Development of Gene-Based SSR Markers in Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.) for Diversity Assessment

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1. Introduction

Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) is a tropical perennial vine species, classified in the family of Fabaceae and subfamily of Papilionoideae, that is cultivated mainly at a subsistence scale in hot and humid countries across India, Southeast Asia, and the Western Pacific islands, with a presence in a number of African countries as well [1–5]. It is grown for its green pods,
tuberous roots, and mature seeds, all of which have received attention for their nutritional content in the past, as comprehensively described in 'The Winged Bean—A high-protein crop for the tropics' from the National Academy of Science in 1981 [1]. Initial interest was drawn to high crude protein levels in seeds, which are comparable to soybean [6–8]. Its vining nature and nitrogen fixation activity have seen it used as a cover crop and also incorporated into rotation or intercropping systems [9–11]. As such, winged bean could be a good candidate for diversifying diets to improve nutritional security, based on complex and more sustainable agricultural systems [12]. Despite its potential, winged bean has received limited research investment for developing molecular tools that can support breeding programmes, until recently. Recent reports include the development of inter-Simple Sequence Repeats (iSSRs) and Randomly Amplified Polymorphic DNA (RAPD) markers for genetic diversity and for clonal fidelity analyses and two small transcriptomic assemblies derived from a mix of leaf, bud, and shoot of Sri Lankan accessions and leaf tissue from a Nigerian accession, respectively [13–17]. Given that winged bean is believed to be largely self-pollinated, heterozygosity would be expected to be low, although a formal assessment is needed and the species does produce large flowers, suggesting a contribution from insect pollination, as recorded by Erskine [18]. Thus, molecular breeding will facilitate utilisation of genetic resources in winged bean breeding, especially among accessions, through combining beneficial traits. Molecular markers that are tightly linked to important agronomic traits are a precondition for undertaking molecular breeding in plants. The genetic basis of traits in winged bean remains largely unexplored, and to date there has not been any genetic linkage map reported for this crop, although controlled crosses have been reported [19–22].

In this study, we generated RNA-seq data from four tissues (leaf, root, reproductive tissues, and pod) of six locally grown accessions, followed by the identification of SSR-containing sequences and validation of a subset of genic SSR markers. To our knowledge, this is the first application of within-species genic SSR markers in winged bean accessions. The data will help to begin the development of comprehensive genetic information and tools to facilitate future breeding programmes, as well as allow the levels of natural inbreeding to be determined, to allow appropriate breeding schemes to be devised. The transcriptome will allow us to gain a better understanding of the phylogenetic relationships between winged bean and other leguminous and model plants.

2. Material and Methods

2.1. Plant Material, RNA Extraction, Complementary DNA (cDNA) Library Construction, and Sequencing

A total of six locally grown winged bean accessions (two derived from Malaysian Agricultural Research and Development Institute (MARDI) and four from local planters) were grown from August to December 2012 at Lady Bird Farm, Broga, Semenyih, Malaysia (Latitude: 2.9394 N; Longitude: 101.8971 E; Altitude: 45m asl). RNA was extracted separately from leaf, root, pod, and reproductive tissue (comprising of bud and flower) by pooling the respective tissues from all the six accessions. Extraction was performed from different tissue groups separately using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) followed by another round of purification using RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) before library preparation.

Total RNA was measured using the Qubit RNA BR assay kit (Thermo Fisher Scientific, Waltham, MA, USA). A total of 5 µg of RNA was used for enrichment of mRNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Beverly, MA, USA). RNA fragmentation was done using NEBNext Magnesium RNA Fragmentation Module (New England Biolabs, Beverly, MA, USA). Illumina stranded whole transcriptome sequencing libraries were prepared through a dUTP approach using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Beverly, MA, USA). Libraries were gel purified using 2% E-Gel SizeSelect (Thermo Fisher Scientific, Waltham, MA, USA) and quality control was performed using bioanalyser HS kit (Agilent biotechnologies, Palo Alto, CA, USA). Quantification was done using qPCR (quantitative polymerase chain reaction) (Kapa Biosystems, Woburn, MA, USA). Equimolar amounts of barcoded libraries were mixed and subjected
to 250 bp paired-end run using MiSeq V2 chemistry (Illumina, San Diego, CA, USA) on Illumina MiSeq sequencing platform according to manufacturer’s instruction.

