

Morphological and phylogenetic analyses reveal the taxonomic distinctiveness between *Nymphaea pygmaea* and *N. tetragona* (Nymphaeaceae)

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Abstract

In this study, we aimed to clarify the taxonomic identity between *Nymphaea tetragona*, which has a red to purple stigmatic disk and the widest distribution range among the genus, and *Nymphaea pygmaea*, which has a yellow stigmatic disk and is ordinarily treated as a synonym of *N. tetragona*, based on detailed morphological measurements and phylogenetic analyses. Through morphological analysis, we assessed 26 quantitative characters of 111 individuals from 15 populations. Seventeen characters were significantly different between the two species; however, these characters overlapped. Principal component analysis based on 13 morphological characters revealed different trends in the morphology of the two species. In DNA analysis, one nuclear ribosomal DNA region, internal transcribed spacer (ITS) region, and one chloroplast DNA region (*rps4-trnF*) were compared. The ITS region showed differences in 18–19 substitutions and two indels between *N. pygmaea* and *N. tetragona*. Additionally, the two species were closely related within the subgenus *Nymphaea*, and *N. tetragona* and *Nymphaea leibergii* formed a robust monophyletic group with *N. pygmaea* as a sister taxon. These results indicate that *N. tetragona* and *N. pygmaea* have differentiated morphologically and genetically, thus making these two species taxonomically distinct.

Introduction

Nymphaea L., commonly known as water lily, is a perennial floating-leaved plant distributed across all continents except Antarctica (Wiersema 1988). Among the six genera of Nymphaeaceae L., comprising approximately 60 species, *Nymphaea* is the largest genus, with 35–40 recognized species (Les 2017; Wiersema 1988). The genus is further divided into five subgenera: *Anecphyra* (Casp.) Conard, *Brachyceras* (Casp.) Conard, *Hydrocallis* (Planch.) Conard, *Lotos* (DC.) Conard, and *Nymphaea* (Les 2017). The subgenus *Nymphaea* consists of seven species, namely *Nymphaea alba* L., *Nymphaea candida* J.Presl & C.Presl, *Nymphaea leibergii* Morong, *Nymphaea loriana* Wiersema, Hellquist & Borsch, *Nymphaea mexicana* Zucc., *Nymphaea odorata* Aiton, and *Nymphaea tetragona* Georgi (Borsch et al. 2014). These species exhibit cold tolerance and are distributed across subtropical to arctic zones (Slocum 2005).

N. tetragona has the widest distribution within the genus (Wiersema 1988). Its key morphological characters include white petals and a tetragon flower bed receptacle (Georgi 1775). This species is known for its wide morphological variation (Borsch et al. 2014; Volkova and Shipunov 2007; Wiersema 1996), with several recognized infraspecific taxa, such as *N. tetragona* var. *angusta* Caspary, var. *erythrostigmatica* Koji Ito, and var. *lata* Caspary. Several taxa are recognized as synonyms, including *N. crassifolia* (Hand.-Mazz.) Nakai (Lee 1996), *Nymphaea fennica* Mela (Wiersema 1996), and *Nymphaea pygmaea* (Salisb.) W.T.Aiton (Caspary 1866; Fu et al. 2001; Lee 1996; Wiersema 1996). Among these, the taxonomic status of *N. pygmaea* is often debated in relation to *N. tetragona*, considering their morphological and flower color differences, distributional ranges, and phylogenetic relationships (Borsch et al. 2014; Dkhar 2010; Fu et al. 2001; Naito and Shiga 2024; Wiersema 1996).

N. pygmaea was described by Richard Anthony Salisbury (1761–1829) as *Castalia pygmaea* Salisb. based on its small leaves (Salisbury 1805–1808). The plants considered as type materials were collected by William Kerr in China and introduced in 1805 from the ship Winchelsea, commanded by Capt. Campbell, under the auspices of the East India Company (Wiersema, 1996). Salisbury (1805–1808) noted that the transplanted plants had pygmy leaves, white petals, and slightly tetragon receptacles. The notable character of *N. pygmaea* is its yellow stigmatic disk and pollen sac (Salisbury 1805–1808), in contrast to *N. tetragona* in North America and Russia, which has red stigmatic disks (Borsch et al. 2014; Wiersema 1996). Subgenus *Nymphaea* comprises three sections: sect. *Chamaenymphaea* (Planch.) Wiersema, sect. *Nymphaea*, and sect. *Xanthantha* (Casp.) Wiersema. *N. pygmaea* serves as the type species for sect. *Chamaenymphaea*, including *N. tetragona* (Planchon 1853; Wiersema 1996). Caspary (1866) treated *N. pygmaea* as a synonym of *N. tetragona* without a detailed description. Subsequently, many taxonomists have followed this treatment (Lee 1996; Wiersema 1996). Fu et al. (2001) also treated *N. pygmaea* as a synonym of *N. tetragona* but noted differences in leaf thickness and distributional ranges. *N. pygmaea* has thicker leaves with impressed veins on the abaxial side, whereas *N. tetragona* has thinner leaves with raised veins on the abaxial side (Fu et al. 2001). Dakhr et al. (2010) performed a molecular phylogenetic analysis of Indian *Nymphaea* species, including *N. tetragona*. The study suggested that the *Nymphaea* specimen with a yellow-colored stigmatic disk is *N. tetragona*, and internal transcribed spacer (ITS) sequences revealed a close association with specimens from China rather than from Russia, although the location of the specimen in China remains unknown. *N. pygmaea* is distributed in China, Japan, and Vietnam, whereas *N. tetragona* occurs at latitudes above 42°N–50°N (Fu et al. 2001; Naito and Shiga 2024). Based on these results, Dakhr et al. (2010) suspected that the specimen could be *N. pygmaea*. Borsch et al. (2014) demonstrated that *N. pygmaea* and *N. tetragona* were phylogenetically distinct based on the molecular phylogenetic analysis of subgen. *Nymphaea*. This study relied solely on one cultivated *N. pygmaea* plant of unknown origin and performed no morphological examination. Thus, the previous studies may not be sufficient to clarify their taxonomic identities.

In Japan, Ito (1968) classified plants with a yellow stigmatic disk as *N. tetragona* var. *tetragona* and described those with red to purple color as *N. tetragona* var. *erythrostigmatica*. These two varieties are widely recognized among *Nymphaea* species distributed in Japan (Kadono 1994, 2014; Shiga 2015). However, outside of Japan, particularly in Europe, North America, and Russia, only *N. tetragona* var. *tetragona* is recognized, without distinguishing infraspecific taxa based on stigmatic disk color (Volkova and Shipunov 2007; Wiersema 1996). Therefore, taxonomic classifications of *N. tetragona*, its infraspecific taxa, and its close relatives may be inconsistent, although detailed examination has not been conducted.

In the present study, we aimed to clarify the taxonomic relationship between *N. pygmaea* and *N. tetragona* by comparing their quantitative morphological traits and conducting phylogenetic analyses based on nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) sequences. We used samples collected from various localities representing the known distribution areas of native *Nymphaea* species in Japan and Korea.

Materials and methods

Plant materials

Nymphaea plants with yellow and red stigmatic disks were defined as *N. pygmaea* and *N. tetragona*, respectively (Fig. 1). Sampling was conducted from June to early October in 2019 and 2020 to cover the known distribution areas of native *Nymphaea* species in Japan and Korea (Kadono 1994, 2014; Lee 1996). *N. pygmaea* was collected from 34 populations spanning from Kyushu to Hokkaido, Japan, and one population in Korea. *N. tetragona* was obtained from six populations in Hokkaido, Japan (Fig. 2 and Table 1).

Table 1

Population codes, coordinates, numbers of samples for morphological analyses (MA), ribo- and haplotypes, accession numbers of nrDNA (internal transcribed spacer [ITS] region) and cpDNA (*rps4-trnF* region) sequences, and voucher information of 40 *Nymphaea* populations in Japan and Korea. One sample for each population was tested for DNA sequencing analysis. All voucher specimens were deposited to The Niigata University Herbarium (NGU).

