International Journal of Agricultural Technology 2024 Vol. 20(4):1463-1482
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Cross amplification of SSR markers to selected Philippine native wax plants (*Hoya* R. Br.) species

Lorico, C. H.[1], Almontero, C. C.[1*], Lalusin, A. G.[1], Gentallan Jr., R. P.[1] and Guevarra, M. L. D.[2]

[1]Institute of Crop Science University of the Philippines Los Baños, College, Laguna, 4031 Philippines; [2]Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna, 4031 Philippines.

Lorico, C. H., Almontero, C. C., Lalusin, A. G., Gentallan Jr., R. P. and Guevarra, M. L. D. (2024). Cross amplification of SSR markers to selected Philippine native wax plants (*Hoya* R. Br.) species. International Journal of Agricultural Technology 20(4):1463-1482.

Abstract Out of 23 Orchidaceae SSR markers, 16 primer pairs were amplified to the 21 accessions of *Hoya* species. These SSR markers elucidated the genetic variations of the samples using different genetic indices such as expected heterozygosity (He), Shannon diversity index (I), and polymorphic information content (PIC). The marker informativeness was further evaluated using fixation index and gene flow, which resulted in moderate to very strong genetic differentiation and medium to high gene flow, respectively. The suggested markers for each *Hoya* species are C 268, PA 10, and AKE 4 for *H. benguetensis*, C 268, IPS 10, PAP 1520, and AKE 6 for *H. soligamiana*, and IPS 10, PAP 1520, AKE 4, and AKE 12 for *H. benvergarai.* Moreover, a UPGMA tree was generated using the Dice dissimilarity coefficient, which grouped the accessions into three clusters. Clustering showed *H. soligamiana* species in one group, while the remaining species were distributed to different clusters. Further studies could include more species of *Hoya* and microsatellite markers to reveal a greater extent of variations for applications such as genetic barcoding and marker-assisted selection.

Keywords: Primers, Genetic variations, Diversity

Introduction

Wax plants (*Hoya* sp. Apocynaceae) were first reported in the book of Robert Brown in 1810 about the Australian flora together with their respective plant characteristics (Brown, 1810). It is described as an epiphytic vine and sometimes shrub that grows on trees and occasionally in rocks in the subtropical montane regions (Zhao *et al.,* 2020). The Philippines is said to have a diverse collection and highest number of *Hoya* species, together with New Guinea (Wanntorp *et al.,* 2014). It has 202 native species of *Hoya* and five species that still need to be named, as listed by Pelser *et al*. (2022) in the Co's Digital Flora of the Philippines.

[*]**Corresponding Author**: Almontero, C.C.; **Email:** ccalmontero@up.edu.ph

According to Kloppenburg (1991), the floral parts of *Hoya* that showed phenotypic distinctions are peduncle, pedicel, calyx, corolla, corona, anther appendages, pollinaria, stigma, ovaries, and pods. Widiarsih *et al*. (2012) utilized vegetative and reproductive characters in the morphological characterization and genetic diversity assessment of 16 accessions of *Hoya*. However, recently, Baltazar and Buot (2019) have noted several circumstances of phenotypic plasticity in *Hoya* and have determined stable characters that could be used for phenotypic evaluation. Maranan (2011), on the other hand, utilized standard gene barcoding regions in plant, the maturase K gene (*matK*) and the 1,5-biphosphate carboxylase oxygenase large subunit gene (*rbcL*) in diversity analysis of gene sequences and to barcode six Philippine endemic *Hoya*s while Maranan and Diaz (2013) employed the same markers in the determination of molecular diversity of five *Hoya* species (*H. crassicaulis* Elmer x Kloppenburg*, H. madulidii* Kloppenburg*, H. pubicalyx* Merill*, H. siariae* Kloppenburg*,* and new species *H.* sp1). Widiarsih *et al*. (2014) made use of microsatellite markers to distinguish sixteen accessions of *H. mindorensis* Schlechter. On the other hand, Chen *et al*. (2016) designed transcriptome derived SSR markers for *H. ledongensis* and successfully amplified it in *H. jianfenglingensis*. These studies prove that molecular research on this crop has been insufficient in the past decade, and established markers are inadequate for a more thorough genetic study.

Orchidaceae, a diverse family of angiosperms similar to Apocynaceae*,* is a large group of monocots and epiphytes consisting of 440 epiphytic genera (Kress, 1986). The shared attributes of the Orchidaceae and Apocynaceae families in relation to evolution and development were studied by Endress (2016). He noted that both families converge in the formation of pollinia and pollinaria which is unique to them. Also, they have the most extreme flower synorganization, the integration of different organs into complex structure, which led to shared prominent features that conditions the synorganization or as a result. Some of these are stability of floral organ, highly regular floral symmetry, thick and firm consistency of floral organs, pollen aggregation to pollinia, pollinia organized to pollinaria, pollinaria with translator, and hidden stigma (Endress, 2016). Unlike *Hoya*, species belonging to Orchidaceae have established a great number of microsatellite markers.

In this study, the established Orchidaceae SSR and EST-SSR from the study of Almontero *et al.* (2022) as well as the polymorphic microsatellite markers from Widiarsih *et al.* (2014) were utilized to increase established *Hoya* markers, especially the accessions of *H. benguetensis, H. soligamiana,* and *H. benvergarai.* Specifically, the study aimed to identify amplifiable markers from the aforementioned studies and to determine the informativeness of the cross-

amplified markers for the initial elucidation of the genetic diversity of the accessions in the collection.

Materials and methods

The study was conducted last November 2022 to April 2023 at the Molecular Plant Breeding Laboratory of the Institute of Crop Science (ICropS), College of Agriculture and Food Science (CAFS), University of the Philippines Los Banos (UPLB), College, Los Banos, Laguna, Philippines. The plant materials were collected in the screenhouse of the Fruit, Ornamental, and Medicinal Crops Section of the Institute of Plant Breeding (IPB), CAFS, UPLB. The species of *Hoya benguetensis, H. soligamiana*, and *H. benvergarai* utilized in this study are presented in Table 1 together with their unique collecting number. The extraction process employed in this study was cetyltrimethylammonium bromide (CTAB) protocol with the findings of Widiarsih *et al.* (2011) which were minipreparation and omission of sodium dodecyl sulfate (SDS). The DNA quantification was done using a spectrophotometer and 1 % agarose gel electrophoresis.

