Mitochondrial Microsatellite Markers for the Australian Ectomycorrhizal Fungus Laccaria sp. A (Hydnangiaceae)

Authors: Sheedy, Elizabeth M., Wouw, Angela P. Van de, Howlett, Barbara J., and May, Tom W.

Source: Applications in Plant Sciences, 2(3)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300086

Applications in Plant Sciences 2014 2(3): 1300086

PRIMER NOTE

MITOCHONDRIAL MICROSATELLITE MARKERS FOR THE AUSTRALIAN ECTOMYCORRHIZAL FUNGUS *LACCARIA* SP. A (HYDNANGIACEAE)[1]

ELIZABETH M. SHEEDY[2,4], ANGELA P. VAN DE WOUW[2], BARBARA J. HOWLETT[2], AND TOM W. MAY[3]

[2]School of Botany, The University of Melbourne, Victoria 3010, Australia; and [3]Royal Botanic Gardens Melbourne, Private Bag 2000, South Yarra, Victoria 3141, Australia

- *Premise of the study:* Microsatellite loci were developed for the ectomycorrhizal fungus *Laccaria* sp. A to investigate the population genetic structure of this fungal symbiont across its fragmented distribution in southeastern Australia.
- *Methods and Results:* A partial genome sequence from an individual collection of *Laccaria* sp. A was obtained by 454 genome sequencing. Eight microsatellite markers were selected from 66 loci identified in the genome. The selected markers were highly polymorphic (4–19 alleles per locus, average 13 alleles) and amplified reproducibly from collections made across the distribution of this species. Five of these markers also amplified reproducibly in the sister species *Laccaria* sp. E (1). All eight of the selected microsatellite loci were from the mitochondrial genome.
- *Conclusions:* The highly polymorphic markers described here will enable population structure of *Laccaria* sp. A to be determined, contributing to research on mycorrhizal fungi from a novel distribution.

Key words: Hydnangiaceae; *Laccaria*; microsatellites; mitochondria; mycorrhizae; *Nothofagus cunninghamii.*

Ectomycorrhizal fungi are diverse and important components of ecosystems and form mutually beneficial associations with plants. Conservatively, there are 7750 species of ectomycorrhizal fungi, although there may be as many as 25,000 (Rinaldi et al., 2008). However, sets of microsatellite markers are only available for around 20 species, most from the Northern Hemisphere (Douhan et al., 2011). Microsatellite markers can be exploited to determine features of the biology and ecology of organisms, including turnover rates of individuals in a population and the gene flow between geographically disjunct populations. These types of information have been reported for very few ectomycorrhizal fungi.

Laccaria Berk. & Broome is a genus of ectomycorrhizal fungi with species in both hemispheres. Multigene phylogeny has recently distinguished considerable cryptic diversity among Australian *Laccaria*, with *Laccaria* sp. A the most readily identifiable species (Sheedy et al., 2013). *Laccaria* sp. A only associates with *Nothofagus cunninghamii* (Hook.) Oerst., the dominant tree in Australian cool temperate rainforests, and these species share a fragmented distribution in the states of Victoria and Tasmania. Microsatellite markers have been designed independently for three Northern Hemisphere species (e.g., Wadud et al., 2006a, 2006b; Labbé et al., 2011); however, Northern Hemisphere species are phylogenetically distant to Australian species (Sheedy et al., 2013). Sixteen markers from *L. bicolor* (Maire) P. D. Orton (Labbé et al., 2011) could not be amplified in Australian species (E. Sheedy, unpublished data). Here we report markers designed specifically for *Laccaria* sp. A.

METHODS AND RESULTS

Collections of *Laccaria* sp. A fruit-bodies were made from across the species distribution (west Tasmania, 96; northeast Tasmania, 44; west Victoria, 54; east Victoria, 56). Selected collections from each population were vouchered at the National Herbarium of Victoria (MEL) (Sheedy et al., 2013; Appendix 1). DNA was extracted from individual fruit-bodies as described in Sheedy et al. (2013).

