Comparison of natural and artificial hybridization in *Potamogeton*

Porovnání přirozené a experimentální hybridizace v rodu *Potamogeton*

Zdeněk K a p l a n & Judith F e h r e r

Institute of Botany, Academy of Sciences of the Czech Republic, CZ-252 43 Průhonice, Czech Republic, e-mail: kaplan@ibot.cas.cz, fehrer@ibot.cas.cz

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

The first attempt to artificially hybridize species of *Potamogeton* resulted in the hybrid *P. perfoliatus* × *P. gramineus*. The morphological features, reproductive behaviour and molecular markers of the offspring of this experimental hybridization were compared with those of the parental species and natural hybrids of the same assumed parentage. A phenotype corresponding to that of the natural hybrid *P. × nitens* was acquired from an experimental cross between *P. perfoliatus* and *P. gramineus*. All plants, both natural and artificial, of this hybrid were consistently sterile. They showed the ITS variants of both parental taxa, which is consistent with biparental inheritance of nuclear DNA. The experimental hybrid was used to test the maternal inheritance of chloroplast DNA in *Potamogeton*. Sequences of a chloroplast intergenic spacer (*rpl*20-*rps*12) were identical with those of the female parent. Then, the directions of the crosses resulting in the natural hybrids were investigated. Of five natural populations of *P. × nitens*, *P. gramineus* was the maternal parent of two and *P. perfoliatus* of three populations. The frequency of hybridization events and rise of hybrids are discussed.

K e y w o r d s : clonality, cpDNA, hybridization, internal transcribed spacer, plant taxonomy, *Potamogeton*, recurrent origin, sterility, variation

Introduction

For more than a century it has been known that hybrids make up an important component of *Potamogeton* diversity. However, for a long time the knowledge on them came only from morphological studies. The identification of many hybrids has been refined over the course of time. In Europe, this knowledge culminated in the monograph of British pondweeds by Preston (1995), which shows that precise analysis of diagnostic characters and detailed knowledge of the range of variation of the species make many hybrids recognizable entities. A recent study by Kaplan & Fehrer (2004), based on a combination of morphological and molecular approaches, proved that careful and detailed morphological examination can indeed lead to the identification of some hybrids and that these can be reliably distinguished from extreme morphotypes of species imitating hybrids. On the other hand, a worldwide revision of the genus by Wiegleb & Kaplan (1998) indicates that the great majority of recognizable hybrids are between rather dissimilar broad-leaved species or have a broad-leaved species as one of their parents. Also, most of the isozyme or DNA-based molecular evidence for hybridization in this cosmopolitan genus comes from a relatively small area of Europe (British Isles, France, Germany, Denmark, Czech Republic and Italy; Hollingsworth et al. 1995, 1996, Preston et al. 1998, King et al. 2001, Fant et al. 2001a, b, 2003, 2005, Kaplan et al. 2002, Kaplan & Fehrer 2004, Kaplan & Wolff 2004, Fant & Preston 2004, Kaplan 2006) and a few solitary records from Japan (Honshu; Iida & Kadono 2002) and the USA (Texas;

Whittall et al. 2004). The incidence of hybridization between linear-leaved species and in the Southern Hemisphere is entirely unknown.

The investigation of stem anatomy patterns was, besides morphological examination, the only method that gave additional information on species differences and relationships (e.g., Raunkiaer 1896, 1903, Fischer 1904, 1905, 1907, Hagström 1916, Ogden 1943, 1974a, b, Symoens et al. 1979, Tur 1982, Wiegleb 1990a, b, c, Kaplan 2001, 2005a, b, Kaplan & Wolff 2004, Kaplan & Symoens 2004, 2005). Biosystematic research based on controlled experiments on living plants was generally not used. Cultivation of selected *Potamogeton* plants by Alfred Fryer was the only notable exception (see his observations in Fryer 1890, 1894, 1898–1900, and Preston 1988 for a review of Fryer's work). Only recently, cultivation experiments were employed by Kaplan (2002) to assess the contribution of phenotypic plasticity to the total morphological variation. An extensive literature search revealed that no one had ever attempted to produce *Potamogeton* hybrids artificially (see also the negative comments in Preston 1988: 22, Les & Philbrick 1993: 185, Hollingsworth et al. 1995: 60, Preston 1995: 42, and Brayshaw 2000: 93). As pointed out by Hollingsworth et al. (1995) the reason was the considerable practical difficulties in growing and crossing *Potamogeton* species.

Biosystematic studies involving experimental hybridization require a lot of time and effort, but can bring substantial insights into the particular group concerned (see the recent discussion on this topic by Crawford et al. 2005, also some recent papers in this journal: Khalaf & Stace 2000, Krahulec et al. 2004, 2005, Jarolímová 2005, Mráz & Paule 2006). This is why experimental hybridization was included in our broadly based long-term research on *Potamogeton*. The aim of this study was to test interspecific crossability of *Potamogeton* species, to compare natural and experimental hybrids using traditional taxonomic and molecular approaches, and to investigate their viability and breeding behaviour. To confirm the identity of the hybrids, PCR-RFLPs of a nuclear marker (the internal transcribed spacer region of ribosomal DNA) were used to demonstrate the contribution of both parental species, and sequences of a chloroplast intergenic spacer (between genes coding for ribosomal proteins L20 and S12) to infer the maternal parent. Analyses of chloroplast DNA regions were already used by Fant et al. (2003, 2005) and Kaplan & Fehrer (2004) to identify the direction of the cross under the general assumption that chloroplast DNA is maternally inherited in the majority of angiosperms (Birky 1995). Another goal of the present study was to test this assumption for *Potamogeton* using an experimentally produced hybrid plant.

