Genetic uniformity in *Amphibolis antarctica*, a dioecious seagrass

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Few detailed studies have been published on genetic variation in seagrasses except those on the monoecious *Zostera marina* L. or the hermaphrodite *Posidonia australis* Hook. f. This paper presents allozyme, RFLP and reproductive biology data on *Amphibolis antarctica* (Labill.) Sonder & Aschers, one of the 75 per cent of all seagrass species which are dioecious. Collections were made from approximately one-third of the species range in Western Australia. Its only congener, *A. griffithii* (J. M. Black) den Hartog, was collected from one site to provide a comparison. Flowering was observed in 25 per cent of the shoots surveyed and the average sex ratio was 3.8: 1 (F:M) which it has been suggested indicates sexual reproduction. No genetic variation was found within or between populations at 14 allozyme loci. 18S RFLPs and M13 DNA fingerprinting gave few satisfactory results but also did not exhibit any variability. Allozyme variation was observed between *A. antarctica* and *A. griffithii*, the only congeneric species. The lack of allozyme and DNA variation within *A. antarctica* indicates a potentially low level of outbreeding, a highly clonal reproductive system or a very efficient genetic system in *A. antarctica*. The hypothesis that the dioecious reproductive system evolved in seagrasses to maximize outbreeding and genetic variability, proposed by several authors, is questioned in light of these data.

Keywords: allozyme, fingerprint, genetic system, genetic uniformity, seagrass.

Introduction

The marine angiosperms (seagrasses) comprise only 58 species in 12 genera worldwide, all from the Alismatidae (Tomlinson, 1982; Kuo & McComb, 1989). Their characteristics may be derived from a common ancestor or may be the result of convergent evolutionary processes (den Hartog, 1970; Les & Haynes, 1995). Their fossil record is old, a seagrass flora being present in the late Cretaceous with extant species identified in the fossil floras of the Eocene (Larkum & den Hartog, 1989). The lack of species diversity and the antiquity of the species both suggest that there has been an extremely slow rate of evolution within the group (den Hartog, 1970; Les, 1988; Larkum & den Hartog, 1989).

The seagrasses present an interesting problem with regard to their evolutionary stability and breeding systems. Approximately 75 per cent of seagrasses are dioecious (den Hartog, 1970; Pettitt *et al.*, 1981; Les, 1988; Cox & Humphries, 1993) compared to only 4 per cent among angiosperms generally

(Richards, 1986). The striking predominance of dioecy amongst seagrasses has not been adequately explained, but has been attributed to the perceived advantage of elevated levels of genetic diversity as a result of outcrossing (den Hartog, 1970; Pettitt *et al.*, 1981). This argument is questioned by Les (1988) who points out that dioecy does not guarantee high outcrossing rates in seagrasses, where sexual reproduction may be sporadic and/or insignificant. The validity of the outcrossing argument therefore needs to be tested by estimating the importance of sexual reproduction and measuring levels of outcrossing in seagrass populations with different breeding systems. It is also possible that the ancestors of the seagrasses may have been primitively unisexual (Cox & Humphries, 1993; Posluszny & Charlton, 1993; Les & Haynes, 1995; Les *et al.*, 1996), so that the present high frequency of dioecy does not reflect adaptation to their present environmental niche.

Most seagrasses have strong vegetative growth, via rhizomes, and can form independent ramets over time allowing long-lived individuals to generate stable communities. Clonal growth has been observed to reduce the frequency of sexual repro-

duction (Cook, 1983, 1985) but the establishment of sexual offspring in clonal populations can be difficult. Whether seagrass species establish new communities mainly clonally or via sexual offspring is still unknown.

In the dioecious seagrasses the frequency of sexual reproduction is variable and the sex ratios of these species are female-biased (den Hartog, 1970; Les, 1988). Les (1988) has summarized the available flowering frequency data which indicate that flowering varies from 3–80 per cent of sampled shoots in different species. The observed ratio of female to male flowering shoots is closer to unity in the more infrequently flowering taxa (Les, 1988). Estimates of unequal sex ratios may result from sampling within clones, a common problem in sampling dispersed, clonal organisms. However, sex ratios in seagrasses have been poorly investigated and the reproductive implications have not been adequately assessed. Genetic and environmental factors as well as clonal sampling effects may affect the estimated frequency of a particular sex (Lloyd & Bawa, 1984; Richards, 1986). Female-dominated sex ratios are often documented among apomicts and thus apomixis cannot be discounted among seagrasses, particularly where genetic variability is low (Richards, 1986; Les, 1988; Asker & Jerling, 1992).

