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Molecular Evidence for a Natural Primary Triple Hybrid in Plants Revealed from Direct Sequencing

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• *Background and Aims* Molecular evidence for natural primary hybrids composed of three different plant species is very rarely reported. An investigation was therefore carried out into the origin and a possible scenario for the rise of a sterile plant clone showing a combination of diagnostic morphological features of three separate, well-defined *Potamogeton* species.
• *Methods* The combination of sequences from maternally inherited cytoplasmic (*rpl*20-*rps*12) and biparentally inherited nuclear ribosomal DNA (ITS) was used to identify the exact identity of the putative triple hybrid.
• *Key Results* Direct sequencing showed ITS variants of three parental taxa, *P. gramineus*, *P. lucens* and *P. perfoliatus*, whereas chloroplast DNA identified *P. perfoliatus* as the female parent. A scenario for the rise of the triple hybrid through a fertile binary hybrid *P. gramineus* × *P. lucens* crossed with *P. perfoliatus* is described.
• *Conclusions* Even though the triple hybrid is sterile, it possesses an efficient strategy for its existence and became locally successful even in the parental environment, perhaps as a result of heterosis. The population investigated is the only one known of this hybrid, *P.* × *torssanderi*, worldwide. Isozyme analysis indicated the colony to be genetically uniform. The plants studied represented a single clone that seems to have persisted at this site for a long time.

Key words: Triple hybrid, interspecific hybridization, *Potamogeton*, Potamogetonaceae, internal transcribed spacer, reproductive isolation, clonal propagation, asexual reproduction.

INTRODUCTION

Interpecific hybridization is a widespread phenomenon that has markedly contributed to diversity and speciation in the plant kingdom (e.g. Arnold, 1997; Rieseberg, 1997; Rieseberg and Carney, 1998; Arnold *et al.*, 1999; Barton, 2001). Most of the literature on hybridization is based on binary hybrids. The use of molecular tools has shown that interspecific hybridization is even more prevalent than indicated by morphological and cytogenetic evidence. Molecular investigations have confirmed the hybrid nature of many species (reviewed by Arnold, 1997; Rieseberg, 1997) and have also revealed many historical hybridization events (e.g. Rieseberg and Soltis, 1991; Wendel *et al.*, 1995; Campbell *et al.*, 1997; Nelson-Jones *et al.*, 2002; Koch *et al.*, 2003; Ritz *et al.*, 2005; Fehrer *et al.*, 2007).

In contrast, recent natural hybrids between three (or more) species are rarely reported (e.g. Stace, 1975; Kirschner and Skalický, 1990; Kitchener, 1997; Štěpánek, 1997; Hodálová, 2002; Bureš, 2004). These records, based primarily on examination of morphology, are confined to only several genera of angiosperms known to produce fertile binary hybrids. Besides primary hybrids, backcross hybrids and introgressants, Holub (1992) describes polyhybrids (triple hybrids arisen from crosses of a primary hybrid with a third species) and superhybrids (hybrids arisen from crosses of two different fertile hybrids) in *Crataegus* (Rosaceae).

There does exist a rich literature on two aspects of triple hybridization in plants. The first includes papers on experimental triple hybrids (e.g. Dionne, 1963; Hermsen and Ramanna, 1973; Kalasa Balicka, 1976, 1980; Bothmer *et al.*, 1988, 1989; Maekawa *et al.*, 1991; Gadella, 1992; Molina *et al.*, 2004; Mráz and Paule, 2006). An outstanding, notable experiment was carried out by Nilsson (1954), who as a result of successive artificial hybridization obtained a hybrid involving 13 different species of *Salix* (Salicaceae). Even more abundant is the literature on past allopolyploid speciation involving ancient hybridizations of at least three species, mainly grasses and grain crops (e.g. Kihara, 1944; McFadden and Sears, 1946; Lilienfield, 1951; Simmonds, 1976; Dvořák *et al.*, 1988, 1993, 1998; Dvořák and Zhang, 1990; Gill *et al.*, 1991; Wang *et al.*, 1997; Dvořák, 1998; Huang *et al.*, 2002; Mason-Gamer, 2004).

In contrast, molecular evidence for three different species contributing to recent natural hybrid individuals is relatively scarce. It seems that the only known examples in plants concern *Aesculus* (dePamphilis and Wyatt, 1990), *Iris* (Arnold, 1993) and *Quercus* (Dodd and Afzal-Rafii, 2004). In these studies, one to several individuals of the populations investigated combined genetic markers (allozyme, RAPD or AFLP) of three species. Interestingly, although hybridization is much more common in plants, a number of cases of natural primary trihybrids have been reported in animals including fur seals (*Arctocephalus* – Lancaster *et al.*, 2006), parthenogenetic lizards (*Cnemidophorus* – Parker and Selander, 1976; Densmore *et al.*, 1989; *Heteronotia* – Hillis *et al.*, 1991), unisexual fish (*Poeciliopsis* – Mateos and Vrijenhoek, 2005), ticks (*Hyalomma* – Rees *et al.*, 2003) and stick insects (*Bacillus* – Mantovani *et al.*, 2001) and also in yeast (*Saccharomyces* – González *et al.*, 2006).

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In plants, *Potamogeton* is a genus well known for the occurrence of interspecific hybrids (e.g. Graebner, 1907; Linton, 1907; Hagström, 1916; Dandy, 1975; Preston, 1995). Wiegleb and Kaplan (1998) identified 50 binary *Potamogeton* hybrids worldwide, some of which are locally frequent and represent clearly circumscribed biological entities. Several hybrids between two species of *Potamogeton* were recently confirmed by molecular techniques such as isozyme analysis (e.g. Hollingsworth *et al.*, 1995, 1996; Preston *et al.*, 1998; Fant *et al.*, 2001*a*, *b*; Iida and Kadono, 2002; Kaplan *et al.*, 2002; Fant and Preston, 2004; Kaplan and Wolff; 2004; Kaplan, 2007) or DNA-based techniques (King *et al.*, 2001; Fant *et al.*, 2003; Kaplan and Fehrer, 2004, 2006; Whittall *et al.*, 2004).

In contrast to many records on binary hybrids, only a few *Potamogeton* plants were interpreted as triple hybrids (Hagström, 1916; Clark, 1942). Among them, *P.* × *torssanderi* was assumed to be a hybrid of *P. gramineus* × *P. lucens* × *P. perfoliatus* (Hagström, 1916). The existence of other alleged triple hybrids seems hardly possible as each case would initially require a fertile binary hybrid. However, almost all *Potamogeton* hybrids are consistently sterile (Wiegleb and Kaplan, 1998). All these morphology-based theories on triple hybrids in *Potamogeton* were later abandoned, and the respective hybrids are no longer recognized in the recent taxonomic literature. For example, in his review of the British Isles, one of the centres of *Potamogeton* hybridization, Dandy (1975) did not recognize any triple hybrid.

Because molecular evidence on the existence of primary triple hybrids in plants is extremely rare, a detailed study was conducted on natural sterile plants of *Potamogeton* × *torssanderi* that were the most promising of being triple hybrids. Some old herbarium collections of this taxon indeed show a combination of typical features of three separate species. The question was if these plants actually represent triple hybrids or so far unrecognized morphological variants of already known binary hybrids.

