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Amplification and DNA Sequencing of the Nuclear Ribosomal Internal Transcribed Spacer Region from Dried Black Pepper Samples for Traceability

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Abstract

Traceability is the ability to follow the circulation of a product through all steps in the supply chain. Traceability will ensure transparency of product origin and quality. Traceability involves product records and labeling. For biological samples, in addition to the commonly used management parameters, a number of specific markers will be used to control the product, such as nucleic acid (DNA), protein, carbonhydrates, etc. Pepper is a product with economic value and is grown in many different countries around the world. Pepper products from different growing regions will have different quality and prices. Tracing the origin of pepper also faces many challenges. Therefore, in this study, appropriate conditions for DNA extraction from dried pepper were determined, including extraction buffer, sample grinding method, and total DNA precipitation conditions. Appropriate conditions to carry out amplification of the internal transcribed spacer (ITS) region were also determined, including the amount of DNA template (100 ng), primer concentration (0.3 µM), primer annealing temperature (55 °C) and number of reaction cycles (35 cycles). The optimal procedure was applied to amplify the ITS region of 19 pepper samples. The ITS region nucleotide sequences of 19 samples were determined and used to build a phylogenetic tree of pepper samples from different growing regions in Vietnam. The genetic diversity of the ITS region of Vietnamese pepper was also evaluated and compared with data originating from some countries, such as Thailand, China, and the US.

Keywords: Dried black pepper, DNA sequence, ITS, phylogenetics, traceability.

1. Introduction

Black pepper (*Piper nigrum* L.) is a tropical plant used as a spice worldwide. The fruit of black pepper can be processed in different ways, so there are final products such as white, black, red, and green pepper, or dried for convenient storage and use. Black pepper contains many substances, such as terpenes, alkaloids, lignans, phenylpropanoids, etc., that are responsible for a number of important biological activities, such as antioxidant, antibacterial, anti-cancer, anti-inflammatory, analgesic, antipyretic, hepatoprotective, bio-enhancing, and enzyme inhibitory activities [1]. Known as the "king of spices," black pepper (*Piper nigrum* L.) is a perennial plant and a very important spice in economic and consumer terms. It is native to southwest India and Southeast Asia and is grown commercially in tropical regions such as Indonesia, Malaysia, Brazil, Thailand, Madagascar, and West Africa. Today, it is widely distributed worldwide [2].

The Internal Transcribed Spacers (ITS) region is a genomic region that codes for a non-functional ribonucleic acid (RNA) segment located in the coding region for nuclear ribosomal RNAs [3]. In eukaryotes, the ITS region is about 655 bp long; the ITS1 region is between the 18S and 5.8S regions and is about 255-261 bp in size; the 5.8S region is about 164 bp long; and the ITS2 region is between the 5.8S and 28S regions and is about 216-233 bp [4, 5]. The prominence of nucleic acid (DNA) sequence data is highlighted by a survey of phylogenetic studies involving comparisons at the genus level or lower, showing that among 244 published articles in the last five years, 66% consisted of ITS data. In fact, 34% of all published phylogenetic hypotheses are based entirely on ITS sequences [6]. The ITS region is a widely sequenced region of DNA that is useful for elucidating phylogenetic relationships, especially in plants and fungi.

As mentioned, pepper is a valuable and staple agricultural product in several countries. The quality of pepper varies depending on the varieties and growing regions. This can lead to commercial fraud. Thus, the traceability of pepper is very important and necessary. In this study, optimal conditions for DNA extraction from dried pepper samples and optimal

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polymerase chain reaction (PCR) conditions for amplification of the ITS region were determined. The nucleotide sequences of the ITS region of nineteen dried pepper samples were determined and used to construct the phylogenetic relationship between pepper varieties from Vietnam and other countries.

2. Materials and Methods

2.1. Materials

Dried pepper samples were collected from different growing regions in Vietnam, including Quang Tri, Dak Lak, Dak Nong, Gia Lai.

The forward and reverse primers were universal primers and shown as ITS1 (5'-TCCGTAGGTGAAC CTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGA TATGC-3'), respectively. These primers were synthesized by IDT Company, USA, and used for PCR reactions to amplify the ITS region. Reagents for PCR reaction including PrimeSTAR HS DNA polymerase, 5X PrimeSTAR Buffer, and dNTP mixture, were purchased from TaKaRa (Japan). The PureLink PCR purification kit was purchased from ThermoFisher Scientific, USA. All chemicals and reagents used in this study were molecular biology grade.

