Development of Microsatellite Markers for the Apomictic Triploid Fern Myriopteris lindheimeri (Pteridaceae)

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Primer Note

Development of microsatellite markers for the apomictic triploid fern *Myriopteris lindheimeri* (Pteridaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed for investigating the population dynamics of *Myriopteris lindheimeri* (Pteridaceae), an apomictic triploid fern endemic to deserts of the southwestern United States and Mexico.
- *Methods and Results:* Using 454 sequencing, 21 microsatellite markers were developed. Of these, 14 were polymorphic with up to five alleles per locus and eight markers amplified in one or more congeneric close relatives (*M. covillei*, *M. fendleri*, *M. aurea*, and *M. rufa*). To demonstrate marker utility, *M. lindheimeri* samples from three Arizona populations were genotyped at nine loci. For each population, diversity measures including percent polymorphic loci, frequency of heterozygotes across all loci, and genotypic diversity were calculated. Across the three populations, on average, 63% of loci were polymorphic, the average frequency of heterozygotes (across all loci) was 0.32, and average genotypic diversity was 0.34.
- *Conclusions:* These markers provide a foundation for future studies exploring polyploidy and apomixis in myriopterid ferns.

Key words: asexual; cheilanthoid; *Myriopteris lindheimeri*; polyploidy; pteridophyte; simple sequence repeats (SSRs).

Myriopteris Fée is an early diverging lineage of cheilanthoid ferns (Pteridaceae) that contains approximately 47 species encompassed within three major clades—all of which, until recently, were circumscribed in the large, polyphyletic genus, *Cheilanthes* Sw. (Grusz and Windham, 2013; Grusz et al., 2014). The covillei clade is the largest subclade in the recently resurrected genus *Myriopteris*, within which *M. lindheimeri* (Hook.) J. Sm. resides (Grusz et al., 2014). *Myriopteris lindheimeri* itself comprises a number of relatively widespread, apomictic triploid lineages ($n = 2n = 90$ chromosomes; Windham and Yatskievych, 2003) derived from a comparatively rare, sexual diploid cytotype through intraspecific whole genome duplication, i.e., autopolyploidy (Grusz et al., 2009). Its distribution spans the southwestern United States (Arizona, New Mexico, Texas) and adjacent Mexico (Windham and Rabe, 1993).

Here, we use 454 next-generation sequencing to develop microsatellite markers for *M. lindheimeri*. Like *M. lindheimeri*, many members of the covillei clade are also apomictic polyploids or, alternatively, sexual diploids that are involved in the formation of downstream polyploid taxa of hybrid origin (Grusz et al., 2009). For this reason, we tested our newly developed markers for cross-amplification in diploid and polyploid taxa spanning the covillei clade, including: *M. aurea* (Poir.) Grusz & Windham (apomictic triploid), *M. covillei* (Maxon) Á. Löve & D. Löve (sexual diploid), *M. fendleri* (Hook.) E. Fourn. (sexual diploid), and *M. rufa* Fée (apomictic triploid) (Windham and Rabe, 1993; Windham and Yatskievych, 2003; Grusz and Windham, 2013; Grusz et al., 2014).

METHODS AND RESULTS

Genomic DNA of a single individual of diploid *M. lindheimeri* (voucher: *Schuettpelz 450* [DUKE], collected from the Tonto National Forest, Pinal Co., Arizona, USA) was extracted from silica gel–dried material using the DNeasy Plant Mini Kit following the manufacturer's protocol (QIAGEN, Valencia, California, USA). Genomic DNA was run on two lanes (1/4 plate = 24 wells) using the Roche 454 GS-FLX Titanium sequencing platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) at the Duke University Center for Genomic and Computational Biology sequencing facility. The 454 run generated 234,428 sequence reads with a median length of 403 bp. Raw data were scanned for di-, tri-, tetra-, penta-, and hexanucleotide perfect microsatellite repeats using MSATCOMMANDER version 0.8.2 (Faircloth, 2008). Of the 234,428 sequence reads searched, 25,295 sequences contained a total of 33,955 repeats. Given the surplus of repeat regions, we focused our efforts on a subset of nonplastid regions (determined by BLASTN against the *M. lindheimeri* chloroplast genome; Wolf et al., 2011) containing di-, tri-, and tetranucleotide repeats with sufficient flanking sequence in which to develop primers (Chakraborty et al., 1997). A total of 159 unlabeled primer pairs were designed in Primer3, using default settings, implemented within MSATCOMMANDER (Rozen and Skaletsky, 1999; Faircloth, 2008).

