



ELSEVIER

Aquatic Botany 72 (2002) 161–174

**Aquatic
botany**

www.elsevier.com/locate/aquabot

Natural hybridization and the imperiled *Nuphar* of western Japan

Donald J. Padgett^{a,*}, Michiko Shimoda^b,
Laura A. Horky^a, Donald H. Les^c

^a Department of Biological Sciences, Bridgewater State College, Bridgewater, MA 02325, USA

^b Laboratory of Biological Research, Towa Kagaku Co., Ltd., Hiroshima 730-0841, Japan

^c Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269-3043, USA

Received 9 April 2001; received in revised form 8 November 2001; accepted 8 November 2001

Abstract

Ponds in the Saijo Basin of western Japan contain three *Nuphar* taxa, two of which are threatened. It has been proposed previously that the rarest of the three, hitherto *Nuphar japonica* var. *saijoensis*, may have originated from natural hybridization between *N. japonica* and *Nuphar pumila* subsp. *oguraensis*. To test this hypothesis, we conducted morphological analyses, pollen and seed fertility tests, and a RAPD analysis of all three pond-lilies. Individuals of the putative hybrid exhibit intermediate morphology, reduced pollen and seed viability, and genetic additivity in comparison to the other species. The putative hybrid is also limited geographically to an area of parental sympatry. Our findings support the hybrid origin of *N. japonica* var. *saijoensis*, which we recognize nomenclaturally as *Nuphar* × *saijoensis*. Loss and degradation of habitat due to urbanization is a major threat to the survival of this taxon. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Nuphar* spp.; Nymphaeaceae; Saijo Basin; Hybridization

1. Introduction

Nuphar Sm. (Nymphaeaceae), the yellow pond-lilies, comprises a small genus of perennial hydrophytes that inhabit primarily north temperate freshwater ponds and streams. Well separated from other water-lily genera, *Nuphar* is most closely allied to the East Asian *Barclaya* (Les et al., 1999). Ten species and seven subspecies have been proposed worldwide, differentiated by qualitative and quantitative features of leaf, flower, fruit and geographic distribution (Padgett, 1997). *Nuphar* species have recently been divided into two

* Corresponding author. Tel.: +1-508-531-2089; fax: +1-508-531-1785.

E-mail address: dpadgett@bridgew.edu (D.J. Padgett).

sections (sects. *Nuphar* and *Astylus*) corresponding to an Old World–New World divergence (Padgett et al., 1999; Padgett, 1999). Natural hybridization has been documented or suspected within, or between, either section and has often hampered the clear delimitation of the proposed parental taxa (Heslop-Harrison, 1953; Les and Philbrick, 1993; Padgett et al., 1998).

In Japan, taxonomic interpretations of *Nuphar* have varied to indicate between two to four indigenous species, namely, *N. pumila* (Timm) DC, *N. japonica* DC, *N. subintegerrima* (Casp.) Makino, and *N. oguraensis* Miki, the latter three being endemic to the East Asian islands (Beal, 1955; Ohwi, 1965; Ohtaki and Ishidoya, 1980; Kadono, 1994). Recent taxonomic studies by Padgett (1997, 1999) recognized only two species in Japan, *N. japonica* and *N. pumila*, treating *N. oguraensis* as a subspecies of *N. pumila* (*N. pumila* subsp. *oguraensis* (Miki) Padgett) and *N. subintegerrima* as a synonym of *N. japonica*.

Both *N. pumila* subsp. *pumila* of northern Japan and the endemic *N. pumila* subsp. *oguraensis* of central and southern locales are listed as threatened in Japan (Environmental Agency of Japan, 2000). Natural hybridization has been suggested as the cause of difficulty in determining the taxonomic identity of some Japanese populations (Kadono, personal communication).

1.1. *Nuphar* of the Saijo Basin

The Saijo Basin, Hiroshima Prefecture, of western Japan is an area ca. 10 km in diameter located 30 km east of Hiroshima City. Surrounded by mountains, the valley landscape is characterized by a high number (i.e. >1000) of artificial ponds. The pond density of the Saijo Basin ranks as one of the highest in the country (Takeuti, 1939). Most of these ponds are utilized for rice field irrigation and are known to support common and rare species of plants and animals (Shimoda, 1985, 1993a).

Conforming to Ohwi's (1965) interpretation of *Nuphar*, Shimoda (1991) described two novel varieties within the Saijo Basin: *N. oguraensis* var. *akiensis* Shimoda and *N. japonica* var. *saijoensis* Shimoda. Thus, populations of *N. pumila* subsp. *oguraensis* in the Saijo Basin (Shimoda's *N. oguraensis* var. *akiensis*) have a distinctive red-colored stigmatic disk rather than the typical yellow one. Likewise, some populations of *N. japonica* are smaller than usual (var. *saijoensis*) and also have a distinctive red-colored stigmatic disk (Kadono, 1994). Plants of this latter variety are deemed rare, as they inhabit only a few waters of the Saijo Basin (Hiroshima Prefecture, 1995). Accordingly, three *Nuphar* taxa occupy the waters of the Saijo Basin: *N. japonica* var. *japonica*, *N. japonica* var. *saijoensis*, and *N. pumila* subsp. *oguraensis* (*N. oguraensis* var. *akiensis*).

When these plants were initially assessed by Shimoda, the possibility was raised that *N. japonica* var. *saijoensis* may represent an interspecific hybrid based on its intermediate leaf morphology and close geographical proximity to both the typical *N. japonica* and *N. pumila* subsp. *oguraensis*. Lacking sufficient empirical data to resolve this question, however, Shimoda (1991) described the unique phenotypes as a novel variety of *N. japonica*, the species which it most resembled.

The present study expands the analyses of *Nuphar* in the Saijo Basin. Our focus was to reevaluate the taxonomic status of *N. japonica* var. *saijoensis* and test the hypothesis of its hybrid origin by: (1) quantifying and evaluating its suggested morphological intermediacy;

(2) evaluating its fertility from seed germination and pollen stainability data and (3) using molecular markers to ascertain whether genetic additivity of parental markers could be observed in the putative hybrid.

