A morphological, anatomical and isozyme study of *Potamogeton* ×*schreberi*: confirmation of its recent occurrence in Germany and first documented record in France

Morfologie, anatomie lodyhy a isozymová spektra u *Potamogeton* ×*schreberi*: potvrzení současného výskytu v Německu a první údaj pro Francii

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A combined study of morphology, stem anatomy and isozyme patterns was used to reveal the identity of sterile plants from two rivers on the Germany/France border. A detailed morphological examination proved that the putative hybrid is clearly intermediate between *Potamogeton natans* and *P. nodosus*. The stem anatomy had characteristics of both species. The most compelling evidence came from the isozyme analysis. The additive "hybrid" banding patterns of the six enzyme systems studied indicate inheritance from *P. natans* and *P. nodosus*. In contrast, other morphologically similar hybrids were excluded: *P.* ×*gessnacensis* (= *P. natans* × *P. polygonifolius*) by all the enzyme systems, *P.* ×*fluitans* (= *P. lucens* × *P. natans*) by AAT, EST and 6PGDH, and *P.* ×*sparganiifolius* (= *P. gramineus* × *P. natans*) by AAT and EST. All samples of *P.* ×*schreberi* are of a single multi-enzyme phenotype, suggesting that they resulted from a single hybridization event and that the present-day distribution of *P.* ×*schreberi* along the Saarland/Moselle border was achieved by means of vegetative propagation and long-distance dispersal. Neither of its parental species occur with *P.* ×*schreberi* or are present upstream, which suggests that this hybrid has persisted vegetatively for a long time in the absence of its parents. The total distribution of this hybrid is reviewed and a detailed account of the records from Germany is given. *P.* ×*schreberi* appears to be a rare hybrid. The risk of incorrect determination resulting from the identification of insufficiently developed or inadequately preserved plant material is discussed.

K e y w o r d s : anatomy, clone, dispersal, distribution, electrophoresis, France, Germany, hybridization, isozymes, morphology, *Potamogeton*, relic, taxonomy, vegetative propagation

Introduction

The group of taxa, which was in central- and west-European literature of the 19th century treated as a broadly defined "*Potamogeton fluitans*", is one of the most difficult complexes of the genus (Kaplan 2001, Kaplan 2002a). It was later split into several taxa, particularly as a result of a detailed investigation of the morphology and stem anatomy by Raunkiaer (1896, 1903) and Fischer (1904, 1905, 1907). Nowadays the group is treated as several distinct taxa. It includes a fertile species called *P. nodosus* and several very similar *P. natans* hybrids, such as *P.* ×*fluitans* (= *P. lucens* × *P. natans*), *P.* ×*schreberi* (= *P. natans* × *P. nodosus*), *P.* ×*gessnacensis* (= *P. natans* × *P. polygonifolius*) and *P.* ×*sparganiifolius* (= *P. gramineus* × *P. natans*). These taxa share the same general appearance and several

taxonomically important morphological characters, such as petiolate submerged leaves with narrowly oblong to oblanceolate-oblong lamina and the capacity to produce floating leaves with a coriaceous lamina, which is generally cuneate at the base and gradually tapering into the petiole. The hybrids often produce transitional leaves, which are in their shape and size intermediate between the very narrow submerged leaves (reduced to phyllodes near the base of the stem) and broad floating leaves. The identification of the members of this complex is extremely difficult. In addition to detailed analysis of morphology, studies of stem anatomy (Raunkiaer 1903, Fischer 1905, Fischer 1907, Kaplan 2001), isozyme analysis (Hollingsworth et al. 1995b, Fant et al. 2001b, Kaplan et al. 2001) or cytological investigations (Preston et al. 1998) are usually necessary for reliable identification. The most important diagnostic characters of these taxa are summarized in Table 1 and selected features of their stem anatomy are given in Table 2.

A plant belonging to this group of taxa but not identical with any of the broad-leaved species was discovered in the river Blies, running along the Germany/France border. It was first recorded at Reinheim, Saarland, Germany, by P. W. in 1976 (Fig. 1) and tentatively considered as an atypical form of *P. nodosus*. This plant was later repeatedly collected there, and it became more and more apparent that it belongs to a morphologically similar hybrid, possibly *P. natans* × *P. nodosus*. Another colony of these plants was discovered by P. W. in the same river at Bliesbruck, ca 2.2 km downstream of Reinheim, but already across the border, in Lorraine, France.

The third site for this plant was found accidentally, which illustrates how easily a hybrid of the "*P. fluitans*"-complex can be overlooked and confused with other similar species. When looking for additional samples of *Potamogeton* species for comparison and isozyme analysis in Saarland and Lorraine in July 2001, P. W. collected also plant material from the river Saar at Hanweiler, ca 0.5 km downstream the mouth of the river Blies. The site was discovered by F. J. Weicherding (pers. comm.) in 2001, who recorded the occurrence there of *P. nodosus*. In the absence of submerged leaves, the plants were confused with *P. nodosus*. However, further examination of the plants in cultivation proved that they belong to the same putative hybrid as that observed in Blies (see the section Morphological evaluation for details).

Despite the original identification as *P. nodosus*, a detailed examination of the plants from both the rivers revealed some characteristics more closely associated with *P. natans*. Judging from the intermediate shape and size of the submerged leaves, the plants may have been a hybrid between *P. natans* and a species with broad submerged leaves. The identity of the other parent was uncertain because of the existence of several hybrids of *P. natans* that share the same general appearance. Although observations suggested the origins to be *P. natans* × *P. nodosus*, some forms of selected other *P. natans* hybrids such as *P.* ×*gessnacensis*, *P.* ×*fluitans* or *P.* ×*sparganiifolius* are not very different. No broad-leaved species is known to occur in Blies at present, so the joint occurrence of possible parental taxa cannot serve as a clue for the identification of the putative hybrid.

Because of the difficulties in identifying taxa of the "*P. fluitans*"-complex, we collected for detailed examination both fresh and herbarium material of the putative hybrid, of both supposed parental species and of the three other species known to produce similar hybrids with *P. natans*. In addition to their morphology, details of stem anatomy and isozyme analysis were used. The stem anatomy proved to be an important source of additional characters for resolving taxonomic difficulties in *Potamogeton* (Raunkiaer 1896, 1903, Fischer

Table 1. – Selected diagnostic characters of *Potamogeton natans*, *P. nodosus* and morphologically similar *P. natans* hybrids (after Dandy 1975, Preston 1995a, b, Preston et al. 1998, Kaplan 2001, and own observations).

