142	154154	175189	230234	144154	179195
P11	127127	142142	154154	175201	230234	154154	195205
P12	127127	142142	163169	175179	230234	144154	179209
P13	127127	142142	154163	175201	230234	144154	195205
P13c	127127	142142	154163	175179	230234	154154	179195
P14	127127	142150	163169	175175	230236	144144	179179
P15	127127	142142	163169	201201	230230	144154	179205
P24	127127	142148	154163	175175	230236	144154	179211
Q2	127127	142142	154169	201201	230230	152154	195205
Q3	121127	148150	154163	189201	230230	154154	195211
Q4	127127	148150	163163	175175	230236	152154	179179
Q5	127127	142148	163169	175187	234234	154154	179209
Q6	121127	142150	163163	175175	234234	144144	179211
Q8	127127	142142	166169	187201	230260	144154	179195
Q9	127127	142142	154154	175189	230234	144154	179195
Q10	127127	142150	154163	175201	234236	144154	179211
Q11	127127	142142	154154	189189	230234	144154	179195
Q12	127127	142142	163163	175179	230230	144154	179209
Q13	127127	148148	163169	175191	230230	144154	195211
Q14	127127	142142	163169	201201	230230	154154	179205

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
R2	127127	142150	154163	175175	230260	144154	195205
R3	127127	142142	154163	175201	230260	154154	179211
R4	127127	150150	163169	175201	234236	144154	195205
R6	127127	142148	169169	175187	234234	144144	179209
R7	127127	142142	154163	175201	230234	144154	195205
R9	127127	142142	154154	175189	230234	144154	179195
R11	127127	142142	154154	189189	230234	144154	179195
R12	127127	142142	163169	201201	230230	144144	179205
R13	127127	142142	154163	175201	230234	144154	195205
R14	127127	142142	154154	179203	236236	152154	179205

QUADRATS

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
1.1	127127	148150	163169	179179	260260	154154	195195
1.2	121127	148150	154163	189201	230230	144154	195211
1.3	121127	148148	154163	189201	230230	144154	195211
1.4	127127	142142	154163	175201	230234	144154	195205
1.5	127127	142142	154163	175201	230234	144154	195205
1.6	121127	148148	154163	189201	230230	144154	195211
1.7	121127	148148	154163	189201	230230	144154	195211
1.8	121127	148150	154163	189201	230230	144154	195211
1.9	127127	142142	154163	175201	230234	144154	195205
1.10	121127	148150	154163	189201	230230	144154	195211
1.11	127127	142142	154163	175175	230234	144154	195205
1.12	127127	142142	154154	189189	230234	144154	179195
1.13	121127	148148	154163	189201	230230	144154	195211
1.14	127127	148148	154163	189201	230230	144154	195211
1.15	127127	142142	154154	189201	230230	144154	179195
1.16	121127	148150	154163	189201	230230	144154	195211
1.17	121127	148150	154163	189201	230230	144154	195211
1.18	127127	148148	154163	189201	230230	144154	195211
1.19	121127	142150	169169	175193	230260	154162	179205
1.20	121127	148150	154163	189201	230260	144154	195211
1.21	121127	148148	154163	189201	230230	144154	195211
1.22	127127	148150	154163	189201	230230	144154	195211
1.23	127127	142150	169169	175193	230260	154162	179205
1.24	121127	142150	169169	175193	230230	154162	179205
1.25	121127	148148	154163	189201	230230	144154	195211
2.1	127127	142142	163169	175201	230234	144154	179195
2.2	127127	142150	154163	189189	230234	144144	179211
2.3	127127	142142	154163	193201	230230	154162	179179
2.4	127127	142142	169169	175193	230260	154162	179205
2.5	127127	142142	154163	175179	230236	154154	179205
2.7	121127	142148	154163	193193	230230	154154	179179
2.8	127127	142148	154154	189201	234234	144154	179195
2.9	127127	142142	163169	175201	230234	144154	179195
2.10	127127	142142	154169	175175	230230	144152	179195
2.11	127127	142142	154163	175175	230230	144144	205211
2.12	127127	142142	154163	175201	230234	144154	195205
2.13	127127	142142	154163	179201	230230	152154	179179
2.14	121127	142148	154163	193193	230234	154154	179179
2.15	127127	142142	154163	175177	230236	154154	179205
2.16	127127	142142	154163	175179	230230	154154	179205
2.17	127127	142142	154163	175179	230234	154154	179195
2.18	127127	142142	163169	175179	230234	144154	179209
2.19	127127	142142	154169	175177	230230	144152	179195
2.20	127127	142142	154163	175179	230234	154154	179195
2.21	121127	142142	163169	175179	230230	144154	179205
2.22	127127	142142	154163	175179	230230	154154	179209
2.23	127127	140150	154169	175175	230230	144152	179195
2.24	127127	142150	154163	175179	230230	154154	179205
2.25	127127	142142	154163	175179	230234	154154	179195
3.1	121127	142148	169169	175189	230230	144154	179195
3.2	127127	142148	154163	175175	230230	144144	195211
3.3	127127	142142	163169	189201	230260	144154	179195
3.4	127127	142150	154163	175201	234236	144154	179211
3.6	127127	142148	163169	175189	230230	144154	209211
3.7	127127	142150	154163	175201	236236	144154	179211
3.8	127127	142150	154163	175201	234236	144154	179211
3.9	127127	142150	154163	175201	230234	144154	179211
3.10	127127	142150	154163	175201	234236	144154	179211
3.11	127127	142150	154163	175201	234236	144154	179211

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
3.12	127127	142150	154163	175201	234236	144154	179211
3.13	127127	142148	154163	175175	230230	144144	195211
3.14	127127	142142	154163	175201	230234	144154	195205
3.15	127127	142148	154163	189189	230234	144144	179211
3.16	127127	142150	154163	175201	230234	144154	179211
3.17	127127	142150	154163	175201	234236	144154	179211
3.18	127127	142150	154163	175201	234236	144154	179211
3.19	127127	142150	154169	177201	234234	154154	179179
3.20	127127	142148	154163	175175	230230	144144	195211
3.21	127127	142148	154163	175175	230230	144144	195211
3.22	127127	142150	154163	175201	234236	144154	179211
3.23	127127	142150	154163	175201	234236	144154	179211
3.24	127127	142150	154163	175201	236236	144154	179211
3.25	127127	142142	154163	175179	230234	144152	179195
4.1	127127	142148	154163	175175	230230	144144	195211
4.2	121127	142148	154163	189207	230234	144154	205211
4.3	127127	142142	154163	175201	230234	144154	179195
4.4	127127	142150	154163	175201	234236	144154	179211
4.5	127127	142148	154163	175175	230230	144144	195211
4.6	127127	142150	163169	193193	234236	154154	195209
4.7	121127	142150	154163	179191	230234	144154	179179
4.8	127127	142150	154163	175201	234236	144154	179211
4.9	127127	142150	154163	175201	234236	144154	179211
4.10	127127	142150	154154	175201	230230	144144	195211
4.11	127127	142142	154154	191191	230234	154154	179179
4.12	127127	142142	154163	189201	230230	154154	195195
4.13	127127	142148	154169	193193	230260	152154	179179
4.14	127127	142150	163169	193193	234236	154154	195209
4.15	127127	142142	154154	189195	230260	144154	179179
4.16	127127	142148	154163	175189	230234	144154	205209
4.17	127127	142142	154163	175201	230234	144154	195205
4.18	121127	142148	154163	175195	230234	144154	205211
4.19	127127	142150	163169	193193	234236	154154	195209
4.20	127127	142150	154163	175201	234236	144154	179211
4.21	127127	142142	163169	175179	230234	144154	179195
4.22	121121	142150	154163	179191	230234	144152	179209
4.23	127127	142148	154169	175201	230230	144144	195211
4.24	121127	142142	154169	177189	230260	144152	179179
4.25	127127	142148	163169	175179	230234	144154	179209
5.1	127127	142150	154163	175175	230236	144154	195211
5.2	127127	142150	154169	175193	230230	154154	179209
5.3	127127	142150	154163	175201	236236	144154	179211
5.4	127127	142150	154163	175201	234236	144154	179211
5.5	127127	142148	154163	175175	230230	144144	179211
5.6	127127	142142	154163	175201	234236	144154	195211
5.7	127127	142150	163163	175191	234260	152152	195209
5.8	127127	142150	154169	175193	230230	154154	179209
5.9	121127	142150	154163	179193	234236	154154	179179
5.10	121127	142142	154154	189193	234260	144144	179205
5.11	121127	142150	154163	179193	234236	154154	179179
5.12	121127	142150	154163	179193	230234	144154	179179
5.13	127127	142148	154163	175175	230230	144144	195211
5.14	127127	142142	154163	175189	230234	144144	179195
5.15	127127	142150	154163	175175	230230	144154	179195
5.16	127127	142148	154154	175201	230230	144144	195211
5.17	127127	142150	154169	177201	234234	154154	179179
5.18	127127	142150	163163	175175	230230	152152	179195
5.19	127127	142150	163169	189201	230230	144154	179179
5.20	127127	142142	154154	189195	230260	144154	179179
5.21	127127	140150	154163	175193	234234	154154	205209
5.22	127127	142148	154163	175175	230230	144144	195211
5.23	121127	142148	154163	179191	230234	144154	179179
5.24	127127	142150	163169	191191	234234	154154	195209
5.25	127127	142142	154163	177179	230230	154154	179209

