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گونهٔ *Vallisneria denseserrulata* Makino از خانوادهٔ Hydrocharitaceae در ایران: رکورد جدید تأییدشده توسط داده‌های nrITS و matK/trnK5´intron

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چکیده

دو نمونه از گونهٔ *Vallisneria denseserrulata* از بخش جنوب شرقی رودخانهٔ سیمره واقع در استان ایلام ایران در جایگاه رکوردی جدید برای فلور ایران به ثبت رسید. تحلیل‌های تبارشناختی با روش شانس بیشینهٔ داده‌های توالی DNA از نشانگر ITS هسته‌ای (nrITS) و مناطق ژنی *mat*K/*trn*K از DNA کلروپلاستی نشان داد این نمونه‌ها به گونهٔ *V. spiralis* که از دیرباز تنها نمایندهٔ جنس *Vallisneria* در ایران قلمداد می شده است ، تعلق ندارند؛ اگرچه ظاهر بسیار مشابه دو تاکسون خواهر ممکن است باعث شناسایی اشتباه این گونه در گذشته شده باشد، در نظر قراردادن این واقعیت که گونه‌های جنس *Vallisneria* در سراسر جهان به‌طور وسیع کشت و تجارت می‌شوند و نیز قرارگرفتن آن درون کلاد شامل نمونه‌های کشت‌شده با هویت نامشخص، فرضیهٔ فرار این گونه از محیط کشت و استقرار آن در طبیعت منطقه را نیز مطرح و محتمل نشان می‌دهد.

واژه‌های کلیدی: Hydrocharitaceae، شناسایی، گزارش جدید، تحلیل تبارشناسی، رودخانهٔ سیمره، *V. spiralis*، *V. denseserrulata*.

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Vallisneria Denseserrulata Makino (Hydrocharitaceae) in Iran: A New Record Confirmed by nrITS and *mat*K/*trn*K 5' Intron Data

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Abstract

Two specimens of *Vallisneria denseserrulata* from the southeastern part of the Seimareh River in the Iranian province of Ilam were documented as new records for the flora of Iran. The maximum likelihood phylogenetic analyses of the specimens using DNA Sequence data from nuclear internal transcribed spacer (nrITS) and the plastid *mat*K and *trn*K 5' intron gene regions revealed that the specimens are not conspecific with *V. spiralis* that has long been regarded to be the sole representative of the genus in Iran. Although their quite similar appearances do not preclude the possibility of the two siblings as being misidentified with each other, the fact that *Vallisneria* species are highly cultivated and traded commercially around the world, as well as its resolution within the clade containing the cultivated material of unknown provenance, left open the possibility of it being an escapee from cultivation which has established and naturalized in the region.

Keywords: Hydrocharitaceae, Identification, New Record, Phylogenetic Analysis, Seimareh River, *V. Denseserrulata*, *V. Spiralis*.

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Introduction

Vallisneria L. (Hydrocharitaceae) species which are commonly named tape grass or water celery (Les, 2020) are obligately submersed, freshwater aquatics which are included in the monocotyledonous plant family Hydrocharitaceae. The genus is usually perennial and dioecious with clustered male and solitary pistillate flowers (Les, 2020). However, annuals, as well as infrequent monecious habits bearing umbellate inflorescences are also observed but rarely (Lowden, 1982). All *Vallisneria* species are categorized with a peculiar mechanism of water pollination among angiosperms considered as type III-B (Cook, 1982) that involves the complete detachment of male flowers, followed by their swimming and subsequent physical contact with pistillate flowers where they deposit their pollen through a specialized process no stage of which involves water (Cook, 1982; Sculthorpe, 1967). *Vallisneria* shares this peculiar phenomenon with three of its Hydrocharit relatives including *Appertiella* C. D. K. Cook & Triest, *Enhalus* Rich., and *Lagarosiphon* Harv. (Les, 2020).

