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PHYLOGENETIC RELATIONSHIPS IN *NUPHAR* (NYMPHAEACEAE): EVIDENCE FROM MORPHOLOGY, CHLOROPLAST DNA, AND NUCLEAR RIBOSOMAL DNA[1]

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The genus *Nuphar* consists of yellow-flowered waterlilies and is widely distributed in north-temperate bodies of water. Despite regular taxonomic evaluation of these plants, no explicit phylogenetic hypotheses have been proposed for the genus. We investigated phylogenetic relationships in *Nuphar* using morphology and sequences of the chloroplast gene *matK* and of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. Two major lineages within *Nuphar* are consistently resolved with the morphological and molecular data sets. One lineage comprises New World taxa and the other represents a primarily Old World lineage. Relationships within the major lineages were poorly resolved by morphology and ITS, yet certain relationships were elucidated by all analyses. Most notable is the strong support for a monophyletic lineage of dwarf taxa and the alliance of the North American *N. microphylla* with the Eurasian taxa. Minor discordance between the independent cladograms is accounted for by hybridization. The common taxonomic practice of uniting all North American and Eurasian taxa under one species is not supported phylogenetically.

Key words: ITS; *matK*; morphology; *Nuphar*; Nymphaeaceae; phylogeny; waterlilies.

Nuphar Sm. is a widespread genus of freshwater ponds and slow streams in the temperate Northern Hemisphere. Contemporary analyses using anato-morphological and chloroplast sequence data firmly associate *Nuphar* with the Nymphaeaceae (Ito, 1987; Les, Garvin, and Wimpee, 1991; Moseley, Schneider, and Williamson, 1993; Les, Schneider, and Padgett, 1997), supporting a long-established taxonomic tradition (Salisbury, 1806; Caspary, 1891; Cronquist, 1981). Recent phylogenetic estimates place *Nuphar* as most basal in the family and indicate the south Asian genus *Barclaya* to be its closest living relative (Les et al., in press). Interestingly, *Nuphar* is the only genus in the family lacking tropical species.

Species of *Nuphar* share several characters that are unique in the Nymphaeaceae and provide evidence for the monophyly of the genus, including a superior gynoecium, abaxial petal nectaries, echinate and anasulcate pollen, emergent fruit maturation, and presence of only a single (outer) satellite vascular bundle in peduncles (Les et al., in press). Because of this combination of characters, Kerner (1891), Nakai (1943), and Takhtajan (1997) proposed that *Nuphar* should be recognized as a separate monogeneric family, but this opinion has been largely ignored.

At the intrageneric level, *Nuphar* is among the most taxonomically troublesome genera in the Nymphaeaceae. Existing treatments (e.g., Morong, 1886; Harz, 1893; Schuster, 1907; Miller and Standley, 1912; Heslop-Harrison, 1955; Beal, 1956) focused mostly on a regional scale and are generally discordant. The most unparalleled classification of the genus was proposed by Beal (1956). The great morphological variability, evidence of hybridization, and uniform chromosome number among then-recognized species led Beal (1956) to treat these 22–25 taxa as one species with nine subspecies. Thus, all *Nuphar* occurring in both North America and Europe were classified as *N. lutea* (L.) Sm. In addition to his polymorphic concept of *N. lutea*, Beal (1955) recognized a second species, *N. japonica* DC., from Japan. Although Beal's (1956) revision met with much dissent (e.g., Sculthorpe, 1967; Hultén, 1971; Voss, 1985; Crow and Hellquist, in press), it remained popular in North America and was adopted in several regional floras and manuals (e.g., Calder and Taylor, 1968; Correll and Correll, 1972, Godfrey and Wooten, 1981; Rhoads and Klein, 1993). Despite the wealth of systematic attention the genus has received, there have been no specific hypotheses of phylogenetic relationships proposed for *Nuphar*. Consequently, precise evolutionary relationships within the genus remain enigmatic.

The genus *Nuphar* is delimited here to comprise 15 taxa recognized tentatively at the rank of species: *N. advena* (Ait.) Ait. f., *N.* × *intermedia* Ledeb., *N. japonica* DC., *N. lutea* (L.) Sm., *N. microphylla* (Pers.) Fern., *N. oguraensis* Miki, *N. orbiculata* (Small) Mill. & Standl., *N. ozarkana* (Mill. & Standl.) Standl., *N. polysepala* En-

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TABLE 1. Characters and character states used in the phylogenetic analysis of *Nuphar* morphology. All multistate characters were unordered.

1. Sepal number: 0 = 5, 1 = 6, 2 = 9
2. Sepal color (adaxial): 0 = green, 1 = red
3. Anther length/filament length: 0 = 0.2–0.7, 1 = 1.0–2.4
4. Anther color: 0 = yellow, 1 = purple
5. Stigmatic disk margin: 0 = lobed, 1 = entire
6. Stigmatic disk color: 0 = yellow/green, 1 = dark red, 2 = brown
7. Stigmatic disk size (disk/fruit diameter): 0 = <0.45, 1 = >0.45
8. Fruit shape: 0 = urceolate, 1 = ovoid
9. Constriction below stigmatic disk: 0 = <9 mm, 1 = >9 mm
10. Constriction/fruit diameter: 0 = <0.25, 1 = >0.25
11. Fruit surface: 0 = smooth, 1 = ribbed
12. Leaf habit: 0 = floating, 1 = emergent, 2 = submersed
13. Leaf blade length: 0 = >15 cm, 1 = < 12 cm
14. Leaf blade shape (length/width): 0 = 1, 1 = 1.5, 2 = 2, 3 = 2.5, 4= >4
15. Blade sinus size (sinus length/blade length): 0 = 0.10–0.25, 1 = 0.26–0.35, 2 = 0.36–0.55
16. Petiole shape (cross-section): 0 = terete, 1 = trigonous, 2 = flattened and winged, 3 = flattened
17. Petiole anatomy: 0 = reticulate arrangement of lacunae, 1 = central lacuna

gelm., *N. pumila* (Timm) DC., *N.* × *rubrodisca* Morong, *N. sagittifolia* (Walt.) Pursh, *N. sinensis* Hand.-Mazz., *N. ulvacea* (Mill. & Standl.) Standl., and *N. variegata* Durand. These 15 taxa, adopted from generally accepted taxonomies (e.g., Heslop-Harrison, 1955; Wiersema and Hellquist, 1997), reflect the complete morphological and geographical variation found within the genus (see Padgett, 1997). The objectives of this study were to present a comprehensive phylogenetic reconstruction of *Nuphar* based on three independent data sets (morphology, chloroplast DNA, and nuclear DNA) and to compare and evaluate the resulting hypotheses of phylogenetic relationships.

