Application of internal transcribed spacer (ITS) sequences for identifying *Anoectochilus setaceus* Blume in Thanh Hoa, Vietnam

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Применение последовательностей внутренних транскрибируемых спейсеров (ITS) для идентификации образцов *Anoectochilus setaceus* Blume в Тханьхоа (Вьетнам)

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Background. The term "DNA barcode" is used extensively in molecular taxonomy. Basically, this technique is based on the use of a DNA sequence (about 400–800 bp) as a standard to identify and determine the species relation of organisms quickly and accurately. Therefore, DNA barcodes not only help taxonomists in classifying and identifying species, but also improve their ability to control, understand and utilize biodiversity. In this study, the authors conducted identification of samples of *Anoectochilus setaceus* Blume collected in Thanh Hoa through the isolated sequence of ITS gene regions. **Materials and methods.** Total DNA was extracted from young leaves of *A. setaceus* samples using CTAB method. The ITS gene segment was amplified by PCR and sequenced. This genetic sequence was analyzed, compared and used to establish a phylogenetic tree using BioEdit, BLAST and DNASTAR programs. **Results and conclusion.** We isolated 4 sequences of the ITS gene region in 4 *A. setaceus* samples collected at Xuan Lien and Pu Luong of Thanh Hoa province; the ITS gene region was 667 nucleotide long. The findings identified the samples as the same species and showed 99% similarity to the ITS gene sequence of *A. roxburghii* (Wall.) Lindl. published in GenBank, GQ328774. This study also demonstrates that the method employing internal transcribed spacer (ITS) sequences is an effective tool to identify *A. setaceus* taxa.

Key words: molecular taxonomy, DNA barcoding, ITS primers, identification of samples.

Актуальность. Термин «DNA barcode» широко используется в молекулярной таксономии. По существу, этот метод основан на использовании последовательности ДНК (около 400–800 пн) в качестве стандарта для быстрой и точной идентификации и определения видовых отношений организмов. Таким образом, ДНК-штрихкоды помогают систематикам не только классифицировать и идентифицировать виды, но и контролировать, понимать и использовать биоразнообразие. В настоящем исследовании авторы провели идентификацию образцов *Anoectochilus setaceus* Blume, собранных в провинции Тханьхоа, на основе анализа последовательностей ITS. **Материалы и методы.** Тотальную ДНК выделяли из молодых листьев образцов *A. setaceus* с использованием бромида цетилтриметиламмония (CTAB-метод). Сегмент гена ITS амплифицировали методом ПЦР и секвенировали. Эту генетическую последовательность анализировали, сравнивали и использовали для построения «филогенетического древа» с помощью программ BioEdit, BLAST, DNASTAR. **Результаты и выводы.** Нами выделены 4 последовательности генной области ITS из 4 образцов *A. setaceus*, собранных в Сюань Лиен и Пу Луонг провинции Тханьхоа; изученный ITS-регион имел длину 667 нуклеотидов. Полученные данные идентифицировали образцы как один и тот же вид и показали 99-процентное сходство с последовательностью ITS *A. roxburghii* (Wall.) Lindl., опубликованной в базе данных GenBank (GQ328774). Исследование также демонстрирует, что использование внутреннего транскрибируемого спейсера (ITS) является эффективным методом для идентификации образцов *A. setaceus*.

Ключевые слова: молекулярная таксономия, баркодирование ДНК, праймеры ITS, идентификация образцов.

Introduction

In medical terms, *Anoectochilus setaceus* Blume is a powerful medicinal orchid which has functions beneficial for human health, i.e., improving blood circulation, producing antibacterial, anti-tracheitis, anti-hepatitis, and anti-neurasthenia effects (Lin et al., 1993; Lin et al., 2000; Wang et al., 2002; Ye et al., 2017). In particular, some active substances in *A. setaceus* are able to stop the growth of cancer cells. The chemical constituents of *A. setaceus* include quercetin, isoharmnetin-3-O-beta-D-glucopyranosid, kaempferol-3-O-beta-D-glucopyranosid, 5-hydroxy-3'-4'-7'-trimethoxyflavonol-3-O-beta-D-rutinosid and isorhamnetin-3-O-beta-D-rutinosid, etc. (He et al., 2006; Ye et al., 2017; Chac et al., 2019). Because of its

medicinal values, *A. setaceus* is popular in the market and sold at a high price. The small population of plants which scatters in a few highland forests, and illegal exploitation have made *A. setaceus* listed in the Vietnam Red Data Book (Vietnam Academy of Science and Technology, 2007). As a result, all exploitation and use of *A. setaceus* for illegal commercial purposes are banned in Vietnam.