Preliminary isozyme analyses (Z. Kaplan and I. Plačková, unpubl. res.) using recently collected plants from the original population of *P.* × *torssanderi* were not fully conclusive, mainly because of too high similarity of isozyme phenotypes between *P. gramineus* and *P. lucens*. However, the dimeric enzyme 6PGDH showed a highly complex banding pattern, which was consistently different from that of typical samples of similar hybrids *P.* × *nitens* (*P. gramineus* × *P. perfoliatus*) and *P.* × *salicifolius* (*P. lucens* × *P. perfoliatus*), and which could have been explained only (*a*) as a hybrid product of crossing *P. perfoliatus* with a highly heterozygous plant of either *P. gramineus* or *P. lucens*, or (*b*) as a triple hybrid. Although most enzyme systems used were sensitive enough to reveal variation between different populations within many *Potamogeton* species and hybrids (Kaplan *et al.*, 2002; Kaplan and Štěpánek, 2003; Kaplan and Wolff, 2004; Kaplan, 2007), the eight plants of *P.* × *torssanderi* investigated were genetically uniform suggesting that they represent a single clone.

Nuclear ribosomal DNA (nrDNA), especially the variable internal transcribed spacer (ITS) region, is frequently employed for the identification of hybrid and allopolyploid origin by RFLP, direct sequencing, cloning, or a combination of these (e.g. Soltis and Soltis, 1991; Sang *et al.*, 1995; O'Kane *et al.*, 1996; Rauscher *et al.*, 2002; Nieto Feliner *et al.*, 2004; Guggisberg *et al.*, 2006). nrDNA data alone can provide direct evidence of reticulate evolution if concerted evolution fails to act across the repeat units contributed by different parent species (e.g. Hughes *et al.*, 2002, and references therein). Here evidence is presented from direct sequencing of the ITS region for the contribution of all three presumed parental species to *P.* × *torssanderi*, and its maternal origin is revealed from sequences of the *rpl*20-*rps*12 chloroplast intergenic spacer. Scenarios for the rise of the triple hybrid and the presumed age of this vegetative clone are discussed on the basis of the results of the molecular analyses, chromosome counts, and knowledge of the breeding behaviour, life history and ecology of species and hybrids of *Potamogeton*.

MATERIALS AND METHODS

Study taxa

All three putative parental species, *Potamogeton gramineus*, *P. lucens* and *P. perfoliatus*, belong to a group of broad-leaved pondweeds. They are morphologically clearly defined as each of them is characterized by a large set of differentiating features (e.g. Preston, 1995; Wiegleb and Kaplan, 1998). Since their first formal description by Linnaeus (1753) they have always been considered as distinct species.

Flowers of all three species are often self-pollinated. However, as they are markedly protogynous, they may occasionally permit cross-pollination. All species are considered to be tetraploids with a chromosome number of $2n = 52$ (Z. Kaplan and V. Jarolímová, unpubl. res.), although different chromosome counts were exceptionally reported for *P. perfoliatus* (Hollingsworth *et al.*, 1998). *Potamogeton* × *torssanderi* was hexaploid with $2n = 78$ (Z. Kaplan and V. Jarolímová, unpubl. res.).

Taxonomic delimitations of species, hybrid formulas for recognized hybrids and nomenclature of all taxa follow Wiegleb and Kaplan (1998), with the exception of *P.* × *torssanderi* (Tiselius) Dörfler, whose concept is defined in this paper.

Plant material

Plant samples of the putative triple hybrid were collected from the type locality of *P.* × *torssanderi*, the only known population of this taxon worldwide. With respect to the clonal population structure of this sterile hybrid (see Results for details), eight ramets were sampled at a distance of at least 3 m between each plant clump to avoid collecting from a single active shoot system. In addition, living specimens of all three putative species were collected in various regions. Plants were cultivated in the experimental garden at the Institute of Botany, Průhonice, Czech Republic, in 180 × 140 × 80 cm water-filled laminate tanks, which

were sunk in the ground in order to prevent overheating of the water in summer. The samples were planted in submerged plastic pots containing previously desiccated pond mud. Herbarium vouchers from the field as well as from cultivation are preserved in the Herbarium of the Institute of Botany, Průhonice (acronym PRA). Specimens included in the molecular analyses are summarized in Table 1. Besides the recent collections of *P.* × *torssanderi*, approx. 110 historical herbarium specimens of this hybrid from the type locality were studied in the herbaria of B, BM, BP, BRNM, C, E, FR, G, LD, LE, M, P, PR, PRA, PRC, S, UPS, W, WU, Z and ZT (acronyms follow Holmgren *et al.*, 1990).

Molecular analyses

DNA was isolated from fresh or CTAB-conserved material according to Štorchová *et al.* (2000).

Chloroplast DNA sequencing was used for identification of the female parent of the hybrids. Maternal transmission of cpDNA in *Potamogeton* was recently ascertained (Kaplan and Fehrer, 2006). In order to find a chloroplast DNA region differentiating between the closely related species *P. gramineus* and *P. lucens*, samples from both species (see Table 1) were sequenced for the *trn*L gene and for the *trn*L-*trn*F, *trn*S-*trn*G, *trn*H-*psb*A, and *rpl*20-*rps*12 intergenic spacers; the *trn*L gene, *trn*L-F and *rpl*20-*rps*12 were also sequenced for some *P. perfoliatus* samples.

Amplification of the *trn*L gene and the *trn*L-*trn*F region was as follows: 25 μL PCR-reactions contained 1·5 mM $MgCl_2$, 200 μM of each dNTP, 0·5 mM of primers c and f (Taberlet *et al.*, 1991), a few nanograms of genomic DNA, 2·5 μL of Mg^{2+}-free reaction buffer and 1 unit *Taq* DNA polymerase (MBI Fermentas). Four minutes of pre-denaturation at 94 °C were followed by 40 cycles of 94 °C/30 s, 50 °C/30 s and 72 °C/1·5 min, and a final

TABLE 1. *Origin, reference and GenBank accession numbers of* Potamogeton *samples included in the study*

Taxon	Ref. no.	Origin and field collection records	ITS	*rpl*20-*rps*12	*trn*L-*trn*F	*trn*H-*psb*A	*trn*S-*trn*G	*rbc*L
P. lucens	317	Czech Republic, Hrobice, 50°06′N, 15°47′E, 9 Sep. 1996, coll. Z. Kaplan 96/627	EF174584	EF174595	EF174577			
	858	The Netherlands, Arcen, approx. 51°28′N, 06°12′E, 1997, coll. P. Denny	EF174583	EF174594	EF174578	EF174573	EF174571	
P. gramineus	885	Czech Republic, Rozkoš Reservoir, 50°23′N, 16°05′E, 22 Aug. 1997, coll. Z. Kaplan 97/829	EF174589	DQ468864		EF174574	EF174572	
	897	Czech Republic, Hradčany u Mimoně, 50°37′N, 14°43′E, 18 Sep. 1996, coll. Z. Kaplan 96/638	DQ468860	DQ468866	EF174575			EF174582
	1285	France, Rémelfing, approx. 49°06′N, 07°04′E, 21 July 2001, coll. P. Wolff	DQ468861	DQ468865	EF174576			EF174581
	1611	USA, Vermont, Bliss Pond, 44°21′N, 72°30′W, 22 July 2005, coll. Z. Kaplan and C. B. Hellquist 05/352	EF174587	EF174590				
	1698	USA, New Hampshire, Ossipee Lake, 43°46′N, 71°08′W, 29 July 2005, coll. Z. Kaplan and C. B. Hellquist 05/421	EF174585	EF174592				
	1705	USA, Maine, Nickerson Lake, 46°06′N, 67°55′W, 2 Aug. 2005, coll. Z. Kaplan and C. B. Hellquist 05/430	EF174586	EF174591				
	1729	USA, Maine, Pushaw Lake, 44°54′N, 68°47′W, 4 Aug. 2005, coll. Z. Kaplan and C. B. Hellquist 05/455	EF174588	EF174593				
P. perfoliatus	979	Switzerland, Bodensee Lake, approx. 47°30′N, 09°33′E, 23 June 1998, coll. Z. Kaplan 98/125	AY529527	DQ468862	EF174579			
	1002	Sweden, Björka, approx. 55°40′N, 13°39′E, 12 Aug. 1998, coll. Z. Kaplan 98/338	AY529526	DQ468863				
	1470	Germany, Ebing, approx. 50°02′N, 10°55′E, 11 June 2003, coll. L. Meierott	AY529525	EF174597	EF174580			
P. × *torssanderi*	1006	Sweden, Sillen Lake, approx. 59°02′N, 17°22′E, 13 Aug. 1998, coll. Z. Kaplan 98/343		EF174596				

