August 31, 2020

Genetic diversity of *Elephantopus mollis* H.B.K. and *Elephantopus scaber* L. based on DNA barcodes

Author's Details:

Nguyen Thi Thuy Nhien[1], Tran Gia Huy[1], Phung Thi Hang[2], Nguyen Trong Hong Phuc[2], Do Tan Khang[1*]

[1]Biotechnology Research and Development Institute, Can Tho University, Vietnam [2]School of Education, Can Tho University, Vietnam [*]Correspondence: Do Tan Khang (dtkhang@ctu.edu.vn), Biotechnology Research and Development Institute, Can Tho University, Vietnam

Received Date: 19-July-2020 Accepted Date: 10-Aug-2020 Published Date: 31-Aug-2020

Abstract

The study was conducted to compare the diversity of three sequences of DNA barcodes (atpF-atpH spacer, ITS region, and rbcL gene) of Elephantopus mollis H.B.K. and Elephantopus scaber L.. Three individuals were represented for each species and were collected at an indigenous location in Bay Nui area, An Giang province, Vietnam. The results showed that DNA barcodes were successfully amplified. The ITS and rbcL regions had no difference in the nucleotide sequences of samples in each species, except that atpF-atpH region had a difference at the 37th nucleotide position of E. mollis H.B.K. The results of sequence polymorphism analysis showed that the ITS region had higher variation than the two regions of atpF-atpH and rbcL through the percent identity of 89.58, 98.99 and 100%, respectively. The result of analyzing phylogenetic trees of DNA sequences through genetic distance and bootstrap values showed that the ITS region could genetically distinguish between E. mollis H.B.K. and E. scaber L. and from some other species higher than the other two regions.

Keywords: *atpF-atpH, E. mollis H.B.K., E. scaber L., ITS, rbcL.*

INTRODUCTION

In the world, species of *Elephantopus* (Asteraceae) are commonly used in traditional (folk) medicine for the treatment of nephritis, edema, dampness, pain in the chest, fever, scabies, and arthralgia due to wound and cough of pneumonia (Kabiru and Por, 2013). In Vietnam, the genus *Elephantopus* was recorded to include two species, *Elephantopus scaber* L. and *Elephantopus mollis* H.B.K. (Ho, 2003; Bien, 2007). The biological activities of these two species have been studied such as antioxidant activity (Ganga *et al*., 2012; Sopan and Vijay, 2016), antifungal, antibacterial (Consolacion *et al*., 2009; Anitha *et al*., 2011), anti-inflammatory (Singh *et al*., 2006), anti-nausea, anti-allergic, anti-cancer (Kabeer and Prathapan, 2014). These two herbal plants in the world literature show a lot of morphological and anatomical similarities (Bunwong *et al*., 2014; Loeuille *et al*., 2015). Hence, the confusion over their names have also been noted (Loi, 2004). Currently in plant taxonomic research, besides methods based on morphological and anatomical characteristics, molecular genetic methods are also applied. In particular, DNA barcodes is one of the most common method approach based on short DNA sequences with an evolutionary rate fast enough to give fast and accurate classification results (Mishra *et al*., 2016). In plants, the commonly used DNA barcodes are sequences of the nucleus and the chloroplast genome (Kress *et al*., 2005; Hollingsworth *et al*., 2009). For *E. scaber* L. and *E. mollis* H.B.K., the nuclear ITS region and plastid *rbc*L gene were used in studies at the genetics of *Elephantopus* genus classification system (Keeley *et al*., 2007; Schilling, 2013; Gong *et al*., 2018). In addition, the *atp*F–*atp*H intergenic sequence of these two species has not been recorded in the National Center for Biotechnology Information (NCBI) database. Until now, such species are commonly used in traditional medicine in Vietnam but their research on DNA barcoding is limited. Therefore, the aim of this study to study used three regions of DNA sequencing (*atp*F-*atp*H spacer, ITS region and *rbc*L gene to supplement the genetic database at the molecular level. The results of this study are to investigate the genetic relationship of the two species and contribute to the development of this medicinal resource locally.

MATERIALS AND METHODS

Experimental material

Three individuals were represented for each *E. mollis* H.B.K. and *E. scaber* L. sample were collected following the method from Thin (2007). The scientific name was identified based on morphological characteristics (Ho, 2003; Bien, 2005) (Fig 1). All individuals were collected at an indigenous location in Bay Nui area, An Giang province (Table 1).

