GENIC MICROSATELLITE LOCI OF A CRITICALLY ENDANGERED DIPTEROCARP, DIPTEROCARPUS CORNUTUS (KERUING GOMBANG), DERIVED FROM TRANSCRIPTOME SEQUENCES

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ABSTRACT

Dipterocarpus cornutus is an important timber tree in Southeast Asia. Population decline of the species due to habitat loss and conversion has threatened its existence and it is listed as Critically Endangered in the IUCN Red List. To aid in the assessment of the species' genetic diversity, we developed microsatellite loci mined from transcriptome sequence data. Specific microsatellite primer pairs obtained after agarose electrophoresis screening were further evaluated for polymorphism through fragment analysis using 24 wild individuals from Pasoh Forest Reserve, Negeri Sembilan. Nineteen loci were isolated for D. cornutus. These loci showed specific amplification and are polymorphic (DcoT01, DcoT02, DcoT03, DcoT04, DcoT06, DcoT14, DcoT22, DcoT25, DcoT26, DcoT27, DcoT28, DcoT31, DcoT32, DcoT35, DcoT36, DcoT44, DcoT45, DcoT59 and DcoT60). The number of alleles ranged from 2 to 19, and the observed and expected heterozygosity ranged from 0.208 to 1.000 and from 0.191 to 0.948,

respectively. Null allele was detected at two loci, namely DcoT06 and DcoT26. Excluding these two loci, we have successfully developed 17 microsatellite loci in D. cornutus, readily available to be applied for its conservation genetics.

Keywords: *Simple sequence repeat, tropical timber, next generation sequencing, conservation genetics, timber tracking.*

INTRODUCTION

Dipterocarpus cornutus Dyer, locally known as Keruing Gombang belongs to the family Dipterocarpaceae. The species is widely distributed across Peninsular Thailand, Peninsular Malaysia, Singapore, Sumatra and Kalimantan (Symington, 1943; Yong et al., 2021). In Malaysia, the species occurs in lowland to hill dipterocarp forests up to 1000 m altitude (Choo et al., 2001). As a valuable timber tree, *D. cornutus* is traded under the name *Keruing* and is suitable for heavy construction, flooring and poles (Choo et al., 2001). Currently, the species is categorized as critically endangered in the IUCN Red List of Threatened Species (Ashton, 1998). Decline in the species' extent of occurrence due to habitat loss and conversion has threatened the species existence and may affect viability of its populations (Ashton, 1998). Despite its high demand and threat, few genetic studies have been conducted on *D. cornutus*.

Microsatellite or simple sequence repeat (SSR) markers have been widely used as a powerful tool in studies of population genetics, genetic structure and molecular breeding (Wickneswari & Ho, 2003; Yoichi et al., 2016). Its characteristics of being highly polymorphic, highly reproducible and co-dominant make microsatellites the marker of choice among other molecular markers (Wang et al., 2018). To aid in genetic diversity assessment, we developed microsatellite loci based on transcriptome sequence of *D. cornutus* obtained via Illumina paired-end sequencing. The use of next generation sequencing (NGS) technology for marker discovery is cost and time efficient compared with conventional methods involving cloning (Chen et al., 2014; Zalapa et al., 2012).

METHODOLOGY

Sample collection and DNA extraction

Leaf materials of 24 wild individuals of *D. cornutus* were collected from Pasoh Forest Reserve, Negeri Sembilan. Total genomic DNA was extracted from approximately 5g of leaf tissues from each individual using a modified CTAB method (Murray & Thompson, 1980) and further purified using High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany). The quantity and quality of the DNA were assessed using electrophoresis and Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

RNA extraction and transcriptome sequencing

Total RNA from fresh leaves of *D. cornutus* was extracted using RNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's manual. The quality of the RNA was checked using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and quantified using Qubit 2.0 (Thermo Fisher Scientific, USA). The RNA integrity was assessed with a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Library preparation and transcriptome sequencing with Illumina NovaSeq 6000 were outsourced to Novogene Co. Ltd.

