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POLYMORPHIC CHLOROPLAST MICROSATELLITE MARKERS IN THE OCTOPOLOID *LEPIDIUM MEYENII* (BRASSICACEAE) AND CROSS-SPECIES AMPLIFICATION IN *LEPIDIUM*

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Abstract

Premise of the study—As a crop and medicinal plant, the octoploid Andean endemic *Lepidium meyenii* suffers from taxonomic uncertainty. Few molecular markers are available to genotype individuals or track gene flow in wild and cultivated material.

Methods and Results—Using available sequence data, eight cpSSR primer pairs were developed for *L. meyenii*. Levels of polymorphism checked in 56 individual *L. meyenii*, including cultivated and wild material, revealed that the number of alleles per locus ranged from three to five, and intrapopulation allele frequencies ranged from 0.071 to 1.0. Polymerase-chain-reaction screens using our cpSSR primers in 27 other *Lepidium* species and three *Coronopus* species suggested a high degree of interspecific amplification.

Conclusions—These polymorphic cpSSR markers should prove useful in characterizing genetic variation among cultivated and wild *L. meyenii*. Additionally, interspecific amplifications suggest that these markers will be useful for the study of related taxa.

Keywords

cpSSR; *Lepidium meyenii*; maca; microsatellite

The Andean domesticate *Lepidium meyenii* Walp. (“maca” syn. *L. peruvianum* Chacón) (Brassicaceae) is one of the highest-elevation crops on earth (National Research Council, 1989). Adapted to cool and arid conditions, it is typically grown in the Junin and Cerro de Pasco Departments of Peru (Bonnier, 1986). *Lepidium meyenii* is the only member of the large, cosmopolitan genus *Lepidium* that forms a fleshy hypocotyl and root, which resemble those of two other cultivated but divergent members of the Brassicaceae, the radish and turnip (Li et al., 2001). Root extracts from *L. meyenii* have high nutrient concentrations and unique bioactive compounds (i.e., glucosinolates, isothiocyanates, macamines, and

macamides; Gonzales et al., 2008) that are noted for their potential antioxidant and hormonal regulatory activities. With increasing interest in the use and preservation of lesser-known crops and the heritability of biomedical compounds comes a need for genetic markers that can be used to investigate the origins, taxonomy, population structure, and links between genotype and phenotype within *L. meyenii*. As a step toward developing a comprehensive set of markers for *L. meyenii*, we used an available chloroplast genome (cpDNA) sequence and a bioinformatics approach to develop eight cpDNA-specific microsatellite markers for the species that also broadly amplify across *Lepidium*.

As a result of their uniparental inheritance and the relatively slowly evolving nature of individual cpDNA microsatellites, cpDNA markers are sometimes considered of limited utility to population genetic studies in plants. However, the application of numerous linked maternally inherited markers (cpDNA microsatellites) has considerable potential for investigating an octoploid taxon of potential hybrid origin (Ebert and Peakall, 2009). In particular, we plan to use these markers in future studies of *L. meyenii* to identify broad population substructures and to better understand the origin and maternal ancestry of this polyploid species in relation to biparentally inherited nuclear markers.

METHODS AND RESULTS

Using the *L. virginicum* complete cpDNA genome sequence (T. Hosouchi et al., unpublished data; available online at <http://ncbi.nlm.nih.gov/nuc-core/139388889>) and the software package CID (Freitas et al., 2008), we proceeded with high-throughput microsatellite (SSR) marker identification and primer design. Applying the default SSR detection parameters (mononucleotide repeats 10; di- repeats 6; tri- repeats 5; tetra-, penta-, hexa-repeats 5; maximum number of nucleotides separating two or more SSRs to establish compound loci = 100), CID identified 64 SSRs, including 20 (A)_x, 39 (T)_x, 4 (AT)_x, and 1 (TA)_x repeat motifs. To focus on the markers most likely to be polymorphic among closely related individuals, the set was reduced to 19 SSRs that included the longest mononucleotide (13) and dinucleotide (6) repeats. Each of these SSRs and ±200 bp of 5' and 3' sequence were then compared to available sequences in NCBI through BLAST searches. BLAST results were used to identify potential variability at each locus and conservation in the 5' and 3' flanking regions. One marker was monomorphic when aligned with other Brassicaceae or *Carica* (Caricaceae), and the flanking region(s) of two others were difficult to align with available *Lepidium* and more divergent Brassicaceae. Primer design for the remaining 16 SSRs prioritized flanking-sequence conservation across *Lepidium* to facilitate interspecific amplifications.

