Plant Syst Evol (2010) 287:57–63
DOI 10.1007/s00606-010-0289-y

ORIGINAL ARTICLE

Identification of hybrids in broad-leaved *Potamogeton* species (Potamogetonaceae) in China using nuclear and chloroplast DNA sequence data

Zhi-Yuan Du · Chun-Feng Yang · Jin-Ming Chen · You-Hao Guo

Received: 10 October 2009 / Accepted: 29 March 2010 / Published online: 25 April 2010

Abstract Hybridization is relatively frequent in the pondweed genus *Potamogeton*. A total of five putative hybrids of broad-leaved *Potamogeton* in China were collected in our recent investigations. We used internal transcribed spacers (ITS) of nuclear ribosomal DNA and chloroplast *rbc*L gene sequences to confirm the origins of the putative hybrids. Using ITS sequence additivity, we confirmed that the five putative hybrids were *P.* × *anguillanus* Koidzumi (*P. wrightii* × *P. perfoliatus*), *P.* × *malainoides* Miki (*P. distinctus* × *P. wrightii*), *P. distinctus* × *P. nodosus*, *P. nodosus* × *P. wrightii*, and *P. distinctus* × *P. gramineus*. The latter four hybrids are new records for China, and *P. distinctus* × *P. gramineus* is a new hybrid combination in *Potamogeton*. We found a new genotype of *P. perfoliatus* in northeast China. Hybrids between the new and a common genotype of *P. perfoliatus* were found in Central China. The maternal parents of the six hybrids were confirmed by chloroplast *rbc*L gene sequence data. The hybrids *P.* × *anguillanus* and *P. distinctus* × *P. gramineus* are reciprocal hybrids. *P.* × *anguillanus* has multiple origins from different populations. *P. distinctus* × *P. gramineus* has multiple origins within a single population.

Keywords Hybridization · *Potamogeton* · ITS · *rbc*L · Broad-leaved

Z.-Y. Du · C.-F. Yang · J.-M. Chen
Key Laboratory of Aquatic Botany and Watershed Ecology, The Chinese Academy of Sciences, Wuhan 430074, Hubei, People's Republic of China

Z.-Y. Du · C.-F. Yang · J.-M. Chen (✉)
Wuhan Botanical Garden, The Chinese Academy of Sciences, Wuhan 430074, Hubei, People's Republic of China
e-mail: jmchen@wbgcas.cn

Y.-H. Guo (✉)
Laboratory of Plant Systematics and Evolutionary Biology, College of Life Sciences, Wuhan University, Wuhan 430072, Hubei, People's Republic of China
e-mail: yhguo@whu.edu.cn

Introduction

The genus *Potamogeton* (Potamogetonaceae) is a "notoriously difficult" group in taxonomy because of its high morphological and ecological diversity (Hagström 1916; Wiegleb 1988). This genus, as traditionally defined, consists of two subgenera, *Potamogeton* and *Coleogeton*. Subgenus *Coleogeton* has long leaf sheaths, characteristic leaf and peduncle anatomy, and a higher ploidy level (hexaploidy) than subgenus *Potamogeton* (generally diploids or tetraploids) (Les and Haynes 1996; Holub 1997; Haynes et al. 1998). Recently, it was suggested that subgenus *Coleogeton* be elevated to the generic level, giving it the correct name *Stuckenia* Börner (Les and Haynes 1996; Holub 1997; Haynes et al. 1998; Lindqvist et al. 2006; Kaplan 2008). Within the current *Potamogeton*, two morphological lineages have been recognized: the broad-leaved species and the linear-leaved species (Iida et al. 2004; Lindqvist et al. 2006; Zhang et al. 2008).

The genus *Potamogeton* is a "classic" example of hybridization in aquatic plants (Les and Philbrick 1993). Preston (1995) reviewed that there were 21 species and 26 hybrids in *Potamogeton* in Great Britain and Ireland (including *Stuckenia*). In Japan, there are 18 species and nine hybrids in *Potamogeton* (Iida et al. 2004). Wiegleb and Kaplan (1998) reviewed the taxonomic treatment of *Potamogeton* and *Stuckenia* worldwide and assessed the validity of all taxa in these genera. At least 69 species

(including 62 species in *Potamogeton*) and more than 50 hybrids (including about 47 hybrids in *Potamogeton*) were considered. According to the description of the *Potamogeton* species in the studies of Wiegleb (1990a, b), Sun (1992), and Wiegleb and Kaplan (1998), we determined that there were about 22 species in *Potamogeton* (excluding *Stuckenia*) distributed in China. Hybridization is relatively frequent in *Potamogeton* and can generate individuals representing a morphological continuum between two species, thereby complicating traditional morphologically based taxonomies (Whittall et al. 2004; Lindqvist et al. 2006). Until now, only a few hybrids were identified in *Potamogeton* in China (e.g., Wang et al. 2007; Zhang et al. 2008; Du et al. 2009).

