

## **A phylogenetic investigation of the taxonomically problematic *Eucalyptus odorata* complex (E. section *Adnataria* series *Subbuxaeales*): evidence for extensive interspecific gene flow and reticulate evolution**

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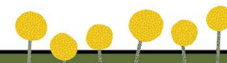
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# A phylogenetic investigation of the taxonomically problematic *Eucalyptus odorata* complex (*E.* section *Adnataria* series *Subbuxuales*): evidence for extensive interspecific gene flow and reticulate evolution

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## ABSTRACT

To investigate the relationships among species in the taxonomically problematic *Eucalyptus odorata* species complex, we generated molecular data using double-digest restriction site-associated DNA sequencing (ddRADseq) and Diversity Arrays Technology sequencing (DARtseq). These data were analysed utilising principal-component analysis (PCA), phylogenetic networks, phylogeny reconstruction and hybridisation tests. Twelve species that are variously recognised in the complex were sampled from across their ranges, along with co-occurring members of *E.* section *Adnataria*, to allow for patterns of hybridisation and gene flow to be identified. Despite the large genetic datasets generated, many relationships within the *E. odorata* complex were poorly resolved, and few species were monophyletic, likely owing to both biological factors including recent speciation and extensive hybridisation and introgression, and potential over-splitting of taxa. We show that multiple taxa with limited distributions are the result of reticulate evolutionary events and that typical *Eucalyptus viridis* R.T.Baker and the possibly con-specific *E. aenea* K.D.Hill are sister to the rest of the complex. The remaining species appeared to represent a discontinuous crescent-shaped cline running from the Flinders Ranges to the south-western slopes region of New South Wales, with limited support for an east–west split in this cline across the Murray River Basin. *Eucalyptus viridis* var. *latiuscula* Blakely, which is not closely related to the typical variety of this species in our data, may represent a northern extension to this cline.

**Keywords:** biogeography, DARtseq, ddRADseq, *Eucalyptus*, hybridisation, phylogenetics, reticulate evolution, south-eastern Australia.

## Introduction

Species in many plant genera will readily hybridise with closely related and co-occurring species despite being morphologically and ecologically distinct with largely separate evolutionary histories (Abbott *et al.* 2013). This makes defining species difficult under some of the most widely applied species concepts such as the biological species concept (BSC), which regards species as the widest populations of organisms that can interbreed, while being unable to interbreed with other populations (Mayr 1963, 2000), and the phylogenetic species concept, which includes all individuals descending from a common ancestor as a species (Baum 1992). In Australia, one large and iconic group where this species definition problem is prevalent is the eucalypts, a diverse clade of woody shrubs and trees comprising >750 recognised species and numerous undescribed entities (Council of Heads of Australasian Herbaria 2016) that are the dominant arboreal taxa across much of the continent. Within the largest eucalypt genus, *Eucalyptus*, infra-sectional hybridisation is a frequently observed phenomenon despite ‘pure’ species

dominating most natural populations (Griffin et al. 1988); however, there is a sharp decline in hybridisation frequency and hybrid fitness at the sectional level (Larcombe et al. 2015). It has previously been suggested that hybridisation is particularly frequent in *E.* subgenus *Symphyomyrtus* (Jones et al. 2016; Flores-Rentería et al. 2017), the subgenus to which all species sampled in this study belong.

The group investigated in this study, the *E. odorata* complex, provides an extra layer of complexity to the problem of defining species, in the scattered yet overlapping distribution of several taxa within the complex (Fig. 1), making it difficult to conclude whether morphologically similar entities that occur hundreds of kilometres apart are conspecific. The complex consists of between 3 and 12 species, depending on which classification system is employed (Table 1) and taxa in the complex are united by their mallee to small tree-like habit, lanceolate to narrowly lanceolate juvenile leaves up to 2.5 cm wide, simple axillary inflorescences, buds with outer operculum intact at anthesis, and cup-shaped to barrel-shaped fruit with three or four locules (Rule 2018). The complex is part of *E.* section *Adnataria* series *Subbuxuales*, along with the grey boxes, four species of lignotuberous but generally single-trunked woodland or forest trees (*E. albens* Benth., *E. microcarpa* (Maiden) Maiden, *E. moluccana* Roxb., *E. woollisiana* R.T.Baker) and two other mallee species that are closely related to the *E. odorata* complex, *E. albopurpurea* (Boomsma) D.Nicolle and *E. froggattii* Blakely (Nicolle 2019). *Eucalyptus froggattii* is recognisable by its terminal inflorescences, square buds and fruit, and tightly crowded oils glands in the adult leaves (Brooker et al. 2015). *Eucalyptus albopurpurea* is less distinct from members of the *E. odorata* complex and may be better placed within the complex because it is known to intergrade with *E. odorata* Behr on Kangaroo Island (Nicolle 2000), but differs in its wider juvenile (up to 4.7 cm) and adult leaves, apparently terminal inflorescences, marginally longer buds and fruit, and often pink to purple flowers (Brooker et al. 2015).

The diversity of the complex is best understood by following its taxonomic history, which starts with the three accepted species described prior to 1910 (Table 1), namely, *E. odorata*, *E. polybractea* R.T.Baker and *E. viridis* R.T.Baker. The first of these, *E. odorata*, is a mallee or tree with variably larger buds and fruit than for the other two, which occurs in temperate woodlands to the west of the Murray Basin in South Australia (SA), although historically it has also been considered to be present in the western Wimmera of Victoria and adjacent areas of south-eastern SA (Brooker et al. 2015). *Eucalyptus polybractea* was described to include mallees that have bluish-green leaves and somewhat pruinose buds and fruit, which occur in the following three highly disjunct areas: on sandstone outcrops in the Victoria Goldfields, around the township of West Wyalong in New South Wales (NSW) and in the Flinders Ranges of SA (Brooker et al. 2015). In 2018, the range of *E. polybractea* was extended into the Victorian Wimmera by the recognition of a new

subspecies, *E. polybractea* subsp. *subcerea*, with reportedly pruinose seedlings and faintly pruinose buds and fruit in this region (Rule 2018). The final of these three long-standing taxa is *E. viridis*, which includes mallees with linear green leaves and non-glaucous buds and fruits, and has historically been considered to have a distribution that encompasses much of that of *E. polybractea*, with a northern extension from West Wyalong to central Queensland along the inland slopes of the Great Dividing Range (GDR), with some small populations in the Hunter Valley (Brooker et al. 2015). The species was also previously considered to occur in the Wimmera and was considered more common in this area than was *E. odorata* (Chippendale 1988; Brooker et al. 2015). This classification broadly held for 90 years despite several varieties and subspecies of these taxa being proposed and some taxonomic confusion regarding the name *E. fruticetorum* F.Muell. ex Miq., which Blakely (1934) applied to *E. polybractea* despite the type material representing *E. odorata* (Pryor and Johnson 1971).

Starting with the publication of *E. wimmerensis* Rule in 1990, there has been a significant increase in the number of described taxa in the group, with eight species published in the past 30 years (Table 1). *Eucalyptus wimmerensis* was described (Rule 1990) to include all populations in the Wimmera region of Victoria and adjacent parts of SA that had previously been considered *E. viridis* (Fig. 1a). These populations differ from typical *E. viridis* largely in their juvenile leaf morphology, but also exhibit wider adult leaves and marginally larger fruits; all traits suggesting a closer affinity with *E. polybractea* and *E. odorata* than with *E. viridis* (Rule 1990; Nicolle 2006). Rule (2018) described five subspecies of *E. wimmerensis*; however, these show minor morphological differences and, given there are many questions regarding species-level taxonomy in the complex, we do not analyse these subspecies in detail in this paper. The next taxonomic change in the complex was again made by Rule, with the separation of all populations east of the Murray Basin (Fig. 1b), formerly considered to be *E. odorata*, into the new taxon *E. silvestris* Rule on the basis of their smaller buds and fruit, lustrous adult leaves, and broader juvenile leaves with longer petioles (Rule 1994). Other authors have suggested these traits may result from hybridisation between *E. odorata* or *E. wimmerensis* and the grey box, *E. microcarpa* (Brooker et al. 2015; Nicolle 2019).

Further populations that had previously been regarded as *E. viridis* were separated into another new taxon, *E. aenea* K.D.Hill, by Hill (1997). This species includes populations in the Goulburn River National Park (NSW) and nearby areas (Fig. 1a) that grow on exposed slopes among taller eucalypt forest and differ from typical *E. viridis* in their broader adult and juvenile leaves, bluish-green juvenile leaves and wholly smooth bark (Hill 1997). Currently, this is the only species in the group not recognised on the Australian Plant Name Index (Council of Heads of Australasian Herbaria 2016), being regarded as a synonym of *E. viridis*. In 2002, a further

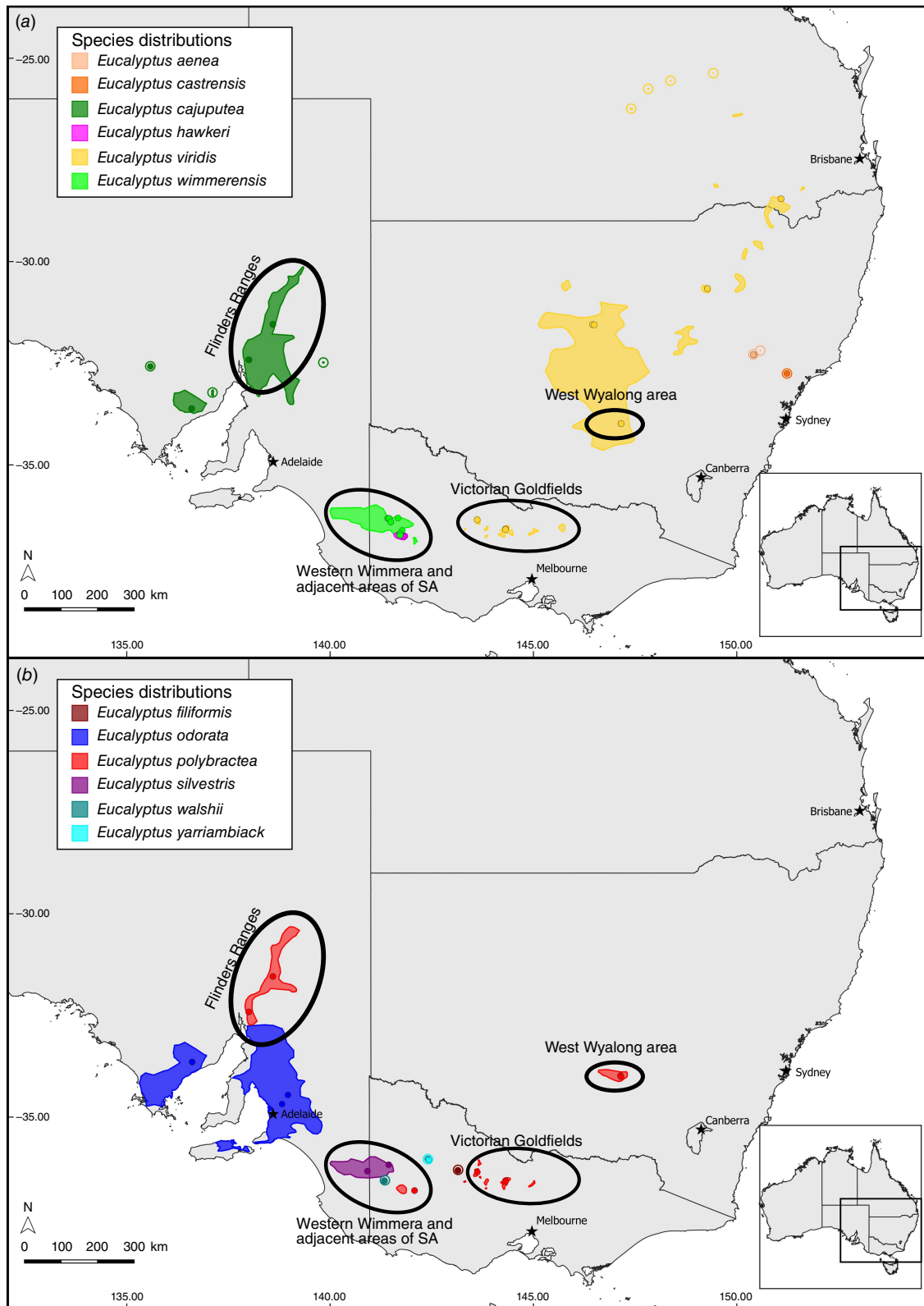


Fig. 1. (Caption on next page)

**Fig. 1.** Distributions of the 12 species of the *E. odorata* complex, using taxonomic concepts employed *a priori* in this study. (a) *E. viridis* and species most commonly considered its closest relatives. (b) *E. odorata*, *E. polybractea* and species commonly considered close relatives of these. Distributions are coloured by species and open circles are used to highlight geographically restricted populations. Closed points indicate the collecting localities and seed provenances for samples used in this study. Black circles indicate major regions where members of the *E. odorata* complex occur as applied in text, which may differ from the actual geographic extent.

population of mallee-boxes with affinities to *E. viridis* and *E. aenea* discovered on the Singleton Army Base (NSW) was described as *E. castrensis* K.D.Hill by Hill and Stanberg (2002) (Fig. 1a). Plants from this population have larger adult leaves, buds and fruit, broader juvenile leaves and a stronger medial constriction of their operculum than does *E. aenea*, while also growing into more robust mallees that retain basal rough bark (Hill and Stanberg 2002). Per Nicolle (2019), the type population of this taxa consists of two forms, one potentially referable to *E. aenea* and the second that matches the type material that may represent *E. aenea* × *E. microcarpa*–*E. moluccana* hybrids, with the concept of the name as applied by Hill and Stanberg (2002) and Copeland and Hunter (2005), including both the potential *E. aenea* and *E. aenea* × *E. microcarpa*–*E. moluccana* hybrid forms.

In a 2004 publication, Rule described the following three further taxa in the complex from Victoria: *E. filiformis* Rule, *E. hawkeri* Rule and *E. walshii* Rule. *Eucalyptus filiformis* is known from only seven individuals that form a copse on Mount Jeffcott in the eastern Wimmera (Fig. 1b) and show morphological affinities with both *E. viridis* and *E. polybractea*, differing primarily from the former in having dull bluish-green leaves with clearly visible venation and larger fruits, and the latter in having persistent thin grey, box bark, narrower adult and juvenile leaves and thin-walled fruit (Rule 2004). The seven known individuals of this species show minimal morphological differentiation. A further individual at this locality was subsequently discovered by Rule and provided to us under the informal name '*E. aff. polybractea* (Mount Jeffcott)' on the basis of its smooth bark, glaucous juvenile leaves and minute, lightly waxy buds, and barrel-shaped fruits (K. Rule, pers. comm., 18 November 2018).

*Eucalyptus walshii* is also known only from a single population with minor morphological differences from the other species in the complex. This species occurs near Broughtons Waterhole in the Little Desert of western Victoria (Fig. 1b), with key morphological features being pale smooth bark above a basal stocking of box bark, a tree-like habit and broad adult leaves with dense reticulate venation (Rule 2004). It was described as showing greatest morphological affinities with *E. odorata* and *E. albopurpurea* (Rule 2004), which both occur only west of the Murray basin under Rule's classification.

The third of the mallee-box species from Rule's (2004) publication, *E. hawkeri*, is somewhat more widespread, occurring in many mallee communities in the southern Wimmera (Rule 2018; Fig. 1b) The primary characters of this species are its slender tree-like habit, lanceolate and often pendulous adult leaves, and substantial stocking of

rough bark. This taxon has been suggested by Nicolle (2019) and Brooker et al. (2015) as being a recurring intergrade between *E. wimmerensis* and *E. microcarpa*.

