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TRACING THE BASIS OF ECOLOGICAL SUCCESS OF WILD RADISH (*RAPHANUS RAPHANISTRUM*) IN DIVERSE ENVIRONMENTS THROUGH A COMPARISON OF LIFE HISTORY TRAITS AND AFLP MOLECULAR MARKERS

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ABSTRACT

Background Wild radish is among the most troublesome weeds in Australia. Perhaps, it was introduced into Australia as a contaminant of agricultural produce, and since that time it has been successfully colonized in a wide range of environments. The present research was planned to test the genetic variation in wild radish through a comparison of life history traits and amplified fragment length polymorphism (AFLP) molecular markers.

Methodology Four populations of wild radish were sampled from south-Western Australia. Genetic variations within and between populations were measured and compared between AFLP and plant life history traits. It was hypothesized that populations from nearby sites would show greater similarity than more widely separated sites, and that populations from environmentally similar sites would show greater similarity than populations from dissimilar sites and that this would be most marked in life history traits.

Results The primer combination *Eco*Rl AAC/*Mse*I CAA resulted in a total of 448 loci, of which 99% of the Merredin, 84% Nukarni, 93% Mullewa and 88% Denmark loci were polymorphic. Principal coordinates analysis and cluster analysis showed that variations within populations (71.7%, Fst = 0.71) were higher than the variations between populations (28.3%, Fst = 0.28). The Nukarni population appeared to be the most distinctive. The Mullewa and Merredin populations showed the greatest level of similarity. The Denmark and Mullewa populations were most distinct in terms of life history traits. The Denmark population had the largest pods and seeds while, the Mullewa population had the smallest. The results of the study supported the hypothesis that environment strongly influenced these traits, especially the seed and pod size. The genetic markers, despite separating the biotypes, did not support the hypothesis that the greatest differences would arise from populations separated most in terms of space and environment.

Conclusion Wild radish was introduced to Western Australia on several occasions; probably the Merredin and Nukarni populations represented the separate introduction. But, the life history traits revealed that evolution since introduction was convergent while environmental conditions were similar and divergent where they differed.

INTRODUCTION

Wild radish (*Raphanus raphanistrum*) is a problematic invasive weed of many crops throughout the globe (Parsons and Cuthbertson 1992; Lu et al. 2019; Goggin et al. 2019). It has been a troublesome weed since its accidental introduction into Australia during the latter part of the last century. It was probably introduced into Australia as a contaminant of agricultural produce (Donaldson 1986), and since

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that time it successfully colonized in a wide range of environments. It causes severe reduction in the yield of crops and thus has become considered as most troublesome agricultural weed (Kebaso et al. 2020). It is an obligate out-breeder, which can respond rapidly to environmental changes. Wild radish, a cross pollinating species of Mediterranean origin, has widely colonized in Western Australia, where it is considered to be a serious weed of cereals and canola (Borger et al. 2012). Seed dormancy varies among naturalized populations. For example, Cheam (1986) found that populations collected in wet areas have greater seed dormancy than those from drier areas, a result that differs from capeweed (*Artcotheca calendula*) in the same region (Dunbabin and Cocks 1999). However, little is known of genetic variation and there have been no measures of variation in DNA among Western Australian populations.

Genetic variation in self-pollinating annual species has been measured by many authors. Woodward and Morley (1974) found that time for flowering of *Trifolium glomeratum* is related to length of growing season in South-Eastern Australian populations. In a more detailed study, Smith et al. (1995) observed a similar relationship for the same species in Western Australia. Most other self-pollinating species that have been examined behave in the same way, for example *H. leporinum* and *H. glaucum* (Cocks et al. 1976), *Bromus diandrus* (Kon and Blacklow 1989) and most annual legumes native to Syria (Ehrman and Cocks 1996). Other traits known to vary between populations are seed dormancy (Groves et al. 1982; Dunbabin and Cocks 1999), response of seedlings to temperature (Groves 1975) and isozymes (Panetta 1990).

Inbreeding species are, for a variety of reasons, likely to demonstrate significant difference between population differentiation (Allard 1965; Allard 1975; Kon and Blacklow 1989; Neuffer and Meyer-Walf 1996). Outbreeders have much less potential to do this and generally show less difference between population differentiations. However, Dunbabin (2001) in his study of the outbreeding capeweed (*Artcotheca calendula*) and the inbreeding barley grass (*H. leporinum*) did not detect variation between populations using life history traits. However such variations were detected using genetic markers. For outcrossing wild radish, one might expect that differentiation between populations in life history traits and in genetic markers will depend on the distance between them.