2. Materials and methods

2.1. Morphological analysis

Morphological data were obtained from herbarium specimens collected in 46 ponds within the Saijo Basin (132°44'E, 34°25'N). Twenty quantitative variables were scored (comprising six vegetative, eight floral, and six fruit features) for a total of 106 operational taxonomic units (OTUs) of *N. pumila* subsp. *oguraensis*, 79 OTUs of *N. japonica* var. *japonica*, and 62 OTUs of *N. japonica* var. *saijoensis*. Vouchers are listed in Appendix A. Leaf measurements were restricted to floating or exposed lamina. In instances where multiple measurements of a feature were made (usually two to three measurements per plant), the average value for that feature was used to represent an individual.

For each taxon, means and standard deviations were calculated for all variables using the SYSTAT (version 5.0) software package (Wilkinson, 1990). Character means were compared between three taxa using an analysis of variance (ANOVA) and a Tukey HSD post hoc test. Principal components analysis (PCA) was performed on a standardized similarity matrix using NTSYS-pc (version 1.80) software (Rohlf, 1993). The PCA dataset included all characters found to be statistically significant ($P < 0.05$) between the taxa except the leaf width variable, so as to avoid a misleading correlation with the leaf length:width ratio variable. Unscorable data were treated as missing.

2.2. Seed analyses

Fifty-six fresh fruits (21 of *N. pumila*, 12 of *N. japonica* var. *japonica*, and 23 of *N. japonica* var. *saijoensis*) were examined for seed studies. Seeds were removed from ovaries and stored in plastic dishes containing tap water. To test their fertility, seeds of each fruit were initially cold stratified (refrigerated for 5 weeks) to help break any dormancy (Smits et al., 1990). Seed samples were then placed under light banks (at room temperature) for a photoperiod of 15.5 h to allow for germination. Light was provided by fluorescent “grow” lights to simulate natural light. Germination was indicated by the presence of a protruding radicle. Germination was recorded every 2 days for 5 weeks.

2.3. Pollen viability analysis

Pollen viability from 26 accessions was estimated from the percentage stainability of ≥ 200 randomly selected grains taken from herbarium specimens (Appendix B). Anthers were removed from herbarium sheets and dissected in aniline blue/lactophenol following Kearns and Inouye (1993). 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.

2.4. RAPD analysis

Forty-one samples of fresh leaf tissue were collected and silica gel-dried for DNA analyses (representing 24 accessions of *N. pumila*, 10 accessions of *N. japonica* var. *japonica*, and 7 accessions of *N. japonica* var. *saijoensis*). Total genomic DNA was extracted using a modified CTAB procedure (Doyle and Doyle, 1987). The DNA concentration of each sample was standardized to ca. 25 ng/ul. Amplification reactions and profiles followed those of Padgett et al. (1998) using 10 random 10-mer primers (OPA-1 through OPA-10; Operon Technologies, Alameda, CA) to amplify DNAs. Amplification products were separated electrophoretically on 1.5% agarose gels. Markers that occurred in all three taxa were excluded from the analysis. Amplifications were repeated to document consistently amplified bands. RAPD data were summarized as the number of markers shared by *N. japonica* var. *saijoensis* and either *N. pumila* or *N. japonica* var. *japonica*.

3. Results

3.1. Morphological data

Nuphar japonica var. *japonica* and *N. pumila* differed significantly ($P < 0.05$) for mean values of 15 of 20 (75%) characters compared (Table 1). For *N. japonica* var. *saijoensis*, the means of 17 (85%) characters were intermediate between those of *N. japonica* var. *japonica* and *N. pumila*. Mean values of all six vegetative characters in *N. japonica* var. *saijoensis* differed significantly from those of the other two taxa. The first three eigenvalues explained 76% of the total variation (46.3, 15.8, and 13.4, respectively) in the PCA analysis (Fig. 1). Variables with the highest correlations to the first PCA axis were leaf length, vein number, and leaf sinus length. Fruit length, seed width, and leaf length to width ratio showed the highest correlations with the second PCA axis. Seed width, anther length, and fruit width showed the highest correlations with the third PCA axis. The PCA (first two axes) clustered the OTUs of *N. japonica* var. *saijoensis* essentially between those of *N. japonica* var. *japonica* and *N. pumila* subsp. *oguraensis* (Fig. 1).

3.2. Seed counts and viability

Seed number ranged from 9 to 130 seeds per fruit among the three taxa. The highest mean seed number per fruit occurred in *N. japonica* var. *japonica* (71 per fruit) and differed significantly ($P < 0.05$) from that of both *N. japonica* var. *saijoensis* and *N. pumila*. Fruits of *N. japonica* var. *saijoensis* possessed the least number of seeds on average (29 per fruit), but did not differ significantly from that of *N. pumila* (40 per fruit).

After 4 weeks, very little additional germination occurred. Eight of the 10 (80%) population samples of *Nuphar japonica* var. *japonica* had at least one seed germinate. Within these eight samples, seed germination ranged from 5 to 38%. In *N. japonica* var.

