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Assessment of genetic variation and clonality in the seagrass *Posidonia australis* using RAPD and allozyme analysis

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ABSTRACT: The genetic structure of a meadow of *Posidonia australis* Hook. f., an endemic seagrass from southern Australian coasts, was investigated at Warnbro Sound, Western Australia. A high level of genetic variation as determined by allozyme and RAPD (random amplified polymorphic DNA) analyses was found within a small area of the meadow. Allozyme analysis identified 15 four-locus genotypes from 43 samples (35%). RAPD analysis identified 16 four-primer genotypes from 22 samples (73%). The levels of genetic variation in *P. australis* are equivalent to or greater than that observed in other hydrophilous species. The intrapopulational genetic variation demonstrated in this study shows that meadows of *P. australis* need not be monoclonal in all cases.

KEY WORDS: Seagrass · Population structure · Clonality · RAPD · Allozyme

INTRODUCTION

Seagrasses can form large 'meadows' by strong vegetative growth through rhizome extension from terminal meristems and by clonal reproduction through rhizome fragmentation. The processes that are involved with the formation of seagrass meadows are reliant on the nature of the ecosystems they dominate. The temperate seagrass species *Posidonia australis* Hook. f. can form large monospecific meadows through strong rhizomatous growth, and these meadows are found throughout southern Australia. *P. australis* ecosystems are known to be highly productive and are apparently slow growing (West 1990). Also, many problems that may be associated with hydrophilous pollination and hydrophilous seed dispersal in seagrasses have not been addressed, but hydrophily itself may have an effect on the ability of these species to form seed and settle in new habitats (Les 1988).

Clonal growth occurs among species in many habitats and is particularly prevalent in aquatic systems (Parker 1979, Richards 1986, Ellstrand & Roose 1987, Les 1991, Grace 1993). This growth habit can greatly influence the structure and genetic diversity of plant populations. Populations can range from a single genetic individual (genet) to multiclonal aggregates (Stoddart 1984, Ellstrand & Roose 1987, Ayre & Willis 1988, Les 1991). Genetic polymorphism exists in many clonal plant populations, providing growing evidence for the common occurrence of multiclonality (Ellstrand & Roose 1987, Murawski & Hamrick 1990, Vasseur et al. 1991, Eckert & Barrett 1993). However, very few studies have looked at the genetic structure of such populations in detail.

To date population genetic structure has been studied in 4 clonal hydrophilous taxa (Les 1991, Laushman 1993). Two species, *Zostera marina* and *Vallisneria americana*, showed considerable within-population diversity where both species exhibited a number of multi-locus allozyme genotypes (Laushman 1993). However, of 2 *Ceratophyllum* species, one, *C. demersum*, was variable within some populations whilst the other, *C. echinatum*, had only monoclonal populations (Les 1991). The lack of variation in *C. echinatum* populations was not attributable to a lack of sexual reproduction as 7 of the 12 populations were described as sexual during the collection period (Les 1991).

Previously, the method of choice for assessing genetic variation within and between populations has been allozyme analysis (see Soltis & Soltis 1989 or Richardson et al. 1985 for review). This method can be performed quickly and relatively cheaply and there is now a large body of literature for comparison (e.g. Hamrick & Godt 1990). However, the ability to detect differences between individuals depends upon the numbers of polymorphic loci. Where low levels of polymorphism are encountered, allozyme analysis may not distinguish between different individuals.

A range of techniques based on direct DNA manipulation for assessing genotypic variation is now available (Schaal et al. 1991). Random amplified polymorphic DNA (RAPD) analysis, a relatively new technique, is becoming widely used (Chalmers et al. 1992, Wilde et al. 1992, Crossland et al. 1993, Huff et al. 1993, Patwary et al. 1993). This method was developed independently by 2 laboratories in 1990 (Welsh & McClelland 1990, Williams et al. 1990), and offers a sensitive technique for obtaining informative data about individual genotypes. RAPDs are a fast and cost-effective method for screening genotypic variation in moderate sized population samples (Ragot & Hoisington 1993).