CORES

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
A1_1	127127	142148	154154	189201	230230	144154	179195
A1_2	127127	142142	163169	175201	230230	144154	195205
A1_3	127127	142148	154154	189201	230230	144154	195205
A1_4	127127	148150	154163	189201	230230	144154	179195
A1_5	121121	142142	163163	175175	230230	144154	179205
A1_6	127127	142142	169169	175179	230230	154154	179195
A1_7	121127	142148	163169	189201	230250	144154	179195
A1_8	127127	142148	154154	189201	230230	144154	179195
A1_9	127127	148150	154154	175193	260260	154154	179179
A1_10	121127	142150	163169	189201	230260	144154	179195
A1_11	127127	142150	154154	189201	230230	144154	179195
A1_12	127127	142150	169169	175193	230230	154162	179205
A1_13	127127	142142	169169	175179	230234	154154	179205
A1_14	127127	142142	154163	175201	230230	154154	179195
A1_15	127127	142148	169169	175191	230230	154162	179195
A1_16	127127	142148	154154	189189	230230	144154	179209
A1_18	127127	142142	163163	175175	234234	144144	195205
A1_20	127127	142142	169169	175179	230234	154154	179195
A1_21	127127	142148	169169	175193	230230	154162	179195
A1_22	127127	142150	169169	175193	230230	154162	179195
A2_1	127127	142148	154163	175175	230230	144154	179179
A2_2	127127	142142	169169	175179	260260	154154	179195
A2_3	121127	148150	154163	189201	230230	144154	195211
A2_4	127127	142142	154163	179201	230230	154154	179211
A2_5	127127	142148	154154	189201	230230	144154	179195
A2_6	127127	142142	163163	175175	230230	144154	179195
A2_7	127127	142148	169169	193201	230230	144154	179195
A2_8	127127	142142	163169	175201	230230	144154	195195
A2_9	127127	142142	163163	175175	230230	144154	179195
A2_10	121127	142142	163163	175179	230230	144144	179195
A2_11	127127	142142	169169	175193	230230	144154	179179
A2_12	121127	148150	154163	189201	230230	144154	195211
A2_13	127127	142148	154169	179203	230230	144154	195211
A2_14	127127	142150	154169	175193	230230	154154	179195
A2_15	127127	142142	163163	175179	230234	144154	195195
A2_16	127127	142148	154163	175175	230230	144154	179179
A2_17	127127	148150	163169	177189	230230	152154	179205
A2_18	127127	142150	154163	175175	234234	144154	179195
A2_19	127127	142150	154169	175193	230260	154154	179195
A2_20	127127	142150	154169	177203	230230	144154	179195
A2_21	127127	142142	169169	175179	230230	154154	179195
A2_22	127127	142142	163163	175179	230234	144154	179195
A2_23	121127	142142	169169	175179	230230	144154	179195
A2_24	127127	142142	163163	175175	230230	144154	195205
A3_1	121127	148150	154163	189201	230230	144154	195211
A3_2	127127	142148	154154	189201	230230	144154	179195
A3_3	121127	148150	154163	189201	230230	144154	195211
A3_4	127127	142148	154154	189201	230230	144154	179195
A3_5	121127	142148	163169	175191	230234	144154	179195
A3_6	127127	150150	163163	175175	230234	144154	195195
A3_7	127127	142148	154154	189201	230230	144154	179195
A3_8	121127	142148	163169	175191	230234	144154	179195
A3_9	121127	142148	163169	175191	230234	144154	179195
A3_10	121127	142150	163169	189201	230260	144154	179179
A3_11	121127	142148	163169	175193	230230	144154	195205
A3_12	127127	142142	154163	175201	230234	144154	195205
A3_13	121127	142142	163169	175175	230234	144154	179209
A3_14	127127	150150	163163	175175	230234	144154	195195
A4_1	127127	142148	163163	175203	230230	144154	179211
A4_2	127127	142142	163163	175175	234234	144154	195205
A4_3	127127	142148	154154	189201	230230	144154	179195
A4_4	127127	142142	163163	175175	234234	144154	195205
A4_5	127127	142148	154163	175175	230260	144154	179179
A4_6	121127	148150	154163	189201	230230	144154	195211
A4_7	127127	142148	154163	175175	230230	144154	179179
A4_8	127127	142142	154163	175201	230230	144154	179195
A4_9	127127	142148	154154	189201	230230	144154	179195
A4_10	121127	148150	154163	189201	230230	144154	195211
A4_11	121127	142150	154163	175201	260260	154154	179179
A4_12	127127	142148	154163	175175	230260	144154	179179
A4_13	121127	142150	163163	175175	230234	154154	179179
A4_14	127127	142148	154154	189201	230230	144154	179195
A4_15	121127	142150	163163	175175	230230	154154	179179

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
A4_16	127127	142148	154154	189201	230230	144154	179195
A4_17	127127	142148	154163	175175	230260	144154	179179
A4_18	127127	142142	154163	177201	230236	154154	179179
A4_19	127127	142150	163163	175193	234234	144154	179205
A4_20	127127	142142	163163	177189	230230	144154	179195
A4_21	127127	142148	154163	175175	230260	144154	179179
A4_22	127127	142142	163163	175175	234234	144154	195205
A4_24	127127	142148	154163	175175	230260	144154	179179
A4_25	127127	142150	163163	175201	230230	154154	195209
A4_26	127127	142148	154163	175201	230234	144144	195211
A5_1	127127	142150	154163	175175	230230	144154	179195
A5_2	127127	142148	154163	175175	230230	144154	179195
A5_3	127127	140150	154163	193193	230230	154154	179179
A5_4	127127	142142	154163	175201	230234	144154	195205
A5_5	127127	142150	169169	175193	230260	154162	179205
A5_6	127127	142148	154154	189201	230230	144154	179195
A5_7	127127	142142	163169	175201	230234	144154	179195
A5_8	127127	142150	169169	175193	230260	154162	179205
A5_9	127127	142148	154154	189201	230230	144154	179195
A5_10	127127	142150	169169	175193	230260	154162	179205
A5_11	121127	148150	154163	189201	230230	144154	195211
A5_12	127127	142150	169169	175193	230260	154162	179205
A5_13	127127	142148	154163	175201	230234	144144	195211
A5_14	127127	142150	169169	175193	230260	154162	179205
A5_15	127127	142150	154163	175175	230230	144154	179195
A5_16	127127	142150	169169	175193	230260	154162	179205
A5_17	127127	142150	169169	175193	230260	154162	179205
A5_18	127127	142150	169169	175193	230260	154162	179205
A5_19	127127	142150	169169	175193	260260	154162	179205
A5_20	127127	142150	169169	175193	230260	154162	179205
A5_21	127127	142148	154163	175201	230234	144144	195211
A5_22	127127	142150	169169	175193	230260	154162	179205
A5_23	127127	142150	169169	175193	230260	154162	179205
A5_24	127127	142148	154154	189201	230230	144154	179195
A5_25	127127	142148	154154	189201	230230	144154	179195
A5_26	127127	142150	169169	175193	230260	154162	179205
A5_27	121127	148150	154163	189201	230230	144154	195211
A5_28	127127	142148	154163	175201	230234	144144	195211
A5_29	127127	142142	163169	175175	230234	144154	209209
A5_30	127127	142148	154154	175193	230230	154162	179205
A5_31	121127	142150	169169	175193	230234	154154	179209
A5_32	127127	142150	154163	175175	230230	144154	179195
A5_33	127127	142142	154163	175201	230234	144154	195205
A5_34	127127	142150	169169	175193	230260	154162	179205
A5_35	127127	142150	154163	175175	230230	144154	179195
A5_36	127127	142150	169169	175193	230260	154162	179205

APPENDIX 2b

Here the Data Matrix used for analyses in Chapter IV is presented

Cymodocea nodosa

Monospecific stands

Sample	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 20*	*Cy 17*	*Cy 18*
1_1	142142	154154	189189	230234	143155	179195
1_2	142142	154154	189189	230236	143155	179195
1_3	142142	154154	189189	230236	143155	179195
1_4	142142	154154	189189	230236	143155	179195
1_6	142142	154154	189189	230236	143155	179195
1_7	142150	154163	175201	230234	143155	179211
1_8	142142	154154	189189	230230	143155	179195
1_9	142142	154154	189189	230234	143155	179195
1_10	142142	154154	189189	230230	143155	179195
1_11	142142	154154	189189	230234	143155	195195
1_12	142142	154154	189189	230234	143155	179195
1_13	142150	163163	175201	230236	143155	179195
1_14	142142	154154	189189	230234	143155	179195
1_15	142148	154163	175201	230234	143143	195211
1_16	142142	154154	189189	230234	143155	179195
1_17	142142	154154	189189	230234	143155	179195
1_18	142142	154163	189189	230234	143143	179179
1_19	142142	154154	189189	230234	143155	179195
1_20	142142	154163	175201	230234	143155	195205
1_21	142142	154154	189189	230234	143155	179195
2_1	142142	154154	189189	230234	143155	179195
2_2	142142	154154	189189	230236	143155	179195
2_3	142142	154154	189189	230234	143155	179195
2_4	142142	154154	189189	230234	143155	179195
2_5	142142	154154	189189	230234	143155	195195
2_6	142148	154163	175175	230230	143143	195211
2_7	142150	154163	175201	234236	143155	179211
2_8	148150	154163	189201	230230	143155	195211
2_9	142142	154154	189189	230234	143155	179195
2_10	142142	154154	189189	230236	143155	179195
2_11	142142	154154	189189	230234	143155	179195
2_12	142142	154154	189189	230234	143155	179195
2_13	142142	154154	189189	230236	143155	179195
2_14	142150	163169	175175	230230	155155	195195
2_15	142150	154163	175201	234236	143155	179211
2_16	142142	163163	175195	234236	143155	209209
2_17	142142	154154	175191	230230	143155	179211
2_18	142150	154163	175201	234236	143155	179211
2_19	142142	154154	189189	230234	143155	179195
2_20	142142	154154	189189	230234	143155	179195
3_1	142142	154163	175201	230234	143153	195205
3_2	142148	154163	175175	230230	143143	195211
3_3	142150	154163	175201	234236	143155	179211
3_4	142142	154163	175201	230236	143155	195205
3_5	142142	154163	175201	230234	143155	195205
3_6	142150	154163	175201	230236	143155	179211
3_7	142150	154163	175201	234236	143155	179211
3_8	142142	154163	175201	230234	143155	195205
3_9	142150	154163	175201	234236	143155	179211
3_10	142142	154163	175201	230234	143155	195205
3_11	142150	154163	175201	234236	143155	179211
3_12	142142	154163	175201	230234	143155	195205
3_13	142150	154163	175201	234236	143155	179211
3_14	150150	154169	175191	230260	153155	179195
3_15	142150	163163	175201	230234	155155	195205
3_16	142142	154163	175201	230234	143155	195205
3_17	142150	163163	175201	230234	143155	179211
3_18	142142	163163	175201	230234	143155	195205
3_19	142150	154163	175201	234236	143155	179211
3_20	142142	154163	175201	230236	143155	195205
3_21	142150	154163	175201	234236	143155	179211

