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Primer Note

Microsatellite markers for the New Zealand endemic *Myosotis pygmaea* species group (Boraginaceae) amplify across species[1]

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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 *M. pygmaea* 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 *M. pygmaea* 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 *M. 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 *M. 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.

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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 McLay, 2011). To generate sufficient template for the requirements of Illumina MiSeq library preparation, extracted DNA was pooled and amplified 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 in equal concentration and sequenced using the paired-end 250-bp chemistry on a MiSeq (Illumina) by the Massey Genome Service. The resulting 2.7 million sequences were trimmed of 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 (Soltis et al., 2011) with the most complete mitochondrial and chloroplast sequences from GenBank. The chloroplast genomes used were: *Nicotiana undulata* Ruiz & Pav. NC_016068 (Solanaceae), *Olea europaea* L. subsp. *maroccana* (Greuter & Burdet) P. Vargas, J. Hess, Muñoz Garm. & Kadereit NC_015623 (Oleaceae), *Coffea arabica* L. NC_008535 (Rubiaceae), 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 perfect di- to hexanucleotide microsatellite

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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)[a]	T_a (°C)	GenBank accession no.
MYPY-4	F: TATGCTCGTACCGAAACAC R: AGTGCTTATGTTTGCCCTC	NED (2)	$(TGT)_8$	248–255	53	KP861356
MYPY-10	F: GCGACATTGCAACTGATAC R: TACCTCATCGCTCAATACC	VIC (1)	$(GAT)_{10}$	312–345	53	KP861353
MYPY-14	F: AAGAACATTTTGCCACAGC R: TTAAATCATTGCACGTCCG	VIC (2)	$(GAA)_7$	211–217	53	KP861350
MYPY-17	F: CCTCTCTCTATATGTCGCG R: GGATTACCTTGAGGCAGTG	VIC (3)	$(ATA)_{12}$	273–311	53	KP861357
MYPY-20	F: GTTGAGAGAGCTCTACTGC R: GTACCCAGCATTAACCAGG	FAM (4)	$(AT)_9$	228–236	53	KP861359
MYPY-26	F: ACTTGGAGAACGATTTGTCCG R: AACCGCCGCAAAATTCAAAC	NED (3)	$(TC)_7$	374–477	53	KP861355
MYPY-28	F: TGACTCTGGACAATGATGAGAGAG R: CGGCTGTTTTAGAACCACCC	VIC (4)	$(TA)_9$	341–357	53	KP861352
MYPY-29	F: GGTTCAGTGATAATGTTCGAGCC R: CACAGGAAGGATCAATGACTGC	FAM (2)	$(AC)_9$	334–342	53	KP861351
MYPY-36	F: GTTGTGCTTGATGGTGACCC R: CCCATCCTTCTTCTCCACCC	NED (4)	$(GAT)_{10}$	259–296	53	KP861360
MYPY-40	F: CTGCCTCATTATTCTCTGGG R: CACGACCATTCCATGTTAAC	FAM (1)	$(AG)_7$	261	53	KP861358
MYPY-41	F: CTTCTTGACGCTTTTGCTAC R: TTCAGAATAGCAATTGTCGC	NED (1)	$(TG)_8$	269–271	53	KP861354
MYPY-48	F: ATTCGACGTAGATCTTGTGC R: AAAGAAAACTGCAGAACGTG	FAM (3)	$(GATGAA)_7$	251–275	53	KP861349

[a] 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.

repeats with a minimum of seven uninterrupted repeat units using a search tool in Geneious (Phobos plugin; Mayer, 2010), which identified 484 repeats. Sequences were removed from consideration if the paired-end sequences were found to be overlapping only in the repeat region, if regions near the microsatellite contained other microsatellite loci or single base pair repeats >4 bp, or if there were greater than 14 repeats. After removing unsuitable loci, primers were designed for 147 microsatellite regions using Primer3 within Geneious (Untergasser et al., 2012). The default settings were used except for: product size = 100–400 bp with a 50-bp buffer on both sides of the target region; primer size = 18 bp (minimum)–20 bp (optimal)–22 bp (maximum); melting temperature (T_m) = 47–55–60°C; 3′ GC content = 40–50–60%; maximum T_m difference = 10°C; GC clamp = 1; max poly N = 4. An M13 tag (CACGACGTTGTAAAACGAC) was added to the 5′-end of the forward primer for each locus, and a PIG-tail sequence (GTTTCTT; Brownstein et al., 1996) was added to the 5′-end of each reverse primer.

For reasons of practicality, 48 primer pairs were chosen to trial a range of: uninterrupted number of repeats, types of microsatellites (e.g., di-, tri-, tetra-, penta-, and hexa-), and PCR product sizes. These 48 were initially trialed on seven individuals from five populations of four *M. pygmaea* group species (Appendix 1). Each locus was amplified individually in 10-μL PCR reactions that contained 1 μL of a 1 : 50 dilution of template DNA (5–50 ng), 0.02 μM forward primer, 0.45 μM reverse primer, 0.45 μM M13 primer (labeled with FAM, NED, or VIC), 1.5 mM $MgCl_2$, 1× buffer BD (Solis BioDyne, Tartu, Estonia), 250 μM of each dNTP, and 1 unit FIREPol *Taq* polymerase (Solis BioDyne). PCRs were carried out with the following cycling program: an initial denaturation of 95°C for 3 min; 40 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. A volume of 0.75 μL of each PCR product for three loci, each with a different fluorophore, was added to 9 μL of Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) premixed with a ROX-labeled CASS ladder (Symonds and Lloyd, 2004) for

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.[a]

	South Island									North Island			
	Coronet Peak (N = 13)			Tapuae-o-Uenuku (N = 14)			Mt. Altimarlock (N = 11)			Ruahine Ranges (N = 15)			Total (N = 53)
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A_T
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	1	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_T = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population.

