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Research Article
A Study of the Medicinal Plants of Genus *Orthosiphon* sp.: Aiming to Expand Valuable Medicinal Resources for Medical Treatment

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Abstract

Background and Objective: *Orthosiphon* sp., contains many active ingredients that are beneficial to health. In addition to prevention, they are also used in treatment. This study aims to evaluate the agronomic characteristics of 9 *Orthosiphon* sp., based on morphology and combine molecular biology methods to determine the genetic relationship based on the "ITS1-4" gene region. **Materials and Methods:** Nine seed samples/species *Orthosiphon* sp., grown in 9 provinces in South Vietnam were collected and planted for survey at the Can Tho experimental farm. The method of observing and describing the external morphology and microdissection of the eye cage is carried out. The DNA extraction was performed at the Molecular Biology Laboratory, Institute of Food and Biotechnology, Can Tho University. **Results:** There are differences in some parameters except leaf width and flower diameter. A simple pedigree chart can classify nine cat breeds into 3 groups: Group I includes An Giang (Râ1), Ca Mau (Râ2) and Can Tho (Râ3) breeds that are genetically close to each other and have only the same quantity, group II includes 2 varieties of Vinh Long (Râ8) and Dong Thap (Râ6), group III includes 4 identical samples in Kien Giang (Râ7), Hau Giang (Râ4), Soc Trang (Râ5) and Tien Giang (Râ9). **Conclusion:** With molecular biology techniques, 9 cat breeds have been identified, all belonging to the species *Orthosiphon aristatus*. However, to have more accurate and complete conclusions, it is necessary to study some specific gene sequences related to the morphology and substances contained in the plant.

Key words: *Orthosiphon* sp., stem height, DNA, flower diameter, root length

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Orthosiphon sp., is a herbaceous plant usually about 30-60 cm tall, with a stem that has edges and few branches. When young, the tree trunk is green and hairy. As it gets older, the tree trunk gradually turns purple. *Orthosiphon* sp., belongs to the mint family (Lamiacea). The *Orthosiphon* genus currently includes 40 species, in Vietnam alone a total of 08 species have been discovered, including *Orthosiphon lanatus* Doan, *Orthosiphon marmoritis* (Hance) Dunn, *Orthosiphon rubicundus* (D. Don) Benth, *Orthosiphon velterii* Doan, *Orthosiphon rotundifolius* (Doan), *Orthosiphon truncates* (Doan) and *Orthosiphon spiralis* (Lour.) Merr.

Previous studies have shown that *Orthosiphon* sp., contains many active ingredients that are beneficial to health. In addition to prevention, they are also used in treatment. The researchers[1-4] used *Orthosiphon stamineus* treatment of hypertension, edema, epilepsy and flu. Adnyana *et al.*[5] in different studies on *Orthosiphon stamineus*, different extracts (50% hydro-alcoholic, distilled water, 50-70% hydroacetone and chloroform extract). *Orthosiphon stamineus* extracts showed potential antioxidant activity, with the highest activity found in hydroacetone extracts.

Many studies shows the important role of *Orthosiphon* sp., for human health[6-8]. Many reliable studies have shown that *Orthosiphon* sp., is good for the liver and kidneys[9,10]. Translated from *Orthosiphon* sp., may help reduce blood sugar levels and increase leptin and healthy HDL levels. It can also prevent platelets in the blood from sticking together, thereby reducing the risk of heart attack and stroke. In addition, the herb *Orthosiphon* sp., can help reduce fat in the body, they affect the enzymes α-amylase and α-glucosidase which help the body absorb increased glucose by inhibiting pancreatic. In addition affects the enzyme angiotensin helps regulate blood pressure and prevents some heart diseases. *Orthosiphon* sp., may help reduce urinary tract infections and kidney stones. It also helps prevent kidney stone formation and protects the kidneys from the harmful effects of certain drugs and reagents. In addition, *Orthosiphon* sp., can be anti-inflammatory. This herb is useful in reducing uric acid levels in the blood, treating rheumatism, gout and increasing bone strength in postmenopausal or menopausal people. Because they are naturally high in potassium and calcium.

Some previous studies showed that the extracts have antioxidant capacity equivalent to some standard antioxidants BHA and quercetin. The application of DNA barcoding relies on using one or more DNA fragments of about 400-800 bp as a standard to quickly and accurately identify species. Coding DNA fragments will be used accordingly[11,12]. However, these studies mainly focus on medicinal properties and pay little attention to the genetic characteristics of the variety/species *Orthosiphon* sp. Sample collection in the Mekong Delta was carried out to determine the genetic relationships between varieties/species of *Orthosiphon* sp., based on agronomic characteristics and the sequence of the "ITS1-4" gene region.

