Folia Geobotanica 39: 431–453, 2004

EVIDENCE FOR THE HYBRID ORIGIN OF *POTAMOGETON* ×*COOPERI* (*POTAMOGETONACEAE*): TRADITIONAL MORPHOLOGY-BASED TAXONOMY AND MOLECULAR TECHNIQUES IN CONCERT

Zdeněk Kaplan & Judith Fehrer

Institute of Botany, Academy of Sciences of the Czech Republic, CZ-252 43 Průhonice, Czech Republic; fax +420 267 750 031, e-mail kaplan@ibot.cas.cz, fehrer@ibot.cas.cz

Abstract: The identity of plants morphologically intermediate between *Potamogeton crispus* and *P. perfoliatus* from two recently discovered sites, one in Moravia, Czech Republic and another in Wales, United Kingdom, was investigated with molecular markers. Evidence from restriction fragment length polymorphism analysis of the nuclear internal transcribed spacer region of ribosomal DNA and of the *trn*K-*trn*Q chloroplast DNA intergenic spacer confirmed the morphology-based determination of two putative hybrid samples as *P.* ×*cooperi*. The hybrids showed the ITS variants of both parental taxa, consistent with the expected biparental inheritance of nuclear DNA. The chloroplast DNA markers indicate *P. crispus* as the female parent in both hybridization events. The hybrid origin of another dubious sample was excluded by the molecular data, in accordance with previous detailed morphological examination. This plant represented an extreme, narrow-leaved form of *P. perfoliatus*, imitating *P.* ×*cooperi* in some characters. The results of the molecular analyses are discussed in relation to the morphology of the plants. They underline that some *Potamogeton* hybrids could indeed be identified by careful and detailed morphological examination and also that these identifications were reliable and confirmed by molecular markers. This study exemplifies that long-term taxonomic expertise usually generates very well-founded specific questions suitable for straightforward treatment by appropriate molecular methods. The process and ecological implications of hybrid formation are also discussed

Keywords: cpDNA, Hybridization, Intra-individual polymorphism, ITS, Plant taxonomy, Variation

Nomenclature and hybrid formulas: WIEGLEB & KAPLAN (1998)

INTRODUCTION

The existence of *Potamogeton* hybrids has been reported in the literature for over a century and is nowadays widely recognized (FRYER 1890, ASCHERSON & GRAEBNER 1897, BAAGØE 1897, RAUNKIAER 1903, FISCHER 1904, 1905, 1907, GRAEBNER 1907, HAGSTRÖM 1916, GLÜCK 1936, OGDEN 1943, DANDY 1975, 1980). As PRESTON (1995: 42) pointed out, despite that most evidence comes from morphological and anatomical studies and from the sterility of putative hybrids, "the existence of hybrids is not likely to be doubted by anyone who is familiar with the morphology of the species". In his recent revision, PRESTON (1995) provided detailed descriptions of 26 hybrids recognized in the British Isles. WIEGLEB & KAPLAN (1998) listed 50 confirmed hybrids worldwide, some of which are locally frequent and represent clearly circumscribed biological entities. Among them, hybrids involving

P. crispus L. or *P. perfoliatus* L. are relatively common (PRESTON 1995, WIEGLEB & KAPLAN 1998).

The hybrid between *P. crispus* and *P. perfoliatus* was mentioned first by FRYER (1890). Originally he described it as a variety, *P. undulatus* var. *cooperi* FRYER (FRYER 1891), under the incorrect assumption that *P. undulatus* WOLFG. is a hybrid with the same parentage. As a result of subsequent investigations by RAUNKIAER (1896) and BAAGØE (1897), which revealed that *P.* ×*undulatus* was actually the hybrid between *P. crispus* L. and *P. praelongus* WULFEN, Fryer recombined his taxon to the rank of a distinct hybrid *P.* ×*cooperi* (FRYER) FRYER (FRYER 1897). Detailed reviews on the history of Alfred Fryer's *Potamogeton* studies, including his pilot observations on *Potamogeton* hybrids were given by PRESTON (1988a,b). Two more binomials for the hybrids derived from *P. crispus* × *P. perfoliatus* were at that time independently proposed also by ASCHERSON & GRAEBNER (1897) and FISCHER (1904); these are *P.* ×*cymatodes* ASCH. et GRAEBN. and *P.* ×*cymbifolius* G. FISCH., respectively (WIEGLEB & KAPLAN 1998).

Nowadays, the existence of *P.* ×*cooperi* is taken as an accepted fact in several European countries and the details concerning this hybrid have been repeatedly discussed during the last few decades (e.g. DANDY 1975, PLOEG 1990, WOLFE MURPHY et al. 1991, PRESTON 1995). The hybrid has even been recorded as relatively widespread in the British Isles (PRESTON 1995, PRESTON & CROFT 1997).

Rather surprisingly, HAYNES (1985) in his revision of the group of clasping-leaved *Potamogeton*, which also includes *P. perfoliatus*, did not discuss any hybrids at all involving this species, even though some of them (e.g. *P.* ×*nitens* WEBER, *P.* ×*salicifolius* WOLFG., *P.* ×*cognatus* ASCH. et GRAEBN., *P.* ×*cooperi*) are frequently cited in Floras and floristic papers. The herbarium of the Botanischer Garten und Botanisches Museum Berlin-Dahlem (acronym B) preserves a specimen collected by G. Fischer at Ebing that was identified by himself as the hybrid *P. perfoliatus* × *P. crispus*. Haynes re-determined this herbarium specimen in 1983 as *P. perfoliatus*. However, the character combination (in particular the shape of the leaves and the reduced number of veins in the main-stem leaves to only 7–9) in our view excludes the identity of this herbarium specimen with this species, as Haynes suggested. In contrast, the character set is well in accordance with *P.* ×*cooperi* as it is understood. LES & PHILBRICK (1993) summarized that "although at least seven different hybrid reports implicate *Potamogeton crispus* as one parent, ... none have been verified".

Because of the immense range of phenotypic plasticity of *Potamogeton* taxa (KAPLAN 2002a) and the occurrence of aneuploids in the genus (KALKMAN & VAN WIJK 1984, HOLLINGSWORTH et al. 1998), some authors were sceptical about identifying *Potamogeton* hybrids morphologically and stressed the need for more convincing evidence (LES & PHILBRICK 1993). Recently, there has been a lot of interest in the use of molecular techniques such as isozyme analysis (e.g. HOLLINGSWORTH et al. 1995b, 1996b, PRESTON et al. 1998b, FANT et al. 2001a,b, IIDA & KADONO 2002, KAPLAN et al. 2002, KAPLAN & WOLFF 2004) or DNA-based techniques (KING et al. 2001, FANT et al. 2003) to confirm *Potamogeton* hybrids.

