

Systematics of *Vallisneria* (Hydrocharitaceae)

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Abstract—Morphology, species delimitation, and interspecific relationships were evaluated in a phylogenetic context in the aquatic monocotyledon genus *Vallisneria* using a combination of morphological and molecular (nrITS, *rbcL*, *trnK* 5' intron) data. Contrary to previous studies that recognized few species worldwide, we distinguished 12 species by molecular data, and an additional 2–3 species by morphological differences within groups that were invariant at the molecular level. Two new *Vallisneria* species (*V. australis*, *V. erecta*) are formally described. Other potentially novel species were detected from the cultivated material examined but require further study to elucidate their taxonomic status. Phylogenetic analyses indicated that vitate (caulescent) species (including *Maidenia rubra*) are not basal, but nested between two groups of rosulate (rosette) species. To preserve *Vallisneria* as monophyletic, a new combination is made (*V. rubra*) that accommodates the transfer of *M. rubra* to *Vallisneria*. Several taxonomic characters associated with the stigma morphology of pistillate flowers were found to represent suites of features related to pollination. In most cases, these character suites corresponded to a particular arrangement of filaments in the staminate flowers. The precise geographical origin of *Vallisneria* remains difficult to determine. However, we conclusively documented the presence of the Old World *V. spiralis* in Texas (United States), which constitutes the first authentic record of this nonindigenous species in North America.

Keywords—Bayesian analysis, chloroplast DNA, *Maidenia*, *Nechamandra*, nuclear ribosomal DNA, phylogenetics.

Vallisneria L. (Hydrocharitaceae Juss.) is a monocotyledon genus of uncertain taxonomic diversity, but includes an estimated four to ten species worldwide (Cook et al. 1974; Cook 1996a). Although *Vallisneria* itself is distributed widely (Sculthorpe 1967), the genus typically exhibits low regional diversity throughout its range, with the highest number of species reported in Australia (Jacobs and Frank 1997). A recent phylogenetic study of Hydrocharitaceae has indicated that *Vallisneria*, *Maidenia* Rendle, and *Nechamandra* Planch. are closely related and resolve as a well-supported and relatively derived clade within subfamily *Hydrilloideae* Luer. (Les et al. 2006b).

Vallisneria species are annual or perennial, dioecious, submersed, freshwater aquatics that are highly valued commercially as ornamental aquarium specimens (Kasselman 2003). Because *Vallisneria* is cultivated so widely, the potential for introductions to nonindigenous regions is high (Lowden 1982); however, a more reliable means of identifying species and cultivars is necessary before this possibility can be evaluated with confidence. Ecologically, the plants are an important source of food for a variety of wildlife. The buds, foliage, fruits, roots and tubers are eaten by many waterfowl (Martin and Uhler 1939; McAtee 1939; Schloesser and Manny 1990; Zhang and Lu 1999) and augment the diets of various freshwater crabs, herbivorous fish, manatees, moose, muskrats and turtles (de Vos 1958; Bengtson 1983; Zu et al. 1999; Armstrong and Booth 2005). The leaves support large populations of aquatic invertebrates, which are eaten by many species of fish (Feldman 2001).

Hydrocharitaceae are intriguing botanically because of their diverse morphology and broad range of reproductive systems. The family is primitively unisexual but contains species with derived bisexual as well as dioecious and monoecious sexual conditions (Les et al. 1997). Hydrocharitaceae also comprise a spectrum of pollination methods including entomophily, hydrophily, and a unique system des-

ignated simply as “type III” (Cook 1982). The latter system, found only in Hydrocharitaceae, involves the complete detachment of staminate flowers from a spathe at the base of the submersed male plants; the flowers rise to the surface, where they open to form free-floating, raft-like structures that disperse on the water surface by wind and currents (Duchartre 1855; Kerner 1891; Cook 1982). Despite its unorthodox nature, the type III system has evolved independently several times within the family, and includes three subtypes (A, B, C), each distinguished by differences in the specific means of pollen delivery (Cook 1982; Les et al. 2006b).

In *Maidenia*, *Nechamandra* and *Vallisneria* (all designated as type III-B), the pollen remains dry within elevated anthers and the stigmas remain dry within the perianth of the pistillate flower. Pollination ensues as the floating staminate flowers aggregate around the solitary pistillate flowers, which orient their opening just at the water surface while remaining attached to the submersed female plants by long, flexuous peduncles. Eventually, the anthers make contact with the stigmas and deposit their pollen, occasionally as entire staminate flowers tumble into the pistillate flower (Wylie 1917; Svedelius 1932; Kausik 1939; Sculthorpe 1967; Cook 1982, 1996a). After fertilization, the peduncle coils into a spiral, effectively pulling the developing fruit under water to complete its maturation (Kausik 1939; Wilder 1974).

Despite the attention focused on these reproductive peculiarities, the taxonomy of *Vallisneria* has remained poorly understood, and species limits require further clarification. For many years, the original Linnaean name *Vallisneria spiralis* was applied indiscriminately to similar rosulate (rosette) plants that inhabited Asia, Australia, Europe and North America. Eventually, numerous differences were detected among plants from all four continents, prompting authors to call for a revision of the genus (Svedelius 1932; Miki 1934; Kausik 1939). More detailed observations have led several authors to distinguish a number of taxa in North America,

Asia and Australia from what originally was regarded as *V. spiralis* (Miki 1934; Marie-Victorin 1943; Kadono 1994; Jacobs and Frank 1997; Haynes 2000).

Rendle (1916) erected the Australian genus *Maidenia*, which resembles *Vallisneria* but differs conspicuously by its vittate (caulescent) habit. There also have been two vittate Australian species (*V. caulescens*, *V. triptera*) assigned specifically to *Vallisneria* (Bailey 1888; Jacobs and Frank 1997). Preliminary phylogenetic studies of Hydrocharitaceae have indicated that *Maidenia* probably is not distinct from *Vallisneria* (Les et al. 2006b); however, additional sampling of taxa is necessary to confidently ascertain the phylogenetic relationships of the vittate plants with respect to the rosulate species.

The only contemporary treatment of *Vallisneria* that approaches a comprehensive study was made by Lowden (1982), who radically revised the taxonomy by recognizing only two species worldwide, each with two varieties. Lowden's taxonomic treatment of *Vallisneria*, which strongly emphasized a few microscopic floral characters, has been followed widely for nearly a quarter of a century. However, the technical difficulty of observing the key features (Jacobs and Frank 1997) has hindered the efforts of taxonomists to evaluate their taxonomic utility, and many specimens worldwide remain difficult to identify. Furthermore, the taxonomic scheme proposed by Lowden (1982) deserves further scrutiny with respect to the status of the vittate species, which were not considered in his study.

Undeniably, very few morphological characters in *Vallisneria* are consistent enough to be useful taxonomically due to extensive phenotypic plasticity. Many readily observable vegetative features such as proportions of leaves and fruits, their color, dentition of leaf margins, etc., vary considerably and have been abandoned by some taxonomists (Lowden 1982). Other features, particularly those relating to floral morphology, have been lost or compacted as a consequence of reduction (Rendle 1916). Yet, some features (e.g. vittate vs. rosulate habit) can provide consistent distinguishing characteristics; thus the morphology of *Vallisneria* should be thoroughly reevaluated.

Few phylogenetic hypotheses have been presented for *Vallisneria*. Svedelius (1932) postulated that *Vallisneria* species with more deeply incised stigmatic lobes were more primitive than those with less-divided lobes. Lowden (1982) extended this concept by suggesting that the species with entirely free lobes and "free" staminodes were even more primitive. Lowden (1982) further remarked that fused stamen filaments "might represent a derived trait" with respect to stamens having completely free filaments. This opinion was echoed by McConchie and Kadereit (1987), who also considered androecial hairs to represent a derived condition.

Lowden (1982) encountered unusual umbellate (occasionally monoecious) inflorescences on some North American *Vallisneria* specimens, but regarded them as aberrant and insignificant taxonomically, and considered them to indicate a primitive condition from which the solitary pistillate inflorescences and dioecious condition had arisen. He believed that the few umbellate plants collected from several tropical/subtropical American localities were "marginal ancestral stocks" that had persisted by means of "very effective" asexual reproduction (Lowden 1982). Lowden also generalized that evolution in *Vallisneria* had proceeded along "a continuous gradient in floral variation" and he regarded the higher frequency of bisexual populations in temperate areas

to indicate a temperate origin of the genus (Lowden 1982). We would add that the vittate growth form found in *Maidenia* and *Nechamandra* (the presumed outgroups of *Vallisneria*) could lead one reasonably to conclude that vittate *Vallisneria* species are primitive in the genus and the rosulate species derived.

However, because no comprehensive phylogenetic framework currently exists for *Vallisneria*, it is impossible to evaluate any of these hypotheses or the potential convergence of morphological characters that have been used taxonomically. Given that there are several outstanding examples of morphological convergence known in Hydrocharitaceae (Les et al. 2006b) the influence of homoplasy on the taxonomic utility of morphological features in the genus should not be underestimated and warrants a comprehensive appraisal using phylogenetic methods.

In this study, we have conducted a phylogenetic analysis of *Vallisneria* using morphology and molecular data obtained from maternally inherited (cpDNA: *rbcL*, *trnK* 5' intron) and biparentally inherited (nuclear: nrITS) genomes. Our survey includes specimens collected throughout the range of the genus, as well as a variety of material in cultivation worldwide. Our main objectives were to 1) establish a sound phylogenetic framework for *Vallisneria* and related genera; 2) use this framework to assess the monophyly of the genus and relationships among the species; 3) determine whether morphological convergence has influenced the taxonomic utility of characters currently used to circumscribe taxa; 4) use the results of phylogenetic analysis to improve the taxonomy of *Vallisneria*; and, 5) determine the origins of cultivated material and evaluate introductions of nonindigenous species where possible.

MATERIALS AND METHODS

Taxon Sampling—When preliminary results indicated that Lowden's (1982) two-species system was untenable, we designated eighteen taxa for analysis (*Nechamandra* + *Maidenia* + 16 *Vallisneria*). Previous studies (Les et al. 1997; 2006b) consistently identified *Nechamandra* as the outgroup to the remaining taxa based on phylogenetic analyses of the entire family Hydrocharitaceae. *Maidenia* (monotypic) is the only other genus in this group to have been distinguished by contemporary authors. Taxa within *Vallisneria* include those entities accepted in current taxonomic treatments as well as those recognized by the present authors as potentially novel.

Of these 18 taxa, we obtained 51 accessions (OTUs) representing field-collected material from Africa, Asia, Australia, Europe, and North America, as well as some cultivated specimens of unknown provenance (Appendix 1). We compared multiple accessions for all but three taxa (*Nechamandra alternifolia*; *Vallisneria asiatica* var. *asiatica*, *V. gracilis*). *Vallisneria asiatica* var. *asiatica*, *V. asiatica* var. *biwaensis*, and *V. gracilis* were sampled only from cultivated material; however, we were confident about the geographical origin of these cultivated specimens. All other taxa were represented by at least one specimen collected directly from the field within their native distributional range. *Nechamandra* was used as the outgroup for all analyses.

Morphological Data and Analysis—We scored states for 26 morphological characters (9 vegetative, 17 reproductive) across all 18 designated taxa (Tables 1, 2) using information compiled from pertinent sources (Richard 1811; Chatin 1855a, 1855b; Parlato 1855; Rendle 1916; Wylie 1917; Fernald 1918; Svedelius 1932; Miki 1934; Witmer 1937; Kausik 1939; Marie-Victorin 1943; Kaul 1970; Aston 1973; Cook et al. 1974; Wilder 1974; Cook 1982; Cook and Lüönd 1982; Lowden 1982; McConchie 1983; McConchie and Kadereit 1987; Sainty and Jacobs 1994; Cook 1996a, 1996b; Jacobs and Frank 1997; Cowie et al. 2000; Haynes 2000). For five characters (leaf length, leaf width, fruit length, peduncle length [all maximal] and seed length and width) continuous (quantitative) data were converted to discrete states using break points that reasonably characterized the salient morphological differences among *Vallisneria* taxa as observed in nature or described in the preceding references. Maximum length and width values for seeds were converted into length: width ratios that were

TABLE 1. Morphological characters and states used in phylogenetic analysis of *Vallisneria*.

Vegetative: 1. habit (perennial = 0; annual = 1); 2. growth form (caulescent = 0; rosulate = 1); 3. mudflat forms (absent = 0; present = 1); 4. max. leaf length (short [4–10 cm] = 0; medium [50–80 cm] = 1; long [100–300 cm] = 2); 5. max. leaf width (narrow [1–7 mm] = 0; medium [10–15 mm] = 1; broad [16–35 mm] = 2); 6. red/brown pigmented leaf striae (absent = 0; present = 1); 7. leaf blades (flat to undulate = 0; strongly twisted spirally = 1); 8. mature leaf apex (acute = 0; obtuse/rounded = 1); 9. apex teeth (sparse [$< 2/\text{mm}$] = 0; dense [$\geq 2/\text{mm}$] = 1).
Reproductive: 10. petal number (δ) (none = 0; one = 1); 11. locules/anther (three or four = 0; two = 1; one = 2); 12. filaments (free/united only at base [‘V-shaped’] = 0; partially/fully fused [‘Y-shaped’] = 1); 13. hairs at base of androeceium (absent = 0; present = 1); 14. hypanthium (shorter than the peduncle = 0; longer than the peduncle = 1); 15. petal number (\varnothing) (three = 0; two = 1; none = 2); 16. staminode number (\varnothing) (three = 0; two = 1; none = 2); 17. free portion of staminode (arising at upper edge of adjacent stigma margins = 0; arising below upper edge of adjacent stigma margins = 1); 18. staminodes (cylindrical = 0; flattened = 1); 19. stigma (divided $> \frac{2}{3}$ to base = 0; divided $< \frac{1}{2}$ to base = 1); 20. max. fruit length (medium [1–15 cm] = 0; long [16–27 cm] = 1); 21. max. peduncle length (sessile [0 cm] = 0; medium [13–80 cm] = 1; long [100–300 cm] = 2); 22. fruit cross-sect. (ovoid = 0; flat/linear = 1; triangular = 2); 23. fruit margin (wingless = 0; two-three-winged/ridged = 1); 24. female flowers (solitary = 0; in umbels = 1); 25. seed length/width ratio (max. mm) (1.7–2.0 = 0; 2.3–2.5 = 1; 3.0–5.0 = 2); 26. seed (wingless = 0; winged = 1).

then assigned to three discrete states (Table 1). Taxa possessing multiple states for a character were coded as polymorphic. All character states were treated as unordered in the analyses.

