Songklanakarin J. Sci. Technol.
44 (5), 1172–1178, Sep. – Oct. 2022

http://www.sjst.psu.ac.th

Original Article

Variation in phenotypic and genotypic characteristics of the soybean collection at Can Tho University

Huynh Ky[1*], Van Quoc Giang[1], Nguyen Loc Hien[1], Nguyen Chau Thanh Tung[1], and Yeap Swee Keong[2]

[1] *Department Genetics and Plant Breeding, College of Agriculture, Can Tho University, Can Tho City, 94115 Vietnam*

[2] *China-ASEAN College of Marine Sciences, Xiamen University Malaysia, Sepang, Selangor, 43900, Malaysia*

Received: 10 February 2022; Revised: 1 July 2022; Accepted: 10 July 2022

Abstract

Soybean (*Glycine max* (L.) Merr.) is a key crop that contributes significantly to the global economy. In this work, 100 soybean varieties were obtained from Can Tho University and analysed for diversity using an ISSR marker and nine yield component characteristics. The findings of principal component analysis indicate that the first axis accounted for the majority of variance in the germplasm (51.4%), followed by the second axis (18.1%). Thereby the seed width (group I), node number (group II), and 100 seed weight (group III) within the axis all have a significant effect on the population's phenotype. Eight agro-morphological characteristics (seed length, seed breadth, seed thickness, plant height, number of nodes, pod number, seed number, and 100 seed weight) varied significantly across the 100 soybean germplasms. Classification of the samples into two clusters was performed using ISSR markers analysis. The first cluster included 95 germplasms. Five distinct varieties comprised the second cluster (V57, V83, V62, V63, V67). Following a model-based structural study, two populations were formed, with some genotypes mixing considerably. The study's findings are instructive for future research targeted at boosting the yields of the 100 soybean varieties in Vietnam.

Keywords: *Glycine max.* (L.) Merr. ISSR marker, phenotypic, soybean, yield

1. Introduction

Soybean (*Glycine max* (L.) Merr.) is a critical crop not only for human consumption, but also for animal feed due to its high protein and vegetable oil contents. By 2020, global soybean fields reached 127.89 million hectares and output 361.82 million metric tons, while Vietnam's production reached around 0.05 million hectares and 0.07 million metric tons (Linh & Gilleski, 2021). The global population is anticipated to quadruple by 2050 (United Nation [UN], 2019), implying that soybean consumption is also expected to increase. Additionally, agricultural land will diminish as a result of urbanization and climate change (Pham Thi, Kappas, & Faust, 2021). As a consequence of the growing demand for high-yielding, environmentally resistant soybean varieties, each country has focused its breeding programs on producing new soybean varieties, necessitating the introduction of variable soybean genotypes as breeding materials.

Genetic diversity study is necessary for crop improvement in breeding programs, and it may be done through morphological and molecular techniques (Govindaraj, Vetriventhan, & Srinivasan, 2015). Throughout the history of genetic diversity research, morphological features such as leaflet form, flower color, growth habit, leaflet texture, leaflet shape, canopy pattern, and seed color have been utilized to differentiate between varieties with distinct characteristics (Huang *et al*., 2016; Shilpashree *et al*., 2021). Due to their reliability for germplasm evaluation and trait-related selection,

*Corresponding author
Email address: hky@ctu.edu.vn

these morphological characteristics are exhibited consistently across various atmospheric conditions, unaffected by environmental variables and enhance the specificity and precision of germplasm screening (Bari *et al*., 2012; Singh *et al*., 2010).

Molecular markers have been widely utilized to assess the genetic diversity of a variety of crops, including rice (Ngangkham *et al*., 2019; Yadav *et al*., 2017), peanut (Samaha, Ahmed, & Abd El-Hameid, 2019), green bean (Sarikamis, Yasar, Bakır, Kazan, & Ergul, 2009), potato (Khidr, Arafa, Eldemery, & Elsanhoty, 2017), and soybean (Jain, Joshi, & Rajpurohit, 2017; Kachare, Tiwari, Tripathi, & Thakur, 2019; Kumawat *et al*., 2015; Singh *et al*., 2010). The current study aimed at assessing the morphological and genomic diversity of 100 soybean varieties in order to improve future breeding efforts.