Opinions on the actual occurrence and frequency of *Potamogeton* hybrids in the field and the possibility of distinguishing them on morphological grounds still differ greatly. This may be due in part to the long tradition of studying *Potamogeton* hybrids in Europe (established

more than 120 years ago by Fryer and developed in the early studies of stem anatomy by Raunkiaer and Fischer, see WIEGLEB 1990 and KAPLAN 2001) in contrast to other parts of the world. Although a significant effort to provide a revision of North-American broad-leaved *Potamogeton* hybrids was made by OGDEN (1943), a recently published account of *Potamogetonaceae* in Flora of North America (HAYNES & HELLQUIST 2000) provides no revised treatment of hybrids (see also the review by PRESTON 2001). The authors confined themselves to listing "all the hybrids that HAGSTRÖM (1916) proposed for species that occur in North America" with a few additions by later authors. All these hybrids are reported only as "have been described", regardless of whether their identities have already been confirmed, interpreted in another way, or are still uncertain. *P.* ×*cooperi* is not listed, although both *P. crispus* and *P. perfoliatus* meet the criterion of species occurring in North America.

We have therefore taken the opportunity of conducting a study on plants morphologically intermediate between *P. crispus* and *P. perfoliatus*. These plant have been recently discovered on two sites, one of which is in Moravia, Czech Republic, and another in Wales, United Kingdom. Samples from both these populations were cultivated and subjected to detailed study. In addition to morphological examination, nuclear ribosomal as well as chloroplast DNA markers were used to test the reliability of our morphology-based identification and to show the respective contributions of the putative parental taxa to the supposed hybrid plants.

Analysis of the chloroplast DNA was supposed to provide information about the direction of the cross in putative hybrids. In cpDNA, the hybrids were supposed to show the pattern of the female parent only. The assumption that cpDNA is maternally inherited in *Potamogeton* like in the majority of angiosperms (BIRKY 1995) was confirmed for an experimentally produced hybrid (KAPLAN & FEHRER, unpubl.).

In contrast, additive patterns reflecting the contribution of both parental taxa were expected from the use of nuclear markers. While gene conversion processes are known to homogenize different copies of nrDNA in the same organism with time (e.g. WENDEL et al. 1995, FRANZKE & MUMMENHOFF 1999) and other, not yet fully understood dynamics may lead to the preferential deletion of the copies of one or other parent (e.g. WENDEL 2000, ÁLVAREZ & WENDEL 2003), in our case, the parental taxa were thought to be sufficiently different and the hybridizations recent enough to have a good chance to detect both copies in the hybrids (e.g. CAMPBELL et al. 1997, VARGAS et al. 1999, BAUMEL et al. 2001).

MATERIAL AND METHODS

Study species

Both putative parental species belong to an informal group of broad-leaved pondweeds, and among them to homophyllous species, which produce only submerged leaves but no floating ones. *Potamogeton crispus* is characterized by (3–)5(–7)-veined leaves, serrate and usually strongly undulate leaf margins, with teeth easily visible with the naked eye, compressed and shallowly grooved stem and fruits adnate at their base, with beak ± half as long as the rest of the fruit. If careful inspection of morphology is applied, this unique combination of features makes *P. crispus* always easily recognizable and the most distinct species within the genus (WIEGLEB & KAPLAN 1998). The species is native in Europe, Africa, Asia and Australia but has also been introduced in New Zealand, North America and southern

South America. The other parent, *P. perfoliatus*, has leaves with 11–33 longitudinal veins and obscurely denticulate, sometimes minutely undulate or ± flat margins, terete stem and fruits free at the base, with a beak much less than half as long as the rest of the fruit. It occurs mainly in the Northern Hemisphere, in Europe, northern and central Africa, Asia and eastern North America, but in some regions it penetrates southwards down to Australia and Central America (WIEGLEB & KAPLAN 1998). Both species are sympatric in much of their ranges, and as they occupy a similar range of habitats such as lakes, water reservoirs and rivers, they are sometimes found growing together at the same site. This holds true also for the Czech Republic and United Kingdom where these species are more or less widespread (see the maps of the respective species in NOVÁKOVÁ 1982 and PRESTON 1995, respectively), although *P. perfoliatus* became very rare in the Czech Republic during the last decades (KAPLAN 2002b) and some decline has been noted also in United Kingdom (PRESTON 1995).

Both *P. crispus* and *P. perfoliatus* are mostly self-pollinated but the protogynous flowers may occasionally permit some cross-pollination. Both species are considered to be tetraploids (perhaps autotetraploids of ancient origin) with chromosome number 2n=52, although also different chromosome counts have exceptionally been reported (HOLLINGSWORTH et al. 1998).

The sample sites of *P. ×cooperi*

The Moravian locality is a small water reservoir closely adjacent to the coast of the upper dam in a cascade of three lowland reservoirs Nové Mlýny, at Pasohlávky village, southern Moravia. This is a relatively new habitat as the construction of the main upper dam was completed in 1978 and the small adjacent reservoir was created at the same time. *Potamogeton ×cooperi* was first discovered there by J. Rydlo in 2002. Both putative parental species have been found together with the hybrid (vouchers in ROZ).

In Wales, the hybrid was collected in the river Solva, growing in running water 10–30 cm deep. It was first collected there in the 1930s (see PRESTON & CHATER 1997 for the history of discovery and earlier collections) and recently recollected by T. D. Dines & C. D. Preston. In contrast to the Moravian site, none of the parental species were found together with *P. ×cooperi* in the river Solva.

Plant material

Besides two individuals of putative *P. ×cooperi* originating from the two localities mentioned above (Moravia and Wales), fresh material of both supposed parental species was collected in various regions particularly in Central Europe. Because the genetic variation between populations is high, but low or absent within populations of *Potamogeton* species (VAN WIJK et al. 1988, HETTIARACHCHI & TRIEST 1991, HOLLINGSWORTH et al. 1995a, 1996a, KAPLAN & ŠTĚPÁNEK 2003), usually only a single individual was taken from a population but a larger number of populations was sampled to cover most of the intraspecific variation. Additional specimens from the type locality of *P. ×cymbifolius* (a synonym of *P. ×cooperi*, proposed independently by FISCHER 1904) in Bavaria, Germany, were kindly collected for this study by L. Meierott: one individual of each *P. crispus*, *P. perfoliatus* and a narrow-leaved fragment tentatively designated as "possibly *P. ×cooperi*", which later proved

Table 1. The origin, reference numbers and GenBank accession numbers of the *Potamogeton* samples included in the study.