	P. natans	*P. nodosus*	*P. ×schreberi*	*P. ×gessnacensis*	*P. ×fluitans*	*P. ×sparganiifolius*
Occurrence of phyllodial leaves	present throughout the stem up to floating leaves	absent	present near the base of the stem	present near the base of the stem	present near the base of the stem	present near the base of the stem
Length:width ratio of lamina of upper submerged leaves	–	(4–) 5–8 (–9)	(6–) 10–40 (–56)	8–175	4–20	16–72
Length of lamina of submerged leaves (mm)	–	(50–) 160–280 (–350)	30–180 (–300)	15–210	60–220	60–520
Width of lamina of submerged leaves (mm)	–	(11–) 22–38	2–15	1–4	8–33	2–12
Number of veins in upper submerged leaves	–	(7–) 11–21	3–11	3–5	7–15	3–13
Length of petiole of submerged leaves (mm)	–	40–210	95–425	45–175	20–70	0–55
Presence of discoloured section between petiole and lamina of floating leaves	almost always present	always absent	absent or rarely trace visible	absent or rarely trace visible	always absent	sometimes present
Shape of dorsal side of stipules	ridged	ridged	ridged	ridged	winged	ridged

Table 2. – A comparison of the most important anatomical characters of five broad-leaved *Potamogeton* species (after Wiegleb & Kaplan 1998). Rare deviations from the respective ordinary patterns were omitted.

	P. natans	*P. lucens*	*P. gramineus*	*P. nodosus*	*P. polygonifolius*
Type of stele	trio or complex oblong	oblong type	oblong type	mostly trio type	proto type
Shape of endodermal cells	U-type	U-type	U-type	O-type	O-type
Occurrence of interlacunar bundles	present in 3–4 circles	present in 1–3 circles	present in 1 circle	absent	absent
Occurrence of subepidermal bundles	present	present	present or absent	absent	present
Presence of pseudohypodermis	present in 1–2 layers	present in 1 layer	absent or present in 1 incomplete layer	absent	present in 1–2 layers

1904, 1905, 1907, Hagström 1916, Ogden 1943, 1974a, 1974b, Symoens et al. 1979, Tur 1982, Wiegleb 1990a, b, Kaplan 2001). Isozymes have been used to explore the relationships and hybridization between many different groups of plants. Isozymes have also been used to identify several hybrids in the genus *Potamogeton* (Hollingsworth et al. 1995b, 1996b, Preston et al. 1998, Fant et al. 2001a, b, Iida and Kadono 2002, Kaplan et al. 2002).

The aim of the present paper is (1) to reveal the identity of the plants of intermediate morphology from the rivers Blies and Saar, (2) test the hypothesis of interspecific hybridization, and (3) establish the parentage of the putative hybrid. This should rule out the possibility that the studied plants of intermediate morphology are only extreme forms of a "true" species. It is known that some species can under certain circumstances produce phenotypes that are very similar to that of hybrids (Kaplan 2002b).

Material and methods

Plant material

In addition to the putative hybrid, specimens of species that could have given rise to the intermediate plant (based on its morphology) were sampled: *Potamogeton gramineus*, *P. lucens*, *P. natans*, *P. nodosus*, and *P. polygonifolius*. Altogether, 61 samples from 39 populations were cultivated and investigated (Table 3). Because the genetic variation between populations is high, but low or absent within populations of *Potamogeton* species (van Wijk et al. 1988, Hettiarachchi & Triest 1991, Hollingsworth et al. 1995a, 1996a, Kaplan & Štěpánek 2003), only 1–3 individuals were taken from each population but more populations were sampled to cover intraspecific variation. Specimens for analyses were collected from a wide area along the Germany/France border where the hybrid was found. Additional samples were collected in other areas of Europe, mainly Central Europe, in order to define more accurately the isozyme diversity of the species.

Samples of the 6 taxa were cultivated in the experimental garden at the Institute of Botany, Průhonice, Czech Republic, from 1996–2003. Plants were cultivated in 180 × 140 × 80 cm water-filled plastic tanks, which were sunk in the ground in order to prevent overheating of the water in summer. The samples were planted in submerged plastic pots containing pond mud, which had previously been desiccated.

Potamogeton polygonifolius is considered to be a diploid, whereas all the other species are tetraploid (Hollingsworth et al. 1998, Z. Kaplan & V. Jarolímová, unpubl.). A rich collection of voucher material from both field and cultivated plants is preserved in the Herbarium of the Institute of Botany, Průhonice (acronym PRA). The nomenclature and hybrid formulas follow Wiegleb & Kaplan (1998).

Investigation of stem anatomy

Fresh samples from all three populations and the cultivated sample 1278 from Blies were examined. Short pieces of stem were cut from the middle of the internode region of the main stem. Transverse sections ± 0.05 mm thin were cut by hand using a razor blade. This was done under a stereomicroscope using reflected light and the sections were stained in a drop of water with toluidine blue. After 1–3 minutes, depending on stainability, the sec-

Table 3. – Origin and reference numbers of the cultivated and isozymatically analysed samples of *Potamogeton* taxa.

Taxon	Ref. no.	Origin of samples and records on the vouchers from the field
P. ×schreberi	1276	France, Lorraine, Dept. Moselle: river Blies at N margin of Bliesbruck, 14 VII 2001, coll. P. Wolff s. n.
	1278	Germany, Saarland: river Blies above the bridge at SE margin of Reinheim, 14 VII 2001, coll. P. Wolff s. n.
	1279	Germany, Saarland: river Blies below the bridge at SE margin of Reinheim, 14 VII 2001, coll. P. Wolff s. n.
	1280	Germany, Saarland: river Saar S of Hanweiler, 14 VII 2001, coll. P. Wolff s. n.
P. gramineus	318	Czech Republic, Bohemia, Distr. Pardubice: fishpond Baroch near Hrobice, 18 IX 1996, coll. Z. Kaplan 96/624
	885	Czech Republic, Bohemia, Distr. Náchod: Rozkoš reservoir by Šeřeč, 22 VIII 1997, coll. Z. Kaplan 97/829
	897	Czech Republic, Bohemia, Distr. Česká Lípa: fishpond Držák near Hradčany, 18 IX 1996, coll. Z. Kaplan 96/638
	1008	Sweden, Prov. Södermanland: Sillen Lake near Vårdinge, 13 VIII 1998, coll. Z. Kaplan 98/345
	1156	France, Brittany, Dept. Morbihan: Scorff River N of Lorient, VI 1998, coll. J. Květ s. n., comm. Z. Kaplan
	1285	France, Lorraine, Dept. Moselle: canal at Rémelfing, 21 VII 2001, coll. P. Wolff s. n.
P. lucens	316	Czech Republic, Bohemia, Distr. Pardubice: fishpond Baroch near Hrobice, 18 IX 1996, coll. Z. Kaplan 96/627
	317	Czech Republic, Bohemia, Distr. Pardubice: fishpond Baroch near Hrobice, 18 IX 1996, coll. Z. Kaplan s. n.
	858	Netherlands, Prov. Limburg: Arcen, cult. P. Denny, 1997 comm. Z. Kaplan
	912	Czech Republic, Bohemia, Distr. Turnov: fishpond at Arnoštice near Žehrov, 18 IX 1997, coll. Z. Kaplan 97/914
	978	Switzerland, canton Sankt Gallen: ditch with running water at Altenrhein near Rorschach, 23 VI 1998, coll. Z. Kaplan 98/123
	1150	Czech Republic, Bohemia, Distr. Turnov: fishpond at Arnoštice near Žehrov, 29 VII 1999, coll. Z. Kaplan s. n.
	1284	France, Lorraine, Dept. Moselle: river Sarre (= Saar) S of Grosbliederstroff, 21 VII 2001, coll. P. Wolff s. n.
P. natans	319	Czech Republic, Bohemia, Distr. Pardubice: fishpond Baroch near Hrobice, 18 IX 1996, coll. Z. Kaplan 96/626
	911	Czech Republic, Bohemia, Distr. Turnov: fishpond at Arnoštice near Žehrov, 18 IX 1997, coll. Z. Kaplan 97/913
	977	Switzerland, canton Sankt Gallen: ditch with running water at Altenrhein near Rorschach, 23 VI 1998, coll. Z. Kaplan 98/122
	1028	Denmark, county Sønderjylland: brook Uge Baek at Hajstrup, 19 VIII 1998, coll. Z. Kaplan 98/380
	1148	Czech Republic, Bohemia, Distr. Turnov: fishpond at Arnoštice near Žehrov, 29 VII 1999, coll. Z. Kaplan s. n.
	1283	Germany, Saarland: pond near Saarbrücken-Am Homburg, 21 VII 2001, leg. F. J. Weicherding s. n., comm. P. Wolff
	1310	Germany, Saarland: pond near Saarbrücken-Rußhütte, 14 VI 2002, coll. P. Wolff s. n.
	1317	Czech Republic, Bohemia, Distr. Cheb: fishpond Nový at Novosedly near Hranice, southern bank, 24 VI 2002, coll. Z. Kaplan s. n.
	1318	Czech Republic, Bohemia, Distr. Cheb: pond at NW margin of Libá, 24 VI 2002, coll. Z. Kaplan 02/119

