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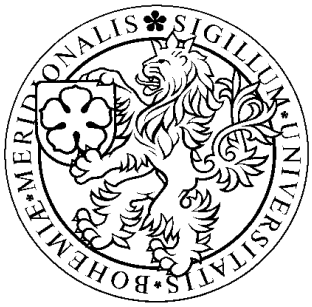
FACULTY OF AGRICULTURE
BIOTECHNOLOGICAL CENTRE



PhD Thesis

Application of different methodological approaches in plant population studies.

Ing. Barbora Kubátová
České Budějovice
2008

ACKNOWLEDGMENTS

Above all I would like to thank to my advisor professor Vladislav Čurn for his support, help and unceasing patience. I acknowledge the help and inspiration of all coauthors of papers I included with their agreement in my PhD thesis. I thank to Jan Suda and Pavel Trávníček for their being.

PROHLÁŠENÍ

Prohlašuji, že jsem předkládanou disertační práci vypracovala sama na základě literárních pramenů uvedených v seznamu publikací a na základě získaných výsledků. Má role při přípravě publikací zahrnutých do této disertační práce byla následující:

1. **Kubátová B., Trávníček P., Čurn V., Suda J. (2008):** Adressing the flow cytometric puzzle: complex ploidy variation in *Gymnadenia conopsea* agg. (*Orchidaceae*). – American Journal of Botany (*submitted*).

   Příprava a vedení studie, odběr vzorků na lokalitách, příprava vzorků pro cytometrické analýzy a část vlastních cytometrických analýz.

2. **Čurn V., Kubátová B., Vávřová P., Suchá O., Čížková H. (2007):** Phenotypic and genotypic variation of *Phragmites australis*: Comparison of populations in two man-made lakes of different age and history. – Aquatic Botany 86: 321-330.

   Izolace DNA, screening primerů, RAPD analýzy, vyhodnocování DNA dat a cytometrické analýzy.

3. **Suchá O., Vávřová P., Čížková H., Čurn V., Kubátová B. (2007):** Phenotypic and genotypic variation of *Phragmites australis*: II. A comparative study of clones originating from two populations of different age. – Aquatic Botany 86: 361-368.

   Izolace DNA, RAPD analýzy, vyhodnocování DNA dat a cytometrické analýzy.

4. **Kubátová B. and Čurn V. (2008):** Phenotypic and genotypic variation of *Phragmites australis*: High level of cryptic variation revealed on the basis of molecular marker analysis non-corresponding to phenotype performance. – Molecular Ecology (*submitted*).

   Příprava vzorků pro AFLP včetně fragmentačních analýz a vyhodnocení dat. Amplifikace cpDNA, klonování a sekvenování vybraných vzorků, úprava a hodnocení sekvenačních dat. Část mikrosatelitových analýz a jejich hodnocení. Příprava vzorků pro cytometrické analýzy a vlastní cytometrické analýzy.

5. **Kubátová B., Trávníček P., Bastlová D., Čurn V., Jarolímová V., Suda J. (2008):** DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale. – Journal of Biogeography 35: 167-176.

   Příprava a vedení studie, vypěstování rostlin pro cytometrické analýzy, příprava vzorků pro cytometrické analýzy a část vlastních cytometrických analýz.

V Českých Budějovicích, dne 29. 4. 2008 .....

CONTENTS

I. Summary (in Czech) - Abstrakt

Předkládaná disertační práce se zabývá aplikací různých metodologických přístupů ve studiu taxonomické problematiky a genetické, cytogenetické a morfologické struktury a variability rostlinných populací. Disertační práce a publikace v ní zahrnuté jsou věnovány studiu komplexu *Gymnadenia conopsea* (L.) R.Br. (pětiprstka žežulník) a populací *Phragmites australis* (Cav.) Trin. ex Steud. (rákos obecný) a *Lythrum salicaria* L. (kyprej vrbice). Mezi základní metodologické přístupy použité pro řešení otázek a objasnění hypotéz dílčích studií obsažených v jednotlivých publikacích, patří morfologické a DNA molekulární markery a průtoková cytometrie.

