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Short title: Diversity of floating heart

Introduction of three cryptic lineages of invasive *Nymphoides cristata* in the southeastern United States

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Abstract

Crested floating heart [*Nymphoides cristata* (Roxb.) Kuntze] is an invasive aquatic plant in the southeastern United States. For clonal plants like *N. cristata*, clonal diversity may influence response to control tactics and/or evolutionary potential. However, little is known about the diversity of introduced *N. cristata*. In this study, we used genotyping-by-sequencing to quantify *N. cristata* diversity in the southeastern U.S. and determine how that diversity is distributed across the invaded range. Our results show that at least three distinct genetic lineages of *N. cristata* are present in the southeastern U.S. Geographic distribution of the lineages varied, with one widespread lineage identified across several states and others only found in a single waterbody. There is also evidence of extensive asexual reproduction, with invaded waterbodies often host to a single genetic lineage. The genetic diversity reported in this study likely results from multiple introductions of *N. cristata* to the southeastern U.S. and should be considered by managers when assessing control tactics such as screening for biocontrol agents or herbicide testing. The extent and distribution of genetic diversity should also be considered by researchers studying the potential for invasive spread of *N. cristata* within the U.S. or hybridization with native *Nymphoides* species.

Key words: aquatic plant, biological invasions, clonal reproduction, genotyping-by-sequencing

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Management Implications

The identification of three distinct *Nymphoides cristata* (crested floating heart) lineages here – and a fourth interspecific hybrid lineage (*N. cristata* × *N. aquatica*) reported elsewhere – has implications for the management of this invasive species. Studies of control techniques, spread, and impacts of *N. cristata* should explicitly consider the genetic diversity identified in this study. For example, herbicides are currently the most common option for controlling *N. cristata*, including submersed applications of diquat, endothall, and florpyrauxifen-benzyl, foliar applications of endothall, imazamox, and imazapyr, and foliar combinations of flumioxazin and glyphosate. However, herbicide testing has not explicitly considered whether *N. cristata* lineages differ in their responses. As differences in herbicide response has been found among distinct clonal genotypes of other aquatic plants (e.g., 2,4-D and fluridone in *Myriophyllum* spp.; fluridone in *Hydrilla verticillata*), we posit that herbicide trials for *N. cristata* should include representatives of the four distinct lineages identified thus far. Similarly, *N. cristata* has been identified as a candidate for biological control. However, there are no biocontrol agents currently in operation. The identification of distinct lineages in the U.S. suggests at least three independent introductions. Genetic survey of *N. cristata* in its native range could help inform the search for biological control agents, especially if introductions can be traced to distinct geographic origins. Further, any biological control agents identified should be tested on the distinct genetic lineages identified in the U.S., as biocontrol efficacy can vary at the subspecific level. Finally, it is possible that the distinct lineages could have distinct environmental preferences or tolerances that could be important for predicting their spread across the landscape.

Introduction

Genetic variation can influence plant invasions and management (i.e., Barrett 1992; Bossdorf et al. 2005; Sakai et al. 2001; Ward et al. 2008). Additionally, genetic variation can facilitate adaptation to new environments encountered after introductions, and during range expansion (e.g., Lee 2002; Prentis 2008). For example, distinct genetic populations or lineages of invasive plants may vary in their response to herbicides (e.g., Kay 1992; Netherland and Willey 2017; Chorak and Thum 2020; Williams et al. 2020; Kurniadie et al. 2021). Similarly, genetic

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lineages can differ in the effect of biological control agents (e.g., Sobhian et al. 2003; Bruckart et al. 2004; Williams et al. 2014; Blossey et al. 2018).