This study describes the population structure of an Australian seagrass species, *Posidonia australis*, within a meadow using a structured sampling programme. The aim of this investigation was to determine if a meadow of *P. australis* was multiclonal or monoclonal, and if multiclonal to determine the extent of genetic variation within the meadow. Also, this study is the first to use both allozymes and RAPDs to assess population structure in an aquatic plant population system.

METHODS

Sampling. An area of *Posidonia australis* meadow was chosen in Warnbro Sound south of Perth, Western Australia (115° 42.3′ E, 32° 18.6′ S) (Fig. 1). Samples were taken from a meadow located 30 m from the beach below Mersey Point at an average depth of 1 m. In this area the *P. australis* was discontinuous along the shore edge and occurred as occasional patches within a larger, more continuous *Posidonia sinuosa* meadow. Shoot densities were recorded and a map of the meadow plotted relative to sampling points (Fig. 2). The *P. australis* meadow was surrounded by mixed *P. sinuosa* and *P. australis* meadow (seaward) and bare sand (shoreward).

Shoot meristems were collected from an area approximately 16 × 20 m. Samples were collected on a grid at 4 m intervals except for one 4 × 4 m square which was sampled at 1 m intervals (Fig. 2). Shoot material (3 to 4 shoots) was obtained at each collection point by pulling up a small piece of rhizome with attatched shoots and placing in a plastic bag. Each shoot was cleaned and any material with epiphytic growth was removed. The basal meristematic region was removed (up to 15 cm of growth), rinsed in filter-sterilised oceanic water, placed in a small plastic bag and heat sealed (for extracting DNA) or diced and placed in microfuge tubes (1.5 ml, for allozyme electrophoresis), then stored at −80 °C. Not all samples from each collection point were attached to the same piece of rhizome. Therefore, material used in allozyme and RAPD analyses were not necessarily from the same rhizome and thus do not represent dependent samples of the same meadow.

Allozyme analysis. Microfuge tubes, containing diced meristematic tissue, were placed on ice and then ground in freshly made buffer [modified from Cheliak & Pitel (1984) and Wendel & Weeden (1989)] containing 0.1 M phosphate buffer, 10% sucrose, 7% PVP-40 (polyvinyl-pyrrolidone, MW 40 000), 1% PVP-360 (polyvinyl-pyrrolidone, MW 360 000), 5 mg ml^{-1} ascorbate, 17 mg ml^{-1} Na_2EDTA, 38 mg ml^{-1} NaH_3SO_4, 80 mg ml^{-1} Borax, 100 mg ml^{-1} BSA-V, 1 mg ml^{-1} DTT, 45 mg ml^{-1} DIECA (sodium diethyl dithiocarbamate), 2 mg ml^{-1} NADP, 4 mg ml^{-1} NAD, 0.2 mg ml^{-1} Pyridoxyl-5-PO_4; the pH was adjusted to 6.8 with Tris. Samples were stored frozen at −80 °C until use.

