The influence of pre- and post-zygotic barriers on interspecific Corymbia hybridization

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INTRODUCTION

Hybridization results in gene flow between individuals and contributes to the evolutionary process of plant speciation (Hegarty and Hiscock, 2005; Poke et al., 2006; Rieseberg and Blackman, 2010). Hybridization can also cause genetic pollution, threatening the integrity of native species via introgression of genes from planted exotic species into sympatric populations (Potts et al., 2003; Barbour et al., 2008, 2010). The eucalypts are genetically diverse, comprising >700 species within three genera: Eucalyptus and sister genera Angophora and Corymbia (Hill and Johnson, 1995). Many eucalypt species are of global economic importance, with >19 Mha established as timber plantations (Iglesias-Trabado and Wiltshire, 2008). Maintenance of species integrity relies on reproductive isolation between interfertile species. Reproductive isolating barriers within the Eucalyptus–Angophora–Corymbia group become more effective with increasing taxonomic distance between parents; for example intraseries crosses are more successful than interseries crosses (Griffin et al., 1988; Ellis et al., 1991). Hence the taxonomic affinity of the two parental eucalypt species predicts the likelihood of two species forming hybrids (Pryor, 1951; Ellis et al., 1991). Cases of unusual natural hybridization (Potts and Wiltshire, 1997; Stokoe et al., 2001) or results from experimental hybridization (Heslop-Harrison, 2010) can indicate areas where taxonomic revision may be required. Species integrity relies on key reproductive isolating barriers to inhibit hybridization. In native eucalypt populations, these barriers are strong, and only 15% of expected combinations have been recorded, often from only a single herbarium record (Griffin et al., 1988). These barriers occur as environmental isolating barriers or endogenous structural and physiological isolating barriers (Potts and Wiltshire, 1997). Environmental isolating barriers include differences in flowering times, dissimilar pollen vectors and geographic distance.

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between otherwise compatible species (Griffin et al., 1988; Potts and Wiltshire, 1997; Shepherd et al., 2008). Endogenous reproductive isolation encompasses both incompatibility and incongruity, which may act simultaneously (De Nettancourt, 1984). Incompatibility involves reproductive failure whereby an active mechanism interferes with an otherwise compatible cross (McClure et al., 2000). Incongruity occurs when the pollen–pistil interaction becomes less successful with increasing taxonomic distance between the two parental species, resulting in greater reproductive failure (Hogenboom, 1975).

Physiological isolation is triggered by genetic combining irregularities between the male and female parents which interrupt or impede the reproductive and development process. This can result in poor adhesion of pollen grains to the stigma surface (Rouger et al., 1988), inhibition of pollen germination and pollen tube growth (Potts and Marsden-Smedley, 1989), the development of abnormal or directionless pollen tubes (Ellis et al., 1991; Tangmitcharoen and Owens, 1997), failure of pollen tubes to penetrate the ovule (Sedgley and Smith, 1989; Ellis et al., 1991), poor development or abortion of embryos (Sedgley and Granger, 1996; Pound et al., 2003; Suitor et al., 2008) and abnormal growth or death in germinated seed and juvenile plants (Potts and Dungey, 2004). Structural isolating mechanisms often arise through disparity in flower size, whereby the pollen tubes of a small flowered species are unable to grow the full length of the style of larger species (Gore et al., 1990). The locations of endogenous reproductive barriers are influenced by parental interactions (Pound et al., 2003; Suitor et al., 2009), with poorly compatible crosses often failing earlier in the reproductive process (Waser and Price, 1991). Pollen tube arrest in the style and failure of pollen tubes to penetrate ovules in the ovary are major locations of interspecific reproductive isolation between eucalypt species (Ellis et al., 1991).

Corymbia (bloodwoods and ghost gums) is a genus that was segregated from Eucalyptus by Hill and Johnson (1995). Rather than accept the new genus, Brooker (2000) lumped bloodwoods and the related genus Angophora into Eucalyptus sensu lato, although he recognized some similar groups at the subgeneric level. Molecular phylogenetic evidence (Ladiges and Udovicic, 2000; Steane et al., 2002; Parra-O et al., 2006, 2009) has shown that Angophora and Corymbia form a clade, sister to Eucalyptus sensu stricto, and the three genera are now accepted by all Australian herbaria.

Hill and Johnson (1995) informally recognized seven Corymbia sections within two infrageneric clades, Blakearia + Ochtraria + Cadagaria + Pollicitaria and Rufaria + Apteria + Fundoria. Taxonomic revision by Parra-O et al. (2009), using molecular and morphological characters, has since provided resolution of relationships within Corymbia and formally recognized new taxonomic categories and names. This formal classification supports the monophyly of Corymbia and elevates the two clades recognized by Hill and Johnson (1995) to subgeneric level: subgenus Blakeella (sections Abbreviata, Naviculares, Torellianae and Maculatae) and subgenus Corymbia (sections Calophyllae, Corymbia and Septentrionales). Sections within subgenus Corymbia were also revised by Parra-O et al. (2009) to recognize monophyletic groups. These authors found that, of Hill and Johnson’s sections, Apteria and Fundoria were nested in Rufaria; the revised classification of subgenus Corymbia now includes three sections, in phyletic sequence: Calophyllae, Corymbia and Septentrionales. A phylogenetic tree illustrating taxonomic relationships between infrageneric Corymbia clades (modified from Parra-O et al., 2009) is included as Fig. 1.

