Evaluation of Genetic Diversity and Identification of *Huperzia* Species Collected in Some Different Areas in Vietnam by Molecular Markers

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Abstract. The objective of this study was to evaluate genetic diversity of 35 *Huperzia* samples collected from the different geographical areas in Vietnam by using *rbc*L markers. The results revealed that the samples were genetically diverse in high genetic similarity which ranged from 98.0 to 99.0%, respectively. Based on the Genbank data in comparison, it was ascertained that only one sample (BK3) is a member of *H.phlegmarioides* group, and others belonged *H. squarrosa* group as divided into two main groups by phylogenetic tree analyses. It suggests that some *Huperzia* samples may plausibly descend from the same origin, evolution and arising relations. This study has provided useful information for further identifying the high-quality genetic plant sources for propagation, development and conservation of the high-valued medicinal materials of *Huperzia* plants in this country.

Introduction

Huperzia species such as *H. squarrosa* (HS) or *Lycopodium squarrosum* (LS) species belongs to the family of Lycopodiaceae. This species is widely distributed in many countries in the world, and available in the tropical and temperate regions with over 415 species in global [1]. They are adapted in mountainous regions with the height from 1000 to 3000 m above sea level [2]. In Vietnam, these plants are well-defined as high tolerance to heat, drought and growing in thin forests. They are also obtainable in some places from the North to the Centre of this country [3] and are easily found in the moist forests and rainforests at high altitude in and amongst mosses and other angiosperms epiphytes [4]. The major morphological characteristics of *H. squarrosa* were narrated as shown in Figure 1. The stem is pendulous and up to 60 cm long, branching regularly dichotomous and isotomous; their leaves microphyllous, spirally arranged, not imbricated and sharply acute, entire, rigid, coriaceous, shining, veins simple with no branches or unions extended to a middle portion (Figure 1 A, B).
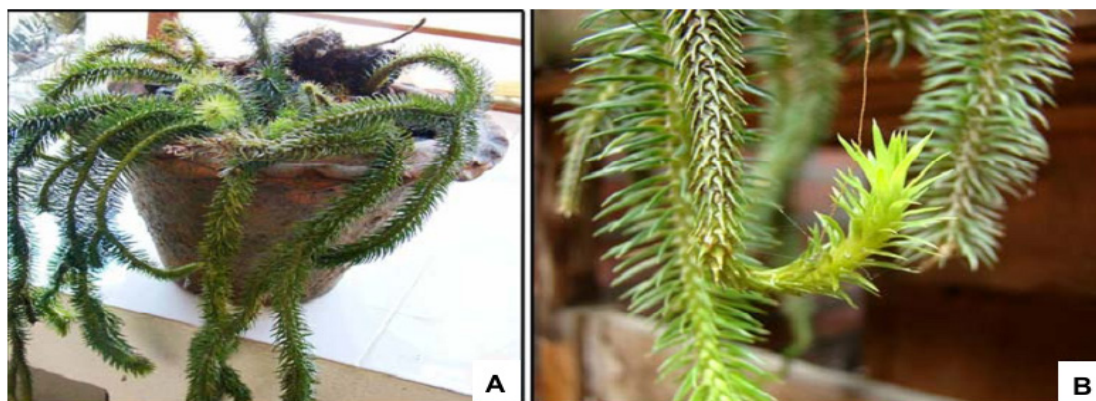

Figure 1. Morphological structures of the sporophytic body in *H. squarrosa* (G.Forst) Trev. A: Habit; B: upward turned stem [4]

H. squarrosa is known as a highly valuable medicinal plant, which contains alkaloids, flavones, triterpenes, phenolic acids, especially including Huperzine A (Hup A), Huperzine B (Hup B), N-methyl-huperzine B, Huperzinine, Lycoporine A, Carina-tumine A, etc [5]. To the best of our knowledge, over 300 Lycopodium alkaloids have been reported [6]. According to the report of Ngoc et al [7], the alkaloids isolated from *H. squarrosa* collected from Lam Dong province – Vietnam includes 6 main compounds: lycosquarosine A, acetylaposerratinine, huperzine A, huperzine B, 8α-hydrophlemariurine B and huperzinine which may account for the effectiveness of this plant in the treatment of several brain diseases [8].

This plant is traditionally used to treat many ailments like cold, fever, bruises, pain, strains, contusion, stasis swelling, rheumatism etc. The numerous studies on pharmacological effects have been done such as anticonvulsant, anti-inflammatory, antioxidant, anti-aoptosis, and organophosphate poisoning myasthenia gravis [9]. Interestingly, this plant has enhanced the neurotransmitters, prevent plaque formation, tangles in the brain, feed and nourish brain cells, which positive response to Alzheimer's, Parkinson's, brain atrophy and dementia and diseases related to brain nerve damage, preventing memory decline or brain atrophy [10-13].