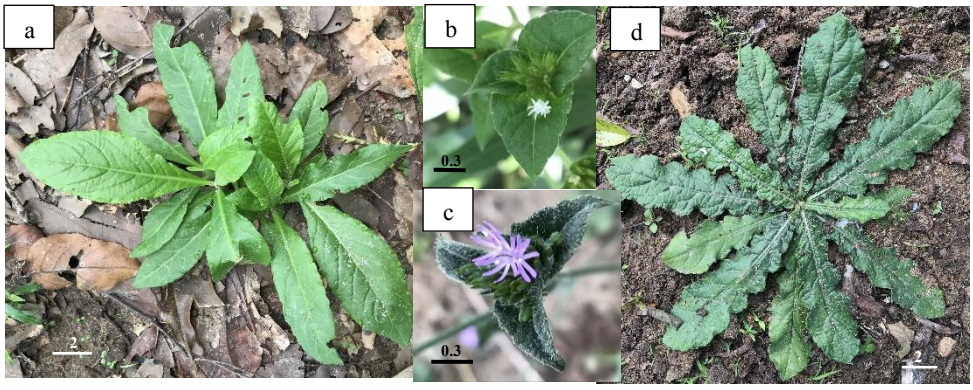

Fig. 1: Morphology of *E. mollis H.B.K. (a and b); E. scaber L. (c and d); Bar: cm*

Table 1: Information on studied samples

Sign	Species	Location of sampling	Coordinates	
			N	E
Mol_1	*Elephantopus mollis* H.B.K.	Medicinal center (Cam Mountain, An Giang)	10º 29'57.38"	105º 00'02.35"
Mol_2		Bo Hong peak (Cam Mountain, An Giang)	10º 29'43.83"	104º 58'57.32"
Mol_3		Tri Ton District, An Giang	10º 25'24.47"	105º 00'12.00"
Sca_1	*Elephantopus scaber* L.	Medicinal center (Cam Mountain, An Giang)	10º 29'57.38"	105º 00'02.35"
Sca_2		To Mountain, An Giang	10º 25'07.59"	104º 58'51.01"
Sca_3		Tri Ton District, An Giang	10º 25'24.59"	104º 56'45.02"

Construction of DNA barcode library for *atp*F-*atp*H, ITS and *rbc*L

Total DNA was isolated following the CTAB-based protocol (Rogers and Bendich, 1988) and modified Dung (2011). DNA purity and intact were assessed by 0.8% (w/v) agarose electrophoresis at 100 V for 20 minutes. Primers and thermal cycles for amplification (ABI 9700, USA) of three DNA barcodes were listed in Table 2. Amplicons underwent 2% (w/v) agarose electrophoresis to confirm the PCR yield and specificity. DNA sequencing was conducted by the Sanger method (ABI 3130, USA).

Table 2: Primer sequences, annealing temperatures and expected product length for three DNA barcode loci; *atp*F-*atp*H, ITS and *rbc*L

Region	Primer name	Sequences (5'-3')	Annealing temperature (Time)	Expected product length	Sources
*atp*F-*atpH*	*atp*F	ACTCGCACACACTCCCTTTCC	51ºC 40s	196-573 bp	Vijayan and Tsou, 2010
	*atp*H	GCTTTTATGGAAGCTTTAACAAT			
ITS	ITS1	TCCGTAGGTGAACCTGCGG	55ºC 30s	500-700 bp	White *et al.*, 1990
	ITS4	TCCTCCGCTTATTGATATGC			
*rbc*L	*rbc*L-aF	ATGTCACCACAAACAGAGACTAAAGC	55ºC 30s	550-600 bp	Kress *et al.*, 2005
	*rbc*L-aR	GTAAAATCAAGTCCACCRCG			

Data analysis

DNA sequences were tested for nucleotide accurate confirmation and aligned by the Clustal W algorithm using Bioedit software (Thompson *et al*., 1994). The Basic Local Alignment Search Tool (BLAST) was used to find the similarity sequence against the NCBI database. The phylogenetic tree was constructed by the

Maximum Likelihood method and genetics distance was conducted by MEGA X (Kumar *et al*., 2018). Bootstrapping with 1000 replication was used for inferring the confidence of phylogenetic tree.

RESULTS AND DISCUSSION

Result of total DNA extraction and cloning DNA barcode fragments using PCR

The electrophoresis results showed that the DNA regions were successfully amplified and consistent with the expected product length (Fig 2). The results on the electrophoresis showed DNA bands are thick, clear, with no additional products, it proves that PCR products are specific, can be purified and directly used to proceed to determine the nucleotide sequence of barcode fragments *E. mollis* H.B.K. và *E. scaber* L..

