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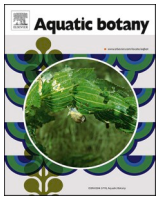

Vallisneria (Hydrocharitaceae): novel species, taxonomic revisions, and hybridization

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ABSTRACT

Vallisneria is a cosmopolitan genus of aquatic plants comprising 14 species within the family Hydrocharitaceae. Previous research suggests *Vallisneria* is a more speciose genus than current taxonomy indicates, and there remains contention on the level of species diversity within the genus. In order to address some of these taxonomic issues, this study estimates phylogenetic relationships within the genus using a previously published molecular dataset augmented with previously unsampled species through the use of maximum likelihood analyses for all molecular data partitions (e.g., nrITS, cpDNA, and combined nuclear and cpDNA datasets). Based on our findings, we recommend the resurrection of two *Vallisneria* species (*V. gracilis* and *V. neotropicalis*), and formally recognize and describe a new species (*V. jacobsii*). Morphological data was shown to be useful for some species delimitation but overall, molecular sequence data provided the best estimates of species identification for cultivated specimens. We also show the presence of naturally occurring putative *Vallisneria* hybrids within Northern Territory, Australia, and give conclusive evidence that non-native hybrids are being used for a restoration project in Crystal River, Florida.

1. Introduction

Vallisneria L. (Hydrocharitaceae) is a genus of submerged, aquatic flowering plants that often form dense stands in natural habitats due to their stoloniferous growth habits. The genus is widely distributed in temperate, tropical, and subtropical regions in Europe, Africa, Asia, Australia, and North America. Except for the three caulescent species endemic to Australia, all other *Vallisneria* species produce linear leaves from a basal rosette. Additionally, most species produce flat to undulate leaves while a few species show strongly spiraled leaf growth. However, both intra and interspecies variation in leaf morphology can be observed within natural populations which makes field identification quite difficult when using vegetative characters. *Vallisneria* species are dioecious with very small (e.g., 3 – 9 mm) solitary, female flowers presented at the surface of the water on long thin stalks. In contrast, male flowers are formed on short stalks and become detached and float to the surface at maturity. The diminutive and ephemeral nature of the flowers, like vegetive features, make their use in field identification difficult. Thus, despite its longstanding presence in the aquarium plant trade, the taxonomy of the genus is problematic and even the correct number of accepted species is unclear (Les et al., 2008). Furthermore, the accidental or intentional introduction of nonindigenous and hybrid *Vallisneria* plants into non-native areas has resulted in these plants establishing populations within multiple North American and Japanese regions (Les et al., 2008; Wasekura et al., 2016; Gorham et al., 2021).

Prior taxonomic work by Lowden in 1982 highlighted the impact that phenotypic plasticity plays when analyzing morphological features in *Vallisneria*. Some of the features he recognized as being "indecisive characters" included leaf length and width, and the presence/absence of leaf margin serrations (Lowden, 1982). Despite *Vallisneria* being host to a number of highly plastic characters, Lowden (1982) was able to determine a handful of consistent, microscopic traits which were mostly associated with floral morphology. These traits are nearly impossible to ascertain from herbarium specimens, as the staminate flowers of *Vallisneria* species are too small and delicate to preserve, and pistillate flowers lose their 3-dimensional structure when dried and pressed. Lowden instead relied on field studies and measurements of living plants, with an emphasis on what he thought were invariant floral traits. Lowden's strict evaluation of *Vallisneria* led him to recognize only two species, *V. spiralis* and *V. americana*, and four varieties (Lowden, 1982).

The two-species system proposed by Lowden (1982) failed to capture the degree of floral and vegetative morphology and biogeography of the

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genus, and a robust estimate of phylogeny was required to begin the study of complex patterns of evolution in *Vallisneria*. The most comprehensive phylogenetic analysis of *Vallisneria* was conducted by Les et al. (2008). Their study employed parsimony and Bayesian analyses of a combined nrITS and plastid (*rbcL* + *trnK* 5′ intron) molecular dataset as well as a matrix of 26 morphological characters to estimate phylogeny for 18 taxa (16 species of *Vallisneria*, *Maidenia*, and *Nechamandra* [outgroup]). The resulting evolutionary framework was used to assess relationships and species limits within the genus. Their phylogenetic analyses resolved the monotypic genus *Maidenia*, and placed this clade nested within *Vallisneria*, and sister to the two caulescent *Vallisneria* species (*V. caulescens* and *V. triptera*). *Maidenia* was subsequently transferred into *Vallisneria* in order to maintain a monophyletic group. With this merger and the identification of two additional Australian *Vallisneria* species (*V. australis* and *V. erecta*), Les et al. (2008) formally recognized 14 distinct species. They further note that based on their phylogenetic results, Australia maintains the highest level of diversity for *Vallisneria* in the world, with five rosulate species (*V. annua*, *V. australis*, *V. erecta*, *V. gracilis*, and *V. nana*) and three caulescent species (*V. caulescens*, *V. rubra*, and *V. triptera*). Although Les et al. (2008) included much of the Australian *Vallisneria* diversity, additional taxa endemic to the Northern Territory were not included in their study and remained unplaced in the genus. Not included in the Les et al. (2008) study were two species described from China (*V. longipedunculata* X.S. Shen, 2000 and *V. anhuiensis* X.S. Shen, 2001) in the early 2000 s. For the present study due to COVID-related import restrictions, we were unable to obtain samples of these species.

The purpose of this present study was to expand upon the work from Les et al. (2008) by adding in data from specimens that had yet to be included in phylogenetic analyses of the genus *Vallisneria*. The resulting evolutionary framework was used to: (1) assess the current taxonomy of *Vallisneria*; (2) give notes on the feasibility of morphological data for species delimitation within the genus and the presence of naturalized hybrids; (3) help identify cultivated plants commonly sold in the aquarium trade; (4) present data to support a putative new species from Northern Territory, Australia; and (5) show evidence that non-native hybrids are being used for a restoration project in Crystal River, Florida.

