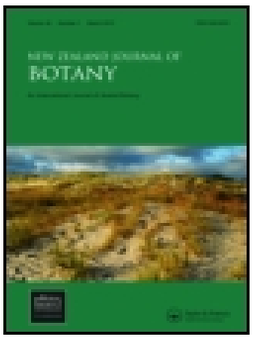

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Genetic and morphological evidence for localised interspecific gene flow in *Phormium* (Hemerocallidaceae)

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Abstract Gene flow between two species of *Phormium*, *P. tenax* and *P. cookianum*, was investigated by analysis of morphology, AFLP profiles, and chloroplast simple sequence repeats (cpSSRs). We examined 44 wild samples of *Phormium*, focusing on Okiwi Bay (Marlborough, New Zealand), where the two species grow in close proximity. Two genetic groups corresponding with *P. tenax* and *P. cookianum* are evident in analyses of AFLP profiles from Okiwi Bay. The profiles of Okiwi Bay plants were also compared with profiles from three hypothetically pure *P. cookianum* populations from Marlborough, and a representative sample of *P. tenax* combined from sites throughout New Zealand. Bayesian analysis of genetic admixture suggests that all of the plants resembling *P. tenax* and most of the plants resembling *P. cookianum* sampled from Okiwi Bay are admixed (i.e., of hybrid origin). Neighbour Net and principal component analyses reveal that *P. cookianum* and *P. tenax* from Okiwi Bay are genetically intermediate between "pure" populations of the species. Further, all of the Okiwi Bay samples share the same chloroplast haplotype. Analysis of morphological characters comparing a small number of Okiwi Bay *P. tenax* plants to selected *P. tenax* plants from throughout New Zealand under common garden conditions confirm that the Okiwi Bay population includes plants with unusually small stature and fruit characters approaching *P. cookianum*. These results support a hypothesis of local introgression between *P. tenax* and *P. cookianum*.

Keywords *Phormium tenax*; *Phormium cookianum*; AFLP; hybridisation; introgression; morphology; New Zealand flora; chloroplast SSR

INTRODUCTION

Plants of the genus *Phormium* (comprising *P. cookianum* and *P. tenax*) are a common and important feature of natural and garden vegetation of New Zealand, and *P. tenax* is both culturally and economically significant (Wehi & Clarkson 2007). Both species are morphologically variable in New Zealand, and some of this variation has been attributed to introgression between them (Wardle 1979). A recent study of *Phormium* used AFLP data to evaluate the current taxonomy and investigate biogeographic patterns in New Zealand (Smissen & Heenan 2007) in order to inform a taxonomic revision of the genus. Although AFLP profiles indicated that *P. cookianum* and *P. tenax* are genetically distinct across their overlapping ranges, a number of wild-collected plants showed evidence of hybridisation between them. These putatively hybrid plants included some from coastal Marlborough and Nelson (referred to as *P.* "Coast") that had fruit morphology intermediate between *P. cookianum* and *P. tenax* (Smissen & Heenan 2007, fig. 1). Plants belonging to a population of *P. tenax* from Okiwi Bay, Marlborough (42°13′S, 173°52′E), lacked some AFLP fragments typical of *P. tenax* and had a number of fragments more typical of *P. cookianum*. Although these plants from Okiwi Bay have the appearance of *P. tenax*, they are atypical in that they are generally smaller and more compact. Plants with this morphology have been observed as far south as Paparoa Point c. 2 km south-west of Okiwi Bay. Smissen & Heenan (2007) suggested that the Okiwi Bay *P. tenax* may have been subject to localised introgression from *P. cookianum* or *P.* "Coast" that are also found in the area. As alternative

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hypotheses, they suggested the Okiwi Bay population of *P. tenax* might represent part of a hybrid species with *P. tenax* and *P. cookianum* as parents, or that the presence of AFLP markers more typical of *P. cookianum* in the Okiwi Bay population of *P. tenax* might represent retained ancestral polymorphism. The presence of natural hybrids in wild populations of *Phormium* prompted Smissen & Heenan (2007) to conclude that a theme for future research is the role of hybridisation in the evolution of the genetic, morphological, and geographic patterns evident in the genus.

In this paper we present analyses of additional data from AFLP and from two highly polymorphic chloroplast simple sequence repeats for the Okiwi Bay population of *P. tenax*, the Okiwi Bay *P. cookianum*, three populations of *P. cookianum* from Marlborough, and selected other *P. tenax* accessions, as a test of the hypothesis that the population is introgressed with genes from the proximate *P. cookianum* population. If this hypothesis is correct, we expect to find a greater similarity in genetic markers between the Okiwi Bay *P. tenax* and proximate *P. cookianum* plants than between the Okiwi Bay *P. tenax* and more geographically distant populations of *P. cookianum*. We also present an analysis of morphological characters for the Okiwi Bay *P. tenax* and other *P. tenax* grown together in a common garden as a test of field observations that the Okiwi Bay population displays morphological characters consistent with introgression from *P. cookianum*.

