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Inferring the Origin of *Potamogeton* ×*inbaensis* (Potamogetonaceae) Using Nuclear and Chloroplast DNA Sequences

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Many hybrid taxa have been described in *Potamogeton*. These hybrids can usually be recognized by morphological intermediacy between parent species but in some cases recognition is difficult due to extensive morphological variation. *Potamogeton* ×*inbaensis* Kadono which was reported as a hybrid between *P. lucens* L. subsp. *sinicus* (Migo) H. Hara var. *teganumensis* (Makino) H. Hara and *P. wrightii* Morong, is one of them. In this study nuclear and chloroplast DNA sequence confirmed this report and inferred *P. wrightii* as the maternal parent of the hybrid. *Potamogeton* ×*inbaensis* was also found together with both parents a few years ago in artificial ponds near Inbanuma Lake, but no hybrid plants from opposite parental pairs were recognized. Considering geographic distance *P.* ×*inbaensis* is inferred to have undergone hybridization events independently in Inbanuma Lake and in Oitoike Pond. Morphological analysis showed that leaf morphological characters partially overlap between *P.* ×*inbaensis* and both of the parental taxa which suggests that identification based on leaf morphology needs careful examination and that molecular analysis is a more useful tool for recognition of hybrids.

Key words: *atpB-rbcL*, hybrid, ITS, morphology, *Potamogeton*.

The genus *Potamogeton* L. (Potamogetonaceae) is one of the largest genera of aquatic plants and is distributed in fresh water areas of the world (Preston 1995, Cook 1996, Wiegleb and Kaplan 1998). It is well known that the genus shows extensive morphological variation at species level and intraspecific level (Kaplan 2002). In the most recent monograph of the genus, 69 species and 50 confirmed hybrids are recognized (Wiegleb and Kaplan 1998). Several studies based on morphology (Preston et al. 1998a, Kaplan 2001) or based on both morphological and molecular analyses (e. g., Hollingsworth et al. 1995, 1996, Preston et al. 1998b, King et al. 2001, Fant et al. 2001a, 2001b, 2003, 2005, Iida and Kadono 2002, Kaplan et al. 2002, Fant and Preston 2004, Kaplan and Fehrer 2004, Kaplan and Wolff 2004, Whittall et al. 2004) investigated the hybrid species and inferred their origin. Of these Kaplan and Fehrer (2004) reported that nuclear ribosomal (nr) DNA markers are a useful tool to identify *Potamogeton* hybrids.

In the flora of aquatic plants in Japan, 18 species and 9 hybrids of the genus were recognized (Kadono 1994). One putative hybrid *Potamogeton* ×*inbaensis* Kadono, which is not recognized in the monograph of

Wiegleb and Kaplan (1998), was originally reported from Inbanuma Lake (Kadono 1983) and is known to be distributed in the Inbanuma and Teganuma Lakes of Chiba prefecture and disjunctly in Kita-Kyushu-shi of Fukuoka Prefecture (Kadono 1994). As the putative hybrid is male sterile and shows an intermediate feature in length of petiole and leaf size between *P. lucens* L. subsp. *sinicus* (Migo) H. Hara var. *teganumensis* (Makino) H. Hara and *P. wrightii* Morong (Kadono 1983), it is considered to be their hybrid. One parent species *P. lucens* subsp. *sinicus* var. *teganumensis* is an endangered species and its distribution is limited to a few localities in Japan (Kadono 1994 Environment Agency of Japan 2000). The other, *P. wrightii*, is widely distributed in East Asia, India, Southeast Asian islands and the Pacific area (Wiegleb 1990b). These putative parent species are known to be clearly distinguished based on nucleotide sequences of chloroplast (cp) DNA (Iida et al. 2004) as well as leaf morphology (Kadono 1983).

Recently for conservation of aquatic plants three artificial ponds (referred to as Pond 1, 2 and 3 in this study) were made on the reclaimed land of Teganuma Lake in 1997 1998 and 2002 respectively (Fig. 1). As was reported in Momohara et al. (2001) several aquatic plants that were germinated from buried seeds in the lake sediments were observed in these ponds. Among them *P. lucens* subsp. *sinicus* var. *teganumensis* and *P. wrightii* were found together with their putative hybrid. However, contrary to the Kadono's (1983) suggestion, it is difficult to critically distinguish these three taxa because of extensive morphological variation.