P. nodosus	839	Czech Republic, Moravia, Distr. Hodonín: Velička navigation canal at the N margin of Strážnice, 25 VI 1997, coll. Z. Kaplan 97/510
	971	Italy, Lombardy, Distr. Sondrio: ditches on N bank of the Lago di Mezzola (lake) NW of Novate Mezzole, 18 VI 1998, coll. Z. Kaplan 98/75
	1131	France, Aquitaine, Dept. Lot-et-Garonne: Le Temple-sur-Lot, cult. Botanischer Garten der Georg-August-Universität, Göttingen, comm. Z. Kaplan
	1281	Germany, Saarland: flooded gravel pit W of Nennig, 22 VII 2001, coll. P. Wolff s. n.
	1282	France, Lorraine, Dept. Moselle: river Saar S of Grosbliederstroff, 21 VII 2001, coll. P. Wolff s. n.
	1309	France, Lorraine, Dept. Moselle: mill-race of river Saar at Welferding near Sarreguemines, 14 VI 2002, coll. P. Wolff s. n.
	1316	Czech Republic, Bohemia, Distr. Nymburk: gravel pit lake NE of Sokoleč, 23 VI 2002, coll. Z. Kaplan s. n.
P. polygonifolius	1272	Germany, Rhineland-Palatinate: ditch near river Glan N of Vogelbach, 14 VII 2001, coll. P. Wolff s. n.
	1308	Germany, Saarland: brook Kirkelerbach W of Kirkel, 16 VI 2002, coll. P. Wolff s. n.
	1313	Germany, Saarland: ditch with running water in reserve "Kühnbruch" in valley of Blies River SE of Niederbexbach, 16 VI 2002, coll. P. Wolff s. n.
	1319	Czech Republic, Bohemia, Distr. Cheb: fishpond Nový at SE margin of Novosedly near Hranice, southern bank, 24 VI 2002, coll. Z. Kaplan s. n.
	1320	Czech Republic, Bohemia, Distr. Cheb: fishpond Nový at SE margin of Novosedly near Hranice, southwestern bank, 24 VI 2002, coll. Z. Kaplan s. n.
	1321	Czech Republic, Bohemia, Distr. Cheb: pool on bank of forest fishpond NW of Novosedly near Hranice, 24 VI 2002, coll. Z. Kaplan 02/118

tions were washed in distilled water and studied under a transmitted light microscope at a magnification 20–60× (general anatomical pattern) or 150–550× (thickening of endodermal cells, occurrence of interlacunar and subepidermal bundles, development of pseudohypodermis).

A detailed review of the use of the stem anatomy in the systematics and identification of species of *Potamogeton* is provided by Wiegleb (1990c). Descriptive terms used in stem anatomy are explained by Wiegleb & Kaplan (1998). Line drawings of important anatomical structures (interlacunar and subepidermal bundles, pseudohypodermis) are given by Symoens et al. (1979) and coloured photographs by Kaplan (2001).

Electrophoresis

Leaf material was collected from cultivated plants early in the morning in the summers of 2002 and 2003 and immediately used for enzyme extraction. The leaves were dabbed free of water, marl and algae. Approximately 60 mg of leaf tissue was mechanically ground with Dowex-Cl (1-X8) and quartz sand and homogenized on ice in 0.75 ml tris-HCl extraction buffer. Two different extraction buffer systems were used: (a) "viola" (0.1 M tris-HCl pH 8.0, 70 mM 2-mercaptoethanol, 26 mM sodium metabisulfite, 11 mM ascorbic acid, 4% polyvinylpyrrolidon) was used to separate isozymes of AAT; and (b) "luzula" (75 mM tris-H_3PO_4 pH 7.5, 13 mM 2-mercaptoethanol, 7.8 mM dithioerythritol, 2.8 mM L-ascorbic acid, 4% polyvinylpyrrolidon) for samples later stained for ADH, EST, GDH, LAP, PGM, SOD, and 6PGDH. The extracts were centrifuged for 10 min at 13,000 rpm

Fig. 1. – Floating leaves and inflorescence of *Potamogeton* ×*schreberi* from the river Saar at Hanweiler, Saarland, Germany, 21 VII 2001, photo by P. Wolff.

Fig. 2. – Phyllodial submerged leaves of a young shoot of *Potamogeton* ×*schreberi* from the river Blies at Bliesbruck, Dept. Moselle, Lorraine, France, 1 IX 1999, photo by P. Wolff.