AFLP were used to determine the genetic variations between and within populations of wild radish. The results were compared with variation in life history traits which might have been expected to be more influenced by environmental factors. Four populations, from Denmark on the South Coast of Western Australia (high rainfall, geographically isolated from the other test populations), two from near Merredin in the Eastern wheat belt (low rainfall, geographically close to each other) and one from Mullewa in the Northern wheat belt (low rainfall, geographically isolated from the other test populations) were chosen. The hypothesis tested was that genetic variation in both AFLP markers and life history traits would be greatest within, and least between populations at the Eastern wheat belt sites, and greatest between populations variation would occur between the isolated populations at Denmark and Mullewa.

MATERIALS AND METHODS

Origin of plant populations

Wild radish (*Raphanus raphanistrum*) populations were collected at Merredin (31º 22'S, 118º 32'E), Nukarni (30 km South of Merredin 31º 23'S, 118º 15'E), Mullewa (28º 33'S, 115º 29'E) and Denmark (34º 58'S, 117º 39'E) (Figure1). These populations were selected because they were representing a range of annual rainfall, temperature and growing season lengths (Table 1).

Measurement of life history traits

The seeds were directly sown into the field where possible 10 plants from each site were planted in a randomized complete block design with two replicates (a total of 20 plants). The numbers of plants surviving to maturity from each site were: Merredin 16, Nukarni 20, Mullewa 18 and Denmark 18. The plots were weeded and fertilized as required and irrigated when necessary. A number of variables were measured on each surviving plant (Table 2). In all analysis, means of the two replicates were used.

The data were analyzed using the method of residual maximum likelihood (REML) (Peterson and Thompson 1971) and canonical variate analysis. REML analysis was used to apportion genetic variation between and within the sites. A general analysis of variance was used to check the levels of significance on the life history traits between populations. Canonical variate analysis was used on the plant traits to separate the four sites with the mean values of each trait at each site used as variates. Mean canonical variants one (CV1) scores were plotted against mean canonical variant two (CV2) scores for each site.

