CHARACTERISTICS OF BARCODE NUCLEOTIDE SEQUENCES *matK* AND ITS OF THE *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 PLANTS COLLECTED IN LUANG NAMTHA PROVINCE OF LAOS

Pham Thi Thanh Nhan[1*], Tangmany SYSOMEPHONE[2]
[1]TNU - University of Education, [2]Research Institute for Educational Sciences - Ministry of Education and Sports, Laos

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ABSTRACT

The *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants have been over-exploited and recorded in Vietnam's Red Data Book. In this paper, we analyze characteristics of *matK, ITS* nucleotide sequences from this species collected in Luang Namtha province of Laos to find out the differences at the molecular level among species, and provide information about barcodes for the identification, exploitation and conservation of their genetic resources. Lengths of the isolated *matK* gene segment and *ITS* region are around 900bp and 500bp, respectively. Their sequences have the similarity with other species in the genus *Anoectochilus*, 100% and 98%, respectively, to *Anoectochilus roxburghii* and *Anoectochilus burmannicus* species in the Genbank. There are 15 positions of nucleotide changes in each gene segment. The *matK* and *ITS* barcodes can be used for detecting the genus *Anoectochilus*. In which, the *ITS* barcode identified the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 species and the *Anoectochilus roxburghii* species with the sequence ID in NCBI of MK451732.1 are in the same branch of the phylogenetic with a bootstrap value of 98%.

ĐẶC ĐIỂM TRÌNH TỰ BARCODE *matK* VÀ ITS Ở CÂY LAN KIM TUYẾN (*Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840) THU THẬP TẠI TỈNH LUANG NAMTHA, LÀO

Phạm Thị Thanh Nhàn[*1], Tangmany SYSOMEPHONE[2]
[1]Trường Đại học Sư phạm – ĐH Thái Nguyên, [2]Viện nghiên cứu Khoa học giáo dục - Bộ Giáo dục và Thể thao, Lào

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TỪ KHÓA

DNA barcode
ITS
Lan Kim tuyến
Lào
matK

TÓM TẮT

Lan Kim tuyến (*Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840) đang bị khai thác quá mức và được ghi vào Sách Đỏ ở Việt Nam. Trong báo cáo này, chúng tôi phân tích đặc điểm trình tự nucleotide barcode *matK, ITS* của loài được thu thập ở tỉnh Luang Namtha, Lào nhằm tìm ra sự khác biệt ở cấp độ phân tử giữa các loài, đồng thời cung cấp thông tin về mã vạch, góp phần hỗ trợ việc định danh, khai thác và bảo tồn nguồn gen loài này. Đoạn gen *matK* và vùng *ITS* phân lập được có chiều dài khoảng 900bp và 500bp. Hai trình tự có độ tương đồng cao với các loài khác trong chi *Anoectochilus*, lần lượt là 100% và 98% với các loài *Anoectochilus roxburghii* và *Anoectochilus burmannicus* trên Genbank. Mỗi đoạn gen có 15 vị trí thay đổi nucleotide. Mã vạch *matK, ITS* có thể được sử dụng để phát hiện chi *Anoectochilus*. Trong đó, cây phát sinh sử dụng trình tự barcode *ITS* xác định được loài *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 nằm cùng nhánh với loài *Anoectochilus roxburghii* (MK451732.1) với hệ số boostrap 98%.

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[*] Corresponding author. *Email: ptnhanbio@tnue.edu.vn*

1. Introduction

The *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants belong to the *Anoectochilus* genus, Orchidaceae family, and Orchidales order, which has a synonym name as *Chrysobaphus roxburghii* Wall. 1826, *Anoectochilus setaceus Blume*. This plant is a rare medicine with very high economic value. According to oriental medicine, it has a sweet taste, cool properties, and tonic yin. It is used to improve health, treat neurasthenia, cool and detoxify, high blood pressure, hepatitis, expectoration, and high fever... [1], [2]. These plants are found in India, Nepal, Bhutan, China, Myanmar, Thailand, Laos, Cambodia, Malaysia, Indonesia and Vietnam. Species with a wide distribution but a small number of individuals, which regenerate slowly and require strict living conditions, are at risk of extinction in the wild. In Vietnam, these species are classified in group IA of Decree 32/2006/ND-CP, which prohibits strict exploitation for commercial purposes. They are also listed in the Red Data Book as the endangered plant group EN A1a,c,d [3].