extension step at 72 °C for 10 min. Products were purified with the QIAquick kit (Qiagen), sequenced in both directions using the PCR primers (GATC Biotech, Konstanz, Germany), and aligned in BioEdit (Hall, 1999). Among approx. 970 aligned characters, only four substitutions occurred between *P. perfoliatus* and both *P. gramineus* and *P. lucens*. Two *P. gramineus* individuals differed from each other by one substitution in the *trn*L intron and three indels in a 25-bp region of the *trn*L-*trn*F intergenic spacer containing tandem repeats and a poly-T stretch. No consistent differences between *P. gramineus* and *P. lucens* were found, and the region was therefore abandoned.

Amplification and sequencing of the other three chloroplast intergenic spacers was done as described previously for *rpl*20-*rps*12 (Kaplan and Fehrer, 2006), using the primers developed by Hamilton (1999). The *trn*H-*psb*A region was only 291 bp long and identical between the sequenced *P. gramineus* and *P. lucens* samples. The *trn*S-*trn*G spacer was about 900 bp long, comparably AT-rich (about 75 %), and contained several long poly-A and poly-T stretches. From one sample (*P. lucens* 858), 855 bp of well-readible sequence could be obtained, another sample (*P. gramineus* 885) yielded only 527 bp of difficult to read sequence due to three 12–14 bp long polynucleotide stretches, all of them longer than the corresponding ones of the *P. lucens* sample. In addition to these differences, two interspecific substitutions were found. This region was nevertheless dismissed for further study because of expected sequencing difficulties. The *rpl*20-*rps*12 intergenic spacer of about 800 bp length sequenced easily, yielded several species-diagnostic characters, and was therefore chosen for further study.

Two divergent samples of *P. gramineus* were additionally sequenced for part of the conservative chloroplast *rbc*L gene. Conditions were as described previously for this gene (Kaplan and Fehrer, 2006) with the exception that newly designed *Potamogeton*-specific primers were used for amplification and sequencing (Po-rbcLf: 5′-tatactcctgaatat gaaacc-3′, Po-rbcLr: 5′-ataaatggttgtgagtttacg-3′).

For identification of nuclear genome contributions to the hybrids, the ribosomal ITS region of all putative parents (two to eight samples per species from different regions) was amplified and sequenced as described previously (Kaplan and Fehrer, 2004). Three separate PCR reactions were performed for *P.* × *torssanderi* and pooled for direct sequencing to ensure representative amplification of the parental copies by reducing PCR drift and the relative effect of potential polymerase-induced errors (Wagner *et al.*, 1994).

GenBank accession numbers of all sequences are provided in Table 1.

RESULTS

Morphological variation and identification

The plants later named *P.* × *torssanderi* were first collected by Axel Torssander and Gustaf Tiselius in 1893 for the famous Tiselius exsiccate collection *Potamogetones suecici exsiccati* (fasc. 2, no. 75, issued in 1895). Soon it was recollected for another exsiccate, Dörfler's *Herbarium normale* (no. 3583, issued in 1898). Plants from these collections best exhibit the combination of typical diagnostic features of all three species. Most of the submerged leaves are sessile and resemble leaves of *P. gramineus* in shape and size, but particularly those of side branches are clearly semi-amplexicaul, which is a feature reminiscent of *P. perfoliatus*. The uppermost submerged leaves often show the characteristic shape, venation and mucronate termination of *P. lucens*. They are also mostly shortly petiolate. Another character of *P. lucens* is the two ribs winged towards the base on the abaxial side of the uppermost stipules, but the stipules from the lower parts of the stem are markedly smaller than is usual in this species, suggesting the influence of the other two species. Also the number of longitudinal veins in submerged leaves (7–17) is intermediate between *P. perfoliatus* and either *P. gramineus* or *P. lucens*. The floating leaves, if present, have a subcoriaceous lamina and clearly indicate influence of the only heterophyllous species, *P. gramineus*.

In contrast to these 'typical', best-developed herbarium specimens, our plants collected recently from the original site of *P.* × *torssanderi* and cultivated in the garden produced only submerged membranous leaves, but no floating subcoriaceous leaves. These phenotypes somewhat resembled narrow-leaved forms of *P.* × *salicifolius* (*P. lucens* × *P. perfoliatus*) or broad-leaved submerged forms of *P.* × *nitens* (*P. gramineus* × *P. perfoliatus*), and their reliable identification based solely on morphology was not conclusive.

Reproductive behaviour of P. × torssanderi

Whereas flowers of fertile *Potamogeton* species open to reveal the dehiscing anthers, the tepals of the cultivated *P.* × *torssanderi* remained tightly closed and hid the anthers in the inner side of the concave tepals. The entire spikes rotted well before fruit could set. This behaviour of floral organs was repeatedly observed in numerous sterile hybrids (Preston, 1995: 46; Preston *et al.*, 1998; Kaplan and Fehrer, 2004, 2006; Kaplan and Wolff, 2004; Kaplan, 2007). No sign of fruiting material has ever been observed among the numerous collections of this hybrid available, although almost all of them were collected with spikes.

Chloroplast DNA

The *rpl*20-*rps*12 intergenic spacer proved to be the best region for distinguishing between the putative parents *P. lucens*, *P. gramineus* and *P. perfoliatus*. Variable positions are summarized in Table 2. Some intraspecific polymorphism was found in *P. perfoliatus* (at one position) and to a larger extent in *P. gramineus* (at three positions). The two most divergent samples of the latter taxon also differed in the very conservative *rbc*L gene, which has been found to be nearly invariant in several distantly related *Potamogeton* species (Les *et al.*, 1997).

