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Genetic evidence supports reclassification of *Agrostis billardierei* var. *filifolia* and *A. aemula* R.Br. var. *setifolia* as a single species, *A. punicea* (Poaceae)

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Abstract

The genetic relationship between *Agrostis billardierei* R.Br. var. *filifolia* Vickery and *A. aemula* R.Br. var. *setifolia* Vickery was investigated using random amplification of polymorphic DNA. Three random primers generated 49 products of which only one was present in all samples. *Agrostis aemula* var. *setifolia* was found to be more similar to *A. billardierei* var. *filifolia* than to *A. aemula* var. *aemula* supporting reclassification of *A. billardierei* var. *filifolia* and *A. aemula* var. *setifolia* as a single species, *A. punicea* A.J.Brown. & N.G.Walsh.

Introduction

Many populations of native grass species exist as isolated remnants because the once extensive grasslands on the western basalt plains in Victoria have declined to less than 1% of their previous extent (Scarlett *et al*. 1992). Interest in the preservation and utilisation of these species in Australia has highlighted some problems in the delineation of some taxa. Before management strategies for maintaining biodiversity can be implemented, there is a need to improve our understanding of the relationships between taxa and the level of diversity within recognised taxa.

A number of native species of *Agrostis* R.Br. are found in lowland Victoria: *A. adamsonii* Vickery, *A. aemula* R.Br., *A. avenacea* J.F.Gmel., *A. billardierei* R.Br., *A. robusta* A.J.Brown & N.G.Walsh, *A. punicea* A.J.Brown & N.G.Walsh, *A. rudis* Roem. & Schult. and *A. venusta* Trin. *Agrostis punicea* is a recently described species based on morphological examination of former varieties of *A. aemula* and *A. billardierei* (Brown & Walsh 2000). Doubts had existed about the legitimacy of *A. billardierei* var. *filifolia* Vickery and *A. aemula* var. *setifolia* Vickery because they are superficially very similar and their separation is based on small morphological differences. When describing the two taxa, Vickery (1941) noted that they showed a strong resemblance with the major difference being the hairy lemma of *A. aemula* var. *setifolia*. Brown and Walsh (2000) found that, besides the difference in lemma hairiness, *A. billardierei* var. *filifolia* and *A. aemula* var. *setifolia* were separated morphometrically, on average, only by the slightly shorter inflorescences and slightly longer lemmas, lemma-setae and awns of the latter taxon.

Brown and Walsh (2000) also found that *A. billardierei* var. *filifolia* and *A. aemula* var. *setifolia* had similar ecological preferences. While populations commonly contain either one of these taxa, populations have been observed where both occur, suggesting that they may interbreed. In contrast, the typical varieties of each species are generally distinct in form and growth habit (Walsh 1994).

The distribution of the taxa is somewhat different (Brown & Walsh 2000). *Agrostis. aemula* var. *setifolia* and *A. billardierei* var. *filifolia* is confined to moist, generally open, lowland environments of southern Victoria, south-east South Australia and Tasmania. *Agrostis aemula* var. *aemula* is fairly common from coastal to subalpine environments

across Victoria, New South Wales, South Australia and Tasmania with isolated occurances in Western Australia and Queensland. *Agrostis billardierei* var. *billardierei* is widely distributed around the coastlines of southern Australia with a few inland occurrences.

This study was undertaken to see whether there was genetic support for the reclassification by Brown and Walsh (2000) of *A. billardierei* var. *filifolia* and *A. aemula* var. *setifolia* as a single species, *A. punicea*. The similarity between these taxa is investigated using RAPDs which are dominant markers thought to sample randomly across the genome (Stammers *et al*. 1995). RAPDs have been found to be useful markers for taxonomic studies at the species level or for species complexes where the number of morphological characters can be insufficient between taxa and other DNA-based methods do not detect sufficient variation (Gonzalez & Ferrer 1993; Kazan *et al*. 1993; Stammers *et al*. 1995).

