

Nymphaea odorata (Water-lily, Nymphaeaceae):
Analyses of molecular and morphological studies

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment in requirements for the degree of
Masters of Science
in
Biology

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February 6, 2003

Blacksburg, Virginia

KEY WORDS: *Nymphaea*, speciation, hybridization, internal transcribed spacer (ITS), *trnL-F*, inter-simple sequence repeats (ISSR), morphological variation

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ABSTRACT

Molecular and morphologic studies were used to determine the evolution, classification and differentiation of *Nymphaea odorata*. Molecular analyses of the nuclear internal transcribed spacer (ITS) region, the chloroplast *trnL-F* region, and inter-simple sequence repeat (ISSR) markers determined the variation present between and within two species of *Nymphaea*. The ITS region resulted in a phylogeny depicting strong separation between species (*N. mexicana* and *N. odorata*) and some separation between *N. odorata*'s subspecies. The ITS region contained polymorphisms, which upon SAHN clustering and principle coordinate (PCOA) and minimum spanning tree (MST) analyses produced groups similar to the clades in the ITS phylogeny. Sixteen accessions were chosen for *trnL-F* analysis, where a subspecies-specific molecular marker was found. In most accessions the marker confirmed the original subspecies classification. Molecular analyses using ISSRs characterized among population variation in *N. odorata* and *N. mexicana* using five primers. ISSR markers among populations were highly variable within a species and were used in UPGMA, PCOA and MST analysis, which resulted in separation between the subspecies.

Both univariate and multivariate analyses were performed on quantitative and qualitative morphological characters. An analysis of variance resulted in six morphological characteristics that were statistically significant ($P < 0.05$), the majority being leaf blade characteristics. Multivariate statistics of principle component analysis and discriminate analysis resulted in groups for each subspecies, both emphasized the importance of quantitative leaf blade characteristics. Overall, both morphology and molecular characteristics supported the classification of subspecies for ssp. *odorata* and ssp. *tuberosa*, due a lack of strong segregation of characteristics.

ACKNOWLEDGEMENTS

I would like to thank Khidir Hilu for his tremendous support, encouragement and critique throughout this research. Thanks also to my committee Lynn Adler and Bruce Turner for fruitful discussions and edits to earlier drafts on my manuscript. Thank you to Thomas Borsch for without his previous work and current dedication to *Nymphaea*, this project would not have been possible. Also, thanks to John Wiersema whose intimate knowledge and life long devotion to *Nymphaea* has made this project outstanding.

Additionally, I would like to thank Susan Neves for training me on molecular analyses and expert advice. Also, for her help with field collections in Tennessee. I am also indebted to Tracey Slotta for her many hours of patience while teaching me frustrating statistical programs.

Thank you to the International Waterlily Society, Virginia Academy of Sciences, Virginia Graduate Student Association, Sigma Xi and the Virginia Tech Biology Department which all provided funding.

Finally, without the unending support of my parents and Jim Woods this work would not have been possible. Further thanks to Jim for help with field collections and graphic support. I would also like to thank Dr. Kimberly Hunter for giving me the tools to succeed and making me believe in myself.

TABLE of CONTENTS

CHAPTER 1. Introduction

1.0 Introduction	1
1.1 Nymphaeaceae	2
1.11 <i>Nymphaea mexicana</i>	3
1.12 <i>Nymphaea odorata</i>	5
1.2 Hybridization and Taxonomic Issues	7
1.3 Objectives and Approach	7
1.4 Choice of Molecular Approach	9
1.5 References	23

CHAPTER 2. Speciation in the North American Water-lily *Nymphaea odorata* Using Molecular Variation in the ITS and *trnL-trnF* Regions

2.1 Introduction	26
2.2 Materials and Methods	27
2.21 Material	27
2.22 DNA Extraction, Amplification and Sequencing	27
2.23 Sequence Alignment and Phylogenetic Analyses	28
2.24 Analysis of Polymorphic Sites	28
2.3 Results	29
2.31 ITS Region	29
2.32 <i>trnL-trnF</i> Region	29
2.33 Polymorphic Sites	30
2.4 Discussion	30
2.41 Implications to the genus <i>Nymphaea</i>	30
2.42 Speciation of <i>Nymphaea odorata</i>	31
2.43 Evidence of Hybridization	34
2.44 Implications to Aquatic Speciation	36
2.45 Conclusions	37
2.5 References	47

CHAPTER 3. Using Morphology and Inter-Simple Sequence Repeats (ISSR) to Analyze the Variation in Populations of *Nymphaea odorata*

3.1 Introduction	49
3.2 Materials and Methods	50
3.21 Morphological Analyses	50
3.22 ISSR Analyses	51
3.3 Results.....	53
3.31 Morphological Analyses.....	53
3.32 ISSR Analyses.....	54
3.4 Discussion.....	55
3.41 Morphological differences between the subspecies.....	55
3.42 Intrapopulation and Interpopulation variation using ISSR markers.....	56

3.43 Conclusions.....	57
3.5 References.....	73
Summary and Conclusions.....	75
Curriculum Vitae.....	78

LIST of FIGURES

- Fig 1.1 Phylogeny of *Nymphaea* based on sequences of the 5.8S gene and ITS 2 using *Cabomba* and *Nuphar lutea* as outgroups. The strict consensus tree based on the six most parsimonious trees (length = 578). Numbers on the branches indicate bootstrap values. Subgenera abbreviated as follows: Lotos = *Lotos*, Hydro = *Hydrocallis*, Brachy = *Brachyceras*, Anec = *Anecphyta*, Nymph = *Nymphaea*. Reproduced from Borsch 2000 figure 56.....12
- Fig 1.2 Distribution of *N. mexicana* throughout the United States. This species is native in North America in the south from Florida to Texas. Populations in California and Arizona are probably from ornamental introductions. Recopied from Wiersema and Hellquist 1997.....13
- Fig 1.3 Distribution of *N. odorata* ssp. *odorata* in North America. Subspecies *odorata* overlaps in distribution with *N. mexicana* and subspecies *tuberosa*. It occurs separate from subspecies *tuberosa* in the extreme south and Nova Scotia.....14
- Fig 1.4 Distribution of *N. odorata* ssp. *tuberosa* in North America. This subspecies has a more restricted distribution than subspecies *odorata*. It occurs separately from ssp. *odorata* in Ohio, Illinois and Indiana. Subspecies *tuberosa* does not overlap in distribution with *N. mexicana*...15
- Fig 1.5 A repeat unit of nuclear ribosomal DNA. This portion of the nrDNA shows the three main coding regions the 18S, 5.8S, and 26S genes. Between the coding regions lie two internal transcribed spacers (ITS 1, ITS 2). The intergenomic spacer (IGS) region is located between the 26S and 18S genes and includes the external transcribed spacer (ETS).....16
- Fig 1.6 The *trnT-trnF* region in the chloroplast genome. The sizes and locations of the primers (A-F) are based on the variability occurring within the genus *Nymphaea*. This figure was replicated from Borsch (2000). The primers correspond to Taberlet et al. (1991)17
- Fig 1.7 The internal transcribed spacer (ITS) region in nuclear ribosomal DNA. The ITS lies between the 18S rDNA and 26S rDNA, and is composed of the transcribed spacers (ITS 1, ITS 2) and 5.8S rDNA. ITS 5 and ITS 4 are universal primers that are anchored in the 18S and 26S gene, respectively (White et al. 1990)18
- Fig 2.1. The geographic distribution of *N. mexicana* and *N. odorata* in North America. *Nymphaea odorata* ssp. *odorata* has the widest distribution, overlapping with both *N. mexicana* and ssp. *tuberosa* (Wiersema and Hellquist 1997)38
- Fig 2.2. Bootstrap tree based on sequences from the ITS region (length = 190, CI = 0.947, RI = 0.977). Numbers on branches indicate bootstrap values based on 500 replicates. *Nymphaea ampla* and *N. elegans* were used as outgroups. Italicized names signify accessions sequenced for the *trnL-trnF* region. The clade marked by an arrow collapsed in a strict consensus analysis. Column A indicates the presence (+) or absence (-) of petiole stripes, and column B depicts leaf blade undersurface color (0=green, 1= mix of green and purple, 2= reddish-purple).....39

Fig 2.3 Tree produced from SAHN clustering of polymorphic ITS sites. Four distinct groups are evident MEX (*N. mexicana* and *N. mexicana* x *N. odorata* putative hybrids), TUB (ssp. *tuberosa*), ODO (ssp. *odorata*) and six accessions belonging to both subspecies (O/T group)...40

Fig 2.4 Principal coordinate analysis with minimum spanning tree superimposed generated from polymorphic ITS sites. All *N. mexicana* and putative hybrid accessions group together, and cluster closest to ssp. *tuberosa*. Several accessions form a gradient from ssp. *tuberosa* to ssp. *odorata*. One accession of ssp. *tuberosa* (KN28) clusters with the main group of ssp. *odorata* accessions. The proportion of total variance comprising each axis was 63.0% for axis I, 11.8% for axis II, and 5.7% for axis II.....41

Fig 2.5 Three possible hypotheses for the origin of the subspecies of *N. odorata* (ssp. *odorata* and ssp. *tuberosa*) from *N. mexicana*.....42

Fig 2.6. Synapomorphies between ssp. *odorata*, ssp. *tuberosa* and *N. mexicana* in the ITS region. The % shared was calculated as the number of accessions sharing a bp with *N. mexicana* per the total accessions of each subspecies. Most (11/12) basepairs are shared with ssp. *tuberosa*.....43

Fig 3.1 Plots (regression line) of leaf blade width by blade length for each subspecies. For subspecies *odorata* (a) $R^2 = 0.96$ and $P < 0.0001$ and ssp. *tuberosa* (b) $R^2 = 0.86$ and $P < 0.0001$59

Fig 3.2 Plot of scores by principal component axes I and II from 26 accessions of *N. odorata* on eleven morphology characters. Accessions from ssp. *odorata* occur mostly on the negative side of both axes, whereas accessions from ssp. *tuberosa* occur mostly on the positive side of both axes.....60

Fig 3.3 Principal coordinate analyses with minimum spanning tree superimposed based on ISSR markers excluding primer 814. *Nymphaea mexicana* and *N. odorata* are clearly segregated; separation between the subspecies of *N. odorata* is less distinct but still evident. Accessions KN17 and 42N have unique placement (see text). The proportion of total variance comprising each axis was 17.8% for axis I, 9.2% for axis II, and 7.2% for axis III.....61

Fig 3.4 Phenogram illustrating genetic relationships among *N. mexicana*, *N. odorata* and putative hybrids (*N. mexicana* x *N. odorata*) generated by the UPGMA cluster analysis (NTSYS-pc) calculated from ISSR markers excluding primer 814. *Nymphaea mexicana* and the putative hybrids group separately from *N. odorata*. Each subspecies is represented by three distinct groups, each group showing geographic partitioning. Numbers in triangles denote the number of accessions in each triangle. Geographic locations are indicated by state abbreviations.....62

Fig 3.5 Illustration of leaf blades for ssp. *odorata* and ssp. *tuberosa* based on the mean measurements of leaf blade size and shape, the presence or absence of petiole stripes, and lobe apex shape. All characteristics were statistically significant in a t-test (see Table 3.5).....63

LIST of TABLES

Table 1.1 A comparison between the two species of <i>Nymphaea</i> and their intermediates. Significant ecological and morphology characteristics are listed for <i>N. mexicana</i> , <i>N. odorata</i> ssp. <i>odorata</i> , <i>N. odorata</i> ssp. <i>tuberosa</i> and the intermediates (Wiersema and Hellquist 1997). Blank areas in the chart indicate unknown information and two letter abbreviations for states are used. LB= leaf blade characteristics.....	19
Table 1.2 The taxonomic varieties associated with <i>Nymphaea odorata</i> . Each variety is listed with a brief description and reference. Currently, the Flora of North America recognizes only var. <i>gigantea</i> and var. <i>rosea</i> (Wiersema and Hellquist 1997).....	21
Table 1.3 Species used for the preliminary analysis. Accessions were chosen to cover the geographic range of both species and subspecies.....	22
Table 2.1. Accessions used along with their classification, geographic location and sources of the material. Accessions classified as <i>N. odorata</i> hybrid without a subspecies affiliation were identified as putative hybrids between the subspecies.....	44
Table 2.2. The identity of the one-basepair molecular marker at position 396 in the <i>trnL-trnF</i> region. Accessions selected (Fig 2.2) were either putative hybrids between the species, representatives of each subspecies, or had unusual placement in the ITS bootstrap tree. Accessions 50N, 33N, KN12, KN29, 1N, KN6 and KN14 are representatives of the subspecies and were included to check the accuracy of this nucleotide position.....	46
Table 3.1. Accessions used along with their classification, geographic location, and collector information. Italics indicate accessions used in morphological analyses. Accessions containing an accession number were used only in molecular analyses. Voucher specimens were deposited at FR, FTG, and VPI (herbarium abbreviations from Holmgren et al. 1990)	64
Table 3.2. Qualitative and quantitative morphological characters measured for each accession.....	65
Table 3.3 ISSR primers used on accessions of <i>N. odorata</i> , <i>N. mexicana</i> and <i>N. mexicana</i> x <i>N. odorata</i> putative hybrids. All primers listed were used on 10 accessions from a Tennessee population to determine intrapopulation variation in <i>N. odorata</i> . * = Primers used on interpopulation analysis.....	66
Table 3.4 ANOVA analyses of the morphological variables that differ among the subspecies (ssp. <i>odorata</i> and ssp. <i>tuberosa</i>). R^2 = correlation coefficient and P = significance value obtained using a student's t-test for quantitative variables and a contingency table for qualitative variables.....	67

Table 3.5 The mean and standard deviation (SD) of leaf blade characters for ssp. <i>odorata</i> and ssp. <i>tuberosa</i> along with the results of a t-test. *= Statistically significant (P < 0.10) using t-test.....	68
Table 3.6 Results of principal components analysis on eleven morphology characters from 26 accessions from <i>N. odorata</i> . Characters arranged in descending order according to loadings on first component.....	69
Table 3.7 Cross-validation summary with the linear discriminant function based on 26 accessions of <i>N. odorata</i> . Percentage of accessions correctly classified using quantitative characteristics of petiole diameter, blade length, blade width, sinus length, and number of leaf veins.....	70
Table 3.8 Comparison of ISSR primers used for interpopulation analysis on 43 accessions from <i>N. odorata</i> , <i>N. mexicana</i> and putative hybrids <i>N. mexicana</i> x <i>N. odorata</i> . The percent polymorphic bands was calculated as the number of polymorphic loci per group (ssp. <i>tuberosa</i> , ssp. <i>odorata</i> , <i>N. mexicana</i> and hybrids) divided by the total number of loci in the primer. * = Primer that displayed intrapopulation variation.	71
Table 3.9 Analysis of molecular variance (AMOVA) based on ISSR markers excluding primer 814. The total data set contains three taxonomic groups (ssp. <i>odorata</i> , ssp. <i>tuberosa</i> and <i>N. mexicana</i> with <i>N. mexicana</i> x <i>N. odorata</i>) and 19 regions (which represent individual states). Statistics include sums of squared deviations (SSD), mean squared deviations (MSD), variance component estimates, and the percentages of the total variance contributed by each component.....	72

1.0 Introduction

The definition of speciation is well understood, but the process is under constant debate. Speciation occurs when one species branches into two or more species (Kottler 1978). The process of speciation must be able to explain diverse phenotypes, use of different ecological resources, and how two species can coexist in sympatry (Barton 2001). While phenotype and ecological characteristics vary between species it is possible to find the same amount of diversity within a species. Speciation must be able to explain why some species can display a large range of phenotypes yet have the same genetic background. The process of speciation must also shed light on why two species with a different genetic background can coexist in the same environment. Understanding how speciation occurs can give insights into relationships between genetics and morphology.

Several modes in which speciation can arise include allopatric, parapatric, and sympatric. Allopatric speciation involves the separation of two species by geographic isolation. It begins when a population is separated into two sub-populations, which continue to evolve in isolated areas (Endler 1977). The separated populations are geographically distinct races that undergo evolution. These races are the beginning of a new species and have the ability to maintain their own morphologic and physiologic variation. Overtime each race slowly develops reproductive isolation, forming two new species. In the case of allopatric speciation reproductive isolation occurs among several populations at once.

Parapatric speciation occurs within a continuous parental species range (Endler 1977). Parental species have wide distributions and substantial variation in character traits. This variation in character traits is found in specific geographic areas, where overlap between the parentals occurs. In areas of overlap, a cline is formed when parents with two different character traits mate (Endler 1977). The populations on either side of the cline then evolve into two separate species through the differentiation of character traits.

In the absence of spatial differences, sympatric speciation may occur (Endler 1977). Sympatric speciation involves the founder effect where a few individuals migrate into a new habitat; here ecological precedes genetic divergence. Changes in ecological differentiation promote reproductive isolation creating two separate species. The new species can then colonize

new environments, which could then force reproductive isolation from its parentals.

Allopatric, parapatric, and sympatric speciation may be possible modes of speciation in the white water-lily *Nymphaea odorata* Aiton. As an aquatic plant, *N. odorata* has unique factors that influence each mode of speciation. Aquatic plants require the presence of standing or slow-moving water in order to survive. This water in the form of rivers, lakes or ponds has a patchy distribution, separated by dry land. Gene flow may be more difficult between these populations, increasing the chance of allopatric speciation.

Nymphaea odorata has a large geographic range and high phenotypic plasticity, both characteristics associated with parapatric speciation. This water-lily has formed two subspecies, *odorata* and *tuberosa*. Hybrids between the subspecies have been reported, however a specific cline has not been discovered. Parapatric speciation may also be influenced by the methods of seed dispersal in this organism. Although the method is unknown in *N. odorata*, studies have suggested dispersal to occur by floatation on water or through animals such as ducks (Cely 1979; Capperino and Schneider 1985). Dispersal by water would limit individuals from invading new habitats, whereas ducks may be a method of long distance dispersal.

The two subspecies of this water-lily have been reported to have ecological preferences and morphological differences (Wiersema and Hellquist 1997). Sympatric speciation could occur within an environmentally unique population to form a new subspecies. This subspecies could then perpetuate throughout the new environment, possibly invading new habitats and increasing the geographic distribution of the species. *Nymphaea odorata* makes a unique case study for aquatic speciation because it may possess all three possible modes of speciation.

1.1 Nymphaeaceae

Nymphaeaceae, the water-lily family, consists of 50 aquatic perennial species. These aquatic herbs have leaves and flowers that emerge from a vertical rhizome and float atop the water (Schneider et al. 1995). The flowers of this family are extremely attractive, and bear numerous petals, giving this plant family a unique ornamental appeal. The flowers are bisexual, ranging in color from white to yellow, and are usually pollinated by beetles (Wiersema and Hellquist 1997). Nymphaeaceae is among the most basal families of angiosperms and contains

six well-resolved genera *Barcalya*, *Euryale*, *Nymphaea*, *Nuphar*, *Ondinea*, and *Victoria* (Borsch 2000; Borsch et al. 1999; Qiu et al. 1999; Zanis et al. 2002). Of the six genera, only *Nymphaea* and *Nuphar* inhabit North America.

The genus *Nymphaea* is the most diverse genus in the family, containing 40 species with an almost worldwide distribution. *Nymphaea* displays primitive flower characteristics; it contains numerous petals that gradually change into stamens toward the flower's center. The flower is distinguished from other genera in the family by the presence of a perianth that spreads at anthesis (Wiersema and Hellquist 1997). Conrad (1905) recognized five subgenera within *Nymphaea*: *Anecphyra*, *Brachyceras*, *Hydrocallis*, *Lotus*, and *Nymphaea*. Each subgenus has a distinct geographic distribution; subgenus *Nymphaea* occurs in the temperate regions and contains seven species.