Each microsatellite region (159 in total) was amplified by PCR from genomic DNA of the individual for which the 454 sequencing was completed (*Schuettpelz 450*). This amplification followed Schuettpelz and Pryer (2007), except that the annealing temperature was set to 60°C to prevent nonspecific primer binding. Amplicons were visualized on a 1% agarose gel using SYBR Safe DNA Gel Stain (Life Technologies, Carlsbad, California, USA), run for 35 min at 75 V. Amplifications that produced a single strong band were purified

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TABLE 1. Characteristics of 21 microsatellite markers developed in *Myriopteris lindheimeri*.[a]

Locus	Primer sequences (5′–3′)[b]	Repeat motif	Allele size range (bp)	T_m (°C)	*n*	*A*	Het	GenBank accession no.
F28JE	F: GCACGTCCATTTGTACTCGG	$(AGC)_4$	271	60.0	16	1	0	KT215756
	R: CTAAACTGGCCTCTGCCAC			58.9				
F9ROB	F: TGGAGCGGAAGGAGAATCG	$(AGG)_6$	177	59.9	27	1	0	KT215757
	R: ACCTTCCTCCACGCATCTG			60.2				
FJIEP	F: GGCGGATCAAATGGACACC	$(ATC)_7$	228–246	59.6	75	5	1	KT215758
	R: TCCATGCTGGATAGCTCCTAC			59.5				
FYM3K	*F: AGAGTGAAACCAGAAACCTGC	$(ATC)_8$	197–203	59.2	84	4	0.99	KT215759
	R: GTGTGCCGCTTAAACAATGAAG			59.8				
GCG5L	*F: GCGAACTTTGGACAACGGG	$(CT)_8$	441–447	60.4	12	3	0.50	KT215760
	R: ACAGAAACCCTATCAAGGCAG			58.4				
HAWY1	F: ACGACTCCGAATAAAGTCTGC	$(AAG)_5$	262–268	58.9	11	2	0.91	KT215761
	R: GAGCATGATGGAACACTGCC			60.0				
HDLR7	F: CCTCGGCCTCTAGTGTAGC	$(ATC)_6$	197–206	59.7	15	2	0.53	KT215762
	R: CCTTTGCAAGACATGCCCG			60.5				
HGGWA	^F: ACCCACGCATGTAAACAGATTG	$(AAC)_6$	182–185	60.3	9	2	0.33	KT215763
	R: ACCATTTCTGTGGGAGGTC			57.4				
HL9PJ	F: CTCACCAACTAAGCTCCTTGAC	$(CT)_6$	411–415	59.4	10	3	0.50	KT215764
	R: GTGAGCTGCAGACGAAAGG			59.6				
HO454	F: AACACAGGTAGAGGCCGTG	$(AGC)_6$	175	60.1	6	1	0	KT215765
	R: TGCTGCTGCCTTGAAATCC			59.5				
HY3SM	^F: TTGTCACTGTGCGACATGC	$(ATGC)_5$	347–363	59.8	18	4	0.17	KT215766
	R: TCTTTCTAGCAATCTCAGAAGACC			58.9				
IAO3P	F: ACAAGTTGACATCCGTTGGG	$(AGAT)_5$	321	59.2	13	1	0	KT215767
	R: AGAGCTCCACCCTTTGACC			59.7				
IMEWX	F: TAGGTTTGCGCATTGCTGG	$(AGAT)_{10}$	352–356	59.9	14	2	0.14	KT215768
	R: CGTTCTGAGTTTCGGTCCC			58.9				
IQLI0	F: ACGCCAATCGATCTCAAGC	$(ACCTCC)_4$	184–214	59.1	31	4	0.68	KT215769
	R: ATGAAGGGAGAATGTCGGC			58.0				
IVYHJ	F: GGGTAGAAAGAATTTCAAGTGAGC	$(AAC)_6$	238	58.8	6	1	0	KT215770
	R: GAATTTGGGTCCGCAGGC			59.9				
J0629	^F: AGGTCGTTTCCGCCATTTC	$(AT)_6$	413–453	59.2	6	3	0.50	KT215771
	R: CACTGCGCTGCAACCTATC			60.0				
JDYSK	^F: GCTTTGTTAGTGGCCTCGC	$(CCG)_4$	280	60.2	75	1	0	KT215772
	R: AGGCTCGGATGAGGTTTGG			60.2				
JGM27	*F: AGCGGGCCTATTCCAGATAC	$(AGC)_7$	261–270	59.8	31	3	0.42	KT215773
	R: CTGTAGGTGGTGCGGAAAC			59.2				
JJUWV	F: AACACAGGTAGAGGCCGTG	$(AGC)_6$	191	60.1	6	1	0	KT215774
	R: TGTCACATCTCCCGGCTG			59.8				
JS90I	*F: CTTAAAGCTGCCTGCGACC	$(CT)_6$	352–355	59.9	10	2	0.70	KT215775
	R: GTTGCTGTCGGCTAAGGAC			59.3				
JW1YD	^F: GATCGTCGGCCGGGAAG	$(CCG)_6$	183–195	60.7	24	3	0.42	KT215776
	R: TCGGATGTGCTACAGGTGG			59.9				