2. Materials and methods

2.1. Taxon sampling

Specimens comprising 16 unique individuals representing eight species and six hybrid or undetermined taxa were field collected or purchased as cultivated material then kept in a living collection at the University of Kansas (Appendix A). Multiple *V. neotropicalis* plants were collected from different locations in Central Florida and although 18 specimens are listed, the *Vallisneria americana* plants collected from Square Lake, MN showed identical molecular sequences, so for combined molecular analyses we only used one individual. Among the 16 taxa, five were from previous collection events in Australia that were cultivated in isolation to avoid possible hybridization (Dave Wilson pers. com.) and imported (USDA permit #P37–21–00284) for use in the present study. Four specimens were field collected in Minnesota (MN DNR permit #2021–1670) and Florida (FWC Special Permit), and the remaining seven were sourced from the aquatic plant trade. Accessioned sequences from additional taxa used for phylogenetic analysis were pulled from GenBank associated with Les et al. (2008) and Gorham et al. (2021).

2.2. Living collection

In order to identify and measure morphological characters from *Vallisneria* specimens growing in identical conditions, a 50-gallon Rubbermaid stock tank was retrofitted with a Fluval FX4 filter, Cerge's CO_2 reactor (attached to CO_2 system), and submerged aquarium heaters to allow the tank to act as a common garden for all specimens in this study. All plants were grown in separate plastic pots (8 cm wide x 9 cm tall) containing a 50/50 mix of silty clay loam and organic raised bed soil and capped with fine grained sand. Nutrient levels were monitored weekly and the addition of KNO_3, KH_2PO_4, K_2SO_4, and Plantex CSM+B to the water followed an Estimative Index dosing regimen (Watson, 2005). Levels of CO_2 were maintained between 30 and 50 mg/L. Water depth was kept at about 27 cm deep and water temperature a constant 82°F/28 °C. RODI water was added weekly to maintain a constant water level.

2.3. Morphological data

Morphological data for vegetative characters were collected after a growing period of six months to ensure plants were given enough time to produce mature leaves, and reproductive characters were collected as flowers became available. Floral traits were examined with both dissecting and compound microscopes. This study was not able to include male and female counterparts for each taxon (except for *Vallisneria neotropicalis*), thus it was not possible to assess all sexual characters. Additionally, female flowers were pruned before full seed development could occur to safeguard from the possibility of hybrid plants mixing with isolated specimens.

2.4. Molecular data and analyses

Leaves were collected from the 16 taxa in the living collection and dried for 24–48 h in plastic bags partially filled with silica beads. Total genomic DNA was extracted using Qiagen DNeasy Plant Kits (Qiagen Inc., Hilden, Germany). Each taxon was concurrently extracted in two columns, then final DNA precipitates were combined to ensure adequate final DNA concentration. A NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) and a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) were used to check DNA products, and extractions were repeated for taxa yielding low-quantity DNA (< 10 ng/μL).

Primers for DNA amplification were obtained for three gene regions based on those used in Les et al. (2008). The forward and reverse primer pairs were: *rbcL* (1 F & 1204 R), *trnK* 5' intron (0067 F, 0510 R, 0468 F, and 1198 R), and nrITS (ITS5, ITS4, and ITS1). All primers were mixed with deionized water to create 100 μM stock solutions then diluted down to 5 μM for use in Polymerase Chain Reactions (PCR).

Amplification of DNA was completed using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Uppsala, Sweden) combined with a mixture of 1 μL (5 μM) forward primer, 1 μL (5 μM) reverse primer, 22 μL RODI water, and 1 μL DNA. Some nrITS amplifications required 1 μL of DMSO due to poor amplification. Thermal cycling involved 96 °C for 5 min, 35 × (45 s at 96 °C, 45 s at 48 °C, 1 min 30 s at 72 °C), then final extension at 72 °C for 10 min. Amplification was verified through agarose gel electrophoresis using 5 μL of PCR product. Products from PCR reactions were sent to ACGT, Inc. (Wheeling, Illinois) for Sanger sequencing. Resulting DNA sequence chromatograms were hand-checked using Chromas (Technelysium, Australia) and single nucleotide polymorphisms (SNPs) were changed to corresponding IUPAC codes. Sequences were assembled using SeaView (Gouy et al., 2021).

Sequence data for *rbcL*, *trnK* 5' intron, and nrITS was generated for all 16 taxa from the living collection and then deposited in GenBank (Appendix A). The specimen names unique to this study are preceded numerically in the figures (e.g., 1 - *V. nana,* 2 - *V. jacobsii*, etc.) whereas specimens previously used in Les et al. (2008) are numbered following specimen names. For the three phylogenetic analyses completed in this study, previously published sequences were pulled from GenBank, then new sequences generated from this study were added to corresponding datasets (Supplemental Table 1). For the nrITS tree, select sequences from Gorham et al. (2021) were pulled and combined with three taxa from this study (Supplemental Table 1). Gene sequences were

concatenated in Mesquite (Maddison and Maddison, 2021) for the two analyses using multiple genes (combined molecular and cpDNA). With all analyses, sequences were aligned using MUSCLE (Edgar, 2004). For tree building, maximum likelihood analysis was completed using IQ-Tree (Trifinopoulos et al., 2016) along with ModelFinder (Kalyaanamoorthy et al., 2017) to select the best-fit model for sequence data. In the combined molecular analysis, ModelFinder suggested combining nrITS and cpDNA sequences into an unpartitioned dataset and using one model of evolution, which was also suggested with the cpDNA analysis. Tree files were visualized using FigTree v1.4.4 (Rambaut, 2018) by rooting *Nechamandra alternifolia* as the outgroup for the combined molecular and cpDNA trees, and *Vallisneria spinulosa* in the nrITS tree. Node support was estimated using 100 standard bootstrap replicates and branch support was conducted using an approximate Bayes test (Anisimova et al., 2011). Nodes were considered highly supported with values > 80 for bootstrap and > 0.95 for aBayes.

2.5. Polymorphic nrITS sequences and putative hybrids

Several taxa used in this study showed polymorphic nrITS sequences ranging from one to 14 SNPs. With the specimens we are identifying as putative hybrids, aligned sequences were paired up with a potential parental type following a "pseudo-clone" method described in Les et al. (2009), Tippery and Les (2013), and Razifard et al. (2017) along with evidence from overall taxa placement within the cpDNA tree (Supplemental Fig. 1) showing relatedness via maternally inherited plastid genes. The left-over sequence was then paired with taxa that complimented nucleotides for those polymorphic sites based on the corresponding IUPAC codes (Fig. 1). The cultivated specimen used in this study labeled as 11 – *Vallisneria* sp. "Leopard" initially presented 50 SNPs when examining chromatogram data, but this was reduced to 14 SNPs after accounting for indels in areas where the sequence had many sequential polymorphic sites (nucleotide positions 217 through 248, and 452 through 464). The two specimens from Northern Territory (3 & 6 *V.* indet.) had zero and two SNPs, which provided no information for putative parental types with sequence data.

