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An investigation of genetic variation in *Banksia integrifolia* (Proteaceae) by the use of the AFLP technique

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Abstract. The *Banksia integrifolia* L.f. species complex has undergone several taxonomic treatments over the past 20 years. In order to gain further insight into phenetic relationships between the taxa of this species, the distribution of genetic variation over the geographic range of *B. integrifolia* was investigated by the amplified fragment length polymorphism (AFLP) technique. Ordination and classification analyses resulted in clusters of individuals that closely conformed to the different taxa of this species. Further ordination and classification analyses of individuals of just *B. integrifolia* subsp. *integrifolia* indicate that there are also geographical patterns within the subspecies, with those individuals from the north of the species' range clustering away from those in the south of the range. A Mantel-test between morphological data collected in a previous study and molecular data collected in this study indicated a highly significant correlation between morphological and genetic variation. This study thus supports the current taxonomic classification of the *B. integrifolia* species complex.

Introduction

Banksia integrifolia L.f., the coast banksia, is one of the largest and most common banksias on Australia's east coast, occurring from eastern Port Phillip Bay in Victoria to north of Mackay in Queensland. Over this range, *B. integrifolia* occupies a wide variety of habitats, from coastal dunes and heathlands at sea level, to the fringes of montane rainforest and cloudforest on tablelands and peaks of the Great Dividing Range (Thiele and Ladiges 1994). With the possible exception of *B. spinulosa*, this is a broader latitudinal, elevational and ecological amplitude than that of any other species in the genus (Thiele and Ladiges 1994).

Taxonomic history of *Banksia integrifolia*

The *B. integrifolia* species complex has undergone several taxonomic treatments over the past 20 years. George (1981) split the complex and described the following three new species: *B. saxicola* A.S.George from Victoria, *B. conferta* A.S.George from New South Wales and Queensland and *B. plagiocarpa* A.S.George from northern Queensland. *Banksia integrifolia sensu stricto* was characterised by an arborescent habit, dentate seedling and juvenile leaves, entire adult leaves, pale yellow, cylindrical inflorescences and small follicles that open spontaneously.

George (1981) also recognised three varieties of *B. integrifolia sensu stricto*, mainly on the basis of differences in adult leaf shape. *Banksia integrifolia* var. *aquilonia* A.S.George has long, narrow, acute leaves that are spirally arranged and follicles that are typically slightly larger than those of the other varieties. Uniquely in *Banksia*, this variety has a fringe of short stiff hairs each side of the midrib on the leaf undersurface. It occurs in northern Queensland between Paluma and Mount Finnigan and is geographically disjunct, by over 200 km, from the other varieties (Fig. 1*a*).

Banksia integrifolia var. *integrifolia* has short, usually obtuse, dull-green leaves and is characteristically found within 2 km of the coast in south-eastern Australia, south of Fraser Island in Queensland (Fig. 1*a*). Both this variety and var. *aquilonia* are morphologically relatively uniform (George 1981).

George (1981) considered *B. integrifolia* var. *compar* to be morphologically and ecologically more variable than the other two varieties. It has a scattered distribution in southern Queensland and north-eastern New South Wales. The type specimen has large, glossy undulate leaves with a relatively obtuse tip and is typical of plants from the coastal lowlands of southern Queensland between Rainbow Beach and Proserpine (Fig. 1*b*; Thiele and Ladiges 1994). In contrast, plants from high elevations on peaks and tablelands of the Great Dividing Range between Lamington Plateau in south-eastern Queensland and Mount Wilson in New South Wales have narrow acute leaves without undulate margins (Fig. 1*b*; Thiele and Ladiges 1994). George (1981) noted that these

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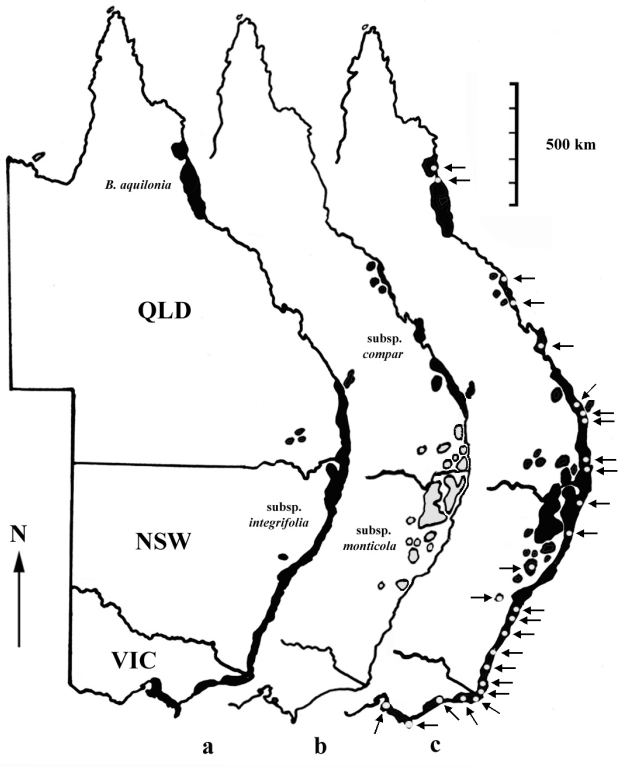

