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# EVIDENCE FOR THE HYBRID ORIGIN OF *NUPHAR* × *RUBRODISCA* (NYMPHAEACEAE)[1]

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Plants intermediate in appearance between *Nuphar microphylla* and *N. variegata* (Nymphaeaceae) have long been assumed to be the result of hybridization. The evidence for this is based primarily on field observations of morphology, poor fruit production, close geographical proximity of presumed parent species, and limited pollen sterility data. Fertile populations of the same plants have also been documented. We employed multivariate analyses of morphology, pollen fertility studies, and random amplified polymorphic DNA (RAPD) markers to test the hypothesis that *Nuphar* × *rubrodisca* represents a natural interspecific hybrid between *N. microphylla* and *N. variegata*. Examination of 15 morphological characters demonstrated the intermediacy of *N.* × *rubrodisca* between *N. microphylla* and *N. variegata*, and the pollen data revealed a markedly lower mean pollen viability in *N.* × *rubrodisca* (23%) compared to the other two species (91 and 86%, respectively). Eight 10-mer primers produced 13 species-specific RAPD markers for *N. microphylla* and nine for *N. variegata*, with all 22 markers present in *N.* × *rubrodisca*. The data from RAPDs are concordant with morphology in implicating *N. microphylla* and *N. variegata* as parents of *N.* × *rubrodisca*.

**Key words:** hybridization; morphology; *Nuphar*; Nymphaeaceae; random amplified polymorphic DNA (RAPD)

Instances of hybridization in aquatic angiosperms remain poorly documented, with few studies presenting even basic statistical or molecular evidence (Les and Philbrick, 1993). Hybridization has been investigated in <20% of aquatic angiosperm genera. From this sample, persuasive evidence of natural hybridization has been presented for 57% of these genera (Les and Philbrick, 1993). Such studies are important because documentation of natural hybrids is the first step to understanding the significance of hybridization in aquatic angiosperms.

Several reports of interspecific hybridization exist for *Nuphar* (Les and Philbrick, 1993). *Nuphar* species occupy a diversity of freshwater habitats including ponds, lakes, streams, and slow-moving rivers. *Nuphar* is distributed in temperate regions of North America from Alaska to Newfoundland south to northeastern Mexico and Cuba. In the Old World, *Nuphar* occurs in temperate Eurasia, throughout Europe south to northern Africa, west to the Kamchatka Peninsula, Russia, and Japan (Beal, 1956).

*Nuphar* species are taxonomically difficult. Although the most recently published revision of *Nuphar* combined all North American taxa under the single species name *N. lutea* (L.) Sm. (Beal, 1956), the present taxonomic reevaluation using morphological and molecular data has failed to support this concept (Padgett, Les, and Crow, 1996). For instance, two North American species with greatly overlapping ranges, *N. variegata* Durand and *N. microphylla* (Pers.) Fern., are readily distinguishable. *Nuphar variegata* has more sepals, a greater number of stigmatic rays, larger fruits, longer anthers, and is generally larger overall than *N. microphylla* (Fassett, 1957; Voss, 1985).

The conspicuous differences of these species led early taxonomists to suspect the occurrence of hybridization between the two following the discovery of what they regarded as morphologically intermediate specimens. Peck (1881) named *Nuphar advena* (Ait.) Ait. f. var. *hybrida* Peck from plants intermediate in morphology between *N. variegata* and *N. microphylla* and suggested its possible hybrid origin from these species. Fletcher (1881) similarly regarded intermediate plants with poorly developed fruits near Ottawa, Canada, as putative hybrids between *N. variegata* and *N. microphylla*. Specimens sent to R. Caspary by Fletcher also were determined as hybrids between *N. variegata* and *N. microphylla* because of their apparent morphological intermediacy and deformed pollen grains (Fletcher, 1882, 1883; Macoun, 1883). Other intermediate plants lacking fruit development and with poorly developed pollen were later observed growing in the proximity of *N. variegata* and *N. microphylla* in the Adirondacks of New York (Morong, 1886).

However, several Vermont populations were known that were similar in appearance to the putative hybrids, but possessed well-developed fruits and viable pollen. Furthermore, they occurred at a considerable distance from populations of either presumed parental species (Morong, 1886). Morong (1886) described these fertile plants as a distinct species, *N. rubrodisca* Morong. Peck

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(1899) later elevated his *N. advena* var. *hybrida* to species status (as *Nymphaea hybrida* (Peck) Peck ). Others, however, retained the hybrid status (e.g., *Nymphaea* × *fletcheri* Lawson) for sterile specimens (Lawson, 1888). (Note that the genus name *Nymphaea* L. was applied to *Nuphar* prior to the conservation of the latter generic name.) Gray (1895) treated all putative hybrids of *Nuphar variegata* and *N. microphylla* in North America as *Nuphar advena* var. *minus* Morong. He regarded this variety as a partially to fully fertile "established hybrid" possibly introgressing with the parental species. Miller and Standley (1912) rejected the hybrid origin of *Nuphar rubrodisca*, a taxon that they believed to be a distinct species with low fertility that was compensated by asexual reproduction. They suggested that more evidence was necessary before a hybrid origin of *N. rubrodisca* could be adequately demonstrated. Contemporary taxonomists continue to differ on whether these plants should be recognized as a distinct species (Hellquist and Crow, 1984; Wiersema and Hellquist, 1997) or merely as hybrids with no distinct nomenclatural status (Voss, 1985; Gleason and Cronquist, 1991).

The present study was undertaken to reevaluate the taxonomic status of *Nuphar* × *rubrodisca* by testing the hypothesis of its hybrid origin and parentage. This was done by (1) examining the geographical distribution of the taxon in comparison to those of the putative parents *N. variegata* and *N. microphylla*, (2) using uni- and multivariate statistics to quantify and evaluate the suggested morphological intermediacy of this taxon with respect to its putative parents, (3) evaluating the fertility of *N.* × *rubrodisca* from pollen stainability data, and (4) using molecular markers to ascertain whether *N.* × *rubrodisca* exhibits additivity of genetic markers that are unique to each of the putative parental species. It was anticipated that the compilation of these data should provide suitable information to determine whether *Nuphar* × *rubrodisca* is a hybrid between *N. variegata* and *N. microphylla*, or a species distinct from both.

## MATERIALS AND METHODS

***Geographical distributions***—Geographical distributions of *Nuphar variegata*, *N. microphylla*, and *N.* x *rubrodisca* were determined from 281 specimens examined from 15 herbaria (BM, DAO, FLAS, IA, MT, NASC, NHA, NCSC, P, TUFT, UC, UNA, US, V, VT). The geographical locality of each specimen was plotted on North American base maps to obtain estimates of the distribution ranges for each taxon (see Appendix 1 for citation of representative specimens).

***Morphological analysis***—Morphological data were obtained from 216 of the herbarium specimens examined for geographical distributions (Appendix 1). Five vegetative and ten reproductive characters were scored for 77 operational taxonomic units (OTUs) of *Nuphar microphylla*, 69 OTUs of *N.* × *rubrodisca*, and 70 OTUs of *N. variegata*. For each taxon, means and standard deviations were calculated for all variables using SYSTAT (version 5.0) software (Wilkinson, 1990). Character means were compared among the three taxa using an analysis of variance (ANOVA) and were evaluated for significant differences by performing a Tukey HSD post hoc test. Data were then arranged in a rectangular matrix for input in principal components analyses (PCA). Unscorable data were treated as missing. The matrix included OTUs of *N. microphylla*, *N. variegata,* and *N.* × *rubrodisca* (216 OTUs × 15 characters; 52% missing data). The PCA was performed using NTSYS-pc (version 1.80) software (Rohlf, 1993). Data were standardized by dividing the difference of each variable and its mean by the standard deviation. Product moment correlations were computed among the standardized variables, the first three principal component axes were extracted from the correlation matrix, and OTUs were projected upon each axis. Results of the PCA were depicted as a scatterplot representing the superimposition of components I and II. The percentage variation explained by each eigenvalue and correlations of variables with eigenvectors were tabulated.

