Microsatellite markers for Corybas (Orchidaceae) species in New Zealand

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PREMISE OF THE STUDY: Microsatellite markers were developed for New Zealand species of Corybas (Orchidaceae) to investigate population genetics and species delimitation.

METHODS AND RESULTS: From sequencing a total genomic DNA library (using Illumina MiSeq), we developed 22 microsatellite markers for C. obscurus. The di- and trinucleotide repeat loci were initially trialed on individuals representing seven Corybas taxa (C. “rimutaka,” C. confusus, C. hypogaeus, C. macranthus, C. obscurus, C. trilobus, and C. walliae) and had one to eight alleles per locus. Twelve polymorphic markers were further tested on six Corybas populations from three of the seven taxa (C. obscurus, C. “rimutaka,” and C. trilobus). Observed and expected heterozygosities ranged from 0–1 and 0–0.859, respectively. The utility of these 12 loci was further validated in five related Corybas species (C. hypogaeus, C. obscurus, C. vitreus, C. walliae, and C. “rimutaka”; 38 individuals) representing populations from across the North and South Islands. The average value for genetic diversity among populations (\(F_{st}\)) of 0.439 shows differentiation among species.

CONCLUSIONS: These markers will be useful for future studies aimed at delimiting species boundaries and examining the genetic diversity of the New Zealand Corybas species.

KEY WORDS: Corybas; Corybas obscurus; microsatellite; New Zealand; Orchidaceae; spider orchid.

Corybas Salisb. is a diverse genus of terrestrial orchids that includes ca. 150 species that are widespread across Australasia and Southeast Asia (Lyon, 2014). These orchids consist of a single leaf and flower, making the entire plant only a few centimeters tall and rather inconspicuous. Some grow in sympatry and can also form dense and extensive intermingling clonal populations where hybridization may occur. In New Zealand, there are currently 21 accepted species of Corybas (Breitwieser et al., 2017). This number is likely to increase as there are several morphologically distinct entities known only by tag names that require genetic assessment to fully confirm their taxonomic status. This situation is not unique to New Zealand Corybas; a number of species aggregates have also been detected in Australian Corybas (Brown et al., 2008).

A recent study described five new species of Corybas endemic to New Zealand (Lehnebach et al., 2016) and identified at least two other morphologically distinct entities. One of the new species, C. obscurus Lehnebach, is considered “At Risk – Naturally Uncommon” in the list of Threatened and Uncommon Plants of New Zealand (de Lange et al., 2018) because it is restricted to a small area in the South Island. The undescribed entity C. “rimutaka” also occurs in this area, but chloroplast and nuclear sequence data were unable to discriminate this taxon from some samples of C. obscurus and the sympatric species C. hypogaeus (Colenso) Lehnebach (Lehnebach et al., 2016). These plants are all part of the C. trilobus (Hook. f.) Rchb. f. species aggregate. Floral characters are particularly variable within this species aggregate, and it is likely that several taxa are included under this name (St. George, 2008). A few of these nameless orchids are also considered “Threatened” or “At Risk” (de Lange et al., 2018).

Understanding the distribution of the morphological variation in New Zealand Corybas and determining taxonomic status is critical to ensure survival and suitable management of their populations. Genetic markers will aid in delimiting species boundaries and linking patterns of morphological and genetic variation. Therefore, we have developed a set of microsatellite markers that will be used in future studies to delimit species boundaries between closely related entities and detect gene flow between co-occurring species.

METHODS AND RESULTS

DNA was extracted from C. obscurus (WELT-SP104152; Appendix 1) using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). From sequencing a total genomic DNA library (using Illumina MiSeq), we developed 22 microsatellite markers for C. obscurus. The di- and trinucleotide repeat loci were initially trialed on individuals representing seven Corybas taxa (C. “rimutaka,” C. confusus, C. hypogaeus, C. macranthus, C. obscurus, C. trilobus, and C. walliae) and had one to eight alleles per locus. Twelve polymorphic markers were further tested on six Corybas populations from three of the seven taxa (C. obscurus, C. “rimutaka,” and C. trilobus). Observed and expected heterozygosities ranged from 0–1 and 0–0.859, respectively. The utility of these 12 loci was further validated in five related Corybas species (C. hypogaeus, C. obscurus, C. vitreus, C. walliae, and C. “rimutaka”; 38 individuals) representing populations from across the North and South Islands. The average value for genetic diversity among populations (\(F_{st}\)) of 0.439 shows differentiation among species.

