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Evaluating Genetic Diversity and Chemical Substances of *Disporopsis longifolia* Craib Samples Collected in Vietnam

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Abstract

Disporopsis longifolia Craib is a valuable medicinal plant and has been recorded in the Red Data Book in many countries, including Vietnam. Fifteen samples of *Disporopsis* (leaves and tubers) were collected in the fifteen different communes of five districts in Ha Giang province. The samples were sequenced by amplifying the *trnH-psbA* locus in the chloroplast. All sequence data were compared with the reference genes published in NCBI. The results showed a significantly high genetic similarity among the fifteen samples, ranging from 98.5%-100%. The genetic similarity factors also ranged from 96.67- 97.48% with reference sample KJ745836.1- D.longifolia, which had been published data. Fifteen samples and references were divided into two main groups. This result shows a close relationship between the studied samples of *Disporopsis* with the genus *D.longifolia*. The main chemical components of this species were initially identified, including polysaccha-

rides, saponins and amino acids. The total polysaccharide content calculated by glucose ranges from 6.94% to 24.0%. This is considerably useful for the conservation, exploitation and effective use of this precious genetic resource in Vietnam.

Keywords: Genetic diversity, *Disporopsis longifolia* Craib, sequence, *trn*H/*psb*A

Introduction

Disporopsis longifolia Craib belongs to the Convallariaceae family and is widely dispersed throughout the tropics from India to the mountains of Southeast Asia and China. In Vietnam, *Disporopsis* is available in some northern mountainous areas. This valuable medicinal plant was listed in Red Data Book in many countries, including Vietnam [1]. Several studies indicated this plant species mainly possesses steroidal saponins and phenolic constituents, which showed various biological activities such as antitumor, antidiabetes, and anti-inflammatory [2-4]. The morphological traits of *D. Longifolia* are illustrated as tubes (rhizome moniliform (1-2 cm thick), the stem arching from 30 to 60 cm, and the longest can reach 110 cm. The leaves are alternate, the petiole is about 5-8 mm; blades of leaves lanceolate to elliptic. The flowers are in the cluster; the perianth is white color (8-10 mm) [3-4]. Corona lobes opposite perianth lobes. The anthers are attached at the hollowed point of the corona lobe apex. In China, roots and stems of *D. longifolia* are used as the decoction by some ethnic minority people for bellyache, adynamia, cough, pneumonia, and asthma [5]. In the Vietnamese Medicinal Plants, *Disporopsis* rhizome has starch, oligosaccharides and polysaccharides used as a tonic to cure fatigue, anorexia, back pain, rheumatism, and dry neck thirst [6]. Furthermore, some species belonging to *Disporopsis* including *D.aspersa,* and *D.pernyl* were reported to be rich in medicinal properties and responsible for anticancer, antifungal, anti-inflammatory, neuritogenic and cytotoxic activities [6-7].

The chloroplast genome is highly conservative and specific to each species. Therefore, using chloroplast genome analysis results in plant phylogenetic research and classification is of great interest to scientists. Based on the available information from phylogenetic studies, some loci are gene segments or genes that indicate potential barcodes for species on earth. The *trnH-psbA* region has been highly capable of identifying the species. Locus *trnH-psbA* has successfully amplified in many angiosperms and gymnosperms. Recently, many studies have used *trnH-psbA* as an independent barcode indicator for plants or in combination with other markers to accurately identify species and generate taxonomic trees [8-12]. Unfortunately, the *Disporopsis* species in Vietnam has a negligible report on its identification and chemical compositions. Therefore, this study aims to assess the genetic diversity of 15 samples of *Disporopsis* species collected in different areas and analyze its main chemical substances to further conserve and develop as well as effectively exploit this species in our country.

