Microsatellite Markers for the New Zealand Endemic Myosotis pygmaea Species Group (Boraginaceae) Amplify Across Species

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Microsatellite markers for the New Zealand endemic
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• Premise of the study: Microsatellite loci were developed as polymorphic markers for the New Zealand endemic Myosotis pygmaea species group (Boraginaceae) for use in species delimitation and population and conservation genetic studies.

• Methods and Results: Illumina MiSeq sequencing was performed on genomic DNA from seedlings of M. drucei. From trimmed paired-end sequences >400 bp, 484 microsatellite loci were identified. Twelve of 48 microsatellite loci tested were found to be polymorphic and consistently scorable when screened on 53 individuals from four populations representing the geographic range of M. drucei. They also amplify in all other species in the Myosotis species group, i.e., M. antarctica, M. brevis, M. glauca, and M. pygmaea, as well as 18 other Myosotis species.

• Conclusions: These 12 polymorphic microsatellite markers establish an important resource for research and conservation of the Myosotis species group and potentially other Southern Hemisphere Myosotis.

Key words: Boraginaceae; forget-me-nots; microsatellites; Myosotis; New Zealand; threatened species.

Forget-me-nots (Myosotis L., Boraginaceae) are found in both the Northern and Southern Hemispheres, with a center of diversity in New Zealand. The Myosotis pygmaea species group (Meudt et al., 2015) comprises M. antarctica Hook. f., M. brevis de Lange & Barkla, M. drucei (L. B. Moore) de Lange & Barkla, M. glauca (G. Simpson & J. S. Thomson) de Lange & Barkla, and M. pygmaea Colenso, all native to New Zealand. Questions persist regarding the delimitation of these morphologically similar species (de Lange et al., 2010), four of which appear on the New Zealand threatened species list (de Lange et al., 2013). Indeed, of the 44 endemic New Zealand Myosotis taxa, 32 are considered threatened or at risk (de Lange et al., 2013). A priority in the conservation management of members of this genus is to both accurately delimit species and understand the levels and structure of genetic diversity present. Low genetic diversity in New Zealand Myosotis, as evidenced by previous studies (Meudt et al., 2013, 2015), suggests that additional molecular markers are needed.

Here we report the development of 12 polymorphic microsatellite markers for the Myosotis pygmaea species group, which will be used in future studies of species delimitation and population genetic research. Additionally, we evaluate the utility of these loci in 18 other Myosotis species.

METHODS AND RESULTS

Sibling individuals were selected from the type locality of M. drucei as the source DNA for marker development (WELT SP100445; Appendix 1). Genomic DNA was extracted from fresh young leaf tissue from 15 seedlings using a modified cetyltrimethylammonium bromide (CTAB) method (Shepherd and McPhee, 2011). To generate sufficient template for the requirements of Illumina MiSeq, library preparation was performed using a REPLI-g kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocol. DNA was quantified using a Qubit 2.0 Fluorometer (Thermo-Fisher Scientific, Waltham, Massachusetts, USA), and a genomic library was prepared using the TruSeq Library Preparation Kit (Illumina, San Diego, California, USA) by the Massey Genome Service (Massey University, Palmerston North, New Zealand). The indexed library was pooled with three other libraries to be sequenced in equal concentration and sequenced using the paired-end 250-bp chemistry on a MiSeq (Illumina) in the Massey Genome Service. The resulting 2.7 million sequences were trimmed to low-quality results using a 0.01 quality cut-off in DynamicTrim in SolexaQA (Cox et al., 2010), which yielded 1,449,369 trimmed paired-end sequences with an average length of 380 bp, ranging in size from 11–492 bp. Paired-end sequences were joined using the program FLASH (Magoc and Salzberg, 2011).