V první práci byla pomocí průtokové cytometrie zjišťována cytogenetická struktura a variabilita ve 36 populacích komplexu *G. conopsea* (pětiprstka žežulník s.l.) pocházejících z Čech, Moravy a Slovenska. V rámci této studie bylo provedeno 2226 cytometrických analýz, při kterých bylo detekováno pět různých ploidních úrovní. Vedle již dříve známých tetra-, okto- a dekaploidů byly také v rámci tohoto komplexu nalezeni jedinci s dosud nepublikovanými ploidními úrovněmi (hexaploidi a dodekaploidi). Současně byl také zjištěn sympatrický růst jedinců různých ploidních úrovní ve stejné populaci u více než poloviny studovaných populací (19 ze 36). V deseti populacích se společně vyskytovaly dvě ploidní úrovně. Na šesti lokalitách byly zjištěny tři ploidní úrovně a konečně společný růst jedinců čtyř rozdílných ploidních úrovní byl zaznamenán na dvou lokalitách. Na jedné lokalitě se dokonce zjistily současně se vyskytující jedinci všech pěti ploidních úrovní. Zatímco tetraploidi a oktoploidi často tvořily monoploidní populace, jedinci vzácných ploidních úrovní se vyskytovaly pouze společně s jedinci jedné nebo druhé majoritní ploidní úrovně. Frekvence výskytu třech zjištěných minoritních ploidních úrovní byly velmi nízké a byly na úrovni 1,3% u hexaploidů, 0,5% u dekaploidů a 0,4% u dodekaploidů. Kromě společného růstu rozdílných cytotypů (náležejících druhu *G. conopsea*) na jedné lokalitě, byl také zaznamenán společný růst rostlin *G. conopsea* a *G. densiflora*, i když oba taxony byly v rámci dané lokality prostorově odděleny. Nejčastěji zjištěná ploidní úroveň byla tetraploidní, přičemž tuto ploidní úroveň mělo 60,0% analyzovaných jedinců, zatímco oktoploidi byly zjištěni u 37,8% analyzovaných jedinců.

Následující tři práce se zabývaly studiem dvou historicky odlišných populací *P. australis*. Lokalita na Opatovickém rybníku reprezentovala populaci zhruba 500 let starou, zatímco populace na pískovně Halámky byla vytvořena přibližně před 30 lety. U obou populací byla studována genetická a morfologická variabilita. Podle předpokladu byla zjištěna nižší genetická variabilita na historicky starší lokalitě Opatovický rybník oproti mladé lokalitě na pískovně Halámky. Vzorky pro tyto studie byly záměrně odebírány z morfologicky homogenních stanovišť a byla testována hypotéza, že tato uniformní stanoviště jsou monoklonální. Tato hypotéza nebyla potvrzena. V jedné ze studií zabývající se *P. australis* bylo u obou populací sledováno, zda podobné morfometrické charakteristiky naměřené v přirozeném porostu budou také zjištěny u jedinců přenesených do kultivačního pokusu, což by ukazovalo na genetickou podstatu těchto sledovaných charakteristik. Rostliny pocházející z mladší populace na Halámkách si uchovávaly podobné morfometrické charakteristiky i po přenesení do experimentálních podmínek. V této práci byla také testována hypotéza, zda robustnost některých morfotypů vyskytující se ve sledovaných populacích není způsobena vyšší ploidní úrovní (oktoploidní), ale při analýzách ploidní úrovně vybraných rostlin byly nalezeni pouze tetraploidi. V poslední části série prací věnovaných rákosu byly použity čtyři typy DNA molekulárních markerů (RAPD, SSR, AFLP a sekvenování cpDNA) k hodnocení genetické variability. Na základě dat z těchto nových analýz a dat získaných v předchozích studiích byla porovnávána využitelnost jednotlivých DNA markerů a morfologických charakteristik a dále pak různé kombinace těchto přístupů. Kombinace více přístupů v hodnocení jak genetické tak morfologické variability v obou populacích rákosu vykazovala jasnější odlišení sledovaných populací.