2. Materials and Methods

2.1 Plant materials

A total of 100 Can Tho University soybean varieties/lines whose collections include samples from Asia, USA, and local landraces, were subjected to the study (Table 1).

Table 1. List of the 100 CTU soybean varieties/ lines used in this study.

No	Registered name	Origin	No	Registered name	Origin
V1	TGX 814-26D	imported	V51	A100	Landrace
V2	TGX 811-27D	imported	V52	Thanh Oai 2	Landrace
V3	VERDA	imported	V53	Van Den Tu Liem	Landrace
V4	SENCA	imported	V54	Nam Can 4 hat den	Landrace
V5	PURGA	imported	V55	So 87	Landrace
V6	GELDULT A	imported	V56	T4	Landrace
V7	TROPICAL	imported	V57	VS 87-C1	imported
V8	MACK 57	imported	V58	VX 87-C2	imported
V9	Lien Xo 4	imported	V59	VX 87-09-2	imported
V10	Lien Xo 6	imported	V60	VX 87-09-1	imported
V11	Ottawa	imported	V61	VX 87-04-4	imported
V12	Nhat Ban 20	imported	V62	Xanh lo	Landrace
V13	Nhat Ban 38	imported	V63	Hong Dinh A	Landrace
V14	Nhat Ban 17A	imported	V64	Thanh Linh	Landrace
V15	EGSY 73	imported	V65	X33	Landrace
V16	IGH 23	imported	V66	Vang Nguyen Duong	Landrace
V17	GC 86040-1	imported	V67	T 84	Landrace
V18	GC 82349-6-1	imported	V68	T 78	Landrace
V19	GC 86026-48	imported	V69	Tan Quyen 1	Landrace
V20	G 12501	imported	V70	My Hung	Landrace
V21	D75-9207	imported	V71	MTĐ 760-4	Improved
V22	G 9556	imported	V72	MTĐ 517-8	Improved
V23	PK 73-49	imported	V73	MTĐ 176	Improved
V24	AGS 79	imported	V74	MTĐ 9	Improved
V25	AGS 299	imported	V75	MTĐ 22	Improved
V26	AGS 9	imported	V76	MTĐ240	Improved
V27	AGS 208	imported	V77	MTĐ 305	Improved
V28	AGS 85	imported	V78	MTĐ 173	Improved
V29	AGS 214	imported	V79	MTĐ 120-2	Improved
V30	Ankur	imported	V80	Cọc chùm x NTC 188	Improved
V31	GAS 73	imported	V81	Santa Maria x V74 d2	Improved
V32	F 5-3	imported	V82	Santa Maria x V74 (d10)	Improved
V33	ALOMA	imported	V83	MTĐ 10	Improved
V34	S1 F1-1	imported	V84	MTĐ 459	Improved
V35	TGX 573-201	imported	V85	Cọc chum x V73	Improved
V36	TGX 573-209D	imported	V86	DT 2000	Landrace
V37	MTĐ 860-1	Improved	V87	3 thang chin DakLak	Landrace
V38	PI462312(Rpp3)	imported	V88	HL 09-5	Landrace
V39	Kokuwase Chamame	imported	V89	HL 09-9	Landrace
V40	Sapporo midori	imported	V90	MTĐ 455-3	Landrace
V41	Umai Chame	imported	V91	Nhat 17a-7	Landrace
V42	MTĐ 878-8	Improved	V92	MTĐ 865-1	Improved
V43	MTĐ 878-15	Improved	V93	MTDD	Improved
V44	MTĐ 760-4	Improved	V94	DT thu thap Daklak	Landrace
V45	Thanh Linh 1	Landrace	V95	Daklak	Landrace
V46	Ban Doc A hat vang	Landrace	V96	MTD 765 (white flower)	Improved
V47	Tho Xuan	Landrace	V97	MTD 765 (purper flower)	Improved
V48	So 81	Landrace	V98	MTD 861	Improved
V49	Hong Dinh B	Landrace	V99	MTD 878-22	Improved
V50	Dau mien trang D2	Landrace	V100	MTD 878-22	Improved

2.2 Phenotypic characteristics

The morphological characteristics of soybeans that contribute to yield, such as seed length, seed width, seed thickness, plant height, node number, branch number, pod number, seed number, and 100 seed weight, were recorded according to the International Board for Plant Genetics Resource recommendations (1984).