***Pollen viability analysis***—Pollen viability from 30 accessions (ten of each taxon) was estimated from the percentage stainability of 100+ randomly selected grains taken from herbarium specimens (Appendix 2). Anthers were removed from herbarium sheets and dissected in aniline blue/lactophenol following Radford et al. (1974). Means (percentage viability) and standard deviations were calculated as above using SYSTAT. Differences among means were determined by ANOVA and Tukey tests as described above.

***RAPD analysis***—Total genomic DNA was extracted from young, submersed leaf tissue representing three accesssions of *Nuphar microphylla*, five accessions of *N.* × *rubrodisca*, and five accessions of *N. variegata* (Appendix 3) using a modified CTAB procedure (Doyle and Doyle, 1987). Amplifications were carried out in 25-μL reactions consisting of 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 0.005% Tween 20, 0.005% NP-40, 2.0 mmol/L $MgCl_2$, 100 μmol/L each of dATP, dCTP, dGTP, and dTTP, 15 ng of primer, 1 μL (~20 ng) DNA, and 0.6 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Connecticut). Eight random 10-mer oligodeoxynucleotide primers (OPF-1, OPF-2, OPF-3, OPF-4, OPF-5, OPF-6, OPF-8, OPF-10; Operon Technologies, Alameda, California) were used to amplify DNAs (each reaction used a single primer). A thermocycle profile of 1 min at 94°C, 2 min at 36°C, and 2 min at 72°C was carried out for 45 cycles followed by a 7-min final extension cycle at 72°C.

Amplification products were separated electrophoretically on 1.5% agarose gels in 0.5x tris-borate-EDTA buffer and were visualized by staining with ethidium bromide. Band sizes were estimated using a standard marker consisting of *Bst*E II-digested Lambda DNA. A preliminary screening was conducted that included several additional *Nuphar* species (*N. japonica*, *N. lutea*, *N. advena*, and *N. polysepala*) to identify RAPD markers specific for either *N. microphylla* or *N. variegata*. Non specific markers, as well as markers that occurred in all three taxa (*N. microphylla*, *N. variegata*, *N.* × *rubrodisca*), were excluded from the analysis. RAPD data were summarized as the number of markers shared by *N.* × *rubrodisca* and either *N. microphylla* or *N. variegata*. Band reproducibility was verified by comparing several replicated amplifications for each marker scored.

## RESULTS

***Geographical distribution***—The geographical distribution of *Nuphar variegata* is wider than that of *N. microphylla*, but the ranges of both species broadly overlap in northeastern North America. *Nuphar* × *rubrodisca* occurs almost entirely in the zone of overlap between *N. variegata* and *N. microphylla* (Fig. 1).

***Morphological analysis***—*Nuphar microphylla* and *N. variegata* differed significantly ($P < 0.05$) for mean values of all characters compared (Table 1). For *N.* × *rubrodisca*, the means of all characters were intermediate between those of *N. microphylla* and *N. variegata*. Means of 13 characters for *N.* × *rubrodisca* differed significantly ($P < 0.05$) from both *N. microphylla* and *N. variegata* (Table 1). The first three eigenvalues explained 90% of the total variance (79.0, 7.6, and 3.3, respectively) in the

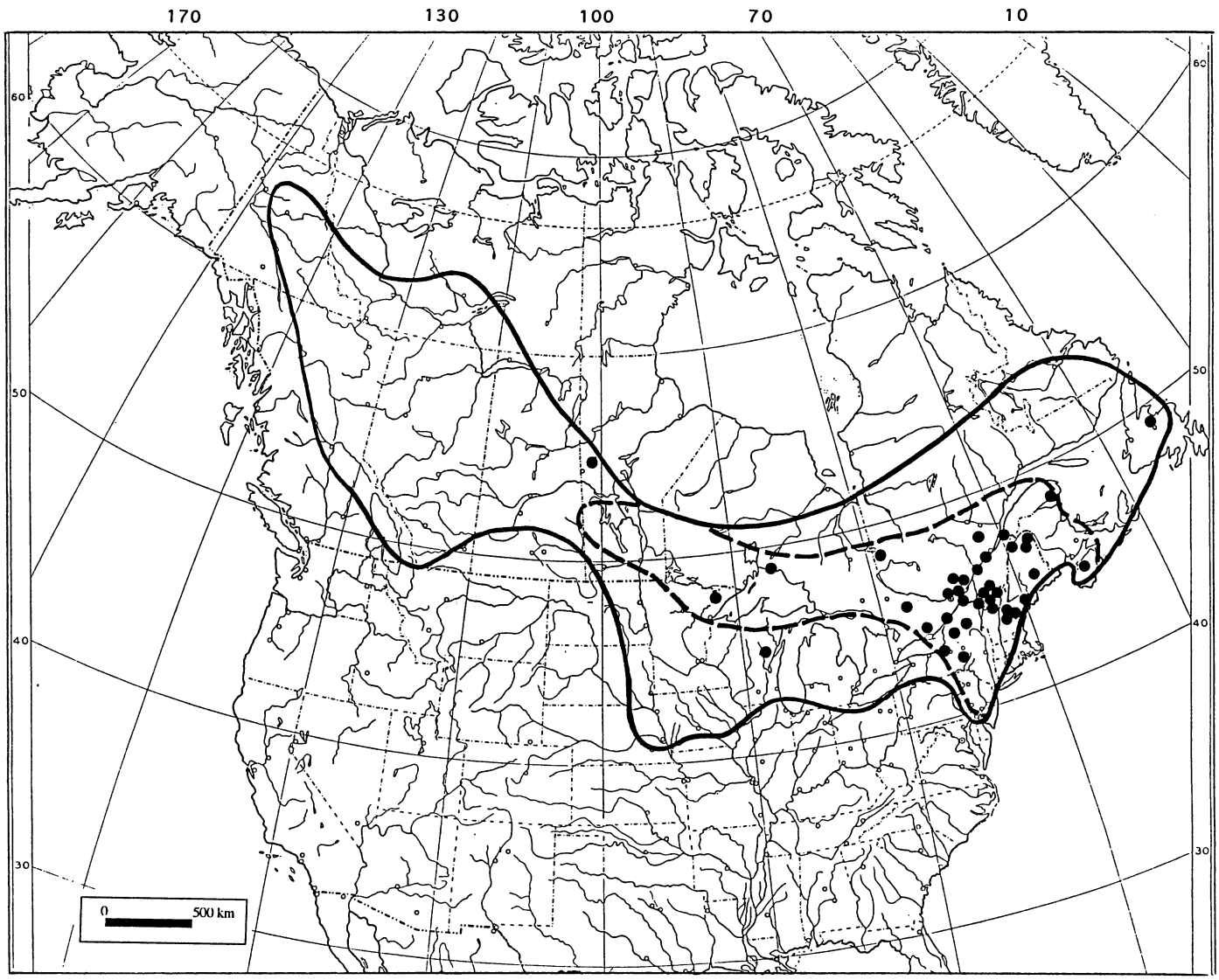



Fig. 1. Distribution of *Nuphar microphylla* (dashed line), *N.* × *rubrodisca* (circles), and *N. variegata* (solid line).

