

## HYDROCHARIS DUBIA (HYDROCHARITACEAE), A NEW RECORD FOR THE FLORA OF IRAN, CONFIRMED BY nrDNA ITS SEQUENCE DATA

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Received 2020. 12. 24; accepted for publication 2021. 02. 12

Abdi, M. & Afsharzadeh, S. 2021. 12. 30: A new record of *Hydrocharis dubia* (Hydrocharitaceae) from Iran, confirmed by nrDNA ITS sequence data. -*Iran. J. Bot.* 27 (2): 191-197. Tehran.

A population of *Hydrocharis dubia* of Hydrocharitaceae was discovered as a new record from the Caspian Sea coast in Mazandaran province. Phylogenetic analyses of both maximum parsimony and the maximum likelihood of DNA sequence data from the internal transcribed spacer region evidently confirmed the occurrence of *H. dubia* in Iran. We speculate that the verification of *H. dubia* in Iran out of its up to now reported range represents a non-indigenous introduction presumably caused by migratory waterfowl visiting the province while facilitating long seed dispersal. Regular monitoring is recommended to be applied in order to follow up its establishment and adaptation in the area, to control it as a potential noxious weed.

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**Keywords:** phylogenetic analysis; invasive; seed dispersal; *Hydrocharis*; Iran

گزارش *Hydrocharis dubia* (Blume) Backer (Hydrocharitaceae) به عنوان رکورد گونه غیر بومی جدید، تأیید شده توسط داده‌های توالی nrDNA ITS برای فلور ایران

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جمعیتی از گونه‌ی *Hydrocharis dubia* متعلق به خانواده‌ی Hydrocharitaceae به عنوان رکورد جدیدی در سواحل دریای خزر در استان مازندران مشاهده شد. تحلیل‌های تبارشناختی با هر دو روش پارسیمونی بیشینه و شانس بیشینه داده‌های توالی DNA از نشانگر ITS هسته‌ای آشکارا حضور گونه‌ی *H. dubia* را در ایران با حمایت قوی تأیید نمود. *H. dubia* در ایران خارج از محدوده‌ی پراکنش کنونی خود گونه‌ای غیربومی است. تفسیر ما بر این است که پرندگان مهاجر آبی که به استان مازندران مهاجرت می‌کنند، به احتمال بسیار سبب پراکنش دانه در مسافت‌های طولانی و ورود و استقرار این گونه در ایران شده‌اند. ردیابی منظم این گونه به دلیل دارا بودن پتانسیل تهاجمی در خارج از قلمرو طبیعی خود به منظور کنترل آن پیشنهاد می‌شود.

### INTRODUCTION

Descriptions of the ranges occupied by particular species at a certain point in time are only static impressions of plant geography. Due to dispersal, colonization, and climatic change, the adventive spread and naturalization of certain hydrophytes over and

above their defined natural ranges can lead to peculiar distributions (Sculthorpe 1967).

Management concerns will arise upon encountering a new alien aquatic in any area. The major challenge in this regard is whether the newcomer should be welcome, helped, and protected to successfully extends

its range, or it should be instantly controlled or eradicated to avoid its invasive potential which can easily hamper the native indigenous flora.

*Hydrocharis* L. commonly named as frog-bit are perennial predominantly floating-leaved, freshwater aquatics which are included in the monocotyledonous plant family Hydrocharitaceae. The genus comprises three species worldwide (Cook & Lüönd 1982); *H. chevalieri* (De Wild.) Dandy, *H. morsus-ranae* L. and *H. dubia* (Blume) Backer, which are quite similar morphologically. All three species have unisexual flowers and are primarily insect pollinated. Along with seed production, vegetative reproduction by means of stolon, tubers and turions are also prevalent.

*Hydrocharis chevalieri* is confined to central Africa and currently has no record of inventory either as a weed or natural expansion of its range outside this area.