DNA of *Laccaria* sp. A (MEL 2359659), from Mt. Michael, Tasmania, was sequenced at the Australian Genome Research Facility (AGRF; Brisbane, Australia) on the next-generation Roche GS-FLX platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). A total of 204,362 sequence reads, with an average length of 383 bp, was obtained from a quarter plate of sequencing. Reads were assembled by AGRF (using the software package Newbler; 454 Life Sciences, a Roche Company) into 13,234 contigs with an average length of 621 bp, totaling 8.2 Mb, and no further processing was done. The closest known genome size is of *L. bicolor* (65 Mb; Martin et al., 2008). Contigs were screened for di-, tri-, tetra-, penta-, and hexanucleotide repeats with a minimum repeat number of six, using the online software WebSat (Martins et al., 2009). A total of 66 microsatellite loci were identified, comprising mainly tri- and dinucleotide repeats (36 and 14, respectively). Using the online interface of Primer3 (version 0.4.0; Rozen and Skaletsky, 2000) and a product size range of 150–300 bp, primers were designed for 25 selected loci that had at least seven repeat units and included a mixture of repeat types (e.g., di- and trinucleotide repeats).

Seven collections of *Laccaria* sp. A were used to test whether the 25 microsatellite markers amplified reproducibly (Appendix 1: Set 1). Reactions were in a volume of 25 µL containing 2.5 µL of 10× buffer, 62.5 mM of $MgCl_2$, 2.5 mM

[1]Manuscript received 15 November 2013; revision accepted 2 December 2013.

The authors thank Joanne Birch and Daniel Murphy for suggesting improvements to the manuscript. This research was supported by the Holsworth Wildlife Research Endowment, the Jim Ross Scholarship of the Cybec foundation, and the Albert Shimmins writing-up award to E.M.S.

[4]Author for correspondence: esheedy@student.unimelb.edu.au

doi:10.3732/apps.1300086

of each dNTP, 10 pmol of each primer, 0.5 unit of *Taq* DNA polymerase (Scientifix, Cheltenham, Victoria, Australia), and 1 µL of DNA template, diluted 1 : 200 or 1 : 100 as required. The cycle conditions were a 3-min denaturation at 94°C; 10 touchdown cycles at 94°C (30 s), 62°C (30 s) (−1°C/cycle), 72°C (30 s); 25 cycles at 94°C (30 s), 52°C (30 s), 72°C (30 s); and a final extension at 72°C (10 min). Of the 25 loci, 12 were selected (based on amplification success, lack of nonspecific binding and presence of polymorphisms) for further testing on a second set of 23 collections (Appendix 1: Set 2). Amplifications of selected loci used a lower concentration of dNTPs so that PCR products could be directly sequenced as purification methods resulted in substantial product loss and poor sequence quality. The following reactions were then set up: 25 µL containing 2.5 µL of 10× buffer, 62.5 mM of $MgCl_2$, 0.2 mg/mL of bovine serum albumin (BSA; New England BioLabs, Ipswich, Massachusetts, USA), 1.25 mM of each dNTP, 10 pmol of each primer, 0.5 unit of *Taq* DNA polymerase (Scientifix), and 1 µL of DNA template, diluted 1 : 200 or 1 : 100 as required. Thermocycling conditions were as described above, but with an annealing time of 1 min and an initial annealing temperature of 60°C.