Sequences of *P.* × *torssanderi* corresponded to that of *P. perfoliatus* samples 979 and 1470 (see Table 2); sample 1002 had a unique mutation at position 590 not

TABLE 2. *Sequence variation in the* rpl*20*-rps*12 intergenic spacer*

Species	Sample	Position in alignment								
		34	71	229	403–410	490–496	525	527	590	728–738
P. lucens	317	T	C	**G**	TTCACAAT	**TTCAAGA**	A	G	C	CATTGATACTT
	858	T	C	**G**	TTCACAAT	**TTCAAGA**	A	G	C	CATTGATACTT
P. gramineus	897	A	C	T	TTCACAAT	–	A	G	C	CATTGATACTT
	1611	A	C	T	TTCACAAT	–	A	G	C	CATTGATACTT
	1698	A	C	T	TTCACAAT	–	A	G	C	CATTGATACTT
	1705	A	C	T	TTCACAAT	–	A	G	C	CATTGATACTT
	1729	A	C	T	TTCACAAT	–	A	G	C	CATTGATACTT
	885	T	C	T	TTCACAAT	–	A	A	C	–
	1285	T	C	T	TTCACAAT	–	A	G	C	–
P. perfoliatus	979	T	**G**	T	**–**	–	**G**	G	C	–
	1002	T	**G**	T	**–**	–	**G**	G	T	–
	1470	T	**G**	T	**–**	–	**G**	G	C	–
P. × *torssanderi*	1006	T	**G**	T	**–**	–	**G**	G	C	–

Species-specific substitutions are shown in bold.

found in any other *Potamogeton* species sequenced so far (>40; J. Fehrer and Z. Kaplan, unpubl. res.). Thus, the female parent of the analysed *P.* × *torssanderi* was determined to be *P. perfoliatus*.

Nuclear DNA

Parental ITS sequences were obtained from two to eight individuals of each species, preferably from different geographic areas (Table 1). *Potamogeton gramineus* and *P. lucens* had very similar sequences that consistently differed from each other only at a single position showing species-specific nucleotide substitutions for all three species. *Potamogeton gramineus* was polymorphic with two samples (1285 and 885) showing as many as six substitutions compared with five other conspecific plants. *Potamogeton lucens* differed at three positions and *P. gramineus* (except 1285 and 885) only at one position from all other sequences. *Potamogeton perfoliatus* was the most divergent with 25 substitutions and two insertions/deletions (indels) relative to *P. gramineus* and *P. lucens*. Most samples additionally showed intra-individual polymorphisms at one to three positions, but none of them involved species diagnostic positions.

Despite the low variation between *P. gramineus* and *P. lucens* sequences, shifts caused by the *P. perfoliatus*-specific indels (1 bp and 2 bp, respectively) resulted in many additional positions in both sequencing directions that allowed for the contributions of all three parents to be traced: 158 positions distinguished between *P. perfoliatus* and *P. gramineus*/*P. lucens*, six positions distinguished *P. gramineus* from *P. lucens*/*P. perfoliatus*, three positions distinguished *P. lucens* from *P. gramineus*/*P. perfoliatus*, and three positions displayed discernable additive peaks of all three species. Fig. 1 shows representative examples of diagnostic sites.

Direct sequencing of *P.* × *torssanderi* revealed that the *P. lucens* ITS was the predominant sequence type. All lower peaks in the electropherogram were predictable from alignments of the parental species (Fig. 1). They corresponded to either *P. perfoliatus* (best recognized as 'tails' of peaks starting downstream of species-specific indels, Fig. 1A, C) or to a particular ITS sequence type of *P. gramineus* (e.g. 897). The contribution of *P. gramineus* copies is illustrated for four positions indicated by asterisks (Fig. 1B, C); two of them additionally reveal a particular *P. gramineus* sequence variant (represented by samples 897, 1611, 1698, 1705 and 1729). The alternative variant that did not contribute to the hybrid (represented by samples 1285 and 885) can also be excluded from a lack of its specific substitutions at four positions which should otherwise be present. Unequivocal hybrid-specific or other mutations different from those of the recent parental taxa were missing.

The noise level in the electropherograms was low. Out of 651 positions analysed, 476 did not show any noise at all (see also Fig. 1); at the remaining positions, the noise level was still considerably lower than the signal of both under-represented sequence types. Direct sequencing was thus suitable to unequivocally reveal contributions of all three presumed parental species in this case and of a particular ITS variant of *P. gramineus*.

Intraspecific variation in P. gramineus

Chloroplast DNA as well as nuclear ITS sequences revealed comparably high intraspecific genetic variation in *P. gramineus*. Particularly specimens 1285 from France and 885 from the Czech Republic shared a rather divergent ITS sequence and also similar, unique cpDNA haplotypes. As the intraspecific genetic variation within *P. gramineus* exceeded the interspecific differences between *P. lucens* and *P. gramineus*, special attention had to be paid to identify correctly *P. gramineus* (or a particular variant of it) as one of the parents of the triple hybrid.

The *P. gramineus* ITS type found in *P.* × *torssanderi* from Sweden is present in samples from the Czech Republic and the USA. The same specimens also share a chloroplast haplotype (Table 2) which additionally occurs in Swedish *Potamogeton* hybrids with *P. gramineus*