In field investigations in China, we found that the morphological characteristics of many broad-leaved *Potamogeton* plants were beyond the description of the species in this genus. Some morphological characteristics of these plants were similar to those of hybrids, which have been reported outside of China (Wiegleb 1990a, b; Iida et al. 2007). Due to the limited studies on the identification of hybrids in this genus in China, whether these plants were hybrids or new species in *Potamogeton* remained unclear. A reliable identification based solely on morphology is not always conclusive. In recent years, molecular techniques have been used to identify some putative *Potamogeton* hybrids (e.g., Kaplan and Wolff 2004; Fant et al. 2005; Kaplan and Fehrer 2006, 2007; Iida et al. 2007; Du et al. 2009). As ITS sequences of hybrids often show additive patterns of both parental species, this region has been widely used to identify *Potamogeton* hybrids (Whittall et al. 2000, 2004; Kaplan and Fehrer 2007). In this study, we use nuclear ITS sequence additivity to provide molecular evidence for hybridization in broad-leaved *Potamogeton* species in China. Maternal inheritance of cpDNA in the genus *Potamogeton* was confirmed experimentally (Kaplan and Fehrer 2006). In this study, we use chloroplast *rbc*L gene sequences to identify the maternal parents of the hybrids.

Materials and methods

Plant material and sampling

In China, 12 broad-leaved *Potamogeton* species (*P. wrightii*, *P. lucens*, *P. perfoliatus*, *P. praelongus*, *P. distinctus*, *P. nodosus*, *P. natans*, *P. gramineus*, *P. alpinus*, *P. polygonifolius*, *P. maackianus*, and *P. crispus*) were reported in a previous study (Sun 1992). In our recent field investigations, we did not collect any samples of *P. praelongus*, *P. alpinus*, or *P. polygonifolius* in the investigated areas. *Potamogeton polygonifolius* was only erroneously recorded from China as this species does not occur anywhere in Asia, and the previous records were based on misidentified plants of *P. distinctus* (Wiegleb and Kaplan 1998). Due to the distinct leaf characteristics of *P. maackianus* and *P. crispus*, the two species were unlikely to be the parental species of the putative hybrids. Thus, the two species were excluded from this study. In total, seven broad-leaved *Potamogeton* species were collected from eight provinces in northeastern, northwestern, central, and southwestern China during 2005 and 2008. Because several plants found in the field had distinct or intermediate characteristics compared with the known *Potamogeton* species, we presumed that these plants might have hybrid origins. A total of five putative hybrids were included in this study.

The localities, taxa, number of samples included in the molecular analyses, and GenBank accession sequence numbers are given in Table 1. Voucher specimens were deposited at Wuhan Botanical Garden under the following series: WH0201–WH0293. About 5 g of fresh leaves were harvested from each plant and dried in a zip-top plastic bag containing about 80 g of silica gel. The samples were stored at room temperature until DNA was isolated in the laboratory.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from dried leaf tissue using the CTAB protocol described by Doyle and Doyle (1990).

The nuclear ITS (internal transcribed spacer) sequences were amplified using primers ITS F (King et al. 2001) and ITS4 (White et al. 1990). The chloroplast *rbc*L gene was amplified using primer 26 and primer 1375 described by Iida et al. (2007). The PCR amplification was performed in 25 μl reaction mixtures containing 20 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, and 1 U of *Taq* DNA polymerase (TaKaRa Biotechnology, Dalian). PCR amplification conditions for the ITS region were as follows: an initial pre-denaturation step at 94°C for 5 min; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; with a final extension step of 10 min at 72°C. PCR amplification conditions for the chloroplast *rbc*L gene were the same as for ITS except that the extension time was 2 min. Amplification of genomic DNA was done in a PTC-100 thermocycler (MJ Research). Amplification products were separated on 1.5% agarose gels run at 80 V in 0.5× TBE, visualized by staining with ethidium bromide, and photographed under ultraviolet light. Sizes of amplification products were estimated using a 200 bp DNA ladder (TaKaRa Biotechnology).