Agrostis avenacea was included for comparison in this study because it is considered a distinct though variable, common and widespread species, found in all regions of Victoria, in all states except the Northern Territory, and in Polynesia (Walsh 1994). Also, it frequently occurs near *A. aemula* and *A. billardierei*. It is distinguished from *A. aemula* by a number of features used collectively, rather than a sharp discontinuity in features, suggesting differences in the genetic basis of many genes which characterise the species. An earlier study on genetic diversity in native *Agrostis* species found that *A. avenacea* was less similar to *A. billardierei* var. *filifolia* than to *A. billardierei* var. *robusta* Vickery or *A. adamsonii* (James & Brown 2000).

Methods

PLANT MATERIAL

Wild populations of the study species were sampled from sites in western Victoria (Fig. 1). Seed collections for the populations, 'HD': Hadden (*Agrostis billardierei* var. *filifolia*), 'EM': East Mortlake (*A. aemula* var. *aemula*), 'WM': West Mortlake and 'SHR': Ballyrogan (*A. avenacea*) were made between December and February 1996/97. *Agrostis avenacea* was represented by both large weeping and small upright forms (WM and SHR respectively). Currently, these forms are not formally recognised. Seed was collected from populations 'SBL': South Bulart and 'LR': Lake Repose, Glenthompson (*A. billardierei* var. *filifolia*) and 'DIG': Dartmoor (*A. aemula* var. *setifolia*) between December and February 1998/99. Seed was obtained from up to 10 individuals in each population, with collections maintained as individual seed-lots. Approximately 20 seeds per individual were placed in tapering tubes in a pinebark-based medium, covered in a thin layer of mix with particle size < 2mm and left to germinate in a glasshouse for up to 10 weeks. A single seedling per individual seed-lot was chosen at random and potted on to provide fresh leaf material for DNA extractions.

DNA ISOLATION

Genomic DNA was extracted from fresh leaf material using acetylmethylammonium bromide (CTAB) method modified from Rogers and Bendich (1985). DNA quality was assessed visually after electrophoresis on a 1.0% agarose gel containing ethidium bromide. To further assess DNA quality, 0.5 µg DNA from a subsample of plants was digested separately with restriction enzymes *Eco*R1 and *Dra*1 in 25 µl volumes in the appropriate buffers at 37°C overnight (c. 17 h) and OD_{260}/OD_{280} ratio was measured.

DNA AMPLIFICATION

Forty synthetic decamer primers (kits A and B) from Operon Technologies, Inc. (Alameda, Calif., U.S.A.) were tested in PCR reactions according to the protocol of Williams *et al*. (1990). Three primers (OPB-8, OPB-12, OPB-20) giving discrete, reproducible amplification products were chosen for further analysis. Reactions were performed in a volume of 25 µl containing 5–10 ng DNA, 1 U Taq polymerase (Gibco-BRL),