CONCLUSIONS: These markers will be useful for future studies aimed at delimiting species boundaries and examining the genetic diversity of the New Zealand Corybas species.

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Germany) with slight modifications of the manufacturer's protocol (0.5% β-mercaptoethanol [BME] added to Buffer AP1; incubated at 65°C for 15 min; chilled on ice for 10 min). A DNA library was prepared using the Illumina TruSeq Library Preparation Kit (Illumina, San Diego, California, USA) following manufacturer's protocols. The indexed library was pooled with three other libraries (Fuchsia excorticata [Onagraceae; Van Etten et al., 2013], Sophora microphylla [Fabaceae; Van Etten et al., 2014], and Korthalsella salicornioides [Salicaceae; S. M. Pearson et al., unpublished]) in equal concentration and sequenced via Illumina MiSeq (Illumina) using 250-bp paired-end chemistry (New Zealand Genomics Limited, Palmerston North, New Zealand). The resulting 2.6 million sequences (991 million base pairs) were trimmed of low-quality results using a 0.01 quality cut-off in DynamicTrim in SolexaQA (Cox et al., 2010), and the remaining sequences were assembled using Velvet version 1.1 (Zerbino and Birney, 2008). The raw data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; accession SRP150798).

Plastid and mitochondrial sequences were removed by performing BLAST searches against related organellar sequences in GenBank (plastid: Phalaenopsis aphrodite Rchb. f. [Orchidaceae], NC_007499; mitochondria: Tripsacum dactyloides (L.) L. [Poaceae], NC_007499).

### TABLE 1. Characteristics of 22 microsatellite loci developed for New Zealand Corybas.

| Locusa | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | GenBank accession no. |
|--------|------------------------|--------------|------------------------|----------------------|
| Corybas-05 | F: CTCTCTGACCTTGGATC (AG)n | (AG)n | 1 333 | 3 331–333 |
| R: TCCCAAGCAAGACTCTGGAG | | | | MF076670 |
| Corybas-07 | F: CCCAGTGACCAAAGTAGAG (AG)n | (AG)n | 1 334 | 3 332–336 |
| R: TCATGGAACCTGGATAGTGG | | | | MF076671 |
| Corybas-09 | F: GCAACCTTGTGTCAATGTC | (AT)n | 1 372 | 2 369–372 |
| R: GCACCTAATGGCATTGG | | | | MF076672 |
| Corybas-12 | F: TCACAACTGGAAATGACCCGAC | (AAG)n | 1 311–320 | 4 311–320 |
| R: AGGCCAACATCAACTC | | | | MF076673 |
| Corybas-16 | F: ACTCATAGGGGCTTGTTG | (AT)n | 1 269 | 4 269–275 |
| R: AGAACAATCAAAATGACCG | | | | MF076674 |
| Corybas-18 | F: AAATGACAGTGAAGGCCAG | (AAG)n | 2 298–301 | 3 298–301 |
| R: TCATAATTGATGGGCTTGG | | | | MF076675 |
| Corybas-19 | F: GTTGGCCCTATCAAATATGC | (AT)n | 1 267 | 3 251–267 |
| R: TCCTCATTGCTGTC | | | | MF076676 |
| Corybas-22 | F: AGATTGGCATGCTGTAG | (AG)n | 2 209–211 | 2 209–211 |
| R: GAAGCTTCTGTCATCTGC | | | | MF076677 |
| Corybas-23 | F: ACAATGATGACTCAAATATGC | (AT)n | 2 342–354 | 2 352–354 |
| R: TGCTATGATGAGTACAGTC | | | | MF076678 |
| Corybas-24 | F: CTTTGGGAGTCTCTGC | (AT)n | 1 247 | 4 245–269 |
| R: CTATGAAATTGCTGAGGG | | | | MF076679 |
| Corybas-27 | F: TTGGCAGCTATGTGAGTACG | (AT)n | 1 240 | 3 238–242 |
| R: TGACAGGTATGAGGAAAACG | | | | MF076680 |
| Corybas-28 | F: GGATGTGGCTGTATTTTG | (AT)n | 1 198 | 3 196–203 |
| R: CAGGTTAAGCCAGATTCC | | | | MF076681 |
| Corybas-32 | F: TTGGCAAGGGTTAATAGTC | (AT)n | 1 162 | 7 212–212 |
| R: ATAAAGAGATCTAGTCACC | | | | MF076682 |
| Corybas-33 | F: TCACGCCATCGAATAGTG | (AG)n | 2 164–166 | 2 164–166 |
| R: AATGTCCATTCACTAGTTCAGG | | | | MF076683 |
| Corybas-36 | F: AGCCCTCTACTATTGTCG | (AT)n | 1 152 | 4 146–152 |
| R: TGACATTTGCTAATCTTC | | | | MF076684 |
| Corybas-41 | F: TATGTGTGGGCGTCTCTGC | (AG)n | 2 155–157 | 2 155–157 |
| R: CGGGATTCTCTCTCTT | | | | MF076685 |
| Corybas-42 | F: AAGGTACCTTGTGAGGTCG | (AG)n | 1 333 | 3 325–345 |
| R: TCTTGTGAGTTGAAGGCC | | | | MF076686 |
| Corybas-44 | F: GTGACGTATGTTGTGCTG | (AT)n | 1 214–218 | 6 218–248 |
| R: TTTCCAGACAGATGCAGTG | | | | MF076687 |
| Corybas-45 | F: CATTTCGGCGACAACCTC | (AG)n | 2 264–266 | 8 262–284 |
| R: ACCAGCGTGATATCAAG | | | | MF076688 |
| Corybas-46 | F: TAGATTGTGAGATCGCGAG | (AT)n | 1 187 | 2 184–187 |
| R: ATGCCAATCATCAGTGG | | | | MF076689 |
| Corybas-47 | F: GTATGATGTTGAGGCGCAG | (AAC)n | 2 332–346 | 8 317–349 |
| R: CCATAGGCGAAAGTTTTGG | | | | MF076690 |
| Corybas-48 | F: ACACCCTAAATAGGGCGAAG | (ATC)n | 1 178–187 | 8 178–247 |
| R: CGGATAAGGAGTGCATTC | | | | MF076691 |