Sample	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 20*	*Cy 17*	*Cy 18*
4_1	142142	154163	175175	230232	153153	179209
4_2	142142	154169	175201	230230	143155	179179
4_3	142142	163163	175175	230236	155155	179195
4_4	142150	154163	179195	230234	143155	179195
4_5	142148	163169	175175	234234	153153	179195
4_6	142148	154163	175175	230230	143143	195211
4_7	142142	154169	177201	230230	143153	179195
4_8	142150	154163	175201	230234	143155	179211
4_9	142150	154163	175201	234236	143155	179211
4_10	142150	154163	175191	230230	143155	179211
4_11	142148	154163	175175	230230	143143	195211
4_12	142150	154163	191201	230230	143155	179195
4_13	142150	154163	189201	234236	143143	179195
4_14	142148	163169	175175	234234	155155	179195
4_15	142150	154163	175175	230230	143155	179195
4_16	142148	154154	175201	230230	143143	195211
4_17	142150	154163	175201	234234	143155	179211
4_18	142150	163169	175175	234234	153153	179195
4_19	148148	154163	175201	230232	143155	179195
4_20	142150	154163	175201	234236	143155	179211
4_21	142150	154163	175201	234234	143155	179211
5_1	142150	154169	175201	230232	153155	179179
5_2	142142	154163	179191	230234	153153	195195
5_3	142150	154169	175201	230230	155155	179179
5_4	142142	154163	175201	230230	155155	179211
5_5	142150	154169	175175	234260	143155	179211
5_6	142142	154163	175201	230230	155155	179179
5_7	142142	154154	189201	234234	143155	179205
5_8	142150	154169	195201	234234	153155	179195
5_9	142142	154163	175201	230230	155155	179179
5_10	142142	154163	175201	230230	155155	179179
5_11	142142	154154	175191	230234	143143	179211
5_12	142142	154163	189189	230234	153153	195195
5_13	142142	154154	175201	230230	143155	195195
5_14	142142	154154	175201	230230	143155	195195
5_15	142142	154154	189201	234234	143155	179205
5_16	142150	154163	175195	234234	143153	179195
5_17	142142	154163	175195	230234	153155	195195
5_18	142142	163163	175175	230236	155155	195205
5_19	142150	154169	175201	230232	153155	179179
5_20	142150	163163	175191	230234	153153	179211
5_21	142142	154169	175195	230234	153153	179179

Mixed Stands

Sample	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 20*	*Cy 17*	*Cy 18*
1_2	142142	154163	175201	230234	143155	195205
1_3	142150	154163	175201	234236	143155	179211
1_4	142150	154163	175201	234236	143155	179211
1_5	142150	154163	175201	234236	143155	179211
1_6	142142	154163	175201	230234	143155	195205
1_7	142142	154163	175201	230234	143155	195205
1_8	142150	154163	175201	230234	143155	179195
1_9	142150	154163	175201	234236	143155	179211
1_10	142150	154163	175201	234236	143155	179211
1_11	142142	154163	175201	230234	143155	195205
1_12	142150	154163	175201	234236	143155	179211
1_13	142150	154163	175201	234236	143155	179211
1_14	148150	163169	175175	230230	153153	195195
1_15	142142	154163	175201	230234	143155	195205
1_16	142142	154163	175201	230234	143155	179195
1_17	142150	154163	175201	234236	143155	179211
1_18	142142	154163	175201	230234	143155	195205
1_19	148150	154163	175201	230236	143155	195211
1_20	142142	154163	175201	230234	143155	195205
1_21	142142	154154	189189	230234	143155	179195
2_1	142150	163169	175191	232234	153155	179195
2_2	142150	163169	175191	232234	153155	179195
2_3	142150	163169	175191	232234	153155	179195
2_4	142150	163169	175191	232232	153153	179195
2_5	142150	163169	175191	232234	153155	179195
2_6	142150	163169	175191	232234	153153	179195
2_7	142150	163163	175191	234234	143143	195205
2_8	142150	163169	175191	232234	153155	179195
2_9	140142	154163	175191	230232	155155	205209
2_10	142150	163169	175191	232234	153155	179195
2_11	140142	154163	175191	230230	155155	205209
2_12	140142	154163	175191	230230	155155	205209
2_13	142150	163169	175191	230234	155155	211211
2_14	142142	154154	175189	230234	143143	179195
2_15	140142	154163	175191	230232	155155	205209
2_16	142150	163169	175191	232234	153155	179195
2_17	142150	163169	175191	232234	153155	179195
2_18	140142	154163	175191	230230	155155	205209
2_19	140142	154163	175191	230232	155155	205209
2_20	142150	163169	175191	232234	153153	179195
2_21	142150	163169	175191	232234	153155	179195
3_1	142142	163169	179195	230230	155155	179195
3_2	150150	154169	189195	230260	153153	179195
3_3	150150	154169	189195	230260	155155	179179
3_4	142142	163169	201201	230232	153153	179205
3_5	142142	154163	175195	234236	143155	179189
3_6	142150	163169	175175	230230	143143	179195
3_7	142142	163163	175175	230236	153155	179195
3_8	142142	163169	179195	230230	155155	179195
3_10	142142	163169	175191	230230	155155	179195
3_11	142142	163169	191201	230232	153153	179205
3_12	142142	163163	175175	230236	153153	179195
3_13	142142	154163	175201	232234	153153	179195
3_14	142142	163169	179195	230230	155155	179195
3_15	142142	154169	175175	230230	143155	179195
3_16	150150	154163	189195	230260	155155	179195
3_17	150150	163169	175191	230236	155155	179195
3_18	142150	163169	175189	230230	143155	179195
3_19	142142	154154	175175	230230	143155	195195
3_20	142150	154154	175195	230234	155155	179195
3_21	142142	163163	175175	230236	155155	179195
4_1	142150	163163	175191	234234	143143	195205
4_2	142142	163163	175189	230234	143143	205211
4_3	142142	163163	195201	230230	153155	195205
4_4	142142	169169	175201	230230	143155	179195
4_5	142142	163169	175189	230234	143155	179209
4_6	142148	163163	175201	234236	143155	205211
4_7	142148	163163	175201	234236	143155	205211
4_8	142142	163163	175189	230234	143143	205211
4_9	142150	163169	175201	230240	143153	179195
4_10	140150	154169	175201	230230	155155	179205

Sample	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 20*	*Cy 17*	*Cy 18*
4_11	142148	163163	175201	234236	143155	205211
4_12	142150	154169	175201	230230	155155	179205
4_13	142142	163163	175189	230234	143143	205211
4_14	142150	163169	175189	230260	155155	179209
4_15	142150	163163	175191	234234	143143	195205
4_16	142142	163163	175189	234260	143155	179195
4_17	142142	163163	175189	230234	143143	205211
4_18	142148	163163	175201	234236	143155	205211
4_19	142148	163163	175201	234236	143155	205211
4_20	142150	154163	175175	234260	153153	179195
5_1	142142	163169	195195	230230	155155	179211
5_2	142142	163163	175195	230234	143143	179179
5_3	142150	154154	191201	230230	143155	179195
5_4	142142	154163	175201	232260	143155	179211
5_5	140142	154163	175195	230230	155155	205211
5_6	142150	154169	175195	230230	155155	179179
5_7	142150	154154	191201	230230	143155	179195
5_8	142150	163163	195195	230234	155155	179205
5_9	142142	163163	175195	230236	143143	179179
5_10	142142	154169	175191	234236	143155	179179
5_11	142142	154163	175195	230230	155155	205211
5_12	142142	154163	175195	230230	155155	205211
5_13	142150	154154	189201	230230	143155	179195
5_14	142150	154154	191201	230230	143155	179195
5_15	142150	163163	177191	230234	143153	195211
5_16	142150	163163	177201	234236	155155	179205
5_17	142150	154169	175195	230230	155155	179179
5_18	142142	154163	175201	230234	143155	195205
5_19	142148	154169	175175	234260	143155	179179
5_20	140142	154163	175195	230232	153155	205211
5_21	142150	154169	175177	230230	153155	179179

