Phylogenetic and Microsatellite Markers for Tulasnella (Tulasnellaceae) Mycorrhizal Fungi Associated with Australian Orchids

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PRIMER NOTE

PHYLOGENETIC AND MICROSATELLITE MARKERS FOR *TULASNELLA* (*TULASNELLACEAE*) MYCORRHIZAL FUNGI ASSOCIATED WITH AUSTRALIAN ORCHIDS[1]

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- *Premise of the study:* Phylogenetic and microsatellite markers were developed for *Tulasnella* mycorrhizal fungi to investigate fungal species identity and diversity. These markers will be useful in future studies investigating the phylogenetic relationship of the fungal symbionts, specificity of orchid–mycorrhizal associations, and the role of mycorrhizae in orchid speciation within several orchid genera.
- *Methods and Results:* We generated partial genome sequences of two *Tulasnella* symbionts originating from *Chiloglottis* and *Drakaea* orchid species with 454 genome sequencing. Cross-genus transferability across mycorrhizal symbionts associated with multiple genera of Australian orchids (*Arthrochilus*, *Chiloglottis*, *Drakaea*, and *Paracaleana*) was found for seven phylogenetic loci. Five loci showed cross-transferability to *Tulasnella* from other orchid genera, and two to *Sebacina*. Furthermore, 11 polymorphic microsatellite loci were developed for *Tulasnella* from *Chiloglottis*.
- *Conclusions:* Highly informative markers were obtained, allowing investigation of mycorrhizal diversity of Tulasnellaceae associated with a wide variety of terrestrial orchids in Australia and potentially worldwide.

Key words: microsatellites; mycorrhizal fungi; orchids; phylogenetic; *Sebacina*; *Tulasnella.*

Identification of species in fungi has always been challenging due to their cryptic nature. Furthermore, universally accepted DNA barcode markers are lacking, with the ribosomal internal transcribed spacer region (ITS) showing the highest probability of successful identification of the broadest range of fungi (Schoch et al., 2012). In orchid mycorrhizae, the ITS as well as the mitochondrial large subunit (LSU) are most commonly used. However, dependence on only two sequence loci for accurate species identification lacks discrimination, especially for fine-scale ecological and evolutionary interaction studies between orchids and their mycorrhizal symbionts.

Tulasnella J. Schröt. (family Tulasnellaceae, order Cantharellales) is an endophytic fungus occurring in the roots, stems, or protocorns of a range of orchids, trees, and liverworts. In Australia, sexually deceptive orchids within the tribe Drakaeinae such as *Chiloglottis* R. Br. and *Drakaea* Lindl. are known to form mycorrhizal associations with narrow groups of monophyletic *Tulasnella* lineages (Roche et al., 2010; Phillips et al., 2011). However, the actual number of *Tulasnella* species associated with the orchid genera has not been fully resolved. Furthermore, population-level studies are rare in orchid–mycorrhizal associations because suitable population-level markers often are unavailable. Consequently, to facilitate investigations into evolutionary interactions among *Tulasnella* species and their orchid hosts, markers are needed at both the species and population levels.

METHODS AND RESULTS

Fungal isolation and DNA extractions were conducted for *Tulasnella* mycorrhizal fungi as described in Roche et al. (2010) from a range of host species within the genera *Arthrochilus* F. Muell., *Chiloglottis*, *Drakaea*, and *Paracaleana* Blaxell (Appendix 1).

Phylogenetic loci development—*Tulasnella* isolates from *Chiloglottis* aff. *jeanesii* (an undescribed taxon most closely related to *C. jeanesii* D. L. Jones) and *D. elastica* Lindl. were grown in liquid culture and DNA extracted as described previously (Roche et al., 2010).

Sequences for each isolate were generated using a 3-kb pair-end sequence library (Roche, 454 Life Sciences, Branford, Connecticut, USA) on a GS FLX 454 platform using GS XL70 sequencing chemistry. The sequences for each *Tulasnella* isolate were separately assembled using CLC Genomics Workbench software (CLC bio, Aarhus, Denmark) with the software's standard assembly parameters.

