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GENETIC RELATIONSHIPS AMONG 40 SPECIES OF *EUCALYPTUS* BASED ON SIMPLE SEQUENCE REPEAT MARKERS

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Characteristics of genetic sequences across 40 *Eucalyptus* and *Corymbia* species, and across individuals within species, were examined based on 20 simple sequence repeat (SSR) loci. Variation of target sequences between species were mainly due to differences in the numbers of repetitions and base mutations on the associated flanking region sequences, whilst variations between individuals within species were mainly due to differences within the repetitive SSR motifs. Estimates obtained across the species examined in this study for indices of genetic diversity, including average expected heterozygosity of 0.48 and average polymorphism information content value of 0.73, were generally consistent with values published from previous studies focused on either single or limited numbers of eucalypt species. Results from analyses of genetic relationships among the 155 genotypes sampled in this study, particularly genetic distances separating species, could be used to help identify potentially compatible hybrid combinations.

Keywords: *Corymbia*, genetic relationship, genetic diversity, polymorphism, taxonomy

INTRODUCTION

The genus *Eucalyptus* includes over 700 specific taxa (Brooker 2000). Where natural ranges of some of these species overlap, naturally occurring interspecific *Eucalyptus* hybrids can sometimes be found (Boland et al. 2006). Artificial interspecific hybrids can also be created relatively easily among some *Eucalyptus* species that belong to the same subgenus (Eldridge et al. 1993). Indeed, artificial hybrid varieties involving *Eucalyptus urophylla*, most commonly with *E. grandis*, are the foundation of substantial commercial forest plantation areas in many tropical and warmer subtropical regions of the world to produce pulpwood, fuelwood, poles, veneer logs and even saw logs (Eldridge et al. 1993, Arnold et al. 2013).

Where *Eucalyptus* species have been planted in exotic environments, taxonomic identification can sometimes be challenging. If records of seed origins are lost or uncertain, traditional taxonomic keys—which for many eucalypt species rely heavily on morphology of flower buds, flowers and/or seed capsules—are sometimes of little use as some species can be shy to flower in some exotic environments. A notable case is *E. dunnii* in central to northern Guangxi and other target plantation environments of southern China and even those in other countries; trees of this species of up to age 10 years rarely flower (Arnold & Xiang 2003). Less well known is that *E. camaldulensis* can sometimes be a shy and fickle flowerer in exotic environments; it flowers on some but not all sites, and where it does flower, this only happens in occasional years (Luo JZ, personal communication). Such lack of reproductive activity can be problematic for taxonomic identification. Even if the species identity of a tree is known, that may not be sufficient for some research questions if the tree of interest bears no reproductive buds and/or seed capsules. For instance, *E. camaldulensis* now has seven recognised subspecies with identification of these subspecific taxa relying largely on flower bud morphology (McDonald et al. 2009).

Examination of DNA sequences might provide an effective way to overcome such taxonomic uncertainties. To date, molecular genetic analyses have provided effective ways to determine both genetic identity and genetic relationships among known taxa within a genus. Nowadays such work is greatly facilitated by reference data available in the open access database GenBank (http://www.ncbi.nlm.nih.gov/genbank/), which includes many *Eucalyptus* nucleotide and

expressed sequence tag (EST) sequences as well as the complete genome sequence of *E. grandis* (Myburg et al. 2014).

Though numerous molecular genetic studies have been carried out to examine relationships within individual species and among closely related species of *Eucalyptus*, relatively few studies have examined genetic relationships among broader groups of *Eucalyptus* species using molecular genetics. Studies that have been published to date include that by Sale et al. (1993) who examined genetic relationships among 24 species representing five subgenera of the genus using restriction fragment length polymorphisms (RFLP) and Steane et al. (2002) who analysed 90 species of *Eucalyptus* along with 28 species representing eight other genera including *Angophora* and *Corymbia*. The former study found significant differences in chloroplast DNA levels among subgenera and among species within subgeneric groupings. Steane et al. (2002) found that *Angophora* and *Corymbia* appeared sufficiently differentiated from *Eucalyptus*, and their results fortified arguments against the 'lumping' of *Angophora* and *Corymbia* into the genus *Eucalyptus*. They also found that species of *Corymbia* could be divided between two clades, one of which seemed closely related to *Angophora*, and that sections *Adnataria* and *Dumaria* of the *Eucalyptus* genus could form a monophyletic group. Their results also suggested that sections *Exsertaria* and *Latoangulatae* of this genus could justifiably be combined into a single section, and that section *Bisectaria* of *Eucalyptus* could be divided into two distinct and 'quite unrelated' groups 'Bisectae I' and 'Bisectae II'.

In a more recent study, Balasaravanan et al. (2005) used inter-simple sequence repeat (ISSR) markers to examine genetic relationships between six *Eucalyptus* species, namely, *E. camaldulensis, Coyrmbia citriodora* (syn. *E. citriodora*), *E. grandis*, *E. pellita*, *E. tereticornis*, and *E. urophylla*. Cluster and principal component analyses revealed wide genetic diversity among populations of *E. tereticornis, E. camaldulensis* and *E. urophylla* and narrow genetic diversity among populations of *C. citriodora* and *E. grandis* (maximum Nei's genetic distance = 0.29). A separate study examining the utility of 930 amplified fragment length polymorphisms (AFLPs) for analysing relationships among Tasmanian taxa of the *Eucalyptus* subgenus *Symphyomyrtus* section *Maidenaria*, found that a combination of phylogenetic and population genetic approaches could offer a good understanding of taxonomic relationships below the sectional level in *Eucalyptus* (McKinnon et al. 2008).

