PART OF A HIGHLIGHT ON GENOMIC EVOLUTION

Landscape and taxon age are associated with differing patterns of hybridization in two *Eucalyptus* (Myrtaceae) subgenera

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• **Background and Aims** Hybridization is an important evolutionary process that can have a significant impact on natural plant populations. *Eucalyptus* species are well known for weak reproductive barriers and extensive hybridization within subgenera, but there is little knowledge of whether patterns of hybridization differ among subgenera. Here, we examine eucalypts of Western Australia's Stirling Range to investigate how patterns of hybridization are associated with landscape and taxon age between the two largest *Eucalyptus* subgenera: *Eucalyptus* and *Symphyomyrtus*. In doing so, we tested a hypothesis of OCBIL (old, climatically buffered, infertile landscape) theory that predicts reduced hybridization on older landscapes.

• **Methods** Single nucleotide polymorphism markers were applied to confirm the hybrid status, parentage and genetic structure of five suspected hybrid combinations for subg. *Eucalyptus* and three combinations for subg. *Symphyomyrtus*.

• **Key Results** Evidence of hybridization was found in all combinations, and parental taxa were identified for most combinations. The older parental taxa assessed within subg. *Eucalyptus*, which are widespread on old landscapes, were identified as well-defined genetic entities and all hybrids were exclusively F_1 hybrids. In addition, many combinations showed evidence of clonality, suggesting that the large number of hybrids recorded from some combinations is the result of long-term clonal spread following a few hybridization events rather than frequent hybridization. In contrast, the species in subg. *Symphyomyrtus*, which typically occur on younger landscapes and are more recently evolved, showed less distinction among parental taxa, and where hybridization was detected, there were high levels of introgression.

• **Conclusions** Reduced hybridization in subg. *Eucalyptus* relative to extensive hybridization in subg. *Symphyomyrtus* affirmed the hypothesis of reduced hybridization on OCBILs and demonstrate that clade divergence times, landscape age and clonality are important drivers of differing patterns of speciation and hybridization in *Eucalyptus*.

Key words: *Eucalyptus*, dartseq, hybrid zone, OCBIL theory, *Symphyomyrtus*, taxon age.

INTRODUCTION

Hybridization is a key biological process that can have a significant impact on the evolution of natural plant populations (Stebbins, 1959; Griffin *et al.*, 1988; Arnold, 1997; Rieseberg *et al.*, 2003; Arnold *et al.*, 2012, 2016; Abbott *et al.*, 2013; Abbott, 2017). Hybridization between divergent lineages generates novel genetic combinations that can influence adaptation, range expansion and speciation (Rieseberg *et al.*, 2003; Arnold *et al.*, 2012) but can also be linked to invasiveness, genetic swamping and extinction (Abbott, 2017; Hopper, 2018; Rutherford *et al.*, 2019). Determining the key factors driving hybridization is therefore important to understanding ecological and evolutionary processes, but also to improve taxonomic knowledge and inform biodiversity conservation.

Recent analyses across broad taxonomic levels have shown that certain plant groups exhibit a greater propensity for hybridization, particularly woody perennials, and that life history,

reproductive traits, habitat disturbance and genome size can also be important determinants for the frequency of hybridization across plant orders, families and genera (Beddows and Rose, 2018; Mitchell *et al.*, 2019). Much work has also been done at fine taxonomic scales, directly assessing hybridization between taxa and the genetic structure of natural hybrid zones. These studies have shown that the occurrence of hybridization is influenced by a complex suite of spatial, temporal, genetic and mechanical barriers to reproduction, as well as factors determining hybrid fitness, should reproduction occur (Widmer *et al.*, 2009; Baack *et al.*, 2015). Indeed, for closely related taxa, the removal of geographical or temporal isolating barriers is often all that is required to achieve natural hybridization (Abbott, 2017). Moreover, the genetic structure of hybrid zones can be used to infer the relative strength of reproductive isolation. For example, weak reproductive barriers can result in a full array of hybrid classes and high introgression through

ongoing breeding between hybrid and parental genotypes, while strong reproductive barriers may generate less complex hybrid zones, limited to sterile F_1 individuals (Abbott, 2017). While hybridization has been investigated at both broad and fine taxonomic scales, there remains a gap in knowledge regarding the factors driving differential patterns of hybridization within large, evolutionarily diverse genera.

Eucalyptus is a large genus that is well known for a high propensity for hybridization (Griffin *et al.*, 1988; Larcombe *et al.*, 2015). It is one of Australia's most iconic plant genera with over 700 species, the majority of which fall into two of eight subgenera: subg. *Symphyomyrtus* (563 species) and subg. *Eucalyptus* (124 species) (Nicolle, 2019). Complete reproductive isolation in *Eucalyptus* has occurred over 21–31 Myr, with isolation strength increasing with increasing genetic distance (Larcombe *et al.*, 2015). Indeed, there are strong reproductive barriers among *Eucalyptus* subgenera; however, gene flow among species within series and sections is relatively common (Potts and Wiltshire, 1997; Walker *et al.*, 2009). While various comparative studies have been published on subgeneric differences in *Eucalyptus*, such as distribution, evolution and diversification (Thornhill *et al.*, 2019), ecological traits (Noble, 1989), and generalist vs. specialist attributes (Anekonda *et al.*, 1999), little is known on whether patterns of hybridization differ between the major subgenera (but see Griffin *et al.*, 1988).