The paired-end sequences were then imported into Geneious 6.1.5 (Biomatters, Auckland, New Zealand), where only sequences >400 bp were retained. Organellar sequences were removed by performing a local BLAST search of the M. drucei sequences against the phylogenetically closest relatives (Solis et al., 2011) with the most complete mitochondrial and chloroplast sequences from GenBank. The chloroplast genomes used were: Nicotiana undulata Ruiz & Pav. NC_016066 (Solanaeaceae), Olea europaea L. subsp. maroccana (Greuter & Burdet) P. Vargas, J. Hess, Muñoz Garn & Kadereit NC_015623 (Oleaceae), Coffea arabica L. NC_008535 (Rubiaeaceae), and Arabidopsis thaliana (L.) Heynh. NC_000932 (Brassicaceae). The mitochondrial genomes used were: N. tabacum L. NC_006581, A. thaliana NC_001284, and Vigna radiata (L.) R. Wilczek NC_015121 (Fabaceae). The remaining 397,224 sequences were split into four groups (due to computer memory constraints), and the first group of 99,999 sequences was searched for perfectly di- to hexanucleotide microsatellite sequences against the phylogenetically closest relatives (Solis et al., 2011) with the most complete mitochondrial and chloroplast sequences from GenBank. The chloroplast genomes used were: Nicotiana undulata Ruiz & Pav. NC_016066 (Solanaeaceae), Olea europaea L. subsp. maroccana (Greuter & Burdet) P. Vargas, J. Hess, Muñoz Garn & Kadereit NC_015623 (Oleaceae), Coffea arabica L. NC_008535 (Rubiaeaceae), and Arabidopsis thaliana (L.) Heynh. NC_000932 (Brassicaceae). The mitochondrial genomes used were: N. tabacum L. NC_006581, A. thaliana NC_001284, and Vigna radiata (L.) R. Wilczek NC_015121 (Fabaceae). The remaining 397,224 sequences were split into four groups (due to computer memory constraints), and the first group of 99,999 sequences was searched for perfectly di- to hexanucleotide microsatellite sequences against the phylogenetically closest relatives (Solis et al., 2011) with the most complete mitochondrial and chloroplast sequences from GenBank. The chloroplast genomes used were: Nicotiana undulata Ruiz & Pav. NC_016066 (Solanaeaceae), Olea europaea L. subsp. maroccana (Greuter & Burdet) P. Vargas, J. Hess, Muñoz Garn & Kadereit NC_015623 (Oleaceae), Coffea arabica L. NC_008535 (Rubiaeaceae), and Arabidopsis thaliana (L.) Heynh. NC_000932 (Brassicaceae). The mitochondrial genomes used were: N. tabacum L. NC_006581, A. thaliana NC_001284, and Vigna radiata (L.) R. Wilczek NC_015121 (Fabaceae). The remaining 397,224 sequences were split into four groups (due to computer memory constraints), and the first group of 99,999 sequences was searched for perfectly di- to hexanucleotide microsatellite
TABLE 1. Primer sequences and characteristics of 12 microsatellite loci developed in *Myosotis drucei*.