The internal transcribed spacer (ITS) regions are localized between nuclear genes coding for 18S, 5.8S and 26S rRNA (Kress et al., 2005). Among the gene units are ITS1 and ITS2 segments, which form a basic gene group. Such gene groups replicate continuously in thousands of copies in the nucleus genes while being separated by the nontranscribed spacer region. In plant classification studies at the species level, ITS region is the most commonly decoded locus (Gigliano, 1999; Li D.Z. et al., 2011; Takamiya et al., 2011; Tripathi et al., 2013). The ITS region has been shown to be highly effective in classification of plants and fungi (except for ferns). This is a locus used for short DNA sequencing (Stoeckle, 2003; Chen, Shiau, 2015). It was demonstrated in most studies that the ITS region had a wide diversity (about 13.6% among closely related species) at the species level (Li M. et al., 2011; Techen et al., 2014; Lv et al., 2015; Hu et al., 2019). One of the advantages of the ITS region is that it can replicate two smaller segments (ITS1 and ITS2) on both sides with the 5.8S locus, which is useful in case of replicating damaged samples. It was also pointed out that the ITS region had low levels of variation within the species (Baldwin et al., 1995; Yang et al., 2012; Wang et al., 2020). That more than 100 thousand ITS sequences (until June 2016) have been published and the new figures continue to be added to the GenBank database is now a valuable source for studies on species.

A. setaceus in Vietnam is in danger of extinction because of its small and sparse population mainly in the forests as well as its overexploitation by humans. Moreover, the regeneration ability of this species is very low in the wild, especially where the ecological environment is devastated. As a result, the conservation and development of this species is a key and necessary issue. Thus, it is important to isolate and read ITS genome sequences and *A. setaceus* genotypes to provide important scientific data for the conservation, development and appropriate exploitation of this rare and precious medicinal herb.

Materials and methods

Plant materials

From March 2018 to May 2018, a total of 4 *Anoectochilus setaceus* samples were collected from Xuan Lien National Park and Pu Luong Nature Reserve in Thanh Hoa Province, Vietnam. Specifically, two samples (XL1 and XL2) were collected in Xuan Lien National Park and the other two (PL1 and PL2) were collected in Pu Luong Nature Reserve. Samples of young leaves were collected, stored in paper bags with silica gel, then moved to the laboratory and kept in a freezer at a temperature of –20°C for DNA extraction. Species identification of collected samples was performed by Associate Professor Tran Minh Hoi (Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology) on the basis of morphological characteristics. In addition, 1 ITS sequence of *A. roxburghii* (Wall.) Lindl. (GQ328774) and 1 ITS sequence of *A. lanceolatus* Lindl. (KT344108) were downloaded from the GenBank database.

Total DNA extraction

Total DNA was extracted from the young leaves of *A. setaceus* samples using CTAB method (Doyle, Doyle, 1987) with some improvement.

Step 1: Put 200 mg of young leaves in liquid nitrogen (–196°C) to grind into a fine powder. Add 6 ml wash buffer (Tris – HCl 100mM, pH = 8.0; EDTA 5 mM, pH = 8.0; NaH_2PO_4 0.4%; sorbitol 350 mM; water). Transfer to 1.5 ml Eppendorf tube. Centrifuge the sample for 15 minutes, at a speed of 12000 rounds per minute at 4°C, remove the fluid, and collect the sediment.

Step 2: Add 500 µl of extraction buffer to each tube (components of the buffer: Tris – HCl 100 mM, pH = 8.0; EDTA 20 mM, pH = 8.0; CTAB 4%; NaCl 1.4 M; water), mix gently, incubate at 65°C for 30 minutes (gently stir every 15 minutes). After finishing the incubation, leave the sample under room temperature for 5 minutes.

Step 3: Centrifuge for 15 minutes, at a speed of 12 000 rounds per minute at 4°C. Transfer the aqueous upper phase to a new 1.5 ml Eppendorf tube.

Step 4: Add 500 µl of C : I (24 chloroform : 1 isoamyl) to remove protein and impurities, stir for 15 minutes. Centrifuge for 15 minutes, at a speed of 12000 rounds per minute at 4°C. Carefully suck 500 µl of clear liquid in the upper phase and transfer to a new 1.5 pl Eppendorf tube (remove precipitate). Repeat this step twice.

Step 5: Add 500 µl of isopropanol, mix gently, precipitate the DNA at –20°C overnight. Then centrifuge for 15 minutes, at a rate of 12000 rounds per minute at 4°C, collect the precipitate.