Note: A = number of alleles.

*Annealing temperature for all loci was 53°C.

*Corybas confusus, C. hypogaeus, C. macranthus, C. obscurus, C. “rimutaka,” C. trilobus, and C. walliae.
| Locus | Site 1 (n = 5) | Site 2 (n = 21) | Site 3 (n = 21) | Site 4 (n = 16) | Site 5 (n = 16) | Total (n = 73) |
|-------|---------------|----------------|----------------|----------------|----------------|---------------|
|       | Allele size   | Allele size   | Allele size   | Allele size   | Allele size   | Allele size   |
|       | A             | H₁           | A             | H₁           | A             | A             |
| Corybas-07 | 305–334 bp  | 305–334 bp  | 305–334 bp  | 305–334 bp  | 305–334 bp  | 305–334 bp  |
| Corybas-12 | 311–314 bp  | 311–314 bp  | 311–314 bp  | 311–314 bp  | 311–314 bp  | 311–314 bp  |
| Corybas-16 | 326–331 bp  | 326–331 bp  | 326–331 bp  | 326–331 bp  | 326–331 bp  | 326–331 bp  |
| Corybas-19 | 267–277 bp  | 267–277 bp  | 267–277 bp  | 267–277 bp  | 267–277 bp  | 267–277 bp  |
| Corybas-23 | 352–354 bp  | 352–354 bp  | 352–354 bp  | 352–354 bp  | 352–354 bp  | 352–354 bp  |
| Corybas-24 | 246–247 bp  | 246–247 bp  | 246–247 bp  | 246–247 bp  | 246–247 bp  | 246–247 bp  |
| Average | 320–332 bp  | 320–332 bp  | 320–332 bp  | 320–332 bp  | 320–332 bp  | 320–332 bp  |

Note: n = number of alleles; A = total number of alleles; H₁ = observed heterozygosity; H_o = expected heterozygosity; m = number of sampled individuals.