Fig. 1. The east coast of Australia, showing the distribution of *Banksia aquilonia* and the three subspecies of *B. integrifolia* [map on the basis of Thiele (1993)]. (*a*) *Banksia aquilonia* and *B. integrifolia* subsp. *integrifolia*. (*b*) *Banksia integrifolia* subsp. *compar* (black) and *B. integrifolia* subsp. *monticola* (grey). (*c*) Distribution of all four taxa and localities of collections (white circles indicated by arrows) used in the molecular analysis.

montane populations are distinctive, but because they could not adequately be distinguished from typical *B. integrifolia* var. *compar* they were provisionally included in that variety. Thiele and Ladiges (1994) analysed distribution and altitude data for *B. integrifolia sensu stricto* on the basis of the considerable information collected on this species (1736 sight records) between 1984 and 1986 as part of the Banksia Atlas Project (Taylor and Hopper 1988). Records of the three varieties suggest a broad sympatry between *B. integrifolia* var. *integrifolia* and *B. integrifolia* var. *compar*. However, Thiele and Ladiges (1994) demonstrated that there is a disjunction between montane (above 650-m altitude) and coastal (below 500-m altitude) populations of *B. integrifolia sensu stricto*. By the use of ordination analysis of morphological characters from both adults (leaves and fruits) and seedlings (leaves; Fig. 2), these authors concluded that the montane populations of *B. integrifolia* var. *compar* comprise a separate taxon, which is phenetically closer to *B. integrifolia* var. *integrifolia* than it is to typical *B. integrifolia* var. *compar*. In addition, *B. integrifolia* var. *aquilonia* is phenetically quite distinct from the remaining taxa.

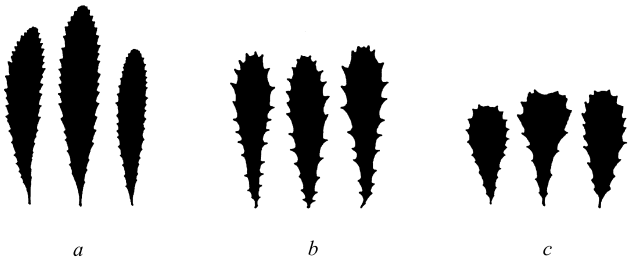

Fig. 2. Examples of seedling leaf outlines (×0.8; from Thiele 1993). Leaves of *B. integrifolia* subsp. *compar* (*a*) are elliptic, with shallow sinuses and straight-sided teeth; those of *B. integrifolia* subsp. *monticola* (*b*) are obovate, with relatively deep sinuses and curve-sided teeth; those of *B. integrifolia* subsp. *integrifolia* (*c*) are obovate, with shallow sinuses and curve-sided teeth.

On the basis of examination of herbarium material, Thiele and Ladiges (1994) suggested that intermediate populations occur, particularly between the montane taxon and the coastal var. *integrifolia*, indicating limited gene flow in some areas where the apparent elevational disjunction of the taxa breaks down. In addition, intermediates are evident where populations of *B. integrifolia* var. *integrifolia* and *B. integrifolia* var. *compar* are sympatric, for example at Rainbow Beach in Queensland. No intermediates between *B. integrifolia* var. *aquilonia* and the other taxa have been reported. Overall, the importance of hybridisation and introgression at the boundaries of taxa is unknown.

Thiele and Ladiges (1994) considered that an infraspecific rank was most appropriate for the four taxa because this would allow indeterminate material to be referred to a species name. As a result, they published the new names and combinations *B. integrifolia* subsp. *monticola* Thiele, *B. integrifolia* subsp. *aquilonia* (A.S.George) Thiele and *B. integrifolia* subsp. *compar* (R.Br.) Thiele. On the basis of the previously described distinctive characteristics, George (1996*a*) later raised *B. integrifolia* subsp. *aquilonia* to a specific rank, *Banksia aquilonia* (A.S.George) A.S.George.

The aim of this paper was to use a molecular method, the AFLP technique (Vos *et al.* 1995), to examine the distribution of genetic variation over the geographic range of *B. integrifolia*. More specifically, the study aims to determine whether the AFLP technique yields further information about the phenetic relationships within *B. integrifolia* and whether it lends support to the current taxonomic status of this species. Hereafter, the three subspecies of *B. integrifolia* will generally be referred to as *B.* subsp. *integrifolia*, *B.* subsp. *monticola* and *B.* subsp. *compar*.

Materials and methods

Seed material

In 1987 and 1988, Thiele, for his analysis of the *B. integrifolia* species complex, collected seeds from 59 populations of all *B. integrifolia* taxa

Table 1. Primer combinations used for selective amplification

Primer combination	*Eco* + 3-primer (5′–3′)	*Mse* + 3-primer (5′–3′)
1	GACTGCGTACCAATTC/ACC	GATGAGTCCTGAGTAA/CTA
2	GACTGCGTACCAATTC/ACC	GATGAGTCCTGAGTAA/CTT
3	GACTGCGTACCAATTC/ACA	GATGAGTCCTGAGTAA/CTA
4	GACTGCGTACCAATTC/ACA	GATGAGTCCTGAGTAA/CTT

and forms then recognised. Populations were chosen so that they covered the ecological and geographic range of the species (Thiele 1993). Generally, three non-adjacent trees were sampled at each site to represent a population, but collections were also made from isolated trees where adequate material was scarce. Even though *B. aquilonia* is no longer considered part of the *B. integrifolia* species complex (George 1996*a*), it was included in this analysis of genetic variation.