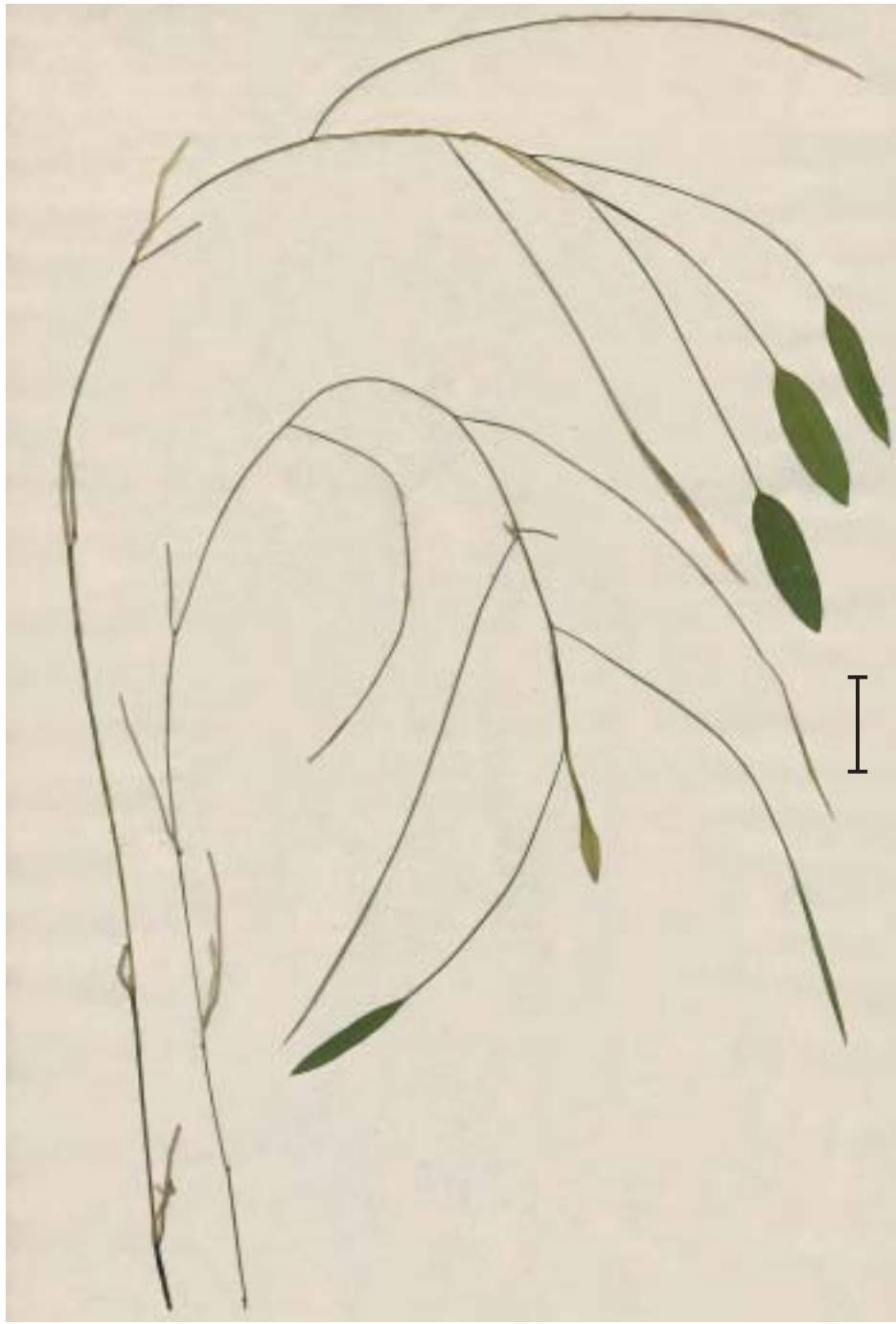

Fig. 3. – Shoots of *Potamogeton* ×*schreberi*, showing *P. natans*-like phyllodial lower submerged leaves, transitional upper submerged leaves and *P. nodosus*-like floating leaves; plants from Blies at Reinheim, Saarland, Germany, cultivated as Z. Kaplan C 1278; scale bar = 3 cm.

and clear supernatants were stored in Eppendorf tubes at –75 °C for up to 16 months until investigated in electrophoresis.

Electrophoresis was run on non-denaturing polyacrylamide gels in a Hoeffer vertical electrophoresis unit at 4 °C. The gels consisted of a separating (8% acrylamide, buffer of 1.82 M tris-HCl, pH 8.9) and stacking gel (4% acrylamide, buffer of 0.069 M tris-HCl, pH 6.9). The electrode buffer consisted of 0.02 M tris and 0.24 M glycine at a pH 8.3. All enzymes migrated anodally and visualized loci were numbered in order of decreasing anodal mobility.

The following eight enzymes were analysed: aspartate aminotransferase (AAT, EC 2.6.1.1), alcohol dehydrogenase (ADH, EC 1.1.1.1), esterase (EST, EC 3.1.1.-), glutamate dehydrogenase (GDH, EC 1.4.1.2), leucine aminopeptidase (LAP, EC 3.4.11.1), phosphoglucomutase (PGM, EC 2.7.5.1), superoxide dismutase (SOD, EC 1.15.1.1), 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44). The staining procedures to visualize ADH and 6PGDH followed Vallejos (1983), and Wendel & Weeden (1989) for PGM, EST, SOD, and GDH, with the following modifications: ADH (20 ml ethanol), 6PGDH (0.1 M tris-HCl pH 8.4, 30 mg 6-phosphogluconic acid), PGM (24 mg $MgCl_2$, 50 mg glucose-1-phosphate, 10 mg NADP), EST (Na-phosphate buffer pH 6.45; 25 mg ß-naphthylphosphate, 50 mg Fast Blue BB), SOD (0.05 M tris-HCl pH 8.2, 4.5 mg EDTA, 5 mg NBT).

Results

Morphological evaluation

Examination of an abundance of material of the putative hybrid from both the field (all three sites) and cultivation revealed variation in morphological features inconsistent with that of similar species, such as *P. natans*, *P. nodosus* or *P. polygonifolius*. The specimens with well preserved submerged leaves show some characteristics of *P. natans*, such as the presence of phyllodes near the base of the stem (Figs 2–4). Upper submerged leaves, if present and not decayed, resemble phyllodes but have a slightly expanded linear-elliptical lamina at the distal end, which have up to 9 lateral veins and the base tapers very gradually into the petiole. The shape is generally intermediate between the phyllodes of *P. natans* and the laminar leaves of a species with broad submerged leaves such as *P. nodosus* or *P. polygonifolius*. These submerged leaves are mostly not preserved on specimens collected later in the season (Fig. 5). Some plants have a few floating leaves with a slightly browner section of the petiole adjacent to the lamina, which may represent a vestige of the flexible junction present in *P. natans*. All floating leaves point in the same direction on the water surface, which is typical of *P. nodosus* and *P. polygonifolius*. In contrast, the floating leaves of *P. natans* growing in standing water usually form a circle on the water surface with their apices pointing outwards. Some plants have transitional leaves, intermediate in shape and size between submerged phyllodes and floating laminar leaves (Figs 3 & 4). On the other hand, the floating leaves most resemble those of *P. nodosus* (Fig. 1), which, in conjunction with the presence of phyllodial leaves, suggests *P. ×schreberi*.

The putative hybrid appears to be intermediate between *P. natans* and *P. nodosus*. However, the submerged leaves of some of the plants are not dissimilar from those of *P. ×sparganiifolius* and some weaker plants resembled *P. ×gessnacensis* or *P. ×fluitans*. The plants of *P. ×sparganiifolius* from running water differ in that the submerged leaves

have longer laminas and shorter petioles. The submerged leaves of *P.* ×*gessnacensis* are all phyllodial, without or with only very narrow lamina. On average, *P.* ×*gessnacensis* is a more slender than *P.* ×*schreberi*, with smaller floating leaves and submerged leaves with short petioles. *P.* ×*fluitans* differs in having stipules with two narrow wings along the length of the dorsal side, which is a feature inherited from one of its parents, *P. lucens*. In contrast, *P.* ×*schreberi* has only unwinged ridges. Both the submerged and floating leaves of *P.* ×*fluitans* have shorter petioles than those of *P.* ×*schreberi*. A selection of the most important diagnostic characters of these hybrids are summarized in Table 1.