MATERIALS AND METHODS

Morphology—Morphological features were surveyed for 13 of the 15 taxa, excluding *N.* × *rubrodisca* and *N.* × *intermedia* (the intermediate morphology of these hybrid taxa confounded character scoring). The characters for cladistic analysis of morphology were assessed from living plants in the field or herbarium specimens. Seventeen characters were selected for analysis because of their presence in most taxa, unequivocal scoring, and potential for phylogenetic informativeness (Table 1). Preliminary studies of both pollen and seed morphology failed to discern discrete characters between taxa (D. Padgett, unpublished data), hence the data set lacks features of these particular elements.

The morphological characters included in the analysis are described below. Enclosed in parentheses is the character number indicated in Table 1. All multistate characters were treated as unordered. Table 2 contains the data matrix.

SEPAL NUMBER (1). The outgroup *Barclaya* and all Eurasian species of *Nuphar* share five sepals. In North America, most *Nuphar* species have six sepals. Exceptions include the North American *N. microphylla*, with five sepals, and *N. polysepala*, typically with nine sepals. There are local variations in sepal number, but these are interpreted as aberrant variations and are rarely encountered.

SEPAL COLOR (2). In general, the abaxial sepal surface in *Nuphar* is yellow. The base of the adaxial sepal surface is green in most species, usually progressing to yellow towards the apex. In *N. ozarkana* and *N. variegata* the adaxial coloration is red to dark purple.

ANTHER/FILAMENT LENGTH RATIO (3). There are two distinct stamen types found in *Nuphar*: anthers equaling or longer than the filaments (most North American taxa) and anthers shorter than the filaments (all Eurasian taxa).

ANTHER COLOR (4). The color of the pollen sacs and surrounding connective tissue varies from yellow (most species) to purple (as in *N. polysepala*).

STIGMATIC DISK MARGIN (5). Stigmatic disks of most *Nuphar* species have essentially an entire margin. In contrast, several species have a distinctly lobed disk margin.

STIGMATIC DISK COLOR (6). Immature pistils among most *Nuphar* species have yellow stigmatic disks. When mature, these disks turn green, but remain somewhat yellowish. Dark carmine-colored disks are found among flowers and fruits of *N. microphylla*.

STIGMATIC DISK SIZE (7). Statistical analysis of *Nuphar* taxa (Padgett, 1997) indicates two size classes of stigmatic disk diameter ratios, relative to the diameter of the mature ovary (disk/fruit diameter). These ratio classes are represented by two discrete character states: broad (>0.45) and narrow (<0.45).

FRUIT SHAPE (8). Mature *Nuphar* are either urceolate or ovoid. Urceolate fruits are usually smaller in size, with a well-defined ovary (''flaggon-shaped'' in the sense of Heslop-Harrison [1955]). Ovoid fruits are larger, and more columnar in appearance.

CONSTRICTION BELOW STIGMATIC DISK (9). Beal (1956) rec-

TABLE 2. Matrix indicating distribution of character states used in morphology-based phylogenetic analysis of *Nuphar* and *Barclaya*. Character descriptions are in Table 1.

	Character number																
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
N. advena	1	0	1	0	1	0	1	1	1	1	1	1	0	1	2	0	0
N. ozarkana	1	1	1	0	1	0	1	1	1	1	1	1	0	1	2	0	0
N. ulvacea	1	0	1	0	1	0	1	1	1	1	1	0	0	3	1	0	0
N. orbiculata	1	0	1	0	1	0	1	1	1	1	1	0	0	0	2	0	0
N. sagittifolia	1	0	1	0	1	0	1	1	1	1	1	0	0	4	0	0	0
N. variegata	1	1	1	0	1	0	1	1	1	1	1	0	0	1	2	2	0
N. polysepala	2	0	1	1	1	0	1	1	1	1	1	0	0	1	2	0	0
N. microphylla	0	0	0	0	0	1	0	0	0	0	0	0	1	1	3	3	0
N. pumila	0	0	0	0	0	0	0	0	0	0	0	0	1	1	3	3	0
N. sinensis	0	0	0	0	0	0	0	0	0	0	0	0	1	1	3	3	0
N. oguraensis	0	0	0	0	0	0	0	0	0	0	0	0	1	1	3	3	1
N. japonica	0	0	0	0	0	0	0	0	0	0	0	1	0	2	1	0	0
N. lutea	0	0	0	0	1	0	0	0	0	0	0	0	0	1	2	1	0
B. rotundifolia	0	1	0&1	?	1	2	1	0	?	?	0	2	0	0	2	0	0

ognized Eurasian *Nuphar lutea* taxa as characterized by a strongly constricted style. Morphometric analyses disclose two discrete size classes of the constriction width (Padgett, 1997) corresponding to either a narrow (2–6 mm) or broad constriction (9–22 mm).

CONSTRICTION/FRUIT DIAMETER RATIO (10). Analysis of constriction widths relative to overall fruit diameters clearly indicates two ratio classes, here treated as discrete character states: constrictions less than one-quarter of the ovary width (<0.25) and constrictions nearly half as wide or greater (>0.40) than the ovary width (Padgett, 1997). When the constriction below the disk is narrow, the constricted region is usually elongated. This is conspicuous in early fruit maturation with the stigmatic disk raised above the sepals.

