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RAPID COMMUNICATION

Note on important and novel findings

DNA barcoding of the invasive alien aquatic weed *Heteranthera reniformis* Ruiz and Pavon using chloroplast DNA markers

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Abstract

Morphology-based species identification may not be accurate and necessitates extensive taxonomic knowledge. Regarding *Heteranthera reniformis* Ruiz and Pavon, an invasive alien species in Japan, it is difficult to find appropriate information on molecular data. DNA barcoding is a convenient method of identifying species based on the nucleotide diversity of conserved sequences. In this study, we aim to assess the suitability of chloroplast *maturase K* (*matK*) and *ribulose-1,5-bisphosphate carboxylase large* (*rbcL*) as DNA barcode markers by collecting molecular data and comparing them with related species available in the gene bank. Both *matK* and *rbcL* markers exhibited a 100% success rate in their amplification and sequencing, with gene sizes of 833 bp and 552 bp, respectively. The subsequent sequence analysis confirmed biological homology with closely related species. The highest homology of 99.52% in the *matK* sequence was found with *H. callifolia*. Moreover, the *rbcL* sequence showed a higher homology of 99% with seven species of the same family. This study highlights the utility of both chloroplast gene regions as barcode primers in elucidating fundamental molecular aspects of *H. reniformis* plant.

Keywords Chloroplast DNA · DNA barcoding · *Heteranthera reniformis* · Invasive weed · Plant morphology

Introduction

Heteranthera reniformis Ruiz and Pavon is an annual or facultatively perennial aquatic plant of the Pontederiaceae family, originally from North, Central, and South America (Gabela 1974; Hill 2006). This species has become problematic in paddy fields because of its high biomass production and reproduction rate, affecting water quality and reducing rice yield by up to 70% (Ferrero 1996). The plant can propagate from stem fragments, developing roots at each node and with long-lasting seed viability; with these characteristics, it rapidly proliferates under favorable conditions (Csurhes 2008; Hill 2006). This plant has already been naturalized in some parts of Europe and Australia (Csurhes 2008; Gussev et al. 2020; Mitrushev et al. 2022; Molina and Blanco 2024). In Japan, *H. reniformis* was first discovered in Shizuoka City in 1996 and was later found in 73 different sites, such as the pesticide-free paddy fields and waterways of Kawajima, Saitama, in 2013 and 2014 surveys (Mineta 2015). Since 2020, we have been observing increasing *H. reniformis* infestations during our visits to Kawajima, especially in herbicide-free paddy fields, ditches, and ponds. In our conversation with organic paddy farmers, we found them struggling with manual weeding owing to the vigorous proliferation of *H. reniformis*. Morphologically, *H. longirachilla* is often confused with *H. reniformis* because of its reniform leaves and a yellow base in the upper perianth lobe. However, distinctions between them become evident during the flowering stage, such as a shorter spathe and 6–14 flowers in *H. longirachilla* compared with 2–8 flowers with a longer spathe in *H. reniformis* (Sousa et al.

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2018a, b). Similar traits of *H. reniformis* to *H. limosa* and *H. multiflora* add to potential confusion in identification (Csurhes 2008; Pellegrini and Horn 2017). Specific characteristics of this plant are discernible primarily in the mature stage, particularly in flowers, making the flowering stage crucial for species-level identification.

DNA barcoding has been an effective method of identification, early detection, monitoring, genetic evaluation, and management of invasive plants (Cross et al. 2011; Pecnikar and Buzan 2013; Van De Wiel et al. 2009). Species identification is possible irrespective of the life stage by utilizing short DNA sequences, enabling comparisons among and within species (Kress et al. 2005; Antil et al. 2023). However, much molecular information on *H. reniformis* is not available. In the case of plants, the chloroplast genome has been widely used as they have the highest universality and can provide information about the evolution, adaptation, and population genetics of plants (Robbins and Kelly 2023; Gao et al. 2019; Thode and Lohmann 2019). In particular, chloroplast genome regions of *maturase K (matK)* and *ribulose-1,5-bisphosphate carboxylase large (rbcL)* are well known as valuable markers for DNA barcoding owing to their high-quality sequencing capacity for preliminary analysis (CBOL Plant Working Group et al. 2009; Ho et al. 2021; Ismail et al. 2020).