Germination of seeds

A subset of seeds collected by Thiele (1993) was chosen to represent as many populations as possible (Fig. 1*c*). Seed from each mother tree at most collection sites had been mixed so it was not possible to determine whether all the seeds collected from any one site were from the same tree or from different trees and this may affect the degree of genetic variation detectable between populations. The seeds were germinated in petri dishes on filter paper moistened with water. Each petri dish represented a separate site, with a maximum of 20 seeds per dish. Petri dishes were placed in a growth cabinet at 20–22°C with a light (16 h) and dark (8 h) cycle, although this was not expected to affect germination. After 6 weeks, healthy seedlings were transferred to pots to be grown in a glasshouse. Leaves were harvested fresh for DNA extraction about 6–8 months after germination.

DNA extraction

Genomic DNA was isolated from 0.5–1.0 g of leaf tissue by the CTAB method described by Doyle and Doyle (1990), with the modifications for seedling leaves suggested by Maguire *et al.* (1994). DNA samples were stored at –20°C in Tris–EDTA buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA].

AFLP procedure

Approximately 250 ng of DNA were used for AFLP analysis, which was performed with the AFLP Analysis System I Kit and the protocol recommended by the manufacturer (Life Technologies). Briefly, genomic DNA was digested to completion with *Eco*RI and *Mse*I and ligated to kit-supplied *Eco*RI and *Mse*I adapters. Two rounds of polymerase chain reaction (PCR) were then performed. Non-selective pre-amplification used primers provided by the manufacturer. During selective amplification, only some of the available restriction fragments are amplified. Selectivity was achieved by the use of primers that annealed to either the *Eco*RI or *Mse*I end of a restriction fragment and have three additional 3′ nucleotides. These primers are referred to as *Eco* + 3 and *Mse* + 3, respectively. The sequences of the *Eco* + 3 primers were 5′-GACTGCGTACCAATTCNNN-3′ (where NNN is either ACC or ACA). The sequences of the *Mse* + 3 primers were 5′-GATGAGTCCTGAGTAANNN-3′ (where NNN is either CTA or CTT). Table 1 lists the four primer combinations used in this study. The *Eco* + 3 primers were end-labelled using [γ^{33}P]-ATP (Amersham) and T4 polynucleotide kinase (Promega).

PCR products were mixed with 10 μL of sequencing loading buffer [98% (v/v) formamide, 10 mM EDTA, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue]. Mixtures were heated for 3 min at 90°C and then quickly cooled on ice. Four microlitres of each sample were electrophoresed in a 5.5% denaturing polyacrylamide sequencing gel at 65 W for about 2 h. After electrophoresis, gels were dried on filter paper and exposed to X-ray film (Kodak or Fuji) for 1–2 days at room temperature.

Classification and ordination

Bands on the autoradiographs were scored as present (1) or absent (0) for each individual plant and the resultant data matrices analysed with the multivariate analysis programme NTSYS-pc (Rohlf 1998). Similarity matrices were constructed by the Jaccard coefficient (Sneath and Sokal 1973). The Jaccard coefficient is defined as the number of (1, 1) matches, i.e. the number of bands common to both individuals, divided by the total number of comparisons, excluding (0, 0) matches (Clifford and Williams 1976). The Jaccard coefficient was chosen because the AFLP technique does not yield information about the number of alleles at a single locus. Therefore, it cannot be assumed that the absence of a band indicates similarity between two individuals at an individual locus.

The two different pattern analysis techniques used were hierarchical classification and ordination. The fusion strategy used for cluster analysis was the unweighted pair group method of arithmetic averages (UPGMA). The accuracy of fit of data to the dendrogram was assessed by calculating the correlation between the phenetic matrix (derived from the dendrogram) and the original similarity matrix. The ordination method used was non-metric multidimensional scaling (NMDS). NMDS is an iterative process by a steepest descent algorithm to minimise stress, the deviation between the final similarity matrix and the original one (Kruskal 1964).

Morphological versus *molecular characters*

Thiele (1993), for his analysis of the *B. integrifolia* species complex, measured the following 18 morphological characters: six adult leaf characters; four adult fruit characters and eight seedling leaf characters. To assess the degree of correlation between morphological and molecular characters, a Mantel-test (Sokal 1979) was calculated between Euclidean distance matrices of the morphological characters from Thiele (1993) and the AFLP molecular markers from this study. An average was calculated for each morphological trait and marker frequency per population. *P*-values indicate the probability of obtaining a more extreme Mantel correlation coefficient by chance alone and significance testing was on the basis of 10000 permutations.

Results

Germination success

Germination of seeds per petri dish was variable, ranging from 0 to 100%, but overall was approximately 60%. DNA was isolated and AFLPs performed on 67 individuals from 26 populations (Table 2). The populations ranged from Cape Schank in southern Victoria to Tully in northern Queensland (Fig. 1*c*).