Vallisneria has cosmopolitan distribution with the highest concentration of species reported to occur in Australia (Jacobs & Frank, 1997). The genus approximately contains 12 to 15 species that are quite similar and highly challenging for taxonomic study (Les et al., 2008). Except for a few features such as vittate vs. rosulate habit, there are nearly no consistent vegetative or morphological characters to effectively serve as independent taxonomical markers capable of delimiting species boundaries within the genus (Les et al., 2008). Many of the distinct vegetative characters (e.g. leaf width as well as the shape of leaf margin either as toothed or entire), have been postulated to be entirely discarded due to their indeterminate nature in terms of taxonomic validity (Lowden, 1982). Likewise, many floral characters such as the color of the flower, the length of sepal, coiling of the female scapes, the curvature of the fruits, the width of male scapes, and the number of fertile stamens have been evaluated as insufficient and irrelevant to taxonomic inference (Lowden, 1982). Furthermore, floral characters that have often been used for distinguishing species in *Vallisneria* are often lost or highly reduced (Rendle, 1916) which leads to the misidentification of taxa in the genus. Moreover, the comprehensive reevaluation of the morphological features of *Vallisneria*, performed by Les et al. (2008) suggested that the relative degree of stigma and staminode fusion, stigmatic incision, and filament fusion which were emphasized taxonomically by Lowden were structurally correlated and did not manifest independent characters. Conversely, molecular data such as cpDNA and nrITS sequences have been rationally proved to perform well in delimiting species in *Vallisneria* (Les et al., 2008).

In Iran, the genus reportedly has been represented by *V. spiralis* (Dandy, 1971; Dinarvand, 2017), the original Linnaean name which for many years was unsystematically applied to the similar rosulate plants that inhabited all four continents (Les et al., 2008). In order to confidently identify *Vallisneria* species in Iran, the authors of the present study performed a molecular phylogenetic analysis of the collected material from different localities all over the country (Table

1). The verification of species identifications for these plants was essential to correctly authenticate the taxonomic status of the genus in Iran. This study can further elucidate the complicated status of its closest sister species that is *V. spiralis* in Iran.

Material and Methods

Sampling and Locations

Vallisneria accessions were collected during fieldwork between 2015-2017, targeting the Iranian distributional range of the genus. 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 at the herbarium of the University of Isfahan (Table 1). The collected specimens were also georeferenced either manually or in the field using a GPS unit. Georeferenced accessions as *V. denseserrulata* were collected from the Seimareh River approximately 4 km located in the northwest of the village of Ghaleh Tasme of the central district of the Darreh Shahr county of Ilam province. The geographical coordinates are provided in Fig 1.