Step 6: Add 500 µl of 70% ethanol. Centrifuge for 10 minutes, 12000 rounds per minute at 4°C, decant the precipitate (repeat 2–3 times). Dry at room temperature and dissolve DNA in 50 µl of distilled water twice. Store in the freezer at –20°C until use.

ITS gene amplification via PCR technique

The ITS gene fragments amplified via PCR technique with ITSF / ITSR specific primers were obtained on T100 Thermal Cycler Bio-Rad PCR machine (Table 1). The amplified ITS gene fragment was expected to be about 700 nucleotides long. The reaction was carried out with a total volume of 25 µl, including 12.5 µl Maxter Mix 2X (Thermo Scientific), 1 µl for each primer, 1 µl DNA template and 9.5 µl deionized water. The PCR cycle included denaturation starting at 94°C for 4 minutes; repeated 25 cycles including: denaturing at 94°C for 30 seconds, attaching primer at 55°C for 40 seconds, synthesizing and lasting at 72°C for 40 seconds. The process ended by exposing it to 72°C for 10 minutes and storing at 4°C.

Table 1. ITS primers used for PCR amplification

Таблица 1. Праймеры, использованные для амплификации района ITS

Primer code		Sequence (5’→3’)	Source
ITS	F	AGGAGAAGTCGTAACAAGGTTTCC	Macrogen (South Korea)
	R	GATATGCTTAAACTCAGCGGGTC	

Electrophoresis for PCR product testing

The PCR products were electrophoresed on 0.8% agarose gel. The gels were then removed from the electrophoresis equipment and gently transfered to a container with ethidium bromide solution, in which they were stained for 10 minutes. The gels were washed by being soaked in water for 2–3 minutes before being put into a machine for ultraviolet (UV) light observation and taking photos.

Purification of PCR products

The next step after amplification should be collecting the genes in a pure and gel-free form. Purification was conducted using Kit GenJET PCR Purification of Thermo Scientific.

Determination of the order of the nucleotides within an ITS gene

The order of the nucleotides within an ITS gene was determined on the ABI PRISM® 3100 Avant Genetic Analyzer, using the BigDye® Terminator v3.1 Cycle Sequencing Kit with specific primers. The gene sequence was analyzed and compared, and a phylogenetic tree was established using BioEdit, BLAST, DNASTAR.

Results and discussion

ITS gene amplification of *A. setaceus* samples

After being amplified using PCR techniques, the ITS gene of *A. setaceus* samples were electrophoresed on 0.8% agarose gel and photographed. PCR results with specific primer pairs are shown in Fig. 1.

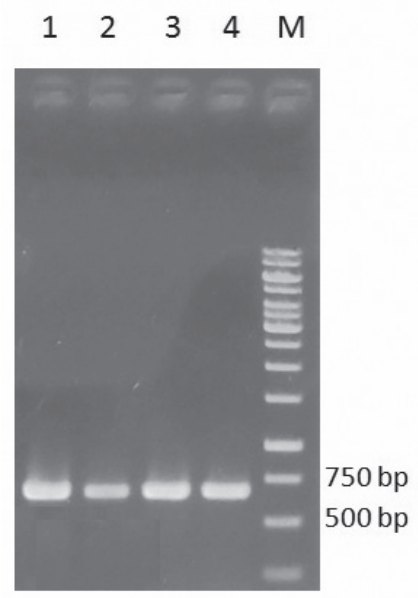

Fig. 1. PCR results with ITS primer pair for 4 *Anoectochilus setaceus* samples.
M – Molecular weight marker;
1 – XL1, 2 – XL2; 3 – PL1; 4 – PL2

Рис. 1. Результаты ПЦР 4-х образцов *Anoectochilus setaceus* с использованием пары ITS-праймеров.
M – Маркер молекулярного веса;
1 – XL1, 2 – XL2; 3 – PL1; 4 – PL2

The result of electrophoresing 4 samples with ITS F/R primers was a single band at about 700 bp. This result was consistent with theoretical calculations and served as a basis in terms of ITS gene sequencing for further research.