For simple sequence repeat (SSR) marker development, a total of nine plants from five accessions—each one representing a geographical origin—from the International Institute of Tropical Agriculture (IITA) genebank and MARDI, were used (as listed in Table 1 below). Two individuals were used per accession, which were collected after a cycle of single seed descent purification from January to June 2013 at the Lady Bird Farm, except for the Malaysian line.

### Table 1. Winged bean accessions (two individuals per origin, except Malaysian line) used and their origins.

| Individuals | Origin          |
|-------------|-----------------|
| Tpt53-9-8   | Bangladesh      |
| Tpt53-9-10  |                 |
| Tpt17-6-3   | Indonesia       |
| Tpt17-6-8   |                 |
| M3-3        | Malaysia        |
| Tpt10-7-5   | Papua New Guinea|
| Tpt10-7-7   |                 |
| SLS319-10-3 | Sri Lanka       |
| SLS319-10-4 |                 |

#### 2.2. De Novo Transcriptome Assembly and Microsatellite Identification

Adaptors and low quality reads (below Q20) were trimmed using Scythe and Sickle [23], respectively, using default settings. Trimmed reads from all tissues were pooled to assemble a combined de novo assembly with Trinity version 2.2.0 pipeline using the strand specificity option. Trinity assembled transcripts were annotated with Trinotate software suite version 1.1 [24], with a blast e-value threshold of $1 \times 10^{-5}$ from NCBI-BLAST [25], HMMER/PFAM [26], SignalP [27], EMBL eggNOG [28], and Gene Ontology (GO) [29] databases. The data is deposited in NCBI Sequence Read Archive (BioProject ID PRJNA374598) under the accession number of SRP099538; SRR5252647 (root), SRR5252647 (reproductive tissue), SRR5252648 (pod), and SRR5252649 (leaf).

This was followed by the identification of microsatellites using MicroSAtellite (MISA) Perl script program, based on a minimum number of repeats of six for di-, five for tri-, tetra-, penta- and hexa-nucleotide repeat motif (monomer repeats were excluded), whilst the maximum number of bases interrupting two SSRs in a compound microsatellite was 100 [30].

#### 2.3. Microsatellite Markers Development and Scoring

Primer pairs were designed from sequences harbouring a minimum of 18-bases long microsatellites (i.e., minimum 9 and 6 repetitions for di- and tri-nucleotide motifs, respectively) (Table S1). Primer3 [31] and PrimerQuest (Integrated DNA Technologies, Coralville, Iowa, USA) were used for oligo design, with the latter used whenever the first was not able to design with standard parameters for the given sequence. Where possible, two pairs of primers were designed for a single target region, so as to have an alternative if the first pair failed to amplify the target region. DNA was extracted from the leaf of nine genotypes (Table 1) using a modified cetyltrimethylammonium bromide (CTAB) method [32]. In addition, an RNase digestion step was added and 1 volume of isopropanol was used instead of 2/3 volume. Primer screening and optimisation was carried out in a three primer system for fluorescent labelling [33] using an equimolar mixture of genotypes. Each 20 µL of PCR reaction consisted of 1× Buffer S, 200 µM dNTPs, 0.02 µM forward primer, 0.18 µM M13-dye labelled primer, 0.2 µM reverse primer, 20 ng of DNA, and 1 U of Taq DNA Polymerase (Vivantis, Subang Jaya, Selangor, Malaysia). The PCR was programmed for 3 min of initial denaturation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C, with a 10 min final
elongation. Initial polymorphism evaluation of primers was performed across all genotypes using a long capillary fragment analyser (Advance Analytical Technologies – AATI, Ankeny, IA, USA) with default parameters, except for using 4 µL of samples, and 4 kV of separation voltage for 180 min per run. Data was then analysed with Advance Analytical PROSize 2.0 v1.3.1.1 (Advance Analytical Technologies – AATI, Ankeny, IA, USA) to identify potential SSR markers based on presence of polymorphic amplicons across genotypes.

