Applications in Plant Sciences

PRIMER NOTE

Molecular markers for *Nassella trichotoma* (Poaceae) to study genetic variation in New Zealand

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PREMISE OF THE STUDY: Simple sequence repeat (SSR) markers were developed for the study of genetic diversity of New Zealand *Nassella trichotoma* (Poaceae) and to support future studies in its native range.

METHODS AND RESULTS: Genomic DNA was extracted from *N. trichotoma* leaf material and subjected to Roche 454 sequencing. From a total of 745 putative SSRs, 48 with di- to pentanucleotide repeats were screened, 32 primer pairs were designed, and 15 polymorphic markers were optimized for multiplex PCR on 105 *N. trichotoma* samples from four New Zealand regions. Each locus resulted in two to six alleles per locus, and four of the loci cross-amplified in *N. tenuissima*. The mean observed and expected heterozygosity ranged from 0.00 to 0.90 and 0.00 to 0.50 per locus, respectively.

CONCLUSIONS: The novel SSR markers are valuable for the study of genetic diversity of *N. trichotoma* and might also be useful for closely related species.

KEY WORDS *Nassella tenuissima*; *Nassella trichotoma*; nassella tussock; New Zealand; Poaceae; simple sequence repeat (SSR) markers.

Nassella trichotoma (Nees) Hack. ex Arechav. (Poaceae), also known as nassella tussock, is an invasive South American weedy grass in New Zealand and Australian grassland areas that is a difficult target for control. The current control method is to manually dig out all plants before they seed each year. This method has resulted in a stable metapopulation of the species in pastures in the Canterbury Region of New Zealand (Lamoureaux et al., 2015). Although this method is more cost-effective in the long term than not removing the plants, a control method resulting in a declining regional metapopulation is desired by many farmers. To this end, this study developed molecular markers to characterize the genetic variation in *N. trichotoma* in New Zealand as a step toward development of potential new control methods.

We used 454 sequencing to develop simple sequence repeat (SSR) markers to study the population genetics of *N. trichotoma* in New Zealand. SSR markers allow population genetic analyses with large sample numbers, are economical, and are highly reproducible. This method has been successfully applied to the detection of the native range of other weeds in the past (Hardesty et al., 2012). *Nassella tenuissima* (Trin.) Barkworth is a closely related species with a similar native range that is also a naturalized weed in New Zealand and Australia and, due to its similar appearance, is often confused with *N. trichotoma* (Jacobs et al., 1998). We included samples of *N. tenuissima* in our study to test if the markers would amplify this species and enable molecular distinction.

Understanding the scale and distribution of the genetic variation of *N. trichotoma* in New Zealand is paramount to identifying the origin of the plants and will guide the search for potential biological control agents and the development of more efficient control methods.

METHODS AND RESULTS

Culm samples of *N. trichotoma* were collected from eight populations in New Zealand: two from the Canterbury Region (North Canterbury and Banks Peninsula), five from the Marlborough Region, and one from the Hawke's Bay Region. The samples were processed immediately or dried in silica gel. To test for cross-species amplification of the markers, five herbarium samples of a closely related species, *N. tenuissima*, were sourced from the Allan Herbarium, Lincoln, New Zealand. Location data and herbarium voucher information are listed in Appendix 1.

Total genomic DNA was extracted from a single fresh *N. trichotoma* culm sample (Nas-library, Appendix 1) using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. This DNA sample was used to generate a genomic library for a Roche 454 GS Junior using Roche Titanium chemistry (Roche, Basel, Switzerland). The Roche 454 GS Junior run resulted in 173,511 reads (average read length 419 bp) and a total yield

TABLE 1. Characteristics of 32 microsatellite loci developed in *Nassella trichotoma*.[a]

