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Genetic variation within and between populations of *Potamogeton pusillus* agg.

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Abstract. Patterns of isozyme variation were examined in 17 populations of *P. pusillus* and *P. berchtoldii*, together with one population of *P. trichoides* taken for comparison. Both *P. pusillus* and *P. berchtoldii* displayed low levels of variation within populations associated with high levels of interpopulation differentiation. This pattern of partitioning of genetic variation within and between populations is attributed to the founder effect, frequent vegetative propagation by turions, dominant self-fertilization and limited seedling recruitment. The mechanism of pollen transfer was investigated in cultivation. Effective pollination takes place in air above the water surface (autogamy, geitonogamy, anemogamy), on the water surface (epihydrogamy) or below water surface (hydroautogamy). The species are self-compatible. The low level of infra-population variation together with rare occurrence of heterozygotes suggest that selfing is the most frequent mode of pollination, although the protogynous flowers may occasionally permit some cross-pollination. Unique enzyme markers were found for *P. pusillus* and *P. berchtoldii*, and also for the single population of *P. trichoides*. All multienzyme phenotypes were species-specific. Isozyme data support the separate position of *P. pusillus* and *P. berchtoldii*. UPGMA dendogram based on enzyme data of 133 plant samples revealed three distinct main enzymatic entities perfectly corresponding to the three morphologically defined species.

Key words: *Potamogeton pusillus*, *Potamogeton berchtoldii*, isozymes, genetic variation, population structure, reproductive systems, clonal growth.

The *Potamogeton pusillus* agg.[1] belongs among the taxonomically most difficult groups in the family Potamogetonaceae. The complex is almost cosmopolitan in its distribution; the only continent from which it is absent is Australia. All morphotypes within this group were by earlier authors referred to as a single species, sometimes divided into a few varieties. The first attempt to arrange all variation in this group in Central Europe and to describe it in terms of formal classification appeared in the Central-European Flora by Ascherson and Graebner (1897) who distinguished five infraspecific taxa. Soon after, Hagström (1901) separated two species, *P. pusillus* (nowadays called *P. berchtoldii* Fieber) and *P. panormitanus* Biv. (true *P. pusillus* L., see Dandy and Taylor 1938, 1940). Fischer (1907) followed Hagström in treating two distinct species and subdivided Bavarian plants into 23 and eight infraspecific taxa, respectively, at the variety

[1] In this paper, *Potamogeton pusillus* agg. is understood as comprising two species in Europe, namely *P. pusillus* L. s. str. (syn. *P. panormitanus* Biv.) and *P. berchtoldii* Fieber.

and form levels. In his worldwide account, Graebner (1907) lumped the two species into *P. pusillus* and recognized 14 units mostly at variety level within it, besides 3-4 closely related South American species. Both species were subdivided into several forms and varieties also by Hagström (1916) in his monograph. Besides these two basic units, he described six additional similar species from this complex mostly from Africa. In a revision of North American linear-leaved *Potamogeton* species, Fernald (1932) adopted both species with several varieties. The concept of many infraspecific taxa has been gradually abandoned in the literature, with the notable exception of Soó (1936, 1938) who proposed many new combinations of highly dubious value. At present, both main taxa are generally recognized as separate species in Eurasia (e.g. Dandy 1980, Kašina 1988, Preston 1995) or either as varieties (Haynes 1974, Hellquist and Crow 1980) or recently as subspecies (Haynes and Hellquist 1996, Haynes and Hellquist 2000) in North America. At the worldwide scale, satisfactory taxonomic solution for this complex is still not available. In a recent global account of *Potamogeton* species (Wiegleb and Kaplan 1998), a broad concept of *P. pusillus* had to be temporarily adopted again to cover the extremely rich mosaic of widespread phenotypes, local taxa and extreme forms of *P. pusillus* agg.

In spite of the fact that the extreme morphological variation in *P. pusillus* agg. has been observed by many authors, its origin has little been studied systematically. Variation due to seasonal development was noted by Haynes (1974) who reported differences in development of lacunae and shape of leaf apex between plants collected in early summer and those collected later in the season. Contribution of variation due to environment has recently been investigated by Kaplan (2002), who observed considerable phenotypic plasticity in general appearance, branching pattern, width and colour of leaves, details of venation, and shape of leaf apex.

The main aim of this study is to provide data on isozyme variation in *P. pusillus* and *P. berchtoldii* at three levels: (1) genetic variation within populations in order to estimate prevailing modes of reproduction, (2) genetic variation between populations within each species to reveal possible contribution of different genotypes to observed morphological differences, and (3) inter-specific differences to test the distinction of *P. pusillus* and *P. berchtoldii* at species level.

Material a methods

Plant material. *Potamogeton pusillus* and *P. berchtoldii* are morphologically very similar. In fact, most recent guides to identification stress only a single morphological feature: structure of stipules. These are closed and tubular when young in *P. pusillus* but open and convolute in *P. berchtoldii*. Hence in this study, sampled plant material of *P. pusillus* agg. was divided into two groups solely according to the stipule shape. Besides similar morphology, both species also share many basic biological features. The two species are largely sympatric in Eurasia and North America, but only *P. pusillus* occurs in most of Africa. The exact position of South American plants that belong to this complex is still insufficiently known. *P. trichoides* Cham. et Schltdl. is a species related to *P. pusillus* agg. as delimited here, which is, however, taxonomically well defined and usually morphologically well distinguishable particularly when fertile material is available (Preston 1995, Wiegleb and Kaplan 1998). A single population of this species was also included in analyses for interspecific comparison. All three species are considered to be diploids with a chromosome number $2n = 26$ (cf. Hollingsworth et al. 1998). Nine chromosome counts have also been determined in plants included in this study, five in *P. pusillus* s. str. and four in *P. berchtoldii*, all with chromosome number $2n = 26$ (Jarolímová and Kaplan, unpubl.).

