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Full Length Article

Phylogenetic Analysis of *Ancistrocladus* Species (Ancistrocladaceae) from Vietnam

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Abstract

Genus *Ancistrocladus* used to be exploiting for medicinal purposes is found wildly growing across Vietnam. Identification at species level of these woody lianas for phytochemical and pharmacological research is difficult because the similarity in morphological structures exists. Therefore, molecular markers can help for studying genetic diversity as well as species determination of genus *Ancistrocladus*. In this study, ITS rebotypic and *matK* haplotypic DNA sequences of 14 *Ancistrocladus* samples collected throughout Vietnam and referred sequences from GeneBank were analyzed based on NJ method for clustering, alignment for homology level, point mutation accountancy, and PM test for genetic diversity of population. Polymorphism of 688bp Internal Transcribed Spacers (ITS) and 1280 bp MaturaseK (*matK*) markers significantly clustered the studied samples into 3 groups on NJ trees according to three geographical regions from the North to the South of Vietnam (57–70% of bootstrap values). Homology level of 99.90–99.98% in each group indicated that all 14 studied lianas belonged to *A. tectorius* species. High genetic diversity of the studied *A. tectorius* population also obtained from NJ analysis, genetic differentiation (0.846–0.913; *P*=0.0005–0.0142) between three groups, and their halotypic and rebotypic diversity (0.76–0.89). ITS and *matK* sequence analysis revealed a higher polymorphic level of ITS compared to *matK*. Results from NJ analysis and PM test suggested that ITS and *matK* could be jointly applied for phylogenetic analysis, species determination and genetic diversity estimation of *Ancistrocladus* genus. © 2017 Friends Science Publishers

Keywords: Phylogenetic analysis; *Ancistrocladus* species; ITS; *matK*; Polymorphism

Introduction

Ancistrocladus is the only genus of Ancistrocladaceae family, mainly distributed in tropical countries of South East Asia, India, Srilanka and Africa. All species are woody lianas, with a series of curved hooks of each representing a shoot closing after a sympodial branching. Their leaves are borne in dense, evergreen rosettes and have short petioles and lack stipules. They have a single wax-secreting trichome in the epidermal pits and glands on the abaxial surface. The flowers are small with a basally connate corolla while the fruit is a nut with often wing-like accrescent sepals (Gereau, 1997; Pham, 2003; Taylor *et al.*, 2005). It is the pharmaceutically useful genus and many different alkaloid compounds have been identified from the members (Bringmann and Pokorny, 1995). The most known compound from *Ancistrocladus* genus is the michellamine-type alkaloid exhibiting the promising activity against HIV (McMahon *et al.*, 1997; Bringmann *et al.*, 2016a). Many other alkaloids from *Ancistrocladus* also show anti-cancer properties (Jiang *et al.*, 2013; Bringmann *et al.*, 2016b; Le *et al.*, 2016; Li *et al.*, 2017), antiviral (Fasina *et al.*, 2013), anti-malaria (White *et al.*, 1999; Bringmann *et al.*, 2013) and anti-oxidant (Cheek, 2000). However, species identification for phytochemical and pharmacological research is difficult because a complex morphological similarity is existed and then diagnostic reproductive features are frequently unavailable.

The reported number of *Ancistrocladus* species ranged from 25 to 30 and divided into African and Asian species. In tropical Africa, 11 *Ancistrocladus* species was described by Gereau (1997) and later by Check (2000). In a comprehensive revision, Taylor *et al*. (2005) reported that

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there were 14 *Ancistrocladus* species typical for Asia in which 12 were found in Southeast Asia. The first species described in 1970 as *Ancistrocladus tectorius* (Lour.) Merr. is the most common population in the continental regions of Cambodia, Laos, Thailand and Vietnam as well as coastal regions of Indonesia and Malaysia. However, existing morphological differentiation within this species created uncertainty in taxonomy. Lastly, 6 species under *A. tectorius* including *A. extensus* Wall. ex Planch., *A. pinangianus* Wall. ex Planch., *A. cochinchinensis* Gagnep., *A. harmandii* Gagnep., *A. hainanensis* Hayata, Icon., and *A. carallioides* Craib, Bull.— were reveaved (Taylor *et al.*, 2005). Based on habitat, phenology and distribution, Pham (1991) reported that Vietnam has three species in the genus *Ancistrocladus*, including *A. cochinchinensis* Gagnep, *A. tertorius* (Lour.) Merr and *A. waliichii*. Another species, *A. scandens* (Lour.) Merr. was added in the list by the same author in 2003 (Pham, 2003).