In the present study we aimed to identify plants from the three artificial ponds and to infer the origin of *P.* ×*inbanensis* based on both morphological and molecular analyses. First we determined molecular markers that can distinguish each taxon using nrDNA and cpDNA sequences in considering insights by Kaplan and Fehrer (2004) and Iida et al. (2004). We collected morphologically typical plants of each taxon as standard and examined their genotypes. Then we compared the genotypes of plants sampled from the artificial ponds to the standard ones. In addition we examined the morphological characters of the plants to consider circumscription of the taxa.

Materials and Methods

Taxon sampling–We collected morphologically typical plants as standard of each taxon to determine molecular markers; one plant of *P. lucens* subsp. *sinicus* var. *teganumensis* from Oitoike Pond, one of *P. wrightii* from Namiki and three of *P.* ×*inbaensis* from Inbanuma Lake the type locality and Oitoike Pond (Table 1, Fig. 1). In addition we collected a total of 20 *Potamogeton* plants from Pond1 and 2 in 2000 and from 3 in 2000 and 2003, respectively (Table 1, Fig. 1). Fresh leaves were stored at –80ºC for molecular analysis. Their voucher specimens were deposited in the Herbarium of The National Science Museum (TNS) and some plants were transplanted to the Tsukuba Botanical Garden, The National Science Museum.

DNA analysis–DNA extraction and PCR amplification: Total DNA was extracted from frozen leaf tissue and purified following the method of Tanaka et al (2003). First, for the standard plants, internal transcribed spacer (ITS) region of nrDNA and the intergenic region between *atpB* and *rbcL* genes (*atpB* - *rbcL*) of cpDNA were amplified by the polymerase chain reaction (PCR). ITS region was amplified using primers ITS4 and ITS5 of Baldwin (1992) and *atpB* - *rbcL* region using primers atpB2F of Manen et al. (1994) and rbcL2R [5' - CAA CAC TTG CTT TAG TCT CT - 3'] newly designed for the present study. The PCR reaction mixture consisted of 1.0 unit of *ExTaq* DNA polymerase (TaKaRa Bio, Ohtsu, Shiga,

Table 1. Location of *Potamogeton* plant material and results of molecular analysis

Species	Locality	Number of individuals	Voucher specimen	ITS	DDBJ Accession number	cpDNA	DDBJ Accession number
P. lucens subsp. *lucens* var. *teganumensis*							
	Oitoike Pond, Fukuoka Prefecture (Control)	1	TNS 9525969	L_{ITS}	AB206990	L_{cp}	AB206987
	Teganuma artificial pond1, Chiba Pref. (POND1)	1	TNS 9525970	L_{ITS}		L_{cp}	
	Teganuma artificial pond2, Chiba Pref. (POND2)	1	TNS 9525971	L_{ITS}		L_{cp}	
	Teganuma artificial pond3, Chiba Pref. (POND3)	9	TNS 9525973-TNS9525981	L_{ITS}		L_{cp}	
P. wrightii							
	Namiki, Ibaraki Prefecture (Control)	1	TNS 9525993	W_{ITS}	AB206991	W_{cp}	AB206988
	Teganuma artificial pond1, Chiba Pref. (POND1)	1	TNS 9525995	W_{ITS}		W_{cp}	
	Teganuma artificial pond2, Chiba Pref. (POND2)	2	TNS 9525996-TNS9525997	W_{ITS}		W_{cp}	
	Teganuma artificial pond3, Chiba Pref. (POND3)	3	TNS 9525999-TNS9526001	W_{ITS}		W_{cp}	
P. ×*inbaensis*							
	Lake Inbanuma, Chiba Prefecture (Control)	1	TNS 9525987	LW_{ITS}		W_{cp}	
	Oitoike Pond, Fukuoka Prefecture (Control)	2	TNS 9525988-TNS9525989	LW_{ITS}		W_{cp}	
	Teganuma artificial pond1, Chiba Pref. (POND1)	1	TNS 9525990	LW_{ITS}		W_{cp}	
	Teganuma artificial pond2, Chiba Pref. (POND2)	1	TNS 9525991	LW_{ITS}		W_{cp}	
	Teganuma artificial pond3, Chiba Pref. (POND3)	1	TNS 9525992	LW_{ITS}		W_{cp}	