Plants for cultivation and analyses were sampled from sites in Central Europe in 1997–2000. The origin of plants used for isozyme analysis is summarized in Table 1. The reference population numbers correspond to numbers of cultivated material and numbers of vouchers; herbarium specimens of individuals from the populations were designated as their fractions (e.g. 307/1, 307/2 etc.). Voucher herbarium specimens from both field and

Table 1. Localities of samples used for isozyme analysis, with population designations used in the text and reference population numbers corresponding to numbers of cultivated material and numbers of voucher herbarium material

Species	Population	No.	Locality
P. pusillus	Untersee	990	Switzerland: Distr. Thurgau: S margin of the Untersee (lake) 1 km ESE of Ermatingen village, 395 m a. s. l., 23 VI 1998
	Bodensee I	987 (P)	Austria: Vorarlberg: Distr. Bregenz: inlet at S margin of Fussacher Bucht (bay) of Bodensee Lake 1.5 km N–NNE of Fußach village, near Höchst, 396 m a. s. l., 23 VI 1998
	Litoměřice	959	Czech Republic: Bohemia: Distr. Litoměřice: small pond in valley at N margin of Litoměřice-Pokratice town suburb, 260 m a. s. l., 27 V 1998
	Rokytňany	1133	Czech Republic: Bohemia: Distr. Mladá Boleslav: pond 0.4 km W of Dolní Rokytňany village, 234 m a. s. l., 11 VI 1999
	Dubovec	1212	Czech Republic: Bohemia: Distr. Jindřichův Hradec: Velký Dubovec pond 1.3 km SSE of Lomnice nad Lužnicí village, 425 m a. s. l., 15 VI 2000
	Panenský	1159	Czech Republic: Bohemia: Distr. Jindřichův Hradec: Velký Panenský fishpond 1.3 km SSE of Lomnice nad Lužnicí village, 420 m a. s. l., 8 IX 1999
	Stružky	1213	Czech Republic: Bohemia: Distr. Jindřichův Hradec: Stružky fishpond 0.8 km W of Břilice village, 445 m a. s. l., 15 VI 2000
	Šimanov	307	Czech Republic: Bohemia: Distr. Jindřichův Hradec: Šimanov pond (above Thierov pond) near Třeboň, 1.4 km NE of Třeboň railway station, 428 m a. s. l., 21 VIII 1996
P. berchtoldii	Thuner See	991	Switzerland: Distr. Bern: Interlaken: small pool on E bank of the Thuner See (lake) W of Unterseen town, 560 m a. s. l., 21 VI 1998
	Bodensee II	987 (B)	Austria: Vorarlberg: Distr. Bregenz: inlet at S margin of Fussacher Bucht (bay) of Bodensee Lake 1.5 km N–NNE of Fußach village, near Höchst, 396 m a. s. l., 23 VI 1998
	Skalná I	1147	Czech Republic: Bohemia: Distr. Cheb: pool in bottom of disused clay-pit called Suchá 1.8 km E–ESE of Skalná, 430 m a. s. l., 29 VII 1999
	Skalná II	1224	Czech Republic: Bohemia: Distr. Cheb: pool in bottom of disused clay-pit near Zelená settlement, 0.8 km SE of Skalná, 440 m a. s. l., 10. VIII. 2000
	Chvojno	1219	Czech Republic: Bohemia: Distr. Pardubice: forest ditch with slowly running water 2.8 km NE of Vysoké Chvojno village, 269 m a. s. l., 31 VII 2000
	Týniště	927	Czech Republic: Bohemia: Distr. Rychnov nad Kněžnou: drainage ditch with slowly running water in forest sand-pit 1.3 km E of Týniště nad Orlicí, 260 m a. s. l., 5 IX 1997
	Litice	930	Czech Republic: Bohemia: Distr. Ústí nad Orlicí: pool in the bottom of disused granite quarry by N margin of Litice nad Orlicí village, 460 m a. s. l., 11 IX 1997
	Žamberk	910	Czech Republic: Bohemia: Distr. Ústí nad Orlicí: fishpond in castle park at SE margin of Žamberk town, 413 m a. s. l., 14 IX 1997

Table 1 (continued)

Species	Population	No.	Locality
	Chropyně	1160	Czech Republic: Moravia: Distr. Přerov: ditch with standing water in meadows at WNW margin of Chropyně village, 194 m a. s. l., 14 IX 1999
P. trichoides	Lomnice	1157	Czech Republic: Bohemia: Distr. Jindřichův Hradec: Velký Panenský fishpond 1.3 km SSE of Lomnice nad Lužnicí village, 420 m a. s. l., 8 IX 1999

cultivation are preserved in the herbarium of the Institute of Botany, Průhonice (PRA).

A population selected for sampling for study of genetic variation within populations had to fulfil the following criteria: (1) water habitat is not too old (either a relatively new one such as a clay pit or a small pond seasonally drawdown recently); (2) the water body does not lie on the main local migration routes of waterfowl; (3) individuals in the population flower freely and produce abundant fruits; and (4) the population is well established. The requirements 1 and 2 should have increased the chance that the variation studied reflects breeding behaviour of individuals within the population rather than complicated history of repeated colonization of propagules from various sources. The third requirement was applied to avoid sampling of vegetatively persisting clones. The last requirement should have excluded too young populations that had no chance to build up within-population variation. Material for analyses was collected at least 2 m between each plant, if possible, in order to avoid collecting from a single shoot system. Ten to fifteen samples were collected from each population, with exception of population Thuner See where only seven samples could be collected without sampling twice from one clump. Additional material was collected from water bodies that had not fulfilled the requirements described above. However, these samples were used only for comparison of variation between populations. Only a few individuals were generally taken from each of these populations.

Cultivation. Plants of *P. pusillus* agg. were cultivated for observations of reproductive behaviour and for isozyme analyses in the experimental garden at the Institute of Botany, Průhonice, Czech Republic, in 1995-2001. Altogether, samples of 11 populations of *P. berchtoldii*, nine populations of *P. pusillus*, and six populations of *P. trichoides* were grown. Plants were cultivated in plastic tanks of two sizes ($180 \times 140 \times 80$ and $200 \times 120 \times 35$ cm) filled with water and sunk in the ground in order to prevent overheating of water in summer. Each sample was planted in a plastic pot with pond mud after desiccation treatment and submerged in a cultivation tank.

Electrophoresis. Leaf material was collected from cultivation early in the morning in summers 1999 and 2000 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, ADH, EST, and PGI; 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 AAT, ADH, EST, GDH, LAP, PGI, PGM, SOD, and 6PGDH. The extracts were centrifuged for 10 min at 13,000 rpm and clear supernatants were stored at −75 °C for up to 16 months until investigated in electrophoresis.

Electrophoresis on non-denaturing polyacrylamide gels was run in a Hoeffer vertical electrophoresis unit at 4 °C. The gels consisted of separating gel (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, pH 8.3.