Table 1
Comparison of 20 morphological characters for *Nuphar* taxa of southwest Japan^a

Character	<i>N. japonica</i> var. <i>japonica</i>		<i>N. japonica</i> var. <i>saijoensis</i>		<i>N. pumila</i>	
	<i>N</i>	Mean (S.D.)	<i>N</i>	Mean (S.D.)	<i>N</i>	Mean (S.D.)
Leaf						
Length (cm)	54	19.03 (3.19) a	28	14.92 (2.78) b	49	8.94 (1.53) c
Width (cm)	54	11.68 (2.19) a	28	9.99 (1.83) b	49	6.77 (1.12) c
Length:width ratio	54	1.64 (0.16) a	28	1.49 (0.09) b	49	1.32 (0.10) c
Sinus to length ratio	54	0.30 (0.03) a	28	0.37 (0.02) b	49	0.41 (0.03) c
Petiole diameter (mm)	54	4.29 (1.19) a	27	2.90 (0.65) b	49	1.32 (0.40) c
Lateral veins (no.)	53	22.04 (3.96) a	28	15.23 (2.79) b	49	10.37 (1.19) c
Flower						
Sepal length (cm)	14	2.28 (0.33) a	15	2.39 (0.39) a	34	2.40 (0.46) a
Sepal width (cm)	14	1.56 (0.33) a	15	1.55 (0.34) a	34	1.34 (0.38) a
Petal length (mm)	8	8.04 (1.69) a	10	8.10 (1.52) a	23	6.02 (1.49) b
Petal width (mm)	8	4.33 (0.77) a	10	3.00 (0.66) b	23	2.05 (0.44) c
Anther length (mm)	12	4.80 (1.00) a	14	5.03 (0.49) a	31	3.84 (0.76) b
Stigmatic disk (mm)	11	6.21 (1.25) a	15	5.90 (1.00) a	34	5.36 (1.27) a
Stigmatic rays (no.)	12	7.30 (2.57) a	16	9.28 (1.59) a	31	7.66 (2.48) a
Peduncle diameter (mm)	11	4.90 (1.44) a	12	4.54 (1.07) a	29	3.37 (0.80) b
Fruit						
Length (cm)	18	3.88 (1.05) a	28	3.45 (0.59) a	51	2.67 (0.57) b
Width (cm)	18	2.66 (0.67) a	28	1.94 (0.33) b	51	1.70 (1.03) b
Stigmatic disk (mm)	18	8.25 (1.75) a	28	7.76 (1.48) a	50	6.42 (1.51) b
Neck diameter (mm)	18	6.50 (1.78) a	28	5.73 (1.15) a	51	3.90 (1.37) b
Seed length (mm)	6	4.50 (1.22) a	6	4.33 (0.81) a	28	3.69 (0.84) a
Seed width (mm)	6	3.50 (0.83) a	6	3.16 (0.51) ab	28	2.54 (0.76) b

^a Letters summarize the results of a Tukey HSD multiple comparisons test. Taxa with the same letters do not differ significantly for that character ($P < 0.05$).

saijoensis, 14 of 23 (61%) samples had some measurable level of germination. Of these samples, germination rates ranged from 3 to 30% for this taxon. Eleven of the 22 (50%) population samples of *N. pumila* germinated. In these samples, germination rates ranged between 2 and 67%.

Of the germinating samples, *N. pumila* exhibited the highest mean seed viability (mean = 22.1%, S.D. = 22.9, $n = 11$) and *N. japonica* var. *saijoensis* had the lowest viability (mean = 14.4%, S.D. = 9.1, $n = 14$). However, there were no statistically significant differences ($P = 0.05$) between the mean germination rates of the three taxa. When the non-germinating samples (i.e. 0% germination) were included in the calculations (data not shown), mean germination rates were slightly reduced among the three taxa and still no significant differences were observed.

3.3. Pollen viability

Pollen viability ranged from 42 to 97% among the three taxa compared. The highest mean pollen viability (mean = 93.9%, S.D. = 3.3, $n = 4$) occurred in *N. japonica* var.

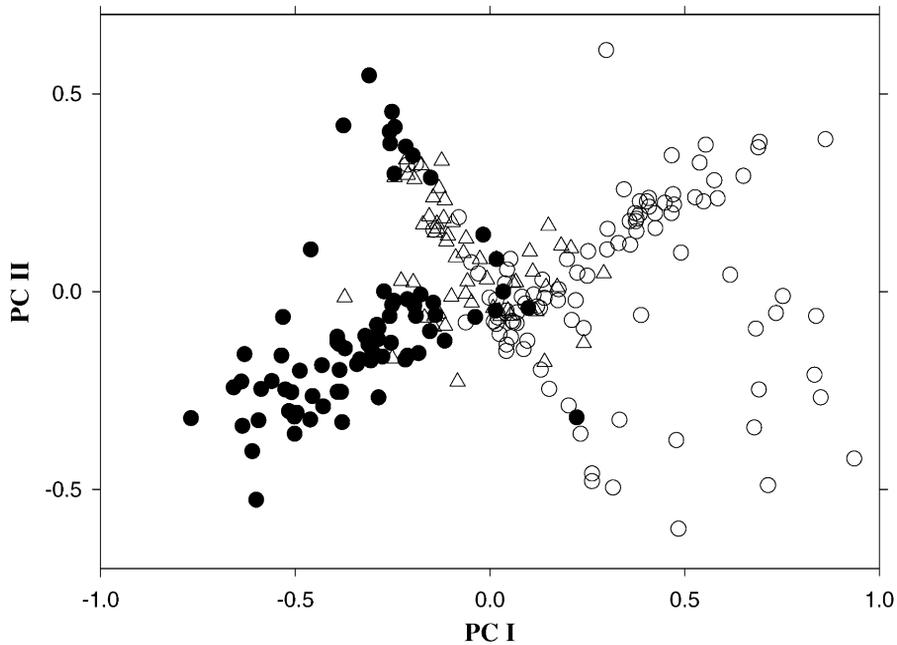


Fig. 1. Principal components plot (first two components) of *N. japonica* var. *japonica* (●), *N. japonica* var. *saijoensis* (△), and *N. pumila* subsp. *oguraensis* (○) based on morphological variables.

japonica, but did not differ significantly from that of *N. pumila* (mean = 87.7%, S.D. = 9.9, $n = 12$). Mean pollen viability of *N. japonica* var. *saijoensis* was markedly reduced (mean = 53.2%, S.D. = 8.0, $n = 10$) and differed significantly ($P < 0.01$) from both *N. japonica* var. *japonica* and *N. pumila*. Several grains among samples of these particular plants were evidently malformed by having smaller and more narrowed grains rather than the normal spheroidal shape.