Other studies reported to date on the interspecific genetic relationships within the *Eucalyptus* genus have mainly concentrated on important commercial plantation species. Zhang et al. (2010) analysed the genetic relationships between 18 provenances of four *Eucalyptus* species using ISSR markers and found *E. camaldulensis* to have close affinities to both *E. grandis* and *E. tereticornis*, but relationships between *E. pellita*, *E. camaldulensis* and *E. tereticornis* were somewhat more distant. Liu et al. (2011) studied internal transcribed spacer (ITS) regions of nuclear ribosomal DNA of 11 species of *Eucalyptus* and found that ITS sequences in *C. citriodora*, *E. pellita* and *E. urophylla* showed close genetic relationships but not as close as that between *E. grandis* and *E. exserta*. Liu et al. (2014) analysed chloroplast DNA of 17 *Eucalyptus* and *Corymbia* species and found that *C. erythrophloia* and *E. shirleyi* (the latter belonging to *Eucalyptus* subgenus *Symphyomyrtus* section *Adnataria*) had a relatively close genetic relationship, but less so than those between *E. camaldulensis*, *E. platyphylla* and *E. haemastoma* (the former two species belonging to subgenus *Symphyomyrtus* section *Exsertaria*, and the latter to subgenus *Eucalyptus* section *Eucalyptus*).

However, phylogenetic analyses of subgenus *Symphyomyrtus* section *Maidenaria* of the genus *Eucalyptus* by McKinnon et al. (2008) found that high homoplasy, intergrading taxa and non-discrete characters together make lower level systematics challenging which can lead to uncertain identification. Many discrete eucalypt taxa have been shown to have very similar or identical ITS sequences and chloroplast DNA sequences (Fladung et al. 2015). An alternative approach used by Barthe et al. (2012) for examining phylogenetics across multiple populations of three tree genera, *Citrus*, *Jacaranda*, and *Quercus*, involved SSR alleles. Their study found that amplicon size variation, SSR variation itself, insertions/deletions (indels) and single nucleotide polymorphisms (SNPs) observed in the flanking regions all contributed significantly to the phylogenetic information and that the best means for differentiation among both populations and individuals within populations was provided by flanking region and SSR regions of SSR markers.

In this current study, 20 pairs of SSR primers were used to explore genetic relationships and genetic diversity among 155 *Eucalyptus* genotypes representing 40 species, including 17 species of subgenus *Symphyomyrtus* section *Maidenaria*, 4 species of subgenus *Symphyomyrtus* section *Exsertaria*, and 7 species of subgenus *Symphyomyrtus* section *Transversaria*. We aimed to analyse the genetic relationships among species and determine whether SSR markers could contribute to deeper taxonomic understanding, and possibly revisions, of the subgenus *Symphyomyrtus*. Multiple genotypes of some species were included to place variation among species and among sections in the context of the variation within species.

MATERIALS AND METHODS

Plant materials and DNA isolation

This study examined 155 genotypes of *Eucalyptus* representing 40 species; details of these are listed in Table 1. Twenty-five species were sourced from field trials at Haikou Forest Farm, Kunming, Yunnan province, China. The other species collected represented commercial plantation taxa from southern China. From each tree sampled, fresh leaf tissue was collected and stored at -80 °C until required for DNA extraction.

Total genomic DNA was extracted from each fresh leaf sample using the CTAB method described by Doyle and Doyle (1990) with modifications suggested by Li (2010). The concentrations and quality of DNA samples were detected by a UV-Vis spectrophotometer and then diluted to 100 ng μL^{-1}.

Analyses of SSR markers

Twenty *Eucalyptus* SSR primers were selected for this study on the basis of being able to propagate stable and specific DNA segments in different species. Information on these 20 SSR primers, designed by the China Eucalypt Research Centre, is provided in Appendix 1.

Polymerase chain reaction (PCR) amplification of SSR loci was carried out in 96-well V-bottom plates. Each reaction was performed in 50 µL volume containing 100 ng template DNA, 0.75 unit of Taq DNA polymerase, 0.2 mmol L^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$ and 50 ng each of forward and reverse primer. PCR amplifications were performed in a thermal cycler with a 96 deep well reaction module using the following protocol: one cycle of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50 to 60 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min.

To evaluate the microsatellite polymorphism and genetic diversity of the 155 genotypes, PCR products were sequenced by Invitrogen Trading (Shanghai) Co. Ltd. after detection by 1.5% agarose gel electrophoresis in our own laboratory.

Data analyses

To ensure the accuracy of sequences, the forward and reverse sequences were assembled and manually adjusted using Geneious 7.1 software (https://www.geneious.com/). MAFFT version 7 and BLAST softwares were used for splice alignment of sequences and sequence retrieval. Genetic parameters obtained for each SSR loci included: DNA polymorphism (*Pi*), haplotype diversity (*Hd*), number of alleles (*Na*), effective number of alleles (*Ne*), Shannon's information index (*I*), expected heterozygosity (*He*) and polymorphic information content (PIC). These genetic parameters were calculated using DnaSP 5.10.01 software and GenAlEx 6.5.01. SSRHunter software was used for searching microsatellites.

