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Gene Flow Between Introduced and Native *Eucalyptus* Species: Morphological Analysis of Tri-Species and Backcross Hybrids Involving *E. nitens*

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Summary

Morphometric analyses were conducted on second-generation tri-species and backcross hybrids in *Eucalyptus*. These hybrids were all produced using pollen from two *E. nitens* x *cordata* F_1 hybrids and controlled pollination techniques. Tri-species hybrids were created with *E. gunnii*, *E. ovata* and *E. viminalis* as females, while backcrosses were produced with *E. cordata*. Multivariate analysis of seedling characteristics indicated that eighty percent of the backcross hybrids fell within the morphological range of *E. cordata*. All three cross combinations of the tri-species hybrids were biased away from *E. nitens* and towards their maternal parent and *E. cordata*. The inclusion of data for first-generation (F_1) hybrids between the pure parental species in the current work showed the F_1's to be easily distinguishable from pure species, compared to second-generation hybrids. The use of morphology for detecting second-generation hybridisation involving exotic plantation species and native eucalypt populations will therefore be

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unreliable, and identifies a need for preventing second-generation hybrids from establish in the wild. The current work, nevertheless, provides further demonstration of the effectiveness of morphological identification of F_1 hybrids. The easy recognition of F_1 hybrids will be useful in identifying sites and species at risk of exotic gene flow and enable the development of weeding programs that focus on removing exotic hybrids in the wild.

Key words: genetic pollution, genetic contamination, introgression, exotic species, pollen dispersal, *Eucalyptus*, plantation forestry.

Introduction

Hybridisation and gene flow between species of eucalypts has been frequently recorded (GRIFFIN *et al.,* 1988), and is likely to have played a significant role in the evolution of the genus (MCKINNON *et al.,* 2004a). The production and morphology of F_1 hybrids in the genus has received considerable attention, and in a number of cases F_1 hybrids in *Eucalyptus* have been shown to reach reproductive maturity (POTTS and DUNGEY, 2004). The potential for second-generation hybridisation such as three-species (tri-species) and backcross (BC_1) hybridisation, however, has received little attention. The morphology and fitness of such hybrids is therefore poorly understood, despite the fact that they are a major step in the process of introgression (ARNOLD, 1997).

Recent interest in second-generation hybridisation has developed due to the potential for pollen-mediated gene flow from exotic eucalypt plantations into native populations in Australia (POTTS *et al.,* 2003). Understanding the potential for hybridisation involving pollen from exotic F_1 hybrids established in the wild (BARBOUR *et al.,* 2003) or from F_1 hybrids used for plantation stock (DALE, 2002), is a key factor in assessing the impacts of pollen flow from commercial plantations (POTTS *et al.,* 2003). In this study we assessed, firstly, whether tri-species and backcross hybrids could be produced with F_1 hybrid pollen from *E. nitens* ♀ x *cordata* ♂ F_1 hybrids, using controlled pollination techniques. Secondly, we compared the seedling morphology of first- and second-generation hybrids relative to pure species parental controls. *Eucalyptus nitens* is a plantation species that is exotic to the island of Tasmania, where the study was conducted (PEDERICK, 1979) and is hybridising with native species such as *E. viminalis* and *E. ovata* (BARBOUR *et al.,* 2003). *Eucalyptus cordata* and *E. gunnii* are two other endemic species on the island (WILLIAMS and POTTS, 1996).

Materials and Methods

The pollen used in this work was collected in 1995 from two full-sibling *E. nitens* x *cordata* F_1 hybrid trees grown in an experimental garden. These trees were the product of controlled pollinations involving *E. cordata* as a pollen parent and were described in Tibbits (1989). The *E. nitens* x *cordata* F_1 hybrid pollen was used as it was the only suitable F_1 hybrid pollen involving *E. nitens* known to exist, and *E. nitens* is the most commonly used species in plantations in Tasmania (WOOD et al., 2001). The F_1 pollen was stored in gelatine capsules,

Table 1. – Summary of controlled pollination success using *E. nitens* x *cordata* F_1 hybrid pollen on *E. cordata*, *E. gunnii*, *E. ovata* and *E. viminalis*. The table shows the number of trees assessed, viable seed per flower pollinated, seed sown, germinants per seed sown and healthy seedlings without abnormality per germinant (Hyb = *E. nitens* x *cordata* hybrid cross, Out = intraspecific outcross).