PCA analysis. Variables with the highest correlations to the first PCA axis were flower width, number of lateral leaf veins and flower length. Fruit width, fruit length, and the length of the leaf sinus showed the highest correlations with the second PCA axis. Anther length, leaf sinus length, and the number of stigmatic rays showed the highest correlations with the third PCA axis (Table 2). The PCA clustered the OTUs of *N.* × *rubrodisca* essentially between those of *N. microphylla* and *N. variegata*, but closer overall to *N. microphylla* (Fig. 2).

***Pollen viability***—Pollen viability ranged from 13 to 99% among the three taxa compared. The highest mean pollen viability occurred in *N. microphylla* (91%), but did not differ significantly from that of *N. variegata* (86%)(Table 3). Mean pollen viability of *N.* × *rubrodisca* was substantially reduced (23%) and differed significantly ($P < 0.001$) from both *N. microphylla* and *N. variegata* (Table 3).

***RAPD analysis***—Eight random RAPD primers yielded 13 reliable markers that were specific to *Nuphar microphylla* and nine markers that were exclusive to *N. variegata* (Fig. 3). All 22 of these markers were detected in the individuals of *N.* × *rubrodisca* surveyed (Table 4).

## DISCUSSION

Gottlieb (1972) discussed several criteria for testing whether a particular diploid taxon originated through hybridization. These features include a geographical distribution in the region of parental sympatry, morphological intermediacy in several characters, partial fertility, and biochemical additivity. Although no single criterion can provide a clear means for testing a hypothesis of hybridization, each criterion that can be fulfilled provides a higher level of support for a hybrid origin (Gottlieb, 1972). It is also essential that these "hybrid" criteria be evaluated carefully because features such as morpholog-

TABLE 1. Comparison of *Nuphar microphylla, N.* × *rubrodisca,* and *N. variegata* for 15 morphological characters. Sample size (*N*), mean, and standard deviation (SD) are given. Superscripts summarize the results of a Tukey HSD multiple comparisons test. Species with the same letters do not differ significantly for that character ($P < 0.05$). Characters are numbered as they are discussed in the text.

| Character | *N. microphylla* | | *N.* × *rubrodisca* | | *N. variegata* | |
|---|---|---|---|---|---|---|
| | *N* | Mean (SD) | *N* | Mean (SD) | *N* | Mean (SD) |
| Leaf | | | | | | |
| 1. Length (cm) | 55 | 7.19 (1.95)[a] | 52 | 12.28 (3.14)[b] | 39 | 21.90 (5.54)[c] |
| 2. Width (cm) | 55 | 5.20 (1.16)[a] | 52 | 8.91 (1.98)[b] | 39 | 15.94 (3.83)[c] |
| 3. Sinus (cm) | 55 | 2.95 (0.73)[a] | 52 | 4.11 (1.08)[b] | 38 | 7.06 (2.43)[c] |
| 4. Petiole diameter (mm) | 45 | 1.29 (0.43)[a] | 47 | 2.65 (0.77)[b] | 36 | 6.68 (1.56)[c] |
| 5. Lateral veins (no.) | 51 | 8.62 (2.08)[a] | 31 | 16.16 (2.03)[b] | 28 | 26.64 (5.42)[c] |
| Flower | | | | | | |
| 6. Length (cm) | 29 | 1.26 (0.23)[a] | 37 | 1.80 (0.31)[b] | 36 | 2.84 (0.36)[c] |
| 7. Width (cm) | 30 | 1.69 (0.43)[a] | 37 | 2.28 (0.38)[b] | 36 | 3.59 (0.37)[c] |
| 8. Anther length (mm) | 29 | 2.00 (0.62)[a] | 42 | 3.26 (0.62)[b] | 44 | 5.72 (1.26)[c] |
| 9. Stigmatic disk (mm) | 27 | 3.93 (0.96)[a] | 42 | 6.86 (1.06)[b] | 45 | 11.44 (3.16)[c] |
| 10. Stigmatic rays (no.) | 29 | 8.06 (1.22)[a] | 44 | 10.93 (1.75)[b] | 45 | 14.71 (2.77)[c] |
| 11. Peduncle diam. (mm) | 30 | 2.58 (0.61)[a] | 31 | 3.77 (0.58)[b] | 35 | 6.27 (1.40)[c] |
| Fruit | | | | | | |
| 12. Length (cm) | 21 | 1.73 (0.60)[a] | 12 | 1.91 (0.48)[a,b] | 27 | 3.13 (0.60)[b] |
| 13. Width (cm) | 20 | 1.45 (0.29)[a] | 12 | 1.69 (0.57)[a] | 27 | 2.65 (0.77)[b] |
| 14. Neck diameter (mm) | 19 | 2.12 (0.46)[a] | 12 | 5.79 (2.14)[b] | 24 | 11.62 (3.39)[c] |
| 15. Stigmatic disk (mm) | 14 | 3.42 (0.73)[a] | 12 | 7.70 (1.76)[b] | 26 | 13.61 (2.85)[c] |

ical intermediacy and sterility are not invariably associated with hybrids (Rieseberg, 1995) and may result from entirely separate processes (Les and Philbrick, 1993). Nevertheless, the Gottlieb (1972) criteria provide a convenient avenue for discussing data that bear on the putative hybrid nature of *Nuphar* × *rubrodisca.*

The distribution of *Nuphar* × *rubrodisca* satisfies the first criterion of occupying a zone of parental sympatry. Except for three accessions, *N.* × *rubrodisca* occurs within the region of overlap of the ranges of *N. microphylla* and *N. variegata* (Fig. 1). Populations of *N.* × *rubrodisca* apparently do not extend beyond the distributional limits of either putative parent. All three taxa occupy similar habitats in lakes, ponds, and sluggish watercourses. *Nuphar* × *rubrodisca* often occurs with either *N. microphylla* or *N. variegata* (occasionally both) in the same body of water. In Lake Champlain, where all three taxa are common, *N. microphylla* tends to colonize deeper waters with *N. variegata* in shallow shoreline waters; *N.* × *rubrodisca* occurs in depths more or less between the two species (D. Padgett, Southwest Missouri State University, personal observation).

