IDENTIFY *CENTROSTACHYS AQUATICA* BY USING DNA BARCODING

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Abstract:
A common floating aquatic plant in An Giang, Vietnam is *Centrostachys aquatica*. The purpose of the study, " Identify *centrostachys aquatica* by using DNA barcoding"is to serve as a foundation for future research to identify specific medicinal properties for the treatment of human ailments. The outcomes demonstrated that the leaves are cool-nosed, green, 1-1.5 cm wide, and 6-7 cm long. The veins are feather-shaped, and the lower surface is hairy while the upper surface is smooth. The inflorescence is 7–10 cm long and is made up of symmetrically growing clusters of several odd-sized flowers that range in size from 0.5–0.8 cm. a floating aquatic herbaceous plant with striped and occasionally red dots that is between 1 and 1.5 cm in size. The roots are short and twisted, and they get smaller from the neck to the tip over time. They have a diameter of 0.2 to 0.5 cm and are 10–15 cm long. There is a 98.35% similarity between the sequence of this RBCL gene and the sequence of *Centrostachys aquatica* that has been reported. As a result, the study uses DNA, a highly reliable and selective technology, to identify the species names of *Centrostachys aquatica* (R.Br.) Wall.

Keywords: *Centrostachys aquatica,* DNA barcoding, morphological indicator, PCR analysis, *Achyranthes aquatica* R.Br.

Introduction

The majority of people on earth have ingrained the use of herbs as medicine into their culture because they have known and trusted traditional medicines for thousands of years to keep them well. Before the advent of modern medicine, human health [1]. Today, despite the fact that medicine has advanced significantly and is accessible in all nations, people continue to use traditional healing techniques using medicinal herbs. In industrialized nations in particular, many choose to employ herbal remedies over modern pharmaceuticals to treat or maintain [2]. Scientists are actively studying the biological processes that underlie the therapeutic benefits of herbs, which has the potential to make them a natural supply of raw materials for the creation of safe and effective human medicines [3].

Centrostachys aquatica (R.Br. Wall ex Moq Tand), also known as *Achyranthes aquatica* R.Br. (Synonym), is a perennial emergent macrophyte that roots at nodes to create meadows in marshy areas and beside rivers. Its stems range in length from 0.3 to 1.5 meters. Although common in tropical Africa, South and East Asia, including India, and Senegal and Nigeria, the species has also been documented there [4] [5]. In the Mekong Delta region of Vietnam, ever since *Centrostachys aquatica* has been handed down by people for the treatment of liver diseases such as melisma and yellow skin, In Bangladesh, water-scratch grass is used by people for liver purification, women's menstrual irregularities, rheumatism, and snakebite detoxification [6].

Identification of species is the first step in quantifying biodiversity and constructing our core understanding of the biological world. The lack of expert categorization knowledge and occasionally the limitations of morphology-based identification provide some challenges to this essential endeavor [7]. The conventional morphometric taxonomy investigation takes a long time because it depends on the organism's growth stage. These studies, which rely on predetermined classifications and expertise, are also time-consuming [8]. DNA barcoding is a modern marker-based approach in molecular systematics research that aims to establish a shared community resource of DNA barcoding for rapid identification, effective discrimination between taxa, and authenticated classification based on molecular data [9]. Scientists apply the DNA barcoding technique by utilizing an exclusive and ultimate source from the genetic structure available in the organisms and providing promising and reproducible results with certainty. It is one of the latest concepts, aiming to afford

rapid, accurate, and automatable species identification techniques using a standardized DNA region as a tag [9] [10] [11]. Identifying one or a few DNA areas that make it easier to discriminate between the majorities of the world's species is the conceptually straight forward purpose of DNA barcoding. These sections should then be sequenced from various sample sets to create a macroscopic reference library of life on earth. DNA barcoding presents an opportunity to utilize biodiversity research in a completely new way by taking into account both established molecular biology techniques and recent breakthroughs in bioinformatics. Numerous applications of DNA barcoding exist to help save biodiversity from concerns like climate change caused by humans and the prevalent illegal trade in animals and animal products [9].