*Hydrocharis morsus-ranae* is widely distributed in the west and central Europe with scattered distribution towards eastern Europe, extending to adjacent parts of Asia where it is sparingly represented in Turkey, the Caucasus (Catling & al. 2003), and the northwestern Iranian shore of the Caspian Sea (Assadi & Wendelbo 1977). Isolated collections have also been reported from central Siberia and Kazakhstan (Cook & Lüönd 1982). The species has been introduced to North America and Canada where it exists as an invasive hard-to-manage exotic weed. The reports of *H. morsus-ranae* from Australia (Bentham 1873) and Japan (Holm & al. 1979) are apparent misidentifications (Catling & al. 2003) with its congener *H. dubia*, and can largely be due to their quite similar appearances.

*Hydrocharis dubia* is prevalent in southern and eastern Asia including Japan, China, Bangladesh, Nepal, Myanmar, Vietnam, Java, and New Guinea. It extends its natural range from Kashmir throughout India, along the Himalayas and is sporadically dispersed in Russia (Cook & Lüönd, 1982). Plausible occurrences of the *H. dubia* has also been reported from north-east Pakistan (Ghafoor 2019, as cited in Efremov & al. 2020). While not considered as exotic, *H. dubia* is recorded as noxious weed in Japan (Oki 1991) and is evidently introduced and neutralized in Australia (Bean 2011). Although the highly similar congeners (i.e. *H. morsus-ranae* and *H. dubia*) have been reported to be clearly allopatric (Cook & Lüönd 1982), a recent floristic inventory in the Kashmir Himalaya revealed both species to grow sympatrically in two wetlands (Ganie & al. 2016). In Iran the sole representative of the genus has long been considered to be *H. morsus-ranae* which has been reported from a few localities in northern provinces of Gilan and Mazandaran (Dinarvand, 2017). No record from any of the other two species of the genus has been reported from the country to date.

Effective management of aquatic habitats

principally relies on the precise application of taxonomic identifications to both native and potentially non-native invasive species (Moody & al. 2008). Nevertheless, convergent evolution and substantial reduction in vegetative and morphological characters throughout the course of evolution have led many aquatic species to gain relatively similar traits which in turn renders them prone to misidentification using traditional taxonomic methods. Conversely, the application of molecular data and phylogenetic analyses have proved to be effective for early detection, correct identification, and clarification of species limits for aquatic plants which exhibit little or no detectable morphological variation (Moody & al. 2008).

During our field collecting trips targeting the distribution of Hydrocharitaceae in Iran, we discovered a very small population of small floating-leaf individuals in a pond between Amol and Fereydunkenar, in Mazandaran province. In order to correctly determine the identity of the discovered plants, we performed a molecular phylogenetic analysis using nrDNA ITS sequence data. The verification of species identification was essential for determining the status of the genus in Iran and to propose recommendations for future monitoring and management.

## MATERIALS AND METHODS

### Sampling and locations

*Hydrocharis* accessions were collected during fieldwork from two localities within the distribution range of the genus in Iran, including Amir kalayeh wetland in Lahijan, Gilan Province, and a pond approximately 220 km farther between Dabudasht of Amol county and Mrzango wetland 11 km southwestern of the Fereydunkenar wetland in Mazandaran province (fig. 1). All collected specimens were dried on herbarium sheets using relevant procedures suggested to treat aquatic plants (fig. 2). Additionally, a corresponding piece of plant material from each accession was designated for subsequent DNA analysis. All voucher specimens are deposited in the University of Isfahan herbarium. Collected specimens were also georeferenced either manually or in the field using GPS unit. The localities, geographical coordinates, and voucher herbarium numbers of the examined specimens are provided in table 1.

### DNA isolation, PCR amplification, sequencing and alignment

Total genomic DNA was extracted from herbarium specimens using a slightly modified CTAB method (Doyle & Doyle 1987). The polymerase chain reaction for amplification and sequencing of the nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) involved using ITS5 and ITS4 primers (Baldwin 1992).