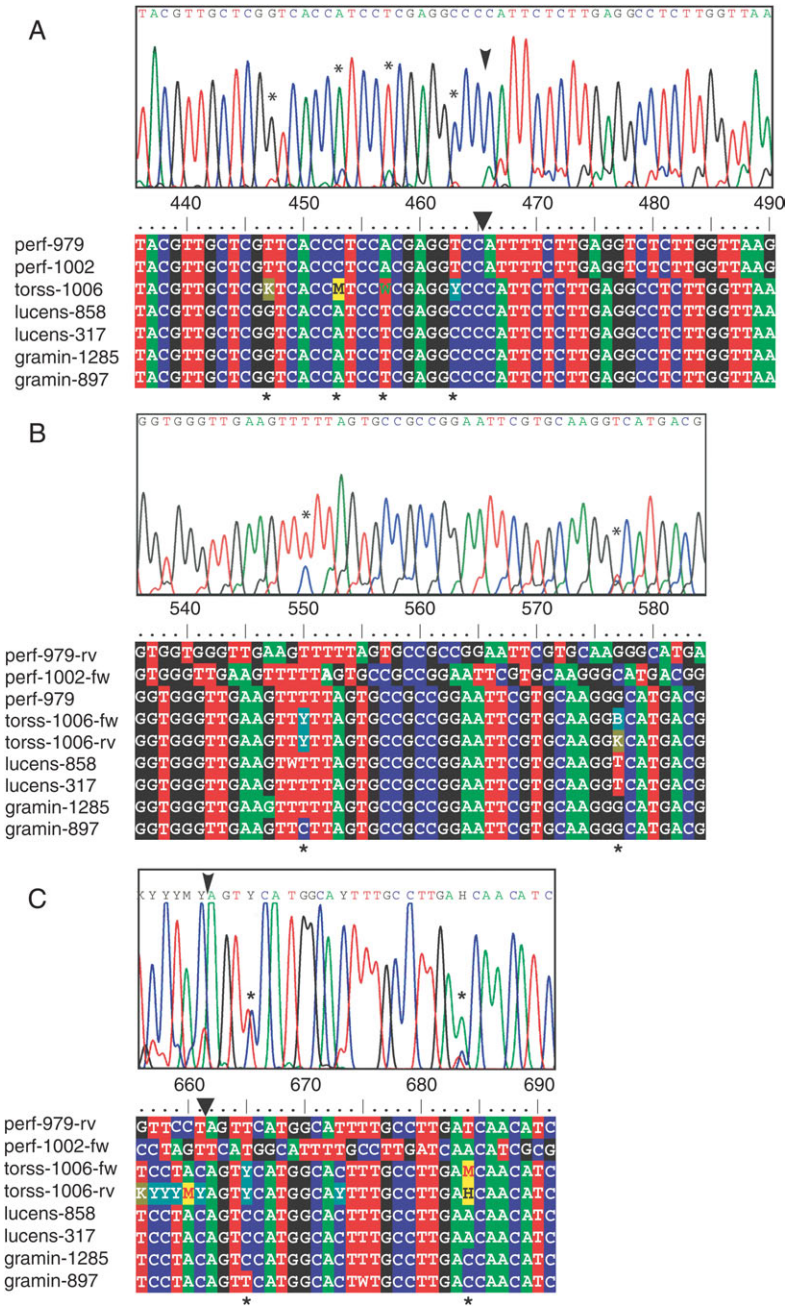

FIG. 1. *Potamogeton* × *torssanderi* triple hybrid and parental ITS sequences. Three diagnostic electropherogram clippings of directly sequenced *P.* × *torssanderi* ITS are shown along with the corresponding alignments of its parental taxa; relative positions are given above the alignments. Electropherograms of (A) and (B) were sequenced with the forward primer, (C) with the reverse primer; the plus strand is indicated in all cases. (A) Asterisks mark substitutions between parents; arrowheads indicate a 1-bp deletion in *P. perfoliatus* relative to *P. lucens*/*P. gramineus.* In the alignment, *P. perfoliatus* is written without a gap despite the indel position to reflect the sequencing reaction of the hybrid sample with the forward primer. (B) The upper *P. perfoliatus* sequence is aligned from the 3'-end without gaps (ignoring a 2-bp indel) reflecting sequencing with the reverse primer; the second is aligned as in (A); the third one is corrected for indels. Two positions with evidence for *P. gramineus* ITS are indicated by asterisks. The 'C' contributed by *P. gramineus* type 897 is recognized as 'Y' (C or T) in *P.* × *torssanderi* in both reading directions; the other position shows character states of all three parental species in forward direction [electropherogram; B (= C, G, or T) in alignment], and of *P. lucens*/others in reverse direction (K = G or T). (C) Sequences are aligned at their 3'-end to reflect sequencing with the reverse primer, only the second *P. perfoliatus* sequence is aligned without gaps from the 5'-direction (forward primer). Arrowheads indicate a 2-bp deletion in *P. perfoliatus* (upper sequence in alignment). At the position marked by the right asterisk, all three parental species have different diagnostic bases, recognizable in reverse direction (electropherogram; H = A, C or T); in forward direction, only 'C' for *P. gramineus* and 'A' for *P. lucens*/*P. perfoliatus* (= M, see alignment) are evident. Equal height of peaks at the left asterisk position indicates added-up 'T'-signals of *P. gramineus* type 897 and *P. perfoliatus*, exceptionally matching the height of the 'C'-signal of the otherwise dominating *P. lucens* sequence.

maternal origin (Kaplan and Fehrer, 2006). Thus, a similar, rather widespread *P. gramineus* genotype has probably contributed to the triple hybrid.

DISCUSSION

Origin of the triple hybrid P. × torssanderi

Contribution of three different parental genomes to the triple hybrid was demonstrated: *P. perfoliatus* could be identified from morphology, chloroplast DNA and a minority of ITS sequence types; *P. gramineus* from morphology and from another under-represented ITS copy type; and *P. lucens* whose contribution was least obvious from morphology provided the predominant ITS sequence variant.

In a triple hybrid, a third parental species requires fertility of a previous hybrid of two other species. Almost all *Potamogeton* hybrids are sterile (Wiegleb and Kaplan, 1998), therefore only a combination of closely related species or species with less effective reproductive barriers can presumably produce a fertile hybrid. Indeed, the primary hybrid of *P. gramineus* and *P. lucens*, *P.* × *angustifolius*, is capable of producing well-developed fruits. Observations on cultivated plants proved that seeds from these fruits germinate and the seedlings grow up to adult F_2 plants (Z. Kaplan, unpubl. res.). In addition, all three binary hybrids between the three putative parental species of *P.* × *torssanderi* are the most frequent *Potamogeton* hybrids in Europe, particularly common in Scandinavia. This suggests that there is a relatively low reproductive isolation between these three species. However, since the other two hybrids, *P.* × *salicifolius* (*P. lucens* × *P. perfoliatus*) and *P.* × *nitens* (*P. gramineus* × *P. perfoliatus*), are sterile (Kaplan and Fehrer, 2006; Kaplan, 2007), none of them could have been involved as the first binary hybrid in the rise of the triple hybrid. Thus, the only probable scenario is that a *P. gramineus* × *P. lucens* fertile hybrid hybridized with a *P. perfoliatus* plant and gave rise to the clone of *P.* × *torssanderi* studied.

As *P.* × *torssanderi* is hexaploid ($2n = 78$), it probably resulted from the combination of an unreduced gamete ($n = 52$) and a normal reduced gamete with 26 chromosomes. Hybrids are more likely to produce unreduced gametes than pure species because of potentially disturbed meiosis. Therefore, it is assumed that *P. perfoliatus* may have contributed a reduced gamete in the second hybridization, which according to cpDNA, must have been the maternal one. An unreduced gamete of *P. gramineus* × *P. lucens* would contain equal amounts of both ITS types so that, theoretically, the hexaploid hybrid should also show equal contributions from these two parents. However, *P. lucens* sequences predominated, suggesting that the binary hybrid contributing to the triple hybrid may have been a later generation hybrid *P.* × *angustifolius* or a backcross to *P. lucens*.

Alternative explanations for the dominance of one sequence type involve hybridization-associated locus loss of nrDNA. However, testing this scenario is currently not possible as the number and genomic organization of nrDNA loci are unknown in *Potamogeton*, and their karyotype, consisting of numerous small chromosomes, is difficult to assess (V. Jarolímová, pers. comm.). Gene conversion which often leads to homogenization of parental ITS copies in hybrids (reviewed by Álvarez and Wendel, 2003) and may therefore also lead to a skewed distribution of sequence types, is thought to be slowed down or absent in asexually reproducing organisms (Baldwin *et al.*, 1995; Campbell *et al.*, 1997). More background information on *Potamogeton* genomes would be needed to test between these possibilities.

Given our present knowledge, we assume that a homoploid (tetraploid) hybrid between *P. lucens* and *P. gramineus*, or its backcross to *P. lucens*, has contributed to the hexaploid triple hybrid analysed, presumably via an unreduced male gamete, whereas *P. perfoliatus* contributed a reduced female (diploid) gamete (in the subsequent hybridization).

Potamogeton × *torssanderi* represents one of very few well-documented examples of a natural primary hybrid involving three species. Although each hybrid is unique and cannot be described in general terms, the cases best comparable with this *Potamogeton* triple hybrid in terms of origin and asexual strategy of survival are represented by several animal systems: In parthenogenic lizards (Hillis *et al.*, 1991), unisexual fish (Mateos and Vrijenhoek, 2005) and stick insects (Scali *et al.*, 1995), a diploid binary hybrid subsequently hybridized with a third species, and the resulting trihybrid became triploid by genome addition and persists by parthenogenetic reproduction. As most broad-leaved species in *Potamogeton* are tetraploid, their genome duplication occurred a long time ago and they meanwhile behave like diploids (for a review on 'diploidization', see Ma and Gustafson, 2005), hence genome addition in *P.* × *torssanderi* went from the tetraploid to the hexaploid level.