PCR products of single genotypes from potentially polymorphic SSR markers were then separated using an ABI Genetic Analyser ABI3730XL using Peak Scanner v2 for scoring (Applied Biosystems, Foster City, CA, USA). Validated markers were subsequently characterised using Power Marker v3.5 [34] for major allelic frequency, alleles per marker, heterozygosity, and polymorphic information content (PIC).

2.4. Cluster Analysis

A hierarchical cluster analysis was performed with the Dice (also known as Nei and Li) similarity coefficient and unweighted pair-group method with arithmetic mean (UPGMA) algorithm in Genstat 18th Edition [35].

3. Results and Discussion

3.1. Transcriptome Assembly and In Silico Identification of Microsatellites

A total of four libraries generated 12.77 million reads of cleaned 250 bp paired ends (Table 2). The de novo assembly derived from all tissues produced a total of 198,554 contigs with an average size of 798 bp and an N50 of 1462 bp.

Table 2. Summary statistics for the de novo assembled transcriptome.

| Tissue            | Leaf        | Pod         | Reproductive Tissue | Root       |
|-------------------|-------------|-------------|---------------------|------------|
| Number of raw read/base (bp) | 3,150,356/1,544,004,822 | 3,973,092/1,868,456,680 | 3,544,968/1,719,303,632 | 3,873,893/1,859,527,511 |
| Numbers of trimmed read/base (bp) | 3,113,502/1,801,766,113 | 3,157,832/1,431,024,141 | 3,199,527/1,461,908,151 | 3,303,324/1,503,324 |
| Number of contigs/base (bp) | 1,438,258,180/798           | 1,301,766,113/439        | 1,431,024,141/439       | 1,461,908,151/439     |
| Average contig size (bp) | 798                     | 1,462                   |                      |             |

Out of 198,554 contigs, 138,958 (70.0%) could be annotated. Among them, 75,308 (54.2%), 69,172 (49.8%), 6499 (4.7%), 60,040 (43.2%), and 70,069 (50.4%) were found in NCBI-BLAST, HMMER/PFAM, SignalP, EMBL eggNOG, and GO databases, respectively, with no significant homology found from tmHMM on the prediction of transmembrane helices (Table S2). Figure 1 illustrates the abundance of transcripts classified based on gene ontology.
In this study, a total of 9682 putative SSR repeat motifs were identified from 8793 SSR containing sequences, which came from 4.4% of the total contig number in this assembly (Table S3). On average, there was one SSR locus for every 16.4 kbp of de novo assembly. After excluding mononucleotide motifs, trinucleotide repeats were the most abundant type (50.1%) (summarised in Table 3). This is consistent with Vatanparast et al.’s study [16], although hexamer motifs were not evaluated in this study. The most frequent dimer motifs were AG/GA/CT/TC type, followed by AT/TA, whereas for trimeric repeats, AAG/AGA/GAA/CTT/TCT/TTC were the most abundant (Figure 2 and Table 4). Both observations on the most common di- and tri-nucleotide repeat motif are in agreement with the winged bean transcriptome from Vatanparast et al. as well as with the soybean, medicago, and lotus Expressed Sequence Tag (EST)-SSR summarised by Jayashree et al. [16,36].