Pop. Code	Sampling locality	Coordinates		Sequence type		Accession no.		MA	Voucher
		Latitude	Longitude	ITS	<i>rps4-trnF</i>	ITS	<i>rps4-trnF</i>		
Nymphaea pygmaea									
NP1	Ebino-shi, Miyazaki, Japan	32.05169	130.93358	nrN1	cpN1	LC775151	LC775191	10	NGU 11657
NP2	Sukumo-shi, Kochi, Japan	32.94522	132.78111	nrN1	cpN1	LC775152	LC775192	0	-
NP3	Ureshino-shi, Saga, Japan	33.06744	129.97645	nrN2	cpN1	LC775153	LC775193	10	-
NP4	Arita-cho, Saga, Japan	33.22014	129.87035	nrN2	cpN1	LC775154	LC775194	0	NGU 11655
NP5	Karatsu-shi, Saga, Japan	33.40537	130.10744	nrN1	cpN1	LC775155	LC775195	0	-
NP6	Kama-shi, Fukuoka, Japan	33.54369	130.75550	nrN1	cpN1	LC775156	LC775196	0	-
NP7	Chikujou-machi, Fukuoka, Japan	33.63237	131.04673	nrN1	cpN1	LC775157	LC775197	0	-
NP8	Kitakyushu-shi, Fukuoka, Japan	33.78669	130.87847	nrN1	cpN1	LC775158	LC775198	0	-
NP9	Imabari-shi, Ehime, Japan	33.99761	133.00780	nrN1	cpN1	LC775159	LC775199	0	NGU 11650
NP10	Nara-shi, Nara, Japan	34.60900	135.97352	nrN1	cpN1	LC775160	LC775200	0	-
NP11	Ono-shi, Hyogo, Japan	34.87907	135.00076	nrN1	cpN1	LC775161	LC775201	10	NGU 8301
NP12	Iinan-cho, Shimane, Japan	35.01391	132.70516	nrN1	cpN2	LC775162	LC775202	0	NGU 8731
NP13	Taga-cho, Shiga, Japan	35.19983	136.30996	nrN1	cpN1	LC775163	LC775203	3	NGU 11317
NP14	Nasukarasuyama-shi, Tochigi, Japan	36.65837	140.11440	nrN1	cpN1	LC775164	LC775204	6	NGU 12378
NP15	Itoigawa-shi, Niigata, Japan	36.91476	137.90033	nrN1	cpN1	LC775165	LC775205	0	NGU 15565
NP16	Katashina-mura, Gunma, Japan	36.92194	139.21053	nrN1	cpN1	LC775166	LC775206	0	NGU 12948
NP17	Katashina-mura, Gunma, Japan	36.92575	139.22365	nrN1	cpN1	LC775167	LC775207	0	-
NP18	Katashina-mura, Gunma, Japan	36.92834	139.22829	nrN1	cpN1	LC775168	LC775208	0	NGU 12954
NP19	Katashina-mura, Gunma, Japan	36.93177	139.23758	nrN1	cpN1	LC775169	LC775209	0	NGU 12952
NP20	Katashina-mura, Gunma, Japan	36.93417	139.22410	nrN1	cpN1	LC775170	LC775210	0	T. Shiga 11434
NP21	Katashina-mura, Gunma, Japan	36.94060	139.22847	nrN1	cpN1	LC775171	LC775211	0	NGU 12950
NP22	Tsunan-machi, Niigata, Japan	36.97462	138.59713	nrN1	cpN1	LC775172	LC775212	5	NGU 11675
NP23	Itoigawa-shi, Niigata, Japan	36.97462	138.59713	nrN1	cpN1	LC775173	LC775213	0	-
NP24	Inawashiro-machi, Fukushima, Japan	37.51094	140.11169	nrN1	cpN1	LC775174	LC775214	0	NGU 11676
NP25	Goseong-gun, Gangwon-do, South Korea	38.25355	128.55626	nrN1	cpN1	LC775175	LC775215	6	NGU 8679
NP26	Murakami-shi, Yamagata, Japan	38.54518	140.36656	nrN1	cpN1	LC775176	LC775216	0	NGU 11672
NP27	Kami-machi, Miyagi, Japan	38.55432	140.68026	nrN1	cpN1	LC775177	LC775217	0	NGU 11659
NP28	Hanamaki-shi, Iwate, Japan	39.41820	141.18929	nrN1	cpN1	LC775178	LC775218	0	NGU

Pop. Code	Sampling locality	Coordinates		Sequence type		Accession no.		MA	Voucher
		Latitude	Longitude	ITS	<i>rps4-trnF</i>	ITS	<i>rps4-trnF</i>		
									11681
NP29	Akita-shi, Akita, Japan	39.82303	140.08411	nrN1	cpN1	LC775179	LC775219	10	NGU 11155
NP30	Tsugaru-shi, Aomori, Japan	40.81180	140.27159	nrN1	cpN1	LC775180	LC775220	0	NGU 4952
NP31	Tsugaru-shi, Aomori, Japan	40.84641	140.30048	nrN1	cpN1	LC775181	LC775221	0	NGU 11670
NP32	Higashidori-mura, Aomori, Japan	41.29451	141.40436	nrN1	cpN1	LC775182	LC775222	8	NGU 11664
NP33	Atuma-cho, Hokkaido, Japan	42.67701	141.83335	nrN1	cpN1	LC775183	LC775223	0	NGU 11648
NP34	Ishikari-shi, Hokkaido, Japan	43.24029	141.34339	nrN1	cpN1	LC775184	LC775224	10	NGU 11678
<i>N. tetragona</i>									
NT1	Taiki-cho, Hokkaido, Japan	42.55410	143.47979	nrN3	cpN4	LC775185	LC775225	0	NGU 11700
NT2	Shibecha-cho, Hokkaido, Japan	43.14960	144.55105	nrN3	cpN4	LC775186	LC775226	6	NGU 11692
NT3	Nemuro-shi, Hokkaido, Japan	43.32029	145.61725	nrN3	cpN4	LC775187	LC775227	5	NGU 11712
NT4	Uryuu-cho, Hokkaido, Japan	43.69968	141.60428	nrN4	cpN3	LC775188	LC775228	2	NGU 11688
NT5	Kamikawa-cho, Hokkaido, Japan	43.93252	142.96418	nrN3	cpN3	LC775189	LC775229	10	NGU 11701
NT6	Sarufutsu-mura, Hokkaido, Japan	45.23455	142.21441	nrN3	cpN3	LC775190	LC775230	10	NGU 11694
Total								111	

Morphological analyses

For morphological analysis, leaves and flowers fixed in 70% ethanol (EtOH, v/v) from 3 to 10 individuals were sampled from 10 populations of *N. pygmaea* and five populations of *N. tetragona*, resulting in a total of 111 individuals from 15 populations being measured. We assessed 26 morphological characters, comprising 11 leaf characters and 15 flower characters, which have been previously considered important for the identification of subgenus *Nymphaea* (Salisbury 1805–1808; Wiersema 1996; Table 2, Fig. S1). Characters L1–L6 were measured using ImageJ 1.53e (Schneider 2012) based on scanned images. Other characters were measured using a micrometer (L7 and L8), caliper (F1), and scale loupe (F2, F3, F5, F6, F8, F9, F10, F11, and F12).

Table 2

Twenty-six morphological characters of *Nymphaea pygmaea* and *Nymphaea tetragona* investigated in this present study. Wilcoxon test (L1, L2, L3, L4, L5, L6, L11, F1, F3, F4, F7, F8, F9, and F13), Student's t-test (L8, L9, L10, F2, F5, F6, F10, F11, F12, F14, and F15), and Welch's t-test (L7) were subjected. Schematic images of the characters are shown in Fig. S2

Characters	<i>Nymphaea pygmaea</i> (n = 78)	<i>N. tetragona</i> (n = 33)	p-value ²	PCA ³
Leaf				
L1: Leaf length (cm)	12.61 ± 4.05 (5.54 – 23.61)	11.48 ± 3.55 (5.95 – 18.06)	ns	
L2: Leaf width (cm)	10.55 ± 3.38 (4.53 – 21.07)	8.77 ± 2.96 (4.26 – 14.78)	**	
L3: Leaf length from apex to base (cm)	6.83 ± 2.30 (2.92 – 12.61)	6.20 ± 2.04 (2.86 – 9.89)	***	+
L4: L1-L3 (cm)	5.78 ± 1.80 (2.47 – 11.01)	5.29 ± 1.55 (3.09 – 8.61)	ns	
L5: Distance between lobe apices (cm)	3.68 ± 1.42 (1.53 – 8.36)	2.17 ± 1.29 (0.69 – 5.03)	ns	+
L6: Overlapping length of lobes (cm)	-0.40 ± 0.58 (- 2.32 – 1.00)	-0.07 ± 0.52 (- 1.06 – 1.50)	**	
L7: Leaf thickness excluding leaf vein (mm)	0.337 ± 0.080 (0.148–0.560)	0.285 ± 0.057 (0.178 – 0.410)	***	+
L8: Leaf thickness including leaf vein (mm)	0.421 ± 0.107 (0.158 – 0.700)	0.410 ± 0.117 (0.220 – 0.672)	ns	+
L9 ¹ : L2/L1	0.838 ± 0.044 (0.747 – 0.963)	0.757 ± 0.047 (0.648 – 0.853)	***	+
L10: L5/L2	0.352 ± 0.088 (0.127 – 0.611)	0.239 ± 0.099 (0.073 – 0.514)	***	+
L11: L3-L4	1.05 ± 0.73 (- 0.36 – 3.21)	0.91 ± 0.74 (- 0.41 – 2.79)	ns	+
Flower				
F1: Diagonal length of receptacle (cm)	11.49 ± 3.52 (4.80 – 24.55)	13.02 ± 3.43 (7.60 – 20.70)	*	
F2: Sepal length (mm)	32.6 ± 6.7 (16.8 – 55.5)	25.7 ± 5.2 (15.7 – 34.3)	***	+
F3: Sepal width (mm)	15.2 ± 5.9 (4.3 – 40.1)	15.4 ± 4.0 (8.6 – 23.5)	***	+
F4: Petal number	11.1 ± 2.6 (5 – 17)	16.4 ± 2.4 (12 – 21)	***	+
F5: Petal length (mm)	29.4 ± 6.2 (15.0 – 46.3)	23.6 ± 4.7 (15.0 – 32.6)	***	
F6: Petal width (mm)	10.9 ± 3.4 (3.8 – 21.1)	11.1 ± 2.9 (6.8 – 16.7)	ns	
¹ These characters underwent arcsine transformation				
² p-value are from statistical tests for each character (ns, p ≥ 0.05; *, < 0.05; **, < 0.01; ***, < 0.001)				
³ “+” indicates that they were used for principal component analysis				