Western Australia is a hotspot for *Eucalyptus* diversity with ~474 species from four subgenera (Nicolle, 2019); we focus here on the two largest subgenera, subg. *Symphyomyrtus* and subg. *Eucalyptus*. Subgenus *Symphyomyrtus* is distributed widely throughout Western Australia, from high-rainfall temperate regions in the south and monsoonal regions in the north, through to the arid interior, while the distribution of subg. *Eucalyptus* is more restricted, confined to areas of the more mesic south-west and southern coastline where water availability is higher (Thornhill *et al.*, 2019). In terms of habitat occupied within these regions, subg. *Eucalyptus* occurs broadly in nutrient-impoverished and infrequently disturbed landscapes (Noble, 1989), which are typically old landforms such as granite outcrops, lateritic uplands and high sandplains (Hopper, 2009). In comparison, members of subg. *Symphyomyrtus* typically dominate younger, more highly disturbed landscapes, such as river valleys or mountain slopes (Noble, 1989). These differences in landscape age and ecological preferences in many ways reflect the evolution of the genus. Subgenus *Symphyomyrtus* (crown age of 26–33 Myr) displays a relatively recent radiation of habitat-generalist taxa, with some sections, including *Glandulosae* to which all sampled taxa of subg. *Symphyomyrtus* in this study belong, showing an acceleration in diversification beginning just 2–3 Mya. In comparison, the older, more habitat-specific taxa of subg. *Eucalyptus* have gradually diversified over the last 31–39 Myr (Noble, 1989; Thornhill *et al.*, 2019).

The differences in distribution, ecological preferences and date of evolutionary diversification between the two eucalypt subgenera present an opportunity to test a key hypothesis proposed in OCBIL theory regarding 'reduced hybridization on older landscapes' (Hopper, 2009, 2018). OCBIL theory was developed to explain the rich biodiversity and endemism that

is commonly observed on old, climatically buffered, infertile landscapes (OCBILs), such as those in the Southwest Australian Floristic Region (SWAFR, *sensu*; Gioia and Hopper, 2017) on which subg. *Eucalyptus* species often occur, and contrasts these with young, often disturbed, fertile landscapes (YODFELs), such as those often occupied by subg. *Symphyomyrtus* species. Reduced hybridization on OCBILs is hypothesized based on two key factors: taxon age and disturbance regimes. Taxon age is a well-established factor influencing rates of hybridization; as lineages diverge, genomic incompatibilities evolve that reinforce reproductive barriers and thus result in lower rates of hybridization (Larcombe *et al.*, 2015). In terms of OCBILs, therefore, the generally older age of taxa that occur on these older landscapes predicts that hybridization capabilities among these more divergent taxa should be low. The effect of disturbance on hybridization is less well understood in the context of OCBILs/YODFELs. The common observation of increased rates of hybridization in areas of high disturbance, particularly in ecotones between the preferred habitats of the parental taxa, however, suggests that environmental perturbations may create niches that are more suited to hybrid phenotypes than those of the parental taxa (Abbott, 2017; Beddows and Rose, 2018; Hopper, 2018). Thus, it is expected that highly disturbed YODFEL environments, coupled with younger, less divergent taxa, are likely to exhibit higher levels of hybridization (Arnold, 2016; Hopper, 2018).

The Stirling Range National Park is a localized hotspot for biodiversity within the SWAFR, with over 1500 plant species, including 80 endemic taxa (Barrett and Cochrane, 2005). The region is also the most topographically diverse part of the SWAFR with a range of OCBIL and YODFEL formations (Fig. 1). Extensive surveys of the Stirling Range in the 1980s found 44 species of *Eucalyptus* in the National Park, as well as a notably rich concentration of interspecific eucalypt hybrids, totalling 21 hypothesized combinations (Hopper, 1993; Department of Biodiversity, Conservation and Attractions, 2019). Subsequent discoveries have revealed further combinations, bringing the total to eight from subg. *Symphyomyrtus* and 16 from subg. *Eucalyptus*. The hybrid identification of all combinations has thus far been determined based on intermediate morphological characters and, in some cases, progeny segregation trials. However, molecular data might prove valuable to confirming their hybrid status and parentage.

Here, we apply high-resolution genomic data to a number of hypothesized hybrid combinations from subg. *Eucalyptus* and *Symphyomyrtus* in the Stirling Range National Park. Within subg. *Eucalyptus*, we focus on *E. erectifolia* (Brooker and Hopper), a formally recognized species (Brooker and Hopper, 1986) throughout the Stirling Range. The species is suspected to be a hybrid because it displays morphological affinity to a number of sympatric taxa (Brooker and Hopper, 1986). In addition, there are other populations that show morphological affinity to *E. erectifolia* but were not included within the taxon due to their dissimilarities and, in some cases, their affinity to other sympatric taxa. It is therefore suspected that there may be a number of hybrid combinations within the morphologically variable *E. erectifolia* complex (Table 1). Within subg. *Symphyomyrtus*, we focus

FIG. 1. Stylized cross-sectional diagram of the Stirling Range showing younger landscape components, such as river valleys and eroding hill slopes (YODFEL) through to older landscape components, such as sandplains and hilltops (OCBIL).

TABLE 1. *The morphology of Eucalyptus erectifolia and selected morphologically similar forms, sympatric taxa to which they show close morphological affinity, the landscape in which they occur, and whether they exhibit evidence of clonality.*

Taxon/population	Diagnostic morphology	Morphological affinity	Landscape	Clonal
<i>E. erectifolia</i> Multiple	Mallee 1–4 m; smooth grey stems; leaves narrowly lanceolate, concolorous, held erect; fruit cupular	<i>E. pachyloma</i> , <i>E. buprestium</i> , <i>E. marginata</i> subsp. <i>marginata</i>	OCBIL/transitional	Y
<i>E. aff. erectifolia</i> Stirling Range Drive, Bluff Knoll	Mallee 1–4 m; rough fibrous bark; leaves lanceolate to falcate, slightly discolourous, held erect; fruit cupular	<i>E. pachyloma</i> , <i>E. buprestium</i> , <i>E. marginata</i> subsp. <i>marginata</i>	OCBIL/transitional	Y
Bluff Knoll Road	Mallee 1–4 m; rough fibrous bark; leaves narrowly lanceolate, concolorous, held erect; fruit cupular to obconical	<i>E. pachyloma</i> , <i>E. buprestium</i> , <i>E. marginata</i> subsp. <i>marginata</i>	OCBIL	Y
Mt Talyuberlup	Mallee 1–3 m; smooth grey stems; leaves narrowly elliptical to lanceolate, concolorous, held erect; fruit truncate-globose.	<i>E. pachyloma</i> , <i>E. buprestium</i> , <i>E. marginata</i> subsp. <i>marginata</i> , <i>E. ligulata</i> subsp. <i>stirlingica</i>	OCBIL	N
Central lookout	Mallee 2 m; smooth grey stems rough at base; leaves elliptical to ovate, concolorous; fruit cupular to obconical	<i>E. pachyloma</i> , <i>E. buprestium</i> , <i>E. marginata</i> subsp. <i>marginata</i> , <i>E. preissiana</i> subsp. <i>preissiana</i> , <i>E. staeri</i>	OCBIL	N