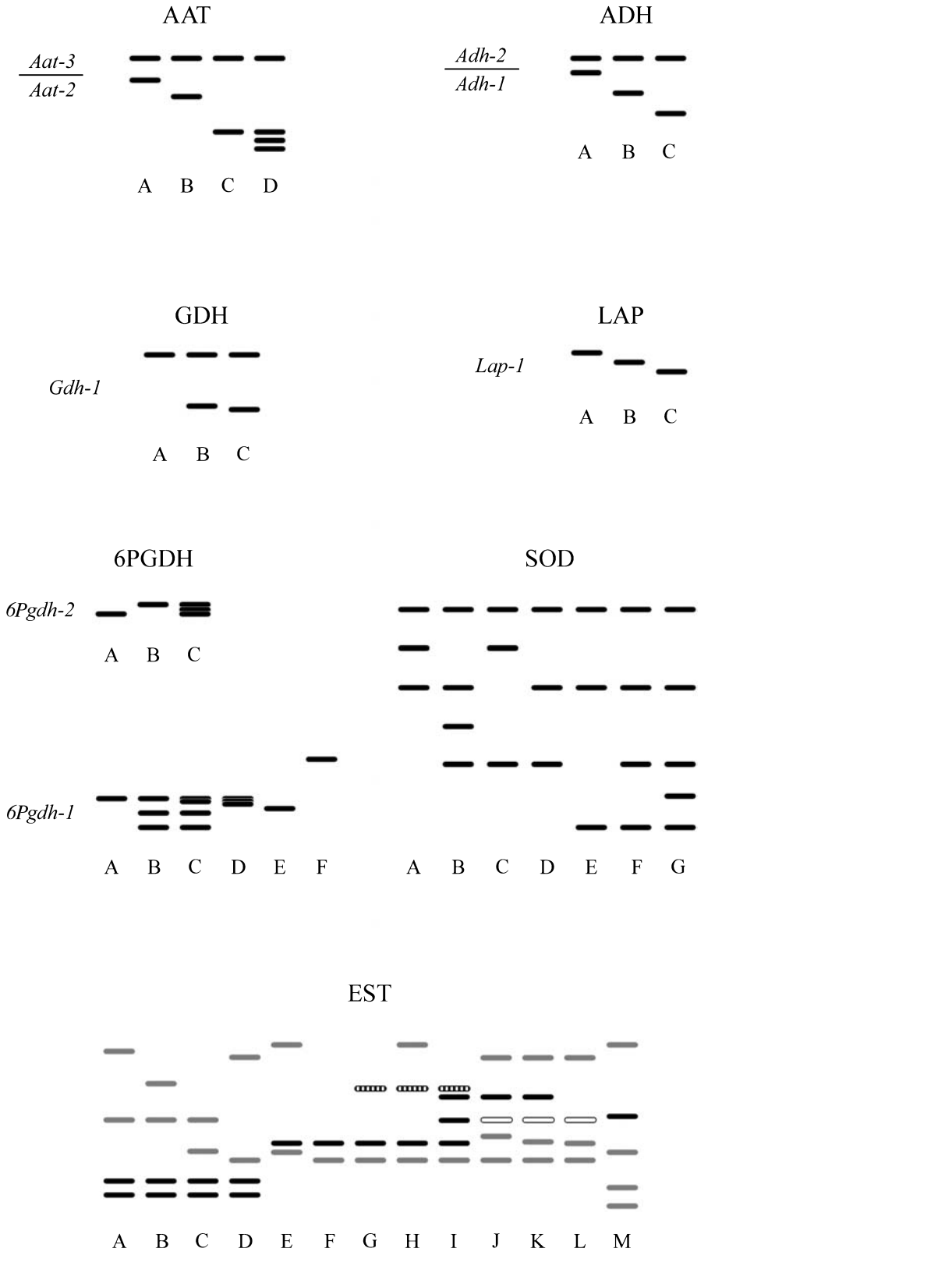
The following nine enzymes were analyzed: aspartate aminotransferase (AAT, EC 2.6.1.1), alcohol dehydrogenase (ADH, EC 1.1.1.1), esterase (EST, EC 3.1.1.-), glutamate dehydrogenase (GDH, EC 1.4.1.2), leucine aminopeptidase (LAP, EC 3.4.11.1), phosphoglucoisomerase (PGI, EC 5.3.1.9), phosphoglucomutase (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 followed Vallejos (1983) to visualize ADH and 6PGDH, and Wendel and Weeden (1989) for PGM, PGI, 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), PGI (10 mg NADP, 24 mg $MgCl_2$), EST (Na-phosphate buffer pH 6.45; 25 mg β-naphthylphosphate, 50 mg Fast Blue BB), SOD (0.05 M tris-HCl pH 8.2, 4.5 mg EDTA, 5 mg NBT).

Enzyme systems AAT and LAP were stained using the following methods. Two staining solutions were prepared for AAT: A (20 ml 0.1 M tris-HCl pH 8.4, 240 mg aspartic acid, 40 mg α-ketoglutaric acid) and B (20 ml 0.1 M tris-HCl pH 8.4, 50 mg Fast Blue BB Salt, 50 mg Fast Violet B). The solution A was prepared at least 15 min before the application. Gel was rinsed in water and then in buffer tris-HCl pH 7. Solutions A and B were mixed and poured on the gel. Gel was incubated in the dark at 32 °C until bands appeared, and then it was rinsed and fixed (1:1:3:5, glycerine, acetic acid, H_2O, methanol). The gel stained for LAP was rinsed in buffer 60 ml 0.2 M tris-maleate pH 6 and incubated 10 min with 40 mg L-leucyl-β-naphthylamide-HCl in 50% acetone and 60 mg $MgCl_2$ (both dissolved in 30 ml buffer). Afterwards solution of 25 mg Fast Black K Salt in 30 ml buffer was added and gel was incubated in the dark at 32 °C until bands appeared.

Data analysis. The data set resulting from the isozyme electrophoresis was analyzed for interspecific relationships by unweighted pair-group method using arithmetic averages (UPGMA) clustering 28 population-multienzyme phenotype units (see Table 3), where alleles were taken for characters and their absence/presence coded as character binary states. All homomeric and monomeric bands showed in Fig. 1 were scored for the UPGMA analysis. Simple Matching and Jaccard coefficients were applied, both used as similarity or distance. The different coefficients gave more or less identical results and only the analysis based on Simple Matching is therefore shown.