| Locus   | Primer sequences (5′–3′) | Fluorescent dye (pooling group) | Repeat motif | Allele size range (bp)* | T<sub>m</sub> (°C) | GenBank accession no. |
|---------|--------------------------|---------------------------------|--------------|--------------------------|-------------------|----------------------|
| MYPY-4  | F: TATGTCGTGACACCACACAC  | NED (2)                        | (TGT)<sub>4</sub> | 248–255                  | 53                | KP861356             |
|         | R: AGTCTTATTTGGCCCTCT    |                                 |              |                          |                   |                      |
| MYPY-10 | F: GGCAGATGCACTGATGAC    | VIC (1)                         | (GAT)<sub>10</sub> | 312–345                  | 53                | KP861353             |
|         | R: TACCTGATGCTGACATCAC   |                                 | (GAC)<sub>9</sub>  | 211–217                  | 53                | KP861350             |
| MYPY-14 | F: AAGAACATTTGGCACACCAC  | VIC (2)                         | (GAA)<sub>4</sub>  | 203–215                  | 53                | KP861356             |
|         | R: TTAATACATGCACTGCG     |                                 |              |                          |                   |                      |
| MYPY-17 | F: CTCCTCTATAATGTGCGG    | VIC (3)                         | (ATA)<sub>12</sub> | 273–311                  | 53                | KP861357             |
|         | R: GGATTACCTTGGGACAGTG    |                                 |              |                          |                   |                      |
| MYPY-20 | F: GTGGAGGAGAGCTCCTGCG   | FAM (4)                         | (AT)<sub>14</sub>  | 328–361                  | 53                | KP861359             |
|         | R: GTACCCGACATTAACAGG     |                                 |              |                          |                   |                      |
| MYPY-26 | F: ACTTGGAGAAGATTGTTGGCC  | NED (3)                         | (TC)<sub>7</sub>   | 374–477                  | 53                | KP861355             |
|         | R: AACGCCGCAAATTTCAACAC  |                                 |              |                          |                   |                      |
| MYPY-28 | F: TGACCTGCAACTATGAGAGAG | VIC (4)                         | (TA)<sub>16</sub>  | 341–357                  | 53                | KP861352             |
|         | R: GCCTGTGTTATGACCCCC    |                                 |              |                          |                   |                      |
| MYPY-29 | F: GTTTCACTGATAATGGTGGGC  | FAM (2)                         | (AC)<sub>18</sub>  | 334–341                  | 53                | KP861351             |
|         | R: CACAGGAGGATCACTGACGGC  |                                 |              |                          |                   |                      |
| MYPY-36 | F: GTTGCCCTGRGCTGGAC     | NED (4)                         | (GAT)<sub>10</sub> | 259–296                  | 53                | KP861360             |
|         | R: CACCATCTTTCCTCCACCC   |                                 |              |                          |                   |                      |
| MYPY-40 | F: CTGCTCATTATTCTCTGCGG  | FAM (1)                         | (AG)<sub>12</sub>  | 261                     | 53                | KP861358             |
|         | R: CACGACATTGCTGTTAACAC  |                                 |              |                          |                   |                      |
| MYPY-41 | F: CTCCTCTGAGCAGTTTCCTAC | NED (1)                         | (TG)<sub>14</sub>  | 269–271                  | 53                | KP861354             |
|         | R: TTTGAGATATGTGGGAGGCG   |                                 |              |                          |                   |                      |
| MYPY-48 | F: ATTTGGAGATATGTGGGAGGCG| FAM (3)                         | (GATGAA)<sub>10</sub> | 251–275                  | 53                | KP861349             |
|         | R: AAGAAGAACATTTCAACGACAGG |                                 |              |                          |                   |                      |

*Note: Fragment size range based on 53 *Myosotis drucei* samples from four populations: WELT SP091599, WELT SP100445, WELT SP100440, and WELT SP100428; voucher information in Appendix 1.*

TABLE 2. Summary statistics of microsatellite polymorphism determined by screening 53 *Myosotis drucei* samples from four populations; three from the South Island and one from the North Island of New Zealand.*

| Locus | South Island | North Island |
|-------|-------------|--------------|
|       | Coronet Peak (N = 13) | Tapuae-o-Uenuku (N = 14) | Mt. Altimarlock (N = 11) | Ruahine Ranges (N = 15) | Total (N = 53) |
|       | A | H<sub>e</sub> | H<sub>r</sub> | A | H<sub>e</sub> | H<sub>r</sub> | A | H<sub>e</sub> | H<sub>r</sub> | A | H<sub>e</sub> | H<sub>r</sub> |
| MYPY-4 | 2 | 0.077 | 0.204 | 2 | 0.000 | 0.375 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 |
| MYPY-10 | 3 | 0.000 | 0.462 | 3 | 0.000 | 0.500 | 2 | 0.091 | 0.351 | 1 | 0.000 | 0.000 | 7 |
| MYPY-14 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.408 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.391 | 3 |
| MYPY-17 | 2 | 0.077 | 0.074 | 3 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 4 |
| MYPY-20 | 2 | 0.000 | 0.153 | 2 | 0.000 | 0.408 | 3 | 0.100 | 0.515 | 1 | 0.000 | 0.000 | 4 |
| MYPY-26 | 2 | 0.000 | 0.142 | 2 | 0.000 | 0.408 | 1 | 0.000 | 0.000 | 3 | 0.000 | 0.561 | 5 |
| MYPY-28 | 2 | 0.000 | 0.500 | 2 | 0.000 | 0.355 | 2 | 0.091 | 0.087 | 1 | 0.000 | 0.000 | 4 |
| MYPY-29 | 2 | 0.000 | 0.165 | 3 | 0.667 | 0.667 | 2 | 1.000 | 0.500 | 2 | 0.600 | 0.420 | 4 |
| MYPY-36 | 3 | 0.077 | 0.210 | 2 | 0.000 | 0.408 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 4 |
| MYPY-40 | 2 | 0.000 | 0.165 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 |
| MYPY-41 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.142 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 2 |
| MYPY-48 | 2 | 0.000 | 0.473 | 2 | 0.000 | 0.408 | 1 | 0.000 | 0.000 | 2 | 0.000 | 0.337 | 4 |