The few published studies of genetic variability in seagrass populations have led to conflicting generalizations about their genetic diversity and recruitment methods (McMillan, 1982, 1991; Les, 1988; Fain *et al.*, 1992; Laushman, 1993; Alberte *et al.*, 1994; Waycott, 1995). Most of these studies have been conducted on monoecious and hermaphrodite seagrasses, principally the northern hemisphere species *Zostera marina*, using allozymes (Gagnon *et al.*, 1980; Laushman, 1993), RFLPs (Fain *et al.*, 1992) and DNA fingerprinting (Alberte *et al.*, 1994). *Zostera marina* has genetically diverse populations which regenerate from a stored seed bank (Orth *et al.*, 1994). A detailed study has been conducted on *Posidonia australis*, an Australian endemic with hermaphrodite flowers (Waycott, 1995). Compared to *Z. marina*, *P. australis* has a similarly diverse population genetic structure (based on allozyme and RAPD data), but cannot rely on a seed store as its mature fleshy fruits contain germinating seeds when released from the parent plant (Waycott, 1995). This seagrass must have formed its genetically diverse meadows through frequent seedling recruitment into meadows or via the intermingling of long-lived ramets from different genets and/or meadows (Waycott, 1995).

Amphibolis antarctica is confined to the west and south coast of Australia (Walker & Cambridge, 1995). The species forms large highly productive monospecific meadows which attain a large biomass (Walker, 1989). This genus has an unusual growth form; the leaves are borne on a lignified stem with new leaves borne in the centre of leaf clusters so that there is a continual turnover of the outer older leaves. *Amphibolis* also has viviparous seedlings, a trait that has led to the seagrasses being considered as having evolved similarly to the mangroves (den Hartog, 1970). Vivipary, in mangroves, is said to be a mechanism promoting dispersal, seedling establishment and survival under strong wave action (Saenger, 1982; Tomlinson, 1986).

Amphibolis was chosen for this study because of its unusual reproductive system, combining dioecy and vivipary, and the observation of regular seedling production. This paper describes the first detailed study of a dioecious seagrass utilizing geographically widespread but locally intensive sampling in order to assess genetic variability and levels of outbreeding.

Methods

Collections

Flowering shoots or shoots with seedlings attached were collected from 13 locations by SCUBA or snorkel diving for the determination of flowering frequency, sex ratios and allozyme analysis (Table 1). Flowering frequency and sex determinations were made by collecting shoots at random across the meadows surveyed, except at Shoalwater Bay site 1 where only flowering shoots were collected (flowering frequency could not be determined at this locality). Samples were kept cool and examined while fresh. For allozyme analysis, shoots carrying viviparous seedlings near maturity or independent seedlings from drift were stored in sea water, on ice or refrigerated, until grinding. The collections for allozyme analysis were made over ≈ 1100 km from populations with very different habitats. These habitats ranged from hypersaline at Shark Bay (Walker, 1985) to a very high wave energy habitat at Sarge Bay and significantly colder water temperatures found at Flinders Bay in the Southern Ocean (Walker & Cambridge, 1995). As a control, samples of the congeneric *Amphibolis* species, *A. griffithii*, were collected from one location near the Shoalwater Bay sites.

Starch gel electrophoresis

Sample grinding and starch gel electrophoresis were carried out according to the methods used in

Table 1 Collection locations, analysis conducted, and the numbers and types of samples of *Amphibolis antarctica* run per population for allozyme analysis

Population	Location		No. samples assayed for allozymes	
	Latitude	Longitude	Maternal	Seedlings
Shark Bay†	25°25′S	113°35′E	—	6
Marmion Lagoon†	31°50′S	115°45′E	5	32
Rottnest‡	32°00′S	115°31′E	—	—
Point Peron†	32°16′S	115°41′E	10	48
Shoalwater Bay, site 1‡§	32°17′S	115°41′E	—	—
Shoalwater Bay, site 2‡¶	32°17′S	115°41′E	—	—
Geographe Bay‡	33°37′S	115°08′E	—	—
Cowaramup Bay†	33°52′S	114°59′E	6	11
Hamelin Bay†‡	34°12′S	115°01′E	6	25
Sarge Bay†	34°22′S	115°08′E	5	25
Flinders Bay†	34°19′S	115°10′E	6	14
Peaceful Bay‡	35°02′S	116°55′E	—	—
Two Peoples Bay‡	34°57′S	118°11′E	—	—
Total no. allozyme samples run			38	161

†Allozyme analysis.
‡Sex ratio determination.
§Towards Point Peron on south side of peninsula.
¶Apex Camp, 300 m south of site 1.