To analyse the genetic relationships among the species, maximum likelihood and maximum parsimony phylogenetic trees were constructed with PhyML-3.1 and Paup-4.10 software, respectively. The best model was selected using jModelTest 2.1.5 software. Phylogenetic trees were viewed using MEGA 6.06 software

RESULTS

Characteristics of sequences

From the 20 *Eucalypt* SSR primers used for PCR and DNA representing 155 genotypes in this study, 2769 PCR products were successfully sequenced. The electrophoresis results of PCR amplification were satisfactory with clear strap, correct place and specificity amplification. The amplification of SSR loci ranged from 115 (loci of gSSR-GR139) to 155 (loci of eSSR-GR071, gSSR-GR053, gSSR-GR119 and gSSR-GU002), with an average of 138 (Table 2).

The sequencing results showed that 22 species of *Eucalyptus* had amplicon sequences in

Table 1 Details of the 155 genotypes of *Eucalyptus* and *Corymbia*, representing 40 species, sampled in this study

Species sample code	Species	Number of individual trees	Locality of collection	Classification		
				Genus	Subgenus	Section
HK001	*E. viminalis*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK002	*E. globulus*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK003	*E. amplifolia*	1	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Exsertaria*
HK004	*E. deanei*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
HK005	*E. fibrosa*	1	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Adnataria*
HK006	*E. neglecta*	2	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK007	*E. badjensis*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK008	*E. parvula*	6	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK009	*E. fraxinoides*	1	HKFF	*Eucalyptus*	*Eucalyptus*	*Renantheria*
HK010	*E. macarthurii*	5	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK011	*E. triflora*	1	HKFF	*Eucalyptus*	*Eucalyptus*	*Renantheria*
HK012	*E. dalrympleana*	5	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK013	*E. laevopinea*	4	HKFF	*Eucalyptus*	*Eucalyptus*	*Renantheria*
HK014	*E. nitens*	2	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK015	*E. dunnii*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK016	*E. camphora*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK017	*E. mannifera*	3	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK018	*E. nova-anglica*	4	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK019	*E. cinerea*	5	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
HK020	*E. smithii*	6	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
YPL021	*E. elata*	1	YPLFF	*Eucalyptus*	*Eucalyptus*	*Renantheria*
YPL022	*E. robusta*	2	YPLFF	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
HM023	*E. saligna*	2	HMFF	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
HM024	*E. cloeziana*	1	HMFF	*Eucalyptus*	*Idiogenes*	-*
HK025	*E. benthamii*	6	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
SX026	*E. exserta*	2	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Exsertaria*
SX027	*E. wetarensis*	6	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
SX028	*E. pellita*	6	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
SX029	*C. torelliana*	6	SCEN	*Corymbia*	*Blakella*	*Torellianae*
SX030	*C. ptychocarpa*	6	SCEN	*Corymbia*	*Corymbia*	*Corymbia*
SX031	*E. tereticornis*	6	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Exsertaria*
SX032	*E. urophylla*	6	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
SX033	*C. citriodora*	4	SCEN	*Corymbia*	*Blakella*	*Maculatae*
SX034	*E. camaldulensis*	6	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Exsertaria*
SX035	*E. grandis*	6	SCEN	*Eucalyptus*	*Symphyomyrtus*	*Transversaria*
HK036	*E. dorrigoensis*	6	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
SX037	*C. variegata*	6	SCEN	*Corymbia*	*Blakella*	*Maculatae*
HK038	*E. pauciflora*	1	HKFF	*Eucalyptus*	*Eucalyptus*	*Renantheria*
HK039	*E. cephalocarpa*	5	HKFF	*Eucalyptus*	*Symphyomyrtus*	*Maidenaria*
YPL040	*E. oblique*	1	YPLFF	*Eucalyptus*	*Eucalyptus*	*Renantheria*

HKFF = Haikou Forest Farm, Kunming, Yunnan, HMFF = Huangmian Forest Farm, Liuzhou, Guangxi, SCEN = South China Experiment Nursery, Zhanjiang, Guangdong, YPLFF = Yipinglang Forest Farm, Chuxiong, Yunnan; Classification is based on Hill and Johnson (1995), Brooker (2000) and Nicolle (2015); *subgenus *Idiogenes* is monotypic, with *E. cloeziana* being the sole species within it

Table 2 Amplification results of 20 SSR primer pairs

Primer code	Number of amplifications	SSR motif[1]	Length of sequences/ bp[2]	Primer code	Number of amplifications	SSR motif	Length of sequences/ bp
eSSR-GR001	126	(AGAAAA)3	244	gSSR-GR109	127	(AG)5–22	217–321
eSSR-GR024	125	(ACCAGC)3–7	260–296	gSSR-GR119	155	(CT)7–22	163–207
eSSR-GR026	154	(GCGGTG)3–6	360–413	gSSR-GR138	119	(CGG)3–10/ (GA)4–12	198–228
eSSR-GR029	154	(CT)4	264–272	gSSR-GR139	115	(TCC)6–8	293–312
eSSR-GR071	155	(GA)3–24	281–330	gSSR-GR150	138	(CTC)3–6	264–277
eSSR-GR076	150	(CT)5–16	250–279	gSSR-GR153	149	(TCT)3–9	260–269
eSSR-GR097	141	(TC)9–12	341–356	gSSR-GR162	127	(CGCCCC)3–4	170–196
eSSR-GR102	134	(TCC)3–12	226–330	gSSR-GU002	155	(ACCAGC)3–7	170–215
gSSR-CA012	126	(CT)9–22	451–488	gSSR-GU031	128	(GCC)3–8	276–297
gSSR-GR053	155	(TC)6–13	305–332	gSSR-UR009	136	(CT)6–23	207–259
Total	2769		6102				