Determination of the ITS gene sequence of *A. setaceus* samples

After electrophoresis, ITS gene sequencing was conducted on the ABI PRISM® 3100 Avant Genetic Analyzer, using the BigDye® Terminator v3.1 Cycle Sequencing Kit with specific primers. The findings were as follows:

Pu Luong 1 ITS sequence (PL1)

GGGAAAAAGACCCTAAAGAGGATGGATGACTTTGGATA
ACACGTGAACATTTGACGGCGGTTGCTGTCTATAAACACCAT
CCATCTATTGGCCCCTCTTGATTGAGGCAACAATAAAAAGAT
GGAGGGAAAAACAACTCGGGCGCAGTTGTGCGCCAAGGAAG
TATGTTGCATTGGCATCGATGACTATTCGCCAAAGCCTGTCG
TGCTTAGCGGAGTGTTGTTGTTGCTTCTTAAGTATTGTATGA
CTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGC
GCAGCGAAATGCGATACGTGGTGTGAATTGCAGAATCCCGTG
AACCATCAAATCTTTGAACGCAAGTTGCGCCTGAGGCCAAT
TGGCTAAGGGCACGTCCGCCTGGGCGTCAAGCATTACATCGC
TTCATTCGACACCAATTGCCCAGTATTTTGCTGTGGTGCTGG
TCTGAATGCGGAGAGTGGCCCTTCGTGCACACTTGTGCGACG
GGTTGAAGAACAATTTGCTTTCCTCTGGCCATGTTTTGATA
AAGGGGTGGTGTATGCAGCCATTAGGCCCACACTATCATCTC
ATTGCCTTGAGGAGGATAAATGTACACATTCGTGGCTGATCA
CCCGATATAAATGTCGCAGGTGACGCCCTGAAATGCGACCC
CA

Pu Luong 2 ITS sequence (PL2)

GGGAAAAAGACCCTAAAGAGGATGGATGACTTTGGATA
ACACGTGAACATTTGACGGCGGTTGCTGTCTATAAACACCAT
CCATCTATTGGCCCCTCTTGATTGAGGCAACAATAAAAAGAT
GGAGGGAAAAACAACTCGGGCGCAGTTGTGCGCCAAGGAAG
TATGTTGCATTGGCATCGATGACTATTCGCCAAAGCCTGTCG
TGCTTAGCGGAGTGTTGTTGTTGCTTCTTAAGTATTGTATGA
CTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGC
GCAGCGAAATGCGATACGTGGTGTGAATTGCAGAATCCCGTG
AACCATCAAATCTTTGAACGCAAGTTGCGCCTGAGGCCAAT
TGGCTAAGGGCACGTCCGCCTGGGCGTCAAGCATTACATCGC
TTCATTCGACACCAATTGCCCAGTATTTTGCTGTGGTGCTGG
TCTGAATGCGGAGAGTGGCCCTTCGTGCACACTTGTGCGACG
GGTTGAAGAACAATTTGCTTTCCTCTGGCCATGTTTTGATA
AAGGGGTGGTGTATGCAGCCATTAGGCCCACACTATCATCTC
ATTGCCTTGAGGAGGATAAATGTACACATTCGTGGCTGATCA
CCCGATATAAATGTCGCAGGTGACGCCCTGAAATGCGACCC
CA

Xuan Lien 1 Sequence (XL1)

GGGAAAAAGACCCTAAAGAGGATGGATGACTTTGGATA
ACACGTGAACATTTGACGGCGGTTGCTGTCTATAAACACCAT
CCATCTATTGGCCCCTCTTGATTGAGGCAACAATAAAAAGAT
GGAGGGAAAAACAACTCGGGCGCAGTTGTGCGCCAAGGAAG
TATGTTGCATTGGCATCGATGACTATTCGCCAAAGCCTGTCG
TGCTTAGCGGAGTGTTGTTGTTGCTTCTTAAGTATTGTATGA
CTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGC
GCAGCGAAATGCGATACGTGGTGTGAATTGCAGAATCCCGTG
AACCATCAAATCTTTGAACGCAAGTTGCGCCTGAGGCCAAT
TGGCTAAGGGCACGTCCGCCTGGGCGTCAAGCATTACATCGC
TTCATTCGACACCAATTGCCCAGTATTTTGCTGTGGTGCTGG
TCTGAATGCGGAGAGTGGCCCTTCGTGCACACTTGTGCGACG
GGTTGAAGAACAATTTGCTTTCCTCTGGCCATGTTTTGATA
AAGGGGTGGTGTATGCAGCCATTAGGCCCACACTATCATCTC
ATTGCCTTGAGGAGGATAAATGTACACATTCGTGGCTGATCA
CCCGATATAAATGTCGCAGGTGACGCCCTGAAATGCGACCC
CA

Xuan Lien 2 Sequence (XL2)

