**Nuphar × fluminalis, a New Hybrid from Central Japan**

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The morphology, allozyme variation and pollen viability in nine populations of *Nuphar japonica*, *N. submersa*, and unidentified intermediate plants were examined. A phenogram based on cluster analysis using 10 morphological characters revealed three cluster groups. Groups 1 and 3 had distinctive morphological characteristics and corresponded to the published descriptions of *N. japonica* and *N. submersa*, respectively, whereas Group 2 showed intermediate values in most characteristics between the two species. In the allozyme study, many morphologically intermediate plants showed additive combinations of species-specific alleles in two loci (*lap1* and *mdh3*) from the two species. Pollen viability was significantly lower in intermediate plants than in the two species. Based on the evidence, we concluded that the intermediate plants were of hybrid origin between *N. japonica* and *N. submersa* and described them as *N. × fluminalis*.

Key words: allozymes, hybridization, *Nuphar japonica*, *Nuphar submersa*, *Nuphar × fluminalis*, pollen fertility

In the genus *Nuphar* Sm. (Nymphaeaceae), natural hybridization and introgression is well known and makes taxonomic delimitation of the species difficult (Heslop-Harrison 1953, Beal 1956, Padgett et al. 1998, 1999, 2002, Shiga and Kadono 2004, 2007). In Japan, the hybrid status of some plants intermediate between named species has been confirmed and the unstable conditions of the habitat have been discussed as playing an important role in hybridization (Shiga and Kadono 2007).

During a survey of *Nuphar submersa* Shiga and Kadono, a newly reported species from central Japan (Shiga et al. 2006), we found some unidentifiable plants. After morphological, allozymic and pollen viability studies, we concluded these plants to be hybrids between *N. japonica* DC., which is widely distributed in Japan (Kadono 1994), and *N. submersa*. In this paper we describe the new hybrid.

**Materials and Methods**

**Plant materials**

We collected 86 individual plants of *Nuphar* from 9 populations in central to eastern Japan (Table 1). The collections included specimens of *Nuphar japonica*, *N. submersa* and unidentified plants. Sampling occurred from August to early September in 2001, 2002, 2004 and 2005.

**Morphological analyses**

For measurements of morphological characteristics, we collected submerged leaves and flowers from 6 to 10 plants of *Nuphar* in each population (Table 1). We assessed the following 13 morphological characteristics that have been considered important in the identification of the taxa of *Nuphar* (Kadono 1994, Padgett et al. 1999, Shiga and
Kadono 2004, 2007, Shiga et al. 2006): length of submerged leaf blade (SL1), width of submerged leaf blade (SL2), shape of submerged leaf blade (SL3; SL1/SL2), sinus depth (SL4), sinus depth/length of blade ratio (SL5; SL4/SL1), maximum petiole width at 5 cm from the base of the blade (SL6), number of veins (SL7), maximum length of stigmatic disk (F1), stigma width (F2), number of stigmas (F3), anther length (F4), filament length (F5), ratio of anther length/filament length (F6; F4/F5). These measurement comprised 7 vegetative (SL1-SL7) and 6 floral (F1-F6) characteristics. All measurements were made using materials fixed in FAA (ethanol, formalin, and acetic acid) in the field.

The data for most quantitative characteristics were continuous, and it was difficult to recognize taxonomic units based on specific key characteristics. We therefore performed cluster analysis for the 9 populations based on the mean values of each characteristic. We chose Ward’s method (Milligan 1980) for the analysis and used standardized variables and Euclidean distances.

Differences among populations and among cluster groups in each morphological trait were tested using nested analysis of variance (nested ANOVA). When the differences were significant among taxonomic groups ($P < 0.05$), we performed multiple comparison using Scheffé’s $F$-test (Scheffé 1953). To analyze morphological relationships among individuals, we performed principal-component analysis (PCA). All statistical tests were performed using JMP ver. 4J (SAS Institute Inc., USA).

To avoid misleading correlations, we excluded three characteristics (used for the calculation of ratios) from the cluster analysis and PCA: width of submerged leaf blade (SL2), sinus depth (SL4) and filament length (F5). As a result, 10 characters were used. Before applying the analyses, we arcsine-transformed the submerged leaf blade shape (SL3).

Allozyme analyses
We prepared extracts from 0.1 g of fresh leaves in 500 µL of grinding buffer (Soltis et al. 1983), and electrophoresed the extracts in 9% starch gels. The buffer systems, investigated enzymes and procedures of electrophoresis and staining were the same as in Shiga and Kadono (2007).