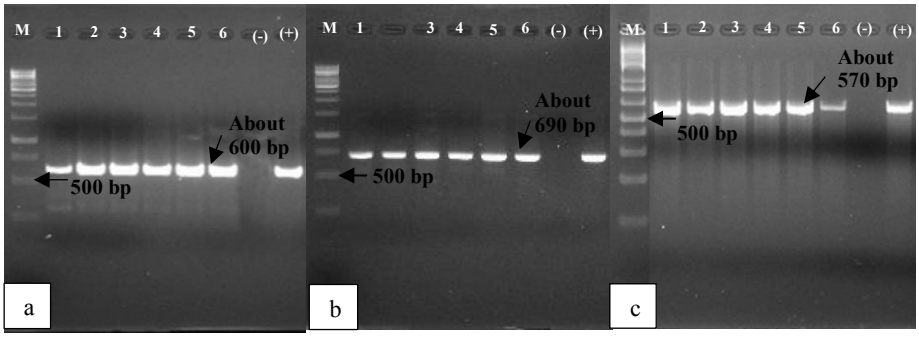

Fig. 2: Results of electrophoresis of the samples

a) PCR result of atpF-atpH gene fragment cloning b) ITS; c) rbcL; 1) Mol_1; 2) Mol_2; 3) Mol_3; 4) Sca_1; 5) Sca_2; 6) Sca_3; M: standard scale: 1 kb (a,b) and 100 bp (c); (-): Negative control; (+): Positive control.

The result of identifying and analyzing the nucleotide sequence of the DNA barcode

The result of the atpF-atpH spacer

The sequence of *atp*F-*atp*H region in the PCR product of the samples was determined with a size of 542 nucleotides (Fig 3). There are differences between the composition of nucleotides of the two studied species (Table 3). Both *E. mollis* H.B.K. and *E. scaber* L. had the highest proportion of Thymine (T) nucleotides, accounting for 37.13%, 37.64% and the lowest proportion was Cytosine (C) nucleotide with 14.96%, 15.13%, respectively. Two species had lower G-C content (33.43% in *E. mollis* H.B.K. vs. 33.46% in *E. scaber* L.) and higher transition. Results showed that the three sequences of *E. scaber* L. did not differ in the nucleotide composition. However, the *atp*F-*atp*H sequence of *E. mollis* H.B.K was different when losing nucleotide A (Adenine) at 37th position of both Mol_2 and Mol_3 samples. In this region, the sequence between such species has five different nucleotide points (corresponding 5 SNPs), accounting for 0.92% of the whole sequence (Fig 3). The result of comparing the *atp*F-*atp*H sequence between the two species showed that the three *E. scaber* L. samples have percent identity of 99.08% with Mol_1 samples and 98.89% similarity with samples Mol_2 and Mol_3 of *E. mollis* H.B.K.. The sequence comparison on Genbank showed that the *atp*F-*atp*H region of two species of *E. mollis* H.B.K. and *E. scaber* L. has not been published. Compared with other species, the samples in this region have the highest BLAST identity of 96.56% with *Lychnophora pinaster* Mart. (code number: MF804924). It belongs to the family Asteraceae and is vulnerable to extinction and found exclusively in the Minas Gerais State-Brazil[26]. In addition, the *atp*F-*atp*H sequence of the two species also resulted in a similarity less than 96% compared to other species (e.g., *Brachylaena huillensis*, *Atractylodes macrocephala* and *Cichorium intybus*).

