Molecular identification of alien species of *Vallisneria* (Hydrocharitaceae) species in Japan with a special emphasis on the commercially traded accessions and the discovery of hybrid between nonindigenous *V. spiralis* and native *V. denseserrulata*

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ABSTRACT

The aquatic plant genus *Vallisneria*, which includes several invasive species, is frequently used as ornamental plants in aquaria. Naturalized populations of *Vallisneria* have been discovered in Japan and are believed to have escaped from aquaria. We identified these naturalized *Vallisneria* using the internal transcribed spacer regions of nuclear ribosomal DNA (nrITS). Two accessions of nonnative *Vallisneria* were determined to have invaded into Japan: one is a hybrid between *Vallisneria spiralis* native to Eurasia and *Vallisneria denseserrulata* native to China and Japan, and another is *Vallisneria australis* native to Australia. The invasive nature of the former accession may have resulted from hybrid vigor. We also sequenced the nrITS regions of the accessions commercially traded in Japan. The two naturalized accessions were genetically identical to two of those circulating in the Japanese market as ornamental plants for aquaria. These invasive accessions propagate vegetatively and would profoundly influence native water ecosystems in Japan. We seek to arouse attention regarding the risks posed by these invasive *Vallisneria* in Japan, as well as other areas.

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1. Introduction

Recent assessments have shown that alien aquatic plants, as compared to terrestrial plants, tend to have greater economic and ecological impacts on freshwater ecosystems (Vilà et al., 2010; Gordon et al., 2012). The growth and proliferation of invasive aquatic plants exert undesirable effects on native ecosystems by accelerating the decomposition rate and the sediment of decaying plants, as well as by diminishing the biodiversity of native flora and fauna. For example, the water hyacinth (*Eichhornia crassipes*), a viciously invasive aquatic plant found in over 50 nonindigenous countries, causes serious ecological and economic problems in freshwater ecosystems (Villamagna and Murphy, 2010). This species is still expanding its distribution range in concert with increasing global warming (Rahel and Olden, 2008)

Vallisneria, an aquatic plant genus, contains some species that are invading many global regions. In Europe, nonnative *Vallisneria*

spiralis is widespread in many countries and is the third most invasive aquatic plant in this part of the world (Hussner, 2012). Another species, *Vallisneria nana*, has also invaded Europe from Australia (Hussner, 2012). *Vallisneria* is also recognized as an invasive plant in New Zealand (de Winton et al., 2009), North America (Les et al., 2008), the Caribbean (Lowden, 1982), and China (Xiao et al., 2011).

In Japan, presumed nonnative *Vallisneria* plants differing morphologically from native species have been discovered (Ando and Murata, 2003; S. Fujii, personal observation). Ando and Murata (2003) treated the presumed alien *Vallisneria* plants collected from the central part of Japan as *Vallisneria gigantea*, although they noted that the species name requires further scrutiny. Some species of *Vallisneria* are extremely valuable as ornamental plants in aquaria and are commercially traded all over the world (Kasselman, 2003), as well as in Japan. Thus, the alien *Vallisneria* species found in Japan have probably escaped from aquaria or were unintentionally released. Similar escapes from aquaria to natural environments in Japan are known for *Cabomba caroliniana*, *Nymphoides aquatica*, and *Pistia stratiotes*, which are nonindigenous aquatic plants and very popular as aquarium ornaments in Japan (Kadono, 1994).
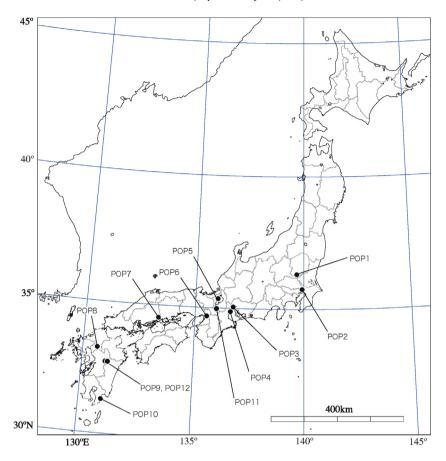
Fig. 1. The locations collected for putatively naturalized alien *Vallisneria* (Pop1–10) and two herbarium specimens (Pop11 and Pop12).

