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Micro-morphological, phytochemical and molecular characteristics of aquatic pennywort (*Hydrocotyle verticillata)* accessions in Vietnam

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Abstract: *Hydrocotyle verticillata*, known as aquatic pennywort, is commonly found in wetlands. Recently, it is widely cultivated as an ornamental plant in Vietnam. In this work, we characterised a four-accession germplasm of *H. verticillata*, gathered across Vietnam with a maximal distance of 620 km. The accessions were identified based on ITS sequencing, using primers ITS_{u1} and ITS_{2R}. The four accessions displayed variations in morphological and micro-morphological traits such as stolon diameter, thickness of runner cortex, runner stele diameter, number of runner cortex cell layer, number of runner vascular bundles, diameter of runner xylem vessel elements, diameter of young root, root cortex thickness, diameter of root stele, number of cortex cell layer of root, number of xylem bundles of root, diameter of xylem vessel elements of root, leaf thickness and palisade mesophyll thickness. In addition, phytochemical contents, including Vitamin C, phenolic, flavonoid and saponin, were found to be significantly different among *H. verticillata* accessions, while reducing sugar, carotenoid and tannin contents were comparable. Finally, genetic diversity was demonstrated within the germplasm using RAPD genotyping. While the number of accessions was small (four accessions), this report showcased the diversity of aquatic pennywort accessions in Vietnam, warranting further studies into this vegetatively propagated species. These findings formed the basis for further research on breeding, cultivation and extraction of bioactive compounds from *H. verticillata*.

Keywords: Aquatic pennywort, *Hydrocotyle verticillata*, morphological, micro-morphological, *RAPD*.

Introduction

Aquatic pennywort (*Hydrocotyle verticillata*), also known as whorled pennywort, is a member of the ginseng family (*Araliaceae*). It is a fast-growing aquatic herb that is also an invasive aquatic weed in water bodies across the world. It can outcompete native plants, affect water quality and alter aquatic populations. While it is strictly managed in some countries, it is sold as an aquarium plant in others. At the same time, aquatic pennywort is considered as an environmental remedy in polluted water bodies as it functions as a natural oxygenator and removes both divalent iron and total iron (Chu et al., 2020). *H. verticillata* is still under-investigated worldwide (Daminar and Bajo, 2013; Emeka et al., 2022; Downie et al., 2014; Lim et al., 2014; Umate and Deogade, 2020).

More recently, *H. verticillate* is considered as a source of medicinal compounds since its extract contained bioactive compounds that are useful in treating leukemia (Daminar and Bajo, 2013; Emeka et al., 2022) and other diseases (Umate and Deogade, 2020). Useful bioactive compounds in *H. verticillata* leaves include 3. beta.17. beta.-dihydroxyestr-4-ene (10.89%); hexadecanoic acid, ethyl ester (7.49%), and 1-Methylbicyclo [3.2.1] octane (7.28%) (Emeka et al., 2022). These compounds may underlie *antibacterial activity against both gram-positive and gram-negative bacteria exhibited by leaf extracts (Dhivya et al., 2023). H. verticillata* was introduced into Vietnam, it is now found near canals, water bodies and is commonly grown as an ornamental plant. Despite their potential to be used as a medicinal plant and as a remedy for environmental pollution, *H. verticillata* remained under-investigated. No effort to characterize *H. verticillata* accessions in Vietnam has been attempted. To understand variations among *H. verticillata* accessions in Vietnam, we collected four accessions with a maximal distance of 620 km (Table 1, Fig. 1). The germplasm was extensively characterized in terms of morphological, micro-morphological traits, phytochemical contents and genetics. While the number of *H. verticillata* accessions in this study was small, this pioneering work forms the basis for future analysis of aquatic pennywort in Vietnam, and their potential to be used in medicine and environmental remedies.

Results

ITS identification

The four accessions collected in this study had been identified as *H. verticillata* based on the ITS sequences (GenBank accessions: OM943954.1, OM943955.1, OM943956.1 and OM943957.1), using primers ITS_{u1} (Cheng et al., 2016) and ITS_{2R} (Chen et al., 2010). The sizes of the sequences were 799-802 bp, with roughly 58% GC. There were two polymorphic sites and a low rate of variable sites, 0.25%.

Morphological characterization

Among aquatic pennywort accessions, there were significant differences in quantitative morphological features but no differences in qualitative morphological traits. With their spreading leaf arrangement, orbicular leaf shape, crenate leaf edge, dark green color, glabrous leaf surface, long, thin, and light green petioles at the base, as well as their white stolons, greenish white flowers, and compound inflorescence, all four accessions exhibited good regenerability. With the exception of dry matter, there were notable variations between aquatic pennywort accessions in all quantitative morphological characteristics (Table 2, Figure 2). HUIB_HV23, an aquatic pennywort accession, had the longest and widest leaves (5.63 cm × 4.15 cm), whereas HUIB_HV17 had the shortest leaves (2.74 cm × 2.55 cm). The leaf

Table 1. Aquatic pennywort accessions collected in Vietnam. These accessions were collected while we were studying the diversity of pennywort (*C. asiatica*) across Vietnam.