FRUIT SURFACE (11). The surface texture of mature ovary walls varies from smooth (as in *Nuphar lutea*) to vertically ribbed with distinct grooves (e.g., *N. variegata*). Occasional furrowing on fruits of smooth ovary taxa is subtle and restricted to just below the stigmatic disk.

LEAF HABIT (12). Although all *Nuphar* species possess submersed basal leaves, exposed leaves are either floating or emergent. Floating lamina occur most commonly in the genus.

LEAF BLADE LENGTH (13). Blade length varies within species and populations, yet several species maintain small-sized leaves when compared to other taxa. The small-leaved taxa are commonly referred to as dwarf *Nuphar*. Character states (<12 cm or >15 cm) reflect a conspicuous morphological gap distinguishing these small-leaved taxa from larger leaved species (Padgett, 1997).

LEAF LENGTH/WIDTH RATIO (14). Blade shape was emphasized by Miller and Standley (1912) and Beal (1956) to distinguish taxa in *Nuphar*. Exposed blades can be ovate (1:1.5), orbicular (1:1), or narrowly lanceolate (1:>4).

BLADE SINUS/LENGTH RATIO (15). Basal sinus length relative to total blade length has been used as a reliable diagnostic character in taxonomic keys and descriptions of *Nuphar* (Beal, 1956; Wiersema and Hellquist, 1997). Most species have a basal sinus length that is about one-third of the blade length. Taxa with elongated leaves possess shorter basal lobes and therefore much shallower sinuses. The diminutive taxa exhibit deeper sinuses in comparison to blade lengths.

PETIOLE SHAPE (16). Despite some minor local variation, petiole shape remains constant within *Nuphar* taxa. Observed cross-sectional shapes of petioles include terete, dorsally flattened (''winged''), trigonous, and dorsally flattened but unwinged variations.

PETIOLE ANATOMY (17). Internal petiole anatomy (lacunar size and arrangement) has been used to distinguish genera (Goleniewska-Furmanowa, 1970; Chen and Zhang, 1992) and/or species groups (Conard, 1905) in the Nymphaeaceae sensu lato. In *Nuphar*, petiole lacunae are small and arranged in a reticulate fashion, similar to that found in *Barclaya*. Most taxa possess similarly sized and randomly arranged lacunae. Petioles of *N. oguraensis* possess a larger, centralized lacuna among the smaller lacunae.

Molecular data sets—Total genomic DNA was isolated from silica gel-dried leaf tissue of 13 field-collected *Nuphar* taxa using a modified CTAB procedure (Doyle and Doyle, 1987). No material was available of either *N.* × *intermedia* or *N. sinensis*. Voucher specimens have been deposited at NHA (see Table 4).

The entire *matK* gene and portions of the flanking 5′ and 3′ *trn*K introns (~2.5 kb total) were amplified from total genomic DNA using the polymerase chain reaction (PCR) and thermostable DNA polymerase. Primers used for amplification were the *trnK*-3914F and *trnK*-2R primers of Johnson and Soltis (1994). The double-stranded amplification products were purified by gel isolation in low melting point agarose followed by a secondary GeneClean II purification (Bio101, La Jolla, California). Direct dideoxy sequencing of purified DNAs was performed using Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) and eight sequencing primers including *trnK*-3914F, *trnK*-2R, and *matK*-1470R of Johnson and Soltis (1994) and five newly designed primers (Table 3). The *matK* gene and flanking intron sequences were obtained from 13 *Nuphar* taxa and from *Barclaya longifolia* (Table 4).

TABLE 3. Base compositions of novel amplification and sequencing primers used in the *matK* study.

Primer	5′ sequence 3′
Forward	
N-2-F	AAT TGA ATC TCG TCA TTA GCA
Reverse	
N-1-R	CGG GTG CGA AGA GTT TGA AGC
N-2-R	CAT CTG GAA ATC TTG CTT
N-3-R	ATG ATT AAA TGA TTC TGT TG
N-7-R	TTC TAG CAC ACG AAA GTC G

The internal transcribed spacer (ITS) region (including ITS 1 and ITS 2 spacer regions, and the 5.8S gene) was amplified from total genomic DNA. PCR and sequencing primers followed Baldwin (1992). In some taxa, when double-stranded products were difficult to sequence, single-stranded amplifications were performed. The double-stranded amplification products were purified by gel isolation in low melting point agarose followed by a secondary GeneClean II purification. Single-stranded DNAs were purified via centrifugal column dialysis (Baldwin, 1992). Direct dideoxy sequencing of purified DNAs was performed as in the *matK* study with four sequencing primers (ITS-2, ITS-3, ITS-4, and ITS-5 according to Baldwin [1992]). Sequences of the ITS region were obtained from the same 13 *Nuphar* accessions as in the *matK* study (Table 4).

Phylogenetic analysis—The boundaries of both the *matK* gene and the ITS region were determined by comparison to published sequences (Sugita, Shinozaki, and Sugiura, 1985; Yokota et al., 1989; Baldwin, 1992). The phylogenetic significance of the morphology and sequence data was assessed by maximum parsimony methods employing the computer program PAUP, v. 3.1.1 (Swofford, 1993). All characters were unweighted and unordered. Most-parsimonious trees were found using heuristic searches, with TBR (tree bisection-reconnection) branch swapping, MULPARS, and steepest descent. In the DNA data sets, indels were treated as an alternative character state. Strict consensus trees were constructed from all most-parsimonious trees. Bootstrap analyses (1000 replicates) were conducted to examine the relative level of support for individual clades on the cladograms of each search (Felsenstein, 1985). Decay indices were used as another measure of the robustness of individual branches (Donoghue et al., 1992).