The amount and distribution of genetic variation in invasive plants will be primarily influenced by the number of introductions, the genetic structure and diversity of source populations, and the extent of admixture, sexual reproduction, and asexual reproduction in the introduced range. At one extreme, introduction of a single genet followed by exclusively asexual reproduction will result in essentially no genetic diversity in the introduced range (e.g., Hollingsworth and Bailey 2000; Le Roux et al. 2007; Loomis and Fishman 2009; Zhang et al. 2010). At the other extreme, multiple introductions from genetically distinct source populations, followed by admixture and sexual reproduction, can generate greater and novel genetic diversity in introduced populations (Facon et al. 2008; Kolbe et al. 2004; Lavergne & Molofsky 2007). Population genetic and genomic descriptions of invasive species can help determine the number of distinct lineages present in the introduced range, and provide insight into the extent or potential for admixture of distinct lineages which in turn can inform studies on herbicidal and biological control development.

Crested floating heart [*Nymphoides cristata* (Roxb.) Kuntze] is a floating-leaved aquatic plant native to Asia that has spread across the southeastern United States. The first introduction of *N. cristata* appears to have occurred in south Florida circa 1996 when plants escaped from cultivation for the aquatic garden trade (Burks 2002). Since then, *N. cristata* has been observed in Louisiana (2012), Mississippi (2016), North Carolina (2017), South Carolina (2006), and Texas (2014), and is considered an invasive/noxious species in several of the inhabited states (Thayer and Pfingsten 2024). The scattered distribution of *N. cristata* populations throughout the southeastern U.S. may reflect multiple introductions (Burks 2002), but no genetic analysis has been conducted to date to test whether introduced *N. cristata* consists of one or more distinct lineages.

Nymphoides cristata is capable of both asexual and sexual reproduction, although the prevailing mode of reproduction remains largely unknown. In the native range, *N. cristata* reproduces sexually through a gynodioecious breeding system derived from heterostyly, where female plants with reduced, sterile stamens, rely on the bisexual-morph plants for pollen (Nair 1973). In addition, bisexual plants from the native range have shown self-compatibility in an experimental setting (Nair 1973). However, asexual reproduction via vegetative propagation is

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likely responsible for the majority of biomass (Nair 1973; Sculthorpe 1967), a strategy common in the *Nymphoides* genus (Gettys et al. 2017; Sivarajan and Joseph 1993). Similarly, *N. cristata* is thought to reproduce primarily through vegetative means in the introduced range (fragmentation, daughter plants, tubers, and rhizomes) (Burks 2002; Willey and Langeland 2011). Spread and range expansion of the species is likely facilitated via fragmentation caused by contact with boat motors, wave action, and mechanical harvesting (Burks 2002; Willey et al. 2014). It is also capable of producing seeds (Burks 2002; Gettys et al. 2017), and has hybridized with a native species, big floating heart [*Nymphoides aquatica* (J.F. Gmel.) Kuntze] (Harms et al. 2021), but the extent of sexual reproduction in U.S. populations remains unknown.

In this study, we conducted a population genomic survey of introduced *N. cristata* in the southern U.S. to determine how much genetic diversity is in the introduced range. For example, if the *N. cristata* invasion results from asexual spread of a single genetic clone, then we would expect to find no population genomic variation in our survey (barring somatic mutation and genotyping error). In contrast, identification of multiple distinct genetic lineages would suggest multiple introductions from different sources, and the potential for intermediate genotypes if distinct lineages have hybridized.

Materials and Methods

Sample collection:

Samples were obtained from a previous study on *N. cristata* (see Harms et al. 2021), using preserved DNA samples and dried plant tissues. Briefly, Harms et al. (2021) collected 1-13 plants per waterbody, depending on the size of the infestation, with small populations minimally sampled (i.e., only a few plants). Within waterbodies, plants were sampled 3-5 meters apart to avoid repeatedly collecting the same plant. The final data set consisted of 62 samples (i.e., leaves from *N. cristata* plants) from 12 different waterbodies throughout the southeast United States, including lakes, ponds, roadside ditches, and canals (Table 1). We acknowledge that our sampling strategy limits out inference regarding diversity within waterbodies. However, the main focus of this study was to evaluate evidence for one versus multiple genetic lineages in the U.S., and given the likelihood of local clonal reproduction, we prioritized the number of waterbodies examined over the number of individuals per waterbody.

