Multiplexed Microsatellite Markers for the Genetic Analysis of *Eucalyptus leucoxylon* (Myrtaceae) and Their Utility for Ecological and Breeding Studies in other *Eucalyptus* Species

K. M. Ottewell, S. C. Donnellan, G. F. Moran, and D. C. Paton

From the Department of Environmental Biology (Ottewell and Paton), Evolutionary Biology Unit and Centre for Evolutionary Biology and Biodiversity (Ottewell and Donnellan), University of Adelaide, North Terrace, Adelaide, SA, Australia 5000; CSIRO Forestry and Forestry Products, P.O. Box E4008, Kingston, ACT, Australia 2614 (Moran).

Address correspondence to D. C. Paton, Department of Environmental Biology, University of Adelaide.

*Eucalyptus leucoxylon* is a widespread woodland tree species found in southeastern Australia that has suffered from, and continues to be, threatened by the impacts of habitat clearance and degradation. Populations now consist predominantly of scattered individuals, and their conservation status is of increasing concern. We report the development and characterization of a set of eight highly polymorphic microsatellite loci for *E. leucoxylon*. The loci can be amplified in three PCR multiplexes and electrophoresed in a single lane, allowing rapid throughput of large numbers of samples. A total of 111 alleles were detected in 68 individuals with an average of 12.3 alleles per locus, a mean expected heterozygosity of 0.83, and a mean observed heterozygosity of 0.72. The combined probabilities of identity and probabilities of paternity exclusion allow an extremely precise level of individual identification, indicating that these microsatellite markers will be ideal for population genetic and parentage-type studies in *E. leucoxylon*. The markers also exhibited an average of 76% conservation within the subgenus *Symphyomyrtus*, to which *E. leucoxylon* belongs, and 53% conservation across other subgenera of *Eucalyptus*, demonstrating the potential of these markers in ecological and breeding studies in a wide range of *Eucalyptus* species.

Introduction

Trees of the genus *Eucalyptus*, which contains over 800 species (Brooker and Kleinig 2001), dominate vegetation associations throughout much of temperate Australia. Since European settlement, eucalypt woodlands have been disproportionately cleared because they occur on land most suitable for agriculture. *Eucalyptus leucoxylon*, a member of the subgenus *Symphyomyrtus* and commonly known as the South Australian blue gum or yellow gum, is an abundant woodland eucalypt with a distribution across much of southern Australia, from east of the Nullarbor Plain to southwestern Victoria. It is often a large woodland tree, 10–20 m in height, monoecious, and pollinated predominantly by birds (Ellis and Sedgley 1993). *E. leucoxylon* has historically suffered and continues to be threatened by extensive clearance for agriculture, viticulture, horticulture, and firewood and timber collection. The population now predominantly comprises scattered single trees, some at densities lower than 1 tree/ha, and to a lesser extent as fragments of intact vegetation of varying quality and size (Paton et al. 1999). Scattered trees, in comparison with trees in intact vegetation, are susceptible to increased mortality due to continued clearance for urban and agricultural development, dieback, land salinization and the impacts of stock and native fauna (Paton et al. 1999). Seedling recruitment is virtually nonexistent in such a modified system due to grazing pressure from introduced and native species, weed invasion, and altered soil regimes (Hobbs 1993; Paton et al. 1999; Yates and Hobbs 1997), causing concerns about the long-term viability and persistence of these trees in rural landscapes.

Scattered trees may be important in the landscape to act as corridors for the movement of pollinators and other animals (Fischer and Lindenmayer 2002; Law et al. 2000; Paton and Eldridge 1994). They provide a food source and shelter in a landscape otherwise devoid of suitable habitat. In this way, scattered trees may provide linkage between population fragments and aid in the transmission of genetic
material between them. Scattered trees may also help ameliorate the effects of inbreeding, genetic drift, and bottlenecking to which small, isolated populations are otherwise susceptible (Young et al. 1996).

