Evaluation of Genetic Diversity of Tongkat Ali (*Eurycoma sp.*) Using Simple Sequence Repeat (SSR) Marker

Nusanisa Chedao[a*] and Nifareesa Chealoh[a],
and Raheema Wamaedeesa[a]

Received: August 6, 2024; Revised: August 20, 2024;
Accepted: August 26, 2024; Published Online: September 9, 2024

Abstract

Tongkat Ali (*Eurycoma sp.*) is a popular medicinal plant in Thailand. Tongkat Ali herb is in the *Eurycoma* genus originates from the southern border provinces and Malaysia. The *Eurycoma* is one of the most important genus, however only few comprehensive research studies have dealt on it and methodogies to propagate this genus are also limited. Here, we study to evaluate the genetic diversity of sixteen *Eurycoma* sp. simple sequence repeat (SSR) markers from southern and northeastern of Thailand in the 18 pairs of microsatellite markers were examined. Authenticated polymorphisms were detected in a total of ten SSR primer pairs and lead to an array of DNA fragments that differed between three band sizes from each fragment, with requiring 1 at least for two states on either side or not. On average, five alleles were recorded for each marker and PIC values varied from 0.26 to 0.68. The genetic similarity coefficients of all samples ranged from 0.63 to 1. Most samples from Narathiwat and Yala provinces were in the same group (similarity coefficient = 0.66), while samples from Amnat Charoen and Ubon Ratchathani provinces were in another group (similarity coefficient = 0.72). This study provides valuable information for establishing a genetic diversity database and guides future studies on active substances in *Eurycoma sp*., which may contribute to the development of new herbal medicine formulations.

Keywords: *Eurycoma sp.*, SSR marker, Genetic diversity

Introduction

Tongkat Ali (*Eurycoma sp.*) is an indigenous medicinal plant belonging to the Simaroubaceae family and is found in southeastern Asia, particularly in Thailand. Throughout many regions, it has been implemented for its medicinal attributes. This genus is especially common in Malaysia and Thailand, Laos, Myanmar...etc. This is proven by the different parts of the plant that have medicinal properties, each part having a slightly different profile of bioactive compounds. The stem of Tongkat Ali contains quasinoides, alkaloids, triterpenes (thyrucalain type), phenolic derivatives of squalane, and tannins, as well as biphenylene esters such as 9-methoxycanthin-6-one11-O-glucopyranside, which are organic contaminants. It also contains biologically active steroids such as novel polysaccharides or glycoprotein urea-peptides. Eurycomalactone, eurycomanone, phenylpropanoids and longilactone the other major bioactive group in the root (and this group was found to be more important than the glycosaponins), were mainly concentrated here. These compounds have a broad spectrum of bioactivities e.g. anti-malarial, antiparasitic and as an anti-inflammatory (Wizneh & Asmawi., 2014). It is also used to increase male sexual activity and hormonal balance (Sathitbut *et al*., 2016; Rehman *et al*., 2016). In addition, Tongkat Ali is also considering the use of Tongkat Ali in animal feeds beyond its human requirements, reflecting a general trend towards improving food safety in livestock production. For example, Pimpa *et al.* (2019) showed that the inclusion of fermented *E. longifolia* root and

[a] Faculty of Agriculture, Princess of Naradhiwas University 96000, Thailand
*Corresponding Author Email: nusanisa.c@pnu.ac.th

galangal in a Total Mixed Ration (TMR) diet for Wagyu crossbred cattle increased protein digestibility to 62.3%, indicating its potential to promote the growth of *E. longifolia* in cattle fed a high fiber-based diet. However, the increasing demand for *E. longifolia*, particularly from wild species, has raised concerns about the potential loss of genetic diversity and risk of future extinction. This underscores the importance of research and conservation of the genetic variety of the *E. longifolia* in Thailand due to the limited available data. The *Eurycoma* genus in Southeast Asia encompass three species: *E. apiculata* A. W. Benn, *E. harmandiana* Pierre and *E. longifolia* Jack. In Thailand, there are two known species: *E. longifolia* Jack which is widespread throughout the country and *E. harmandiana* Pierre which is mostly restricted to the northeastern region, especially in Ubon Ratchathani Province. The similarities in appearance between these species make precise classification based solely on physical characteristics difficult.