DNA sequencing:

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We used next-generation, genotyping-by-sequencing (GBS) to generate a single nucleotide polymorphism (SNP) data set. Prior to sequencing, DNA was extracted from plant tissues using the Qiagen DNeasyPlant Kit (Qiagen Corporation, 27220 Turnberry Lane, Suite 200, Valencia, CA 91355) following the standard plant protocol. A Qubit fluorometer (ThermoFisher Scientific, 168 Third Ave., Waltham, MA, 02451) was used to confirm genomic DNA content was high enough for sequencing (≥60ng total gDNA), then all extracts were sent to the University of Minnesota Genomic Center for library assembly and sequencing. The sequencing library was prepared for double digest restriction-site associated DNA sequencing (ddRAD; Peterson et al. 2012) using the restriction enzyme pair *Pst*I and *Msp*I and size-selected for 101bp fragments using the PippinHT system. The library was sequenced on an Illumina NovaSeq 6000 (Illumina, Inc., 5200 Illumina Way, San Diego, CA, 92122) system targeting approximately 2.5M single-end reads per sample.

Bioinformatics and filtering:

Following sequencing, we processed the raw sequence data using a bioinformatics pipeline to produce a SNP data set for downstream diversity analyses. First, the reads were demultiplexed by barcode and adapters were trimmed using *gbstrim*, a custom script designed to pre-process GBS data generated by UMGC (Garbe 2022). The demultiplexed reads were then passed to *Stacks* v2.55 (Catchen et al. 2013), where the *process_radtags* module removed low-quality reads (i.e., "Phred" score < 25). Next, the core de novo pipeline was executed in *Stacks* with the minimum stack depth (*m*), mismatch distance between loci within an individual (*M*), and number of mismatches between loci in the catalog (*n*) parameters set at 5, 4, 4, respectively. These were selected following a parameter optimization procedure similar to that outlined in Paris et al. (2017). After catalog creation, SNP identification and genotyping were also performed in *Stacks*, retaining only one biallelic SNP per RAD-locus. The resulting variant-calling files were exported for further filtering and analysis. All bioinformatic work in this study was performed on the Montana State University Tempest computing cluster.

To ensure only high-quality variants were included in downstream analysis, the genetic data were filtered with the R package *vcfR* v1.13.0 (Knaus and Grunwald 2017). To reduce the amount of missing data, we excluded loci that were absent in >25% of the individuals and removed any individuals with >75% missing genotype calls. We also removed loci with unusual read depth (under 10^{th} percentile or over 90^{th} percentile) and set the minimum minor allele

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frequency to 0.05. After filtering, the final genetic data set contained 62 *N. cristata* samples genotyped at 2,242 SNPs.

Data analysis:

To avoid violating any model assumptions associated with the clonality of *N. cristata*, we used a model-free approach to explore the amount and distribution of genetic diversity. A Euclidean genetic distance matrix was generated in *adegenet* v2.1.7 (Jombart and Ahmed 2011) for use in Principal Components Analysis (PCA). The PCA was completed in the *ade4* package (Dray and Dufour 2007) to visualize genetic variation among samples and determine whether distinct genetic groups of *N. cristata* exist. We also generated a genetic dissimilarity matrix using *poppr* v.2.9.3 (Kamvar et al. 2014) to summarize the actual number of SNP differences between individuals. The distribution of *N. cristata* across the invaded range was mapped with *ggplot2* (Wickham 2016). All data analyses were conducted in R version 4.2.1 (R Core Team 2021).