Recently, Borsch (2000) has resolved several major clades within *Nymphaea* based on sequence analyses of nuclear internal transcribed spacer (ITS) region. In this phylogeny, species belonging to the temperate subgenus *Nymphaea* appear in a well-defined clade (Figure 1.1). Within this clade *N. mexicana* Zuccarini appears in a sister clade to *N. odorata* Aiton with strong support (Figure 1.1). These two species have several characteristics in common; they are native to the Americas and can form large clonal populations due to the production of long runners (*N. mexicana*) or thick rhizomes (*N. odorata*). The species overlap in distribution in the southern United States, where hybrids between them have been reported (Wiersema 1996; Wiersema and Hellquist 1997). Although the two species hybridize, they remain morphologically distinct; *N. mexicana* retains its yellow petal color and distinct stolons, whereas *N. odorata* has white petals and lacks stolons. While these two species remain distinct, *N. odorata* has formed two subspecies, which are morphologically similar and hybridize (Wiersema and Hellquist 1997). With the current knowledge of a basic phylogeny, a detailed morphology, and the formation of hybrids, these species represent a good system to study aquatic plant speciation.

1.11 *Nymphaea mexicana*

Nymphaea mexicana, the yellow water-lily, occurs in warm temperate and subtropical regions. Its distribution extends from Mexico north into Texas and east to Florida (Figure 1.2).

It was first collected in Mexico around 1827 and since then has appeared throughout the southern United States (Conrad 1905). Currently, only seven states list *N. mexicana* as native whereas several other states such as Arizona, California, Indiana, and the Carolinas report it as adventive (Crovello et al. 1983; Radford et al. 1968; Wiersema and Hellquist 1997).

As the only member of the section *Zanthantha* within the subgenus *Nymphaea*, *N. mexicana* is morphologically distinct from other members of the subgenus. *Nymphaea mexicana* has a unique root system of unbranched rhizomes that form overwintering roots. The roots also function as vegetative propagules, which are often termed “nodules or tiny bananas” (Wiersema and Hellquist 1997). The leaves have a glabrous petiole, are purple abaxially and have blades that are ovate to elliptic in shape. The leaf blade contains 11 to 22 radiate, centrally impressed veins with an entire or sinuate margin. The flower ranges in size from 6 to 11 cm and has 12 to 30 yellow petals with 50 to 60 yellow stamens. *Nymphaea mexicana* blooms from the spring until early fall and is mainly pollinated by beetles (Wiersema and Hellquist 1997). The flowers bloom for two consecutive days, and open each day around 11 a.m. and close around 4 p.m. (Capperino and Schneider 1985). After pollination, the flower produces large globose seeds in a berry-like fruit; these seeds are the largest (0.5 cm) among *Nymphaea* species. The seed number in the fruit is inversely proportional to the size, and can range in color from light to dark green (Capperino and Schneider 1985). *Nymphaea mexicana* is considered a diploid, with $2n=56$ (Wiersema and Hellquist 1997).

Ecologically, *N. mexicana* inhabits still alkaline waters on the outer coastal plain and can occur in ponds with depths ranging from one to 1100 meters. The vegetative propagules are an important source of food for canvasback ducks and, consequently, the distribution of *N. mexicana* follows the winter migration of these ducks (Cely 1979). Furthermore, this species is also distributed using its long runners. Within a population, *N. mexicana* can quickly propagate and rapidly cover an aquatic habitat, resulting in increased evapotranspiration rates (Capperino and Schneider 1985). Increasing evapotranspiration will cause a reduced air and water supply in the wetland. Therefore, *N. mexicana* is considered invasive in many areas and is termed a weedy plant in the Flora of North America (Wiersema and Hellquist 1997).

1.12 *Nymphaea odorata*

Nymphaea odorata, or the white water-lily, is sister to *N. mexicana* (Fig. 1.1). The species was first described in 1789 and was named for its strong, sweet odor. This water-lily is native to the United States but was introduced to Europe in the late 1700s (Conrad 1905). As the most common water-lily in the United States, it extends in distribution from Florida to Newfoundland, Nova Scotia. Two subspecies are recognized in *Nymphaea odorata*: ssp. *odorata* and ssp. *tuberosa*. The former overlaps in distribution with *N. mexicana* whereas the latter has a more northern distribution (Wiersema and Hellquist 1997).

Nymphaea odorata has a unique and highly variable morphology (Table 1.1). The root system of *N. odorata* is composed of long, branched rhizomes. From the rhizomes, both leaf blades and flowers arise independently of each other. The leaf blades are ovate to nearly orbiculate in shape with entire margins, and contain 6 to 27 radiate, centrally impressed veins. The flower ranges in diameter from 6 to 19 cm and has 17 to 43 white or sometimes pink petals with 35 to 120 yellow stamens. However, the pink flowered varieties are thought to be from hybrids between natural populations and escaped ornamentals. The berry-like fruit in this species produces seeds that are ovoid in shape and vary in size from 1.5 to 4.8 mm (Wiersema and Hellquist 1997).

Ecologically, *N. odorata* prefers slightly acidic conditions and is found in ponds up to 1700 m deep (Cely 1979). This water-lily is weedy, and in one season can quickly propagate vegetatively to cover an entire pond. Additionally, *N. odorata* may spread throughout a pond through allelopathy. *Nymphaea odorata* serves as a host for the fungus *Sclerotium hydrophilum*, which is pathogenic to other aquatic plants (Bowerman and Goos 1991). The combination of allelopathy and pathogenesis provides a strong means for dominance of this species in the aquatic community. In contrast, another fungus, *Dichotomophthoropsis nymphaearum*, was found to be slightly pathogenic to *N. odorata* and could possibly be used to control its population growth, although never tested (Charudattan and Walker 1982).

Subspecies *odorata* has a broad distribution that encompasses major portions of the United States and extends into Canada (Figure 1.3). This subspecies is also found outside of the contiguous United States in Mexico, the Bahamas, and South America. *Nymphaea odorata* ssp. *odorata* is morphologically distinct from ssp. *tuberosa*, having smaller seeds (1.5 to 2.5 mm),

and leaves that are greenish or reddish purple with a deep red-purple underside (Table 1.1). Flowering occurs from the spring to early fall, but peaks mainly in the summer farther north. In response to the length of daylight, flowering is more variable in the north where flowers open slightly later in the morning and close much later in the afternoon compared to subspecies *tuberosa*. The somatic number of this subspecies may be $2n=56$ or 84 (Wiersema and Hellquist 1997).

Ecologically subspecies *odorata* inhabits acidic or alkaline still water, and is found in ponds with depths ranging from sea level to 1700 m (Wiersema and Hellquist 1997). *Nymphaea odorata* ssp. *odorata* displays phenotypic plasticity in both flower and leaf blade size. Environmental conditions do not seem to cause the plasticity, as plants of the same size have been reported under a variety of conditions (Wiersema and Hellquist 1997). Due to the large variability and wide geographical distribution, this subspecies is considered more of an invasive weed compared with subspecies *tuberosa*.

Conrad (1905) describes *N. tuberosa* Paine as a species based on its tuber-like branches, odorless flower and striped petiole. Some of these characteristics are still used define ssp. *tuberosa*. Wiersema and Hellquist (1994) reclassified *N. tuberosa* as a subspecies of *Nymphaea odorata* because of the morphologic variation found throughout the geographic range of both subspecies.

Subspecies *tuberosa* has a more limited distribution, extending mainly from the central United States toward the northeast (Figure 1.4). Most significantly, its current distribution does not overlap with *N. mexicana*. Morphologically, subspecies *tuberosa* differs in several aspects from ssp. *odorata*. Subspecies *tuberosa* has larger seeds, 2.8 to 4.5 mm, and the leaf petiole is green with brown-purple stripes and green or faintly purple underside (Table 1.1). This subspecies blooms from late spring through the summer. Ecologically, ssp. *tuberosa* inhabits mostly alkaline still water and occurs in ponds of depths from 100 to 400 m (Wiersema and Hellquist 1997). While each subspecies has distinct morphological characteristics, hybrids are formed where their distributions overlap. The hybrids can be intermediate or have a mixed morphology, making classification at the subspecies level difficult.

1.2 Hybridization and Taxonomic Issues

Hybrids are formed when two parentals interbreed and create genetically distinct or intermediate individuals. The parents can be from different species or subspecies. Hybrids are distinguished from their parents by having a mixed genotype. The resultant hybrid often may be sterile or fertile depending on the genetic compatibility of the parentals. The presence of fertility between two parental taxa may indicate gene flow between parental populations.

Hybrids occur between both subspecies (ssp. *odorata* and ssp. *tuberosa*) and between *N. mexicana* and *N. odorata* ssp. *odorata* (Table 1.1). The hybrids between *N. mexicana* and ssp. *odorata* occur in the southern United States where populations of both species overlap. They have increased vigor, are morphologically intermediate and sterile (Wiersema and Hellquist 1997). Hybrids between ssp. *tuberosa* and ssp. *odorata* have been reported where their populations overlap in Connecticut, Illinois, Maine, Massachusetts, Minnesota, Michigan, Nebraska, New Hampshire, New York, Pennsylvania, Wisconsin, Vermont, and areas in Canada. The hybrids can exhibit a mixture of morphology from the two parental subspecies, or intermediate morphological characteristics, which makes them impossible to classify to either subspecies (Wiersema and Hellquist 1997). In addition, several taxonomic varieties have been described for *N. odorata*, two recognized by the Flora of North America (Table 1.2). Taxonomically, *N. odorata* and its subspecies can be difficult to classify because of the presence of hybrids. While the Flora of North America recognizes several definitive morphologic characteristics that discriminate between the subspecies (presence of stripes, color of leaf blade under surface, and seed size); these characteristics do not clearly separate when hybrids are formed (Wiersema and Hellquist 1997).

1.3 Objectives and Approach

The goal of this project was to examine patterns of variation and speciation in the water-lily *Nymphaea odorata* using morphological and molecular information. Morphological characteristics were determined for 31 individuals from both subspecies, and analyzed using analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), principal

coordinate analysis (PCOA), principal components analysis (PCA) and discriminate analyses. For the molecular analyses three unique regions of DNA were used to analyze 47 accessions from both species (*N. mexicana* and *N. odorata*) and subspecies (*odorata* and *tuberosa*).

Two types of populations were studied, populations where each species and subspecies exist sympatrically, and populations where they occur allopatrically. We hypothesized that in areas of sympatry, each subspecies should exhibit distinct morphologies, due to a lack of gene flow between the subspecies in sympatric areas. In areas of allopatry, hybrids between species and subspecies will occur and, thus, morphological distinctness may be blurred (Turelli et al. 2001). In allopatry hybrids will form, allowing for gene flow to possibly occur between the species and subspecies. The hybrids between species will be identified based on a mixed morphology, sharing some characteristics from each species. Hybrids between subspecies may have either a mixed morphology (sharing some characteristics from each subspecies) or an intermediate morphology, a blending of morphological characteristics between the subspecies. Therefore, hybrids may not be morphologically identifiable but subspecies can still be distinguished. The main hypothesis for the morphological study was that subspecies could be distinguished based on specific morphological characteristics.

Using several different molecular techniques will give insights into the evolution of *N. odorata*. Areas in the DNA sequences that differ by the presence of indels or nucleotide substitution represent genetic differentiation. In the current phylogeny (Borsch 2000; Figure 1.1), *N. mexicana* is sister to *N. odorata*, and the two have distinct nuclear ITS sequences, differing by several basepair (bp) insertions. Therefore, *N. odorata* may have a common ancestry with *N. mexicana* in southern North America and, thus, the southern populations of ssp. *odorata* are expected to have an ancestral genotype that shares more synapomorphies with *N. mexicana*. As *N. odorata* invaded new habitats east and north it would acquire unique mutations that distinguish its DNA sequence from *N. mexicana*. In more northern regions, *N. odorata* has genetically diverged from *N. mexicana*, leading to the emergence of another subspecies, *tuberosa*. The subspecies should contain unique molecular markers specific to each subspecies, yet still share some synapomorphies because they have a common ancestry and continue to hybridize. In contrast, subspecies *tuberosa* does not overlap in distribution with *N. mexicana* and the two are expected to be genetically distinct. The genetic distance between *N. mexicana*

and ssp. *tuberosa* should be greater than between *N. mexicana* and ssp. *odorata*. The hypotheses for the molecular studies are that ssp. *odorata* was the first subspecies to evolve from *N. mexicana*.

An alternative hypothesis is that *N. mexicana* could share a common ancestor to both subspecies, i.e. they evolved from different plants of *N. mexicana*. Since there are no current hybrids between *N. mexicana* and ssp. *tuberosa*, the hybrids could have become extinct. In this case the genetic distance between *N. mexicana* and ssp. *odorata* would be the same as between *N. mexicana* and ssp. *tuberosa*. To evaluate the evolution of *N. odorata* from *N. mexicana*, molecular markers that showed variation at the population level were used.

1.4 Choice of Molecular Approach

To understand gene flow among populations, the most common markers used are obtained from DNA. Within a cell, DNA is located in the mitochondria, the chloroplast, and the nucleus; the latter two are most frequently used to resolve phylogenetic relationships in plants. Both types are uniquely inherited in angiosperms; chloroplast DNA (cpDNA) is usually maternally inherited whereas nuclear is bi-paternally inherited, having equal parts from the mother and father (Soltis et al. 1996). However, specific inheritance patterns for mitochondrial and chloroplast DNA are unknown in *Nymphaea*. Finding a genomic region that can provide the appropriate amount of variation at the population level has been difficult. A preliminary study was done on four separate DNA regions to determine which region had the greatest amount of variation at the level of interest. For this preliminary study four samples were chosen so that the geographical range of both species and subspecies was represented (Table 1.3; also see Borsch 2000).

The first region chosen was the 5S ribosomal gene that occurs in tandem repeats, varying from hundreds to thousands of copies. The spacer region between the repeats has been shown to vary at the individual level, using specific loci or restriction enzyme analysis (Rogers and Bendich 1987). To determine variation between the selected accessions, attempts were made to amplify the region with polymerase chain reaction (PCR) (Table 1.3) using primers from Appels and Clarke (1992). Amplification was not successful and, thus, this aspect of the work was terminated.

The second region chosen was the nuclear external transcribed spacer region (ETS) region located between the 26S and 18S ribosomal genes (Fig. 1.5). This region has been effective in population studies due to its high rate of mutation compared to the internal transcribed spacer (ITS) (Baldwin and Markos 1998, Linder et al. 2000). Since the ETS region is difficult to amplify, it has mostly been used to supplement ITS data, especially in Asteraceae where specific primers have been designed. Furthermore, ETS data has recently been useful in studies of newly evolving lineages where ITS lacks variation (Clevinger and Panero 2000). The ETS region lacks universal primers because of a large amount of variability found within this region among angiosperms (Volkov et al. 1996). Due to the problems of choosing a conserved region within ETS, the entire intergenomic spacer (IGS) region is first amplified and then primers are designed specifically for the family or genus studied (Baldwin and Markos 1998).

In the preliminary study, long PCR that amplifies a large genomic region, was attempted in order to isolate the IGS region (Fig. 1.5). After the IGS was amplified, the primer in the 18S gene was used for sequencing due to its proximity to the ETS region. After the region was sequenced, a primer was designed specifically for the ETS region, about 800 bp downstream of the 18S gene. Amplification was not successful for all the four accessions. Further attempts were made to design a new primer and sequence farther into the IGS region, but all failed. The ETS region was too difficult to amplify and was abandoned for a different region.

The third region chosen for molecular studies was the chloroplast *trnT-trnF* region. In the Nymphaeales, this region is 2712 bp long and was amplified in two sections: (1) the *trnT* gene and the *trnL*-intron, and (2) the *trnL*-intron to the *trnF*-gene (Figure 1.6). This region was previously used by Borsch (2000) to generate a phylogeny of *Nymphaea*, showing variability in the *trnL-trnF* region at the subspecies level. The *trnL-trnF* region was amplified in all accessions (Table 1.3), and the alignment revealed specific markers for each species and subspecies. Between the two species (*N. mexicana* and *N. odorata*) there were several sequence differences in the form of indels and bp substitutions, and between the subspecies there was a one bp substitution. This region did not have enough information to continue with the rest of the subspecies sampling.

The fourth region attempted was the ITS which has been shown to be variable at the subspecies level within *Nymphaea* (Borsch 2000). This region, as described earlier, lies between

the 18S and 26S rDNA and is easily amplifiable with two universal primers ITS 4 and ITS 5 (Figure 1.7). All accessions (Table 1.3) were easily amplified and upon alignment differences were observed between the species and subspecies. There were many nucleotide substitution differences between the two subspecies and, therefore, this region was chosen for subsequent analysis.

The aims of this study were to determine the variation present between ssp. *odorata* and ssp. *tuberosa*, and which subspecies shares a common ancestry with *N. mexicana*. This project combines both molecular and morphological analyses to evaluate the above objectives. An understanding of the variation present within and between subspecies will give insight into the process of speciation in *N. odorata*. Furthermore, this variation may give indirect evidence for gene flow in these *Nymphaea* species. The patterns of variation present in these aquatic plants may give us a comprehensive understanding of the method of evolution of *N. odorata* from *N. mexicana*.

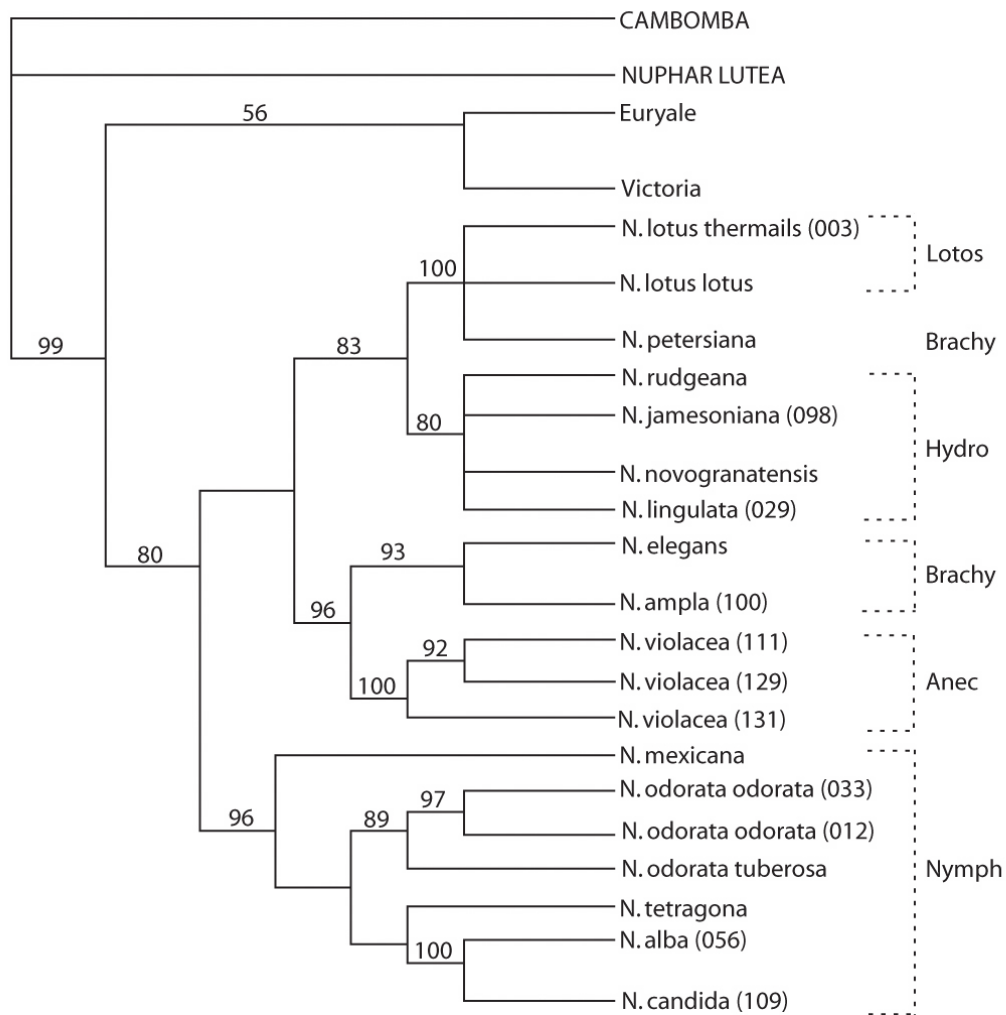


Fig 1.1 Phylogeny of *Nymphaea* based on sequences of the 5.8S gene and ITS 2 region using *Cabomba* and *Nuphar lutea* as outgroups. The strict consensus tree based on the six most parsimonious trees (length = 578). Numbers on the branches indicate bootstrap values.

