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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI FROM *ARTHROPODIUM CIRRATUM* (ASPARAGACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for *Arthropodium cirratum* (Asparagaceae) to study population genetic structure and translocation of this species. These markers were tested for cross-amplification in two other *Arthropodium* species.
- *Methods and Results:* Sixteen microsatellite markers were developed from a genomic library and tested in three populations of *A. cirratum.* The loci exhibited one to five alleles per locus, with private alleles present in each of the populations. Cross-amplification tests in the two other New Zealand *Arthropodium* species revealed that many of the loci amplify and demonstrate polymorphism in *A. bifurcatum*.
- *Conclusions:* These markers will be useful for determining genetic structure in *A. cirratum* and for determining the origins of translocated populations of this species.

Key words: *Arthropodium*; Asparagaceae; nuclear microsatellites; rengarenga; translocation.

Arthropodium R. Br. (Asparagaceae) is a genus of nine species (Heenan et al., 2004) of perennial, lily-like herbs found in Australia, New Zealand, New Caledonia, New Guinea, and Madagascar. Within New Zealand, there are three endemic species: *A. candidum* Raoul (small renga lily), *A. cirratum* (G. Forst.) R. Br., and *A. bifurcatum* Heenan, A. D. Mitch. & de Lange (the latter two species both have the common names rengarenga and New Zealand rock lily).

Arthropodium cirratum was cultivated as a food source for Māori and translocated beyond its natural range (Shepherd et al., 2016). A recent phylogeographic study of *A. cirratum* using chloroplast sequences revealed a very high level of structuring, with many populations fixed for unique chloroplast haplotypes (Shepherd et al., 2016). Microsatellite markers will be useful for testing whether the nuclear genome demonstrates the high genetic structuring found for the chloroplast genome (Shepherd et al., 2016). They will also aid in examining the origins of the populations that derive from translocation by Māori and testing proposed hybridization between *A. cirratum* and *A. bifurcatum* (Heenan et al., 2004).

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METHODS AND RESULTS

We extracted DNA from leaf tissue of four *A. cirratum* individuals (Appendix 1), each from different populations, using a modified cetyltrimethylammonium bromide (CTAB) method (steps 1, 3–7 from table 1 in Shepherd and McLay, 2011). The extracted DNA was pooled and amplified using a REPLI-g kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol to generate sufficient template for library construction. An Illumina paired-end genomic library was constructed using the TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, California, USA) following the manufacturer's instructions. The library was sequenced in a single lane using the Illumina MiSeq platform to generate 2 × 250-bp reads at the Massey Genome Service (Massey University, Palmerston North, New Zealand).

We assembled the resulting 10,955,497 paired sequence reads using MEGAHIT (Li et al., 2015), as this software required sequence reads of the same length. A set of four assembly parameters was tried with the reads, and the resulting contigs were merged to make a set of longest unique contigs. This resulted in 1.589 Gb of assembled sequence, comprising 2,618,361 contigs, with a maximum length of 18,007 bp, average GC content of 34.74%, and an N50 of 1513 bp when analyzed using QUAST (Gurevich et al., 2013). The SSR_pipeline (Miller et al., 2013) was used to detect di- and tetranucleotide repeats on this contig set with a minimum of 250-bp flanking sequence on each side to allow for PCR primer design. We used WebSat (Martins et al., 2009) to develop primers for 33 loci, which had at least eight tetra- or 15 dinucleotide repeat units. An M13 tag (TGTAAAACGACGGCCAGT) was added to the 5′ end of the forward primer of each locus. These primer pairs were tested on five samples, which included three samples of *A. cirratum* and one sample each of *A. candidum* and *A. bifurcatum*. Each locus was initially amplified individually in 10-µL PCR reactions that contained 1 µL of diluted template DNA, 0.02 µM forward primer, 0.8 µM reverse primer, 0.8 µM M13 primer (labeled with FAM, NED, PET, or HEX), 1× MyTaq mix (Bioline, London, United Kingdom), and 0.1 M betaine. PCR thermocycling conditions were an initial denaturation of 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; followed by eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 15 min.