Fig. 1. *The in vitro and natural Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 *plants*

The genus *Anoectochilus* has about 12 species [1]. Identifying plants in general, and medicinal plants in particular, can be based on many various methods. The traditional methods such as analysis and comparison of anatomical, morphological, biochemical, or physiological characteristics... have been reported successfully in many plants, such as *Fissstigma pallens* (Fin. & Gagnep.) Merr. [4], *Brachiaria mutica* [5], *Pluchea indica* (L.) LESS. and *Pluchea pteropoda* HELMS. [6]… However, species identification is not always effective, especially for species belonging to the same subgenus or whose body parts are not intact.

Day to day, DNA barcodes are used regularly and widely to provide supplementary evidence for taxonomic studies at the molecular level. Consequently, the trend of a combination genetic markers and morphological characteristics in species identification becomes very significant for systematic studies. In which, DNA barcodes become one of the most efficient tools. Some barcoding loci consisting of *rpoC1, matK, ITS, rbcL, trnH-psbA*… have been applied effectively in plant identification [7]. Especially, the matK gene in the chloroplasts has been successfully applied in many plant species [8]. On one hand, numerous other studies have also demonstrated the significance of the ITS region. This region is in the cell nucleus, consisting of the *ITS1-5.8S-ITS2* sequence, and is used for classifying animal and plant species with highly accurate rates. Previous studies determined the relationships of plant species based on its nucleotide sequence in *Dalbergia cochinchinensis, Dalbergia oliveri* and *Dalbergia tonkinensis* plants [9], *Scrophularia* and many other species are effective evidence of using *ITS* region in plant identification [10]. Nguyen Thi Phuong Trang et al. (2014) used a combination of three chloroplast gene segments *rbcL, matK*, and *trnH-psbA* to study the nucleotide sequences of three *Hopea* species belonging to the genus *Hopea* that are at risk of extinction in Vietnam. including Hon Gai Star (*Hopea chinensis*), Hainan Star (*Hopea hainanensis*) and Devil's Face Star (*Hopea mollissima*) [11]. Nguyen Hung Manh et al. (2021) compared the *rbcL* and *trnH-psbA* gene segments of the *Abies delavayi subsp. fansipanensis (Q.P. Xiang) Rurhforth* with the Chinese species, and showed that there was a different position at the 455th nucleotide [12].

This study presents the characteristics of DNA barcode *matK* and *ITS* from the *Anoectochilus setaceus Blume* plant collected in Luang Namtha Laos to thoroughly understand differences in the

molecular level between species, contributing to providing information about DNA barcode for this plant species.

2. Research methods

2.1. Plant materials

The *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 samples collected in Luang Namtha province of Laos were used for revealing characteristics of *matK* and *ITS* sequences. These plants were classified based on morphological characteristic at the laboratory of plant cell technology, cultured *in vitro* and grown in the experimental garden belonging to the faculty of Biology, Thai Nguyen University of Education.

Chemicals used in this study were from Invitrogen (ThermoFisher Scientific, USA) and Merck (Darmstadt, Germany) such as chloroform, isoamyl, CTAB, PCR Master mix ...

2.2. DNA extraction, PCR amplification, and sequencing methods

Total genomic DNA was extracted and purified from young fresh leaves according to the protocol of Shaghai-Maroof MA. *et al*. (1984) [13]. The sequences of *ITS* region and *matK* gene in the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants were amplified by the PCR method with the primer pairs listed in Table 1.

The PCR amplification was performed with a final volume of 25 µL including 1.5 µL reverse primers (10 pmol. μL^{-1}), 1.5 µL forward (10 pmol. μL^{-1}), 1.0 µL template genomic DNA (500 ng. mL^{-1}), 12.5 µL 2x Master mix, and 8.5 µL deion water. The thermus cycle of PCR amplification consisted of 4 min at 94°C for initial denaturation, 28 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 54°C, extension for 1 min 30 s at 72°C, and a final extension step for 10 mins at 72°C. The PCR products were tested by the 1.0% agarose gel electrophoresis.