Table 1. List of *Hoya* species collected in the IPB nursery together with their collecting number

ID	COLLECTING NUMBER	SPECIES/ VARIETY	PLACE COLLECTED FROM
A	2020-004	*H. benguetensis*	Los Baños, Laguna
B	2021-035	*H. benguetensis*	Los Baños, Laguna
C	2022-174	*H. benguetensis* Cagayan	Baggao, Cagayan
D	2022-173	*H. benguetensis* 'maruja'	Baggao, Cagayan
E	2022 - 003	*H. benguetensis*	Los Baños, Laguna
F	2022 - 056	*H. benguetensis*	San Juan, Batangas
G	2022-018	*H. soligamiana* yellow	Los Baños, Laguna
H	2022-013	*H. soligamiana* var	Los Baños, Laguna
I	2022-112	*H. soligamiana* (yellow)	Los Baños, Laguna
J	2022 - 060	*H. soligamiana* (Bukidnon)	San Juan, Batangas
K	2022-123	*H. benguetensis* Cgy 12	Baggao, Cagayan
L	2022-114	*H. benguetensis* Baggao #6	Baggao, Cagayan
M	2022-120	*H. benguetensis* (dark red)	Baggao, Cagayan
N	2022-170	*H. benguetensis* (orange pomelo)	Baggao, Cagayan
O	2022-127	*H. benguetensis*	Baggao, Cagayan
P	2022 - 082	*H. benvergarai*	Cagayan
Q	2022-094	*H. benguetensis*	
R	2022-157	*H. benguetensis*	Baggao, Cagayan
S	2022-179	*H. benvergarai*	Baggao, Cagayan
T	2022-145	*H. benguetensis* (kayumanggi)	Baggao, Cagayan
U	P1	*H. benguetensis* from Microgrow	

PCR amplification was performed using the nineteen microsatellite markers (Table 2) from the study of Almontero *et al.* (2022) together with the four primer pairs acquired from the study of Widiarsih *et al*. (2014). The products were separated using a 2% agarose gel with a reference 100bp DNA ladder. Primers with amplified PCR products were evaluated and considered as a successful amplification of markers from *Orchidaceae* to Hoya. It was then scored using GenAnalyzer. Different genetic diversity indices were calculated using Genetic Analysis in Excel (GenAlEx), Online Marker Efficiency Calculator (iMEC), and XLStat. The parameters were genotypic and allelic frequencies, number of different allele (Na), number of effective alleles (Ne), observed (Ho) and expected heterozygosity (He) per marker, Shannon Information Index (*I*), Fixation Index (*F* and F_{st}), Gene flow (*Nm*), and Polymorphic Information Content (PIC). For cluster analysis, a dendrogram was generated using the Dice dissimilarity coefficient and the Unweighted Pair Group Method Analysis (UPGMA) clustering.

Table 2. Profile of SSR markers utilized in determining the genetic diversity of the selected accessions of *Hoya*

Marker Name	Primer Sequence (5'→ 3')	Repeat Motif	GenBank Accession Number
C 32[a]	AATGGACCTTCTTTGCATTAC ATTACCGTTCATTTCTGGTGC	$(GT)_{40}(GA)_{27}$	FJ539050
C 208[a]	TCATTGATGTTGGGAGCCTAA CTTGCCCTCTATCTTTCTCTT	$(TA)_3(GT)_{42}$ $(GA)_{10}$	FJ539052
C 268[a]	TGGAAATGCATGTTGCCCGA ACTGAGTGACCTTGGAAGAC	$(GT)_{17}(GA)_{39}$	FJ539054
IPS 10[a]	AGAGAGAGAAAGAGAGAGATGC CTACGCCTGATTTGATTTCTA	$(AG)_7$	AJ566356
IPS 13[a]	GCTAGAGATAGAGAGAGAAAGAG CTACGGCTGATTTGATTTCTA	$(AG)_6$	AJ566353
IPS 52[a]	GCAATGGAGAAAAAGGATTTA GCTCCACTCACCTGTTAGTTA	$(TTG)_6$	AY378151
PA 10[a]	TCTTCAGTCCCTCACTCATC ACAAAGCGGTGGAGAATATG	$(CT)_{14}$	
PA 21[a]	TCTCTCACTTTGTCACTCGC AAAGGGAAGTAGGGAAGGAG	$(CT)_{14}$	
PA 24[a]	TTGATCTCTCTGGCACCCAC AAGAGAGAGTTAGTTGGAGAT	$(TC)_{36}$	
PA 32-1[a]	CTCTTCCTGCTTTTCCTAGG AAGAGGGTGTGAGGAAGAGG	$(CT)_{25}$	
PA 36[a]	CTCCACTTTATCTCTCTACC ATTGAGCGAGATAAAACTAG	$(TC)_{39}$	

Table 2. Profile of SSR markers utilized in determining the genetic diversity of the selected accessions of *Hoya.* (Continued)

Marker Name	Primer Sequence (5'→ 3')	Repeat Motif	GenBank Accession Number
PGA 06[a]	GTGAAAGACACACACACACACA GGTTGTACGCCTTTGTCGAT	$(CA)_{10}$	EF462862
PGA 16[a]	TGAACGAACACACACACACACA TTGGCCTTAAGGATAATACATCAA	$(AC)_{10}$	EF462863
PAP 1520[b]	ATCAGCCTTCATGATCTTCTT AACTCTACCACCATCAGCAG	$(GCT)_8$	
PAP 3222[b]	GAGTATTGAATCCCCAAGTTT TTCAGAATCATCTTTCTCCTG	$(GAG)_8$	
PAP 3268[b]	TAACTCGCCTTCTCGTCTTA TTTTTCCATTACTGTTTGATGA	$(AAC)_9$	
PAP 3754[b]	AGTCTGAAGCTTCTTCTTGCT CAATATAGAGGAGGAGCAGGT	$(TCC)_8$	
PAP 4282[b]	CTATGCTTCCCACAGAAACC CTGTGATCCACCATCCTTAC	$(AGA)_8$	
PAP 4825[b]	ACCAGCTTCTACATTTCCAAT AAGATCTTCATTGATCCTTTTG	$(AGA)_8$	
AKE 4[c]	CAGTTTCTTTGGATGGTG CAATCAGATAGGCAACGAG	$(CT)_{18}$	AY312442
AKE 5[c]	CGTAGACGATAGCCTTGATAGC CACTCCTGGATGCTTTCA	$(CT)_{16}$ … $(CA)_6(TA)_2$	AY312443
AKE 6[c]	CAGAGACAATGATAAGACCACAAT ATACCATGAAAAGGCTGCTC	$(CT)_{13}$ … $(CT)_2(CA)_9$	AY312444
AKE 12[c]	GTGTTTGGGTTTTAAGGAAGAA AAAGCCCAACTAAATATAACTAAT	$(GA)_{30}$	AY312450

Results

Marker amplification

Cross amplification of molecular markers has been utilized on a variety of crops to overcome the cost of development of primers that could delineate certain species. The study employed a total of 23 microsatellites and EST-SSRs previously applied to Orchidaceae and *Hoya* which resulted in varying percentages of amplifiable markers (Table 3) and their respective profiles (Table 4).