*Note: A = number of alleles; A<sub>T</sub> = total number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>r</sub> = observed heterozygosity; N = sample size for each population.*

*South Island: Coronet Peak = WELT SP091599, Tapuae-o-Uenuku = WELT SP100440, Mt. Altimarlock = WELT SP100442; North Island: Ruahine Ranges = WELT SP100445. See Appendix 1 for voucher information.*

http://www.bioone.org/loi/apps

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### Table 3. Cross-amplification of 12 novel microsatellite loci in 22 *Myosotis* species.

| Species name | Voucher no. | N Location | MYPY-4 | MYPY-10 | MYPY-14 | MYPY-17 | MYPY-20 | MYPY-26 | MYPY-28 | MYPY-29 | MYPY-36 | MYPY-40 | MYPY-41 | MYPY-48 |
|--------------|-------------|-----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| *Myosotis pygmaea* | | | | | | | | | | | | | | |
| *M. arnoldii* | SP100473 | 3 NZ | 6 | 8 | 5 | 6 | 1 | 2 | + | 2 | 3 | 2 | 3 | — | 4 |
| *M. cheesemani* | SP092210 | 1 NZ | + | + | + | + | + | + | + | + | + | + | — | — |
| *M. colensoi* | SP092419 | 1 NZ | + | — | — | + | + | + | + | — | — | — | — | — |
| *M. forsteri* | SP089691 | 1 NZ | 2 | 1 | 2 | 2 | — | 2 | — | 2 | 3 | 1 | 1 | 1 |
| *M. glabrescens* | SP089801 | 1 NZ | + | + | 2 | + | — | — | — | + | + | + | — | — |
| *M. macrantha* | SP100468 | 3 NZ | 3 | 7 | 4 | 4 | 2 | 1 | 2 | 3 | 4 | 2 | 3 | 3 |
| *M. pansa* | SP089670 | 2 NZ | 2 | 1 | 2 | 2 | — | — | 1 | 1 | — | 1 | — | — |
| *M. petiolata* | SP089853 | 3 NZ | 2 | 1 | 2 | 2 | — | — | — | 1 | 1 | 1 | 1 | 1 |
| *M. potosiana* | SP089687 | 2 NZ | 1 | 2 | 1 | 2 | — | 1 | 1 | 1 | — | 2 | 1 | — |
| *M. pulvinaris* | SP092196 | 1 NZ | — | 2 | + | + | + | — | 2 | + | + | + | + | — |
| *M. small white* | SP090247 | 1 NZ | 2 | 2 | 1 | 1 | 2 | — | 1 | — | 2 | 3 | 1 | 1 |
| *M. tenericuluis* | SP090251 | 1 NZ | — | 2 | — | + | + | + | — | 2 | — | — | — | — |
| *M. tenericulis* | SP092404 | 1 NZ | 2 | — | + | + | — | + | — | + | + | + | + | — |

**Note:** N = number of individuals trialed from each population.

a Number of amplified alleles are indicated, + = amplified with unknown levels of polymorphism as only one allele in one individual amplified, — = no amplification.

b See Appendix 1 for voucher information.

c Aust = Australian native; CI = Campbell Island native; Euro = European native growing in New Zealand; NZ = New Zealand endemic.
subsequent fragment separation on an ABI 3730 Genetic Analyzer (Applied Biosystems) by the Massey Genome Service.