Amplification products were sequenced to confirm the presence of the targeted microsatellites. Eight primer pairs consistently produced polymorphic bands (Table 1). These primers were subsequently synthesized with a fluorescent tag (VIC, NED, PET, or FAM; Applied Biosystems, Foster City, California, USA) attached to the 5′ end of the forward primer and a GTTTCTT 'pigtail' appended to the 5′ end of the reverse primers. Microsatellite alleles were separated by capillary electrophoresis using an ABI 3730 DNA Analyzer (Applied Biosystems) at AGRF. Alleles were scored based on size (position of peaks) using Geneious version 5.6.4 (Drummond et al., 2012). From the 29 test collections, 27 haplotypes were detected, with duplicates being of fruit-bodies from the same fairy-ring, indicating a high level of resolution (Appendix 1). The number of alleles and unbiased haploid diversity were determined using GenAlEx (version 6.5; Peakall and Smouse, 2012). For 120 collections, the number of alleles per locus was 4–19 and the unbiased haploid diversity was 0.345–0.905 (Table 2).

All eight loci produced single alleles with a range of sizes. A BLAST search of the contigs containing the loci and the *L. bicolor* nuclear and mitochondrial genomes (genome version 1.0, MycorWeb; http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/blast.html) revealed the eight loci were mitochondrial. Of the original 66 loci, 27 were in the mitochondrial genome, 23 did not match nuclear or mitochondrial genomes (most likely due to the high level of sequence divergence between species), and 16 matched to sequences in the nuclear genome.

The eight primer pairs that amplified polymorphic microsatellites in *Laccaria* sp. A were also trialed on 18 other Australian species of Hydnangiaceae (Sheedy et al., 2013) under the amplification and capillary separation conditions listed above. Five loci (ESLA06, ESLA19, ESLA41, ESLA24, and ESLA39) amplified in other species of Australian Hydnangiaceae (Appendix 2), and the species with the most consistent amplification was *Laccaria* sp. E (1), the sister species of *Laccaria* sp. A (Sheedy et al., 2013).

CONCLUSIONS

The eight mitochondrial microsatellite markers reported here are highly polymorphic, making them useful for differentiating between genetic individuals as well as for investigating the population genetic structure of *Laccaria* sp. A. Mitochondrial markers were not deliberately sought; the markers were selected because

Table 1. Characteristics of microsatellite loci in *Laccaria* sp. A, derived from partial sequencing of the genome of collection MEL 2359659. Annealing temperature for all loci was a 'touchdown' of 60–50°C.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	Fluorescent label[a]	GenBank accession no.
ESLA06*	F: GGACAAGTGGCAACTGCAT	$(TTA)_{17}$	240	FAM	KF736239
	R: CGCTTTAATTGCAGCCTTGA				
ESLA12*	F: AATAAAAACCTAGACACCGCTACG	$(AATGAT)_9$	151	VIC	KF736240
	R: AAAGCAAATATGAATGTGCCAAT				
ESLA19*	F: AGCACAAGCACAAGCACAAG	$(ACAATA)_{19}$	230	VIC	KF736241
	R: AGTAGCTCCCACCATTCTAGG				
ESLA24*	F: GCTGCAACTTGTGATCAAACC	$(TTA)_{12}N_{21}(TATTC)_{18}(AATTC)_5$	224	FAM	KF736242
	R: GCTACGGCTTGCTTGTGTCT				
ESLA38*	F: TTTCAGTAGTGTTCTTTGCCAAT	$(AATGAG)_7(TATGAG)_5N_{27}(ATA)_{19}$	348	VIC	KF736243
	R: CCCCCTTATTTTGCCTATTAAC				
ESLA39*	F: TGCCGTTCTAACCATTGAACT	$(TAT)_7$	248	NED	KF736244
	R: AGCGAAGCAAACAGCGTAAT				
ESLA41*	F: CCACTGCTGCTTTTTACGC	$(ACAAT)_{11}$	167	NED	KF736245
	R: AACAATTGTAAGAAAAACAAGAAACAA				
ESLA66*	F: TTCGCAGAGACAAACAATAGC	$(TATCCT)_{15}$	228	PET	KF736246
	R: TGGGTTGGCTTTACTGGTGT				
ESLA31†	F: GGATGAACACGAAAGTGCAG	$(AC)_8$	288		
	R: AGGACCGCGGTATTCAATTT				
ESLA45	F: TTTTATCGAATGGCTGAGCA	$(TAT)_{10}$	182		
	R: AAACCTACTTTTGTGATTACTCCTGA				
ESLA50‡	F: CATCAATCACCCTTCGCTTT	$(TCC)_9$	226		
	R: GGATGGCCAATCTACGACAT				
ESLA54‡	F: TGCATAGCACAAAAATGCTG	$(AC)_8$	156		
	R: ACTCGAGGTACCGGGATTG				
ESLA56‡	F: CAGATGCCCTGTTCTTGCTC	$(TCT)_8$	152		
	R: AACAGGAGAAGCAGGTTCCA				
ESLA58‡	F: GGAATCTCACGGAAAAACCA	$(GA)_7$	153		
	R: TTGGCATCACCTTCCTTCAT				
ESLA61	F: AATCCTAGCTAGTAGTGCTATCTTCC	$(TAATA)_{13}N_{66}(ATTAAT)_6$	234		
	R: TTAAGCCCTAGCTAGCCCACT				
ESLA64	F: TTGAATTTTGAATTAGCAATTAGCA	$(TAA)_{11}$	213		
	R: TTGGTAAGCCTAAGAAGATTTGAA				