Of the 33 primer pairs tested, 16 amplified in at least two species and were polymorphic. These 16 loci were subsequently screened using 63 samples from three populations of *A. cirratum* and additional samples of *A. bifurcatum* and

TABLE 1. Primer sequences and thermal cycling conditions for 16 microsatellite loci developed for *Arthropodium cirratum*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye (Pooling group)	GenBank accession no.
ArtCir1	F: AAAACACAGCAGACAAACACA	$(CCTC)_7$	351–359	52	FAM (3)	KY907147
	R: ATTGTACTCCGCTCATTGTTCC					
ArtCir4	F: CAGTTCGCTAAAGGACGGAG	$(TATT)_7$	207–219	55	FAM (3)	KY907148
	R: TAATTGGACCTCTCTCATCGGT					
ArtCir7	F: AATTGCCTTCAACGTCTTTAGC	$(AATA)_7$	200–215	55	FAM (1)	KY907149
	R: CGAATACGAACCCCATATTGAC					
ArtCir9	F: GCCGAAGCTGACAATGAAA	$(TCTT)_7$	255–267	55	FAM (2)	KY907150
	R: CCCACATAATCAAAACCTCCAT					
ArtCir12	F: CCTACCTGCATCTTGACCTTGT	$(TTTG)_8$	360–372	55	NED (3)	KY907151
	R: GTTGAGAGAATGACACTTGGGC					
ArtCir13	F: TTCGATAGAGAGTGGTGACGAG	$(TATT)_7$	260–272	55	HEX (1)	KY907152
	R: AAATCAATCCCCTCCGTTAGAT					
ArtCir18	F: CTTGTAAAGTCAAGCTCATCGGT	$(TAAA)_{11}$	318–336	55	HEX (1)	KY907153
	R: ACCGGACATCCAACAATTAGAA					
ArtCir22	F: ACATCTTTTCATACACGGGCTT	$(ATAA)_9$	382–405	52	PET (1)	KY907154
	R: CTCTCAAGGATCACAAGGAACC					
ArtCir23	F: CGAAAACGACTAACGTGAAGAA	$(ATGT)_7$	344–360	55	FAM (2)	KY907155
	R: TATGTGTTGGTTGAAGGAGAGC					
ArtCir26	F: GGGCCACTCATATTTCATTTTC	$(CATA)_7$	380–401	52	NED (2)	KY907156
	R: GTAGGTGCTATCCTCCCTTCCT					
ArtCir32	F: CCGTACCTTCTCTCTGTTTGTGT	$(TAAA)_7$	367–385	56	NED (1)	KY907157
	R: ACCCAACCCTCATTTTATCTCC					
ArtCir38	F: AGCTATGCCCCTCTTTTAGTCA	$(ATTT)_7$	274–289	55	HEX (2)	KY907158
	R: ACCAAGATTGCTCCATCAAAGT					
ArtCir43	F: TAAAGGAGGAAATGGGTAGGT	$(TA)_{18}$	388–409	55	PET (2)	KY907159
	R: TCTTCTACAACAACACCGAGAA					
ArtCir48	F: TTCGCAAAGGATATTAGGTGTG	$(AT)_{20}$	305–409	55	PET (2)	KY907160
	R: TACGAGAACAAGGGAGGGATTA					
ArtCir50	F: GGCTAATTTTAATGTGCTTGGC	$(AT)_{18}$	358–403	55	NED (3)	KY907161
	R: ATGGATGAGAGAGAAAGGACCA					
ArtCir59	F: CTATCTCACCATATCGCGTGC	$(AT)_{18}$	290–305	55	PET (1)	KY907162
	R: TCGTTTCAAGACAGAAGGCAT					

Note: T_a = annealing temperature.