3. Results

3.1. Molecular analysis: ML with additional taxa

With the addition of new taxa, the ML analyses on the combined molecular dataset showed only one topological change when compared to the Les et al. (2008) phylogeny. The placement of *Vallisneria caulescens* resolved with low support (Fig. 2; BS = 62, aBayes 0.61). This analysis concurs with previously known issues of finding high support for the placement of this taxon, along with the support for the relationships between the caulescent taxa (Les et al., 2008). Consistent with Les et al. (2008), our results showed that the combined molecular dataset provided for the highest number of parsimony informative characters and in turn produced the highest phylogenetic signal of the phylogeny estimates (Supplemental Table 1).

3.2. Molecular analysis: cultivated specimens, hybrids, and novel species

In the combined molecular dataset, taxa with polymorphic nrITS sequences (>3 SNPs) were omitted. There was high support for the inclusion of the cultivated specimen 4 - *V.gracilis* within the group containing *V. annua*, *V. erecta*, and *V. gracilis* (BS = 100, aBayes = 0.98; Fig. 2). There was also high support for the placement of the cultivated 9 - *V. natans* within the group containing *V. natans* and its two varieties (BS = 100, aBayes = 1.0). With the addition of the three Northern Territory specimens 3 & 6 - *V.* indet., and the novel species we are calling *V. jacobsii*, the *V. australis* clade originally designated by Les et al. (2008) separated into two subclades with high support (BS = 92, aBayes = 0.97; BS = 80, aBayes = 1.0). Within the North American clade (BS = 95, aBayes = 1.0), adding taxa from both Minnesota and Florida continued to support the separation of *V. americana* and *V. neotropicalis* + *V.* sp. (umbellate) (BS = 100, aBayes = 1.0; BS = 58, aBayes = 0.94). Additionally, the cultivated specimen 12 - *V. neotropicalis* resolved within its respective group.

The cultivated specimens 8 - *V. spiralis*, 10 - *V.* sp., and the specimen collected from Crystal River, Florida labled as 17 - *V.* sp. "Rock Star" resolved within the *V. denseserrulata* + *V. spiralis* clade with high support (BS = 100, aBayes = 1.0) in the cpDNA (*trnK* 5' intron + *rbcL*) dataset (Supplemental Fig. 1). With the addition of these three taxa to the dataset established by Gorham et al. (2021) for use in detecting non-native hybrids within the southeastern United States, they resolved

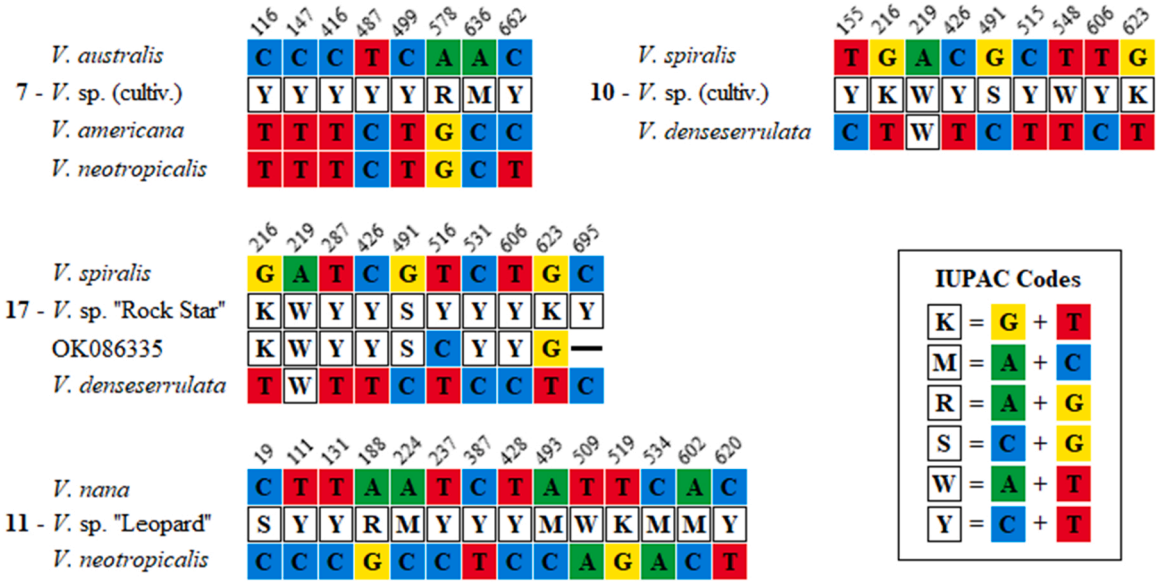

Fig. 1. Potential hybrid taxa from this study presenting > 3 SNPs in nrITS sequences, and their putative parental types. Polymorphic sites are designated as IUPAC codes and their nucleotide position within aligned sequences are given (above).