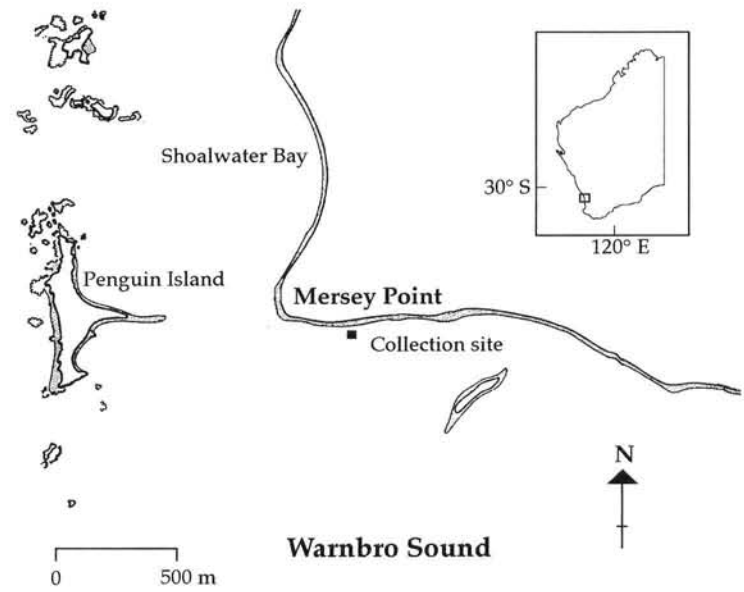

Fig. 1. Warnbro Sound, Western Australia, and relative position of sampled *Posidonia australis* meadow

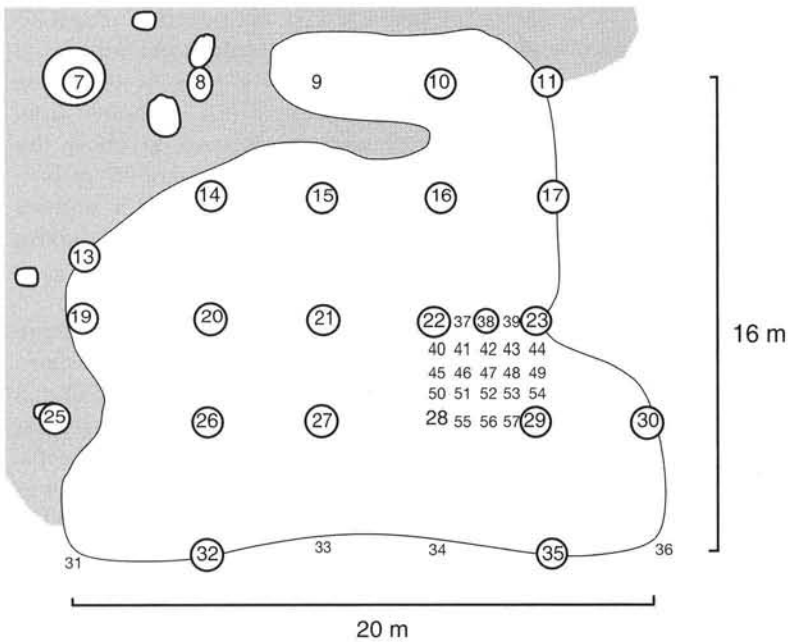

Fig. 2. Population sampling points on a plot of the *Posidonia australis* meadow at Mersey Point, Warnbro Sound, Western Australia. Numbers represent sample number used in collection and subsequent allozyme analysis. Circles around numbers represent samples used in RAPD analysis. The shaded area is predominantly *P. sinuosa* meadow with patchy *P. australis*

Horizontal starch gel electrophoresis was conducted according to Wendel & Weeden (1989), using 2 buffer systems stained for the corresponding enzyme systems: Histidine Citrate (Moran & Hopper 1983) — GPI (E.C. 5.3.1.9), IDH (NADP) (E.C. 1.1.1.42), PGM (E.C. 5.4.2.2), DIA (E.C. 1.6.99.–); and Morpholine Citrate pH 4.7 (Clayton & Tretiak 1972) — PGD (E.C. 1.1.1.44), MDH (E.C. 1.1.1.37). Only clearly defined, scorable loci were recorded for the 43 samples assayed. Polymorphic loci were tested for conformance to Hardy-Weinberg equilibrium using the program BIOSYS-1 (Swofford & Selander 1981). Polymorphic loci were used to generate a list of shared multilocus genotypes.

RAPD analysis. DNA was extracted using the method of Huff et al. (1993), then further purifed by extraction with equal volumes of phenol (Tris pH 8.0, equilibrated):chloroform:isoamylalcohol (25:24:1) twice, followed by extraction with chloroform:isoamylalcohol (24:1) twice, then ethanol precipitation and resuspension of the dried pellet in sterile water. Each DNA sample was then diluted to 10 ng μl^{-1} immediately before RAPD analysis was performed.