A number of studies have now described the impact of habitat fragmentation on levels of pollen-mediated gene flow in tropical (e.g., Dick et al. 2003; Stacy et al. 1996) and temperate (e.g., Dow and Ashley 1998; Sork et al. 2002) tree species. Highly polymorphic microsatellite markers have proved ideal for these types of studies for a number of reasons. They are easily assayed by polymerase chain reaction (PCR) amplification using primers designed to match unique sequences flanking tandem repeat arrays, and high-throughput automation is relatively easily achieved. The generally high levels of variability observed at microsatellite loci allow precise identification of individuals, especially useful for detecting the paternal contribution in parentage and mating system studies in natural populations, as well as the estimation of fundamental genetic parameters for the genetic conservation and management of ecologically important species (Blouin 2003; Jamne and Lagoda 1996; Sunnucks 2000).

In recent years, microsatellite markers have been developed for a number of Eucalyptus species, including E. nitens (Byrne et al. 1996), E. grandis and E. urophylla (Brondani et al. 1998, 2002), and E. globulus (Moran, unpublished data). Although Byrne et al. (1996) and Brondani et al. (2002) reported successful transfer of microsatellite primers from their target species to closely related species within the subgenus Symphyomyrtus (in the order of 95–100%), the risk of mutations in primer, flanking, and microsatellite sequences generally increases with the evolutionary distance among taxa (Peakall et al. 1998). Because the species from which microsatellites have been isolated to date are in different sections of the subgenus Symphyomyrtus from E. leucoxylon, we anticipated a lower transfer rate than that reported by Byrne et al. (1996) and Brondani et al. (2002). Indeed, a trial of 14 E. globulus microsatellite primers in the more distantly related E. leucoxylon found only four microsatellite loci to be polymorphic (Ottewell, unpublished data), an insufficient number of loci for the detailed analysis of parentage and patterns of pollen-mediated gene flow in this species. Therefore, it was necessary to specifically isolate microsatellite loci for our Eucalyptus species. Furthermore, E. leucoxylon belongs to a section of Eucalyptus (Adnataria) that contains many ecologically important and threatened species for which microsatellite markers have not yet been developed. Thus molecular markers developed for E. leucoxylon have a higher potential than previously published markers to be useful for genetic studies in other species from this group of eucalypts, whose species are not necessarily of interest to the forestry industry. The present study reports the development and characterization of a set of eight highly variable microsatellite loci for E. leucoxylon. To facilitate the large-scale analysis of mating system parameters and parentage in scattered E. leucoxylon individuals, we optimized a multilocus genotyping system based on fluorescent detection of multiplexed loci. The eight loci were tested for Mendelian inheritance and linkage in an E. nitens test cross and located on an existing E. nitens linkage map (Byrne et al. 1995), to determine their usefulness for QTL analysis. Furthermore, we examined the conservation of these microsatellite markers in a range of ecologically and commercially important forest and woodland eucalypts of increasing evolutionary distance from E. leucoxylon.

The set of eight microsatellite markers that we optimized for rapid-throughput genotyping will allow investigation of the ecological impacts of deforestation on populations of E. leucoxylon and potentially a wide range of codistributed forest and woodland eucalypt species in southern Australia. They also provide additional markers for selective breeding programs and QTL analysis in commercially important species.

Materials and Methods

Plant Material and DNA Isolation

Total genomic DNA was extracted from leaves of a single E. leucoxylon individual (E15602, Flaxley, South Australia) using the protocol of Byrne et al. (1993) and subsequently used to develop a (CA)n and (CAG)n genomic-enriched library. (CA)n repeats have previously been isolated in Eucalyptus species and have been shown to be widely dispersed through the genome (Brondani et al. 1998). Though trinucleotide microsatellites are typically less abundant than dinucleotides in eukaryotic genomes (Lagercrantz et al. 1993; Toth et al. 2000), we chose to also isolate a trinucleotide repeat because they are more easily and reliably scored (Scotti et al. 2002).