Controversy in species determination and genetic diversity of *Ancistrocladus* genus when using morphological characteristics and chemical constituents could be unraveled with DNA markers. The nucleotide sequence comparison of the chloroplast DNA (cpDNA) *trnK* intron, the nuclear internal transcribed spacers (ITS) and the inter-simple sequence repeat (ISSR) finger-printings separated 75 samples from *A. tectorius* populations in Thailand, Laos and Malaysia. The variability in nucleotide sequences of *trnK* and ITS of samples from Southeast Asia also offered similar value as data matrix comprising the West African and Indian taxa (Meimberg *et al.*, 2010). Therefore, polymorphic levels of *matK* and ITS sequences could be DNA markers to classify and reveal phylogeny of *A. tectorius* complex in Vietnam. In this paper, species determination, genetic diversity and phylogeny of *Ancitrocladus* genus in Vietnam was reported for the first time based on polymorphism of *matK* and ITS markers using fourteen *Ancitrocladus* samples of six different locations from Southern to Northern Vietnam. These results could provide useful information for conservation and phytochemical and pharmacological research of these woody lianas in the country.

Materials and Methods

Experimental Materials

Fourteen samples of *Ancistrocladus* genus were collected from six provinces from the Northern to the Southern of Vietnam (Table 1; Fig. 1) and traditional classification by Prof. Ninh Khac Ban and Dr. Luu Dam Ngoc Anh (also co-authors in this report). Fresh young leaves were collected, preserved in silica gel or frozen at -80ºC for DNA extraction. Voucher specimens were deposited at Department of Biological resources, Institute of Marine Biochemistry and Natural Conservation Department, Vietnam National museum of Nature (VAST).

PCR and DNA Sequencing

Total DNA of the collected samples was extracted using CTAB-based lysis buffer according to Doyle and Doyle (1990). The concentration of DNA was quantified using a NanoDrop 1000 instrument (Thermo Scientific, USA) and by electrophoresis in 0.8% agarose gel.

The *matK* and ITS fragments were amplified from total DNA and primer pairs specialized for each fragment, including TQ_ITSF: 5'-CCTGCGGAAGGATCATTGTC-3' and TQ_ITSR: 5'-CAAGGATTCCCCTAGTAACG-3' for ITS and TQ_MATKF: 5'-TCGAATGTATCGACAG AATC-3' and TQ_MATKR: 5'-TAGGTCCTCTATATAACC TC-3' for matK.

PCR amplification of ITS was carried out under the following conditions: 95°C 3 min; 95°C 30 s, 60°C 40 s, 72°C 40 s (30 cycles); 72°C 7 min while the thermocycle for *matK* was 95°C 3 min; 95°C 40 s, 50°C 30 s, 72°C 60 s (30 cycles); 72°C 7 min. Both PCR components were 5 µL 10X PCR Buffer, 10 mM dNTPs, 2 mM primers, 50 ng of genomic DNA, 1 unit of *Taq* Polymerase and 2 mM of $MgCl_2$ and H_2O up to total 50 µL. Size of PCR products was verified by 1.0% agarose gel electrophoresis in TAE buffer and purified using QIAquick® PCR purification kit (Qiagen), cloned in TA® cloning vector and sequenced by Macrogen Inc. (Korea). Nucleotide sequences of 14 *Ancistrocladus* specimen were registered to GeneBank accession nos. from KY435683 to KY435696 for ITS fragments and from KY442767 to KY442780 for matK fragments.

