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Author: Chapman, Mark A.

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Applications in Plant Sciences

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Genomic Resources Note

Transcriptome sequencing and marker development for four underutilized legumes[1]

Mark A. Chapman[2,3,4]

[2]Centre for Biological Sciences, University of Southampton, Southampton SO17 1BJ, United Kingdom; and [3]Centre for Underutilised Crops, University of Southampton, Southampton SO17 1BJ, United Kingdom

- *Premise of the study:* Combating threats to food and nutrition security in the context of climate change and global population increase is one of the highest priorities of major international organizations. Hundreds of species are grown on a small scale in some of the most drought/flood-prone regions of the world and as such may harbor some of the most environmentally tolerant crops (and alleles).
- *Methods and Results:* In this study, transcriptomes were sequenced, assembled, and annotated for four underutilized legume crops. Microsatellite markers were identified in each species, as well as a conserved orthologous set of markers for cross-family phylogenetics and comparative mapping, which were ground-truthed on a panel of diverse legume germplasm.
- *Conclusions:* An understanding of these underutilized legumes will inform crop selection and breeding by allowing the investigation of genetic variation and the genetic basis of adaptive traits to be established.

Key words: beans; drought; legumes; microsatellites; simple sequence repeats (SSRs); transcriptome.

Threats to food security, resulting from climate change and a growing population, are the focus of major international organizations such as the Food and Agriculture Organization of the United Nations (von Braun et al., 2003; FAO et al., 2012). It is clear that major changes will be needed to the way we produce, process, and transport food. Only a handful of crops provide the basic nutritional intake for the majority of the human population; however, dozens of species are cultivated at a smaller scale, and several thousand more have a variety of food and nonfood uses (Mangelsdorf, 1966). Many of the less-studied crops have advantages in terms of environmental tolerances and unique nutritional profiles when compared to the widely cultivated crops. As such, investigations into less-studied crops (hereafter referred to as "underutilized crops") have the potential to reveal crops, crop varieties, and unique alleles that could be applied in future agriculture and crop breeding (Varshney et al., 2010).

Legumes are a vitally important source of protein in developing countries, where meat protein is consumed infrequently. They are also often grown in poor soils, because of their associated atmospheric N_2 fixation, and where water availability is low or unpredictable. With this motivation, this study reports transcriptome sequences for four underutilized legumes: hyacinth bean (*Lablab purpureus* (L.) Sweet), grasspea (*Lathyrus sativus* L.), winged bean (*Psophocarpus tetragonolobus* (L.) DC.), and Bambara groundnut (*Vigna subterranea* (L.) Verdc.). Hyacinth bean is a very versatile crop, likely originating in Africa, and is cultivated throughout the tropics, often as forage for livestock. The leaves and flowers can be eaten by humans raw or cooked, and the roots can be eaten cooked; notably, this species is one of the most drought-tolerant legumes (Ewansiha and Singh, 2006; Maruthi et al., 2006). Grasspea is widely grown in East Africa and Asia and is often considered an "insurance crop" because of its ability to grow under drought conditions even when other crops fail (Polignano et al., 2005). The seed, while being a good source of protein (Abd El-Moneim et al., 2000), contains the neurotoxin ODAP (β-*N*-Oxalyl-L-α,β-diaminopropionic acid), which can cause health problems if it is the sole or major protein source in the diet. Winged bean (*P. tetragonolobus*) is very versatile, with all parts of the plant (leaves, flowers, tubers, and seeds) edible (National Academy of Sciences, 1975). Finally, Bambara groundnut (*V. subterranea*) grows similarly to the peanut in that its flowers develop into pods underground. It is thought to have originated in West Africa and is widely cultivated in sub-Saharan Africa, as well as in some regions of Asia, and can be grown on marginal soils where other legumes fail to grow (Jørgensen et al., 2010).