Materials and Methods

Material collection

A total of 15 samples of *Disporopsis* species were collected from 15 different communities of five districts in Ha Giang province, a mountainous area in north Vietnam. The sample information is listed in Table 1. The main rhizome (tubers) were collected in the same areas as shown in Table 1. All samples were washed, cut into small pieces, dried and stored at 45℃ in an incubator for 5 days, then sealed in plastic bags and kept in a dry place.

Table 1: List of the collected *Disporopsis* samples used in this study

No	District	Commune	Sample code
1		Thuong Son	HTT3
2	Vi Xuyen	Phong Quang	HTT4
3		Tung Ba	HTT5
4		Phuong Đo	HTT8
5	Tp Ha Giang	Tran Phu	HTT9
6		Ngoc Đuong	HTT10
7		Bat Đai Son	HTT13
8	Quan Ba	Minh Son	HTT14
9		Yen Phu	HTT15
10		Khuon Lung	HTT18
11	Xin Man	Na Chi	HTT19
12		Thu Ta	HTT20
13		Lac Nong	HTT23
14	Bac Me	Yen Phu	HTT24
15		Minh Ngoc	HTT25

Total DNA extraction, amplification of *trnH-psbA*, ITS sequencing and phylogenetic analysis

The fresh leaves of 15 samples of *Disporopsis* species were collected and rapidly transferred to our laboratory for DNA extraction. The DNA extract was conducted following the CTAB method [13] with some minor modifications [14]. The final extracted DNA products were confirmed and recorded by using Spectrophotometer. The primers used in this study were *trnH-psbA*. Specifically, the forward *trnH-psbA*-F primer was: 5'-ACTGCCTTGATCCACTTGGC-3'; and the reveturn *trnH-psbA*-R primer was: 5'-CGAAGCTCCATCTACAAATGG-3, respectively. The DNA amplification was done in a polymerase chain reaction (PCR). The total volume of the PCR reaction was 15 µl included: 1,5 µl PCR buffer (with Mg +) 25 mM; 0.3 µl dNTP 10mM; 0.2 µl Taq DNA polymerase 5U; 1.0 µl bait of 10mM (each primer); 2.0 µl DNA; 9 µl distilled water twice deionized. The PCR reactions were carried out in the thermal cycle: 94°C (5 min), 35 replicate cycles [94°C (1 min), 54°C (45s), 72°C (50s)] and finished at 72°C (7 min). All DNA samples were sent to Macrogen company (Korea) for ITS sequencing. Sequencing was done by using ABI PRISM 3700-DNA Analyzer (Applied Biotech).

Chemical substance evaluation

All tubers of each sample species were evaluated for *in vitro* reactions of flavonoid, coumarin, saponin, alkaloid, polysaccharide and amino acids following the previously described method [15] with some minor modifications. Specifically, the polysaccharide content (%) was done following the formula:

$$X\ (\%) = \frac{A_t \times C_c \times 100 \times 100}{A_C \times C_{bd} \times (1 - B)}$$

Of which:

A_t: optical absorbance of the test solution
A_c: optical absorbance of the standard solution
C_c: concentration of standard (mg / ml).
C_{bd}: initial concentration of the test sample (mg / ml).
B: moisture of test sample (%).

Statistical Analyses

The data were analyzed by Excel ver 2016 and using MEGA 5.1 software to generate a phylogenetic tree with neighbor-joining (NJ) methods. BLAST tool was used to compare the sequences from National Center for Biotechnology Information databases (NCBI).

Results and Discussion

Identification of *Diporopsis* samples based on ITS region sequences

The DNA extraction of 15 leaf samples of *Diporopsis* showed high concentration and purity. The results of PCR reaction with *trnH-psbA* primer revealed that all samples were in mono-forms with sizes in the range of 600-620 bp (Fig.1).