A previous study by Sathitbut *et al*. (2016) employed Amplified Fragment Length Polymorphism (AFLP) markers to study the genetic diversity of *E. longifolia* across 29 provinces in Thailand, identifying two distinct species. However, AFLP markers have limitations, including their complexity, the need for specialized equipment, and their dominant nature, which prevents differentiation between homozygous and heterozygous states (Vos *et al*., 1995; Mueller & Wolfenbarger, 1999). In contrast, Simple Sequence Repeat (SSR) markers, or microsatellites, offer several advantages for genetic studies. As co-dominant markers, SSRs can distinguish between homozygous and heterozygous alleles, providing more detailed genetic information. Their high polymorphism and multiple alleles per locus make them particularly useful for studying genetic diversity, population structure, and genetic mapping (Ellegren, 2004; Varshney *et al*., 2005). SSR markers have been successfully applied in genetic diversity analyses of various plants, including indigenous rice in India (Singh *et al*., 2016), napier grass (Wang *et al*., 2017), sweet potato (Ngoria *et al*., 2019), and cabbage (Yin *et al*., 2023).

This study focuses on the importance of Tongkat Ali and aims to assess the genetic diversity of *Eurycoma sp.* in Thailand through SSR molecular markers and aiming to identify the species and construct a phylogenetic tree from it, also to provide insights for research and conservation of medicinal plants in Thailand.

Materials and Methods

Sample Collection and Preparation

Eurycoma samples were obtained from its natural environments in four Thai provinces: Narathiwat and Yala in the south, and Amnat Charoen and Ubon Ratchathani in the northeast. A total of 16 samples were collected, of which 9 samples were from the southern region of Thailand and 7 samples were from the northeastern region of Thailand. The focus is on collecting young, healthy leaves from each *Eurycoma* plant to ensures high-quality genetic material for analysis. These leaves underwent a careful preparation process to maintain their genetic integrity, starting with gentle cleaning to remove any dirt or debris. The washed leaves were cut into small pieces for further grinding in liquid nitrogen. The finely ground leaf powder was then transferred to sterilized microcentrifuge tubes and stored at -20°C until further use.

DNA Extraction

DNA extraction was performed using a modified CTAB (cetyltrimethylammonium bromide) method based on Doyle and Doyle (1987). The procedure was as follows: fifty milligrams of the ground tissue were transferred to a microcentrifuge tube. One milliliter of CTAB extraction buffer (20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 2% CTAB, 2% PVP-40, 1.4 M NaCl, and 2% β-mercaptoethanol) was added to the sample. The mixture was incubated at 65°C for 60-90 min with gentle mixing from time to time. Following incubation, 500 µl of chloroform was added, and the sample was centrifuged at 12,000 rpm at 4°C for 2 minutes. The aqueous phase (approximately 700 µl) was carefully transferred to a new 1.5 mL microcentrifuge tube. This chloroform extraction step was repeated once more to

ensure thorough purification.

To remove RNA contamination, 2 µl of RNase A was added to the aqueous phase and incubated at 37°C for 30 minutes. Subsequently, 700 µl of cold isopropanol was added to precipitate the DNA. The tube was gently inverted several times to mix the contents thoroughly. The sample was then centrifuged at 12,000 rpm at 4°C for 10 minutes to pellet the DNA. The DNA pellet was washed twice with 1 mL of 70% ethanol to remove residual salts and other contaminants. After careful removal of the ethanol, the DNA pellet was air-dried at 55°C until all traces of ethanol had evaporated. The dried DNA pellet was then resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). Finally, the extracted DNA samples were stored at -20°C until further use.

