Applications in Plant Sciences

Applications in Plant Sciences 2014 2(7): 1400029

Primer Note

Microsatellite primers for the fungi *Rhizopogon kretzerae* and *R. salebrosus* (Rhizopogonaceae) from 454 shotgun pyrosequencing[1]

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- *Premise of the study: Rhizopogon kretzerae* and *R. salebrosus* (Rhizopogonaceae) are ectomycorrhizal fungi symbiotic with pines and the mycoheterotrophic plant *Pterospora andromedea* (Ericaceae). Microsatellite loci will allow population genetic study of fungal hosts to *P. andromedea*.
- *Methods and Results:* Shotgun pyrosequencing of *R. kretzerae* DNA resulted in primer development of 23 perfect microsatellite loci and screened across two populations each for *R. kretzerae* and *R. salebrosus*. Twelve loci were polymorphic in *R. kretzerae* populations, and 11 loci cross-amplified in *R. salebrosus* populations. For *R. kretzerae* and *R. salebrosus*, number of alleles was one to eight and one to nine, respectively, and observed heterozygosity ranged from 0.00–0.57 and 0.00–0.70, respectively.
- *Conclusions:* These are the first microsatellite loci developed for any species within *Rhizopogon* subgenus *Amylopogon.* These microsatellite loci will be used in conservation genetic studies of rare to endangered eastern populations and to compare plant and fungal population genetic structure at different hierarchical levels.

Key words: conservation genetics; GS-FLX Titanium; microsatellites; rare fungi; *Rhizopogon kretzerae*; *Rhizopogon* subgenus *Amylopogon.*

Rhizopogon Fr. (Rhizopogonaceae) is a fungal genus of species that form ectomycorrhizal symbioses with conifers and are pivotal components of temperate forest ecosystems (Molina et al., 1999). Four species (*R. kretzerae* Grubisha, Dowie & Mill., *R. salebrosus* A. H. Sm., *R. ellenae* A. H. Sm., and *R. arctostaphyli* A. H. Sm.) in *Rhizopogon* subgenus *Amylopogon* (A. H. Sm.) Grubisha & Trappe are obligately ectomycorrhizal with several pine species and are the symbiotic mycobiont host to the mycoheterotrophic plant, *Pterospora andromedea* Nutt. (Ericaceae; Bidartondo and Bruns, 2002; Dowie et al., 2011, 2012; Hazard et al., 2012; Grubisha et al., 2014), a North American endemic divided into eastern and western populations (Bakshi, 1959). *Pterospora andromedea* primarily associates with two closely related *Rhizopogon* species: western *P. andromedea* are symbiotic primarily with *R. salebrosus* while eastern populations are found with *R. kretzerae* (Grubisha et al., 2014). *Rhizopogon kretzerae* is currently only known from collections of *P. andromedea* roots and only found in association with *Pinus strobus* L. (Hazard et al., 2012; Grubisha et al., 2014). Eastern *P. andromedea* populations have undergone recent decreases in population size and possible local extinction from a variety of anthropogenic factors (Schori, 2002).

[1]Manuscript received 22 March 2014; revision accepted 20 May 2014.

Funding for this research has been provided by the National Science Foundation (DEB-1050315 to M.R.K. and DEB-1050292 to S.L.M.).

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doi:10.3732/apps.1400029

Copopulation genetic studies of the fungal host and mycoheterotrophic plant will (1) provide information about levels of genetic variation and population structure at different hierarchical levels in plant and fungal populations, and (2) be important in developing conservation management plans for the rare to endangered eastern populations of both species. While microsatellite loci have been previously developed for distantly related species in three of the five *Rhizopogon* subgenera (Kretzer et al., 2000, 2004; Grubisha et al., 2005), the microsatellite loci reported here, which were developed from *R. kretzerae* and *R. salebrosus*, are the first for any species in *Rhizopogon* subgenus *Amylopogon*.