The most recently described species in the group was *E. yarriambiack* Rule from a small population restricted to a narrow roadside strip in an otherwise extensively cleared agricultural area near Yarriambiack Creek, north of the town of Brim in the southern Murray Mallee of Victoria (Fig. 1a). This population lies outside the current distribution of all other species of the *E. odorata* complex, and differs from them in its tree-like growth habit, box bark coverage of its main branches, and smaller fruit (Rule 2012). Nicolle (2019) noted that the type material may represent a hybrid between *E. largiflorens* F.Muell. and *E. odorata*, despite the lack of other *E. odorata* complex members in the area.

The final member of the group is *E. cajuputea* Miq., a name considered a synonym of *E. odorata* as early as 1867 (Council of Heads of Australasian Herbaria 2016) and resurrected by Nicolle and Roberts (2013) to include all populations previously regarded as *E. viridis* in the Flinders Ranges, where the type material for the species was collected, and northern Eyre Peninsula, with outlying populations in the Gawler Ranges and on Oulnina Hill (Fig. 1a). These populations have wider juvenile and adult leaves that lack the lustrous colouration of *E. viridis* (Nicolle and Roberts 2013). A detailed overview of the morphological variation in the group is provided by Rule (2018).

In this study, we aim to use and compare genomic data generated from double-digest restriction-associated DNA sequencing (ddRADseq; Peterson et al. 2012) and Diversity Array Technologies sequencing (DARTseq; Sansaloni et al. 2010) to build a systematic understanding of the taxa in the *E. odorata* complex. The DARTseq approach was designed using eucalypts (Sansaloni et al. 2010) and has been successfully used to investigate both phylogenetic and population genetic relationships in various groups of eucalypts in the past (Steane et al. 2011, 2017; Jones et al. 2016; Rutherford et al. 2016, 2018, 2019, 2020; Jordan et al. 2017), whereas ddRADseq has seen very little use in eucalypts (Aguirre et al. 2019), despite widespread use in phylogenetic studies of other taxa (Yang et al. 2016; Guo et al. 2020). Both techniques use restriction-enzyme digestion to fragment genomic DNA; however, DARTseq is a proprietary method performed on a fee-for-service basis only by Diversity Array Technologies (Canberra, ACT, Australia), whereas ddRADseq can be performed 'in-house' by researchers at a lower cost per sample. Thus, by using both, we aim to show the costs and advantages of each approach in phylogenetic studies of



**Table 1.** An overview of the classification schemes of the *E. odorata* complex published by authors in the past 100 years.

Species	Blakely (1934)	Pryor and Johnson (1971)	Chippendale (1988)	Brooker et al. (2015)	Australian Plant Census (Council of Heads of Australasian Herbaria 2016)	Rule (2018)	Nicolle (2019)
<i>E. odorata</i> Behr (1847)	Accepted. Distribution: SA, Flinders Ranges, Eyre Peninsula, Mount Lofty Ranges, Bordertown area; Vic., Wimmera and Goldfields	Accepted. Distribution: SA; Vic.	Accepted. Distribution: SA, Flinders Ranges, Eyre Peninsula, Mount Lofty Ranges, Bordertown area. Vic., north-western Wimmera.	Accepted. Distribution: SA, Flinders Ranges, Eyre Peninsula, Mount Lofty Ranges, Bordertown area; Vic., Wimmera.	Accepted	Accepted. Distribution: SA, ?not Bordertown area.	Accepted. Distribution: SA, southern Flinders Ranges, Eyre Peninsula, Mount Lofty Ranges, Bordertown area; Vic., Wimmera
<i>E. cajuputea</i> Miq. (1856)	Synonym of <i>Eucalyptus odorata</i> Behr	Untreated: presumably accepted Blakely's synonymisation with <i>E. odorata</i> Behr.	Synonym of <i>Eucalyptus odorata</i> Behr	Per type synonym of <i>Eucalyptus odorata</i> Behr. Concept of Nicolle (2014) is considered a synonym of <i>E. viridis</i> .	Accepted	Untreated	Accepted. Distribution: SA, Flinders Ranges, northern Eyre Peninsula
<i>E. viridis</i> R.T.Baker (1900)	Accepted. Distribution: SA, Flinders Ranges; Vic., Goldfields; NSW, widespread on inland slopes of GDR north of West Wyalong; Qld, Inglewood.	Accepted. Distribution: SA; Vic.; NSW; Qld.	Accepted. Distribution: SA, Flinders Ranges; Vic., Wimmera and Goldfields; NSW, widespread on inland slopes of GDR north of West Wyalong, Hunter Valley; Qld, south-eastern corner inland of GDR.	Accepted. Distribution: SA, Flinders Ranges and Bordertown area; Vic., Wimmera and Goldfields; NSW, widespread on inland slopes of GDR north of West Wyalong; Qld, south-eastern corner inland of GDR.	Accepted	Accepted. Distribution: Untreated	Accepted. Distribution: Vic., Goldfields; NSW, widespread on inland slopes of GDR north of West Wyalong; Qld, south-eastern corner inland of GDR.
var. <i>latiuscula</i> Blakely (1934)	Erected: 'It is readily distinguished from the typical form by the broader leaves and coarser buds and fruits.' Distribution: NSW, Minore; Qld, Inglewood.	Hybrid: <i>E. viridis</i> × <i>E. woollsiana</i>	Hybrid: <i>E. microcarpa</i> × <i>E. viridis</i> .	Reputed hybrid: <i>E. microcarpa</i> × <i>E. viridis</i> but authors state it bears a strong resemblance to populations described as <i>E. wimmerensis</i> by other authors, which they regard as a synonym of <i>E. viridis</i> .	Synonym of <i>E. viridis</i> R.T.Baker	Untreated	Hybrid: <i>E. viridis</i> × <i>E. woollsiana</i>

(Continued on next page)

**Table 1.** (Continued)

Species	Blakely (1934)	Pryor and Johnson (1971)	Chippendale (1988)	Brooker et al. (2015)	Australian Plant Census (Council of Heads of Australasian Herbaria 2016)	Rule (2018)	Nicolle (2019)
<i>E. polybractea</i> R.T.Baker (1901)	Synonym of <i>E. fruticetorum</i> F.Muell. ex Miq.	Accepted. Distribution: Vic.; NSW.	Accepted. Distribution: Vic., Goldfields; NSW, West Wyalong.	Accepted. Distribution: SA, Flinders Ranges; Vic., Goldfields; NSW, West Wyalong.	Accepted	Accepted.	Accepted. Distribution: SA, Flinders Ranges; Vic., Goldfields; NSW, West Wyalong.
subsp. <i>subcerea</i> Rule (2019)	–	–	–	Untreated	Untreated	Erected. Vic., southern Wimmera.	Synonym of <i>E. wimmerensis</i> Rule
<i>E. wimmerensis</i> Rule (1990)	–	–	–	Synonym of <i>Eucalyptus viridis</i> R.T.Baker	Accepted	Accepted.	?Accepted: possibly conspecific with <i>E. cajuputea</i> . Distribution: SA, Bordertown area; Vic., Wimmera.
subsp. <i>arapilensis</i> Rule (2019)	–	–	–	Untreated	Untreated	Erected.	Synonym of <i>E. wimmerensis</i> Rule
subsp. <i>parviformis</i> Rule (2019)	–	–	–	Untreated	Untreated	Erected.	Synonym of <i>E. wimmerensis</i> Rule
subsp. <i>pallida</i> Rule (2019)	–	–	–	Untreated	Untreated	Erected.	Synonym of <i>Eucalyptus odorata</i> Behr
subsp. <i>grata</i> Rule (2019)	–	–	–	Untreated	Untreated	Erected.	Synonym of <i>Eucalyptus odorata</i> Behr
<i>E. silvestris</i> Rule (1994)	–	–	–	Intergrade:	<i>E. microcarpa</i> × <i>E. odorata</i>	Accepted	Accepted: Closer to <i>E. microcarpa</i> than <i>E. odorata</i> complex. Distribution: SA, Bordertown area; Vic., north-western Wimmera.
Synonym of <i>Eucalyptus odorata</i> Behr							

(Continued on next page)

Table 1. (Continued)

Species	Blakely (1934)	Pryor and Johnson (1971)	Chippendale (1988)	Brooker et al. (2015)	Australian Plant Census (Council of Heads of Australasian Herbaria 2016)	Rule (2018)	Nicolle (2019)
<i>E. aenea</i> K.D.Hill (1997)	–	–	–	Synonym of <i>Eucalyptus viridis</i> R.T.Baker	Synonym of <i>E. viridis</i> R.T.Baker	Untreated	Accepted but questioned: possibly conspecific with <i>E. wimmerensis</i> . Distribution: NSW, Hunter Valley.
<i>E. castrensis</i> K.D.Hill (2002)	–	–	–	?Synonym of <i>Eucalyptus viridis</i> R.T.Baker	Accepted	Untreated	Hybrid (type material): <i>E. aenea</i> × <i>E. microcarpa</i> . taxonomic synonym of <i>E. aenea</i>
<i>E. filiformis</i> Rule (2004)	–	–	–	Synonym of <i>Eucalyptus polybractea</i> R.T.Baker	Accepted	Accepted. Distribution: Vic., one copse at Mount Jeffcott.	Synonym of <i>E. polybractea</i> R.T.Baker
<i>E. walshii</i> Rule (2004)	–	–	–	Synonym of <i>Eucalyptus viridis</i> R.T.Baker	Accepted	Accepted. Distribution: Vic., ~30 individuals at Broughton's Waterhole.	Hybrid: <i>E. wimmerensis</i> × <i>E. microcarpa</i>
<i>E. hawkeri</i> Rule (2004)	–	–	–	Intergrade: <i>E. microcarpa</i> × <i>E. viridis</i>	Accepted	Accepted. Distribution: Vic., southern Wimmera.	Intergrade: <i>E. wimmerensis</i> × <i>E. microcarpa</i>
<i>E. yarriambiack</i> Rule (2012)	–	–	–	Reputed hybrid: <i>E. viridis</i> × <i>E. odorata</i>	Accepted	Accepted. Distribution: Vic., ~150 individuals on Yarriambiack Creek, Brim.	Hybrid (type material): <i>E. odorata</i> × <i>E. largiflorens</i> . Synonym of <i>Eucalyptus odorata</i> Behr

The status of each taxon in each scheme is outlined and details of the distribution of taxa provided where authors provided this information. A number of infraspecific taxa erected within *E. odorata* and subsequently synonymised with this species or assigned to other species prior to receiving widespread recognition, are excluded, along with *E. fruticetorum*, which has been misapplied to *E. polybractea*, and *E. acacioides* A.Cunn. ex Maiden, a superfluous name for *E. viridis*. Locality abbreviations: GDR, Great Dividing Range; NSW, New South Wales; Qld, Queensland; SA, South Australia; Vic., Victoria.



eucalypts, as well as exploring the value of combined DArTseq/ddRADseq data. We sample all 12 species within the *E. odorata* complex from multiple locations throughout their ranges to test monophyly, and resolve relationships and patterns of hybridisation among them. This represents the first molecular systematics investigation focussed on the group and the first direct comparison between ddRADseq and DArTseq data in answering phylogenetic and taxonomic questions in *Eucalyptus*.

## Materials and methods

### Sample collection and preparation

The classification system of Nicolle (2019) was followed in this study, with the exception of the consideration of all 12 species in the *E. odorata* complex outlined in Table 1 as potentially valid. Where possible, samples of each species were collected from a representative set of wild populations, and this was supplemented, when necessary, with cultivated specimens from Currency Creek Arboretum, the Royal Botanic Gardens Victoria, and the private seedling experiments of Kevin Rule (Table 2). All species in the complex were represented by at least two samples except for *E. castrensis*, which grows only on Department of Defence managed land (Hill and Stanberg 2002) and for which only a single cultivated specimen, which is more referable to the putative *E. aenea* form than the putative hybrid typical material, was accessible during this study. In addition to two samples of *E. hawkeri* from Mount Arapilies in the southern Wimmera, we identified an additional specimen from the northern Wimmera as *E. hawkeri*, on the basis of its single-trunked habit, substantial stocking of rough bark and somewhat weeping foliage, providing the first record of this species north of the Little Desert. Furthermore, two samples associated with the *E. odorata* complex, but of uncertain identity, were included, namely, a seedling grown from seed of Rule's '*E. aff. polybractea* (Mount Jeffcott)', and a sample that could not be positively assigned to either *E. dumosa* A.Cunn. ex J.Oxley or *E. polybractea* from Wychitella Nature Conservation Reserve in the Victorian goldfields that was suspected to be a hybrid. In addition to the members of the *E. odorata* complex, sampling was extended to include many co-occurring species of *E. section Adnataria* to test for possible hybridisation. A sample each of *E. dumosa* (*E. section Dumaria*) and *E. oleosa* F.Muell. ex Miq. (*E. section Bisectae*), were collected as outgroups for phylogenetic analysis (Table 2).

Approximately 10 leaves were collected from each sampled individual and placed immediately into a coffee filter nested in silica beads to dehydrate before storage at the University of Melbourne. Voucher specimens were collected for at least one individual of each species at each collection site and were lodged at the University of Melbourne Herbarium (MELU; Table 2).

Because DArTseq is performed in 96 sample plates and was undertaken after the generation of preliminary results from the ddRADseq investigation (91 samples), there were some changes in sampling between the two datasets. Namely, as discussed below, the wild population of *E. filiformis* was found to be clonal, so six of the eight samples included in the ddRADseq dataset were excluded from the DArTseq run, and an extra two samples of *E. polybractea*, five of *E. viridis*, one *E. aenea* and a third outgroup sample of *E. leptophylla* F.Muell. ex Miq. were included to bring the total sample number in the DArTseq dataset to 94.

Field collections for this study were completed under scientific collecting permits granted by Department of Environment, Land, Water and Planning, Government of Victoria (10008557), NSW National Parks & Wildlife Service, Office of Environment & Heritage, Government of New South Wales (SL102100) and Department for Environment and Water, Government of South Australia (Q26766-1).

### DNA isolation, library preparation and sequencing

The modified CTAB protocol of Schuster et al. (2018) was employed for total DNA isolation from 70 to 80 mg of dry leaf material for each of the 100 samples. A Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to check extraction purity and a Qubit fluorometer (ver. 2.0, Thermo Fisher Scientific) to quantify the concentration of extracted DNA. Informed by the results of Yang et al. (2016) *in silico* tests of digestion of the *E. grandis* W.Hill genome, the restriction enzymes EcoR1-HF and Msp1 were chosen for genome digestion to create the ddRADseq library. The library preparation and pooling followed the protocol of Fahey et al. (2021). The resulting pool was quantified on a 2200 Tape Station (Agilent) using a D1000 kit and sequencing was undertaken at the Walter and Eliza Hall Institute of Medical Research (WEHI) Genomics Hub, Melbourne, Vic., Australia, on an Illumina NextSeq. 500 using a 300 cycle, 2× paired-end reads kit. For DArTseq, extractions were standardised to a concentration of 50 ng μL<sup>-1</sup> and sent to Diversity Array Technologies Pty Ltd (Canberra, ACT, Australia) for high-density sequencing using their proprietary DArTseq pipeline (Kilian et al. 2012). Filtered read data as well as DArT SNP data were returned for analysis.