SSR Marker Selection

For this study Microsatellite (SSR) marker primers were used according to Tnah *et al*. (2010). We selected 18 SSR markers that had previously shown efficacy in discriminating different *Eurycoma* species (Table 1)

Table No.1 Presents the primers used for the genetic classification of *Eurycoma* species in this study.

Primer	Base sequence	Nucleotide sequence (5'-3')	Annealing temperature (°C)	Size (base pairs)
Eu002	$(GA)_{23}$	F-ATGCCTGCACATAAAGCTCACACG	60	244-281
		R-TCATCGGGAGAGACGGTTTC		
Eu004	$(CT)_{14}$	F-AAACAGAAGGCAACACCATTAGAA	55	379-396
		R-GAACAAGAAGCCGAAACAT		
Eu005	$(CT)_{14}$	F-GGCGGATTGGAAGTTATGA	55	243-285
		R-TAAATGAGGGGTTTCTGAATCTAA		
Eu025	$(CT)_{14}$	F-TCAAGGACCCGTTAGATTA	55	294-326
		R-AGCATTGGAGGTAAGGTCTTGTAA		
Eu026	$(CT)_{25}$	F-TTTCCCATCACTGCTCTTGTATA	55	218-254
		R-CGGCACTTGTATATGTAGAT		
Eu044	$(GA)_{14}$	F-ATTTCTTCCCTCAACAACATACTC	60	233-264
		R-ACTTCCTCCACCATCGTCTTCT		
Eu048	$(CT)_{17}$	F-TTCATTGGCAATTATCAAGTTTCA	55	73-110
		R-CCTGCGGTCTCATATCAAG		
Eu054	$(GA)_{13}$	F-TTGATTGTTGGCTTATACCT	55	151-163
		R-TAATGTCTCACAAATCTCATGTGC		
Eu055	$(GA)_{15}$	F-GTCCAAAACCAACGTCTATTATTC	55	98-115
		R-GCTGCCAAATCAATGTCACT		
Eu066	$(GA)_{15}$	F-AGCCCTTTCAGCTCCAACTTAACAT	60	274-298
		R-GCCCATCCTATAACGACTCTTAG		
Eu085	$(GT)_{7}$	F-GAGGGGAAGATGAAGGGACAAT	60	199-205
		R-GGGCAATTATTCTCACCGTACAA		
Eu098	$(GT)_{6}$	F-ATTTACATTCTTATTGGCACTCA	55	133-200

Table No.1 (Continue)

Primer	Base sequence	Nucleotide sequence (5'-3')	Annealing temperature (°C)	Size (base pairs)
		R-GACTAAATTAACTCAGCGTACAGA		
Eu099	$(CT)_{14}$	F-CTTTTCCGTGTTCGATACTTGTCC	60	129-145
		R-CTCCAGTTTGAAACCCATTACCC		
Eu104	$(GT)_{11}$	F-CCTTCTATTCTCCGCAAAC	55	214-237
		R-TATATCAATGCCAAAGACTATCCC		
Eu112	$(GT)_{7}$	F-CCGCCAGGATACTCGTTTCT	55	161-167
		R-AAAGCCCAACAAACCCTAAAAG		
Eu130	$(CA)_{11}$	F-AGAAACAGGCAAGCATGAGCAAGC	60	218-228
		R-TTGAGGCCAGTATCGAGGACC		
Eu199	$(GA)_{14}$	F-TTATCAAGTCCACAAGAAACCCTA	55	102-122
		R-TAATCCACTACTTCAATTCCC		
Eu205	$(CT)_{16}$	F-AACGGCGTCAATAATAGGAG	60	230-258
		R-CAGATGTAAGAGCGTGGGGATTCA		