Microsatellite identification and marker development

The quality of paired-end reads was assessed using FastQC (Andrews, 2010). A pre-processing analysis to discard low quality sequences using Trimmomatic v0.32 (Bolger et al., 2014) was executed. *De novo* transcriptome assembly of the cleaned paired-end reads was performed using Trinity RNA-Seq v2.8.6 (Grabherr et al., 2011). Microsatellite-containing sequences were mined using MIcroSAtellite identification tool (MISA) (Thiel et al., 2003), with the minimum number of repeats set at 10 for mono-nucleotide repeats, six for di-nucleotide motifs, and five for tri-, tetra-, penta- and hexa-nucleotide repeats. Primers were designed using Primer 3 (Rozen & Skaletsky, 2000) via an in-house script. Selection of primers was based on the criteria: primer length of 18 to 24 bases, GC content of 40 to 60% and the expected PCR product sizes of 100 to 380 bp.

Microsatellite screening and validation

Sixty pairs of microsatellite primers were selected based on the criteria mentioned and screened on four *D. cornutus* individuals via PCR amplification and electrophoresis at 2% agarose. The product size was estimated using 100-bp DNA ladder (New England Biolabs). Successful primer pairs that produce clear single band were selected for 5'-fluorescent labelling of either HEX (green) or 6-FAM (blue) at the forward primers. The labelled primers were further screened for polymorphisms on 24 *D. cornutus* individuals from Pasoh Forest Reserve, Negeri Sembilan. The PCR amplifications were conducted in a 10 µl reaction mixture containing 1 x GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.3 µM forward and 0.3 µM reverse primers, 0.2 mM of each dNTP, 0.5 U of GoTaq Flexi DNA polymerase (Promega Corporation) and 10 ng of genomic DNA. The PCR was run in SimpliAmp Thermal Cycler (Applied Biosystems) for an initial denaturing step at 94 °C for 4 min, 40 cycles of 94 °C for 1 min, 55 °C annealing temperature for 30 s and 72 °C for 40 s, followed by final extension of 72 °C for 30 min. The PCR products were subjected to fragment analysis using an ABI 3500xL Genetic Analyzer (Applied Biosystems, USA) with ROX 400 as an internal size standard. Genotyping was performed using GeneMarker v2.6.4 (SoftGenetics, 2010).

Evaluation of microsatellite markers

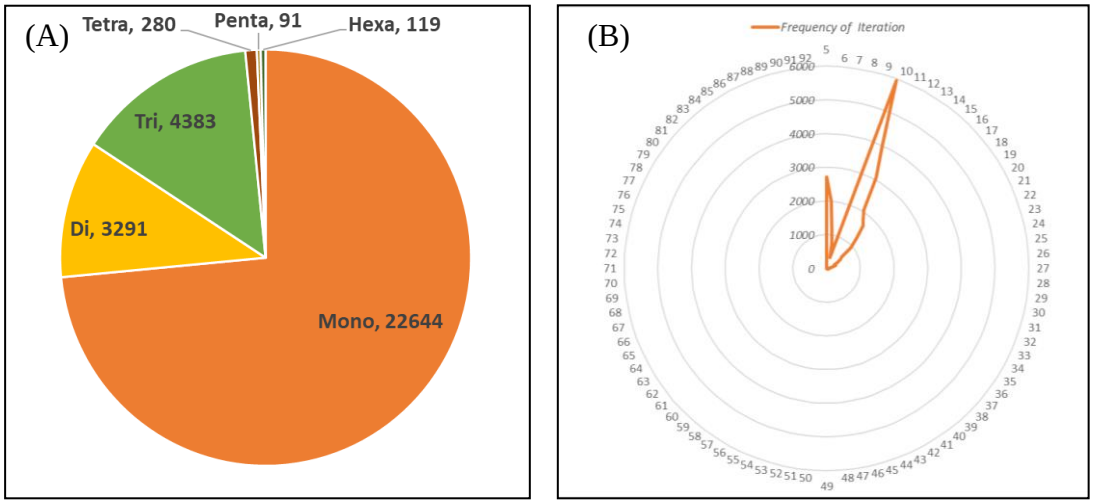
The 24 *D. cornutus* samples were used for microsatellite markers evaluation. Analysis of data was performed using Microsatellite Toolkit (Park, 2008). Parameters calculated include number of alleles (*A*), observed (H_o) and expected heterozygosity (H_e) as well as polymorphic information content (PIC). MICROCHECKER v2.2.3 (van Oosterhout et al., 2004) was used to check for scoring errors caused by stutters or large-allele dropouts and to estimate null-allele frequencies.