Characters	<i>Nymphaea pygmaea</i> (n = 78)	<i>N. tetragona</i> (n = 33)	p-value ²	PCA ³
F7: Stamen number	40.4 ± 9.3 (24 – 71)	61.6 ± 22.8 (32 – 116)	***	+
F8: Stamen length (mm)	11.1 ± 2.0 (7.2 – 17.6)	8.9 ± 1.9 (6.2 – 12.7)	***	
F9: Anther length (mm)	5.1 ± 1.2 (3.1 – 7.6)	3.6 ± 0.9 (1.8 – 5.4)	***	+
F10: Maxmam filament width (mm)	2.2 ± 0.6 (1.1 – 3.7)	2.2 ± 0.4 (1.5 – 2.9)	ns	
F11: Length from maxmam filament width position to apex (mm)	7.4 ± 1.5 (4.8 – 10.8)	5.5 ± 1.2 (3.6 – 7.5)	***	
F12: Carpellary appendage length (mm)	5.1 ± 5.6 (1.5 – 4.2)	4.8 ± 1.1 (3.2 – 7.5)	ns	
F13 ¹ : F3/F2	0.46 ± 0.11 (0.1 – 0.7)	0.60 ± 0.08 (0.4 – 0.8)	***	+
F14: F9/F8	0.46 ± 0.05 (0.34 – 0.59)	0.40 ± 0.05 (0.28 – 0.49)	***	
F15: F1×F1/2	72.1 ± 47.0 (11.5 – 301.4)	90.5 ± 47.9 (28.9 – 214.2)	ns	
¹ These characters underwent arcsine transformation				
² p-value are from statistical tests for each character (ns, p ≥ 0.05; *, < 0.05; **, < 0.01; ***, < 0.001)				
³ “+” indicates that they were used for principal component analysis				

The following statistical analyses were conducted to compare morphological characters between the two *Nymphaea* groups: Wilcoxon test for L1, L2, L3, L4, L5, L6, L11, F1, F3, F4, F7, F8, F9, and F13; Student's t-test for L8, L9, L10, F2, F5, F6, F10, F11, F12, F14, and F15; and Welch's t-test for L7. Additionally, a preliminary principal component analysis (PCA) was conducted using 25 characters to select the morphological characters for the main PCA. When a character exhibited a high correlation ($r > 0.8$) with other characters, the one with the highest loading on the first three principal components was chosen. Characters with a low contribution (< 0.4) to the first three components were excluded. Consequently, 13 characters were retained for PCA (see Table 2). These statistical analyses were performed using JMP v.11.2.0 (SAS Institute Inc., Cary, North Carolina, USA).

DNA analyses

For DNA analyses, 40 individuals from 40 populations were used. Total genomic DNA was extracted from 5 × 5 mm sections of silica gel–dried leaves, taken from one individual in each population, using the cetyltrimethylammonium bromide method (Milligan 1992). One nrDNA region (ITS region: ITS1, 5.8S rRNA, and ITS2) and one cpDNA region (*rps4-trnF* region: *rps4-trnT* spacer, *trnT*, *trnT-trnL* spacer, *trnL* 5′-exon, *trnL* intron, *trnL* 3′-exon, and *trnL-trnF* spacer) were amplified via PCR. Amplification primers for these regions were as follows: ITS5 and ITS4 (ITS region; White et al. 1990), *trnL* (USS) 5′ exonC and *trnF* (GAA) (Taberlet et al. 1991), *rps4*-5R (Sauquet et al. 2003), *trnL*110R (Borsch et al. 2003), and new primers (Nt_rps4-trnF_F1: CTTCTTCTTATAACTGGCAAGG, Nt_rps4-trnF_R1: ATTGAGAACACAAGACTTAGCT, and Nt_rps4-trnF_F2: CTTAACGCCTATGTAGTTACTA) designed using Primer3Plus (Untergasser et al. 2007) for the *rps4-trnF* region (Table S1). Nt_rps4-trnF_F2 was used for direct sequencing.

PCR was performed using a TaKaRa PCR Thermal Cycler TP650 Standard (TaKaRa Bio Inc., Tokyo, Japan) and an EmeraldAmp™ PCR Master Mix Kit (Takara Bio Inc., Tokyo, Japan). The PCR profile was as follows: one cycle at 94°C for 4 min (preliminary denaturation), followed by 35 cycles at 94°C for 1 min (denaturation), 53°C (ITS) and 52°C (*rps4-trnF*) for 1 min (annealing), 72°C for 2 min (extension), and a final step at 72°C for 7 min (final extension). PCR products were purified using the exoSAP method (Dugan et al. 2002). Purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, USA) with the same primers on an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster, USA). Some samples were outsourced to Eurofin Genomics Co., Ltd. (Tokyo, Japan) DNA Sequence Service for sequencing. Chromatograms of nucleotide sequences were edited and assembled using Geneious software version 10.0.8 (Biomatters, Auckland, New Zealand). The resulting sequences were deposited in the DDBJ/GenBank/EMBL-Bank database (Table 1).

To reveal the genetic relationships among *N. pygmaea*, *N. tetragona*, and closely related species, their haplotypes or genotypes were compared. In addition to the sequences determined in this study, 21 sequences of 12 *Nymphaea* taxa in the ITS and *rps4-trnF* regions were obtained from Borsch et al. (2014), Dakhr et al. (2010), and Woods et al. (2007) (Table S2). *N. tetragona* sequences with unknown stigmatic disk color were excluded from our analyses (e.g.,

accession nos.: AY707899, EU428056). Because the voucher specimen of *N. tetragona* (FJ597745) has a yellow stigmatic disk (Dakhr et al. 2010), it was treated as *N. pygmaea*. *N. loriana* was also excluded due to its hybrid origin. This species has two ITS region sequences, one matching *N. leibergii* and the other *N. odorata* (Borsch et al. 2014). The sequences of nrDNA (ITS region: 664–686 bp) and cpDNA (*rps4-trnF* region: 1,678–1,679 bp) were aligned using MUSCLE ver. 3.5 (Edgar 2004). For phylogenetic analysis based on the ITS region, the sequences were trimmed at both ends, and the same range was used as accession no. “AY7718480 (*N. odorata*)” (603–626 bp). Regarding the *rps4-trnF* region, a sequence range equivalent to the sequence trimmed at both ends (1–115 bp and 1,795–1,904 bp) of “LN_590041 (*N. tetragona*)” was determined. Additionally, an insertion/deletion (indel) segment of simple sequence repeats, corresponding to 50,344–51,792 bp in “NC_006050 (*N. alba*)”, was removed from this region. Following these procedures, a sequence range of 1,374–1,383 bp was obtained for phylogenetic analysis.

To determine the phylogenetic position of *N. pygmaea* and *N. tetragona* within subgen. *Nymphaea*, *Nymphaea amazonum* Mart & Zucc., *Nymphaea jamesoniana* Planch., *Nymphaea lingulata* Wiersema, and *Nymphaea novogranatensis* Wiersema (subgen. *Hydrocallis*, Borsch et al. 2014) were selected as outgroup taxa. For analyzing the ITS region, the sequences of *N. jamesoniana* (FR717597) and *N. lingulata* (HG518070) were used. For analyzing the *rps4-trnF* region, the sequences of *N. novogranatensis* (AM422034) and *N. amazonum* (AM422026) were used.