on three separate combinations that form suspected hybrid zones on the southern slope of Mt Talyuberlup, a peak near the centre of the Stirling Range. In each case, a morphological transition is observed over some 200 m between parental taxa that occur at the lower and upper slopes, with the hypothesized hybrids occurring on the mid-slope between the preferred habitat of each putative parental species (Table 2). In investigating these taxa in both subgenera, we have two major aims. The first was to test their hypothesized hybrid status and confirm their parentage. Our second aim was to characterize the range of hybrid classes present within each combination to assess differences in hybridization between the two subgenera that may align with phylogenetic context, as proposed in OCBIL theory. Specifically, we hypothesized that given subgeneric differences, such as diversification rates and taxon age and their occurrence on different landscape ages, subg. *Symphyomyrtus* parental species will be less genetically distinct and show higher rates of introgressive hybridization than members of subg. *Eucalyptus*.

MATERIALS AND METHODS

Sampling scheme

Across the whole study, leaf samples were collected from eight trees to represent each taxon (putative parental species or hybrid) within any given population (Fig. 2). Where populations contained fewer than eight trees for any taxon, all individuals present were sampled. Voucher specimens were collected to represent all putative hybrids and parents from each sampling locality; these have been lodged with the Western Australian Herbarium (PERTH). Information regarding sample sizes and sampled locations are provided in [Supplementary Data Tables S1A](#) (subg. *Eucalyptus*) and [S1B](#) (subg. *Symphyomyrtus*).

For subg. *Eucalyptus*, typical *E. erectifolia* and its putative parents (*E. buprestium*, *E. marginata* subsp. *marginata* and *E. pachyloma*) were sampled from three populations (Bluff Knoll Road, Formby South Road, Red Gum Pass) within the Stirling Range (Fig. 2). Because all the relevant taxa are

TABLE 2. The diagnostic morphology of the hypothesized *Eucalyptus* subg. *Symphyomyrtus* hybrids at Mt Talyuberlup and their putative parents, the landscape in which they occur, and whether they exhibit evidence of clonality.

Taxon/population	Diagnostic morphology	Morphological affinity/ sympatric taxa	Landscape	Clonal
<i>E. medialis</i> × <i>E. wandoo</i> subsp. <i>wandoo</i> Mt Talyuberlup	Mallee 7 m, thick trunk (>10 cm), smooth grey bark, rough stocking shedding in strips	<i>E. medialis</i> , <i>E. wandoo</i> subsp. <i>wandoo</i>	YODFEL	N
<i>E. occidentalis</i> × <i>E. sporadica</i> Mt Talyuberlup	Mallee to 4 m, smooth bark, sometimes with rough stocking; fruits cylindrical to campanulate	<i>E. occidentalis</i> , <i>E. sporadica</i> , <i>E. astringens</i> subsp. <i>redacta</i>	YODFEL	N
<i>E. lehmannii</i> subsp. <i>parallela</i> × <i>E. talyuberlup</i> Mt Talyuberlup	Mallee 4 m; unfused hypanthium, long slender opercula	<i>E. lehmannii</i> subsp. <i>parallela</i> , <i>E. talyuberlup</i>	YODFEL	N

FIG. 2. Google Earth (2019) image of the Stirling Range National Park showing the *Eucalyptus* subg. *Eucalyptus* (●) and *E. subg. Symphyomyrtus* (▲) sampling locations.

sympatric at each of the three populations with no spatial segregation, sampling of each species was conducted randomly. In addition, each parental species was also sampled from further away (>5 km) to serve as genetically pure, allopatric reference populations. The sampling of reference *E. marginata* subsp. *marginata* populations includes those from both montane and lower slope locations because these populations exhibit differences in habit and fruit form that may represent different taxonomic entities. Populations of *E. aff. erectifolia* that differed from typical *E. erectifolia* in bark morphology (Bluff Knoll, Bluff Knoll Road and Stirling Range Drive) and fruit morphology (Central Lookout and Mt Talyuberlup) were sampled in the same manner to investigate their potential hybrid origin, as well as their genetic relationship to *E. erectifolia*. In addition to the putative parental taxa named above, several other

taxa were sampled as putative parents for *E. aff. erectifolia*, based on morphology and their being sympatric at each population; *E. ligulata* subsp. *stirlingica* (Mt Talyuberlup), and *E. preissiana* subsp. *preissiana* and *E. staeri* (Central Lookout). Finally, both typical *E. erectifolia* and *E. aff. erectifolia* can occur in large circular clumps that suggest clonality. We tested this by sampling from either end of large clumps in populations at Bluff Knoll, Bluff Knoll Road, Red Gum Pass and Stirling Range Drive.

