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Molecular phylogeny of Annonaceae species from Binh Chau-Phuoc Buu Nature Reserve based on two chloroplast gene regions

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ABSTRACT

In this study, based on molecular biology techniques, the *mat*K and *trn*L-F regions of ten Annonaceae species grown in Binh Chau-Phuoc Buu Nature Reserve, Vietnam were successfully amplified and sequenced for the first time. Along with other highly homologous DNA sequences from the GenBank database, the molecular phylogeny of ten studied species was also established. By using the alignment tool of NCBI database, the percentage of identity among sequences of studied species was also presented. The study aims to partially contribute to the further understanding of the evolutionary relationships among Annonaceae species.

Keywords: Annonaceae, *mat*K and *trn*L-F regions, phylogeny, Binh Chau-Phuoc Buu Nature Reserve

INTRODUCTION

Annonaceae is one of the large families belonging to the phylum Magnoliophyta. The family currently includes 135 genera with around 2,500 species being distributed mainly in tropical and subtropical regions. Only a few species were found in temperate regions (Tran *et al.*, 2014). As a large family, Annonaceae has many species that are widely used as timber, food or ornamental plants. Besides, Annonaceae is also a rich family in natural medicinal plant resources, many species of which have been used to treat skin diseases, flu, bone and joint diseases or liver diseases (Do, 2004). According to the book "The Flora of Vietnam" published in 2000, Prof. Hoang Ho Pham reported that Annonaceae has 155 species belonging to 28 genera (Pham, 2000). However, many botanists have discovered and published many new species for science as well as recorded some new species for Vietnam flora since 2000, bringing the total number of Annonaceae species in Vietnam to around 183 species belonging to 29 genera, 2 subspecies and 21 varieties (Tran *et al.*, 2014; Tagane *et al.*, 2015; Nguyen *et al.*, 2016; Bui *et al.*, 2016; Ly, 2017; Do *et al.*, 2018; Ly *et al.*, 2019).

Up to now, many Annonaceae species have been studied worldwide in terms of their genetic diversity, chemical composition and biological activity (Costa *et al.*, 2015; Araujo *et al.*, 2017; Tamfu *et al.*, 2019; Bhardwaj *et al.*, 2019). However, in Vietnam, aside from the investigation of species composition, many studies about Annonaceae mainly focused on the chemical composition of essential oils extracted from species of this family (Tran *et al.*, 2013; Do, 2013) while very few studies related to genetic traits have been conducted as far as we know. To fill the gap in the literature, this study for the first time successfully sequenced *mat*K and *trn*L-F region sequences and built a phylogenetic tree for 10 species of the Annonaceae collected at the Binh Chau-Phuoc Buu nature reserve.

MATERIALS AND METHODS

Plant Samples Collection

Specimens of ten Annonaceae species were collected from Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng Ward, Xuyen Moc District, Ba Ria-Vung Tau Province, Vietnam

(Figure 1 & 2). All vouchered specimens were deposited at the Herbarium of Binh Chau-Phuoc Buu Nature Reserve. The information of the vouchered numbers and collection sites of ten studied species were presented in Table 1. In addition, *mat*K and *trn*L-F sequences of twenty-seven Annonaceae and one Magnoliaceae species from the Genbank database were used for the establishment of the phylogenetic tree (Table 2).

Morphological Taxonomy

The method described in the Royal Botanic Gardens, Kew, was utilized to prepare and process the specimens (Bridson & Forman, 1999). Specimens were identified at the species level based on the comparison among Annonaceae members in terms of morphological vegetative and reproductive characteristics described in published works (Pham, 2000; Tran *et al*., 2014; Tagane *et al*., 2015; Nguyen *et al*., 2016; Bui *et al*., 2016; Ly, 2017; Do *et al*., 2018; Ly *et al*., 2019).

