

## Molecular identification of hybrids from a former hot spot of *Potamogeton* hybrid diversity

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### ARTICLE INFO

#### Article history:

Received 14 December 2011

Received in revised form 8 November 2012

Accepted 20 November 2012

Available online 20 December 2012

#### Keywords:

Hybridization

Taxonomy

*Potamogeton*

Molecular identification

Diversity

Aquatic vegetation

### ABSTRACT

More than a century ago, an extremely high diversity of *Potamogeton* hybrids was recorded in several rivers and streams in Jutland, Denmark. Accuracy of their identification was doubted by some later authors who were sceptical about the existence and co-occurrence of so many hybrids. Selected original localities were investigated for the presence of morphologically matching plants after more than 100 years. In spite of profound changes in landscape and considerable deterioration of aquatic habitats during the 20th century resulting in a significant decline of submerged vegetation, three of ten previously recorded hybrids were recently found that had persisted at their original localities. Two of them, whose existence had not been proved previously, were subjected to molecular analyses. RFLP, direct sequencing and cloning of the ITS region confirmed their previous morphological identification: *P. × undulatus* as *P. crispus* × *P. praelongus* and *P. × cognatus* as *P. perfoliatus* × *P. praelongus*. Chloroplast DNA sequencing identified *P. praelongus* as the maternal parent in both crosses. The existence of most of the other hybrid combinations recorded from Jutland was proved by means of molecular analyses conducted on plants from other regions. Their morphologies perfectly correspond to old herbarium vouchers from Jutland and support their original identifications. These observations indicate that Jutland rivers and streams hosted a high species and hybrid diversity still in the late 19th century, but most of this richness has meanwhile disappeared.

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### 1. Introduction

Hybridization is one of the key processes contributing to the taxonomic complexity in Potamogetonaceae (Kaplan et al., 2009; Kaplan, 2010a; Kaplan and Fehrer, 2011; Kaplan and Uotila, 2011). Although the occurrence of hybrids in *Potamogeton* was first suggested more than a century ago (see the reviews in Wiegleb et al., 2008; Kaplan et al., 2009), molecular studies have only recently provided firm evidence for the existence of many of them (e.g., King et al., 2001; Fant et al., 2003, 2005; Kaplan and Fehrer, 2004, 2006, 2009; Ito et al., 2007; Wang et al., 2007; Du et al., 2009; Kaplan et al., 2009; Zalewska-Gałosz et al., 2009; Zalewska-Gałosz and Ronikier, 2010). DNA sequencing and RFLPs have contributed to the discovery and/or exact identification of several entirely new hybrid combinations (Kaplan et al., 2009, 2011; Du et al., 2010; Zalewska-Gałosz et al., 2010; Kaplan and Fehrer, 2011; Zalewska-Gałosz and Ronikier, 2011) and even confirmed the existence of a triple hybrid in *Potamogeton* (Kaplan and Fehrer, 2007). Hybridization thus markedly increases the taxonomy-relevant diversity in *Potamogeton* (Kaplan, 2010a).

The geographic distribution of *Potamogeton* hybrids is highly uneven. Wiegleb (1988) identified Great Britain, Denmark, Sweden, and Japan as centres of hybridization in *Potamogeton*. High hybrid diversity was recently identified also in the north-eastern U.S.A. (Kaplan et al., 2009). Although at least 86 hybrid combinations have been convincingly documented in the Potamogetonaceae, almost all were recorded in a relatively few regions of the Northern Hemisphere (for a review, see Kaplan, 2010a). Only recently, a *Potamogeton* hybrid was identified also in the Southern Hemisphere, in Australia (Kaplan et al., 2011).

In Europe, there is a clear gradient of hybrid occurrence towards higher latitude: both hybrid diversity and the number of localities with hybrids increase towards northern Europe (Kaplan, 2007, 2010a; Kaplan et al., 2009). This distribution pattern is typically associated with suitable habitats occurring in a previously glaciated landscape (Kaplan, 2007). The northern regions host the highest diversity with about 25–30 hybrids (Kaplan, 2010a).

Some areas of Denmark used to be rich in *Potamogeton* hybrids in the past. Johannes Schönberg Baagøe (1838–1905), a pharmacist in Næstved, was an extremely active field botanist and particularly enthusiastic about *Potamogeton*. In the period from 1893 to 1905 he collected several thousands of specimens, professionally preserved them for his herbarium and identified many of them as hybrids. He widely distributed his duplicates to various herbaria

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**Table 1**

List of herbarium specimens collected by Baagøe in 1895–1905 and distinguished by Hagström (1916) as *Potamogeton* hybrids. The hybrids re-discovered within this study are given in bold. Nomenclature of species given in hybrid formulas has been updated according to recent nomenclature. Localities in the River Gudenå near Randers and Kongensbro and in the River Ribeå near Ribe, which were investigated in this study, are given in bold; collections from parts of Denmark other than Jutland are given in brackets. Collections cited by Hagström (1916) as probably hybrid but uncertain are indicated by question marks. The third column provides references to molecular studies (or recently revised hybrids of which material for molecular analysis is not available) that proved the existence of the particular hybrid combinations, using mostly material of different geographic origin.