Species	Female tree no.	n seed/flower		n seed sown		n germs/seed sown		n norms/germs	
		Out	Hyb	Out	Hyb	Out	Hyb	Out	Hyb
E. cordata	1	1.4	0.7	20	13	1.00	0.92	0.90	0.85
	2	0.0	1.4		10		1.00		0.50
E. gunnii	1	6.6	1.1	24	18	0.83	0.61	0.83	0.39
E. ovata	1	1.0	0.4	25	12	0.92	0.50	0.92	0.50
E. viminalis	1	4.2	1.0	12	23	1.00	0.91	1.00	0.78
	2	1.6	0.4	18	4	0.78	0.00	0.61	

within vials containing silica gel, and frozen. At pollination, the F_1 pollen was found to have low viability when tested (< 1%, agar technique with 20% boron, POTTS and MARSDEN-SMEDLEY, 1989), possibly due to lengthy storage. Controlled crossing using this pollen was conducted on two *E. cordata*, one *E. gunnii*, one *E. ovata* and two *E. viminalis* trees between May 2001 and March 2004 (*Table 1*). The *E. cordata* grandparent used to produce the *E. nitens* x *cordata* F_1 hybrid pollen parents, and the *E. cordata* used in our backcrossing experiment, were unrelated, but all were of the western form of the species (POTTS, 1989). The cut-style pollination technique was used for crossing onto the *E. cordata*, *E. gunnii* and *E. ovata*, while stigma-pollination was used for crossing onto *E. viminalis*. The later method involved pollination of receptive stigma while the former involved removing the stigma by cutting two-thirds up the length of the style using a scalpel and placing pollen on the newly exposed style surface (see BARBOUR *et al.,* 2005 for further description of techniques). On each, tree individual branches were selected, and on each branch, an average of 16 flowers were emasculated and treated with pollen from individual pollen-parents. Each female tree typically had two branches treated separately with the two *E. nitens* x *cordata* pollen parents and three branches treated with various unrelated intraspecific outcross pollens collected from the same or more distant populations. Following pollination, isolation was achieved using terylene bags, which were removed after three weeks once flowers were no longer receptive.

Seed capsules were collected approximately one year after pollination. Capsules produced from open-pollinated flowers were also collected from each tree. Seed was extracted from the capsules, and viable and inviable seed were counted (see BARBOUR *et al.,* 2005 for methods). Portions of this seed were germinated and grown under glasshouse conditions (*Table 1*). Included with this was an uncounted small amount of open-pollinated seed from a selection of 22 *E. nitens* females, providing a representative sample of the genetic diversity within the species. In general, seedlings from each female tree (second-generation, outcross, open-pollination and F_1's (described below)) and an even division of the *E. nitens* seedling were kept together as a block. The position of

seedling-type within block and block within glasshouse was randomised. The number of seed that germinated and the number of germinants that grew to healthy seedlings without abnormality were counted. Normal seedlings were those that grew without loss of apical dominance, structural deformities, or stunting (i.e. runts). Once seedlings of all cross-types had developed 10 or more nodes with fully expanded leaves, a range of apical bud (2 characters), leaf (11 characters), stem (8 characters) and general growth characters (5 characters) were assessed (*Table 2*). These characters were assessed for all second-generation hybrids that grew to be healthy seedlings and 10 outcross seedlings from each tree used as a maternal seed parent (evenly selected from across pollen parents). When enough outcross seedlings were not available from a particular tree, seedlings grown from open-pollinated seed of these trees were used. One seedling from each of 22 *E. nitens* families was also assessed (previously published in Barbour *et al.,* 2005). Raw data were transformed where necessary to optimise normality and homogeneity of variance. A canonical discriminant analysis was then conducted that aimed to maximise the differences between the parental species of the second-generation hybrid types. This was done using the DISCRIM procedure of SAS (version 9.1, Cary NC, USA). These discriminant func-