Subgenera abbreviated as follows: Lotos = *Lotos*, Hydro = *Hydrocallis*, Brachy = *Brachyceras*, Anec = *Anecphyra*, Nymph = *Nymphaea*. Reproduced from Borsch 2000 figure 56.

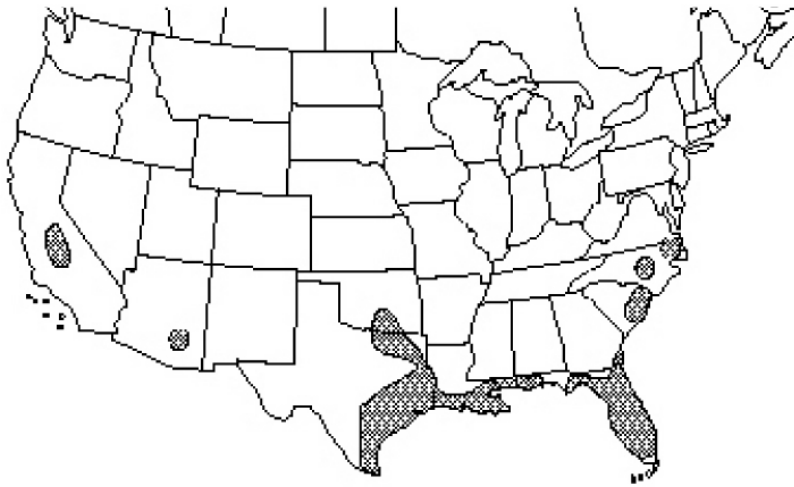


Fig 1.2 Distribution of *N. mexicana* throughout the United States. This species is native in North America in the south from Florida to Texas. Populations in California and Arizona are probably from ornamental introductions. Recopied from Wiersema and Hellquist 1997.

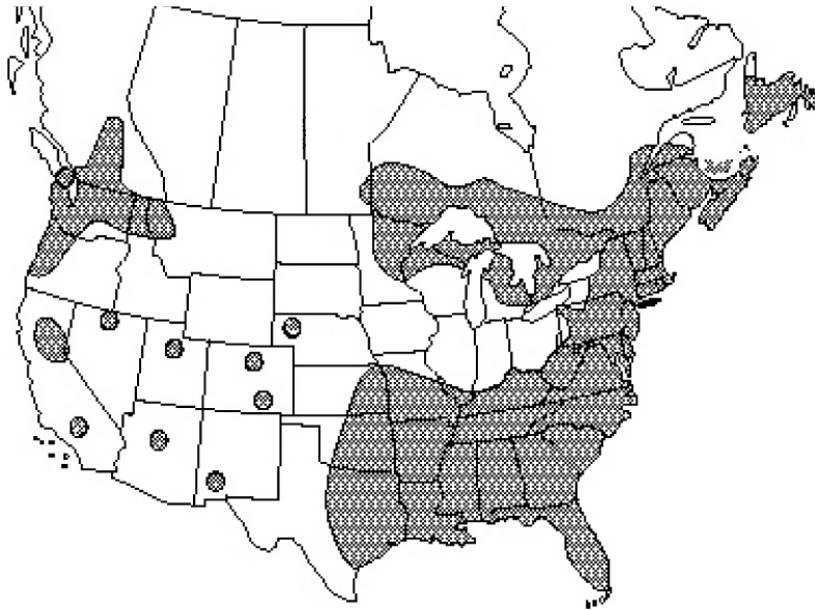


Fig 1.3 Distribution of *N. odorata* ssp. *odorata* in North America. Subspecies *odorata* overlaps in distribution with *N. mexicana* and subspecies *tuberosa*. It occurs separate from subspecies *tuberosa* in the extreme south and Nova Scotia.

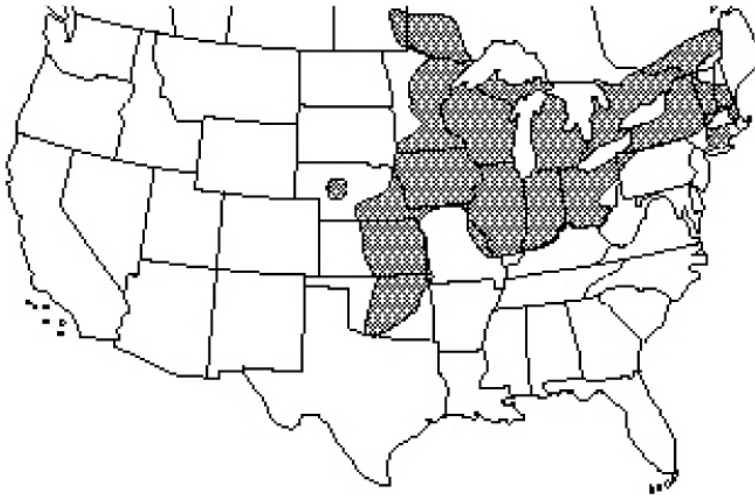


Fig 1.4 Distribution of *N. odorata* ssp. *tuberosa* in North America. This subspecies has a more restricted distribution than subspecies *odorata*. It occurs separately from ssp. *odorata* in Ohio, Illinois and Indiana. Subspecies *tuberosa* does not overlap in distribution with *N. mexicana*.



Fig 1.5 A repeat unit of nuclear ribosomal DNA. This portion of the nrDNA shows the three main coding regions the 18S, 5.8S, and 26S genes. Between the coding regions lie two internal transcribed spacers (ITS 1, ITS 2). The intergenomic spacer (IGS) region is located between the 26S and 18S genes and includes the external transcribed spacer (ETS).



Fig 1.6 The *trnT-trnF* region in the chloroplast genome. The sizes and locations of the primers (A-F) are based on the variability occurring within the genus *Nymphaea*. This figure was replicated from Borsch (2000). The primers correspond to Taberlet et al. (1991).

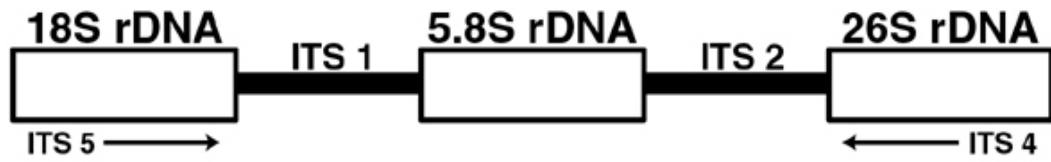


Fig 1.7 The internal transcribed spacer (ITS) region in nuclear ribosomal DNA. The ITS lies between the 18S rDNA and 26S rDNA, and is composed of the transcribed spacers (ITS 1, ITS 2) and 5.8S rDNA. ITS 5 and ITS 4 are universal primers that are anchored in the 18S and 26S gene, respectively (White et al. 1990).

Table 1.1 A comparison between the two species of *Nymphaea* and their intermediates. Significant ecological and morphology characteristics are listed for *N. mexicana*, *N. odorata* ssp. *odorata*, *N. odorata* ssp. *tuberosa* and the intermediates (Wiersema and Hellquist 1997). Blank areas in the chart indicate unknown information and two letter abbreviations for states are used. LB= leaf blade characteristics.

	<i>Nymphaea mexicana</i>	Intermediates between <i>N. mexicana</i> and <i>N. odorata</i>	<i>Nymphaea odorata</i> ssp. <i>odorata</i>	Intermediates between ssp. <i>odorata</i> and ssp. <i>tuberosa</i>	<i>Nymphaea odorata</i> ssp. <i>tuberosa</i>
Distribution	Southeastern United States from TX to FL. In Mexico this species is less robust.	Areas where populations overlap in FL, GA, LA, TX.	Throughout the East coast spreading in the south to TX.	Areas where populations overlap in the north Manitoba, CT., IL, ME, MA, MN, MI, NE, NH, NY, PA, VT, WI.	North Eastern United States up into Canada.
Rhizomes	Unbranched, erect, stolons elongate	Resemble one or other parent	Branched, stolons absent, not constricted at branch joints		Branched, constricted at branch joints
Petiole	Petiole glabrous		Petiole glabrous greenish or reddish purple		Petiole green with brown-purple stripes
LB- Color	Abaxially purplish		Abaxially deeply reddish purple		Abaxially green or faintly purple
LB- Shape	Ovate to elliptic		Ovate to nearly orbiculate		Ovate to nearly orbiculate
LB- Margins	Entire or sinuate		Entire		Entire
LB- Venation	Radiate centrally		Radiate centrally		Radiate centrally
LB- Principal veins	11-22		6-27		6-27
Seeds	5 mm, globose		1.5-2.5 mm, ovoid		2.8-4.8 mm, ovoid
Stamens	50-60, yellow		35-120, yellow		35-120, yellow
Petals	12-30, yellow		17-43, White, rarely pink		17-43, White, rarely pink
Flower	Floating or immersed		Floating		Floating
Flower- Size	6-11 cm		6-19 cm		6-19 cm
Flower- Pistil	7-10 locular		10-25 locular		10-25 locular
Flowering Time	Spring-fall, summer farther north		Spring-early fall, summer farther north		Late spring-summer
Habitat	Outer coastal plain, alkaline ponds, lakes		Acidic or alkaline ponds, lakes		Alkaline ponds, lakes

Lake Depth	1-1100 m		0-1700 m		100-400 m
Sterility		Sterile		Fertile	
Vigor		Increased			
Ploidy	2n=56		2n=56, 84		2n=84
Weediness	W2		W2		W1

Table 1.2 The taxonomic varieties associated with *Nymphaea odorata*. Each variety is listed with a brief description and reference. Currently, the Flora of North America recognizes only var. *gigantea* and var. *rosea* (Wiersema and Hellquist 1997).

Variety	Description	Reference
<i>rosea</i>	Pink-flowered, due to ornamental introduction	Wiersema and Hellquist 1997
<i>gigantea</i>	Large flowers, sepals 6-10 cm long, petioles and peduncles thick, leaf margin upturned	Godfrey and Wooten 1981
<i>godfreyi</i>	Small flowers, sepals 3.5-6 cm long, petioles and peduncles thin	Godfrey and Wooten 1981
<i>minor</i>	Flowers 5-9 cm wide, occurs from NJ to LA	Godfrey and Wooten 1981
<i>stenopetala</i>	Found in shallow waters, dismal swamps	Harvill et al. 1992

Table 1.3 Species used for the preliminary analysis. Accessions were chosen to cover the geographic range of both species and subspecies.

Species	Geographic Location	Accession Number
<i>N. mexicana</i>	Florida	69N
<i>N. odorata</i> ssp. <i>odorata</i>	Michigan	KN7
<i>N. odorata</i> ssp. <i>odorata</i>	Florida	33N
<i>N. odorata</i> ssp. <i>tuberosa</i>	Manitoba, Canada	KN6

1.5 References

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Chapter 2: Speciation in the North American Water-lily *Nymphaea odorata* Using Molecular Variation in the ITS and *trnL-trnF* Regions

2.1 Introduction

Nymphaea (L.) is the most diverse genus in the water-lily family Nymphaeaceae. Borsch (2000) has resolved five major clades within *Nymphaea* based on sequence variation of nuclear internal transcribed spacer (ITS) region. One clade in this phylogeny consisted of the temperate subgenus *Nymphaea*. Within this subgenus, *N. mexicana* Zuccarini appeared in a clade sister to *N. odorata* Aiton with 100% bootstrap (BS) support. These two species are confined to the Americas and overlap in distribution throughout Florida, Georgia, Louisiana and Texas, where hybrids have been reported (Wiersema 1996, Wiersema and Hellquist 1997). Despite possible gene flow, each species remains morphologically distinct. *Nymphaea mexicana* retains its yellow petal color and prominent stolons, compared with the white petals and absence of stolons in *N. odorata*.

Nymphaea odorata has been classified into ssp. *odorata* and ssp. *tuberosa* primarily on the basis of petiole stripes, color of leaf blade undersurface, and seed size (Wiersema and Hellquist 1997). In subspecies *odorata*, the petiole lacks stripes, the abaxial surface of leaf blade is reddish-purple, and the seeds are 1.5 - 2.5 mm long. In contrast, ssp. *tuberosa* has a striped petiole, an abaxially green leaf blade, and 2.8 - 4.5 mm long seeds. Differences in geographic distribution are also evident (Fig 2.1). Morphological characters have been used to identify specimens to each subspecies. However, hybrids between these subspecies can be difficult to identify based on morphology and are classified as *N. odorata* without a subspecies affiliation (Wiersema and Hellquist 1997).

The goal of this project was to use sequence information from the nuclear ITS and chloroplast *trnL-F* regions to assess the diversification, classification and evolution of *N. odorata*. Both genomic regions are variable in *Nymphaea* (Borsch 2000). The ITS region has been useful in phylogenetic studies at the species level and was used to determine the variation present in *N. mexicana* and *N. odorata*. Previous analysis of the *trnL-trnF* region in *N. odorata* revealed a molecular marker that is phylogenetically informative at the subspecies level (Borsch 2000).

2.2 Materials and Methods

2.21 Material

Forty-seven accessions from across the North American distribution of *N. mexicana* and *N. odorata* were used for analyses of the ITS region (Table 2.1). The morphological characters in the Flora of North America were used to classify accessions to a subspecies (Wiersema and Hellquist 1997). *Nymphaea ampla* Salisbury and *N. elegans* Hooker were used as outgroups since they are members of the sister subgenus *Brachyceras* (Borsch 2000). Voucher specimens for outgroups were deposited at MEXU for *N. ampla* and FR for *N. elegans* (Holmgren et al. 1990). The in-group voucher specimens, accessions of *N. odorata* and *N. mexicana*, were deposited at FR, FTG, and VPI (Holmgren et al. 1990).

2.22 DNA Extraction, Amplification and Sequencing

DNA was extracted from silica gel-dried or frozen leaf tissue using a modified CTAB protocol (Borsch 2000). ITS and *trnL-F* amplification followed Borsch (2000). The ITS region was amplified and sequenced for all accessions using primers ITS 4 and ITS 5 (White et al. 1990). The *trnL-F* region was chosen because it contains a subspecies-specific marker that was used to check the morphological identification of the accessions sequenced. The accessions chosen for *trnL-F* sequencing (1) were classified as *N. mexicana* X *N. odorata* putative hybrids, (2) showed unusual placement in the ITS phylogeny or (3) were selected as representatives for each subspecies (defined by having typical morphological characteristics and ITS sequence of the subspecies) (Fig 2.2). Primers C and F (Taberlet et al. 1991) were used to amplify and sequence the *trnL-F* part of the *trnT-trnF* region, due to the presence of a one basepair (bp) molecular marker in the former.

The ITS and *trnL-F* amplified products were resolved on a 1.5% agarose gel, then excised and column cleaned using the QiaQuick gel extraction kit (QIAGEN, Inc., Valencia, California). The cleaned products were sequenced with the amplification primers utilizing the ABI Prism™ BigDye Terminator cycle sequencing Ready reaction kit (Perkin Elmer, Norwalk, Connecticut). Samples were then resolved on an ABI 377 Automated DNA Sequencer, and resulting chromatograms were manually edited using EditView version 1.0.1 (Applied Biosystems, Foster City, California).

2.23 Sequence Alignment and Phylogenetic Analyses

ITS sequences including outgroups were aligned using Clustal X (as implemented by QuickAlign, Muller 2002). The alignment required the insertion of 35 gaps varying from one to eleven basepairs. For parsimony analysis, a heuristic tree search was employed with all characters equally weighted, their states unordered and gaps treated as missing data. The search options consisted of random sequence addition for 500 replicates, holding 500 trees, using TBR branch swapping, MULPARS on, and steepest descent off. Bootstrap values (Felsenstein 1985) based on 500 replicates were calculated as measures of support for individual clades, following the same search conditions as the parsimony analysis. The data set was also analyzed with a Neighbor-Joining (NJ) approach, utilizing Kimura (1980) 2-parameter distance estimate. All analyses were performed in PAUP* version 4.0b10 (Swofford 2002, Sunderland, MA).

2.24 Analysis of polymorphic sites

Polymorphisms, an accession where two basepairs are present at the same position, were found in the ITS region. The presence of a polymorphism was checked by two methods, (1) confirmation of both basepairs in the polymorphic site in the forward and reverse primer sequence and (2) by re-amplification and sequencing of the accession.

In parsimony analyses the polymorphic sites were treated as uncertainty and, thus, information from these positions did not affect those analyses. To determine relationships among accessions possessing polymorphic sites, a data matrix was constructed using only polymorphism sites. Outgroups, *N. elegans* and *N. ampla*, did not contain polymorphic sites and were excluded from the matrix. At each polymorphic site in the alignment, the accessions were scored for the presence (1) or absence (0) of the multi-state characters contained in the polymorphism. In accessions that contained a polymorphism at a specific site, both multi-state characters were scored as present. The data matrix was analyzed with sequential, agglomerative, hierarchal, and nested (SAHN) clustering, principal coordinate analysis (PCOA), and minimum spanning tree (MST); the latter was superimposed on the PCOA. NTSYS-pc package of computer programs version 2.02k was used for these analyses (Rohlf 1998).

2.3 Results

2.31 ITS region

The alignment of the ITS region including the outgroups is 712 bp long. The ITS region is 656 bp in *N. mexicana* and 647 bp in *N. odorata*. There are 163 (24.8%) variable characters, of which 133 (18.7%) are parsimony informative. Excluding outgroups, there are 44 parsimony informative characters, including 12 between ssp. *odorata* and ssp. *tuberosa*. In an initial parsimony analysis with only 30 accessions, a strict consensus tree based on 10,000 trees resolves the subspecies in two clades with each clade having 52% BS support (tree not shown). After additional collecting, 17 accessions (KN15-KN33) were added and a new strict consensus tree was calculated, and a polytomy was evident for the ssp. *tuberosa* clade.

A parsimony analysis, including all 47 accessions, produces a bootstrap tree with a tree score of 190, indicating the number of steps taken to create the tree (Fig 2.2). This bootstrap tree has a *N. mexicana* and putative hybrids (*N. mexicana* X *N. odorata*) clade, a ssp. *odorata* clade and a ssp. *tuberosa* clade. *Nymphaea mexicana* and the putative hybrids appear in a sister clade to *N. odorata* with 100% BS support. *Nymphaea odorata* is monophyletic, in the analysis including all 47 accessions, with 55% and 65% BS support for ssp. *tuberosa* and ssp. *odorata* clades, respectively. The ssp. *odorata* clade contains one accession (KN28) classified as ssp. *tuberosa*. Additionally, accessions classified as *N. odorata* hybrid, thought to be putative hybrids between the subspecies (Table 2.1), appeared in the ssp. *tuberosa* clade. The overall topology of the neighbor-joining analysis (47 accessions used, not shown) is congruent with the topology of the bootstrap consensus tree.

2.32 trnL-trnF region

Previous analysis resulted in one variable site (T or G) at position 396 in the *trnL-trnF* region, representing a molecular marker that discriminates between the two subspecies (Borsch 2000). This marker is a T in *N. mexicana* and ssp. *odorata*, and G in ssp. *tuberosa* (Table 2.2). Accession KN7 was identified morphologically by Borsch, Wiersema and Hellquist (Table 2.1) as ssp. *odorata* but, results in a G at this molecular marker. This region was also sequenced in the *N. mexicana* x *N. odorata* putative hybrid (accession KN20). This putative hybrid has a distinct *N. mexicana* sequence, sharing many indels with *N. mexicana*, but also has a

synapomorphy with *N. odorata* at position 623 (not shown).

