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Authors: Ruibal, Monica P., Peakall, Rod, Smith, Leon M., and Linde, Celeste C.

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PHYLOGENETIC AND MICROSATELLITE MARKERS FOR *TULASNELLA* (*TULASNELLACEAE*) MYCORRHIZAL FUNGI ASSOCIATED WITH AUSTRALIAN ORCHIDS¹

MONICA P. RUIBAL^{2,3}, ROD PEAKALL², LEON M. SMITH², AND CELESTE C. LINDE²

²Evolution, Ecology and Genetics, Research School of Biology, Australian National University, Canberra, Australian Capital Territory 0200, Australia

- **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 Cantharrellales) is an endophytic fungus occurring in the roots, stems, or protocorms 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 *Basidiomycota* 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.).

³Author for correspondence: monica.ruibal@anu.edu.au

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 R: ATTGCGTAVGAGAGCTGRAC | Methionine adenosyltransferase ¹ | 5e-57 | 66–48 | ~300 | JX524464 JX524465 |
| C14436* | F: ATGGACGGTACYGADGGTCTYG R: CACGGAAGTAYTCNGCRATGG | ATP | 1e-150 | 66–48 | ~600 | JX524466 JX524467 |
| C4102 | F: ATCAARTAYGCTGGCCTKCCTTGG R: CGRCCGCCWGTTCATGTACTCGCA | Glutamate synthase ¹ | 0.0 | 66–48 | ~1000 | JX524468 JX524469 |
| C3304 | F: TTGAAGTCACCGGGAAGAAC R: CGGCGTTACGCTTSGTCT | ATP helicase ¹ | 1e-159 | 66–48 | ~800 | JX524470 JX524471 |
| C4722 | F: ATGARTGGAARGGGTGGATGC R: GCGTTKCGMAGGACGATGAA | O-acetyltransferase (CAS1) ¹ | 4e-14 | 66–48 | ~800 | JX524472 JX524473 |
| C12424 | F: AGGCRAACCGRATGATRCGCTCCG R: GGTGCAGARATYACRGACTC | Isocitrate dehydrogenase ¹ | 9e-49 | 66–48 | ~500 | JX524474 JX524475 |
| C10499 | F: CCCTCCGYTCYACTCYTAYAC R: ACTGATTCARCAACTCCAACATCG | 26S proteasome regulatory complex ² | 7e-157 | 55 | ~500 | JX524476 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 MgCl₂, 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 R: GACCACAACCACAACCACAA | (GTC) ₉ | NED | 66–48 | 252–270 | JX514904 |
| Tul 4 | F: CCCCTACCCCTCCACACTT R: TTGGTGACGAAGGTCTTTCC | (TC) ₁₁ | PET | 66–48 | 223–231 | JX514905 |
| Tul 11 | F: ACTGACACCGGAGAATTTTCG R: AAGCCCACAACCATAACGAC | (GTC) ₉ | VIC | 66–48 | 165–204 | JX514906 |
| Tul 12 | F: GTACTCGATCTCGCCGATGT R: GGTGAAGTTCATGGCTTCGT | (GTC) ₈ | NED | 54 | 305–323 | JX514907 |
| Tul 13 | F: GAACCTATCAGCGCCTGTGT R: TTTGTGTTGGTTGTCCGAGA | (CT) ₈ | PET | 66–48 | 198–216 | JX514908 |
| Tul 16 | F: GGAATCAGACACCAAGAGG R: ACTCCCCAGATGCGTTAGAA | (GT) ₈ | VIC | 54 | 250–298 | JX514909 |
| Tul 17 | F: ACTCGACACGACGAGGAATG R: GGACGACTTTCGTGGCAGTAT | (AG) ₈ | PET | 66–48 | 310–322 | JX514910 |
| Tul 23 | F: TGTGATGTGGCCACCAAAAT R: AGTTGCCGTCCATCAGTTTC | (TTC) ₈ | PET | 54 | 363–396 | JX514911 |
| Tul 24 | F: ATAGGGATTTTCGGCCAACTT R: CTGCTGGGAAAGGTGAAGAG | (TCG) ₈ | VIC | 66–48 | 326–344 | JX514912 |
| Tul 65 [†] | F: CCTAATCTTCAGTTTACCGC R: TTGTTTGACCCGTATCCCGTC | (CAA) ₅ N ₃₆ (CAG) ₇ | FAM | 66–48 | 313–328 | JX514913 |
| Tul TGC6 [†] | F: GTAAGTTGGGCCATTTGCAT R: TGTTTAACGCCCAACAACAA | (CTG) ₃ N ₁₉ (TGC) ₅ | NED | 52 | 239–266 | JX514914 |

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 hexanucleotide 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- μ L reactions containing 1 \times PCR buffer (1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3 final concentration; QIAGEN), 0.2 μ M dNTPs, 5 μ g 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.