Plants of intermediate vegetative morphology have long been cited as evidence of interspecific hybridization between *Nuphar microphylla* and *N. variegata*, although most reports have been anecdotal rather than empirically founded. Even though hybrids often express parental rather than intermediate characters (Rieseberg, 1995), the numerical evaluation of both vegetative and floral fea-

TABLE 2. Correlations of variables with each of the first three axes extracted from a principal components analysis. Superscripts identify those variables with the three highest correlations to each axis.

| Variable | PC I | PC II | PC III |
|---|---|---|---|
| 1. Leaf length | −0.91479 | 0.32286 | −0.11006 |
| 2. Leaf width | −0.92767 | 0.30474 | −0.08937 |
| 3. Leaf sinus | −0.83430 | 0.36650[3] | −0.35379[2] |
| 4. Petiole diameter | −0.91568 | 0.26058 | 0.04452 |
| 5. Lateral vein no. | −0.94302[2] | 0.21296 | 0.03220 |
| 6. Flower length | −0.93851[3] | 0.04166 | 0.10297 |
| 7. Flower width | −0.95942[1] | 0.04883 | 0.05997 |
| 8. Anther length | −0.85970 | 0.15266 | 0.45588[1] |
| 9. Stigmatic disk | −0.85695 | −0.28863 | −0.15764 |
| 10. Stigmatic rays | −0.83511 | −0.15426 | −0.20081[3] |
| 11. Peduncle diameter | −0.86614 | 0.00333 | 0.17863 |
| 12. Fruit length | −0.85272 | −0.44725[2] | 0.09636 |
| 13. Fruit width | −0.75731 | −0.51872[1] | −0.13549 |
| 14. Fruit neck diameter | −0.91429 | −0.20324 | −0.01400 |
| 15. Fruit stigmatic disk | −0.93690 | −0.22108 | 0.04546 |

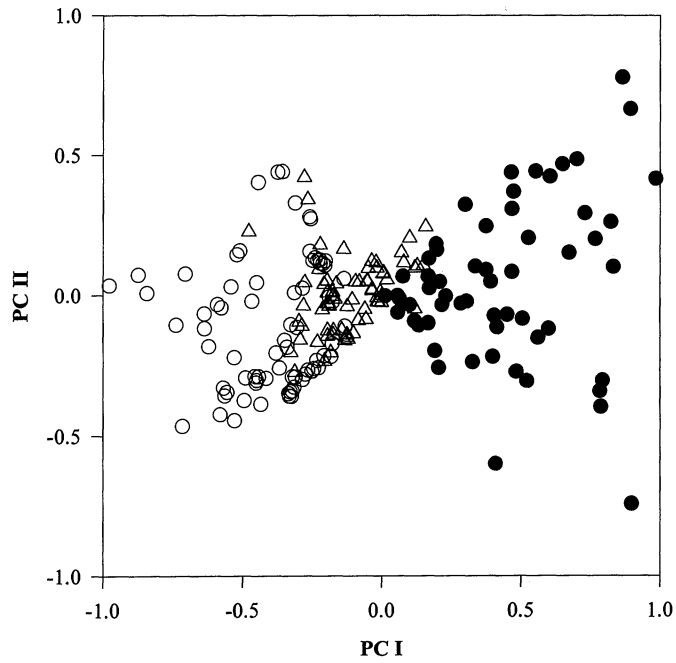



Fig. 2. Principal components plot (first two components) of *Nuphar microphylla* (open circles), *N.* × *rubrodisca* (triangles), and *N. variegata* (solid circles) based on morphological variables.

TABLE 3. Summary of aniline blue pollen viability analysis. $N = 10$ for all species. Means with different superscripts differ significantly ($P = 0.01$).

| Species | Viability range (%) | Mean % viability (SD) |
|---|---|---|
| *Nuphar microphylla* | 82–98 | 91.10[a] (4.93) |
| *Nuphar* × *rubrodisca* | 13–50 | 23.20[b] (11.40) |
| *Nuphar variegata* | 69–99 | 86.00[a] (12.22) |

tures indicated a consistent pattern of morphological intermediacy for the majority of traits examined. In a selected set of 15 characters, those of *N.* × *rubrodisca* were all quantitatively intermediate, being smaller than those of *N. variegata* and larger than those of *N. microphylla* (Table 1). The morphological intermediacy of *N.* × *rubrodisca* is compelling given that both putative parents represent discrete size extremes for these features (Fig. 2). The OTUs of *N.* × *rubrodisca* clustered between the three-dimensional character space of the two putative parents, although were somewhat closer associated to *N. microphylla* (Fig. 2). Thus, the statistical analyses corroborate what various authors have long suggested, i.e., *N.* × *rubrodisca* is intermediate morphologically to *N. microphylla* and *N. variegata*. Gottlieb (1972) viewed morphological intermediacy as a primary criterion of hybridity.

The morphological intermediacy of *Nuphar* × *rubrodisca* is also evident in features (qualitative or overlapping parental traits) that were excluded from the formal numerical analysis. *Nuphar microphylla* typically has five sepals, whereas *N. variegata* has six. Not surprisingly, *N.* × *rubrodisca* has either five or six sepals, depending on the population. Intermediacy can also be observed in several qualitative traits. The stigmatic disk of *N. microphylla* is dark red, in *N. variegata* it is yellow, and in *N.* × *rubrodisca* it is bright red. The margins of the stigmatic disk differ among the three taxa in a similar fashion—deeply lobed in *N. microphylla*, essentially entire to undulate in *N. variegata*, and crenate in *N.* × *rubrodisca*. Additionally, Britton (1901) reported that the number of stamen rows differed among these taxa, with five to seven rows in *N. variegata*, three or four rows in *N. microphylla*, and about five in *N.* × *rubrodisca*.

The fertility of *Nuphar* × *rubrodisca* is reduced markedly in comparison to both *N. microphylla* and *N. variegata*. The pollen viability of both putative parental species did not differ significantly, and did not fall below 69% (Table 3). Pollen viability of *N.* × *rubrodisca* ranged from 13 to 50%, but was significantly lower than the mean of either putative parent (Table 3). Pollen stainability data indicate that *N.* × *rubrodisca* satisfies the criterion of "partial fertility", but also that fertility may be retained at fairly high levels in some instances. Variable fertility among populations of *N.* × *rubrodisca* has been observed previously (Morong, 1886). Although low pollen fertility is evident in many populations of *N.* × *rubrodisca*, little quantitative data on fruit production or seed viability exist. Morong (1886) found only a single fruit with two or three seeds in a survey of New York populations of *N.* × *rubrodisca*. In Vermont, however, he found an abundance of fruit, but gave no indication of

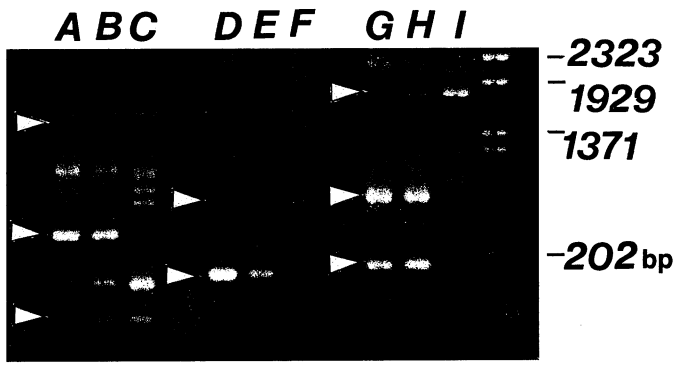



Fig. 3. Amplified bands of *Nuphar* DNA with three different 10-mer primers. Lanes C, F, and I are *N. variegata* (DNA templates from *Padgett 491*, *488*, and *491*, respectively). Lanes B, E, and H are *N.* × *rubrodisca* (*Padgett 481*, *481*, and *479*, respectively). Lanes A, D, and G are *N. microphylla* (*Padgett 397*). Refer to Appendix 3 for information regarding collection numbers. Bands in lanes A-C were produced from primer OPF-4, lanes D-F from primer OPF-3, and G-I from primer OPF-2. Arrows indicate species-specific bands of either *N. variegata* or *N. microphylla* present in *N.* × *rubrodisca*.

seed number (Morong, 1886). Four fruits collected recently from Vermont contained only five, six, eight, and nine seeds each, with numerous undeveloped ovules (D. Padgett, Southwest Missouri State University, unpublished data). Compared to the numerous seeds typically found on herbarium specimens of *N. microphylla* and *N. variegata*, the level of seed set in *N.* × *rubrodisca* appears to be extremely low. Some herbarium specimens of *N.* × *rubrodisca* also contain fruits with numerous, well-developed seeds, although their viability remains to be demonstrated.