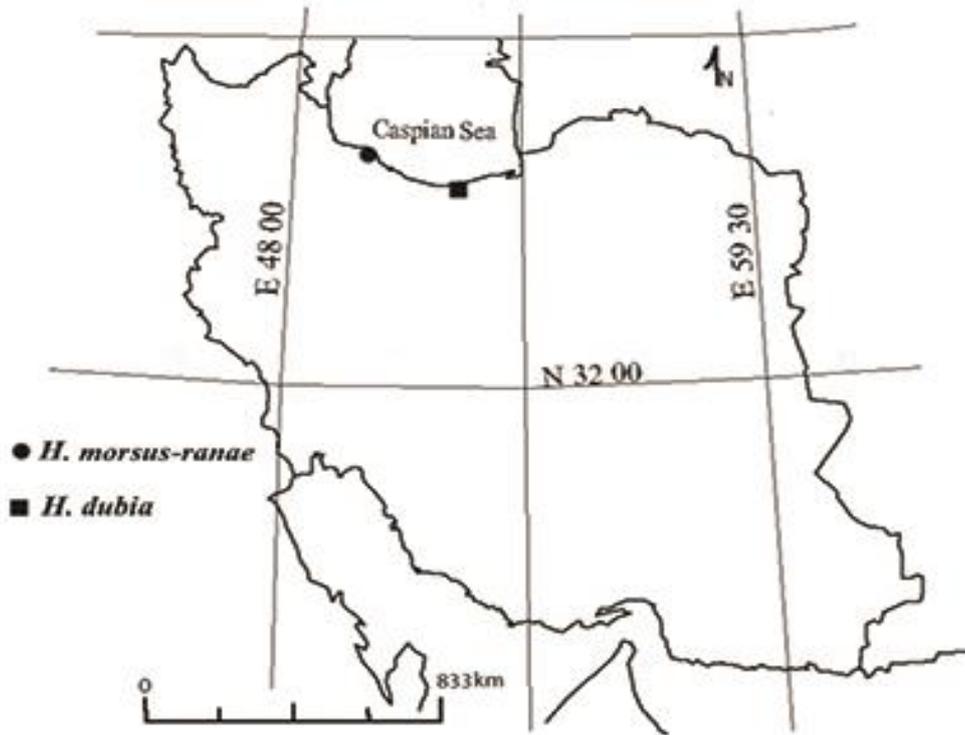


Fig. 1. Map of Iran showing *Hydrocharis* species collections documented by molecular analyses.