Detection of different ITS copy types

In the triple hybrid, the contribution of particular nuclear parental genomes was deduced from direct sequencing, which has been successfully applied to the identification of hybrid/allopolyploid genome composition in other plant families (e.g. Sang *et al.*, 1995; Campbell *et al.*, 1997; Whittall *et al.*, 2000; Nieto Feliner *et al.*, 2004; Guggisberg *et al.*, 2006). Average relative peak heights at polymorphic sites have been shown to represent accurately the proportions of products in a mixture obtained by PCR amplification (Rauscher *et al.*, 2002). Especially when electropherograms indicate almost no noise (see Fig. 1, polymorphism-free positions) and care has been taken to avoid PCR drift (see Materials and methods), direct sequencing is both sensitive and reliable in detecting minority sequence types. In our specific case, apart from the quality of the sequencing reaction, the high similarity between *P. gramineus* and *P. lucens* sequences as well as the underrepresentation of the most divergent *P. perfoliatus* ITS copies made it possible to simultaneously discriminate between three parental sequences, which is not often feasible.

Approximate age of P. × torssanderi

The triple hybrid was first collected in 1893 in Lake Sillen and was still confirmed as common there in 1998.

Since *P.* × *torssanderi* is sterile, it must have persisted vegetatively at this site for more than a century, but presumably for a considerably longer period. Already at the time of its discovery, the hybrid must have produced an extensive clonal colony rich in individual ramets. The first collectors (e.g. Axel Torssander, Gustaf Tiselius, Sigfrid Almquist, Amandus Ekström, Johan Gustaf Laurell) collected altogether hundreds of specimens at that site for their herbaria and for widely distributed exsiccate collections without any serious attenuation of the existence of the clone. The hybrid may well be a relic from the early postglacial period. The association of hybrids with environments severely affected by the glacial cycles of the Late Pleistocene is well documented (Kerney, 2005). Several observations suggest that seedling recruitment is generally rare in established *Potamogeton* populations (Brux *et al.*, 1987, 1988; van Wijk, 1989; Kautsky, 1991; Hollingsworth *et al.*, 1996; Kaplan *et al.*, 2002; Kaplan and Štěpánek, 2003; Kaplan and Fehrer, 2004). That is why the opportunities for new hybrid genotypes were certainly greater in the open habitats of a postglacial landscape than they are in the present-day lakes with rich established plants communities. Although hybrids between separately adapted populations are on average less fit than either of their parents, *P.* × *torssanderi* seems to possess an efficient strategy for its existence and became locally successful even in the parental environment. The vigour of this hybrid may be associated with heterosis of sterile clonal hybrid lineages (Rieseberg and Carney, 1998).

Potential and limitations of morphological identification

Several previous molecular studies (Hollingsworth *et al.*, 1995, 1996; Preston *et al.*, 1998; Fant *et al.*, 2001*a*, *b*, 2003; King *et al.*, 2001; Kaplan *et al.*, 2002; Kaplan and Fehrer, 2004, 2006; Kaplan and Wolff, 2004) demonstrated that many *Potamogeton* hybrids can be reliably identified morphologically as long as adequate inspection of key features is adopted. However, *P.* × *torssanderi* belongs to a group of hybrids that can only be morphologically identified if the particular plant is optimally developed and shows diagnostic features of all three species involved in hybridization. Numerous herbarium specimens from Lake Sillen collected by various botanists since 1892 include both 'typical' *P.* × *torssanderi* as well as plants that may be triple hybrids but cannot be unequivocally distinguished from the binary hybrids of the three parental species involved (i.e. from *P.* × *angustifolius*, *P.* × *nitens* and *P.* × *salicifolius*).

As in many other aquatic plants, phenotypic plasticity plays a large role in plant morphology in *Potamogeton* (Kaplan, 2002). The extensive range of phenotypic plasticity obscures morphological differences between taxa. Some extreme forms of one taxon may easily mimic another taxon in such case. This makes even some entire taxa difficult to delimit morphologically from other similar taxa. Due to phenotypic plasticity, distinguishing between all four different hybrid combinations of the three parental species is difficult. Thus, morphological identification of *P.* × *torssanderi* will always have to be done with utmost care. In general, experimental proof of the identity of questionable plants with molecular markers is always advisable, particularly because character expression in hybrids is largely unpredictable (Rieseberg and Ellstrand, 1993).

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LITERATURE CITED

Álvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* **29**: 417–434.

Arnold ML. 1993. *Iris nelsonii* (Iridaceae): origin and genetic composition of a homoploid hybrid species. *American Journal of Botany* **80**: 577–583.

Arnold ML. 1997. *Natural hybridization and evolution.* Oxford: Oxford University Press.

Arnold ML, Bulger MR, Burke JM, Hempel AL, Williams JH. 1999. Natural hybridization: how low can you go and still be important? *Ecology* **80**: 371–381.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**: 247–277.

Barton N. 2001. The role of hybridization in evolution. *Molecular Ecology* **10**: 551–568.

Bothmer R, Bengtsson M, Flink J, Linde-Laursen I. 1988. Complex interspecific hybridization in barley (*Hordeum vulgare* L.) and the possible occurrence of apomixis. *Theoretical and Applied Genetics* **76**: 681–690.

Bothmer R, Claesson L, Flink J, Linde-Laursen I. 1989. Triple hybridization with cultivated barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **78**: 818–824.

Brux H, Todeskino D, Wiegleb G. 1987. Growth and reproduction of *Potamogeton alpinus* Balbis growing in disturbed habitats. *Archiv fur Hydrobiologie, Beihefte* **27**: 115–127.

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

Bureš P. 2004. *Cirsium* Mill. In: Slavík B, Štěpánková J, Štěpánek J eds. *Květena České republiky [Flora of the Czech Republic]*. Praha: Academia, **7**: 385–419.

Campbell CS, Wojciechowski MF, Baldwin BG, Alice LA, Donoghue MJ. 1997. Persistent nuclear ribosomal DNA sequence polymorphism in the *Amelanchier* agamic complex. *Molecular Biology and Evolution* **14**: 81–90.

Clark WA. 1942. Pondweeds from North Uist (V.-C. 110), with a special consideration of *Potamogeton rutilus* Wulfg. and a new hybrid. *Proceedings of the University of Durham Philosophical Society* **10**: 368–373.

Dandy JE. 1975. *Potamogeton* L. In: Stace CA ed. *Hybridization and the flora of the British Isles*. London/New York/San Francisco: Academic Press, 444–459.

dePamphilis CW, Wyatt R. 1990. Electrophoretic confirmation of interspecific hybridization in *Aesculus* (Hippocastanaceae) and the genetic structure of a broad hybrid zone. *Evolution* **44**: 1295–1317.

Densmore LD, Wright JW, Brown WM. 1989. Mitochondrial-DNA analyses and the origin and relative age of parthenogenetic lizards (genus *Cnemidophorus*). II. *C. neomexicanus* and the *C. tesselatus* complex. *Evolution* **43**: 943–957.