RESULTS AND DISCUSSION

Microsatellite distribution in transcriptome sequences of *D. cornutus*

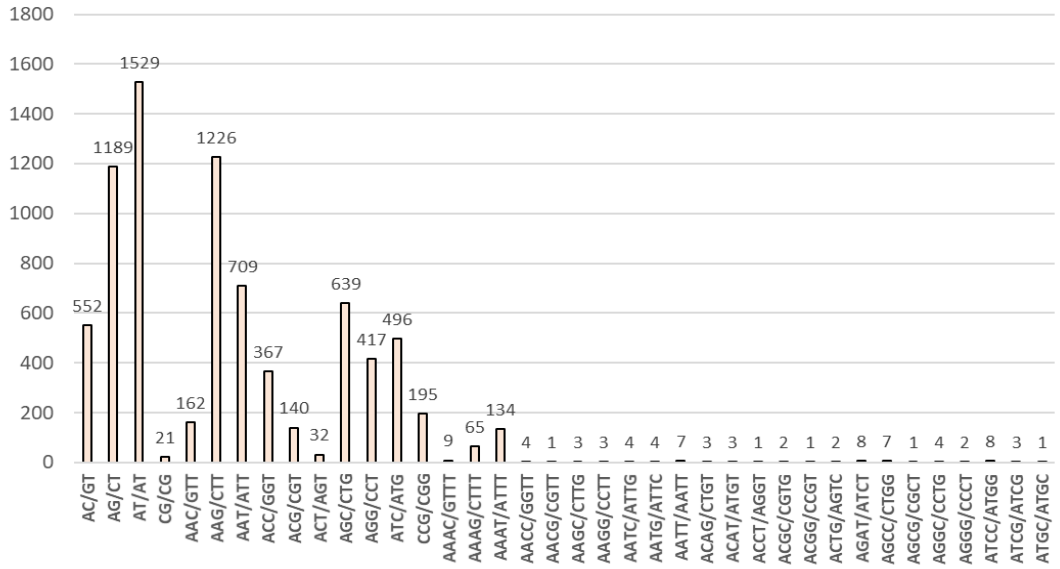
The quality of *D. cornutus* transcriptome sequences was assessed using FastQC and 'trimmed' via Trimmomatic v0.32. A total of 29,521,672 clean paired-end reads were obtained. The clean reads were *de novo* assembled into 161,656 contigs and were used for mining potential microsatellite motifs. From the total number of sequences examined, 30,808 microsatellites were identified with mononucleotide motifs being the most enriched (22,644; 73.5%), followed by trinucleotides (4,383; 14.23%) and dinucleotides (3,291; 10.68%). When excluding mononucleotides, trinucleotides are the most abundant among the repeat type. Studies in plants such as *Brassica rapa (*Zheng et al., 2016), *Chrysanthemum indicum (*Han et al., 2018) and *Pseudostellaria heterophylla* (Xu et al., 2023) similarly observed trinucleotide repeats as predominant sequence repeat. In EST-derived microsatellites, such observation is considered common, as changes in trinucleotide repeat number will not cause frameshift mutations (Fan et al., 2019). In contrast, repeat motifs of tetra- (280; 0.91%), hexa- (119; 0.39%) and penta- (91; 0.3%) have low frequency of less than 1%, respectively (**Figure 1A**). From the generated data, the number of iterations of a given repeat motifs varied from 5 to 92 (the minimum limit set for microsatellite marker discovery was 5). Iterations with the highest frequency was 10 (5,948; 19.31%) followed by 11 (3,961; 12.86%), 12 (3,066; 9.95%) and 5 (2,723; 8.84%) iterations (**Figure 1B**). Repeat motifs with more than 20 iterations were infrequent with occurrence rate of < 1%.