Optimisation of the RAPD procedure involved testing different primer and $MgCl_2$ concentrations initially. Reproducibility was tested using multiple extractions from the same individual, and repeated runs under the same conditions were performed. All reactions were set up using standard sterile conditions and solutions. Final, reproducible RAPD reactions contained 1 U *Tth* plus DNA polymerase (Biotech International), 1× reaction buffer (Biotech International, 10× containing 670 mM Tris pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton X-100, 2 mg ml^{-1} gelatin), 1 mM $MgCl_2$, 0.1 mM dNTPs (Promega), 0.2 µM primer (University of British Columbia), 10 ng total genomic DNA. The following cycling profile was used for all reactions; 2 min 94 °C, 1 min 37 °C, 1 min 72 °C for 1 cycle; 30 s 94 °C, 30 s 37 °C, 1 min 72 °C for 45 cycles followed by 5 min at 72 °C. The PCR products (5 µl) were visualised either on 1 mm, 6%, 40:1 polyacrylamide [made in 1× TBE (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA)] gels then silver stained, or on 1.2 to 2% agarose gels then ethidium bromide stained and visualised on a UV light box. The polyacrylamide gels were run in a Biorad Protean II xi gel tank, 25 samples per gel, for 800 volt hours and were stained using the Biorad silver stain kit at half of the recommended concentrations and 25 to 50% longer incubations.

RAPD bands were generated from nine 10-base primers. Of these, 4 primers were chosen that showed interpretable, reproducible banding patterns (Table 1). RAPD gels were scored for presence and absence of all bands. The total banding pattern, relative to all other individuals, was interpreted as the RAPD phenotype for each individual. This information was treated in the same way as the allozyme loci where individuals sharing multilocus RAPD phenotypes were grouped together as having apparently identical genotypes.

Table 1. 10-mer primers, with their nucleotide sequence, used in the RAPD survey of *Posidonia australis* shoots. All primers were obtained from The University of British Columbia Biotechnology Department

Primer name	Primer sequence
UBC-222	AAGCCTCCCC
UBC-262	CGCCCCCAGT
UBC-228	GCTGGGCCGA
UBC-243	GGGTGAACCG

RESULTS

Shoot densities within the area of *Posidonia australis* meadow sampled were approximately 550 shoots m^{-2}, resulting in approximately 200 000 shoots in the area samples. From these only 43 shoots were used in allozyme analysis (0.02% of shoots present) and 22 shoots for RAPD analysis (0.01% of shoots present). Several multi-locus genotypes were identified. Allozyme analysis gave 15 differences out of the 43 samples; RAPD analysis identified 16 different phenotypes amongst 22 samples.

Twelve allozyme loci were scored, of which 4 (PGM-1, DIA-1, MNR-1, 6PGD-2) were polymorphic with 2 alleles each. Fifteen genotypes (out of a maximum possible of 16) were found in the 43 samples; all 4 loci conformed to Hardy-Weinberg equilibrium with no significant excess of heterozygotes (Table 2). The distribution of the multilocus allozyme genotypes within the meadow is shown in Fig. 3. There were 6 samples with unique genotypes, 5 genotypes common to 2 or 3 samples and 4 genotypes common to 5 or more samples. The distribution of shared multilocus genotypes shows some grouping, but there are also samples with similar genotypes at opposite corners of the sampling area (e.g. samples 7 and 35 from genotype 'g' in Table 2).