For future investigations, including population genetic and linkage or quantitative trait locus (QTL) mapping, transcriptome sequences can be mined for simple sequence repeats (SSRs, or microsatellites). These markers are typically highly polymorphic and are often transferable to closely related species; for example, 45–91% of 127 *Vigna radiata* (L.) R. Wilczek SSRs successfully amplified in other *Vigna* species (Tangphatsornruang et al., 2009). Furthermore, a comparative analysis of transcriptome sequences can be carried out to isolate markers with the potential to amplify in multiple species. These are typically PCR-based markers (i.e., a conserved orthologous set [COS], sensu Fulton et al., 2002) and are designed to amplify across a range of species, typically within a botanical family, for comparative

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[4]Author for correspondence: m.chapman@soton.ac.uk

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TABLE 1. Summary statistics for the four de novo assembled transcriptomes.

Descriptor	Species (accession)			
	Lablab purpureus (AusTRCF309497)	*Lathyrus sativus* (REWA2)	*Psophocarpus tetragonolobus* (Ibadan Local-1)	*Vigna subterranea* (KAHEMBA)
USDA PI no.	666239	345525	477137	241982
Country of origin	Malawi	India	Nigeria	Africa
No. of reads	16,190,774	15,779,854	16,625,155	7,887,745
No. of normalized reads	3,803,885	3,350,019	3,444,907	2,821,597
No. of genes	32,446	33,042	33,595	34,401
No. of transcripts	52,019	49,280	52,083	47,759
Total no. of assembled bases	51,997,858	41,878,949	46,788,260	37,938,088
% GC	41.79	40.42	41.52	42.82
N50 all transcripts	1570	1309	1420	1212
Median length all transcripts	682	572	584	516
Mean length all transcripts	999.59	849.82	898.34	794.37
Total no. of SSRs	2567	1139	1884	1305
No. of simple SSRs	2529	1117	1853	1292
No. of compound SSRs	38	22	31	13
No. of contigs with SSRs	2427	1106	1814	1265
No. of loci with 1 SSR	2287	1073	1744	1225
No. of loci with 2 SSRs	118	33	62	36
No. of loci with 3 SSRs	9	0	4	2
No. of loci with 4 SSRs	1	0	0	0
No. of BLAST hits to *Pvul*	42,478	29,701	37,006	38,961
% BLAST hits to *Pvul*	82	60	71	82
No. of *Pvul* hits to each	19,171	13,417	17,135	18,640
% *Pvul* hits to each	70	49	63	69

Note: PI no. = Plant Introduction number; *Pvul* = *Phaseolus vulgaris*; SSR = simple sequence repeat; USDA = U.S. Department of Agriculture.

QTL analyses and/or phylogenetics (e.g., in the Asteraceae [Chapman et al., 2007], Brassicaceae [Jeong et al., 2014], Pinaceae [Liewlaksaneeyanawin et al., 2009], Rosaceae [Cabrera et al., 2009], and Solanaceae and allies [Wu et al., 2006]). So far, however, there has been no attempt to develop COS markers for the Fabaceae, despite the large size of the family and the variety of economically important species within (Stevens, 2006; Meyer and Purugganan, 2013).

In this study, seedling transcriptomes from these four underutilized crops were sequenced using Illumina technology, assembled, annotated, and mined for SSRs and COS markers. The resultant data have been made publicly available as a valuable resource for investigations into gene expression and genetic variation in these and other leguminous crops.

METHODS AND RESULTS

Seeds were obtained from the U.S. Department of Agriculture (Ames, Iowa, USA; Table 1). The seeds were soaked overnight in 0.5 mg·mL^{-1} gibberellic acid, then rinsed and placed on damp filter paper at 22°C until germination. Germinated seeds were transferred to a 2 : 1 mixture of Levington's F2+S and vermiculite in a greenhouse with 16-h days, supplemented with artificial light. RNA extraction was carried out from true leaves of seedlings using a QIAGEN RNeasy Kit (QIAGEN, Crawley, United Kingdom) following the manufacturer's protocol, with on-column DNase digestion (RNase-free DNase, QIAGEN). Three micrograms of RNA was sent to the Wellcome Trust Centre for Human Genetics (Oxford, United Kingdom) for library preparation using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, California, USA) and sequencing on a partial lane of an Illumina HiSeq 2500 with 100-bp paired-end (PE) reads.

