*Available online www.jsaer.com*

***Journal of Scientific and Engineering Research*, 2019, 6(10):24-32**

**Research Article**

**ISSN: 2394-2630**
**CODEN(USA): JSERBR**

# Genetic Diversity and Identification of the Local *Podocarpus* Samples by Molecular Markers at Moutainous Areas of Yen Tu, Quan Ninh Province, Vietnam

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**Abstract** *Podocarpus* is a genus of conifers which is belonging to Podocarpaceae family. *Podocarpus* comprises of over 100 species and widely spreading in many areas of the world. *Podocarpus* plants are available in nature and distributed in subtropical and tropical forest areas in Vietnam. This plant is grown as an ornamental tree with potential economic benefits in whole of this country. The objective of this study was to evaluate genetic diversity and identification of 12 local *Podocarpus* samples collected in the mountainous areas of Yen Tu, Quang Ninh province by ITS markers. The results showed that the samples were genetically diverse with high genetic similarity, of which the greatest homogenetic was 98.74%, while the lowest was 87.52%, respectively. Noteworthily, the nearest genetic distance was 0.01, whereas, the farthest was 0.14. It implies that some samples of *Podocarpus* may have an identical origin, evolution and relationships. We have also successfully identified 12 *Podocarpus* samples belonging the *Podocarpus macropyllus* based on the published reference on Genbank (NCBI). This study may provide useful information to further conservation efforts and development as well as exploitation of the genetic resource *Podocarpus* in this country.

**Keywords** Genetic diversity, Podocarpaceae family, Genbank, species

## Introduction

*Podocarpus* is a genus of conifers which is belonging to Podocarpaceae family. *Podocarpus* comprises of over 100 species and widely spreading in the subtropical and tropical areas in the world [1]. Presently, there are are 27 Podocarpaceae that are listed in the red list and 17 species are critically in danger [2,3]. Numerous medicinal secondary metabolites of Podocarpaceae belonging to flavonoids, phenolics, sequiterpenoids, morditerpene dilactones, triterpenoids and steroids have been isolated and reported that benefit to animals and humans. They are bioactive compounds and responsible for antioxidant, antimicrobial, anticancer, insecticidal, antifeedant, herbicidal, fungicidal and cardioprotective activities [4]

*The Podocarpus* species are known to be native to southern Japan and eastern China. The morphological characteristics were narrated as the medium size evergreen shrubs or trees and are usually reaching from 1 to 20 meters tall [5]. The leaves are shaped with a strap and ranged from 6 to 12 cm long. The cones are borne on a short stem and have 2-4 scales, usually only one (sometimes two) fertile, each fertile scale bearing a single apical seed 10–15 mm. When mature, the scales swell up and become reddish-purple, fleshy and berry-like, 10–20 mm long; they are then eaten by birds, which disperse the seeds in their droppings. It was reported that *Podocarpus macrophyllus* adapts to grow in forests, open thickets, and roadsides from near sea level to 1000 m [5].

In Vietnam, generally, *Podocarpus* plants are available in nature and distributed in subtropical and tropical the forest areas in Vietnam such as Quang Ninh, Nghe An, Ha Tinh, Tuyen Quang provinces. This plant is grown as an ornamental tree with potential economic benefits in the whole of this country. Yen Tu National Forest is rich in flora and fauna with 981 species where *Podocarpus* species are naturally grown. Lam et al., [7] reported that 15 species belonging to *Podocarpus* including *P. Chinensis* and *P. pilgeri.* were pinpointed at 800 – 1000 m above the sea level. *Podocarpus* plants are well adapted in the altitude of over 2000 m [6]. According to the report of Center of Plant Data of Vietnam [7], *Podocapus* in this area is *P. brevifolius* species and sparely found in the mountainous areas of Yen Tu, Quang Ninh province. The total areas of Yen Tu National Forest are 2.783 ha and located in Quang Ninh province and consist of 2 communes Thuong yen cong and Phuong Dong, Uong Bi city where *Podocarpus* species are naturally grown. A decoction of this plant leaves is used as Vietnamese traditional medicines for rheumatism and a wide range ailments [14].