To manage alien species adequately, one must not only recognize the morphological differences between natives and alien species, but also reveal their identities and origins (Douglas et al., 2009). In addition, their invasion pathways often comprise essential information for their control (Hulme, 2009). In the case of *Vallisneria*, because few morphological diagnostic characters are available (Kadono, 1994), taxonomic treatments and species identification have often been confused (Lowden, 1982; Jacobs and Frank, 1997), and molecular identifications are expected to be more useful. Les et al. (2008) determined the internal transcribed spacers of nuclear ribosomal DNA (nrITS) in most *Vallisneria* species, and provided the simple DNA barcodes of the species. In this study, we sequenced the ITS regions of plants presumed to be alien in Japan and determined their identities. Furthermore, to clarify their invasion pathways, we sequenced the same ITS regions in the accessions circulating as ornamental aquarium plants in Japanese markets, and examined whether alien species occurring in Japan in the wild are identical to such commercial accessions.

2. Materials and methods

2.1. Plant materials

Our preliminary survey revealed that two types of alien *Vallisneria* may have become naturalized in Japan: one was reported as *V. gigantea* by Ando and Murata (2003) and the other is a "*Vallisneria asiatica*-like plant," which had often been confused with *V. asiatica* native to Japan. These plants were distinguished from the two native species in Japan (i.e., *V. asiatica* and *Vallisneria denseserrulata*) based on the morphological characters of the stolon, the stem of

the rosette, and leaves (S. Fujii, personal observation). Based on the information of "newly found" populations from amateur botanists and our survey both in the herbarium and in the field, we sampled a total of 10 populations of presumed alien *Vallisneria* species from Japan: the "*V. gigantea*-like plant" was collected from a single population (Pop1), and the *V. asiatica*-like plant was gathered from nine populations (Fig. 1, Table 1). The number of individuals collected for each population is given in Table 2. We sampled each individual at least a few meters apart to prevent the collection of the same genets. The leaf materials were stored in a refrigerator (−25 °C) in Ziplock® plastic bags until DNA extractions were carried out. We also extracted DNA from two herbarium specimens of presumed alien individuals collected from two populations (Pop11 and Pop12) and examined them using the molecular identification methods above.

In addition to the sampling of naturally occurring alien *Vallisneria*, we purchased accessions commercially circulating as aquarium ornaments from nurseries (Table 4). We cultivated these accessions in aquaria until DNA extractions were performed.

2.2. DNA sequencing and homology search

Total genomic DNA was isolated from 50 to 100 mg of leaf tissues or a small portion of dried leaf from the specimens based on the method of Maki et al. (2002). Polymerase chain reaction (PCR) amplification was performed in a total reaction volume of 15 μL containing 10–20 ng of total DNA, 0.15 μM of each primer, 0.1 mM deoxynucleotide triphosphates (dNTPs), 50 mM KCl, 2 mM MgCl$_2$, 10 mM Tris–HCl (pH 8.3), and 0.375 units of Taq DNA polymerase (Amplicon Inc., Irvine, CA, USA). Double-stranded DNA was

Table 1
Locality/habitat and first recoded year of populations, sexual organ observed and voucher specimens.

Population	Locality (habitat)	First recorded year	Sexual organ	Voucher specimen
Pop1	Sano-shi, Tochigi Pref. (farm drain)	2005	Male flower	T. Shiga 3575 (OSA)
Pop2	Matsudo-shi, Chiba Pref. (Saka River)	2005	Female flower	S. Fujii 15496 (KYO, OSA, TUS)
Pop3	Tushima-shi, Aichi Pref. (Nagara River)	2010	Female flower	S. Fujii 14955 (KYO, OSA, TUS)
Pop4	Suzuka-shi, Mie Pref. (farm drain)	2006	Female flower	S. Fujii 15550 (KYO, OSA, TUS)
Pop5	Takashima-shi, Shiga Pref. (farm drain)	1999	Female flower	S. Fujii 14285 (KYO, OSA, TUS)
Pop6	Osaka-shi, Osaka Pref. (Bandaiji River)	2011	Female flower	S. Fujii 15332 (KYO, OSA, TUS)
Pop7	Fukuyama-shi, Hiroshima Pref. (Ashida River)	2013	Not observed	S. Fujii 16041 (KYO, OSA, TUS)
Pop8	Saga-shi, Saga Pref. (farm drain)	2007	Female flower	S. Fujii 15811 (KYO, OSA, TUS), S. Fujii 15648 (KYO, OSA, TUS)
Pop9	Hakusui-mura, Kumamoto Pref. (Shirakawa River, spring water)	2001	Not observed	S. Fujii & M. Maki 16020 (KYO, OSA, TUS)
Pop10	Higashi-kushira-cho, Kagoshima Pref. (Hami River)	2012	Female flower	S. Fujii 15663 (KYO, OSA, TUS)
Pop11	Kusatsu-shi, Shiga Pref. (Isaza River)	2002	Male flower	S. Fujii 15731 (KYO, OSA, TUS)
Pop12	Hakusui-mura, Kumamoto Pref. (Shirakawa River, spring water)	2001	Not observed	C. Sato 95120 (MAK, TNS)