Results

Enzyme analysis. A total of 133 plants from Central Europe was analyzed using isozyme electrophoresis. Gels were stained for 9 enzyme systems (AAT, ADH, EST, GDH, LAP, PGI, PGM, SOD, 6PGDH). PGI and PGM could not be interpreted because 6PGDH was stained in the same place on the gels. Two other isozyme systems (EST, SOD) showed patterns too complex for analysis. Their individual loci could not be determined reliably without additional tests of determination of the actual genetic basis of each. Hence the electrophoretic profiles were used only for comparison of polymorphism on the level of isozyme phenotypes, but were not scored for allele frequencies because it was not possible to infer reliably which constitution was heterozygous. The remaining 5 enzyme systems showed 9 isozyme loci. Among them, *Aat-1* could not


AAT
Aat-3
Aat-2
A B C D
ADH
Adh-2
Adh-1
A B C
GDH
Gdh-1
A B C
LAP
Lap-1
A B C
6PGDH
6Pgdh-2
6Pgdh-1
A B C D E F
SOD
A B C D E F G
EST
A B C D E F G H I J K L M

be analyzed because of low enzyme activity in some samples, *Aat-3* and *Adh-2* gave no variation for the material tested. Observed isozyme phenotypes of the 8 isozyme loci that displayed interpretable activity and legible bands (*Aat-2*, *Aat-3*, *Adh-1*, *Adh-2*, *Gdh-1*, *Lap-1*, *6Pgdh-1*, *6Pgdh-2*), together with the 2 enzymes systems difficult to interpret in terms of loci (EST, SOD), are given in Fig. 1.

Variation at individual enzyme systems and loci. AAT gave patterns consisting with 3 loci, of which *Aat-1* was insufficiently stained and could not be scored in all individuals. The slowest products *Aat-3* produced a single invariant phenotype of one band representing one homozygous allele. Altogether 5 bands appeared at *Aat-2* that gave 4 different phenotypes (Fig. 1). The *P. berchtoldii* plants showed phenotypes A and B; A was unique to this species, B was shared with *P. trichoides*. The middle band of *Aat-2* was diagnostic for *P. pusillus* and appeared in all samples of this species. Most populations of this species had homozygous phenotype C, the heterozygous phenotype D appeared in samples of only one population. *P. pusillus* and *P. berchtoldii* shared no band and therefore no enzyme phenotype at *Aat-2* (Table 2).

The pattern of ADH consisted of two loci. *Adh-2* showed a homozygous phenotype of a single band in all studied samples. Additional faint secondary bands sometimes appeared above the single band. The faster locus *Adh-1* was variable. Three different phenotypes were observed, each of a single band (Fig. 1). All plants of *P. pusillus* and of *P. trichoides* shared phenotype B. The *P. berchtoldii* plants had mostly phenotypes C, but one plant with phenotype A was detected in a variable population. *P. pusillus* and *P. berchtoldii* were consistently different in this locus (Table 2).

The gel stained for GDH displayed one locus with three phenotypes (Fig. 1). The most common phenotype of *Gdh-1*, designated A, consisted of a single band and was detected in all three studied species. Each of the lower two bands was unique to one species: that of phenotype B to *P. berchtoldii*; that of phenotype C to *P. pusillus*. Up to five additional faint bands appeared between the two bands of phenotypes B and C. These had probably risen due to post-translation modification of a minority portion of molecules of the subunit. The number of these faint heteromeric bands indicated that GDH was hexameric in the studied plants.

The single displayed locus of LAP gave three different phenotypes, each homozygous of one allele. A "second" band of secondary origin appeared closely above the "main" one in all lines. Phenotype B with the band in the middle position was most frequent and was found in all three species. Phenotype A with the slowest band was unique to three populations of *P. berchtoldii*, whereas phenotype C appeared only in three populations of *P. pusillus*.

Two loci were detected in 6PGDH. Three phenotypes were found in the slower area of activity with locus *6Pgdh-2*. Two of them, A and B, were homozygous, each consisting of a single band that presented a subset of the pattern seen in phenotype C (Fig. 1). The phenotype A was most frequent in all three species. The phenotype B was confined to one population of *P. pusillus*, whereas the heterozygous phenotype C was unique to one plant of *P. berchtoldii*. In *6Pgdh-1*, altogether seven

Fig. 1. Interpreted enzyme phenotypes of AAT, ADH, GDH, LAP, 6PGDH, EST and SOD observed in samples of *P. pusillus*, *P. berchtoldii* and *P. trichoides*. All supposed secondary bands are omitted. Size of bands and distances between them within an enzyme system (or each locus of 6PGDH) are printed in 100% of actual size as the appeared on the gel. Alphabetical codes below the banding patterns denote the different locus or enzyme phenotypes of polymorphic enzymes. At AAT and ADH, these codes refer to phenotypes of loci *Aat-2* and *Adh-1*, respectively. Black, shaded, striped and empty bands in EST indicate brown, violet, green and yellow bands on gels, respectively

Table 2. Occurrence of locus/enzyme phenotypes in plant material studied, with number of analyzed plants from each population (N), number of plants with particular multienzyme phenotype among the plants studied (n), and code of multienzyme phenotype (MEP). Each multienzyme phenotype is defined by the unique combination of the eight locus/enzyme phenotypes

Species	Population	N	n	MEP	Aat-2	Gdh-1	Adh-1	Lap-1	6Pgdh-2	6Pgdh-1	SOD	EST
P. pusillus	Untersee	3	2	U	C	A	B	C	A	F	D	A
			1	X	C	C	B	C	A	F	B	A
	Bodensee I	3	3	V	C	C	B	B	A	F	D	C
	Litoměřice	12	12	Y	C	C	B	C	A	F	D	C
	Rokytňany	1	1	T	C	A	B	B	A	F	D	C
	Dubovec	10	7	Q	C	A	B	B	A	F	B	C
			2	R	C	A	B	B	A	F	C	C
			1	P	C	A	B	B	A	F	A	B
	Panenský	10	10	Z	D	A	B	B	A	F	B	D
	Stružky	10	6	T	C	A	B	B	A	F	D	C
			1	S	C	A	B	B	A	F	D	A
			3	O	C	A	B	B	A	F	A	A
	Šimanov	1	1	W	C	C	B	C	B	F	A	C
P. berchtoldii	Thuner See	7	7	E	A	B	C	B	A	A	D	K
	Bodensee II	2	1	G	A	B	C	B	A	A	E	L
			1	D	A	B	C	A	A	E	E	L
	Skalná I	10	7	I	A	B	C	B	A	D	F	G
			1	J	A	B	C	B	A	D	F	H
			1	H	A	B	C	B	A	B	G	I
			1	C	A	B	A	B	C	D	F	G
	Skalná II	15	14	K	B	A	C	B	A	A	D	K
			1	L	B	B	C	A	A	A	D	K
	Chvojno	11	11	A	A	A	C	A	A	A	E	J
	Týniště	11	11	B	A	A	C	B	A	C	D	E
	Litice	1	1	F	A	B	C	B	A	A	E	F
	Žamberk	1	1	K	B	A	C	B	A	A	D	K
	Chropyně	13	13	M	B	B	C	B	A	A	D	K
P. trichoides	Lomnice	12	12	N	B	A	B	B	A	A	D	M