The populations included 41 individuals of *B. integrifolia* subsp. *integrifolia*, nine of *B. integrifolia* subsp. *monticola*,

Table 2. Populations represented and the number of individuals from each in the AFLP analysis of *Banksia integrifolia*

Populations are ordered from south to north within each taxon. The codes are the same as those used by Thiele (1993)

Taxon	Location	Thiele's code	No. individuals
B. subsp. *integrifolia*	Cape Schank, Vic. (38°29′S, 144°54′E)	KRT1912 & 1913	3
	Lakes Entrance, Vic. (37°52′S, 151°09′E)	KRT1864	2
	East Cape Conran, Vic. (37°49′S, 148°44′E)	KRT1909 & 1910	3
	Mallacoota, Vic. (37°34′S, 149°45′E)	KRT1908	3
	Green Cape, NSW (37°16′S, 150°02′E)	KRT1899	3
	Eden, NSW (37°04′S, 149°54′E)	KRT1896	3
	Wallaga Lake, NSW (36°21′S, 150°04′E)	KRT1893	3
	Broulee Beach, NSW (35°51′S, 150°10′E)	KRT1890	3
	Lake Burrill, NSW (35°23′S, 150°26′E)	KRT1886	3
	Otford, NSW (34°14′S, 150°59′E)	KRT1879	3
	Gerroa, NSW (34°14′S, 150°59′E)	KRT1882	3
	Hungry Head, NSW (30°17′S, 153°09′E)	KRT1813	3
	Brunswick Heads, NSW (28°33′S, 153°33′E)	KRT1802	2
	Bribie Island, Qld (27°04′S, 153°12′E)	KRT1801	3
	Peregian Beach, Qld (26°26′S, 153°06′E)	KRT1796	1
B. subsp. *monticola*	Blue Mountains NP, NSW (33°30′S, 50°24′E)	KRT1871 & 1872	3
	Ebor, NSW (30°30′S, 152°10′E)	KRT1705 & 1706	3
	New England NP, NSW (30°29′S, 152°24′E)	KRT1711	3
B. subsp. *compar*	Rainbow Beach, Qld (25°55′S, 153°06′E)	KRT1789	2
	Coonarr Beach, Qld (24°59′S, 152°24′E)	KRT1777	2
	Miriam Vale, Qld (24°20′S, 151°34′E)	KRT1773	3
	Yeppoon, Qld (23°09′S, 150°44′E)	KRT1770	3
	Eungella, Qld (21°12′S, 148°31′E)	KRT1722 & 1724	3
	Conway NP, Qld (20°15′S, 148°46′E)	KRT1726	1
B. aquilonia	Mt Leach Range, Qld (18°28′S, 146°08′E)	KRT1734	1
	Tully, Qld (17°45′S, 146°01′E)	KRT1764	2
Total	26		67

Table 3. Total number of scorable bands, number of polymorphic bands and percentage of polymorphic bands per primer pair for the 67 *Banksia integrifolia* and *B. aquilonia* individuals

Primer combination	No. scorable bands	No. polymorphic bands	% Polymorphism
1	36	18	53
2	47	28	66
3	52	37	71
4	49	23	59
Total	184	106	74

14 of *B. integrifolia* subsp. *compar* and three of *B. aquilonia*. The discrepancy of frequencies between taxa was due to differential germination success and the original number of populations of each taxon sampled by Thiele (1993).

AFLP analysis

The set of 106 polymorphic markers selected was on the basis of our analysis of genetic variation in *B. integrifolia* at Wilson's Promontory National Park (K. M. Evans, E. Newbigin, P. Ades, unpubl. data). Table 3 shows the total number of scorable bands, the number of polymorphic bands scored and the percentage of polymorphic bands per primer pair for the three *B. integrifolia* taxa and *B. aquilonia*.

Classification and ordination

The codes in the cluster analyses and ordinations (Figs 3–6) indicate the taxon and geographical location of the population. The first letter represents the taxon as follows: I for *B. integrifolia* subsp. *integrifolia*; M for *B. integrifolia* subsp. *monticola*; C for *B. integrifolia* subsp. *compar*; and A for *B. aquilonia*. The second letter represents the state within Australia in which the population was located as follows: V for Victoria; N for New South Wales; and Q for Queensland. The four-number code is the collection number used by Thiele (1993; see Table 2). For example, sample MN1871 is from a *B. integrifolia* subsp. *monticola* plant from population 1871 in New South Wales.