Zostera noltii

	Monospecific stands					
Sample	**F11**	**F8**	**B8**	**B3**	**B1**	**D6**
1.02	283285	197210	139149	180212	106116	185221
1.03	283285	197210	139149	180212	106116	185221
1.04	283285	197210	139149	180212	106116	185221
1.05	283285	197210	139149	180212	106116	185221
1.08	283285	197210	139149	180212	091091	225225
1.09	283285	197210	139149	180212	106116	185221
1.10	283285	197210	139149	180212	106116	185221
1.11	283285	197210	139149	180212	106116	185221
1.12	283293	210216	145149	180192	091116	221221
1.13	293297	210216	145149	180192	091116	221221
1.14	293297	210216	145149	180192	091116	221221
1.15	283293	210216	145149	180192	091116	221221
1.16	283293	210216	145149	180192	091116	221221
1.17	283293	210216	145149	180192	091116	221221
1.18	283293	210216	145149	180192	091116	221221
1.19	283285	197210	139149	180212	106116	185221
1.20	283285	197210	139149	180212	106116	185221
1.21	293297	210216	145149	180192	091116	221221
2.01	283293	197197	145149	180192	091116	221221
2.02	293297	210216	145149	180192	091116	221221
2.03	293297	210216	145149	180192	091116	221221
2.04	293297	210216	145149	180192	091116	221221
2.05	293297	210216	145149	180192	091116	221221
2.06	293297	210216	145149	180192	091116	221221
2.07	293297	210216	145149	180192	091116	221221
2.08	293297	210216	145149	180192	091116	221221
2.09	293297	210216	145149	180192	091116	221221
2.10	293297	210216	145149	180192	091116	221221
2.11	293297	210216	145149	180192	091116	221221
2.12	293297	210216	145149	180192	091116	221221
2.13	293297	210216	145149	180192	091116	221221
2.14	293297	210216	145149	180192	091116	221221
2.16	293297	210216	145149	180192	091116	221221
2.17	293297	210216	145149	180192	091116	221221
2.18	293297	210216	145149	180192	091116	221221
2.19	293297	210216	145149	180192	091116	221221
2.20	293297	210216	145149	180192	091116	221221
2.21	293297	210216	145149	180192	091116	221221
3.01	285293	210216	145149	180192	091116	221223
3.02	285293	210216	145149	180192	091116	221223
3.03	285293	210216	145149	180192	091116	221223
3.04	283285	197210	139149	180212	106116	185221
3.05	285293	210216	145149	180192	091116	221223
3.06	283285	197210	139149	180212	106116	185221
3.07	283285	197210	139149	180212	106116	185221
3.08	283285	197210	139149	180212	106116	185221
3.09	283285	197210	139149	180212	106116	185221
3.10	285293	210216	145149	180192	091116	221223
3.11	285293	210216	145149	180192	091116	221223
3.12	285293	210216	145149	180192	091116	221223
3.13	285293	210216	145149	180192	091116	221223
3.14	285293	210216	145149	180192	091116	221223
3.15	285293	210216	145149	180192	091116	221223
3.16	285293	210216	145149	180192	091116	221223
3.17	285293	210216	145149	180192	091116	221223
3.18	285293	210216	145149	180192	091116	221223
3.19	285293	210216	145149	180192	091116	221223
3.20	285293	210216	145149	180192	091116	221223
3.21	283285	197210	139149	180212	106116	185221
4.01	283285	197210	139149	180212	106116	185221
4.02	283285	197210	139149	180212	106116	185221
4.03	283285	197210	139149	180212	106116	185221
4.04	283285	197210	139149	180212	106116	185221
4.05	283285	197210	139149	180212	106116	185221
4.06	283285	197210	139149	180212	106116	185221
4.07	283285	197210	139149	180212	106116	185221
4.08	283285	197210	139149	180212	106116	185221
4.09	283285	197210	139149	180212	106116	185221

4.10	283285	197210	139149	180212	106116	185221
4.11	283285	197210	139149	180212	106116	185221
4.12	283285	197210	139149	180212	106116	185221
4.13	283285	197210	139149	180212	106116	185221
4.14	283285	197210	139149	180212	106116	185221
4.15	283285	197210	139149	180212	106116	185221
4.16	283285	197210	139149	180212	106116	185221
4.17	283285	197210	139149	180212	106116	185221
4.18	283285	197210	139149	180212	106116	185221
4.19	283285	197210	139149	180212	106116	185221
4.20	283285	197210	139149	180212	106116	185221
4.21	283285	197210	139149	180212	106116	185221
5.01	283285	197210	139149	180212	106116	185221
5.02	283285	197210	139149	180212	106116	185221
5.03	283285	197210	139149	180212	106116	185221
5.04	283285	197210	139149	180212	106116	185221
5.05	283285	197210	139149	180212	106116	185221
5.06	283285	197210	139149	180212	106116	185221
5.08	285293	197210	145149	180192	091116	221223
5.09	283285	197210	139149	180212	106116	185221
5.10	283285	197210	139149	180212	106116	185221
5.11	283285	197210	139149	180212	106116	185221
5.12	283285	197210	139149	180212	106116	185221
5.13	283285	197210	139149	180212	106116	185221
5.14	283285	197210	139149	180212	106116	185221
5.15	283293	197210	145149	180192	091116	221221
5.16	283285	197210	139149	180212	106116	185221
5.17	285293	210216	145149	180192	091116	221223
5.18	283285	197210	139149	180212	106116	185221
5.19	283285	197210	139149	180212	106116	185221
5.20	283285	197210	139149	180212	106116	185221

		Mixed stands				
Sample	**F11**	**F8**	**B8**	**B3**	**B1**	**D6**
1.01	283293	210216	145149	180192	091116	221223
1.08	283285	197210	139149	180212	106116	185221
1.09	283285	197210	139149	180212	106116	185221
1.10	283285	197210	139149	180212	106116	185221
1.12	283285	197210	139149	180212	106116	185221
1.13	283285	197210	139149	180212	106116	185221
1.14	283285	197210	139149	180212	106116	185221
1.15	283285	197210	139149	180212	106116	185221
1.16	283285	197210	139149	180212	106116	185221
1.17	283285	197210	139149	180212	106116	185221
1.18	283285	197210	139149	180212	106116	185221
1.19	283285	197210	139149	180212	106116	185221
2.01	283293	197216	139145	180212	106116	221221
2.02	283293	197216	139145	180212	106116	221221
2.03	283293	197216	139145	180212	106116	221221
2.04	283293	197216	139145	180212	106116	221221
2.05	283293	197216	139145	180212	106116	221221
2.06	277283	197203	139139	194223	103105	208212
2.07	283293	197216	139145	180212	106116	221221
2.08	277283	197203	139139	194223	103105	208212
2.09	283293	197216	139145	180212	106116	221221
2.10	283293	197216	139145	180212	106116	221221
2.11	283293	197216	139145	180212	106116	221221
2.12	283293	197216	139145	180212	106116	221221
2.13	283293	197216	139145	180212	106116	221221
2.14	283293	197216	139145	180212	106116	221221
2.15	283293	197216	139145	180212	106116	221221
2.16	283293	197216	139145	180212	106116	221221
2.17	283293	197216	139145	180212	106116	221221
2.18	283293	197216	139145	180212	106116	221221
2.19	283293	197216	139145	180212	106116	221221
2.20	283293	197216	139145	180212	106116	221221
2.21	283293	197216	139145	180212	106116	221221
3.01	283285	197210	139149	180212	106116	185221
3.02	283285	197210	139149	180212	106116	185221
3.03	283285	197210	139149	180212	106116	185221
3.04	283285	197210	139149	180212	106116	185221
3.05	283285	197210	139149	180212	106116	185221
3.06	285293	210216	145149	180192	091116	221223
3.07	283285	197210	139149	180212	106116	185221

3.08	285293	210216	145149	180192	091116	221223
3.09	283285	197210	139149	180212	106116	185221
3.10	283285	197210	139149	180212	106116	185221
3.11	283285	197210	139149	180212	106116	185221
3.12	283285	197210	139149	180212	106116	185221
3.13	283285	197210	139149	180212	106116	185221
3.15	283285	197210	139149	180212	106116	185221
3.16	283285	197210	139149	180212	106116	185221
3.17	283285	197210	139149	180212	106116	185221
3.18	283285	197210	139149	180212	106116	185221
3.19	283285	197210	139149	180212	106116	185221
3.20	283285	197210	139149	180212	106116	185221
3.21	285293	210216	145149	180192	091116	221223
4.01	277283	197203	139139	194223	103105	208212
4.02	277283	197203	139139	194223	103105	208212
4.03	277283	197203	139139	194223	103105	208212
4.04	277283	197203	139139	194223	103105	208212
4.05	277283	197203	139139	194223	103105	208212
4.06	277283	197203	139139	194223	103105	208212
4.07	277283	197203	139139	194223	103105	208212
4.08	277283	197203	139139	194223	103105	208212
4.09	277283	197203	139139	194223	103105	208212
4.10	277283	197203	139139	194223	103105	208212
4.11	283293	197216	139145	180212	106116	221221
4.12	283293	197216	139145	180212	106116	221221
4.13	277283	197203	139139	194223	103105	208212
4.14	277283	197203	139139	194223	103105	208212
4.15	283293	197216	139145	180212	106116	221221
4.16	277283	197203	139139	194223	103105	208212
4.17	277283	197203	139139	194223	103105	208212
4.18	277283	197203	139139	194223	103105	208212
4.19	277283	197203	139139	194223	103105	208212
4.20	283293	197216	139145	180212	106116	221221
5.01	283293	197216	139145	180212	106116	221221
5.02	283293	197216	139145	180212	106116	221221
5.04	283293	197216	139145	180212	106116	221221
5.06	283285	197210	139149	180212	106116	185221
5.07	283293	197216	139145	180212	106116	221221
5.08	283293	197216	139145	180212	106116	221221
5.09	283293	197216	139145	180212	106116	221221
5.10	283293	197216	139145	180212	106116	221221
5.11	283293	197216	139145	180212	106116	221221
5.13	283285	197210	139149	180212	106116	185221
5.15	283285	197210	139149	180212	106116	185221
5.16	283285	197210	139149	180212	106116	185221
5.17	283293	197216	139145	180212	106116	221221
5.18	283285	197210	139149	180212	106116	185221
5.19	283293	197216	139145	180212	106116	221221
5.20	283293	197216	139145	180212	106116	221221
5.21	283285	197210	139149	180212	106116	185221