Close relationships between infrageneric Corymbia clades are reflected in the unusually high propensity for Corymbia species to form hybrids across taxonomic groups (Griffin et al., 1988), with many examples of intersectional, interseries and intraseries hybrids (Hill and Johnson, 1995; Queensland Herbarium, 2002; Sedgley and Delaporte, 2004; Lee et al., 2009; Dickinson et al., 2010). Wide crosses between species from subgenera Blakeella and Corymbia are recognized from natural populations (Septentrionales × Maculatae; Hill and Johnson, 1995) and controlled pollination studies (Septentrionales × Maculatae, Septentrionales × Naviculares and Maculatae × Septentrionales; Sedgley and Delaporte, 2004). Interest in Corymbia hybrids is increasing as they offer great promise for hardwood plantation forestry in Australia and overseas (Assis, 2000; Lee et al., 2007, 2009, 2010; Verma and Sharma, 2011).

Corymbia hybrids for forestry use are principally derived using C. torelliana (sect. Torellianae) as the maternal parent and spotted gum taxa (sect. Maculatae), primarily C. citriodora subsp. citriodora, C. citriodora subsp. variegata or C. henryi, as the pollen parent (Dickinson et al., 2010). These hybrids have advantages over their parental species, including resilience to the disease Quambalaria (Lee, 2007; Lee et al., 2009), which is a major impediment to Corymbia plantations across eastern Australia (Dickinson et al., 2004; Pegg et al., 2009). Manipulated Corymbia hybrids derived using the large-flowered species C. ptychocarpa and C. ficifolia (Henry, 1995; Sedgley and Delaporte, 2004) also have commercial value for amenity horticulture and the floriculture industry.

![Fig. 1. Phylogenetic tree of infrageneric Corymbia clades, based on a combined analysis of nuclear rDNA (ETS + ITS) and morphological characters (modified from Parra-O et al., 2009).](https://academic.oup.com/aob/article-abstract/109/7/1215/101509)
This study examines the effects of taxonomic affinity on the hybridization success of *C. torelliana* with 16 related species, representing six of the seven *Corymbia* sections, both *Corymbia* subgenera and single species from *Angophora* and *Eucalyptus*. Pollen–pistil interactions were also examined from selected crosses, to identify the locations of pre-zygotic or post-zygotic isolating barriers, which may inhibit successful hybridization. The results of this study provide further understanding of the relationships between infrageneric *Corymbia* clades and possible new genetic combinations with characteristics desirable for plantation forestry, floriculture or amenity uses.

**MATERIALS AND METHODS**

*Parental plants*

From 2005 to 2008, a total of 16 intra- and interspecific crosses were conducted across five experiments, via controlled pollination (Table 1). In all crosses, *Corymbia torelliana* was used as the maternal parent and the *C. torelliana × C. torelliana* cross was used as a control. In expts 1, 2, 3, 4 and 5, a total of two, three, four, five or five maternal parent trees were used, respectively. All maternal parent trees were large (>8 m tall) and were selected from amenity plantings of unknown genetic origin, near Mareeba, 17.00°S, 145.43°E, Queensland, Australia.

Experiment 1 identified the location of barriers to interspecific reproductive success via pollen, pollen tube, embryo and seed measurements, using selected pollen parents (*C. torelliana, C. citriodora* subsp. *citriodora, C. tessellaris* and *C. intermedia*) from four *Corymbia* sections. Experiments 2–5 assessed the reproductive success of many interspecific crosses, via measurement of capsule retention and seed yields. Pollen parents included 14 *Corymbia* taxa, representing six of the seven *Corymbia* sections, and one species each from the related genera, *Angophora* and *Eucalyptus*.

Pollen from each paternal parent was collected from 2–6 individuals per taxa. Flowers were collected prior to opening, placed in vases in the laboratory and anthers harvested after operculum shedding. The pollen was then extracted, dried for 72 h in a silica-gel desiccator and stored in gel capsules at 4°C until required. A pollen polymix was made for each parent species, with pollen viability confirmed 2 weeks prior to pollination, using the methods described by Moncur (1995).