For the three proposed hybrid combinations of subg. *Symphyomyrtus* (*E. lehmannii* subsp. *parallela* × *E. talyuberlup*, *E. medialis* × *E. wandoo* subsp. *wandoo* and *E. occidentalis* × *E. sporadica*) at Mt Talyuberlup (Fig. 2), putative parental species were sampled from either end of the slope, whilst morphologically intermediate individuals (i.e. putative hybrids) were

sampled from the mid-slope. In the case of *E. lehmannii* subsp. *parallela* × *E. talyuberlup*, individuals were also sampled from Mt Trio where a similar transition is observed. Additional populations of each putative parent were also sampled from further away to serve as genetically pure allopatric reference populations. Although not observed at the Mt Talyuberlup site, *E. astringens* subsp. *redacta* was also considered as a possible parental species in the *E. occidentalis* × *E. sporadica* hybrid combination and was sampled from a nearby location (Central Lookout; ~5 km). Note that *E. medialis*, a Stirling Range endemic, was synonymized with a widespread species, *E. hebetifolia*, by French (2012). However, there are morphological differences between them and for clarity when referring to the morphological attributes of the hybrids in this study, we refer to the Stirling Range populations as *E. medialis*.

SNP genotyping and quality filtering

DNA was extracted from freeze-dried leaf material using a modified CTAB method (Doyle and Doyle, 1987) with 10 mM sodium sulphite (Byrne et al., 2001) and 2 % (w/v) polyvinylpyrrolidone added to the extraction buffer. Following extraction, all samples were sent to Diversity Array Technologies Pty Ltd (DART; Canberra, Australia) for DARTseq analysis. Library preparation followed Sansaloni et al. (2010) with complexity reduction testing resulting in the use of restriction enzymes *Pst*I and *Hpa*II. Amplification of ligated fragments was conducted in 30 cycles of PCR, with an initial denaturation at 94 °C for 1 min followed by 30 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 45 s, and a final step at 72 °C for 7 min. Following PCR and quantification, amplification products were standardized and pooled for sequencing (single read) on an Illumina HiSeq2500 sequencer for 77 cycles. Bioinformatic analyses were conducted on four separate datasets, one for the *E. erectifolia* complex (subg. *Eucalyptus*) and one for each of the three subg. *Symphyomyrtus* combinations. For each dataset, sequences were processed in two stages using DART proprietary pipelines; the primary pipeline for quality control and assembly, and the secondary pipeline (DARTsoft14) for SNP calling. These pipelines make use of technical replicates (~25 % of samples) for a measure of genotyping reproducibility and Mendelian inheritance patterns to filter sequencing errors and remove paralogous regions.

We undertook further filtration of each dataset using the ‘dartR’ package (Gruber et al., 2018) in R (R Core Team, 2016). Each dataset was first reduced to a single SNP per read, followed by further filtering on reproducibility (>0.99), call rate (>0.99), minor allele frequency (>0.02) and heterozygosity (>0.02). These steps reduced the *E. erectifolia* dataset to 4056 loci, the *E. lehmannii* subsp. *parallela* × *E. talyuberlup* dataset to 7071 loci, the *E. medialis* × *E. wandoo* subsp. *wandoo* dataset to 9229 loci, and the *E. occidentalis* × *E. sporadica* dataset to 9827 loci. Due to the complex array of relationships identified among the various hybrid groups and parental taxa in the *E. erectifolia* complex (see Results), this dataset was further subset to provide greater analytical resolution. In this case, an additional filtering step was applied to remove any monomorphic loci that may have resulted from

subsetting the data. All resulting datasets were converted for use in other programs using PGDSPIDER v.2.1.0.0 (Lischer and Excoffier, 2012).

Clonality

Clonal assessment was based on the technical replicates generated by DART, where randomly selected DNA samples were genotyped in duplicate. Calculating twice the average genetic distance among known replicates gives an estimate of sequencing error and provides a threshold with which to apply to the genetic distances among all samples to identify clonemates. Datasets with confirmed clonality were reduced to a single sample per genetic individual prior to quality filtering and subsequent analyses.

Hybrid and parent identification

To test the morphological identification of hybrid taxa and confirm parental taxa for each combination, the genetic relationships among putative hybrid and parental taxa were assessed using two clustering methods. Principal coordinates analysis (PCoA) was performed in R using the ‘ade4’ package v.2.0.1 (Jombart, 2008). PCoA is a multivariate statistical approach that provides a useful method for exploring evidence of hybridization because hybrids would be expected to align midway between two clearly separated parental species. Once likely parental taxa were identified using PCoA, these relationships were further tested using the Bayesian clustering program STRUCTURE v.2.3.4 (Pritchard et al., 2000) to assign individuals to a *K* number of genetically homologous clusters with no prior information regarding identity or location. Analyses were run using the correlated frequency model, 100 000 burnin, 100 000 Markov chain Monte Carlo replications and five repetitions for each *K* value up to a limit of five. For this analysis, expectations for hybridization would result in *K* = 2, with putative hybrids admixed between the two parental taxa.

Characterization of hybrid population structure

Once parent–hybrid relationships were established through PCoA and STRUCTURE, ‘dartR’ was used to reduce each dataset to those loci exhibiting fixed differences between the two parent species for use in NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002). This program uses a Bayesian clustering framework to estimate the relative likelihood that each individual belongs to one of six hybrid classes (Parent 1, Parent 2, *F*₁, *F*₂, and backcrosses to either parent) based on allelic differences between the parents. The analysis for each dataset was run with defined parental species, 100 000 burnin and 100 000 sweeps. For each of the *Symphyomyrtus* datasets, the fixation rate was lowered to 90 % as the parental taxa exhibited low numbers of loci with fixed differences. Furthermore, NEWHYBRIDS analysis was not conducted for the ‘*E. occidentalis* × *E. sporadica*’ dataset because the parentage of the putative hybrids could not be confidently determined (see Results).

RESULTS

Subgenus *Eucalyptus*

Clonality. Evidence of clonality was found in all mallee clumps where clonality was suspected. These clonal clumps occurred in typical *E. erectifolia* sampled from Red Gum Pass, where the four mallee clumps analysed ranged from 13.4 to 24.4 m in diameter at the widest point, as well as the rough barked forms of *E. aff. erectifolia* occurring at Bluff Knoll (8.3 m), Bluff Knoll Road (18.8 m) and Stirling Range Drive (25.2 m). Separate mallee clumps within populations showed distinct genotypes.