Table 1. *Characteristics of ITS, matK primer pairs*

Type of primer	Abbreviations	Nucleotide sequence 5'->3'	Theoretical length (bp)
ITS	*ITS*-F	ACGAATTCATGGTCCGGTGAAGTGTTCG	500
	ITS-R	TAGAATTCCCCGGTTCGCTCGCCGTTAC	
matK	*matK*-F	CGATCTATTCATTCAATATTTC	900
	matK-R	TCTAGCACACGAAGTCGAAGT	

The *ITS and matK* segments were sequenced by the ABI PRISM® 3100 Avant Genetic Analyzer machine with a specific primer pair and Kit BigDye® Terminator v3.1 Cycle Sequencing. The nucleotide sequence data were analyzed by the tools of Bioedit, BLAST (Basic Local Alignment Search Tool), and MEGA 11.

3. Results and discussion

3.1. PCR amplification of the ITS region and matK gene

The total genomic DNA was extracted and purified from leaves tissues of the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants, and then assessed via the agarose gel electrophoresis and spectrophotometer (Fig. 2).

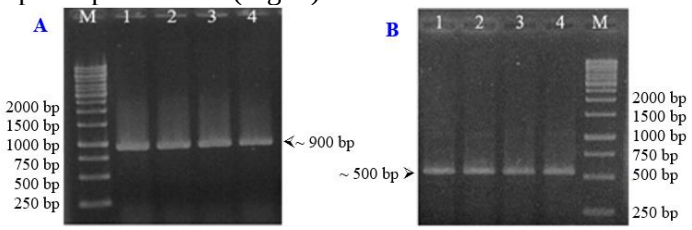


Fig. 2. *PCR amplification of matK gene (A) and ITS region (B).*
(1-4: PCR products of matK/ITS segment; M: Marker 1kb)

The result showed that there was a specific and clean band. The ratios of OD_{260}/OD_{280} ranged from 1.8 to 2.0 which demonstrated that there was little contamination of RNA and protein (data not shown). The *ITS* region and *matK* gene of the genomic DNA were amplified by PCR using primer pairs *ITS-F/ITS-R* and *matK-F/matK-R*, respectively. The results of the PCR product electrophoresis revealed a DNA fragment of the *ITS* region and *matK* gene with the expected size of approximately 500 bp and 900 bp, respectively (as seen in Fig. 2B). The PCR products of the *ITS* sequence and *matK* gene of *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 collected in Luang Namtha province of Laos were purified and sequenced in the ABI PRISM® 3100 automated sequencer. After editing, these sequences were analyzed by using BLAST on the NCBI website to compare the similarity, and identity level and identify the genus and species names based on isolated genes.

3.2. Nucleotide sequence analysis of the matK gene

The nucleotide sequence of the *matK* gene fragment isolated from *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 in Laos was similar to 100 sequences of species belonging to the genus *Anoectochilus* and Goodyerinae in the GenBank (Fig. 3).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Anoectochilus burmannicus chloroplast, complete genome	Anoectochilus b...	1164	1164	100%	0.0	100.00%	152868	NC_066958.1
Anoectochilus pingbianensis maturase K (matK) gene, complete cds; chloroplast	Anoectochilus p...	1158	1158	100%	0.0	99.84%	1612	MK451793.1
Anoectochilus chapaensis voucher TXDor13 chloroplast, complete genome	Anoectochilus c...	1158	1158	100%	0.0	99.84%	152395	MW589500.1
Anoectochilus elatus voucher MH 177135 maturase K gene, partial cds; chloroplast	Anoectochilus e...	1153	1153	99%	0.0	100.00%	845	KU687098.1
Anoectochilus elatus voucher MH 177133 maturase K gene, partial cds; chloroplast	Anoectochilus e...	1153	1153	99%	0.0	100.00%	845	KU687097.1
Anoectochilus elatus voucher MH 177136 maturase K gene, partial cds; chloroplast	Anoectochilus e...	1153	1153	99%	0.0	100.00%	845	KU687096.1
Anoectochilus elatus voucher MH 128042 maturase K gene, partial cds; chloroplast	Anoectochilus e...	1153	1153	99%	0.0	100.00%	845	KU687095.1
Anoectochilus elatus voucher MH 177137 maturase K gene, partial cds; chloroplast	Anoectochilus e...	1153	1153	99%	0.0	100.00%	845	KU687094.1
Anoectochilus elatus voucher MH 177134 maturase K gene, partial cds; chloroplast	Anoectochilus e...	1153	1153	99%	0.0	100.00%	845	KU687093.1
Anoectochilus hainanensis maturase K (matK) gene, complete cds; chloroplast	Anoectochilus h...	1136	1136	100%	0.0	99.21%	1612	MK451792.1
Anoectochilus emeiensis chloroplast DNA, complete genome	Anoectochilus e...	1136	1136	100%	0.0	99.21%	152650	NC_033895.1
Anoectochilus formosanus chloroplast, complete genome	Anoectochilus f...	1136	1136	100%	0.0	99.21%	151414	NC_061756.1
Anoectochilus zhejiangensis voucher MH Li or098 chloroplast, complete genome	Anoectochilus z...	1136	1136	100%	0.0	99.21%	152509	NC_054353.1
Anoectochilus roxburghii maturase K (matK) gene, complete cds; chloroplast	Anoectochilus r...	1134	1134	99%	0.0	99.21%	1612	MK451794.1

