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Isolation and characterization of 52 polymorphic EST-SSR markers for *Callitris columellaris* (Cupressaceae)[1]

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- *Premise of the study:* We developed simple sequence repeat (SSR) markers from expressed sequence tags (ESTs) for *Callitris columellaris* sensu lato (s.l.) to elucidate population genetic structure and detect outlier loci by genome scan.
- *Methods and Results:* mRNA from an individual seedling was subjected to cDNA synthesis and then de novo pyrosequencing. Two hundred and nineteen primer pairs bordering sequence regions were designed from the obtained sequence data. In total, 52 showed polymorphism within 16 individuals representative of the species' entire range, with the number of alleles per locus and expected heterozygosity ranging from two to 10 and 0.06 to 0.84, respectively.
- *Conclusions:* The EST-SSR markers developed in this study will be useful for evaluating the range-wide genetic structure of *C. columellaris* s.l. and detecting outlier loci under selection, as well as providing useful markers to investigate the conservation genetics and reproductive ecology of the species.

Key words: *Callitris columellaris*; Cupressaceae; expressed sequence tag; genetic structure; microsatellite; pyrosequencing.

Callitris columellaris F. Muell. sensu lato (s.l.) (Cupressaceae) is the most widespread conifer species in Australia (Farjon, 2005). This species is sometimes divided into three species (i.e., *C. columellaris* sensu stricto [s.s.], *C. glaucophylla* Joy Thomps. & L. A. S. Johnson, and *C. intratropica* R. T. Baker & H. G. Sm.) based on foliage color, cone size, tree habit, and geographic range (Bowman and Harris, 1995; Hill, 1998), although there is some continuity in such morphological traits between intraspecific taxa (Farjon, 2005). The distribution of *C. columellaris* s.l. (Fig. 1) extends from the arid interior of the continent to more mesic habitats near the eastern coast and to northern monsoonal areas with annual precipitation of more than 2000 mm (Prior et al., 2011). This wide ecological range, coupled with the large morphological variation across the species' range, would indicate that genetic adaptation could be an important factor allowing this species to occupy greatly differing environments in the continent. To understand the genetic basis of adaptation to local environments, it is necessary to investigate numerous nuclear genes with highly variable levels of divergence. Adaptive genes under divergent selection and genome regions tightly linked to them are expected to show stronger genetic divergence than neutral regions with weak or no linkage to such loci. Genome scan approaches basically detect adaptive loci as outliers by comparing the relative levels of differentiation among large numbers of unlinked genomic regions, thus requiring many markers (Storz, 2005; Tsumura et al., 2007). Expressed sequence tag–simple sequence repeat (EST-SSR) markers are useful in detecting outlier loci by genome scan because a large number of polymorphic markers can be developed with relative ease using EST data and they are less susceptible to null alleles than anonymous SSRs (Ellis and Burke, 2007). Neutral EST-SSR markers can also be useful for elucidating species' genetic structure, which can control for associations between adaptive genes and environments. In this study, we developed EST-SSR markers for *C. columellaris* s.l. from expressed sequence data with the aid of pyrosequencing techniques, and evaluated their polymorphisms and transferability among intraspecific taxa and to other species of Cupressaceae.

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METHODS AND RESULTS

Total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Le Provost et al., 2007) from a one-month-old seedling of *C. intratropica*, which was grown from a seed collected from a population in Pine Creek, Northern Territory, Australia (Fig. 1). A voucher specimen of a sibling individual is deposited at Kyoto University Herbarium