ITS (Internal Transcribed Spacer) of nuclear ribosomal DNA is one of the most DNA markers used for plant species identification, diversity evaluation, life history, ecological studies and forensic analyses. This technique has been greatly applied since its first report in 2013 [8,9]. Among the DNA regions, ITS like ITS2 is widely applied for DNA fragments in plant molecular systematics at the generic and species levels due to its potentially high solution of inter and intraspecific relationship [9]. Presently, in this country, most studies to evaluate the genetic diversity of *Podocarpus* species have been mainly applied based on its morphological characteristics. Hence, the objective of this study was to assess genetic diversity and identification of the local *Podocarpus* samples by ITS markers.

## Materials and Methods

In this study, 12 local *Podocarpus* samples species were kindly provided by the Fruit and Vegetable Research Institute in 2018. The samples were gathered at Thuong yen cong commune, Uong bi-district, Ninh Binh province. The collected information was presented in Table 1.

**Table 1:** The list of 12 *Podocarpus* samples used in this study

| No | Collected area | | Sample code | No | Collected areas | | Sample code |
|---|---|---|---|---|---|---|---|
| | District | Commune | | | District | Commune | |
| 1 | Uong bi | Thuong yen cong | N1 | 7 | Uong bi | Thuong yen cong | N7 |
| 2 | Uong bi | Thuong yen cong | N2 | 8 | Uong bi | Thuong yen cong | N8 |
| 3 | Uong bi | Thuong yen cong | N3 | 9 | Uong bi | Thuong yen cong | N9 |
| 4 | Uong bi | Thuong yen cong | N4 | 10 | Uong bi | Thuong yen cong | N10 |
| 5 | Uong bi | Thuong yen cong | N5 | 11 | Uong bi | Thuong yen cong | N11 |
| 6 | Uong bi | Thuong yen cong | N6 | 12 | Uong bi | Thuong yen cong | N12 |

## Total DNA extraction, PCR and DNA samples sequencing

The fresh leaves of all samples were collected and intermediately transferred to the laboratory for DNA extraction. In this study, DNA extract was done following CTAB method (cetyltrimethylammonium bromide) of Hoyle and Doyle [10] with some minor modifications. The yielded DNA products were then determined by the use of spectrophotometer [11]. PCRs and DNA sequencing: nuclear ribosomal Internal Transcribed Spacers including ITS1 and ITS8 with nucleotide sequence following to the method of Trung et al [12] as shown in Table 2.

**Table 2:** The information of two primers used in this study

| No | Nucelotide sequence |
|---|---|
| *ITS1* | GGTTCAAGTCCCTCTATCCC |
| *ITS8 F(GAA)* | ATTTGAACTGGTGACACGAG3 |

Amplification was made in a polymerase chain reaction (PCR) in the tuber 0.2ml which contained 9.0 µl, 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 50ng/µl (1.0 µl), and total volume was 15 µl, respectively. Amplification reactions of PCR program were established and presented in Table 3.

**Table 3:** PCR reaction procedures used in this study

| Step | Temperature (°C) | Time |
|---|---|---|
| 1 | 94 | 5 min |
| 2 | 94 | 1 min |
| 3 | 56 | 45 s |
| 4 | 72 | 50 s |
| 5 | Repeat the second step, | 35 cyles |
| 6 | 72 | 7 min |
| 7 | 4 | ∞ |

**Statistical Analysis**

The sequences were aligned and analyzed by using MEGA v5.1 program to generate the phylogeny.

**Results and Discussion**

**Molecular markers application to identify *Podocarpus* species based on ITS region sequences**

In the fact that it is often faced with difficulty to accurate identify *Podocarpus*, in this study,we used the ITS1/ITS8 primers which is helpful to provide and accurate texonomic identification using specific DNA region, the ITS region was successfully amplified by PCR. The product was then examined on the gel agarose (1.5%) The obtained DNA was indicated to be high quality with the appearance of only one band with the length size ranged from 700- 800 bp (Figure 1). Our result was coincide with some previous reports who amplified the ITS region of the plant samples [13]. To emphasize that the obtained bands were the obvious and correct size, which are enough quality to use for sequencing.