GGGAAAAAGACCCTAAAGAGGATGGATGACTTTGGATA
ACACGTGAACATTTGACGGCGGTTGCTGTCTATAAACACCAT
CCATCTATTGGCCCCTCTTGATTGAGGCAACAATAAAAAGAT
GGAGGGAAAAACAACTCGGGCGCAGTTGTGCGCCAAGGAAG
TATGTTGCATTGGCATCGATGACTATTCGCCAAAGCCTGTCG

TGCTTAGCGGAGTGTTGTTGTTGCTTCTTAAGTATTGTATGA
CTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGC
GCAGCGAAATGCGATACGTGGTGTGAATTGCAGAATCCCGTG
AACCATCAAATCTTTGAACGCAAGTTGCGCCTGAGGCCAAT
TGGCTAAGGGCACGTCCGCCTGGGCGTCAAGCATTACATCGC
TTCATTCGACACCAATTGCCCAGTATTTTGCTGTGGTGCTGG
TCTGAATGCGGAGAGTGGCCCTTCGTGCACACTTGTGCGACG
GGTTGAAGAACAATTTGCTTTCCTCTGGCCATGTTTTGATA
AAGGGGTGGTGTATGCAGCCATTAGGCCCACACTATCATCTC
ATTGCCTTGAGGAGGATAAATGTACACATTCGTGGCTGATCA
CCCGATATAAATGTCGCAGGTGACGCCCTGAAATGCGACCC
CA

Based on 4 sequences from the isolation, we found that the nucleotide order of the studied samples were equal (667 nucleotides); then we made a comparison with the ITS gene sequence published in GenBank as a basis for further research.

Analysis of the studied ITS sequences in comparison with the ITS sequences published in GenBank

DNASTAR software was used to compare the studied ITS sequences with the ITS sequences published in the GenBank database. The results are shown in Fig. 2.