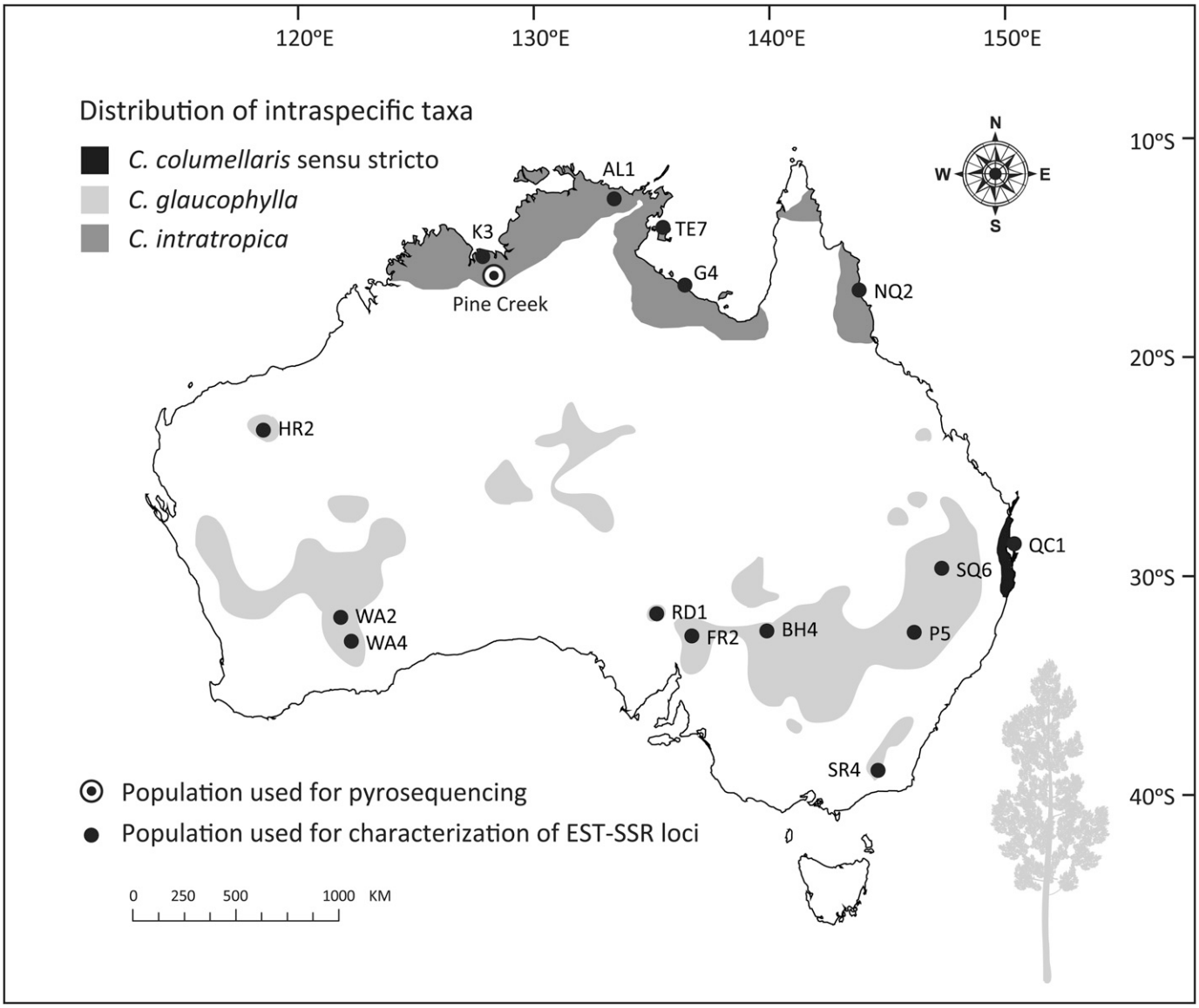

Fig. 1. Distribution of *Callitris columellaris* s.l. and location of a population used for pyrosequencing and populations for characterization of the EST-SSR loci. Biophysical information and latitude/longitude data of these populations are available in the supplementary data of Prior et al. (2011).

(accession number KYO20443). Purification of total RNA was conducted using the SV Total RNA Isolation System kit (Promega, Madison, Wisconsin, USA). Further cDNA synthesis and de novo pyrosequencing were performed by staff at the biotechnology company Hokkaido System Science (HSS Co., Ltd., Hokkaido, Japan), with the aid of a Genome Sequencer FLX (Roche Applied Science, Branford, Connecticut, USA). Pyrosequencing of this cDNA pool produced 386 753 reads with an average length of 355 bp. The Cross-Match program (http://bozeman.mbt.washington.edu/phrap.docs/phrap.html) and TIGR SeqClean sequence trimming pipeline (http://compbio.dfci.harvard.edu/tgi/software/) were used to remove poly A and adapter sequences from the cDNA sequences prior to further analyses. We used an EST sequence-assembler MIRA (Chevreux et al., 2004) to run de novo assemblies, resulting in 36 999 contigs (hereafter referred to as unigenes) in total. Using the resultant unigene library, a similarity search against the National Center for Biotechnology Information (NCBI) nr database was conducted by BLASTX (Altschul et al., 1990) algorithm with an *E*-value cutoff of 1E-5. PCR amplicon primers were designed using MISA (Thiel et al., 2003) and Primer3 (Rozen and Skaletsky, 2000) software, after trimming low quality regions using the qualityTrimmer command in Euler-SR package (Chaisson and Pevzner, 2008).