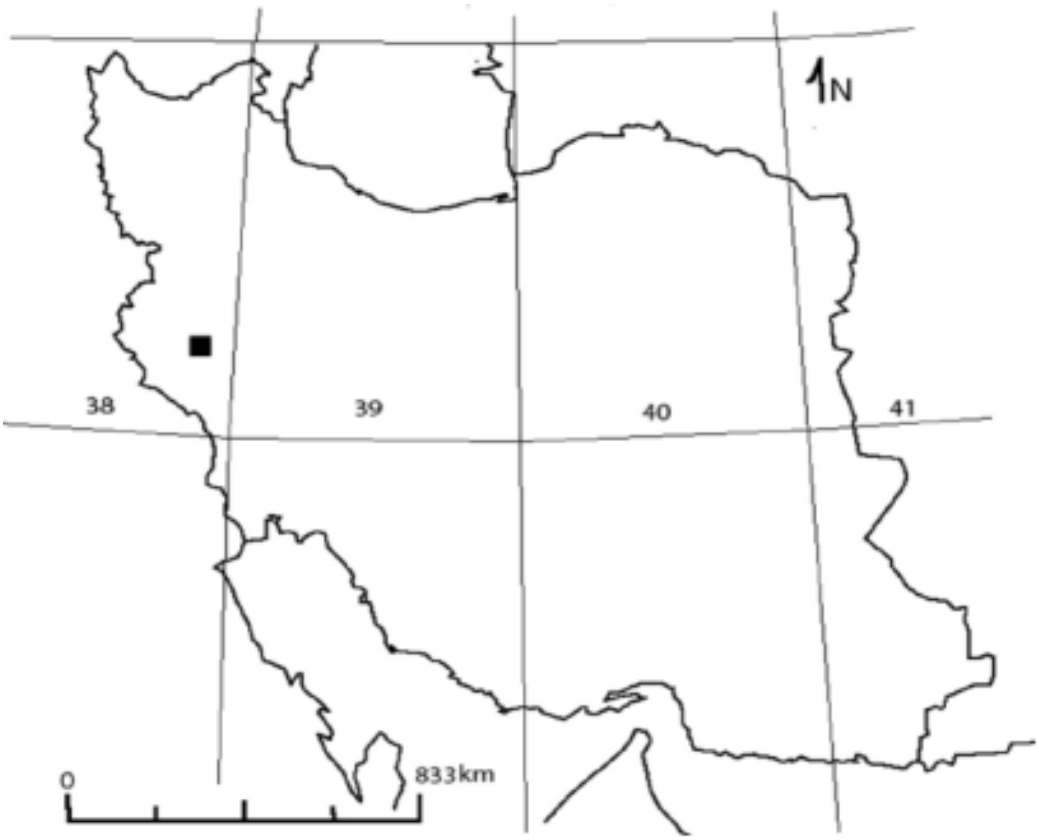

Figure 1. Map of Iran showing V. denseserrulata collection site documented by molecular analyses. The accessions are georeferenced as having 33° 13' 33" N and 47° 08' 03" E coordinates.

Table 1. Localities, Geographical Coordinates, Voucher, and Herbarium Numbers of the Specimens (for which DNA data were newly generated in this study)

Specimen	Locality	Voucher no.	Herbarium identification no.	Geographical coordinates
V. denseserrulata	Ilam: Darreh Shahr, 4 km northwest of the village of Ghaleh Tasme	05K	23021	47° 08' 03" E 33° 13' 33" N
V. denseserrulata	Ilam: Darreh Shahr, 4 km northwest of the village of Ghaleh Tasme	03B	23022	47° 08' 03" E 33° 13' 33" N
V. spiralis	Khuzestan, Soush, Shavur river	063C4	23023	48° 14' 42.40" E 32°11' 40.16" N
V. spiralis	Lorestan, Seymareh river	071B5	23024	47° 3' 22.82" E 33° 40' 56.69" N
V. spiralis	Lorestan, Seymareh river	71-1C5	23025	47° 3' 22.82" E 33° 40' 56.69" N
V. spiralis	Gilan, Badar Anzali, Anzali wetland	066A4	23026	49° 18' 52.68" E 37° 30' 8.84" N
V. spiralis	Lorestan, Seymareh river	071-2A5	23027	47° 3' 22.82" E 33° 40' 56.69" N
V. spiralis	Khuzestan: Dezful, Dez river	070E5	23028	48° 20' 5.22" E 32° 14' 25.48" N
V. spiralis	Gilan, Badar Anzali, Anzali wetland	067F1	23029	49° 18' 44.01" E 37° 29' 4.71" N
V. spiralis	Gilan, Badar Anzali, Anzali wetland	66-1H6	23030	49° 21' 13.21" E 37° 28' 8.01" N

Figure 2. Photographs of the herbarium specimen identified as *Vallisneria denseserrulata*

DNA Isolation, PCR Amplification, Sequencing, and Alignment

Total genomic DNA was extracted from either herbarium specimens or silica gel dried leaves using a slightly modified CTAB method (Doyle & Doyle, 1987). The polymerase chain reaction for the amplification and sequencing of the nuclear ribosomal internal transcribed spacer (nrITS) involved using ITS5 and ITS4 primers (Baldwin, 1992). For the amplification and sequencing of *mat*K/*trn*K gene region, four sets of primers were used: 0067F and 1198R as external primers and 0468F and 0510R as internal primers of forward and reverse reactions, respectively (Les et al., 2008).

PCR amplifications were carried out in either 12.5 or 25 µL reaction volume containing 0.25 µM of each primer, 0.19 µM dNTPs (Promega, Madison, WI, USA), 1.25 µL 10x Titanium Taq® reaction buffer with 0.065 µL Titanium Taq® polymerase (View, CA, USA Clontech, Mountain), and 20 ng genomic DNA. For minimizing secondary structure problems, 1.25 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to the amplification reaction of nrITS region. The final volume in each reaction case was adjusted with Molecular Biology grade Water.

PCR conditions for the amplification of nrITS fragments contained 2 min initial denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C (40 sec), annealing at 52 °C (40 sec) and extension at 72 °C (1 min), with a final extension at 72 °C for 10 min. Thermal cycling conditions for chloroplast *mat*K/*trn*K regions originally consisted of 3 min initial denaturation at 94 °C, then 29 cycles of denaturation at 94 °C (40 sec), annealing at 52 °C (40 sec), and extension at 72 °C (1 min), with a final extension at 72 °C for 10 min. PCR products were purified with ExoSAP-IT (Bell, 2008) and were sent for sequencing (Macrogen Inc, USA).