Note: *A* = number of alleles; Het = frequency of heterozygotes; *n* = number of individuals surveyed; T_m = melting temperature.

[a]Voucher information for surveyed individuals is listed in Appendix 1.

[b]Asterisks (*) and hats (^) indicate that a 6-FAM (blue) or HEX (green) fluorescent label was used, respectively, when markers were multiplexed for population-wide genotyping (Tables 3 and 4); for all single-sample genotyping reactions, a 6-FAM (blue) fluorescent label was used.

TABLE 2. Amplification of microsatellite markers in taxa closely related to *Myriopteris lindheimeri*, together with their corresponding fragment lengths.[a,b]

Locus	*Myriopteris covillei* (*S2x*)	*Myriopteris fendleri* (*S2x*)	*Myriopteris rufa* (*A3x*)	*Myriopteris aurea* (*A3x*)
F9ROB	193, 205	205	175, 199	208
FYM3K	194	194, 200	203	—
HAWY1	—	423, 435	—	—
HDLR7	—	206	209	206
HY3SM	357	354	355	361
J0629	221, 316*	313	—	—
JJUWV	—	—	—	207
JW1YD	190	184	190	187, 193

Note: — = failed amplification.

[a]Ploidy levels are indicated in parentheses after taxon names (*S2x* = sexual diploid, *A3x* = apomictic triploid; Windham and Rabe, 1993); fragment lengths in bp.

[b]Detailed voucher information listed in Appendix 2.

*Fragment analysis produced stutter peaks.

TABLE 3. Population summary for nine highly polymorphic, newly developed microsatellite loci surveyed across three populations of *Myriopteris lindheimeri*.[a]

	Carr Canyon (n = 8)		Jacobson Canyon (n = 15)		Paradise (n = 13)	
Locus	*A*	% Heterozygous	*A*	% Heterozygous	*A*	% Heterozygous
FYM3K	2	87.5	2	13.3	2	84.6
GCG5L	2	12.5	3	100	2	8.3
HGGWA	2	12.5	1	0.0	2	7.7
HY3SM	1	0.0	2	86.7	3	15.4
J0629	1	0.0	1	0.0	1	0.0
JDYSK	1	0.0	1	0.0	1	0.0
JGM27	1	0.0	1	0.0	2	7.7
JS90I	2	87.5	3	100	3	100
JW1YD	2	12.5	2	86.7	2	30.8

Note: % Heterozygous = proportion of individuals heterozygous at each locus for each population; *A* = number of alleles per population per locus; n = number of individuals per population.

[a]Voucher information listed in Appendix 2. Geographic coordinates: Carr Canyon (31.4394°N, 110.2861°W); Jacobson Canyon (32.6834°N, 109.7632°W); Paradise (31.9590°N, 109.2116°W).

and sequenced also following the protocol of Schuettpelz and Pryer (2007). Clean sequence fragments (assumed to represent ca. single-copy markers) were assembled in Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and examined to confirm the presence of the anticipated microsatellite repeat. For regions with the repeat, new forward primers were designed with a CAG nucleotide tag (5′-CAGTCGGGCGTCATCA-3′) incorporated at the 5′ end of the primer sequence—to be used in combination with a complementary, fluorescently labeled nucleotide tag in subsequent genotyping reactions (Schuelke, 2000).