METHODS AND RESULTS

Genomic DNA (gDNA) was isolated from 60 mg of silica gel–dried pure cultures of *R. kretzerae* isolate Miller207 (Michigan; Table 1) and *R. salebrosus* isolate Miller322 (Washington; 46.20545°N, 117.7704°W) separately using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). These two cultures were obtained from *P. andromedea* monotropoid roots following Grubisha et al. (2014). Each culture was ground in liquid nitrogen to a fine powder. Twice the recommended volume of buffer AP1, RNaseA, and buffer AP2 were used. Library construction and 454 shotgun pyrosequencing were performed by the Advanced Genetic Technologies Centre (AGTC) at the University of Kentucky (Lexington, Kentucky, USA). Libraries for each species were prepared using the GS FLX+/XL+ Titanium Rapid Library Preparation Kit (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). Instead of nebulizing, gDNA was sheared using a Bioruptor NGS (UCD-600TS; Diagenode, Denville, New Jersey, USA) by sonicating at 4°C with six cycles of 5 s on and 90 s off. Shotgun 454 pyrosequencing was performed on 25% of a

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Table 1. Genetic properties of 17 microsatellite loci developed from *Rhizopogon kretzerae* and one locus from *R. salebrosus*, and potential applicability in *R. arctostaphyli* and *R. ellenae*.

Locus	*R. kretzerae* Quebec[a] (n = 14)			*R. kretzerae* Michigan[a] (n = 11)			*R. salebrosus* Oregon[a] (n = 20)			*R. salebrosus* Wyoming[a] (n = 20)			*Ra* (n = 2)[a]	*Re* (n = 2)[a]
	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e		
Rkre3	3	0.14	0.14	1	0.00	0.00	I	—	—	I	—	—	M	—
Rkre5	2	0.07	0.19	2	0.18	0.17	1	0.00	0.00	2	0.15	0.14	M	—
Rkre6	2	0.50	0.44	2	0.36	0.46	7	0.45	0.61	6	0.70	0.76	—	—
Rkre14	3	0.29	0.25	1	0.00	0.00	S	—	—	S	—	—	—	—
Rkre15	2	0.21	0.19	3	0.18	0.17	5	0.65	0.72	5	0.55	0.72	P	M
Rkre17	S	—	—	S	—	—	4	0.00	0.27	4	0.00	0.55	P	M
Rkre18	1	0.00	0.00	3	0.27	0.24	S	—	—	S	—	—	P	M
Rkre20	1	0.00	0.00	1	0.00	0.00	2	0.10	0.18	2	0.05	0.05	P	M
Rkre23	2	0.29	0.34	2	0.27	0.48	3	0.05	0.14	4	0.35	0.67	P	M
Rkre24	4	0.43	0.47	2	0.55	0.40	4	0.35	0.44	4	0.40	0.72	M	P
Rkre27	2	0.43	0.41	1	0.00	0.00	3	0.25	0.59	3	0.35	0.56	P	M
Rkre29	1	0.00	0.00	1	0.00	0.00	3	0.10	0.10	2	0.25	0.22	—	—
Rkre34	4	0.57	0.64	3	0.18	0.17	6	0.25	0.69	5	0.40	0.73	M	—
Rkre36	5	0.43	0.55	5	0.45	0.62	5	0.10	0.48	5	0.25	0.72	—	—
Rkre37	4	0.29	0.26	2	0.00	0.20	I	—	—	I	—	—	—	—
Rkre38	1	0.00	0.00	1	0.00	0.00	5	0.35	0.72	5	0.45	0.58	M	M
Rsal2	—	—	—	—	—	—	1	0.00	0.00	3	0.10	0.10	M	—

Note: — = no PCR amplification; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; I = does not amplify in all isolates; M = monomorphic; n = sample size; P = polymorphic; *Ra* = *R. arctostaphyli*; *Re* = *R. ellenae*; S = stutter.