The newly-generated DNA sequences were primarily edited using the program Codon code Aligner (Codon Code Corporation, Dedham, Massachusetts) and assembled into contigs in Geneious v.6.1.6 (Kearse et al., 2012). The contigs were then exported into Geneious and were re-edited and extracted as 14 consensus sequences. Additionally, the authors retrieved previously published alignment for *Vallisneria* from extracted sequences (Les et al., 2008) and realigned with the newly-generated sequences using Geneious alignment algorithm with default parameters followed by manual adjustment. The newly-generated DNA sequences for *V. denseserrulata* and a subset of sequences for *V. spiralis* were deposited in the GenBank. A total of 44 and 22 DNA sequences were subsequently selected for nrITS and *mat*K/*trn*K analyses, respectively.

Phylogenetic Analysis

Phylogenetic analyses were performed using the maximum likelihood method as an optimality criterion. Optimal models for maximum likelihood analyses were assessed using Akaike Information Criteria (AIC) as implemented in Modeltest (Posada & Crandall, 2001). Maximum likelihood bootstrap analyses were then performed with 1000 replicates using PhyML (Guindon et al., 2010) as implemented in the program Geneious with the GTR model for nucleotide sequence evolution. Tree searching was set to be obtained with subtree pruning and regrafting (SPR). ML trees were rooted using *Nechamandra* as the outgroup following (Les et al., 2008). Weakly supported nodes depicted on *mat*K/*trn*K tree were collapsed using Geneious consensus tree builder by choosing a support threshold of 50.

Results

The maximum likelihood nrITS tree of the best score (Fig. 2) resolved all but two Iranian *Vallisneria* accessions as sisters to *V. spiralis* accessions with negligible support (results not shown). Nevertheless, two *Vallisneria* accessions collected from Seymareh river of Ilam province clearly embedded within *V. denseserrulata* accessions with strong support (99 %). The *V. denseserrulata* clade was further depicted as sister to the material of unknown provenance which was grown in cultivation.