Dionne LA. 1963. Studies on the use of *Solanum acaule* as a bridge between *Solanum tuberosum* and species in the series *Bulbocastana*, *Cardiophylla* and *Pinnatisecta*. *Euphytica* **12**: 263–269.

Dodd RS, Afzal-Rafii Z. 2004. Selection and dispersal in a multispecies oak hybrid zone. *Evolution* **58**: 261–269.

Dvořák J. 1998. Genome analysis in the *Triticum-Aegilops* alliance. In: Slinkard AE, ed. *Proceedings of the 9th International Wheat Genetics Symposium*, Saskatoon, Saskatchewan, Canada. Saskatoon: University Extension Press, 1: 8–11.

Dvořák J, Zhang HB. 1990. Variation in repeated nucleotide sequences sheds light on the phylogeny of the wheat B and G genomes. *Proceedings of the National Academy of Sciences of the USA* **87**: 9640–9644.

Dvořák J, McGuire PE, Cassidy B. 1988. Apparent sources of the A genomes of wheat inferred from polymorphism in abundance and restriction fragment length of repeated nucleotide sequences. *Genome* **30**: 680–689.

Dvořák J, di Terlizzi P, Zhang HB, Resta P. 1993. The evolution of polyploid wheats: identification of the A genome donor species. *Genome* **36**: 21–31.

Dvořák J, Luo M-C, Yang Z-L, Zhang H-B. 1998. The structure of the *Aegilops tauschii* gene pool and the evolution of hexaploid wheat. *Theoretical and Applied Genetics* **97**: 657–670.

Fant JB, Preston CD. 2004. Genetic structure and morphological variation of British populations of the hybrid *Potamogeton* × salicifolius. *Botanical Journal of the Linnean Society* **144**: 99–111.

Fant JB, Preston CD, Barrett JA. 2001*a*. Isozyme evidence for the origin of *Potamogeton* × *sudermanicus* as a hybrid between *P. acutifolius* and *P. berchtoldii*. *Aquatic Botany* **71**: 199–208.

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

Fant JB, Kamau EA, Preston CD. 2003. Chloroplast evidence for the multiple origins of the hybrid *Potamogeton* × *sudermanicus* Hagstr. *Aquatic Botany* **75**: 351–356.

Fehrer J, Gemeinholzer B, Chrtek J Jr, Bräutigam S. 2007. Incongruent plastid and nuclear DNA phylogenies reveal ancient intergeneric hybridization in *Pilosella* hawkweeds (*Hieracium*, Cichorieae, Asteraceae). *Molecular Phylogenetics and Evolution* **42**: 347–361.

Gadella TWJ. 1992. Notes on some triple and inter-sectional hybrids in *Hieracium* L. subgenus *Pilosella* (Hill) S. F. Gray. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen* **95**: 51–63.

Gill KS, Lubbers EL, Gill BS, Raupp WJ, Cox TS. 1991. A genetic linkage map of *Triticum tauschii* (DD) and its relationship to the D genome of bread wheat (AABBDD). *Genome* **34**: 362–374.

Gonzáles SS, Barrio E, Gafner J, Querol A. 2006. Natural hybrids from *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces kudriavzevii* in wine fermentations. *FEMS Yeast Research* **6**: 1221–1234.

Graebner P. 1907. *Potamogeton* (Tourn.) L. In: Engler A ed. *Das Pflanzenreich, Regni vegetabilis conspectus*. Berlin, Germany, **31**(IV.11): 39–142, 161–162.

Guggisberg A, Bretagnolle F, Mansion G. 2006. Allopolyploid origin of the Mediterranean endemic, *Centaurium bianoris* (Gentianaceae), inferred by molecular markers. *Systematic Botany* **31**: 368–379.

Hagström JO. 1916. Critical researches on the Potamogetons. *Kungliga Svenska Vetenskapsakademiens Handlingar* **55**: 1–281.

Hall TA. 1999. BioEdit, a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hamilton MB. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**: 521–423.

Hermsen JGTh, Ramanna MS. 1973. Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. *Euphytica* **22**: 457–466.

Hillis DM, Moritz C, Porter CA, Baker RJ. 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. *Science* **251**: 308–310

Hodálová I. 2002. A new hybrid *Senecio* × *slovacus* from the *S. nemorensis* group (Compositae) in the West Carpathians. *Biologia (Bratislava)* **57**: 75–82.

Hollingsworth PM, Preston CD, Gornall RJ. 1995. Isozyme evidence for hybridization between *Potamogeton natans* and *P. nodosus* (Potamogetonaceae) in Britain. *Botanical Journal of the Linnean Society* **117**: 59–69.

Hollingsworth PM, Preston CD, Gornall RJ. 1996. Isozyme evidence for the parentage and multiple origins of *Potamogeton* × *suecicus* (*P. pectinatus* × *P. filiformis*, Potamogetonaceae). *Plant Systematics and Evolution* **202**: 219–232.

Hollingsworth PM, Preston CD, Gornall RJ. 1998. Euploid and aneuploid evolution in *Potamogeton* (Potamogetonaceae): a factual basis for interpretation. *Aquatic Botany* **60**: 337–358.

Holmgren PK, Holmgren NH. 1990. Index Herbariorum. Part I. The Herbaria of the World. Ed. 8. *Regnum Vegetabile* 120: 1–693.

Holub J. 1992. *Crataegus* L. In: Hejný S, Slavík B, Kirschner J, Křísa B eds. *Květena České republiky [Flora of the Czech Republic]*. Praha: Academia, **3**: 488–525.

Huang XQ, Borner A, Roder MS, Ganal MW. 2002. Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. *Theoretical and Applied Genetics* **105**: 699–707.

Hughes CE, Bailey CD, Harris SA. 2002. Divergent and reticulate species relationships in *Leucaena* (Fabaceae) inferred from multiple data sources: insights into polyploid origins and nrDNA polymorphism. *American Journal of Botany* **89**: 1057–1073.

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

Kalasa Balicka M. 1976. The triple hybrid (*Solanum tuberosum* L. × *S. vernei* Bitt. et Wittm.) × *S. bulbocastanum* Dun. *Genetica Polonica* **17**: 165–169.

Kalasa Balicka M. 1980. Meiosis and microspore formation in the triple hybrid of (*Solanum tuberosum* L. × *Solanum vernei* Bitt. et Wittm.) × *Solanum bulbocastanum* Dun. *Genetica Polonica* **21**: 425–431.

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

Kaplan Z. 2007. First record of *Potamogeton* × *salicifolius* for Italy, with isozyme evidence for plants collected in Italy and Sweden. *Plant Biosystems* **141**: in press.

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

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

Kaplan Z, Štěpánek J. 2003. Genetic variation within and between populations of *Potamogeton pusillus* agg. *Plant Systematics and Evolution* **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.

Kautsky L. 1991. *In situ* experiments on interrelationships between six brackish macrophyte species. *Aquatic Botany* **39**: 159–172.

Kearney M. 2005. Hybridization, glaciation and geographical parthenogenesis. *Trends in Ecology and Evolution* **20**: 495–502.

Kihara H. 1944. Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare*. *Agricultural Horticulture* **19**: 889–890 [In Japanese].

King RA, Gornall RJ, Preston CD, Croft JM. 2001. Molecular confirmation of *Potamogeton* × *bottnicus* (*P. pectinatus* × *P. vaginatus*, Potamogetonaceae) in Britain. *Botanical Journal of the Linnean Society* **135**: 67–70.