2.3 Genomic DNA extraction

Young leaves were collected and genomic DNA extracted using the cetyltrimethylammonium bromide (CTAB) technique (Carolina *et al*., 2015). To determine the quality and quantity of DNA, electrophoresis of genomic DNA was performed using an 0.8 percent (w/v) agarose gel and a nanodrop 2000 spectrophotometer (ThermoFisher Scientific, USA), respectively. The qualifying DNA samples were diluted with dH_2O to a final concentration of 50 ng/l and stored at -20℃ for further PCR analysis.

2.4 ISSRs analysis

A panel of 10 ISSR markers were selected for genetic diversity assessment. In PCR reaction mixture of 20 ml comprising 1x reaction buffer, 1 unit of Taq DNA polymerase, 200 mM each of the dNTPs mix, 0.5 µL/reaction of forward and reverse primers, and 50 ng of template DNA, reproducible and distinct banding patterns were produced. The PCR was performed on a programmable GeneAmp PCR 2700 (ABI, USA) with the following cycling parameters: initial denaturation (94℃) for 5 minutes, denaturation (95℃) for 30 seconds, primer annealing (45℃ - 65℃) for 30 seconds, primer extension (72℃) for 30 seconds (40 cycles), final primer extension (72℃) for 5 minutes, and a hold temperature of 10℃. Following the PCR process, the amplified products were visualized after a run on a 1.5 percent agarose gel stained with ethidium bromide (EtBr) dye in 1x TAE buffer. The size of the amplified DNA fragments was determined using standard 100 bp and 1 kb DNA ladders (Thermo, USA). DNA fragments were visualized and photographed using a UV-transilluminator and a digital camera. The amplicons obtained were scored based on the presence (used as 1) or absence (used as 0) of bands for each primer. Only distinct and unambiguous bands were assessed for each primer's banding pattern.

2.5 Data analysis

The results were scored in binary data and processed by the distance matrix method to connect the relationships among individuals. The PIC (Polymorphism Information Content) of the ISSR markers was calculated using the formula for dominant markers and the mean of the PIC of each allele (De Riek, Calsyn, Everaert, Van Bockstaele, & De Loose, 2001). The Hopkins statistic was used to test whether the dataset is significantly clusterable (Hopkins & Skellam, 1954; Lawson & Jurs, 1990). Furthermore, RStudio version 1.4.1717 was used to run statistical processing such as PCA, ANOVA, and heatmap for clustering (RStudio Team, 2015). Additionally, the population structures of the accessions of interest were quantified and visualized using Structure version 2.3.4 (Pritchard, Stephens, & Donnelly, 2000).

3. Results and Discussion

3.1 Diversity of phenotypic characteristics

Frequency distributions of nine growth and agronomic traits are presented in Figure 1. According to Figure 2A, the Hopkins statistic is close to 1 (H = 0.73) so we can reject the null hypothesis and conclude that the dataset is significantly clusterable. Using principal component analysis (Figure 2B and 2C), the first axis mainly accounted for the variation in the germplasm (51.4%), followed by the second axis (18.1%). This result indicates that seed width (group I) and number of nodes (group II) within the axes exhibit great influence on the phenotype of the population. Moreover, the first five components accounted for 92.80% of the total variation, with components PC3, PC4, and PC5 contributing 11.6%, 7.9%, and 3.8%, respectively. Thus, phenotypic traits can be used to identify germplasm GenBank material (Islam *et al*., 2018; Liu *et al*., 2016; Nguyen & Norton, 2020). The findings from this study have the potential to be applied to the Mekong delta of Vietnam in the future as part of a higher yield soybean molecular breeding program.