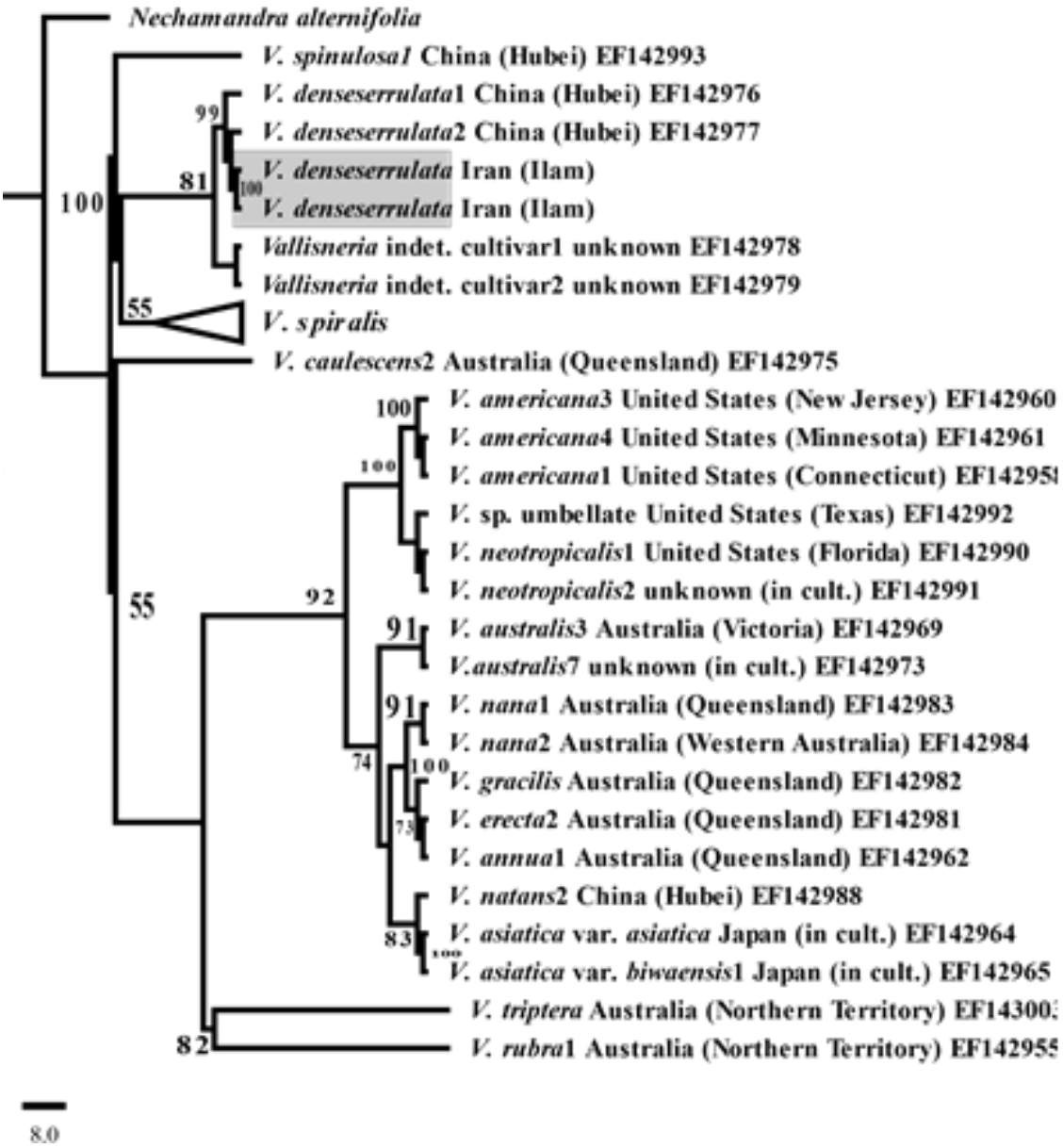

Figure 3. The optimal tree of maximum likelihood (ML) analysis of internal transcribed spacer. *Vallisneria* species collected and identified as *V. spiralis* from Iran and the nine *V. spiralis* accessions extracted from Les et al. (2008) alignment are deliberately collapsed to a single terminal. *V. denseserrulata* accessions from Iran are differentiated as gray-highlighted. Numbers above or below branches represent nodal support values obtained from 1000 bootstrap replicates. Support values less than 50% are not given.

Similar to the nrITS data, the two above-mentioned accessions collected from the Seymareh River, fell within *V. denseserrulata* on the *mat*K/*trn*K sequence-data tree with strong support. *Vallisneria denseserrulata* accessions were genetically distinct from *V. spiralis* accessions from the same locality. The support value for the inclusion of the two Iranian accessions as *V. denseserrulata* on the *mat*K/*trn*K tree was slightly lower than the nrITS tree. Likewise, the depiction of the other Iranian *Vallisneria* accessions on the *mat*K/*trn*K tree was more closely related to *V. spiralis* with negligible support. Depiction of *V. denseserrulata* from Iran as embedded within the clade comprising natural populations of *V. denseserrulata* and the cultivated material of unknown origin was another notable result of the analyses.