The following analyses were carried out for each of the four crops separately. Sequence data were trimmed of adapters and low-quality sequences ($q < 10$) using cutadapt (http://code.google.com/p/cutadapt/; Martin, 2011), and the Perl script resyncMates.pl (https://github.com/percyfal/ratatosk.ext.scilife/blob/master/scripts/resyncMates.pl) was used to maintain proper read pairing where one of a pair was removed by cutadapt. The resulting clean reads were used in transcriptome assembly with Trinity ver. r20140717 (Grabherr et al., 2011). Libraries were normalized to a kmer coverage of 30 so as to reduce computation time, and then assembled using Trinity with the settings *–min_kmer_cov 2* to increase the stringency for reads being assembled together, and *–max_diffs_same_path 4–max_internal_gap_same_path 15*, which allowed for more divergent reads (up to four nucleotide differences and up to a 15-bp gap) to

TABLE 2. Primer sequences for 12 COS markers tested in 12 members of the Fabaceae.

F primer name	F primer sequence	R primer name	R primer sequence
COS1_F245	ARCAAAAGCATGGAAGAAGTGAA	COS1_R728	CYACATCTCCATTGTTMACACT
COS2_F267	CAAGAAGARGGAGGAAGAGGC	COS2_R754	GATTTCTTCCAWAGCTTCCAWAT
COS3_F1596	GCTAGAGGAGGAGARACGCC	COS3_R2073	AGGRCATCTGAAYCTTTCG
COS4_F645	GAGAARCTTGGAGGACCWGTT	COS4_R844	AAATGTYCGCATTTCATAYTGCT
COS5_F319	TTCCTCACAACGAGTCTRTYGA	COS5_R406	AATACCATCCAACWATGATTTCYT
COS6_F104	AAGGTTYACGAGYTGAGGCA	COS6_R347	CKRCGAATAGCCCTGGTCTTCTT
COS7_F633	AAACAAACTTTTCCATTTTCAAGG	COS7_R787	CCRGCATTNGCAAACCTATTA
COS8_F291	GCCATYCGHGAAATCATGTT	COS8_R610	ATATCCTTGCAAGYCCAAAGT
COS9_F448	CGVACWAATTTCARYTCCATAA	COS9_R530	AGCAATGCTCTTCAAGCTAACTG
COS10_F295	GRGAAAGGAGTTCYGATGA	COS10_R537	TGCTARGARGASYGRGAAGA
COS11_F419	TCCTTTGTTTCTTTRGCTGG	COS11_R674	GCACAATTTACATTKACYGATTT
COS12_F446	TTKCAAGAAAGAAGATSVYMTCAT	COS12_R550	TCMAGATAWCCTCCWCCCA

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be assembled into the same contig, i.e., taking into account the potential heterozygosity of the species. Heterozygosity was assessed by mapping the reads back to the respective transcriptomes, parsing the data through SAMtools (Li et al., 2009; mpileup settings -q 3 -Q 20), vcf2fq (settings -d 3), seqtk, and outputting .fa files. Putative functions of transcripts were assigned based on comparisons to transcripts derived from the common bean (*Phaseolus vulgaris* L.) genome sequencing project (Schmutz et al., 2014; http://www.phytozome.net/commonbean.php) with BLASTn and an *E*-value cut-off of e-30. SSRs were identified in the transcriptomes by running misa.pl (http://pgrc.ipk-gatersleben.de/misa/) with the minimum number of repeats set at 8, 6, and 4 for dinucleotides, trinucleotides, and tetranucleotides, respectively.