PCR amplification products of both ITS region and *rbc*L gene of each sample were purified using PCR product purification kits (SBS Genetech, Shanghai). All the

Table 1 Localities, taxa, number of samples, and GenBank accession sequence numbers for sampled *Potamogeton* specimens

Locality	Taxon	Number	ITS	*rbc*L
Lotus pond, Mishan, Heilongjiang; 45°30′N, 131°51′E	*P. distinctus*	3	FJ956759	FJ956814
	P. perfoliatus	3	FJ956772	FJ956827
	P. lucens	3	FJ956774	FJ956829
Xiaoxingkai Lake, Heilongjiang; 45°21′N, 132°18′E	*P. wrightii*	3	FJ956762	FJ956817
Pond, Zhiyi, Mishan, Heilongjiang; 45°29′N, 130°38′E	*P. natans*	3	FJ956776	FJ956831
Pond, Wanbao, Antu, Jilin; 42°52′N, 128°19′E	*P. natans*	3	FJ956777	FJ956832
Changning River, Weinan, Shanxi; 35°33′N, 109°50′E	*P. wrightii*	11	FJ956763	FJ956818
	P. perfoliatus	12	FJ956770	FJ956825
	P. × *anguillanus*	30	FJ956867 & FJ956868	FJ968680
Shitou River, Taibai, Shanxi; 34°03′N, 107°22′E	*P. distinctus*	3	FJ968813	FJ968814
Xijian River, Linbao, Henan; 34°23′N, 110°46′E	*P. wrightii*	11	FJ956764	FJ956819
	P. perfoliatus	11	FJ956771	FJ956826
	P. × *anguillanus*	21	FJ956869 & FJ956870	FJ968681
	P. × *perfoliatus*	23	FJ956873 & FJ956874	FJ968683
Tucheng River, Yichang, Hubei; 30°39′N, 111°05′E	*P. wrightii*	11	FJ956765	FJ956820
	P. × *anguillanus*	29	FJ956871 & FJ956872	FJ968682
Pond, Baoxia, Yunxian, Hubei; 32°41′N, 110°22′E	*P. nodosus*	3	FJ956767	FJ956822
Anlei River, Danjiang, Hubei; 32°30′N, 110°22′E	*P. nodosus*	2	FJ956768	FJ956823
Pond, Songbai, Shenlongjia, Hubei; 31°44′N, 110°41′E	*P. distinctus* × *P. nodosus*	1	FJ956875 & FJ956876	FJ968684
Pond, Heqing, Yunnan; 26°36′N, 100°10′E	*P. distinctus*	6	FJ956760	FJ956815
	P. natans	3	FJ956778	FJ956833
	P. lucens	3	FJ956775	FJ956830
	P. wrightii	2	FJ956766	FJ956821
	P. nodosus × *P. wrightii*	28	FJ956883 & FJ956884	FJ968688
	P. × *malainoides*	3	FJ956881 & FJ956882	FJ968687
	P. distinctus × *P. nodosus*	2	FJ956877 & FJ956878	FJ968685
Yuhe River, Lijiang, Yunnan; 26°52′N, 100°13′E	*P. nodosus* × *P. wrightii*	14	FJ956885 & FJ956886	FJ968689
Tianshenqiao, Zhongdian, Yunnan; 27°48′N, 99°48′E	*P. distinctus*	13	FJ956761	FJ956816
	P. nodosus	2	FJ956769	FJ956824
	P. distinctus × *P. nodosus*	10	FJ956879 & FJ956880	FJ968686
	P. gramineus	9	FJ956773	FJ956828
Pond, Pudacuo, Zhongdian, Yunnan; 27°46′N, 99°52′E	*P. distinctus* × *P. gramineus*	34	FJ956887 & FJ956888	FJ968690 FJ968691

samples in Table 1 were directly sequenced in both directions using the amplification primers. Sequencing was carried out using the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and performed on an ABI 3730 sequencer (Applied Biosystems). Sequences were assembled using the program SeqMan 5.01 (DNASTAR, Madison, WI) and aligned using CLUSTAL X (Thompson et al. 1997). The alignments were then adjusted manually using the program EditSeq 5.01 (DNASTAR).

For the putative hybrids, ITS polymorphism at variable sites that differed between the parental species was identified as superimposed nucleotides (additive patterns) from chromatograms of direct sequences, and indel polymorphisms in the ITS region were determined according to the site where there was a shift in the direct sequencing reading frame between the two ITS types (Whittall et al. 2000, 2004; Kaplan and Fehrer 2007). For each hybrid population, the purified ITS PCR products of one hybrid sample were cloned into the pUC19 vector (TaKaRa) following the manufacturer's instructions. For each of them, five positive clones were sequenced.