MATERIALS AND METHODS

Materials: Nine seed samples/species *Orthosiphon* sp., grown in provinces such as Angiang, Camau, Can Tho, Dongthap, Soctrang, Tiengiang, Haugiang, Kiengiang, Vinhlong were collected and planted for survey at the Can Tho Experimental Farm. This study was carried out from March, 2022 to July, 2022. The seed samples were arranged in a completely randomized design with three replications. The treatment was the cultivar/species *Orthosiphon* sp., collected in nine provinces of the Mekong Delta.

Morphological and agronomic methods: The method of observing and describing the external morphology and microdissection of the eye cage is carried out based on the botanical research method of Khonsung *et al.*[13], with improvements. Parts described include: Stem, leaves, roots, flowers, fruits and seeds. The measurement method is carried out as follows.

Stem height (cm) measure the entire stem above the ground, from the ground to the end of the bud, the method was presented in Fig. 1a. Meanwhile, leaves measure two indicators: Leaf length from petiole to leaf tip and the width of the largest leaf blade (Fig. 1b). Root length measured from the underground part (mature zone) to the root tip (Fig. 1c). Measuring the inflorescence axis length (Fig. 1d) indicates flower diameter and describes the color and shape of the corolla. Fruit size is measured by the diameter of the fruit as shown in Fig. 1e and also describes the color and shape of the fruit. All agronomic indicators were measured and averaged over 5 sample plants.

Molecular methods: The 5 fresh leaf samples were collected and frozen. The DNA extraction was performed at the Molecular Biology Laboratory, Institute of Food and Biotechnology, Can Tho University. Total DNA of samples of genus/species *Orthosiphon* sp., extracted from fresh leaf samples according to the extraction procedure using the improved CTAB method[14]. After purification, check DNA quality by electrophoresis on a 1% agarose gel; after electrophoresis, the gel was stained with redsafe dye (Biobasic, UK). The PCR amplification was performed with primer pairs and sequencing of the ITS1-4 gene region the sequence of the primer pair is as follows: F: 5'-TCCGTAGG TGAACCTGCGG-3'; R: 5'-TCCTCCGCTTATTGATATGC-3'.

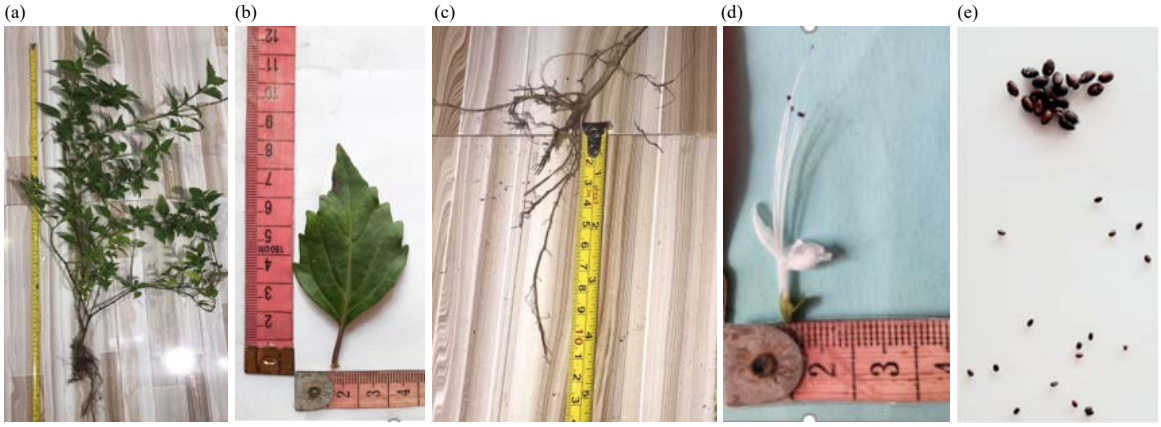

Fig. 1(a-e): Morphology of *Orthosiphon* sp., (a) Stem of *Orthosiphon* sp., (b) Leaves of *Orthosiphon* sp., (c) Root system of *Orthosiphon* sp., (d) Flower diameter and (e) Fruit and seeds of *Orthosiphon* sp.