[1]Capital letters in brackets represent SSR motifs, numbers after the bracket represent SSR repeat numbers, with ranges in numbers indicated thus: 3–7, [2]ranges in lengths of sequences are indicated thus: 3–7

20 SSR loci, with 20 of these species belonging to subgenus *Symphyomyrtus* and the other two species, *E. fraxinoides* and *E. obliqua*, belonging to subgenus *Eucalyptus*. Meanwhile, the four *Corymbia* species included in this study, *C. torelliana*, *C. ptychocarpa*, *C. citriodora* and *C. variegata*, had ampliconic sequences in only 10 SSR loci and the amplification results showed that these 10 loci efficiently transferred to subgenus *Symphyomyrtus*.

Polymorphism of microsatellite sequences

In total 2769 sequences were detected from polymorphisms of the 20 SSR microsatellite loci across the 155 *Eucalyptus* genotypes in this study (Table 3). The variation of microsatellite motifs (SSR variation) and the repeat number (amplicon size variation) showed some associations with genetic relationships among species. For instance, for the ampliconic sequences in eSSR-GR026, six species belonging to subgenus *Eucalyptus*, namely, *E. fraxinoides*, *E. triflora*, *E. laevopinea*, *E. elata*, *E. pauciflora* and *E. obliqua*, had the same motif (GGC) with SSR repeat numbers ranging from 5 to 9. Four *Corymbia* species, *C. citriodora*, *C. ptychocarpa*, *C. torelliana* and *C. variegata*, had the same motif (GCG) with SSR repeat numbers ranging from 3 to 4. For ampliconic sequences in eSSR-GR076, 32 species, including the four *Corymbia* species and *Eucalyptus* subgenus *Symphyomyrtus* species, had the same SSR motif (CT), and their repeat numbers ranged from 5 to 16, whilst the motif of the six *Eucalyptus* subgenus *Eucalyptus* species along with *E. cloeziana* was TC with repeat numbers ranging from 9 to 10. For the ampliconic sequences in gSSR-GR139, four species (*E. fraxinoides*, *E. triflora*, *E. elata* and *E. obliqua*) had the same motif (CTC) with repeat numbers ranging from 7 to 9. Meanwhile, 26 species of subgenus *Symphyomyrtus* had the same motif (TCC), with repeat numbers ranging from 6 to 8. The genomic SSR GU002 did not amplify in any of the *Corymbia* species; however, it produced amplicons with three repeats of the TC motif in six species of *Eucalyptus*.

Some discrepancies were found in detecting microsatellites among individuals of some of the species examined in this study. For instance, for two ampliconic sequences of *E. benthamii* in gSSR-GU002, the SSR motifs were CCAGCA in one genotype and in the other five genotypes the sequences were ACCAGC. Differences within species of *Eucalyptus* were also evident in microsatellite ampliconic sequences. While 13 species—*E. viminalis*, *E. macarthurii*, *E. dalrympleana*, *E. dunnii*, *E. camphora*, *E. mannifera*, *E. cinerea*, *E. smithii*, *E. saligna*, *E. urophylla*, *C.*

Table 3 Summary statistics of 20 SSR loci

SSR loci	*Na*	*Ne*	*I*	*He*	PIC	*Pi*	*Hd*
eSSR-GR001	1	0.75	0.00	0.00	0.00	0.01	0.77
eSSR-GR024	19	3.33	1.09	0.50	0.88	0.02	0.73
eSSR-GR026	32	6.63	1.48	0.59	0.95	0.02	0.82
eSSR-GR029	2	1.06	0.10	0.05	0.05	0.01	0.64
eSSR-GR071	30	7.32	1.65	0.61	0.94	0.04	0.97
eSSR-GR076	25	5.52	1.52	0.61	0.91	0.01	0.89
eSSR-GR097	15	4.11	1.29	0.58	0.87	0.04	0.95
eSSR-GR102	25	5.21	1.46	0.61	0.93	0.01	0.62
gSSR-CA012	31	7.89	1.54	0.58	0.96	0.02	0.93
gSSR-GR053	17	3.70	1.35	0.60	0.82	0.02	0.90
gSSR-GR109	30	5.74	1.52	0.60	0.94	0.03	0.88
gSSR-GR119	32	8.67	1.81	0.66	0.95	0.05	0.96
gSSR-GR138	25	6.46	1.57	0.63	0.93	0.01	0.50
gSSR-GR139	7	1.33	0.58	0.33	0.42	0.01	0.83
gSSR-GR150	11	2.71	0.96	0.46	0.83	0.02	0.91
gSSR-GR153	4	1.62	0.65	0.40	0.45	0.03	0.90
gSSR-GR162	6	1.59	0.67	0.39	0.54	0.01	0.53
gSSR-GU002	18	3.58	1.13	0.52	0.85	0.08	0.86
gSSR-GU031	6	1.49	0.50	0.30	0.51	0.01	0.84
gSSR-UR009	24	3.54	1.21	0.51	0.85	0.02	0.82
Average	18	4.11	1.11	0.48	0.73	0.02	0.81
Standard deviation	10.6	2.39	0.51	0.18	0.29	0.02	0.14