V páté a poslední práci byla zjišťována variabilita ploidní úrovně populací *L. salicaria* pocházejících z původního a sekundárního areálu tohoto druhu, kde se projevuje jeho výrazný invazní charakter. V této studii bylo analyzováno 1884 potomků pocházejících od téměř 600 mateřských rostlin, jejichž semena byla nasbírána na 124 lokalitách v primárním a sekundárním areálu výskytu *L. salicaria*. Kromě již dříve známých diploidů a tetraploidů byly poprvé analyzování hexaploidní jedinci. Při analýzách ploidní úrovně byli také potvrzeni triploidi. V primárním areálu byly nalezeni di-, tri-, tetra- a hexaploidi s jasnou převahou výskytu tetraploidů. V populacích z Izraele byly

zjištěni pouze diploidi a v tureckých populacích se sympatricky vyskytovaly diploidní a hexaploidní jedinci. Triploidní potomstvo bylo nalezeno v populaci pocházející z Maďarska. Oproti variabilitě ploidní úrovně zjištěné v primárním areálu, invazní populace byly striktně tetraploidní, čímž nebyla prokázána hypotéza, že invaznost rostlin *L. salicaria* v jejím sekundárním areálu (převážně Severní Amerika) je způsobena posunem v ploidní úrovni.

II. Overview

The aim of my PhD thesis is to use a global approach to the study of plant populations. Initially, my aim was to apply my thesis only to the study of molecular markers in plant populations. I soon realized that a global approach that combines techniques from different fields of biological sciences could reveal more of the complexity inherent in plant populations.

The first and main reason why I decided to combine multiple approaches was that the combination of different methods gives us more opportunities to interpret, explain and apply our results.

The second reason was that I was mostly interested in wild plant populations. Plant population studies based on molecular genetics usually require previous knowledge about the genome of studied species, unless I would want to use only non-specific DNA markers such as RAPD, AFLP or costly sequencing of cpDNA or ribosomal DNA which is not so effective when it is used at the species level. There is no such requirement for these techniques for initial sequence knowledge. It is well known that RAPD technique is the easiest method to use however, it has many limitations when it is used as the sole DNA marker. A study using RAPD as a single method is no longer publishable in scientific journals. To be scientifically valid, one would need to add either another reliable DNA method or morphological data. Although molecular biology is presently very popular, there is still little known about the genetics of wild plant species. Thus the combination of different approaches reveals more about the studied species.

I will demonstrate that a combination of **classic biology** (e.g. field and greenhouse experiments, morphological measurements) linked with **molecular biology** (e.g. specific and non-specific DNA markers), and recently more often (frequently) used **plant flow cytometry** is a useful way to study plant populations.

The main goal of my PhD thesis was not only to provide new information about the studied species, but also to compare different approaches when they are used as a single method or when they are combined with several other methods. I believe that my results and conclusions derived from a combined scientific approach have greater value for

advancing scientific knowledge in the field of biology than those based only on a single method. My PhD thesis includes several projects aimed mostly at plant population structure of one certain species or genus in case of the *Gymnadenia* complex.

I believe that it is more useful to use a multi-discipline approach to advance our knowledge of the complexity inherent in plant populations.

III. Aims of the thesis

The aim of my PhD thesis is to utilize different methodological approaches in plant population studies. I subjected three different plant species/taxa to evaluation of their variability and genetics structure using three methodological approaches and/or their combination. My aim is to study plant populations form several views and apply variable approaches to gain new complex knowledge about studied objects.

In this thesis I included three papers and two manuscripts focused on responding of partial questions and hypothesis.

Manuscript I. - Addressing the flow cytometric puzzle: complex ploidy variation in *Gymnadenia conopsea* complex (*Orchidaceae*).

I.a What is the population cytotype structure based on representative sampling?

I.b Do ploidy-mixed populations occur? If so, what is their frequency and which cytotypes are involved?

I.c Which minority cytotypes can be found and under which conditions?

I.d What is the exact distribution pattern of different cytotypes within a population? Do the data suggest genuine sympatry or rather a certain habitat segregation?

Paper II. - Phenotypic and genotypic variation of *Phragmites australis*: Comparison of populations in two man-made lakes of different age and history.

II.a The number of clones per unit of population area is lower in the older population than the younger population.

II.b The maximum area covered by a single clone is larger in the older population than the younger one.

II.c Morphologically homogeneous stands are monoclonal.

II.d Morphological variability is a good indicator of the genetic variability in multiclonal stands and populations.

Paper III. - Phenotypic and genotypic variation of *Phragmites australis*: A comparative study of clones originating from two populations of different age.

III.a Are the growth and morphometric characteristics of morphotypes originating from the two populations also preserved in the culture?

III.b Have the growth characteristics of the robust morphotypes a genetic basis?

Manuscript IV. - Phenotypic and genotypic variation of *Phragmites australis*: High level of cryptic variation revealed on the basis of molecular marker analysis non-corresponding to phenotype performance.