DNA extraction

Twenty-five plants of each population were grown in

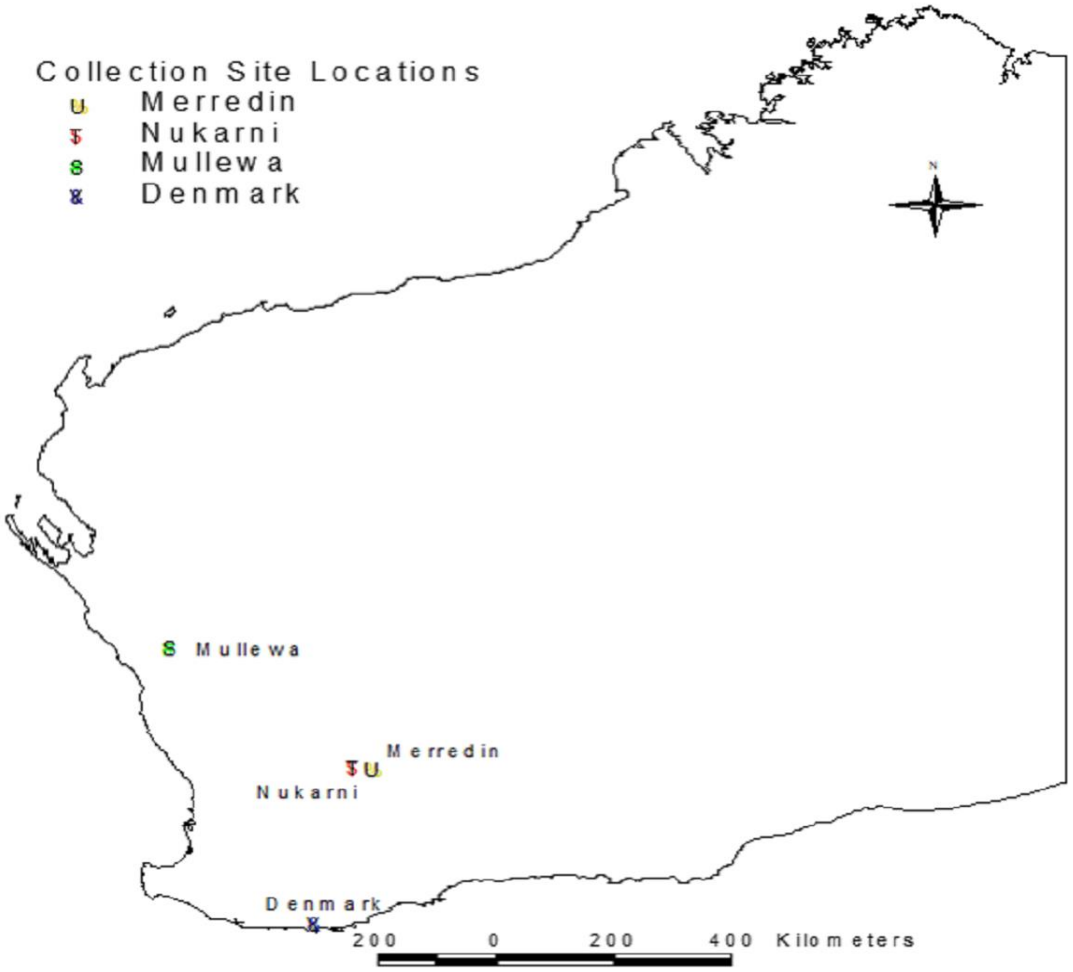

Figure 1 Showing the sites at which the four populations of wild radish were collected around Western Australia

Table 1 Mean maximum and minimum temperature in January and July and mean annual rainfall at Merredin, Nukarni, Mullewa and Denmark

Location	Maximum January Temperature (°C)	Maximum July Temperature (°C)	Minimum January Temperature (°C)	Minimum July Temperature (°C)	Annual mean Rainfall (mm yr^{-1})
Merredin	34	16	17	5	314
Nukarni	34	16	18	6	328
Mullewa	37	19	19	7	340
Denmark	25	16	13	7	1001

Meteorological data were provided by Commonwealth Bureau of Meteorology, Canberra Australia

a glasshouse, and 300 mg of fresh young leaf material was sampled from each plant for the DNA extraction. It was not always possible to extract DNA successfully; in our case DNA was successfully extracted from 18 Merredin plants, 15 Nukarni plants, 21 Mullewa plants and 20 Denmark plants. The reason for the difficulty may be associated with compounds in the leaf tissue, which are often found in broad-leaved plants, making extraction of high quality DNA difficult (Kim et al. 1997). Three extraction methods were tested; DNAzol from Life Technologies Australia Pty Ltd (Chomczynski et al. 1997), Sarkosyl Ultrapure (GIBCO) (Doyle and Doyle 1987) and the modified CTAB method (Bennett and Mathews, 2003). The best method was found to be the modified CTAB method, which resulted in high quality DNA.

AFLP molecular markers

The GIBCO BRL AFLP™ (Life Technologies, Melbourne, Australia) analysis system 1 and starter primer kit was used for the AFLP analysis. The procedure used was modified slightly from the manufacturer's instructions. For wild radish, the only modification required was that the undiluted pre-amplification template was used at the selective amplification step instead of the dilution listed. The rest of the Life Technologies procedure was followed according to the instructions Total ten primer combinations were screened to determine which gave the clearest AFLP fingerprints. *Eco*RlAAC/*Mse*I CAA was the best primer combination because it produced the most bands that could easily be scored. As AFLP markers were dominant, alleles were scored as either present or absent at each AFLP fragment. A DNA ladder (30-700 bp) was included on each gel to estimate the size of fragments. Where bands were scored outside this range, their size was estimated by extrapolation from the ladder. In order to assess the reliability of the AFLP procedure, one DNA sample from each site was selected for parallel AFLP analysis with one primer combination. Identical AFLP analysis fingerprints were obtained each time the same DNA template was used, indicating consistent and reliable results from the technique. The AFLP data were analyzed using the University of Geneva's Arlequin programme (Schneider et al. 2000). Within population, variation was estimated using two methods. The percentage of polymorphic loci was used as a measure of allelic richness within each site. The average gene diversity was the probability that homologous loci, randomly chosen from two individuals, were different. This latter statistic reflected the overall diversity of the loci, not simply the number of loci, which were polymorphic. The division of total variation within and between populations was measured using the analysis of molecular variance (AMOVA).