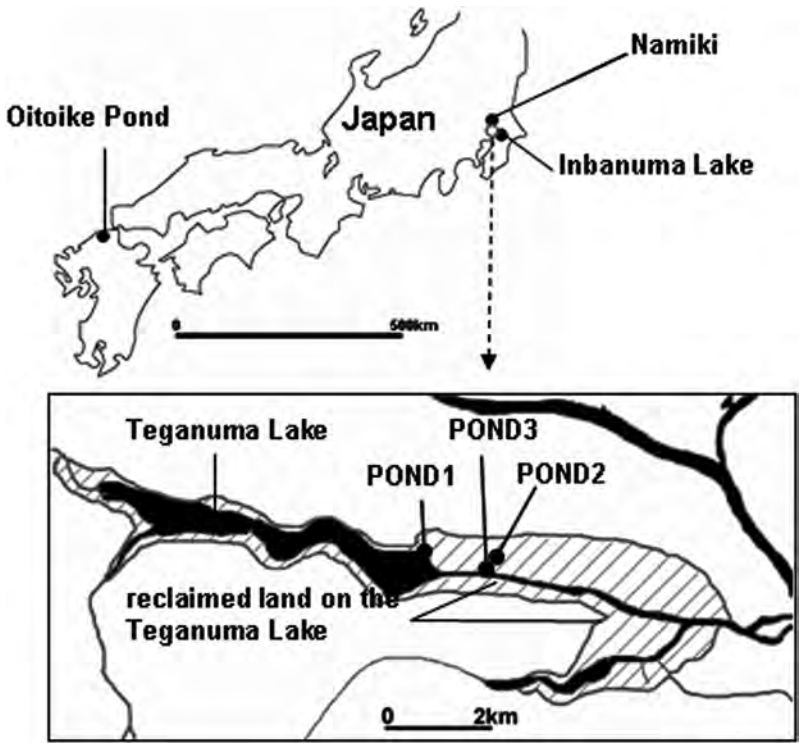

Fig. 1. Sampling locations. Shaded areas show the reclaimed land near Teganuma Lake.

Japan), 1 μL of 10 % *ExTaq* buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 0.8 μL of 0.2 mM dNTP solution, 0.5 μL of each 10 pM primer and 10–30 ng of genomic DNA in a total volume of 11 μL. PCR cycling conditions for amplification of ITS region and *atpB* - *rbcL* region were 96°C for 2 min then 35 cycles of 96°C for 45 sec, 52°C for 30 sec, 72°C for 1 min, and finally 72°C for 5 min.

Direct sequencing and cloning for the standard samples–Nucleotide sequences of PCR products were determined by the direct sequencing or cloning methods. Template DNA fragments were amplified using the ABI PRISM Big Dye Terminator v 3.1 (Applied Biosystems, Foster, California, USA) with the same primers as those used for PCR. DNA sequencing was performed using an ABI PRISM 3100 (Applied Biosystems). The resultant electopherograms of direct sequencing for the ITS region of plants of *P.* ×*inbaensis* showed superimposed waves at the same sites for both complementary strands. These products were ligated into *lac*Z sites of pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) using Ligation High (TOYOBO, Osaka, Japan) and then the recombinant plasmids were transformed into JM109 competent cell (Promega). Ninety-six clones per sample were picked from clones grown on Luria-Bertani agar plates containing ampicillin. Plasmids including the target region were used as template DNA in PCR and their nucleotide sequences were determined.

Sequencing for samples from the artificial ponds–Based on results for the standard plants genotypes of ITS and cpDNA for plants, from the artificial ponds were determined. Nucleotide sequencing of ITS and *atpB* - *rbcL* regions of samples from the artificial ponds were determined using PCR and direct sequencing methods. On sequencing for samples including heterogeneous sequences in ITS region sequencing primers ITS3 of Baldwin (1992) and ITS3_inba2 [5' - GTG GAG ATT GAC CCT CCA TT - 3'] newly designed for the present study were used to separate alleles from PCR products without cloning method.

Morphological analysis–Kadono (1983) used leaf morphological characters to distinguish *P. lucens* subsp. *sinicus* var. *teganumensis*, *P. wrightii* and *P.* ×*inbaensis*. We measured length of petiole, and width and length of leaf lamina for all collected samples (Fig. 2). The ratio of length/width of leaf lamina was used to evaluate the leaf shape.