The PCR reaction includes 35 heating cycles, a denaturation period of 5 minutes at 95°C, 60 sec at 95°C, an annealing time of 50 sec at 54°C, an extension time of 90 sec at 72°C, an extension chain length of 5 min at 72°C and the product was stored at 10°C for 20 min. Electrophoresis of PCR products was then purified using the Wizard SV gel kit and PCR Clean-up System (Promega), based on the Sanger *et al.*[15] method. Sent to the Molecular Biology Laboratory. The sequence of the ITS gene region was solved by the Korean Sequencing Company. Molecular data related to the sequence of the "ITS" gene region were analyzed and clustered using Mega X software[16].

Methods of analyzing and processing data: To determine the genetic relationship between nine accessions/species *Orthosiphon* sp., constructed a family tree using Mega. The sequences of the "ITS" gene region of nine accessions/species *Orthosiphon* sp., used to build the genealogical diagram are aligned with BioEdit software is a biological sequence alignment editor and remove some noisy sequences at the two ends of each sequence. The obtained data such as stem height, leaf length and width were calculated as average, standard deviation value, Analysis of Variance (ANOVA) and compared for significant differences between cultivar samples by test. Duncan at the 5% significance level using Microsoft Excel 2013 and SPSS 20.0 software.

RESULTS AND DISCUSSION

Agronomic characteristics: Observe the plant *Orthosiphon* sp., is a type of single-leaf culture, with short petioles, located opposite each other in a cross-shaped shape. The leaves have a tapered shape, about 4-8 cm long and about 2-4 cm wide, with serrated edges on the upper 2/3 of the leaf. Leaf size among *Orthosiphon* sp., shows that leaf length fluctuates more than width. Agronomic characteristics of *Orthosiphon* sp., were presented in Table 1. The survey results were consistent with the studies of Tezuka *et al.*[17] and Dieu *et al.*[12].

Recorded results (Table 1) show that body height varies from 33.5 cm (Tiengiang) to 77.1 cm (Angiang), this is the variable with the most change, results of statistical analysis. Showed a difference at the 1% significance level using the Duncan's test. The phenotypic and genotypic variances were equivalent, which suggests that stem height is contributed by genes. Therefore, improving varieties is effective with genetic progress parameters reaching over 100%, this is also consistent with the research of Muhammad *et al.*[18]. The longest leaf length is 6.75 cm (Can Tho) and the shortest leaf length is 4.31 cm (Kiengiang).

Analysis of the coefficient of variation between phenotype and genotype of *Orthosiphon* sp., are equivalent and the heritability is relatively high, many other studies have also made similar observations Woerdenbag *et al.*[7]. Root observation showed that the root length between the *Orthosiphon* sp., changed from 12.02 cm (Soc Trang) to 20.45 cm (An Giang), the Duncan test showed a difference at the 5% significance level. Flowers of *Orthosiphon* sp., grows in clusters of 6-10 rings at the top of the tree, each ring has 6 flowers. The flowers are usually white, the stamens extend outward 2-3 times longer than the petals. Research by Dieu *et al.*[8], the recorded flower color of *Orthosiphon* sp., the same, while the inflorescence axis length has a difference at the 5% significance level. Fruit diameters vary from 1.18 cm (Ca Mau) to 1.75 cm (Dong Thap), statistical analysis shows that there is a difference at the 5% significance level.

Table 1: Agronomic characteristics of 9 varieties of *Orthosiphon* sp. (unit: cm)

Provinces/samples	Stem height	Leaf length	Leaf width	Root length	Flower length	Fruit diameter
Angiang (Râ1)	77.1	5.27	3.18	20.45	12.39	1.22
Camau (Râ2)	35.5	6.34	3.03	12.02	15.00	1.18
Can Tho (Râ3)	44.3	6.75	3.48	17.03	16.00	1.75
Dongthap (Râ6)	42.3	4.75	2.68	18.23	13.00	1.55
Soctrang (Râ5)	34.5	5.84	2.53	12.02	15.40	1.33
Tiengiang (Râ9)	33.5	6.18	2.93	14.02	13.25	1.24
Haugiang (Râ4)	61.4	5.62	3.38	16.13	11.13	1.36
Vinhlong (Râ7)	48.1	4.57	2.78	18.45	12.39	1.52
Kiengiang (Râ3)	55.5	4.31	2.23	16.22	13.00	1.34
Average	48.01	5.51	2.91	16.06	12.07	1.39
Accreditation	**	*	**	*	**	*
Var. G	8987.00	20.03	0.52	9.02	0.19	0.09
Var. P	9055.00	23.08	1.65	13.16	0.32	0.27
GCV	0.75	0.35	0.09	0.13	0.16	0.14
PCV	0.75	0.37	0.15	0.16	0.20	0.24
h^2 (%)	0.99	0.86	0.32	0.69	0.59	0.33
GA (%)	194.06	8.51	84.68	5.16	68.76	35.32

Var. G: Variance of genotype, Var. P: Variance of phenotype, GCV: Genotypic coefficient of variation, PCV: Phenotypic coefficient of variation), h^2: Heritability in broad sense, GA: Genetic advance and *Significance at 5% while **Significance at 1%

Table 2: Identification results of 9 samples of *Orthosiphon* sp.