Table 3. Occurrence of multienzyme phenotypes (A–Z) among 50 samples of eight populations of *P. pusillus*, 71 samples of nine populations of *P. berchtoldii* and 12 samples of one population of *P. trichoides*. Each crossing symbol represents one population-multienzyme phenotype unit used in UPGMA analysis

Species	Population	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
P. pusillus	Untersee																					×			×		
	Bodensee I																						×				
	Litoměřice																									×	
	Rokytňany																				×						
	Dubovec																×	×	×								
	Panenský																										×
	Stružky															×				×	×						
	Šimanov																							×			
P. berchtoldii	Thuner See					×																					
	Bodensee II				×			×																			
	Skalná I			×					×	×	×																
	Skalná II											×	×														
	Chvojno	×																									
	Týniště		×																								
	Litice						×																				
	Žamberk											×															
	Chropyně													×													
P. trichoides	Lomnice														×												

bands gave six different phenotypes, three of them heterozygous (Fig. 1). Phenotype F with the remote slowest band was diagnostic to *P. pusillus*, being found in all samples of this species. In *P. berchtoldii*, this locus was highly variable. Homozygous phenotype A was most frequent. Heterozygous phenotypes were detected in both mixed populations with homozygous plants (B, D, E) and uniform population (C). *P. trichoides* was homozygous with phenotype A (Table 2).

Isozyme system SOD was too complex to allow distinguishing of loci and heterozygous constitution. Seven isozyme phenotypes were observed (Fig. 1); of those A, B and C were unique to *P. pusillus*, and E, F and G to *P. berchtoldii*. The most common phenotype D appeared in some plants of all three species.

EST gave a very complicated pattern of bands of at least four colours (Fig. 1). Thirteen enzyme phenotypes were distinguished. They showed very high variation between populations and most of them (B, D, E, F, G, H, I, J, L, M) were peculiar to a single population or to some plants of a population. Phenotypes A and C appeared in two and six populations of *P. pusillus*, respectively, and phenotype K was found in four populations of *P. berchtoldii*. The two fastest bands in phenotypes A, B, C and D were diagnostic for *P. pusillus*. No diagnostic band was found for *P. berchtoldii* although the fastest band was present in 84% of studied plants of this species (Table 2).

Multienzyme phenotypes. A total of 22 multienzyme phenotypes was observed (Table 2). In the eight populations of *P. pusillus*, 12 multienzyme phenotypes were found, whereas 13 multienzyme phenotypes were detected among plants from the nine populations of *P. berchtoldii*. Among populations studied for infra-population variation, two of four were uniform in *P. pusillus*, and four of six were invariable in *P. berchtoldii* (Table 4). Genetic variation within populations were estimated by the number of multienzyme phenotypes detected in population (V), proportion of loci polymorphic (p), mean number of alleles per locus (A), and mean proportion of heterozygotes per locus (H). Average values of these parameters were V = 2.00, p = 0.095, A = 1.095, H = 0.033 for *P. pusillus* and V = 1.67, p = 0.125, A = 1.317, H = 0.045 for *P. berchtoldii*. The single population of *P. trichoides* was uniform. All multienzyme phenotypes were species-specific (Table 3).

In *P. pusillus*, four of the distinguished loci were polymorphic and two uniform, with two more enzyme systems that were polymorphic (Table 2). Twelve multienzyme phenotypes were observed (Table 3). Eleven of them were confined to a single population, except multienzyme phenotype T which was found in two populations. Among the recognized loci, seven alleles were unique to *P. pusillus* (4 in *Aat-2*, 1 in *Gdh-1*, 1 in *Lap-1*, and 1 in *6Pgdh-1*), three of them diagnostic for this species (2 in *Aat-2*, 1 in *6Pgdh-1*). *Adh-1* also reliably distinguished *P. pusillus* from *P. berchtoldii*. Additionally five unique bands were recognized in EST, two of them diagnostic, and two unique bands in SOD.

All loci/enzyme systems of *P. berchtoldii* were polymorphic (Table 2). Thirteen multienzyme phenotypes were found (Table 3). Twelve of them were detected in only one population but K was located in two populations. The distinguished loci displayed eleven alleles unique to *P. berchtoldii* (1 in *Aat-2*, 1 in *Gdh-1*, 2 in *Adh-1*, 1 in *Lap-1*, 1 in *6Pgdh-2*, and 5 in *6Pgdh-1*); two other alleles were shared with *P. trichoides* but not found in *P. pusillus* (1 in *Aat-2*, and 1 in *6Pgdh-1*). Additional eight and two unique bands were recognized in EST and SOD, respectively.

The single population of *P. trichoides* was uniform in all enzyme systems (Table 2). Unique enzyme markers were found only in EST where 3 bands confined to this species were observed.

In the UPGMA dendrogram (Fig. 2), variation among populations of *P. pusillus* is found at a similarity coefficient of 0.73–1.00, and variation within populations at a coefficient 0.92–1.00. Values of a similarity coefficient for *P. berchtoldii* are 0.76–1.00 for variation among populations and 0.83–1.00

for that within populations. The single population of *P. trichoides* was invariable.

Relationships between the species. The investigated material represents three distinct enzymatic entities perfectly corresponding to the three morphologically defined putative species. In the UPGMA dendogram, all populations of each of *P. pusillus* and *P. berchtoldii* were clustered together and separated from populations of the other species and from that of *P. trichoides* (Fig. 2). In this analysis, *P. berchtoldii* is the most distinct species separated from the others at a similarity coefficient of 0.64. The *P. trichoides* cluster is separated from *P. pusillus* at a coefficient of 0.73. Exclusion of *P. trichoides* material from the analysis did not change the mutual relationships between *P. pusillus* and *P. berchtoldii*.