For phylogenetic analysis, samples representing a single haplotype were treated as a single operational taxonomic unit (OTU). For analyses of nrDNA and cpDNA, aligned data, excluding indels, consisted of 642 characters representing 12 OTUs from nine species in the ITS region and 1,401 characters representing 10 OTUs from nine species in the *rps4-trnF* region. These datasets were subjected to maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) analyses using MEGA 10.0.5 (Kumar et al., 2018). For ML analyses of nrDNA and cpDNA, K2 + G model (Kimura 2-parameter; Kimura, 1980) and T92 model (Tamura 3-parameter; Tamura, 1992) were employed, respectively. For NJ analyses of nrDNA and cpDNA, a T92 + G model was applied to the same alignment data. MP analyses of nrDNA and cpDNA were conducted using Subtree-Pruning-Regrafting (Nei and Kumar 2000). The robustness of each clade was tested using bootstrap analysis with 1,000 replications.

Results

Morphological analyses

Six leaf characters (L2, L3, L6, L7, L9, and L10) and 11 flower characters (F1, F2, F3, F4, F5, F7, F8, F9, F11, F13, and F14) were significantly different between *N. pygmaea* and *N. tetragona* ($p < 0.05$; Table 2). Regarding leaf shape ($L9 = L2/L1$), *N. pygmaea* exhibited wider floating leaves than *N. tetragona* (*N. pygmaea*, $L9 [L2/L1] = 0.838 \pm 0.044 [0.747–0.963]$; *N. tetragona*, $0.757 \pm 0.047 [0.648–0.853]$) (mean $\pm$ SD and [minimum–maximum values]) ($p < 0.001$) (Table 2). In terms of flower characters, *N. pygmaea* displayed more elongated sepals than *N. tetragona* (*N. pygmaea*, $F13 [F3/F2] = 0.46 \pm 0.11 [0.1–0.7]$; *N. tetragona*, $0.60 \pm 0.08 [0.4–0.8]$) ($p < 0.001$). *N. pygmaea* had fewer ($F4 = 11.1 \pm 2.6 [5–17]$) and longer petals ($F5 = 29.4 \pm 6.2 \text{ mm} [15.0–46.3]$) than *N. tetragona* ($F4 = 16.4 \pm 2.4 [12–21]$; $F5 = 23.6 \pm 4.7 \text{ mm} [15.0–32.6]$) ($p < 0.001$). *N. pygmaea* also had fewer ($F7 = 40.4 \pm 9.3 [24–71]$) and longer stamens ($F8 = 11.1 \pm 2.0 \text{ mm} [7.2–17.6]$) than *N. tetragona* ($F7 = 61.6 \pm 22.8 [32–116]$; $F8 = 8.90 \pm 1.92 \text{ mm} [6.20–1.92]$) ($p < 0.001$). Additionally, *N. pygmaea* had a higher ratio of anther length to total stamen length than *N. tetragona* (*N. pygmaea*, $F14 [F9/F8] = 0.46 \pm 0.05 [0.34–0.59]$; *N. tetragona*, $0.40 \pm 0.05 [0.28–0.49]$) ($p < 0.001$).

The PCA of 13 morphological characters (L3, L5, L7, L8, L9, L10, L11, F2, F3, F4, F7, F9, and F13) revealed well-defined morphological relationships between the two species (Fig. 3). Although *Nymphaea* plants were scattered continuously along principal component (PC) axis 1, the two species could be clearly distinguished on PC axis 2 (Fig. 3a). The ranges of PCA values along axis 1 were $-4.584–7.195$ (mean $\pm$ SD; 0.267 ± 2.321) for *N. pygmaea* and $-4.220–4.159$ (-0.630 ± 2.177) for *N. tetragona*. The ranges of PCA values along axis 2 were $-1.705–2.336$ (0.971 ± 1.028) for *N. pygmaea* and $-3.841–0.352$ (-2.294 ± 0.810) for *N. tetragona*. Additionally, a scatter plot, averaging the PC scores for each population, showed a clear distinction between the two species on PC axis 2 (Fig. 3b).

The first three PCs accounted for 74.8% of the variance. PC axis 1 explained 40.9% of the total variance, attributed to the length from leaf tip to base (L3) and sepal width (F3) (Table S3). PC axis 2 explained 24.4% of the total variance, attributed to the number of petals (F4) and sepal shape (F13) (Table S3).

DNA sequencing and phylogenetic analysis

nrDNA analysis

Among the ITS region sequences (684–686 bp) from 40 samples of *Nymphaea* plants, four ribotypes were distinguished (nrN1, nrN2, nrN3, and nrN4; Table 1, Fig. 4). The ITS sequences of *N. pygmaea* were essentially identical (nrN1, $n = 32$), except for two samples from Saga Prefecture (Pref.) (NP3 and NP4; nrN2). Conversely, *N. tetragona* had nrN3 ($n = 5$), except for one sample from NT4 (nrN4). The sequence differences between *N. pygmaea* (nrN1 and nrN2) and *N. tetragona* (nrN3 and nrN4) were 18–19 substitutions and two indels (Table 3). The difference between nrN1 and nrN2 was one substitution, which was a heterozygous peak observed in the sequencing chromatogram in nrN2. nrN1 and nrN2 were similar to *N. pygmaea* sequences (nrN12, HG518076; nrN13, FJ597745), with zero to three substitutions and one indel between them. The difference between nrN3 and nrN4 was one substitution, which was a heterozygous peak in nrN4. nrN3 was identical to the ribotype of *N. tetragona* collected from Canada (HG518081).

Table 3

Variable sites of four ribotypes of internal transcribed spacers (ITS 1 and 2) and 5.8S rRNA regions in Sect. *Chamaenymphaea* (*Nymphaea pygmaea*, *Nymphaea leibergii*). Numbers in parentheses after the sequence type indicate the number of samples

		1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	4	4	4	4	4	4	4	5	5
		3	3	4	6	7	8	1	9	2	3	3	4	4	6	7	7	7	7	2	5	5	6	8	9	9	2	–	4
Species	Ribotype	0	8	4	0	1	0	8	8	9	0	7	3	7	9	1	9	9	0	3	4	5	7	2	3	7			1
<i>Nymphaea pygmaea</i>	nrN1 (32)	T	C	A	T	T	G	A	G	-	A	A	T	-	T	-	C	C	T	A	-	C	A	C	-				
	nrN2 (2)	T	C	A	T	T	A/G	A	G	-	A	A	T	-	T	-	C	C	T	A	-	C	A	C	-				
	nrN12 (1)	T	C	A	T	T	G	A	G	-	A	A	T	-	T	-	C	C	T	A	-	C	A	C		insertion ²			
	nrN13 (1) ¹	T	C	A	T	C	G	A	G	-	A	A	T	-	T	-	C	T	T	A	-	C	A	C	-				
<i>N. tetragona</i>	nrN3 (6)	C	C	C	G	T	G	C	A	-	G	C	C	A	C	-	C	C	C	T	C	C	C	C	A	-			
	nrN4 (1)	C	C	C	G	T	G	C	A	-	G	C	C	A	C	-	C/T	C	C	T	C	C	C	C	A	-			
<i>N. leibergii</i>	nrN6 (1)	C	T	C	T	T	G	A	G	G	G	T	C	A	C	T	C	C	T	A	C	A	C	A	A	-			
¹ The sequence deposited for accession FJ597745 is shorter than the other ribotype sequences, lacking the terminal region from positions 1 to 13 observed sequences																													
² TTTTCGTCGGCGTTG																													

The topologies of ML, NJ, and MP trees were identical (Fig. 4, Supplementary Information 1). The phylogeny of subgenus *Nymphaea* comprised two clades. One clade included *N. mexicana* (nrN7 and nrN8) and *N. odorata* (nrN9, –nrN11), whereas the other consisted of five species (nrN1–nrN6, nrN12, and nrN13). Within the latter clade, two subclades were identified, comprising *N. alba* (nrN5) and *N. candida* (nrN5), as well as *N. leibergii* (nrN6), *N. tetragona* (nrN3 and nrN4), and *N. pygmaea* (nrN1, nrN2, nrN12, and nrN13). Regarding sect. *Chamaenymphaea* (*N. pygmaea*, *N. tetragona*, and *N. leibergii*), the section was monophyletic. In terms of inner relationships, *N. tetragona* and *N. leibergii* formed a monophyletic group, with *N. pygmaea* identified as their sister clade. All clades in these phylogenetic trees were supported by high bootstrap values (> 90%; Fig. 4).

cpDNA analysis

Among the *rps4-trnF* region from 40 *Nymphaea* samples, four haplotypes were distinguished (Tables 1 and S4). The *rps4-trnF* region of *N. pygmaea* was identical (cpN1, n = 34), except for one sample from Shimane Pref. (NP12; cpN2). *N. tetragona* exhibited two haplotypes (cpN3, n = 3; cpN4, n = 3). The differences among these four haplotypes were as follows: one substitution and two indels between cpN1 and cpN2, one substitution and one indel between cpN1 and cpN3 and between cpN2 and cpN4, two substitutions and one indel between cpN1 and cpN4, one indel between cpN2 and cpN3, and one substitution between cpN3 and cpN4 (Table S4). cpN1 was identical to *N. pygmaea* sequences (LN590038) deposited in DNA sequence databases. cpN3 and *N. tetragona* collected from Canada (HG518081) exhibited the same sequence.