A total of 219 EST-SSR primer pairs bordering sequence regions with more than four di- to hexanucleotide repeats were designed. For each primer pair, genomic DNA from one individual was used to check PCR amplification. PCR reaction was carried out following the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany), in a final volume of 10 µL, which contained approximately 5 ng of DNA, 5 µL of 2× Multiplex PCR Master Mix, and 0.2 µM of each primer. The PCR thermal profile involved denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min; and a final 7-min extension step at 72°C. PCR products were labeled with Chromatide Alexa Fluor 488-5-dUTP (Invitrogen, Carlsbad, California, USA) according to Kondo et al. (2000), and then loaded onto an autosequencer (3100 Genetic Analyzer; Applied Biosystems, Carlsbad, California, USA) to assess fragment lengths using GENOTYPER software (Applied Biosystems). For the 78 primer pairs that exhibited clear microsatellite peaks at the expected fragment length (Table 1), extracted DNA of 16 individuals of *C. columellaris* s.l. representative of the species' range (Fig. 1) was used to evaluate EST-SSR polymorphism and transferability among the three intraspecific taxa using the same PCR conditions described above. In addition, these primers were applied to two other Cupressaceae conifers, *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. (subfamily Cupressoideae) and *Cryptomeria japonica* (Thunb. ex L. f.) D. Don (subfamily

TABLE 1. Characteristics of the 78 EST-SSR markers for *Callitris columellaris* s.l. Shown for each primer pair are forward and reverse primer sequence, repeat motif, putative functional annotation by the NCBI nr database search with *E*-value, and the GenBank accession number.