Variation at microsatellite loci was surveyed in 68 individuals from 2 natural populations of E. leucoxylon, located at Flaxley and Ngarkat Conservation Park in South Australia. Genomic DNA of these individuals was extracted using the MasterPure Plant Leaf DNA Purification Kit per the manufacturer’s protocol. Genomic DNA from progeny and parents from a mapping pedigree of E. nitens were extracted using the methods of Byrne et al. (1993). Leaves from Eucalyptus species in which microsatellites were tested for conservation of loci were collected from trees in the Currency Creek Arboretum, Waite Arboretum, or the Adelaide Botanic Gardens, South Australia. Species were selected on the basis of the classification of Brooker and Kleinig (2001) and their phylogenetic relationships (Steane et al. 2002). Leaves were collected from two individuals of each species and genomic DNA extracted using the MasterPure Plant Leaf DNA Purification Kit.

Microsatellite Marker Development

Microsatellite enriched libraries were prepared with modifications of the method of Gardner et al. (1999). Sau3A-cut DNA was ligated to adaptors (Refseth et al. 1997) and size-fractionated on 2.5% NuSieve gel (FMC Bioproducts). Fragments in the size range 300–1000 bp were then probed
using biotinylated (CA)$_{11}$ and (CAG)$_{8}$ oligonucleotide probes. Streptavidin-coated magnetic beads (Magnesphere Paramagnetic Particles, Promega) were used to capture microsatellite-containing fragments during the enrichment process. Eluted fragments were then PCR-amplified using a long-range protocol. PCR products were amplified in a volume of 50 µl (1× Gibco BRL eLONgase Buffer A, 1× Buffer B [gives a final Mg concentration of 1.5 mM], 0.2 mM of each dNTP, 0.2 µM primer, 1 U Gibco BRL eLONgase polymerase) with the following cycling conditions: 92°C for 1 min (1 cycle) and 92°C for 30 s, 60°C for 1 min, 72°C for 15 min (30 cycles).

Cloning of microsatellite-enriched PCR products was achieved using the plasmid pGEM 5Zf(+) cut with EcoR V (pGEM-T Vector System, Promega) following the manufacturer’s instructions. Plasmid and insert were transformed into competent JM109 cells (Promega). Recombinant clones were detected using blue/white screening (Sambrook et al. 1989) and screened for the presence of microsatellites using the (CA)$_{11}$ and (CAG)$_{8}$ biotinylated probes and DIG-labeling following the manufacturer’s instructions (Boehringer-Mannheim). Hybridization of probes to target DNA was performed at 55°C for (CA)$_{11}$ and 65°C for (CAG)$_{8}$.

Positive clones identified during the screening process were sequenced using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems) and analyzed on an ABI-Prism 377 automated sequencer. Sequences were aligned by eye in SeAl (version 1.0) to check for duplicate clones.

Multiplexed Fluorescence-Based Analysis

Primer pairs were designed, using the program Oligo (version 4.0), from the flanking sequence of (CA)$_{11}$ and (CAG)$_{8}$ microsatellites to meet the following conditions: $T_m$ of 58–60°C; GC content 40–60%; no CC/GG ends, no significant hairpins or duplexes. To allow screening of microsatellite primers for polymorphism without directly fluorescingly labeling each primer pair, the protocol of Schuelke (2000) was used. One primer of the microsatellite primer pair is designed with a universal M13 tail attached, and a third primer is the reverse complement of M13 with a fluorescent dye label attached at the 5’ end (e.g., FAM, TET, HEX). The product was amplified by a nested PCR protocol in 20 µl containing: 1× Promega Taq Gold PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl), 2–4 mM MgCl$_2$, 0.2 µM fluorescently labeled M13 primer, 0.2 µM Primer 1, and 0.05 µM Primer 2 (with M13 tail), 0.2 mM of each dNTP and 0.02 U Promega Taq Gold. A touchdown PCR program was used with a cycling profile of 95°C for 9 min, 65°C for 45 s and 72°C for 1 min (1 cycle); 94°C for 45 s, 58°C for 45 s, and 75°C for 1 min (1 cycle); 94°C for 45 s, 55°C for 45 s, and 75°C for 1 min (8 cycles); 94°C for 45 s, 52°C for 45 s, and 75°C for 1 min (32 cycles); and a final extension step at 72°C for 12 min. Fluorescently tagged products were then separated on a 5% denaturing acrylamide gel (Long Ranger, 6 M Urea/1× TBE) run on an ABI-Prism 377 automated sequencer using the GeneScan application. The output was analyzed using Genotyper software (ABI). Products were sized using the TAMRA labeled (ABI) size standard.