Figure 1. Microsatellites distribution in transcriptome sequences of *D. cornutus*. (A) Distribution of mono-, di-, tri-, tetra-, penta- & hexa-nucleotides; (B) Frequency of iteration in repeat motifs.



A total of 38 different types of motifs were identified which belonged to four types of dinucleotide repeats, 10 types of trinucleotides and 24 types of tetranucleotide repeats (**Figure 2**). The most common type of di-repeat was AT/AT (1529), which accounted for 46.5% of the repeats, followed by AG/CT (1189; 36.1%), AC/GT (552; 16.8%) and CG/CG (21; 0.6%). For the trinucleotide motifs, AAG/CTT motif (1226; 28%) was the most abundant tri-repeats followed by AAT/ATT (709; 16.2%), AGC/CTG (639; 14.6%) and ATC/ATG (496; 11.3%). Among tetra-repeats, AAAT/ATTT was the most abundant (134; 47.9%) followed by AAAG/CTTT (65; 23.2%). The dominant trinucleotide repeat AAG/CTT motif was consistent with studies on *Dipteronia* (Zhou et al., 2016) and rubber tree (Li et al., 2012). For most species, dinucleotide repeat motif of AG/CT was the most abundant (Triwitayakorn et al., 2011; Alias et al., 2021).

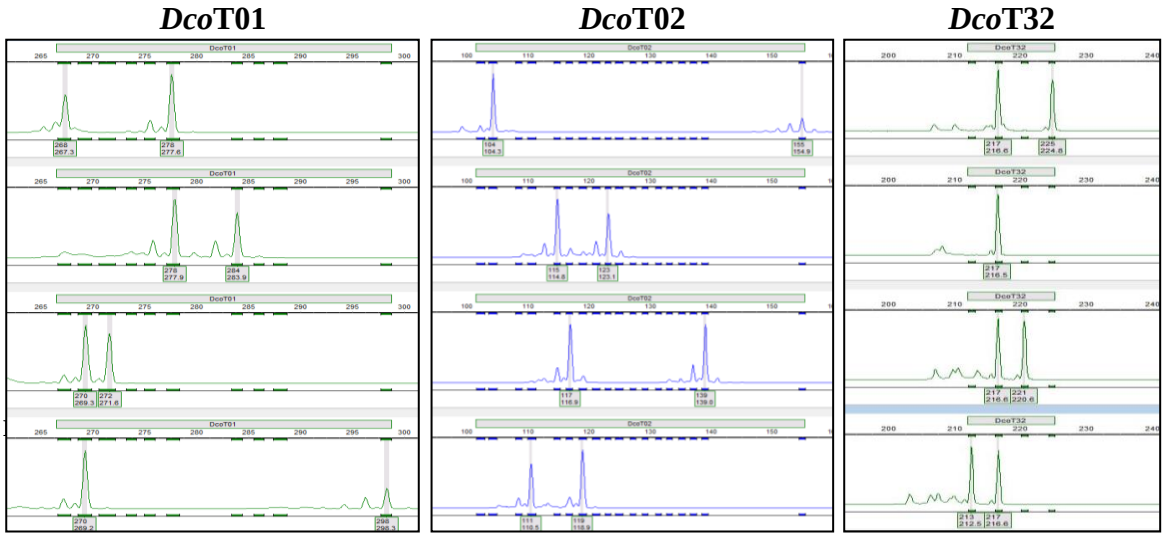
Figure 2. Distribution of microsatellite repeat motifs in *D. cornutus* comprising of di-, tri- and tetranucleotides with sequence complementary.



Screening of microsatellite primers

Sixty pairs of microsatellite primers with 15 di-, 15 tri- and 30 tetranucleotides were selected for screening. Mononucleotides and compound microsatellites were excluded from selection. Out of 60, 43 primer pairs (71.7%) showed specific-single band from electrophoresis screening, whereas 17 primer pairs were excluded from further analysis due to multiple bands or low amplification. For fragment analysis, 21 out of 43 primer pairs yielded consistent and scorable genotypes. Two loci were identified as monomorphic (*Dco*T39 and *Dco*T57). Nineteen loci showed specific amplification and are polymorphic (*Dco*T01, *Dco*T02, *Dco*T03, *Dco*T04, *Dco*T06, *Dco*T14, *Dco*T22, *Dco*T25, *Dco*T26, *Dco*T27, *Dco*T28, *Dco*T31, *Dco*T32, *Dco*T35, *Dco*T36, *Dco*T44, *Dco*T45, *Dco*T59 and *Dco*T60). **Figure 3** illustrates the electropherograms of three polymorphic loci.