Locus	Primer sequences (5′–3′)[b]	Repeat motif	Allele size range (bp)	GenBank accession no.
NasSSR4[c]	F: ATGGCGACAGGATGAGTGC R: CACTCCGTTTCGCCCATTC	$(CT)_{11}$	194–197	MH301024
NasSSR5[c]	F: CGACGAGTGAGGTCTCCG R: AGCCCATGAATGGTTTGAGC	$(AAGAG)_4$	270, 274	MH301025
NasSSR13[c]	F: TGTCTCGCTGTTACAAATCGC R: CCACTCGAACCCTAGTCCC	$(CCG)_9$	311–323	MH301031
NasSSR15[c]	F: GGCACGAAATTGACCTGGC R: GACCATGATATGTTGCTCCGTC	$(CT)_{14}$	349–353	MH301033
NasSSR16[c]	F: TGATTCGATCCCGGAGGTG R: AGCCCAAGACGTTCAGAGG	$(CT)_{11}$	419–432	MH301034
NasSSR17[c]	F: AAGGACAGCCACTCGTACC R: TGTGCTTCAGACATGCTAATGG	$(AT)_{11}$	298–353	MH301035
NasSSR20[c]	F: TAAGCCAATCCGGTTCTAGCC R: GATTTGAGAGGCGATCGGTG	$(GCCGT)_4…(AAAGG)_6…(AGCCG)_6$	149–167	MH301037
NasSSR23[c]	F: ATGTAGGAGCTAGCCACGG R: AAGCAAGAGGATGGCAACG	$(AG)_{11}$	311–341	MH301038
NasSSR27[c]	F: CGGGACAATCTCCTCGGG R: TTCCAGAAAGCTCCACGGG	$(AC)_{10}$	169–188	MH301041
NasSSR29[c]	F: GCTGAATCTTTAACTGTTGTCACG R: CGTCCAATTGTTTCGATGCG	$(AG)_{10}$	245–262	MH301042
NasSSR35[c]	F: AGCGCACCGTTATTTCCATC R: CGGGAAACCCTAGACAGGC	$(CT)_{12}$	161–200	MH301046
NasSSR36[c]	F: GGGACGAATCAAACGCGAC R: AGAACGATTATGCATCTGAACG	$(CTT)_{14}$	245–263	MH301047
NasSSR37[c]	F: AGAACTGGACAGGATCGATTAAC R: TAGTGGTCAGAGCATGCCG	$(AG)_{13}$	188–199	MH301048
NasSSR40[c]	F: TGTGTTTCTCAGTCATACGAGC R: CAACGCAGCCATATCCACC	$(AC)_{13}$	169–172	MH301050
NasSSR45[c]	F: ATCGTGGAGGTAACGTCGG R: ACTCTGAATTGAAACACGTATGC	$(ATCT)_8$	366, 382	MH301052
NasSSR1[d]*	F: AGCTCGTCATCCATTTCAACG R: CTGGCTGTGTCGCATGAAC	$(AATCT)_4$	454	MH301023
NasSSR6[d]*	F: CAATGAACGTCCGATCCCG R: AATCAAGATAAACTCATCCGAGC	$(CTTTT)_4$	256	MH301026
NasSSR7[d]*	F: ACGCAACGCGCAGTATTTC R: TGCATGCGTATCAATTAACCTG	$(GATT)_7$	395	MH301027
NasSSR10[d]*	F: AGTGACGTGTGCTACCTCC R: TGGGACACATATCCGAGGC	$(CCTCT)_4$	399	MH301028
NasSSR11[d]*	F: CACTGGGTCACTGGTTCGG R: AGCAGTATAGTACGGTTGTTAAATGG	$(AC)_{11}$	193	MH301029
NasSSR12[d]*	F: CGCTTTAAACGCAAACCCG R: TGGCCTACTAGCTTTGTCCC	$(CGTGT)_4$	254	MH301030
NasSSR14[d]*	F: CTACGGCCTCGATATCCGC R: GCTTGTTCCTCGGATCTGTTG	$(GCT)_8$	419	MH301032
NasSSR19[d]*	F: GAAAGAATCGCGCCGGAAG R: GGACGGGTCACCTCATGG	$(AGC)_8$	164	MH301036
NasSSR25[d]*	F: TTCGTTCCGGGCTCTTCC R: TTAGAGCATGGCACCGAGG	$(ACGGC)_5$	330	MH301039
NasSSR26[d]*	F: ACGGTTTCAAGTACATGCCAG R: ACCCGGACCATCGATCAAG	$(CATCT)_4$	201	MH301040
NasSSR31[d]*	F: AGCCGAACGCGATAATTCC R: AGTTCCGATTGCCTGGTTG	$(AC)_{10}$	190	MH301043
NasSSR32[d]*	F: GCGCGTAAGCACCAGTTC R: GGTATGCAATGCGACGGAC	$(GTTTT)_5$	262	MH301044
NasSSR34[d]*	F: GCAGTGGCCCTAGGATTGG R: GCAGCACAAGGTAACACGG	$(CGG)_8$	262	MH301045
NasSSR38[d]*	F: TCCAGAAGAGTTCCCACGG R: TGCACGTGGGTTAAGAAAGG	$(AAAAG)_4$	287	MH301049
NasSSR42[d]*	F: GGCCATGAGGCAAATGGAG R: CCAAGTCCTGCTCACATCG	$(ATC)_8$	182	MH301051
NasSSR47[d]*	F: TCCGTATCGTGACAGCTTG R: GCCAACTTTCGTGACGGG	$(AACC)_6$	202	MH301053
NasSSR48[d]*	F: CAGCTGCACAGTCACATGG R: TTCCGATCGAGCACCATTC	$(GCGT)_6$	419	MH301054

