Statement of work

Introduction

Nuphar sagittifolia (Walter) Pursh (Nymphaeaceae; Fig. 1), Cape Fear spatterdock, is an aquatic macrophyte endemic to the Atlantic Coastal Plain from Virginia to South Carolina (Padgett 2007). Populations of *N. sagittifolia* are known from a small area in central coastal Virginia and from southeastern North Carolina into contiguous South Carolina, separated by a gap in southeastern Virginia and northeastern North Carolina. It is state listed in Virginia and a federal species of special concern. The United States Fish and Wildlife Service (USFWS) has been petitioned to consider *Nuphar sagittifolia* for federal listing, due to apparent contraction of its already narrow range and the possibility that saltwater intrusion and pressure from aquatic invasive plants are negatively impacting populations (USFWS, pers. comm.). In order to make an informed decision, a range-wide status assessment is prerequisite.

Figure 1: *Nuphar sagittifolia* flower

However, a key requirement for status assessment and conservation strategy development is a solid understanding of the taxonomic units involved. Unfortunately, the taxonomic limits of *Nuphar sagittifolia* remain woefully unclear. In fact, numerous populations, due to their intermediate morphology, cannot be reliably and conclusively identified to this or any other species of *Nuphar*, using the most recent revision of the genus (i.e., Padgett 2007). The primary morphological character used to date to distinguish *N. sagittifolia* from sympatric *N. advena* (Aiton) W.T. Aiton is the leaf length:width ratio. A large portion of reported populations of *Nuphar sagittifolia* are morphologically intermediate between *N. advena* and *N. sagittifolia*, and exhibit at least some leaves considerably shorter than "typical" *N. sagittifolia*, but have the abundant submerged foliage characteristic of the species (B. Sorrie[1], pers. comm., J. Gray[2], pers. comm., K. Culatta, pers. obs.). It is likely that more intermediate populations will be documented as surveys continue. Populations of unresolved taxonomic identity are shown in red on Figure 2.

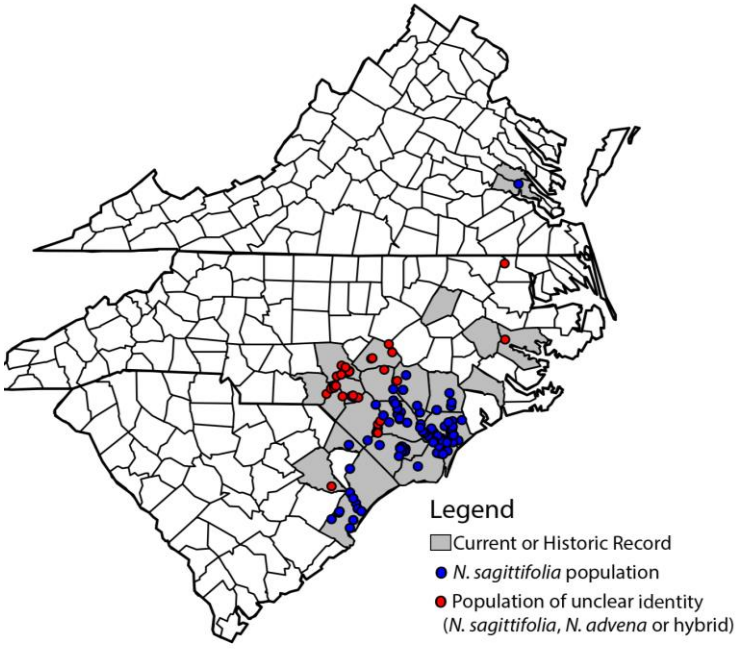

Figure 2: *Nuphar sagittifolia* county distribution and known occurrence. Data sources: NC Natural Heritage Program; USFWS; Virginia Dept. of Conservation and Recreation; Herbarium records via sernecportal.org; pers. obs. Map created by K. Culatta.