Table 2
Population code in Fig. 1 and Table 1, number of the samples sequenced for ITS, taxa hit with the highest homology (accessions deposited in DDBJ/EMBL/GenBank databases), homology (number of nucleotide differences), and accessions of the sequences determined in this study.

Population	Number of samples	Taxon hit with the highest homology	Accession	No. of nucleotide differences	Accession
Pop1	3	*Vallisneria* sp. 'australis'	EF142973	0	LC002671
Pop2	3	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002672
Pop3	3	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002673
Pop4	3	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002674
Pop5	3	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002675
Pop6	2	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002676
Pop7	5	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002677
Pop8	5	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002678
Pop9	3	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002679
Pop10	3	*Vallisneria* sp. *Christensen s. n.*	EF142979	6	LC002680
Pop11	1	*Vallisneria* sp. 'australis'	EF142973	0	LC002681
Pop12	1	*Vallisneria* sp. 'australis'	EF142973	0	LC002682

Pop11 and Pop12 were based on herbarium specimens.

amplified after incubation at 94 °C for 3 min, followed by 30 cycles of incubation at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 15 min. We amplified the nrITS regions and sequenced the fragments with a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye™ Terminator Cycle Sequencing Kit (version 3.1; Applied Biosystems). For sequencing, we used the same primers used for amplification. All sequences have been deposited in the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases. The homology search was carried out with BLAST using blastn at the DDBJ Web site (http://www.ddbj.nig.ac.jp/).

Because some presumed alien plants occurring in the wild and a few commercially traded accessions had heterozygous sites in their ITS regions, the ITS fragments of such plants were ligated into the pGEM-T vector (Promega, Madison, WI, USA) and transformed into competent cells (competent high DH5α; Toyobo, Tokyo, Japan). We cloned the ITS fragments of only three presumed alien plants (one each from Pop 2, Pop4, and Pop8) and a commercially traded plant (from the plant supplier 'Tropiland' in Table 4) because all of the heterozygous sequences were identical in the electrogram. The electrograms of the heterozygous individuals showed entirely a same pattern, suggesting that the ITS sequences of all the heterozygous plants were identical. After blue/white screening, we sequenced at least eight positive colonies via direct sequencing as above. We used the M13F primer (CAGGAAACAGGATGAC) and M13R primer (CAGGAAACAGCTATGAC) for DNA sequencing of the target fragments.

3. Results

We sequenced a total of 35 individuals of presumed alien *Vallisneria* in Japan from 10 naturally occurring populations and two herbarium specimens. After alignments, the total sequence length was 427 or 420 bp, of which 87 bp was 5.8S ribosomal DNA and the remainder was ITS1 or ITS2. The sequences were deposited in DDBJ/EMBL/GenBank; the accession numbers are shown in Table 2. Of these sequences, two were different: one was amplified from all naturalized populations except one, and the other from a single naturalized population (Pop1) and two herbarium specimens (Pop11 and Pop12; Table 2). Hereafter, the former and the latter were designated genotypes A and B, respectively.