2.33 Polymorphic sites

Thirty polymorphic sites were found in the ITS region, of which 17 are in ITS 1, four in the 5.8S gene, and nine in ITS 2. Of the 47 accessions scored, 29 contain one to nine polymorphic sites. Four accessions (KN7, KN17, KN31, and KN37) have the same character status for the polymorphic sites at five positions, two of these accessions are ssp. *odorata* and two are ssp. *tuberosa* (Table 2.1).

SAHN clustering and PCOA analyses of the polymorphic matrix produces groups identical to the clades resolved in the bootstrap tree of the ITS region (Fig 2.2). In SAHN clustering, the *N. mexicana* and putative hybrid group (MEX) appear more similar to the ssp. *tuberosa* group (TUB) than to the ssp. *odorata* group (ODO) (Fig 2.3). The MEX and TUB groups form one group, while the ODO group and accessions from both subspecies (the O/T group) form another group. Similar to the bootstrap tree, the ODO group contains one accession of ssp. *tuberosa* (KN28). A PCOA analysis with a MST superimposed provides similar results to SAHN clustering (Fig 2.4) where three groups are resolved representing *N. mexicana* and the putative hybrids, ssp. *tuberosa*, and ssp. *odorata* (Fig 2.4). The PCOA reiterates the association between *N. mexicana* and ssp. *tuberosa*, evident by the MST connecting the two groups. In the PCOA the MST also connects the ssp. *tuberosa* group through a gradation of accessions to the ssp. *odorata* group. The accessions forming the gradation (KN15, KN25, KN37, KN17, and KN16) are the same accessions in the O/T group in the SAHN analysis and are unresolved in the bootstrap tree.

2.4 Discussion

2.41 Implications to the genus *Nymphaea*

The parsimony analysis in this study supported the previous phylogeny (Borsch 2000), showing *N. mexicana* ancestral to *N. odorata*. In a phylogenetic analysis *N. mexicana* and *N. odorata* formed sister clades, with 100% BS support for each clade (Fig 2.2), indicating a distinction between the species.

This study represented the first time that putative hybrids between *N. mexicana* and *N.*

odorata were included in a phylogenetic analysis. Classification of the putative hybrids by Borsch (pers. comm.) was based on having intermediate leaf morphology, as no flowers were present. In the ITS based phylogeny, the putative hybrids appeared in the *N. mexicana* clade and shared many indels in this region with *N. mexicana*. Furthermore, the hybrids between the subspecies of *N. odorata* contained polymorphisms in the ITS region. In contrast, there were no polymorphisms present in the *N. mexicana* x *N. odorata* putative hybrids in the ITS region. A possible explanation of the absence of polymorphisms is that concerted evolution could have worked to conserve the ITS region of the hybrids between *N. odorata* and *N. mexicana* toward *N. mexicana*. In addition, the only hybrid (KN22) analyzed for the *trnL-F* region had a sequence similar to *N. mexicana*, except for one basepair, which was shared with *N. odorata*. It is expected that the *trnL-F* region would be identical to the maternal parent, if that parent was *N. mexicana*. The molecular evidence presented here does not strongly support a hybrid origin for accessions KN20 and KN22.

2.42 Speciation of *Nymphaea odorata*

The ITS region was effective in resolving *N. mexicana* and *N. odorata* but less effective at the subspecies level. Results from the ITS phylogeny supported a weak separation between ssp. *tuberosa* and ssp. *odorata*, with 55% and 65% BS support, respectively (Fig 2.2). Additional evidence for the discrimination between the subspecies was found in the *trnL-F* region (Table 2.2). Most accessions revealed a subspecies-specific molecular marker. However, accession KN7 was classified as ssp. *odorata* but possessed a G, the molecular marker for ssp. *tuberosa*. Placement in the ssp. *odorata* clade in the ITS phylogeny and the morphology support a ssp. *odorata* classification for accession KN7. The *trnL-F* marker may represent maternal gene flow (from ssp. *tuberosa*) or possibly a back mutation in this accession, reiterating problems related to subspecies classification. Additional support of a subspecies classification was provided by the polymorphic site analyses in the ITS region, and resulted in separation of each subspecies in the SAHN clustering, PCOA and MST analyses (Fig 2.3, Fig 2.4). In each analysis, distinct ssp. *odorata* and ssp. *tuberosa* clusters were present. In the SAHN clustering, the O/T group contained accessions from both subspecies, indicating possible gene flow between

the subspecies. Molecular analyses supported a classification of subspecies, as gene flow between the subspecies was evident, but some discreteness was still apparent from the ITS and *trnL-F*.

Morphological characters of petiole striping and color of leaf blade undersurface mostly support the subspecies classification based on molecular information. Morphological characters defining each subspecies were mapped onto the ITS phylogeny (Fig 2.2). These characters, determined by Wiersema and Hellquist (1997) in the Flora of North America (FNA), showed the morphological variation present within a subspecies. There were several accessions (KN3, KN4, KN5, KN15, KN37) with a mixed morphology having petiole striping (indicating ssp. *tuberosa*) and reddish-purple leaf blade undersurface (indicating ssp. *odorata*). Additionally, 12 accessions displayed a purplish-green leaf blade, an intermediate between ssp. *odorata*'s reddish-purple leaf blade and ssp. *tuberosa*'s green leaf blade. The morphological characteristics used by the FNA may not clearly define each subspecies (Chapter 3). Poor separation of the two subspecies on the basis of morphological characteristics supports a classification of subspecies instead of separate species. Overall, evidence from the ITS and *trnL-F* regions gave more conclusive results than morphology.

Although previous analysis has shown *N. mexicana* as sister to *N. odorata*, it remains unclear which subspecies in *N. odorata* evolved first. Three current hypotheses are possible: (a) ssp. *odorata* has a common ancestry with *N. mexicana*, (b) ssp. *tuberosa* has a common ancestry with *N. mexicana*, or (c) each subspecies share a common ancestry with *N. mexicana* (Fig 2.5).

Evidence to support subspecies *odorata* evolving from *N. mexicana* includes parsimony analyses of the ITS, *trnL-F* evidence, and ploidy information. In the parsimony analyses, ssp. *odorata* forms a moderately resolved clade in the strict consensus tree (65% BS, Fig 2.2). This clade occurs in the strict consensus tree because it appears in 100% of the 10,000 trees generated by a heuristic search, whereas the ssp. *tuberosa* clade appears in only 82% of the trees. Additional support is the presence of a synapomorphy between *N. mexicana* and ssp. *odorata* in the *trnL-F* region (Table 2.1). Although only one basepair, it is a reliable molecular marker for ssp. *odorata*. Ploidy information provides further evidence to support this hypothesis. Löve and Löve (1982) have reported the chromosome number of *N. mexicana* as $2n=56$, ssp. *odorata* as

2n= 56 or 84, and ssp. *tuberosa* as 2n= 84. Since ssp. *tuberosa* has the largest chromosome number it is probably the most derived. Therefore, ssp. *odorata* could be ancestral to ssp. *tuberosa* and the first to evolve from *N. mexicana*.

On the other hand, the ITS region may indicate another possible scenario in which ssp. *tuberosa* evolved from *N. mexicana* and then subsequently gave rise to ssp. *odorata*. Evidence from synapomorphies from the ITS region, ITS polymorphic site analyses, and morphology support this hypothesis. Within the ITS region, there were 12 bp that were variable between the subspecies of *N. odorata* (Fig 2.6). Eleven of the 12 bp are synapomorphies between *N. mexicana* and ssp. *tuberosa*, whereas ssp. *odorata* and *N. mexicana* shared only one bp in all accessions. These synapomorphies could have evolved by random mutation, yet it is highly doubtful that a random mutation would occur 11 times at the same site in both *N. mexicana* and ssp. *tuberosa*. Additional support for this hypothesis comes from the polymorphic sites in the ITS region. *Nymphaea mexicana* is connected to ssp. *tuberosa* via the MST in the PCOA (Fig 2.4), reinforcing the presence of shared molecular characters between *N. mexicana* and ssp. *tuberosa*. Further evidence for this hypothesis is provided by morphology. *Nymphaea mexicana* has the largest seed size in *Nymphaea*, and ssp. *tuberosa* has the larger seed size of the two subspecies. Both *N. mexicana* and ssp. *tuberosa* frequently reproduce by detachable tubers. However, reproductively the tubers are quite different, and are probably not homologous (John Wiersema pers. comm.). The tubers in *N. mexicana* are stolons that are located at the end of long runners, whereas the tubers in ssp. *tuberosa* occur directly on the rhizome. Ecologically, both *N. mexicana* and ssp. *tuberosa* prefer alkaline environments compared to ssp. *odorata*, which prefers acidic environments (Wiersema and Hellquist 1997).

While the evidence for the speciation of ssp. *tuberosa* from *N. mexicana* is strong, additional molecular evidence and life history characteristics supports an independent speciation of each subspecies from *N. mexicana*. In this hypothesis, both subspecies would have the same genetic distance from *N. mexicana*, assuming the biological clock. Initially, evidence from the ITS parsimony analysis indicates that ssp. *odorata* has an increased genetic distance from *N. mexicana* compared to ssp. *tuberosa*, because accessions from ssp. *odorata* form a moderately resolved clade (65% BS) in the strict consensus tree (not shown). Accessions of ssp. *tuberosa* form a polytomy in the strict consensus tree. However, life history characteristics of ssp.

tuberosa may provide an explanation for the increased divergence of *ssp. odorata*. In populations of *ssp. odorata*, seed set is almost always found, whereas in populations of *ssp. tuberosa*, seed set is rare. It is hypothesized that *ssp. tuberosa* frequently reproduces asexually (by tubers), possibly allowing for increased time between generations that result from sexual reproduction (Wiersema pers. comm.). The increased time between generations may decrease the chance of mutations to become fixed, making it difficult for this subspecies to accumulate unique mutations compared to an organism with a quicker generation time, as in *ssp. odorata*.

2.43 Evidence of Hybridization

There was a marked difference in sequence variability in the plastid and nuclear regions studied. The chloroplast region contained only one variable site (Table 2.2), whereas the ITS region contained 40 variable sites between the subspecies. Numerous studies have found chloroplast DNA less variable due to low substitution rates (Wolfe et al. 1987; Franzke and Hurka 2000; Stanford et al. 2000; Sang et al. 1997). However, other studies have shown the chloroplast DNA to be more informative compared to nuclear DNA (Koch et al. 1998; Martinsen et al. 2001; Hilu and Melillo unpublished). Furthermore, Sang et al. (1997) found hybrids to have fixed nuclear DNA but cpDNA inherited from only one parent. However, this is not true for the regions studied in *N. odorata*, as the cpDNA was similar in all accessions. Therefore, no conclusions about hybridization in *N. odorata* can be drawn based on the chloroplast region. Determining the genome that is most informative may depend on the organism of interest.

Species that are perennial, outcrossing breeders and have the ability to asexually reproduce are the most likely to form hybrids (Rieseberg 1997). *Nymphaea odorata* possess all of these characteristics, making it highly likely to form hybrids. Hybrids between the species (*N. mexicana* and *N. odorata ssp. odorata*) and subspecies of *N. odorata* have been reported (Wiersema and Hellquist 1997). Hybrids between the two species are sterile, however, intraspecific hybrids in *N. odorata* are fertile, and can be difficult to classify based on morphology alone (Wiersema and Hellquist 1997). Borsch and Wiersema (pers. comm.) classified three accessions (KN1, KN3, KN4) as putative hybrids because they had combined morphological characteristics from both subspecies (Fig 2.2), and they occur in a population where both subspecies were present. However, molecular analyses characterized these

accessions as members of *ssp. tuberosa* based on ITS synapomorphies shared with *ssp. tuberosa*, and as part of a cluster with other accessions of *ssp. tuberosa* in ITS phylogeny, SAHN clustering, PCOA and MST analyses. Therefore in *Nymphaea*, it is difficult to classify hybrids based on morphology alone. Yet, it may be possible to classify hybrids based on molecular evidence from the ITS region.

Hybrids can have a unique effect in parsimony analyses. They can form polytomies between sister and non-sister taxa, or result in a basal trichotomy showing no relationship among ancestral taxa (Humphries 1983). In the ITS phylogeny and polymorphic site analyses, five accessions (KN15, KN25, KN37, KN17, KN16) did not group with either subspecies. In the strict consensus tree (not shown) these accessions, along with others from *ssp. tuberosa*, formed a polytomy at the base of the *ssp. odorata* clade. Since these accessions contained conflicting morphological characters with both subspecies and have a unique placement in ITS phylogeny, they could be hybrids between the subspecies.

Polymorphisms detected in the ITS region provided support for the hybrid origin of accessions KN7, KN17, KN31, and KN37. DNA of the hybrid is generally thought to reflect a combination of both parents' nuclear DNA. However, DNA of hybrids may be unique where concerted evolution could silence one parents' contribution to the genome (McDade 1995). In this study, the presence of a novel polymorphism in *N. odorata* may indicate a lack of concerted evolution. Recently, in the ITS region concerted evolution has been questioned by the presence of novel polymorphisms (Barkman and Simpson 2002, Franzke and Mummenhoff 1999, Sang et al. 1995, Wendel et al. 1995). In our study, four accessions KN7, KN17, KN31, and KN37 shared five character states; all were polymorphic. Among the accessions that share loci, two of them KN17 (*ssp. tuberosa* from Ohio) and KN37 (*ssp. odorata* from Virginia), never group with either subspecies in the ITS parsimony analyses. These accessions may possess molecular characters of both subspecies.

Polymorphic sites in the ITS region can also suggest relationships between hybrids and parental taxa. Franzke and Mummenhoff (1999) studied a unique system of allopolyploid speciation where both parents and hybrids were known. They determined that the presence of polymorphisms suggested rapid genomic change in allopolyploids and possible multiple origins for hybrids. Therefore, recent hybrids contain polymorphic sites in the ITS region, and the

basepairs in the polymorphism are present in the parents of the hybrid. In this study polymorphisms were analyzed by both SAHN clustering and PCOA with MST analyses. The SAHN clustering analysis is based on average distance between accessions, whereas the MST analysis depicts relationships between nearest neighbors. Therefore, the MST is a better indication of the relationships between accessions of *N. odorata*. The MST resulted in a ssp. *odorata* group and a ssp. *tuberosa* group. There were more connections within each group than between the ssp. *tuberosa* and ssp. *odorata* groups (Fig 2.4). This may indicate that potential gene flow between populations most likely occurs within a subspecies. The MST pattern observed here may also be an indication of the potential gene flow between the subspecies. The ssp. *odorata* and ssp. *tuberosa* groups were connected through a gradation of accessions (KN15, KN25, KN37, KN16, KN7, and KN17) in the MST. Potential gene flow between the subspecies may be seen by connections from accessions of Virginia (KN25, KN37) to accessions of Ohio (KN15, KN17). Therefore, the current distribution of each subspecies suggests that the potential gene flow between Virginia and Ohio populations may be ancient.

2.44 Implications to Aquatic Speciation

This study represents a rare glimpse into aquatic plant speciation. The geographic distribution and morphological characteristics of hybrids in *N. odorata* may give insights into speciation. Evidence from the ITS region, specifically the phylogeny and polymorphic site analyses, suggested accessions KN15, KN17, KN25, KN37, and KN16 as hybrids. Accessions KN15 and KN17 are both ssp. *tuberosa* from Ohio, and display mixed morphologies. Petiole stripes are present with a reddish-purple leaf blade in KN15 but no stripes and a intermediate (green and reddish-purple) leaf blade in KN17. These Ohio populations represent the morphological variation present within a relatively small geographic area. Additionally, they are morphologically unique compared to other accessions because they display a purple-pink flower. Conrad (1905) describes a similar colored *N. odorata* as an endemic of Putnam County, West Virginia. Due to geographic proximity of Ohio and West Virginia, it is possible that these accessions have a common descendant with the ancient West Virginia population mentioned by Conrad (1905).

Accessions KN25, KN37 and KN16 are within the same geographic proximity but have

unique morphological characteristics. Accession KN25, a ssp. *odorata* from Tennessee, and KN37, a ssp. *odorata* from Virginia, are in ponds that have both light pink and white flowers. The light pink flowers are distinct from the purple-pink flowers in Ohio, as they are thought to be from hybridization between natural and ornamental water-lilies. Accession KN16, a ssp. *odorata* from the Delaware population, is a morphologically unique coastal variety that was previously classified as var. *gigantea* (Wiersema and Hellquist 1997). Variety *gigantea* has an increased leaf and flower size, but a hybrid origin for this variety has not been documented.

The five accessions are possibly ancient hybrids that have been sustained due to their geographic isolation from other populations of *N. odorata*. Their geographic location, throughout the central eastern United States, suggests a possible ancient hybrid zone between the two subspecies where gene flow was occurring. In the absence of gene flow, it is expected that each subspecies will continue to diverge, eventually forming distinct species. However, additional molecular studies at the population level are still needed to support a theory that the subspecies are diverging.

2.45 Conclusions

Based on ITS data, *N. odorata* is a monophyletic group, and *N. mexicana* is sister to *N. odorata*. The speciation of *N. odorata* from *N. mexicana* is apparent, and the most likely subspecies to have a common ancestry with *N. mexicana* is probably ssp. *tuberosa*. The strongest evidence to support ssp. *tuberosa*'s speciation from *N. mexicana* is the presence of synapomorphies in the ITS region. Within *N. odorata*, polymorphic sites were detected and indicate possible hybrids between the subspecies. The geographic location of these hybrids suggests a possible ancient hybrid zone. Overall, evidence supports the segregation of ssp. *odorata* and ssp. *tuberosa*, with limited gene flow between the subspecies.

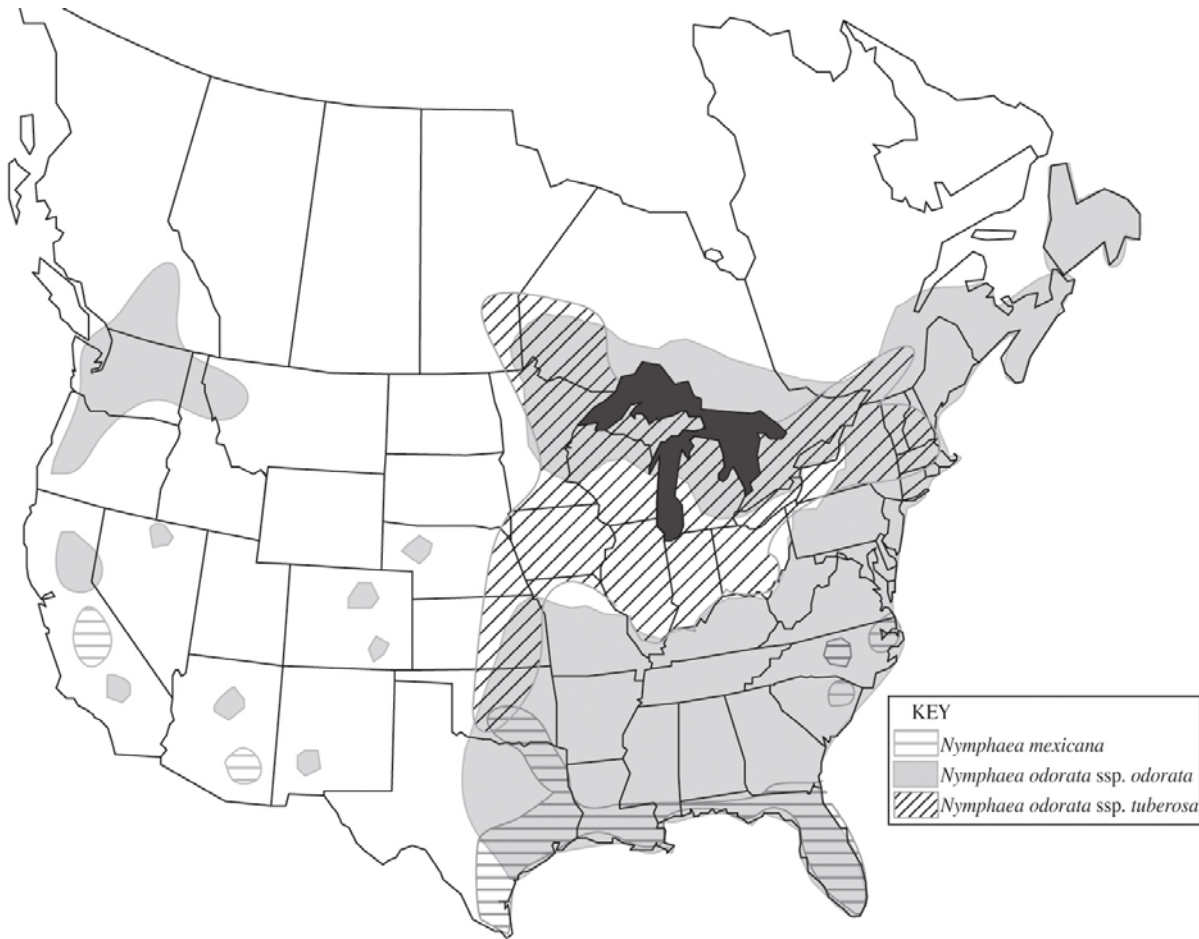


Fig 2.1. The geographic distribution of *N. mexicana* and *N. odorata* in North America. *Nymphaea odorata ssp. odorata* has the widest distribution, overlapping with both *N. mexicana* and ssp. *tuberosa* (Wiersema and Hellquist 1997).