A. candidum (Appendix 1). For this trial, some loci were coamplified in the same PCR reaction (ArtCir13 with ArtCir18, ArtCir9 with ArtCir23, and ArtCir43 with ArtCir48). For these combined PCR reactions, 1 µL of diluted template DNA was combined with 0.02 µM each forward primer, 0.8 µM each reverse primer, 1.2 µM M13 primer (labeled with FAM, NED, PET, or HEX), 1× MyTaq mix (Bioline), and 0.075 M betaine. The PCR annealing temperatures are reported in Table 1. Genotyping was performed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) at the Massey Genome Service. Alleles were sized using the internal size standard GeneScan 500 LIZ (Applied Biosystems) and scored using Geneious version 10.0.2 (Biomatters Ltd., Auckland, New Zealand).

The number of alleles and observed and expected heterozygosities for the three *A. cirratum* populations were determined using GenAlEx 6.5 (Peakall and Smouse, 2012). Observed and expected heterozygosities ranged from 0.000

TABLE 2. Genetic diversity measures for three populations of *Arthropodium cirratum*.[a]

	Maunganui Bluff (N = 22)			Matapouri Bay (N = 20)			Hick's Bay (N = 21)			Total (N = 63)
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A_T
ArtCir1	1	0.000	0.000	2	0.100	0.261	1	0.000	0.000	2
ArtCir4	1	0.000	0.000	2	0.100	0.455*	2	0.048	0.046	3
ArtCir7	1	0.000	0.000	3	0.100	0.261	1	0.000	0.000	4
ArtCir9	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1
ArtCir12	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2
ArtCir13	1	0.000	0.000	1	0.000	0.000	2	0.286	0.444	3
ArtCir18	2	1.000	0.500*	2	0.800	0.480*	2	1.000	0.500*	4
ArtCir22	1	0.000	0.000	2	0.000	0.495*	1	0.000	0.000	2
ArtCir23	1	0.000	0.000	2	0.100	0.320*	2	0.143	0.278	4
ArtCir26	1	0.000	0.000	2	0.100	0.180	2	0.000	0.172	5
ArtCir32	1	0.000	0.000	1	0.000	0.000	2	0.095	0.091	3
ArtCir38	1	0.000	0.000	2	0.850	0.489*	3	0.952	0.544*	5
ArtCir43	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2
ArtCir48	1	0.000	0.000	2	0.100	0.095	1	0.000	0.000	3
ArtCir50	1	0.000	0.000	1	0.000	0.000	2	0.810	0.495	2
ArtCir59	2	0.045	0.044	2	0.150	0.139	1	0.000	0.000	3

Note: A = number of alleles; A_T = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of samples.

[a]Locality and voucher information are provided in Appendix 1.

*Significant departure from Hardy–Weinberg equilibrium (HWE) at $P < 0.05$ following sequential Bonferroni correction.

TABLE 3. Cross-amplification of 16 *Arthropodium cirratum* microsatellites in *A. bifurcatum* and *A. candidum*, showing fragment sizes of each allele.

Locus	*A. bifurcatum* (*N* = 6)	*A. candidum* (*N* = 3)	*A. cirratum* (*N* = 63)	A_T (*N* = 72)
ArtCir1	351, 355, 359	363	351, 359	4
ArtCir4	207, 215, 219	219	208, 211, 219	5
ArtCir7	200, 208, 212	189	200, 212, 213, 215	6
ArtCir9	267, 256	267	255	3
ArtCir12	360, 363, 372	368	363, 368	4
ArtCir13	268	—	260, 268, 272	3
ArtCir18	336	—	318, 334, 336, 325	4
ArtCir22	386, 389, 402, 405	—	382, 386	5
ArtCir23	344, 355	—	345, 348, 357, 360	6
ArtCir26	380, 388, 390, 401	—	383, 387, 393, 397, 399	9
ArtCir32	375, 379	—	367, 369, 385	5
ArtCir38	281, 276	—	274, 281, 282, 285, 289	6
ArtCir43	388, 394, 403	391	408, 409	6
ArtCir48	313	—	303, 313, 315	3
ArtCir50	358, 377, 385	403	358, 403	4
ArtCir59	290	290	290, 297, 305	3