To identify COS loci, the four transcriptomes were BLASTed against each other in a "round-robin" fashion (*Lablab* vs. *Lathyrus*, *Lathyrus* vs. *Psophocarpus*, *Psophocarpus* vs. *Vigna*, and *Vigna* vs. *Lablab*) and reciprocal best BLAST hits retained. Where all four comparisons successfully produced a reciprocal best BLAST hit this was considered a potential COS locus. For 12 such loci (picked at random, except for avoiding short loci, <300 bp), the sequences were aligned using Clustal Omega (Sievers and Higgins, 2014) and checked for presences of introns by comparison to the bean genome (Schmutz et al., 2014; http://www.phytozome.net/commonbean.php); degenerate primers were then designed to flank the introns using Primaclade (Gadberry et al., 2005; Table 2). These were then used to PCR amplify DNA extracted from the four species used in the transcriptome assembly as well as eight other legume taxa (*Crotalaria brevidens* Benth., *Lupinus mutabilis* Sweet, *Mimosa pudica* L., *Phaseolus coccineus* L. [runner bean], *Pisum sativum* L. [field pea], *Vigna aconitifolia* (Jacq.) Marechal [moth bean], *V. unguiculata* subsp. *unguiculata* (L.) Walp. [black-eyed pea], and *V. unguiculata* subsp. *sesquipedalis* (L.) Verdc. [yardlong bean]). DNA extraction was carried out with a cetyltrimethylammonium bromide (CTAB)–based method, and PCR amplification took place using a touchdown program with 55°C final annealing temperature (see Chapman and Burke, 2012 for details). PCR products were resolved on agarose gels stained with GelRed (Biotium, Hayward, California, USA).

Between 7.9 and 16.6 million paired-end 100-bp reads were generated for each of the bean samples (reads have been deposited in the National Center for Biotechnology Information [NCBI] Sequence Read Archive [http://www.ncbi.nlm.nih.gov/sra] under BioProject ID PRJNA273585). After normalization, the number of paired reads to assemble was 2.8 to 3.8 million (Table 1). Assembly of the transcriptomes results in between 32,446 and 34,401 genes, corresponding to 47,759 to 52,083 transcripts (Table 1; Fig. 1A). The number of genes and number of transcripts was similar across species. In these four species, there are therefore approximately 1.4 to 1.6 isoforms per gene, a value comparable to the proportion of *Arabidopsis* intron-containing genes that have more than one alternative transcript (ca. 42%; Filichkin et al., 2010). The FASTA-formatted transcriptome sequences are available from the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.k9h76; Chapman, 2015). N50, mean, and median transcript lengths were largely comparable across species; however, they were lowest for *V. subterranea*, for which the smallest number of reads was obtained (Fig. 1B). In line with the above statistics, the distribution of transcript lengths was qualitatively similar across species; however, more of the longer transcripts were resolved for *Lablab* and more of the shorter transcripts for *Vigna* (Fig. 1C). BLASTn searches against the common bean (*P. vulgaris*) coding DNA sequences (one sequence per common bean locus) resulted in between 71% and 92% of transcripts having a hit (Appendix S1). Heterozygosity was low, as predicted for an inbred accession from a gene bank, varying from 0.014% in *Lablab* and *Vigna* to 0.028% in *Psophocarpus* and 0.149% in *Lathyrus*.

Mining for SSRs identified between 1106 (*Lathyrus*) and 2427 (*Lablab*) SSR-containing loci (with one to four SSRs per locus) per transcriptome (Table 1; Appendix S2). This corresponds to 4.7%, 2.2%, 3.5%, and 2.6% of the transcripts containing at least one SSR for *Lablab*, *Lathyrus*, *Psophocarpus*, and *Vigna*, respectively. A large proportion of the SSRs (36–43% per transcriptome) were found in the first or last 50 bp of the sequence, which may hamper primer design for this subset of loci. The proportions of di-, tri-, and tetranucleotide repeat SSRs was similar across *Lablab*, *Lathyrus*, and *Psophocarpus*, with di- and trinucleotide repeat SSRs each making up a similar proportion of total SSRs (36–47% each) and tetranucleotide SSRs only making up 16–20% of the total SSRs. In *Vigna*, however, dinucleotide SSRs were much less common than trinucleotide SSRs (29% vs. 55% of total SSRs). The most common di-, tri-, and tetranucleotide motifs are AG/CT (25–32% of SSRs), AAG/CTT (9–19% of SSRs), and TTTC/GAAA (2–4% of SSRs), respectively.