To assess the utility of each marker across the genus and within *L. meyenii*, we tested amplification of the 16 primer pairs in 28 North and South American *Lepidium* species, including *L. meyenii* accessions (Appendix 1). DNA samples were isolated from fresh greenhouse-grown material using the protocol of Alexander et al. (2007). For difficult-to-extract herbarium samples, this protocol was modified to include 30 µL of Qiagen proteinase K (>600 mAU/mL stock solution) in the grind buffer, followed by an overnight room-temperature incubation on a horizontal shaker. Polymerase chain reaction (PCR) amplifications were performed using 10 µL total reaction volumes prepared using 1 µL of genomic DNA with a 9-µL mix containing 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂), 100 µM of each dNTP, 0.5 µM of each forward and reverse primer, and 1.5 units of *Taq*. Samples were amplified with a denaturing step of 94°C for 3 min, followed by 15–35 cycles of 94°C for 30 s, annealing temperature and time (Table 1), and 72°C for 30 s, followed by one final extension cycle of 72°C for 7 min. Success of amplifications was judged using UV transillumination after electrophoresis in 1% agarose gels stained with

EtBr. Eight of the primer pairs (see Table 1) consistently amplified single bands in *L. meyenii* (Table 2) and individuals from other *Lepidium* species (see Appendix).

The forward primer for each of the eight cpSSR markers was 5' labeled with HEX or 6-FAM to enable the use of Applied Biosystems dye set D and the Genescan 500 ROX standard (Applied Biosystems, Foster City, California, USA) on the Laboratory for Evolutionary and Ecological Genetics (NMSU) ABI 3100 DNA sequencer. Unlabeled LeviCp138773 primers consistently amplified in many samples; however, this marker became difficult to amplify after the addition of a 6-FAM. Allele sizes and frequencies for all other microsatellites applied to 56 samples of *L. meyenii* (see Appendix) are given in Table 2. The number of alleles per locus across all samples ranged from three to five, and intrapopulation allele frequencies per cpSSR ranged from 0.077 to 1.0. The range of allele sizes and frequencies recovered from the "Red" hypocotyl and "Yellow" hypocotyl cultivated "populations" from Horizon Herbs & Seeds, LLC, in Oregon suggest that these markers may indeed contain sufficient variation for characterization of larger samples of cultivated material.

CONCLUSIONS

These cp microsatellites detect greater levels of polymorphism within *L. meyenii* than previously reported for population-level markers (RFLPs and RAPDs; Toledo et al., 1998) and show considerable promise for studying many other *Lepidium* species (see Appendix). These markers should be useful for population genetic applications and should inform future investigations of population structure, maternal inheritance, gene flow, and the identification of *Lepidium* plant material. In combination with existing marker systems, the cpSSRs identified here should shed new light on population-level processes in *L. meyenii* and in the genus overall.

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Appendix. Taxa used in this study and voucher information. Greenhouse-grown specimens cultivated at the NMC greenhouse are noted after the voucher information. Interspecific amplification of cpDNA microsatellite markers is shown in the table at the end of the appendix

Taxon; Voucher specimen, Collection locale; Herbarium.

Coronopus didymus (L.) Sm.; 175005, Peru, Lima; USM. *C. rhytidocarpus* MacL.; 220, Argentina, Buenos Aires; BA. *C. serratus* Desv.; Peterson, Argentina, Corrientes; LP.

Lepidium aletes Macbr.; Zardini et al., Argentina, Coreintes; LP. *Lepidium bipinnatifidum* Desv.; Nunez & Vargas 13757, Peru, Apurimac; MO. *L. bonariense* (L.); Beck, Bolivia, Oropesa; MO. *L. chichicara* Desv.; 04594519, Bolivia, La Paz; MO; 193700, Bolivia, La Paz; USM.