**Table 1. Pollen parent details for the 16 crosses made with the *C. torelliana* maternal parent in expts 1–5**

<table>
<thead>
<tr>
<th>Genus</th>
<th>Subgenus</th>
<th>2009 system</th>
<th>1995 system</th>
<th>Species and authority</th>
<th>Provenance</th>
<th>Expt no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Torelliana</td>
<td>Cadagaria</td>
<td><em>C. torelliana</em> (F. Muell.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Amenity (Mareeba, Qld.)</td>
<td>X X X X X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Maculatae</td>
<td>Politaria</td>
<td><em>C. citriodora</em> (Hook.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Herberton, Qld.</td>
<td>X X X X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Maculatae</td>
<td>Politaria</td>
<td><em>C. citriodora</em> subsp. <em>variegata</em> (F. Muell.) A.R. Bean &amp; M.W. McDonald</td>
<td>Woondum, Qld.</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Maculatae</td>
<td>Politaria</td>
<td><em>C. henryi</em> (S.T. Blake) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Seed orchard (Tuan, Qld.)</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Maculatae</td>
<td>Politaria</td>
<td><em>C. maculata</em> (Hook) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Seed orchard (Holbrook, NSW)</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Abbreviatae</td>
<td>Blakearia</td>
<td><em>C. dallachiana</em> (Benth.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Mareeba, Qld.</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Abbreviatae</td>
<td>Blakearia</td>
<td><em>C. tessellaris</em> (F. Muell.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Tolga, Qld.</td>
<td>X X X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Blakella</em></td>
<td>Naviculares</td>
<td>Ochtraria</td>
<td><em>C. leichhardtii</em> (F.M. Bailey) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Watsonville, Qld.</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Corymbia</em></td>
<td>Septentronics</td>
<td>Apteria</td>
<td><em>C. trachyploia</em> (F. Muell.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Clairview, Qld.</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Corymbia</em></td>
<td>Septentronics</td>
<td>Rafaria</td>
<td><em>C. erythrophloia</em> (Blakely) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Watsonville, Qld.</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Corymbia</em></td>
<td>Septentronics</td>
<td>Rafaria</td>
<td><em>C. intermedia</em> (R.T. Baker) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Yungaburra, Qld.</td>
<td>X X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Corymbia</em></td>
<td>Septentronics</td>
<td>Rafaria</td>
<td><em>C. pytchocarpa</em> (F. Muell.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Amenity (Mareeba, Qld.)</td>
<td>X</td>
</tr>
<tr>
<td><em>Corymbia</em></td>
<td><em>Corymbia</em></td>
<td>Calophyllae</td>
<td>Rafaria</td>
<td><em>C. ficifolia</em> (F. Muell.) K.D. Hill &amp; L.A.S. Johnson</td>
<td>Amenity (Adelaide, SA)</td>
<td>X</td>
</tr>
<tr>
<td><em>Angophora</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td><em>A. floribunda</em> (Smith) Sweet</td>
<td>Seed orchard (Ravenshoe, Qld.)</td>
<td>X</td>
</tr>
<tr>
<td><em>Eucalyptus</em></td>
<td><em>Symphyomyrtus</em></td>
<td>–</td>
<td>–</td>
<td><em>E. pellita</em> F. Muell</td>
<td>Seed orchard (Cardwell, Qld.)</td>
<td>X</td>
</tr>
</tbody>
</table>

*Corymbia* sections are listed using the formal classification system of Parra-O et al. (2009) and the informal names assigned by Hill and Johnson (1995).
Pollinations

Controlled pollinations were carried out between August and October, using the conventional pollination method (Van Wyk, 1977; Dickinson et al., 2010) for expt 1 and the one-stop pollination method (Harbard et al., 1999; Dickinson et al., 2010) for expts 2–5. All flowers were accessed using an 8 m elevated platform. In expt 1, each cross treatment was conducted on three flower bunches on each of the two maternal parents. Samples were collected 1 and 5 weeks after pollination by randomly harvesting 3–4 capsules per bunch. These were pooled to give ten samples per cross treatment for each maternal parent, thus giving 20 replicates per harvest. All remaining capsules for each maternal parent were harvested at maturity and assessed individually. In expt 2, each cross treatment was conducted on three flower bunches on each of the three maternal parents, giving nine replicates. In expts 3–5, each cross treatment was conducted on a single flower bunch on each maternal parent, giving four, five or five replicates, respectively.

Flower bunches were selected prior to pollination, if most buds were yellow and within 0–3 d of natural operculum lift. Open flowers and immature, over-ripe and excessive buds were then removed, retaining approx. 50–100 buds per bunch. The retained flowers were emasculated using pollination pliers. Flower bunches pollinated using the conventional pollination method (expt 1) were covered with an exclusion bag. They were pollinated approx. 7 d later when the stigmas had visible exudate. Exclusion bags were re-applied and retained for a further 7 d, then removed. Flower bunches pollinated using the one-stop pollination method (expts 2–5) had the styles cut below the stigma using a scalpel blade and the pollen applied to the cut style surface with a match stick. Pollinated flowers were covered with a polyester exclusion bag for 14 d, to prevent pollen contamination from other sources.

Pistil sampling and measurement

Experiment 1 samples were collected 1 week after pollination for measurement of pollen adhesion and germination, pollen tube growth and embryo fertilization. Ten flowers per cross treatment were collected for each maternal parent, fixed in Carnoy’s solution [60 % ethyl alcohol (95 %), 30 % chloroform, 10 % glacial acetic acid], and stored at 4 °C, until assessment. Samples were rinsed in distilled water three times then softened by autoclaving at 121 °C for 20 min in a solution of 0.8 N NaOH. Samples were then rinsed in distilled water and stained in a solution of decolourized aniline blue (DAB), for a minimum of 48 h.

Pistils were dissected from the developing capsule and cut longitudinally using a scalpel blade, exposing the transmitting tissue and pollen tubes for pollen tube measurement. Pistils were then squashed onto slides with the cut surface of both halves facing upright and viewed under fluorescence using a Zeiss Axioskop (2 MOT) microscope. A sub-section comprising 33 % of the stigma surface was examined for each sample, the number of germinated and non-germinated pollen grains counted (Fig. 2A) and the percentage germination calculated. The transmitting tissue in the middle section of the style was then examined (Fig. 2B) and the number of pollen tubes counted. One of the three locules in each flower was randomly selected and the ovules dissected and counted using a stereo microscope. Ovules were then mounted on a slide and viewed using fluorescent microscopy. Fertilized ovules were identified where pollen tubes were observed to have penetrated the ovule micropyle (Fig. 2C).
TABLE 2. Pre-zygotic reproductive success for pollen adhesion, germination, pollen tube growth and ovule penetration (7 d after pollination) and post-zygotic reproductive success for embryo development (5 weeks after pollination) and seed production (12 weeks after pollination), for the four crosses made with the C. torelliana maternal parent in expt 1

<table>
<thead>
<tr>
<th>Pollen parent</th>
<th>Subgenus, section</th>
<th>Pre-zygotic</th>
<th>Post-zygotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pollen grains on stigma</td>
<td>Pollen germination (%)</td>
</tr>
<tr>
<td>C. torelliana</td>
<td>Blakella, Torellianae</td>
<td>326&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. citriodora</td>
<td>Blakella, Maculatae</td>
<td>223&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>55.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. tessellaris</td>
<td>Blakella, Abbreviatae</td>
<td>275&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>49.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. intermedia</td>
<td>Corymbia, Septentrionales</td>
<td>160&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.033</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Treatment means with different letters are significantly different (P < 0.05).