IV.a Different molecular markers and morphological characteristics can better reveal genetic structure of studied populations than single marker focused approach.

IV.b The patterns of genotypic variability of two reed populations inhabiting sites differing in both succession stage and substrate conditions are distinct.

IV.c The morphological variability of studied populations/stands reflects their genotypic variation and the morphological variability should be an indicator of their genetic variability.

Paper V. - DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale.

V.a What is the level of ploidy variation in both primary and secondary ranges of *L. salicaria* distribution?

V.b What is the cytotype structure within both native and invasive *L. salicaria* populations?

V.c Might the switch in ploidy level trigger invasive spread in secondary area of distribution?

V.d Is there a difference in genome size between native and invasive populations?

IV. Introduction

1. Characteristics of studied species and their scientific background

1.1. Studied models

There were three different models of plant species involved in my PhD thesis. The first one is ***Gymnadenia conopsea* (L.) R.Br. complex** (Pětiprstka žežulník - fragrant orchid, *Orchidaceae*). It represents rare and endangered species and taxonomically problematic group. *G. conopsea* complex is an ideal system to study biological processes in plant populations due the presence of the extensive variation observed in traits that may be involved in speciation and the fact that multiple taxa are characterized by groups of these traits (Soliva and Widmer, 1999). There has been many biological approaches used in study of this complex e.g. morphology, phenology, ecology, molecular genetics, phylogenetics or karyology. *G. conopsea* complex still attract an attention of plant systematics, population geneticists, and evolutionary biologists. It is a relatively young plant group where many questions remain unsolved.

Second and third studied species both belong to native plant species of European wetlands, which successfully invade North America wetlands. These are ***Lythrum salicaria* L.** (Kyprej vrbice - purple loosestrife, *Lythraceae*) and ***Phragmites australis* (CAV.) TRIN. ex STEUD** (Rákos obecný - common reed, *Poaceae*). While *L. salicaria* is without any doubt described as an invasive species there are some queries about origin of invasive populations of *P. australis* in North America. It was commonly considered to be an exotic species in North America. However, there is clear evidence of the presence of common reed in this area long ago before first European colonizers entered the continent (Saltonstall, 2002, 2003a). It is now known that the North American native forms of *Phragmites* are markedly less vigorous than European forms (Saltonstall, 2002, 2003a). Whereas *P. australis* invades the wetlands in North America there was recorded rapid decline of plants of *P. australis* from shoreline habitats of European lakes.

A lot of studies were occupied with ecology, ecophysiology or morphology of common reed, in last years also genetic structure of both native and invasive populations was studied. But it still seems that only complex approach and combining of ecological

and molecular data can discover cryptic variation and/or invasion and maybe predict behavior of reed populations.

While purple loosestrife only exceptionally becomes dominant in Eurasian wetland communities, it often becomes an important constituent or dominant species in North American plant communities invaded by it. It was probably introduced into North America from Europe in the early 1800s through ship ballast and seed sales (Mack, 1991), but it was not recognized as invasive until the 1930s, when it began to form monospecific stands in the floodplain pastures of the St. Lawrence River in Quebec (Louis-Marie, 1944). Since then, it has followed a distinct pattern of invasion across the United States and Canada.

L. salicaria has been subjected to many studies. Currently, there are a lot of information about morphology, phenology and ecophysiology of this species but there are very little knowledge about cytogenetics, genetic structure and variability of this species in both areas of distribution but these information are very important to understanding of invasive behavior; not only for this species.

1.2. *Gymnadenia conopsea* (L.) R.Br. complex (*Orchidaceae*)

The family *Orchidaceae* is one of the most species rich families in the plant world, with the estimated number of species ranging from 19,300 (Wells and Willems, 1991; Dressler, 1993) up to perhaps 25,000 (Nilsson, 1992) distributed among 725 genera of which only about 115 - 200 are growing in Europe (Wells and Willems, 1991; Gustafsson, 2003). The vast majority of orchid species are found in tropical areas, where they often are epiphytic, whereas all European orchid species are ground-dwelling (Gustafsson, 2003). Most of the species are perennial and many are long-lived, ages of 15-30 years has been recorded in some northern species (Tamm, 1972; Wells and Willems, 1991). Orchids occupy different types of habitats. Epiphytes are primarily found in trees, and tropical species often occur high up in the tree tops (Mossberg and Nilsson, 1985). The majority of the ground-dwelling species grow in open areas like grazed meadows, marshes and shores, even though some species also are found deep in the forest (Mossberg and Nilsson, 1985). Tropical orchid species exhibit a tremendous variation in size, shape and colour, whereas ground-living species have a more or less uniform construction with flowers on a leafy stem, either single or multi-flowered organized as a spike or a raceme (Mossberg and Nilsson, 1985).