Hybrid formula	Listed localities	Molecular evidence (or recent morphological and anatomical revision)
<i>P. acutifolius</i> × <i>P. compressus</i>	(Hjulby mose, Fyen)	Zalewska-Gałosz and Ronikier (2010)
<i>P. alpinus</i> × <i>P. crispus</i>	<b>Lilleå at Gudenå</b> ; (Vigersdalså, Sjælland)	
<i>P. berchtoldii</i> × <i>P. pusillus</i>	? Nybro kro	Fehrer and Kaplan (unpublished)
<i>P. berchtoldii</i> × <i>P. trichoides</i>	(Villa Gallina, Sjælland)	
<i>P. compressus</i> × <i>P. trichoides</i>	<b>Varmingsö; Nibså</b>	Kaplan and Fehrer (2004)
<i>P. crispus</i> × <i>P. perfoliatus</i>	<b>Gudenå</b>	This study
<b>P. crispus</b> × <b>P. praelongus</b>	<b>Varmingsö; Nibså; Gudenå</b> ; Kongeå	Hollingsworth et al. (1996), McMullan et al. (2011)
<i>P. filiformis</i> × <i>P. pectinatus</i>	Kongeå; (Susa, Sjælland)	
<i>P. friesii</i> × <i>P. pusillus</i>	Skjernå; Skaber Möllesö	Fehrer and Kaplan (unpublished)
<i>P. gramineus</i> × <i>P. lucens</i>	(Susa ad Hjelmsølle, Sjælland;? Glænø, Sjælland)	Fehrer and Kaplan (unpublished)
<i>P. gramineus</i> × <i>P. natans</i>	<b>Gudenå</b> ; Handbjergå	Kaplan and Fehrer (2006), Kaplan et al. (2009)
<i>P. gramineus</i> × <i>P. perfoliatus</i>	Skiveå; Skærum river; (Ulse lake, Sjælland; Ringsted River, Sjælland)	
<i>P. lucens</i> × <i>P. natans</i>	<b>Gudenå</b> ; Skjernå; Vardeå; Skalså; (Odenseå, Fyen)	Fant et al. (2001, 2003), Kaplan et al. (2002)
<i>P. lucens</i> × <i>P. nodosus</i>	<b>Gudenå at Kongensbro</b>	(Zalewska-Gałosz, 2010)
<b>P. lucens</b> × <b>P. perfoliatus</b>	<b>Gudenå at Kongensbro</b>	Fant and Preston (2004), Kaplan (2007), Bobrov and Sinjushin (2008)
<i>P. lucens</i> × <i>P. praelongus</i>	<b>Gudenå near Randers; Nibså; Varming Lake</b> ; Sneumå; Skalså; (Odenseå, Fyn)	(Zalewska-Gałosz, 2010)
<b>P. perfoliatus</b> × <b>P. praelongus</b>	<b>Varming Lake; Nibså near Ribe</b>	This study

and to contemporary *Potamogeton* experts. His material was further evaluated by the German botanist Georg Fischer and the Swedish monographer Johan Oskar Hagström who accepted almost all his hybrids and, based on his material, they distinguished a few additional ones (Fischer, 1907; Hagström, 1916). Altogether, Baagøe collected 14–17 currently recognized hybrids from his relatively small sampling area (see Table 1). The extraordinary co-occurrence of five *Potamogeton* hybrids involving seven species discovered in a single Danish river by Baagøe found its place even in textbooks (e.g. Hutchinson, 1975). However, some authors were sceptical about the accuracy of morphological identification of *Potamogeton* hybrids and doubted the existence of such hybrid diversity. Fernald (1932) believed that hybridization is actually uncommon in *Potamogeton*. Les and Philbrick (1993) considered hybridization "a dogmatic attribute of the genus" not supported by convincing evidence. Because of the lack of expert attention and failure to apply adequate approaches, *Potamogeton* hybrids are in many regions of the world neglected and remain undetected (Kaplan et al., 2009, 2011).

Previous studies demonstrated that due to extensive phenotypic plasticity, some *Potamogeton* hybrids can mimic species, and vice versa (Kaplan, 2002, 2005a,b; Kaplan and Fehrer, 2004; Kaplan and Wolff, 2004; Kaplan and Symoens, 2005; Kaplan et al., 2009). Other hybrids can be identified morphologically only when specific key structures are carefully examined (Preston, 1995; Preston et al., 1999; Kaplan, 2008; Kaplan et al., 2009) or if the particular plant is fully developed and shows diagnostic features of the species involved in the hybridization (Kaplan and Wolff, 2004; Kaplan and Fehrer, 2007). The exact taxonomic identity of some *Potamogeton* hybrids can be revealed only by molecular analysis (Kaplan et al., 2009; Zalewska-Gałosz et al., 2010; Kaplan and Fehrer, 2011). A recent study showed that some hybrids that were considered well recognized morphologically were misunderstood and actually have different parentages (Kaplan and Fehrer, 2011). It is thus advisable to have molecular proof of the identity of questionable plants (Kaplan and Fehrer, 2007, 2009, 2011; Zalewska-Gałosz and Ronikier, 2010; Kaplan et al., 2009, 2011), particularly if a hybrid combination previously not analyzed with molecular methods is considered.

The aims of this study are: (1) to test by molecular analyses whether intermediate plants recorded by Baagøe and still extant at their localities are really of hybrid origin, (2) to identify the precise parentages of the detected hybrids, (3) to reveal the direction of the crosses, and (4) to provide an assessment of the previous and extant diversity at selected Baagøe's localities in Jutland.

## 2. Methods

### 2.1. Plant material

Localities of the putative hybrids were identified mainly from herbarium labels of Baagøe's specimens preserved in the separate Baagøe herbarium kept at C. Duplicates were investigated in various other European herbaria examined for a monograph of Potamogetonaceae within the framework of the Species Plantarum Project – Flora of the World (for the complete list of studied herbaria, see Kaplan, 2008, 2010a,c; Kaplan and Marhold, 2012). Distribution records given by Hagström (1916) were also considered.