Ovule sampling and measurement

In expt 1, ten developing capsules per cross treatment were harvested at 5 weeks after pollination, fixed in FPA50 [90 % ethyl alcohol (50 %), 5 % formalin, 5 % propionic acid] and stored at 4 °C until required. Capsules were dissected and the number and size of developing embryos were assessed. Developing embryos were distinguished from other embryos by size (>700 μm) and appearance, with developing embryos well hydrated and light brown/yellow in colour (Fig. 2D).

Capsule retention and seed yields

Capsules in expt 1 were harvested at maturity 12 weeks after pollination, dried individually, seed extracted and the seed number per capsule measured. Capsules in expts 2–5 were assessed for capsule retention per bunch, 2 weeks after pollination and at maturity 10–14 weeks after pollination. At maturity, capsules were harvested and air-dried for a minimum of 7 d. Seed was then extracted and the seed number per capsule calculated. The results of capsule retention at maturity and seed number per capsule were used to calculate seed number produced per capsule pollinated. Seed viability was assessed, with available seed (maximum of 100 seeds per bunch) sown onto germination trays, placed into a germination cabinet and incubated at 25 °C for 10 d. The germinated seed were then counted, and viable seed per capsule pollinated was calculated.

Statistical analysis

Prior to analysis, all data were screened for assumptions of normality and homogeneity of variance. Where necessary, percentage data were arcsine-transformed to meet the assumptions of parametric tests, and numerical data were log-transformed to correct for unequal variances. Statistical analysis was conducted using Genstat 9.2 statistical software (Genstat, 2007). For all experiments, data were analysed via analysis of variance (ANOVA). Where F-values were significantly different (P < 0.05) pairwise comparisons between means were conducted using Tukey’s multiple comparison test.

RESULTS

Pre-zygotic reproductive isolating barriers were observed within the C. torelliana maternal parent at all four pre-zygotic fertilization stages, with significant differences (P < 0.05) between crosses for the parameters of pollen adhesion to stigma, pollen germination, pollen tube growth through the style and pollen tube penetration of the ovule micropyle (Table 2). Differences in reproductive success between crosses were immediately apparent at the first stage of fertilization, with the number of pollen grains that adhered to the stigma for the interspecific C. intermedia cross significantly lower (P < 0.05) than for the intraspecific C. torelliana cross (Table 2). A further barrier to reproductive success was observed at the second stage of fertilization, with the germination percentage of adhered pollen grains on the stigma surface significantly lower (P < 0.05) for the C. intermedia cross than for the C. torelliana cross (Table 2). There were no differences in pollen to stigma adhesion or pollen germination between the C. torelliana, C. citriodora subsp. citriodora and C. tessellaris crosses.

The number of pollen tubes observed in the middle style region for the interspecific C. intermedia and C. tessellaris crosses was significantly lower (P < 0.001) than the intraspecific C. torelliana cross (Table 2). Pollen tube numbers were not significantly different between the C. citriodora subsp. citriodora and C. torelliana crosses. At the fourth stage of fertilization, the number of ovules penetrated by pollen tubes was lowest (P < 0.001) for the C. intermedia and C. tessellaris crosses, intermediate for the C. citriodora subsp. citriodora cross and highest for the C. torelliana cross (Table 2).

Five weeks after pollination, the intraspecific C. torelliana cross had significantly more developing embryos per capsule (P < 0.01) than the interspecific C. citriodora subsp. citriodora, C. tessellaris and C. intermedia crosses (Table 2). The C. citriodora subsp. citriodora cross also had a significantly higher number of developing embryos than the C. tessellaris and C. intermedia crosses. Embryo size at 5 weeks after pollination was greatest in crosses where embryo numbers were low (Fig. 2D). Embryos within the C. intermedia and C. tessellaris crosses were significantly larger (P < 0.01) than embryos...
within the *C. torelliana* and *C. citriodora* subsp. *citriodora* crosses (Table 2). At capsule maturity, the *C. torelliana* cross had a significantly higher (*P* < 0.001) seed number per capsule than all crosses (Table 2). The *C. citriodora* subsp. *citriodora* cross also had significantly higher seed yields than the *C. tessellaris* and *C. intermedia* crosses. Seed yields for both the *C. intermedia* and *C. tessellaris* crosses were very low and not significantly different from each other.

**Interspecific hybridization**

Reproductive output for the *C. torelliana* maternal parent varied widely between crosses, with significant differences (*P* < 0.05) for capsule retention at maturity, seed number per capsule, seed per capsule pollinated (Table 3) and viable seed per capsule pollinated (Figs 3–6). In all experiments, the highest capsule retention rates and seed yields were recorded for the intraspecific *C. torelliana* cross. Capsules and seeds were produced for interspecific hybrid crosses between *Corymbia* species from different sections and subgenera: sections *Torellianae* × *Maculatae* and *Torellianae* × *Abbreviatae* in subgenus *Blakella*, and *Torellianae* × *Septentroniales* in subgenus *Corymbia*. Crosses between species from different genera (*Corymbia* × *Angophora*, *Corymbia* × *Eucalyptus*) were unsuccessful.