APPENDIX 2c

Here the Data Matrix used for analyses in Chapter V is presented

Sample	Cy 1	Cy 3	Cy 4	Cy 16	Cy 17	Cy 18	Cy 20
Anc1	123123	144144	163169	177177	230234	149153	195209
Anc2	123123	144152	163169	177177	230234	153163	185209
Anc3	123123	144152	163169	177177	230234	153163	185209
Anc4	123123	144152	163169	177177	230234	153163	185209
Anc5	123123	144152	163169	177177	230234	153163	185209
Anc6	123123	144144	163169	177177	230234	149153	195209
Anc7	123123	144152	163169	177177	230234	153163	185209
Anc8	123123	144152	163169	177177	230234	153163	185209
Anc9	123123	144152	163169	177177	230234	153163	185209
Anc10	123123	144152	163169	177177	230234	153163	185209
Anc11	123123	144152	163169	177177	230234	153163	185209
Anc12	123123	144152	163169	177177	230234	153163	185209
Anc13	123123	144152	163169	177177	230234	153163	185209
Anc14	123123	144152	163169	177177	230234	153163	185209
Anc15	123123	144152	163169	177177	230234	153163	185209
Can1	129129	142142	154154	193193	234234	141143	197197
Can2	123123	142142	154154	193193	234234	141143	197197
Can3	123123	142142	154154	193193	234234	141143	197197
Can4	123123	142142	154154	193193	234234	141143	197197
Can5	123123	142142	154154	193193	234234	141143	197197
Can6	123123	142142	154154	193193	234234	141143	197197
Can7	123123	142142	154154	193193	234234	141143	197197
Can8	123123	142142	154154	193193	234234	141143	197197
Can9	129129	142142	154154	193193	234234	141143	197197
Can10	123123	142142	154154	193193	234234	141143	197197
Can11	123123	142142	154154	193193	234234	141143	197197
Can12	123123	142142	154154	193193	234234	141143	197197
Can13	123123	142142	154154	193193	234234	141143	197197
Can14	123123	142142	154154	193193	234234	141143	197197
Can15	123123	142142	154154	193193	234234	141143	197197
Can16	123123	142142	154154	193193	234234	141143	197197
Can17	123123	142142	154154	193193	234234	141143	197197
Can18	123123	142142	154154	193193	234234	141143	197197
Can19	123123	142142	154154	193193	234234	141143	197197
Can20	123123	142142	154154	193193	234234	141143	197197
Cap1	129129	144144	163169	179189	230230	155155	209209
Cap2	129129	142144	163169	179189	230230	155155	209209
Cap3	123129	142144	166169	179189	230230	155155	209209
Cap4	123129	142144	166169	179189	230230	155155	209209
Cap5	123129	142144	166169	179189	230230	155155	209209
Cap6	123129	142144	166169	179189	230230	155155	209209
Cap7	123129	142144	166169	179189	230230	155155	209209
Cap8	123129	142144	166169	179189	230230	155155	209209
Cap9	123129	142144	166169	179189	230230	155155	209209
Cap10	123129	142144	166169	179189	230230	155155	209209
Cap11	123129	142144	166169	179189	230230	155155	209209
Cap12	123129	142144	166169	179189	230230	155155	209209
Cap13	123129	142144	166169	179189	230230	155155	209209
Cap14	123129	142144	166169	179189	230230	155155	209209
Cap15	123129	142144	166169	179189	230230	155155	209209
Cap17	123129	144144	166169	179189	230230	155155	209209
Cap18	123129	142144	166169	179189	230230	155155	209209
Cap19	123129	142144	166169	179189	230230	155155	209209
Cap20	123129	142152	169169	179189	230230	155155	209209
Cap21	123129	142152	169169	179189	230230	155155	209209
Cap22	129129	144152	154163	189189	230260	143155	195197
Cap23	129129	144152	154163	189189	230260	143155	195197
Cap24	129129	144152	154163	189189	230260	143155	195197
Cap25	129129	144152	154163	189189	230260	143155	195197
Cap26	129129	144144	163166	175175	230234	143155	195197
Cap27	129129	144144	163166	177189	228236	143155	179179
Cap28	129129	144144	163166	177189	228236	143155	179179
Cap29	129129	144144	163166	177189	228236	143155	179179
Cap30	129129	144144	163169	179189	230230	143155	179209
Cap31	129129	144152	163169	177189	230234	143155	195209
Cap32	129129	144144	163169	179189	230230	143155	179209

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
Cap33	129129	144144	163169	179189	230230	143155	179209
Cap34	129129	144144	163169	179189	230230	143155	179209
Cap35	129129	144144	163169	179189	230230	143155	179209
Cap36	129129	144144	166169	179179	230230	155155	195209
Cap37	129129	144144	166169	177189	230234	143155	195195
Cap38	129129	144144	166169	177189	230234	143155	195195
Cap39	129129	144144	166169	179179	230230	143155	179209
Cms13	123129	144148	154163	179179	230234	153155	179179
Cms14	123129	144148	154163	179179	230234	153155	179179
Cms15	123129	144148	154163	179179	230234	153155	179179
Cms5	129129	144148	163163	175179	230234	141155	179179
Cms18	129129	144152	163163	175179	230234	141155	179179
Cms1	129129	144148	163169	179179	230234	143155	179211
Cms3	123123	148148	163169	175177	234234	153155	179179
Cms4	129129	144148	163169	179179	230234	143155	179211
Cms6	129129	144148	163169	179179	230234	143155	179211
Cms7	129129	144148	163169	179179	230234	143155	179211
Cms8	129129	144148	163169	179179	230234	143155	179211
Cms10	129129	144148	163169	179179	230234	143155	179211
Cms11	129129	144148	163169	179179	230234	143155	179211
Cms12	129129	144148	163169	179179	230234	143155	179211
Cms16	129129	144148	163169	175179	230234	141155	179179
Cms17	129129	144148	163169	179179	230234	143155	179211
Cms19	129129	144148	163169	179179	230234	143155	179211
Cms20	129129	144148	163169	179179	230234	143155	179211
ICR1	129129	150152	154163	193195	234234	143155	179179
ICR3	129129	150152	154163	193195	234234	143155	179213
ICR4	129129	150152	154163	193195	234260	143155	179213
ICR5	123129	144144	163166	175203	230230	143155	179213
ICR2	129129	144152	163169	189189	234234	143143	179189
ICR6	129129	144144	154154	175203	230236	155155	179195
ICR7	129129	144144	154154	175203	230236	155155	179195
ICR8	129129	144144	154163	175203	230234	143155	179195
ICR9	129129	144144	163169	201203	230230	155155	195209
Mes28	123129	144152	154163	175177	230230	143155	179195
Mes29	123129	144152	154163	175177	230230	143155	179195
Mes30	123129	144152	154163	175177	230230	143155	179195
Mes31	123129	144152	154163	175177	230230	143155	179195
Mes32	123129	144152	154163	175177	230230	143155	179195
Mes33	123129	144152	154163	175177	230230	143155	179195
Mes34	123129	144152	154163	175177	230230	143155	179195
Mes35	123129	144152	154163	175177	230230	143155	179195
Mes36	123129	144152	154163	175177	230230	143155	179195
Mes37	123129	144152	154163	175177	230230	143155	179195
Mes38	123129	144152	154163	175177	230230	143155	179195
Mes39	123129	144152	154163	175177	230230	143155	179195
Mes40	123129	144152	154163	175177	230230	143155	179195
Mes1	123123	144144	163166	175175	230236	143143	195197
Mes2	123123	144144	163166	175175	230236	143143	195197
Mes3	123123	144144	163166	175175	230236	143143	195197
Mes4	123123	144144	163166	175175	230236	143143	195197
Mes5	123123	144144	163166	175175	230236	143143	195197
Mes6	123123	144144	163166	175175	230236	143143	195197
Mes7	123123	144144	163166	175175	230236	143143	195197
Mes8	123123	144144	163166	175175	230236	143143	195197
Mes9	123123	144144	163166	175175	230236	143143	195197
Mes10	123123	144144	163166	175175	230236	143143	195197
Mes11	123123	144144	163166	175175	230236	143143	195197
Mes12	123123	144144	163166	175175	230236	143143	195197
Mes13	123123	144144	163166	175175	230236	143143	195197
Mes14	123123	144144	163166	175175	230236	143143	195197
Mes15	123123	144144	163166	175175	230236	143143	195197
Mes16	123123	144144	163166	175175	230236	143143	195197
Mes17	123123	144144	163166	175175	230236	143143	195197
Mes18	123123	144144	163166	175175	230236	143143	195197
Mes19	123123	144144	163166	175175	230236	143143	195197
Mes20	123123	144144	163166	175175	230236	143143	195197
Mes21	123123	144144	163166	175175	230236	143143	195197
Mes22	123123	144144	163166	175175	230236	143143	195197
Mes23	123123	144144	163166	175175	230236	143143	195197
Mes24	123123	144144	163166	175175	230236	143143	195197
Mes25	123123	144144	163166	175175	230236	143143	195197
Mes26	123123	144144	163166	175175	230236	143143	195197

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
Mes27	123123	144144	163166	175175	230236	143143	195197
ICT1	129129	144144	154154	189189	230234	143155	179195
ICT2	129129	144144	154154	179203	236236	143155	179205
ICT3	119129	144144	154154	179203	236236	143155	179195
ICT4	129129	144148	154154	175201	230234	143143	195211
ICT5	129129	144144	154154	179203	236236	153155	179205
ICT6	129129	144144	154154	175189	230234	155155	179195
ICT7	129129	144144	154154	189189	230234	143155	179195
ICT8	129129	144144	154154	175189	230234	141143	179195
ICT9	129129	144144	154154	175189	230234	143143	179195
ICT10	129129	148148	154154	189189	234236	143155	179211
ICT11	129129	144152	154163	201201	236260	155155	179211
ICT12	129129	144152	154163	175201	234236	143155	179211
ICT13	129129	144148	154163	175175	230230	155155	195205
ICT14	129129	144152	154163	175201	234236	143155	179211
ICT15	129129	148152	154163	175201	230234	143143	195211
ICT16	129129	144144	154163	175201	230234	143155	195205
ICT17	129129	144144	154163	175201	230234	143155	195205
ICT18	129129	144152	154163	175201	234236	143155	195211
ICT19	129129	144152	154163	175201	234236	143155	179211
ICT20	129129	144152	154163	175201	234236	143155	179211
ICT21	129129	144152	154163	175201	234236	143155	179211
ICT22	129129	144148	154163	175201	230234	143155	195211
ICT23	129129	144144	154163	175201	230234	143155	195205
ICT24	129129	144144	154163	175201	236236	143155	179211
ICT25	129129	144152	154163	175201	234236	143155	179211
ICT26	123129	144148	154163	189207	230234	143155	205211
ICT27	129129	144152	154163	175201	234236	143153	179211
ICT28	129129	144152	154163	175201	234236	143155	179211
ICT29	129129	144144	154169	175201	230230	143155	179205
ICT39	123129	144144	154169	179189	230260	143143	179179
ICT31	129129	144144	154169	175189	230260	143155	179179
ICT32	129129	148152	154169	189201	230234	143155	195209
ICT33	123129	144144	154169	179189	230260	143143	179179
ICT34	129129	144152	163163	175201	230234	143155	195205
ICT35	129129	144144	163169	201201	230230	143155	179205
ICT36	129129	144144	163169	201201	230230	155155	179205
ICT37	129129	144152	163169	175189	230234	143153	187211
ICT38	129129	144144	163169	175201	230234	143155	179195
ICT39	129129	144144	163169	175201	230234	143155	179195
ICT40	129129	144144	166169	189201	230260	143143	179195
IMR13	129129	144144	154154	175175	230234	155155	179195
IMR33	129129	144144	154154	175175	234234	155155	179195
IMR36	129129	144144	154154	175193	230236	155155	179195
IMR01	129129	144144	154163	175193	228260	155155	179195
IMR02	129129	144144	154163	175175	230230	143155	179195
IMR04	129129	152152	154163	175175	230230	155163	179179
IMR07	129129	144144	154163	175193	230236	155155	195195
IMR08	129129	150152	154163	175193	230260	143155	179195
IMR09	129129	144152	154163	181193	230260	143155	195195
IMR14	129129	150152	154163	175193	234260	155155	179195
IMR17	129129	152152	154163	175175	230230	155155	179179
IMR18	129129	150152	154163	175175	230260	155155	179195
IMR19	129129	144144	154163	175175	230230	143155	179195
IMR20	129129	144144	154163	175175	230230	143155	179195
IMR28	129129	150152	154163	175193	230260	155155	179195
IMR32	129129	150152	154163	175193	230260	155155	179195
IMR34	129129	144144	154163	175175	230230	143155	179195
IMR37	129129	144144	154163	175175	230230	143155	179195
IMR38	129129	144144	154163	175175	230234	143155	179195
IMR41	129129	144144	154163	175175	230260	155155	179179
IMR42	129129	144144	154163	175193	230234	143155	179195
IMR43	123129	144144	154163	175175	230230	143155	179195
IMR45	129129	144144	154163	175175	230230	143155	179195
IMR12	123129	144152	154169	175175	230234	155163	195195
IMR15	123129	144152	154169	175175	230234	155163	195195
IMR27	123129	144152	154169	175175	234234	155163	195195
IMR29	123129	144152	154169	175175	230234	155155	195195
IMR30	129129	144144	154169	175175	234234	155155	195195
IMR48	123129	144152	154169	175175	234234	155163	195195
IMR49	123129	144152	154169	175175	234234	155163	195195
IMR46	129129	144144	163163	175175	230236	143155	195195
IMR10	129129	144144	163166	175181	230236	143155	195195