Four parsimony analyses were performed: an analysis of morphology, an analysis of *matK* sequences, an analysis of ITS sequences, and a combined analysis of morphology, *matK*, and ITS data. The *matK* and morphology analyses used the closely related *Barclaya longifolia* and *B. rotundifolia*, respectively, as outgroups. Initially, representatives of Cabombaceae (*Brasenia* and *Cabomba*) and Nymphaeaceae (*Nymphaea* and *Barclaya*) were selected as outgroups for the ITS analysis. Partial ITS sequences were obtained for the first three of these genera (available upon request) but could not be readily aligned with any *Nuphar* ITS sequence. Despite repeated efforts, ITS sequences of *Barclaya* were not attainable. Thus, the ITS search utilized mid-point rooting.

RESULTS

Morphology—The 17 morphological characters used in the cladistic analysis included seven fruit characters, four floral characters, and six vegetative characters (Table 1). Of these 17 characters, 14 (82%) were found to be phylogenetically informative (Table 2).

Cladistic analysis of the morphological data produced 190 equally most parsimonious trees (Fig. 1), with a length of 32 and CI (consistency index) of 0.84 [CI ex-

TABLE 4. Sources of plant materials used in the phylogenetic analysis of DNA nucleotide sequence of *Nuphar* Sm. Collection information is available in Padgett (1997).

Species	Sources/vouchers	GenBank no.[a]			
		ITS (ITS-1/ITS-2)	*matK*	5′ *trnK*	3′ *trnK*
Barclaya longifolia Wall.	Suwannee Laboratories, FL		GBAN-AF092982	GBAN-AF117638–9	GBAN-AF117640
Nuphar advena (Ait.) Ait. f.	*Padgett 402* (NHA)	GBAN-AF077582/GBAN-AF067583	GBAN-AF117075–6	GBAN-AF117077	GBAN-AF117078
N. japonica DC.	*Shimoda 5507* (HIRO)	GBAN-AF067595/GBAN-AF067596	GBAN-AF117091	GBAN-AF117092	GBAN-AF117093
N. lutea (L.) Sm.	*Crow et al. 93-327* (NHA)	GBAN-AF067593/GBAN-AF067594	GBAN-AF117100	GBAN-AF117101	GBAN-AF117102
N. microphylla (Pers.) Fern.	*Padgett 482* (NHA)	GBAN-AF067597/GBAN-AF067598	GBAN-AF117094	GBAN-AF117095	GBAN-AF117096
N. oguraensis Miki	*Shimoda 5506* (HIRO)	GBAN-AF067592 (ITS-2)	GBAN-AF117103	GBAN-AF117104	GBAN-AF117105
N. orbiculata (Small) Standl.	*Padgett 464* (NHA)	GBAN-AF067588/GBAN-AF067589	GBAN-AF117071–2	GBAN-AF117073	GBAN-AF117074
N. ozarkana (Mill. & Standl.) Standl.	*Padgett 473* (NHA)	GBAN-AF067584/GBAN-AF067585	GBAN-AF117082	GBAN-AF117083	GBAN-AF117084
N. polysepala Engelm.	*Wiersema s.n.* (NHA)	GBAN-AF067576/GBAN-AF067577	GBAN-AF117085	GBAN-AF117086	GBAN-AF117087
N. pumila (Timm) DC.	*Crow et al.* (NHA)	GBAN-AF067599/GBAN-AF067600	GBAN-AF117088	GBAN-AF117089	GBAN-AF117090
N. rubrodisca Morong	*Padgett 483* (NHA)	GBAN-AF06580/GBAN-AF067581	GBAN-AF117097	GBAN-AF117098	GBAN-AF117099
N. sagittifolia Walt.	*Padgett 441* (NHA)	GBAN-AF067590/GBAN-AF067591	GBAN-AF117079	GBAN-AF117080	GBAN-AF117081
N. ulvacea (Mill. & Standl.) Standl.	*Padgett 469* (NHA)	GBAN-AF067586/GBAN-AF067587	GBAN-AF117068	GBAN-AF117069	GBAN-AF117070
N. variegata Durand	*Padgett 485* (NHA)	GBAN-AF067578/GBAN-AF067579	GBAN-AF092979	GBAN-AF092980	GBAN-AF092981

[a] The prefix GBAN- has been added for linking the online version of *American Journal of Botany* to GenBank and is not part of the actual Genbank accession number.

Fig. 1. One of 190 most parsimonious cladograms from a cladistic analysis of 17 morphological characters (length = 32, CI = 0.84, RI = 0.91) showing distribution of characteristics. Character numbers (above the branch) and states (below the branch) correspond to those in Table 1.

cluding uninformative characters = 0.82, RI (retention index) = 0.91]. The large number of trees differed mostly within the large clade of *Nuphar advena, N. ozarkana, N. variegata, N. ulvacea, N. orbiculata, N. sagittifolia,* and *N. polysepala.* Also, *N. microphylla* was often aligned as a sister taxon to the remaining dwarf species (*N. pumila, N. sinensis,* and *N. oguraensis*).

The strict consensus tree (Fig. 2) showed that all 190 cladograms agreed in the following respects: (1) two monophyletic clades are formed corresponding largely to a New World/Old World divergence, (2) the North American *Nuphar microphylla* appeared in the Eurasian clade,

Fig. 2. Strict consensus tree of 190 most parsimonious trees based on 17 characters of morphology for species of *Nuphar* and the outgroup *Barclaya rotundifolia.* Length = 32, CI = 0.84, RI = 0.91. Bootstrap values above 50% based on 1000 replicates are shown above each branch and decay values are shown below.