Table 3. Percent amplification of primers from Orchidaceae and *Hoya* to the selected accessions of Philippine *Hoya*

Source	Marker Type	Total Number of Markers	Percent Amplification (%)		
			H. benguetensis	*H. soligamiana*	*H. benvergarai*
Orchidaceae	SSR	13	69.23	69.23	61.54
	EST-SSR	6	50.00	50.00	50.00
Hoya	SSR	4	100.0	100.0	100.0
Average			**73.08**	**73.08**	**70.51**

Table 4. The marker profile of 16 successfully amplified SSR and EST-SSR primers with respect to the different species of *Hoya*

PRIMER	Published Ta (°C)	Optimized Ta (°C)	Expected Band Size (bp)	Observed Band Size (bp)	Number of alleles		
					S1	S2	S3
C 208	50.0	47.7	140-390	120-205	9	2	2
C 268	46.0	46.0	100-300	80-335	12	3	2
PA 10	56.9	56.9	160-300	325-820	13	3	1
PA 21	54.0	54.0	130-310	150-480	8	5	0
PA 32-1	54.0	54.0	160-180	435-760	4	3	1
PGA 06	51.0	47.0	160-330	300-1795	6	4	2
PGA 16	58.1	48.7	360	490-715	8	2	2
IPS 10	45.0	45.0	110-200	450-1340	6	5	3
IPS 52	48.1	48.1	110-450	330-490	9	2	1
PAP 1520	58.1	47.2	135-170	210-880	12	6	4
PAP 3222	55.0	55.0	155-180	45-180	6	3	1
PAP 3754	55.0	48.4	150-185	250-515	9	1	2
AKE 4	44.0	46.5	179	535-780	14	5	3
AKE 5	48.0	47.1	232	215-250	4	2	2
AKE 6	46.0	47.1	169	280-575	9	6	2
AKE 12	41.5	42.8	226	285-385	11	4	3

Ta, Annealing temperature; S1, *H. benguetensis*; S2, *H. soligamiana*; S3, *H. benvergarai.*

Cross-family amplification of SSR markers (69.23%) from Orchidaceae to *Hoya* was found to be higher as compared to EST-SSR primers (50.0%). The species under *H. benvergarai* failed to amplify 1 SSR marker (70.51%) which made its mean amplification percentage relatively lower than the other two species (73.08%). There are seven primers (C 268, PA 10, PA 21, PA 32-1, IPS 10, IPS 52, and PAP 3222) that readily amplified across the different accessions while the remaining nine had to be optimized. Moreover, the primer PA 21 was not able to cross-amplify to the 2 species of *H. benvergarai* causing the slight differences in amplification percentage (Table 3) of the species. The expected and observed band sizes was noted to have significant differences specifically in the cases of PA 10, PA 32-1, PGA 06, PGA 16, IPS 10, PAP 1520, PAP 3754, AKE 4, and AKE 6 where it did not satisfy the expected range of values. Four primers (PA 10, PA 32-1, IPS 52, PAP 3222) showed monomorphism of the 2 species belonging to *H. benvergarai* since the three markers (PA 10, IPS 52, PAP 3222) only amplified to one species under *H. benvergarai* and the SSR primer PA 32-1 generated one type of allele for both species. In the case of *H. soligamiana,* only one species was able to amplify the primer PAP 3754 hence, one type of allele was recorded. The number of alleles per species recorded has a range of 4 (PA 32-1) to 14 (AKE 4) for *H. benguetensis,* 1 (PAP 3754) to 6 (PAP 1520 and AKE 6) for *H. soligamiana*, and 1 (PA 10, PA 32-1, IPS 52, PAP 3222) to 4 (PAP 1520) for *H. benvergarai.* The mean allele per species is 8.75, 3.50, and 1.94 for *H. benguetensis, H. soligamiana,* and *H. benvergarai*, respectively. The electrophoretograms (Figure 1) of different primer pairs in 2% agarose gel electrophoresis (AGE) presented the differences in a banding pattern relative to the DNA ladder (100 bp.)

Microsatellite marker analysis

The ability of markers to delineate the collection was analyzed using a variety of diversity indices. Table 5 to Table 7 showed the summary of the different measures of diversity with respect to the markers and species utilized in this study which includes the expected (He) and observed (Ho) heterozygosity, Shannon diversity index (I), and polymorphic information content (PIC). Moreover, for every marker group, mean of each index are indicated for greater comparison between primer source and type. The distribution of alleles for each species of *Hoya* per marker was illustrated (Figure 2).

