Development of Phylogenetic Markers for Sebacina (Sebacinaceae) Mycorrhizal Fungi Associated with Australian Orchids

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Primer Note

Development of phylogenetic markers for *Sebacina* (Sebacinaceae) mycorrhizal fungi associated with Australian orchids[1]

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- *Premise of the study:* To investigate fungal species identity and diversity in mycorrhizal fungi of order Sebacinales, we developed phylogenetic markers. These new markers will enable future studies investigating species delineation and phylogenetic relationships of the fungal symbionts and facilitate investigations into evolutionary interactions among *Sebacina* species and their orchid hosts.
- *Methods and Results:* We generated partial genome sequences for a *Sebacina* symbiont originating from *Caladenia huegelii* with 454 genome sequencing and from three symbionts from *Eriochilus dilatatus* and one from *E. pulchellus* using Illumina sequencing. Six nuclear and two mitochondrial loci showed high variability (10–31% parsimony informative sites) for Sebacinales mycorrhizal fungi across four genera of Australian orchids (*Caladenia*, *Eriochilus*, *Elythranthera*, and *Glossodia*).
- *Conclusions:* We obtained highly informative DNA markers that will allow investigation of mycorrhizal diversity of Sebacinaceae fungi associated with terrestrial orchids in Australia and worldwide.

Key words: mycorrhizal fungi; orchids; phylogenetics; *Sebacina.*

Lack of macro- and microscopic characters in fungi severely hampers species identification. This is particularly true for the inconspicuous species complex known as *Sebacina vermifera* (Oberw.) P. Roberts (family Sebacinaceae, order Sebacinales, informally assigned as subgroup B [Weiss et al., 2004]). These fungi are not known to form basidiocarp or hymenial layers (Oberwinkler et al., 2013). However, DNA sequencing of samples taken from soil or root samples indicates that there is extensive diversity within this species complex (Weiss et al., 2004). Thus, it is likely that there are many species yet to be determined in this group. Australian terrestrial orchids within the genera *Caladenia* R. Br., *Glossodia* R. Br., *Elythranthera* (Endl.) A. S. George, and *Eriochilus* R. Br. form mycorrhizal associations with fungi of the *S. vermifera* subgroup B species complex. However, the actual number of *Sebacina* species associated with these orchids is presently unknown. Two commonly used markers for fungal species identification, the ribosomal internal transcribed spacer (ITS) and mitochondrial large subunit (mLSU), both suggest that species-level differences exist among orchid mycorrhizal isolates extracted from these orchid genera (Weiss et al., 2004; Wright et al., 2010). Here we report the development of additional loci to aid accurate species identification of orchid mycorrhizal fungi allied to *S. vermifera*.

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METHODS AND RESULTS

Partial genome sequencing for genetic marker development—A *Sebacina* isolate from *C. huegelii* Rchb. f. and *Eriochilus pulchellus* Hopper & A. P. Br., as well as three isolates from *Eriochilus dilatatus* Lindl., were grown in liquid culture and DNA was extracted as described previously (Roche et al., 2010). For the *C. huegelii* isolate, sequences were generated with a 3-kb mate-pair library on the GS FLX 454 platform with GS XL70 sequencing chemistry (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). CLC Genomics Workbench software (CLC bio, Aarhus, Denmark) was used to separately assemble the *C. huegelii* sequences using the default assembly parameters. Sequences for the isolates from *Eriochilus* were produced with a HiSeq2500 (Illumina, San Diego, California, USA) using paired-end libraries with 350-bp fragments. Reads were quality filtered, and the adapters were removed using libngs (https://github.com/sylvainforet/libngs) using a minimum quality of 25 bp and a minimum read size of 150 bp. The genomic assemblies were carried out with SPAdes (Bankevich et al., 2012) using the following options: "-k 21,33,55,77,99,127–careful -t 16 -m 64".