TABLE 5. Mean pairwise distances (as calculated in PAUP) between nucleotide sequences of *matK* (including portions of the 5′ and 3′ introns of *trnK*) of *Nuphar* species and *Barclaya longifolia.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *N. ulvacea*	—	0.000	0.000	0.002	0.003	0.001	0.002	0.004	0.005	0.004	0.004	0.004	0.005	0.021
2. *N. orbiculata*		—	0.001	0.003	0.003	0.002	0.002	0.005	0.005	0.004	0.004	0.004	0.005	0.020
3. *N. advena*			—	0.003	0.003	0.001	0.001	0.003	0.003	0.003	0.003	0.003	0.004	0.019
4. *N. variegata*				—	0.000	0.003	0.002	0.005	0.005	0.004	0.004	0.004	0.005	0.021
5. *N. sagittifolia*					—	0.003	0.003	0.005	0.005	0.004	0.004	0.004	0.005	0.021
6. N. ozarkana						—	0.001	0.004	0.004	0.003	0.003	0.003	0.004	0.020
7. *N. polysepala*							—	0.003	0.004	0.003	0.003	0.003	0.004	0.019
8. *N. pumila*								—	0.001	0.000	0.000	0.000	0.001	0.019
9. *N. japonica*									—	0.000	0.001	0.000	0.000	0.019
10. *N. microphylla*										—	0.000	0.000	0.001	0.019
11. *N.* × *rubrodisca*											—	0.000	0.001	0.019
12. *N. lutea*												—	0.001	0.019
13. *N. oguraensis*													—	0.019
14. *B. longifolia*														—

(3) *N. lutea* occupied a basal position in the Eurasian clade, (4) *N. japonica* appeared as a sister taxon to the four dwarf taxa, and (5) the dwarf taxa appear monophyletic. The six synapomorphies for the New World clade were six sepals (character 1), short filaments (character 3), ovoid-shaped fruit (character 8) with ribbed walls (character 11), and broad stigmatic disks slightly constricted below (characters 9 and 10). This clade was moderately supported by bootstrap (69%) but unresolved (Fig. 2). The highest bootstrap value (92%) was found supporting the dwarf clade (Fig 2).

cpDNA phylogeny—All species of *Nuphar* have a *matK* gene measuring 1518 bp in length (1515 bp in *Barclaya longifolia*). Partial sequences of the 5′ *trnK* intron (332 bp) and the 3′ *trnK* intron (333 bp) were obtained and incorporated into the data set. The total number of variable sites between all species is 50, 12 of which (24%) are potentially informative phylogenetically. Mean pairwise distances (as calculated by PAUP) between taxa varied from 0.0 to 0.6% within *Nuphar* to 2.2% between *Nuphar* and *Barclaya*. Pairwise distances ranged from 0.0 to 0.5% in *Nuphar* to 2.1% with *Barclaya* in the combined *matK*-intron data set (Table 5). The parsimony analysis of the *Nuphar matK*-intron data set yielded 18 trees of 62 steps (CI = 0.96 and 0.90 excluding uninformative characters, RI = 0.94). The strict consensus of the 18 most parsimonious trees shows two major clades (Fig. 3) splitting the *Nuphar* taxa roughly into New World and Old World groups, a topology similar to that of the morphology-based cladogram. Compared to morphology, greater resolution is offered in the New World clade with *matK* data. *Nuphar ulvacea, N. orbiculata, N. advena,* and *N. ozarkana* form a monophyletic, though weakly supported, group (Fig. 3). The best supported element (97% bootstrap) in the topology was the association of the boreal *N. variegata* and the mid-Atlantic *N. sagittifolia*. These taxa had identical *matK* sequences, each sharing three synapomorphic point mutations. The position of *N. polysepala* within the New World clade was uncertain.

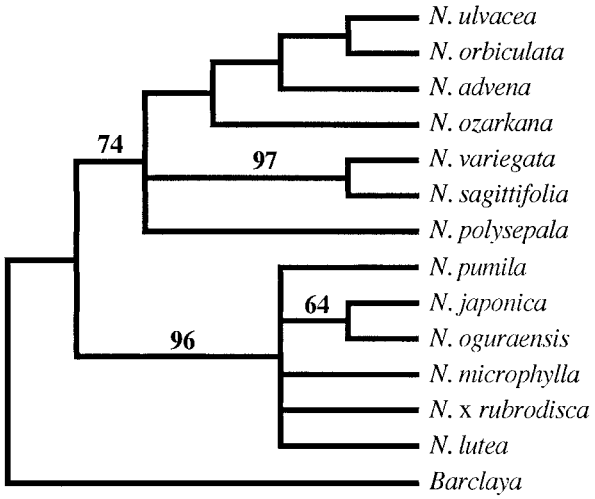


Fig. 3. Strict consensus tree of the 18 equally parsimonious trees inferred from analysis of *Nuphar matK* (and portions of the flanking *trnK* intron). Length = 62, CI = 0.96, RI = 0.94. Numbers above each branch represent bootstrap values above 50% based on 1000 replicates.

Although well supported by bootstrap (96%), the Old World clade is otherwise poorly resolved (Fig. 3). Within this clade, a subclade containing the Japanese *Nuphar japonica* and *N. oguraensis* is supported by a 64% bootstrap value. *Nuphar* × *rubrodisca* and one of its parent species, *N. microphylla*, both North American in distribution, are positioned in the Old World clade. The alignment of *N. microphylla* in this group was also found in the morphology-based cladogram (which excluded *N.* × *rubrodisca*). When *N.* × *rubrodisca* is removed from the data set, the same overall topology is found.

nrDNA phylogeny—Among most *Nuphar* species, ~85% (233–235 bp) of the total ITS 1 was determined. Complete ITS 2 sequences of *Nuphar* measured 242–250 bp in length. Several indels ranging in size from 1 to 5 bp were detected. Partial sequences of 5.8s (61 nucleotides mainly at the 3′ end) were obtained for most species and incorporated into the data set. A total of only 252 bp of sequence, mostly of ITS 2, were obtained for *N. oguraensis.* In *N. sagittifolia*, 86 (~30%) bp were not sequenced from the 3′ end of ITS 1. The total number of variable sites between all species was 37, with 16 (43%) of these in ITS 1, 20 (54%) in ITS 2, and 1 (3%) in 5.8s.