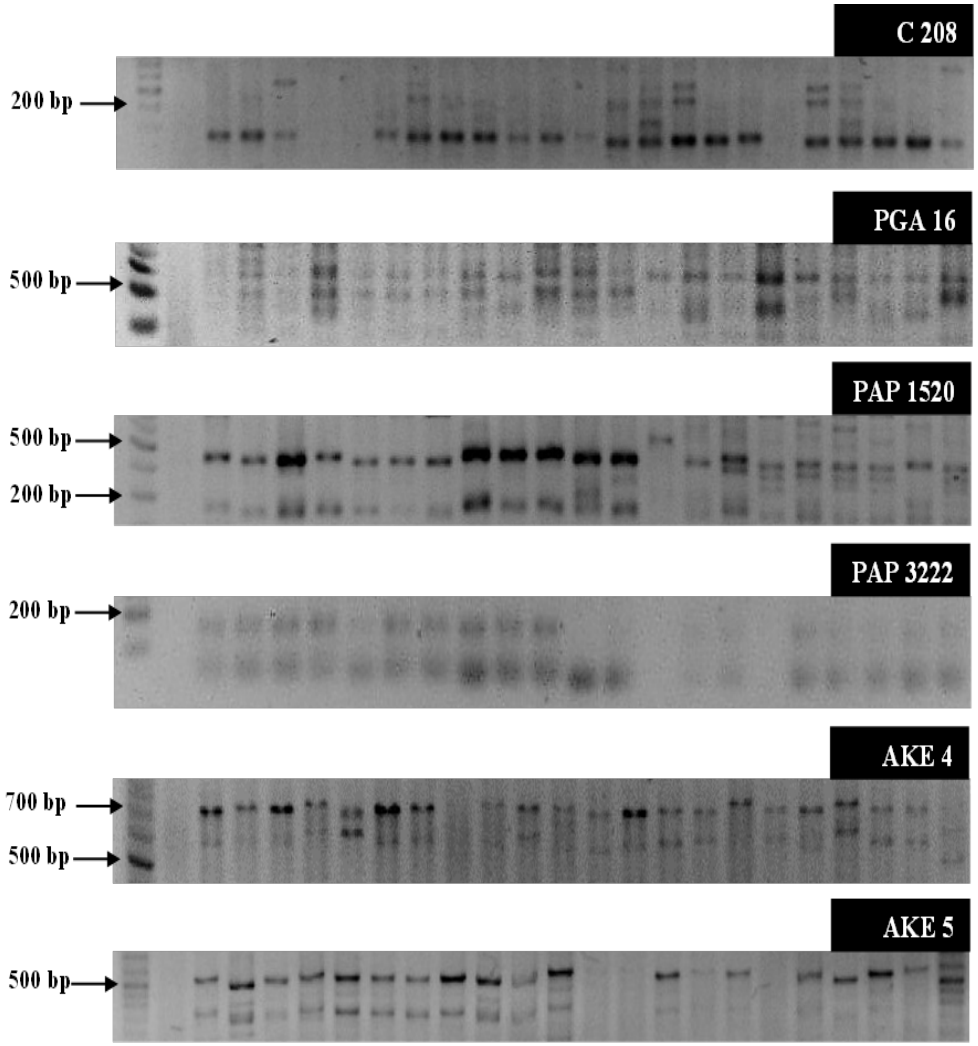

Figure 1. Representative electrophoretogram of 21 accessions of *Hoya* species with 100 bp ladder (Lane 1), control (Lane 2), and samples A- U (Lane 3- 23) for markers C 208, PGA 16, PAP 1520, PAP 3222, AKE 4, and AKE 5 in 2% AGE. The respective species of *Hoya* and their ID is in Table 1.

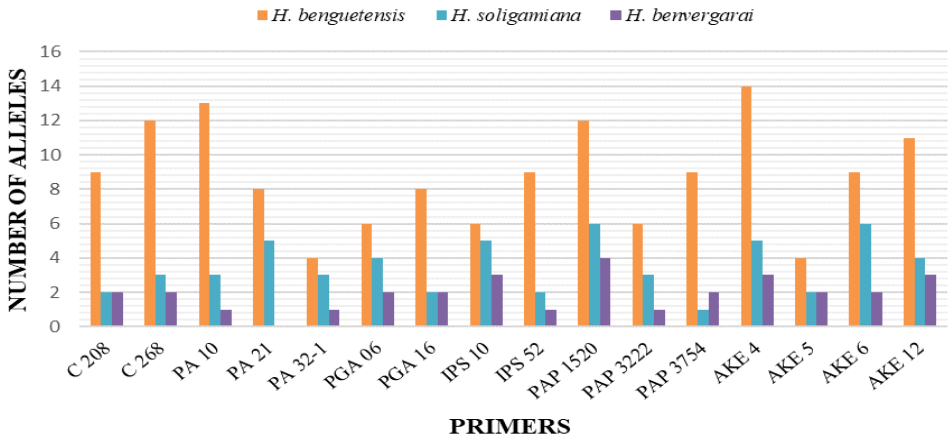

Figure 2. Distribution of alleles per marker of the 3 species of *Hoya* using 16 microsatellite and EST-SSR markers

Table 5. Diversity indices of the 16 primer pairs used in the amplification of *Hoya benguetensis* species

Markers	Ne	Ho	He	I	PIC
C 208	9.00	0.00	0.88	2.14	0.38
C 268	12.00	0.47	0.89	2.33	0.40
PA 10	13.00	0.42	0.91	2.46	0.38
PA 21	8.00	0.15	0.74	1.72	0.39
PA 32-1	4.00	0.00	0.65	1.17	0.35
PGA 06	6.00	0.31	0.77	1.59	0.37
PGA 16	8.00	0.00	0.83	1.94	0.38
IPS 10	6.00	0.30	0.80	1.67	0.34
IPS 52	9.00	0.09	0.87	2.12	0.37
Mean	8.33	0.19	0.82	1.90	0.37
PAP 1520	12.00	1.00	0.89	2.35	0.40
PAP 3222	6.00	0.62	0.73	1.50	0.36
PAP 3754	9.00	0.10	0.87	2.10	0.34
Mean	9.00	0.57	0.83	1.98	0.37
AKE 4	13.00	0.87	0.90	2.43	0.40
AKE 5	4.00	0.00	0.73	1.35	0.37
AKE 6	9.00	0.66	0.84	1.97	0.38
AKE 12	11.00	0.93	0.86	2.14	0.38
Mean	9.25	0.62	0.83	1.97	0.38

Ne, Number of Effective Allele; Ho, Observed Heterozygosity; He, Expected heterozygosity; I, Shannon Diversity Index.

The number of effective alleles for *H. benguetensis* ranges from 4 (PA 32-1 and AKE 5) to 13 (PA 10 and AKE 4) with a mean of 8.33, 9.00, and 9.25 for marker types SSR and EST-SSR from Orchidaceae, and microsatellite from *Hoya.* The observed heterozygosity was 0.00 (C 208, PA 32-1, AKE 5) which entails that no heterozygotes were present in this marker to 1.00 (PAP 1520), completely heterozygotes. The lowest mean observed heterozygosity (0.19) was found in SSR markers from Orchidaceae. As for the expected heterozygosity, the mean of each marker type was relatively the same and PA 10 (0.91) had the highest value for this parameter. Similar to expected heterozygosity, the values for the Shannon diversity index were relatively the same for the marker types ranging from 1.90 to 1.98. Species richness was detected highest using marker PA 10 (2.46) while lowest with primer PA 32-1 (1.17) but still in the accepted range of 1.5 to 3.5. All the markers are reasonably informative (0.50 > PIC > 0.25) with values ranging from 0.34 (PAP 3754 and IPS 10) to 0.40 (AKE 4 and C 268).

Table 6. Diversity indices of the 16 markers used in the amplification of *Hoya soligamiana* species

Markers		Ne	Ho	He	I	PIC
C208		2.00	0.00	0.38	0.56	0.42
C268		3.00	0.75	0.66	1.08	0.41
PA 10		3.00	0.33	0.50	0.87	0.38
PA 21		5.00	1.00	0.75	1.49	0.35
PA 32-1		3.00	0.00	0.63	1.04	0.37
PGA 06		4.00	0.50	0.66	1.21	0.37
PGA 16		2.00	0.00	0.50	0.69	0.34
IPS 10		5.00	0.50	0.75	1.49	0.41
IPS 52		2.00	0.00	0.44	0.64	0.38
	Mean	3.22	0.34	0.59	1.01	0.38
PAP 1520		6.00	1.00	0.81	1.73	0.41
PAP 3222		3.00	1.00	0.63	1.04	0.34
PAP 3754		1.00	0.00	0.00	0.00	0.38
	Mean	3.33	0.67	0.48	0.92	0.38
AKE 4		5.00	1.00	0.78	1.56	0.38
AKE 5		2.00	0.00	0.44	0.64	0.37
AKE 6		6.00	1.00	0.81	1.73	0.41
AKE 12		4.00	0.50	0.72	1.32	0.42
	Mean	4.25	0.63	0.69	1.31	0.40

Ne, Number of Effective Allele; Ho, Observed Heterozygosity; He, Expected heterozygosity; I, Shannon Diversity Index.