### Dataset filtering, construction, and analysis

The Cutadapt (ver. 2.8) python script (Martin 2011) was used to trim raw reads from ddRADseq to remove restriction-site residues and bases added for adaptor diversity with a maximum error rate of 0.5. The assembly pipeline ipyrad (ver. 0.9.62, see <https://ipyrad.readthedocs.io/en/master/>, Eaton and Overcast 2020) was used to reconstruct loci by mapping reads to the *Eucalyptus grandis* reference genome (Myburg et al. 2014); this allowed us to avoid problems

**Table 2.** Sample collection details and herbarium accession numbers for specimen used in this sample.

Species	Sample	Herbarium accession	Datasets present in	Longitude	Latitude	Location
<i>Eucalyptus aenea</i>	PSF90A	MELUD122656a	Both	150.4117	-32.29352	Goulburn River NP, Hunter Valley, NSW
<i>Eucalyptus aenea</i>	PSF90H	–	DARtseq only	150.41022	-32.29282	Goulburn River NP, Hunter Valley, NSW
<i>Eucalyptus aenea</i>	PSF90J	MELUD122657a	Both	150.40936	-32.29303	Goulburn River NP, Hunter Valley, NSW
<i>Eucalyptus albens</i>	PSF88	MELUD122655a	Both	148.77977	-32.30312	Wongarbon NR, central-western NSW
<i>Eucalyptus albobarpurea</i>	CC560	MELUD122661a	Both	137.74009	-35.80631	American River lookout, Kangaroo Island, SA
<i>Eucalyptus albobarpurea</i>	FI of DN3180	MELUD122663a Parent: CANB892303.1	Both	135.73306	-34.83306	Cultivated: Currency Creek Arboretum. Seed provenance: Sleaford Mere, Eyre Peninsula, SA
<i>Eucalyptus baueriana</i>	PSF68A	MELUD122642a	Both	144.49664	-37.66121	Long Forest NCR, Bacchus Marsh, Vic.
<i>Eucalyptus behriana</i>	PSF47C	–	Both	135.68701	-34.46946	South of Edillilie, Eyre Peninsula, SA
<i>Eucalyptus behriana</i>	PSF47I	MELUD122610a	Both	135.71107	-34.37423	Roadside, south of Edillilie, Eyre Peninsula, SA
<i>Eucalyptus behriana</i>	PSF69E	MELUD122643a	Both	144.50092	-37.66377	Long Forest NCR, Bacchus Marsh, Vic.
<i>Eucalyptus behriana</i>	PSF75H	MELUD122648a	Both	147.27818	-33.92756	Roadside, east of West Wyalong, South-West Slopes, NSW
<i>Eucalyptus behriana</i>	PSF75J	–	Both	147.2863	-33.92861	Roadside, east of West Wyalong, South-West Slopes, NSW
<i>Eucalyptus cajuputea</i>	FI of DN5929	MELUD122665a Parent: PERTH8797374	Both	135.58083	-32.58056	Cultivated: Currency Creek Arboretum. Seed provenance: Chillunie campground, Gawler Ranges, SA
<i>Eucalyptus cajuputea</i>	PSF45H	MELUD122601a	Both	136.59554	-33.61663	Yeldulknie CP, Eyre Peninsula, SA
<i>Eucalyptus cajuputea</i>	PSF48B	–	Both	138.0014	-32.4154	Devils Peak, southern Flinders Ranges, SA
<i>Eucalyptus cajuputea</i>	PSF48H	MELUD122613a	Both	137.99721	-32.41522	Devils Peak, southern Flinders Ranges, SA
<i>Eucalyptus cajuputea</i>	PSF50D	–	Both	138.5919	-31.54312	Wilpena Pound, northern Flinders Ranges, SA
<i>Eucalyptus cajuputea</i>	PSF50P	MELUD122620a	Both	138.59294	-31.54344	Wilpena Pound, northern Flinders Ranges, SA
<i>Eucalyptus castrensis</i>	FI of DN5263	MELUD122664a	Both	151.23861	-32.75611	Cultivated: Currency Creek Arboretum. Seed provenance: Singleton Military Base, Hunter Valley, NSW
<i>Eucalyptus dumosa</i>	PSF34A	MELUD122581a	Both	140.78767	-36.12536	North of Bordertown, south-eastern SA
<i>Eucalyptus fasciculosa</i>	PSF36A	MELUD122584a	Both	138.81891	-34.67973	Para Wirra CP, southern Mount Lofty Ranges, SA
<i>Eucalyptus filiformis</i>	PSF100B	MELUD122676a	Both	143.1427778	-36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.

(Continued on next page)

Table 2. (Continued)

Species	Sample	Herbarium accession	Datasets present in	Longitude	Latitude	Location
<i>Eucalyptus filiformis</i>	PSF100C	–	ddRADseq only	143.1427778	–36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus filiformis</i>	PSF100D	–	ddRADseq only	143.1427778	–36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus filiformis</i>	PSF100E	–	ddRADseq only	143.1427778	–36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus filiformis</i>	PSF100F	–	ddRADseq only	143.1427778	–36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus filiformis</i>	PSF100G	–	ddRADseq only	143.1427778	–36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus filiformis</i>	PSF100H	–	ddRADseq only	143.1427778	–36.31213889	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus filiformis</i>	PSF73	–	Both	–	–	Cultivated: Royal Botanical Gardens Victoria. Seed provenance: Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus froggattii</i>	PSF99A	MELUD122673a	Both	143.5694444	–36.39333333	Roadside, north-west of Wedderburn, Victorian goldfields, Vic.
<i>Eucalyptus froggattii</i>	PSF99B	MELUD122674a	Both	143.5655556	–36.39294444	Roadside, north-west of Wedderburn, Victorian goldfields, Vic.
<i>Eucalyptus hawkeri</i>	KRule5370	MELUD122690a	Both	141.8383	–36.7508	Mount Arapiles-Tooan NP, Wimmera, Vic.
<i>Eucalyptus hawkeri</i>	PSF54A	MELUD122625a	Both	141.8075	–36.76486	Mount Arapiles-Tooan NP, Wimmera, Vic.
<i>Eucalyptus hawkeri</i>	PSF105E	MELUD122687a	Both	141.4527778	–36.31858333	Roadside, Diapur, Wimmera, Vic.
<i>Eucalyptus largiflorens</i>	PSF102	MELUD122678a	Both	143.1436111	–36.28586111	Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus largiflorens</i>	PSF103	MELUD122679a	Both	142.4130556	–36.05597222	Roadside, north of Brim, Wimmera, Vic.
<i>Eucalyptus largiflorens</i>	PSF64	MELUD122636a	Both	142.01988	–36.23524	Roadside, north of Antwerp, Wimmera, Vic.
<i>Eucalyptus leptophylla</i>	PSF221	–	DARtseq only	143.62355	–36.348394	Wychitella NCR, Victorian goldfields, Vic.
<i>Eucalyptus leucoxydon</i>	PSF98	MELUD122672a	Both	143.61	–36.36444444	Wychitella NCR, Victorian goldfields, Vic.
<i>Eucalyptus melliodora</i>	PSF106A	MELUD122698a	Both	144.9557	–37.78963	Royal Park, Melbourne, Vic.
<i>Eucalyptus microcarpa</i>	PSF59	–	Both	141.72267	–36.73057	Jane Duff Highway Park, Wimmera, Vic.
<i>Eucalyptus microcarpa</i>	PSF71D	MELUD122646a	Both	144.50787	–37.66367	Long Forest NCR, Bacchus Marsh, Vic.
<i>Eucalyptus moluccana</i>	PSF94	–	Both	150.77838	–34.08188	Roadside, Glen Alpine, Sydney, NSW
<i>Eucalyptus odorata</i>	PSF36B	MELUD122585a	Both	138.81903	–34.68059	Para Wirra CP, South Mount Lofty Ranges, SA
<i>Eucalyptus odorata</i>	PSF38	MELUD122587a	Both	138.96329	–34.45696	Roadside, north-west of Nurioopta, Barossa Valley, SA
<i>Eucalyptus odorata</i>	PSF45C	MELUD122596a	Both	136.60605	–33.64548	Yeldulknie CP, Eyre Peninsula, SA

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Table 2. (Continued)

Species	Sample	Herbarium accession	Datasets present in	Longitude	Latitude	Location
<i>Eucalyptus odorata</i>	PSF45E	MELUD122598a	Both	136.60661	-33.64596	Yeldulknie CP, Eyre Peninsula, SA
<i>Eucalyptus odorata</i>	PSF45F	MELUD122599a	Both	136.60653	-33.64603	Yeldulknie CP, Eyre Peninsula, SA
<i>Eucalyptus oleosa</i>	PSF41B	MELUD122591a	Both	138.10168	-33.03633	Telowie Gorge NP, southern Flinders Ranges, SA
<i>Eucalyptus polyanthemos</i>	PSF70B	MELUD122645a	Both	144.50594	-37.66137	Long Forest NCR, Bacchus Marsh, Vic.
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF21C	MELUD122569a	DARtseq only	143.61945	-36.35311	Wychitella NCR, Victorian goldfields, Vic.
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF27A	MELUD122575a	Both	144.311692	-36.567858	Greater Bendigo NP, Victorian goldfields, Vic.
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF27H	–	Both	144.311802	-36.588538	Greater Bendigo NP, Victorian goldfields, Vic.
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF49C	MELUD122615a	Both	138.00116	-32.4155	Devils Peak, southern Flinders Ranges, SA
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF49F	MELUD122616a	Both	137.99759	-32.4152	Devils Peak, southern Flinders Ranges, SA
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF50B	MELUD122618a	Both	138.59196	-31.54206	Wilpena Pound, northern Flinders Ranges, SA
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF50E	MELUD122619a	Both	138.59187	-31.54297	Wilpena Pound, northern Flinders Ranges, SA
<i>Eucalyptus polybractea</i> subsp. <i>subcerea</i>	PSF56B	MELUD122628a	Both	142.07626	-36.80765	Roadside, south-east of Lower Norton, Wimmera, Vic.
<i>Eucalyptus polybractea</i> subsp. <i>subcerea</i>	PSF56C	–	Both	142.07581	-36.80931	Roadside, south-east of Lower Norton, Wimmera, Vic.
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF74A	MELUD122647a	Both	147.14005	-33.9847	Charcoal Tank Reserve, South-West Slopes, NSW
<i>Eucalyptus polybractea</i> subsp. <i>polybractea</i>	PSF74J	–	Both	147.15279	-34.00844	Roadside, south of West Wyalong, South-West Slopes, NSW
<i>Eucalyptus polybractea</i> × <i>E. dumosa</i>	PSF96A	MELUD122670a	DARtseq only	143.60444	-36.36528	Wychitella NCR., Victorian goldfields, Vic.
<i>Eucalyptus populnea</i>	PSF79	MELUD122651a	Both	147.26711	-33.92327	Roadside, east of West Wyalong, South-West Slopes, NSW
<i>Eucalyptus porosa</i>	PSF39	MELUD122588a	Both	138.66748	-34.13232	Roadside, north-west of Rhynie, Mid-North SA
<i>Eucalyptus porosa</i>	PSF45D	MELUD122597a	Both	136.60658	-33.6459	Yeldulknie CP, Eyre Peninsula

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Table 2. (Continued)

Species	Sample	Herbarium accession	Datasets present in	Longitude	Latitude	Location
<i>Eucalyptus sideroxylon</i>	PSF95	MELUD122697a	Both	144.95621	-37.79237	Cultivated: Royal Park, Melbourne, Vic. Seed provenance: Unknown
<i>Eucalyptus silvestris</i>	F1 of DN445	MELUD122662a Parent: AD 162852	Both	140.91667	-36.33333	Cultivated: Currency Creek Arboretum. Seed provenance: 15.2 km west of Bordertown, south-eastern SA
<i>Eucalyptus silvestris</i>	PSF61A	MELUD122632a	Both	141.44124	-36.17295	Roadside, south of Yanac, Wimmera, Vic.
<i>Eucalyptus</i> aff. <i>polybractea</i> Mt. Jeffcott	KRule3912	MELUD122694a	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Mount Jeffcott FFR, Victorian goldfields, Vic.
<i>Eucalyptus tricarpa</i>	PSF107	MELUD122700a	Both	144.94822	-37.78965	Cultivated: Royal Park, Melbourne, Vic. Seed provenance: Unknown
<i>Eucalyptus viridis</i>	PSF22B	MELUD122570a	DArTseq only	143.61636	-36.35474	Wychitella NCR., Victorian goldfields, Vic.
<i>Eucalyptus viridis</i>	PSF28C	MELUD122576a	Both	144.311673	-36.572918	Greater Bendigo NP, Victorian goldfields, Vic.
<i>Eucalyptus viridis</i>	PSF28I	–	Both	144.311686	-36.591507	Greater Bendigo NP, Victorian goldfields, Vic.
<i>Eucalyptus viridis</i>	PSF76D	MELUD122649a	Both	147.15471	-33.98636	Charcoal Tank Reserve, South-West Slopes, NSW
<i>Eucalyptus viridis</i>	PSF76E	MELUD122650a	Both	147.15714	-33.98701	Roadside, east of West Wyalong, South-West Slopes, NSW
<i>Eucalyptus viridis</i>	PSF86A	MELUD122653a	DArTseq only	146.45175	-31.5491	Roadside, Canbelego on Barrier Highway, Canbelego Downs, NSW
<i>Eucalyptus viridis</i>	PSF86I	–	DArTseq only	146.49374	-31.55663	Roadside, Canbelego on Barrier Highway, Canbelego Downs, NSW
<i>Eucalyptus viridis</i>	PSF91B	MELUD122658a	DArTseq only	149.27872	-30.66369	Pilliga NP, Pilliga, NSW
<i>Eucalyptus viridis</i>	PSF91E	–	DArTseq only	149.28014	-30.66465	Pilliga NP, Pilliga, NSW
<i>Eucalyptus viridis</i>	PSF93B	MELUD122660a	Both	151.08972	-28.45646	Roadside, south of Inglewood, Darling Downs, Qld
<i>Eucalyptus viridis</i>	PSF93H	–	Both	151.08911	-28.4562	Roadside, south of Inglewood, Darling Downs, Qld
<i>Eucalyptus walshii</i>	F1 of DN6866	MELUD122666a	Both	141.33806	-36.56222	Cultivated: Currency Creek Arboretum. Seed provenance: Little Desert NP, Lowan Mallee, Vic.
<i>Eucalyptus walshii</i>	PSF72	MEL 2384948A	Both	–	–	Cultivated: Royal Botanical Gardens Victoria. Seed provenance: Little Desert NP, Lowan Mallee, Vic.
<i>Eucalyptus wimmerensis</i> (subspecies unknown)	KRule0025	MELUD122695a	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Roadside, north of Nhill, Wimmera, Vic.

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Table 2. (Continued)

Species	Sample	Herbarium accession	Datasets present in	Longitude	Latitude	Location
<i>Eucalyptus wimmerensis</i> subsp. <i>pallida</i>	KRule1012	MELUD122692a	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Roadside, west of Diapur, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>grata</i>	KRule10312	MELUD122691a	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Roadside, west of Diapur, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>parviformis</i>	KRule1714	MELUD122688a	Both	141.714899	–36.689886	Nurcong Flora Reserve, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> (subspecies unknown)	KRule2014	MELUD122693a	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Nurcong Flora Reserve, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> (subspecies unknown)	KRule3012	–	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Roadside, west of Cooack, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> (subspecies unknown)	KRule4708	MELUD122696a	Both	–	–	Cultivated: Kevin Rule's private collection. Seed provenance: Nurcong Flora Reserve, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>wimmerensis</i>	PSF105C	MELUD122685a	Both	141.4513889	–36.32038889	Roadside, Diapur, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>arapilensis</i>	PSF58A	MELUD122630a	Both	141.72247	–36.73059	Jane Duff Highway Park, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>arapilensis</i>	PSF58D	–	Both	141.72237	–36.73011	Jane Duff Highway Park, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>wimmerensis</i>	PSF60A	MELUD122631a	Both	141.49132	–36.40552	Mallee Dam Bushland Reserve, Wimmera, Vic.
<i>Eucalyptus wimmerensis</i> subsp. <i>wimmerensis</i>	PSF60G	–	Both	141.49142	–36.40309	Mallee Dam Bushland Reserve, Wimmera, Vic.
<i>Eucalyptus woollsiana</i>	PSF92A	MELUD122659a	Both	151.09396	–28.47847	Roadside, south of Inglewood, Darling Downs, QLD
<i>Eucalyptus yarriambiack</i>	PSF104A	MELUD122680a	Both	142.4041667	–36.02708333	Roadside, north of Brim, Wimmera, Vic.
<i>Eucalyptus yarriambiack</i>	PSF104B	MELUD122681a	Both	142.4030556	–36.02708333	Roadside, north of Brim, Wimmera, Vic.
<i>Eucalyptus yarriambiack</i>	PSF104C	MELUD122682a	Both	142.4147222	–36.04919444	Roadside, north of Brim, Wimmera, Vic.
<i>Eucalyptus yarriambiack</i>	PSF65A	MELUD122637a	Both	142.42645	–36.05482	Roadside, north of Brim, Wimmera, Vic.