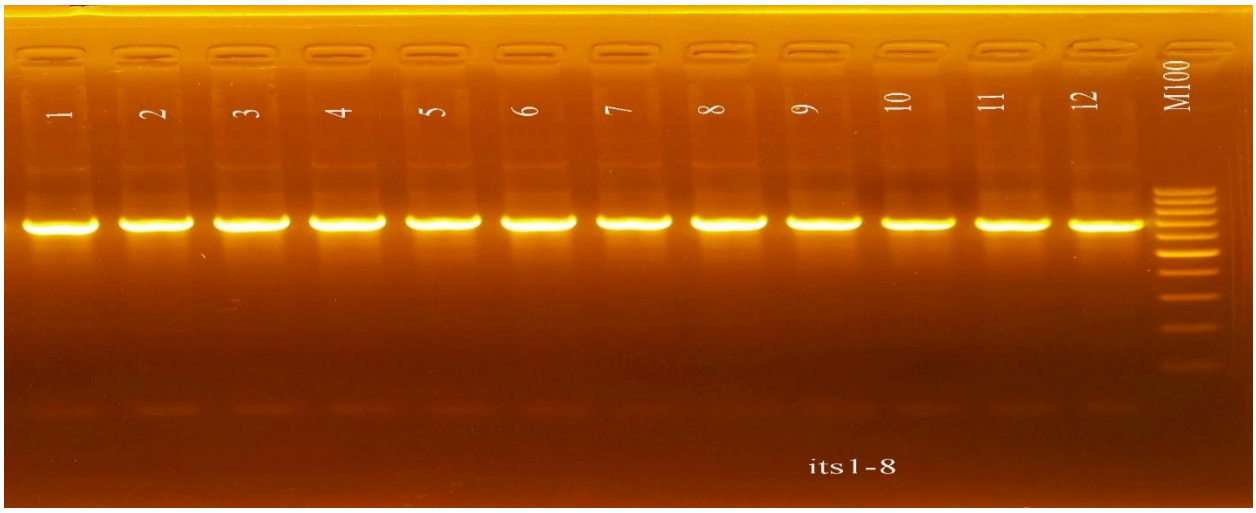



*Figure 1: Electrophoresis of amplification ITS segments on the 12 Podocarpus samples by PCR with ITS1 and ITS8 primers*

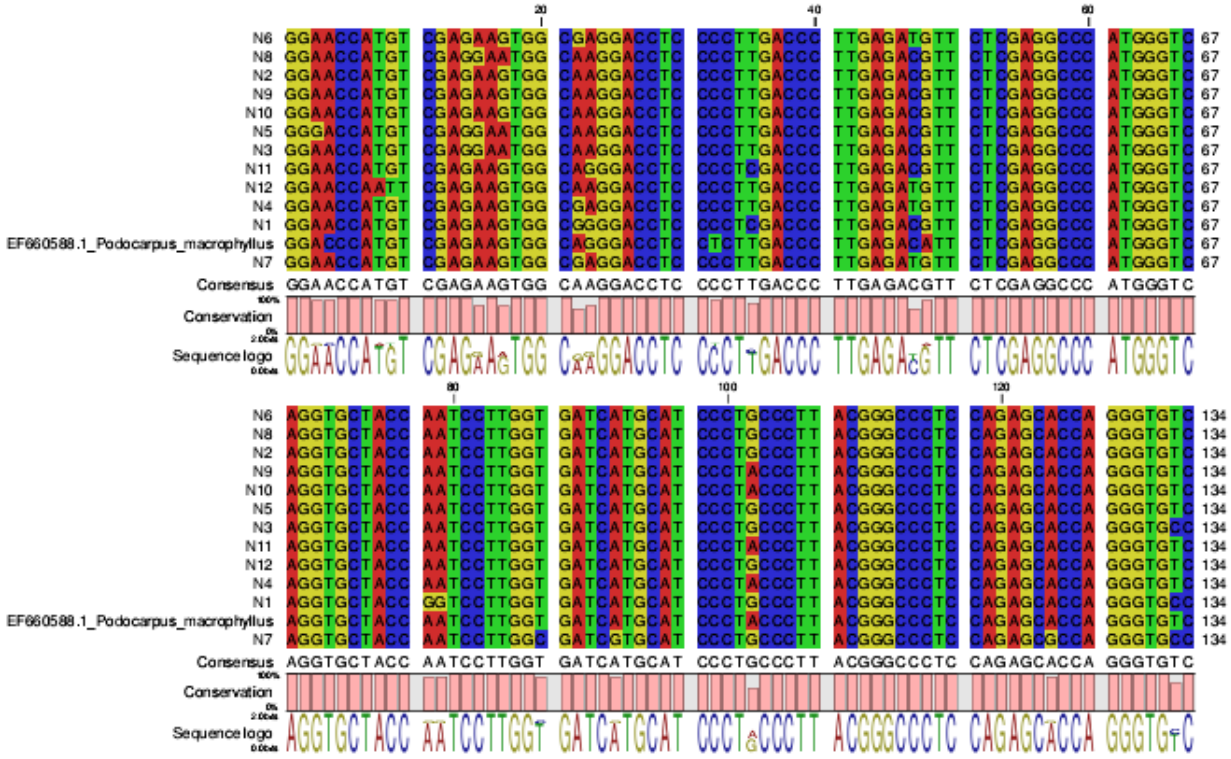
**Comparison of sequencing regions of *ITS1-5,8S-ITS2* of the *Podocarpus* samples**