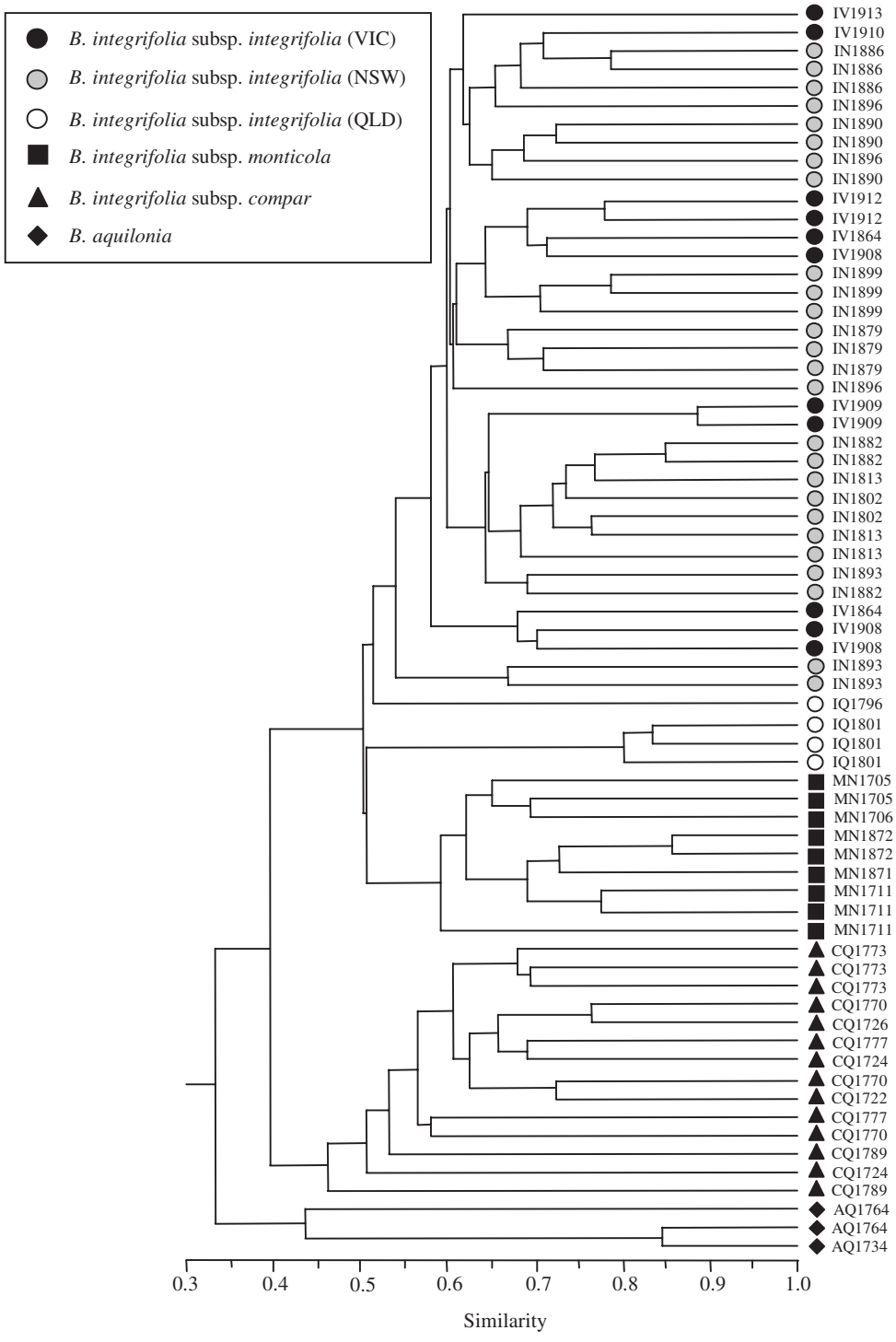

Fig. 3. UPGMA dendrogram of 67 *Banksia integrifolia* and *B. aquilonia* plants grown from seeds collected by Thiele (1993). The dendrogram was generated from a Jaccard similarity matrix of 106 polymorphic AFLP markers.

Classification and ordination analyses were performed on the following two groups of individuals: first, all 67 individuals that were grown from seed collected by Thiele (named KRT individuals); second, just the 41 individuals of *B.* subsp. *integrifolia* (named *B.* subsp. *integrifolia* individuals).