DNA Analysis

Phylogenetic relationships and genetic diversity of the *Ancistrocladus* samples were analyzed based on polymorphism of ITS rebotypic and matK haplotypic fragment sequences of 14 samples (Table 1) and referred sequences from GeneBank (Figs. 2 and 3) using ClustalW method of Mega 3.1 (Kumar *et al.*, 2004). Phylogeny reconstruction was done with tree inferences using Neighbor Joining method from Mega 3.1 with Bootstrap test of 1000 replicates. For species determination, homology level after aligning DNA sequences of the studied samples and references in the same groups on NJ trees obtained from phylogeny reconstruction analysis was estimated using Multiple Sequence Alignment method of DNAMAN 4.15 (Lynnon BioSoft). Specific point mutations within ITS rebotypic and matK haplotypic sequences were indicated using Align of ClustalW and Sequence Data Explorer methods from Mega3.1. Grouping the studied *Ancistrocladus* samples on the NJ trees and comparatively polymorphic levels between ITS rebotypic and matK haplotypic sequences were interpreted from point mutation analysis. Genetic diversity of populations and genetic differentiation between populations, which were branched on NJ trees, were estimated with Permutation test of Hudson *et al.* (1992) using DnaSP4.15 (Rozars *et al.*, 2003).

Table 1: ITS and *matK* sequences of the studied *Ancistrocladus* samples

Name of Sample	ITS rebotype[a]	*matK* haplotype[a]	Provincial localities[b]	Regions[b]
Vinhphuc1	VinhphucI1	VinhphuM1	Vinhphuc	Northern
Bacgiang1	BacgiangI1	BacgiangM1	Bacgiang	Northern
Quangtri1	QuangtriI1	QuangtriM1	Quangtri	North-Central
Hue1	HueI1	HueM1	Thuathien-Hue	Central
Hue2	HueI2	HueM2	Thuathien-Hue	Central
Hue3	HueI3	HueM3	Thuathien-Hue	Central
Hue4	HueI4	HueM4	Thuathien-Hue	Central
Hue5	HueI5	HueM5	Thuathien-Hue	Central
Nhatrang1	NhatrangI1	NhatrangM1	Khanhoa	South-Central
Nhatrang2	NhatrangI2	NhatrangM2	Khanhoa	South- Central
Nhatrang3	NhatrangI3	NhatrangM3	Khanhoa	South- Central
Phuquoc1	PhuquocI1	PhuquocM1	Kiengiang	South Western
Phuquoc2	PhuquocI2	PhuquocM2	Kiengiang	South Western
Phuquoc3	PhuquocI3	PhuquocM3	Kiengiang	South Western

[a]) Accession numbers in the text; [b]) details given in Fig. 1

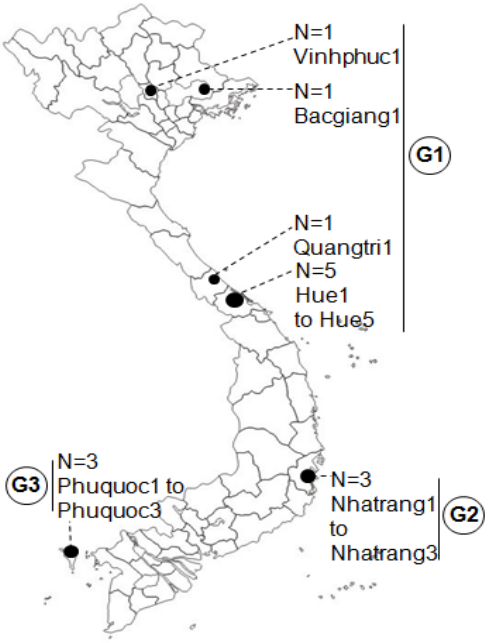

Fig. 1: Map of area under investigation from the North to the South of Vietnam. Each site included number and name of samples taken after the name of localities where samples were collected. G1-G3: three groups of studied samples (see the results)