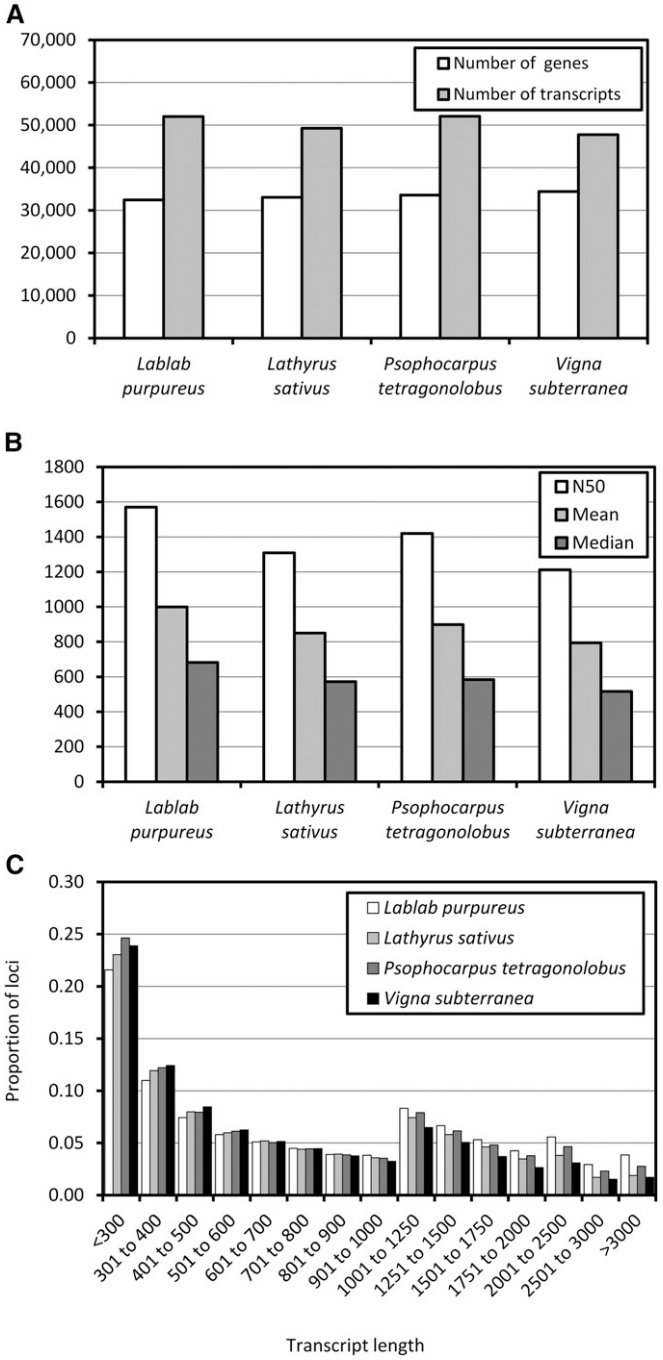


Fig. 1. Summary statistics for the four de novo transcriptome assemblies. (A) Number of genes and number of transcripts assembled for each species. (B) N50, mean, and median transcript length. (C) Distribution of transcript lengths for the four transcriptomes (note the change in bin size along the x axis).