Total Genomic DNA Extraction and PCR Amplification

Gene Jet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA) and the protocol given by the manufacturer were used for the extraction of total genomic DNA from fresh leaves of ten studied species. The *mat*K and *trn*L-F regions were respectively amplified on Mastercycler machine (Eppendorf, Germany) using primers (forward: 5'ACCCAGTCCATCTGGAAATCTTGGTTC3', reverse: 5'CGTACAGTACTTTTGTGTTTACGAG3'), and primers (forward: 5'CGAAATCGGTAGACGCTACG3', reverse: 5'ATTTGAACTGGTGACACGAG3') (Taberlet *et al*., 1991). A total volume of 25µL PCR mixture was used, in which 12.5µL Go-Taq green master mix (Promega, USA), 1.25µL of each forward and reverse primers (10 µM), 9.5µL nuclease-free deionized water and 0.5µL DNA template (25µg/mL) were included. The PCR process was programmed to comprise three major cyclic reactions, including 3 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C); and a final extension at 72°C for 10 min. The amplicons were then subjected to purification prior to sequencing on ABI 3130 XL Sequencer.

Sequencing Data Analysis

The *mat*K and *trn*L-F sequences of studied specimens were blasted against the NCBI Genbank database. The *mat*K and *trn*L-F sequences of this study along with their homologous sequences on the database were aligned and concatenated using ClustalW (Thompson *et al*., 1994). Phylogenetic analyses of the concatenated sequences of *mat*K and *trn*L-F were conducted using PAUP*4.0a146 (Swofford, 2002) with *Magnolia kobus* (Magnoliaceae) as the outgroup (Guo *et al*., 2017). Cluster

Figure 1: Annonaceae species in this study. a-c: *Artabotrys hexapetalus*, d-f: *Desmos cochinchinensis*, f-h: *Mitrephora thorelii*, i-j: *Goniothalamus touranensis*, K: *Polyalthia luensis.* Photos: Van Son Le.

Figure 2: Annonaceae species in this study. a-b: *Uvaria grandiflora,* c-d: *Sphaerocoryne affinis*, e-g: *Uvaria littoralis,* h-j: *Uvaria micrantha*, k-l: *Xylopia pierrei.* c-k: Van Son Le; a,b and l: Nga Nguyen-Phi.

supports were obtained by performing the bootstrap values of 50% or higher. The alignment tool of the NCBI database was employed to calculate the percentage of identity among sequences.

RESULTS AND DISCUSSION

The lengths of *trn*L-F sequences of 10 studied samples are ranging from 728 to 906 bps while those of *mat*K regions are ranging from 793 to 818 bps. The lengths of a combined data set (*trn*L-F and *mat*K) ranged from 1521 to 1724 bps. The *trn*L-F and *mat*K sequences of 10 studied species, including *A. hexapetalus*, *D. cochinchinensis*, *G. touranensis*, *M. thorelii*, *P. luensis*, *S. affinis*, *U. grandiflora*, *U. littoralis*, *U. micrantha* and *X. pierrei* were registered in NCBI database with the accession numbers of OL505587 and OL604142, OL505588 and OL604143, OL505589 and OL604144, OL505590 and OL604145, OL505591 and OL604146, OL505592 and OL604147, OL505593 and OL604148, OL505594 and OL604149, OL505595 and OL604150, OL505596 and OL604151, respectively. Based on these data, the phylogenetic trees showed that relationship among ten studied species collected from Binh Chau-Phuoc Buu Nature Reserve and other

Annonaceae plants from Vietnam were firstly established by using *mat*K (Figure 3), *trn*L-F (Figure 4) and combined *trn*L-F and *mat*K (Figure 5).