Material and methods

Plant material

Samples from 173 populations of 39 *Potamogeton* species from various parts of the world were cultivated in the Experimental Garden at the Institute of Botany, Průhonice, Czech Republic (for the list of taxa grown from 1995 to 2000 see Table 1 in Kaplan 2002). Plants were grown in water-filled laminate tanks of two sizes ($180 \times 140 \times 80$ and $200 \times 120 \times 35$ cm), which were sunk in the ground in order to prevent overheating of the water in summer. The samples were planted in submerged plastic pots containing previously desiccated pond mud.

Table 1. – Origins and reference numbers of *Potamogeton* samples used in crossing experiments.

Taxon	Ref. no.	Origin and field collection records
P. alpinus	338	Czech Republic, Malšova Lhota, 8 X 1996, coll. Z. Kaplan 96/681
P. berchtoldii	910	Czech Republic, Žamberk, 14 IX 1997, coll. Z. Kaplan 97/907
	927	Czech Republic, Týniště nad Orlicí, 5 IX 1997, coll. Z. Kaplan 97/852
	1224	Czech Republic, Skalná, 10 VIII 2000, coll. Z. Kaplan 00/178
P. gramineus	318	Czech Republic, Hrobice, 9 IX 1996, coll. Z. Kaplan 96/624
	331	Czech Republic, Hradčany u Mimoně, 18 IX 1996, coll. Z. Kaplan 96/638
P. pectinatus	841	Czech Republic, Strážnice, 25 VI 1997, coll. Z. Kaplan 97/509
P. perfoliatus	840	Czech Republic, Ostrožská Nová Ves, 25 VI 1997, coll. Z. Kaplan 97/524
P. praelongus	249	Czech Republic, Malšova Lhota, 18 VII 1996, coll. Z. Kaplan 96/396
P. pusillus	307	Czech Republic, Třeboň, 21 VIII 1996, coll. Z. Kaplan 96/627
	987/2	Austria, Bodensee, 23 VI 1998, coll. Z. Kaplan
	1159	Czech Republic, Lomnice nad Lužnicí, 8 IX 1999, coll. Z. Kaplan
P. trichoides	828	Slovakia, Malacky, 15 VI 1997, coll. Z. Kaplan 97/340

Because of the difficulties of cultivating *Potamogeton* plants and of getting two particular plants to flower at the same time, only those of the cultivated plants that flowered simultaneously were finally cross-pollinated. The species used for crossing experiments and the origins and reference numbers of the respective specimens are summarized in Table 1. The hybrid resulting from the crosses was compared using molecular markers with a representative collection of parental species and natural hybrids of the same parentage; these samples are listed in Table 2. The taxonomic delimitations and nomenclature of the species follow Wiegleb & Kaplan (1998); the species concept for the European taxa of the *P. pusillus* agg. was updated in Kaplan & Štěpánek (2003).

Crossing

Because of their structure, the bisexual flowers of *Potamogetonaceae* cannot be emasculated before crossing is performed (Guo & Cook 1990, Kaplan & Štěpánek 2003). The anthers are enclosed on the inner side of the concave tepals, and their filaments are fused with the bases of the tepals. When the tepals open, the anthers have already released pollen grains, many of which stick on the stigma. As the flowers are self-compatible, self-fertilization is effected. This is the most frequent and effective mode of pollination in *Potamogeton* (Kaplan & Štěpánek 2003), which precludes experimental crossing at full flowering. Unfortunately, any attempt to remove the anthers from young flower buds fatally damages the flowers. For this reason, attempts were made to utilize the occurrence of protogyny in *Potamogeton* flowers and to pollinate the stigma before the anthers dehisce. During the early female phase, the tepals are closed and protect the anthers. However, the stigma protrudes through the tepals and is ready to receive pollen one day (or perhaps even a few days) before the anthers of the same flower open. At this stage of flower development the stigmas were artificially covered with pollen from flowers of another species.

The crossing experiments were performed in the experimental garden during the summers of 1997–2004. Pollen was transfered between the following species (the first named species in a pair acted as the female plant): *P. alpinus* × *P. gramineus*, *P. alpinus* × *P. pusillus*, *P. berchtoldii* × *P. pusillus*, *P. berchtoldii* × *P. trichoides*, *P. gramineus* × *P. berchtoldii*, *P. perfoliatus* × *P. alpinus*, *P. perfoliatus* × *P. gramineus*, *P. perfoliatus* ×

P. pectinatus, *P. perfoliatus* × *P. praelongus*, and *P. pusillus* × *P. alpinus*. Any fruits that developed were collected when they appeared to be fully mature and kept over winter in a refrigerator at a temperature of 2–6 °C in order to break seed dormancy. In the following spring, the fruits were placed in small water-filled plastic containers at about 20 °C. This sudden increase in temperature usually stimulates germination of the seeds (Kaplan & Štěpánek 2003). Young seedlings were then transferred to the cultivation tanks in the garden and grown to the adult stage.

Evaluation of progeny

The progeny from cross-pollinations involving broad-leaved species were first analysed morphologically. This was possible particularly for offspring from crosses of very dissimilar species (a broad-leaved crossed with a linear-leaved species, which occurred most frequently). The directions of the crosses between broad-leaved species were chosen so that the more distinctive species was the maternal plant. The offspring were first propagated clonally to produce several ramets and then grown to the adult stage. Their morphological features and, where appropriate, stem anatomy (for the importance of anatomical features for identification see the references in the introduction) and reproductive behaviour were analysed.

Because of their greatly reduced morphology and the similarity of the taxa within the *P. pusillus* agg., isozyme analysis (see e.g. Kaplan et al. 2002, Kaplan & Štěpánek 2003, Kaplan & Wolff 2004, Kaplan 2006) was used to evaluate the progeny of crosses between the linear-leaved species (the crosses *P. berchtoldii* × *P. pusillus* and *P. berchtoldii* × *P. trichoides*). Isozyme phenotypes of these three species were surveyed by Kaplan & Štěpánek (2003), who identified species-specific banding patterns for each of these species.

The offspring that was found to be a true hybrid was further analysed using nuclear and chloroplast DNA markers.