Nearly 1800 potential COS loci were identified by carrying out BLAST comparisons between the four transcriptomes (Fig. 2; Appendix S3). These loci represent those for which a reciprocal best BLAST hit was identified in all pairwise comparisons (see above). To test the efficacy of this strategy in producing reliably amplifiable cross-family loci, primers were designed for the first 12 loci (avoiding three very short loci; Table 2) and PCR amplified from DNA from 12 legume species (see above). Of the 12 primer pairs, one failed to amplify and four amplified in at least 10 of the 12 species. The remainder successfully amplified between two and nine samples (Fig. 3).

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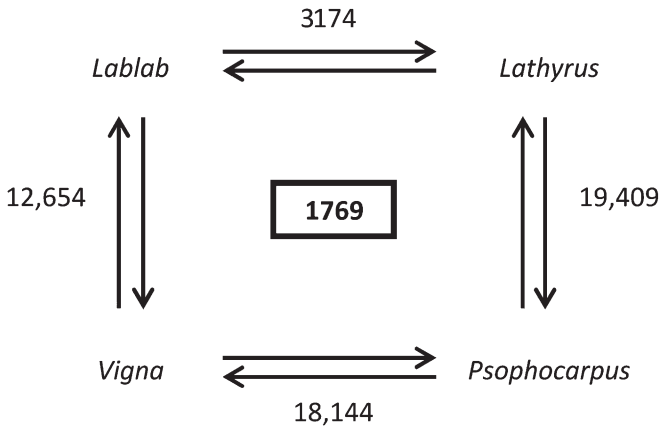


Fig. 2. Results of the pairwise BLAST comparisons of the four de novo transcriptomes. Values between pairs of taxa indicate the number of reciprocal best BLAST hits; the value in the center is the total number of loci for which all four pairwise comparisons resulted in reciprocal best BLAST hits (i.e., the number of potential COS markers).

DISCUSSION

To identify, understand, and benefit from crops with adaptive stress tolerances, it is important to characterize the genetic variation within the crop, with one goal being to relate genetic to phenotypic variation (Burke et al., 2007; Meyer and Purugganan, 2013). Legumes are vitally important crops due to their high protein content, especially in countries where meat protein is rarely consumed (they are sometimes called the "meat of the poor"; de Jager, 2013). The aim of this study was not simply to provide a comprehensive set of transcripts from these species but to pave the way, using SSR and COS markers, for further investigations including population genetics, QTL mapping, and marker-assisted selection.

The percentage of transcripts with a putative orthologue from the fully sequenced *P. vulgaris* was quite high (60–82%; Table 1; Appendix S1), and was highest for the two species most closely related to *Phaseolus* (*Lablab* and *Vigna*) and lowest for *Lathyrus*, which is found in a different clade within the Papilionoideae legumes. The percentage of 27,197 *P. vulgaris* transcripts (taking just the longest transcript from each locus) with a BLAST hit in these four legumes was lower (49–70%; Table 1); however, this is not unexpected given the relatively shallow sequencing effort undertaken. Again, the percentage of hits was highest for *Lablab* and *Vigna* and lowest for *Lathyrus* (Table 1).

The development of cross-family markers to use the legume genomic resources in other species with limited resources would be of great benefit. Between 1139 and 2567 SSRs were identified in 1106 to 2427 transcripts (Table 1; Appendix S2) corresponding to approximately 2.2–4.7% of transcripts containing at least one SSR, a value similar to other studies (2.5–21.1% in a review by Ellis and Burke, 2007). In this paper, approximately