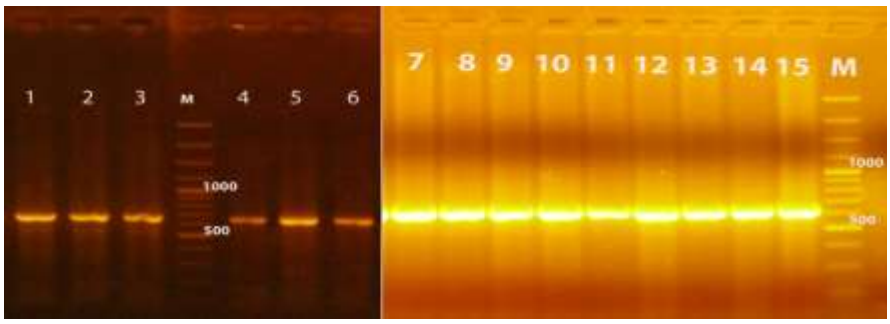

Fig 1: Electrophoresis of PCR products of *Diporopsis* samples with *trnH-psbA* primers; M: Marker 1kb plus DNA; lane 1-15: *Diporopsis* samples

The data shown in Table 2 disclosed that the samples had consistent nucleotide lengths, as described in Fig.1, approximately 600bp. The nucleotide sequence length of the samples was slightly varied from 596 nucleotides in the

samples of HTT4, HTT5, HTT8, HTT10, HTT14, HTT15 and HTT18 to 599 nucleotides (HTT20). The number of nucleotides of each sequence fragment varies among the samples. It may explain due to the fragmentation or addition (InDels) at some locations on the gene segment. The sequence lengths of the 15 *Diporopsis* samples have more than 15-18 nucleotides compared to the reference sample of KJ745836.1_*D. longifolia* and the 8-11 nucleotides more than the reference samples belonging to the genus *Polygonatum*, asparagus family on the database of NCBI.

Table 2: The sequence length of 15 *Disporopsis* samples

No	Sample code	Total of nucleotides	No	Sample code	Total of nucleotides
1	HTT3	597	11	HTT19	597
2	HTT4	596	12	HTT20	599
3	HTT5	596	13	HTT23	597
4	HTT8	596	14	HTT24	597
5	HTT9	598	15	HTT25	597
6	HTT10	596	16	KJ745836.1	581
7	HTT13	597	17	MK855008.1	589
8	HTT14	596	18	MK855000.1	589
9	HTT15	596	19	MK855005.1	589
10	HTT18	596	20	KJ745879.1	588

To compare the sequences of 15 samples with the reference genes published in NCBI, there was a difference between the studied samples and the reference gene KJ745836.1_*D.longifolia* with reference genes belonging to the genus *Polygonatum*. At the position 428-436, the samples and the reference gene KJ745836.1_*D.longifolia* has had mutations by missing eight nucleotides, the differences from the other four references of the genus *Polygonatum*. From position 461-468, there was a nucleotide replacement at four references of the *Polygonatum* genus compared to the *Diporopsis* samples and the reference gene KJ745836.1_*D.longifolia*. In positions 461, 466 and 468, there were nucleotide substitutions from A to G, A to C, and C to T, respectively. From position 462 to 6464, there was a nucleotide replacement from G, A, A to T. However, from position 589-608, the reference gene KJ745836.1_*D. longifolia* and four references of the genus *Polygonatum* have had mutations that lost 19 nucleotides, respectively which are distinct from the 15 studied samples. At these locations, by using the *trnH-psbA* primer, the polymorphisms of the 15 samples were determined.