TABLE 6. Mean pairwise distances (as calculated in PAUP) between nucleotide sequences of ITS region (including ITS-1, ITS-2, and portion of 5.8 gene) of *Nuphar*.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *N. ulvacea*	—	0.005	0.002	0.004	0.002	0.002	0.011	0.041	0.040	0.043	0.002	0.033	0.051
2. *N. orbiculata*		—	0.007	0.006	0.004	0.007	0.016	0.039	0.042	0.041	0.004	0.035	0.055
3. *N. advena*			—	0.006	0.004	0.004	0.013	0.043	0.040	0.045	0.004	0.035	0.051
4. N. variegata				—	0.002	0.006	0.015	0.041	0.044	0.043	0.002	0.037	0.051
5. *N. sagittifolia*					—	0.002	0.013	0.042	0.048	0.044	0.000	0.040	0.047
6. *N. ozarkana*						—	0.013	0.043	0.043	0.045	0.004	0.035	0.052
7. *N. polysepala*							—	0.044	0.044	0.046	0.013	0.037	0.047
8. *N. pumila*								—	0.011	0.002	0.039	0.007	0.012
9. *N. japonica*									—	0.013	0.042	0.007	0.024
10. *N. microphylla*										—	0.041	0.009	0.012
11. *N.* × *rubrodisca*											—	0.035	0.047
12. *N. lutea*												—	0.020
13. *N. oguraensis*													—

Potentially phylogenetically informative sites numbered 9 (41%) in ITS 1, 12 (54%) in ITS 2, and 1 (5%) in 5.8s. Mean pairwise distances (as calculated by PAUP) of the ITS sequences between taxa varied from 0.0 to 5.5% within *Nuphar* (Table 6).

With the partial sequence of *N. oguraensis* removed from the data set, a single most parsimonious tree was obtained with a length of 36 steps (CI = 0.94, RI = 0.97) (Fig. 4). Three equally shortest trees (39 steps, CI = 0.92, RI = 0.96) were found with the inclusion of *N. oguraensis.* The three trees differed in respect to the positioning of *N. oguraensis* among the other two dwarf taxa (*N. microphylla* and *N. pumila*), but otherwise identical in topology to the tree derived without *N. oguraensis.* The most parsimonious ITS tree shows two clades separating the species into largely New and Old World groups (Fig. 4), as produced in the previous analyses of morphology and cpDNA. The New World clade has *N. polysepala* as the basal, sister species to the remaining species. Within this largely unresolved clade is a weakly supported clade containing *N. orbiculata, N. variegata, N. sagittifolia,* and *N.* × *rubrodisca.* The Old World clade depicts *N. lutea* at the base of the remaining species and *N. japonica* as a sister species to the dwarf species (Fig. 4). This clade is identical (but excluding *N. sinensis* and *N. oguraensis* from this analysis) to the one produced in the morphology-based consensus tree.

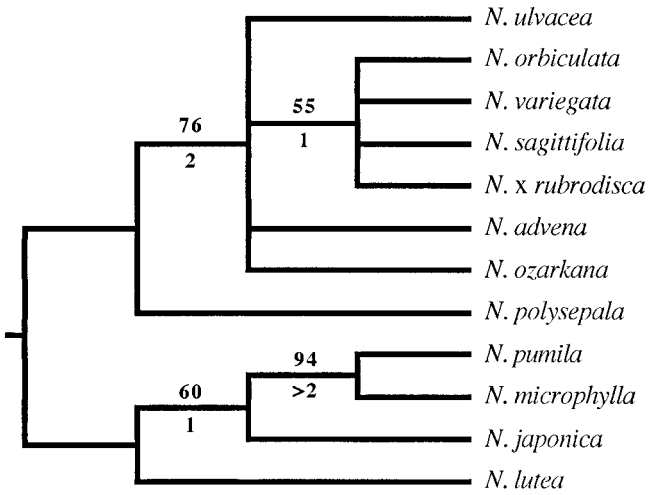


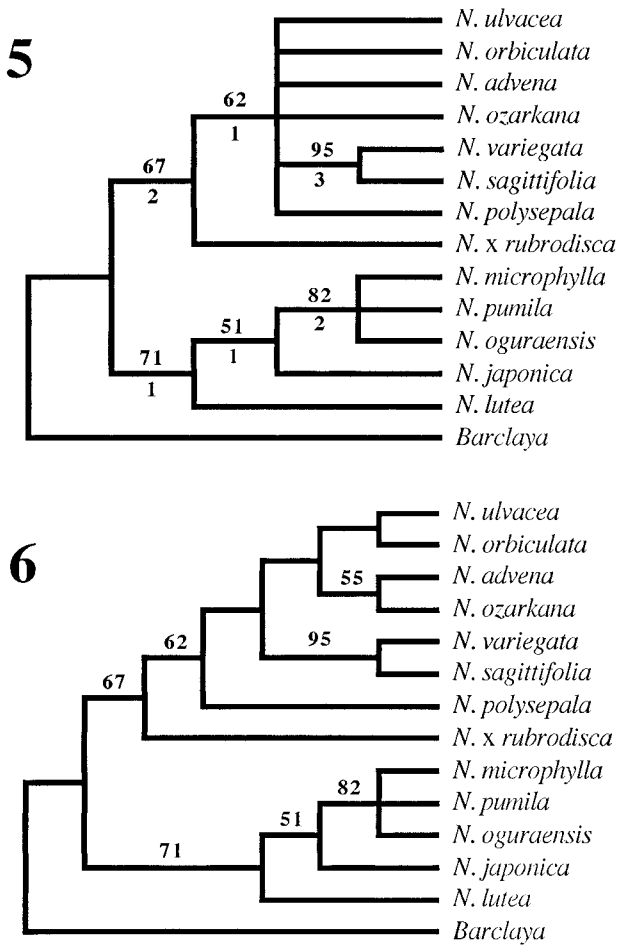
Fig. 4. Single most parsimonious tree inferred from nucleotide sequences of *Nuphar* ITS region (and partial 5.8s) using mid-point rooting. Tree length = 36, CI = 0.94, and RI = 0.97. Numbers above and below each branch represent bootstrap and decay indices, respectively.