The Internal Transcribed Spacer (ITS) is one of the most popular tools and widely used DNA fragments in plant molecular systematics at the genetic and molecular species levels due to its potentially high resolution of inter-and intraspecific relationship [14]. The ITS region is argued to be useful for low-level phylogenetic analysis because of its relatively fast rate of evolution [15]. Moreover, ITS is considered as one of the most feasible molecular markers for angiosperm phylogenetic inference and genetics relatedness which is a proven useful source of characteristics for phylogenetic studies in the plant kingdom [16]. In the current application, DNA sequences from a standardized gene region are possibly generated from a small tissue sample taken from the known species [17]. Typically, ITS sequences have provided the molecular evidence to evaluate the phylogeny of several taxonomic groups from the genus and subfamily of the plants.

In the world, *Huperzia* is listed in a red book as an endangered plant [18]. In Vietnam, this is one of the rarest species carrying precious genetic resources, which are being degraded and also included in the red book that needs to be protected, developed and appropriately exploited. However, in Vietnam, customarily, the botanists have identified and classified the relatedness of the valuable species based on the morphological and cytological characteristics. The disadvantages of these methods are restricted due to the environmental effects and diagnostic resolution. Hence, the objective of this study was to assess the genetic diversity and identification of 35 samples collected from different areas by using *rbc*L markers. In this study, the sequences of the chloroplast gene ribulose-1-5-bisphosphate carboxylase/oxygenase (*rbc*L) were to use for genetic variation and identification of the *Huperzia* samples.

Materials and Methods

Materials collection

A total of 35 samples of *Huperzia* species was collected in the different provinces in Vietnam. The detail information of the samples as shown in Table 1.

Table 1. List of the collected *Huperzia* samples used in this study

No	Origin	Code	No	Origin	Code
1	Cho Moi – Bac Kan	BK1	19	Tram Tau – Yen Bai	YB1
2	Na Ri – Bac Kan	BK2	20	Van Tran – Yen Bai	YB2
3	Cho Don – Bac Kan	BK3	21	Luc Yen – Yen Bai	YB3
4	Thai An – Quan Ba – Ha Giang	HG1	22	Lam Binh – Tuyen Quang	TQ1
5	Vung Tai – Quan Ba – Ha Giang	HG2	23	Na Hang-Tuyen Quang	TQ2
6	Viet Lam – Vi Xuyen – Ha Giang	HG3	24	Ham Yen-Tuyen Quang	TQ3
7	Tra Linh – Cao Bang	CB1	25	Sa Pa- Lao Cai	LC1
8	Nguyen Binh – Cao Bang	CB2	26	Van Ban- Lao Cai	LC2
9	Trung Khanh – Cao Bang	CB3	27	Muong Khuong- Lao Cai	LC3
10	La Hien – Vo Nhai – Thai Nguyen	TN1	28	That Khe-Lang Son	LS1
11	Quy Ky – Thai Nguyen	TN2	29	Huu Lung -Lang Son	LS2
12	Sang Moc – Vo Nhai – Thai Nguyen	TN3	30	Loc Binh -Lang Son	LS3
13	Doan Hung – Phu Tho	PT1	31	Tay Thien-Vinh Phuc	VP1
14	Tan Son – Phu Tho	PT2	32	Phong Nha-Ke Bang-Quang Binh	QB1
15	Yen Lap – Phu Tho	PT3	33	Quynh Nhai-Son La	SL1
16	Sin Ho – Lai Chau	LCH1	34	Hoa Binh	HB1
17	Muong Te – Lai Chau	LCH2	35	Vang Vieng - Laos	TDL
18	Tam Duong – Lai Chau	LCH3			

Total DNA extraction and amplification of rbcL

The fresh samples were collected and intermediately transferred to the laboratory for DNA extraction. In this study, DNA extract was done following the CTAB method of Doyle and Doyle [19] with some minor modifications. The yielded DNA products were then confirmed and determined by the use of Spectrophotometer [20]. The forward primer *rbc*L-F was 5''-ATGTCACCACAAACAGAGACTAAAGC-3' and the reverse primer *rbc*L-R was 5'-CTTCTGCTACAAATAAGAATCGATCTC-3', respectively.

Amplification was made in a polymerase chain reaction (PCR) in the tuber 0.2 ml with total volume (15 µl), of which contained 9.0 µl deionized distilled water, buffer Mg+ 25 Mm (1.5 µl), dNTPs 10 Mm (0.3 µl), Taq ADN polymerase 5 U/µl (0.2 µl), ITS1 10 µM (1.5 µl), ITS8 10 µM (1.5 µl) and DNA 50 ng/µl (1.0 µl), respectively. PCR program was performed as the method described by Trung et al [15]. Briefly, denaturation was made for 94ºC, 56ºC for annealing, and 72ºC for extension and 72ºC for the final extension. Gel removal Qiagen Kit was applied for PCR products.