Discussion

Pattern of genetic variation within and between populations. In the preceding paragraphs, it has been shown that most genetic variation in *P. pusillus* and *P. berchtoldii* is distributed between rather than within populations (Fig. 2, Tables 2 and 4). At least half of the populations studied for infra-population variation were found to be uniform in both species, while others generally presented low levels of genetic variation as estimated by the number of multienzyme phenotypes detected in population (V), proportion of loci polymorphic (p), mean number of alleles per locus (A), and mean proportion of heterozygotes per locus (H) (Table 4). This low diversity within populations was revealed in spite of the fact that only freely flowering and fruiting populations were sampled for the analysis to avoid collecting clones obviously persisting vegetatively. In contrast, the differences between populations were very high. With one exception in both species, each multienzyme phenotype was specific to a single population. The pattern of distribution of genetic variation in populations of *P. pusillus* and *P. berchtoldii* revealed in this study is in accordance with published studies on isozyme variation in other species of *Potamogeton*. Isozyme variation was very high between populations but low or absent within populations of *P. pectinatus* L. in a study by van Wijk et al. (1988). In an extensive isozyme survey made by Hettiarachchi and Triest (1991), most of 18 *Potamogeton* species showed low infra-population variation and high inter-population differentiation. Isozyme variation was found in two of twelve populations of *P. coloratus* Hornem. by Hollingsworth et al. (1995a). New Zealand samples of *P. ochreatus* Raoul, *P. cheesemanii* A. Benn. and *P. crispus* L. showed little genetic diversity between populations but even less variation within them (Hofstra et al. 1995). More variation was found to be distributed between than within populations of *P. pectinatus* L. and *P. filiformis* Pers. by Hollingsworth et al. (1996a). These observations correspond also to the general trend in many aquatic plants (e.g. Les 1988, Laushman 1993, Philbrick and Les 1996).

Role of reproduction modes in the genetic variation pattern. A species' breeding system often plays a central role in determining the distribution of genetic variation within and between populations. In *P. pusillus* agg., vegetative propagation seems to be the principal mean of maintenance of populations. Colonies in deep, shaded or turbulent water often do not flower. Then, the individuals reproduce solely by turions. These are short stems with reduced leaves modified into vegetative buds. They are produced in quantity in late summer in the leaf axils (typically in *P. pusillus*) or on the apices of the main shoot and axillary branches (in *P. berchtoldii*). These turions are dormant and serve mainly to carry the colony over the winter since the rest of the plant is annual and dies down in autumn. The clonal growth is the only opportunity to survive for the populations that fail to flower at all. However, even if plants in some populations flower and fruit freely and a rich seed bank is produced, seedling recruitment is generally rare in established populations. In spring, the faster growth of new stems from turions causes failure of the smaller and

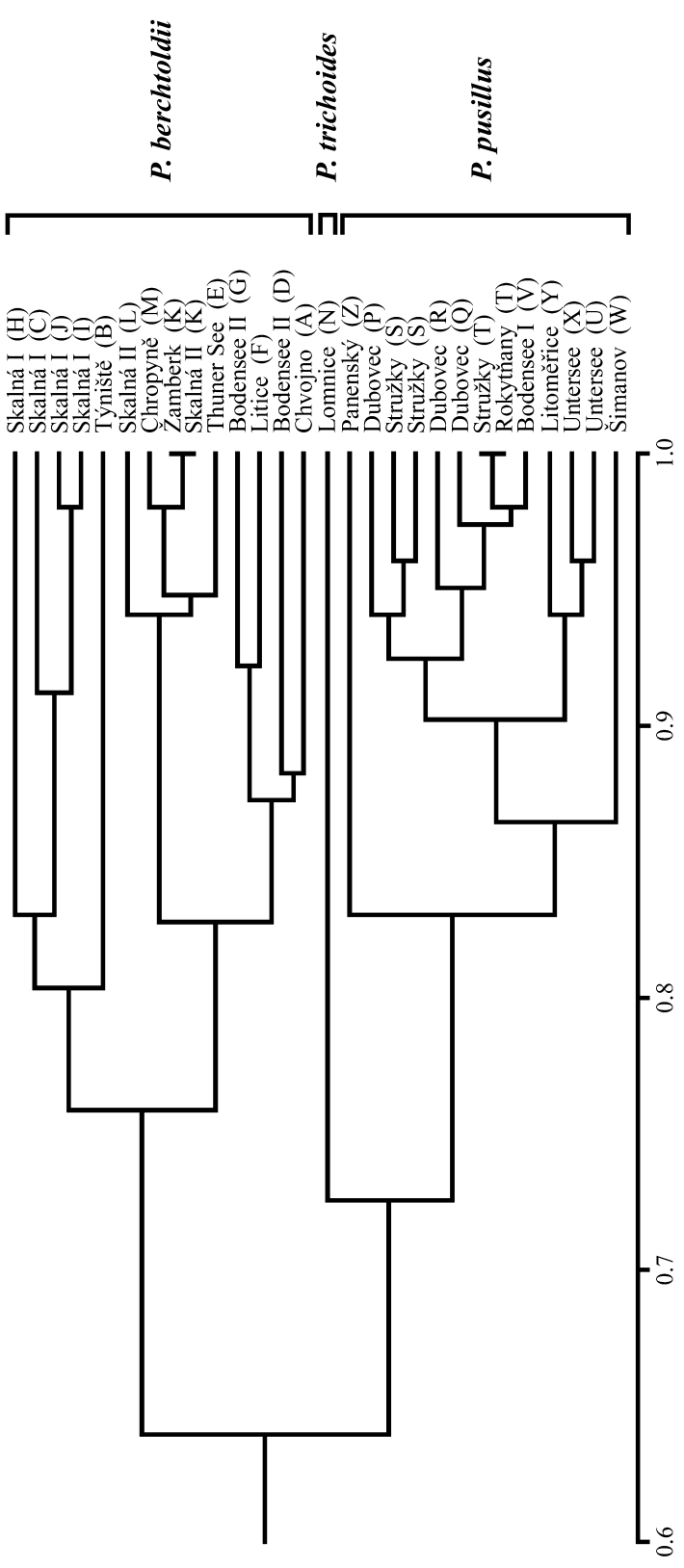


Fig. 2. An UPGMA dendrogram based on presence-absence data of 51 enzyme markers, constructed for the 28 population-multienzyme phenotype units (altogether representing 133 plant individuals) listed in Table 3. Simple Matching was applied as a similarity coefficient

Table 4. Genetic variation within populations of *P. pusillus*, *P. berchtoldii* and *P. trichoides* based on isozyme data: number of individuals sampled (N), number of multienzyme phenotypes detected in population (V), proportion of loci polymorphic (p), mean number of alleles per locus (A), mean number of alleles per polymorphic locus (A_p), mean proportion of heterozygotes per locus (H), range of similarity coefficient between individuals within the population (S)