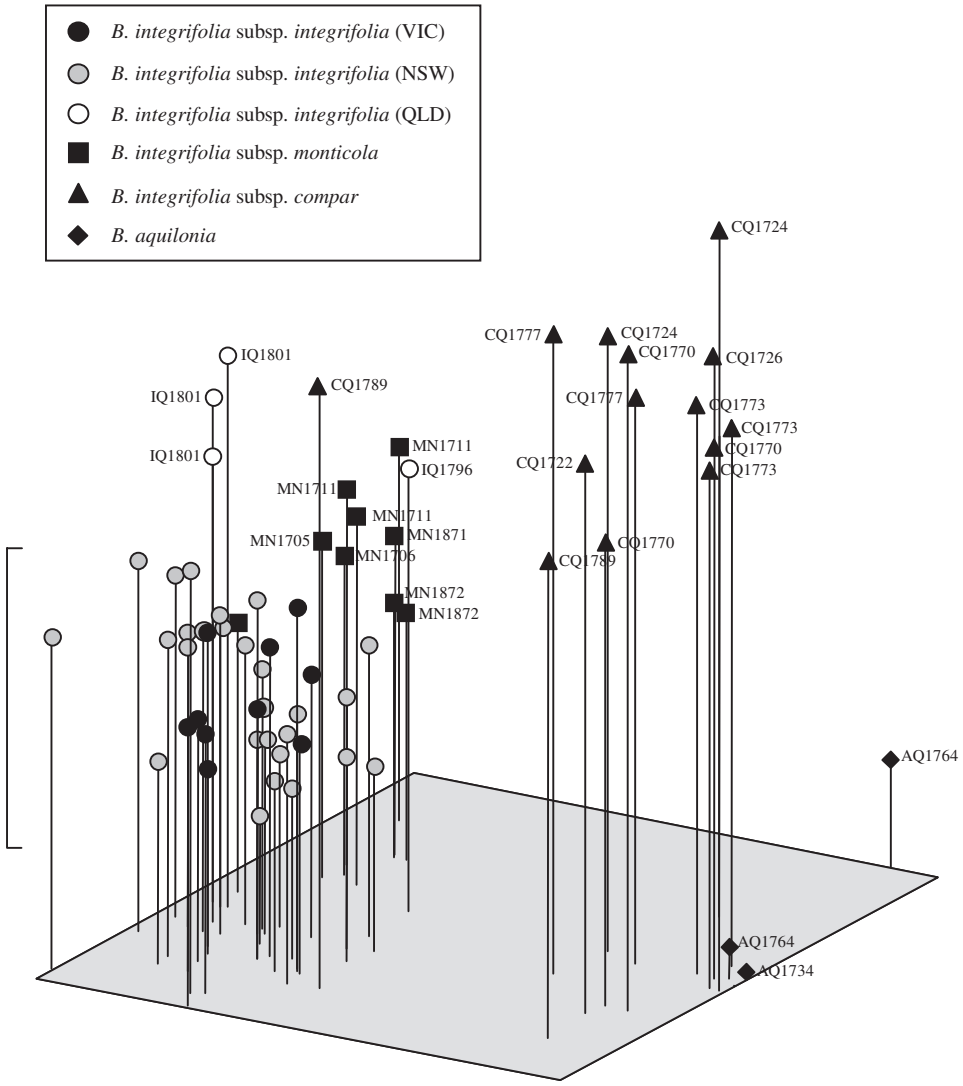

Fig. 4. NMDS in three dimensions of 67 *Banksia integrifolia* and *B. aquilonia* plants grown from seeds collected by Thiele (1993). The ordination was generated from a Jaccard similarity matrix of 106 polymorphic AFLP markers. Unlabelled individuals in the bracketed area include IV1912, IV1913, IV1864, IV1909, IV1910, IV1908, IN1899, IN1896, IN1893, IN1890, IN1886, IN1879, IN1882, IN1813, IN1802 and MN1705.

KRT individuals

Cluster analysis of the AFLP data for all 67 individuals of *B. integrifolia* and *B. aquilonia* by UPGMA resulted in a dendrogram that very closely conformed to the following four different taxa: *B. integrifolia* subsp. *integrifolia*, *B. integrifolia* subsp. *compar*, *B. integrifolia* subsp. *monticola* and *B. aquilonia* (Fig. 3). The co-phenetic correlation coefficient between the similarity matrix and the dendrogram was 0.903, indicating that the information presented in the dendrogram is a good representation of the original similarity data (Rohlf 1998). The only exceptions are the three *B.* subsp. *integrifolia* individuals from Bribie Island in Queensland (IQ1801), which cluster with individuals of *B.* subsp. *monticola*, although with a very short branch length. Individuals from the same population tend to cluster, although this is not always the case.

NMDS in two dimensions converged with a stress one value of 0.191 and so the ordination was re-run in three dimensions, the stress value reducing to 0.138. Figure 4 shows that eight of the nine *B.* subsp. *monticola* individuals cluster near the *B.* subsp. *integrifolia* individuals and that the remaining individual lies within the *B.* subsp. *integrifolia* cluster. The three plants of *B.* subsp. *integrifolia* from Bribie Island (IQ1801) form a group closer to *B.* subsp. *integrifolia* than *B.* subsp. *monticola*. The *B.* subsp. *integrifolia*

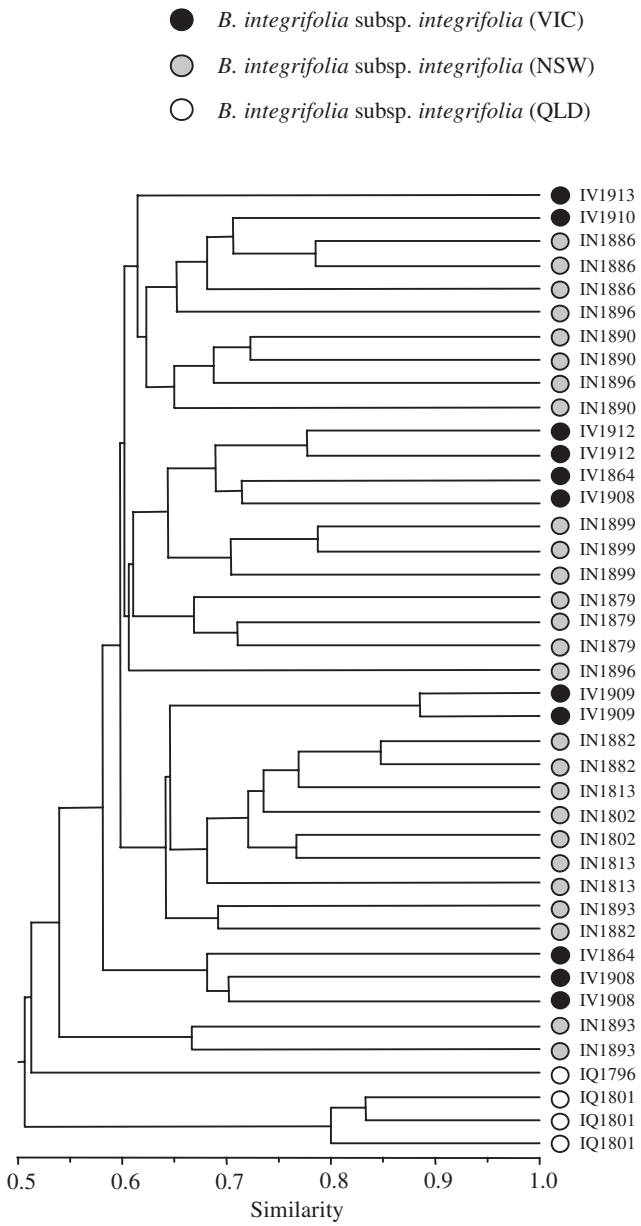

Fig. 5. UPGMA dendrogram of 41 *Banksia integrifolia* subsp. *integrifolia* plants grown from seeds collected by Thiele (1993). The dendrogram was generated from a Jaccard similarity matrix of 106 polymorphic AFLP markers.

individual from Peregian Beach in Queensland (IQ1796) and one of the *B*. subsp. *compar* individuals from Rainbow Beach (CQ1789) group with the individuals of *B*. subsp. *monticola* in the ordination space intermediate between *B*. subsp. *integrifolia* and *B*. subsp. *compar*. With that one exception, both *B*. subsp. *compar* and *B*. *aquilonia* cluster away from the other taxa.

