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METHODS AND RESOURCES

A PCR assay for the detection of introduced *Vallisneria spiralis*, *V. denseserrulata* and their hybrids

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Abstract

The southern varieties of the American eelgrass, *Vallisneria americana*, are vital contributors to aquatic ecosystems throughout Florida and other areas within the southeastern United States. Recent discovery of cryptic non-native *Vallisneria* in natural waterbodies of central and southeast Florida has raised concerns for the biosecurity of native American eelgrass populations, which have been lost from many historical locations and are the subject of multiple, intensive restoration/reintroduction efforts throughout the state. The non-native grasses were found to be derivatives of a hybrid *V. spiralis* × *V. denseserrulata*, which is ubiquitous within the global aquarium trade and represents a worldwide invasive threat. Concern for the native taxon and difficulties in visually distinguishing it from non-native taxa prompted the development of a molecular screening assay. The method leverages parsimony-informative insertion/deletion-based variation within a fluorescently labeled amplified segment of the nuclear ribosomal internal transcribed spacer region. Based on comparisons to sequenced specimens (n = 96), fragment length polymorphisms for the amplicon, visualized by capillary electrophoresis, reliably diagnosed the Florida non-native grasses with 100% accuracy, sensitivity, and specificity. With additional ground-truthing to confirm intraspecific stability, this assay has the potential for broader application within the genus, including detection of the invasive *V. australis* (cf. *V. gigantea*), another ubiquitous aquarium product, as well as identification of first-generation hybrid forms.

Keywords Eelgrass · Non-native · Cultivar · Molecular detection · Insertion/deletion

Introduction

Eelgrasses of the genus *Vallisneria* are important constituents of aquatic ecosystems throughout the world, providing carbon cycling, nutrient uptake, and sediment stabilization, as well as food and shelter for a diverse array of faunal and infaunal communities (Wigand et al. 2000). Although there are 13 widely accepted species and perhaps 3–5 distinct varieties, the taxonomy is unsettled. For example, the Integrated Taxonomic Information System only recognizes three nominal species (Retrieved June 24, 2022, from the Integrated Taxonomic Information System, ITIS, on-line database, http://www.itis.gov), whereas Plants of the World Online (http://www.plantsoftheworldonline.org/) recognizes 14 species but omits the widely accepted *V. gracilis* (POWO 2022). More than a decade ago, Les et al. (2008) identified three phylogenetically discrete North American lineages within the American eelgrass complex: a broadly distributed northern lineage representing the nominal *V. americana* (Michaux 1803); a southern lineage referred to as '*neotropicalis*' (Marie-Victorin 1943); and a novel unnamed lineage associated with a distinct '*umbellate*' morphotype of uncertain provenance. However, insufficient taxonomic sampling and limited biogeographic information has thus far precluded further taxonomic consideration of the two North American lineages; the designation '*neotropicalis*' is currently considered a junior synonym of *V. americana* (POWO 2022).

American eelgrass populations are typically found in shallow waters of sandy or mucky bottomed lakes, less often in deeper waters, flowing rivers and streams, and occasionally in oligohaline areas of estuaries (Kraemer et al. 1999; Bortone and Turpin 2000). In Florida, eelgrass populations serve as an important food source for several imperiled

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species, such as the Florida manatee (*Trichechus manatus latirostris*) but can also be impacted by them due to overgrazing (Lefebvre and Powell 1990; Hauxwell et al. 2004). Because of various natural and anthropogenic stressors (Kemp 2000), many *Vallisneria* populations have experienced significant declines (Moore et al. 2004; Buzzelli et al. 2017; Orth et al. 2017). As a result, restoration and reintroduction efforts have intensified across Florida and elsewhere (Lloyd et al. 2011; Engelhardt et al. 2014).

In the recent genetic study of Gorham et al. (2021), which included material from the southern U.S. states of Florida, Mississippi, Alabama, and Kentucky, members of the '*neotropicalis*' and unnamed '*umbellate*' lineages were found within native thickets. However, a cryptic non-native hybrid *Vallisneria* was also detected from multiple sites in central and southeast Florida and one location in central Alabama. The parental species of this hybrid, *V. spiralis* and *V. denseserrulata*, are indigenous to southern Europe, the Middle East, Asia, and Africa (Lowden 1982) and do not occur naturally in North America. Upon finding non-native specimens in natural waterbodies, state biologists in Florida initiated a broad, sample-intensive molecular survey to determine the scope and scale of the introduction, with a secondary objective of ascertaining its origin(s).