The phylogenetic distribution of morphological character state data was analyzed using unweighted maximum parsimony (MP) as implemented in PAUP* v4.0b10 (Swofford 2002). Searches were completed using the branch-and-bound algorithm (furtherest addition sequence; Multrees option; multistate characters treated as polymorphisms) with *Nechamandra* as the outgroup (Les et al. 2006b). Results yielding multiple trees of equal length were depicted as strict consensus trees. Internal support for nodes was estimated using a bootstrap (BS) analysis (full heuristic search; 1,000 replicates; maxtrees increased automatically).

Molecular Data and Analyses—Total genomic DNA was extracted from 50 accessions representing 18 taxa of *Maidenia*, *Nechamandra*, and *Vallisneria* (Table 1). Source material for DNA extraction was dried in silica, preserved in CTAB (Rogstad 1992; Thomson 2001), or obtained from live plants. Our extraction method followed Doyle and Doyle (1987), adjusted to accommodate smaller volumes and with the following modifications: samples were incubated in CTAB buffer overnight at 65°C;

RNase A was added at the end of the incubation step; chloroform alone (without isoamyl alcohol) was employed to isolate nucleic acids, and two chloroform extractions were done; DNA precipitation involved one aliquot of isopropanol with 0.9 M ammonium acetate, followed by addition of 100% ethanol. Samples were kept at -20°C overnight to precipitate DNA.

Primers for DNA amplification were obtained for each of the three target gene regions: *rbcL*, *trnK* 5' intron, and the nuclear ribosomal ITS region (nrITS). For *rbcL*, the flanking primers were 1F (5'-ATGTCACCA-CAACAGAACTAAAGC-3') and 1204R-m1 (5'-CCTAAGGGTGTCT-TAAAGTTTCTCC-3'). For nrITS, the ITS5/ITS4 primer pair was used (Baldwin 1992). External amplification/sequencing primers for the *trnK* intron were: 0067F (5'-GATCCTGAAAGGTAATGAATGG-3') and 1198R (5'-CCTTCTCTCCATTGTTGG-3'); in addition, internal sequencing primers were: (0468F: 5'-ATAAGTGTGTATAAGAAAC-3'; 0510R: 5'-TCTTCTTTTCATCAGTAT-3').

Target concentrations for each DNA amplification were 100 ng genomic DNA, 500 nM each primer, 2.5 mM MgCl_2 , and 0.5 units of heat-stable polymerase in a 25 μL reaction. Dimethyl sulfoxide (DMSO) was included at one-tenth the final volume to minimize secondary structure. Prior to the addition of polymerase, samples were heated at 94°C for 2 min to denature potentially interfering enzymes. Thermal cycling involved 2 min initial denaturation at 94°C , then 29 cycles of 40 s at 94°C , 52°C for 40 s, and 72°C for 1 min for every 1 kb of target amplification; final extension was completed at 72°C for 10 min. Amplification products were cleaned using the QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, California).

Cycle sequencing mix consisted of 3–5 μL cleaned amplification product, 2 μL BigDye® Terminator v1.1 (Applied Biosystems, Foster City, California), 0.5 μL DMSO, 1.25 mM primer, and water to bring the total volume to 10 μL . Samples underwent 40 cycles of 20 s at 94°C , 15 s at 50°C , and 4 min at 60°C , after an initial 2 min denature at 94°C . Amplified products were cleaned using Sephadex® columns (Sigma-Aldrich, St. Louis, Missouri). Sequencing was conducted on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). DNA Sequence chromatograms were proofread using the program 4Peaks v1.6 (Griekspoor and Groothuis 2005) and exported in FASTA format. Contigs were assembled for each accession and aligned manually to other sequences using MacClade 4.06 (Maddison and Maddison 2000).

We generated nrITS sequence data for all 51 OTUs and *trnK* 5' intron data for 47 OTUs, which included all accessions that exhibited different ITS sequences. Because of the very low level of variability observed for *rbcL*, data for this locus were obtained from only one exemplar for each of the 18 designated taxa (0.56% missing data cells). In addition to sequence data, matrices of insertion/deletion (indel) events were compiled for nrITS and *trnK* 5' intron (*rbcL* sequences lacked indels). Indels were scored as present or absent, with an indel that spanned several consecutive nucleotides treated as a single character with states corresponding to the length of the indel. The indel matrices were complete for all taxa (no missing data). A “core group” of 18 OTUs was defined, which consisted

TABLE 2. Matrix of morphological character states used in phylogenetic analysis of *Vallisneria*. ? = data missing; na = data not applicable; * = outgroup.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
<i>Maidenia rubra</i> Rendle	1	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	1	0	1	0	1	0	0	0
<i>Nechamandra alternifolia</i> Planch.*	0	0	0	0	0	0	0	0	0	1	1	0	0	1	2	2	na	na	0	0	0	0	0	0	0	0
<i>Vallisneria americana</i> Michx.	0	1	0	2	1	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	1	0	0	0	2	0
<i>V. annua</i> S. W. L. Jacobs & K. A. Frank	1	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	0	0	2	0
<i>V. asiatica</i> Miki var. <i>asiatica</i>	0	1	0	1	1	0	0	0	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	0	2	0
<i>V. asiatica</i> var. <i>bivaensis</i> Miki	0	1	0	1	1	0	1	0	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	0	2	0
<i>V. australis</i> [sp. nov.]	0	1	0	2	2	0	0	1	0,1	1	1	1	0	0	0	0	0	0	1	1	2	0	0	0	1	0
<i>V. caulescens</i> F. M. Bailey & F. Muell.	1	0,1	0	0	2	0	0	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	0
<i>V. denseserrulata</i> Makino	0	1	0	2	1	0	0	0	0	1	2	0	0	0	0	0	1	0	0	2	1	0	0	0	2	0
<i>V. erecta</i> [sp. nov.]	0	1	1	1	2	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	2	0	0	0	1	0
<i>V. gracilis</i> F. M. Bailey	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0	?	1	0	0	0	?	?
<i>V. nana</i> R.Br.	0	1	0	2	2	0	0	0	0,1	1	1	0,1	0	0	0	0	0	0	1	0	2	0	0	0	2	0
<i>V. natans</i> (Lour.) Hara	1	1	0	2	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	1	1	1	0	0	2	0
<i>V. neotropialis</i> Marie-Vict.	0	1	0	2	2	1	0	1	1	1	?	1	1	0	0	0	0	0	1	0	2	0	0	0	?	?
<i>V. sp.</i> (umbellate plants)	0	1	0	1	2	1	0	1	1	1	?	0	1	0	0	0	0	0	1	0	1	0	0	1	?	?
<i>V. spinulosa</i> S. Z.Yan	0	1	0	2	1	0,1	0	0	0	1	2	0	0	0	0	0	1	0	0	0	2	2	1	0	2	1
<i>V. spiralis</i> L.	0	1	0	2	1	0,1	0	1	0	1	2	0	0	0	0	0	1	0	0	0	1	0	0	0	2	0
<i>V. triptera</i> S. W. L. Jacobs & K. A. Frank	1	0	0	0	1	0	0	1	1	1	0	1	1	0	0	0	0	0	1	0	1	2	1	0	1	0

of a complete set of sequences (nrITS, *rbcL*, *trnK* 5' intron), indel matrices, and morphological data.

The *trnK* sequences for *V. spinulosa* were polymorphic on initial sequencing, so the cleaned amplicon (accession 1, Table 1) was subcloned using the TOPO TA Cloning Kit with pCR2.1-TOPO Vector (Invitrogen Corporation, Carlsbad, California), and then amplified and sequenced as above. Two distinct sequence variants were isolated that together accounted for the initial polymorphic sequence (data not shown). Multiple clones of each sequence were compared against each other and the original polymorphic sequence to correct for cloning artifacts. Both variants localized to the same branch of the *trnK* 5' intron tree, however, one of these was far more divergent. The divergent sequence was deposited in GenBank (accession number EF143057), and subsequently removed from analysis, and the less divergent sequence was retained. Because the two accessions of *V. spinulosa* had identical polymorphic sequences, the retained clone sequence was used for both accessions.

Data from *rbcL*, *trnK* 5' intron, and nrITS region were partitioned to facilitate different permutations of combined analysis. A partition-homogeneity test (incongruence length-difference test or ILD) was implemented to evaluate the homogeneity of different data partition subsets (including morphology) using PAUP* v4.0b10 (Swofford 2002). We removed invariant and uninformative sites prior to each analysis following the modified method suggested by Lee (2001). The test implemented 1,000 replicates (heuristic search, simple addition sequence, TBR, maxtrees = 1,000). Comparisons were made between different cpDNA data partitions (*rbcL* vs. *trnK* 5' intron), cpDNA vs. nrDNA (*rbcL* + *trnK* 5' intron vs. nrITS), morphological data vs. individual molecular data partitions (*rbcL*; *trnK* 5' intron; nrITS), and morphological data vs. combined molecular data (*rbcL* + *trnK* 5' intron + nrITS). All comparisons were evaluated using a threshold of $p < 0.001$.

With the ILD test indicating the combinability of all molecular data (see Results below), the following MP analyses were conducted using PAUP* (Swofford 2002): a heuristic search (simple addition sequence; MulTrees option; maxtrees increased automatically) was used to analyze the nrITS data (including indels) for all 51 OTUs and the *trnK* 5' intron data (with indels) for a subset of 47 OTUs. A branch & bound search (furthest addition sequence) was used for all analyses restricted to the 18 core taxa including the combined cpDNA data (*rbcL* + *trnK* 5' intron with indels), the combined molecular data (nrITS with indels + *rbcL* + *trnK* 5' intron with indels), and the combined morphological and molecular data. Although the ILD test found the morphological data to be only marginally congruent with the molecular data (see Results below), we executed a combined MP analysis (as above) that included all characters to evaluate the effect of adding the nonmolecular data to the combined molecular analysis. To facilitate this analysis, all multistate sites (including morphological data) were treated as uncertainties. For all MP analyses, internal support for nodes was estimated by bootstrap analysis (BS), implemented as described for morphological data above (full heuristic search; 1,000 replicates; maxtrees = 100,000).

All data were analyzed by Bayesian methods using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). An appropriate model of evolution (under the AIC criterion) was selected for each data partition using the program Modeltest v3.4 (Posada and Cran-

dall 1998; Posada and Buckley 2004). We employed models that were closest to (nrITS: GTR + Γ) or the same as (*rbcL*: HKY + I; *trnK* 5' intron: GTR + I) those indicated by Modeltest. All combined analyses were conducted while retaining the appropriate model for each data partition, with nonnucleotide data analyzed using the "datatype = standard" option of MrBayes using all default parameters. Four separate runs were made with each data set to compare the resulting likelihood scores. Markov Chain Monte Carlo was implemented with four heated chains and trees were sampled every 1,000th generation for two-million generations. We discarded as burn-in the first 25% of the total number of generations. We generated a 50% majority rule consensus tree from the remaining trees, in which the percentage of nodes recovered represented their posterior probability (PP). Because the tree topologies resulting from Bayesian analyses recovered essentially the same well-supported nodes as those resolved using MP, only the MP trees (strict consensus) with nodal support indicated by both BS and PP values are presented here.

To indicate relative phylogenetic signal (Hillis 1991), we computed skewness (g_1) values for all data sets (18 taxa) using PAUP* (Swofford 2002) to evaluate a sample of 1,000,000 random trees for each.

We used the consensus tree topology resulting from the MP analysis of combined molecular data to map the state distributions for four floral characters (Table 1; characters 12, 13, 17, 19) emphasized in recent taxonomic treatments (e.g. Lowden 1982); in each case the "1" state has been postulated as the derived condition. The character states were mapped for the 18 core taxa using PAUP* (Swofford 2002). We did not use the combined morphological/molecular data tree for this analysis in order to provide an independent assessment of the character state distributions.

All DNA sequence data have been deposited in GenBank under the following accession numbers: EF142954–EF143066, EF155532, EF694962–EF694964 (Appendix 1). A copy of the complete data matrix has been deposited in TreeBASE (study number S1824).

RESULTS

Morphological Analysis—Statistics for the all analyses are summarized in Table 3. The morphology cladogram was poorly supported (Fig. 1). Only four of the 12 nodes resolved had both bootstrap values > 50% and Bayesian posterior probabilities > 0.50 (Fig. 1).