ITS sequencing and phylogenetic analysis

Sequencing was carried out at Macrogen company (Korea) by applying ABI PRISM 3700 DNA Analyzer (Applied Biotech). The data were used to analyze by MEGA 5 software to generate

a phylogenetic tree with neighbour-joining (NJ) methods. BLAST tool was used to compared the obtained sequences to National Center for Biotechnology Information databases (NCBI).

Results and Discussion

Molecular markers application to identify Huperzia samples based on ITS region sequences

Recently, the advances in molecular systematics in the plants have been significantly improved. The chloroplast gene ribulose-1-5-bisphosphate carboxylase/oxygenase for the large subunit of *rbc*L located on the chloroplast genome which is a proper use for the interference of phylogenetic relationships at the higher taxonomic levels [21]. Moreover, it is conspicuous that the molecular approach has been powerful and accuracy for phylogenetic analysing in the plant which is arduous to decipher by phenotyping. Hence, in this study, a total of 35 samples were collected and analysed by *rbc*L markers. The DNAs of 35 samples of *Huperzia* species had enough high concentration and quality for further experiments. All bands were clean and enough quality for further experiment. PCR reaction was performed with the primers *rbc*L. The results showed that 35 samples of *Huperzia* were a single-form band with the size of 650 bp (Figure 1), which was a resemblance to the report of Sundary and Papuangan [22] who applied the *rbc*L gene for analysing of *Syzygium aromaticum*.

The purified PCR products with the primers *Rbc*L were directly analyzed by use of the ABI PRISM 3700 DNA Analyzer (Applied Biotech) of Macrogen company (Korea) and MEGA v5.1 software. The sequences of leaf samples were compared to the published NCBI reference sequences (corresponding to each species) using Blast tool (Basic Local Alignment Search Tool).
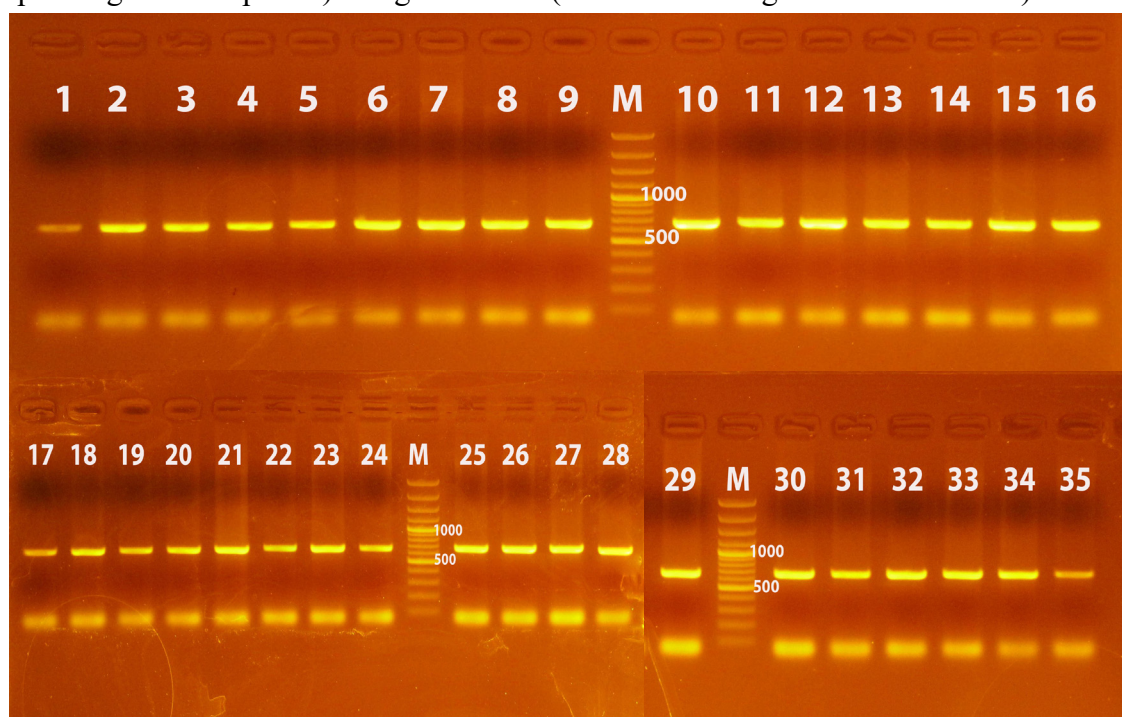
Figure 2. PCR products of the *H. squarrosa* samples with pair primers *Rbc*L-F/*Rbc*L-R; M: Marker 100bp plus DNA

The results showed that the sequences of 35 samples had a similar percentage from 98.0% to 99.0% in comparison to NCBI. In particular, 02 *Huperzia* samples having 98.0% of similarity to NCBI references (PT2 and LC3) were identified. The remaining 33 samples showed a similar percentage of 99,0% in comparing to Genbank (DQ464235.1 and AJ133896.1) as indicated in Table 1. Our obtained results were similar to some previous studies to use *rbc*L to identify the higher taxonomic levels [23-24].