Figure 3. Electropherograms of *D. cornutus* polymorphic loci for *Dco*T01, *Dco*T02 and *Dco*T32.



The genetic diversity parameters of the 19 polymorphic microsatellite loci based on 24 wild samples from Pasoh Forest Reserve, Negeri Sembilan are shown in **Table 1**. A total of 145 alleles were observed. The mean number of alleles are 7.6, ranging from 2 (*Dco*T31) to 19 (*Dco*T03). The mean observed (H_o) and expected heterozygosity (H_e) are 0.618 and 0.658, ranging from 0.208 (*Dco*T31 & *Dco*T60) to 1.000 (*Dco*T02 & *Dco*T28) and from 0.191 (*Dco*T31) to 0.948 (*Dco*T03), respectively. The population exhibited moderately high level of genetic diversity and was comparable with other tropical trees such as the lowland *Aquilaria malaccensis* (0.545, Tnah et al. 2012) and upper hill dipterocarp, *Shorea platyclados* (0.680, Ng et al. 2019). Polymorphic information content (PIC) ranged from 0.169 (*Dco*T31) to 0.923 (*Dco*T03) with an average of 0.612. According to Botstein et al. (1980), PIC value greater than 0.5 generally indicates high polymorphism, whereas a PIC of 0.25 to 0.5 considered as moderate, and a PIC of less than 0.25 indicate as low polymorphism. In this study, 13 out of 19 loci (68.4%) displayed PIC value greater than 0.5, whereas four loci (21.1%) exhibited moderate polymorphism (*Dco*T25, *Dco*T32, *Dco*T35 & *Dco*T45). The remaining two loci (*Dco*T31 & *Dco*T60) showed low polymorphisms. Mutation at the priming site usually results in null allele. Null allele was detected at two loci, namely *Dco*T06 and *Dco*T26, by indication of excess homozygosity. Excluding these two loci, 17 transcriptomic microsatellite loci were successfully developed.

Table 1. Genetic diversity parameters for 19 polymorphic microsatellites in D. cornutus. Number of alleles (A), observed (HO) and expected heterozygosity (HE) and polymorphic information content (PIC) were estimated based on 24 individuals. *Sign of null allele by indication of excess homozygote at loci DcoT06 & DcoT26.

Locus	Repeat motif	Size range (bp)	*A*	H_O	H_E	PIC
*Dco*T01	$(TG)_{22}$	268-298	10	0.917	0.842	0.807
DcoT02	$(AG)_{16}$	102-155	18	1.000	0.944	0.920
*Dco*T03	$(AG)_{23}$	105-152	19	0.917	0.948	0.923
*Dco*T04	$(AG)_{14}$	332-379	6	0.792	0.744	0.681
*Dco*T06	$(CA)_{15}$	333-378	11	0.583*	0.807	0.767
*Dco*T14	$(TC)_{19}$	114-166	18	0.833	0.934	0.909
*Dco*T22	$(CTC)_{11}$	123-141	6	0.542	0.651	0.594
*Dco*T25	$(ATA)_{12}$	163-173	6	0.458	0.430	0.402
*Dco*T26	$(AAC)_{11}$	205-214	4	0.318*	0.665	0.595
*Dco*T27	$(GAA)_{11}$	305-311	5	0.667	0.706	0.644
*Dco*T28	$(TGA)_{11}$	281-302	8	1.000	0.851	0.812
*Dco*T31	$(TTAT)_5$	229-233	2	0.208	0.191	0.169
*Dco*T32	$(TTAT)_5$	213-225	4	0.417	0.568	0.459
*Dco*T35	$(TTTA)_8$	320-344	4	0.583	0.499	0.452
*Dco*T36	$(AAGA)_5$	281-287	5	0.750	0.748	0.690
*Dco*T44	$(ACAG)_7$	137-161	7	0.583	0.749	0.697
*Dco*T45	$(TATT)_5$	201-213	4	0.292	0.409	0.358
*Dco*T59	$(TTTG)_5$	266-282	5	0.667	0.629	0.570
*Dco*T60	$(CAGC)_5$	176-188	3	0.208	0.194	0.178
		Mean	7.6	0.618	0.658	0.612