Na = number of alleles, *Ne* = effective number of alleles, *I* = Shannon's information index *He* = expected heterozygosity, PIC = polymorphic information content, *Pi* = DNA polymorphism, *Hd* = haplotype diversity

citriodora, *E. grandis* and *E. dorrigoensis*—had the same SSR motifs among individual genotypes within each of these species, the repeat numbers varied between and within each of these species. Moreover, similar differences were found in other SSR loci between and within species.

Genetic diversity of SSR Loci

Results from analyses conducted with the software DnaSP 5.10.01 showed that the haplotype diversity values (*Hd*) of ampliconic sequences in the SSR loci were high and ranged from 0.50 (gSSR-GR138) to 0.97 (eSSR-GR071), with an average of 0.81 (± 0.14 standard deviation) (Table 3). DNA polymorphism (*Pi*) of SSR loci ranged from 0.01 (eSSR-GR029) to 0.08 (gSSR-GU002), with an average value of 0.02 (± 0.02). Analyses of the genetic polymorphism of 20 combined ampliconic sequences from DnaSP 5.10.01 provided an estimate of overall DNA polymorphism of 0.04 and overall *Hd* of 0.97. When compared with the averages estimated from twenty SSR loci among 40 eucalypt species (Table 3), the overall *Hd* and the *Pi* estimates were higher than the respective average values of *Hd* and *Pi*.

The SSR loci examined in this study had different contributions to the total genetic diversity observed among 155 genotypes (Table 3). Apart from monomorphic loci of eSSR-GR001, the PIC of the other SSR loci were all relatively high with an average of 0.73 (± 0.29), and the maximum value of 0.96 was obtained for

the locus gSSR-CA012. The average of Shannon's information index (*I*) was 1.11 (± 0.51) with the highest value being 1.81 for locus gSSR-GR119. The effective number of alleles (*Ne*) ranged from 0.75 (locus eSSR-GR001) to 8.67 (locus gSSR-GR119), with an average value of 4.11 (± 2.39). The highest value for expected heterozygosity (*He*) was 0.66 (locus gSSR-GR119), with an average of 0.48 (± 0.18). Based on maximum values of the seven indices examined, the loci eSSR-GR026, eSSR-GR071, gSSR-CA012, gSSR-GR119 and gSSR-GR138 proved to have the greatest contribution to overall genetic diversity.

Genetic relationships among *Eucalyptus* species

To assess genetic relationships between species, DNA data from 20 SSR primer pairs of the 155 genotypes was used to generate a maximum likelihood and maximum parsimony phylogenetic tree (Figure 1). The best model of the maximum likelihood phylogenetic tree was GTR + I + G based on the 20 SSR loci, and the gamma distribution shape parameter was 0.48. The value of the consistency index by maximum parsimony method was 0.37, and the retention index was 0.90. This phylogenetic analysis clearly grouped the 155 genotypes into two distinct groups. The first group comprised 133 genotypes representing 36 species of the genus *Eucalyptus* (Group I), and included three subgroups, *Symphyomyrtus*, *Idiogenes* and subgenus *Eucalyptus*. The genetic distances between species within this group showed close agreement with subgeneric classifications recognised by Brooker (2000) and Nicolle (2015). For example, *E. amplifolia* and *E. tereticornis* belonged to *Eucalyptus* subgenus *Symphyomyrtus* series *Exsertae* subseries *Tereticornosae* (A in the Figure 1). Similarly, *E. pellita*, *E. wetarensis* and *E. urophylla* of the subgenus *Symphyomyrtus* were all closely located in the phylogenetic tree and these species belonged to subgenus *Symphyomyrtus* section *Transversaria* series *Resiniferae* (B in the Figure 1). Within the subgroup *Symphyomyrtus*, *E. grandis* and *E. robusta* should have belonged to series *Transversaria*, but the results from this current study indicated that they had a closer genetic relationship with species of series *Maidenaria*. In addition, *E. exserta* and *E. camaldulensis* should have belonged to series *Exsertaria*, yet our results indicated they were closer genetically to species of *Maidenaria*.

The second group (Group II) on the phylogenetic tree included 22 genotypes of the four *Corymbia* species: *C. citriodora*, *C. ptychocarpa*, *C. torelliana* and *C. variegata*. Of these, *C. torelliana*, *C. citriodora* and *C. variegata* belonged to the subgenus *Blakella* of *Corymbia* and formed a clade, but *C. ptychocarpa* stood apart within Group II.