Among the cpDNA trees, the topologies of ML, NJ, and MP trees were nearly identical (Fig. S2, Supplementary Information 2). Similar to the ITS results, subgenus *Nymphaea* consisted of two clades: one included *N. mexicana* (cpN8 and cpN9) and *N. odorata* (cpN10–cpN12), whereas the other comprised five species, namely *N. alba* (cpN5), *N. candida* (cpN6), *N. leibergii* (cpN7), *N. tetragona* (cpN3 and cpN4), and *N. pygmaea* (cpN1 and cpN2). Although these cpDNA trees did not resolve the phylogenetic relationships among *N. alba*, *N. candida*, *N. mexicana*, and *N. odorata*, the monophyly among sect. *Chamaenymphaea* species (*N. leibergii*, *N. pygmaea*, and *N. tetragona*) was robust (Bootstrap values, 80–90%; Fig. S2).

Discussion

Taxonomic status of *N. pygmaea* and *N. tetragona*

N. pygmaea and *N. tetragona* were morphologically and phylogenetically distinct (Figs. 3 and 4 and Tables 2, 3, and S4). In our nrDNA analysis, although we did not investigate reproductive isolation between the two taxa, we observed no individuals or populations with intermediate morphologies between the two species and no heterozygous peaks in the species-character sites of ITS region sequences. Moreover, the distinct distribution ranges of the two species suggest that they are reproductively well-isolated (Naito and Shiga 2024). Our results support the assertion that *N. pygmaea* should be treated as a distinct species and not a synonym of *N. tetragona*. However, one taxonomic issue remains unresolved: the type specimens of *N. pygmaea* have not been identified to date (Wiersema 1996). In our study, we were unable to examine the type specimens morphologically and genetically. To further clarify the taxonomic status of *N. pygmaea* and its variations, it is necessary to conduct comparisons using samples from southern China, which is considered the species' type locality (Wiersema 1996).

The morphological differences between the two species noted by Fu et al. (2001), namely stigmatic disk color and leaf thickness, are supported by the findings of the present study. Thus, the distribution of the two species in Eurasia and adjacent countries could be explained as suggested by Fu et al. (2010). In Japan, *N. tetragona* is unevenly distributed in the northern and eastern areas above 42°N in Hokkaido, with its distribution rarely overlapping with that of *N.*

pygmaea, except in some areas. Only *N. pygmaea* is found south of Hokkaido (Naito and Shiga 2024). Consequently, a clear distributional boundary exists between the two species, indicating a high selective pressure that may have influenced each species' distribution. This selection pressure could involve differences in ecological traits, particularly optimal growth temperature (Cox et al. 2016), and other factors such as pollinator and reproductive interference (Kuno 1992; Waser 1978). Comparative physiological, ecological, and population genetic studies of these two species can help elucidate the mechanisms underlying the formation of distribution patterns.

Relationships with infraspecific taxa

In the Korean peninsula, Nakai (1938) noted that *N. tetragona* is not distributed. He described the Korean *Nymphaea* as a variety, *N. pygmaea* var. *minima* Nakai (Nakai 1938), which he later reclassified as a species, *Nymphaea minima* (Nakai) Nakai (Nakai 1952). The key morphological characters of this taxon are primarily determined by leaf size (length = 1.8–5.5 cm, width = 1.6–4.3 cm) and flower size (sepal length = 1.7 cm, petal length = 1.5 cm), distinguishing it as smaller than *N. pygmaea* var. *pygmaea* (Nakai 1938). In the present study, small *Nymphaea* plants with yellow stigmatic disks were collected in Japan (leaf length = 2.9 cm, leaf width = 2.5 cm, sepal length = 1.4 cm, petal length = 1.3 cm [NGU 12124]) and Korea (leaf length = 4.8 cm, leaf width = 3.8 cm, sepal length = 2.0 cm, petal length = 1.5 cm [NGU 8679]); however, these populations also included larger individuals with a wide range of morphological variations, without genetic differences between Japanese and Korean *N. pygmaea* (Figs. 3 and 4). Therefore, it is reasonable to treat *N. minima* (= *N. pygmaea* var. *minima* Nakai) as a synonym of *N. pygmaea*.

Caspary (1866) considered *N. pygmaea* to be synonymous with *N. tetragona*, and he described two infraspecific taxa, var. *lata* and var. *angusta*, without mentioning their stigmatic disk colors. var. *lata* was reported to be distributed in northern and eastern Asian regions (Southeastern Siberia, Kamchatka, and Manchuria), whereas var. *angusta* was found in China, Japan, and India (Caspary 1866). The distributional patterns of these two infraspecific taxa were similar to those of *N. tetragona* and *N. pygmaea*, respectively. According to Caspary (1866), the morphological differences between the two infraspecific taxa were in sepal and petal shapes (length: width = 1.16–2.7:1 [*lata*], 2.0–3.61:1 [*angusta*]) and leaf shape (leaf length: lobe length = 2.27:1 [*lata*], 1.9:1 [*angusta*]). However, the present study confirmed that sepal shapes (1.3–2.4:1 [*N. tetragona*]; 1.4–3.7:1 [*N. pygmaea*]) and leaf shapes (1.90–2.51:1 [*N. tetragona*]; 1.91–2.47:1 [*N. pygmaea*]) completely overlapped between the two species. Thus, var. *lata* and var. *angusta* did not correspond well to *N. tetragona* and *N. pygmaea*, respectively. Distinguishing the morphology between the two infraspecific taxa is difficult. Additionally, it remains unclear whether the two infraspecific taxa are synonymous with *N. pygmaea* and *N. tetragona*.

In the present study, *N. tetragona* plants collected from both Canada and Japan exhibited identical DNA sequences. Our investigation of samples collected from the paratype locality of *N. tetragona* var. *erythrostigmatica* revealed DNA sequences and morphology identical to those of European *N. tetragona* var. *tetragona*, although *N. tetragona* var. *erythrostigmatica* plants are extinct in their holotype locality (Naito and Shiga 2021). Therefore, treating *N. tetragona* var. *erythrostigmatica* as synonymous with *N. tetragona* var. *tetragona* would be appropriate.

Interspecific hybrids in subgen. *Nymphaea*

Nymphaea species are known to readily crossbreed with each other, leading to many cultivated plants derived from artificial hybridization (Slocum 2005; Wiersema 1988). In subgenus *Nymphaea*, interspecific hybridization has been observed in areas where species distributions overlap, resulting in several reported hybrids: *N. candida* (*N. alba* × *N. tetragona*; Volkova et al. 2010), *N. loriana* (*N. leibergii* × *N. odorata*; Borsch et al. 2014), *N. leibergii* × *N. tetragona*, and *N. mexicana* × *N. odorata* (Borsch et al. 2014). In this study, hybrids between *N. pygmaea* and *N. tetragona* were not found. In Hokkaido Island, Japan, the two species grow allopatrically, except for one mire (Sasaki 2004, 2020). Naito and Shiga (2024) reported that the localities of collected specimens of *N. pygmaea* and *N. tetragona* were distinctly separated in Japan. Additionally, Fu et al. (2001) noted that *N. tetragona* was limited to boreal regions above 50°N latitude in North America and Europe. Even if the two species can cross, their hybrids may be rare due to their different distributional patterns. More detailed surveys regarding morphological variations and gene flows among the populations of these two species distributed at the boundaries of their distributional areas, as well as artificial crossing tests, are needed to confirm their isolation patterns and hybridization events.

Taxonomic treatment

Nymphaea pygmaea (Salisb.) W.T.Aiton, Hort. Kew., ed. 2, 3: 293 (1811)(Fig. 1a–f). ≡ *Castalia pygmaea* Salisb., Parad. Lond. 1(2): 68 (1805–8). Type: unknown (see Wiersema 1996).

= *Nymphaea pygmaea* var. *minima* Nakai, Bot. Mag. (Tokyo) 14(11): 746 (1938), syn. nov. ≡ *Nymphaea minima* (Nakai) Nakai, Bull. Nat. Sci. Mus., Tokyo 31: 25 (1952) ≡ *Nymphaea tetragona* var. *minima* (Nakai) W.Lee, Lineamenta Florae Koreae: 360 (1996). Holotype: South Korea; South Hwanghae Prov., 29 Jul. 1929, T. Nakai 12758 (TI!).

= *Nymphaea japono-koreana* Nakai, J. Jap. Bot. 14: 751–752 (1938), nom. nud.

= "*Nymphaea tetragona* Georgi var. *tetragona*" auct. non Georgi: Ohwi and Kitagawa, New Fl. Jap. 672–673 (1983); Kadono, Aquat. Pl. Jap. 109 (1994); Ito, Fl. Jap. 2a: 358 (2006); Choi H, Gen. Vas. Pl. Korea 161–162 (2007); Kadono, Fld. Guide Aquat. Pl. Jap. 50 (2014); Shiga, Wild Fl. Jap. 1: 48. pl. 37 (2015).