Unfortunately, morphological characters in this group are highly variable, and the most consistent diagnostic characters are centered on microscopic traits that can be phenotypically plastic (Jacobs and Frank 1997; MacFarland 2006). Molecular testing is required for robust species assignment, but sequencing hundreds of specimens can be time consuming and costly, both in terms of consumables and labor. Therefore, a rapid and cost-effective screening procedure that distinguishes *V. spiralis*, *V. denseserrulata*, and their hybrids from Florida's native eelgrass is urgently needed for conservation and management of this important resource.

To address this need, we developed a fluorescent polymerase chain reaction (PCR)/capillary electrophoresis screening assay, centered on multiple parsimony-informative insertion/deletion variants (InDels) within a small segment of the nuclear ribosomal DNA internal transcribed spacer (nrITS) region (Fig. 1). Our procedures yielded fragment length polymorphisms (reflecting various combinations of gap character states) for a novel PCR amplicon, which we have confirmed differentiate Florida native lineages from the recently discovered non-native lineages. Moreover, with confirmation of intraspecific stability, the amplicon fragment lengths inferred in silico from homologous character states in GenBank-accessioned sequences could provide other species diagnoses and delineations among *Vallisneria* congeners.

Materials and methods

From the National Center for Biotechnology Information (NCBI) Nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore), we downloaded the *Vallisneria* nrITS region GenBank accessioned sequences that appeared in Les et al. (2008), which were products of the ITS5-F/ITS4-R primer pair from Baldwin (1992). We aligned these using ClustalW with default options as implemented in Mega X (Kumar et al. 2018), retaining a 706 base-pair (bp) segment for analysis. The outgroup species *Nechamandra alternifolia*, which is closely related to *Vallisneria*, was included in the alignment. We reconstructed a maximum-likelihood phylogenetic tree, adopting the Tamura 3-parameter substitution model (Tamura 1992), with uniform rates among sites, using all data and the nearest-neighbor heuristic method of inference. Reliabilities of branch lengths were established with 1000 bootstrap replicates (Felsenstein 1985). We omitted two specimens classified as "indeterminate" in Les et al. (2008), which were posited by Wasekura et al. (2016) and Gorham et al. (2021) to be hybrid cultivars of *V. spiralis* × *V. denseserrulata*.

Comparisons of multiple parsimony-informative gap characters across aligned sequences within a segment of the first transcribed spacer, ITS1 (Fig. 1), revealed the potential utility of a PCR fragment length polymorphism assay. Incorporating proximal nucleotide (nt) sequence information, we designed a PCR primer pair spanning this region of interest (Table 1). All alignment-based nt positions referenced herein begin with the 5′-terminal nt of the original ITS5-F primer, end with the reverse complement of the new 5′-terminal nt of Val-ITS-R and include the outgroup sequence. The region of interest occurred between nt positions 34 to 210 (Fig. 2). Presumptive gap-state profiles and fragment lengths for all taxa, as inferred from accessioned data, are provided in Table S1.

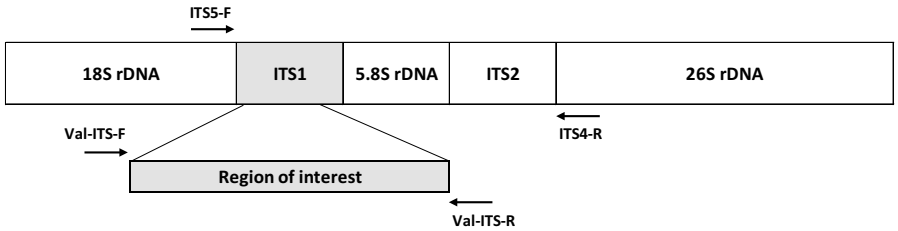

Fig. 1 Schematic (unscaled) structure of the nuclear ribosomal DNA cistron that shows the region of interest within the first internal transcribed spacer (ITS1)

Table 1 Primer specifications for the nrITS region sequence analysis and PCR assay to delineate *Vallisneria* taxa

Primer name	Sequence (5′–3′)	nt	GC content (%)	T_m (°C)
ITS5-F[a]	CGAAGTAAAAGTCGTAACAAGG	22	41	52
ITS4-R[a]	TCCTCCGCTTATTGATATGC	20	45	53
Val-ITS-F[b]	GTAAAAGTCGTAACAAGGTTTCCGTAGG	28	43	59
Val-ITS-R[b]	GTAATCCTTGACGCRATCCGCGC	23	56–60	64–66

Val-ITS-F contains 18 of 22 nt (grey segment) of ITS5-F, which attaches to the beginning of the ITS1 spacer. *GC content* (((guanine nt + cytosine nt)/total nt) × 100), T_m melting temperature

