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Gene flow between introduced and native *Eucalyptus* species: crossability of native Tasmanian species with exotic *E. nitens*

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Abstract. *Eucalyptus nitens* (Deane & Maiden) Maiden has been extensively introduced to the island of Tasmania for plantation purposes. Natural hybridisation with two native species has already been confirmed and this study aimed to determine which other Tasmanian native species could potentially hybridise with *E. nitens*. Controlled and supplementary pollinations with *E. nitens* pollen were undertaken on all Tasmanian native species that are potentially at risk of exotic gene flow and hence genetic pollution. Across the seven species tested by using controlled pollinations, seed set per flower, following *E. nitens* pollinations, was significantly less than for intraspecific outcross pollinations. No significant differences were evident in the percentage of seed that germinated or the percentage of germinants that grew into healthy seedlings in the glasshouse. Hybridity was verified by morphometric analyses and F_1 hybrid seedlings were clearly differentiated from parental species and generally intermediate in morphology. Supplementary *E. nitens* pollination of open-pollinated native flowers was conducted to simulate natural pollination where pollen competition would occur. Seven of the fifteen species tested produced F_1 hybrids in this case; however, further crossing is required to verify failed cross combinations. Although *E. nitens* can potentially hybridise with many native species, the results from both supplementary and controlled pollinations suggest the presence of post-pollination barriers of varying strength that need to be considered in assessing the risk of exotic gene flow from plantations.

Introduction

The high number of exotic species being translocated around the world through human activities (Drake *et al*. 1989) is placing an increasing number of native species at risk of hybridisation and introgression of exotic genes (Rhymer and Simberloff 1996; Vila *et al*. 2000; Schierenbeck *et al*. 2005). Such pollen-mediated gene flow can result in the dilution of native-species gene pools, causing the loss of their genetic integrity and in cases causing their extinction through complete replacement (Rhymer and Simberloff 1996; Vila *et al*. 2000). Rare, isolated or small populations are of particular concern because of the potential for pollen swamping by more abundant exotics (Ellstrand 1992; Levin *et al*. 1996). The introgression of exotic genes may cause phenotypic alterations to native species, resulting in additional community-wide impacts (Anttila *et al*. 1998; Whitham *et al*. 2003). In the case of species used in agriculture or forestry, the conservation of their native gene pools is important as they can provide an important source of genetic diversity for infusion into domesticated lineages (Jana and Nevo 1991; Nevo 1998; Yawen *et al*. 2001). Understanding the potential for pollen-mediated gene flow from an exotic species is therefore an important aspect of exotic-species management, especially in agriculture (Brown *et al*. 1997; Brown and Brubaker 2000; Baltazar *et al*. 2005) or forestry (Commonwealth of Australia 1998, 2003) where introductions are intentional and often occur on a large scale.

Although there is little doubt that pre-pollination barriers such as flowering time are important in restricting interspecific hybridisation in plants, major barriers can occur after pollination (Levin 1978). Such post-pollination barriers may prevent pollen from fertilising ovules, the development of viable F_1 hybrid seed or the successful germination and growth of F_1 hybrids (Levin 1978; Potts *et al*. 2003). These crossability barriers are often detected when hybridising species for breeding purposes (Wiersma 2003; Potts and Dungey 2004) and can prevent the escape of transgenes from agricultural systems (Hoffman 1990; Raybould and Gray 1994; Brown *et al*. 1997). Crossability barriers are also well known to prevent gene flow among sympatric species in the wild (Klips 1999; Williams *et al*. 2001; Ramsey *et al*. 2003). Assessment of these barriers is therefore an important step in identifying native species at risk of exotic gene flow.

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In Australia, the *Eucalyptus* plantation estate has rapidly expanded in the last decade and now covers over 675 000 ha (Wood *et al*. 2001; National Forest Inventory 2004). This expansion has raised concerns about the potential for exotic pollen-mediated gene flow into native eucalypt populations (Wardell-Johnson *et al*. 1997; Strauss 2001; Potts *et al*. 2003), as most eucalypt plantations are established out of their natural distribution as locally exotic species (Wood *et al*. 2001; Potts *et al*. 2003). This is certainly the case on the island of Tasmania, where *E. nitens* has been extensively introduced from continental Australia for plantation purposes (Pederick 1979; Wood *et al*. 2001). *E. nitens* belongs to subgenus *Symphyomyrtus* section *Maidenaria*, along with 17 of Tasmania's 29 native species (Williams and Potts 1996; Brooker 2000). The remaining native species belong to subgenus *Eucalyptus* (Brooker 2000) and are reproductively isolated from *E. nitens* because of strong physiological barriers (Griffin *et al*. 1988; Ellis *et al*. 1991). Among the *Symphyomyrtus* species, populations of most species have been found in close spatial proximity to *E. nitens* plantations (Barbour 2004). Some of these species are abundant in Tasmania, i.e. *E. brookeriana*, *E. ovata* and *E. viminalis*; however, some are rare such as *E. perriniana* (Williams and Potts 1996). Because interspecific hybridisation within section *Maidenaria* is common (Griffin *et al*. 1988; Williams and Potts 1996), *E. nitens* is expected to be able to hybridise with most Tasmanian *Symphyomyrtus* species. Artificial pollinations have shown that *E. nitens* can act as a seed parent with a number of these species (Tibbits 1989), and there seems little reason why *E. nitens* cannot act as a pollen parent in these cases. However, verification of crossability with *E. nitens* pollen is necessary, as unilateral post-pollination barriers to F_1 hybridisation do exist amongst species of the same taxonomic section. For example, whereas hybrids between the Tasmanian native *E. globulus* and *E. nitens* can be produced by using *E. nitens* as a female, the cross is not successful in the reverse direction as the pollen tubes of *E. nitens* are unable to grow the full length of the style of the large-flowered *E. globulus* (Gore *et al*. 1990). In addition, most crossability studies have been undertaken by using controlled pollination in the absence of competition between intraspecific and interspecific pollen which may exaggerate cross success compared with that found under natural pollination (Klips 1999; Vanden Broeck *et al*. 2003).

This study aimed to assess the crossability of all native Tasmanian *Symphyomyrtus* species, except *E. globulus*, with pollen of the exotic *E. nitens*. Two types of artificial pollination were undertaken on females of native species; the first involved using isolation techniques (controlled pollination), the second involved adding *E. nitens* pollen to open-pollinated flowers and thus in the presence of intraspecific pollen (supplementary pollination). The supplementary pollination technique provided the best simulation of natural pollination and an assessment of the ability of exotic *E. nitens* pollen to compete with naturally occurring pollen. In this case, hybridity was verified by morphometric analyses. This work is part of a series of publications assessing the risks of exotic gene flow from *E. nitens* plantations in Tasmania (Barbour *et al*. 2002, 2003, 2005; Barbour 2004), which includes a broad review of the risks of exotic gene flow in *Eucalyptus* (Potts *et al*. 2003).