Principal coordinates analysis was used to group individuals. A similarity matrix was generated from all the recorded loci for each individual, calculated using the simple matching method. Cluster analysis was calculated using amplified fragment length polymorphism data of all the individuals at the four sites. For this method, a similarity matrix was generated using the Euclidean distance method, and the plants clustered using the group average method.

RESULTS

Life history traits

Data presented in Table 3 showed the percentage of variation both between and within populations for all life history traits. REML analysis revealed that variation between sites was greater than within the sites for seed weight, pod weight and flowering date; for the remainder of variables, within site variation was substantially greater than that between sites. However, apart from leaf shape and leaf area, the traits differed significantly between sites. The first canonical variate accounted for 84.5% of the variation, and the second for 14.0%. Only non-significant amounts of variation were accounted for higher order variates. The first canonical variate consisted of inputs from variables associated with pod and seed size, the variates becoming larger as pod and seed size (Table 4). The second variate was negatively associated with pod width and seed size and included an element of leaf shape.

In Figure 2, plants from the four populations grouped together, with plants from the geographically close sites, Merredin and Nukarni, showing the greatest similarity. The figure showed that plants from Denmark produced larger pods than plants from Mullewa in particular, with the sites at Merredin and Nukarni intermediate. Plants from Nukarni and Denmark produced the largest seeds.

AFLP molecular markers

The combination *EcoRl*AAC/*Mse*I CAA was the best primer combination of the ten tested. This showed the clearest bands in all four populations resulting in 112 scoreable bands at Merredin, 82 at Nukarni, 100 at Mullewa, and 110 at Denmark. Data presented in Table 5 showed the results of the gene diversity indices and the AMOVA for each population. The results indicated that up to 99% of the loci were polymorphic with average gene diversity ranging from 0.32±0.16 at Denmark to 0.39±0.20 at Merredin. Overall, 72% of the variation was found within sites and 28% between sites.

The dendrogram of the cluster analysis is shown in Figure 3, with almost all individuals group at the site levels. The majority of the population from Mullewa separated from the majority of the Denmark population at the 56% similarity level (but one of the Mullewa population differed from the remainder at 52% level and two individuals from the Denmark population separated from the remainder at 49% level); the population from Merredin differed from the Mullewa and Denmark populations at the 48% similarity level, and the Nukarni population differed from all other populations at 40% similarity level. One individual from Merredin separated from the Nukarni population at 47% level and from the remainder of the Merredin population at 40% level. Thus, the cluster analysis suggested that the two most closely related populations were from Denmark and Mullewa, and the population from Nukarni was the least closely related to the others. In general, the

Table 2 Description of the 12 life history traits measured on each plant from the four experimental sites

Plant trait	**Description**	**Units**
Flowering time	The number of days from sowing until anthesis of the first flower	
Number of leaves	Number of leaves on the main stem when the first flowers opened	
Plant height	Length of the main stem from ground to shoot apex	mm
Plant width	Mean of plant width in the North–South, East-West directions	mm
Leaf area	Area of the leaf subtending the first flowering branch	cm^2
Ratio of leaf length to width	Ratio of leaf length to width	
Number of primary branches	The number of branches at maturity	
Pod length	Mean length of 10 pods per plant	mm
Pod width	Mean width of 10 pods	µm
Number of pod segments	Mean number of pod segments/pod in 10 pods	
Seed weight	Weight of 100 seeds per plant	g
Pods weight	Weight of 10 pods per plant	g

Table 3 Mean trait value over all sites, the percentage of variation residing between and within sites

Plant traits	**Mean trait values over all sites**	*Variation between sites (%)*	**Variation within sites (%)**
Flowering time	236.2	55.40***	44.60
Number of leaves	3.4	18.87**	81.13
Plant height	23.4	6.92*	93.08
Plant width	41.1	1.44*	98.56
Ratio of leaf length to width	2.3	0.00n.s	100.00
Leaf area	35.9	0.00n.s	100.00
Number of primary branches	8.1	20.69**	79.31
Pod length	38.9	41.61***	58.39
Pod width	0.2	27.85***	72.15
Number of pod segments	5.0	25.31**	74.69
100 Seed weight	0.6	77.69***	22.31
10 pods weight	1.5	67.51***	32.49

The significance level of the difference between populations for 12 traits P<0.001(***), P<0.005(**) and P<0.05(*).