I was studying only populations of *Gymnadenia conopsea* complex and results of this study are a part of my PhD thesis. There is a lot of contradiction in its taxonomy and several concepts are described for it in the literature, depending on the author's opinion. Where some authors use the term taxa the others may use term subspecies or even species. It may be confusing for unconcerned readers and I will explain current approach of the taxonomy in *G. conopsea* complex later. In advance, I want to point out that the taxonomical terms applied in this review are used as they were used in the original sources according the author's sence.

1.2.1. Species description, distribution and habitats

The genus *Gymnadenia* (*Orchidaceae*) belongs to terrestrial orchids and it is part of a closely-related clade of Eurasian orchid genera (Campbell et al., 2002). *Gymnadenia* is "sister" to the larger genus *Dactylorhiza*, and hybridization between species of these two

genera is not uncommon (Farrington and Bateman, 1989; Stace, 1997). *G. conopsea* s.l. is a complex that shows considerable variation in morphology, phenology, genetics and karyology across its distributional range (e.g. Jongepierová and Jongepier, 1989; Mrkvička, 1993; Gustafsson, 2003; Marhold et al., 2005; Campbell et al., 2007). Two recognized taxa of *G. conopsea* that differ in flowering phenology, karyology and morphology may grow in sympatry in central Europe (Reinhard et al., 1991; Kubátová et al., 2008). Where different taxa/phenological variants occur in sympatry, they may form mixed populations, which are mainly separated in flowering time: lowland populations of ssp. *conopsea* flowering in May and June and those of ssp. *densiflora* in July and August (e.g. Jongepierová and Jongepier, 1989; Soliva and Widmer, 1999). The flowers are heavily scented and pollinators (different species of Lepidoptera, diurnal and nocturnal moths and hawkmoths) are rewarded with an abundant amount of nectar (Van der Cingel, 1995; Proctor et al., 1996; Jersáková and Kindlmann, 2004). The species of genus *Gymnadenia* are self-compatible but pollinator-dependent for fruit setting (Gustafsson, 2000). However, inflorescences contain many simultaneously open flowers (Lang, 1990), so that geitonogamous pollination is probably frequent. Capsules can contain 2000 or more seeds, each weighing approximately 1 mg (Harper, 1977), and seeds may be dispersed by wind over distances of at least 5–10 km (Rasmussen, 1995). Like in several other ground orchids, roots of *Gymnadenia* are associated with mycorrhizal fungi (Harvais and Hadley, 1967). *Gymnadenia* is generally found in agricultural landscapes, in open grassland, grazed and subalpine meadows and close to marshes and fens (Gustafsson and Thorén, 2001). Habitat preferences may differ slightly between the taxa, with ssp. *densiflora* preferring slightly wetter habitats (Soliva and Widmer, 1999; Gustaffson and Lönn, 2003). Both taxa, *G. conopsea* and *G. densiflora*, are widely distributed from lowland habitats to subalpine meadows (Reinhard et al., 1991). The number of populations have decreased during the last few decades, mostly due to habitat destruction and changed practices in agriculture and forestry (Gustafsson and Thorén, 2001) but it can be very abundant where it occurs (Campbell et al., 2007). The whole complex is widespread over most of Europe with the exception of the extreme southwest and southeast (Moore, 1980).

1.2.2. Taxonomical and nomenclatorical problematics in *G. conopsea* complex

In recent years, there has been much debate about taxonomic status in *Gymnadenia* (Campbell et al., 2007). Traditionally in *G. conopsea*, two varieties with distinct morphological separation have been identified, one early-flowering, var. *conopsea*, and one late-flowering, var. *densiflora* (e.g. Möseler, 1987; Soliva and Widmer, 1999; Lönn et al., 2006). But in the study by Gustafsson and Lönn (2003) individuals morphologically similar to var. *conopsea*, but-late flowering as var. *densiflora* were observed (Lönn et al., 2006). *G. conopsea* complex is definitely an example of taxonomically problematic group.