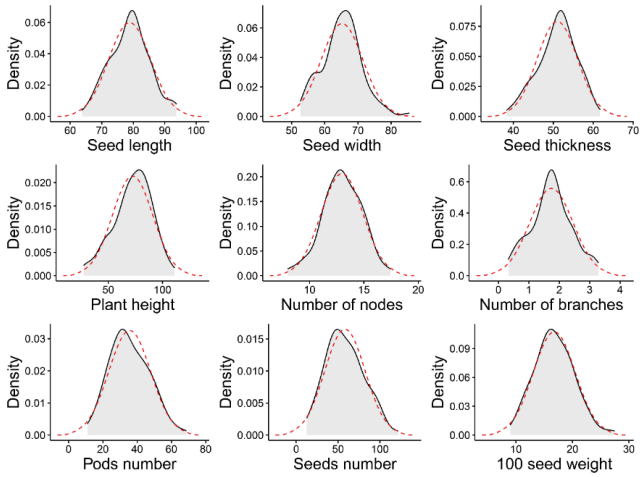

Figure 1. Normal distribution of nine phenotypic characteristics of 100 soybean samples using in the study

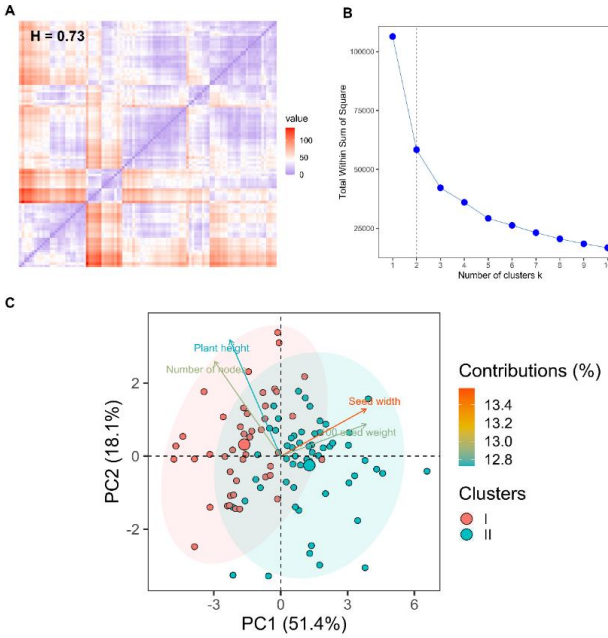

Figure 2. Cluster analysis of 100 soybean germplasms (A) Hopkins statistical testing for nine traits (B) K-means cluster analysis (C) Principal component analysis of 100 soybean samples based on morphological traits.

The frequency distributions of 9 quantitative morphological traits were divided among two groups obtained from cluster analysis (Figure 3). Eight agro-morphological traits (seed length, seed width, seed thickness, plant height, number of nodes, pods number, seeds number, 100 seed weight) demonstrated significant variation among the 100 soybean germplasms. According to Figure 3, group I had shorter seed length, seed width, seed thickness, and 100 seed weight distribution than group II. Group I, on the other hand, presented larger plant height, number of nodes, number of branches, pods number and seeds number.

3.2 ISSR polymorphism among 100 soybean varieties/lines

In this study, 10 ISSR primers were evaluated in order to acquire highly informative ISSR markers. As illustrated in Figure 4, the similarity coefficients Nei and Li (1979) for relationships in this study ranged between 0.79 and 1.00. Additionally, 100 soybean germplasms have been classified into two distinct clusters. 95 germplasms were contained in the first cluster. The second cluster contained five varieties (V57, V83, V62, V63, V67). As can be seen from the graph, V75 and V76, V85 and V86, V94 and V95, V97 and V98 shared 100 percent similar coefficient relationship in cluster I, indicating that these varieties are closely related. They may have descended from the same ancestor but were given different names due to their origins (Table 1). In comparison to Jaccard's coefficient (SJ), the Nei and Li coefficients vary only by the double weight applied to the frequency of bands in each of the two genotypes studied (Duarte, Santos, & Melo, 1999; Mohammadi & Prasanna, 2003), indicating that the Nei and Li coefficient is more appropriate for the type of analysis described in this study. Because phenotypic characteristics are derived from genotypic characteristics, this analysis is highly desirable, valuable, and useful for selection.