Banksia integrifolia *subsp*. integrifolia *individuals*

In order to examine more closely the distribution of genetic variation between individuals of *B*. subsp. *integrifolia*, cluster analysis and ordination were carried out on the 41 *B*. subsp. *integrifolia* individuals alone. Cluster analysis resulted in a dendrogram that shows a distinct group of individuals from Bribie Island in Queensland (IQ1801; Fig. 5). The co-phenetic correlation coefficient was lower than for the previous dendrogram, $r = 0.771$.

NMDS in two dimensions for the 41 individuals of *B*. subsp. *integrifolia* converged with a stress one value of 0.269; ordination in three dimensions converged with a stress one value of 0.187. Figure 6 shows that the four individuals from Queensland (IQ1796 and IQ1801) cluster away from the other individuals of *B*. subsp. *integrifolia*. Although there is overlap between *B*. *integrifolia* individuals from different locations, those from further north (e.g. Brunswick Heads, IN1802; Hungry Head, IN1813; and Gerroa, IN1882) generally cluster towards the individuals from Queensland and those from southern New South Wales and Victoria (e.g. Lakes Entrance, IV1864; Mallacoota, IV1908; Green Cape, IN1899; and Eden IN1896) generally cluster together.

Morphological versus *molecular characters*

Only those populations for which both morphological (Thiele 1993) and molecular data were available could be used for this analysis. This meant that the three *B*. *aquilonia* individuals and the three individuals from population IN1896 could not be included. In total, 61 individuals from 23 populations and three taxa were represented. It is important to note that different plants were used in the molecular and morphological analyses. However, some of the morphological analyses were carried out on seedlings that may be siblings of the seedlings used in the molecular analysis.

The Mantel-test of the association between the Euclidean distance matrix of the morphological data and the Euclidean distance matrix of the molecular data was highly significant; $r = 0.6148$; $P = 0.0002$. This is consistent with the cluster analyses and ordinations that show distinct clusters of each *B*. *integrifolia* taxon.

Discussion

By the use of the AFLP technique to investigate the distribution of genetic variation over the geographic range of *B*. *integrifolia*, it was found that there was a highly significant correlation between morphological and genetic variation. This study therefore supports the morphological analyses of Thiele and Ladiges (1994) and the number of taxa recognised within the *B*. *integrifolia* species complex (George 1996*b*).

Thiele and Ladiges (1994) concluded that *B*. subsp. *monticola* is phenetically closer to *B*. subsp. *integrifolia* than to *B*. subsp. *compar*, a conclusion supported by the molecular data shown here. Eight of the nine individuals of *B*. subsp. *monticola* formed a cluster that was closer to the *B*. subsp. *integrifolia* cluster than to the *B*. subsp. *compar*