[a] Fluorescent labels are from Applied Biosystems.

* These loci were used to examine population characters of *Laccaria* sp. A (Table 2). The remaining loci were not used due to inconsistent amplification (loci ESLA54, ESLA61, ESLA64) or lack of variation (all other loci).

† Locus is nuclear; all other loci are mitochondrial, unless otherwise marked.

‡ Genomic origin of locus is unknown.

TABLE 2. Characteristics of eight polymorphic microsatellite markers in collections from four populations of *Laccaria* sp. A.[a]

	WV (n = 19)		EV (n = 25)		WT (n = 50)		NET (n = 26)		All (n = 120)	
Locus	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*
ESLA06	2	0.491	2	0.280	3	0.117	3	0.394	4	0.345
ESLA12	3	0.205	7	0.833	11	0.771	7	0.809	12	0.752
ESLA19	6	0.836	5	0.513	9	0.811	10	0.877	11	0.840
ESLA24	8	0.766	6	0.710	13	0.896	8	0.828	15	0.855
ESLA38	8	0.766	7	0.540	13	0.887	9	0.837	15	0.834
ESLA39	5	0.532	3	0.227	7	0.733	5	0.649	10	0.735
ESLA41	10	0.912	7	0.637	14	0.927	11	0.902	19	0.905
ESLA66	5	0.620	5	0.363	15	0.914	11	0.892	18	0.842

Note: *A* = number of alleles; *h* = unbiased haploid diversity; *n* = sample size.
[a] Population: WV = west Victoria; EV = east Victoria; WT = west Tasmania; NET = northeast Tasmania.

they were polymorphic and amplified consistently. The reason for the preponderance of mitochondrial markers is unknown. Due to the uniparental inheritance of the mitochondria and the presence of single alleles at each locus, the alleles are straightforward to score. These markers will significantly contribute to research on ectomycorrhizal fungi, as they allow investigation of fungal populations from a species (and close relatives) from the Southern Hemisphere, from a previously unstudied host tree (*N. cunninghamii*) and ecosystem (cool temperate rainforest).

LITERATURE CITED

DOUHAN, G. W., L. VINCENOT, H. GRYTA, AND M.-A. SELOSSE. 2011. Population genetics of ectomycorrhizal fungi: From current knowledge to emerging directions. *Fungal Biology* 115: 569–597.

DRUMMOND, A. J., B. ASHTON, S. BUXTON, M. CHEUNG, A. COOPER, C. DURAN, M. FIELD, ET AL. 2012. Geneious v5.6.4 created by Biomatters. Website http://www.geneious.com [accessed 12 February 2014].