Taxon	Reference no.	Origin + collection records	GenBank accession number
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
	985	Austria, Fußach, 23.VI.1998, coll. Z. KAPLAN 98/131	
	1002	Sweden, Björka, 12.VIII.1998, coll. Z. KAPLAN 98/338	AY529526
	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 (the sample tentatively designated as "possible *P.* ×*cooperi*")	AY529525
	1471	Germany, Ebing, 11.VI.2003, coll. L. MEIEROTT	
	1479	Switzerland, Altenrhein, 23.VI.1998, coll. Z. KAPLAN 98/125	
	1480	Czech Republic, Týn nad Vltavou, 27.VII.2003, coll. Z. KAPLAN 03/159	
	1481	Czech Republic, Staré Splavy, 3.VIII.2003, coll. Z. KAPLAN 03/161	
P. ×*cooperi*	1248	United Kingdom, Wales, Lower Solva, 8.VI.2001, coll. T.D. DINES & C.D. PRESTON	
	1420	Czech Republic, Pasohlávky, 21.VI.2002, coll. J. RYDLO	
P. crispus	1463	Czech Republic, Bohuslavice, 7.VI.2003, coll. Z. KAPLAN 03/121	
	1464	Czech Republic, Velká Jesenice, 7.VI.2003, coll. Z. KAPLAN 03/122	
	1465	Czech Republic, Nahořany, 7.VI.2003, coll. Z. KAPLAN 03/124	
	1466	Czech Republic, Uhřínovice, 7.VI.2003, coll. Z. KAPLAN 03/126	
	1472	Germany, Ebing, 11.VI.2003, coll. L. MEIEROTT	AY529523
	1473	Czech Republic, Poděbrady, 22.VI.2003, coll. Z. KAPLAN 03/142	AY529524
	1476	Czech Republic, Lomnice nad Lužnicí, 8.IX.1999, coll. Z. KAPLAN 99/154	
	1477	Czech Republic, Mláka, 15.VI.2000, coll. Z. KAPLAN 00/13	
	1478	Czech Republic, Mostov, 10.VIII.2000, coll. Z. KAPLAN 00/182	
	1483	Czech Republic, Chudíř, 6.VIII.2003, coll. Z. KAPLAN 03/173	
	1485	Czech Republic, Hrobice, 10.VIII.2003, coll. Z. KAPLAN 03/180	

Table 2. A comparison of the most important diagnostic characters of *Potamogeton crispus*, *P. perfoliatus* and *P.* ×*cooperi*, compiled from the relevant literature (DANDY 1975, PRESTON 1995, WIEGLEB & KAPLAN 1998) and modified according to our experience.

	P. crispus	*P.* ×*cooperi*	*P. perfoliatus*
Stem	compressed and shallowly grooved	slightly compressed and shallowly grooved	terete
Leaf shape	linear to linear-oblong	linear-lanceolate to ovate	narrowly lanceolate to orbicular-ovate
Leaf width (mm)	(4–)6–12	8–25	(7–)12–42
Leaf length : width ratio	5–9(–13)	(1.9–)2.3–6.2	(1.0–)1.3–5.3(–10)
Number of longitudinal leaf veins	(3–)5(–7)	7–11(–13)	11–33
Shape of leaf margin	serrate and usually strongly undulate	denticulate to irregularly serrulate and sometimes slightly undulate	denticulate and sometimes minutely undulate or ± flat
Shape of leaf apex	plane	sometimes slightly but distinctly hooded	sometimes slightly but distinctly hooded
Shape of leaf base	broadly cuneate to auriculate, never amplexicaul	semi-amplexicaul	amplexicaul
Length of spike (mm)	5–16	4–13	13–25
Number of flowers	3–8	4–15	9–20
Capacity to produce well-formed fruits	present	absent	present

to be a form of *P. perfoliatus* (see below). Besides this fresh material, four dried samples (1476, 1477, 1478, 1479) were taken from herbarium specimens recently collected by Z. Kaplan. Altogether 11 populations of each *P. crispus* and *P. perfoliatus* were sampled. Specimens included in the study are summarized in Table 1. The details on the artificially produced hybrid *P. perfoliatus* L. × *P. gramineus* L., which provided the evidence on maternal inheritance of cpDNA in *Potamogeton*, will be given in a separate paper.

Fresh samples of most of the *P. perfoliatus* and all the putative *P.* ×*cooperi* were cultivated in the experimental garden at the Institute of Botany, Průhonice, Czech Republic, where they were grown from 1997–2003. Plants were cultivated in 180 × 140 × 80 cm water-filled plastic tanks, which were sunk in the ground in order to prevent overheating of water in summer. The samples were planted in plastic pots with pond mud that underwent desiccation treatment and were submerged in a cultivation tank. Leaf material from each plant was sampled in summer 2003 and immediately used for DNA extractions. Plants of *P. crispus* were collected in the field and preserved in transparent PET bottles filled with water for up to 2 weeks before DNA isolation. Voucher herbarium specimens from both field and cultivated plants are preserved in the Herbarium of the Institute of Botany, Průhonice (acronym PRA).

DNA isolation

DNA isolations were performed from leaves as described in ŠTORCHOVÁ et al. (2000), but the fresh leaves were crushed in liquid nitrogen and the first centrifugation step was performed at 9000 rpm for better sedimentation of the material. Quality and yield of the isolated DNA were checked on agarose gels. From the dry samples (1476–1479) only degraded DNA was obtained so that they were excluded from molecular analyses.

Analysis of chloroplast DNA

The *trn*K-*trn*Q intergenic spacer of chloroplast DNA that proved to be useful in a previous study on *Potamogeton* hybrids (KING et al. 2001) was PCR-amplified as follows: Reaction volumes of 50 µl contained 5 µl of Mg^{2+}-free reaction buffer, 2 mM $MgCl_2$, 100 µM of each dNTP, a few nanograms of genomic DNA, 1 unit of Taq DNA-polymerase (MBI Fermentas) and 0.2 mM of each primer (*trn*K2 and *trn*Qr; DUMOLIN-LAPEGUE et al. 1997). After an initial pre-denaturation at 94 °C for 5 min, 30–35 cycles of 94 °C/30s, 48 °C/30s and 72 °C/2.5 min were performed followed by a final extension at 72 °C for 10 min. One *P. perfoliatus* (1480) and one *P. crispus* (1465) sample did not produce any amplified products and were also excluded from further molecular studies.

Two samples of each putative parental taxon were initially screened for distinguishing RFLP patterns, subjecting the fragments of approx. 3100 bp length to restriction digests with six different enzymes (4 and 5 bp-cutters and AT-rich 6 bp-cutters, accounting for the usually observed AT content of cpDNA). *Tru* I (*Mse* I) produced differential patterns between the parental species and was subsequently used for all samples. 30–50 ng of PCR product were digested overnight at 65 °C using 5 U of enzyme and 1/10 of the reaction volume of the manufacturer's reaction buffer (MBI Fermentas). Products were separated on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

Analysis of nuclear ribosomal DNA

The expectation was to detect copies of both parental species in the putative hybrids. As an efficient way to do this without subcloning PCR products and sequencing different clones, we designed RFLPs for the internal transcribed spacer (ITS) region that allowed the simultaneous detection of both variants in the hybrids. First, two samples of different geographic origin from each of the parental species were chosen for sequencing in order to facilitate the search for appropriate restriction enzymes and to assess intraspecific variation to some extent. Additionally, the sample first tentatively designated as possible *P.* × *cooperi* (1470) but later suspected to be only a narrow-leaved form of *P. perfoliatus*, was included (see Table 1). The entire ITS region (ITS1, 5.8S, ITS2) was amplified using the primers ITS F (KING et al. 2001) and ITS 4 (WHITE et al. 1990). Conditions were as described for cpDNA except that the reaction volume was 25 µl, annealing temperature 55 °C and extension time 1.5 min. PCR products were sequenced (GATC Biotech AG, Konstanz, Germany) using the ITS F primer; one of them (979) was additionally sequenced with ITS 4 because of intra-individual polymorphism (see below). Original ABI-files were obtained and proof read manually. Sequences were deposited in the GenBank database (accession numbers

Fig. 1. A common form of *Potamogeton crispus* with linear-oblong leaves, which are undulate at margin (Z. KAPLAN 99/154); scale bar = 3 cm.