Fig. 3. *The BLAST result of the obtained matK gene fragment in NCBI*

The results demonstrated that nucleotide sequence similarities between them ranged from 94.93% to 100% and isolated *matK* sequence had the highest values of identity (100%) with *Anoectochilus burmannicus* in both Query cover and Per. Identity, even higher than those in *Anoectochilus roxburghii* in the NCBI. It was a difference in isolated length that resulted in these BLAST results. These results determined that the *matK* gene sequence in this study was the sequence in the genus *Anoectochilus*.

Table 2. *The differences in SNP positions of matK gene segments*

SNP positions	1-6	165	178	202	336	385	424	429	430	449
Anoectochilus_matK	TTTTCA	G	G	T	T	T	G	A	A	G
A.roxburghii (EU817409.1)	TTTTCA	G	G	T	G	-	G	G	G	G
A. burmannicus (NC066958.1)	TTTTCA	G	G	T	T	T	G	A	A	G
A.pingbianensis (MK451793.1)	TTTTCA	G	G	T	G	T	G	A	A	G
A.chapaensis (MW589500.1)	TTTTCA	G	G	T	G	T	G	A	A	G
A.hainanensis (MK451792.1)	TTTTCA	T	A	T	G	T	G	G	G	G
A.emeiensis (NC033895.1)	TTTTCA	T	G	A	G	T	G	A	G	T
A.formosanus (NC061756.1)	TTTTCA	T	G	A	G	T	G	A	G	T
A.zhejiangensis (NC054353.1)	TTTTCA	T	G	A	G	T	G	A	G	T
A.koshunensis (EU797512.1)	TTTTCA	T	G	A	G	T	-	A	G	T
A.elatus (KU687098.1)	-	G	G	T	T	T	G	A	A	G
Ludisia discolor (NC030540.1)	-	G	G	T	G	T	G	A	A	G

Note: Anoectochilus_matK is the matK sequence of the Anoectochilus roxburghii (Wall.) Wall. ex Lindl. 1840 *plants collected in Laos. The numbers and capital letters in parentheses are accesion numbers of Anoectochilus species published in the GenBank.*

In the continuous step, the isolated *matK* nucleotide sequence was compared with others of eleven various species belonging to the genus *Anoectochilus* in the Genbank, such as *Anoectochilus burmannicus, Anoectochilus pingbianensis, Anoectochilus chapaensis, Anoectochilus elatus, Anoectochilus hainanensis, Anoectochilus emeiensis, Anoectochilus formosanus, Anoectochilus zhejiangensis, Anoectochilus koshunensis, Anoectochilus sikkimensis and Ludisia discolor*, in which there are ten species with the highest sequence similarity. The data showed that there are a total of 15 different positions of single nucleotide polymorphism (SNP) between them (as seen in Table 2). In which, the sequence of the *matK* gene is isolated from *A. roxburghii* (Wall.) Wall. ex Lindl. 1840 plants has the least difference with that of the *A. burmannicus* plants (NC066958.1).