**Table 3.** In silico identification of microsatellites from the mixed tissue assembly. SSR, simple sequence repeat.

| Total number of sequences examined | 198,554 |
|-----------------------------------|---------|
| Total size of examined sequences (bp) | 158,382,439 |
| Total number of identified SSRs | 9682 |
| Number of SSR containing sequences | 8793 |
| Number of sequences containing more than one SSR | 780 |
| Number of SSRs present in compound formation | 352 |
| Number of dimer-repeat | 4500 |
| Number of trimer-repeat | 4855 |
| Number of tetramer-repeat | 279 |
| Number of pentamer-repeat | 48 |
Figure 2. The number distribution of different microsatellite motif types identified. SSR, simple sequence repeat.
Table 4. Frequency distribution of di- and tri-nucleotide motif repeat in this de novo assembly.

| Number of Repeat Motif | Total  | %   |
|------------------------|--------|-----|
| Di-nucleotide          |        |     |
| AC/GT/CA/TG            | 5      | 6   | 7   | 8   | 9   | 10  | >10 |
| AG/CT/GA/TC            | -      | 256 | 138 | 63  | 37  | 12  | 10  |
| AT/TA                  | -      | 995 | 543 | 330 | 391 | 434 | 189 |
| CG/GC                  | -      | 407 | 201 | 1167| 113 | 107 | 68  |
| Total                  | -      | 1696| 883 | 560 | 541 | 553 | 267 |
| Tri-nucleotide         |        |     |
| AAC/ACA/CAA/GTT/TGT/TTG| 279    | 131 | 50  | 17  | 0   | 0   | 0   |
| AAG/AGA/GAA/CTT/TCT/TTC| 612    | 405 | 351 | 11  | 0   | 0   | 0   |
| AAT/ATA/TAA/TAA/TAT/ATT| 305    | 145 | 112 | 15  | 0   | 0   | 0   |
| ACC/CAC/CCA/GGT/GTG/TGG| 307    | 62  | 55  | 10  | 0   | 0   | 0   |
| ACG/CGA/GCA/CGT/CTC/GTC| 90     | 66  | 12  | 7   | 0   | 0   | 0   |
| ACT/CTA/TAC/AGT/AGG/CTT| 36     | 8   | 3   | 3   | 0   | 0   | 0   |
| AGC/GAG/GGA/GAG/CTG/CCT| 271    | 105 | 38  | 9   | 0   | 0   | 0   |
| AGG/GGA/GAG/GCC/GCG/GCC| 247    | 115 | 75  | 11  | 0   | 0   | 0   |
| ATC/CAT/TCA/GAT/AGT/TGA| 311    | 83  | 24  | 34  | 0   | 0   | 0   |
| CCG/CGC/CAG/GAG/GTC/CGG| 247    | 130 | 55  | 8   | 0   | 0   | 0   |
| Total                  | 2705   | 1250| 775 | 125 | 0   | 0   | 0   | 4855
3.2. Development of SSR Markers and Cluster Analysis

A total of 56 (targeting 42 dimer-repeat regions) and 78 (targeting 53 trinucleotide SSR) primer pairs were designed. Subsequently, 20 dinucleotide SSR primers and 26 trinucleotide SSR primers gave good amplification products at the expected size. After polymorphism evaluation using all genotypes in this study, 18 validated SSR markers (8 for di-nucleotide and 10 for tri-nucleotide repeated motifs; Table S1) were scored and are summarised in Table 5. The low validation rate of polymorphic markers pairs were designed. Subsequently, 20 dinucleotide SSR primers and 26 trinucleotide SSR primers gave reasonable, with the accessions used in this study is limited, they cover a broader range of geographical origins. Residual heterozygosity could still be observed within each accession (shaded values in Table 5), even where a cycle of line purification in a controlled environment has been carried out, indicating that further cycles are needed to obtain homozygous lines, in particular for Tpt10, Tpt53, and M3. This data, along with the winged bean large flower size, also suggest that such a purification process may need to be carried out under an insect-proof enclosed environment. Using these markers, an average of 2.5 and 2.4 alleles per locus for di- and tri-nucleotide SSRs, respectively, was observed (Table 6). Individual PIC values varied from 0.16 to 0.67, which is comparable to recent legume studies in pigeonpea [37], mungbean [38], and common bean [39], although lower than in cowpea [40] and bambara groundnut [41].