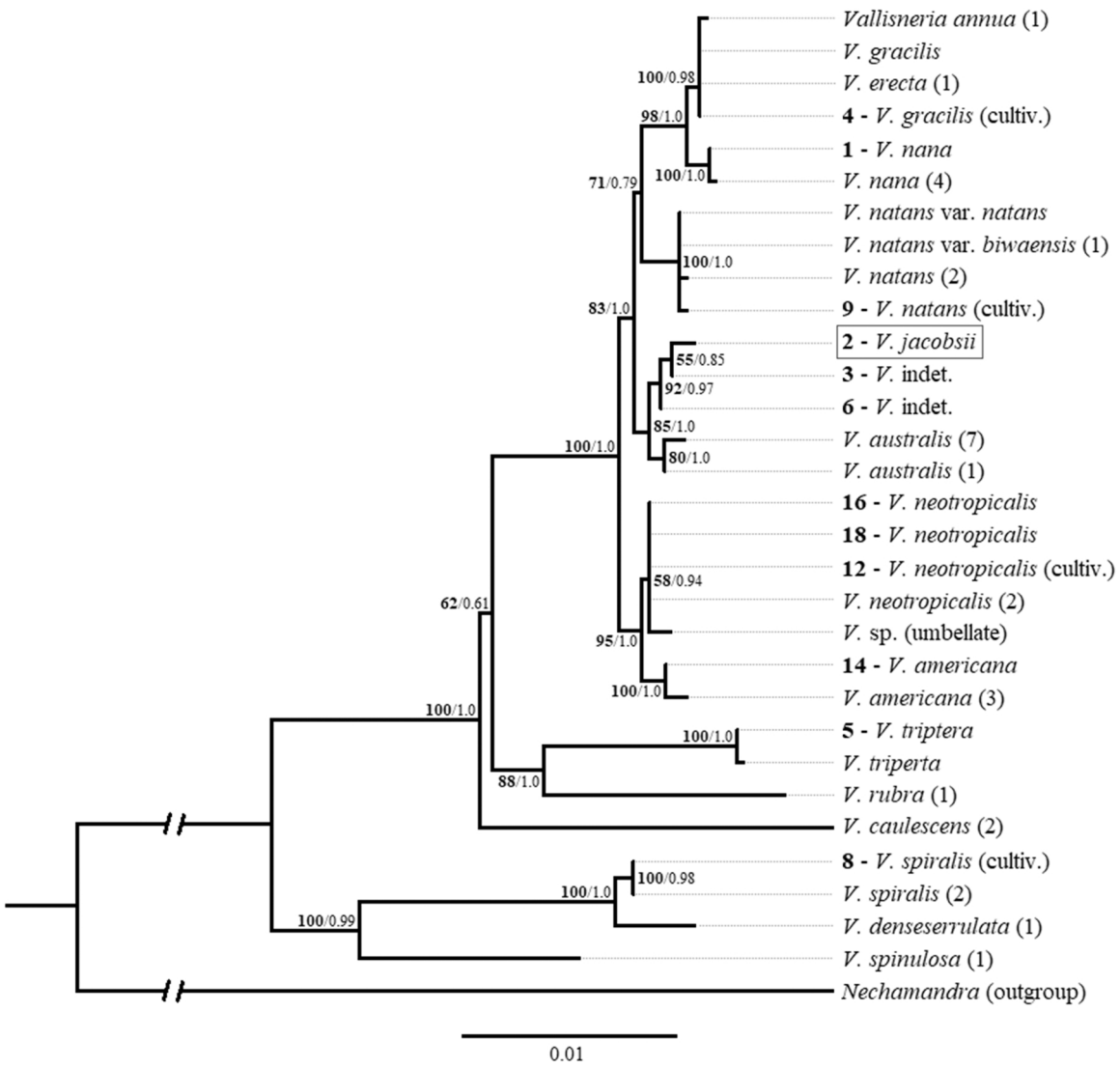

Fig. 2. Phylogram estimated from combined molecular data from both Les et al. (2008) dataset and new taxa from this study. Numbers before taxon indicate new specimens from this study in Appendix A. Branch support is indicated by bootstrap values (left) and aBayes for ML (right) for each node. Scale for branch lengths is indicated.

within the same clade with high support (Fig. 3, BS = 100, aBayes = 1.0). Additionally, the specimen 17 - *V.* sp. "Rock Star" resolved amongst the group containing the non-native hybrid specimens from Gorham et al. (2021) along with the two indeterminate specimens from Les et al. (2008).

The four putative hybrid taxa in this study with > 3 SNPs in nrITS sequences showed an overall high number of informative sites when paired with potential parental types (Fig. 1). For three of the taxa there was each one polymorphic site left unpaired with parental types; 10 - *V.* sp. (cultiv.) nucleotide position 548, and 11 - *V.* sp. "Leopard" nucleotide position 19, and 17 - *V.* sp. "Rock Star" nucleotide position 695 as nrITS sequences from Gorham et al. (2021) did not include sequence data at this region.

3.3. Morphological characters

Several taxa in the common garden experiment showed leaves with horizontal/diagonal striation patterns (Fig. 4a). The novel species we are calling *Vallisneria jacobsii* grew leaves with alternating vertical green/red striping patterns and bright yellow midveins, along with basal growth appearing distichous (Fig. 5). The specimens 3 & 6 - *V.* indet. grew stiff, opaque leaves that extended above the surface of the water and had antrorse bristles on the abaxial and adaxial sides of leaf apices, which are autapomorphic features of *V. erecta*. After a period of over one year growing together, 1 - *V. nana* and 4 - *V. gracilis* continued to show both size and leaf coloration differences (Fig. 4b). Leaf growth differences were also maintained between the Minnesota *V. americana* and Florida *V. neotropicalis* specimens (Fig. 4c). Two taxa in the collection showed strongly spiraled leaf growth: 9 - *V. natans* (cultiv.) and 10 - *V.* sp. (cultiv.); however, their overall appearance was quite different. Leaf edge serrations on 9 - *V. natans* (cultiv.) extended from the apex all the way to the base of the plant, while 10 - *V.* sp. (cultiv.) only had serrations on leaf apices. Additionally, the leaves of 9 - *V. natans* (cultiv.) felt stiff and brittle and were less malleable when compared to the leaves of 10 - *V.* sp. (cultiv.).

4. Discussion

4.1. Phylogenetics using molecular data

The combined molecular dataset provided the highest phylogenetic signal of the tree estimates conducted in this study (Fig. 2 & Supplemental Table 1). We thus consider these results to be the best hypothesis for interpreting phylogenetic relationships amongst the sampled taxa.

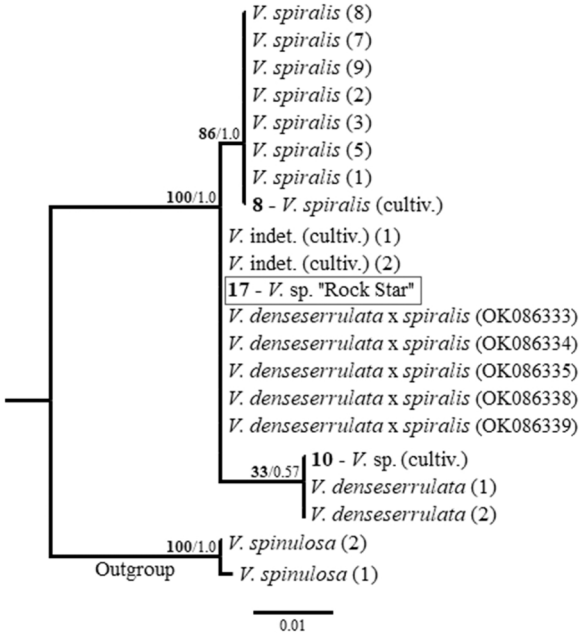

Fig. 3. Phylogram estimated from nrITS data from both Gorham et al. (2021) dataset and three new taxa from this study. Branch support is indicated by bootstrap values (left) and aBayes (right) for each node. Numbers before taxon indicate new specimens from this study in Appendix A. Scale for branch lengths is indicated.