Table 2
Summary of RAPD analysis of *N. japonica* var. *saijoensis* and putative parents^a

Primer	Total number of additive markers in <i>N. japonica</i> var. <i>saijoensis</i>	Markers unique to <i>N. japonica</i> var. <i>japonica</i> shared with <i>N. japonica</i> var. <i>saijoensis</i>	Markers unique to <i>N. pumila</i> subsp. <i>oguraensis</i> shared with <i>N. japonica</i> var. <i>saijoensis</i>
OPA-1	1		1
OPA-2	1	1	
OPA-5	1	1	
OPA-8	2	1	1
OPA-9	2	1	1
OPA-10	2	2	
All primers	9	5	4

^a Total number of markers unique to each parent yet showing additivity in *N. japonica* var. *saijoensis* are given.

3.4. RAPD analysis

Six RAPD primers yielded five reliable markers that were specific to *N. japonica* var. *japonica* and four markers that were exclusive to *N. pumila*. All nine of these markers were detected in the individuals of *N. japonica* var. *saijoensis* surveyed (Table 2).

4. Discussion

Hybridization may occur frequently in *Nuphar*. *Nuphar* hybrids have been reported or suspected between several species, yet many have not been studied in any detail (Les and Philbrick, 1993; Padgett et al., 1998). In a comprehensive study of a putative *Nuphar* hybrid taxon in North America, Padgett et al. (1998) successfully employed the criteria offered by Gottlieb (1972) to test whether a plant taxon arose through hybridization. Principal criteria among these are: geographical distribution in area of overlap of parental species, morphological intermediacy, partial fertility, and biochemical additivity. These same criteria were applied in this analysis of a putative *Nuphar* hybrid of the Saijo Basin, Japan.

Nuphar japonica var. *japonica* ranges throughout most of Japan while *N. pumila* subsp. *oguraensis* is found from central to southern Japan. All populations of *N. japonica* var. *saijoensis* are positioned within the area of overlap between these two taxa (Fig. 2). This distribution of *N. japonica* var. *saijoensis* satisfies the first criterion of occupying a zone of parental sympatry. All three taxa occupy similar habitats in ponds (Shimoda, 1993b).

The intermediate vegetative and floral morphology among plants of *N. japonica* var. *saijoensis* led Shimoda (1991) to hypothesize its possible hybrid origin. Our numerical evaluation of vegetative, floral, and fruit features indicates a consistent pattern of morphological intermediacy for many of the traits examined. In a set of six vegetative characters, those of *N. japonica* var. *saijoensis* were all intermediate quantitatively, in most instances being smaller than the nominal variety and larger than those of *N. pumila* (Table 1). The OTUs of *N. japonica* var. *saijoensis* clustered between the three-dimensional character space of the two putative parents. Thus, the comprehensive statistical analyses corroborate the findings of Shimoda's (1991) cursory study, i.e. *N. japonica* var. *saijoensis* is morphologically intermediate to *N. japonica* var. *japonica* and *N. pumila* subsp. *oguraensis*.

The morphological intermediacy of *N. japonica* var. *saijoensis* is also evident in features (qualitative, or overlapping parental traits) that were excluded from the formal numerical analysis. For example, *N. japonica* var. *japonica* has a filament to anther length ratio from 1 to 2, whereas *N. pumila* has a ratio of 2–4 (Table 3). Plants of *N. japonica* var. *saijoensis* have a filament to anther length ratio ranging from 1 to 3.5. Overlapping parental traits can be observed in several qualitative features also. The leaf habit of both *N. japonica* varieties is either floating or emersed, while in *N. pumila*, it is strictly floating. Petiole shape and lacunar arrangement are also similar in both varieties of *N. japonica* and different from *N. pumila* (Table 3). Yet the stigmatic disk color of Saijo Basin *N. pumila* is similar to that of *N. japonica* var. *saijoensis*, and different from *N. japonica* var. *japonica*.

The pollen fertility of *N. japonica* var. *saijoensis* is reduced markedly in comparison to both *N. japonica* var. *japonica* and *N. pumila*. The pollen viability of both putative parental