Table 4 Component loadings for life history traits in the canonical variate analysis, as well as the amount of variation accounted for by the first and second canonical variants (CV1 and CV2)

Plant traits	**CV1**	**CV2**
% Variation	84.5	14.0
Number of primary branches	-0.421	-0.148
Flowering time	-0.064	-0.057
Plant height	-0.04	0.094
Ratio of leaf length to width	1.19	1.153
Leaf area	0.014	0.021
Number of leaves	1.107	0.059
Number of pod segments	0.862	-0.221
Pod width	8.028	-2.332
Pod length	-0.052	0.008
10 pods weight	2.205	0.261
100 seed weigh	2.086	-7.133
Plant width	-0.006	-0.007

principal coordinate's analysis supported the cluster analysis (Figure 4). The first three coordinate loadings contained respectively, PC1: 22.8% of the total variation, PC2: 12.4% of the total variation and PC3: 10.4%. Individuals were grouped according to their sites of origins, Nukarni was genetically the most distinct; and Merredin and Mullewa are genetically the closest. In contrast to the cluster

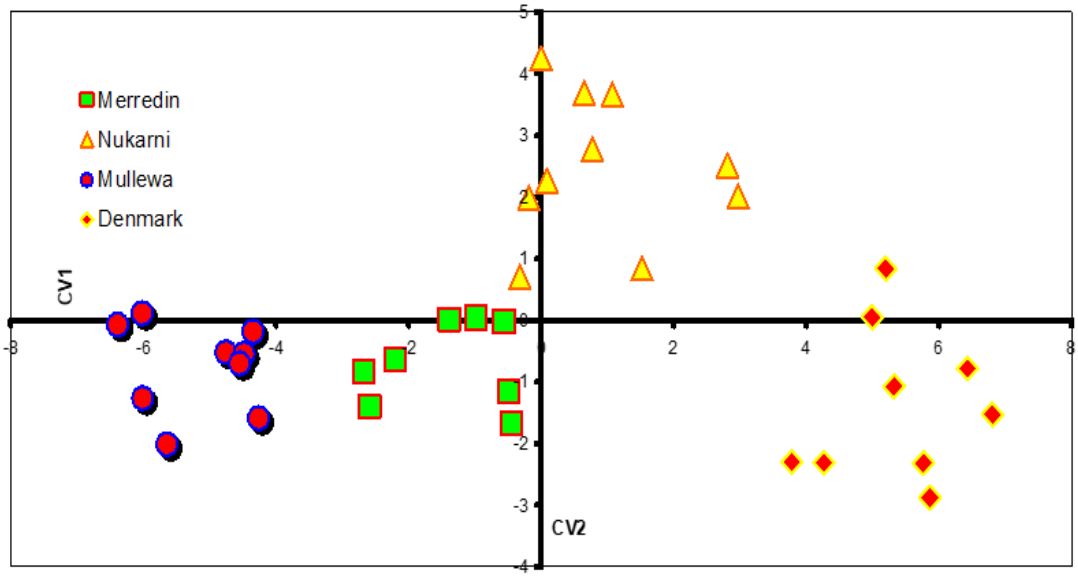

Figure 2 A plot of canonical variate 1 (CV1) against canonical variate 2 (CV2) for each of the plants in which life history traits were measured. Plants with similar symbols originate from the same site as indicated on the figure (Merredin, Nukarni, Mullewa and Denmark)

