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SPECIES DELINEATION OF *Kaempferia galanga* L. AND *Kaempferia pulchra* L. IN THE PHILIPPINES USING *psbA-trnH* AND *petA-psbL-psbJ*

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ABSTRACT - There are two *Kaempferia* species commonly found in the Philippines, *K. galanga* and *K. pulchra*. The former is known as a medicinal herb while the latter is an ornamental crop and was identified in the country as an introduced species. Proper delineation of the two species is needed to avoid adulteration of *K. galanga*-based herbal products with *K. pulchra*. In this study, five local accessions of *K. galanga* and four of *K. pulchra* were subjected to molecular analysis utilizing chloroplast intergenic spacers, *psbA-trnH* and *petA-psbL-psbJ*. The derived sequences were also compared to the sequences of *K. galanga* and *K. pulchra* from Thailand and some related species as outgroups. This can feasibly evaluate the quality of *K. galanga*-based medicinal products and assess diversity of the local *Kaempferia* species. Results showed that the combined sequences of the two intergenic spacers can be used to differentiate local *K. galanga* and *K. pulchra* as observed in the generated consensus phylogenetic tree having bootstrap values of 55 and 63%, respectively. Intraspecific sequence variation was observed among *K. galanga* as Negros Occidental accessions separated from the group with bootstrap value of 68%. In addition, it was observed that local *K. galanga* is closely related to Thailand species while local *K. pulchra* is distinct to their Thailand counterpart.

Keywords: Kaempferia galanga, Kaempferia pulchra, psbA-trnH, petA-psbL-psbJ

INTRODUCTION

Kaempferia is an aromatic and rhizomatous herb under family Zingiberaceae which is composed of about 70 species. It is widely distributed in the South East Asia including the Philippines. Quisumbing (1978) recognized two *Kaempferia* species in the country, *K. galanga* (gisol) and *K. rotunda* (gisol na bilog). Both species were cited as important medicinal herbs used in different regions of the country. Decoction of *K. galanga* rhizomes, an official entry in the French Pharmacopoeia, is used as a tonic, carminative, and gargle. In the Visayas, it is given to mother after childbirth as tonic. It can also be used as a remedy for common cold, bronchitis, tuberculosis, headache, dyspepsia and malaria (Ibrahim 1999; Lee et al 2005). Pharmacological studies showed that the plant has anticancer (Mathew et al 2010), antioxidant (Sumazian et al 2010) and wound healing (Shanbhag et al 2006) activities. The plant was once considered to be an endangered species (Kochuthressia et al 2012). *K. rotunda*, on the other hand, is used as treatment for gastric disorders (Quisumbing 1978; Ibrahim 1999; Lee et al 2005). However, on the duration of the study, no *K. rotunda* was found. Instead, another species was identified which is *K. pulchra*. In comparison with *K. galanga* and *K. rotunda*, *K. pulchra* is an introduced species and mainly

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used as an ornamental plant because of its distinct markings on its foliage and purple flowers. The plant is also widely distributed from India and Myanmar to Thailand and Peninsular Malaysia where its leaves are edible and the rhizomes are medicinal (Picheansoonthon and Koonterm 2008).

Adulteration is one of the issues being raised with regards to the use of herbal drugs. Because of biological relationship of *K. pulchra* to *K. galanga*, it can be used as an adulterant to the latter's raw and herbal preparations. Therefore, proper authentication and accurate delineation of the two species must be done.

The two plants are morphologically different. *K. galanga* has short stature, pale brown tuberous roots, golden-brown rhizomes, short leaf sheath, and thick, shiny leaves (Fig. 1A). On the other hand, *K. pulchra* has yellowish tuberous roots, purplish rhizomes, long leaf sheath, and thin leaves having distinct markings (Fig. 1B). However, these differences may not be sufficient to differentiate the two species especially when they are already processed as herbal products. In addition, problem with regards to intraspecific variation may arise. Indrayan and colleagues (2007) reported two known varieties of *K. galanga* in India, 'Kasthuri' and 'Rajani'. Presence of these variants was not documented for the Philippine species.

These problems can be resolved with the use of molecular markers. In 2010, Techaprasan and colleagues assessed the genetic variation of different *Kaempferia* species in Thailand using chloroplast intergenic spacers, *psbA-trnH* and *petA-psbL-psbJ*. Using the technique, species delineation of available accessions of *K. galanga* and *K. pulchra* in the Philippines was done in this study. Distinct string sequences were provided to differentiate the two species. This can be used to evaluate the quality of any *K. galanga*-based herbal products in terms of their chemical constituents, uniformity, potency, safety, and efficacy. Local species were also compared to their Thailand counterparts to have a more robust analysis and to situate the study to global scenario.