In comparison with the previous *Hoya* species (*H. benguetensis*), here, the effective number of alleles is significantly lower with values ranging from 1 (PAP 3754) to 6.00 (PAP 1520 and AKE 6). The microsatellite markers from *H.*

mindorensis Schleter were the highest with a mean value of 4.25. Complete homozygosity was accounted for C208, PA 32-1, PGA 16, IPS 52, PAP 3754, and AKE 5 by the parameter observed heterozygosity while all heterozygotes were detected by PA 21, PAP 1520, PAP 3222, AKE 4, and AKE 6. The genic SSR markers from Orchidaceae had the greatest mean (0.67) among the marker source and types however, this was not translated for expected heterozygosity since it only got a mean value of 0.48, the lowest among the three. The Shannon diversity index in this species of *Hoya* varied greatly. PAP 3754 (0.00) is the lowest due to non-amplification of the primer to the 3 accessions (75.0%) of *H. soligamiana.* On the other hand, PAP 1520 and AKE 6 had the highest value for *I* (1.73) which entails more species richness was elucidated. Comparison of marker types for Shannon showed that *H. mindorensis* primers have the highest index (1.31). The PIC values of the accessions of *H. soligamiana* with regards to the markers employed were also reasonably informative (0.50 > PIC > 0.25). The highest recorded PIC for these accessions were 0.42 (C208 and AKE 12) to 0.34 (PGA 16 and PAP 3222). The mean PIC per marker type was not significantly different from each other ranging from 0.38 (Orchidaceae primers) to 0.40 (markers from *Hoya*).

The *H. benvergarai* species had only two accessions unlike the two previously discussed species. The information of the two species collected were analysed using the diversity indices, the same procedure with the other species and the results were presented (Table 7). With that being said, the macros GenAlEx noted that results should be treated with caution since sample population size was significantly lower than the prescribed one (n>5). Hence, non-amplification would greatly affect the results of the analysis, especially in this case.

Average Ne ranges from 1.75 (Orchidaceae SSR) to 2.5 (*Hoya* SSR) with IPS 52 (1.00) having the lowest and PAP 1520 (4.00) containing the highest number of effective alleles. Eleven primers (73.33%) showed complete homozygosity between the two accessions of *H. benvergarai* with 3 markers having missing data (PA 10, IPS 52, PAP 3222) for one sample. Both the accessions were considered heterozygotes for PAP 1520 and AKE 12. A similar pattern was indicated for expected heterozygosity (0.00) for markers PA 10, IPS 52, and PAP 3222 since only one *H. benvergarai* accession was able to amplify while PA 32-1 had only one banding pattern. The highest expected heterozygosity was recorded in PAP 1520 (0.75) and *Hoya* primers having the greatest mean of 0.57. For Shannon diversity index, markers PA 10, PA 32-1, IPS 52, and PAP 3222 were not able to account for any species diversity (0.00). The same as expected heterozygosity, PAP 1520 had the highest index (1.37) and *H. mindorensis* Schleter markers had 0.87 mean value for *I,* significantly lower

than the previous species. The polymorphic information content was still in the reasonably informative range however it is substantially lower than the other two *Hoya* species. AKE 6 (0.35) had the maximum value while PA 10 and IPS 52 were on the minimum with a value of 0.25. The PIC values were not significantly different in terms of marker type.

Table 7. Diversity indices of the 16 SSR and EST-SSR primers employed in the amplification of *Hoya benvergarai* accessions

Markers	Ne	Ho	He	I	PIC
C208	2.00	0.00	0.50	0.69	0.34
C268	2.00	0.00	0.50	0.69	0.35
PA 10	1.00	0.00	0.00	0.00	0.25
PA 21	-	-	-	-	-
PA 32-1	1.00	0.00	0.00	0.00	0.29
PGA 06	2.00	0.00	0.50	0.69	0.32
PGA 16	2.00	0.00	0.50	0.69	0.34
IPS 10	3.00	0.50	0.63	1.04	0.33
IPS 52	1.00	0.00	0.00	0.00	0.25
Mean	1.75	0.06	0.33	0.48	0.31
PAP 1520	4.00	1.00	0.75	1.37	0.33
PAP 3222	1.00	0.00	0.00	0.00	0.27
PAP 3754	2.00	0.00	0.50	0.69	0.34
Mean	2.33	0.33	0.42	0.69	0.31
AKE 4	3.00	0.50	0.63	1.04	0.34
AKE 5	2.00	0.00	0.50	0.69	0.29
AKE 6	2.00	0.00	0.50	0.69	0.35
AKE 12	3.00	1.00	0.63	1.04	0.32
Mean	2.50	0.38	0.57	0.87	0.33

Ne, Number of Effective Allele; Ho, Observed Heterozygosity; He, Expected heterozygosity; I, Shannon Diversity Index.

Marker informativeness among Hoya species

The genetic variations among the species in a sample can be elucidated using these markers with the aid of diversity parameters. The parameters fixation index and gene flow (Table 8) were then calculated to show genetic differentiation and transfer, respectively.

The fixation index (F_{st}) is the measure for the genetic differentiation of a population and using the standard of Bird *et al*. (2017) which interprets $F_{st} = 0$ as no differentiation within the population and $F_{st} = 1$ for complete differentiation as well as designates little differentiation ($F_{st} < 0.05$), moderate ($0.15 > F_{st} > 0.05$), strong ($0.25 > F_{st} > 0.15$), and very strong differentiation ($F_{st} > 0.25$). There is a very strong differentiation for this collection of *Hoya* using the markers C 208, PA 10, PA 21, PA 32-1, IPS 52, PAP 3222, and PAP 3754 while strong differentiation was demonstrated by C 268, PGA 06, and PGA 16 and the rest

showed moderate differentiation. On the other hand, the gene flow (Nm) measures the rate of migration to understand population genetics and dynamics. In this parameter, the classification of Wright (1965) divides the grade into high gene flow (Nm ≥ 1.00), medium gene flow (0.99 ≥ Nm ≥ 0.25), and low gene flow (0.24 ≥ Nm). The markers accounted for high (PGA 06, IPS 10, PAP 1520, AKE 4, AKE5, AKE 6, AKE 12) to medium gene flow (remaining markers). Moreover, PAP 1520 (2.105) has the highest value while PA 21 (0.297) has the lowest among the molecular markers.