tions could then be used as a hybrid index along which the positions of the hybrids could be calculated to assess their morphological relationship to the parental species seedlings. Included in the analyses were data for F_1 hybrids (presented previously in Barbour *et al.,* 2005) produced from *E. nitens* pollen and an *E. gunnii* (tree 1 of current study), an *E. ovata* and an *E. viminalis*. These were included to compare the inheritance of morphological characters in first-generation (F_1) hybrids relative to second-generation hybrids.

Results

Pollination of *E. cordata*, *E. gunnii*, *E. ovata* and *E. viminalis* with *E. nitens* x *cordata* F_1 hybrid pollen was successful in producing second-generation hybrid seed and seedlings (*Table 1*). The different pure parental species seedlings were highly differentiated in each of the discriminant analyses. The tri-species hybrids fell within or close to the morphometric ranges described for *E. cordata* and/or their maternal seed parent and were clearly distinct from *E. nitens*. Tri-species hybrids with *E. ovata* fell intermediate between *E. ovata* and *E. cordata* to just outside the range of *E. cordata* (*Figure 1*), while those with *E. viminalis* were heavily biased to the maternal parent *E. viminalis* with 28% of the seedlings

Table 2. – Characters used in the morphometric analysis of the first (F_1) and second-generation hybrids and their pure species controls. Included are the transformations (Transf.) used for the analysis (Log, logarithmic; Sqrt, square root).