Alleles were visualized and scored using GeneMapper version 3.7 (Applied Biosystems). Of the 48 primer pairs tested, 25 were polymorphic, two were monomorphic, seven were unscorable, and 14 did not amplify. Twenty-four of the polymorphic loci were further tested using the above PCR conditions on 15 individuals from five Myosotis species. The 12 markers (Table 1) with the best amplification rates were selected for further investigation using four populations of M. drucei to demonstrate the utility of the markers in a population genetic framework. For these four populations, Table 2 shows the number of alleles, and observed ($H_o$) and expected ($H_e$) heterozygosities, which were determined using GenAlEx (Peakall and Smouse, 2012). The average number of observed alleles per locus was 3.75, and average $H_o$ was 0.059 (Table 2). $H_e$ was typically lower than $H_o$, which matches the hypothesized mostly selfing nature of the M. pygmaea species group (Robertson and Lloyd, 1991; Brandon, 2001). The 12 markers amplified well across the other four species (one population each) in the M. pygmaea group (voucher information in Appendix 1) and were also trialed in an additional 18 species of Myosotis, 14 endemic to New Zealand, one from Australia, and three introduced to New Zealand from Europe. Amplification rates and polymorphism are reported in Table 3.

**CONCLUSIONS**

We describe 12 polymorphic microsatellite loci that will be useful for exploring species limits within the M. pygmaea species group, as well as determining the population genetic variation within and among other species of Southern Hemisphere Myosotis.

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### APPENDIX 1. Voucher and location information for all *Myosotis* populations used in this study.

| Species | Location | Voucher no. |
|---------|----------|-------------|
| *Myosotis pygmaea* species group | | |
| *Myosotis antarctica* Hook. f. | New Zealand, Campbell Island, cliffs near Menhir | WELT SP102775 |
| *Myosotis brevis de Lange & Barkla* | New Zealand, Coastal Taranaki, Puakapu Rd. end* | WELT SP090361 |
| *Myosotis brevis de Lange & Barkla* | New Zealand, Coastal Taranaki, Stent Rd. | WELT SP090543 |
| *Myosotis drucei* (L. B. Moore) de Lange & Barkla | New Zealand, North Island, Ruahine Ranges, near Mt. Maungamahue* | WELT SP100445 |
| *Myosotis drucei* (L. B. Moore) de Lange & Barkla | New Zealand, South Island, Marlborough, Tapuae-o-Uenuku | WELT SP100440 |
| *Myosotis drucei* (L. B. Moore) de Lange & Barkla | New Zealand, South Island, Central Otago, Coronet Peak | WELT SP091599 |
| *Myosotis drucei* (L. B. Moore) de Lange & Barkla | New Zealand, South Island, Marlborough, Mt. Altimarlock* | WELT SP100428 |
| *Myosotis glauca* (G. Simpson & J. S. Thomson) | New Zealand, South Island, Central Otago, Nevis Valley* | WELT SP093284 |
| *Myosotis pygmaea* Colenso | New Zealand, North Island, Coastal Taranaki, Opunake treatment ponds | WELT SP090540 |
| *Myosotis pygmaea* Colenso | New Zealand, North Island, Northwest Nelson, near Sandhill Creek river mouth* | WELT SP100460 |