The majority of *Potamogeton* hybrids were recorded by Baagøe in Jutland, Denmark, mainly in the River Gudenå near Randers and Kongensbro and in the River Ribeå (also called Nibså or Varmingsö in their sections) near Ribe (Table 1). Within this study, the original localities in these two rivers were first visited in 1998, a century after Baagøe's studies, and investigated for the occurrence of *Potamogeton* species and putative hybrids. Unfortunately, because of the considerable changes in landscape and habitats (see Section 4), only one population of plants corresponding to that from Baagøe's collection (*P. × undulatus* = *P. crispus* × *P. praelongus*, samples no. 1024 and 1025) was re-discovered in 1998. Another putative hybrid from there was found and was contributed to this study by C.D. Preston in 2000 (*P. × cognatus* = *P. perfoliatus* × *P. praelongus*, sample no. 1226). The same hybrids were recorded in these sites again in 2012, when this paper was already under review. In addition to the populations detected previously, *P. × undulatus* was also found in some stretches of the River Gudenå between Ulstrup and Randers. Another hybrid (*P. × salicifolius* = *P. lucens* × *P. perfoliatus*) was located in the same river on that

occasion. All these are hybrids between broad-leaved species of the genus.

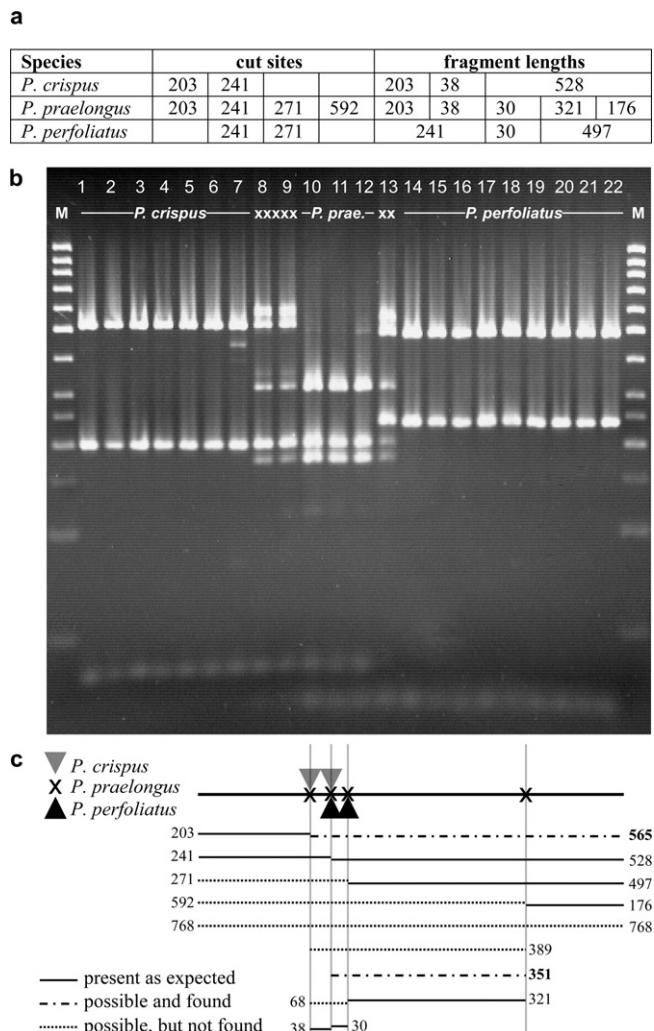
The putative hybrids *P. × undulatus* and *P. × cognatus* were cultivated in the experimental garden at the Institute of Botany, Průhonice, Czech Republic, in 1998–2011 or 2000–2011, respectively, and used for tests of fertility and phenotypic plasticity. Accessions of the putative hybrids were consistently sterile. Besides the putative hybrids, the presumed parental species were included in the molecular analyses for comparison. Each species was represented by 3–9 accessions of different geographic origin. All accessions are summarized in Table 2. All studied species and hybrids are tetraploids (Z. Kaplan and V. Jarolímová, unpublished data; Hollingsworth et al., 1998). Taxonomic delimitations of species and nomenclature follow Wieglob and Kaplan (1998) with the exception of two recently re-evaluated hybrids (Zalewska-Gałosz, 2010, 2011) and the *P. pusillus* agg., which was updated and refined by Kaplan and Štěpánek (2003) and Kaplan (2010b). Voucher specimens are preserved in the herbarium of the Institute of Botany, Průhonice (acronym PRA).

## 2.2. Molecular analyses

DNA isolations, PCR amplification and sequencing of the ITS region and the *rpl20-rps12* spacer were done as described previously (Kaplan and Fehrer, 2004, 2006). The origins of *P. × cognatus* (presumed *P. paelongus* × *P. perfoliatus*) and *P. × undulatus* (presumed *P. paelongus* × *P. crispus*) were assessed by character additivity in direct sequencing (complete ITS 1, 5.8S, and begin of ITS 2 until a diagnostic indel between *P. perfoliatus* and *P. crispus*/*P. paelongus*) and documented by PCR-RFLPs. The restriction enzyme *Rsa* I was selected based on the sequence alignment of several accessions of each parental species as it permitted discrimination between all three species in a single digest and produced well-separable fragments. PCR-RFLPs of the ITS region were conducted as described in Kaplan et al. (2009). The patterns suggested the partial loss of restriction sites in the hybrids. Therefore, PCR products of one accession of each hybrid combination were cloned as described in Fehrer et al. (2009) and 4–7 clones per accession were sequenced. Substitutions in cloned sequences that occurred in only one clone or were not accounted for by direct sequencing and differed from all three species were considered as polymerase errors and corrected (0–2 per clone). The sequences were submitted to GenBank and complemented by previously published sequences of the parental species. Accession numbers are given in Table 2.