| Code	Description	Transf.
Bud characters		
BUDFUS	Type of bud fusing (0-2); 0 = no fusing, 1 = partial fusing, 2 = complete fusing, see *E. nitens* in Fig. 1 of Barbour *et al.* (2005) for example of fusing	None
LFUS	Length of fusing of the apical bud leaves (mm)	None
Leaf characters		
CRENM	Crenulate margins (0-2); 0 = none, 2 = max.	None
CORD	Length of extension of the cordate lobes of the lamina past the base of the petiole/lamina join (mm)	$X^{0.25}$
LAML	Length of lamina (mm)	Log
LAMW	Lamina width at widest point (mm)	None
LA	Leaf angle (°); the axillary angle made by the mid-rib and the stem	None
LGL	Leaf glaucousness (0-7); 0 to 1 = green, 2 to 4 = sub-glaucous and 5 to 7 = glaucous, Converted from Cauvin *et al.* (1987)	None
LP	Leaf plane (°); cross-sectional angle of the leaf from horizontal	None
LTA	The acute angle of the leaf tip (°)	Log
LWP	Length along mid-rib to widest point (mm)	Sqrt
MRRED	Mid-rib redness (0-2); 0 = none, 2 = max.	None
PETL	Length of petiole (mm)	None
Plant characters		
HT10	Height to node 10 (mm)	Log
LAT05	Number of laterals from nodes 0 to 5 (n)	None
LAT610	Number of laterals from node 6 to 10 (n)	None
LLLAT	Length of longest lateral (mm)	None
NLLAT	Node of longest lateral (0-10)	None
Stem characters		
INTER10	Inter-node length between node 9 and 10 (mm)	None
INTRA10	Intra-node length at node 10 (mm)	None
SGL	Stem glaucousness (0-2); 0 = absent, 2 = max.	None
SRE	Stem rectangularity (mm); SD1 / SD2, see Barbour *et al.* (2003)	None
SRED	Stem redness (0-2); 0 = none, 2 = max.	None
SRO	Stem roundness (mm); SD1 / SD3, see Barbour *et al.* (2003)	None
SV	Stem verrucae (0-2); 0 = absent, 2 = max.	None
WW	Waviness of stem wings (0-3); 0 = no wings, 1 = non-wavy wings, 3 = maximum waviness, see *E. nitens* Fig. 1 of Barbour *et al.* (2005) for example of wavy wings	None
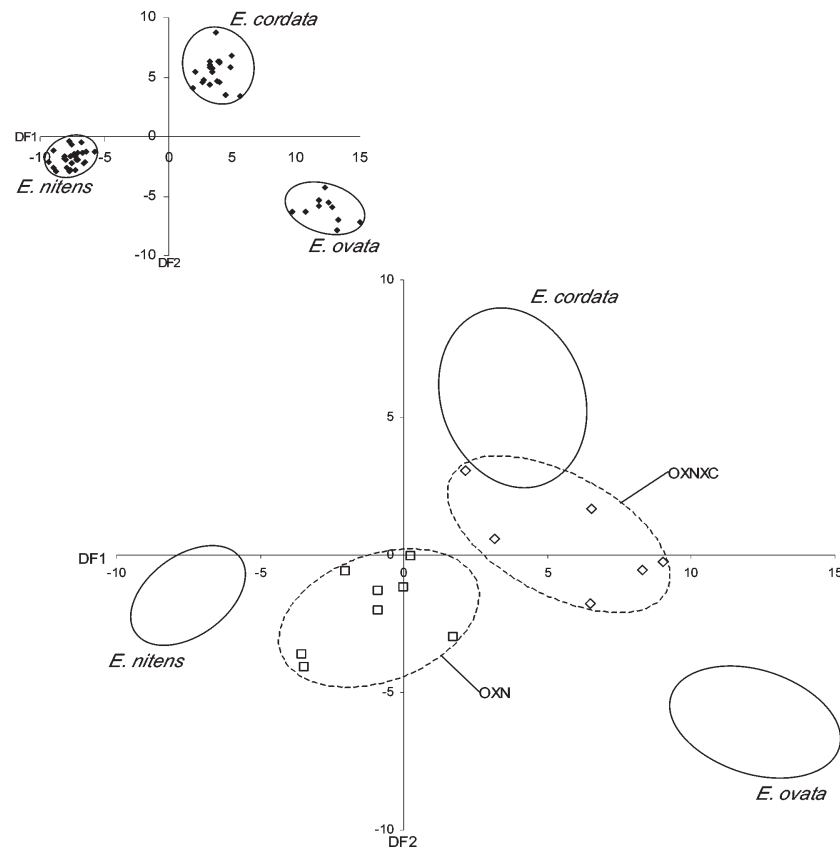
Figure 1. – Discriminant analysis of *E. ovata* x (*nitens* x *cordata*) tri-species hybrids (◇, OXNXC) compared to their pure parental species (◆, plotted separately above and envelope (◯) shown below). The multivariate distribution of F_1 hybrids between *E. ovata* and *E. nitens* produced in Barbour *et al.* (2005) is also provided (□, OXN). DF1 and DF2 are the first and second discriminant functions respectively, separating the three pure species.

falling within its range (*Figure 2*). Tri-species hybrids with *E. gunnii* ranged from a resemblance to *E. gunnii* through to well within the range of *E. cordata*, no doubt due to the similarity in characteristics of *E. cordata* and *E. gunnii* (*Figure 3*). Some of the *E. cordata* x (*nitens* x *cordata*) hybrids ranged from being intermediate between *E. nitens* and *E. cordata*, however, 80% fell within the range of *E. cordata* (*Figure 4*). In comparison to both the tri-species and backcross second-generation hybrids, the F_1 hybrids displayed discrete distributions relative to the pure parental species and generally fell intermediate between the parent species (*Figure 1, 2, 3*).