Significance of deviation from Hardy-Weinberg equilibrium: *P < 0.05, **P < 0.01, ***P < 0.001.

Primers were tested initially on seven individuals from a range of named species and one tag-named entity from New Zealand (C. "rimutaka," C. confusus Lehnebach, C. hypogaeus, C. macranthus (Hook. f.) Rchb. f., C. obscurus, C. trilobus, and C. walliae Lehnebach; Appendix 1). DNA was extracted from silica-dried leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). The 10-μL PCR cocktail contained 5–50 ng of DNA, 0.02 μM of forward primer, 0.45 μM of reverse primer, 0.45 μM of M13 primer (labeled with FAM, NED, or VIC), 1.5 mM of MgCl₂, 1× buffer BD (Solis BioDye, Tartu, Estonia), 250 μM of each dNTP, and 0.5 units of Firepol Taq polymerase (Solis BioDye). The PCR cycling program had an initial denaturation of 95°C for 3 min; 35 cycles of 95°C for 30 s, 53°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products (0.14–1.25 μL) for two to three loci of distinguishable sizes and labeled with different fluorophores were co-loaded and added to 9 μL of Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) and 1 μL of CASS ladder (Symonds and Lloyd, 2004) for subsequent fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) (Massey Genome Service at Massey University, Palmerston North, New Zealand). Alleles were visualized and scored using GeneMapper version 3.7 (Applied Biosystems).

Of the 48 primer pairs trialed, seven did not amplify, three were unscorable, four were monomorphic, 31 were polymorphic within an individual, and three were polymorphic among species. Twenty-two loci (Table 1) successfully amplified across all taxa. The number of alleles per locus ranged from one to two in the C. obscurus sample and from two to eight in the six other taxa. From these, the 12 most polymorphic loci were used for preliminary population genetic analyses on three populations of C. obscurus, one population of C. “rimutaka,” and two populations of C. trilobus (Table 2, Appendix 1); DNA extraction and locus amplification were as described above. We aimed to sample 15–20 individuals per population, but because of the small and precarious nature of the C. obscurus populations, only five individuals per population were included of that species. The total number of alleles, observed heterozygosity (H₁), and expected heterozygosity (H_e) were determined using GenAIEx 6.501 (Peakall and Smouse, 2006). Deviation from Hardy–Weinberg equilibrium (HWE) was determined using the Markov chain method provided by Web version 4.2 of GENEPOP software (Rousset, 2008). The number of alleles ranged from 3–22 (average of 8.8) per locus, H₁ from 0–1 (average of 0.452), and H_e from 0–0.859 (average of 0.390) (Table 2). All loci except Corybas-19 deviated significantly from HWE in at least one population. These deviations were usually a lower than expected H_e suggesting population substructure due to genetic drift.
C. walliae
C. vitreus
C. obscurus

Locality and voucher information are provided in Appendix 1.

Note: A = number of alleles; A = total number of alleles; n = number of sampled individuals.

To test the transferability of the markers for use in species delimitation, 38 individuals from both the North and South Island were chosen, representing four species and one tag-named entity (C. hypogaeus, C. obscurus, C. vitreus Lehnebach, C. walliae, and C. "rimutaka"); 3–16 individuals per taxon; Appendix 1). For these, we amplified the 12 novel microsatellite markers and genotyped them as described above. GenAlEx 6.501 was used to determine the percentage of successful amplifications per locus and FST. Amplification success rate was 95.8% on average, ranging from 81.58% to 100% amplification across all taxa (Table 3). Alleles ranged from 2.8–5 per species, with an average of 8.4 across all loci and species.

CONCLUSIONS

We developed 22 polymorphic microsatellite markers from C. obscurus that amplified to varying degrees in seven congeneric species and one undescribed entity. Twelve markers amplified reliably across seven species and were further tested on multiple populations and species to test their amplification across species and potential utility for population genetics. Due to the high success rate of amplification and the number of polymorphic loci, these markers will be informative for population genetics, mating system analysis, species delimitation, and determining the extent of hybridization within populations of mixed species. As such, these markers will facilitate the development of a conservation strategy for these species in New Zealand, as well as Australia.

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DATA ACCESSIBILITY

The raw data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession SRP150798); primer sequences were uploaded to GenBank, and accession numbers are provided in Table 1.