Phylogenetic analysis of morphological data (Fig. 1) resolved *Maidenia rubra* within *Vallisneria*, in a weakly-supported clade with *V. triptera* and *V. caulescens* (BS = 33; PP = 0.16). The basal position of this "vittate clade" indicated a single derivation of the rosulate habit (remaining *Vallisneria* taxa). Morphological analyses indicated multiple clusters of Australian taxa, which were dispersed among species from Asia and North America. The two varieties of *V. asiatica* along with their sister species *V. natans* formed a clade (BS = 61; PP = 0.73). The umbellate *Vallisneria* taxon and *V. neo-*

TABLE 3. Summary statistics for maximum parsimony [MP] and Bayesian analyses of single and combined datasets. Data reported for nrDNA include ITS-1, 5.8s, ITS-2 (with indel characters); *trnK* = 5' intron (with indel characters). Morph = morphology; CI = consistency index; CI_(exc) = consistency index excluding uninformative sites; RI = retention index; lnL = natural log-likelihood score (harmonic mean); α = the mean of alpha, the shape parameter of the Γ distribution; p invar. = proportion of invariant sites; s.d. = standard deviation. Na = not applicable to analysis.

	morph	nrITS	<i>trnK</i>	<i>rbcL</i>	<i>trnK</i> + <i>rbcL</i>	<i>trnK</i> + <i>rbcL</i> + nrDNA	all data
# characters/alignment length	26	674	1115	1106	2221	2895	2921
% missing data cells	2.4	0.07	2.9	0.56	0.59	0.46	0.48
skewness (g_1)	-0.40	-1.03	-1.43	-1.32	-1.39	-1.21	-1.18
# (%) variable characters	26 (100)	216 (32)	131 (12)	49 (4)	176 (8)	392 (14)	418 (14)
# (%) informative characters [MP]	19 (73)	124 (18)	65 (6)	16 (1)	56 (3)	142 (5)	161 (6)
# trees [MP]	3	10	8394	4	34	2	2
tree length [MP]	71	317	159	53	204	519	595
CI [MP]	0.56	0.83	0.89	0.98	0.93	0.86	0.81
CI _(exc) [MP]	0.51	0.75	0.82	0.95	0.83	0.72	0.65
RI [MP]	0.64	0.95	0.97	0.98	0.90	0.81	0.75
lnL	-270.15	-2575.14	-2461.03	-1918.38	-4186.77	-6673.75	-7085.75
α (s.d.) [nrDNA]	na	0.2828 (0.0449)	na	na	na	0.3540 (0.0954)	0.4126 (0.1001)
p invar. (s.d.) [<i>trnK</i>]	na	na	0.6547 (0.0397)	na	0.3765 (0.1562)	0.6808 (0.0381)	0.6906 (0.0316)
p invar. (s.d.) [<i>rbcL</i>]	na	na	na	0.7947 (0.1064)	0.8729 (0.0271)	0.8908 (0.0108)	0.8855 (0.0100)

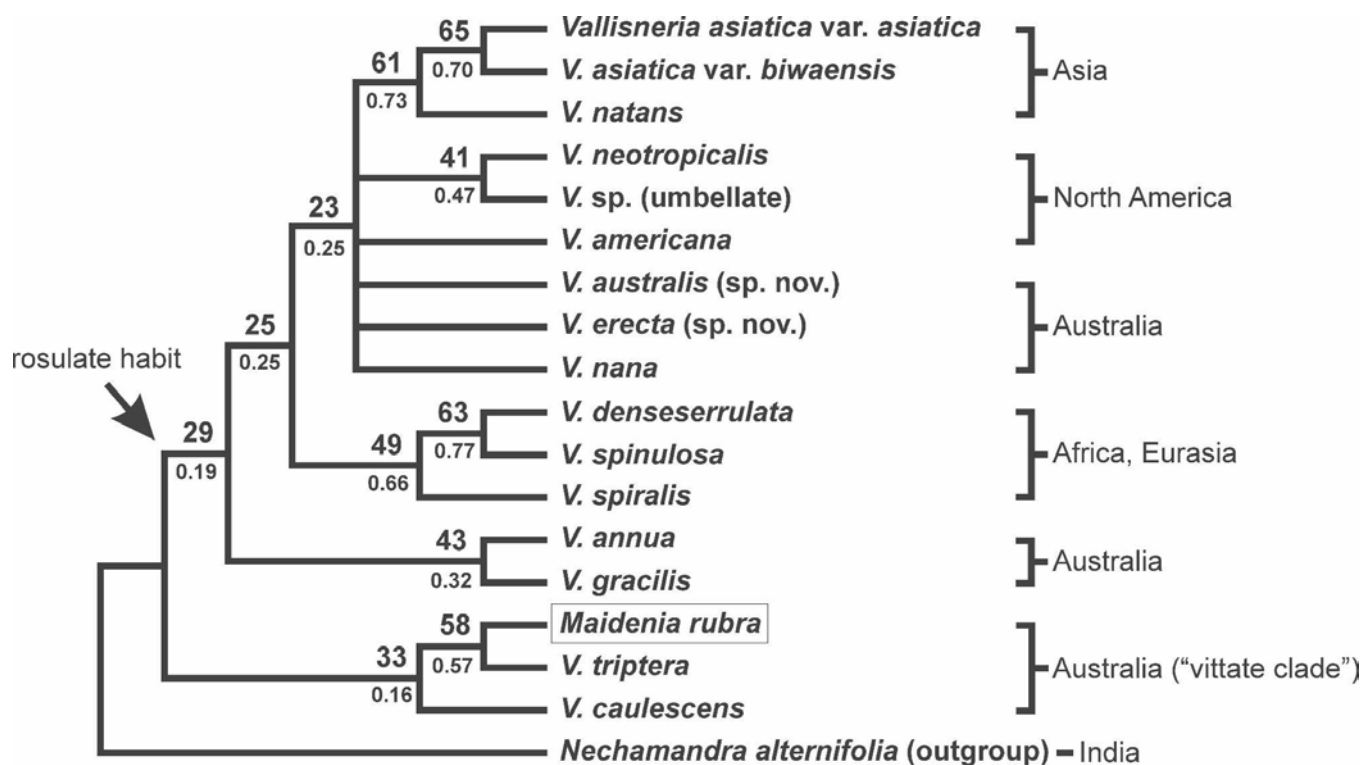


FIG. 1. Strict consensus tree derived from MP analysis of morphological data for *Vallisneria* and *Maidenia rubra* (boxed). Geographical distributions are indicated to right. Bootstrap values (upper) and Bayesian posterior probabilities (lower) are shown for all nodes. A clade of rosulate taxa is indicated by the arrow.

tropicalis associated as a weakly-supported clade (BS = 41; PP = 0.47; Fig. 1).

Molecular Analyses—NUCLEAR RIBOSOMAL DATA—Similar to the morphological analysis, the nrITS tree (Table 3, Fig. 2) also embedded *Maidenia rubra* within *Vallisneria* among the vittate *V. triptera* and *V. caulescens*, although the precise interrelationships of these taxa were not well-supported. However, the nrITS cladogram differed fundamentally by placing *V. spinulosa* as the sister to all remaining *Vallisneria* taxa and *Maidenia*, but with no statistical support (BS = 40; PP = 0.48).

As was the case with morphology, the nrITS data recognized several independent clades of Australian taxa; however, unlike morphology, nrITS data resolved a strongly-supported clade (BS = 100; PP = 1.00) consisting of the tropical Australian *V. annua*, *V. erecta*, *V. gracilis*, and *V. nana*. This clade remained distinct from a more widespread Australian taxon that we eventually recognized as *V. australis*, a distinct and novel species (Fig. 2). Consistent with results from morphology, nrITS data recovered a clade consisting of *V. asiatica* (both varieties) and *V. natans* but with strong support (BS = 94; PP = 1.00). The nrITS data also clearly indicated the distinct nature of *V. americana* and *V. neotropica*, as well as the association of the umbellate material with the latter (Fig. 2). *Vallisneria spiralis* was strongly supported as the sister species of *V. denseserrulata* (BS = 100; PP = 1.00; Fig. 2), a result conflicting with the morphological data, which weakly resolved *V. denseserrulata* and *V. spinulosa* as sisters (Fig. 1). The nrITS data also indicated two genetically distinct elements within the *V. denseserrulata* clade (Fig. 2), a group of naturally occurring plants (Appendix 1; accessions #1–2) and two accessions comprising plants of unknown provenance that were sampled from material distributed in cultivation (Appendix 1; accessions “*V. indet. [cultiv.]*” #1–2). Interpopula-

tional variation in *V. australis* and *V. caulescens* also was evident from the nrITS data (Fig. 2).

CPDNA DATA—The *trnK* 5' intron data (Table 3) provided strong support for only three clades involving different taxa (Fig. 3): 1) *Vallisneria denseserrulata* (and associated cultivated material) + *V. spiralis* (BS = 100; PP = 1.00); 2) *Maidenia* and all *Vallisneria* taxa excluding *V. denseserrulata* (and associated cultivated material), *V. spinulosa* and *V. spiralis* (BS = 99; PP = 1.00); and 3) the previous clade less the vittate *Maidenia rubra*, *V. caulescens* and *V. triptera* (BS = 89; PP = 1.00). A clade with weak support (BS = 69; PP = 0.87) included *V. asiatica* (both varieties) and *V. natans* but was not resolved in the strict consensus. The *trnK* 5' intron data supported an association of *Maidenia rubra* and *V. triptera* (BS = 64; PP = 0.99); however, that result also was not resolved by the strict consensus tree. A clade consisting of *V. denseserrulata*, *V. spinulosa* and *V. spiralis* was resolved, but only with weak support (BS = 57; PP = 0.49). Most multiple taxon accessions had identical *trnK* 5' intron sequences with the exception of *Maidenia rubra* (three haplotypes) and *V. spiralis* material from Africa, which differed from the European accessions by a single substitution.

The *rbcL* data (Table 3; Fig. 4a) resolved the same three clades (labeled 1–3 above) as did the *trnK* 5' intron data, also with high support, i.e.: 1) BS = 100; PP = 1.00; 2) BS = 93; PP = 0.92; and 3) BS = 96; PP = 1.00 respectively. In addition, the *V. denseserrulata* + *V. spinulosa* + *V. spiralis* clade received higher support from *rbcL* (BS = 72; PP = 0.88) than from *trnK* 5' intron data. A clade of three Australian species (*V. annua*, *V. erecta*, *V. gracilis*) received moderate support (BS = 64; PP = 0.91; Fig. 4a).

The ILD test indicated no significant incongruence ($p = 1.000$) between the *trnK* 5' intron and *rbcL* data partitions, a



FIG. 2. Phylogram representing one of ten shortest trees derived from MP analysis of nrITS data (sequences and indel characters) for *Vallisneria* taxa and *Maidenia rubra* (boxed). Geographical distributions are indicated to right. Bootstrap values (upper) and Bayesian posterior probabilities (lower) are shown for nodes that are resolved in all 10 MP trees and for nodes that collapse in the strict MP consensus tree (indicated by smaller arrows). Numbers after each taxon name indicate the specific accession as indicated in Appendix 1. Scale for branch lengths is indicated. Clades of rosulate taxa are indicated by larger arrows.

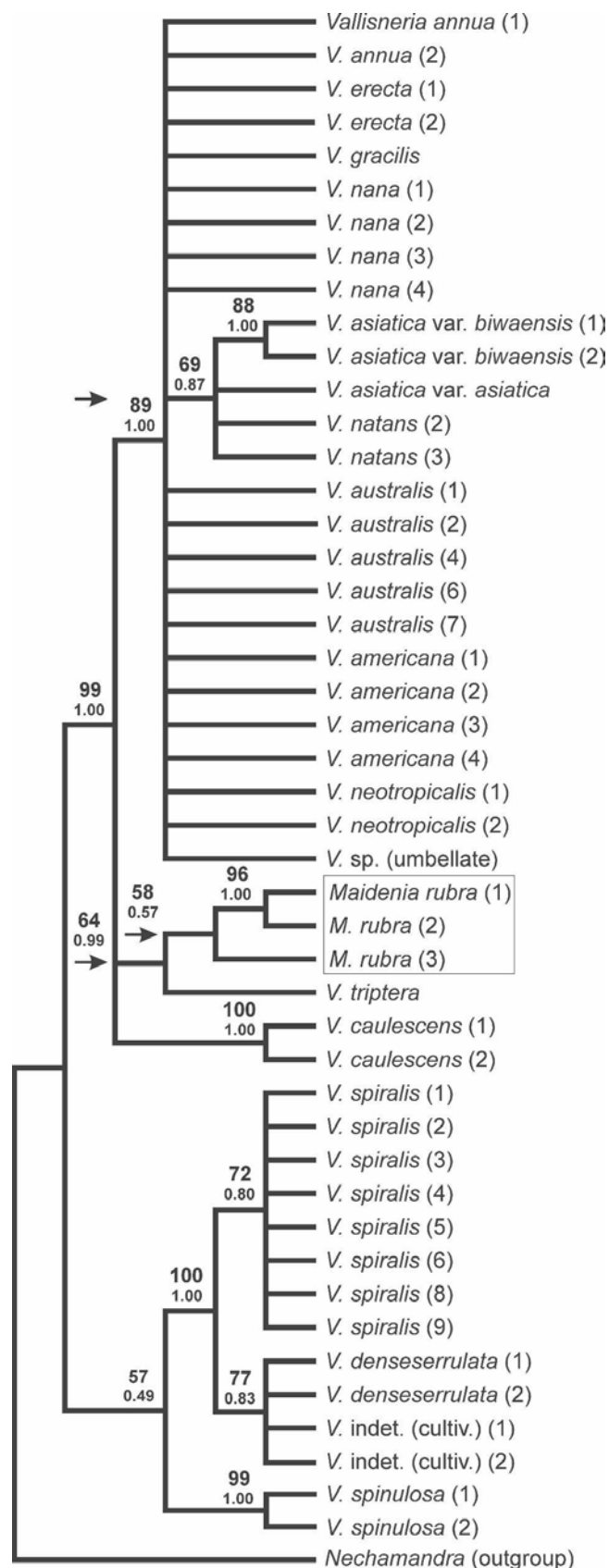


FIG. 3. Strict consensus tree derived from MP analysis of *trnK* 5' intron data (sequences and indel characters) for *Vallisneria* taxa and *Maidenia rubra* (boxed). Branch support is indicated by bootstrap values (upper) and Bayesian posterior probabilities (lower). Arrows identify clades that have > 50% bootstrap support but collapse in the strict consensus tree. Numbers after each taxon name indicate the specific accession as indicated in Appendix 1.

result that supports a combined cpDNA analysis. In addition to the same three well-supported clades mentioned previously for the separate analyses, the combined *trnK* 5' intron and *rbcL* data (Fig. 4b) resolved the clade of *V. asiatica* (both varieties) and *V. natans* (BS = 84; PP = 1.00) and a clade of *V. australis* and *V. nana* (BS = 85; PP = 1.00). The clade of *V. annua*, *V. erecta*, and *V. gracilis* that was resolved by *rbcL* data was retained with comparable support (BS = 64; PP = 0.94). Mixed support was provided for *Maidenia rubra* + *V. triptera* (BS = 59; PP = 1.00); however, the clade was not resolved in the strict consensus tree. Support for the *V. denseserrulata* + *V. spinulosa* + *V. spiralis* clade increased marginally (BS = 76; PP = 0.91).