Results and Discussion

Our survey of *N. cristata* diversity identified three distinct genetic groups (Figure 1). We refer to those groups as genetic lineages, collections of closely related individuals distinguished by the genomic variants (i.e., SNPs) inherited from a common ancestor. Genetic variation was found both within- and between lineages, although the amount of between-lineage variation was far greater than within. Within lineages, individuals averaged approximately 35 SNP differences, while individuals compared across lineages differed by an average of 1028 – 1514 differences (Table 2).

The CFH-2 lineage was the most common and widespread; it was found in Florida, Louisiana, and Texas, and occurred in 10 of the 12 waterbodies sampled overall (Figure 2). In contrast, CFH-3 was only found in one waterbody (Lake Marion, SC), and CFH-1 was only found in two waterbodies, both in southeast Florida (Figure 2).

The differences in relative abundance among lineages may be due to introduction dynamics and/or ecological differences among lineages. *N. cristata* was likely brought to the U.S. for trade as an aquatic garden ornamental and remains available for purchase from vendors in the industry. Although it is now illegal to possess, import, or distribute *N. cristata* in several southern states (FL, SC, NC, TX), the ornamental industry is a likely candidate for initial introduction(s) and subsequent range expansion. The relative abundance of the CFH-2 lineage

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may indicate that it was a preferential/popular lineage in the industry, or that it was the first to escape cultivation. Indeed, *N. cristata* is clonally propagated for sale in the industry, although it was not possible to include commercially available ornamental samples in this study due to time and funding constraints. In addition, CFH-2 may be more vigorous and widely adapted in the introduced range than others, facilitating its spread across the southern states while CFH-1 and CFH-3 remained isolated. Further investigation would be necessary to determine whether CFH-2 presents a greater management challenge. We recognize that the number of waterbodies sampled and number of individuals sampled within a waterbody was limited and there may be more diversity in the introduced range than we detected.

Burks (2002) suggested multiple introductions, possibly escaped from ornamental water gardens, based on the scattered distribution of *N. cristata* across southern Florida. Indeed, we identified two distinct lineages in Florida: one that was restricted to southeastern Florida, and a second lineage that is widespread across Florida and the U.S. Gulf Coast (Figure 2). These may represent two independent introductions in Florida followed by range expansion of CFH-2. It is also possible that CFH-2 has been repeatedly introduced across the Gulf Coast states, with its widespread distribution representing numerous independent introductions. Further, we identified a third unique lineage found only in South Carolina (CFH-3), which could represent another introduction.

We cannot rule out with certainty the alternative hypotheses of a single introduction from a genetically variable source, or accumulation of new mutations following introduction. However, we would have expected more within-waterbody lineage diversity if *N. cristata* was introduced from a genetically variable source (although we recognize that within waterbody sample sizes were low). Similarly, we find clonal evolution post-introduction unlikely because of the relatively large number of allelic differences (1028 - 1514) separating the three distinct lineages combined with the relatively recent introduction (~1996). Plant invasions resulting from multiple introductions are common (Dlugosch and Parker 2008), particularly for ornamental species like *N. cristata* where the plant trade increases the likelihood of repeated introductions (Dehnen-Schmutz et al. 2007). Sampling efforts in the native range (i.e., southeastern Asia), along with additional sampling in the U.S., could help clarify the number and location(s) of sources of *N. cristata* introduction. In addition, *N. cristata* has been identified as a good candidate for biological control, although natural enemies/potential agents have yet to be

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described (Harms and Nachtrieb 2019). Identification of source populations in the native range might also yield natural enemies of this plant that could be tested as potential biocontrol agents (Bossdorf et al. 2005; Gaskin et al. 2011).