In three phylogenetic trees (Figure 3, 4 and 5), all ten studied species in this study were classified in the group of their genus,

Table 1: Detailed information of ten studies species collected from Binh Chau-Phuoc Buu Nature Reserve

Scientific names	voucher numbers	Collected sites
Artabotrys hexapetalus	AH	10⁰ 33′17.2″-107⁰ 31′19.3″
Desmos cochinchinensis	DC	10⁰ 32′57.4″-107⁰ 28′58.1″
Goniothalamus touranensis	GT	10⁰ 37′12.3″-107⁰ 32′14.3″
Mitrephora thorelii	MT	10⁰ 32′55.9″-107⁰ 28′57″
Polyalthia luensis	PL	10⁰ 32′58.4″-107⁰ 28′58.2″
Sphaerocoryne affinis	SA	10⁰ 32′43.2″-107⁰ 30′34.0″
Uvaria micrantha	UM	10⁰ 32′09.9″-107⁰ 28′29.2″
Uvaria littoralis	UL	10⁰ 32′37.8″-107⁰ 28′57.5″
Uvaria grandiflora	UG	10⁰ 32′18.0″-107⁰ 26′51.6″
Xylopia pierrei	XP	10⁰ 31′40.2″-107⁰ 27′46.9″

which implied the sequencing data were accurate. The data showed that the classification of species in the phylogenetic trees based on *mat*K sequences was similar to that of *trn*L-F sequences. However, when considering the individual gene regions (*mat*K or *trn*L-F) (Figures 3 and 4), some species of genera *Mitrephora* and *polyalthia* did not separate into two distinct groups in phylogeny analysis, *Mitrephora maingayi* and *M. thorelii* species, for example, tended to nest within species of genus *Polyalthia* instead. This is probably because those two genera are thought to have close morphological and genetic relationships (Chatrou et al., 2012; Guo et al., 2017). For instance, *Mitrephora* and *polyalthia* were grouped in the same tribe Miliuseae of the subfamily Malmeoideae (Guo et al., 2017) and placed closely in a phylogenetic tree constructed based on the combination of the three regions *rbc*L, the *trn*L intron and *trn*L-F (Chatrou et al., 2012).

Meanwhile, the result of phylogeny analysis based on the combination of *mat*K and *trn*L-F sequence regions (Figure 5)