Therefore, this study aims to identify based on the morphological characteristics and perform DNA barcoding of *H. reniformis* using chloroplast DNA markers (*matK* and *rbcL*) to compare it with closely related species.

Materials and methods

Sample collection and species identification

Heteranthera reniformis plants were collected in May 2021 from herbicide-free paddy field, where *H. reniformis* grows abundantly in Kawajima, Saitama (35° 59′ 19″ N, 139° 29′ 21″ E). The morphological characteristics of the obtained plant samples were identified using the morphological information from past studies (Hill 2006; Horn 2020; Sousa et al. 2018a, b; Pellegrini and Horn 2017). They were cultivated in a glasshouse at Saitama University, and the morphological characteristics of their flowers and seeds were confirmed to be *H. reniformis* (Csurhes 2008; Hill 2006; Horn 2020; Sousa et al. 2018a, b; Pellegrini and Horn 2017).

DNA extraction, polymerase chain reaction (PCR) amplification, and DNA sequencing

After harvest, the leaves and seeds of *Heteranthera reniformis* were ground finely using a sterilized mortar and pestle. Total genomic DNA was extracted from a 100 mg of sample using a DNA™ SPIN kit for soil (MP Biomedical) following the manufacturer's instructions.

DNA concentration was determined using a WPA Bio wave DNA Life Science UV/Vis spectrophotometer (Biochrom Cambridge, UK). Total genomic DNA was employed in PCR to amplify the chloroplast DNA markers *matK* and *rbcL* using KOD FX Neo (TOYOBO Co., Ltd., Osaka, Japan). The gene-specific primer sequences and reaction conditions used in this study are listed in Table 1. The PCR mixture contained 10 µl of 2 × PCR buffer, 0.4 µl each of forward and reverse primers (0.2 µM), 4 µl of Deoxynucleotide triphosphates, 0.4 µl of KOD FX NEO, 100 ng of DNA template, and ddH_2O was added to a final volume of 20 µl.

PCR products were separated by electrophoresis on 2% agarose gel using a 100 bp DNA ladder as a size marker. Gel images were visualized with an Imager, and PCR products were purified using a Fast Gene Gel/PCR Extraction kit (Nippon Genetics, Japan) following the manufacturer's protocol.

Table 1 Gene-specific primers, sequences, and their reaction conditions (CBOL Plant Working Group et al. 2009)

Locus	Name of primer	Primer sequence 5–3′	Reaction condition
matK	1R_KIM f 3F_KIM r	ACCCAGTCCATC TGGAAATCT TGGTTC CGTACAGTACTT TTGTGTTTA CGAG	a. 94 °C–2 min (initial denaturation)—1 cycle b. [98 °C–10 s (denaturing), 55 °C–30 s (annealing), 68 °C–30 s (extension)]—30 cycles c. 68 °C–5 min (final extension)—1 cycle d. 15 °C (hold)
rbcL	*rbcL*a_F *rbcL*a_R	ATGTCACCACAA ACAGAGACT AAAGC GTAAAATCAAGT CCACCRCG	