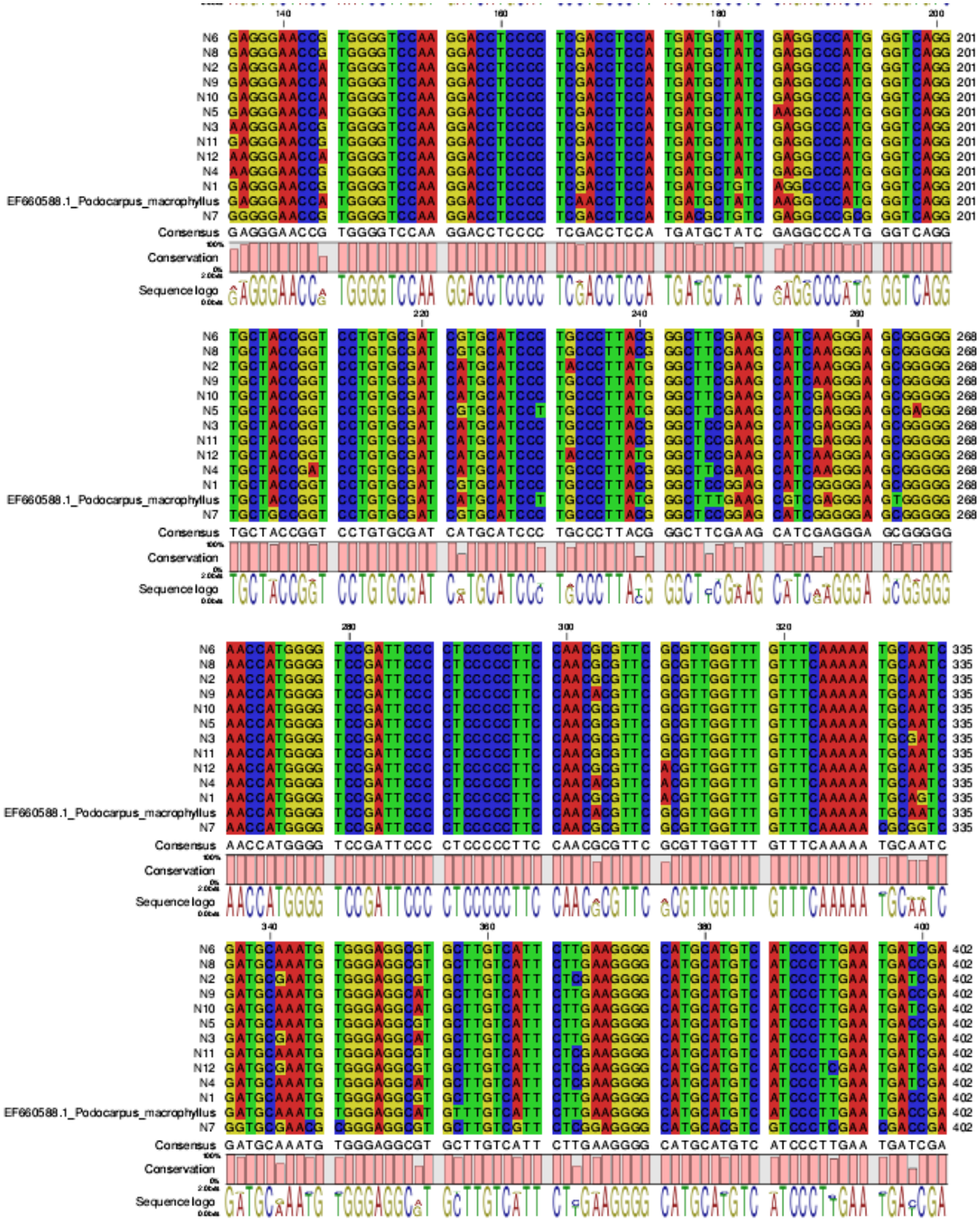
A total of 12 PCR products of 12 *Podocarpus* samples was to sequence by Macrogen company (Korea). The collected data were analysed and compared by using the ClustalW of the software MEGA v6 and CLC 8.0. The results revealed the difference between the sequences which were mainly single polymorphic positions (SNP), of which one nucleotide was replaced by another nucleotide in the sequencing region of ITS1-5.8S – ITS2 of the *Podocarpus* samples as shown in Figure 2.