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
IMR03	129129	144152	163169	175193	230234	155163	195195
IMR05	129129	144144	163169	175181	230236	155155	195195
IMR06	129129	144144	163169	193193	228260	143155	179195
IMR11	129129	144144	163169	193193	230234	143155	195195
IMR16	129129	144144	163169	193193	230230	143155	195195
IMR21	129129	144144	163169	193193	230236	143155	195195
IMR22	129129	144152	163169	175193	230234	155163	179195
IMR23	129129	144144	163169	175193	232234	143155	179201
IMR24	129129	144144	163169	193193	230236	143155	195195
IMR25	129129	144144	163169	175175	230234	155163	195195
IMR26	129129	144144	163169	175193	232234	143155	179179
IMR31	129129	144144	163169	193193	230236	143155	195195
IMR35	129129	144144	163169	193193	230236	143155	195195
IMR39	129129	144144	163169	193193	230230	143155	195195
IMR40	129129	144144	163169	193193	230234	143155	195195
IMR44	129129	144152	163169	175193	230234	155163	179195
IMR47	129129	144152	163169	175175	230234	155163	179195
IMR50	129129	144144	163169	175193	232234	143155	179179
Mlt2	129129	148148	154163	181187	230232	143155	197201
Mlt13	129129	144144	154163	177187	234234	143155	195197
Mlt19	123129	144152	154163	177199	230230	143155	197201
Mlt18	129129	144144	154166	177199	230230	143155	197201
Mlt34	129129	144148	154166	181199	230234	143155	201209
Mlt25	129129	148152	154172	177187	234234	143155	201209
Mlt4	129129	152152	163163	177199	230234	143155	209209
Mlt9	129129	144148	163163	177199	230232	143155	201201
Mlt12	129129	152152	163163	179199	230234	143155	201209
Mlt17	129129	152152	163163	179199	234234	143155	201209
Mlt20	129129	152152	163163	177199	230234	143155	201209
Mlt22	123123	144144	163163	181181	230234	143155	209209
Mlt24	129129	144144	163163	181187	230234	143155	197209
Mlt32	129129	144148	163163	181199	230230	143155	209209
Mlt36	129129	144144	163163	199199	230234	143155	197201
Mlt38	123129	148152	163163	181187	230234	143155	195203
Mlt8	129129	144144	163166	181199	234234	143155	195209
Mlt11	129129	152152	163166	181187	234234	143155	197201
Mlt28	129129	144144	163166	181181	234234	143155	201209
Mlt35	123123	148152	163166	177199	232234	143155	195209
Mlt39	123129	144144	163166	177187	230234	143155	179197
Mlt40	129129	144144	163166	177199	230232	143155	179195
Mlt5	123129	144152	163169	177199	232234	143155	197199
Mlt6	129129	148148	163169	187197	230230	143155	197197
Mlt15	129129	144152	163169	177199	234234	143155	195195
Mlt23	129129	144148	163169	187199	230234	143155	209209
Mlt26	129129	148148	163169	177187	230234	143155	209209
Mlt27	129129	144148	163169	187199	230234	143155	197201
Mlt3	129129	148152	166169	199199	232234	143155	197197
Mlt29	123129	152152	166169	199199	234234	143155	197197
Mlt1	129129	148148	169169	199199	232234	143155	197201
Mlt10	129129	148148	169169	199199	232234	143155	197201
Mlt14	129129	148148	169169	199199	232234	143155	197201
Mlt21	129129	144152	169169	177181	230234	143155	201209
Mlt30	129129	144152	169169	177181	230232	143155	201209
Mlt31	129129	148148	169169	199199	234234	143155	197201
Mlt33	129129	144144	169169	181181	232234	143155	201209
Mlt37	123129	148152	169169	177177	230234	143155	179195
Mlt7	129129	152152	169172	187199	230234	143155	197201
Mlt16	129129	148148	169172	181199	230234	143155	195201
Ors7	123129	144144	154154	187187	230230	141155	195209
Ors11	123129	144152	154166	187201	232260	143155	195209
Ors26	123129	144152	154166	187187	228234	143147	209209
Ors23	123123	144152	154169	175187	230260	153155	195209
Ors6	123129	144152	163163	187187	230232	143153	195205
Ors18	123123	144144	163166	187201	230230	141145	205209
Ors20	123129	144152	163166	187187	230230	143153	209209
Ors21	129129	144152	163166	187187	228230	155155	209209
Ors24	123129	142144	163166	187187	230230	141141	197209
Ors25	123123	152152	163166	175187	230232	143153	195209
Ors3	129129	142144	163169	175187	228230	143155	195195
Ors1	123123	142144	166166	175201	230230	143155	205209
Ors2	123123	144152	166166	175187	230260	153153	195209
Ors4	123123	144144	166166	179187	232232	143153	195209
Ors5	123123	144152	166166	175187	228260	153153	195209

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
Ors12	123123	144144	166166	187201	230260	143155	195197
Ors13	123123	144152	166166	179201	228230	141153	195205
Ors14	123123	144152	166166	187201	230230	143153	195209
Ors15	123123	142142	166166	179201	230260	153153	197209
Ors16	129129	144152	166166	191201	260260	153155	193205
Ors27	123129	144152	166166	179187	230230	143155	195209
Ors28	123129	144152	166166	179201	230230	153155	195209
Ors29	123123	144152	166166	187201	230230	141145	205209
Ors8	123129	144152	169169	179201	230232	141153	197197
Ors30	123123	144152	169169	175187	234260	153155	195209
Kop1	123123	144144	163163	177187	230230	130149	209209
Kop3	123123	144144	163163	177177	230230	163163	195195
Kop4	123123	142152	163163	177177	230230	145145	195195
Kop6	123123	142152	163163	177177	230230	145145	195195
Kop8	123123	144152	163163	177177	230230	143149	195209
Kop9	123123	144144	163163	177187	230230	143143	195209
Kop10	123123	144152	163163	177177	230230	143143	195195
Kop11	123123	144152	163163	177177	230230	143143	195209
Kop2	123123	144144	163169	177177	230230	143149	195209
Kop5	123123	144152	163169	177177	230230	143149	195209
Kop7	123123	144152	163169	177177	230230	143149	195209
Lec1	123129	144152	163163	175177	230230	143145	195195
Lec2	123129	144152	163163	175177	230230	143145	195195
Lec3	123129	144152	163163	175177	230230	143145	195195
Lec4	123129	144152	163163	175194	230234	143145	209211
Lec5	123129	144152	163163	175177	230230	143145	195195
Lec6	123129	144152	163163	175177	230230	143145	195195
Lec7	123129	144152	163163	175194	230234	143145	209211
Lec8	123129	144152	163163	175177	230230	143145	195195
Lec9	123129	144152	163163	175177	230230	143145	195195
Lec10	123129	144152	163163	175194	230234	143145	209211
Lec11	123129	144152	163163	175177	230230	143145	195195
Lec12	123129	144152	163163	175177	230230	143145	195195
Lec13	123129	144152	163163	175177	230230	143145	195195
Lec14	123129	144152	163163	175177	230230	143145	195195
Lec15	123129	144152	163163	175194	230234	143145	209211
Lec16	123129	144152	163163	175194	230234	143145	209211
Lec17	123129	144152	163163	175177	230230	143145	195195
Lec19	123129	144152	163163	175194	230234	143145	209211
Lec20	123129	144152	163163	175194	230234	143145	209211
Tun15	119123	142152	154163	175181	230234	143143	195207
Tun17	123123	152152	154163	173175	230234	143155	195201
Tun22	129129	142148	154163	173199	234234	137143	197201
Tun27	123129	144152	154163	183209	230230	143143	201201
Tun34	123123	152154	154163	179179	230236	143155	201207
Tun3	129129	152152	154166	175189	234234	143155	195201
Tun7	119129	144152	154166	175183	230234	143149	195201
Tun8	123129	144148	154166	175185	230234	137145	195207
Tun9	129129	148154	154166	179185	230230	143155	195201
Tun10	123129	152152	154166	177177	230234	143143	195195
Tun11	123129	144152	154166	177177	230234	143149	195201
Tun13	123129	152152	154166	177177	230234	143155	195195
Tun16	119123	142142	154166	173199	230234	143143	201201
Tun23	123129	152154	154166	175183	232234	143147	195195
Tun25	119123	152152	154166	177189	234234	143143	201201
Tun31	129129	142142	154166	179201	234234	137143	195197
Tun37	129129	144152	154166	175175	230230	143143	201201
Tun38	123129	144154	154166	177177	230230	143155	201201
Tun4	123123	142152	154169	175179	234236	137155	201201
Tun26	123123	152152	154169	177199	230230	143155	195207
Tun35	123129	152152	154169	175177	234234	143143	195207
Tun21	123123	142142	163163	189201	234234	137143	195195
Tun2	123129	142142	163166	175175	230234	137143	201201
Tun5	119129	144152	163166	175183	230234	143149	195201
Tun12	123123	152154	163166	179179	230236	143155	201207
Tun14	123123	144152	163166	173209	230230	143155	195201
Tun18	123129	152152	163166	183199	230234	143143	195201
Tun20	123123	152152	163166	175197	230234	145145	201201
Tun28	123123	148152	163166	177177	230236	143143	201207
Tun29	123123	142154	163166	175177	230234	137143	195201
Tun36	129129	142152	163166	177183	230234	143143	207207
Tun19	123129	152152	166166	177177	230234	143143	195195
Tun24	119129	152154	166169	175187	230234	143143	195201