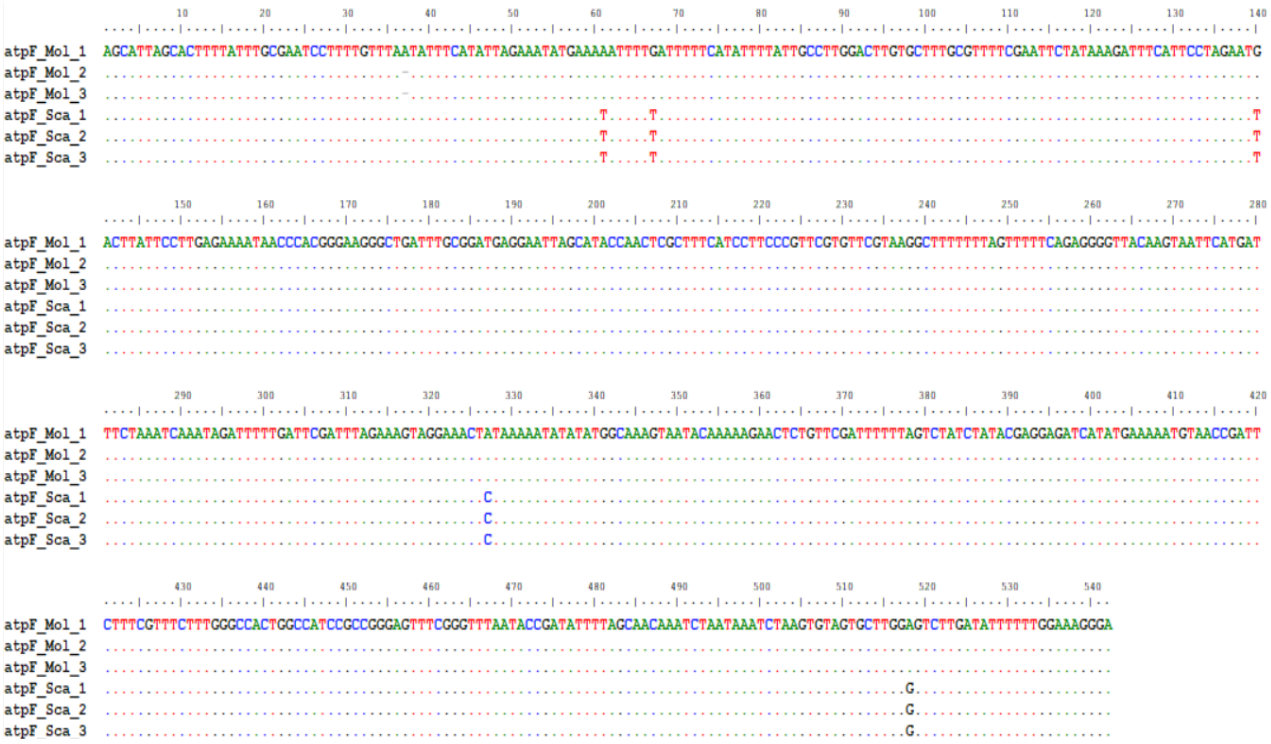

Fig. 3: The results of alignment sequences with the *atp*F-*atp*H region

The result of the ITS region

The ITS region of nuclear ribosomal DNA is important molecular markers in phylogenetic analyses and is often used to classify molecular levels for terrestrial plants, with a high rate of evolution and sequence variation (China Plant BOL Group *et al*., 2011). The ITS sequence region selected for analysis has the lengths of 647 nucleotides (Fig 4). The comparison results showed that in the same species, there was no difference in the nucleotide sequences of samples. The proportion of nucleotides in these two species is different (Table 3). In two species of *E. mollis* H.B.K. and *E. scaber* L. both had Guanine (G) nucleotides, accounting for 27.09%, 27.86%, respectively. The lowest nucleotide content was Adenine (A) nucleotides for *E. mollis* H.B.K. accounting for 23.37% and *E. scaber* L. was Thymine (T) nucleotides accounting for 22.29%. Both species had higher G-C content (52.79% in *E. mollis* H.B.K. vs. 54.80% in *E. scaber* L.) and lower transition. The percent identity of the ITS region between the two species was 89.58%, with 72 different positions between the sequences of the two species (accounting for 11.13% of the whole sequence). The sequence polymorphism in this region was relatively high, with differences such as addition, loss and nucleotide changes occurring between sequences (Fig 4). The comparison with the Genbank database, for the ITS region, the sequences of the two studied species are highly similar and consistent with the sequence of the corresponding species. Specifically, the three samples of *E. mollis* H.B.K. were high similarity with the gene sequence code HQ158400 (99.70%) and the samples of *E. scaber* L. were 97.5% similarity to the corresponding gene sequence (code number: KP052671). These two similar sequences were collected in Thailand and China. However, their article information has not been published yet.

Fig. 4: The results of alignment sequences with the ITS region

The result of the rbcL gene sequence

The *rbc*L gene region sequences with a length of 536 nucleotides were analyzed, the results showed no difference between all the sequences, 100% similarity (Table 3). Both species had the highest ratio of Thymine (T) nucleotides with 30.22% and the lowest was Cytosine (C) nucleotides with 19.78%, content G-C ratios (43.47%) lower than the content A-T ratios (56.53%). The sequences had the highest similarity with 98.95% with the gene sequence code JQ933320 of *E. scaber* L. on Nepal. With the advantages studied, the *rbc*L gene region is commonly used in analyzing genetic relationships in plants (Newmaster *et al*., 2006). However, the level of variation is low and the ability to distinguish species is not high (Kress *et al*., 2005). According to Li *et al*. (2012) that have demonstrated that in *E. scaber* L. there was no phenotypic trait variation of rbcL gene region with intra-specific variation was 0.0%. Hence, in this region it is difficult to distinguish between *E. mollis* H.B.K. and *E. scaber* L.