[a] Geographic coordinates for the populations are: Oregon = 44.86375°N, 118.40122°W; Wyoming = 41.18207°N, 106.13342°W; Michigan = 46.6427°N, 89.1787°W; and Quebec 45.4947°N, 76.3786°W. Due to the rare to endangered status of the eastern populations, locations are approximate. *Rhizopogon arctostaphyli*: Oregon = 44.49815°N, 120.38706°W; Washington = 46.24940°N, 117.76921°W. *Rhizopogon ellenae*: Wyoming = 45.08520°N, 109.35103°W; Washington = 44.78683°N, 118.36720°W.

plate on a Titanium GS-FLX (454 Life Sciences, a Roche Company) yielding a total of 152,007 reads with an average length of 414 bp for *R. kretzerae* and a total of 92,252 reads with an average length of 517 bp for *R. salebrosus*. At the AGTC, raw sequence reads were initially processed using prinseq-lite.pl (http://prinseq.sourceforge.net/) and involved removing all (1) reads with >2% length with uncalled bases (N), (2) duplicate reads, (3) reads with Q < 17, (4) reads too short in length [average length – 2(standard deviation)], and (5) reads too long in length [average length + 2(standard deviation)]. Processed reads were assembled de novo using Newbler version 2.8 (454 Life Sciences, a Roche Company) at the AGTC, which produced 10,190 contigs for *R. kretzerae* (average contig size = 1502 bp, N50 = 1701 bp) and 5178 contigs for *R. salebrosus* (average contig size = 1302 bp, N50 = 1428 bp).

Perfect microsatellite repeats were identified from the assembled contigs using MSATCOMMANDER version 1.0.8 (Faircloth, 2008) for *R. kretzerae* and *R. salebrosus*, yielding 43 and 21 dinucleotides with at least six repeat units, 137 and 48 trinucleotides with at least five repeat units, 34 and 12 tetranucleotides with at least four repeat units, five and 11 pentanucleotides with at least four repeat units, and five and 11 hexanucleotides with at least four repeat units, respectively. Within MSATCOMMANDER, Primer3 (Rozen and Skaletsky, 2000) was used to generate primers for all loci using default parameters except that difference in acceptable primer melting temperature was 2°C with an optimum of 60°C; maximum poly X was set to three; GC clamp = yes; and a target PCR product size of 90–210 bp. Selection of loci for initial screening was based on Primer3 output tabulated in a spreadsheet by MSATCOMMANDER, and those that had no lowercase letters in the primer sequences that would reflect nucleotide mismatches across reads in assembled contigs. Forty-eight loci (38 identified from *R. kretzerae* and 10 from *R. salebrosus*) were initially screened across four samples (two *R. kretzerae*, two *R. salebrosus*; Table 1) for positive PCR products using agarose gel electrophoresis. Loci were PCR amplified using 0.1 μM of each forward and reverse primer in a 10-μL reaction volume that included 1× CoralLoad PCR Buffer with 1.5 mM $MgCl_2$ (QIAGEN), 200 μM each dNTP, 2.5 units *Taq* DNA Polymerase (QIAGEN), and 1.0 μL of 1 : 10 diluted genomic DNA. Thermocycler parameters were 94°C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension for 10 min at 72°C. PCR products were visualized in a 2% agarose gel stained with GelRed (Phenix Research Products, Candler, North Carolina, USA) on a UV transilluminator. Of the 48 loci assessed, 27 loci (23 from *R. kretzerae* and four from *R. salebrosus*) yielded no more than one or two bright PCR product bands within the approximate expected size range (target size + 40 bp, −10 bp) and no PCR products outside the expected size range. The lower threshold was set to target size −10 bp due to the relatively low number of repeats.