Results

Phylogenetic Analysis

The ITS and *matK* fragments were amplified from the total DNA of *Ancistrocladus* leaves. After a brief blast and alignment to all nucleotide sequences of *Ancistrocladus* genes available from GeneBank, the 688bp of ITS fragments and 1280bp of *matK* fragments were chosen for phylogenetic analysis. High similarities in both ITS and *matK* sequences revealed that 14 *Ancistrocladus* linas from different locations in Vietnam belonged to *A. tectorius* complex. The polymorphism of ITS sequences clustered 14 woody lianas under investigation into three groups from G1 to G3 (Fig. 2). G1 consists of eight samples in Vinhphuc, Bacgiang, Quangtri and Hue provinces belonging to the North and the Central of Vietnam. This group is positioned as sister to *A. tectorius* specimens in the Eastern Thailand with bootstrap value of 70%. Group 2 comprises samples from Nhatrang province, the South-Central of Vietnam supporting with bootstrap of 61% as sisterhood of specimens in Laos and Southern Thailand. G3 are samples collected from Phuquoc island in South Western of Vietnam and they form the bootstrap value of 62% with *A. tectorius* in South-Eastern Thailand (Fig. 2). Polymorphic levels in *matK* sequences divided 14 *Ancistrocladus* samples in our study into three similar groups of G1 to G3 with confidence intervals of bootstrap values ranging from 57–62% (Fig. 3). Additionally, three groups in both ITS and *matK* phylogenetic trees were also in accordance to 3 different regions in Vietnam from the North-Central to South-Central and South-Western (Fig. 1; Table 1). This indicated high genetic diversity of the *Ancistrocladus* liana population depending on geographical region where the plants habituated.

Species Determination

Homologous analyses of ITS and *matK* sequences between the studied lianas and referred specimens showed that all 14 *Ancistrocladus* samples in Vietnam belonged to *A. tectorius* complex, which correlated to the phylogenic analyses (Fig. 2 and 3) that all *Ancistrocladus* samples in this study were clustered into 3 groups together with *A. tectorius* specimens in Laos and Thailand. Comparative analyses by alignment of ITS sequences revealed the 99.62% similarity of 8 samples in G1 clustered with 5 referenced samples from Eastern Thailand. The other 3 samples of each group G2 and G3 were related to *A. tectorius* from Laos and 5 from South and South-Eastern Thailand with the homology level of 99.90% and 99.97%, respectively. The *matK* sequences of 8 samples in G1, 3 in G2 and 3 in G3 were also found high homologous degree of 99.95, 99.96 and 99.98%, respectively, with those of *A. tectorius* from Eastern and South-Eastern Thailand.

Fig. 2: Neighbor Joining analysis of targeted and referred ITS sequences of *Ancistrocladus* species. *Ancistrocladus* samples in Vietnam were branched with other South-East Asian countries into 3 groups (G1-G3). Isolate names of the studied samples were detailed in Table 1. Bootstrap values from phylogeny test between branches and groups with less than 50% were hided. *T. pentatum* GeneBank accession no. HM204913 as outgroup

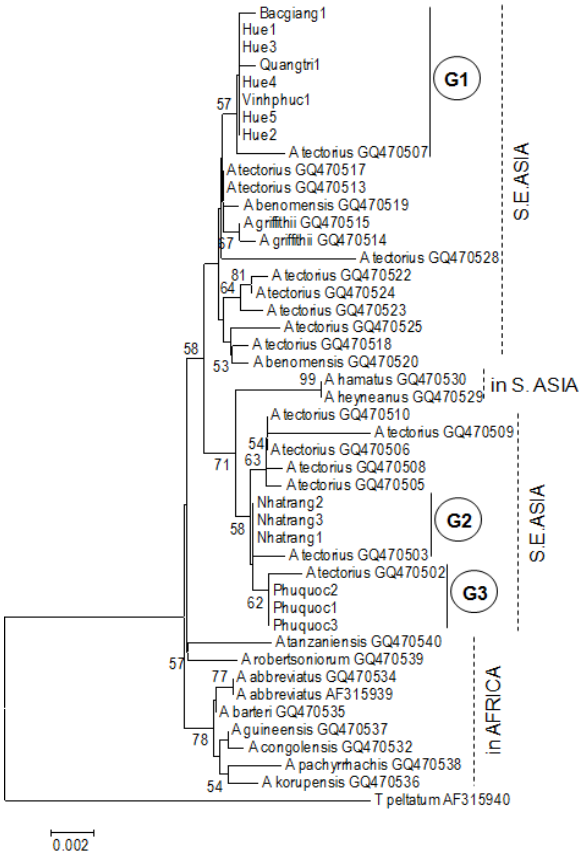

Fig. 3: Neighbor Joining analysis of targeted and referred *matK* sequences of *Ancistrocladus* species. *Ancistrocladus* samples in Vietnam were branched with other South-East Asian countries into 3 groups (G1-G3). Isolate names of the studied samples were detailed in Table 1. Bootstrap values from phylogeny test between branches and groups with less than 50% were hided. *T. pentatum* GeneBank accession no. AF315940 as outgroup