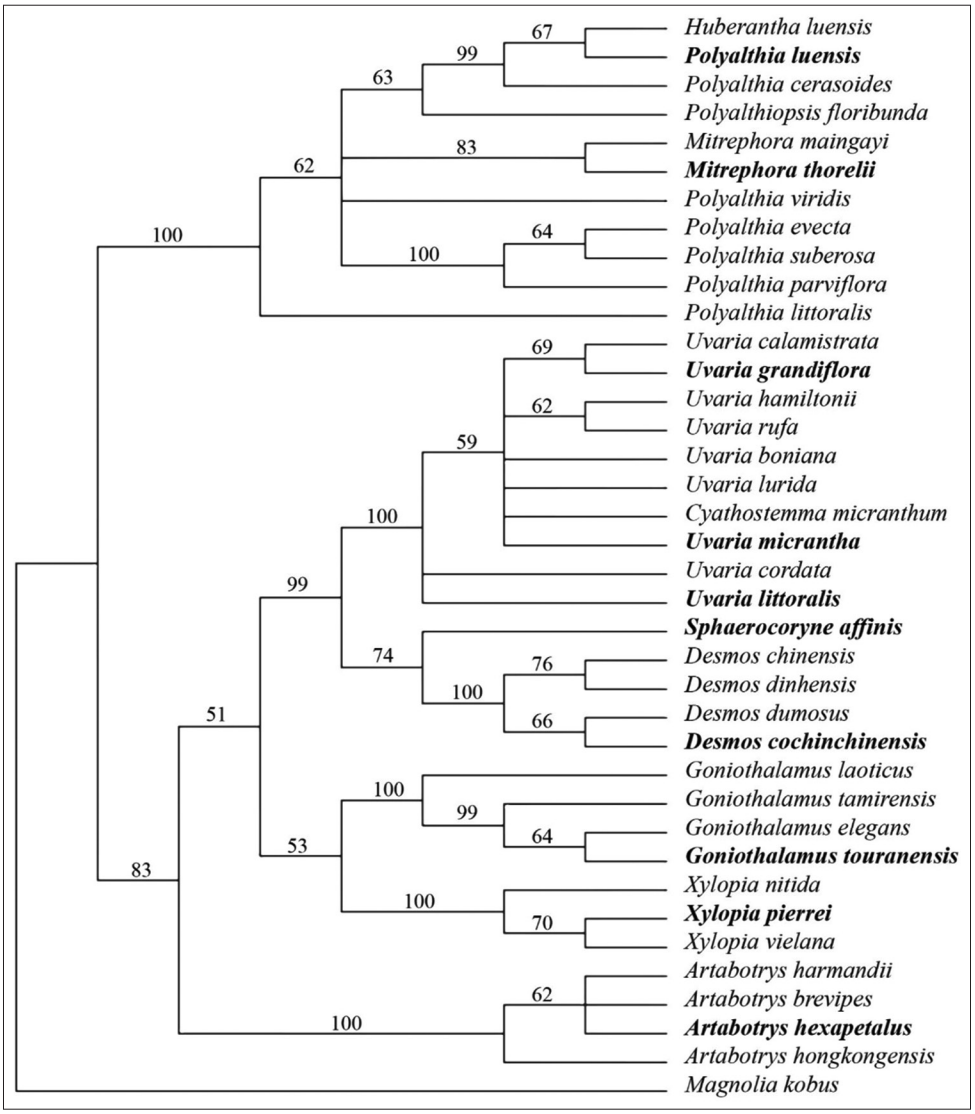

Figure 3: One of the largest parsimonious trees obtained based on the combined *trn*L-F IGS data sets. Gaps were treated as missing data. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes

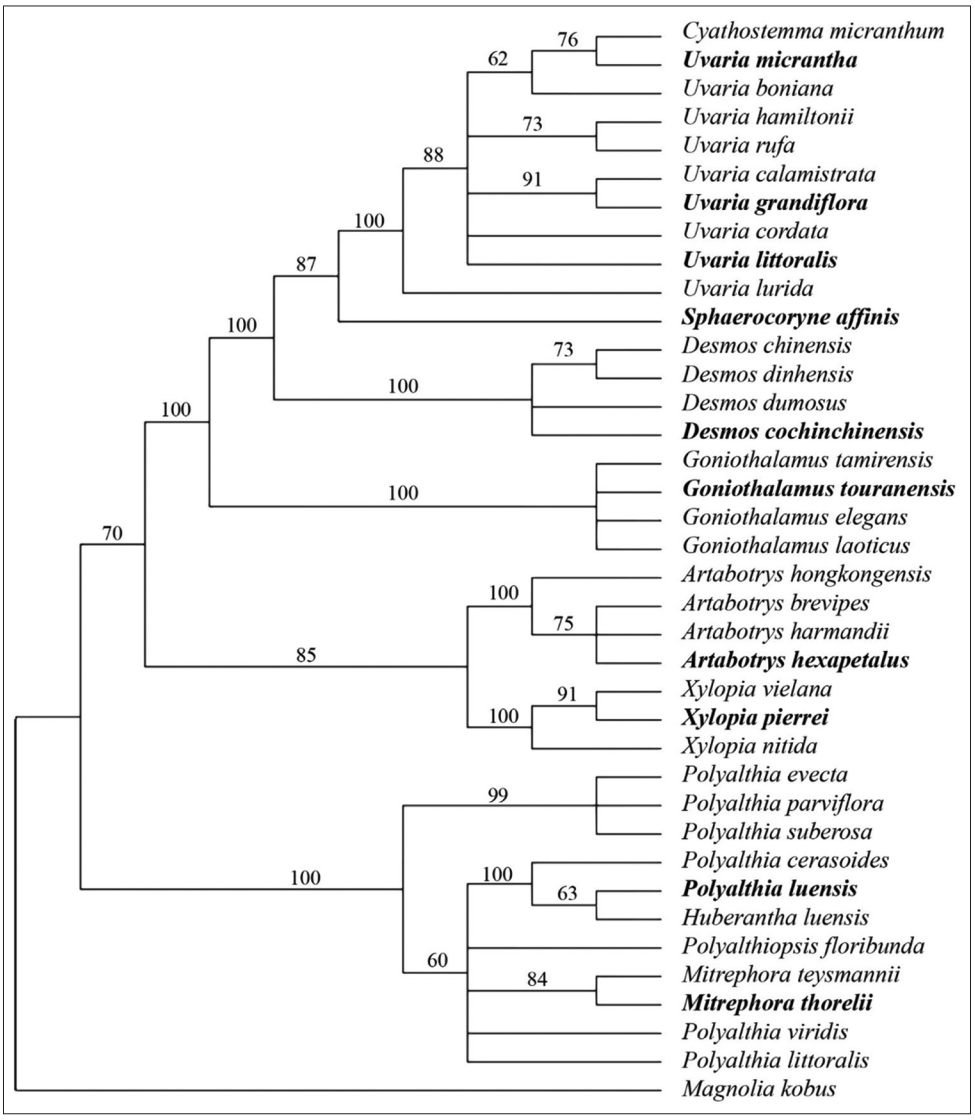

Figure 4: One of the largest parsimonious trees obtained based on the *mat*K data sets. Gaps were treated as missing data. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes

Table 2: Sequences of twenty-seven Annonaceae and one Magnoliaceae species from GenBank database used in this study

Scientific names	Accession numbers (*mat*K/*trn*L-F)	Scientific names	Accession numbers (*mat*K/*trn*L-F)
Artabotrys brevipes	MN207316/MN207411	*Polyalthia parviflora*	JX227892/JX227868
Artabotrys harmandii	KM924838/KM924936	*Polyalthia suberosa*	AY518833/AY319152
Artabotrys hongkongensis	HG004941/KM924937	*Polyalthia viridis*	AY518784/AY319154
Desmos chinensis	KP093297/JQ762415	*Uvaria boniana*	FJ743757/FJ743864
Desmos dinhensis	JQ768569/JQ768729	*Uvaria calamistrata*	FJ743759/FJ743866
Desmos dumosus	HG005013/JQ768730	*Uvaria cordata*	AB924906/JN175213
Goniothalamus elegans	EU715069/KM818850	*Uvaria hamiltonii*	FJ743765/FJ743871
Goniothalamus laoticus	EU715073/KM818881	*Uvaria lurida*	FJ743769/FJ743875
Goniothalamus tamirensis	EU715080/KM818866	*Uvaria rufa*	FJ743772/FJ743878
Mitrephora maingayi (Syn.: *M. teysmannii*)	AY518856/AY319109	*Xylopia nitida*	KX998984/MK797736
Polyalthia cerasoides	AY518854/AY319131	*Xylopia vielana*	KX998990/KM924964
Polyalthia evecta	AB924818/JX227861	*Huberantha luensis*	MG264586/MG264578
Polyalthiopsis floribunda	MG264583/MG264575	*Cyathostemma micranthum*	FJ743745/FJ743854
Polyalthia littoralis	AY518835/AY319140	*Magnolia kobus*	AY743476/AY743457

showed that the genus *Mitrephora* no longer nested within genus *Polyalthia*, but tended to separate although they were still closely placed on the phylogenetic tree. The result was consistent with many previous studies on the phylogeny analysis of Annonaceae in particular and in plants in general. In fact, the longer the DNA sequences used in the phylogeny analysis are, the more