Results

Nuclear ITS ribotypes

The boundaries of the ITS region were determined by comparison with previously published sequences (Wang

et al. 2007). The ITS region (including 5.8S rDNA) of *P. perfoliatus* was 646 bp in length. The length of *P. wrightii*, *P. distinctus*, and *P. nodosus* ITS sequences was 647 bp. The length of the ITS region of *P. gramineus*, *P. natans*, and *P. lucens* was 649 bp. No intra- or inter-population variation was detected within each pure species. For each population, the sequence of only one sample of each species was submitted to GenBank (Table 1). Each of the seven pure species had species-specific ITS sequences. The sequences of the seven species had too many different sites, which could not be shown in a single table, so only pairwise comparisons were carried out. Fifteen substitutions and one indel distinguished the *P. perfoliatus* sequences from the *P. wrightii* sequences (Table 2). The plants of *P. perfoliatus* from Mishan population of Helongjiang province are a new genotype different from other populations (Z. Kaplan and J. Fehrer, personal communication). The ITS sequences of this genotype and the common genotype differed by five substitutions (Table 2). Twenty-five substitutions and three indels distinguished the *P. gramineus* sequences from the *P. distinctus* sequences (Table 3). Six variable sites distinguished the *P. distinctus* or *P. nodosus* sequences from the *P. wrightii* sequences, and two different sites distinguished the sequences of *P. distinctus* and *P. nodosus* (Table 4).

The putative hybrids had superimposed nucleotides of both parental species from chromatograms of direct sequences. For each hybrid population, all the samples showed the same additive pattern in direct sequencing, and one hybrid sample was cloning sequenced. Each of them had five cloned sequences belonging to two types of ITS sequences corresponding to two pure species. For example, one or two sequences were identical to one pure species, and the other four or three were identical to another pure species. Sometimes one clone was a recombined sequence of two species, which was caused by PCR-mediated recombination, and was disregarded for comparison. The type 1 and type 2 ITS sequences of the hybrids in Tables 2, 3, and 4 were based on the cloned sequences and were submitted to GenBank. This phenomenon suggested that the two pure species should be the parental species of the hybrids. The five hybrids were *P.* × *anguillanus* Koidzumi (*P. wrightii* × *P. perfoliatus*), *P.* × *malainoides* Miki (*P. distinctus* × *P. wrightii*), *P. distinctus* × *P. nodosus*, *P. nodosus* × *P. wrightii*, and *P. distinctus* × *P. gramineus*. In the Linbao population, 11 *P. perfoliatus* samples are of a common genotype in China, and the other 23 samples are hybrids between the common genotype and the Mishan genotype.

Chloroplast *rbc*L haplotypes

An 890 bp region of the *rbc*L gene sequence was obtained. The variable nucleotide sites in *rbc*L sequences for

Table 2 Variable nucleotide sites in ITS sequences for *P. wrightii*, two genotypes of *P. perfoliatus*, and their hybrids

Taxon	015	022	035	056	067	074	136	165	187	218	402	412	433	480	481	517	554	559	594	613
P. wrightii (647 bp)	G	A	A	A	A	C	C	G	T	T	C	T	C	A	T	T	C	A	G	C
P. perfoliatus (646 bp)	T	C	T	T	T	T	T	G	A	–	T	A	T	T	C	A	C	G	A	T
P. perfoliatus (Mishan)	T	C	T	T	T	T	C	T	A	–	T	A	T	T	C	A	T	G	C	C
P. × *anguillanus* (type 1)	G	A	A	A	A	C	C	G	T	T	C	T	C	A	T	T	C	A	G	C
P. × *anguillanus* (type 2)	T	C	T	T	T	T	T	G	A	–	T	A	T	T	C	A	C	G	A	T
P. × *perfoliatus* (type 1)	T	C	T	T	T	T	T	G	A	–	T	A	T	T	C	A	C	G	A	T
P. × *perfoliatus* (type 2)	T	C	T	T	T	T	C	T	A	–	T	A	T	T	C	A	T	G	C	C

Table 3 Variable nucleotide sites in ITS sequences for *P. distinctus*, *P. gramineus*, and their hybrids