## 3. Results

The identity of *Potamogeton × undulatus* as *P. crispus* × *P. paelongus* and *Potamogeton × cognatus* as *P. perfoliatus* × *P. paelongus* was confirmed by additive banding patterns in PCR-RFLPs (Fig. 1). One or two additional bands were observed in the hybrid samples. Their lengths suggested the partial loss of one or two restriction sites. Incomplete digests are unlikely because the reaction conditions corresponded to manifold overdigests (excess of restriction enzyme, overnight incubation), and similar quantities of PCR products were used for parental as well as for hybrid samples. Direct sequencing showed that both parental sequences were present in equal amounts in all hybrid accessions, but no indication of partial loss of restriction sites could be found. One cloned sequence of *P. × undulatus* (accession 1025) was identical to *P. crispus*, two to *P. paelongus*, and one was recombinant, ITS 1 corresponding to *P. paelongus*, ITS 2 to *P. crispus* (Table 3). Whether this indicates intragenomic recombination or PCR recombination cannot be decided based on these data. No intraspecific variation was found in any of the parental sequences. Three clones of *P. × cognatus*



**Fig. 1.** PCR-RFLP of *P. × cognatus*, *P. × undulatus*, and their parents. (A) Cut sites (*Rsa*I) inferred from ITS sequences and resulting fragment lengths. (B) PCR-RFLPs of *P. crispus*, *P. paelongus*, *P. perfoliatus*, and their respective hybrids ('xxx'). The faint additional band at about 450 bp in sample 7 is a PCR artefact. All hybrids show additive patterns of their respective parental species plus 1–2 additional bands (white arrowheads). Samples (lines 1–22): 1463, 1464, 1466, 1473, 1483, 1485, 1472, 1024, 1025, 249, 881, 1530, 1226, 840, 1467, 1469, 1481, 1470, 985, 979, 1531, 1002 (for details, see Table 2). (C) Restriction map with relative positions of cut sites (bold upper line). Below, all possible fragment lengths that can theoretically occur if restriction sites are lost are summarized. Two of these ("possible and found") correspond to the additional fragments in (B).

(accession 1226) were identical to *P. perfoliatus* except that one character (position 58) matched *P. paelongus* in one of these clones. Four clones of *P. × cognatus* corresponded to the *P. paelongus* ribotype; two identical ones showed two *P. perfoliatus* characters (positions 182 and 714). They may result from polymerase errors occurring in earlier rounds of the PCR reaction or indicate some gene conversion activity. Evidence for the partial loss of restriction sites indicated by the PCR-RFLPs could be found in none of these hybrids, either in direct sequencing or in clones. Perhaps the digested products were inadequately separated in electrophoresis for some reason. ITS sequences of all other European broad-leaved species of sect. *Potamogeton* markedly differ from those of the parental species of the studied hybrids (Kaplan and Fehrer, 2011) and can therefore be easily excluded as potential parents.

The variation in the *rpl20-5' rps12* spacer of chloroplast DNA was rather low, but sufficient to reveal that the maternal parent of all hybrid accessions was *P. paelongus* (Table 4).

**Table 2**

Accessions used for molecular analyses and GenBank accession numbers.

Taxon	Reference number	Origin and voucher collection records	ITS	rpl20-5' rps12
<i>P. crispus</i>	1463	Czech Republic: Bohemia, Bohuslavice (coll. Z. Kaplan 03/121)	GU814242 <sup>a</sup>	GU814253 <sup>a</sup>
	1464	Czech Republic: Bohemia, Velká Jesenice (coll. Z. Kaplan 03/122)	GU814243 <sup>a</sup>	GU814254 <sup>a</sup>
	1466	Czech Republic: Bohemia, Uhřínovice (coll. Z. Kaplan 03/126)		
	1472	Germany: Bavaria, Ebing, Main River (coll. L. Meierott, cult. & coll. Z. Kaplan 1472)	AY529523 <sup>b</sup>	GU814255 <sup>a</sup>
	1473	Czech Republic: Bohemia, Poděbrady (coll. Z. Kaplan 03/142)	AY529524 <sup>b</sup>	GU814256 <sup>a</sup>
	1483	Czech Republic: Bohemia, Chudíř (coll. Z. Kaplan 03/173)		
	1485	Czech Republic: Bohemia, Hrobice (coll. Z. Kaplan 03/180)		
<i>P. × undulatus</i> (= <i>P. crispus</i> × <i>P. praelongus</i> )	1024	Denmark: Jutland, Ribe, Varming, Ribeå (coll. Z. Kaplan 98/374)		JQ241253
	1025	Denmark: Jutland, Ribe, Varming, Ribeå (coll. Z. Kaplan 98/376)	JQ241256–JQ241258	JQ241254
<i>P. praelongus</i>	249	Czech Republic: Bohemia, Malšova Lhota (coll. Z. Kaplan 96/396)		
	881	Germany: Mecklenburg-Vorpommern, Güstrow, Dobbin, Lake Gültz (coll. Z. Kaplan 97/824)	HQ263528 <sup>c</sup>	HQ263469 <sup>c</sup>
	1530	Italy: Trentino-Alto Adige, Prov. Bolzano, San Valentino alla Muta, Lake Muta (coll. Z. Kaplan & J. Štěpánková 04/62)	HQ263529 <sup>c</sup>	HQ263479 <sup>c</sup>
<i>P. × cognatus</i> (= <i>P. perfoliatus</i> × <i>P. praelongus</i> )	1226	Denmark: Jutland, Randers, Gudenå (coll. C.D. Preston, cult. & coll. Z. Kaplan 1226)	JQ241259–JQ241265	JQ241255
<i>P. perfoliatus</i>	840	Czech Republic: Moravia, Ostrožská Nová Ves (coll. Z. Kaplan 97/524)		
	979	Switzerland: St. Gallen, Altenrhein, Lake Constance (coll. Z. Kaplan 98/125)	AY529527 <sup>b</sup>	DQ468862 <sup>d</sup>
	985	Austria: Vorarlberg, Fußbach, Lake Constance (coll. Z. Kaplan 98/131)	HQ263520 <sup>c</sup>	HQ263462 <sup>c</sup>
	1002	Sweden: Prov. Skåne, Björka River (coll. Z. Kaplan 98/338)	AY529526 <sup>b</sup>	DQ468863 <sup>d</sup>
	1467	Czech Republic: Bohemia, Martinov (coll. J. Hummel, in herb. Z. Kaplan 03/130)		
	1469	Czech Republic: Doubrava (coll. J. Rydlo, in herb. Z. Kaplan 03/139)		
	1470	Germany: Bavaria, Ebing, Main River (coll. L. Meierott, cult. & coll. Z. Kaplan 1470)	AY529525 <sup>b</sup>	EF174597 <sup>e</sup>
	1481	Czech Republic: Bohemia, Staré Splavy (coll. Z. Kaplan 03/161)		
	1531	Italy: Trentino-Alto Adige, Prov. Bolzano, San Valentino alla Muta, Lake Muta (coll. Z. Kaplan & J. Štěpánková 04/63)		