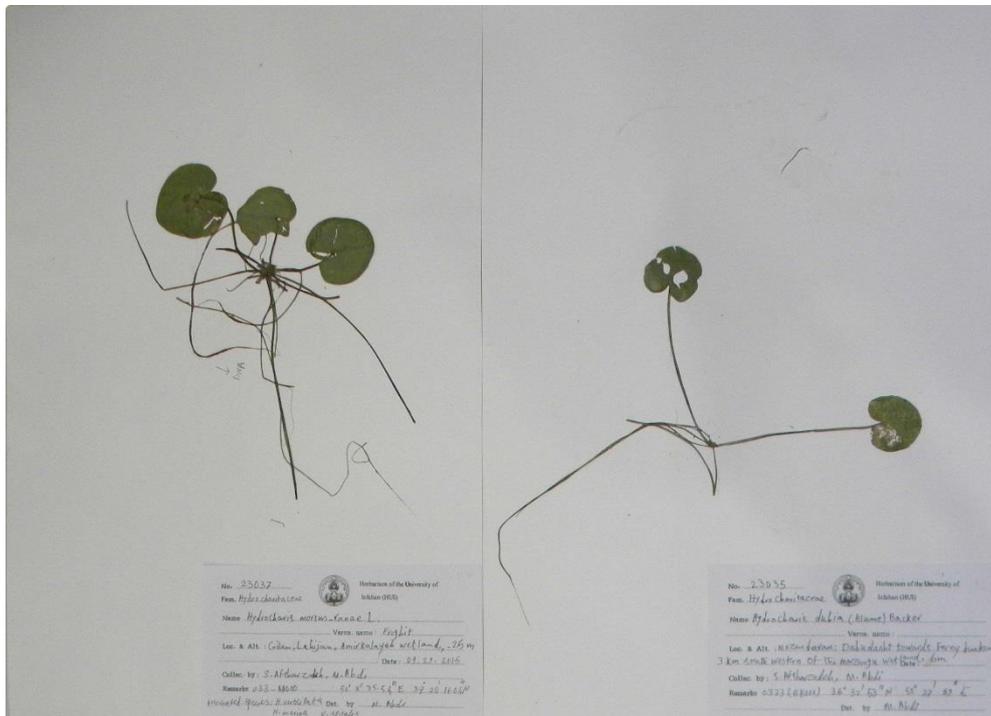


Fig. 2. Photographs of two herbarium specimens identified as *Hydrocharis morsus-ranae* (left) and *Hydrocharis dubia* (right).

Table 1. Localities, geographical coordinates and voucher herbarium numbers of the specimens for which DNA data were newly generated in this study.

Specimen	Locality	Voucher number	Herbarium identification no.	GenBank accession no.	Geographical coordinates
<i>H. dubia</i>	Mazandaran: Dabudasht towards Fereydunkenar, 3 km south western of the Marzangu wetland, 6m.	0333(BB000)	23035	MW498412	36° 32' 53" N 52° 27' 09" E
<i>H. dubia</i>	Mazandaran: Dabudasht towards Fereydunkenar, 3 km south western of the Marzangu wetland, 6m.	0833(E6001)	23034	MW517573	36° 32' 53" N 52° 27' 09" E
<i>H. morsus-ranae</i>	Gilan, Lahijan, Amir kalayeh wetland, -25m.	033(AA000)	23032	MW498978	50° 11' 35.54" E 37° 20' 16.04" N
<i>H. morsus-ranae</i>	Gilan, Lahijan, Amir kalayeh wetland, -25m.	093(B41A85F)	23031	MW503696	50° 11' 41.13" E 37° 19' 42.42" N

PCR amplifications and thermal cycling conditions for amplification of ITS region was done according to usual methods. PCR products were purified with ExoSAP-IT (Bell, 2008) and were sent for sequencing (Macrogen Inc, USA).

Eight newly generated forward and reverse ITS sequences were primarily edited and assembled in to four contigs using program Codon code Aligner V.1.2.1 (CodonCode Corporation, Dedham, Massachusetts), and exported in to Geneious (Kearse & al. 2012) where extracted as four consensus sequences (two for *H. dubia* and two for *H. morsus-ranae*). Additionally, we retrieved five previously published sequences of *H. morsus-ranae*, *H. dubia* and *Limnobia spongia* (Bosc) Steud. from GenBank (AY335963, MG215991, AY335962, HQ687166 and JF975450). *Limnobia spongia* (Bosc) Steud. was selected as the outgroup following several previous phylogenetic analyses of Hydrocharitaceae which consistently resolved *Limnobia* and *Hydrocharis* as sister taxa (Les & al. 2006; Ross & al. 2016; Chen & al. 2012; Les & Tippery 2013). A total of ten sequences were aligned using Geneious alignment algorithm with default parameters. The new alignments were exported as nexus files to be further analyze with the program PAUP. Novel DNA sequences (generated in this study) for *H. morsus-ranae* and *H. dubia* have been deposited in GenBank (Accession numbers are provided in table 1).