The history of human treatment has gone through many periods and today, people are back to the early days with the active search for medicinal herbs to serve as a source of raw materials for making medicines to replace modern medicines. From the given causes and based on folk experiences, the study " Identify *centrostachys aquatica* by using DNA barcoding " is proposed as the first step in the research on identification of the plant in order to contribute to biodiversity, the source of medicinal materials derived from nature.

Materials and Methods

Materials

The samples of *Centrostachys aquatica* were collected in the Mekong River area, An Giang province, Vietnam.

Chemicals for DNA extraction: CTAB Buffer (2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl), β-mercaptoethanol, Chloroform : Isoamyl alcohol (24:1), Enzyme RNase, Isopropanol and ethanol (70%).

Chemicals for PCR analysis: PCR Mix (NEXpro, Korea), purified agarose, Ethidium bromide, TAE 1X, Loading dye 6x, Ladder 2-log and TE.

Methods

Morphological indicator

Observe and measure plant parts (roots, stems, leaves, flowers) [12].

PCR analysis

DNA from leaf samples was extracted by the CTAB method [13]. Weigh 100 mg of leaf sample into a mortar and grind it finely in 1 ml of CTAB 2X solution, which has been incubated at 65°C for 15 minutes. Pour all the solution into a new tube and proceed to inject more CTAB. Adjust to the 1.5 ml mark. Mix well and centrifuge at 13000 rpm for 10 min. After centrifugation, withdraw each tube with 1000µl of the supernatant and place it in a new tube. Then add to each tube 10µl of β-mercaptoethanol. Incubate at 65°C for 60 minutes (mix the sample every 10 minutes). 500 µl of chloroform was added to each tube, mixed well, and centrifuged at 13000 rpm for 10 min. Withdraw 750 µl of the above solution into a new tube, then continue to add 500 µl of chloroform, mix well, and centrifuge at 13000 rpm for 10 minutes. Withdraw 550 µl of the supernatant and place it in a new tube, then add 500 µl of chloroform to each tube and centrifuge at 13000 rpm for 10 min. Withdraw 350 µl of the supernatant into a new tube, then add 5 µl of RNase to each tube, shake well, and incubate the sample at 37°C for 2 h. After 2 hours of incubation, continue to add 300 µl CTAB 2X and 500 µl chloroform to each tube. The sample was centrifuged at 13000 rpm for 10 min. Next, withdraw 400 µl of the supernatant from each tube and put it into a new tube. At the same time, add 400 µl of isopropanol (1:1 ratio), mix well, and incubate at -20°C for 30 minutes. The sample was centrifuged at 13000 rpm for a minute, carefully discarding the upper solution, leaving the precipitate deposited below. Add 500 µl of 70% ethanol to each tube and centrifuge at 13000 rpm for 5 min to rinse the sample, then discard the alcohol and leave the precipitate. Add 500 µl of 70% ethanol further to each tube to rinse the sample a second time, and centrifuge at 13000 rpm for 5 min. Then discard the alcohol and leave the precipitate. Use a micropipette to suck up the remaining alcohol in each tube and let the sample dry (under a ceiling fan) for 1 hour. Finally, add 30 µl TE to each tube (pH = 8.0) to dissolve the DNA and refrigerate at -20 °C. DNA, after being extracted and purified, will

be checked by electrophoresis on a 1% agarose gel. PCR reaction (Polymerase Chain Reaction), also known as DNA amplification reaction, consists of 50 µl using the PCR KIT kit (NEXproTM Diagnostics), including components 10X e-Taq Buffer, 10 mM dNTP, e-Taq DNA Polymerase, purified water, primer pair RbcL, and DNA. All were mixed well before being added to the GeneAmp PCR System 2700 PCR machine. This reaction was carried out in 35 heating cycles, including: 5 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C; chain elongation for 5 minutes at 72°C; and product storage at 10°C for 20 minutes. PCR products were electrophoresed and purified using the Wizard SV Gel kit and PCR Clean-up System (Promega), and then sent for sequencing using the Sanger method [14]. The molecular weight was calculated using GelAnalyzer software. Sequencing results were stored in FASTA format and analyzed using the latest BioEdit software version 7.0.5 [15]. Then, by the BLAST method, the NCBI gene bank system (National Center for Biotechnology Information) was used for species identification.