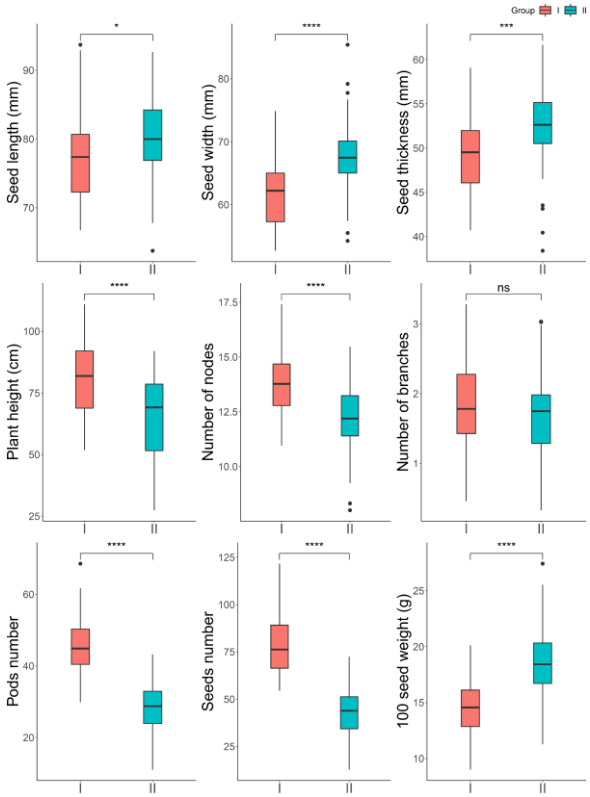

Figure 3. Distribution of nine quantitative morphological traits in two groups derived from cluster analysis. Asterisks represent statistical significance (**p<0.05; ***p<0.001; ****p<0.0001); ns indicates no statistical significance.