Fig. 2. A common form of *Potamogeton perfoliatus* with ovate leaves (cultivated as Z. KAPLAN 840); scale bar = 3 cm.

Fig. 3. Vegetative shoots of *Potamogeton* ×*cooperi* from Pasohlávky, Moravia, Czech Republic (cultivated as Z. KAPLAN 1420); scale bar = 3 cm.

Fig. 4. An adult flowering plant of *Potamogeton* ×*cooperi* from Pasohlávky, Moravia, Czech Republic (cultivated as Z. KAPLAN 1420); scale bar = 3 cm.

Fig. 5. Vegetative shoots of *Potamogeton* ×*cooperi* from Lower Solva, Wales, United Kingdom (cultivated as Z. KAPLAN 1248); scale bar = 3 cm.

Fig. 6. A narrow-leaved form of *Potamogeton perfoliatus*, which mimics *P.* ×*cooperi* (cultivated as Z. KAPLAN 1470); scale bar = 3 cm.

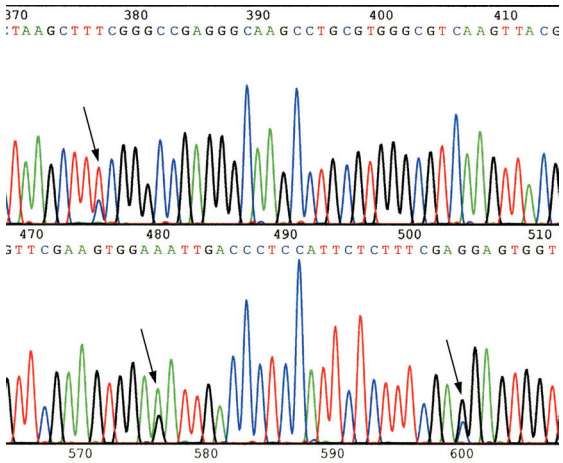

Fig. 7. Intra-individual polymorphism in *P. perfoliatus* ITS sequences. Arrows indicate the three polymorphic sites found in one of our samples (979). The higher peaks in the chromatogram corresponded to the nucleotide states also found in an unpublished GenBank sequence of this species (AY330703); the alternative nucleotide states (lower peaks) were present in our samples 1002 and 1470.

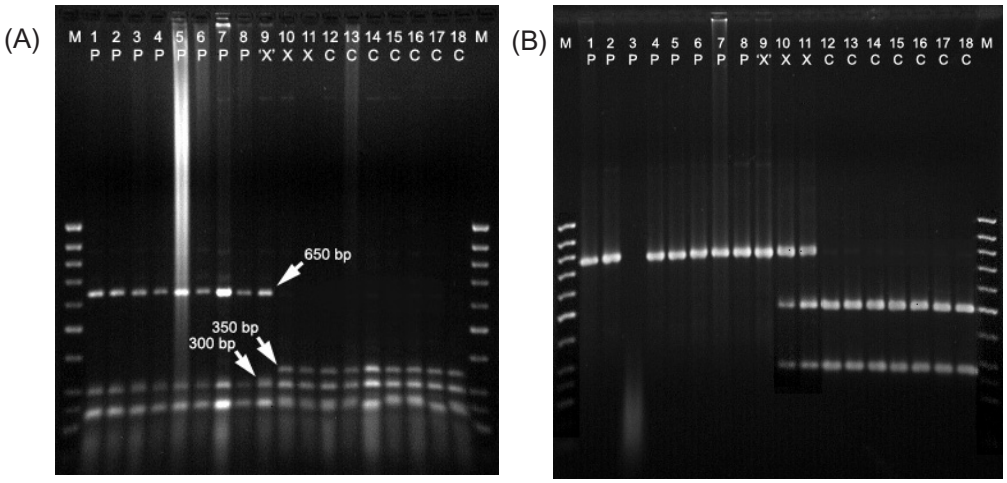

Fig. 8. **(A)** – RFLP of the *trn*K-*trn*Q intergenic spacer of chloroplast DNA. The *P. perfoliatus*-specific (P) fragment (650 bp) is cut into two fragments in the *P. crispus*-specific (C) samples; the smaller one (300 bp) runs – according to the relative brightness of bands in samples with similar DNA concentration (e.g. compare 1–4 to 15–18 or 7 to 14) – along with a fragment of similar length that is present in both species. The sample 1470 originally suspected to be *P.* ×*cooperi* but later identified as a narrow-leaved form of *P. perfoliatus* ("X") in track 9 shows the *P. perfoliatus* pattern (for discussion see text). The two true hybrids (X) show the *P. crispus* haplotype indicating their maternal origin from this species in both cases. Identity of samples (compare Table 1): 1 – 1002, 2 – 979, 3 – 985, 4 – 1467, 5 – 1469, 6 – 840, 7 – 1481, 8 – 1471, 9 – 1470, 10 – 1248, 11 – 1420, 12 – 1463, 13 – 1485, 14 – 1483, 15 – 1464, 16 – 1466, 17 – 1473, 18 – 1472. **(B)** – RFLP of the internal transcribed spacer. 1–8 (P): *P. perfoliatus*; 9 ("X"): sample originally incorrectly suspected to be *P.* ×*cooperi* (see text) showing only the *P. perfoliatus* pattern (uncut PCR product, 768 bp); 10–11 (X): true hybrids showing the ITS variants of both parental taxa; 12–18 (C): *P. crispus* (273 and 495 bp fragments, respectively). Sample 3 was degraded for unknown reasons, but also had the *P. perfoliatus* pattern (not shown). Identity of samples (compare Table 1): 1 – 1002, 2 – 979, 3 – 985, 4 – 1467, 5 – 1469, 6 – 840, 7 – 1481, 8 – 1471, 9 – 1470, 10 – 1248, 11 – 1420, 12 – 1463, 13 – 1485, 14 – 1483, 15 – 1464, 16 – 1466, 17 – 1473, 18 – 1472.

AY529523-AY529527, see Table 1). Alignment was performed with CLUSTAL_X (THOMPSON et al. 1997) and was unambigous for all positions.

Initial screening for RFLP-generating enzymes was done by visual inspection of palindromic motifs present in only one of the species. Determination of cut sites of differentiating enzymes and lengths of the resulting fragments was facilitated using the respective features of BioEdit (HALL 1999). Due to the rather high divergence between ITS sequences of *P. perfoliatus* and *P. crispus*, many differentiating restriction sites could be inferred from the sequences. Among these, a *Dra* I site was chosen because it produced the easiest readable RFLPs, i.e., not too many or too small fragments, and because its 6-bp recognition site differed by three nucleotide substitutions between *P. perfoliatus* and *P. crispus* so that the likelihood of it arising twice independently was extremely low.