Genotyping reactions used 10× PCR buffer IV containing $MgCl_2$ (ABgene, Epsom, United Kingdom) combined with 2.4 mM dNTPs, 100 µg/mL bovine serum albumin (BSA), 5 U/µL *Taq* polymerase, 2 µM reverse primer, 10 µM CAG-tagged forward primer, 10 µM fluorescently labeled CAG complementary primer, plus 1 µL of DNA template for a 12-µL reaction. Each reaction entailed an initial denaturation step (94°C for 7 min), followed by 10 denaturation, annealing, and elongation cycles (94°C for 30 s, 62°C [−1°C per cycle] for 30 s, 72°C for 30 s, respectively) and 27 additional denaturation, annealing, and elongation cycles (94°C for 30 s, 51°C for 30 s, 72°C for 30 s, respectively) with a final elongation step (72°C for 12 min). Fragment analyses were run using a GeneScan 500 LIZ Size Standard on a 3730xl DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). The resulting data were visualized using GeneMarker 2.2.0 (SoftGenetics, State College, Pennsylvania, USA). Of the 159 primer pairs tested, 138 failed to amplify, amplified multiple bands, or produced poor fragment peaks (due to stutter, multiple peaks, or inconsistent amplification) and were discarded, leaving 21 markers that amplified well in *M. lindheimeri*.

To determine the utility of these 21 markers, we surveyed each new locus across multiple individuals spanning the northern range of *M. lindheimeri* (Table 1; Appendix 1). Fragment analysis revealed alleles ranging from 175–453 bp in length; of the 21 markers assessed, 14 were heterozygous within or polymorphic across individuals of *M. lindheimeri* (number of alleles ranging from two to five; Table 1), and eight amplified in one or more closely related taxa (Table 2). Population-level diversity measures were assessed for three populations of *M. lindheimeri* in Arizona, USA: Carr Canyon (n = 8 individuals; 31.4394°N, 110.2861°W), Jacobson Canyon (n = 16 individuals; 32.6834°N, 109.7632°W), and Paradise (n = 12 individuals; 31.9590°N, 109.2116°W) (Table 3). Individuals from each population were genotyped for a subset of our newly developed, polymorphic microsatellite loci (nine loci total) using a multiplex approach: for each individual, all nine loci were amplified individually and the resulting fluorescently labeled amplicons were pooled in two separate multiplex reactions (Table 1). The resulting fragment data were used to calculate percentage of polymorphic loci (P), heterozygote frequency over all loci (Het), and genotypic diversity ($G = 1 - \Sigma g_i^2$, where g_i is the frequency of the i^{th} genotype; Table 4); all measures were calculated manually using Microsoft Excel version 14.4.8 (Microsoft, Redmond, Washington, USA). Samples from Carr Canyon and Jacobson Canyon were polymorphic at 55.6% of loci surveyed and each had relatively low genotypic diversity (0.25); however, Jacobson Canyon had a higher heterozygote frequency compared to Carr Canyon (0.43 vs. 0.24, respectively). Samples surveyed from Paradise were polymorphic at 77.8% of loci; and, while heterozygote frequency over all loci was relatively low (0.28), genotypic diversity was high (0.52), indicating a relative abundance of unique genotypes compared to other populations sampled.

TABLE 4. Population-level genetic diversity statistics for nine highly variable microsatellite loci for three populations of *Myriopteris lindheimeri*.

Genetic diversity statistic	Carr Canyon (n = 8)	Jacobson Canyon (n = 15)	Paradise (n = 13)
% P	55.6	55.6	77.8
Het	0.24	0.43	0.28
G	0.25	0.25	0.52

Note: % P = % polymorphic loci; G = genotypic diversity; Het = heterozygote frequency over all loci; n = number of individuals sampled per population.

CONCLUSIONS

In this study, we developed 14 polymorphic microsatellite loci for *M. lindheimeri*, eight of which amplify well in one or more congeneric species. *Myriopteris* is notorious for its high incidence of polyploidy, hybridization, and apomixis (Windham et al., 2009; Grusz et al., 2014), yet little is known about the influence of these processes on population dynamics in the genus (or in ferns in general). Given that polyploidy, hybridization, and apomixis are intimately linked to the evolution of many fern lineages, it is our hope that by refining our understanding of these processes in *Myriopteris*, we will improve our understanding of these phenomena in ferns as a whole.