Table 2. *Posidonia australis*. Allozyme multilocus genotypes for all samples surveyed; each row represents samples with the same genotype for each different technique and the corresponding code used in Fig. 3. Note that allozyme and RAPD samples are not necessarily from the same rhizome and therefore represent independent samples from each sampling point. Allozyme alleles were scored with the number 1 representing the fastest cathodal allele

Code	Sample number	Allozyme genotype			
		PGM-1	DIA-1	MNR-1	PGD-1
g	7, 42, 32, 33, 35, 51, 54, 57	22	11	11	11
c	8, 14, 29	12	11	11	11
j	9	12	11	11	12
e	10, 11	12	12	11	11
f	15, 16	12	12	12	11
k	17	11	12	12	11
b	19, 25	11	11	12	12
a	20, 21, 26	11	11	11	12
l	22	12	11	12	11
d	23, 27, 41, 43, 48	12	11	22	11
i	28, 36, 38, 47, 50, 56	22	11	22	11
h	30, 31, 40, 46, 49	22	11	12	11
m	44	11	11	12	11
n	45	12	11	12	12
o	34	22	11	12	22
Test for Hardy-Weinberg equilibrium:					
	χ^2 =	1.388	0.0	3.364	0.040
	p =	0.239	1.0	0.067	0.841

The choice of primers and optimisation of RAPD conditions gave reproducible results from replicated plant samples. Up to 6 separate runs of the same DNA samples and replicate DNA samples from the same individual were performed to show the reproducibility of each primer for both ethidium bromide and silver staining procedures. The 4 primers chosen generated a total of 45 different banding positions of which 25 were polymorphic (55.6%) (e.g. Fig. 4).

RAPD analysis identified a greater phenotype (overall banding pattern) variation than demonstrated by the allozyme analysis. Seventeen of the 23 samples assayed had different multi-primer phenotypes; only 4 sets of samples shared bands. The distribution of RAPD multi-primer phenotypes was similar to that identified by allozyme analysis. There were 16 different RAPD phenotypes identified in the 22 samples assayed (73%). These samples show a loose grouping (Fig. 3) but most represent unique phenotypes. These results cannot be compared with the allozyme data directly as samples were not necessarily from the same plant, but they do show similarities with the allozyme results.

DISCUSSION

Allozyme and RAPD variation were both detected within the sampled population of *P. australis* with more diversity among RAPD samples: 74% unique multilocus genotypes versus 35% unique multilocus allozyme genotypes. That is, more than 1 individual occurs in this meadow. These different individuals were detected despite the small area of meadow surveyed and the small number of shoots sampled. The allozyme and RAPD genotypes were not clumped but dispersed throughout the meadow. However, both techniques showed similar mixed distribution of genotypes.

RAPDs are expected to reveal more diversity as they sample the DNA more directly but do not discriminate between coding and non-coding regions of the genome. Allozymes are transcribed, active proteins and represent a discrete genomic component. In one of the few studies using allozyme and RAPD markers, Lui & Furnier (1993) also found a higher level of variation using RAPDs in 2 aspen species. However it is important to take care with the methodology and to assess reproducibility and consistency before using

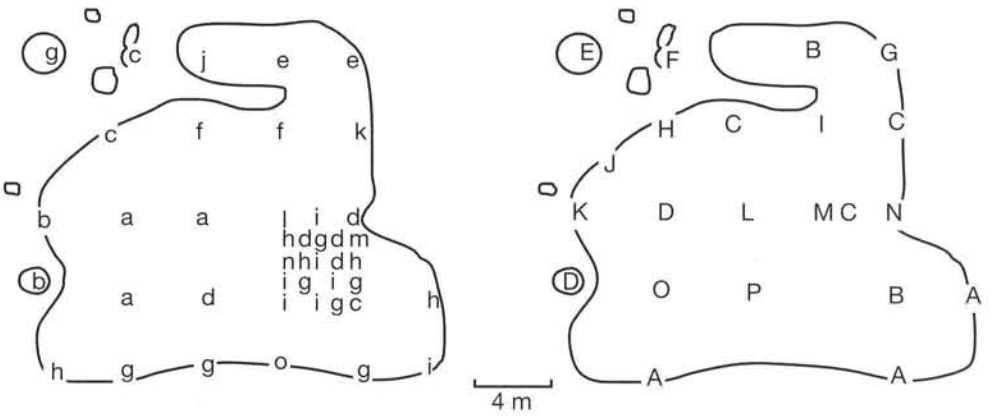

Fig. 3. *Posidonia australis*. Overlay plot of allozyme and RAPD multilocus genotypes onto an outline of the meadow (refer to Fig. 2). Left side: Allozyme genotypes are indicated by lowercase letters. Right side: RAPD phenotypes are indicated by uppercase letters