Hybrid and parental identification. The PCoA of the full dataset for subg. *Eucalyptus* presented a triangular arrangement of parental taxa across the first two axes (52.6 % of the total variation), with all putative hybrid samples falling midway between three groups of differing parental combinations (Fig. 3A) – Group 1: *E. buprestium*, *E. erectifolia*, *E. aff. erectifolia*, *E. ligulata* subsp. *stirlingica*, *E. pachyloma* and *E. preissiana*

subsp. *preissiana* (Mt Talyuberlup) (Fig. 3B); Group 2: one sample of each *E. erectifolia* (ERRG12) and *E. aff. erectifolia*, *E. marginata* subsp. *marginata*, *E. pachyloma*, *E. preissiana* subsp. *preissiana* and *E. staeri* (Bluff Knoll Road and Central Lookout) (Fig. 3C); Group 3: *E. buprestium*, *E. aff. erectifolia*, *E. marginata* subsp. *marginata* and *E. staeri* (Bluff Knoll and Stirling Range Drive) (Fig. 3D). Due to the large number of taxa and complex relationships in this dataset, subsetting into each of these three groups provided better resolution in the PCoA to clearly identify the parental taxa for each hybrid combination. Subsetting the data further into each putative hybrid and its identified parents, the STRUCTURE analyses were consistent with the PCoA results, finding $K = 2$ for all hybrid combinations with each parental species assigned to separate genetic clusters and the hybrid individuals admixed between the two parental clusters (Fig. 4A–N).

Based on the PCoA and STRUCTURE analyses, a total of seven hybrid combinations were identified for the *E. erectifolia* complex. (1) Typical *E. erectifolia* was identified as *E. buprestium* × *E. pachyloma* (Fig. 4A, B). The only specimens

FIG. 3. Principal coordinates analyses (PCoA) of subg. *Eucalyptus* hybrids (○) and their putative parents (●) across the first two axes. (A) Overall PCoA of all sampled subg. *Eucalyptus* individuals, with the ellipses indicating data subsets used to undertake further analysis. (B–D) PCoAs showing taxonomic relationships among subsets of pure species and putative hybrid individuals.

FIG. 4. Cluster analyses performed on each of seven subg. *Eucalyptus* hybrids (○) and their putative parents (●) collected from the Stirling Range, Western Australia. (A, C, E, G, I, K, M) Principal coordinates analyses across the first two axes. (B, D, F, H, J, L, N) STRUCTURE results ($K = 2$) with the vertical bar plots showing the proportion of each genetic cluster assigned to each specimen sampled at each location. Dark bars indicate separate populations/species (sampling sites: BK, Bluff Knoll; BR, Bluff Knoll Road; CL, Central Lookout; FR, Formby Road; KP, Kalgan Plains; MT, Mt Talyuberlup; TR, Mt Trio; RG, Red Gum Pass; SC, Salt Creek; SR, Salt River Road; SD, Stirling Range Drive). *The parental genotype reference populations used for each analysis.

that did not follow this pattern (ERFR8 and ERRG12) were found to be misidentifications and the result of two different hybrid combinations: (2) *E. erectifolia* × *E. ligulata* subsp. *stirlingica* (ERFR8; Fig. 4C, D) and (3) *E. marginata* subsp. *marginata* × *E. pachyloma* (ERRG12; Fig. 4E, F). The single rough-barked clump at Bluff Knoll Road was also identified as *E. marginata* subsp. *marginata* × *E. pachyloma* (Fig. 4E, F). (4) The rough barked forms occurring at Bluff Knoll and Stirling Range Dr were both identified as *E. buprestium* × *E. marginata* subsp. *marginata* (Fig. 4G, H). (5) The smooth barked form found at Mt Talyuberlup was identified as *E. buprestium* × *E. ligulata* subsp. *stirlingica* (Fig. 4I, J); a single sample collected as *E. buprestium* from Mt Talyuberlup (BUMT4) was also indicated to belong to this hybrid combination. (6) The single specimen occurring at Central Lookout was determined as *E. marginata* subsp. *marginata* × *E. preissiana* subsp. *preissiana* (Fig. 4K, L). (7) The final hybrid combination identified was *E. ligulata* subsp. *stirlingica* × *E. pachyloma* that occurred as a single specimen originally sampled as *E. pachyloma* from the Formby South Road population (PAFR3; Fig. 4M, N).

Characterization of hybrid population structure. Assessment of hybrid genealogical classes indicated that the majority of hypothesized hybrids identified within the *E. erectifolia* complex were first-generation products of crosses (i.e. F_1 s), whilst all parents showed full assignment to their respective parental classes (Fig. 5). The only exception to this was the single individual identified as *E. ligulata* subsp. *stirlingica* × *E. pachyloma* (PAFR3), which was identified as a backcross to *E. pachyloma* (Fig. 5G).

Genetic divergence in *E. marginata*. A stand-alone PCoA of the eight *E. marginata* subsp. *marginata* populations showed no genetic differentiation between the stunted, small-fruited mallees from montane sites and the lower slope trees with larger fruits (Fig. 6).

Subgenus *Symphyomyrtus*

Hybrid and parental identification. Similar clustering patterns were present among the three *Symphyomyrtus* combinations (Fig. 7A–E). Within the *E. medialis* × *E. wandoo* subsp. *wandoo* dataset, the first axis of the PCoA (15.7 % of the total variation) separated the reference populations of the parental species with putative hybrids falling midway (Fig. 7A). The two *E. wandoo* subsp. *wandoo* populations (local and reference) were clustered together. Most *E. medialis* individuals from the transition zone were clustered with the hypothesized hybrids, as well as a single individual from the *E. medialis* reference population; a further two individuals fell midway between the hybrid group and the remainder of the reference population. The second axis (4.3 %) separated the two *E. wandoo* subsp. *wandoo* populations, whilst the third axis (3.7 %) separated the two *E. medialis* populations with the putative hybrids clustered with *E. medialis* individuals from the transition zone. STRUCTURE analysis ($K = 2$) followed a similar pattern with both *E. wandoo* subsp. *wandoo* populations assigned primarily to a single genetic cluster and most individuals from the *E. medialis* reference population, as well as two from the transition zone, assigned primarily to the other genetic cluster (Fig. 7B). The putative hybrids and the

remainder of the *E. medialis* transition zone population showed equal assignment to each cluster, whilst three individuals from the reference population also showed some admixture.