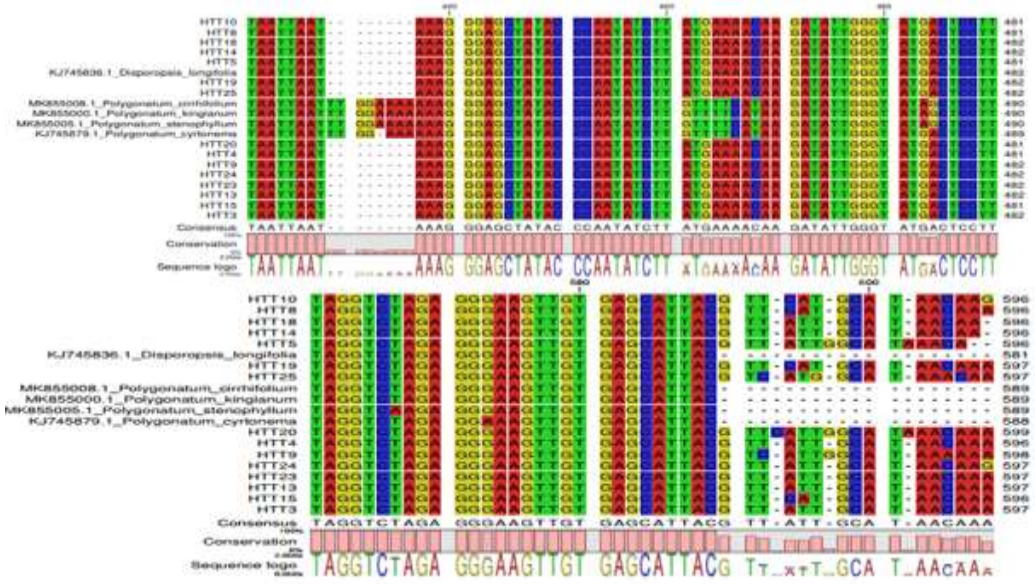

Fig 2. Sequences and location of different nucleotides of the *Diporopsis* samples compared to the references published in NCBI

Remarkably, at positions 595 and 606, only the HTT25 sample has changed from G and C nucleotides; the remaining samples have changed into T and A nucleotides, respectively. At positions 596 and 597, only the HTT20 sample has a change to T and G nucleotides, the remaining samples have a shift in G nucleotide or lose one nucleotide like five reference genes. Similarly, at position 607, only the HTT5 mutation loses a nucleotide like the five reference genes; the remaining mutants have an additional nucleotide A (Fig. 2). In the previous studies, some reports evaluated the genetic diversity and successfully identified the medicinal plant species, including *Huperiza*, *Panax* by using molecular markers [8-11].

Genetic diversity and genetic relationship analyses among 15 samples of *Diporopsis*

The data presented in Table 3 showed significantly high similarity among the 15 sequences of the samples. The lowest genetic similarity coefficient is 98.5% (between HTT5 and HTT25). The highest genetic similarity coefficient is 100% (between pairs of models HTT14 - HTT18, HTT3 - HTT13, HTT3 - HTT23, HTT13 - HTT23 and HTT8 - HTT15). The results comparing genetic similarities between reference samples and 15 research samples indicated that: 15 samples of *Diporopsis* have had the highest genetic correlation coefficient with reference sample KJ745836.1_*D. longifolia*, ranging from 96, 67% (HTT20 model) to 97.48% (HTT14 model; HTT18). Genetic similarity coefficient between 15 studied samples and four reference samples MK855008.1_*P.cirrhifolium*, MK855000.1_*P. kingianum* (Red Star), MK855005.1_*P.stenophyllum* and MK745879.1_*P.cyrtonema* range from 93.91-95.02 %. It showed that 15 samples were in the same branch with the reference sample KJ745836.1_*D. longifolia*; the

remaining branches were four reference samples MK855008.1_*P.cirrhifolium*, MK855000.1_*P. kingianum*, MK855005.1_*P.stenophyllum* and MK745879.1_*P. cyrtonema*. Fifteen research samples were divided into two groups as follows:

Group I: The first group: consisting of two samples of *Diporopsis* were HTT9 (collected in Tran Phu, Ha Giang city, Ha Giang) and HTT25 (in Minh Ngoc commune, Bac Me district, Ha Giang). These two samples shared coefficients nucleotide sequence similarity of 99.5%. The similarity coefficient between the two samples in this group with the reference sample KJ745836.1_*D. longifolia* ranged from 97.16% (HTT9 and KJ745836.1) to 97.32% (between HTT25 and KJ745836.1).