Combined analysis—Because the overall topologies of three independent phylogenies were congruent, the data sets were combined for a final analysis. The analysis of a combined morphology-*matK*-ITS data matrix for 13 taxa of *Nuphar,* plus the *Barclaya* outgroup, resulted in 39 most parsimonious trees, with a length of 158 steps (CI = 0.85, CI excluding autapomorphies = 0.74, RI = 0.86). In both strict and 50% majority-rule consensus trees, once again two major clades were revealed with moderate support (Figs. 5, 6). In the Old World clade, *N. lutea* was the sister species to the clade comprising *N. japonica* and the dwarf taxa. The dwarf taxa clade (excluding *N. sinensis* here) was supported (82% bootstrap) as monophyletic (Fig. 5).

The combined analysis placed *Nuphar* × *rubrodisca* at the base of the North American clade. The inclusion of this hybrid taxon in the New World clade was moderately supported (67% bootstrap, decay index = 2). Relationships of the remaining New World species were virtually unresolved in the strict consensus tree (Fig. 5), except for the high support (95% bootstrap and decay index = 3) associating *N. variegata* with *N. sagittifolia.* The 50% majority-rule consensus tree offers more resolution in the New World clade, but with weakly supported branches (Fig. 6). In this tree, *N. polysepala* is a sister species to the clade of the remaining North American species. Among these species, *N. variegata* and *N. sagittifolia* form a basal clade. The remaining four species are divided into two clades, with *N. advena* and *N. ozarkana* as sister taxa in one, and *N. ulvacea* and *N. orbiculata* in the other (Fig. 6).

DISCUSSION

Phylogenetic analyses of over 2100 bp of cpDNA sequences, 500 bp of nrDNA sequences, and 17 morphological characters of 13 *Nuphar* taxa provide congruent phylogenies. The morphology, *matK,* and ITS phylogenies reveal two well-supported clades within the genus, corresponding to a New World/Old World divergence.



Figs. 5–6. Phylogenetic trees inferred from a combined analysis of *Nuphar* morphology, *matK* (and introns), and ITS (and partial 5.8s) data. Length = 158, CI = 0.85, and RI = 0.86. Bootstrap values and decay indices are shown above and below each branch respectively. **5.** Strict consensus tree of the 39 equally parsimonious trees. **6.** Fifty percent majority-rule consensus tree of 39 equally most parsimonious.

The three data sets also support the monophyly of Old World dwarf taxa and the inclusion of the North American *N. microphylla* as a part of that lineage.

Although phylogenetic analyses of each data set revealed some well-supported groups of species, relationships among taxa within the New World clade remain poorly resolved. The lack of synapomorphic characters in the morphology data set resulted in a complete polytomy of this group. The *matK* phylogeny only weakly supported relationships among the largely southern U.S. taxa (*Nuphar ozarkana, N. advena, N. orbiculata,* and *N. ulvacea*). Within the New World clade, the *matK*-based topology did indicate a strong relationship between *N. variegata* and *N. sagittifolia,* but failed to elucidate the position of *N. polysepala* (Fig. 3). The ITS phylogeny placed the northwestern North American *N. polysepala* at the base of the New World clade, but provided little additional information within this lineage (Fig. 4). Overall, the data sets indicate that the New World taxa are extremely closely related, but distinct from Old World lineage representatives.

Relationships are better resolved within the Old World clade, at least with morphology and ITS data (Figs. 2, 4). From these data sets, the widespread Eurasian *Nuphar lutea* is resolved at the base of the lineage, followed by the Japanese endemic *N. japonica.* A monophyletic dwarf *Nuphar* lineage (*N. microphylla, N. pumila, N. oguraensis,* and *N. sinensis*) is revealed by both morphology and ITS data. The *matK* data offer little phylogenetic information within the Old World clade, except for an indication that *N. japonica* and *N. oguraensis* are sister taxa.

Discordance among phylogenies—Discordance between nuclear- and chloroplast-based phylogenies has been detected within several plant groups (e.g., Soltis and Kuzoff, 1995; Soltis, Johnson, and Looney, 1996; Kellogg, Appels, and Mason-Gamer, 1996). Explanations for the cause of incongruence between phylogenetic hypotheses based on cpDNA and other data sets often implicate hybridization and introgression, particularly at lower taxonomic levels in groups noted for interfertility (Doyle, 1992; Rieseberg and Brunsfield, 1992; Soltis and Kuzoff, 1995). Chloroplast capture via hybridization provides a species with a foreign chloroplast genome, thus profoundly affecting cpDNA-based phylogenetic reconstructions.

Discordance between the independent *Nuphar* phylogenies is mainly a result of hybridization. The Nymphaeaceae are noted for hybridization (Les and Philbrick, 1993) and hybridization has indeed been documented in *Nuphar* (Heslop-Harrison, 1953; Padgett, Les, and Crow, 1998). A major difference between the ITS and *matK* topologies involves the placement of *N.* × *rubrodisca.* In the tree based on ITS data, *N.* × *rubrodisca* is within the New World clade (Fig. 4), whereas *matK* data place *N.* × *rubrodisca* within the Old World clade (Fig. 3). *Nuphar* × *rubrodisca* is a known hybrid taxon between the New World *N. variegata* and the Old World allied *N. microphylla* (Padgett et al., 1998). Identical cpDNA sequences between *N. microphylla* and *N.* × *rubrodisca* indicate chloroplast inheritance from the former. At least from the plants sampled, ITS data identify *N. variegata* as the pollen donor of the cross (neither *N. orbiculata* nor *N. sagittifolia* are potential parents; see Padgett, Les, and Crow, 1998).