Where given for cultivated specimen, collection coordinates represent those of the wild maternal tree where the seed was sourced from. Reserve type abbreviations: CP, Conservation Park; FFR, Flora and Fauna Reserve; NCR, Nature Conservation Reserve; NP, National Park.



related to inclusion of paralogous loci as *ipyrad* discards any reads that do not map uniquely to the reference genome. Various values for the statistical and majority rule base call minimum (6, 20), clustering threshold (0.85, 0.9, 0.95) and minimum number of samples with data at each locus (four samples, 25, 50, 75% of total samples) were tested to find the most informative dataset. The dataset presented in this paper is that derived using base call minimums of six, clustering threshold of 0.85% and a minimum of four samples returned per locus; however, the loci reconstructed using each of the different input variables showed little variation beyond the increasing number of loci retained when the minimum sample threshold was decreased (Supplementary Table S1).

Principal-component analyses (PCA) and network analyses were performed on both the ddRADseq and DArTseq SNP datasets individually. Because the total size of the ddRADseq alignment produced by *ipyrad* was too large to feasibly analyse, we converted the single-nucleotide polymorphism (SNP) dataset produced by *ipyrad* to a NEXUS alignment to perform these analyses. Additionally, using the *DArTR* R package (ver. 1.9.9.1, see <https://CRAN.R-project.org/package=dartR>; Gruber et al. 2018) we converted the DArTseq data to an alignment of SNPs for network analysis. Networks were created in Splitstree (ver. 4.16.1, Universität Tübingen, Tübingen, Germany; Huson and Bryant 2006) using the NeighbourNet algorithm and Uncorrected-P distances ignoring ambiguities and with outgroups excluded.

Principal-component analysis is sensitive to missing data and uneven sampling (McVean 2009). So, we created a further SNP dataset from our ddRADseq data utilising *VCfTools* (ver. 0.1.16, see <https://vcftools.github.io/index.html>; Danecek et al. 2011), where we allowed maximum missing data per SNP of 10%, filtered for one SNP per 5000-bp window of the *E. grandis* reference genome, and included only samples in *E. series Subbuxaeales* and excluded seven of the eight clonal *E. filiformis* samples and two *E. wimmerensis* samples with significantly above-average missing data, namely, KRule1714 and KRule3012 with 3215 and 1413 loci respectively in the final assembly, against an average of 10 313 loci for all other samples. Similar filtering was applied to the DArTseq SNPs utilising the *DArTR* package in R (R Foundation for Statistical Computing, Vienna, Austria), including filtering for a single SNP per locus, reproducibility of >0.98, callrate >0.90 and a minor allele frequency of >0.02. PCAs were performed in R utilising the *VCfR* (ver. 1.12.0, <https://CRAN.R-project.org/package=vcfR>; Knaus and Grünwald 2017) package to load ddRADseq VCF data, and the *dudi.pca* function of *adegenet* (ver. 2.1.5, <https://CRAN.R-project.org/package=adegenet>; Jombart and Ahmed 2011), before being graphed using *ggplot2* (ver. 3.3.5, <https://CRAN.R-project.org/package=ggplot2>; Wickham 2016).

The PHYLIP alignment produced by *ipyrad* was used to estimate a maximum-likelihood (ML) phylogeny in RAxML

(ver. 8.2.12, see <https://cme.h-its.org/exelixis/web/software/raxml/>; Stamatakis 2014) by using the rapid hill-climbing tree-search algorithm and performing 100 rapid bootstraps. Because of the anonymous nature of the data, no partitioning was used, along with a GTR substitution matrix. Additionally, a maximum-parsimony (MP) analysis was undertaken using PAUP (ver. 4.0a, see <https://paup.phylosolutions.com/>; Swofford 2002), with 100 bootstrap replicates and multistate characters interpreted as polymorphisms. Owing to the size and complexity of the dataset, the required computational resources proved prohibitive for Bayesian phylogeny estimation. Trees were visualised in TreeGraph 2 (ver. 2.15, see <http://treegraph.bioinfweb.info/>; Stöver and Müller 2010) to build figures. The same phylogenetic analyses were performed on an alignment of the DArTseq data generated using the *gl2fasta* function of the *DArTR* R package. The resultant phylogenies are available in the supplementary material of this article (Supplementary Fig. S1–S4).

Although minor differences in relationships among samples existed between the two individual data sources, so as to build the most robust phylogeny possible, a further *ipyrad* run was performed utilising similar settings as for the ddRADseq dataset. The only change to those settings was to treat the data as RAD reads rather than ddRAD and this was undertaken on the combined DArT reads and the forward reads from ddRADseq to reconstruct the largest alignment possible while not incidentally including any loci twice. Although previous studies have highlighted that different techniques to generate molecular data for phylogenetic studies can generate different results (Collins and Hrbek 2015; Kirschner et al. 2021), in this case, both techniques are enzymatic reduced-representation library-generation techniques and therefore there should be limited causes of bias in the representation of the genome between them. Both MP and ML phylogenetic analyses were performed on the combined dataset, as they were on the individual datasets. Additionally, a tetrad analysis with 100 bootstrapping replicates was performed in the *ipyrad* toolbox (Eaton and Overcast 2020) on the combined dataset and is presented in the supplementary material (Supplementary Fig. S5).

To investigate the patterns of introgression in samples that showed unexpected or unstable placement in the phylogenetic analyses and PCAs, we undertook ABBA-BABA tests utilising the combined ddRAD and DArT data. ABBA-BABA tests were performed using the *ipyrad* analysis toolbox. In all tests, species and samples of interest were treated as P2 individuals and the sample PSF41B (*E. oleosa*) was used as the outgroup. For those samples where the ABBA-BABA tests indicated hybridisation between parental species we had sampled, we also performed NewHybrid tests (ver. 1.1, see <https://github.com/eriqande/newhybrids>; Anderson and Thompson 2002) to determine the hybrid generations. NewHybrids was performed using the *gl.nhybrids* function from the *DArTR* R package. A filtered version of the combined SNP dataset with a minimum of 75% of

samples represented at each locus and one SNP per 5000-bp window of the *E. grandis* reference genome was used for NewHybrids analyses.

The full dataset included several suspected hybrids and introgressed samples that caused reduced support values throughout the phylogeny; therefore, we completed additional ML and MP analyses on both the individual ddRADseq and DArTseq datasets, and on the combined dataset, with 10 samples being removed on the basis of the findings of earlier analyses and hybridisation tests. Seven samples that were suspected to be introgressed *a priori*, showing unexpected placement in the initial tree and support for introgression in ABBA-BABA tests, were removed, including the suspected *E. dumosa* × *E. polybractea* intersectional hybrid PSF96A, the suspected *E. filiformis* × *E. leucoxydon* F.Muell. interserial hybrid KRule3912, and the five samples of *E. hawkeri* and *E. silvestris* (DN445, KRule5370, PSF54A, PSF61A and PSF105E). Additionally, three samples that were oddly placed in the initial analyses and networks, but for which patterns of introgression could not be established in ABBA-BABA tests, were also excluded, including *E. cajuputea* PSF50P, *E. polybractea* PSF50B and *E. aenea* PSF90J. Because of a lack of supported relationships in the reduced tree within the core of the *E. odorata* complex, which is defined as including, from this point forward, *E. odorata*, *E. cajuputea*, *E. wimmerensis*, *E. walshii*, *E. yarriambiack*, *E. filiformis*, *E. polybractea*, and *E. viridis* samples from south-eastern Queensland, an isolation by distance test was undertaken in R using the *mantel* function from the *vegan* package (ver. 2.5-6, J. Oksanen, F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlenn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, and H. Wagner, see <https://CRAN.R-project.org/package=vegan>) on uncorrelated-P distances calculated from the SNP alignment used in MP analyses.

## Results

### Datasets

The final ddRAD dataset used in network analyses contained 1 032 139 SNPs across 89 samples, with a missing data proportion of 65.85% and was reduced to 2287 SNPs across 59 samples for PCA analysis. The DArTseq network dataset contained 60 991 SNPs, with 43.6% missing data across 90 samples, whereas the PCA version contained 68 samples and 2681 SNPs. The combined alignment used for ML phylogeny reconstruction contained 119 489 loci and was a total of 12 430 010 bp in length, with the 975 539 SNPs present in the dataset being used for MP phylogeny reconstruction.

### ddRADseq and DArTseq networks and PCAs

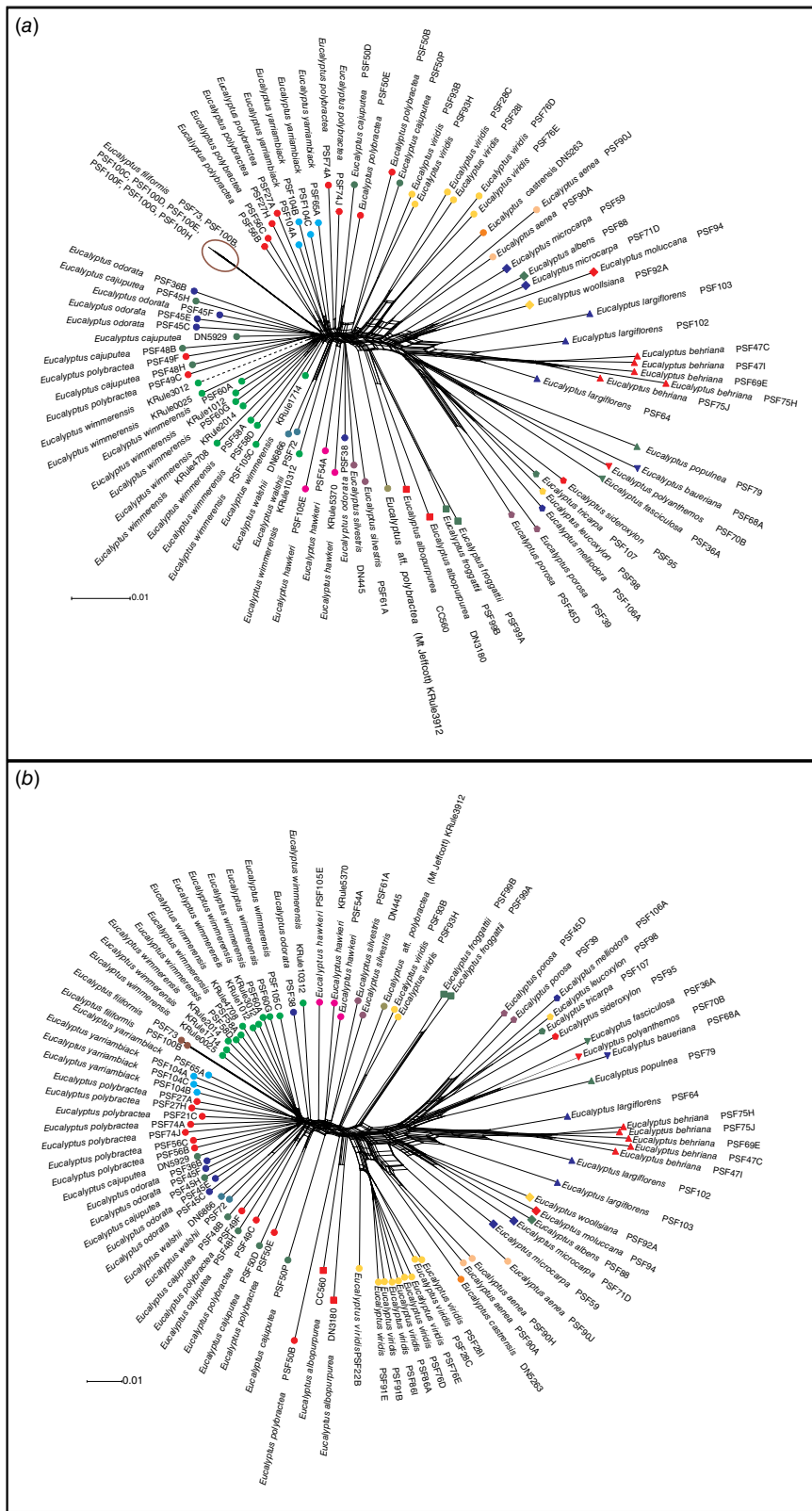
In both the ddRAD and DArT networks (Fig. 2), the clustering of all samples other than the mallee species in *E. series Subbuxales* (the *E. odorata* complex, *E. albopurpurea*, and

*E. froggattii*) was identical and matched the relationships established in subsequent phylogenetic analyses and are discussed in regard to them. Relationships within the *E. odorata* complex are not particularly clear and varied marginally between the datasets (Table 3); however, the clustering of the samples in the core *E. odorata* complex is much clearer in the DArT network than the ddRAD network. A comparison between the relationships of these samples is provided in Table 3; however, PCAs, phylogenies and ABBA-BABA tests provided greater insight into the relationships in this group.

In Fig. 3, we see that although PC3 in the ddRAD analysis matches PC2 from the DArT one, overall, the PCA plots produced are very similar and fit with the patterns observed in the networks and phylogenies. PC2 in the ddRADseq analysis predominately separates the two *E. froggattii* samples from the other samples and has only a marginally higher eigenvalue than does PC3 (3.80 v. 3.35%). It is likely that the difference in sampling between the two datasets, namely the extra six *E. viridis* samples in the DArTseq analysis, is responsible for this difference. In both analyses, the following three main groupings form: the grey boxes, *E. viridis* and allied species, and the remainder of the *E. odorata* complex. Both *E. froggattii* and *E. albopurpurea* fall towards the centre of the plot using the two selected PCs, although the former segregates on PC2 and PC3 in the ddRAD and DArT analyses respectively (not shown). In both plots, the *E. viridis* segregates *E. aenea* and *E. castrensis* sits between *E. viridis* and the grey boxes, and the most northerly population of *E. viridis* sampled sits between the other *E. viridis* samples and the rest of the *E. odorata* complex. Additionally, the two suspected *E. wimmerensis*–*E. microcarpa* intergrades, *E. hawkeri* and *E. silvestris*, sit between the *E. odorata* complex and the main *E. odorata* complex cluster, albeit closer to the former. One sample of the grey-box *E. microcarpa* (PSF59) collected at a locality where *E. wimmerensis* is also present, separates from the other grey-box samples towards the *E. odorata* complex cluster in the DArT plot. All these results suggest possible hybridisation and genetic introgression between these taxa that we investigate using ABBA-BABA tests. There is no separation of the remaining seven species in the *E. odorata* complex that form the main cluster, although *E. yarriambiack* and *E. walshii* sit towards the end of the cluster closest to the grey boxes, which may suggest some introgression into these populations as has previously been hypothesised, a finding we test in our ABBA-BABA tests.

### Combined data phylogeny

There were no supported differences between ML and MP phylogenies in either the full-sample (Fig. 4) or reduced-sample (Fig. 5) analyses at an 80% bootstrap threshold, with the reduced sampling analyses including more nodes that met this threshold (50 in the complete dataset, 55 in the



**Fig. 2.** Neighbour-net networks of *E. odorata* complex taxa and co-occurring members of *E.* section *Adnataria* created in Splitstree V.4 using uncorrelated-P distances of SNPs generated by A. ddRADseq and B. DArTseq. Tips are coloured by species, with shapes used to distinguish different major groups: *E.* series *Heterophloiae* (inverse triangles), *E.* series *Melliodorae* (hexagons), *E.* series *Buxuales* (triangles), the grey-box taxa (diamonds), mallee members of *E.* series *Subbuxuales* not in the *E. odorata* complex (squares), and the *E. odorata* complex (circles) coloured consistent with Fig. 1.

reduced sampling dataset). The following discussion focusses primarily on the reduced sample phylogeny, unless otherwise specified.