These 27 loci were screened in 65 isolates from two populations each for *R. kretzerae* (n = 14 and n = 11; Table 1) and *R. salebrosus* (n = 20 both; Table 1), and for cross-amplification in two distantly related *Rhizopogon* subgenus *Amylopogon* species that are also hosts for *P. andromedea*: *R. arctostaphyli* (n = 2 for both; Table 1) and *R. ellenae* (n = 2 for both; Table 1). *Pterospora andromedea* ectomycorrhizal roots were collected and fungal DNA was isolated as previously described (Dowie et al., 2012; Grubisha et al., 2014). Due to the extremely small sample of rootball collected, voucher specimens in herbaria were not made except for samples from Michigan (DAOM 242738) and Quebec (DAOM 242739) that were previously deposited at the Canadian National Mycological Herbarium (Grubisha et al., 2014). The forward primer was 5′ end-labeled with one of four dyes in the ABI DS-33 dye set (Table 2). Loci were PCR amplified in multiplex reactions using the QIAGEN Multiplex PCR Kit in a 10-μL volume with 1× QIAGEN Multiplex PCR Master Mix, 50 nM each primer, 0.25–0.5× Q-solution, and 1.0 μL of 1 : 10 diluted genomic DNA. Touchdown thermocycler conditions were: 95°C for 15 min; 10 cycles of 94°C for 30 s, 67°C for 90 s, decreasing 1°C each cycle, and 72°C for 30 s; 25 cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 30 s; with a final extension of 45 min at 60°C. Primers with a higher or lower touchdown annealing profile are listed in Table 2. Fragment analysis was conducted using the GeneScan 500 LIZ Size Standard (Applied Biosystems, Foster City, California, USA) on an ABI 3730 DNA Analyzer (Applied Biosystems) by the Biotechnology Resource Center (BRC) at Cornell University. Allele sizes were called manually using the Microsatellite Plugin in Geneious version R6.1.5 (Drummond et al., 2011). Loci selected for the final set were PCR amplified independently to verify allele size ranges.

Of the 23 loci developed in *R. kretzerae* and screened with fluorescently labeled primers in *R. kretzerae* populations, 12 loci were polymorphic (Table 1), seven loci were monomorphic, and four loci had excessive stutter. In *R. salebrosus* populations, 21 of the 23 loci developed in *R. kretzerae* successfully PCR amplified and 11 loci were polymorphic, four of which were monomorphic in *R. kretzerae* populations (Table 1). The four loci isolated in *R. salebrosus* did not PCR amplify in *R. kretzerae* populations screened. In *R. salebrosus* populations, only one locus (Rsal2) was polymorphic (Table 1). A few loci

Table 2. Characteristics of 12 polymorphic and five monomorphic microsatellite loci developed in *Rhizopogon kretzerae* and one polymorphic locus developed in *R. salebrosus*.