Our study included multiple accessions of previously undetermined replicate taxa of the same species, and although the combined molecular phylogeny was useful in determining species identity for some taxa, and they were not always useful in increasing nodal support at the intra-specific level (e.g., *Vallisneria neotropicalis*).

With the exception of the caulescent taxa (i.e., *Vallisneria rubra, V. triptera*, and *V. caulescens*) and the rosulate taxa (*V. denseserrulata*, *V. spinulosa*, and *V. spiralis*), all other *Vallisneria* taxa showed relatively low sequence divergence for the data used in this study (Fig. 2). The caulescent taxa are endemic to Australia and are placed as sequential sisters to a strongly supported (BS = 100, aBayes = 1.0) subclade of taxa that are exclusively rosulate. In our phylogenetic estimates, the consistent internal placement of the caulescent taxa, along with the placement of other rosulate species (Fig. 2) was in agreement with the results from Les et al. (2008); neither analysis resolved a single clade comprising only the caulescent taxa.

Within the *V. australis* group, there is strong support (BS = 92, aBayes = 0.97) for a clade comprising two undetermined taxa and a morphologically distinct taxon from Northern Territory that we recognize as a new species (*V. jacobsii*). *Vallisneria jacobsii* and the two undetermined taxa represent the first instance of taxa closely related to *V. australis* being found in the Australian tropics of Northern Territory. The distributional range for *V. australis* is well documented within herbarium collections, and database information found on Atlas of Living Australia (2022) points to a species deeply rooted in South Australia, Victoria, New South Wales, and a few outlying specimens in Queensland. While morphological data suggests the two undetermined taxa are potentially hybrids, molecular data in this study provided no support for this hypothesis. The novel species *V. jacobsii* distinguishes itself through a

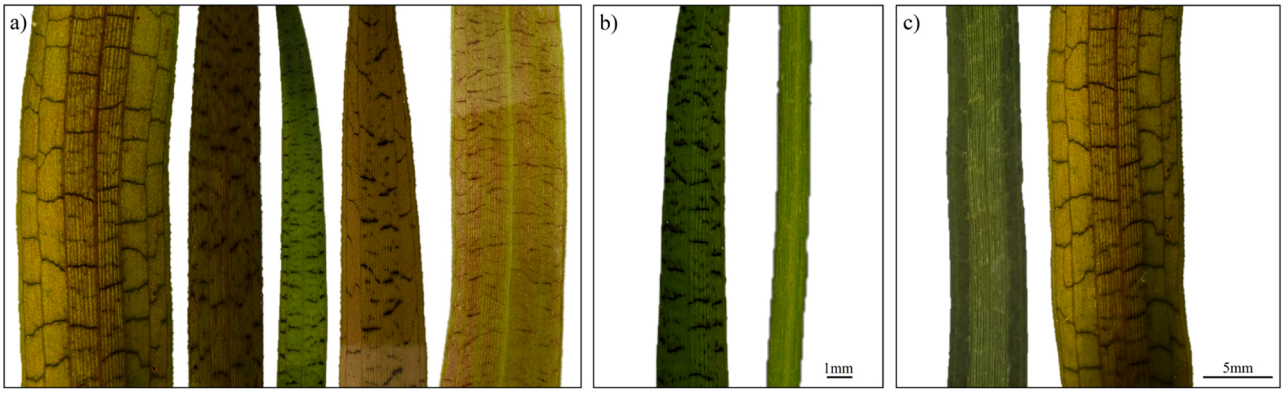

Fig. 4. a) Horizontal and diagonal striation patterns on *Vallisneria* leaves. From left to right: **18 -** *V. neotropicalis*, **3 -** *V.* indet, **1** - *V. nana*, **11** - *V.* sp. "Leopard", and **2** - *V. jacobsii.* Leaf coloration and size differences between b) **1** - *Vallisneria nana* (left) and **4** - *V. gracilis* (right); c) **14** - *Vallisneria americana* (left) and **18** - *V. neotropicalis* (right).

Fig. 5. a) Leaf coloration plasticity for *Vallisneria jacobsii* in different growing conditions. b) Phyllotaxy of *Vallisneria jacobsii* appearing distichous.

combination of morphological characters (i.e., alternating vertical red/green striping pattern and horizontal striations on leaves, and a basal growth form appearing distichous) which are entirely unique to this taxon when compared to other *Vallisneria* species.

The East Asian *V. natans* and accompanying varieties resolved as sister to the group containing *V. annua*, *V. erecta*, *V. gracilis*, and *V. nana* with high support (BS = 100, aBayes = 1.0; Fig. 2). While there is overlap in the natural distribution of *V. natans*, *V. densesserulata*, and *V. spinulosa*, their placement in the phylogenetic reconstruction suggests that they are not closely related, and the occurrence of *V. natans* represents a more derived group from a recent radiation. Consistent with the results from Les et al. (2008), we found no strong evidence to argue for the resurrection of *V. asiatica* (represented in the dataset as *V. natans* var. *natans*, and *V. natans* var. *biwaensis*) as a separate species and a dissolution of its synonymy with *V. natans*.

4.2. *Vallisneria* morphology

The general consensus of experienced aquatic botanists has been that *Vallisneria* is a difficult group to work with in terms of systematics and taxonomy (Don Les, pers. com., Christel Kasselmann pers. com., and Dave Wilson pers. com.). *Vallisneria* is known to present phenotypic plasticity that is dependent on various growing conditions like water depth (Li et al., 2018) and substrate/sediment type (Xie et al., 2005; Xiao et al., 2006; Wang and Yu, 2007). In this study, a common garden experiment was conducted to reduce as much as possible phenotypic plasticity associated with differences in growing conditions that could affect plant growth. By controlling for light, nutrients, and water quality, any true differences in morphological characters would be attributed to genetics instead of environment (Sultan, 2010). Some of the justifications for the synonymy of certain *Vallisneria* species, such as *V. gracilis*/*V. nana*, and *V. americana*/*V. neotropicalis* were directly related to the assumption that different environmental conditions are the main source for phenotypic variation (Lowden, 1982; Jacobs and Frank, 1997), so this common garden approach would shed some light on these opinions. Understandably, obtaining the specimens from Les et al. (2008) was impossible, and obtaining live plants from those specific collection points would be equally unlikely.