<sup>a</sup> From Kaplan et al. (2011).<sup>b</sup> From Kaplan and Fehrer (2004).<sup>c</sup> From Kaplan and Fehrer (2011).<sup>d</sup> From Kaplan and Fehrer (2006).<sup>e</sup> From Kaplan and Fehrer (2007).

The identity of *Potamogeton × salicifolius* as *P. lucens* × *P. perfoliatus* was confirmed by direct sequencing of the ITS region as described in Kaplan and Fehrer (2007). As this hybrid has already been studied thoroughly in previous studies (e.g., Fant and Preston, 2004; Kaplan, 2007; Bobrov and Sinjushin, 2008), the details on the molecular prove are not presented in this paper.

#### 4. Discussion

The molecular analysis provided proof of the identity of the two investigated hybrids as *P. × undulatus* = *P. crispus* × *P. praelongus* and *P. × cognatus* = *P. perfoliatus* × *P. praelongus*. These identifications are in accordance with their morphological determination (Baagøe, 1897; Hagström, 1916; Dandy, 1975; Preston, 1995; Kaplan and Zalewska-Gałosz, 2004; Alix and Scribailo, 2006; Kaplan, 2010a). Because of the considerable decline of aquatic vegetation during the 20th century (see below), recent material of the rest of Baagøe's hybrids from his original sites is not available for DNA analyses. However, the existence of most of the recorded hybrid combinations was proved with molecular analyses conducted on morphologically matching material of different geographic origin (see Table 1). Morphologies of these hybrids, often very characteristic, perfectly correspond to Baagøe's voucher herbarium specimens. Another two of Baagøe's hybrids

were recently revised in detailed morphological and anatomical studies and accepted as sufficiently documented (see Table 1).

Previous studies demonstrated that hybrid colonies can persist at a locality for a considerable period, even hundreds or thousands of years (Hollingsworth et al., 1996; Preston et al., 1998; King et al., 2001; Kaplan and Wolff, 2004; Kaplan and Fehrer, 2007, 2011; Kaplan, 2010c), provided that the ecological conditions remain suitable. Long-term persistence of already established hybrid clones is therefore more likely in rivers than their recurrent rise. As the three hybrids sampled at the localities where they were recorded more than a century ago are consistently sterile – like the great majority of hybrids involving broad-leaved *Potamogeton* species (Hagström, 1916; Dandy, 1975; Preston, 1995; Wieglob and Kaplan, 1998; Kaplan, 2001, 2010a; Kaplan and Fehrer, 2007; Kaplan et al., 2009; Bobrov and Chemeris, 2006) – and no recombination or back-crossing is therefore possible, the morphologically matching plants may even be genetically identical with Baagøe's herbarium specimens.

The molecular proves of the existence of many *Potamogeton* hybrids and the possibility of reliable morphological identifications of many of them indicate that the scepticism expressed by some authors (see Section 1) about the real existence of the hybrid diversity recorded from Jutland was overly distrustful. Morphological and anatomical data coupled with molecular evidence support the results of the early authors that Jutland rivers and streams had

**Table 3**

Character states in the ITS region differing between the parents, and cloned sequences of hybrids.