RAPDs. In this study some primers showed a high degree of variability between runs. It may be possible to eradicate this variability by altering the conditions of the RAPD reactions, but in this study only primers that gave consistent, reproducible results with the standard conditions were used.

The levels of allozyme variation in *Posidonia australis* described here are equivalent to, or greater than, other hydrophilous species (Les 1991, Laushman 1993). In his discussion on population structure in hydrophiles, Les (1988) suggests that until studies such as this have been conducted, the breeding system and population structure in hydrophiles will not be understood. Seagrasses are generally reported to be genetically depauperate, at least on the basis of allozymes (McMillan 1991). This study has shown that in this population of *P. australis* there exists a multiple number of genotypes. However, though this variation occurs, it cannot be said to be the result of recent sexual reproduction, as will be discussed.

One factor in meadow structure and dynamics in the genus *Posidonia* is the apparent lack of seedling establishment into meadows (Kirkman 1985, Kirkman & Kuo 1990). This genus has an unusual disjunct distribution, most species occurring along the southern coast of Australia but with 1 Mediterranean species (den Hartog 1970). The fruits of *Posidonia* are positively buoyant and when mature are released and rise to the surface. Later the fruit splits, releasing the seed which usually has germinated already (Kuo 1982, author's pers. obs.). The seed is negatively buoyant with a winged keel and sinks to the bottom rapidly (Kuo 1982). Thus *Posidonia* does not develop a seed bank in the sediment, but sends young seedlings away from the parent meadow to survive elsewhere only if both habitat and conditions permit. Seedling recruitment is not observed on a year-to-year basis, perhaps as conditions suitable for establishment are infrequent or rare. Thus, although genotypic variation occurs, it apparently does not result from recent sexual reproduction.

The genotypic diversity of the *Posidonia australis* meadow was much greater than expected. Before this analysis it was thought that the small area of meadow studied would be a single plant, or perhaps only a few plants. More intensive sampling between the 4 m and 1 m collections might show a greater frequency of common genotypes. In any case, the identification of so many different genotypes (and presumably therefore different individuals) suggested a far greater recruitment into this population than had been previously

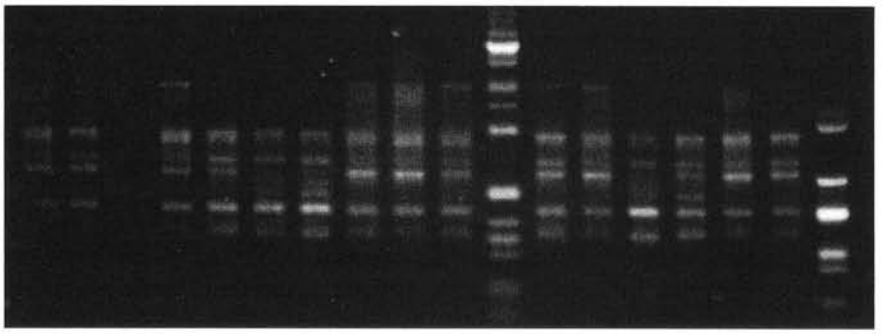

Fig. 4. *Posidonia australis*. RAPD run with primer UBC-222 run on a 1.5% agarose gel and stained with ethidium bromide. There are 4 phenotypes represented on this gel; sample numbers from left are (phenotype designation in parentheses): 7(2), 8(3), –, 10(4), 11(3), 13(1), 14(1), 15(4), 16(4), 17(4), Standard (Molecular weight marker X, Boehringer Mannheim), 19(4), 20(4), 21(1), 22(1), 23(4), 25(4), Standard (pGem3Z, Promega™ Hinf I cut)