[1] North Carolina Natural Heritage Program
[2] Endangered Species Botanist, Fort Bragg NC

Hybridization is well documented among *Nuphar* species (Padgett et al. 1998, 2002), including cross pollination between *N. sagittifolia* and *N. advena* resulting in a 17.4% fruit set (DePoe and Beal 1969). Fernald described a hybrid of *N. sagittifolia* and *N. advena*, *Nuphar* x *interfluitans*, from the Chickahominy River in Virginia (1942), but the taxon has not been recognized in subsequent treatments. Beal (1956) recognized *N. sagittifolia* as one of nine intergrading subspecies of a single polymorphic *N. lutea* (L.) Sm. spanning Europe and North America, but this circumscription has been rejected by most North American taxonomists (Wiersema and Hellquist 1993; Haines et al. 2011; Weakley 2015). Though most current taxonomists agree *Nuphar sagittifolia* should be treated at the rank of species, relationships among species of *Nuphar* remain unclear (Padgett et al. 1999).

The unresolved taxonomic circumscription of *N. sagittifolia* presents a serious barrier in assessing the conservation needs of the species, as the number of populations considered true members of the species cannot be reliably determined. In order to address this need, the objectives of the current study are to:

1. Conduct a taxonomic analysis of the *N. sagittifolia* complex using a combined genetic and morphological approach.
2. Assess genetic diversity and population structure in true *Nuphar sagittifolia.*
3. Conduct a status assessment of true *Nuphar sagittifolia.*

Methods

Leaf tissue and morphometric data will be collected from a minimum of 20 populations throughout the range of *N. sagittifolia*, including populations with typical *N. sagittifolia* morphology, typical *N. advena* morphology, and hybrid morphology. Leaf tissue and leaf morphology data will be collected from 30 individuals in each population, and flower and fruit morphology will be documented in a subset of individuals (minimum 3 flower, 3 fruit per population). Voucher specimens will be collected at each population and deposited at the herbarium of North Carolina State University. Leaf tissue will be collected from a small section along the margin of each leaf, leaving the leaf mostly intact to minimize damage to the individual.

Populations will be assessed for total extent, percent cover of *N. sagittifolia* vegetation at the water surface, and presence and quantity of flowers and fruit. Environmental data will be collected, including stream width, stream flow, canopy condition, and water transparency. Nearby occurrence of invasive species, especially Alligatorweed (*Alternanthera philoxeroides*), and *Hydrilla verticillata* will be documented. Surveys for new populations will be completed in counties with current or historic *N. sagittifolia* occurrence and bordering counties, especially in the gap between the North Carolina and Virginia records, where one unconfirmed population has been reported.

The proposed project will combine morphometric and population genetic techniques for a new approach to the taxonomic problem outlined above. Previous studies have demonstrated correlation between morphometric and genetic characteristics in plants using RAPD (Persson and Gustavsson 2001) and AFLP (Assogbadjo et al. 2006) genetic markers. The proposed project will utilize microsatellite markers, which target sequences that are highly variable due to relatively fast mutation rates, and have been

used correlate morphology and geography in plants (Sarri et al. 2006). Four microsatellite markers developed in *Nuphar submersa* Shiga & Kadono (Yokogawa et al. 2012) and four developed in *N. japonica* DC. (Kondo et al. 2016) have been selected based on similarity to published portions of the *N. advena* genome (NCBI accession SRX1060236). The use of pseudo-multiplexed fluorescently labeled primers as in Schuelke (2000) will allow four PCR products to be genotyped in each well, cutting sequencing costs. Through an integrative approach, connections between geographic, morphological, and genetic characteristics of populations can be evaluated in informative ways. Of particular interest is potential correlation between morphological and genetic distance among individuals, which will give insight into how morphological differences reflect population structure. A population genetic assessment of *N. sagittifolia* will describe genetic structure, diversity within and among populations, and determine the relative importance of clonal and sexual reproduction. It is unclear if large stands of *N. sagittifolia* represent multiple genotypes, or if the rhizomatous habit allows large areas to be dominated by one genetic individual. The extent of clonality, extent of gene flow within and among river systems, and level of genetic diversity within populations will clarify which populations are of highest priority for conservation. Population genetic information has been used to inform conservation decisions in numerous plant taxa, including *Nuphar submersa* in Japan (Shiga et al. 2017).