Table 2. Comparison of ITS sequences of 35 *Huperzia* samples on Blast
(http://blast.ncbi.nlm.nih.gov/Blast.cgi)

No	Code	Taxon	GenBank Accession No.	ID (%)
1	BK1	*Huperzia squarrosa*	DQ464235.1	99.0
2	BK2	*Huperzia squarrosa*	DQ464235.1	99.0
3	BK3	*Huperzia phlegmarioides*	AJ133896.1	99.0
4	HG1	*Huperzia squarrosa*	DQ464235.1	99.0
5	HG2	*Huperzia squarrosa*	DQ464235.1	99.0
6	HG3	*Huperzia squarrosa*	DQ464235.1	99.0
7	CB1	*Huperzia squarrosa*	DQ464235.1	99.0
8	CB2	*Huperzia squarrosa*	DQ464235.1	99.0
9	CB3	*Huperzia squarrosa*	DQ464235.1	99.0
10	TN1	*Huperzia squarrosa*	DQ464235.1	99.0
11	TN2	*Huperzia squarrosa*	DQ464235.1	99.0
12	TN3	*Huperzia squarrosa*	DQ464235.1	99.0
13	PT1	*Huperzia squarrosa*	DQ464235.1	99.0
14	PT2	*Huperzia squarrosa*	DQ464235.1	98.0
15	PT3	*Huperzia squarrosa*	DQ464235.1	99.0
16	LCH1	*Huperzia squarrosa*	DQ464235.1	99.0
17	LCH2	*Huperzia squarrosa*	DQ464235.1	99.0
18	LCH3	*Huperzia squarrosa*	DQ464235.1	99.0
19	YB1	*Huperzia squarrosa*	DQ464235.1	99.0
20	YB2	*Huperzia squarrosa*	DQ464235.1	99.0
21	YB3	*Huperzia squarrosa*	DQ464235.1	99.0
22	TQ1	*Huperzia squarrosa*	DQ464235.1	99.0
23	TQ2	*Huperzia squarrosa*	DQ464235.1	99.0
24	TQ3	*Huperzia squarrosa*	DQ464235.1	99.0
25	LC1	*Huperzia squarrosa*	DQ464235.1	99.0
26	LC2	*Huperzia squarrosa*	DQ464235.1	99.0
27	LC3	*Huperzia squarrosa*	DQ464235.1	98.0
28	LS1	*Huperzia squarrosa*	DQ464235.1	99.0
29	LS2	*Huperzia squarrosa*	DQ464235.1	99.0
30	LS3	*Huperzia squarrosa*	DQ464235.1	99.0
31	VP1	*Huperzia squarrosa*	DQ464235.1	99.0
32	QB1	*Huperzia squarrosa*	DQ464235.1	99.0
33	SL1	*Huperzia squarrosa*	DQ464235.1	99.0
34	HB1	*Huperzia squarrosa*	DQ464235.1	99.0
35	TDL	*Huperzia squarrosa*	DQ464235.1	99.0

As the results attained, it demonstrated that 35 samples collected from different geographical provinces were accurately the *Huperzia* species. The next step was to confirm at the molecular level by comparing the sequences of each species by using the corresponding reference species in both location and length of nucleotides applying the MEGA v5.1 software (Table 3 and Figure 3).

Table 3. Sequence length analyses of total nucleotides of the 35 *Huperzia* samples

No	Sample code	Total of Nucleotides	No	Sample code	Total of Nucleotides
1	BK1	615	19	YB1	614
2	BK2	615	20	YB2	613
3	BK3	616	21	YB3	616
4	HG1	616	22	TQ1	613
5	HG2	616	23	TQ2	615
6	HG3	616	24	TQ3	613
7	CB1	614	25	LC1	615
8	CB2	613	26	LC2	614
9	CB3	614	27	LC3	614
10	TN1	615	28	LS1	613
11	TN2	615	29	LS2	614
12	TN3	616	30	LS3	615
13	PT1	614	31	SL1	616
14	PT2	613	32	HB1	615
15	PT3	613	33	TDL	614
16	LCH1	615	34	VP1	614
17	LCH2	615	35	QB1	613
18	LCH3	618			