All *V. asiatica*-like plants were found to be of genotype A. This genotype was not identical to any wild *Vallisneria* species in homology searches, but had the highest homology (98.6%) to the sequence of *Vallisneria* sp. Christensen (EF142979) in GenBank (Table 2). According to Les et al. (2008), the origins of *Vallisneria* sp. Christensen were unknown; they were in cultivation as *V. spiralis*. Therefore, the plants in Japan with genotype A are genetically very close to the cultivars. Of the difference between the sequences of the Japanese nonindigenous plants (*V. asiatica*-like plants) and the cultivars used in a previous molecular phylogenetic study (*Vallisneria* sp. Christensen), 1.4% (6 of 427 bp) resulted from the heterozygous sites (treated as "N") in the ITS region of the former plants (Table 3, Fig. 2). The same heterozygous sites were found in the sequences of all *V. asiatica*-like plants in Japan. This suggests that the former nonindigenous plants may be of hybrid origin. By cloning the heterozygous fragments, we obtained a sequence almost fully identical to those of *V. spiralis* and *V. denseserrulata* by homology search, suggesting that the heterozygous individuals of the naturalized aliens (i.e., *V. asiatica*-like plants) are hybrids of *V. spiralis* and *V. denseserrulata*, which are resolved as sister species in the previous phylogenetic tree (Les et al., 2008) and morphologically treated as intraspecific taxa (Lowden 1982).

Table 3
Nucleotide differences between *Vallisneria asiatica*-like plants and *V.* sp. *Christensen s. n.* (EF142979).

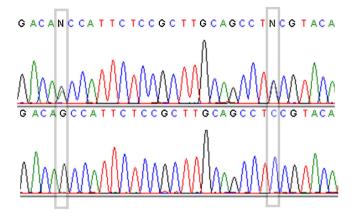| Sample	Nucleotide positions					
	92	95	161	300	383	398
V. asiatica – like plant	G/T	A/T	T/C	C/T	C/T	C/T
V. sp. *Christensen s. n.* EF142979	G	A	T	C	C	C

Fig. 2. A part of the electrogram for nrITS sequencing. Upper and lower panels showed the sequences of individuals from Pop2, putatively naturalized alien *Vallisneria*, and *V. spiralis* purchased from a plant supplier (Charm, Oura), respectively. The two boxes show the heterozygous sites in the upper sequences.

Another genotype B sequence had the highest (100%) homology to that of *Vallisneria australis*, suggesting that the population in Tochigi Prefecture (Pop1) and two individuals from the herbarium specimens (Pop11 and Pop12) were genetically identical to *V. australis*, which was recently described by Les et al. (2008) for the plants occurring in Australia.

The genotypes A and B populations consisted only of female and male individuals, respectively (Table 1).

From the commercially available plants, we obtained 14 nrITS sequences. Of these sequences, three (one of *Vallisneria caulescens* and two of *V. spiralis*) were identified correctly as those of the same species, but the remaining ones were identified as those of the different species (Table 4), indicating that the latter species were traded with taxonomically incorrect names. Our data on the nrITS sequences revealed that the ornamental *Vallisneria* traded as *V. spiralis* or *Vallisneria* sp. comprised two accessions: one had the nrITS sequences identical to that of *V. spiralis* itself, and the other had the heterozygous nrITS same as the ones possessed by the non-indigenous *V. asiatica*-like plants in Japan. As a result, the latter

also displayed the highest homology to *Vallisneria* sp. *Christensen*, as seen in the *V. asiatica*-like plants in Japan (see above), and had fully identical nrITS sequence of the nonindigenous *V. asiatica*-like plants. Therefore, some accessions of *Vallisneria* circulated commercially in Japan were also hybrids between *V. spiralis* and *V. denseserrulata*. On the other hand, considering the results of the homology search (Table 4), the nrITS sequences of the accessions traded commercially as *V. gigantea* were identified as that of *V. australis* (Table 4).

4. Discussion

Our molecular identification of presumed alien *Vallisneria* individuals revealed that two alien accessions have naturalized in Japan. One, the *V. asiatica*-like plant of genotype A, was revealed to be a hybrid between *V. spiralis* and *V. denseserrulata*. The morphological distinction between this accession and the native congener *V. asiatica* in Japan becomes relatively clear if examined in detail; in comparison to the native *V. asiatica*, the hybrid *V. asiatica*-like plant has rosettes and an often elongating stem, and its leaves have a round apex (S. Fujii, personal observation).