Results and Discussion

Morphological indicator

Centrostachys aquatic was shown in Figure 1. The results showed that the leaves are green, cool-nosed, 1-1.5 cm wide, and 6-7 cm long. The upper surface is smooth, the lower surface is hairy, and the veins are feather-shaped (Figure 2). The inflorescence is 7–10 cm long, consisting of many flowers growing symmetrically, with odd flowers 0.5–0.8 cm in size (Figure 3). A floating aquatic herbaceous plant, 1-1.5 cm large, with striped and sometimes red spots (Figure 4). The roots are small and curved, gradually getting smaller from the root neck to the root tip. They are 10–15 cm long and 0.2–0.5 cm in diameter (Figure 5). The characteristics examined are almost compatible with the studies of *Centrostachys aquatic* [16].

Figure 1. *Centrostachys aquatic*

Figure 2. *Centrostachys aquatic* leaf (front side of leaf and back side of leaf)

Figure 3. *Centrostachys aquatic* inflorescence and flower

Figure 4. *Centrostachys aquatic* stem Figure 5. *Centrostachys aquatic* root

PCR analysis

The sequence of RbcL primer pairs used in PCR is shown in Table 1 [17].

Table 1. List of rbcL primer pairs used for PCR amplification and sequencing [18]

RbcL.F	ATGTCACCACAAACAGAGACTAAAGC
RbcL.R	GTAAAATCAAGTCCACCRCG

PCR reactions with primers RbcL were shown in Table 2.

Table 2. PCR reaction with a volume of 50 µl

Chemicals	Volume (µl)
H_2O	25
My taq Mix 2X	20
Primer F	1
Primer R	1

DNA	3
Total	50

Table 3. The steps of PCR reactions

Step	Name	Cycle	T (^{0}C)	Time (second)
1	denaturation	35	95	5
			95	60
2	primer pair	35	56	40
3	Stretch - stabilize the product	35	72	70
			72	5
4	Product preservation		10	Foever

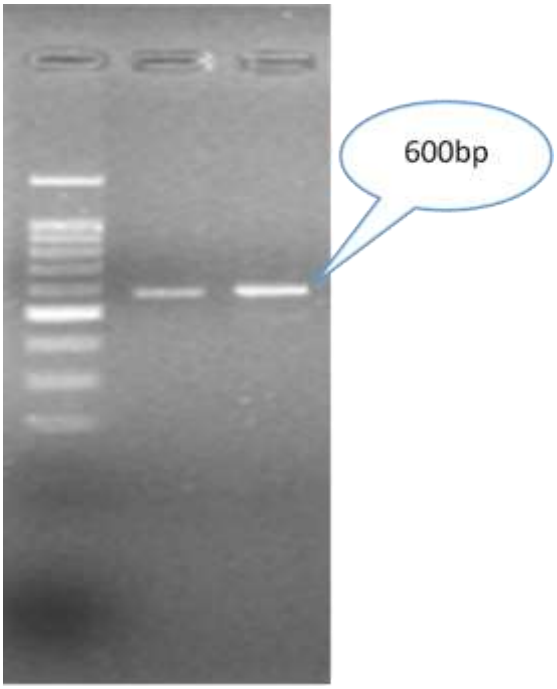

Figure 6. Electrophoresis spectra of PCR products from *Centrostachys aquatic* on 1.5% Agarose gel

PCR reaction with primer ITS 1-4:

ITS 1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS 4: 5'-TCCTCCGCTTATTG ATATGC-3'

Table 4.

Chemicals	Volume (µl)
H_20	25
My taq Mix 2X	20
Primer F	1
Primer R	1

DNA	3
Total	50

Table 5.

Step	Name	Cycle	T (^{0}C)	Time (second)
1	denaturation	35	95	5
			95	50
2	primer pair	35	54	30
3	Stretch - stabilize the product	35	72	70
			72	5
4	Product preservation		10	Foever