As a total of 12 *Camellia* samples were a remarkable difference in the ITS1-5.8S – ITS2 sequence regions and the component of nucleotides. Based on the differentiation of nucleotides sequences, it is possible to identify the collected samples as shown in Table 4. The results demonstrated the differentiation of Guanin, Cytosine, Adenine and Thymine composition of the samples. Generally, Guanin and Cytosine ratios were higher than the Adenine and Thymine rations. On the other hand, the percentage (%) of GC was greater than the (%) of AT, respectively. Strikingly, the N7 sample had the highest G+C component by 62.2%, while the lowest A+T component was 37.8%. The average percentage of component G+C in all 12 samples was reached 54.2%, and A+T was 45.8%, respectively (Table 4).

**Table 4:** Nucleotide components of 12 *Podocarpus* samples

| No | Sample | T(U) | C | A | G | %GC | %AT |
|---|---|---|---|---|---|---|---|
| 1 | N1 | 22.8 | 29.1 | 20.3 | 27.7 | 56.9 | 43.1 |
| 2 | N2 | 24.3 | 27.4 | 22.4 | 25.9 | 53.2 | 46.8 |
| 3 | N3 | 23.8 | 27.9 | 21.4 | 26.9 | 54.7 | 45.3 |
| 4 | N4 | 24.6 | 27.4 | 23.1 | 25.0 | 52.3 | 47.7 |
| 5 | N5 | 24.3 | 27.2 | 22.1 | 26.4 | 53.6 | 46.4 |
| 6 | N6 | 24.3 | 27.5 | 21.9 | 26.2 | 53.7 | 46.3 |
| 7 | N7 | 20.9 | 30.8 | 16.9 | 31.4 | 62.2 | 37.8 |
| 8 | N8 | 24.1 | 27.7 | 22.2 | 26.0 | 53.7 | 46.3 |
| 9 | N9 | 24.3 | 27.5 | 22.7 | 25.5 | 53.0 | 47.0 |
| 10 | N10 | 24.2 | 27.6 | 22.2 | 26.0 | 53.6 | 46.4 |
| 11 | N11 | 23.7 | 27.9 | 22.3 | 26.1 | 54.0 | 46.0 |
| 12 | N12 | 24.2 | 27.6 | 22.6 | 25.6 | 53.2 | 46.8 |
| **Average** | | 23.9 | 27.8 | 21.8 | 26.4 | 54.2 | 45.8 |