As expected, the outgroups formed two clades, a basal one containing species of *E.* section *Bisectae* and a further one containing the sample of *E. dumosa* (*E.* section *Dumaria*)

**Table 3.** A summary of the clustering of mallee species in *E. series Subbuxaeales* in both our ddRADseq and DArTseq networks (Fig. 2).

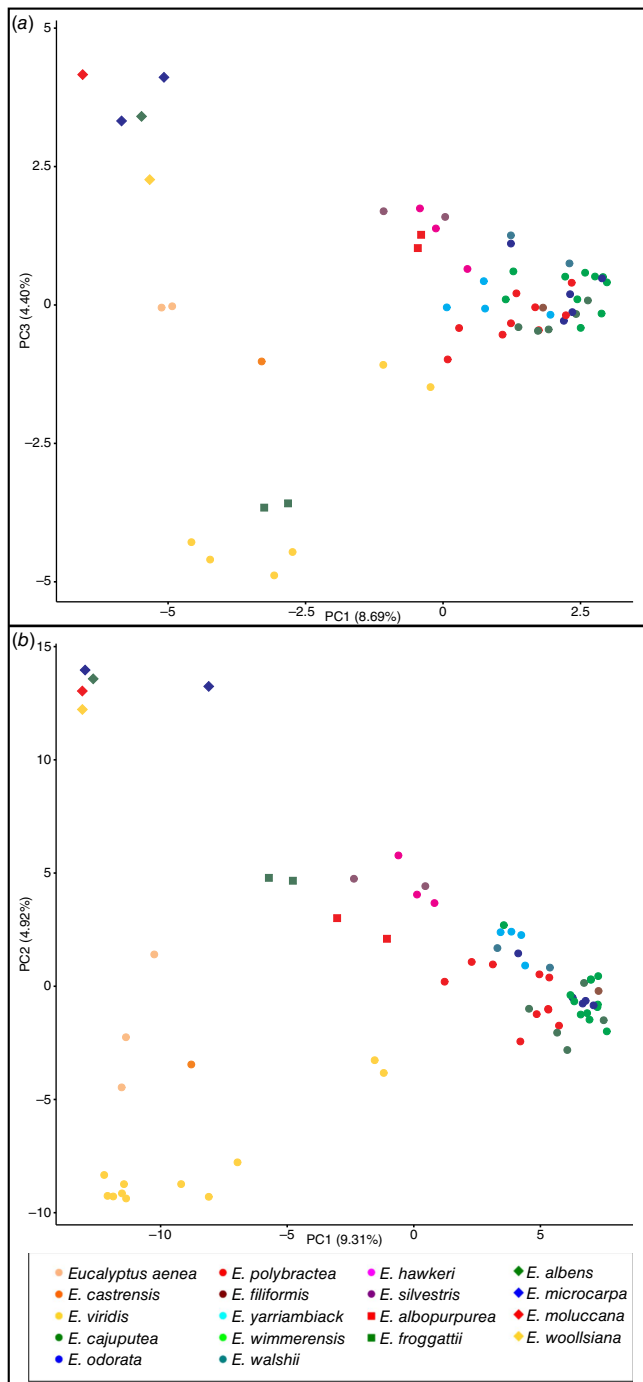
Species	Monophyletic cluster ddRADseq	Monophyletic cluster DArTseq	Same clustering in both datasets?
<i>E. albopurpurea</i>	Yes	Yes	No – with <i>E. froggattii</i> in ddRADseq network and as a distinct cluster in DArTseq network
<i>E. froggattii</i>	Yes	Yes	No – with <i>E. albopurpurea</i> in ddRADseq network and as a distinct cluster in DArTseq network
<i>E. viridis</i>	No – northern samples outside main <i>E. viridis</i> , <i>E. aenea</i> , <i>E. castrensis</i> cluster	No – northern samples outside main <i>E. viridis</i> , <i>E. aenea</i> , <i>E. castrensis</i> cluster	Yes
<i>E. aenea</i>	Yes	Yes – however, one sample (PSF90J) shows greater separation from other samples and links to the grey boxes	No
<i>E. castrensis</i>	Single sample clustered with <i>E. aenea</i>	Single sample clustered with <i>E. aenea</i>	Yes
<i>E. cajuputea</i>	No – Flinders Ranges samples cluster with <i>E. polybractea</i> samples from Flinders Ranges and the remaining samples cluster intermingled with <i>E. odorata</i>	No – Flinders Ranges samples cluster with <i>E. polybractea</i> samples from Flinders Ranges and the remaining samples cluster intermingled with <i>E. odorata</i>	Yes
<i>E. odorata</i>	No – sample PSF38 clusters with <i>E. silvestris</i> , remaining samples being intermingled with <i>E. cajuputea</i> not from Flinders Ranges	No – sample PSF38 clusters with <i>E. wimmerensis</i> , remaining samples being intermingled with <i>E. cajuputea</i> not from Flinders Ranges	No – sample PSF38 falls with <i>E. silvestris</i> in ddRAD and <i>E. wimmerensis</i> in DArT
<i>E. polybractea</i>	No – Flinders Ranges samples cluster with <i>E. cajuputea</i> samples also from Flinders Ranges	No – Flinders Ranges samples cluster with <i>E. cajuputea</i> samples also from Flinders Ranges	No – narrow clusters hold, but relationships among them vary
<i>E. filiformis</i>	Clonal	Clonal	No – with <i>E. polybractea</i> and <i>E. yarriambiack</i> in ddRAD, and <i>E. wimmerensis</i> in DArT
<i>E. yarriambiack</i>	Yes	Yes	Yes
<i>E. wimmerensis</i>	No – subsp. <i>grata</i> sample (KRule10312) outside main cluster	No – subsp. <i>grata</i> sample (KRule10312) outside main cluster	Yes
<i>E. silvestris</i>	Yes	Yes	Yes
<i>E. hawkeri</i>	No – PSF105E and PSF54A cluster together, KRule5370 clusters with <i>E. silvestris</i> and <i>E. odorata</i> PSF38	No – KRule5370 and PSF54A cluster together, PSF105E on lone branch	No
<i>E. walshii</i>	Yes	Yes	No – with <i>E. wimmerensis</i> subsp. <i>grata</i> in ddRAD, distinct cluster in DArT
<i>E. aff. polybractea</i> (Mount Jeffcott) KRule3912	Lone branch outside core <i>E. odorata</i> complex	Lone branch outside core <i>E. odorata</i> complex	Yes
Genetically distinct Flinders Ranges samples ( <i>E. polybractea</i> PSF50B and <i>E. cajuputea</i> PSF50P)	Cluster together linked to other Wilpena Pond samples	Separate branches outside core <i>E. odorata</i> complex not closely linked to other Wilpena Pond samples	No

A comparison between the clustering of these datasets is also provided.

and the putative *E. polybractea* × *E. dumosa* hybrid sample (PSF96A), which was included only in the full dataset analysis. The first clade to diverge in *E. section Adnataria* included the three sampled species from *E. series Heterophloiae* (*E. baueriana* Schauer, *E. polyanthemos* Schauer and

*E. fasciculosa* F.Muell.) and *E. populnea* from *E. series Buxaeales*, although the inclusion of the latter in this clade is not supported in the MP analysis. A relationship between *E. populnea* F.Muell. and *E. ser. Heterophloiae* makes *E. series Buxaeales* polyphyletic in our phylogeny. The next





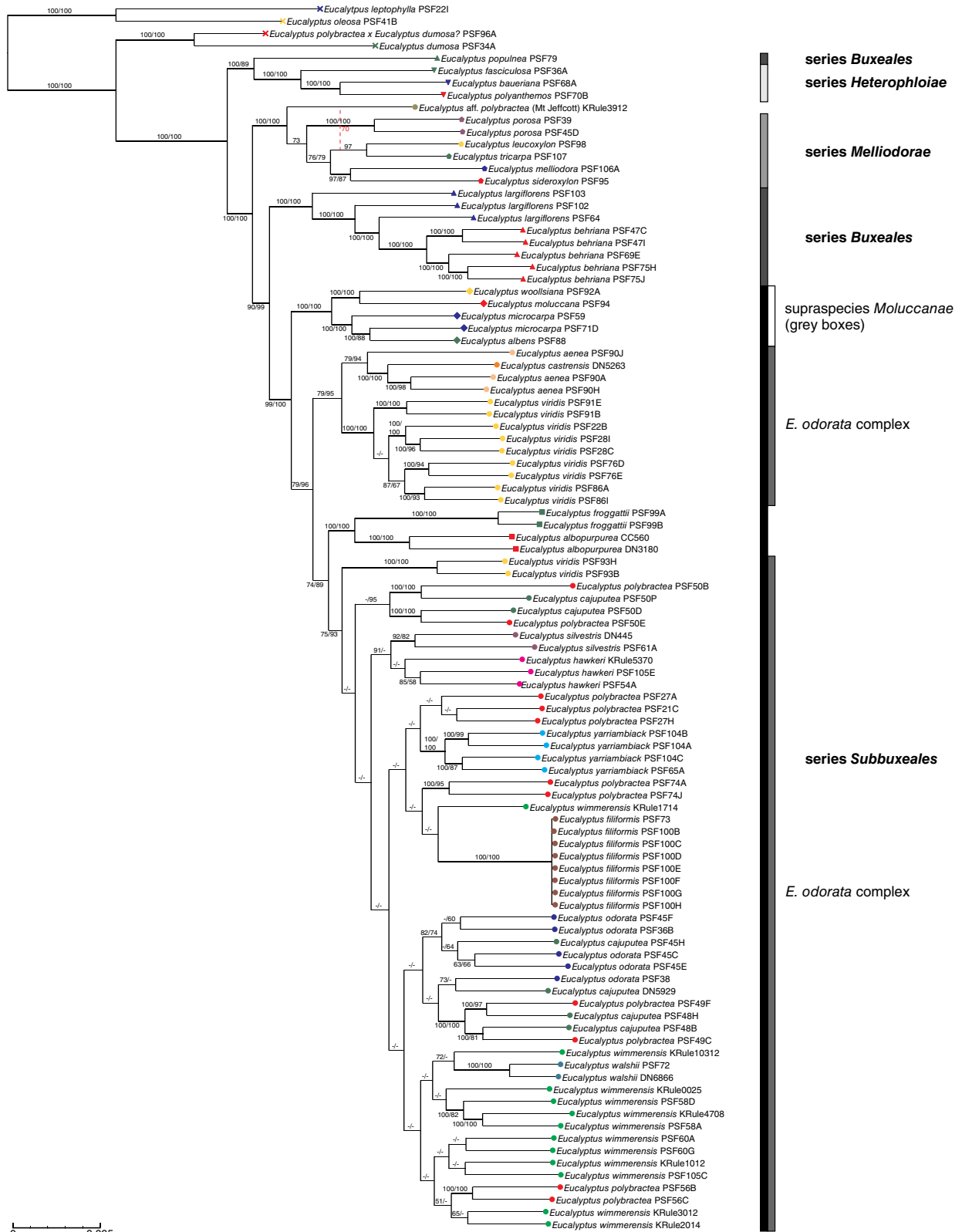
**Fig. 3.** Plot of PCA analyses of SNPs generated using (a) ddRADseq and (b) DArTseq. Points are coloured by species consistent with Fig. 1, with shapes used to distinguish different major groups: the grey-box taxa (diamonds), mallee members of *E. series Subbuxaeles* not in the *E. odorata* complex (squares), and the *E. odorata* complex (circles).

clades to diverge are *E. series Melliodorae*, which contains both samples of *E. porosa* F. Muell. ex Miq. as sister to the rest of the series, and the '*E. aff. polybractea* (Mount Jeffcott)' seedling, followed by a clade containing *E. behriana* and

*E. largiflorens* from the polyphyletic *E. series Buxaeles*, which is resolved as sister to a monophyletic *E. series Subbuxaeles*. Within the main *E. series Buxaeles* clade, *E. largiflorens* formed a grade that subtends a monophyletic *E. behriana* clade. Within *E. series Subbuxaeles*, the four grey-box species were supported as a monophyletic clade sister to the mallee species (*E. odorata* complex, *E. albopurpurea* and *E. froggattii*). Within the grey boxes, the only species represented by more than one sample, *E. microcarpa*, was not monophyletic, with one sample, PSF59, being the earliest diverging member of the clade matching the PCA and network findings. *Eucalyptus albopurpurea* and *E. froggattii* were supported as sister species and, in turn, as the sister clade to the *E. odorata* complex. Although the *E. odorata* complex was supported as a monophyletic group, there were very few resolved relationships within the complex. None of the widespread species (*E. cajuputea*, *E. odorata*, *E. polybractea*, *E. viridis* and *E. wimmerensis*) was found to be monophyletic, although, in most cases, where multiple samples from the same site were included, they were supported as each other's closest relatives.

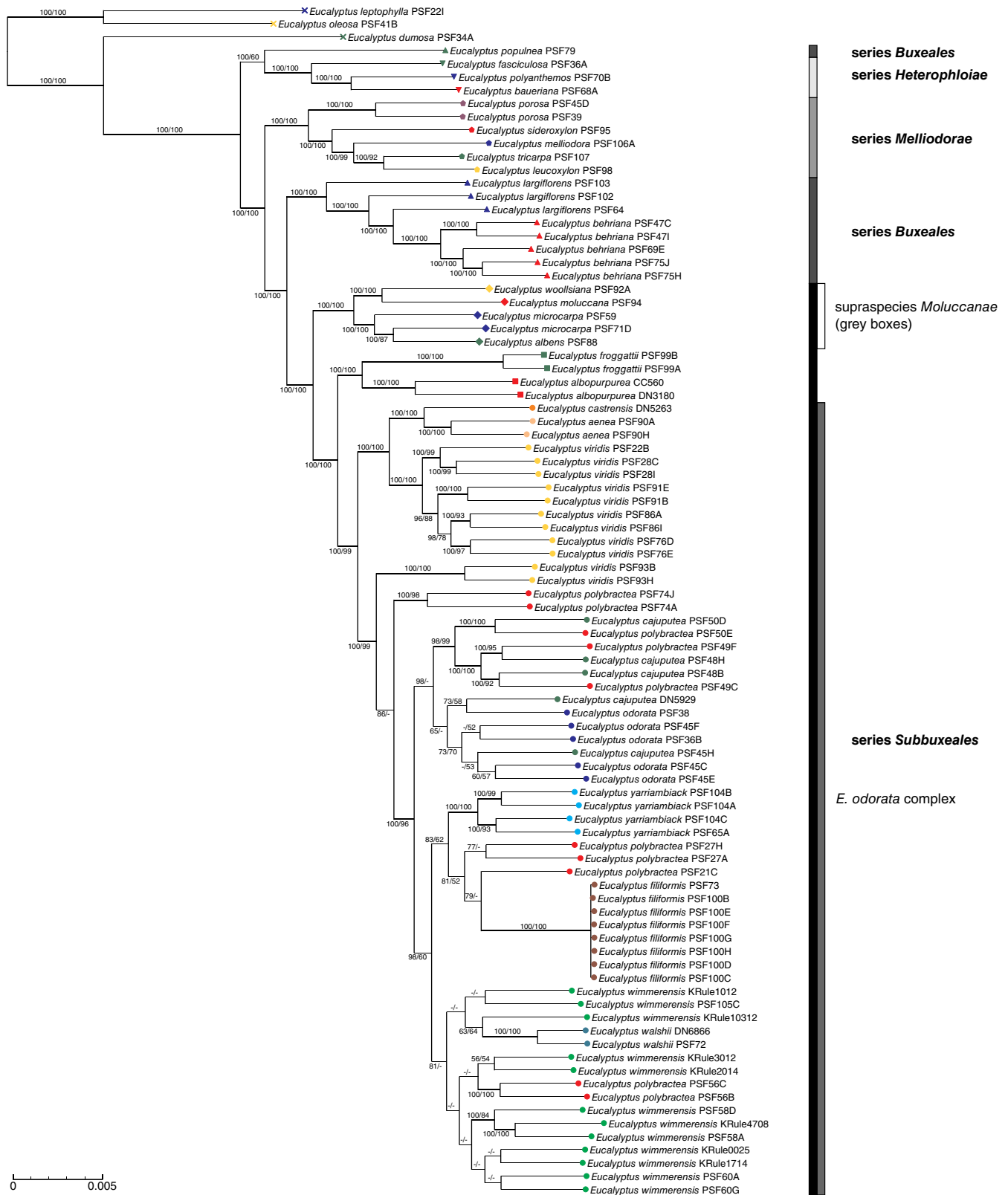
The majority of *E. viridis* samples, including all from Victoria and the central west of NSW, were supported as a monophyletic lineage, which was sister to a clade containing the two species from the Hunter Valley, *E. aenea* and *E. castrensis*. Within this main *E. viridis* clade, the three Goldfields samples were supported as a sister lineage to the samples from north of the Murray River, with the samples from each of the three locations in the latter group forming sister pairs and the Pilliga population diverging first from a West Wyalong and Candelego clade. The two samples of *E. viridis* from the Inglewood area on the Qld–NSW border were not supported as part of this clade; rather, these samples were the next clade to diverge with respect to the rest of the *E. odorata* complex, followed by a clade containing the two samples of *E. polybractea* from the West Wyalong area, rendering the latter species polyphyletic also.