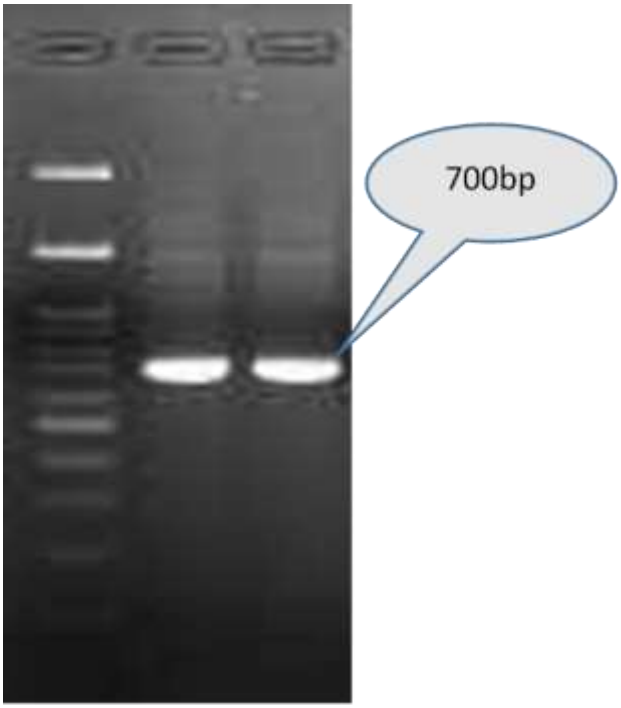

Figure 7. Electrophoresis spectra of PCR products from *Centrostachys aquatic* on 1.5% Agarose gel

Sample of grass tested named Centrostachys aquatica, 98.35% similarity.

TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTGCCTAGCAGAACGACTAGTGA
ACATGTTTACATCTTMCACTGGGAAGGGGGTGCTGGCTTGTCTAGTCCCTTCCCAACGTTGG
GGAGCGTCCCCTTTATTGGTGGTGCTTACCAACACATTAACGAACCCCGGCGTGATATACGC
CAAGGAATAAAATTGAGTGTGTGTGCGTATTTCTATTCGAATTTTCGGATGTGGATGCTAGCAC
CCAAACTAAGTCATTAGATGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAAC
GTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGC
AAGTTGCGCCTGAAGCCTTTTGGCCAAGGCACGTCTGCCTGGGCGTCACGCATAGCGTCTCT
CCCCATCTCGCAAGWGTGAAGGGGAGAGGATGATGGTCTCCCATGTCTCATTGAACATGGAT

GACCTAAATTAGGAAGCCACAGGATATGAGATGCTGCGGCGAATGGTGGTTGTATATATGGC
CTTTCCTCGCGTCGTGTATCACATAGTTCACATGGCCTCGTAGGACCCTTTAAAACCTTTGCG
ACCCCAGGTCAGGCGGGGTTACCCGCTGAGTTTAA

NCBI loading sequence:

AAAGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACCTGCCTAGCAGAACGACTAGTGAAC
ATGTTTTTACATCTTMCACTGGGAAGGGGGTGCTGGCTTGTCTAGTCCCTTCCCAACGTTGGG
GAGCGTCCCCTTTATTGGTGGTGCTTACCAACACATTAACGAACCCCGGCGTGATATACGCCA
AGGAATAAAATTGAGTGTGTGT

GCGTATTTCTATTCGAATTTTCGGATGTGGATGCTAGCACCCAAACTAAGTCATTAGATGACTC
TCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTG
TGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCTGAAGCCTTTTGG
CCAAGGCACGTCTGCCTGGGCGTCACGCATAGCGTCTCTCCCCATCTCGCAAGWGTGAAGG
GGAGAGGATGATGGTCTCCCATGTCTCATTGAACATGGATGACCTAAATTAGGAAGCCACAG
GATATGAGATGCTGCGGCGAATGGTGGTTGTATATATGGCCTTTCCTCGCGTCGTGTATCACA
TAGTTCACATGGCCTCGTAGGACCCTTTAAAACCTTTGCGACCCCAGGTCAGGCGGGGTTAC
CCGCTGAGTTTTTGGGCC

The research results also confirmed the identification of *Centrostachys aquatic* [16] [19]. After extraction, DNA isolation, and gene sequencing of *Centrostachys aquatic* leaf samples resulted in gene sequencing. A comparison of this RBCL gene sequence with the published sequence of *Centrostachys aquatica* showed a 98.35% similarity. Therefore, the study determines the names of *Centrostachys aquatica* (R.Br.) Wall species by DNA, which is a method with high reliability and selectivity. The results of the study have created a reference for documents on the morphology of parts of *Centrostachys aquatic* plants with illustrations. In order to complete the data set on the morphology of *Centrostachys aquatic* plants, it is necessary to expand the scope of research on microsurgery and *Centrostachys aquatic* powders.