MATERIALS AND METHODS

Plant material

Plants included in this study were *a priori* assigned to *P. tenax*, the putatively introgressed *P. tenax* population at Okiwi Bay (hereafter referred to as *P.* aff. *tenax*: OKT), and *P. cookianum*. A broad circumscription of *P. cookianum* is adopted here, and includes plants that are referable to *P. cookianum* from Limestone Stream (LS), Clarence River (CR), Okiwi Bay (OKC), and Woodside Creek (WC), Marlborough. For consistency and clarity throughout this paper, the population of *P. cookianum* from Okiwi Bay is referred to as *P. cookianum* Okiwi Bay. Plants from Woodside Creek were called *P.* "Coast" in Smissen & Heenan (2007) on the basis of growth habit and fruit morphological characters.

Phormium cookianum Okiwi Bay (OKC) and *P.* aff. *tenax* (OKT) were each sampled from natural populations at Okiwi Bay, Marlborough. In the area of coastal Marlborough to the north and south of Okiwi Bay, *P. tenax* is somewhat sporadic in occurrence, and numerically large wild populations are uncommon. Therefore, we selected plants of *P. tenax* of known wild origin, representing the geographic range of the species in New Zealand, from the research garden, Landcare Research (Table 1: T1–T7), to serve as a proxy population for comparison with the Okiwi Bay population. This sampling included two plants from a small population found by the authors c. 10 km north of Okiwi Bay: these plants

Table 1 Origin of *Phormium* samples included in AFLP analysis.

Species	Location	Field tissue collections	Landcare garden accessions
P. cookianum	Woodside Creek	WC1–WC6	
	Limestone Stream		LS1 (72/95A), LS2 (72/95B)
	Clarence River		CR1 (134/05B), CR2 (134/05C), CR3 (134/05D)
P. cookianum Okiwi Bay	Okiwi Bay	OKC1–OKC11	
P. tenax	Northland, Karikari Beach		T1 (19417A)
	Taranaki, New Plymouth, Paritutu Rock		T2 (9955)
	Westland, Ten Mile Creek		T3 (208/94C)
	Canterbury, Wakanui Beach		T4 (112/94A)
	Southland, Te Anau Downs		T5 (19428)
	Marlborough, Clifford Bay		T6 (28/95)
	Marlborough, north of Clarence River		T7 (145/05B)
P. aff. *tenax*		OKT1–OKT12	
P. aff. *tenax* Paparoa Point			PP1 (143/05B), PP2 (142/05)
Putative F1 hybrid		OKx	

might have originated from a historical Māori planting (J. Clayton-Green pers. comm.). Vouchers for accessions from the research garden are deposited in the Allan Herbarium (CHR).

When plants were collected from the Okiwi Bay site in March 2007, few were in flower or fruit. Therefore, individual sample vouchers were not taken and morphological observations of the taxonomically important inflorescence characters for the individual plants not made. Vouchers for the *P.* aff. *tenax* (CHR 505351, 505354, 505355, 505357, 505362, 505303) and *P. cookianum* Okiwi Bay (CHR 505353, 505356) were collected in November 2005 by two of the authors (PBH and RDS) and are deposited in the Allan Herbarium. Based on our 2005 characterisation of the Okiwi Bay site, *P. cookianum* is found growing north of the Mororimu Stream, on a railway cutting and rocky outcrop. *Phormium* aff. *tenax* is found on the south side of the Mororimu Stream on a rocky ridge and steep hillside, on a rocky platform immediately by the sea, and sporadically by the sea to the south-west as far as Paparoa Point (see below). Collections were specifically made from rock outcrops north and south of the Mororimu Stream where the two forms are at their closest. We also collected tissue from a plant (OKx) growing amongst the *P.* aff. *tenax* that had been observed in fruit the previous year and showed intermediacy in fruit characters suggesting it may be an F_1 or recent generation hybrid between the *P. cookianum* Okiwi Bay and *P.* aff. *tenax*.

Two plants from Paparoa Point (PP1 and PP2), c. 2 km south-west of Okiwi Bay, were also included in the study. One of these was identified in the field at the time of its collection as *P.* aff. *tenax*, while the other was identified as a possible hybrid between *P.* aff. *tenax* and typical *P. tenax*. Paparoa Point represents the southern extreme of the known range of *P.* aff. *tenax*.

DNA data and analyses

DNA extractions, digestions, PCR reactions, and gel electrophoresis for the generation of AFLP profiles (Vos et al. 1995) follow Smissen & Heenan (2007). Duplicate samples in repeated preliminary tests showed no variation in AFLP profiles, indicating that AFLP profiles were reproducible when conducted under the DNA extraction and reaction conditions used. The primer combinations used were: Eco+AGG and Mse+CTA; Eco+ACC and Mse+CAT; Eco+ACG and Mse+CTC; Eco+ACG and Mse+CAG; Eco+AGG and Mse+CAC. Polymorphic bands were scored as present (1) or absent (0) to produce a binary character matrix. The data matrix was subjected to Neighbour Net analysis using SplitsTree 4.0 (Huson & Bryant 2006). Principal components analysis was conducted using GenStat 6 (VSN International), and the data were subjected to Bayesian inference of population structure and admixture using the program Structure 2.2 (Pritchard et al. 2000; Falush et al. 2007). Run length was 100 000 after a "burn-in" of 50 000 replications using the admixture model. Other parameter settings were default except that an ancestry model with separate alpha inferred for each population and an allele frequency model with independent allele frequencies and lambda inferred separately for each population were selected. The bar plot of ancestry estimates from Structure and the Neighbour Net graph were redrawn using Freehand MX (Macromedia). Marker fragments presented in Table 3 were selected as those having the highest absolute value

Table 2 Collection and voucher information for *Phormium* samples used in the morphological analysis.