L. costaricense Thell.; 9502; OSBU. *L. cyclocarpum* Thell.; 161158; Peru, Chancay; USM. *L. densiflorum* Schrad.; Doan 465, USA, Arizona; NMC. *L. depressum* Thell.; Solomon & Moraes 13409, Bolivia, La Paz; MO. *L. dictyotum* Gray; Rose 40192, USA, California; NMC. *L. filisegmentum* Hitchc.; Julien, Argentina; LP. *L. fremontii* Watson; Strother 894; NMC. *L. gelidum* Wedd.; s.n. UPS. *L. gracile* Boelcke; ex Museum Botanicum Hauniense (C) Plantae Argentinae No. 1821; UPS. *L. johnstonii* Hitchc.; Taylor et al. 04571853, Chile, Antofagasta; MO. *L. kalenbornii* Hitchc.; Leon et al. 162976, Peru, Ancash; USM. *L. lasiocarpum* Nutt.; Alexander 532, USA, New Mexico; NMC. *L. latifolium* (L.); McIntosh 3038, USA, New Mexico; NMC. *L. meyenii* Walp.; Garcia et al. 973; Bolivia, La Paz; MO; Solomon 13184; Bolivia, La Paz; MO; Quiros 92B498-8, Peru, Carahuamayo; NA; Quiros 92B374-18L, Peru, Cerro de Pasco; NA; Quiros 92B495-8, Peru, Huancayo; NA; Quiros 92B497-11, Peru, Huayre; NA; 'Red' cultivated NMC; 'Yellow' cultivated; NMC. *L. montanum* Nutt.; Alexander 116, USA, New Mexico; Welsh 27081, USA, Utah; NMC. *L. nitidum* Nutt.; Spellenberg 13503, USA, California; NMC. *L. oblongum* Small; Peterson 97-10, USA, New Mexico; NMC. *L. parodii* Thell.; Cabrera, Argentina, Buenos Aires; LP. *L. quitense* Turcz.; 3792042, Ecuador, Tungurahua; MO. *L. racemosum* Giseb.; Moore 2581, Argentina, Feuerland; UPS. *L. solomonii* Al-Shehbaz; 3780661, Bolivia, La Paz; MO. *L. spicatum* Desv.; Dawson, Argentina, Buenos Aires; LP. *L. tayloriae* Al-Shehbaz; Taylor 4355023, Chile, Atacama; MO. *L. virginicum* var. *medium* (Greene) Hitchc.; Alexander 563, USA, New Mexico; NMC.

Greenhouse-grown specimens

L. meyenii Walp.; Garcia et al. 973; Bolivia, La Paz; MO; Solomon 13184; Bolivia, La Paz; UPS; Quiros 92B498-8, Peru, Carahuamayo; NA; Quiros 92B374-18L, Peru, Cerro de Pasco; NA; Quiros 92B495-8, Peru, Huancayo; NA; Quiros 92B497-11, Peru, Huayre; NA; 'Red' cultivated NMC; 'Yellow' cultivated; NMC.

Table 1

Characterization of eight microsatellite loci identified from the *Lepidium virginicum* chloroplast genome sequence.

Locus	Repeat motif	Primer sequence (5'-3')	Size (bp)	T _a (°C) Time
LeviCp46399-46582	(A) ₁₃	F: 6-FAM -CCATTGCACATTGTAGAACTARGTTTC R:CATCATYTTTGTATTTGCAATTCAATATGG	167-182	57 1'15"
LeviCp51882-52091	(T) ₁₀ ...(T) ₁₂	F: 6-FAM -CGGCGYGGAGTCATTGGTTCAAATCC R:CRACACGAGTSGAGGCTMTCAATACG	205-208	60 30"
LeviCp99902-100077	(T) ₁₃	F: HEX -GCAAGTTACAAATTCTGTTTCGG R:GTTTCCACGCCCTTTAAGACCCCG	172-178	60 30"
LeviCp108436-108713	(AT) ₇	F: HEX -CCCTCCTCGCCCAACCGGCC R:CTGCCCAAGCCCGGCTGAGGAAG	273-278	60 1'15"
LeviCp114238-114554	(A) ₁₄	F: 6-FAM -GAGCATTTAGGGTTTGTCTTTGGG R:CGTGTCTACCAATTTCAACCATAGCGG	309-315	60 30"
LeviCp127143-127397	(T) ₁₆ ...(T) ₁₁	F: 6-FAM -GAAGCTATCCCAAGTTTCTGCAATTTTC R:GGATCCATGCGTTCCCAAGGCGTAAAC	235-252	60 30"
LeviCp130060-130273	(AT) ₇	F: HEX -GCTATTCTGCCCAAGCCCGGCTGAGGAAG R:GATAGCGGACTCAAAGCTATGGAAC	204-211	60 1'15"
*LeviCp138682-138882	(A) ₁₃	F: 6-FAM -CCATTCAAGAGTTCTTATGTGTTTCCACGCCCC R:GGATAGCAAGTTACAAATTCTGTTTCGGTAGG	205, 207	60 30"