Within the rest of the *E. odorata* complex, there was support in the ML analysis for an east–west divide across the Murray River Basin, further rendering *E. polybractea* polymorphic, although the MP analysis did not corroborate this. In the western clade, all samples from the Flinders Ranges, both of *E. polybractea* and *E. cajuputea* (but excluding the two samples removed because of suspected introgression from other taxa, namely PSF50B and PSF50P) formed a monophyletic lineage, with the two sampling locations (Wilpena Pound and Devils Peak) being represented by supported clades. There were no supported relationships between any of the other *E. cajuputea* and *E. odorata* samples in the western clade. Additionally, there was also support for a division between samples from the western Wimmera and those from the eastern Wimmera and Victorian goldfields in the eastern clade, although, again, this was supported only in the ML analysis. There were minimal supported relationships in the western Wimmera

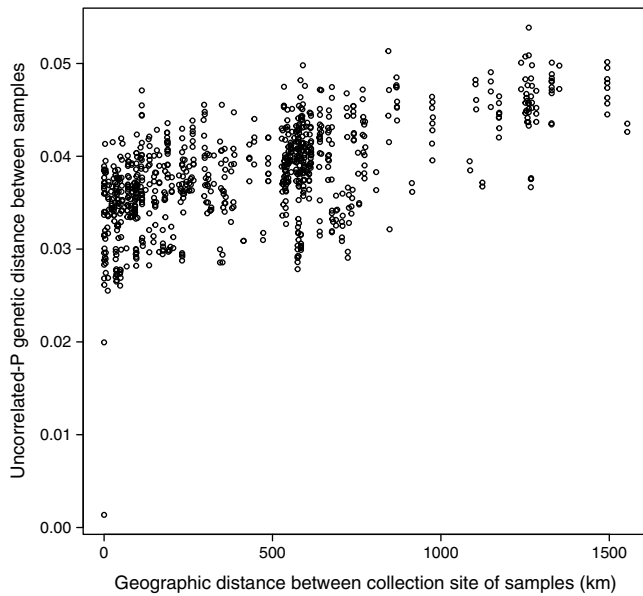


**Fig. 4.** Maximum-likelihood phylogeny generated using RAXML including all samples in the combined ddRADseq and DArTseq dataset. Support values on branches are those from the ML and MP analysis, with branches with greater than 80% bootstrapping support in both analyses thickened. Series are labelled as per [Nicolle \(2019\)](#).





**Fig. 5.** Maximum-likelihood phylogeny generated using RAXML, excluding samples with strong evidence for hybridisation and introgression in the combined ddRADseq and DArTseq dataset. Support values on branches are those from the ML and MP analysis, with branches with greater than 80% bootstrapping support in both analyses thickened. Series are labelled as per [Nicolle \(2019\)](#).



**Fig. 6.** Isolation by distance plot of core *E. odorata* complex samples (*E. odorata*, *E. cajuputea*, *E. wimmerensis*, *E. walshii*, *E. yarriambiack*, *E. filiformis*, *E. polybractea* and *E. viridis* from south-eastern Queensland). Geographic distances are kilometres between collection coordinates and genetic distances are uncorrelated-P distances.

clade, with only samples from a single collecting locality being supported as monophyletic groups, including the samples of *E. walshii* and *E. polybractea* subsp. *subcerea*. In the eastern Wimmera and Goldfields clade, the four samples of *E. yarriambiack* were supported as a clade, with the ML analysis placing them sister to the samples of *E. polybractea* from this region and the clonal *E. filiformis*. Our test showed significant IBD in this core clade (Mantel statistic  $r$ : 0.597, significance:  $1 \times 10^{-4}$ , Fig. 6).

### Hybridisation tests

There was a number of ABBA-BABA tests on samples with unexpected and unstable placement in other analyses that showed significant introgression at the commonly used threshold Z-score of three (Zheng and Janke 2018; Table 4). Two tests showed very high Z-scores and  $D$ -statistics, corresponding to the two samples not being assigned to existing species, namely the *E. dumosa*  $\times$  *E. polybractea* individual (PSF96A) and the seedling of *E. aff. polybractea* (Mount Jeffcott) (KRule3912), which is an apparent *E. filiformis*  $\times$  *E. leucoxydon* hybrid. For all tests for introgression from *E. microcarpa* into *E. hawkeri*, *E. silvestris*, *E. walshii* and *E. wimmerensis* subsp. *grata* with respect to *E. wimmerensis*, the Z-scores were greater than three. The individual tests of the former two returned higher  $D$ -statistics, ranging from 0.147 to 0.209, matching their more intermediary placement in phylogenetic analyses and PCAs, whereas *E. walshii* and *E. wimmerensis* subsp. *grata* returned lower  $D$ -statistics of 0.131 and 0.110 respectively, as expected on the basis of

their more consistent placement with *E. wimmerensis* samples in other analyses. Support for introgression of *E. wimmerensis* into the *E. microcarpa* sample PSF59 was also found with a lower  $D$ -statistic of 0.111. No support for introgression of *E. largiflorens* nor *E. microcarpa* into *E. yarriambiack*, when compared with *E. polybractea* from the Victorian goldfields, was found.

Although reaching the threshold Z-score of three,  $D$ -statistics for *E. aenea* (excluding PSF90J) and *E. castrensis* being introgressed from the co-occurring grey boxes *E. albens* and *E. moluccana* were low (0.071–0.089). There was no support for our sample of *E. castrensis* being more introgressed from the grey-box species than is *E. aenea*. Tests of the aberrant *E. aenea* sample PSF90J being more introgressed by the grey boxes than other *E. aenea* samples returned the opposite result, with negative  $D$ -statistics suggesting that both *E. aenea* and *E. viridis* share more derived alleles with *E. albens* and *E. moluccana* than this sample. A similar situation is observed for the two samples from the Flinders Ranges that show aberrant placement (PSF50B and PSF50P), which showed negative  $D$ -statistics, some with a Z-score greater than three, when compared with all other taxa (not shown) except *E. populnea*, which has a positive  $D$ -statistic but fails to reach the threshold Z-score of three. This suggests that a species we have not sampled, potentially one genetically related to *E. populnea*, is the source of introgression into these samples.

The NewHybrid tests (Table 5) suggested that none of the samples that showed evidence of hybridisation and introgression was an F1 hybrid. The seedling of *E. aff. polybractea* (Mount Jeffcott; KRule3912), the *E. dumosa*  $\times$  *E. polybractea* individual (PSF96A) and the *E. microcarpa* sample with *E. wimmerensis* introgression (PSF59) were all assigned F2 status. The last of these was an unexpected result, given its consistent placement with the grey-box samples in other analyses and a low  $D$ -statistic in the ABBA-BABA analysis. As expected, all *E. hawkeri* and *E. silvestris* samples were hybrids backcrossed with *E. wimmerensis*, although both *E. walshii* samples and the *E. wimmerensis* subsp. *grata* sample were assigned to the parental *E. wimmerensis*. The two *E. aenea* and one *E. castrensis* sample tested were both assigned to *E. viridis* rather than to a hybrid generation, supporting there being only historic introgression into these populations from one or both grey-box species with which they co-occur.

## Discussion

### Comparison of ddRADseq and DArTseq

In the context of phylogenetic studies of *Eucalyptus*, we have shown that there is minimal difference in the phylogenetic signal in the data generated through ddRADseq (a hands-on, in-house reduced-representation genetic-library approach)

**Table 4.** Results of ABBA-BABA tests for hybridisation and introgression among taxa and samples utilising the *ipyrad* toolbox.

PI	Hybrid	P2	Z-score	D-statistic	bootstrapping standard deviation	ABBA	BABA
<i>Eucalyptus viridis</i>	<i>Eucalyptus aenea</i>	<i>Eucalyptus albens</i>	4.68	0.089	0.019	1097.447	918.1224
<i>Eucalyptus viridis</i>	<i>Eucalyptus aenea</i>	<i>Eucalyptus moluccana</i>	4.67	0.089	0.019	960.6352	804.2073
<i>Eucalyptus aenea</i>	<i>Eucalyptus aenea</i> PSF90J	<i>Eucalyptus moluccana</i>	7.53	-0.173	0.023	707.6875	1002.938
<i>Eucalyptus aenea</i>	<i>Eucalyptus aenea</i> PSF90J	<i>Eucalyptus albens</i>	8.32	-0.173	0.021	789.5938	1120.531
<i>Eucalyptus viridis</i>	<i>Eucalyptus aenea</i> PSF90J	<i>Eucalyptus moluccana</i>	5.60	-0.112	0.020	953.6173	1195.259
<i>Eucalyptus viridis</i>	<i>Eucalyptus aenea</i> PSF90J	<i>Eucalyptus albens</i>	5.99	-0.109	0.018	1001.853	1248.203
<i>Eucalyptus filiformis</i>	<i>Eucalyptus</i> aff. <i>polybractea</i> (Mount Jeffcott)	<i>Eucalyptus leucoxydon</i>	20.21	0.447	0.022	1269.691	485.7629
<i>Eucalyptus cajuputea</i>	<i>Eucalyptus cajuputea</i> PSF50B	<i>Eucalyptus populnea</i>	1.90	0.062	0.033	486.1563	429.4688
<i>Eucalyptus cajuputea</i>	<i>Eucalyptus cajuputea</i> PSF50P	<i>Eucalyptus populnea</i>	0.12	0.003	0.024	677.375	673.375
<i>Eucalyptus cajuputea</i>	<i>Eucalyptus cajuputea</i> PSF50B	<i>Eucalyptus porosa</i>	1.26	-0.036	0.029	560.3906	602.7031
<i>Eucalyptus cajuputea</i>	<i>Eucalyptus cajuputea</i> PSF50P	<i>Eucalyptus porosa</i>	1.38	-0.031	0.023	831.2969	884.4844
<i>Eucalyptus aenea</i>	<i>Eucalyptus castrensis</i>	<i>Eucalyptus moluccana</i>	0.55	-0.014	0.025	657.5	676.25
<i>Eucalyptus aenea</i>	<i>Eucalyptus castrensis</i>	<i>Eucalyptus albens</i>	0.89	-0.021	0.024	763.7813	796.9688
<i>Eucalyptus viridis</i>	<i>Eucalyptus castrensis</i>	<i>Eucalyptus moluccana</i>	3.19	0.071	0.022	884.7159	767.3656
<i>Eucalyptus viridis</i>	<i>Eucalyptus castrensis</i>	<i>Eucalyptus albens</i>	4.05	0.078	0.019	985.5534	843.1403
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus hawkeri</i> KRule5370	<i>Eucalyptus microcarpa</i>	8.65	0.186	0.021	930.9141	639.4598

(Continued on next page)

Table 4. (Continued)

PI	Hybrid	P2	Z-score	D-statistic	bootstrapping standard deviation	ABBA	BABA
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus hawkeri</i> PSF105E	<i>Eucalyptus microcarpa</i>	8.27	0.165	0.020	880.2864	630.975
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus hawkeri</i> PSF54A	<i>Eucalyptus microcarpa</i>	8.93	0.183	0.020	898.7345	620.7579
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus microcarpa</i> PSF59	<i>Eucalyptus wimmerensis</i>	5.00	0.111	0.022	819.8536	656.4888
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus silvestris</i> DN445	<i>Eucalyptus microcarpa</i>	7.16	0.147	0.021	849.9266	632.2463
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus silvestris</i> PSF61A	<i>Eucalyptus microcarpa</i>	9.86	0.209	0.021	983.7544	643.8333
<i>Eucalyptus polybractea</i> (West Wyalong)	<i>Eucalyptus viridis</i> (Inglewood, Qld)	<i>Eucalyptus woollsiana</i>	0.88	0.019	0.022	788.4688	758.8438
<i>Eucalyptus viridis</i>	<i>Eucalyptus viridis</i> (Inglewood, Qld)	<i>Eucalyptus woollsiana</i>	0.32	-0.006	0.019	888.4217	899.399
<i>Eucalyptus polybractea</i> (West Wyalong)	<i>Eucalyptus viridis</i> (Inglewood, Qld)	<i>Eucalyptus viridis</i>	0.39	-0.006	0.017	1248.456	1264.504
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus walshii</i>	<i>Eucalyptus microcarpa</i>	7.09	0.131	0.018	858.9831	660.5651
<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus wimmerensis</i> subsp. <i>grata</i> KRule10312	<i>Eucalyptus microcarpa</i>	5.51	0.110	0.020	733.5381	588.7356
<i>Eucalyptus polybractea</i> (Victorian goldfields)	<i>Eucalyptus yarriambiack</i>	<i>Eucalyptus microcarpa</i>	1.07	-0.021	0.020	793.026	827.026
<i>Eucalyptus polybractea</i> (Victorian goldfields)	<i>Eucalyptus yarriambiack</i>	<i>Eucalyptus largiflorens</i>	0.97	0.017	0.017	1079.777	1044.688
<i>Eucalyptus dumosa</i>	<i>Eucalyptus dumosa</i> × <i>Eucalyptus polybractea</i> PSF96A	<i>Eucalyptus polybractea</i> (Victorian goldfields)	37.20	0.653	0.018	1096.146	229.9375