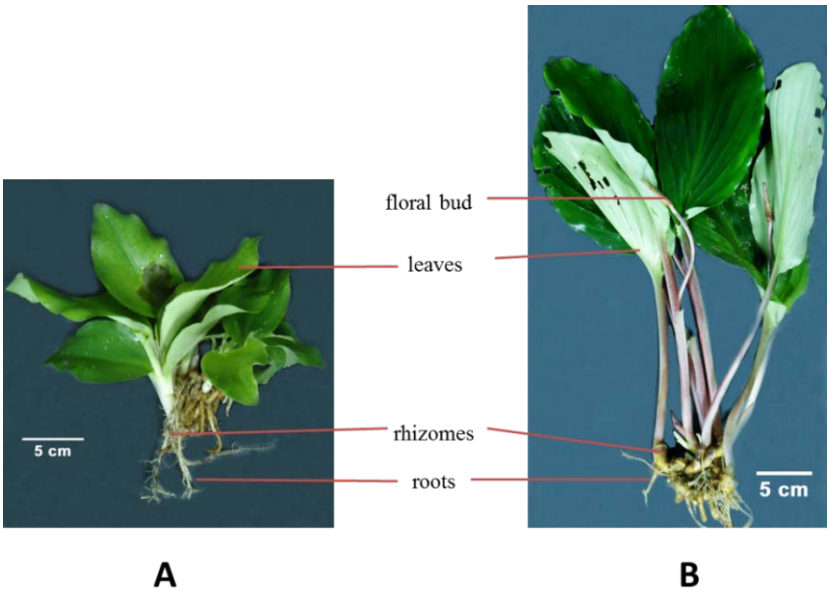

Figure 1. General morphology of *K. galanga* (A) and *K. pulchra* (B).

MATERIALS AND METHODS

Sample Collection

Five *Kaempferia galanga* accessions were used in the study. These are from Los Baños, Laguna (LB_{KG}), Ecosystem Research and Development Bureau – Los Baños Experimental Station (ERDB-LBES; ($ERDB_{KG}$), Lemery, Batangas (BAT_{KG}) and two from Negros Occidental: Herbanext® Laboratories, Inc., Bago City ($HeNO_{KG}$) and Sum-ag, Bacolod City ($SmNO_{KG}$). While for *K. pulchra*, four accessions were collected from University of the Philippines-College of Agriculture and Food Science (UPLB-CAFS) Ornamental Crops Nursery (LB_{KP}), UPLB Museum of Natural History (MNH_{KP}), Tagaytay City, Cavite (CAV_{KP}) and Herbanext® Laboratories, Inc., Bago City, Negros Occidental ($HeNO_{KP}$). External morphology of the plant samples used was provided in Figure 2.

DNA Isolation

Genomic DNA was extracted from 100 mg young leaf samples of the different accessions using Vivantis® GF-1 Plant DNA Extraction Kit with some modifications. Thereafter, DNA concentrations and quality were assessed by electrophoresis on 1% agarose gel (Vivantis®) at a constant power of 220 volts using Mupid-2® mini gel migration trough. Concentrations and quality of the extracted DNA were also measured by taking their absorbance at 260/280 nm using Epoch® microplate spectrophotometer.

Polymerase Chain Reaction (PCR) Amplification

The *psbA-trnH* and *petA-psbJ-psbL* sites of each accession were amplified using the primer pairs developed by Techaprasan and colleagues in 2010 (*psbA*-1F: 5′-CTTGGTATGGAAGTAATGCA-3′ and *trnH*-1R: 5′-ATCCACTTGGCTACATCCG-3′, and *petA*-F: 5′-AGGTTCAATTGTMCGAAATG-3′ and *psbL*-R: 5′-GTACTTGCTGTTTTATTTTC-3′). Primer pairs were synthesized in dry and desalted form through AITbiotech PTE LTD in 50 nM concentrations.