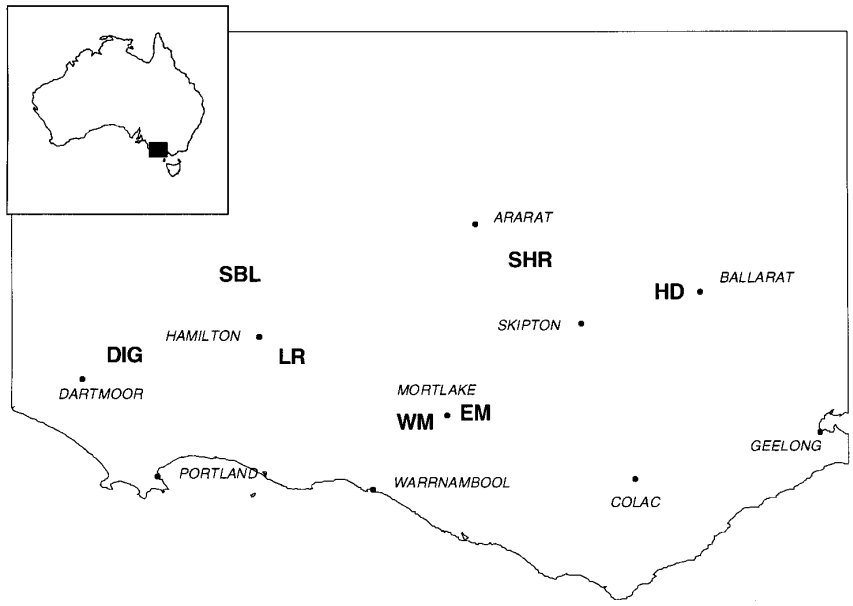

Figure 1. Distribution of *Agrostis* study sites in Victoria's Western District. *A. aemula* var. *aemula*: Wooriwyrite (EM); *A. aemula* var. *setifolia*: Digby (DIG); *A. avenacea*: Ballyrogan (SHR), Connewarren (WM); *A. billardierei* var. *filifolia*: Haddon (HD), Lake Repose (LR), South Bullart (SBL).

1 × Taq polymerase buffer (Gibco-BRL), 160 µM each dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$ and 15ng primer, using a Corbett Research FTS 960 thermocycler. For the PCR, initial strand separation and amplification was initiated with one cycle of 94°C for 5 min, 35°C for 2 min, 72°C for 1 min, followed by 40 cycles of 94°C for 30 sec, 38°C for 30 sec (except for primer OPB-08 where annealing temperature was 44°C), 72°C for 30 sec. and a final extension step of 72°C for 3 min. Amplified products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide and run in TAE buffer at 80-100 v for 2 – 4 h. Images were visualised and photographed using a Sci Tech CCD video camera module and saved to computer using UVIdoc image capture software.

DATA ANALYSIS

RAPD products were scored as either present (1) or absent (0) for each individual, based on the assessment of a minimum of two independent PCR runs. Fragments migrating at the same size were considered to be homologous.

Genstat 5 version 4.1 (PC/Windows NT) (Lawes Agricultural Trust Rothamstead) was used for data analysis. Chi-squared tests were performed to determine which band frequencies were different between populations. Two similarity matrices were calculated using simple-matching coefficient (SMC) (Gordon 1981). The first compared individuals and was used as the basis for ordination by principle co-ordinate analysis (Gower 1966). The second compared populations. A dendrogram (average-link) was constructed based on the second, reduced similarity matrix, to compare populations. Diversity indices were calculated using the Shannon-information index (Russell *et al*. 1993) and presented as a single index, averaged over all loci, to provide an average per locus diversity (Chakraborty & Rao 1991). The amount of variation partitioned within and among populations was calculated from the diversity indices (King & Schaal 1989).

Results

Genetic variation was detected by RAPD PCR. RAPDs generated reliable and reproducible polymorphic patterns that enabled an assessment of the relationship between the former species: *A. aemula* (including both var. *aemula* and var. *setifolia*), *A. billardierei* (only var. *filifolia* represented) and *A. avenacea*.

Scorable RAPD fragments ranging from 0.38 to 2.5 kb in size were amplified by three decamer primers (OPB-08, -12 and -20). They generated a total of 49 products with an average of 16 products per primer. A fourth primer, whilst successfully amplifying DNA for most samples did not reliably amplify DNA from population 'EM' and so was eliminated from the analyses. The three primers revealed polymorphisms among the three species examined and only one amplification product was present in all individuals regardless of species. Polymorphic products per population varied from 0% (SBL) to 81.3% (SHR), and per species varied from 54.5% (*A. billardierei*) to 96.4% (*A. aemula*) (Table 1). The high level of polymorphism in *A. aemula* was due to the small number of RAPD fragments (3) that occurred in both the 'DIG' and 'EM' populations.

Table 1. Number of RAPD bands (% polymorphic), range in no. band differences between RAPD phenotypes* for each population and each species. *(populations only)

Species		Popn	Within populations		Within species
			No. RAPD bands (% polymorphic)	Range in no. band differences between RAPD phenotypes	No. RAPD bands (% polymorphic)
A. billardierei	var. *filifolia*	HD	14 (14.3)	1–2	
	var. *filifolia*	SBL	15 (0.0)	0–0	22 (54.5)
	var. *filifolia*	LR	20 (45.0)	1–9	
A. avenacea	upright form	SHR	17 (82.3)	1–11	24 (87.5)
	weeping form	WM	19 (63.2)	1–6	
A. aemula	var. *aemula*	EM	18 (66.7)	1–10	28 (96.4)
	var. *setifolia*	DIG	13 (15.4)	1–2	

Similarity between individuals ranged from 41.5–100%. Seventy-nine percent of RAPD PCR products varied significantly ($P = 0.01$) in frequency between populations. The average similarity between species was 56.2% for *A. billardierei* vs *A. avenacea*, 66.7% for *A. billardierei* vs *A. aemula* and 70.5% for *A. avenacea* vs *A. aemula*. The number of phenotypes present in populations is listed for each population (Table 2).