#### Other New Zealand *Myosotis*

| Species | Location | Voucher no. |
|---------|----------|-------------|
| *Myosotis arnoldii* L. B. Moore | New Zealand, South Island, Marlborough, Mt. Benmore | WELT SP100439 |
| *Myosotis arnoldii* L. B. Moore | New Zealand, South Island, Northwest Nelson, Hoary Head | WELT SP100473 |
| *Myosotis cheesemani* Petrie | New Zealand, South Island, Central Otago, Pisa Range | WELT SP092210 |
| *Myosotis colensoi* (Kirk) J. F. Machr. | New Zealand, cultivated (Origin: South Island, Canterbury, Castle Hill) | WELT SP092419 |
| *Myosotis forsteri* Lehmn. | New Zealand, North Island, Kaweka Ranges | WELT SP089828 |
| *Myosotis forsteri* Lehmn. | New Zealand, North Island, Raukumara, Waioeka Conservation Area | WELT SP089691 |
| *Myosotis forsteri* Lehmn. | New Zealand, South Island, Northwest Nelson, Kahurangi National Park | WELT SP092179 |
| *Myosotis glabrescens* L. B. Moore | New Zealand, South Island, Central Otago, Hector Mountains | WELT SP089801 |
| *Myosotis macrantha* (Hook. f.) Benth. & Hook. f. | New Zealand, South Island, Central Otago, Queenstown, Moke Creek | WELT SP100494 |
| *Myosotis pansa* (L. B. Moore) Meudt, Prebble, R. J. Stanley & Thorsen subsp. *pansa* | New Zealand, North Island, Auckland Region, Anawhata stream | WELT SP089670 |
| *Myosotis pansa* (L. B. Moore) Meudt, Prebble, R. J. Stanley & Thorsen subsp. *pansa* | New Zealand, North Island, Auckland Region, Parahaka Valley | WELT SP089674 |
| *Myosotis pansa* subsp. *praeceps* Meudt, Prebble, R. J. Stanley & Thorsen | New Zealand, North Island, Taranaki, Paraninhi/White Cliffs | WELT SP089686 |
| *Myosotis pansa* subsp. *praeceps* Meudt, Prebble, R. J. Stanley & Thorsen | New Zealand, North Island, Waikato, Ngarupupu Point | WELT SP089685 |
| *Myosotis petiolata* Hook. f. | New Zealand, North Island, Hawkes Bay, Te Waka Range | WELT SP089853 |
| *Myosotis pottsiana* (L. B. Moore) Meudt, Prebble, R. J. Stanley & Thorsen | New Zealand, North Island, Bay of Plenty, Ohiu Stream | WELT SP089689 |
| *Myosotis pottsiana* (L. B. Moore) Meudt, Prebble, R. J. Stanley & Thorsen | New Zealand, North Island, Bay of Plenty, Waikokopu Stream | WELT SP089687 |
| *Myosotis pulvinaris* Hook. f. | New Zealand, South Island, Central Otago, Pisa Range | WELT SP092196 |
| *Myosotis* “small white” | New Zealand, South Island, Northwest Nelson, Kahurangi National Park | WELT SP090251 |
| *Myosotis spathulata* G. Forst. | New Zealand, North Island, Hawkes Bay | WELT SP090628 |
| *Myosotis spathulata var. radicata* L. B. Moore | New Zealand, cultivated, origin Kaweka Ranges, North Island | WELT SP092757 |
| *Myosotis tenericaulis* Petrie | New Zealand, South Island, Northwest Nelson, Kahurangi National Park | WELT SP092404 |
| *Myosotis uniforma* Hook. f. aff. | New Zealand, South Island, Central Otago, Pisa Flats | WELT SP089883 |

#### Other *Myosotis*

| Species | Location | Voucher no. |
|---------|----------|-------------|
| *Myosotis arvensis* (L.) Hill | New Zealand, North Island, Wellington, Karori | WELT SP094173 |
| *Myosotis australis* R. Br. | Australia, New South Wales, Barrington Tops National Park | MPN 44757 |
| *Myosotis discolor* Pers. | New Zealand, South Island, Central Otago, Ranfurly Holiday Park | WELT SP089930 |
| *Myosotis laxa* Lehmn. | New Zealand, South Island, Canterbury, Arthurs Pass | WELT SP090206 |

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*a* A written description of the population location is included rather than GPS locations due to the threatened status of these species. An * indicates the five populations on which the markers were initially trialed.

*b* One voucher was collected for each population used; all vouchers are deposited in the herbaria of the Museum of New Zealand Te Papa Tongarewa (WELT) or Massey University (MPN).