Group II: includes 13 seed samples, divided into 2: Sub-group 2.1 included 4 models: HTT8, HTT10, HTT15, HTT19 and reference sample KJ745836.1_*D. longifolia*. The samples in this subgroup have had very high homologous nucleotide sequences, ranging from 99.66% (between HTT10 and HTT19) to 100% (between HTT8 and HTT15), respectively. The samples in this group have had a similarity in the nucleotide sequence with the reference sample KJ745836.1 ranging from 97.15% (between HTT8, HTT10, HTT15 and KJ745836.1) to 97.32% (between HTT19 sample and KJ745836.1). These four samples were collected in the three different districts of Ha Giang, of which HTT8 and HTT10 samples were collected from Ha Giang city, Ha Giang province.

Table 3. Coefficients of genetic diversity among 15 *Diporopsis* samples

	HTT10	HTT8	HTT18	HTT14	HTT5	KJ745836.1	HTT19	HTT25	MK855008.1	MK855000.1	MK855005.1	KJ745879.1	HTT20	HTT4	HTT9	HTT24	HTT23	HTT13	HTT15	HTT3
HTT10																				
HTT8	99.83																			
HTT18	99.33	99.33																		
HTT14	99.33	99.33	100																	
HTT5	99	99	99.33	99.33																
KJ745836.1_Disporopsis_longifolia	97.15	97.15	97.48	97.48	97.15															
HTT19	99.66	99.83	99.5	99.5	98.83	97.32														
HTT25	98.66	98.83	99.16	99.16	98.5	97.32	98.99													
MK855008.1_Polygonatum_cirrhifolium	94.55	94.55	94.87	94.87	94.55	97.28	94.71	94.71												
MK855000.1_Polygonatum_kingianum	94.55	94.55	94.87	94.87	94.55	97.28	94.71	94.71	100											
MK855005.1_Polygonatum_stenophyllum	94.38	94.38	94.7	94.7	94.38	97.11	94.55	94.55	99.83	99.83										
KJ745879.1_Polygonatum_cyrtonema	94.7	94.7	95.02	95.02	94.7	97.45	94.87	94.87	99.15	99.15	98.98									
HTT20	99	99.17	99.17	99.17	99.5	96.67	99	98.67	94.08	94.08	93.91	94.23								
HTT4	99.5	99.66	99.66	99.66	99.33	97.15	99.5	99.16	94.55	94.55	94.38	94.7	99.5							
HTT9	98.83	99	99.33	99.33	99	97.16	99.16	99.5	94.55	94.55	94.39	94.71	99.17	99.33						
HTT24	99.5	99.33	99.83	99.83	99.17	97.32	99.5	99.16	94.71	94.71	94.55	94.87	99.17	99.66	99.33					
HTT23	99.33	99.5	99.83	99.83	99.17	97.32	99.66	99.33	94.71	94.71	94.55	94.87	99.33	99.83	99.5	99.83				
HTT13	99.33	99.5	99.83	99.83	99.17	97.32	99.66	99.33	94.71	94.71	94.55	94.87	99.33	99.83	99.5	99.83	100			
HTT15	99.83	100	99.33	99.33	99	97.15	99.83	98.83	94.55	94.55	94.38	94.7	99.17	99.66	99	99.33	99.5	99.5		
HTT3	99.33	99.5	99.83	99.83	99.17	97.32	99.66	99.33	94.71	94.71	94.55	94.87	99.33	99.83	99.5	99.83	100	100	99.5	