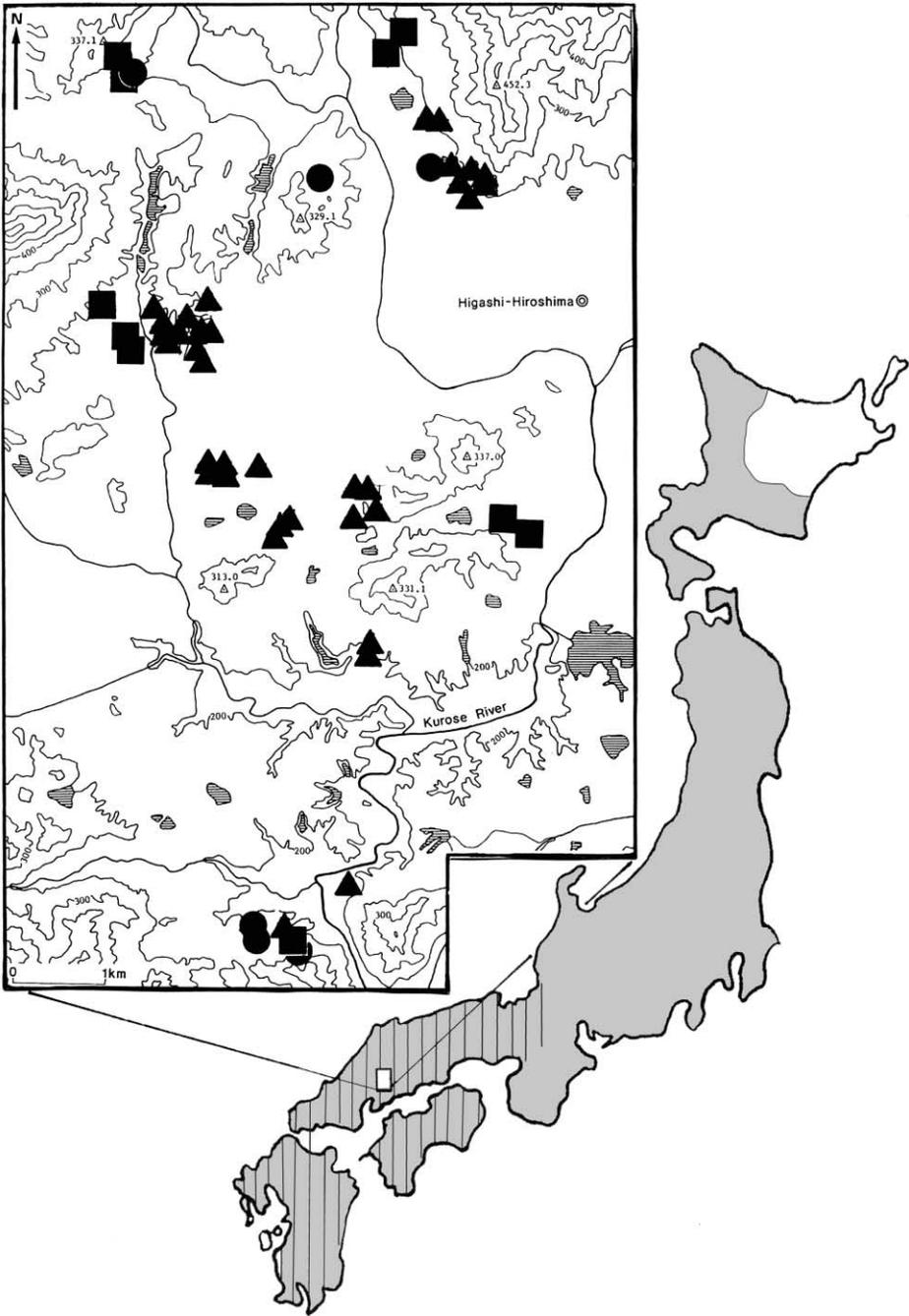


Fig. 2. Distribution of *Nuphar japonica* var. *saijoensis* (●), *N. pumila* subsp. *oguraensis* (▲), and *N. japonica* var. *japonica* (■) within the Saijo Basin. Map of Japan shows the overall distribution of *N. japonica* var. *japonica* (shaded) and *N. pumila* subsp. *oguraensis* (vertical lines).

Table 3

A comparison of selected qualitative and quantitative characters in *Nuphar* of the Saijo Basin, western Japan, adapted from Shimoda (1991) and Padgett (1997)

Character	<i>N. japonica</i> var. <i>japonica</i>	<i>N. japonica</i> var. <i>saijoensis</i>	<i>N. pumila</i> subsp. <i>oguraensis</i>
Leaf habit	Floating or emersed	Floating or emersed	Floating
Blade shape	Narrowly ovate to ovate	Ovate	Broadly ovate or broadly elliptic
Petiole shape	Terete	Terete	Flattened
Petiole lacunae arrangement	Reticulate	Reticulate	Single central lacuna
Stigmatic disk	Yellow	Red-tinged	Red-tinged
Filament:anther length ratio	1–2	1–3.5	2–4

taxa did not differ significantly, and did not fall below 59%. Pollen viability of *N. japonica* var. *saijoensis* ranged from 42 to 68%, but was significantly lower than the mean of either putative parent. *Nuphar japonica* var. *saijoensis* also had the lowest seed set compared to the putative parent taxa. The low mean number of seeds per fruit for *N. japonica* var. *saijoensis* (29) differed significantly from *N. japonica* var. *japonica* (71), but not from the small-fruited *N. pumila*.

When fertility was assessed using seed germinability, again *N. japonica* var. *saijoensis* exhibited the lowest percent viability. In comparison to germination studies of other *Nuphar* taxa within a similar 5-week period, however, germination averages seem low overall for the Japanese *Nuphar*. After a comparable treatment of *N. lutea* seeds, Smits et al. (1990) reported about 60% germination. While germination rates of *N. pumila* did reach almost 67%, neither *N. japonica* var. *japonica* nor var. *saijoensis* reached 40%. Despite the seemingly low germination rates overall, and the lowest found among *N. japonica* var. *saijoensis* plants, none of the mean germination percentages differed statistically. Several samples were visibly infected with fungal hyphae which may have affected germination rates.

RAPD data clearly indicate molecular additivity in *N. japonica* var. *saijoensis*. The putative parental taxa, *N. japonica* var. *japonica* and *N. pumila*, each possessed several unique RAPD DNA markers from a survey of six primers (Table 2). The surveyed plants of *N. japonica* var. *saijoensis* combined all nine markers that distinguished the putative parental taxa. Thus, the DNA data provide compelling evidence that plants referable to *N. japonica* var. *saijoensis* indeed represent an interspecific hybrid between *N. japonica* (var. *japonica*) and *N. pumila* subsp. *oguraensis*.

All four criteria of hybridity that were assessed in this study have been demonstrated. We believe the evidence presented here demonstrates a high degree of confidence for the hybrid origin of *N. japonica* var. *saijoensis*. Additional features of *N. japonica* var. *saijoensis* are also consistent with this interpretation. Some populations of *N. japonica* var. *saijoensis* are found in the same body of water as *N. japonica* var. *japonica* and these are always close geographically to populations of *N. pumila* (Fig. 2). These taxa are mostly insect-pollinated based on floral studies of other related species (Ervik et al., 1995; Lippok and Renner, 1997) and all three taxa have bisexual flowers and are likely to be outcrossing. Like all

other *Nuphar* taxa, *N. japonica* var. *saijoensis* is strongly rhizomatous, which would allow for an almost indefinite perpetuation of sterile hybrid offspring. Furthermore, the hybrids appear to retain at least partial fertility as evidenced by pollen and seed viability. All of these factors can be viewed as conditions that would not deter hybridization.