No.	Accession code	Location of collection	Coordinate
1	HUIB_HV17	Ngo May, Kon Tum	14°21'25.4"N 107°59'59.5"E
2	HUIB_HV22	Hung Nguyen, Nghe An	18°40'14.9"N 105°36'42.8"E
3	HUIB_HV23	Quynh Luu, Nghe An	19°10'51.2"N 105°39'39.9"E
4	HUIB_HV24	Tinh Gia, Thanh Hoa	19°32'20.3"N 105°47'40.0"E

Table 2. Quantitative morphological traits observed in four aquatic pennywort accessions.

Accession code	Leaf length (cm)	Leaf width (cm)	Leaf petiole length (cm)	Number of primary lateral veins	Runner length (cm)	Fresh yield at the first harvest (g)	Plant weight (g)	Dry matter (%)
HUIB_HV17	2.74 ± 0.27^{c}	2.56 ± 0.24^{b}	13.79 ± 1.09^{b}	13.63± 0.93^{b}	8.65 ± 0.82^{a}	542.41 ± 88.02^{a}	1.24 ± 0.04^{b}	16.800^{a} ± 0.400
HUIB_HV22	3.81 ± 0.36^{b}	2.80 ± 0.29^{b}	16.95 ± 1.64^{a}	13.17 ± 1.12^{b}	5.75 ± 0.77^{c}	549.82 ± 38.86^{a}	1.26 ± 0.04^{b}	16.267^{a} ± 0.231
HUIB_HV23	5.63 ± 0.62^{a}	4.15 ± 0.70^{a}	13.21 ± 1.54^{b}	16.03 ± 1.47^{a}	6.65 ± 0.71^{b}	474.24 ± 13.31^{a}	0.69 ± 0.01^{c}	16.467^{a} ± 0.306
HUIB_HV24	5.50 ± 0.60^{a}	4.25 ± 0.67^{a}	10.97 ± 1.01^{c}	16.37 ± 1.50^{a}	6.29 ± 0.64^{b}	295.58 ± 11.29^{b}	2.02 ± 0.02^{a}	16.400^{a} ± 0.400

The same upper-case letters within columns indicate the lack of significant difference (p ≥ 0.05). Errors represent standard deviation.

petioles of HUIB_HV22 and HUIB_HV24 were the longest and shortest, respectively (16.95 cm vs. 10.97 cm).

There were 13.17 to 16.37 main lateral veins in total. With 16.37 primary lateral veins, HUIB_HV24 had the greatest, while HUIB_HV22 had the fewest (13.17). At the first harvest, there were significant differences in both plant weight and fresh yield, which ranged from 0.69 g to 2.02 g and 295.58 g to 549.82 g, respectively. At the first harvest, HUIB_HV22 (549.82 g) had the highest fresh yield while HUIB_HV24 (295.58 g) had the lowest. HUIB_HV23 had the lowest plant weight (2.02 g and 0.69 g, respectively), while HUIB_HV24 had the highest plant weight. across 16.27% and 16.80%, there was no discernible variation in dry matter across the four aquatic pennywort accessions.

Micro-morphological identification

The anatomical features of leaves, runners and roots of four aquatic pennywort accessions were showed in Figure 3. The epidermis (1) consisted of a layer of rectangular cells with uneven thickness. Inside the epidermis lied the cortex of the runner and root (2). The runner cortex comprised approximately 9-13 layers of cells, with an outer layer consisting of 1-2 collenchyma cells having thick walls, followed by parenchyma cells (8-11 layers) that were either round or polygonal, with slightly curved edges and varying sizes. The stem cortex contained large air spaces. The root cortex consisted solely of parenchyma cells, typically with 3-5 cell layers, with small air spaces. The innermost layer of the root cortex was the endodermis (6). Inside the cortex was the cylindrical region of the runner and root. The xylem (3) and phloem (4) were arranged in vascular bundles in the runner and alternate in the root. Toward the innermost part of the runner lied the pith (5), composed of parenchyma cells with thin walls, exhibiting diverse shapes and sizes. The runner typically contained 10-11 vascular bundles, while the root had 2-3 xylem branches alternating with phloem. In leaves, both the upper and lower epidermal layers consisted of flattened or oval-shaped cells with uneven dimensions. Beneath the upper epidermal cell layer was the palisade mesophyll (9), which comprised two layers of elongated, rectangular cells arranged vertically. The spongy mesophyll (10), characterized by large intercellular spaces and a variety of cell shapes and sizes, lied immediately below the palisade mesophyll.