Fluorescently tagged microsatellite markers were initially screened on a panel of seven E. leucoxylon individuals sampled from across the broad study region to ensure microsatellites were polymorphic across the region of interest, not just in the focal populations. The microsatellite markers that showed clearly interpretable polymorphisms and that amplified products of suitable sizes for lane multiplexing were then chosen for direct fluorescent dye labeling. One primer of each pair was labelled at the 5’ end with one of the following dyes: 6-FAM, TET, or HEX. Loci for direct labeling were chosen on the basis that their allele size ranges allowed for eight loci to be multiplexed and electrophoresed in a single lane. Microsatellite loci were PCR-multiplexed using a step-down program 94°C for 9 min (1 cycle); 94°C for 30 s, 65–55°C (step down 2°C for each cycle) for 30 s, 72°C for 45 s (10 cycles); 94°C for 30 s, 55°C for 30 s, 72°C for 45 s (20 cycles); final extension 72°C for 12 min. PCR multiplex 1 contained E128, E116, and E123/ E129; multiplex 2 contained E107, E114, and E118; and multiplex 3 contained E801 and E113 with amplification under the following conditions: 1× Promega Taq Gold PCR buffer, 2 mM MgCl$_2$, 0.14–0.2 µM of each primer, 0.2 mM each dNTP and 0.03 U Promega Taq Gold in a total volume of 15 µl.

Genetic Analyses

Microsatellites were tested for Mendelian inheritance and pairwise linkage in a mapping pedigree of E. nitens using Linkem (Vowden and Ridout 1994). Microsatellite loci were positioned on the E. nitens map (Byrne et al. 1995) using the Outmap program (Butcher et al. 2002). Microsatellite locus allele frequencies and descriptive locus statistics (A, H$_o$, H$_e$, F$_S$) in the two natural populations of E. leucoxylon surveyed were calculated in Genepop (Raymond and Rousset 1995) available online at www.biomed.curtin.edu.au/genepop/index.html. Genetic information content was estimated by the single-locus and multilocus probability of genetic identity (P$_ID$) (Paetkau et al. 1995) and the paternity exclusion probability (E) (Weir 1996), implemented in the program Identity (Wagner and Sefc 1999). Identity was also used to detect identical genotypes in the two study populations.

Cross-Species Amplification of Loci

Cross-species amplification of loci was attempted in a range of Eucalyptus species of increasing evolutionary distance from E. leucoxylon. Representative species from five of the most speciose sections of Symphyomyrtus, from four of the five subgenera of Eucalyptus, and from the two Eucalyptus-like genera, Angophora and Corymbia, were chosen for testing (note that the status of Angophora and Corymbia is still unclear, but we choose to follow the relationships of Eucalyptus demonstrated in Steane et al. 2002). Two individuals from
each species were tested using the stepdown PCR program described previously but with an incremental step down from 65°C to 50°C. We confirmed the presence of a locus (as defined by the presence of closely colocated primer binding sequences to allow standard PCR amplification) in nontarget species by visualizing PCR products on a 1.5% agarose gel. A PCR product of approximately the same size as detected in E. leucoxylon was considered to be consistent with the presence of a microsatellite at that locus in the nontarget taxon but would need to be confirmed by sequencing or screening for length polymorphism.