[a]From Baldwin (1992), [b]developed for this study

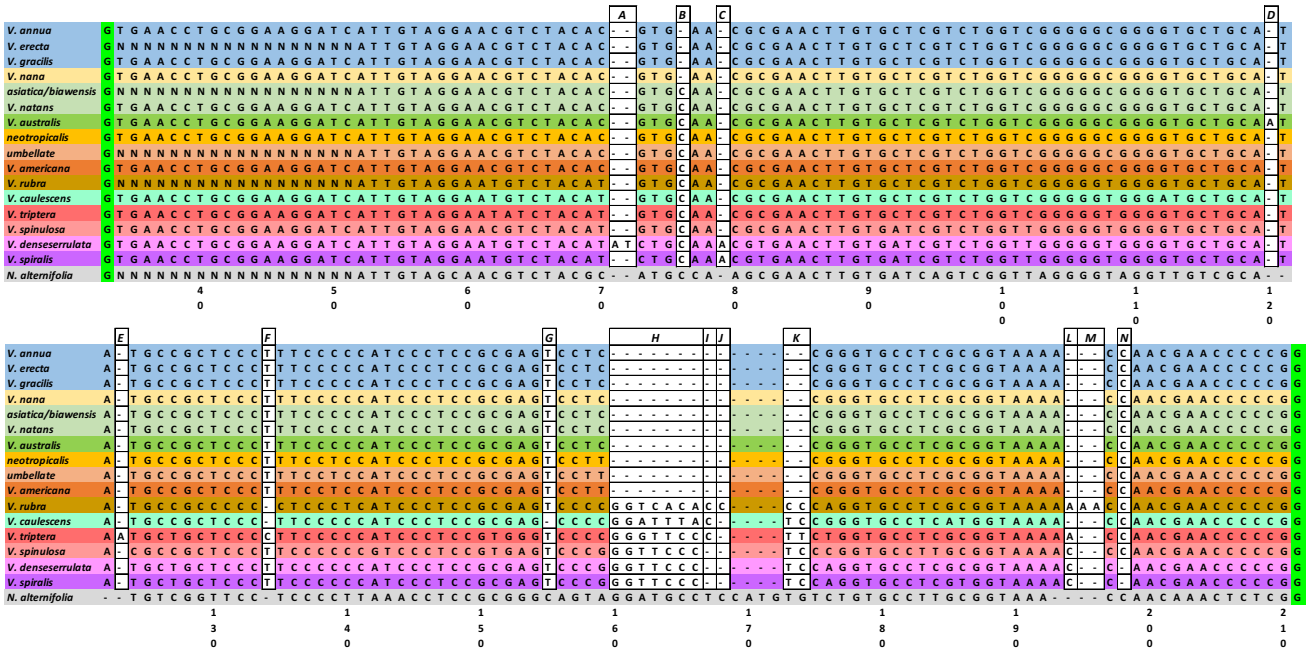

Fig. 2 Aligned portions of the nrITS region sequences from Les et al. (2008). The region of interest for *Vallisneria* occurs within nt 34 and nt 210 of ITS1 (Fig. 1). Parsimony-informative gap characters *A*–*N* are shown in white boxes. Terminal nucleotides for forward and reverse primers are highlighted in bright green. Alignment-based nt positions within ITS1 are numbered below the sequence. Taxa with distinct sequences are colored separately. Sequence for the outgroup species is shown but was not considered in gap-variant characterization. Missing data symbol = (N); (partial) gap symbol = (−)

Based on the observed alignment, the combined presence or absence of a hepta-nucleotide InDel (GGDYHYM) beginning at position 160 (gap *H*) and a dinucleotide InDel (YY) beginning at position 173 (gap *K*) separated all six basal lineages from derived lineages (Fig. 2). *V. denserrulata* also possessed a species-specific dinucleotide InDel (AT) at position 71 (gap *A*). Thus, given codominant marker polymorphism and depending on post-introduction mating histories, some *V. spiralis* × *V. denserrulata* could display heterozygous 214/216 bp fragment lengths while others could display a homozygous parental fragment length. *V. caulescens* and *V. rubra* also possessed additional gap states that, in silico, yielded species-specific fragment lengths for each. Disparate gap-state profiles led to identical fragment lengths in some instances (e.g., *V. spinulosa* and *V. spiralis*). Among derived lineages, the gap-state profile of *V. australis* possessed a unique mononucleotide InDel (A) at position 120 (gap *D*) and can be expected to yield a species-specific 205 bp fragment. *V. nana* and the closely related group of *V. annua*, *V. gracilis*, and *V. erecta* all shared a common gap-state profile, leading to a shared 203 bp fragment length that delineates members of this foursome from congeners. Whereas there were two parsimony-informative nucleotide substitutions (positions 139 and 159) within the region of interest, all gap states shared by *V. natans* and '*asiatica*' were also shared by all three American eelgrass lineages and thus are expected to yield identical 204 bp fragment lengths.