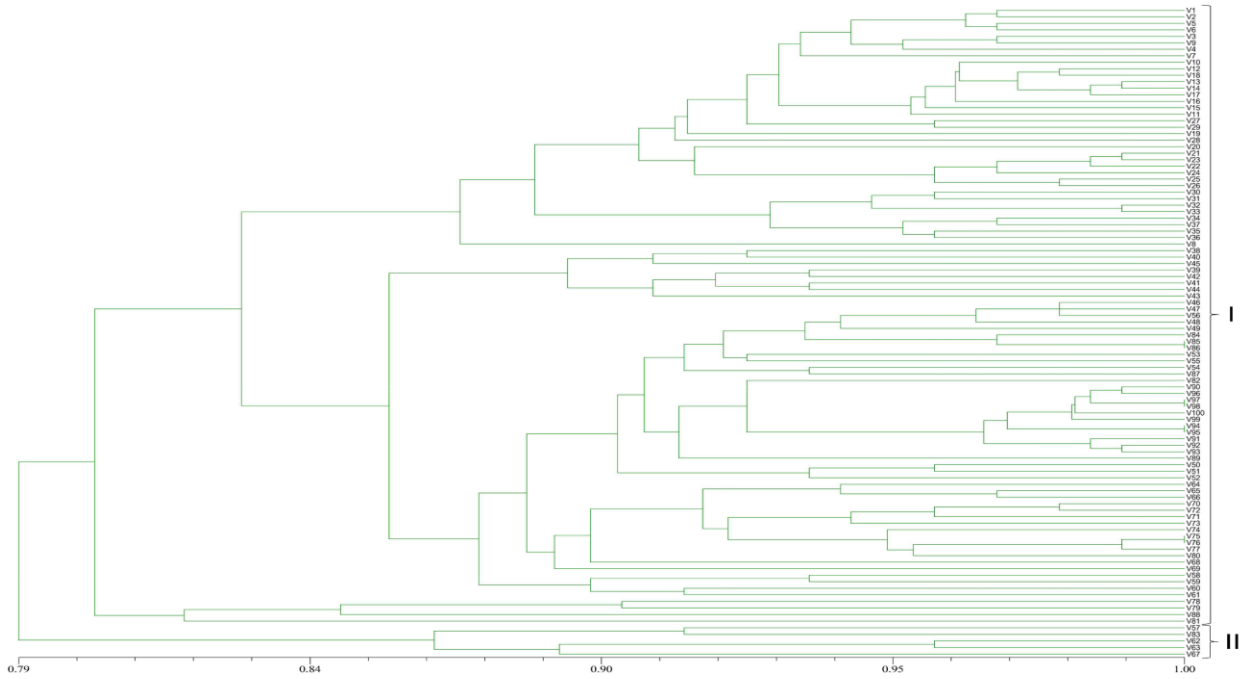

Figure 4. The dendrogram illustrates the similarity coefficient of Nei and Li for 100 soybean germplasms obtained from 10 ISSR markers based on UPGMA method.

3.3 Population structure in model-based analysis

Additionally, a model-based structure analysis was performed to determine the number of populations that could be generated using 100 genotypes and 10 ISSR markers (Figure 5). Both the LnP(D) and Evanno's K values indicated the existence of two genetically distinct groups (K = 2). Structure simulations were conducted by varying K from 1 to 10 and performing 5 runs for each K using all 100 genotypes, with the highest likelihood obtained at K = 2. As a result, two populations were obtained with some genotypes mixing slightly, as illustrated in Figure 5. Additionally, as observed by UPGMA cluster analysis, this population structure analysis (Figure 5) confirmed the grouping of genotypes (Figure 4). Thus, the effectiveness of any crop breeding program is contingent upon the genetic diversity present within the desired improvement characteristics and the degree to which these characteristics are inherited (Adjebeng-Danquah *et al*., 2020; Patel *et al*., 2013). From this perspective, studying genetic variation is extremely beneficial for pre-breeding programs because it enables the selection of parental lines with various desirable characteristics. Despite the fact that molecular markers have been widely used to study genetic diversity, these markers have been linked to genomic regions associated with agronomic traits, these molecular characteristics may be unaffected by environmental factors. In this study, the structure analysis of agro-morphological characteristics classified all varieties into two groups (Figure 5). Following molecular analysis with 10 ISSR markers, two major clusters were identified. At K = 2, all 100 varieties/lines were divided into two populations, indicating genetic differentiation in the overall varieties. The study's findings

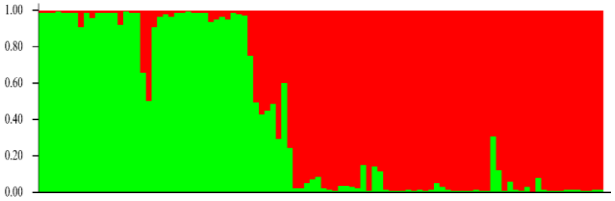

Figure 5. Population structure of 100 genotypes using 10 ISSR markers was determined using STRUCTURE 2.3.4 software

provide useful information for future work aimed at increasing the yields of 100 indigenous soybean varieties in Vietnam.

4. Conclusions

Variation in phenotypic characteristics was informative in distinguishing the population. This is an important trait for contributing to soybean yield. Additionally, the use of the regular panel ISSR marker has been described in sufficient detail and may be used to assess the Mekong Delta soybean population's genetic diversity. It is critical for Can Tho University to collect soybean varieties with a diverse genetic resource that will aid in the improvement of yields for Mekong Delta soybean production.

Acknowledgements

This study was funded in part by the Can Tho University Improvement Project VN14-P6 supported by a Japanese ODA loan. Thank to SES proofreading service.