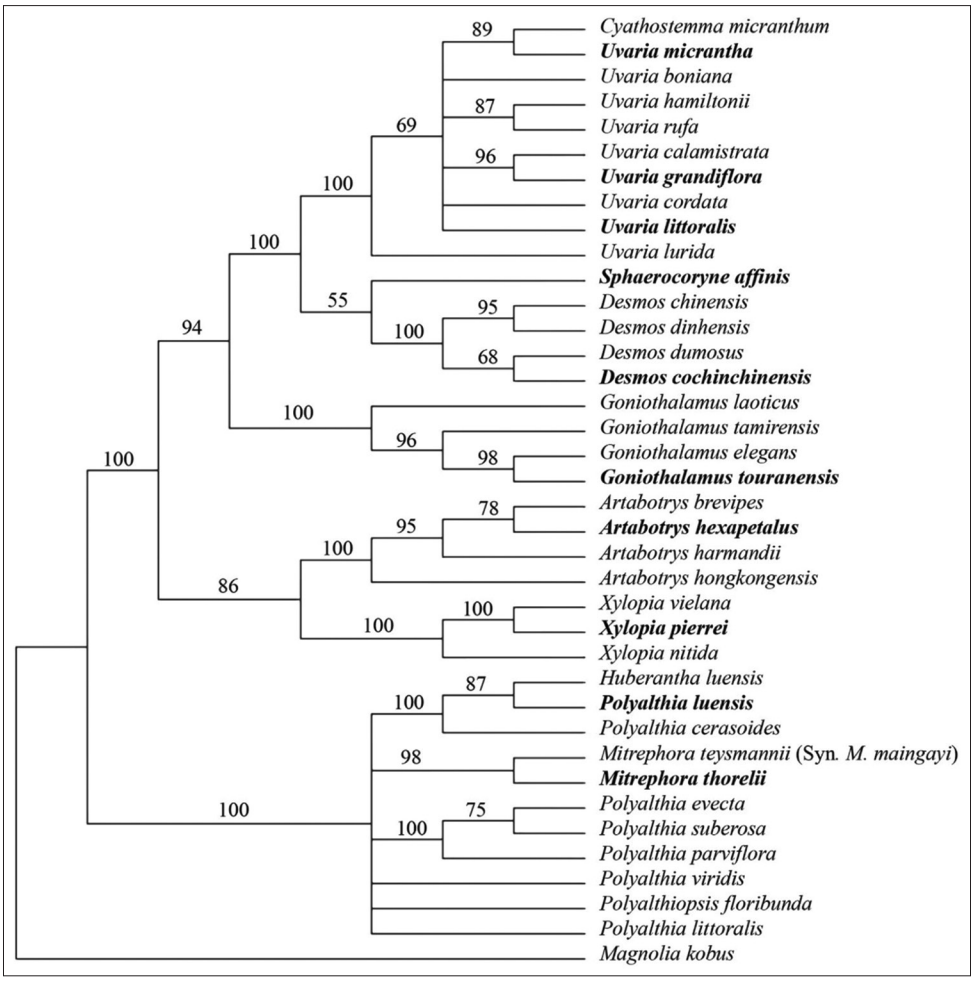

Figure 5: One of the largest parsimonious trees obtained based on the combined *trn*L-F and *mat*K data sets. Gaps were treated as missing data. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes

accurate the arrangement of species on the phylogenetic tree is (Doyle & Thomas, 1997; Rodriguez *et al*., 2016; Guo *et al*., 2017; Chaowasku, 2020).

Of the 10 Annonaceae species whose *mat*K and *trn*L-F regions were sequenced in this study, *Mitrephora thorelii* is supposed to be a synonym of *M. tomentosa*, while *M. maingayi* is a synonym of *M. teysmannii* (Weerasooriya & Saunders, 2005). Accordingly, *M. thorelii* (synonymous with *M. tomentosa*) was morphologically similar to *M. teysmannii* in some traits, such as petals densely pubescent abaxially; carpels 10-17 per flower (Weerasooriya & Saunders, 2005). On the phylogenetic tree (Figure 5), *M. thorelii* and *M. teysmannii* were placed in a same branch with a bootstrap value of 98%. And a detailed comparison of the *mat*K and *trn*L-F sequence regions of the two species *M. thorelii* and *M. teysmannii*, revealed a 100% similarity in the *mat*K region and a 99% similarity in the *trn*L-F region with 6 differences in 884 nucleotides.