PCR was undertaken using Vivantis® DNA Amplification Product (2X Taq Master Mix) in G-Storm® or BIO-RAD® thermocyclers. The reaction volume is 30 µl composed of 1X Vibuffer, 1.5mM $MgCl_2$, 0.20 mM dNTPs, 0.1 mM of each primer, 50 ng total DNA templates and 1 U Taq DNA polymerase. Polymerase chain reaction was done with an initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 2 sec, annealing at 55°C for 1 min and extension at 72°C for 30 sec. The final extension was carried out for 7 min at 72°C. PCR products were resolved in 1% Vivantis® agarose gel in Tris-base: acetic acid: EDTA (TAE) buffer at a constant power of 220 volts (Enduro™ Gel XL).

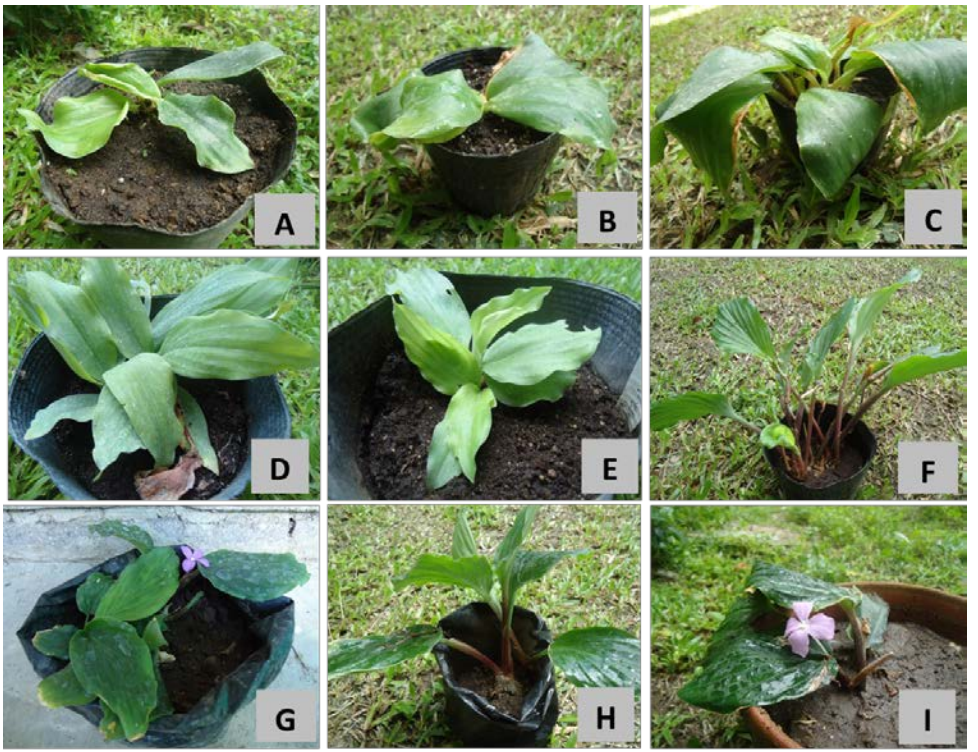

Figure 2. External morphology of *K. galanga* (A-E) and *K. pulchra* (F-I) used in the study. *K. galanga* collected from Los Baños, Laguna (LB_{KG}; A), Ecosystem Research and Development Bureau – Los Baños Experimental Station ($ERDB_{KG}$; B), Lemery, Batangas (BAT_{KG}; C), Herbanext® Laboratories, Inc., Bago City, Negros Occidental ($HeNO_{KG}$; D), and Sum-ag, Bacolod City, Negros Occidental ($SmNO_{KG}$; E). *K. pulchra* from University of the Philippines-College of Agriculture and Food Science (UPLB-CAFS) Ornamental Crops Nursery (LB_{KP}; F), UPLB Museum of Natural History (MNH_{KP}; G), Tagaytay City, Cavite (CAV_{KP}; H) and Herbanext® Laboratories, Inc., Bago City, Negros Occidental ($HeNO_{KP}$; I).

Sequence Analysis

The amplified psbA-trnH and petA-psbJ-psbL were direct-sequenced for both forward and reverse directions through AITbiotech PTE LTD. Raw sequences were trimmed using PreGap 4 and Trev 1.9 softwares. Conserved regions of the genes per accession were generated using Gap 4 by aligning the forward and reverse sequences and manual editing of any mismatch observed. These softwares were included in Staden® Package (Staden et al 2000).