Table 3: The composition nucleotides of the studied sequences

Region sequences	The ratio of nucleotides (%)				Total Nu	Region sequences	The ratio of nucleotides (%)				Total Nu
	A	C	G	T			A	C	G	T	
ITS											
Mol_1	23.37	25.7	27.09	23.84	646	Sca_1	22.91	26.93	27.86	22.29	646
Mol_2	23.37	25.7	27.09	23.84	646	Sca_2	22.91	26.93	27.86	22.29	646
Mol_3	23.37	25.7	27.09	23.84	646	Sca_3	22.91	26.93	27.86	22.29	646
***atp*F-*atp*H**											
Mol_1	29.52	14.94	18.45	37.08	542	Sca_1	28.97	15.13	18.27	37.64	542
Mol_2	29.39	14.97	18.48	37.15	541	Sca_2	28.97	15.13	18.27	37.64	542
Mol_3	29.39	14.97	18.48	37.15	541	Sca_3	28.97	15.13	18.27	37.64	542
***rbc*L**											
Mol_1	26.31	19.78	23.69	30.22	536	Sca_1	26.31	19.78	23.69	30.22	536
Mol_2	26.31	19.78	23.69	30.22	536	Sca_2	26.31	19.78	23.69	30.22	536
Mol_3	26.31	19.78	23.69	30.22	536	Sca_3	26.31	19.78	23.69	30.22	536

The sequences in the three genomic regions were compared through analysis indices: conserved sites, variable sites, parsimony informative sites, singleton sites (Table 4). In all three regions, the sequence shows that the number of conserved sites is higher than the variable sites and the singleton sites are not recorded. In the *rbc*L gene region, because the analysis sequences were 100% similar to each other, the remaining indices were equal to zero. The ITS region has the lowest number of conservation sites (89.16%), so the number of variable sites and the parsimony-informative sites accounts for 10.84%, higher than *atp*F-*atp*H (0.92%). In

the study of Li *et al*. (2012) also showed that informative polymorphic characters of *rbc*L region was lower than ITS region in *E. scaber* L.. However, atpF-atpF spacer and *E. mollis* B.H.K. were not mentioned in this study. The sequence comparison results showed that ITS region had a higher sequence and species variation than *rbc*L gene sequence and *atp*F-*atp*H spacer.

Table 4: The comparison indexes between sequences

Region	conserved sites	Ratio (%)	variable sites	Ratio (%)	Parsim-info sites	Ratio (%)	Singleton sites	Ratio (%)
*atp*F-*atp*H	537/542	99.08	5/542	0.92	5/542	0.92	0/542	0
ITS	576/646	89.16	70/646	10.84	70/646	10.84	0/646	0
*rbc*L	356/356	100	0/356	0	0/356	0	0/356	0

The result of analyzing phylogenetic trees of DNA sequences

From the results of sequence analysis, the phylogenetic tree showed the genetic relationship of the two studied species with the species in the *Elephantopus* genus and some other species in the family are compared. The Maximum Likelihood analysis method on MEGA X software with a bootstrap value of 1000 replication was used to check the correlation and accuracy of the diagram. The results showed that the *atp*F-*atp*H region were classified into two main branches, the first consisting of two samples of *Lychnophora pinaster* Mart. (code number: MF804924), *Brachylaena huillensis* O. Hoffm. (code number: EU626809) and the second branch of the study sample. The genetic distance between the two main branches was about 0.6. Six sequences *atp*F-*atp*H region of two species of *E. mollis* H.B.K. and *E. scaber* L. were arranged in two sub-branches corresponding to each species with genetic distance of 0.002 (Fig 5a).