Restriction digests were performed using 4 µl of PCR product, 2 U of *Dra* I and 1/10 reaction volume of *Dra* I buffer (MBI Fermentas) and incubated overnight at 37 °C according to the manufacturer's instructions. The products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

RESULTS

Morphological evaluation

Three samples thought by their collectors to be the hybrid between *P. crispus* and *P. perfoliatus* (1248, 1420, 1470) have been included in this study. The parental species themselves are quite distinct morphologically (Figs. 1 and 2) and are only rarely confused with one another. Their hybrid combines the characters of both parents (Table 2). There are no other similar species or hybrids among European members of the genus. Some hybrids sometimes confused with *P. ×cooperi* (such as *P. ×undulatus*, *P. ×cognatus* or *P. ×nitens*) still show several distinguishing features not observed in our specimens. The identity of the three intermediate samples with other *Potamogeton* taxa could therefore be excluded. The only other possibility was that these plants were extreme forms of one of the two putative parental species.

The Moravian plants (1420) are intermediate in many characters. Specimens collected early in the season are more *P. crispus*-like in the general appearance and shape of leaves (Fig. 3). The complete plants collected in mid summer are the most easy to recognize as they have (5–)7–veined linear-oblong basal leaves, i.e., *P. crispus*-like, whereas the mid- and upper-stem leaves are ovate, clearly *P. perfoliatus*-like in shape (Fig. 4). The well developed leaves of adult shoots have 7–11, exceptionally up to 13 longitudinal leaf veins. Throughout the stem, the range of leaf width is (6–)9–19(–21) mm, that of leaf length : width ratio is 2.6–6.2. The spikes are 6–10 mm long, with 6–8 flowers. We identified this sample as *P. ×cooperi*.

A similar pattern of morphological features was also observed in the Welsh plant (1248). The stem is compressed and grooved but less markedly than is usual in *P. crispus*. The leaves are linear-lanceolate to ovate, 9–16 mm wide, 1.9–4.3 times as long as wide, with 7–9(–11) longitudinal veins (Fig. 5). The leaf apex is sometimes slightly hooded as in *P. perfoliatus*. The spikes are 5–12 mm long, with 4–10 flowers. Particularly the intermediate number of leaf

veins, but also the other characters when considered in conjunction, clearly indicate that the plant is intermediate between the putative parents and we determined it as *P.* ×*cooperi*.

These two samples of *P.* ×*cooperi* cultivated in tanks in the experimental garden produced abundant spikes and flowered each summer. Nevertheless, no fruit development has been observed in cultivation in spite of normal flowering. The development of flowers differed from that of true species. The tepals of these plants remained tightly closed and the stigmas protruded through them. The entire spikes rot after flowering instead of setting fruit. This behaviour is typical of sterile hybrids (PRESTON 1995: 46, PRESTON et al. 1998a, KAPLAN & WOLFF 2004). In contrast, the tepals of fertile plants open to reveal the anthers and the carpels.

In contrast, the Bavarian plant (1470) showed a somewhat different pattern of features. The fragments collected in the field and some of the specimens obtained from cultivation were superficially similar to the two hybrid samples (Fig. 6). The resemblance is best expressed by the leaves, which are lanceolate to ovate and 8–19 mm wide. However, although these characters mimic *P.* ×*cooperi*, other features such as the high number of longitudinal leaf veins (11–17) precludes the plant from being this hybrid. Further we did not notice any of the characters of *P. crispus* usually observed in *P.* ×*cooperi*, such as the compression of the stem or the typical linear-oblong basal leaves with reduced number of lateral veins. The general appearance of the adult plant is also in accordance with some other collections that we consider to be one of many forms of *P. perfoliatus*. Unfortunately, this sample did not flower in 2003, possibly because the sample was planted in the cultivation tank in late July when the season was too advanced. The sample failed to produce sufficient vegetative mass to enable the developpemnt of generative organs. We considered this to be a narrow-leaved form of *P. perfoliatus* mainly because of the high number of lateral veins and the lack of any conclusive evidence of introgression of *P. crispus*.

Analysis of chloroplast DNA

Restriction digests of the amplified fragment between the second exon of *trn*K and *trn*Q with *Tru* I produced two well distinguished patterns for *P. crispus* and *P. perfoliatus*, designated as C and P, respectively (Fig. 8a). These were uniform for all plants tested in both species. The *P. perfoliatus*-specific fragment was cut into two fragments in the *P. crispus*-specific samples; the smaller one running along with a fragment of similar length that is present in both species. The two putative hybrids determined as *P.* ×*cooperi* (samples 1420 and 1248, designated X in the figure) showed cpDNA of the *P. crispus* type. The third sample first suspected of being *P.* ×*cooperi* (1470, designated "X" in the figure), which according to its morphology was later identified to be merely a narrow-leaved form of *P. perfoliatus*, showed the *P. perfoliatus*-type of chloroplast DNA. This did not yet necessarily exclude a hybridogenous origin of the sample but in any case *P. perfoliatus* provided the cpDNA. According to the maternal inheritance of cpDNA, in both cases of our confirmed natural hybrids *P.* ×*cooperi* (1420, 1248), *P. crispus* was the female parent.

Analysis of nuclear ribosomal DNA

The ITS sequences of the two species were rather divergent; about 7.5% and 10.2% nucleotide differences in ITS 1 and ITS 2, respectively. Exact figures cannot be provided

because the exact positions of rRNA genes and ITS are unclear except for the 18S-ITS1 boundary. Even the most similar sequences and those of the closest relatives available in GenBank (e.g. *Liliopsida*) are too divergent and the treatment of the boundaries with different authors too inconsistent (9–11 bases variation at each boundary) to infer the positions properly. With the exception of two unpublished *Potamogeton* sequences (see below), no other GenBank sequence was similar enough to allow unambiguous alignment of ITS with our samples.

Our two *P. crispus* sequences (samples 1472 and 1473 – AY529523 and AY529524) were identical; the two *P. perfoliatus* samples (1002 and 979 – AY529526 and AY529527) were polymorphic at three positions (0.4%), one of them situated in the 5.8S rDNA region. The sequence of the narrow-leaved *P. perfoliatus* (1470 – AY529525) was identical to one of our typical samples of *P. perfoliatus* (1002) confirming that it indeed belonged to this species. Our second *P. perfoliatus* (979) was identical to an unpublished sequence of *P. perfoliatus* (GenBank AY330703) except that the latter showed a number of unclear positions at both ends indicating bad sequence quality of the latter. The only other *Potamogeton* sequence available in GenBank (*P. nodosus* POIR., AF102273, unpubl.) was more similar to *P. crispus* than to *P. perfoliatus*, but clearly different from both species. Closer examination of the three polymorphic sites found in *P. perfoliatus* revealed intra-individual polymorphism of ITS in one of our samples (979), exhibiting both respective character states of the intra-specific polymorphism at all three positions (Fig. 7).

In all the PCR-RFLPs, it was possible to distinguish clearly *P. crispus* from *P. perfoliatus* by their ITS-RFLP phenotypes, designated as C and P, respectively (Fig. 8b). No variation was detected within the species. The restriction enzyme *Dra* I did not cut the *P. perfoliatus* PCR product (768 bp), but cut the *P. crispus* samples once producing bands of 495 and 273 bp, respectively. The sample 1470 (designated "X" in the figure) originally thought to be the hybrid but later considered as a narrow-leaved form of *P. perfoliatus* showed only the *P. perfoliatus* pattern as expected. In contrast, the presumed hybrids (1248 and 1420, designated X in the figure) exhibited additive RFLP patterns of both the parental taxa, consistent with the biparental inheritance of nuclear DNA. The fact that the Welsh hybrid also showed *P. crispus* patterns with both types of molecular markers even though only parental reference material from Central Europe was used for comparison indicates that intraspecific variation of this species was neglectable in our study.