The cross between the *C. torelliana* (sect. *Torellianae*) maternal parent and *C. tessellaris* (sect. *Abbreviatae*), both subgenus *Blakella*, was the most successful interspecific hybrid in expt 2. This cross had a similar capsule retention rate to the intraspecific *C. torelliana* cross; however, seed number per capsule, seed per capsule pollinated (Table 3) and viable seed per capsule pollinated (Fig. 3) were significantly lower (*P* < 0.001). Intersubgeneric crosses between the *C. torelliana* (subgenus *Blakella*) maternal parent and *C. intermedia* or *C. ptychocarpa* (subgenus *Corymbia*, sect. *Naviculares*) were unsuccessful.

**TABLE 3. Capsule (cap.) and seed yields for the 16 crosses made with the *C. torelliana* maternal parent in expts 2–5**

<table>
<thead>
<tr>
<th>Pollen parent</th>
<th><em>Corymbia</em> subgenus, section</th>
<th>Cap. no. (0 weeks)</th>
<th>Cap. % (2 weeks)</th>
<th>Cap. % (maturity)</th>
<th>Seed/cap.</th>
<th>Seed/cap. pollinated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. torelliana</em></td>
<td><em>Blakella, Torellianae</em></td>
<td>60.8</td>
<td>61.0</td>
<td>8.2</td>
<td>17.2</td>
<td>1.34</td>
</tr>
<tr>
<td><em>C. tessellaris</em></td>
<td><em>Blakella, Abbreviatae</em></td>
<td>67.3</td>
<td>61.3</td>
<td>5.7</td>
<td>5.4</td>
<td>0.40</td>
</tr>
<tr>
<td><em>C. intermedia</em></td>
<td><em>Corymbia, Septentroniales</em></td>
<td>64.3</td>
<td>47.1</td>
<td>2.0</td>
<td>8.2</td>
<td>0.11</td>
</tr>
<tr>
<td><em>C. ptychocarpa</em></td>
<td><em>Corymbia, Septentroniales</em></td>
<td>63.9</td>
<td>62.3</td>
<td>0.4</td>
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<td><em>P</em>-value</td>
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<td>0.675</td>
<td>0.193</td>
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<td><em>C. torelliana</em></td>
<td><em>Blakella, Torellianae</em></td>
<td>65.8</td>
<td>59.9</td>
<td>22.4</td>
<td>24.1</td>
<td>5.57</td>
</tr>
<tr>
<td><em>C. citriodora</em> subsp. <em>citriodora</em></td>
<td><em>Blakella, Maculatae</em></td>
<td>68.2</td>
<td>50.6</td>
<td>14.4</td>
<td>15.0</td>
<td>1.97</td>
</tr>
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<td><em>C. dallachiana</em></td>
<td><em>Blakella, Abbreviatae</em></td>
<td>61.0</td>
<td>49.8</td>
<td>2.0</td>
<td>3.7</td>
<td>0.04</td>
</tr>
<tr>
<td><em>C. clarksoniana</em></td>
<td><em>Corymbia, Septentroniales</em></td>
<td>70.0</td>
<td>67.3</td>
<td>20.3</td>
<td>9.1</td>
<td>2.51</td>
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<td><em>C. erythrophyloia</em></td>
<td><em>Corymbia, Septentroniales</em></td>
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<td>59.1</td>
<td>12.1</td>
<td>10.8</td>
<td>1.20</td>
</tr>
<tr>
<td><em>C. trachyphyloia</em></td>
<td><em>Corymbia, Septentroniales</em></td>
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<td>59.9</td>
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<tr>
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<td>0.006</td>
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<td><em>C. torelliana</em></td>
<td><em>Blakella, Torellianae</em></td>
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<td>27.0</td>
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<td>62.9</td>
<td>23.8</td>
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<td>70.2</td>
<td>5.6</td>
<td>5.7c</td>
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<td><em>C. leichardtii</em></td>
<td><em>Blakella, Naviculares</em></td>
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<td>70.7</td>
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<td>–</td>
<td>0.00</td>
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<td><em>C. ficifolia</em></td>
<td><em>Corymbia, Calophyllae</em></td>
<td>57.0</td>
<td>43.1</td>
<td>5.2</td>
<td>0.7d</td>
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<td>48.3</td>
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<td>0.00</td>
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<td><em>E. pellita</em></td>
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<td><em>C. torelliana</em></td>
<td><em>Blakella, Torellianae</em></td>
<td>71.4</td>
<td>61.4</td>
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<td>24.8</td>
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<td><em>C. citriodora</em> subsp. <em>variegata</em></td>
<td><em>Blakella, Maculatae</em></td>
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<td>58.6</td>
<td>12.1</td>
<td>15.0</td>
<td>1.63</td>
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<td><em>C. henryi</em></td>
<td><em>Blakella, Maculatae</em></td>
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<td>18.1</td>
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</table>

Treatment means with different letters are significantly different (*P* < 0.05).