Taxon	012	038	049	084	156	165	187	215	218	228	402	408	418	421	425	437	445	482	488	504	518	549	556	562	596	614	615	628	639
P. distinctus (647 bp)	T	C	T	C	T	G	T	T	T	A	C	C	T	–	T	A	G	T	G	T	T	T	T	G	T	–	–	T	T
P. gramineus (649 bp)	C	G	G	T	G	T	A	C	–	G	G	A	C	C	C	C	A	C	A	C	A	C	C	A	C	T	A	C	C
P. distinctus × *P. gramineus* (type 1)	T	C	T	C	T	G	T	T	T	A	C	C	T	–	T	A	G	T	G	T	T	T	T	G	T	–	–	T	T
P. distinctus × *P. gramineus* (type 2)	C	G	G	T	G	T	A	C	–	G	G	A	C	C	C	C	A	C	A	C	A	C	C	A	C	T	A	C	C

Table 4 Variable nucleotide sites in ITS sequences for *P. wrightii*, *P. distinctus*, *P. nodosus*, and their hybrids

Taxon	015	022	056	426	436	444	480	561
P. wrightii (647 bp)	G	A	A	C	C	A	A	A
P. distinctus (647 bp)	T	C	T	C	A	G	T	G
P. nodosus (647 bp)	T	C	T	G	C	G	T	G
P. × *malainoides* (type 1)	G	A	A	C	C	A	A	A
P. × *malainoides* (type 2)	T	C	T	C	A	G	T	G
P. distinctus × *P. nodosus* (type 1)	T	C	T	C	A	G	T	G
P. distinctus × *P. nodosus* (type 2)	T	C	T	G	C	G	T	G
P. nodosus × *P. wrightii* (type 1)	G	A	A	C	C	A	A	A
P. nodosus × *P. wrightii* (type 2)	T	C	T	G	C	G	T	G

P. wrightii, *P. perfoliatus*, *P. perfoliatus* (Mishan genotype), *P. distinctus*, *P. gramineus*, and *P. nodosus* are given in Table 5. No intra- or interpopulation variation was identified within each pure species. For each population, the sequence of only one sample of each species was submitted to GenBank (Table 1). All the 30 *P.* × *anguillanus* samples from the Weinan population had *rbc*L sequences identical to *P. wrightii*, and all the *P.* × *anguillanus* samples from the Yichang and Linbao populations had *rbc*L sequences identical to *P. perfoliatus*. The hybrids *P. distinctus* × *P. gramineus* from a pond of Zhongdian population had either *P. distinctus* or *P. gramineus rbc*L sequences. Twenty-three samples had *P. distinctus* sequences, and 11 samples had *P. gramineus* sequences. The hybrids *P.* × *anguillanus* and *P. distinctus* × *P. gramineus* should therefore be reciprocal hybrids that originated from crosses in both directions. Intrapopulation variation and reciprocal hybridization was not detected in the other four hybrids. *Potamogeton* × *malainoides* accessions from the Heqing population had *rbc*L sequences identical to *P. distinctus*. The *rbc*L sequences of *P. nodosus* × *P. wrightii* from the Heqing and Lijiang populations were identical to *P. nodosus*. As the *rbc*L sequences of *P. distinctus* and *P. nodosus* were identical, it was impossible to identify the maternal parent of their hybrid. In the Linbao population, all the *P. perfoliatus* hybrids between the common genotype and the Mishan genotype had *rbc*L sequences identical to the common genotype.

Table 5 Variable nucleotide sites in *rbc*L sequences for two genotypes of *P. perfoliatus*, *P. wrightii*, *P. distinctus*, *P. nodosus*, *P. gramineus*, and their hybrids

Taxon	042	220	236	281	355	529
P. perfoliatus	C	A	C	G	A	T
P. perfoliatus (Mishan)	T	A	C	G	A	T
P. wrightii	T	A	C	C	T	G
P. distinctus	T	A	C	G	T	G
P. nodosus	T	A	C	G	T	G
P. gramineus	T	C	G	G	T	T
P. × *anguillanus* (Weinan)	T	A	C	C	T	G
P. × *anguillanus* (Yichang & Linbao)	C	A	C	G	A	T
P. distinctus × *P. gramineus* (type 1)	T	A	C	G	T	G
P. distinctus × *P. gramineus* (type 2)	T	C	G	G	T	T
P. × *perfoliatus* (Linbao)	C	A	C	G	A	T
P. × *malainoides*	T	A	C	G	T	G
P. distinctus × *P. nodosus*	T	A	C	G	T	G
P. nodosus × *P. wrightii*	T	A	C	G	T	G