The data shown in Table 3 disclosed that the sequence lengths of the samples were consistent with the results of the PCR band size as presented in Figure 3.
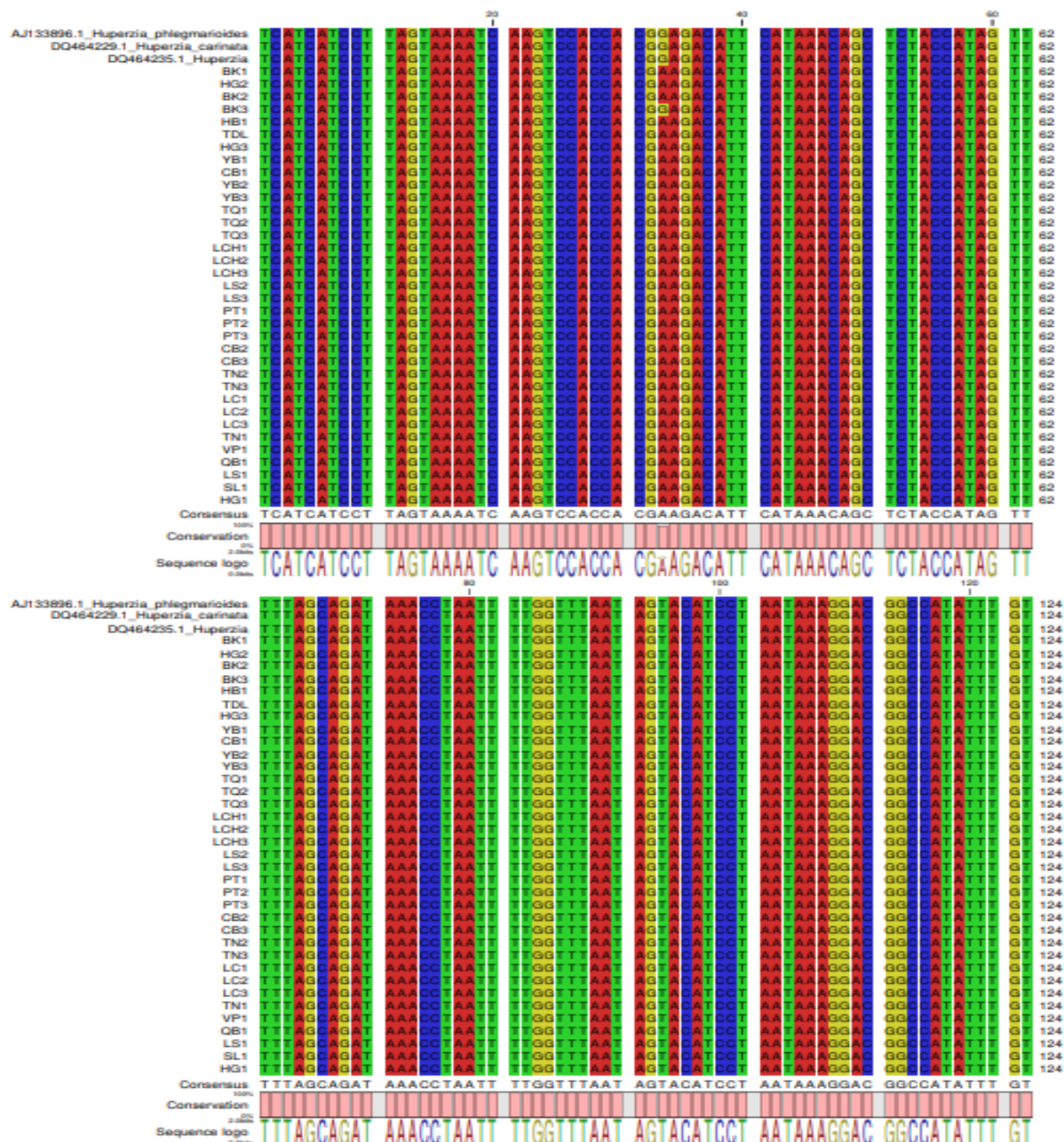
Figure 3. Comparison of a nucleotide sequence of 35 *Huperzia* samples with the reference sequence species

In comparing to the database from the NCBI Genbank, the results of 35 *Huperzia* samples were highly similar to the corresponding sequences. Additionally, they were consistent with the results when comparing nucleotide sequences to find the corresponding species via Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Among them, there was only one sample (BK3) belonging to *H.phlegmarioides* group, and the others of the 34 samples were clustered in *H.squarrosa* group, respectively (Figure 3 and Figure 4). In the fact that the available databases on the *rbc*L gene are very useful and facilitated to identify phylogenetic relationships of many plant species at interfamilial levels [25, 22]. Dubisson [26] successfully applied *rbc*L sequences of 18 species of the fern genus *Trichomanes* for assessing their systematic relationships which showed a more accurate reflection of the generic concept applied to other fern and angiosperms.