In the study of Gorham et al. (2021), nrITS regions of 35 *Vallisneria* shoots from Florida were sequenced, yielding 9 hybrids of *V. spiralis* and *V. denseserrulata*. From

ongoing work, we sequenced an additional 419 shoots from Florida. *Vallisneria* beds were identified and opportunistically selected from waterbodies throughout Florida—one bed per waterbody. In most cases, a single ramet or connected set of ramets (i.e., a genet) comprised the sample from each waterbody; any subsequent grabs were spaced at least 5 m apart to reduce the possibility of resampling a clone (Engelhardt et al. 2014). All samples were shipped or transported to the Fish and Wildlife Research Institute in St Petersburg, Florida, and a single blade was selected per sample. Each blade was scraped to remove epibionts and preserved at room temperature in 90% non-denatured absolute ethyl alcohol (EtOH, 99.5% purity). Tissue handling, DNA extraction, and nrITS sequencing procedures for the new shoots followed those described in Gorham et al. (2021) with one exception—during DNA extraction, tissues were incubated in the lysis buffer for 1 h at 55 °C prior to chilling and homogenization. Cycle sequencing was performed in both directions. Sequencing products were processed using an Applied Biosystems (ABI) 3130XL Genetic Analyzer; sequence reads were assembled and edited with Sequencher (Version 4.9; Gene Codes Corporation, Ann Arbor, MI) and aligned using ClustalW as previously described. Sequences from the new shoots expanded our library of 'known' material for native and non-native taxa.

After validating that Val-ITS-F/R yielded quality amplicons via agarose gel electrophoresis, the 5′-end of Val-ITS-F was labeled with 6-FAM fluorescent dye (Thermo Fisher Scientific, Waltham, MA). We implemented the new fluorescent PCR/capillary electrophoresis assay to screen 48 native and 48 non-native specimens from our in-house sequence library of 'known' individuals. The same template DNA stocks used in sequencing reactions were used for this assay. Amplifications were conducted in total reaction volumes of 20 µl, each containing 75 ng of template DNA, 50 µM of dNTP mix, 0.25 µl of 0.1 mg/ml BSA, 5 µl of GoTaq DNA (5×) Polymerase buffer (Promega Corporation, Madison, WI), 2.5 mM MgCl2, 0.0017 µl of a 100 µM solution of forward and unlabeled reverse primers, and 1.5 units of GoTaq DNA polymerase (Promega Corporation, Madison, WI). Reactions were performed according to the following thermal profile: initial denaturation at 94 °C for 2 min; 30 cycles of 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. NTC (no-template-control) PCR reactions were included in assays. For capillary electrophoresis, 1.0 µl of each PCR product was mixed with 12.5 µl Hi-Di formamide (Applied Biosystems) and 0.25 µl of GeneScan 500 ROX size standard (Applied Biosystems) in a 96-well plate, denatured at 98 °C for 2 min and immediately chilled on ice for 3 min. Fragments were processed on the ABI 3130XL and sized using GeneScan software (Applied Biosystems). Assay performance was evaluated in terms of accuracy, sensitivity, and specificity, reflecting within-specimen comparisons of fragment-based diagnoses to sequencing results.

To pre-qualify our procedures for the detection of F1 native × non-native hybrids, should they be encountered, we carefully quantified the template DNAs of eight library specimens identified as '*neotropicalis*' having expected 204 bp fragment lengths and eight *V. spiralis* × *V. denseserrulata* library specimens with expected homozygous 216 bp fragment lengths. Then, 1:1 template admixtures (35 ng of DNA per taxa) were created from these specimen DNAs to mimic eight native × non-native first-generation (F1) hybrid genomes. Admixed templates were subjected to PCR amplification as described above.

Results

The maximum likelihood phylogenetic analysis recovered the evolutionary lines of descent among *Vallisneria* taxa posited within Les et al. (2008). Basal and derived lineages were robustly partitioned within the genus. The tree with the highest log likelihood is shown in Fig. 3. With the exception of *V. annua*, *V. gracilis*, and *V. erecta*, which together formed a single clade, the hypothesized monophyly of each nominal species was generally well supported. The southern lineages of American eelgrass '*neotropicalis*' and '*umbellate*' also formed a well-supported monophyletic group distinct from the northern lineage *V. americana*. The node separating hypothesized basal and derived taxa was recovered in ≥ 990 of 1000 bootstrap trees. Observed relationships provided an interpretive framework for our new assay.