The occurrence of natural hybridization between *V. spiralis* and *V. denseserrulata* is unlikely because the distributions of the two species do not overlap; *V. spiralis* is found in southern Europe and the Near East, and *V. denseserrulata* in the Middle East and East Asia (Lowden, 1982). Rather, hybridization likely occurred in a nursery treating both of the species, or in the area in which one of the species naturalized, while the other is native. On-site hybridization is implausible in Japan because no naturalized population of *V. spiralis* exists in the country.

In Japan, the first invasion of nonindigenous *V. asiatica*-like plants into the wild was believed to occur in the 1990s because the oldest record of a herbarium specimen was collected from Pop5 in 1999, and the invasive accession was scattered widely from central to western Japan in the 2000s and 2010s. The fact that this hybrid accession is commercially traded in Japan suggests that it had escaped from aquaria. In Japan, many nonindigenous plants that escaped from aquaria have recently become problematic; some aquatic species, such as *Gymnocoronis spilanthoides*, *Myriophyllum aquaticum*, and *P. stratiotes*, are included on the List of Regulated Living Organisms under Japan's Invasive Alien Species Act (http://www.env.go.jp/nature/intro/1outline/files/siteisyu_list_e.pdf).

All populations of the nonindigenous *V. asiatica*-like plants examined in this study consisted only of female individuals, suggesting that the populations do not regenerate by sexual reproduction but via vegetative propagation, and they may outcompete indigenous submerged aquatic species and rapidly occupy areas in rivers or drainages. In the lower reaches of the Nagara River

Table 4
Distributed names of the commercially traded accessions sequenced in this study, suppliers, taxa hit with the highest homology (accessions deposited in DDBJ/EMBL/GenBank databases), homology (number of nucleotide differences), and accessions of the sequences determined in this study.

Distributed name	Supplier	Taxon hit with the highest homology	Accession	No. of nucleotide differences	Accession
V. caulescens	Charm, Oura	*V. caulescens*	EF142974	1	LC002683
V. neotropicallis	Charm, Oura	*V. spiralis*	EF142986	0	LC002684
V. americana	Aqua Art Gallery, Matusyama	*V.* sp. '*australis*'	EF142973	2	LC002685
V. natans	Aqua Art Gallery, Matusyama	*V. gracilis*	EF142982	4	LC002686
V. nana	Tropiland, Tokyo	*V. gracilis*	EF142982	2	LC002687
V. nana	Charm, Oura	*V. gracilis*	EF142982	2	LC002688
V. spiralis	Aqua Art Gallery, Matusyama	*V. spiralis*	EF694962	0	LC002689
V. spiralis	Charm, Oura	*V. spiralis*	EF694962	0	LC002690
V. spiralis	Tropiland, Tokyo	*V.* sp. *Christensen s. n.*	EF142979	6	LC002691
V. sp.	Shin-Takataki, Osaka	*V.* sp. *Christensen s. n.*	EF142979	6	LC002692
V. asiatica var. *biwaensis*	Charm, Oura	*V. natans*	EF142989	0	LC002693
V. asiatica var. *biwaensis*	Aqua Art Gallery, Matusyama	*V. natans*	EF142989	0	LC002694
V. gigantea	Aqua Art Gallery, Matusyama	*V.* sp. '*australis*'	EF142973	0	LC002695
V. gigantea	Tropiland, Tokyo	*V.* sp. '*australis*'	EF142973	0	LC002696

(Pop3), the hybrid *Vallisneria* grows thickly in the kilometer ranges (S. Fujii, personal observation) and overgrows exclusively in Pop 2, 4, 5, and 8, although the inhabiting ranges are at present restricted to tens or hundreds of meters (H. Wasekura, S. Fujii, and M. Maki Maki, personal observation). It usually grows densely as carpet-like population covering bottoms of rivers, and looks to become exclusive against other submerged water plants. The lignified stem may contribute to forming dense and permanent populations. The actual situation of this submerged plant and its impact on water ecosystems must be evaluated as soon as possible. Because interspecific or intraspecific hybridization often leads to invasive success via hybrid vigor and results in the hybrid becoming prevalent in non-native areas (Tiebre et al., 2007; Ross and Auge, 2008; Travis et al., 2010), we should be apprehensive of invasion by this submerged hybrid. From the standpoint of preventive measures, their sale or trade must be restricted and their release into natural environments avoided.