The PCoA of *E. lehmannii* subsp. *parallela* × *E. talyuberlup* clearly separated the reference populations for the two parental species on the first axis, representing 21.9 % of the variation (Fig. 7C). The two *E. talyuberlup* transition zone populations were clustered with the *E. talyuberlup* reference population, whilst the Mt Trio *E. lehmannii* subsp. *parallela* and putative hybrid individuals clustered with the *E. lehmannii* subsp. *parallela* reference population. The Mt Talyuberlup *E. lehmannii* subsp. *parallela* and putative hybrid populations were clustered together and positioned midway between these main groups. The second and third axes separated the *E. talyuberlup* reference populations from the Mt Talyuberlup and Mt Trio populations (Fig. 7C). Both axes also showed minor separation of Mt Trio *E. lehmannii* subsp. *parallela* and hybrid populations from the reference population. STRUCTURE analysis ($K = 2$) showed a similar result to the first axis of the PCoA (Fig. 7D). All *E. talyuberlup* populations were assigned primarily to a single genetic cluster. The Mt Trio *E. lehmannii* subsp. *parallela* and hybrid individuals and the *E. lehmannii* subsp. *parallela* reference population were assigned primarily to the other genetic cluster. The Mt Talyuberlup *E. lehmannii* subsp. *parallela* and hybrid individuals showed equal assignment to both clusters.

For the *E. occidentalis* × *E. sporadica* hybrid dataset, the first axis of the PCoA represented 9.7 % of the variation and separated the *E. occidentalis* and *E. sporadica* reference populations (Fig. 7E). The three Mt Talyuberlup populations were positioned midway between these clusters, and *E. astringens* subsp. *redacta* was situated closer to the *E. sporadica* reference cluster. Among the Mt Talyuberlup populations, *E. occidentalis* and *E. sporadica* showed some separation, with hybrid individuals positioned between these clusters and showing some overlap with both species. The second axis separated the Mt Talyuberlup populations from the *E. occidentalis* and *E. sporadica* reference populations with *E. astringens* subsp. *redacta* positioned midway between the two *E. sporadica* populations. The third axis further separated the three Mt Talyuberlup populations, with hybrid individuals falling midway between *E. occidentalis* and *E. sporadica*. STRUCTURE analysis ($K = 3$) assigned *E. occidentalis* and *E. sporadica* reference populations each to a single genetic cluster (Fig. 7F). *Eucalyptus sporadica* at Mt Talyuberlup was assigned primarily to a third cluster with *E. occidentalis*, and hybrid individuals showing majority assignment to this cluster with some admixture with the *E. occidentalis* reference population. *Eucalyptus astringens* subsp. *redacta* showed majority assignment to the *E. sporadica* reference cluster and some admixture with the Mt Talyuberlup *E. sporadica* cluster.

Characterization of hybrid population structure. NEWHYBRIDS analysis of *E. medialis* × *E. wandoo* subsp. *wandoo* found evidence of F_1 and advanced generation hybrids (Fig. 8A). The two individuals sampled as hybrids each showed full assignment to the F_1 and *E. medialis* backcross (BC Pa2) categories. The majority of individuals (6/8) sampled as *E. medialis* from the Mt Talyuberlup site showed full or mixed assignment to the F_1 and F_2 clusters, with only a single specimen fully assigned to the *E. medialis* parental

FIG. 5. NEWHYBRIDS analysis of the seven hybrid groups identified from Figs 1 and 2 with vertical bars indicating the proportion of each hybrid class assigned to each individual (Pa1, parent 1; Pa2, parent 2; F_1 , first-generation hybrid; F_2 , second-generation hybrid; BC Pa1, parent 1 backcross; BC Pa2, parent 2 backcross). The fixation rate and number of loci used for each analysis are shown to the right of each plot. *The parental genotype reference populations used for each analysis.

FIG. 6. PCoA of eight *E. marginata* subsp. *marginata* populations collected from montane (▲) and lower slope (●) sites of the Stirling Range that exhibit differing morphology. For context, also included is a reference population (■), considered morphologically typical of the taxon, from outside of the Stirling Range.

cluster. The remaining individual showed full assignment as a backcross to *E. medialis* (BC Pa2). All *E. wandoo* subsp. *wandoo* from the hybrid zone showed full assignment to the *E. wandoo* subsp. *wandoo* parental class. Additionally, three individuals from the *E. medialis* reference population were genetically separated from the main grouping. Treatment of these individuals as unknowns in the NEWHYBRIDS analysis indicated they were backcrosses to *E. medialis*, with all showing full or majority assignment to the backcross (BC Pa2) cluster.

Analysis of the *E. lehmannii* subsp. *parallela* × *E. talyuberlup* dataset using NEWHYBRIDS showed all individuals except the hypothesized hybrids and *E. lehmannii* subsp. *parallela* from Mt Talyuberlup to have full assignment to their respective parental clusters (Fig. 8B). *Eucalyptus lehmannii* subsp. *parallela* and hybrid individuals from Mt Talyuberlup showed similar parentage assignment with the majority showing full assignment to the F_2 cluster and others showing full or partial assignment to the *E. lehmannii* subsp. *parallela* backcross cluster (BC Pa2). No F_1 or *E. talyuberlup* backcross hybrids were indicated in the sampled populations.