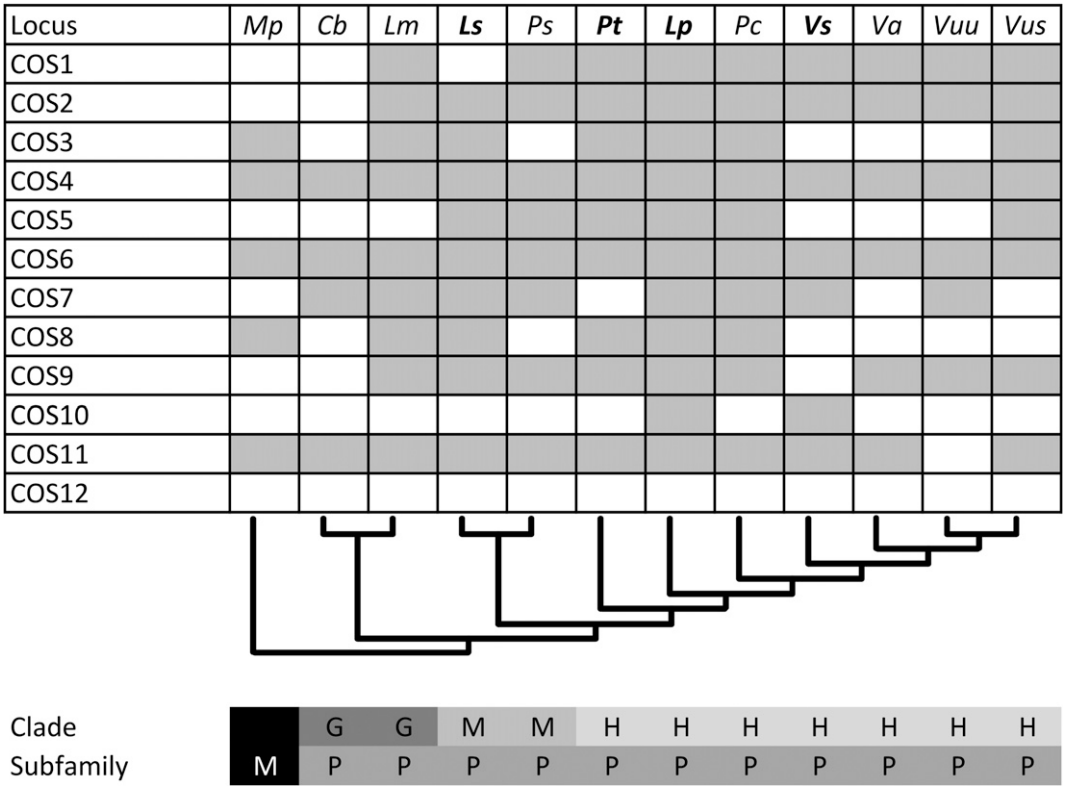


Fig. 3. Results of the PCR test of the first 12 COS loci. Loci are listed on the left and species acronyms across the top. *Mp* = *Mimosa pudica*; *Cb* = *Crotalaria brevidens*; *Lm* = *Lupinus mutabilis*; *Ls* = *Lathyrus sativus*; *Ps* = *Pisum sativum*; *Pt* = *Psophocarpus tetragonolobus*; *Lp* = *Lablab purpureus*; *Pc* = *Phaseolus coccineus*; *Vs* = *Vigna subterranea*; *Va* = *Vigna aconitifolia*; *Vuu* = *Vigna unguiculata* subsp. *unguiculata*; *Vus* = *Vigna unguiculata* subsp. *sesquipedalis*. Species names in bold are the four from which transcriptomes were sequenced. Shaded boxes indicate successful amplification. A skeleton phylogenetic tree based on Wojciechowski et al. (2004) and Delgado-Salinas et al. (2011) is given beneath the table, as are the subfamilies (M = Mimosoideae; P = Papilionoideae) and clades within (G = Genistoids; M = Millettioids; H = Hologalegina) to which the species belong.

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1800 potential COS markers were identified (Fig. 2; Appendix S3), and, although only a small subset was tested for cross-family amplification, these markers show promise for fulfilling the aims of COS loci, i.e., they cross-amplify in a diverse assemblage of species and are thus useful in anchoring QTL maps or for phylogenetic reconstructions of the members of the family. This study demonstrates that a modest sequencing effort in selected taxa can lead to very large resources for future crop-specific and comparative analyses.

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