Similarity between populations ranged from 50.0-94.3% (Table 3). Of note, is the low similarity (62.0%) between the populations DIG and EM formerly considered to be different varieties of *A. aemula*. Likewise, similarity between EM (*A. aemula* var. *aemula*) and *A. billardierei* var. *filifolia* is only 51.8%. In contrast, the similarity between DIG (*A. aemula* var. *setifolia*) and *A. billardierei* var. *filifolia* is 81.7%.

The relationship between populations is depicted in Fig 2. The population of *A. aemula* var. *setifolia* (DIG) clearly clusters with the populations of *A. billardierei* var. *filifolia* (HD, SBL and LR). Importantly, DIG shows a greater affinity with HD (84.0%) rather than EM (62.0%) despite HD and DIG being separated by almost twice the geographic distance separating DIG and EM (Fig. 1).

Table 2. Sample numbers, population size and genetic diversity indices for each population.

Variety		Population (popn size)	No. samples	No RAPD phenotypes	H_O
A. billardierei	var. *filifolia*	HD (100-250)	9	4	0.0097
	var. *filifolia*	SBL (500-1000)	7	1	0.0000
	var. *filifolia*	LR (>2500)	7	6	0.0487
A. avenacea	upright form	SHR (100-250)	10	9	0.0808
	weeping form	WM (50-100)	10	9	0.0539
A. aemula	var. *aemula*	EM (100-250)	10	10	0.0648
	var. *setifolia*	DIG (>2500)	10	4	0.0068

Table 3. Reduced similarity matrix based on RAPD data comparing all populations. * = no. plants sampled.

Species	*A. billardierei*			*A. avenacea*		*A. aemula*	
Popn	HD[9]*	SBL[7]	LR[7]	SHR[10]	WM[10]	EM[10]	DIG[10]
HD	–						
SBL	85.6	–					
LR	84.8	94.3	–				
SHR	59.5	56.2	56.3	–			
WM	58.0	54.8	52.4	85.7	–		
EM	52.6	52.7	50.0	78.0	78.2	–	
DIG	84.0	81.2	79.8	62.5	63.1	62	-

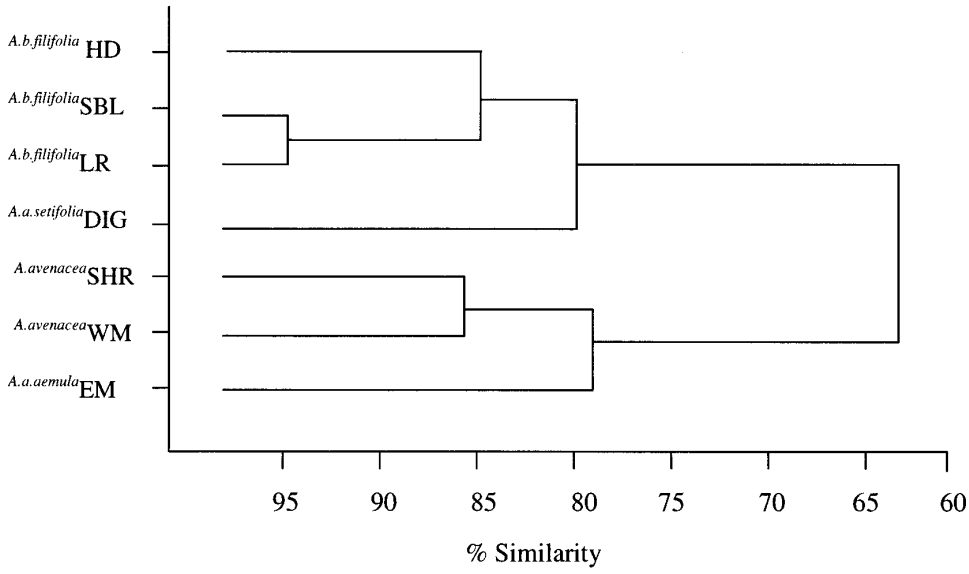

Figure 2. Cluster of populations based on reduced similarity matrix (simple matching coefficient).