Kirschner J, Skalický V. 1990. Violaceae Batsch. In: Hejný S, Slavík B, Hrouda L, Skalický V eds. *Květena České republiky [Flora of the Czech Republic]*. Praha: Academia, **2**: 394–431.

Kitchener GD. 1997. A triple hybrid willowherb: *Epilobium ciliatum* × *E. hirsutum* × *E. parviflorum. BSBI News* **75**: 66–67.

Koch M, Dobeš C, Mitchell-Olds T. 2003. Multiple hybrid formation in natural populations: concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). *Molecular Biology and Evolution* **20**: 338–350.

Lancaster ML, Gemmell NJ, Negro S, Goldsworthy S, Sunnucks P. 2006. Ménage á trois on Macquarie Island: hybridization among three species of fur seal (*Arctocephalus* spp.) following historical population extinction. *Molecular Ecology* **15**: 3681–3692.

Les DH, Cleland MA, Waycott M. 1997. Phylogenetic studies in Alismatidae. II. Evolution of marine angiosperms (seagrasses) and hydrophily. *Systematic Botany* **22**: 443–463.

Lilienfield FA. 1951. H. Kihara: genome analysis in *Triticum* and *Aegilops*. Concluding review. *Cytologia* **16**: 101–123.

Linnaeus C. 1753. *Species plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas.* Holmiae.

Linton EF. 1907. Hybrids among British phanerogams. *Journal of Botany* **45**: 296–304.

McFadden ES, Sears ER. 1946. The origin of *Triticum spelta* and its free-threshing hexaploid relatives. *Journal of Heredity* **37**: 81–89, 107–116.

Ma X-F, Gustafson JP. 2005. Genome evolution of allopolyploids: a process of cytological and genetic diploidization. *Cytogenetic and Genome Research* **109**: 236–249.

Maekawa M, Ha S, Kita F. 1991. Identification of reciprocal translocations observed in several *Melilotus* species (subgenus *Eumelilotus*) by interspecific triple crossings. *Euphytica* **54**: 255–261.

Mantovani B, Passamonti M, Scali V. 2001. The mitochondrial cytochrome oxidase II gene in *Bacillus* stick insects: ancestry of hybrids, androgenesis, and phylogenetic relationships. *Molecular Phylogenetics and Evolution* **19**: 157–163.

Mason-Gamer RJ. 2004. Reticulate evolution, introgression, and intertribal gene capture in an allohexaploid grass. *Systematic Biology* **53**: 25–37.

Mateos M, Vrijenhoek RC. 2005. Independent origins of allotriploidy in the fish genus *Poeciliopsis. Journal of Heredity* **96**: 32–39.

Molina MD, García MD, López CG, Ferrero VM. 2004. Meiotic pairing in the hybrid (*Zea diploperennis* × *Zea perennis*) × *Zea mays* and its reciprocal. *Hereditas* **141**: 135–141.

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

Nelson-Jones EB, Briggs D, Smith AG. 2002. The origin of intermediate species of the genus *Sorbus. Theoretical and Applied Genetics* **105**: 953–963.

Nieto Feliner G, Gutiérrez Larena B, Fuertes Aguilar J. 2004. Fine-scale geographical structure, intra-individual polymorphism and recombination in nuclear ribosomal internal transcribed spacers in *Armeria* (Plumbaginaceae). *Annals of Botany* **93**: 189–200.

Nilsson NH. 1954. Über Hochkomplexe Bastardverbindungen in der Gattung *Salix*. *Hereditas* **40**: 517–522.

O'Kane SL Jr, Schaal BA, Al-Shebaz IA. 1996. The origins of *Arabidopsis suecica* (Brassicaceae) as indicated by nuclear rDNA sequences. *Systematic Botany* **21**: 559–566.

Parker ED Jr, Selander RK. 1976. The organization of genetic diversity in the parthenogenetic lizard *Cnemidophorus tesselatus. Genetics* **84**: 791–805.

Preston CD. 1995. *Pondweeds of Great Britain and Ireland.* London: Botanical Society of the British Isles.

Preston CD, Hollingsworth PM, Gornall RJ. 1998. *Potamogeton pectinatus* L. × *P. vaginatus* Turcz. (*P.* × *bottnicus* Hagstr.), a newly identified hybrid in the British Isles. *Watsonia* **22**: 69–82.

Rauscher JT, Doyle JJ, Brown AHD. 2002. Internal transcribed spacer repeat-specific primers and the analysis of hybridization in the *Glycine tomentella* (Leguminosae) polyploid complex. *Molecular Ecology* **11**: 2691–2702.

Rees DJ, Dioli M, Kirkendall LR. 2003. Molecules and morphology: evidence for cryptic hybridization in African *Hyalomma* (Acari: Ixodidae). *Molecular Phylogenetics and Evolution* **27**: 131–142.

Rieseberg LH. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28**: 359–389.

Rieseberg L, Carney S. 1998. Plant hybridization. *New Phytologist* **140**: 599–624.

Rieseberg LH, Ellstrand NC. 1993. What can molecular and morphological markers tell us about plant hybridization. *Critical Reviews in Plant Sciences* **12**: 213–241.

Rieseberg LH, Soltis DE. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* **5**: 65–84.

Ritz CM, Schmuths S, Wissemann V. 2005. Evolution by reticulation: European dogroses originated by multiple hybridization across the genus *Rosa. Journal of Heredity* **96**: 4–14.

Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. *Proceedings of the National Academy of Sciences of the USA* **92**: 6813–6817.

Scali V, Tinti F, Mantovani B, Marescalchi O. 1995. Mate recognition and gamete cytology features allow hybrid species poduction and evolution in *Bacillus* stick insect. *Bollettino di Zoologia* **62**: 59–70.

Simmonds NW. 1976. *Evolution of crop plants.* London: Longman.

Soltis PS, Soltis DE. 1991. Multiple origins of the allotetraploid *Tragopogon mirus* (Compositae): rDNA evidence. *Systematic Botany* **16**: 407–413.

Stace CA, ed**. 1975.** *Hybridization and the flora of the British Isles.* London/New York/San Francisco: Academic Press.

Štěpánek J. 1997. *Knautia* L. In: Slavík B, Chrtek J, Tomšovic P eds. *Květena České republiky [Flora of the Czech Republic]*. Praha: Academia, **5**: 543–554.

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.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.

van Wijk RJ. 1989. Ecological studies on *Potamogeton pectinatus* L. III. Reproductive strategies and germination ecology. *Aquatic Botany* **33**: 271–299.

Wagner A, Blackstone N, Cartwright P, Dick M, Misof B, Snow P, *et al*. 1994. Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. *Systematic Biology* **43**: 250–261.

Wang GZ, Miyashita NT, Tsunewaki K. 1997. Plasmon analyses of *Triticum* (wheat) and *Aegilops*: PCR-single-stranded conformational polymorphism (PCR-SSCP) analyses of organellar DNAs. *Proceedings of the National Academy of Sciences of the USA* **94**: 14570–14577.

Wendel JF, Schnabel A, Seelanan T. 1995. An unusual ribosomal DNA sequence from *Gossypium gossypioides* reveals ancient, cryptic, intergenomic introgression. *Molecular Phylogenetics and Evolution* **4**: 298–313.

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

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

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