Materials and methods

Pollination techniques

Artificial pollinations of Tasmanian *Symphyomyrtus* species were conducted from March 2000 through to May 2002. One to four trees of each native species were used as females; 39 were from natural stands and four were ornamentals (Appendix 1, available as accessory material on the web). Two techniques were used for controlled pollination with *E. nitens* pollen; these were stigma (Moncur 1995) and cut-style (adapted from Williams *et al*. 1999) pollination. Both controlled-pollination techniques involved isolation of receptive flowers from all pollen other than that applied, therefore producing seedlings of known genotype. The stigma-pollination method involved the application of pollen directly to the sigma, whereas the cut-style method involved cutting off the top third of the style, thereby removing the stigma and creating an immediately receptive surface for pollination to be conducted in the same manner as for the stigma pollination (Patterson *et al*. 2004). Most trees treated with controlled pollinations received the cut-style treatment because of the shorter time required for the procedure, with the conventional stigma pollination undertaken on only five trees. Isolation was achieved by using terylene bags placed over each branch, which were removed 3 weeks after pollination. The technique for supplementary pollination was from Patterson *et al*. (2004) and involved applying *E. nitens* pollen to stigma of open-pollinated flowers that were not emasculated nor isolated. The flowers that were used for this technique ranged in development from having expanded stamen with undehisced anthers, through to the stamen just starting to wilt with fully dehisced anthers, ensuring that at least some flowers were receptive (Patterson *et al*. 2004).

Inter- and intraspecific pollinations were undertaken by using pollen from individual *E. nitens* trees or pollen from trees of the same native species as the female, respectively, in a randomised single-pair mating design. For *E. nitens*, a total of 32 pollen parents was used from the main provenances of *E. nitens* (Pederick 1979). These trees were located in Gunns Ltd seed orchards. The pollen for intraspecific pollinations was collected from 3–13 trees from different populations for each native species. Pollen extraction followed the techniques of Moncur (1995), with all pollen kept frozen for storage. Pollen viability tests were conducted on *E. nitens* pollen (agar assessment with 20% sucrose, Potts and Marsden-Smedley 1989). Only pollen with greater than 5% *in vitro* germination prior to pollination was used. Intraspecific pollen was not tested as it was collected just before its use.

For each native tree, individual branches were prepared, with ~16 flowers for treatment with a specific pollen parent and pollination technique. For each cross type (*E. nitens* or intraspecific outcross), three branches were generally treated for cut-style pollination, eight branches for stigma pollination and 10 branches for supplementary pollination per tree. The allocation of pollen parent to branch was random (only constrained by pollen availability and viability), with no pollen used twice per mother tree or pollination technique. Pollen was applied with a matchstick, with enough being applied so that it was visible on the stigma or cut-style (Moncur 1995).

Seed collection, processing, germination and seedling growth

Seed capsules were generally left to ripen for 8–15 months after pollination before being harvested (Appendix 1). Capsules were assessed as being ripe if they had well developed valves. The capsules from each pollination technique and individual pollen parent/female tree combination were kept separate, and their seed maintained as individual seedlots throughout the study. After harvesting, capsules were dried at room temperature and their contents extracted by hand, allowing for viable and inviable seeds to be counted (see Drake 1975). Viable seeds were those that were black, round and heavier, and contained white embryonic tissue that could be seen if the seed was squashed. Squashing of seeds to test for viability was done on just a few seeds per species, to verify the characteristics of viable seeds for that species. Immature viable seeds were similar in shape, but were cream to light brown in colour. These immature seeds were treated as mature seeds in the assessment of viable-seed yields. Inviable seeds were black, thin and lighter in weight, and empty when squashed. Open-pollinated seed capsules were also collected from each tree; however, the seeds in capsules were not counted as they were only for use in the morphometric study and not for assessment of relative cross success.

Seedlots were germinated in a glasshouse maintained at 24°C where they were grouped by female and then randomly arranged within these female groups. Each seedlot was sown in a punnet containing a layer of vermiculite over potting mix, and kept moist. For each species assessed with controlled pollination, a generally even number of seeds was randomly selected from each seedlot for sowing. The quantity of seeds sown per species depended on availability. In cases where large quantities of seeds were gained, enough was sown so that a representative population could be assessed for their seedling morphology, i.e. 50–100 seeds (see below). Comparable amounts of outcross to F_1 hybrid seeds were sown. For the supplementary pollinations, generally all seedlots with more than six viable seeds were sown. An average of 17 seeds per tree were germinated from the open-pollinated seeds collected from each native tree. Small, uncounted quantities of open-pollinated seeds from 26 of the 32 *E. nitens* pollen parents were also germinated for the morphometric study. Despite having apparently well formed valves, capsules from five of the higher-altitude species either did not open (*E. johnstonii*) or produced a high proportion of immature seeds (*E. archeri*, *E. subcrenulata*, *E. urnigera* and *E. vernicosa*). In these cases, most of the mature seeds were sown to obtain plants for the morphometric analysis but the germination was poor.

The number of viable seeds that germinated was scored for each seedlot once seedlings developed fully expanded cotyledons. Seedlings were then pricked into individual pots ($40 \times 40 \times 70$ mm) containing potting mix. Seedlings from each seedlot were kept together in the glasshouse and seedlots from the same female grouped together in a nested design. The arrangement of seedlots within the female block was random as was the position of the female block in the glasshouse. A four-seedling plot of each of the 26 *E. nitens* seedlots was randomly allocated to a native female block. After 6 months, when most seedlings had at least 10 or more nodes, the number of seedlings that were healthy, abnormal or dead was recorded for each control pollinated seedlot. An abnormal seedling was one that had lost apical dominance, was structurally deformed or substantially reduced in size (i.e. dwarf).

The relative success of *E. nitens* compared with intraspecific controlled outcross pollination was assessed by using the numbers of capsules per flower, seeds per capsule and seeds per flower as well as the percentage of viable seeds that germinated and the percentage of germinants that grew to healthy seedlings. The significance of the difference between the inter- and intraspecific pollinations was tested with paired *t*-tests conducted on the means for each native tree. Paired *t*-tests were undertaken on both the combined stigma and cut-style pollination results and on just the cut-style results. Trees were removed from analyses if there was a missing value for either of the pollen types owing to a failure at a previous stage (i.e. germination not assessed because of a complete capsule abortion) and the *E. johnstonii* data was also removed as the capsules proved to be immature after harvest.