Table 8. Fixation index and gene flow of the 16 SSR and EST-SSR markers employed across 21 *Hoya* accessions

Markers	Fixation Index (Fst)	Gene Flow (Nm)
C208	0.280	0.643
C268	0.205	0.968
PA 10	0.419	0.346
PA 21	0.457	0.297
PA 32-1	0.298	0.590
PGA 06	0.166	1.257
PGA 16	0.224	0.868
IPS 10	0.117	1.892
IPS 52	0.442	0.316
PAP 1520	0.106	2.105
PAP 3222	0.296	0.594
PAP 3754	0.325	0.520
AKE 4	0.121	1.818
AKE 5	0.117	1.881
AKE 6	0.193	1.043
AKE 12	0.107	2.086
Mean	**0.242**	**1.076**

Another important aspect of marker informativeness is its ability to delineate species diversity in a collection. Using the same parameters for marker analysis, observed and expected heterozygosity, Shannon diversity index, and fixation index, together with polymorphic loci, the species *H. benguetensis, H. soligamiana,* and *H. benvergarai* have been compared (Table 9).

Table 9. Genetic diversity indices between species of *Hoya* in the collection

SPECIES	Sample Size	PPL (%)	Ho	He	I	F
H. benguetensis	15	100.00	0.37	0.82	1.94	0.56
H. soligamiana	4	93.75	0.47	0.59	1.07	0.29
H. benvergarai	2	68.75	0.19	0.38	0.58	0.59
Mean		**87.50**	**0.34**	**0.60**	**1.20**	**0.47**

PPL, Percent of Polymorphic Loci; Ho, Observed Heterozygosity; He, Expected Heterozygosity; I, Shannon Diversity Index; F, Fixation Index

Among the three species of *Hoya*, *H. benguetensis* had a completely polymorphic loci having 100% PPL while *H. benvergarai* only had 68.75% and *H. soligamiana* (93.75%) was relatively near the mean (87.50%). In cases of lowest value of 0.19 and 0.38, respectively. The *H. benguetensis* (0.82) had the highest expected value for He while for Ho, *H. soligamiana* (0.47) had the greatest among the three species. The pattern of He and PPL was also observed for Shannon's diversity Index with the order of *H. benguetensis* (1.94), *H. soligamiana* (1.07), and *H. benvergarai* (0.58) and a mean of 1.20. The fixation index for all the species indicates a very strong differentiation with the highest value of 0.59 (*H. benvergarai*) and the smallest of 0.29 (*H. soligamiana*). Since the informativeness of the markers employed in this study was established with respect to the *Hoya* species utilized, specific markers that elucidate species diversity with high polymorphic content are proposed (Table 10) for a more discriminative identification and analysis.

Table 10. Microsatellite and EST-SSR markers recommended for *Hoya* species delineation

SPECIES	SSR MARKERS
H. benguetensis	C 268, PA 10, AKE 4
H. soligamiana	C 268, IPS 10, PAP 1520, AKE 6
H. benvergarai	IPS 10, PAP 1520, AKE 4, AKE 12

The clustering and differentiation in this collection was generated using all the cross-compatible markers to maximize the polymorphism elucidated by the markers. A dendrogram was generated using Dice dissimilarity coefficient and UPGMA clustering (Figure 3). The accessions per cluster is noted (Table 11) for ease of visualization and analysis.

Table 11. *Hoya* accessions per cluster as generated in the UPGMA tree with 0.8 Dice dissimilarity coefficient

CLUSTER I (n=11)		CLUSTER II (n=4)	CLUSTER III (n=6)
H. benguetensis **(2020-004)**	*H. soligamiana* (2022-018)	*H. benguetensis* (2021-035)	*H. benguetensis* (2022-173)
H. benguetensis **(2022-120)**	*H. soligamiana* (2022-013)	*H. benguetensis* (2022-174)	*H. benguetensis* (2022-123)
H. benguetensis **(2022-170)**	*H. soligamiana* (2022-112)	*H. benguetensis* (2022 - 003)	*H. benguetensis* (2022-114)
H. benguetensis **(2022-157)**	*H. soligamiana* (2022-060)	*H. benguetensis* (2022 - 056)	*H. benguetensis* (2022-127)
H. benguetensis **(2022 - 145)**	*H. benvergarai* (2022 - 179)		*H. benguetensis* (2022-094)
H. benguetensis **(P1)**			*H. benvergarai* (2022-082)