Description: Herbs, perennial, aquatic. Rhizomes erect, cylindrical, unbranched, stolons absent (Fig. 1f). Leaves dimorphic. Submerged leaves: leaf blade deltoid, sagittate, orbicular, 6–24 × 5–21 cm, membranous, margin entire, base cordate. Floating leaves: leaf blade obovate to orbicular, 5.5–24 × 4.5–21 cm, subcoriaceous, abaxially glabrous, base deeply cordate and basal lobes parallel to overlapping, thicker, 0.2–0.6 mm, impressed veins abaxially, margin entire (Fig. 1a, e). Flowers floating, 3–6 cm in diameter (Fig. 1c). Calyx insertion on receptacle tetragonal (Fig. 1d). Sepals four, narrowly deltoid, 1.7–5.6 × 0.4–4.0 cm, obscurely veined, persistent (Fig. 1b). Petals 5–17, white, broadly lanceolate, oblong, or narrowly obovate, 1.5–4.6 × 0.4–2.1 cm, transition to stamens gradual (Fig. 1b). Stamens 24–71, 0.7–1.8 × 0.1–0.4 cm, yellow; filament of outer stamens wider than the anther, connective apically appendaged slightly

(Fig. 1b, c). Carpels completely united, walls between locules of the ovary single. Stigma rays 5–11, yellow (Fig. 1c); carpellary appendages broadly lanceolate, 0.2–0.4 cm, yellow (Fig. 1c). Fruit globose, 2.8–7.7 × 1.2–2.9 cm. Seeds ellipsoid to ovoid, 1.9–3.6 × 1.3–2.6 mm, smooth. Fl. Jun.–Oct.

Regional name: Hitsuji-gusa (Japan: Ito 2016) and Kkoma-Suryeon (Korea: National Institute of Botanical Resources 2023)

Distribution and habitat

China, India, Japan, Korea, Nepal, and Vietnam. In ponds, lakes, marshes, moors, and paddy fields.

Specimens examined: JAPAN. Hokkaido Pref.: Ebetsu-shi, 14 Jun. 2016, N. Nitta s.n. (SAPS); Iburi-no-kuni, Aug. 1893, Y. Tokubuchi s.n. (SAPS); 13 Jul. 1895, N. Date s.n. (SAPS); 21 Aug. 1899, J. Hanazawa s.n. (SAPS); 22 Aug. 1899, J. Hanazawa s.n. (SAPS); 17 Jun. 1975, A. Namimatsu s.n. (SAPS) (two specimens); 22 Jun. 1975, S. Nakayama s.n. (SAPS) (six specimens); Kuromatsunai-cho, 13 Jun. 1989, K. Ito s.n. (SAPS); Mukawa-cho, 17 Aug. 1964, Y. Takahashi s.n. (SAPS); 12 Jun. 1999, K. Oita 99-5930 (SAPS); 27 Aug. 2000, K. Oita 00-6048 (SAPS) (two specimens); 15 Jul. 2001, K. Oita 01-6609 (SAPS); Nanae-cho, 3 Aug. 1925, S. Miki s.n. (OSA230084); Oshamanbe-cho, 8 Aug. 2008, H. Takahashi s.n. (SAPS); Oshima-no-kuni, Aug. 1886, S. Nozawa s.n. (SAPS); 9 Jul. 1916, F. C. Grcatrex 189 (SAPS); Jun. 1917, S. Tanaka s.n. (SAPS); Sapporo-shi, 16 Aug. 1988, M. Hara 5758, 5990 (SAPS); 30 Aug. 1989, M. Hara 1256 (SAPS); Shiraoi-cho, 31 Jul. 1906, K. Saida 198 (TNS5549); 19 Aug. 1976, M. Hara 144 (SAPS); Tomakomai-shi, 29 Aug. 1969, M. Hara 132 (SAPS); 29 Aug. 1972, M. Hara 130 (SAPS); 28 Jun. 1974, S. Nakayama s.n. (SAPS); 27 Jun. 1987, J. Oginiawa s.n. (TNS988649); 17 Aug. 1989, Y. Kadono 6146 (OSA); 24 Aug. 1994, H. Takahashi 17548 (SAPS); 23 Aug. 1998, H. Takahashi 25789 (SAPS); Junsai-numa, 17 Aug. 1888, Y. Tokubuchi s.n. (SAPS). Aomori Pref.: Higashidori-mura, 28 Aug. 2019, H. Naito et al. 271, 272 (NGU11664, 11665); 19 Jun. 2022, J. Sasaki s.n. (NGU14396); 12 Aug. 2022 (S. Kato s.n.), NGU14398; Tsugaru-shi, 26 Aug. 2019, H. Naito et al. 273 (NGU11668–11970). Iwate Pref.: Hanamaki-shi, 27 Aug. 2019, H. Naito et al. 267–269 (NGU11681–11684). Miyagi Pref.: Kami-machi, 28 Aug. 2019, H. Naito et al. 270 (NGU11659–11661). Akita Pref.: Akita-shi, 23 Aug. 2019, H. Naito et al. AM43, 44 (NGU11155, 11161, 11162, 11165). Yamagata Pref.: Murayama-shi, 28 Aug. 2019, S. Kato et al. TN176 (NGU10875). Fukushima Pref.: Inawashiro-machi, 23 Sep. 2019, S. Kato s.n. (NGU11676–11677). Tochigi Pref.: Nasukarasuyama-shi, 23 Sep. 2019, H. Naito et al. TN14, 15 (NGU12378–12380). Gunma Pref.: Katashina-mura, 10 Sep. 2019, H. Naito et al. 276, 277, 304, 306 (NGU12948–12955). Niigata Pref.: Tsunan-machi, 11 Aug. 2019, S. Kato et al. s.n. (NGU11675); Jouetsu-shi, 14 Sep. 2019, H. Naito et al. 222 (NGU11654, 11715). Gifu Pref.: Mizunami-shi, 7 Sep. 2020, H. Naito et al. 497, 498 (NGU11756, 11758). Aichi Pref.: Toyota-shi, 9 Sep. 2020, H. Naito et al. 601–604 (NGU11757, 11761, 11762). Mie Pref.: Tamaki-cho, 10 Sep. 2020, H. Naito et al. 662 (NGU11722). Shiga Pref.: Taga-cho, 7 Jul. 2019, T. Shiga 11317 (NGU10595, 10596). Hyogo Pref.: Ono-shi, 19 Sep. 2019, T. Shiga et al. 11229 (NGU8301, 8302). Shimane Pref.: Iinan-cho, 23 Jul. 2018, K. Tsubota & S. Midorikawa 232 (NGU 8731). Ehime Pref.: Imabari-shi, 22 Jul. 2019, Y. Fujiwara s.n. (NGU11650–11653). Saga Pref.: Arita-cho, 23 Jul. 2019, H. Naito et al. 228 (NGU11655, 11656). Miyazaki Pref.: Ebino-shi, 24 Jul. 2019, H. Naito et al. 227 (NGU11657–11658). KOREA. Gangwon-do: Goseong-gun, 8 Jun. 2019, T. Shiga et al. 10665, 10666 (NGU8679, 8680).

Nymphaea tetragona Georgi, Bemerk. Reise Russ. Reich 1: 220 (1775)(Fig. 1g–l). $\equiv$ *Castalia tetragona* (Georgi) G. Lawson, Proc. & Trans. Roy. Soc. Canada 6, Sect. 4: 112 (1888). Lectotype: Angara, Siberia, Russia, Jul. 1772, J. Georgi (LE1009632!) (see Byalt and Krupkina 2017).

= *Nymphaea tetragona* var. *erythrostigmatica* Koji Ito, J. Geobot. 16: 100 (1968), syn. nov.; Kadono, Aquat. Pl. Jap. 112 (1994); Kadono, Fld. Guide Aquat. Pl. Jap. 51 (2014); Shiga, Wild Fl. Jap. 1: 48 (2015). Holotype: Sarufutsu-mura, Kimomanuma, Japan, 21 Jul. 1967, Ko. Ito s.n. (SAPS72107!).

= *Nymphaea fennica* Mela, Acta Soc. Fauna Fl. Fenn. 14(3): 1 (1897). Syntype: Several syntypes at H (see Wiersema 1996).