![Fig. 3. Viable seed per capsule pollinated for the four crosses made with the *C. torelliana* maternal parent in expt 2. Treatment means with different letters are significantly different (*P* < 0.001).](https://academic.oup.com/aob/article-abstract/109/7/1215/101509)
Septentrionales) had low reproductive output. These crosses had a significantly lower ($P < 0.05$) capsule retention rate, seed number per capsule, seed yield per capsule pollinated (Table 3) and viable seed per capsule pollinated (Fig. 3) than the intraspecific C. torelliana cross. Seed number per capsule for the C. ptychocarpa cross was also significantly lower than for the C. intermedia cross.

Good interspecific reproductive success was achieved between the C. torelliana (sect. Torellianae) maternal parent and the C. citriodora subsp. citriodora (sect. Maculatae) cross in expt 3, with percentage capsule retention, seed yields (Table 3) and viable seed per capsule pollinated (Fig. 4) similar to those of the intraspecific C. torelliana cross. Intersubgeneric crosses between the C. torelliana (subgenus Blakella) maternal parent and C. erythrophloia or C. clarksoniana (subgenus Corymbia, sect. Septentrionales) were also successful. These crosses had similar capsule retention at maturity to the intraspecific C. torelliana cross, with C. erythrophloia also having a similar seed number per capsule and C. clarksoniana a similar seed per capsule pollinated (Table 3) to the C. torelliana cross. Viable seed per capsule pollinated (Fig. 4) were, however, significantly lower ($P < 0.01$) for these crosses than for the C. torelliana cross. The reproductive successes of the C. dallachiana (sect.
Abbreviatae) and C. trachyphloia (sect. Septentrionales) crosses were poor, with significantly lower ($P < 0.05$) capsule retention at maturity and seed yields than the intraspecific C. torelliana cross.

High reproductive success of the C. torelliana (sect. Torellianae) maternal parent with the C. citriodora subsp. citriodora (sect. Maculatae) cross was observed again in expt 4, within a large taxonomic range of hybrid crosses. The C. citriodora subsp. citriodora cross had similar capsule retention at maturity to the intraspecific C. torelliana cross, and significantly greater ($P < 0.001$) seed number per capsule, seed per capsule pollinated (Table 3) and viable seed per capsule pollinated (Fig. 5), than all other interspecific crosses. The C. maculata (sect. Maculatae) and C. tessellaris (sect. Abbreviatae) crosses had low reproductive output and the C. leichardtii (sect. Naviculares) cross did not produce seed. The intersubgeneric cross between C. torelliana (subgenus Blakella) and C. ficifolia (subgenus Corymbia, sect. Callophyllae) had low reproductive output. The intergeneric crosses between C. torelliana and either Angophora floribunda or Eucalyptus pellita did not produce seed; however, capsules were retained to maturity, particularly within the E. pellita cross (10.2% capsule retention).

Hybrid crosses between the C. torelliana (sect. Torellianae) maternal parent and C. citriodora subsp. citriodora, C. citriodora subsp. variegata or C. henryi (sect. Maculatae) in expt 5 had high reproductive output (Table 3, Fig. 6). The C. citriodora subsp. citriodora cross was highly successful, with similar reproductive output to the intraspecific C. torelliana cross for all seed parameters measured, except viable seed per capsule pollinated ($P < 0.05$). Reproductive output of the C. citriodora subsp. variegata and C. henryi crosses was lower than that of the intraspecific C. torelliana cross for the parameters of seed number per capsule pollinated and viable seed per capsule pollinated. Seed number per capsule for the C. henryi cross was also lower than for the intraspecific C. torelliana cross. Reproductive output between the C. citriodora subsp. citriodora, C. citriodora subsp. variegata and C. henryi crosses was not significantly different for any parameter measured.

**DISCUSSION**

**Reproductive isolating barriers**

Interspecific C. torelliana hybridization was controlled by pre-zygotic reproductive isolating barriers. Reproductive isolation occurred immediately for the C. intermedia cross, with few pollen grains adhering to the stigma. Pollen adhesion is the first cellular event that occurs when pollen is deposited on the stigma (Heizmann et al., 2000) and is reliant on a successful interaction between the pollen grain and adhesion molecules on the stigma (Heslop-Harrison, 2000; Lord, 2000). This process acts as an initial defensive mechanism preventing entry of pathogens, but can also act as a physiological isolating mechanism, which can inhibit adhesion of undesirable self-pollen (Heslop-Harrison, 2000) and interspecific pollen (Rougier et al., 1988).

Low pollen germination for the C. intermedia cross suggests activity of another reproductive isolating barrier. Pollen germination on the stigma surface occurs after successful rehydration by watery stigma secretions (Lord, 2000) which are regulated by both physiochemical and genetic controlling factors (Heslop-Harrison, 1987). Genotypes of different compatibility hydrate at different rates, possibly determined by pollen surface molecular interactions, which results in variable pollen germination rates (Zuberi and Dickinson, 1985).

Optimum conditions for pollen hydration and germination vary between species within the eucalypts, particularly in the levels of lipids, proteins, carbohydrates and boric acid within the stigmatic exudate (Potts and Marsden-Smedley, 1989). Differences in pollen germination are expected for different interspecific crosses, with high variation identified between pollen parents in a Eucalyptus hybridization experiment involving 21 pollen parent species (Ellis et al., 1991). In our study, the lower germination of the C. intermedia pollen on the C. torelliana style may be due to a physiological isolating mechanism inhibiting pollen germination on the style.