indicated by studies of seagrass community dynamics (Kirkman 1985, Kirkman & Kuo 1990). It is possible that the sampling procedure failed to detect a greater number of common genotypes because of the distance between sampling points. Only a small proportion of the total number of shoots within the meadow were analysed (0.02 and 0.01% for allozymes and RAPD samples respectively), which may explain the diversity of genotypes identified. Sampling all the shoots within a small quadrat or along a transect may identify any smaller-scale pattern of variation.

Only 1 other reported study has determined genetic variation within populations of a seagrass (Laushman 1993). Allozyme electrophoresis of *Zostera marina* showed within-population genetic variation and a high degree of clonality. In this seagrass there is capacity for sexual reproduction, seedling recruitment and gene flow between populations. It appears that *Posidonia australis* has a similar capacity. If this is the case then meadow formation and maintenance may be a complicated process involving a myriad of factors.

In *Posidonia australis* meadows the diversity, as indicated in this study, must result from either a genetically diverse founding population (indicating extreme longevity) or else considerable gene flow into populations (indicating more frequent recruitment). However, there are 2 alternative hypotheses that could explain how a population of this structure might arise if there is little or no recruitment. The population may have been formed from a large number of seedlings with different genotypes. This would give rise to a large area with an intermingled pattern of different genotypes. Another possible mechanism which might evolve this population structure could be that the individuals, over a long time period, might form the observed pattern of genotypes by the meeting of, and eventual interspersion of, growing faces of different 'clumps' formed by different individuals. This latter process would require a long time to occur as the rhizome extension rate of *P. australis* is very slow, approximately 2.5 cm yr^{-1} (West 1990). In fact, if seedlings were established 5 m apart, the time taken before the rhizomes of the 2 clumps met would be approximately 100 yr and it would take 200 yr for the rhizomes of each clump to reach the starting point of the other clump. In this scenario the formation of a meadow such as that sampled in this study would take thousands of years. It would be difficult to ascertain the cause of meadow formation and maintenance, short of observing significant seedling recruitment into existing meadows.

There is an inherent difficulty in studying clonal organisms due to their often dispersed nature, and in *Posidonia* this is enhanced by the large meadow-forming nature of the populations. In animals, the study of clones has relied on assessing the genotypic diversity within populations using allozyme diversity (Parker 1979, Ayre 1990). In the corals *Pavona cactus* and *Pocillopora damicornis*, multi-locus allozyme genotypes show that clonality is common in some populations (Stoddart 1984, Ayre & Willis 1988). The sea anemone *Actinia tenebrosa* has a highly clonal population structure, with most settling juveniles being asexually derived progeny from nearby clones (Ayre 1983). Among plant populations there is a similar situation in the bromeliad *Aechmea magdalenae*; nearest-neighbour ramets are clonal but populations contain a number of clones (Murawski & Hamrick 1990). Alternatively, in the emergent aquatic *Decodon verticillatus* some populations exhibit large numbers of clones while others are monoclonal (Eckert & Barrett 1993). More studies that assess the fine-scale population structure of clonal species will be needed to gauge the levels of diversity within clonal populations and to indicate the sorts of sampling strategies that are necessary for studying clonal species.

Genetic diversity is present in *Posidonia australis* and the meadow studied is multiclonal. This is significant in that multiclonal populations have a greater probability of outbreeding and of successful sexual reproduction. It is of great interest therefore to determine the levels of outbreeding in populations of this species and to evaluate the success of sexual reproduction in multiclonal and monoclonal populations if they occur. Further studies of other populations and other species may reveal very different population structures. Multiclonal populations also have important implications for meadow rehabilitation. Where severe disturbances have occurred it may be important to determine the diversity of material re-seeded or transplanted to determine if long-term meadow success is optimised.

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