Sample symbol	Identification results	Similar ratio (%)
Râ1	*Orthosiphon aristatus*	99.48
Râ2	*Orthosiphon aristatus*	99.02
Râ3	*Orthosiphon aristatus*	99.20
Râ4	*Orthosiphon stamineus*	99.37
Râ5	*Orthosiphon aristatus*	99.18
Râ6	*Orthosiphon stamineus*	99.34
Râ7	*Orthosiphon aristatus*	100.00
Râ8	*Orthosiphon aristatus*	98.29
Râ9	*Orthosiphon aristatus*	99.87

Sequence of gene region "ITS1-4": Amplification of the "ITS1-4" gene region of about 600 bp shows the results in Fig. 2. The electrophoresis results of the PCR products recorded that the gene segment was successfully amplified in all 09 Cat's Whiskers samples. In all samples, a clear, bright DNA band was obtained, with no additional DNA bands appearing, consistent with the theoretical size of the ITS gene segment expected to be cloned. The results were consistent with the study of White *et al.*[19]. The PCR product is then genetically sequenced.

Research by Son *et al.*[20] used the primer pair ITS1-4 with the same sequence as the primer pair used in the study, but on some subjects belonging to other plant groups, the ITS gene segment was also recorded. In other groups of plants, the size is also about 600 bp. This result was like the result of cloning the ITS segment in the 9 Cat's Whiskers samples studied. From there, the size of the ITS1-4 fragment is a common primer in different plant species when using the same primer pair for cloning. However, this result was only similar in size, but to confirm the diversity of the nucleotide sequence, it is necessary to sequence the gene segment and do a comparative analysis.

Table 2 shows that there were 9 sequences of *Orthosiphon* sp., compared with the sequences on the NCBI gene bank, nine varieties were recorded with results consistent with the sequence of *Orthosiphon aristatus* with similarity coefficients from 98.00 to 100%. This shows that the reliability of the "ITS" sequence is very high. In addition, only sample Râ7 has 100% similarity with the species. Based on the sequences of these nine varieties, the genetic relationship between them was determined through the construction of a phylogenetic tree in the sequence of the "ITS" gene region, the results were presented in Fig. 3.

Decoded ITS sequences of 9 plant samples *Orthosiphon* sp., corrected by removing noise signals at both ends of the sequence, after alignment with a length of 572 bp, the data was used to build a genealogical diagram using the maximum likelihood method. Based on the pedigree diagram, the 9 samples in the study are grouped into 2 branches: Branch I includes 5 samples Râ4, Râ3, Ra5, Râ6 and Râ2; Branch

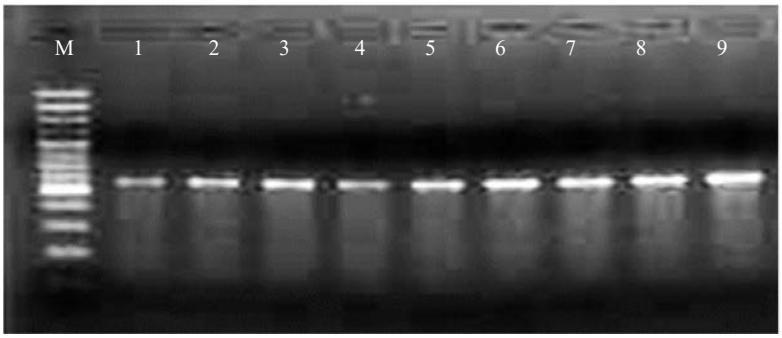

Fig. 2: PCR product of the ITS1-4 gene region of a 9 *Orthosiphon* sp.