We also examined the relationship between evolutionary distance and the degree of microsatellite primer conservation among the taxa compared. Because of the uncertainty surrounding evolutionary relationships amongst eucalypts and that “taxonomic distance” is not a good surrogate for evolutionary distance, we used sequences of the nuclear rDNA cistron comprising the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA gene to construct a genetic distance matrix to determine the evolutionary distances among eucalypt species. The majority of sequences were obtained from Steane et al. (2002), available through GenBank (www.ncbi.nlm.nih.gov). Species for which rDNA sequence data were not already available (E. leucoxylon and E. petiolaris) were sequenced using the ITS4 and ITS5 primers of White et al. (1990), using sequencing conditions already described. A neighbor-joining tree constructed from pairwise Kimura two-parameter distances was used to visually display evolutionary distances among the taxa.

Results and Discussion
Microsatellite Marker Development

Of the 592 microsatellite clones from the (CA)n enrichment screened, 58 (10%) strong positives were identified. Of these, 46 clones were sequenced. Six clones were CA-enriched but contained no microsatellite, and five clones were duplicates of two other clones; the rest of the microsatellite sequences were unique. In addition, 519 microsatellite clones from the (CAG)n enrichment were screened and 52 (10%) strong positives identified, of which 23 were sequenced. Four clones were false positives, and no duplicate sequences were found. Primer pairs for 20 (CA)n microsatellites and 10 primer pairs from (CAG)n microsatellites were designed from the sequences flanking the microsatellite regions. All unique sequences are deposited with GenBank (Table 1).

Of the 30 primer pairs designed, 11 were discontinued (36%) because they showed nonspecific amplification of secondary bands or they were monomorphic when screened across a panel of seven E. leucoxylon individuals. Of the remaining 19 primer pairs, 8, E105, E107, E113, E114, E116, E118, E123, and E128, were selected for further use, based on their level of polymorphism, repeatability, lack of stutter, and appropriate size range for lane multiplexing (Tables 1 and 2). After testing the eight-locus multiplex across two populations of E. leucoxylon individuals, it was found that E123 produced spurious banding patterns that were likely due to coamplification of a second locus. E123 was subsequently replaced by E129 without necessitating the rearrangement of the remaining seven loci.

Mendelian Inheritance and Genetic Diversity of Microsatellite Loci

Of the 19 microsatellite primer pairs available, a total of 13 were assayed in parents of cross E. nitens (Table 1). First, the eight-locus multiplex was screened across the E. nitens pedigree. Although E07 and E129 failed to amplify, E01 and E116 proved monomorphic in the full sib cross and E13, E14, E18, E23, and E28 were polymorphic. Another four loci, E305, E306, E317, and E327, not used in the E. leucoxylon population study, were also screened and found to be polymorphic. A total of 9 loci were used to provide segregation and inheritance data from 280 E. nitens progeny. All loci tested exhibited Mendelian inheritance of alleles (Table 1).

Five of the nine microsatellite loci described in Table 2 are perfect repeats; the remaining four are compound repeats. Compound repeats were significantly more polymorphic (average 15.5 alleles/locus) than the perfect repeats (9.8 alleles/locus) (t = 3.0095, df 7, P = .02). Across the 9 microsatellite loci screened in the 68 individuals of E. leucoxylon, a total of 111 alleles were identified, with an average of 12.3 alleles per locus (range 8–20) (Table 2).