The genetic relationship among the species of the *Anoectochilus* genus was analyzed based on the *matK* nucleotide sequence by using the MEGA 11 software. The low divergence of the *matK* nucleotide sequence of the analyzed twelve species revealed that the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plant is a member of the *Anoectochilus* genus. The genetic relationship among various *Anoectochilus* species based on the *matK* nucleotide sequences was presented in a phylogenetic tree by using the Maximum Likelihood method constructed in the MEGA 11 software. The results show that there are two main branches. The first one consists of *Anoectochilus* species, in which the *matK* gene fragment of the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants located in the same branch as *Anoectochilus burmannicus* with a bootstrap value of 73% (as seen in Fig. 4). Meanwhile, *Macode petola* and *Ludisia discolor* belong to two distinct genus located in another branch with a bootstrap value of 100%. Therefore, compared with the results of analyzing the morphological characteristics of these plants, the *matK* barcode is only significant for determining the *Anoectochilus* genus.

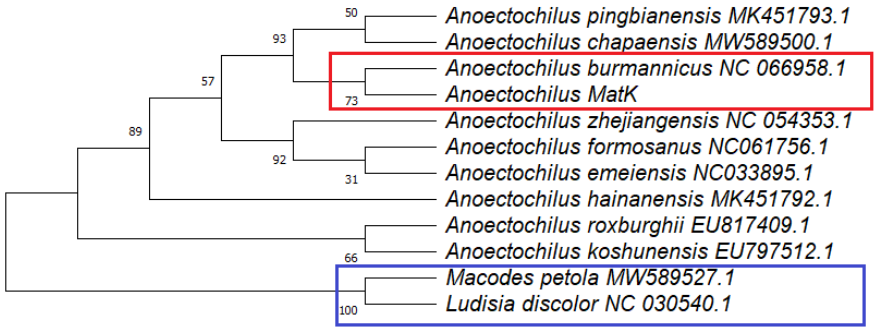
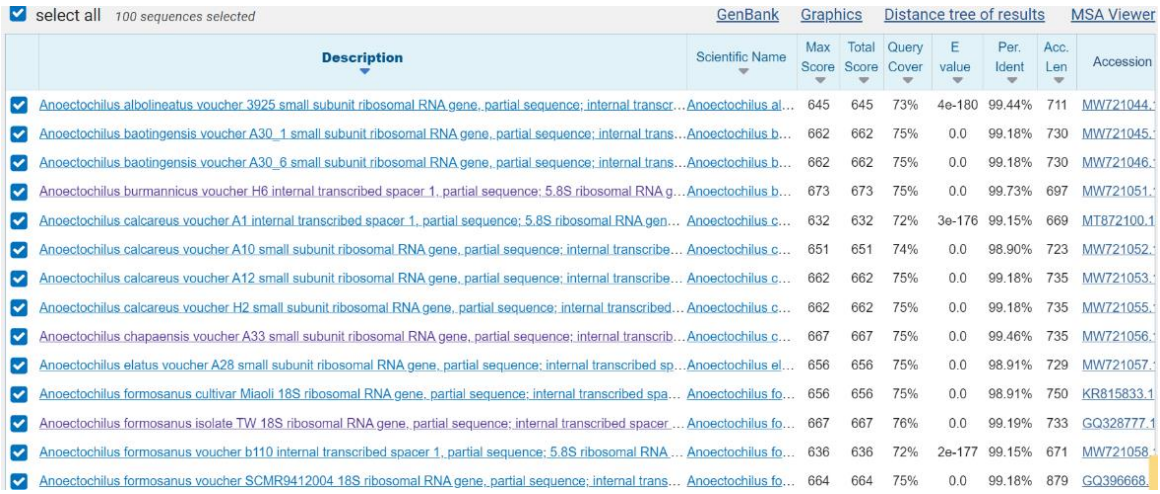


Fig. 4. *The phylogenetic tree of twelve Anoectochilus species constructed on the matK nucleotide sequences. Bootstrap values are above the nodes. The numbers and capital letters are accesion numbers of Anoectochilus species published on the GenBank. Anoectochilus MatK is the matK sequence of the Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 *plants collected in Laos*