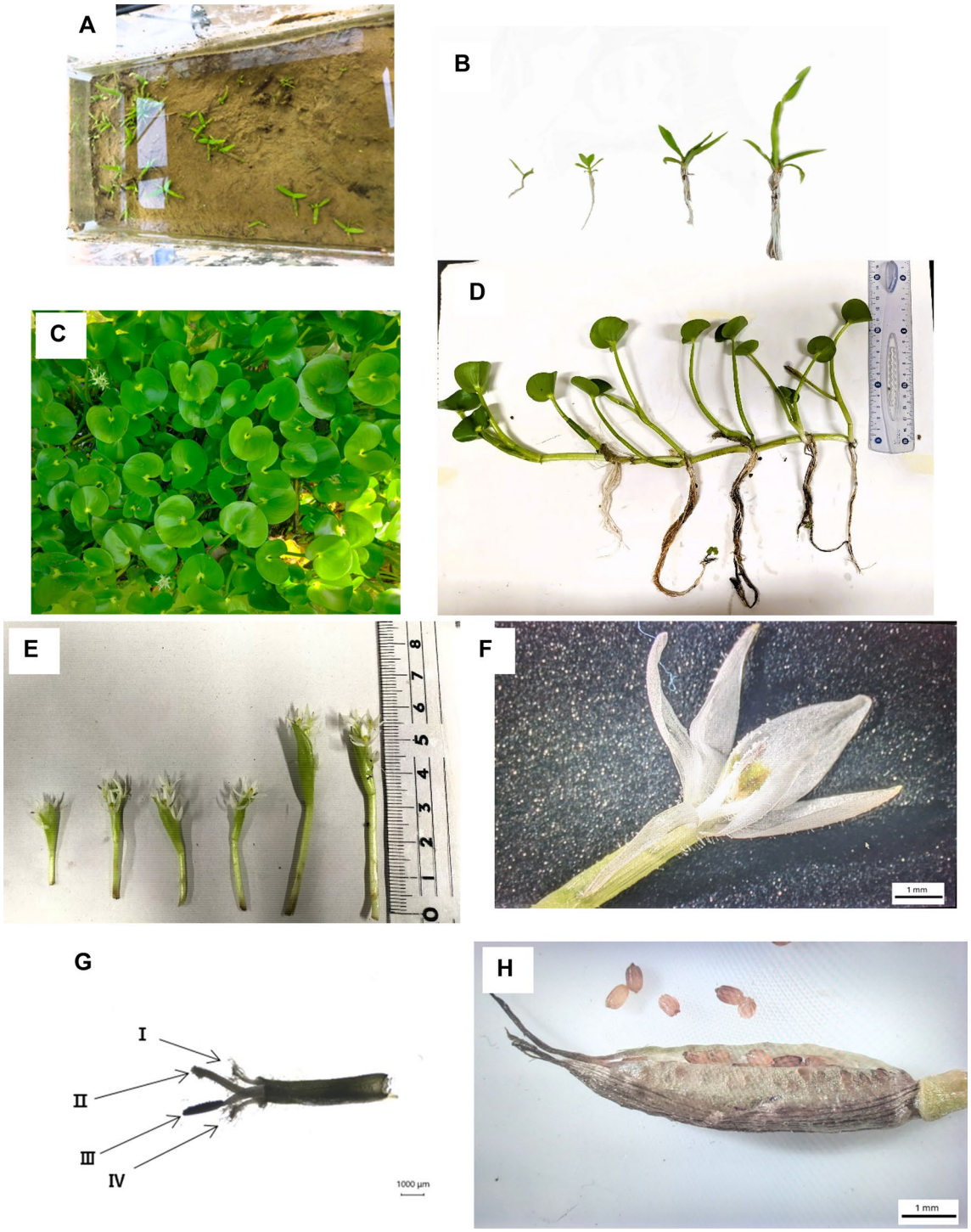


Fig. 1 *Heteranthera reniformis*: **A** and **B** seedlings; **C** and **D** habit; **E** inflorescences; **F** flower; **G** stamens (I and IV—lateral stamens, III—central stamen) and gynoecium(II); H capsule

Sequence analyses

The amplified PCR products of the above genes were sequenced by Eurofins Genomics (Eurofins Genomics GmbH; Eurofins Genomics Inc). The sequences were assembled and trimmed using CLC Main Workbench 21. The sequences of two DNA barcodes of *Heteranthera reniformis* were submitted to the GenBank nucleotide sequence database (i.e., DDBJ), and their accession numbers are listed as LC726247.2 (*matK*) and LC726246.1 (*rbcL*). The