Locus	Primer sequence (5′–3′)	Repeat motif	BLASTX top hit description	*E*-value	GenBank Accession No.
Ccol_c5260	F: GTTGAGGAGGCCAATGGTAATGATGAG R: ATTAAAGCAGGAAACAGCACTCCTTTG	$(GAT)_7GGTGAG(GAT)_5$	Kaurene synthase	3.29E-30	FX176414
Ccol_c6531*	F: GTTCCTCATTAATGCCTCTACATCCGC R: ATTTGAAATACAGGTTTGTGAAGTGGCA	$(TAT)_5$	—		FX176415
Ccol_c6704*	F: GTTTTTCATCTGTGTATGTGCCATCCC R: ATTGCAGGTACACCAATATGGGGAGAG	$(GCT)_5$	Unnamed protein	7.64E-35	FX176416
Ccol_c7379*	F: GTTTTGTGAGCACAAGCTTGAGTCTAGG R: ATTGTTCTTGCTCTTGGTGCACTTGAA	$(AG)_6$	ABI1 protein phosphatase 2c–like protein	1.41E-09	FX176417
Ccol_c9327	F: GTTTCTTTGAAGAGGGCAATCTCCTTG R: ATTTTCGTGATGCCAATGGAGATAATG	$(TCC)_5$	Chromatin binding	8.74E-48	FX176418
Ccol_c9389*	F: GTTGTTTGGGTTGGAAATGTACGTGGT R: ATTATCATAAGCTGCCAATTCTTCCCA	$(TC)_6$	Triacylglycerol lipase 2	1.18E-52	FX176419
Ccol_c17194	F: GTTGGCTTTTAGGCATTGTTTGGACTG R: ATTTATGCTAAGTGAAGCAACCGCAGA	$(TCT)_5$	GAUT7 LGT7	7.36E-39	FX176409
Ccol_c27784*	F: GTTCTCCAAGTTCTGCCCAGTTTCCTA R: ATTGGTAGGGATTGAATTGCATCTTTATGG	$(CT)_6$	—		FX176410
Ccol_c30466	F: GTTTGTAAAAGCTCCGAACCACCATTT R: ATTGAAAGGCACTTCAGAGGAGCAGAG	$(GAT)_7$	Noninducible immunity 1	7.16E-17	FX176411
Ccol_c34264*	F: GTTTGAAATGTGGGAGTGAGAGTGAGG R: ATTCTGTAAGACGAAGATGACTCCGCA	$(ATA)_5$	Unnamed protein	3.68E-44	FX176412
Ccol_c34786	F: GTTGGTTTTAGAGATGATGCCTTACTCGC R: ATTGTGACATTGGTCTTTCATTTTGCG	$(AAAG)_5$	—		FX176413
Ccol_rep_c412*	F: GTTGTTGTTAGGATGCCTGTGAAGGCT R: ATTTCTAGTTGTGGCAGACTTTGGCAG	$(AGC)_7$	—		FX176468
Ccol_rep_c723	F: GTTGATTCTTCTAGACAACCGGTCCCA R: ATTGAGAGCTTAAGGATGTGGATGCGT	$(AAG)_5$	Aquaporin	1.01E-146	FX176475
Ccol_rep_c723_2	F: GTTGCTTAGATTTGTGGGTTGCTACGG R: ATTGGATATTCTGGGTGGGTCCATTTT	$(TGC)_5$	—		FX176476
Ccol_rep_c1012*	F: GTTTGATAAGGAGGGTAAGCACCCTGA R: ATTCAAAGAAGGTAAGCACGAGCCTGT	$(AGG)_5$	Unnamed protein	2.69E-70	FX176420
Ccol_rep_c1487*	F: GTTCCTTCTGAGCATTTTCTTTCCGTG R: ATTAAGAACCTGAGCGTCAACCAGAAC	$(TC)_6$	Stress-induced protein sti1–like protein	3.84E-78	FX176434