The combined cpDNA cladogram was consistent with results from nrITS data analysis by placing *Maidenia rubra* within *Vallisneria* among the other caulescent taxa. These vitate taxa resolved as a polytomy situated between two clades of rosulate species (Figs. 3, 4a, b).

COMBINED NRDNA AND CPDNA—We performed a combined analysis of all the molecular data when the ILD test indicated that the nrDNA and cpDNA data partitions were not significantly incongruent ($p = 0.130$). The topology of the combined molecular data MP cladogram (Fig. 4c) was similar to that generated by nrITS data (Fig. 2) with two exceptions: *V. spinulosa* resolved with *V. denseserrulata* and *V. spiralis* as a basal clade in *Vallisneria* (BS = 53; PP = 0.42) and secondly, *V. triptera* was sister to *Maidenia rubra* (BS = 61; PP = 1.00). Neither result was consistently well-supported. The combined molecular data (Fig. 4c) resolved *M. rubra*, *V. caulescens* and *V. triptera* as a clade with low support (BS = 32; PP = 0.18). Otherwise, significant Bayesian support (PP > 0.95) characterized all branches with high MP bootstraps (BS > 80).

COMBINED MORPHOLOGICAL AND MOLECULAR DATA—The morphological data showed variable degrees of congruence with the different molecular data partitions as indicated by p values generated using the ILD test: vs. *rbcL* ($p = 0.181$); vs. *trnK* ($p = 0.015$); vs. nrITS ($p = 0.002$); vs. combined *rbcL* + *trnK* + nrITS data ($p = 0.002$). Although all comparisons produced p values above the 0.001 threshold, the nrITS partition resulted in nearly significant values when combined with morphological data. There were two major topological differences between the trees generated from morphology and those from molecular data: 1) the placement of the vitate taxa (a sister clade to all rosulate *Vallisneria* by morphological data; nested within rosulate *Vallisneria* by molecular data) and 2) the position of *V. annua* and *V. gracilis* (near the vitate taxa by morphology; with *V. erecta* by molecular data).

The MP strict consensus topology resulting from combined morphological and molecular data (Fig. 4d) differed only slightly from that obtained using combined molecular data (Fig. 4c). Notable exceptions were: 1) *V. caulescens* did not resolve as a clade with *Maidenia* and *V. triptera*; and 2) *V. australis* resolved in a clade containing all of the rosulate Australian *Vallisneria* taxa (also including *V. annua*, *V. erecta*, *V. gracilis*, *V. nana*). The combined data analysis also recovered two additional clades: 1) the two varieties of *V. asiatica* (BS = 60; PP = 0.97); and 2) *V. annua* + *V. gracilis* (BS = 97; PP = 1.00).

Phylogenetic signal (as indicated by negative g_1 values) was highest in the cpDNA data, somewhat lower in nrITS data, and considerably weaker in the morphological data (Table 3).

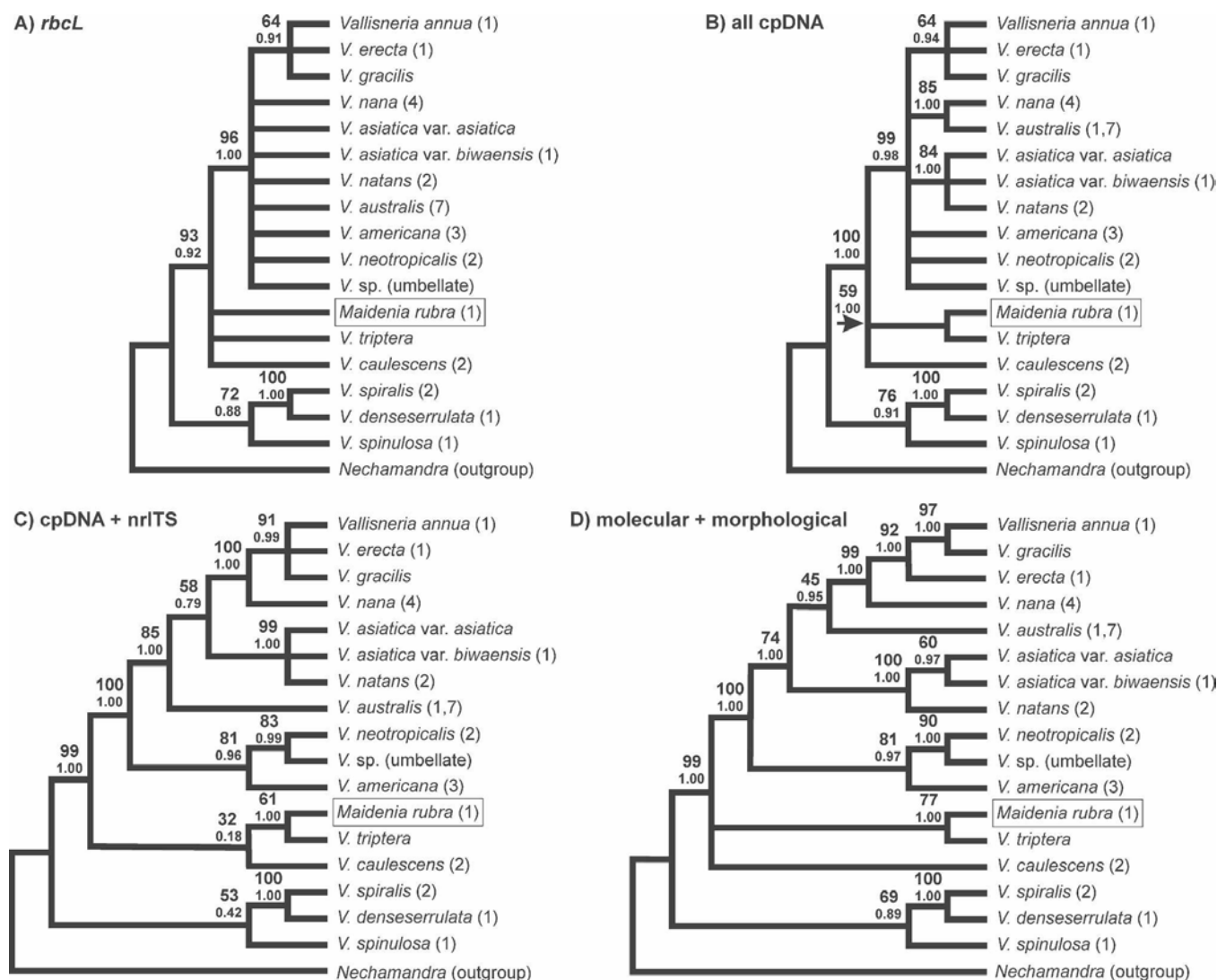


FIG. 4. A-D. Phylogenetic analyses of “core” *Vallisneria* taxa (*Maidenia rubra* is boxed). In each case, the MP strict consensus tree is shown with bootstrap values (upper) and Bayesian posterior probabilities (lower) indicated for each of the resolved nodes. Numbers after each taxon name indicate the specific accession as indicated in Appendix 1. A. Cladogram generated using *rbcL* sequence data. B. Cladogram from combined cpDNA sequence data. Arrow indicates a node with > 50% bootstrap support that collapses in the strict consensus. C. Cladogram from combined molecular data. D. Cladogram from combined molecular and morphological data.

Character Mapping—Using the cladogram constructed from combined DNA sequence data as a reference, the *V. spiralis* clade resembled the outgroup (*Nechamandra*) but could be delimited from the majority of ingroup taxa by a relatively distinct distribution of states for a suite of floral characters (Fig. 5). These features consisted of filament fusion (character 12), androecial hairs (character 13), staminode position (character 17) and stigma incision (character 19; Tables 1, 2). Exceptions to this general pattern included *V. annua*, *V. caulescens* and *V. gracilis*. All ingroup taxa possessed alternative states for characters 17 and 19, which indicated that they were strongly correlated. The states for characters 12 and 19 correlated in all taxa except *V. erecta* and the umbellate material (Fig. 5). The states for androecial pubescence (character 13), were distributed more diffusely on the cladogram.

DISCUSSION

Vallisneria presents an unusually difficult challenge for taxonomic study due to various confounding factors. The

genus itself has a cosmopolitan distribution and the species are quite similar and often distinguished only by minute floral characters. Moreover, the dioecious sexual condition has resulted in collections that are biased toward fruiting stages of female plants (which are most readily observed) and contain few examples of male flowers, which detach from plants and are water dispersed. Typically, it is difficult to observe both sexes of a plant without extensive field study in order to provide a thorough taxonomic evaluation. Vegetative material is notoriously difficult to identify.

Morphological Variation—The interpretation of morphological differences among *Vallisneria* species is challenging even when adequate material is available. Staminate *Vallisneria* flowers are modified from a more typical monocotyledonous condition: the three petals have been reduced to a single rudiment and the three stamens to one pair and a staminode. Some teratological forms have been observed bearing three fertile stamens (Richard 1811; Chatin 1855a) or 4-merous flowers (Chatin 1855a); one species (*V. caulescens*)

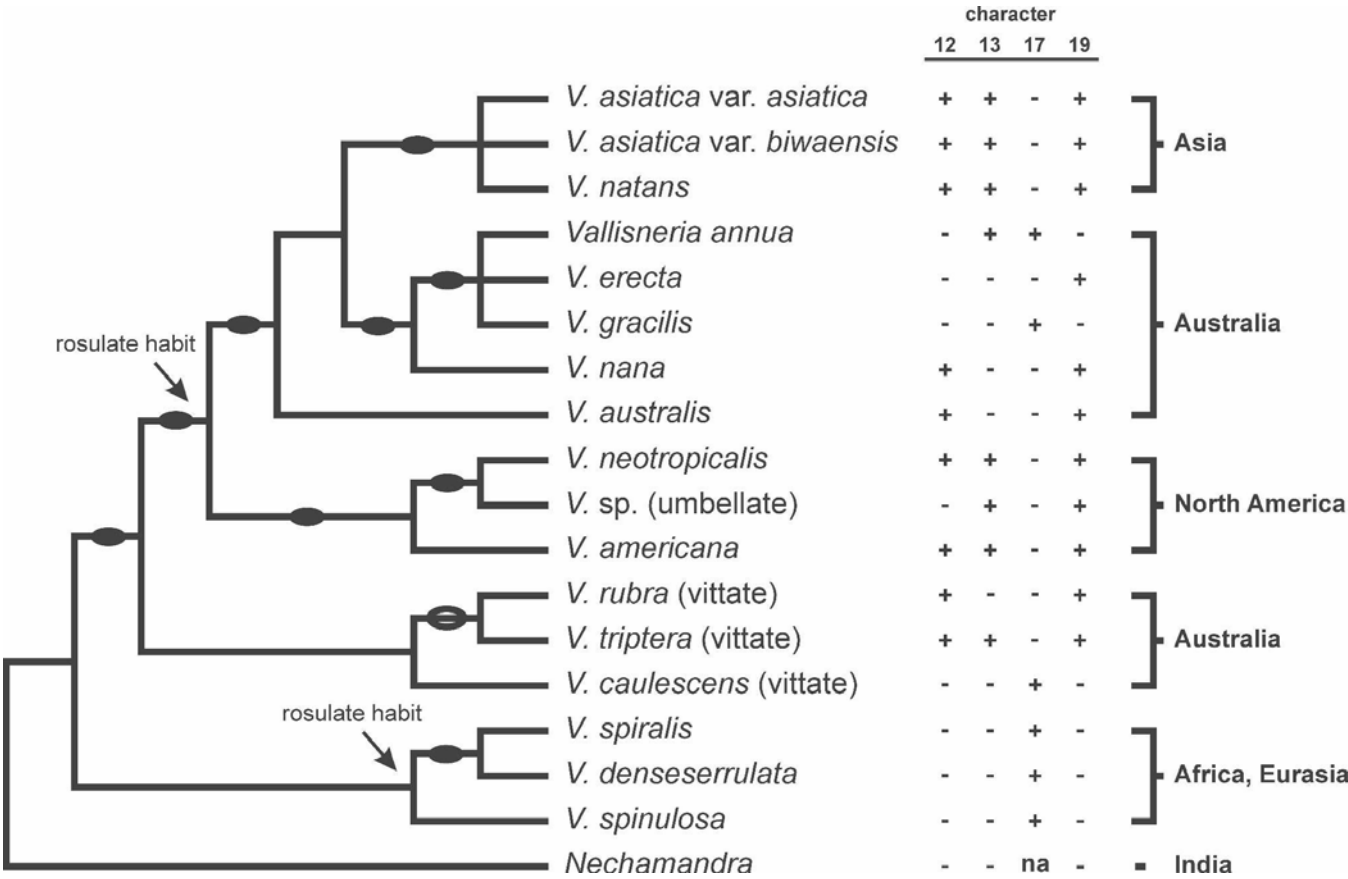


FIG. 5. Distribution of selected reproductive traits (characters 12, 13, 17, 19 in Tables 1, 2) on MP strict consensus tree from combined molecular data (Fig. 4c). “+” = state regarded as derived; “-” = state regarded as basal; na = character not present. Strong branch support (BS > 80; PP > 0.95) is indicated by closed symbols; mixed support (BS < 80; PP > 0.95) is indicated by the open symbol. Clades of rosulate taxa are indicated by arrows.

consistently has a 2-merous perianth (McConchie and Kade-reit 1987). Umbellate material can include both 2- and 3-merous flowers (Lowden 1982). Filament fusion in some species led several earlier researchers to misinterpret the staminate flowers as having a single stamen (Miki 1934) where actually there were two (Witmer 1937).

The number of anther locules also is difficult to assess. As Witmer (1937) explained, the anther of *Vallisneria* is derived from a tetralocular condition with an early developmental sterilization typically resulting in two locules per anther. However, incomplete sterilization of sporogenous cells can occur within a single species resulting in two, three or even four locules per anther (Witmer 1937). In other cases (e.g. *Maidenia rubra*), three locules consistently are formed (McConchie 1983). Parlature (1855) described the anther of *V. spiralis* as bilocular but with contiguous chambers (which would appear as one when mature). Chatin (1855b) also described the anther of *V. spiralis* as bilocular, but 4-valved upon dehiscence.