Species	Location	Vouchers
P. aff. *tenax*	Okiwi Bay, Marlborough	CHR 567919, 567911, 567876, 567868, 567869, 567870, 567872; 4 samples with no voucher
P. tenax	Three Kings Islands, North Auckland	CHR 567885, 567902
	Poor Knights Islands, North Auckland	CHR 567892, 567894; one sample with no voucher
	Karikari Peninsula, North Auckland	CHR 567897
	Kaipara, North Auckland	CHR 567901
	Ten Mile Creek, Westland	CHR 567924
	Chesterfield, Westland	CHR 567879, 567878, 567900
	Muritai, Marlborough	CHR 567873, 567874, 567917, 567875, 567826
	Wakanui, Canterbury	CHR 567832, 567850
	Port Hills, Canterbury	CHR 567830
	Chatham Island	CHR 567822, 567838, 567860

for their latent vectors for principal component 1 in the principal components analysis.

In addition to AFLP data, we surveyed size variation in two simple sequence repeats (SSRs) located in the *trn*L-F intergenic spacer and *trn*L intron. We used the tabc and tabf primers of Taberlet et al. (1991) along with two novel primers, tabcSSR (ttgagtctctgcacctatcc) and tabfSSR (aacctaagaaattcatgaac) to amplify DNA regions containing these SSRs. PCR conditions were an initial denaturation at 96°C for 120 s followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 60 s and then a final extension step at 72°C for 30 min. Amplification products were separated on a 6% PAGE sequencing gel, visualised by silver staining, and sized by comparison with previously sequenced samples.

Morphological characters

Seven morphological characters (five measurements of each character for each plant) were scored for 11 *P.* aff. *tenax* and 22 *P. tenax* from plants grown at Landcare Research, Lincoln (Table 2). The characters are: leaf length, leaf width, scape length, scape width immediately below the lowest lateral branch, fruit length, fruit width, and seed length. The data were summarised for each plant and analysed by principal components analysis using MVSP v.3.1 (Kovach 2005). Variation in each individual character for *P.* aff. *tenax* and *P. tenax* was expressed in terms of mean ± standard deviation (SD) and compared using an analysis of variance (ANOVA: Statistical Sciences 1998).

RESULTS

AFLP

A total of 186 fragments were scored, 154 of which were polymorphic, to produce a binary character matrix. Two samples of *P. tenax*, collected from a small number of plants north of the Clarence River, had identical AFLP profiles and were assumed to be ramets of the same genotype. Only one sample from this population (T7) was included in further analysis. A principal component analysis (Fig. 1) using this matrix generally separated *P. cookianum* and *P. tenax* along the first component axis (which accounted for 16% of the variation in the data matrix). However, samples from Okiwi Bay showed considerable variation in their principal component 1 scores, spanning the range from *P. cookianum* to *P. tenax*. One sample from Paparoa Point (PP2), which had similar

Table 3 Occurrence in samples of the most informative AFLP characters (see text for selection of these). *Phormium tenax* markers are shown as *, *P. cookianum* markers as +. N_C is the number of *P. cookianum* markers present in each sample, N_T is the number of *P. tenax* markers. Within taxa, samples are in order, top to bottom, of their N_C–N_T. T, *P. tenax* samples; PP, Paparoa Point *P.* aff. *tenax* or *P. tenax*; OKT, Okiwi Bay *P.* aff. *tenax*; OKC, Okiwi Bay *P. cookianum*; OKx, putative hybrid *P. cookianum* × *P.* aff. *tenax*; CR, Clarence River *P. cookianum*; WC, Woodside Creek *P. cookianum*; LS, Limestone Stream *P. cookianum*.