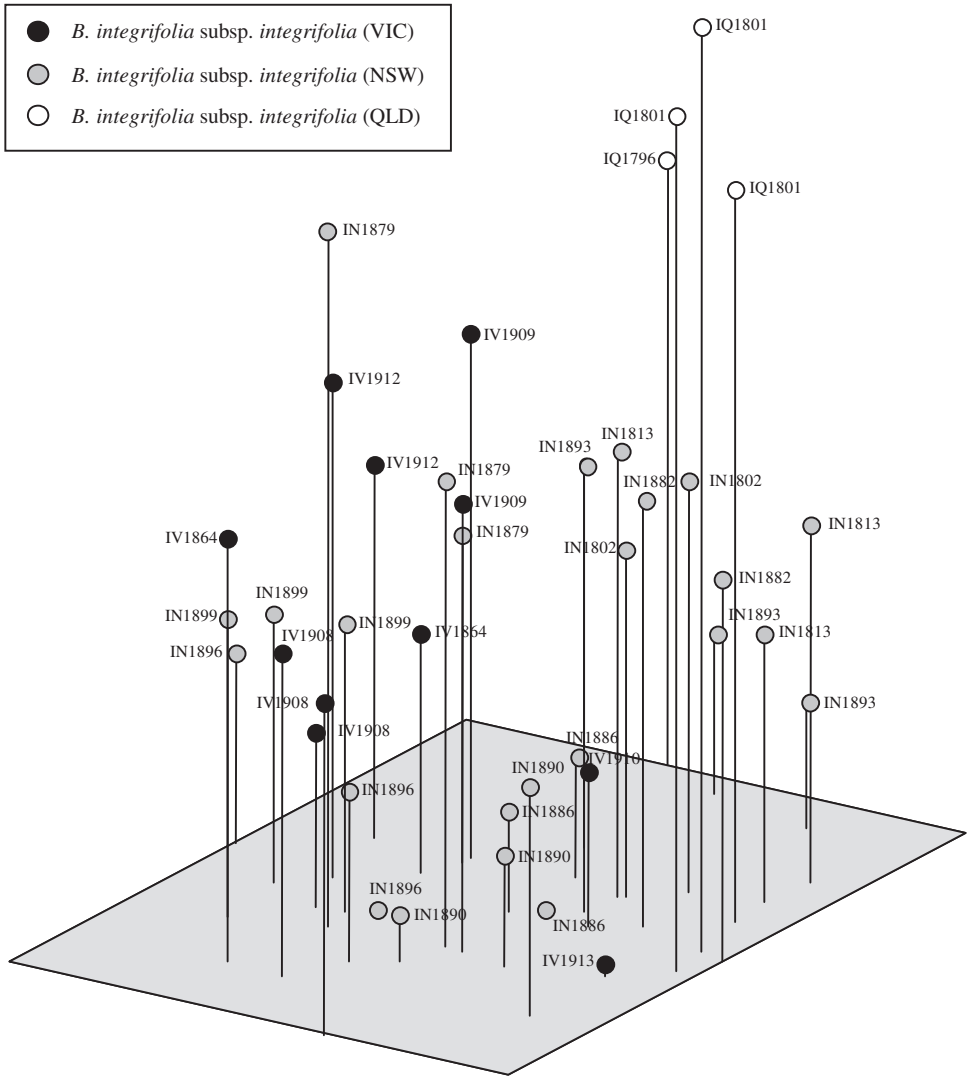

Fig. 6. NMDS in three dimensions of 41 *Banksia integrifolia* subsp. *integrifolia* plants grown from seeds collected by Thiele (1993). The ordination was generated from a Jaccard similarity matrix of 106 polymorphic AFLP markers.

cluster. One individual of *B*. subsp. *integrifolia* (IQ1796) and one individual of *B*. subsp. *compar* (CQ1789) also clustered with these eight individuals of *B*. subsp. *monticola*. There is the possibility that these two individuals are hybrids or intermediates of *B*. subsp. *monticola*. Indeed, Thiele and Ladiges (1994) noted that some populations studied for their morphological analysis from inland northern New South Wales and southern Queensland were intermediate, at least in adult morphology, between *B*. subsp. *monticola* and *B*. subsp. *integrifolia*. However, this hypothesis is unlikely for two reasons. First, the direct distance between the individuals of *B*. subsp. *monticola* in these analyses and the populations of IQ1796 and CQ1789 is over 500 km. In addition, these two seedlings do not display the dentate leaves with deep sinuses and sharp, spinose teeth that are characteristic of *B*. subsp. *monticola*.

Although IQ1796 and CQ1789 do cluster with individuals of *B*. subsp. *monticola*, this ordination space is also intermediate between *B*. subsp. *integrifolia* and *B*. subsp. *compar*. More plausibly, these individuals may in fact have little association with *B*. subsp. *monticola* and by their combination of characters share the ordination space where hybrids or intermediates between individuals of *B*. subsp. *integrifolia* and individuals of *B*. subsp. *compar* would be expected. The coastal location of these two populations close to the boundary between *B*. subsp. *integrifolia* and *B*. subsp. *compar* makes it likely that they are evidence of hybridisation and introgression between these

two taxa. Thiele and Ladiges (1994) reported that where populations of *B.* subsp. *integrifolia* and *B.* subsp. *compar* are sympatric, for example at Rainbow Beach in Queensland (CQ1789), morphological intermediates are evident.

With the one exception already discussed, *B.* subsp. *compar* formed a distinct cluster away from other individuals of *B.* subsp. *integrifolia*. Although only a small number of individuals of *B. aquilonia* were included in the analyses, they were genetically distinct from all other taxa. Also, there was no evidence of hybridisation between individuals of *B. aquilonia* and any of the *B. integrifolia* taxa. In order to investigate more fully the importance of hybridisation and introgression at the boundaries of taxa, more specific sampling and a larger sample size would be needed.

Also a geographical pattern from south to north is evident within *B.* subsp. *integrifolia*. Populations represented in the molecular analyses occur from Cape Schank in Victoria to Peregian Beach in Queensland, a direct distance of about 1500 km and considerably further along the coast. Those individuals from further south clustered away from those individuals from further north. For example, individuals from Mallacoota (IV1908), Green Cape (IN1899) and Eden (IN1896) clustered away from individuals from Hungry Head (IN1813), Brunswick Heads (IN1802) and Bribie Island (IQ1801). The four individuals from Queensland (IQ1796 and IQ1801) formed their own group away from most individuals of *B.* subsp. *integrifolia*. It is likely that if more populations from northern New South Wales and southern Queensland had been sampled, then individuals from these populations would have filled the ordination space between plants from Queensland and the bulk of the individuals of *B.* subsp. *integrifolia*. Most of the populations of *B.* subsp. *integrifolia* used in these analyses are located in Victoria and southern New South Wales, with only four of the 15 populations being from the northern half of New South Wales and southern Queensland.

For all taxa, individuals from the same population usually cluster together, although this is not always the case. This is probably because only a maximum of three individuals were included from each of the populations and so only a subset of the genetic variation present in the entire population is represented. As stated in the Materials and methods section, it is not known whether all the seeds were from the same mother tree or from different trees in the population and this could have affected the degree of genetic variation detected between populations.

Conclusion

This study has demonstrated the significant degree of congruency between morphological and molecular characters of subspecies of *B. integrifolia* and *B. aquilonia* and thus lends strong support to the current taxonomic classification of the *B. integrifolia* species complex. This study also provides some evidence of hybridisation or introgression occurring between the different taxa of *B. integrifolia*.

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