Discussion

A total of six hybrids of broad-leaved species were successfully identified. In our previous study, we confirmed that *P.* × *anguillanus* from the Shanxi and Hubei provinces in China originated from hybridization between *P. perfoliatus* and *P. wrightii* (Du et al. 2009). *Potamogeton wrightii* and *P. perfoliatus* are clearly differentiated by leaf shape. The leaves of *P. perfoliatus* are sessile with lanceolate blades and amplexicaul at the base, whereas those of *P. wrightii* are petiolate with oblong linear blades and cuneate at the base. In our recent field investigation, in Linbao of the Henan province, we found some *Potamogeton* plants similar to *P.* × *anguillanus*. Their morphological characteristics were intermediate between the putative parental species (*P. wrightii* and *P. perfoliatus*). Their leaves were sessile, linear-lanceolate, and cuneate at the base. Their blade margin was undulate and the blade was introflexed. Their cloned ITS sequences had two types of sequences corresponding to either *P. wrightii* or *P. perfoliatus*, which indicated that these samples were the hybrid *P.* × *anguillanus*. Using chloroplast *rbc*L gene sequences, we found that the maternal parent of the hybrid *P.* × *anguillanus* from the Linbao and Yichang populations was *P. perfoliatus*, and *P. wrightii* was the maternal parent of *P.* × *anguillanus* from the Weinan population. Both parental species were found in the Weinan and Linbao populations. The maternal parent *P. perfoliatus* was not found in the Yichang population, but it appeared at the nearby sites. *Potamogeton* × *anguillanus* is a reciprocal hybrid with multiple origins from different populations.

In the genus *Potamogeton*, *P. distinctus* and *P. wrightii* have an East and Southeast Asian distribution, and *P. distinctus* is closely related to *P. nodosus* (Wiegleb 1990a, b). The ITS sequences of *P. distinctus* and *P. nodosus* had only two different sites, while six variable

sites distinguished the *P. distinctus* or *P. nodosus* sequences from the *P. wrightii* sequences. The blade shapes of *P. distinctus* and *P. nodosus* are very similar, e.g., they have lanceolate floating leaves and their submerged leaves are membranous and narrowly lanceolate. Their main difference is that *P. nodosus* usually has four carpels and *P. distinctus* usually has two carpels. The three species, *P. distinctus*, *P. wrightii*, and *P. nodosus*, could create three hybrids (Wiegleb 1990a, b). In this study, we identified several collections from the Heqing population of the Yunnan province as the hybrid *P.* × *malainoides* (*P. distinctus* × *P. wrightii*), several collections as the hybrid *P. nodosus* × *P. wrightii*, and several collections as the hybrid *P. distinctus* × *P. nodosus*. The parental species *P. distinctus* and *P. wrightii* were found there. We did not collect any *P. nodosus* samples from the Heqing population, but it may appear at the nearby sites. The hybrid *P. nodosus* × *P. wrightii* was also found in the Lijiang population of the Yunnan province. Neither parent was found in the sampling river, but they appeared at the nearby pond. The hybrid *P. distinctus* × *P. nodosus* was also identified from the Tianshenqiao population of the Yunnan province and from the Shengnongjia population of the Hubei province. Both parents were found in these populations. These three confirmed hybrids belong to the hybrids that have been reviewed by Wiegleb (1990a, b), and these are their first records in China.

The hybrid *P.* × *malainoides* from the Heqing population had both floating and submerged leaves, but there was no obvious difference between the two types of leaves. They had a blade shape similar to *P. wrightii* with long petiole, parallel margins, and acute apex, and both of them were subcoriaceous. When they were taken out of the water, it was difficult to distinguish them. The hybrid *P.* × *malainoides* from the Heqing population had four carpels as does *P. wrightii*. The hybrid *P. nodosus* × *P. wrightii* had similar morphology to *P.* × *malainoides*. The two populations of *P. nodosus* × *P. wrightii* were found in fast running waters, and no leaves were floating on the water surface. The hybrids *P. distinctus* × *P. nodosus* were in the vegetative state. They had the same leaf morphology as the parental species and could not be identified morphologically.