We detected 15 putative loci from the variation in banding patterns of the six enzymes. These loci were distributed as follows: a single locus for LAP, two loci each for PGI and PMI, three loci each for PGM and TPI, and four loci for MDH. Of these, 12 loci were genetically interpretable. All loci except lap1 and mdh3 could not be used for species-specific markers, so we determined the genotypes of lap1 and mdh3 and compared them with the different morphological groups.

Pollen viability
Pollen viability was estimated based on the
stainability of the pollen grains stained with a cotton blue staining solution (20% phenol, 20% lactic acid, and 40% glycerin aqueous solution to which 1% cotton blue aqueous solution was added) for 1 h. We removed three anthers from each flower and collected the pollen grains on a glass slide. We counted more than 500 pollen grains from each flower, and calculated pollen viability as the number of stained pollen grains divided by the total number of pollen grains examined.

In all analyses, we examined the same individuals (Table 1).

Results

Cluster groups

The Ward’s phenogram revealed two major clusters (Fig. 1); the second cluster contained two distinct subclusters. We used these three clusters as operational taxonomic units and named them Groups 1, 2 and 3.

We found significant differences among the three cluster groups in all measured characteristics (nested ANOVA, \( P < 0.05 \); Table 2). Group 1 differed significantly from the other morphological group in 9 characteristics. It had the highest values in 9 characteristics (SL1, SL3, SL5, SL6, SL7, F1, F3, F4, F6). Group 3 differed significantly from the other morphological groups in 6 characteristics. Group 3 had the lowest values in 6 characteristics (SL1, SL5, SL7, F2, F4, F6). Group 2 differed significantly from the other groups in 5 characteristics, but had neither the highest nor lowest values in the measured characteristics. The values were thus intermediate between those in Groups 1 and 3 in 5 characteristics (SL1, SL3, SL7, F4, F6).

The plants in Groups 1 and 3 corresponded well to the descriptions of Nuphar japonica and N. submersa, respectively (De Candolle 1821, Shiga et al. 2006). Hereafter, we refer to Groups 1, 2, and 3 as N. japonica, intermediate plants, and N. submersa, respectively.

Morphological relationships among two species and intermediate plants

The PCA revealed well-expressed morphological relationships among the three morphological groups (Fig. 2a). The two species (Groups 1 and 3) and intermediate plants (Group 2) could be clearly distinguished along the PC axis 1 (Fig. 2a). The ranges of PCA values along axis 1 were 1.105 to
FIG. 2. Results of principal-components analysis (PCA) of 10 morphological characteristics in three cluster groups. (a) three cluster groups, (b) genotype of lap1, (c) genotype of mdh3, and (d) pollen viability superimposed on PCA (on PC axis 1 only). Symbols indicate phenotypes (a) and (d)) or genotypes (b) and c)). In figure a), broken lines encircle same populations. For population code, see Table 1.

4.933 for Nuphar japonica (Group 1) and -4.397 to -2.951 for N. submersa (Group 3). Data for the intermediate plants (Group 2) were scattered between the ranges of values for N. japonica and N. submersa (-2.019 to 0.107). Morphological relationships were not well resolved in PC2 and PC3.

The first three principal components (PCs) accounted for 88.7% of the variance. PC1 explained 74.6% of total variance, and all characteristics except for SL3 contributed to this variance. PC2, which explained 7.4% of the total variance, was contributed to by none of the characteristics.

Genotype of lap1 and mdh3

Two polymorphic loci (lap1 and mdh3) showed distinctive genotype frequencies among Nuphar japonica, N. submersa, and the intermediate plants (Figs. 2b, c, Table 3). The genotypes of N. submer-
and *N. japonica* were fixed as “aa” and “bb”, respectively. In contrast, morphologically intermediate plants were not homozygous in the two loci, but most of the intermediate plants were “ab” heterozygous.

**Pollen viability**

Pollen viability was 93.2% ± 4.6% (mean ± SD) in *Nuphar japonica*, 93.1% ± 3.5% in *N. submersa*, and 35.5% ± 14.1% in the intermediate plants (Fig. 2d, Table 3). It was significantly lower in the intermediate plants than in either of the two species (Kruskal-Wallis test, P < 0.001).

In the intermediate plants, the pollen viability of the plants of heterozygous genotype in the two loci (30.6% ± 14.2%, n = 17) was lower than that of the plants, which is homozygous at one locus (44.4% ± 11.7%, n = 9) (Mann-Whitney’s U test, P = 0.031).