RAPD data clearly indicated molecular additivity in *Nuphar* × *rubrodisca*. The putative parental species *N. microphylla* and *N. variegata* each possessed several unique RAPD markers from a survey of eight primers (Table 4). The surveyed plants of *N.* × *rubrodisca* combined all 22 markers that distinguished the putative parental species (Table 4; Fig. 3). Because a wider survey of other *Nuphar* species indicated that these genetic markers were apparently restricted to the two putative parental species, it is difficult to accept any other explanation for their shared presence in *N.* × *rubrodisca* other than as a result of hybridization. Thus, the RAPD data

TABLE 4. Summary of RAPD analysis of *Nuphar* × *rubrodisca* and putative parents. Total number of markers unique to each parent yet showing additivity in *N.* × *rubrodisca* are given.

| Primer | Total number of additive markers in *N.* × *rubrodisca* | Markers unique to *N. microphylla* shared with *N.* × *rubrodisca* | Markers unique to *N. variegata* shared with *N.* × *rubrodisca* |
|---|---|---|---|
| OPF-1 | 2 | 1 | 1 |
| OPF-2 | 2 | 1 | 1 |
| OPF-3 | 2 | 1 | 1 |
| OPF-4 | 2 | 1 | 1 |
| OPF-5 | 6 | 4 | 2 |
| OPF-6 | 2 | 1 | 1 |
| OPF-8 | 3 | 2 | 1 |
| OPF-10 | 3 | 2 | 1 |
| All primers | 22 | 13 | 9 |

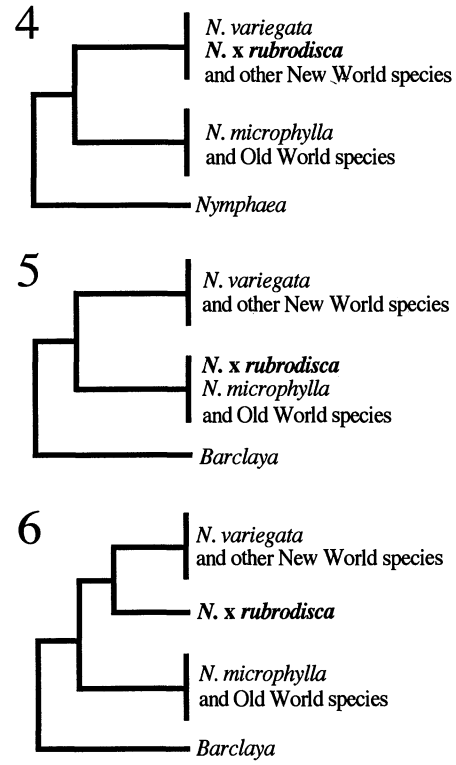
provide compelling evidence that *N.* x *rubrodisca* indeed represents an interspecific hybrid of *N. microphylla* and *N. variegata.*

All four criteria of hybridity that were addressed in this study have been demonstrated. Although other criteria remain untested (e.g., synthetic hybrid production), we believe that the evidence presented demonstrates a high degree of confidence for the hybrid origin of *Nuphar* × *rubrodisca.* Additional features of *N.* × *rubrodisca* are also consistent with this interpretation. *Nuphar* × *rubrodisca* is frequently found in the same body of water as *N. microphylla* and/or *N. variegata,* and these are most likely insect-pollinated based on floral studies of other related species (Schneider and Moore, 1977; Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997). All three species have bisexual flowers and are likely to be outcrossing. Like all *Nuphar* species, *N.* × *rubrodisca* is strongly rhizomatous, which would allow for an almost indefinite perpetuation of sterile hybrid offspring. The chromosome number ($2n = 34$) of both parental species (and for all *Nuphar* species examined) is identical and constant (Les and Philbrick, 1993). All of these factors can be viewed as conditions that would not deter hybridization.

Although the present evidence strongly suggests that *Nuphar* × *rubrodisca* is a hybrid derived from *N. microphylla* and *N. variegata,* it is difficult to determine whether this taxon should be recognized as a discrete hybrid species. There is some evidence that would support the discrete hybrid species status of *N.* × *rubrodisca. Nuphar* × *rubrodisca* is distinct morphologically from *N. microphylla* and *N. variegata,* at least for 13 of the characters evaluated statistically (Table 1). Pollen fertility and seed production are high in some populations of *N.* × *rubrodisca.* The presence of *N.* × *rubrodisca* in localities where neither parent occurs indicates that some effective dispersal and establishment of new populations is possible (although extirpation of the parental species cannot be ruled out in such instances). *Nuphar* × *rubrodisca* proliferates vegetatively, and its establishment within aquatic systems may be the result of drifting rhizome fragments. Additionally, waterfowl transport of small rhizomes may also take place. It remains to be demonstrated whether *N.* × *rubrodisca* can propagate sexually through self-fertilization.

We have not observed any evidence to indicate that *Nuphar* × *rubrodisca* has diverged from either *N. microphylla* or *N. variegata.* Morphology and RAPD markers show intermediacy or additivity rather than any features unique to *N.* × *rubrodisca* that might indicate the presence of a functional isolating barrier between it and the other two species. Instead, the observations presented strongly suggest that hybrids between *N. microphylla* and *N. variegata* may occur repeatedly, and that *N.* × *rubrodisca* does not appear to represent a stabilized hybrid or a monophyletic assemblage derived from a single ancestral hybridization event. In accordance with this interpretation, we designate these hybrids nomenclaturally as *N.* × *rubrodisca,* a "nothospecies" (Greuter et al., 1994).

Evidence from phylogenetic analyses has been shown to increase the suspicion and/or detection of the hybrid nature of taxa (Rieseberg and Brunsfeld, 1992; Rieseberg, 1995). As part of a larger phylogenetic study of




Figs. 4–6. Simplified phylogenetic trees of *Nuphar* and placement of *N.* × *rubrodisca.* **4.** Tree derived from nucleotide sequence data of internal transcribed spacer regions (nrDNA). **5.** Tree derived from nucleotide sequence data of *matK* gene (cpDNA). **6.** Tree derived from combined nuclear and chloroplast data.

*Nuphar,* parsimony analyses of morphological and molecular data have indicated a New World/Old World divergence largely congruent with current geographical distributions (Padgett, 1996, 1997). Among the two lineages, *N. microphylla* is positioned within the Old World lineage and *N. variegata* within the New World lineage (Figs. 4–6). Analyses of nuclear and chloroplast DNA sequences have offered different alliances for *N.* × *rubrodisca* with either lineage. Analysis of nuclear DNA places the *N.* × *rubrodisca* in the "New World" clade with *N. variegata* (Fig. 4), while chloroplast DNA positions this taxon in the "Old World" clade with *N. microphylla* (Fig. 5). The phylogenetic position of *N.* × *rubrodisca* in a combined analysis is as a sister taxon to the "New World" lineage (Fig. 6). The discordance between the independent phylogenies favors the hybrid origin of *N.* × *rubrodisca* and illustrates the effect of hybridization on phylogeny. This outcome also emphasizes the need to survey both nuclear and organellar genomes in phylogenetic studies (Swofford et al., 1996).