Another accession of the alien *Vallisneria* in Japan, which was identified as *V. gigantea* by Ando and Murata (2003), was found in only a few localities and identified to be the recently described species *V. australis*, which had been previously treated as *V. nana* (Les et al., 2008). This species has also invaded Hungary from Australia (Hussner, 2012). The biological reason why the accession had successfully naturalized in Japan is unclear, although the species is also traded commercially as ornamental plants for aquaria (Table 4). More attention to the species is warranted in Japan, as well as other countries. This accession is also clearly distinct from the Japanese indigenous species in that the former has evergreen, coriaceous, and larger leaves (14–24 mm wide). Only male plants were found, which also likely propagate vegetatively. It occupied or covered large areas of the rivers (Pop1 and Pop11; H. Wasekura, S. Fujii, and M. Maki, personal observation). The invasion by the accession may be at an early stage, judging from the number of localities (Table 2), and these rhizomes should be eradicated as soon as possible.

5. Morphology and taxonomic treatment

For morphological identification and its taxonomic treatment, the putative hybrid plant is described as below.

Vallisneria × pseudorosulata S. Fujii et M. Maki, hybrid nov.

Typus: JAPAN, Honshu: Takashima-shi, Shiga Pref., Nov. 13, 2013, S. Fujii 15,996 (holo-OSA, iso-KYO, MAK, TI, TNS, TUS)

This plant was revealed to be a hybrid between *V. denseserrulata* (Makino) Makino and *V. spiralis* L. by the heterozygous sites in the ITS region; one of the sequence was supposed to have originated from *V. denseserrulata* and the other from *V. spiralis*.

Diagnosis: This hybrid is evergreen; apex of leaf usually round and finely serrate; vertical stems of rosettes often elongating and lignified; flowers usually in middle to late autumn. *Vallisneria denseserrata* is deciduous, apex of leaf acute to acuminate, strongly serrate; stolons with minute acicles, bearing tubers; flowers in late autumn. The morphological difference between the hybrid and *V. spiralis* is not clear but the following characters are useful for identification; elongating and lignified stems of rosettes, leaf width larger in well grown plants, i.e. ca. 10 mm or more in hybrid, though up to 10 mm in *V. spiralis* (Dandy, 1980). We have another species of *Vallisneria* in Japan; i.e. *V. asiatica* is usually deciduous except in southern Japan, apex acute; vertical stems of rosettes neither lignified nor elongated.

Description: Submerged rosulate, stoloniferous, evergreen perennial. Vertical stems of rosettes often lignificate to some extent and sometimes elongate up to 15 cm; stolons horizontally elongating, surface smooth, usually without leaves, bears no tubers. Fascicled leaves linear, tape shaped, up to ca. 0.7 m long, 3–13 mm wide, margins toothed obscure or entire, apex usually round to obtuse, sometimes acute, finely serrate. Pistillate flower sessile; scape 0.25–0.7 m long, thick coiling after flowering; spathe thin, translucent, 8–12 mm long, enclosing usually 1 flower; ovary 10–20 mm long; sepals 3, ascending, 2–3 mm long; stigmatic lobes shallowly or deeply split; staminodia adonate to the cleft apex of the fused stigmatic lobes. Male flowers unknown. Mature fruits and seeds not observed.

Distribution: Commercially distributed as '*V. spiralis*'. Naturalized Honshu and Kyushu Islands in Japan. Parent species show disjunct distribution; *V. spiralis* in southern Europe and Near East Asia, while *V. denseserrulata* in Middle and East Asia. Originated place of the hybrid is unknown.

Habitat: Farm courses, creeks, rivers, especially where flows are slow and water fluctuation is little or stable. Growing in 0.1–1.0 m (or more?) depth.

Ecology: Only female plants are known, probably regenerating vegetatively. Usually grows as dense and carpet like populations on the bottom. Lignified stems supposed to contribute to make such populations exclusive against other aquatic plants. Many populations in small size, but widely scattered population (more than 5000 m) was found in Nagara River. Flowers bloom from (September) October to November.

Note: *V. spiralis* was reported to be introduced northern Europe (Lowden 1982; Hussner 2012), North America (Les et al., 2008), New Zealand (ISSG Global invasive species database http://www.issg.org/database/welcome/) and China (Xiao et al., 2011) but some of those populations might be confused with the hybrid. Detail examination will be need.

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