2.2. Methods

2.2.1. Grinding dried pepper

The powder of dried berries was prepared by four different methods: (1) the dried berries were submerged in the extraction buffer, followed by grinding with a mortar and pestle; (2) the dried berries were ground in liquid nitrogen into fine powder; (3) the dried berries were ground in a mortar and pestle into fine powder, followed by sieving with a pore size of 1 mm; (4) the dried berries were ground in a mortar and pestle into fine powder without sieving.

2.2.2. Total DNA extraction from dried pepper

Total DNA was extracted from dry ground pepper and sieved finely using the CTAB method as described by Doyle and Doyle (1990) [7] and the method developed in this study: 0.1 g of fine powder was added to 1 mL of extraction buffer 1 (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 1% SDS, and 150 mM NaCl) and extraction buffer 2 (100 mM Tris-HCl, pH 8.0, 20 mM NaCl, 2 M NaCl, 1% PVP, and 3% CTAB), supplemented with 10 µL of proteinase K, and incubated at 37 °C for 30 minutes and 65 °C for 30 minutes. The 5 M CH_3COONa solution was added to the sample mixture with a volume ratio of 1:3 and then incubated on ice for 1 hour. Next, the mixture was centrifuged at 8000 rpm for 20 minutes at 4 °C to collect the supernatant. The sample was extracted with the same volume of CIAA mixture (chloroform: isoamyl alcohol, 24:1). An equal volume of isopropanol was added to the sample and incubated on ice for 1 hour. Then, the mixture was centrifuged at 8000 rpm for 20 minutes at 4 °C to collect the DNA pellet. The DNA pellet was washed with 70% ethanol twice. Finally, the DNA was air-dried and dissolved in 50 µL of deionized water. The concentration and purity of total DNA are determined by spectrophotometry and checked by agarose gel electrophoresis. The DNA sample was stored at -20 °C.

2.2.3. PCR reaction

A 50 µL reaction mixture using the PrimeSTAR HS DNA Polymerase PCR kit (Takara Bio, Japan) includes: 10 µL 5X Buffer; 4 µL dNTPs; 3 µL ITS1/ITS4 primer (5 µM); 0.5 µL DNA Polymerase; and 30.5 µL water. The thermal cycle includes 35 cycles: 98 °C/20s; 55 °C/30s; 72 °C/45s; and finally 72 °C/7 min. The PCR products were checked by 0.8% agarose gel electrophoresis and purified using the PureLink PCR purification kit for DNA sequencing.

2.2.4. DNA sequencing of PCR product

The nucleotide sequence of the PCR product was determined by the Sanger method and outsourced for the 1st BASE company.

2.2.5. Analysis of DNA sequences

The DNA sequence data was analyzed by BioEdit software. The analysis of the phylogenetic relationship of nineteen pepper samples and six published data was carried out by the Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) software. Six referent sequences were collected from the NCBI database.

3. Results and Discussions

3.1. Optimization for DNA Extraction from the Dried Black Pepper Samples

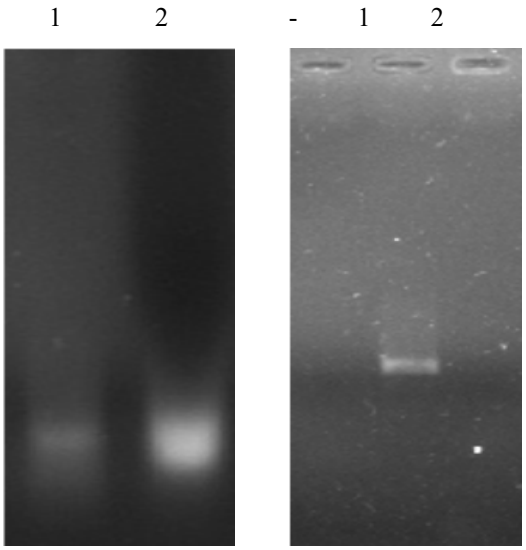
3.1.1. Selection of extraction buffer

Dried black pepper contains a complex of carbohydrates (37.4%), proteins (25.5%), fibres (23.6%), fat (5.3%), polyphenolic compounds, and other metabilites [8]. These components significantly affect the efficiency and quality of the DNA extraction [9]. Alternatively, drying pepper can also cause damage to DNA integrity. Therefore, the extraction of total DNA will face many challenges. A few previous literature mentioned the DNA extraction from dried pepper samples so far. In this study, two extraction buffers were used to extract total DNA from a dried pepper sample, including buffer 1 (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 1% SDS, and 150 mM NaCl) and buffer 2 (100 mM Tris-HCl, pH 8.0, 20 mM NaCl, 2 M NaCl, 1% PVP, and 3% CTAB). Buffer 1 was constructed in this study, while buffer 2 was followed in a previous study [9]. The quantity of DNA extracts indicated that the total DNA mount extracted with buffer 2 was higher than that of