Another species is *Uvaria littoralis*, which has a synonym of *U. cordata* (Meade & Parnell, 2018). The results from the three phylogenetic trees (Figures 3, 4 and 5) all revealed that the two species were in the same group, revealing a genetic similarity between them. The detailed comparison of the *mat*K and *trn*L-F sequence regions showed a 100% similarity in *mat*K region and a 99.88% similarity in *trn*L-F region with only 1 difference found in a total of 852 nucleotides. This result initially showed that there was a difference, at least in the *trn*L-F sequence region, between the two taxa (*Uvaria littoralis* and *U. cordata*) which are thought to be synonymous with each other. From the above results, we suggest that more other gene regions need to be studied to check for the differences (if any) between those 2 taxa to have a correct classification. Meanwhile, the other two species of the genus *Uvaria*, *U. micrantha* and *U. grandiflora*, were grouped with *U. boniana* and *U. calamistrata*, respectively. This finding was in line with the studies of Zhou et al. (2010) and Meade & Parnell (2018).

In addition, Sinclair (1955) transferred *Uvaria micrantha* to the genus *Cyathostemma* with another name of *Cyathostemma micranthum* (Meade & Parnell, 2018). However, *U. micrantha* is now an accepted species while *C. micranthum* is considered a synonym of *U. micrantha* (Meade & Parnell, 2018). The results from phylogeny analysis showed (Figure 5) that *U. micrantha* and *C. micranthum* were in the same group with a bootstrap value of 89%, supporting that those two species had genetic a similarity.

However, based on the detailed comparison of *mat*K and *trn*L-F sequence regions of *U. micrantha* species of in this study and *C. micranthum* species from the GenBank database, we found that *mat*K region had 2 differences in the total 785 nucleotides (99.75%) while the similarity in the *trn*L-F region was 99.87% (1 difference out of the total 757 nucleotides). The result also showed that there was a difference in genetic characteristics between the two taxa that are considered to be homologous.

Polyalthia luensis is now considered the synonym of *Huberantha luensis* (Pierre) Chaowasku. This species is a rare species and has only been discovered so far in Vietnam (Chaowasku *et al.*, 2018). The results presented on the phylogenetic tree (Figure 5) showed that *P. luensis* was grouped with *H. luensis* with a bootstrap value of 87%. The detailed comparison of the *mat*K and trnL-F sequence regions between *H. luensis* and *P. luensis* showed only one difference in the lengths of 790 and 891 pbs, respectively (with similarities of 99.87% and 99.89%). Furthermore, *Xylopia pierrei*, a rare species found only in Vietnam and Cambodia (Pham, 2000; Turner, 2018), has never been studied for genetic characteristics before. The results presented on the phylogenetic tree (Figure 5) showed that X. *pierrei* was grouped with X. *vielana* species (a species only distributed in Indochina) with a bootstrap value of 100%. The results of a detailed comparison of *mat*K and *trn*L-F sequences of the two species revealed similarities of 99.62% and 98.02%, respectively. Finally, the remaining species in this study, including *A. hexapetalus*, *S. affinis*, *D. cochinchinensis* and *G. touranensisis* had the arrangements on the phylogenetic tree similar to those of previous studies (Doyle & Thomas, 1997; Wang *et al.*, 2012; Tang *et al.* 2015a; Tang *et al.* 2015b; Guo *et al.*, 2017; Xue & Saunders, 2020).

CONCLUSION

In this study, the *mat*K and *trn*L-F sequences of ten Annonaceae species collected from Binh Chau-Phuoc Buu Nature Reserve were successfully amplified and sequenced. Notably, *trn*L-F regions of *P. luensis*, *G. touranensis*, *M. thorelii*, *U. micrantha* as well as X. *pierrei* (*mat*K and *trn*L-F) in this study were firstly published on the GenBank database. Along with other DNA sequences from the GenBank database, the phylogenetic trees for Annonaceae species from Vietnam have been established. The percentage of identity among sequences of studied species was also presented.

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