Micro-morphological traits varied among the four aquatic pennywort accessions except for NRCOCL, ISCRu, ISCR and NPMCL (Table 3). SD varied from 1.26 mm (HUIB_HV23) to 1.72 mm (HUIB_HV17). TRC ranged from 305.00 µm (HUIB_HV22) to 502.25 µm (HUIB_HV17). RSD was the largest (802.50 µm) in HUIB_HV24 and smallest (578.50 µm) in HUIB_HV23. All aquatic pennywort accessions had 1.5 runner collenchyma cell layer (NRCOCL). NRCCL ranged from 9.50 (HUIB_HV24) to 13.00 (HUIB_HV22). The ISCRu was present in all four aquatic pennywort accessions. HUIB_HV24 had 11 NRVB and the rest had 10 NRVB. HUIB_HV17 had the thinnest DXVE and DYR (31.55 µm and 0.17 mm, respectively) while the thickest DXVE and DYR were observed in HUIB_HV24 (48.48 µm and 0.26 mm, respectively). RCT ranged from 0.06 µm (HUIB_HV22) to 0.08 µm (HUIB_HV17 and HUIB_HV23). DRS ranged from 0.05 µm (HUIB_HV17) to 0.08 µm (HUIB_HV24). HUIB_HV17 and HUIB_HV24 had the highest NCCLR (4.5) and HUIB_HV22 had the lowest one (3.5). HUIB_HV17 having two xylem bundles of root (NXBR) while the rest had three NXBR. All the aquatic pennywort accessions had DXVER of 0.01 µm. The presence of ISCR was observed in most accessions except HUIB_HV22. LT ranged from 0.13 mm (HUIB_HV23) to 0.18 mm (HUIB_HV24). All aquatic pennywort accessions had two palisade mesophyll cell layers (NPMCL) and 0.07 µm of PMT, except HUIB_HV23 (0.06 µm of PMT).

Phytochemical analysis

We found significant differences in the contents of Vitamin C, phenolic, flavonoid and saponin but not in reducing sugar, carotenoid and tannin contents among four aquatic pennywort accessions (Tables 4 and 5). HUIB_HV23 had the highest Vitamin C content (0.401% of dry weight), while HUIB_HV22 had the lowest (0.337% of dry weight). Reducing sugar ranged from 7.639% of dry weight (HUIB_HV22) to 8.534% of dry weight (HUIB_HV24). Carotenoid ranged from 0.65% of dry weight (HUIB_HV24) to 0.71% of dry weight (HUIB_HV17). Tannin content varied from 3.37% of dry weight (HUIB_HV17) to 3.81% of dry weight (HUIB_HV22). The phenolic content varied from 0.31% (HUIB_HV23) to 0.48% (HUIB_HV24) of dry weight. HUIB_HV24 had the highest flavonoid content (6.590% of dry weight), while HUIB_HV17 had the lowest flavonoid content (4.914% of dry weight). The saponin content varied from 1.541 mg GYE/g of dry weight (HUIB_HV22) to 2.339 mg GYE/g of dry weight (HUIB_ HV17).

Genetic diversity analysis

A total of 100 UBC RAPD primers were used to screen four aquatic pennywort accessions. Out of eighteen polymorphic RAPD primers, only four primers (Table 6) yielding clear DNA bands were selected to genotype the germplasm (Fig. 4).

A total of 160 DNA bands were recorded. The highest number of bands were observed in HUIB_HV22, with 49 DNA bands (30.6%). On the other hand, the lowest number of bands were observed in HUIB_HV17 with 33 DNA bands (20.6%). The total number of amplified DNA bands with each primer ranged from 28 (UBC#384) to 45 (UBC#376) DNA bands, averaging from 7 to 11 DNA bands per primer. A total of 20 polymorphic DNA bands were obtained using four polymorphic RAPD primers from the

Table 3. Micro-morphological features observed in four aquatic pennywort accessions collected in Vietnam.

	SD	TRC	RSD	NRCOCL	NRCCL	ISCRu	NRVB	DXVE	DYR
HUIB_HV17	1.72 ± 0.061	502.25±0.816	773.25±2.573	1.5±0	12.50±0.424	P	10±0	31.55±0.374	0.17±0.007
HUIB_HV22	1.43±0.007	305.00±2.449	783.00±1.633	1.5±0	13.00±0.00	P	10±0	43.58±0.383	0.19±0.002
HUIB_HV23	1.26±0.009	337.75±1.225	578.50±1.633	1.5±0	11.50±0.00	P	10±0	37.80±0.408	0.23±0.002
HUIB_HV24	1.60±0.002	435.75±0.816	802.50±0.816	1.5±0	9.50±0.408	P	11±0	48.48±2.419	0.26±0.016
p (Kruskal-Wallis)	0.015	0.016	0.016	1	0.021	1	0.012	0.016	0.015
	RCT	**DRS**	**NCCLR**	**NXBR**	**DXVER**	**ISCR**	**LT**	**PMT**	**NPMCL**
HUIB_HV17	0.08±0.003	0.05±0.002	4.5±0	2±0	0.01±0.001	P	0.17±0.005	0.07±0.004	2±0
HUIB_HV22	0.06±0.002	0.06±0.001	4.0±0	3±0	0.01±0.000	A	0.17±0.002	0.07±0.00	2±0
HUIB_HV23	0.08±0.003	0.06±0.002	3.5±0	3±0	0.01±0.000	P	0.13±0.001	0.06±0.001	2±0
HUIB_HV24	0.07±0.008	0.08± 0.008	4.5±0	3±0	0.01±0.001	P	0.18±0.008	0.07±0.002	2±0
p (Kruskal-Wallis)	0.03	0.022	0.012	0.012	1	0.012	0.027	0.432	1