DISCUSSION

The detailed morphological analysis leads to the conclusion that the Moravian and Welsh samples 1420 and 1248, respectively, are clearly intermediate between *P. crispus* and *P. perfoliatus*. All important diagnostic features are in accordance with the character pattern considered to be typical for *P.* ×*cooperi*. The observed behaviour of floral organs indicates full sterility. Still, this does not rule out the possibility that these plants are sterile aneuploid variants rather than interspecific hybrids. However, the molecular analyses confirmed the identity of the supposed hybrid samples 1420 and 1248 with *P.* ×*cooperi*, the hybrid between *P. crispus* and *P. perfoliatus*. The chloroplast DNA markers identified *P. crispus* as the maternal parent of both hybrid plants.

As described above, *P.* ×*cooperi* was found accompanied with both its putative parents in the Moravian locality. The respective water reservoir was completed and filled in 1978. None of the parental taxa were among the first species colonizing the new habitat and it may therefore be concluded that hybridization between *P. crispus* and *P. perfoliatus* took place in the locality quite recently, very probably not more than 10 years ago. In contrast, none of the parental species were found together with *P.* ×*cooperi* in the Welsh locality. The 70-year-old history of repeated collecting of the hybrid in the river Solva implies that the local colony of *P.* ×*cooperi* has been established a long time ago and the hybrid seems to be orphaned there (PRESTON & CHATER 1997). The occurrence of a *Potamogeton* hybrid in the absence of one or both its parents is repeatedly documented (e.g. DANDY & TAYLOR 1946, HOLLINGSWORTH et al. 1996b, PRESTON & CHATER 1997, PRESTON et al. 1998a,b, 1999, KING et al. 2001, KAPLAN & WOLFF 2004).

Since the plants of *P.* ×*cooperi* are sterile, their ability to spread is confined to vegetative propagation within a relatively limited area. Turions produced in the leaf axils as well as detached stem and rhizome fragments provide the main means of dispersal. These propagules may be transported by rivers, particularly during spring floods with rapid water flow. However, natural long-distance dispersal outside a water body such as a water reservoir or a river can almost be excluded. As the distance between the Moravian and Welsh localities is approximately 1600 km, we consider the two hybrid populations as results of two different hybridization events.

The literature on *Potamogeton* taxonomy (mainly Floras) often claims that hybridization within this genus is frequent. However, the exact pattern of the process of hybridization is only rarely discussed (notable exception is PRESTON 1995). Results of our studies, together with additional field observations indicate that cross-pollination may be a relatively frequent event if two *Potamogeton* species with the same pollination system grow together and flower at the same time. However, the critical point in development of hybrid plants seems to be the limited germination of the seeds.

In the Czech Republic, each of the six recently discovered sites of *P.* ×*fluitans* ROTH (= *P. lucens* L. × *P. natans* L.) was a fishpond that had been seasonally dry a few years ago. This confirmed the previous conclusion about limiting factors in establishing of this hybrid in Central-European ponds (KAPLAN et al. 2002). Most Central-European lowland and shallow water reservoirs filled for many years and exhibiting eutrophic conditions are relatively poor in species of water macrophytes. The bottom is usually covered by thick organic-rich sediment (sapropel), which is often toxic because of anaerobic conditions. Water transparency is generally low and seed germination is inhibited. Populations of colonized plants are relatively stabilized. If present, species of *Potamogeton* mostly persist vegetatively. However, when the pond bottom is seasonally exposed, the sediment is oxidized and mineralized. After the fishpond is filled again, high concentration of nutrients is released from the flooded soil while water transparency is still high. This enables explosive development of macrophyte vegetation, including seedling recruitment from the seed bank and establishing of new hybrid plants. However, the hybrids usually soon disappear after the habitat condition are not longer favourable, which is often connected with intensive fish breeding. The fact that *P.* ×*fluitans* was not known from the Czech Republic before its recent discovery (KAPLAN

2001) but it has been recently detected in six fishponds seasonally dried during previous summers indicates that the seed bank contains hybrid seeds quite frequently, very probably in most of the sites where *P. lucens* and *P. natans* grow, flower and set seed together. These observations may also explain the rather early and quick rise of *P. ×cooperi* in the newly constructed reservoir in the southern Moravia.

Under optimal conditions, the establishment of new hybrid plants really may be a frequent event. A detailed field study of PRESTON et al. (1999) led to the discovery of *P. ×suecicus* K. RICHT. (= *P. filiformis* PERS. × *P. pectinatus* L.) in 15 of 17 lochs in the Outer Hebrides from which one or both its parents had been recorded. Although these observations were not universal for all British areas studied, they supported the view of HESLOP-HARRISON & CLARK (1941) that the hybrid "seems to occur wherever the parent species clash". A detailed isozyme study confirmed multiple origins of several of these clones (HOLLINGSWORTH et al. 1996b).

When the hybrid seed overcomes the critical stage of seedling recruitment, the new hybrid colony can persist in the locality for a considerably long period, even for hundreds or thousands of years, provided that the ecological conditions of the habitat remain suitable. Thus, several colonies of *Potamogeton* hybrids (e.g. *P. ×lanceolatifolius*, *P. ×nitens*, *P. ×salicifolius*, *P. ×undulatus*) were still confirmed during an extensive fieldwork in 1998 in Denmark and Sweden as persisting in the absence of their parents on the sites where they were first recorded in 1895–1897 mainly by I. Baagøe and G. Tiselius (KAPLAN, unpubl.). The distribution of *P. ×suecicus* in England, south of the present limit of distribution of *P. filiformis*, suggests that these clones may be relics from glacial periods (HOLLINGSWORTH et al. 1996b). The occurrence of *P. ×bottnicus* HAGSTR. (= *P. pectinatus* L. × *P. vaginatus* TURCZ.) in Britain indicates that this hybrid may have persisted in the islands for thousands of years. One of its parents, *P. vaginatus*, is now restricted in Europe to Scandinavia and in Britain it is documented only from deposits which date back to the first British glacial period (PRESTON et al. 1998b, KING et al. 2001). Besides the long history of some hybrid clones, sometimes they occupy considerable areas and even can produce dominant stands. These factors make *Potamogeton* hybrids a significant component of aquatic communities.

In contrast to the confirmed hybrid origin of the Moravian and Welsh samples, the Bavarian sample 1470, intentionally collected at a site with previously recorded occurrence of *P. ×cooperi*, but identified as a narrow-leaved form of *P. perfoliatus*, was confirmed with molecular data as really belonging to this species, not to the hybrid. Although both parental species still seldom occur at the river Main near Ebing, the hybrid seems to be extinct there at present. However, the past occurrence of "true" *P. ×cooperi* in pools of the river Main at Ebing is well documented by numerous specimens collected there by G. Fischer from 1900 to 1906 and widely distributed in many herbaria.