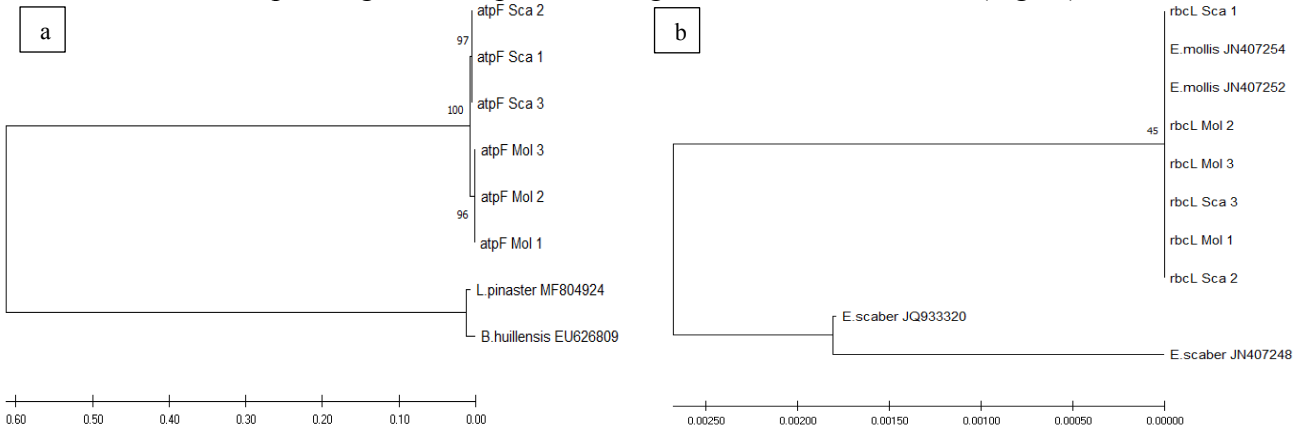

Fig. 5: The phylogenetic tree depicting the genetic relationship between *atp*F-*atp*H region samples (a) and *rbc*L region samples (b) were constructed by Maximum Likelihood method by MEGA X software, bootstrapping with 1000 replication

Four *rbc*L gene region sequences of two species of *E. mollis* H.B.K. and *E. scaber* L. were compared[30] (Fig 5b). The six studied samples were grouped with the sequences JN407252 and JN407254 of *E. mollis* H.B.K.. The gene sequences code JQ933320 and JN407248 of *E. scaber* L. were about to be grouped. The genetic distance of these two main branches was relatively low at 0.0036. The results showed that the genetic distance of *E. mollis* H.B.K. and *E. scaber* L. in the rbcL region is 0.00 (Fig 6a). Therefore, it is difficult to distinguish the two species in this region and moreover, the *rbc*L gene region has had poor classification results in the genus *Elephantopus* (Saslis-Lagoudakis *et al*., 2012). The concatenated sequence tree of *atp*F-*atp*H and *rbc*L region was established to show more clearly the genetic distance between the two studied species. The results showed that the genetic distance of the two species was 0.0023 and higher than the construction based on the single sequence of the atpF-atpH region (0.002) and *rbc*L gene region (0.00). The overall increase in sequence length would lead to smaller variances for evolutionary distances and other parameters in Maximum Likelihood analysis method (Gadagkar *et al*., 2005).

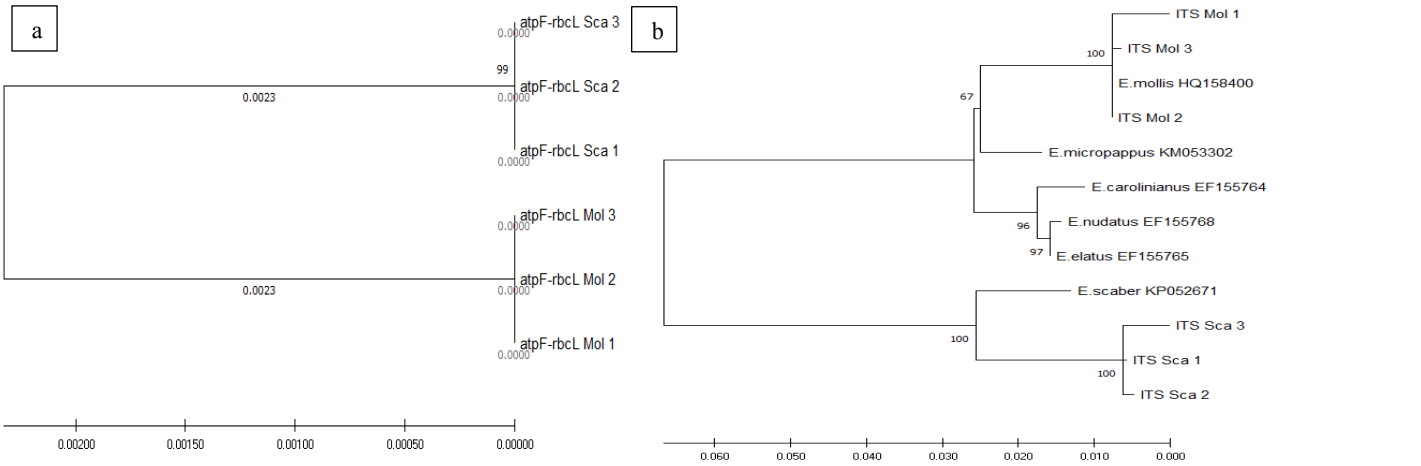

Fig. 6: The phylogenetic tree depicting the genetic relationship between concatenated *atp*F-*atp*H + *rbc*L of six studied samples (a) and ITS region samples (b) was constructed by Maximum Likelihood method by MEGA X software, bootstrapping with 1000 replication.