Phylogenetic analysis was carried out with the use of MEGA® v5.05 (Tamura et al 2011). Using ClustalW® incorporated in the software, the derived sequences of all accessions were aligned and compared with *psbA-trnH* and *petA-psbJ-psbL* sequences of *K. galanga* (JT2008-12) and *K. pulchra* (TT10144) Thailand species accessed from GenBank®. In addition, sequences of other relative species, *Curcuma longa* and *Hedychium longicornutum* from Zingiberaceae, and *Musa acuminata* of Musaceae, were also included in the analysis as outgroups. Details of the accessed sequences were summarized in Table 1.

Nucleotide sequence divergence between pairs of taxa was calculated in accordance to Tamura 3-parameter similarity index without indel consideration. The evolutionary history was inferred by phylogeny reconstruction using the Maximum Likelihood method. Gaps were also treated as missing data. Bootstrapping (1000 replicates) was performed and its values were superimposed on the phylogenetic tree to illustrate confidence level of relationships among *Kaempferia* species and outgroups.

Table 1. Accession numbers and number of base pairs of the sequences derived from GenBank®.

SPECIES	*psbA-trnH*		*petA-psbJ-psbL*	
	Accession number	**No. of base pairs**	**Accession number**	**No. of base pairs**
Kaempferia galanga, JT2008-12	GQ385977.1	736	GQ386060.1	694
Kaempferia pulchra, TT10144	GQ386028.1	726	GQ386111.1	661
Hedychium longicornutum	HM749002.1	735	HM749013.1	662
Curcuma longa	FJ687416.1	758	JF730291.1	691
Musa acuminata	FJ871864.2	775	EU017000.1	960

RESULTS AND DISCUSSION

Isolation of Genomic DNA

Genomic DNA from young leaves of different accessions of *K. galanga* and *K. pulchra* was successfully isolated using Vivantis® GF-1 Plant DNA Extraction Kit. The extraction method used yielded relatively high DNA content with $SmNO_{KG}$ (302.803 ng/µl) recorded the highest concentration. The 260 nm/280 nm absorbance of DNA extracts, which is indicative of purity, suggested that the samples were in good quality as they registered values in the ideal range of 1.8-2.0.

Analysis of *psbA-trnH* and *petA-psbJ-psbL* Sequences

The extracted gDNA were used for Polymerase Chain Reaction. PCR products of *psbA-trnH* and *petA-psbJ-psbL* spacers in *K. galanga* and *K. pulchra* accessions were approximately 800 and 1200 bp in length, respectively. Only the partial *petA-psbJ-psbL* was considered for analyses as the nucleotide sequences at the 3′ end of spacers were missing as observed also by Techaprasan et al (2010). Sequence characteristics and nucleotide sequence divergence of *psbA-trnH* and partial *petA-psbJ-psbL* among *Kaempferia* accessions and outgroup references were noted after multiple sequence alignment. These are summarized in Table 2.

Table 2. Sequence characteristics and nucleotide sequence divergence of *psbA-trnH* and partial *petA-psbJ-psbL* across *Kaempferia* accessions and outgroups, *Curcuma longa*, *Hedychium longicornutum*, and *Musa acuminata*.

	psbA-trnH	***petA-psbJ-psbL***	**COMBINED SEQUENCES**
Number of nucleotides (bp)	672-792	628-960	1300-2013
Number of conserved sites	348	326	668
Number of uninformative-variable sites	478	473	923
Number of informative characters	18	24	40
% of interspecific sequence divergence within *Kaempferia*	0.000-0.0120	0.000-0.007	0.000-0.007
% of interspecific and intergeneric sequence divergence	0.000-1.037	0.000-1.084	0.000-1.188
% of intraspecific sequence divergence			
K. galanga	0.000-0.008	0.000	0.000-0.001
K. pulchra	0.000-0.005	0.000-0.007	0.000-0.007
Curcuma longa	0.000	0.000	0.000
Hedychium longicornutum	0.000	0.000	0.000
Musa acuminata	0.000	0.000	0.000

The nucleotide sequences of *psbA-trnH* within *Kaempferia* accessions ranged between 672 bp (LB_{KP}) and 794 bp (BAT_{KG}) and came up with 1058 bp after alignment with the outgroups. Likewise, the partial *petA-psbJ-psbL* sequences ranged from 628 bp (LB_{KP}) to 771 bp ($HeNO_{KG}$) in length and resulted to multiple alignment of 963 bp. For the combined sequences, number of nucleotides ranged from 1300 bp (LB_{KP}) to 1564 bp ($HeNO_{KG}$) and brought about 2063 bp after alignment.