DISCUSSION

Microsatellite polymorphisms among SSR loci

SSR marker technology has proved to be a valuable tool in the characterisation and evaluation of genetic diversity within and among species (Ballesta et al. 2015, Contreras-Solo et al. 2016). In the present study, polymorphisms were analysed on SSR loci among and within a broad group of *Eucalyptus* and *Corymbia* species. As flanking regions of DNA sequences appearing in amplicons on either side of the SSR sequences tend to be highly conserved, many SSR loci are identical in species that have close genetic relationships (Wang et al. 2008). Indeed, results from our study showed some of the SSR loci were transferrable across species within and even across some subgenera of *Eucalyptus*, which concurs with Cupertino (2011) and Liu et al. (2018) who had previously found that microsatellite transferability across species of subgenus *Symphyomyrtus* could vary from 80 to 100%.

Mean PIC of genomic-SSR was higher (0.75) compared with the value recorded by EST-SSR markers (0.69). The relatively low level of polymorphism in EST-SSR markers may be due to the location of these markers in more conserved and expressed sequences compared with genomic sequences which are spread throughout the genome (Parthiban et al. 2018). Due to high discrimination power and high polymorphism, genomic SSR primers can be used effectively in constructing genetic linkage maps and parental identification. EST-SSR markers have been found to be moderately polymorphic when compared with genomic-SSRs because of DNA sequences being more conserved in transcribed regions (Filho et al. 2010, Parthiban et al. 2018). Liu et al. (2017) reported a higher PIC value of 0.49 with genomic-SSR primers compared with 0.47 generated by EST-SSR primers with six species of eucalypt. PIC values ranging from 0.06 to

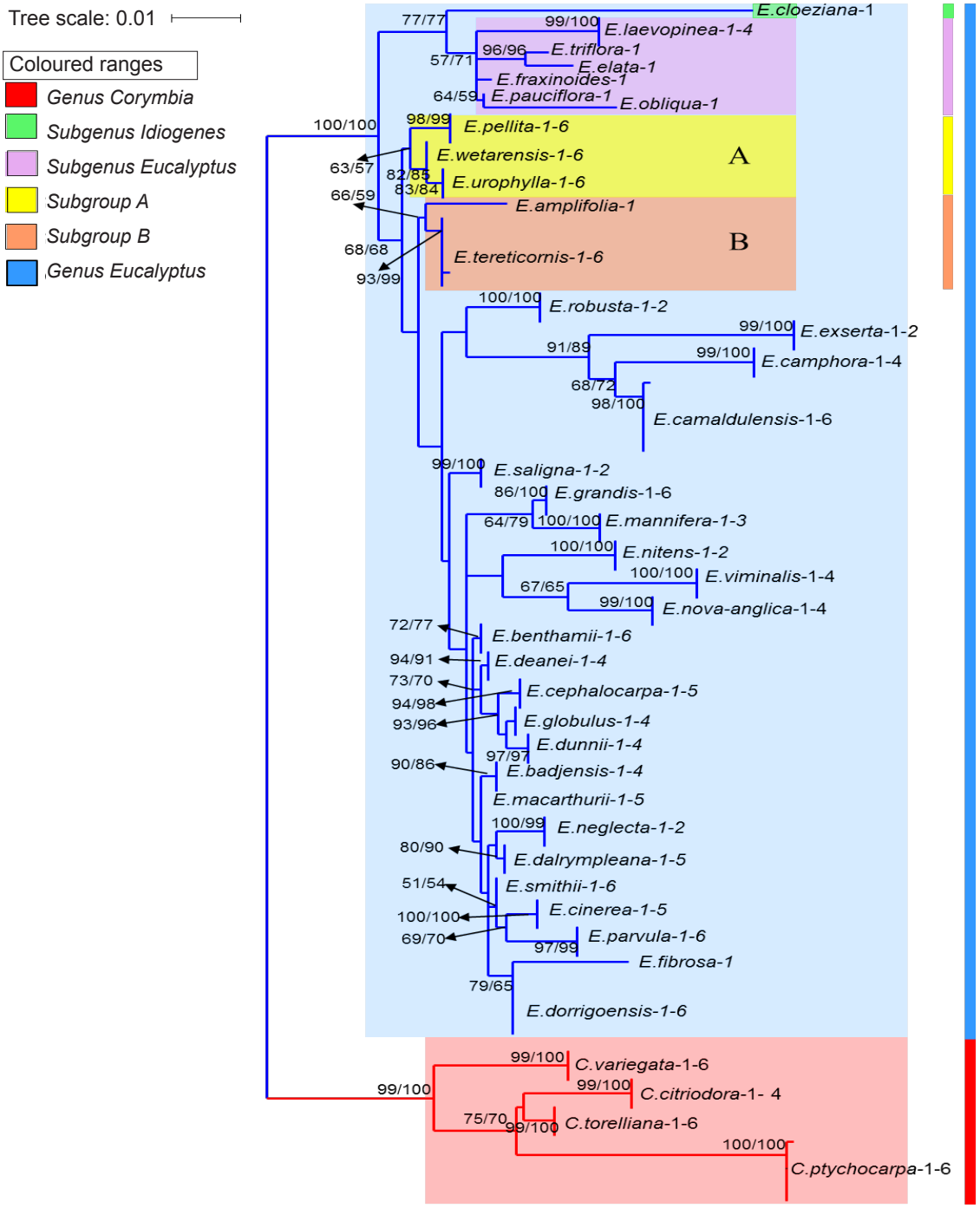

Figure 1 Maximum likelihood phylogenetic tree generated for 155 genotypes of *Eucalyptus* and *Corymbia* using ampliconic sequences of four SSRs; the numbers on branch points represent percentages obtained from heuristic searching of maximum parsimony/maximum likelihood methods

0.89 were obtained using 97 *Corymbia citriodora* individuals with 13 SSR primers (Liu et al. 2016). High variation in the PIC values ranging from 0.67 to 0.79 have also been reported from a group of 20 eucalypt genotypes by He et al. (2015).