Kadono (1983) also reported the number of vascular bundles and its arrangement in the stem stele for the three taxa. Wiegleb (1990a) classified the *Potamogeton* species based on the shape of the stem stele. We examined the shape of the transverse section of stem stele using transplanted plants (Fig. 3). Internodes from each shoot were sliced by razor and transverse sections were stained in 0.05 % toluidine blue for a few minutes. Stained samples were observed under a microscope.

Results and Discussion

Molecular markers to identify taxa and hybrid origin–From ITS sequences of the standard samples, two sequence types were distinguished. These types differed from each other at a total 30 sites; 27 substitutions and three indels (Table 2). Each standard plant of *P. lucens* subsp. *sinicus* var. *teganumensis* and *P. wrightii* has a specific sequence, L_{ITS} type (644 bp) and W_{ITS} type (642 bp), respectively. The standard samples of *P.* ×*inbaensis* include L_{ITS} and W_{ITS} types together so that it showed heterozygosity (LW_{ITS} type).

From *atpB* - *rbcL* sequences of the standard samples two sequence types were

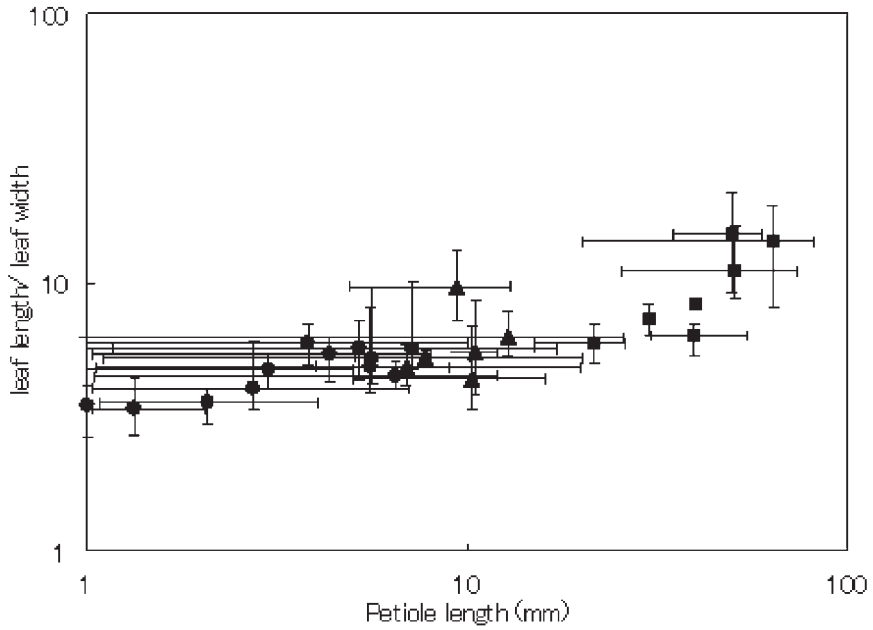

Fig. 2. Comparison of leaf morphological characters i. e., length of petiole and length/ width of leaf lamina that have been used as identification characters since Kadono (1983). The three taxa were identified based on molecular data. Round, square and triangle symbols indicate *Potamageton lucens* subsp. *sinicus* var. *teganumens*, *P. wrightii* and *P.* ×*inbaensis*, respectively. Bars indicate maximum and minimum values in each plants.

found. These types were distinguished by one substitution and two indels (Table 3). The standard sample of *P. lucens* subsp. *sinicus* var. *teganumensis* has a specific sequence L_{CP} type (779 bp) but those of *P. wrightii* and *P.* ×*inbaensis* shared an identical W_{CP} type (783 bp). No *P.* ×*inbaensis* plants with L_{CP} type were observed.

By combining the sequence types of ITS and cpDNA, three taxa are likely to be distinguished based on genotypes. *Potamageton lucens* subsp. *sinicus* var. *teganumensis*, *P. wrightii* and *P.* ×*inbaensis* showed L_{ITS}/L_{CP} genotype, W_{ITS}/W_{CP} genotype and LW_{ITS}/W_{CP} genotype, respectively. The LW_{ITS}/W_{CP} genotype of the standard samples of *P.* ×*inbaensis* supports the view that it originated from a cross between *P. lucens* subsp. *sinicus* var. *teganumensis* and *P. wrightii*. In addition, considering the maternal inheritance of the chloroplast genome, sharing of W_{CP} types suggested that *P. wrightii* is the maternal parent of the standard samples of *P.* ×*inbaensis* and *P. lucens* subsp. *sinicus* var. *teganumensis* is the paternal parent.