The genetic distance between *P. distinctus* and *P. gramineus* was large. A total of 25 substitutions and three indels distinguished the *P. gramineus* ITS sequences from the *P. distinctus* sequences. They also had many morphological differences. The floating leaves of *P. gramineus* were much smaller than those of *P. distinctus*. *Potamogeton gramineus* had sessile small lanceolate submersed leaves. *Potamogeton distinctus* usually has two carpels and *P. gramineus* usually has four carpels. No previous study has found hybridization between these two species. However, using ITS sequences, we identified the collections from the Pudacuo population in the Yunnan province as the hybrid *P. distinctus* × *P. gramineus*. The hybrids had floating leaves medium size between *P. distinctus* and *P. gramineus*. Their submerged leaves were similar to *P. distinctus* and decayed early. The hybrid plants had four carpels as does *P. gramineus*. Neither parent appeared in the sampling pond, but they appeared at the nearby pond in Tianshenqiao where *P. distinctus* × *P. gramineus* was not found. Both parental species could be the maternal parent of the hybrid *P. distinctus* × *P. gramineus* from the Pudacuo population. This indicates that *P. distinctus* × *P. gramineus* is a reciprocal hybrid and has multiple origins within a single population, which is not a typical pattern in *Potamogeton*. Most of the hybrids seemed sterile. Some were in the vegetative state without flowers, some produced flowers with abortive carpels, and some could set a few fruits with unmature embryos. More long-term population investigations need to be done to find if there are some fertile hybrids. The fruits of the hybrids need to be anatomized to find whether the embryos are abortive or mature. Seed germination experiments could tell whether the hybrids are truly fertile.

In this study, we found a new genotype of *P. perfoliatus* in northeastern China (Z. Kaplan and J. Fehrer, personal communication). Its leaves were thinner than other *P. perfoliatus* samples. The common genotype of *P. perfoliatus* is distributed over most of China. The hybrids between these two genotypes were collected in central China. Their leaves and fruits were similar to the common genotype and could not be distinguished morphologically. This is the only hybrid we found between two genotypes of a species. Their maternal parents were the common genotype. The paternal genotype was not found in the Linbao population.

Several confirmed broad-leaved *Potamogeton* hybrids, such as *P. natans* × *P. lucens*, *P. natans* × *P. nodosus*, *P. alpinus* × *P. nodosus*, *P. perfoliatus* × *P. gramineus*, and *P. perfoliatus* × *P. lucens*, have been reported in Europe based on molecular evidence (Hollingsworth et al. 1995; Fant et al. 2001; Kaplan et al. 2002; Kaplan and Wolff 2004; Kaplan and Fehrer 2006, 2009; Kaplan 2007; Zalewska-Galosz et al. 2009). All parental species of these hybrids are distributed in China, and several species often grow together, which provides the opportunity for hybridization. However, these hybrids were not found in the current study, which might be due to our limited collection of samples. In future work, more samples from more regions should be included, which would be valuable for understanding the extent of hybridization in *Potamogeton*.

Acknowledgments The authors would like to thank Dr. Zdenek Kaplan and Dr. Judith Fehrer for giving comments on this article and for identifying the two genotypes of *P. perfoliatus*. We also thank

Xiao-Lin Zhang, Tao Wan, and Yi-Hong Zhen for their help in the fieldwork. This study was supported by grants from the National Natural Science Foundation of China (No. 30430050 and No. 30800061).

References

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Du ZY, Yang CF, Chen JM, Guo YH (2009) Nuclear and chloroplast DNA sequences data support the origin of *Potamogeton intortusifolius* J.B. He in China as a hybrid between *P. perfoliatus* Linn. and *P. wrightii* Morong. Aquat Bot 91:47–50

Fant JB, Preston CD, Barrett JA (2001) Isozyme evidence of the parental origin and possible fertility of the hybrid *Potamogeton* × *fluitans* Roth. Plant Syst Evol 229:45–57

Fant JB, Kamau E, Preston CD (2005) Chloroplast evidence for the multiple origins of the hybrid *Potamogeton* × *fluitans*. Aquat Bot 83:154–160

Hagström JO (1916) Critical researches on the *Potamogeton*. Kungl Svenska Vetenskapsakad Handl 55:1–281

Haynes RR, Les D, Král M (1998) Two new combinations in *Stuckenia*, the correct name for *Coleogeton* (Potamogetonaceae). Novon 8:241

Hollingsworth PM, Preston CD, Gornall RJ (1995) Isozyme evidence for hybridization between *Potamogeton natans* and *P. nodosus* (Potamogetonaceae) in Britain. Bot J Linn Soc 117:59–69

Holub J (1997) *Stuckenia* Börner: the correct name for *Coleogeton* (Potamogetonaceae). Preslia 69:361–366