P: Present, A: Absent; Stolon diameter (mm): SD; Thickness of runner cortex (µm): TRC; Runner stele diameter (µm): RSD Number of runner collenchyma cell layer: NRCOCL Number of runner cortex cell layer: NRCCL Intercellular spaces in cortex of runner: ISCRu Number of runner vascular bundles: NRVB Diameter of runner xylem vessel elements (µm): DXVE Diameter of young root (mm): DYR Root cortex thickness(µm): RCT Diameter of root stele (µm): DRS Number of cortex cell layer of root: NCCLR Number of xylem bundles of root: NXBR Diameter of xylem vessel elements of root (µm): DXVER Intercellular spaces in cortex of root: ISCR Leaf thickness (mm): LT Palisade mesophyll thickness (µm): PMT Number of palisade mesophyll cell layer: NPMCL

Table 4. Vitamin C content, reducing sugar and carotenoid contents in four aquatic pennywort accessions.

No.	Accession	Vitamin C content (% dry weight)	Reducing sugar (% dry weight)	Carotenoid (mg/100 g dry weight)
1	HUIB_HV17	$0.380^{b} \pm 0.009$	$7.883^{a} \pm 1.338$	$0.707^{a} \pm 0.074$
2	HUIB_HV22	$0.337^{a} \pm 0.012$	$7.639^{a} \pm 0.899$	$0.666^{a} \pm 0.073$
3	HUIB_HV23	$0.401^{b} \pm 0.013$	$8.331^{a} \pm 0.694$	$0.685^{a} \pm 0.051$
4	HUIB_HV24	$0.346^{a} \pm 0.020$	$8.534^{a} \pm 1.084$	$0.650^{a} \pm 0.005$

The same lower-case letters within columns indicate the lack of significant difference ($p \geq 0.05$). Errors represent standard deviation.

Table 5. Tannin, phenolic, flavonoid and saponin contents in four aquatic pennywort accessions.

No	Accession	Tannin content (% dry weight)	Phenolic content (mg GAE/g of dry weight)	Flavonoid content (mg CE/g dry weight)	Saponin content (mg GYE/g of dry weight)
1	HUIB_HV17	$3.370^{a} \pm 0.254$	$17.379^{a} \pm 0.495$	$4.914^{a} \pm 0.644$	$2.339^{c} \pm 0.046$
2	HUIB_ HV22	$3.813^{a} \pm 0.096$	$18.110^{ab} \pm 0.792$	$4.990^{a} \pm 0.555$	$1.541^{a} \pm 0.033$
3	HUIB_HV23	$3.592^{a} \pm 0.440$	$17.332^{a} \pm 0.235$	$5.314^{a} \pm 0.649$	$1.593^{a} \pm 0.062$
4	HUIB_HV24	$3.658^{a} \pm 0.240$	$18.951^{b} \pm 0.548$	$6.590^{b} \pm 0.144$	$1.939^{b} \pm 0.038$

The same lower-case letters within columns indicate that the lack of significant difference ($p \geq 0.05$). GAE: gallic acid equivalents, CE: catechin equivalents, GYE: gypenoside XVII equivalents. Error bars represent standard deviation.

four different aquatic pennywort accessions (the average number of polymorphic DNA bands per primer was 11.75). The band size ranged from 200 to 1600 bp.

Theses polymorphic DNA bands were employed in the development of lineage trees and the investigation of genetic diversity. According to the PIC values (Table 7) indicated that primers UBC#354, UBC#362 and UBC#376 were able to detect polymorphisms at medium levels in four aquatic pennywort accessions, however primer UBC#384 showed low level polymorphism detection. The highest values were obtained with primer UBC#354, according to the results of the MI index (the capacity to identify polymorphic loci between genotypes in each primer) and the Rp index (which is based on the distribution of alleles in the sampled genotypes; primers with the larger polymorphic bands, the higher the Rp value). As a result, this primer was the best option for identifying polymorphism in the population. Examination of each person's genetic diversity coefficient within the population. The examined samples had a high degree of diversity, as evidenced by the analysis of the genetic diversity coefficient of individuals in the population (h), which ranged from 0.049 (UBC#384) to 0.238 (UBC#354), with an average of 0.135. Between UBC#384 to UBC#354, the Shannon genetic diversity index (I) varied from 0.136 to 0.347. The four RAPD primers have a mean of 1.426 and a range of observed alleles (na) of 1.250 (UBC#384) to 1.600 (UBC#354). Furthermore, there was variation in the number of effective alleles (ne) between primers, with an average value of 1.220 and a range of 1.130 (UBC#362) to 1.431 (UBC#354). To summarize, out of the four aquatic pennywort accessions that were gathered throughout Vietnam, primer UBC#354 displayed the highest level of genetic diversity.

Individuals of aquatic pennywort accessions had genetic similarity coefficients ranging from 0.67 to 1.00 (Fig. 5). The phylogenetic tree separated the four aquatic pennywort accessions into two major groups according to the genetic similarity coefficient. The remaining aquatic pennywort accessions were in group II, while the single aquatic pennywort accession in group I was HUIB_HV22. The aquatic pennywort accessions in this group were split into two subgroups with a genetic separation of 0.896. HUIB_HV17 was found in the first grouping, and the genetic distances of the other two subgroups (HUIB_HV23 and HUIB_HV24) were identical (Fig. 5).