To design phylogenetic markers that are broadly applicable, we focused primer design on common regions of high sequence homology. Initially, we compared the four *Sebacina* isolates from *Eriochilus* against the *C. huegelii* isolate within CLC Genomics Workbench software. This allowed us to target the high homology sequence regions shared across the five isolates for positioning the primers. Out of the 58,295 successful reads of *Sebacina* from *C. huegelii*, there were 1251 shared reads with *E. pulchellus* (average length 285 bp) and 950 to 1605 shared reads with *E. dilatatus* isolates (average length

Table 1. Characteristics of phylogenetic primers developed for *Sebacina* isolates in this study.

Locus	Primer sequence (5′–3′)	Top BLAST hit to annotated or predicted gene	*E*-value of top hit in GenBank	Fragment length (bp)	GenBank accession no.
C2754	F: GAAGRCAATCYTCGGCTCCATA R: CGATGGGTAGRCAGTTGAA	Serine/threonine protein phosphatase	2e-57	~951	KJ410224–KJ410227
C5129[#1]	F: TATGCGTCGTCATCGCTAAG R: TCTCCTTCGGCATCAAAGTC	Ca^{2+} binding actin-bundling protein	5e-110	~1270	KJ410219–KJ410223
C16699[#1]	F: ATCAAGWTCCATCCTGAACTTTA R: ACCRAGGACAAGRGTCTTCT	Ca^{2+} binding actin-bundling protein	2e-49	~895	KJ410214–KJ410218
C19981[2]	F: ATCAGTCTCTSCGYCARAAG R: AGCCCATATARCCTTCATTTCC	D-xylulose kinase	9e-12	~425	KJ410209–KJ410213
C28586[2]	F: ACAACGARAACTGCTGGGAC R: AGGAGGAAATCRCGCAAGTAGAC	Septin ring protein	8e-40	~575	KJ410204–KJ410208
C43566[1]	F: ACGCCYACYTTYCCGTATCC R: CGAYCTYCATTTCAGCGTCT	Phosphatase DCR2	7e-18	~1150	KJ410200–KJ410203
C11488[*3]	F: GACTTTCAGGATTTTACTC R: ACTCCGTTTCCRAATAATTCTC	NAD5	0.0	~840	KJ410195–KJ410199
C11804[*3]	F: CRGCAAATGGWATTAAAG R: CCTAGTTCWTAYTCTATTGCAC	ATP synthase	2e-65	~500	KJ410190–KJ410194

[*] Mitochondrial marker.
[#] Loci C5129 and C16699 partially overlap.
[1] Source for gene identification: Martin et al. (2008).
[2] Source for gene identification: Stajich et al. (2010).
[3] Source for gene identification: Zuccaro et al. (2011).

375 and 588 bp). GenBank BLAST searches (http://www.ncbi.nlm.nih.gov/) were conducted on 159 of *C. huegelii* sequences with high homology with at least two *Eriochilus* isolates. A total of 31 sequences (out of the 159) were identified as being associated with annotated or predicted genes from *Basidiomycota* R. T. Moore or *Ascomycota* (Berk.) Caval.-Sm. fungi. Priming sites were selected for 19 consensus sequences using Primer3 (Rozen and Skaletsky, 2000); selection was based on product length, ease of primer design, and gene designation.

Genetic marker amplification success and sequence diversity—Successful fungal isolation, following Roche et al. (2010), was obtained for several *Sebacina* isolates from host species within the Australian orchid genera *Caladenia* (14 spp.), *Glossodia* (2 spp.), *Eriochilus* (3 spp.), and *Elythranthera* (1 sp.). A total of 31 isolates were evaluated for DNA sequence diversity with the new fungal primers described below (Tables 1 and 2, Appendix 1). This set represented one to four isolates each from the 13 most strongly supported phylogenetic clades as revealed by a preliminary screen with ITS (bootstrap support >0.78; data not shown) of *Sebacina* isolates associated with several Australian orchids.