N6
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EF660588.1_Podocarpus_macrophyllus
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Conservation
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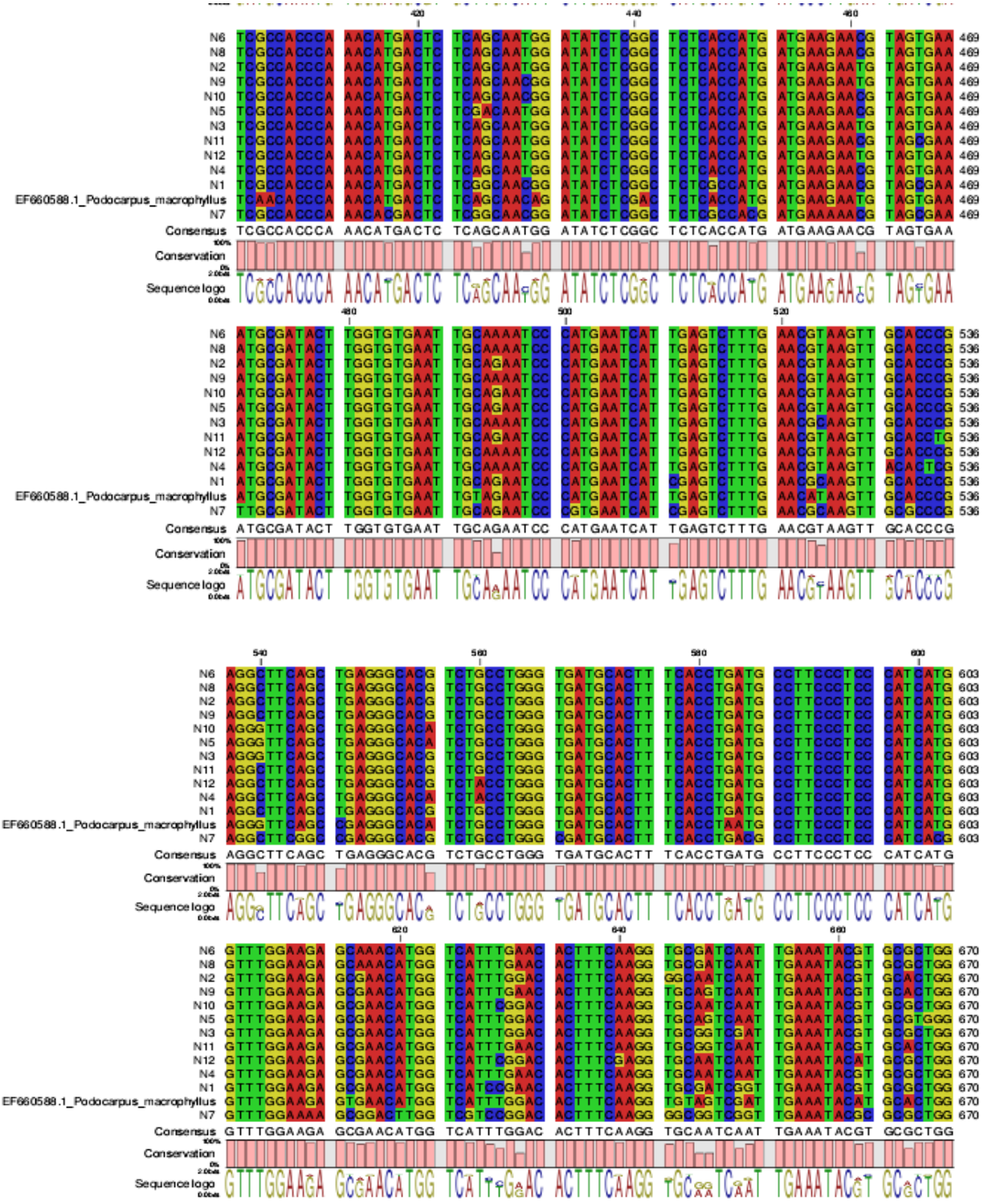
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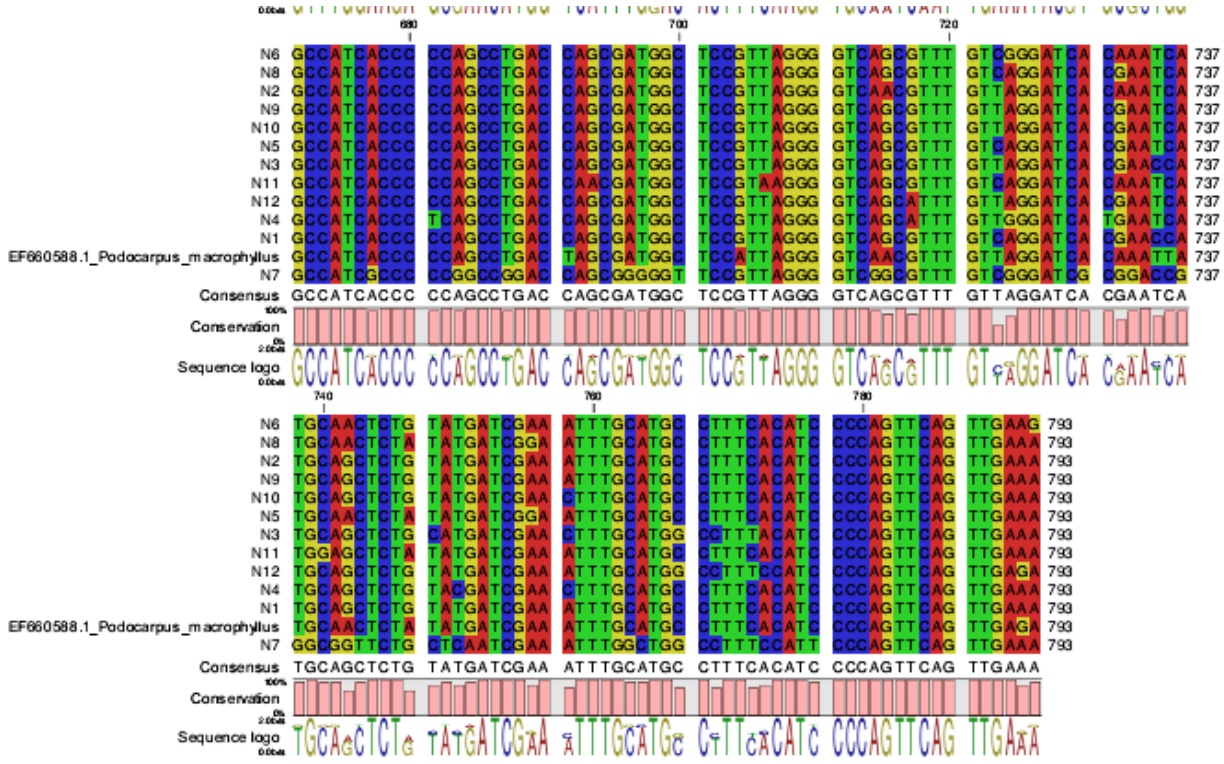