Discussion

This study is the first characterization of micro-morphology, phytochemicals and molecular diversity of aquatic pennywort (*H. verticillata*) accessions collected across Vietnam. The phytochemical characterization of the four *H. verticillata* accessions collected in this study showed that Vitamin C, reducing sugar, carotenoid, saponins, tannins, flavonoids and phenolic contents were comparable with the previous study (Emeka et al., 2022) and similar to those in *C. asiatica* (Truong et al., 2024), an important medicinal plant that was used to treat a range of ailments (Brinkhaus et al., 2000).

Interestingly, Dhivya and co-workers (2023) reported that *H. verticillata* extracts inhibited the growth of bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and

Table 6. The sequence of RAPD polymorphic primers.

No.	Primer name	Sequence
1	UBC#354	CTAGAGGCCG
2	UBC#362	CCGCCTTACA
3	UBC#376	CAGGACATCG
4	UBC#384	TGCGCCGCTA

Table 7. Genetic diversity of four aquatic pennywort accessions.

Primer	*na**	*ne**	*h**	*I**	PIC index	Rp coefficient	MI coefficient
UBC#354	1.600	1.431	0.238	0.347	0.238	3.500	1.164
UBC#362	1.427	1.130	0.100	0.169	0.161	3.000	0.413
UBC#376	1.400	1.194	0.123	0.192	0.158	3.500	0.380
UBC#384	1.250	1.163	0.049	0.136	0.090	1.000	0.047
Average	**1.426**	**1.220**	**0.135**	**0.208**	-	-	-
SD	**0.500**	**0.327**	**0.177**	**0.260**	-	-	-

Note: na, Number of observed alleles; ne, Number of effective alleles (Kimura and Crow, 1964); h, Nei's genetic diversity (1973); I, Shannon genetic diversity index (Lewontin, 1972).

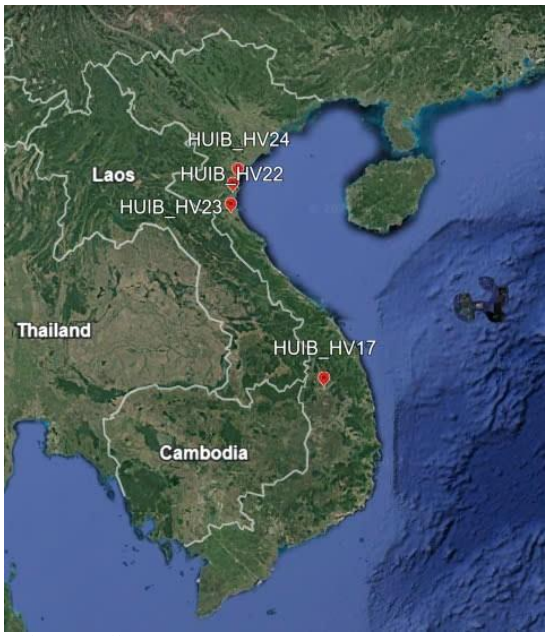

Figure 1. Collection sites of four *Hydrocotyle verticillata* accessions: HUIB_HV17, HUIB_HV22, HUIB_HV23 and HUIB_HV24.

Klebsiella pneumoniae. Therefore, *H. verticillata* may contain valuable medicinal compounds that warrant further investigations. Furthermore, *H. verticillata* is highly tolerant to waterlogging and thrives in flooding areas (Kozeko et al., 2023). Genetic investigation into the mechanism of waterlogged resistance in *H. verticillata* will be useful for breeding programs aiming to generate waterlogged tolerant vegetables.

Genetic diversity was assessed among the four *H. verticillata* accessions. Among the 100 RAPD primers used, four primers revealed high polymorphism. Primer UBC#376 showed the highest number of amplified DNA bands (45 DNA bands) while UBC#384 primer yielded the least number of amplified DNA bands (28 DNA bands) (Table 7). All primers amplified six polymorphic bands, except for primer UBC#382 with two polymorphic bands. According to Nei (1978), the greater the number of amplified DNA bands, the greater the ability to distinguish samples on the pedigree tree and the more accurate the pedigree tree can be built. Genetic diversity was detected among the *H. verticillata* accessions. The four aquatic pennywort accessions were divided into two groups. Group I contained only one accession **(**HUIB_HV22) and group II contained three accessions (HUIB_HV17, HUIB_HV23 and HUIB_HV24; Fig. 5). While accessions HUIB_HV22 and HUIB_HV23 were collected in the same province in central Vietnam, they differed in terms of genetics, micro-morphological traits and phytochemical contents. On the other hand, HUIB_HV17 was genetically closer to HUIB_HV23 and HUIB_HV24, even though it was distributed roughly 600 km away from HUIB_HV23 and HUIB_HV24.

Overall, genetic diversity is consistent with the diversity in quantitative morphological and micro-morphological traits, and phytochemical contents.