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Appendix 1. *Myriopteris lindheimeri* vouchers used to survey allelic variation for 21 newly designed microsatellite loci (Table 1). COUNTRY: State—Fern DNA Database no. (https://fernlab.biology.duke.edu); *Collector no.*; specific locality; voucher herbarium; SSR loci (0 = not amplified, 1 = amplified). For each individual, SSR loci are listed in the following order: F28JE, F9ROB, FJIEP, FYM3K, GCG5L, HAWY1, HDLR7, HGGWA, HL9PJ, HO454, HY3SM, IAO3P, IMEWX, IQLI0, IVYHJ, J0629, JDYSK, JGM27, JJUWV, JS90I, JW1YD. For example, "11111111111111111111" indicates data from a given individual contributed to the allele summary for all loci.

MEXICO: Baja California Sur—DB7262; *Moran 18821*; Mpio. Ensenada, Valle de Guadelupe; ASU; 00110000000000010000. DB7260; *Rebman 2334*; Mpio. Mulege, NW of Sierra de San Francisco; ASU; 00110000000000000000. Chihuahua—DB7264; *Reeves 5707*; Mpio. Camargo, Sierra de la Ranchera; ASU; 00110000000000010000. DB9127; *Atwood 21622*; Mpio. Chihuahua, Ojito Pass S of Ciudad Chihuahua; BYU; 00001100100000000000. DB7261; *Baker 14364*; Mpio. Temosachic, Yepachic; ASU; 00110000000000010000. DB7259; *Reeves 4851*; Mpio. Nuevo Casas Grandes, NW of Galeana; ASU; 00110000000000010000. No DB; *Atwood 1176*; Mpio. Nuevo Casas Grandes, E slope of Sierra Madre Occidental; UT; 00001000000000000000. DB8594; *Correll 21581*; not available; NY; 01110011000111001101. DB8592; *Johnston 11334*; not available; NY; 10110000000010010000. Durango—DB8593; *Díaz 189*; not available; NY; 00110100100000010010. Guanajuato—DB6522; *Rothfels 3084*; Mpio. Allende, NE of San Miguel de Allende; DUKE; 00110000000000010000. DB8584; *Rzedowski 52144*; not available; NY; 01010000001001000001. Jalisco—DB8585; *McVaugh 16758*; not available; NY; 00110000000000010010. Querétaro—DB6804; *Rothfels 3476*; Mpio. Colon; DUKE; 00100000000000000000. San Luis Potosí—DB6509; *Rothfels 3066*; Mpio. Santa Maria del Rio, S of Santo Domingo; DUKE; 11111101111101111111. Sonora—DB7263; *McGill 6441*; Mpio. Fronteras, N of Esqueda; ASU; 00010000000000010000. DB7266; *Doan 1218*; Mpio. Imuris, NE Imuris; ASU; 00110000000000010000. DB8586; *Doan 1218*; Mpio. Imuris, NE Imuris; NY; 01110000001001001101. DB8595; *van Devender s.n.*; not available; NY; 11010000001001001101.

