A systematic study of Nuphar (Nymphaeaceae) in Japan with special reference to the role of hybridization

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A systematic study of *Nuphar* (Nymphaeaceae) in Japan with special reference to the role of hybridization

コウホネ属（スイレン科）の分類学的再検討および種間交雑に関する研究

February, 2007

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CONTENTS

SUMMARY

Nuphar Sm. (Nymphaeaceae), yellow water-lily, is a genus of perennial freshwater macrophytes and is distributed in the temperate zone of the Northern Hemisphere. The genus is one of the most taxonomically problematic aquatic plants. I investigated the morphological and genetic variations of *Nuphar* in Japan and adjacent countories and, based on the results, I tried the taxonomical revision of *Nuphar* in Japan.

In Chapter 1, I gave an outline of the taxonomical, morphological, genetical and ecological characterstics of the genus *Nuphar*, with special reference to the possibility of homoploid hybrid speciation.

In Chapter 2, I investigated the morphological variations and genetic relationships of a total of 56 populations of *Nuphar japonica* DC., *N. subintegerrima* (Casp.) Makino, *N. oguraensis* Miki and unidentified intermediate plants in central to western Japan.

The phenogram of cluster analysis based on 15 morphological characters revealed five cluster groups (Groups 1-5). Groups 1, 3 and 5 corresponded well to the description of *N. japonica*, *N. oguraensis* and *N. subintegerrima sensu stricto*, respectively. On the other hand, Groups 2 and 4 showed intermediate values in most of their characters. Morphological relationships suggested that the two intermediate groups were of hybrid origin between *N. japonica* and *N. oguraensis* and between *N. japonica* and *N. subintegerrima*, respectively.

Allozyme analyses (analysis of allele frequencies and principal coordinate analysis based on shared allele distance among the 162 multi-locus genotypes) showed the three *Nuphar* species to be well distinguished. The intermediate plant groups were also shown genetically to be of hybrid origin. However, many of the

intermediate plants did not show additive combinations of parental allozymes, which were expected in F_1 hybrids. The absence of characteristic parental bands suggested that sexual reproduction had occurred within the hybrids. Crossability analysis supported that reproductive isolation might be weak in *Nuphar* species and promote introgressive hybridization.

In Chapter 3, I investigated morphological and allozyme variation and pollen viability in 22 populations of *Nuphar japonica*, *N. pumila* (Timm) DC., and unidentified intermediate plants in Hokkaido to assess hybridization and introgression between the two species and the potential existence of homoploid hybrid speciation.

A phenogram based on cluster analysis using 15 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. pumila*, respectively, whereas Group 2 showed intermediate values in most characteristics. In the allozyme study, many morphologically intermediate plants showed additive combinations of species-specific alleles from the two species. In the DNA analysis, Pollen viability was significantly lower in intermediate populations than in the two species. Intermediate populations were found within the area of overlap between the ranges of the two species. On these evidences, I concluded that the intermediate plants represent hybrids between the two species. Relationships among species-specific AFLP bands, cpDNA haplotypes and morphology indicated that the hybrids have backcrossed to *N. japonica* repeatedly and genomes of *N. pumila* have been introduced to *N. japonica* as nuclear genes.

Four hybrid populations showed different relationships between morphology and pollen viability. Hybrids in artificially disturbed populations had discontinuously

intermediate morphology between their parental species and were completely fertile in some cases. It is probable that ecological selection has contributed to the discontinuous morphological patterns. Homoploid hybrid speciation may occurr in these *Nuphar* hybrids.

In Chapter 4, I investigated morphology, allozyme variation and pollen viability in 9 populations of *Nuphar japonica*, *N. submersa* Shiga & Kadono, and unidentified intermediate plants in central to eastern Japan. A phenogram based on cluster analysis using 10 morphological characters revealed three cluster groups. Groups 1 and 3 corresponded to *N. japonica* and *N. submersa*, respectively, whereas Group 2 showed intermediate values in most characteristics between the two species. In 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 these evidences, I concluded that the intermediate plant was of hybrid origin between the two species.

In Chapter 5, I tried to solve phylogenetic relationships and geographical patterns of *Nuphar* species, using AFLPs and allozyme markers.

In phylogenetic analysis, the neighbor-joining topology based on AFLP data supported interspecific relationships which were proposed by previous phylogenetic study, except for *N. subintegerrima*. Asian *Nuphar* plants were monophyletic group with 83% bootstrap values in the NJ tree. Within Asian *Nuphar* taxa, four strongly supported monophyletic groups were recognized; the first cluster consisted of *N. oguraensis*, *N. shimadae* Hayata and *N. submersa*; the other three clusters corresponded to *N. pumila*, *N. japonica* and *N. subintegerrima*, respectively. The phylogenetic tree showed both *N. pumila* and *N. oguraensis* were sister taxa but well

separated phylogenetically. In *N. oguraensis* group, two subclusters were recognized. The first subcluster consisted of *N. submersa* and the second subcluster consisted of *N. oguraensis* and *N. shimadae*. *Nuphar oguraensis* and *N. shimadae* should be considered conspecific.

In the AFLP analysis, the two clades of *N. japonica* showed significant correlation each other between geographic distance and genetic distance. The geographical distribution ranges of the two AFLP groups did not overlap and the two cpDNA haplotypes indicated almost the same distributional patterns. Furthermore, the plants of the two groups had significantly different morphological traits. Allopatric speciation may be in progress in *N. japonica*.

Nucleotide diversity, genetic differentiation and genotypic diversities within and among populations lower in the populations of all the species distributed in east to north regions than those of central to south regional ones. It is possible that the eastern to northern populations of *Nuphar* had experienced bottleneck effect. Present distributional patterns and geographical genetic variation of Japanese *Nuphar* may result from the distribution of refugia in glacial periods.

In Chapter 6, I proposed a taxonomic revision of Japanese *Nuphar* taxa based on the results of previous chapters and ca. 800 sheets of herbarium specimens, including some European specimens. Here I revised the Japanese *Nuphar* into six species and three hybrid taxa, as follows:

N. japonica DC.,

N. pumila (Timm) DC.,

N. saikokuensis Shiga & Kadono, sp. nov.,

N. shimadae Hayata,

N. subintegerrima (Casp.) Makino,

N. submersa Shiga & Kadono, sp. nov.,

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

N. ×*hokkaiensis* Shiga & Kadono, hybr. nov.,

N. ×*saijoensis* (Shimoda) Shiga & Kadono, comb. nov..

I reduced taxonomic rank of the taxa characterized by the red stigmatic disc to form.

Natural hybridization and introgression frequently occur in *Nuphar*. Aquatic species with high morphological variability may consist of a complex of homoploid hybrid species and parental species. In this study, I conclude that repeated hybridization and introgression caused high morphological variability of Japanese *Nuphar* and geographic isolation is an important factor for divergence and speciation in the genus.

CHAPTER 1

General Introduction

High morphological variability in aquatic plants is well known and seriously constrains taxonomic resolution in some cases (Sculthorpe 1967, Santamaría 2002). The variations are caused by phenotypic plasticity but the role of hybridization and geographical variations are also considerable (Les and Philbrick 1993, Avise 2000, Lowe *et al.* 2004). Natural hybrids are common and frequent in many plant groups (Arnold 1997, Rieseberg 1997). In aquatic plants, hybridization has been reported in 26% of 177 aquatic genera, except for single-species genera (Les and Philbrick 1993). Les and Philbrick (1993) suggested that detailed studies including molecular techniques of aquatic plants revealed a higher incidence of morphological variations attributed to hybridization and geographical variation.

Hybridization and introgression have played a significant role in speciation, and have resulted in the production of new and evolutionarily stable lineages (Stebbins 1950, Dejoode and Wendel 1992, Rieseberg *et al.* 1995). Hybrid species can be classified into two types: allopolyploid, which involves a change in ploidy, and homoploid, in which the hybridization occurs with no change in chromosome number (Grant 1983, Rieseberg 1997, Gross and Rieseberg 2005). In allopolyploid hybrid complexes, the derived species can be distinguished from the parental ones based on cytogenetic features such as differences in the chromosome number or size. However,

it is very difficult to detect hybridization events in homoploid hybrid complexes because the hybrid swarms retain the original ancestral genetic system (Grant 1983).

Homoploid hybrid speciation has been confirmed with molecular markers in only eight plant genera and two animal genera (Rieseberg 1997, Gross and Rieseberg 2005). However, cryptic hybridization and hybrid speciation may occur among many plant species with the same level of ploidy. Natural hybridization has also obscured the morphological and genetic discontinuities between ancestral traits (Harrison 1993, Arnold 1997) and has sometimes made it difficult to recognize species delimitations. It is possible that species that exhibit highly polymorphic morphology consist of a complex of homoploid hybrid species and parental species.

Nuphar Sm. (Nymphaeaceae), yellow water-lily, is a genus of perennial freshwater macrophytes and is distributed in the temperate zone of the Northern Hemisphere. The genus is one of the most problematic aquatic macrophytes to identify and seven to 20 species have been recognized worldwide, according to Cook (1990), Padgett (1999) and Padgett *et al.* (1999). In Japan four species with one or two varieties or formas have been recognized in various Japanese floras (Kitamura and Murata 1961, Ohwi 1965, Tamura 1982). In 1991, Shimoda described two new varieties in two species. Kadono (1994) enumerated four species, *N. japonica* DC., *N. oguraensis* Miki, *N. subintegerrima* (Casp.) Makino and *N. pumila* (Timm) DC. with three varieties and one form. The former three species are endemic to eastern Asia and *N. pumila* is distributed widely in the Old World (Hara 1951, Beal 1956, Ohwi 1965, Tamura 1982, Kadono 1994). Natural hybridization and introgression have been considered to cause the indeterminable taxonomic delimitation (Beal 1956, Padgett *et al.* 1998, 1999, 2002). Heslop-Harrison (1953), Les and Philbrick (1993)

and Padgett *et al.* (1998, 2002) described typical examples of natural hybridization among *Nuphar* species.

The chromosome number of *Nuphar*, including natural hybrids, is uniform (2n = 34) and the genus has thus been interpreted as diploid with x = 17 (Langlet and Søderberg 1927, Heslop-Harrison 1953, Ohga *et al.* 1962, Okada and Tamura 1981); the absence of specimens with different chromosome numbers suggests that homoploid hybrid speciation may have occurred in the genus *Nuphar*. Some hybrids have been considered to represent stabilized lineages, including *N.* ×*spenneriana* Gaudin (Heslop-Harrison 1953), *N.* ×*rubrodisca* Morong (Padgett *et al.* 1998), and *N.* ×*saijoensis* (Shimoda) Padgett and Shimoda (Padgett *et al.* 2002). However, how and under what environmental conditions these hybrid lineages are stabilized is not fully known.

The plants of this genus grow clonally with rhizome and the fragments may be transported by water flow and give rise to new ramets (Heslop-Harrison 1955, Smits *et al.* 1989). It is considered that seed-dispersal of *Nuphar* species also depends on water flow (Smith *et al.* 1989, Hart and Cox 1995, Ouborg *et al.* 2000). Smith *et al.* (1990) studied germination for *N. lutea* (L.) Sm., which evidenced that seeds could not germinate after chewing by waterfowls or after dehydration treatment. Pollinators of *Nuphar* have been known to be beetles and flower flies, which pollinated in short range (Lippok *et al.* 1997, 2000). Hence, in *Nuphar* species, it seems that geographical patterns of watershed strongly affect the gene flow and distribution of genealogical lineages. Furthermore, it is needed to understand morphological, genetic relationships and geographical patterns to solve variable morphology and patterns of hybrid zones of *Nuphar* species.

In this study, I investigated the inter and intraspecific morphological

relationships, genetic variation, as revealed by allozyme analysis, amplified fragment length polymorphism (AFLP), and cpDNA sequence variations, and pollen viability to evaluate whether taxonomic delimitations of genus *Nuphar* are affected by the hybridization events and the geographical variations. The aims of the present study are (1) to confirm whether hybridization and introgression has occurred among Japanese *Nuphar* taxa (Chapters 2, 3, and 4), (2) to compare putative hybrid populations to elucidate the potential for subsequent homoploid hybrid speciation (Chapters 3 and 4), (3) to evaluate phylogenetic relationships (Chapter 5), (4) to discuss the geographic variation and importance of allopatric speciation (Chapter 5) and (5) to revise the taxonomy of the genus *Nuphar* in Japan (Chapter 6).

CHAPTER 2

Morphological variations and genetic relationships of *Nuphar japonica*, *N. oguraensis*, *N. subintegerrima*, and unidentified plants in central to western Japan

INTRODUCTION

In Japan, Kadono (1994, 1995) reported intermediate forms that were difficult to identify to known species in Japan. He also addressed a problem regarding the delimitation of *Nuphar subintegerrima* (Casp.) Makino. *Nuphar subintegerrima* was first described as *N. japonica* DC. var. *subintegerrima* Casp. by Caspary (1866). Makino (1910) later recognized it as a distinct species, *N. subintegerrima* (Casp.) Makino. He described *N. subintegerrima* as being a dwarf plant with emergent or floating leaves of 5-11 cm long and 4-8.5 cm wide. Plants with floating leaves longer than 20 cm, which are widely distributed in central to western Japan, have also been treated as *N. subintegerrima*. Kadono (1994) doubted that these plants were conspecific with *N. subintegerrima* in a strict sense as described by Makino (1910). Furthermore, some plants are intermediate in size and morphology between *N. subintegerrima sensu lato* and *N. japonica*. Because herbarium specimens of *N. subintegerrima* are difficult to distinguish from *N. japonica*, Padgett (1999) and

Padgett *et al.* (2002) treated *N. subintegerrima* as a synonym of *N. japonica*

Natural hybridization and introgression have been considered to cause the indeterminable taxonomic delimitation (Beal 1956, Padgett *et al.* 1998, 1999, 2002). Heslop-Harrison (1953), Les and Philbrick (1993) and Padgett *et al.* (1998, 2002) described typical examples of natural hybridization among *Nuphar* species. Molecular markers such as allozyme are powerful tools to detect hybridization event and, so far, have identified many hybrid species (cf. Arnold *et al.* 1990, Nason *et al.* 1992). These genetic data from allozymes can supply successfully species-specific markers, which provide the quantification of introgression (Arnold *et al.* 1990, Szymura and Barton 1991, Rieseberg and Ellstrand 1993).

In the second chapter, I describe the morphological variations, with special reference to populations in central to western Japan where delimitation of the species of *Nuphar* is most problematic. Then, I will try to evaluate the hypothesis of hybrid origin of unidentified intermediate plants and discuss some related problems based on the allozyme study and crossability analysis in *N. japonica*, *N. oguraensis* and *N. subintegerrima sensu stricto*. I will focus on the following two topics: (1) Are the unidentified intermediate plants conspecific with *N. subintegerrima*?, (2) Are the unidentified intermediate plants of hybrid origin?, (3) How is the genetic relationship among these *Nuphar* taxa?

MATERIALS AND METHODS

Plant materials

Plant materials of *Nuphar* were collected from 53 localities from central to western Japan (Table 1). Some northern populations were included to represent typical *N. japonica* for comparison. Sampling was conducted from late July to early October in

2001 and 2002. In each of the three localities where I recognized apparently different forms of plants growing in neighboring stands I collected the different forms separately and designated them as A, B and C (Table 1). As a result, I sampled 56 populations in total. They included populations of *N. japonica*, *N. subintegerrima s. l.*, *N. oguraensis* and the unidentified intermediate plants. The *Nuphar* plants are predominantly clonal and it is probable that only one genet constitutes a local population. The rhizome length of Japanese *Nuphar* was about 3 to 5 m in the fields (Nakamura and Yamatou 1986). So I got samples at an interval of 5 m or more to collect different genets.

Morphological analysis

For measurement of morphological characters, more than ten emergent or floating leaves, flowers and fruits were collected from each population. Twenty-seven morphological characters, comprising 9 vegetative, 12 floral and 6 fruit characters, that have been used as important characters for the identification of the taxa of *Nuphar* (Ohtaki and Ishido 1980, Tamura 1982, Kadono 1994, Padgett *et al.* 1999), were investigated (see Table 2). Qualitative features (L9, Fl4 and Fl9) were scored as shown in Table 2 and treated as quantitative characters.

All measurements were made using the materials fixed in FAA (ethanol - formalin - acetic acid) in the field.

Allozyme analyses

Extracts were prepared from 0.1g fresh emerged or floating leaves in 500 μl of grinding buffer (Soltis *et al.* 1983) and electrophoresed on 9 % starch-gels. Two buffer systems, Histidine-criate, pH 6.5 (system 1) and Tris-Borate-EDTA, pH 8.6 (system

2) were used to resolve the following six enzymes (Soltis *et al.* 1983). System 1 was run for three enzymes: malate dehydogenase (MDH, EC 1.1.1.37), phosphoglucose isomerase (PGI, EC 5.3.1.9) and phosphoglucomutase (PGM, EC 5.4.2.2), and system 2 for three enzymes: leucine aminopeptidase (LAP, EC 3.4.11.1), triose-phosphate isomerase (TPI, EC 5.3.1.1) and phosphomannose isomerase (PMI, EC 5.3.1.8). Electrophoresis was run at 40 mA for 7 h (system1) and at 60 mA for 6h (system 2). Staining recipes were taken from Akiyama and Suzuki (1998) modified from Wendel and Weeden (1989).

Genetic interpretations of zymograms were conducted from isozyme number and subunit structures of each enzyme (Gottlieb 1982, Weeden and Wendel 1989). Putative loci were numbered sequentially, beginning with the most anodal migrating band; putative alleles were labeled alphabetically, also beginning with the most anodal band. The numbers and letters corresponded to inferred encoding alleles. Genetic analysis of inheritance patterns has supported my genetic interpretations of zymograms (data not shown).

Crossability analysis

Plants of five populations, NI-1 (Group 1), HY-8 (Group 3), GI-1 and MI-1 (Group 5), and HY-1 (Group 4), were selected to use in artificial cross experiments in a green house and in the fields. A total of 25 cross combinations were carried out among cluster groups, that is NI-1×NI-1, NI-1×HY-8, NI-1×GI-1, NI-1×MI-1, NI-1×HY-1, HY-8×NI-1, HY-8×HY-8, HY8×GI-1, HY8×MI-1, HY-8×HY-1, GI-1×NI-1, GI-1×HY-8, GI-1×GI-1, GI-1×MI-1, GI-1×HY-1, MI-1×NI-1, MI-1×HY-8, MI-1×GI-1, MI-1×MI-1, MI-1×HY-1, HY-1×NI-1, HY-1×HY-8, HY-1×GI-1, HY-1×MI-1 and HY-1×HY-1, during the years 2002 to 2005. To investigate a possibility of apomixis, I

also emasculated the buds of some flowers in each population and bagged. One month later after artificial crossing, the fruits were harvested in the green house and the fields.

Seeds obtained from artificial crossing were tested for germination rate. Germination studies by Smith *et al.* (1989) had shown that optimal germination of seeds of *N. lutea* (L.) Sibth. & Sm. was obtained when the seeds were subjected to a cold treatment and subsequently incubated in hypoxic tap water. Thus, in order to obtain a maximal germination response, the seeds were stored at 4 °C for 5 weeks. Subsequently, seeds were incubated in 30 ml screw bottles filled with tap water. Germination was checked after the seeds had been incubated for 4 weeks at ca. 20 °C with a 14 h photoperiod. Light was provided by fluorescent white lamps.

Data analyses

Most quantitative data were continuous for all characters, making them difficult to use for recognizing taxonomic units based on specific key characters. Cluster analysis was therefore carried out for the 52 populations based on the averages of each character. The Ward's method was chosen for this analysis, using standardized variables and the squared Euclidean distance.

Before applying cluster analysis, principal components analysis (PCA) was performed to select morphological characters to use in cluster analysis. When a character showed a high correlation ($r>0.7$) with other characters, one character that showed the highest proportion on the first three principal components was selected. As a result, the following 15 characters were used for cluster analysis: leaf blade length (L1), leaf blade shape (L3), distance to maximum width of blade/total length of blade ratio (L7), presence or absence of central lacuna in the petiole (L9), maximum

length of the stigmatic disc (Fl1), min./max. ratio of the stigmatic disc length (Fl3), stigma width (Fl6), stigma length/width ratio (Fl7), shape of stigma (Fl9), anther length (Fl10), anther length/filament length ratio (Fl12), fruit length (Fr1), length/width ratio of the fruit (Fr3), seed length (Fr4), seed width (Fr5).

Differences among populations and among cluster groups for each morphological trait were tested using nested analysis of variance (nested ANOVA). When the differences were significant ($P<0.05$), a multiple comparison was performed with Scheffe's F-test. To analyze morphological relationships among each cluster groups, PCA was performed, based on the same data set of cluster analysis. All statistical tests were performed using JMP ver. 4J (SAS Institute Inc., USA).

To examine genetic relationships of Japanese *Nuphar*, shared allele distance (DSA; Jin and Chakraborty 1993) between multi-locus genotype (MLG) was calculated by using the program Populations version 1.2.28 (Langella 2002). In clonal plants, analyses based on allele frequencies in populations such as Nei's genetic distance (Nei 1972) may show incorrect genetic similarity by populations. Relationships among all MLGs were assessed by a principal coordinate analysis (PCO) using the program MVSP version 3.1 (Kovach 1999).

RESULTS

Morphological variations

The Ward's method phenogram showed three major clusters (Fig. 1). An additional subcluster was recognized within each of the second and the third clusters. These five cluster groups were recognized as operational taxonomic units (OTUs) and named Groups 1, 2, 3, 4, and 5.

Significant differences among the cluster groups and populations were found in

all of the measured variables by the nested ANOVA (P<0.05; Table 3). Group 1 was significantly different from the other cluster groups in 17 characters. It had the highest values in 14 characters (L1-L4, L6-L8, Fl1, Fl2, Fl5, Fl8, Fl10, Fr1, Fr2) and the lowest values in two characters (L5, Fl3). Group 3 was significantly different in 13 characters and had the lowest values in six characters (L7-L9, Fl6, Fl10, Fl12). Except for the only one sample, this group was especially distinct in the presence of central lacuna in the petiole (L9=0.02±0.14). Group 5 was significantly different from other cluster groups in 19 characters. Group 5 had the highest values in four characters (Fl6, Fl12, Fr4, Fr5) and the lowest in 14 characters (L1-L4, L6, Fl1, Fl2, Fl4, Fl5, Fl7-Fl9, Fl11, Fr1). Its roundish leaves (L3=1.12±0.06 and L7=0.55±0.03) was useful for distinguishing Group 5 from the other groups, although some plants of Group 3 had roundish leaves.

Group 2 was significantly different in 12 characters and Group 4 was significantly different in 11 characters. But Groups 2 and 4 did not have diagnostic characters, except for Fr3 and Fr6 in Group 4. The values of most of the characters of both groups were intermediate among Groups 1, 3 and 5. The values of Group 2 were intermediate between Groups 1 and 3 in 17 characters (L1-L8, Fl1, Fl2, Fl5, Fl6, Fl8, Fl10, Fl12, Fr5, Fr6), between Groups 1 and 5 in 13 characters (L1-L8, Fl1, Fl2, Fl5, Fl8, Fr1) and between Groups 3 and 5 in four characters (Fl6, Fl12, Fr5, Fr6). The values of Group 4 were also intermediate between Groups 1 and 3 in 10 characters (L1-L3, L5-L8, Fl8, Fl10, Fr2), between Groups 1 and 5 in 19 characters (L1-L8, Fl1, Fl2, Fl4, Fl5, Fl7-Fl11, Fr1, Fr2) and between Groups 3 and 5 in six characters (Fl4, Fl6, Fl7, Fl11, Fl12, Fr5).

The PCA based on the same data set with cluster analysis provided well-expressed morphological relationships among five cluster groups (Fig. 2). The

two intermediate cluster groups were separated by principal component 2. Group 2 was intermediate between Groups 1 and 3 and Group 4 was intermediate between Groups 1 and 5. The first three principal components were responsible for 68.5% of the variance. Principal component 1 accounted for 37.4% of total variance, which was contributed to by leaf characters (L1, L3, L7, L9), characters of stamen (Fl10, Fl12) and fruit length (Fr1). Principal component 2, accounting for 22.1% of total variance, was contributed to by characters of stigma (Fl6, Fl7, Fl9) and the width of the seed (Fr5) (Table4).

The geographic distribution patterns of each cluster group are shown in Fig. 3. The populations of Group 1 are widely distributed from northern Japan to Kyushu. Populations of Groups 2 and 4 are distributed from central to western Japan. Populations of Group 3 are distributed in western Japan. The distribution range of Group 5 is limited to the Tokai Region of central Japan.

Isozyme loci

Fifteen putative loci were detected from variation in banding patterns of six enzymes (Fig. 4). These loci were distributed as follows: single locus for LAP, two loci for PGM and PMI, three loci for PGI and TPI, and four loci for MDH. The 12 loci, excluding *pgm2*, *pgi1* and *tpi1*, were genetically interpretable (Fig. 4). All loci except for *mdh2* were polymorphic. All dimeric enzymes, MDH, PGI and TPI, had heterodimeric bands between loci (*mdh1-mdh2*, *mdh3-mdh4*, *pgi1-pgi2* and *tpi2-tpi3*). Although putative null alleles were detected in two loci, *lap1* and *pmi2*, these putative loci could be interpreted by the density of the bands.

Variation between morphological groups

Nine polymorphic loci (*lap1*, *mdh1*, *mdh3*, *pgi2*, *pgi3*, *pmi1*, *pmi2*, *tpi2* and *tpi3*) showed the patterns of allele frequencies that were distinctive among Groups 1, 3 and 5, respectively (Table 5). In Group 3, the allele for one of the three diagnostic loci (*mdh3*, *mdh4* and *tpi2*) was fixed. Group 5 had four diagnostic loci (*lap1*, *pmi1*, *pmi2* and *tpi3*) and, among them, *pmi1* was fixed. Although Group 1 had two specific alleles in *mdh3* and *pgi3* with low frequency, they did not display a clear pattern like those of Groups 3 and 5. Allele frequencies in Group 2 were intermediate to those of Groups 1 and 3 in *mdh3*, *mdh4* and *pgm1*. But the allele frequencies were intermediate between those of Group 5 and the other two Groups 1 and 3 in *lap1*, *pmi1* and *pmi2*. Some MLGs of Group 2, J-O 1, 2, 5, 6, 10, 18 and 20 (see Appendix 1), had the alleles, which were species-specific both to Group 3 (*mdh3a*) and Group 5 (*pmi1a*) in four populations (NI-5, TO-1, TO-2 and TO-3b). Group 4 had intermediate allele frequencies between Groups 1 and 5 in all loci diagnostic to Group 5 (*lap1*, *pmi1*, *pmi2* and *tpi3*). In the two intermediate morphological Groups (2 and 4), specific allele frequency detected in *pgi1* (FU-1, GI-2) was low.

Principal coordinate analysis (PCO)

Figure 5 shows the result of PCO analysis of 162 MLGs based on 11 isozyme loci. The Groups 1, 3 and 5 could be distinguished and two continuous intermediate Groups (2 and 4) scattered along axis 1 (46.2% of total variance) (Fig. 5a). The ranges of axis 1 of the three species were 0.048 to 0.252 (Group 1), 0.219 to 0.327 (Group 3) and –0.284 to –0.144 (Group 5). The ranges of MLGs of Group 2 (–0.093 to 0.268) and Group 4 (–0.358 to 0.099) were wider than the range of the other three Groups. The MLGs of Group 4 were mostly scattered between Groups 1 and 5, although only one

MLG was plotted out of these ranges. In contrast, the distribution range of MLGs of Group 2 was over the range from Group 1 to Group 3. There was no distinct separation along axis 2 (31.1%) and axis 3 (16.8%; not shown).

Genetic variations of each population were shown in Figs. 5b-d. In Groups 1, 3, and 5, genotypic variation was low and well circumscribed in a given area. On the other hand, the populations of Groups 2 and 4 showed various patterns: some populations scattered between Groups 1 and 5 or Groups 1 and 3 and same as the morphological relationships (HY-1, HY-5, GI-2, KA-1, KA-2, KA-5, NA-1, OK-3 and SI-1), some were more or less overlapped with Groups 1, 3, and 5 (FU-2, GI-3, HI-2a, HY-7, NI-5, OH-1, OK-1, OK-6, TO-1, TO-2 and TO-3b), and others were not distinguishable for the morphological relationships (FU-1, FU-2, FU-3, GI-3, HI-4, HI-5, HY-3, KA-4, MY-1b, MY-1c and TO-3a).

Cross incompatibility

All cross combinations produced fruits and emasculated flowers produced no fruits in all treatments (Table 6).

Seeds of most of the combinations showed the germination rate between 39.5-78.5% (Table 7). In the case of Group 3, however germination rate was significantly reduced, when pollen donor was other taxa（Kruskal-Wallis test, $P<0.001$）.

DISCUSSION

The morphological variations and relationships among Nuphar *in central to western Japan*

The phenogram based on 15 morphological characters revealed five cluster groups.

The cluster groups are well distinguishable each other by many morphological characters. Groups 1, 3 and 5 in particular exhibited some remarkable characters. In contrast, Groups 2 and 4 showed intermediate values in most of their characters.

Plants of Groups 1, 3 and 5 correspond well to the classical descriptions of *N. japonica*, *N. oguraensis* and *N. subintegerrima*, respectively (De Candolle 1821, Delessert 1823, Caspary 1866, Makino 1910, Miki 1934). Group 1 includes the biggest plants in many quantitative characters and, therefore, it is obviously identifiable as *N. japonica*, one of the largest species of *Nuphar* in the Old World (Padgett *et al.* 1999, Padgett 2003). Group 3 is characterized by thin petiole (L8=3.33 ±0.89), the presence of a central lacuna in the petiole (L9=0.02±0.14) and the lowest anther to filament length ratio (Fl12=0.51±0.08) and identifiable as *N. oguraensis* (Miki 1934). Group 5 includes the smallest plants with roundish leaves. These features correspond to those of *N. subintegerrima* as described by Makino (1910). Hereafter, this taxon is expressed as *N. subintegerrima s. s.*.

Recent taxonomic studies of worldwide *Nuphar* by Padgett (1999, 2003) and Padgett *et al.* (2002) recognized only two species in Japan, *Nuphar japonica* and *N. pumila* (Timm) DC., and treated *N. oguraensis* as a subspecies of *N. pumila* and *N. subintegerrima* as a synonym of *N. japonica*. My study shows that three species are distinctly distinguishable. *Nuphar subintegerrima s. s.* is distinct in the small, roundish leaves. Intermediate plants which have so far been identified as *N. subintegerrima*, are not conspecific with *N. subintegerrima s. s.* The intermediate plants may have caused confusion in the treatment of Padgett (1999) and Padgett *et al.* (2002).

As can be seen from phenetic relationships among cluster groups (Table 3, Fig. 2), the occurrences of two groups of intermediate plants, Groups 2 and 4, is confirmed.

Although morphological variables of both cluster groups widely overlapped, some floral features differ from each other. Plants of Group 2 are intermediate between *N. japonica* and *N. oguraensis* in some floral characters (Fl1, Fl2, Fl5, Fl6, Fl8, Fl10, Fl12). Group 4 is intermediate between *N. japonica* and *N. subintegerrima s. s.* in such characters as Fl1, Fl2, Fl4, Fl5, and Fl7-Fl11. The PCA indicated that Group 2 was intermediate between Groups 1 and 3 and Group 4 was intermediate between Groups 1 and 5. Principal component 2, summarizing variations in some floral characters, provided evidence for differentiation between Groups 2 and 4. These findings support the recognition of two different intermediate groups by their morphology and suggest hybrid origin for them from different parental combinations; Group 2 between *N. japonica* and *N. oguraensis* and Group 4 between *N. japonica* and *N. subintegerrima*. In *Nuphar*, natural hybridization and introgression are known to cause difficulty in the taxonomic delimitation of species (Heslop-Harrison 1953, Beal 1956, Les and Philbrick 1993, Padgett *et al.* 1998, 2002). It is probable that natural hybridization occurs widely in Japan.

Geographic distribution patterns of Groups 2 and 4 (Fig. 3) agree well with the range of distribution of the indeterminable specimens of *Nuphar* that were provisionally identified as *N. subintegerrima* (Kadono 1994). The populations of Group 2 were within the range of overlap of *N. japonica* (Group 1) and *N. oguraensis* (Group 3) in western Japan. In the Hokuriku Region, however, *N. oguraensis* is not located. In contrast, populations of Group 4 are distributed more widely than one of the putative parents, *N. subintegerrima s. s.* (Group 5). *Nuphar subintegerrima s. s.* differs from other taxa in large seeds (Table 3, Chapter 6). Miki (1960) reported reliquiaes of large seed of *Nuphar*, which was similar that of *N. subintegerrima*, in Shimane Prefecture. Hence, the past distributinal range of this species might be

wider than present distributional range. Further molecular and historical analysis of the distribution pattern of each taxon is needed to determine the origin of the intermediate groups.

The genetic relationship and origin of intermediate groups

In allele frequencies and PCO analysis, the plants of Group 4 were intermediate between *N. japonica* and *N. subintegerrima* and the plants of Group 2 were intermediate among three species (Table 5, Fig. 5). Although the intermediate groups had one specific allele (*pgi1c*) in low frequency, allele frequencies of Group 4 were intermediate between those of *N. japonica* and *N. subintegerrima* and these of Group 2 were intermediate among three species (Table 5). Furthermore, many plants of the intermediate groups had species-specific alleles of putative parental species (Appendix 1). The PCO analysis indicated that three species were well identified and two morphologically intermediate groups (Groups 2 and 4) were plotted continuously between *N. subintegerrima* and *N. oguraensis* in principal coordinate axis 1 (Fig. 5). The plants of Group 4 were genetically intermediate between *N. japonica* and *N. subintegerrima* as was the case with intermediate morphology. In contrast to Group 4, the plants of Group 2 were genetically intermediate among three species.

Natural hybridization in *Nuphar* has been reported with some combinations (Les and Philbrick 1993). Most of putative hybrids were considered natural crosses between dwarf taxa and large-leaved taxa (Heslop-Harrison 1953, Padgett *et al.* 1998, Padgett *et al.* 2002). In Japan, *N.* ×*saijoensis* (Shimoda) Padgett and Shimoda was reported to be of hybrid origin between *N. japonica* and *N. pumila* subsp. *oguraensis* (Miki) Padgett (=*N. oguraensis* var. *akiensis* Shimoda), which has a reddish stigmatic disk (Shimoda 1991, Kadono 1994), based on morphology, pollen and seed

fertility tests and randomly amplified DNA (RAPD) analysis (Padgett *et al.* 2002). From morphological analyses, I hypothesized that natural hybridization occurred widely in Japan and the two intermediate groups recognized by morphological characters were of hybrid origin between *N. japonica* and *N. oguraensis* and between *N. japonica* and *N. subintegerrima*, respectively. The genetic evidences obtained in this study supported my hypothesis that Group 4 is of hybrid origin between *N. japonica* and *N. subintegerrima*. On the contrary, Group 2 is genetically intermediate among three species. Some populations of Group 2 had MLGs, which had alleles specific to both *N. oguraensis* and *N. subintegerrima*. Therefore, natural hybridization may have occurred not only between *N. japonica* and *N. oguraensis* but also between *N. oguraensis* and *N. subintegerrima*.

In addition, many intermediate plants did not show additive combinations of parental allozymes, which would be expected in F_1 hybrids. The absence of some characteristic parental bands suggests that sexual reproduction has occurred among the hybrid plants. I suppose that genetic variations within and among the intermediate populations are likely to be due to repeated hybridization events, such as intercrosses between parent species or between independently produced hybrids, and backcrossing of hybrids with one of the parent species.

During my field study, I observed that some populations of Groups 2 (GI-3, OK-6, KA-2) and 4 (NI-5, HI-2a, TO-2, TO-3b, MY-1bc) showed lower fruit set and pollen stainability than their putative parent species (data not shown). This observation agrees well with the fact that interspecific hybrids are often less fertile than their parents (Gottlieb 1972, Metz *et al.* 1994, Arnold 1997), and suggests that the Groups 2 and 4 are of hybrid origin.

All of the combinations of crosses among *N. japonica*, *N. oguraensis*, *N.*

subintegerrima and intermediate plants (HY-1; Group 4) produced F_1 progeny in my crossability analysis (Table 6, 7). The chromosome number of *Nuphar* is 2n=34 and was interpreted as diploid based on x=17 (Langlet and Søderberg 1927, Heslop-Harrison 1953, Ohga *et al.* 1962, Okada and Tamura 1981). Reproductive isolation mechanism might be weak in *Nuphar* species and promote introgressive hybridization among these plants, although hybridization events between *N. oguraensis* and other taxa may be directional as suggested by germination experiment (Table 7).

Some plants of intermediate populations had the same MLGs with putative parental species. It is possible that some intermediate populations include plants of putative parental species, because I identified the two intermediate morphological groups by averages of morphological characters of each population in this chapter. Further morphological and molecular analyses based on individual data are needed to discuss the hybridizaion and introgression events in each population. In the next chapter, I will discussed hybridization event in Hokkaido Island based on individual population data.

The three Japanese *Nuphar* species were well distinguished by patterns of allele frequencies and PCO analysis (Table 5, Fig. 5). Although *N. japonica* had species-specific alleles with low frequencies, *N. oguraensis* and *N. subintegerrima* had diagnostic loci and were distinctly distinguishable. Although a recent taxonomic study of *Nuphar* worldwide treated *N. subintegerrima* as a synonym of *N. japonica* (Padgett *et al.* 1999, 2002, Padgett 2003), the present results indicate that *N. subintegerrima* is a distinct taxon.

Several isozymes in my study (MDH, PGI and TPI) revealed duplicated loci (Fig. 2). Isozyme duplication has also been reported in isozyme studies of *N. pumila* var.

ozeensis (Miki) H.Hara and intermediate plants, identified as "*N. subintegerrima*" (Suzuki *et al.* 1997, Murayama *et al.* 1998). Padgett *et al.* (2002) discussed that intermediate populations of *Nuphar* in Japan are of hybrid origin, based on allozyme data of Suzuki *et al.* (1997) in which enzyme loci of an intermediate population were duplicated. However, my allozyme analysis revealed that isozyme duplication was not restricted to intermediate plants, but also occurred in MDH, PGI and TPI in *N. japonica*, *N. oguraensis* and *N. subintegerrima*. Furthermore, these isozymes were also duplicated in *N. pumila,* (Chapter 3), and *N. lutea* (data not shown). Although the *Nuphar* species have been considered diploid (Langlet and Søderberg 1927, Okada and Tamura 1981), these isozyme duplications suggest that the sect. *Nuphar* (Padgett 1999) is genetically polyploid. It is probable that these duplications occurred early in the evolution of the genus *Nuphar*. To understand evolutionary history of *Nuphar* I conducted further molecular study based on AFLP fragments, which is described in Chapter 5.

CHAPTER 3

Cryptic hybridization and introgression between *Nuphar japonica* and *N. pumila*: Homoploid hybrid speciation in progress?

INTRODUCTION

In northern Japan, the distribution ranges of *N. japonica* DC. and *N. pumila* (Timm) DC. overlap, but the two species have different morphological and ecological traits. *Nuphar japonica*, which is distributed in Japan and Korea, is an emergent-leaved plant and one of the largest *Nuphar* species. It is mainly a lowland plant found in lakes, slow rivers, and canals (Lee 1989, Kadono 1994, Padgett *et al.* 1999, Padgett 2003). On the other hand, *N. pumila* is a small floating-leaved species found in lakes and moors from sea level to high elevations, and is distributed from East Asia to Europe (Hara 1951, Beal 1956, Padgett *et al.* 1999). Padgett *et al.* (2002) recognized two subspecies, subsp. *pumila* and subsp. *oguraensis*, in *N. pumila* in Japan. However, *N. oguraensis* Miki could be well recognized as a distinct species from morphology, genetic features and distribution ranges (Chapters 2, 5 and 6). I will treat *N. pumila* subsp. *pumila* sensu Padgett *et al.* (2002) as *N. pumila* in this paper. In Hokkaido, Japan, unidentified plants intermediate in morphology between *N. japonica* and *N. pumila* have been reported (Ito 1967), and the chromosome numbers

of these intermediate plants were the same as those in all *Nuphar* species (Keiko Murayama and Yasuro Kadono, unpublished data). Such intermediate plants may have originated through homoploid hybridization between the two species.

In this chapter, I investigate the morphological relationships, allozyme variation, AFLP fragment variation, cpDNA haplotype and pollen viability of putative hybrid populations to evaluate the hypothesis that homoploid hybridization and subsequent hybrid speciation has occurred between the two species. The goals of my study are (1) to confirm whether hybridization and introgression have occurred between *N. japonica* and *N. pumila*, and (2) to compare putative hybrid populations to elucidate the potential for homoploid hybrid speciation.

MATERIALS AND METHODS

Plant materials

I collected a total of 423 individual plants from 22 populations in Hokkaido, Japan (Table 8). These included specimens of *N. japonica*, *N. pumila*, and unidentified intermediate plants. As *Nuphar* plants are predominantly clonal, it is possible that local populations may comprise a single genet. Because Japanese *Nuphar* has a rhizome length of 3 to 5 m (Nakamura and Yamatou 1986), I collected the samples at intervals of at least 5 m to minimize the risk of collecting multiple specimens of the same genet. Sampling was conducted from August to early September in 2004.

Artificial cross experiment was performed between the plants of *N. japonica* (ATT) and *N. pumila* (BEB) and I obtained six synthetic F_1 hybrids. The six hybrids were checked for genetic composition of nuclear DNA and chloroplast DNA by allozyme and AFLP markers and *trnL* intron sequences.

Morphological analyses

For measurements of morphological characteristics, I collected emergent or floating leaves, flowers, and fruits from 3 to 60 *Nuphar* plants in each population (Table 8). I assessed the 21 morphological characteristics (Table 9) that have been considered important in the identification of *Nuphar* taxa (Ohtaki and Ishido 1980, Kadono 1994, Padgett *et al.* 1999, Chapter 2). These comprised 11 vegetative, 6 floral, and 4 fruit 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. Thus, I performed cluster analysis for the 22 populations based on the mean values of each characteristic. I chose Ward's method for this 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 ($P<0.05$), I performed a multiple comparison using Scheffé's F'-test. To analyze morphological relationships among individuals, I performed principal-components analysis (PCA). All statistical tests were performed using JMP ver. 4J (SAS Institute Inc., USA).

To avoid misleading correlations, I excluded six characteristics (used for the calculation of ratios) from the cluster analysis and PCA: leaf blade width (L2), sinus depth (L4), length from the leaf tip to the point of maximum blade width (L6), minimum petiole width at 5 cm from the base of blade (L9), filament length (F15), and seed width (Fr3). As a result, the remaining 15 characters were used. Before applying these analyses, I arcsine-transformed the maximum blade width

position/total length of blade (L7), the min./max. petiole diameter ratio (L10), and the length/width of seed ratio (Fr4).

Allozyme analyses

I 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 Chapter 2.

Fifteen putative loci were detected from variation in banding patterns of six enzymes. These loci were distributed as follows: single locus for LAP, two loci for PGM and PMI, three loci for PGI and TPI, and four loci for MDH. The 12 loci, excluding *pgm2*, *pgi1* and *tpi1*, were genetically interpretable (for locus names, refer to those of Chapter 2). All loci except for *mdh3* were monomorphic. So I determined genotypes of *mdh3* and compared among different morphological groups.

DNA extraction

Total DNA was extracted from 50-100 mg dry mass of leaves of each accession by the CTAB method (Stewart and Via 1993) with some modificaitons and approximately 200 ng of total DNA per sample was obtained.

Chloroplast DNA analysis

The portion of the *trnL* intron region was amplied from total genomic DNA using the polymerase chain reaction (PCR) and thermostable DNA polymerase. Primers used for amplification of the *trnL* (UAA) intron were primers C (5'-CGAAATCGGTAGACG CTACG-3') and D (5'-GGGGATAGAGGGACTTGAAC-3') by Taberlet *et al.* (1991).

More than one sample was selected from each population and sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an ABI Prism 3100 automated sequencer (Applied Biosystems) with its associated DNA sequencing analysis software.

The cpDNA sequences revealed three haplotypes and I designated a new primer pair (Forward 5'-CAACCCGAATCCTTATTTTT-3', Reverse 5'-TCTCCCAGTCTCATA CTCTG-3') to recognize the haplotypes by PCR product size (Fig. 9). PCR product sizes were determined for 22 samples of *N. japonica*, 80 samples of intermediate populations, 34 samples of *N. pumila* and 6 artificial crosses using the ABI Prism 3100 automated sequencer and GenScan 3.7 analysis software (Applied Biosystems).

AFLP analysis

AFLP was performed for the same samples with cpDNA analysis according to Vos *et al.* (1995) with some modifications. Sample DNA was restricted with the endonucleases *Eco*RI and *Mse*I at 37℃ in 1.5 hours and ligated to appropriate double-stranded adapters at 20℃, overnight. Two steps of amplification followed, that is, a preselective amplification in which I used primers with one base pair (bp) extension, and a second amplification in which primers with 3 bp extensions were used, thereby further reducing the number of fragments. For the second amplification, I initially tried 16 different primer combinations. From these, I selected four combinations, -ACA(FAM)/-CTA, -ACG(FAM)/-CTA, -ACA(JOE)/-CTG and -ACG(JOE)/-CTG, for the extensions to the *Eco*RI and *Mse*I sites, respectively.

The PCR products from the selective amplification were visualized using an ABI Prism 3100 automated sequencer. Fragments from 50 to 500 bp in size were sized with GeneScan 500 ROX Size Standard (Applied Biosystems). Sizing and

quantification of AFLP bands with GenScan 3.7 (Applied Biosystems) and the electoropherograms were imported into Genotyper 3.7 (Applied Biosystems). Assignment of AFLP bands of size categories was performed by manual evaluation of all electropherograms using Genotyper 3.7. The AFLP bands were aligned after size categories in a data matrix and either value 0 or 1 (0=fragment absent; 1=fragment present) was assigned. If an AFLP band fixed among all plants of putative parental species examined, I defined it as a species-specific band.

Pollen viability

Pollen viability was estimated based on the stainability of the pollen grains with cotton blue staining solution (20% phenol, 20% lactic acid, and 40% glycerine aqueous solution to which 1% cotton blue aqueous solution was added) for 1 h. I removed three anthers from each flower and collected the pollen grains on a glass slide. I 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 this analysis, I examined the same individuals used in other analyses, including examined all samples of morphologically intermediate specimens in the cluster analysis.

Data analysis

AFLP is a dominant marker (Vos *et al.* 1995). Thus, if F_1 hybrids backcross to one parent repeatedly, other parental AFLP bands are reduced. To analyze patterns of introgression, I judged that it is originated by backcrossing, if a hybrid did not have species-specific markers of both parental species with same ratio, it is originated by backcrossing.

I superimposed number of specific bands of putative parental species on morphological variations to compare patterns of introgression of specific markers.

All statistical tests were performed using JMP ver. 4J (SAS Institute Inc., USA).

RESULTS

Cluster analysis

The Ward's phenogram revealed two major clusters (Fig. 6), and the first cluster contained two distinct subclusters. I used these three clusters as operational taxonomic units and named them Groups 1, 2 and 3.

I found significant differences among cluster groups and populations in all measured characteristics (nested ANOVA, $P<0.05$; Table 10). Group 1 differed significantly from the other cluster groups in 11 characteristics. It had the highest values in 9 characteristics (L1, L7, L8, L10, L11, Fl2, Fl6, Fr1, Fr4) and the lowest values in two characteristics (L3, L5). Group 3 differed significantly from the other cluster groups in 12 characteristics. This group had the highest values in two characteristics (L3, L5) and the lowest values in 10 characteristics (L1, L7, L8, L10, L11, Fl1, Fl2, Fl4, Fl6, Fr4). Group 2 differed significantly from the other groups in 10 characteristics, but had neither highest nor lowest values in the measured characteristics. The values for Group 2 were thus intermediate between those in Groups 1 and 3 in 10 characteristics (L1, L3, L5, L7, L10, L11, Fl2, Fl6, Fr4).

The plants in Groups 1 and 3 corresponded well to the descriptions of *N. japonica* and *N. pumila*, respectively (Timm 1792, De Candolle 1821). Hereafter, I thus refer to Groups 1, 2, and 3 as *N. japonica*, intermediate populations, and *N. pumila*, respectively.

Morphological relationships among cluster groups

The PCA, based on the same data used in the cluster analysis, revealed well-expressed morphological relationships among the three cluster groups (Fig. 7a). The two species (Groups 1 and 3) could be clearly distinguished, and the intermediate populations (Group 2) were scattered continuously along PC axis 1 (Fig. 7a). The ranges of PCA values along axis 1 were 1.545 to 6.951 for *N. japonica* (Group 1) and –5.248 to –0.780 for *N. pumila* (Group 3). The range for plants in the intermediate populations (–2.366 to 5.642) was wider than the range of either of the two parent species. Data for plants in the intermediate populations (Group 2) were scattered continuously within the ranges of values for *N. japonica* and *N. pumila*.

The first three principal components (PCs) accounted for 80.4% of the variance (Table 11). PC1 explained 67.0% of total variance, and all characteristics except for Fl3 and Fr2 contributed to this variance. PC2, which explained 7.4% of the total variance, was contributed to only by characteristic Fl3.

Genotype of mdh3

One polymorphic locus (*mdh3*) showed distinctive genotype frequencies among *N. japonica*, *N. pumila*, and the intermediate populations (Fig. 7b; Table 12). The genotypes of *N. pumila* and *N. japonica* were nearly fixed as “aa” and “bb”, respectively. In contrast, most morphologically intermediate plants were “ab”.

The geographic distributions of the different genotypes of *mdh3* and of the morphological groups are shown in Fig. 8. I have added data for some northern Honshu populations of *N. japonica* for comparison (see Chapter 2). Intermediate populations were distributed within the area of overlap between the ranges of *N. japonica* and *N. pumila*. The "ab" heterozygotic populations of *N. japonica* and *N.*

pumila (SAA, TAO, TOB and TOJ) were located near the other different morphological groups.

Chloroplast DNA analysis

Three cpDNA lengths were detected at the partial *trnL* intron region as 234, 238 and 241 bp (Table 13). Sequence analyses confirmed that these haplotypes differed by 12 indels (total 48 bp) together with 4 substitution sites.

The haplotypes detected at *trn*L intron region showed that haplotype length of 238 bp was specific to *N. japonica* and 234 bp was specific to *N. pumila* except for one population (URU), which had the haplotype of 241 bp. Most of intermediate populations had the *N. japonica* haplotype except for one in TST and three in NNO that had *N. pumila* haplotype (234 bp) (Table 13). All of the synthetic F_1 hybrids had a haplotype of the maternal parent (Table 13).

AFLP analysis

AFLP bands discriminated 108 genotypes; 22 genotypes from seven populations of *N. japonica*, 34 genotypes from 11 populations of *N. pumila*, and 52 genotypes from four intermediate populations (Table13). All of the plants of *N. japonica* and *N. pumila* were revealed to have unique genotypes and each intermediate population consist of more than two genotypes. *Nuphar japonica* and *N. pumila* had 25 and 16 species-specific bands, respectively (Table 13). Both sets of these specific bands were additively observed in four intermediate populations; 72-100 % of *N. japonica* specific bands and 0-100 % of *N. pumila* specific bands (Table 13). All of the synthetic F_1 hybrids had the whole parental bands with complete additive combination (Table 13). Although the plants of SAS showed almost complete additive combination pattern of

parental AFLP bands, the plants in other intermediate populations indicated various combination patterns (Fig. 10). Each population except for SAS did not have species-specific markers of both *N. japonica* and *N. pumila* with same ratio and species-specific bands of *N. pumila* were mostly reduced.

Rlationships among species-specific AFLP bands and morphology (PC1) of hybrid populations were shown in Fig. 11. Some plants morphologically similar to *N. japonica*, which comprised 25 to 75 percentiles of morphological variation (PC1) of *N. japonica*, had one to 10 *N. pumila* specific bands. There were no plants morphologically similar to *N. pumila*.

Pollen viability

The pollen viability averaged 92.6%±5.5% (mean±SD) in *N. japonica* (Group 1), 92.7%±9.8% in *N. pumila* (Group 3), and 64.7%±33.1% in the intermediate populations (Group 2); it was significantly lower in the intermediate populations than in either of the two species (Kruskal–Wallis test, $P<0.001$).

Relationships between pollen viability and morphology (PC axis 1) were shown in Fig. 7c. Pollen viability varied widely in the intermediate populations (from 0% to 99.2%). In particular, morphologically intermediate plants with PC1 ranging from about –2 to 2 had lower pollen viability than other plants in the intermediate populations that resembled to *N. japonica* morphologically.

Relationships between pollen viability and AFLP marker were also shown in Fig. 12. Intermediate plants with many species-specific markers of both *N. japonica* and *N. pumila* exhibited lower pollen viability than others.

Comparison of intermediate populations

The four intermediate populations (SAK, SAS, NNO, and TST) showed different patterns of morphology, *mdh3* genotype, and pollen viability (Fig. 13). Morphological variations (PC1) were continuous from the intermediate populations to the range of *N. japonica* in SAK, but were discontinuous among *N. pumila*, intermediate plants, and *N. japonica* in TST and NNO (Fig. 13). In terms of the *mdh3* genotypes, some plants of *N. japonica* and *N. pumila* with typical morphology were heterozygotes in SAK and TST. Some intermediate plants were homozygotes in SAK, TST, and NNO, although most of the genotypes were heterozygotes (Figs. 8, 13).

Pollen viability of morphologically intermediate plants varied among populations from low viability in SAS (14.4% to 19.5%) to high viability in SAK where the plants resembled to *N. japonica* (all>80%). It was widely variable in TST (8.4% to 95.0%) and NNO (0% to 96.7%). All plants with typical *N. japonica* and *N. pumila* morphology showed high pollen viability.

DISCUSSION

Hybridization between N. japonica *and* N. pumila

In a comprehensive study of putative *Nuphar* hybrid taxa, Padgett *et al.* (1998, 2002) successfully employed the criteria offered by Gottlieb (1972) to test whether a plant taxon had arisen through hybridization. The principal criteria were morphological intermediacy, biochemical additivity of both the parental species, reduced fertility compared with the parent species, and geographical distribution in the area of overlap of the parental species. I applied the same criteria to the present analysis of intermediate populations that were putative *Nuphar* hybrids.