Polymerase chain reaction

The genetic diversity analysis of *Eurycoma* samples was conducted using Polymerase Chain Reaction (PCR). DNA amplification was performed in a total reaction volume of 10 µl, containing 10 ng of DNA template, 5U of Taq PCR Master Mix, and 10 pmol each of forward and reverse primers. The PCR thermal cycling program began with primary denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at primer-specific temperatures for 30 seconds, and extension at 72°C for 1 minute. The process ends with a final extension step at 72°C for 7 minutes. After amplification, PCR products were initially screened using 1.5% agarose gel electrophoresis. The successfully amplified DNA markers were then used for all *Eurycoma* samples and further analyzed using electrophoresis on a 6% denaturing polyacrylamide gel (PAGE). Preparation of polyacrylamide gels involves meticulously cleaning two glass slides with 95% ethanol. One slide is coated with a silane adhesion agent to ensure gel adhesion, while the other is coated with a silane repellent to prevent gel adhesion. These slides are formed with 0.35–0.40 mm spacers with the coated surfaces facing inward. Carefully pour the 5% polyacrylamide gel solution between the assembled plates, taking care to avoid air bubble formation. A comb was inserted at the top, and allow the gel to polymerize for approximately 1.5 hours. After polymerization, the comb was gently removed, and the gel assembly was placed into the electrophoresis apparatus. The electrode chamber is filled with 1x TBE buffer and connected to a power supply to prepare the system for sample loading and electrophoresis. This detailed and precise protocol ensures high-resolution separation of SSR markers, facilitating accurate genetic diversity analysis of the *Eurycoma* samples.

PCR products were denatured by denaturing polyacrylamide gel electrophoresis analysis. DNA samples were denatured single-stranded at 95°C for 5 minutes and then rapidly cooled to 0°C by immediately placing on ice. The denatured DNA samples were then loaded onto 5% polyacrylamide gels in 1X TBE buffer. Electrophoresis was performed at 50 volts for 1.5-2 hours, maintaining a constant temperature of approximately 50°C. After electrophoresis, the gels are stained using a silver staining method to reveal DNA

bands. The process involved agitating the gel plates in 10% acetic acid solution for 20 minutes to remove urea, a component of the polyacrylamide gel. The plates were then rinsed twice with distilled water for 2 minutes each. Staining was performed using a silver stain solution (1 g $AgNO_3$, 1.5 mL of 37% formaldehyde, 1000 mL dH_2O) for 30 minutes. A quick rinse with water (approximately 10 seconds) was performed to remove excess silver stain. The plates were then agitated in a developer solution (30 g sodium carbonate, 200 µl of 10 mg/mL sodium thiosulfate, 1.5 mL of 37% formaldehyde, 1000 mL dH_2O) to reveal the DNA bands. Once the DNA bands were clearly visible, the reaction was stopped using 10% acetic acid. Finally, the plates were rinsed with distilled water for 10 minutes. After drying, the number of DNA bands was counted to obtain genotype data for further analysis.

Data analysis

The DNA fingerprints obtained from SSR markers were analyzed by comparing the DNA bands of each sample. Data were recorded and analyzed based on the presence and absence of DNA bands in all 16 *Eurycoma* samples. Each primer was used to analyze the Polymorphism Information Content (PIC) and Similarity index. Subsequently, the coefficients were used in cluster analysis and dendrograms were generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). This analysis was performed using NTSYS-pc version 2.1 software, and the results were presented in the form of a phylogenetic tree.

Results and Discussion

The genetic relatedness of 16 *Eurycoma* samples from natural sources was studied using SSR molecular markers. Out of 18 SSR primers tested, 17 primers (94.44%) successfully amplified DNA in a PCR reaction. Eleven primer pairs (64.70%) produced detectable DNA bands on a 5% denaturing polyacrylamide gels. Among these, 10 primer pairs (90.90% of the amplifiable primers) revealed polymorphic DNA bands. A total of 51 polymorphic alleles were detected across all 16 *Eurycoma* samples. The number of alleles per primer ranged from 3 to 7, with an average of 5.10 alleles per primer. Three SSR primers (Eu002, Eu005, and Eu055) produced 7 alleles each, while one primer (Eu054) produced 6 alleles. Another primer (Eu066) generated 5 alleles, and four primers (Eu004, Eu026, Eu104, and Eu199) produced 4 alleles each. Lastly, one primer (Eu098) produced 3 alleles. These results demonstrate the effectiveness of SSR markers in revealing genetic diversity among the sampled *Eurycoma* populations.