buffer 1, but the quality of the DNA extract carried out by buffer 1 was better than that by buffer 2 (Table 1).

Table 1. The quality of DNA extracts with different extraction buffers

	Buffer 1	Buffer 2
A260/A230	1.00	0.64
A260/A280	1.43	1.08
Concentration (ng/μL)	120	650



(a) (b)

Fig. 1. Electrophoresis pattern of DNA extracts (a) and respective PCR products (b). 1, DNA sample extracted with buffer 1; 2, DNA sample extracted with buffer 2; (-), negative control.

The electrophoresis pattern of total DNA showed that a smear appeared at lane 1, while there was no smear in lane 2, indicating a complete degradation of DNA with the extraction buffer 2 (Fig. 1a). The DNA extracts were used as templates to amplify the target gene in the ITS region by PCR. The results showed that the PCR product only appeared in extract 1 (Fig. 1b, lane 1). The obtained results demonstrated that buffer 1 was suitable for total DNA extraction from a dried pepper sample.

3.1.2. Sample grinding methods

In this study, the sample used was dried berries, so the method of grinding the sample will affect the integrity of the DNA as well as the efficiency of total DNA extraction. Four different grinding methods were applied, and a better DNA extract was obtained with method 3. The spectrophotometric measurements of the DNA extract at 260 and 280 nm achieved a ratio (A260/A280) of 1.84, indicating a high purity of the total DNA extract (Table 2).

Table 2. The quality of DNA extracts with different grinding methods

Method	1	2	3	4
A260/A230	1.68	1.89	1.55	1.25
A260/A280	1.84	1.60	1.84	1.79
Concentration (ng/μL)	106	204.3	115.8	161.0

Fig. 2. Electrophoresis pattern of DNA extracts with the different grinding methods.

A clear band of high molecular weight DNA was observed on the agarose electrophoresis pattern (Fig. 2, lane 3), indicating the presence of genomic DNA in the DNA extract. The yield of DNA was 58 μg/g of dried black pepper sample, which was higher than that from the previous report [9]. The obtained results indicated that the ground powder of dried black pepper berries should be sieved with a pore size of 1 mm, giving better DNA quality and yield.

3.1.3. Precipitation of DNA

Dark-coloured DNA precipitation was observed in this study and previous reports when extracting DNA from plant samples [9, 11]. This may be due to polyphenolic compounds and polysaccharides co-precipitated with DNA during precipitation. Dhanya *et al*. indicated that precipitation of DNA with PEG resulted in improved purity of DNA extract compared to isopropanol or ethanl precipitation [9]. However, Aboul-Maaty and Oraby [10] demonstrated that precipitation of DNA with ice-cold isopropanol in higher concentrations of sodium chloride and potassium acetate removed polysaccharides. In this study, DNA was precipitated by both polyethylene glycol (PEG) and isopropanol methods. The quality of DNA extracts was checked by spectrophotometric measurement and the PCR method. The result showed

that precipitation with isopropanol yielded a higher DNA concentration than precipitation with PEG (Table 3).

Table 3. The quality of DNA extracts with different precipittation methods

Precipitation method	PEG precipitation		Isopropanol precipitation	
Sample	8198-02	8198-19	8198-02	8198-19
A260/A230	1.99	1.62	1.48	1.41
A260/A280	1.92	1.83	1.81	1.83
Concentration (ng/μL)	87.5	65.4	120.1	122.4

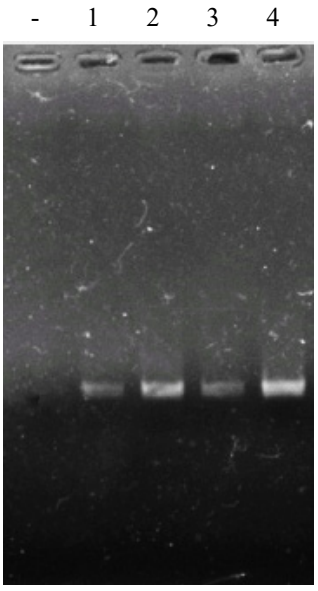


Fig. 3. Electrophoresis pattern of PCR products with the different DNA precipitation methods. (-), negative control; lane 1, 3, total DNA precipitation with 3% PEG; lane 2, 4, total DNA precipitation with isopropanol.