Discussion

This is the first detailed morphological analysis of second-generation hybrids in *Eucalyptus*, with the only previous studies being conducted on a single morphological trait (leaf glaucousness, Paton, 1981; Cauvin et al., 1987). Both the tri-species (*E. nitens* x *cordata* on *E. gunnii, E. ovata* and *E. viminalis*) and backcross (*E. nitens* x *cordata* on *E. cordata*) second-generation hybrids showed considerable bias in morphology away from *E. nitens*, with all but the *E. ovata* x (*nitens* x *cordata*) overlapping in morphometric space with one or two of the other parents. The bias observed in the back-

cross (BC_1) hybrids occurred with the maternal *E. cordata* parent, which was expected since 75% of the genetic material within these hybrids comes from *E. cordata*. Such BC_1 hybrids would be typically found in hybrid swarms between species that have recently come into contact and can represent a step in the process of introgression and assimilation of conspecific genes into the recurrent population (Potts and Reid, 1988; Arnold, 1997). The degree of morphological similarity between the *E. cordata* BC_1 hybrids and the parental *E. cordata* in the current work, has demonstrated that just one generation of backcrossing can result in difficulties in morphological identification of hybrids. In the case of the tri-species hybrids, however, patterns of cross-pollination and directions of further gene flow are uncertain due to the potentially poor viability of such crosses (see below) and the fact that three pure parental species are involved. Interestingly, however, the bias seen in the morphology of the tri-species hybrids was always away from *E. nitens* and towards the maturnal parent and *E. cordata*. This may reflect endogenous barriers to *E. nitens* gene flow, as no evidence of dominance in the maternal parent characteristics was observed in the F_1 hybrids between *E. nitens* and *E. gunnii*, and *E. nitens* and *E. ovata*. Such barriers may occur through imbalance in successful recombination and production of F_1

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hybrid pollen or through pre-zygotic (i.e. pollen tube growth and ovule penetration) or post-zygotic (i.e. seed abortion) selection against of *E. nitens* genetic material (MYBURG *et al.,* 2004).

Very little published data exists on the production and fitness of tri-species hybrids in *Eucalyptus*. While there are some records of their existence (PILIPENKA, 1969; YORKE and ASHTON, 1982; CAUVIN *et al.,* 1987; DE ASSIS,
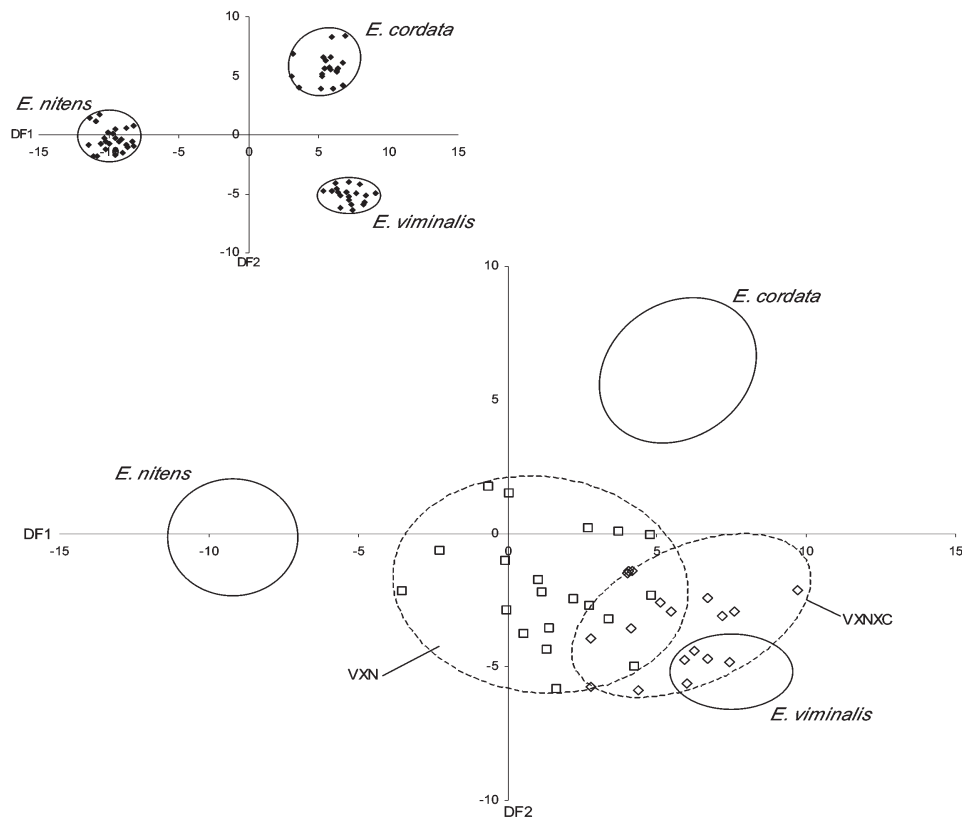