Seedling morphology

Because the supplementary pollination technique involved applying *E. nitens* pollen to open-pollinated flowers, seedlings gained from this method were of mixed genotype, being either self, intra- or interspecific crosses. Consequently, once healthy seedlings had grown to 10 or more nodes the number of putative *E. nitens* F_1 hybrids within these seedlots was visually assessed. This involved identifying seedlings that were outside the phenotypic range of the female species and intermediate between *E. nitens* and the maternal native parent. *E. nitens* is morphologically distinct from most native Tasmanian species in seedling morphology, particularly in the fusing of its apical bud, the large size and pronounced basal lobing of its sessile leaves and large flanges along the stem (Fig. 1). The generally intermediate nature of interspecific F_1 hybrids therefore makes them very distinct from pure species half-sibs in such cases (Tibbits 1988; Barbour *et al*. 2002, 2003). When interspecific hybrids were produced through controlled pollination techniques, these assisted in visually detecting the same hybrid phenotypes in supplementary pollinated seedlots.

Following the identification of putative *E. nitens* F_1 hybrids in the supplementary seedlots, a morphometric analysis was conducted to verify their hybridity. This analysis used seedlings of each cross-type and pollination technique randomly selected as follows: (i) 10 pure native seedlings from each female tree (from controlled outcross and/or open-pollination); (ii) one seedling from each open-pollinated *E. nitens* family; (iii) as many as 10 F_1 hybrids per pollination technique (controlled and supplementary) from each native female tree when available; (iv) as many as 10 atypical seedlings from supplementary seedlots of each native female tree when these occurred; (v) 10 randomly selected seedlings from across the supplementary seedlots of each native female tree, when F_1 hybrids were not evident in the supplementary seedlots or available from controlled pollination to verify that no hybridisation had occurred. The atypical seedlings appeared morphologically different from typical pure-species seedlings and were likely naturally occurring hybrids among local native species (i.e. not involving *E. nitens*) or extremes of the morphological range of the maternal species. Atypical seedlings were sampled to verify that they were not *E. nitens* F_1 hybrids. These atypical seedlings were not the same as abnormal seedlings as they did not lack vigour or display any form of structural deformity. Abnormal seedlings were not used in the analyses as they were not expected to survive beyond the seedling stage and therefore unlikely to contribute to the population.

Twenty-six morphological characters were used in the morphometric analysis, including all characters used by Barbour *et al*. (2003) as well as five additional characters (Appendix 2, available as accessory material on the web). Cross-type means were calculated for all characters. Raw data were then transformed where necessary to optimise normality and homogeneity of variance (Appendix 2). One-way ANOVAs were conducted to compare each native species with *E. nitens* for each character, using the transformed data. This was followed by a canonical discriminant analysis which aimed to maximise the difference among pure species cross-types. This was done by using the DISCRIM procedure of SAS (version 9.1, Cary, NC, USA). Analyses were conducted in a pair-wise manner to produce a single discriminant function that maximised the differences between each native pure