The two most commonly accepted taxa in recent treatments (e.g. Marhold et al., 2005) are:

- *G. conopsea* (L.) R. Br. s.s. (harbouring tetraploid and octoploid cytotypes plus at least three minority ploidies – see further, all based on x = 10)
- *G. densiflora* (Wahlenb.) A. Dietr. (plants with wider leaves, many-flowered inflorescence and an intense pleasant smell; 2n = 4x = 40)

G. conopsea (L.) R. Br. was originaly described by Carl Linne in 1753 under the name *Orchis conopsea* (or *conopea*). The genus *Orchis* was later divided and Robert Brown denominated this taxon *Gymnadenia conopsea* (L.) R. Br. In original conception this taxon included also *G. densiflora* (Wahlenb.) A. Dietr. Conception of this taxon is still heterogeneous and even in modern floras *G. conopsea* includes *G. densiflora* (Sebald et al., 1998). Wisskirchen and Haeupler (1998) recognize two subspecies in the frame of *Gymnadenia conopsea* (L.) R. Br. - ssp. *conopsea* and ssp. *densiflora.* Marhold et al. (2005) and Kubát (2002) and other authors accepted "split" of *G.conopsea* s.l. and adopted two individual and distinct species *G. conopsea* and *G.densiflora*.

G. densiflora (Wahlenb.) A. Dietr. was described already in 1806 from Sweden by Wahlenberg (1806) under the name *Orchis conopsea* var. [β] *densiflora*. This name was later transferred to the genus *Gymnadenia* and treated either as *G. densiflora* (Wahlenb.) A. Dietr., *G. conopsea* subsp. *densiflora* (Wahlenb.) K. Richt. or *G. conopsea* var. *densiflora* (Wahlenb.) Hartm. Wahlenberg´s name was recently lectotypified by Moberg and Nilsson (1991) by a specimen from Thunberg´s herbarium in Uppsala (UPS-THUNB no. 21193), providing the base for an unequivocal interpretation of this

name. Although in the past the taxon corresponding to *G. densiflora* was accepted in many floras (e.g. Ascherson and Graebner, 1905-1907; Soó, 1973), it is mentioned neither in Flora Europaea (Moore, 1980) nor in some recent Central European floras or identification keys (e.g. Adler et al., 1994). As a separate taxon it was accepted by Bisse (1963), Möseler (1987), Mrkvička (1993), and recently also by Wisskirchen and Haeupler (1998). However, there is a lot of confusion surrounding this name.

In addition, several other, more or less confusing names e.g. *G. conopsea* subsp. *montana* Bisse, *G. conopsea* subsp. *alpina* (Rchb. f.) Soó, the latter of which refers to (sub)alpine populations of the Alps and the Carpathians and subsp. *borealis* (Druce) M. Bateman, Pridgeon and M. W. Chase can be found in the literature (e.g. Soliva and Widmer, 1999; Delforge, 2006; Campbell et al., 2007).

Subspecies *borealis* (Druce) R. M. Bateman, Pridgeon and M. W. Chase (heath fragrant orchid; Rose, 1991; Foley and Clarke, 2005) is mainly found on hill pastures in northern England and Scotland at altitudes up to 700 m, especially in grassland dominated by *Molinia caerulea* (Campbell et al., 2002; Campbell et al., 2007). It flowers in July, later than *G. conopsea* but earlier than *G. densiflora*. It is smaller in mean stature than *G. conopsea* and *G. densiflora*, and has small, dark-pink flowers that smell of cloves (Campbell et al., 2007). All these taxa, *borealis*, *conopsea*, and *densiflora* cannot always be reliably distinguished on the basis of morphological characteristics, as many of these overlap and are variable within each taxon (Campbell et al., 2007). Some authorities refer to *borealis*, *conopsea*, and *densiflora* as ecotypes (Scacchi and de Angelis, 1989), some as subspecies (Stace, 1997), and others as species (Bateman et al., 1997; Pridgeon et al., 1997; Bateman, 2006), whereas one recent study using microsatellite markers on ten Swedish populations treated all plants as belonging to the same unspecified taxonomic unit (Gustafsson, 2000).