Species	Population	N	V	p	A	A_p	H	S
P. pusillus	Litoměřice	12	1	0.00	1.13	–	0.00	1.00
	Stružky	10	3	0.25	1.00	1.00	0.00	0.92–1.00
	Panenský	10	1	0.00	1.25	–	0.13	1.00
	Dubovec	10	3	0.13	1.00	1.00	0.00	0.92–1.00
P. berchtoldii	Chvojno	11	1	0.00	1.00	–	0.00	1.00
	Thuner See	7	1	0.00	1.13	–	0.00	1.00
	Týniště	11	1	0.00	1.63	–	0.13	1.00
	Skalná I	10	4	0.50	1.88	3.33	0.14	0.83–1.00
	Skalná II	15	2	0.25	1.13	1.50	0.00	0.94–1.00
	Chropyně	13	1	0.00	1.13	–	0.00	1.00
P. trichoides	Lomnice	12	1	0.00	1.00	–	0.00	1.00

weaker seedlings in the competition for light. In waters with very low light transparency, the seedlings die soon after germination. Also, seeds of several species exhibit prolonged dormancy[2] and they are likely to be covered with sediment before they start to germinate. In contrast, vegetative propagation is very effective. It has been reported that a single turion of another species of the genus, *P. crispus* L., planted at the start of the season grew into a plant that produced 23,520 turions by the end of the year (Yeo 1966). It may be concluded that the main function of fruits in *P. pusillus* agg. seems to be in dispersal and long-term survival during unfavourable periods. Similar observations on the low importance of seeds in maintaining established populations have been made in other species of *Potamogeton*. Brux et al. (1987, 1988) noted only rare establishment of plants of *P. alpinus* Balb. from seed in the wild despite a rich seed bank and found that the colonies are maintained almost entirely by means of vegetative reproduction. Van Wijk (1989) concluded that maintenance of populations of *P. pectinatus* L. was almost entirely due to vegetative persistence and reproduction. Kautsky (1991) recorded no seedlings in the site where *P. pectinatus* and *P. perfoliatus* L. had been the most abundant angiosperms. Hollingsworth et al. (1996a) concluded that seedling recruitment rather than the frequency of sexual reproduction itself is an important factor in controlling the observed levels of variation in *P. pectinatus*.

The propagules of *P. pusillus* agg. (seeds, in shorter distances also turions and shoot fragments) are easily dispersed between standing water bodies by waterfowl. Almost every abandoned clay pit or stone quarry with a water body in Central Europe is quickly colonized by these linear-leaved pondweeds. The new population is often established by a single or a few genotypes and this founder effect determines low initial genetic variation. Plants in these shallow waters usually flower freely and regularly set fruit in quantity. Next spring, when dormancy of a portion of the seed bank is broken by low temperature during the winter, seeds start to germinate and their success is often high in the new habitat with almost optimal conditions such as sufficient nutrients, clear water and low competition.

[2] Several hundreds of fruits of *P. berchtoldii* from a single population were collected in 1993. They did not germinate during the first season and have been preserved in a refrigerator at about 4 °C. Each spring, several tens of them are removed and kept at room temperature. Some germinating seedlings have been observed each year. Now, in 2002, the experiment still continues.

However, the low genetic diversity or even uniformity is usually retained for many subsequent generations. These plants also exhibit high heterozygote deficiency. This pattern of genetic variation coincides well with what one would expect in a self-fertile inbreeder.

The dominance of self-fertilization was also observed in cultivation. The plants were flowering freely in summer when grown in shallow standing water. No differences in pollination have been observed between *P. pusillus* and *P. berchtoldii*. Both species share the same floral structures. The plants have several-flowered spikes on peduncles longer than the spikes. When in flower, the inflorescence usually projects above water surface. The flowers are bisexual and exhibit strong protogyny. The pollen grains are hydrophobic and spherical in shape.

The following mutually non-exclusive modes of effective pollination according to the pattern of pollen transfer from anthers onto the stigma have been observed by Z. K. in cultivation. In natural populations, more than one of these modes are likely to operate simultaneously. They may also be considered as different aspects of the overall pollination system:

1. Autogamy. When petals open, a lot of pollen is released from the anthers on the stigma of the same flower. Most of the pollen stuck on the stigma seems to originate from the same flower and within-flower self-pollination would be the most frequent mode of pollination. To test self-compatibility, several spikes were each enclosed in a small bag before the flowers opened. The isolation within the bag not only prevented pollination by pollen from a different plant, but served also as a barrier against wind that could transfer pollen between flowers. Although this did not exclude that some lower flowers in a spike were pollinated by the pollen fallen from the apical part of the spike, there was obviously no mechanism that would bring pollen upwards. Development to mature fruits was observed in all spikes, including their apical flowers. Full fruit set was also repeatedly observed in cultivation in the first spike when no other source of pollen for crossing had been available.

2. Geitonogamy. In *P. pusillus* agg., several flowers are usually open simultaneously in the same spike and transfer of pollen falling from upper flowers to a stigma of flowers below seems to be the most common case of between-flower pollination within an individual.

3. Anemogamy. The inflorescences usually project above the water surface before flowering and the peduncles bend upwards under negative geotropism, which seems to be an adaptation to anemogamy. The pollen from dehisced anthers may be then easily blown away by wind. The concave shape of the petals arising close to the anthers gives rise to small turbulences that enhance the chance that the pollen grains are caught in the airstream. Some of them may then accidentally reach the stigma of another flower.

4. Epihydrogamy. Even though flowering spikes are mostly emerged, they sometimes get in contact with the water surface, particularly when water level has risen or at a late phase of flowering. The pollen is hydrophobic and pollen grains float on the water surface. In sheltered places, the water surface may be covered by a carpet of floating pollen grains. When pollen comes in contact with a flower, pollen grains stick on the stigmas.

5. Hydroautogamy. This term has been proposed by Philbrick and Anderson (1987) for self-pollination in submerged flowers of *Potamogeton*. The selfing takes place via bubbles that are produced when the anthers dehisce. These bubbles provide a surface on which pollen moves from the anther to the stigma of the same flower. Hydroautogamy is suggested as an intermediate stage between aerial and true water pollination in aquatic plants (Philbrick and Anderson 1987, Philbrick 1988, Philbrick and Les 1996).

Each of the above pollination modes gave rise to mature fruit that was later able to germinate. It is therefore assumed that fertilization by the acquired pollen follows each of the pollination modes.