PCR reactions were performed in 30-µL reactions containing 2 µL of 20–100 ng of template DNA, 14.75 µL of H_2O, 6 µL Mango*Taq* 5× PCR buffer (Bioline, Sydney, Australia), 1 µL dNTPs (2.5 mM), 1.5 µL of $MgCl_2$ (50 mM), 2 µL bovine serum albumin (BSA; 10 mg/mL), 1.5 µL of each primer (10 µM), and 1 unit of Mango*Taq* polymerase (Bioline). A touchdown thermal profile was used consisting of a 3-min denaturation at 94°C; followed by 12 touchdown cycles at 94°C (30 s) with the first annealing temperature at 66°C (40 s) (−3°C/second cycle) and a primer extension at 72°C (1 min); then 30 cycles at 94°C (30 s), 48°C (40 s), 72°C (1 min); with a final extension at 72°C for 20 min. Products were sequenced bidirectionally with ABI PRISM BigDye Terminator version 3.1 sequencing kit on an ABI 3100 automated sequencer (Applied Biosystems, Carlsbad, California, USA). Sequences were edited using the program Sequencher version 4.7 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned in Geneious Pro version 6.1.6 (Drummond et al., 2011). Consistent and high-quality amplification of all tested *Sebacina* isolates occurred for eight primer sets (Table 2). Estimates of variability across the alignment of up to 31 sequences were performed within MEGA version 5.2 (Tamura et al., 2011). The eight markers revealed 15–40% nucleotide diversity and 11–31% parsimony informative sites across these sequences (Table 2).

Table 2. Characteristics of markers in *Sebacina* mycorrhizal fungi from Australian orchids.

Locus	Amplification success (%)	Aligned length (bp)	No. of variable sites (%)	Parsimony informative sites (%)
C2754	95	814	20.2	17.9
C5129	90	1161	29.3	25.4
C16699	100	781	40.5	29.7
C5129+C16699	90	1592	29.4	25.3
C19981	100	382	35.3	31.4
C28586	100	516	26.6	23.6
C43566	94	928	34.3	30.7
C11488	87	740	14.6	10.7
C11804	77	444	29.7	25.5

Phylogenetic analyses—A multiple sequence alignment was constructed using the alignment tool in Geneious Pro version 6.1.6 (Drummond et al., 2011) before performing manual checks and minor adjustments. Phylogenies of individual and concatenated loci were estimated with a maximum likelihood (ML) analysis using RAxML 7.0.3 (Stamatakis et al., 2008). Support for nodes was assessed for ML trees using 1000 pseudoreplicates of nonparametric bootstrapping in RAxML. A GTR+G substitution model was used for all analyses as all other models are nested inside this model. Trees were visualized using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) and midpoint rooted. Among the 31 isolates, eight to 12 well-supported clades were identified with the newly developed loci (Fig. 1, Appendices S1–S7).

CONCLUSIONS

We successfully designed polymorphic markers for eight putative gene coding loci that successfully amplified across the mycorrhizal fungi species complex *S. vermifera.* These new markers will allow investigations of the species diversity, phylogenetic relationships, and the specificity of orchid mycorrhizal associations for a wide range of Australian terrestrial orchids.