*Figure 2: Comparison of nucleotides sequences of the 12 Podocarpus samples*

The variation in the sequence of ITS1-5,8S-ITS2 regions between the samples was shown via similarity coefficients of each sample by use of the genetic distance tool CLC v8 software (Table 5). Based on the data of analyses by ITS1-5,8S-ITS2 sequences, the *Podocarpus* samples collected in mountainous areas of Yen Tu was shown to be genetically diverse with high genetic similarity among the samples. Of which the greatest homogenetic was 98.74%, while the lowest was found by 87.52%. Noteworthily, the nearest genetic distance was 0.01, whereas, the farthest was 0.14. It implies that some samples of *Podocarpus* may have an identical origin, evolution and relationships.

**Table 5:** Correlation coefficients and genetic distance of 12 *Podocarpus* samples

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N6 | 1 | | 0.01 | 0.03 | 0.03 | 0.03 | 0.04 | 0.04 | 0.03 | 0.04 | 0.03 | 0.04 | 0.12 |
| N8 | 2 | 98.74 | | 0.03 | 0.02 | 0.03 | 0.02 | 0.03 | 0.03 | 0.04 | 0.04 | 0.04 | 0.13 |
| N2 | 3 | 97.48 | 97.23 | | 0.02 | 0.02 | 0.04 | 0.04 | 0.03 | 0.03 | 0.04 | 0.05 | 0.13 |
| N9 | 4 | 97.48 | 97.73 | 98.23 | | 0.02 | 0.03 | 0.03 | 0.03 | 0.04 | 0.03 | 0.05 | 0.13 |
| N10 | 5 | 97.23 | 97.23 | 97.98 | 98.49 | | 0.03 | 0.03 | 0.03 | 0.04 | 0.03 | 0.05 | 0.13 |
| N5 | 6 | 96.47 | 97.73 | 96.47 | 96.72 | 97.23 | | 0.04 | 0.04 | 0.05 | 0.05 | 0.05 | 0.14 |
| N3 | 7 | 96.22 | 96.72 | 96.47 | 96.72 | 96.97 | 95.71 | | 0.04 | 0.04 | 0.05 | 0.05 | 0.11 |
| N11 | 8 | 97.23 | 97.23 | 97.23 | 97.23 | 96.97 | 95.96 | 95.96 | | 0.05 | 0.04 | 0.04 | 0.13 |
| N12 | 9 | 95.96 | 95.71 | 97.23 | 96.47 | 96.47 | 94.70 | 96.22 | 95.21 | | 0.04 | 0.07 | 0.13 |
| N4 | 10 | 97.10 | 96.34 | 96.34 | 97.10 | 97.10 | 94.83 | 95.59 | 95.84 | 96.09 | | 0.06 | 0.14 |
| N1 | 11 | 95.71 | 95.71 | 94.70 | 95.21 | 95.46 | 94.70 | 94.96 | 95.71 | 93.69 | 94.07 | | 0.10 |
| N7 | 12 | 88.90 | 88.40 | 88.40 | 88.15 | 88.40 | 87.64 | 89.66 | 88.40 | 88.40 | 87.52 | 90.67 | |