An unexpected result in the *matK* phylogeny is the close relationship indicated between *Nuphar variegata* and *N. sagittifolia.* The alliance of these two taxa is evidenced by three substitutions, which represents the highest level of shared *matK* sequence variation between any two taxa in the data set. The identical *matK* sequences of these two taxa is perplexing, since their ranges are widely allopatric. *Nuphar variegata* is a boreal species in North America occurring mainly north of the glacial boundary, while *N. sagittifolia* is a coastal plain species limited to Virginia, North Carolina, and South Carolina. Morphologically, the species differ markedly, most obviously by their leaf morphology. To rule out any possibility that DNA contamination might account for this unusual result, several extractions were repeated from different accessions and re-analyzed, always with the same result. Morphology-based or ITS phylogenies fail to elucidate the positions of these two taxa. The cpDNA data suggest that these taxa may have historically occurred in closer proximity where, perhaps, populations of

N. sagittifolia had captured the chloroplast genome of *N. variegata* (or ancestor) following an ancient hybridization event. *Nuphar variegata* is known to hybridize.

Another unexpected result of the *matK* phylogeny was the resolution of a clade containing *Nuphar japonica* and *N. oguraensis.* This clade is not revealed in the morphological phylogeny. Although both taxa are included in the Old World lineage, *N. oguraensis* comprises part of the well-supported clade of dwarf species in the morphology cladogram. When the partial ITS sequence of *N. oguraensis* is included in the analysis, this taxon also is positioned in the dwarf clade. Hybridization as the cause of this discordance between phylogenies is highly tenable. The two species are endemic to Japan and overlap in distribution. Also, interspecific hybridization has been well documented between *N. pumila* (a dwarf species) and *N. lutea,* and between the dwarf *N. microphylla* and *N. variegata* (Padgett, Les, and Crow, 1998). Interspecific hybridization involving *N. japonica* and *N. oguraensis* has been suggested previously (M. Shimoda, Towa Kagaku Co., personal communication).

Combined phylogeny—The phylogeny based on the combined data set adds internal support for certain lineages and clarifies one of the unexpected *matK* relationships. Both the strict consensus tree and the majority-rule consensus tree reconcile the phylogenetic position of *N.* × *rubrodisca*. In both trees this hybrid taxon is at the base of the remaining New World members. The combined phylogeny likewise places *N. oguraensis* back in the monophyletic clade of dwarf species with *N. microphylla* and *N. pumila.* The association of *N. variegata* and *N. sagittifolia* remains highly supported in the combined phylogeny.

Few hypotheses concerning evolutionary relationships within *Nuphar* have been advanced for comparison. The reconstructed relationships within *Nuphar* presented here fail to corroborate Beal's (1955, 1956) hypothesis of a wide-ranging, polymorphic species (as *N. lutea*) in the New and Old Worlds, that is distinct from *N. japonica*. All evidence indicates that Beal's *N. lutea* is not monophyletic, seriously calling into question a taxonomic scheme that has found wide acceptance for decades. Hultén (1971) remarked on a close relationship of the Eurasian *N. lutea* sensu stricto with *N. polysepala* and *N. variegata* of North America and considered them to form a species complex. A close relationship between these taxa is not supported by either morphological or molecular data, although a relationship between the latter two taxa is evident. The low divergence between *Nuphar* taxa overall, however, is in agreement with Beal's general concept of closely related lineages within the genus.

The resulting phylogenies estimated herein have an intriguing phytogeographical implication. The analyses of all data place *Nuphar microphylla,* a boreal North American species, in the same clade as all the Eurasian species. This dwarf species may have migrated from Eurasia via a land bridge following the divergence of the two larger lineages. Without further information, the geographical origin (western Europe or eastern Asia) of the ancestor of this taxon is speculative. The low molecular divergence of *N. microphylla* from other dwarf taxa supports a more recent dispersal. Given the relatively large size of *Nuphar* rhizomes and intolerance of seeds to drying or digestion, long-distance dispersal does not seem plausible.

The phylogeny of *Nuphar* offers a baseline framework to study the evolution of morphological characters. Several floral and fruit features can be evaluated between the two major clades that support two largely geographic groups of species. Species of the Old World group share fruits with elongated necks (''styles'' of some authors) and narrow stigmatic disks. Except for *N. lutea,* the margins of the stigmatic disks of these species are incised to the extent of being lobed. Furthermore, Old World taxa have five sepals per flower and short anthers supported by relatively long filaments (Padgett, 1997).

In contrast, species of the New World group lack the neck in their fruits and have much broader stigmatic disks that are essentially entire-margined. Flowers of the New World taxa are showier, with more sepals than Old World species (up to 14 in *Nuphar polysepala*). The anthers in this group are more elongate than those in the Old World group, which possess shorter filament lengths.

The evolution of these reproductive features may be related to certain pollination mechanisms and/or pollinators. Floral biology studies of representative taxa in both clades indicate that all taxa are self-compatible and protogynous, with floral odor and color the primary means of attracting pollinators (Schneider and Moore, 1977; Ervik, Renner, and Johanson, 1995). Pollination studies of the Old World *Nuphar lutea* and *N. pumila* indicate these species are visited by an array of flies, bees, and beetles but are mainly pollinated by flies, and not by beetles (Van der velde et al., 1978; Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997). Studies of the New World *N. advena* and *N. polysepala* reveal pollination predominantly by beetles, but visitation by bees and flies (Schneider and Moore, 1977, and references therein). Schneider and Moore (1977) asserted that the overall floral structure of *Nuphar advena* (e.g., broad, flat stigmatic disks, and numerous stamens) assures beetle pollination. It remains to be seen whether the markedly different anther lengths between taxa of the Old and New World groups may influence pollination or pollinator effectiveness or selection.

The lack of phylogenetic information within *Nuphar* precluded Lippok and Renner (1997) from hypothesizing ancestral floral features in the genus. Yet, based on floral features, they suggested that the ancestral floral morphology probably favored fly and bee pollination (Lippok and Renner, 1997).

In conclusion, our study provides insight into the evolution of two major lineages within *Nuphar*. As illustrated above, the relationships depicted from both morphological and molecular data are in conflict with more recent taxonomic evaluations of the genus. These results provide support for a new classification of *Nuphar* to accommodate these two phylogenetically distant lineages.

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