Description: Herbs, perennial, aquatic. Rhizomes erect, cylindrical, rarely branched; stolons absent (Fig. 1l). Leaves dimorphic. Submerged leaves: leaf blade deltoid, sagittate, orbicular, 6–18 × 4–15 cm, membranous, margin entire, base cordate. Floating leaves: leaf blade cordate-ovate to ovate-elliptic, 6–18 × 4–15 cm, subcoriaceous, abaxially glabrous, base deeply cordate and basal lobes parallel to overlapping, thinner, 0.2–0.4 mm, raised veins abaxially, margin entire (Fig. 1g, k). Flowers floating, 3–6 cm in diameter (Fig. 1i). Calyx insertion on the receptacle prominently tetragonal (Fig. 1j); sepals 4, broadly deltoid to narrowly ovate, 1.6–3.4 × 0.9–2.4 cm, veined, persistent (Fig. 1h). Petals 12–21, white, rhombic to obovate, 1.5–3.3 × 0.7–1.7 cm, transition to stamens gradual (Fig. 1h). Stamens 32–116, 0.6–1.3 × 0.2–0.3 cm, yellow–orange, abaxially usually tinged with red to purple in the inner stamens; filament of outer stamens wider than anther very prominent, connective apically appendaged slightly (Fig. 1h, i). Carpels completely united, walls between locules of the ovary single. Stigma rays 4–12, dark red to purple (Fig. 1i); carpellary appendages broadly lanceolate, 0.3–0.8 cm, yellow or dark red to purple, apically tinged with red to dark red (Fig. 1i). Fruit globose, 2.6–3.7 × 1.0–2.2 cm. Seeds ellipsoid to ovoid, 2.5–2.9 × 1.5–1.9 mm, smooth. Fl. Jul.–Aug.

Regional name: Pygmy water-lily (North America: Wiersema and Hellquist 1997), Small white water-lily (North America: Wiersema and Hellquist 1997), Nymphéa tetragonal (North America: Wiersema and Hellquist 1997), Shui lian (China: Fu et al. 2001), Ezo-Beni-Hitsuji-gusa (Japan: Ito 1968), and Suryeon (Korea: National Institute of Botanical Resources 2023)

Distribution and habitat

China, Japan, Russia; North America, Europe. In ponds, lakes, marshes, and moors.

Specimens examined: JAPAN. Hokkaido Pref.: Akkeshi-cho, 14 Aug. 1925, S. Miki s.n. (OSA230085); Ikeda-cho, 20 Aug. 1934, H. Yokoyama s.n. (SAPS); Hamanaka-cho, 7 Aug. 2020, H. Naito et al. 398 (NGU11704); Kamikawa-cho, 14 Aug. 1947, M. Tatewaki 35570 (SAPS) (three specimens); 9 Jul. 1950, M. Kurashige 233 (SAPS); 1 Aug. 1967, K. Ito & A. Umezawa s.n. (SAPS) (two specimens); 2 Aug. 1967, K. Ito & A. Umezawa s.n. (SAPS); Kitami-shi, 10 Aug. 1925, collector unknown 231 (SAPS); 2 Aug. 1967, K. Ito & A. Umezawa s.n. (SAPS) (two specimens); 13 Aug. 2019, H. Naito et al. 214–216 (NGU11701–11703); 4 Jun. 2022, J. Sasaki s.n. (NGU14393, 14394); Kushiro-shi, 13 Aug. 1884, K. Miyabe s.n. (SAPS); 11 Jul. 1886, M. Nakamura B.149 (SAPS); 13 Aug. 1887, S. Nogawa s.n. (SAPS); 10 Aug. 1951, M. Tatewaki s.n. (SAPS) (two specimens); 28 Jun. 1967, K. Seto 16511 (OSA); 25 Aug. 1975, Y. Kadono 1641 (OSA); 17 Jul. 1987, Y. Kadono 4689 (OSA); 7 Aug. 1991, Y. Kadono et al. 18 (OSA); 9 Aug. 1991, Y. Kadono et al. s.n. (OSA); Nemuro-shi, 28 Jul. 1928, M.

Tatewaki & Y. Tokunaga s.n. (SAPS); 29 Aug. 2004, T. Shiga & S. Takebayashi 65 (OSA220192); 29 Aug. 2004, T. Shiga & S. Takebayashi 52 (OSA220147); collection date unknown, S. Yamane s.n. (SAPS); Sarufutsu-mura, 28 Jul. 1966, K. Ito s.n. (SAPS); 28 Jul. 1966, K. Ito s.n. (SAPS16635); 21 Jul. 1967, K. Ito s.n. (SAPS16636–16638); 21 Jul. 1967, K. Ito s.n. (SAPS); 17 Jul. 1975, Y. Kadono 1977 (OSA); 21 Jul. 1987, Y. Kadono 4779 (OSA) (four specimens); 21 Jul. 1987, Y. Kadono 4784 (OSA); 5 Aug. 1998, H. Takahashi 25535 (SAPS15970); 26 Aug. 1989, J. Oginiawa 10643, 39380 (TNS960643, 989380); 15 Aug. 2019, H. Naito & T. Shiga 209–211, 301 (NGU11693–11696); 5 Aug. 2020, H. Naito et al. 281, 282, 558, 559 (NGU11711–11714, 11716); 5 Aug. 2020, H. Naito et al. 558, 559 (NGU11711–11714, 11716); 6 Aug. 2020, H. Naito et al. 333, 356 (NGU11697, 11698); 11 Jul. 2022, J. Sasaki s.n. (NGU14395); Shibeche-cho, 9 Aug. 1969, N. Fujita 51 (TNS788665); 29 Aug. 1992, H. Takahashi & Y. Takashima 15143, 15162 (SAPS); 29 Jul. 2019, S. Kato s.n. (NGU11692); Taiki-cho, 28 Jul. 1979, E. Miki & M. Ito s.n. (TNS395745); 17 Jul. 1985, Y. Kadono & G. Wiegler 859 (OSA) (two specimens); 22 Jun. 2019, K. Shutoh et al. 3681 (SAPS55004); 26 Jun. 2019, K. Shutoh s.n. (NGU11700); 8 Aug. 2020, H. Naito et al. 429 (NGU11699); Takinoue-cho, 3 Aug. 1966, K. Ito s.n. (SAPS); Toyokoro-cho, 9 Aug. 2020, H. Naito et al. 457, 560 (NGU11705–11708); Toyotomi-cho, 13 Jun. 1938, K. Miyabe & H. Yokoyama s.n. (SAPS); 2 Aug. 1968, K. Ito s.n. (SAPS); 16 Aug. 1980, K. Harusawa s.n. (OSA34528); Tsukigata-cho, 7 Aug. 2020, K. Katagiri K1, 2 (NGU11709, 11710); Uryu-cho, 8 Jul. 1998, H. Takahashi 25199 (SAPS16630); 23 Jul. 1998, H. Takahashi 25291, 25294, 25308 (SAPS16631, 16632, 16634); 12 Aug. 1998, H. Takahashi 25642 (SAPS); 6 Jun. 2004, M. Yamazaki s.n. (NGU7915); 6 Jun. 2004, M. Yamazaki s.n. (NGU); 12 Aug. 2009, M. Yamazaki s.n. (NGU13468); 23 Jul. 2019, K. Shutoh et al. 3658, 3659 (SAPS54921–54923); 11 Aug. 2020, H. Naito et al. 523–527, 532 (NGU11687–11691). Kurils: Furukamappu, 20 Aug. 1931, J. Ohwi 923 (TNS222126) (two specimens); Kunashiri, 26 Aug. 1923, M. Tatewaki 3411 (SAPS25573); 20 Aug. 1936, M. Tatewaki 25573 (SAPS); Tomari-mura, Aug. 1933, A. Tanaka s.n. (OSA230088). Sakhalin: Korsakov District, 29 Jul. 2001, H. Tatewaki 29516 (SAPS) (four specimens); Kusunnai, 21 Jun. 1906, T. Miyake s.n. (SAPS); Nyiwo, 15 Aug. 1922, Y. Kudo & M. Tatewaki 6584 (SAPS) (four specimens); Okhinsky District, 4 Aug. 2001, V.Y. Barkalov 10195 (SAPS); 4 Aug. 2001, T. Fukuda 1501 (SAPS); 11 Aug. 2001, T. Fukuda 1981 (SAPS); Shisuka-cho, 15 Aug. 1906, T. Miyake s.n. (SAPS); 29 Aug. 1929, collector unknown (TNS1066643); 1933, Y. Tanaka s.n. (OSA230087); 16 Aug. 1935, S. Sugawara 30155, 30157 (SAPS); collection date and collector unknown (SAPS) (two specimens); Shisuka-gun, 18 Jul. 1937, B. Yoshimura & M. Hara s.n. (TNS268313); Tominai-cho, 3 Aug. 1930, H. Otani & Y. Imai s.n. (SAPS); Ushiro-mura, 24 Jun. 1934, F. Fujita s.n. (OSA230086).

Declarations

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Author contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were mainly performed by Hanaka Naito and Takashi Shiga. The first draft of the manuscript was written by Hanaka Naito, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

Fig. 1 (Naito et al.)