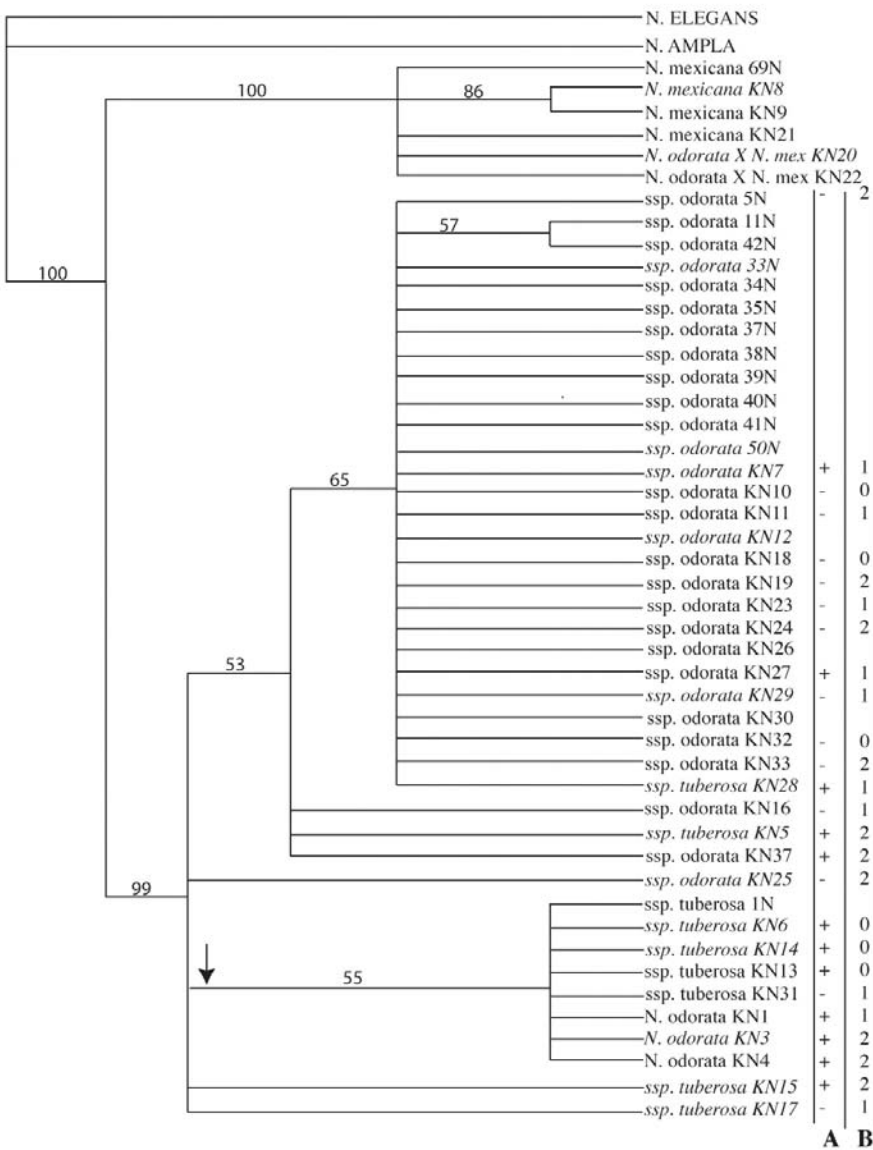


Fig 2.2. Bootstrap tree based on sequences from the ITS region (length = 190, CI = 0.947, RI = 0.977). Numbers on branches indicate bootstrap values based on 500 replicates. *Nymphaea ampla* and *N. elegans* were used as outgroups. Italicized names signify accessions sequenced for the *trnL-trnF* region. The clade marked by an arrow collapsed in a strict consensus analysis. Column A indicates the presence (+) or absence (-) of petiole stripes, and column B depicts leaf blade undersurface color (0=green, 1= mix of green and purple, 2= reddish-purple).

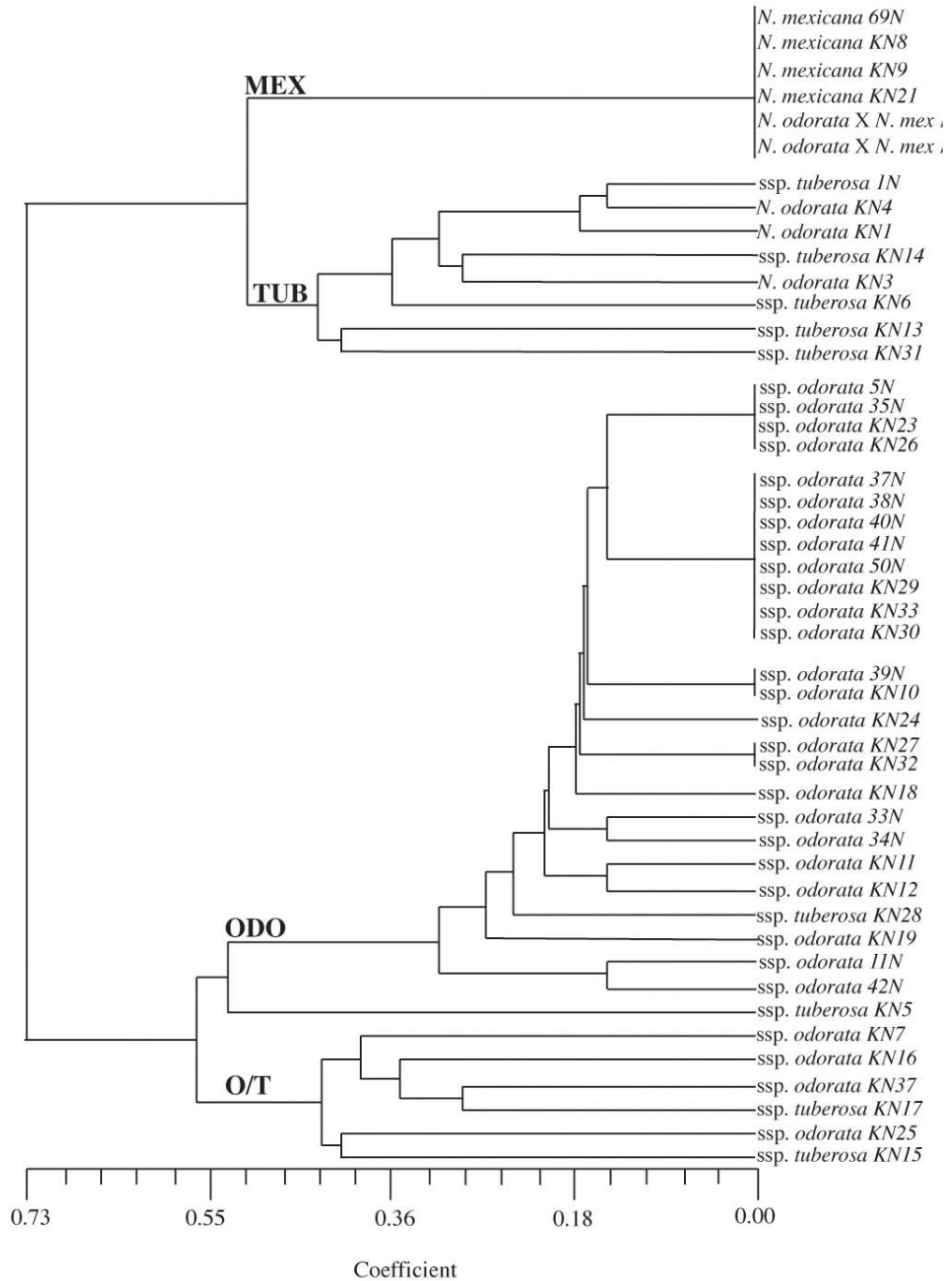


Fig 2.3 Tree produced from SAHN clustering of polymorphic ITS sites. Four distinct groups are evident MEX (*N. mexicana* and *N. mexicana* x *N. odorata* putative hybrids), TUB (*ssp. tuberosa*), ODO (*ssp. odorata*) and six accessions belonging to both subspecies (O/T group).

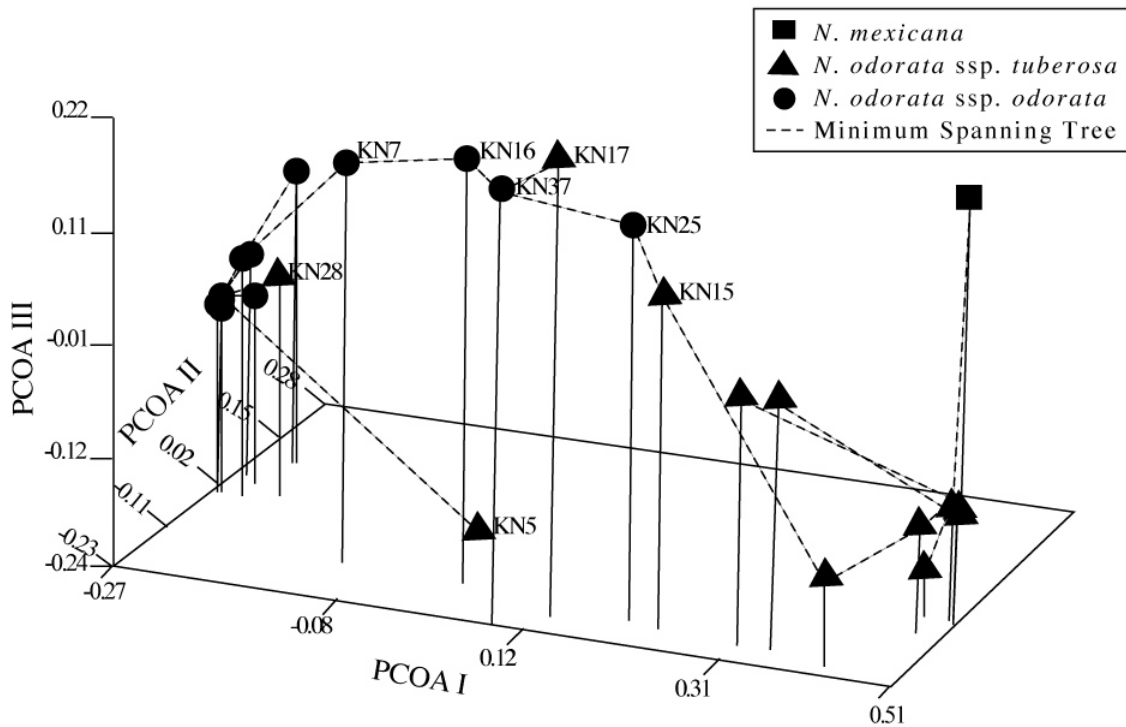


Fig 2.4 Principal coordinate analysis with minimum spanning tree superimposed generated from polymorphic ITS sites. All *N. mexicana* and putative hybrid (*N. mexicana* x *N. odorata*) accessions group together, and cluster closest to ssp. *tuberosa*. Several accessions form a gradient from ssp. *tuberosa* to ssp. *odorata*. One accession of ssp. *tuberosa* (KN28) clusters with the main group of ssp. *odorata* accessions. The proportion of total variance comprising each axis was 63.0% for axis I, 11.8% for axis II, and 5.7% for axis III.

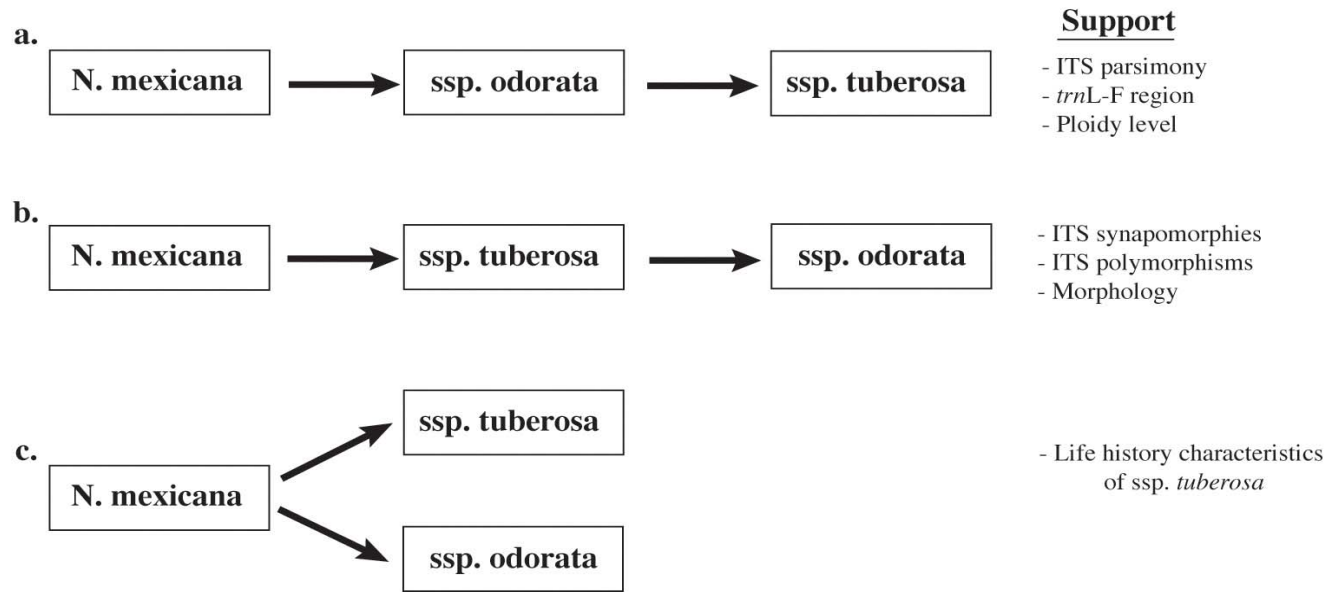


Fig 2.5 Three possible hypotheses for the origin of the subspecies of *N. odorata* (*ssp. odorata* and *ssp. tuberosa*) from *N. mexicana*.

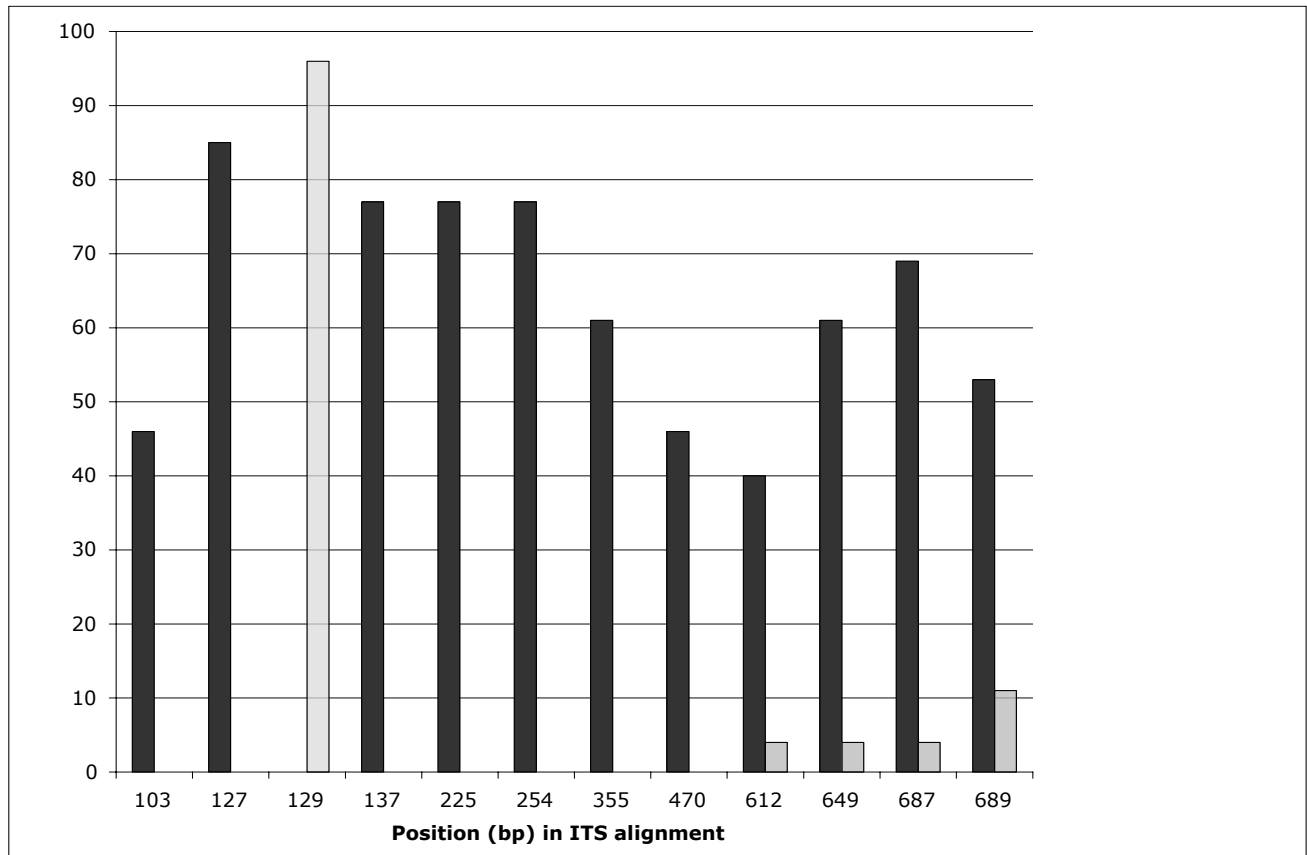


Fig 2.6. Synapomorphies between *ssp. odorata*, *ssp. tuberosa* and *N. mexicana* in the ITS region. The % shared was calculated as the number of accessions sharing a bp with *N. mexicana* per the total accessions of each subspecies. Most (11/12) basepairs are shared with *ssp. tuberosa*.

Table 2.1. Accessions used along with their classification, geographic location and sources of the material. Accessions classified as *N. odorata* hybrid without a subspecies affiliation were identified as putative hybrids between the subspecies.