Taxon	Position in alignment <sup>a</sup>
	11112222222244444444444555555566666666666667
	55788912380244556704455566667777112236790013455556667881
	58014530022279011322727803460179343847152635134580124564
<i>P. perfoliatus</i> 979	TTATCTTCTGGACTTAYGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. perfoliatus</i> 985	TTATCTTCTGGACTTACGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. perfoliatus</i> 1002	TTATCTTCTGGACTTACGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. perfoliatus</i> 1470	TTATCTTCTGGACTTACGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. ×cognatus</i> 1226 clone 10	TTATCTTCTGGACTTACGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. ×cognatus</i> 1226 clone 1	TTATCTTCTGGACTTACGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. ×cognatus</i> 1226 clone 2	TCATCTTCTGGACTTACGTCACATC-TTGTC-CGTGTTGCGATCGC--TTTCC
<i>P. ×cognatus</i> 1226 clone 7	TCAAGGACTTGGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAC
<i>P. ×cognatus</i> 1226 clone 5	TCAAGGACTTGGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAC
<i>P. ×cognatus</i> 1226 clone 4	TCAAGGACTCAGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAT
<i>P. ×cognatus</i> 1226 clone 12	TCAAGGACTCAGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAT
<i>P. praelongus</i> 881	TCAAGGACTCAGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAT
<i>P. praelongus</i> 1530	TCAAGGACTCAGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAT
<i>P. ×undulatus</i> 1025 clone 14	TCAAGGACTCAGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAT
<i>P. ×undulatus</i> 1025 clone 1	TCAAGGACTCAGGACTCACTGCTCACCCCCGCCT-CATACCATAG---CCACCCAT
<i>P. ×undulatus</i> 1025 clone 3 <sup>b</sup>	TCAAGGACTCAGGACTCATGATTCT-TTACTTTA-GCCATGGTCCA-ACCCCT
<i>P. ×undulatus</i> 1025 clone 4	CCTAGGACTCACTTTATGTGATTCT-TTACTTTA-GCCATGGTCCA-ACCCCT
<i>P. crispus</i> 1463	CCTAGGACTCACTTTATGTGATTCT-TTACTTTA-GCCATGGTCCA-ACCCCT
<i>P. crispus</i> 1464	CCTAGGACTCACTTTATGTGATTCT-TTACTTTA-GCCATGGTCCA-ACCCCT
<i>P. crispus</i> 1472	CCTAGGACTCACTTTATGTGATTCT-TTACTTTA-GCCATGGTCCA-ACCCCT
<i>P. crispus</i> 1473	CCTAGGACTCACTTTATGTGATTCT-TTACTTTA-GCCATGGTCCA-ACCCCT

<sup>a</sup> Positions are numbered from the first base after the forward primer<sup>b</sup> Recombinant clone (not submitted to GenBank)

hosted a high species and hybrid diversity still in the late 19th century. Unfortunately, much of this richness largely disappeared with habitat deterioration during the 20th century. Sand-Jensen et al. (2000) compared species lists of submerged plants compiled from sites distributed across Denmark at the end of the 19th century with their own recent studies on the vegetation of small lakes and streams. Both the old and the recent studies included some of Baagøe's localities. Most of the lakes which contained diverse submerged vegetation 100 years ago now exhibit considerable eutrophication and have lost all or most of their submerged species. Species richness has also declined markedly in the studied streams. A significant decline was identified in the diversity of *Potamogeton* species over all sites. Species typical for oligotrophic conditions (e.g. *P. filiformis* and *P. polygonifolius*) as well as a group of slow-growing species (e.g. *P. alpinus*, *P. lucens*, *P. praelongus*, and *P. compressus*) were once common, but are now infrequent. Other species have

remained as rare as they used to be a century ago (e.g. *P. acutifolius*, *P. coloratus*, and *P. rutilus*). Because these aquatic habitats are sometimes difficult to access and investigate thoroughly, we believe (and hope) that more detailed screening in suitable habitats will reveal some more surviving populations of the rare hybrid taxa.

A similar decline in the diversity of species and hybrids of *Potamogeton* was noted also in the Czech Republic by Kaplan (2010a,b). The traditional cycle of fishpond management, which included regular summer draining, was largely abandoned and fish farming became more intensive. Fishponds not drained for many years become eutrophic, with cloudy water and bottoms covered with thick organic-rich sediment (sapropel), which is often toxic because of anaerobic conditions. Many lowland sections of rivers were channelled in the late 19th and 20th century, which dramatically affected the distribution and diversity of river vegetation. As a result, most localities of uncommon *Potamogeton* species and hybrids in rivers no longer contain them. Drastic changes in the composition of macrophyte communities of lakes and rivers were identified also in Germany. Wieglob et al. (1991) noted that nearly all species showed a decline in frequency, which was particularly recognizable in *Potamogeton*. Severe eutrophication, channelization of rivers and streams, and changes of hydrological and hydraulic factors were identified as the most important factors responsible for the decline of macrophyte vegetation.

In general, freshwater habitats in cultivated and densely populated lowland regions of Europe have experienced profound changes during the last 100 years (Sand-Jensen et al., 2000). Straightening of stream meanders, weed cutting, organic pollution and eutrophication are among the most important impacts on streams and their vegetation. Habitat destruction has markedly restricted aquatic species that were previously widely distributed. Many of them presently survive in low abundance in a few populations (Riis and Sand-Jensen, 2001, 2002; Sand-Jensen et al., 2006).

**Table 4**Character states in the *rpl20-5'rps12* chloroplast region differing between the parents and comparison with their hybrids.

Taxon	Position in alignment <sup>a</sup>				
	97	250	261–271	517	707
<i>P. perfoliatus</i> 979	G	A	TAAAATAAATA	T	C
<i>P. perfoliatus</i> 985	G	A	TAAAATAAATA	T	C
<i>P. perfoliatus</i> 1002	G	A	TAAAATAAATA	T	C
<i>P. perfoliatus</i> 1470	G	A	TAAAATAAATA	T	C
<i>P. × cognatus</i> 1226	C	A	TAAAATAAATA	G	C
<i>P. praelongus</i> 881	C	A	TAAAATAAATA	G	C
<i>P. praelongus</i> 1530	C	A	TAAAATAAATA	G	C
<i>P. × undulatus</i> 1024	C	A	TAAAATAAATA	G	C
<i>P. × undulatus</i> 1025	C	A	TAAAATAAATA	G	C
<i>P. crispus</i> 1463	C	T	—	T	A
<i>P. crispus</i> 1464	C	T	—	T	A
<i>P. crispus</i> 1472	C	T	—	T	A
<i>P. crispus</i> 1473	C	T	—	T	A

<sup>a</sup> Positions are numbered from the first base after the forward primer.