There are several other reports of hybridization in *Nuphar* (Beal, 1956; Wood, 1959). *Nuphar* × *intermedia* is a natural European hybrid between *N. lutea* and *N. pumila* (Timm) DC. (Caspary, 1869, 1870, 1879; Heslop-Harrison, 1953). As with *N.* × *rubrodisca,* individuals of *N.* × *intermedia* show morphological intermediacy and reduced (~15%) pollen fertility (Heslop-Harrison, 1975). Artificial crosses of *N. lutea* and *N. pumila* yielded hy-

brids that closely resembled *N.* × *intermedia* and possessed highly sterile pollen (Caspary, 1869, 1870). Interestingly, the geographical range of *N.* × *intermedia* extends beyond the northern limit of either *N. lutea* or *N. pumila* and the hybrid reportedly ripens its fruits the earliest of the three (Kerner von Marilaun, 1895; Heslop-Harrison, 1953).

*Nuphar* × *interfluitans* Fern. was described by Fernald (1942) as a hybrid between *N. advena* and *N. sagittifolia* Walt. It also displays morphological intermediacy, is highly sterile (lacks fruits), and occurs within the proximity of the putative parents. Experimental $F_1$ hybrids between *N. advena* and *N. sagittifolia* yielded only 17.4% fruit set and poor seedling viability (DePoe and Beal, 1969). More study of the plants from the localities where *N. sagittifolia* and *N. advena* overlap is needed to permit a more accurate interpretation of this putative hybrid. The hybrids described in each of these instances appear to represent spontaneous $F_1$ plants such as those that we recognize as *N.* × *rubrodisca*. All have also been designated nomenclaturally as nothospecies rather than as stabilized hybrid species.

Additional *Nuphar* hybrids are suspected (e.g., *N. variegata* × *N. polysepala*; *N. variegata* × *N. advena*), but these have not been studied in any detail (Wood, 1959; Brayshaw, 1993). *Nuphar oguraensis* Miki var. *saijoensis* Shimoda (Shimoda, 1991) was initially perceived to be a hybrid between *N. japonica* and *N. oguraensis* (M. Shimoda, Towa Kagaku Co., personal communication) and deserves renewed study. Likewise, the occurrence of a number of taxonomically "difficult" intermediate plants have suggested the possibility of hybridization between *N. oguraensis* and both *N. japonica* and *N. subintegerrimum* (Casp.) Makino (Y. Kadono, Kobe University, personal communication). A more detailed evaluation of these complexes may turn up further evidence of hybridization in *Nuphar*.

Hybridization may occur frequently in *Nuphar*. Here we have provided evidence to support the interpretation of *N.* × *rubrodisca* as a hybrid nothospecies that spontaneously results from the natural crossing of *N. microphylla* and *N. variegata*. Most other putative *Nuphar* hybrids that have been studied in any detail display similar characteristics. Our conclusions fail to corroborate Miller and Standley (1912) who did not accept the hybrid origin of *N.* × *rubrodisca*. Multivariate analyses indicate that flower size and the number of leaf veins are the most effective characters for separating *N. microphylla*, *N. variegata*, and *N.* × *rubrodisca*, with fruit size and leaf sinus length of secondary importance (Table 2).

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APPENDIX 1. Voucher specimens of *Nuphar* material used in morphological analyses.

*N. microphylla*

CANADA. **Manitoba**: Parker Bog, *Parker 85-775* (DAO). **New Brunswick**: Fredericton, *Fowler* s.n., 30 Jul 1892 (US); Madawaska Co., *Roberts & Bateman 64-3220* (MT); Northumberland Co., *Webster & Fielding 178* (DAO); Restigauche Co., McDougall Lake, *Roberts & Drury 63-1882* (DAO); St. John's River, *Hay 98* (BM). **Ontario**: Corry Lake, *Breitung 6818* (MT); Glengarry Co., west of Alexandria, *Dore 21444* (DAO); Kenora District, Lake of the Woods, *Macins 39-67* (DAO); Lac James, Chalk River, *Vladykon v-3* (DAO); Renfrew Co., Westmeath, *Darbyshire & Dore 1639* (DAO); Buckanan, Ottawa River, *Breitung 7060* (DAO); Thunder Bay District, Black Sturgeon Lake, *Garton 12532* (DAO). **Quebec**: Baie des Chaleurs, Comte de Gaspé, *Marie-Victorin et al. 44324A* (MT); Becancour, *Houle 76-992* (MT); Iberville Co., Henryville, *Adrien 2092* (MT); Nominingue, Labelle, *Roy 1693* (MT); Oka, *Dansereau 194* (MT); Rigaud, Comte de Vaudreuil, *Roy 3343* (DAO); Sainte-Rose, Laval, *Marie-Victorin & Rolland-Germain 44307* (DAO); St. Eustache, *Victorin s.n.* (UC).

U.S.A. **Connecticut**: New Haven Co., Milford, *Eames 1798* (CONN). **Maine**: Androscoggin Co.: Auburn, 13 Jul 1875 (NHA). Aroostook Co.: Round Pond T13, R12, *Lawe 19445* (NHA); St John River, *Fernald* s.n. (CONN); St. Francis, *Fernald 10* (NHA); St. Francis, *Evans 16001* (NHA); Littleton-Houlton line, *Hellquist 13842* (NASC); Presque Isle, *Chamberlain 2126* (UC); Presque Isle, *Hellquist et al. 13873* (NASC); Washburn, *Hellquist 5971* (NASC); Washburn, *Crow 2941* (NHA); Leanwell, Girard Pond, *Norton 8275* (NHA); Houlton, *Crow et al. 2932* (NHA); Oxford Co., Gilead, *Moore 1119* (UC); Somerset Co., Township VI, *St. John & Nichols 2291* (US); Washington Co., Edmunds, *Pike et al. s.n.* (NHA); York Co., Alfred, *Cleonique-Joseph 6165* (MT). **Massachusetts**: Berkshire Co., Sheffield, *Weatherbee 3743* (NHA); Hampden Co., Holyoke, *Lumsden s.n.* (UC); Middlesex Co., Concord, Sudbury River, *Worthen s.n.* (US). **Minnesota**: Lake Co., Basswood Lake, *Lakela 8960* (DAO); St. Louis Co.: Palo, *Lakela 9174* (DAO); Lac La Croix, *Lakela 16597* (DAO); **New York**: Cortland Co., Willow Grove, *Wiegand 6430* (NCSC); Herkimer Co., Gray, *House s.n.* (US); McDonough, *Coville s.n.* (US); Saratoga Co., Coveville, *Muenscher & Lindsey 3316* (UC); St. Lawrence Co., Canton, *Phelps s.n.* (NCSC); Lonesome Bay, *Muenscher & Maguire 2254* (UC); Ulster Co., Stoney Ridge, *Manning s.n.* (FLAS); Washington Co., Whitehall, Lake Champlain, *Carpenter s.n.* (VT). **Vermont**: Addison Co.: Addison, *Wodehouse s.n.* (VT); Ferrisburg, *Hellquist 5665* (NASC); Ferrisburg, Lewis Creek, *Padgett 480* (NHA); Ferrisburg, Little Otter Creek, 16 Aug 1896 (VT); Ferrisburg, *Grout s.n.*, 16 Aug 1896 (VT); Ferrisburg, *Eggleston 2543* (VT); Hancock, *Dutton s.n.* (VT); Hancock, Lost Pleiad Pond, 18 Jul 1879 (VT); Caledonia Co.: East Barnet, *Blanchard s.n.* (UC); Danville, *Grout s.n.* (VT); Chittenden Co.: Burlington, *Flynn s.n.* (VT), Shelburne, *Pringle s.n.,* 24 Jul 1862 (VT); Shelburne, *Pringle s.n.,* 15 Jul 1878 (VT); Shelburne, La Platte River, *Padgett 482* (NHA); Colchester, *Zika 1760* (VT); Colchester, *Flynn s.n.* (VT); Franklin Co., Highgate, *Jesup s.n.* (NHA); Orleans Co.: Barton, Crystal Lake, *Hellquist 5082* (NASC); Irasburg, *Hellquist 2766* (NASC); Irasburg *Hellquist 2765* (NASC); Washington Co.: East Montpelier, *Tower 6891* (VT); *Pringle s.n.,* 23 Feb 1909 (UC).