Aquatic pennywort remains largely under-investigated worldwide. In the genus *Hydrocotyle*, *H. sibthorpioides* is widely used in traditional medicine to treat fever, edema, psoriasis and liver diseases (Huang et al., 2013a). It contained valuable compounds that displayed anti-tumour, liver-protective and neuro-protective effects (Yu et al., 2007; Huang et al., 2013b; Hazarika et al., 2022). While preliminary work by Emeka and co-workers (2022) demonstrated the abundance of tannins and alkaloids in *H. verticillata* leaf extracts, it remains to be explored whether *H. verticillata* contained other valuable compounds that can be used or serve as precursors for drug development programs. Towards this direction, this study contributes to a greater understanding of aquatic pennywort in Vietnam and more broadly in Asia.

Materials and methods

Plant collection, cultivation and identification using ITS sequencing

Four aquatic pennywort accessions were collected from different locations in Vietnam (Table 1). The soil that used for planting collected aquatic pennywort accessions was taken from Quang Tho, Thua Thien Hue (16°32'06.2"N, 107°31'39.7"E), a popular pennywort producing location in Vietnam (Truong et al., 2024). A mother plant was planted in a plastic tray (W40 cm x L65 cm

Figure 2. Variations in leaf morphological traits and runner length of four aquatic pennywort accessions. (A) HUIB_HV17, (B) HUIB_HV22, (C) HUIB_HV23 and (D) HUIB_HV24.

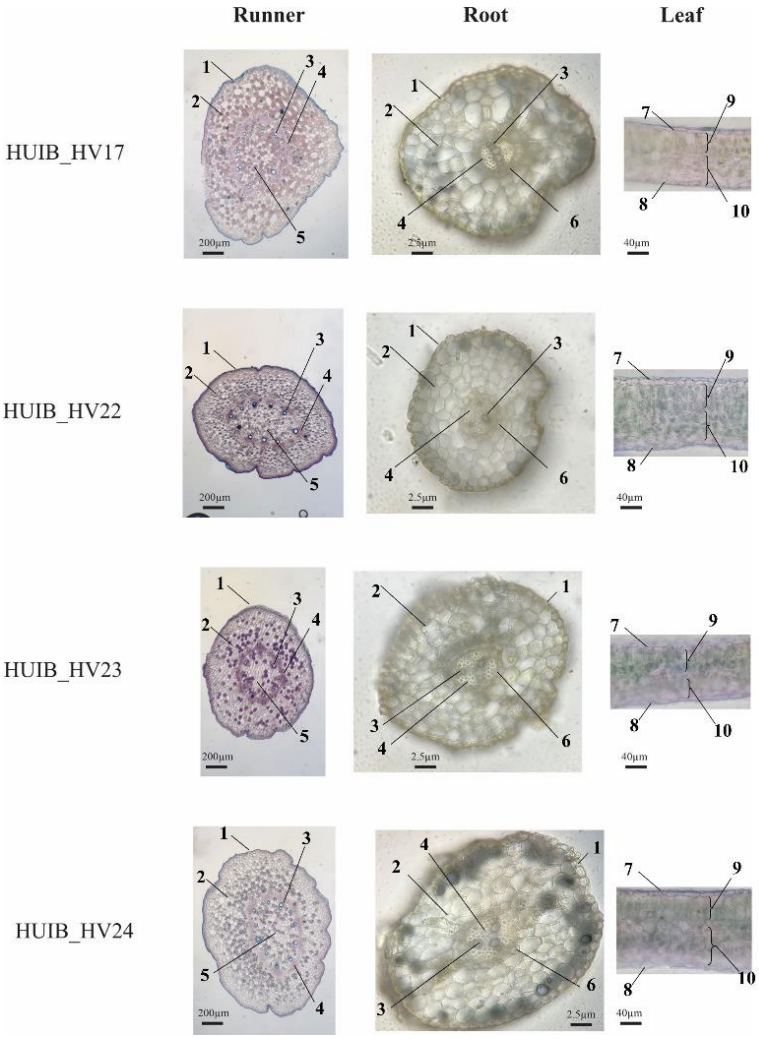

Figure 3. Anatomical features of leaves, runners and roots of the four aquatic pennywort accessions collected throughout Vietnam. (1) Epidermis, (2) Cortex, (3) Xylem, (4) Phloem, (5) Pith, (6) Endodermis, (7) Upper epidermis, (8) Lower epidermis, (9) Palisade mesophyll and (10) Spongy mesophyll.

x H18 cm) filled with 30 kg of soil and maintained water level daily up to marked point of 5 cm from tray surface. The trays were placed in full sun position (sunlight from roughly 6 AM to 6 PM) at the Institute of Biotechnology (16°29'35.7"N, 107°36'20.1"E) in Hue city, Vietnam with temperature ranging from 20°C to 35°C during the day. After five months, nine runner cuttings of each accession were planted to a new W40 cm x L65 cm x H18 cm tray with three replications for each accessions. The water level in the trays was maintained daily up to the marked point of 5 cm from the tray surface.