Pollent tube growth was inhibited into and throughout the style for the C. intermedia and C. tessellaris crosses, providing further evidence of barriers to interspecific reproduction. Disruption of pollen tube growth often occurs soon after pollen germination, either during penetration of the stigma cuticle or during early growth within the transmitting tract of the style (Ellis et al., 1991; Wallwork and Sedgley, 2005). Isolation can also occur in the lower style, with pollen tube growth disrupted within the first millimetre of styles cut using the one-stop pollination method (Suitor et al., 2009). Disrupted pollen tubes are often characterized by non-directional growth and/or abnormal appearance (thickened walls, bulbous growth, forking). In eucalypts, the greater the taxonomic distance between the two parent species, the greater the chances of divergent evolution and incongruity, resulting in a greater number of malfunctions in pollen tube growth (Ellis et al., 1991). Within the four Corymbia species investigated in our study, pollen tube numbers in the style were lowest in the C. intermedia cross, intermediate in the C. tessellaris cross and similar between the C. citriodora subsp. citriodora and C. torelliana crosses.

Large differences in reproductive success were observed between crosses at the fourth stage of pre-zygotic fertilization: pollen tube penetration of the ovule micropyle. Differences in
Interspecific hybridization

Corymbia torelliana formed hybrids with species from numerous Corymbia sections, confirming the close relationships of infrageneric clades within this genus. This included species from sections within both Corymbia subgenera, particularly sections Maculatae, Abbreviatae and Septentrionales. This is consistent with many records of natural and manipulated interspecific Corymbia hybridization, with Griffin et al. (1998) identifying Corymbia as an atypical eucalypt group with an unusually high rate of intersectional and interseries hybrids. The use of the one-stop pollination method in expts 2–5, whereby the stigma is decapitated and pollen applied directly to the cut style surface, circumvented some early pre-zygotic reproductive barriers (Ellis et al., 1991), whereas in more closely related crosses, including intraspecific outcross and self-pollen, reproductive success is primarily controlled by late pre-zygotic or post-zygotic barriers (Pound et al., 2002).

Corymbia torelliana (sect. Torellianae) hybridized most readily with species from section Maculatae, particularly C. citriodora subsp. citriodora, C. citriodora subsp. variegata and C. henryi. Only the C. maculata cross had low success. The southerly occurring C. maculata is genetically distinct from the northern Maculatae taxa, which has been attributed to an isolation-by-distance model (Shepherd et al., 2008; Ochieng et al., 2010). Poorer interspecific reproductive success between the northerly occurring C. torelliana and C. maculata may also indicate an isolation-by-distance effect. Concurrently, C. citriodora subsp. citriodora occurs naturally in forests adjacent to C. torelliana and had high interspecific reproductive success. The C. citriodora subsp. variegata and C. henryi crosses achieved similar capsule retention rates to C. torelliana, and produced moderate seed yields. These results concur with numerous records of natural spontaneous C. torelliana × C. citriodora subsp. citriodora, × C. citriodora subsp. variegata and × C. henryi hybrids (Hill and Johnson, 1995; Nikles et al., 2000) and with the results of controlled pollination studies (Lee et al., 2009; Dickinson et al., 2010), suggesting a close relationship between species within the Torellianae and Maculatae sections.

Hybrid crosses between C. torelliana (sect. Torellianae) and species from section Abbreviatae had variable reproductive success. The best results were obtained for the C. torelliana × C. tesellaris cross where small quantities of seed were produced. Natural interspecific hybrids between C. torelliana and C. tesellaris are known (Hill and Johnson, 1995); however, these are rare considering both species coexist at numerous locations in north Queensland. Active reproductive isolation between Corymbia species and species from section Abbreviatae may be due to evolutionary diversion of their reproductive systems. Species within section Abbreviatae (the paper-fruiting bloodwoods) have characteristic vegetative and floral morphological attributes that distinguish them from other Corymbia species (Brooker, 2000). Floral differences between C. torelliana and species within section Abbreviatae may have contributed to incompatibility or incongruity effects, driven by physiological or structural barriers, which resulted in greater reproductive isolation.

The creation of intersubgeneric hybrids between C. torelliana (subgenus Blakella, sect. Torelliana) and either C. clarksoniana or C. erythrophloia (subgenus Corymbia, sect. Septentrionales) confirms the close relationships between infrageneric Corymbia clades. Natural intersubgeneric Corymbia hybrids are recognized: C. gummifera × C. maculata (subgenus Corymbia, sect. Corymbia × subgenus Blakella, sect. Maculatae) and C. intermedia × C. maculata (subgenus Corymbia, sect. Septentrionales × subgenus Blakella, sect. Maculatae) (Hill and Johnson, 1995). Numerous intersubgeneric Corymbia hybrid combinations have also been created via reciprocal manipulated pollination of species from subgenus Corymbia, sections Septentrionales or Calophyllae, with species from subgenus Blakella, sections Maculatae or Naviculares (Sedgley and Delaporte, 2004). Historically, eucalypt subgenera have been regarded as reproductively isolated (Griffin et al., 1988; Potts and Wiltshire, 1997; Potts and Dungey, 2004). Cases of unexpected intersubgeneric eucalypt hybridization including subgenera Idiogenes × Eucalyptus (E. acmenoides × E. cloeziania; Stokoe et al., 2001) have prompted further phylogenetic and taxonomic investigation (Steane et al., 2002).