Table 2 BLAST results of *matK* sequence of invasive weed *Heteranthera reniformis*

Species name (accession number)	Query coverage (%)	*E* value	Percent identity (%)
H. callifolia (NC063306.1)	100	0	99.52
Pontederia crassipes (NC046773.1)	100	0	97.48
P. africana (NC063314.1)	100	0	97.24
P. vaginalis (MN750360.1)	100	0	97.24
P. elata (MN750352.1)	100	0	97.24
P. paniculata (NC063305.1)	100	0	97.24
P. valida (NC063313.1)	100	0	97.12
P. hastata (NC063309.1)	100	0	97.12
P. cordata (MH616141.1)	100	0	97.00
P. cyanea (LC536960.1)	100	0	96.88
P. azurea (LC536957.1)	100	0	96.88
P. plantaginea (MN750358.1)	100	0	96.76
P. australasica (NC063307.1)	100	0	96.6
H. gardneri (KU127495.1)	96	0	97.64
H. dubia (MK520176.1)	91	0	98.69
P. korsakowii (KX526757.1)	94	0	96.68

Table 3 BLAST results of *rbcL* sequence of invasive weed *Heteranthera reniformis*

Species name (accession number)	Query coverage (%)	*E* value	Percent identity (%)
H. dubia (MG225632.1)	100	0	99.64
H. callifolia (NC063306.1)	99	0	99.64
Pontederia australasica (NC063307.1)	100	0	99.46
H. limosa (U41583.1)	99	0	99.64
H. oblongifolia (U41584.1)	99	0	99.45
P. cyanea (U41588.1)	99	0	99.45
H. zosterifolia (U41587.1)	99	0	99.27
H. seubertiana (U41586.1)	99	0	98.91
H. gardneri (U41582.1)	99	0	98.91
P. meyeri (U41577.1)	99	0	98.91
P. vaginalis (LC655239.1)	99	0	98.73
P. hastata (KF724351.1)	99	0	98.73
P. africana (NC063314.1)	99	0	98.73
P. valida (NC063313.1)	99	0	98.73
P. elata (MN750352.1)	99	0	98.73
P. paradoxa (U41579.1)	99	0	98.72
P. plantaginea (MN750358.1)	99	0	98.55
P. paniculata (NC063305.1)	100	0	98.37
P. korsakowii (MN750356.1)	99	0	98.18
P. cordata (NC044968.1)	100	0	98.01
P. azurea (LC536889.1)	99	0	98.18
H. rotundifolia (U41585.1)	99	0	97.63
P. heterosperma (U41576.1)	99	0	97.99
P. sagittate (L20128.1)	99	0	97.82
P. crassipes (NC046773.1)	99	0	97.64

sequences obtained from our samples were aligned in the NCBI database using the Basic Local Alignment Search Tool (BLAST) to compare them with those of related genera or species available in the NCBI database.

Results and discussion

Morphological trait description

Seedlings of *Heteranthera reniformis* have narrow linear leaves at the start of their growth and later broaden into kidney-shaped leaves (Fig. 1A and B). Leaves are sessile, alternate, and reniform with obtuse leaf blade apex (Fig. 1C). The leaf blade width is 10–40 mm, and the leaf blade length is 10–30 mm, with the width usually larger than the length. The petiole is 4–14 cm long and glabrous (without hair) with distal internodes (5–50 mm) and mucilaginous sheaths (Fig. 1D). Peduncles are 5–42 mm long and glabrous. *H. reniformis* has a raceme inflorescence with 2–8 glandular flowers that are 5–10 mm long with six white lobes and short spathes (mucronate) (Fig. 1E). The central broad lobe has a distinct yellowish or green region at the bottom, and the remaining lower lobes are 3.0–6.5 mm long and arranged at the apex of the flower (Fig. 1F). The flowers open about a few hours after sunrise and wither after that. There are three stamens of two types. One has a central non glabrous stamen of 2.0–4.7 mm length and a 0.6–1.8 mm long yellow or blue anther. The other type has two shorter white lateral non glabrous stamens with a length of 0.9–2.2 mm and 0.2–0.8 mm long yellow anthers (Fig. 1G). The gynoecium is white and non-glabrous (Fig. 1F and G). The capsule is elongated by 0.5–1 cm length. The seeds have lengths and widths of 0.5–0.9 mm and 0.3–0.5 mm, respectively, and have 8–14 longitudinal wings (Fig. 1H).