The hybrid *P. ×cooperi* has so far been recorded from Great Britain, Ireland, Denmark, the Netherlands, Germany, former Czechoslovakia, Lithuania and Romania (FISCHER 1904, 1907, HAGSTRÖM 1916, HEJNÝ 1950, GALINIS 1963, ȚOPA 1966, DANDY 1975, PLOEG 1990, PRESTON 1995). However, some records are possibly erroneous. For example, the specimens from Romania issued as the exsiccate collection Fl. Rom. Exs. no. 2705 and identified as this hybrid actually belong to *P. perfoliatus* (freely fruiting plants with 17-veined

leaves!). We have, however, recently identified herbarium specimens of *P.* ×*cooperi* also from France and Russia. Still, *P.* ×*cooperi* is rare outside the British Islands and there are only some 10 confirmed records from the Continent.

Besides the compelling evidence of the hybrid origin of the Moravian and Welsh samples provided by this study, these results also show that reliable identification of some *Potamogeton* hybrids is, under certain circumstances, possible with their morphology alone. This is particularly true for the hybrids between very dissimilar species, such as between a broad-leaved and a narrow-leaved species. Similar agreement between identifications from a morphological and isozyme perspectives was reported by PRESTON et al. (1999) based on British samples of *P.* ×*suecicus*. In contrast to this rather optimistic view, morphological recognition of some hybrids between narrow-leaved species may be impossible due to overall similarity between these plants and to their simple structure, which did not provide enough characters. The hybrid *P.* ×*cooperi* may lie just in the middle between easy recognizable and unrecognizable hybrids. We would like to stress, however, that identification of hybrids must always be done with utmost care. Some extreme phenotypes of true species may mimic hybrids and only careful and detailed examination of well developed material can reveal their true identity (see the example of sample 1470 discussed in this study or other cases described by KAPLAN 2002a). The recognition of hybrids requires some experience and great prudence should be exercised whenever a putative hybrid is investigated.

Acknowledgements: We are particularly grateful to Jaroslav Rydlo, Chris D. Preston, Lenz Meierott and Jirka Hummel who kindly provided us with plant material. We also would especially like to thank Ludmila Kirschnerová who performed the PCR-RFLPs for this study. Mária Loncová, Radmila Dvořáková and Marie Stará helped with the labwork. We wish to express our thanks to Jitka Štěpánková for her help during fieldwork and to Ondřej Dvořák and Vojtěch Zavadil for taking care of the cultivated *Potamogeton* material. John Bailey kindly improved the English of an earlier version of the manuscript. We gratefully acknowledge an anonymous reviewer for helpful comments. The research was supported by grants no. 206/03/P156 and 206/02/0773 from the Grant Agency of the Czech Republic, institutional long-term research plan no. AV0Z6005908 from the Academy of Sciences of the Czech Republic, and grant no. KSK6005114 from the Grant Agency of the Academy of Sciences of the Czech Republic.

REFERENCES

ASCHERSON P. & GRAEBNER P. (1897): *Synopsis der mitteleuropäischen Flora 1*. Wilhelm Engelman, Leipzig.

ÁLVAREZ I. & WENDEL J.F. (2003): Ribosomal ITS sequences and plant phylogenetic inference. *Molec. Phylogenet. Evol.* 29: 417–434.

BAAGØE J. (1897): *Potamogeton undulatus* WOLFGANG. *Bot. Tidsskr*. 21: 221–236, pl. 7.

BAUMEL A., AINOUCHE M.L. & LEVASSEUR J.E. (2001): Molecular investigations in populations of *Spartina anglica* C.E. HUBBARD (*Poaceae*) invading coastal Brittany (France). *Molec. Ecol.* 10: 1689–1701.

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

CAMPBELL C.S., WOJCIECHOWSKI M.F., BALDWIN B.G., ALICE L.A. & DONOGHUE M.J. (1997): Persistent nuclear ribosomal DNA sequence polymorphism in the *Amelanchier* agamic complex. *Molec. Biol. Evol.* 14: 81–90.

DANDY J.E. (1975): *Potamogeton* L. In: STACE C.A. (ed.), *Hybridization and the flora of the British Isles*, Academic Press, London, pp. 444–459.

DANDY J.E. (1980): 1. *Potamogeton* L. In: TUTIN T.G., HEYWOOD V.H., BURGES N.A., MOORE D.M., VALENTINE D.H., WALTERS S.M. & WEBB D.A. (eds.), *Flora Europaea 5*: Alismataceae *to* Orchidaceae (Monocotyledones), Cambridge University Press, Cambridge, pp. 7–11.

DANDY J.E. & TAYLOR G. (1946): An account of × *Potamogeton suecicus* RICHT. in Yorkshire and the Tweed. *Trans. & Proc. Bot. Soc. Edinburgh* 34: 348–360.

DUMOLIN-LAPEGUE S., PEMONGE M.-H. & PETIT R.J. (1997): An enlarged set of consensus primers for the study of organelle DNA in plants. *Molec. Ecol.* 6: 393–397.

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*. *Aquatic 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. *Pl. Syst. Evol.* 229: 45–57.

FANT J.B., KAMAU E.M. & PRESTON C.D. (2003): Chloroplast evidence for the multiple origins of the hybrid *Potamogeton ×sudermanicus* HAGSTR. *Aquatic Bot.* 75: 351–356.

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.

FRANZKE A. & MUMMENHOFF K. (1999): Recent hybrid speciation in *Cardamine* (*Brassicaceae*) – conversion of nuclear ribosomal ITS sequences in statu nascendi. *Theor. Appl. Genet.* 98: 831–834.

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

FRYER A. (1891): On a new British *Potamogeton* of the *nitens* group. *J. Bot.* 29: 289–292, t. 313.

FRYER A. (1897): *Potamogeton undulatus*, WOLF. = *P. perfoliatus* × *crispus*. *Rep. Bot. Soc. Exch. Club Brit. Isles* 1: 497.

GALINIS V. (1963): 1 šeima. Pludiniai – *Potamogetonaceae* ENGL. In: NATKEVIČAITĖ-IVANAUSKIENĖ M (ed.), *Lietuvos TSR Flora (Flora of Lithuania) 2*, Lietuvos TSR Mokslu Akademija Botanikos Institutas, Vilnius, pp. 35–84 & 677.

GLÜCK H. (1936): Pteridophyten und Phanerogamen. In: PASCHER A. (ed.), *Süßwasserflora Mitteleuropas 15*, Jena.

GRAEBNER P. (1907): 4. *Potamogeton* (TOURN.) L. In: ENGLER A. (ed.), *Das Pflanzenreich*, *Regni vegetabilis conspectus 31 (IV.11)*, Berlin, pp 39–142 & 161–162.

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

HALL T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Series* 41: 95–98.

HAYNES R.R. (1985): A revision of the clasping-leaved *Potamogeton* (*Potamogetonaceae*). *Sida* 11: 173–188.

HAYNES R.R. & HELLQUIST C.B. (2000): 195. *Potamogetonaceae* DUMORTIER. In: FLORA OF NORTH AMERICA EDITORIAL COMMITTEE (eds.), *Flora of North America north of Mexico 22*, Oxford University Press, New York, pp. 47–74.