| Sample | Fragment presence or absence | | |

OKT3	*	*	*			*		*	*	+				*	*			+	*	+		*	*		+	+	+	*	*			12	6	6
OKT4	*	*		+		*	+	*	*	+					*		*	+	*	+		*			+	+	+		*	+	+	10	9	1
OKT5	*	*	*			*		*	*				+	*	*	*	*	+	*	+		*		*		+	+	+	*	*			15	5	10
OKT6	*	*	*	+	+	*		*	*					*	*			+		+					+	+	+	*				9	7	2
OKT7	*		*	+		*	+		*				+	*	*		*	+		+						+	+	*	*			9	7	2
OKT8	*	*	*			*		*	*	+					*	*	*	+		+			*		+	+			*			11	5	6
OKT9		*	*		+	*	+		*	+			+		*	*	*	+					*				+	*	*			10	6	4
OKT10	*	*	*		+	*		*	*	+					*	*	*	+	*	+		*	*			+	+	*	*			14	6	8
OKT11	*	*	*		+	*			*	+					*	*	*	+	*			*	*	*		+	+	*	*			14	5	9
OKT12		*	*				+				+			*	*					+			*	*	+			*				7	4	3
P. cookianum Okiwi Bay																																		
OKC1		*	*	+			+		*	+			+					+							+			*				4	6	−2
OKC2				+	+					+			+	*	*			+		+		*		*		+	+	*		+	+	5	8	−3
OKC3		*	*	+			+			+			+					+		+		*		*	+	+		*				4	8	−4
OKC4			*	+	+					+			+	*	*			+		+			*	+	+	+		*				4	9	−5
OKC5			*	+	+				*	+	+	+	+	*				+		+				+	+	+		*				4	11	−7
OKC6				+	+		+		*	+	+	+	+				*	+		+			*	+	+	+		*		+		4	13	−9
OKC7					+		+			+			+					+		+			*	+	+	+		*			+	2	9	−7
OKC8					+		+			+	+	+	+	*			*	+		+	+			+	+	+		*		+	+	3	13	−10
OKC9				+	+		+			+	+	+	+				*	+		+				+	+	+			*	+	+	2	13	−11
OKC10				+	+		+			+	+	+	+					+		+	+			+	+	+	*		+			1	14	−13
OKC11		*		+			+		*	+	+	+	+				*	+		+					+	+					+	3	10	−7
Putative F1 hybrid																																		
OK×		*		+	+			*		+	+		+	*		*	*			+	+			*	+	+	+	*			+	7	10	−3
P. cookianum																																		
WC1				+	+		+				+	+	+		*			+		+						+	+					1	10	−9
WC2				+	+		+			+	+	+	+					+		+	+					+					+	0	10	−10
WC3				+	+		+			+			+		*			+		+				+	+	+	*					2	10	−8
WC4				+	+		+			+			+		*			+		+	+				+							1	9	−8
WC5				+	+		+		*	+	+	+	+					+			+				+	+	*				+	2	10	−8
WC6				+	+		+			+	+	+	+		*			+						+	+	+	*					2	11	−9
LS1			*	+	+						+		+		*			+		+				+	+	+					+	2	9	−7
LS2				+	+				*	+	+		+		*			+		+				+	+	+						2	10	−8
CR1				+	+		+		*	+			+	*	*			+		+	+			+	+				+			3	11	−8
CR2				+	+		+			+	+	+	+					+		+	+					+	+	*		+		1	13	−12
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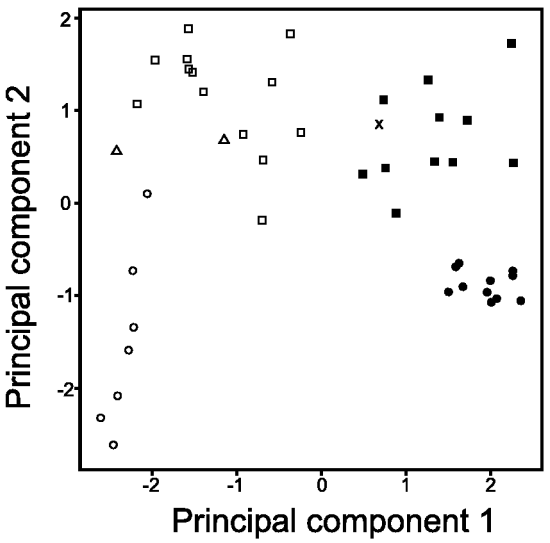


Fig. 1 Scatter plot of principal component scores for analysis of AFLP profiles. ○, *Phormium tenax*; □, *P.* aff. *tenax*; ●, *P. cookianum*; ■, *P. cookianum* Okiwi Bay; △, *Phormium* from Paparoa Point; ×, putative F1 hybrid between *P.* aff. *tenax* and *P. cookianum* Okiwi Bay.

morphology to *P.* aff. *tenax*, was grouped within the *P.* aff. *tenax* samples, supporting its field identification. The second principal component shown in the graph accounted for 8% of the variation in the data and weakly separated the Okiwi Bay samples from others. A third principal component accounting for 6% of the variation separated the Limestone Stream samples from the remainder (not shown). Table 3 records the distribution among samples of AFLP fragments with the greatest absolute value for their latent vectors for the first principal component. We divided these fragments into two classes: those most frequently found in samples *a priori* identified as *P. tenax*, and those most frequently found in samples *a priori* identified as *P. cookianum*. For each genotype, we counted the number of *P. tenax* markers and subtracted the number of *P. cookianum* markers to produce a crude hybrid index. Samples identified *a priori* as *P. tenax* had values for this index of 10 or 11, the *P.* aff. *tenax* samples had values ranging from 1 to 10, the samples identified *a priori* as *P. cookianum* ranged from −7 to −12, the *P. cookianum* Okiwi Bay genotypes ranged from −2 to −13, and the putative F_1 or recent generation hybrid −3. The sample from Paparoa Point identified as *P.* aff. *tenax* had a value of −4, while the sample tentatively identified as *P.* aff. *tenax* × *P. tenax* had a value of 11.