The UPGMA cluster analysis of *Eurycoma* samples revealed two main groups (Figure1). The genetic similarity coefficients in the 16 samples were relatively high, ranging from 0.63 to 1.00. This high similarity is normal when analyzing genetic similarity coefficients via cluster analysis. The samples were divided into two main groups with a genetic similarity level of approximately 0.48, corresponding to *E. longifolia* and *E. harmandiana* samples. Within the E. longifolia group, two subgroups were identified at a genetic similarity level of about 0.66. One subgroup consisted of NRW1, NRW2, NRW3, and NRW6, while the other included NRW4, YL7, YL8, and YL9. Similarly, the E. harmandiana group was divided into two subgroups at a genetic similarity level of approximately 0.72, comprising AN10, AN11, AN12, AN13 in one subgroup, and UBP14, UBP15, UBP16 in the other. The genetic similarity matrix for all 16 *Eurycoma* samples ranged from 0.41 to 1.00. The highest similarity index of 1.00 was observed between samples UBP15 and UBP16. The lowest similarity indices of 0.41 were found between several pairs: AN10 and YL7, AN12 and NWT6, AN13 and NWT3, AN13 and NWT6, AN13 and YL8, UBP15 and NWT3, and UBP16 and NWT3.

Eurycoma samples from Narathiwat and Yala provinces, characterized by thin, slender leaves, yielded high-quality DNA when extracted using a modified Doyle & Doyle (1987) method. The extracted DNA was clear, colorless, and had a concentration ranging from 20-60 ng/µl with high purity. In contrast, samples from Amnat Charoen province had thicker leaves compared to those from the southern provinces. This resulted in less finely ground leaf samples during the grinding process, resulting in DNA fragmentation and lower DNA yields. These finding results are consistent with the study by