First, as can be seen from the phenetic relationships among the three cluster groups based on morphological characteristics, intermediate populations were widely

scattered between those of *N. japonica* and *N. pumila*. Second, the *mdh3* genotype of many morphologically intermediate plants was an "ab" heterozygote, which is considered to be the progeny resulting from a cross between “aa” (*N. pumila*) and “bb” (*N. japonica*). In AFLP analysis, intermediate plants also had both AFLP fragments specific to the two species. Third, the plants of intermediate populations showed low mean pollen viability, although some intermediate plants in the NNO, TST, and SAK populations exhibited high pollen viability. So far as my observations were concerned, fruit and seed set ratios were also low in intermediate plopulations except for some intermediate plants in a few populations. Fourth, all intermediate populations were found within the area of overlap between the distributions of the two species. In addition, some populations of *N. japonica* and *N. pumila* located near the other morphological groups (SAA, TAO, TOB and TOJ) had allozyme alleles from the other species. These results support the hypothesis that the morphologically intermediate plants represent hybrids between *N. japonica* and *N. pumila*.

The variation in pollen viability among the putative hybrids (Figs. 7c, 13) suggests that the hybrids with heterozygote genotypes are not F_1 progeny but rather later progeny. Some hybrids did not show additive combinations of parental allozyme bands, as would be expected in F_1 hybrids. And many hybrids did not have all species-specific AFLP bands of both parental species. This suggests that sexual reproduction has occurred among the hybrid plants.

Introgression and pollen viability restoration

Many hybrids (93.8% of total hybrids) had *N. japonica*’s cpDNA haplotype. It is probable that a hybridization event in which a female parent was *N. japonica* and a male parent was *N. pumila* occurred. The artificial cross experiment in Chapter 2

showed the case that, when a female parent was *N. oguraensis*, germination rate was reduced. Because *N. oguraensis* is closely related to *N. pumila* (Chapter 5), hybrid seeds with a female parent of *N. pumila* may be inferior in germination ability in natural habitats.

Reduction of species-specific AFLP bands of *N. pumila* in all of the hybrid populations, except for SAS, strongly suggests that hybrids have backcrossed to *N. japonica* repeatedly. Hybrid populations are distributed in lowland lakes (Table 8). *Nuphar pumila*'s habitats such as highland lakes and marshes are generally oligotrophic environments. Hybrids may not be able to grow in oligotorophic environment, and thus backcrossing to *N. pumila* is limited. Further field and greenhouse experiments are needed to discuss about backcross events in this hybrid zone.

Relationships among species-specific AFLP bands, cpDNA haplotypes and morphology (PC1) indicated that genomes of *N. pumila* have been introduced to *N. japonica* as nuclear genes (Figs. 10, 11). In hybridization study, maternal or paternal genome marker, such as cpDNA and mtDNA, are used as a sign of intorogressive hybridization event (Rieseberg and Wendel 1993, Sutton *et al.* 1994, Watano *et al.* 1996, Senjo *et al.* 1999). In this study, I could not detect introgression of cpDNA genome in this hybrid zone of *Nuphar*. Therefore, Many introgressions of nuclear genomes may occur in cryptic hybrid events of other plant species.

It has been shown that fertile hybrid lineages can be obtained after a small number of generations of selfing or backcrossing, even if F_1 hybrids were almost completely sterile in *Helianthus* species (Rieseberg 1997, Rieseberg and Noyes 1998). Backcrosses and crosses between hybrids have restored pollen viability in the hybrid *Nuphar* populations in my study (NNO, TST, and SAK).

Potential of homoploid hybrid speciation in Nuphar

As hybrid backcrosses occur repeatedly, morphological 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 considered to be restored (Rieseberg and Noyes 1998). The results in two hybrid populations (SAA and SAK) support these hypotheses and also suggest the occurrence of introgression in SAK.

However, although the NNO and TST hybrids were evidently intermediate morphologically between their parental species, they were fertile. The habitats of the two populations were artificially disturbed environments, in which strong ecological selection had presumably operated. One environment (TST) is used as an irrigation pond and the other (NNO) is a famous sight-seeing location in Hokkaido where many ferries and powerboats disturb the *Nuphar* population. In contrast, the habitats of SAS and SAK were lakes in a natural condition.

The known processes of homoploid hybrid speciation include recombinational speciation with chromosomal sterility barriers and hybrid speciation with external barriers, including ecological prezygotic barriers (Grant 1983, Rieseberg 1997). Recent theoretical and empirical studies supported the hypothesis that ecological selection is a major factor in promoting homoploid hybrid speciation (Buekle *et al.* 2000, Gross and Rieseberg 2005). It is possible that ecological selection contributed to the discontinuous morphological patterns observed in NNO and TST. Homoploid hybrid speciation may thus occur in the populations under ecological selection.

The case is similar in *N.* ×*saijoensis*, a hybrid between *N. japonica* and *N. oguraensis*. *Nuphar* ×*saijoensis* usually inhabits irrigation ponds in western Japan (Shimoda 1991). Irrigation ponds were the habitats liable to large water level

fluctuations. The habit of *N.* ×*saijoensis* is similar to *N. japonica* × *N. pumila* and the hybrids change their growth form from emergent-leaved in shallow water to floating-leaved in deep water. Artificially disturbed environments may be essential to stabilizing hybrid lineages of *Nuphar* as distinct taxa.

An artificial hybrid between *N. japonica* and *N. pumila* has been well known among aquatic plant cultivators, but natural hybrids of this combination have not been reported (Swindells 1983). Thereby, I described the hybrid formerly in Chapter 6.

CHAPTER 4

Hybridization between *Nuphar japonica* and *N. submersa* in central Japan: A case in lotic water

INTRODUCTION

In the genus *Nuphar* Sm. (Nymphaeaceae), natural hybridization and introgression are well known and have been understood to account for the difficulty in taxonomic delimitation of *Nuphar* species (Heslop-Harrison 1953, Beal 1956, Padgett *et al.* 1998, 1999, 2002; Chapters 2 and 3). In Japan hybrid status of some intermediate plants has been confirmed and the unstable habitat conditions have been discussed to play an important role to the hybrid speciation (Chapter 3). In previous chapters (Chapters 2 and 3), I mainly discussed about hybridization events in lentic water, such as irrigation ponds, however.

Nuphar submersa Shiga and Kadono is a new species reported from central Japan in my study (see Chapter 6). During the course of my survey of the new species, I found some unidentified plants intermediate in morphology in rivers and streams. As the results of morphological, allozymic and pollen viability studies, I concluded the intermediate plants to be a hybrid between *N. japonica* DC., which is widely distributed in Japan (Kadono 1994), and *N. submersa*. In this chapter, I will investigate the new hybrid and discuss the hybridization event in lotic water.

MATERIALS AND METHODS

Plant materials

I collected a total of 86 individual plants from 9 populations in central to eastern Japan (Table 14). These included specimens of *Nuphar japonica*, *N. submersa*, and unidentified intermediate plants. Sampling occurred from August to early September in 2001, 2002, 2004 and 2005.

Morphological analyses

For measurements of morphological characteristics, I collected submerged leaves and flowers from 6 to 10 *Nuphar* plants in each population (Table 14). I assessed the following 13 morphological characteristics that have previously been considered important in the identification of *Nuphar* taxa (Kadono 1994, Padgett *et al.* 1999, Chapters 2, 3 and 5): submerged leaf blade length (SL1), submerged leaf blade width (SL2), submerged leaf blade shape (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 stigma (F3), anther length (F4), filament length (F5), anther length/filament length ratio (F6=F4/F5). These comprised 7 vegetative (SL1-SL7), 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. Thus, I performed cluster analysis for the 9 populations based on the mean values of each characteristic. I chose Ward's method for this 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 ($P<0.05$), I performed a multiple comparison using Scheffé's F-test. To analyze morphological relationships among individuals, I performed principal-components analysis (PCA). All statistical tests were performed using JMP ver. 4J (SAS Institute Inc., USA).

To avoid misleading correlations, I excluded three characteristics which were used for the calculation of ratios from the cluster analysis and PCA: submerged leaf blade width (SL2), sinus depth (SL4) and filament length (F5). As a result, the remaining 10 characters were used. Before applying these analyses, I arcsine-transformed the submerged leaf blade shape (SL3=SL1/SL2).

Allozyme analyses

I 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 the procedures of electrophoresis and staining were the same as in Chapters 2 and 3.

I 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 a species-specific marker, so I determined the genotypes of *lap1* and *mdh3* and compared them among the different morphological groups. Locus names were referred to as in those of Chapter 2.

Pollen viability

Methods of estimation of pollen viability were the sme as in Chapter 3. In all analyses, I examined the same individuals used in morphological and allozyme analyses (Table 14).

RESULTS

Cluster groups

The Ward's phenogram revealed two major clusters (Fig. 14), and the second cluster contained two distinct subclusters. I used these three clusters as operational taxonomic units and named them Groups 1, 2 and 3.

Significant differences were found among the three cluster gruops in all measured characteristics (nested ANOVA, $P < 0.05$; Table 15). Group 1 differed significantly from the other morphological groups 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. This species 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 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 *N. japonica* and *N. submersa*, respectively (De Candolle 1821, Chapter 6). Hereafter, I thus 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. 15a). The two species and intermediate plants could be clearly distinguished along PC axis 1 (Fig. 15a). The ranges of PCA values along axis 1 were 1.105 to 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. pumila* (–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 (Table 16). 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. 15b-c, Table 17). The genotypes of *N. japonica* were fixed as "aa" in *lap1* and "bb" in *mdh3*. On the other hand, the genotypes of *N. submersa* were fixed as "cc" in *lap1* and "aa" in *mdh3*. In contrast, morphologically intermediate plants were not homozygous in the two loci, but most of the intermediate plants were heterozygous like "ac" and "ab".

Pollen viability

The pollen viability was 93.2%±4.6% (mean ± SD) in *N. japonica*, 93.1%±3.5% in

N. submersa, and 35.5%±14.1% in the intermediate plants (Fig. 15d, Table 17). 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 of genotype, which is homozygous in 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 those of *Nuphar japonica* and *N. submersa*. Secondly, the *lap1* and *mdh3* genotypes of many morphologically intermediate plants were "ac" and "ab" heterozygotes, which are considered to be the progeny resulting from a cross between "aa" and "cc" in *lap1* and "aa" and "bb" in *mdh3*. Third, the plants of intermediate populations showed low pollen viability, although some intermediate plants exhibited moderate pollen viability. Fruit setting ratio and seed setting ratio of the intermediate populations were low in a natural habitat (e.g. Figs. 16E-F). Fourth, all intermediate populations were found within Tochigi Prefecture, where the distributions of the two putative parent species overlap. *Nuphar japonica* is distributed in Japan and Korea (Lee 1989, Kadono 1994) and *N. submersa* is endemic to Tochigi Prefecture (Chapter 6). These results indicate that the morphologically intermediate plants represent hybrids between *N. japonica* and *N. submersa*.

Some intermediate plants did not show additive combinations of parental allozyme bands, as would be expected in F_1 hybrids. This suggests that sexual

reproduction has occurred among the hybrid plants. The pollen viability of the putative later progeny was higher than putative F_1 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, morphological 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). The 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 have not occurred in this hybrid complex.

All of the hybrid populations were located in the irrigation ditches with seasonal water level fluctuation (Takashi Shiga and Yasuro Kadono, unpublished data). In Japanese *Nuphar* hybrids, some populations of *N.* ×*saijoensis* (Shimoda) Padgett and Shimoda and *N.* ×*hokkaiensis* Shiga & Kadono (=*N. japonica* × *pumila*; see Chapter 6) occurred in artificially fluctuating environments such as irrigation ponds (Shimoda 1991, Chapter 3). The hybrid between *N. japonica* and *N. submersa* may also adapt to such a changing lotic water environment and hybrid speciation may be in progress.

I described this hybrid as *N.* ×*fuluminalis* Shiga & Kadono in Chapter 6.

CHAPTER 5

Inter and intraspecific phylogeny and geographic variation of *Nuphar* species in Japan and adjacent countries based on the AFLP fragment and allozyme data

INTRODUCTION

Recent theoretical and empirical advances in the field of molecular genetics have provided many insights into the assessments of evolutionary genetic relationships among populations for a number of species (Avise 2000). Phylogeographic studies interpret the historical processes that may have left their evolutionary signatures on the present geographic distributions of genetic traits (Lowe *et al.* 2004). Aquatic plants are particularly suitable for historical biogeographic studies due to their limited dispersal capacity. It is supported that land is a barrier to their dispersal and thus local populations are confined to their own watershed and isolated from one another (Johansson and Nilsson 1993).

The species of the genus *Nuphar* Sm. are perennial aquatic vascular plants in north temperate regions. A plant of this genus grow clonally with rhizome and the fragments may be transported by water flow and give rise to new ramets (Heslop-Harrison 1955, Smits *et al.* 1989). It is considered that seed-dispersal of

Nuphar species also depend on water flow (Smith *et al.* 1989, Hart and Cox 1995). Smith *et al.* (1990) have studied germination traits for *N. lutea* (L.) Sm., which evidenced that seeds could not germinate after chewing by waterfowls or after dehydration treatment. Pollinators were beetles and flower flies, which pollinated in short range (Lippok *et al.* 1997, 2000). Hence, in *Nuphar* species, it seems that geographical patterns of watershed strongly affect the gene flow and distribution of genealogical lineages. Furthermore, it is needed to understand genetic relationships and geographical patterns to solve variable morphology and patterns of hybrid zones of *Nuphar* species.

In this chapter, I investigated the genetic features in Japanese, Korean and Taiwanese populations of *Nuphar* species, that is, *N. japonica* DC., *N. oguraensis* Miki, *N. pumila* (Timm) DC., *N. shimadae* Hayata and *N. subintegerrima* (Casp.) Makino, by AFLP and allozyme analyses in order to clarify genetic relationships of genealogical lineages and its geographic patterns. Genetic data were also used to elucidate the congruence between phylogeny and current taxonomy of the species and varieties based on morphological and geographic patterns. To investigate phylogeographic patterns, genetic studies based on molecular markers such as allozymes and organelle and nuclear genome sequences are effective. The slow rate evolution of non cording and intron region of cpDNA and mtDNA sequence, however, conspire to limit the resolution of phylogeographic patterns revealed in studies of plants (Schaal and Olsen 2000). Thus, I tried to solve phylogenetic relationships and geographical patterns of *Nuphar* species, using AFLPs, which are high variability nuclear markers. And I also used allozyme markers to estimate and compare genotypic diversities among and within the populations for each species.

MATERIALS AND METHODS

Nuphar species and its distribution

Five species and some intraspecific taxa distribute in Japan, Korea and Taiwan, according authors to Ohwi (1965), Tamura (1981), Lee (1989), Kadono (1994) and Fu (2001). *Nuphar japonica*, which is a large emergent species, distributes in overall Japanese archipelago and Korean peninsular (Lee 1989, Kadono 1994; Chapters 2 and 3). *Nuphar oguraensis* is a floating leaved species and distributes in Western Japan (Kadono 1994; Chapter 2). Some specimens from Korea had a petiole with central lacuna (*Kadono s.n.* in herbarium of Kobe Univ.; Table 18), which is a diagnostic character of *N. oguraensis* (Miki 1934), although only *N. japonica* and *N. pumila* are recognized in Korea (Lee1989). *Nuphar oguraensis* is also confirmed to be distributed in Korea in the present study. Distribution range of *N. pumila* is wide from Lapland to East Asia (Hara 1951) and this species is floating leaved taxon. In Japan, *N. pumila* distribute in Hokkaido Island and north Japan (Kadono 1994; Chapter 3). One endemic species, *N. shimadae*, is distributed in Taiwan (Hayashi 2002), although taxonomic delimitation between *N oguraensis* and *N. shimadae* is indefinite (Miki 1934, 1937). A present distribution of *N. subintegerrima*, which is a small plant, is endemic to Tokai District, Japan (Makino 1910; Chapter 2).

Plant materials

A total of 65 *Nuphar* plants were collected from 60 localities in Japan, Korea, Taiwan and Slovakia; 29 of *N. japonica*, nine of *N. oguraensis* var. *oguraensis*, four of *N. oguraensis* var. *akiensis*, one of *N. shimadae*, two of a new submerged taxon (Chapter 6) that I provisory named "*N. submersa*" in this chapter, 11 of *N. pumila* var. *pumila*, 3 of *N. pumila* var. *ozeensis* (Table 18). European taxon, *N. lutea*, was added to

compare with the Asian taxa. Based on the results of a molecular phylogenetic study by Les *et al.* (1999), *Barclaya longifolia* Wall. was chosen as the out-group taxon in the phylogenetic study. In this chapter, I treated each *Nuphar* plants as operational taxonomic units (OTUs).

AFLP analysis

The methods of AFLP analysis were the same as in Chapter 3.

Chloroplast DNA analysis

The method of DNA extraction and cpDNA analysis were the same as in Chapter 3. I identified haplotypes of *trnL* intron region of *N. japonica.*

Allozyme analysis

I 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. Sampling numbers, the buffer systems, investigated enzymes and procedures of electrophoresis and staining were the same as in Chapters 2, 3 and 4. Sampling numbers were the same as in Chapters 2 and 3 (Tables 3, 10).

Morphological analysis

If cpDNA and AFLP analyses showed clear intraspecific group in *N. japonica*, I measured morphological characters, which were used in Chapters 2, 3, and 4, to elucidate whether morphological differentiation have occurred. All populations except for IK-1, SI-3, and WA-1 were investigated. Sampling numbers were the same as in Chapters 2, 3, and 4 (Tables 3, 10, 15).

Data analysis

Phylogenetic analysis was performed using PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein 1993). A phylogenetic tree based on nucleotide diversity as a measure of genetic distance (nucleotide substitution per nucleotide site, *d*; Nei and Miller 1990, Innan *et al*. 1999) was constructed using neighbor-joining method (Saitou and Nei 1987). These were exported to NEIGHBOR program from the PHYLIP package.

To estimate the historical process of dispersal, pairwise distance comparison between genetic and geographical distance were conducted within a species or population (Hutchison and Templeton 1999). Geographic distance was taken as the minimum distance between two locations, and calculated from latitude and longitudes of sampling locations by the GRS80 method (http://vldb.gsimc.go.jp/sokuchi/surveycalc/bl2stf.html). The significance of correlation between genetic and geographic distance was assigned by Mantel test (10000 permutations; Mantel 1967) using IBD program version 1.52 (Bohonak 2002).

I applied the criteria described by Good and Wake (1992) to evaluate whether different cohesive groups exist within species. This method analyzes genetic differentiation as a function of spatial separation, based on a plot of pairwise genetic distance versus geographical distances. A regression line is fitted to a set of points representing pairs of populations from a priori defined subsets of samples, and if the regression line passes through the origin, the samples are interpreted as being conspecific, because this pattern is most readily explained by gene flow with isolation-by-distance. Conversely, the regression for samples that include genetically isolated groups will deviate significantly from a 0 origin, because genetic divergence

among samples is expected to be independent of their degree of geographical separation. This analysis was performed for *N. japonica*.

In order to evaluate genetic diversity, the proportion of shared fragments for each pair of samples, F (Nei and Li 1979, Innan *et al.* 1999) was calculated for AFLP data. From the value of F obtained for each taxon, I estimated the nucleotide diversity, π, i.e., the average number of nucleotide substitutions per nucleotide site, using the method of Innan *et al.* (1999) for AFLP data. This method takes into account relative probabilities of evolutionary events such as mutations in the restriction sites or in the additional selective nucleotides, and appearance of new restriction sites within the fragments. The nucleotide diversity was estimated about *N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima*.

Genotypic diversity, which is a diversity of genotype, within and between populations was surveyed in all Japanese taxa except for *N. submersa*. The genotypic diversity within populations was estimated by mean numbers of MLGs per population, a ratio of polymorphic populations, the Shimpson's diversity index (D) (Peet 1974). About the genotypic diversity among populations, mean numbers of populations per MLG, a ratio of unique MLGs in a population and a ratio of populations with the commonest MLG were calculated.

All statistical tests except for Mantel test were performed using JMP ver. 4J (SAS Institute Inc. USA).

RESULTS

The number of fragments scored is summarized in Table 19. A total of 835 different AFLP fragment size categories were observed from four selective primer pairs (Appendixes 2-5). For each primer pairs, of the total 194-236 fragments scored, 4-33

(1.7-17.0%) were unique for the outgroup taxon. Of the total 772 fragments scored in *Nuphar* species, 749 (97.0%) were found to be polymorphic. For each of the four species, *N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima*, 196-237 fragments were scored per samples on average (Table 20).

All the 66 plants examined were revealed to be unique genotypes (Appendix 2-5).

Phylogenetic relationships among species

Excluding European taxon, *N. lutea*, Asian *Nuphar* plants were monophyletic group with 83% bootstrap values in the NJ tree (Fig. 17; pairwise genetic distance is shown in Appendix 6). Within Asian *Nuphar* taxa, four main monophyletic groups were recognized; the first cluster consisted of *N. oguraensis*, *N. shimadae* and *N. submersa*; the other three clusters corresponded to *N. pumila*, *N. japonica* and *N. subintegerrima*, respectively. These four clusters were strongly supported with high bootstrap values (94-100%).

About the relationships among four main monophyletic groups, the plants of *N. pumila* and *N. oguraensis*, including *N. shimadae* and *N. submersa*, were sister groups with 88% bootstrap value. The relationships among the plants of *N. japonica* and *N. subintegerrima* were poorly resolved with low support value (53%), although the cluster group of *N. subintegerrima* was the ancestral position (Fig. 17). In *N. oguraensis* group, two subclusters were recognized. The first subcluster of two OTUs with 100% support value consisted of *N. submersa* and a group of *N. oguraensis* and *N. shimadae* (Fig. 17). Thirteen fragments were fixed in TG-1 and TG-2 as species-specific fragments of *N. submersa.*

Phylogenetic relationships within species

There was congruence between phylogenetic position and geographical origins of *Nuphar* plants. The cluster group of *N. japonica* consisted of two main subclusters (Clade-1 and 2) with high bootstrap values (98% and 81%; Fig. 17). The subclusters identified well corresponded to the geographical positions of the samples; one subcluster, Clade-1, from East to North Japan and another, Clade-2, from West to South Japan (Fig. 18). Two cpDNA hapolotypes were detected in *N. japonica* (Fig. 9) and these distributional patterns were very similar to the clade distributions (Fig. 18).

In the plants of *N. oguraensis*, two clusters consisting of more than one OTU were identified, although the branching orders among these two clusters and eight OTUs (GI-4, HY-8, HI-2b, HI-7, HHS, FO-1, MY-2 and MY-8) were not solved (Fig. 17). These clusters identified distributed in Korea and Taiwan (*N. shimadae*) and in Kochi Pref., Japan (Fig. 17).

The plants of *N. pumila* in Hokkaido Island were revealed to be a monophyletic group (Fig. 17). One subcluster and three OTUs were recognized within Hokkaido Island group. This subcluster consisted of two groups; the group of seven OTUs (KUT, TSC, NET, NEN, HAK, AKT and BEB) with 97% bootstrap value corresponded to plants from East Hokkaido Island (Table 18, Fig. 17) and the other of three OTUs were from Uryu-numa Moor (URU).

Within the cluster of *N. subintegerrima*, two subclusters were recognized. These subclusters distributed in Gifu, Aichi and in Mie Pref., respectively.

The plants of intraspecific taxa were not monophyletic (Fig.17). Three plants of *N. oguraensis* var. *akiensis* and three plants of *N. pumila* var. *ozeensis* did not belong to the same cluster together. These OTUs closely related with the geographically close OTUs rather than the OTUs of same intraspecific taxa (Table 18, Fig. 17).

Correlation between genetic and geographic distances

The correlation between genetic and geographic distances was demonstrated for *N. japonica*, *N. pumila*, *N. subintegerrima*, and *N. oguraensis* including *N. shimadae* in Fig. 19. In four species examined, a significant positive correlation (P<0.01) was found for *N. japonica*, *N. oguraensis* and *N. pumila*.

Figure 20 shows the correlation between genetic and geographic distance for intraspecific groups of *N. japonica*. A significant positive correlation was revealed for Clade-1 (P<0.01) and 2 (P<0.05). But the regression line of relationship between Clade-1 and Clade-2 deviated from a 0 origin.

Genetic variation within a species

The intraspecific genetic variation of the four *Nuphar* species was estimated using two parameters, proportion of shared AFLP fragments between individuals F and nucleotide diversity π (Table 20). There were two groups with regard to F and π; one had high F and low π and included *N. japonica* and *N. pumila*, and the other had low F and high π and included *N. oguraensis* and *N. subintegerrima*. The proportion of shared fragments between individuals was different significantly (H=161.2, P<0.0001).

In intraspecific groups of *N. japonica*, Clade-1 had higher F and lower π (F=0.89, π=0.0073) than those of Clade-2 (F=0.85, z=5.68, P < 0.0001; π=0.0102; Table 20), while the distributional range of the former group was much greater than that of the latter (Fig. 18).

Genotypic diversity

Statistics summarizing genotypic diversity within and among populations based on the frequencies of MLGs were shown in Table 21. The ratio of distingishable genotypes (MLG/N) of central to south regional taxa and clade, that is, *N. oguraensis*, *N. subintegerrima* and Clade-1 of *N. japonica*, were higher than those of east to north regional taxa and clade, *N. pumila* and Clade-2 of *N. japonica*.

In terms of diversity within populations, mean number of MLGs per population, the number of polymorphic populations, the diversity index (D) were higher in central to south regional taxa and clade than in others as was the case with MLG/N, although the differences in these statistics were not significant (Kruskal-Wallis test, $P>0.05$). As for diversity among populations, genetic variation among populations was also high in central to south regional taxa and clade than in others, which was indicated by low mean number of populations per MLG, many locally unique MLGs and low ratio of the commonest MLG, although there were no significant differences statistically (Kruskal-Wallis test, $P>0.05$). In *N. japonica*, *N. oguraensis*, and *N. pumila* the commonest MLGs (e.g. Jap 16, Ogu 6) had wide geographic distribution patterns (Appendix 1).

Morphological analysis

To evaluate morphological differentiation between the two clades of *N. japonica*, I measured 28 morphological characters; 7 emergent leaf, 7 submerged leaf, 8 floral, 6 fruit characters (Table 22). Significant differences between the two clades of *N. japonica* were found in 19 characters (t-test, $P<0.05$). Clade-1 had the higher values in 8 characters and the lowest values in 11 characters. The plants of Clade-1 were larger in plant size than those of Clade-2. In leaf morphology, Clade-1 had long

narrowly oblong leaves and narrowly ovoid fruits and seeds. On the other hand, Clade-2 had oblong to ovate leaves, in which sinus is deep like floating leaves and ovoid fruits and seeds.

DISCUSSION

Phylogenetic relationships among species

The neighbor-joining topology based on AFLP data supported interspecific relationships, which were proposed by previous phylogenetic study based on morphology and nrDNA sequences of ITS and *matK* (Padgett *et al.* 1999), excepted for *N. subintegerrima*. Padgett *et al.* (1999)'s phylogenetic tree was poorly resolved as to relationships among dwarf *Nuphar* species and their phylogenetic tree was biased for morphological data. As AFLP data provided clear phylogenetic relationships of Japanese *Nuphar*, further study by AFLP is needed to elucidate phylogeny of the genus *Nuphar*.

The phylogenetic tree showed both *N. pumila* and *N. oguraensis* were sister taxa but well separated phylogenetically. Although Padgett *et al.* (2002) treated *N. oguraensis* as a subspecific rank of *N. pumila*, their distribution ranges do not overlap and the central lacuna of petiole distinguished *N. oguraensis* from *N. pumila* (Kadono 1994, Chapters 2 and 6).

In *N. oguraensis* group, two subclusters were recognized. The first subcluster consisted of *N. submersa* and the second one included *N. oguraensis* and *N. shimadae*, which are morphologically very similar (Miki 1934), with 100% support value. It is probable that *N. oguraensis* and *N. shimadae* are conspecific.

The phylogenetic study also showed both *N. submersa* and *N. oguraensis* to be sister taxa, but well separated phylogenetically. *Nuphar submersa*, which is a new

species from Tochigi Prefecture, is a submerged plant in most cases inhabiting rivers and streams (Chapter 6). When *N. submersa* was cultivated in lentic water, the plants rarely formed incomplete floating leaves with a partially cuticularized leaf blade (data not shown, Yoichi Komine personal communications). It is probable that *N. submersa* lost the genetic ability to form floating leaves. This result is a case that submerged species has evolved from floating-leaved species in Japan.

Genetic and morphological differentiation of geographic lineage

In my AFLP analysis, the two clades of *N. japonica* showed significant correlation each other between geographic distance and genetic distance. But the regression line of relationship between Clade-1 and Clade-2 deviated from a 0 origin (Fig. 20). This is an expected pattern when two compared groups do not form a single genetically cohesive group (Good and Wake 1992, Sites Jr. and Marshall 2003). The geographical distribution ranges of the two AFLP groups did not overlap and the two cpDNA haplotypes indicated almost the same distributional patterns (Fig. 18). Furthermore, the plants of the two groups had significantly different morphological traits (Fig. 21, Table 20). Hence these results provide that the two groups are separating from the same cohesive group (Templeton 1989), and allopatric speciation may be in progress in *N. japonica*.

It is suggested that geographic patterns of species and interspecies lineages of aquatic life, which depended on hydrochory for dispersal, were affected by geographic pattern of water system (e.g. Avise 2000, Takehana *et al.* 2003). Geographic distribution of subclades of *N. japonica* and those of *N. pumila* and *N. oguraensis*, which are closely related species, showed similar pattern (Fig. 24). The distribution of southern clade and species, Clade-2 of *N. japonica* and *N. oguraensis*, is limited to

the region south from Kinki and Tokai district. Geographical border may be existent in central Japan in *Nuphar* species.

Genetic diversity, differentiation and genotypic variation

Nucleotide diversity, genetic differentiation and genotypic diversities within and among populations of east to north regional clade and species were also lower than those of central to south regional clade and species, although there were no significant differences statistically. It is possible that the eastern to northern populations of *Nuphar* had experienced bottleneck effect. Glacial-interglacial cycles during the Pleistocene have had profound effects on the evolutionary history and the distribution pattern of northern temperate plants (Avise 2000, Petit *et al.* 2002, Sawada *et al.* 2003). Present distributional patterns and geographical genetic variation of Japanese *Nuphar* may result from distribution of refugia in glacial periods. According to some authors (Beal 1956, Cook 1990, Padgett 1999, Padgett *et al.* 1999), only two species, *N. lutea* and *N. pumila*, are distributed widely in the northern Eurasian Continent. However I recognized more species in Japan (see Chapter 6). The evidences in the previous chapters suggest that genetic and morphological divergence among them, and speciation in Japan. Further molecular study including other old world species and new world species is needed to know detailed evolutional history of the genus *Nuphar*.

Nuphar subintegerrima showed high level of genetic differentiation, nucleotide and genotypic diversities (Tables 20, 21), regardless the present restricted distribution (Fig. 3). Some studies have indicated an association between the distributional range and genetic variation. That is, broadly distributed species showed higher genetic diversity than the species with restricted distribution.

Endemic species, in particular, showed very low isozyme diversity within and among populations (Lesica *et al*. 1988, Hamrick and Godit 1989, Santamaría 2002). Genetic differentiation in *N. subintegerrima* may reflect the isolated situation caused by the disappearance of formerly distributed many populations.

It seems common in aquatic plants that genetic and genotypic variation within populations is low and high among populations (Les 1991, Laushman 1993, Hollingworth *et al*. 1996, Kaplan and Štěpánek 2003). Similar tendency was confirmed in all *Nuphar* species (Table 21). The dominance of clonal reproduction results in low genotypic diversity within a population. The species of *Nuphar* grow clonally with rhizome and the fragments may be transported by water flow and give rise to new ramets (Heslop-Harrison 1955, Smits *et al*. 1989). No or few seedlings of *N. lutea* and *N. polysepala* Engelm. were found in the field and vegetative reproduction was suggested to be dominant in these species (Hart and Cox 1995, Barrat-Segretain 1996). On the other hand, in *N. pumila* var. *ozeensis* seedling establishment had been reported in Ozegahara Moor of central Japan (Murayama *et al*. 1998, Kanai 2002). In this study, many populations of *N. japonica*, *N. oguraensis* and *N. subintegerrima* consisted of several genets, but the number of the clones was limited in many cases (Table 21). In the AFLP analysis, the populations of *N. japonica* and *N. pumila* in Hokkaido consisted of some genets (Table 13, Chapter 3). In addition, I observed the seedling establishment in the populations of *N. japonica* (YA-1), *N. oguraensis* (HY-8), and *N. pumila* (URU) (data not shown). Hence seedling establishment may be frequently in Japanese *Nuphar* species, although vegetative reproduction must be dominant.

The commonest MLGs of *N. japonica*, *N. oguraensis*, *N. pumila* (e.g. Jap 16, Ogu 6; Appendix 1) were distributed widely. In clonal plants, widespread genotypes

indicated that vegetative organ is dispersed in other sites and the same clones spread widely (Hollingsworth *et al.* 1996, Kadono *et al.* 1997) or the total number of clones detected is an underestimate (Ellstrand and Roose 1987). In *Nuphar*, the rhizome is dispersed to the same water body but difficult to disperse to other distant water bodies (Smits *et al.* 1989, Hart and Cox 1995, Barrat-Segretain 1996). Moreover, these MLGs are homozygote in all loci (Appendix 1). Therefore, it is likely that the widespread MLGs are not the same genet.

CHAPTER 6

A taxonomic revision of genus *Nuphar* Sm. in Japan

INTRODUCTION

The genus *Nuphar* Sm. is taxonomically the most problematic genus in the Nymphaeaceae because of its polymorphic morphology (Beal 1956, Padgett *et al.* 1999). Taxonomic interpretations of *Nuphar* have varied and two to ca. 20 species have been recognized in the world according to authors (Beal 1956, Cook 1990, Padgett 1999, Padgett *et al.* 1999). Recent phylogenetic study based on morphological data, cpDNA and nrDNA sequences gave evidence to the presence of two major lineages within this genus, although relationships within the major lineages were poorly resolved (Padgett *et al.* 1999). One lineage comprises primarily the Old World taxa and the other represents the New World taxa.

In Japan, taxonomic treatments of *Nuphar* have generally recognized four species, that is, *N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima*, and the taxa except for *N. pumila* are endemic to the East Asia (e.g. Ohwi 1965, Tamura 1982, Kadono 1994). Many taxonomic studies of *Nuphar* have been conducted in Europe and North America (Morong 1886, Harz 1893, Schuster 1907-1908, Miller and Standley 1912, Heslop-Harison 1955, Beal 1956). However, there have been rather few studies in Asia including Japan. Recent taxonomic studies based on a

restricted number of herbarium specimens by Padgett (1999, 2003) recognized only two species, *N. japonica* and *N. pumila*, in Japan.

Taxonomy of *Nuphar* is problematic because of not only variable morphology but also incomplete herbarium specimens. Herbarium specimens of *Nuphar* are notoriously incomplete and measurements of dried specimens are considered too inaccurate to allow a critical analysis of variation (Heslop-Harrison 1955, Beal 1956, Padgett 1999). So, intensive studies based on fresh materials of many populations of *Nuphar* are needed.

In this chapter, I will propose a taxonomic revision of Japanese *Nuphar* taxa based on the results of previous chapters. I investigated morphology, using fresh materials, and genetic features of many populations in Chapters 1-5. In addition, I also examined ca. 800 sheets of herbarium specimens, including some European specimens. Specimens in the herbarium of Hiroshima University (HIRO), The Kyoto University Museum (KYO), Makino Herbarium, Tokyo Metropolitan University (MAK), Osaka Museum of Natural History (OSA), The Hokkaido University Museum (SAPS), Botanical Garden of Hokkaido University (SAPT), Taiwan Forest Research Institute (TAIF), The University of Tokyo (TI), National Science Museum, Tokyo (TNS), Tochigi Prefectural Museum (TOCH), Herbarium Tsudanum, Tohoku University (TUS) and Kobe University (tentatively abbreviated as KOBE) were examined. Here six species (Table 23), including four forms, and three nothospecies are enumerated from Japan based on morphological, genetical, ecological and geographical studies. Measurement values refer to fresh materials.

KEY TO THE JAPANESE SPECIES OF NUPHAR

A. Leaves always submerged under natural conditions; submerged leaves narrowly

oblong-triangular, base slightly cordate-sagittate.....1. *N. submersa*

A. Leaves both emergent or floating as well as submerged; base of leaves with a deep sinus

B. Leaves emergent

C. Emergent leaves widely ovate to oblong, 10-50 cm. Seeds 3.0-5.5 mm long

D. Emergent leaves narrowly ovate to oblong, 25-50 cm long

..... 2. *N. japonica*

D. Emergent leaves widely ovate to narrowly ovate, 10-30 cm long

..... 3. *N. saikokuensis*

C. Emergent leaves rounded, 4-17 cm long; seeds 5.5-6.5 mm long

.....4. *N. subintegerrima*

B. Leaves floating

C. Anther and filament length ratio 1:1 to 1:2

D. Floating leaves rounded, 4-17 cm long; seeds 5.5-6.5 mm long

..... 4. *N. subintegerrima*

D. Floating leaves widely ovate to narrowly ovate, 10-30 cm long; seeds 3.0-4.5 mm long ..... 3. *N. saikokuensis*

C. Anther and filament length ratio 1:2 to 1:3

D. Petiole without lacuna ..... 5. *N. pumila*

D. Petiole with central lacuna ..... 6. *N. shimadae*

TAXONOMY

1. **Nuphar submersa** Shiga & Kadono, **sp. nov**. (Figs. 24, 25, 26).

=*Nuphar* sp., Plants of Utsunomiya City 146, tab. 34 (2001).

="*Nuphar subintegerrima*" auct. non Makino: Sekimoto, Index of Plant of Tochigi

Prefecture 19 (1951); Plant Society of Tochigi Prefecture, List of Plants in Tochigi Prefecture 126 (1968); Hasegawa, Vegetation and Flora of Tochigi Prefecture, tab. 155 (1983); Komine, Plants of Tochigi **1**: 199 (2003).

="*Nuphar japonica*" auct. non DC.: Komine, Plants of Tochigi **1**: 199 (2003), p. p.; Komine, Plants of Tochigi **2**; 142 (2003), p. p.

Haec species nova *Nuphari japonicae* DC. et *N. oguraensi* Miki proxima est, sed ab eis foliis submersis anguste oblongis triangulatis, non sinuosis, antheris rufescentibus et fructibus rubris differt.

Typus: JAPAN; Tochigi Pref.; Koshiro, Imaichi-shi, alt. 240 m, Sept. 29, 2004, *T. Shiga 3480* (Holotype OSA, Isotypes KYO, TNS).

Description: Perennial aquatic herbs. Rhizomes slender, procumbent, branching. Leaves submerged or rarely floating in lentic water; submerged leaves narrowly oblong-triangular, 10-18 cm long, 2-5 cm wide (Figs. 25A, 26), membranaceous, undulate, without sinus; floating leaves narrowly ovate, base slightly cordate-sagittate. Petiole flattened, usually with central lacuna (Fig. 22H). Peduncle raised above the water. Flowers yellow, June to October, 2-3 cm across, protogynous; sepals 5, obovate, apex rounded, 1-2 cm long (Figs. 25C-D); petals spatulate, 5-7 mm long (Fig. 25E); anthers strongly recurved after anthesis (Figs. 24B, 25G), 1.5-2.5 mm long, the length ratio of anther to filament 1:2 to 1:3, pollen sack tinged orange to red (Figs. 24C, 25F); carpels many, fused; stigmatic disc tinged reddish to dark red, 4-8 mm across, 7-9 rays, shallowly dentate, stigmatic rays usually regularly arranged, 2-3 mm long (Fig. 24A); fruit reddish, ovoid, 2-3 cm long (Fig. 23); seeds numerous, narrowly ovoidal to ovoidal, 3.5-4.5 mm long, 2.5-3.5 mm wide (Fig. 25H).

Japanese name: Shimotsuke-kōhone (nov.)

Distribution: Japan (Tochigi Pref.). Endemic.

Hab.: Rivers and streams.

Note: *Nuphar submersa* is a new submerged species inhabiting rivers and streams, which resemble *N. japonica* in the morphology of submerged leaves and *N. oguraensis* in the flower morphology and petiole anatomy. Narrow oblong submerged leaves and reddish stigmatic disc of this species have been often confused with *N. japonica* or *N. pumila* var. *ozeensis* (Hasegawa 1982, Botany Section of Natural Environment Research Group of Tochigi Prefecture 2003ab), although Hasegawa (2001) suggested this species was different from *N. japonica* and the the other *Nuphar* species.

The phylogenetic study (Chapter 5) showed both *N. submersa* and *N. oguraensis* (= *N. shimadae*) were sister taxa but well separated phylogenetically. Diagnostic morphology and life form of *N. submersa* with submerged leaves alone in lotic water differ from *N. oguraensis*. Furthermore, *N. submersa* is isolated ca. 300 km east from easternmost locality of *N. oguraensis* (Aichi Pref.). Therefore, I described *N. submersa* as a new species. Further studies about relationships of dwarf *Nuphar* species is needed to know whether this species should be treated as a subspecies or varietiy of *N. pumila*.

The investigation of herbarium specimens showed that distribution range of *N. submersa* was restricted to Tochigi Pref. (Fig. 27). In my field research, only two small populations were located in Tochigi Pref. and most of the populations have been extinct to date; population size of TG-1 was 0.5m × 30m and TG-2 was fragmented in 1m × 100m range. It has proven to be one of the species most urgently in need of protection in the conservation programs of Japanese flora.

The *Nuphar* plant called “Nagaba-beni-kōhone” by Japanese aquatic plant cultivators is sometimes this species.

Specimens examined: **JAPAN: Tochigi Pref.**: Hosyakuji-mura, Jul. 31, 1951, *Y. Matsumura s.n.* (TNS 011391, 016444); Mine-cho, Utsunomiya-shi, Sept. 18, 1947, (TOCH 121889); Shimokawai, Minaminasu-machi, alt. 130 m, Sept. 6, 1995, *Y. Komine 81551* (TOCH); Nov. 2004, *H. Hirayama s.n.* (KOBE); Aug. 6, 2005, *T. Shiga 3560-3562* (KOBE, OSA); Koshiro, Imaichi-shi, alt. 240 m, Jul. 26, 2003. *M. Komakura & T. Sugawara s.n.* (TOCH 139148); Sept. 30, 2004, *T. Shiga 3479* (KOBE, OSA), Aug. 6, 2005, *T. Shiga 3595* (KOBE); Sakazura, Kawachi-cho, Kawachi-gun, Aug. 17, 1980, *T. Noguchi s.n.* (KOBE); Nomoto-gawa River, Higashimizunuma, Haga-cho, Haga-gun, Aug. 8, 1988, *Y. Kadono 5528* (KOBE).

2. **Nuphar japonica** DC., Syst. Nat. (Candolle) **2**: 62 (1821); Icon. Select. Pl. **2**: 3. tab. 6 (1823); Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 82. tab. 3. F., fig. 48. A-E (1937); Makino, Makino's New Ill. Fl. Jap. tab. 653 (1961); Ohwi, Fl. Jap. 437. fig. 5 (1965); Kitamura & Murata, Col. Ill. Herb. Pl. Jap. **2**: 252. tab. 56, fig 113-1 (1972); Ohtaki & Ishido, Ill. Jap. Water Pl. 91. tab. 41 (1980); Tamura, Wild Fl. Jap. **2**: 94. pl. 93-4 (1982); Lee, Ill. Fl. Korea 339 (1989); Kadono, Aquat. Pl. Jap. 112, 114 (1994). ≡*Nymphaea lutea* sensu Thunberg, Fl. Jap. 223 (1784). non. L. ≡*Nuphar japonicum* DC. var. *crenatum* Casp. subvar. *luteum* (= *flava*) Casp., Ann. Mus. Bot. Lugduno-Batavi. **2**: 254, tab. Ⅷ, fig. 11-22 (1865); Makino, Bot. Mag. (Tokyo) **11**: 279 (1897). ≡*Nymphaea japonica* (DC.) Kuntze, Rev. Gen. Pl. **1**: 12 (1891). ≡*Nymphozanthus japonicus* (DC.) Fernald, Rhodora **21**: 187 (1919). **Lectotype [designated here]**: Icon. Select. Pl. **2**: 3. tab. 5 (1823).

=*Nuphar japonicum* DC. var. *stenophyllum* Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 82. tab. 3. G., fig. 48. F-G (1937). nom. nud.

Description: Perennial aquatic herbs, procumbent. Rhizomes branching, stout. Leaves spirally arranged, emergent and submerged, rarely floating in deep water; submerged leaves narrowly oblong, 10-50 cm long, 7-20 cm wide, membranaceous, margin undulate, or submerged leaves sometimes absent in well grown populations in stagnant water; emergent and floating leaves subcriaceous, narrowly ovate to

oblong, 20-52 cm long, 10-26 cm wide, base with a deep sinus, entire, apex obtuse to rounded, upper surface glabrous, lower surface slightly pubescent. Petiole usually terete to more of less flattened above (Fig. 22A). Peduncle raised above water. Flowers yellow, June to October, 4-6 cm across, protogynous; sepals 5, obovate-orbicular, apex rounded, 2-3 cm long, subcoriaceous, yellow, becoming green in fruit; petals inconspicuous, obovate-cuneate, ca. 8 mm long; anthers many, 3-8 mm long, ratio of pollen sack to filament length ca. 1:1 to 1:2; pistil 1, carpels, many, fused; stigmatic rays 3-6 mm long; disc of stigma yellow, 7-16 mm across, 8-23 rays, dentate; fruit green, ovoid to subglobose, 3-6 cm long (Fig. 23); seeds numerous, ovoid, 4.0-5.5 mm long, 2.5-4.5 mm wide.

Nuphar japonica DC. f. **japonica**

Japanese name: Kōhone.

Distribution: Japan (Hokkaido, Honshu, Shikoku and Kyusyu) and Korea

Hab.: Lakes, ponds, rivers and streams.

Note: De Candolle (1821) did not designate type specimen of *N. japonica* DC. in his description. After that, in Icones Selectae Plantarum vol. 2 (Delessert 1823), Turpin illustllated *N. japonica* (tab. 6) based on original material which was used for description. Thus, I selected the illustration to serve as the lectotype. This plate clearly shows the diagnostic anther and filament ratio, which is ca. 1:1, and narrowly oblong emergent leaf with a deep sinus.

In my genetic and morphological study, two geographical lineages of *N. japonica* are cleary recognizable (Chapter 5). Although these groups seems to be geographically separating from same cohesive groups and differ in many morphological traits, preliminary artificial cross between the two groups provides

fertile seeds (data not shown) and the morphology between the groups is continuous (Fig. 20). Therefore I did not admit intraspecific rank of *N. japonica*. Miki (1937) reported narrowly oblong submerged leaf plants as *N. japonica* var. *stenophylla*, which is naked name. North distributional group (Clade-1) includes plants of this morphological type (Fig. 20).