Locus[a]	5′ end-labeled dye	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
Rkre3	NED	F: TACCTTGCCCACTATCGACG R: TAATCGATGGACCCGCTGAG	$(TGC)_5$	200–221	60	KJ443650
Rkre5	VIC	F: AGACCAATACGCGTGAACAC R: CAGAGCAATCCGAACACGAG	$(CG)_6$	179–183	57	KJ443651
Rkre6	PET	F: CCGTGACAATCCAACTGCAC R: GGCAACACTGAATCTGGAGC	$(ACG)_6$	218–221	57	KJ443652
Rkre14	VIC	F: AATGCTGGACTTCTTGCACG R: TGGGCATCATGGTGTAGGAC	$(AG)_6$	192–196	60	KJ443653
Rkre15	6-FAM	F: TGTTTGGTCGTGATCTTGTAGC R: ACCTTCGTAGTCCAGTGCTG	$(AT)_7$	169–181	52	KJ443654
Rkre17	PET	F: AAGCAATAAAGGAATCTGCAGC R: CCGGATGTCTAACACTGGTC	$(AT)_{11}$	176	57	KJ443659
Rkre18	6-FAM	F: TCAATGACCGGTCCACTCTC R: GTCAGTAGGTGTATTCTTGCTGG	$(ATGC)_5$	120–128	60	KJ443655
Rkre19	PET	F: GATGCAAGGCTCGTTGTCTC R: GCAAGTTCTGGAGCAAGGAC	$(TCT)_{10}$	202	57	KJ443660
Rkre20	VIC	F: TGCTTCTCAAACAACCGAGG R: GGTGGTAGCGATTGAGGGAC	$(ACC)_6$	114	57	KJ443661
Rkre23	PET	F: ACTTTGATGCAGGCGAGAAC R: TGACCGTCACAAATCGTTCC	$(AGC)_5$	129–132	57	KJ443656
Rkre24	NED	F: TCAGGGTTGGACTCGTGAAG R: CGCGCCTGAAGTTTGGAC	$(AGC)_7$	117–132	52	KJ443657
Rkre27	6-FAM	F: ATGGAGGATGAGAGTGCGG R: CGTCGTGACATGAGTGCTTG	$(ACG)_6$	108–111	57	KJ443658
Rkre29	PET	F: AGGTTGGCTGTGCATGAATG R: ACACTAGCCTGCTTCCTCAG	$(AT)_6$	188	57	KJ443662
Rkre34	PET	F: TATAGACTCGGACGCACACC R: AGGTTCCCGTGAGTGATATCG	$(CA)_{10}$	119–131	57	KJ443663
Rkre36	NED	F: AAGCAGGGTACAGACTCACC R: TCTGGATCGTTTGCAATGCC	$(TC)_{11}$	173–215	57	KJ443664
Rkre37	VIC	F: TGGTGGTGGGTATGAGTTGG R: TTTGTCAAAGCAGGCTACGC	$(TGG)_9$	183–217	52	KJ443665
Rkre38	NED	F: ATGCAGGTAGACGGGACTTG R: TACTCGTCGCTACAGTCCG	$(GTG)_6$	209	57	KJ443666
Rsal2	NED	F: TGTCCTCAAGAGATCCGCAG R: AGATTCATCGAAACCGCACG	$(TCG)_5$	110–119	57	KJ443667

Note: T_a = annealing temperature.
[a] Rkre = isolated from *R. kretzerae*; Rsal = isolated from *R. salebrosus*.

cross-amplified in *R. arctostaphyli* and *R. ellenae* isolates and may prove to be useful when screened at a larger scale (Table 1). Locus Rkre19 had stutter problems in both *R. kretzerae* and *R. salebrosus* but was polymorphic in *R. arctostaphyli* and *R. ellenae* (Table 1).

Number and size range of alleles, and observed (H_o) and expected heterozygosities (H_e) were calculated in GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012; Table 1). When both populations for each species were considered (*R. kretzerae*, n = 25; *R. salebrosus*, n = 40), for the loci isolated in *R. kretzerae* (Rkre3–Rkre38; Table 1), there were one to eight alleles, and H_e ranged from 0.00 to 0.64 in *R. kretzerae* populations (Table 1); and there were one to nine alleles and H_e values of 0.00 to 0.76 in *R. salebrosus* populations (Table 1). For locus Rsal2 there were one to three alleles, and H_e ranged from 0.00 to 0.10. Scoring errors due to stuttering, large allele dropout, and null alleles were checked using MICROCHECKER 2.2.3 (van Oosterhout et al., 2004). For *R. kretzerae* populations, two loci showed signs of possible null alleles (Rkre5 in Quebec and Rkre37 in Michigan). In the combined *R. salebrosus* populations, possible null alleles and scoring errors due to stuttering were detected at four loci (Rkre3, Rkre17, Rkre27, Rkre34).

CONCLUSIONS

These are the first microsatellite loci developed for any species in *Rhizopogon* subgenus *Amylopogon*. Presently we are using these loci to assess the conservation genetic status of *R. kretzerae*, which will be important for developing conservation management plans for eastern *P. andromedea*. We are also conducting copopulation genetic studies to determine if this fungal host and mycoheterotrophic plant share similar patterns of population genetic structure and variation at different hierarchical levels. Cross-amplification of loci isolated in *R. kretzerae* with *R. salebrosus* and two distantly related *Rhizopogon* subgenus *Amylopogon* species suggests they may be useful in studies of other species in this subgenus.

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