Among the 419 in-house nrITS region sequences, character states (presence or absence) of gaps *A*-*N* (Table S1) were invariant within 296 newly identified native '*neotropicalis*' and '*umbellate*' specimens. Within 123 newly identified non-native *V. spiralis* and/or *V. denseserrulata* specimens, gap states for *B*-*N* were also invariant, while the state of gap *A* varied depending on hybrid background. Gap states for *A*, *C*, *H*, *K*, *L*, and *N* differed between native and non-native specimens. The same pattern of within-taxon gap stability was observed among the 35 native and 9 non-native specimens sequenced in prior work. It should be noted that much of our early sampling effort was concentrated in the Osborne-Ida Chain of Lakes system in an area of southeast Florida that is known from our prior work to harbor non-natives. The high incidence of non-natives in this one system is not indicative of a high state-wide occurrence rate.

The labeled PCR primers also yielded quality amplification products. In capillary electrophoresis, all fragment lengths binned in a manner consistent with sequence expectations, with native specimens displaying the expected homozygous peak at 204 bp and non-native specimens displaying heterozygous 214/216 bp peaks or homozygous

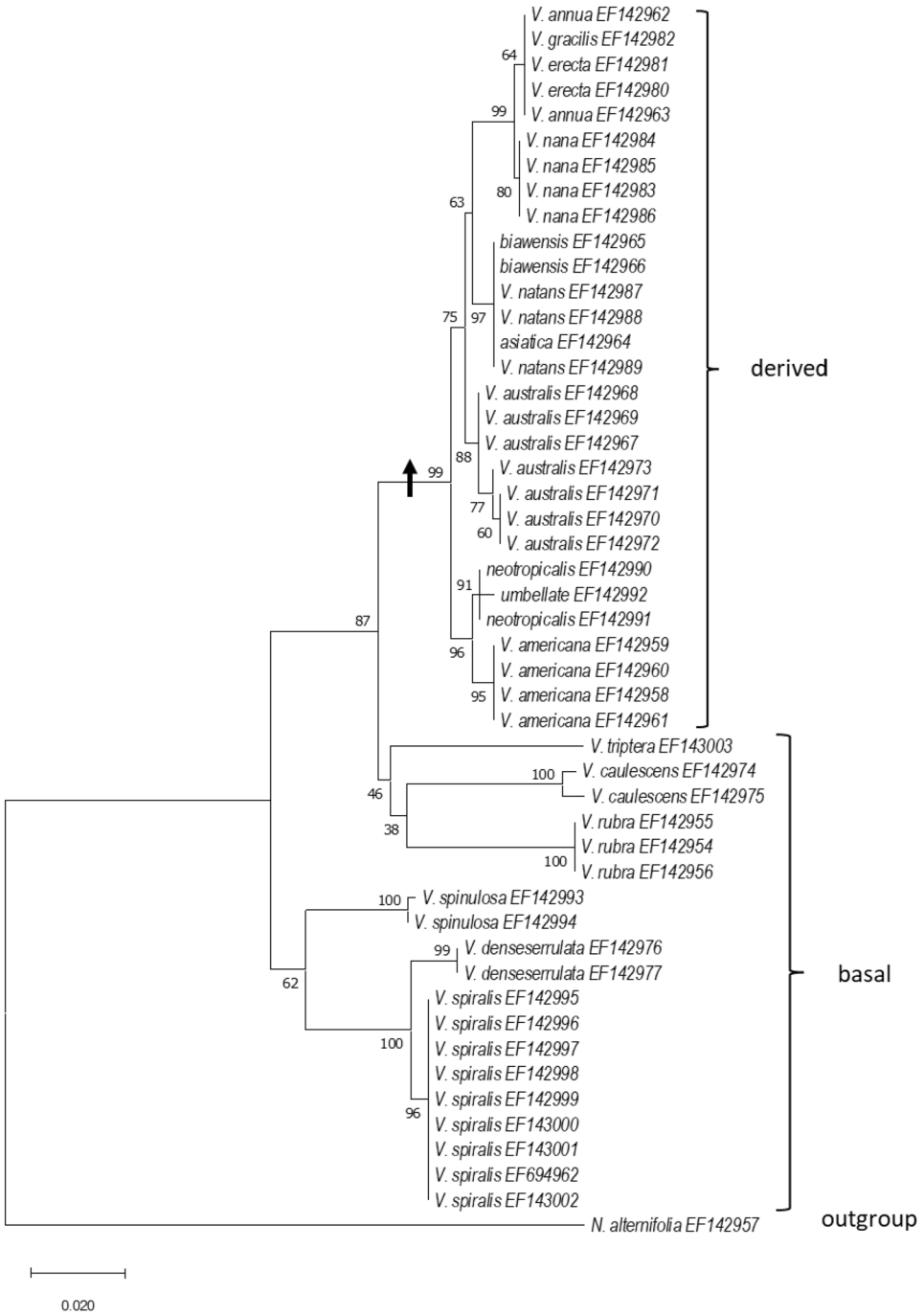

Fig. 3 Phylogenetic reconstruction of accessioned nrITS region sequences from Les et al. (2008). Basal and derived taxon groups within the genus *Vallisneria* are delineated. The arrow indicates a hypothetical common ancestor to the derived group in which gaps *H* and *K* were gained

216 bp peaks (Fig. 4a–c), depending on their hybrid background. Homozygous 214 bp peaks were not expected based on the analyzed set of 48 non-native sequences. A minor forward stutter accompanied homozygous 216 bp fragments (Fig. 4c), which is a common PCR artefact that did not impact assay performance but could likely be reduced or eliminated with use of a high-fidelity polymerase (Yamanoi et al. 2021). No secondary products suggestive of underlying paralogy were observed (Buckler et al. 1997). All 96 native and non-native specimens were correctly diagnosed as 'derived *Vallisneria*' or 'basal *Vallisneria*', respectively, and accuracy, sensitivity, and specificity for the assay were 100%. Results of assays in which native and non-native DNA templates were admixed prior to PCR confirmed that both the small and large fragment length polymorphisms co-amplify in detectable fashion; electropherograms displayed the expected fragment-length-polymorphism pattern with peaks at both 204 and 216 bp (Fig. 4d).