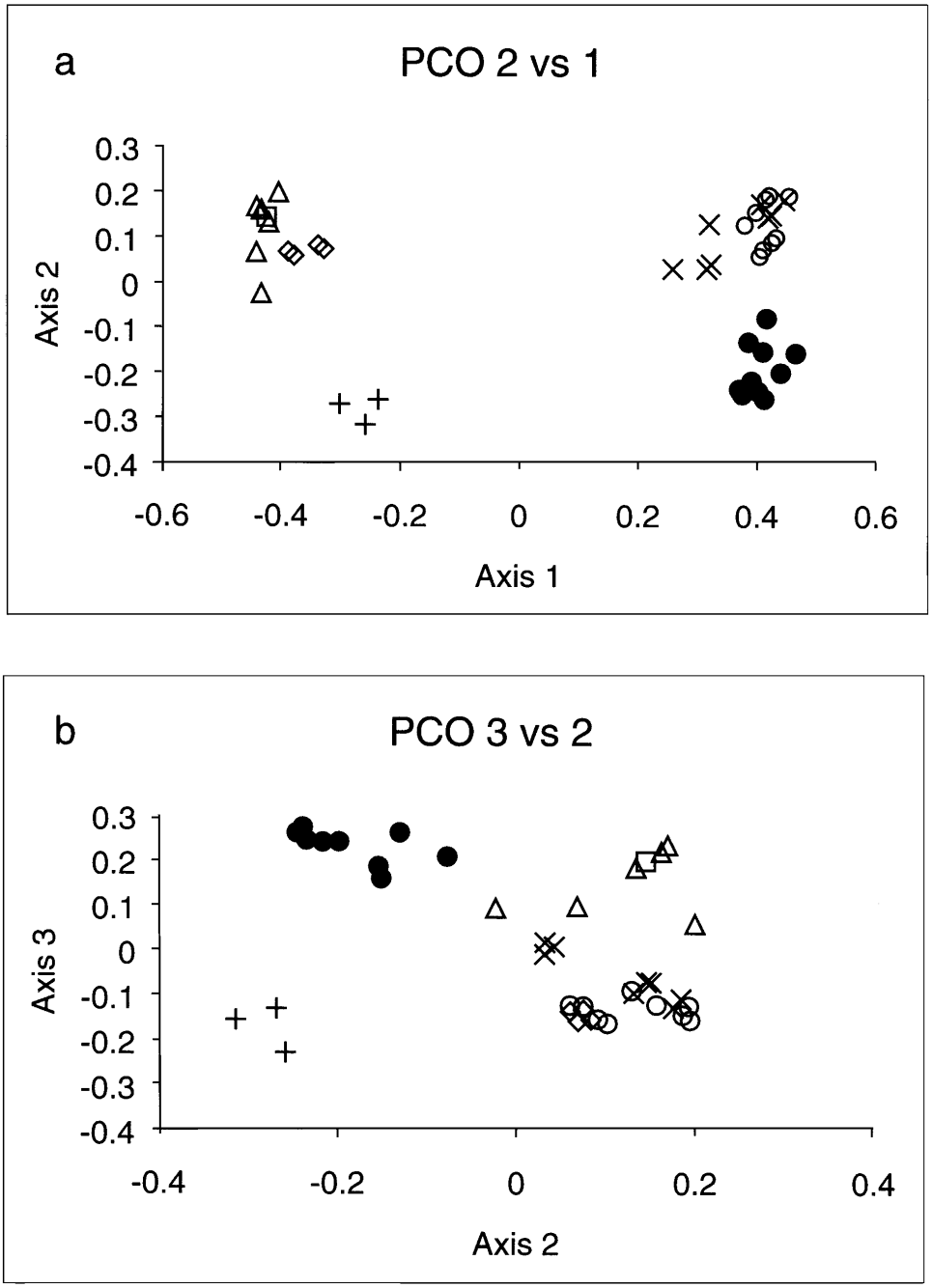

Figure 3. Principle co-ordinate analysis of RAPD phenotypes for all *Agrostis* individuals. Axes 1, 2 and 3 account for 73.8 % of variation. **a.** PCO vector 2 vs 1; **b.** PCO vector 3 vs 2. ◇HD, □SBL, △LR, ×SHR, ○WM, ●EM, +DIG. (Number of symbols does not equal number of individuals due to some individuals sharing phenotypes).

PRINCIPAL COORDINATE ANALYSIS OF INDIVIDUALS

The first two axes of the principal co-ordinate analysis accounted for 52.4% and 11.3% of total variation, respectively, with a further 9.9% accounted for by the third axis. Individuals within the populations of *A. billardierei* var. *filifolia* (HD, SBL and LR) group together and the populations form a discrete cluster (Fig 3a). Clustering for *A. avenacea* is similar, although some separation of the SHR population has occurred. However, for *A. aemula*, whilst the individuals within each variety cluster together (populations EM and DIG), the two varieties form two distinct and quite separate clusters in both principal co-ordinate plots (Figs 3a, b).

DIVERSITY INDICES

Estimates of individual population diversity (H_O) were derived from RAPD phenotypes (Table 2). The highest value of H_O=0.0808 was for population SHR of *A. avenacea*. No variation was detected in SBL and low levels were found in HD and DIG. While levels of diversity were variable depending on populations, they were not necessarily correlated with population size.

Average diversity for each species was calculated separately as ${}^{Abill}H_{SP}$=0.1526 for *A. billardierei*, ${}^{Aaven}H_{SP}$=0.2281 for *A. avenacea* and ${}^{Aaem}H_{SP}$=0.2722 for *A. aemula* (Table 4). The lower value for *A. billardierei* reflects the low population diversity values for populations HD and SBL. The comparatively high value for *A. aemula* results from the two populations sharing very few RAPD characters, whereas for *A. avenacea*, it results from high diversity within each population.