Flowering plants of *P.* ×*schreberi* were present at all the sites: Reinheim, Bliesbruck and Hanweiler. However, there was no sign of fruit development. In cultivation, all the *P.* ×*schreberi* plants produced spikes and flowered each summer, but did not produce fruit. The development of flowers differed from that in the species. The tepals of the *P.* ×*schreberi* plants remained tightly closed and the stigmas protruded through them. The entire spikes died after flowering instead of setting fruit. This is typical of sterile hybrids (Preston 1995a:46, Preston 1995b, Preston et al. 1998). In contrast, in fertile species the tepals open to reveal the anthers and carpels.

The identification of underdeveloped or inadequately preserved specimens of *P.* ×*schreberi* could be misleading. The plants in Saar at Hanweiler in July 2001 had *P. nodosus*-like floating leaves whereas the submerged ones were at that time decayed and entirely disappeared. The plants were considered to be *P. nodosus*. However, a cultivated sample (1280) from this population produced new shoots with phyllodial leaves near the base of the stem and the upper submerged leaves expanded above the petiole into a very narrow lamina. Only then did it become apparent that the plant is not *P. nodosus* but perhaps the same putative hybrid as that observed in Blies. These observations were confirmed in the field the following year. The plants examined in June 2002, i.e. a month earlier than in the previous year, still had some typical phyllodes and phyllode-like submerged leaves, which disappeared within a month in Saar whereas some were still present on plants in Blies. This may be because of the higher water temperature in Saar resulted in faster decomposition of the submerged leaves.

Examination of stem anatomy

The examined samples of the putative hybrid had the following stem anatomy: stele is of trio type (Fig. 6); endodermis of U-type, with some cells of intermediate O-U-type, with rather indistinct thickening of cell walls; 1–2 circles of interlacunar bundles, of which the inner circle is incomplete, with scattered faint cells; scattered and weak subepidermal bundles; and 1 layer of pseudohypodermis, which is locally discontinuous for short stretches (Fig. 7).

The stele in the putative hybrid is in accordance particularly with *P. natans* and *P. nodosus*, or a hybrid of these (Table 2). The shape of the endodermal cells is as in *P. natans*, *P. lucens* or *P. gramineus*, i.e. U-type, with thick cell walls on the inner and lateral faces and a thin one next to the cortex. However, the thickening is rather indistinct, which indicates influence of a species with O-type endodermis, such as *P. nodosus* or *P. polygonifolius*, in which the walls of the endodermal cells are variously (sometimes strongly but sometimes almost not at all) but evenly thickened.

Interlacunar bundles arranged in 1–2 circles is typical of weakly growing individuals of *P. lucens* (Table 2). However, the identity of the studied samples with this species may be

Fig. 4. – Shoots of *Potamogeton* ×*schreberi* showing *P. natans*-like phyllodes near the base of the stem, transitional upper submerged leaves and *P. nodosus*-like floating leaves; plants from Blies at Bliesbruck, Dept. Moselle, Lorraine, France, 14 VII 2001, coll. P. Wolff s. n.; scale bar = 3 cm.

Fig. 5. – Adult flowering plants of *Potamogeton* ×*schreberi*, submerged leaves of which have decayed; sample from Blies at Reinheim, Saarland, Germany, cultivated as Z. Kaplan C 1278; scale bar = 3 cm.

easily excluded, particularly because of the capacity to produce floating leaves, which are always absent in *P. lucens*. The number and arrangement of the interlacunar bundles (numerous in the outer circle and scattered or almost absent in the inner one) may be a consequence of hybridization between a species with many bundles and one lacking them. Of the possible hybrid combinations, this intermediate pattern would be expected as a result of hybridization between *P. natans* or *P. lucens* and *P. nodosus* or *P. polygonifolius*. Similarly, the incomplete circle of weak subepidermal bundles is intermediate between that of several species pairs, mostly involving *P. nodosus*, although it must be admitted the pattern observed in *P. gramineus* is not very different.

The presence of one partly incomplete layer of pseudohypodermis is typical of *P. gramineus* and perhaps allowed also for *P. lucens* (Table 2). However, both these species may be excluded on morphological grounds. The observed pattern is more likely derived from the hybridization of a species with a well developed pseudohypodermis and one lacking this structure. In this case, *P. nodosus* must have been one of the parents. The other species could be *P. natans* or *P. polygonifolius*, but also *P. lucens* cannot be entirely excluded. Again, the involment of the last-named species is not supported by morphological data.

In summary, the anatomical observations support the identity of the putative hybrid as *P. ×schreberi*. The stem anatomy combines the characters of *P. natans* and *P. nodosus*. Although the hypothetic combination *P. lucens* × *P. nodosus* (which has never been proved to exist) could possibly also explain the anatomical structure it is excluded on morphological grounds.

Isozyme analysis

Gels were stained for 8 enzyme systems (AAT, ADH, EST, GDH, LAP, PGM, SOD, 6PGDH). PGM could not be interpreted because 6PGDH was unintentionally stained in the same place on the gels. The phenotypes of the enzyme GDH varied little between the taxa. Because of the variable staining of the bands between samples and the difficulty of reliable interpreting of phenotypes of this hexameric enzyme, the GDH data were not included in the analysis. The phenotypes of the remaining 6 enzyme systems were sufficiently variable and legible (Fig. 8).

The pattern of AAT (dimer) consisted of 3 visualized loci. *Aat-1* was highly variable in the visualized samples but the bands of about half of the individuals were insufficiently stained and not consistently scorable. Thus this locus was omitted from the analysis. The slower *Aat-2* and *Aat-3* were polymorphic, with 4 and 3 different isozyme phenotypes, respectively (Fig. 8). The slowest band of *Aat-2*, representing one homozygous allele, appeared solitary only in *P. nodosus* (phenotype A) and contributed to the banding set of the putative hybrid (phenotype B). Another homozygous band was observed in *P. natans*, *P. gramineus* and *P. lucens* (phenotype C) and also contributed to the banding pattern of the putative *P. ×schreberi*. This hybrid showed an additive profile, composed of three bands consistent with a heterozygote pattern (phenotype B). In addition, there was a novel band in the middle between the two bands observed in other species. This was interpreted as a heterodimer. The third, fastest homozygous band was observed only in *P. polygonifolius* (phenotype D). Of the three isozyme phenotypes of *Aat-3*, the fastest band of homozygous constitution (phenotype B) was present in some samples of *P. nodosus*, and in all of

P. natans and the putative *P.* ×*schreberi*. The slowest band appearing solitary (phenotype C) was found only in *P. polygonifolius*. The heterozygous phenotype consisting of three bands (phenotype A) was observed in some samples of *P. nodosus* and all of *P. gramineus* and *P. lucens*.