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
Edr1	129129	144144	154169	177177	234234	149149	205205
Edr2	129129	144144	154169	177177	234234	149149	205205
Edr3	129129	144144	169173	177177	230236	151151	197205
Edr4	129129	142144	170173	177177	236236	149149	195205
Edr5	129129	142144	170173	177177	236230	149149	195205
Edr6	129129	142144	170173	177177	236236	149149	195205
Edr7	129129	142144	170173	177177	236236	149149	195205
Edr8	129129	142144	170173	177177	236236	149149	195205
Edr9	129129	142144	170173	177177	236236	149149	195205
Edr10	129129	142144	170173	177177	236236	149149	195205
Edr11	129129	142144	170173	177177	236236	149149	195205
Edr12	129129	142144	170173	177177	236236	149149	195205
Edr13	129129	142144	170173	177177	236236	149149	195205
Edr14	129129	142144	170173	177177	236236	149149	195205
Edr15	129129	142144	170173	177177	236236	149149	195205
Edr16	129129	144144	170176	177177	230230	149151	205205
Edr17	129129	144144	170176	177177	230230	149151	205205
Edr18	129129	144144	170176	177177	230230	149151	195205
Edr19	129129	144144	170176	177177	230230	149151	195205
Edr20	129129	144144	170176	177177	230230	149151	195205
Edr21	129129	144144	170176	177177	230230	149151	195205
Edr22	129129	142144	173176	177177	230230	149151	195195
Edr23	129129	144144	173176	177177	230230	149151	205205
Edr24	129129	142144	173176	177177	230230	149151	195205
Liv1	129129	142152	166166	177201	230230	141141	179197
Liv2	129129	144152	166166	175201	230230	141141	179197
Liv3	129131	144144	169169	179201	230234	141153	179213
Liv4	123129	144144	166166	173173	230234	141143	195211
Liv5	129129	144152	163163	175201	230230	141163	179213
Liv6	123123	144152	166166	173175	230230	143143	197209
Liv7	123123	144152	166166	173175	230230	143143	197209
Liv8	123123	144144	166169	175177	230230	141141	179197
Liv9	129129	144144	163163	187177	230234	141141	211211
Liv10	129129	144144	166166	175177	230230	141155	197197
Liv11	129129	144152	163163	173201	230234	141143	179197
Liv12	123129	144152	166166	185185	230230	143153	209211
Liv13	129129	142152	163166	175201	230230	143143	197209
Liv14	123129	152152	163163	173185	234234	143143	197197
Liv15	129129	142152	163166	177201	230230	143143	197209
Liv16	131131	144152	163163	175185	230230	143143	211211
Liv17	131131	144152	163163	175177	230230	141143	179197
Liv18	125131	144152	163163	175201	230230	143143	173197
Liv19	131131	144144	163163	177201	230234	141153	177211
Liv20	125125	152152	163163	173185	230230	143153	197211
Liv21	125129	144152	166166	177179	230234	143143	197209
Liv22	125129	144152	163169	177179	234234	143143	211211
Liv23	125129	144152	163169	175179	234234	143143	211211
Liv24	125129	144152	163169	177177	234234	143143	211211
Liv25	129129	144152	163166	171185	230230	143143	195209
Liv26	125129	152152	163166	175179	230230	143163	197213
Liv27	129129	148152	163163	175175	230230	141141	179211
Liv28	129129	144144	166166	175201	230230	141141	197211
Liv29	129129	144144	166166	173201	230234	143143	197211
Liv30	125129	146146	166166	171201	230234	143143	197209
Liv31	129129	146152	163166	171201	230230	143153	197211
Liv32	129129	146152	163166	173201	230230	141153	197213
Liv33	123129	144144	163163	175201	230234	141141	197211
Liv34	129129	144144	163166	177179	230234	143155	179195
Civ1	125125	146152	163166	177179	230230	143143	179179
Civ2	125125	146152	163166	171185	230230	143143	195211
Civ3	125125	146152	163166	171185	230230	143143	195211
Civ4	125125	146146	166166	177177	230230	141143	179211
Civ5	125125	146146	163163	175177	230230	141141	195213
Civ6	125125	144144	163163	175177	230230	141141	195213
Civ7	125129	144144	163163	175177	230230	141141	195211
Civ8	123123	146146	163169	179179	230230	141143	179211
Civ9	125125	146146	154163	179179	230230	143143	179213
Civ10	125125	144144	154163	179179	230230	143143	179213
Civ11	125125	144144	163166	177179	230230	143143	177209
Civ12	125125	144144	154163	177179	230230	141143	179213
Civ13	125125	144144	166169	179179	230230	141143	179211
Civ14	125125	144144	154169	179179	230230	143143	179213
Civ15	125125	146152	163166	175175	230230	141143	195211

Sample	*Cy 1*	*Cy 3*	*Cy 4*	*Cy 16*	*Cy 17*	*Cy 18*	*Cy 20*
Civ16	125125	144152	163169	175179	230230	141143	177195
Civ17	123123	146146	163166	175175	230230	143143	195213
Civ18	125125	146146	166169	177179	230230	141143	177209
Civ19	125125	146146	154166	177177	230230	141143	177177
Civ20	125125	146152	154166	173175	230230	141143	179213
Civ21	125125	144152	163166	175175	230230	141143	195213
Civ22	125125	146146	163166	175175	230230	143143	195211
Civ23	125125	146144	166169	175187	230230	141143	179195
Civ24	123123	144146	154166	175175	230230	141143	177177
Civ25	125125	144146	154163	175177	234234	141143	177209
Civ26	125125	144146	154163	177177	234234	141141	179211
Civ27	125125	146146	154163	175177	234234	143143	179211
Civ28	123123	144144	163163	177177	230230	141143	179195
Civ29	125125	144144	166166	185185	230230	141143	179211
Civ30	125125	146152	166166	187187	230230	141143	179209
Civ31	125125	146146	163166	179187	230230	141141	179195
Civ32	125125	144144	163169	179187	230230	141143	195195
Civ33	125125	144146	154166	175177	230234	141143	195213
Civ34	125125	146146	154166	175177	230230	143145	179213
Civ35	125125	144144	163169	179187	230230	143143	195195
Civ36	125125	146146	154166	175177	230234	141141	195213
Civ37	125125	144144	154166	175177	230234	141143	195211
Civ38	125125	146146	154166	175177	230234	141143	195211
Civ39	125125	144146	154163	175177	230234	143143	195213
Civ40	125125	144144	163166	179187	230230	141143	195195
Civ41	125125	144144	163163	175177	230230	141143	195213
Civ42	125125	144144	163163	175177	230230	141143	195213
Lbr1	125125	144148	163163	177177	230234	153153	197209
Lbr2	125125	152152	166166	177187	234234	141153	197197
Lbr3	125125	144152	163163	177201	230230	141143	197197
Lbr4	125125	148152	166166	177223	230234	143153	197197
Lbr5	125125	148152	166166	177223	230234	143153	197197
Lbr6	125125	144144	163163	201201	230234	141143	197213
Lbr7	125125	148152	166166	201223	230234	143153	197197
Lbr8	125125	148152	166166	177223	230234	143153	197197
Lbr9	125125	144152	169169	177223	230230	141143	197197
Lbr10	125125	144148	163163	177177	230230	141141	197197
Lbr11	125125	144144	163169	175177	230230	141141	197197
Lbr12	125125	144148	163163	177177	230230	141141	197197
Lbr13	125125	148148	163163	177177	230234	143153	213213
Lbr14	125125	148148	166169	177177	230234	143153	197197
Lbr15	125125	152152	169169	175175	230234	141143	197213
Lbr16	125125	148148	163169	177177	230234	143143	197213
Lbr17	125125	148148	163163	177177	230234	143153	213213
Lbr18	125125	144148	166166	177187	230234	143153	197197
Lbr19	125125	144144	169169	175177	230230	143153	197197
Lbr20	125125	144148	169169	177187	230230	143153	197209
Lbr21	125125	144152	166166	177187	230230	143153	197209
Lbr22	125125	148152	166166	177187	230230	143153	197197
Lbr23	125125	148152	169169	175177	230230	143143	197213
Lbr24	125125	152152	166166	177177	230230	153153	197209
Lbr25	125125	152152	166166	177177	230230	153153	197209
Lbr26	125125	144152	166166	177187	230230	143153	197209
Lbr27	125125	152152	166166	177177	230230	153153	197209
Lbr28	125125	144152	169169	177187	230230	143153	197209
Lbr29	125125	152152	166169	177177	230230	153153	197209
Lbr30	125125	152152	166169	177177	230230	153153	197209
Lbr31	125125	144144	163169	177177	230236	141143	213213
Lbr32	125125	152152	166169	177177	230230	153153	197209
Lbr33	125125	144144	163163	177187	230230	141153	197197
Lbr34	125125	152152	166169	177177	230230	143153	197209
Lbr35	125125	144150	163163	177187	230230	153153	197197
Lbr36	125125	144148	169169	175177	230230	141153	197213
Lbr37	125125	144150	163163	177187	230230	153153	197197
Lbr38	125125	148152	163166	175177	230234	141143	197209
Lbr39	125125	144150	163163	177187	230230	153153	197197
Lbr40	125125	144150	163163	177187	230230	153153	197197
Lbr41	125125	144150	163163	177187	230230	153153	197197
Lbr42	125125	144150	163163	177187	230230	153153	197197
Lbr43	125125	144150	163163	177187	230230	153153	197197
Lbr44	125125	144150	163163	177187	230230	153153	197197
Lbr45	125125	144150	163163	177187	230230	153153	197197
Lbr46	125125	144150	169169	177187	230236	141143	209209

Appendix 3

Here a list of softwares used for the genetic analyses conducted is presented.