Genotyping of plants from the artificial ponds–As it has been reported that maternal or paternal species are not always fixed for other *Potamogeton* hybrid taxa (Fant et al. 2003, 2005), we need to analyze more samples to clarify the process of hybridization occurring *P.* ×*inbaensis*.

Among 20 plants from the artificial ponds, L_{ITS} and W_{ITS} types in ITS and L_{CP} and W_{CP} types in *atpB - rbcL* were detected. Of these, 11 plants showed the L_{ITS}/L_{CP} genotype, six plants the W_{ITS}/W_{CP} genotype and three plants

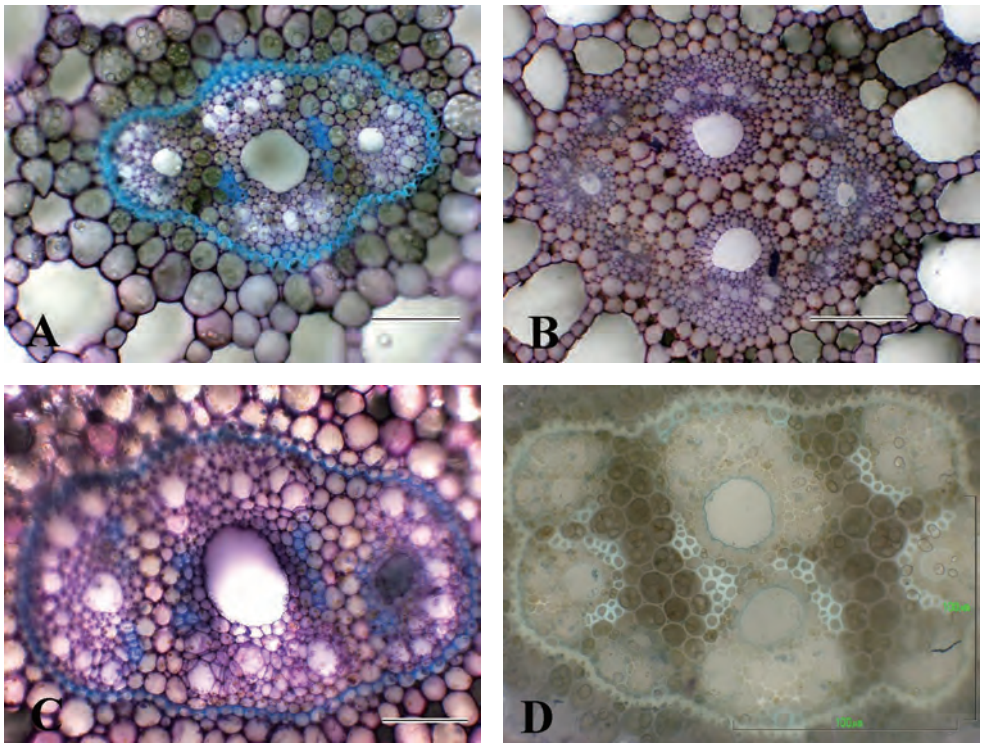

Fig. 3. Stem transverse section of the three taxa of *Potamageton* showing the part of stem stele. A, C: Oblong type with three vascular bundles. B, D: Eight bundle type. A. *P. lucens* subsp. *sinicus* var. *teganumens* from Pond 3 (TNS 9525975) B. *P. wrightii* from Pond 3 (TNS 9526000). C. *P.* ×*inbaensis* from Oitoike Pond (TNS 9525989) D. *P.* ×*inbaensis* from Inbanuma Lake (TNS 9525987). Scale bar = 100 μm.

Table 2. Comparison of the ITS sequences of the *Potamogeton* individuals examined. Only the sites that differed among taxa are shown. 217 and 418 are one base gaps, 613–614 is a two-base gap

Position		1	1	2	3	4	5	8	1 5	1 8	2 1	2 4	2 7	4 0	4 1	4 7	4 7	4 8	4 2	4 8	4 9	4 9	5 1	5 3	5 4	5 5	5 6	5 9	6 1	6 1	6 1	6 2	6 3
		1	4	1	7	8	5	3	5	6	4	7	7	1	7	7	8	4	9	0	6	7	0	8	5	6	5	3	4	9	7	8
Type	L_{ITS}	C	T	C	G	G	T	T	G	A	C	–	G	G	A	C	C	C	T	C	A	A	T	C	C	C	C	T	A	C	C	A
	W_{ITS}	T	G	A	C	T	A	C	T	T	T	T	A	C	C	T	–	T	A	T	G	T	G	T	T	T	T	–	–	T	T	T