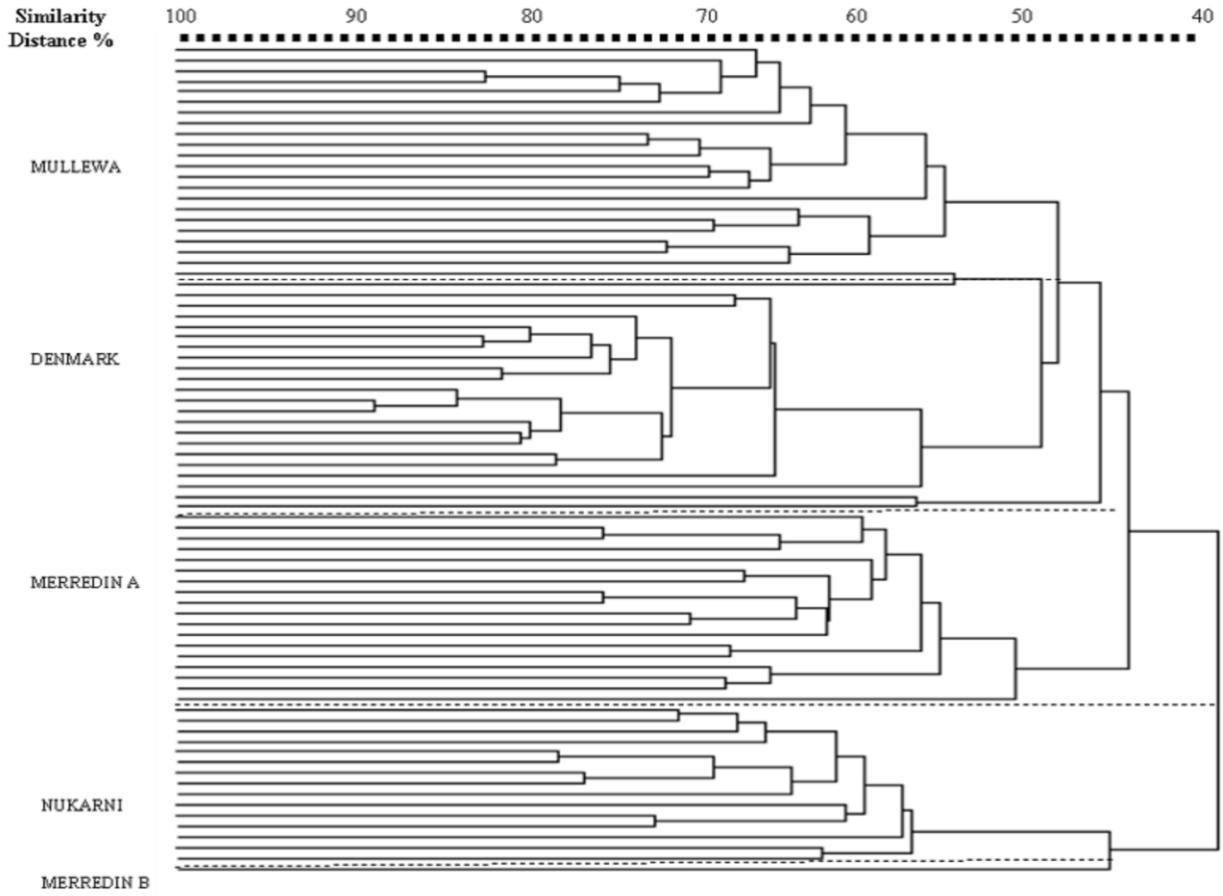

Figure 3 Dendrogram from cluster analysis of wild radish (*Raphanus raphanistrum*) populations analysed for genetic variation using AFLP technique