The unique banding pattern of *P. polygonifolius* for these isozymes excludes it as a potential parent of the hybrid as this species consistently has an allele absent in the hybrid. In contrast, *P. nodosus* is a likely parent as the slowest allele present in the hybrids is unique to this species. Either *P. natans*, *P. lucens* or *P. gramineus* may have been the other species, judging from the *Aat-2* pattern. However, the last two are excluded by *Aat-3*, because they consistently have two alleles not present in the hybrid. The combination *P. natans* × *P. nodosus* is the most likely explanation for the isozyme pattern observed in the hybrid.

Gels stained for ADH (dimer) show a single continuous zone of activity, which possibly represents two overlapping loci. Four isozyme phenotypes were detected (Fig. 8). In many samples there was a second band of secondary origin just above the homomeric bands. These secondary bands were excluded from the interpretation (and not shown in the figure). A homozygous phenotype of the single fastest allele (phenotype A) was present in *P. nodosus* and *P. gramineus*. Another homozygous phenotype, consisting of a single band (phenotype C), was present in *P. gramineus*, *P. natans* and *P. lucens*. The putative hybrid showed an additive "hybrid" profile, which consisted of the two bands observed in these species with an additional heterodimer band between them (phenotype B). A different triple-banded phenotype is unique to *P. polygonifolius* (phenotype D).

The ADH patterns support either *P. nodosus* or *P. gramineus* as one of the parental species, and either *P. natans*, *P. lucens* or *P. gramineus* as the other. Only *P. polygonifolius* can be excluded as it has two additional unique alleles.

EST (monomer or dimer) gave a very complicated pattern of bands of four colours (brown, violet, green, yellow) in three zones of activity consisting of a fully unresolved number of loci (at least 5 distinguished, 4 of them overlapping on the gels). Enzyme activity was variable both between and within samples. Most bands were insufficiently stained in the fastest zone (altogether up to 5–6 different bands observed in the visualized samples) and in the narrow slowest zone (consisting of ca 3 bands). Thus, only the middle zone with a total of 9 bands could be interpreted. This zone includes 10 phenotypes (A–J), each specific to a given taxon (Fig. 8). Two different phenotypes are present in *P. nodosus* and *P. natans*, three in *P. lucens*. The enzyme phenotypes of these species consisted of 2–4 bands, while those of the hybrid consisted of 7 bands (phenotype C) each found in some of the species.

The EST enzyme pattern of the putative hybrid has the additive profile consistent with the parentage of *P. nodosus* as one parent and *P. natans* as the other one. No other parental combination can explain the enzyme pattern of the hybrid. This excludes *P. gramineus* and *P. polygonifolius* from consideration, together with *P. lucens*, which consistently has an allele not observed in the hybrid.

Three zones of activity, representing three loci, were observed in gels stained for LAP (monomer), but only the middle one, designated as *Lap-2*, was adequately stained for interpretation (Fig. 8). Three allelic variants generating five isozyme phenotypes were observed at this locus. The homozygous phenotype of the slowest band (phenotype D) was unique to *P. polygonifolius* but the band is a subset of the double-banded pattern (phenotype E) seen in *P. lucens*. Other homozygotes were represented by the middle band (phe-

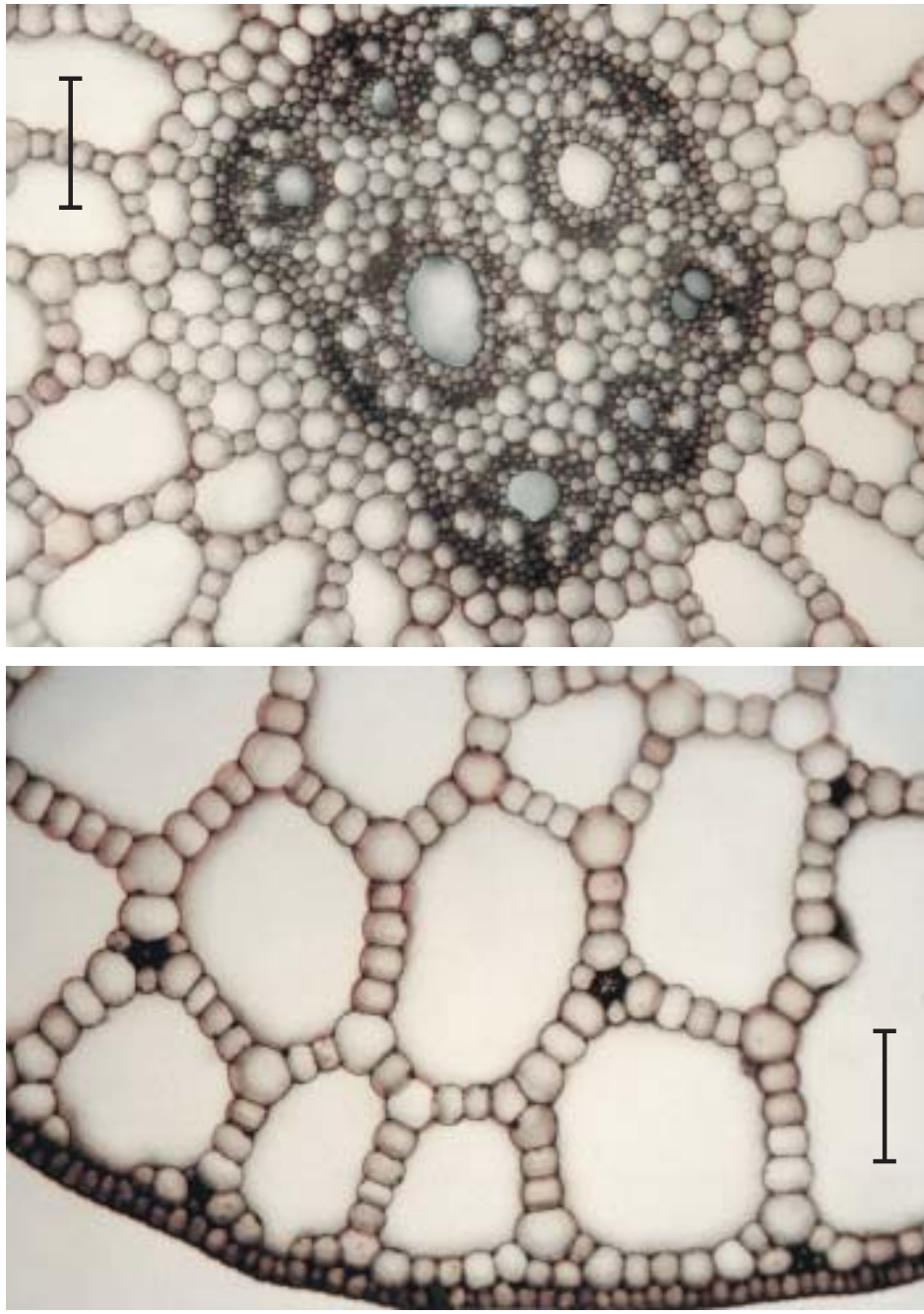

Fig. 6, 7. – Stem anatomy of *Potamogeton* ×*schreberi*: stele of trio type, endodermis of U-type, with rather indistinct thickening of cell walls (above), scattered interlacunar and subepidermal bundles, pseudohypodermis present in 1 layer, locally discontinuous (below), see text for details; sample from Blies at Reinheim, Saarland, Germany, cultivated as Z. Kaplan C 1278; scale bars = 100 µm.