Genetic diversity and genetic relationships between the 35 samples of Huperzia samples

The data in Table 3 showed that the sequences of 35 *Huperzia* samples had a high similarity. The phylogenetic tree of 35 *Huperzia* samples was generated by using the Mega 6.0 software following the Maximum likelihood method (Figure 4). The results of the polygenetic relationship analysis have revealed that the phylogenetic tree divided 35 *Huperzia* samples into two main different clusters:

The first cluster included the sequences of 34 *Huperzia* samples and reference species of *H.squarrosa* (DQ464235.1 on GenBank). The samples in this group had very high genetic similarity in comparing to the reference species in a range from 95.47% (between the LCH3 sample with the reference species) to 96.25% (between the samples YB2, TQ1, TQ3, PT2, PT3, CB2, QB1 and LS1 with reference species), respectively.
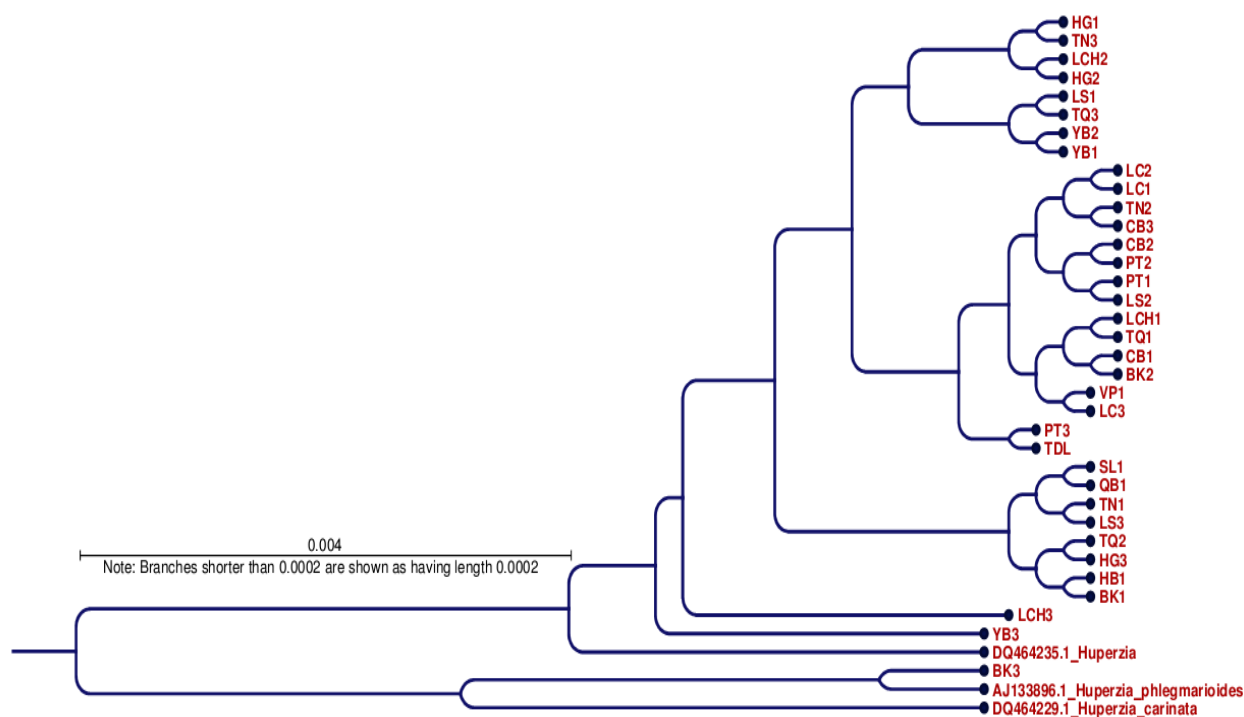
The second cluster comprised the sequence of the *Huperzia* sample of BK3 (collected from the Cho Don - Bac Kan) which was matching with *H. phlegmarioides* (AJ133896.1 on GenBank) and *H.carinata* (DQ464229.1 on GenBank) in the same branch. The genetic similar coefficient of this sample was 95.62% in comparing to the reference species *H. phlegmarioides* was 94.81% to compare with the reference species *H.carinata*.

To the best our knowledge, over 251 plant species have been successfully applied and reached 100% by use of two primary types [27, 22], and acquired a greater length (around 1.428 to 2500 bp) by applying full-length *rbc*L [28]. Additionally, Kesanakuthi et al [29] used *rbcL* sequence alone to investigate 85% plant species groups of all root samples, and spelling out the ecological factors that contributed to the subterranean spatial organization of plant diversity in nature. By using datasets of Genbank, some works used the blast algorithm to examine the ability of *rbc*L as well as various combination of the nuclear ribosomal ITS and 5.8S regions to make accurate identification in land plants species [30-31]. Moreover, ptDNA markers, *ITS* such as ITS2 has had great effective DNA barcoding marker with over 92% successful identification in 6600 plant samples [32]. Also, some recent reports have ascertained that using *rbc*L+ *matK* as the standard barcode for the flora of moderate phylogenetic dispersion was up to 92% of the species that could be differentiated the plant kingdom [33-34].