Estimates obtained in this study on SSR loci indices relating to genetic diversity and relatedness are consistent with previous studies focused on either single eucalypt species or limited numbers of eucalypt species. For example, Payn et al. (2008) observed high level of genetic diversity throughout the geographic range (*He* = 0.703 to 0.776) of *E. urophylla* using 12 SSR loci. Ribeiro et al. (2011) obtained a *He* of 0.85 in *E. globulus* using 16 SSR markers. The average polymorphism information content reported

from eight microsatellite markers in candidate genes for *E. globulus* (0.718) (Acuna et al. 2012) was also similar to the value obtained in the current study (0.729).

Genetic relationships among species of *Eucalyptus*

According to the phylogenetic tree produced by this study, phylogenies of the 40 eucalypt species examined are in close agreement with the *Eucalyptus* taxonomic classifications of Brooker (2000) and Nicolle (2015). Both these authors conceptually based their taxonomies on the classification of Pryor and Johnson (1971) and Hill and Johnson (1995), which considered extensive observation of heritable phenotypic characters in seedling and adult plants.

The groupings and genetic distances between taxa indicated that the species examined aligned into two distinct groups and the relationships implied were somewhat similar to those found by Steane et al. (2007) and Bayly et al. (2013). Our results support the hypothesis of Bayly et al. (2013), who analysed chloroplast genomes of 39 eucalypt species (of *Eucalyptus*, *Corymbia* and *Angophora*) and found that *E. umbra* (subgenus *Eucalyptus*) and *E. cloeziana* were placed in close genetic proximity to each other (at node 16). The relatively close genetic proximity between *E. cloeziana* and species of *Eucalyptus* subgenus *Eucalyptus* in our phylogenetic tree has also been suggested by other previous studies. According to Wang (2010), the genetic distance separating the subgenera *Idiogenes* and *Eucalyptus* is the shortest of all genetic separations among *Eucalyptus* subgenera. In addition, the published taxonomic classifications by Nicolle (2015) and Brooker (2000) designate this subgenus as being monotypic.

Hill and Johnson (1995) created a new milestone in *Eucalyptus* taxonomy when they 'split' the genus and introduced a new genus, *Corymbia*, comprising ghost gums and bloodwoods. However, the other species of the genus *Eucalyptus* remained untreated at this level in a formal taxonomic sense. Brooker (2000) took a different view and maintained the eucalypts as single genus but divided this into seven polytypic subgenera (*Angophora, Corymbia, Blakella, Eudesmia, Symphyomyrtus, Minutifructa* and *Eucalyptus*) and six monotypic subgenera (*Acerosa, Cruciformes, Alveolata, Cuboidea, Idiogenes* and *Primitiva*). Brooker's (2000) classification was intentionally phylogenetic in that it proceeded from what was generally assumed to be more primitive subgenera (*Eucalyptus* subgenus *Angophora* and subgenus *Corymbia*) to the most advanced and modified group, the monocalypts (*Eucalyptus* subgenus *Eucalyptus*). However, the rank of subgeneric taxa suggested by analyses of SSR sequences in our study support the earlier taxonomy of Hill and Johnson (1995) and a recent one presented by Nicolle (2015) which placed *Corymbia* as a separate genus rather than just a subgenus.

The genetic grouping of the four *Corymbia* species represented in this study, with their separation into two subgenera, *Blakella* and *Corymbia*, is consistent with previous phylogenetic analyses reported by Steane et al. (2002) and Bayly et al. (2013). However, not all studies agree with *Corymbia* being considered paraphyletic. The studies of Parra-O et al. (2009) and Ochieng et al. (2007) provide evidence that the genus *Corymbia* is in fact monophyletic. Results presented from the current study also support a controversial classification of *Symphyomyrtus* species, with their separation into three sections: *Maidenaria*, *Exsertaria* and *Transversaria*. Pryor and Johnson (1971) considered the assignment of *Symphyomyrtus* species among these sections to be subjective and the study reported by Steane et al. (2002) has questioned the validity of designating *Transversaria* and *Exsertaria* as separate sections.

It is well known that two species of *Eucalyptus* belonging to the same subgenus, section and series can, in general, more easily be hybridised with each other than those from different sections/series and/or subgenera (Eldridge et al. 1993, Potts & Dungey 2004). Indeed, some of the more successful eucalypt hybrid combinations commonly used in commercial plantations around the world, including *E. urophylla* × *grandis*, *E. grandis* × *urophylla* and some hybrids involving *E. pellita* (Eldridge et al. 1993), involve combinations of species from within series *Transverae* within subgenus *Symphyomyrtus*.

Viability of interspecific eucalypt combinations tends to be lower for intersectional crosses, compared with intrasectional crosses and decreases with increasing genetic distance between parent species (Potts & Dungey 2004). Even so, localised genetic exchange has been found to occur among *Eucalyptus*

species belonging to different series based on observations of natural interspecific hybridisation among co-occurring species (Steane et al. 2002, McKinnon et al. 2008). Duncan (1989) recorded that all 17 Tasmanian species of section *Maidenaria* have at least one natural hybrid combination, and most of these species have several natural hybrid combinations, including combinations across series.