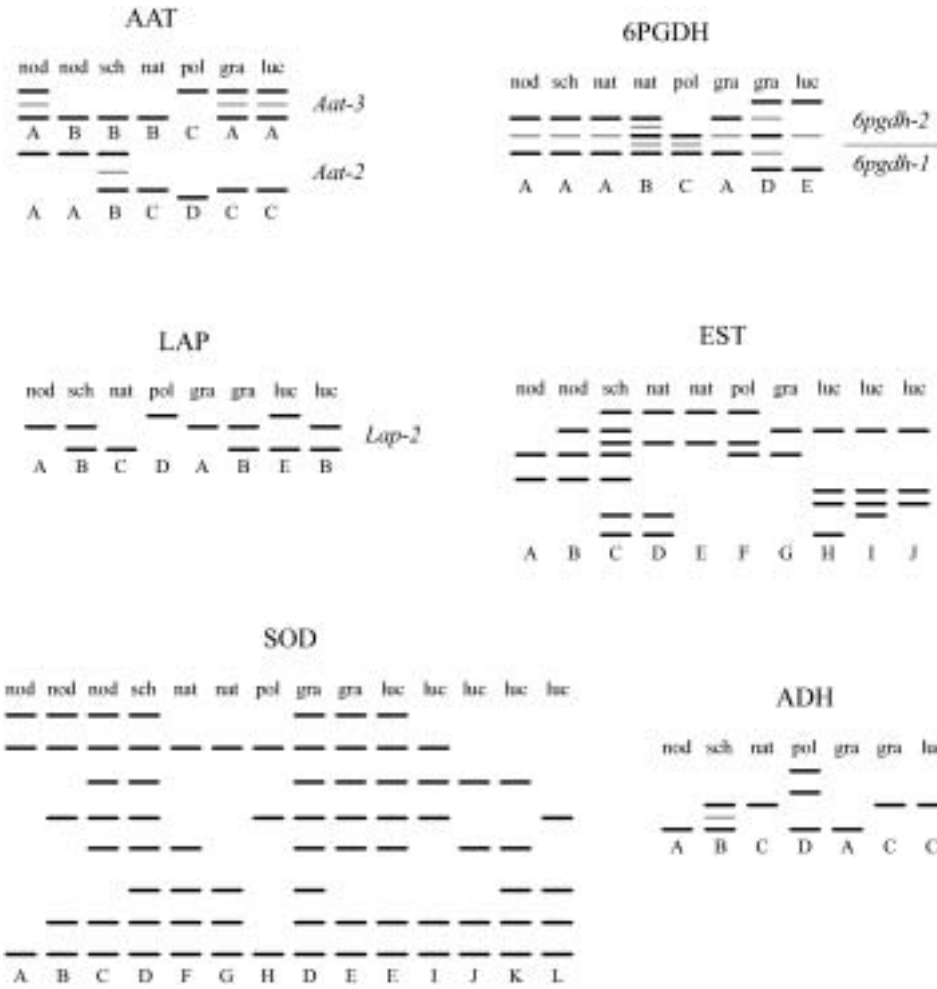

Fig. 8. – Enzyme phenotypes of AAT, ADH, LAP, EST, SOD and 6PGDH present in samples of *Potamogeton nodosus* (nod), *P.* ×*schreberi* (sch), *P. natans* (nat), *P. polygonifolius* (pol), *P. gramineus* (gra) and *P. lucens* (luc). All enzymes migrated anodally (towards the bottom of the figure). Size of the bands and distances between them within an enzyme system are printed in 72% of the actual size they were on the gel. In the dimeric systems AAT, ADH and 6PGDH, supposed mono- and homomeric bands are illustrated in black, bands corresponding to supposed heterodimers are given in grey. The secondary bands that appeared in some samples at ADH and SOD are not shown. Alphabetical codes below the banding patterns denote the different locus or enzyme phenotypes of polymorphic enzymes.

notype A, in *P. nodosus* and *P. gramineus*) and the fastest band (phenotype C, in *P. natans*). Heterozygous phenotype composed of these alleles possessed a double-banded isozyme profile (phenotype B) in *P. gramineus*, *P. lucens* and the putative hybrid.

Several parental combinations could have resulted in the *Lap-2* patterns, including *P. natans* × *P. nodosus*, *P. natans* × *P. lucens* and *P. natans* × *P. gramineus*. The enzyme phenotype of the putative hybrid also occurs in some plants of *P. lucens* and *P. gramineus*.

However, these species may be easily excluded on morphological grounds. Only *P. polygonifolius* is entirely excluded from the consideration, as it has completely different bands than the putative hybrid.

A single more or less continuous zone of activity was revealed for SOD (dimer or tetramer) (Fig. 8). Though it probably consisted of three loci, they were difficult to separate and the area of activity was therefore interpreted as one complex locus with a total of 8 bands. Between the third and the sixth homomeric bands of the complete banding pattern there were several additional bands in some samples of *P. natans*, which were interpreted as secondary bands (possibly heterotetramers) and omitted from further consideration. Twelve isozyme phenotypes were observed. Most of them were species-specific, with only phenotype E common to some samples of *P. gramineus* and *P. lucens*, and the "full" enzyme profile, designated as phenotype D, to some samples of *P. gramineus* and all of the putative *P. ×schreberi*.

The enzyme patterns of SOD were very similar in the species. No species-specific alleles, which would unambiguously identify the parents of the hybrid, were found. The slowest band of phenotypes A, B, C, D and E was the most informative (Fig. 8). Besides the putative hybrid, this band was present in *P. nodosus*, *P. gramineus* and one sample of *P. lucens*. Thus, one of these species must have been involved in the hybridization. There are several parental combinations that would explain the pattern observed in the putative *P. ×schreberi* (phenotype D), including a cross between phenotype C of *P. nodosus* and phenotype F of *P. natans*.

Two loci were detected in 6PGDH (dimer), which formed intergenic heterodimers giving rise to multibanded isozyme patterns (Fig. 8). Altogether 7 bands gave 5 different phenotypes. Three of them were homozygous in both loci: phenotype A, found in *P. nodosus*, *P. natans*, *P. gramineus* and the supposed *P. ×schreberi*, phenotype C, unique to *P. polygonifolius*, and phenotype E, in samples of *P. lucens*. The remaining enzyme phenotypes B and D were heterozygous at least for one of the loci. The heterozygotes were present samples of *P. natans* and *P. gramineus*.

The 6PGDH banding pattern of the putative *P. ×schreberi* is the same as that observed in *P. nodosus* and some samples of *P. natans* and *P. gramineus*. Thus, any of these species may be the parental species of the hybrid. In contrast, the enzyme profiles of *P. polygonifolius* and *P. lucens* exclude them as parents of the hybrid plants.