Progress to date

Surveys and data collection began in the 2017 field season. Surveys have been completed in four North Carolina counties, and started in nine more (representing 76% of counties with current or historic *N. sagittifolia* records in the state). Plants were found absent at three previously reported localities, present at twelve others, and eight new populations were documented within the known range.

Morphological data and leaf tissue for DNA extraction have been collected from three populations, along with leaf tissue from four additional locations for use in microsatellite marker testing. DNA has been successfully extracted from 65 individuals for marker testing, including one *Nuphar japonica* individual, provided by the Denver Botanic Gardens, to serve as a control.

Timeline

Marker testing and DNA extraction from the three populations already collected will be completed by Spring 2018. During the 2018 growing season, tissue and morphometric data will be collected from 17 more populations, and surveys and status assessment of all known populations will be completed. Lab work (DNA extraction, PCR with microsatellite markers) will be completed by Spring 2019, and results prepared and submitted for publication in 2019.

How the study will benefit coastal wetlands

A better understanding of the population genetic characteristics, habitat, and proper management of this charismatic, endemic member of Southeastern Coastal Plain flora will encourage protection of wetland habitat and foster broader interest in aquatic communities. In addition, documentation of invasive plant occurrences and other potential threats to federally listed species benefit wetlands as a whole. Conservation of

Nuphar sagittifolia populations will also have a locally desirable impact on wetland communities, as the presence of aquatic macrophytes is correlated with increased fish density and fish species richness (Randall et al. 1996) and increased invertebrate abundance (Cyr and Downing 1988) compared to unvegetated areas. The presence of aquatic vegetation supports fish communities by providing complex structure for foraging and predator avoidance (Killgore et al. 1989). This study will provide a baseline measurement of *Nuphar sagittifolia* population distribution, size, and density essential for quantitative tracking of population changes and the extent of range contraction.

Funds Requested

Funding will be used for field and laboratory costs as follows:
Fluorescent tagged M13 primers: $412 (four 10,000 pmol vials, $103 each); **Sanger Sequencing at NCSU Genomic Sciences Laboratory: $450** (fifteen 96 well plates, $30 each) **Micropipet tips: $369.90** (six cases, $61.65 each); **96-well PCR plates: $322.98** (six cases, $53.83 each); **Miscellaneous laboratory consumables: $400** (Reagents, gloves, filters, etc.); **Four-wheel drive vehicle rental for remote surveys: $1,020** ($0.68/mile from NCSU Motor Pool: 6 trips, 250 miles each); **Motorized boat rental for large river surveys: $900** (3 days, $300 each from Beach House Boat Rentals, Murrell's Inlet SC); **Gas reimbursement for data collection in personal vehicle: $802.50** (1500 miles at standard rate of 53.5 cents/mile); **Miscellaneous field consumables: $300** (Silica gel, vials, camera batteries, etc.)

Total Funds Requested: $4,977

Dissemination of Results

Results of the study will be shared with interest groups whose members frequently encounter *N. sagittifolia* in work or recreational setting. Such local communities of interest (e.g. kayak clubs, agency field scientists, etc.) have already been involved in the survey efforts and valuable location reports have been received from citizens. Opportunities for sharing the results of this study through conference presentations include the meetings of The Association of Southeastern Biologists, The Botanical Society of America, and the Society of Wetland Scientists. I anticipate three scientific publications resulting from the proposed work: (1) Taxonomic analysis the *N. sagittifolia* complex (target journal: Systematic Botany), (2) Conservation genetics of *N. sagittifolia* (target journal: Conservation Genetics), and (3) Status assessment of *N. sagittifolia* (technical report to the USFWS). *Nuphar sagittifolia* is tracked by state agencies throughout its range, and new or updated element occurrence records will be submitted to each appropriate agency. Information provided to USFWS and state agencies will aid in educating landowners and recreational boaters.

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