References

[1] Kaur, H.P., Kaur, S., Prasad, B., Manu Priya and Anjali, "Phytochemical, Antioxidant and Antibacterial Studies on Bambusa arundinacea and Mangifera indica," *INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE,* vol. 3, no. 3, pp. 87-93, 2015.

[2] Tiwari, S., "Plants: A Rich source of herbal medicine," *Journal of Natural Products,* vol. 1, pp. 27-35, 2008.

[3] Nikhal, S. B., "Hydroalcoholic extraction of Mangifera indica (leaves) by Soxhletion," *International Journal of Pharmaceutical Sciences,* vol. 2, no. 1, pp. 30-32, 2010.

[4] JG, Baker, "Flora of Tropical Africa," *Royal Botanic Gardens, Kew,,* vol. 6, p. 14, 1913.

[5] CC, Townsend, "Flora Zambesiaca," *Royal Botanic Gardens, Kew,* vol. 9, p. 28, 1988.

[6] Moliur Rahman, R. T., "Floristic diversity of dhamrai upazila of dhaka with emphasis on medicinal plants," *Bangladesh J. Bot,* vol. 41, no. 1, pp. 71-85, 2012.

[7] Chase, M. W., Salamin, N., Wilkinson, M., Dunwell, J. M., Kesanakurthi, R. P., Haidar, N., & Savolainen, V., "Land plants and DNA barcodes: Short-term and long-term goals,"

Philosophical Transactions of the Royal Society B: Biological Sciences, vol. 360, no. 1462, p. 1889–1895, 2005.

[8] Costion, C., Ford, A., Cross, H., Crayn, D., Harrington, M., & Lowe, A., "Plant DNA barcodes can accurately estimate species richness in poorly known floras," *PLoS ONE,* vol. 6, no. 11, p. 1–9, 2011.

[9] Bandyopadhyaya, S., Vankayalapati, R., Rajanna, L., & Kulkarni, S., "DNA barcoding and its applications - A critical review," *Cmrims J. Res. and Dev,* vol. 1, no. 1, p. 77–81, 2013.

[10] Paul D N Hebert, Alina Cywinska, Shelley L Ball, Jeremy R deWaard, "Biological identifications through DNA barcodes," *Proc Biol Sci,* vol. 270, no. 1512, pp. 313-21, 2003.

[11] Paul D. N. Hebert, Sujeevan Ratnasingham and Jeremy R. deWaard, "Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species," *Proc Biol Sci,* vol. 270, pp. 96-99, 2003.

[12] Thin, Nguyen Nghia, "Plant research methods," *Education publisher,* 2006.

[13] Doyle, JJ, "Isolation of Plant DNA from Fresh Tissue," *American Journal of Plant Sciences,* vol. 12, no. 1, pp. 13-15, 1990.

[14] Sanger, S., Nicklen, S., and Coulson, A.R, "DNA sequencing with chain-terminating inhibitors," *Proc Natl Acad Sci U S A,* vol. 74, no. 12, p. 5463–5467, 1977.

[15] Hall, Thomas, "BioEdit is a biological sequence alignment editor written for Windows 95/98/NT/2000/XP," *Janus-I Science,* vol. 41, pp. 95-98, 1999.

[16] Võ Văn Chi, "Từ điển cây thuốc Việt Nam tập 1," *Hà Nội: Nhà xuất bản Y học,* 2018.

[17] Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, et al., "Multiple Multilocus DNA Barcodes from the Plastid Genome Discriminate Plant Species Equally Well," *PLoS ONE,* vol. 3, no. 7, 2008.

[18] Levin, R.A., Wagner, W.L., Hoch, P.C., et al., "Family-level relationships of Onagraceae based on chloroplast rbcL and ndhF data," *American Journal of Botany,* vol. 90, pp. 107-115 107-115 (modified from Soltis P et al. (1992) Proceedings of National Academy of Sciences USA, 89: 449-451), 2003.

[19] Đỗ Tất Lợi, Những cây thuốc và vị thuốc Việt Nam, Nhà xuất bản Y học , 2006.