Molecular analyses of experimental and natural hybrids

DNA isolations were performed using the sorbitol extraction method (Štorchová et al. 2000) employing the slight modifications described in Kaplan & Fehrer (2004).

M a t e r n a l   t r a n s m i s s i o n   o f   c p D N A

In order to test for maternal inheritance of the chloroplast DNA in *Potamogeton*, sequences of the experimentally produced hybrid *P. perfoliatus* × *P. gramineus* (907) were compared with those of its respective parents. As the *P. gramineus* parental plant was not available for molecular study, parental sequences of the conservative *rbc*L already available in GenBank (accession numbers U80723 and U80724, Les et al. 1997) were used for comparison. A 576 bp-fragment of the *rbc*L gene was PCR-amplified using primers RH1S (Petersen & Seberg 2003) and J556R (Drábková et al. 2003) under the following conditions: 50 μl-reactions contained 5 μl of Mg^{2+}-free reaction buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, a few nanograms of genomic DNA, 1 unit of *Taq* DNA-polymerase (MBI Fermentas) and 0.5 mM of each primer. 35 cycles were done at 94 °C–30 s, 45 °C–45 s, 72 °C–60 s with an initial pre-denaturation at 94 °C for 4 min and a final extension at 72 °C for 10 min. The product was purified, sequenced in both directions and the sequence submitted to GenBank (accession no AY545465, see also Table 2).

Table 2. – Origins, reference numbers and GenBank accession numbers of *Potamogeton* samples used for the molecular comparison. Boldface numbers indicate identical cpDNA sequence between a particular *P. gramineus* sample and *P. × nitens*; numbers in italics indicate cpDNA sequence identity between *P. perfoliatus* samples and *P. × nitens*. The *rbc*L sequence of the experimental hybrid is identical to a GenBank sequence of *P. perfoliatus* (U80724).

Taxon	Ref. no.	Origin and field collection records	GenBank accession no.		
			ITS	*rbc*L	*rpl*20–*rps*12
P. gramineus	885	Czech Republic, Rozkoš Reservoir, 22 VIII 1997, coll. Z. Kaplan 97/829			DQ468864
	897	Czech Republic, Hradčany u Mimoně, 18 IX 1996, coll. Z. Kaplan 96/638	DQ468860		**DQ468866**
	1156	France, Scorff River, VI 1998, coll. J. Květ			
	1285	France, Rémelfing, 21 VII 2001, coll. P. Wolff	DQ468861		DQ468865
	1489	Czech Republic, Vysoká u Jihlavy, 14 VIII 2003, coll. Z. Kaplan 03/188			
P. perfoliatus	840	Czech Republic, Ostrožská Nová Ves, 25 VI 1997, coll. Z. Kaplan 97/524			
	979	Switzerland, Altenrhein, 23 VI 1998, coll. Z. Kaplan 98/125	AY529527		*DQ468862*
	985	Austria, Fußach, 23 VI 1998, coll. Z. Kaplan 98/131			
	1002	Sweden, Björka, 12 VIII 1998, coll. Z. Kaplan 98/338	AY529526		*DQ468863*
	1467	Czech Republic, Martinov, 6 VI 2003, coll. J. Hummel, in herb. Z. Kaplan 03/130			
	1469	Czech Republic, Doubrava, 13 VI 2003, coll. J. Rydlo, in herb. Z. Kaplan 03/139			
	1470	Germany, Ebing, 11 VI 2003, coll. L. Meierott	AY529525		
	1481	Czech Republic, Staré Splavy, 3 VIII 2003, coll. Z. Kaplan 03/161			
	1531	Italy, Lago di Muta, 8 VI 2004, coll. Z. Kaplan & J. Štěpánková 04/63			
P. × nitens from the field	879	Germany, Gültz See, 15 VIII 1997, coll. Z. Kaplan 97/828			*DQ468868*
	999	Sweden, Björka, 12 VIII 1998, coll. Z. Kaplan 98/335			**DQ468871**
	1000	Sweden, Björka, 12 VIII 1998, coll. Z. Kaplan 98/336			**DQ468873**
	1003	Sweden, Björka, 12 VIII 1998, coll. Z. Kaplan 98/339			**DQ468874**
	1007	Sweden, Sillen Lake, 13 VIII 1998, coll. Z. Kaplan 98/344			*DQ468870*
	1015	Sweden, Metsjön Lake, 15 VIII 1998, coll. Z. Kaplan 98/358			**DQ468875**
	1016	Sweden, Metsjön Lake, 15 VIII 1998, coll. Z. Kaplan 98/360			**DQ468872**
	1019	Sweden, Stora Ullevifjärden Lake, 15 VIII 1998, coll. Z. Kaplan 98/363			*DQ468869*
P. × nitens from experimental crossing	907	artificially synthesized hybrid from the cross 840 × 318		AY545465	*DQ468867*

Internal transcribed spacer (ITS)

Amplification, sequencing of two samples from each parental species and the choice of an RFLP (restriction fragment length polymorphism) marker that distinguished between the respective species was done as described in Kaplan & Fehrer (2004). The restriction enzyme *Taq*I was found to produce several species-specific bands and well-distinguishable fragment sizes. A larger selection of samples from each parent and all hybrids from different localities (Table 2) were subjected to RFLPs of the PCR-amplified ITS region. Restriction digests were done using 8–20 µl of PCR product (corresponding to approx. 200 ng of amplified DNA) in order to visualize even small fragments. Reactions were performed with 5 units of enzyme and 1/10 of the respective buffer (MBI Fermentas) at 65 °C for 12 hours. Fragments were separated on a 3% high resolution agarose gel, and 350 ng of DNA size standard per lane were used.