## Acknowledgements

We are grateful to C.D. Preston for providing us with one of the hybrids, to Jitka Štěpánková, Bjarne Moeslund, Kaj Sand-Jensen, Jens Christian Schou, Peter Holm and Jan Prančl for their help during fieldwork, to Marie Stará, Abdolreza Yadollahi and Veronika Bambasová for performing the molecular labwork, and to curators of the visited herbaria who allowed us to study their collections. The research was supported by grant no. 206/09/0291 from the Czech Science Foundation and by the long-term research development project no. RVO 67985939 from the Academy of Sciences of the Czech Republic.

## References

- Alix, M.S., Scribailo, R.W., 2006. First report of *Potamogeton × undulatus* (*P. crispus* × *P. praelongus*, Potamogetonaceae) in North America, with notes on morphology and stem anatomy. *Rhodora* 108, 329–346.
- Baagøe, J., 1897. *Potamogeton undulatus* Wolfgang. *Bot. Tidsskr.* 21, 221–236, pl. 7.
- Bobrov, A.A., Chemeris, E.V., 2006. *Potamogeton × vespicus* (Potamogetonaceae)—novyi gibridnyi rdest iz Verchnego Povolzhya (*Potamogeton × vespicus* (Potamogetonaceae)), a new hybrid pondweed from the Upper Volga region. *Bot. Zhurn.* 91 (1), 71–84.
- Bobrov, A.A., Sinjushin, A.A., 2008. Morphological and molecular confirmation of the hybrid *Potamogeton × salicifolius* (*P. lucens* × *P. perfoliatus*, Potamogetonaceae) in Upper Volga region (Russia). *Komarovia* 6, 71–79.
- 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.
- Du, Z.-Y., Yang, C.-F., Chen, J.-M., Guo, Y.-H., 2009. Nuclear and chloroplast DNA sequences data support the origin of *Potamogeton intortusfolius* J.B. He in China as a hybrid between *P. perfoliatus* Linn. and *P. wrightii* Morong. *Aquat. Bot.* 91, 47–50.
- Du, Z.-Y., Yang, C.-F., Chen, J.-M., Guo, Y.-H., 2010. Identification of hybrids in broad-leaved *Potamogeton* species (Potamogetonaceae) in China using nuclear and chloroplast DNA sequence data. *Plant Syst. Evol.* 287, 57–63.
- 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., 2001. Isozyme evidence of the parental origin and possible fertility of the hybrid *Potamogeton × fluitans* Roth. *Plant Syst. Evol.* 229, 45–57.
- 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.
- Fehrer, J., Krak, K., Chrtk Jr., J., 2009. Intra-individual polymorphism in diploid and apomictic polyploid hawkweeds (*Hieracium*, Lactuceae, Asteraceae): disentangling phylogenetic signal, reticulation, and noise. *BMC Evol. Biol.* 9, 239.
- Fischer, C., 1907. Die bayerischen Potamogetonen und Zannichellien. *Ber. Bayer. Bot. Ges.* 11, 20–162.
- Fernald, M.L., 1932. The linear-leaved North American species of *Potamogeton*, section *Axillares*. *Mem. Am. Acad. Arts Sci.* 17, 1–183.
- Hagström, J.O., 1916. Critical researches on the Potamogetons. *Kungl. Svenska Vetenskapsakad. Handl.* 55 (5), 1–281.
- 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.
- Hollingsworth, P.M., Preston, C.D., Gornall, R.J., 1998. Euploid and aneuploid evolution in *Potamogeton* (Potamogetonaceae): a factual basis for interpretation. *Aquat. Bot.* 60, 337–358.
- Hutchinson, G.E., 1975. A Treatise on Limnology, vol. 3: Limnological Botany. John Wiley & Sons, New York, London, Sydney and Toronto.
- Ito, Y., Tanaka, N., Uehara, K., 2007. Inferring the origin of *Potamogeton × inbaensis* (Potamogetonaceae) using nuclear and chloroplast DNA sequences. *J. Jap. Bot.* 82, 20–28.
- 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. Neotyping 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., 2007. First record of *Potamogeton × salicifolius* for Italy, with isozyme evidence for plants collected in Italy and Sweden. *Plant Biosyst.* 141, 344–351.
- Kaplan, Z., 2008. A taxonomic revision of *Stuckenia* (Potamogetonaceae) in Asia, with notes on the diversity and variation of the genus on a worldwide scale. *Folia Geobot.* 43, 159–234.
- Kaplan, Z., 2010a. Hybridization of *Potamogeton* species in the Czech Republic: diversity, distribution, temporal trends and habitat preferences. *Preslia* 82, 261–287.
- Kaplan, Z., 2010b. Potamogetonaceae Dumort.—rdestovité. In: Štěpánková, J., Chrtk Jr., J., Kaplan, Z. (Eds.), Květena České republiky (Flora of the Czech Republic), vol. 8. Academia, Praha, pp. 329–384.
- Kaplan, Z., 2010c. Tiselius' *Potamogeton* exsiccates: changes in taxonomy and nomenclature from one-century perspective. *Ann. Bot. Fenn.* 46, 373–393.
- 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., Fehrer, J., 2006. Comparison of natural and artificial hybridization in *Potamogeton*. *Preslia* 78, 303–316.