A number of loci exhibited very positive FIS values and an excess of homozygotes in the two E. leucoxylon populations (Table 2, E007, E116, E123, E129). Although this may reflect high levels of inbreeding in the populations, it may also be an artifact caused by the presence of null alleles at these loci. However, as mentioned previously, E123 was discontinued due to spurious banding patterns and the positive FIS for E129 is potentially due to small sample size. In preliminary mating system studies of E. leucoxylon, null alleles were not
detected for any of the loci across a small number of open-pollinated families. In addition, the loci tested in the E. nitens pedigree exhibited Mendelian inheritance of alleles.

For the final eight-locus multiplex (i.e., excluding El23 and including El29), the mean expected heterozygosity was 0.83 and the mean observed heterozygosity was 0.72 (Table 2). The paternity exclusion probability (E) was high for most loci, with the exception of El29, perhaps due to a small sample size. The combined probability of paternity exclusion using all eight loci was 0.9990, indicating a 99.90% chance of

**Table 1.** Microsatellite markers developed for *Eucalyptus leucoxylon*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat motif</th>
<th>Primer sequence (5’→3’)</th>
<th>Product length (bp)</th>
<th>Observed segregation ratio</th>
<th>Segregation probability</th>
<th>Linkage group</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>El01</td>
<td>(GT)10</td>
<td>(F) FAM-CACCTAGTTGGCTTCAGAC</td>
<td>362</td>
<td>M</td>
<td>—</td>
<td>—</td>
<td>AY390559</td>
</tr>
<tr>
<td>El05</td>
<td>(TA)2(CA)8(TA)2</td>
<td>(F) TGTGATGCTCATCTTTATTG</td>
<td>312</td>
<td>127:153</td>
<td>1</td>
<td>2</td>
<td>AY390563</td>
</tr>
<tr>
<td>El06</td>
<td>(AC)12</td>
<td>(F) TCTGGCAAGTTTTATGTACTC</td>
<td>134</td>
<td>130:140</td>
<td>1</td>
<td>1</td>
<td>AY390564</td>
</tr>
<tr>
<td>El07</td>
<td>(GT)13</td>
<td>(F) TAC-TGAGATGTCAGGCCAAC</td>
<td>121</td>
<td>DNT</td>
<td>—</td>
<td>—</td>
<td>AY390565</td>
</tr>
<tr>
<td>El13</td>
<td>(TC)17(AC)10</td>
<td>(F) HEX-CAGAGTGCACGCAAGCC</td>
<td>190</td>
<td>57.78:79.66:0.19</td>
<td>12</td>
<td>El29</td>
<td>AY390571</td>
</tr>
<tr>
<td>El14</td>
<td>(GT)13(AG)15</td>
<td>(F) ACCTTAGAAGGCTGAGCATC</td>
<td>184</td>
<td>78.66:57.77:0.23</td>
<td>12</td>
<td>El29</td>
<td>AY390572</td>
</tr>
<tr>
<td>El16</td>
<td>(AT)6(GT)20</td>
<td>(F) HEX-GATTATACCTACTTTGTGC</td>
<td>246</td>
<td>M</td>
<td>—</td>
<td>—</td>
<td>AY390574</td>
</tr>
<tr>
<td>El17</td>
<td>(GT)16(GA)9</td>
<td>(F) CIAAATAATCTGGCAGCGAACAG</td>
<td>180</td>
<td>68.67:77.65:0.75</td>
<td>5</td>
<td>El29</td>
<td>AY390575</td>
</tr>
<tr>
<td>El18</td>
<td>(TC)16(AC)9(CA)2</td>
<td>(F) ACCCCACACCTTGTTTC</td>
<td>288</td>
<td>153:127</td>
<td>0.12</td>
<td>6</td>
<td>AY390576</td>
</tr>
<tr>
<td>El23</td>
<td>(GCT)7</td>
<td>(F) TET-CAGATGCATGAGGCAAG</td>
<td>281</td>
<td>57.78:72.63:0.33</td>
<td>4</td>
<td>El29</td>
<td>AY390580</td>
</tr>
<tr>
<td>El27</td>
<td>(TGG)3(AGC)12</td>
<td>(F) GCTTTTCAGAGGGCGATTTC</td>
<td>307</td>
<td>127:153</td>
<td>0.04</td>
<td>El29</td>
<td>AY390584</td>
</tr>
<tr>
<td>El28</td>
<td>(CAG)8</td>
<td>(F) TCTGAGGAGGGGCTGACGAC</td>
<td>215</td>
<td>139:137:0.90</td>
<td>11</td>
<td>El29</td>
<td>AY390585</td>
</tr>
<tr>
<td>El29</td>
<td>(GA)14</td>
<td>(F) FAM-CCTTTCATGCTTCCACATC</td>
<td>268</td>
<td>M</td>
<td>—</td>
<td>—</td>
<td>AY390586</td>
</tr>
</tbody>
</table>