Frustrated by the extensive variability of vegetative structures in the genus, Lowden (1982) relied mainly on floral characters, which he believed to be more consistent, to separate *Vallisneria* species. He recognized only two species, one delimited as possessing stamens with free filaments not subtended by hairs, and fringed, deeply divided stigma lobes (i.e. *V. spiralis*) and another with fused filaments, androecial hairs, and an unfringed, shallowly lobed stigma (i.e. *V. americana*). He subdivided each of these species into two varieties, depending on whether the minute staminodia 1) originated

near the apex (*V. spiralis* var. *spiralis*) or 2) nearer the base (*V. spiralis* var. *denseserrulata* Makino) of the fused adjacent stigma lobes, 3) were adnate to the styles (*V. americana* var. *americana*), or 4) were “free” from reduced styles (*V. americana* var. *biwaensis* (Miki) Lowden).

Although Lowden (1982) clearly defined *Vallisneria* taxa using this combination of characters, his taxonomic scheme was difficult to implement. A major shortcoming is that it did not consider any of the vittate *Vallisneria* taxa (only *V. caulescens* had been described at that time and *Maidenia* was not regarded as a member of the genus). Both *Maidenia rubra* and *V. triptera* possess fused filaments (McConchie 1983; Jacobs and Frank 1997), yet both taxa differ considerably from *V. americana* by their vittate habit and other features. The cooccurrence of fused filaments in such morphologically distinct taxa raises doubts regarding the taxonomic value of this character. Furthermore, the free filaments of *V. caulescens* indicate that heterogeneity in this trait occurs even among vegetatively similar (i.e. vittate) species.

Other studies indicate that the floral features selected by Lowden are not as invariant as he had presumed. The degree of filament fusion differs considerably in *V. nana*; single collections may have virtually free filaments in some flowers but filaments fused up to 75% of their length in others (Jacobs and Frank 1997). Intraspecific variation in this character also is indicated by Lowden’s (1982) circumscription of *V. americana*: filaments range from “partially connate” in var. *americana* to “connate nearly to apex” in var. *biwaensis*. A discrepancy occurs with Lowden’s placement of umbellate *Vallisne-*

ria material in *V. americana*, despite having staminate flowers with free filaments (Lowden 1982).

Stigma lobe morphology is difficult to interpret in *Vallisneria* because individual lobes as well as adjacent lobes vary in their degree of fusion/incision. To interpret the morphology correctly, it is important to evaluate each stigmatic unit in the context of the staminodes or sepal lobes that alternate with them (Fig. 6a). In some taxa, two fused adjacent lobes can appear as a single, incised lobe (Fig. 6c). Individual stigma sections can be shallowly or deeply incised, and adjacent lobes can fuse variously with the staminodes (at their base, partway up their margins, or at the upper margin of the point of contact between adjacent stigma lobes). The apparent extent of fusion between adjacent lobes also is influenced by the degree to which the style extends from the hypanthium.

Although Lowden (1982) relied on the type of staminode fusion in the pistillate flowers to delimit varieties of *Vallisneria*, it is difficult to apply this feature as an effective taxonomic character. Notably, the structures often are absent or so reduced that they cannot easily be discerned even after a careful examination (Kausik 1939; Jacobs and Frank 1997). Interpretation of staminode arrangement also is difficult even when the structures are clearly visible. Because all *Vallisneria* species possess epigynous flowers, some degree of fusion occurs in all staminodes. The essential difference in staminode arrangement is their relative degree of fusion, a characteristic that Lowden (1982) emphasized. The degree of staminode fusion is evaluated with respect to the free terminal portion, which can appear to arise from various positions on adjacent stigma sections or the style. However, the relative extension of the style varies below the stigma, making it difficult to determine by superficial observation whether a staminode arises from the style itself or from a pair of fused, adjacent stigma sections. We did not attempt to make the latter discernment in our evaluation, especially given that

staminode morphology was scored in several instances from relatively simple drawings.

Several factors raise interpretive issues regarding staminode morphology. We observed that staminodes in *V. australis* can vary in insertion from the upper edge of adjacent stigma sections, to just below the upper margin of their fused bases. Also, we maintain that the difference in staminode arrangement between *V. asiatica* var. *asiatica* (= *V. americana* var. *americana* sensu Lowden) and *V. asiatica* var. *biwaensis* (= *V. americana* var. *biwaensis*) is not whether they are fused to or free from the style, respectively (e.g. Lowden 1982), but simply that a reduced style (e.g. in var. *biwaensis*) can present the appearance of free staminodes. Thus it is possible for this feature to converge in *Vallisneria* taxa where style length varies. A thorough anatomical study of these characters would be necessary to provide more definitive evidence bearing on the question of where the staminodes arise.

Our results lead us to suggest that the relative degree of stigma and staminode fusion, stigmatic incision and even filament fusion (all emphasized taxonomically by Lowden) may be correlated structurally. In all of the cases we examined, taxa having their adjacent stigma bases fused also exhibit deeply incised stigmas (Fig. 5; characters 17, 19 respectively). Different patterns of staminode fusion are related directly to differences in stigma lobe adnation, given that the staminodes always occur directly between adjacent lobes where fusion potentially occurs. Thus, their configuration is a consequence of adnation and does not represent an independent character.

The different floral conditions are functionally significant. Stigmas with bases that are not fused to their neighbors ordinarily will reflex between the sepals to receive pollen (Fig. 6b). When adjacent stigmas are fused basally, they might not be able to reflex sufficiently to present their receptive surfaces at the proper orientation relative to the elevated stamens of the floating male flowers. However, a deeply incised

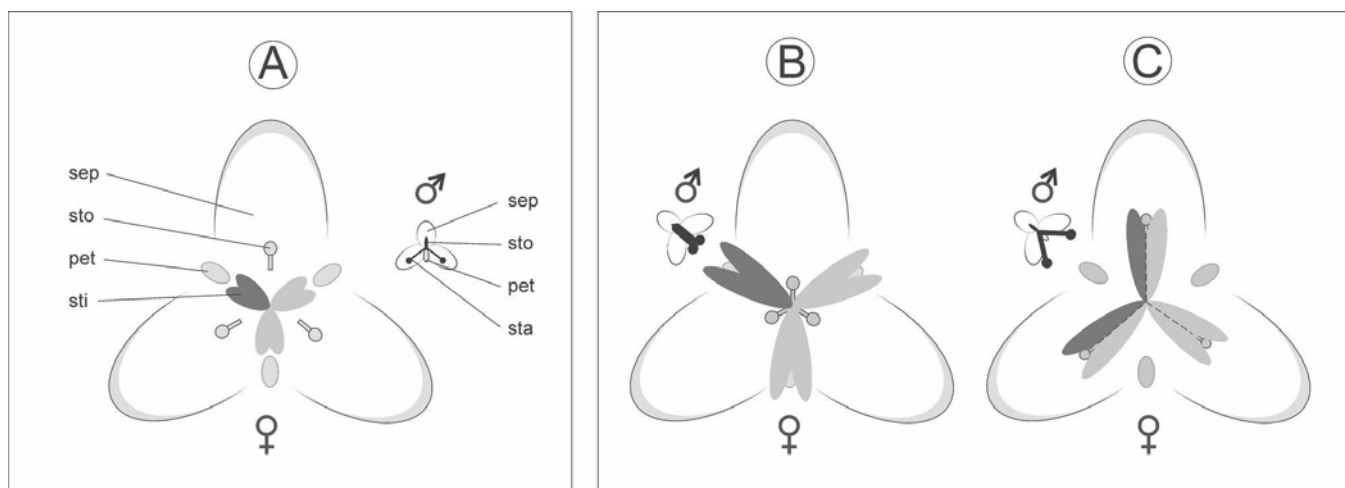


FIG. 6. Reproductive modifications in *Vallisneria*. A. Generalized flowers of *Vallisneria* showing arrangement of sepals (sep), staminodes (sto), petals (pet), and stigma units (sti) in the pistillate flower (♀) and sepals (sep), staminode (sto), petal rudiment (pet) and stamens (sta) in the staminate flower (♂). A single lobed stigma unit (opposite the petal) is darkly colored; B. Corresponding floral modifications (e.g. *V. americana*) where (♀) each stigma unit (darkly colored) is shallowly lobed and situated between the divergent sepals; the bases of adjacent stigma units are free and the tips of the staminodes extend between them. Filaments of the free-floating staminate flowers (♂) are fused, bringing together the pollen masses of both anthers into a single presentation unit, which eventually contacts the stigma; C. Corresponding floral modifications (e.g. *V. spiralis*) where (♀) each stigma unit (darkly colored) is deeply lobed and the bases of adjacent units are fused together (along dashed lines) with the intervening staminodes. Here the filaments of the free-floating staminate flowers (♂) are free and widely divergent, a configuration corresponding with the more widely divergent lobes of each stigma unit.

stigma could facilitate curvature of a pair of fused adjacent stigma lobes, as a compensatory change to preserve functionality of the pollination mechanism (Fig. 6c). This change would have the added benefit of placing the receptive stigma surfaces directly over the sepals, thus minimizing their contact with the water. Such a stigma lobe orientation is particularly conspicuous in *V. caulescens*, the only *Vallisneria* species with a dimerous floral condition (McConchie and Kadereit 1987).

Furthermore, fused filaments probably deliver pollen more efficiently to lobed stigmas that are oriented between sepals (Fig. 6b); whereas, free filaments (with divergent anthers), would be more effective at placing pollen along the broadly spreading lobes of adjacent stigmas at the point of contact between the sepals (Fig. 6c). In this sense, the relative degree of filament fusion would be optimized in proportion to the relative degree of stigma incision, which correlates with the other characters mentioned. In *Vallisneria*, pollen tubes do not penetrate any stigmatic tissue but grow through an open canal that forms in the center of the flower between the stigma lobes (Witmer 1937). Presumably, these modifications provide the optimal placement of pollen to facilitate subsequent pollen tube growth down the stylar canal. The influence of these floral variants on pollination success certainly deserves further study.

One exception in filament morphology occurs in the umbellate *Vallisneria*, which has free filaments but shallowly-lobed stigmas oriented between the sepal lobes. However, this taxon appears to be monoecious (Lowden 1982) and its pollination mechanism remains to be elucidated. A few other exceptions occur within the Australian *V. annua* group (Fig. 5), an indication that some intermediate conditions retain functionality. If stigma fusion, associated staminode fusion, stigma incision and filament fusion often are linked and related to pollination, then convergence in the different configurations potentially would be high. *Vallisneria annua* and *V. gracilis* (species not considered by Lowden) provide a conspicuous example of convergence in stigmatic traits (Fig. 5).

As Lowden (1982) applied these floral traits taxonomically, he did not consider their potential correlation with the pollination mechanism. Rather, he treated the traits as a suite of independent markers that arguably discriminated between two main taxa, which he consequently recognized as species. Using the combined molecular cladogram (Fig. 4c) to map these morphological traits phylogenetically (Fig. 5), it is apparent that Lowden's taxonomic rationale is supported only generally. The different suites of states for characters 12 (filament fusion), 17 (staminode arrangement) and 19 (stigma incision) delimit two basic groups within *Vallisneria* that correspond roughly to the two species recognized in Lowden's treatment, but there are several exceptions. It also is evident that such a circumscription accounts for only a small proportion of the phylogenetic diversity within each of those major groups and it clear that these should be subdivided to include additional species.

As one example, Lowden (1982) reduced *V. asiatica* var. *biwaensis* to a variety of *V. americana* principally on the basis of their similar pattern of filament fusion. However, our phylogenetic analysis (Fig. 4c) indicated that a substantial amount of genetic divergence distinguishes *V. americana* from *V. asiatica*. Similarly, Lowden's placement of *V. natans*

in synonymy with *V. denseserrulata* is inconsistent with every analysis that we have conducted.

Another floral character that differentiates some groups of *Vallisneria* species is the presence of androecial hairs (character 13; Fig. 5), a feature also emphasized taxonomically by Lowden (1982). The function of these hairs is not known, but they are regarded as representing a derived feature. Phylogenetically, the presence of androecial hairs does appear to be relatively derived (they are absent from the basal clade), but they are distributed too inconsistently among clades to serve reliably as a taxonomic marker above the species level (Fig. 5).

A conspicuous reproductive trait in *Vallisneria* is the presence of umbellate, pistillate flower-bearing inflorescences in some specimens occurring in the Gulf coastal plain of North America. In contrast with the solitary pistillate flowers reported for *Vallisneria* (and *Nechamandra*), this feature arguably would inspire taxonomic recognition at some level. However, umbellate inflorescences occasionally have been observed (among predominately solitary pistillate-flowered plants) in *Vallisneria* from Australia (S. W. L. Jacobs, pers. obs.) and Asia (L. Chen, pers. obs.). In the North American material, the umbels contain male flowers in a monoecious condition (Lowden 1982), which is unique in this otherwise dioecious genus. Lowden (1982) assigned umbellate *Vallisneria* plants either to *V. americana* var. *americana* or to *V. americana* var. *biwaensis* depending on whether their staminodes were adnate to the style or free, respectively. The results of our present phylogenetic analyses (both morphological and molecular data) indicate that the umbellate *Vallisneria* material from North America is related closely to *V. neotropicalis* (unfortunately, umbellate material from Asia or Australia was not available for analysis). Because the umbellate condition appears sporadically in different *Vallisneria* species from different geographical regions, the appropriate taxonomic disposition of such unusual material is difficult to determine and will be considered further in a subsequent study.

Phylogenetics of *Vallisneria*—MORPHOLOGY—The morphological data were of limited value for phylogenetic reconstruction in *Vallisneria*. We were able to score few characters across all of the taxa and the phylogenetic signal expressed in our resulting dataset was considerably lower than the values obtained for any of the molecular datasets (Table 3). The low phylogenetic signal was not due entirely to the small number of characters surveyed, given that the *rbcl* data yielded fewer informative characters than morphology but produced a much stronger phylogenetic signal (Table 3).