The quality of the DNA extract was also checked by PCR and showed a stronger signal band for the PCR product using the DNA template from isopropanol precipitation compared with PEG precipitation (Fig. 3).

3.2. Optimization of PCR Conditions for Applyfing the ITS Region

3.2.1. Determination of a suitable amount of DNA template

In theory, the success of a PCR technique is proportional to the amount of DNA template used. A higher amount of DNA template will result in a better PCR product. However, the use of a huge amount of DNA template may cause a reverse effect due to increasing contaminants. In this study, different amounts of DNA template ranging from 20 to 100 ng were added to a 25-µl PCR mixture. The DNA electrophoresis pattern showed the appearance of a significant signal band at all three concentrations of DNA template (20, 60, and 100 ng/25 µl reaction). The obtained result indicated that 20 ng DNA template was enough to get a positive PCR result, and 100 ng DNA template did not reduce the efficiency of the reaction due to the risk of containing more inhibitors (Fig. 4). There was not much difference in PCR results for the three amounts of template DNA used. However, the total DNA sample extracted from dried black pepper may contain a lot of broken DNA; therefore, the recommended amount of template DNA to use is 100 ng to ensure successful amplification of the target gene.

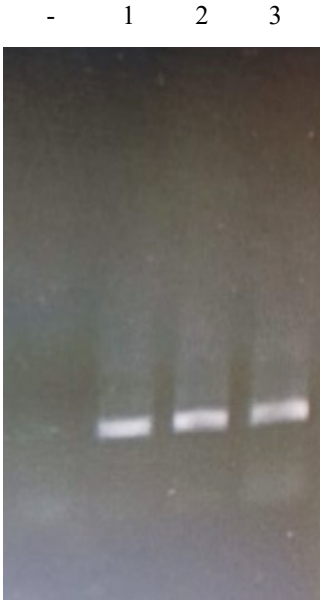


Fig. 4. Amplification of the ITS region with different amounts of DNA template (20, 60, and 100 ng). Lane 1, 0 ng; lane 2, 20 ng; lane 3, 60 ng; and lane 4, 100 ng.

3.2.2. Determination of a suitable concentration of primers

The optimization of primer concentrations is necessary to enhance the specific hybridization between the primers and target genes and also eliminate spurious primer dimers [10]. Primers can self-anneal or anneal to the other primer in the reaction to form a double-strand structure called primer dimers. If the primer concentration is in excess of the template concentration, then the primers will be more likely to anneal to themselves or each other over the DNA template. If the primer concentration is too low, it will not be enough to carry out the reaction. Both cases result in a reduction in PCR efficiency. In the present study, a range of primer concentrations of 0.2 - 0.5 µM was applied in PCR. The result showed that the signal intensity of the target band was proportional to the increasing primer concentrations (Fig. 5).

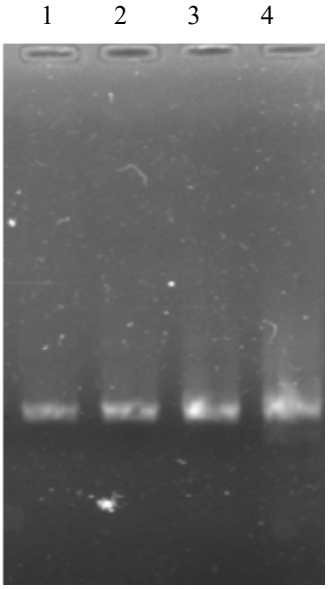


Fig. 5. Amplification of the ITS region with different concentration of primers. Lane 1, 0.2 µM; lane 2, 0.3 µM; lane 3, 0.4 µM; and lane 4, 0.5 µM.

There was no significant difference in the signal intensity of the target bands at the primer concentrations of 0.4 µM and 0.5 µM; therefore, 0.4 µM primers could be used for further experiments.