Neighbour Net analysis using SplitsTree 4.8 largely grouped individual *P. cookianum* genotypes into populations, and separated these from samples of *P. tenax* by numerous small splits (Fig. 2). Genotypes from Okiwi Bay were spread through the graph, with *P. cookianum* Okiwi Bay appearing closer to the other *P. cookianum* genotypes and *P.* aff. *tenax* generally closer to the *P. tenax* genotypes.

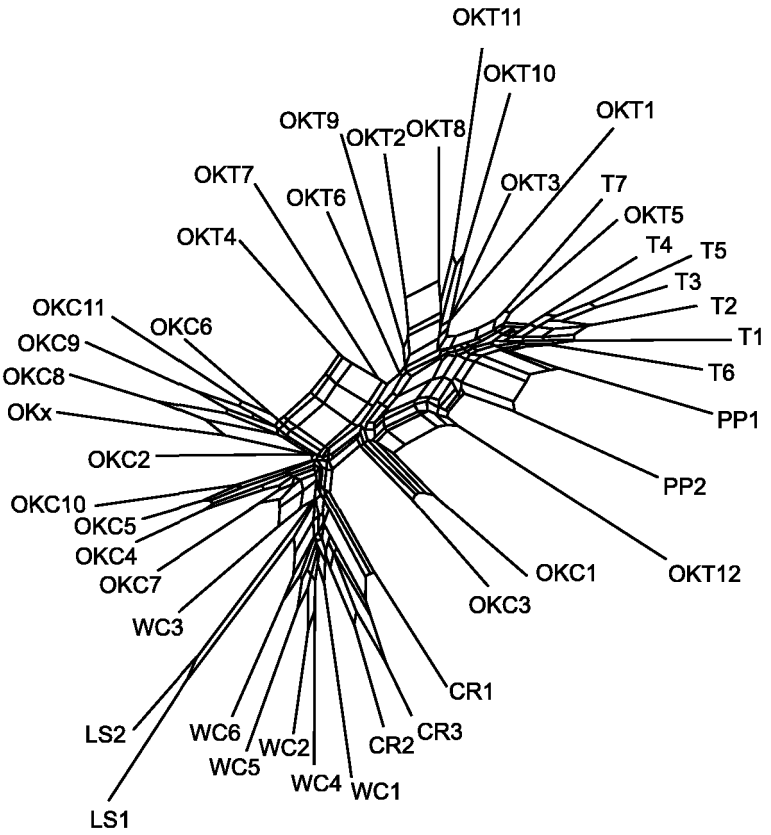
We used the program Structure 2.2 to estimate the degree of admixture between *P. cookianum* and *P. tenax* in this sample from their AFLP profiles. To do this we assumed two populations and allowed the program to assign individuals to these. A bar graph representing the admixture estimates from structure is shown in Fig. 3. These estimates of admixture for individual plants must be treated with some caution, however, as 95% probability intervals for the estimate of admixture proportions were typically large, particularly so for the samples from Okiwi Bay. Also, we treated a number of geographically isolated samples of *P. tenax* as a single population. The admixture estimates for which the 95% probability interval excluded a pure ancestry are indicated on Fig. 3. In the analysis using Structure, samples of *P. cookianum* and *P. tenax* were assigned to the two populations. The Okiwi Bay samples were estimated to have a varying degree of admixture, with the *P.* aff. *tenax* samples deriving a greater portion of their genotypes from *P. tenax*, and the *P. cookianum* Okiwi Bay samples a greater fraction of their genotypes from *P. cookianum*. Although 95% probability intervals for T6 and T7 (*P. tenax* samples from north of the Clarence River and Clifford Bay, both in Marlborough) exclude a pure ancestry, this is only just the case (95% probability intervals for membership of the *P. tenax* group are 0.709–0.999 for T6 and 0.647–0.998 for T7).

cpSSRs

Results of fragment sizing for the two cpSSRs sampled are shown in Table 4. None of the populations from which more than one genotype was sampled showed any polymorphism. All the Okiwi Bay *Phormium* samples and the two Paparoa Point samples share a cpSSR haplotype, which was also sampled from the *P. tenax* from north of the Clarence River (T7).