[a] South Island: Coronet Peak = WELT SP091599, Tapuae-o-Uenuku = WELT SP100440, Mt. Altimarlock = WELT SP100428; North Island: Ruahine Ranges = WELT SP100445. See Appendix 1 for voucher information.

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

Species name	Voucher no.[b]	*N*	Location[c]	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* species group**															
M. antarctica	SP102775	12	CI	2	1	2	1	2	1	2	1	1	1	1	1
M. brevis	SP090361	25	NZ	1	1	1	1	2	2	1	1	1	1	1	1
M. glauca	SP093284	17	NZ	1	1	1	1	1	1	2	1	1	2	1	1
M. pygmaea	SP090540	13	NZ	1	1	1	1	1	1	2	1	1	1	1	1
Other New Zealand *Myosotis*															
M. arnoldii	SP100473	3	NZ	6	8	5	6	1	2	+	3	2	3	—	4
	SP100439	3													
M. cheesemanii	SP092210	1	NZ	+	+	+	+	—	—	—	+	+	+	—	—
M. colensoi	SP092419	1	NZ	+	—	—	+	—	—	—	+	+	+	—	—
M. forsteri	SP089691	1	NZ	2	1	2	2	—	2	—	2	3	1	1	1
	SP089928	1													
	SP092179	1													
M. glabrescens	SP089801	1	NZ	+	+	2	+	—	—	—	+	+	+	—	—
M. macrantha	SP100468	3	NZ	3	7	4	4	2	1	2	3	4	2	3	3
	SP100494	3													
M. pansa subsp. *pansa*	SP089670	2	NZ	2	1	2	2	—	—	1	1	—	1	—	—
	SP089674	1													
M. pansa subsp. *praeceps*	SP089685	2	NZ	2	1	3	—	—	—	—	1	—	2	—	—
	SP089686	1													
M. petiolata	SP089853	3	NZ	2	1	2	2	—	—	—	1	—	1	1	—
M. pottsiana	SP089687	2	NZ	1	2	1	2	—	1	1	1	—	2	1	—
	SP089689	1													
M. pulvinaris	SP092196	1	NZ	—	2	+	+	—	—	+	2	+	+	+	+
M. "small white"	SP090247	1	NZ	2	1	1	2	—	1	—	1	3	1	1	—
	SP090251	1													
M. spathulata	SP090628	2	NZ	2	1	1	1	—	—	1	1	—	2	1	—
	SP092757	1													
M. tenericaulis	SP092404	1	NZ	2	—	+	+	—	—	—	+	—	+	—	—
Other *Myosotis*															
M. arvensis	SP094173	1	Euro	—	—	+	+	—	—	—	—	—	—	—	—
M. australis	MPN44757	2	Aust	1	—	1	2	—	—	—	—	1	2	—	—
M. discolor	SP089930	1	Euro	—	—	—	+	—	—	+	+	—	+	—	—
M. laxa	SP090206	1	Euro	—	—	+	+	—	—	—	—	—	—	—	—

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.

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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_o was typically lower than H_e, 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[a]	Voucher no.[b]
***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, Puketapu 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) de Lange & Barkla	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, South Island, Northwest Nelson, near Sandhill Creek river mouth*	WELT SP100460
Other New Zealand *Myosotis*		
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 cheesemanii Petrie	New Zealand, South Island, Central Otago, Pisa Range	WELT SP092210
Myosotis colensoi (Kirk) J. F. Macbr.	New Zealand, cultivated (Origin: South Island, Canterbury, Castle Hill)	WELT SP092419
Myosotis forsteri Lehm.	New Zealand, North Island, Kaweka Ranges	WELT SP089928
Myosotis forsteri Lehm.	New Zealand, North Island, Raukumara, Waioeka Conservation Area	WELT SP089691
Myosotis forsteri Lehm.	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 macrantha (Hook. f.) Benth. & Hook. f.	New Zealand, South Island, Northwest Nelson, Lake Peel	WELT SP100468
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, Pararaha Valley	WELT SP089674
Myosotis pansa subsp. *praeceps* Meudt, Prebble, R. J. Stanley & Thorsen	New Zealand, North Island, Taranaki, Paraninihi/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, Ohutu 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 “small white”	New Zealand, South Island, Northwest Nelson, Kahurangi National Park	WELT SP090247
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 uniflora Hook. f. aff.	New Zealand, South Island, Central Otago, Pisa Flats	WELT SP089883
Other *Myosotis*		
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 Lehm.	New Zealand, South Island, Canterbury, Arthurs Pass	WELT SP090206

[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).

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