The introduction of three distinct lineages provides opportunities for genetic admixture and the generation of novel genetic variants in the introduced range (e.g., Facon et al. 2008; Kolbe et al. 2004; Lavergne & Molofsky 2007). Although interspecific hybrids between *N. cristata* and native *N. aquatica* have been identified in the Santee Cooper Reservoir system in South Carolina (Harms et al. 2021), we did not find any evidence for sexual reproduction among the three lineages, as evidenced by the lack of genetically intermediate individuals (Figure 1). In addition, the average pairwise genetic distances between individuals were approximately 97% greater when comparing between lineages versus within lineages. It is possible that these lineages have sexually reproduced with one another, but that we did not sample them. Alternatively, it is possible that the distinct lineages are capable of sexual reproduction but have not had sufficient opportunity, yet. The three lineages were largely allopatric, but there was some overlap between two of them in south Florida. Finally, it is possible that the different lineages have some reproductive barriers (e.g., pre or post mating, pre or post zygotic) that limit sexual reproduction between them. Additional study of reproductive potential among the introduced lineages is warranted.

While we cannot rule out some degree of sexual reproduction within lineages, we hypothesize that the low genetic variation observed within each of the lineages primarily reflects sequencing/genotyping errors, and that the *N. cristata* lineages identified here have primarily reproduced asexually throughout the southeastern United States. Although individuals within each lineage were not genetically identical, per se, clonal genotypes are not expected to have identical genotypes across thousands of SNPs generated by ddRAD due to sequencing/genotyping error and somatic mutations (da Cuhna et al. 2021; Reynes et al. 2021). The interpretation of the low within lineage variation as clonal reproduction is consistent with previous field observations and reports of reproductive biology of *N. cristata* that hypothesize prolific vegetative propagation and spread in the invaded range (Burks 2002; Harms and Nachtrieb 2019; Willey and Langeland 2011). Inbreeding could also account for the within-lineage variation we observed. *Nymphoides cristata* has been proven to be self-compatible through artificial pollination of bisexual plants in an experimental setting (Nair 1973). However,

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heterostyly provides a morphological based incompatibility system in *N. cristata*. Although incomplete, this system is thought to promote reproduction between the female and bisexual plant morphs (i.e., dioecism) in the native range (Nair 1973). Further sampling and detailed description of flower morphology in the introduced range could help decipher the reproductive capabilities of the introduced populations. Finally, we acknowledge that the low sample sizes within waterbodies preclude an understanding of the relative extent of sexual versus asexual reproduction in any single population of *N. cristata*.

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Table 1. Waterbodies where *Nymphoides cristata* were collected for use in this study, including genetic lineage assignments (Lineage ID) and number of individuals (*N*) collected from each waterbody.

Waterbody	Latitude	Longitude	County	State	Lineage ID	*N*
Lake Fairview	28.6005	-81.4128	Orange	FL	CFH-2	1
JW Corbett WMA	26.8579	-80.4165	Palm Beach	FL	CFH-2	2
Roadside canal	26.6548	-80.1747	Palm Beach	FL	CFH-1, CFH-2	2, 6
Flying Cow ditch	26.6348	-80.3001	Palm Beach	FL	CFH-1	6
Business pond	26.0057	-80.3018	Broward	FL	CFH-2	2
Flat Lake	30.2817	-90.8182	Ascension	LA	CFH-2	13
Private pond	30.4043	-91.1576	Avoyelles	LA	CFH-2	1
Lake Marion	33.5372	-80.428	Berkeley	SC	CFH-3	7
Caddo Lake	32.7195	-94.1198	Harrison	TX	CFH-2	10
Lake Conroe	30.5643	-95.6358	Montgomery	TX	CFH-2	3
Houston Arboretum	29.7618	-95.4498	Harris	TX	CFH-2	7
Roadside ditch	30.4399	-94.7201	Hardin	TX	CFH-2	2

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Table 2. Summary of individual-based genetic distance within and between lineages. Reported values along the diagonal are the absolute number of SNP differences between individuals within a lineage. Between lineage values represent the average number of SNP differences observed across lineages.

	CFH-1	CFH-2	CFH-3
CFH-1	30	-	-
CFH-2	1514	31	-
CFH-3	1099	1028	41

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Figure 1. Principle component analysis of 62 *Nymphoides cristata* samples. Principle components 1 and 2 account for 96.1% of cumulative variation. Colored ellipses (non-statistical) were added *a posteriori* to denote putative genetic lineages identified by ordination.