In this study, based on region sequences, a total of 35 *Huperzia* samples were successfully in analysed their species-level identification by using *rbc*L primers and compared NCBI Genbank databases. However, due to the lack of species-level variation which may hinder species-level identification. Therefore, it should be applied *rbc*L combining ITS, *mat*K, *trn*H-*psb*A for species-level accuracy identification of the plants. Moreover, some specifically different DNA regions from both the plastid genome such as *rbc*L, *rpo*B, *rpo*C1, *mat*K, *ycf*1 and *trn*H-*psb*A and nuclear genome ITS should be further applied for accurate identification of various species.

Table 4. Genetic homology coefficients between the 35 *Huperzia* samples

	AJ133896	DQ464229	DQ464423	TN1	HG2	BK2	BK3	HB1	TDL	HG3	YB1	QB1	YB2	YB3	TQ1	TQ2	TQ3	LCH1	LCH2	LCH3	LS2	LS3	PT1	PT2	PT3	QB2	QB3	TN2	TN3	LC1	LC2	LC3	TH1	VP1	QB1	LS1	SL1	HG1
AJ133896.1_Huperzia_phlegmarioides																																						
DQ464229.1_Huperzia_carinata	97.64																																					
DQ464235.1_Huperzia	97.31	98.65																																				
BK1	94.15	94.63	95.93																																			
HG2	93.99	94.48	95.78	99.51																																		
BK2	94.15	94.63	95.93	99.51	99.68																																	
BK3	95.62	94.81	94.27	98.38	98.06	98.54																																
HB1	94.15	94.63	95.93	100	99.51	99.51	98.38																															
TDL	94.3	94.79	96.09	99.19	99.35	99.67	98.21	99.19																														
HG3	93.99	94.48	95.78	99.84	99.35	99.35	98.22	99.84	99.03																													
YB1	94.3	94.79	96.09	99.51	99.51	99.51	98.38	99.51	99.19	99.35																												
QB1	94.3	94.79	96.09	99.51	99.51	99.84	98.38	99.51	99.51	99.35	99.67																											
YB2	94.45	94.94	96.25	99.35	99.35	99.35	98.21	99.35	99.35	99.19	99.04	99.51																										
YB3	94.15	94.63	95.93	99.03	99.19	99.19	98.05	99.03	99.19	98.87	98.86	99.19	99.02																									
TQ1	94.45	94.94	96.25	99.35	99.35	99.67	98.21	99.35	99.67	99.19	99.04	99.51	99.84	99.67	99.35																							
TQ2	94.15	94.63	95.93	100	99.51	99.51	98.38	100	99.19	99.84	99.51	99.51	99.35	99.03	99.35																							
TQ3	94.45	94.94	96.25	99.35	99.35	99.35	98.21	99.35	99.35	99.19	99.04	99.51	100	99.02	99.67	99.35																						
LCH1	94.15	94.63	95.93	99.51	99.68	100	98.54	99.51	99.67	99.35	99.51	99.84	99.35	99.19	99.67	99.51	99.35																					
LCH2	94.15	94.63	95.93	99.35	99.04	99.51	98.7	99.35	99.51	99.19	99.35	99.35	99.51	99.35	99.51	99.35	99.51	99.51																				
LCH3	93.69	94.17	95.47	98.87	98.71	99.03	97.58	98.87	98.71	99.03	98.71	99.03	98.54	98.55	98.87	98.87	98.54	99.03	98.55																			
LS2	94.3	94.79	96.09	99.51	99.51	99.84	98.38	99.51	99.51	99.35	99.67	100	99.51	99.19	99.84	99.51	99.51	99.84	99.35	99.03																		
LS3	94.15	94.63	95.93	100	99.51	99.51	98.38	100	99.19	99.84	99.51	99.51	99.35	99.03	99.35	100	99.35	99.51	99.35	98.87	99.51																	
PT1	94.3	94.79	96.09	99.51	99.51	99.84	98.38	99.51	99.51	99.35	99.67	100	99.51	99.19	99.84	99.51	99.51	99.84	99.35	99.03	100	99.51																
PT2	94.45	94.94	96.25	99.19	99.35	99.67	98.21	99.19	99.67	99.03	99.19	99.51	99.35	99.19	99.67	99.19	99.35	99.67	99.51	98.71	99.51	99.19	99.51															
PT3	94.45	94.94	96.25	99.35	99.35	99.67	98.21	99.35	99.51	99.19	99.51	99.84	99.35	99.03	99.67	99.35	99.35	99.67	99.19	98.87	99.04	99.35	99.04	99.35														