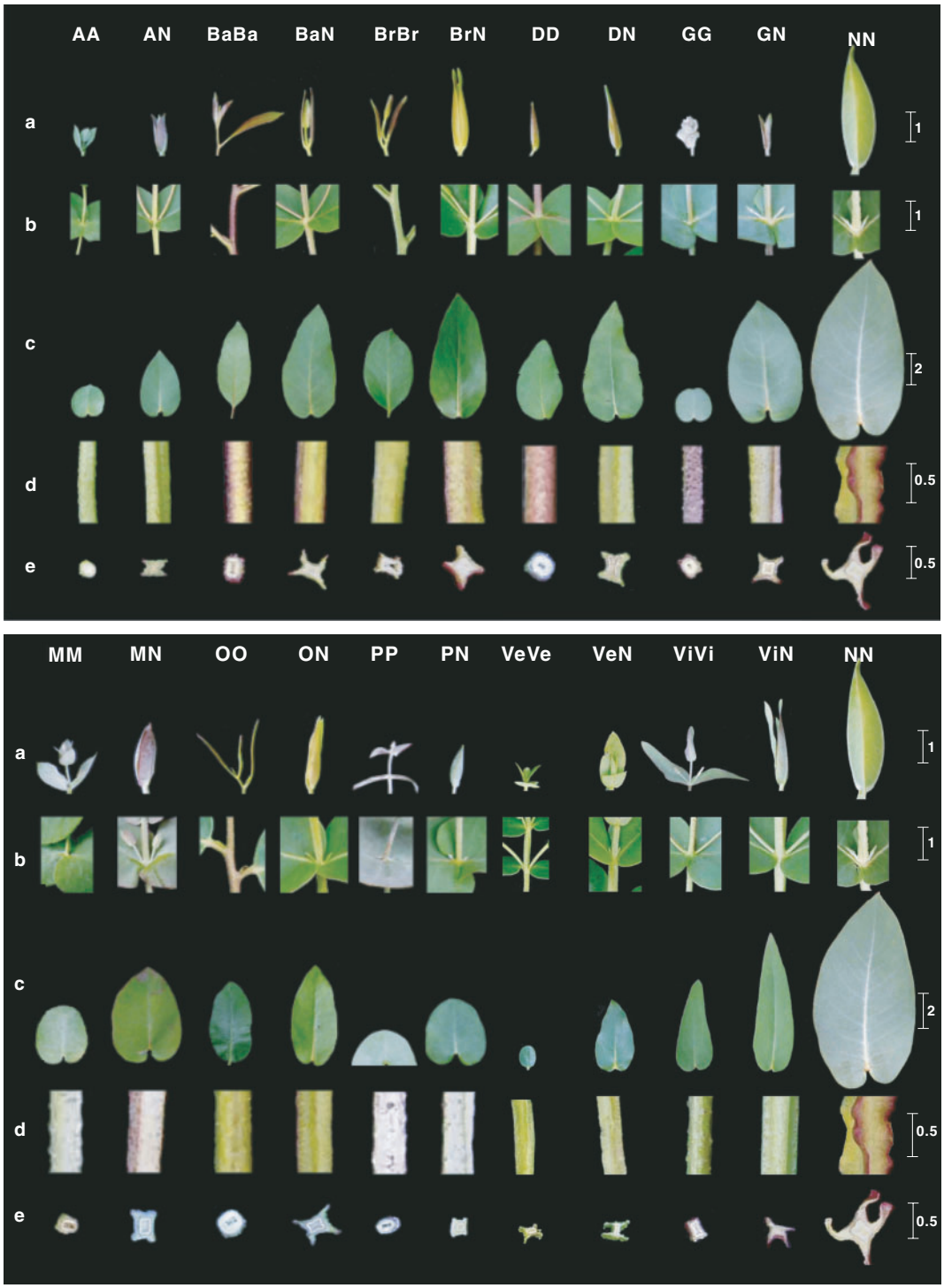

Fig. 1. Seedling morphology (at Node 10) of *Eucalyptus nitens* (NN), native Tasmanian *Symphyomyrtus* species and their F_1 hybrids with *E. nitens* as the pollen parent. (*a*) Variation in bud, (*b*) node (leaves truncated), (*c*) leaf, (*d*) longitudinal stem and (*e*) cross-sectional stem morphology is shown (scale = cm's). *Eucalyptus archeri* (AA), *E. barberi* (BaBa), *E. brookeriana* (BrBr), *E. dalrympleana* (DD), *E. gunnii* (GG), *E. morrisbyi* (MM), *E. ovata* (OO), *E. perriniana* (PP), *E. vernicosa* (VeVe) and *E. viminalis* (ViVi) are shown, combined with their F_1 hybrids (i.e. ViN = *E. viminalis* × *nitens*) produced through controlled and/or supplementary pollination.

species ((i) above) and *E. nitens* ((ii) above) for use as a hybrid index (see Barbour *et al*. 2003). The positions of the hybrids, atypical and other seedlings (i.e. (iii), (iv) and (v) above) along these discriminant functions were then calculated to determine their parentage. Some characters were dropped from the analysis of particular species combinations because of the absence of variance in one or both of the pure species (Appendix 3, available as accessory material on the web).

Results

Controlled pollination

In total, 7 of the 13 species assessed for their crossability with *E. nitens* pollen under controlled pollination produced viable hybrid seedlings (Table 1). However, across all native trees, the success of *E. nitens* pollination was significantly lower than intraspecific outcross pollination. Paired *t*-tests across species and pollination techniques indicated significantly lower number of capsules per flower ($t_{15} = 2.5$, $P < 0.05$; Out [intraspecific outcross; mean ± s.e.] = 0.31 ± 0.06; $F_1 = 0.15 \pm 0.06$), seed per capsule ($t_9 = 2.8$, $P < 0.05$; Out = 8.3 ± 1.9, $F_1 = 6.7 \pm 2.2$) and seed per flower ($t_{14} = 3.4$, $P < 0.01$; Out = 3.1 ± 0.8; $F_1 = 1.4 \pm 0.7$) with *E. nitens* than with intraspecific outcross pollination. The same significant trends were obtained when the few stigma pollination treatments were removed, ensuring that significance was not a result of the possible confounding influence of pollination technique. Across all the native trees and controlled pollination types, seed set per flower following *E. nitens* pollinations was reduced on average to 44% of that of intraspecific outcross pollinations. The poor success of interspecific hybridisation through these stages of fertilisation and seed development were seen for *E. urnigera*, for example, which produced 24 capsules from pure species outcrossing on two trees and none from pollination with *E. nitens*. In comparison, there were no significant differences between *E. nitens* and intraspecific outcrosses for the percentage of viable seeds that germinated ($t_8 = 0.6$, $P = 0.54$; Out = 73.5 ± 10.2, $F_1 = 70.5 \pm 6.9$) nor the percentage of germinants that grew to healthy seedlings ($t_7 = 1.7$, $P = 0.14$; Out = 83.3 ± 6.3, $F_1 = 66.0 \pm 8.6$). Notable, however, was the markedly lower seedling survival of the F_1 hybrids (24%) than the outcross seedlings (89%) for the one *E. dalrympleana* tree tested, which appeared to be due to cessation of growth beyond the first cotyledon.

The *E. nitens* seedlings were morphologically clearly differentiated from those of all native species, and pair-wise comparisons of means were significant for most characters assessed in the morphometric analysis (Appendices 3, 4, available as accessory material on the web). Visually, the F_1 hybrid seedlings from controlled pollinations appeared to be outside the phenotypic range of the pure-species phenotypes and intermediate between them (Fig. 1) and this was clearly evident in the discriminant analysis (Fig. 2). However, there were exceptions to the F_1 hybrid intermediacy. The *E. ovata* × *nitens* hybrids were biased towards *E. nitens* and the *E. viminalis* × *nitens* hybrids were biased towards *E. viminalis* in the discriminant space. This bias in the case of the *E. ovata* hybrids made them very distinct from their pure *E. ovata* half-sibs but even the *E. viminalis* hybrids could be visually differentiated from pure *E. viminalis* seedlings (Fig. 1).

Supplementary pollination

In this section we verify the morphology of the exotic F_1 hybrids from supplementary pollinated seedlots, so that their frequency can be calculated with certainty. The distinctive nature of the *E. nitens* F_1 hybrid morphology made for ready identification of seedlings produced from flowers that were supplementary-pollinated with *E. nitens* pollen (Figs 1, 2). The results from the supplementary pollinations were summarised by classifying the species into four groups. The first group consisted of species that produced *E. nitens* F_1 hybrids through both controlled and supplementary pollination; these were *E. archeri*, *E. barberi*, *E. gunnii* and *E. ovata*. All the putative hybrids from supplementary pollination fell within or next to the morphometric ranges described for the known hybrids from controlled pollination, therefore verifying their classification (Fig. 2). These putative F_1 hybrids fell outside the range of parental species, and the atypical seedlings from the supplementary seedlots of *E. archeri* and *E. barberi* were clearly differentiated from the F_1's and in a position consistent with them being pure species.