*N.* × *rubrodisca*

CANADA. **Manitoba**: S. of Sheridan, *Foster 73* (DAO). **New Brunswick**: Northumberland Co., Pond near Waye's Bridge, *Webster & Fielding 213* (DAO); Sackville, *Dore 45-1039* (DAO). **Newfoundland**: Grand Falls, *Fernald & Wiegand 5417* (US). **Nova Scotia**: Springfield, *Smith et al. 2536* (DAO). **Ontario**: Algonquin Park, Red Pine Lake, *Macoun 23261* (US); Torbolton, Constance Creek, *Senn 1941* (DAO); Marmora, *Dore 1944* (DAO); Ottawa, *Fletcher 96.5* (DAO); Ottawa, *Fletcher s.n.,* 3 Aug 1881(DAO); Ottawa, *Fletcher s.n.,* Jul 1902 (US); Schreiber, Lake Rongie, *Hellquist 2251* (NASC). Quebec. d'Hebecourt, Lac Duparquet, *Bergeron et al. 81-82* (MT); Pontiac, *Marie-Victorin et al. 43995* (DAO); Brigham's Creek, Ottawa River, *Fletcher s.n.,* 1 Aug 1882 (US); Chandler, *Marie-Victorin et al. 44553* (UC); Compton Co., Dell Lake, *Calder 1174* (DAO); Duparquet, *Baldwin & Breitung 4209* (MT); Gatineau Co.: Aylwin Trop, *Jenkins et al.* 3646 (DAO); Hull, *Dore & Calder 47-1102* (DAO); Hull, *Scott 97* (DAO); Hull, *Thomson 1924* (BM); Hull, *Rolland 16173* (UC); Nominique, Labelle, *Roy 1368* (DAO); Ile Perrot, Montreal Island, *Dore & Cody 13941* (DAO); Templeton, *Calder et al. 1638* (DAO); Rigaud, *Roy 4005* (DAO); Rigaud, *Roy 3999* (DAO); St. Francis River, *Eggleston 3010* (ANS).

USA. **Maine**: Aroostook Co.: Cross Lake, *Kendall s.n.,* 12 Jul 1903 (US); Garfield, *Norton 16609* (NHA); Fort Fairfield, *Hellquist 7745* (NASC); Fort Fairfield, *Padgett 490* (NHA); Washburn, *Hellquist 7659* (NASC). **Minnesota**: St. Louis Co.: Crooked Lake, near Curtain Falls, *Lakela 11589* (DAO); Namakan Lake, *Lakela 14439* (DAO); Clear Lake, southwest of Ely, *Lakela 17873* (DAO). **New York**: Little Tupper Lake, *Morong s.n.,* 3–9 Aug 1884 (VT); Adirondacks, *Morong s.n.,* Aug 1884 (BM); Newcomb, *House 9068* (UC); Newcomb, *House 15375* (MT); Lisbon, *Phelps 445* (US); Onondago Co., Fabius, *House s.n.,* Aug 1903 (US); *Caspary s.n.* (IA); **Vermont**: Addison Co.: Ferrisburg: Dead Creek, *Hellquist 5502* (NASC); Dead Creek, *Hellquist 5503* (NHA); Lake Champlain, *Hellquist 5462* (NASC); Lake Champlain, *Morong s.n.,* 11 Aug 1885 (BM); Lewis Creek, *Hellquist 15610* (NASC); Lewis Creek, *Padgett 481* (NHA); mouth of Lewis Creek, *Cooley s.n.,* 23 Jul 1966 (VT); mouth of Otter Creek, *Hellquist 5558* (NASC); Little Otter Creek, *Crow & Hellquist 3046* (NHA); Little Otter Creek, *Padgett 479* (NHA); *Brainerd s.n.,* 7 Aug 1879 (VT); North Ferrisburg, Lake Champlain, *Hellquist 13202* (NASC); Orwell, Lake Champlain, *Padgett 398* (NHA). Caledonia Co.: Barnet, *Hellquist 6452* (NASC); Danville, *Grout s.n.*, 5 Jul 1894 (VT); Peacham, *Hellquist 9783* (NASC). Chittenden Co.: Colchester, *Griffin s.n.* (VT); Colchester, *Flynn s.n.,* 26 Jun 1899 (VT); Shelburne, La Platte River, *Pringle s.n.,* 24 Jul 1879 (VT). Essex Co.: Brunswick, *Fernald 1023* (VT); Canaan, *Hellquist 6258* (NASC). Lamoille Co.,Wolcott, *Hellquist 13090* (NASC); Orleans Co., Westmore, *Hellquist 2606* (NASC); Rutland Co., east of Benson, *Hellquist & Popp 15917* (NASC); Lake Champlain, *Pringle s.n.,* 24 Jul 1879 (US); Groton, White Mountain Pond, 23 Jun 1902 (VT). **Wisconsin**: Washington, *Hotchkiss & Koehler 4308* (US).

APPENDIX 1. Continued.

*N. variegata*

CANADA. **Alberta**: Ma-Me-O Beach, *Turner 7429* (MT). **British Columbia**: Prince George, *Brayshaw 5089* (V); Swan Lake, *Brayshaw 5282* (V); Jaffray, *Brayshaw s.n.*, 3 Jul 1972 (V). **Newfoundland**: Lewisporte District, *Crow et al. 82-430* (NHA). **Northwest Territory**: northeast of Fort Resolution, Simpson Island Group, *Preble 242* (US). **Nova Scotia**: Cape Breton, Scatari Island, *Smith et al. 5239* (DAO); Sable Island, *St. John 1288* (US). **Ontario**: Frontenac Co., between Hart Lake and Lake Opinicon, *Soper 5588* (MT); Glengarry Co., northeast of Summerstown, *Gogo 274* (DAO); Carleton Co.: Torbolton, *Senn 1941* (MT); mouth of Jock River *Cody & Calder 625* (BM); Strathroy, *Wood s.n.,* 29 May 1934 (DAO); Point Dubuc, *Dubois 193* (UC). **Quebec**: Chenaux, *Morency 557* (MT); Saint-Adolphe, *Rolland-Germain 2851* (MT); Senneterre, *Baldwin & Breitung 4390* (MT); Gatineau Park, Brown Lake, *Gillett & Seaborn 13662* (V); Nomingue, *Lucien 424* (US); Nouveau-Liverpool, Chaudiere, *Rouleau 627* (MT); Chertsey, *Hamel & Forget h-19* (MT); Buckingham, *Cleonique 7259* (MT); Laurentides National Park, Lac Tremblay, *Gauthier 11262* (MT); Smoky Hills, *Dutilly & Lepage 11161* (MT); Weedon, *Hamel & Brisson 15211* (DAO). **Saskatchewan**: Cumberland House, *Argus 4014* (DAO); Lake Athabasca, east of William River, *Argus 341-62* (DAO). **Yukon Territory**: northwest of Mayo, *Calder 4056* (US).