<i>Nymphaea</i> species	Accession Number	Geographic Location	Collector and collector number
<i>N. elegans</i>	6N	Florida	Borsch and Wilde 3084
<i>N. ampla</i>	100N	Mexico	Novelo, R.A. et al. 1295
<i>N. mexicana</i>	69N	Florida	Borsch & Summers 3227
<i>N. mexicana</i>	KN8	Texas	Woods & Borsch KN0701
<i>N. mexicana</i>	KN9	Louisiana	Woods & Borsch KN1101
<i>N. mexicana</i>	KN21	Mexico	Anonymous
<i>N. mexicana X N. odorata</i>	KN20	Florida	Borsch & Summers 3213
<i>N. mexicana X N. odorata</i>	KN22	Florida	Borsch & Summers 3214
<i>N. odorata ssp. odorata</i>	5N	Maryland	Borsch, Hilu & Wiersema 2361
<i>N. odorata ssp. odorata</i>	11N	Florida	Borsch & Wilde 3128
<i>N. odorata ssp. odorata</i>	42N	Florida	Borsch & Wilde 3099
<i>N. odorata ssp. odorata</i>	33N	Florida	Borsch & Wilde 3101
<i>N. odorata ssp. odorata</i>	34N	Florida	Borsch & Wilde 3125
<i>N. odorata ssp. odorata</i>	35N	Florida	Borsch & Wilde 3127
<i>N. odorata ssp. odorata</i>	37N	Georgia	Borsch & Wilde 3131
<i>N. odorata ssp. odorata</i>	38N	Georgia	Borsch & Wilde 3133
<i>N. odorata ssp. odorata</i>	39N	Georgia	Borsch & Wilde 3134
<i>N. odorata ssp. odorata</i>	40N	Georgia	Borsch & Wilde 3135
<i>N. odorata ssp. odorata</i>	41N	Georgia	Borsch & Wilde 3136
<i>N. odorata ssp. odorata</i>	50N	Georgia	Borsch & Wilde 3132
<i>N. odorata ssp. odorata</i>	KN7	Michigan	Borsch, Wiersema & Hellquist 3398
<i>N. odorata ssp. odorata</i>	KN10	Texas	Woods & Borsch KN0801
<i>N. odorata ssp. odorata</i>	KN11	Louisiana	Woods & Borsch KN0901
<i>N. odorata ssp. odorata</i>	KN12	Louisiana	Woods & Borsch KN1001
<i>N. odorata ssp. odorata</i>	KN18	South Carolina	Wiersema KN0601
<i>N. odorata ssp. odorata</i>	KN19	Virginia	Woods KN1201
<i>N. odorata ssp. odorata</i>	KN23	Vermont	Borsch, Wiersema & Hellquist 3331
<i>N. odorata ssp. odorata</i>	KN24	North Carolina	Woods KN1401
<i>N. odorata ssp. odorata</i>	KN26	Tennessee	Woods & Neves KN1701
<i>N. odorata ssp. odorata</i>	KN27	Vermont	Borsch, Wiersema & Hellquist 3322
<i>N. odorata ssp. odorata</i>	KN29	Vermont	Borsch, Wiersema & Hellquist 3330
<i>N. odorata ssp. odorata</i>	KN30	Florida	Borsch & Summers 3215
<i>N. odorata ssp. odorata</i>	KN32	Vermont	Borsch, Wiersema & Hellquist 3323
<i>N. odorata ssp. odorata</i>	KN33	Vermont	Borsch, Wiersema & Hellquist 3324
<i>N. odorata ssp. odorata</i>	KN16	Delaware	Wiersema KN0401
<i>N. odorata ssp. odorata</i>	KN25	Tennessee	Woods & Neves KN1501
<i>N. odorata ssp. odorata</i>	KN37	Virginia	Woods KN1301
<i>N. odorata ssp. tuberosa</i>	KN28	Vermont	Borsch, Wiersema & Hellquist 3329
<i>N. odorata ssp. tuberosa</i>	KN5	Wisconsin	Borsch, Wiersema & Hellquist 3396
<i>N. odorata ssp. tuberosa</i>	1N	New York	Borsch 3156
<i>N. odorata ssp. tuberosa</i>	KN6	Canada	Borsch, Wiersema & Hellquist 3389
<i>N. odorata ssp. tuberosa</i>	KN14	Michigan	Wiersema KN0201
<i>N. odorata ssp. tuberosa</i>	KN15	Ohio	Wiersema KN0301
<i>N. odorata ssp. tuberosa</i>	KN13	Pennsylvania	Wiersema KN0101
<i>N. odorata ssp. tuberosa</i>	KN31	Vermont	Borsch, Wiersema & Hellquist 3325
<i>N. odorata ssp. tuberosa</i>	KN17	Ohio	Wiersema 2384

<i>N. odorata</i> hybrid	KN1	Michigan	Borsch & Wiersema 3399
<i>N. odorata</i> hybrid	KN3	Michigan	Borsch & Wiersema 3401
<i>N. odorata</i> hybrid	KN4	Michigan	Borsch & Wiersema 3402

Table 2.2. The identity of the one basepair molecular marker at position 396 in the *trnL-trnF* region. Accessions selected (Fig 2.2) were either putative hybrids between the species, representatives of each subspecies, or had unusual placement in the ITS bootstrap tree. Accessions 50N, 33N, KN12, KN29, 1N, KN6 and KN14 are representatives of the subspecies and were included to check the accuracy of this nucleotide position.

Accession	Identified As:	Location	Results
KN8	<i>N. mexicana</i>	Texas	T
KN20	<i>N. mexicana x N. odorata</i>	Florida	T
50N	<i>N. odorata</i> ssp. <i>odorata</i>	Georgia	T
33N	<i>N. odorata</i> ssp. <i>odorata</i>	Florida	T
KN12	<i>N. odorata</i> ssp. <i>odorata</i>	Louisiana	T
KN29	<i>N. odorata</i> ssp. <i>odorata</i>	Vermont	T
KN7	<i>N. odorata</i> ssp. <i>odorata</i>	Michigan	G
KN25	<i>N. odorata</i> ssp. <i>odorata</i>	Tennessee	T
1N	<i>N. odorata</i> ssp. <i>tuberosa</i>	New York	G
KN3	<i>N. odorata</i> hybrid	Michigan	G
KN5	<i>N. odorata</i> ssp. <i>tuberosa</i>	Wisconsin	G
KN6	<i>N. odorata</i> ssp. <i>tuberosa</i>	Manitoba	G
KN14	<i>N. odorata</i> ssp. <i>tuberosa</i>	Michigan	G
KN15	<i>N. odorata</i> ssp. <i>tuberosa</i>	Ohio	G
KN17	<i>N. odorata</i> ssp. <i>tuberosa</i>	Ohio	G
KN28	<i>N. odorata</i> ssp. <i>tuberosa</i>	Vermont	G

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Chapter 3: Using Morphology and Inter-Simple Sequence Repeats (ISSR) to Analyze the Variation in Populations of *Nymphaea odorata*

3.1 Introduction

Nymphaea odorata is the most common water-lily in North America. Its geographical distribution extends from Florida north to Nova Scotia, Newfoundland and east to Texas (Wiersema and Hellquist 1997). This water-lily has been divided into two subspecies: ssp. *odorata* and ssp. *tuberosa*. In the Flora of North America, Wiersema and Hellquist (1997) characterized each subspecies as having distinct morphologies and unique geographical distributions. Subspecies *tuberosa* is distinguished from ssp. *odorata* by the presence of petiolar stripes, a green leaf blade undersurface, and 2.8 - 4.5 mm long seeds (Wiersema and Hellquist 1997). This subspecies distribution extends from the central United States northeast into Canada. Subspecies *odorata* has no striping on its petiole, a reddish-purple leaf blade undersurface, and 1.5 - 2.5 mm long seeds. This subspecies has a widespread geographic distribution encompassing major portions of the United States, from the south extending into Canada.

The objectives of this study were to quantitatively evaluate the morphological differentiation between the subspecies and then assess the genetic relationship among populations of *N. odorata* using inter-simple sequence repeat (ISSR) markers. This study will also examine if the morphological characters used by Wiersema and Hellquist (1997) adequately separate the subspecies and whether other morphological characters support this separation.

Previous results (Chapter 2) based on the nuclear internal transcribed spacer (ITS) region have suggested that the morphological characteristics in the Flora of North America (FNA; Wiersema and Hellquist 1997) might not clearly distinguish each subspecies. Lack of segregation may be due to the presence of fertile hybrids found in Canada, Minnesota, Michigan, New York, Vermont, and Wisconsin where subspecies distributions overlap. Identification of these hybrids can be difficult. Hybrids can display a mixed morphology, having characteristics of both subspecies, or they can have morphological characteristics that are intermediate between the subspecies. The morphological variation within *N. odorata* was investigated using univariate and multivariate statistical approaches.

ISSR markers have been used to construct phylogenetic relationships by determining the

amount of genetic variation among species (Rania et al. 2001). Markers are based on a microsatellite motif, e.g., (CA)_x and are anchored on the 5' or 3' end with one to three nucleotides. The advantages of ISSR markers over random amplified polymorphic DNA (RAPD) are that they result in greater band reproducibility, have more variation, and are more cost effective (Wolfe and Liston 1998). Each band generated represents a diallelic marker where the fragment size represents a locus (Wolfe and Randle 2001). ISSR data was used in this study to quantify the amount and distribution of genetic variation within and among populations of *N. odorata*.

3.2 Materials and Methods

3.21 Morphological Analyses

Accessions were identified by the collectors (Table 3.1) on the basis of morphological characters listed for each subspecies in the FNA (Wiersema and Hellquist 1997). If an accession could not be keyed out to a subspecies it was treated as *N. odorata* hybrid without a subspecies affiliation. Thirty-one accessions of *N. odorata* from across its geographic range were measured for 27 morphological characters (Tables 3.1, 3.2). Quantitative and qualitative morphological characters were chosen from current taxonomic treatments of *N. odorata* (Borman et al. 1999; Conrad 1905; Crovello et al. 1983; Godfrey and Wooten 1981; Harvill et al. 1992; Jones et al. 1997; Radford et al. 1968; Wiersema and Hellquist 1997). Qualitative characters were scored for a specific character state (Table 3.2) and quantitative characters were measured using a ruler or counted.

Differences among morphological characters were sought using univariate and multivariate statistical approaches. Univariate analysis was performed on each character to determine if a statistically significant difference existed between subspecies, as classified based on characteristics in the FNA (Wiersema and Hellquist 1997). Accessions identified as *N. odorata* hybrid were included in univariate analysis, multivariate analysis of variance (MANOVA), and discriminate analysis as accessions of ssp. *tuberosa*, based on previous analysis of the ITS region (Chapter 2). An analysis of variance (ANOVA) was generated for each morphological character using a student's t-test for quantitative characters and a

contingency table for qualitative characters. Additionally, a linear regression analysis for each subspecies was used to look for an association between any of the quantitative morphological characters. All univariate analyses were performed in JMP, version 4 (SAS Institute, Inc.).

Multivariate methods were used to determine if a grouping of subspecies was found based on no prior assumption of subspecies classification. These were sequential, agglomerative, hierarchical, and nested (SAHN) clustering, principal coordinate analysis (PCOA), and minimum spanning tree (MST), all performed in NTSYS-pc computer program version 2.102 (Rohlf 1998, Exeter Publishers Ltd.). SAHN clustering was performed to allow for an objective classification into groups using an average distance between accessions (Boonkerd et al. 2002). The MST was superimposed on the PCOA and groups of accessions in these analyses emphasize relationships between nearest neighbors.

Four different combinations of morphological characters were analyzed to determine the separation present between the two subspecies based on different data sets: (1) all morphological characters, (2) qualitative characters, (3) quantitative characters, and (4) characters that were significant to $P < 0.10$ in the ANOVA analyses. SAHN clustering, PCOA, and MST were performed on matrices 1-4 (all 31 accessions included). SAHN clustering was performed using the unweighted pair-group method with arithmetic averages (UPGMA). For PCOA and MST analyses a similarity matrix was generated. For matrix 2, comprised entirely of qualitative characters, the simple matching coefficient was used to generate a similarity matrix.

In addition, discriminant analysis, MANOVA, and principal component analysis (PCA) were performed on 26 accessions of *N. odorata* using SAS version 8.0 (SAS Institute 1999-2001). For these analyses, accessions with missing values (KN26, KN10, KN18, KN14, *N. tuberosa* Lectotype) were excluded, as well as variables with few entries (qualitative characters 7-13 and quantitative 6-14; Table 3.2). All remaining quantitative variables were log (base 10) transformed because they were not normally distributed. PCA was based on a correlation matrix, and discriminant analysis and MANOVA were performed on only quantitative characters 1-5 (Table 3.2).

3.22 ISSR Analyses

An ISSR survey was done to determine the genetic variation both within and among

populations of *N. odorata*. Ten primers (Table 3.3) were used on ten accessions from a southern Tennessee population to assess intrapopulation variation. These accessions were chosen randomly at equal distances along the circumference of the pond.

To determine interpopulation variation, 37 accessions of *N. odorata*, two accessions of putative hybrids *N. mexicana* x *N. odorata* and four accessions of *N. mexicana* were examined (Table 3.1). DNA was extracted from silica-dried or frozen leaf tissue by a modified CTAB method (see details in Chapter 2). After extraction, single primer PCRs were conducted using five ISSR primers (Table 3.3). Four primers were chosen having no intrapopulation variation, and one primer was chosen that displayed intrapopulation variation. The primer with intrapopulation variation was randomly chosen from the primers in the intrapopulation study (Table 3.3). PCR reaction volumes of 25µl consisted of 0.4µM primer, 1X *Taq* polymerase buffer, 0.2µM dNTPs, 0.2 U *Taq* DNA polymerase (Promega, Madison, Wisconsin), 1.5 mM MgCl₂, and 1µl of DNA. The thermocycler program was performed following the Wolfe and Randle (2001) protocol. Replicate experiments were done to check the accuracy of the bands produced with ISSR primers. Additionally, all experiments included negative controls by replacing DNA with sterile water.

The ISSR PCR products were analyzed on 1.5% agarose gels in 1X TAE buffer following the conditions in Wolfe and Randle (2001). Gels were stained with ethidium bromide to visualize the banding patterns and digital images were captured using Alpha Digital Image System (Alpha Innotech Corp., San Leandro, California). Each image was printed in 8x10 format and fragment sizes were analyzed by comparing the accessions to a 1 KB ladder standard (Promega, Madison, Wisconsin) included in the gels. The bands were scored as band present = 1 or band absent = 0 for all accessions and each primer. Only reproducible bands were scored for subsequent analyses.

For each primer, the number of loci and genotypes were determined. Additionally, the percent polymorphic bands was calculated for three taxonomic groups: (1) *N. odorata* ssp. *tuberosa*, (2) *N. odorata* ssp. *odorata* and (3) *N. mexicana* and *N. mexicana* x *N. odorata* putative hybrids. *Nymphaea mexicana* and the putative hybrids were consolidated into one taxonomic group based on results from the ITS region (Chapter 2). The percentage of polymorphic bands was calculated by dividing the number of polymorphic bands per taxonomic group by the total number of bands produced by the primer

(Li and Ge 2001).

The matrix constructed from the presence or absence of ISSR bands was utilized to generate a similarity matrix with the DICE coefficient (Pearson et al. 2002). The similarity matrix was then used to produce a phenogram using the UPGMA analysis. To examine the clustering pattern between all individuals a PCOA was performed using the similarity matrix. To detect local distortion in the PCOA an MST was also computed on a similarity matrix. All analyses were performed in NTSYS-pc computer program version 2.102 (Rohlf 1998, Exeter Publishers Ltd.).

An analysis of molecular variance (AMOVA) was computed to determine the genetic structure within and among *N. odorata* and *N. mexicana*. Initially, the presence/absence matrix was input into AMOVAprep computer program version 1.55 (Miller 1998), which created a distance matrix. This matrix was then used in the AMOVA computer program version 1.55 (Excoffier 1995) to estimate the variation among individuals/within regions, among regions/within species, and among species (Excoffier et al. 1992). Regions were defined as populations within an individual state (Table 3.1) and species were defined as the three taxonomic groups (ssp. *odorata*, ssp. *tuberosa*, *N. mexicana* and putative hybrids).

3.3 Results

3.3.1 Morphological Analyses

Based on ANOVA, eight of the 27 vegetative morphological characters are moderately significant ($P < 0.10$) between the subspecies (Table 3.4). Among the significant characters, seven are leaf blade characteristics, with each subspecies having distinct leaf blade characters (Table 3.5). The linear regression analysis for each subspecies was only statistically significant for blade width by blade length (Fig 3.1). MANOVA analysis based on quantitative characteristics 1-5 was not statistically significant (Wilks' Lambda = 0.64; $F = 1.45$; $df = 7$; $P = 0.245$).

SAHN clustering, PCOA, and MST based on each of the four morphological data matrices (see Materials and Methods) did not result in a significant grouping by subspecies or geography (not shown). However, some separation of the subspecies is shown by the PCA,

which used the data set excluding missing characters (Fig 3.2). Coordinates PCI and PCII separate accessions of *ssp. odorata* from accessions of *ssp. tuberosa*. The PCA also provides insight into the morphological variation present. The first three derived components account for 66.14% of the total variance (Table 3.6). Most of the variation in component one is contributed by quantitative leaf blade characteristics (blade width, blade length, blade sinus length, petiole diameter), whereas most of the variation in component two is contributed by qualitative leaf blade characteristics (petiole stripes, petiole hairs, and sinus overlap). Component three has low loadings for most variables, except for lobe apex. The discriminant analysis based on quantitative characteristics 1-5 classifies 2/3 of the specimens correctly with an average error rate of 31% (Table 3.7).

3.32 ISSR

The preliminary study of intrapopulation variation conducted on ten samples from the Tennessee population yielded four primers showing variability and six primers showing no variability (Table 3.3). Based on results of the intrapopulation study, five primers were chosen for the interpopulation study (Table 3.3). Four of these primers showed no intrapopulation variation and were used to decrease the detection of genetic variability among populations. The fifth primer, 814, was used in the interpopulation study to determine if primers variable at the population level would be applicable on a larger scale (among populations).

The PCR fragments for all primers range from 300 bp to 2000 bp. Seventy-two loci were scored for the five primers, which resulted in 30-41 genotypes for each primer (Table 3.8). The wide range of percent polymorphic bands between taxonomic groups shows high genetic variability within and among species. The primer displaying variability within a population (primer 814) produces the largest number of genotypes, 41, even though it contained the smallest number of loci. Due to the large amount of variability produced by primer 814, it was excluded from PCOA, MST, UPGMA and AMOVA analyses.

The PCOA and MST reveal accessions from *N. mexicana* and *N. odorata*, forming two discrete groups (Fig 3.3). Within the *N. odorata* group, the two subspecies show additional segregation. Accession 42N of *ssp. odorata* (Florida) is distinct from other accessions. Accession KN17 of *ssp. tuberosa* (Ohio) displays odd placement in the PCOA by grouping in the center of *ssp. odorata* accessions.

The UPGMA analysis of the ISSR markers produces results analogous to the PCOA results (Fig 3.4), where three main groups that represent each subspecies are evident. Some geographic pattern is apparent; for example three accessions of *ssp. tuberosa* from a Michigan population cluster together and five accessions of *ssp. odorata* from southern populations (Florida and Georgia) cluster together.

The AMOVA shows higher variation within a region (an individual state) than between different taxonomic groups (Table 3.9). Of the total genetic diversity, 3% is attributed to differences among taxonomic groups, 14% to differences among regions within a taxonomic group, and 89% to differences within a region.

3.4 Discussion

3.4.1 Morphological differences between the subspecies

In their treatment of *Nymphaea odorata* for the Flora of North America (FNA), Wiersema and Hellquist (1997) recognized *ssp. odorata* and *ssp. tuberosa* based on differences in petiole stripes, color of leaf blade undersurface and seed size. Univariate analysis of the 27 morphological characters examined here showed eight characters to be statistically significant between the subspecies, of which only petiole stripes was used to discriminate between the subspecies by Wiersema and Hellquist (1997). Surprisingly, the color of the leaf blade undersurface was not statistically significant between the two subspecies. This is probably due to the variation found in the color of leaf blade undersurface, as 12 accessions were intermediate in this characteristic, having a mix of reddish-purple (*ssp. odorata*) and green (*ssp. tuberosa*) color. The majority of the statistically significant morphological characters were leaf blade characteristics. Previous authors (Borman et al. 1999; Conrad 1905; Crovello et al. 1983; Godfrey and Wooten 1981; Harvill et al. 1992; Jones et al. 1997; Radford et al. 1968; Wiersema and Hellquist 1997) have not used leaf blade characters of length, width, length of sinus and number of leaf veins to discriminate between the two subspecies. In general, *ssp. tuberosa* has a larger leaf blade length/width ratio, longer blade sinus, more leaf veins, and a pointed lobe apex compared to *ssp. odorata*.