Chloroplast DNA (cpDNA)

In the course of another study (Z. Kaplan & J. Fehrer, in preparation), the chloroplast *rpl*20-5'*rps*12 intergenic spacer was among several regions analyzed found to be the most suitable part of cpDNA for studying species relationships in *Potamogeton* and for distinguishing between very closely related species. PCRs were performed in 50 µl reaction volumes containing 2.5 mM $MgCl_2$, 120 µM of each dNTP, 0.4 mM of primers rpl 20 and 5'-rps 12 (Hamilton 1999), 1/10 of $MgCl_2$-free reaction buffer and 1 unit of *Taq* DNA-polymerase (MBI Fermentas). A few nanograms of genomic DNA were used for amplification. Pre-denaturation of 5 min at 94 °C was followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 2.5 min. Final extension was done at 72 °C for 10 min with subsequent cooling of the reaction to 4 °C. PCR products were purified and sequenced (details in Kaplan & Fehrer 2004) with either rpl 20 or 5'-rps 12 as sequencing primers. Sequences for 2–3 plants of each parent and of all hybrid samples were obtained (see Table 2 for a list of samples and for GenBank accession numbers).

Results

Experimental hybridization

No plants were obtained from the crosses between *P. alpinus* × *P. gramineus*, *P. alpinus* × *P. pusillus*, *P. perfoliatus* × *P. alpinus* and *P. perfoliatus* × *P. praelongus*, because the seeds failed to germinate. This is not surprising for seeds from crosses in which *P. alpinus* was the maternal plant, as the normal behaviour of seeds of this species always shows low germinability (Z. Kaplan, unpublished data). Brux et al. (1987, 1988) also noted that seed of this species rarely produce plants in the wild, despite a rich seed bank, and that the colonies were maintained almost entirely by vegetative reproduction. The fruits collected from *P. perfoliatus* flowers pollinated by *P. alpinus* or *P. praelongus* pollen were superficially well-shaped but may not have contained a well-developed viable embryo.

The progenies of the crosses *P. gramineus* × *P. berchtoldii*, *P. perfoliatus* × *P. pectinatus*, and *P. pusillus* × *P. alpinus* were morphologically identical with the maternal parents and did not show any character of the other, very dissimilar parent. All these plants obviously arose via autogamy. The offspring of the crosses of the morphologically

similar species pairs *P. berchtoldii* × *P. pusillus* and *P. berchtoldii* × *P. trichoides* were subjected to isozyme analysis. All offspring plants showed banding patterns corresponding exclusively to one of the species, in each case it was that of the maternal plant. Thus, none of these plants were actual hybrids, but had developed from selfed flowers.

The only plant that morphologically appeared to be a hybrid resulted from crossing *P. perfoliatus* (sample 840) × *P. gramineus* (sample 318). This specimen (designated 907) was cultivated in 2000–2001 and subjected to further investigation.

Morphological evaluation of the cross *P. perfoliatus* × *P. gramineus*

The natural hybrid *P.* × *nitens*, assumed to have resulted from a cross between *P. gramineus* and *P. perfoliatus*, is generally intermediate between its parents, showing a combination of parental characters. Most phenotypes are more similar to *P. gramineus*, resembling this species in its general appearance and shape of leaves. These are relatively easy to distinguish from *P. perfoliatus*, as the hybrid has narrower submerged leaves with fewer longitudinal veins, more acute and sometimes apiculate leaf apex, more persistent stipules and in at least some clones the capacity to produce floating leaves. Unlike *P. gramineus*, the hybrid has a more sparingly branched stem, semi-amplexicaul bases of submerged leaves, a more obtuse apex and more numerous longitudinal veins. Both parental species differ from *P.* × *nitens* in their capacity to produce well-formed fruits; in contrast, the hybrid is consistently sterile. The most important diagnostic characters of all three taxa are summarized in Table 3.

The experimental offspring 907, which has *P. perfoliatus* as its seed parent, is clearly not a product of autogamy. In fact, the hybrid is more similar to the other species, *P. gramineus*, resembling this parent particularly in the shape and size of submerged leaves. The leaves are 7–9 (–11)-veined, acute or indistinctly apiculate. However, the semi-amplexicaul bases, particularly apparent on young leaves on side branches, are clearly derived from *P. perfoliatus*. Only 5–7 mm long and gradually decaying and disappearing stipules are also similar to *P. perfoliatus*. Petiolate coriaceous floating leaves with 9–13-veined lamina, demonstrating influence of *P. gramineus*, were also produced on some ramets. In summary, the experimental hybrid 907 shows a combination of characters of *P. gramineus* and *P. perfoliatus*, and its morphology falls within the variation range of the natural hybrid *P.* × *nitens*.

Potamogeton × *nitens* shows a high level of morphological variation. This can be partly attributed to the considerable phenotypic plasticity shown by many other *Potamogeton* taxa (Kaplan 2002). However, a high level of genetic variation may also be expected between different plants of this hybrid as a result of the different allelic combinations of the rather distantly related parental taxa. Indeed, each of the six different clones included in this study showed a specific pattern of variation even though they were all cultivated under the same conditions.

Reproductive behaviour

In cultivation both natural and artificial hybrid plants produced also reproductive organs. In contrast to those of pure species, the spikes of *P.* × *nitens* were markedly shorter than those of either of the parental species. Unlike flowers of fertile plants, which open to reveal the dehiscing anthers, the tepals of *P.* × *nitens* remained tightly closed. The entire abortive

Table 3. A comparison of the most important diagnostic characters of *Potamogeton gramineus*, *P. perfoliatus* and *P. × nitens*, compiled from the literature (Dandy 1975, Preston 1995, Wiegleb & Kaplan 1998), and modified and supplemented according to personal experience. Rare extremes were omitted.