The second group consisted of those species that produced *E. nitens* hybrids from supplementary *E. nitens* pollination only, and not through controlled *E. nitens* pollinations; these were *E. brookeriana*, *E. perriniana* and *E. vernicosa*. For this group, the pattern of differentiation of the putative hybrids was consistent with that exhibited by the group-one hybrids, which provided verification of their hybridity, despite having no known hybrids for comparison. The putative hybrids all fell well outside the morphological range of their parental species and were generally intermediate (Fig. 2). The atypical seedlings that were identified in the supplementary seedlots of *E. brookeriana* were clearly differentiated from those phenotypes identified as F_1's and fell within the range of *E. brookeriana*. In the case of *E. perriniana*, the putative F_1's clustered together as a distinct phenotypic group. One atypical seedling fell in close proximity to this group, but its narrow and glaucous leaves suggested that it was a natural hybrid between *E. perriniana* and *E. viminalis*. The seedlots from supplementary pollination of *E. vernicosa* displayed no atypical seedlings, but the one putative F_1 hybrid showed strong bias towards the *E. nitens* and was clearly distinct from both parents, verifying its classification. No seedlings resembling the *E. nitens* F_1 hybrids identified

Table 1. Controlled pollinations using exotic *Eucalyptus nitens* pollen on native Tasmanian *Symphyomyrtus* species

For each intraspecific outcross (Out) and interspecific cross with *E. nitens* (F_1), the table shows the number of female trees and flowers pollinated, the number of capsules and viable seed set, the number of viable seed per flower and sown, the percentage of viable seed that germinated and the percentage of germinants that grew into healthy seedlings without abnormalities under glasshouse conditions (% germinants to normal seedlings). *Symphyomyrtus* species not tested with controlled pollinations were *E. globulus*, *E. perriniana*, *E. rodwayi* and *E. subcrenulata*. Superscripts indicate the number of trees pollinated by using the stigma-pollination technique, the remainder were pollinated by the cut-style technique. Discrepancies in the number of seed produced compared with that sown and germinated are explained in the text

Female species	No. of female trees	No. of flowers pollinated		No. of capsules		No. of viable seeds		No. of viable seeds per flower		No. of viable seeds sown		Percentage seed germination		Percentage germinants to normal seedlings	
		Out	F_1	Out	F_1	Out	F_1	Out	F_1	Out	F_1	Out	F_1	Out	F_1
E. archeri	1	61	62	35	8	252	27	4.1	0.4	6	27	0	26		71
E. barberi	1	41	34	20	29	154	172	3.8	5.1	93	109	63	56	63	39
E. brookeriana	1	83	81	0	0			0.0	0.0						
E. cordata	3	69	90	6	0	80		1.2	0.0	39		95		92	
E. dalrympleana	1[1]	279	334	26	21	101	70	0.4	0.2	42	32	86	66	89	24
E. gunnii	1	54	49	40	13	338	53	6.3	1.1	45	45	62	64	93	93
E. johnstonii	1	48	34	9	2	0	0	0.0	0.0						
E. morrisbyi	2[2]	194	306	94	110	1786	2121	9.2	6.9	128	140	94	89	89	81
E. ovata	2	111	208	19	8	83	26	0.7	0.1	25	20	92	75	100	53
E. rubida	1	0	81		3		1		0.01		1		0		
E. urnigera	2	46	96	24	0	150		3.3	0.0	78		15		58	
E. vernicosa	1	34	53	4	0	13		0.4	0.0	0					
E. viminalis	3[2]	609	767	61	90	292	245	0.5	0.3	106	137	81	85	77	79

in the supplementary seedlots were recorded among the seedlings grown from open-pollinated seeds from each tree, indicating they were unlikely to be natural hybrids (also applies for species in group one).

The species in the third group, *E. dalrympleana* and *E. viminalis*, were those that produced *E. nitens* F_1 hybrids through controlled pollination but not through supplementary pollination with *E. nitens* pollen (Table 2). None of the randomly selected or atypical seedlings from these supplementary-pollinated seedlots fell within the phenotypic range of the known F_1 hybrids (Fig. 2). This pattern of crossability could arise where competition experienced in supplementary pollination prevents interspecific hybridisation. However, in these specific cases the evidence for a barrier under supplementary pollination is weak as different individual trees were used for the control and supplementary pollinations, and the number of seedlings grown was small.

The fourth group consisted of species that did not produce *E. nitens* F_1 hybrids, either by using controlled or supplementary methods, and again required verification that no *E. nitens* hybrids were present in the supplementary seedlots. This group consisted of *E. cordata*, *E. johnstonii*, *E. rodwayi*, *E. rubida*, *E. subcrenulata* and *E. urnigera.* Morphometric analysis of the atypical and randomly selected seedlings from the supplementary seedlots of each species indicated that they were very similar to the pure native species. A few seedlings from the *E. johnstonii*, *E. rodwayi* and *E. rubida* supplementary seedlots deviated slightly towards *E. nitens*. Nevertheless, these plants were very biased towards the maternal parents and represented extremes of a continuous distribution rather than a distinct phenotype. This left little doubt of their classification. Combined with this, F_1 hybrids between *E. rubida* and *E. nitens*, for example, would be expected to be morphologically similar to those between *E. gunnii* and *E. nitens* because of the similar juvenile morphology of *E. rubida* and *E. gunnii*. However, no seedlings resembling this F_1 phenotype were present.

Only 7 of the 15 species assessed for their crossability with *E. nitens* through supplementary pollination produced *E. nitens* F_1 hybrids (Table 2). The highest proportion of F_1 hybrid seedlings was obtained for *E. brookeriana* (32%) and *E. ovata* (26%), with both trees of each species tested producing hybrids. These species belong to the subsection *Triangulares* (Brooker 2000). No hybrids were found with *E. rodwayi*, and only one hybrid seedling was found with *E. barberi*, yet both species also belong to this subsection. However, only a small quantity of seeds from one *E. rodwayi* female was tested. The other species tested were taxonomically more closely related to *E. nitens* as they belonged to the same subsection (subsection *Euryotae*) but displayed low levels (0–11%) of hybridisation when supplemented with *E. nitens* pollen. However, in some cases the sample sizes tested were low (Table 2). Indeed, the subalpine species *E. vernicosa*, *E. subcrenulata* and *E. johnstonii* were poorly tested owing to difficulties in judging when capsules were ripe.

Discussion

Artificial pollination has shown that the exotic *E. nitens* pollen can hybridise with 10 of Tasmania's 17 native *Symphyomyrtus* species. Seven of these species were successfully crossed under supplementary pollination, indicating that *E. nitens* can effectively compete with natural pollen in these cases. F_1 hybrids derived from pollination of *E. gunnii* and *E. perriniana* females with *E. nitens* pollen have previously been reported, as have F_1 hybrids from pollination of *E. nitens* females with *E. cordata*, *E. dalrympleana*, *E. gunnii*, *E. johnstonii*, *E. morrisbyi*, *E. ovata*, *E. perriniana*, *E. rodwayi*, *E. rubida*, *E. urnigera* and *E. viminalis* (Table 3). Two of the hybrid combinations produced with *E. nitens* pollen in the current work, *E. ovata* × *nitens* and *E. viminalis* × *nitens*, have been recorded as naturally occurring exotic hybrids. The *E. nitens* F_1 hybrids with *E. archeri*, *E. barberi*, *E. brookeriana* and *E. vernicosa* have not been previously reported, and hybrids with *E. dalrympleana* and *E. morrisbyi* have not been reported with *E. nitens* as the pollen parent. This study has shown that at least half of Tasmania's native *Symphyomyrtus* species have the potential to successfully hybridise to some extent with *E. nitens*, and are therefore potentially at risk of exotic gene flow if pollinated by *E. nitens*. However, further assessments by the supplementary-pollination technique are now necessary to better characterise crossability under natural conditions when *E. nitens* pollen is competing with intraspecific pollen. This is particularly the case where no or low levels of hybridisation were found in the current study.