Despite their low phylogenetic signal, the morphological data resolved several clades (Fig. 1) that also appeared in molecular analyses. Examples include a vittate clade (including *Maidenia*) also resolved by combined molecular data (Fig. 4c), an African/Eurasian clade of rosulate species (*V. denseserrulata*, *V. spinulosa*, *V. spiralis*) resolved by all but the nrITS data (Figs. 2–4) and a clade of *V. neotropicalis* and umbellate North American material also resolved by combined molecular data (Fig. 4c). However, the basal position of the vittate clade and the separation of *V. annua* and *V. gracilis* from other rosulate Australian taxa (Fig. 1) were indicated only by the morphological data. This result was as a consequence of vegetative characters (shorter, narrower leaves) that were convergent with the caulescent outgroup *Nechamandra* (characters 4, 5; Table 1, 2).

Although we present an analysis of combined nonmolecular and molecular data (Fig. 4d), we are reluctant to accept the resulting topology as our best estimate of relationships in the group given that the congruency of the morphological and molecular data partitions varied considerably (e.g. $p = 0.181$ vs. *rbcl*; $p = 0.002$ vs. combined molecular data) and because of the weak phylogenetic signal contributed by morphology. The congruence values are difficult to interpret because the ILD test can be unreliable when the data partitions have few informative sites (Darlu and Lecomte 2002), which was the case with our morphological and *rbcl* data sets (Table 3).

The combination of morphological and molecular data (Fig. 4d) produced a topology similar to that generated by the combined molecular data (Fig. 4c), differing mainly by the positions of *V. australis* and *V. caulescens*, which were not particularly well-resolved by any of the individual analyses. The combined molecular and nonmolecular data also provided resolution of some additional nodes (e.g. *V. annua/V. gracilis*; *V. asiatica* varieties), an advantage emphasized by Wortley and Scotland (2006).

MOLECULAR DATA—Because all three molecular datasets were highly congruent and contributed strong phylogenetic signal, the combined molecular data provided the most reasonable reconstruction of phylogenetic relationships in *Vallisneria*. In contrast to morphology (Fig. 1), all molecular data resolved species with a rosulate growth form as basal elements in *Vallisneria* and embedded the vittate taxa between two groups of rosulate species with strong support (Figs. 2–4). Because all of our analyses consistently placed *Maidenia* within *Vallisneria*, we have concluded that the monophyly of *Vallisneria* can be maintained either by merging *Maidenia* with *Vallisneria*, or by splitting the whole complex into several genera. As the latter option is suboptimal, and is likely to have severe practical implications, the appropriate new combination to accommodate the taxonomic transfer of *Maidenia rubra* to *Vallisneria* (as *V. rubra*) is provided in the taxonomic section below.

Each of the vittate *Vallisneria* taxa is characterized by substantial molecular divergence (Fig. 2), a factor known to reduce the phylogenetic reliability of methods such as unweighted parsimony, which we used in many of our analyses. As one example, the position of *V. rubra* (= *Maidenia*) was not resolved consistently among the MP molecular analyses, with nrITS data placing it as the sister to *V. caulescens* (Fig. 2), *trnK* 5' intron data placing it as the sister to *V. triptera* (Fig. 3) and *rbcl* data placing it ambiguously among both species (Fig. 4a). The combined cpDNA data (Fig. 4b) and combined molecular data (Fig. 4c) resolved *V. rubra* and *V. triptera* as sister species (supported well by Bayesian analyses and poorly by MP), a result that was in agreement with morphological analyses (Fig. 1).

It is surprising that hybrids involving *V. rubra* and *V. caulescens* have been reported (McConchie 1983) despite their apparently high degree of genetic differentiation. However, Jacobs and Frank (1997) pointed out that many populations attributed to *V. caulescens* by McConchie actually are *V. triptera*, and only *V. rubra* and *V. triptera* (but not *V. caulescens*) are known to grow in the vicinity of hybrid populations. Our examination of the purported hybrid specimens indicated that they probably are robust forms of *V. rubra*.

The precise pattern of habit evolution in *Vallisneria* cannot

readily be ascertained. Despite the indication of a single transition from vittate to rosulate habit from the morphological cladogram (Fig. 1), the analyses of all molecular data consistently resolved rosulate species as basal in *Vallisneria*, with the vittate taxa embedded among other rosulate species (Figs. 2–4). Analyses of the combined molecular data (Fig. 4c) resolved a vittate clade containing *V. rubra*, *V. triptera* and *V. caulescens*, but with weak support (BS = 32; PP = 0.18). If this topology is correct, then one could reconstruct either a single origin of rosulate species (from a vittate ancestor similar to *Nechamandra*) with one reversion back to the vittate growth form in the ancestor of the three caulescent taxa or two independent origins of the rosulate habit (each interpretation requires two evolutionary steps).

Some ambiguity also exists with respect to interrelationships among the basal rosulate elements of *Vallisneria*. Although nrITS data yielded a topology where *V. spinulosa* was sister to all ingroup taxa (Fig. 2), other molecular data (including all combined data sets) resolved *V. spinulosa* in a basal clade with *V. spiralis* and *V. denseserrulata* (Figs. 3, 4a–c). There was not particularly high internal support for either configuration. It is likely that *V. spinulosa* and *V. spiralis/V. denseserrulata* diverged quite early in the phylogenetic history of the genus as evidenced by the relatively large branch lengths associated with these species (Fig. 2).

Except for the placement of *V. australis*, there was high internal support for the remaining clades resolved by the combined molecular data (Fig. 4c). In this analysis, the Asian *V. asiatica* and *V. natans* represented a relatively derived group that apparently originated within the clade of rosulate Australian taxa. The combined morphological/molecular analysis (Fig. 4d) indicated a slight topological variation where all rosulate Australian species resolved as a sister clade to the *V. asiatica/V. natans* clade.

The basal placement of rosulate *Vallisneria* species of principally Eurasian distribution is consistent with Lowden's (1982) hypothesis that the genus originated in temperate regions. However, it is also worth noting that despite the occurrence of several distinct Australian clades, our results do not preclude the possibility that *Vallisneria* originated in Australia with three subsequent non-Australian radiations (Africa/Eurasia, Asia, North America; Fig. 5). Because Australia contains both temperate and tropical species, a more refined analysis would be necessary to evaluate this question satisfactorily.

FLORAL EVOLUTION—There have been various interpretations regarding the polarity of floral characters in *Vallisneria*. Floral character states that are presumed to represent primitive conditions (Svedelius 1932; Lowden 1982; McConchie and Kadereit 1987) include unfused stamen filaments (character 12), the lack of androecial hairs (character 13), and shallowly incised stigmas (character 19). Because all of these conditions occur in the outgroup (*Nechamandra*), their interpretation as primitive is reasonable. It is noteworthy that these states occur uniformly in the basal clade that includes *V. spiralis* (Fig. 5). However, Svedelius (1932) and Lowden (1982) interpreted the pattern of fusion between adjacent stigma lobes (character 17) as derived in *V. spiralis*. Because our interpretation of states for this character is based on the relative position of staminodes (which are absent in *Nechamandra*), the polarity is difficult to ascertain. Given that we have shown this and other floral characters (i.e. characters 12,

19) to be strongly correlated, one could infer that *Nechaman-dra* ancestrally would have possessed the same state for character 17 as *V. spiralis* (followed by a secondary loss of stamnodes). Under this interpretation, the assessment of polarity for this character by Svedelius (1932) and Lowden (1982) appears to be incorrect.

DELIMITATION OF TAXA—Our selection of molecular data performed reasonably well in delimiting taxa in *Vallisneria*. Generally, the cpDNA data (Figs. 3, 4a, b) lacked sufficient variability to distinguish many taxa as compared to nrITS sequences (Fig. 2). The distinctness of *V. spiralis* and *V. denseserrulata* (merged by some authors) was one notable feature supported by both cpDNA and nrITS data (Figs. 2–4). Furthermore, nrITS data delimited two quite divergent taxa within the sister clade to *V. spiralis* (Figs. 2, 3); one representing natural populations of *V. denseserrulata* and another consisting of cultivated material. With further study, it may be possible to distinguish these taxa as distinct species; however, at this time we are not even certain of the geographic origins of the cultivated material. It is also possible that the cultivated material is of hybrid origin. Although we did not observe polymorphic nrITS sequences for the cultivated material (often indicative of hybridization), this possibility merits further consideration.

Lowden (1982) placed the African *V. aethiopica* Fenzl in synonymy with *V. denseserrulata* (as *V. spiralis* var. *denseserrulata*). We analyzed African material (*V. spiralis* #1; Appendix 1) that was collected roughly 1,100 km south of the type locality of *V. aethiopica* (Fenzl 1865). This material differed from European accessions of *V. spiralis* by only a single nucleotide substitution (in the *trnK* 5' intron) and clearly resolved within the clade containing *V. spiralis* (Figs. 2, 3). Additional sampling would be necessary to determine whether *V. denseserrulata* also occurs in Africa and if it truly is conspecific with *V. aethiopica*.

The nrITS data clearly resolved an isolated clade of Australian material that we have designated as the new species *V. australis*. This taxon, along with *V. nana*, had been included previously under *V. spiralis* (Aston 1973), a result that is not supported by any of the analyses we conducted (Figs. 1–4). Hartog (1957) recognized all rosulate Australian *Vallisneria* as *V. gigantea* Graebn., a taxon later subdivided into a tropical segregate (*V. nana*) and a more temperate segregate that initially was designated as *V. americana* (Lowden 1982; Jacobs and Frank 1997). During the course of this study it became clear to us that the “temperate” Australian segregate (extending into subtropical Australia under cultivation) was distinct from both *V. americana* and *V. nana* and it is this material that we have recognized as a new species. Because our inspection of type material indicates that the name *V. gigantea* is most likely a synonym of *V. nana*, we formally describe the new species *V. australis* in the taxonomic section below.

Similar to the situation with *V. denseserrulata*, the nrITS data distinguished cultivated material of unknown provenance (accessions #4–7) from material collected in natural populations of *V. australis* (accessions #1–3; Queensland and Victoria). Once the geographical origin of the cultivated material is determined, renewed study may indicate the need for further taxonomic subdivision of this species. Because collectors who seek ornamental species for aquariums are attracted to unusual phenotypes, it would not be surprising for selections of cultivated material to reflect novel taxa.

Although we found the nrITS data generally to be useful taxonomically, they did not differentiate *V. natans* from *V. asiatica* (either variety) and failed to delimit species in the *V. annua* group (Fig. 2). Morphologically (Tables 1, 2), *V. natans* differs from *V. asiatica* by possessing longer and broader leaves (characters 4, 5) with reddish pigmentation (character 6) and a shallowly-lobed stigma (character 19). All of these features are characterized by rather extensive variability and it is understandable that some authors (e.g. Kadono 1994) have merged *V. asiatica* with *V. natans*. *Vallisneria asiatica* var. *biwaensis* is somewhat more distinct and differs conspicuously from *V. asiatica* var. *asiatica* by its strongly spirally-twisted leaves (Tables 2, 3); it also exhibits minor sequence divergence in the *trnK* 5' intron (Fig. 3). Although our study did not focus specifically on these three taxa, we can provide no strong argument either for or against the merger of *V. natans* with *V. asiatica* as done by Hara (1974) and Kadono (1994). However, we would recommend that at least two taxa be retained as varieties of *V. asiatica* until their distinctness can be evaluated more critically (material of *V. asiatica* var. *higoensis* Miki, potentially a third variety, was unavailable for analysis). Because the oldest available epithet is ‘*natans*’ (*Physium natans* Lour., 1790) the proper combinations would be those made by Hara (1974): *Vallisneria natans* var. *natans* (= *V. asiatica* var. *asiatica*) and *Vallisneria natans* var. *biwaensis* (Miki) Hara (= *V. asiatica* var. *biwaensis*). If *V. asiatica* var. *asiatica* was to be retained as distinct from *V. natans* var. *natans*, then an additional new combination would be required.

Species of the *V. annua* group are very closely related, as evidenced by their molecular similarity (Figs. 2, 3, 4a–c). However, these taxa can be distinguished morphologically using a combination of characters. Although none of the molecular data sets provided resolution among these three species, a sister relationship between *V. annua* and *V. gracilis* (and more distant relationship to the taxon we indicate as *V. erecta*) is supported (Tables 2, 3) by their much narrower leaves (character 5), staminode morphology (characters 17, 18), deeply-lobed stigmas (characters 19), and shorter peduncles. Also, the formation of mud-flat forms has been observed only in *V. erecta*. It is for these reasons that we have elected to recognize *V. erecta* as a new species, which is distinct from but closely related to *V. annua* and *V. gracilis*. *Vallisneria annua* differs from *V. gracilis* by its facultative annual habit (character 1), longer leaves (character 4) with sparsely-toothed apices (character 9) and presence of androecial hairs (character 13).

Although some authors (Godfrey and Wooten 1979; Lowden 1982; Haynes 2000) have merged *V. neotropalis* and *V. americana*, we have found these taxa to be well differentiated by both morphological (Fig. 1) and molecular characters (Figs. 2, 4c). Thus, *V. neotropalis* is not simply a larger growth form of *V. americana* resulting from the prolonged growing season of southern latitudes as Godfrey and Wooten (1979) concluded, but a genetically distinct lineage, and we highly recommend that the two species be maintained. The distinguishing characteristics compared by Marie-Victorin (1943) provide a good means of differentiating these species morphologically. Even vegetatively, *V. neotropalis* can be separated from *V. americana* by its foliage, which is suffused with reddish pigment striations. We also have observed that the conspicuous broad lacunal band, so typical of *V. ameri-*

cana (Crow and Hellquist 2000), is absent on specimens of *V. neotropicalis*.

Our study indicates the need to adopt a taxonomic concept of *Vallisneria* substantially revised from the two-species system recommended by Lowden (1982). The nrITS sequences could distinguish 12 of the 15 putatively distinct *Vallisneria* species considered in our study. If *V. natans* and *V. asiatica* are merged taxonomically (see above), then the only case where nrITS data did not provide interspecific delimitation was among the closely-related members of the *V. annua* group. Consequently, the nrITS sequences generated by this study can be used effectively as a resource for identifying most unknown specimens of *Vallisneria* worldwide to the species level. The nrITS data also were effective at establishing the derivation of cultivated material, either as belonging directly to an existing species or as specimens closely-related to an existing species (where further taxonomic study is warranted). It is possible that further study may reveal the existence of new species from among the various specimens of *Vallisneria* material presently in cultivation or perhaps from other geographical regions not represented in our survey.