**Table 5.** Results of NewHybrids analyses of suspected hybrid samples on the basis of ABBA-BABA analyses.

P0	PI	Hybrid	P0	PI	F1	F2	F1 × P0	F1 × PI
<i>Eucalyptus viridis</i>	<i>Eucalyptus albens</i> and <i>Eucalyptus moluccana</i>	<i>Eucalyptus aenea</i> PSF90A	0.95	0	0	0.01	0.04	0
<i>Eucalyptus viridis</i>	<i>Eucalyptus albens</i> and <i>Eucalyptus moluccana</i>	<i>Eucalyptus aenea</i> PSF90H	0.99	0	0	0	0.01	0
<i>Eucalyptus viridis</i>	<i>Eucalyptus albens</i> and <i>Eucalyptus moluccana</i>	<i>Eucalyptus castrensis</i> DNicolle5263	0.99	0	0	0	0	0
<i>Eucalyptus filiformis</i>	<i>Eucalyptus leucoxylon</i>	<i>Eucalyptus</i> aff. <i>polybractea</i> (Mount Jeffcott) KRule3912	0	0	0	1	0	0
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus hawkeri</i> KRule5370	0	0	0	0	0	1
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus hawkeri</i> PSF105E	0	0	0	0	0	1
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus hawkeri</i> PSF54A	0	0	0	0	0	1
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus microcarpa</i> PSF59	0	0	0	1	0	0
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus silvestris</i> DN445	0	0	0	0	0	0.99
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus silvestris</i> PSF61A	0	0	0	0.10	0	0.90
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus wimmerensis</i> subsp. <i>grata</i> KRule10312	0	1	0	0	0	0
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus walshii</i> DNicolle6866	0	1	0	0	0	0
<i>Eucalyptus microcarpa</i>	<i>Eucalyptus wimmerensis</i>	<i>Eucalyptus walshii</i> PSF72	0	1	0	0	0	0
<i>Eucalyptus polybractea</i>	<i>Eucalyptus dumosa</i>	<i>Eucalyptus dumosa</i> × <i>Eucalyptus polybractea</i> PSF96A	0	0	0	1	0	0

Assignment probabilities for the suspected hybrids are shown for the parental groups (P0 and PI), F1 hybrids (F1), F2 hybrids (F2) and hybrids backcrossed with either parent (F1 × P0 and F1 × PI).

and DArTseq (a proprietary service gaining popularity in genetic studies of eucalypts and other plants), with each having benefits over the other. Unlike ddRADseq, DArTseq is designed to deal with highly repetitive genomes, such as those of many eucalypts (Sansaloni *et al.* 2010). Additionally, the data generated by DArTseq have the benefits of reproducibility and this approach offers the ability to expand and combine datasets generated at different times as part of separate sequencing runs because of the targeted nature of the loci genotyped (Sansaloni *et al.* 2010). Reproducibility and combinability are more problematic in ddRADseq datasets developed at different times, where there is a degree of stochasticity in which loci are returned each run owing to slight differences in library preparation and sequencing (O'Leary *et al.* 2018). In addition, we had significantly more even coverage across our samples in the DArTseq than in the ddRADseq data, which allows more confidence in the results of analyses, despite the fact there were fewer overall loci and SNPs. Where ddRADseq comes out ahead is the benefits of performing the library preparation in house, which gives flexibility in the number of samples included, the length of the loci in the library and the sequencing platform used, all for a lower cost. Ultimately, these two techniques may be best suited to different situations, DArTseq is preferable if you will want to combine data sequenced at different times, or across different studies, but ddRADseq might provide a cheaper alternative for a one-off dataset, where all samples are processed at once. Both of our datasets appear to be at the point where simply adding more genetic data does not improve resolution and support of phylogenetic relationships, given they show similar patterns individually (Fig. 2, 3) and we do not increase nor change the signal by combining them (Fig. 4, 5). This is in line with the findings of Luo *et al.* (2018) who performed case studies of molecular species delimitation techniques and found simply adding more loci to datasets often did not increase the discriminatory power of the analyses.

### Phylogeny of *E.* section *Adnataria*

Although this study was focussed on the *E. odorata* complex, we have also produced one of the most resolved phylogenies for section *Adnataria* (Fig. 5). Previous phylogenetic studies of section *Adnataria* have either relied on plastid DNA (Flores-Rentería *et al.* 2017; Alwadani *et al.* 2019), which does not resolve phylogenetic relationships in the eucalypts because of a large cyto-nuclear discrepancy (Steane *et al.* 1998; Jackson *et al.* 1999), internal transcribed spacer sequences, which do not provide enough resolution to reconstruct relationships within the section (Steane *et al.* 2002, 2007; Thornhill *et al.* 2019), or have not resolved any relationships between series and species with support (Woodhams *et al.* 2013). However, because our sampling was comprehensive only for taxa that commonly co-occur with members of the *E. odorata* complex, we sampled only

four of the nine series in *E.* section *Adnataria* that are recognised in the classification of Nicolle (2019). Three of the sampled series (*Heterophloiae*, *Melliodorae* and *Subbuxaeales*) were monophyletic, with the placement of *E. populnea* as sister to *E.* series *Heterophloiae*, rendering *E.* series *Buxaeales* polyphyletic (Fig. 5). A possible explanation for the placement of this sample is introgression from *E. conica* H.Deane & Maiden, a member of *E.* series *Heterophloiae*, which occurs at the collection locality and that we did not sample in this study. As we sampled only 3 of the 15 species placed in *E.* series *Buxaeales* by Nicolle (2019), including a single *E. populnea* individual, and as the group shares several morphological traits (Brooker 2000), we recommend further phylogenetic investigation of the series to test its monophyly.

Brooker (2000) placed *E.* series *Melliodorae* in *E.* subsection *Terminales* along with *E.* series *Heterophloiae* and several series not sampled in our study, although he placed *E.* series *Buxaeales* and *Subbuxaeales* in *E.* subsection *Apicales*. This does not match our phylogeny, which shows *E.* series *Melliodorae* as more closely related to the last two series than to *E.* series *Heterophloiae* (Fig. 5). No previous phylogenetic studies have resolved these subsections to be reciprocally monophyletic (Woodhams *et al.* 2013; Flores-Rentería *et al.* 2017), suggesting a re-assessment of the subsectional classification within *E.* section *Adnataria* may be needed. Brooker's *E.* subsection *Terminales* was defined as including species with inflexed stamens and an outer ring of staminodes (Brooker 2000), which, if our phylogeny is a true representation of evolutionary relationships, suggests that these traits may either be the ancestral state of *E.* section *Adnataria* or independently derived in both series, especially given the placement of *E. porosa*, which lacks staminodes, in *E.* series *Melliodorae*. The close relationship between *E.* series *Buxaeales* and *Subbuxaeales* is consistent with established classifications, with some authors not recognising *E.* series *Subbuxaeales* as distinct from *E.* series *Buxaeales* (Brooker 2000; Brooker *et al.* 2015).

We are able to confirm that *E. porosa* is best placed in *E.* series *Melliodorae* (Fig. 5), despite morphological similarities to members of the *E. odorata* complex (*E.* series *Subbuxaeales*), including its typically mallee growth habit, lack of an operculum scar and axillary inflorescence arrangement (Brooker *et al.* 2015). This aligns with the classification of Nicolle (2019), but not that of Brooker (2000) who considered this species a member of *E.* series *Buxaeales*, which included the *E. odorata* complex in their classification. *Eucalyptus* series *Melliodorae* is united by several key morphological traits that *E. porosa* also shares, namely, the outer operculum being held to anthesis, axillary inflorescences, an intramarginal vein remote from the leaf edge, and a tardily deciduous, broad staminal ring. Only the first two of these characteristics are shared with members of *E.* series *Subbuxaeales* (Brooker *et al.* 2015), whereas *E. froggattii* is the only member of *E.* series *Subbuxaeales* with a remote intramarginal vein (Brooker and Nicolle 2013). A final trait shared by the other members of *E.* series



*Meliodorae* we sampled but lacked by *E. porosa* and two other members of the series we have not sampled (*E. bosistoana* F.Muell. and *E. argophloia* Blakely) is a sterile outer ring of stamens, which fits with the placement of *E. porosa* in this study as sister to the rest of the series (Fig. 5). Additional support for the placement of *E. porosa* in *E. series Meliodorae* comes from hybrids of *E. porosa* × *E. leucoxydon* being common (Nicolle 2014), but no *E. porosa* × *E. odorata* complex hybrids having been recorded.

The seedling of '*E. aff. polybractea* (Mount Jeffcott)' was also part of the *E. series Meliodorae* clade in our full dataset phylogeny (Fig. 4). The maternal parent of this seedling is found at Mount Jeffcott within 100 m of the only known stand of *E. filiformis*, from which it differs primarily in its glaucous juvenile foliage and smooth bark (K. Rule, pers. comm., 22 November 2018). Of the other *E. section Adnataria* taxa that occur at the site (*E. microcarpa*, *E. largiflorens* and *E. leucoxydon*), *E. leucoxydon* is by far the most abundant species perhaps explaining why, despite the extensive gene-flow between *E. microcarpa* and members of the *E. odorata* complex in the Wimmera, our results, both the phylogeny (Fig. 4) and ABBA-BABA tests (Table 4), showed that this seedling is a *E. filiformis* × *E. leucoxydon* hybrid. This finding fits with the observations of its morphology (K. Rule, pers. comm., 3 February 2021), although, because we did not sample the maternal parent tree, we cannot say if it is also a *E. leucoxydon* hybrid, potentially explaining its unique morphology.

The two clades within *E. series Subbuxales* (Fig. 5) are supported by morphology, as the typically single-trunked grey-box species and mallee box species form reciprocally monophyletic clades. This is in line with previous studies that have shown that the mallee growth form may be tied to genetic lineages in other eucalypt groups (Hines and Byrne 2001). Although grey boxes are very morphologically similar and establishing geographic boundaries among the taxa are difficult because they intergrade with one another (Bean 2009; Flores-Rentería et al. 2017), *E. albopurpurea* and *E. froggattii* have not previously been hypothesised to be closely related in the literature. *Eucalyptus albopurpurea* has often been regarded as more morphologically similar to members of the *E. odorata* complex, in particular *E. odorata* itself, with which it intergrades with on Kangaroo Island (Nicolle 2000, 2014), than to *E. froggattii*, which is set apart morphologically by its square buds and fruits (Brooker et al. 2015). However, these two species do have morphological similarities not shared by the members of the *E. odorata* complex, including apparently terminal compound inflorescences, generally broader leaves, and slightly larger buds and fruit (Brooker et al. 2015). More work is needed to clarify the relationship between these two species and the *E. odorata* complex.

### Relationships within the *E. odorata* complex

Relationships within the *E. odorata* complex are largely unresolved in our phylogeny, with very little bootstrap

support in both the MP and ML analyses, and no species with multiple populations sampled being resolved as monophyletic. However, our PCA (Fig. 3) and hybridisation tests (Tables 4, 5) have shed some light on the possible patterns of relatedness and introgression that explain this lack of resolution. There is strong support for the idea that *E. viridis* and the two segregate species from the Hunter Valley, *E. aenea* and *E. castrensis*, form a clade sister to the rest of the complex if northern (Queensland) populations currently regarded as *E. viridis* are excluded. Although *E. viridis* co-occurs with *E. polybractea* at multiple locations and the two may hybridise on occasion (but no evidence of this was seen in this study), the former is the most morphologically distinct species in the complex, given its linear juvenile leaves and its narrow, green adult leaves, and, at most sites, the two are easily recognisable and morphologically distinct. The two Hunter Valley segregates of *E. viridis*, *E. aenea* and *E. castrensis*, form a sister lineage to *E. viridis* in our phylogeny. However, the PCA (Fig. 3) and hybridisation tests (Tables 4, 5) give weight to the hypothesis that these populations have experienced introgression from a grey-box species, which may account for their morphological distinctness, although given the results of our ABBA-BABA tests (Table 4), *E. albens* appears the probable parent rather than *E. moluccana* as was hypothesised by Nicolle (2019). As the NewHybrid analysis assigns these samples to *E. viridis* rather than to a hybrid generation, this finding of introgression from a grey box is likely to be the result of historic introgression. The genetically distinct sample of *E. aenea* (PSF90J) was recognised as differing from smooth-barked typical *E. aenea* in the field because of its stocking of rough bark that reached ~3 m up the trunks. Although genetically distinct from the other *E. aenea* samples in our dataset, this is not due to more substantial genetic input from a grey box as hypothesised. Although grey boxes are the most closely related species to occur in the vicinity of *E. aenea*, ironbark species of *E. section Adnataria* (*E. crebra* F.Muell., and *E. fibrosa* F.Muell.) dominate the site and may be the culprit for this genetic distinctness, although our dataset does not allow us to test this. As our *E. castrensis* sample is more referable to the broader application of the name per Hill and Stanberg (2002) that may represent *E. aenea*, and we have not sampled material from the putative *E. aenea* × *E. microcarpa*–*E. moluccana* hybrids, which match the type material of the species (Nicolle 2019), we cannot say anything further regarding the distinction of *E. castrensis* from *E. aenea*.

Previous authors have noted that Queensland populations in the vicinity of Inglewood, where the two northern *E. viridis* samples included in this study were collected, and Durikai State Forest have broader leaves than typical for *E. viridis*, with Blakely (1934) classifying these populations as *E. viridis* var. *latiuscula* Blakely. Chippendale (1988) suggested that these populations may be hybrids of *E. viridis* and the grey-box *E. woollsiana*, whereas Brooker et al. (2015)

believed that these populations show greater morphological similarities to *E. wimmerensis*, which they regard as a subspecies of *E. viridis*, than to the typical form of *E. viridis* found in the Victorian goldfields and scattered populations in NSW. In the more resolved reduced sampling phylogeny (Fig. 5), the northern samples are placed as the next clade to diverge in the complex after the main *E. viridis* clade, with the samples of *E. polybractea* from the most northerly population diverging next, although the node between these clades is supported only in the ML analysis. The ABBA-BABA tests also do not support introgression from the co-occurring grey-box *E. woollsiana* into these samples compared with *E. viridis* and *E. polybractea* from West Wyalong (Table 4), as was previously hypothesised (Chippendale 1988). Along with the placement of these samples in the PCA analyses (Fig. 3), this raises the question of whether rather than an *E. viridis* × *E. woollsiana* hybrid, they may be *E. viridis* × *E. polybractea* hybrids, although given the lack of *E. polybractea* populations nearby (the nearest known population at West Wyalong being over 820 km away), they would have to be phantom hybrids, which have been observed in eucalypts previously, albeit without such a large geographic distance to the phantom parent (Kirkpatrick *et al.* 1973; Hopper and Wardell-Johnson 2004). However, our ABBA-BABA tests showed no support for this hypothesis (Table 4), with the northern *E. viridis* samples showing a similar number of shared alleles with typical *E. viridis* as our samples of *E. polybractea* from the most northerly population of this species at West Wyalong. This leaves the simplest explanation as the most probable, in that these northern populations previously regarded as *E. viridis* var. *latiuscula* represent a currently unrecognised distinct entity that is sister to the core *E. odorata* complex rather than being closely related to typical *E. viridis*, which fits with the observation of morphological similarities to *E. wimmerensis* of seedlings from populations previously ascribed to this variety at Inglewood and Durakai in Queensland by Brooker *et al.* (2015).

Relationships between the few supported clades and the majority of samples in the core *E. odorata* complex are unsupported, despite the topology returned broadly reflecting geography. Only the taxa known from a single site, namely *E. yarriambiack* and *E. filiformis*, are supported as monophyletic (Fig. 5), and because we have sampled all known wild individuals of *E. filiformis*, we can say with confidence on the basis of the lack of genetic differences between them, that the species represents a single clonal colony. Previously it has been noted that *E. filiformis* does not appear to not readily reproduce (Rule 2004), although specimens grown at the Royal Botanic Gardens Victoria from seed show differences in adult morphology from the wild individuals (Rule 2018). We have sampled one of these cultivated individuals (PSF73) and shown it to also be clonal, suggesting there may be occasional pollination occurring in the wild population and, for this individual at least, any morphological differences from the wild plants are not due to genetic differences.

*Eucalyptus polybractea* is polyphyletic in the phylogeny, although relationships among populations are not fully resolved, especially between the type population at West Wyalong (samples PSF74A and PSF74J) and the Victorian goldfields populations (samples PSF21C, PSF27A and PSF27H). Although our phylogenetic analyses suggest that the two samples from the Wimmera (samples PSF56B and PSF56C) are potentially conspecific with *E. wimmerensis* and the West Wyalong population is sister to the rest of the core *E. odorata* complex (Fig. 5), in our network analyses collections from the Victorian goldfields and West Wyalong were more genetically similar to one another, and to *E. yarriambiack* and *E. filiformis*, than to the Wimmera samples (Fig. 2). On the basis of the supported Flinders Ranges clade, which contains both *E. polybractea* and *E. cajuputea* samples (Fig. 5), we suggest that *E. polybractea* should not be considered to occur west of the Murray Basin.