Genomic DNA was extracted from leaves using CTAB (cetyltrimethylammonium bromide) extraction protocol as described previously (Nguyen et al., 2023). DNA was further purified using silica columns (Biobasic, Canada). The quality of the DNA was examined on agarose gel. $ITS_{u1\text{-}2R}$ primer (ITS_{u1}: GGAAGGACAAGTCGTAACAAGG (Cheng et al., 2016); ITS_{2R}: GACGCTTCTCCAGACTACAAT (Chen et al., 2010)) were used for molecular identification of aquatic pennywort accessions. PCR were performed with ITS primers in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA). Each 15 µL reaction mixture included 7.5 µL of 2x MyFi Mix (Meridian bioscience, USA), 10 pmol of $ITS_{u1\text{-}2R}$ primers, 10-20 ng of template DNA. The thermocycling program involved a cycle of initial denaturation (94°C for 5 min), 30 cycles of amplification (94°C for 30 s, 60°C for 30 s and 72°C for 45 s) and a final extension (72°C for 5 min). PCR products were examined on 1% agarose gels and sequenced using Sanger sequencing method. Raw sequences for the $ITS_{u1\text{-}4}$ gene region were edited in BioEdit v7.2.5 prior to depositing on GenBank (Table 2). Edited sequences were then aligned by

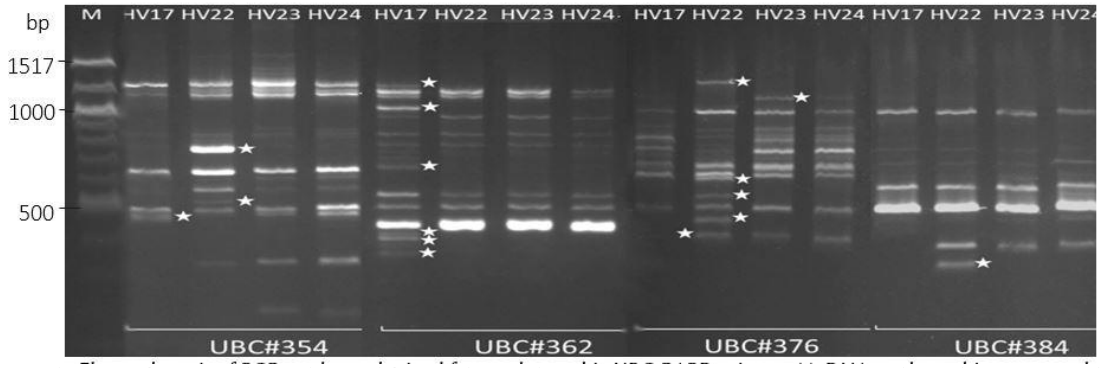

Figure 4. Electrophoresis of PCR products obtained from polymorphic UBC RAPD primers. M: DNA marker; white stars mark polymorphic bands. HV17 (HUIB_HV17), HV22 (HUIB_HV22), HV23 (HUIB_HV23), HV24 (HUIB_HV24).

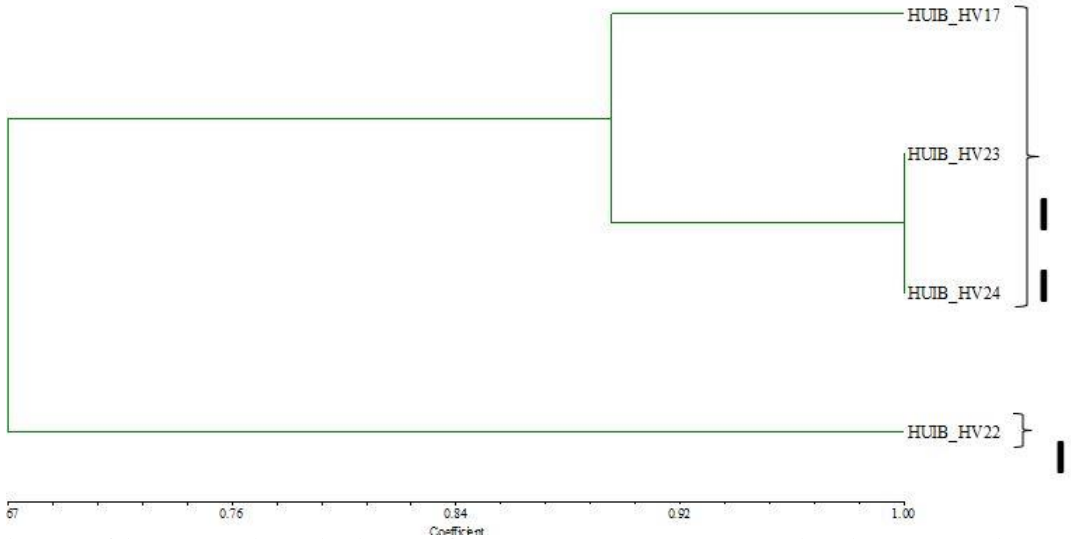

Figure 5. Dendrogram of the genetic relationship between four aquatic pennywort accessions, based on RAPD analysis.

ClustalW in MEGA X and the non-overlapping sequence regions at the 5′- and 3′-ends were trimmed (Kumar et al., 2018).