Specimens examined: **JAPAN**: **Hokkaido Pref.**: Sarobetsu, Teshio-gun, Sept. 1966, *M. Tohyama s.n.* (SAPS); Toikanbetsu, Horonobe-cho, Teshio-gun, Jul. 20, 1956, *H. Hara s.n.* (TI); Jul. 20, 1956, *H. Hara & S. Kurosawa s.n.* (TI); Shimonuma, Horonobe-cho, Teshio-gun, Aug. 3, 1940, *S. Sugawara 9909* (SAPT); Otoi, Horonobe-cho, Teshio-gun, Sept. 1, 2004, *T. Shiga & S. Takebayashi 89* (KOBE, OSA); Wakkasakanai, Toyotomo-cho, Teshio-gun, Sept. 14, 1982, *F. G. Meyer, S. G. March, M. Kawase, D. G. Nielsen and H. Takahashi 18951* (SAPT); Jul. 23, 1987, *Y. Kadono 4826* (KOBE); Nakagawa-cho, Nakagawa-gun, Aug. 10, 1918, *S. Sugawara 9910* (SAPT); Lake Kimoma-numa, Sarufutsu-mura, Souya-gun, Sept. 4, 1966, *S. Hayashi s.n.* (SAPS); Asaginodaichi, Sarufutsu-mura, Souya-gun, Aug. 25, 1999 *H. Tachibana & M. Yamazaki s.n.* (SAPT); Aug. 31, 2004, *T. Shiga & S. Takebayashi 84* (KOBE, OSA); Lake Kamuito-numa, Asagi, Sarufutsu-mura, Souya-gun, Sept. 2, 2004, *T. Shiga & S. Takebayashi 91* (KOBE, OSA); Lake Mokeuni-numa, Sarufutsu-mura, Souya-gun, Jul. 26, 1998, *H. Fujita 9800655* (SAPT); Takigawa-shi, Aug. 29, 1988, *Y. Gouda 127* (KYO); Tsukigaumi, Tsukigata-cho, Kobato-gun, Aug. 10, 1991, *Y. Kadono 7145* (KOBE); Sept. 4, 2004, *T. Shiga 3467* (KOBE, OSA); Horomui, Iwamizawa-shi, Jul. 13, 1884, *K. Miyabe s.n.* (SAPS); Jul. 27, 1887, *K. Miyabe s.n.* (SAPS); May 27, 1906, *S. Ito s.n.* (SAPS); Bibaitappu, Kita-mura, Sorachi-gun, Sept. 4, 2004, *T. Shiga 3469* (KOBE, OSA); Shintotsukawa-mura, Sorachi-gun, Aug. 26, 1960, *Y. Matsumura s.n.* (KYO); Tsuru-numa Pond, Naganuma-cho, Yubari-gun, Jul. 24, 1967, *T. Sasaki s.n.* (SAPS); Sapporo-shi, Jul. 1878 (SAPS); Nakanuma-cho, Higashi-ku, Sapporo-shi, Sept. 10, 2004, *T. Shiga 3474* (KOBE, OSA); Shirakawa-ku, Sapporo-shi, Oct. 8, 1939, *T. Inoue s.n.* (SAPS); Shinoro River, Barato, Kita-ku, Sappro-shi, Jul. 14, 1987, *Y. Kadono 4630* (KOBE); Porotokotan, Shiraoi-cho, Shiraoi-gun, Aug. 26, 1971, *M. Hara 142, 143* (SAPS); Toasa-gawa River, Toasa, Hayakita-cho, Yufutsu-gun, Jul. 15, 1985, *Y. Kadono & G. Wiegleb 876* (KOBE); Toasa-gawa River, Hayakita-cho, Yufutsu-gun, Aug. 25, 1988, *Y. Kadono 5631* (KOBE); Tomino, Atsuma-cho, Yufutsu-gun, Aug. 23, 2004, *T. Shiga 3444* (KOBE, OSA); Tomakomai-shi, Aug. 21, 1899, *J. Hanzawa s.n.* (SAPS); Lake Utonai-ko, Tomakomai-shi, Aug. 18, 1975, *S. Nakayama et al. s.n.* (SAPS); Yufutsu, Tomakomai-shi, Aug. 25, 1892 (SAPS); Aug. 22, 1899, *J. Hanzawa s.n.* (SAPS); Yufutsu River, Tomakomai-shi, Aug. 24, 1994, *H. Takahashi 17543* (SAPS); Bisawa, Tomakomai-shi, Aug. 23, 2004, *T. Shiga 3446* (OSA); Bibi-gawa River, Bisawa, Tomakomai-shi, Jul. 14, 1985, *Y. Kadono & G. Wigleb 863* (KOBE); Bibigawa River, Uenae, Tomakomai-shi, Aug. 4, 1991, *H. Takahashi 11215* (SAPS); Oikomanai, Taiki-cho, Hiroo-gun, Jul. 26, 1979, *E. Miki & M. Ito 93* (KYO, TNS); Lake Oikamanai-numa, Bansei, Taiki-cho, Hiroo-gun, Aug. 24, 2004, *T. Shiga 3457* (KOBE, OSA); Shizukari Moor, Oshamanbe-cho, Yamakoshi-gun, Aug. 9, 1986, *Y. Kadono 3958* (KOBE); Utazai , Kuromatsunai-cho, Suttsu-gun, Aug. 9, 1986, *Y. Kadono 3945* (KOBE); Esa-cho, Hiyama-gun, Jul. 10, 1950, *S. Sugawara 9914* (SAPT); Hakodate-shi, 1861 (TNS); Jul. 1877 (TI); Aug. 1886, *S. Nozawa s.n.* (SAPS); 1903, *U. Faurie 6221* (KYO). **Aomori Pref.**: Lake Ane-numa, Kamikita-cho, Kamikita-gun, Aug. 6, 1986, *Y. Kadono 3854* (KOBE); Itako-numa, Noheji-cho, Kamikita-gun, Jul. 3, 1984, *O. Tominaga s.n.* (OSA); Lake Usoriyama, Osore-zan, Ohata-cho, Shimokita-gun, Aug. 7, 1986, *Y. Kadono 3905* (KOBE); Fukuhara, Kizukuri-cho, Nishitsugaru-gun, Aug. 20, 1989, *Y. Kadono 6203* (KOBE);

Kanzawa-tameike Pond, Koshimizu, Kizukuri-cho, Nishitsugaru-gun, Aug. 20, 1989, *Y. Kadono 6218* (KOBE); Kizukuri-cho, Nishitsugaru-gun, Jul. 3, 1984, *Y. Miyatake & O. Tominaga s.n.* (OSA); Lake Oni-numa, Dekishima, Kizukuri-cho, Nishitsugaru-gun, Aug. 16, 2001, *T. Shiga 3639* (OSA), *3640* (KOBE, OSA); Ohtaki-numa, Tateoka-mura, Nishitsugaru-gun, Aug. 1, 1954, *I. Yokouchi s.n.* (MAK); Wakado-cho, Hirosaki-shi, Sept. 22, 1991, *T. Nakamura 626* (KOBE); Saruka, Onoe-cho, Minamitsugaru-gun, Aug. 22, 1880 (TI). **Iwate Pref.**: Lake Ohnuma, Hachimantai, Hachimantai-shi, Jul. 22, 1953, *H. Hara s.n.* (TI); Nagai, Tamayama-mura, Iwate-gun, Sept. 20, 1991, *T. Nakamura 607* (KOBE); Shimo-nukazuka, Ezuriko-mura, Waga-gun, Sept. 2, 1985, *G. Wiegleb & Y. Kadono 846* (KOBE); Takekoma-cho, Rikuzentakata-shi (Cult.), Aug. 5, 1903, *G. Toba s.n.* (MAK). **Miyagi Pref.**: Wakuya-cho, Tooda-gun, Sept. 18, 1991, *T. Nakamura 457* (KOBE); Gamou, Miyagi-shi, Jul. 2, 1915, *Y. Ogura s.n.* (TI). **Akita Pref.**: Hinai-cho-ohgida, Odate-shi, Jun. 14, 1920, *M. Itsumi 257* (KYO, MAK); Akita-shi, 1930 (TI); Aug. 22, 2004, *T. Shiga 3438* (OSA); Kamikitatesaruta, Akita-shi, Aug. 15, 2001, *T. Shiga 3637* (OSA), *3638* (KOBE, OSA); Lake Ohnuma, Umesawa, Tazawako-cho, Senboku-gun, Jun. 9, 2002, *T. Shiga 3118* (KOBE, OSA), *3119* (OSA); Mizuhataya, shimizu, Nakasen-cho, Senboku-gun, Sept. 2, 1985, *Y. Kadono & G. Wiegleb 877* (KOBE); Otogoe, Yuwa-cho, Minamiakita-gun, Aug. 24, 1989, *Y. Kadono 6326* (KOBE); Takenari-gata, Konoura-cho, Yuri-gun, Aug. 25, 1989, *Y. Kadono 6347* (KOBE). **Yamagata Pref.**: Tazawa, Murayama-shi, Jun. 9, 2002, *T. Shiga 3120* (KOBE, OSA), *3121* (OSA); Naikawa, Tsuruoka-shi, Jul. 27, 1933, *S. Murai s.n.* (KYO); Shimo-nagabashi, Yuza-cho, Akumi-gun, Aug. 25, 1989, *Y. Kadono 6353* (KOBE). **Fukushima Pref.**: Nozawa, Sekisawa, Iitate-mura, Souma-gun, Aug. 23, 2002, *T. Shiga 3250* (KOBE, OSA); Lake Inawashiro, Shidahama, Nakakomatsu, Inawashiro-machi, Yama-gun, Aug. 23, 2002, *T. Shiga 3249* (KOBE, OSA); Tairanumanouchi, Iwaki-shi, Aug. 24, 2002, *T. Shiga 3252* (KOBE, OSA). **Ibaragi Pref.**: Lake Kasumigaura, Toyoshima, Azuma-cho, Inashiki-gun, Aug. 27, 1924, *Y. Narita s.n.* (TI); Lake Kasumigaura, Takasaki, Tamari-mura, Aug. 26, 2001, *T. Shiga 3641, 3642* (KOBE, OSA); Ono-gawa River, Shimone-cho, Ushiku-shi, Aug. 25, 2002, *T. Shiga 3254* (KOBE, OSA); Sotonasakaura?, Itako-shi, 1900, *Y. Suzuki s.n.* (MAK); Tsutchiura-shi, Jul. 28, 1883, *S. Matsuda s.n.* (KYO). **Tochigi Pref.**: Hitomarujinjya, Konaka-cho, Sano-shi (Cult.?), Aug. 7, 2005, *T. Shiga 3572* (KOBE, OSA); Kanuma-shi, Jul. 23, 1901, *T. Makino s.n.* (MAK). **Gunma Pref.**: Morinji-numa Pond, Horiku-cho, Tatebayasi-shi, Oct. 7, 1957, *H. Tanaka s.n.* (TNS). **Chiba Pref.**: Lake Inba-numa, Jul. 24, 1961, *Yano s.n.* (MAK); Kashiwa-shi, Sept. 15, 1958, *K. Nagata s.n.* (TNS); Mama, Ichikawa-shi, Aug. 1, 1893, *K. Miyabe s.n.* (SAPS); Mobara-shi, Sept. 22, 1920, *Osaka Women's college (= Osaka Women's Univ.) s.n.* (MAK); Near Ryufuku-ji?, Asahi-shi, Jul. 1939, *T. Makino s.n.* (MAK). **Tokyo Pref.**: Tokyo, Jun. 14, 1899, *T. Makino? s.n.* (KYO); Kokyo-higashigyoen, Chiyoda-ku (Cult.), Jul. 19, 2005, *T. Shiga 3539* (KOBE, OSA); Meiji-jingu, Shibuya-ku, 1970, *N. Kato 11596, 11600, 11604* (MAK); 1970, *N. Kato & Yano 11601* (MAK); Minami-ike, Meiji-jingu, Shibuya-ku, Oct. 16, 1970, *N. Kato 11606-11608* (MAK); Ohmiyahachiman, Wadamura, Suginami-ku, Nov. 6, 1904, *T. Makino s.n.* (KYO, MAK); Oizumi, Nerima-ku, Jul. 1933, *T. Makino s.n.* (MAK); Sanpouji-ike Pond, Shakujii, Nerima-ku, *C. Kataoka 604* (OSA); Aug. 17, 1900, *S. Matsuda s.n.* (KYO); Sept. 27, 1939, *T. Makino s.n.* (MAK); Jun. 13, 1948, *T. Makino s.n.* (KYO, MAK); Aug. 19, 1948, *J. Ohwi & T. Maruyama s.n.* (TNS); Jul. 2, 2004, *T. Shiga 3412* (KOBE, OSA); Inokashira, Mitaka-shi, 1914, *T. Makino s.n.* (KYO, MAK); Ushihama, Fussa-shi, Jul. 7, 1947, *T. Kawasaki 6624* (TNS). **Kanagawa Pref.**: Nakashinden, Ebina-shi, Jul. 2, 2004, *T. Shiga 3413, 3414* (KOBE, OSA), *3415* (OSA). **Niigata Pref.**: Lake Fukushima-gata, Toyosaka-shi, Jul. 30, 1980, *Y. Kadono 1101* (KOBE); Lake Fukushima-gata, Shinbana, Toyosaka-shi, Jun. 11, 2002, *T. Shiga 3122* (KOBE, OSA); Aug. 5, 2005, *T. Shiga 3553* (OSA); Hacchyo-oki, Nagaoka-shi, Oct. 4, 1896, *T. Arai s.n.* (SAPS); Sekihara, Nagaoka-shi, Jul. 28, 1989, *I. Ito s.n.* (KOBE); Nov. 22, 2002, *T. Shiga 3643* (OSA); Nagamine, Yoshikawa-machi, Nakakubiki-gun, Oct. 10, 2002, *T. Shiga 3320* (KOBE, OSA); Lake Amaga-ike, Iwanoko-shinden, Oogata-machi, Nakakubiki-gun, Jun. 8,

2002, *T. Shiga 3127-3130* (KOBE, OSA); Oct. 11, 2002, *T. Shiga 3318* (KOBE, OSA); Kanda, Sanwa-mura Nakakubiki-gun, August 1, 2003, *T. Shiga 3189* (KOBE, OSA). **Toyama Pref.**: Midarashi Pond, Miyada, Himi-shi, Aug. 4, 1996, *H. Marui 1242* (OSA). **Ishikawa Pref.**: Lake Katano-kamoike, Katano-cho, Kaga-shi, Aug. 10, 2001, *T. Shiga 3645* (OSA). **Fukui Pref.**: Kita-gawa River, Nishinonaka, Mikuni-cho, Sakai-gun, Aug. 28, 1991, *Y. Kadono 7150* (KOBE); Sonobe, Takahama-cho, Ooi-gun, Aug. 11, 1970, *M. Kuwashima 19532* (OSA); Tsuruga-shi, Sept. 21, 1978, *Y. Kadono 1862* (KOBE). **Nagano Pref.**: Lake Nakatsuna, Oomachi-shi, Jul. 26, 1973, *S. Misyo & S. Tsugaru 1030* (TNS); Lake Nakatuna, Yanaba, Ohmachi-shi, Aug. 18, 1975, *K. Seto 21595* (OSA); Aug. 1, 1980, *Y. Kadono 1129* (KOBE); Jul. 27, 1990, *Y. Kadono 6787* (KOBE). **Gifu Pref.**: Hiwada, Takane-mura, Oono-gun, Oct. 4, 1986, *H. Nagase s.n.* (KOBE); Somaga-ike Pond, Hiwada-kougen, Takane-mura, Oono-gun, Oct. 24, 1999, *K. Okuda & M. Kitano 523-15* (SAPT); Kukuri, Kani-shi, Jun. 8, 2001, *T. Shiga 3684* (OSA). **Shizuoka Pref.**: Tsurugaike Pond, Shinohara, Iwata-shi, Sept. 5, 1983, *Kurosawa 430* (TNS); Lake Tsuruga-numa, Iwai, Iwata-shi, Jun. 10, 2001, *T. Shiga 2764* (OSA); Ukishima-numa, Shizuoka-shi, Sept. 6, 1925, *H. Muramatsu s.n.* (TI). **Mie Pref.**: Shiraki-cho, Kameyama-shi, Jul. 5, 1990, *M. Kuribayashi 371* (OSA); Kamishiraki, Kameyama-shi, Jul. 24, 1991, *K. Seto 36535* (OSA); Katsuta, Tamaki-cho, Watarai-gun, Jul. 17, 2002, *T. Shiga 3644* (KOBE, OSA); Maeno, Meiwa-cho, Taki-gun, Oct. 14, 1991, *T. Nakamura 715* (KOBE). **Shiga Pref.**: Shiozu, Nishiazai-cho, Ika-gun, Oct. 20, 1988, *Y. Kadono 5804* (KOBE); Sakai-gawa River, Imazu-cho, Takashima-gun, Jul. 12, 1988, *Y. Kadono 5423* (KOBE); South east of Aiba, Shinasahi-machi, Takashima-gun, Jul. 19, 1996, *S. Fujii & C. Karasaki 5312* (OSA); Zensho, Ohtsu-shi, Aug. 18, 1942, *C. Hashimoto 9888* (KYO). **Kyoto Pref.**: Kameyama Botanical Garden, Kameoka-shi (Cult.), Jun. 20, 1998, *M. Watanabe, K. Kadowaki & M. Miyahara 44* (KYO, OSA). **Osaka Pref.**: Amagataki-cho, Kishiwada-shi, Aug. 14, 1942, *T. Nakazima s.n.* (OSA); Bessho, Sennan-shi, Sept. 16, 1962, *T. Nakazima s.n.* (OSA); Nariai, Kumatori-cho, Sennan-gun, Sept. 23, 1961, *K. Seto 11047* (OSA). **Hyogo Pref.**: Kinashi, Toyooka-shi, Jul. 14, 2002, *H. Marui 5154* (OSA); Akasaka-cho, Aioi-shi, Aug. 23, 1976, *N. Fukuoka & N. Kurosaki 670* (KYO, OSA). **Wakayama Pref.**: Nishinoyama, Naga-cho, Naga-gun, Nov. 3, 2001, *T. Shiga 2833* (OSA). **Okayama Pref.**: Kosaka, Saeki-cho, Wake-gun, Sept. 3, 2001, *T. Shiga 3671* (KOBE, OSA); Nov. 4, 2001, *T. Shiga 2835* (OSA); Shiroo, Kojima, Kurashiki-shi, Sept. 2, 2001, *T. Shiga 3669* (KOBE, OSA). **Hiroshima Pref.**: Misonou, Higashihiroshima-shi, Aug. 16, 2002, *T. Shiga 3241* (KOBE, OSA); Hara, Hachihonmatsu-cho, Higashihirosima-shi, Aug. 16, 2002, *T. Shiga 3243* (KOBE, OSA). **Yamaguchi Pref.**: Osaba, Yamaguchi-shi, Jun. 10, 1893, *S. Nikaidoh 204* (TI). **Kochi Pref.**: Kochi-shi (Cult.), Sept. 1892, *T. Makino s.n.* (MAK); Jun. 1893, *T. Makino s.n.* (MAK); Kusaka-gawa River, Shimobun, Hidaka-mura, Takaoka-gun, Nov. 5, 2003, *T. Shiga 3371, 3372* (KOBE, OSA); Ryu, Usa-cho, Tosa-shi, Nov. 5, 2003, *T. Shiga 3373* (KOBE, OSA). **Kumamoto Pref.**: Sagara-mura, Kuma-gun, May 31, 1987, *Y. Kadono 4520* (KOBE). **Miyazaki Pref.**: Hasetani, Yasuhisa-machi, Miyakonojo-shi, Nov. 7, 1989, *T. Minamitani s.n.* (KOBE); Hasetani, Toyomitsu-cho, Miyakonojo-shi, Oct. 2, 2002, *T. Shiga 3279* (KOBE, OSA); Matsuo, Kushima-shi, Oct. 2, 2002, *T. Shiga 3283* (KOBE, OSA). **Kagoshima Pref.**: Katayama Flower Park, Katayama, Ibusuki-shi (Cult.?), May 28, 1987, *Y. Kadono 4481* (KOBE).

Nuphar japonica DC. f. **rubrotincta** (Casp.) Kitam., Acta Phytotax. Geobot. **20**: 204 (1962); Kitamura & Murata, Col. Ill. Herb. Pl. Jap. **2**: 252 (1972); Tamura, Wild Fl. Jap. **2**: 94 (1982); Kadono, Aquat. Pl. Jap. 112 (1994). ≡*Nuphar japonicum* DC. var.

crenatum Casp. subvar. *rubrotincutum* (= *rubropicta*) Casp., Ann. Mus. Bot. Lugduno-Batavi. **2**: 254, tab. Ⅷ, fig. Ⅰ-Ⅵ (1865); Makino, Bot. Mag. (Tokyo) **11**: 280 (1897). ≡*Nuphar subintegerrimum* (Casp.) Makino f. *rubrotinctum* (Casp.) Makino, Bot. Mag. (Tokyo) **24**: 142 (1910). ≡*Nuphar japonicum* DC. var. *rubrotinctum* (Casp.) Ohwi, Fl. Jap. 437 (1965). nom. nud.; Ohtaki & Ishido, Ill. Jap. Water Pl. 91 (1980). **Lectotype [designated here]**: Ann. Mus. Bot. Lugduno-Batavi. **2**: 254, tab. Ⅷ, fig. Ⅰ-Ⅵ (1865)

Description: Sepals at first yellow then reddish.

Japanese name: Beni-kōhone

Distribution: Japan (only in cultivation)

Note: Type of *N. japonica* var. *crenatum* subvar. *rubrotinctum* was not designated by Caspary (1865). Thus, I selected one illstration of Caspary (1865; tab. Ⅷ, fig. Ⅰ-Ⅵ) on which the description was based, to serve as the lectotype. This plate clearly shows the diagnostic reddish sepals.

Specimen examined: **JAPAN**: **Tokyo Pref.**: Koishikawa Botanical Garden, Bunkyo-ku (Cult.), Jun. 28, 1880 (TNS).

Key to the forms of *Nuphar japonica*:

A. Sepals yellow ..... f. *japonica*

A. Sepals tinged with orange to red ..... f. *rubrotincta*

3. **Nuphar saikokuensis** Shiga & Kadono, **sp. nov.** (Fig. 28)

="*Nuphar subintegerrimum* (Casp.) Makino pro parte" sensu Tamura, Wild Fl. Jap. **2**: pl. 94-1 (1982).

="*Nuphar subintegerrimum* (Casp.) Makino pro parte" sensu Kadono, Aquat. Pl. Jap. fig. 114-115 (1994).

="*Nuphar subintegerrimum*" acut. non Makino: Suzuki *et al.*, Nat. Hum. Activities **2**: 52. pl. 1-2 (1997).

Haec species nova *Nuphari japonicae* DC. et *N. subintegerrimae* (Casp.) DC. et *N. oguraensi* Makino propinqua; ab eis foliis ovatis oblongis, caulibus teretiusculis non lacunaribus differet.

Typus: JAPAN, Pref. Hyogo; Oda-cho, Ono-shi, May 30, 2001, *T. Shiga 3223* (OSA).

Description: Perennial aquatic herbs. Rhizomes procumbent, branching. Leaves submerged and floating or emergent; submerged leaves roundish to ovate, 7-30 cm long, 5-20 cm wide, membranaceous, margin undulate; floating and emergent leaves ovate to widely ovate, 10-30 cm long, 7-20 cm wide, base cordate, apex rounded, upper surface glabrous, lower surface slightly pubescent. Petiole usually terete to more of less flattened above (Fig. 22). Peduncle raised above water. Flowers yellow, June to October, 3-4 cm across, protogynous; sepals 5, obovate-orbicular, apex rounded, 1.5-2.5 cm long, subcoriaceous, yellow; petals inconspicuous, obovate-cuneate, 5-8 mm long; anthers many, 4-6 mm long, ratio of pollen sack to filament length ca. 1:1 to 1:2; pistil 1, carpels, many, fused; stigmatic rays 2.5-4 mm long; disc of stigma yellow, 4-11 mm across, 5-17 rays, shallowly toothed; fruit green, narrowly ovoid to ovoid (Fig. 23); seeds numerous, ovoid, 3.5-5 mm long, 3-4.5 mm wide.

Japanese name: Saikoku-hime-kōhone (nov.)

Distribution: Central to Western Japan

Note: *Nuphar saikokuensis* is morphologically and genetically intermediate among *N. japonica*, *N. oguraensis* and *N. subintegerrima* and considered to be of hybrid origin between *N. japonica* and *N. subintegerrima* or among these three

species (mainly Group 4 in Chapter 2). Because this species is more or less fertile and distributes widely in central to western Japan, I treat this taxon as species rank.

Specimens examined: **JAPAN**: **Niigata Pref.:** Lake Uwasekigata, Maki-machi, Nishikanbara-gun, Jul. 22, 1979, *H. Kariya s.n.* (KOBE); Ikenodaira, Koshiji-machi, Santoh-gun, Aug. 27, 1958, *S. Iwano 11769* (TUS); Jun. 22, 1973, *H. Hujino 15521* (SAPS, TUS); Aug. 6, 1986, *H. Hujino 19291* (TUS); Jul. 5, 1989, *I. Ito s.n.* (KOBE); Ikenodaira, Ojiya-shi, Oct. 13, 2002, *T. Shiga 3331* (KOBE, OSA); Lake Asahiike, Uchigango, Ogata-cho, Nakakubiki-gun, Sept. 5, 1988, *M. Sasagawa s.n.* (KOBE). **Fukui Pref.:** Kunugi, Kanazu-cho, Sakai-gun, Aug. 10, 2001, *T. Shiga 3650, 3651* (KOBE, OSA); Heisenji-cho, Katsuyama-shi, Aug. 10, 2001, *T. Shiga 3648* (KOBE, OSA), *3649, 3652* (OSA); Tsuruga, Echizen, May 5, 1933, *Z. Tashiro s.n.* (KYO); Ikenokochi, Tsuruga-shi, Jul. 18, 1966, *K. Seto 15577* (OSA); Ikenodaira Moor, Ikenokouchi, Tsuruga-shi, Aug. 9, 2001, *T. Shiga 3646, 3647* (OSA). **Gifu Pref.:** Mt. Wada, Itoshiro, Shirotori-cho, Gujo-gun, Jul. 1933, *G. Itoshiro s.n.* (TNS); Jul. 2, 1933, *G. Koidzumi s.n.* (KYO); Jun. 9, 2001, *T. Shiga 3222* (KOBE); Oct. 17, 2003, *T. Shiga 3357* (KOBE, OSA); Kashio, Kawabe-cho, Kamo-gun, Jun. 17, 1997, *T. Umehara 8087* (OSA); Higashitabirako, Kani-shi, Jul. 18, 1996, *H. Takahashi 16543* (KYO); Oct. 1, 2002, *T. Shiga 3654* (KOBE, OSA). **Aichi Pref.:** Tenpaku-cho, Toyohashi-shi, May 22, 1949, *K. Torii s.n.* (KYO). **Mie Pref.:** Suzuka-shi (Iino-mura, Ise), Sept. 8, 1951, *G. Nakai 5651* (KYO); Asauda, near Shijuku, Ueno-shi, Jun. 9, 1968, *I. Hiura s.n.* (OSA); Tsuge, Iga-cho, Ayama-gun, Sept. 2002, *T. Yamaji s.n.* (OSA); Otanuma, Umase, Miyama-cho, Kitamuro-gun, Aug. 6, 1962, *K. Seto 11595* (OSA); May 17, 1987, *K. Seto 32545* (OSA); Oct. 13, 1995, *S. Fujii 4718* (OSA); Oct. 13, 1994, *T. Fujii 4957* (OSA). **Shiga Pref.:** Hamabun, Imazu-cho, Takashima-gun, Aug. 24, 1994, *T. Fujii 3973* (OSA); Lake Biwa, Hamabun, Imazu-cho, Takashima-gun, Oct. 6, 1997, *S. Fujii 5981* (OSA); Lake Hamabun-numa, Hamabun, Imazu-cho, Takashima-gun, Jun. 7, 2002, *T. Shiga 3126* (KOBE, OSA); Warasono, Shinasahi-machi, Takashima-gun, Jun. 7, 2002, *T. Shiga 3124* (KOBE, OSA); Aug. 9, 2001, *T. Shiga 3661* (KOBE, OSA); Aug. 23, 2002, *T. Shiga 3662* (OSA); Lake Biwa, Fukamizo and Harie, Shinasahi-cho, Takashima-gun, Sept. 22, 1994, *S. Fujii 4093* (OSA); Lake Biwa, Haginohama, Takashima-cho, Takashima-gun, Sept. 18, 1994, *T. Fujii 4048* (OSA); Ohmimaiko, Shiga-cho, Shiga-gun, Dec. 16, 1975, *Y. Kadono 1630, 2000* (KOBE); Oct. 21, 1975, *Y. Kadono 1856* (KOBE); Jun. 13, 1975, *Y. Kadono 1972* (KOBE); Jun. 28, 1976, *Y. Kadono 6024* (KOBE); Lake Biwa, Ohmimaiko to Kitahira, Shiga-cho, Shiga-gun, Jun. 30, 1962, *G. Murata 16461* (MAK); Shiga-cho, Shiga-gun (Komatsu-mura, Ohmi), Aug. 15, 1937, *C. Hashimoto 4531* (KYO, TNS); Sept. 26, 1941, *C. Kataoka s.n.* (OSA); Lake Nishinoko, Azuchi-cho, Gamo-gun, Sept. 14, 1975, *K. Nagai s.n.* (KYO, OSA); Tsuda-cho, Ohmihachiman-shi, Aug. 5, 2002, *S. Fujii 9260* (KYO, OSA); Ichinobe, Yokaichi-shi, Sept. 21, 1951, *M. Hutoh 5271* (OSA); Shin-nunobiki River, Youkaichi-shi, Sept. 2, 1988, *Y. Kadono 5659* (KOBE); Lake Biwa, Chuzu-cho, Yasu-gun, Sept. 23, 1994, *S. Fujii 4121* (OSA); Noda, Chuzu-cho, Yasu-gun, Jun. 27, 1996, *K. Seto 46144* (OSA); Nasaka, Minakuchi-cho, Koka-gun, Oct. 15, 1988, *Y. Kadono 5755* (KOBE). **Kyoto Pref.:** Sunako-ike Pond, Osada, Fukuchiyama-shi, Oct. 13, 1975, *Y. Kadono 1980* (KOBE); May 14, 1976, *Y. Kadono 1999* (KOBE); May 29, 1977, *Y. Kadono 25* (KOBE); Jul. 4, 1981, *Y. Miyatake s.n.* (OSA); Naka-ike Pond, Ikejiri, Kameoka-shi, Sept. 19, 1976, *Y. Kadono 1658, 1928* (KOBE); Kamigamo, Kita-ku, Kyoto-shi, Aug. 2, 1932, *J. Ohtuka 4* (KYO); Oharano-Minamikasuga-cho, Nishigyo-ku, Kyoto-shi, Jul. 30, 1998, *S. Tsugaru et al. 26637* (KYO, TUS); Daigo, Fushimi-ku, Kyoto-shi, Oct. 10, 1995, *G. Murata 71619* (KYO); Sept. 21, 1977, *G. Murata et al. 12* (KYO); Aug. 18, 1979, *Z. Sato s.n.* (TI); Oct. 11, 1977, *Y. Kadono 308* (KYO), *1925* (KOBE); Aug. 18, 1978, *Y. Kadono 518* (KYO), *1555* (KOBE); Jun. 9, 1977, *Y. Kadono 1558* (KOBE); Jul. 4, 1978, *Y. Kadono 1564* (KOBE); Oct. 16, 1977, *Y. Kadono 1922* (KOBE); Apr. 25, 1978, *Y. Kadono 531* (KYO); Sept. 11, 2002, *S. Tsugaru et al. 31890*

(KYO); Sept. 14, 1963, *S. Kitamura & G. murata 2270* (MAK); Jul. 23, 1977, *S. Kitamura s.n.* (KYO); May 26, 1921, *N. Kinashi s.n.* (KYO); May 30, 1979, *M. Ito 701* (KYO); Jul. 18, 1980, *K. Seto 26229* (OSA); Jun. 2, 1968, *K. Nagai 10639* (KYO); May 22, 1931, *J. Ohwi s.n.* (KYO); Jul. 30, 1977, *G. Murata 32688* (KYO). **Osaka Pref.:** Botanical Gardens Faculty of Science Osaka City Univ., Kisaichi, Katano-shi (cult.), Aug. 5, 2000, *G. Murata 72887* (KYO). **Hyogo Pref.:** Sasayama-shi (Tanba-sasayama), Sept. 9, 1954, *Z. Tashiro s.n.* (TNS); Aonogahara-cho, Kasai-shi, Oct. 2, 1980, *Y. Kadono 1244* (KOBE); Asazuma-cho, Kasai-shi, Sept. 10, 1995, *N. Kurosaki & K. Akai 1723* (KYO, MAK, OSA); Tamaoka-cho, Kasai-shi, Sept. 12, 2002, *T. Shiga 3229* (KOBE, OSA); Abiki-cho, Kasai-shi, May 17, 2001, *T. Shiga 3221* (KOBE); Ureshino, Yashiro-cho, Katoh-gun, Jun. 23, 1987, *Y. Kadono 4536* (KOBE); Kamikume, Yashiro-cho, Katoh-gun, Aug. 17, 1990, *Y. Kadono 6805* (KOBE); Yamashiro, Yashiro-cho, Katoh-gun, Jul. 15, 2000, *T. Kobayashi 34764* (OSA); Ochihara, Sanda-shi, Sept. 11, 1986, *Y. Kadono 3978* (KOBE); Fujiga-oka, Sanda-shi, May 29, 1998, *T. Fujii 9538* (OSA); Aug. 28, 1998, *T. Fujii 10214* (OSA); Tokura, Sanda-shi, Aug. 11, 1998, *T. Fujii 10031* (OSA); Sakai-cho to Daikai-cho, Ono-shi, Sept. 19, 1983, *Y. Kadono 3193* (KOBE); Ohhiraki-cho, Ono-shi, Oct. 4, 1986, *T. Umehara 1349, 1350* (OSA); Oct. 4, 1986, *T. Fujii T-0243* (OSA); Oda-cho, Ono-shi, May 30, 2001, *T. Shiga 3223, 3234, 3226* (KOBE); May 27, 2002, *T. Shiga 3099* (KOBE, OSA); Aug. 26, 2001, *T. Shiga 3665* (OSA); Ohharanoishii, Takarazuka-shi, Sept. 14, 1997, *K. Seto 48072* (OSA); Ohharano, Takarazuka-shi, Sept. 12, 1999, *H. Kondo 99091201* (OSA); Misaka, Miki-shi, Aug. 26, 1982, *Y. Kadono 2113* (KOBE); Iwaya, Shijimi-cho, Miki-shi, Oct. 2, 1994, *S. Miyake 3170* (KYO); Aug. 19, 1995, *S. Miyake 4059* (OSA); Aug. 5, 1995, *S. Miyake 4338* (MAK); Near Nishimino River, Miki-shi, May 19, 1987, *I. Yamamoto s.n.* (KOBE); Near Ono-shi, Miki-shi, May 19, 1987, *I. Yamamoto s.n.* (KOBE); Kuchiyokawa-cho, Miki-shi, Jul. 26, 1968, *G. Murata & H. Nishimura 301* (KYO); Shijimi-cho, Miki-shi, Sept. 22, 1968, *G. Murata & H. Nishimura 368* (KYO); Higashiiwao, Kamiso-cho, Kakogawa-shi, Oct. 19, 1981, *Y. Kadono 1380* (KOBE); Aug. 21, 1983, *Y. Kadono 3117* (KOBE); Aug. 31, 1984, *Y. Kadono 3590* (KOBE); Sept. 23, 1984, *Y. Kadono 3393, 3397* (KOBE); Nobuka, Kakogawa-shi, Sept. 27, 1987, *Y. Kadono 5076* (KOBE); Kamiso-cho, Kakogawa-shi, Sept. 23, 1984, *N. Kurosaki 14638* (KYO); Hirano-cho to Kande-cho, Nishi-ku Kobe-shi, Jul. 27, 1982, *Y. Kadono 2301* (KOBE); Iwaoka-cho, Nishi-ku, Kobe-shi, Aug. 22, 1984, *Y. Kadono 3416* (KOBE); Shinshinden to Hirano-cho, Nishi-ku, Kobe-shi, Aug. 25, 1984, *Y. Kadono 3533* (KOBE); Hirano-cho, Nishi-ku, Kobe-shi, May 20, 1990, *Y. Kadono 6667* (KOBE); Midorigaoka, Hyogo-ku, Kobe-shi, Aug. 15, 1969, *T. Muroi s.n.* (TNS); Tadokoro, Goshiki-cho, Tsuna-gun, May 24, 2001, *T. Shiga 3220, 3663* (KOBE, OSA); Sept. 12, 2001, *T. Shiga 3664* (OSA). **Nara Pref.:** Saki-cho, Nara-shi, May 22, 1949, *M. Hutoh 4177* (OSA); Hokkeji, Nara-shi, Aug. 11, 2002, *T. Shiga 3230* (KOBE, OSA). **Wakayama Pref.:** Kamitonda-cho, Nishimuro-gun, May, 1925, *J. Nakajima s.n.* (TI); Sou, Nachikatsuura-cho, Higashimuro-gun, Sept. 22, 1996, *K. Ohora s.n.* (OSA). **Okayama Pref.:** Goishi, Mitsuishi, Bizen-shi, Sept. 2, 2001, *T. Shiga 3668* (KOBE, OSA); Kojima, Kurashiki-shi, Sept. 1, 1991, *Y. Obata s.n.* (KOBE); Shiroo, Kojima, Kurashiki-shi, Sept. 2, 2001, *T. Shiga 3670* (KOBE, OSA); Sugisawa, Saeki-machi, Wake-gun, Sept. 3, 2001, *T. Shiga 3672* (OSA). **Yamaguchi Pref.:** Yamaguchi-shi (Miyano-mura, Yoshiki-gun, Suoh), Sept. 23, 1889, *S. Nikaidoh 204* (TNS). **Tokushima Pref.:** Kandase-cho, Komatsushima-shi, Aug. 17, 2002, *T. Shiga 3246* (KOBE, OSA); Higaino-cho, Komatsushima-shi, Sept. 3, 1990, *T. Nakamura 104* (KOBE); Shibafu-cho, Komatsushima-shi, Aug. 18, 2002, *T. Shiga 3673* (OSA), *3674* (KOBE, OSA); Sept. 13, 2001, *T. Shiga 3675* (OSA), *3676* (KOBE, OSA); Taura-cho, Komatsushima-shi, Sept. 12, 2001, *T. Shiga 3681* (KOBE, OSA). **Kagawa Pref.**: Sakamoto, Hiketa-cho, Okawa-gun, Sept. 13, 2001, *T. Shiga 3655* (OSA); Tsuruwa, Tsuda-cho, Okawa-gun, Sept. 14, 2001, *T. Shiga 3657* (OSA), *3683* (KOBE, OSA); Gomyo, Shirotori-cho, Okawa-gun, Sept. 13, 2001, *T. Shiga 3656, 3682* (OSA); Sakaiba, Nishiwake, Ayakami-cho, Ayauta-gun, Sept. 14, 2001, *T. Shiga 3658* (KOBE, OSA); Tomikuma, Ayauta-cho, Ayauta-gun, Sept. 14, 2001, *T. Shiga 3659* (KOBE, OSA); Sept. 14, 2001,

T. Shiga 3660 (OSA). **Ehime Pref.:** Daima, Matsumae-cho, Iyo-gun, Nov. 6, 2003, *T. Shiga 3367, 3368* (KOBE, OSA). **Kumamoto Pref.:** Hitoyoshi (Hitoyoshi, Higo), Aug. 20, 1939, *K. Mayebara 3011* (TI, TNS); Sagara-mura, Kuma-gun, Jun. 10, 1923, *K. Mayebara 2551* (TI, TNS); Hirabaru, Sagara-mura, Kuma-gun, May 31, 1987, *Y. Kadono 4510* (KOBE); Nishiki-cho, Kuma-gun (Nishinomura, Higo), Sept. 5, 1953, *K. Mayebara 5116* (TI, TNS). **Ohita Pref.:** Usa-jingu, Minamiusa, Usa-shi, Jun. 12, 1990, *T. Nakamura & Y. Kadono 12* (KOBE); Sept. 30, 2002, *T. Shiga 3317* (KOBE, OSA). **Miyazaki Pref.:** Tomiyoshi, Yamanokuchi-cho, Kitamorokata-gun, Oct. 2, 2002, *T. Shiga 3282* (KOBE, OSA); Tomita, Shintomi-cho, Koyu-gun, Nov. 8, 1988, *Y. Kadono 5891* (KOBE); Hirata, Kawanami-cho, Koyu-gun, May 26, 1987, *Y. Kadono 4461* (KOBE). **Kagoshima Pref.:** Fumoto, Sendai-shi, Oct. 3, 1965, *Hatushima & Sako 29832* (MAK).

4. **Nuphar subintegerrima** (Casp.) Makino, Bot. Mag. (Tokyo) 24: 141 (1910)(Fig. 4); Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 84. tab. 3. E., fig. 50. H-O (1937); Ohwi, Fl. Jap. 437 (1965); Ohtaki & Ishido, Ill. Jap. Water Pl. 93. tab. 42. (1980); Tamura, Wild Fl. Jap. **2**: 94. pro parte (pl. 94-1 is *N. saikokuensis*). (1982); Kadono, Aquat. Pl. Jap. 113-115. pro parte (1994). ≡*Nuphar japonicum* DC. var. *subintegerrimum* Casp., Ann. Mus. Bot. Lugduno-Batavi. **2**: 254, tab Ⅷ, fig. 1-10 (1865); Makino, Bot. Mag. (Tokyo) **11**: 280 (1887). ≡*Nymphozanthus subintegerrimus* (Casp.) Fernald, Rhodora 21: 187 (1919). **Lectotype [designated here; Fig. 29]**: JAPAN, Prov. Mikawa, Near Toyohashi, Oct. 25, 1893, *T. Makino s.n.* (MAK 59655!). **Syntypes**: JAPAN, Prov. Musashi, Tokyo, cult.?, Jul. 10, 1879 (TI?, non vidi). Prov. Iwashiro, Near Shiokawa in Aizu, Aug., 1879, *J. Matsumura s.n.* (TI?, non vidi). Prov. Tōtōmi, Hamamatsu (TI?, not vidi).

Description: Perennial aquatic herbs. Rhizomes procumbent, branching. Leaves submerged and floating or emergent; submerged leaves roundish to widely ovate, 5-16 cm long, 5-15 cm wide, membranaceous, margin undulate; floating and emergent leaves roundish, 4-17 cm long, 4-15 cm wide, base cordate, apex rounded, upper surface glabrous, lower surface slightly pubescent. Petiole usually terete to more of less flattened above (Fig. 22C). Peduncle raised above water. Flowers yellow,

June to October, 2-3.5 cm across, protogynous; sepals 5, obovate-orbicular, apex rounded, 1.5-2.5 cm long, subcoriaceous, yellow; petals inconspicuous, obovate-cuneate, 3-6.5 mm long; anthers many, 2-3.5 mm long, ratio of pollen sack to filament length ca. 1:1; pistil 1, carpels, many, fused; stigmatic rays 7-11 mm long, broad, apex rounded; disc of stigma yellow or orange, 4-5 mm across, 8-12 rays, shallowly toothed; fruit green or dark red (Fig. 23), ovoid to subglobose, 2-4.5 cm; seeds numerous, widely ovoid, 5.5-6.5 mm long, 3.5-5 mm wide.

Japanese name: Hime-kōhone

Distribution: Japan (Tokai Dist., Fukushima Pref. and Tokyo Pref. (only in cultivation?))

Note: Larger plants with ovate to oblong floating or emergent leaves of 15-30 cm long have often been identified as *N. subintegerrima* (Kadono 1994). But morphological and allozyme studies (Chapter 2) have revealed these plants to be of hybrid origin between *N. japonica* and *N. subintegerrima* or *N. oguraensis* or among these three species. *Nuphar subintegerrima s. s.* is easily distinguishable from other taxa.

Natural populations of this species now distribute only in Tokai Distinct, although herbarium specimens were collected in Tokyo, which were presumed to be cultivated ones. *Nuphar subintegerrima s. l.* including *N. saikokuensis* have been designated as endangered species in Japan (Environmental Agency of Japan 2000). My study shows that *N. subintegerrima s. s.* is particularly endangered. I located only five populations, all limited to Tokai Region. Most of its populations are extinct today (Study Group of Flora of Aichi Prefecture 1996). It has proved to be one of the most urgent species in need of protection in the conservation of Japanese flora.

Specimens examined: **JAPAN**: **Tokyo Pref.:** Fukiage-gyoen, Ohte-machi, Chiyoda-kub (Cult.?), Jun.

6, 1987, *S. Kitamura s.n.* (KYO); Kokyo-higashigyoen, Chiyoda-ku (Cult.), Jul. 19, 2005, *T. Shiga 3540-3542* (OSA); Shiroganedai-cho, Meguro-ku, Tokyo (Cult.?), Aug. 18, 1946, *T. Yamazaki s.n.* (TI); Institute for Nature Study, National Science Museum, Meguro-ku (Cult.?), Jul. 7, 1947, *G. Hiyama s.n.* (TNS); Meguro-ku (Cult.?), Aug. 1946, *I. Furusawa s.n.* (TI); Tokyo, Sept. 14, 1934, *K. Hisauchi s.n.* (TI). **Gifu Pref.:** Matsuki-cho, Takayama-shi, Sept. 17, 1985, *H. Nagase s.n.* (KOBE); Hino, Gifu-shi, May 24, 1992, *K. Yoshida s.n.* (TNS); Tachibokubora, Gifu-shi, Sept. 15, 2002, *T. Shiga 3228* (KOBE); Oct. 1, 2002, *T. Shiga 3653* (KOBE, OSA). **Aichi Pref.:** Gakuden-chiku, Inuyama-shi, Sept. 30, 1999, *K. Okuda & M Kitano 575-3* (SAPS); Ikenodai, Inuyama-shi, Oct. 6, 2002, *T. Shiga 3349* (KOBE); May 10, 2004, *T. Shiga 3378* (KOBE); Ueda, Owari, Jul. 12, 1891, *T. Ito s.n.* (TNS); Nagoya, Jul. 1959, *K. inami s.n.* (TNS); Narumi-cho, Midori-ku, Nagoya-shi, Aug. 22, 1968, *S. Hamashima s.n.* (TNS); Nagoya Univ., Higashiyama, Nagoya-shi, Jun. 29, 1946, *F. Maekawa 166B91* (TI); Tashiro-cho, Nagoya-shi, Aug. 13, 1917, *T. Makino 59656* (MAK); Kamishidami, Moriyama-ku, Nagoya-shi, Oct. 6, 2002, *T. Shiga 3275* (KOBE, OSA). **Mie Pref.:** Oshibuchi, Nansei-cho, Watarai-gun, Oct. 14, 1995, *S. Fujii 4633* (OSA); Sept. 12, 1995, *K. Seto 44776* (OSA); Aug. 11, 2002, *T. Shiga 3234* (KOBE); Sept. 14, 2002, *T. Shiga 3227* (KOBE); Kokai, Isobe-cho, Shima-gun, Sept. 12, 1995, *K. Seto 44751-44753* (OSA); Ugata, Ago-cho, Shima-gun, Sept. 12, 1995, *K. Seto 44758* (OSA): Oct. 16, 2003, *T. Shiga 3355* (KOBE).

5. **Nuphar pumila** (Timm) DC., Syst. Nat. (Candolle). **2**: 61 (1821); Sato, Illust. Aquatic Pl. Manchuria, 74 (1942); Ohwi, Fl. Jap. 437 (1965); Kitamura & Murata, Col. Ill. Herb. Pl. Jap. **2**: 253. fig 113-4 (1972); Tamura, Wild Fl. Jap. **2**: 94. pl. 94-3 (1982); Zhu, Plantae Medicinales Chinae Breali-rientalis, 352 (1989); Kadono, Aquat. Pl. Jap. 116, 118 (1994); Fu & Padgett, Fl. China, 115. fig. 110 (2001). ≡*Nympaea lutea* β. *pumila* Timm, Magaz. F. Naturk. Mecklenb. 2: 256 (1792). ≡*Nymphaea pumila* (Timm) Hoffm., Deuts. Fl. ed. 2, **1**: 241 (1800). ≡*Nymphozanthus pumilus* (Timm) Fernald, Rhodora **21**: 186 (1919). ≡*Nuphar luteum* (L.) Sibth. & Sm. subsp. *pumilum* (Timm) Beal, J. Elisha Mitchell Sci. Soc. **72**: 325 (1956). **Neotypes** [Beal, J. Elisha. Mitchell. Sci. Soc. 72: 325 (1956)]: Savonia borealis, par. Maaninka, in sinu Juurikkalahti lacus Pöljänjärvi, in fundo limosa, 1918, *O. Kyyhkynen*, Pl. Fin. Exs. 1170 (P, MO, NY, P, UC, non vidi), Herb. Delessert (G, non vidi).

=*Nympaea lutea* β. *minima* Willd., Sp. Pl. **2**: 1151 (1799). ≡*Nymphaea minima* (Willd.) Smith, Engl. Bot. 32: tab. 2292 (1811).

=*Nuphar subpumilum* Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 82.

tab. 3. B., fig. 50. A-J (1937). nom. nud.

Description: Perennial aquatic herbs. Rhizomes slender, procumbent, branching. Leaves submerged and floating, rarely emergent in high density or shallow water; submerged leaves roundish to widely ovate, 9-15 cm long, 9-13 cm wide, membranaceous, margin undulate; floating leaves widely ovate to ovate, 8-24 cm long, 6-18 cm wide, base with a deep sinus, entire, apex obtuse to rounded, upper surface glabrous, lower surface slightly pubescent or hirsute. Petiole usually slightly flattened. Vascular bundles of petiole arranged symmetrically with central vascular bundles (Fig. 22B). Peduncle raised above water. Flowers yellow, July to Augst, ca. 2.5 cm across, protogynous; sepals 5, obovate, apex rounded, 1.2-2.5 cm long, subcoriaceous, yellow; petals narrowly obovate, 4-7 mm long; anthers strongly recurved after anthesis, 1.5-4 mm long, ratio of pollen sack to filament length 1:2 to 1:3; pistil 1, carpels, many, fused; stigmatic disc yellow or red, 4-10 mm across, 6-16 rays, deeply lobed; fruit green or dark red ovoid to subglobose, 2-4 cm long; seeds numerous, narrowly ovate to ovate, 3-5 mm long, 1.5-3 mm wide.

Note: Beal (1956) determined six specimens of two localities as neotypes of *N. pumila*. I checked Kyyhkynen's material collected at Lake Pöljänjärvi in TNS (duplicate of neotypes?) and this specimen was well concordant with original description.

Some authors have considered that *N. pumila* has great morphological variability (Heslop-Harrison 1955, Beal 1956). This species closely allied with *N. microphylla* (Pres.) Fern. in North America, *N. oguraensis* and *N. sinensis* Hand.-Mazz. distributed in South China, although phylogenetic relationships among these taxa were poorly resolved (Padgett *et al.* 1999). These taxa called "dwarf *Nuphar*" are characterized by small plant size, deep blade sinus size and flat petiole

(Padgett *et al.* 1999). Padgett (1999) regarded Asian dwarf *Nuphar*, *N. oguraensis* and *N. sinensis*, as subspecies of *N. pumila*. But Japanese *N. pumila* and *N. shimadae* (= *N. oguraensis*) were morphologically and genetically (Chapter 5) distinguishable and geographically isolated each other (Fig. 27). Hence, I accept both taxa as species in this study. Taxonomic revision of dwarf *Nuphar*, *N. pumila*, *N. shimadae*, *N. sinensis* and *N. microphylla*, based on morphological and genetic data is needed.

Nuphar pumila (Timm) DC. f. **pumila**

Japanese name: Nemuro-kōhone

Distribution: Boreal region of the Old World (from Lapland to east Asia, south to the Baltic areas and local in Scotland, southwestern Poland, Switzerland, adjacent areas of France and Germany, Russia including Sakhalin and Kurils, China and Korea in Eurasia. In Japan, Hokkaido and north Honshu).

Hab.: Lakes, ponds and mires

Specimens examined: **JAPAN**: **Hokkaido Pref.**: Lake Meguma-numa, Keihoku, Wakkanai-shi, Sept. 1, 2004, *T. Shiga & S. Takebayashi 213* (OSA); Shimomasuhoro, Wakkanai-shi, Aug. 17, 1980, *K. Harusawa s.n.* (OSA); Shimotonbetsu, Hamatonbetsu-cho, Esashi-gun, Jul. 20, 1987, *Y. Kadono 4759* (KOBE); Lake Oh-numa (Lake Otatomari?), Rishiri Isl., Aug. 17, 1954, *S. Sugawara 9917* (SAPT); Lake Otatomari, Rishiri Isl., Rishirifuji-cho, Rishiri-gun, Aug. 9, 1934, *M. Tatewaki s.n.* (SAPS); Funadomari, Rebun Isl., Rebun-cho, Rebun-gun, Jul. 24, 1933, *M. Tatewaki, T. Nakai & C. Cho 19495* (SAPS); Jul. 25, 1933, *M. Tatewaki, T. Nakai & C. Cho 19439* (SAPS); Lake Chimikeppu, Tsubetsu-cho, Abashiri-gun, Aug. 9, 1953, *Class s.n.* (SAPS); Aug. 18, 1957, *Matsuda 19* (SAPS); Jul. 4, 1970? (MAK); Jul. 25, 1972 (MAK); Aug. 25, 2004, *T. Shiga 3597, 3598* (OSA); Lake Akan-ko, Akan-cho, Akan-gun, Aug. 23, 1897, *Kawakami s.n.* (SAPS); Uryu-numa Moor, Uryu-cho, Uryu-gun, Aug. 23, 1959, *M. Xommer s.n.* (OSA); Aug. 12, 1998, *H. Takahashi 25654* (SAPS); Sept. 6, 2004, *T. Shiga 3678* (OSA); Shinsen-numa Moor, Kyowa-cho, Iwanai-gun, Aug. 14, 1938, *K. Togashi s.n.* (SAPS); Sept. 17, 1966, *Ko. Ito s.n.* (SAPS); Sept. 5, 2004, *T. Shiga 3599* (OSA); Koinuma, Atsuma-cho, Yufutsu-gun, Aug. 23, 2004, *T. Shiga 3447, 3448* (KOBE, OSA); Iwaobetsu, Syari-cho, Syari-gun, Jul. 14, 1951, *Class s.n.* (SAPS); Jul. 30, 1958 (SAPS); Lake Barasanto, Honbekkai, Bekkai-cho, Bekkai-gun, Aug. 30, 2004, *T. Shiga & S. Takebayashi 73* (KOBE, OSA); Nemuro-shi, Aug. 2, 1937, *T. Terasaki s.n.* (TNS); Jul. 22, 1888, *K. Miyabe s.n.* (SAPS); Lake Chohboshi-ko, Chohboshi, Nemuro-shi, Aug. 3, 1979, *Y. Kadono 842* (KOBE); Aug. 30, 2004, *T. Shiga & S. Takebayashi 72* (OSA); Hanasaki, Nemuro-shi, Aug. 1-2, 1928, *S. Saito 959* (TI); Lake

Nanbuto, Katsuragi, Nemuro-shi, Jul. 4, 1987, *T. Sasaki 109* (KYO, OSA, TNS); Jul. 3, 1987, *T. Sasaki 110* (KYO, SAPS); Jul. 21, 1998, *T. Sasaki 124* (KYO, OSA); Aug. 21, 1988, *Y. Kadono 5596* (KOBE); Aug. 29, 2004, *T. Shiga & S. Takebayashi 55, 69* (KOBE, OSA), *70* (OSA); Near Nanbuto Lake, Katsuragi, Nemuro-shi, Jul. 23, 1987, *Y. Inoue & T. Sasaki 6896* (KYO, OSA, TNS); Ochiishi, Nemuro-shi, Jul. 15, 1931, *J. Ohwi s.n.* (KYO, TNS); Tomoshiri, Nemuro-shi, Aug. 6, 1884 (TI); 1894, *S. Yamane s.n.* (SAPS); Aug. 4, 1884, *K. Miyabe s.n.* (SAPS); Aug. 6, 1912, *D. Hoshi s.n.* (SAPS); Aug. 2, 1957, *K. Yoshida s.n.* (SAPS); Jul. 16, 1961, *M. Kikuchi s.n.* (TNS); Aug. 16, 1979, *M. Hara 147* (SAPS); Kiritappu Moorland, Shinkawa, Hamanaka-cho, Akkeshi-gun, Jun. 15, 1968, *Ko. Ito s.n.* (SAPS); Kiritappu Moor, Hamanaka, Hamanaka-cho, Akkeshi-gun, Aug. 29, 2004, *T. Shiga & S. Takebayashi 211* (KOBE, OSA); Lake Tokotan-numa, Tokotan, Honmachi, Akkeshi-cho, Akkeshi-gun, Aug. 29, 2004, *T. Shiga & S. Takebayashi 15* (KOBE, OSA), *210* (OSA); Akanuma, Kushiro Moor, Kushiro-shi, Jul. 17, 1987, *Y. Kadono 4664* (KOBE); Ashirisechiri River, Kushiro-shi, Jul. 11, 1886, *M. Nakamura s.n.* (SAPS); Shinkushiro River, Kushiro Moor, Kushiro-shi, Jul. 17, 1987, *Y. Kadono 4691* (KOBE); Lake Takkobu-numa, Hosooka, Kushiro-cho, Kushiro-gun, Aug. 25, 1975, *Y. Kadono 1639* (KOBE); Aug. 5, 1979, *Y. Kadono 861* (KOBE); Jul. 18, 1987, *Y. Kadono 4724* (KOBE); Aug. 19, 1988, *Y. Kadono 5576* (KOBE); Aug. 9, 1991, *Y. Kadono, T. Nakamura & K. Watanabe 46* (KOBE); Aug. 27, 2004, *T. Shiga 3466* (KOBE, OSA); Lake Shirarutoro, Kayanuma, Shibecha-cho, Kawakami-gun, Aug. 9, 1951, *M. Tatewaki 41983* (SAPS); Aug. 7, 1991, *Y. Kadono 7* (KOBE); Lake Toro, Shibecha-cho, Kawakami-gun, Jul. 9, 1975, *Y. Kadono 1645* (KOBE); Aug. 29, 1992, *H. Takahashi & Y. Takashima 15150* (SAPS); Lake Shikaribetsu, Shikaoi-cho, Kato-gun, Jul. 21, 1935, *M. Hirano s.n.* (OSA); Ohtsu, Toyokoro-cho, Nakagawa-gun, Aug. 24, 2004, *T. Shiga 3680* (OSA); Ukishima-koen, Kitahiyama-cho, Setana-gun, Jun. 24, 1998, *H. Takahashi 24936* (SAPS). **Aomori Pref.**: Minamihakkoda,1965 (TNS); Mt. Kushiga-mine, Minamihakkoda, Aug. 11, 1953, *K. Hoshoi s.n.* (TNS); Ouse-yachi, Minamihakkoda, Towada-shi, Jul. 21, 1957, *K. Hosoi s.n.* (TI); Jul. 18, 1962, *H. Ohashi s.n.* (TI). **Akita Pref.**: Hachimantai, Aug. 1933, *S. Muramatsu s.n.* (TI); Lake Naganuma, Hachimantai, Kazuno-shi, Aug. 8. 1931, *M. Matsuda s.n.* (TNS); Aug. 5, 1951, *S. Kurosawa s.n.* (TI); Jul. 22, 1953, *H. Hara s.n.* (TI). **KURILS**: Toshimoe, Ubetsu, Etorohu Isl. (Iturup Isl.), Aug. 27, 1898, *Kawakami 131* (SAPS); Aug. 25, 1928, *S. Saito s.n.* (TI); Aug. 8, 1939, *B. Shimura s.n.* (SAPS); Kunashiri Isl., Aug. 7, 1923, *M. Tatewaki 3642* (SAPS); 1935, *J. Ohwi? s.n.* (KYO); Tofutsu, Kunashiri Isl., Sept. 17, 1894, *C. Endou 25574* (SAPS); Aug. 20, 1936, *M. Tatewaki 25574* (SAPS); Furukappu, Kunashiri Isl., Jul. 22, 1930, *Y. Matsumura s.n.* (KYO); Aug. 20, 1931, *J. Ohwi s.n.* (KYO); Notoro, Shikotan Isl, Jun. 22, 1934, *M. Tatewaki 20653* (SAPS). **SAKHALIN**: Fukakusa (Uglezavodsk), *S. Sugahara s.n.* (KYO); Chipisani (Ozerskii), *G. Nakahara s.n.* (TI); Aug. 1906, *G. Nakahara s.n.* (TI, TNS). **CHINA**: Shangzhi, Heilongjiang, Aug. 13, 2005, *Y. Zhu s.n.* (OSA). **SWEDEN**: Hälsingländ, Arbrä, Lillbotjärn, Jul. 1885, *N. Söderlund s.n.* (TNS); Orsa parish, the church-villege, Jul. 19, 1899, *A. Björkman s.n.* (TNS); Tarendo parish, Koivuniemi, Jul. 23, 1959, *C. G. Alm s.n.* (TNS); Säver, Skeppsvik Västerbotten, 1888, *N. L. Andersson s.n.* (KYO). **FINLAND**: par. Kirkknummi, Jul. 3, 1963, *J. Merilainen & P. Isoviita s.n.* (TNS); Savonia borealis, par. Maaninka, in sinu Juurikkalahti lacus Pöljänjärvi, in fundo limosa, Aug. 4, 1918, *O. Kyyhkynen s.n.* (TNS; dupulicate of Beal's neotypes?); par. Maaninka, in sinu Juurikkalahti lacus Poljanjarvi, Aug. 4, 1918, *O. Kyyhkynen s.n.* (TI); par. Vehkalahti, Jul. 6, 1960, *L. Fagerstörm s.n.* (TNS); Pohjois-Pohjanmaa, Pudsjärvi, Lätväjärvi, Jul. 26, 1973, *T. Ulvinen & E. Ohenoja s.n.* (TNS); Sydösterbotten, Krsholm s:n, Karperö, träsk, Aug. 11, 1952, *R. Bäck s.n.* (TNS). **POLAND**: Pomerania, Aug. 12, 1938, *H. Greinert s.n.* (KYO); Rybnik, Jul. 30, 1893 (TI).