CONCLUSION

To the best of our knowledge, this is the first report on the development of genic-microsatellites in *D. cornutus*. The 17 polymorphic microsatellite markers are useful tool to estimate the species' genetic diversity and population genetic structure, and to provide important genetic information to the forest managers in formulating *in situ* and *ex situ* conservation programmes of *D. cornutus* and related species. In order to safeguard the evolutionary viability and sustainability of the species, genetic aspects should not be neglected.

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REFERENCES

Alias, N. N., Lee, C. T., Zakaria, N. F., Lee, S. L., Ng, K. K. S., Ng, C. H., Tnah, L. H. & Norlia, B. (2021). Development of genic SSR markers from transcriptome sequences of *Baeckea frutescens* (Cucur Atap) for genetic diversity assessment. *Journal of Tropical Plant Physiology*, 13, 15-25.

Andrews, S. (2010). *FastQC: a quality control tool for high throughput sequence data.* Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc [Accessed on 7 January 2022].

Ashton, P. (1998). *Dipterocarpus cornutus*. The IUCN Red List of Threatened Species 1998: e.T33072A9747216. http://dx.doi.org/10.2305/IUCN.UK.1998.RLTS.T33072A9747216.en [Accessed on 27 March 2023].

Bolger, A. M., Lohse, M. & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114-2120.

Botstein, D., White, R. L., Skolnick, M. & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32(3), 314–331.

Chen, C. M., Sio, C. P., Lu, Y. L., Chang, H. T., Hu, C. H. & Pai, T. W. (2014). Identification of conserved and polymorphic STRs for personal genomes. *BMC Genomics*, 15 (suppl 10), S3.

Choo, K. T., Lim, S. C. & Gan, K. S. (2001). Timber Technology Bulletin. *Timber Notes – Medium Hardwoods II*. No.19.2001. Kuala Lumpur: Timber Technology Centre, FRIM. ISSN: 139-258.

Fan, M., Gao, Y., Gao, Y., Wu, Z., Liu, H., & Zhang, Q. (2019). Characterization and development of EST-SSR markers from transcriptome sequences of Chrysanthemum (*Chrysanthemum ×morifolium* Ramat.), *HortScience horts* 54(5), 772-778.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I. & Regev, A. (2011). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nature Biotechnology*, 29(7), 644-652.

Han, Z., Ma, X., Wei, M., Zhao, T., Zhan, R., & Chen, W. (2018). SSR marker development and intraspecific genetic divergence exploration of *Chrysanthemum indicum* based on transcriptome analysis. *BMC Genomics*, 19: 291.

Li, D. J., Deng, Z., Qin, B., Liu, X. H. & Men, Z. H. (2012). De novo assembly and characterization of bark transcriptome using Illumina sequencing and development of EST-SSR markers in rubber tree (*Hevea brasiliensis* Muell. Arg.). *BMC Genomics*, 13: 192.

Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight DNA. *Nucleic Acids Research*, 8, 4321-4325.

Ng, C. H., Lee, S. L., Tnah, L. H., Ng, K. K. S., Lee, C. T., Diway, B. & Khoo, E. (2019). Genetic diversity and demographic history of an upper hill Dipterocarp *(Shorea platyclados*): implications for conservation. *Journal of Heredity*, 1–13.

Park, S. D. E. (2008). *Excel Microsatellite Toolkit. Computer program and documentation distributed by the author.* Available at: http://animalgenomics.ucd.ie/sdepark/ms-toolkit/ [Accessed on 10 November 2022].

Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Misener, S. & Krawetz, S. A. (Eds.) *Bioinformatics Methods and Protocols. Methods in Molecular Biology™, vol 132.* New Jersey, Humana Press.