To design phylogenetic markers, we performed a de novo assembly within CLC with both *Tulasnella* isolates. This provided a consensus sequence to target high-homology sequence regions shared by both *Tulasnella* isolates. Some 3300 reads were shared out of approximately 20000 reads per species. To maximize sequence length only contigs of both species >200 bp (418 in total) were investigated further. To increase amplification success across the range of *Tulasnella*-orchid host species, GenBank BLAST searches (http://www.ncbi.nlm.nih.gov/) were conducted on the 418 contigs to locate other related sequences. The top BLAST hits to annotated or predicted genes from *Basidomycota* and *Ascomycota* fungi were downloaded and included in alignments within the program Geneious version 5.5.6 (Drummond et al., 2012) for 83 consensus sequences. Of these, we selected 30 consensus sequences to design primers; selection was based on product length, ease of primer design, and gene identity. Primers were designed using

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We thank our collaborators who kindly provided mycorrhizal isolates for cross-species transferability tests: Ryan Phillips (isolates from *Drakaea* and *Paracaleana*), Mark Clements and Chris Howard (isolates from *Corybas*, *Cryptostylis*, and *Rhizanthella*), Zoe Smith (*Tulasnella* isolates from *Diuris*), and Magali Wright (*Sebacina* isolates from *Caladenia*). This work was supported by the Australian Research Council (DP0451374 to R.P., LP098338 and LP110100408 to R.P. and C.C.L.).

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TABLE 1. Characteristics of phylogenetic primers for *Tulasnella* isolates in this study.

Locus	Primer sequences (5′–3′)	Top BLAST hit to annotated or predicted gene	*E*-value of top hit in GenBank	T_a (°C)	Fragment length (bp)	GenBank accession no.
C9522	F: AGATTCAACCKTCKGGTCGTTT	Methionine adenosyltransferase[1]	5e-57	66–48	~300	JX524464
	R: ATTGCGTAVGAGAGCTGRAC					JX524465
C14436*	F: ATGGACGGTACYGADGGTCTYG	ATP	1e-150	66–48	~600	JX524466
	R: CACGGAAGTAYTCNGCRATGG					JX524467
C4102	F: ATCAARTAYGCTGGCCTKCCTTGG	Glutamate synthase[1]	0.0	66–48	~1000	JX524468
	R: CGRCCGCCWGTCATGTACTCGCA					JX524469
C3304	F: TTGAAGTCACCGGGAAGAAC	ATP helicase[1]	1e-159	66–48	~800	JX524470
	R: CGGCGTTACGCTTSGTCT					JX524471
C4722	F: ATGARTGGAARGGGTGGATGC	O-acetyltransferase (CAS1)[1]	4e-14	66–48	~800	JX524472
	R: GCGTTKCGMAGGACGATGAA					JX524473
C12424	F: AGGCRAACCGRATGATRCGCTCCG	Isocitrate dehydrogenase[1]	9e-49	66–48	~500	JX524474
	R: GGTGCAGARATYACRGACTC					JX524475
C10499	F: CCCTCCGYTCYGACTCYTAYAC	26S proteasome regulatory complex[2]	7e-157	55	~500	JX524476
	R: ACTGATTCARCAACTCCAACATCG					JX524477

Note: T_a = annealing temperature.

* Mitochondrial marker.

[1] Source for gene identification: Stajich et al. (2010).

[2] Source for gene identification: Martin et al. (2008).

Primer3 (Rozen and Skaletsky, 2000). Twenty-one (70%) of the 30 primer pairs amplified in both target species. Consistent and high quality cross-genus amplification occurred for seven primer sets in *Tulasnella* from *Arthrochilus*, *Chiloglottis*, *Drakaea*, and *Paracaleana* (Table 1).