Nuphar pumila (Timm) DC. f. **ozeensis** (H. Hara) Shiga & Kadono, **comb. nov**.

[Basionym] *Nuphar pumilum* (Timm) DC. var. *ozeense* (Miki) H. Hara, Bot. Mag. (Tokyo) **64**: 79 (1951); Ohwi, Fl. Jap. 437. (1965); Kitamura & Murata, Col. Ill. Herb. Pl. Jap. **2**: 253. tab. 56. (1972); Tamura, Wild Fl. Jap. **2**: 94. pl. 93-5. (1982); Kadono, Aquat. Pl. Jap. 116, 118 (1994). **Holotype**: JAPAN, Honshu, Prov. Kodzuke, in stagnum paludis Nakatashiro, Ozegahara, ca. 1400m alt., Jul. 26, 1950, *H. Hara s.n.* (TI!)

=*Nuphar ozeensis* Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 82. tab. 3. A., fig. 50. K-T (1937). nom. nud.

Description: Stigmatic disc tinged red and fruit green

Japanese name: Oze-kōhone

Distribution: Japan (Hokkaido and Honshu (Tohoku Dist.))

Hab.: Lakes and mires

Note: The red tinged stigmatic disc has been used as a diagnostic character to classify the various taxa of *Nuphar* (Beal 1956, Padgett 1998). On the other hand, the stigmatic disc varies in color even within a population (Mochizuki 1972). In the AFLP analysis (Chapter 4), *N. pumila* var. *ozeensis* investigated in two localities, GUM and URY, was polyphyletic taxon. Hence, I reduced taxonomic rank of *N. pumila* var. *ozeensis* to form.

Specimens examined: **JAPAN**: **Hokkaido Pref.**: Uryu-numa Moor, Uryu-cho, Uryu-gun, Aug. 23, 1959, *M. Hotta 1670* (KYO); Jul. 27, 1970, *Ko. Ito s.n.* (SAPS); Aug. 11, 1984, *K. Tatsumi & S. Sakai 4090* (KYO, TI); Jul. 8, 1998, *H. Takahashi 25785, 25187* (SAPS); Jul. 23, 1998, *H. Takahashi 25295, 25300, 25648* (SAPS); Aug. 12, 1998, *H. Takahashi 25639, 25641, 25649* (SAPS); Sept. 6, 2004, *T. Shiga 3679* (KOBE, OSA). **Yamagata Pref.**: Mt. Gassan, Jul. 19, 1964, *Y. Yuki s.n.* (TNS); Jul. 27, 1964, *T. Yamazaki s.n.* (TI); Midagahara, Mt. Gassan, Aug. 4, 1948, *M. Sato s.n.* (TI). **Gunma Pref.**: Ozegahara Moor, Aug. 17, 1914, *D. Hoshi s.n.* (TI); Jul. 26, 1922, *D. Hoshi 9929* (SAPT); 1924, *Takeda & Tatewaki 935* (SAPS); Jul. 16, 1924, *H. Takeda s.n.* (TNS); 1929, *T. Makino s.n.* (MAK); Jul. 26, 1932, *Z. Tashiro s.n.* (KYO); Jul. 22, 1934, *Noguchi s.n.* (TNS); Aug. 17, 1941, *D. Hoshi s.n.* (KYO); Aug. 1, 1949, *M. Hutoh 2211* (OSA); Aug. 1950, *M. Mizushima s.n.* (TI); Jul. 16, 1952, *M. Tohyama s.n.* (SAPS); Jul. 21, 1952, *K. Maeda 14538* (OSA); Jul. 27, 1955, *C. Ookawa s.n.* (TNS); Kamitashiro, Ozegahara Moor, Aug. 6, 1946, *M. Furuse s.n.* (KYO); Jul. 20, 1950, *M. Togashi 59978* (TI); Jul. 8, 1951, *I. Harusawa s.n.* (TI); Sept. 4, 1978, *H. Hara & S. Kurosawa s.n.* (TI); Aug. 30, 1979, *H. Hara & S. Kurosawa s.n.* (TI);

Nakatashiro, Ozegahara Moor, Jul. 23, 1946, *M. Furuse 79* (KYO); Aug. 8, 1949 (TNS); Jul. 16, 1950, *M. Mizushima s.n.* (TI); Jul. 17, 1952, *M. Tohyama s.n.* (SAPS); Sept. 18, 1978, *H. Kanai s.n.* (TNS); Sept. 6, 1978, *H. Kanai s.n.* (TNS); Senaka-aburi-tashiro, Ozegahara Moor, Aug. 30, 1979, *H. Hara & S. Kurosawa s.n.* (TI).

Nuphar pumila (Timm) DC. f. **rubro-ovaria** Koji Ito ex Hideki Takah., M. Yamaz. & J. Sasaki, J. Jap. Bot. 80: 51. fig. 2 (2005). **Holotype**: JAPAN, Hokkaido, Sorachi-dhicho, Uryu-gun, Uryu-cho. Uryu-numa mire, alt. 840-850 m, pond J-16. Jul. 23, 1998, *H. Takahashi 25288* (SAPS 628!). **Paratypes**: JAPAN, Hokkaido, Prov. Ishikari, Uryu, Uryunuma bog. Jul. 27, 1970, *Ko. Ito s.n.* (SAPS 626!). Sorachi-shicho, Uryu-gun, Uryu-cho. Uryu-numa mire, alt. 840-850 m, pond G-04. Jul. 23, 1998, *H. Takahashi 25318* (SAPS 627!); Uryu-numa mire, pond I-129. Aug. 2, 2001, *M. Yamazaki, M. Mochida & Y. Fujimura 01447* (SAPS 629!).

Description: Stigmatic disc and fruit tinged red

Japanese name: Uryu-kōhone

Distribution: Japan (Hokkaido)

Hab.: Mires

Specimens examined: **JAPAN**: **Hokkaido Pref.**: Uryu-numa Moor, Uryu-cho, Uryu-gun, Jul. 23, 1998, *H. Takahashi 25655* (SAPS); Sept. 6, 2004, *T. Shiga 3477, 3478* (KOBE, OSA).

Key to the forms of *Nuphar pumila*:

A. Stigmatic disc yellow ..... f. *pumila*

A. Stigmatic disc tinged with red

B. Fruit green ..... f. *ozeensis*

B. Fruit red ..... f. *rubro-ovaria*

6. **Nuphar shimadae** Hayata, Ic. Pl. Formos. **6**: 2. tab. I , fig. 1-11 (1916); Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 84. tab. 3. C., fig. 49. L-T. (illustration

of petiole with lacuna) (1937); Lin, Water Pl. Taiwan **1**: 89 (2002); Fu, Fl. China 115 (2001). pro syn. **Typus**: FORMOSA, Shiuchikuchō, Shimpo, Dec. 1915, *Y. Shimada s.n.* (Holotype TI!; Isotypes TAIF!).

=*Nuphar oguraensis* Miki var. *akiense* Shimoda, J. Phytogeogr. Taxon. 39: 3. fig. 3-4, 9 (1991); Kadono, Aquat. Pl. Jap. 113 (1994). **Holotype**: JAPAN, Pref. Hiroshima, Kamijiike, Saijo-cho, Higashi-Hiroshima City (Pond 13 in Fig. 7 of Shimoda (1991)), Sept. 24, 1986, *M. Shimoda 4713* (HIRO!).

Description: Perennial aquatic herbs. Rhizomes slender, procumbent, branching. Leaves submerged and floating; submerged leaves roundish to ovate, 10-19 cm long, 9-16 cm wide, membranaceous, margin undulate, many submerged leaves remaining even at floating-leaved stage; floating leaves widely ovate to ovate, 9-19 cm long, 8-15 cm wide, base deeply cordate-sagittate, apex rounded, upper surface glabrous, lower surface slightly pubescent or hirsute. Petiole flattened, with central lacuna or without central vascular bundle (Figs. 22D, E). Peduncle raised above water. Flowers yellow, June to November, 2-3 cm across, protogynous; sepals 5, obovate, apex rounded, 1-2 cm long; petals, spatulate, 5-7 mm long; anthers strongly recurved after anthesis, 2.5-4 mm long, ratio of pollen sack to filament length 1:2 to 1:3; pistil 1, carpels, many, fused; stigmatic disc yellow or red, 5-10 mm across, 8-15 rays, shallowly dentate, stigmatic rays usually regularly arranged; fruit green, ovoid, 2-4.5 cm long (Fig. 23); seeds numerous, narrowly ovate to ovate, 3.5-5 mm long, 2-3.5 mm wide.

Note: Miki (1934) indicated that *N. oguraensis* and *N. shimadae* were morphologically resemble each other, especially in the petioles of both species with central lacuna in his illustration (Miki 1937), although yellow stigmatic disc and glabrous lower surface of floating leaves of *N. oguraensis* differ from *N. shimadae*,

which have red stigmatic disc and hirsute floating leaves on the lower surface. In this study, I observed that the floating leaves were much hirsute on lower surface in many specimens of *N. oguraensis* and the AFLP analysis (Chapter 5) provided strong monophyletic relationship of *N. oguraensis* and *N. shimadae*. Furthermore, *N. shimadae* and *N. oguraensis* var. *akiensis* were not separable from *N. oguraensis* var. *oguraensis*. Thus, I consider *N. oguraensis* and *N. shimadae* are conspecific and *N. oguraensis* var. *akiensis*, which have red stigmatic disc (Shimoda 1991), is a synonym of *N. shimadae*.

Further studies about relationships of dwarf *Nuphar* species is needed to determine whether *N. shimadae* should be treated as a subspecies or variety of *N. pumila*.

Nuphar shimadae Hayata f. **shimadae**

Japanese name: Beni-ogura-kōhone (= Taiwan-kōhone)

Distribution: Western Japan (Honshu; Chugoku Dist., Shikoku Dist. and Kyushu Dist.), Korea and Formosa

Hab.: Ponds, rivers and streams

Specimens examined: **JAPAN**: **Hiroshima Pref.**: Kihata, Saijo-cho, Higashihiroshima-shi, Sept. 7, 1990, *T. Nakamura 210* (KOBE); Shitami, Saijo-cho, Higashihiroshima-shi, Aug. 16, 2002S, *T. Shiga 3244* (KOBE, OSA); Sako-ike Pond, Shimomi, Saijo-cho, Higashihiroshima-shi, Aug. 2, 1987, *Y. Kadono 4891* (KOBE). **Kochi Pref.**: Koda, Kochi-shi, Oct. 1892, *T. Makino s.n.* (MAK); Koda-gawa River, Koda, Kochi-shi, Nov. 5, 2003, *T. Shiga 3364-3366* (KOBE, OSA); Okuda-gawa River, Hata-cho, Ino-machi, Agawa-gun, Nov. 5, 2003, *T. Shiga 3362, 3363* (KOBE, OSA). **KOREA**: Aug. 2004, *Y. Kadono s.n.* (KOBE). **FORMOSA**: Nankan, Toyen, Oct. 1924, *S. Sasaki s.n.* (TNS); Xinzhu (Sintiku), Jul. 28, 1922, *T. Ito 9777* (TNS); Sinpo, Sintiku, Nov. 15, 1915, *S. Sasaki s.n.* (TNS); Oct. 20, 1923, *S. Sasaki s.n.* (TNS); Taoyuan Hsein, Hsiaoli, Jun. 11, 1928, *S. Saito s.n.* (TI).

Nuphar shimadae Hayata f. **oguraensis** (Miki) Shiga & Kadono, **comb. nov.**

[Basionym] *Nuphar oguraensis* Miki, Bot. Mag. (Tokyo) **48**: 334. fig. 7 (1934);

Miki, Stud. Hist. & Nat. Monuments in Kyotohu **18**: 84. tab. 3. D., fig. 49. A-K (1937); Ohwi, Fl. Jap. 437 (1965); Kitamura & Murata, Col. Ill. Herb. Pl. Jap. **2**: 253. fig. 113-3 (1972); Tamura, Wild Fl. Jap. **2**: 94. pl. 94-2 (1982); Kadono, Aquat. Pl. Jap. 113, 115 (1994). ≡*Nuphar pumila* (Timm) DC. subsp. *oguraensis* (Miki) Padgett, sida 18: 825 (1999). **Lectotype [designed here; Fig. 30]**: JAPAN, Hondo, Prov. Yamashiro, Mukojima in Lake Ogura, Jul. 3, 1926, *S. Miki s.n.* (OSA!). **Syntypes**: JAPAN, Hondo, Prov. Yamashiro, Mukojima in Lake Ogura, 1926, *S. Miki s.n.* (OSA!); Prov. Kawachi, Hirakata, Aug., 1933, *G. Ikeo s.n.* (OSA?, non vidi); Prov. Hizen, The tatara river in Kasuyagori, Sept. 7, 1933, *R Koketsu et S. Hanada s.n.* (OSA!); Prov. Satsuma, Pond Imuta, Aug., 24, 1927, *S. Imamura s.n.* (OSA?, non vidi).

="*Nuphar subintegerrima*" acut non Makino: Miki, Oekol. Stud. Wasserg. Ogura-teich 143 (1927).

="*Nuphar* sp. (Japanese name: Gifu-Hime-Kōhone)" Takahashi *et al.*, Acta Phytotax. Geobot. **46**: 213-215 (1995).

Description: Stigmatic disc yellow

Japanese name: Ogura-kōhone

Distribution: Western Japan (Honshu; Tokai Dist., Kansai Dist. and Chugoku Dist., Shikoku, Kyushu) and Korea

Hab.: Ponds, rivers and streams

Note: Phylogenetic analysis based on the AFLP data (Chapter 5) indicated that *N. oguraensis* var. *oguraensis*, taxon with yellow stigmatic disc, and *N. shimadae*, taxon with reddish stigmatic disc including *N. oguraensis* var. *akiensis*, were polyphyletic. Hence, I propose this new combination.

Specimens examined: **JAPAN**: **Gifu Pref.**: Hora, Gifu-shi, Jul. 2, 1995, *H. Takahashi 16174* (KYO); Oct. 16, 2003, *T. Shiga 3356* (OSA); May 10, 2004, *T. Shiga 3379* (KOBE, OSA). **Aichi Pref.**: Yatsukaho, Shinshiro-shi, Oct. 19, 1997, *T. Umehara 7547* (OSA); Oct. 19, 1997, *T. Fujii 9014* (OSA). **Kyoto Pref.**:

Prov. Yamashiro, Oct. 19, 1948, *M. Hutoh 2433* (OSA); Kyaike, Osada, Fukuchiyama-shi, Aug. 21, 1965, *M. Togashi s.n.* (TI); Yagi-cho, Funai-gun, Sept. 8, 1957, *G. Murata 10774* (TNS); Ohno, Higashibetsuin-cho, Kameoka-shi, Sept. 1, 1995, *S. Tsugaru & T. Takahashi 22552* (OSA); Kameyama Botanical Garden, Kameoka-shi (Cult.), May 27, 1996, *S. Tsugaru & T. Takahashi 23232* (OSA); Yodo River, Yodo, Fushimi-ku, Kyoto-shi, Jul. 13, 1958, *K. Seto 10458* (OSA, TNS); Shimoueno, Ooyamasaki-cho, Otokuni-gun, Jul. 1, 1938, *H. Yamamoto s.n.* (TNS); Lake Ogura-ike, Uji-shi, Jul. 21, 1929, *O. Kitadani s.n.* (OSA); Jul. 5, 1933, *Y. Araki 291* (KYO, TI). **Osaka Pref.**: Yodo River, Kamishima, Hirakata-shi, Jun. 13, 1960, *K. Seto 9290* (OSA). **Hyogo Pref.**: Joraku, Hikami-cho, Hikami-gun, Jun. 9, 1991, *Y. Kadono 7021* (KOBE); Sept. 4, 1991, *Y. Kadono 7166* (KOBE); Kako-gawa River, Inahata, Hikami-cho, Hikami-gun, Oct. 18, 1986, *Y. Kadono 4158* (KOBE); Ihara, Sannan-cho, Hikami-gun, Sept. 7, 1988, *Y. Kadono 5518, 5730* (KOBE); Sept. 17, 1984, *Y. Kadono 3462* (KOBE); Ichijima, Ichijima-cho, Hikami-gun, Aug. 4, 1961, *H. Koyama 1054* (KYO, MAK, TNS); Atarino, Tannan-cho, Taki-gun, Dec. 15, 1998, *T. Fujii 10801* (OSA); Fukada-Park, Fujiga-oka, Sanda-shi, May 9, 1998, *T. Fujii 9496* (OSA); May 29, 1998, *T. Fujii 9537* (OSA); Aug. 28, 1998, *T. Fujii 10215* (OSA); Hinodesaka, Sanda-shi, Jun. 30, 2002, *T. Shiga 3148* (KOBE, OSA); Hirono-Kaitaku, Sanda-shi, Aug. 28, 1999, *T. Fujii 11587* (OSA); Kawahara, Sanda-shi, Aug. 7, 2000, *S. Fujii & T. Fujii 8097* (OSA); Namita, Sanda-shi, Sept. 5, 1996, *T. Fujii 6610* (OSA); Jul. 25, 1999, *T. Fujii 11422* (OSA); Sept. 1, 2001, *K. Katsura s.n.* (MAK, OSA); Jun. 30, 2002, *T. Shiga 3149* (KOBE, OSA), *3152*, *3153* (OSA); Ohara, Sanda-shi, Aug. 25, 1996, *S. Miyake 5176* (MAK); Aug. 25, 1996, *T. Fujii 5176* (OSA); Seikanji, Kamo, Sanda-shi, Sept. 9, 1997, *T. Fujii 8567* (OSA); Muko-gawa River, Tono, Tannan-cho, Sasayama-shi, Sept. 28, 2001, *T. Shiga 2787* (OSA); Jun. 3, 2002, *T. Shiga 3100* (KOBE, OSA); Tono, Sasayama-shi, Aug. 28, 1999, *T. Fujii 11585* (OSA); Sept. 27, 2001, *T. Yamazaki 1346* (OSA). **Hiroshima Pref.**: Seranishi-cho, Sera-gun, Aug. 16, 2002, *T. Shiga 3240* (KOBE, OSA); Kamitsuda, Seranishi-cho, Sera-gun, Aug. 15, 2002, *T. Shiga 3238* (OSA); Kurokawa to Kamitsuda, Seranishi-cho, Sera-gun, Jul. 30, 1988, *Y. Kadono s.n.* (KOBE); Gion, Fukuda, Daiwa-cho, Kamo-gun, Aug. 19, 2001, *K. Katsura s.n.* (OSA); Ohara, Shimotokura, Daiwa-cho, Kamo-gun, Aug. 19, 2001, *K. Katsura s.n.* (OSA); Hina, Nomi, Toyosaka-cho, Kamo-gun, Sept. 14, 1999, *K. Okuda & M. Shimoda 580-5* (SAPT); Nakayadani, Toyosaka-cho, Kamo-gun, Oct. 2, 1979, *N. Fukuoka, N. Kurosaki & M. Ito 2875* (KYO). **Tokushima Pref.**: Aratano, Anan-shi, Aug. 17, 1965, *Abe 19333* (TNS); Ohtsuda-gawa River, Nagaike-cho, Anan-shi, Aug. 3, 1997, *T. Umehara 7464* (OSA); Yoshino, Kainan-cho, Kaifu-gun, Sept. 25, 1909, *S. Fukui s.n.* (MAK). **Fukuoka Pref.**: Aso-ike Pond, Ikenoyama, Hoshino-mura, Yame-gun, Oct. 3, 2002, *T. Shiga 3666, 3667* (OSA). **Kumamoto Pref.**: Konoe, Higo, May 3, 1953, *K. Mayibara 4983* (TI, TNS), *4984* (TI); Nakaharu, Higo, Aug. 20, 1939, *K. Mayibara 3012* (TI, TNS). **Miyazaki Pref.**: Kawasaka-gawa River, Nagai, Kitagawa-cho, Higashiusuki-gun, Sept. 30, 2002, *T. Shiga 3314* (KOBE, OSA); Kirihara, Takanabe-cho, Koyu-gun, Aug. 3, 1984, *T. Minamitani 36298* (KOBE); Takanabe-cho, Koyu-gun, Jun. 2, 1958, *M. Nagasawa s.n.* (TNS); Ohyamada-ike Pond, Minou, Saito-shi, Oct. 1, 1989, *T. Minamitani 36315* (KOBE); Hamamiya, Takagi-cho, Miyakonojo-shi, Oct. 2, 2002, *T. Shiga 3281* (KOBE, OSA). **Kagoshima Pref.**: Prov. Satsuma, 1899, *T. Makino s.n.* (MAK); Hazuki, Okuchi-shi, Oct. 8, 1934, *Muramatsu s.n.* (TI). **KOREA**: Aug. 2004, *Y. Kadono s.n.* (KOBE).

Key to the forms of *Nuphar shimadae*:

A. Stigmatic disc tinged with red ..... f. *shimadae*

A. Stigmatic disc yellow ..... f. *oguraensis*

NATURAL HYBRIDS

1. **Nuphar ×fluminalis** Shiga & Kadono, **hybr. nov**. (*N. japonica* × *N. submersa*; Figs. 16B, D-F, 31)

Haec planta *Nuphari japonicae* DC. propinqua est, sed ab eis stigmatibus et antheris rufescentibus differt. Affinis *N. submersae* Shiga & Kadono, sed ad eis petiolis solidis et foliis sinuosis distat.

Typus: JAPAN, Pref. Tochigi, Sai-kawa River, Saikawa-bashi, Ohashi-cho, Sano-shi, Alt. 30 m, Aug. 7, 2005, *T. Shiga 3584* (Holotype OSA, Isotype TNS).

Description: Intermediate between *N. japonica* (Fig. 16C) and *N. submersa* (Fig. 16A) in morphology (Fig. 16B). Submerged leaves narrowly ovate, base with a sinus. Similar to *N. japonica*, but anthers strongly recurved after anthesis (Fig. 16B, D). Stigmatic disc and fruit tinged with red (Fig. 16D).

Japanese name: Nagare-kōhone (nov.)

Distribution: Japan (Tochigi pref.). Endemic.

Hab.: Rivers and streams

Note: The *Nuphar* plant called “Nagaba-beni-kohone” by Japanese aquatic plant cultivators is this nothospecies in some cases.

Further taxonomic studies about *N. pumila* and *N. submersa* is needed to determine whether this nothospecies should be treated as a nothosubspecies or nothovariety of *N.* ×*saijoensis* (Shimoda) Padgett and Shimoda

Specimens examined: **JAPAN**: **Tochigi Pref.**: Uba-gawa River, Mizuhono-cho, Ashikaga-shi, May 2, 2003, *H. Kawauchi s.n.* (TOCH); Kurahone, Ohtawara-shi, May 30, 1966, *H. Kato s.n.* (TOCH 121885); Kuwa-mura, Oyama-shi, Sept. 9, 1934, *K. Moritani 1359* (TOCH 12884); Kikusawa-gawa River, Funatsu-cho, Sano-shi, May 26, 2003, *H. Kawauchi s.n.* (TOCH); Kikusawa-gawa River, Horigome-cho, Sano-shi, Nov. 7, 2002, *J. Hasegawa s.n.* (TOCH 137486), Aug. 8, 2005, *T. Shiga 3587-3590, 3596* (OSA); Sept. 22, 2006, *T. Shiga 4015-4017* (OSA); Ishida, Meiji-mura, Kawachi-gun, Jul. 23, 1942, *K. Izawa s.n.* (TNS 70899); Egawa River, Shimokomagi, Mooka-shi, Alt. 60m, Aug. 7, 2005, *T. Shiga 3568-3571* (OSA);

Sept. 22, 2006, *T. Shiga 4021* (OSA); Sai-kawa River, Ohashi-cho, Sano-shi, Alt. 30 m, Aug. 7, 2005, *T. Shiga 3578-3583* (OSA); Sept. 22, 2006, *T. Shiga 4018-4020* (OSA).

2. **Nuphar ×hokkaiensis** Shiga & Kadono, **hybr. nov**. (*N. japonica* × *N. pumila*; Fig. 32)

="*Nuphar pumila* (Timm) DC. var. *ozeensis*" acut non H. Hara: Ito, Jour. Jap. Bot. **42**: 242 (1967).

="*N. japonica* × *pumila*" Swindells, Waterlilies 134 (1983).

Haec planta *Nuphari japonicae* DC. et *N. pumilae* (Timm) DC. propinqua est, sed ab eis foliis emersis ovatis oblongis, stigmatibus luteis vel rubris, fructibus chloris vel rubris differet.

Typus: JAPAN, Pref. Hokkaido; Lake Konuma, Oshima-shicho, Nanae-cho. alt. 120-130m, Sept. 9, 2004. *T. Shiga 3471* (Holotype OSA, Isotype SAPS).

Description: Intermediate between *N. japonica* and *N. pumila* in morphology. Similar to *N. japonica*, but anthers strongly recurved after anthesis. Stigmatic disc is yellow or tinged with red. Fruit is green or tinged with red.

Japanese name: Hokkai-kōhone (nov.)

Distribution: Japan (Hokkaido)

Note: Although an artificial hybrid between *N. japonica* and *N. pumila* was well known among aquatic plant cultivators, natural hybrid of this combination had not been reported (Swindells 1983). Because some *N.* ×*hokkaiensis* have red stigmatic disc, this hybrid species has often been identified as *N. pumila* var. *ozeensis* in Hokkaido, Japan (Ito 1967). *Nuphar* ×*hokkaiensis* has partly to fully fertile pollens (Chapter 3).

Further taxonomic studies about *N. pumila* and *N. shimadae* is needed to make clear whether this nothospecies should be treated as a nothosubspecies or

nothovariety of *N.* ×*saijoensis* (Shimoda) Padgett and Shimoda.

Specimens examined: **JAPAN**: **Hokkaido Pref.**: Asajino Moor, Sarufutsu-mura, Souya-gun, Aug. 26, 1999, *M. Yamazaki 9964, 9965* (SAPT); Lake Kamuito-numa, Asajino, Sarufutsu-mura, Souya-gun, Jul. 21, 1987, *Y. Kadono 4782* (KOBE); Sept. 2, 2004, *T. Shiga & S. Takebayashi 92* (KOBE, OSA); Lake Kimamo-numa, Sarufutsu-mura, Souya-gun, Jul. 28, 1966, *Ko. Ito s.n.* (MAK, SAPS, TI); Aug. 28, 1966, *S. Hayashi s.n.* (SAPS); Sept. 4, 1966, *S. Hayashi s.n.* (SAPS); Jul. 21, 1967, *Ko. Ito s.n.* (SAPS); Jul. 21, 1987, *Y. Kadono 4778* (KOBE); Lake Sansen-numa, Kamisarufutsu, Sarufutsu-mura, Souya-gun, Sept. 2, 2004, T. *Shiga & S. Takebayashi 212* (KOBE, OSA); Lake Mokeuni-numa, Sarufutsu-mura, Souya-gun, Jul. 26, 1998, *H. Fujita 9800653* (SAPT); Aug. 5, 1998, *H. Takahashi 25534* (SAPS); Soarobetsu Mire, Toyotomi-cho, Teshio-gun, Aug. 3, 1995, *H. Fujita 9500442, 9500443* (SAPT); Tsukigaumi, Tsukigata-cho, Kabato-gun, Jul. 13, 1982, *H. Takahashi & H. Kariya 2712* (SAPS); Sept. 4, 2004, *T. Shiga 3468* (KOBE, OSA); Oshima, Kameda (SAPS); Lake Jyunsai-numa, Nanae-cho, Kameda-gun, Aug. 17, 1888 (SAPS); Sept. 1913, *S. Nishida s.n.* (SAPS); Lake Konuma, Nanae-cho, Kameda-gun, Aug. 8, 1986, *Y. Kadono 3926* (KOBE); Aug. 18, 1989, *Y. Kadono 6173* (KOBE); Sept. 9, 2004, *T. Shiga 3470, 3472* (KOBE, OSA); Lake Ohnuma, Nanae-cho, Kameda-gun, Aug. 9, 1906, *K. Saida s.n.* (TNS); Jul. 9, 1916 (SAPS); Aug. 15, 1916, *F. C. Greatrex 170* (SAPS); Jul. 10, 1956, *T. Yamazaki s.n.* (TI); Sept. 15, 1980, *M. Hara 137, 138, 145* (SAPS); Jul. 20, 1980, *M. Hara 141* (SAPS); Aug. 16, 1984, *M. Hara s.n.* (SAPS); Aug. 8, 1986, *Y. Kadono 3923* (KOBE); Sept. 9, 2004, *T. Shiga 3473* (KOBE, OSA); Lake Kimonto-numa, Taiki-cho, Hiroo-gun, Aug. 9, 1998, *H. Takahashi 25628* (SAPS).

3. **Nuphar** ×**saijoensis** (Shimoda) Shiga & Kadono, **comb. nov**. (*N. japonica* × *N. shimadae*)

[Basionym] *Nuphar japonica* DC. var. *saijoense* Shimoda, J. Phytogeogr. Taxon. 39: 5. fig. 5-6, 10 (1991); Kadono, Aquat. Pl. Jap. 112 (1994). ≡*Nuphar* × *saijoensis* (Shimoda) Padgett and Shimoda (= *N. japonica* × *N. pumila* subsp. *oguraensis* (Miki) Padgett), Aquat. Bot. 72: 171 (2002). **Holotype**: JAPAN, Hiroshima Pref., Gouso, Saijo-cho, Higashi-hiroshima City (Pond 50 in Fig. 7 of Shimoda (1991)). Jun. 27, 1989, *M. Shimoda 4742* (HIRO!).

Description: Intermediate between *N. japonica* and *N. shimadae* in morphology. Similar to *N. japonica*, but anthers strongly recurved after anthesis. Stigmatic disc is yellow or tinged with red.

Japanese name: Saijō-kōhone

Distribution: Western Japan.

Note: Shimoda (1991) described this taxon as a variety of *N. japonica* and suggested hybrid origin between *N. japonica* and *N. oguraensis* var. *akiensis*. Padgett *et al.* (2002) investigated *Nuphar* plants in Saijo Basin, Hiroshima Pref., including type localities of *N. oguraensis* var. *akiensis* and *N. japonica* var. *saijoensis* and determined *N. japonica* var. *saijoensis* to be of hybrid origin between *N. japonica* and *N. oguraensis* var. *akiensis*. Then, they treated with *N. oguraensis* as a subspecies of *N. pumila*. Thus, *N.* ×*saijoensis* (Shimoda) Padgett and Shimoda is applied to hybrid between *N. japonica* and *N. pumila*. Hence, I proposed a new combination as hybrid between *N. japonica* and *N. shimadae* (= *N. oguraensis* var. *akiensis*).

Specimens examined: **JAPAN**: **Kyoto Pref.**: Ten-noh, Tanabe-cho, Tuzuki-gun, Sept. 14, 1980, *Y. Kadono 1139* (KOBE). **Hiroshima Pref.**: Kamitsuda, Seranishi-cho, Sera-gun, Aug. 15, 2002, *T. Shiga 3239* (KOBE, OSA); Seranishi-cho, Sera-gun, Aug. 16, 2002, *T. Shiga 3242* (KOBE, OSA); Nitanda, Seranishi-cho, Sera-gun, Aug. 15, 2002, *T. Shiga 3677* (KOBE, OSA); Yamato-cho, Kamo-gun, Aug. 3, 1987, *O. Sekino & I. Yamamoto s.n.* (KOBE); Gouso, Saijo-cho, Higashihiroshima-shi, August 16, 2002, *T. Shiga 3245* (KOBE, OSA); Hachihonmatsu, Higashihiroshima-shi, May 13, 1978, *Y. Kadono 1855* (KOBE). **Kochi Pref.**: Hizume-gawa River, Satokaida, Nangoku-shi, Nov. 5, 2003, *T. Shiga 3359-3361* (KOBE, OSA); Kusaka-gawa River, Shimobun, Hidaka-mura, Takaoka-gun, Nov. 5, 2003, *T. Shiga 3369, 3370* (KOBE, OSA). **Fukuoka Pref.**: Ohkado, Syonai-cho, Kaho-gun, Jun. 7, 1989, *S. Tsutsui s.n.* (KOBE). **Miyazaki Pref.**: Eda, Kitagawa-cho, Higashiusuki-gun, Nov. 25, 1989, *T. Minamitani s.n.* (KOBE); Sept. 30, 2002, *T. Shiga 3315* (KOBE, OSA), *3316* (KOBE); Jizo-ike Podn, Oose-cho, Miyazaki-shi, Oct. 28, 1989, *T. Minamitani s.n.* (KOBE); Oct. 1, 2002, *T. Shiga 3287* (KOBE, OSA).

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TABLE 1. Localities of 59 populations of *Nuphar*. Sampling size for morphological analysis (Mor.) and for allozyme analysis (Allo.) and number of multi-locus genotypes (MLGs; G) are also shown.

Pop. Code	Sampling locality	Latitude, N/ longitude, E	Mor.	Allo.	G
N. japonica **(Group 1)**					
AO-1	Aomori Pref.: Dekijima, Kizukuri-machi	40°50'/140°18'	21	25	1
AK-1	Akita Pref.: Kamikitatesaruta, Akita-shi	39°40'/140°10'	20	20	1
YA-1	Yamagata Pref.: Tazawa, Murayama-shi	38°33'/140°22'	26	35	1
FS-1	Fukushima Pref.: Shidahama, Inawashiro-machi	37°30'/140°9'	10	13	7
FS-2	Fukushima Pref.: Nozawa, Iitate-mura	37°41'/140°45'	10	32	2
FS-3	Fukushima Pref.: Hiranumanouchi, Iwaki-shi	37°0'/140°58'	10	15	1
IB-1	Ibaragi Pref.: Takasaki, Tamari-mura	36°10'/140°19'	10	15	1
IB-2	Ibaragi Pref.: Shimone-cho, Ushiku-shi	36°0'/140°10'	10	22	3
NI-1	Niigata Pref.: Niihana, Toyosaka-shi	37°54'/139°15'	21	30	2
NI-3	Niigata Pref.: Iwanokoshinden, Ogata-machi	37°13'/139°20'	10	n.d.	n.d.
NI-4	Niigata Pref.: Nagamine, Yoshikawa-machi	37°14'/138°22'	6	10	2
SI-2	Shiga Pref.: Hamabun, Imazu-cho	35°26'/136°3'	17	n.d.	n.d.
MI-2	Mie Pref.: Katsuta, Tamaki-machi	34°28'/136°37'	13	17	1
OK-2	Okayama Pref.: Kojimashirao, Kurashiki-shi	34°30'/133°52'	20	30	2
OK-4	Okayama Pref.: Kosaka, Saeki-machi	34°50'/134°3'	15	15	1
HI-10	Hiroshima Pref.: Hachihonmatsu, Higashihiroshima-	34°25'/132°41'	5	10	5
MY-1a	Miyazaki Pref.: Nagai, Kitagawa-cho (A)	32°40'/131°43'	10	n.d.	n.d.
MY-6	Miyazaki Pref.: Toyomitsu-cho, Miyakonojo-shi	31°41'/131°6'	16	23	2
N. oguraensis **(Group 3)**					
HY-8	Hyogo Pref.: Namita, Sanda-shi	34°58'/135°10'	10	24	1
HI-2b	Hiroshima Pref.: Kamitsuda, Seranishi-machi (B)	34°39'/132°54'	10	10	2
HI-7	Hiroshima Pref.: Oguni, Seranishi-machi	34°38'/132°56'	10	25	1
TO-4	Tokushima Pref.: Noe, Kaifu-cho	33°36'/134°20'	n.d.	10	1
FO-1	Fukuoka Pref.: Ikenoyama, Hoshino-mura	33°15'/130°45'	10	13	2
MY-2	Miyazaki Pref.: Nagai, Kitagawa-cho	32°40'/131°43'	10	17	2
MY-8	Miyazaki Pref.: Takagi-cho, Miyakonojo-shi	31°47'/131°6'	10	10	2
N. subintegerrima **(Group 5)**					
GI-1	Gifu Pref.: Tachibokubora, Gifu-shi	35°26'/136°48'	23	20	4
AI-1	Aichi Pref.: Nakashitami, Nagoya-shi	35°15'/137°2'	n.d.	8	1
AI-2	Aichi Pref.: Ikenodai, Inuyama-shi	35°21'/137°1'	19	25	3
MI-1	Mie Pref.: Oshihuchi, Nansei-cho	34°20'/136°38'	21	20	1
MI-3	Mie Pref.: Ukata, Ago-cho	34°20'/136°50'	n.d.	12	1
japonica-oguraensis **intermediate plants (Group 2)**					
NI-5	Niigata Pref.: Ikenodaira, Ojiya-shi	37°13'/138°50'	10	10	3
FU-1	Fukui Pref.: Ikenokouchi, Turuga-shi	35°39'/136°8'	14	20	8
FU-3	Fukui Pref.: Kunugi, Kanatsu-cho	36°12'/136°17'	20	30	2
HY-3	Hyogo Pref.: Tadokoro, Goshiki-machi	34°25'/134°49'	10	18	1
OK-1	Okayama Pref.: Mitsuishigokoku, Bizen-shi	34°47'/134°17'	28	20	3
HI-2a	Hiroshima Pref.: Kamitsuda, Seranishi-machi (A)	34°39'/132°54'	18	45	1
HI-4	Hiroshima Pref.: Nitanda, Seranishi-machi	34°36'/132°57'	n.d.	10	7
HI-5	Hiroshima Pref.: Nitanda, Seranishi-machi	34°36'/132°57'	n.d.	20	4
KA-4	Kagawa Pref.: Nishiwake, Ayakami-cho	34°12'/133°56'	31	10	1
TO-1	Tokushima Pref.: Taura-cho, Komatsushima-shi	34°0'/134°34'	22	10	5

TABLE 1. (continued)

Pop. Code	Sampling locality	Latitude, N/ longitude, E	Mor.	Allo.	G
TO-2	Tokushima Pref.: Kandase-cho, Komatsushima-shi	34°0'/134°35'	30	10	1
TO-3a	Tokushima Pref.: Shibahu-cho, Komatsushima-shi (A)	34°0'/134°35'	22	10	1
TO-3b	Tokushima Pref.: Shibahu-cho, Komatsushima-shi (B)	34°0'/134°35'	21	15	3
MY-1b	Miyazaki Pref.: Nagai, Kitagawa-cho (B)	32°40'/131°43'	10	13	1
MY-1c	Miyazaki Pref.: Nagai, Kitagawa-cho (C)	32°40'/131°43'	10	8	2
japonica-subintegerrima **intermediate plants (Group 4)**					
FU-2	Fukui Pref.: Heisenji-cho, Katuyama-shi	36°2'/136°32'	22	30	11
GI-2	Gifu Pref.: Higashitabirako, Kani-shi	35°24'/137°1'	28	30	12
GI-3	Gifu Pref.: Itoshiro, Shiratori-machi	36°0'/136°46'	42	30	8
SI-1	Shiga Pref.: Warasono, Shinasahi-machi	35°21'/136°4'	17	30	13
NA-1	Nara Pref.: Hokkeji-cho, Nara-shi	34°42'/135°48'	10	25	4
HY-1	Hyogo Pref.: Ota-cho, Ono-shi	34°52'/135°0'	39	30	18
HY-5	Hyogo Pref.: Abiki-cho, Kasai-shi	34°52'/134°53'	15	24	4
HY-7	Hyogo Pref.: Tamaoka-cho, Kasai-shi	34°55'/134°51'	15	16	4
OK-3	Okayama Pref.: Kojimashirao, Kurashiki-shi	34°29'/133°52'	20	20	2
OK-6	Okayama Pref.: Sugisawa, Saeki-machi	34°52'/134°8'	40	15	1
KA-1	Kagawa Pref.: Sakamoto, Hiketa-cho	34°12'/134°26'	16	10	1
KA-2	Kagawa Pref.: Gomyo, Shirotori-cho	34°12'/134°16'	n.d.	20	1
KA-5	Kagawa Pref.: Tomikuma, Ayauta-machi	34°15'/133°52'	27	25	3
OH-1	Ohita Pref.: Minamiusa, Usa-shi	33°31'/131°23'	21	28	21

TABLE 2. Twenty seven morphological characters investigated. Characters marked with asterisk were used for cluster analysis.

Character code	Characters measured
Emergent and floating leaf	
L1	Leaf blade length (cm)*
L2	Leaf blade width (cm)
L3	Leaf blade shape (L1/L2)*
L4	Sinus depth (cm)
L5	Sinus/length of blade ratio (L4/L1)
L6	Length to the maximum blade width position from the blade (cm)
L7	Maximum blade width position/total length of blade ratio (L6/L1)*
L8	Petiole diameter at 5 cm from the base of blade (mm)
L9	Presence or absence of central lacuna in petiole* : 0 = present, 1 = absent
Flower	
Fl1	Maximum length of stigmatic disk (mm)*
Fl2	Minimum length of stigmatic disk (mm)
Fl3	Min./max. ratio of disk length (Fl2/Fl1)*
Fl4	Dentation on the margin of stigmatic disk : 0 = entire, 1 = dentate
Fl5	Stigma length (mm)
Fl6	Stigma width (mm)*
Fl7	Length/width ratio of stigma (Fl5/Fl6)*
Fl8	Number of stigma
Fl9	Apical shape of stigma* : 1 = roundish, 2 = obtuse, 3 = acute
Fl10	Anther length (mm)*
Fl11	Filament length (mm)
Fl12	Anther length/filament length ratio (Fl10/Fl11)*
Fruit	
Fr1	Fruit length (mm)*
Fr2	Fruit width (mm)
Fr3	Length/width ratio of fruit (Frl/Fr2)*
Fr4	Seed length (mm)*
Fr5	Seed width (mm)*
Fr6	Length/width ratio of seed (Fr4/Fr5)

TABLE 3. Comparison of morphological characters for five cluster groups (mean ± SD). Different letters indicate significant differences by Tukey HSD multiple comparison test ($P < 0.05$).

A. Emergent and floating leaf characters

	Cluster group 1			2			3			4			5		
Character	mean ± SD			mean ± SD			mean ± SD			mean ± SD			mean ± SD		
L1 (cm)	30.7 ± 4.72	**a**		20.8 ± 4.26	**b**		14.3 ± 2.09	**c**		18.8 ± 3.28	**d**		11.6 ± 2.90	**e**	
L2 (cm)	18.4 ± 2.58	**a**		13.5 ± 2.33	**b**		11.2 ± 1.47	**c**		12.7 ± 2.64	**b**		10.4 ± 2.53	**d**	
L3 (L1/L2)	1.68 ± 0.18	**a**		1.54 ± 0.15	**b**		1.28 ± 0.10	**c**		1.51 ± 0.21	**d**		1.12 ± 0.06	**e**	
L4 (cm)	8.1 ± 1.30	**a**		6.6 ± 0.97	**b**		6.1 ± 0.78	**c**		6.1 ± 1.00	**c**		4.8 ± 0.80	**d**	
L5 (L4/L1)	0.27 ± 0.04	**a**		0.32 ± 0.05	**b**		0.43 ± 0.02	**c**		0.32 ± 0.03	**b**		0.42 ± 0.04	**c**	
L6 (cm)	20.4 ± 3.75	**a**		12.7 ± 3.28	**b**		7.5 ± 1.31	**c**		11.6 ± 2.21	**d**		6.4 ± 1.77	**e**	
L7 (L6/L1)	0.66 ± 0.06	**a**		0.61 ± 0.05	**b**		0.53 ± 0.03	**c**		0.61 ± 0.05	**b**		0.55 ± 0.03	**d**	
L8 (mm)	9.1 ± 1.36	**a**		6.1 ± 1.59	**b**		3.3 ± 0.89	**c**		5.9 ± 1.22	**b**		4.0 ± 1.12	**d**	
L9	1.00 ± 0.00			1.00 ± 0.00			0.02 ± 0.14			1.00 ± 0.00			1.00 ± 0.00		
Sample no.	205			222			54			313			62		

TABLE 3. (continued)

B. Flower characters

Character	Cluster group 1				2				3				4				5			
	mean	±	SD		mean	±	SD		mean	±	SD		mean	±	SD		mean	±	SD	
Fl1 (mm)	10.3	±	1.71	**a**	8.2	±	1.35	**b**	7.0	±	1.29	**c**	7.5	±	1.35	**c**	6.3	±	0.79	**d**
Fl2 (mm)	9.2	±	1.51	**a**	7.7	±	1.24	**b**	6.6	±	1.08	**c**	7.0	±	1.29	**c**	6.0	±	0.79	**d**
Fl3 (Fl2/Fl1)	0.90	±	0.09	**a**	0.94	±	0.05	**b**	0.94	±	0.05	**b**	0.93	±	0.05	**b**	0.95	±	0.04	**b**
Fl4	0.94	±	0.18	**a**	0.94	±	0.17	**a**	0.97	±	0.12	**a**	0.85	±	0.31	**b**	0.73	±	0.33	**c**
Fl5 (mm)	4.0	±	0.66	**a**	3.3	±	0.55	**b**	2.8	±	0.52	**c**	3.0	±	0.62	**c**	2.5	±	0.33	**d**
Fl6 (mm)	1.0	±	0.14	**a**	0.8	±	0.13	**b**	0.7	±	0.17	**c**	1.0	±	0.19	**a**	1.2	±	0.15	**d**
Fl7 (Fl5/Fl6)	4.36	±	1.20	**ab**	4.14	±	0.92	**a**	4.10	±	1.04	**b**	3.08	±	0.58	**c**	2.11	±	0.37	**d**
Fl8 (no.)	13.74	±	2.55	**a**	12.14	±	1.92	**b**	10.48	±	1.57	**c**	11.42	±	2.37	**d**	8.37	±	1.27	**e**
Fl9	2.96	±	0.14	**a**	2.98	±	0.07	**a**	2.75	±	0.44	**b**	2.75	±	0.36	**b**	2.21	±	0.37	**c**
Sample no.	142				109				55				118				30			
Fl10 (mm)	5.4	±	0.73	**a**	4.4	±	0.75	**b**	3.2	±	0.39	**c**	4.7	±	0.72	**d**	4.2	±	0.75	**b**
Fl11 (mm)	5.8	±	0.91	**a**	6.2	±	1.28	**b**	6.5	±	1.50	**b**	5.2	±	1.01	**c**	4.4	±	1.18	**d**
Fl12 (Fl10/Fl11	0.94	±	0.14	**a**	0.73	±	0.16	**b**	0.51	±	0.08	**c**	0.92	±	0.17	**a**	1.02	±	0.24	**d**
Sample no.	162				130				60				129				30			

TABLE 3. (continued)

C. Fruit characters

Character	Cluster group 1				2				3				4				5			
	mean	±	SD		mean	±	SD		mean	±	SD		mean	±	SD		mean	±	SD	
Fr1 (mm)	41.9	±	6.23	**a**	32.4	±	5.53	**b**	32.3	±	4.99	**b**	31.2	±	4.92	**b**	28.5	±	5.47	**c**
Fr2 (mm)	30.5	±	4.21	**a**	22.0	±	5.04	**b**	20.7	±	3.61	**b**	24.1	±	3.86	**c**	20.2	±	3.04	**b**
Fr3 (Fr1/Fr2)	1.39	±	0.20	**a**	1.53	±	0.30	**b**	1.58	±	0.19	**b**	1.31	±	0.21	**c**	1.42	±	0.21	**a**
Fr4 (mm)	4.8	±	0.30	**a**	4.8	±	0.44	**b**	4.5	±	0.31	**b**	4.6	±	0.41	**b**	5.7	±	0.35	**c**
Fr5 (mm)	3.6	±	0.31	**a**	3.3	±	0.45	**b**	2.9	±	0.28	**c**	3.7	±	0.38	**a**	4.3	±	0.35	**d**
Fr6 (Fr4/Fr5)	1.36	±	0.10	**a**	1.47	±	0.14	**b**	1.55	±	0.17	**c**	1.26	±	0.09	**d**	1.33	±	0.11	**a**
Sample no.	139				96				51				118				25			

TABLE 4. Loadings of 15 morphological characters for the first three factors from the analysis of 52 populations of *Nuphar* in central to western Japan.

Character	PCA 1	PCA 2	PCA 3
L1	0.8848	0.2381	-0.0423
L3	0.7840	0.3377	0.1413
L7	0.8311	-0.0602	0.0638
L9	0.6335	-0.3639	0.2433
Fl1	0.8207	0.3461	-0.0913
Fl3	-0.4259	-0.1377	0.5294
Fl6	0.2950	-0.8234	-0.1975
Fl7	0.2544	0.8055	0.0539
Fl9	0.4146	0.6626	0.3978
Fl10	0.8778	-0.1412	0.0263
Fl12	0.6320	-0.5681	0.2187
Fr1	0.6811	0.2461	-0.5330
Fr3	-0.3631	0.4036	-0.2587
Fr4	0.1544	-0.3653	-0.5573
Fr5	-0.3989	0.6414	-0.2343
Eigenvalue	5.6169	3.3224	1.3388
Variance %	37.44%	22.14%	8.93%
Cumulative % of variance	37.44%	59.59%	68.52%

TABLE 5. Mean allele frequencies for five *Nuphar* species or groups according to morphological type for 12 scorable loci.

Locus	allele	Cluster group				
		3	2	1	4	5
lap1	A	1.000	0.944	1.000	0.801	0.329
	B	0.000	0.056	0.000	0.106	0.377
	null	0.000	0.000	0.000	0.094	0.294
mdh1	A	0.092	0.066	0.000	0.000	0.000
	B	0.789	0.934	1.000	1.000	1.000
	C	0.119	0.000	0.000	0.000	0.000
mdh2	A	1.000	1.000	1.000	1.000	1.000
mdh3	A	1.000	0.281	0.000	0.000	0.000
	B	0.000	0.639	0.960	1.000	1.000
	C	0.000	0.080	0.040	0.000	0.000
mdh4	A	0.000	0.446	0.154	0.561	0.529
	B	1.000	0.554	0.846	0.439	0.471
pgi1	A	0.000	0.000	0.010	0.000	0.471
	B	1.000	0.998	0.990	0.916	0.529
	C	0.000	0.002	0.000	0.084	0.000
pgi2	A	1.000	0.972	0.997	1.000	1.000
	B	0.000	0.028	0.003	0.000	0.000
pgm1	A	0.661	0.245	0.139	0.578	0.553
	B	0.339	0.755	0.861	0.422	0.447
pmi1	A	0.000	0.000	0.000	0.013	0.024
	B	0.000	0.233	0.072	0.634	0.977
	C	1.000	0.767	0.928	0.352	0.000
pmi2	A	0.000	0.221	0.000	0.726	1.000
	null	1.000	0.779	1.000	0.274	0.000
tpi2	A	0.849	1.000	1.000	1.000	1.000
	B	0.151	0.000	0.000	0.000	0.000
tpi3	A	0.000	0.000	0.000	0.117	0.529
	B	1.000	1.000	1.000	0.866	0.377
	C	0.000	0.000	0.000	0.017	0.094

TABLE 6. Fruit set after crossing among five *Nuphar* populations.

A. Green house experiment

Female parent	Pollen donor: Group 1 NI-1		Group 3 HY-8		Group 5 GI-1		Group 5 MI-1		Group4 HY-1		Emasculation	
NI-1	66.7	(6)	54.5	(11)	61.5	(11)	66.7	(13)	62.5	(8)	0	(8)
HY-8	25.0	(4)	50.0	(2)	100.0	(1)	0.0	(1)	n.d.		0	(1)
GI-1	55.6	(9)	50.0	(6)	46.2	(13)	27.3	(11)	27.3	(11)	0	(4)
MI-1	66.7	(6)	42.7	(7)	50.0	(8)	75.0	(4)	33.3	(9)	0	(2)
HY-1	50.0	(2)	n.d.		50.0	(2)	60.0	(5)	20.0	(5)	0	(1)

Values show means (%). Sample size in parenthes.

B. Field experiment

Female parent	Pollen donor: Group 1 NI-1		Group 3 HY-8		Group 5 GI-1		Group 5 MI-1		Group4 HY-1		Emasculation	
NI-1	87.5	(8)	100.0	(5)	100.0	(2)	100.0	(1)	100.0	(4)	0	(2)
HY-8	83.3	(12)	63.6	(11)	75.0	(8)	70.0	(10)	88.9	(9)	0	(7)
GI-1	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
MI-1	100.0	(1)	n.d.		n.d.		100.0	(4)	100.0	(1)	n.d.	
HY-1	88.9	(9)	50.0	(4)	40.0	(5)	57.1	(7)	53.8	(13)	0	(3)

Values show means (%). Sample size in parenthes.

TABLE 7. Germination rate after crossing among five *Nuphar* populations (means ± SD (%)). Different letters indicate significant differences by Tukey HSD multiple comparison test ($P < 0.05$).

Female parent	Pollen donor					Difference among pollen donors
	Group 1	Group 3	Group 5		Group4	
	NI-1	HY-8	GI-1	MI-1	HY-1	
NI-1	71.4 ± 29.6	79.7 ± 20.9	60.1 ± 32.3	37.9 ± 28.9	68.6 ± 34.1	H=1.444 **NS**
HY-8	8.5 ± 14.8 **a**	52.9 ± 28.7 **b**	4.8 ± 5.8 **a**	5.3 ± 11.4 **a**	6.7 ± 6.4 **a**	H=16.413 ***P*<0.01**
GI-1	44.2 ± 34.1	45.9 ± 35.0	66.2 ± 27.9	43.4 ± 49.5	49.2 ± 45.0	H=5.946 **NS**
MI-1	65.2 ± 23.0	60.1 ± 32.3	77.3 ± 25.3	78.5 ± 22.3	63.3 ± 15.3	H=0.748 **NS**
HY-1	56.9 ± 32.6	41.0 ± 22.0	46.9 ± 12.3	59.7 ± 30.6	39.5 ± 29.8	H=2.956 **NS**

TABLE 8. Localities of 22 *Nuphar* populations in Hokkaido used in this study. Numberes of samples for morphological measurements (Morph.), allozyme analyses (Allo.) and pollen stainability (pollen) are also listed.