4.1. Taxonomic implications

The taxonomic implications of this study are appreciable. Because the present evidence strongly suggests that plants of *N. japonica* var. *saijoensis* are hybrids derived from a cross between *N. japonica* var. *japonica* and *N. pumila*, their current nomenclatural designation (as a variety of one parental species) is inappropriate. However, it is difficult to determine whether these plants should be recognized as a discrete hybrid species. There is some evidence that would support the discrete hybrid species status of *N. japonica* var. *saijoensis*. *Nuphar japonica* var. *saijoensis* is distinct morphologically from *N. japonica* var. *japonica* and *N. pumila*, at least for 17 of the characters evaluated statistically (Table 1). Pollen and seed production and fertility are moderate in some populations of *N. japonica* var. *saijoensis*. The presence of *N. japonica* var. *saijoensis* 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).

We have not observed any evidence to indicate that *N. japonica* var. *saijoensis* has diverged from either *N. japonica* var. *japonica* or *N. pumila*. Morphology and RAPD markers show intermediacy or additivity rather than any features unique to *N. japonica* var. *saijoensis* that might indicate the presence of a functional isolating barrier between it and the other two taxa. Instead, the observations presented here indicate that hybrids between *N. japonica* sensu stricta and *N. pumila* may occur repeatedly, and that *N. japonica* var. *saijoensis* does not appear to represent a monophyletic assemblage derived from a single ancestral event.

In accordance with this interpretation, we advocate an alternative view of the *Nuphar* hybrids of the Saijo Basin, in which the nomenclature clearly reflects their hybrid origin. We therefore propose to nomenclaturally designate these hybrids *Nuphar* × *saijoensis*, a “nothospecies” (Greuter et al., 1994). *Nuphar* × *saijoensis* represents a third well-documented natural interspecific hybrid in the genus. Notably, it represents yet another hybrid implicating a “dwarfed” taxon as a parental species. The North American *Nuphar* × *rubrodisca* has the dwarf *N. microphylla* as a parent (Padgett et al., 1998) and the Eurasian *Nuphar* × *intermedia* involves the diminutive *N. pumila* subsp. *pumila* (Heslop-Harrison, 1953). Evidently, there is a propensity for dwarfed taxa to hybridize with other nearby taxa. *Nuphar pumila* subsp. *sinensis* (Hand.-Mazz.) Padgett is the only dwarf taxon to date not known to naturally hybridize. Collectively, the dwarfed taxa (i.e. those much smaller in stature) represent a monophyletic lineage in Sect. *Nuphar* most closely related to *N. japonica* and *N. lutea* (Padgett, 1998; Padgett et al., 1999).

4.2. Conservation

A concern regarding this unique hybrid taxon in Japan relates to its conservation. *Nuphar* × *saijoensis* is extremely rare and imperiled (Hiroshima Prefecture, 1995). Limited to the Saijo Basin (including Kurose Basin component), it is currently confirmed from only eight

bodies of water (excluding a few more unconfirmed sightings). Notably, one population has recently become extirpated since the plant's original varietal description in 1991. Furthermore, one of its two parents (*N. pumila* subsp. *oguraensis*) is also endangered throughout its range (Environmental Agency of Japan, 2000).

The impact of increasing levels of urbanization within and around the Saijo Basin region is suspected to be the primary threat to populations of *Nuphar* × *saijoensis*, as well as other aquatic plant species (Shimoda, 1991; Shimoda et al., 2001). Land development and associated water pollution in irrigation ponds of the Saijo Basin has already been correlated with species diversity decline and even the extirpation of a rare species (Shimoda, 1993a; Shimoda and Hashimoto, 1993). Despite *Nuphar* being tolerant of varied nutrient levels, evidence suggests that populations do not endure heavily polluted waters in Japan (Shimoda, 1993b).

Irrigation ponds outside the Saijo Basin with natural populations of other imperiled Japanese *Nuphar* (i.e. *N. subintegerrima* of some authors) have also experienced severe impacts due to development (Suzuki et al., 1997). In order to afford *Nuphar* × *saijoensis* some assured protection, both the ponds it inhabits and the surrounding buffer areas must be protected (Shimoda, 1997).

More studies are needed to document hybridization in *Nuphar* and its potential implication in the taxonomic problems within the genus. Within Japan, populations have been notoriously difficult to distinguish taxonomically and could be the result of hybridization events. For example, the endangered Japanese endemic *N. subintegerrima* (as accepted by Kadono, 1994) is difficult to recognize from herbarium specimens (Padgett, 1997). Evidence from allozyme data (Suzuki et al., 1997) and cursory geographic distribution and morphology data is suggestive of a hybrid origin yet this hypothesis is in need of further testing.

Nuphar × *saijoensis* (Shimoda) Padgett and Shimoda, comb. et stat. nov.—*N. japonica* var. *saijoensis* Shimoda, J. Phytogeogr. & Taxon. 39: 5. 1991.—Type: Japan, Hiroshima pref., Higashi-Hiroshima City, 27 June 1989, Shimoda 4742 (holotype: HIRO).

5. Conclusions

Nuphar japonica var. *saijoensis* is an imperiled taxon endemic to the waters within the Saijo Basin, western Japan. Studies using geographical, morphological, molecular, and pollen and seed viability data provide evidence that this taxon represents an interspecific hybrid. The parental taxa implicated in the hybridization are the widely distributed *N. japonica* var. *japonica* and rare central-southern *N. pumila* subsp. *oguraensis* of Japan. This information is used to advocate the recognition of this morphologically unique hybrid taxon as a nothospecies, *Nuphar* × *saijoensis* to clearly reflect its origin.

Acknowledgements

This work was supported by a grant from the Society of Fundamental Research of Cultural Assets, Higashi-Hiroshima City, Japan. The following people are thanked for

providing valuable assistance: Edward Brush, Elizabeth Chappuis, Garrett Crow, Takashi Fujioka, John Gabel, Frank Gorga, Frances Jeffries, Yoshiaki Kameyama, Kathleen McCauley, Bonnie Troupe, and Donna Whitney.