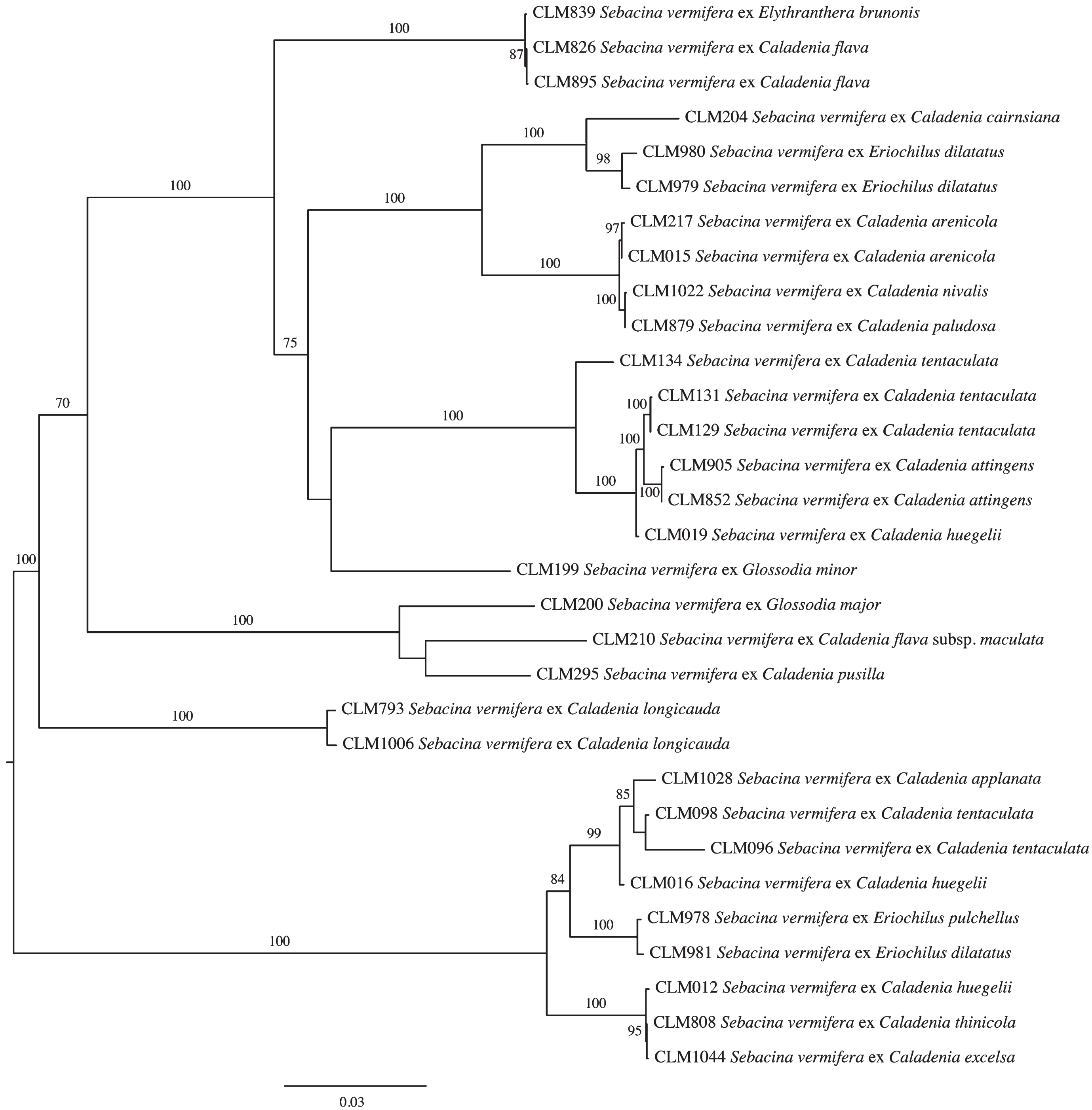

Fig. 1. Midpoint-rooted maximum likelihood tree for *Sebacina vermifera* obtained for eight concatenated loci (two mitochondrial and six nuclear loci). The tree with the highest log likelihood is shown. The numbers above the branches are maximum likelihood bootstrap values. Bootstrap values of ≥70% are shown. The branch length is proportional to the inferred divergence level. The bar indicates substitutions per site.

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Appendix 1. Voucher information for *Sebacina* isolates used in this study.