Table 2 Enzyme systems, number of loci identified and the corresponding gel buffers used for these systems

Enzyme	Code	EC no.	No. loci
Diaphorase†	DIA	1.8.1.4	2
Glucose-6-phosphate isomerase†	GPI	5.3.1.9	1
Glutamate oxaloacetate transaminase†	GOT	2.6.1.1	1
Isocitrate dehydrogenase†	IDH	1.1.1.42	1
Malate dehydrogenase‡	MDH	1.1.1.37	3
Peroxidase‡	PRX	1.11.1.7	1
Phosphoglucomutase†	PGM	5.4.2.2	2
Phosphogluconate dehydrogenase‡	PGD	1.1.1.44	2
Shikimic acid dehydrogenase‡	SDH	1.1.1.25	1
Total no. loci			14

†Histidine citrate (Moran & Hopper, 1983).
‡Morpholine citrate (Clayton & Tretiak, 1972).

Waycott (1995) except that samples were first ground in liquid nitrogen to pulverize the very fibrous tissues before grinding buffer was added. Only young shoot apices still contained within the older leaf sheaths were used to provide the most actively growing tissue and to prevent contamination of the homogenate by epiphyte proteins. Enzyme systems and gel buffers used are outlined in Table 2.

Many other enzyme systems were tested but exhibited little or no activity.

RFLP analysis and DNA fingerprinting

DNA was extracted from uncontaminated shoot apices using the method of Doyle & Doyle (1987) with minor modification (Waycott, 1995). DNA

fingerprinting was carried out using an M13 finger-print probe (Nybom *et al.*, 1990). DNA was digested with *Hae*III and the probe was prepared by PCR amplification of the M13 bacteriophage minisatellite sequence according to Rogstad (1993). *Eco*RI-digested DNA was analysed for 18S rDNA RFLPs using a 1 kb fragment of wheat 18S rDNA (Appels & Dvorak, 1982).

Results

The percentage of shoots collected which were found to be flowering varied from 11 per cent to 50 per cent in different populations. On average, flowering occurred in ≈25 per cent of shoots across all populations surveyed (Table 3). Sex ratios were female-biased, averaging 3.8:1 (F:M) over all populations (Table 3) although individual populations varied from being male-biased (0.2:1, F:M) to 100 per cent female.

All allozyme loci showed no variability within or between populations. All loci appeared to be homozygous, with some loci having shadow bands (Fig. 1). DNA fingerprinting with the M13 probe and rDNA was successful only on six samples but these were observed to be uniform with both probes.

When compared to *A. griffithii* there were obvious allozyme differences between species at *MDH* (Fig. 2) and *GPI*. Other loci were homozygous and indistinguishable from *A. antarctica*, except for *PRX* which was difficult to score reliably owing to the presence of many bands and differing intensities between samples.

Discussion

Flowering was observed in 25 per cent of *A. antarctica* shoots, well within the range of other seagrasses, namely 12 per cent in *Thalassodendron ciliatum* to 83 per cent in *Phyllospadix scouleri* (den Hartog, 1970; Les, 1988). There was a distinct predominance of flowering shoots in females compared to males in five of the seven populations, the two remaining having a male bias. However, in all populations within-clone sampling may have biased the ratio in favour of the clone sampled. Although there appears to be a greater number of female clones in the material sampled, these results indicate that *A. antarctica* is a sexual species, and not an obligate dioecious apomict, in which males would be expected to be extremely rare.