Appendix A

Specimens examined for the analysis of morphological variation. All specimens collected from the Hiroshima Prefecture, Saijo Basin, Higashi-Hiroshima City, Japan, and are deposited at Herbarium of Towa Kagaku Co., Hiroshima City. Collection numbers are those of Shimoda and pond numbers refer to those in Fig. 7 of Shimoda (1991).

Nuphar japonica var. *japonica*

Pond 1: 4686, 4689, 4737, 4747, 4749–4750; Pond 4: 4753–4754; Pond 4': 4833, 4755;
 Pond 5: 4756–4758, 4831–4832; Pond 16: 4759–4761, 4763–4764, 4857–4860;
 Pond 17: 4837–4840, 4843–4845; Pond 18: 4841–4842, 4854–4856;
 Pond 46: 4719–4720, 4769, 4861–4862; Pond 46': 4825–4826; Pond 47: 4716–4718,
 4770–4771, 4827–4830, 4863–4865; Pond 47': 4823–4824, 4836, 5267;
 Pond 53: 4727, 4736, 4738–4740; Pond 54: 4730, 4735, 4746

Nuphar japonica var. *saijoensis*

Pond 2: 4683, 4685, 4752, 4852; Pond 3: 4682, 4688, 4690, 4848, 4851, 4853;
 Pond 8: 4706–4707, 4715; Pond 11: 4703; Pond 15: 4725–4726, 4733, 4765–4768,
 4850; Pond 39: 4687; Pond 42: 4875; Pond 50: 4694, 4729, 4731, 4866, 5508;
 Pond 54: 4728, 4744

Nuphar pumila subsp. *oguraensis*

Pond 6: 4812; Pond 7: 4708, 4813, 4815–4817; Pond 9: 4704–4705;
 Pond 10: 4709–4711, 4871; Pond 11: 4699, 4700–4703; Pond 13: 4814;
 Pond 14: 4712; Pond 19: 4679, 4778–4783; Pond 20: 4807; Pond 21: 4292, 4734,
 4777; Pond 22: 4776; Pond 23: 4375; Pond 24: 4774–4775; Pond 26: 4772–4773;
 Pond 27: 4678; Pond 30: 4279; Pond 31: 4677, 4681; Pond 32: 4376; Pond 33: 4818,
 4868; Pond 36: 4811, 4873–4874; Pond 37: 4721; Pond 38: 2913; Pond 39:
 3761–4763, 4092–4793, 4684, 4687, 4691–4693, 4786–4787, 4869–4870;
 Pond 40: 3635, 3638; Pond 42: 4809–4810, 4876; Pond 43: 3895;
 Pond 45: 4675–4676; Pond 48: 4696, 4698, 4732, 4784–4885, 4806, 4819–4821;
 Pond 48': 3887; Pond 49: 3897; Pond 52: 4695; Pond 55: 4697, 4741

Appendix B

Nuphar specimens collected from the Saijo Basin, Higashi-Hiroshima City, Japan used in pollen viability analysis. Collection numbers are those of Shimoda and pond numbers refer to those in Fig. 7 of Shimoda (1991). All specimens are deposited at herbarium of Bridgewater State College, Bridgewater, MA.

Nuphar japonica var. *japonica*

Pond 16: 5535; Pond 17: 5536; Pond 18: 5537; Pond 46': 5564

Nuphar japonica var. *saijoensis*

Pond 2: 5550–5553; Pond 8: 5566–5567; Pond 15: 5554; Pond 50: 5557, 5559;
Pond 51: 5560

Nuphar pumila subsp. *oguraensis*

Pond 14: 5568; Pond 20: 5538–5539; Pond 26: 5543; Pond 27: 5542;
Pond 28: 5540–5541; Pond 34: 5545–5546; Pond 42: 5547; Pond 44: 5548;
Pond 52: 5562

Appendix C

Voucher *Nuphar* specimens of those used in RAPD analysis. All specimens are deposited at the Herbarium of Towa Kagaku Co., Hiroshima (except *Shimoda* 5560, 5540, 5544, and 5545 deposited at Bridgewater State College, Bridgewater, MA). Collection numbers are those of Shimoda and pond numbers refer to those in Fig. 7 of Shimoda (1991).

Nuphar japonica var. *japonica*

Pond 1: 4686; Pond 4: 4753; Pond 4': 4755; Pond 16: 4759; Pond 17: 4837;
Pond 18: 4841; Pond 46: 4861; Pond 47: 4864; Pond 47': 4822; Pond 53: 4736;
Pond 54: 4746

Nuphar japonica var. *saijoensis*

Pond 2: 4683; Pond 8: 4706; Pond 15: 4725; Pond 50: 4866; Pond 50': 4867;
Pond 51: 5560; Pond 54: 4744

Nuphar pumila subsp. *oguraensis*

Pond 7: 4708; Pond 9: 4704; Pond 10: 4709; Pond 14: 4712; Pond 19: 4778;
Pond 20: 4807; Pond 21: 4777; Pond 26: 4772; Pond 27: 4678; Pond 28: 5540;
Pond 29: 4275; Pond 30: 4279; Pond 31: 4677; Pond 32: 4376; Pond 34: 5545;
Pond 35: 5544; Pond 42: 4876; Pond 45: 4877; Pond 48: 4820; Pond 52: 4695;
Pond 55: 4697

References

- Beal, E.O., 1955. Taxonomic revision of the genus *Nuphar* Sm. Ph.D. Dissertation, State University of Iowa, Iowa City.
- Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19, 11–15.
- Environmental Agency of Japan, 2000. Threatened Wildlife of Japan: Red Data Book, 2nd Edition. Japan Wildlife Research Center, Tokyo.
- Ervik, F., Renner, S.S., Johanson, K.A., 1995. Breeding system and pollination of *Nuphar luteum* (L.) Smith (Nymphaeaceae) in Norway. *Flora* 190, 109–113.