**Discussion**

As can be seen from the phenetic relationships among the three cluster groups based on morphological characteristics, intermediate plants were scattered between *Nuphar japonica* and *N. submersa*. Secondly, the lap1 and mdh3 genotype of many morphologically intermediate plants was “ab” heterozygous, which is considered to be a reflection of crosses between “aa” and “bb” types (*N. japonica* and *N. submersa*). Third, intermediate plants showed low pollen stainability, although some intermediate plants exhibited moderate pollen stainability. The fruit set and seed set ratios of the intermediate populations were low in natural habitats (e.g. Figs. 3A-B). In addition, all intermediate populations were found within Tochigi Prefecture, where the two putative parental species occur sympatrically. *Nuphar japonica* is distributed in Japan and Korea (Lee et al. 1989, Kadono 1994) and *N. submersa* is endemic to Tochigi Prefecture (Shiga et al. 2006). Our results indicate that the morphologically intermediate plants represent hybrids between *N. japonica* and *N. submersa*.

Some hybrid plants did not show additive combinations of the parental allozyme bands, as would be expected in F1 hybrids, suggesting that sexual reproduction has occurred among the hybrids. The pollen viability of the putative later progeny was higher than in the putative F1 hybrids. It is probable that backcrosses and/or crosses between hybrids have restored pollen viability in the hybrid *Nuphar* populations.

As hybrid backcrosses occur repeatedly, mor-

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**TABLE 3.** Frequencies of multi-locus genotypes and mean pollen viability (%) for 9 populations of *Nuphar* from central to eastern Japan.

<table>
<thead>
<tr>
<th>Population</th>
<th>Multi-locus genotype (lap1 and mdh3)</th>
<th>Pollen viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. japonica populations (Group 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YA1</td>
<td>1.000</td>
<td>94.1 (3.2)</td>
</tr>
<tr>
<td>FS2</td>
<td>1.000</td>
<td>93.1 (4.1)</td>
</tr>
<tr>
<td>JB2</td>
<td>1.000</td>
<td>90.9 (6.6)</td>
</tr>
<tr>
<td>NI4</td>
<td>1.000</td>
<td>94.6 (3.5)</td>
</tr>
<tr>
<td>Intermediate plants (Group 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG3</td>
<td>0.333</td>
<td>28.4 (29.2)</td>
</tr>
<tr>
<td>TG4</td>
<td>0.667</td>
<td>38.5 (4.3)</td>
</tr>
<tr>
<td>TG5</td>
<td>0.200</td>
<td>36.8 (4.2)</td>
</tr>
<tr>
<td>N. submersa populations (Group 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>1.000</td>
<td>94.6 (2.4)</td>
</tr>
<tr>
<td>TG2</td>
<td>1.000</td>
<td>91.5 (3.9)</td>
</tr>
</tbody>
</table>
Fig. 3. *Nuphar ×fluminalis* Shiga & Kadono, hybr. nov. from TG4. A-B: Fruit. C: Holotype, *T. Shiga 3384* (OSA). Scale in A and B indicates 1 cm.
phological traits are believed to shift from intermediate values closer to the parental values (Arnold 1997, Lowe et al. 2004, Rosenthal et al. 2005) and pollen viability is assumed to be restored (Rieseberg and Noyes 1998). Our morphological study in three hybrid populations, however, indicated that morphological characters of the intermediate population strictly ranged between Nuphar japonica and N. submersa. Extensive introgression may not have occurred in this hybrid complex.

All of the hybrid populations were located in the irrigation ditches with seasonal water level fluctuation (T. Shiga and Y. Kadono, unpublished data). In Nuphar hybrids in Japan some populations of N. × satyoensis (Shimoda) Padgett and Shimoda and N. × hokkaïensis Shiga & Kadono occur in artificially fluctuating environments, such as in irrigation ponds (Shimoda 1991, Shiga and Kadono 2007). The hybrid between N. japonica and N. submersa may also be adapted to such a changing environment and speciation may be in progress.

**Taxonomy**

*Nuphar × fluminalis* Shiga & Kadono, **hybr. nov.**

(N. *japonica × N. submersa*) (Fig. 3)

Haec planta *Nuphario japonicae* DC. propinquaque est, sed ab ea stigmatibus et antheris rufescenibus differt. Affinis *N. submersae* Shiga & Kadono, sed ab ea petiolis solidiis et foliis sinuosis distant.


Intermediate between *N. japonica* and *N. submersa* in morphology. Submerged leaves narrowly ovate, base with a sinus (Fig. 3C). Filaments moderately recurved after anthesis. Stigmatic disc, pollen sack and fruit tinged with orange or red.

*Japanese name*. Nagare-kōhone (nov.)


*Habitat*. Rivers and streams.


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**References**


— & —. 1999. Phylogenetic relationships in *Nuphar* (Nymphaeaceae): evidence from morphology, chloroplast DNA, and nuclear ribosomal DNA.
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