The means of the leaf blade characteristics (Table 3.5) were used to generate a typical leaf blade for each subspecies (Fig 3.5). Compared to *ssp. odorata*, the leaf area of *ssp. tuberosa*

is larger, circular in size, has a striped petiole and a pointed lobe apex. In contrast, *ssp. odorata* has smaller leaf blade area, ovate in size, does not have a striped petiole, and has a rounded lobe apex.

Multivariate analyses were used to determine if morphological characters can group accessions into each subspecies, and then if the groups are congruent with geography and subspecies designation. SAHN clustering, PCOA, and MST produced no grouping by subspecies or geography. In these analyses lack of grouping may be due to using a combination of flower and leaf blade morphology. In contrast, the PCA results supported a distinction between the subspecies and emphasized the importance of leaf blade characteristics (Fig 3.2). Component 1 explained the majority of the variation found between the subspecies (35.57%) and loaded high for quantitative leaf blade characteristics (Table 3.6). Furthermore, a discriminant analysis using only leaf blade characteristics, showed a distinction between the subspecies (Table 3.7). This analysis classified most accessions to the correct subspecies, 71.43% for *ssp. odorata* and 66.67% for *ssp. tuberosa*.

Therefore, PCA, discriminant analysis and ANOVA results point to distinctness between the two subspecies. These analyses suggest that *ssp. tuberosa* be recognized by the presence of petiole stripes, a circular leaf blade, and a pointed lobe apex and *ssp. odorata* be recognized by having no petiole stripes, an ovate leaf blade and a rounded lobe apex. Morphological analyses on leaf blade characteristics suggested a distinction between the subspecies.

3.42 Intrapopulation and interpopulation variation using ISSR markers

Nymphaea odorata can reproduce both asexually and sexually. As previously proposed in *N. mexicana* (Wiersema and Hellquist 1997) asexual reproduction via rhizomes may allow the plant to quickly cover an entire pond. ISSR markers displayed intrapopulation variation in the Tennessee population containing plants of *N. odorata ssp. odorata* (Table 3.3). There were four variable markers, indicating that a population may not be comprised of one clone. However, further studies of additional populations of both subspecies and larger intrapopulation samples are required to adequately test the clonality of *N. odorata*.

Interpopulation variation showed a distinction between the subspecies using four ISSR primers. The majority of accessions grouped according to subspecies designation in the PCOA, MST and UPGMA analyses (Fig 3.3, 3.4). Additionally, ISSR markers have shown geographic

segregation. In the UPGMA analysis, *ssp. tuberosa* formed three main groups, one of which encompasses three morphologically distinct accessions from a population in Michigan. However, the other two groups of *ssp. tuberosa*, comprised of four and six accessions, do not show any geographic grouping. Within *ssp. odorata* geographic segregation is again evident in two groups. One group of accessions is from Vermont, Virginia, and Tennessee, whereas the other group is from Florida and Georgia. However, for each subspecies the three main groups did not group together, which may indicate hybridization between subspecies.

ISSR markers displayed high interpopulation variation in *N. odorata* (Table 3.3). Polymorphic bands ranged from 70-80% in *ssp. tuberosa*, and 82-100% in *ssp. odorata*. While *ssp. odorata* may appear more variable, the higher percentage of polymorphic bands is most likely an artifact of the larger sampling size used for *ssp. odorata*. Previous studies have shown that small sample sizes may underestimate the genetic diversity present within a species (Wolfe and Randle 2001). Furthermore, AMOVA studies show that most genetic variation in *N. odorata* is distributed within a state rather than between states (Table 3.9). This indicates a highly variable species with relatively unrestricted boundaries to gene flow between states. This high variability found in *N. odorata* is comparable to previous studies of obligate outcrossing organisms (Huff et al. 1993, Hamrick and Godt 1996, Sales et al. 2001).

3.43 Summary and Conclusions

A comparison of morphology and molecular evidence can give insight into speciation in *Nymphaea odorata*. This study supports the classification of *N. tuberosa* as a subspecies of *N. odorata* (Wiersema and Hellquist 1994). The characters used by Wiersema and Hellquist (1997) to characterize each subspecies were the presence or absence of petiolar stripes, color of leaf blade undersurface, and seed size. However, in this study only the presence or absence of petiolar stripes was statistically significant. Univariate and multivariate analyses show some distinction between the subspecies, but not the magnitude of separate species. The strongest evidence for morphological separation between subspecies comes from differences in leaf blade characteristics. ANOVA analyses used both quantitative and qualitative characters, analyzing each character individually. The ANOVA was based on *a priori* knowledge of subspecies classification, and was biased to the characters used to classify the subspecies (petiole stripes). MANOVA and discriminant analyses were also based on previous subspecies classification.

Both analyses were performed on reduced sets of the original data, excluding missing characters and using only quantitative characteristics. Using the reduced data set discriminant analysis was able to classify 2/3 of accessions correctly. SAHN, PCOA, MST and PCA analyses used a combination of quantitative and qualitative characteristics. The benefit to using these analyses is that clusters were generated based on no knowledge of subspecies classification. Only the PCA was able to show some separation between the subspecies. The PCA used a reduced data set and excluded missing characters compared to SAHN, PCOA and MST. The most informative morphological information in this study comes from analyses based on reduced data sets.

Molecular evidence has provided some support for the treatment of two separate subspecies. Each subspecies was divided into three groups; some of the groups show a geographic pattern. The distinction between the subspecies based on ISSR data is also supported by ITS data (Chapter 2). In the phylogeny, based on ITS data, each subspecies clustered in a distinct clade. Furthermore, three populations (TN, VA, MI) clustered together based on ISSRs, but did not always cluster together based on ITS data. ISSR markers are highly variable within *N. odorata*, and may be best used on small-scale population analysis. Overall, ISSR data and morphologic analyses indicates speciation is occurring but there is not a complete distinction between *ssp. odorata* and *ssp. tuberosa*.

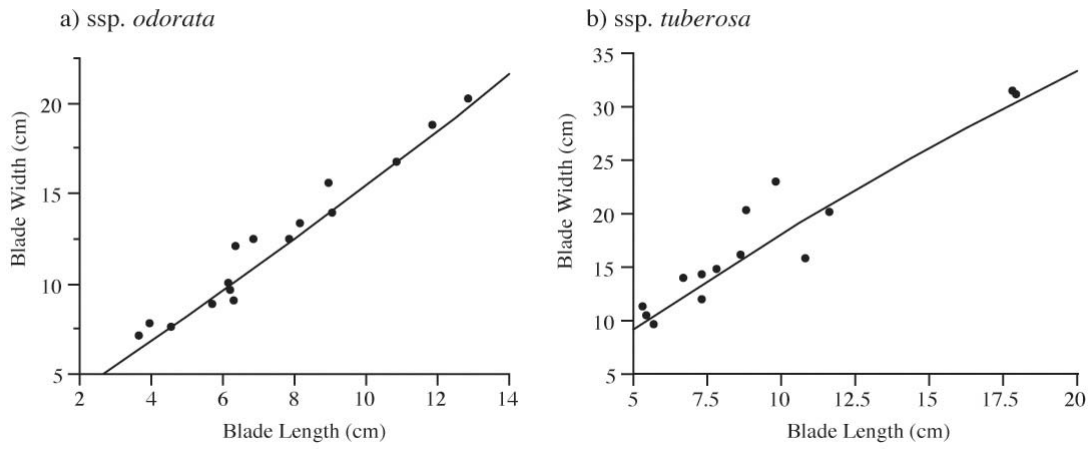


Fig 3.1 Plots (regression line) of leaf blade width by blade length for each subspecies. For subspecies *odorata* (a) $R^2 = 0.96$ and $P < 0.0001$ and *tuberosa* (b) $R^2 = 0.86$ and $P < 0.0001$.

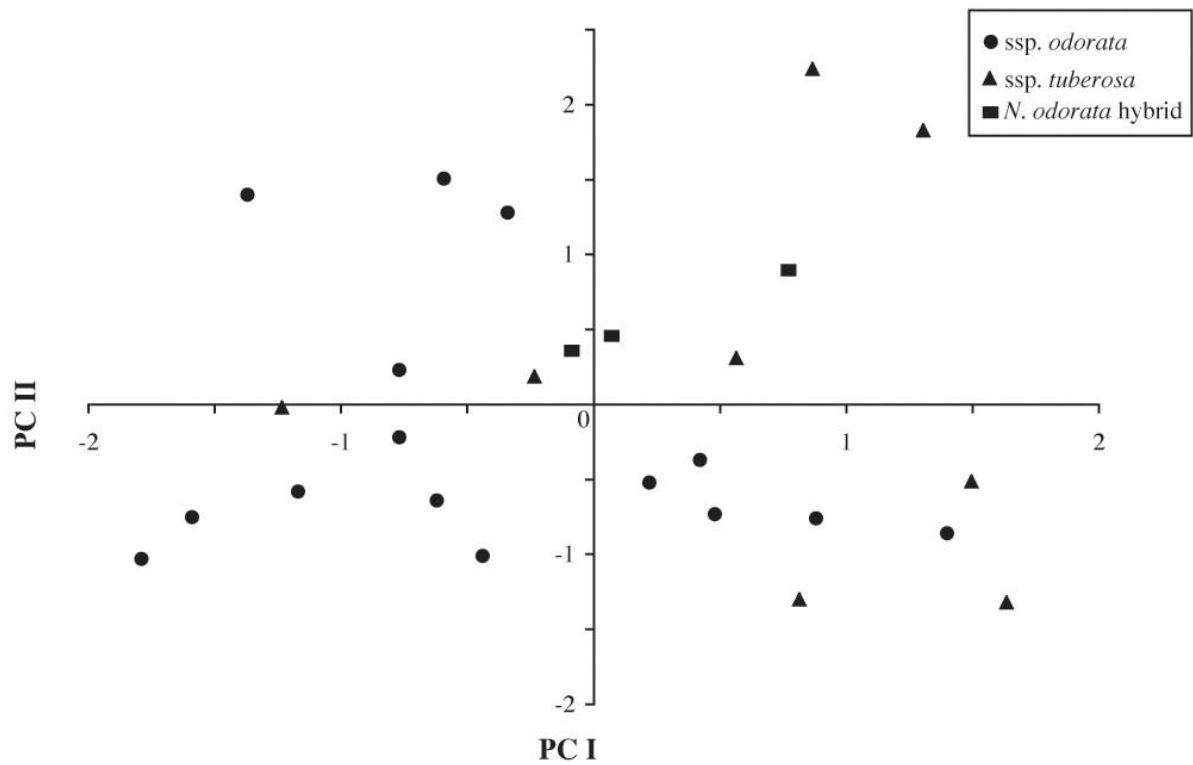


Fig 3.2 Plot of scores by principal component axes I and II from 26 accessions of *N. odorata* on eleven morphology characters. Accessions from *ssp. odorata* occur mostly on the negative side of both axes, whereas accessions from *ssp. tuberosa* occur mostly on the positive side of both axes.

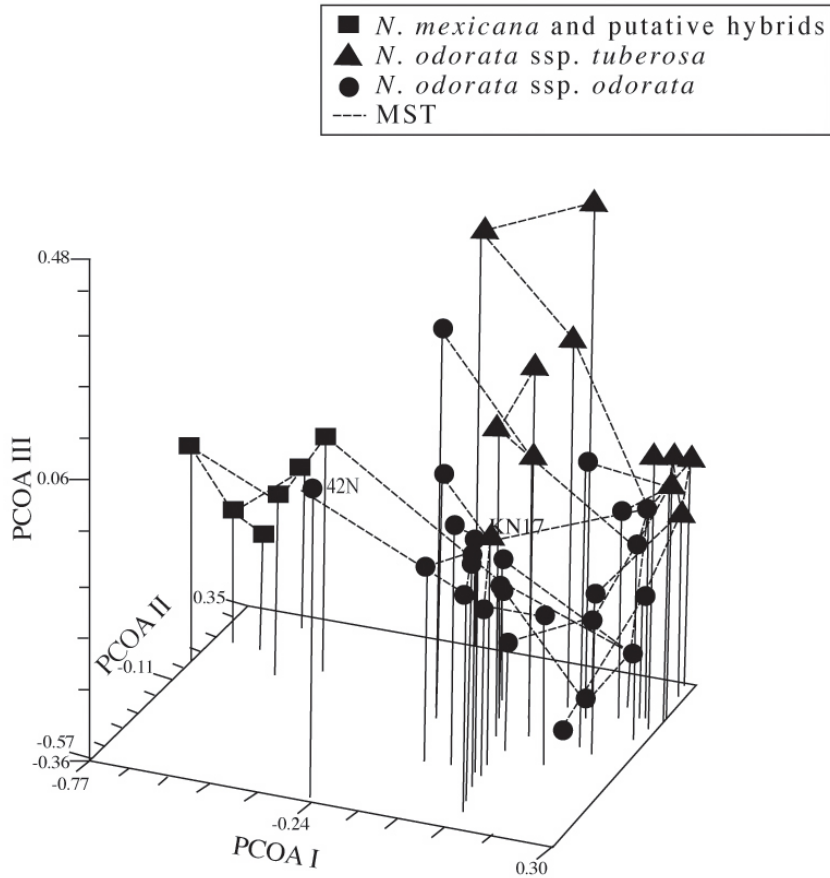


Fig 3.3 Principal coordinate analyses with minimum spanning tree superimposed based on ISSR markers excluding primer 814. *Nymphaea mexicana* and *N. odorata* are clearly distinguished; separation between the subspecies of *N. odorata* is less distinct but still evident. Accessions KN17 and 42N have unique placement (see text). The proportion of total variance comprising each axis was 17.8% for axis I, 9.2% for axis II, and 7.2% for axis III.

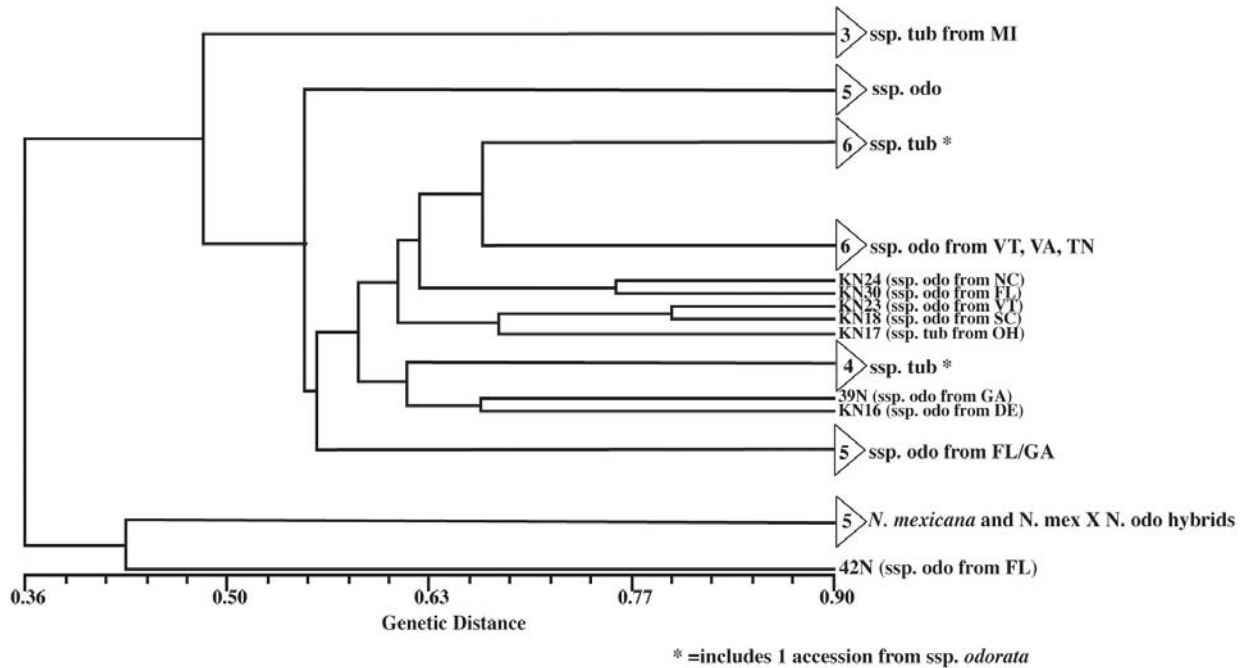
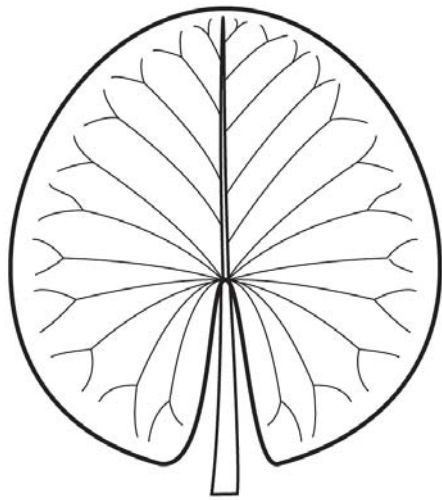
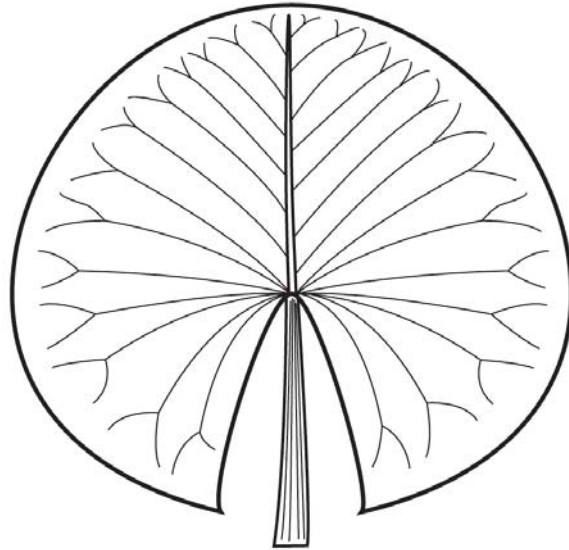


Fig 3.4 Phenogram illustrating genetic relationships among *N. mexicana*, *N. odorata* and putative hybrids (*N. mexicana* x *N. odorata*) generated by the UPGMA cluster analysis (NTSYS-pc) calculated from ISSR markers excluding primer 814. *Nymphaea mexicana* and the putative hybrids group separately from *N. odorata*. Each subspecies is represented by three distinct groups, each group showing geographic partitioning. Numbers in triangles denote the number of accessions in each triangle. Geographic locations are indicated by state abbreviations.



a. Subspecies *odorata*



b. Subspecies *tuberosa*

Fig 3.5 Illustration of leaf blades for ssp. *odorata* and ssp. *tuberosa* based on the mean measurements of leaf blade size and shape, the presence or absence of petiole stripes, and lobe apex shape. All characteristics were statistically significant in a t-test (see Table 3.5).

Table 3.1. Accessions used along with their classification, geographic location, and collector information. Italics indicate accessions used in morphological analyses. Accessions containing an accession number were used only in molecular analyses. Voucher specimens were deposited at FR, FTG, and VPI (herbarium abbreviations from Holmgren et al. 1990).