M: Standard scale is 100 bp, Well1: Sample Râ1, Well 2: Sample Râ2; Well 3: Sample Râ4, Well 5: Râ5 sample, Well 6: Râ6 sample, Well 7: Râ7 sample, Well 8: Râ8 sample and Well 9: Râ9 sample

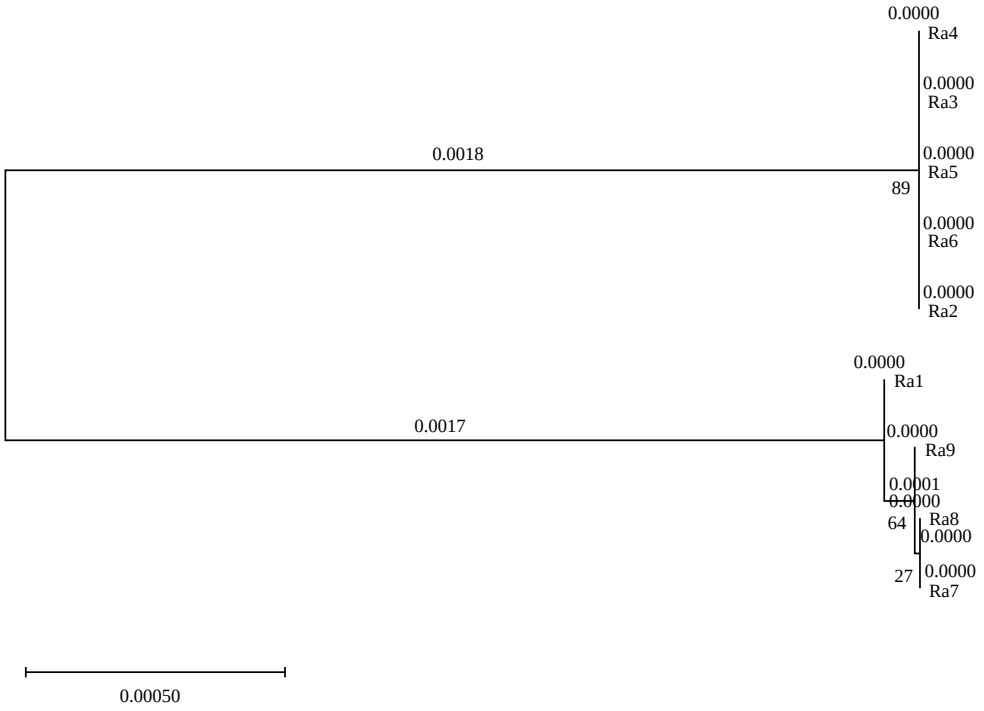

Fig. 3: Family tree diagram of 9 *Orthosiphon* sp.

II includes 4 samples Râ1, Râ9, Râ8 and Râ7. The genetic distance between the two branches is 0.0035. For polymorphisms within each group, the data show that individuals within the same group have nearly identical ITS sequences with negligible differences. The reliability of the diagram is shown by the bootstrap index of the two branches which are 89 and 64%, respectively. The results were consistent with the research of Nguyen *et al.*[6] which also suggested that, based on the sequence of the gene region "ITS 1-4", the species name of *Orthosiphon* sp. The above results reflect the polymorphism of ITS sequences in individuals of the same species showing that individuals in different geographical regions can accumulate variations in DNA sequences, which is also a premise for researching the correlation between differences in gene sequences and specific biological characteristics of organisms such as medicinal and antibacterial properties.

The research results are important in providing a scientific basis for preserving and replicating precious medicinal plants in Vietnam. The data collected are intended to recommend localities to expand cultivation areas for these plants. However, currently there are many difficulties in determining the compatibility between ecological regions and biological characteristics of trees.

CONCLUSION

Results of morphological surveys as well as agronomic characteristics of nine varieties/species of *Orthosiphon* sp., showed differences in some indicators such as stem height, leaf length, root length and fruit diameter. The sequencing of the ITS1-4 gene region on 9 varieties of *Orthosiphon* sp., identified samples of genus/species *Orthosiphon* sp., identical to the species *Orthosiphon aristatus*. Heritability in a broad sense is high for the two traits stem height and leaf length, so it can be considered that these two traits are controlled by genes, this will help improve the variety effectively because of the trait. This is not affected by the environment. In addition, it is also necessary to investigate the sequences of other specialized genes related to morphological and biochemical characteristics such as the content of substances in leaves and fruits to have more accurate conclusions about this species.

SIGNIFICANCE STATEMENT

This study mainly focus on medicinal properties and pay little attention to the genetic characteristics of the variety/species *Orthosiphon* sp. Sample collection in the Mekong Delta was carried out to determine the genetic relationships between varieties/species of *Orthosiphon* sp., based on agronomic characteristics and the sequence of the "ITS1-4" gene region. This study will help researchers explore important areas of *Orthosiphon* sp., such as medicinal herbs and biodiversity. Therefore, research is very necessary, especially for pharmaceutical raw material areas in Southern Vietnam, where there is no data on the international pharmaceutical raw material market.

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