Table 3. Comparison of the *atpB-rbcL* sequences from the *Potamogeton* individuals examined. Only the sites that differed among taxa are shown. 179−186 is an eight-base gap, 375−378 is a four-base gap

		1	1	1	1	1	1	1	1	2	3	3	3	3
Position		7	8	8	8	8	8	8	8	7	7	7	7	7
		9	0	1	2	3	4	5	6	8	5	6	7	8
Type	L_{cp}	–	–	–	–	–	–	–	–	T	A	T	T	T
	TW_{cp}	T	C	A	T	T	C	A	A	C	–	–	–	–

the LW_{ITS}/W_{CP} genotype. No plants with LW_{ITS}/L_{CP} type were observed. Based on the results for the standard samples plants with L_{ITS}/L_{CP} genotype, those with W_{ITS}/W_{CP} genotype and those with LW_{ITS}/W_{CP} genotype were identified as *P. lucens* subsp. *sinicus* var. *teganumensis*, *P. wrightii* and *P.* ×*inbaensis*, respectively (Table 1). In all three ponds the three genotypes were found which indicated that three *Potamogeton* taxa occur together.

Potamogeton ×*inbaensis* with LW_{ITS}/W_{CP} observed in the artificial ponds may have originated from buried seeds in the lake sediments or newly produced by crossing *in situ* between *P. lucens* subsp. *sinicus* var. *teganumensis* and *P. wrightii*. Because the three artificial ponds are quite recent the former hypothesis seems to be reasonable.

It is reported that multiple hybridization events have occurred in some *Potamogeton* hybrids (Fant et al. 2003, 2005). In our case, considering the very long geographic distance between the localities of *P.* ×*inbaensis*, hybridization events are supposed to have occurred independently in Inbanuma Lake, a type locality of *P.* ×*inbaensis*, and separately in Oitoike Pond.

Assessment of morphological characters– We assessed morphological variation of the plants identified by the molecular markers (Fig. 2). *Potamogeton lucens* subsp. *sinicus* var. *teganumensis* have shorter petioles and wider leaves than those of *P. wrightii* and these two species are distinguished from each other by petiole length, although these taxa showed extensive morphological variation even within a single individual. Morphological variation of *P.* ×*inbaensis* shows intermediate features and partly overlapps with both of the parental species.

For the characteristic of stem stele, Wiegleb (1990a) suggested that *P. lucens* subsp. *sinicus* var. *teganumensis* had the oblong laminas with three vascular bundles and *P. wrightii* had the eight-bundle type (Fig. 3). However, one plant of *P.* ×*inbaensis* from Inbanuma Lake had the oblong lamina with three vascular bundles while those from other areas, including the artificial ponds, have the eight-bundle type (Fig. 3).

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伊藤　優[a, *]，田中法生[b]，上原浩一[a]：**核 DNA および葉緑体 DNA 塩基配列を用いた *Potamogeton* ×*inbaensis*（ヒルムシロ科）の起源推定**

ヒルムシロ属には，多くの種間雑種が認識されている．これら雑種は一般に両親種の中間的形質を示すが，ヒルムシロ属では特に形態形質の変異が大きいために，両親種の推定や両親種からの識別が容易ではないものが多い．外部形態形質，特に葉形態形質に基づいてガシャモクとササバモの雑種として報告されたインバモ *Potamogeton* ×*inbaensis* Kadono もその一つである．本研究では，核 DNA と葉緑体 DNA の塩基配列を用いて解析した結果，インバモがササバモを母親とし，ガシャモクを父親とする組み合わせで生じた雑種であると推定した．近年増設された印旛沼近くの人工池でも両親種とともにインバモ 3 個体が確認されたが，それらもすべてササバモを母親とする組み合わせで生じていた．地理的距離を考慮すると，雑

種形成は千葉県印旛沼と福岡県お糸池の二ヶ所で独立に起こったと推定される．外部形態形質を比較した結果，インバモの葉形態形質は両親種との間で部分的に重なりが見られ，形態だけでは認識ができない雑種個体があることがわかった．葉形態形質による雑種の識別には注意が必要であり，分子遺伝学的情報はより有益な手段であると考えられる．

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