U.S.A. **Connecticut**: Hartford Co., Windsor, *Clark 1898* (CONN); Tolland Co.: Mansfield, *Anderson s.n.,* 28 May 1994 (CONN); Union, Brown's Brook, *Mehrhoff 12815* (CONN). **Iowa**: Allamakee Co., near New Albin, *Jolstead s.n.,* 29 Jun 1933 (UC); Cedar Co., west of Cedar Valley, *Fay 704* (IA); Delaware, *Rickey 1224* (IA). Emmet Co.: Cheever, *Thorne 13013* (IA). Hamilton Co.: Goose Lake, *Johnson 51* (IA). **Maine**: Aroostook Co.: Fischer Lake, Fort Fairfield, *Padgett 489* (NHA); Leanwell, *Norton 8377* (NHA); Pettiquaggamas Lake, *Fernald 9* (UC); Portage Lake at Mesquito Brook, Portage Lake, *Padgett 487* (NHA); Presque Isle, north of Westfield, Echoe Lake, *Padgett 484* (NHA); Cumberland Co., Brunswick, *Swallow s.n.* (NHA); Sagadahoc Co., Phippsburg, *Norton 9381* (NHA). **Massachusetts**: Norfolk Co., Wellesley, *Steiger s.n.,* 4 Sept 1936 (NHA); Norfolk Co., Canton, *Judd 1640* (FLAS). **Michigan**: Alger Co. Sable Lake, *Dodge s.n.,* 26 Aug 1916 (US); Allegan Co., Swan Lake, *Wight 5* (US); Keweenaw Co., La Belle, *Richards 4052* (DAO). **Minnesota**: Anoka Co., Cedar Creek Bog, *Buell 665* (NCSC); Cass Co., Big Thunder Lake, *Richards 1087* (F); Morrison Co., Lake Alexander, *Sparrow 001* (UNA); St. Louis Co., Rainy Lake, *Lakela 14716* (DAO). **Nebraska**: Greenwood, *Williams s.n.,* 16 Jul 1890 (US). **New Hampshire**: Belknap Co., Squam Lake, *Allaire 124a* (NHA); Carroll Co., Tamworth, *Hellquist 3529* (NHA); Coos Co., Shelburne, *Deane s.n.,* 11 Aug 1926 (NHA); Cumberland Co., Cape Elizabeth, *Norton 6526* (NHA); Rockingham Co., Windham, *Harris 175* (NHA). **New Jersey**: Tom's River, *Lyon s.n.,* 11 Aug 1902 (US); Spring Lake, *Lyon s.n.,* 30 Jul 1902 (US). **New York**: "New York", *Eaton s.n.,* 1828 (PH); Dutchess Co., Rudd Pond, *Elias 6776* (NHA); Jefferson Co., South Bay, *Robinson & Maxon 74* (US); Madison Co., Peterboro, *Miller s.n.,* 22 May 1904 (US); Washington Co., Carter Lake, *Muenscher & Lindsey 3306* (UC). **Pennsylvania**: Pocono Plateau, *Harshberger s.n.* (US). **Vermont**: Addison Co., Orwell, Lake Champlain, *Padgett 399* (NHA); Bennington Co., Sunderland, *Atwood s.n.,* 29 Jul 1969 (VT); Chittenden Co., Colchester, *Charette 216* (VT). **Wisconsin**: Barron Co., Pickerel Lake, *Davenport 1376* (UNA).

APPENDIX 2. *Nuphar* specimens used in pollen viability analysis.

*N. microphylla*

CANADA. **Ontario**: Whitewater Lake, *Soper 3602* (DAO). **Quebec**: Chambly Co., Chambly Canal, *DuBoulay 2715* (DAO); Nominingue, *Ducharme 375* (DAO).

U.S.A. **Maine**: Franklin Co., Jerusalem, *Norton 13193* (NHA); Aroostook Co., St. Francis, *Fernald 10* (NHA). **Massachusetts**: Berkshire Co., Sheffield, *Churchill s.n.* (NHA); York Co., near Fredericton, *Bassett & Mulligan 2865* (DAO). **Vermont**: Franklin Co., Highgate, *Jesup s.n.* (NHA); Addison Co., Ferrisburg, *Padgett 480* (NHA); Chittenden Co., Shelburne, *Padgett 482* (NHA).

*N.* × *rubrodisca*

CANADA. **Ontario**, Ottawa River, Brigham's Creek, *Fletcher s.n.* (US).

U.S.A. **Maine**: Aroostook Co., Fort Fairfield, *Padgett 490* (NHA); Allegosh Falls, *Lawe 19446* (NHA); Garfield, *Norton 16609* (NHA); Cumberland Co.: Sabago Lake, *Norton 6527* (NHA); Kennebec Co.: Mt. Vernon, *Norton 16800* (NHA). **New York**, Herkimer Co., Wilmurt Lake, *House s.n.* (US). **Vermont**: Addison Co., Ferrisburg, *Padgett 479* (NHA), Ferrisburg, *Padgett 481* (NHA); Caledonia Co., Barnet, *Hellquist 6452* (NASC).

*N. variegata*

CANADA. **Newfoundland**: Fortune-Hermitage District, Seal Cove, *Crow & Hellquist 86-83* (NHA).

U.S.A. **Maine**: Aroostook Co., Presque Isle, *Padgett 484* (NHA), Saint Francis, *Fernald 9* (NHA); Knox Co., Isle Au Haut, *Wise 453* (NHA). **Massachusetts:**, Berkshire Co., Stockbridge, *Padgett 474* (NHA). **New Hampshire**: Carroll Co., Wolfeboro, *H. E. S. s.n.* (NHA); Hillsborough Co., Goffstown, *Batchelder s.n.* (NHA); Strafford Co., Barrington, *Philbrick 790* (NHA). **Vermont**: Rutland Co., Sudbury, *Padgett 477* (NHA); Addison Co., Ferrisburg, *Padgett 478* (NHA).

APPENDIX 3. Sources of *Nuphar* DNA for RAPD analysis. Voucher specimens deposited at NHA.

*N. microphylla*

U.S.A. **Vermont:** Addison Co., Hancock, *Padgett 397*; Ferrisburg, *Padgett 480;* Chittenden Co., Shelburne, *Padgett 482.*

*N.* × *rubrodisca*

U.S.A. **Maine**: Aroostook Co., Fort Farfield, *Padgett 490.* **Vermont**: Addison Co., Orwell, *Padgett 398*; Ferrisburg, *Padgett 479*; Ferrisburg, *Padgett 481*; Orleans Co., Coventry, *Padgett 483.*

*N. variegata*

U.S.A. **Maine**: Aroostook Co., Sinclair (T17 R4 WELS), *Padgett 485*; Portage Lake, *Padgett 488.* **Massachusetts**: Berkshire Co., Stockbridge, *Padgett 474.* **New Hampshire**: Rockingham Co., Rye, *Padgett 491.* **Vermont**: Rutland Co., Sudbury, *Padgett 477.*