[a]Annealing temperatures as per the Methods and Results section.
[b]M13 tag (TGTAAAACGACGGCCAGT) added to the 5′ end of each forward primer.
[c]Values based on 105 samples representing eight New Zealand populations (N = 10–15 for each).
[d]Values based on seven samples for primer tests (see Appendix 1).
*Monomorphic marker.

TABLE 2. Genetic properties of the 15 newly developed polymorphic microsatellite markers of *Nassella trichotoma* in New Zealand and cross-amplification results in *N. tenuissima*.[a]

	SI-NC (*N* = 15)			SI-MB-1 (*N* = 10)			SI-MB-2 (*N* = 10)			SI-MB-3 (*N* = 10)			SI-MB-4 (*N* = 15)			SI-MB-5 (*N* = 15)			NI-HB (*N* = 15)			SI-CB (*N* = 15)			Total	Mean		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*N. ten*
NasSSR4	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.12	3	0.00	0.04	–
NasSSR5	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.05	+
NasSSR13	3	1.00	0.56	2	0.20	0.18	2	0.10	0.10	2	0.80	0.48	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.07	0.13	4	0.27	0.18	–
NasSSR15	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.00	0.05	–
NasSSR16	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.93	0.56	4	0.12	0.12	–
NasSSR17	3	1.00	0.56	2	1.00	0.50	2	1.00	0.50	3	1.00	0.55	2	1.00	0.50	2	1.00	0.50	2	1.00	0.50	3	0.07	0.13	5	0.88	0.47	+
NasSSR20	3	1.00	0.56	2	1.00	0.50	2	1.00	0.50	2	0.90	0.50	2	1.00	0.50	2	1.00	0.50	2	1.00	0.50	5	0.31	0.44	5	0.90	0.50	–
NasSSR23	3	1.00	0.56	2	1.00	0.50	2	1.00	0.50	4	1.00	0.59	2	1.00	0.50	2	1.00	0.50	2	1.00	0.50	3	0.07	0.13	6	0.88	0.47	–
NasSSR27	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.93	0.50	5	0.12	0.11	–
NasSSR29	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.93	0.56	3	0.12	0.07	–
NasSSR35	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.12	3	0.00	0.04	+
NasSSR36	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	4	0.00	0.05	–
NasSSR37	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.93	0.56	4	0.12	0.10	+
NasSSR40	2	0.00	0.23	1	0.00	0.00	1	0.00	0.00	2	0.00	0.18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	3	0.93	0.56	3	0.12	0.12	–
NasSSR45	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	0.00	–

Note: + = successful cross-amplification; — = unsuccessful cross-amplification; *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals analyzed, *N. ten* = *Nassella tenuissima*.

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

of 72.8 Mbp of sequence. The data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRR7125326). DNA from all other samples were extracted using a JANUS work station (PerkinElmer, Waltham, Massachusetts, USA) and the NucleoSpin Plant II kit (PL1 buffer; Macherey-Nagel, Düren, Germany) or the Maxwell DNA Extraction Kit (Promega Corporation, Madison, Wisconsin, USA) following the manufacturer's instructions. DNA quality and quantity were determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Kentucky, USA).