QB2	94.45	94.94	96.25	99.35	99.35	99.67	98.21	99.35	99.67	99.19	99.51	99.84	99.67	99.35	100	99.35	99.67	99.84	99.35	98.87	99.84	99.35	99.84	99.67	99.67													
QB3	94.3	94.79	96.09	99.35	99.51	99.84	98.38	99.35	99.84	99.19	99.35	99.67	99.51	99.35	99.84	99.35	99.51	99.84	99.35	98.71	99.67	99.35	99.67	99.84	99.51	99.84												
TN2	94.15	94.63	95.93	99.51	99.68	100	98.54	99.51	99.67	99.35	99.51	99.84	99.35	99.19	99.67	99.51	99.35	100	99.51	99.03	99.84	99.51	99.84	99.67	99.67	99.84	99.84											
TN3	93.99	94.48	95.78	99.51	100	99.68	98.06	99.51	99.35	99.35	99.51	99.51	99.51	99.35	99.51	99.51	99.35	99.68	99.04	98.71	99.51	99.51	99.51	99.35	99.35	99.51	99.68											
LC1	94.15	94.63	95.93	99.51	99.68	100	98.54	99.51	99.67	99.35	99.51	99.84	99.35	99.19	99.67	99.51	99.35	100	99.51	99.03	99.84	99.04	99.67	99.67	99.67	99.84	100	99.68										
LC2	94.3	94.79	96.09	99.51	99.51	99.84	98.38	99.51	99.35	99.35	99.67	100	99.51	99.19	99.84	99.51	99.51	99.84	99.35	99.03	99.51	99.04	99.04	99.67	99.04	99.51	99.84			99.84								
LC3	94.3	94.79	96.09	99.51	99.51	99.84	98.38	99.51	99.51	99.35	99.67	100	99.51	99.19	99.84	99.51	99.51	99.84	99.35	99.03	100	99.51	100	99.51	99.35	99.67	99.84	99.51	99.84	100								
TH1	94.15	94.63	95.93	100	99.51	99.51	98.38	100	99.19	99.84	99.51	99.51	99.35	99.03	99.35	100	99.35	99.51	99.35	98.87	99.51	100	99.51	99.19	99.35	99.35	99.35	99.51	99.51	99.51	99.51	99.51						
VP1	94.3	94.79	96.09	99.51	99.51	99.84	98.38	99.51	99.51	99.35	99.67	100	99.51	99.19	99.84	99.51	99.51	99.84	99.35	99.03	99.84	99.84	99.67	99.84	99.51	99.84	100	100	99.51								
QB1	94.45	94.94	96.25	99.67	99.19	99.19	98.05	99.67	99.19	99.51	99.19	99.19	99.35	99.02	99.35	99.67	99.35	99.19	99.35	98.54	99.19	99.67	99.19	99.51	99.82	99.35	99.35	99.19	99.19	99.19	99.19	99.19	99.67	99.19				
LS1	94.45	94.94	96.25	99.35	99.35	99.19	98.05	99.35	99.35	99.19	99.04	99.51	100	99.02	99.67	99.35	100	99.82	99.51	98.54	99.51	99.35	99.35	99.35	99.35	99.35	99.51	99.51	99.51	99.51	99.35	99.67	99.19				
SL1	93.99	94.48	95.78	99.84	99.35	99.35	98.22	99.84	99.03	100	99.35	99.35	99.19	98.87	99.19	99.84	99.19	99.03	99.35	99.04	99.19	99.84	99.19	99.19	99.19	99.35	99.35	99.35	99.35	99.84	99.35	99.51	99.19				
HG1	93.99	94.48	95.78	99.51	100	99.68	98.06	99.51	99.35	99.35	99.51	99.51	99.35	99.19	99.35	99.51	99.35	99.68	99.04	98.71	99.51	99.51	99.51	99.35	99.35	99.51	99.68	100	99.68	99.51	99.51	99.51	99.51	99.51	99.19	99.35	99.35	

HG1
TN3
LCH2
HG2
LS1
TQ3
YB2
YB1
LC2
LC1
TN2
CB3
CB2
PT2
PT1
LS2
LCH1
TQ1
CB1
BK2
VP1
LC3
PT3
TDL
SL1
QB1
TN1
LS3
TQ2
HG3
HB1
BK1
LCH3
YB3
DQ464235.1_Huperzia
BK3
AJ133896.1_Huperzia_phlegmarioides
DQ464229.1_Huperzia_carinata

0.004
Note: Branches shorter than 0.0002 are shown as having length 0.0002

Figure 4. Generated phylogenetic tree species of 35 *Huperzia* samples with the reference species

Conclusions

All the *Huperzia* samples collected from some different geographical provinces in Vietnam had similar ITS sequences in compare to NCBI corresponding library reference. Via the application of chloroplast region sequence with the *Rbc*L-F /*Rbc*L-R primers, a total of 34 samples was identified as the group of *H. squarrosa* species (DQ464235.1 on GenBank) with the range from 95.47% to 96.25%. The *Huperzia* sample of BK3 collected from Cho Don - Bac Kan was grouped as *H. phlegmarioides* species with a similar coefficient of 95.62%.

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