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APPENDIX 1. Voucher and locality information for the New Zealand Corybas samples used in this study.

| Species/form          | n   | Voucher no. | Population                                           |
|-----------------------|-----|-------------|-----------------------------------------------------|
| Corybas "rimutaka"    | 1   | WELT-SP105870* | North Island, Rimutaka Forest Park                  |
| Corybas "rimutaka"    | 1   | WELT-SP105871* | North Island, Rimutaka Forest Park                  |
| Corybas "rimutaka"    | 21  | WELT-SP104172d | North Island, Wellington, East Harbour Regional Park |
| Corybas "rimutaka"    | 3   | WELT-SP105940* | North Island, Wellington, QEII Covenant, Eastborne  |
| Corybas "rimutaka"    | 1   | WELT-SP104159* | South Island, Mt. Cook National Park                |
| Corybas "rimutaka"    | 1   | WELT-SP105872* | South Island, Nelson Lakes, Six Mile Creek          |
| Corybas confusus Lehnebach | 1 | WELT-SP104160* | South Island, Mt. Cook National Park                |
| Corybas hypogaeus (Colenso) Lehnebach | 1 | WELT-SP104185* | North Island, Hawke’s Bay, Boundary Stream Mainland Island |
| Corybas hypogaeus      | 1   | WELT-SP104417* | North Island, Te Urewera National Park              |
| Corybas hypogaeus      | 1   | WELT-SP104177* | North Island, Ohakune, Tongariro National Park      |
| Corybas hypogaeus      | 1   | WELT-SP104416* | South Island, Nelson Lakes National Park            |
| Corybas hypogaeus      | 1   | WELT-SP105873* | South Island, Nelson Lakes National Park            |
| Corybas hypogaeus      | 1   | WELT-SP105874* | South Island, Nelson Lakes National Park, Rainbow Ski field |
| Corybas macranthus (Hook. f.) Rchb. f. | 1 | WELT-SP105875* | South Island, Nelson Lakes National Park            |
| Corybas obscurus Lehnebach | 1 | WELT-SP104152* | South Island, Nelson Lakes National Park            |
| Corybas obscurus       | 5   | WELT-SP104152* | South Island, Nelson Lakes National Park, Site 1    |
| Corybas obscurus       | 5   | WELT-SP106571* | South Island, Nelson Lakes National Park, Site 3    |
| Corybas obscurus       | 5   | WELT-SP106570* | South Island, Nelson Lakes National Park, Site 4    |
| Corybas trilobus (Hook. f.) Rchb. f. | 21 | WELT-SP104195* | North Island, Whanganui, Gordon Park Scenic Reserve |
| Corybas trilobus       | 16  | WELT-SP104181* | North Island, Whanganui, Gordon Park Scenic Reserve |
| Corybas vitreus Lehnebach | 1 | WELT-SP105876* | South Island, Richmond Forest Park, Inwood Lookout  |
| Corybas vitreus        | 1   | WELT-SP107154* | South Island, Glenorchy, Glacier Burn track         |
| Corybas vitreus        | 1   | WELT-SP107155* | South Island, Nelson Lakes, Rainbow Station         |
| Corybas walliae Lehnebach | 2 | WELT-SP104410* | North Island, Egmont National Park                  |
| Corybas walliae        | 1   | WELT-SP104178* | North Island, Ruahine Ranges Forest Park            |
| Corybas walliae        | 1   | WELT-SP104175* | North Island, Tongariro National Park               |
| Corybas walliae        | 1   | WELT-SP105877* | South Island, Glen Hope Scenic Reserve              |
| Corybas walliae        | 2   | WELT-SP104391* | South Island, Kahurangi National Park               |
| Corybas walliae        | 1   | WELT-SP104151* | South Island, Nelson Lakes National Park            |

Note: n = number of individuals genotyped.

*One voucher was collected from each population used; vouchers are deposited in WELT. Latitude and longitude are not provided to suppress detailed locality information.

Additional notes:
- Individuals used for library construction.
- Individuals used for individual tests of amplification and polymorphism.
- Individuals used for population analyses.
- Individuals used to test markers on different forms and a wider range of sampling across the North and South Islands.