Ccol_rep_c1683*	F: GTTTTCGTAAGTGATGCACCTGATGGT R: ATTGCAGCGAAACCTCGAAAAAGTAGA	$(TGC)_7$	Unnamed protein	7.21E-32	FX176437
Ccol_rep_c1934*	F: GTTTCGATCTCGGAATATTGTTTGCCT R: ATTTGCAGGATACAACAGCGAATTTCT	$(TC)_6$	Transposon protein	1.41E-10	FX176442
Ccol_rep_c1953*	F: GTTTTATATGGATTGTTTGCCACAGCG R: ATTTAAATTGTACCACATCAGCCCACG	$(AGA)_5$	—		FX176443
Ccol_rep_c2424*	F: GTTGCGTCCTAATCCATTTCATCAAGC R: ATTCTTCTCCGCCTAAATATGCACCAC	$(TGG)_6$	Flavanone 3-beta-hydroxylase	9.99E-22	FX176447
Ccol_rep_c2457*	F: GTTAATGAGTCGCACTTCCAACAACTG R: ATTCTGCAAGATGCCGAACTTTCACTA	$(AAC)_6$	NADH-plastoquinone oxidoreductase subunit 5	1.57E-34	FX176448
Ccol_rep_c2513*	F: GTTTGAAATGTTCTTGAGCATGGTGGT R: ATTAAGCTTCAAAGGGTTCCTACCCAG	$(TTTA)_5$	—		FX176450
Ccol_rep_c2836	F: GTTCAAATCACCACCTCCACCAATACA R: ATTAGTAGGGAGTGCCAGGTTTGACTG	$(CAC)_6$	Unnamed protein	1.88E-09	FX176454
Ccol_rep_c3173*	F: GTTCTTTGAAAATGACAGAGGCTTGGG R: ATTACCATTCTGTGCTGTTCCATTCAA	$(AT)_7$	Pectate lyase	1.36E-171	FX176459
Ccol_rep_c3942*	F: GTTGGGATCGCAACCTCTTTTCTTCTT R: ATTCACCGACTTCACAATTTGTTCTGC	$(AG)_7$	Peroxidase 72	7.00E-30	FX176467
Ccol_rep_c4868*	F: GTTATGGTTTCCACTATTGCAAGCAGC R: ATTCAAGGGGACTACGTAACAACGAGG	$(TTC)_8$	Plastid protein	5.96E-53	FX176469
Ccol_rep_c5445	F: GTTAGAAGAGGAAAGCATTACCATGCG R: ATTGGAACAGCCGTATAGATCTGCCAT	$(GCC)_6$	—		FX176470
Ccol_rep_c6122*	F: GTTAGAGACATTGTTGAATGCAGCCAC R: ATTTCAACAAGCCCTGTGAATTTTCTG	$(TCC)_6$	—		FX176471
Ccol_rep_c6518	F: GTTACTGCAACTGCATCTTGAAAGCAC R: ATTTCTCACAATGACCCTGTCTCTGCT	$(CAT)_5$	BOP NPR1 NIM1–like regulatory protein	3.50E-20	FX176472
Ccol_rep_c7067*	F: GTTATTCAAGGTTTGTGGGATGCAGTT R: ATTTCGGCTACGAGTCAATTTCATACA	$(TA)_9$	—		FX176473
Ccol_rep_c7203*	F: GTTGGCACCCTCCTCCTAAACTGTCTT R: ATTCAATGCTGCTGCATCTTGCTATCT	$(CTT)_5$	—		FX176474
Ccol_rep_c7306*	F: GTTAGGAGAGGAGGTACAGGAGGGAGA R: ATTTTTCATCCTCATCGCTGTTTCAGA	$(GAA)_6$	Tudor domain-containing protein nuclease family protein	1.47E-31	FX176477
Ccol_rep_c7383	F: GTTTAATAGCGGAGCTTGTTTGTGGGT R: ATTTACAGAAGCAGGCTTGCAAATGAG	$(TCC)_6$	Unnamed protein	3.24E-14	FX176478