The ITS region sequences of other species in the genus *Elephantopus* (Keeley *et al*., 2007) were compared with the six samples of the study (Fig 6b). The results of phylogenetic trees showed that the studied sequences are closely related and grouped with the sequence of each species respectively on Genbank, the gene sequence code KP052671 (of *E. scaber* L.) and the gene sequence code HQ158400 (of *E. mollis* H.B.K.) with genetic distances of 0.027 and 0.01, respectively. These branches all had a bootstrap value of 100% (Fig). High bootstrap values showed the accuracy and reliability of each branch in the phylogenetic tree (Efron *et al*., 1996). In addition, the sequences of *E. mollis* H.B.K showed a close relationship with *Elephantopus micropappus* (code number: KM053303) with a genetic distance of 0.025.

When comparing three phylogenetic trees using three DNA barcoding including *atp*F-*atp*H, *rbc*L and ITS combined results of nucleotide sequence comparison, the results showed that the ITS sequence region showed the genetic distance of *E. mollis* H.B.K. and *E. scaber* L. was the highest with 0.049, followed by the *atp*F-*atp*H sequence region (0.002) and the lowest was the *rbc*L sequence region (0.00). These results were consistent with the related barcode DNA studies in the genus *Elephantopus* (Li *et al*., 2012; Tnah *et al*., 2019). Therefore, the ITS region allowed the distinction between *E. mollis* H.B.K. and *E. scaber* L. and distinguished from some other species higher than the other two regions.

CONCLUSION

The study successfully amplified three DNA barcodes including the intergenic spacer *atp*F-*atp*H, ITS sequence region and *rbc*L gene sequence of *E. mollis* H.B.K. and *E. scaber* L. in Bay Nui area, An Giang province. Results of sequence polymorphism analysis showed that there was no difference in the nucleotide sequence of ITS and *rbc*L in samples in the same species. For the *atp*F-*atp*H spacer, *E. mollis* H.B.K. was different when losing a nucleotide of type A (Adenine) at the 37th nucleotide position of Mol_2 and Mol_3 samples. The percent identity between the two species in the ITS region was lowest (89.58%) and the highest was 100% in the *rbc*L region. Therefore, the number of variable sites and parsimony-informative sites in the ITS region accounted for 10.84% higher than *atp*F-*atp*H and *rbc*L region. The result of the sequence comparison on GenBank showed that the *atp*F-*atp*H region of *E. mollis* H.B.K and *E. scaber* L has not been published. The comparison results from the phylogenetic trees of the three DNA barcodes through genetic distance and bootstrap values showed that the ITS region allowed the distinction between *E. mollis* H.B.K. and *E. scaber* L. and distinguished from some other species higher than the other two regions. These suggest that the ITS region was better able to distinguish species than *atp*F-*atp*H and *rbc*L region.

ACKNOWLEDGMENT

The research was supported by funding research projects at Can Tho University in 2019.

REFERENCES

i. *Anitha, V.T., Marimuthu, J., and Antonisamy, S.J., 2011. Anti-bacterial studies on Hemigraphis colorata (Blume) H.G. Hallier and Elephantopus scaber L. Asian Pacific Journal of Tropical Medicine. (2012): 52-57.*

ii. *Bien, L.K., 2007. Flora of Vietnam, Vol 7. Science and Technics Publishing House. Hanoi, 746 pages (In Vietnamese).*

iii. *Bunwong, S., Chantaranothai, P., and Keeley, S.C., 2014. Revisions and key to the Vernonieae (Compositae) of Thailand. PhytoKeys. 37: 25-101.*

iv. *CBOL Plant Working Group, A DNA barcode for land plants, Proceedings of the National Academy of Sciences, 106(31) 12794-12797.*

v. *China Plant BOL Group, Li, D.Z., Gao, L.M. et al., 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proceedings of the National Academy of Sciences. 108(49): 19641-19646.*

vi. *Consolacion, Y.R., Agnes, B.A., and Chang, S.C., 2009. Antimicrobial terpenoids from Elephantopus mollis. NRCP Research Journal. 10(1): 33-38.*

vii. *Dung, T.N., 2011. Molecular Biology Lab Manual. Can Tho University Publishing House. Can Tho, 169 pages (In Vietnamese).*

viii. *Efron, B., Halloran, E., and Holmes, S., 1996. Bootstrap confidence levels for phylogenetic trees. PNAS. 3(23): 13429.*

ix. *Gadagkar, S. R., Rosenberg, M. S., and Kumar, S., 2005. Inferring species phylogenies from multiple genes: Concatenated sequence tree versus consensus gene tree. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution. 304B(1): 64–74.*

x. *Ganga, R.B., Venkateswara, R.Y., Pavan, S., and Dasari, V.S.P., 2012. Qualitative and quantitative phytochemncal screening and in vitro anti oxidant and anti microbial activitles of E. seaber Linn. Recent Res. Sci. Technol. (4): 15-20.*