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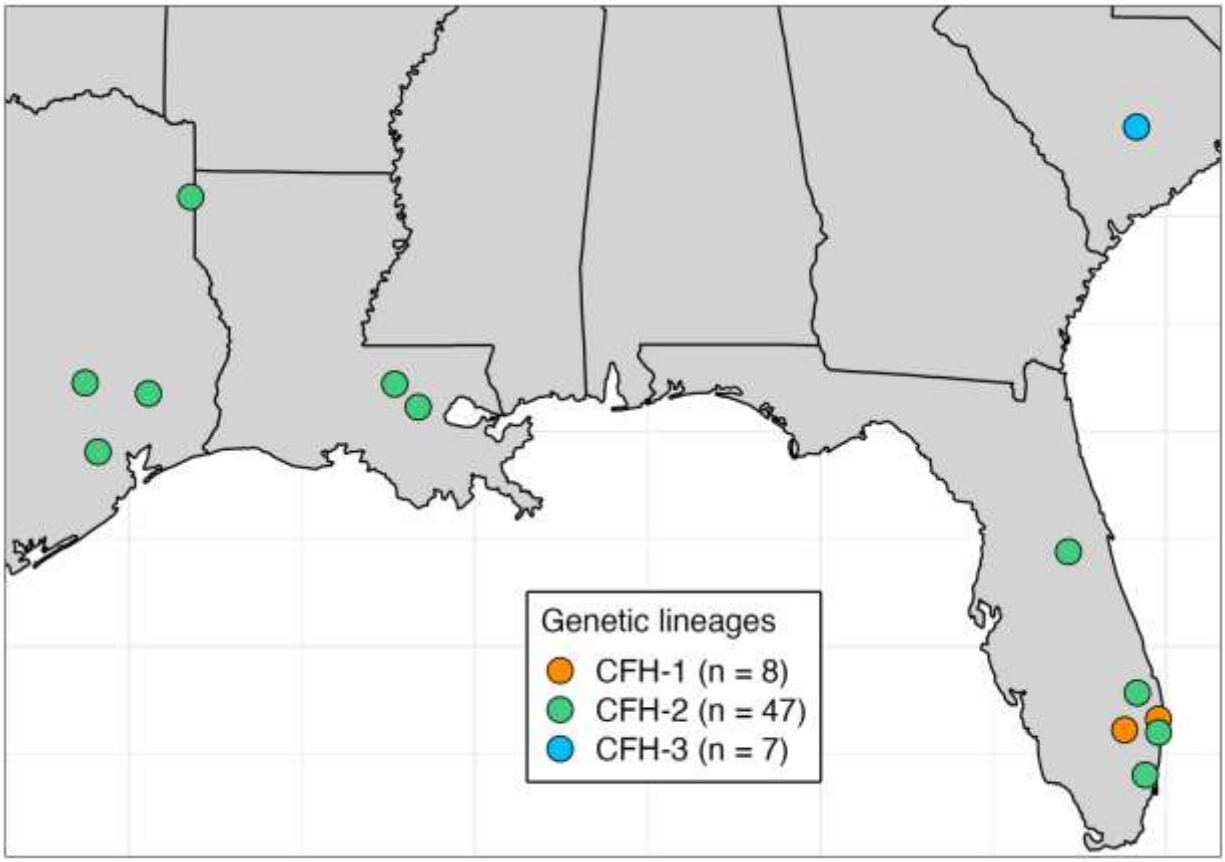

Figure 2. Distribution of *Nymphoides cristata* lineages across the introduced range. Points represent waterbodies where samples were collected and colors denote the genetic lineage(s) present, assigned according to the PCA analysis in Figure 1. **Note there are two overlapping points representing the roadside canal in south Florida where CFH-1 and CFH-2 co-occurred.*

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