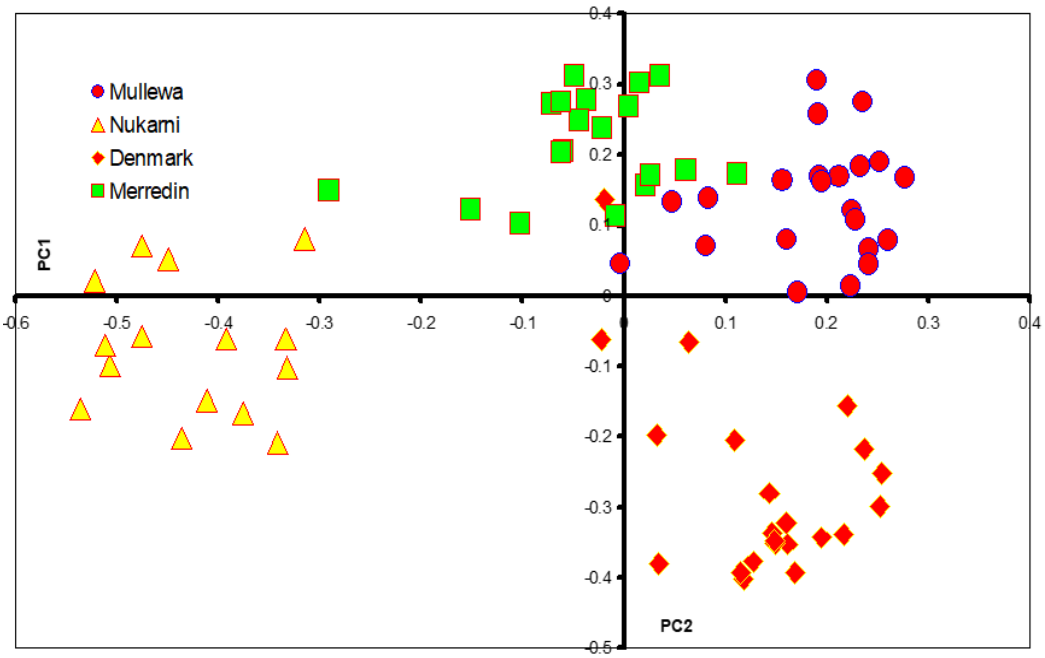

Figure 4 A plot of principal coordinates 1 (PC1) against principal coordinate 2 (PC2) for each of the plants in which DNA was assayed using AFLP markers. Plants with similar symbols originate from the same site as indicated on the Figure (Mullewa, Nukarni, Denmark and Merredin).

Table 5 The number of polymorphic sites, the number of loci recorded, the percentage of polymorphic loci, the average gene diversity and the apportionment of variation between and within sites of wild radish populations collected at Merredin, Nukarni, Mullewa and Denmark

Parameters	Merredin	Nukarni	Mullewa	Denmark
Number of polymorphic sites	110	70	85	97
Number of loci recorded	112	112	112	112
Number of usable loci	111	83	91	110
Average gene diversity[1] (%)	0.39± 0.20	0.35± 0.18	0.36± 0.19	0.32±0.16
Variation between sites[2] (%)	28.3			
Variation within sites[2] (%)	71.7			

[1]Probability that two randomly chosen homologous nucleotides are different (± I SD), [2]Measured by AMOVA

analysis, the principal coordinate analysis separated the Denmark population more clearly from the Merred in population. However, one individual from Merredin separated from all and one individual from Denmark lie within the Merredin population.

DISCUSSION

It was hypothesized that populations from nearby sites (Merredin and Nukarni) would show greater similarity than more widely separated sites, and that populations from environmentally similar sites (Mullewa and Merredin) would show greater similarity than populations from dissimilar sites (Merredin and Denmark). The results from the life history study supported the hypothesis, showing that environment strongly influenced these traits, especially the seed and pod size. The genetic markers, although separating the biotypes, did not support the hypothesis that the greatest differences would arise from populations separated most in terms of space and environment. The explanation may lie in the random nature of genes selected during the DNA analysis compared with the strong selective pressures acting on the life history traits.

The distinctive molecular patterns of the populations from Merredin and Nukarni (Figure 3 and 4) suggested that they had different origins and were introduced to the central wheat belt on separate and unrelated occasions. Such an explanation of the results agreed with the interpretation of Gladstones (1966), who used leaf markers to hypothesise that there were a number of separate introductions of subterranean clover to Western Australia early in the colony's history. Leaf markers were unlikely to possess any adaptive value and therefore behaved in

a similar way to the molecular markers used here. It was found that subterranean clover was introduced from different parts of Mediterranean and northern Europe in the 50 years leading to the building of the railway network in the wheat belt. Evolution of subterranean clover since that time appeared to have hybridisation and natural selection, with relatively few leaf markers predominating, but with the distinctly different ecophysiological behaviour of selected genotypes strongly related to environmental conditions at the sites of collection (Cocks and Phillips 1979; Cocks et al. 1982)

Annual pasture plants and weeds were common in Southern Australia, and this fact indicated repeated introductions. For example, at least two species of barley grass (*H. leporinum* and *H. glaucum*) were present in different parts of South Australia (Cocks et al. 1976). These barley grasses came from different parts of the Mediterranean basin. Capeweed (*Arctotheca calendula*), which was found throughout Southern Australia (Arnold et al. 1985), was a native of South Africa. Numerous annual legumes, mainly from the Mediterranean basin, were also found in Southern Australia. It was inconceivable that these were the results of single introductions.

It is widely recognized that the majority of life history traits expressed by plants were adaptive and thus acted upon by natural selection (Marshall and Allard 1970; Lande 1977; Barrett 1982; Barrett 1988). Though, the adaptive significance of a polymorphism may be unclear in some cases, a more thorough investigation revealed its significance. For example, New (1958) studied *Spergula arvensis* in the United Kingdom and demonstrated variation in seed coat morphology between populations, the significance of which was initially unclear. However, New and Herriot (1981) demonstrated that a particular seed coat morphology had direct adaptive value in allowing seeds to germinate under moisture regimes typical of the areas in which these existed. Thus the patterns of variation found in life history traits were likely to be the result of adaptation to prevailing environmental conditions.