Average genus diversity (calculated for all populations combined) was ${}^{ALL}H_{GEN}$=0.2559. Average species diversity (calculated for all populations combined) was ${}^{ALL}H_{SP}$=0.2176.

H_{POP} provided measures of the average population diversity for each species, ranging from 0.0427 to 0.1540, and for the genus, 0.0378.

PARTITIONING OF VARIATION

Partitioning of variation within and among populations and species can provide an insight into their genetic structure. When variation is partitioned within and among populations (H_{POP}/H_{GEN}), using all seven populations in the analysis, only 14.8% of the observed variation is found within populations (Table 4). The proportion of diversity varies with species (Table 4). For *A. billardierei*, 28.0% of variation was present within populations whereas only 21.8% was present within populations of *A. aemula*. When indices are calculated for *A. billardierei* var. *filifolia* and *A. aemula* var. *setifolia* as a single species, the within-population variation decreases to 17.4%. For *A. avenacea*, 67.5% of variation was found within populations.

Table 4. Genetic diversity indices and partitioning of variation for species analysed separately and also combined.

Species	Diversity index	Partitioning of variation	
		Within popns	Among popns
Agrostis spp. combined	${}^{ALL}H_{POP}$=0.0378 ${}^{ALL}H_{GEN}$=0.2559	14.8%	85.2%
A. billardierei	${}^{Abill}H_{POP}$=0.0427 ${}^{Abill}H_{SP}$=0.1526	28.0% (17.4%)*	72.0% (82.6%)*
A. avenacea	${}^{Aaven}H_{POP}$=0.1540 ${}^{Aaven}H_{SP}$=0.2281	67.5%	32.5%
A. aemula	${}^{Aaem}H_{POP}$=0.0593 ${}^{Aaem}H_{SP}$=0.2722	21.8%	78.2%

*Partitioning of variation when population DIG is included with *A. billardierei*.