PCR reactions were performed in 30-µL reactions containing 1× PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3 final concentration; QIAGEN Gmbh, Hilden, Germany), 0.2 µM dNTPs, 5 µg bovine serum albumin (BSA) (New England Biolabs, Ipswich, Massachusetts, USA), 200 nM of each primer, 1 U of *Taq* polymerase (QIAGEN), and 20–100 ng of template DNA. A touchdown thermal profile was used for initial testing consisting of a 3-min denaturation at 94°C; followed by 12 touchdown cycles at 94°C (30 s), 66°C (40 s) (−3°C/second cycle), 72°C (1 min); 30 cycles at 94°C (30 s), 48°C (40 s), 72°C (1 min); and final extension at 72°C for 20 min. One locus performed better at a fixed annealing temperature of 55°C (Table 1). Products were sequenced bidirectionally with an ABI PRISM BigDye Terminator version 3.1 sequencing kit (Applied Biosystems, Carlsbad, California, USA) on an ABI 3100 automated sequencer. Sequences were edited using the program Sequencher version 4.7 (GeneCodes, Ann Arbor, Michigan, USA), aligned in Geneious, and estimates of variability were performed with MEGA5 (Tamura et al., 2011). The seven markers showed between 24% and 35% nucleotide diversity and 15% and 29% parsimony informative sites (Appendix 2). Amplification was also achieved for some loci in *Tulasnella* from other orchid genera including *Cryptostylis* R. Br., *Corybas* Salisb., and *Diuris* Sm., as well as for *Sebacina* Tul. & C. Tul. from *Caladenia* R. Br. and *Glossodia* R. Br., and *Ceratobasidium* D. P. Rogers from *Rhizanthella* R. S. Rogers (Appendix 2).

TABLE 2. Characteristics of microsatellite primers for *Tulasnella* isolates in this study.

Locus	Primer sequences (5′–3′)	Repeat motif	M13 label	T_a (°C)	Size range (bp)	GenBank accession no.
Tul 2	F: ACATCCGAAATCGAAACCAG	$(GTC)_9$	NED	66–48	252–270	JX514904
	R: GACCACAACCACAACCACAA					
Tul 4	F: CCCCTACCCTCTCCACACTT	$(TC)_{11}$	PET	66–48	223–231	JX514905
	R: TTGGTGACGAAGGTCTTTCC					
Tul 11	F: ACTGACACCGGAGAATTTCG	$(GTC)_9$	VIC	66–48	165–204	JX514906
	R: AAGCCCACAACCATAACGAC					
Tu1 12	F: GTACTCGATCTCGCCGATGT	$(GTC)_8$	NED	54	305–323	JX514907
	R: GGTGAAGTTCATGGCTTCGT					
Tul 13	F: GAACCTATCAGCGCCTGTGT	$(CT)_8$	PET	66–48	198–216	JX514908
	R: TTTGTGTTGGTTGTCCGAGA					
Tul 16	F: GGAATCACGACACCAAGAGG	$(GT)_8$	VIC	54	250–298	JX514909
	R: ACTCCCCAGATGCGTTAGAA					
Tul 17	F: ACTCGACCAGACGAGGAATG	$(AG)_8$	PET	66–48	310–322	JX514910
	R: GGACGACTTCGTGGCAGTAT					
Tul 23	F: TGTTGATGTGGCACCAAAAT	$(TTC)_8$	PET	54	363–396	JX514911
	R: AGTTGCCGTCCATCAGTTTC					
Tul 24	F: ATAGGGATTTCGGCCAACTT	$(TCG)_8$	VIC	66–48	326–344	JX514912
	R: CTGCTGGGAAAGGTGAAGAG					
Tul 65[†]	F: CCTCAATCTTCAGTTTACCGC	$(CAA)_5N_{36}(CAG)_7$	FAM	66–48	313–328	JX514913
	R: TTGTTTGACCCGTATCCCGTC					
Tul TGC6[†]	F: GTAAGTTGGGCCATTTGCAT	$(CTG)_3N_{19}(TGC)_5$	NED	52	239–266	JX514914
	R: TGTTTAACGCCCACAAACAA					

Note: T_a = annealing temperature.

[†] Loci obtained from a genomic library enriched for CAG repeats. Voucher information for isolates ran the 454 library: voucher no. CLM309, host *Chiloglottis* aff. *jeanesii*; voucher no. CLM009, host *Drakaea elastica*. Culture collections are located in the C. Linde laboratory at the Australian National University, Canberra (ANU).

***Microsatellite primer design*—**The 454 reads of *Tulasnella* from *C.* aff. *jeanesii* were screened for di-, tri-, tetra-, penta-, and hexanuclotide repeats using the online software MSATFINDER (http://www.genomics.ceh.ac.uk/msatfinder/). A total of 800 contigs with simple sequence repeats (SSR) were detected. Using the criterion of at least eight repeat units in the sequence, we designed 24 primer pairs using Primer3 (Rozen and Skaletsky, 2000). Two additional SSR loci were obtained via a genomic library enriched for CAG repeats with target clones identified by PCR using published methods (Adcock et al., 2005).