Sub-group 2.2 included 9 varieties: HTT3, HTT4, HTT13, HTT14, HTT18, HTT20, HTT23 and HTT24. Samples in this subgroup have had similar nucleotide sequences ranging from 99.17% (between pairs of HTT5 - HTT3, HTT5 - HTT13, HTT5 - HTT23, HTT5 - HTT24, HTT14 - HTT20, HTT18 - HTT20 and HTT20 - HTT24) to 100% (between pairs of models like HTT3 - HTT13, HTT3 - HTT23, HTT13 - HTT23 and HTT14 - HTT18). Samples in this group have a similarity coefficient on nucleotide sequence with the reference sample KJ745836.1 ranging

from 97.15% (between HTT4, HTT5 and KJ745836.1) to 97.48% (between HTT14 and HTT18 samples) and KJ745836.1). These 9 samples were collected in 4 different districts of Ha Giang province, including 03 samples collected in Vi Xuyen district (HTT3, HTT4, HTT5), 02 samples collected in Quan Ba district (HTT13, HTT14), 02 samples were collected in Xin Man district (TT18, HTT20) and 02 samples were collected in Bac Me district (HTT23, HTT24).

The taxonomic phylogenetic tree showed that the reference sample KJ745836.1_*D. longifolia* manifested the relationship between the studied samples, with a high genetic similarity coefficient (96, 67-97, and 48%). This result showed a close relationship between the 15 samples with the genus *D.longifolia* (Fig 3.) Regardless, the *trnH-psbA* is a DNA barcode indicator that has been widely used to identify species in different taxonomic groups [16]. An alternative way is based on the chloroplast gene sequences, and comparing the sequence differences to design the specific markers has been reported [10, 12].

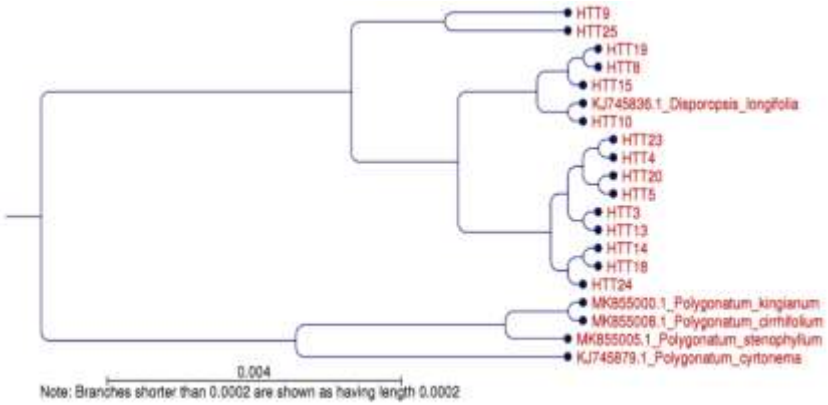

Fig 3. Taxonomic phylogenetic tree of 15 *Diporopsis* samples with the reference samples

Detection of the main active chemical substances contained in the *Diporopsis* samples

As presented in Table 4, within our initial test reaction, *Diporopsis* samples have main saponin and amino acid as well as polysaccharide groups. However, there are no coumarin, alkaloid and flavonoid groups. Among them, the polysaccharide shows a clear positive reaction. These substance groups are announced to have many valuable effects, such as increasing immunity, fighting viruses, and lowering blood sugar. Therefore, our team has conducted a quantification of this group [3,5].

Table 4. Qualitative results obtained by chemical reaction of 15 samples

Substance	Qualitative reaction	Result	Conclusion
Flavonoid	Cyanidin reaction	-	No flavonoid group
	Reaction with NaOH 10%	-	
	Reaction with $FeCl_3$ 5%	+	
	Diazo reaction	-	
Saponin	Frothing phenomenon	++	Yes
Amino acid	Reaction with TT Ninhydrin	++	Yes
Coumarin	Coumarin Lacton opening and closing reaction	-	No
Alkaloid	Reaction with TT. Mayer	-	No
	Reaction with TT. Dragendorff	-	
Polysacharid	Polysacharid Lugol	+++	Yes