Although relationships among *E. wimmerensis* samples in the phylogeny remain unclear, they show little genetic differentiation, although the species' boundaries remain unclear, given the uncertainty regarding the identity of the *E. polybractea* populations in the Wimmera. Although *E. wimmerensis* subsp. *grata* and *Eucalyptus walshii* were not genetically distinct from *E. wimmerensis* in our analyses, the PCA (Fig. 3) and ABBA-BABA tests (Table 4) suggested a low level of introgression from *E. microcarpa*, which may be responsible for the more robust stature of plants in these populations. The other species found only in the Wimmera and adjacent areas of SA, namely *E. silvestris* and *E. hawkeri*, both appear to represent more recent geneflow between *E. wimmerensis* and the co-occurring *E. microcarpa* on the basis of our ABBA-BABA and NewHybrid tests (Table 4). This largely fits with the classifications of both Nicolle (2019) and Brooker *et al.* (2015), although those authors suggested *E. odorata* as being the *E. odorata* complex parent in the case of *E. silvestris*. We have some hesitancy ruling out this hypothesis, given our sampling, because we have shown that there is little genetic distinction between *E. odorata* and *E. wimmerensis*, and we cannot rule out there being populations in the Wimmera or adjacent areas of SA that better fit in *E. odorata* that we have not sampled.

The distinctions between the western taxa, *E. cajuputea*, *E. odorata*, and South Australian populations of *E. polybractea*, are unresolved in our study and require further investigation; however, it is clear what we have called *E. polybractea* in the Flinders Ranges has minimal genetic links to the typical *E. polybractea* of Victoria and NSW and may be best considered conspecific with *E. cajuputea*. Samples from the Flinders Ranges, identified as both *E. cajuputea* and *E. polybractea*, form a single clade in our phylogeny when the two aberrant samples are excluded (Fig. 5), suggesting that there is a single lineage in this region that is distinct from other populations from west of the Murray Basin. The two aberrant samples, one each identified as *E. polybractea* and *E. cajuputea*, showed

significant negative results for most comparisons in our ABBA-BABA tests (Table 4), suggesting that introgression from a species we have not sampled is possible. The only other *E.* section *Adnataria* species that occur at Wilpena Pound and with which hybridisation may be occurring are *E. porosa*, which we have sampled and can therefore rule out, and *E. intertexta* R.T.Baker, which we have not sampled as part of this study. *Eucalyptus intertexta* (*E.* series *Buxeales*) is related to *E. populnea* and *E. largiflorens*, potentially explaining the single positive *D*-statistic with *E. populnea* in our ABBA-BABA tests, although the unresolved placement of the *E. populnea* sample in our phylogenies confounds this because we are unable to establish the relationship between this species and other members of *E.* series *Buxeales*. The mallee species *E. leptophylla* also co-occurs with the population these samples were sourced from and is superficially morphologically similar to members of the *E. odorata* complex, despite being in *E.* section *Bisectae*. This species may be the unknown parent, with its comparatively distant relationship to the *E. odorata* complex potentially explaining the significant negative *D*-statistics. Although we have a single sample of this species in our dataset, as part of the most divergent outgroup clade, we were not able to use it as an ingroup in ABBA-BABA tests to test this hypothesis because these require the inclusion of an outgroup with an evolutionary divergence point prior to the divergence of the three ingroup samples (Durand et al. 2011). The other samples of *E. cajuputea* and *E. odorata* formed a polytomy along with the Flinders Ranges clade in the ML analysis, which may support a lack of distinctness of these two species outside the Flinders Ranges.

### Genetic variation within the core *E. odorata* complex as a cline

Our findings of extensive introgression among members of the *E. odorata* complex and co-occurring box species is not unexpected given that previous studies on *E.* section *Adnataria* have shown extensive hybridisation leading to morphological taxa not forming genetic clades (Flores-Rentería et al. 2017). However, in regard to the taxonomy of the *E. odorata* complex, the nature of the core of the clade as a discontinuous cline of morphological and genetic variation running from the Flinders Ranges, south and then east through south-eastern SA, east through the Wimmera and then the Goldfields of Victoria and then north to West Wyalong in NSW also plays a significant role in disagreements among authorities over where taxonomic boundaries should be drawn. This clinal genetic variation has been taxonomically divided by different authors at different points on the basis of different factors they consider most important for classification and, in this paper, we have, *a priori*, broken it into the seven largely geographically distinct species (*E. odorata*, *E. cajuputea*, *E. wimmerensis*, *E. polybractea*, *E. filiformis*, *E. walshii* and *E. yarriambiack*).

We see evidence for this cline in the most distal populations (Flinders Range and West Wyalong) being the most distinct, and in relationships between neighbouring populations fluctuate between analyses. This includes, for instance, *E. filiformis* clustering with *E. wimmerensis* in one network (Fig. 2b) but being more closely related to *E. polybractea* from the Victorian goldfields in other analyses (Fig. 2a, 5), and the swapping of relationships between the three main groups (samples from west of the Murray River Basin, *E. wimmerensis* and allied samples, and eastern *E. polybractea* and allied samples) in the core group between analyses, even if the alternate relationships are not supported (Fig. 4). This may be indicative of recent rapid diversification of a widespread ancestral population that has undergone vicariance at multiple locations at approximately congruent times. Clines along which diversification has occurred, often resulting in morphologically distinct species with a hybrid zone between them, have previously been observed in other eucalypt groups, including *E. populnea* and *E. brownii* Maiden & Cabbage (Holman et al. 2003), *E. melanophloia* F.Muell. and *E. whitei* Maiden & Blakely (Holman et al. 2011), and the green ashes (*E.* sect. *Eucalyptus*; Rutherford et al. 2018). The strong support for IBD (Fig. 6,  $r$ : 0.597) in our data is congruent with the existence of a genetic cline.

### Taxonomy within the *E. odorata* complex

With the extensive level of interspecific gene flow, our dataset has provided evidence for, and the lack of resolved relationships among populations in the *E. odorata* complex; we do not feel that it is appropriate to make major taxonomic changes on the basis of our study without further work to investigate patterns of diversity in finer detail. However, we see several approaches that could be taken to clarify the taxonomy of the group as outlined in Table 6. The simplest is to uphold the current morphology-based species classification, with adjustments to species distributions where necessary to match resolved phylogenetic relationships. This approach, essentially applying the morphological species concept, would require accepting the recognition of hybrid entities as species, which is being realised as a major driver of plant evolution (Mayr 2000). The one clear change that is well supported in our data is that populations currently regarded as *E. polybractea* in the Flinders Ranges are not related to the eastern populations of that species and the circumscription of *E. cajuputea* should be expanded to include these populations.

The second approach is to greatly reduce the number of species in the complex to only those that are supported as monophyletic, strictly applying the phylogenetic species concept (Baum 1992). This approach would likely reduce the complex back to two or three species, depending on what future study reveals regarding the distinctness of the northern populations of *E. viridis*. *Eucalyptus aenea* and

**Table 6.** An overview of the three possible classification schemes for the *E. odorata* complex outlined on the basis of the findings of the phylogenetic study present here.

Current taxon	Morphology-driven classification	Hybrid classification	Strict phylogenetic classification
<i>E. viridis</i>	<i>E. viridis</i>	<i>E. viridis</i>	<i>E. viridis</i>
<i>E. aenea</i>	<i>E. aenea</i>	<i>E. aenea</i> = <i>E. viridis</i>	= <i>E. viridis</i>
<i>E. castrensis</i>	Potential mixed hybrid population – further sampling needed	Potential mixed hybrid population – further sampling needed	Potential mixed hybrid population – further sampling needed
<i>E. viridis</i> var. <i>latiuscula</i>	Potential new taxon – further sampling needed	Potential new taxon – further sampling needed	Potential new taxon – further sampling needed
<i>E. odorata</i>	<i>E. odorata</i> (distribution reduced to west of Murray Basin and south of the Flinders Ranges)	<i>E. odorata</i> (distribution reduced to west of Murray Basin and south of the Flinders Ranges)	<i>E. odorata</i>
<i>E. cajuputea</i>	<i>E. cajuputea</i> (distribution reduced to only Flinders Ranges)	<i>E. cajuputea</i> (distribution reduced to only Flinders Ranges)	= <i>E. odorata</i>
<i>E. polybractea</i>	<i>E. polybractea</i> (distribution reduced to only central Victoria and West Wyalong region of NSW, populations of <i>E. polybractea</i> subsp. <i>subcerea</i> of uncertain classification between <i>E. polybractea</i> and <i>E. wimmerensis</i> )	<i>E. polybractea</i> (distribution reduced to only central Victoria and West Wyalong region of NSW, populations of <i>E. polybractea</i> subsp. <i>subcerea</i> of uncertain classification between <i>E. polybractea</i> and <i>E. wimmerensis</i> )	<i>E. polybractea</i> (distribution reduced to only central Victoria and West Wyalong region of NSW, populations of <i>E. polybractea</i> subsp. <i>subcerea</i> of uncertain classification between <i>E. polybractea</i> and <i>E. wimmerensis</i> )
<i>E. filiformis</i>	<i>E. filiformis</i>	= <i>E. polybractea</i>	= <i>E. polybractea</i>
<i>E. yarriambiack</i>	<i>E. yarriambiack</i>	<i>E. yarriambiack</i>	= <i>E. polybractea</i>
<i>E. wimmerensis</i>	<i>E. wimmerensis</i>	<i>E. wimmerensis</i>	<i>E. wimmerensis</i>
<i>E. walshii</i>	<i>E. walshii</i>	= <i>E. wimmerensis</i>	= <i>E. wimmerensis</i>
<i>E. hawkeri</i>	<i>E. × silvestris</i>	<i>E. × silvestris</i>	<i>E. × silvestris</i>
<i>E. silvestris</i>	<i>E. × silvestris</i>	<i>E. × silvestris</i>	<i>E. × silvestris</i>

The morphological scheme largely accepts the current morphology-based species with minor adjustments on the basis of genetic findings, whereas the strict phylogenetic classification reduces the group to only those species with genetic evidence for representing distinct lineages. The final hybrid classification strikes a balance by synonymising species where there is evidence against them representing distinct lineages, but respects the morphological classification where there is a lack of evidence for distinctness.

*E. castrensis* would be synonymised with *E. viridis* and the cline of the rest of the complex broken up as follows: *E. odorata* to include populations west of the Murray Basin, *E. wimmerensis* to cover all populations in the western Wimmera of Victoria and adjacent areas of SA, and *E. polybractea* to accommodate populations from the eastern Wimmera, Victorian goldfields and around West Wyalong. Additionally, the name *E. viridis* var. *latiuscula* may need to be resurrected and its taxonomic rank re-assessed to accommodate the northern *E. viridis* populations that may represent a further extension of this cline. We would advocate that this approach is less than optimal, because each of these taxa would cover a large range of morphological variation that is somewhat correlated with geography and there are outstanding questions regarding the monophyly of these taxa. For these reasons, at this point, we would advocate a looser application of the phylogenetic concept and using the framework of integrative taxonomy to consider both the morphological and molecular evidence, synonymising species with molecular evidence against them being distinct entities, while maintaining morphological taxa

with inconclusive molecular support for their status as distinct populations.

Following this reasoning, *E. hawkeri* and *E. silvestris* may need to be synonymised because both represent intergrades between *E. wimmerensis* and *E. microcarpa*. Although also showing introgression from *E. microcarpa*, *E. walshii* is perhaps better synonymised with *E. wimmerensis*, given its much closer genetic ties to this species. Recognition of *E. filiformis* is problematic, because it is clonal and, given its close placement to *E. polybractea* in our phylogeny and networks (Fig. 2, 5), possibly an outlying population of *E. polybractea* that has unique morphology owing to the small population size causing bottlenecks and genetic drift. A similar problem exists for *E. yarriambiack* because our data suggest that it is not experiencing introgression from the co-occurring *E. largiflorens*, but does not represent a distinct lineage from *E. polybractea*, rather being another potential small, isolated population undergoing genetic drift or a genetic bottleneck that should be synonymised with this species. However, in the case of *E. yarriambiack*, the population is not clonal, holds greater genetic diversity than does



the clonal *E. filiformis* and is far enough outside the range of *E. polybractea* that there is likely to be no ongoing gene flow with populations of that species, all suggesting that the species status for *E. yarriambiack* is not unreasonable. For *E. viridis*, *E. aenea* and *E. castrensis*, we recommend that further phylogenetic studies are undertaken before taxonomy is re-assessed, because, although we have shown these three taxa are each other's closest relatives, we have not sampled widely enough to determine whether *E. aenea* and *E. castrensis* are distinct lineages from *E. viridis* or isolated populations experiencing introgression from the co-occurring and more locally abundant grey-box species *E. albens*. In addition, further work is needed to investigate the relationships of the Queensland populations currently regarded as *E. viridis* to that species and to *E. polybractea*, or whether the name *E. viridis* var. *latiuscula* needs to be resurrected and given the rank of species.

## Future directions

In the case of the *E. odorata* complex, this study has seemingly reached the extent of informativeness for data generated using restriction site-associated DNA sequencing. To get a clearer view of the evolutionary history of the group, other data types are likely to be needed to increase resolution, such as target capture data for gene sequences that have the potential to be better correlated with the observed morphological variation. Conversely, given the lack of divergence among species, population genetic approaches such as denser sampling at each site and Structure analyses (Pritchard *et al.* 2000), or its spatially explicit cousin, ConStruct (Bradburd *et al.* 2018), may help resolve species boundaries in the complex. However, it may not be possible to establish a clear and widely accepted species taxonomy in the *E. odorata* complex because speciation is incomplete and ongoing, and we can access only a single point in time without knowing the future trajectory of diverging populations. Classifying organisms into discrete groups is a very human task and such discrete groups may not reflect the processes of the natural world, which are much more continuous in nature and, thus, this task will always be problematic.

## Conclusions

Overall, we have been able to confirm the boundaries of the *E. odorata* complex, clarified the classification of some taxa and resolved the relationships among a limited number of species. We have also shown that ABBA-BABA tests are a valuable tool for understanding relationships in the eucalypts, and these results together with our NewHybrids analysis have shown that there is clearly a large amount of historic and recent hybridisation happening in this group, especially with the closely related grey boxes. We showed

that *E. viridis*, *E. aenea* and *E. castrensis* represent a distinct lineage within the complex, and *E. viridis* var. *latiuscula* from southern Qld may represent a valid taxon. Given the substantial amount of genetic data we have employed in this study, the lack of resolution within the core *E. odorata* complex may reflect the evolutionary history of the group rather than insufficient phylogenetic signal. The core group of species, namely *E. cajuputea*, *E. odorata*, *E. wimmerensis*, *E. yarriambiack*, *E. filiformis*, *E. walshii* and *E. polybractea*, possibly represents a semi-circular-shaped genetic cline that runs from the Flinders Ranges south-east through south-eastern SA, east through inland Victoria before turning north toward the West Wyalong area of NSW, which different authors have divided into separate taxa at different points. Further work on this group is required to answer the questions that our results have raised; however, it seems likely that finding suitable taxonomic solutions may remain challenging.

## Supplementary material

Supplementary material is available [online](#).

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**Data availability.** The data that support this study are available in Dryad at <https://doi.org/10.5061/dryad.prr4xgxm>.

**Conflicts of interest.** David Cantrill and Mike Bayly are both editors for *Australian Systematic Botany*. Despite this relationship, they did not at any stage have editor-level access to this manuscript while in peer review, as is the standard practice when handling manuscripts submitted by an editor to this journal. *Australian Systematic Botany* encourages its editors to publish in the journal and they are kept totally separate from the decision-making processes for their manuscripts. The authors have no further conflicts of interest to declare.

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