3.2.3. Determination of optimal primer annealing temperature

Annealing temperature is considered one of the most important factors strongly affecting PCR efficiency. The ptimal annealing temperature will enhance the PCR reaction and should be first considered to be optimized.

The theoretical primer annealing temperature can be deduced from its melting temperature, which is calculated based on its nucleotide sequences. In this study, universal primer pairs, including ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS region. The melting temperatures of ITS1 and ITS4 were 61.9 ºC and 53.6 ºC, respectively. Therefore, three different annealing temperatures of 53, 55, and 57 ºC were set up to perform the PCR reaction in this study. The result showed that the PCR reaction was successfully carried out at three different annealing temperatures (Fig. 6).

3.2.4. Determination of the number of amplication cycles

The quantity of PCR product is determined according to the function 2^n (*n* is number of cycles). In this study, PCR products will be used to determine the sequence of the ITS region according to the Sanger method, so the quantity and quality of PCR products will be taken into account to obtain the best sequencing results. The obtained results showed that the strongest signal intensity of the target band was observed after 35 cycles, compared with 25 and 30 cycles (Fig. 7).

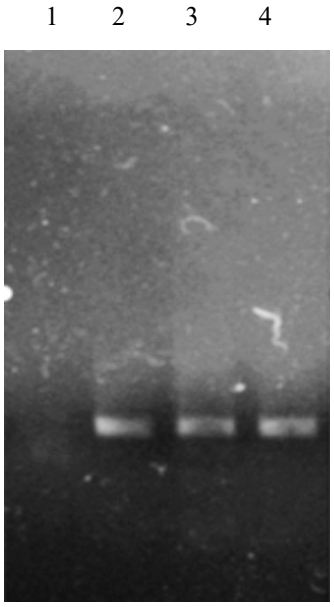


Fig. 6. Amplification of the ITS region with different primer annealing temperatures. Lane 1, negative control; lane 2, 53 ºC; lane 3, 55 ºC; and lane 4, 57 ºC.

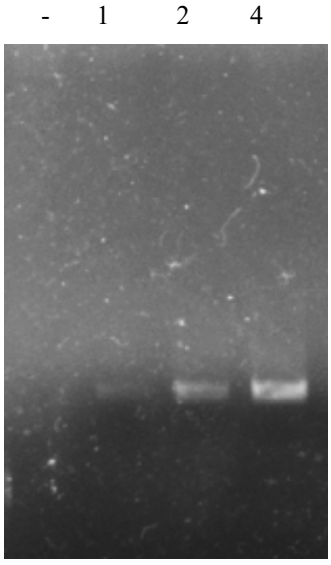


Fig. 7. Amplification of the ITS region with different number of cycles. Lane 1, negative control; lane 2, 25 cycles; lane 3, 30 cycles; and lane 4, 35 cycles.

3.3. Prepration of PCR Products for Sequencing

Optimal conditions for total DNA extraction and PCR reaction were applied for the amplification of ITS region from nineteen different dried black pepper samples. The result showed a successful amplification of the target gene for all nineteen different samples (Fig. 8).

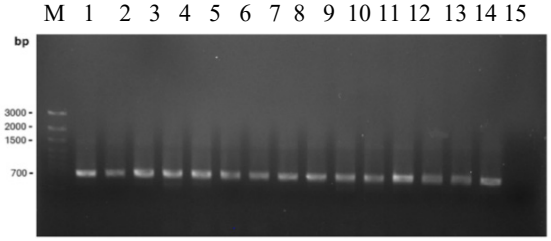


Fig. 8. PCR products of the ITS region with different dried black pepper samples. M, DNA ladder; lanes 1-15, PCR products from fifteen representative samples, respectively; (-) negative control