The results presented here clearly demonstrated that adaptive evolution had occurred since wild radish was introduced to Western Australia. Thus, the genetically dissimilar populations at Nukarni and Merredin had converged to form similar populations in terms of life history traits, while the genetically more similar populations at Denmark and Mullewa had diverged dramatically in terms of their life history traits. Evolution was a potent force on these populations since their introduction, although insufficient time was elapsed for this to be expressed in terms of molecular markers, where genetic drift rather than selection would act to differentiate the populations.

Evolution resulted in ecotypic differentiation of many other annual species introduced to Australia since early in the 19th century. These include *Trifolium glomeratum* (Woodward and Morley 1974), *Hordeum leporinum* and *H. glaucum* (Cocks et al. 1976), *Trifolium subterraneum* (Cocks and Phillips 1979), *Artotheca calendula* (Dunbabin 2001) and others. In the case of *Trifolium subterraneum*, differentiation occurred over 30 years or less (Cocks 1992), with the suggestion that, where hybridisation is possible (this species is mainly self-fertilizing), ecotypic differentiation took place almost immediately. It was therefore not surprising that wild radish showed such strong differentiation in populations as distant as that of Denmark from the wheat belt populations.

Among most of these annual species, one of the most commonly demonstrated ecotypic differences between populations was the flowering time (Woodward and Morley 1974; Cocks and Phillips 1979; Ehrman and Cocks 1996; Bennett 1997). At a site where the growing season was short, flowering and seed set must occur early to ensure seed set before the beginning of dry summer. Where the season was longer, a greater vegetative period provided the maximum opportunity for dry matter accumulation and hence potential for the largest seed set. In the present study, wild radish from areas with high rainfall and low temperature flowered later than strains from low rainfall and high temperature areas.

The results of both approaches used here (AFLPs and life history traits) indicated that the amount of within population variation was much greater than between populations. Kercher and Conner (1996) studied isozymes in wild radish and found that the amount of within population variation was much greater than between populations. Man Kyu Huh and Ohmi Ohnishi (2002) studied in *Raphanus sativus var hortensis f raphanistroides* and found high variation within population and less variation between populations. It was likely that out-crossing species contained significantly more variation within and less between population than inbreeding species (Levin 1977). For example, an isozyme study of the outbreeding colonizer *Silene vulgaris* by Runyeon and Prentice (1996) found that only 2% of the genetic variation occurred between populations. In another study, Sun (1997) found that *Centaurea solstitialis* in North America exhibited only 1% of its variation between populations. Other outbreeders which exhibit this arrangement of genetic diversity included weedy rye (Sun and Corke 1992) and *Echium plantagineum* (Barrett 1982).

Variation in the genetic structure of plant populations was the result of a balance between gene flow, genetic drift and selection pressure (Hayward et al. 1997). Selection pressure was expressed in plant life history traits, which were of direct survival value to the plant (Marshall and Allard 1970; Lande 1977; Barrett 1982; Barrett 1988). This helped to balance the high gene flow between the outcrossing wild radish populations, reducing within population variation and increasing between population variations. However, amplified fragment length polymorphism (AFLPs) and other molecular markers were assumed to be selectively neutral or nearly so (Isabel et al. 1995; Nissen et al. 1995; Hartl and Seefelder 1998), so their distribution was determined largely by gene flow and drift. The high within population variation of wild radish populations was the result of high levels of gene flow, which also limited between population differentiations. There was little or no selection acting to balance this high gene flow.

Although, the assessment of a large and random fraction of the genome could be desirable in some circumstances (Westman and Kresovich 1997; Petersen and Seberg 1998), the random nature of the markers under examination was a distinct disadvantage in assessing the adaptation of a species to its environment. Unlike life history traits, the majority of DNA polymorphisms and probably isozymes were selectively neutral.

CONCLUSIONS

Wild radish (*Raphanus raphanistrum*) widely colonized in Western Australia, and considered to be a serious weed of cereals. Outcrossing and self-fertilization were common within the genus Brassica. Spontaneous hybridization between male-sterile canola and wild radish generated 3,734 hybrid seeds/m^2. High chromosome pairing was observed and the hybrid pollen fertility was measured at 0-30%. Further research on the spontaneous outcrossing and seed production between various male-sterile and transgenic lines of canola with wild radish led to the discovery that the seeds produced showed size dimorphism. Perhaps, more likely is the use of these traits in wild radish for wide crosses with closely related species such as canola or mustard.

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