There appears to be independent origins of horizontal leaf striations present within the genus. *Vallisneria jacobsii*, *V. nana*, *V. neotropicalis*, and the two undetermined taxa from Northern Territory were the only plants in this study that exhibited this leaf patterning. These taxa do not form a monophyletic lineage which suggests horizontal leaf striations are homoplastic and restricted to certain tropical taxa. Les et al. (2008) observed striations in some specimens of *V. natans*, *V. spinulosa*, and *V. spiralis*; however, we did not observe these striations in *V. natans* and *V. spiralis* in this study. Included within the two subclades consisting of 1) *V. australis* + *V. jacobsii* + two undetermined *V.* spp. from Northern Territory, and 2) *V. americana* + *V. neotropicalis* + *V.* sp. (umbellate) (Fig. 2), we see what appears to be segregation of specimens based on both climate and plant coloration. The plants located in the tropics of North America (*V. neotropicalis*) and Australia (*V. jacobsii* and *V. nana*) show brown/reddish leaf coloration, and the presence of horizontal striations. The North American *Vallisneria neotropicalis* has brown leaf color, with red and black longitudinal and transverse veins. The Australian species *Vallisneria jacobsii*, *V. nana*, and the two undetermined specimens from Northern Territory show brown and red leaf colors, but the black horizontal/diagonal striations do not appear to strictly follow longitudinal or transverse veins and are scattered throughout leaf tissue (Fig. 4a). Additionally, the presence of a conspicuous band of lighter colored cells surrounding the leaf midvein called a lacunal band is found on the temperate *V. americana* from Minnesota and *V. australis* distributed in the southern parts of Australia but appears to be absent on those tropical taxa previously mentioned. This convergence on brown/red leaf color with horizontal/diagonal striations within tropical taxa is strictly speculative and would require additional research, but the divergence within molecular data for tropical taxa is sufficient evidence for the resurrection of *V. neotropicalis* and the recognition of a novel species (*V. jacobsii*) in the tropics of Northern Territory, Australia.

4.3. Species recognition

Researchers routinely disagree on the number of and limits to *Vallisneria* species, which is best illustrated by the differences between Lowden (1982) who recognized only two species and Les et al. (2008) who recognized 14 species. The most recent assessment of Hydrocharitaceae was completed by Govaerts (2011), but it seemed to neglect the results from Les et al. (2008) when it came to taxonomic revisions surrounding *Vallisneria gracilis*, and *V. neotropicalis*. The assessment did however conclude that the addition of *V. australis*, *V. erecta*, and the transfer of *Maidenia* into *Vallisneria* was warranted. In the upcoming sections, we will address each instance where molecular and morphological data suggest taxonomic changes which are consistent with both the results from Les et al. (2008), and the results from this current study.

Vallisneria gracilis synonymized with *V. nana* – Looking at the family level assessment for Hydrocharitaceae completed by Govaerts (2011), this statement was used as the justification: "These plants [*V. gracilis*] from or near to the type locality are all strongly perennial, produce very few flowers, no fruits have been found and, after cultivation for a few years, grow into plants indistinguishable from *V. nana*." (Jacobs and Frank, 1997). Additionally, Govaerts (2011) failed to cite Les et al. (2008) who recognized *V. gracilis* as a distinct species. For this study, we used a cultivated specimen of *V. gracilis*, so it is possible that morphological features we observed were the result of artificial selection for this phenotype within the aquatic plant trade (Fig. 4b). Our combined molecular data show that *V. gracilis* is not monophyletic. The lack of resolution within this subclade can likely be attributed to rapid radiation and a disconnect between available molecular data and observable morphological variation (Baldwin et al., 1998). Even if we disregard the use of the cultivated *V. gracilis* specimen, our data concurs with Les et al. (2008) results that show *V. gracilis* as more closely related to *V. erecta* and *V. annua* than to *V. nana* (Fig. 2). Thus, here we conclude that *V. gracilis* should not be in synonomy with *V. nana* and suggest that additional work be conducted to test more robustly the monophyly of *V. gracilis*.

Vallisneria neotropicalis synonymized with *V. americana* – The assessment in Les et al. (2008) that the synonymy of *V. neotropicalis* and *V. americana* should be dissolved is supported by both molecular sequence data and morphological data and suggests that these taxa should be recognized as two distinct North American species. Although the growing environment in the tropics of Florida can provide for much larger *Vallisneria* plants, the common garden experiment showed that the Florida plants maintain both coloration differences in the leaves, and overall size differences compared to plants originating from Minnesota (Fig. 4c). With the addition of sequence data from the present study into the dataset from Les et al. (2008), it is clear that the North American *Vallisneria* taxa are closely related with strong support (BS = 99, aBayes = 1), and each taxon is placed into distinct subclades that represent geography: 1) *V. americana* (BS = 100, aBayes = 1.0) in the temperate United States and Canada; and 2) *V. neotropicalis* + *V. sp.* (umbellate) (BS = 58, aBayes = 0.94) in the tropical and subtropical regions of North America (Fig. 2). From experience with field observations in both central Florida and Minnesota, taxa in these regions are very morphologically distinct; however, biogeographic analyses are required to see if there is a clear dividing line delimiting these North American species, or if a gradient exists where the morphological and molecular differences become less apparent.

Although there was strong support for the monophyly of *Vallisneria americana* (BS = 100, aBayes = 1.0; Fig. 2), the four accessions of *V. neotropicalis* were left unresolved as this group also contained the umbellate taxon *V.* sp. (umbellate). This *V.* sp. (umbellate) taxon is

unique in that unlike the rest of *Vallisneria* taxa with solitary pistillate flowers, it produces an umbellate inflorescence. Further morphological, molecular, and population genetics work are required to test if *V.* sp. (umbellate) is worthy of taxonomic recognition. Work completed by Rohal et al. (2021) indicates that these plants are dispersed within populations of other solitary pistillate *V. neotropicalis* in central Florida, but there is uncertainty in the heritability of this unique floral trait, and it may occur because of meristem damage during floral development, or as a response to varying environmental conditions. Similar to the findings of Les et al. (2008) this study observed umbellate flowers being produced on predominantly solitary pistillate-flowered specimens in both *V. neotropicalis* specimens collected in central Florida.