Figure 2. – Discriminant analysis of *E. viminalis* x (*nitens* x *cordata*) tri-species hybrids (◇, VXNXC) compared to their pure parental species (◆, plotted separately above and envelope (◯) shown below). The multivariate distribution of F_1 hybrids between *E. viminalis* and *E. nitens* produced in BARBOUR *et al.* (2005) is also provided (□, VXN). DF1 and DF2 are the first and second discriminant functions respectively, separating the three pure species.
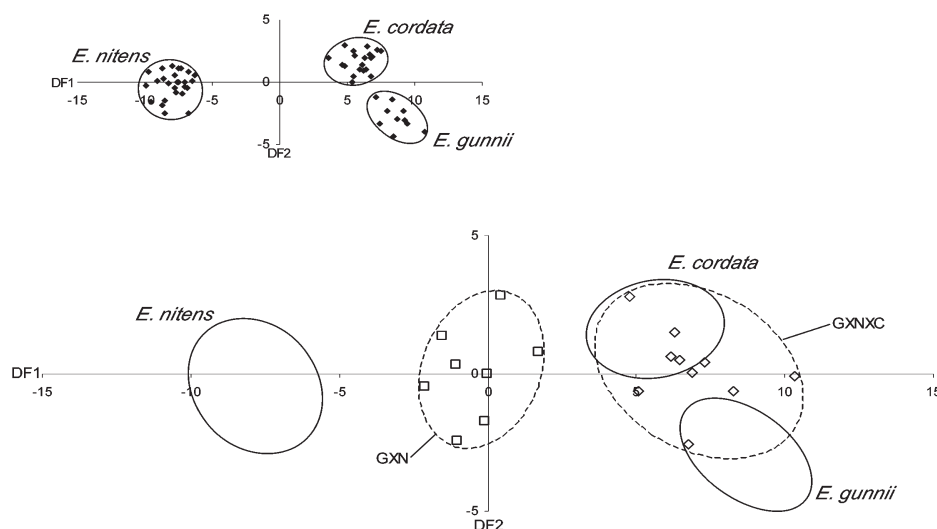