Screening of the loci was performed on several *Tulasnella* individuals from eight *Chiloglottis* species sourced from one or two geographic locations per species (Appendix 3). Forward primers had universal M13 tails added as per the method of Schuelke (2000). PCRs were performed in 30-μL reactions containing 1× PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3 final concentration; QIAGEN), 0.2 μM dNTPs, 5 μg BSA (New England Biolabs), 200 nM of nonlabeled primer, 50 nM of M13-labeled primer, 100 nM of 21M13 primer (labeled with FAM, NED, or VIC; Applied Biosystems), and 20–100 ng of template DNA. PCR was performed using the touchdown thermal profile described earlier. Some loci required further optimization of annealing temperatures (Table 2). Fragment analysis was performed on an ABI 3100 sequencer with amplified products mixed with a 500 LIZ (Applied Biosystems) size ladder, and genotyping determined using GeneMapper version 3.7 software (Applied Biosystems). Eleven loci (two loci from the genomic library) (Table 2) amplified reliably and were polymorphic for *Tulasnella* in all eight *Chiloglottis* host species. Allelic diversity and genotype analyses were performed using GenAlEx version 6.5 (Peakall and Smouse, 2012). All loci resulted in two alleles per locus, consistent with a dikaryotic haploid nature of related genera. Twenty-four genotypes were found among 42 *Tulasnella* isolates assayed. Genotypes were not shared among *Tulasnella* isolates from eight *Chiloglottis* species, or between sites within a host species (Appendix 4).

CONCLUSIONS

We successfully designed polymorphic coding and noncoding markers for *Tulasnella* mycorrhizal fungi from numerous species within four genera of Australian orchids. Some loci are also useful at higher taxonomic levels because they amplify and provide useful sequences for *Sebacina* and/or *Ceratobasidium*. We found that the microsatellite markers are sufficiently polymorphic to investigate species and population-level diversity of *Tulasnella* from *Chiloglottis* hosts. These newly developed polymorphic markers will be useful to investigate diversity, phylogenetic relationships, and specificity of the mycorrhizal–orchid associations.

LITERATURE CITED

Adcock, G. J., N. E. Langmore, R. A. Mulder, and R. M. Kilner. 2005. Microsatellite loci for population and behavioural studies of Horsfield's bronze-cuckoo (*Chalcites basalis*: Aves). *Molecular Ecology Notes* 5: 619–621.

Drummond, A. J., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, et al. 2012. Geneious v5.5.6 created by Biomatters. Website http://www.geneious.com [accessed 28 February 2012].

Martin, F., A. Aerts, D. Ahren, 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.

Peakall, R., and P. Smouse. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics (Oxford, England)* 28: 2537–2539.

Phillips, R. D., M. D. Barrett, K. W. Dixon, and S. D. Hopper. 2011. Do mycorrhizal symbioses cause rarity in orchids? *Journal of Ecology* 99: 858–869.

Roche, S. A., R. J. Carter, R. Peakall, L. M. Smith, M. R. Whitehead, and C. C. Linde. 2010. A narrow group of monophyletic *Tulasnella* (Tulasnellaceae) symbiont lineages are associated with multiple species of *Chiloglottis* (Orchidaceae): Implications for orchid diversity. *American Journal of Botany* 97: 1313–1327.

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

Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, et al. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences, USA* 109: 6241–6246.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Stajich, J. E., S. K. Wilke, D. Ahrén, C. H. Au, B. W. Birren, M. Borodovsky, C. Burns, et al. 2010. Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). *Proceedings of the National Academy of Sciences, USA* 107: 11889–11894.

Tamura, K., D. Peterson, N. Petersom, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.

Appendix 1. *Tulasnella*, *Sebacina*, and *Ceratobasidium* isolates from several Australian orchids used in this study to test cross transferability of phylogenetic markers.