References

Adjebeng-Danquah, J., Manu-Aduening, J., Asante, I. K., Agyare, R. Y., Gracen, V., & Offei, S. K. (2020). Genetic diversity and population structure analysis of Ghanaian and exotic cassava accessions using simple sequence repeat (SSR) markers. *Heliyon*, *6*(1), e03154-e03154. doi:10.1016/j.heliyon.2019.e03154

Bari, A., Street, K., Mackay, M., Endresen, D., De Pauw, E., & Amri, A. (2012). Focused identification of germplasm strategy (FIGS) detects wheat stem rust resistance linked to environmental variables. *Genetic Resources and Crop Evolution*, *59*(7). doi:10.1007/s10722-011-9775-5

Carolina, A., Mesquita, O., De, A., Figueiró, A., Karla, Santos, K., . . . Marinho Juliatti, B. (2015). Optimization of soybean DNA extraction under different storage and development periods otimização de extração de DNA de soja sob diferentes períodos de armazenamento e desenvol vimento. *Bioscience Journal*, *31*, 1102-1106. doi:10.14393/BJ-v31n4a2015-28343

De Riek, J., Calsyn, E., Everaert, I., Van Bockstaele, E., & De Loose, M. (2001). AFLP based alternatives for the assessment of Distinctness, Uniformity and Stability of sugar beet varieties. *Theoretical and Applied Genetics*, *103*(8), 1254-1265. doi:10.1007/s001220100710

Duarte, J. M., Santos, J. B. D., & Melo, L. C. (1999). Comparison of similarity coefficients based on RAPD markers in the common bean. *Genetics and Molecular Biology*, *22*, 427-432. doi:10.1590/S1415-47571999000300024.

Govindaraj, M., Vetriventhan, M., & Srinivasan, M. (2015). Importance of genetic diversity assessment in crop plants and its recent advances: An overview of its analytical perspectives. *Genetics Research International*, *2015*, 431487. doi:10.1155/2015/431487

Hopkins, B., & Skellam, J. G. (1954). A new method for determining the type of distribution of plant individuals. *Annals of Botany*, *18*(2), 213-227. doi:10.1093/oxfordjournals.aob.a083391

Huang, J., Guo, N., Li, Y., Sun, J., Hu, G., Zhang, H., . . . Qiu, L. (2016). Phenotypic evaluation and genetic dissection of resistance to Phytophthora sojae in the Chinese soybean mini core collection. *BMC Genetics*, *17*(1), 85. doi:10.1186/s12863-016-0383-4

International Board for Plant Genetics Resource. (1984). *Description for soybean*. Rome, Italy: International Board for Plant Genetic Resources.

Islam, M. Z., Khalequzzaman, M., Prince, M. F. R. K., Siddique, M. A., Rashid, E. S. M. H., Ahmed, M. S. U., . . . Ali, M. P. (2018). Diversity and population structure of red rice germplasm in Bangladesh. *PloS One*, *13*(5), e0196096. doi:10.1371/journal.pone.0196096

Jain, R., Joshi, A., & Rajpurohit, D. (2017). ISSR based molecular characterization of soyabean (*Glycine max* (L.) Merrill) genotypes. *Bulletin of Environment, Pharmacology and Life Sciences*, *7*(1), 46-54.

Kachare, S., Tiwari, S., Tripathi, N., & Thakur, V. V. (2019). Assessment of genetic diversity of soybean (*Glycine max*) genotypes using qualitative traits and microsatellite markers. *Agricultural Research*, *9*(2). doi:10.1007/s40003-019-00412-y

Khidr, Y., Arafa, M., Eldemery, D. S., & Elsanhoty, R. (2017). Molecular and morphological evaluation of potato genotypes cultivated in sandy soil. *Egyptian Journal of Genetics and Cytology*, *46*, 1-17. doi:10.21608/ejgc.2018.9487

Kumawat, G., Singh, G., Gireesh, C., Shivakumar, M., Arya, M., Agarwal, D. K., & Husain, S. M. (2015). Molecular characterization and genetic diversity analysis of soybean *(Glycine max* (L.) Merr.) germplasm accessions in India. *Physiology and Molecular Biology of Plants : An International Journal of Functional Plant Biology*, *21*(1), 101-107. doi:10.1007/s12298-014-0266-y

Lawson, R. G., & Jurs, P. (1990). New index for clustering tendency and its application to chemical problems. *Journal of Chemical Information and Computer Sciences*, *30*, 36-41.