Population Code	Sampling locality	Latitude (N)	Longitude (E)	Morph.	Allo.	Pollen
NNO	Ohnuma-cho, Nanae-cho	41°59'18"	140°39'52"	26	26	26
TAO	Bansei, Taiki-cho	42°33'54"	143°28'3"	20	20	3
ATT	Tomino, Atsuma-cho	42°38'50"	141°52'45"	20	20	6
ATO	Koinuma, Atsuma-cho	42°38'11"	141°53'39"	12	20	3
TOJ	Ohtsu, Toyokoro-cho	42°40'46"	143°37'54"	3	6	1
TOB	Bisawa, Tomakomai-shi	42°44'26"	141°43'18"	16	20	3
AKT	Honmachi, Akkeshi-cho	43°00'13"	144°51'59"	20	20	3
HAK	Hamanaka, Hamanaka-cho	43°06'37"	145°06'09"	3	7	1
KIO	Bibaitappu, Kita-mura	43°13'07"	141°40'15"	16	16	3
TST	Tsukigaumi, Tsukigata-cho	43°18'28"	141°37'26"	40	40	40
NEN	Katsuragi, Nemuro-shi	43°19'11"	145°37'02"	20	20	3
NET	Makinouchi, Nemuro-shi	43°19'45"	145°37'21"	14	20	3
BEB	Honbekkai, Bekkai-cho	43°25'26"	145°14'56"	20	20	6
TSC	Numasawa., Tsubetsu-cho	43°38'08"	143°52'38"	6	20	5
URU	Uryu-numa Moor, Uryu-cho	43°42'00"	141°36'06"	27	60	5
KUT	Takkobu, Kushiro-shi	43°6'18"	144°29'07"	12	12	3
SAM	Nakanuma-cho, Higashi-ku, Sapporo-shi	43°7'38"	141°26'12"	6	7	3
HOO	Otoi, Horonobe-cho	45°00'09"	141°41'45"	6	10	1
SAS	Kamisarufutsu, Sarufutsu-mura	45°09'31"	142°07'35"	3	3	3
SAA	Asaginodaichi, Sarufutsu-mura	45°12'51"	142°15'21"	20	20	3
SAK	Asagi, Sarufutsu-mura	45°14'07"	142°12'35"	16	29	16
WAM	Keihoku, Wakkanai-shi	45°24'03"	141°49'01"	3	7	1

TABLE 9. Twenty one morphological characters investigated. Characters marked with asterisk were used for cluster and principal component analyses.

Character code	Characters measured
Emergent and floating leaf	
L1	Leaf blade length (cm)*
L2	Leaf blade width (cm)
L3	Leaf blade shape (L1/L2)*
L4	Sinus depth (cm)
L5	Sinus/length of blade ratio (L4/L1)*
L6	Length from the maximum blade width position of the blade (cm)
L7	Maximum blade width position/total length of blade ratio (L6/L1)*
L8	Maximum petiole width at 5 cm from the base of blade (mm)*
L9	Minimum petiole width at 5 cm from the base of blade (mm)
L10	Min./max. of petiole width ratio (L9/L8)*
L11	Number of vein*
Flower	
Fl1	Maximum length of stigmatic disk (mm)*
Fl2	Stigma width (mm)*
Fl3	Number of stigma*
Fl4	Anther length (mm)*
Fl5	Filament length (mm)
Fl6	Anther length/filament length ratio (Fl4/Fl5)*
Fruit	
Fr1	Fruit length (mm)*
Fr2	Seed length (mm)*
Fr3	Seed width (mm)
Fr4	Length/width ratio of seed (Fr2/Fr3)*

TABLE 10. Comparison of morphological characters for three cluster groups (mean ± SD). Different letters indicate significant differences by Tukey HSD multiple comparison test ($P < 0.05$).

	Cluster group 1				2				3			
Character	mean	±	SD		mean	±	SD		mean	±	SD	
Emergent and floating leaf characters												
L1 (cm)	32.81	±	5.48	**a**	27.46	±	7.15	**b**	14.7	±	3.11	**c**
L2 (cm)	17.84	±	3.59	**a**	16.90	±	3.44	**b**	10.8	±	2.00	**c**
L3 (L1/L2)	0.54	±	0.05	**a**	0.62	±	0.05	**b**	0.74	±	0.05	**c**
L4 (cm)	7.16	±	1.78	**a**	8.25	±	1.43	**b**	5.5	±	0.99	**c**
L5 (L4/L1)	0.22	±	0.03	**a**	0.31	±	0.04	**b**	0.38	±	0.03	**c**
L6 (cm)	21.63	±	3.42	**a**	16.99	±	5.09	**b**	8.1	±	1.99	**c**
L7 (L6/L1)	0.66	±	0.05	**a**	0.61	±	0.03	**b**	0.54	±	0.03	**c**
L8 (mm)	10.44	±	1.67	**a**	8.19	±	2.39	**b**	4.98	±	1.13	**c**
L9 (mm)	8.97	±	1.57	**a**	5.82	±	2.55	**b**	2.72	±	0.62	**c**
L10 (L9/L8)	0.86	±	0.05	**a**	0.69	±	0.13	**b**	0.55	±	0.07	**c**
L11 (no.)	79.16	±	12.28	**a**	58.30	±	14.21	**b**	35.41	±	6.43	**c**
Sample no.	112				115				219			
Flower characters												
Fl1 (mm)	9.94	±	1.88	**a**	9.90	±	1.65	**b**	6.85	±	1.29	**c**
Fl2 (mm)	1.01	±	0.16	**a**	0.86	±	0.26	**b**	0.70	±	0.16	**c**
Fl3 (no.)	13.22	±	3.15	**a**	12.75	±	2.71	**ab**	10.77	±	1.87	**b**
Fl4 (mm)	4.17	±	0.61	**a**	4.15	±	0.54	**a**	2.77	±	0.38	**b**
Fl5 (mm)	5.88	±	0.93	**a**	7.64	±	1.46	**ab**	7.78	±	1.49	**b**
Fl6 (Fl4/Fl5)	0.72	±	0.13	**a**	0.56	±	0.14	**b**	0.37	±	0.08	**c**
Sample no.	104				88				152			
Fruit characters												
Fr1 (mm)	44.51	±	7.26	**a**	36.62	±	7.46	**b**	30.32	±	5.26	**b**
Fr2 (mm)	4.62	±	0.39	**a**	4.13	±	0.41	**ab**	4.13	±	0.35	**b**
Fr3 (mm)	3.48	±	0.33	**a**	2.81	±	0.48	**b**	2.34	±	0.28	**c**
Fr4 (Fr5/Fr4)	0.75	±	0.04	**a**	0.68	±	0.08	**b**	0.57	±	0.05	**c**
Sample no.	109				88				193			

TABLE 11. Factor loadings of 15 morphological characters to the first three components of the PCA for 22 populations of *Nuphar* in Hokkaido. Eigenvalues and percentages of variance and cumultative variance to the total variance for the first three principal components are also listed.

Character	PCA 1	PCA 2	PCA 3
L1	0.9331	-0.0751	0.0848
L3	-0.8737	0.1337	-0.1068
L5	-0.9081	0.1541	-0.0287
L7	0.8151	-0.0039	0.1007
L8	0.9097	-0.0131	0.0375
L10	0.8673	-0.1952	-0.0895
L11	0.9388	-0.1243	-0.0038
Fl1	0.7763	0.5095	0.2023
Fl2	0.7674	0.2630	-0.1394
Fl3	0.5723	0.7108	0.2185
Fl4	0.8070	-0.1097	0.1424
Fl6	0.8262	-0.2921	0.0709
Fr1	0.7816	-0.0229	-0.3235
Fr2	0.4974	0.2210	-0.7850
Fr4	0.8679	-0.1594	0.0707
Eigenvalue	10.0487	1.1070	0.8982
Variance %	66.99%	7.38%	5.99%
Cumulative % of variance	66.99%	74.37%	80.36%

TABLE 12. Frequencies of genotypes of *mdh3* and Mean pollen viability (%) for 22 *Nuphar* populations in Hokkaido, Japan.

Population Code	Genotype of *mdh3*				Pollen viability (%)	
	aa	ab	bb	bc	mean	(SD)
N. japonica						
TAO		0.050	0.950		95.1	(2.8)
ATT			1.000		94.6	(3.5)
TOB		0.125	0.875		93.3	(2.1)
KIO			1.000		90.3	(3.2)
SAM				1.000	96.3	(2.3)
HOO			1.000		87.2	
SAA	0.050	0.050	0.900		85.7	(11.0)
Intermediate populations						
NNO		0.692	0.308		49.8	(37.8)
TST	0.050	0.625	0.325		73.7	(19.2)
SAS		1.000			17.6	(2.8)
SAK		0.793	0.207		63.5	(41.3)
N. pumila						
ATO	1.000				71.1	(15.7)
TOJ	0.500	0.500			86.4	
AKT	1.000				86.2	(16.4)
HAK	1.000				89.1	
NEN	1.000				97.7	(0.6)
NET	1.000				99.2	(0.0)
BEB	1.000				98.5	(0.7)
TSC	1.000				95.0	(1.5)
URU	1.000				94.3	(1.9)
KUT	1.000				94.6	(5.7)
WAM	1.000				91.0	

TABLE13. Chloroplast DNA (cpDNA) haplotypes, AFLP genotypes and additive patterns of AFLP bands for *N. japonica* and *N. pumila.* N shows the number of samples used for analyses.

Taxa	Population ID	N	cpDNA	Genotype	AFLP analyses			
					Specific bands for *N. japonica* (A)	Observed ratio of A	Specific bands for *N. pumila* (B)	Observed ratio of B
N. japonica	SAA	3	238	1-3	25	1.00	---	---
	HOO	1	238	4	25	1.00	---	---
	SAM	3	238	5-7	25	1.00	---	---
	KIO	3	238	8-10	25	1.00	---	---
	TOB	3	238	11-13	25	1.00	---	---
	ATT	6	238	14-19	25	1.00	---	---
	TAO	3	238	20-22	25	1.00	---	---
Hybrid populations	SAS	1	238	23	25	1.00	15	0.94
		3	238	24	25	1.00	16	1.00
	SAK	1	238	25	25	1.00	6	0.38
		2	238	26	25	1.00	9	0.56
		3	238	27	25	1.00	10	0.63
		2	238	28-29	24	0.96	11	0.69
		1	238	30	23	0.92	12	0.75
		1	238	31	25	1.00	11	0.69
		3	238	32	24	0.96	12	0.75
		1	238	33	25	1.00	12	0.75
		1	238	34	24	0.96	13	0.81
		1	238	35	25	1.00	13	0.81
	TST	1	238	36	25	1.00	---	---
		2	238	37	24	0.96	---	---
		2	238	38	23	0.92	---	---
		1	238	39	22	0.88	---	---
		2	238	40	24	0.96	1	0.06
		1	238	41	23	0.92	2	0.13
		1	238	42	20	0.80	5	0.31
		4	238	43	24	0.96	2	0.13
		1	238	44	21	0.84	5	0.31
		1	238	45	19	0.76	8	0.50
		1	238	46	20	0.80	9	0.56
		1	238	47	25	1.00	5	0.31
		1	238	48	22	0.88	8	0.50
		1	238	49	21	0.84	10	0.63
		1	238	50	21	0.84	11	0.69
		1	238	51	19	0.76	13	0.81
		1	234	52	23	0.92	10	0.63
		2	238	53	22	0.88	11	0.69
		1	238	54	24	0.96	10	0.63
		3	238	55	23	0.92	11	0.69
		1	238	56	22	0.88	12	0.75
		1	238	57	23	0.92	12	0.75
		7	238	58	23	0.92	12	0.75
		1	238	59	25	1.00	12	0.75

TABLE 13. (continued)

Taxa	Pop. ID	N	cpDNA	AFLP analyses				
				Genotype	Specific bands for *N. japonica*	Observed ratio of A	Specific bands for *N. pumila*	Observed ratio of B
Hybrid population	NNO	2	238	60	25	1.00	---	---
		1	238	61	24	0.96	---	---
		2	238	62	24	0.96	1	0.06
		1	238	63	25	1.00	1	0.06
		1	238	64	18	0.72	8	0.50
		1	234	65	23	0.92	8	0.50
		1	234	66	22	0.88	9	0.56
		1	234	67	23	0.92	10	0.63
		1	238	68	20	0.80	13	0.81
		1	238	69	23	0.92	11	0.69
		1	238	70	21	0.84	14	0.88
		1	238	71	24	0.96	12	0.75
		4	238	72	23	0.92	13	0.81
		1	234	73	23	0.92	13	0.81
		2	238	74	24	0.96	13	0.81
N. pumila	WAM	1	234	75	---	---	16	1.00
	KUT	3	234	76-78	---	---	16	1.00
	URU	5	241	79-83	---	---	16	1.00
	TSC	5	234	84-88	---	---	16	1.00
	BEB	6	234	89-94	---	---	16	1.00
	NET	3	234	95-97	---	---	16	1.00
	NEN	3	234	98-100	---	---	16	1.00
	HAK	1	234	101	---	---	16	1.00
	AKT	3	234	102-104	---	---	16	1.00
	TOJ	1	234	105	---	---	16	1.00
	ATO	3	234	106-108	---	---	16	1.00
Artificial hybrid	ATT×BEB	3	234		25	1.00	16	1.00
(male × female)	BEB×ATT	3	238		25	1.00	16	1.00

TABLE 14. Localities of the 9 *Nuphar* populations used in this study. Numbers of samples (N) for morphological measurements, allozyme analyses, and pollen viability are shown. All voucher specimens are deposited in OSA.

Population Code	Sampling locality	Latitude (N)	Longitude (E)	N	Voucher specimen
Nuphar japonica **(Group 1)**					
YA-1	Yamagata Pref.: Tazawa, Murayama-shi	38°32'39"	140°22'12"	10	*T. Shiga 3120*
FS-2	Fukushima Pref.: Sekisawa, Iitate-mura	37°40'49"	140°45'00"	10	*T. Shiga 3250*
IB-2	Ibaragi Pref.: Tamasaki, Tamari-mura	36°09'32"	140°19'00"	10	*T. Shiga 3252*
NI-4	Niigata Pref.: Nagamine, Yoshikawa-cho	37°14'44"	138°21'58"	10	*T. Shiga 3320*
N. submersa **(Group 3)**					
TG-1	Tochigi Pref.: Koshiro, Nikko-shi	36°39'	139°43'	10	*T. Shiga 3480*
TG-2	Tochigi Pref.: Shimokawai, Nasukarasuyama-shi	36°42'	146°06'	10	*T. Shiga 3560*
Intermediate plants (Group 2)					
TG-3	Tochigi Pref.: Shimokomagi, Mooka-shi	36°26'10"	139°58'56"	6	*T. Shiga 3571*
TG-4	Tochigi Pref.: Ohashi-cho, Sano-shi	36°19'00"	139°33'22"	10	*T. Shiga 3584*
TG-5	Tochigi Pref.: Horigome-cho, Sano-shi	36°20'05"	139°34'05"	10	*T. Shiga 3587*

TABLE 15. Comparison of morphological characteristics of the three groups. Values within a row followed by different alphabets differ significantly (Tukey's HSD multiple-comparison test, $P < 0.05$).

	Cluster group											
	1				2				3			
Characteristic	Mean	±	SD		Mean	±	SD		Mean	±	SD	
Submerged leaf characteristics												
SL1 (cm)	32.3	±	5.8	**a**	21.3	±	4.8	**b**	13.1	±	3.1	**c**
SL3 (SL2/SL1)	0.43	±	0.08	**a**	0.33	±	0.06	**b**	0.29	±	0.03	**b**
SL5 (SL4/SL1)	0.19	±	0.04	**a**	0.14	±	0.03	**b**	0.04	±	0.04	**c**
SL6 (mm)	5.5	±	1.4	**a**	2.2	±	0.4	**b**	1.6	±	0.4	**b**
SL7 (no.)	64.8	±	6.1	**a**	39.5	±	3.5	**b**	29.1	±	5.5	**c**
Flower characteristics												
F1 (mm)	10.6	±	2.3	**a**	7.6	±	1.3	**b**	6.4	±	0.4	**b**
F2 (mm)	1.02	±	0.11	**a**	0.86	±	0.07	**a**	0.57	±	0.06	**b**
F3 (no.)	14.3	±	2.6	**a**	9	±	2.1	**b**	7.5	±	0.9	**b**
F4 (mm)	5.4	±	0.5	**a**	4	±	0.6	**b**	2.3	±	0.2	**c**
F6 (Fl4/Fl5)	0.95	±	0.09	**a**	0.68	±	0.14	**b**	0.35	±	0.06	**c**
Sample no. (n)	40				26				20			

TABLE 16. Factor loadings of 15 morphological characteristics in the first three principal components (PC) of the PCA for 8 populations of *Nuphar* in Tochigi Prefecture. Eigenvalues and percentages of total variance and cumulative variance for the first three principal components are shown.

Characteristic	PCA 1	PCA 2	PCA 3
SL1	0.886	-0.118	-0.215
SL3	0.686	0.431	0.563
SL5	0.83	0.372	0.018
SL6	0.897	-0.166	0.226
SL7	0.927	-0.015	-0.097
F1	0.838	-0.437	0.206
F2	0.849	0.16	-0.295
F3	0.872	-0.35	0.149
F4	0.938	0.025	-0.183
F6	0.888	0.196	-0.229
Eigenvalue	7.463	0.744	0.662
Proportion of total variance (%)	74.6	7.4	6.6
Cumulative % of total variance	74.6	82.1	88.7

TABLE 17. Frequencies of multi-locus genotypes and mean pollen viability (%) for nine *Nuphar* populations from central to eastern Japan.

Population code	Multi-locus genotype (*lap1* and *mdh3*)					Pollen viability (%)	
	1 (aa, bb)	2 (ac, bb)	3 (ac, ab)	4 (aa, ab)	5 (cc, aa)	Mean	(SD)
***N. japonica* populations (Group 1)**							
YA1	1.000					94.1	(3.2)
FS2	1.000					93.1	(4.1)
IB2	1.000					90.9	(6.6)
NI4	1.000					94.6	(3.5)
Intermediate populations (Group 2)							
TG3		0.333	0.667			28.4	(29.2)
TG4			1.000			38.5	(4.3)
TG5		0.200	0.300	0.500		36.8	(4.2)
***N. submersa* populations (Group 3)**							
TG1					1.000	94.6	(2.4)
TG2					1.000	91.5	(3.9)

TABLE 18. Localities of 66 specimens of *Nuphar* and *Barclaya*. Herbarium abbreviation in parenthese.

Pop. Code	Sampling locality	Latitude (N)	Longitude (E)	Voucher for DNA
Nuphar japonica				
SAA	Hokkaido Pref.: Asaginodaichi, Sarufutsu-mura	45°12'51"	142°15'21"	*T. Shiga & S. Takebayashi 84* [OSA]
HOO	Hokkaido Pref.: Otoi, Horonobe-cho	45°00'09"	141°41'45"	*T. Shiga & S. Takebayashi 89* [OSA]
SAM	Hokkaido Pref.: Nakanuma-cho, Higashi-ku, Sapporo-shi	43°7'38"	141°26'12"	*T. Shiga 3474* [OSA]
KIO	Hokkaido Pref.: Bibaitappu, Kita-mura	43°13'07"	141°40'15"	*T. Shiga 3469* [OSA]
TOB	Hokkaido Pref.: Bisawa, Tomakomai-shi	42°44'26"	141°43'18"	*T. Shiga 3446* [OSA]
ATT	Hokkaido Pref.: Tomino, Atsuma-cho	42°38'11"	141°53'39"	*T. Shiga 3444* [OSA]
TAO	Hokkaido Pref.: Bansei, Taiki-cho	42°33'54"	143°28'3"	*T. Shiga 3457* [OSA]
AO-1	Aomori Pref.: Dekijima, Kizukuri-cho	40°50'6"	140°18'4"	*T. Shiga 3639* [OSA]
AK-1	Akita Pref.: Kamikitatesaruta, Akita-shi	39°40'23"	140°10'18"	*T. Shiga 3637* [OSA]
YA-1	Yamagata Pref.: Tazawa, Murayama-shi	38°32'39"	140°22'12"	*T. Shiga 3120* [KOBE, OSA]
FS-1	Fukushima Pref.: Shidahama, Inawashiro-cho	37°30'8"	140°8'41"	*T. Shiga 3249* [OSA]
FS-2	Fukushima Pref.: Nozawa, Iitate-mura	37°40'49"	140°45'1"	*T. Shiga 3250* [OSA]
FS-3	Fukushima Pref.: Tairanumanouchi, Iwaki-shi	37°0'29"	140°58'17"	*T. Shiga 3252* [OSA]
IB-1	Ibaragi Pref.: Takasaki, Tamari-mura	36°9'32"	140°19'1"	*T. Shiga 3641* [OSA]
IB-2	Ibaragi Pref.: Shimone-cho, Ushiku-shi	35°59'41"	140°9'36"	*T. Shiga 3254* [OSA]
TK-1	Tokyo Pref.: Syakujii, Nerima-ku	35°44'16"	139°35'42"	*T. Shiga 3412* [OSA]
KN-1	Kanagawa Pref.: Nakashinden, Ebina-shi	35°26'20"	139°23'10"	*T. Shiga 3413* [OSA]
NI-1	Niigata Pref.: Shinbana, Toyosaka-shi	37°54'19"	139°14'41"	*T. Shiga 3122* [OSA]
NI-4	Niigata Pref.: Nagamine, Yoshikawa-cho	37°14'28"	138°22'11"	*T. Shiga 3320* [OSA]
IK-1	Ishikawa Pref.: Katano-Kamoike, Kaga-shi	36°19'19"	136°17'21"	*T. Shiga 3645* [OSA]
SI-3	Shiga Pref.: Shiozu, Shiozu-cho	35°30'55"	136°9'44"	*K. Murayama & Y. Kadono s.n.* [KOBE]
MI-2	Mie Pref.: Katsuta, Tamaki-cho	34°27'51"	136°37'17"	*T. Shiga 3644* [OSA]
WA-1	Wakayama Pref.: Nishinoyama, Naga-cho	34°16'54"	135°26'33"	*T. Shiga 2833* [OSA]
OK-2	Okayama Pref.: Kojimashirao, Kurashiki-shi	34°29'31"	133°51'38"	*T. Shiga 3669* [OSA]
OK-4	Okayama Pref.: Kosaka, Saeki-cho	34°50'3"	134°3'5"	*T. Shiga 2835* [OSA]
HI-10	Hiroshima Pref.: Hachihonmatsu, Higashihiroshima-shi	34°25'26"	132°41'6"	*T. Shiga 3243* [OSA]
KO-4	Kochi Pref.: Usa-cho, Tosa-shi	33°25'41"	133°27'15"	*T. Shiga 3373* [OSA]
MY-5	Miyazaki Pref.: Matsuo, Kushima-shi	31°28'11"	131°13'16"	*T. Shiga 3283* [OSA]
MY-6	Miyazaki Pref.: Toyomitsu-cho, Miyakonojo-shi	31°41'23"	131°5'47"	*T. Shiga 3279* [KOBE, OSA]

TABLE 18. (continued)

Code	Sampling locality	Latitude (N)	Longitude (E)	Voucher
N. lutea				
	SLOVAKIA: Podunajská Rovina: Kĺúčovec, Zátonoský les	47°47'00"	17°41'44"	*H. Kudoh et al. 03-507* [OSA]
N. oguraensis* var. *oguraensis				
GI-4	Gifu Pref.: Hora, Gifu-shi	35°28'32"	136°43'5"	*T. Shiga 3356* [OSA]
HY-8	Hyogo Pref.: Namita, Sanda-shi	34°57'50"	135°9'47"	*T. Shiga 3149* [KOBE, OSA]
HI-2b	Hiroshima Pref.: Kamitsuda, Seranishi-cho	34°38'46"	132°53'46"	*T. Shiga 3238* [OSA]
HI-7	Hiroshima Pref.: Oguni, Seranishi-cho	34°38'2"	132°55'50"	*T. Shiga 3240* [KOBE, OSA]
FO-1	Fukuoka Pref.: Ikenoyama, Hoshino-mura	33°14'47"	130°45'21"	*T. Shiga 3666* [KOBE, OSA]
MY-2	Miyazaki Pref.: Nagai, Kitagawa-cho	32°40'11"	131°43'17"	*T. Shiga 3314* [KOBE, OSA]
MY-8	Miyazaki Pref.: Takagi-cho, Miyakonojo-shi	31°47'16"	131°6'3"	*T. Shiga 3281* [OSA]
KRE1a	KOREA:			*Y. Kadono s.n.* [KOBE]
KRE2	KOREA:			*Y. Kadono s.n.* [KOBE]
N. oguraensis* var. *akiensis				
HHS	Hiroshima Pref.: Shitami, Higashihiroshima-shi	34°24'30"	132°43'9"	*T. Shiga 3244* [KOBE, OSA]
KO-1	Kochi Pref.: Kohda, Kochi-shi	33°32'30"	133°31'5"	*T. Shiga 3364* [KOBE, OSA]
KO-2	Kochi Pref.: Hata, Ino-cho	33°31'34"	133°26'15"	*T. Shiga 3362* [KOBE, OSA]
KRE1b	KOREA:			*Y. Kadono s.n.* [KOBE]
N. pumila* var. *pumila				
WAM	Hokkaido Pref.: Keihoku, Wakkanai-shi	45°24'03"	141°49'01"	*T. Shiga 3488* [KOBE, OSA]
KUT	Hokkaido Pref.: Takkobu, Kushiro-shi	43°6'18"	144°29'07"	*T. Shiga 3466* [KOBE, OSA]
URUa	Hokkaido Pref.: Uryu-numa Moor, Uryu-cho	43°42'00"	141°36'06"	*T. Shiga 3678* [OSA]
TSC	Hokkaido Pref.: Numasawa., Tsubetsu-cho	43°38'08"	143°52'38"	*T. Shiga 3597* [OSA]
BEB	Hokkaido Pref.: Honbekkai, Bekkai-cho	43°25'26"	145°14'56"	*T. Shiga & S. Takebayashi 73* [KOBE, OSA]
NET	Hokkaido Pref.: Makinouchi, Nemuro-shi	43°19'45"	145°37'21"	*T. Shiga & S. Takebayashi 69* [KOBE, OSA]
NEN	Hokkaido Pref.: Katsuragi, Nemuro-shi	43°19'11"	145°37'02"	*T. Shiga & S. Takebayashi 55* [KOBE, OSA]
HAK	Hokkaido Pref.: Hamanaka, Hamanaka-cho	43°06'37"	145°06'09"	*T. Shiga & S. Takebayashi 211* [KOBE, OSA]
AKT	Hokkaido Pref.: Honmachi, Akkeshi-cho	43°00'13"	144°51'59"	*T. Shiga 3685* [OSA]
KWS	Hokkaido Pref.: Shinsen-numa Moor, Kyowa-cho	42°54'13"	140°35'27"	*T. Shiga 3599* [OSA]
TOJ	Hokkaido Pref.: Ohtsu, Toyokoro-cho	42°40'46"	143°37'54"	*T. Shiga 3680* [OSA]
ATO	Hokkaido Pref.: Koinuma, Atsuma-cho	42°38'11"	141°53'39"	*T. Shiga 3447* [KOBE, OSA]

TABLE 18. (continued)

Pop. Code	Sampling locality	Latitude (N)	Longitude (E)	Voucher
N. pumila* var. *ozeensis				
URUb	Hokkaido Pref.: Uryu-numa Moor, Uryu-cho	43°42'00"	141°36'06"	*T. Shiga 3679* [OSA]
GM-1	Gunma Pref.: Kamitashiro, Katashina-mura	36°55'11"	139°12'12"	Cultivation at Kobe Univ.
N. pumila* var. *ozeensis f. rubro-ovaria				
URUc	Hokkaido Pref.: Uryu-numa Moor, Uryu-cho	43°42'00"	141°36'06"	*T. Shiga 3477* [OSA]
N. shimadae				
	Taiwan: Xinzhu, Taoyuan	25°03'	121°13'	Cultivation at Kobe Univ.
N. subintegerrima				
GI-1	Gifu Pref.: Tachibokubora, Gifu-shi	35°25'41"	136°47'45"	*T. Shiga 3228* [KOBE]
AI-1	Aichi Pref.: Nakashitami, Nagoya-shi	35°14'39"	137°2'27"	*T. Shiga 3275* [OSA]
AI-2	Aichi Pref.: Ikenodai, Inuyama-shi	35°21'13"	137°2'27"	*T. Shiga 3349* [OSA]
MI-1	Mie Pref.: Oshihuchi, Nansei-cho	34°19'36"	136°37'58"	*T. Shiga 3234* [KOBE]
MI-3	Mie Pref.: Ukata, Ago-cho	34°20'18"	136°49'39"	*T. Shiga 3355* [OSA]
N. submersa				
TG-1	Tochigi Pref.: Imaichi-shi	36°39'	139°43'	*T. Shiga 3480* [KYO, OSA, TNS]
TG-2	Tochigi Pref.: Minaminasu-machi	36°42'	140°6'	*H. Hirayama s.n.* [KOBE]
Barclaya longifolia				
	East Asia: Garden plants obtained form garden center			Cultivation at Kobe Univ.

TABLE 19. AFLP variations revealed by four AFLP primer pairs in 65 *Nuphar* plants. Data included that of *Barclaya longifolia* are also shown in parenthes.

Primer combination		Fragment size (bp)	Number of amplified fragments	Number of monomorphic fragments	Number of polymorphic fragments
*Eco*RI	*Mse*I				
ACA	CTA	50-370	178 (197)	8 (2)	170 (195)
ACG	CTA	53-389	201 (208)	5 (2)	196 (206)
ACA	CTG	60-471	232 (236)	8 (4)	224 (232)
ACG	CTG	52-467	161 (194)	2 (0)	159 (194)
Total			772 (835)	23 (8)	749 (827)

TABLE 20. Number of AFLP fragments scored and genetic diversity in *N. japonica*, *N. oguraensis*, *N. pumila* and *N. subintegerrima*. *F*; mean proportion of shared fragments between individuals; π, estimated nucleotide diversity.

Taxa	N	Average no. of fragments ±SD	Total AFLP fragments	Number of polymorphic fragments	*F*±SD	π±SD(×1000)
N. japonica	29	232.1 ± 11.8	425	352	0.84 ± 0.06	11.3 ± 4.3
Clade-1	20	230.2 ± 7.7	368	299	0.89 ± 0.02	7.3 ± 1.6
Clade-2	9	236.3 ± 17.7	329	292	0.85 ± 0.05	10.2 ± 3.3
N. oguraensis	13	197.5 ± 14.7	414	306	0.74 ± 0.04	18.9 ± 3.5
N. oguraensis (including *N. shimadae*)	14	196.6 ± 14.5	423	314	0.74 ± 0.04	18.5 ± 3.4
N. pumila	14	217.6 ± 9.1	397	293	0.83 ± 0.08	12.1 ± 6.3
N. subintegerrima	5	236.8 ± 11.0	376	299	0.74 ± 0.07	18.8 ± 5.5

TABLE 21. Genotypic diversity within and among populations as expressed by frequency of multi-locus genotypes (MLGs) and mean values of Simpson's diversity (D). Allozyme data were summarized from Chapters 2 and 3.

	Species					
Taxa	*N. japonica*			*N. pumila*	*N. ogura-ensis*	*N.subinte-gerrima*
Region	Total	East to North (Clade-1)	West to South (Clade-2)	North	West to South	Central
Populations	23	17	6	12	7	5
Sample size (N)	452	347	105	229	109	85
No. of MLGs recoverd	21	13	10	4	8	9
MLG / N	0.046	0.037	0.095	0.017	0.073	0.106
Diversity within populations						
Mean no. of MLGs per population (SD)	1.957 (1.461)	1.941 (1.478)	2.167 (1.472)	1.083 (0.289)	1.571 (0.535)	2.000 (1.414)
Polymorphic populations (%)	12 (52.2)	8 (47.1)	4 (66.7)	2 (16.7)	4 (57.1)	2 (40.0)
Simpson's D (SD)	0.212 (0.285)	0.175 (0.261)	0.317 (0.348)	0.096 (0.096)	0.196 (0.226)	0.377 (0.332)
Diversity among populations						
Mean no. of populations per MLG (SD)	2.143 (3.245)	2.62 (4.073)	1.5 (0.756)	3.5 (5)	1.375 (0.744)	1.111 (0.333)
Unique MLGs (%)	13 (61.9)	5 (38.5)	6 (60.0)	2 (50.0)	6 (75.0)	8 (88.9)
Populations with the commonest MLG (%)	16 (69.6)	16 (94.1)	3 (50.0)	11 (91.7)	3 (42.9)	1 (40.0)

TABLE 22. Comparison of morphological characters for two clades of *N. japonica* (mean ± SD). Significance of difference by t-test was expressed by ns, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Character	*N. japonica* Clade 1	Clade 2	Difference between the clades
	mean ± SD	mean ± SD	
Emergent leaf characters			
Leaf blade length (cm)	32.47 ± 5.07	28.73 ± 4.52	***
Leaf blade width (cm)	18.18 ± 3.16	18.07 ± 2.59	ns
Length/width of leaf blade ratio	0.56 ± 0.06	0.63 ± 0.05	***
Sinus depth (cm)	7.55 ± 1.63	8.36 ± 1.20	***
Sinus/length of blade ratio	0.23 ± 0.04	0.29 ± 0.02	***
Maximum blade width position/total length of blade ratio	0.67 ± 0.06	0.64 ± 0.04	***
Petiole width at 5 cm from the base of blade (mm)	10.03 ± 1.62	8.28 ± 0.78	***
Sample no.	230	76	
Submerged leaf characters			
Leaf blade length (cm)	26.08 ± 6.28	19.83 ± 3.92	***
Leaf blade width (cm)	11.91 ± 2.82	12.01 ± 1.91	ns
Length/width of leaf blade ratio	0.47 ± 0.11	0.62 ± 0.10	***
Sinus depth (cm)	5.10 ± 1.55	5.37 ± 0.82	ns
Sinus/length of blade ratio	0.20 ± 0.06	0.27 ± 0.03	***
Maximum blade width position/total length of blade ratio	0.72 ± 0.09	0.67 ± 0.05	***
Petiole width at 5 cm from the base of blade (mm)	5.57 ± 1.31	5.13 ± 0.92	*
Sample no.	190	62	
Flower characters			
Length of stigmatic disc (mm)	10.27 ± 1.90	9.26 ± 1.01	**
Width of stigmatic disc (mm)	8.84 ± 1.55	8.79 ± 0.96	ns
Length/width of stigmatic disc ratio	0.87 ± 0.10	0.95 ± 0.04	***
Stigma length (mm)	3.92 ± 0.61	3.53 ± 0.39	***
Number of stigma	13.73 ± 2.97	12.75 ± 1.80	ns
Anther length (mm)	4.78 ± 0.91	5.52 ± 0.75	***
Filament length (mm)	5.82 ± 0.85	6.09 ± 1.14	ns
Anther length/filament length ratio	0.84 ± 0.18	0.92 ± 0.15	**
Sample no.	202	32	
Fruit characters			
Fruit length (mm)	43.61 ± 6.88	42.49 ± 6.52	ns
Fruit width (mm)	28.10 ± 5.25	32.16 ± 3.37	***
Length/width ratio of fruit	0.65 ± 0.11	0.77 ± 0.10	***
Seed length (mm)	4.72 ± 0.37	4.84 ± 0.29	ns
Seed width (mm)	3.52 ± 0.32	3.65 ± 0.32	*
Length/width ratio of seed	0.75 ± 0.05	0.75 ± 0.05	ns
Sample no.	199	38	

TABLE 23. Comparison of *Nuphar japonica*, *N. shimadae, N. pumila, N. saikokuensis, N. subintegerrima* and *N. submersa* in the diagnostic characters. Data of *N. japonica*, *N. shimadae*, *N. pumila* and *N. subintegerrima* were cited from Chapters 2, 3, 4, and 6.

	N. japonica	*N. pumila*	*N. saikokuensis*	*N. shimadae*	*N. subintegerrima*	*N. submersa*
Life form	emergent	floating-leaved	floating-leaved	floating-leaved	emergent or floating-leaved	submerged
Emergent or floating leaf	narrowly ovate	widely ovate to ovate	widely ovate to ovate	widely ovate to ovate	roundish	narrowly ovate*
Submerged leaf	narrowly ovate to ovate	roundish to ovate	widely ovate to ovate	roundish to ovate	roundish to ovate	narrowly oblong-triangular
Sinus of submerged leaf	deep	deep	deep	deep	deep	shallow or absent
Central lacuna of petiole	absent	absent	absent	present	absent	usually present
Stigmatic disc color	yellow	red or yellow	yellow	red or yellow	orange or yellow	red
Anther and filament length ratio	1:1~1:2	1:2~1:3	1:1~1:2	1:2~1:3	1:1	1:2~1:3
Anther color	yellow	yellow	yellow	yellow	yellow	red
Fruit color	green	red or green	green	green	red or green	red

*Leaves of *Nuphar submersa* are always submerged in natural populations. They are rarely floating-leaves in lentic water.

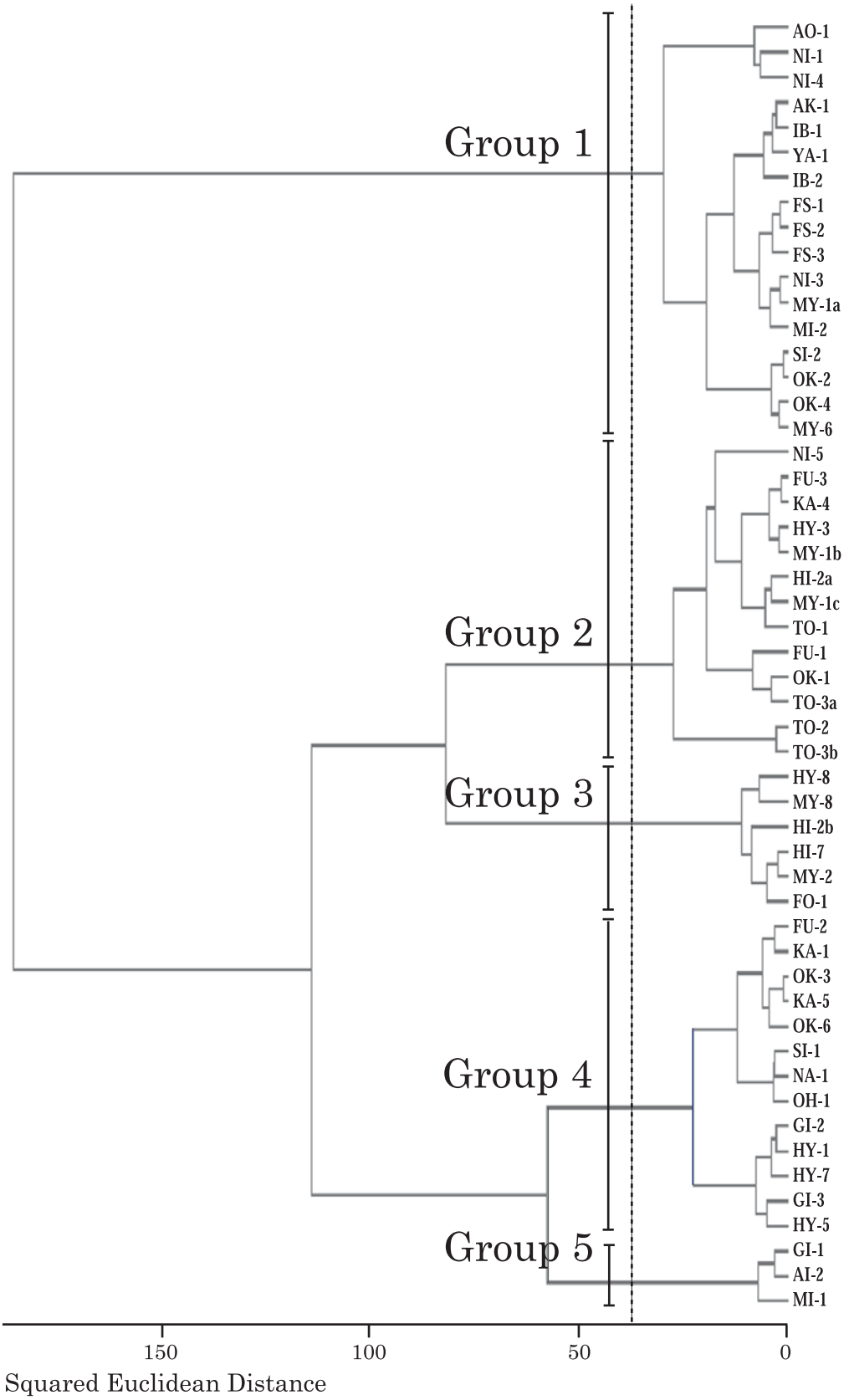

Fig. 1. Phenogram of cluster analysis of 52 *Nuphar* populations in central to western Japan based on 15 morphological characters (Ward method). The population codes are refered to in Table 1.

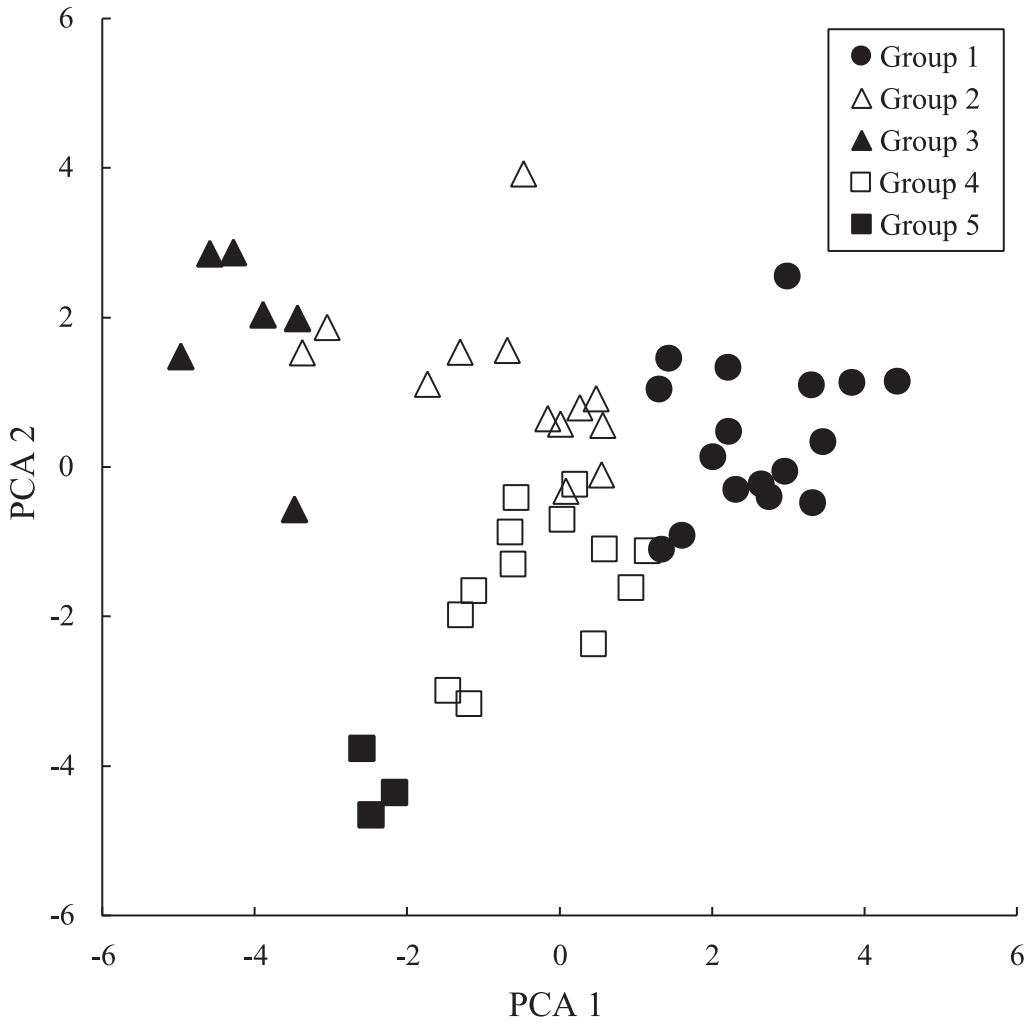

Fig. 2. The 2-D scatter plots by principal components 1 and 2 of a principal component analysis using the same data set as cluster analysis.

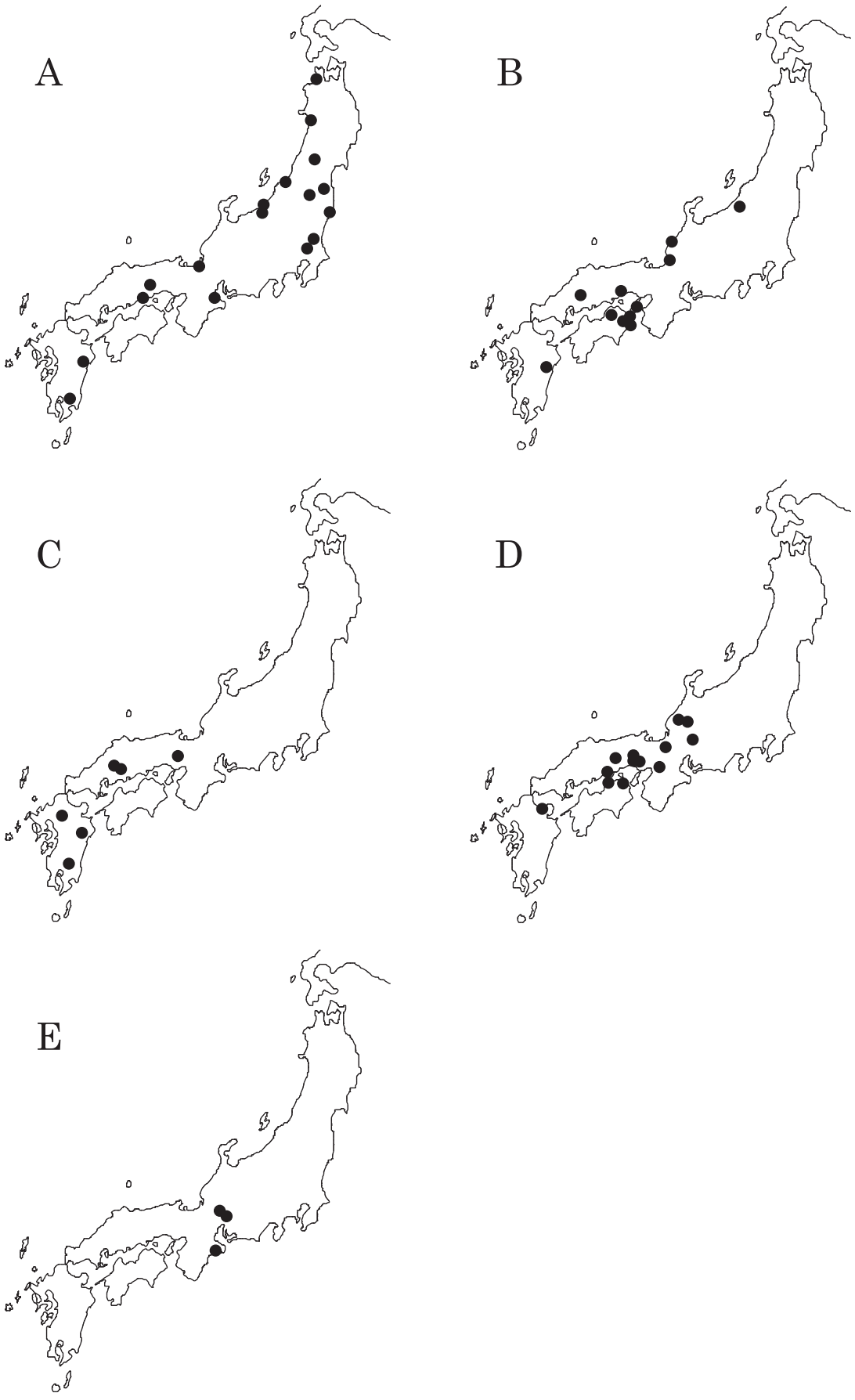

Fig. 3. Distribution of the populations of five cluster groups in Chapter 2. A. Group 1; B. Group 2; C. Group 3; D. Group 4; E. Group 5

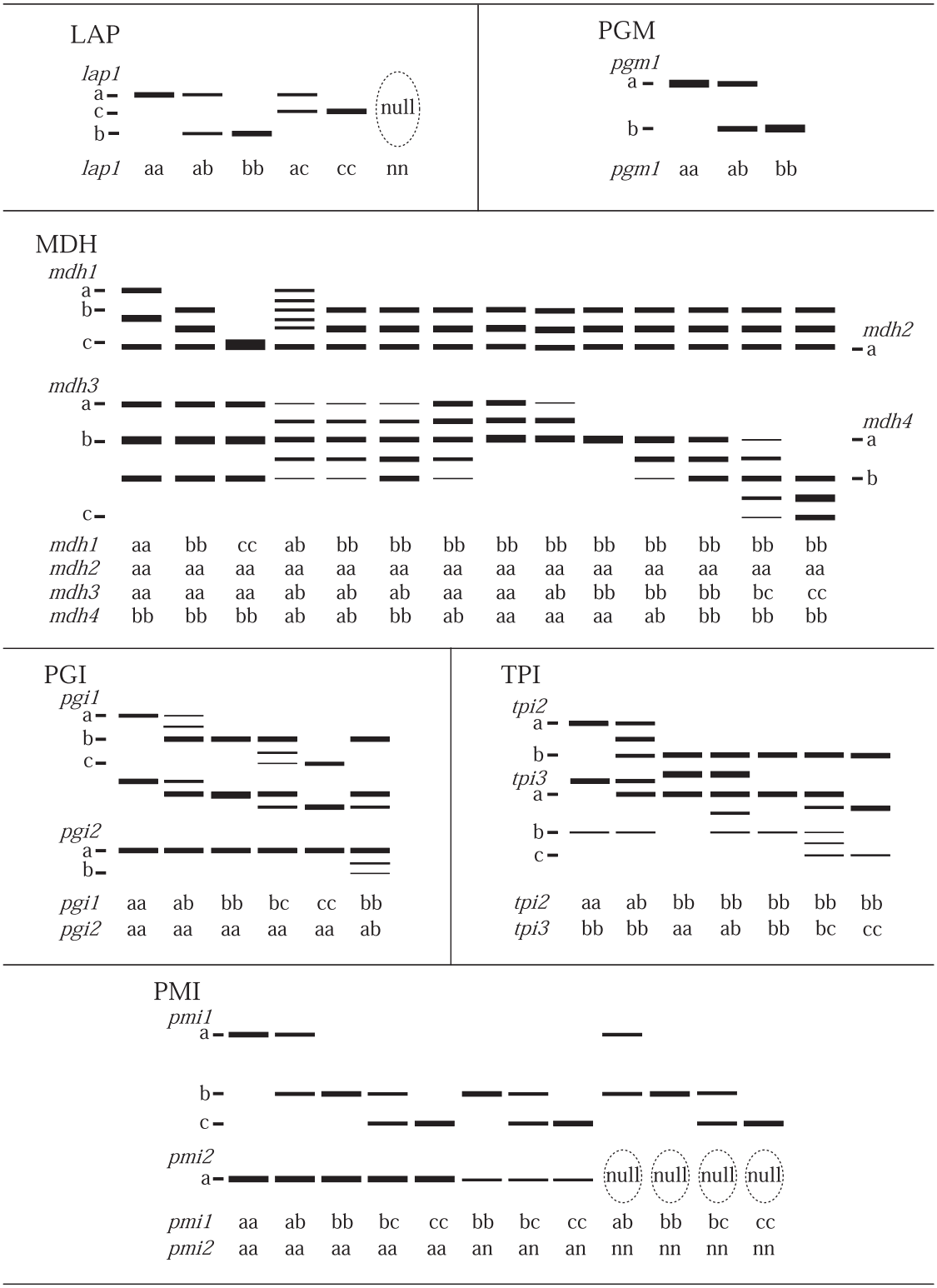

Fig. 4. Schematic illustration of electrophoretic phenotypes observed in Japanese *Nuphar*. Alphabetical codes below the banding patterns denote genotypes of each putative locus. Putative null alleles are indicated by botted line and "n". Anode is toward the top of the figure.

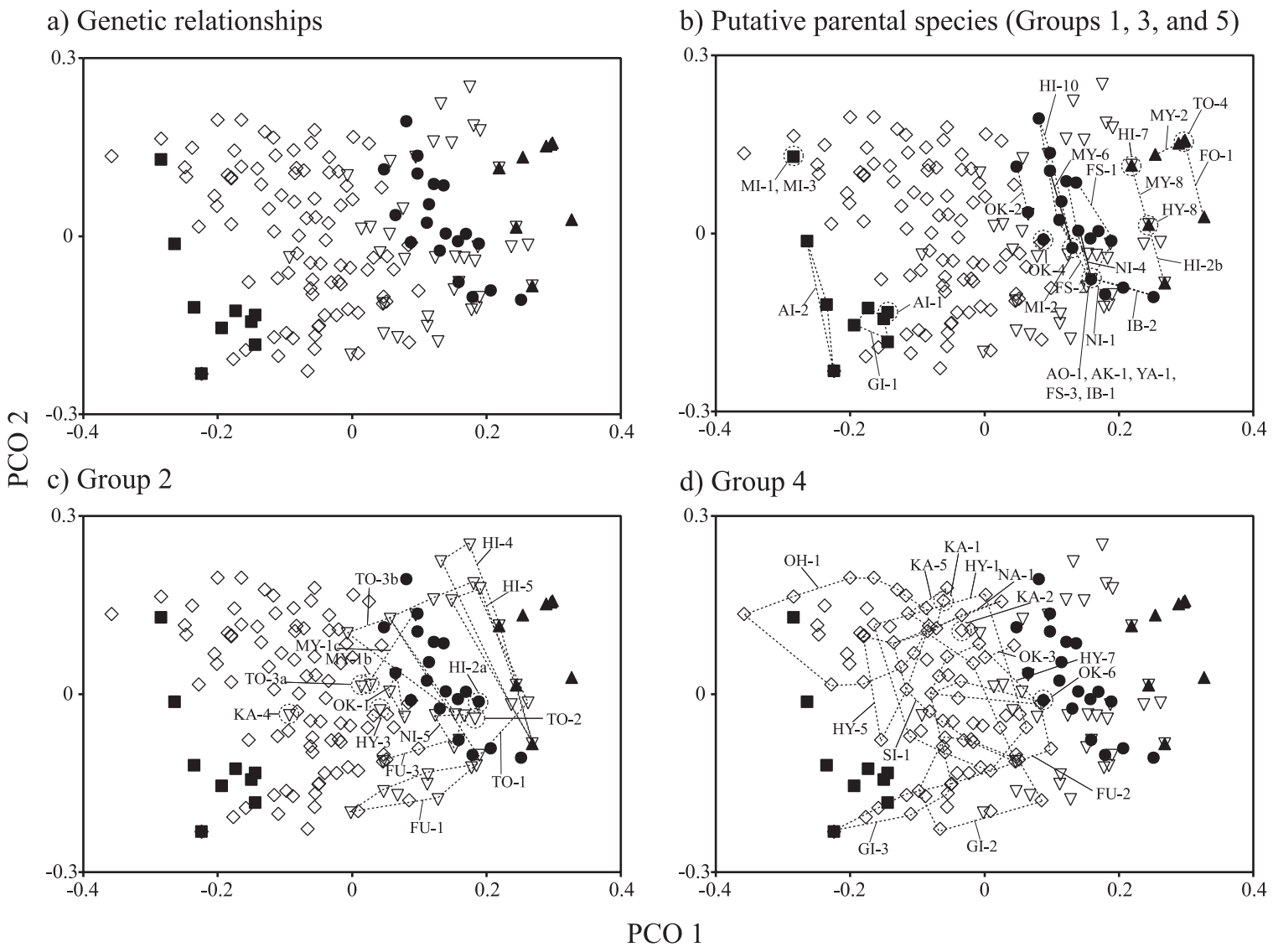

Fig. 5. Principal coordinate analysis of 162 MLGs of *N. japonica* (Group 1; solid circle), *N. oguraensis* (Group 3; solid triangle), *N. subintegerrima* (Group 5; solid square), Group 2 (open inverse triangle), Group 4 (open diamond) based on shared allele distance between each MLG. The first two coordinate axes account for 46.2% and 31.1% of the variation. In the figures b)-d), broken lines encircle the same populations. For population code, see Table 1.