	P. gramineus	*P. × nitens*	*P. perfoliatus*
Shape of lamina of sub-merged leaves	linear-oblong to narrowly lanceolate	oblong-lanceolate to broadly lanceolate or ovate	narrowly lanceolate to broadly ovate
Width of leaf lamina (mm)	(2–) 4–8 (–10)	5–23	12–42
Length:width ratio of lamina	5–21	3–9	1.3–5.3
Number of longitudinal leaf veins	5–7 (–9)	7–17	(13–) 19–33
Shape of lamina apex	acute to obtuse and mucronate	acute to obtuse and indistinctly apiculate	obtuse to rounded, or sub-acute, never mucronate
Shape of lamina base	cuneate, never amplexicaul	broadly cuneate to rounded, mostly semi-amplexicaul	rounded to cordate, amplexicaul
Presence of coriaceous floating leaves on adult fertile plants	mostly present, particularly in standing water	sometimes present	absent
Persistence of stipules	persistent	persistent or present only on upper part of the stem and disappearing at older leaves	fugacious, present mostly only at young leaves and disappearing early
Length of stipule (mm)	10–28	5–25	3–20
Length of spike (mm)	12–35	5–13	13–32
Capacity to produce well-formed fruits	present	absent	present

spikes soon rotted instead of setting fruit. This behaviour of floral organs has been repeatedly observed in various sterile hybrids (Preston 1995: 46, Preston et al. 1998, Kaplan & Wolff 2004, Kaplan & Fehrer 2004, Kaplan 2006).

Genetic analyses

Contribution of both parents: nuclear DNA evidence

Two *P. perfoliatus* samples (1002, 1470) had identical ITS sequences; plant 979 had three sites showing intra-individual polymorphism that corresponds to the known intraspecific variation (Kaplan & Fehrer 2004). Between the two *P. gramineus* samples sequenced (897, 1285) an almost 1% sequence divergence was found (four substitutions in ITS1, three in ITS2). The restriction enzyme chosen did not distinguish between parental variants.

RFLP patterns of all the plants of each parental species from different localities were identical, i.e., any intraspecific variation potentially exceeding that of the sequenced individuals was not detected, confirming that the markers were species-specific. All hybrids had additive patterns (Fig. 1) demonstrating the contribution of both parents, *P. gramineus* and *P. perfoliatus*, to *P. × nitens*. Natural and experimental hybrids did not differ in this respect.
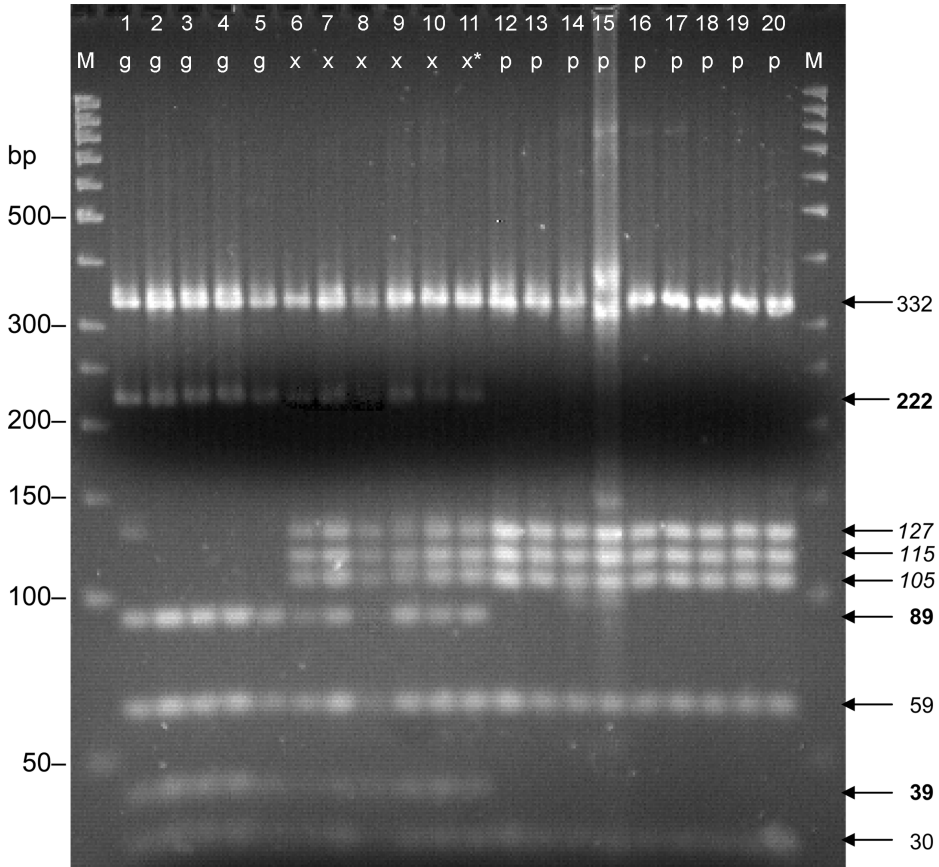
Fig. 1. – PCR-RFLP of the ITS region. *P. gramineus* (g) and *P. perfoliatus* (p) samples from different geographic regions flank the natural (x) and experimental (x*) hybrids. To the right, the exact fragment sizes are indicated (boldface: *P. gramineus*-specific, italics: *P. perfoliatus*-specific). All hybrids show additive patterns. Sample 15 contained a PCR artifact but was identical to the other *P. perfoliatus* samples when repeated. The dark horizontal area in the middle is due to xylene cyanol in the loading dye. Sample details (compare Table 2): 1–885, 2–897, 3–1489, 4–1156, 5–1285, 6–1007, 7–1016, 8–1019, 9–999, 10–879, 11–907, 12–840, 13–1467, 14–1469, 15–1481, 16–1470, 17–979, 18–985, 19–1531, 20–1002.

Maternal inheritance of chloroplast DNA

For determination of the transmission mode of cpDNA in *Potamogeton*, differences in the conservative *rbc*L gene were employed in order to suppress potential intraspecific variation. The respective *rbc*L fragment showed five differences between *P. perfoliatus* and *P. gramineus*, all of them representing unique substitutions in comparison to five other *Potamogeton* species (Les et al. 1997). Three of the five mutations were *P. gramineus*-specific, two *P. perfoliatus*-specific. The experimental hybrid *P. × nitens* (907) matched its maternal parent, *P. perfoliatus*, in all five differentiating sites, which accords with maternal inheritance of cpDNA in this genus.