3.3. Nucleotide sequence analysis of the ITS region

The nucleotide sequence of the *ITS* region from the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plant was similar to 100 sequences of species in the *Anoectochilus* genus and Goodyerinae in the GenBank (full data not shown). These results indicated that ITS nucleotide sequence similarities among them ranged from 98.89% to 99.73% (Fig. 5). These results proved that the isolated *ITS* region sequence in this study was the sequence of the genus *Anoectochilus*, and the highest similarity was found between the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants and *Anoectochilus roxburghii* species (MK451732.1) with 98% of Query cover value, 98.39% of Per. Identity value, and 682 of the total score.



select all 100 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Anoectochilus albolineatus voucher 3925 small subunit ribosomal RNA gene, partial sequence; internal transcr…	Anoectochilus al…	645	645	73%	4e-180	99.44%	711	MW721044.
Anoectochilus baotingensis voucher A30_1 small subunit ribosomal RNA gene, partial sequence; internal trans…	Anoectochilus b…	662	662	75%	0.0	99.18%	730	MW721045.
Anoectochilus baotingensis voucher A30_6 small subunit ribosomal RNA gene, partial sequence; internal trans…	Anoectochilus b…	662	662	75%	0.0	99.18%	730	MW721046.
Anoectochilus burmannicus voucher H6 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g…	Anoectochilus b…	673	673	75%	0.0	99.73%	697	MW721051.
Anoectochilus calcareus voucher A1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gen…	Anoectochilus c…	632	632	72%	3e-176	99.15%	669	MT872100.1
Anoectochilus calcareus voucher A10 small subunit ribosomal RNA gene, partial sequence; internal transcribe…	Anoectochilus c…	651	651	74%	0.0	98.90%	723	MW721052.
Anoectochilus calcareus voucher A12 small subunit ribosomal RNA gene, partial sequence; internal transcribe…	Anoectochilus c…	662	662	75%	0.0	99.18%	735	MW721053.
Anoectochilus calcareus voucher H2 small subunit ribosomal RNA gene, partial sequence; internal transcribed…	Anoectochilus c…	662	662	75%	0.0	99.18%	735	MW721055.
Anoectochilus chapaensis voucher A33 small subunit ribosomal RNA gene, partial sequence; internal transcrib…	Anoectochilus c…	667	667	75%	0.0	99.46%	735	MW721056.
Anoectochilus elatus voucher A28 small subunit ribosomal RNA gene, partial sequence; internal transcribed sp…	Anoectochilus el…	656	656	75%	0.0	98.91%	729	MW721057.
Anoectochilus formosanus cultivar Miaoli 18S ribosomal RNA gene, partial sequence; internal transcribed spa…	Anoectochilus fo…	656	656	75%	0.0	98.91%	750	KR815833.1
Anoectochilus formosanus isolate TW 18S ribosomal RNA gene, partial sequence; internal transcribed spacer …	Anoectochilus fo…	667	667	76%	0.0	99.19%	733	GQ328777.1
Anoectochilus formosanus voucher b110 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA …	Anoectochilus fo…	636	636	72%	2e-177	99.15%	671	MW721058.
Anoectochilus formosanus voucher SCMR9412004 18S ribosomal RNA gene, partial sequence; internal trans…	Anoectochilus fo…	664	664	75%	0.0	99.18%	879	GQ396668.

Fig. 5. *The BLAST result of the obtained ITS region sequence in NCBI*

At the next step, the isolated *ITS* nucleotide sequence was compared with those of seventeen species belonging to the *Anoectochilus* genus in the Genbank, such as *Anoectochilus hainanensis, Anoectochilus burmannicus, Anoectochilus pingbianensis, Anoectochilus chapaensis, Anoectochilus formosanus, Anoectochilus sikkimensis, Anoectochilus medogensis, Anoectochilus calcareus, Anoectochilus baotingensis, Anoectochilus setaceus, Anoectochilus nandanensis*... The results revealed there are mainly 15 different positions about the single nucleotide polymorphism (SNP) among them, and base lengths are different in various species, the shortest is *A.albolineatus* (MW721044.1) with 355 bp while the highest is *Anoectochilus-ITS* with 486 bp after removing primer sequences. In which, the *ITS* sequence isolated from the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plant has the lowest difference from the *A.roxburghii* plants (MK451732.1) (as seen in Table 3).