TABLE 1. Continued.

Locus	Primer sequence (5′–3′)	Repeat motif	BLASTX top hit description	*E*-value	GenBank Accession No.
Ccol_rep_c7650*	F: GTTAAGCATGCGAGACAGCAGTGTTAG R: ATTATCAAACAAGACACGGGAACACAA	$(TTA)_5$	Unnamed protein	1.96E-25	FX176479
Ccol_rep_c8300*	F: GTTTTTCACCTCATCCACATCCAGAAA R: ATTTACGCCTGAGACACCAGAAATTCA	$(AG)_6$	Unnamed protein	3.06E-19	FX176480
Ccol_rep_c8954*	F: GTTCTGATTCATGCTAGATGGGCAATG R: ATTGCAACGACAGCAACCACTAAATTG	$(CCT)_5$	AT4G10340-like protein	7.32E-35	FX176481
Ccol_rep_c9022	F: GTTTGGTTTCTCAATGTCTTCGGATCA R: ATTGGCAGTCCGAATCTGTAAAATTGC	$(GAT)_7$	—		FX176482
Ccol_rep_c9058*	F: GTTGAATCGAATACAGCAGGGGAAGTG R: ATTTCATCCAGGAATTTGAGCCTGTTT	$(AAG)_5$	Unnamed protein	3.89E-19	FX176483
Ccol_rep_c9179	F: GTTGTAAGTTCCATGGATGTTGCTCCC R: ATTGGGAAGACATTGAACAATCTGAACG	$(GAT)_5$	3-Phosphoinositide-dependent protein kinase	1.08E-34	FX176484
Ccol_rep_c9756	F: GTTGGGGGAGAAGTTAGTGACGAGGAA R: ATTCTCTCCACCATATGCTTGCATGAC	$(AGA)_5$	Unnamed protein	2.39E-06	FX176485
Ccol_rep_c9985	F: GTTACGAAAGAGTTAAGGAGTTCGGGG R: ATTGCTTCATAGACAGGACGAGGTGGT	$(CCT)_5$	Unnamed protein	1.36E-17	FX176486
Ccol_rep_c10619*	F: GTTCTGGTATATCTTGCCGACTTTGCC R: ATTTTGAAGCGAAAGAGAGGACGAT	$(GTT)_5$GGATGTGGAA GAGGCGGCTCCCAA ACCTGCCACCA$(CTT)_5$	Unnamed protein	1.54E-25	FX176421
Ccol_rep_c10782*	F: GTTATTGATTTGGTTTGGCTGGCATAG R: ATTAAGGGTGATAACGTCATTGACAACA	$(TTGGTG)_6$	Glutathione peroxidase–like protein	1.01E-49	FX176422
Ccol_rep_c10836*	F: GTTGTCGAGGGAAAGTCATATGGTGCT R: ATTTTCCTCAATCAAATCGGTCTCCAT	$(TG)_7$	—		FX176423
Ccol_rep_c10919	F: GTTTCAAATCTCCTGCAACTCCTCCTC R: ATTGAAGCAGGCAAGAGGAAAGGAAAT	$(CCT)_6$	Homeodomain-like protein	3.00E-35	FX176424
Ccol_rep_c11123	F: GTTGTTCATATTCCTTGCATCGCCATT R: ATTCAACAACCACGGTGACAATAGGAA	$(TTG)_7$	MTA SAH	5.18E-09	FX176425
Ccol_rep_c11543*	F: GTTGAGATGCAAGGGCATCCATTTTAG R: ATTACTCCTCATACCACCATTCTCCCA	$(AG)_8$	—		FX176426
Ccol_rep_c11943*	F: GTTATTTGAGACAGCTTGTCACCTCCC R: ATTGGATTGAGGAAGACACATGAGAGGA	$(CT)_7$	—		FX176427
Ccol_rep_c12028	F: GTTAGGAGCCAGTTCCATGTCAACAAT R: ATTTGACGACAATGAGGATGAAGGAAA	$(TCA)_5$	—		FX176428
Ccol_rep_c12296	F: GTTCTGATGCATAATGGACAAGCACAA R: ATTCTGTCATTGATGCAACTGCATGTC	$(TACA)_7$	—		FX176429
Ccol_rep_c12645	F: GTTTCAAGCAAGCTTCAAGAGACATGG R: ATTAGGGCTAAACCTAGCTGTTGGTCA	$(AG)_8$	—		FX176430
Ccol_rep_c12796*	F: GTTTCAAACTTGACACCTGCATTTGCT R: ATTGGCTTGAGGAGGAGGAAGAAAGAG	$(CTC)_5$	—		FX176431
Ccol_rep_c13243*	F: GTTCAATGGTCGTGGCTTACAAATCAG R: ATTAAGAAGGTTTTGGATCTCGAAGGG	$(AC)_7$	—		FX176432
Ccol_rep_c14347*	F: GTTTGCGTGTGGACACAAATAAGGAGT R: ATTTGCCAATATGGTGATTTGACCAAG	$(GAA)_5$	—		FX176433
Ccol_rep_c15619*	F: GTTTGAAATGTTCTTGAGCATGGTGGT R: ATTAAGCTTCAAAGGGTTCCTACCCAG	$(TTTA)_6$	—		FX176435
Ccol_rep_c16171*	F: GTTATGTGATACCCACTTCGATTGTGC R: ATTCAAATCTGAACAGGGAGAATGGCT	$(TCT)_5$	—		FX176436
Ccol_rep_c16880*	F: GTTGACTTGAGCGCCTTCTTTGATCTT R: ATTCCAGAGATGATGGGGATGAAGAGT	$(TCA)_6$	—		FX176438
Ccol_rep_c17604*	F: GTTATCTTGCTAGAAAAGGGCCACCTC R: ATTGTGAGGCATTGAGAGGCTTGTTTT	$(CTT)_5$	—		FX176439
Ccol_rep_c18889*	F: GTTCAAATCACCACCTCCACCAATACA R: ATTAGTAGGGAGTGCCAGGTTTGACTG	$(CAC)_6$	—		FX176440
Ccol_rep_c18989	F: GTTCAACTTCCTCCTCCTCCTCATCCT R: ATTTAGCGAGGATCAAGAATCACACGA	$(TCA)_7$	—		FX176441
Ccol_rep_c20465	F: GTTTGAGAATGTCTCCAATGTCCTCCA R: ATTCTCTGCTGCCTGTACCATCACAGT	$(ATT)_5$	—		FX176444
Ccol_rep_c21013	F: GTTAATCCCAGTTCCATGCTTCACAAT R: ATTTCACCCAGGAAGCAAGTCAATTTT	$(ATC)_5$	GTP cyclohydrolase	1.04E-25	FX176445
Ccol_rep_c21814*	F: GTTTGTTGTGGCTAGTTTTTCTCGCAA R: ATTGGCTGAATGGATTTAGAATTCTCTGC	$(AT)_6$	—		FX176446
Ccol_rep_c25116*	F: GTTATTTAGTGCGGCTGAAAAGGATCA R: ATTCATCTTCCGATTCTCCTCCCTCTT	$(ATG)_5$	—		FX176449
Ccol_rep_c25312*	F: GTTGGCAGATTCCAAGGTATGTCCCTA R: ATTAGGGCCTCCTTCCTACCTAGTGTG	$(TTC)_5$	—		FX176451
Ccol_rep_c25627*	F: GTTGGGTTTTATGAATACGAGGATGCG R: ATTAAATCGCTCCGTCCAATAAATCCT	$(TA)_6$	—		FX176452
Ccol_rep_c27051*	F: GTTGGCATTTCCAAGCACATTTACTCC R: ATTCATGCTCCTTTGTGGTAGATTTTGG	$(TG)_{10}$	—		FX176453

TABLE 1. Continued.