Notes: Primers are given in 5' to 3' orientation, including each 5' dye label 6-FAM or HEX, as they would be ordered for oligonucleotide synthesis. "F" and "R" designate forward and reverse primers, respectively. "Size" refers to the nucleotide length range of the amplified microsatellite fragment in *L. meyenii*, "T_a" refers to annealing temperature for PCR reactions, and

* designates LeviCp138682-138882 as an optimized primer pair prior to 6-FAM dye labeling; however, this marker was difficult to consistently amplify after adding 6-FAM to the forward primer.

Microsatellite allele frequencies sampled within six populations of *L. meyenii* (n = number of individuals). Alleles are designated by an allele fragment size followed by the proportion in which the allele occurred within samples from the designated population.

Table 2

	Carahuamayo, Peru ($n = 6$)	Cerro de Pasco, Peru ($n = 9$)	Huancayo, Peru ($n = 7$)	Huayre, Peru ($n = 6$)	'Red'* ($n = 14$)	'Yellow'* ($n = 14$)
LeviCp46399-46582	-	-	-	-	-	a ₁₆₇ :0.286
	-	-	-	-	a ₁₇₇ :0.143	a ₁₇₇ :0.286
	a ₁₇₈ :1.0	a ₁₇₈ :1.0	a ₁₇₈ :1.0	a ₁₇₈ :1.0	a ₁₇₈ :0.714	a ₁₇₈ :0.286
	-	-	-	-	-	a ₁₈₀ :0.143
	-	-	-	-	a ₁₈₂ :0.143	-
LeviCp51882-52091	-	a ₂₀₅ :0.111	a ₂₀₅ :0.429	-	a ₂₀₅ :0.071	-
	a ₂₀₆ :0.6	-	a ₂₀₆ :0.143	a ₂₀₆ :0.5	a ₂₀₆ :0.143	a ₂₀₆ :0.214
	a ₂₀₇ :0.2	a ₂₀₇ :0.667	a ₂₀₇ :0.143	-	a ₂₀₇ :0.571	a ₂₀₇ :0.5
	a ₂₀₈ :0.2	a ₂₀₈ :0.222	a ₂₀₈ :0.286	a ₂₀₈ :0.5	a ₂₀₈ :0.214	a ₂₀₈ :0.286
LeviCp99902-100077	a ₁₇₂ :0.333	-	a ₁₇₂ :0.714	-	a ₁₇₂ :1.0	a ₁₇₂ :0.857
	a ₁₇₄ :0.167	a ₁₇₄ :1.0	a ₁₇₄	a ₁₇₄ :1.0	-	-
	a ₁₇₈ :0.5	-	a ₁₇₈ :0.286	-	-	a ₁₇₈ :0.143
LeviCp108436-108713	-	-	a ₂₇₃ :0.143	-	a ₂₇₃ :0.214	a ₂₇₃ :0.143
	-	a ₂₇₄ :0.111	-	-	a ₂₇₄ :0.786	a ₂₇₄ :0.714
	-	a ₂₇₅ :0.222	-	-	-	a ₂₇₅ :0.143
	a ₂₇₆ :0.167	-	a ₂₇₆ :0.143	-	-	-
	a ₂₇₈ :0.833	a ₂₇₈ :0.667	a ₂₇₈ :0.714	a ₂₇₈ :1.0	-	-
LeviCp114238-114554	-	a ₃₀₉ :0.556	a ₃₀₉ :0.571	-	a ₃₀₉ :0.429	a ₃₀₉ :0.143
	a ₃₁₀ :0.167	a ₃₁₀ :0.222	a ₃₁₀ :0.286	a ₃₁₀ :0.167	a ₃₁₀ :0.5	a ₃₁₀ :0.571
	a ₃₁₁ :0.167	a ₃₁₁ :0.222	a ₃₁₁ :0.143	-	a ₃₁₁ :0.071	a ₃₁₁ :0.286
	a ₃₁₂ :0.667	-	-	a ₃₁₂ :0.667	-	-
	-	-	-	a ₃₁₄ :0.167	-	-
LeviCp127143-127397	-	-	-	-	-	a ₂₄₈ :0.143
	-	-	a ₂₅₁ :0.714	-	-	a ₂₅₁ :0.143
	a ₂₅₂ :1.0	a ₂₅₂ :1.0	a ₂₅₂ :0.286	a ₂₅₂ :1.0	a ₂₅₂ :1.0	a ₂₅₂ :0.714