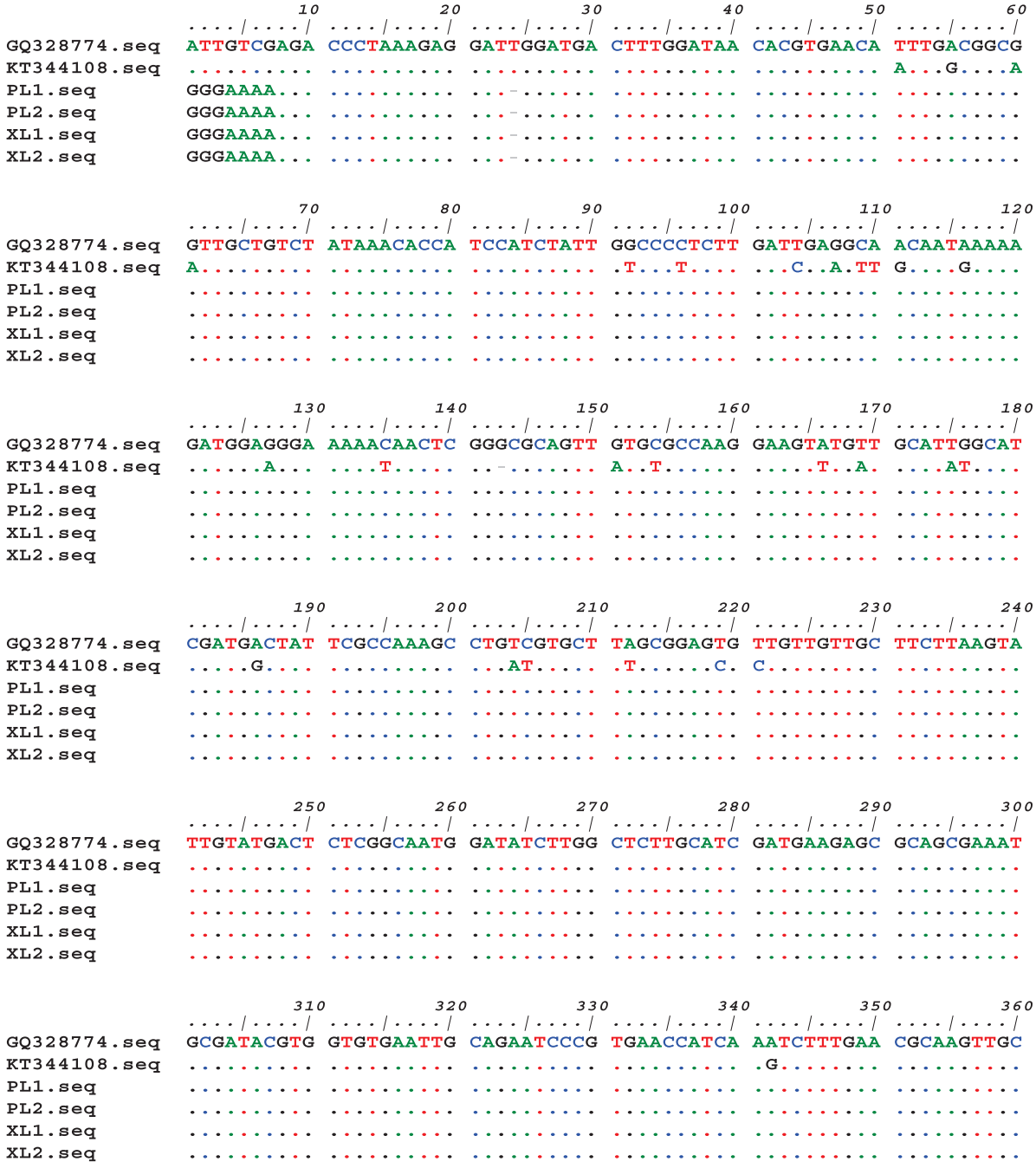

Fig. 2. Comparison among the studied ITS sequences (XL1, XL2, PL1 and PL2) with the ITS sequences in the GenBank database (GQ328774 and KT344108)

Рис. 2. Сравнительный анализ изучаемых ITS-последовательностей (XL1, XL2, PL1 и PL2) и ITS-последовательностей из базы данных GenBank (GQ328774 и KT344108)