Morphological identification

Quantitative morphological traits included leaf length, leaf width, leaf petiole length, number of primary lateral veins, runner length, fresh yield at the first harvest, plant weight and dry matter. Qualitative morphological traits included plant regenerability, leaf arrangement, leaf shape, leaf margin, leaf colour, leaf surface, petiole length, petiole thickness, petiole pigmentation at the base, stolon colour, flower colour, type of inflorescence. These traits were scored at full foliage stage (three plants per accession) at five months post transplanting. Results represent averages of three repeats, with ten randomly selected leaves each repeat.

Micro-morphological identification

Quantitative micro-morphological traits included stolon diameter (SD), thickness of runner cortex (TRC), runner stele diameter (RSD), number of runner collenchyma cell layer (NRCOCL), number of runner cortex cell layer (NRCCL), number of runner vascular bundles (NRVB), diameter of runner xylem vessel elements (DXVE), diameter of young root (DYR), root cortex thickness (RCT), diameter of root stele (DRS), number of cortex cell layer of root (NCCLR), number of xylem bundles of root (NXBR), diameter of xylem vessel elements of root (DXVER), leaf thickness (LT), palisade mesophyll thickness (PMT), number of palisade mesophyll cell layer (NPMCL). Qualitative micro-morphological traits included intercellular spaces in cortex of runner (ISCRu) and intercellular spaces in cortex of root (ISCR). Cross-sections of the runners, root and leaves were prepared and stained as described by Tran et al. (2022). Briefly, the cross-sections were immersed in 5% sodium hypochlorite for 20 minutes before being rinsed with distilled water. The cross-sections were then soaked in 1% acetic acid for about 2 minutes and rinsed with distilled water several times. The cross-sections were stained with 1% methylene blue solution for about 15-30 seconds and rinsed with distilled water several times. The sections were further stained with 10% carmine red for about 30 minutes and rinsed with distilled water several times. The stained samples were observed, photographed, and the microscopic morphological features were described using a light microscope. Microscopic dimensions were measured using a microscope eyepiece measurement. For roots with a small diameter, cross-sections were directly observed and described under a microscope. Image analysis was performed in CorelDRAW.

Phytochemical analysis

Intact healthy leaves were washed with tap water, air-dried before being dried in an oven (50^{o}C) until the moisture content was less than 10%. The dried samples were then ground in a mortar and filtered through a sieve (pore size ≤ 1 mm). The fine powder was subject to phytochemical analysis. The total phenolic content of aquatic pennywort leaves was determined using the Folin–Ciocalteu assay as previously described (Singleton & Rossi, 1965); the total flavonoid content was measured by the aluminium chloride colorimetric assay as previously described (Zhishen et al., 1999); the total saponins content of pennywort leaves was determined using the vanillin-sulphuric acid assay as previously described (Le et al., 2018); the Vitamin C content was performed as previously described (Satpathy et al., 2021); the reducing sugar content (RSC) was determined using the 3,5-dinitrosalicylic acid (DNSA) assay as previously described (Krivorotova and Sereikaite, 2014); the total carotenoid content in aquatic pennywort leaves was determined

using a colorimetric assay as previously described (Biswas et al., 2011); the total tannin content in aquatic pennywort leaves was measured as previously described (Atanassova & Christova-Bagdassarian, 2009). All the methods mentioned above were used with modifications as described in detail by Truong and co-workers (2023).

RAPD analysis

An initial screen was carried out using 100 RAPD UBC (University of British Columbia) primers. Preliminary screen showed that four RAPD primers yielded high polymorphism among aquatic pennywort accessions. PCR mixture (15 µL) included 5x buffer (3 µL), 25 mM $MgCl_2$, 10 mM dNTP, 10 pmol of RAPD primer, 0.2 µL of Taq polymerase and 50 ng DNA template. The thermocycling program involved an initial denaturation (94 °C for 3 min), 40 cycles of amplification (94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min) and a final extension at 72°C for 7 min. PCR products were examined by gel electrophoresis (2% agarose gel in 0.5X TBE). The RAPD-PCR products electrophoresis spectrum of samples with primers were analyse according to the principle of the presence ("1") or absence ("0") of bands. The genetic diversity coefficient was calculated as described by Verma and co-workers (2007). Coefficient of PIC (Polymorphism Information Content), Rp (Resolving power) and MI (Marker Index) of each RAPD primer were calculated as described by Serrote and co-workers (2020). The level of genetic diversity was analyse in PopGen 3.2. The pedigree chart was built in NTSYS 2.1.

Data analysis

ANOVA analysis of variance was performed, and Duncan's test was employed to compare the mean of morphological traits. Statistical significance ($p < 0.05$) of micro-morphological traits was evaluated with Kruskal-Wallis test. All statistical analyses were performed in SPSS (Version 20).

Conclusion

In this work, a four-accession *H. verticillata* germplasm collected throughout Vietnam was characterized. The germplasm displayed variations in morphological, micro-morphological traits, phytochemical contents and genetics. This study forms the basis for further understanding *H. verticillata* botany, phenotypes and genotypes in Asia, where the species remains largely under-investigated.

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Conflict of interest

The authors declare no conflicts of interest.

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