	Carahuamayo, Peru (n = 6)	Cerro de Pasco, Peru (n = 9)	Huancayo, Peru (n = 7)	Huayre, Peru (n = 6)	'Red',* (n = 14)	'Yellow',* (n = 14)
LeviCp130060–130273	–	–	–	–	a ₂₀₅ :0.571	a ₂₀₅ :0.429
	a ₂₀₆ :0.333	–	–	–	a ₂₀₆ :0.429	a ₂₀₆ :0.571
	a ₂₁₀ :0.667	–	a ₂₁₀ :0.857	a ₂₁₀ :1.0	–	–
	–	–	a ₂₁₁ :0.143	–	–	–

Asterisks denote cultivated populations.

Species	LvCp46399	LvCp51882	LvCp99902	LvCp108436	LvCp114238	LvCp127143	LvCp130060	LvCp138682
<i>Coronopus didymus</i>	+		+	+	+	+	+	+
<i>C. rhytidocarpus</i>				+				+
<i>C. serratus</i>				+				+
<i>Lepidium aletes</i>	+		+		+		+	+
<i>L. bipinnatifidum</i>	+		+	+	+			+
<i>L. bonariense</i>	+		+				+	+
<i>L. chichicara</i>	+		+	+	+		+	+
<i>L. costaricense</i>	+	+	+		+		+	+
<i>L. cyclocarpum</i>	+	+	+	+		+		+
<i>L. densiflorum</i>	+	+	+	+	+	+		
<i>L. depressum</i>	+	+	+	+	+	+		+
<i>L. dictyotum</i>	+	+	+	+	+	+	+	+
<i>L. filisegmentum</i>		+		+	+	+		+
<i>L. fremontii</i>	+	+	+	+	+	+	+	+
<i>L. geldum</i>	+	+	+	+	+	+	+	+
<i>L. gracile</i>	+	+	+	+	+	+		+
<i>L. johnstonii</i>	+	+	+	+	+	+	+	+
<i>L. kalenbornii</i>	+	+	+	+	+	+		+
<i>L. lastocarpum</i>	+	+	+	+	+	+	+	
<i>L. latifolium</i>	+	+	+	+	+	+	+	
<i>L. meyenii</i>	+	+	+	+	+	+	+	+
<i>L. montanum</i>	+	+	+	+	+	+	+	+
<i>L. nitidum</i>	+	+	+	+	+	+	+	+
<i>L. oblongum</i>	+	+	+	+	+	+	+	+
<i>L. parodii</i>		+					+	+
<i>L. quitense</i>	+	+	+	+	+	+	+	+
<i>L. racemosum</i>	+	+		+	+	+	+	+
<i>L. solomonii</i>	+			+			+	
<i>L. spicatum</i>	+			+		+		
<i>L. tayloriae</i>	+			+			+	

Species	LvCp46399	LvCp51882	LvCp99902	LvCp108436	LvCp114238	LvCp127143	LvCp130060	LvCp138682
<i>L. virginicum</i>	+	+	+	+	+	+	+	+

Note: + indicates successful PCR amplification.