USA: Arizona—DB3165; *Schuettpelz 458*; Cochise Co., Coronado National Forest; DUKE; 10111000000001101101. DB5749; *Grusz 30*; Cochise Co., Cochise Stronghold; DUKE; 00110000000000010000. DB8588; *Perry s.n.*; Cochise Co., Texas Canyon; NY; 00110000000010011000. DB8590; *Pray 3224*; Cochise Co., Chiricahua National Monument; NY; 11110000000010011001. DB5759; *Grusz 40*; Coronado National Forest, Arizona Trail Post; DUKE; 00110000000000010000. DB5760; *Grusz 41*; Coronado National Forest, Arizona Trail Post; DUKE; 00110000000000010000. DB5761; *Grusz 42*; Coronado National Forest, Arizona Trail Post; DUKE; 00110000000000010000. DB5762; *Grusz 43*; Coronado National Forest, Arizona Trail Post; DUKE; 00110000000000010000. DB5764; *Grusz 45*; Coronado National Forest, Arizona Trail Post; DUKE; 00110100100000010010. DB5766; *Grusz 47*; Coronado National Forest, Arizona Trail Post; DUKE; 00011000000000000000. DB5767; *Grusz 48*; Coronado National Forest, Arizona Trail Post; DUKE; 00110000000000010000. DB3809; *Metzgar 156*; Gila Co., Tonto National Forest; DUKE; 10011000000000010000. DB3812; *Metzgar 159*; Gila Co., Tonto National Forest; DUKE; 10011000000000010000. DB6316; *Grusz 163*; Gila Co., Parker Creek Canyon; DUKE; 00110000000000010000. DB6317; *Grusz 164*; Gila Co., Parker Creek Canyon; DUKE; 00110000000000010000. DB5737; *Grusz 18*; Graham Co., Jacobson Canyon; DUKE; 00110000000000010000. DB5738; *Grusz 19*; Graham Co., Jacobson Canyon; DUKE; 00110100100000010010. No DB; *Grusz 19A*; Graham Co., Jacobson Canyon; DUKE; 00001000000000000000. No DB; *Grusz 19B*; Graham Co., Jacobson Canyon; DUKE; 00001000000000000000. DB5739; *Grusz 20*; Graham Co., Jacobson Canyon; DUKE; 00110000000000010000. DB5740; *Grusz 21*; Graham Co., Jacobson Canyon; DUKE; 00110000000000010000. DB5741; *Grusz 22*; Graham Co., Jacobson Canyon; DUKE; 00110000000000010000. DB5744; *Grusz 25*; Graham Co., Wett Canyon; DUKE; 00110000000000000000. DB8601; *Keil 10251*; Graham Co., Swift Trail junction 666; NY; 01110000001001001101. DB5735; *Grusz 16*; Greenlee Co., AZ Hwy. 78; DUKE; 00110000000000000000. DB3205; *Schuettpelz 498*; Pima Co., Coronado National Forest; DUKE; 11111011111001110011011. DB3790; *Metzgar 137*; Pima Co., Coronado National Forest; DUKE; 10011000000000000010000. DB3796; *Metzgar 143*; Pima Co., Coronado National Forest; DUKE; 10011000000000000010000. DB8587; *Hitchcock 25751*; Pima Co., Baboquivari Mts.; NY; 00110000000000010000. DB8589; *Pray 3275*; Pima Co., Santa Catalina Mts.; NY; 01110011000101001101. DB8591; *Higgins 11728*; Pima Co., Santa Catalina Mts.; NY; 11110011000111001101. DB8602; *Holmgren 6756*; Pima Co., Baboquivari Mts.; SRSC; 11110000001001100111001. No DB; *Cottam 12886*; Pima Co., Alamo Canyon; UT; 10000100000000000000000. No DB; *Higgins 11766*; Pima Co., Santa Catalina Mts.; NY; 00001000000000000000. DB3157; *Schuettpelz 450*; Pinal Co., Tonto National Forest; DUKE; 10111001100101100111001. DB3490; *Windham 97-015*; Pinal Co., SE of Oracle; DUKE; 01110011001111001101. DB3798; *Metzgar 145*; Pinal Co., SSE of Oracle; DUKE; 00110000000000010000. DB3799; *Metzgar 146*; Pinal Co., SSE of Oracle; DUKE; 00110000000000010000. DB3800; *Metzgar 147*; Pinal Co., SSE of Oracle; DUKE; 00110000000000010000. DB5704; *Beck 1082*; Pinal Co., Tonto National Forest; DUKE; 10011000000000000010000. DB5756; *Grusz 37*; Santa Cruz Co., Madera Canyon; DUKE; 00110000000000010000. DB8600; *Franklin 5376*; Santa Cruz Co., Sycamore Canyon; NY; 01110110011010011001. DB8596; *Franklin 4532*; Santa Cruz Co.; NY; 01110000010000011001. DB3147; *Schuettpelz 440*; Yavapai Co., Tributary of Black Canyon; DUKE; 11110110111111111111. New Mexico—DB5380; *Rothfels 2507*; Dona Ana Co., Organ Mts.; DUKE; 00110000000000010000. DB8598; *Rusby s.n.*; Grant Co., Burro Mts.; NY; 11110000000001001100. DB3782; *Metzgar 128*; Hidalgo Co., Peloncillo Mts.; DUKE; 11110011001111001001. DB7257; *Worthington 23487*; Hidalgo Co., Little Hatchet Mts.; ASU; 00010000000000010000. DB8599; *Spellenberg s.n.*; Hidalgo Co., Peloncillo Mts.; NY; 00110000000010011000. DB8580; *Worthington 12682*; Hidalgo Co., Little Hatchet Mts.; NY; 01010000001001000101. DB9131; *Worthington 20617*; Hidalgo Co., Little Hatchet Mts.; UTEP; 00001100100001000010. DB8581; *Worthington 16522*; Luna Co., Windmill Canyon; NY; 01110000001000001101. DB8582; *Worthington 19958*; Luna Co.; NY; 11110110111011101111. DB4577; *Worthington s.n.*; Otero Co., Jarilla Mts.; DUKE; 00110000000000010000. DB3785; *Metzgar 131*; Hidalgo Co., Peloncillo Mts., Granite Gap; DUKE; 00110000000000010000. Texas—DB5632; *Rothfels 2488*; Brewster Co., Big Bend National Park; DUKE; 00110000000000010000. DB8603; *Warnock 19065*; Brewster Co., Glass Mts.; SRSC;