Sequence analyses

In our study, both *matK* (883 bp) and *rbcL* (552 bp) were amplified successfully from both leaves and seeds of *Heteranthera reniformis*. We successfully accumulated the DNA information of *H. reniformis*. The alignment of the sequences of *H. reniformis* and related species for comparison revealed a few nucleotide variations among species of the same genera (Tables 2 and 3). The *matK* and *rbcL* sequences retrieved from the GenBank accessions indicated query coverages of 91 to 100% and 99 to 100%, respectively. The obtained *matK* sequence showed 99% similarity of *H.*

Table 4 Some morphological characters of *Heteranthera reniformis* and closely related species

Characters	*H. reniformis* (this study)	*H. callifolia*[a, b]	*H. dubia*[c, d]	*H. limosa*[c]	*H. oblongifolia*[e]
Leaf blade apex	Obtuse to rounded	Ovate to lanceolate	Acute	Obtuse to slightly acute	Acute to obtuse
Leaf blade form	Reniform	Heart-shaped or rounded	Linear	Oblong to oval	Cordate
Number of flowers	2–8	5–20	1	1	1–2
Perianth lobe color	White	White, blue, mauve or violet	Yellow	Blue or white	Blue or white
Perianth lobe length (mm)	5–10	4–5	4–11	5.2–26.3	7
Lateral stamen and anther length (mm)	0.9–2.2 (stamen) 0.2–0. 8 (anther)	2 (stamen) 1.5–2 (anther)	2–10 (stamen) 0.9–3.9 (anther)	2.3–7.8 (stamen) 0.9–3.3 (anther)	2–3 (stamen)
Central stamen and anther length (mm)	2.0–4.7 (stamen) 0.6–1.8 (anther)			3.3–7.2 (stamen) 1.3–3.9 (anther)	3 (stamen)
Seeds (mm)	0.5–0.9	0.2–1	1–1.6	0.5–0.8	0.8

Superscript letters are the cited papers. a—Okezie and Agyakwa (1989); b—WFO (2024); c—Horn (1998); d—Sousa et al. (2016); e—Sousa et al. (2018a, b)