DISCUSSION

Using high-resolution genomic data, the present study confirmed the hybrid status of several hypothesized combinations of *Eucalyptus* taxa in a biodiversity hotspot of the SWAFR. Moreover, the differing patterns of hybridization found between the two eucalypt subgenera align closely with expectations of taxon age and support a hypothesis for reduced hybridization on older landscapes, as proposed in OCBIL theory (Hopper, 2009, 2018). While hybridization is known to be extensive in *Eucalyptus*, our study provides specific insight into the differing patterns of hybridization within the genus.

Differing patterns of hybridization

Based on known differences in the evolutionary history of the subgenera *Eucalyptus* and *Symphyomyrtus*, particularly taxon age, our data supported the OCBIL theory hypothesis that hybridization patterns would also differ between them. Subgenus *Eucalyptus* has shown a gradual diversification of species over the last 31–39 Myr (Thornhill et al., 2019) and commonly occurs on older landscapes in the SWAFR. It thus follows that our study found this subgenus to exhibit parental species as well-defined genetic entities with large numbers of fixed allelic differences, and although first-generation hybridization was common for some combinations, there was no evidence of further introgression. This indicates strong reproductive and genetic divergence that has developed over a much longer timeframe in these older taxa (Abbott, 2017). In comparison, the sampled taxa of subg. *Symphyomyrtus* have evolved in a more recent (2–3 Mya) radiation of taxa on younger, more disturbed landscapes. In our study, this subgenus showed less genetic distinction among parental species with low numbers of fixed allelic differences, and more complex hybrid zones with high levels of introgression. This is consistent with expectations of weak reproductive and genetic divergence that have not yet had time to evolve among the younger taxa of this subgenus.

While taxon age is probably the predominant contributor to the disparity in patterns of hybridization observed here between *Eucalyptus* subgenera, other factors relevant to OCBIL theory may also play a role. The steep slope of Mt Talyuberlup, where most of our subg. *Symphyomyrtus* hybrids were sampled, constitutes a disturbed landscape where the preferred habitats of each parental species are in close proximity but not overlapping. Here, it is possible that hybrid genotypes are more suited to the disturbed, mid-slope environment and have a fitness advantage over parental genotypes (Arnold, 2016; Abbott, 2017). While the Mt Talyuberlup site represents a known hybridization hotspot for the subg. *Symphyomyrtus* taxa sampled here, it also is possible that

FIG. 7. Cluster analyses performed on three subg. *Symphyomyrtus* hybrid populations and their putative parents collected from the Stirling Range, Western Australia. (A, C, E) Principal coordinates analysis across the three axes. (B, D, F) STRUCTURE results with the vertical bar plots showing the proportion of each genetic cluster assigned to each specimen sampled at each location. Dark vertical lines indicate separate populations/species (sampling sites: BT, Bluff Knoll Turnoff; BR, Bluff Knoll Road; CL, Central Lookout; MT, Mt Talyuberlup; TO, Toolbrunup Road; MH, Mt Hassel; TR, Mt Trio; MM, Mt Maggog; PC, Paper Collar Creek). *The parental genotype reference populations used for each analysis.

FIG. 8. NEWHYBRIDS analysis of the *Eucalyptus medialis* × *E. wandoo* subsp. *wandoo* (A) and *E. lehmannii* subsp. *parallela* × *E. talyuberlup* (B) hybrid groups. Vertical bars indicate the proportion of each hybrid classes assigned to each individual (Pa1, parent 1; Pa2, parent 2; F_1 , first-generation hybrid; F_2 , second-generation hybrid; BC Pa1, parent 1 backcross; BC Pa2, parent 2 backcross). The fixation rate and number of loci used for each analysis are shown to the right of each plot. *The parental genotype reference populations used for each analysis.

similar hybrid zones occur on other disturbed slopes of the Stirling Range. Morphological evidence of this is seen in the *E. lehmannii* subsp. *parallela* × *E. talyuberlup* combination at Mt Trio and although our limited sampling did not identify hybrids in the genetic analysis, continued survey at Mt Trio and elsewhere may uncover further hybridization. In comparison, most of the sampled subg. *Eucalyptus* hybrids fell within the parental habitat and thus the disturbance hypothesis is not relevant to these taxa. For subg. *Eucalyptus*, however, the presence of long-lived clonal individuals on OCBILs supports the ‘Ultimate-self Hypothesis’, whereby the genotypes of some taxa are so suited to environmental challenges that there is reduced need for genetic variation to respond to environmental change (Hopper, 2009). This hypothesis is inherently linked with OCBIL theory as OCBILs are naturally environmentally buffered landscapes that can allow individuals to persist for long periods of time.

Combinations in subgenus *Eucalyptus*

Our genomic data found seven hybrid combinations within the sampled *E. erectifolia* complex that involved six parental taxa. While typical *E. erectifolia* (*E. buprestium* × *E. pachyloma*) is relatively common and found at multiple sites, the remaining hybrid combinations were few in number (Supplementary Data Table S1A), indicating that successful hybridization between most of these parental taxa is rare. Possible pre-mating barriers, such as flowering time and spatial distribution, largely overlap among the sampled parental taxa, suggesting that the observed limitation in hybridization is probably a result of well-developed

post-mating barriers (Larcombe *et al.*, 2015). Furthermore, the distinct lack of post- F_1 hybrids indicates either the sterility of F_1 hybrids or the presence of strong post-zygotic mechanisms that prevent ongoing hybridization. Indeed, it was observed in the field that all subg. *Eucalyptus* hybrids produced fruit in quantities as high as in each respective parental species and thus it seems likely that seed viability in the *E. erectifolia* complex is poor. This is supported by progeny segregation trials that have previously been conducted on typical *E. erectifolia* that resulted in the germination of only a single individual that did not survive past node 8 (Slee *et al.*, 2015).