Analyses of genetic relationships and particularly genetic distance of separation among *Eucalyptus* species might provide means for predicting the potential compatibility of novel interspecific combinations for creating viable F_1 hybrids. Indeed, the phylogenetic map generated in this study suggested possible, new hybrid combinations such as *E. viminalis* × *nova-anglica*, *E. parvula* × *cinerea*, *E. mannifera* × *grandis* and *E. camphora* × *camaldulensis*. All these combinations were more closely related than the parent species of *E. urophylla* × *grandis*, and each of these pairs involved species which, as suggested by this current study, belonged to separate sections within subgenus *Symphyomyrtus*. However, just because a pair of species of *Eucalyptus* are separated by only a short genetic distance does not necessarily mean they will be biologically compatible for creating viable F_1 hybrids. Nor does it mean that, if they can in fact produce viable F_1 hybrid progeny, that such progeny will be of any commercial value (Potts & Dungey 2004). Once viable F_1 hybrid progeny are produced, they need to be subject to intense selection for important commercial traits, considering the fact that many viable interspecific eucalypt hybrids have failed to make it into commercial plantations.

CONCLUSIONS

Despite clear evidence of complex evolution and high levels of homoplasy within the genus *Eucalyptus* and of past interspecific genetic exchanges among many *Eucalyptus* species, the analyses carried out in this study were able to identify differentiated genetic groupings within the genus *Eucalyptus* and subgenus *Symphyomyrtus*. This study also found that variation of sequences among species of eucalypts were mainly due to differences in the numbers of SSR motif repetitions and base mutations on the associated flanking region sequences, whilst variations between individuals within species were mainly due to differences in the actual SSR motifs. These analyses will help provide greater understanding of genetic relationships among and within eucalypt species and could contribute to fundamental understanding for preservation and effective utilisation of eucalypt germplasm resources.

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Appendix 1 The characteristics of the 20 eucalypt SSR primers used in this study

Primer code	Forward (F) primer sequence/reverse (R) primer sequence	Target SSR	Target length/bp
eSSR-GR001	F: GGAAGTGCCCTCTGAAGT R: TCTTAGTTGTCCCATCCTG	(AGAAAA)3	245
eSSR-GR024	F: AATCGTGGCAGCAGAATG R: TGTTACGCCCGAACCTCT	(ACCAGC)5	279
eSSR-GR026	F: AGAGGCTGCTGAGAAAGA R: CACCAAGGGAAGGGAACT	(GTGGCG)4	230
eSSR-GR029	F: CAAGCAGACTATTCCGTGAG R: CATAAGCAACCAGCGATC	(CT)10	275
eSSR-GR071	F: GTATCTCCCACGCCTTAG R: ATTGGCTTGCCTTTCTTG	(GA)11	313
eSSR-GR076	F: ACTCGTTGTGAATGGTGGAA R: CCCGACGAAGGAATGAAG	(CT)12	264
eSSR-GR097	F: TACAGGGAGAAGAGGAAGAAC R: AGATCAGGCAACGGTCAG	(TC)10	344
eSSR-GR102	F: CGGCAACGGAGAAGAATAGGA R: GCCAGCGAGAAGGAAGGACA	(TCC)10	240
gSSR-CA012	F: CAATACTTCTGCCTCCAC R: ACATCCAGCATCCTTACA	(CT)10	452
gSSR-GR053	F: AAAGATGACCTCAGAAGGCACA R: TCAAGCACAACGGCAACA	(TC)10	307
gSSR-GR109	F: GGTCCTCTGTTGCTTTACT R: GCCTAGAAGGGTTATTGTT	(GA)14	230
gSSR-GR119	F: CTCCACTATGCCAAGAACG R: ATGAGGACAGTGCCAAGC	(CT)12	174
gSSR-GR138	F: CGATTGGCTGTATGACGC R: AGGAAGGTCCCTCGGTTT	(CGG)9(GA)8	217
gSSR-GR139	F: AGAGCGACCCAAGAGTGTTTCA R: GCGGCTTCTTCAGGCTTAGTG	(TCC)6	304
gSSR-GR150	F: GGACTCATCCACCTCTTT R: CCATCACCCTGGTCTACT	(CTC)6	274
gSSR-GR153	F: TGATGGGTGCTTTGACTG R: GGATGGCAATGTCTGAAT	(TCT)6	266
gSSR-GR162	F: CAGCAGTCCTTCTGGCAGTT R: TGTTGTCGTGGTGGTTGTAGTT	(CGCCCC)3	190
gSSR-GU002	F: CGTCCCTCAATACCCGAATG R: TGTTACGCCCGAACCTCT	(ACCAGC)5	188
gSSR-GU031	F: CTTTGCCGTACCTTGTCA R: TTCCCTGCTTGCGTTCAT	(GCC)7	293
gSSR-UR009	F: GGGGTTTGTGCTAGTGGA R: GCTCAGCGGTAGAATGGA	(CT)18	249

Capital letters in brackets represent SSR motifs, numbers after the brackets represent SSR repeat numbers