- Kaplan, Z., Fehrer, J., 2007. Molecular evidence for a natural primary triple hybrid in plants revealed from direct sequencing. *Ann. Bot.* 99, 1213–1222.
- Kaplan, Z., Fehrer, J., 2009. An orphaned clone of *Potamogeton × schreberi* in the Czech Republic. *Preslia* 81, 387–397.
- Kaplan, Z., Fehrer, J., 2011. Erroneous identities of *Potamogeton* hybrids corrected by molecular analysis of plants from type clones. *Taxon* 60, 758–766.
- Kaplan, Z., Marhold, K., 2012. Multivariate morphometric analysis of the *Potamogeton compressus* group (Potamogetonaceae). *Bot. J. Linn. Soc.* 170, 112–130.
- 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., Uotila, P., 2011. *Potamogeton × exilis* (*P. alpinus* × *P. natans*), a new hybrid pondweed from Finland. *Nord. J. Bot.* 29, 477–483.
- 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., Zalewska-Gałosz, J., 2004. *Potamogeton* taxa proposed by J.F. Wolfgang and his collaborators. *Taxon* 53, 1033–1041.
- Kaplan, Z., Fehrer, J., Hellquist, C.B., 2009. New hybrid combinations revealed by molecular analysis: the unknown side of North American pondweed diversity (*Potamogeton*). *Syst. Bot.* 34, 625–642.
- Kaplan, Z., Fehrer, J., Hellquist, C.B., 2011. *Potamogeton × jacobsii* (Potamogetonaceae) from New South Wales, Australia—the first *Potamogeton* hybrid from the Southern Hemisphere. *Telopea* 13, 245–256.
- Kaplan, Z., Pláčková, 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 hybridization and chromosome number variation in aquatic angiosperms: evolutionary implications. *Aquat. Bot.* 44, 181–228.
- McMullan, J.J., Gornall, R.J., Preston, C.D., 2011. ITS rDNA polymorphism among species and hybrids of *Potamogeton* subgenus *Coleogeton* (Potamogetonaceae) in north-western Europe. *N. J. Bot.* 1, 111–115.
- Preston, C.D., 1995. Pondweeds of Great Britain and Ireland. B.S.I. Handbook no. 8. 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.
- 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.
- Riis, T., Sand-Jensen, K., 2001. Historical changes in species composition and richness accompanying perturbation and eutrophication of Danish lowland streams over 100 years. *Freshw. Biol.* 46, 269–280.
- Riis, T., Sand-Jensen, K., 2002. Abundance-range size relationships in stream vegetation in Denmark. *Plant Ecol.* 161, 175–183.
- Sand-Jensen, K., Riis, T., Vestergaard, O., Larsen, S.E., 2000. Macrophyte decline in Danish lakes and streams over the past 100 years. *J. Ecol.* 88, 1030–1040.
- Sand-Jensen, K., Friberg, N., Murphy, K. (Eds.), 2006. Running Waters. Historical Development and Restoration of Lowland Danish Streams. National Environmental Research Institute, Copenhagen.
- Wang, Q.D., Zhang, T., Wang, J.B., 2007. Phylogenetic relationships and hybrid origin of *Potamogeton* species (Potamogetonaceae) distributed in China: insights from the nuclear ribosomal internal transcribed spacer sequence (ITS). *Plant Syst. Evol.* 267, 65–78.
- Wieglob, G., 1988. Notes on pondweeds—outlines for a monographical treatment of the genus *Potamogeton* L. *Feddes Repert.* 99, 249–266.
- Wieglob, G., Kaplan, Z., 1998. An account of the species of *Potamogeton* L. (Potamogetonaceae). *Folia Geobot.* 33, 241–316.
- Wieglob, G., Brux, H., Herr, W., 1991. Human impact on the ecological performance of *Potamogeton* species in northwestern Germany. *Vegetatio* 97, 161–172.
- Wieglob, G., van de Weyer, K., Bolbrinker, P., Wolff, P., 2008. *Potamogeton*-Hybriden in Deutschland. *Feddes Report* 119, 433–448.
- Zalewska-Gałosz, J., 2010. *Potamogeton × subrufus* Hagstr.: a neglected Potamogeton hybrid. *Ann. Bot. Fennici* 47, 257–260.
- Zalewska-Gałosz, J., 2011. *Potamogeton × jutlandicus*, a binomial for the hybrid between *P. lucens* and *P. praelongus* (Potamogetonaceae). *Nord. J. Bot.* 29, 473–476.

- Zalewska-Gałosz, J., Ronikier, M., 2010. Are linear-leaved *Potamogeton* hybrids really so rare? Molecular evidence for multiple hybridizations between *P. acutifolius* and *P. compressus* in central Europe. *Nordic J. Bot.* 28, 257–261.
- Zalewska-Gałosz, J., Ronikier, M., 2011. *Potamogeton × maemetsiae*: a new hybrid of linear-leaved pondweeds from Central Europe. *Preslia* 83, 265–279.
- Zalewska-Gałosz, J., Ronikier, M., Kaplan, Z., 2009. The first European record of *Potamogeton × subobtusus* identified using ITS and cpDNA sequence data. *Preslia* 81, 281–292.
- Zalewska-Gałosz, J., Ronikier, M., Kaplan, Z., 2010. Discovery of a new, recurrently formed *Potamogeton* hybrid in Europe and Africa: molecular evidence and morphological comparison of different clones. *Taxon* 59, 559–566.