The cluster analysis from the SSR scores (Figure 3) showed a few clusters with the accessions originating from Papua New Guinea closely related to the Sri Lankan accession, but sharing the least similarity with the Malaysian and Indonesian materials, comparatively. To our knowledge, the genetic relationship between germplasm from Bangladesh and Malaysia are here investigated for the first time with molecular markers, and place the Bangladesh origin closer to the Sri Lankan and Papua New Guinean germplasm. Although the number of accessions used in this study is limited, they cover a reasonable range of germplasm from different origins.

![Dendrogram](image)

**Figure 3.** A dendrogram of the genetic relationship between genotypes from Papua New Guinea (Tpt10), Sri Lanka (SLS319), Bangladesh (Tpt53), Indonesia (Tpt17), and Malaysia (M3).
Table 5. Scores of 18 SSR markers from nine winged bean individuals.

| Marker | Papua New Guinea | Indonesia | Bangladesh | Sri Lanka | Malaysia |
|--------|------------------|-----------|------------|-----------|----------|
|        | Tpt10-7-5 | Tpt10-7-7 | Tpt17-6-3 | Tpt17-6-8 | Tpt53-9-8 | Tpt53-9-10 | SLS319-10-3 | SLS319-10-4 | M3-3 |
| P27.2  | 205  | 199/205 | 199 | 205 | 205 | 205 | 205 | 205 | 205 |
| P43.2  | 199 | 199 | 195 | 195 | 197 | 199 | 199 | 199 | 195 |
| Ptt1   | 335 | 335 | 339 | 339 | 339 | 335/339 | 335 | 335 | 339 |
| Ptt10  | 226/228 | 228 | 226 | 226 | 228 | 228 | 228 | 228 | 228 |
| Ptt14  | 358 | 358 | 352 | 352 | 350 | 350 | 358 | 358 | 354 |
| Ptt24  | 219 | 217/219 | 217 | 217 | 219 | 219 | 219 | 219 | 217 |
| Ptt7.2 | 426/432 | 426 | 426 | 426 | 428 | 428 | 426 | 426 | 426/428 |
| WB17   | 198 | 198 | 198 | 198 | 198 | 198/198 | 198 | 198 | 198 |
| Ptt53  | 315 | 309 | 315 | 315 | 309/315 | 309/315 | 312 | 312 | 315 |
| Ptt58  | 255/261 | 255/261 | 261 | 261 | 261 | 261 | 261 | 261 | 261 |
| Ptt65.1| 273 | 273 | 267 | 267 | 267 | 267 | 267 | 267 | 267/273 |
| Ptt67.1| 293 | 293 | 296 | 296 | 293 | 293 | 296 | 296 | 293/296 |
| Ptt68.1| 226 | 226 | 229 | 229 | 226 | 223/226 | 223 | 223/226 | 226/235 |
| Ptt76.1| 203 | 203 | 203 | 203 | 209 | 209 | 209 | 209 | 209 |
| Ptt8.1 | 306/309 | 306/309 | 306 | 306 | 309 | 309 | 306 | 306 | 309 |
| Ptt85.1| 276/279 | 276/279 | 276 | 276 | 276 | 276 | 276 | 276 | 279 |
| Ptt93.1| 266 | 266 | 272 | 272 | 266/272 | 272 | 266 | 266 | 276 |
| Ptt99.2| 189/195 | 189/195 | 195 | 195 | 189 | 189 | 189 | 189 | 195 |
Table 6. A summary of data analysis of 18 SSR markers. PIC, polymorphic information content.