Locus	Primer sequence (5′–3′)	Repeat motif	BLASTX top hit description	*E*-value	GenBank Accession No.
Ccol_rep_c28703*	F: GTTTTGCTAGAGCTCATGATGTTGATG	$(GTT)_6$	—		FX176455
	R: ATTGTCAATGGCTCCAAGAACTTACATA				
Ccol_rep_c28779*	F: GTTGGTGGATAAGACTGGCAATTGAGG	$(TTC)_5$	Unnamed protein	4.74E-07	FX176456
	R: ATTCATTCAAGAGGGGACTGTCCAAGT				
Ccol_rep_c29861	F: GTTGTATGTAGAGGCGGCATGGAAATC	$(GTG)_5$	ABC transporter-like protein	7.66E-10	FX176457
	R: ATTCCCAAGGATAGCCTACCACCTACC				
Ccol_rep_c29904*	F: GTTTGACATACACAACAGATCCGGACA	$(AT)_7$	—		FX176458
	R: ATTTTATGGGTTGAGATGCCATGTGAG				
Ccol_rep_c33402*	F: GTTGCTTTGTCACATCAGCACCAAGTC	$(AT)_7$	—		FX176460
	R: ATTTTGGTGATACTCCTTCACCAAGGG				
Ccol_rep_c34519*	F: GTTCGTAACGGAGAAATGTGTTGTTTGA	$(AG)_8$	Oxygen-evolving enhancer protein 2	1.93E-39	FX176461
	R: ATTGCCAGGAAGTTTGTTGAAAGTGCT				
Ccol_rep_c35103*	F: GTTAGACCTTTAATCGCTGTGTCTCGC	$(GAA)_6$	Unnamed protein	7.86E-24	FX176462
	R: ATTCGAACTCCTCAAACTCATCCACCT				
Ccol_rep_c35787*	F: GTTCCTGATTGGTCGGAGAAGAAGAAA	$(AG)_6$	Unnamed protein	7.66E-41	FX176463
	R: ATTTCGCAATTCCCTCTCATACAGAGC				
Ccol_rep_c35983*	F: GTTTACGCCTGAGACACCAGAAATTCA	$(TA)_7$	—		FX176464
	R: ATTTTTCACCTCATCCACATCCAGAAA				
Ccol_rep_c36209	F: GTTGGGTGACAGTGAAGGGAATGGTAG	$(ACC)_7$	Inorganic pyrophosphatase	8.27E-35	FX176465
	R: ATTTGAATCCTTTTTCACCTCCTCTGC				
Ccol_rep_c36352	F: GTTATCACAGAAAACGCACCTCCAAAT	$(AC)_7$	CER1 protein	2.87E-08	FX176466
	R: ATTATGAGTGCCGAGAATAGGGAACTG				

Note: — = no significant hits were found in the NCBI nr database; * = loci that exhibited allelic variation.

Taxodioideae), to evaluate the potential use of these markers for population genetic studies in these economically important conifers. To characterize each EST-SSR marker, four genetic diversity statistics below were calculated using FSTAT 2.9.3 software (Goudet, 1995): number of alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}). In addition, the significance of Hardy–Weinberg equilibrium and genotypic equilibrium were tested by 1000 randomizations with adjustment of resulting *P* values by sequential Bonferroni correction, using FSTAT 2.9.3.

Fifty-two primer pairs (as denoted with asterisks in Table 1) successfully PCR-amplified all three intraspecific taxa of *C. columellaris* s.l. and were

TABLE 2. Characteristics of the 52 polymorphic EST-SSR markers for *Callitris columellaris* s.l. The markers with no allelic variation are not shown in this table. Shown for each locus are number of individuals genotyped (N), size range of fragment (bp), number of alleles per locus (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}).