LABBÉ, J., C. MURAT, E. MORIN, F. LE TACON, AND F. MARTIN. 2011. Survey and analysis of simple sequence repeats in the *Laccaria bicolor* genome, with development of microsatellite markers. *Current Genetics* 57: 75–88.

MARTIN, F., A. AERTS, D. AHRÉN, A. BRUN, E. G. J. DANCHIN, F. DUCHAUSSOY, J. GIBON, ET AL. 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature* 452: 88–92.

MARTINS, W. S., D. C. S. LUCAS, K. F. S. NEVES, AND D. J. BERTIOLI. 2009. WebSat: A Web software for microsatellite marker development. *Bioinformation* 3: 282–283.

PEAKALL, R., AND P. E. SMOUSE. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* 28: 2537–2539.

RINALDI, A. C., O. COMANDINI, AND T. W. KUYPER. 2008. Ectomycorrhizal fungal diversity: Separating the wheat from the chaff. *Fungal Diversity* 33: 1–45.

ROZEN, R., AND H. J. SKALETSKY. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SHEEDY, E. M., A. P. VAN DE WOUW, B. J. HOWLETT, AND T. W. MAY. 2013. Multigene sequence data reveal morphologically cryptic phylogenetic species within the genus *Laccaria* in southern Australia. *Mycologia* 105: 547–563.

WADUD, M. A., C. L. LIAN, K. NARA, AND T. HOGETSU. 2006a. Isolation and characterization of five microsatellite loci in an ectomycorrhizal fungus *Laccaria laccata. Molecular Ecology Notes* 6: 700–702.

WADUD, M. A., C. L. LIAN, K. NARA, T. A. ISHIDA, AND T. HOGETSU. 2006b. Development of microsatellite markers from an ectomycorrhizal fungus, *Laccaria amethystina*, by a dual-suppression-PCR technique. *Molecular Ecology Notes* 6: 130–132.

Appendix 1. Collections of *Laccaria* sp. A used to assess amplification and allelic variation of microsatellite loci.

Collection	Voucher[a]	Latitude	Longitude	Site	Population	Set[b]	Hap.[c]
ES087_7.14.1	2360498	–41.47213	145.46077	Philosopher Falls	West Tasmania	1	1
ES198_A4	2359650	–37.48794	145.83394	The Beeches	East Victoria	1	2
ES210_A11	2359652	–38.42517	146.57214	Tarra-Bulga	East Victoria	1	3
ES232_A18	2359653	–38.69858	143.47771	Aire River Crossing	West Victoria	1	4
ES270_A46	2359660	–41.82715	145.50443	Mt. Read	West Tasmania	1	5
ES291_A57	2359661	–42.69452	146.46721	Mt. Michael	Northeast Tasmania	1	6
ES295_A66*	2359659	–41.18582	148.01033	Mt. Michael	Northeast Tasmania	1	7
ES011_4.1.1	2359645	–41.47582	145.46074	Philosopher Falls	West Tasmania	2	8
ES199_A5	2359651	–37.48841	145.83418	The Beeches	East Victoria	2	9
ES261_A37	2359656	–41.62717	145.07750	Savage River	West Tasmania	2	10
ES263_A39		–41.36459	145.58067	Hellyer Gorge	West Tasmania	2	11
ES267_A43	2359655	–41.50199	145.64804	Mt. Pearse	West Tasmania	2	12
ES268_A44		–41.82732	145.50516	Mt. Read	West Tasmania	2	13
ES272_A47		–41.82744	145.50430	Mt. Read	West Tasmania	2	14
ES274_A48		–41.95921	145.45747	Lyell Highway	West Tasmania	2	15
ES276_A49		–42.21491	146.01804	Franklin River	West Tasmania	2	16
ES278_A50		–42.21498	146.01830	Franklin River	West Tasmania	2	17
ES288_A54		–42.83745	146.37706	Scotts Peak	West Tasmania	2	18
ES293_A59		–41.19500	148.00627	Sunflat Road	Northeast Tasmania	2	19
ES295_A66*		–41.18582	148.01033	Mt. Michael	Northeast Tasmania	2	7
ES300_A66		–41.18582	148.01033	Mt. Michael	Northeast Tasmania	2	7
ES306_A66		–41.18582	148.01033	Mt. Michael	Northeast Tasmania	2	7
ES312_A69	2359658	–41.21628	147.93832	Weldborough Pass	Northeast Tasmania	2	20
ES315_A72		–41.25240	147.85489	Rattler Ranges	Northeast Tasmania	2	21
ES316_A73		–41.25349	147.85458	Rattler Ranges	Northeast Tasmania	2	22
ES319_A74	2359662	–41.06765	147.73357	Mt. Horror	Northeast Tasmania	2	23
ES320_A75		–41.32225	147.45370	Diddleum Plains	Northeast Tasmania	2	24
ES322_A76	2359663	–41.35117	147.71608	Ben Ridge Road	Northeast Tasmania	2	25
ES325_A79		–41.25946	146.77177	Holwell Gorge	West Tasmania	2	26
ES333_A83		–41.21770	147.93846	Weldborough Pass	Northeast Tasmania	2	27