Voucher specimen accession no.[1]	Host species	Origin	Collector[2]	GPS
CLM012	*Caladenia huegelii* Rchb. f.	Kings Park Botanical Gardens Collection, WA	NS, KD	31.9704°S, 115.822°E
CLM015	*Caladenia arenicola* Hopper & A. P. Br.	Kings Park Botanical Gardens Collection, WA	NS, KD	
CLM016	*Caladenia huegelii* Rchb. f.	WA	MMW	
CLM019	*Caladenia huegelii* Rchb. f.	WA	MMW	
CLM096	*Caladenia tentaculata* Schltdl.	Maldon, VIC	MMW	36.997°S, 144.067°E
CLM098	*Caladenia tentaculata* Schltdl.	Maldon, VIC	MMW	
CLM129	*Caladenia tentaculata* Schltdl.	Inverleigh, VIC	MMW	38.030°S, 144.050°E
CLM131	*Caladenia tentaculata* Schltdl.	Inverleigh, VIC	MMW	
CLM134	*Caladenia tentaculata* Schltdl.	Wonthaggi, VIC	MMW	38.689°S, 145.589°E
CLM204	*Caladenia cairnsiana* F. Muell.	WA	RP	33.523°S, 118.463°E
CLM210	*Caladenia flava* subsp. *maculata* Hopper & A. P. Br.	WA	RP	28.083°S, 114.222°E
CLM217	*Caladenia arenicola* Hopper & A. P. Br.	Kings Park Glasshouse Collection, WA	RP	31.970°S, 115.822°E
CLM295	*Caladenia pusilla* W. M. Curtis	Bluff Hill, TAS	NS	
CLM793	*Caladenia longicauda* Lindl.	Nyerilup Rd, WA	MW	33.866°S, 118.778°E
CLM808	*Caladenia thinicola* Hopper & A. P. Br.	Gracetown, WA	MW	33.857°S, 114.995°E
CLM826	*Caladenia flava* R. Br.	Gracetown, WA	MW	
CLM852	*Caladenia attingens* Hopper & A. P. Br.	Gracetown, WA	MW	
CLM879	*Caladenia paludosa* Hopper & A. P. Br.	Yalingup, WA	MW	33.658°S, 115.034°E
CLM895	*Caladenia flava* R. Br.	Milyeanup Rd, WA	MW	34.283°S, 115.275°E
CLM905	*Caladenia attingens* Hopper & A. P. Br.	Milyeanup Rd, WA	MW	
CLM1006	*Caladenia longicauda* Lindl.	Toolbrunup Rd, WA	MW	34.099°S, 117.788°E
CLM1022	*Caladenia nivalis* Hopper & A. P. Br.	Moses Rock, WA	MW	33.768°S, 114.994°E
CLM1028	*Caladenia applanata* Hopper & A. P. Br.	Moses Rock, WA	MW	
CLM1044	*Caladenia excelsa* Hopper & A. P. Br.	Wilybrup Rd, WA	MW	33.805°S, 115.019°E
CLM199	*Glossodia minor* R. Br.	Near Bendalong, NSW	CCL	35.114°S, 150.315°E
CLM200	*Glossodia major* R. Br.	Near Bendalong, NSW	CCL	
CLM839	*Elythranthera brunonis* (Endl.) A. S. George	Gracetown, WA	MW	33.857°S, 114.995°E
CLM978	*Eriochilus pulchellus* Hopper & A. P. Br.	Tindele Rd, WA	RP	
CLM979	*Eriochilus dilatatus* Lindl.	Leeman/Dongara, WA	RP	29.520°S, 114.935°E
CLM980	*Eriochilus dilatatus* Lindl.	Leeman/Dongara, WA	RP	
CLM981	*Eriochilus dilatatus* Lindl.	Leeman/Dongara, WA	RP	

Note: NSW = New South Wales; TAS = Tasmania; VIC = Victoria; WA = Western Australia.

[1] Culture collections are stored in water at 5°C on fungal isolation media (FIM) slants (Clements and Ellyard, 1979) and located in the author's laboratory at the Australian National University (ANU).

[2] Collectors: CCL = Celeste C. Linde; KD = Kingsley Dixon; MMW = Magali M. Wright; MW = Michael Whitehead; NS = Nigel Swartz; RP = Ryan Phillips.