Symington, C. F. (1943). *Foresters' manual of dipterocarps*. Malayan Forester Records 16. Kuala Lumpur, University of Malaya Press.

Thiel, T., Michalek, W., Varshney, R. K. & Graner, A. (2003). Exploiting EST databases for the development and characterisation of gene-derived SSR markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics*, 106, 411-422.

Tnah, L. H., Lee, C. T., Lee, S. L., Ng, K. K. S., Ng, C. H., Nurul-Farhanah, Z, Lau, K. H. & Chua L. S. L. (2012). Isolation and characterization of microsatellite markers for an important tropical tree, *Aquilaria malaccensis* (Thymelaeaceae). *American Journal of Botany*, e1-e3.

Triwitayakorn, K. Chatkulkawin, P. Kanjanawattanawong, S. Sraphet, S. Yoocha, T. Sangsrakru, D. Chanprasert, J. Ngamphiw, C. Jomchai, N. Therawattanasuk, K. & Tangphatsornruang, S. (2011). Transcriptome Sequencing of *Hevea brasiliensis* for development of microsatellite markers and construction of a genetic linkage map. *DNA Research*, 18(6), 471–482.

van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shipley P. (2004). Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4, 535-538.

Wang, J., Ge, Q., Li, J., Gao, H., Li, J. & Zhao, F. (2018). Identification of novel EST-SSR markers by transcriptome sequencing in ridgetail white prawn *Exopalaemon carinicauda*. *Genes & Genomics*, 40, 207-215.

Wickneswari, R. & Ho, S. (2003). Determination of genetic relatedness of selected individual trees of *Shorea leprosula Miq.* and *Dipterocarpus cornutus* Dyer in forest seed production areas. *Tropics*, 13 (2), 139-149.

Xu, L., Li, P., Su, J., Wang, D., Kuang, Y., Ye, Z. & Chen, M. (2023). EST-SSR development and genetic diversity in the medicinal plant *Pseudostellaria heterophylla* (Miq.) Pax. *Journal of Applied Research on Medicinal and Aromatic Plants*, 23: 100450.

Yoichi, W., Sakaguchi, S., Ueno, S., Tomaru, N. & Uehara, K. (2016). Development and characterization of EST-SSR markers for the genus *Rhododendron* section Brachycalyx (Ericaceae). *Plant Species Biology*, 32(4), 455-459.

Yong, W.S.Y., Chua, L.S.L., Lau, K.H., Siti-Nur Fatinah, K., Cheah, Y.H., Yao, T.L., Rafidah, A.R., Lim, C.L., Syahida-Emiza, S., Ummul-Nazrah, A.R., Nor-Ezzawanis, A.T., Chew, M.Y., Siti-Munirah, M.Y., Julius, A., Phoon, S.N., Sam, Y.Y., Nadiah, I., Ong, P.T., Sarah-Nabila, R., Suhaida, M., Muhammad-Alif Azyraf, A., Siti-Eryani, S., Yap, J.W., Jutta, M., Syazwani, A., Norzielawati, S., Kiew, R. & Chung, R.C.K. (2021). *Malaysia Red List: Plants of Peninsular Malaysia*. Vol. 1, Part II. Research Pamphlet No. 151. Kepong, Forest Research Institute Malaysia.

Zalapa, J. E., Cuevas, H., Zhu, H., Steffan, S., Senalik, D., Zeldin, E., McCown, B., Harbut, R. & Simon, P. (2012). Using next-generation sequencing approaches to isolate simple sequence repeat (SSRs) loci in the plant sciences. *American Journal of Botany*, 99, 193-208.

Zheng, J. S., Sun, C. Z., Zhang, S. N., Hou, X. L., & Bonnema, G. (2016). Cytogenetic diversity of simple sequences repeats in morphotypes of *Brassica rapa* ssp. *chinensis*. *Frontiers in Plant Science*, 7:1049.

Zhou, T., Li, Z. H., Bai, G. Q., Feng, L., Chen, C., Wei, Y., Chang, Y. X., & Zhao, G. F. (2016). Transcriptome sequencing and development of genic SSR markers of an endangered Chinese endemic genus *Dipteronia* Oliver (*Aceraceae*). *Molecules*, 21(3): 166.