HEJNÝ S. (1950): Čeleď 138. *Potamogetonaceae*. Rdestovité – Rudavcovité. In: DOSTÁL J. (ed.), *Květena ČSR (Flora of the Czechoslovakia) 2*, ČSBS, Praha, pp. 1722–1737.

HESLOP-HARRISON J.W. & CLARK W.A. (1941): Hybrid Potamogetons on the Isle of Benbecula. *Occas. Notes Dept. Bot., King's Coll., Newcastle upon Tyne* 2: 1–4.

HETTIARACHCHI P. & TRIEST L. (1991): Isozyme polymorphism in the genus *Potamogeton* (*Potamogetonaceae*). *Opera Bot. Belg.* 4: 87–114.

HOLLINGSWORTH P.M., GORNALL R.J. & PRESTON C.D. (1995a): Genetic variability in British populations of *Potamogeton coloratus* (*Potamogetonaceae*). *Pl. Syst. Evol.* 197: 71–85.

HOLLINGSWORTH P.M., PRESTON C.D. & GORNALL R.J. (1995b): 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. (1996a): Genetic variability in two hydrophilous species of *Potamogeton*, *P. pectinatus* and *P. filiformis* (*Potamogetonaceae*). *Pl. Syst. Evol.* 202: 233–254.

HOLLINGSWORTH P.M., PRESTON C.D. & GORNALL R.J. (1996b): Isozyme evidence for the parentage and multiple origins of *Potamogeton ×suecicus* (*P. pectinatus* × *P. filiformis*, *Potamogetonaceae*). *Pl. Syst. Evol.* 202: 219–232.

HOLLINGSWORTH P.M., PRESTON C.D. & GORNALL R.J. (1998): Euploid and aneuploid evolution in *Potamogeton* (*Potamogetonaceae*): a factual basis for interpretation. *Aquatic Bot.* 60: 337–358.

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

KALKMAN L. & VAN WIJK R.J. (1984): On the variation on chromosome number in *Potamogeton pectinatus* L. *Aquatic Bot.* 20: 343–349.

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

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

KAPLAN Z. (2002b): 147. *Potamogetonaceae* DUM. – rdestovité. In: KUBÁT K., HROUDA L., CHRTEK J. jun., KAPLAN Z., KIRSCHNER J. & ŠTĚPÁNEK J. (eds.), *Klíč ke květeně České republiky (Key to the flora of the Czech Republic)*, Academia, Praha, pp. 736–743.

KAPLAN Z. & ŠTĚPÁNEK J. (2003): Genetic variation within and between populations of *Potamogeton pusillus* agg. *Pl. 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.

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.

LES D.H. & PHILBRICK C.T. (1993): Studies of hybridisation and chromosome number variation angiosperms: evolutionary implications. *Aquatic Bot.* 44: 181–228.

NOVÁKOVÁ H. (1982): Rozšíření vybraných širokolistých rdestů (*Potamogeton* L.) v ČSR (Distribution of selected broad-leaved pondweeds (*Potamogeton* L.) in the Czech Republic). *Práce Stud., Přír. (Pardubice)* 13–14: 49–71.

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

PLOEG D.T.E. VAN DER (1990): De Nederlandse breedbladige Fonteinkruiden. *Wetensch. Meded. Kon. Ned. Natuurhist. Ver.* 195: 1–98.

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

PRESTON C.D. (1988b): The *Potamogeton* taxa described by Alfred Fryer. *Watsonia* 17: 23–35.

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

PRESTON C.D. (2001): [review] Flora of North America north of Mexico. Volume 22 *Magnoliophyta*: *Alismatidae*, *Arecidae*, *Commelinidae* (in part), and *Zingiberaceae*. *Watsonia* 23: 458–459.

PRESTON C.D., BAILEY J.P. & HOLLINGSWORTH P.M. (1998a): A reassessment of the hybrid *Potamogeton* ×*gessnacensis* G. FISCH. (*P. natans* × *P. polygonifolius*, *Potamogetonaceae*) in Britain. *Watsonia* 22: 61–68.

PRESTON C.D. & CHATER A.O. (1997): *Potamogeton* ×*cooperi* (FRYER) FRYER in Wales. *Bot. Soc. Brit. Isles Welsh Bull.* 62: 11–12.

PRESTON C.D. & CROFT J.M. (1997): *Aquatic plants in Britain and Ireland*. Harley Books, Colchester.

PRESTON C.D., HOLLINGSWORTH P.M. & GORNALL R.J. (1998b): *Potamogeton pectinatus* L. × *P. vaginatus* TURCZ. (*P.* ×*bottnicus* HAGSTR.), a newly identified hybrid in the British Isles. *Watsonia* 22: 69–82.

PRESTON C.D., HOLLINGSWORTH P.M. & GORNALL R.J. (1999): The distribution and habitat of *Potamogeton* ×*suecicus* K. RICHT. (*P. filiformis* PERS. × *P. pectinatus* L.) in the British Isles. *Watsonia* 22: 329–342.

RAUNKIAER C. (1896): *De Danske blomsterplanters naturhistorie (Natural history of Danish flowering plants). I.* Helobiae. Kjobenhavn.

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

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.

THOMPSON J.D., GIBSON T.J., PLEWNIAK F., JEANMOUGIN F. & HIGGINS D.G. (1997): The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quailty analysis tools. *Nucl. Acids Res.* 25: 4876–4882.

ŢOPA E. (1966): Fam. 112. *Potamogetonaceae* A.L. JUSS. In: SĂVULESCU T. (ed.), *Flora republicii socialiste Romania (Flora of Romania) 11*, Acad. Reip. Soc. Rom., Bucuresti, pp. 54–88.

VAN WIJK R.J., VAN GOOR E.M.J. & VERKLEY J.A.C. (1988): Ecological studies on *Potamogeton pectinatus* L. II. Autecological characteristics, with emphasis on salt tolerance, intraspecific variation and isoenzyme patterns. *Aquatic Bot.* 32: 239–260.

VARGAS P., MCALLISTER H.A., MORTON C., JURY S.L. & WILKINSON M.J. (1999): Polyploid speciation in *Hedera* (*Araliaceae*): phylogenetic and biogeographic insights based on chromosome counts and ITS sequences. *Pl. Syst. Evol.* 219: 165–179.

WENDEL J.F. (2000): Genome evolution in polyploids. *Pl. Molec. Biol.* 42: 225–249.

WENDEL J.F., SCHNABEL A. & SEELANAN T. (1995): Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). *Proc. Natl. Acad. Sci. USA* 92: 280–284.

WHITE T.J., BRUNS T., LEE S. & TAYLOR J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: INNIS M.A., GELFAND D.H., SNINSKY J.J. & WHITE T.J. (eds.), *PCR Protocols: A guide to methods and applications*, Academic Press, San Diego, pp. 313–322.

WIEGLEB G. (1990): 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.

WOLFE MURPHY S.A., SMITH S.J. & PRESTON C.D. (1991): Irish pondweeds: 1. A recent record of *Potamogeton ×cooperi* (FRYER) FRYER from Co. Antrim. *Irish. Nat. J.* 23: 457–458.

Received 19 September 2004, revision received and accepted 9 October 2004