Nonindigenous Species—Our molecular data were useful in establishing at least one instance of an introduction of a nonindigenous *Vallisneria* species. Material of *Vallisneria* plants collected in one Texas locality (*V. spiralis* [accession #8]; Table 1; Fig. 2) clearly associated with native Old World specimens of *V. spiralis* (*V. spiralis* [accessions #1–5]; Table 1; Fig. 2). This result documents the first authentic record of *V. spiralis* plants established in North America (earlier reports of *V. spiralis* for the region were based on misidentifications of *V. americana*). Lowden's (1982) survey revealed no evidence of this species in the Americas although he acknowledged that "the possibility is not excluded." He did report minor introductions of *V. spiralis* into Jamaica and Cuba (Lowden 1982); however, we did not examine material from either region and were unable to verify those reports. The comparative use of molecular data in this fashion has proven to be an effective means of identifying introductions of nonindigenous aquatic plants, especially when thorough surveys of aquatic genera have been conducted (Les et al. 2006a).

TAXONOMIC TREATMENT

Vallisneria australis S. W. L. Jacobs & D. H. Les, sp. nov.—

TYPE: AUSTRALIA. New South Wales: Southern Tablelands: Weddenjerry Lake, ca. 14 km S of Bombala on the Cann River Highway, 37°01'10" S 149°15'33" E 760 m. Ephemeral lake with *Eleocharis sphacelata* and *Myriophyllum simulans*. Submerged in water ca. 30 cm deep in deep soft mud. (NSW391440), 19 Feb 1993, S. W. L. Jacobs 6814 (holotype (♀): NSW!, isotype: MEL!). There is a collection of male plants from the same population, S. W. L. Jacobs 6813 (NSW). Illustrations: G. R. Sainty and S. W. L. Jacobs, Waterplants of N.S.W. (as *V. gigantea*) 250 (1981).

Vallisneria spiralis var. *procera* Rodway (1896: 53–54).—TYPE: Jordan River above Bridgewater (HO). [Probable synonym].

V. americanae similis sed foliis plantarum maturarum ampliorum longioribus latioribusque, plantis frigore extremo entectis, differt.

Submerged tufted, stoloniferous perennial. Leaves basal, to 3 m long, 11–35 mm wide; apex obtuse; 5–7 major longi-

tudinal nerves. Male spathe ovate, 10–25 mm long; flowers < 0.5 mm long; sepals 3, subequal, curved, ca. 0.6 mm long; anthers 2; filaments fused for ca. 50% of their length (like 'Y'); staminode minute. Female spathe enclosing 1(–4) sessile flowers, thin, translucent, 10–30 mm long; flowers (1.9–) 2.5–4 mm long; sepals 3, triangular to semiorbicular, ca. 3 mm long; petal rudiments minute or absent; stigmas 3, ca. 3 mm long, bifid for 50–70% of their length; staminodes absent or ca. 0.2 mm long, just below notch on outer surface of stigma. Fruit cylindrical, (15–)20–160 mm long, ca. 2–5 mm wide. Seed narrow ovoid to ellipsoid, 1.5–2.0 mm long, (0.2–) 0.4–0.8 mm diam., smooth surface with a dense coat of short-medium length hairs.

Distribution and Habitat—Grows at the higher altitudes of the eastern coast south from Theodore, Queensland (possibly introduced there), Armidale (Northern Tablelands of New South Wales) and south of the Williams River on coastal N.S.W., and south of the Qld border west of the ranges in N.S.W., to Victoria, South Australia, and Tasmania; introduced in Western Australia. Habitats include more or less perennial creeks, rivers, billabongs and dams. Flowers and fruits are produced during the summer months.

Representative Specimens Examined—AUSTRALIA. Queensland: ca. 3 km SE of Theodore, Cracow road, 22 Apr 1994, Jacobs 7085 (NSW). New South Wales: North Coast: Seaham Weir Pool, 24 Feb 1996, Jacobs 7944, 7945, (NSW). Central Coast: Creek, ca. 2 km E of Gees Lagoon, Upper Colo, 9 Dec 1977, Jacobs 3241, 3242 (NSW, MEL). South Coast: Bondi Lake, Tathra, 7 Oct 1968, Goodrick Q225, (NSW). Northern Tablelands: Dangars Lagoon, 2 Feb 1985, Hight 8 & Jacobs (NSW). Central Tablelands: Duckmaloi River, 30 Nov 1982, Lapinuro 68 & Jacobs (NSW). Southern Tablelands: Lake Bathurst, 11 Feb 1991, Papassotiriou 34, 35 & Jacobs (NSW). North Western Plains: Talmoi Lagoon, ca. 48 km NW of Moree K.L. Wilson 869 (NSW). South Western Plains: South Hanwood, 27 Apr 1992, Sainty s.n. (NSW282253). Victoria: ca. 12 km SE of Kerang, Echuca rd, 23 Apr 1992, Jacobs 6528 (NSW); about 22 miles [35.4 km] direct line WSW of Bendigo, Loddon River at Laanecoorie township, Dec 1967, Aston 1626, 1627 (MEL, NSW). South Australia: Gal Gal Reach, River Murray, 8 May 1987, Symon 14337 (NSW, BRI, AD). Tasmania: 'Blendon', on Jordan River, between Brighton and Broadmarsh, Jacobs 6435 (NSW). Western Australia: Canning River, upstream of the Brookton road bridge, Jacobs 6958 (NSW, PERTH, MEL).

This was one of two Australian species included in either *V. spiralis* L. or *V. gigantea* Graebn. The first is an extant name for what is basically a Eurafrikan species. The second is a later name with the type collected from New Guinea; it is most likely a synonym of *V. nana* R.Br. *Vallisneria australis* was included in *V. americana* by Lowden (1982) and this subsequently has been followed by several authors, mainly due to a lack of alternatives. Although it is clear that the Australian species differs from *V. americana* ecologically and physiologically there are few morphological characters that clearly distinguish them. The Australian species can produce longer and wider leaves than *V. americana* but this character is only visible in the largest of plants and, unfortunately, these are rarely represented in herbaria. *Vallisneria americana* also has hairs at the base of the androecium (absent in *V. australis*), and narrower seeds.

Vallisneria erecta S. W. L. Jacobs, sp. nov.—TYPE: AUSTRALIA. Queensland. Douglas Creek, Daintree River, 16°16.121' S 145°18.626' E. On mudbank, some plants exposed and looking similar to plants in water (leaves stiff and brittle), some plants in 10 cm water, 21 Oct 1999, S.W.L. Jacobs 8584 & D. Les 607, (holotype (♀): NSW!, isotypes: BRI!, CONN!, Z!). Male plants (S.W.L. Jacobs 8583) were collected from the same population (NSW!, BRI!, CONN!).

V. nanae similis sed foliis crassioribus, foliis plantarum in aquis non profundis rigentibus erectisque, perseverantibus ubi aquis recessis expositis, foliis juvenilibus acutis sed foliis maturis obtusis, differt.

Submerged or emergent tufted, stoloniferous, dioecious perennial. Leaves basal, to ca. 80 cm long, 5.5–17 mm wide, on stranded plants the leaves are stiff and nondrooping; apex acute when leaves are small, becoming broad-acute on larger leaves; ca. 5 major longitudinal nerves; margins entire or with sparse minute antrorse bristles. Male spathe ovate, 7–8 mm long; flowers < 0.5 mm long, perianth segments 3, subequal, ca. 0.2 mm long, curved, anthers 2, filaments free (like a 'V'). Female spathe usually enclosing 1 sessile flower ca. 1 cm long, on a long peduncle 0.7–1 mm thick coiling at maturity to retract developing fruit; flowers 2–3 mm long; sepals 3; petal rudiments 3; staminodes inconspicuous or absent; stigmas 3, shortly bifid. Fruit and seeds not observed.

Distribution and Habitat—The species grows on the northeastern coast of Queensland and so far has been collected only from the Daintree River and its tributaries where it inhabits flowing water of perennial creeks and rivers. Flowers (and presumably fruits) are produced in the dry season. The most characteristic feature of this species is that the rosettes exposed by the fluctuating water levels have comparatively stiff, erect leaves. Leaves in deeper permanent water are longer and lose this stiffness but still appear thicker than those of other *Vallisneria* species.

Representative Specimens Examined—AUSTRALIA. Queensland: Daintree River area, 21 Oct 1999, *Jacobs 8585*, (BRI, CONN, NSW); 4 Jul 2001, *Jacobs 8679, 8681*, (NSW, BRI); CULTIVATED, Kelso, Townsville, by L. Smith, originally from Daintree area, 17 Oct, 1999, *Jacobs 8531 & Les 557*, (BRI, CONN, NSW), 17 Oct 1999, *Jacobs 8558*, (NSW, BRI).

***Vallisneria rubra* (Rendle) D. Les & S. W. L. Jacobs, comb. nov.** *Maidenia rubra* A. B. Rendle, *Journal of Botany* 54: 313–316, t. 545 (1916).—TYPE: West[ern] Australia, nr. King River, East Kimberley, W. V. Fitzgerald, October 1906 (*n.v.*).

Although the name *Vallisneria rubra* has not been validly published previously, it is widespread in the aquarium trade where it seems to be used more in the sense of a cultivar name applied primarily to *V. spiralis*; however, it has been (mis)used for several taxa characterized by reddish foliage.

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LITERATURE CITED

- Armstrong, G. and D. T. Booth. 2005. Dietary ecology of the Australian freshwater turtle (*Elseya* sp.: Chelonia: Chelidae) in the Burnett River, Queensland. *Wildlife Research* 32: 349–353.
- Aston, H. I. 1973. *Aquatic plants of Australia*. Carlton, Victoria, Australia: Melbourne University Press.
- Bailey, F. M. 1888. *A synopsis of the Queensland flora; containing both the phaenogamous and cryptogamous plants. Second supplement*. Brisbane: James C. Beal.
- Baldwin, B. G. 1992. Phylogenetic utility of the internal transcribed spacers of ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* 1: 3–16.
- Bengtson, J. L. 1983. Estimating food consumption of free ranging manatees in Florida. *The Journal of Wildlife Management* 47: 1186–1192.
- Chatin, A. 1855a. *Mémoire sur le Vallisneria spiralis, L., considéré dans son organographie, sa végétation, son organogénie, son anatomie, sa tératologie et sa physiologie*. Paris: Mallet-Bachelier.
- Chatin, A. 1855b. Sur le fleurs males du *Vallisneria spiralis*. *Bulletin de la Société Botanique de France* 2: 293–295.
- Cook, C. D. K. 1982. Pollination mechanisms in the Hydrocharitaceae. Pp. 1–15 in *Studies on aquatic vascular plants. Proceedings of The International Colloquium on Aquatic Vascular Plants (Brussels, 23–25 January, 1981)*, eds. J. J. Symoens, S. S. Hooper, and P. Compère. Brussels: Royal Botanical Society of Belgium.
- Cook, C. D. K. 1996a. *Aquatic plant book (revised edition)*. Amsterdam: SPB Academic Publishing.
- Cook, C. D. K. 1996b. *Aquatic and wetland plants of India: a reference book and identification manual for the vascular plants found in permanent or seasonal fresh water in the subcontinent of India south of the Himalayas*. New York: Oxford University Press.
- Cook, C. D. K. and R. Löönd. 1982. A revision of the genus *Nechamandra* (Hydrocharitaceae). *Aquatic Botany* 13: 505–513.
- Cook, C. D. K., B. J. Gut, E. M. Rix, J. Schneller, and M. Seitz. 1974. *Water plants of the world: a manual for the identification of the genera of freshwater macrophytes*. The Hague: W. Junk.
- Cowie, I. D., P. S. Short, and M. Osterkamp Madsen. 2000. *Floodplain flora—A flora of the coastal floodplains of the Northern Territory, Australia*. Flora of Australia Supplementary Series No. 10. Canberra: Australian Biological Resources Study.
- Crow, G. E. and C. B. Hellquist. 2000. *Aquatic and wetland plants of north-eastern North America* vol. 2. Madison, Wisconsin: The University of Wisconsin Press.
- Darlu, P. and G. Lecointre. 2002. When does the incongruence length difference test fail? *Molecular Biology and Evolution* 19: 432–437.
- de Vos, A. 1958. Summer observations on moose behavior in Ontario. *Journal of Mammalogy* 39: 128–139.
- Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.
- Duchartre, M. P. 1855. Quelques mots sur la fécondation chez la Vallisnerie. *Bulletin de la Société Botanique de France* 2: 289–293.
- Feldman, R. S. 2001. Taxonomic and size structures of phytophilous macroinvertebrate communities in *Vallisneria* and *Trapa* beds of the Hudson River, New York. *Hydrobiologia* 452: 233–245.
- Fenzl, E. 1865. Diagnoses praeviae Pemptadis stirpium aethiopicarum novarum. *Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften. Mathematisch-naturwissenschaftliche Classe. Abteilung I* 51: 138–141.
- Fernald, M. L. 1918. The diagnostic character of *Vallisneria americana*. *Rhodora* 20: 108–110.
- Godfrey, R. K. and J. W. Wooten. 1979. *Aquatic and wetland plants of South-eastern United States. Vol. I: Monocotyledons*. Athens: University of Georgia Press.
- Griekspoor, A. and T. Groothuis. 2005. 4Peaks v1.6. (<http://mekentosj.com/4peaks/>).
- Hara, H. 1974. New or noteworthy flowering plants from eastern Himalaya. *Journal of Japanese Botany* 49: 129–137.
- Hartog, C. den. 1957. Hydrocharitaceae. Pp. 381–413 in *Flora Malesiana series 1*, vol. 5, ed. C. G. G. J. van Steenis. The Netherlands: Noordhoff-Kolff N.V.
- Haynes, R. R. 2000. Hydrocharitaceae Jussieu. Tape-grass or frog-bit family. Pp. 26–38 in *Flora of North America north of Mexico* vol. 22, ed. Flora of North America Editorial Committee. New York: Oxford University Press.
- Hillis, D. M. 1991. Discriminating between phylogenetic signal and random noise in DNA sequences. Pp. 278–294 in *Phylogenetic analysis of DNA sequences*, eds. M. M. Miyamoto, and J. Cracraft. New York: Oxford University Press.
- Huelsenbeck, J. P. and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics (Oxford, England)* 17: 754–755.
- Jacobs, S. W. L. and K. A. Frank. 1997. Notes on *Vallisneria* (Hydrocharitaceae) in Australia, with descriptions of two new species. *Telopea* 7: 111–118.
- Kadono, Y. 1994. *Aquatic plants of Japan*. Tokyo: Bun-ichi Sogo Shuppan Co., Ltd.
- Kasselmann, C. 2003. *Aquarium plants*. Malabar, Florida: Krieger Publishing Co.
- Kaul, R. B. 1970. Evolution and adaptation of inflorescences in the Hydrocharitaceae. *American Journal of Botany* 57: 708–715.
- Kausik, S. B. 1939. Pollination and its influences on the behavior of the pistillate flower in *Vallisneria spiralis*. *American Journal of Botany* 26: 207–211.