Morphology

In the PCA of seven morphological characters the *P.* aff. *tenax* plants from Okiwi Bay tend to have lower scores for principal component 1, reflecting their small stature compared with the sampling of *P. tenax* (Fig. 4). The first axis of the principal components



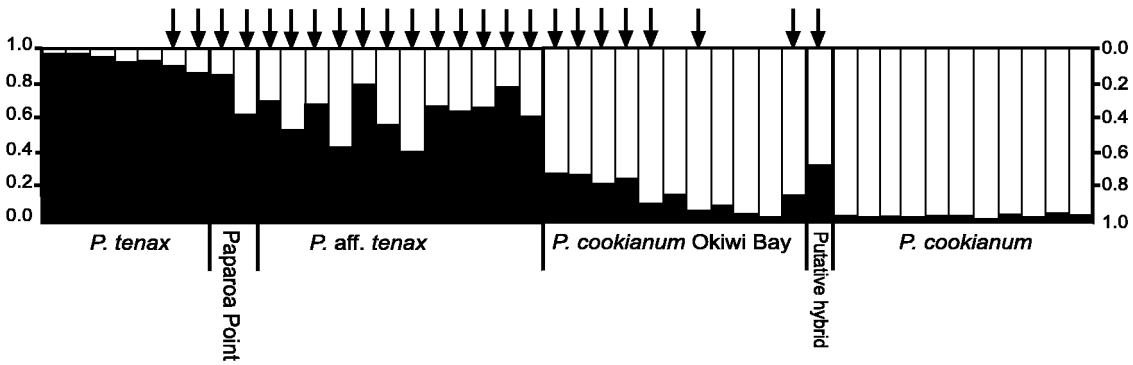


Fig. 2 Neighbour Net graph derived from *Phormium* AFLP profiles. T, *P. tenax* samples; OKT, Okiwi Bay *P.* aff. *tenax*; PP, Paparoa Point *P.* aff. *tenax* or *P. tenax*; OKC, Okiwi Bay *P. cookianum*; OKx, putative hybrid *P. cookianum* × *P.* aff. *tenax*; CR, Clarence River *P. cookianum*; WC, Woodside Creek *P. cookianum*; LS, Limestone Stream *P. cookianum*.

Fig. 3 Bar plot of group membership proportions from Structure. The black portion of each bar represents membership of group 1 (corresponding to *Phormium tenax*); the white portion of each bar represents membership of group 2 (corresponding to *P. cookianum*). Arrows indicate individuals for which 95% probability intervals reported from Structure do not include a pure ancestry.

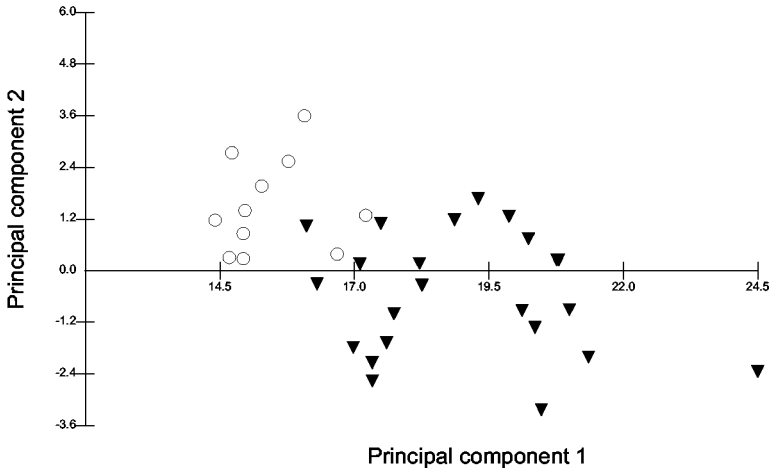


Fig. 4 Scatter plot of principal components scores for morphological analysis. ○, *Phormium* aff. *tenax*; ▼, *P. tenax*.

Table 4 Distribution of cpSSR haplotypes in samples. T, *Phormium tenax* samples; OKT, Okiwi Bay *P.* aff. *tenax*; PP, Paparoa Point *P.* aff. *tenax* or *P. tenax*; OKC, Okiwi Bay *P. cookianum*; OKx, putative hybrid *P. cookianum* × *P.* aff. *tenax*; CR, Clarence River *P. cookianum*; WC, Woodside Creek *P. cookianum*; LS, Limestone Stream *P. cookianum*.

Inferred repeat numbers in cpSSRs (*trn*L intron/*trn*L-F intergenic spacer)					
10/10	10/12	11/11	11/12	12/10	13/12
LS1–LS2	OKC1–OKC11	T3	WC1–WC6	T4	T1–T2
T6	OKT1–OKT12		CR1–CR3		
	OKx		T5		
	PP1–PP2				
	T7				

Table 5 Comparison of characteristics of *Phormium tenax* (n = 23 plants) and *P.* aff. *tenax* (n = 11 plants). n.s. = not significant.

	P. tenax	*P.* aff. *tenax*	ANOVA *P*
Leaf length (m)	1.74 ± 0.30	1.27 ± 0.19	<0.0001
Leaf width (mm)	86.53 ± 11.16	63.63 ± 5.35	<0.0001
Scape length (m)	2.61 ± 0.53	1.72 ± 0.30	<0.0001
Scape width (mm)	18.71 ± 3.54	12.78 ± 1.82	<0.0001
Fruit length (mm)	62.43 ± 8.50	59.51 ± 6.24	n.s.
Fruit width (mm)	11.04 ± 1.14	9.20 ± 0.88	<0.0001
Seed length (mm)	10.45 ± 1.25	8.93 ± 0.58	<0.001

analysis accounts for 99.1% of the variation and component 2 explained only 0.8%. Six of the seven characters had significantly different means using the ANOVA (Table 5).