[a] Herbarium voucher numbers for the National Herbarium of Victoria (MEL).

[b] 'Set' identifies collections used in the first and second sets of microsatellite amplification testing.

[c] 'Hap.' identifies unique haplotypes in these collections.

* Collection ES295_A66 was included in both sets as a positive control.

Appendix 2. Cross-amplification of five microsatellite loci, designed from *Laccaria* sp. A, in species of Australian Hydnangiaceae.

		Laccaria sp. A loci[a]				
Species[b]	*n*[c]	ESLA06	ESLA19	ESLA24	ESLA39	ESLA41
L. sp. E (1)	5	+	+	+	+	+
L. sp. E (2)	1	—	—	—	—	—
L. canaliculata (1)	3	+	+/–	—	+/–	+/–
L. canaliculata (2)	1	+	—	—	+	+
L. canaliculata (3)	1	—	—	—	—	—
L. canaliculata (4)	1	—	—	—	—	—
L. lateritia (1)	3	—	—	—	—	—
L. lateritia (2)	1	+	—	—	—	—
L. sp. B (1)	4	+/–	—	—	+	+/–
L. sp. B (2)	2	+	—	—	—	+/–
L. sp. B (3)	3	+/–	—	—	—	—
L. sp. B (4)	1	+	—	—	—	+
L. sp. D	2	+/–	—	—	+/–	—
L. masoniae (1)	3	—	—	—	+/–*	+/–*
L. masoniae (2)	1	—	—	—	—	—
Hydnangium carneum (1)	4	+/–	—	—	—	+/–
H. carneum (2)	1	+	—	—	—	—
H. sublamellatum	1	+	—	—	—	—

Note: + = locus amplified in every collection tested; +/– = locus amplified in at least one but not all collections tested, for example, one or two of three collections amplified; — = locus did not amplify; *n* = sample size.

[a] Of the eight loci tested, three (ESLA12, ESLA38, ESLA66) did not amplify in any species other than *Laccaria* sp. A; these loci are not included in this table.

[b] Species are as delimited in Sheedy et al. (2013) from a multigene (ITS, *rpb2*, and *tef*-1α) phylogeny, with cryptic phylogenetic species within morphological species indicated by numbers in parentheses. Described morphological species are *L. lateritia* Malençon, *L. canaliculata* (Sacc.) Massee, *L. masoniae* G. Stev., *Hydnangium carneum* Wallr., and *H. sublamellatum* Bougher, Tommerup & Malajczuk.

[c] Voucher information for all collections is provided in Sheedy et al. (2013).

* Only one allele size was obtained for two loci sequenced in the same run with the same fluorescent tag; therefore, the locus from which this allele was amplified cannot be identified.