Figure 3. – Discriminant analysis of *E. gunnii* x (*nitens* x *cordata*) tri-species hybrids (◇, GXNXC) compared to their pure parental species (◆, plotted separately above and envelope (◯) shown below). The multivariate distribution of F_1 hybrids between *E. gunnii* and *E. nitens* produced in BARBOUR *et al.* (2005, same *E. gunnii* female as the OXNXC hybrids) is also provided (□, GXN). DF1 and DF2 are the first and second discriminant functions respectively, separating the three pure species.
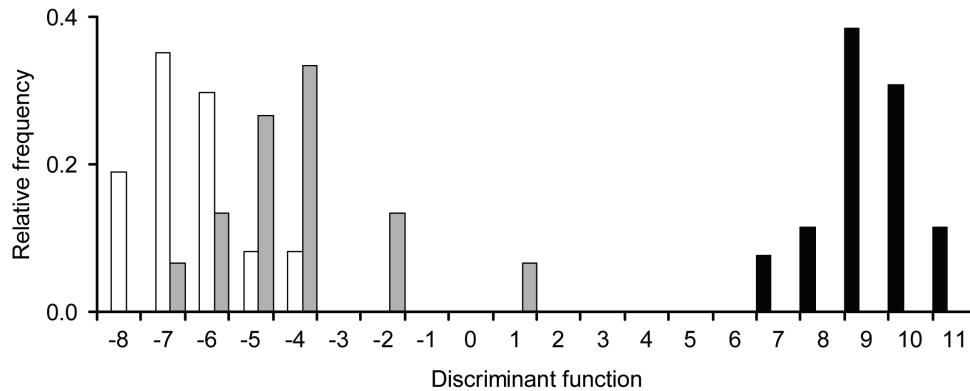
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Figure 4. – Frequency distribution of *E. cordata* x (*nitens* x *cordata*) backcross hybrids (grey) relative to the pure parental species (*E. nitens* = black, *E. cordata* = white) along the single discriminant function separating the pure species.

2000; BARBOUR, 2004a), assessment of their fitness relative to suitable controls is limited (DE ASSIS, 2000; BARBOUR, 2004a). Because of the likely break-up of co-adapted gene complexes the fitness of tri-species hybrids is nevertheless expected to be poor (POTTS and DUNGEY, 2004). In comparison, initial assessments of backcross (BC$_1$) hybrids in *Eucalyptus* (GRIFFIN *et al.,* 2000; POTTS *et al.,* 2000; POTTS *et al.,* 2003) and other plant genera (reviewed in ARNOLD and HODGES, 1995; ARNOLD *et al.,* 1999) suggest that improved fitness relative to parental F$_1$ hybrids can occur (see POTTS, 1986; CHAPARRO *et al.,* 1996; DE ASSIS, 2000; GRIFFIN *et al.,* 2000; MCCOMB *et al.,* 2000; MYBURG *et al.,* 2003, for records of backcross hybrids in *Eucalyptus*). Further research into the performance of second-generation cross-types in *Eucalyptus*, i.e. backcross, tri-species, and F$_2$ generations, is clearly necessary. Such information would provide valuable insights into an important evolutionary process within the genus (MCKINNON et al., 2004a), greatly assist the development of strategies in hybrid breeding programs (POTTS and DUNGEY, 2004) and enhance the risk assessment of pollen flow from exotic eucalypt plantations (POTTS et al., 2003).

As the majority of Australia's plantation estate has only recently been planted (WOOD E*t al.,* 2001), the first generation (F$_1$) of exotic hybrids produced through pollen dispersal into native eucalypt populations are yet to, or are only just beginning to establish (BARBOUR *et al.,* 2003). Because of the clearly distinguishable morphological characteristics of F$_1$ hybrids produced from morphologically distinct parent species in *Eucalyptus,* reliable morphological identification of F$_1$'s can typically be conducted (*Figure 1, 2, 3*; WILTSHIRE and REID, 1987; TIBBITS, 1988; DELAPORTE *et al.,* 2001). The development of morphological monitoring programs aimed at the identification of F$_1$ hybrids will therefore be an effective tool for identifying sites and species at risk of introgression of exotic plantation genes. In the current example, the likelihood of hybridisation between plantation grown *E. nitens* and natural populations of *E. cordata* is low, due the existence of barriers to F$_1$ hybridisation such as spatial isolation and flowering asynchrony (BARBOUR, 2004b; BARBOUR *et al.,* 2005; BARBOUR *et al.,* 2006). The cross combinations produced using the pollen from this F$_1$ hybrid cross-type, however, have clearly demonstrated the difficulties in identifying second-generation hybrids compared to first-generation (F$_1$) hybrids from pure parental species based on morphology. Preventing second- and later-generation hybrids from being created or from establishing in the wild should therefore be an important consideration in the risk management of pollen dispersal from exotic plantations.

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