Identification of exotic F_1 hybrids is a key issue when using the supplementary pollination procedure to assess crossability. In *Eucalyptus*, F_1 hybrids are generally intermediate in morphology, and hybrids between parents that differ substantially in morphology are easily identified from pure parental species (Wiltshire and Reid 1987; Tibbits 1988; Delaporte *et al*. 2001*a*, 2001*b*, 2001*c*; Barbour *et al*. 2003). These characteristics have been frequently used as a tool for their identification among pure-species siblings (Pryor 1976; Wiltshire and Reid 1987; Potts and Reid 1988, 1990; Delaporte *et al*. 2001*c*; Barbour *et al*. 2002, 2003). F_1 hybrids that are slightly biased in overall morphology towards one parent, such as seen in *E. ovata* and *E. viminalis*, have also been reported (Tibbits 1988; Delaporte *et al*. 2001*b*; Barbour *et al*. 2003), but in all cases the F_1 hybrids still lay outside the morphological range of their parents. This easy detection allows for simple monitoring and field identification of exotic hybrids

(Barbour *et al*. 2003). However, for F_1 hybrids between species with similar morphology, such as *E. nitens* × *globulus*, the distinction between the F_1 hybrids and their parent species becomes less clear (Tibbits 1988), making reliable visual identification of such hybrids difficult (Espejo *et al*. 2004). In such cases, molecular (Barbour *et al*. 2002) or chemical (Espejo *et al*. 2004) identification of F_1 hybrids may prove necessary. Indeed, the likelihood

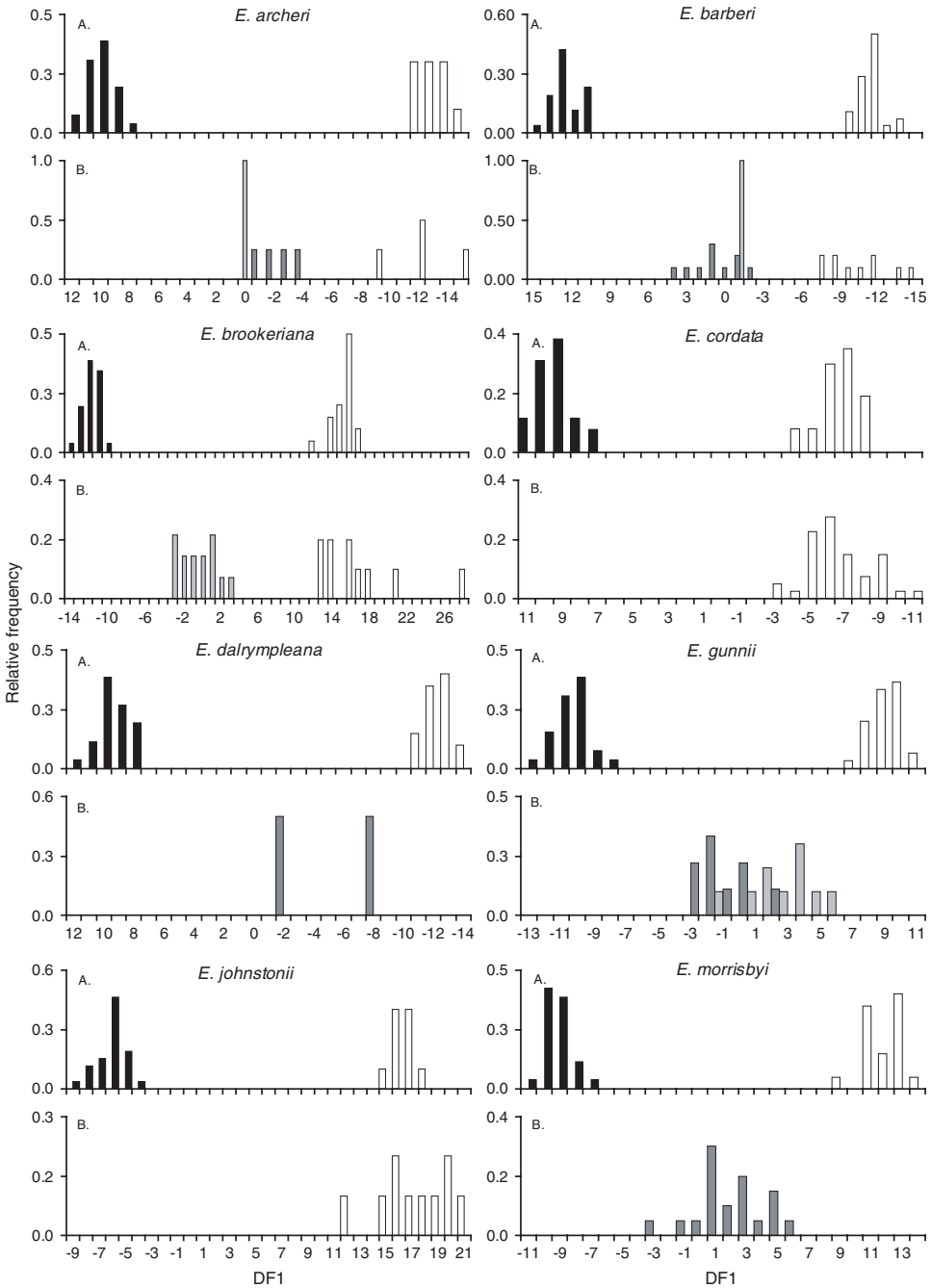

Fig. 2. Discriminant functions differentiating seedlings of *Eucalyptus nitens* and native Tasmanian *Symphyomyrtus* species. The histograms show the relative frequency distribution of seedlings of each cross-type along discriminant function (DF1) separating the pure species. (*a*) Each analysis was conducted pair-wise comparing *E. nitens* (black bars) with each native species (open bars). (*b*) The position of F_1 hybrids from controlled (dark grey bars) and supplementary pollination (light grey bars) and any atypical or randomly selected seedlings from supplementary seedlots (open bars) along the discriminant function was then calculated.