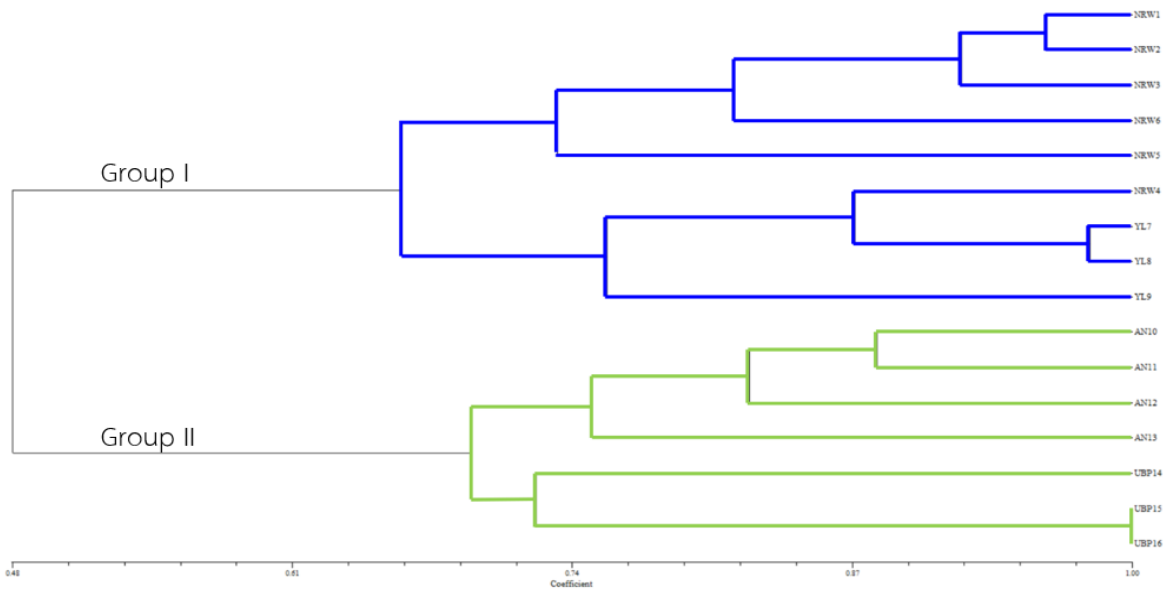

Figure 1 The UPGMA dendogram in 16 *Eurycoma* spp. genetic materials by SSR analysis using Jaccard's similarity matrices.

Safeena *et al*. (2021), which demonstrated low DNA yield and quality when extracting DNA from date palm (*Phoenix dactylifera*) leaves due to its high fiber content, making it difficult to grind. Similarly, Somwong *et al*. (2016) compared DNA extraction methods for Nepenthes mirabilis, a pitcher plant species and found that CTAB concentration in the extraction buffer and leaf freshness significantly affected DNA quantity and quality. Their results showed that DNA extraction from fresh pitcher plant leaves using 2% CTAB concentration yielded the best DNA quantity and quality.

The differences in DNA yield and quality observed in our study could be explained by differences in leaf structure and composition in *Eurycoma* samples from different regions. These findings highlight the importance of improving DNA extraction protocols based on plant tissue characteristics to ensure high-quality DNA for molecular marker analysis.

SSR molecular markers have been widely used and have high efficiency for analyzing plant genetic diversity, which is consistent with the study of Chen et al. (2019), which employed SSR markers to analyze genetic diversity in citrus cultivars in China. Their research showed high genetic diversity among citrus cultivars and clearly distinguishes different citrus groups, highlighting the power of SSR markers in studying plant genetic diversity. SSR markers are characterized by the presence of repetitive sequences at specific positions in the genome. Each repeat unit consists of very short base sequences, typically not exceeding 10 base pairs. These markers exhibit high polymorphism due to their tendency to easily change in the number of repeat units. Furthermore, SSR markers are co-dominant, allowing the distinction between heterozygous and homozygous states. These markers are distributed throughout the genome, making them useful for genome mapping and various applications in molecular genetics.

SSR markers are considered to be a useful tool for assessing genetic diversity in crop species due to their high polymorphism rate (highly informative-ness), their synergistic genome-wide distribution, their relative coverage and reproducibility compared to RFLP etc. across the genomes; which makes SSR a valid tool as it can be amplified from all genomic DNA types described above in the same manner, regardless of their application in many other crop species. These properties make SSR markers a powerful method for assessing genetic diversity and population structure, relationships between different plant populations or within species such as *Eurycoma*. SSR markers have

been used to assess genetic structure and diversity in plant populations across diverse ecosystems due to their high resolution, which allows them to capture subtle genetic differences. The Cophenetic correlation coefficient (r) of 0.96 in the *Eurycoma* clustering analysis shows excellent clustering performance. As stated by Oliya *et al*. (2023), the Cophenetic correlation values are interpreted as follows: values ≤ 0.7 are considered unreliable, values 0.7-0.8 are considered moderately reliable, values 0.8-0.9 are considered reliable, and values 0.9-1.0 are considered very reliable for clustering. Thus, our result of 0.96 indicates a very high level of reliability in clustering the *Eurycoma* samples. The genetic similarity value effectively distinguishes the diversity, which is consistent with the findings of Alam et al. (2023) in their study of maize (Zea mays L.) cultivars in Bangladesh. They reported similarity coefficients ranging from 0.45 to 0.89, which allowed clear grouping of maize varieties based on different genetic characteristics. In this study, the genetic similarity index among the 16 *Eurycoma* samples ranged from 0.41 to 1.00, indicating a wide genetic diversity. This index range effectively covers both closely related and distantly related groups within the samples. High values of this range (1.00) between samples UBP15 and UBP16 indicate a high potential for clonal or pure-line individuals, while lower values (e.g., 0.41) between sample pairs indicate significant genetic divergence. These results underline the power of SSR markers in revealing genetic relationships and diversity patterns within *Eurycoma* populations. The clear separation of clusters and subclusters in this dendrogram, together with the high Cophenetic correlation coefficients found that the SSR markers used have proven to be sufficiently powerful for genetic diversity analyses to be performed in *Eurycoma.* The efficacy of these molecular markers in discriminating genetic diversity suggests that they may be applicable to such analyses in other plant species. These markers could provide a useful resource for a variety of applications, including population genetics studies, conservation efforts, and selection/breeding programs designed to maintain or increase genetic diversity within plant populations.