Another experiment with the aim to exclude theoretical possibility of occurrence of pseudogamy or agamospermy in our studied plants was unsuccessful. Because of their

structure, the flowers were always damaged during attempts to remove all anthers from young flower buds. The same difficulties with emasculation were noted by Guo and Cook (1990) in experiments with *Groenlandia densa* (L.) Fourr. However, because of the occurrence of polymorphic populations, the high number of isozyme phenotypes detected in this study and the occurrence of interspecific hybrids (cf. Preston 1995, Wiegleb and Kaplan 1998), we consider the occurrence of gametophytic apomixis in *P. pusillus* agg. unlikely.

Philbrick (1984) observed reduced seed set in the American population of *P. berchtoldii* he studied and suggested that the pollen seemed to be incompatible with the stigma of the same flower. However, this first interpretation of self-incompatibility has been abandoned in his later works.

Modes 1, 2 and 5 described above always result in selfing. Only in modes 3 and 4 there is some space for crossing between different genets. However, as shown above, natural populations of *P. pusillus* agg. are often genetically uniform so that pollination by a different ramet can equally result in selfing. Also the low proportion of heterozygous plants ($H = 0.00$–0.14, see Table 4) indicates that cross-pollination is rather a rare event in *P. pusillus* agg.

On the other hand, crossing may be favoured by protogyny. At early female phase, the petals are closed and protect the anthers. The stigma is ready to receive pollen a few days before the anthers dehisce. When the petals liberate the pollen, the stigma may have already been fertilized by pollen from a different plant. The released pollen grains may be caught in the airstream and reach another stigma. Cook (1988) considers petals as a pollen-arresting mechanism that liberates the pollen only after the female receptive phase. Our observations suggest that the stigma is still receptive at the male phase unless it has already been fertilized by different pollen. Several bagged inflorescences produced full seed set from all flowers (see above) and thus protogyny here is an inefficient barrier to self-pollination. Despite the dominance of selfing, the occurrence of polymorphic populations, heterozygous plants and interspecific hybrids (cf. Preston 1995, Wiegleb and Kaplan 1998) indicate that outcrossing does sometimes occur.

Relation between habitat conditions and genetic variation pattern

Among the freely flowering and fruiting populations studied for infra-population variation in this study, no isozyme variation was detected in 50% of populations of *P. pusillus* and in 66% of populations of *P. berchtoldii*. 73% of populations consisted only of homozygous plants. The typical habitat of these populations with dominance of selfing is a small and relatively shallow pond, a pool in the bottom of a disused clay-pit or a meadow ditch with standing water. These habitats seem to offer optimal conditions for abundant flowering, fruiting and seed germination.

In contrast, some populations consisted of mixed homozygous and heterozygous plants or even entirely of heterozygotes in one of the loci. All plants of *P. pusillus* from the population Panenský displayed heterozygotes at *Aat-2*. The samples were invariable in all observed enzyme systems. This pattern suggests that the population is actually a clone. In spite of the fact that the plants did flower and set abundant fruits, sexual reproduction must have been ineffective and the population persisted there solely by vegetative reproduction. This is probably because the water in this pond is relatively deep and of low transparency, preventing fruit germination or successful seedling recruitment. Invariable occurrence of heterozygotes at *Aat-2* indicates either the founder effect, with the population having been established from a single turion, or considerable bottle-neck, in both cases followed exclusively by vegetative reproduction.

Similarly, all plants of the population Týniště were heterozygous at *6Pgdh-1* and uniform in all studied enzyme systems. The population grows in a drainage ditch with slowly running water and the absence of genetic vari-

ation in this site may be explained by abundant organic sediment that covers the plants. Small and weak seedlings are likely to be stifled under this sediment whereas stronger turions are probably able to escape by faster growth.

Most likely a combined mode of generative and vegetative reproduction occurs in the population Skalná I. Four multienzyme phenotypes have been revealed among ten plants from this site and a high proportion of heterozygotes was found at *6Pgdh-1*. The locality is a pool in the bottom of a disused clay-pit and the plants grow there in spatially rather isolated clumps and set fruits freely. An alternative interpretation of the genetic structure within this population is that the collected and studied plants may have represented samples of up to four different clones coexisting in the site.

Relationships between species

Species-specific isozyme markers have been found in several studies on *Potamogeton*. Multienzyme phenotype patterns were diagnostic at the species level in the study by Hettiarachchi and Triest (1991) and could serve for identification. Isozyme markers that separate *P. pectinatus*, *P. filiformis*, *P. vaginatus* Turcz. and South American *P. striatus* Ruiz et Pav. were detected by Hollingsworth et al. (1996a, 1996b). Clear interspecific differences in isozyme band pattern were observed by Hofstra et al. (1995) between *P. ochreatus*, *P. cheesemanii* and *P. crispus*. Consistently different isozyme patterns have been repeatedly utilized for identification of hybrids, some of which are otherwise difficult to prove on the morphological grounds only (Hollingsworth et al. 1995b; Hollingsworth et al. 1996b; Preston et al. 1998; Fant et al. 2001a, 2001b; Iida and Kadono 2002; Kaplan et al. 2002).

Unique enzyme markers were found also for *P. pusillus*, *P. berchtoldii*, and for the single population of *P. trichoides* investigated in this study. Sharp genetic differentiation was revealed in several enzyme systems as well as between multienzyme phenotypes of these species. In the UPGMA analysis, *P. pusillus*, *P. berchtoldii* and the single population of *P. trichoides* were divided into three distinct main clusters. In spite of little morphological differences between *P. pusillus* and *P. berchtoldii*, enzyme markers provide convincing support for treating them as two separate species. Identification of sampled plant material on the basis of stipule structure corresponded perfectly with the distinct enzymatic entities.

The isozyme results of this study are in accordance with the separate position of *P. pusillus* and *P. berchtoldii* given by Hettiarachchi and Triest (1991) who compared inter-specific isozyme variation between 18 species of *Potamogeton*. In their study, the cluster analysis (l.c., Fig. 4) divided the diploid linear-leaved species of *P. pusillus* group (sensu Wiegleb 1988) in two subgroups that perfectly corresponded with the division proposed by Hagström (1916). The species with tubular stipules (*P. pusillus*, *P. friesii* Rupr.) appeared in one cluster whereas those with convolute stipules (*P. obtusifolius* Mert. et W. D. J. Koch, *P. trichoides*, *P. berchtoldii*) were placed in another cluster. However, the relationships of *P. pusillus* group to other groups of the genus were quite different from those Hagström assumed. Although results of both isozyme studies support the separate position of *P. pusillus* and *P. berchtoldii*, a different topology was recovered with respect to *P. trichoides*: samples of this species were clustered with *P. berchtoldii* in the Hettiarachchi and Triest study, but the single population examined in this study was placed in the basal position of *P. pusillus* cluster.

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