Tulasnella isolated from												*Sebacina* isolated from			
Arthrochilus		*Chiloglottis*		*Drakaea*		*Paracaleana*		*Diuris*		Others		*Glossodia*		*Caladenia*	
9	*A. oreophilus*	3	*C.* aff. *jeanesii*	2	*D. concolor*	1	*P. hortiorum*	1	*D.* aff. *amplissima*	1	*Cryptostylis erecta*	2	*G. minor*	8	*Caladenia* spp.
		2	*C. diphylla*	3	*D. confluens*	1	*P. lyonsii*	1	*D. chryseopsis*	1	*C. hunteriana*	6	*G. major*		
		2	*C. formicifera*	3	*D. elastica*	5	*P. minor*	2	*D. fragrantissima*	1	*Corybas dowlingii*				
		1	*C. reflexa*	3	*D. glyptodon*	1	*P. terminalis*	2	*D. punctata*	1	*Rhizanthella*				
		4	*C. seminuda*	2	*D. gracilis*	1	*P. triens*	1	*D. pulchella*		*slateri**				
		11	*C. trapeziformis*	1	*D. isolata*			5	*Diuris* spp.						
		3	*C. trilabra*	3	*D. livida*										
		2	*C. valida*												

*A *Ceratobasidium* spp.

Appendix 2. Characteristics of phylogenetic markers in *Tulasnella* and *Sebacina* mycorrhizal fungi for three groups of orchid genera.[a]

	Group 1				Group 2				Group 3			
Locus	AS (%)	AL (bp)	Var. (%)	PI (%)	AS (%)	AL (bp)	Var. (%)	PI (%)	AS (%)	AL (bp)	Var. (%)	PI (%)
C9522[1]	100	299	29.1	15.7	94	247*	38.5	26.7	75	255	23.9	18.8
C14436[2]	95	625*	34.4	22.7	94	555*	56.4	40.0	100	555	10.8	6.7
C4102[3]	97	917*	35.3	26.8	NA[#]	919*	35.4	31.8				
C3304[4]	97	769	32.0	28.0	NA[#]	769	33.8	28.2				
C4722[3]	81	779	34.8	28.2	NA[#]	795	41.0	28.9				
C12424	98	440	33.4	28.9								
C10499	97	500	24.6	22.6								

Note: AL = aligned length; AS = amplification success; PI = parsimony informative sites; Var. = number of variable sites.

[a] Group 1 = *Tulasnella* from *Arthrochilus*, *Chiloglottis*, *Drakaea*, and *Paracaleana*; Group 2 = *Tulasnella* from *Arthrochilus*, *Chiloglottis*, *Drakaea*, *Paracaleana*, and other genera (locus dependent, see footnotes 1 to 4 below); Group 3 = *Sebacina* from *Glossodia major*, *G. minor*, and *Caladenia*. See Appendix 1 for the number of isolates amplified per genera.

* Small intronic regions that were difficult to align were removed in the alignment for final analyses.

[#] Sequencing not attempted for all isolates of each genera.

[1] Also sequenced *Tulasnella* from *Cryptostylis erecta*, *Corybas dowlingii*, *Diuris* aff. *amplissima*, *D. chryseopsis*, *D. fragrantissima*, *D. pulchella*, *D. punctata*, *Diuris* sp., and *Sebacina* from *Glossodia minor*, *G. major*, and *Caladenia* spp.

[2] Also sequenced *Tulasnella* from *Cryptostylis erecta*, *C. hunteriana*, *Corybas dowlingii*, *Diuris* aff. *amplissima*, *D. chryseopsis*, *D. fragrantissima*, *D. pulchella*, *D. punctata*, *Diuris* spp., and *Sebacina* from *Glossodia minor*, *G. major*, and *Caladenia* spp. and *Ceratobasidium* from *Rhizanthella slateri*.

[3] Also sequenced *Tulasnella* from *D. punctata*.

[4] Also sequenced *Tulasnella* from *Cryptostylis erecta*.

Appendix 3. Collection details and microsatellite genotypes of *Tulasnella* isolates from *Chiloglottis*.