Table 3. *The differences in SNP positions of ITS gene regions*

SNP positions	1-3	4	35	70	82	88	103	107	159	173	228	242	278	End
Anoectochilus-ITS	ATC	G	A	A	C	A	T	T	C	A	A	C	G	486
A.roxburghii (MK451732.1)	ATC	A	A	A	C	A	T	T	C	A	A	C	G	372
A.hainanensis (OP787946.1)	-	-	-	A	C	A	T	G	C	A	T	C	A	380
A.pingbianensis (MK451731.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	G	372
A.formosanus (GQ328777.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	G	370
A.burmannicus (MW721051.1)	ATC	A	A	A	C	A	T	T	C	A	A	C	G	367
A.chapaensis (MW721056.1)	ATC	A	A	A	C	A	T	T	T	A	A	C	G	367
A.sikkimensis (MW721078.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	G	367
A.medogensis (MW721065.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	G	367
A.calcareus (MW721055.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	G	367
A.baotingensis (MW721046.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	G	367
A.nandanensis (*MW721069.1)*	ATC	A	A	A	C	A	T	G	C	A	T	C	A	367
A.malipoensis (MW721063.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	A	367
A.elatus (MW721057.1)	ATC	A	A	A	C	G	T	G	C	A	T	C	G	367
A.zhejiangensis (MT872106.1)	ATC	A	A	A	C	A	T	G	C	A	T	C	A	365
A.setaceus (MT872105.1)	ATC	A	A	A	C	A	T	T	T	C	A	C	G	365
A.koshunensis (KT334331.1)	ATC	A	A	-	-	A	C	G	C	A	T	C	A	361
A.albolineatus (MW721044.1)	ATC	A	A	A	C	A	T	T	T	A	A	-	G	355

Note: Anoectochilus-ITS is the ITS sequence of the Anoectochilus roxburghii (Wall.) Wall. ex Lindl. 1840 *plants collected in Laos. The numbers and capital letters in parentheses are accesion numbers of Anoectochilus species published in the GenBank*