Mature capsules of *P. cookianum* and *P. tenax* differ in orientation, the extent of twisting of the valves, and the angle of the valves at the capsule apex (Fig. 5). The capsules of *P. tenax* are upright and with straight valves that have obtuse apices, whereas those of *P. cookianum* are pendulous and the valves are strongly twisted and with acute apices. All of the 11 *P.* aff. *tenax* plants from Okiwi Bay included in the morphometric study have upright capsules like those of *P. tenax*, but they are variable

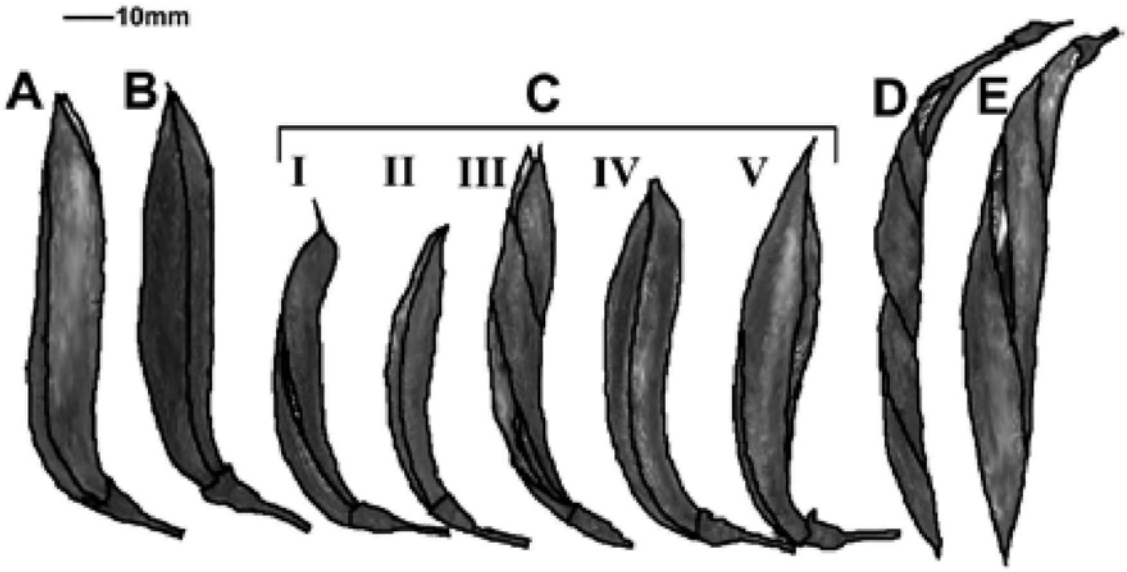


Fig. 5 Fruit. **A**, *Phormium tenax* (Muritai, Marlborough); **B**, *P. tenax* (Port Hills, Canterbury); **C**, *P.* aff. *tenax* (samples from five plants, Okiwi Bay, Marlborough); **D**, *P. cookianum* subsp. *hookeri* (Volcanic Plateau, Wellington); **E**, *P. cookianum* subsp. *cookianum* (Mt Peel, Canterbury).

in the amount of twisting of the valves and the angle of the valve apex. Two of the plants of *P.* aff. *tenax* exhibited twisting of the valves similar to that of *P. cookianum*, with one of these having an obtuse valve apex like *P. tenax* (Fig. 5CI) and the other an acute valve apex like *P. cookianum* (Fig. 5CIII). Other plants of *P.* aff. *tenax* had straight valves and either obtuse apices (Fig. 5CIV) like *P. tenax* or acute apices also more typical of *P. cookianum* (Fig. 5CII, V).

DISCUSSION

The results from the principal component and Neighbour Net analyses of AFLP profiles and the Bayesian estimation of admixture between populations using Structure show a striking similarity in the pattern recovered. In accord with our hypothesis of local introgression at Okiwi Bay, the AFLP profiles of putatively introgressed *P.* aff. *tenax* at Okiwi Bay are more similar to the profiles of *P. cookianum* Okiwi Bay plants than to those of *P. cookianum* plants from other populations. The *P. cookianum* and *P.* aff. *tenax* plants at Okiwi Bay also share a cpSSR haplotype (Table 4). This haplotype was also detected in the two plants sampled from Paparoa Point (see below) and in the two *P. tenax* samples with identical AFLP profiles from north of the Clarence River. However, we note that sampling of these chloroplast markers from throughout New Zealand indicates that this chloroplast lineage may be more widespread, as it was also found in two North Island samples of *P. tenax* and a *P. cookianum* sample from elsewhere in coastal Marlborough (Smissen unpubl. data).