Isolate	Host species	Site	GPS	SSR genotype no.
CLM362	*C. trapeziformis*	Black Mountain, ACT	S35.2749	12
CLM367	*C. trapeziformis*		E149.0976	12
CLM366	*C. trapeziformis*			12
CLM371	*C. trapeziformis*			12
CLM372	*C. trapeziformis*			10
CLM405	*C. trapeziformis*	Australian National Botanical Gardens, ACT	S35.2751	9
			E149.1097	
CLM377	*C.* aff. *jeanesii*	Kanangra Boyd National Park, NSW	S33.9409	22
CLM380	*C.* aff. *jeanesii*		E150.0552	23
CLM389	*C.* aff. *jeanesii*			22
CLM309	*C.* aff. *jeanesii*			24
CLM150	*C.* aff. *jeanesii*	Tallaganda State Forest, NSW	S35.5056	16
CLM310	*C.* aff. *jeanesii*		E149.5351	16
CLM312	*C.* aff. *jeanesii*			17
CLM395	*C. valida*	Kanangra Boyd National Park, NSW	S33.9409	20
CLM396	*C. valida*		E150.0552	20
CLM393	*C. valida*			19
CLM394	*C. valida*			19
CLM305	*C. formicifera*	Upper Kangaroo Valley, NSW	S34.6537	3
CLM306	*C. formicifera*		E150.6016	4
CLM308	*C. formicifera*			4
CLM058	*C. seminuda*	Mt. Werong, NSW	S34.1385	18
CLM059	*C. seminuda*		E149.9722	18
CLM061	*C. seminuda*			18
CLM047	*C. seminuda*			11
CLM064	*C. seminuda*			13
CLM313	*C. seminuda*	Penrose Forest, Exeter, NSW	S34.6295	14
CLM316	*C. seminuda*		E 150.1539	14
CLM317	*C. seminuda*			14
CLM044	*C. seminuda*	Fitzroy Falls, NSW	S34.6477	8
CLM057	*C. seminuda*		E150.480	21
CLM155	*C. trilabra*	Mt. Werong, NSW	S34.1385	15
CLM156	*C. trilabra*		E149.9722	15
CLM157	*C. trilabra*			15

Appendix 3. Continued.

Isolate	Host species	Site	GPS	SSR genotype no.
CLM160	*C. trilabra*			15
CLM068	*C. diphylla*	Bilpin, NSW	S33.5154	1
CLM069	*C. diphylla*		E150.4886	1
CLM341	*C. diphylla*			6
CLM361	*C. diphylla*			7
CLM152	*C. reflexa*	Mt. Wilson, NSW	S33.5211	2
CLM153	*C. reflexa*		E150.3707	2
CLM346	*C. reflexa*			2
CLM353	*C. reflexa*	Mt. Wilson, NSW	S33.4997 E150.4144	5

Note: ACT = Australian Capital Territory; NSW = New South Wales.

Appendix 4. Characteristics of 11 microsatellite loci developed for *Tulasnella* mycorrhizal fungi from the orchid host genus *Chiloglottis*.

	C. trapeziformis (*n* = 6)		*C.* aff. *jeanesii* (*n* = 7)		*C. valida* (*n* = 4)		*C. formicifera* (*n* = 3)		*C. seminuda* (*n* = 10)		*C. trilabra* (*n* = 4)		*C. diphylla* (*n* = 4)		*C. reflexa* (*n* = 4)	
Locus	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*	Size range (bp)	*A*
Tul 2	252	1	252–261	3	255–270	4	252	1	252–261	4	252	1	252	1	252	1
Tul 4	223–224	2	223–231	3	NA	NA	223	1	223–225	3	223	1	223	1	223	1
Tul 11	165–189	2	165–174	3	168–174	3	165	1	165–204	5	165	1	165	1	165	1
Tul 12	305–317	4	305–323	5	308–323	4	311	1	308–317	4	311	1	311	1	311	1
Tul 13	204–210	3	206–210	2	204–210	3	206	1	198–216	5	206	1	202–208	3	204–206	2
Tul 16	252–298	5	270–298	5	270–298	2	256–268	2	270–298	6	279–286	2	256–298	3	250–296	4
Tul 17	310–316	2	312–322	3	312–320	3	316	1	312–316	3	316	1	310–318	3	310–316	2
Tul 23	364–387	5	363–381	5	364–367	2	364–384	2	363–384	4	366–381	2	366–381	2	364–396	4
Tul 24	338–343	2	338–344	2	338	1	338	1	326–341	3	338	1	338	1	338	1
Tul 65	313–328	2	313–314	2	313	1	313	1	313	1	313	1	316	1	313	1
Tul TCG6	242–252	3	251–252	2	251–252	2	251–252	2	239–257	6	251–252	2	242–260	4	239–266	2
No. of genotypes*	3		5		2		2		6		1		3		2	

Note: *A* = number of alleles; *n* = number of isolates studied for each orchid species.
* Number of *Tulasnella* genotypes observed in each of the orchid species studied for *n* isolates.