The numbers of parsimoniously uninformative sites and informative characters of the combined sequences are higher (923 and 40) than the individual *psbA-trnH* (478 and 18) and partial *petA-psbJ-psbL* (473 and 24) sequences. In the same manner, pairwise nucleotide divergence of the combined sequences within *Kaempferia* accessions and across all examined taxa were also higher (0.000-0.007% and 0.000-1.188%) compared to *psbA-trnH* (0.000-0.012 % and 0.002-1.037 %) and partial *petA-psbJ-psbL* (0.000-0.007% and 0.000-1.084%). These values indicated that the combined sequences can give better variation data about the given samples than the individual *psbA-trnH* and partial *petA-psbJ-psbL* sequences.

Phylogenetic Tree Analysis

Bootstrap consensus phylogenetic tree showing the relationship of *Kaempferia* accessions and outgroups based on combined sequences was presented in Figure 3. Taxa were clustered in nodes having bootstrap values representing the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test. Branches having values >50 % infer genetic variations between populations and can be considered as separate groups or clades.

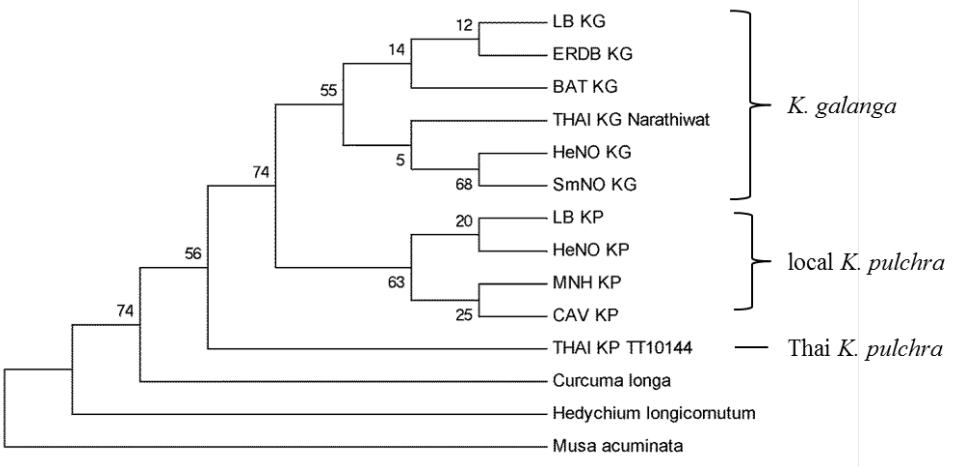

Figure 3. Bootstrap consensus phylogenetic tree showing the relationship of *Kaempferia* species and outgroups (*Curcuma longa, Hedychium longicornutum,* and *Musa acuminata*) based on combined *psbA-trnH* and partial *petA-psbJ-psbL* sequences.
Legend:

KG - *K. galanga* accessions; KP - *K. pulchra* accessions; LB – Los Baños; BAT - Batangas; CAV - Cavite;
ERDB-Ecosystem Research and Development Bureau; HeNO – Herbanext®, Negros Occidental;
SmNO - Sum-ag, Negros Occidental; MNH - Museum of Natural History.

The phylogenetic tree showed that *Kaempferia* species clustered together and separated from outgroups with bootstrap value of 56%. The group was then separated into *K. galanga* and *K. pulchra* accessions with bootstrap values of 55 and 63%, respectively. This grouping, however, excluded *K. pulchra* accession from Thailand. It suggests intraspecific sequence variation within *K. pulchra* which was also observed by Techaprasan et al (2010) in Thailand *K. pulchra* accessions. Moreover, intraspecific sequence variation among *K. galanga* was also observed as Negros Occidental (NO) accessions had bootstrap value of 68%.

These observations suggest that the combined *psbA-trnH* and partial *petA-psbJ-psbL* sequences can be used to distinguish intergeneric, interspecific and even intraspecific sequence variations among the given taxa.

Variations observed were due to different polymorphic sites and indels found in psbA-trnH (Fig. 4A) and partial petA-psbJ-psbL (Fig. 4B) sequences. Using psbA-trnh, single base substitution at sites #221 (T - C) and #332 (A - T) as well as insertion of "TTTTTTTT" at position #333 differentiated Thailand K. pulchra to the rest of given Kaempferia accessions. In addition, insertion of "T" at #857 differentiated Thailand K. pulchra to local accessions. It was also observed that psbA-trnh alone was not able to delineate local K. galanga and K. pulchra but it was able to distinguish intraspecific sequence variation among K. galanga accessions. The two Negros Occidental accessions had single base substitutions at sites #169 (C - T), #385 (A - G), #390 (T - A), #391 (G - A), #392 (T - C), #400 (C - G), and #472 (T - C). Insertions of "TTT" at #396, "TT" at #412, and "TAG" at #415 were also distinct.