Principal component analysis (Fig. 1), the admixture estimates from Structure (Fig. 3), and review of the 30 AFLP fragments selected following principal component analysis (Table 3) suggest that gene flow between the populations of *P. cookianum* and *P.* aff. *tenax* at Okiwi Bay has been bidirectional. These two populations at Okiwi Bay currently have discrete distributions, and our *a priori* assignment of plants to *P. cookianum*, *P.* aff. *tenax*, or in one case to a putative F1 or recent generation hybrid, was entirely concordant with clustering based on principal component analysis and the estimation of genetic admixture by Structure. This suggests that the two populations may be partially reproductively isolated. However, because the history of these populations is unknown, we cannot exclude a hypothesis that insufficient time has elapsed since they came into contact to allow homogenisation of their gene pools, or that the numerically smaller *P. cookianum* Okiwi Bay population is on a trajectory toward extinction and replacement with the introgressed *P.* aff. *tenax*.

Further support for introgression of *P. cookianum* alleles into *P.* aff. *tenax* is provided by morphological characters. For example, capsules of some of the *P.* aff. *tenax* plants have twisted valves and/or valves with acute apices, both characteristics of *P. cookianum* (Fig. 5). There appears to be no variability in the orientation of the capsules in *P.* aff. *tenax*: all of the plants examined have strictly erect capsules like *P. tenax*. Our initial field observation that *P.* aff. *tenax* is small statured compared with typical *P. tenax* was upheld by the PCA and ANOVA of quantitative morphological characters of plants in a common garden.

The two plants we sampled from Paparoa Point (c. 2 km south-west of Okiwi Bay) were collected as "small tenax" (i.e., *P.* aff. *tenax*: PP2), and "possible hybrid, tenax × small tenax" (PP1). Both of these samples shared the 10/12 chloroplast SSR haplotype with the Okiwi Bay populations, and the plant collected as "small tenax" (PP2) groups among the *P.* aff. *tenax* genotypes from Okiwi Bay in the principal component analysis of AFLP profiles occupies a similar position to them in Neighbour Net graphs, and displays a similar level of admixture in the Structure analysis. The other plant ("possible hybrid", PP1) is more similar in AFLP profiles to *P. tenax*, but has a notable affinity with PP2 in the Neighbour Net graph. Occasional plants that are more typical of *P. tenax* occur throughout the area studied (including at least one at Paparoa Point), and, although we do not know the origin of these, they may include planted as well as wild material. We suggest, in line with our field observation, that the *Phormium* at Paparoa Point includes genotypes related closely to *P.* aff. *tenax* from Okiwi Bay, other unrelated genotypes perhaps most similar to *P. tenax*, and possibly hybrids between these.

In general, patterns of morphological and genetic variation in wild *Phormium* are complex (Smissen & Heenan 2007), as might be expected in broadly sympatric, interfertile species with overlapping ecological tolerances. Crossing experiments in *Phormium* have been conducted and include representative genotypes from *P.* aff. *tenax* (Houliston unpubl. data). These indicate that the *P.* aff. *tenax* plants are fertile and reciprocally crossable with *P. tenax* and *P. cookianum*. Other biological attributes adding to the complexity include dispersal of the winged seed by wind and bird pollination, with both processes likely to facilitate seed and pollen movement over considerable distances. These conditions make a positive proof of gene flow between species of *Phormium* difficult, but we consider the data presented here and in Smissen & Heenan (2007) to represent compelling circumstantial evidence favouring the hypothesis of introgression at Okiwi Bay. Given that gene flow between *P.* aff. *tenax* and *P. cookianum* is likely to be ongoing at Okiwi Bay, and continuing gene flow between *P.* aff. *tenax* and *P. tenax* is also likely (at least at a low level), we do not consider *P.* aff. *tenax* at Okiwi Bay to constitute a hybrid species.

Previously in New Zealand, *Podocarpus totara* var. *waihoensis* was shown to be a stable introgressant between *P. acutifolius* and *P. totara*, with *P. acutifolius* the dominant species in the hybrid combination (Wardle 1972; Webby et al. 1987). These examples of introgression in *Phormium* and *Podocarpus* highlight an often discussed but little documented pathway in the evolution of the New Zealand flora. This study of DNA variation, and that of Webby et al. (1987) using flavonoid markers, tested hypotheses generated from careful field observation and highlights the importance of field study in the interpretation of genetic data.

In this paper, we have presented genetic and morphological data as evidence of introgression in a small and reasonably discrete *Phormium* population from Okiwi Bay, Marlborough. However, there remain a number of important gaps in our understanding of gene flow between *P. cookianum* and *P. tenax*. In particular, studies focusing on spatial structuring of genetic variation and paternity-testing at Okiwi Bay could give important insight into what extent the co-existence of the two currently distinct populations is stable, and perhaps the level and direction of natural selection on hybrid genotypes. Morphological analysis of a larger number of *P.* aff. *tenax* plants and genotyping of these may allow insight into the inheritance of the morphological characters that distinguish the species. An understanding of the importance of hybridisation in the evolution of *Phormium* generally will also require examination of plants from other parts of New Zealand where the outcome of hybridisation may be different from Okiwi Bay.

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