Amphibolis antarctica appears to be genetically

Table 3 Sex ratios in flowering material of *Amphibolis antarctica* from seven locations in Western Australia

| Population | No. shoots surveyed | No. flowering shoots | | Flowering frequency | Ratio female to male shoots |
		Female	Male		
Rottnest	79	2	10	0.18	0.2:1
Shoalwater Bay, site 1	NC	18	0	NC	—†
Shoalwater Bay, site 2	80	25	5	0.37	5:1
Geographe Bay	150	14	2	0.11	7:1
Hamelin Bay	45	16	0	0.35	—†
Peaceful Bay	39	2	3	0.13	0.6:1
Two Peoples Bay	18	7	2	0.5	3.5:1
Totals	429	84	22	0.25	3.8:1

NC, data not collected.
†All flowering shoots collected were female.

Fig. 1 Starch gel electrophoretograms of the enzyme system GPI demonstrating homozygosity with shadow bands at these two loci. Samples of *Amphibolis antarctica* were from Shark Bay, Marmion Lagoon and Hamelin Bay on this gel.
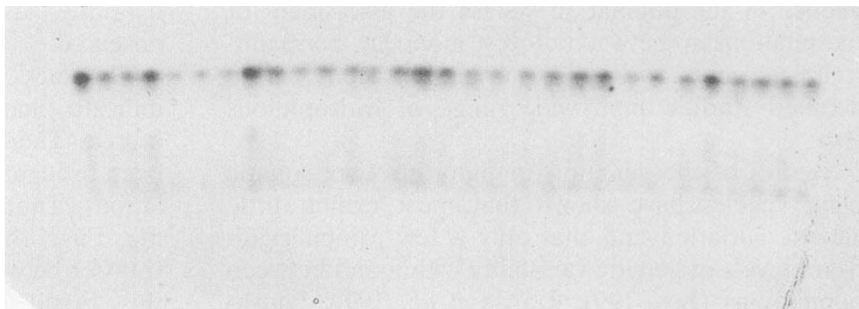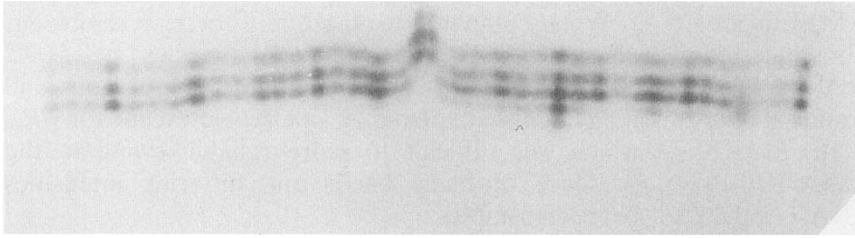
Fig. 2 Starch gel electrophoretogram of MDH showing the three loci with all samples of *Amphibolis antarctica*, except the two central samples of *A. griffithii* which demonstrate the allelic differences between species.

invariant across a wide geographical range. This finding is based on allozyme data but includes some within-population M13 fingerprinting and between-population rDNA RFLP testing. Analyses in this study were conducted on seedling arrays from different populations. Whereas RFLP analysis using ribosomal probes may be expected to reveal levels of variation comparable to those revealed by allozyme analysis, the M13 fingerprinting method may be expected to reveal differences between individuals (Vassart *et al.*, 1987; Rogstad *et al.*, 1988; Rogstad, 1993; Alberte *et al.*, 1994). However, maternal parent and offspring plant tissue as well as several different seedlings from other parental shoots were tested but gave no detectable differences.

Amphobilis antarctica and *A. griffithii* showed allozyme differences at two loci, demonstrating fixed and readily detectable differences between the two species. McMillan (1991) described nearly identical allozyme patterning between sympatric populations of *A. antarctica* and *A. griffithii* but with some differences detected in a peroxidase locus.

The hypothesis that the dioecious reproductive system evolved in seagrasses to maximize outbreeding and genetic variability clearly needs reassessing in the light of these results. Early hypotheses were formulated without an adequate phylogenetic framework to discuss the evolutionary relationship between dioecy and hydrophilous pollination. A molecular phylogeny of the seagrasses is now available and demonstrates that dicliny may be primitive in the marine angiosperms and other members of the subclass Alismatidae (Les & Haynes, 1995; Waycott & Les, 1996; Les *et al.* 1996). Although dioecy may not have evolved as a direct consequence of the pollination system the assessment of the relationship between dioecy, monoecy, hermaphroditism and hydrophily urgently needs more detailed studies on a wide range of hydrophilous taxa.