Molecular markers for *N. trichotoma* were prepared following the method of Abdelkrim et al. (2009) with modifications. The *Nassella* sequence reads were searched for di- to hexanucleotide repeat regions flanked by appropriate regions for primer design using MSATCOMMANDER (Faircloth, 2008), resulting in a total of 745 SSR regions. Primers were designed via the default settings of Primer3 (Rozen and Skaletsky, 1999) as implemented in MSATCOMMANDER with the following user specifications: amplification regions of 100–500 bp, an optimal oligo melting temperature range of 57–62°C, GC content range of 20–80% with an optimum rate of 50%, low levels of self- or pair-complementarity, and a maximum end-stability (ΔG) of 8.0 (Faircloth, 2008). According to these criteria, 48 primer pairs were chosen for screening. These 48 primer pairs were tested on the library sample (Nas-library) and six samples from the Marlborough Region (Appendix 1). An M13 tag (TGTAAAACGACGGCCAGT) on the 5′ end of the forward primer enabled the use of fluorescent-labeled M13F probes to be included in the second step of the PCR amplification, prior to separation via capillary electrophoresis (Schuelke, 2000; Abdelkrim et al., 2009). PCRs and capillary electrophoreses were performed as described in Goeke et al. (2017), using the same PCR conditions for all markers: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 30 s; followed by 10 cycles of 95°C for 20 s, 51°C for 15 s, and 72°C for 30 s; and final extension at 72°C for 10 min. Fragments were sized and scored using GeneMarker version 2.7.0 (SoftGenetics, State College, Pennsylvania, USA) and Geneious 10.1.3 (Biomatters Ltd., Auckland, New Zealand), and polymorphism and repeatability of each locus were assessed.

Of the 48 tested SSR markers, 32 loci could be successfully amplified and showed no more than two alleles per individual. Of the 48 loci tested, 17 produced monomorphic and 15 produced polymorphic fragments (Table 1). The screening of further samples

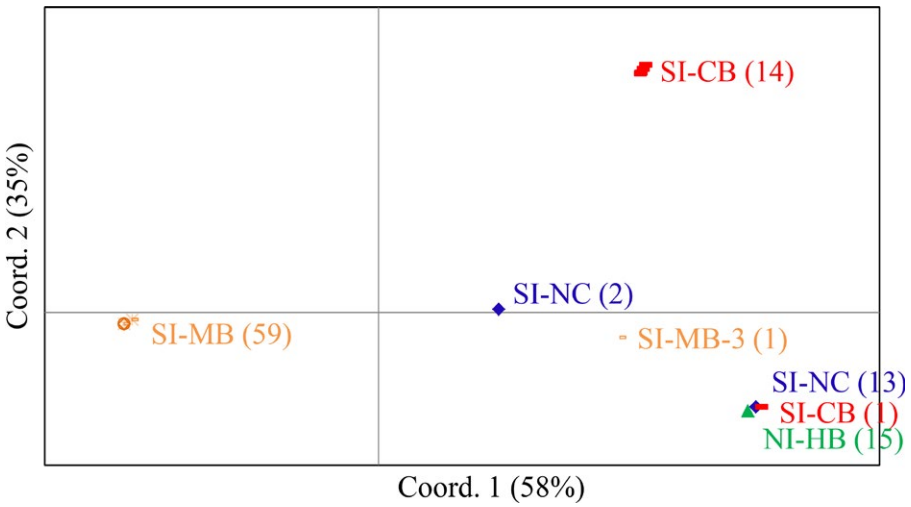

FIGURE 1. Principal coordinates analysis of SSR marker data from 105 *Nassella trichotoma* individuals from eight populations and four regions of New Zealand. Numbers in parentheses represent sample numbers. Coordinate 1 explains 58% of the variation. Coordinate 2 explains 35% of the variation. NI = North Island; SI = South Island; CB = Canterbury (red); HB = Hawke's Bay (green); MB = Marlborough (orange); NC = North Canterbury (blue).