<i>Nymphaea</i> species	Accession Number	Geographic Location	Collector and collector number
<i>N. mexicana</i>	69N	Florida	Borsch & Summers 3227
<i>N. mexicana</i>	KN8	Texas	Woods & Borsch KN0701
<i>N. mexicana</i>	KN9	Louisiana	Woods & Borsch KN1101
<i>N. mexicana</i>	KN21	Mexico	Anonymous
<i>N. mexicana</i> X <i>N. odorata</i>	KN20	Florida	Borsch & Summers 3213
<i>N. mexicana</i> X <i>N. odorata</i>	KN22	Florida	Borsch & Summers 3214
<i>N. odorata</i> ssp. <i>odorata</i>		Maryland	Borsch, Hilu & Wiersema 2361
<i>N. odorata</i> ssp. <i>odorata</i>	11N	Florida	Borsch & Wilde 3128
<i>N. odorata</i> ssp. <i>odorata</i>	42N	Florida	Borsch & Wilde 3099
<i>N. odorata</i> ssp. <i>odorata</i>	33N	Florida	Borsch & Wilde 3101
<i>N. odorata</i> ssp. <i>odorata</i>	34N	Florida	Borsch & Wilde 3125
<i>N. odorata</i> ssp. <i>odorata</i>	37N	Georgia	Borsch & Wilde 3131
<i>N. odorata</i> ssp. <i>odorata</i>	38N	Georgia	Borsch & Wilde 3133
<i>N. odorata</i> ssp. <i>odorata</i>	39N	Georgia	Borsch & Wilde 3134
<i>N. odorata</i> ssp. <i>odorata</i>	40N	Georgia	Borsch & Wilde 3135
<i>N. odorata</i> ssp. <i>odorata</i>	KN7	Michigan	Borsch, Wiersema & Hellquist 3398
<i>N. odorata</i> ssp. <i>odorata</i>	KN10	Texas	Woods & Borsch KN0801
<i>N. odorata</i> ssp. <i>odorata</i>	KN11	Louisiana	Woods & Borsch KN0901
<i>N. odorata</i> ssp. <i>odorata</i>	KN12	Louisiana	Woods & Borsch KN1001
<i>N. odorata</i> ssp. <i>odorata</i>	KN18	South Carolina	Wiersema KN0601
<i>N. odorata</i> ssp. <i>odorata</i>	KN19	Virginia	Woods KN1201
<i>N. odorata</i> ssp. <i>odorata</i>	KN23	Vermont	Borsch, Wiersema & Hellquist 3331
<i>N. odorata</i> ssp. <i>odorata</i>	KN24	North Carolina	Woods KN1401
<i>N. odorata</i> ssp. <i>odorata</i>	KN26	Tennessee	Woods & Neves KN1701
<i>N. odorata</i> ssp. <i>odorata</i>	KN27	Vermont	Borsch, Wiersema & Hellquist 3322
<i>N. odorata</i> ssp. <i>odorata</i>	KN29	Vermont	Borsch, Wiersema & Hellquist 3330
<i>N. odorata</i> ssp. <i>odorata</i>	KN30	Florida	Borsch & Summers 3215
<i>N. odorata</i> ssp. <i>odorata</i>	KN32	Vermont	Borsch, Wiersema & Hellquist 3323
<i>N. odorata</i> ssp. <i>odorata</i>	KN33	Vermont	Borsch, Wiersema & Hellquist 3324
<i>N. odorata</i> ssp. <i>odorata</i>	KN16	Delaware	Wiersema KN0401
<i>N. odorata</i> ssp. <i>odorata</i>	KN25	Tennessee	Woods & Neves KN1501
<i>N. odorata</i> ssp. <i>odorata</i>	KN37	Virginia	Woods KN1301
<i>N. odorata</i> ssp. <i>odorata</i>		Michigan	Borsch & Wiersema 3400
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN28	Vermont	Borsch, Wiersema & Hellquist 3329
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN5	Wisconsin	Borsch, Wiersema & Hellquist 3396
<i>N. odorata</i> ssp. <i>tuberosa</i>		Vermont	Borsch, Wiersema & Hellquist 3326
<i>N. odorata</i> ssp. <i>tuberosa</i>		Manitoba, Canada	Borsch, Wiersema & Hellquist 3392
<i>N. odorata</i> ssp. <i>tuberosa</i>	1N	New York	Borsch
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN6	Manitoba, Canada	Borsch, Wiersema & Hellquist 3389
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN14	Michigan	Wiersema KN0201
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN15	Ohio	Wiersema KN0301
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN13	Pennsylvania	Wiersema KN0101
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN31	Vermont	Borsch, Wiersema & Hellquist 3325
<i>N. odorata</i> ssp. <i>tuberosa</i>	KN17	Ohio	Wiersema 2384
<i>N. odorata</i>	KN1	Michigan	Borsch & Wiersema 3399
<i>N. odorata</i>	KN3	Michigan	Borsch & Wiersema 3401
<i>N. odorata</i>	KN4	Michigan	Borsch & Wiersema 3402
<i>N. tuberosa</i> Paine		Ontario, Canada	Lectotype

Table 3.2. Qualitative and quantitative morphological characters measured for each accession.

Qualitative characters	Character States	Quantitative characters
1. Petiole stripes	1 = present; 0 = absent	1. Petiole diameter
2. Petiole hairs	1 = present; 0 = absent	2. Blade length/ Blade width
3. Sinus overlap	1 = present; 0 = absent	3. Blade sinus length/Blade length
4. Leaf apex	1 = retuse; 0 = rounded	4. Length of blade sinus
5. Lobe apex	1 = ovate; 0 = rounded	5. Number of leaf veins
6. Color leaf blade undersurface	2 = red; 1 = reddish green; 0 = green	6. Diameter of rhizome
7. Tubers	1 = present; 0 = absent	7. Diameter of peduncle
8. Sepal apex	1 = ovate; 0 = rounded	8. Length of sepal
9. Sepal veins	1 = apparent; 0 = not apparent	9. Width of sepal
10. Longest petal's widest point	2 = top 1/3; 1 = middle; 0 = bottom 1/3	10. Number of petals
11. Longest petal apex	1 = ovate; 0 = rounded	11. Length of the longest petal
12. Shortest petal widest point	2 = top 1/3; 1 = middle; 0 = bottom 1/3	12. Width of the longest petal
13. Shortest petal apex	1 = ovate; 0 = rounded	13. Length of shortest petal/ Width of shortest petal

Table 3.3 ISSR primers used on accessions of *N. odorata*, *N. mexicana* and *N. mexicana* x *N. odorata* putative hybrids. All primers listed were used on 10 accessions from a Tennessee population to determine intrapopulation variation in *N. odorata*. * = Primers used on interpopulation analysis.

Primer	Sequence	Within population variation
GACAv _{ar}	(GACA) ₄ GATA-CATA	yes
AGC ₄	(AGC) ₄ -CY	yes
GACA-GATA	(GACA-GATA) ₂	yes
844	(CT) ₈ -RC	yes
898	(CA) ₆ -RY	yes
814*	(CT) ₈ -TG	yes
901*	(GT) ₆ -YR	no
843*	(CT) ₈ -RA	no
CA8*	(CA) ₈ -RG	no
899*	(CA) ₆ -RG	no

Table 3.4 ANOVA analyses of the morphological variables that differ among the subspecies (ssp. *odorata* and ssp. *tuberosa*). R^2 = correlation coefficient and P = significance value obtained using a student's t-test for quantitative variables and a contingency table for qualitative variables.

Character	R^2	P
Petiole stripes	0.54	0.001
Blade length/ Blade width	0.22	0.008
Shortest petal length/Shortest petal width	0.13	0.01
Petiole hairs	0.16	0.03
Length of blade sinus	0.16	0.03
Lobe apex	0.2	0.05
Blade sinus length/ Blade length	0.12	0.06
Number of leaf veins	0.11	0.07

Table 3.5 The mean and standard deviation (SD) of leaf blade characters for *ssp. odorata* and *ssp. tuberosa* along with the results of a t-test. *= Moderately statistically significant ($P < 0.10$) using t-test.

	<i>ssp. odorata</i> (N=16)		<i>ssp. tuberosa</i> (N=13)	
	Mean	SD	Mean	SD
Blade Length/ Blade Width *	0.63	0.05	0.56	0.08
Blade Length	7.6	2.71	8.88	3.38
Blade Width	11.97	4.03	15.95	6.05
Blade Sinus *	5.16	1.66	6.71	2.61
No. Leaf Veins *	24.44	3.33	26.85	4.1

Table 3.6 Results of principal components analysis on eleven morphology characters from 26 accessions from *N. odorata*. Characters arranged in descending order according to loadings on first component.

	1	2	3
Eigenvalues	3.91	2.01	1.34
Component loadings			
Blade width	0.970	-0.100	0.037
Blade length	0.934	-0.126	0.080
Length of blade sinus	0.933	-0.105	0.210
Petiole diameter	0.858	0.079	-0.281
No. leaf veins	0.581	-0.145	0.106
Leaf apex	0.295	0.086	-0.606
Petiole hairs	0.187	0.753	-0.301
Lobe apex	0.127	0.243	0.814
Sinus overlap	0.010	0.670	-0.082
Color leaf blade undersurface	0.059	0.530	0.003
Petiole stripes	0.046	0.768	0.270
Percent of total variance explained	35.57	18.36	12.21

Table 3.7 Cross-validation summary with the linear discriminant function based on 26 accessions of *N. odorata*. Percentage of accessions correctly classified using quantitative characteristics of petiole diameter, blade length, blade width, sinus length, and number of leaf veins.

From subspecies	Classified in subspecies	
	<i>odorata</i>	<i>tuberosa</i>
<i>odorata</i>	71.43	28.57
<i>tuberosa</i>	33.33	66.67

Table 3.8 Comparison of ISSR primers used for interpopulation analysis on 43 accessions from *N. odorata*, *N. mexicana* and putative hybrids *N. mexicana* x *N. odorata*. The percent polymorphic bands was calculated as the number of polymorphic loci per group (*ssp. tuberosa*, *ssp. odorata*, *N. mexicana* and hybrids) divided by the total number of loci in the primer.

* = Primer that displayed intrapopulation variation.

Primer	# loci	# Genotypes	% bands polymorphic within:		
			<i>ssp. tuberosa</i> (N=13)	<i>ssp. odorata</i> (N= 24)	<i>N. mexicana</i> and hybrids (N= 6)
843	16	35	88	88	6
899	14	36	78.5	100	71
901	17	30	70	82	88
CA8	13	34	77	92	38
814*	12	41	83	92	75
Total	72	176			

Table 3.9 Analysis of molecular variance (AMOVA) based on ISSR markers excluding primer 814. The total data set contains three taxonomic groups (*ssp. odorata*, *ssp. tuberosa* and *N. mexicana* with *N. mexicana* x *N. odorata*) and 19 regions (which represent individual states). Statistics include sums of squared deviations (SSD), mean squared deviations (MSD), variance component estimates, and the percentages of the total variance contributed by each component.

Source of Variation	df	SSD	MSD	Variance component	% Total Variance
Among taxa	2	17.9	8.96	-0.34	-3.16
Among regions within taxa	20	239.7	12.0	1.5	13.97
Within regions	17	162.1	9.5	9.5	89.2

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Summary and Conclusions

The goals of this project were to determine the molecular and morphological variation between two subspecies of *N. odorata* (ssp. *odorata* and ssp. *tuberosa*) and the patterns of evolution of the species in relation to *N. mexicana*. Combining morphological and molecular analyses allowed us to determine the differentiation that exists between the subspecies.

First molecular analyses of data from the nuclear internal transcribed spacer (ITS) and the chloroplast *trnL-F* regions were performed to determine the variation present within and between species. A phylogeny from the ITS region resulted in a *N. mexicana* clade and a *N. odorata* clade each having 100% bootstrap support. The subspecies of *N. odorata* grouped into two separate clades, a ssp. *odorata* clade with 65% bootstrap support and a ssp. *tuberosa* clade with 55% bootstrap support. Hybrids between *N. mexicana* and *N. odorata* grouped with accessions of *N. mexicana* in the ITS phylogeny. Furthermore, the ITS region resulted in the presence of polymorphisms in *N. odorata* accessions. A matrix generated based on polymorphisms was subjected to SAHN clustering, principle coordinate (PCOA), and minimum spanning tree (MST) analyses. All analyses produced groups similar to clades found in the ITS phylogeny, showing some distinction between the subspecies. Additionally, several accessions classified morphologically as ssp. *tuberosa* and ssp. *odorata*, did not cluster into the groups representing each subspecies in the SAHN clustering, PCOA, MST and ITS phylogeny, and were considered possible hybrids. The geographic location of the hybrids suggests an ancient hybrid zone, where gene flow was occurring between the subspecies. Selected accessions were chosen from the ITS analyses for *trnL-F* amplification and sequencing. Previous work on this region revealed a subspecies-specific molecular marker (Borsch 2000). In the *trnL-F* region 16 accessions were examined and each correlated with their expected basepair except for one. In this accession the subspecies-specific marker did not match the original subspecies classification. Hybrids between *N. mexicana* and *N. odorata* also had a maternal sequence of *N. mexicana* based on *trnL-F* results.

The ITS region suggested an apparent speciation of *N. odorata* from *N. mexicana*, and probably ssp. *tuberosa* as the most likely subspecies to evolve from *N. mexicana*. The strongest evidence to support the speciation of ssp. *tuberosa* from *N. mexicana* was the presence of

eleven synapomorphies in the ITS region. Outcomes from the molecular evidence suggest a segregation of *ssp. odorata* and *ssp. tuberosa*, with limited gene flow occurring between the subspecies.

The second part of this project compared morphological characters and inter-simple sequence repeat (ISSR) markers to analyze each subspecies. Statistical methods using univariate and multivariate approaches were performed on 14 quantitative and 13 qualitative morphological characters. Accessions were classified to each subspecies based on the characteristics in the Flora of North America (Wiersema and Hellquist 1997). An analysis of variance (ANOVA) showed eight morphological characteristics to be moderately significant ($P < 0.10$), of which seven were leaf blade characteristics. Each subspecies had a distinctive leaf blade based on size, petiole stripes, and shape. The multivariate statistics performed on morphological characteristics were multivariate analysis of variance (MANOVA), discriminate analysis, SAHN clustering, principle component analysis (PCA), PCOA and MST analyses. SAHN clustering, MANOVA, PCOA and MST analyses resulted in no significant grouping based subspecies or geography. However, both discriminate analysis and PCA showed a segregation of the subspecies and emphasized the importance of quantitative leaf blade characteristics. Leaf blade characteristics were not previously used to by Wiersema and Hellquist (1997) discriminate between the subspecies. Therefore, we recommended using leaf blade size, petiole striping, and lobe apex shape to discriminate between the subspecies.

Molecular analysis using ISSR markers provided results similar to the ITS and *trnL-F* regions, and gave additional insight into the variation between subspecies. Within and among population variation was characterized with ISSR markers. Intrapopulation variation was assessed on ten accessions from a Tennessee population, and produced primers with and without variation. Interpopulation analyses were conducted on 43 accessions of *N. odorata* and *N. mexicana* using five primers, four showing no intrapopulation variation and one with intrapopulation variation. As expected, the primer having intrapopulation variation was highly variable among populations, and was excluded from subsequent analyses. ISSR markers among populations were highly variable between and within a species, evident by analysis of molecular variance (AMOVA) results. The matrix based on ISSR markers was analyzed with UPGMA, PCOA and MST. All analyses resulted in a separation of the species (*N. odorata* and *N.*

mexicana) and some segregation between the subspecies of *N. odorata*. Each subspecies resulted in three clusters in the UPGMA analysis, and some of the clusters were correlated with geography. Overall, both morphology and molecular characteristics supported the classification of ssp. *odorata* and ssp. *tuberosa*, but not their representation as distinct species as some had proposed, due to a weak segregation of characters.

Nymphaea odorata is a highly variable species both morphologically and in molecular characters. Both characteristics have given insights into speciation in *N. odorata*. All analyses conducted here, in which *N. mexicana* was included, depicted a clear separation between *N. mexicana* and *N. odorata*. At the species level in *Nymphaea*, molecular analyses clearly segregate each species. In contrast, the subspecies level resulted in moderate segregation, indicated by the lower bootstrap values in the ITS phylogeny. Analyses from the ITS region resulted in more synapomorphies between ssp. *tuberosa* and *N. mexicana*. Therefore, ssp. *tuberosa* may be the first subspecies to evolve from *N. mexicana* compared to ssp. *odorata*. In areas of sympatry, each subspecies has been kept somewhat morphologically and molecularly distinct. However, in areas of allopatry gene flow between the subspecies has occurred, making each subspecies unable to be genetically and morphologically distinct from one another.

Curriculum Vitae for Kristi Y. Woods

Education

Masters of Science
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University of Maryland
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Major: Environmental

Research Experience

- Research on speciation in water-lilies (*Nymphaea*) using molecular and morphological analyses with Dr. Khidir Hilu. 2000- 2003
- Conducted research at Smithsonian Environmental Research Center on ecological relationships of terrestrial orchids with Dr. Dennis Whigham. Summer 2000
- Polyploid research comparing physiological and genetic differences in several varieties of alfalfa (*Medicago*) with Dr. Kimberly Hunter. 1997-2000

Work Experience

- Graduate Teaching Assistant 2000- 2002
 - Lead plant taxonomy students through field and classroom labs.
 - Planned lectures and guided biology majors through laboratories at Virginia Tech.
- Biology Teacher for Upward Bound Program Summer 2001
 - Created and taught biology curriculums for a summer program.
 - Developed and executed labs with students.
 - Led classes and labs for grades 9-12.
- Collaborative Research Assistant for the Smithsonian Institute. Spring 2000
 - Formulated research on the genetic differences in Paw paw trees (*Asimina*).
 - Supervised and delegated work pertaining to the progress of the project.

Presentations

- July 2002: Presented "Speciation in the water-lily *Nymphaea odorata* (Nymphaeaceae): A molecular and morphological analysis of North American populations" at Evolution Society Meeting.

- March 2002: Presented poster entitled “Speciation in Water-Lilies: Evidence from Morphology and the Internal Transcribed Spacer (ITS)” at Virginia Tech Graduate Research Symposium.
- February 2002: Presented “Speciation a case study in the water-lily genus *Nymphaea*” at Virginia Tech Biology Dept. Botany Seminar.
- August 2000: Presented Orchid research at Smithsonian Environmental Research Center.
- Presented Alfalfa research at:
 - Evolution Society Meeting June 1999
 - Henson School of Science Symposium May 1999
 - National Conference of Undergraduate Research April 1998

Conferences Attended

- Evolution Society Meeting Champaign-Urbana, IL Summer 2002
- Botanical Society of America Albuquerque, NM Summer 2001
- Evolution Society Meeting Bloomington, IN Summer 2000
- Evolution Society Meeting Madison, WI Summer 1999
- National Conference of Undergraduate Research Rochester, NY Spring 1999
- National Conference of Undergraduate Research Salisbury, MD Spring 1998
- Botanical Society of America Baltimore, MD Summer 1998

Funding

- Graduate grants
 - National Science Foundation Graduate Fellowship; Amount requested \$16,000
 - Sigma Xi Grant; Amount requested and received \$600
 - International WaterLily Society Grant; Amount requested and received \$500
 - Virginia Academy of Science; Amount requested and received \$1250
 - Adkins Arboretum Grant; Amount requested \$2000
- 79 -
- Graduate Research Development Project (GRDP); Amount requested and received \$300
- Undergraduate grants
 - Dual Degree grant; Amount requested and received \$250
 - Henson School of Science; Amount requested and received \$375

Computer Skills

- Microsoft programs, JMP IN statistical analysis, PAUP, Kodak Digital Analysis, Gel Electrophoresis, SAS, NTSYS-pc, AMOVA and AMOVA-prep program, Fluent in both Mac and IBM formats.

Awards and Affiliations

- Member Botanical Society of America 2001-2003
- Member American Society of Plant Taxonomist 2001-2003
- Recipient of Young Botanist Award, Botanical Society of America 2000
- Recipient Who's Who Among Students in American Universities 1998
- Recipient of the Key Achievement Award 1998
- The National Dean's List 1997-2000
- Recipient of All American Scholar 1998, 1999
- Member of Sigma Xi, Research Honor Society 1998-2003