**The phylogenetic trees based on nucleotide sequences of ITS1-rRNA-ITS2 regions among the12 *Podocarpus* samples**

We have generated the phylogenetic tree based on the ITS1-rRNA-ITS2 sequencing regions by using the Mega 6.0 software and the Maximum like methods. The results showed that 12 *Podocarpus* samples were divided into 2 main groups as follows:

Group I: It consisted of only a single N7 sample and was then checked on the Genbank (NCBI), which was similar to the *Podocarpus neriifolius*. This species was found in some countries such as India, Brunei, Cambodia, Fiji, Indonesia, Laos, Malaysia, Myanmar, Nepal, Thailand, China and Vietnam.

Group II: There were 11 samples. The data were compared with the Genbank database (NCBI). It was found that this group was similar to *Podocarpus macrophyllus* which was originated in China and Japan, respectively.

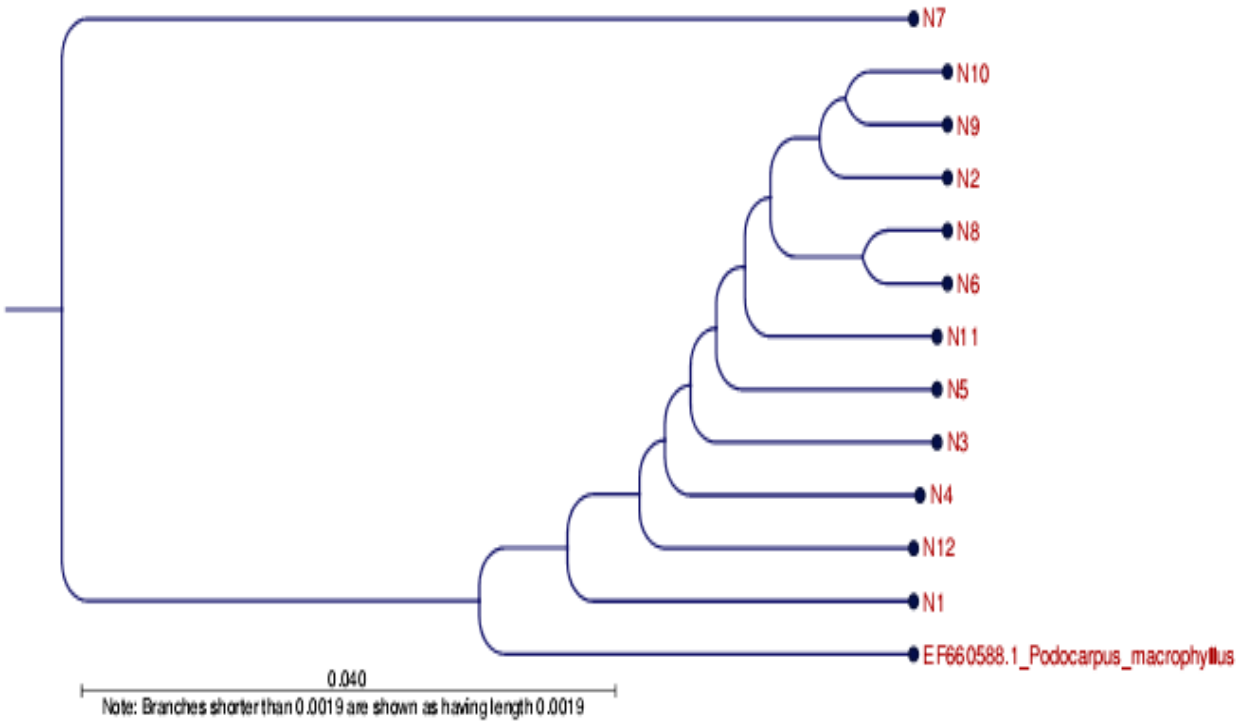


*Figure 3: The phylogenetic trees of 12 Podocarpus samples*

In summary, the 12 *Podocarpus* samples collected in Yen Tu mountainous areas were sequenced which were similar to the published reference on NCBI (code: EF660588.1). We have successfully identified 12 *Podocarpus* samples by use of a sequence of chloroplast with the primer ITS1/ITS8 and concluded that they belong the *Podocarpus macropyllus*. The genetic similarity coefficients among the samples were rather high and ranged from 86.52% to 98.74%, respectively. This information may be useful to further conserve and develop as well as exploit the *Podocarpus* genetic resource in this country.

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