Discussion

Each of the methods indicate that the putative hybrid resulted from a cross between *P. natans* and *P. nodosus*. Although some less well developed specimens could not be reliably determined, a detailed morphological examination of an extensive collection of plant material confirmed that the putative hybrid is clearly intermediate between *P. natans* and *P. nodosus*. The stem anatomy has characteristics of these two species. The enzyme data confirmed the morphology- and anatomy-based determination of the putative hybrid as *P. ×schreberi*. All isozyme phenotypes of the hybrid could have been inherited from *P. natans* and *P. nodosus*. The hybrid has no unique allele and has an additive "hybrid" isozyme pattern for those loci where the parental alleles differ. The involvement of *P. nodosus* is particularly well supported since it is the only species that could have con-

tributed the unique *Aat-2* and EST alleles to the hybrid. In addition to *P. nodosus*, only *P. natans* could have been the other parent and so maintain the homozygous single-banded phenotype of *Aat-3*. In contrast, the involvement of other species in the hybrid was excluded: *P. polygonifolius* by all the systems, *P. gramineus* by *Aat-3* and EST, and *P. lucens* by *Aat-3*, EST and 6PGDH. In addition, the additive "hybrid" banding patterns, particularly at *Aat-2*, ADH and EST, exclude the possibility that the studied plants were aberrant forms of one of the parental species rather than a hybrid.

Even though within-species enzyme variation was detected in both *P. natans* (in EST, SOD, 6PGDH) and *P. nodosus* (in AAT, EST, SOD), all the four populations of *P.* ×*schreberi* from both Blies and Saar had the same invariable multienzyme phenotype. This suggests that all colonies of this hybrid in the area are the progeny of a single hybridization event. As described above, the hybrid appears to be sterile. Since pondweeds are generally well adapted to vegetative propagation, the present-day distribution of *P.* ×*schreberi* along the Saarland/Moselle border was probably achieved by means of long-distance dispersal of stem and rhizome fragments. These are easily detached and transported by rivers, particularly during spring floods with rapid water flow. The new colonies persist by the clonal spread of rhizomes. As none of the parental species were found with *P.* ×*schreberi* or upstream in the river Blies for at least the past several decades, the hybrid seems to have been established there a long time. The occurrence of a *Potamogeton* hybrid in the absence of one or both its parents is repeatedly documented (e.g. Hollingsworth et al. 1996b, Preston & Chater 1997, Preston et al. 1998, King et al. 2001).

Besides the present study, *P.* ×*schreberi* is enzymatically confirmed for Great Britain (Hollingsworth et al. 1995b). The British plants of this hybrid also seemed to be uniform. Only a single multi-enzyme phenotype of *P.* ×*schreberi* was detected over a 4 km stretch of a river, indicating that it was most likely a single clone that had spread vegetatively. Similarly, neither of its parents or any other broad-leaved *Potamogeton* species were found by the British botanists with *P.* ×*schreberi*, although *P. nodosus* was locally frequent downstream.

The hybrid is recorded from Great Britain, Germany, Switzerland and the central part of European Russia (Fischer 1905, Fischer 1907, Koch 1933, Koch 1934, Preston 1995a, b, Bobrov & Reshetnikova 2002). The records for Germany are reviewed here:

B a v a r i a : Brook Seebach near Möhrendorf (Fischer 1907). Brook Vils near Hahnbach (Fischer 1907, Preston 1995b). Brook Vils between Neumühle and Amberg (Preston 1995b). Brook Vils between Vilseck and Amberg (Sonntag et al. 2000, Kohler et al. 2003). Brook Zusam near Donauwörth (Fischer 1907). – Hessen: Brook Nidda near Ilbenstadt and Assenheim; Brook Wetter near Ossenheim, Bauernheim, Dorheim and Schwalheim (Ludwig 1966). – Rhineland-Palatinate: River Lahn between Bad Ems and Lahnstein (Weyer 1997).

The hybrid has not been recently confirmed at most of the cited sites in Germany and it is very probably extinct there. The colonies we studied possibly represent the few extant sites of *P.* ×*schreberi* in Central Europe.

The hybrid has never been previously reported from France. Our find from Bliesbruck is therefore the first record for this country. So far, *P.* ×*schreberi* has not been reported from the German state Saarland.

Besides the compelling evidence for the identity of *P.* ×*schreberi*, this study also illustrates why it is difficult to identify a member of the "*P. fluitans*"-complex when only morphological data from a single sample are available. External factors such as the time of collection of plant material and abiotic factors, such as temperature and nutrient conditions,

affect identification. The typical phyllodes and phyllode-like submerged leaves of *P. ×schreberi* disappear early in the season (Fig. 5). As a consequence, a key diagnostic feature of the hybrid is absent and the plant may be misidentified as *P. nodosus*. Similar phenotypic plasticity obscuring the identity of another *P. natans* hybrid, *P. ×fluitans*, was described by Kaplan (2002b). In the case of the population of *P. ×schreberi* in Saar, the most important plant structures for determination, i.e. inflorescences and submerged leaves, are mostly not present at the same time. This stresses the necessity to repeatedly examine putative hybrid plants, either in the field or cultivation.

The correct determination of herbarium material is therefore very limited. According to our experience, herbaria often contain material that possibly belongs to hybrids or otherwise noteworthy plants. However, their exact identity cannot be established because of insufficiently developed or inadequately preserved important plant structures. This clearly illustrates the importance of collecting rich and well developed voucher collections whenever possible and their careful preparation.

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Souhrn

Taxonomicky mimořádně složitý komplex zahrnující druh *Potamogeton nodosus* a několik morfologicky velmi podobných kříženců druhu *P. natans* patří mezi nejobtížnější skupiny rodu. Tři makrolokality předpokládaného křížence z této skupiny byly nedávno nalezeny ve dvou řekách (Blies, Saar) v pohraniční oblasti německého Sárska a v přilehlém lotrinském departmentu Moselle ve Francii. K identifikaci rostlin byla použita kombinace tří metod. Detailní analýza morfologických znaků bohatého materiálu z terénu i z kultivací prokázala intermediární postavení mezi druhy *P. natans* a *P. nodosus*. Struktura uspořádání znaků v anatomii lodyhy předpokládaného křížence odpovídá kombinaci znaků obou druhů. Nejpřesvědčivější důkazy o hybridním původu rostlin však přinesla analýza isozymů. Isozymová spektra křížence kombinují znakové sady druhů *P. natans* a *P. nodosus*. Uspořádání proužků enzymů elektroforeticky separovaných na gelech dále vylučuje, že by nalezené rostliny mohly patřit k některému jinému morfologicky podobnému taxonu z komplexu okolo druhu *P. nodosus*. Přestože jistá variabilita byla detekována mezi vzorky obou rodičovských druhů, všechny studované rostliny křížence patřily k jedinému genotypu. Tato uniformita naznačuje, že rostliny ze všech tří makrolokalit jsou pravděpodobně produkty jediné hybridizace, která se uskutečnila v minulosti. Studovaný kříženec je zcela sterilní. Přežívání a šíření jeho rostlin je podmíněno klonálním růstem a vegetativním rozšiřováním na nové vhodné lokality. *P. ×schreberi* patří mezi nejvzácnější křížence rdestů. Existují údaje o jeho výskytu ve Velké Británii, Německu, Švýcarsku, Rusku a nyní nově i Francii. Většina údajů však patří minulosti.

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