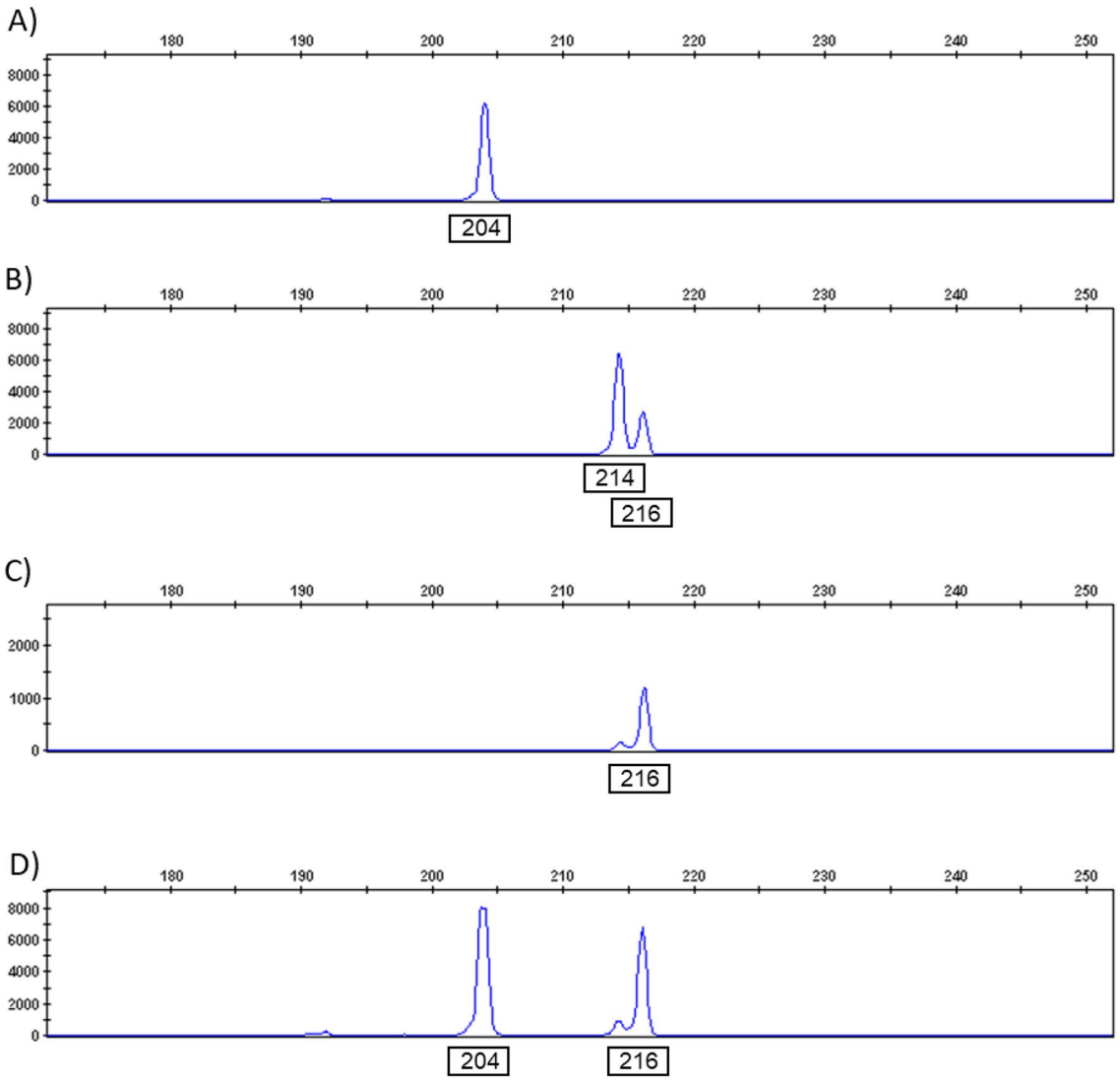

Fig. 4 Amplified DNA fragments for native and non-native *Vallisneria* in Florida as visualized on an ABI 3130XL Genetic Analyzer. See text for PCR reaction conditions and Table S1 for a listing of diagnostic fragment sizes. The *x*-axes indicate fragment size (bp); the y-axes indicate peak intensity. **A**: native specimen '*neotropicalis*'. **B**: non-native specimen *V. spiralis* × *V. denseserrulata* with heterozygous gap *A*. **C**: non-native specimen *V. spiralis* × *V. denseserrulata* with homozygous gap *A*. **D**: 1:1 template admixture of native '*neotropicalis*' and non-native specimen *V. spiralis* × *V. denseserrulata* with homozygous gap *A*

Discussion

The proposed PCR assay provides a convenient diagnostic tool for differentiating American eelgrass specimens from non-native *V. spiralis* × *V. denseserrulata* specimens, which have been recently discovered in Florida and Alabama. Specifically, we have shown via DNA sequencing and PCR assay that the native Florida '*neotropicalis*' and '*umbellate*' lineages, which are derived within the genus, do not share the gap-state profiles and larger amplicon fragment lengths that characterize the parental taxa *V. spiralis* and *V. denseserrulata*, respectively, nor those expected to characterize the other basal *Vallisneria* lineages (Table S1). Our observation that accessioned sequence for the outgroup species *N. alternifolia* also contained a similar multi-nucleotide segment at positions 160–166 and a nearby dinucleotide segment at positions 173, 174, similar to those possessed by basal lineages (Fig. 2), suggests that these two features were lost in a common ancestor of the derived lineages (Fig. 3).