GIMLET v.1.3.2	Number of distinct genotypes Probability of Identity (P_i)
GENETIX v.4.02	Number of Alleles / Locus Observed, expected and non-biased heterozygosity (H_{obs}, H_{exp}, H_{nb}) Inbreeding coefficient (f) Pairwise genetic differentiation (θ)
GENEPOP v. 3.3	Hardy-Weinberg equilibrium
SPAGEDI	Autocorrelation of kinship coefficient (f_{ij}) Slope of the regression kinship/geographic distance (b_F)
FSTAT v. 2.9.1	Inbreeding coefficient (f) Genetic differentiation (θ) Gene Diversity (H_T)
PHYLIP Package v.3.57c	Bootstrap on allelic frequencies Pairwise Cavalli-Sforza & Edward's chord distance Neighbour-joining algorithm for phylogenetic reconstruction
GENECLASS v.1.0.02	Assignment test
BAPS	Bayesian analysis of populations' genetic structure

Appendix 4

This appendix presents a reprint of the paper published in *Molecular Ecology Notes* (2004).

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PRIMER NOTE

Polymorphic microsatellite loci for the marine angiosperm *Cymodocea nodosa*

M. V. RUGGIERO,* T. B. H. REUSCH† and G. PROCACCINI*

Laboratorio di Ecologia del Benthos, Stazione Zoologica 'Anton Dohrn', Punta San Pietro, 80077 Ischia (Naples), Italy, †Max-Planck-Institut für Limnologie, August-Thienemann-Str. 2, 24306 Plön, Germany

Abstract

The seagrass *Cymodocea nodosa* (UCRIA) Ascherson represents a good model to assess the relative contribution of clonal and sexual reproduction to genetic structure in marine plant populations. Seven microsatellite loci with repeat units consisting of one trinucleotide, four simple dinucleotides and two complex dinucleotides are described here. The seven loci are characterized by high number of alleles (from three to 13) and high heterozygosity (H_O ranging from 0.240 to 0.860) in the tested populations. Conditions for multiplex polymerase chain reactions are also described.

Keywords: clonal reproduction, *Cymodocea nodosa*, microsatellite loci, seagrass.

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Cymodocea nodosa (UCRIA) Ascherson is a dioecious marine angiosperm, widely distributed in the Mediterranean Sea, and extending in the Atlantic Ocean from Southern Portugal to the Northern coasts of Africa (den Hartog 1970). It grows in dense meadows, often in association with other seagrasses (Buia & Mazzella 1991).

Cymodocea nodosa is characterized by high rates of both sexual reproduction and clonal propagation through rhizome elongation (Caye & Meinesz 1985; Duarte & Sand-Jensen 1990). It could represent a good model to study how the two modes of reproduction affect the population's genetic structure and to assess genet dynamics and gene flow at different spatial scales.

Only two published studies up to now have dealt with the genetic variability in *C. nodosa*, in which random amplified polymorphic DNA (RAPD) molecular markers revealed very different levels of polymorphism in two distinct populations (Procaccini & Mazzella 1996; Alberto *et al.* 2001). The development of more appropriate molecular markers for this species is thus becoming essential. Microsatellite loci can be considered markers of choice in population genetic studies due to their high polymorphism and codominant mode of inheritance.

Here, seven new polymorphic microsatellite loci for *C. nodosa* are described, characterized by different types of repeat units with respect to others that have been recently selected (Alberto *et al.* 2003).

For genomic library development, high-quality genomic cetyltrimethyl ammonium bromide (CTAB)-extracted DNA (as in Procaccini *et al.* 1996) was digested overnight at 37 °C by blunt-end restriction enzymes (*Alu*I, *Hae*III and *Rsa*I, Amersham). Then 300–600 bp fragments were ligated into a p-BlueScript plasmid vector (Stratagene), followed by transformation into *Escherichia coli* electrocompetent cells. Cells were plated on Luria-Bertani (LB) Ampicillin selective 25 cm Ø plates and incubated overnight at 37 °C. Fifteen plates allowed a total of ~30 000 colonies. A colony-hybridization protocol was then followed. Colonies from each plate were transferred by lifting on Hybond N+ Nylon membranes (Amersham). Five ^{32}P-labelled probes consisting of repeated di- or trinucleotide motifs were used in the hybridization procedure: $(ATT)_8$, $(ACT)_8$, $(AT)_{12}$, $(GA)_{12}$ and $(CA)_{12}$. Hybridization was carried out overnight. After several washes, membranes were exposed to autoradiographic films and developed after 8–10 h exposure. Hybridization and prehybridization of membranes were carried out in Denhart's buffer with Salmon Sperm DNA added (as in Sambrook *et al.* 1989).

Positive colonies were transferred to LB + ampicillin growth medium and incubated at 30 °C overnight. Plasmids were then purified and inserts were sequenced using universal M13 primers through automated sequencing (CEQ 2000XL DNA Analysis System, Beckman Coulter).

Correspondence: Maria Valeria Ruggiero. Fax: + 39 081 984 201; E-mail: ruggiero@szn.it

Table 1 Primer sequences, number of alleles found in Ischia and other nine tested populations, size of alleles, observed (H_O) and expected (H_E) heterozygosities in the Ischia population, and GenBank Accession nos for seven *Cymodocea nodosa* microsatellite loci

Locus	Primer sequence (5′–3′)	Microsatellite repeat	Number of alleles: Ischia ($N = 50$)	Number of alleles: Nine populations ($N = 60$)	Size range of alleles	H_O	H_E	GenBank Accession no.
Cy 1	F: GGAGCAAGTCCGAAGAAGAG R: GAGGAGGAAGGAATGGCTG*	$(CT)_{16}$	3	6	119–133	0.240	0.250	AY559051
Cy 3	F: CGTGGCTCTTTCCGTAAATC* R: CACGCACCCAACAGAAAAG	$(GA)_{12}$	3	6	140–152	0.600	0.562	AY559052
Cy 4	F: GGCTTCAATAATGATGCGGT* R: CACAAGAACCATTCACCCCT	$(TAA)_9$	4	8	154–169	0.720	0.648	AY559053
Cy 16	F: ACTTTCACACTTGCCGTGGT* R: CACCTCGACCAAAACTCCAT	$(CA)_8(CT)_{22}$	11	13	175–207	0.860	0.766	AY559054
Cy 17	F: CTGCTGGCAGGTGAAGAAAT* R: CCGAAGTTGTGCTTTGATCC	$(CT)_{17}CG(AT)_{10}$	4	5	228–260	0.660	0.623	AY559055
Cy 18	F: CGCTCCTTCTTCTACCAGCA* R: CTGCGGGTGCGTCTCT	$(CA)_{16}$	4	9	141–163	0.600	0.641	AY559056
Cy 20	F: ACATGCTTTGGTTGCACAGA* R: ACTCCCACATCTCCCTCAAA	$(TC)_{19}$	5	10	179–211	0.820	0.710	AY559057

*Labelled primer.

A total of 15 positive clones were obtained and primers were designed using the web-based software PRIMER3 (website: www-genome.wi.mit.edu). In order to test for polymorphism, 50 individual *C. nodosa* shoots from a population in the Island of Ischia (Gulf of Naples, Italy) and 60 individuals from nine other geographically distinct populations from the Mediterranean Sea were genotyped. ^{32}P-labelled primers were used in the polymerase chain reactions (PCR) and products were run on a denaturing 6% acrylamide–bisacrylamide gel and visualized by autoradiography.

PCR conditions were as follows: 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM each primer, 0.5 U *Taq* DNA polymerase (Roche) in a total volume of 10 μL. Template DNA can be as low as 2 ng. PCR cycles were as follows: an initial denaturation step of 4 min at 94 °C; 35 cycles consisting of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C, followed by a final extension step of 7 min at 72 °C. All PCR reactions were conducted in a GeneAmp 9700 Thermocycler (PE Applied Biosystems).

More than two alleles were found in seven out of the 15 microsatellite loci. The seven loci were characterized by a total of 57 alleles among all analysed populations, of which 34 were found in Ischia. Features of loci and primer sequences are shown in Table 1. For these seven loci, PCR conditions were optimized for genotyping through automated fragment analysis (CEQ 2000XL DNA analysis system, Beckman Coulter). PCR conditions for multiplexing are shown in Table 2.

Heterozygosity values were tested using the software GENETIX (Belkhir K *et al.* 1996–2002; website: www.univ-montp2.fr/~genetix/genetix/intro.htm).

Alberto *et al.* (2003) found a deficit in heterozygosity of microsatellite loci among seedlings of *C. nodosa*, possibly due to a Wahlund effect in the tested population. In contrast, our newly described loci show a significant excess of heterozygosity in the Ischia population (Table 1), possibly due to a selective heterozygote advantage.

Table 2 PCR conditions for multiplexing. One quadruplex (a) and one triplex (b) are described. Primer concentrations are provided. All other conditions and annealing temperature are as in the text. Starting template DNA can be as low as 2 ng

Locus	Dye	Multiplex	Primer concentration (μM)
Cy 1	IRD700	a	0.4
Cy 3	IRD700	a	0.2
Cy 4	CY5	a	0.05
Cy 16	CY5	a	0.05
Cy 17	CY5	b	0.4
Cy 18	CY5	b	0.4
Cy 20	IRD700	b	1

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