Three groups based on polymorphism 688bp of ITS fragments were separated with 15 point mutations, of which there were 11 specific points (Fig. 4a). *A. tectorius* of G2 was separated from G1 and G3 with 8 specific sites along ITS fragments while 3 samples in G3 were genetically distant from G1 and G3 with three points at positions of nt43, nt236 and nt533. Although longer in length than ITS fragments, there were only 6 specific sites of total 8 point mutations between 14 woody lianas in Vietnam based on 1280 bp of *matK* sequences. Along *matK* fragments, 5 specific point mutations break up *A. tectorius* in G1 from G2 and G3 while G3 was distant from others with only one point at position nt713 (Fig. 4b). The shorter in analyzed fragments and the more point mutations revealed the more polymorphic of ITS than *matK* marker. As a results of point mutation analysis, the *A. tectorius* population in Vietnam in this study was separated into 3 groups with significant genetic differentiation based on estimation of ITS rebotype and *matK* haplotype polymorphism as Kst = 0.864 (P of χ^2 = 0.0142) and Kst = 0.913 (P of χ^2= 0.0005), respectively (Table 2). In addition, high haplotypic and allelic diversity from 0.76 to 0.89 indicated high genetic diversity of the whole studied population.

Discussion

In Vietnam, three or four species in the genus *Ancistrocladus* were considered including *A. tectorius*, *A. cochinchinensis* and *A. waliichii* (Nguyen, 2003) or *A.*

Table 2: Genetic indexes of *A. tectorius* in Vietnam based on ITS rebotype and MatK haplotype polymorphism

Sequences	Group 1 (G1)	Group 2 (G2)	Group 3 (G3)	Total
1) ITS rebotypes (688 sites)				
No of sequences	8	3	3	14
No of rebotypes	5	1	2	8
Repotypic diversity	0.79	0.00	0.67	0.89
Genetic differentiation estimates	*Kst* = 0.846 with χ^2=28.000; *P* of χ^2= 0.0142			
2) *matK* haplotypes				
No of sequences	8	3	3	14
No of halotypes	3	1	1	5
Haplotypic diversity	0.46	0.00	0.00	0.76
Genetic differentiation estimates	*Kst* = 0.913 with χ^2=28.000; *P* of χ^2= 0.0005			

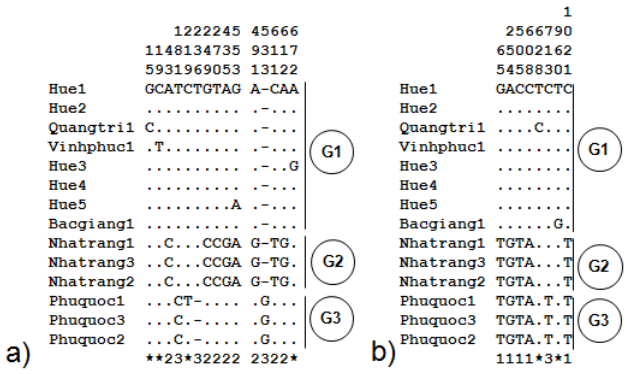

Fig. 4: Point mutation along targeted ITS (a) and *matK* (b) fragments separating *A. tectorius* samples from the North to the South of Vietnam into 3 groups (G1-G3). Vertical numbers on the tops: polymorphic sites along the targeted fragments. 1–3 on the rows at the bottoms: specific point mutation of G1, G2 and G3, respectively; *) point mutation within group