Widespread clonality detected in the *E. erectifolia* complex also inflates the apparent frequency of hybridization in this group. Clonal propagation can allow individuals to survive for hundreds to thousands of years and can result in a census population size that is disproportional to the number of distinct genetic individuals (Bradbury *et al.*, 2016). Our data demonstrated that the circular clumps formed by typical *E. erectifolia*, which was by far the most numerous hybrid combination sampled, were large clonal stands. Tyson *et al.* (1998) estimated the radial expansion of clonal eucalypts to be between 1.5 and 3.4 mm per year. Based on these values, the largest of the clonal clumps sampled here (*E. buprestium* × *E. marginata* subsp. *marginata*; 25.2 m diameter) would be estimated to be between 3706 and 8400 years old. Thus, while our data have shown the *E. erectifolia* hybrids to be relatively common, it is likely that these hybrids have persisted and expanded in clump size over a long time period, such that the actual frequency of sexual hybridization events in this combination is lower than it appears based on census counts.

Combinations in subgenus *Symphyomyrtus*

For recently diverged or diverging lineages, reproductive barriers are often not fully developed and can be easily broken down allowing populations to hybridize (Larcombe *et al.*, 2015). Such appears to be the case found here within subg. *Symphyomyrtus*, where all combinations displayed complex hybrid zones with greater abundance of hybrids and extensive backcrossing to one or both parental species. The presence of all hybrid classes in the *E. medialis* × *E. wandoo* subsp. *wandoo* combination indicates that introgression is not encumbered by any incompatibilities. However, there were some notable hybrid classes missing in other combinations. For example, we found no evidence of backcrossing to either *E. talyuberlup* or *E. wandoo* subsp. *wandoo* in their respective combinations, suggesting uni-directional reproductive barriers. Such barriers are often seen in other hybridizing eucalypts; for example, fertilization between *E. globulus* and *E. nitens* can only occur in one direction because flower size and pollen–pistil size incompatibilities prevents the small-flowered *E. nitens* pollen from pollinating large *E. globulus* flowers (Larcombe *et al.*, 2016). In addition, given the range of hybrid classes found in the sampled *E. lehmannii* subsp. *parallela* × *E. talyuberlup* combination, the lack of F_1 hybrids was surprising. It may be that this represents a situation where F_1 s are able to reach reproductive age but have poor fitness and are relatively short-lived. Alternatively, it may be that our sampling did not capture all of the hybrid classes in this combination. Finally, the high level of admixture among the putative parental species and hybrids of the *E. occidentalis* × *E. sporadica* group on the Mt Talyuberlup slope made confirmation of parental taxa uncertain. It is possible that all these individuals represent hybrids and that sampling further up and downslope would find ‘pure’ individuals of both *E. occidentalis* and *E. sporadica*, but it may also be that these are simply not the correct parental taxa. Given the clear genetic distinction of their respective allopatric reference populations, we do not hold any taxonomic concerns with regard to species boundaries between *E. occidentalis* and *E. sporadica*. The varied results from these three combinations highlight the difficulty of using morphology alone to identify hybrids and parental sources among young, closely related taxa. Further investigation of these hybrid combinations with reproductive trials would be useful in determining the success rate and survivability of each hybrid class.

Taxonomic considerations

Following the confirmation of nine hybrid combinations (seven subg. *Eucalyptus* and two subg. *Symphyomyrtus*) and their parental taxa, these entities should be recognized taxonomically. In particular, *E. erectifolia*, which is currently listed by the Western Australian Herbarium as a distinct species, should be recognized as the nothospecies *E. × erectifolia* to acknowledge its hybrid status. It is also worth noting that we found no genetic divergence within *E. marginata* subsp. *marginata* between the montane mallees with small fruits and the lower slope trees with larger fruits in the Stirling Range, suggesting that they simply represent local variants and not distinct taxonomic entities. Finally, what we recognize in this

study as *E. medialis* is considered an accurate parental assignment to that hybrid combination within the Stirling Range; however, taxonomic clarity regarding the relationship between *E. medialis* and *E. hebetifolia* would benefit from molecular analysis with comprehensive population sampling of both entities.

CONCLUSIONS

Our data have shown considerable differences in patterns of hybridization between the *Eucalyptus* subgenera *Eucalyptus* and *Symphyomyrtus* and support a hypothesis for reduced hybridization on older landscapes as presented in OCBIL theory (Hopper, 2009, 2018). These patterns are intrinsically linked to taxon age, with data showing a decrease in hybridization ability with increasing genetic distance as found in subg. *Eucalyptus*, and higher levels of hybridization with ongoing introgression in more recently diverged taxa such as those of subg. *Symphyomyrtus*. Given that the populations sampled here grow in sympatry and have shown evidence of at least F_1 hybridization, postzygotic mechanisms are considered to be limiting hybridization but are less restrictive for recently diverged lineages such those of subg. *Symphyomyrtus* where levels of introgression were found with one or both parental species. Evidence of widespread clonality in some subg. *Eucalyptus* combinations suggests the apparent high capacity for first-generation hybridization may be disproportional to the actual rate of recruitment, and hybridization events within the subgenus are likely to be comparatively rare. Disturbance regimes are also considered to be playing a role in patterns within subg. *Symphyomyrtus*, with disturbed areas in younger landscapes offering an opportunity for hybrids to prosper over parental genotypes. In comparison, on older, less frequently disturbed landscapes, long-term persistence of individuals provides a more beneficial strategy. While the taxa investigated here represent only a small number of *Eucalyptus* species, our results highlight that clade divergence times, landscape age and clonality are important drivers of differing patterns in species divergence and hybridization patterns in *Eucalyptus*. Further investigation of this hypothesis elsewhere is recommended.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Table S1A: Locations and total number of individuals sampled per population for putative hybrids and parental taxa in the Stirling Range National Park, Western Australia, for subgenus *Eucalyptus*. Table S1B: Locations and total number of individuals sampled for three combinations of putative hybrids and parental taxa on Mt Talyuberlup and Mt Trio in the Stirling Range National Park, Western Australia, for subgenus *Symphyomyrtus*.

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