a Size of PCR product in original target individual EL5602.

b Segregation of genotypes and probabilities from $\chi^2$ tests for Mendelian segregation ratios in an *E. nitens* full-sib family with 280 progeny, M = monomorphic, DNT = did not transfer to *E. nitens*.

c Linkage groups after Byrne et al. (1995) and Thamarus et al. (2002).

d Additional microsatellite sequences are available on GenBank (AY390560–62, AY390566–570, AY390573, AY390577–579, AY390581–583, AY390587). Primer details for these loci are available from the senior author.

detected for any of the loci across a small number of open-pollinated families. In addition, the loci tested in the *E. nitens* pedigree exhibited Mendelian inheritance of alleles.

For the final eight-locus multiplex (i.e., excluding El23 and including El29), the mean expected heterozygosity was 0.83 and the mean observed heterozygosity was 0.72 (Table 2). The paternity exclusion probability (E) was high for most loci, with the exception of El29, perhaps due to a small sample size. The combined probability of paternity exclusion using all eight loci was 0.9990, indicating a 99.90% chance of

**Table 2.** Genetic diversity parameters at microsatellite loci of *E. leucoxylon*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele size range (bp)</th>
<th>No. of alleles (n)</th>
<th>$H_s$</th>
<th>$H_o$</th>
<th>$F_{IS}$</th>
<th>E</th>
<th>$P_{ID}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>El01</td>
<td>350–406</td>
<td>9 (64)</td>
<td>0.74</td>
<td>0.64</td>
<td>+0.132</td>
<td>0.570</td>
<td>0.140</td>
</tr>
<tr>
<td>El07</td>
<td>107–165</td>
<td>14 (37)</td>
<td>0.89</td>
<td>0.70</td>
<td>+0.213</td>
<td>0.582</td>
<td>0.111</td>
</tr>
<tr>
<td>El13</td>
<td>172–204</td>
<td>13 (65)</td>
<td>0.81</td>
<td>0.71</td>
<td>+0.122</td>
<td>0.649</td>
<td>0.084</td>
</tr>
<tr>
<td>El14</td>
<td>166–208</td>
<td>16 (64)</td>
<td>0.92</td>
<td>0.86</td>
<td>+0.067</td>
<td>0.839</td>
<td>0.023</td>
</tr>
<tr>
<td>El16</td>
<td>222–248</td>
<td>13 (63)</td>
<td>0.86</td>
<td>0.68</td>
<td>+0.207</td>
<td>0.742</td>
<td>0.055</td>
</tr>
<tr>
<td>El18</td>
<td>279–319</td>
<td>20 (68)</td>
<td>0.90</td>
<td>0.93</td>
<td>−0.024</td>
<td>0.804</td>
<td>0.032</td>
</tr>
<tr>
<td>El23</td>
<td>270–317</td>
<td>9 (43)</td>
<td>0.85</td>
<td>0.47</td>
<td>+0.452</td>
<td>0.621</td>
<td>0.106</td>
</tr>
<tr>
<td>El28</td>
<td>191–236</td>
<td>9 (65)</td>
<td>0.70</td>
<td>0.71</td>
<td>−0.011</td>
<td>0.518</td>
<td>0.176</td>
</tr>
<tr>
<td>El29</td>
<td>158–282</td>
<td>8 (16)</td>
<td>0.77</td>
<td>0.56</td>
<td>+0.276</td>
<td>0.235</td>
<td>0.413</td>
</tr>
<tr>
<td>Mean (All loci)</td>
<td>12.3</td>
<td>0.83</td>
<td>0.70</td>
<td>0.99996 (total)</td>
<td>6.77 × 10^{-11} (total)</td>
<td></td>
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</tr>
<tr>
<td>Mean (excluding El23)</td>
<td>12.75</td>
<td>0.823</td>
<td>0.723</td>
<td>0.99990</td>
<td>6.37 × 10^{-10}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Locus name, allele size range (bp), number of alleles detected per locus (number of individuals genotyped), expected heterozygosity ($H_s$), observed heterozygosity ($H_o$), inbreeding coefficient ($F_{IS}$), probability of paternity exclusion (E), and probability of genetic identity ($P_{ID}$).
correctly excluding a random nonparent individual tree in the population. The single-locus probability of genetic identity (P(ID)) varied from 0.023 to 0.413 (PID), allowing a very high level of individual identification. All multilocus genotypes identified in the two study populations were unique. Thus the eight-locus multiplex provides a very high level of discrimination, which makes these markers ideal for parentage-type studies in Eucalyptus. They provide sufficient genetic information for the estimation of patterns of pollen-mediated gene flow, including determination of the average number of mating partners.