xi. *Gong, L., Qiu, X.H., Huang, J. et al., 2018. Constructing a DNA barcode reference library for southern herbs in China: A resource for authentication of southern Chinese medicine. PLoS ONE. 13(7): e0201240.*

xii. *Ho, P.H., 2003. An Illustrated Flora of Vietnam, Vol 2. Tre Publishing. Ho Chi Minh, 951 pages (In Vietnamese).*

xiii. *Kabeer, F. A., and Prathapan, R., 2014. Phytopharmacological Profile of Elephantopws scaber. Research article pharmacologia. 5(8): 272-285.*

xiv. *Kabiru, A., and Por, L.Y., 2013. Elephantopus Species: Traditional Uses, Pharmacological Actions and Chemical Composition. Advances in Life Science and Technology. 15: 2225-2239.*

xv. *Keeley, S.C., Forsman, Z., and Chan, R., 2007. A phylogeny of the "evil tribe" (Vernonieae: Compositae) reveals Old/New World long distance dispersal: Support from separate and combined congruent datasets (trnL-F, ndhF, ITS). Molecular Phylogenetics and Evolution. 44(1): 89-103.*

xvi. *Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A., and Janzen, D.H., 2005. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences of the United States of America. 102(23): 8369-8374.*

xvii. *Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution. 35:1547-1549.*

xviii. *Li, M., Wong, K.L., Chan, W.H. et al., 2012. Establishment of DNA barcodes for the identification of the botanical sources of the Chinese 'cooling' beverage. Food Control. 25(2): 594-600.*

xix. *Loeuille, B., Keeley, Sterling C.S., and Pirani, J.R., 2015. Systematics and Evolution of Syncephaly in American Vernonieae (Asteraceae) with Emphasis on the Brazilian Subtribe Lychnophorinae. Systematic Botany. 40(1): 286–298.*

xx. *Loi, D.T., 2004. Vietnamese Medicinal Plants and Herbs. Medical Publishing House. Ha Noi, 1274 pages (In Vietnamese).*

xxi. *Máthé, Á., 2015. Medicinal and Aromatic Plants of the World. Medicinal and Aromatic Plants of the World. 3: 309-316.*

xxii. *Mishra, P., Kumar, A., Nagireddy, A. et al., 2016. DNA barcoding: an efficient tool to overcome authentication challenges in the herbal market. Plant Biotechnol. J. 14: 8–21.*

xxiii. *Newmaster, S.G., Fazekas, A.J., and Ragupath, S., 2006. DNA barcoding in land plants: evaluation of rbcL in a multigene tiered approach. Canadian Journal of Botany. 84(3): 335-341.*

xxiv. *Rogers, S.O., and Bendich, A.J., 1988. Extraction of DNA from plant tissue. In plant molecular biology manual. A6: 1-10.*

xxv. *Saslis-Lagoudakisa, C.H., Vincent, S., Williamson, E.M. et al., 2012. Phylogenies reveal predictive power of traditional medicine in bioprospecting. Proc Natl Acad Sci. 109: 15835-15840.*

xxvi. *Schilling, E.E., 2013. Barcoding the Asteraceae of Tennessee, tribes Cardueae and Vernonieae. Phytoneuron. 19(2013): 1–7.*

xxvii. *Singh, S.D.J., Krishana, V., Mankini, K.L., Manjunatha, B.K., Vidya, S.M., and Manohara, Y.N., 2004. Wound healing activity of the leaf extracts and deoxyelephantopin isolated from Elephantopus scaber Linn. Indian J Phanmacol. 37(4): 238 – 242.*

xxviii. *Sopan, N.K., and Vijay, D.M., 2016. Synthesis, characterization and studies on antioxidant activity of silver nanoparticles using Elephantopus scaber leaf extract. Materials Science and Engineering. (62): 719–724.*

xxix. *Thin, N.N., 2008. Methods of plant research. Hanoi National University Publishing House. Hanoi, 165 pages (In Vietnamese).*

xxx. *Thompson, J.D., Higgins, D.G., and Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties amd weight matrix choice. Nucleic Acids Research. 22(22): 4673-80.*

xxxi. *Tnah, L.H., Lee, S.L., Tan, A.L. et al., 2019. DNA barcode database of common herbal plants in the tropics: a resource for herbal product authentication. Food Control. 95: 318-326.*

xxxii. *Vijayan, K., and Tsou, C.H., 2010. DNA barcoding in plants: taxonomy in a new perspective. Current science. 99: 1530-1540.*

xxxiii. *White, T.J., Bruns, T., Lee, S., and Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to methods and Application, eds. Academic Press, Inc., New York. 5: 315-322.*