Chatin, A. 1855a. *Mémoire sur le Vallisneria spiralis, L., considéré dans son*

- Kerner, A. 1891. *Pflanzenleben*, II. 2nd ed. Leipzig: Bibliographisches Institut.
- Lee, M. S. Y. 2001. Uninformative characters and apparent conflict between molecules and morphology. *Molecular Biology and Evolution* 18: 676–680.
- Les, D. H., R. S. Capers, and N. P. Tippery. 2006a. Introduction of *Glossostigma* (Phymaceae) to North America: a taxonomic and ecological overview. *American Journal of Botany* 93: 927–939.
- Les, D. H., M. A. Cleland, and M. Waycott. 1997. Phylogenetic studies in Alismatidae, II: evolution of marine angiosperms (seagrasses) and hydrophily. *Systematic Botany* 22: 443–463.
- Les, D. H., M. L. Moody, and C. L. Soros. 2006b. A reappraisal of phylogenetic relationships in the monocotyledon family Hydrocharitaceae. Pp. 211–230 in *Monocots: comparative biology and evolution, excluding Poales*, eds. J. T. Columbus, E. A. Friar, J. M. Porter, L. M. Prince, and M. G. Simpson. Claremont, California: Rancho Santa Ana Botanic Garden.
- Lowden, R. M. 1982. An approach to the taxonomy of *Vallisneria* L. (Hydrocharitaceae). *Aquatic Botany* 13: 269–298.
- Maddison, D. R. and W. P. Maddison. 2000. MacClade 4: analysis of phylogeny and character evolution. Sunderland: Sinauer Associates.
- Marie-Victorin, F. 1943. *Les Vallisnères américaines*. Contributions de l'Institut Botanique de l'Université de Montréal, No. 46. Montréal, Canada: Institut Botanique de l'Université de Montréal.
- Martin, A. C. and F. M. Uhler. 1939. *Food of game ducks in the United States and Canada*. U.S. Department of Agriculture Technical Bulletin No. 634. Washington, D.C.: Government Printing Office.
- McAtee, W. L. 1939. *Wildfowl food plants*. Ames, Iowa: Collegiate press, Inc.
- McConchie, C. A. 1983. Floral development of *Maidenia rubra* Rendle (Hydrocharitaceae). *Australian Journal of Botany* 31: 585–603.
- McConchie, C. A. and J. W. Kadereit. 1987. Floral structure of *Vallisneria caulescens* Bailey & F. Mueller. *Aquatic Botany* 29: 101–110.
- Miki, S. 1934. On fresh water plants new to Japan. *The Botanical Magazine (Tokyo)* 48: 326–337.
- Parlatore, P. 1855. Note sur le *Vallisneria spiralis*. *Bulletin de la Société Botanique de France* 2: 299–303.
- Posada, D. and T. Buckley. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* 53: 793–808.
- Posada, D. and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics (Oxford, England)* 14: 917–918.
- Rendle, A. B. 1916. *Maidenia*: a new genus of Hydrocharitaceae. *Journal of Botany* 54: 313–316.
- Richard, L. C. 1811 [1812]. Mémoire sur les Hydrocharidées; c'est-à-dire, sur les plantes qui, avec l'*Hydrocharis*, constituent la famille naturelle de ce nom. *Mémoires de la classe des sciences mathématiques et physiques de l'Institut de France* [s.n.]: 1–81.
- Rodway, L. 1896. Botanical notes. *Papers and Proceedings of the Royal Society of Tasmania* 1895: 51–54.
- Rogstad, S. H. 1992. Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analyses. *Taxon* 41: 701–708.
- Ronquist, F. and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics (Oxford, England)* 19: 1572–1574.
- Sainty, G. R. and S. W. L. Jacobs. 1994. *Waterplants in Australia*. Ed. 3. Darlinghurst, Australia: Sainty & Associates.
- Schlosser, D. W. and B. A. Manny. 1990. Decline of wild celery buds in the lower Detroit River, 1950–85. *The Journal of Wildlife Management* 54: 72–76.
- Sculthorpe, C. D. 1967. *The biology of aquatic vascular plants*. London: Edward Arnold (Publishers) Ltd.
- Svedelius, N. 1932. On the different types of pollination in *Vallisneria spiralis* L. and *Vallisneria americana* Michx. *Svensk Botanisk Tidskrift* 26: 1–12.
- Swofford, D. L. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland: Sinauer Associates.
- Thomson, J. A. 2001. An improved non-cryogenic transport and storage preservative facilitating DNA extraction from 'difficult' plants collected at remote sites. *Telopea* 9: 755–760.
- Wilder, G. J. 1974. Symmetry and development of pistillate *Vallisneria americana* (Hydrocharitaceae). *American Journal of Botany* 61: 846–866.
- Witmer, S. W. 1937. Morphology and cytology of *Vallisneria spiralis* L. *American Midland Naturalist* 18: 309–333.
- Wortley, A. H. and R. W. Scotland. 2006. The effect of combining molecular and morphological data in published phylogenetic analyses. *Systematic Biology* 55: 677–685.
- Wyllie, R. B. 1917. The pollination of *Vallisneria spiralis*. *Botanical Gazette (Chicago, Ill.)* 63: 135–145.
- Zhang, J.-X. and J.-J. Lu. 1999. Feeding ecology of two wintering geese species at Poyang Lake, China. *Journal of Freshwater Ecology* 14: 439–446.
- Zu, G., Y. Guan, G. Hou, H. Li, Y. Chen, J. Tuo, and G. Zhou. 1999. Utilization and protection of the submerged plant resources by pen-crab culturing in Nushan Lake. *Journal of Lake Sciences* 11: 91–96.

APPENDIX 1. List of taxa, vouchers and GenBank accession numbers for material examined. Multiple accessions for a taxon are numbered. For cultivated material ("in cult."), a locality is provided if the provenance of the specimen is confidently known; otherwise "origin unknown" is designated. GenBank accession numbers for each specimen are provided sequentially: nrITS, *trnK* 5' intron, *rbcL*; NA = no sequence.

Maidenia rubra (1), Wilson 8871 (NSW), Australia (Northern Territory), (EF142954, EF143020, EF143004); *M. rubra* (2), Wilson 8872 (NSW), Australia (Northern Territory), (EF142955, EF143021, AY870370); *M. rubra* (3), Hellquist s.n., 1997 (CONN), Australia (Northern Territory; in cult.), (EF142956, EF143022, NA); *Nechamandra alternifolia*, Cook s.n., 5 Oct 1995 (Z), India (Kota, Rajasthan), (EF142957, EF143023, U80706); *Vallisneria americana* (1), Les 510 (CONN), United States (Connecticut), (EF142958, EF143024, NA); *V. americana* (2), Les & Weiss s.n., 21 Sep 2005 (CONN), United States (Connecticut), (EF142959, EF143025, NA); *V. americana* (3), Les & Capers s.n., 31 Oct 2005 (CONN), United States (New Jersey), (EF142960, EF143026, EF143005); *V. americana* (4), Moody 148 (CONN), United States (Minnesota), (EF142961, EF143027, NA); *V. annua* (1), Jacobs 8578 & Les 602 (CONN, NSW), Australia (Queensland), (EF142962, EF143028, EF143006); *V. annua* (2), Jacobs 8586 & Les 608 (CONN, NSW), Australia (Queensland), (EF142963, EF143029, NA); *V. asiatica* var. *asiatica*, Jacobs 8874 (CONN, NSW), Japan (in cult.), (EF142964, EF143030, EF155532); *V. asiatica* var. *biwaensis* (1), Jacobs 8873 (CONN, NSW), Japan (in cult.), (EF142965, EF143031, EF143007); *V. asiatica* var. *biwaensis* (2), Gabel s.n. (CONN), origin unknown (in cult.), (EF142966, EF143032, NA); *V. australis* (1), Jacobs 8548 & Les 574 (CONN, NSW), Australia (Queensland; in cult.), (EF142967, EF143033, NA); *V. australis* (2), Jacobs 8554 & Les 580 (CONN, NSW), Australia (Queensland; in cult.), (EF142968, EF143034, NA); *V. australis* (3), Moody 432 (CONN), Australia (Victoria), (EF142969, NA, NA); *V. australis* (4), Wilstermann s.n., 14 Oct 2005 (CONN), origin unknown (in cult. as 'gigantea'), (EF142970, EF143035, NA); *V. australis* (5), Wilstermann s.n., 5 Dec 2005 (CONN), origin unknown (in cult. as 'gigantea'), (EF142971, NA, NA); *V. australis* (6), Wilstermann s.n., 30 Jan 2006 (CONN), origin unknown (in cult. as 'gigantea neotropalis'), (EF142972, EF143036, NA); *V. australis* (7), Wilstermann s.n., 10 Oct 2005 (CONN), origin unknown (in cult. as 'gigantea'), (EF142973, EF143037, EF143008); *V. caulescens* (1), Jacobs 8764 (NSW), Australia (Queensland), (EF142974, EF143038, NA); *V. caulescens* (2), Jacobs 8765 (NSW), Australia (Queensland), (EF142975, EF143039, EF143009); *V. denseserrulata* (1), Chen 20 (WBG), China (Hubei), (EF142976, EF143040, EF143010); *V. denseserrulata* (2), Chen 21 (WBG), China (Hubei), (EF142977, EF143041, NA); *V. erecta* (1), Jacobs 8531 & Les 557 (CONN, NSW), Australia (Queensland; in cult.), (EF142980, EF143044, EF143011); *V. erecta* (2), Jacobs 8584 & Les 607 (CONN, NSW), Australia (Queensland), (EF142981, EF143045, NA); *V. gracilis*, Jacobs 8549 & Les 575 (CONN, NSW), Australia (Queensland; in cult.), (EF142982, EF143046, EF143012); *V. nana* (1), Jacobs 8511 & Les 538 (CONN, NSW), Australia (Queensland), (EF142983, EF143047, NA); *V. nana* (2), Martine 863 & W.R. Barker s.n. (CONN), Australia (Western Australia), (EF142984, EF143048, NA); *V. nana* (3), Christensen s.n., 23 Jan 2006 (CONN), origin unknown (in cult. as 'Striped' or 'Tiger'), (EF142985, EF143049, NA); *V. nana* (4), Gabel s.n. (CONN), origin unknown (in cult. as *V. spiralis*), (EF142986, EF143050, EF143013); *V. natans* (1), Chen 1 (WBG), China (Hubei), (EF142987, NA, NA); *V. natans* (2), Chen 2 (WBG), China (Hubei), (EF142988, EF143051, EF143014); *V. natans* (3), Chen 4 (WBG), China (Hubei), (EF142989, EF143052, NA); *V. neotropalis* (1), Harms s.n., 22 Jun 2006 (CONN), United States (Florida), (EF142990, EF143053, NA); *V. neotropalis* (2), Padgett s.n., 23 Mar 2006 (CONN), origin unknown (in cult. as 'Giant'), (EF142991, EF143054, EF143015); *V. spinulosa* (1), Chen 10 (WBG), China (Hubei), (EF142993, EF143056, EF143017); *V. spinulosa* (2), Chen 11 (WBG), China (Hubei), (EF142994, EF143058, NA); *V. spiralis* (1), Bogner 2910 (M), Africa (Lake Edward), (EF694962, EF694964, EF694963); *V. spiralis* (2), Karlick s.n., spring 2005 (CONN), Austria, (EF142995, EF143059, EF143018); *V. spiralis* (3), Hussner s.n., Oct 2005 (CONN), Ger-

many (Erft), (EF142996, EF143060, NA); *V. spiralis* (4), *Weicherding s.n.*, 26 Oct 2005 (CONN), Germany (Nennig), (EF142997, EF143061, NA); *V. spiralis* (5), *Weicherding s.n.*, 26 Oct 2005 (CONN), Germany (Palzem), (EF142998, EF143062, NA); *V. spiralis* (6), *Wilstermann s.n.*, 10 Oct 2005 (CONN), origin unknown (in cult.), (EF142999, EF143063, NA); *V. spiralis* (7), *Gabel s.n.* (CONN), origin unknown (in cult. as *V. natans*), (EF143000, EF143064, NA); *V. spiralis* (8), *Owens & Doyle s.n.* (CONN), United States (Texas), (EF143001, NA, NA); *V. spiralis* (9), *Jacobs 8555 & Les 581* (CONN,

NSW), origin unknown (in cult. as 'Contortionist'), (EF143002, EF143065, NA); *V. triptera*, *Wilson s.n.*, 6 Mar 2004 (NSW), Australia (Northern Territory), (EF143003, EF143066, EF143019); *V. indet.* (cultiv.) (1), *Wilstermann s.n.*, 22 Oct 2005 (CONN), origin unknown (in cult. as *V. spiralis*), (EF142978, EF143042, NA); *V. indet.* (cultiv.) (2), *Christensen s.n.* (CONN), origin unknown (in cult. as *V. spiralis*), (EF142979, EF143043, NA); *V. sp.* (umbellate inflorescence) *Owens & Doyle s.n.*, Feb 2001 (CONN), United States (Texas), (EF142992, EF143055, EF143016).