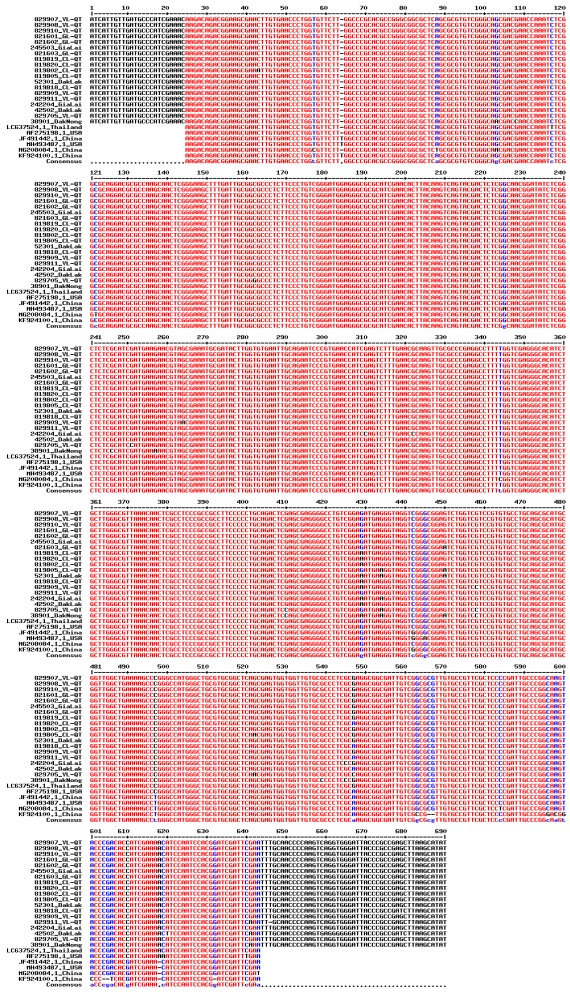


Fig. 9. DNA alignment of ITS sequences from nineteen pepper samples and six publised data.

3.4. Analysis of the DNA Sequence of the ITS Region

The PCR products from nineteen samples were used to determine the nucleotide sequence of the ITS region by the Sanger method. Nineteen obtained sequences and published sequences were aligned, as shown in Fig. 9. The result presented different site mutations in the ITS region between samples. In this study, nineteen pepper samples were collected from different growing regions in Vietnam, such as Daklak, DakNong, Gia Lai, and Quang Tri. To evaluate the genetic relationships between pepper varieties in different growing regions in Vietnam and other countries, the nucleotide sequences of the ITS region were used to build a phylogenetic tree using the Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11) software. The results showed that the nineteen pepper samples in this study were grouped closely together. The six matched reference samples are divided into two different groups (Fig. 10). This shows that there are differences in the gene sequence of the ITS region. This data can be used to distinguish pepper varieties originating from Vietnam compared to those from other countries. The obtained results implicated that the nucleotide sequence of the ITS region can be used as bias to trace the origin of peppers.

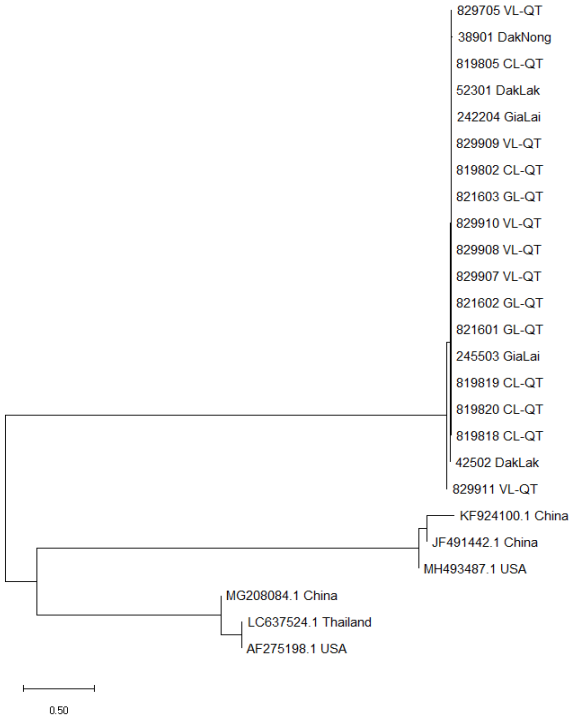


Fig. 10. Phylogenetic analysis of ITS sequences from nineteen pepper samples and six publised data. VL-QT, Vinh Linh - Quang Tri; CL-QT, Cam Lo - Quang Tri; GL-QT, Gio Linh - Quang Tri.

4. Conclusion

Pepper is a valuable and staple agricultural product in several countries, with different quality depending on the production region. The traceability of this item is very important and necessary. This study has established conditions for DNA extraction from dried pepper, and the obtained DNA can be used to amplify the ITS region using the PCR technique followed by DNA sequencing. Sequence analysis of the ITS region showed that there were differences between pepper varieties grown in Vietnam and some other countries. This is evidence for using DNA data to trace the origin of dried pepper.

Acknowledgments

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