Fluorescent PCR/capillary electrophoresis is a powerful and efficient technique for visualizing various types of DNA fragment length polymorphisms (Wenz et al. 2001), including those arising from InDel variants (Väli et al. 2008; Yu et al. 2014; Ramlee et al. 2015; Qu et al. 2020; Carrington et al. 2022). The new assay can be utilized in Florida for

biological field surveys and geospatial mapping of introduced *V. spiralis*, *V. denseserrulata* and *V. spiralis* × *V. denseserrulata* lineages. It can also be used in monitoring applications (Richardson and Pyšek 2012; Oswalt et al. 2021) to trace the spread of previously documented introductions. Learning if and where the non-native populations are naturalized and whether the Florida introduction was an isolated event that spread over time or occurred as a result of multiple events are of interest to managers but requires a detailed multilocus study of tokogenetic relationships between non-native specimens, which is planned to follow the statewide survey. The assay can be used to 'flag' those specimens quickly and cost-efficiently for further analyses.

There is valid concern that introduced grasses may have unknowingly found their way into restoration sites (Gorham et al. 2021), possibly through the use of cultivated materials that were not fully provenanced and/or from outplanting strategies based on transplanting vegetative material from one waterbody to another. Thus, for biosecurity purposes, the assay could be applied to retrospectively examine previously outplanted areas, and screen prospective donor populations in future translocation activities.