Linh, N., & Gilleski, S. (2021). Oilseeds and products annual (Report No. VM2021-0030).

Liu, W., Shahid, M. Q., Bai, L., Lu, Z., Chen, Y., Jiang, L., . . . Lu, Y. (2016). Evaluation of genetic diversity and development of a core collection of wild rice (*Oryza rufipogon* Griff.) populations in China. *PloS One*, *10*(12), e0145990. doi:10.1371/journal.pone.0145990

Mohammadi, S. A., & Prasanna, B. M. (2003). Analysis of genetic diversity in crop plants-salient statistical tools and considerations. *Crop Science*, *43*(4), 1235-1248. doi:10.2135/cropsci2003.1235

Nei, M., & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(10), 5269-5273. doi:10.1073/pnas.76.10.5269

Ngangkham, U., Dash, S., Parida, M., Samantaray, S., Nongthombam, D., Yadav, M. K., . . . Bose, L. K. (2019). The potentiality of rice microsatellite markers in assessment of cross-species transferability and genetic diversity of rice and its wild relatives. *3 Biotech*, *9*(6), 217-217. doi:10.1007/s13205-019-1757-x

Nguyen, G. N., & Norton, S. L. (2020). Genebank phenomics: A strategic approach to enhance value and utilization of crop germplasm. *Plants (Basel, Switzerland)*, *9*(7), 817. doi:10.3390/plants9070817

Patel, S., Ravikiran, R., Chakraborty, S., Macwana, S., Neetiyath, S., Trivedi, R., & Aher, B. (2013). Genetic diversity analysis of colored and white rice genotypes using Microsatellite (SSR) and Insertion-Deletion (INDEL) markers. *Emirates Journal of Food and Agriculture*, *26*(5). doi:10.9755/ejfa.v26i5.15722

Pham Thi, N., Kappas, M., & Faust, H. (2021). Impacts of agricultural land acquisition for urbanization on agricultural activities of affected households: A case study in Huong Thuy Town, Thua Thien Hue Province, Vietnam. *Sustainability*, *13*(15), 8559.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

RStudio Team. (2015). RStudio: Integrated development for R. RStudio, PBC, Boston, MA Retrieved from http://www.rstudio.com/.

Samaha, G. M., Ahmed, M. A., & Abd El-Hameid, A. R. (2019). Assessment of growth and productivity of five peanut cultivars and genetic diversity using RAPD markers. *Bulletin of the National Research Centre*, *43*(1), 168. doi:10.1186/s42269-019-0201-z

Sarikamis, G., Yasar, F., bakır, M., Kazan, K., & Ergul, A. (2009). Genetic characterization of green bean (*Phaseolus vulgaris*) genotypes from eastern Turkey. *Genetics and Molecular Research: GMR*, *8*, 880-887. doi:10.4238/vol8-3gmr605

Shilpashree, N., Devi, S. N., Manjunathagowda, D. C., Muddappa, A., Abdelmohsen, S. A. M., Tamam, N., . . . Janhavi, V. (2021). Morphological charac terization, variability and diversity among vegetable soybean (*Glycine max* L.) genotypes. *Plants (Basel, Switzerland)*, *10*(4), 671. doi:10.3390/plants10040671

Singh, R. K., Bhatia, V. S., Bhat, K. V., Mohapatra, T., Singh, N. K., Bansal, K. C., & Koundal, K. R. (2010). SSR and AFLP based genetic diversity of soybean germplasm differing in photoperiod sensitivity. *Genetics and Molecular Biology*, *33*(2), 319-324. doi:10.1590/S1415-47572010005000024

United Nation. (2019). *World population prospects 2019*. New York, NY.

Yadav, M. K. S. A., Ngangkham, U., Shubudhi, H. N., Bag, M. K., Adak, T., Munda, S., . . . Jena, M. (2017). Use of molecular markers in identification and characterization of resistance to rice blast in India. *PloS One*, *12*(4), e0176236-e0176236. doi:10.1371/journal.pone.0176236