Conclusion

The genetic diversity of 16 *Eurycoma sp.* samples was evaluated using SSR markers. Out of the primers tested, 10 samples successfully amplifiable DNA, representing 90.90% of the primers that could amplifiable DNA. A total of 51 polymorphic alleles were detected across all 16 samples. Cluster analysis revealed two main groups with a genetic similarity level of approximately 0.48. The first group, identified as *E. longifolia*, consisted of samples NRW1, NRW2, NRW3, NRW4, NRW6, YL7, YL8, and YL9. This group was further subdivided into two subgroups at a genetic similarity level of about 0.66. The second main group consisted of samples AN10, AN11, AN12, AN13, UBP14, UBP15, and UBP16. The genetic similarity coefficients among all samples ranged from 0.41 to 1.00. DNA fingerprinting analysis using 10 pairs of SSR primers revealed distinct DNA banding patterns that could effectively discriminate the *Eurycoma* groups based on their collection sites. This genetic difference may be due to local adaptations to specific environments. Studies have shown that *Eurycoma* plants distributed over larger geographical distances exhibited more distinct genetic characteristics. Based on these results, future research should focus on collecting *Eurycoma* samples from natural forests bordering Malaysia to further study the relationship between geographic distribution and genetic diversity. This approach will help determine whether geographical distance can serve as a reliable indicator of genetic diversity in *Eurycoma* populations. This study demonstrates the efficacy of SSR markers in assessing the genetic diversity and relationships among *Eurycoma* populations, providing valuable information for potential conservation strategies and breeding programs.

Acknowledgements

This research was funded by the Agricultural Research Development Agency (Public Organization). We extend our gratitude to the staff of Phu Sing-Phu Pha Phueng Forest Park in Amnat Charoen Province and Pha Taem National Park in Ubon Ratchathani Province for their generous support and assistance throughout this research project.

References

Alam, M. M., Haque, M. S., & Rafii, M. Y. (2023). Genetic diversity analysis of maize (*Zea mays* L.) inbred lines using SSR markers. *Journal of Plant Biology, 66*(1), 45-58. https://doi.org/10.1007/s12374-022-09342-8.

Chen, C., Zhang, Y., Ding, Z., Li, C., & Chen, Z. (2019). Genetic diversity and population structure analysis of Citrus accessions based on SSR markers. *Molecular Breeding*, *39*(8), 1-14. https://doi.org/10.1007/s11032-019-1006-9.

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, *19*(1), 11-15.

Ellegren, H. (2004). Microsatellites: Simple sequences with complex evolution. *Nature Reviews Genetics, 5*(6), 435-445.

Lee, C. T., Norlia, B., Tnah, L. H., Lee, S. L., Ng, C. H., Ng, K. K. S., Nor-Hasnida, H., Nurul-Farhanah, Z., & Suryani, C. S., & Nur-Nabilah, A. (2018). Isolation and characterisation of SSR markers in tongkat ali (*Eurycoma longifolia*) using next-generation sequencing approach. *Journal of Tropical Forest Science, 30*(3): 279–291.

Mueller, U. G., & Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution, 14*(10), 389-394.