Direction of the cross in natural hybrids: cpDNA evidence

The *rpl*20-*rps*12 intergenic spacer that was used here for the first time in *Potamo-getonaceae* amplified well and produced excellently readible sequences. PCR fragments ranged in size between 834 bp (*P. perfoliatus*) and 853 bp (*P. gramineus*), which corresponds to the approximate size range found in other plant families (Hamilton 1999). Two *P. perfoliatus* sequences analyzed were identical, but *P. gramineus* was polymorphic; samples 885 and 1285 showed one substitution, whereas sample 897 differed from both by one substitution and one indel.

Five of the field-collected hybrids (999, 1000, 1003, 1015, 1016) had sequences identical to the latter *P. gramineus* sample. Three natural (879, 1007, 1019) and, expectedly, the experimental hybrid (907) had the *P. perfoliatus* sequence. Thus, *P. × nitens* originated several times independently from the same parental species and both directions of the cross are possible.

Discussion

The natural hybrid *P. × nitens* was first described as early as in the late 18th century by Weber (1787), well before the great majority of *Potamogeton* species were recognized. It was first considered to be a species because at that time interspecific crosses in plants were rarely recognized by botanists. Almquist (1889) was probably the first who suggested that *P. nitens* might actually be a hybrid between *P. gramineus* and *P. perfoliatus*. Since the detailed treatment of this hybrid in Hagström's worldwide study of the genus (Hagström 1916), the identity of *P. × nitens* has become widely accepted. In spite of this rather long taxonomic tradition, many specimens of *P. × nitens* preserved in herbaria are identified incorrectly, being mostly confused with *P. gramineus*.

In this study, *P. perfoliatus* and *P. gramineus* were confirmed as parents of natural plants of *P. × nitens* by two independent approaches, morphological and molecular. At first, a phenocopy corresponding to *P. × nitens* was acquired from an experimental cross of the two assumed parental species. The final compelling evidence came from the additive patterns of the nuclear DNA markers.

Intraspecific genetic variation of the parental species differed. *Potamogeton perfoliatus* was rather uniform; no differences were found in the *rpl*20-*rps*12 spacer of cpDNA between samples from quite distant localities (Switzerland, Sweden) and variation in the nuclear ITS region was also low (0.0–0.4%). In contrast, the *P. gramineus* sequences from France (1285) and the Czech Republic (897) differed by nearly 1% in their ITS, which corresponds to the level of sequence divergence between closely related species in this genus (Z. Kaplan & J. Fehrer, unpublished data). This was even more pronounced in the less variable cpDNA: differences between *P. gramineus*-897 on the one hand and specimens 1285 or 885 (the latter also from the Czech Republic) fall in the range typical for interspecific variation (Z. Kaplan & J. Fehrer, unpublished data). All hybrids with *P. gramineus* cpDNA (from Sweden) had sequences identical to specimen 897 despite the considerable geographic distance. This suggests that *P. gramineus*-897 may represent a more widespread variant of the species that not only occurs in Central Europe but also in Scandinavia and, as we recently discovered, North America (Z. Kaplan & J. Fehrer, in preparation). This genetic variation may coincide with the extreme morphological varia-

tion in this species. However, no substantial morphological differences were identified between the different *P. gramineus* genotypes. This topic requires further investigation based on more representative material.

Although the usual case in angiosperms, maternal inheritance of cpDNA has not been proved before for *Potamogeton*, due to considerable difficulties in producing experimental hybrids. The successful production of one such hybrid allowed us to test whether chloroplast DNA is also maternally inherited in *Potamogeton*. As the actual *P. gramineus* plant used for hybrid production was not available for DNA analyses, we used published *rbc*L sequences for this species assuming that this gene is so conservative that potential intraspecific variation would not be detected. Indeed, the sequence obtained from the experimental hybrid matched the GenBank sequence for *P. perfoliatus* although our seed parent was from the Czech Republic and the conspecific sample was from Vermont, USA (D. H. Les, personal communication). Given that the comparably variable *rpl*20-*rps*12 intergenic spacer also did not differ between *P. perfoliatus* plants from Sweden and Switzerland, the identity of the experimental and several natural hybrid sequences with that of *P. perfoliatus* is not surprising.

Plants from the five natural populations of *P.* × *nitens* showed two different chloroplast haplotypes corresponding to either *P. perfoliatus* or *P. gramineus*, which indicates the independent origin of the populations. At two of the Swedish localities, different ramets were sampled in discrete colonies (999, 1000 and 1003 from Björka, and 1015 and 1016 from Metsjön). These individual plants were genetically identical within the sites. This pattern of partitioning of genetic variation within and between populations of *P.* × *nitens* is consistent with the expected recurrent origin of the populations, very limited long-distance dispersal, observed total sterility of the plants and prevailing clonal propagation in established populations.

The high percentage of unsuccessful crosses in this experiment could indicate that hybridization is a rare event in *Potamogeton*. However, it must be taken into account that under the experimental conditions the pollen was transferred to only a very limited number of flowers and only once. The stigma may not have been in the optimal phase of receptivity at the time when the crosses were performed. In this context one has to keep in mind the wise comment of Davis & Heywood (1963: 444) that "nature has more time and more material for her experiments than man". The frequency of hybrids in those habitats and those parts of the world, which offer the best natural conditions for germination of seeds (typically the lakes in previously glaciated areas of the Northern Hemisphere, see Kaplan 2006), clearly documents that hybridization events are frequent if two or more *Potamogeton* species grow together. And if adult plants of *Potamogeton* hybrids are relatively common there, the frequency of effective interspecific pollen transfers and the production of hybrid seeds must be even higher.