Iida S, Kosuge K, Kadono Y (2004) Molecular phylogeny of Japanese *Potamogeton* species in light of noncoding chloroplast sequences. Aquat Bot 80:115–127

Iida S, Yamada A, Amano M, Ishii J, Kadono Y, Kosuge K (2007) Inherited maternal effects on the drought tolerance of a natural hybrid aquatic plant, *Potamogeton anguillanus*. J Plant Res 120:473–481

Kaplan Z (2007) First record of *Potamogeton* × *salicifolius* for Italy, with isozyme evidence for plants collected in Italy and Sweden. Pl Biosyst 141:344–351

Kaplan Z (2008) A taxonomic revision of *Stuckenia* (Potamogetonaceae) in Asia, with notes on the diversity and variation of the genus on a worldwide scale. Folia Geobot 43:159–234

Kaplan Z, Fehrer J (2006) Comparison of natural and artificial hybridization in *Potamogeton*. Preslia 78:303–316

Kaplan Z, Fehrer J (2007) Molecular evidence for a natural primary triple hybrid in plants revealed from direct sequencing. Ann Bot 99:1213–1222

Kaplan Z, Fehrer J (2009) An orphaned clone of *Potamogeton* × *schreberi* in the Czech Republic. Preslia 81:387–397

Kaplan Z, Wolff P (2004) A morphological, anatomical and isozyme study of *Potamogeton* × *schreberi*: confirmation of its recent occurrence in Germany and first documented record in France. Preslia 76:141–161

Kaplan Z, Plačková I, Štěpánek J (2002) *Potamogeton* × *fluitans* (*P. natans* × *P. lucens*) in the Czech Republic. II. Isozyme analysis. Preslia 74:187–195

King RA, Gornall RJ, Preston CD, Croft JM (2001) Molecular confirmation of *Potamogeton* × *bottnicus* (*P. pectinatus* × *P. vaginatus*) (Potamogetonaceae) in Britain. Bot J Linn Soc 135:67–70

Les DH, Haynes R (1996) *Coleogeton* (Potamogetonaceae), a new genus of pondweeds. Novon 6:389–391

Les DH, Philbrick CT (1993) Studies of hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. Aquat Bot 44:181–228

Lindqvist C, Laet JD, Haynes RR, Aagesen L, Keener BR, Albert VA (2006) Molecular phylogenetics of an aquatic plant lineage, Potamogetonaceae. Cladistics 22:568–588

Preston CD (1995) Pondweeds of Great Britain and Ireland. Botanical Society of the British Isles, London

Sun XZ (1992) Flora of China (Tomus 8). Science Press, Beijing

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 24:4876–4882

Wang QD, Zhang T, Wang JB (2007) Phylogenetic relationships and hybrid origin of *Potamogeton* species (Potamogetonaceae) distributed in China: insights from the nuclear ribosomal internal transcribed spacer sequence (ITS). Plant Syst Evol 267:65–78

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Whittall JB, Liston A, Gisler S, Meinke RJ (2000) Detecting nucleotide additivity from direct sequences is a SNAP: an example from *Sidalcea* (Malvaceae). Plant Biol 2:211–217

Whittall JB, Hellquist CB, Schneider EL, Hodges SA (2004) Cryptic species in an endangered pondweed community (*Potamogeton*, Potamogetonaceae) revealed by AFLP markers. Am J Bot 91:2022–2029

Wiegleb G (1988) Notes on pondweeds—outlines for a monographical treatment of the genus *Potamogeton* L. Feddes Repert 99:249–266

Wiegleb G (1990a) A redescription of *Potamogeton distinctus* including remarks on the taxonomy of the *Potamogeton nodosus* group. Plant Syst Evol 169:245–259

Wiegleb G (1990b) A redescription of *Potamogeton wrightii* (Potamogetonaceae). Plant Syst Evol 170:53–70

Wiegleb G, Kaplan Z (1998) An account of the species of *Potamogeton* L. (Potamogetonaceae). Folia Geobot 33:241–316

Zalewska-Gałosz J, Ronikier M, Kaplan Z (2009) The first European record of *Potamogeton* × *subobtusus* identified using ITS and cpDNA sequence data. Preslia 81:281–292

Zhang T, Wang QD, Li W, Cheng Y, Wang JB (2008) Analysis of phylogenetic relationships of *Potamogeton* species in China based on chloroplast *trn*T-*trn*F sequences. Aquat Bot 89:34–42