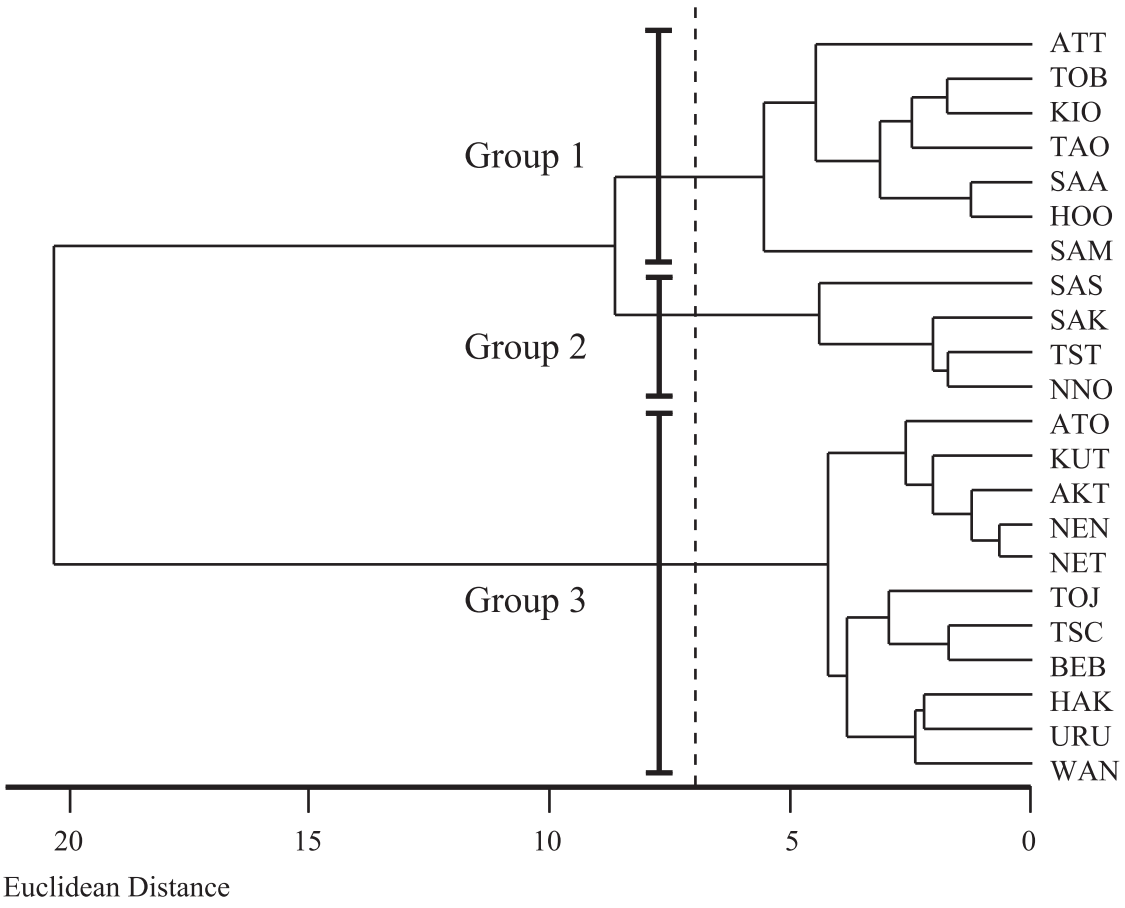

Fig. 6. Phenogram drawn from a cluster analysis (Ward method) for 22 *Nuphar* populations in Hokkaido, Japan. Fifteen morphological characters were analyzed (see Table 9). The population codes are referred to in Table 8.

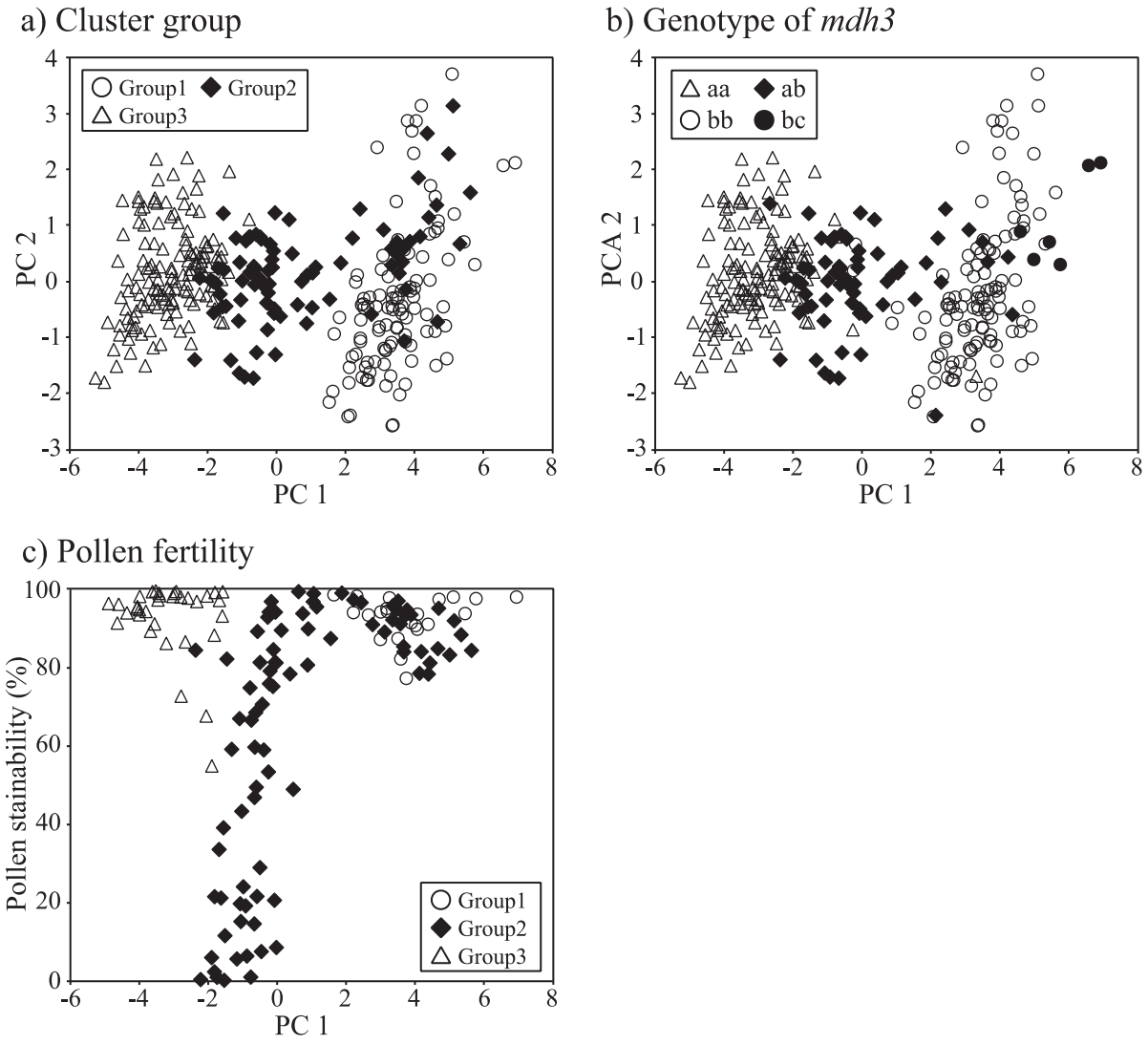

Fig. 7. Principal component analysis (PCA) of *N. japonica* (Group 1), intermediate populations (Group 2) and *N. pumila* (Group 3) from Hokkaido, Japan. Fifteen morphological characters were analyzed (see Table 9). Data for cluster groups (a), genotype of *mdh3* (b) and fertility (c) are superimposed on the PCA (c on axis 1 only). Symbols indicate phenotype (a and c) or genotype (b).

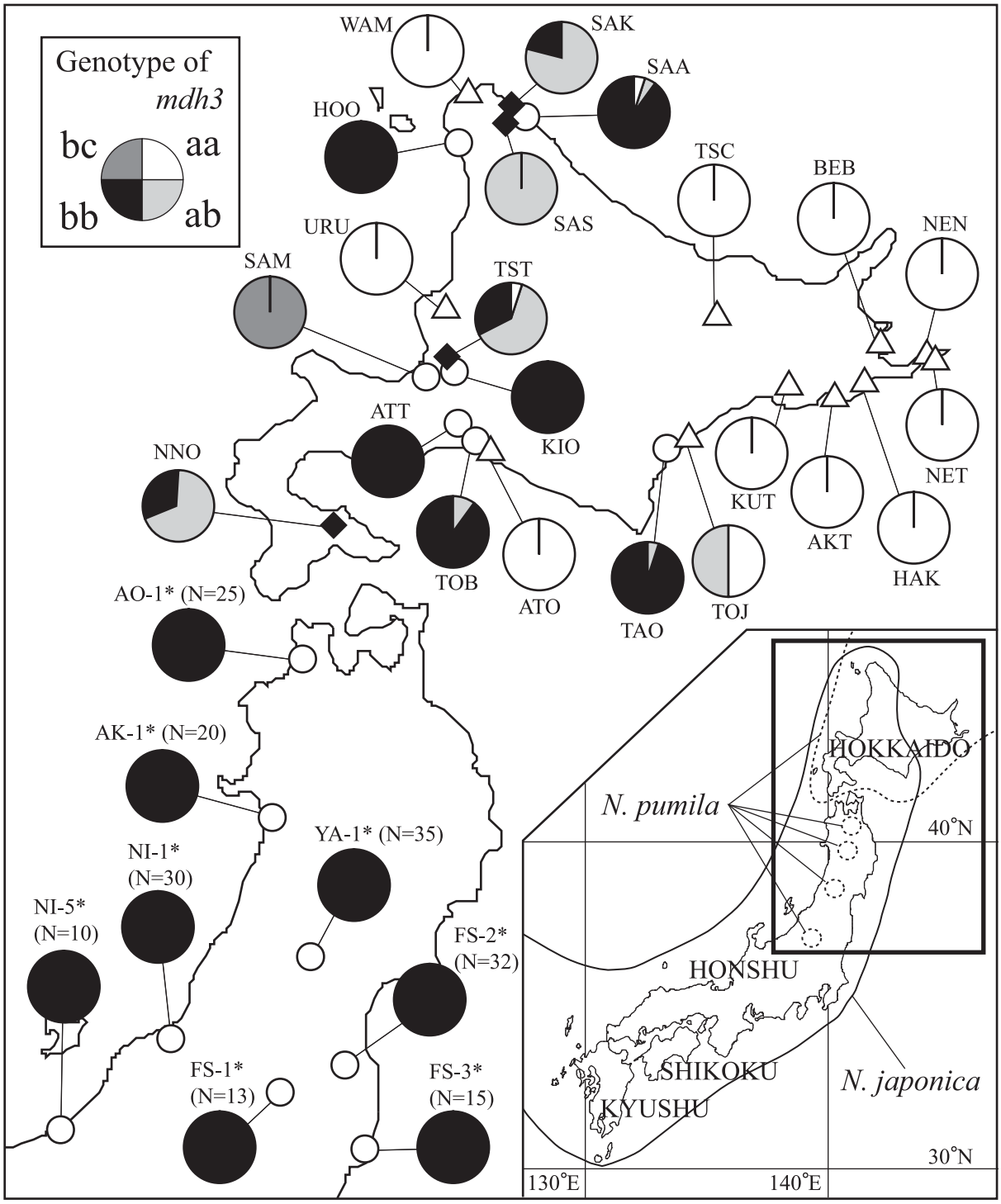

Fig. 8. Geographic distributions of different genotypes of *mdh3*. Symbols show morphological groups of each population: *N. japonica* (Group 1; open circle), intermediate populations (Group 2; solid diamond) and *N. pumila* (Group 3; open triangle). Some northern Honshu populations of *N. japonica* with asterisk are added for comparison (see Chapter 2). Distribution patterns of *N. japonica* and *N. pumila* are also shown.

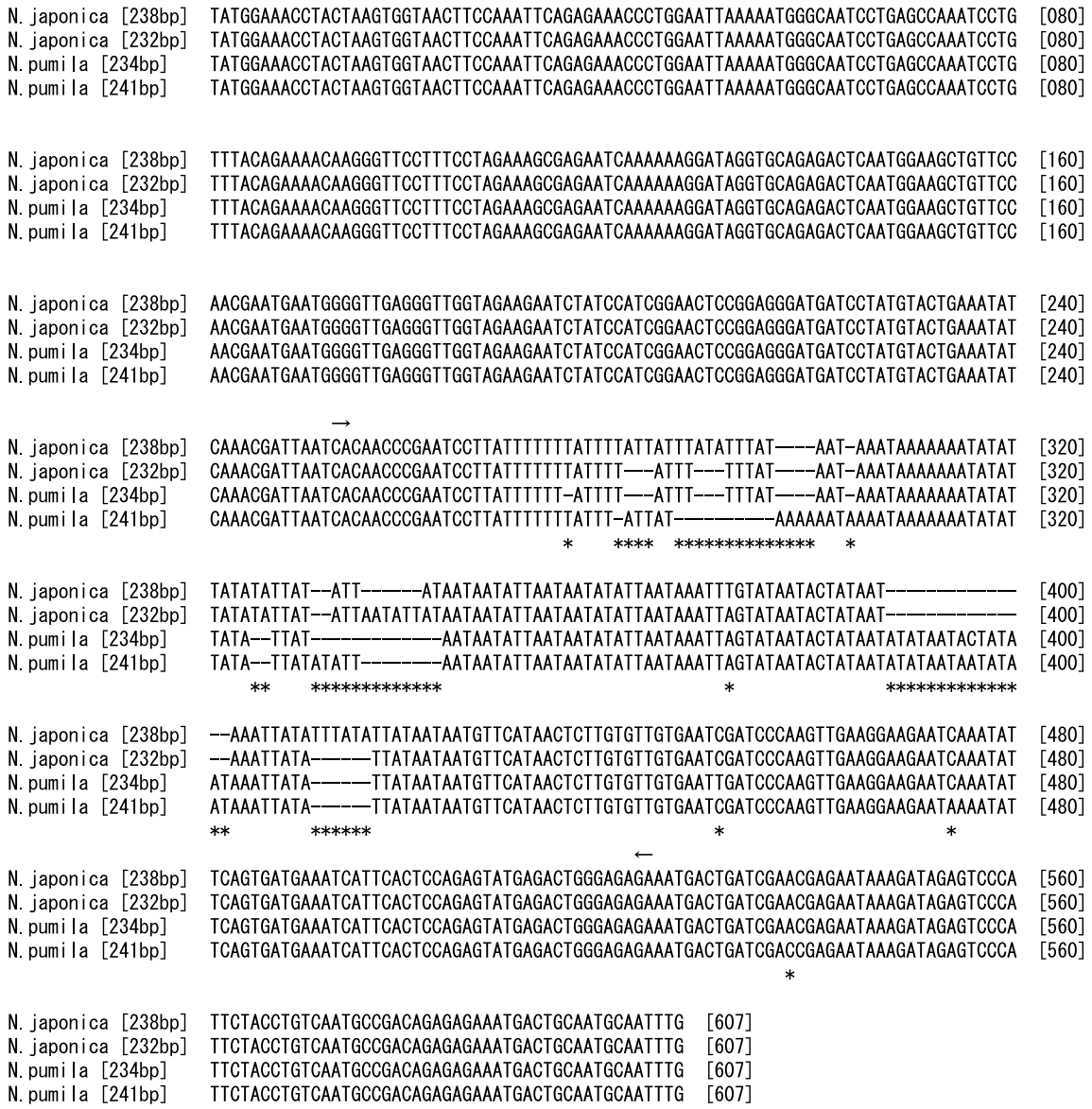

Fig. 9. Partial nucleotide sequences of intron of *Trn*L in *Nuphar japonica* and *N. pumila*. Number of nucleotide from 5' end is indicated on the right. Asterisks show variable sites. Tips of arrows indicate the 3' ends of the new discriminate primers. A fragment size amplified by new primers was also shown in parenthesis.

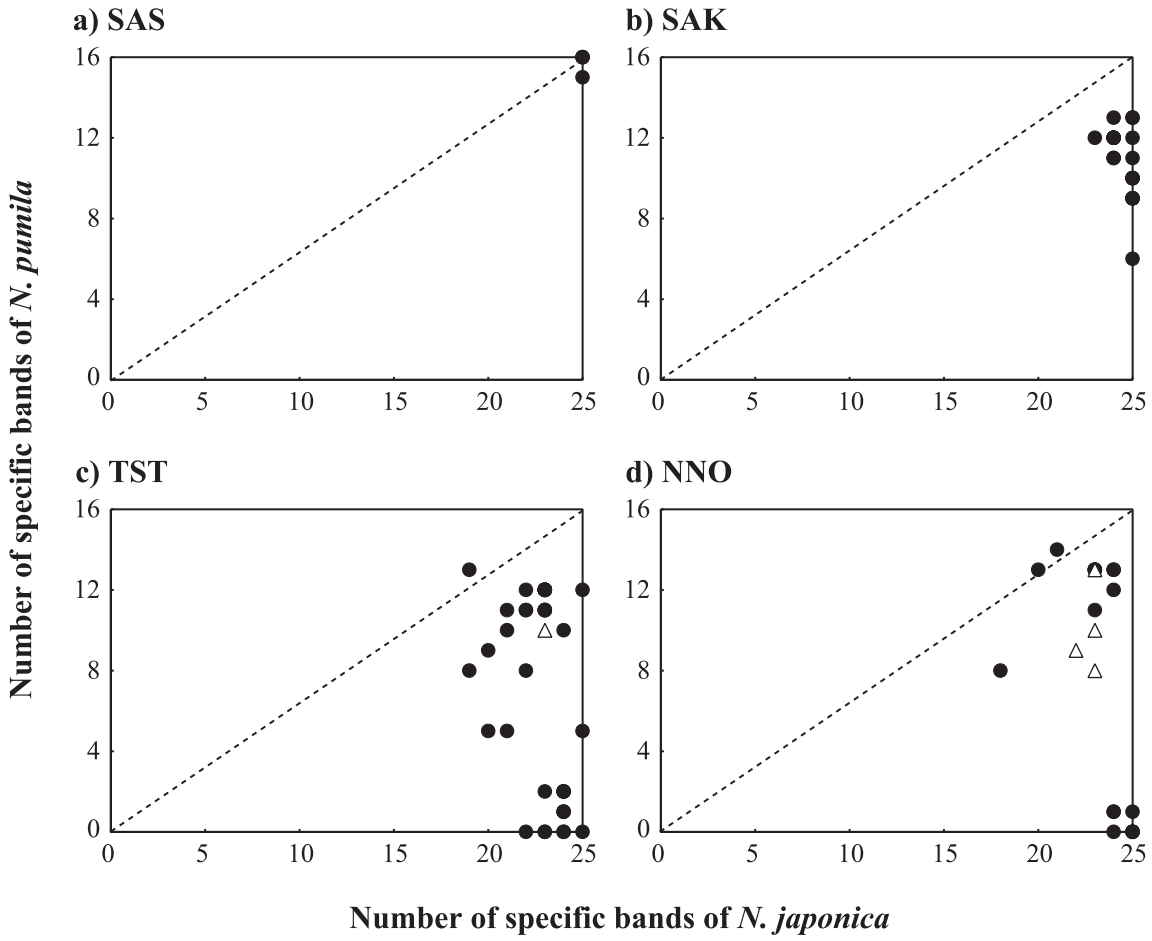

Fig. 10. Scatter diagrams of the number of specific bands for four hybrid populations. CpDNA haplotypes of *N. japonica* type (238; solid circle) and *N. pumila* type (234; open triangle) are also shown. Broken lines show the case in which the hybrids have species-specific markers of both species with the same ratio.

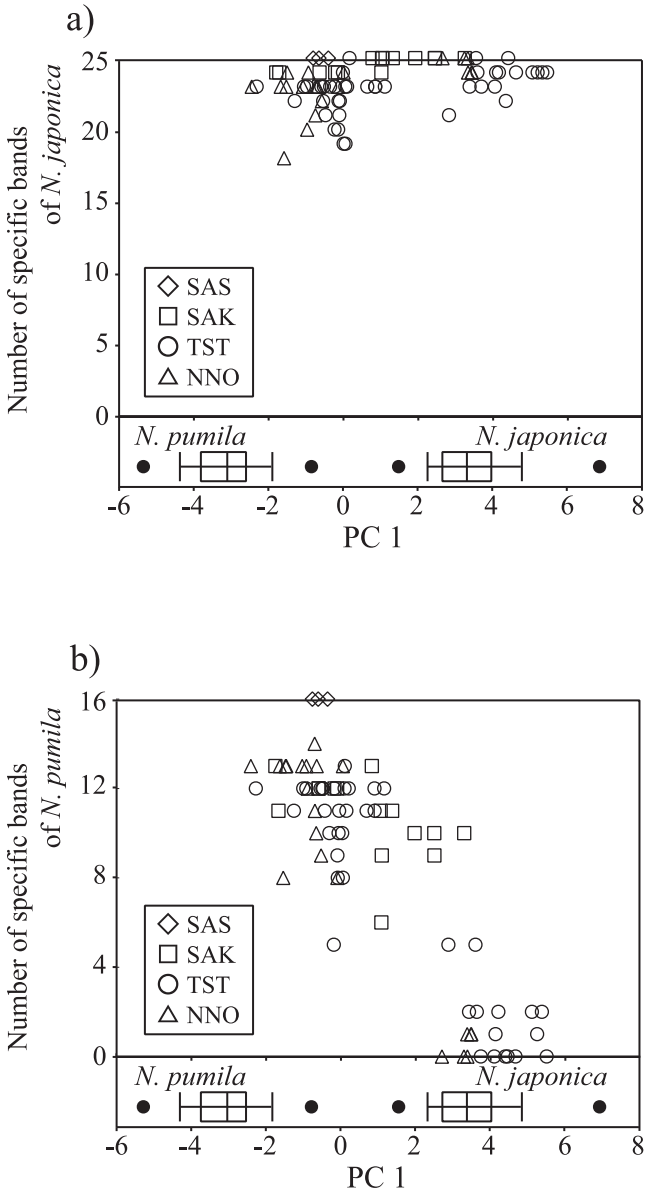

Fig. 11. Number of specific bands of putative parental species and morphological variations (PC axis 1). Different symbols show four hybrid populations, respectively. The box-plot shows the median as the vertical line within the box, the limits of the box represent the 25th and 75th percentile, the limits of the whisker plot the 10th and 90th percentile. Maximum and minimum morphological values (PC axis 1) of *N. japonica* and *N. pumila* are indicated by solid circles.

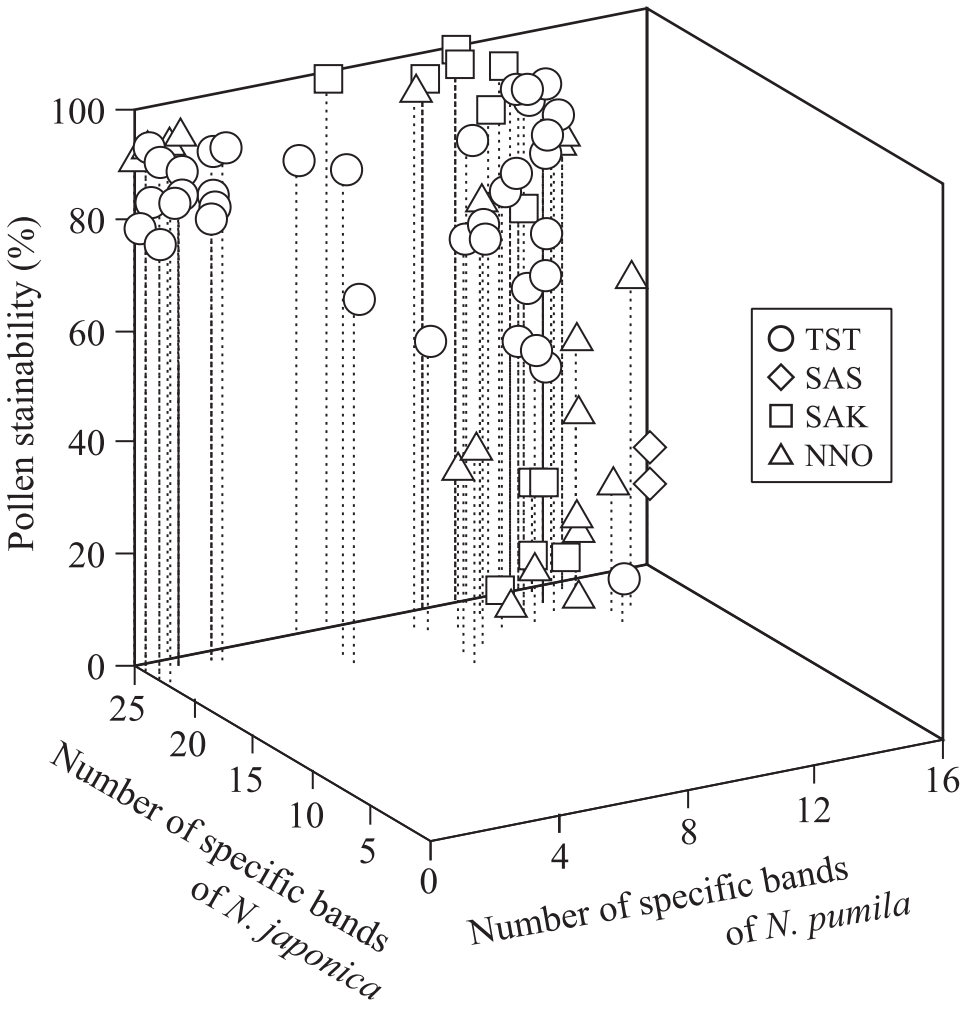

Fig. 12. 3-D scatter diagram of relationship between the number of specific bands and pollen stainability. Different symbols show four hybrid populations, respectively.

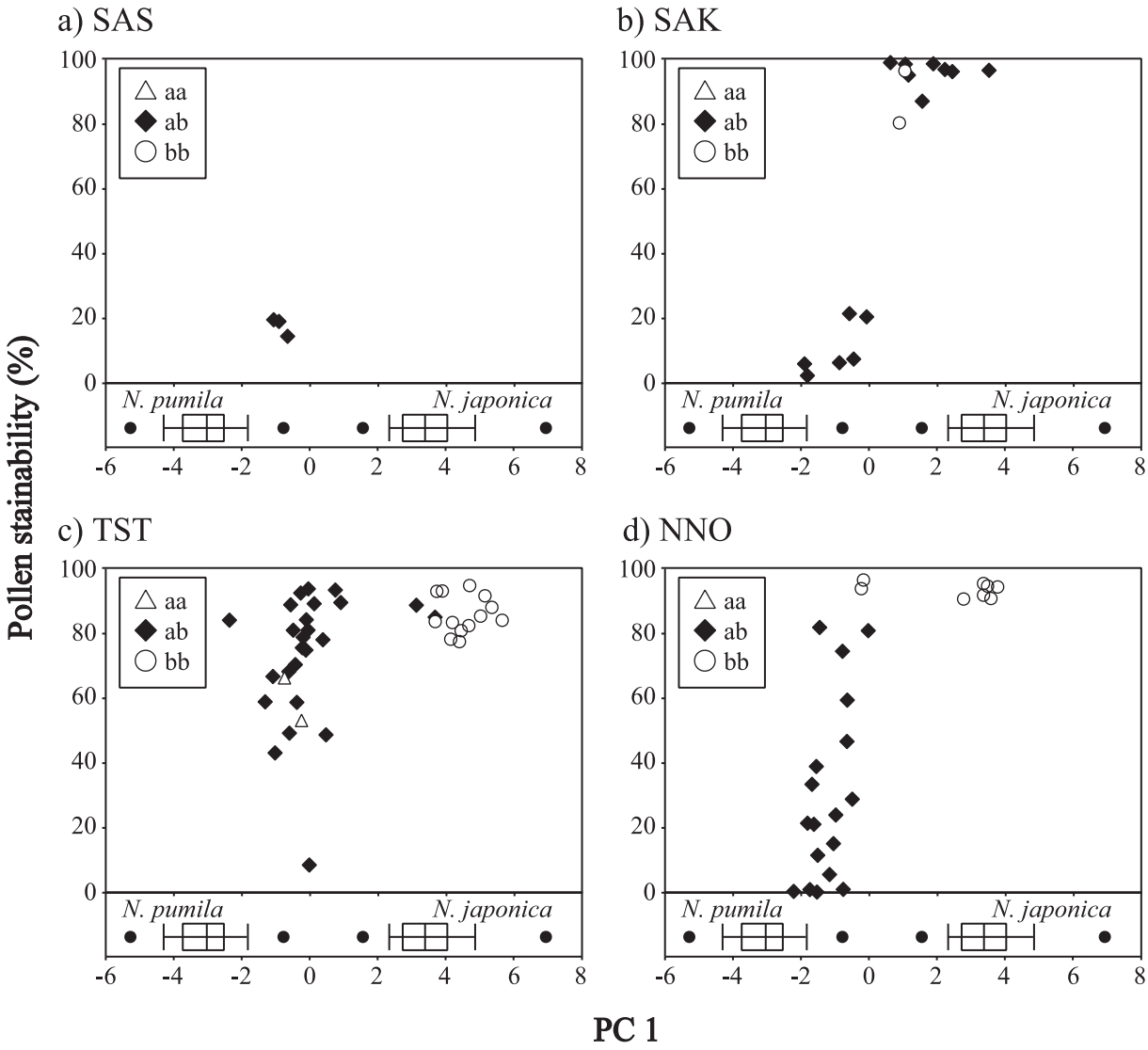

Fig. 13. Pollen stainability (%) of four putative hybrid populations are superimposed on the PCA axis 1. Symbols indicate the *mdh3* genotypes . The box-plot shows the median as the vertical line within the box, the limits of the box represent the 25th and 75th percentile, the limits of the whisker plot the 10th and 90th percentile. Maximum and minimum morphological values (PC axis 1) of *N. japonica* and *N. pumila* are indicated by solid circles.

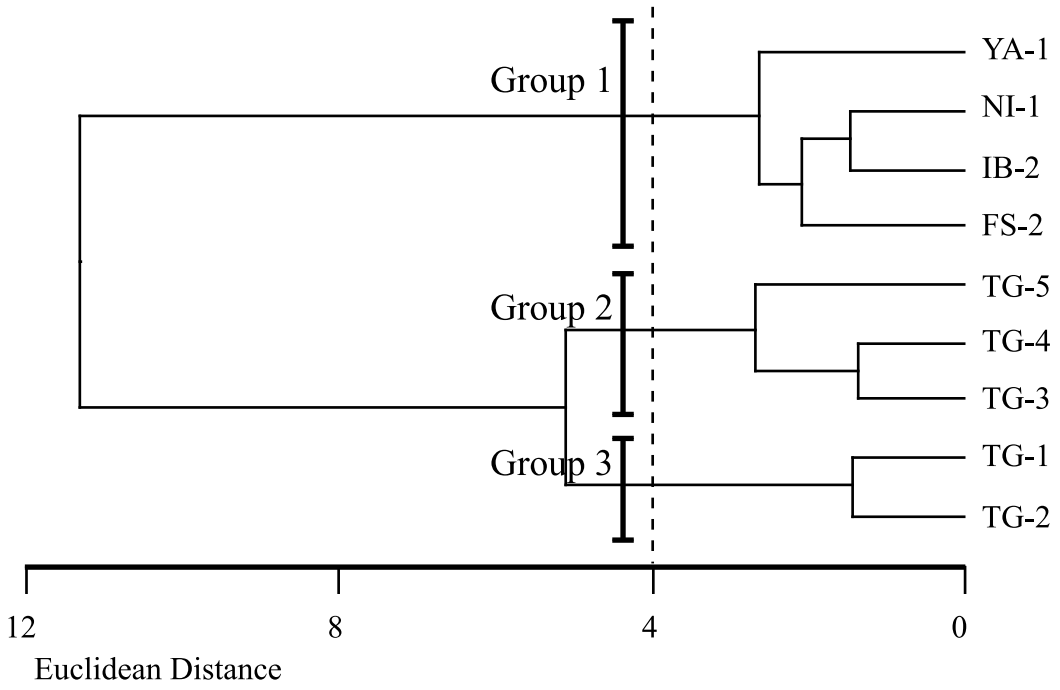

Fig. 14. Phenogram based on the results of cluster analysis (Ward's method) of 9 *Nuphar* populations in central to eastern Japan. Ten morphological characteristics were analyzed. The population codes are referred to in Table 14.

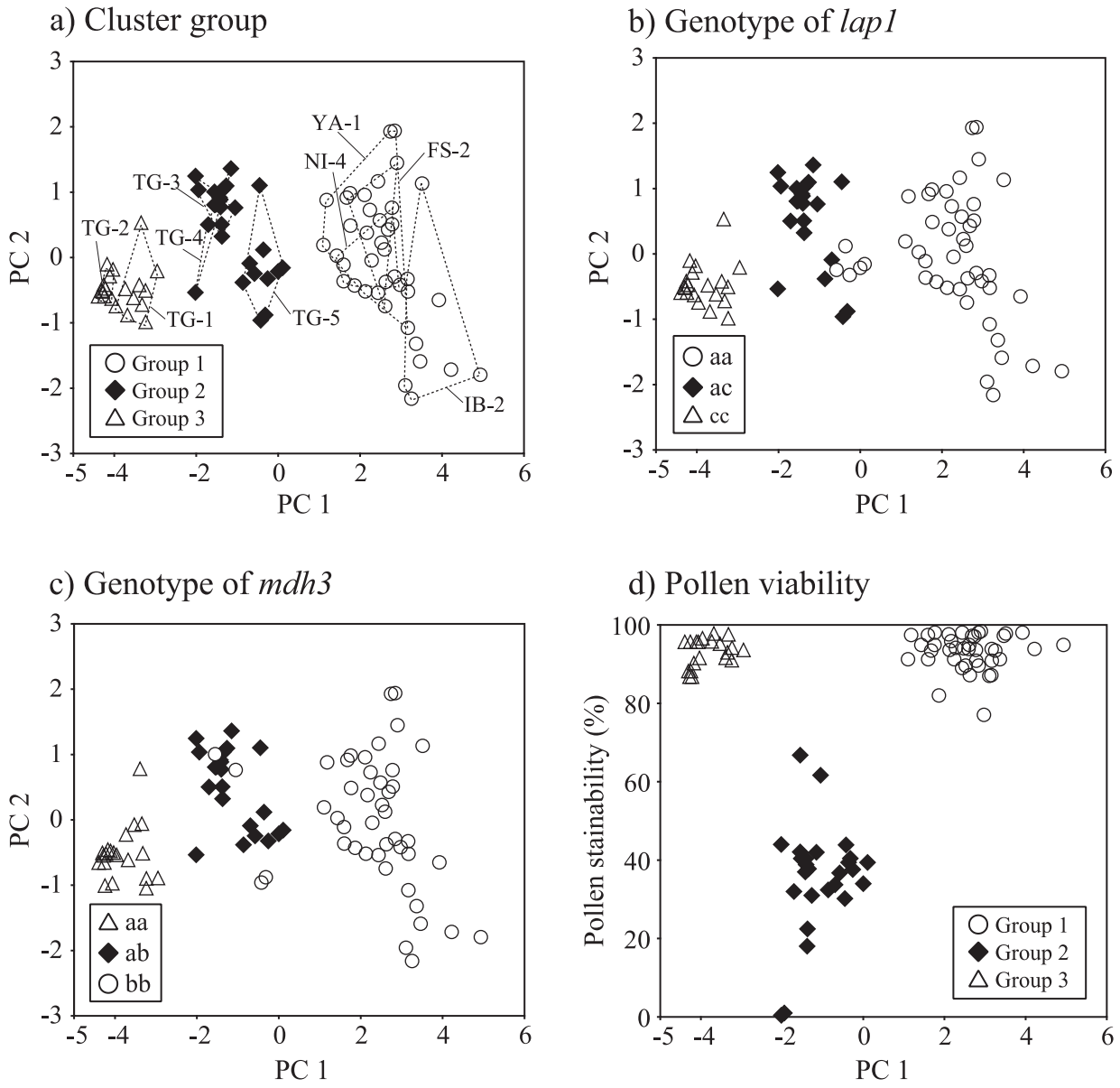

Fig. 15. Results of principal components analysis (PCA) of the plants of the three cluster groups based on 10 morphological characteristics. (a) the three cluster groups, (b) the genotype of *lap1*, (c) the genotype of *mdh3*, and (d) pollen viability superimposed on the PCA (on PC axis 1 only). Each symbol indicates phenotypes ((a) and (d)) or genotypes ((b) and (c)). In the figure a); broken lines encircle the same populations. For population code, see Table 14.

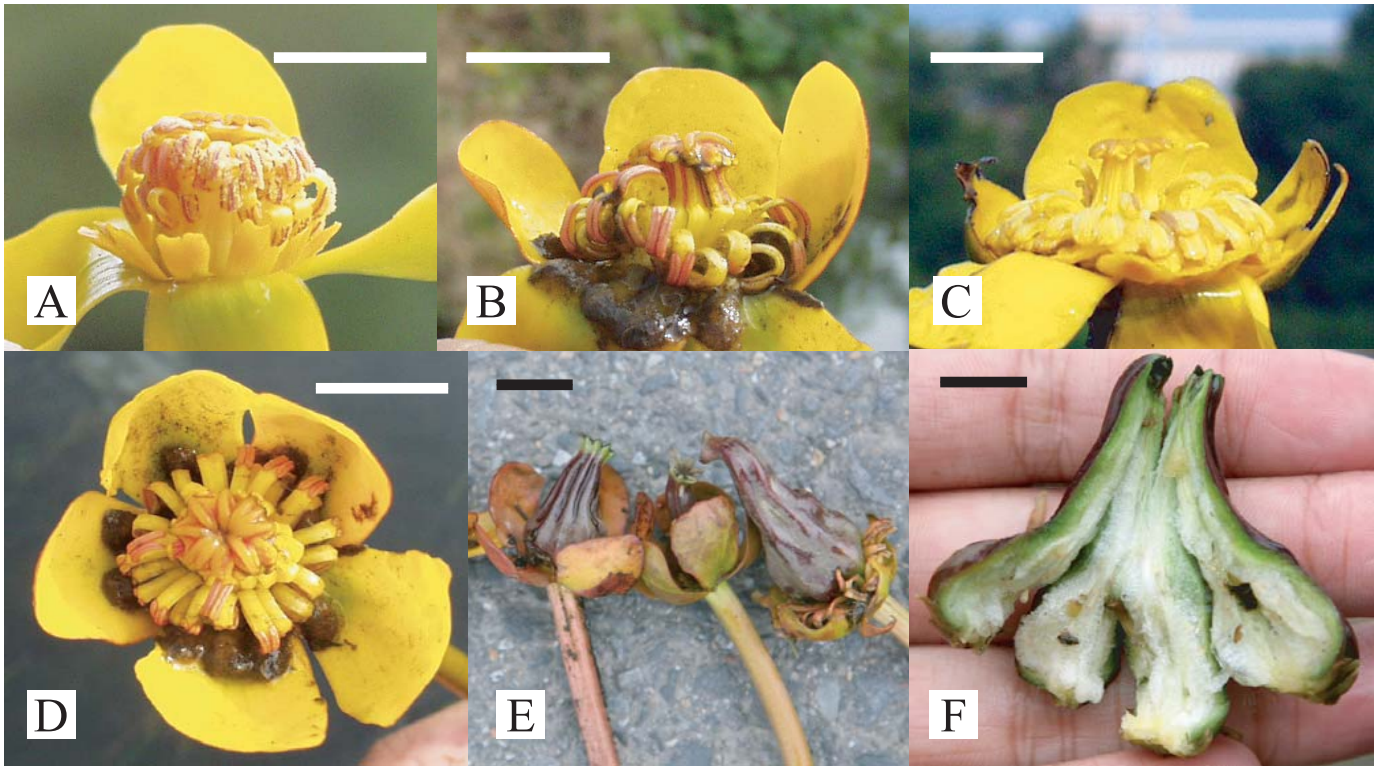

Fig. 16. *Nuphar* x*fluminalis* Shiga & Kadono, hybr. nov. from TG-4 (B, D-F), *N. submersa* (A; *T. shiga 3595* (OSA)) and *N. japonica* (C; *T. shiga 3243* (OSA)). A-D: Flower. E-F: Fruit. Scale indicates 1 cm.

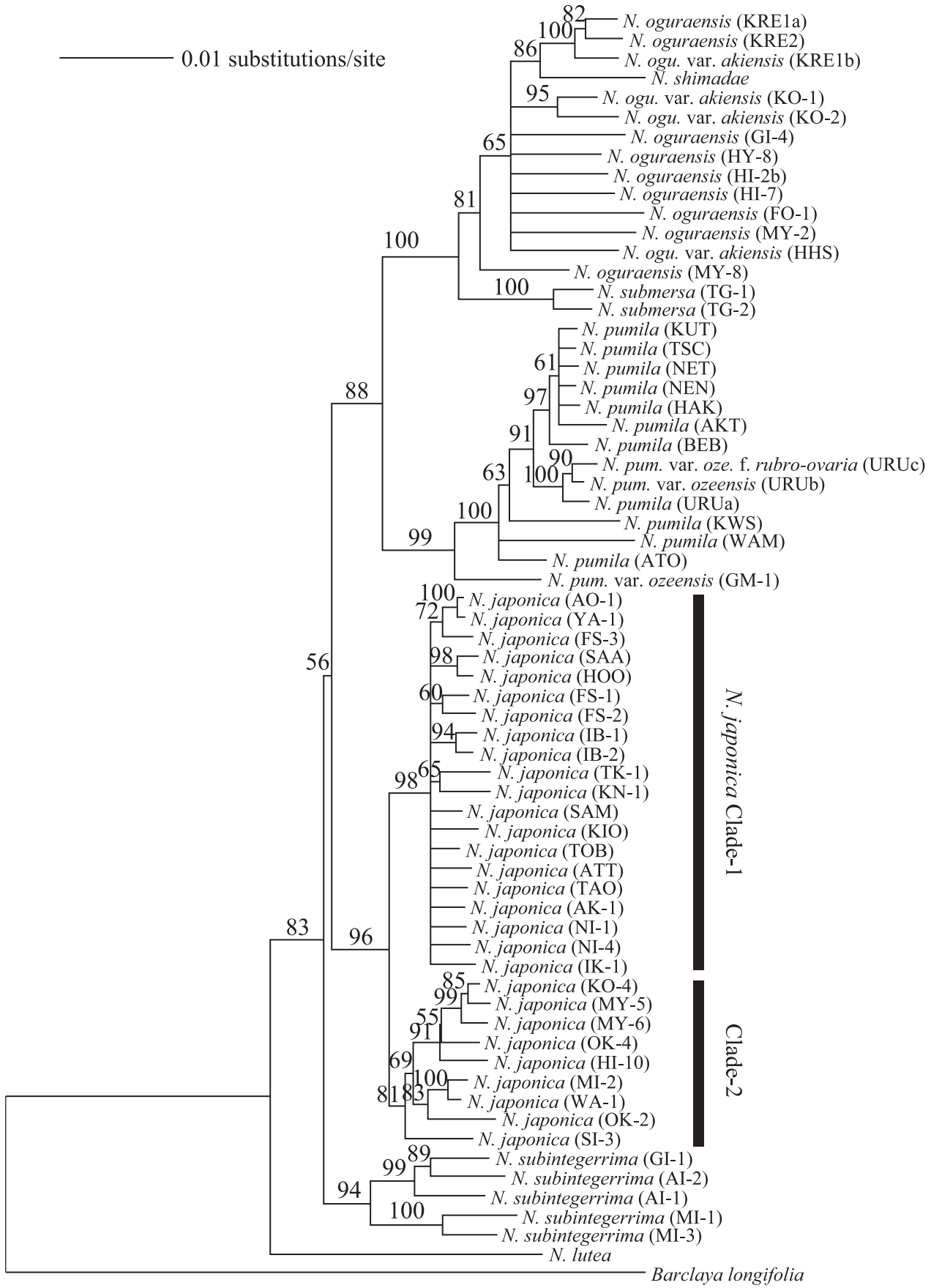

Fig. 17. Phylogenetic relationshipos of *Nuphar* species. Neibor-joining tree based on Nei and Li's genetic distance (1979) for 848 AFLP fragments. Bootstrap values (>50%) based on 1000 replicates are shown above branches.

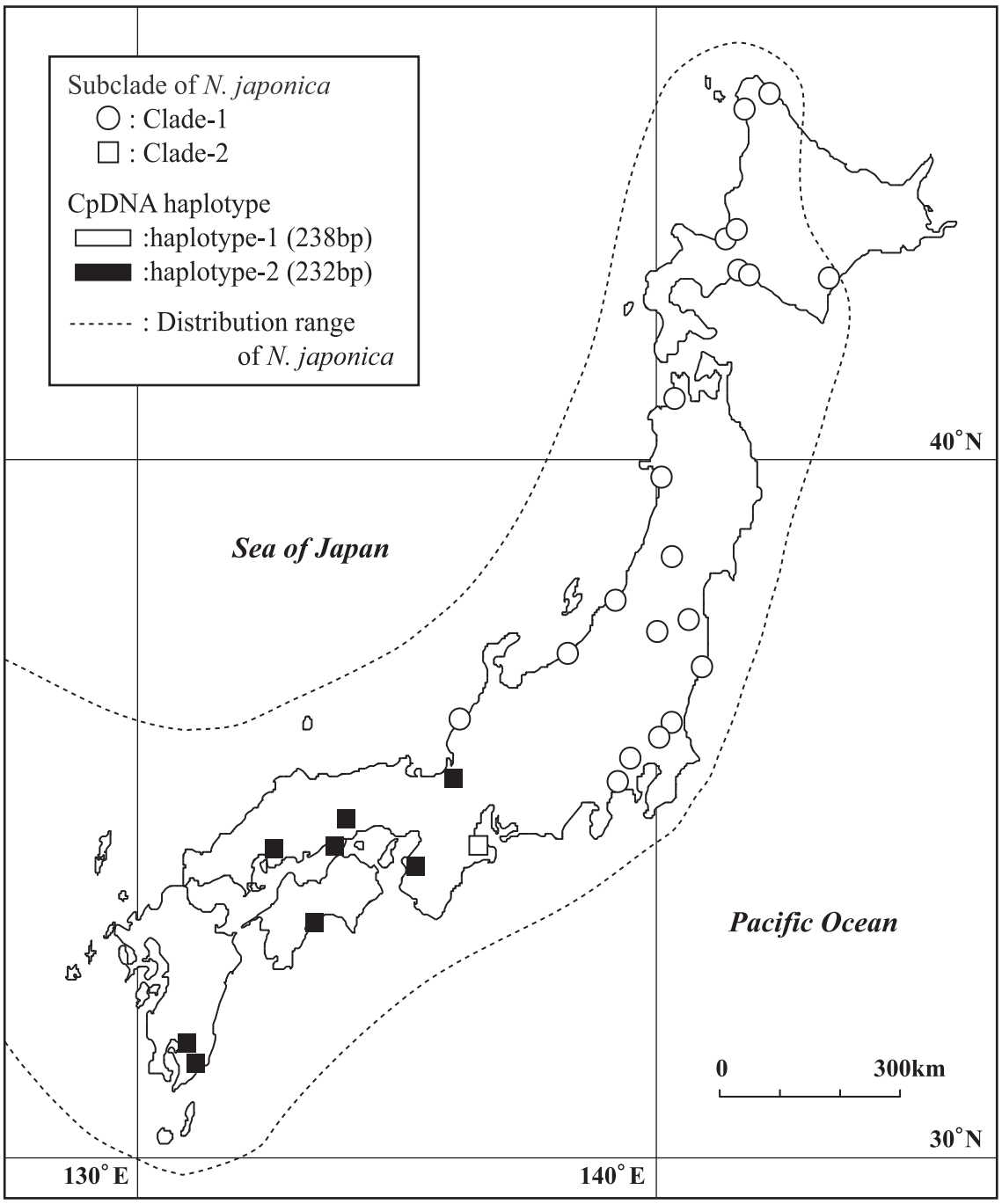

Fig. 18. Geographic distribution of plants of two clades of *N. japonica* and two clades inferred from NJ tree (Fig. 16) and cpDNA, *Trn*L intron region, haplotype. The sequences of each cpDNA haplotype were shown in Fig. 9. One symbol shows one plant. The overall distribution range of *N. japonica* is also shown by broken line.

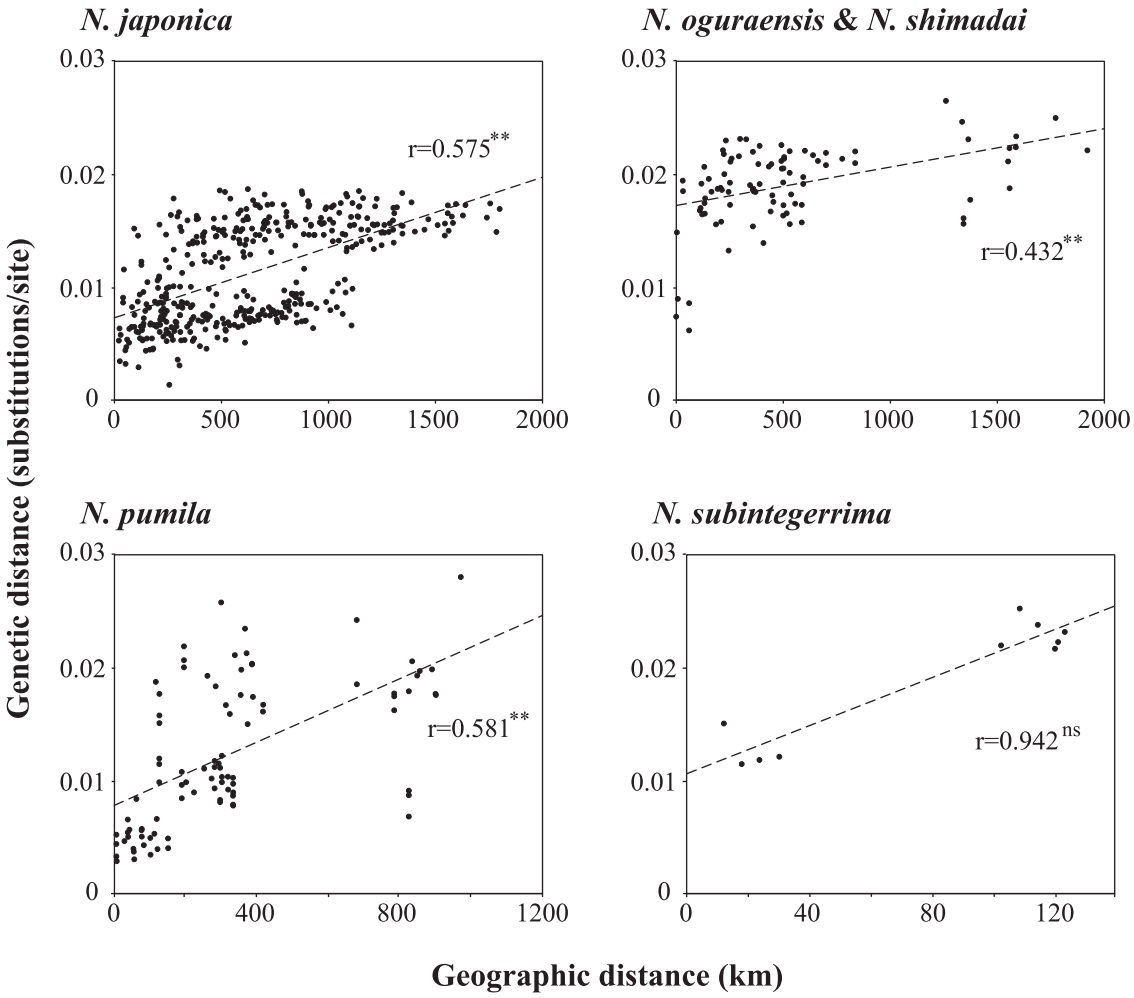

Fig. 19. Scatter diagrams of genetic distance (substitutions/site, *d*; Innan *et al.* 1999) based on the AFLP fragment data against geographic distances between sampling localities, for pairwise comparison of individuals in *N. japonica, N. oguraensis* included *N. shimadai*, *N. pumila* and *N. subintegerrima*. Broken lines are the regression lines. Significance of correlation by Mantel test was expressed by **P<0.01 and ns, P>0.05 on the right shoulders of the corelation coefficients (r).