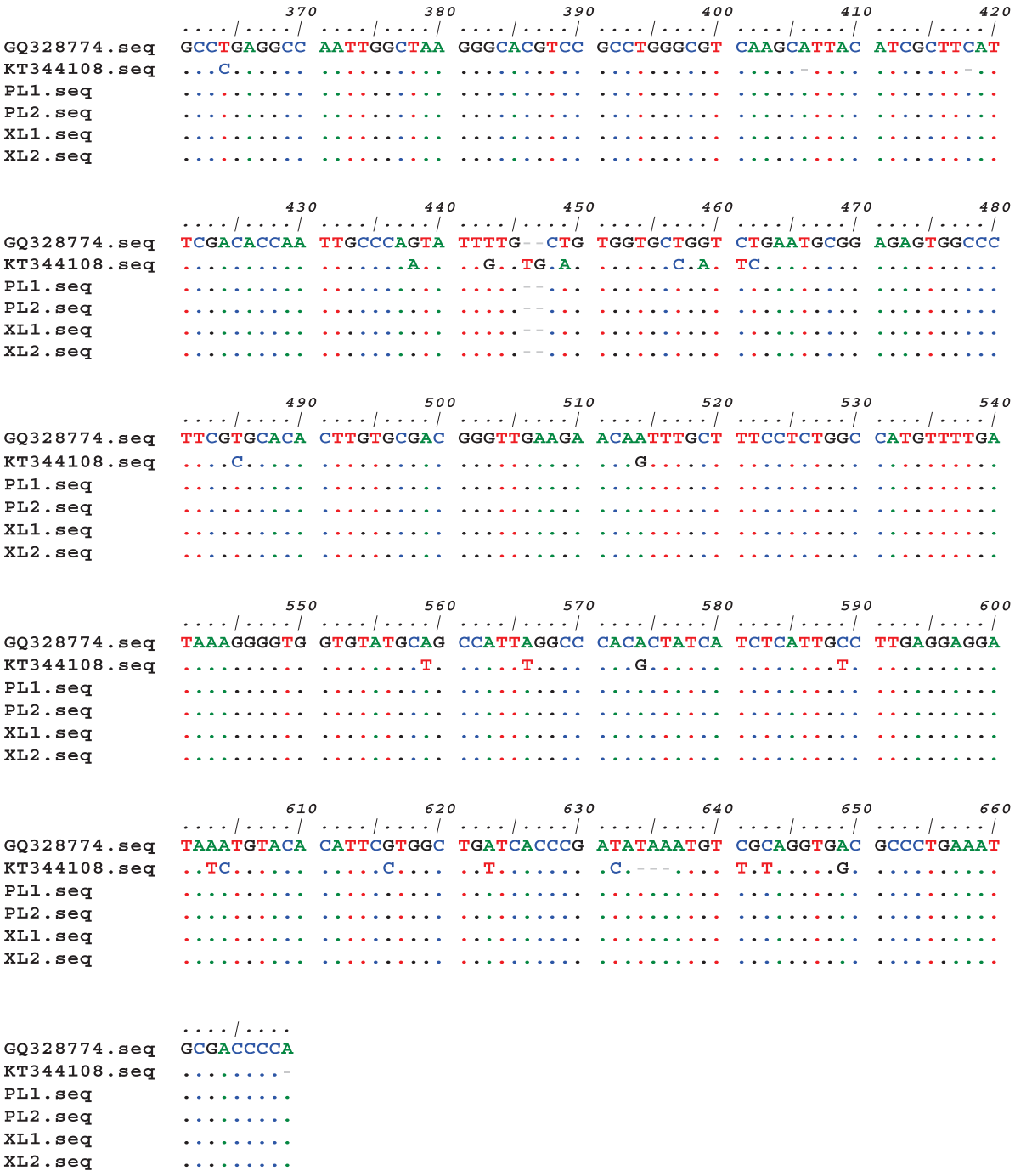

Fig. 2 (continued). **Comparison among the studied ITS sequences (XL1, XL2, PL1 and PL2) with the ITS sequences in the GenBank database (GQ328774 and KT344108)**

Рис. 2 (продолжение). **Сравнительный анализ изучаемых ITS-последовательностей (XL1, XL2, PL1 и PL2) и ITS-последовательностей из базы данных GenBank (GQ328774 и KT344108)**

It can be seen that *A. setaceus* samples collected in Pu Luong (PL1 and PL2) and Xuan Lien (XL1 and XL2) had the same nucleotide order of the ITS region (Table 2), which reconfirm that the studied samples collected in Pu Luong and Xuan Lien were the same species. However, the studied ITS sequences were markedly different from an ITS sequence coded KT344108 published in GenBank. The details are presented in Table 2.

Table 2 shows that there is a different position between the studied ITS gene sequence and the one coded GQ328774 in GenBank. To be more specific, at the 24th position of GQ328774 there was T, while G was recognized at this position of the 4 *A. setaceus* samples. However, there were huge differences between the 4 studied ITS gene sequences and KT344108 as there were up to 50 different positions (Table 2). Therefore, it was possible to conclude that the *A. setaceus* samples and *A. roxburghii* species whose ITS gene sequence published in Genbank, GQ328774, were the same species. This also showed that the variability of the conservative nucleotide sequence of a nuclear gene (ITS) was not high among individuals of the same species (Álvarez, Wendel, 2003).

Table 2. **Differences between the studied ITS sequences (XL1, XL2, PL1 and PL2) and the ITS gene sequences in the GenBank database (GQ328774 and KT344108)**

Таблица 2. **Различия между изучаемыми ITS-последовательностями (XL1, XL2, PL1 и PL2) и ITS-последовательностями из базы данных GenBank (GQ328774 и KT344108)**

No.	Location	GQ328774	XL1	XL2	PL1	PL2	KT344108
1	24	T	G	G	G	G	T
2	51	T	G	G	G	G	A
3	55	A	A	A	A	A	G
4	60	G	G	G	G	G	A
5	91	G	G	G	G	G	T
6	96	C	C	C	C	C	T
7	104	T	T	T	T	T	C
8	107	A	A	A	A	A	G
9	109	C	C	C	C	C	T
10	110	A	A	A	A	A	T
11	101	A	A	A	A	A	T
12	111	A	A	A	A	A	G
13	116	A	A	A	A	A	G
14	127	G	G	G	G	G	A
15	135	C	C	C	C	C	T
16	151	G	G	G	G	G	A
17	154	C	C	C	C	C	T
18	166	A	A	A	A	A	T
19	169	T	T	T	T	T	A
20	175	T	T	T	T	T	A
21	176	G	G	G	G	G	T
22	186	A	A	A	A	A	G
23	204	T	T	T	T	T	A
24	205	C	C	C	C	C	T
25	212	A	A	A	A	A	T
26	219	T	T	T	T	T	C
27	221	T	T	T	T	T	C
28	342	A	A	A	A	A	G
29	364	T	T	T	T	T	C
30	438	G	G	G	G	G	A
31	443	T	T	T	T	T	G
32	449	T	T	T	T	T	A
33	457	T	T	T	T	T	C
34	459	G	G	G	G	G	A

Table 2. The end

Таблица 2. Окончание

No.	Location	GQ328774	XL1	XL2	PL1	PL2	KT344108
35	461	C	C	C	C	C	T
36	462	T	T	T	T	T	C
37	485	T	T	T	T	T	C
38	553	A	A	A	A	A	G
39	559	A	A	A	A	A	T
40	566	A	A	A	A	A	T
41	574	A	A	A	A	A	G
42	589	C	C	C	C	C	T
43	603	A	A	A	A	A	T
44	604	A	A	A	A	A	C
45	616	G	G	G	G	G	C
46	623	A	A	A	A	A	T
47	632	T	T	T	T	T	C
48	641	C	C	C	C	C	T
49	643	C	C	C	C	C	T
50	649	A	A	A	A	A	G

The genetic relationship among the studied ITS gene sequences and the ones published in GenBank was analyzed using BioEdit software. The results are shown in Table 3 and Fig. 3.