The current molecular dataset available for phylogenetic analysis does well to differentiate specific clades within *Vallisneria*, but it lacks the resolution to assess many of the relationships within these clades robustly. To resolve this issue, modern genomic techniques (e.g., multiplexed shotgun genotype sequencing) are needed to obtain higher resolution of phylogenetic relationships like in other taxonomically difficult plant groups (Wessinger et al., 2016; Mort et al., 2015, 2022). Feng et al. (2017) attempted whole genome assembly of *V. spinulosa* and although sequence data was insufficient for whole-genome assembly, they set a foundation for possible genome elements to be used in future phylogenetics research. Additional gene regions like *trnL*, *rpl*16, or *trnH-psbA* may offer the opportunity for better tree resolution when using Sanger sequences (e.g., Mort et al., 2007; Uttgaard et al., 2021), but due to the lack of DNA samples from Les et al. (2008) those regions were not added to this study.

4.4. *Vallisneria hybridization*

Vallisneria appear to naturally hybridize in some cases where there is a direct overlap in the distribution of closely related taxa like *V. rubra* and *V. caulescens* (McConchie, 1983). The suspected hybrids collected by L.A Craven in 1981 mentioned by McConchie were analyzed by Les et al. (2008) and were originally thought to represent a robust form of *V. rubra*, but we think are more likely *V. rubra* x *triptera* given the specimens were collected in an area very close to the type locality of *V. triptera*. These specimens were examined at the Missouri Botanical Garden Herbarium for this study (*L.A. Craven 6576*, MO#3841455). In the collector notes, Craven mentions seeing both male and female plants of *V. rubra* and *V. triptera* (previously *V. caulescens*) growing together in Nanambu Creek, so it is reasonable to see the opportunity for hybridization of these taxa. Photographs of known *V. rubra* x *triptera* hybrids from cultivation (Supplemental Fig. 2) exhibit an intermediate morphology between *V. rubra* and *V. triptera* that is very similar to those shown in Craven's herbarium specimens.

Although hybridization can play an important role in the evolution of plants, there can be harmful effects where introgression leads to postzygotic barriers (Allendorf et al., 2001; Bomblies & Weigel, 2007). Except for the cultivated specimen 7 - *V.* sp., the other putative *Vallisneria* hybrids in this study (3 & 6 - *V.* indet, 10 - *V.* sp., 11 - *V.* sp. "Leopard", and 17 - *V.* sp. "Rock Star") either did not produce flowers, or produced malformed flowers not capable of achieving pollination, which could be evidence of hybrid sterility. They did, however, rapidly produce healthy clonal vegetative growth. The cultivated specimen 7 - *V.* sp. was originally purchased as *V. americana*, but molecular evidence suggests a parental cross consisting of *V. australis* and either *V. americana* or *V. neotropicalis*.

The two undetermined Northern Territory *Vallisneria* plants listed in this study as 3 - *V.* indet. and 6 - *V.* indet. showed growth habits in the common garden experiment consisting of thick, opaque leaves whereas all other *Vallisneria* taxa have a ribbon-like, translucent nature to their leaves. When grown in an aquarium these plants produce wider, slightly more translucent dark green to brown leaves with black horizontal striations (Fig. 4a). They also exhibit antrorse bristles on both abaxial and adaxial sides of leaf apices, an autapomorphic feature unique to *V. erecta*. These plants showed zero and two SNPs in nrITS sequences, which provided no usable molecular evidence for this hypothesis, but given the morphological traits, we suggest that these plants are naturally occurring hybrids. An additional study would need to be conducted in these two rivers to identify which *Vallisneria* species are present together to further justify this claim.

A *Vallisneria* plant known as "Leopard Val" in the aquarium trade was obtained for this study and listed as 11 - *V.* sp. "Leopard" (Appendix A), and our molecular data suggest this plant to be hybrid in origin. The nrITS sequence for this plant was highly polymorphic with 50 SNPs originally presented in chromatogram data as double-peaking nucleotides, although this count was reduced to 14 SNPs after accounting for indels. This molecular evidence for hybridization is consistent with previous studies that have addressed issues with polymorphic nrITS sequences (Rauscher et al., 2002; Les et al., 2009; Kitani et al., 2011; Tippery and Les, 2013; Razifard et al., 2017; Liang et al., 2018). Sequence data for this specimen was ultimately not used for phylogenetic analyses in the combined molecular dataset but given this taxon's position within the combined cpDNA tree (Supplemental Fig. 1) along with assessing putative parental types (Fig. 1) it is possible that the maternal parent in the hybrid cross originated from Australia. The coloration and striation patterns on its leaves look similar to those of *V. nana* from Queensland, Australia (Fig. 4) which is another plant labeled "Leopard Val" in the aquarium trade. Although cpDNA, and parsing out polymorphic nucleotides offers some insight into teasing apart parental types from putative hybrid specimens, sequence cloning methods would need to be implemented for more definitive results.

Hybrid specimens collected around the southeastern United States in Gorham et al. (2021) were confirmed using nrITS sequence data and were most likely caused by aquarium trade-based introductions into nature. The specimens in the present study that are listed as 10 - *V.* sp. (cultiv.) and 17 - *V.* sp. "Rock Star" (collected from Crystal River, Florida) showed eight and ten polymorphic sites in nrITS data and were resolved within the *Vallisneria denseserrulata* + *V. spiralis* clade (Fig. 3 & Supplemental Fig. 1). Additionally, matching putative parental types (Fig. 1) supports the hypothesis that these specimens are the result of hybridization. We therefore consider this to be sufficient evidence to suggest that these two specimens, and those collected by Gorham et al. (2021) are all *V. denseserrulata* x *spiralis* hybrids.

Historically in King's Bay, Florida there were large beds of native *Vallisneria americana* plants that were both salt-tolerant, and herbivore-tolerant as they were noted to be a favorite food item for migrating manatees coming into the bay during winter months where the saltwater from the Gulf of Mexico meets with the freshwater springs of the Crystal River (Hartman, 1971; Etheridge et al., 1985). Due to multiple factors including a prolonged increased salinity in the bay from the "No Name Storm" in 1993, nearly all of this original *V. americana* population disappeared and was eventually replaced with cyanobacteria (Araiza, 2011). In order to maintain the food source for manatees, and to push back harmful algal blooms in the Crystal River, the King's Bay Restoration Project employed the help from the University of Florida and an aquatic restoration company called Sea & Shoreline, LLC (Winter Garden, Florida) to both remove harmful algae/cyanobacteria, and replant *Vallisneria*. According to the project website (https://kingsbayrestorationproject.com), two "Florida native" salt-tolerant *V. americana* plants were developed and labeled as "Rock Star" and "Salty Dog" which were then used in the large-scale replanting project. It is unknown which of these two plants were collected and used in this current study, but our molecular evidence suggests that the plants being used in this project are the same as the widespread, non-native hybrids documented by Wasekura et al. (2016) and Gorham et al. (2021).