-: Negative reaction +: Positive reaction ++: Clearly positive reaction

Quantifying the total polysaccharide in *Diporopsis samples*

The total polysaccharides are quantified by ultraviolet and visible absorption spectrophotometric methods. The results are shown in Table 5 and Fig 4. The total polysaccharide content in the samples of *Diporopsis* is relatively high, ranging from 6.94% to 24.0%. However, the fluctuation range between samples collected in different areas in Ha Giang is quite extensive. The sample with the highest polysaccharide content was HTT13 collected at Bat Dai Son commune of Quan Ba district (24.0%); while the sample with the lowest content is HTT 25 collected in Tran Phu commune of Hà Giang (6.94%). The average value was 13.66%. Generally, the samples in the districts have different degrees of variation due to the influence of climatic conditions. Different elevations could lead to different quality. For instance, Quan Ba district has a high altitude of 800m or more above sea level, followed by districts of Xi Man and Bac Me, Vi Xuyen and Ha Giang province, with the average height sampling from 300-500m above the sea level.

Table 5: The relationship between glucose concentration and absorption

Glucose concentration (µg/mL)	Absorbance (ABS)
7.9	0.794
6.3	0.685
5.2	0.555
2.6	0.265
1.0	0.120

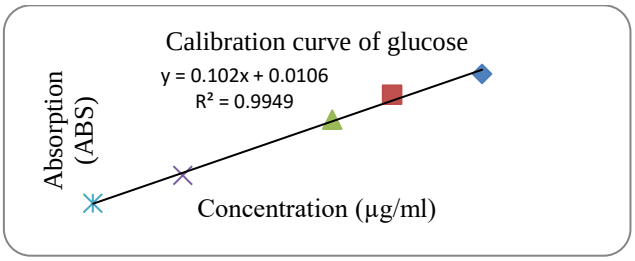

Fig 4. Glucose curve showing the relationship between concentration and absorption

The quantitative results of polysaccharides in the samples are presented in the following table.

Table 6: Polysacharid content in *Diporopsis* samples

No	District	Commune	Notation	Result (*) (%)	Average by district
1	Vi Xuyen	Thuong Son	HTT3	9.02	10.12
2		Phong Quang	HTT4	8.68	
3		Tung Ba	HTT5	12.67	
4	Ha Giang	Phuong Đo	HTT8	8.55	7.95
5		Tran Phu	HTT9	6.94	
6		Ngoc Đuong	HTT10	8.36	
7	Quan Ba	Bat Đai Son	HTT13	24.0	20.40
8		Minh Son	HTT14	19.40	
9		Yen Phu	HTT15	17.81	
10	Xin Man	Khuon Lung	HTT18	15.56	16.34
11		Na Chi	HTT19	14.06	
12		Thu Ta	HTT20	19.40	
13	Bac Me	Lac Nong	HTT23	17.35	15.91
14		Yen Phu	HTT24	16.25	
15		Minh Ngoc	HTT25	14.14	
	Average				13.67

A polysaccharide is a group of substances that have been announced to have many beneficial health effects, such as increasing the body's immunity. The polysaccharides in Ganoderma have been proven to have anticancer properties through immunomodulatory, anti-proliferating and metastatic mechanisms [17] Saponin-rich segments and polysaccharides extracted from *Diporopsis* also used to cure type II diabetes [18].

Conclusions

In summary, the genetic similarity of the 15 *Diporopsis* samples was also identified, ranging from 98.5% (between HTT5 and HTT25) to 100% in other samples which had a genetic similarity coefficient ranging from 96.67- 97.48% with the reference sample KJ745836.1_*D.longifolia* published in NCBI. Two main

groups were generated based on the phylogenetic tree of the samples and the reference sample. The total polysaccharide content calculated by glucose ranges from 6.94% to 24.0%. This result also contributes to controlling the quality of *Diporopsis.*

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