was conducted with the 15 polymorphic loci, of which 11 could be combined into three multiplex PCR reactions. Multiplex combinations were: set 1 (NasSSR5, NasSSR15, NasSSR16, and NasSSR35), set 2 (NasSSR4, NasSSR23, NasSSR36, and NasSSR40), and set 3 (NasSSR17, NasSSR29, and NasSSR45). Initial testing of these mixes with four samples confirmed the applicability of the multiplexed primer pairs for detection of correct genotypes. The three mixes and four individual primer pairs were then applied to the remaining *N. trichotoma* DNA samples for genotyping and to five *N. tenuissima* samples to test cross-species amplification. The 15 developed novel markers could successfully be applied to genotype 105 *N. trichotoma* samples. Summary statistics and principal coordinates analysis (PCoA) were prepared using GenAlEx 6.501 (Peakall and Smouse, 2006). Among the 15 polymorphic loci, the total number of alleles per locus ranged from two to six, mean levels of observed heterozygosity ranged from 0.00 to 0.90, and mean levels of expected heterozygosity ranged from 0.00 to 0.50. The mean percentage of polymorphic loci across all populations was 42.5%. Four of the 15 markers (27%) successfully cross-amplified the related species *N. tenuissima* (Table 2). The fragment sizes were monomorphic, identical for all five samples, and different from *N. trichotoma* (NasSSR5 = 267 bp, NasSSR17 = 327 bp, NasSSR35 = 169 bp, and NasSSR37 = 182 bp). The PCoA analysis separated the three regions on the South Island: Canterbury (Banks Peninsula), North Canterbury (Benger Farm), and Marlborough (Fig. 1). The five populations from Marlborough formed one group. The Hawke's Bay population on the North Island was similar to the North Canterbury population. One individual sample from Banks Peninsula, Canterbury, also belonged to this combined group. Two individual samples from North Canterbury and one from Marlborough did not group with any population. The genotype of the single sample used for generating the sequencing library (from Amberley, North Canterbury) was similar to the North Canterbury/Hawke's Bay group.

CONCLUSIONS

We developed 32 SSR markers for *N. trichotoma* in New Zealand, based on Roche 454 sequencing of total genomic DNA. Fifteen polymorphic markers were optimized for genotyping of eight populations from one region in the North Island and three regions in the South Island of New Zealand. Genetically distinct populations were identified, and the results will be valuable for intraspecific phylogenetic and population structure studies to identify the overseas origin of *N. trichotoma* in New Zealand and to source potential biological control agents. Cross-amplification of the markers in *N. tenuissima* suggests their suitability for other closely related species. The monomorphic markers could also potentially be useful for the study of closely related species.

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DATA ACCESSIBILITY

Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1.

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APPENDIX 1. Location data and herbarium voucher information for the *Nassella* specimens included in this study.

Species	Sample/voucher ID	Collection locality	Sampling region	Geographic coordinates[a] E	N
N. tenuissima (Trin.) Barkworth	CHR614137	Takaka	Golden Bay, South Island, NZ	2493305	6031797
N. tenuissima	CHR688701	Christchurch	Canterbury, South Island, NZ	2479963	5738940
N. tenuissima	CHR640050	Taihape	Manawatu-Whanganui, North Island, NZ	2749161	6165585
N. tenuissima	CHR553902	Hamilton	Waikato, North Island, NZ	2714714	6376380

(continued)

APPENDIX 1. (Continued)

Species	Sample/voucher ID	Collection locality	Sampling region	Geographic coordinates[a] E	N
N. tenuissima	CHR525821	Masterton	Wairarapa, North Island, NZ	2733007	6023282
N. trichotoma (Nees) Hack. ex Arechav.	Nas-library[b,c]	Amberley	North Canterbury, South Island, NZ	2492817	5782876
N. trichotoma	SI-NC_01–15	Mt. Benger Farm	North Canterbury, South Island, NZ	2499892	5814003
N. trichotoma	SI-MB-1_01–10[b]	Ridgetop Rise	Marlborough, South Island, NZ	1672879–1672902	5401380–5401391
N. trichotoma	SI-MB-2_01–10[b]	Schollum	Marlborough, South Island, NZ	1649686–1649697	5394415–5394422
N. trichotoma	SI-MB-3_01–10[b]	Lulworth	Marlborough, South Island, NZ	1692747–1692827	5362141–5362188
N. trichotoma	SI-MB-4_01–15	Kemp's Farm	Marlborough, South Island, NZ	2596843	5956999
N. trichotoma	SI-MB-5_01–15	Dashwood	Marlborough, South Island, NZ	2598237	5953444
N. trichotoma	NI-HB_01–15	Craggy Range Rd., Tuki Tuki	Hawke's Bay, North Island, NZ	2851386	6162322
N. trichotoma	SI-CB_01–15	Banks Peninsula Scarborough Reserve	Canterbury, South Island, NZ	2489771	5734862

Note: CB = Canterbury; CHR = vouchers deposited at Allan Herbarium, Lincoln, New Zealand; HB = Hawke's Bay; MB = Marlborough; NC = North Canterbury; NI = North Island; SI = South Island.

[a]Coordinates are given in New Zealand Map Grid coordinates.

[b]The Nas-library sample from Amberley and two samples each from Ridgetop Rise, Schollum, and Lulworth were used for the initial primer screen.

[c]Used for library construction.