5. Conclusion

Similar to the conclusions of Les et al. (2008), additional studies will need to be conducted to capture new data from *Vallisneria* plants in

additional geographical areas not included within this study. Most notably, *V. anhuiensis* and *V. longipedunculata* are currently two unsequenced species and their current phylogenetic placement within *Vallisneria* is unknown. These two species are not included in any phylogenetic studies so in order to get the full placement of accepted *Vallisneria* species, we would need to include these taxa. There are currently no sequence data for either species on GenBank. We hypothesize that since both were described near the type locality of *V. spinulosa* and share similar morphological features, they are likely closely related.

Despite having high overall support for the tree resulting from maximum likelihood with combined molecular data, we hesitate to accept the topology as the best representation of interspecific relationships within *Vallisneria*. The limited number of informative characters provided by the three gene regions used in this study, along with a resulting lack of resolution for multiple clades within the tree indicates that a more robust dataset needs to be established before we can produce a more accurate phylogenetic reconstruction. Additionally, the method of direct sequencing nuclear ribosomal DNA appears to fail at capturing intra-genomic variation that has been shown within nrITS sequences in other plant species (Song et al., 2012), which could affect overall tree topology within phylogenetic analyses.

6. Taxonomic treatment

Vallisneria jacobsii A. P. Martin & D. N. Wilson, sp. nov.

Type: Australia. Northern Territory: East Arnhem, Arafura Basin 12°39'30.1"S 135°11'26.0"E. Madaylanga billabong/oxbow lake near Gulbuwangay River. Collected by Ian Morris and Dave Wilson in 2007, then grown in isolated cultivation. Vouchered: 23 June 2022, *A. P. Martin #5* (holotype (♀): KANU! #431096; isotype: MO!). There were no male plants collected. Name chosen to honor Surrey Jacobs, a distinguished botanist whose passion for Australian aquatic plants will remain largely unrivaled.

Perennials, dioecious, stoloniferous. **Leaves** basal, submerged, appearing distichous, longitudinally striped alternating green and red, with dark red to black horizontal/diagonal striations, blade linear, 65–100 × 1–1.8 cm, concavo-convex in cross-section, longitudinally nerved with 5 major veins, distal margins serrulate, apex acute to slightly obtuse. **Inflorescences** axillary, 1-flowered; peduncle 0.8–1 mm wide; spathe thin, translucent. **Flowers** unisexual, sessile; pistillate 1–1.3 cm long; sepals 3, apex rounded to slightly acute; petal rudimentary 3, alternate with sepals; stigmas crimson color, pubescent, hairs white; stigmatic lobes arise alternate to sepals; staminate flowers unknown.

Molecular analyses from the present study resolve *Vallisneria jacobsii* most closely related to *V. australis*. *Vallisneria jacobsii* differs greatly from *V. australis* in certain morphological features including basal growth form (distichous vs. rosette), leaf coloration pattern (green/red striping absent lacunal band vs. solid green with lacunal band), horizontal/diagonal striations (present vs. absent), female flower size, and stigma color (crimson vs. white). Dave Wilson describes this plant as being one of the largest leafed *Vallisneria* species he has observed in the Northern Territory, but the leaf length and width measurements vary due to phenotypic plasticity. Leaf coloration and striation patterns vary depending on the growing conditions (Fig. 5).

Distribution: only known to occur in a billabong/oxbow lake named Madayalanga by the local indigenous people (Yolŋu) in East Arnhem, Northern Territory, Australia.

CRediT authorship contribution statement

Alex P. Martin: Conceptualization, Data Curation, Investigation, Resources, Validation, Visualization, Writing – Original Draft. **Mark E. Mort:** Funding acquisition, Supervision, Writing – Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A

List of taxa, collection locations (when known), and GenBank accession numbers for specimens used in this study. Material sourced from aquarium trade is listed as "cultivated." GenBank accession numbers for each specimen are given sequentially: nrITS, *trnK* 5' intron, *rbcL*.

- *Vallisneria nana*, Little Yabba Creek, Queensland, Australia (ON863731, ON863713, ON863749)
- *V. jacobsii*, Madayalanga, Northern Territory, Australia (ON863732, ON863714, ON863750)
- *V.* indet., Daly River, Northern Territory, Australia (ON863733, ON863715, ON863751)
- *V. gracilis*, cultivated, origin unknown (ON863734, ON863716, ON863752)
- *V. triptera*, Arnhem Hwy, Northern Territory, Australia (ON863735, ON863717, ON863753)
- *V.* indet., Roper River, Northern Territory, Australia (ON863736, ON863718, ON863754)
- *V.* sp., cultivated, origin unknown (ON863737, ON863719, ON863755)
- *V. spiralis*, cultivated, origin unknown (ON863738, ON863720, ON863756)
- *V. natans*, cultivated, origin unknown (ON863739, ON863721, ON863757)
- *V. denseserrulata* x *spiralis*, cultivated, origin unknown (ON863740, ON863722, ON863758)
- *V.* sp. "Leopard", cultivated, origin unknown (ON863741, ON863723, ON863759)
- *V. neotropicalis*, cultivated, origin unknown (ON863742, ON863724, ON863760)
- *V. americana*, Square Lake, Minnesota, United States, (ON863743, ON863725, ON863761)
- *V. americana*, Square Lake, Minnesota, United States, (ON863744, ON863726, ON863762)
- *V. americana*, Square Lake, Minnesota, United States, (ON863745, ON863727, ON863763)
- *V. neotropicalis*, Lake Hernando, Florida, United States, (ON863746, ON863728, ON863764)
- *V.* sp. "Rock Star", Crystal River, Florida, United States, (ON863747, ON863729, ON863765)

- *V. neotropicalis*, Little Jones Creek, Florida, United States, (ON863748, ON863730, ON863766)

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2023.103669.

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