Cross-Species Amplification

The evolutionary distances among Eucalyptus taxa in which E. leucoxylon microsatellites were tested and the number of loci conserved in each species. The neighbor-join tree was constructed using ITS and 5.8S rRNA sequences available from GenBank: Arillastrum gummiferum (outgroup) AF058454; Corymbia calophylla AF390460; Angophora costata AF058455; E. eudesmoides AF390468; E. baileyana AF390467; E. dojiana AF058462; E. sieberi AF058495; E. obliqua AF058484; E. marginata AF390530; E. melliodora AF390514; E. petidiaris AY388998; E. leucoxylon AF388997; E. grandis AF058475; E. camaldulensis AF058473; E. salmonophloia AF390509; E. nitens AF058472; E. globulus AF058463.

Figure 1. Evolutionary relationships of Eucalyptus taxa in which E. leucoxylon microsatellites were tested and the number of loci conserved in each species. The neighbor-joining tree was constructed using ITS and 5.8S rRNA sequences available from GenBank: Arillastrum gummiferum (outgroup) AF058454; Corymbia calophylla AF390460; Angophora costata AF058455; E. eudesmoides AF390468; E. baileyana AF390467; E. dojiana AF058462; E. sieberi AF058495; E. obliqua AF058484; E. marginata AF390530; E. melliodora AF390514; E. petidiaris AY388998; E. leucoxylon AF388997; E. grandis AF058475; E. camaldulensis AF058473; E. salmonophloia AF390509; E. nitens AF058472; E. globulus AF058463.

myrtus (Figure 1). All E. leucoxylon primer pairs amplified a product of expected size (100%) in species within the section Adnataria. Across sections within the subgenus Symphyomyrtus, an average of 76% of loci were conserved. Across the Eucalyptus subgenera tested, 53% of loci were conserved, but only 22% of loci were conserved in the more distantly related genera Angophora and Corymbia.

The majority of woodland eucalypt species in southern Australia that are under threat from habitat fragmentation and ongoing deforestation are classified in the subgenus Symphyomyrtus. The transferability of loci within this subgenus means that E. leucoxylon microsatellites may be used relatively easily in studies involving a range of key Eucalyptus species in threatened woodlands in southern Australia. Moreover, our multiplexing of the amplification and genotyping of these loci provides a highly efficient system for analyzing numerous individuals with minimal time and cost.

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