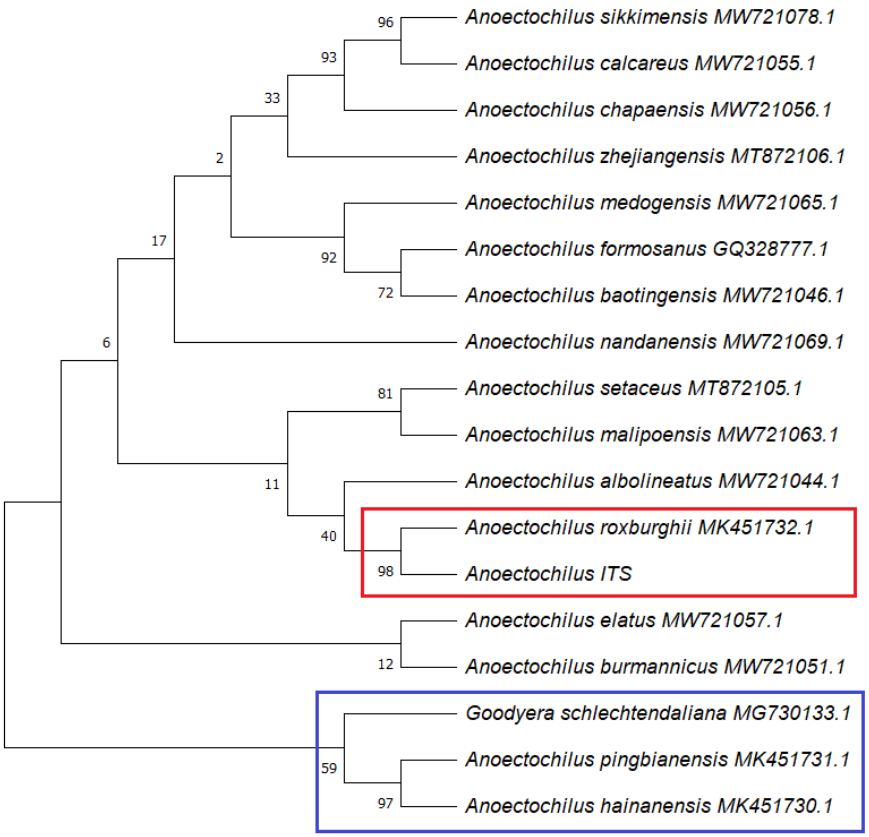


Fig. 5. *The phylogenetic tree of eighteen Anoectochilus species constructed on the ITS nucleotide sequences. Bootstrap values are above the nodes. The numbers and capital letters are accesion numbers of the Anoectochilus species published on the GenBank. Anoectochilus ITS is the ITS region sequence of the Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 *plants collected in Laos*

The genetic relationship among the species of the *Anoectochilus* genus was determined based on the *ITS* region sequences by using the MEGAX software. The pairwise distance matrix about *ITS* region sequences of the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants and eighteen *Anoectochilus* species and their phylogenetic tree were constructed by using MEGA 11 and the Maximum Likelihood method (as shown in Fig. 5). The results indicated that there are also two main groups. The first consists of *Anoectochilus* species, in which the *ITS* region sequence of the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plants located in the same branch as *Anoectochilus roxburghii* (MK451732.1). Meanwhile, the second divided into two branches with a bootstrap value of 59%, one is *Goodyera* schlechtendaliana (sequence ID MG730133.1), and the others are *A.pingbianensis* (MK451731.1), and *A.hainanensis* (MK451730.1) with a bootstrap value of 97%. In general, the coefficients of the bootstrap of the *ITS* sequence are lower than those of the *matK*, but the *ITS* barcode results in locating in the same branch of the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 species and the *Anoectochilus roxburghii* species (Sequence ID: MK451732.1) with a bootstrap value of 98% when using the BLAST tool and the MEGAX software.

Along with this approach in the same species, Lo Thi Mai Thu et al. (2019) used the sequence of *ITS* region and *rpoC1* gene fragments for *Anoectochilus* species identification collected in Thuan Chau, Son La. Their lengths were 666 bp and 628 bp, respectively. The BLAST result identified the *Anoectochilus* sample LKT-SL as *Anoectochilus setaceus* species. The genetic distances between this sample and the species on the GenBank based on the *ITS* and *rpoC1* sequences were low, at 0.2%.

In the study of Huynh Huu Duc et al. (2019), nine DNA barcodes *matK, rbcL, rpoB1, rpoB2, rpoC1, rpoC2, ITS, ITS1, and ITS2* were used for genetic analysis. The results indicated that the amplifications of these barcodes were different, ranging from 50%-100%, such as *ITS1* and *ITS2* 100%, *ITS* 83.3%, *rbcL* 50%, *matK* from 66.7 - 83.3%, *rpoB* and *rpoC* 83.3% in detail. Based on *ITS1* and *ITS2* sequences, the phylogenetic tree was constructed to show the relationship and discrimination between *Anoectochilus* and *Lusidia* species. They concluded successful uses of these DNA barcodes and opened opportunities for conservation and sustainable genetic resources [15]. Thus, these values are lower than ours. Whereas, Viet The Ho et al. (2021) demonstrated that the discrimination power of *rbcL* and *matK* markers in the jewel orchid study showed various efficiency levels. The *rbcL* barcode was more reliable than *matK* barcode or the combination of both these genes in distinguishing potential [16].

4. Conclusion

Molecular markers (or DNA barcodes) effectively support the morphology method in species identification and taxonomy. The *ITS* region and *matK* gene segment were isolated from leaves of the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 plant have base lengths of around 500bp and 900bp, respectively. Their sequences have the highest similarity with other species in the genus *Anoectochilus*, 100% and 98%, respectively, to *Anoectochilus roxburghii* and *Anoectochilus burmannicus* species in the Genbank. There are 15 positions of nucleotide changes in each gene segment. The *matK* and *ITS* barcodes can be used for detecting the genus *Anoectochilus*. In which, the *ITS* barcode identified the *Anoectochilus roxburghii* (Wall.) Wall. ex Lindl. 1840 species and the *Anoectochilus roxburghii* species in NCBI (Sequence ID: MK451732.1) are in the same branch of the phylogenetic.

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