Souhrn

Přestože je existence kříženců v rodu *Potamogeton* známa již více než století, experimentální důkaz jejich existence cílenou hybridizací dosud chyběl. Pokusy s křížením rdestů vyústily ve vznik hybridní rostliny z kombinace *P. perfoliatus* × *P. gramineus*. Její morfologická výbava, reprodukční chování a molekulární markery byly porovnány s odpovídajícími charakteristikami rodičovských druhů a přirozených kříženců stejné hybridní kombinace. Fenotyp přirozeného křížence *P.* × *nitens* odpovídal fenotypu experimentálního křížence *P. perfoliatus* × *P. gramineus*. Všechny rostliny byly zcela sterilní; přestože vytvářely květy, okvětní lístky zůstávaly uzavřené, plody se nevyvíjely a celé květenství záhy odumíralo. Analýza jaderné DNA potvrdila přítomnost specifických sekvencí obou předpokládaných rodičovských druhů. Experimentální kříženec byl analyzován k potvrzení dědičnosti chloroplastové DNA po mateřské linii. Následně byly studovány rostliny z přírodních populací křížence *P.* × *nitens* s cílem odhalit, který z rodičovských druhů byl mateřskou rostlinou. Bylo zjištěno, že v přírodě dochází k hybridizaci v obou směrech, tedy oplozením vajíčka na rostlině druhu *P. gramineus* pylem *P. perfoliatus* i naopak.

References

Almquist S. (1889): 1. *Potamogéton* Tourn. – In: Krok Th. O. B. N., C. J. och C. Hartmans handbok i Skandinaviens flora, Ed. 12, p. 42–56, Stockholm.

Birky C. W. Jr. (1995): Uniparental inheritance of mitochondrial and chloroplast genes: Mechanisms and evolution. – Proc. Natl. Acad. Sci. USA 92: 11331–11338.

Brayshaw T. C. (2000): Pondweeds, bur-reeds and their relatives of British Columbia: Aquatic families of monocotyledons. Ed. 2. – Royal British Columbia Museum, Victoria.

Brux H., Todeskino D. & Wiegleb G. (1987): Growth and reproduction of *Potamogeton alpinus* Balbis growing in disturbed habitats. – Arch. Hydrobiol., Beih., 27: 115–127.

Brux H., Herr W., Todeskino D. & Wiegleb G. (1988): A study on floristic structure and dynamics of communities with *Potamogeton alpinus* Balbis in water bodies in the northern part of the Federal Republic of Germany. – Aquat. Bot. 32: 23–44.

Crawford D. J., Mort M. E. & Archibald J. K. (2005): Biosystematics, chromosomes and molecular data: melding the old and the new. – Taxon 54: 285–289.

Davis P. H. & Heywood V. H. (1963): Principles of angiosperm taxonomy. – Oliver and Boyd, Edinburgh & London.

Drábková L., Kirschner J., Seberg O., Petersen G. & Vlček C. (2003): Phylogeny of the Juncaceae based on *rbc*L sequences, with special emphasis on *Luzula* DC. and *Juncus* L. – Plant Syst. Evol. 240: 133–147.

Fant J. B., Kamau E. A. & Preston C. D. (2003): Chloroplast evidence for the multiple origins of the hybrid *Potamogeton* × *sudermanicus* Hagstr. – Aquat. Bot. 75: 351–356.

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

Fant J. B. & Preston C. D. (2004): Genetic structure and morphological variation of British populations of the hybrid *Potamogeton* × *salicifolius*. – Bot. J. Linn. Soc. 144: 99–111.

Fant J. B., Preston C. D. & Barrett J. A. (2001a): Isozyme evidence for the origin of *Potamogeton* × *sudermanicus* as a hybrid between *P. acutifolius* and *P. berchtoldii*. – Aquat. Bot. 71: 199–208.

Fant J. B., Preston C. D. & Barrett J. A. (2001b): Isozyme evidence of the parental origin and possible fertility of the hybrid *Potamogeton* × *fluitans* Roth. – Plant Syst. Evol. 229: 45–57.

Fischer G. (1904): Beitrag zur Kenntnis der bayerischen Potamogetoneen IV. – Mitt. Bayer. Bot. Ges. 1: 356–366 & 375–388.

Fischer G. (1905): Beitrag zur Kenntnis der bayerischen Potamogetoneen. V. – Mitt. Bayer. Bot. Ges. 1: 471–475.

Fischer G. (1907): Die bayerischen Potamogetonen und Zannichellien. – Ber. Bayer. Bot. Ges. 11: 20–162.

Fryer A. (1890): Supposed hybridity in *Potamogeton*. – J. Bot. 28: 173–179.

Fryer A. (1894): *Potamogeton polygonifolius* v. *pseudo-fluitans*. – J. Bot. 32: 97–100.

Fryer A. (1898–1900): The Potamogetons (pond weeds) of the British Isles. – London.

Guo Y. & Cook C. D. K. (1990): The floral biology of *Groenlandia densa* (L.) Fourreau (*Potamogetonaceae*). – Aquat. Bot. 38: 283–288.

Hagström J. O. (1916): Critical researches on the Potamogetons. – Kungl. Svenska Vetenskapsakad. Handl. 55(5): 1–281.

Hamilton M. B. (1999): Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. – Molec. Ecol. 8: 521–423.

Hollingsworth P. M., Preston C. D. & Gornall R. J. (1995): Isozyme evidence for hybridization between *Potamogeton natans* and *P. nodosus* (*Potamogetonaceae*) in Britain. – Bot. J. Linn. Soc. 117: 59–69.

Hollingsworth P. M., Preston C. D. & Gornall R. J. (1996): Isozyme evidence for the parentage and multiple origins of *Potamogeton × suecicus* (*P. pectinatus × P. filiformis*, *Potamogetonaceae*). – Plant Syst. Evol. 202: 219–232.

Iida S. & Kadono Y. (2002): Genetic diversity and origin of *Potamogeton anguillanus* (*Potamogetonaceae*) in Lake Biwa, Japan. – J. Plant Res. 115: 11–16.

Jarolímová V. (2005): Experimental hybridization of species in the genus *Rorippa*. – Preslia 77: 277–296.