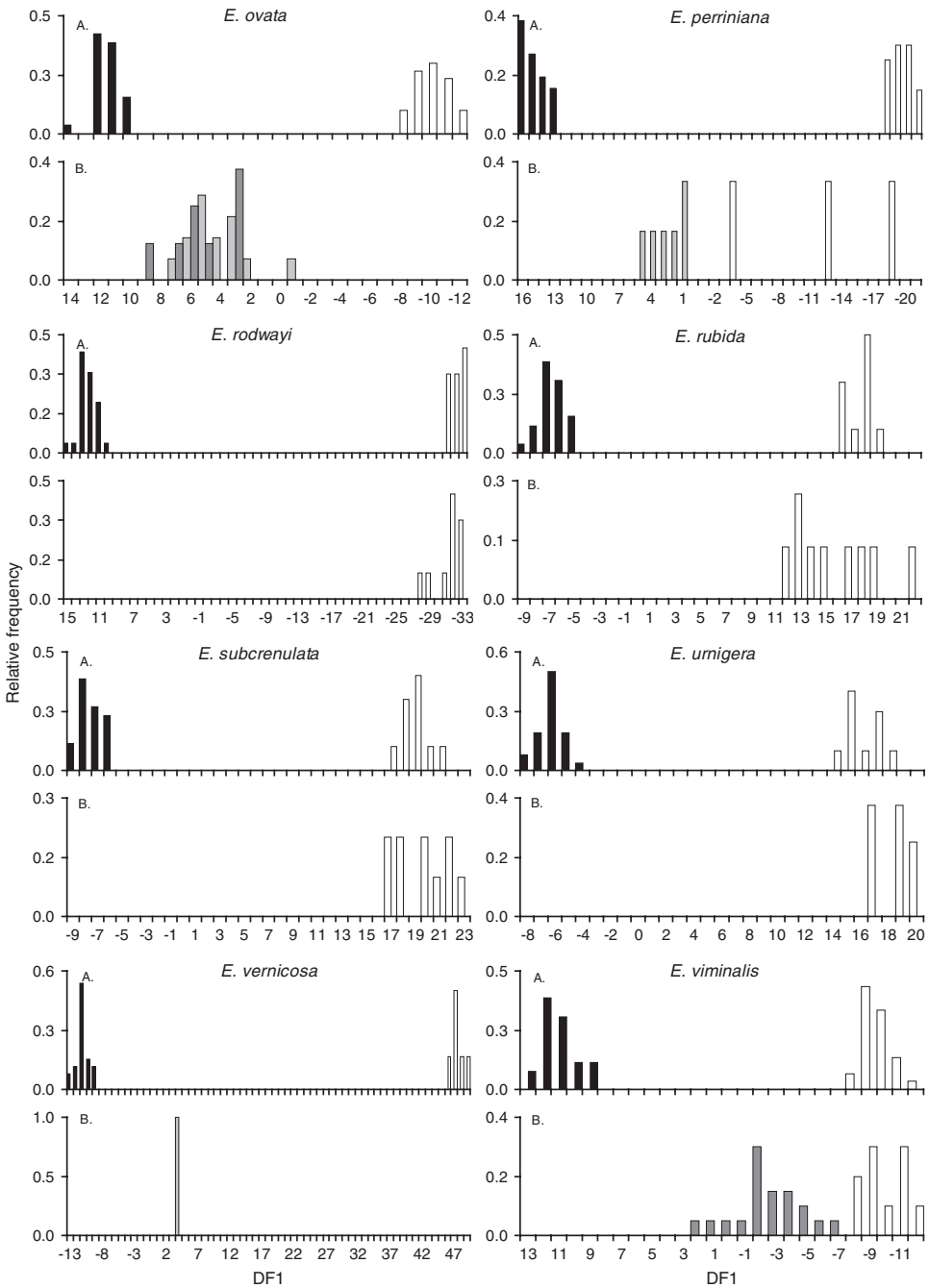

Fig. 2. (*continued*)

of detecting species-specific markers that differentiate an exotic from a local native species is high (Steane *et al*. 1998), as they would have evolved in allopatry.

Interestingly, two of the four species taxonomically classified as more distantly related to *E. nitens* (subsection *Euryotae*), namely *E. brookeriana* and *E. ovata* (subsection *Triangulares*) (Brooker 2000), displayed higher levels of F_1 hybridisation under supplementary pollination than species within the same subsection as *E. nitens*. This contrasts with previous reports of a trend for poorer cross success with increasing taxonomic distance between parents (Griffin *et al*. 1988; Ellis *et al*. 1991; Tibbits 2000; Delaporte *et al*. 2001*b*). However, this trend is typically based on comparisons involving crosses between more distantly related species than assessed in the present study.

Despite F_1 hybrid seedlings being obtained following pollination of most native Tasmanian *Symphyomyrtus* species with *E. nitens*, there appears to be a partial

Table 2. Supplementary pollination using exotic *Eucalyptus nitens* pollen on native Tasmanian *Symphyomyrtus* species

The table shows the number of female trees and flowers pollinated, capsules and viable seed produced, viable seed sown, and seedlings that grew to an assessable height (*n* seedlings). The percentage of seedlings that were F_1 hybrids with *E. nitens*, and the number of female trees that produced such hybrids compared with how many were tested, is also shown. The number of female trees pollinated and the number tested differ in some cases, as mature seed was not obtained from some trees. The taxonomic series of each species following Brooker (2000) is shown in superscript ([F]*Foveolatae*, [O]*Orbiculares*, [S]*Semiunicolores*, [V]*Viminales*). Seedlots with low seed number were not sown. The only two *Symphyomyrtus* species not tested were *E. globulus* and *E. morrisbyi*

Species	No. of female trees	No. of flowers pollinated	No. of capsules	No. of viable seed	No. of viable seed sown	No. of seedlings	Percentage of *E. nitens* hybrid seedlings	No. of ♀ trees hybridising/no. of ♀ trees tested
E. archeri[O]	3	259	124	98	96	20	5.0	1/2
E. barberi[F]	2	200	82	459	405	294	0.3	1/2
E. brookeriana[F]	2	186	89	289	283	232	31.5	2/2
E. cordata[O]	4	279	87	913	855	575	0.0	0/4
E. dalrympleana[V]	2	272	81	187	139	111	0.0	0/1
E. gunnii[O]	4	382	220	1133	1028	748	6.1	1/3
E. johnstonii[S]	3	186	77	156	155	42	0.0	0/2
E. ovata[F]	2	283	50	104	99	86	25.6	2/2
E. perriniana[O]	2	155	109	418	413	334	1.8	2/2
E. rodwayi[F]	3	322	68	43	36	32	0.0	0/1
E. rubida[V]	1	70	30	31	31	19	0.0	0/1
E. subcrenulata[S]	1	67	67	176	148	31	0.0	0/1
E. urnigera[O]	1	33	2	23	23	7	0.0	0/1
E. vernicosa[S]	2	101	29	40	37	9	11.1	1/2
E. viminalis[V]	1	118	58	74	72	66	0.0	0/1