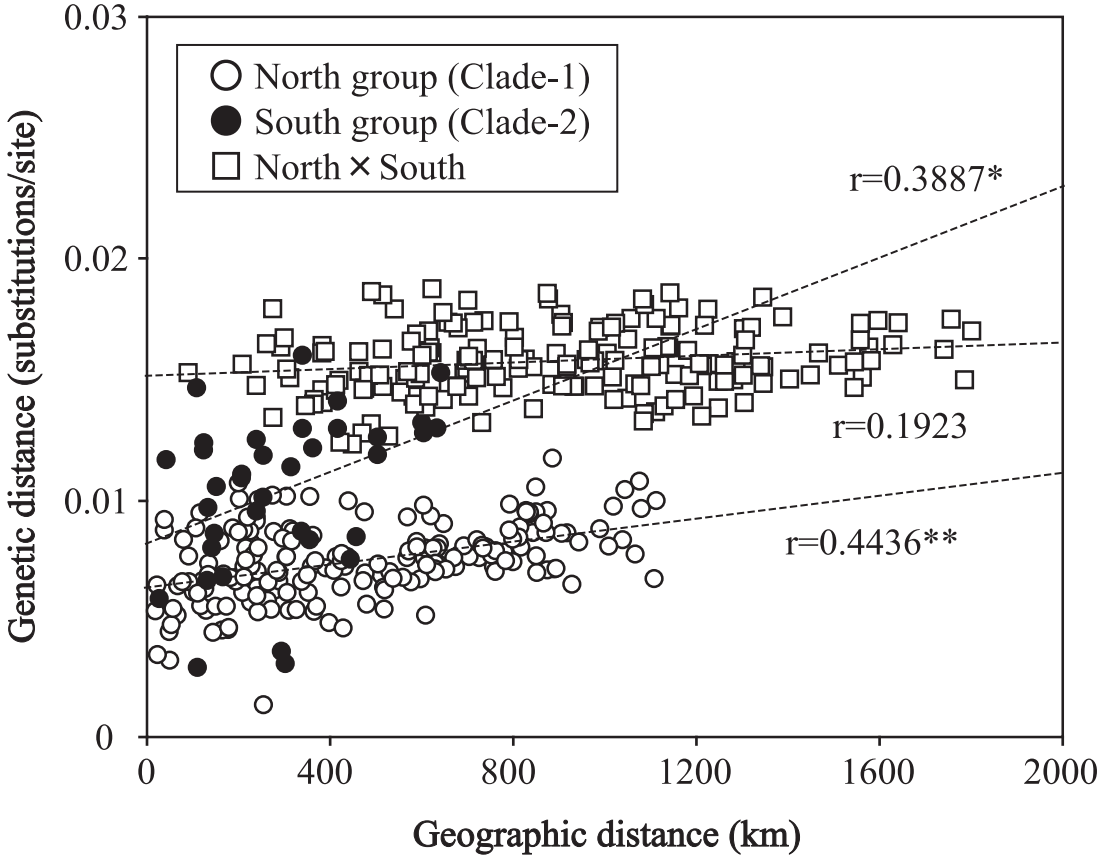

Fig. 20. Scatter diagrams of genetic distance based on the AFLP fragment data against geographical distances between sampling localities, for pairwise comparison of individuals in the two geographic clades of *N. japonica* and relationships between two clades. Broken lines are the regression lines. Significance of correlation by Mantel test was expressed by *P<0.05 and **P<0.01 on the right shoulders of the corelation coefficients (r).

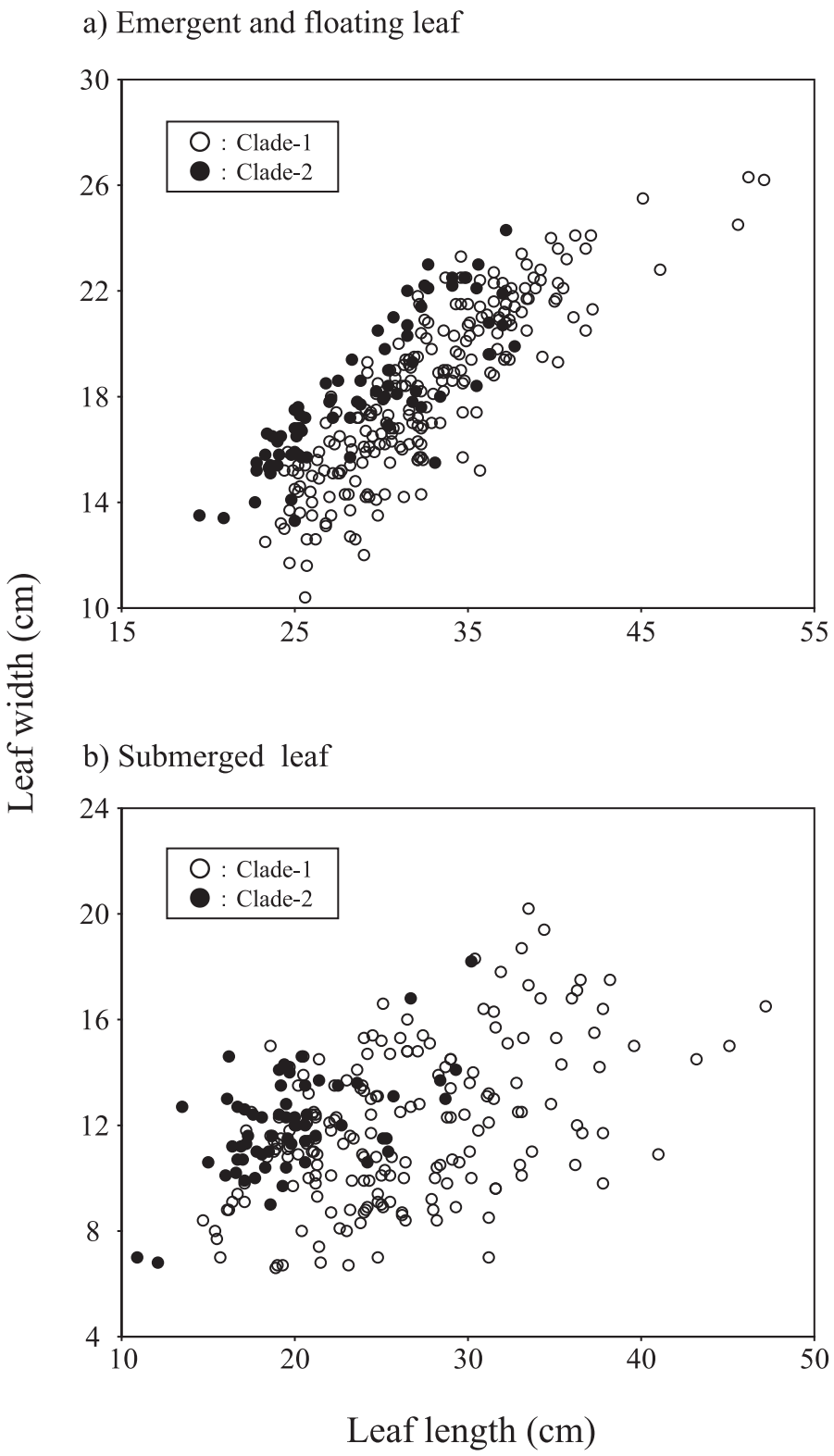

Fig. 21. Comparison of leaf morphology of the *N. japonica*'s two phylogenetic clades. Symbols indicate plants of Clade-1 (open circle) and Clade-2 (solid circle).

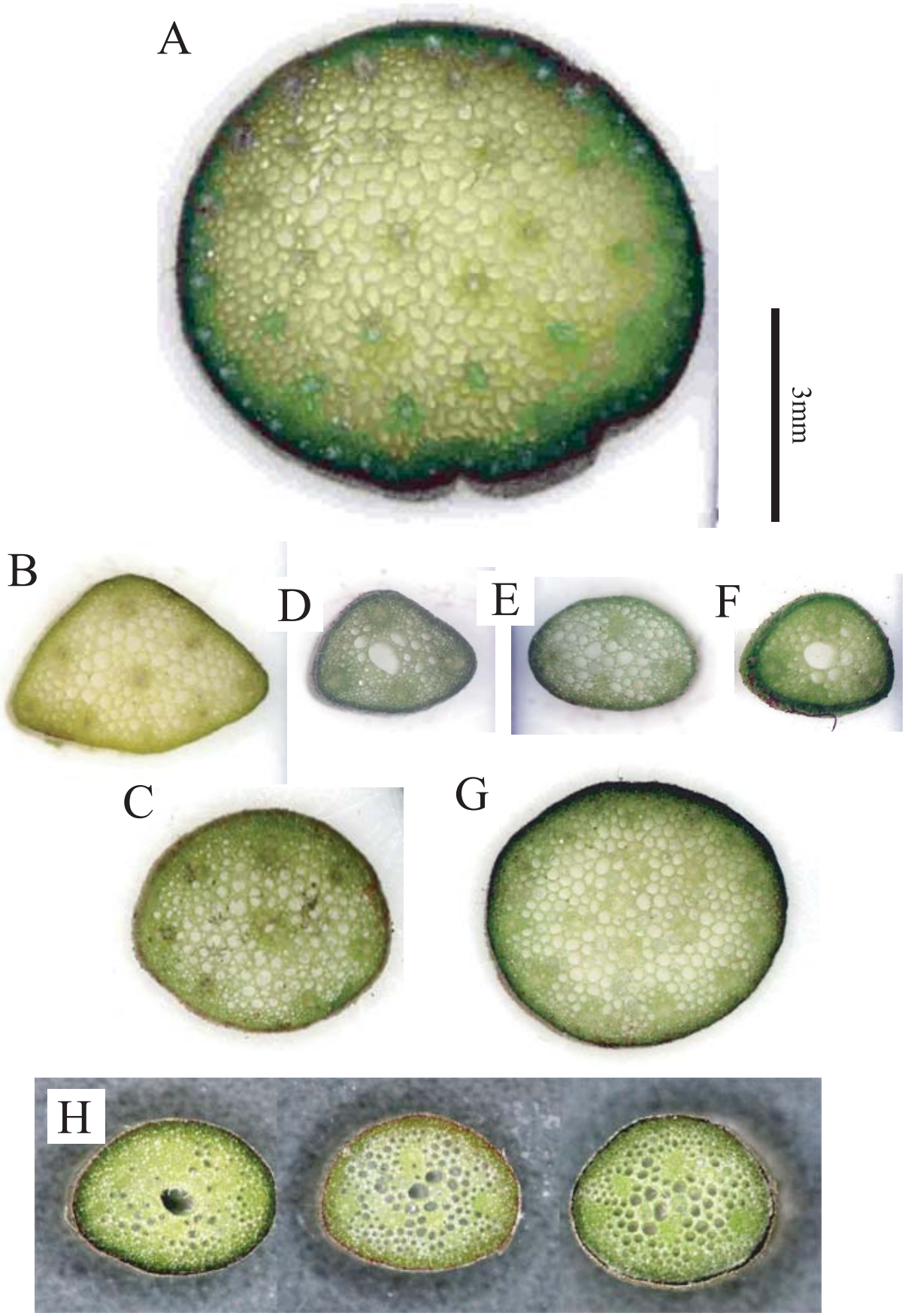

Fig. 22. Comparison of petiole anatomy; A) *N. japonica* (NI-1), B) *N. pumila* (NNA), C) *N. subintegerrima* (MI-1), D) *N. oguraensis* var. *oguraensis* (HY-8), E) *N. oguraensis* var. *akiensis* (HHS), F) *N. shimadae* (Taiwan), G) *N. saikokuensis* (GI-3) and H) *N. submersa* (TG-1).

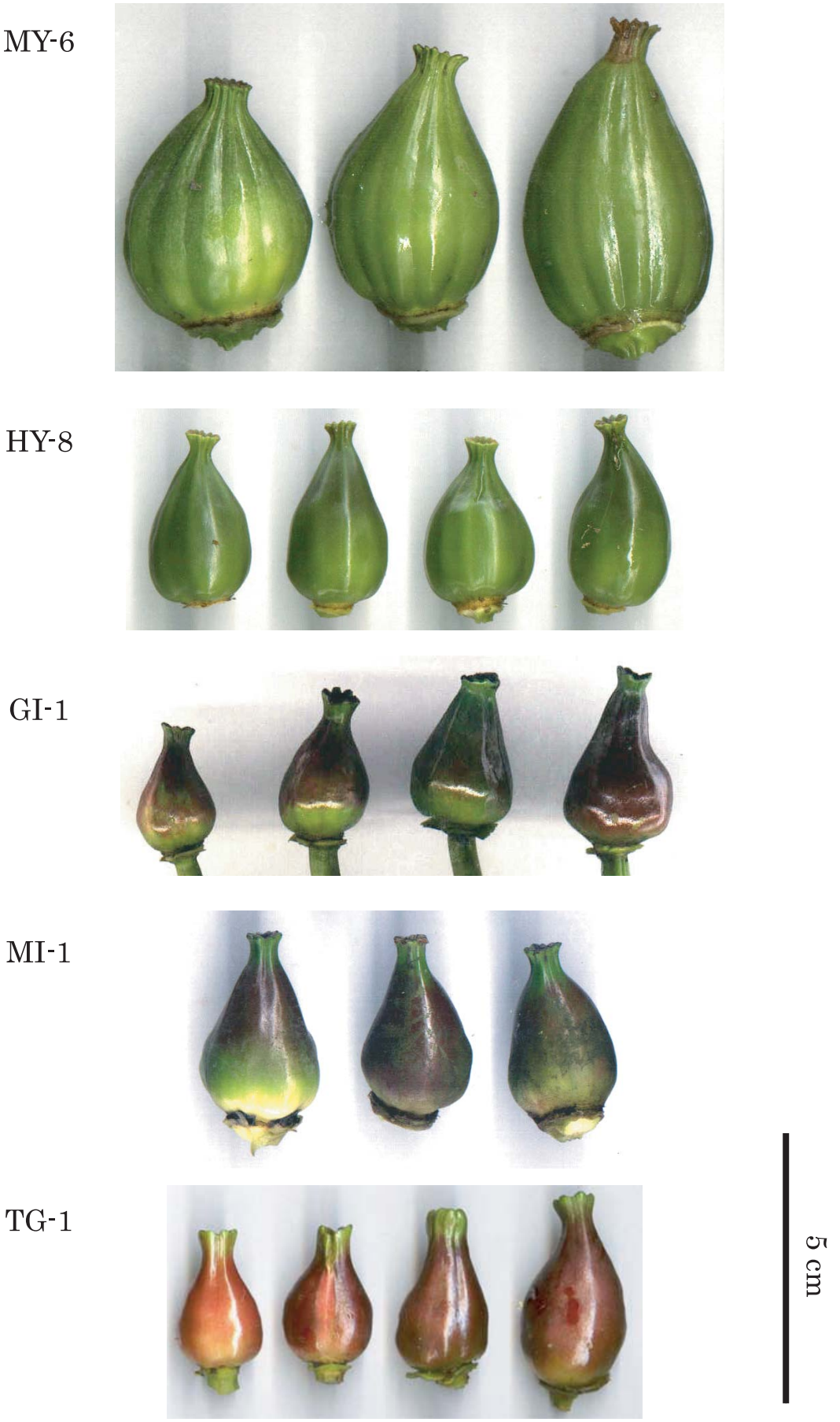

Fig. 23. Comparison of fruit color of *N. japonica* (MY-6), *N. oguraensis* (HY-8), *N. subintegerrima* (GI-1, MI-1) and *N. submersa* (TG-1)

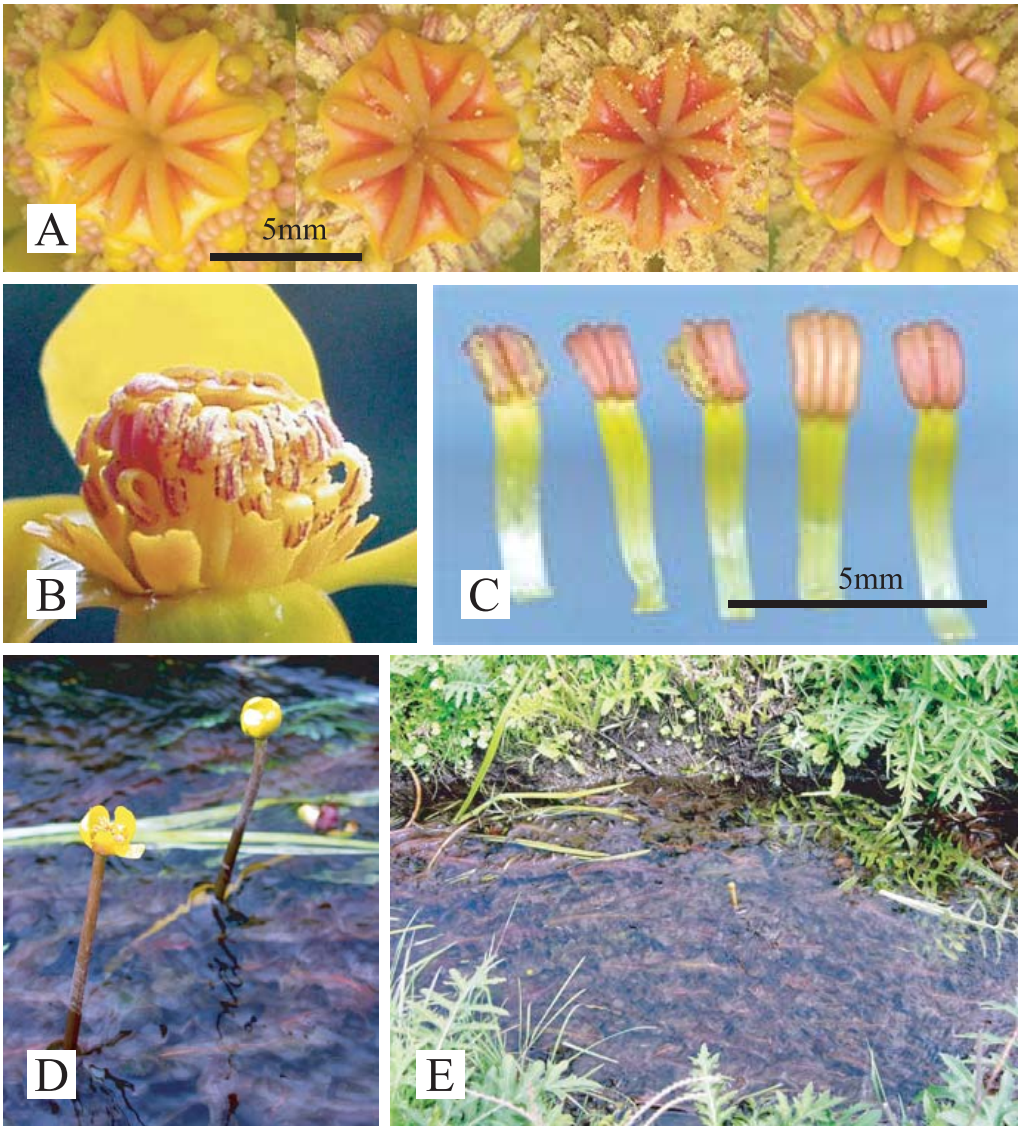

Fig. 24. *Nuphar submersa* Shiga & Kadono, sp. nov. from TG-1. A: Stigmatic disc. B: Flower. C: Stamen. D-E: Habit.

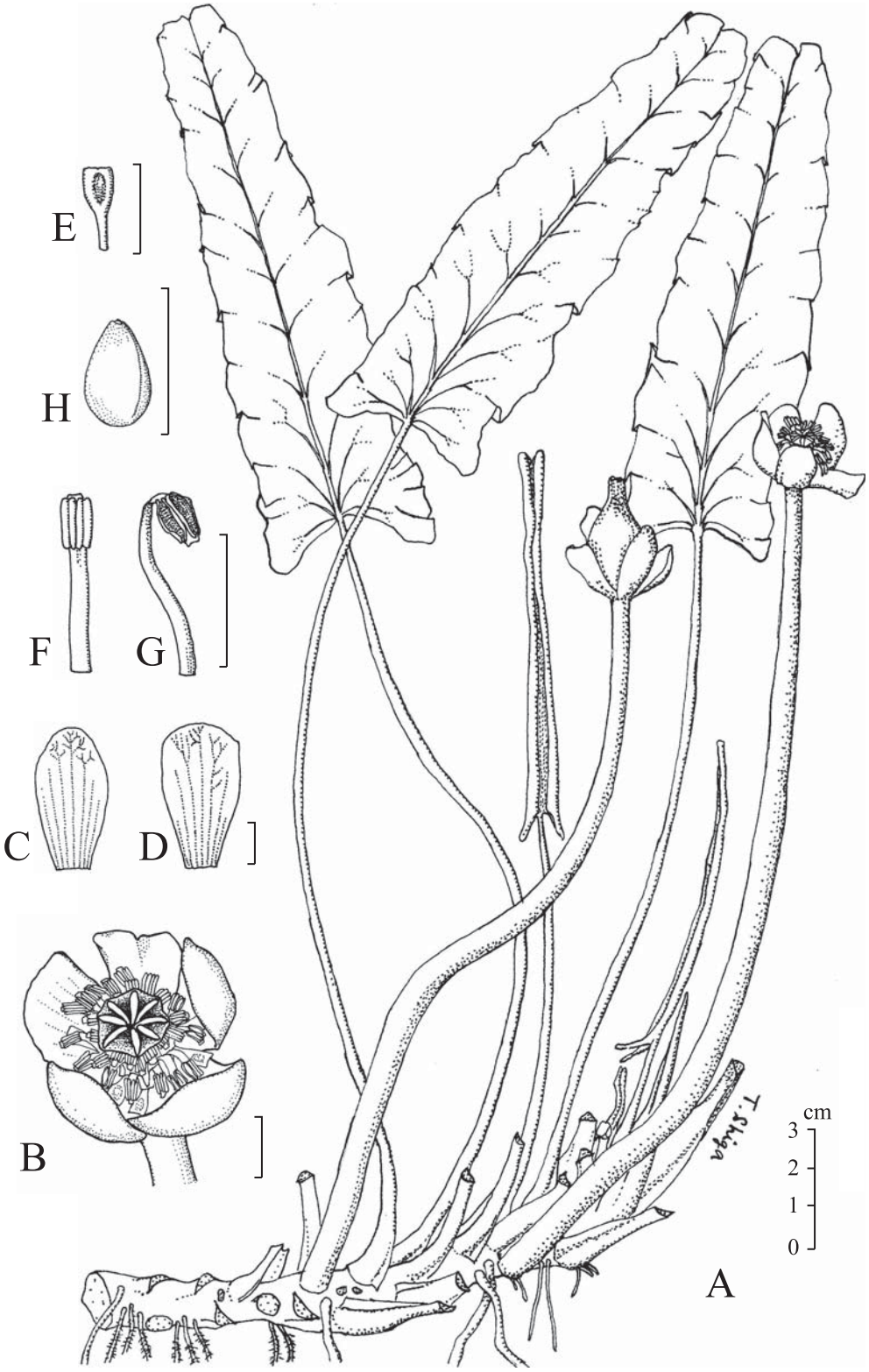

Fig. 25. *Nuphar submersa*. A: Whole plant. B: Flower. C-D: Sepal. E: Petal. F: Stamen. G: Stamen after anthesis. H: seed. A, F-G from *T. Shiga 3480* (OSA). B-E, H from *T. Shiga 3479* (OSA). Scale indicates 5 mm except A.

Fig. 26. Holotype of *Nuphar submersa* Shiga & Kadono (*T. Shiga 3480* (1/3), OSA). Bar indicates 5cm.

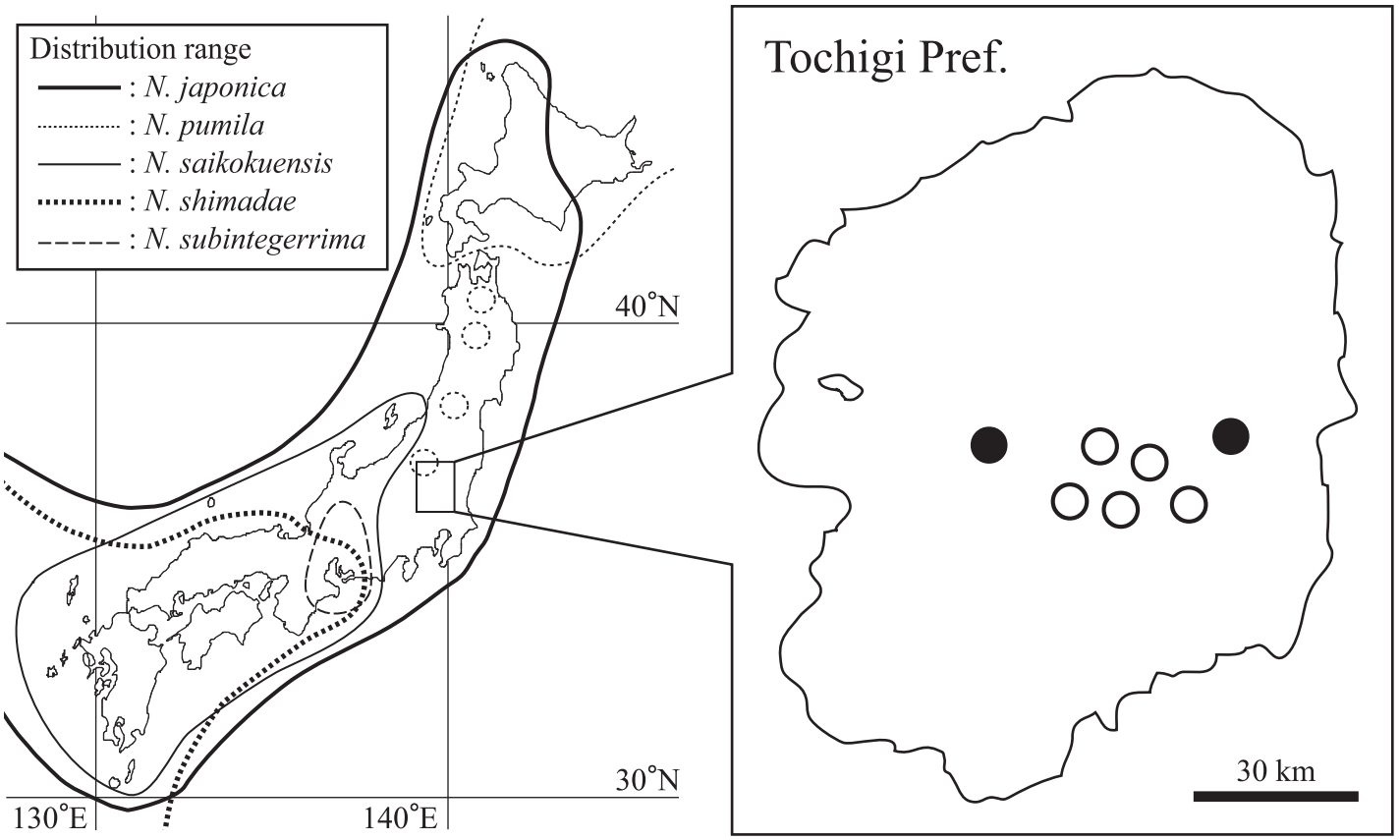

Fig. 27. Distribution of *N. submersa* based on herbarium specimens. Symbols indicate harbarium specimen of extant (●) and extinct (○) populations. Distribution ranges of five other species of Japanese *Nuphar* are also shown.

Fig. 28. Holotype of *Nuphar saikokuensis* Shiga & Kadono (*T. Shiga 3225*, OSA). Bar indicates 5cm.

Fig. 29. Lectotype of *Nuphar subintegerrima* (Casp.) Makino (*T. Makino 59655*, MAK). Bar indicates 5cm.

Fig. 30. Lectotype of *Nuphar oguraensis* Miki (*S. Miki s.n.*, OSA). The letters, "Lake Ogura July 3 1926", on newspaper were written by Shigeru Miki.

Fig. 31. Holotype of *Nuphar* x*fluminalis* Shiga & Kadono (*T. Shiga 3584*, OSA). Bar indicates 5cm.

Fig. 32. Holotype of *Nuphar* x*hokkaiensis* Shiga & Kadono (*T. Shiga 3471*, OSA). Bar indicates 5cm.

APPENDIX 1. Multi-locus genotypes (MLGs) of five *Nuphar* groups based on 11 loci in Chapter 2.

MLG	*lap1*	*mdh1*	*mdh3*	*mdh4*	*pgi1*	*pgi2*	*pgm1*	*pmi1*	*pmi2*	*tpi2*	*tpi3*	Population code
N. japonica **(Group 1)**												
Jap 1	aa	bb	bb	aa	bb	aa	aa	cc	nn	bb	bb	HI-10
Jap 2	aa	bb	bb	aa	bb	aa	ab	cc	nn	bb	bb	MY-6
Jap 3	aa	bb	bb	aa	bb	aa	bb	cc	nn	bb	bb	HS-10, HI-10
Jap 4	aa	bb	bb	ab	bb	aa	aa	bc	nn	bb	bb	OK-2
Jap 5	aa	bb	bb	ab	bb	aa	aa	cc	nn	bb	bb	HI-10
Jap 6	aa	bb	bb	ab	bb	aa	ab	bc	nn	bb	bb	OK-2
Jap 7	aa	bb	bb	ab	bb	aa	ab	cc	nn	bb	bb	HI-10
Jap 8	aa	bb	bb	ab	bb	aa	bb	cc	nn	bb	bb	MI-2, HI-10, MY-6
Jap 9	aa	bb	bb	bb	ab	aa	aa	cc	nn	bb	bb	FS-1
Jap 10	aa	bb	bb	bb	ab	aa	ab	cc	nn	bb	bb	FS-1
Jap 11	aa	bb	bb	bb	ab	aa	bb	cc	nn	bb	bb	NI-1
Jap 12	aa	bb	bb	bb	ab	ab	ab	cc	nn	bb	bb	FS-1
Jap 13	aa	bb	bb	bb	bb	aa	aa	cc	nn	bb	bb	FS-1
Jap 14	aa	bb	bb	bb	bb	aa	ab	bc	nn	bb	bb	OK-4
Jap 15	aa	bb	bb	bb	bb	aa	ab	cc	nn	bb	bb	FS-1, NI-4
Jap 16	aa	bb	bb	bb	bb	aa	bb	cc	nn	bb	bb	AO-1, AK-1, YA-1, FS-1, FS-2, FS-3, NI-1, NI-4, IB-1, IB-2
Jap 17	aa	bb	bb	bb	bb	ab	ab	cc	nn	bb	bb	FS-1
Jap 18	aa	bb	bc	bb	bb	aa	bb	cc	nn	bb	bb	IB-2
Jap 19	aa	bb	cc	bb	bb	aa	bb	cc	nn	bb	bb	IB-2
N. oguraensis **(Group 3)**												
Ogu 1	aa	aa	aa	bb	bb	aa	aa	cc	nn	bb	bb	TO-4
Ogu 2	aa	bb	aa	bb	bb	aa	aa	cc	nn	bb	bb	HI-7, MY-8
Ogu 3	aa	bb	aa	bb	bb	aa	aa	cc	nn	ab	bb	MY-2
Ogu 4	aa	bb	aa	bb	bb	aa	aa	cc	nn	aa	bb	MY-2
Ogu 5	aa	bb	aa	bb	bb	aa	ab	cc	nn	bb	bb	HI-2b
Ogu 6	aa	bb	aa	bb	bb	aa	bb	cc	nn	bb	bb	HY-8, HI-2b, MY-8
Ogu 7	aa	cc	aa	bb	bb	aa	aa	cc	nn	bb	bb	FO-1
Ogu 8	aa	cc	aa	bb	bb	aa	ab	cc	nn	bb	bb	FO-1
N. subintegerrima **(Group 5)**												
Sub 1	aa	bb	bb	ab	ab	aa	bb	aa	aa	bb	aa	GI-1
Sub 2	aa	bb	bb	ab	ab	aa	bb	bb	aa	bb	aa	GI-1
Sub 3	aa	bb	bb	ab	bb	aa	bb	aa	aa	bb	cc	AI-1
Sub 4	aa	bb	bb	ab	bb	aa	bb	bb	aa	bb	aa	GI-1
Sub 5	aa	bb	bb	bb	bb	aa	bb	bb	aa	bb	aa	GI-1
Sub 6	bb	bb	bb	aa	aa	aa	aa	bb	aa	bb	bb	MI-1, MI-3
Sub 7	nn	bb	bb	bb	bb	aa	aa	bb	aa	bb	aa	AI-2
Sub 8	nn	bb	bb	bb	bb	aa	ab	bb	aa	bb	aa	AI-2
Sub 9	nn	bb	bb	bb	bb	aa	bb	bb	aa	bb	aa	AI-2
japonica-oguraensis **intermediate (Group 2)**												
J-O 1	aa	ab	ab	ab	bb	aa	aa	bc	aa	bb	bb	TO-3b
J-O 2	aa	ab	ab	ab	bb	aa	aa	bc	an	bb	bb	TO-1, TO-3b
J-O 3	aa	ab	ab	ab	bb	aa	aa	bc	nn	bb	bb	TO-1
J-O 4	aa	ab	ab	ab	bb	aa	aa	cc	nn	bb	bb	TO-1
J-O 5	aa	ab	ab	ab	bb	aa	bb	cc	an	bb	bb	TO-3b
J-O 6	aa	ab	ab	ab	bb	ab	bb	cc	an	bb	bb	TO-2
J-O 7	aa	ab	ab	ab	bb	ab	bb	cc	nn	bb	bb	TO-1

APPENDIX 1. (continued)

MLG	*lap1*	*mdh1*	*mdh3*	*mdh4*	*pgi1*	*pgi2*	*pgm1*	*pmi1*	*pmi2*	*tpi2*	*tpi3*	Population code
J-O 8	aa	bb	aa	aa	bb	aa	aa	cc	nn	bb	bb	HI-4
J-O 9	aa	bb	aa	ab	bb	aa	aa	cc	nn	bb	bb	HI-4, HI-5
J-O 10	aa	bb	aa	ab	bb	aa	bb	cc	an	bb	bb	NI-5
J-O 11	aa	bb	aa	ab	bb	aa	bb	cc	nn	bb	bb	HI-4
J-O 12	aa	bb	aa	bb	bb	aa	aa	cc	nn	bb	bb	HI-4
J-O 13	aa	bb	aa	bb	bb	aa	ab	cc	nn	bb	bb	HI-5
J-O 14	aa	bb	aa	bb	bb	aa	bb	cc	nn	bb	bb	HI-4, HI-5
J-O 15	aa	bb	ab	aa	bb	aa	aa	cc	nn	bb	bb	HI-4
J-O 16	aa	bb	ab	ab	bb	aa	aa	bc	nn	bb	bb	MY-1c
J-O 17	aa	bb	ab	ab	bb	aa	aa	cc	nn	bb	bb	HI-4, HI-5
J-O 18	aa	bb	ab	ab	bb	aa	bb	cc	an	bb	bb	NI-5
J-O 19	aa	bb	ab	ab	bb	aa	bb	cc	nn	bb	bb	HI-2a
J-O 20	aa	bb	ab	bb	bb	aa	bb	cc	an	bb	bb	NI-5
J-O 21	aa	bb	ab	bb	bb	ab	bb	bc	nn	bb	bb	TO-1
J-O 22	aa	bb	bb	aa	bb	aa	bb	bc	nn	bb	bb	OK-1
J-O 23	aa	bb	bb	ab	bb	aa	ab	bb	nn	bb	bb	MY-1b, MY-1c
J-O 24	aa	bb	bb	ab	bb	aa	ab	bc	an	bb	bb	TO-3a
J-O 25	aa	bb	bb	ab	bb	aa	ab	bc	nn	bb	bb	OK-1
J-O 26	aa	bb	bb	ab	bb	aa	bb	bc	nn	bb	bb	OK-1
J-O 27	aa	bb	bb	bb	bb	aa	bb	cc	aa	bb	bb	FU-3
J-O 28	aa	bb	bb	bb	bb	aa	bb	cc	nn	bb	bb	FU-3
J-O 29	aa	bb	cc	bb	bb	aa	bb	bb	aa	bb	bb	FU-1
J-O 30	aa	bb	cc	bb	bb	aa	bb	bb	an	bb	bb	FU-1
J-O 31	aa	bb	cc	bb	bb	aa	bb	bc	aa	bb	bb	FU-1
J-O 32	aa	bb	cc	bb	bb	aa	bb	bc	an	bb	bb	FU-1
J-O 33	aa	bb	cc	bb	bb	aa	bb	bc	nn	bb	bb	FU-1
J-O 34	aa	bb	cc	bb	bb	aa	bb	cc	aa	bb	bb	FU-1
J-O 35	aa	bb	cc	bb	bb	aa	bb	cc	an	bb	bb	FU-1
J-O 36	aa	bb	cc	bb	bc	aa	bb	bc	an	bb	bb	FU-1
J-O 37	ab	bb	bb	aa	bb	aa	bb	bb	an	bb	bb	KA-4
J-O 38	ab	bb	bb	ab	bb	aa	bb	cc	an	bb	bb	HY-3
japonica*-*subintegerrima **intermediate (Group 4)**												
J-S 1	aa	bb	bb	aa	bb	aa	aa	ab	aa	bb	bc	HY-1
J-S 2	aa	bb	bb	aa	bb	aa	aa	ab	nn	bb	bb	HY-1
J-S 3	aa	bb	bb	aa	bb	aa	aa	bb	aa	bb	aa	OH-1
J-S 4	aa	bb	bb	aa	bb	aa	aa	bb	aa	bb	bb	HY-1, HY-5, NA-1
J-S 5	aa	bb	bb	aa	bb	aa	aa	bb	aa	bb	bc	HY-5
J-S 6	aa	bb	bb	aa	bb	aa	aa	bb	an	bb	bb	HY-1, KA-5
J-S 7	aa	bb	bb	aa	bb	aa	aa	bc	aa	bb	ab	OH-1
J-S 8	aa	bb	bb	aa	bb	aa	aa	bc	aa	bb	bb	SI-1, NA-1
J-S 9	aa	bb	bb	aa	bb	aa	aa	cc	aa	bb	ab	OH-1
J-S 10	aa	bb	bb	aa	bb	aa	aa	cc	aa	bb	bb	NA-1
J-S 11	aa	bb	bb	aa	bb	aa	aa	cc	an	bb	bb	NA-1
J-S 12	aa	bb	bb	aa	bb	aa	ab	bb	aa	bb	bb	SI-1, HY-1, HY-5
J-S 13	aa	bb	bb	aa	bb	aa	ab	bb	an	bb	bb	HY-1
J-S 14	aa	bb	bb	aa	bb	aa	ab	bb	nn	bb	bb	HY-1, OK-3
J-S 15	aa	bb	bb	aa	bb	aa	ab	bc	aa	bb	bb	SI-1
J-S 16	aa	bb	bb	aa	bb	aa	ab	bc	nn	bb	bb	OK-3
J-S 17	aa	bb	bb	aa	bb	aa	ab	cc	aa	bb	bb	SI-1

APPENDIX 1. (continued)

MLG	*lap1*	*mdh1*	*mdh3*	*mdh4*	*pgi1*	*pgi2*	*pgm1*	*pmi1*	*pmi2*	*tpi2*	*tpi3*	Population code
J-S 18	aa	bb	bb	aa	bb	aa	bb	ab	aa	bb	bb	HY-1
J-S 19	aa	bb	bb	aa	bb	aa	bb	bb	aa	bb	bb	HY-1
J-S 20	aa	bb	bb	aa	bb	aa	bb	bb	aa	bb	bc	HY-5
J-S 21	aa	bb	bb	aa	bb	aa	bb	bb	an	bb	bb	HY-1, SI-1
J-S 22	aa	bb	bb	aa	bb	aa	bb	bc	aa	bb	bb	SI-1
J-S 23	aa	bb	bb	aa	cc	aa	bb	bb	an	bb	bb	GI-2
J-S 24	aa	bb	bb	ab	bb	aa	aa	ab	aa	bb	bb	HY-1
J-S 25	aa	bb	bb	ab	bb	aa	aa	bb	aa	bb	aa	OH-1
J-S 26	aa	bb	bb	ab	bb	aa	aa	bb	nn	bb	bc	HY-1
J-S 27	aa	bb	bb	ab	bb	aa	aa	bc	aa	bb	ab	OH-1
J-S 28	aa	bb	bb	ab	bb	aa	aa	bc	aa	bb	bb	SI-1, HY-7
J-S 29	aa	bb	bb	ab	bb	aa	aa	cc	aa	bb	bb	HY-7
J-S 30	aa	bb	bb	ab	bb	aa	ab	bb	aa	bb	bb	SI-1
J-S 31	aa	bb	bb	ab	bb	aa	ab	bc	aa	bb	bb	SI-1
J-S 32	aa	bb	bb	ab	bb	aa	bb	ab	aa	bb	bb	HY-1
J-S 33	aa	bb	bb	ab	bb	aa	bb	ab	nn	bb	bb	HY-1
J-S 34	aa	bb	bb	ab	bb	aa	bb	bb	an	bb	bb	SI-1, HY-1
J-S 35	aa	bb	bb	ab	bb	aa	bb	bc	aa	bb	bb	SI-1
J-S 36	aa	bb	bb	ab	bb	aa	bb	bc	an	bb	bb	SI-1
J-S 37	aa	bb	bb	ab	cc	aa	bb	bb	aa	bb	bb	GI-2
J-S 38	aa	bb	bb	ab	cc	aa	bb	bb	an	bb	bb	GI-2
J-S 39	aa	bb	bb	ab	cc	aa	bb	bb	nn	bb	bb	GI-2
J-S 40	aa	bb	bb	ab	cc	aa	bb	bc	aa	bb	bb	GI-2
J-S 41	aa	bb	bb	bb	bb	aa	aa	bb	aa	bb	bb	FU-2, HY-7
J-S 42	aa	bb	bb	bb	bb	aa	aa	bc	aa	bb	bb	FU-2
J-S 43	aa	bb	bb	bb	bb	aa	ab	bb	aa	bb	bb	GI-2
J-S 44	aa	bb	bb	bb	bb	aa	ab	bb	an	bb	bb	FU2, HY-1
J-S 45	aa	bb	bb	bb	bb	aa	ab	bb	nn	bb	bb	HY-1
J-S 46	aa	bb	bb	bb	bb	aa	ab	bc	aa	bb	bb	FU-2
J-S 47	aa	bb	bb	bb	bb	aa	ab	bc	nn	bb	bb	OK-6
J-S 48	aa	bb	bb	bb	bb	aa	ab	cc	aa	bb	bb	FU2
J-S 49	aa	bb	bb	bb	bb	aa	ab	cc	an	bb	bb	FU-2, HY-7
J-S 50	aa	bb	bb	bb	bb	aa	bb	bb	aa	bb	bb	GI-2, FU-2
J-S 51	aa	bb	bb	bb	bb	aa	bb	bb	an	bb	bb	FU-2, HY-1
J-S 52	aa	bb	bb	bb	bb	aa	bb	bc	aa	bb	bb	FU-2
J-S 53	aa	bb	bb	bb	bb	aa	bb	bc	an	bb	bb	FU-2
J-S 54	aa	bb	bb	bb	bb	aa	bb	cc	aa	bb	bb	SI-1
J-S 55	aa	bb	bb	bb	bb	aa	bb	cc	an	bb	bb	FU-2
J-S 56	aa	bb	bb	bb	bc	aa	ab	bc	aa	bb	bb	GI-2
J-S 57	aa	bb	bb	bb	bc	aa	bb	bb	aa	bb	bb	GI-2
J-S 58	aa	bb	bb	bb	cc	aa	bb	bb	aa	bb	bb	GI-2
J-S 59	aa	bb	bb	bb	cc	aa	bb	bb	an	bb	bb	GI-2
J-S 60	aa	bb	bb	bb	cc	aa	bb	bb	nn	bb	bb	GI-2
J-S 61	ab	bb	bb	aa	bb	aa	aa	bb	aa	bb	ab	OH-1
J-S 62	ab	bb	bb	aa	bb	aa	aa	bb	an	bb	ab	OH-1
J-S 63	ab	bb	bb	aa	bb	aa	aa	bb	an	bb	bb	KA-5
J-S 64	ab	bb	bb	aa	bb	aa	aa	bb	nn	bb	bb	KA-5
J-S 65	ab	bb	bb	aa	bb	aa	aa	bc	aa	bb	aa	OH-1
J-S 66	ab	bb	bb	aa	bb	aa	aa	bc	an	bb	ab	OH-1

APPENDIX 1. (continued)

MLG	*lap1*	*mdh1*	*mdh3*	*mdh4*	*pgi1*	*pgi2*	*pgm1*	*pmi1*	*pmi2*	*tpi2*	*tpi3*	Population code
J-S 67	ab	bb	bb	aa	bb	aa	aa	bc	an	bb	bb	KA-1
J-S 68	ab	bb	bb	aa	bb	aa	aa	cc	aa	bb	ab	OH-1
J-S 69	ab	bb	bb	ab	bb	aa	aa	bb	aa	bb	ab	OH-1
J-S 70	ab	bb	bb	ab	bb	aa	aa	bb	an	bb	aa	OH-1
J-S 71	ab	bb	bb	ab	bb	aa	aa	bc	an	bb	ab	OH-1
J-S 72	ab	bb	bb	ab	bb	aa	aa	bc	an	bb	bb	KA-2
J-S 73	ab	bb	bb	bb	bb	aa	aa	bb	aa	bb	aa	OH-1
J-S 74	ab	bb	bb	bb	bb	aa	aa	bb	aa	bb	ab	OH-1
J-S 75	ab	bb	bb	bb	bb	aa	aa	bc	aa	bb	ab	OH-1
J-S 76	bb	bb	bb	aa	bb	aa	aa	bb	aa	bb	aa	OH-1
J-S 77	bb	bb	bb	aa	bb	aa	aa	bc	aa	bb	aa	OH-1
J-S 78	bb	bb	bb	aa	bb	aa	aa	bc	an	bb	aa	OH-1
J-S 79	bb	bb	bb	aa	bb	aa	aa	cc	aa	bb	ab	OH-1
J-S 80	bb	bb	bb	ab	bb	aa	aa	bc	aa	bb	ab	OH-1
J-S 81	nn	bb	bb	bb	bb	aa	bb	bb	aa	bb	aa	OH-1
J-S 82	nn	bb	bb	bb	bb	aa	bb	bb	aa	bb	ac	GI-3
J-S 83	nn	bb	bb	bb	bb	aa	bb	bb	aa	bb	cc	GI-3
J-S 84	nn	bb	bb	bb	bb	aa	bb	bc	aa	bb	aa	GI-3
J-S 85	nn	bb	bb	bb	bb	aa	bb	bc	aa	bb	ac	GI-3
J-S 86	nn	bb	bb	bb	bb	aa	bb	bc	aa	bb	cc	GI-3
J-S 87	nn	bb	bb	bb	bb	aa	bb	cc	aa	bb	aa	GI-3
J-S 88	nn	bb	bb	bb	bb	aa	bb	cc	aa	bb	ac	GI-3

APPENDIX 2. Data matrix of absence (not shown) or presence (1) of the AFLP fragments in ACG-CTA primer combination.

Genotype	Taxa	Pop. Code	Fragment size (bp) 53	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	80	94	99	102	103	104	105	106	107	111	112	113	114	115	116	118	123	126	128
1	*N. japonica*	SAA		1	1							1		1	1					1			1		1		1	1		1							1			1	1		1
2		HOO		1	1							1		1	1					1			1		1		1	1		1							1			1	1		1
3		SAM		1	1							1		1	1					1			1		1		1	1		1										1	1		1
4		KIO		1	1							1		1	1					1			1		1		1	1		1							1			1	1		1
5		TOB		1	1	1						1		1	1					1			1		1		1	1		1										1	1		1
6		ATT		1	1									1	1					1			1		1		1	1		1							1			1	1		1
7		TAO		1	1		1					1		1	1					1			1		1		1	1		1							1			1	1		1
8		AO-1		1	1	1						1		1	1			1		1			1	1	1		1	1		1							1			1	1		1
9		AK-1		1	1		1					1		1	1					1			1		1		1	1		1							1			1	1		1
10		YA-1		1	1	1						1		1	1			1		1			1	1	1		1	1		1							1			1	1		1
11		FS-1		1	1	1						1		1	1	1		1		1			1	1	1		1	1		1							1			1	1		1
12		FS-2		1	1	1						1		1	1	1		1		1			1	1	1		1	1		1							1			1	1		1
13		FS-3		1	1	1						1		1	1	1		1		1			1	1	1		1	1		1									1	1	1		1
14		IB-1		1	1	1						1		1	1	1		1		1			1	1	1		1	1		1						1				1	1		1
15		IB2-		1	1							1		1	1	1				1			1		1		1	1		1							1			1	1		1
16		TK-1		1	1	1								1	1					1			1		1		1	1		1										1	1		1
17		KN-1		1	1		1							1	1	1				1			1	1	1		1	1		1											1		1
18		NI-1		1	1	1						1		1	1	1		1		1			1		1		1	1		1											1	1		1
19		NI4		1	1							1		1	1	1		1		1			1	1	1		1	1		1									1		1	1		1
20		IK-1		1	1							1		1	1	1				1			1		1		1	1		1							1			1	1		1
21		SI-3		1	1		1							1	1	1				1			1		1		1	1	1						1		1			1	1		1
22		MI-2		1	1	1	1	1				1		1	1	1		1		1			1		1		1	1	1	1		1					1			1	1		1
23		WA-1		1	1			1						1	1	1				1			1	1	1		1	1	1	1		1					1			1	1		1
24		OK-2		1	1									1	1	1				1			1	1	1		1	1	1	1			1				1			1	1		1
25		OK-4		1	1			1						1	1					1			1		1		1	1	1	1							1			1	1		1
26		HI-10		1	1		1	1				1		1	1					1			1		1		1	1	1	1							1			1	1		1
27		KO-4		1	1		1	1						1	1					1			1		1		1	1	1	1		1					1			1	1		1
28		MY-5		1	1			1						1	1					1			1		1		1	1	1	1							1			1	1		1
29		MY-6		1	1			1						1	1					1			1		1		1	1	1	1							1			1	1		1
30	*N. oguraensis*	GI-4			1		1		1			1	1							1		1		1	1				1			1		1			1						
31		HY-8			1				1			1	1							1			1		1																		
32		HI-2b			1				1			1	1					1		1			1	1	1										1								
33		HI-7			1	1			1			1	1							1			1		1			1		1				1						1		
34		FO-2			1				1			1	1							1			1		1									1							1	
35		MY-2			1		1					1	1							1			1		1									1							1	
36		MY-8			1				1		1	1		1	1					1			1		1			1	1					1						1	1	
37		Ko-1y			1				1			1	1							1			1		1	1								1							1	
38		Ko-2y			1				1			1	1							1			1		1									1							1	
39	*N. ogu.* var. *akiensis*	HHS	1		1				1		1		1	1	1				1	1			1	1	1		1							1							1	1	
40		KO-1		1	1				1	1		1	1	1					1	1			1		1																		1
41		KO-2			1				1	1		1	1	1						1			1		1				1		1										1		
42		Ko-1r			1				1			1	1							1			1		1																1		
43	*N. shimadai*				1				1			1	1			1				1			1	1	1																		
44	*N. submersa*	TG-1			1		1			1		1	1	1					1	1			1		1	1								1	1								
45		TG-2			1		1			1		1	1	1					1	1			1		1	1									1								
46	*N. pumila*	WAM			1	1							1	1						1	1			1	1				1											1		
47		KUT			1							1	1	1						1	1				1				1													
48		URUa			1							1	1	1				1		1	1				1				1											1		
49		TSC			1							1	1	1						1	1				1				1													
50		BEB			1							1	1	1					1	1	1				1				1													
51		NET			1							1	1	1			1	1	1	1	1			1	1				1											1		
52		NEN			1	1						1	1	1				1		1	1			1	1				1											1		
53		HAK			1							1	1	1				1		1	1				1				1													
54		AKT			1							1	1	1				1		1	1				1				1		1											
55		KWS			1							1	1	1	1	1		1		1				1	1				1													
56		ATO			1							1	1	1						1	1				1			1	1											1		1
57	*N. pum.* var. *ozeensis*	URUb			1							1	1	1						1	1				1				1											1		
58		GM-1			1	1						1		1	1					1	1		1	1	1		1	1		1											1		
59		URUc			1							1	1	1						1	1				1				1											1		
60	*N. subintegerrima*	GI-1			1			1						1	1	1				1			1		1			1	1									1		1		
61		AI-1			1			1						1	1					1			1		1		1	1	1									1		1		
62		AI-2			1			1						1	1	1		1		1			1	1	1		1	1	1									1		1		
63		MI-1		1	1			1						1	1	1		1		1			1	1	1			1	1						1			1		1		
64		MI-3			1			1						1	1	1			1	1			1	1	1			1	1						1			1		1		
65	*N. lutea*			1	1	1						1	1	1	1		1		1			1		1	1								1	1					1	1	
66	*Barclaya longifolia*			1		1			1					1				1							1																1		

Appendix 2. (continued)

Fragment size (bp)

Genotype	129	132	135	136	137	139	140	142	143	148	151	152	154	158	159	163	164	167	168	173	174	175	177	179	180	181	182	184	187	190	191	194	195	196	197	198	200	206	209	210	212	214	216	220	223	224	226	228	230	232	234	236	237	239	240
1			1		1		1								1		1			1				1	1	1				1		1				1		1				1										
2			1		1		1								1		1			1				1	1	1				1		1				1		1				1										1
3			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
4			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
5			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
6			1		1		1								1		1			1				1	1	1						1				1		1				1				1					1	1	1
7			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
8			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
9			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
10			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
11			1		1		1								1		1			1				1	1	1						1				1		1				1				1							1
12			1		1		1								1		1			1				1	1	1						1	1			1		1				1				1							1
13			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
14			1		1		1								1		1			1				1	1	1						1				1		1				1				1							1
15			1		1		1								1		1			1				1	1	1						1				1		1				1				1							1
16			1		1		1								1		1			1				1		1		1				1				1		1				1				1						1	1
17			1		1		1								1		1			1				1		1		1				1				1		1				1				1						1	
18			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
19			1		1		1								1		1			1				1	1	1						1			1	1		1				1				1						1	1
20			1		1		1								1		1			1				1	1	1						1				1		1				1				1						1	1
21			1		1		1								1		1			1				1		1								1		1	1		1				1				1		1							1
22			1		1		1								1		1			1	1			1	1	1	1						1	1		1	1		1				1				1		1							1
23			1		1		1								1		1			1	1				1	1	1						1	1		1	1		1				1				1		1							1
24			1		1		1								1		1			1	1				1		1						1	1		1	1		1				1				1											1
25			1		1		1								1		1			1	1				1		1						1	1		1	1		1				1				1		1							1
26			1		1		1								1		1			1	1				1	1	1						1	1			1		1				1				1		1							1
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66									1	1								1		1	1							1																					1														

Appendix 2. (continued)

Fragment size (bp)

Genotype	241	242	244	245	246	247	248	249	250	251	252	253	254	255	256	258	260	261	262	263	264	266	267	268	270	271	272
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4			1													1									1		
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9			1				1									1								1	1		
10			1		1		1		1							1							1		1		
11			1				1				1		1			1		1			1	1	1		1		
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16			1		1											1		1		1			1		1		
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18			1		1		1						1			1							1		1		
19			1				1									1			1		1				1		
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27			1				1	1							1	1								1	1		
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66										1																	

Genotype	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	293	294	295	296	297	298	299	301	303
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66				1		1		1			1	1																

Appendix 2. (continued)

	Fragment size (bp)																																																						
Genotype	304	305	306	308	309	310	311	312	313	314	316	317	319	321	322	324	325	327	330	334	335	339	340	341	343	344	345	346	348	352	353	354	356	358	361	364	365	366	367	370	372	373	375	376	377	378	379	380	381	382	383	384	385	387	388	389
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66							1			1																																														

APPENDIX 3. Data matrix of absence (not shown) or presence (1) of the AFLP fragments in ACA-CTA primer combination.