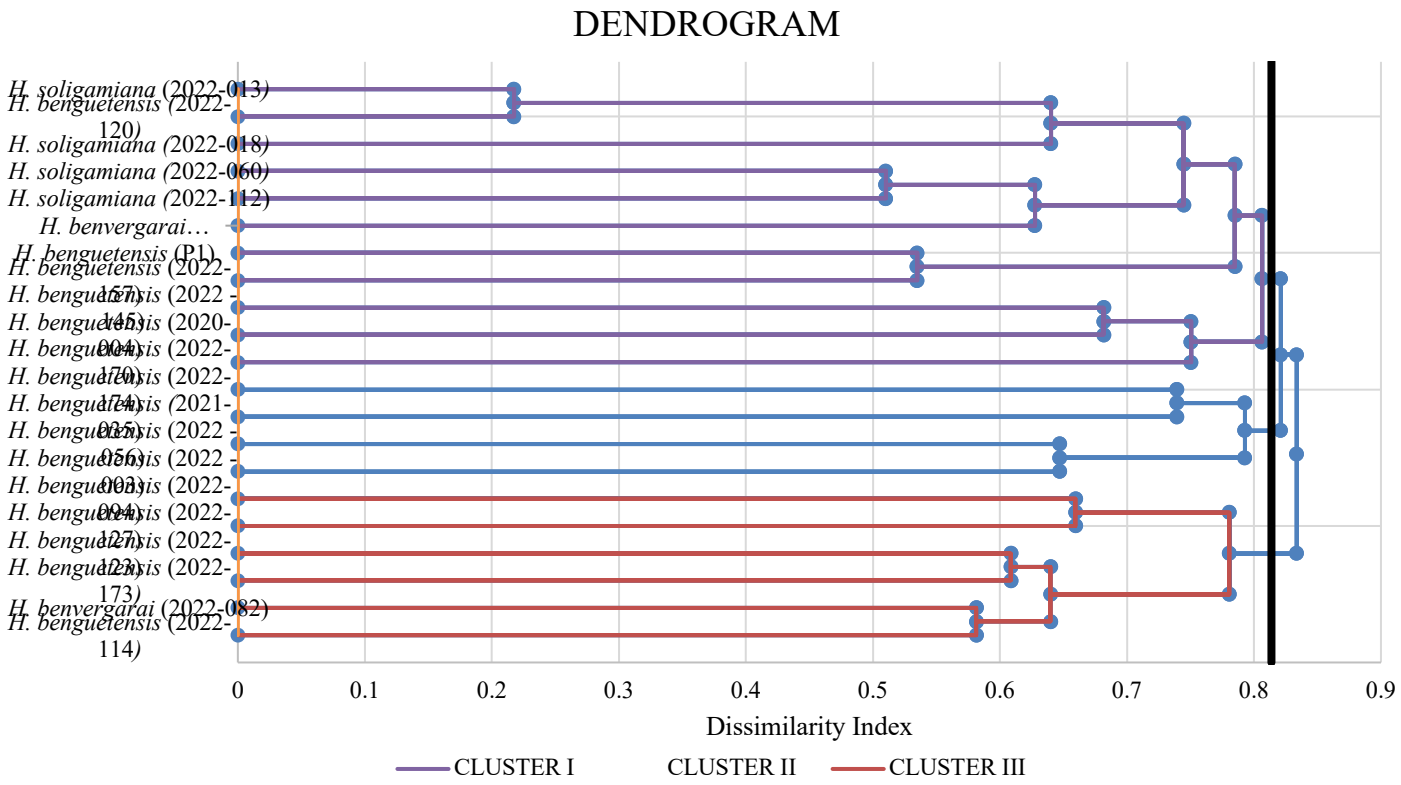

Figure 3. Dendrogram generated using Dice dissimilarity coefficient of 0.8 and UPGMA clustering across the 21 accessions of selected Philippine *Hoyas*.

The generated dendrogram grouped the 21 accessions of *Hoya* into three clusters with 0.80 dissimilarity coefficient. The first cluster (Cluster I) had 11 species of *Hoya* consisting of 6 *H. benguetensis* (2020-004, 2022-157, 2022-145, 2022-112, 2022-120, P1), 4 *H. soligamiana* (2022-018, 2022-013, 2022-112, 2022-060), and 1 *H. benvergarai* (2022-179). The *H. benguetensis* accessions (2021-035, 2022-174, 2022-003, 2022-056) comprise Cluster II while Cluster III is predominantly *H. benguetensis* (2022-173, 2022-127, 2022-123, 2022-114, 2022-094) and 1 *H. benvergarai* (2022-082). Based on location, the species from Cluster I were collected from different areas as well as Cluster II (Batangas, Cagayan, and Laguna) while Cluster III species were all collected in Cagayan. Further sub-grouping of Cluster I at 70% dissimilarity diverged the cluster into five (5) sub-clusters. The first subgroup of Cluster I consists of 2 *H. soligamiana* (2022-013 and 2022-018) and 1 *H. benguetensis (*2022- 120) while the second subgroup has the remaining *H. soligamiana* (2022-060 and 2022-112) and 1 *H. benvergarai* (2022-179). Both subgroups 3 and 4 have two accessions of *H. benguetensis*, P1 and 2022-157, and 2022-145 and 2022-004, respectively. The last subgroup of Cluster I had only one accession of *H. benguetensis* (2022-170). Subgrouping for the second cluster resulted in three (3) subgroupings with one sample each for subgroups 1 (2022-174) and 2 (2021-035). The other two accessions were clustered for the last subgroup (2022-003 and 2022-056). The last cluster (Cluster III) had only two subclusters with two and four samples for subgrouping 1 and 2, respectively. The first subgrouping has 2 *H. benguetensis* (2022-094 and 2022-127) while the second had the other *H. benvergarai* (2022-082) and 3 *H. benguetensis* (2022-123, 2022-173, 2022-114).

Discussion

The marker amplification deviates from the findings of the study of Lebedev *et al.* (2020) and He (2006) which had a higher percentage transferability of EST-SSR than microsatellite markers. The varieties under *H. benguetensis* and *H. soligamiana* had amplified the 9 microsatellite markers of Orchidaceae while all the three species had amplified 3 EST-SSRs. All the polymorphic markers previously evaluated for *Hoya mindorensis* Schleter (Widiarsih *et al*., 2014) were cross-compatible with the selected *Hoya* species. The complete amplification of SSR markers is due to the close distance between the *H. mindorensis* and this section of *Hoya.* Moreover, the amplified markers were able to detect polymorphism between species, however showed some differences in properties from the published studies. One circumstance is the difference in annealing temperature from the published studies and observed which had been the case with Marin *et al*. (2020) where they tested the samples to a range of temperatures until it resulted in a successful amplification. Another

instance is transference, a term that describes positive amplification of expected band size (Savadi *et al*., 2012) in transferability studies, which was not satisfied in this study. This is possibly due to the apparent phylogenetic distance between Orchidaceae and *Hoya* for markers in the cross-family amplification.

In comparison with the study of Widiarsih *et al*. (2014), the number of alleles of *H. benguetensis* and *H. benvergarai* deviates from the ones noted in their study for AKE 4, 5, 6, and 12 which are 7, 3, 7, and 8, respectively. This could possibly indicate that there are more regions in the genome that complement the primer sequence. Moreover, the findings of Almontero *et al*. (2022) in terms of the amplified band (1 to 8 alleles) were not mirrored for accessions under *H. benguetensis* while species *H. soligamiana* and *H. benvergarai* satisfied the expected range. Furthermore, the PIC (0.662 – 0.829) noted in their study was significantly higher than the ones reported here. With that, this section of *Hoya* is less diverse than the aforementioned studies.

There are three suggested markers for *H. benguetensis* which are C 268, PA 10, and AKE 4 which showed high polymorphism, genetic differentiation, and gene migration. Also, this is highly discriminative as compared to the markers recommended for the remaining species since the values recorded were significantly higher. Moreover, this is affected by the sample size wherein the *H. soligamiana* and *H. benvergarai* were restricted. With that, the markers proposed for the *H. soligamiana* and *H. benvergarai* species had the highest value among the 16 primer pairs. The two species had 4 microsatellite and EST-SSR markers. They can be both distinguished by IPS 10 (microsatellite) and PAP 1520 (EST-SSR) while *H. soligamiana* has an additional C 268 and AKE 6, and *H. benvergarai* has AKE 4 and 12.

It is important to note that *H. soligamiana* 2022-013 and *H. benguetensis* 2022-120, two taxonomically different *Hoya* species, had approximately 80% similarity with each other using these 16 primer pairs. This entails that there is a highly conserved region between the two samples of the two different species. The *H. soligamiana* 2022-013 was collected from Los Baños, Laguna, and was said to be a yellow-green variant while *H. benguetensis* 2022-120 came from Baggao, Cagayan, and has a dark red color based on the limited passport data given. Also, all the *H. soligamiana* accessions have been grouped in one cluster while the *H. benvergarai* species were placed in different clusters. Limited information about the background of the species such as its morphological characteristics and geographical origin, no correlation and further interpretation of the results can be made.