Kaplan Z. (2001): *Potamogeton ×fluitans* (*P. natans × P. lucens*) in the Czech Republic. I. Morphology and anatomy. – Preslia 73: 333–340.

Kaplan Z. (2002): Phenotypic plasticity in *Potamogeton* (*Potamogetonaceae*). – Folia Geobot. 37: 141–170.

Kaplan Z. (2005a): Neotypification of *Potamogeton ×fluitans* Roth and the distribution of this hybrid. – Taxon 54: 822–826.

Kaplan Z. (2005b): *Potamogeton schweinfurthii* A. Benn., a new species for Europe. – Preslia 77: 419–431.

Kaplan Z. (2006): First record of *Potamogeton × salicifolius* for Italy, with isozyme evidence for plants collected in Italy and Sweden. – Plant Biosyst. (in press).

Kaplan Z. & Fehrer J. (2004): Evidence for the hybrid origin of *Potamogeton ×cooperi* (*Potamogetonaceae*): traditional morphology-based taxonomy and molecular techniques in concert. – Folia Geobot. 39: 431–453.

Kaplan Z. & Symoens J.-J. (2004): (1638) Proposal to conserve the name *Potamogeton schweinfurthii* A. Benn. (*Potamogetonaceae*) with a conserved type. – Taxon 53: 837–838.

Kaplan Z. & Symoens J.-J. (2005): Taxonomy, distribution and nomenclature of three confused broad-leaved *Potamogeton* species occurring in Africa and on surrounding islands. – Bot. J. Linn. Soc. 148: 329–357.

Kaplan Z. & Štěpánek J. (2003): Genetic variation within and between populations of *Potamogeton pusillus* agg. – Plant Syst. Evol. 239: 95–112.

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.

Khalaf M. K. & Stace C. A. (2000): Breeding systems and relationships of the *Cerastium tomentosum* group. – Preslia 72: 323–344.

King R. A., Gornall R. J., Preston C. D. & Croft J. M. (2001): Molecular confirmation of *Potamogeton × bottnicus* (*P. pectinatus × P. vaginatus*, *Potamogetonaceae*) in Britain. – Bot. J. Linn. Soc. 135: 67–70.

Krahulec F., Kaplan Z. & Novák J. (2005): *Tragopogon porrifolius × T. pratensis*: the present state of an old hybrid population in Central Bohemia, the Czech Republic. – Preslia 77: 297–306.

Krahulec F., Krahulcová A., Fehrer J., Bräutigam S., Plačková I. & Chrtek J. jun. (2004): The Sudetic group of *Hieracium* subgen. *Pilosella* in the Krkonoše Mts: a synthetic view. – Preslia 76: 223–243.

Les D. H., Cleland M. A. & Waycott M. (1997): Phylogenetic studies in *Alismatidae*, II: Evolution of marine angiosperms (seagrasses) and hydrophily. – Syst. Bot. 22: 443–463.

Les D. H. & Philbrick C. T. (1993): Studies of hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. – Aquat. Bot. 44: 181–228.

Mráz P. & Paule J. (2006): Experimental hybridization in the genus *Hieracium* s. str.: crosses between diploid taxa. – Preslia 78: 1–26.

Ogden E. C. (1943): The broad-leaved species of *Potamogeton* of North America north of Mexico. – Rhodora 45: 57–105, 119–163 & 171–214.

Ogden E. C. (1974a): Anatomical patterns of some aquatic vascular plants of New York. – Bull. New York State Mus. Sci. Serv. 424: 1–133.

Ogden E. C. (1974b): *Potamogeton* in New York. – Bull. New York State Mus. Sci. Serv. 423: 1–20.

Petersen G. & Seberg O. (2003): Phylogenetic analyses of the diploid species of *Hordeum* (*Poaceae*) and a revised classification of the genus. – Syst. Bot. 28: 293–306.

Preston C. D. (1988): Alfred Fryer and the study of the genus *Potamogeton* of the British Isles. – Arch. Nat. Hist. 15: 15–33.

Preston C. D. (1995): Pondweeds of Great Britain and Ireland. – Botanical Society of the British Isles, London.

Preston C. D., Hollingsworth P. M. & Gornall R. J. (1998): *Potamogeton pectinatus* L. × *P. vaginatus* Turcz. (*P. × bottnicus* Hagstr.), a newly identified hybrid in the British Isles. – Watsonia 22: 69–82.

Raunkiaer C. (1896): De danske Blomsterplanters Naturhistorie. I. Enkimbladede [= Helobiae]. – Kjøbenhavn.

Raunkiaer C. (1903): Anatomical *Potamogeton*-studies and *Potamogeton fluitans*. – Bot. Tidssk. 25: 253–280.

Symoens J. J., van de Velden J. & Büscher P. (1979): Contribution a l'étude de la taxonomie et de la distribution de *Potamogeton nodosus* Poir. et *P. thunbergii* Cham. et Schlechtend. en Afrique. – Bull. Soc. Roy. Bot. Belgique 112: 79–95.

Štorchová H., Hrdličková R., Chrtek J. Jr., Tetera M., Fitze D. & Fehrer J. (2000): An improved method of DNA isolation from plants collected in the field and conserved in saturated NaCl/CTAB solution. – Taxon 49: 79–84.

Tur N. M. (1982): Revisión del género *Potamogeton* L. en la Argentina. – Darwiniana 24: 217–265.

Weber G. H. (1787): Ordinis medicorum … Supplemento florae holsaticae. – Kilonii.

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. (1990c): The importance of stem anatomical characters for the systematics of the genus *Potamogeton* L. – Flora 184: 197–208.

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

Whittall J. B., Hellquist C. B., Schneider E. L. & Hodges S. A. (2004): Cryptic species in an endangered pondweed community (*Potamogeton*, *Potamogetonaceae*) revealed by AFLP markers. – Amer. J. Bot. 91: 2022–2029.