Ngoria, S., Kansup, J., Ruangwised, B., Chanroj, W., Amawan, S., & Wongtiem, P. (2019). The study of genetic diversity of cassava (*Manihot esculenta*) using SSR markers. *Thai Agricultural Research Journal*, *37*(1), 2-13. (in Thai)

Oliya, B. K., Maharjan, L., & Pant, B. (2023). Genetic diversity and population structure analysis of *Paris polyphylla* Sm. revealed by SSR marker. *Heliyon, 9*(7), e18230. https://doi.org/10.1016/j.heliyon. 2023.e18230

Pimpa, O., Khwangkaew, S., Pimpa, B., Khamseekhiew, B., & Pastsart, U. (2019). Effect of herbal fermented juice supplementation on intake and digestibility of Wagyu crossbred cattle. *Khon Kaen Agricutlure Journal, 47* Suppl(1), 127-130. (in Thai)

Rehman, S. U., Choe, K. & Yoo, H. H. (2016). Review on a traditional herbal medicine, *Eurycoma longifolia* Jack. (Tongkat Ali): Its traditional uses, chemistry, evidence- based pharmacology and toxicology. *Journal Molecules, 21*(331), 1-31.

Safeena, M. I. S., Dissanayake, Y., Zakeel, M. C. M., Warnakula, L., Cooray, R., & Dayarathna, D. A. R. K. (2021). An improved method for efficient recovery of high quality DNA from date palm (*Phoenix dactylifera* L; Arecaceae). *MethodsX, 8*, 101384. https://doi.org/10.1016/j.mex.2021.101384

Sathitbut, V., Keeratinijakal, V., & Peyachoknagul, S. (2016). Genetic diversity of *Eurycoma longifolia J*ack in Thailand using AFLP markers. In *Proceedings of the 54th Kasetsart University Annual Conference*, February 2-5, 2016. Bangkok: Kasetsart University. (in Thai)

Singh, N., Choudhury, D.R., Tiwari, G., Singh, A.K., Kumar, S., Srinivasan, K., Tyagi, R.K., Sharma, A.D., Singh, N.K. and Singh, R. (2016). Genetic diversity trend in Indian rice varieties: an analysis using SSR markers. *BMC Genetics,* 17(127).

Somwong, B., Kanjanasopa, D., Ninwichian, P., Thitithanakul, S., Onsanit, S., & Sontikun, Y. (2016). DNA extraction of *Nepenthes mirabilis*. *Songklanakarin Journal of Plant Science, 3*(2), 28-33. (in Thai).

Tnah, L. H., Lee, C. T., Lee, S. L., Ng, K. K. S., Ng, C. H., & Hwang, S. S. (2011). Microsatellite markers of an important medicinal plant, *Eurycoma longifolia* (Simaroubaceae), for DNA profiling. *American Journal of Botany*, 98(5), e130-e132. https://doi.org/10.3732/ajb.1000469

Varshney, R. K., Graner, A., & Sorrells, M. E. (2005). Genic microsatellite markers in plants: Features and applications. *Trends in Biotechnology, 23*(1), 48-55.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., ... & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research, 23*(21), 4407-4414.

Wang, J., Chen, Z., Jin, S., Hu, Z., Y., Huang and Y. Diao. 2017. Development and characterization of simple sequence repeat (SSR) markers based on a full-length cDNA library of Napier Grass (*Pennisetum purpureum* Schum). *Gene Genomic*, *39*(3), https://doi.org/10.1007/s13258-017-0536-5

Wizneh, F. M. and Asmawi, M. Z. (2014). *Eurycoma longifolia* Jack (Simarubaceae); Advances in Its Medicinal Potentials. *PHCOG J. 6*, 1-98. https://doi.org/10.5530/pj.2014.6.1

Yin, J., Zhao, H., Wu, X., Ma, Y., Zhang, J., Li, Y., Shao, G., Chen, H., Han, R., & Xu, Z. (2023). SSR marker based analysis for identification and of genetic diversity of non-heading Chinese cabbage varieties. *Frontiers in Plant Science, 14*, 1112748. https://doi.org/10.3389/fpls.2023.1112748