Discussion

Grass species have often been difficult to define because of minor differences in morphology, phenotypic plasticity in response to the environment, and frequently, a degree of intergradation between species. An advantage of using RAPDs is that they can be used to determine the affiliation of plants showing morphological characters with values common to more than one taxon. This approach has been used successfully for a number of grass species: *Agrostis adamsonii* (James & Brown 2000), *Hordeum* (Gonzalez & Ferrer 1993), *Lolium*/*Festuca* complex (Stammers *et al*. 1995; Pašakinskienė *et al*. 2000). The use of a molecular method in this study complements the set of characters used in the morphological analysis of Brown and Walsh (2000).

COMPARISON OF *AGROSTIS AEMULA* VAR. *SETIFOLIA* AND *A. BILLARDIEREI* VAR. *FILIFOLIA*

Agrostis aemula var. *setifolia* has been shown to be morphologically distinct from *A. aemula* var. *aemula* (Brown & Walsh 2000) and *A. billiardierei* var. *filifolia* has been shown to be both morphologically and genetically distinct from other varieties of *A. billiardierei* (Brown & Walsh 2000; James & Brown 2000; Ryan, unpublished data). This study shows a high degree of genetic similarity between *A. aemula* var. *setifolia* and *A. billardierei* var. *filifolia*.

Hierarchical clustering of populations (Fig. 2) shows three main clusters. The three populations of *A. billardierei* var. *filifolia* plus the population of *A. aemula* var. *setifolia* form a cluster that separates from *A. avenacea* and *A. aemula* var. *aemula* at a similarity of 62.0%.

In the principal co-ordinate analysis, *A. aemula* var. *setifolia* (DIG) individuals are clearly separated from populations of *A. billardierei* var. *filifolia*, although the most similar population is always HD. The clustering of the *A. billardierei* var. *filifolia* and *A. aemula* var. *setifolia* populations with 79.8% similarity is strong evidence for their reclassification as a single species or at least as individual species; both separate from *A. billardierei* and *A. aemula*. Cluster analysis using morphological characteristics found the same relative clustering pattern for *A. billardierei* var. *filifolia*, *A. aemula* var. *setifolia* and *A. aemula* var. *aemula* but at greater similarities (87% between the first two taxa and 79% between the first two and the third) (Brown & Walsh 2000).

The two populations of *A. avenacea* (WM and SHR) cluster at 85.7% similarity, and can be regarded as a single species, despite showing morphological variation and being separated by 70 km. *Agrostis avenacea* shows a similarity of 78.2% to *A. aemula* and indicates a closer similarity than would be normally accepted as delineating species. As there is no sharp morphological separation between *A. avenacea* and *A. aemula* (Walsh 1994), their taxonomic status requires further investigation, using a wider range of populations than is reported on here.

PARTITIONING OF VARIATION

The genetic structure of species is determined by the evolutionary and ecological history of individual species. The breeding systems of grasses influence the partitioning of variation, with selfing species having more genetic divergence (nearly half) among populations than mixed-mating and outcrossing species (Godt & Hamrick 1998). For outcrossing grass species, most diversity is found within populations. For example, in *Hordeum spontaneum*, which is known to have high levels of selfing, 43% of variation was found within populations (Dawson *et al*. 1993) whereas 72.9–80.5% within-population-variation was found within *Buchloe dactyloides*, a diploid out-crossing species (Huff *et al*. 1993).

Only 14.8% of observed variation in the combined *Agrostis* species genetic analysis is found within populations (Table 4). This is similar to the value (14.3%) found in another study of *Agrostis* species (James & Brown 2000) and is consistent with the low level of gene flow which is expected if the populations studied, comprise different species.

The proportion of variation partitioned within-populations for *A. billardierei* var. *filifolia* (28.0%) and *A. aemula* (21.8%) is much lower than the value of 73% found for other grass species (Godt & Hamrick 1998), the 78% reported for outcrossing species (Hamrick

et al. 1991) or the near 50% values reported for selfing species. The results for these *Agrostis* species could be due to self-fertility and the result of limited gene exchange among populations due to fragmentation of habitats, or some asexual reproduction. The low levels of variation in populations HD and DIG (Table 2) support the low levels commonly found in species with restricted ranges (Hamrick *et al*. 1991) but the complete absence of variation in SBL also indicates possible differences in reproductive strategies.