#### Phylogenetic analysis

Phylogenetic analyses were performed using both equally weighted maximum parsimony and maximum likelihood methods as optimality criterions. Exhaustive maximum parsimony (MP) searches were performed using the program PAUP v4.0b 4a with default settings, treating all characters as unordered and gaps either as a 5<sup>th</sup> character state or as missing data. Strict consensus

tree was used to summarize the agreement between multiple equally parsimonious solutions. Nodal support was obtained using non-parametric bootstrap analysis obtained from 1,000 replicates. Maximum likelihood bootstrap analysis was performed with 1000 replicates using PhyML (Guindon & al. 2010) as implemented in the program Geneious with the GTR model of nucleotide substitution. Tree searching were set to be obtained with subtree pruning and regrafting (SPR). Both MP and ML trees were rooted using *Limnobia* as the outgroup. Scrutiny of the specimens identified as *H. morsus-ranae* and *H. dubia* indicated little reliable variation among the two congeners to be used as diagnostic non-overlapping features. However, an identification key, based on more consistent verifying features of the examined specimens, is provided for comparison between *H. dubia* and *H. morsus-ranae* in Iran

#### RESULTS

We sequenced 672 and 682 nucleotides of the internal transcribed spacer region of *H. dubia* and *H. morsus-ranae* respectively. Phylogenetic analyses of 683 bp of nrDNA ITS sequences yielded congruent branching patterns across maximum likelihood and maximum parsimony tree topologies. Sequences obtained for plant material collected in Amir kalayeh wetland resolved as a strongly supported clade with *H. morsus-ranae* accessions retrieved from GenBank. The Iranian sequences had only one indel difference of an insertion of a nucleotide "A" in position 79 of 298 bp of the internal transcribed spacer I region. Whereas, the two accessions associated with the material collected from the pond between Amol and Fereydunkenar formed a clade with *H. dubia* accessions with strong support (fig. 3).

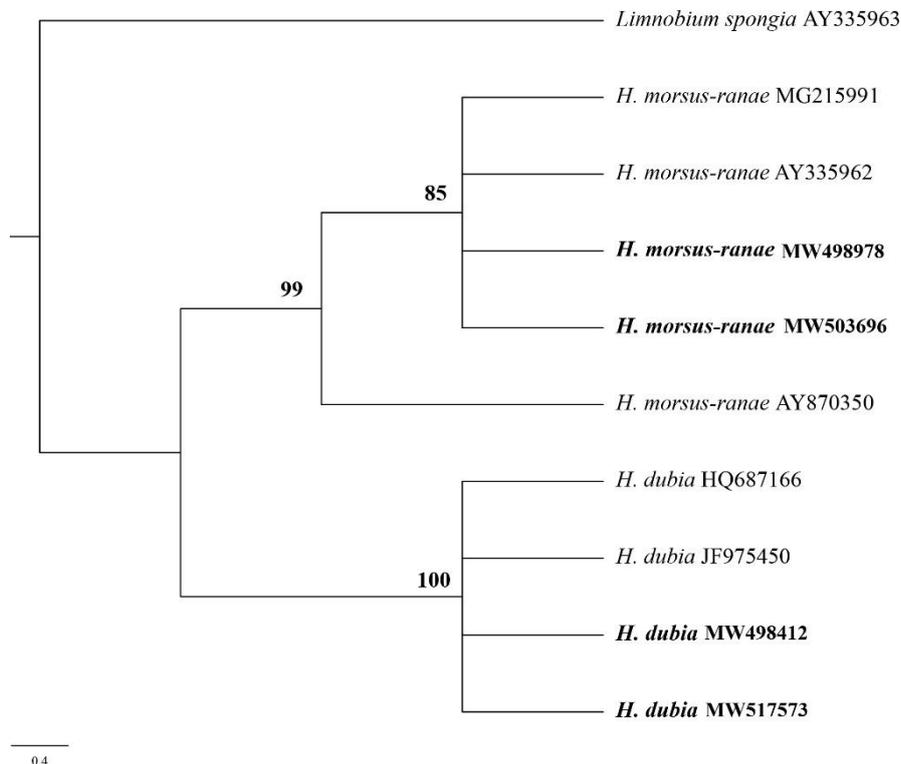


Fig. 3. Strict consensus tree of three equally most parsimonious solutions (TL = 60, CI = 1, RI = 1) resulted from internal transcribed spacer sequences of *Hydrocharis* species from Iran along with associated accessions. *Hydrocharis* species collected from Iran are differentiated in boldface text. Values above each branch are bootstrap support values obtained from 1000 replicates.