Introduced species become especially problematic when their gametes are reproductively compatible with those of native congeners (Vilà et al. 2000). Normally, a substantial reservoir of genetic diversity is needed for rapid adaptation to novel environments (Pertoldi et al. 2007; Leimu and Fischer 2008), but with introgression, pre-adapted characteristics and phenotypic plasticity can combine with early hybrid vigor to circumvent the need, resulting in aggressive range expansion and genetic assimilation by non-native species (Ward et al. 2008). Crossbreeding between native and non-native *Vallisneria* has not been detected in natural Florida waterbodies, despite the fact that they have been shown to co-occur within a few meters of each other in some locations (Gorham et al. 2021; Tringali et al. unpublished data). Nonetheless, there is not yet sufficient information or empirical evidence to discount the possibility. Our results with 1:1 mixed-template DNAs strongly suggested that F1 offspring of native × non-native parents, should they arise, as well as the portion of F2 and backcross specimens that by chance carry the heterozygous *H*/*K* polymorphism would be readily detectable by the current assay given adequate peak signal intensity. However, if crossbreeding occurred in previous generations such that F1 hybrids are no longer living but F2 and/or backcrossed hybrid progeny exist, only one quarter of F2 progeny would be detectable with a single-locus screen and the ability to detect hybrid progeny backcrossing to native eelgrass would diminish with each generation. Therefore, if it is learned that native × non-native reproductive exchange occurs or has occurred, the assay will need to be expanded to include additional unlinked markers.

It remains possible that non-native *Vallisneria* species from the derived clade could be introduced into Florida over time, and this has implications for natural surveys and population monitoring. As with American eelgrass lineages, accessioned sequences of other derived congeners did not share the larger fragment lengths shown for *V. spiralis* and *V. denseserrulata* nor those expected for the other basal lineages (Fig. 2; Table S1). With the exception of *V. natans*, '*asiatica*', and '*biawensis*' (the latter two are considered to be cultivated varieties of *V. natans*), all derived species appear to possess parsimony-informative gap states within the interrogated amplicon that, in silico, allow either positive diagnosis or delineation from members of the American eelgrass complex. For example, *V. australis* (cf. *gigantea*; Les et al. 2008) has been domesticated under the trade name 'Gigantea' for the global aquarium trade. Like cultivars of *V. spiralis* and *V. denseserrulata*, this Australian native is readily available for online purchase and shipping throughout the United States. To date, there are no documented introductions in the U.S. (The PLANTS Database, Retrieved July 5, 2022; version 5.1.1, http://plants.usda.gov), but without wide-scale genetic testing, this should not be taken as 'evidence of absence'. Sequence data for the six accessioned specimens suggest that the fragment length of Gigantea should differ from those of American eelgrass lineages by 1 bp, which in our experience would be detectable by capillary electrophoresis (see also Yang et al. 2015). *V. natans* and the Japanese varieties '*asiatica*' and '*biawensis*' have also been domesticated for marketing in the U.S. under the trade names 'Contortion/Contortionist' and 'Corkscrew' tape grass but appear to be indistinguishable from American eelgrass with the current assay. Thus, we are seeking an additional marker to multiplex within the proposed assay to allow delineation of North American eelgrass from *V. natans* and its '*asiatica*' and '*biawensis*' varieties. Sequence-based ground-truthing with additional derived-taxon specimens, especially these particular non-natives, will be required.

Lastly, cultivars of *V. spiralis*, *V. denseserrulata*, and *V. australis* are ubiquitous within the domestic and global aquarium trade and reportedly constitute threats to native grasses worldwide (Martin and Coetzee 2011; Willby 2007; Hussner 2012). Therefore, this simple but effective PCR screening assay could find utility in conservation, resource management, and restoration programs throughout North America and in northern Europe, the United Kingdom, Australia, New Zealand, Japan, and other potentially impacted regions (Hussner and Lösch 2005; Wasekura et al. 2016).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12686-023-01311-9.

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Author contributions MDT: conceptualization, visualization, methodology, formal analysis, resources, writing—original draft. SBG: conceptualization, investigation, data curation, resources, writing—review & editing. SS: methodology, formal analysis, investigation. CP: investigation, writing—review & editing. MSB: investigation, data curation. btf: conceptualization, resources, writing—review & editing. CM: conceptualization, funding acquisition, writing—review & editing.

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Data availability Sequence alignments for accessioned nrITS region data in FASTA format are given in Supplementary Material Seq File S1. All nrITS 'region of interest' sequences generated in house and analyzed for assay performance ($n = 96$) are available from the corresponding author on reasonable request.

Declarations

Competing interest The authors declare that they have no known competing financial or non-financial interests that could have influenced the work reported in this paper.

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