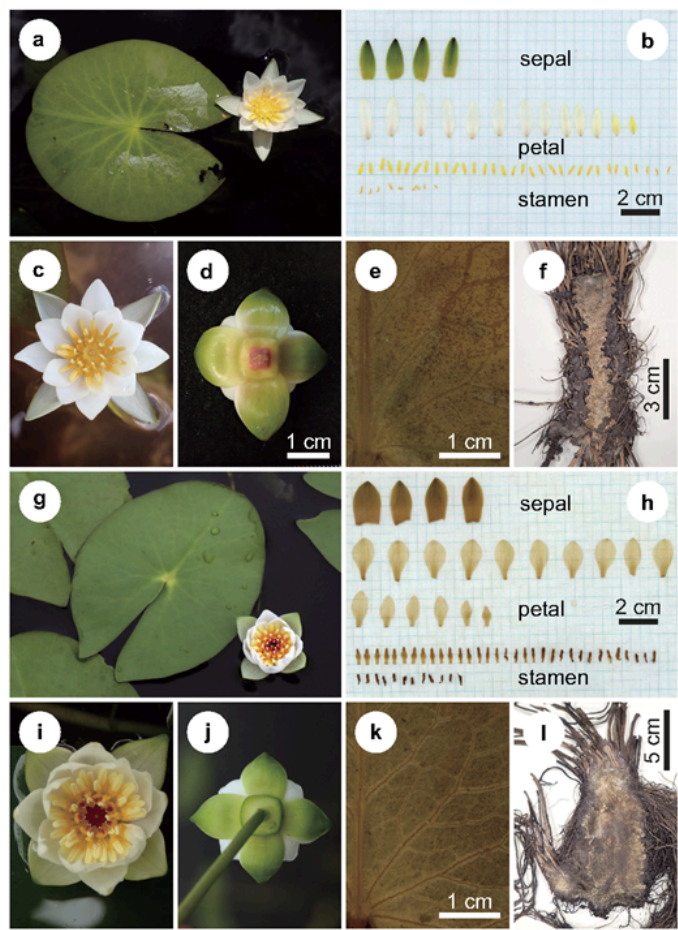


Figure 1

Nymphaea pygmaea (a–f) and *Nymphaea tetragona* (g–l). a, g, whole plants; b, h, sepals, petals, and stamens; c, i, flower; d, j, receptacle; e, k, veins of undersurface of leaf; f, l, vertical section of rhizome. Samples shown in e, h, and k were fixed with 70 % EtOH (v/v). Samples shown in f and l were from

Fig. 2(Naito et al.)

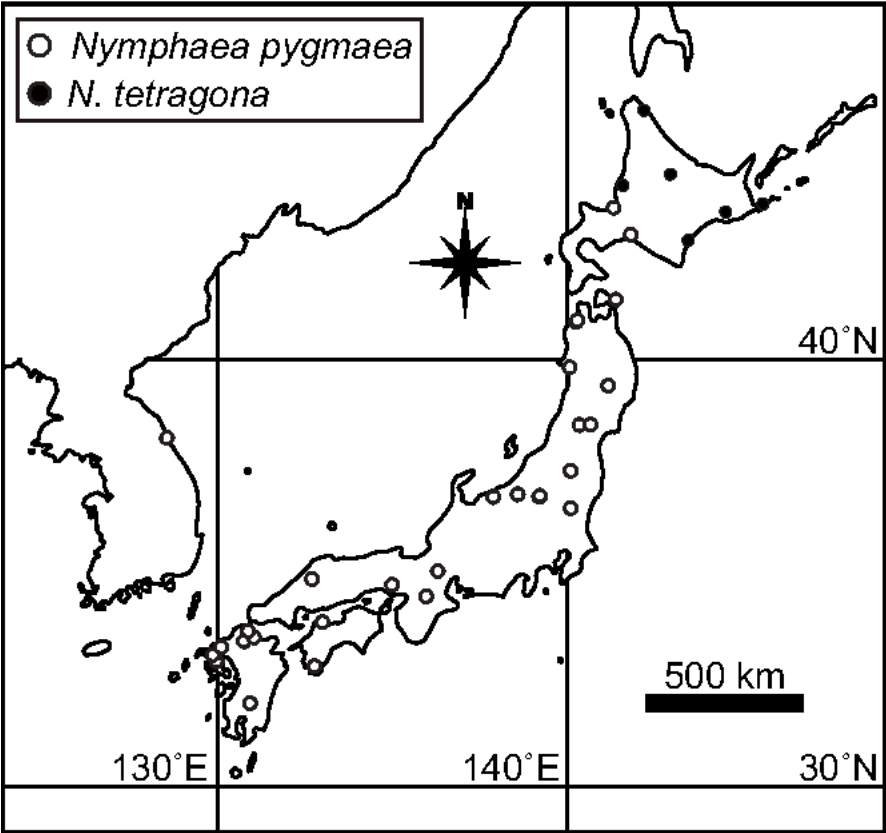


Figure 2

Locations of 40 populations of *Nymphaea pygmaea* and *Nymphaea tetragona* collected for morphological and genetic analyses

Fig. 3 (Naito et al.)

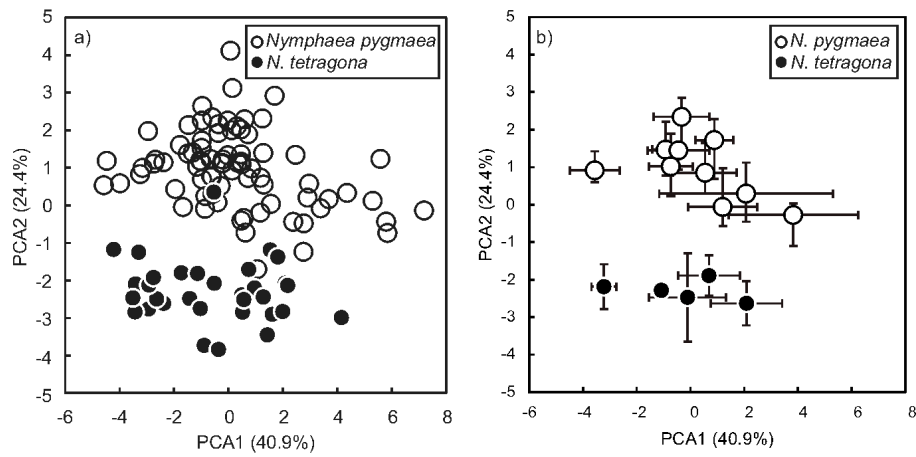


Figure 3

Results of principal component analysis (PCA) of *Nymphaea pygmaea* and *Nymphaea tetragona* based on 11 morphological characters. Symbols indicate individual (a) or population (b) averages

Fig. 4 (Naito et al.)

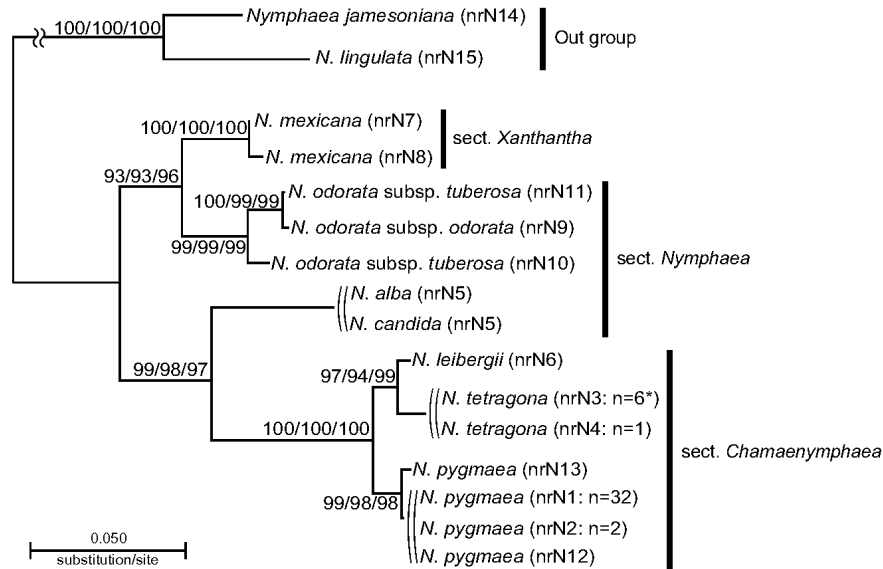


Figure 4

Neighbor-joining (NJ) tree based on 642 aligned characters of nuclear ribosomal DNA (internal transcribed spacer [ITS]1, 5.8S, and ITS2) of 15 ribotypes representing 10 *Nymphaea* taxa. Bootstrap values (>50 %) of each tree (maximum likelihood [ML], NJ, maximum parsimony [MP]) are shown above or under branches. Samples representing identical sequences in 642 characters of alignment matrix were treated as a single operational taxonomic unit. The classification of sections given by Wiersema (1996) are indicated on the right side of each clade. *: Including five samples from this study and one sequence from GenBank

Supplementary Files

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