Key to the species of *Hydrocharis* in Iran

- 1. Leaves usually with two lateral stipules, leaf blades mostly horizontal and floating, with the surface usually uniform and rarely showing honeycomb-like pad, with primary veins usually diverging from the midrib at an angle of 75-90; roots usually hanging free in the water ..... ***H. morsus-ranae***
- Leaves usually with one median stipule, leaf blades usually erect and aerial, with the surface usually forming a spongy honey-come pad, with primary veins usually diverging from the midrib at an angle of 50-85; roots usually bottoming in sediments..... ***H. dubia***

**DISCUSSION**

ITS sequences data clearly confirmed the occurrence of *Hydrocharis dubia* in the Iranian province of Mazandaran 220 km apart from its congener *Hydrocharis morsus-ranae* which its occurrence was documented with our ITS sequence data in Amir kalayeh wetland of the Gilan province. Using nrDNA (ITS) sequence data for the purpose of identification, proved useful for successful documentation of such aquatic genera which exhibit little or no detectable variation among species.

The plausible source of the relatively unusual manifestation of *H. dubia* in Iran out of its up to now reported range is not ascertainable with confidence. However, two possibilities exist: It is plausible that *H. dubia* which is among aquarium and ornamental plants and is cultivated worldwide for the purpose of being used in ponds and water gardens, has somehow managed to escape from cultivation and has subsequently established and reproduced by means of vegetative propagules. Alternatively, seeds and seedlings of *H. dubia* can be dispersed throughout long distances by waterfowl (Les 2020) and the seeds, fragments and turions can locally disperse the plant by means of swimming, birds, fishing equipment and human activities such as boating (Les 2020). Given the presence of *H. dubia* in Russia and considering the proximity of the pond where we observed *H. dubia* to the wetlands of the province which host migratory waterfowl from Russia, (i.e. Fereydunkenar wetland, Marzango wetland, Miankaleh wetland, etc.), it is also possible that such migratory waterfowl have introduced the seeds by their regular seasonal visits where they have established or dispersed locally through water.

At this point we are not certain when the

introduction occurred in the past. However, the observed population of *H. dubia* comprised of relatively small individuals which appeared to be initially in seedling stage. Although we examined the neighboring areas near the pond where the population was first observed, we were not able to locate further populations. However, the possibility still exists that other populations of it here or elsewhere at the vicinity of the observed population have gone unnoticed. *Hydrocharis morsus-ranae* has formerly been reported from 2 km east of Mahmudabad from north of Amol (Uotila 1984) which is quite close to the area where we collected *H. dubia*. We did neither verify that report in our survey nor find populations of *H. morsus-ranae* in the designated location despite extensive field investigation. If the identification of that material as *H. morsus-ranae* had been correct, the species is either extinct in the region or had gone unobserved in our survey. The latter scenario violates the assumption that the two congener do not overlap geographically (Cook & Lüönd 1982). Co-occurrence of both species is additionally reported in Miragund and Haigam wetlands in Kashmir, India (Ganie & al. 2016).

Compared to the congener i.e. *H. morsus-ranae*, *H. dubia* appears to be less noxious out of its natural range. Nevertheless, the potential invasive nature of the species and the climatic conditions of the area (Mazandaran province) which is appropriate for its invasion, also the fact that the plant can easily be amplified and dispersed by means of vegetative propagules post introduction, leaves open the possibility that it has the potential to become established, or even to become a more frequent invasive element of the northern Iran flora. Therefore, careful monitoring is recommended to be applied in order to control it as a potential noxious weed.

## ACKNOWLEDGEMENTS

The authors thank the University of Isfahan for supporting the project. We also acknowledge the guide and the lab facilities provided by professor D.H. Les at the University of Connecticut for conducting molecular work.

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