Genotype	Taxa	Pop. Code	Fragment size (bp) 51	52	53	54	55	56	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	75	76	77	78	80	81	82	83	84	86	88	89	91	93	94	95	96	97	98	99
1	*N. japonica*	SAA		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
2		HOO		1	1		1			1	1		1	1	1	1	1	1				1		1							1				1		1					1	1
3		SAM	1	1	1		1				1		1	1	1	1	1	1			1			1							1				1							1	1
4		KIO		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
5		TOB		1	1		1			1	1		1	1	1	1	1	1				1		1							1				1							1	1
6		ATT		1	1		1			1	1		1	1	1	1	1	1				1		1							1				1							1	1
7		TAO		1	1		1			1	1		1	1	1	1	1	1				1									1				1							1	1
8		AO-1		1	1		1				1		1	1	1	1	1	1				1									1				1							1	1
9		AK-1	1	1	1	1					1		1	1	1	1	1	1			1				1						1				1			1				1	1
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13		FS-3		1	1		1			1	1		1	1	1	1	1	1				1		1							1				1			1				1	1
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19		NI4		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
20		IK-1		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
21		SI-3		1			1				1		1	1	1	1	1	1				1									1				1							1	1
22		MI-2		1	1		1			1	1		1	1	1	1	1	1			1										1				1							1	1
23		WA-1		1	1		1			1	1		1	1	1	1	1	1			1										1				1							1	1
24		OK-2		1			1			1	1		1	1	1	1	1	1													1				1							1	1
25		OK-4		1			1			1	1		1	1	1	1	1	1													1				1							1	1
26		HI-10		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
27		KO-4		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
28		MY-5		1			1			1	1		1	1	1	1	1	1				1									1				1							1	1
29		MY-6		1			1			1	1		1	1	1	1	1	1													1				1							1	1
30	*N. oguraensis*	GI-4		1		1	1				1		1	1		1	1	1		1		1									1				1		1						
31		HY-8		1			1			1	1	1	1	1		1	1			1					1					1	1				1		1						
32		HI-2b	1	1	1	1					1		1	1		1	1			1	1	1	1		1					1	1				1		1	1					
33		HI-7		1	1		1				1		1	1		1	1			1					1					1	1				1		1	1					
34		FO-2		1			1				1		1	1		1				1					1						1				1		1						
35		MY-2		1						1	1		1	1		1					1				1						1				1		1						
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38		Ko-2y		1			1			1	1		1	1		1				1					1					1	1				1	1		1					
39	*N. ogu.* var. *akiensis*	HHS		1		1	1				1		1	1		1				1		1	1							1	1				1		1	1					
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43	*N. shimadai*			1			1				1		1	1		1				1		1			1					1	1				1	1		1					1
44	*N. submersa*	TG-1		1	1		1			1	1		1	1		1						1						1		1				1	1		1					1
45		TG-2		1	1		1			1	1		1	1		1	1			1		1						1		1				1	1		1	1				1
46	*N. pumila*	WAM		1	1		1			1	1		1	1		1	1					1			1	1				1	1	1	1	1	1		1					1
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57	*N. pum.* var. *ozeensis*	URUb		1			1			1	1		1			1	1					1	1		1					1	1			1	1						1	1	1
58		GM-1		1			1			1	1		1		1	1						1			1					1	1			1	1								
59		URUc		1	1		1			1	1		1			1	1					1	1		1						1	1			1	1						1	1	1
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63		MI-1	1	1	1	1	1				1		1	1		1	1			1		1				1			1		1				1							1	1
64		MI-3		1			1				1		1	1		1	1					1							1		1				1						1	1	1
65	*N. lutea*		1		1	1					1		1	1	1	1	1				1	1			1	1	1	1			1				1			1			1		1	1
66	*Barclaya longifolia*							1	1				1		1			1	1	1				1	1							1			1				1				1	

Appendix 3. (continued)

	Fragment size (bp)																																																					
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Appendix 3. (continued)

	Fragment size (bp)																																																					
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61				1	1	1				1						1							1		1	1	1												1		1	1										1			
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65		1						1		1	1					1						1			1	1													1			1										1			
66							1				1			1	1			1	1	1	1		1										1										1	1											

Appendix 3. (continued)

	Fragment size (bp)																																											
Genotype	272	273	274	275	276	277	278	281	282	283	284	286	287	288	289	290	291	296	297	298	299	301	302	305	312	314	317	329	332	334	337	342	345	347	352	354	355	357	360	361	362	363	369	370
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24		1				1		1		1			1								1			1			1	1		1		1						1						1
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32		1		1				1		1			1		1				1					1					1	1		1						1					1	
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37															1									1					1	1		1						1						
38															1									1					1	1		1						1						
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44		1				1																		1					1	1		1				1		1						
45		1				1		1					1											1					1	1		1				1		1						
46						1		1					1						1		1	1		1			1		1	1		1			1		1							
47		1				1							1						1		1			1					1	1		1					1							
48						1		1					1						1		1			1					1	1		1			1		1		1					
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51		1				1							1						1		1			1					1	1		1					1							
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56						1							1						1		1			1			1		1	1		1					1	1						
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65		1																		1		1						1		1		1						1						
66							1			1		1		1		1																		1				1						

APPENDIX 4. Data matrix of absence (not shown) or presence (1) of the AFLP fragments in ACA-CTG primer combination.

Genotype	Taxa	Pop. Code	Fragment size (bp) 60	61	62	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	91	92	93	94	95	97	99	101	102	104	105	106	107
1	*N. japonica*	SAA	1	1		1			1	1		1		1		1										1	1			1		1	1	1			1					
2		HOO	1	1		1			1	1		1		1		1										1	1			1		1	1	1			1					
3		SAM	1	1	1					1		1		1		1								1	1	1	1			1		1	1	1			1					
4		KIO	1	1	1			1	1	1	1	1	1	1		1							1		1	1	1			1		1	1	1			1					
5		TOB	1	1	1				1	1	1	1	1	1		1								1	1	1	1			1		1		1			1	1				
6		ATT	1	1						1		1	1	1		1								1	1	1	1			1		1	1	1			1					
7		TAO	1	1	1					1		1		1		1								1	1	1	1			1		1	1	1			1					
8		AO-1	1	1	1					1		1	1	1		1								1	1	1	1			1		1	1	1			1					
9		AK-1	1	1	1					1		1	1	1		1								1	1	1	1			1		1	1	1			1	1				
10		YA-1	1	1	1					1		1	1	1		1								1	1	1	1			1		1	1	1			1					
11		FS-1	1	1	1					1		1	1	1		1								1	1	1	1			1		1	1	1			1					
12		FS-2	1	1						1		1	1	1		1								1	1	1	1			1		1	1	1			1					
13		FS-3	1	1						1		1	1	1		1								1	1	1	1			1		1	1	1			1					
14		IB-1		1						1		1		1		1								1	1	1	1			1		1	1	1			1					
15		IB2-	1	1						1		1		1		1								1	1	1	1			1		1	1	1			1					
16		TK-1	1	1						1		1		1		1								1	1	1	1			1		1	1	1	1		1					
17		KN-1	1	1						1		1		1		1								1	1	1	1			1		1	1	1			1					
18		NI-1	1	1	1				1	1	1			1		1							1	1		1	1			1		1	1	1			1					
19		NI4		1						1		1		1		1								1	1	1	1			1		1		1			1					
20		IK-1	1	1						1		1		1		1								1	1	1	1			1		1	1	1			1					
21		SI-3	1	1					1	1	1		1			1							1		1	1	1			1		1	1	1				1				
22		MI-2	1	1	1	1			1	1	1			1		1		1					1	1		1				1		1	1	1			1	1				
23		WA-1	1	1					1	1	1		1			1		1					1	1		1				1		1	1	1			1	1				
24		OK-2	1	1	1				1	1	1			1		1		1					1	1		1	1			1		1	1	1			1					
25		OK-4	1	1	1					1		1		1		1								1	1	1	1			1		1	1	1			1	1				
26		HI-10	1	1	1				1	1		1	1	1		1										1	1					1	1	1			1	1				
27		KO-4	1	1	1					1		1		1		1										1	1			1		1	1	1			1	1				
28		MY-5	1	1	1					1		1		1		1										1	1			1		1	1	1			1					
29		MY-6	1	1	1					1		1		1		1										1	1			1		1	1	1			1	1				
30	*N. oguraensis*	GI-4	1	1					1	1			1	1		1					1					1	1					1		1		1			1			1		
31		HY-8	1	1					1	1			1	1		1					1			1		1	1					1		1		1			1			1		
32		HI-2b	1	1					1	1			1	1		1					1		1			1			1			1		1		1			1		1			
33		HI-7	1	1					1	1		1		1		1					1			1	1	1					1		1		1			1					
34		FO-2	1	1					1	1				1		1					1				1	1				1	1		1	1	1			1			1	1	
35		MY-2	1	1					1	1				1		1					1			1	1	1					1		1		1			1			1		
36		MY-8	1	1			1		1	1				1		1					1					1	1				1		1		1			1					
37		Ko-1y	1	1						1			1	1		1										1	1			1	1	1	1	1			1			1		
38		Ko-2y	1	1						1			1	1		1								1	1	1				1	1		1	1			1			1		
39	*N. ogu.* var. *akiensis*	HHS	1		1					1	1				1		1					1			1		1			1	1		1				1			1		
40		KO-1	1	1					1	1				1		1					1				1	1				1	1		1	1	1			1			1	1	
41		KO-2	1	1					1	1				1		1					1				1	1				1	1		1	1	1			1			1	1	
42		Ko-1r	1	1						1			1	1		1								1	1	1				1	1		1	1	1			1			1		
43	*N. shimadai*		1		1						1				1		1						1			1		1			1		1	1	1			1			1		
44	*N. submersa*	TG-1	1	1					1	1		1		1	1										1	1		1			1		1		1			1					
45		TG-2	1	1					1	1		1		1		1									1	1	1				1		1		1			1					
46	*N. pumila*	WAM	1	1	1						1			1	1		1	1	1	1	1	1	1		1	1		1			1		1	1	1		1		1				
47		KUT	1	1	1					1				1		1					1				1					1	1		1	1	1	1		1	1				
48		URUa		1	1					1			1			1			1	1	1	1		1		1			1		1		1	1	1	1		1					
49		TSC	1	1	1					1				1		1								1		1				1	1		1	1	1	1		1					
50		BEB	1	1	1	1			1	1				1		1								1		1				1	1		1	1	1	1		1	1				
51		NET	1	1	1					1				1		1								1		1				1	1		1	1	1	1		1	1				
52		NEN	1	1	1					1				1		1								1		1				1	1		1	1	1	1		1					
53		HAK	1	1	1					1				1		1								1		1				1	1		1	1	1	1		1	1				
54		AKT	1	1	1	1				1				1		1								1		1				1	1		1		1	1		1					
55		KWS	1	1	1					1	1		1			1						1	1			1					1		1		1	1		1	1				
56		ATO	1	1	1					1				1		1					1	1			1	1	1	1			1		1	1	1	1		1	1				
57	*N. pum.* var. *ozeensis*	URUb		1	1					1			1			1							1		1	1	1	1	1		1		1	1	1	1		1					
58		GM-1	1	1	1					1			1			1						1	1	1		1					1	1	1		1			1					
59		URUc		1	1					1			1			1							1		1	1			1		1		1	1	1	1		1					
60	*N. subintegerrima*	GI-1		1	1					1			1	1	1										1	1	1				1		1	1	1	1							
61		AI-1	1	1	1					1		1	1	1	1									1	1	1				1		1	1	1			1					
62		AI-2		1						1	1		1	1	1	1								1		1	1	1			1	1		1	1								
63		MI-1	1	1		1			1	1	1			1		1		1								1	1	1			1	1			1			1					
64		MI-3	1	1	1		1		1	1		1		1		1			1							1	1		1		1		1		1			1					
65	*N. lutea*			1						1			1			1	1									1	1			1		1	1	1	1	1			1				
66	*Barclaya longifolia*						1						1										1		1						1					1		1			1		1

Appendix 4. (continued)

Fragment size (bp)

Genotype	108	109	110	111	112	113	114	115	116	117	118	120	121	122	124	125	126	127	128	129	130	131	132	134	135	136	137	138	141	142	143	144	145	147	150	151	152	153	156	157	158	159	161	162	163	165	166	167	169	170	172	173	175	178	179
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2			1	1	1		1			1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
3			1	1	1		1			1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
4			1	1	1		1			1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
5			1	1	1		1			1	1			1	1	1					1			1		1		1	1				1									1			1	1	1		1	1		1		1		1
6			1	1	1		1			1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
7			1	1	1		1			1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
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10			1	1			1			1	1	1		1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		
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12		1	1	1			1	1		1	1			1		1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		
13	1		1	1			1			1	1			1	1	1								1		1		1	1				1									1	1		1	1	1		1	1		1		1		
14		1	1	1			1	1		1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		
15			1	1			1	1		1	1			1		1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		
16		1	1	1			1	1		1	1			1	1	1					1			1		1		1	1				1									1	1		1		1		1	1		1		1		1
17			1	1	1		1	1		1	1			1	1	1				1				1		1		1	1				1									1	1		1	1	1		1	1		1		1		
18			1	1			1			1	1			1		1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
19			1	1	1		1			1	1			1	1	1								1		1		1	1				1									1	1		1	1	1		1	1		1		1		1
20			1	1	1		1	1		1	1			1		1						1	1	1		1		1	1				1									1	1		1	1	1		1	1		1		1	1	
21		1	1	1			1	1		1	1			1	1	1					1			1		1		1	1				1									1	1		1	1	1		1	1		1		1		
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APPENDIX 4. (continued)

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APPENDIX 4. (continued)

Fragment size (bp), 258–298

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Fragment size (bp), 299–359

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3			1								1		1						1			1	1		1		1	
4			1								1		1						1			1	1		1		1	
5			1								1		1						1			1	1		1	1	1	
6			1								1		1		1				1			1	1		1		1	
7			1								1		1		1				1			1	1		1		1	
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9			1								1		1		1				1			1	1		1	1	1	
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23			1								1		1		1			1	1			1	1		1		1	
24											1		1					1	1			1	1		1		1	
25			1								1		1		1				1			1			1			
26																						1			1			
27																						1			1			
28																						1			1			
29																						1			1			
30													1						1					1	1			
31													1						1						1		1	
32									1				1						1					1	1		1	
33									1			1	1						1					1	1		1	
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35									1			1	1						1						1		1	
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42						1							1						1					1	1		1	
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45									1			1	1					1	1					1	1		1	
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56			1	1		1							1		1				1			1		1	1		1	
57			1										1		1				1					1	1		1	
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59			1										1		1				1					1	1		1	
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62											1		1		1				1		1		1		1		1	
63											1		1		1			1	1			1	1		1		1	
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65							1			1			1					1					1		1		1	
66																												

APPENDIX 4. (continued)

Genotype	Fragment size (bp)																								
	360	361	362	372	374	378	386	387	388	389	396	397	399	410	411	416	419	425	429	431	444	449	453	454	456	460	464	465	471
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2					1																				1				
3					1																				1				
4					1						1														1				
5					1																				1				
6					1																				1				
7					1																				1				
8					1																				1				
9					1						1														1				
10					1																				1				
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12					1																				1				
13					1																				1				
14					1						1														1				
15					1						1														1				
16					1																				1				
17	1				1											1									1				
18					1																				1				
19					1																				1				
20					1																				1				
21					1						1														1				
22					1						1											1			1				
23					1						1											1			1				
24					1						1											1			1				
25					1						1											1			1				
26					1						1											1			1				
27					1						1											1			1				
28					1						1											1			1				
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31					1					1		1					1							1		1		1		
32					1					1		1													1				
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37					1							1								1					1				
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39					1				1	1		1													1				
40					1							1													1				
41					1							1													1				
42					1				1	1		1								1					1				
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46		1		1											1				1		1	1	1		1				
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64					1																	1			1			1	
65					1											1									1	1			
66	1				1																								

APPENDIX 5. Data matrix of absence (not shown) or presence (1) of the AFLP fragments in ACG-CTG primer combination.

Genotype	Taxa	Pop. Code	Fragment size (bp) 52	53	54	55	56	57	60	62	63	65	66	67	68	69	70	71	73	74	75	76	77	78	80	81	82	83	84	85	86	87	89	90	91	92	93	94	95	97	99	104	106	107
1	*N. japonica*	SAA	1				1		1	1						1	1			1		1				1	1				1	1												
2		HOO	1				1		1	1				1		1	1			1		1				1	1				1	1												
3		SAM	1				1		1	1				1		1	1			1		1				1	1				1	1												
4		KIO	1				1			1						1	1			1		1				1	1				1												
5		TOB	1				1		1	1				1		1	1			1		1				1	1				1												
6		ATT	1				1		1	1				1			1			1		1				1	1				1	1												
7		TAO	1				1		1	1						1	1			1		1				1	1				1												
8		AO-1	1				1		1	1						1	1			1		1				1	1				1												
9		AK-1	1				1		1	1				1		1	1			1		1				1	1				1												
10		YA-1	1				1		1	1						1	1			1		1				1	1				1												
11		FS-1	1				1		1	1				1		1	1			1		1				1	1				1		1										
12		FS-2	1				1		1	1				1		1	1			1		1				1	1				1	1												
13		FS-3	1				1			1				1		1	1			1		1				1	1				1												
14		IB-1	1				1		1	1				1		1	1			1		1				1	1				1												
15		IB2-	1				1			1				1		1	1			1		1				1	1				1												
16		TK-1	1				1	1						1		1	1			1		1				1	1				1												
17		KN-1	1				1		1	1				1		1	1			1		1				1	1				1	1											
18		NI-1	1				1			1				1			1			1		1				1	1		1		1												
19		NI4	1				1			1				1		1	1			1		1				1	1				1	1											
20		IK-1	1				1			1				1		1	1			1		1				1	1				1	1										1	
21		SI-3	1			1	1		1	1				1			1	1		1		1				1	1				1											
22		MI-2	1			1	1			1				1			1			1		1				1	1				1											
23		WA-1	1			1	1			1				1			1			1		1				1	1				1											
24		OK-2	1			1	1			1				1	1	1	1					1				1	1				1											
25		OK-4	1			1	1		1	1				1			1			1	1	1				1	1				1											
26		HI-10	1		1	1	1		1	1				1		1	1			1		1				1	1				1											
27		KO-4	1			1	1		1	1				1		1	1			1		1				1	1				1	1										
28		MY-5	1			1	1							1		1	1					1				1					1	1										
29		MY-6	1			1	1		1	1				1			1			1		1				1	1				1	1										
30	*N. oguraensis*	GI-4					1	1		1							1					1				1						1										1
31		HY-8				1	1		1	1							1	1				1				1						1										
32		HI-2b	1				1		1	1				1								1				1			1			1										1
33		HI-7					1	1	1	1								1				1				1						1										1
34		FO-2				1	1			1				1								1				1						1										
35		MY-2					1	1	1	1							1					1				1						1										1
36		MY-8				1	1		1	1				1								1				1						1										
37		Ko-1y					1	1	1	1												1				1						1										1
38		Ko-2y					1	1	1	1												1				1				1		1										1
39	*N. ogu.* var. *akiensis*	HHS					1	1	1	1				1			1	1				1				1						1										1
40		KO-1	1				1	1	1	1							1	1				1				1				1	1										1
41		KO-2	1				1	1	1	1							1	1				1				1				1	1	1										1
42		Ko-1r					1	1	1	1												1				1				1		1		1								1
43	*N. shimadai*						1	1		1							1									1						1										1
44	*N. submersa*	TG-1					1	1		1												1				1				1		1								1		1
45		TG-2					1	1		1												1				1				1	1	1								1		1
46	*N. pumila*	WAM	1		1		1		1	1								1				1				1				1	1	1									1	
47		KUT	1		1		1			1		1	1					1	1			1				1																
48		URUa	1	1			1			1			1	1				1	1			1				1				1	1		1									
49		TSC	1		1		1			1					1	1		1				1				1					1										1	
50		BEB	1		1		1			1		1	1					1	1			1				1					1				1							
51		NET	1		1		1			1				1	1	1		1	1			1				1					1											
52		NEN	1	1			1			1				1	1	1		1	1			1				1					1		1							1	1		
53		HAK	1		1		1			1					1	1		1	1			1				1																
54		AKT	1		1		1			1					1	1		1	1			1				1																
55		KWS	1				1			1		1						1	1			1				1																
56		ATO	1		1		1		1	1		1	1					1	1			1				1					1	1										
57	*N. pum.* var. *ozeensis*	URUb	1	1			1			1		1	1					1	1			1				1					1	1	1									
58		GM-1	1	1			1		1	1				1					1			1				1	1					1										1	
59		URUc	1		1		1			1		1	1					1	1			1				1																
60	*N. subintegerrima*	GI-1	1		1	1	1	1												1		1	1			1																
61		AI-1			1	1		1									1			1		1				1					1				1							
62		AI-2	1		1	1	1	1												1		1	1			1																
63		MI-1	1			1	1															1				1					1											
64		MI-3	1			1																1				1					1											
65	*N. lutea*				1		1	1									1		1	1		1				1			1	1					1	1						
66	*Barclaya longifolia*										1	1				1	1							1	1			1		1		1				1						

Appendix 5. (continued)

Genotype	109	110	112	115	116	117	118	120	121	122	123	124	127	129	131	132	133	134	135	136	137	141	143	148	150	151	153	157	159	161	162	163	165	168	169	170	172	173	174	175	177	179	181	182	183	186	187	188	189	191	193	194	200	201	203
	Fragment size (bp)																																																						
1				1											1									1							1							1											1			1		1	
2				1											1									1							1							1											1			1		1	
3		1		1											1									1							1							1											1			1		1	
4				1											1									1							1							1											1			1	1		
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6	1			1		1		1		1			1		1									1							1					1		1											1			1		1	
7				1											1									1							1																		1			1	1		
8	1	1		1											1									1							1																		1			1		1	
9	1	1		1											1									1							1						1												1			1		1	
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11		1		1											1									1							1																		1			1		1	
12				1		1									1									1							1							1											1			1		1	
13				1											1									1							1									1									1			1		1	
14		1		1		1									1									1							1																	1	1			1		1	
15		1		1		1									1									1							1									1								1	1			1		1	1
16				1		1									1									1							1						1	1											1	1		1		1	
17		1		1											1									1				1			1								1	1									1			1		1	
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19		1		1											1									1							1							1											1			1		1	
20		1		1											1									1							1									1									1			1	1		
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24						1									1									1							1								1	1								1		1		1		1	
25						1									1									1							1																	1	1	1		1		1	
26						1									1																1																	1	1	1		1		1	
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34									1						1			1																																	1	1	1	
35			1		1				1						1									1												1				1	1					1		1			1		1	
36									1						1			1																			1										1			1		1	
37			1		1				1						1									1												1		1		1	1					1		1			1		1	
38			1		1				1						1									1		1												1			1					1		1			1		1	
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57			1		1										1			1						1														1	1			1			1			1			1		1	
58	1		1	1	1				1						1			1						1														1							1			1			1		1	
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65					1								1		1				1					1							1								1	1						1	1			1	1			1	
66					1			1				1		1		1	1					1					1		1	1															1	1		1	1			1			

APPENDIX 5. (continued)

	Fragment size (bp)																																																					
Genotype	206	209	211	213	217	218	219	220	221	222	224	226	229	230	235	236	238	240	241	242	246	247	249	254	255	257	258	259	263	264	265	266	268	269	271	272	276	281	282	283	284	287	288	289	291	294	301	302	308	310	312	318	319	327	328
1					1					1							1			1									1				1						1		1		1										1		
2					1					1							1			1									1				1						1		1		1										1		
3					1					1			1				1			1									1				1						1		1		1										1		
4	1				1					1			1				1			1									1				1						1		1		1	1									1		
5	1				1					1							1			1									1				1						1		1		1	1									1		
6					1					1		1					1			1									1				1						1		1			1									1		
7	1				1					1							1			1													1						1		1		1										1		
8					1					1							1			1									1				1						1		1		1	1			1						1		
9					1					1							1			1									1				1						1		1		1	1									1		
10					1					1							1			1									1				1						1		1		1	1			1						1		
11					1					1							1			1													1						1		1		1	1								1			
12					1					1							1			1									1				1						1		1			1								1			
13					1					1							1			1									1				1						1		1		1										1		
14					1					1							1			1													1						1		1			1									1		
15	1				1					1							1			1									1				1						1		1			1									1		
16					1					1							1			1									1				1						1		1			1									1		
17					1					1							1			1									1	1			1						1		1											1			
18					1					1							1			1									1				1						1		1		1	1											
19					1					1							1			1									1				1	1					1		1			1								1			
20					1					1							1			1									1				1						1		1		1	1				1				1			
21					1					1							1			1					1				1				1	1					1		1			1								1			
22	1				1					1	1						1			1					1				1				1	1					1		1			1									1		
23	1				1					1	1						1			1					1				1				1	1					1		1			1									1		
24	1				1						1						1								1				1				1	1					1		1			1								1			
25					1					1	1						1			1													1	1					1		1			1									1		
26	1				1					1	1						1			1									1				1						1		1			1									1		
27					1					1	1						1			1					1				1				1	1					1		1			1								1			
28					1					1	1				1					1					1				1				1						1		1											1			
29					1					1	1						1			1					1				1				1	1					1		1			1								1			
30	1									1						1				1								1	1				1	1	1																				
31									1	1										1													1	1	1						1														
32										1							1			1								1	1				1	1	1						1														
33					1					1							1			1								1	1				1	1	1																				
34									1	1							1																1	1	1						1														
35									1	1							1			1								1					1	1	1																				
36	1								1	1							1			1														1	1						1														
37				1						1							1			1								1	1				1	1	1																				
38				1						1			1				1			1								1	1					1	1																				
39										1	1						1			1								1	1				1		1																				
40										1							1			1								1	1				1	1	1				1																
41										1							1			1								1	1				1	1	1																				
42										1							1			1								1	1				1	1	1																				
43	1									1							1			1								1	1				1		1																				
44				1						1										1								1	1					1	1						1														
45				1						1										1								1	1					1	1						1														
46		1							1	1							1			1															1					1	1	1													
47	1	1				1			1	1							1			1															1					1	1	1													
48	1	1				1			1	1							1			1															1					1	1	1													
49		1				1			1	1							1			1															1					1	1	1													
50		1				1			1	1							1			1															1					1	1	1													
51		1				1			1	1							1			1															1					1	1	1													
52		1				1			1	1							1			1															1					1	1	1													
53		1				1			1	1							1			1															1					1	1	1													
54						1			1	1							1			1															1					1	1	1													
55	1	1								1							1			1												1			1					1	1	1													
56			1		1	1			1	1							1			1													1		1					1	1	1													
57		1				1			1	1							1			1															1					1	1	1													
58		1			1	1			1	1							1			1									1			1	1		1					1	1	1		1								1			
59						1			1	1			1				1			1															1					1	1	1													
60	1				1					1	1										1				1				1				1	1		1	1		1		1	1													
61	1				1					1	1														1				1				1	1		1			1		1												1		
62	1				1					1	1										1				1				1				1	1		1	1		1		1	1													
63	1										1														1				1				1	1				1			1												1		
64	1									1	1														1				1		1		1	1							1														
65					1	1	1	1	1			1		1				1				1					1		1				1	1	1			1		1	1	1									1				1
66					1							1							1				1	1		1					1	1					1	1							1	1			1	1			1	1	

Appendix 5. (continued)

Genotype	Fragment size (bp) 329	332	335	338	354	360	369	370	378	390	391	393	394	395	397	399	402	403	406	410	412	416	418	426	427	428	431	433	436	441	444	445	446	447	448	450	453	455	457	461	464	467
1											1							1		1					1						1					1		1				
2											1							1		1					1						1					1		1				
3											1							1		1					1						1					1		1				
4											1							1		1																1		1				
5											1							1		1					1						1					1		1				
6											1							1	1	1					1						1					1		1				
7											1							1		1																1		1				
8											1							1		1					1						1					1		1				
9											1							1		1											1					1		1				
10											1							1		1											1					1		1				
11											1							1								1					1					1		1				
12											1							1		1						1					1					1		1				
13											1							1		1												1				1		1				
14											1							1																		1		1				
15											1							1		1					1						1					1		1				
16									1		1							1		1											1					1		1				
17									1		1							1		1	1	1									1					1		1				
18											1							1		1																		1				
19											1							1		1						1					1					1		1				
20											1							1		1			1			1					1					1		1				
21											1							1		1											1					1		1		1		
22											1							1		1																1		1		1		
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24											1							1		1						1					1					1		1		1		
25											1							1																		1		1		1		
26											1							1																		1		1				
27											1							1		1						1					1					1		1		1		
28											1							1		1						1					1					1		1		1		
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46	1											1									1	1																1				
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49	1										1							1		1											1							1				
50	1										1							1		1		1									1							1				
51	1										1							1		1		1									1							1				
52	1										1							1		1		1									1							1				
53	1										1							1		1											1							1				
54	1										1							1		1											1							1				
55	1										1																				1							1				
56	1										1							1													1							1				
57	1										1							1		1					1						1							1				
58	1			1							1							1		1					1						1					1		1				
59	1										1							1		1											1							1				
60	1										1							1		1											1					1		1		1		
61											1																							1			1		1			
62	1										1							1		1											1					1		1		1		
63	1						1				1									1					1						1					1		1				1
64											1							1		1																1		1				1
65	1							1				1		1			1																1		1			1			1	
66		1	1			1			1	1					1	1							1	1					1	1												

APPENDIX 6. Pairwise genetic distance (substitutions/site) among 66 AFLP genotypes.

Genotype	Taxa	Pop. Code	1	2	3	4	5	6	7	8	9	10	11
1	*N. japonica*	SAA	---										
2		HOO	0.003	---									
3		SAM	0.006	0.006	---								
4		KIO	0.006	0.007	0.006	---							
5		TOB	0.006	0.006	0.004	0.005	---						
6		ATT	0.006	0.006	0.005	0.006	0.005	---					
7		TAO	0.005	0.006	0.004	0.004	0.004	0.005	---				
8		AO-1	0.007	0.007	0.005	0.007	0.005	0.006	0.005	---			
9		AK-1	0.008	0.007	0.005	0.008	0.005	0.007	0.006	0.005	---		
10		YA-1	0.007	0.007	0.006	0.007	0.006	0.006	0.005	0.001	0.006	---	
11		FS-1	0.009	0.008	0.008	0.009	0.007	0.008	0.007	0.005	0.007	0.005	---
12		FS-2	0.009	0.008	0.008	0.009	0.007	0.007	0.008	0.006	0.008	0.006	0.005
13		FS-3	0.008	0.007	0.008	0.008	0.007	0.007	0.007	0.005	0.007	0.005	0.006
14		IB-1	0.010	0.009	0.008	0.010	0.008	0.008	0.008	0.006	0.007	0.006	0.005
15		IB2-	0.008	0.008	0.007	0.008	0.007	0.008	0.007	0.007	0.007	0.007	0.007
16		TK-1	0.011	0.010	0.009	0.010	0.008	0.009	0.009	0.009	0.010	0.009	0.010
17		KN-1	0.010	0.009	0.009	0.012	0.009	0.009	0.009	0.010	0.009	0.010	0.009
18		NI-1	0.009	0.009	0.005	0.008	0.006	0.008	0.007	0.006	0.007	0.006	0.008
19		NI4	0.008	0.008	0.007	0.008	0.007	0.007	0.008	0.007	0.009	0.007	0.008
20		IK-1	0.007	0.008	0.007	0.007	0.007	0.008	0.006	0.007	0.007	0.007	0.007
21		SI-3	0.014	0.015	0.016	0.015	0.015	0.015	0.016	0.015	0.014	0.015	0.015
22		MI-2	0.016	0.015	0.014	0.015	0.014	0.015	0.015	0.015	0.015	0.015	0.015
23		WA-1	0.015	0.014	0.014	0.014	0.013	0.013	0.014	0.014	0.013	0.014	0.014
24		OK-2	0.017	0.018	0.018	0.016	0.017	0.018	0.018	0.018	0.017	0.017	0.017
25		OK-4	0.015	0.017	0.015	0.016	0.015	0.015	0.015	0.016	0.015	0.015	0.016
26		HI-10	0.015	0.015	0.015	0.014	0.013	0.015	0.015	0.016	0.015	0.016	0.016
27		KO-4	0.015	0.016	0.015	0.016	0.015	0.015	0.015	0.016	0.015	0.015	0.016
28		MY-5	0.017	0.017	0.016	0.017	0.016	0.017	0.017	0.017	0.017	0.017	0.017
29		MY-6	0.015	0.016	0.015	0.016	0.015	0.016	0.016	0.016	0.015	0.016	0.017
30	*N. oguraensis*	GI-4	0.039	0.040	0.038	0.039	0.038	0.040	0.039	0.039	0.037	0.040	0.040
31		HY-8	0.036	0.038	0.035	0.037	0.035	0.037	0.035	0.037	0.036	0.036	0.036
32		HI-2b	0.038	0.035	0.035	0.038	0.036	0.038	0.038	0.035	0.034	0.035	0.035
33		HI-7	0.037	0.036	0.036	0.038	0.035	0.038	0.036	0.036	0.036	0.036	0.037
34		FO-2	0.040	0.041	0.039	0.040	0.039	0.042	0.039	0.040	0.039	0.039	0.039
35		MY-2	0.040	0.041	0.041	0.041	0.039	0.042	0.039	0.042	0.039	0.041	0.041
36		MY-8	0.034	0.033	0.033	0.033	0.032	0.034	0.032	0.034	0.032	0.034	0.033
37		Ko-1y	0.038	0.039	0.038	0.038	0.036	0.039	0.039	0.036	0.036	0.036	0.037
38		Ko-2y	0.040	0.039	0.039	0.040	0.039	0.040	0.040	0.038	0.038	0.038	0.038
39	*N. ogu.* var. *akiensis*	HHS	0.038	0.037	0.036	0.038	0.036	0.038	0.038	0.036	0.036	0.035	0.036
40		KO-1	0.033	0.032	0.033	0.036	0.035	0.034	0.035	0.034	0.032	0.034	0.035
41		KO-2	0.036	0.037	0.037	0.038	0.038	0.038	0.038	0.037	0.038	0.037	0.037
42		Ko-1r	0.040	0.040	0.038	0.040	0.037	0.039	0.040	0.036	0.037	0.036	0.037
43	*N. shimadai*		0.042	0.042	0.040	0.040	0.039	0.043	0.041	0.039	0.040	0.039	0.040
44	*N. submersa*	TG-1	0.050	0.036	0.036	0.037	0.034	0.037	0.037	0.037	0.035	0.037	0.037
45		TG-2	0.036	0.053	0.035	0.037	0.034	0.036	0.036	0.036	0.035	0.036	0.036
46	*N. pumila*	WAM	0.041	0.038	0.050	0.041	0.038	0.039	0.039	0.038	0.039	0.038	0.036
47		KUT	0.036	0.034	0.033	0.052	0.032	0.034	0.032	0.034	0.033	0.034	0.033
48		URUa	0.036	0.033	0.034	0.036	0.056	0.034	0.034	0.033	0.035	0.034	0.034
49		TSC	0.034	0.032	0.032	0.036	0.033	0.055	0.033	0.032	0.034	0.033	0.033
50		BEB	0.035	0.032	0.034	0.037	0.033	0.034	0.054	0.034	0.034	0.034	0.032
51		NET	0.035	0.032	0.033	0.036	0.032	0.034	0.033	0.057	0.034	0.032	0.031
52		NEN	0.034	0.032	0.032	0.035	0.031	0.033	0.032	0.031	0.056	0.031	0.030
53		HAK	0.035	0.033	0.032	0.036	0.032	0.034	0.032	0.032	0.033	0.057	0.032
54		AKT	0.038	0.037	0.037	0.039	0.035	0.038	0.036	0.036	0.037	0.036	0.052
55		KWS	0.040	0.038	0.039	0.039	0.037	0.040	0.039	0.037	0.038	0.037	0.036
56		ATO	0.031	0.029	0.030	0.033	0.029	0.030	0.030	0.030	0.031	0.030	0.029
57	*N. pum.* var. *ozeensis*	URUb	0.036	0.035	0.035	0.036	0.034	0.035	0.036	0.035	0.037	0.036	0.035
58		GM-1	0.027	0.027	0.027	0.029	0.025	0.026	0.028	0.026	0.027	0.026	0.026
59		URUc	0.037	0.034	0.033	0.036	0.033	0.034	0.034	0.034	0.034	0.034	0.033
60	*N. subintegerrima*	GI-1	0.029	0.029	0.025	0.027	0.028	0.028	0.027	0.028	0.026	0.028	0.029
61		AI-1	0.029	0.026	0.025	0.027	0.027	0.027	0.026	0.026	0.025	0.027	0.028
62		AI-2	0.032	0.031	0.028	0.030	0.030	0.030	0.030	0.029	0.029	0.030	0.030
63		MI-1	0.033	0.032	0.029	0.033	0.032	0.032	0.032	0.031	0.030	0.032	0.032
64		MI-3	0.032	0.032	0.029	0.030	0.031	0.032	0.030	0.030	0.031	0.031	0.032
65	*N. lutea*		0.047	0.043	0.042	0.046	0.044	0.042	0.045	0.043	0.041	0.042	0.043
66	*Barclaya longifolia*		0.097	0.100	0.092	0.092	0.092	0.093	0.095	0.092	0.094	0.092	0.096
Genotype			1	2	3	4	5	6	7	8	9	10	11

APPENDIX 6. (continued)

Genotype	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
12	---														
13	0.006	---													
14	0.005	0.006	---												
15	0.006	0.007	0.003	---											
16	0.009	0.009	0.008	0.007	---										
17	0.009	0.009	0.009	0.009	0.009	---									
18	0.007	0.007	0.007	0.008	0.010	0.010	---								
19	0.008	0.008	0.009	0.009	0.011	0.009	0.009	---							
20	0.007	0.008	0.008	0.007	0.010	0.008	0.008	0.007	---						
21	0.014	0.016	0.014	0.014	0.015	0.016	0.016	0.013	0.015	---					
22	0.015	0.015	0.014	0.014	0.015	0.018	0.012	0.014	0.015	0.012	---				
23	0.014	0.014	0.013	0.013	0.014	0.016	0.012	0.012	0.015	0.010	0.003	---			
24	0.017	0.018	0.017	0.015	0.018	0.018	0.016	0.015	0.017	0.012	0.010	0.008	---		
25	0.017	0.017	0.015	0.015	0.016	0.018	0.016	0.014	0.016	0.011	0.009	0.008	0.012	---	
26	0.015	0.016	0.015	0.014	0.018	0.019	0.016	0.016	0.016	0.016	0.012	0.012	0.014	0.010	---
27	0.016	0.017	0.016	0.015	0.016	0.017	0.016	0.014	0.015	0.013	0.011	0.011	0.012	0.007	0.007
28	0.017	0.018	0.017	0.016	0.017	0.018	0.017	0.015	0.016	0.015	0.013	0.012	0.013	0.008	0.008
29	0.016	0.018	0.017	0.016	0.017	0.018	0.017	0.015	0.016	0.013	0.013	0.012	0.014	0.007	0.009
30	0.040	0.040	0.040	0.040	0.044	0.041	0.039	0.038	0.040	0.039	0.039	0.038	0.041	0.040	0.039
31	0.036	0.037	0.036	0.036	0.036	0.038	0.034	0.035	0.036	0.034	0.034	0.033	0.036	0.034	0.036
32	0.035	0.037	0.035	0.036	0.038	0.039	0.033	0.037	0.038	0.036	0.035	0.036	0.039	0.038	0.039
33	0.036	0.037	0.036	0.036	0.036	0.039	0.036	0.036	0.037	0.035	0.036	0.036	0.040	0.034	0.038
34	0.040	0.041	0.039	0.039	0.041	0.041	0.038	0.040	0.040	0.039	0.040	0.041	0.041	0.038	0.041
35	0.042	0.042	0.040	0.040	0.043	0.042	0.040	0.039	0.041	0.039	0.039	0.040	0.041	0.038	0.042
36	0.033	0.034	0.033	0.032	0.037	0.035	0.033	0.031	0.034	0.029	0.030	0.030	0.031	0.029	0.031
37	0.038	0.037	0.039	0.038	0.039	0.039	0.038	0.037	0.038	0.036	0.039	0.039	0.040	0.038	0.040
38	0.038	0.040	0.039	0.039	0.040	0.040	0.039	0.039	0.040	0.036	0.040	0.039	0.040	0.038	0.041
39	0.037	0.037	0.036	0.035	0.038	0.038	0.036	0.037	0.039	0.035	0.038	0.037	0.037	0.037	0.037
40	0.034	0.036	0.035	0.034	0.037	0.035	0.034	0.035	0.034	0.034	0.036	0.035	0.038	0.034	0.039
41	0.037	0.038	0.038	0.037	0.040	0.039	0.038	0.038	0.036	0.036	0.040	0.039	0.039	0.037	0.041
42	0.038	0.038	0.039	0.039	0.039	0.039	0.037	0.037	0.039	0.037	0.038	0.038	0.040	0.039	0.040
43	0.041	0.040	0.041	0.040	0.042	0.043	0.039	0.040	0.042	0.040	0.041	0.041	0.043	0.040	0.041
44	0.036	0.037	0.037	0.036	0.038	0.038	0.036	0.034	0.037	0.037	0.038	0.037	0.040	0.038	0.038
45	0.036	0.036	0.038	0.037	0.038	0.038	0.036	0.035	0.037	0.036	0.036	0.036	0.039	0.036	0.037
46	0.039	0.038	0.038	0.039	0.040	0.037	0.038	0.040	0.040	0.042	0.040	0.038	0.043	0.041	0.041
47	0.036	0.034	0.036	0.034	0.035	0.034	0.033	0.035	0.033	0.037	0.034	0.034	0.038	0.035	0.036
48	0.036	0.034	0.037	0.035	0.037	0.035	0.033	0.035	0.035	0.037	0.036	0.035	0.039	0.040	0.037
49	0.034	0.033	0.035	0.034	0.035	0.033	0.033	0.035	0.032	0.037	0.035	0.035	0.039	0.037	0.037
50	0.035	0.033	0.036	0.035	0.035	0.033	0.033	0.035	0.034	0.038	0.034	0.034	0.039	0.037	0.037
51	0.034	0.032	0.034	0.034	0.034	0.032	0.033	0.033	0.032	0.038	0.034	0.034	0.038	0.037	0.037
52	0.033	0.031	0.033	0.034	0.033	0.032	0.031	0.032	0.033	0.038	0.034	0.034	0.038	0.038	0.038
53	0.035	0.033	0.035	0.034	0.035	0.033	0.032	0.034	0.033	0.038	0.035	0.035	0.039	0.037	0.037
54	0.038	0.036	0.039	0.038	0.039	0.037	0.037	0.037	0.036	0.041	0.037	0.038	0.041	0.040	0.040
55	0.048	0.037	0.040	0.040	0.041	0.039	0.037	0.039	0.040	0.041	0.039	0.038	0.043	0.044	0.041
56	0.031	0.059	0.031	0.032	0.032	0.032	0.030	0.031	0.030	0.032	0.031	0.031	0.037	0.031	0.033
57	0.038	0.036	0.048	0.037	0.038	0.036	0.036	0.036	0.035	0.037	0.038	0.037	0.040	0.038	0.038
58	0.028	0.028	0.030	0.060	0.032	0.030	0.027	0.027	0.028	0.030	0.030	0.029	0.032	0.032	0.033
59	0.036	0.034	0.037	0.036	0.051	0.035	0.034	0.035	0.035	0.036	0.036	0.035	0.040	0.038	0.036
60	0.029	0.029	0.029	0.028	0.028	0.060	0.027	0.028	0.028	0.024	0.022	0.022	0.025	0.026	0.029
61	0.030	0.027	0.029	0.029	0.029	0.032	0.065	0.027	0.029	0.026	0.023	0.022	0.025	0.026	0.026
62	0.031	0.030	0.031	0.031	0.031	0.034	0.028	0.061	0.030	0.026	0.023	0.023	0.027	0.028	0.030
63	0.031	0.032	0.032	0.031	0.034	0.034	0.031	0.031	0.054	0.027	0.021	0.022	0.025	0.027	0.030
64	0.031	0.031	0.033	0.032	0.033	0.034	0.030	0.027	0.032	0.069	0.022	0.023	0.026	0.025	0.028
65	0.041	0.041	0.044	0.045	0.044	0.045	0.042	0.044	0.046	0.046	0.042	0.043	0.047	0.047	0.046
66	0.098	0.095	0.093	0.096	0.093	0.098	0.094	0.095	0.098	0.094	0.096	0.016	0.098	0.094	0.097
Genotype	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26

APPENDIX 6. (continued)

Genotype	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
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28	0.003	---													
29	0.004	0.006	---												
30	0.039	0.040	0.040	---											
31	0.036	0.037	0.037	0.019	---										
32	0.038	0.041	0.038	0.019	0.019	---									
33	0.037	0.039	0.035	0.019	0.016	0.015	---								
34	0.040	0.042	0.038	0.022	0.018	0.021	0.017	---							
35	0.041	0.043	0.042	0.017	0.014	0.018	0.013	0.017	---						
36	0.031	0.033	0.031	0.021	0.016	0.022	0.015	0.018	0.017	---					
37	0.038	0.041	0.038	0.021	0.021	0.021	0.017	0.019	0.016	0.020	---				
38	0.039	0.041	0.037	0.021	0.022	0.021	0.017	0.019	0.017	0.020	0.006	---			
39	0.036	0.036	0.035	0.021	0.023	0.018	0.019	0.022	0.019	0.023	0.021	0.021	---		
40	0.036	0.038	0.036	0.018	0.022	0.017	0.018	0.021	0.019	0.023	0.019	0.018	0.016	---	
41	0.038	0.040	0.037	0.018	0.020	0.021	0.016	0.019	0.015	0.021	0.016	0.016	0.019	0.009	---
42	0.038	0.041	0.039	0.022	0.022	0.021	0.020	0.022	0.019	0.022	0.007	0.009	0.022	0.020	0.017
43	0.040	0.041	0.039	0.022	0.025	0.023	0.022	0.024	0.023	0.026	0.016	0.018	0.019	0.022	0.021
44	0.038	0.039	0.039	0.026	0.027	0.029	0.023	0.030	0.023	0.027	0.020	0.019	0.027	0.027	0.026
45	0.037	0.037	0.037	0.026	0.028	0.028	0.024	0.032	0.025	0.029	0.024	0.023	0.028	0.026	0.027
46	0.041	0.042	0.042	0.042	0.042	0.045	0.043	0.048	0.045	0.042	0.041	0.041	0.040	0.038	0.042
47	0.036	0.036	0.037	0.037	0.035	0.040	0.038	0.040	0.039	0.034	0.036	0.036	0.042	0.038	0.038
48	0.037	0.036	0.038	0.038	0.038	0.039	0.042	0.045	0.042	0.038	0.040	0.039	0.043	0.037	0.040
49	0.035	0.035	0.037	0.037	0.034	0.039	0.038	0.041	0.039	0.034	0.036	0.035	0.040	0.036	0.037
50	0.036	0.037	0.038	0.040	0.036	0.039	0.040	0.042	0.042	0.036	0.038	0.039	0.042	0.037	0.040
51	0.036	0.036	0.037	0.039	0.036	0.039	0.040	0.042	0.041	0.036	0.038	0.038	0.041	0.038	0.039
52	0.036	0.036	0.037	0.039	0.036	0.037	0.040	0.041	0.041	0.037	0.038	0.038	0.040	0.036	0.039
53	0.035	0.036	0.037	0.038	0.035	0.038	0.040	0.041	0.041	0.037	0.038	0.037	0.042	0.037	0.039
54	0.038	0.038	0.040	0.039	0.037	0.041	0.041	0.044	0.041	0.038	0.039	0.038	0.044	0.040	0.040
55	0.041	0.041	0.043	0.038	0.039	0.037	0.042	0.045	0.042	0.038	0.040	0.041	0.039	0.038	0.042
56	0.032	0.032	0.033	0.037	0.035	0.037	0.036	0.041	0.038	0.035	0.036	0.035	0.039	0.033	0.036
57	0.037	0.036	0.037	0.040	0.037	0.043	0.041	0.044	0.041	0.037	0.038	0.037	0.043	0.040	0.039
58	0.032	0.032	0.030	0.040	0.036	0.040	0.035	0.042	0.038	0.032	0.036	0.035	0.041	0.037	0.038
59	0.036	0.036	0.038	0.038	0.037	0.039	0.040	0.043	0.041	0.036	0.038	0.037	0.042	0.038	0.040
60	0.026	0.026	0.028	0.041	0.041	0.038	0.041	0.043	0.046	0.037	0.042	0.042	0.040	0.039	0.043
61	0.027	0.026	0.029	0.041	0.039	0.040	0.038	0.042	0.044	0.037	0.041	0.042	0.038	0.038	0.042
62	0.028	0.027	0.031	0.041	0.041	0.037	0.042	0.044	0.045	0.039	0.043	0.044	0.040	0.039	0.042
63	0.029	0.027	0.031	0.039	0.040	0.040	0.043	0.045	0.046	0.036	0.047	0.046	0.042	0.041	0.045
64	0.026	0.025	0.027	0.037	0.036	0.040	0.038	0.042	0.041	0.032	0.042	0.041	0.040	0.039	0.041
65	0.047	0.048	0.049	0.052	0.053	0.052	0.053	0.057	0.056	0.049	0.054	0.055	0.052	0.051	0.057
66	0.096	0.095	0.098	0.105	0.106	0.109	0.110	0.112	0.114	0.107	0.110	0.109	0.110	0.107	0.110
Genotype	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41

APPENDIX 6. (continued)

Genotype	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
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42	---														
43	0.016	---													
44	0.022	0.024	---												
45	0.024	0.028	0.007	---											
46	0.040	0.041	0.038	0.038	---										
47	0.035	0.039	0.032	0.032	0.021	---									
48	0.038	0.042	0.036	0.034	0.020	0.009	---								
49	0.034	0.039	0.032	0.031	0.019	0.004	0.010	---							
50	0.036	0.041	0.035	0.033	0.020	0.006	0.010	0.007	---						
51	0.037	0.041	0.033	0.032	0.020	0.003	0.009	0.004	0.005	---					
52	0.036	0.040	0.034	0.032	0.020	0.005	0.008	0.005	0.007	0.003	---				
53	0.037	0.042	0.033	0.033	0.021	0.003	0.008	0.004	0.006	0.004	0.004	---			
54	0.038	0.042	0.035	0.034	0.023	0.005	0.011	0.005	0.008	0.005	0.006	0.005	---		
55	0.040	0.039	0.038	0.037	0.026	0.016	0.016	0.018	0.017	0.017	0.016	0.015	0.018	---	
56	0.034	0.040	0.033	0.032	0.017	0.009	0.012	0.010	0.011	0.009	0.010	0.010	0.011	0.019	---
57	0.036	0.040	0.034	0.034	0.022	0.009	0.005	0.011	0.012	0.010	0.010	0.011	0.012	0.018	0.011
58	0.038	0.041	0.036	0.034	0.028	0.018	0.018	0.019	0.020	0.018	0.018	0.020	0.020	0.024	0.018
59	0.036	0.040	0.034	0.033	0.021	0.007	0.004	0.008	0.010	0.008	0.009	0.008	0.009	0.015	0.010
60	0.042	0.041	0.037	0.038	0.044	0.038	0.037	0.037	0.039	0.039	0.039	0.038	0.040	0.039	0.036
61	0.041	0.042	0.038	0.038	0.041	0.038	0.037	0.038	0.038	0.039	0.039	0.038	0.042	0.041	0.037
62	0.042	0.042	0.044	0.043	0.044	0.040	0.038	0.039	0.040	0.039	0.039	0.040	0.041	0.039	0.038
63	0.046	0.045	0.042	0.041	0.042	0.042	0.038	0.041	0.041	0.042	0.040	0.041	0.042	0.040	0.039
64	0.042	0.042	0.036	0.037	0.041	0.038	0.037	0.038	0.038	0.038	0.037	0.038	0.040	0.039	0.036
65	0.052	0.052	0.050	0.048	0.049	0.047	0.049	0.048	0.048	0.047	0.049	0.048	0.051	0.047	0.047
66	0.109	0.111	0.114	0.112	0.117	0.109	0.105	0.110	0.114	0.107	0.106	0.106	0.105	0.105	0.099
Genotype	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56

APPENDIX 6. (continued)

Genotype	57	58	59	60	61	62	63	64	65	66
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58	0.016	---								
59	0.003	0.017	---							
60	0.039	0.037	0.037	---						
61	0.040	0.037	0.037	0.012	---					
62	0.040	0.038	0.037	0.012	0.015	---				
63	0.041	0.041	0.040	0.023	0.025	0.022	---			
64	0.038	0.038	0.037	0.022	0.022	0.024	0.011	---		
65	0.049	0.049	0.046	0.044	0.041	0.046	0.043	0.047	---	
66	0.101	0.104	0.105	0.096	0.094	0.098	0.098	0.104	0.102	---
Genotype	57	58	59	60	61	62	63	64	65	66