00110000000000010000. DB8604; *Warnock 20502*; Brewster Co., SE of Marathon; SRSC; 00110000000000010000. DB8605; *Johnston 3601*; Brewster Co., S of Marathon; SRSC; 00010000000000010000. DB8583; *Worthington 9233*; El Paso Co., Franklin Mts.; NY; 00010000000110011000. DB8615; *Warnock 16183*; El Paso Co., Hueco Tanks; SRSC; 00110000000000010000. DB8616; *Warnock 9992*; El Paso Co., McKelligen Canyon; SRSC; 00010000000000000000. DB5364; *Rothfels 2490*; Jeff Davis Co., Fort Davis State Park; DUKE; 11110110011011111101. DB8609; *Keough 235*; Jeff Davis Co., Fern Canyon; SRSC; 00110000000000000000. DB8610; *Sperry s.n.*; Jeff Davis Co., Fern Canyon; SRSC; 11110000000110011000. DB8611; *Whitehouse 11199*; Jeff Davis Co., W of Ft. Davis; SRSC; 11000110000000000000. DB8612; *Warnock 22632*; Jeff Davis Co., NW boundary of Davis Mts. State Park; SRSC; 00110000000010011000. DB8613; *Manning 969*; Jeff Davis Co., SE of Ft. Davis on Hwy. 118; SRSC; 11110110111011111111. DB8614; *Bridges s.n.*; Jeff Davis Co., S of arboretum; SRSC; 11111110011011111101. DB7258; *Reeves 6344*; Llano Co., Enchanted Rock; ASU; 00110000000000010000. DB8606; *Warnock 176*; Presidio Co., Tinaja Prieta Canyon; SRSC; 00110000000000010000. DB8607; *Warnock 543*; Presidio Co., Pelillos Arroyo; SRSC; 00110000000110011000. DB8608; *Warnock 511*; Presidio Co., San Antonio Canyon; SRSC; 00110000000110011000.

Appendix 2. Vouchers used to examine genetic diversity across populations of *Myriopteris lindheimeri* and to test amplification success in taxa closely related to *M. lindheimeri*. *Taxon—Collector no.*; locality; sample size (*n*). All vouchers deposited at DUKE.

Myriopteris aurea—Beck 1192; Mexico: Guerrero, Mpio. de Leonardo Bravo, approx. 1.4 km S of Corral de Bravo on road to Cruz de Cote and Hierba Santa; *n* = 1. *M. covillei—Schuettpelz 442*; USA: Arizona, Maricopa Co., Tonto National Forest, Sycamore Creek near Sugarloaf Mountain; *n* = 1. *M. fendleri—Windham 3408*; USA: Arizona, Bernalillo Co., Embudito Canyon; *n* = 1. *M. lindheimeri—Grusz 171*; USA: Arizona, Graham Co., Jacobson Canyon; *n* = 15. *Grusz 173*; USA: Arizona, Cochise Co., Paradise; *n* = 13. *Grusz 180*; USA: Arizona, Cochise Co., Carr Canyon; *n* = 8. *M. rufa—Metzgar 161*; USA: Arizona, Gila Co., Sierra Ancha, Parker Creek Canyon; *n* = 1.