Locus	N	Range (bp)	N_a	H_o	H_e	F_{IS}	Locus	N	Range (bp)	N_a	H_o	H_e	F_{IS}
Ccol_c6531	16	137–140	2	1.00	0.50	−1.00	Ccol_rep_c10619	16	163–172	4	0.31	0.44	0.30
Ccol_c6704	16	246–255	3	0.31	0.36	0.14	Ccol_rep_c10782	16	80–104	5	0.56	0.72	0.22
Ccol_c7379	16	206–209	2	0.19	0.45	0.58	Ccol_rep_c10836	16	133–153	9	0.63	0.84	0.26
Ccol_c9389	16	112–116	3	0.25	0.32	0.22	Ccol_rep_c11543	16	216–243	9	0.31	0.81	0.61*
Ccol_c27784	16	184–196	5	0.31	0.57	0.46	Ccol_rep_c11943	16	137–141	3	0.19	0.17	−0.08
Ccol_c34264	16	170–173	2	0.13	0.12	−0.07	Ccol_rep_c12796	16	278–296	5	0.31	0.28	−0.11
Ccol_rep_c412	16	248–254	3	0.13	0.12	−0.05	Ccol_rep_c13243	16	101–105	3	0.38	0.54	0.31
Ccol_rep_c1012	16	239–248	3	0.19	0.17	−0.08	Ccol_rep_c14347	16	278–287	4	0.13	0.23	0.45
Ccol_rep_c1487	16	107–109	2	0.06	0.06	−0.03	Ccol_rep_c15619	16	342–348	3	0.38	0.43	0.13
Ccol_rep_c1683	16	163–184	8	0.69	0.72	0.05	Ccol_rep_c16171	16	106–118	4	0.38	0.32	−0.16
Ccol_rep_c1934	16	173–176	2	0.13	0.12	−0.07	Ccol_rep_c16880	16	231–234	2	0.06	0.34	0.82
Ccol_rep_c1953	16	136–142	3	0.19	0.27	0.31	Ccol_rep_c17604	16	86–104	5	0.19	0.58	0.68*
Ccol_rep_c2424	16	225–258	7	0.06	0.62	0.90*	Ccol_rep_c18989	16	130–148	5	0.44	0.67	0.35
Ccol_rep_c2457	16	204–219	5	0.19	0.63	0.70*	Ccol_rep_c21814	16	162–174	4	0.25	0.41	0.39
Ccol_rep_c2513	16	341–349	4	0.19	0.23	0.18	Ccol_rep_c25116	16	139–154	2	0.31	0.26	−0.19
Ccol_rep_c3173	16	162–174	7	0.50	0.75	0.33	Ccol_rep_c25312	16	100–112	3	0.25	0.22	−0.11
Ccol_rep_c3942	16	222–240	10	0.69	0.80	0.14	Ccol_rep_c25627	16	96–99	3	0.13	0.32	0.61
Ccol_rep_c4868	16	218–221	2	0.06	0.06	−0.03	Ccol_rep_c27051	16	157–173	7	0.38	0.63	0.40
Ccol_rep_c6122	16	224–233	3	0.06	0.17	0.64	Ccol_rep_c28703	16	118–127	4	0.75	0.63	−0.19
Ccol_rep_c7067	16	280–294	7	0.44	0.66	0.34	Ccol_rep_c28779	16	257–260	2	0.06	0.06	−0.03
Ccol_rep_c7203	16	204–222	5	0.31	0.67	0.53	Ccol_rep_c29904	16	154–160	4	0.19	0.43	0.57
Ccol_rep_c7306	16	103–106	2	0.13	0.12	−0.07	Ccol_rep_c33402	16	93–99	2	0.06	0.06	−0.03
Ccol_rep_c7650	16	223–226	2	0.06	0.06	−0.03	Ccol_rep_c34519	16	100–106	4	0.38	0.64	0.41
Ccol_rep_c8300	16	177–195	5	0.00	0.73	1.00*	Ccol_rep_c35103	16	215–221	3	0.19	0.51	0.63
Ccol_rep_c8954	16	300–303	2	0.00	0.47	1.00*	Ccol_rep_c35787	16	117–135	6	0.81	0.72	−0.13
Ccol_rep_c9058	16	213–246	4	0.25	0.37	0.32	Ccol_rep_c35983	16	179–195	4	0.00	0.70	1.00*

Note: * = significant deviation of F_{IS} value from zero tested with 1000 randomizations ($P < 0.01$).

shown to be polymorphic with N_a ranging from two to 10, while H_o and H_e ranged from 0.0 to 1.0 and 0.06 to 0.84, respectively (Table 2). Significant departures from Hardy–Weinberg equilibrium were detected in seven loci, while no significant genotypic disequilibrium for any pair of loci was detected (Table 2). Forty-one loci showed high similarity to proteins in the NCBI nr database, with *E*-values smaller than 1E-5 (Table 1). Only two markers (Ccol_rep_c723 and Ccol_rep_c18989) in *Chamaecyparis obtusa* and one (Ccol_rep_c412) in *Cryptomeria japonica* amplified target regions, all of which showed no allelic variation when range-wide samples (N = 16) were used for evaluation of the marker polymorphism.

CONCLUSIONS

We developed 52 polymorphic EST-SSR markers for *C. columellaris* s.l. from a cDNA library, all of which are transferable in all three intraspecific taxa but not to related genera. These markers will be useful for evaluating the range-wide genetic structure of *C. columellaris* s.l. and, by genome scanning, for detecting outlier loci under selection, as well as providing useful markers to investigate the conservation genetics and reproductive ecology of the species.

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