reniformis to *H. callifolia*. Furthermore, 97% similarity was found with *Pontederia crassipes, P. africana, P. vaginalis, P. elata, P. paniculate, P. valida, P. hastata and P. cordata* (Table 2). Similarly, the obtained *rbcL* sequence showed 99% sequence similarity of *H. reniformis* to *H. callifolia, H. dubia, P. australasica, H. limosa, H. oblongifolia, P. cyanea and H. zosterifolia* (Table 3). Although *H. callifolia* and *H. reniformis* have several distinct morphological characteristics, as shown in Table 4, *H. callifolia* showed the highest homology with *H. reniformis* (Tables 2 and 3). Additionally, from the available data, *H. callifolia* is closest to *H. reniformis* in the phylogenetic tree, and the *Heteranthera spp.* are clustered together (Supplementary material, Figs. S1 and S2). *H. reniformis* can be separated from *H. callifolia* by the leaves (reniform vs cordiform leaves), the leaf blade apex (obtuse to rounded vs ovate to lanceolate), and the number of flowers (2–8 vs 5–20). However, even though there are significant morphological variations in leaves or flowers when compared with the species of the same genera, they still have high sequence homology in *rbcL* (Tables 3 and 4). Dong et al. (2014) analyzed *rbcL* for the DNA barcoding of chlorophytes, charophytes, bryophytes, lycophytes, ferns, gymnosperms, and angiosperms. They found discrimination success rates of 5.19–96.33% for *rbcL*a, and 22.09–98.43% for *rbcL*b in species-rich families, and 0–88.73% for *rbcL*a and 2.04–100% for *rbcL*b in species-rich genera (Dong et al. 2014). Similarly, Shimada (2000) reported a sequence divergence of four base pairs (0.3% divergence) in *rbcL* between *Gelidium linoides* (Gelidiaceae) and *G. tenuifolium* (Gelidiaceae) despite their classification as distinct species. Likewise, variation levels among species in the same genera in a sequence of *matK* also differ from species to species. Some species, such as *Monoecious* and *Saraca* spp. have enough variation in the *matK* sequence to discriminate them (Ramesh et al. 2022; Sil et al. 2021). In our study, the genetic variations between *H. reniformis* and other related species were relatively low for both *matK* and *rbcL* sequences (Tables 2, 3, S1, and S2). Therefore, finding the best gene region in plants with nucleotides sufficiently varied to distinguish all species but sufficiently conserved to have a relatively slight variation across species is challenging.

The ITS region is also the better option for phylogenetic classification with a higher resolution capacity as the variation of nucleotides can be found (Devi et al. 2022; Jamdade et al. 2022; Mursyidin and Setiawan 2023). However, some results showed that the use of ITS region as a marker can be difficult owing to incomplete concerted evolution and incomplete lineage sorting (Vázquez 2019; Wang et al. 2023). Therefore, many studies show successful species identification and discrimination results with the barcode combination analysis of different genes (Cahyaningsih et al. 2022; Prasetya et al. 2022; Nguyen et al. 2022). The ongoing infra familial classification adjustments in Pontederiaceae have resulted in the consolidation of genera, with former *Eichhornia*, *Monochoria*, and *Reussia* now classified as *Pontederia*, whereas *Eurystemon*, *Hydrothrix*, *Schlleropsis*, and *Zosterella* have been reclassified as *Heteranthera* (Pellegrini 2017; Pellegrini et al. 2018; Pellegrini and Guarnier 2022). These changes reflect efforts to refine and streamline taxonomic groupings within the family. Furthermore, additional sequencing of different loci (e.g., the ITS region) and analysis by combining their results with current results can provide more comprehensive data on this plant for future species identification and phylogenetic research.

Moreover, early detection of alien species such as *H. reniformis* is essential for preventing their spread and mitigating ecosystem damage. Environmental DNA (eDNA)

technology is an effective tool for detecting alien species in the early stages of their spread (Pecnikar and Buzan 2013; Miyazono et al. 2023; Sahu et al. 2023). Our research findings can contribute to providing data for future eDNA studies focused on the early detection of *H. reniformis*.

Conclusion

DNA barcoding is a valuable tool for taxonomists for rapid and accurate species identification. The DNA barcoding of *Heteranthera reniformis* with *matK* and *rbcL* markers was successful. The sequences obtained from DNA barcoding possibly contribute to improving nucleotide data in databases, enabling rapid identification and recognition in the introduced regions even if morphological information is poor. Furthermore, more extensive molecular analysis is needed for a robust taxonomy system and precisely resolved phylogenetic research.

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Author contributions TF and STM conceptualized and designed the experiment. Experiments and data analyses were performed by STM under the supervision of TF and RS. ST and RS assisted in the DNA analysis. STM wrote the first draft of the manuscript. All authors edited and provided feedback on the manuscript.

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Declarations

Conflict of interest The authors declare that they have no financial or non-financial conflicts of interests to disclose.

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