- Gottlieb, L.D., 1972. Levels of confidence in the analysis of hybridization in plants. *Ann. Missouri Bot. Gard.* 59, 435–446.
- Greuter, W., Barrie, F.R., Burdet, H.M., Chaloner, W.G., Demoulin, V., Hawksworth, D.I., Jørgensen, P.M., Silva, P.C., Trehane, P., McNeill, J., 1994. *International Code of Botanical Nomenclature (Tokyo code)*. Koeltz Scientific Books, Königstein.
- Heslop-Harrison, Y., 1953. *Nuphar intermedia* Ledeb., a presumed relict hybrid, in Britain. *Watsonia* 3, 7–25.
- Hiroshima Prefecture, 1995. *Threatened wildlife of Hiroshima Prefecture: Red Data Book Hiroshima*. Hiroshima Environment and Health Association, Hiroshima.
- Kadono, Y., 1994. *Aquatic Plants of Japan*. Bun-ichi Sogo Shuppan, Co., Ltd., Tokyo.
- Kearns, C.A., Inouye, D.W., 1993. *Techniques for Pollination Biologists*. University Press of Colorado, Niwot, CO.
- Les, D.H., Philbrick, C.T., 1993. Studies of hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. *Aquat. Bot.* 44, 181–228.
- Les, D.H., Schneider, E.L., Padgett, D.J., Soltis, P.S., Soltis, D.E., Zanis, M., 1999. Phylogeny, classification and floral evolution of water lilies (Nymphaeaceae; Nymphaeales): a synthesis of non-molecular, *rbcL*, *matK*, and 18S rDNA data. *Syst. Bot.* 24, 28–46.
- Lippok, B., Renner, S.S., 1997. Pollination of *Nuphar* (Nymphaeaceae) in Europe: flies and bees rather than *Donacia* beetles. *Plant Syst. Evol.* 207, 273–283.
- Ohtaki, S., Ishidoya, T., 1980. *Illustrated Japanese Water Plants*. Hokuryukan Co., Ltd., Tokyo.
- Ohwi, J., 1965. *Flora of Japan*. Smithsonian Institution, Washington, DC.
- Padgett, D.J., 1997. A Biosystematic Monograph of the Genus *Nuphar* Sm. (Nymphaeaceae). Ph.D. Dissertation, University of New Hampshire, Durham, NH.
- Padgett, D.J., 1998. Phenetic distinction between the dwarf yellow water-lilies: *Nuphar microphylla* and *N. pumila* (Nymphaeaceae). *Can. J. Bot.* 76, 1755–1762.
- Padgett, D.J., 1999. Nomenclatural novelties in *Nuphar* (Nymphaeaceae). *Sida* 18, 823–826.
- Padgett, D.J., Les, D.H., Crow, G.E., 1998. Evidence for the hybrid origin of *Nuphar* × *rubrodisca* (Nymphaeaceae). *Amer. J. Bot.* 85, 1468–1476.
- Padgett, D.J., Les, D.H., Crow, G.E., 1999. Phylogenetic relationships in *Nuphar* (Nymphaeaceae): evidence from morphology, chloroplast DNA, and nuclear ribosomal DNA. *Amer. J. Bot.* 86, 1316–1324.
- Rohlf, F.J., 1993. *NTSYS-pc, Numerical Taxonomy and Multivariate Analysis System*. Exeter Software, New York, NY.
- Shimoda, M., 1985. Phytosociological studies on the vegetation of irrigation ponds in the Saijo Basin, Hiroshima Prefecture, Japan. *J. Sci. Hiroshima Univ., Ser. B, Div. 2* (19), 237–297.
- Shimoda, M., 1991. *Nuphar* from the Saijo Basin, Hiroshima Prefecture, western Japan. *J. Phytogeogr. Taxon.* 39, 1–8 (in Japanese, with English abstract).
- Shimoda, M., 1993a. Effect of urbanization on pond vegetation in the Saijo Basin, Hiroshima Prefecture, Japan. *Hikobia* 11, 305–312.
- Shimoda, M., 1993b. Habitats of the genus *Nuphar* Sm. in the Saijo Basin, Hiroshima Prefecture, western Japan. *J. Phytogeogr. Taxon.* 41, 107–111.
- Shimoda, M., 1997. Differences among aquatic plant communities in irrigation ponds with differing environments. *Jpn. J. Limnol.* 58, 157–172.
- Shimoda, M., Hashimoto, T., 1993. Short-term changes in aquatic vegetation and water quality in a small irrigation pond. *J. Phytogeogr. Taxon.* 41, 103–106 (in Japanese, with English abstract).
- Shimoda, M., Otakara, Y., Fujioka, T., 2001. Habitats of *Nuphar japonicum* var. *saijoense* in the Saijo Basin, Hiroshima Prefecture, western Japan. In: *Studies on the Vegetation of Alluvial Plains. The Association to Commemorate the Retirement of Prof. Dr. Shigetoshi Okuda, Yokohama, Japan*, pp. 93–106 (in Japanese, with English abstract).
- Smits, A.J.M., Van Avesaath, P.H., Van der Velde, G., 1990. Germination requirements and seed banks of some nymphaeid macrophytes: *Nymphaea alba* L., *Nuphar lutea* (L.) Sm. and *Nymphoides peltata* (Gmel.) O. Kuntze. *Freshwater Biol.* 24, 315–326.
- Suzuki, T., Murayama, K., Kadono, Y., Nagayoshi, T., 1997. Allozyme variation of *Nuphar subintegerrimum* (Nymphaeaceae) at Kita-Nagaike pond, Kakogawa City, Hyogo Prefecture, Japan. *Nat. Hum. Activities* 2, 47–52.
- Takeuti, Z., 1939. Distribution of irrigation ponds in Japan proper (except Hokkaido), *Geogr. Rev. Jpn.* 15, 283–300 (in Japanese).
- Wilkinson, L., 1990. *SYSTAT: The System for Statistics*, version 5.0. SYSTAT, Inc., Evanston, IL.