Table 3. Published F_1 hybrid combinations involving *Eucalyptus nitens* as either the seed or pollen parent with the native Tasmanian eucalypt species

Successful hybridisation and verified crossing incompatibilities are provided (numbers refer to citations in table footer), as well as the pollination technique used in the current work that resulted in successful hybridisation (controlled, C; supplementary, S). All previously published hybrid combinations were produced through controlled stigma pollination, with the exception of those in Barbour *et al*. (2002) which were from natural pollination and detected among open-pollinated seedlots. 1, Cauvin *et al*. (1987); 2, Griffin *et al*. (1988); 3, Tibbits (1989); 4, Gore *et al*. (1990); 5, Ellis *et al*. (1991); 6, Potts *et al*. (1992); 7, Espejo *et al*. (1995); 8, Tibbits (2000); 9, Barbour *et al*. (2002); 10, Barbour *et al*. (2003); 11, Barbour *et al*. (2005)

Tasmanian native species	*E. nitens*	
	Female (seed parent)	Male (pollen parent)
Subgenus Eucalyptus		
All 12 species	Incompatible, 2, 5	Incompatible, 2, 5
Subgenus Symphyomyrtus		
E. archeri		C, S
E. barberi		C, S
E. brookeriana		S
E. cordata	3	
E. dalrympleana	3	C
E. globulus	3, 6, 7, 8	4 (incompatible)
E. gunnii	3, 8	1, C, S
E. johnstonii	3	
E. morrisbyi	3	C
E. ovata	3, 8	9, 10, 11, C, S
E. perriniana	8	8, S
E. rodwayi	3	
E. rubida	8	
E. subcrenulata		
E. urnigera	8	
E. vernicosa		S
E. viminalis	3, 10	10, C

barrier to hybrid seed set. Across all species, controlled *E. nitens* pollinations produced significantly less viable seed per flower than intraspecific outcross pollinations. This reduction was evident even in our controlled crosses with *E. ovata*, despite this combination displaying some of the highest levels of hybridisation following supplementary pollination. Reduced seed set in interspecific *v.* intra-specific crosses has been reported in eucalypts. Such reduced seed set may be due to pre-fertilisation barriers arising from an inability of *E. nitens* pollen tubes to reach the ovaries because of structural (Gore *et al*. 1990; Delaporte *et al*. 2001*b*) or physiological incongruity (Ellis *et al*. 1991), or because of post-fertilisation barriers causing zygote or seed abortion (Sedgley and Granger 1996). Structural style-length barriers may be limiting the crossability of larger flowered species such as *E. cordata* and *E. urnigera* with *E. nitens* pollen, as these species produced no hybrids under controlled or supplementary pollination, yet crosses have been successful using *E. nitens* as the female (Tibbits 1989, 2000). Most pollinations undertaken in the current work used the cut-style technique, which involved removal of the stigma and upper-style. Therefore, this method may underestimate post-pollination barriers between species, as stigmatic and upper-stylar barriers have not been assessed. Nevertheless, most barriers to seed set between species from the same eucalypt subgenera occur after this point (Ellis *et al*. 1991; Sedgley and Granger 1996). Even when controlled pollinations are successful, other barriers may be revealed under natural pollination owing to pollen or zygote competition with intraspecific crosses (Klips 1999; Ramsey *et al*. 2003; Vanden Broeck *et al*. 2003); however, such barriers have not been studied in *Eucalyptus*.

There was little overall evidence of reduced seed germination and early seedling growth in F_1 hybrids compared with the native species outcrosses. Some F_1 hybrid crosses did show atypically poor germination (e.g. *E. dalrympleana* and *E. ovata*) or high levels of seedling abnormality and death (e.g. *E. barberi*, *E. dalrympleana* and *E. ovata*). However, there was insufficient power in our experiment to statistically test specific differences. Significant levels of seedling abnormality and mortality have been reported in other interspecific F_1 hybrids of *Eucalyptus* (Potts *et al*. 1992; Lopez *et al*. 2000; Meddings *et al*. 2003). This was the case for a number of the F_1 hybrid crosses involving *E. nitens* females and Tasmanian *Symphyomyrtus* species (Tibbits 1988: mean % of seedlings dead or grown with abnormalities, F_1 crosses = 14%, intraspecific outcrosses = 6%). In these studies and in the current work, pre-dispersal stages of gene flow were tested under natural conditions as developing capsules on trees in the wild or in gardens, whereas the post-dispersal stages were assessed in the glasshouse and/or nursery as germinating seeds and young seedlings. Such growing conditions place little exogenous (environmental) pressure on the performance of each cross-type, and may overestimate the relative fitness of the hybrids compared with those in the wild (Arnold 1997; Potts *et al*. 2003).

The current work provides biological data on the crossability of *E. nitens* pollen with native Tasmanian *Symphyomyrtus* species. This shows that a number of native species can potentially hybridise with *E. nitens*. However, crossability represents just one step in the process of gene flow and introgression of exotic plantation genes into native-species gene pools. Assessments of other steps in the process of gene flow such as the pollen dispersal patterns from plantations (Barbour *et al*. 2005), spatial proximity (Barbour 2004), flowering asynchrony (Barbour 2004) and the fitness of first- and later-generation hybrids relative to native eucalypt species (Barbour *et al*. 2003; Potts *et al*. 2003) have also been conducted or are currently underway. As barriers to gene flow can occur at one or more of these steps, future work aims to combine these studies in an overall assessment of the risks of exotic gene flow from *E. nitens* plantations in Tasmania. For example, of the 10 native *Symphyomyrtus* species shown to hybridise with *E. nitens* pollen, populations of *E. gunnii*, *E. brookeriana*, *E. ovata* and *E. perriniana* occur in close proximity to *E. nitens* plantations. These species also overlap in their flowering time with *E. nitens* (Barbour 2004), indicating they are at risk of exotic gene flow. However, other species such as *E. barberi* and *E. morrisbyi* that were also found to hybridise with *E. nitens* following artificial pollination, are spatially and temporally (because of flowering asynchrony) isolated from *E. nitens*, and are therefore at low risk of exotic gene flow.

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