| Marker | SSR Motif | Major Allele Frequency | No. of Alleles | Heterozygosity | PIC  |
|--------|-----------|------------------------|----------------|---------------|------|
| P27.2  | TA        | 0.83                   | 2              | 0.11          | 0.24 |
| P43.2  | TA        | 0.56                   | 3              | 0             | 0.49 |
| Pt1.1  | CT        | 0.5                    | 2              | 0.11          | 0.38 |
| Pt10   | TC        | 0.72                   | 2              | 0.11          | 0.32 |
| Pt14   | TG        | 0.44                   | 4              | 0             | 0.64 |
| Pt24   | GT        | 0.61                   | 2              | 0.11          | 0.36 |
| Pt7.2  | TC        | 0.67                   | 3              | 0.22          | 0.4  |
| WB17   | GA        | 0.94                   | 2              | 0.11          | 0.1  |
| Average dimer SSR markers | | 0.66 | 2.5 | 0.1 | 0.37 |
| Pt53   | CGC       | 0.56                   | 3              | 0.22          | 0.53 |
| Pt58   | TAG       | 0.89                   | 2              | 0.22          | 0.18 |
| Pt65.1 | CAG       | 0.72                   | 2              | 0.11          | 0.32 |
| Pt67.1 | AGA       | 0.5                    | 2              | 0.11          | 0.38 |
| Pt68.1 | AAC       | 0.5                    | 4              | 0.33          | 0.59 |
| Pt76.1 | CGC       | 0.56                   | 2              | 0             | 0.37 |
| Pt78.1 | AAC       | 0.56                   | 2              | 0.22          | 0.37 |
| Pt85.1 | GCG       | 0.78                   | 2              | 0.22          | 0.29 |
| Pt93.1 | TGT       | 0.5                    | 3              | 0.11          | 0.5  |
| P99.2  | TTC       | 0.56                   | 2              | 0.22          | 0.37 |
| Average trimer SSR marker | | 0.61 | 2.4 | 0.18 | 0.39 |

4. Conclusions

A set of validated functional winged bean genic-SSR markers is reported here for the first time, to our best knowledge. The reported residual heterozygosity across screened genotypes has suggested that further investigation needs to be carried out on the rate of natural outcrossing in winged bean, in order to understand how genetic materials should be maintained, improved, and introduced into breeding programmes. The cluster analysis provides an initial insight into the potential for these markers to be used on a larger number of winged bean accessions, to carry out a more comprehensive diversity analysis with the evaluation of germplasm from genebanks and from commonly cultivated lines. Finally, this set of 18 microsatellite markers could also be used to contribute to genetic linkage maps in winged bean, with the integration of single nucleotide polymorphisms (SNPs) markers for higher density. Such a map would be the first backbone for linkage analysis and the genetic dissection of traits with agronomic importance in this legume.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/8/3/100/s1, Table S1: the sequences of forward and reverse SSR-primers used in this study; Table S2: Functional annotation of assembled transcripts; Table S3: Identified microsatellites.

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Conflicts of Interest: The authors declare no conflict of interest.
References

1. NAS. *The Winged Bean: High-Protein Crop for the Humid Tropics*, 2nd ed.; National Academy Press: Washington, DC, USA, 1981.

2. Klu, G.Y.P. Induced mutations for accelerated domestication—a case study of winged bean. *West Afr. J. Appl. Ecol.* 2000, 1, 47–52.

3. Khan, T.N. Papua New Guinea: A centre of genetic diversity in winged bean (*Psophocarpus tetragonolobus* (L.) Dc.). *Euphytica* 1976, 25, 693–705. [CrossRef]

4. Harder, D.K. Chromosome Counts in *Psophocarpus*. *Kew Bull.* 1992, 47, 529–534. [CrossRef]

5. Harder, D.K.; Smartt, J. Further evidence on the origin of the cultivated winged bean, *Psophocarpus tetragonolobus* (L.) DC. *Econ. Bot.* 1992, 46, 187–191. [CrossRef]

6. Prakash, D.; Misra, P.N.; Misra, P.S. Amino acid profile of winged bean (*Psophocarpus tetragonolobus* (L.) DC.): A rich source of vegetable protein. *Plant Foods Hum. Nutr.* 1987, 37, 261–264. [CrossRef] [PubMed]

7. Kadam, S.S.; Salunkhe, D.K. Winged bean in human nutrition. *Crit. Rev. Food Sci. Nutr.* 1984, 21, 1–40. [CrossRef] [PubMed]

8. Okezie, B.O.; Martin, F.W. Chemical composition of dry seeds and fresh leaves of winged bean varieties grown in the U.S. and Puerto Rico. *J. Food Sci.* 1980, 45, 1045–1051. [CrossRef]