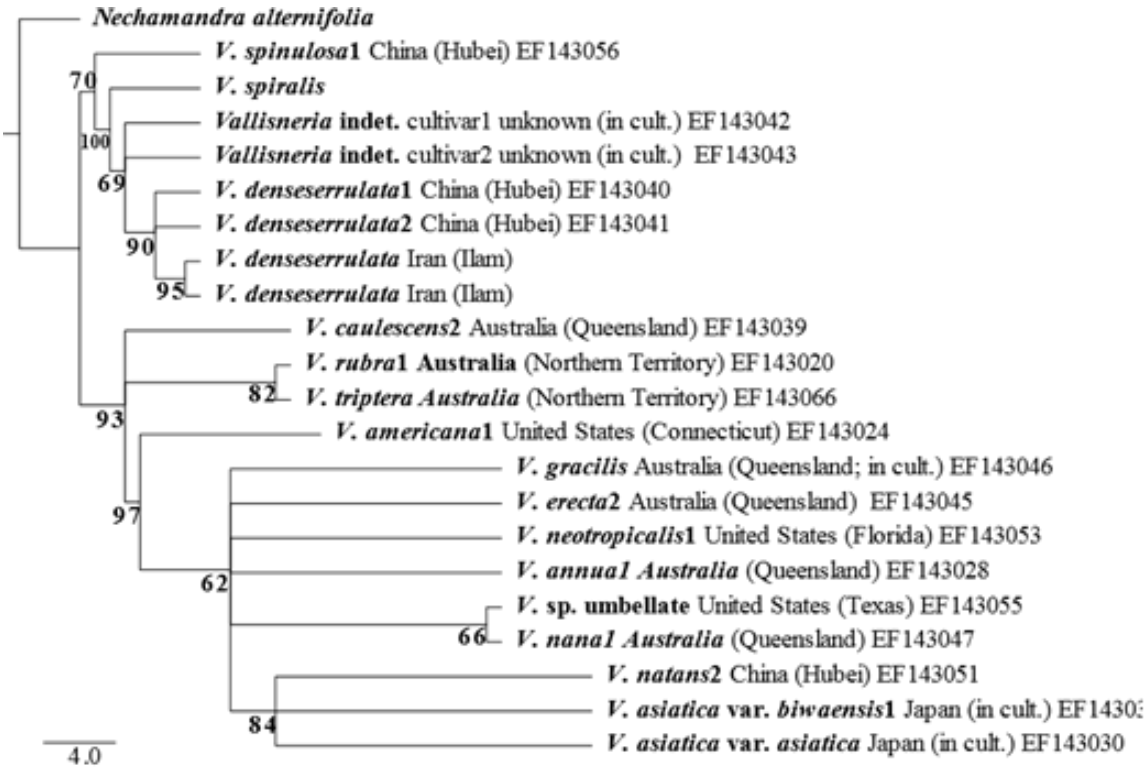

Figure 4. The optimal tree of the Maximum Likelihood (ML) analysis of *mat*K/*trn*K data. *V. denseserrulata* accessions from Iran are differentiated as gray-highlighted. *Vallisneria* species collected and identified as *V. spiralis* from Iran and the two *V. spiralis* accessions extracted from Les et al. (2008) alignment are deliberately collapsed to a single terminal. Numbers above or below branches represent nodal support values obtained from 1000 bootstrap replicates. Weakly supported nodes are collapsed by choosing a support threshold of 50.

Discussion

Both nrITS and *mat*K/*trn*K 5' intron sequences data evidently confirmed the occurrence of *V. denseserrulata* in the Seimareh River of the Ilam province. *Vallisneria denseserrulata* was once reduced to the lower rank of subspecies and as conspecific within *V. spiralis* in a two-species classification system designed by Lowden (1982) who also placed it in synonymy with the distantly-related congener *V. natans* (Lour.) Hara. A taxonomic treatment which was largely based

on the floral feature of minute staminodia whose origination was rather more basal in *V. denseserrulata* relative to its origination in *V. spiralis* var. *spiralis* that was rather more apical. Nevertheless, the most comprehensive study regarding the systematics of *Vallisneria* performed by Les et al., (2008) evidently indicated a sufficient amount of genetic variation among the three (i.e. *V. spiralis*, *V. denseserrulata,* and *V. natans*) to retain them as distinct species.

Our maximum likelihood phylogenetic analyses largely confirmed the results of Maximum parsimony and Bayesian analyses performed by (Les et al., 2008). Nevertheless, one notable difference was observed on the nrITS tree which depicted *V. spinulosa* as sister to the clade comprising *V. spiralis*, *V. denseserrulata* and the cultivated material of unknown provenance with full support. A result that was consistent with the maximum parsimony analysis of *trn*K 5' data in their analyses albeit of them with negligible support.

Although it is highly probable that the quite similar appearance of the two congeners has led the sisters to be misidentified with each other, given the fact that *Vallisneria* species are broadly cultivated for the purpose of being used in aquarium trade around the world along with its resolution within the clade containing the cultivated material of unknown provenance, leaves open the possibility that it has escaped from cultivation and has subsequently established and naturalized in the region. *Vallisneria denseserrulata* has been reported from several areas in Asia including the neighboring country Pakistan (Lowden, 1982). Although *V. denseserrulata* has been designated to be a conspecific of *V. aethiopica* (the latter as a synonym) by Lowden (1982), the verification of the occurrence of *V. denseserrulata* in Africa requires further investigation (Les et al., 2008).

Future scrutiny of the material collected as *Vallisneria* from other localities in Iran may shed light on the origin of *V. denseserrulata* as well as the cultivated material of unknown provenience. Likewise, it may clarify the complicated status of *V. spiralis* in Iran. Table 2 provides a comparison between *V. denseserrulata* and *V. spiralis* which appeared to be more consistent among the examined specimens.

Table 2. Morphological comparisons between two *Vallisneria species* (*V. spiralis* and *V. denseserrulata*)

V. spiralis	***V. denseserrulata***
peduncle length 13-80 cm	peduncles usually long (100-300 cm)
fruit cross-section usually ovoid	fruit cross-section usually linear
leaves sometimes have brown or red pigmentation	brown or red pigmentation on leaves is rare
mature leaf apex usually rounded or obtuse	mature leaf apex usually acute

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