Studies of the genetic variability in other aquatic plant species have shown that most exhibit little genetic variation and that only a few exhibit significant levels of genetic variability within and between populations (Les, 1991; Harris *et al.*, 1992; Laush-

man, 1993). Terrestrial clonal plants show a greater tendency for multiclonality (as identified by allozyme variation) with widespread clones infrequent (Ellstrand & Roose, 1987). A striking exception to this generalization is *Typha latifolia* which exhibited no genetic variability in allozymes across 74 populations at 19 allozyme loci (Mashburn *et al.*, 1978). Low genetic variability in aquatic plants is usually ascribed to highly clonal growth and asexual reproduction or to founder effects (Les, 1988, 1991; Triest, 1991). In addition to these mechanisms, high levels of inbreeding may also promote genetic homogeneity within populations where there are no secondary outbreeding mechanisms (postzygotic seed selection) (James, 1992).

In *A. antarctica* the lack of variability can be explained by neither clonality nor founder effects. Although both may result in local homogeneity they would not be expected to result in genetic uniformity across the species range. In this study samples were taken from widespread geographical locations and diverse environmental conditions, suggesting that the genetic homogeneity observed is a characteristic of the species and not a locally induced sampling phenomenon. Frequent and copious seedling formation has been observed in this species suggesting that it is unlikely that the whole species is composed of a single clone. The seedlings themselves could be clonal as seedling formation has not been shown to be sexual by embryological studies and may be apomictic. However, pollination has been shown to be successful both in culture and in the field (Ducker *et al.*, 1978; Verduin *et al.*, 1996). In addition, high frequencies of male plants persist in some populations, and no evidence of fixed hybridity, as is characteristic of apomicts, was observed.

The lack of observable genetic variability may indicate that *A. antarctica* is sexual, but highly inbred. There is the possibility that inbreeding has been brought about through the structuring of populations. There are several causes for such structuring. First, there is local clonal growth, and pollen transfer between genets may be restricted because of this clonal growth. The ability of seagrasses to

achieve successful submarine or even surface pollination over long distances is untested, and according to Cox & Humphries (1993), improbable. This means that local pollination would be the main mechanism for generating sexual offspring. This type of breeding system constraint has been observed in the sedge *Carex platyphylla*, a wind-pollinated, clone forming terrestrial plant (Handel, 1985). Secondly, population structuring may result from bottleneck effects associated with catastrophic habitat upheaval leaving only a few isolated pockets of individuals which have survived to recolonize to the distribution observed today. The Australian coastline has been subject to many changes in habitat since its Gondwanan origins, including dramatic sea-level falls and rises since the rifting of Australia from the Antarctic during the Cretaceous (Quilty, 1994), with considerable opportunity for bottleneck effects to occur. However, inbreeding would merely promote local homogeneity, and could be expected to promote between-population divergence. In *A. antarctica*, this is not observed; the genetic homogeneity appears to be species-wide.

If the success of an organism, or group of organisms, is defined as persistence in an environment over a long period of time, we might view the seagrasses as a highly successful group. They have an extremely persistent fossil record which dates back approximately to the origin of the angiosperms (Larkum & den Hartog, 1989). *Amphibolis* has achieved a considerable geographical distribution, is highly productive and is commonly the dominant species, particularly in southern and western Australian temperate oceanic waters. If *A. antarctica* is viewed as successful, then it must possess a genetic system with evolutionary capability enabling it to persist over long periods of time, to meet the environmental challenges that have occurred, and yet to exhibit only minor levels of genetic variation, and perhaps none at all. Perhaps *A. antarctica* has evolved a remarkably capable and adequate genetic system that combines optimal levels of vegetative growth and asexual reproduction with highly exogamous sexual reproduction and dispersal in the marine environment.

A more detailed study across the entire range of *A. antarctica*, perhaps in conjunction with the congeneric *A. griffithii*, may help to unravel the problem of why there appears to be so little genetic variability in this species. If the species is able to evolve, then genetic recombination must be possible, and should be current and detectable. There is no need to suppose that such capability must be reflected in diversity at the allozyme level, but it should be

reflected in DNA sequence diversity. It is therefore necessary to develop more sensitive and reliable molecular methods for this species to search for its sequence diversity, before the significance of the apparent lack of genetic variation we have observed can be properly understood.

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