Note: A_T = total number of alleles; *N* = number of samples.

to 1.000 and 0.044 to 0.544, respectively (Table 2). Alleles per locus ranged from one to five in *A. cirratum* (mean = 3). All three of the *A. cirratum* populations exhibited private alleles, and 14 of the loci had private alleles in at least one of the three populations. Tests of pairwise linkage disequilibrium were performed using GENEPOP 4.2 (Rousset, 2008). No significant linkage disequilibrium was detected among paired loci comparisons after sequential Bonferroni correction (Holm, 1979). Deviation from Hardy–Weinberg equilibrium was tested for each locus with GenAlEx 6.5. Following sequential Bonferroni correction, significant deviation from Hardy–Weinberg equilibrium was observed for five loci (Table 2). This is unsurprising for a species with delayed autonomous self-pollination (Zhou et al., 2012). ArtCir18 showed fixed heterozygote genotypes for all the screened individuals in the Maunganui Bluff and Hick's Bay populations, but each population was fixed for different alleles.

All 16 loci amplified in the closely related species *A. bifurcatum*, and 12 of these were polymorphic (Table 3). Eight loci amplified in the more distantly related *A. candidum*, but none of the three samples screened were polymorphic at these loci.

CONCLUSIONS

We developed 16 variable microsatellite markers for *A. cirratum* using Illumina MiSeq data. Although most of the markers had a low number of alleles, many showed fixed allelic differences between the populations examined. These markers will be useful for characterizing genetic diversity and structure in *A. cirratum* and for examining the translocation of this species.

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Appendix 1. Location and voucher information for *Arthropodium* species used in this study.

Species	*n*	Voucher no.[a]	Location	Geographic coordinates
Arthropodium cirratum (G. Forst.) R. Br.	1	WELT SP103437[b]	In cultivation, ex. Surville Cliffs, Northland, NZ	–34.3956, 173.0124
	1	WELT SP104032[b]	Waikawa, East Cape, NZ	–37.6783, 177.7483
	1	AK 311376[b]	Haparapara, East Cape, NZ	–37.7929, 177.6679
	1	CHR 473343[b]	Papanui Point, Waikato, NZ	–37.8898, 174.7636
	21	AK 311414	Hick's Bay, East Cape, NZ	–37.5683, 178.2866
	22	AK 308946	Maunganui Bluff, Northland, NZ	–35.7783, 173.5703
	20	WELT SP103515	Matapouri Bay, Northland, NZ	–35.5623, 174.5094
A. bifurcatum Heenan, A. D. Mitch. & de Lange	1	WELT SP103440	In cultivation, ex. Hen Island, Northland, NZ	–35.8917, 174.7274
	2	WELT SP103512	In cultivation, ex. Poor Knights Islands, Northland, NZ	–35.4688, 174.7365
	1	WELT SP103511	In cultivation, ex. Surville Cliffs, Northland, NZ	–34.3956, 173.0124
	1	AK 309832	Surville Cliffs, Northland, NZ	–34.3956, 173.0124
	1	WELT SP103534	Great Island, Three Kings, Northland, NZ	–34.1575, 172.1387
A. candidum Raoul	2	WELT SP103527	Golden Bay, NW Nelson, NZ	–40.8873, 172.8122
	1	—	Lake Wakatipu, Otago, NZ	–40.0424, 168.63704

Note: *n* = number of sampled individuals; NZ = New Zealand.

[a]Vouchers are deposited in the herbaria of Auckland Museum (AK), Auckland, New Zealand; Landcare Research (CHR), Lincoln, New Zealand; or the Museum of New Zealand (WELT), Wellington, New Zealand. One representative voucher sample was collected per population.

[b]Samples used for initial library construction.