It can be pointed out that the ITS gene sequences of *A. setaceus* samples collected at Xuan Lien (XL1 and XL2) and Pu Luong (PL1 and PL2) of Thanh Hoa were the same and similar to GQ328774 of *A. roxburghii*. The differences were very small. The genetic relationship among the samples and KT344108 (*A. lanceolatus*) was relatively large, as shown in Fig. 3.

The findings show that the ITS gene sequences of *A. setaceus* samples collected at Xuan Lien (XL1 and XL2) and Pu Luong (PL1 and PL2) and ITS gene sequence of A. *roxburghii* (GQ328774) were related to the same species and had a relatively large dissociation coefficient with *A. lanceolatus* (KT344108). The ITS gene sequences of *A. setaceus* samples collected at Xuan Lien (XL1 and XL2) were not genetically different from those collected in Pu Luong (PL1 and PL2). It is also proved that the method employing internal transcribed spacer (ITS) sequences is an effective tool to identify *A. setaceus*.

Table 3. Comparison among the studied ITS gene sequences (XL1, XL2, PL1 and PL2) with the ITS gene sequences in the GenBank database (GQ328774 and KT344108)

Таблица 3. Сравнительный анализ изучаемых ITS-последовательностей (XL1, XL2, PL1 и PL2) и ITS-последовательностей из базы данных GenBank (GQ328774 и KT344108)

	Percent Identity							
	1	2	3	4	5	6		
1		92.6	98.9	98.9	98.9	98.9	1	GQ328774.seq
2	7.9		91.5	91.5	91.5	91.5	2	KT344108.seq
3	1.1	9.1		100.0	100.0	100.0	3	PL1.seq
4	1.1	9.1	0.0		100.0	100.0	4	PL2.seq
5	1.1	9.1	0.0	0.0		100.0	5	XL1.seq
6	1.1	9.1	0.0	0.0	0.0		6	XL2.seq
Divergence	1	2	3	4	5	6		

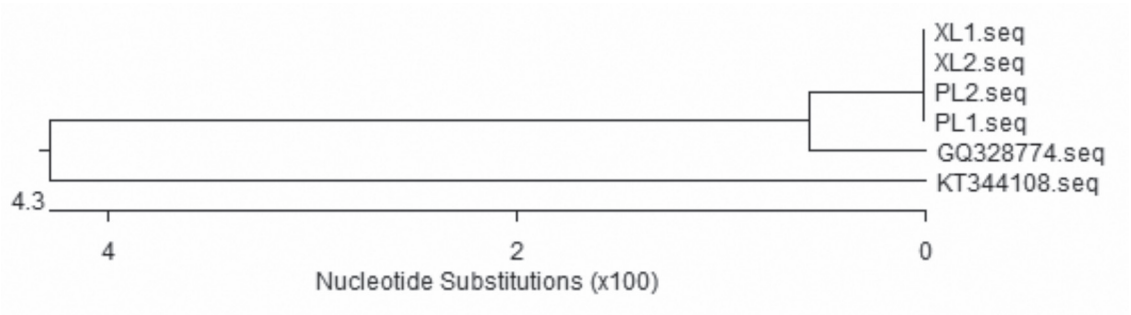

Fig. 3. Tree diagram showing the genetic relationship between the studied ITS gene sequences (XL1, XL2, PL1 and PL2) and the ITS gene sequences published in the GenBank database

Рис. 3. «Филогенетическое древо», построенное на основе сравнения изучаемых ITS-последовательностей (XL1, XL2, PL1 и PL2) и ITS-последовательностей из базы данных GenBank

Conclusion

The ITS gene sequences of *A. setaceus* in Thanh Hoa were successfully isolated and read in this study; the studied ITS gene was 667 nucleotides long and 99% similar to the ITS gene sequence of *A. roxburghii* published in GenBank, GQ328774. The study results also showed that the ITS gene sequences of *A. setaceus* samples collected at Xuan Lien (XL1 and XL2) and Pu Luong (PL1 and PL2) of Thanh Hoa province were not different, which meant the samples belonged to the same species.

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