In *A. avenacea*, the variation within populations (67.5%) is similar to that found previously for *A. avenacea* (57.9%) using RAPDs (James & Brown 2000). Although the level of selfing in *A. avenacea* is not known, the variation within populations is lower but still comparable to an out-crossing species. *Agrostis avenacea* is a widespread, more common species than the others studied here and although some selfing may occur, its gene flow may not be as restricted.

POPULATION SIZE, GENETIC DIVERSITY AND BREEDING SYSTEM IMPLICATIONS

The mating system of plants plays an important role in determining the genetic structure and diversity of populations. Compared to other plants, grasses, in general, have higher levels of genetic diversity but there is more genetic differentiation among populations. For example, about 27% of total genetic variation is partitioned among grass populations compared with 22% for other plant species (Godt & Hamrick 1998).

Differences in the amount of variation in the populations of *A. billardierei* var. *filifolia* suggest that there may be variations in the breeding system within this taxon. It also suggests a flexible breeding system. It has been found that selfing species of grasses are genetically depauperate, relative to mixed mating and outcrossing species, both within populations and within species and in general, most are annuals whose population sizes often fluctuate (Godt & Hamrick 1998). It is possible that some of the *Agrostis* populations are ephemeral and sourced from larger, permanent populations where reproduction is mainly sexual, but apomixis occurs periodically. Population LR has a high level of diversity (H_O=0.0487) and could consist of plants which almost always reproduce sexually. On the other hand, populations HD (H_O=0.0097) and SBL (H_O=0.0) may have breeding systems with more emphasis on apomixis or selfing. SBL plants were grown from seed collected from different parents and the lack of variation in the sample suggests that seed may be apomictic in origin and that the population was founded by seed from a single parent plant. Similarly for DIG, there may be years where seed production is largely apomictic, leading to a cohort of genetically identical seedlings. Alternatively, the low diversity in DIG may be a result of repeated genetic bottlenecks if plant numbers fluctuate widely over time.

If apomictic populations of *Agrostis* species do occur, or if apomixis is a component of the breeding system, the genetic structure of populations may change rapidly. These results highlight the need for detailed breeding system studies on individual species or groups to understand their genetic structure.

Conclusions

Genetic variation detected with RAPD-PCR supports the conclusions resulting from a morphological study by Brown and Walsh (2000) placing *A. aemula* var. *setifolia* and *A. billardierei* var. *filifolia* in a new species, *A. punicea*. Other taxonomic issues still exist in the lowland *Agrostis* species in south-eastern Australia and a critical appraisal of additional closely related taxa, including assessment of reproductive structures, ploidy levels and breeding systems is still required to resolve them.

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Note added in proof: Since completion and submission of the study reported in this paper, the genus *Lachnagrostis* has been recognised to occur in Australia (Jacobs, *Telopea* **9**, 439-448, 2001; Jacobs, *Telopea* **9**, 837-838, 2002). The current names for taxa referred to in the text, with synonyms in brackets, are *L. aemula* (R.Br.) Trin. (syn. *Agrostis aemula*), *L. billardierei* (R.Br.) Trin. (syn. *A. billardierei*), *L. filiformis* (Forst.) Trin. (syn. *A. avenacea*), *L. scabra* (Beauv.) Nees. ex. Steudel (syn. *A. rudis*), *L. adamsonii* (Vickery) S.W.L.Jacobs (syn. *A. adamsonii*), *L. robusta* (Vickery) S.W.L.Jacobs (syn. *A. billardierei* var. *robusta*), *L. punicea* ssp. *punicea* (A.J.Brown & N.G.Walsh) S.W.L.Jacobs (syn. *A. punicea* var. *punicea*, *A. aemula* var. *setifolia*) and *L. punicea* ssp. *filifolia* (Vickery) S.W.L.Jacobs (syn. *A. punicea* var. *filifolia*, A. *billardierei* var. *filifolia*).