Poor reproductive output was measured for a number of crosses between C. torelliana and species from sections across both Corymbia subgenera. These included intrasubgeneric crosses between C. torelliana and C. maculata (sect. Maculatae), C. dallachiana (sect. Abbreviatae) or C. leichardtii (sect. Naviculares) and intersubgeneric crosses between C. torelliana and C. intermedia, C. ptychocarpa,
C. trachyploia (sect. Septentrionales) or C. ficifolia (sect. Callophyllae). Evolutionary diversion of the reproductive systems of hybrid parent species, even in closely related taxa, can trigger physical or physiological reproductive isolation. In Eucalyptus, unilateral reproductive isolation between the closely related species E. nitens and E. globulus is attributed to simple differences in flower morphology (Gore et al., 1990). Our results suggest that while taxonomic affinity is a useful guide to predict the likelihood of two species forming hybrids (Pryor, 1951; Ellis et al., 1991), within genus Corymbia where intersubgeneric crosses are successful, other factors are also important.

Specific genetic combining interactions between individual parents, known as specific hybridizing ability (SHA; Nikles and Newton, 1991), has a great influence on the reproductive success of interspecific hybrids. Variation in SHA between different F1 hybrid families of the same interspecific cross is well recognized, resulting in highly variable reproductive success and field performance of different F1 hybrid families of Pinus elliottii (Brawner et al., 2003), E. globulus (Volker et al., 2008) and C. torelliana (Lee et al., 2009). In our study, the interspecific crosses undertaken utilized only a small number of maternal or paternal parents. It is possible that SHA may have contributed to cases of unexpectedly poor reproductive success, such as occurred in the C. torelliana × C. maculata cross.

The two intergeneric crosses Corymbia torelliana × Angophora floribunda and Corymbia torelliana × Eucalyptus pellita did not yield seed; however, mature capsules were produced for both crosses. Successful hybridization between eucaulpyt genera is highly improbable. A review of natural and manipulated eucalypt hybrids by Griffin et al. (1988) was unable to verify any records of intergeneric eucalypt hybrids. Manipulated intergeneric crosses between Eucalyptus and Corymbia have also been unsuccessful in manipulated pollination studies (Ellis et al., 1991; Paramathma et al., 1997). The production of seedless mature capsules in our study may be due to pre-zygotic parthenocarpy, whereby fruit growth is stimulated by pollen–pistil interactions or by post-zygotic stenospornocarpy, whereby embryos abort following fertilization yet fruit growth continues to maturity (Sedgley and Griffin, 1989; Suior et al., 2008). Ellis et al. (1991) identified early pre-zygotic intergeneric hybrid reproductive success with observations of pollen germination, stigma penetration and pollen tube growth through the style for six different Eucalyptus × Corymbia crosses and pollen tube penetration of the ovule for one Eucalyptus × Corymbia cross. In our study, early pollination success may have stimulated capsules to develop through to maturity, despite reproductive failure during pre-zygotic or post-zygotic stages.

The hybridizing propensity of Corymbia provides encouragement to explore new Corymbia hybrid combinations for commercial plantation forestry and ornamental use. Manipulated pollination methods involving stigma decapitation (Dickinson et al., 2010) may eliminate certain pre-zygotic isolating barriers, facilitating the creation of previously unknown Corymbia hybrid combinations. In Australia, high land prices in higher rainfall zones (>1000 mm year⁻¹) have resulted in an expansion of hardwood plantations into lower rainfall zones, which have been traditionally regarded as marginal for production forestry (Lee et al., 2010). Corymbia species are a significant component of woodlands and forests across northern Australia, including semi-arid and arid regions of the continent (Hill and Johnson, 1995). Interspecific Corymbia hybrids derived from species adapted to marginal environments could provide many new desirable phenotypes suitable for plantation forestry in low rainfall zones. A number of large-flowered Corymbia species, including C. ficifolia and C. ptychocarpa, are also recognized for their ornamental merit and potential for hybridization (Henry, 1995; Sedgley and Delaporte, 2004). New interspecific Corymbia hybrids could provide phenotypes suitable for the amenity horticulture and floriculture industry.

In Australia, genetic pollution from spotted gum plantations (sect. Maculatae) into native Corymbia forests is considered likely (Barbour et al., 2008). The highest risk for interspecific gene flow is between spotted gum species, with interspecific gene flow between species from different Corymbia sections considered the lowest risk. In our study, intraspecific C. torelliana hybridization was more successful than interspecific hybridization; however, incomplete reproductive isolation was observed between species from a number of Corymbia sections, particularly sections Torelliana and Maculatae. Interspecific reproductive success between Corymbia species generally decreased with increasing taxonomic distance between parents. Our results suggest that interspecific pollen-mediated gene flow from Corymbia plantings into sympatric native Corymbia populations is a valid concern. Improved understanding of hybridizing potential between species from different Corymbia sections will assist the development of gene flow risk assessment frameworks (Potts et al., 2003) for Corymbia plantations, allowing the implementation of strategies to minimize the risks and consequences of genetic pollution to natural Corymbia populations.

Interspecific Corymbia hybrids were successfully created between C. torelliana and species from both Corymbia subgenera, confirming the close relationships of infrageneric clades within this genus. Pre-zygotic isolating barriers were identified at the four stages: pollen adhesion to the stigma; pollen germination; pollen tube growth in the style; and pollen tube penetration of the ovule micropyle. Post-zygotic isolating barriers were not identified for any of the crosses examined. Increasing activity of reproductive isolating barriers was identified with increasing taxonomic distance between parents, suggesting that interspecific incongruity is broadly applicable to Corymbia. The poor success of some closely related crosses suggests that factors in addition to incongruity can also influence interspecific Corymbia hybridization.

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LITERATURE CITED