Single nucleotide polymorphism of partial petA-psbJ-psbL at sites #230 (G - A), #325 (C - T) and #520 (C - T) differentiated Thailand K. pulchra to the rest of Kaempferia accessions. Moreover, the local K. pulchra accessions can be distinguished to Thailand accession by single base substitution at #491 (C - T). K. galanga accessions can be distinguished to local K. pulchra by having single base substitution at #298 (G - A) and #491 (T - C). Insertion of "AATAC" at #709 and "AGAATTAGTA" at #436 also distinguished K. galanga accessions to local K. pulchra but the latter sequence was found to be absent in Negros Occidental accessions. In addition, single nucleotide polymorphism at sites #93 (G - A), #94 (G - T), #95 (T - C), #96 (G – T), and #102 (C - T) and insertion of "CC" at #103 differentiated Negros Occidental accessions to the rest of K. galanga.

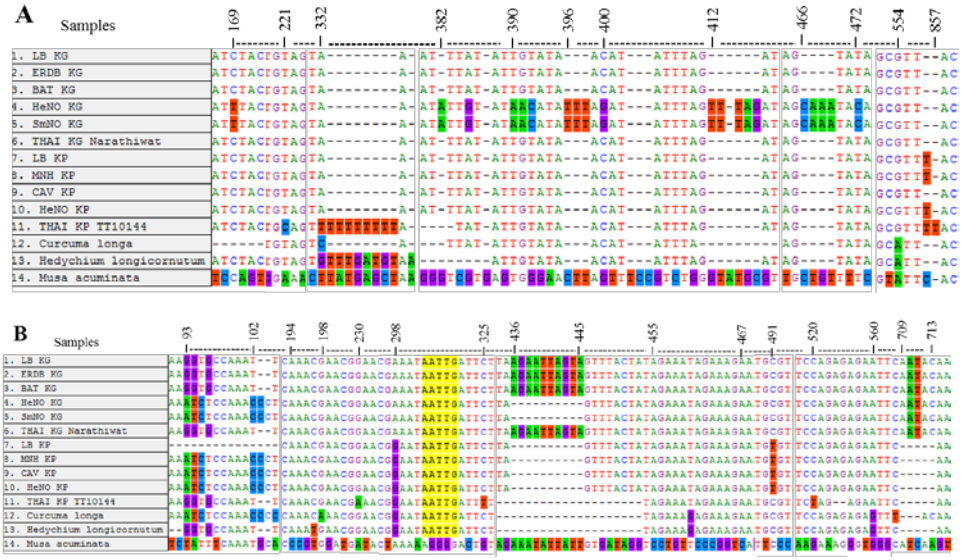

Figure 4. Polymorphic sites and indels (color-filled nucleotides) of *psbA-trnH* (A) and partial *petA-psbJ* (B) sequences used for delineating *K. galanga* (KG) and *K. pulchra* (KP) accessions from the Philippines and Thailand as well as outgroup species: *Curcuma*, *Hedychium*, and *Musa*.

CONCLUSIONS AND RECOMMENDATIONS

In this study, local *Kaempferia galanga* was differentiated from its closely related species, *K. pulchra*, based on the two chloroplast intergenic spacers, *psbA-trnH* and *petA-psbJ-psbL*. The constructed phylogenetic tree based on the combined sequences, showed the separation of local *K. galanga* and *K. pulchra* accessions. Within the group of *K. galanga*, strong relationship between the two accessions from Negros Occidental was noted suggesting intraspecific sequence variation. The local accessions were also compared to their Thailand counterparts. It was observed that local *K. galanga* is closely related to Thailand species while local *K. pulchra* is distinct to their Thailand counterpart. The analysis suggested that the combined data of the two intergenic spacers can be used to differentiate local *K. galanga* and *K. pulchra*. Moreover, it is recommended to have higher number of samples including accessions from Mindanao, if available, as well as local *K. rotunda* to have a more robust analysis.

STATEMENT OF AUTHORSHIP

The first author initiated the concept, conducted the literature search, prepared the conceptual framework, performed the laboratory work, formulated recommendations, and prepared the paper for publication. The second author identified some issues, formulated recommendations, and reviewed the paper.

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