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

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

*A *Ceratobasidium* spp.

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APPENDIX 2. Characteristics of phylogenetic markers in *Tulasnella* and *Sebacina* mycorrhizal fungi for three groups of orchid genera.^a

| Locus | Group 1 | | | | Group 2 | | | | Group 3 | | | |
|---------------------|---------|---------|----------|--------|-----------------|---------|----------|--------|---------|---------|----------|--------|
| | 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.

^aGroup 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 | <i>C. trapeziformis</i> | Black Mountain, ACT | S35.2749 | 12 |
| CLM367 | <i>C. trapeziformis</i> | | E149.0976 | 12 |
| CLM366 | <i>C. trapeziformis</i> | | | 12 |
| CLM371 | <i>C. trapeziformis</i> | | | 12 |
| CLM372 | <i>C. trapeziformis</i> | | | 10 |
| CLM405 | <i>C. trapeziformis</i> | Australian National Botanical Gardens, ACT | S35.2751 | 9 |
| | | | E149.1097 | |
| CLM377 | <i>C. aff. jeanesii</i> | Kanangra Boyd National Park, NSW | S33.9409 | 22 |
| CLM380 | <i>C. aff. jeanesii</i> | | E150.0552 | 23 |
| CLM389 | <i>C. aff. jeanesii</i> | | | 22 |
| CLM309 | <i>C. aff. jeanesii</i> | | | 24 |
| CLM150 | <i>C. aff. jeanesii</i> | Tallaganda State Forest, NSW | S35.5056 | 16 |
| CLM310 | <i>C. aff. jeanesii</i> | | E149.5351 | 16 |
| CLM312 | <i>C. aff. jeanesii</i> | | | 17 |
| CLM395 | <i>C. valida</i> | Kanangra Boyd National Park, NSW | S33.9409 | 20 |
| CLM396 | <i>C. valida</i> | | E150.0552 | 20 |
| CLM393 | <i>C. valida</i> | | | 19 |
| CLM394 | <i>C. valida</i> | | | 19 |
| CLM305 | <i>C. formicifera</i> | Upper Kangaroo Valley, NSW | S34.6537 | 3 |
| CLM306 | <i>C. formicifera</i> | | E150.6016 | 4 |
| CLM308 | <i>C. formicifera</i> | | | 4 |
| CLM058 | <i>C. seminuda</i> | Mt. Werong, NSW | S34.1385 | 18 |
| CLM059 | <i>C. seminuda</i> | | E149.9722 | 18 |
| CLM061 | <i>C. seminuda</i> | | | 18 |
| CLM047 | <i>C. seminuda</i> | | | 11 |
| CLM064 | <i>C. seminuda</i> | | | 13 |
| CLM313 | <i>C. seminuda</i> | Penrose Forest, Exeter, NSW | S34.6295 | 14 |
| CLM316 | <i>C. seminuda</i> | | E 150.1539 | 14 |
| CLM317 | <i>C. seminuda</i> | | | 14 |
| CLM044 | <i>C. seminuda</i> | Fitzroy Falls, NSW | S34.6477 | 8 |
| CLM057 | <i>C. seminuda</i> | | E150.480 | 21 |
| CLM155 | <i>C. trilabra</i> | Mt. Werong, NSW | S34.1385 | 15 |
| CLM156 | <i>C. trilabra</i> | | E149.9722 | 15 |
| CLM157 | <i>C. trilabra</i> | | | 15 |

APPENDIX 3. Continued.

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

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*.

| Locus | <i>C. trapeziformis</i> (n = 6) | | <i>C. aff. jeanesii</i> (n = 7) | | <i>C. valida</i> (n = 4) | | <i>C. formicifera</i> (n = 3) | | <i>C. seminuda</i> (n = 10) | | <i>C. trilabra</i> (n = 4) | | <i>C. diphylla</i> (n = 4) | | <i>C. reflexa</i> (n = 4) | |
|-------------------|------------------------------------|---|------------------------------------|---|-----------------------------|----|----------------------------------|---|--------------------------------|---|-------------------------------|---|-------------------------------|---|------------------------------|---|
| | 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.