tectorius, *A. cochinchinensis*, *A. waliichii* and *A. scandens* (Pham, 2003). In our previous study in isolating and structural determinating chemical compounds from *Ancistrocladus* genus in Vietnam, the sample collected in Vinhphuc province (Vinhphuc1; Table 1) was morphologically indentified as *A. cochinchinensis*. In this study, 14 collected specimens across Vietnam were identified as *A. tectorius* according to both ITS and *matK* sequences (Fig. 2 and 3), including the Vinhphuc1 sample. Almost all studied samples were closely related to this species in South and South East Thailand of South East Asia (Meimberg *et al.*, 2010). The ISSR as well as ITS or *trn*K intron sequence analysis supported the results of morphological examinations as consideration that the *A. tectorius* is a complex of six species including *A. cochinchinensis* (Taylor *et al.*, 2005; Meimberg *et al.*, 2010). Therefore, *A. cochinchinensis* mentioned previously by Nguyen *et al.* (1997) and our study (Le *et al.*, 2016) is one species of *A. tectorius* complex in Vietnam. Acorrding to Pham (2003), *A. scandens* is common species of *Ancistrocladus* in Vietnam and can be found in many different areas while *A. waliichii* is located in Dongnai and Tayninh, South Eastern of Vietnam. However, neither *A. scandens* nor *A. waliichii* was collected in all six targeted locations as well as no *Ancistrocladus* species in Dongnai and Tayninh were found in our study. Taylor *et al.* (2005) described *A. waliichii* being a "dubious identity" species; therefore this is another reason for the absence of the species in its habitat as well as other areas in Vietnam. A wide distribution of *A. scandens* reported in Vietnam (Pham, 2003) and dominant of *A. tectorius* complex in six different locations in our investigation rised a hypothesis that *A. scandens* could be one species in *A. tectorius* complex of Vietnam.

Nguyen (2003) indicated in The Flora List of Vietnam that the distribution of *Ancistrocladus* genus was mainly in the South of Vietnam. In this study however, the genus was found spreading from North to Centre as well as South of Vietnam. Although all specimens are indicated as *A. tectorius*, our study indicated that all 14 *Ancistrocladus* samples collected throughout Vietnam clustered into 3 groups in NJ trees together with the referred samples in South-East Asia when also based on their ITS and *matK* DNA polymorphisms (Fig. 2 and 3), inferring the South-East Asian origins of the studied lianas in Vietnam. These results supported the findings of Meimberg *et al.* (2010) that all *Ancistrocladus* samples in Thailand, Malaysia, Indonesia, Laos and Vietnam were grouped with significantly genetic distances from those in other countries on phylogenetic trees when analyzed based on ITS and *trnK* intron sequences. In addition to the results of NJ analysis, significant genetic differentiation and high halotypic and rebotypic diversity (Table 2) between 3 groups, all together, revealed high genetic diversity of the studied *A. tectorius* population in Vietnam.

Recently, *matK* (maturaseK) has emerged as an invaluable gene because of its high phylogenetic signal compared with other genes and the universal *matK* primers can be used for DNA barcoding of angiosperms (Turmel *et al.*, 2006; Barthet and Hilu, 2007; Yu *et al.*, 2011). In addition to *matK*, the most popular sequences for phylogenetic inference at the generic and infrageneric levels in plants is the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron (Alvarez and Wendel, 2003). The *matK* is nested in the group II intron between the 50 and 30 exons of *trnK* in the large single copy region of the chloroplast genome of most green plants. Sequence analysis of the nuclear ITS region

and chloroplast *trnK* gene within *A. tectorius* in Southeast Asia revealed genetic differentiation in this population (Meimberg *et al.*, 2010). A higher similarity of individuals of one group to other populations than to neighboring plants indicated the existence of several species in each population such as *A. pinangianus* and *A. cochinchinensis* in Southern Thailand and Malaysia; *A. griffithii* and *A. pinangianus* in southern Thailand and Laos; *A. cochinchinensis*, *A. harmandii* and *A. hainaniensis* in Central Thailand and Hainan. In our study, the 688bp-ITS and 1280bp-*matK* markers identified all 14 samples being *A. tectorius* and highly homologous to the species in Thailand. The pairwise distance of 14 samples using ITS and *matK* sequences were 15 and 8, respectively. Analysis of point mutations in ITS and *trnK* indicated that the *A. tectorius* population of Vietnam was less diverse compared to Southeast Asia samples (24 and 14 points) and Africa samples (26 and 21 points). Agreement to previous studies, the more polymorphic of IST than *matK* sequences also was found.

Conclusion

The diversity of *Ancistrocladus* spp. in Vietnam revealed that all 14 studied lianas belonged to *A. tectorius* complex. A high genetic diversity was found in collected liana populations, which fell into three groups according to three geographical regions from the North to South of Vietnam. matK haplotypes and ITS rebotypes can be applied for species determination and genetic diversity of *Ancistrocladus* and other related genera.

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