The taxonomic classification of organisms relies on their morphological characteristics and their phylogenetic similarities with other existing and established species (Viscosi and Cardini, 2012). Phenotype, however, is greatly

influenced by environmental factors especially in the case of *Hoya* as discussed by Baltazar and Buot (2019) which addressed the concerns regarding phenoplasticity and parataxonomists in *Hoya* nomenclature. They stated that the morphology of *Hoya* changes with different environmental conditions, however, two plant features, leaf venation, and pollinarium, remain stable. They recommended the use of these characters in delineating species and for easier taxonomic work. In this case, the other viable solution for proper and easier taxonomic identification is barcoding at the genotypic level. Here, the effect of varying environments and its interaction with the crop is not accounted for hence, classification is based solely on the DNA sequence of the plant. The use of DNA barcoding is more informative because it would elucidate the species diversity at the genetic level and could identify clones in a population.

This study concluded that cross-amplification of SSR markers from the Orchidaceae family to *Hoya* as well as *H. mindorensis* Schleter to *H. benguetensis, H. soligamiana,* and *H. benvergarai* was attainable. It was able to capture genetic differences among the accessions of *Hoya* and initially elucidated the genetic diversity using informative markers. Another study may be conducted with more samples and primer pairs to further delineate the native *Hoya* species in the country. Moreover, incorporating sequencing of amplicons and its annotation in molecular databases would improve the genetic studies of *Hoya* and other related species.

Acknowledgments

The authors would like to extend their gratitude to the Department of Science and Technology (DOST) for funding the research, the Molecular Plant Breeding Laboratory of ICropS for the laboratory equipment and resources, the IPB for the plant materials collected through the DOST-PCAARRD funded project "Varietal Development of Philippine Native *Hoya*s".

References

Almontero, C., Hernandez, J., Lalusin, A. G., Genaleen, M., Diaz, Q., Ocampo, T. M. and Hernandez, J. E. (2022). Fingerprinting of Philippine Waling-Waling (*Vanda sanderiana* Rchb.f.) Accessions Using Simple Sequence Repeat (SSR) Markers. Philippine Journal of Crop Science, 2022;https://www.researchgate.net/publication/368882476

Baltazar, A. M. and Buot, I. J. (2019). Controversies on Hoya R. Br. Taxonomy. The Thailand Natural History Museum Journal, 59-68.

Bird, K., An, H., Gazave, E., Gore, M., Pires, J., Robertson, L. and Labate, J. (2017). Population Structure and Phylogenetic Relationships in a Diverse Panel of Brassica rapa L. Frontiers in Plant Science, 8:doi: 10.3389/fpls.2017.00321

Brown, R. (1810). Prodromus florae Novae Hollandiae et Insulae Van-Diemen. London: R. Taylor et socii. doi:https://doi.org/10.5962/bhl.title.3678

Chen, Z., Deng, Y., Zhou, R. and He, S. (2016). Development of transcriptome-derived SSR markers for *Hoya ledongensis* (Apocynaceae) and cross-amplification in a congener. Applications in Plant Sciences, 4.

Endress, P. K. (2016). Development and evolution of extreme synorganization in angiosperm flowers and diversity: a comparison of Apocynaceae and Orchidaceae. Annals of Botany, 117:749-767.

He, G., Woullard, F. E., Marong, I. and Guo, B. Z. (2006). Transferability of Soybean SSR Markers in Peanut (*Arachis hypogaea* L.). Peanut Science, 33:22-28.

Kloppenburg, D. (1991). Philippine Hoya species: a monograph. New York. Retrieved from https://archive.org/details/phillipinehoyasp00klop/page/n3/mode/2up

Kress, W. J. (1986). The Systematic Distribution Of Vascular Epiphytes: An Update. Selbyana, 9:2-22.

Lebedev, V. G. (2019). Transferability and Polymorphism of SSR Markers Located in Flavonoid Pathway Genes in Fragaria and Rubus Species. Genes. doi:https://doi.org/10.3390/genes11010011

Maranan, F. (2011). Molecular diversity, DNA barcode identification, and ecological notes of selected Philippine endemic Hoya species (Apocynaceae). Agris.

Maranan, F. S. and Diaz, M. G. (2013). Molecular diversity and DNA barcode identification of selected Philippine endemic Hoya species (Apocynaceae). Philippine Agricultural Scientist, 86-92.

Marin, A. L., Costa-Silva, C., Rivas, P. M., Bonifacio-Anacleto, F., Santos, L. G., Filho, R. M., and Martinez, C. A. (2018). Diagnostic fingerprints ISSR/SSR for tropical leguminous species *Stylosanthes capitata* and *Stylosanthes macrocephala*. Scientia Agricola. doi:http://dx.doi.org/10.1590/1678-992X-2018-0252.

Pelser, P., Barcelona, J. and Nickrent, D. (2022). Apocynaceae. Retrieved from Co's Digital Flora of the Philippines: https://www.philippineplants.org/Families/Apocynaceae.html

Savadi, S. B., Fakrudin, B., Nadaf, H. L. and Gowda, M. V. C. (2012). Transferability of Sorghum Genic Microsatellite Markers to Peanut. American Journal of Plant Sciences, 03:1169-1180.

Viscosi, V. and Cardini, A. (2012). Leaf Morphology, Taxonomy and Geometric Morphometrics: A Simplified Protocol for Beginners. PLOS ONE, 7(3). doi:10.1371/annotation/bc347abe-8d03-4553-8754-83f41a9d51ae.

Wanntorp, L., Grudinski, M., Forster, P. I., Muellner-Riehl, A. N. and Grimm, G. W. (2014). Wax plants (Hoya, Apocynaceae) evolution: Epiphytism drives successful radiation. International Association for Plant Taxonomy, 63:89-91.

Widiarsih, S., Siar, S. V. and Lalusin, A. G. (2011). Optimization of DNA Extraction Protocol for *Hoya mindorensis* Schlechter. Philippine Journal of Crop Science, 36:63-66.

Widiarsih, S., Siar, S. V. and Lalusin, A. G. (2014). Molecular Characterization of *Hoya mindorensis* Schlechter by Microsatellite Markers. Philippine Journal of Crop Science, 58-64.

Widiarsih, S., Siar, S., Lalusin, A., Carandang, J. and Borromeo, T. (2012). Genetic diversity assessment in vegetative and reproductive characters of *Hoya mindorensis* Schlechter. Philippine Journal of Crop Science, 23-29.

Zhao, M. X., Wang, H. Y., Yin, Z. J., Wang, M.-J., Yang, J. C. and Tan, Y. H. (2020). Hoya gaoligongensis (Apocynaceae, Asclepiadoideae), is a new species from Yunnan, SW China. PhytoTaxa, 219-226.

(Received: 10 October 2023, Revised: 28 June 2024, Accepted: 30 June 2024)