9. Anugroho, F.; Kitou, M.; Kinjo, K.; Kobashigawa, N. Growth and nutrient accumulation of winged bean and velvet bean as cover crops in a subtropical region. *Plant Prod. Sci.* 2010, 13, 360–366. [CrossRef]

10. Banerjee, A.; Bagchi, D.K.; Si, L.K. Studies on the potential of winged bean as a multipurpose legume cover crop in tropical regions. *Exp. Agric.* 2008, 44, 297–301. [CrossRef]

11. Hikam, S.; MacKown, C.T.; Poneleit, C.G.; Hildebrand, D.F. Growth and N accumulation in maize and winged bean as affected by N level and intercropping. *Ann. Bot.* 1991, 68, 17–22. [CrossRef]

12. FAO. *Coping with Climate Change—The Roles of Genetic Resources for Food and Agriculture*; FAO: Rome, Italy, 2015.

13. Mohanty, C.S.; Verma, S.; Singh, V.; Khan, S.; Gaur, P.; Gupta, P.; Nizar, M.A.; Dikshit, N.; Pattanayak, R.; Shukla, A.; et al. Characterization of winged bean (*Psophocarpus tetragonolobus* (L.) DC.) based on molecular, chemical and physiological parameters. *Am. J. Mol. Biol.* 2013, 3, 187–197. [CrossRef]

14. Koshy, E.P.P.; Alex, B.K.K.; John, P. Clonal fidelity studies on regenerants of *Psophocarpus tetragonolobus* (L.) DC. using RAPD markers. *Bioscan* 2013, 8, 763–766.

15. Chen, D.; Yi, X.; Yang, H.; Zhou, H.; Yu, Y.; Tian, Y.; Lu, X. Genetic diversity evaluation of winged bean (*Psophocarpus tetragonolobus* (L.) DC.) using inter-simple sequence repeat (ISSR). *Genet. Resour. Crop Evol.* 2015, 62, 823–828. [CrossRef]

16. Vatanparast, M.; Shetty, P.; Chopra, R.; Doyle, J.J.; Sathyanarayana, N.; Egan, A.N. Transcriptome sequencing and marker development in winged bean (*Psophocarpus tetragonolobus*; Leguminosae). *Sci. Rep.* 2016, 6, 29070. [CrossRef] [PubMed]

17. Chapman, M.A. Transcriptome sequencing and marker development for four underutilized legumes. *Appl. Plant Sci.* 2015, 3, 1400111. [CrossRef] [PubMed]

18. Erskine, W. Measurements of the cross-pollination of winged bean in Papua New Guinea. *SABRAO J. Breed. Genet.* 1980, 12, 11–14.

19. Eagleton, G.E. Evaluation of Genetic Resources in the Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.) and Their Utilisation in the Development of Cultivars for Higher Latitudes. Ph.D. Dissertation, University of Western Australia, Perth, Australia, 1983.

20. De Silva, H.N.; Omran, A. Diallel analysis of yield and yield components of winged bean (*Psophocarpus tetragonolobus* (L.) D.C.). *J. Agric. Sci.* 1986, 106, 485–490. [CrossRef]

21. Erskine, W.; Khan, T.N. Inheritance of pigmentation and pod shape in winged bean. *Euphytica* 1977, 26, 829–831. [CrossRef]

22. Erskine, B.Y.W. Heritability and combining ability of vegetative and phenological characters of winged beans (*Psophocarpus tetragonolobus* (L.) D.C.). *J. Agric. Sci.* 1981, 96, 503–508. [CrossRef]

23. Joshi, N.A.; Fass, J.N. *Sickle: A Sliding-Window, Adaptive, Quality-Based Trimming Tool for FastQ Files*, version 1.33; Software; 2011. Available online: https://github.com/najoshi/sickle (accessed on 13 March 2013).
24. Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q.; et al. Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nat. Biotechnol.* 2011, 29, 644–652. [CrossRef] [PubMed]

25. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinformatics.* 2009, 10, 421. [CrossRef] [PubMed]

26. Finn, R.D.; Bateman, A.; Clements, J.; Coggill, P.; Eberhardt, R.Y.; Eddy, S.R.; Heger, A.; Hetherington, K.; Holm, L.; Mistry, J.; et al. Pfam: The protein families database. *Nucleic Acids Res.* 2014, 42, D222–D230. [CrossRef] [PubMed]

27. Petersen, T.N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nat. Methods* 2011, 8, 785–786. [CrossRef] [PubMed]

28. Powell, S.; Forslund, K.; Szklarczyk, D.; Trachana, K.; Roth, A.; Huerta-Cepas, J.; Gabaldón, T.; Rattei, T.; Creevey, C.; Kuhn, M.; et al. eggNOG v4.0: Nested orthology inference across 3686 organisms. *Nucleic Acids Res.* 2014, 42, D231–D239. [CrossRef] [PubMed]

29. Gene Ontology Consortium. The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res.* 2004, 32 (Suppl. 1), D258–D261.

30. Thiel, T.; Michalek, W.; Varshney, R.K.; Graner, A. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* 2003, 106, 411–422. [CrossRef] [PubMed]

31. Koressaar, T.; Remm, M. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 2007, 23, 1289–1291. [CrossRef] [PubMed]

32. Doyle, J. DNA Protocols for Plants. In *Molecular Techniques in Taxonomy*; Hewitt, G.M., Johnston, A.W.B., Young, J.P.W., Eds.; Springer: Berlin/Heidelberg, Germany, 1991; pp. 283–293.

33. Schuelke, M. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* 2000, 18, 233–234. [CrossRef] [PubMed]

34. Liu, K.; Muse, S.V. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* 2005, 21, 2128–2129. [CrossRef] [PubMed]

35. VSN International. *GenStat for Windows*, 18th ed.; VSN International: Hemel Hempstead, UK, 2015.

36. Jayashree, B.; Punna, R.; Prasad, P.; Bantte, K.; Hash, C.T.; Chandra, S.; Hoisington, D.A.; Varshney, R.K. A database of simple sequence repeats from cereal and legume expressed sequence tags mined in silico: Survey and evaluation. *In Silico Biol.* 2006, 6, 607–620. [PubMed]

37. Dutta, S.; Kumawat, G.; Singh, B.P.; Gupta, D.K.; Singh, S.; Dogra, V.; Gaikwad, K.; Sharma, T.R.; Raje, R.S.; Bandhopadhyay, T.K.; et al. Development of genic-SSR markers by deep transcriptome sequencing in pigeonpea (*Cajanus cajan* (L.) Millspaugh). *BMC Plant Biol.* 2011, 11, 17. [CrossRef] [PubMed]

38. Gupta, S.K.; Bansal, R.; Gopalakrishna, T. Development and characterization of genic SSR markers for mungbean (*Vigna radiata* (L.) Wilczek). *Euphytica* 2014, 195, 245–258. [CrossRef]

39. Blair, M.W.; Hurtado, N.; Chavarro, C.M.; Muñoz-Torres, M.C.; Giraldo, M.C.; Pedraza, F.; Tomkins, J.; Wing, R.; Varshney, R.; Graner, A.; et al. Gene-based SSR markers for common bean (*Phaseolus vulgaris* L.) derived from root and leaf tissue ESTs: An integration of the BMc series. *BMC Plant Biol.* 2011, 11, 50. [CrossRef] [PubMed]

40. Gupta, S.K.; Gopalakrishna, T. Development of unigene-derived SSR markers in cowpea (*Vigna unguiculata*) and their transferability to other Vigna species. *Genome* 2010, 53, 508–523. [PubMed]

41. Molosiwa, O.O.; Aliyu, S.; Stadler, F.; Mayes, K.; Massawe, F.; Kilian, A.; Mayes, S. SSR marker development, genetic diversity and population structure analysis of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landraces. *Genet. Resour. Crop Evol.* 2015, 62, 1225–1243. [CrossRef]