The short-spurred fragrant orchid, *Gymnadenia odoratissima* (L.) L.C.M. Richard, species well recognized and differentiated from *G. conopsea* (*G. conopsea* complex including *G. densiflora*), has a geographic distribution restricted to Europe and, except for the area from northwest Italy to Rumania where it is fairly common, its populations are scattered with large gaps (Gustafsson and Sjörgen-Gulve, 2002). This species is often use as an out-group e.g. in phylogenetic studies of *G. conopsea* complex for its close relationship to this complex.

1.2.3. Genetic and cytogenetic variation in *G. conopsea* complex

Several studies aimed at elucidating genetic variation using various molecular markers. Scacchi and Angelis (1989) classified plants as *G. conopsea* subsp. *conopsea* and *G. conopsea* subsp. *densiflora* in Italy using allozyme analysis. Combination of allozyme, cpDNA evidence and flower morphology was used in the study from Soliva and Widmer (1999) in Switzerland. In both cases there are no data on chromosome numbers of studied plants and their morphology which makes the unequivocal interpretation of their results difficult. Nevertheless, the Italian study showed clear heterogeneity within *G. conopsea* in respect of the allozymes. Scacchi and de Angelis (1989) called for further study of "dry" and "humid" ecotypes in the area of their study. They suggested that the ecotypes might be of some taxonomic significance. This is in accordance with observations from Marhold et al. (2005) that different taxa or different cytotypes in the complex of *G. conopsea* might differ also in ecological requirements. Soliva and Widmer (1999) found considerable heterogeneity among populations of what they considered *G. conopsea* subsp. *conopsea*, in comparison with their *G. conopsea* subsp. *densiflora*. They have found that the gene flow among populations of *G. conopsea* subsp. *conopsea* was higher than among those of subsp. *densiflora*, and that the gene flow between these two subspecies is low. They found four cpDNA haplotypes, which differed in the number of microsatellite repeats.

Much more controversial are studies published by Dworschak (2002). Without providing any substantial data or argumentation he published several species and subspecies new for science for the area of south Bavaria. Their value remains to be checked using standard taxonomic methods.

In addition, microsatellite primes were developed for fragrant orchid (Gustaffson, 2000; Gustafsson and Thorén, 2001), which opened up the way for detailed parentage analyses and identification of direction and amount of gene flow between individuals and populations (Gustaffson, 2000; Campbell et al., 2007). Also nuclear sequences of ITS were used to study the genetic divergence in *Gymnadenia* (Gustafsson and Lönn, 2003; Bateman et al., 2003; Lönn et al., 2006). Gustafsson and Lönn (2003) found low variability in ITS sequences in both flowering-time variants of *G. conopsea* and in *G. odoratissima* as well as Bateman et al., (2003) reported that *G. conopsea* and *G. odoratissima* have identical or

near-identical ITS sequences, despite their clear and reliable morphological differences. According Gustafsson and Lönn (2003) *Gymnadenia* sequences formed two distinct major clusters with high support from bootstrap analyses, with the late-flowering variant and *densiflora* in one cluster and the early-flowering variant and *G. odoratissima* with almost identical ITS sequences in another. Also Lönn et al., (2006) found the flowering time to clearly dividing the species into two groups, using slowly evolving ITS sequences and, in comparison, fast-evolving microsatellite markers. These data support the results gained by Soliva and Widmer (1999) and Scacchi and Angelis (1989) who also discovered differentiation between two subspecies *G. conopsea* ssp. *conopsea* and ssp. *densiflora* using allozymes analyses, as mentioned above.

Campbell et al. (2007) used five microsatellite loci to seek the genetic differentiation and restricted gene flow that would support the taxonomic division of *Gymnadenia* into three species *G. borealis*, *G. conopsea* and *G. densiflora* in United Kingdom. Three main conclusions were formed in this study. Firstly, there was a moderately high level of overall genetic variation despite proximity to the range edge for the genus, whereas, in many species, genetic diversity tends to be relatively low, especially in small and isolated populations. Secondly, universally high F_{IS} values indicated that *Gymnadenia* has an unusual population structure, probably with a high level of inbreeding. Thirdly, there was clear evidence of genetic differentiation compatible with earlier morphological classification of *G. conopsea* into three distinct taxonomic units.

From the above overview it is clear that there is a large amount of controversy in taxonomy of *G. conopsea* complex. Therefore, more sophisticated approach is needed, which includes testing all plants in a population sample for their ploidy level, their morphometric evaluation, in combination with the use of methods of molecular systematics. Considerable variation in the number of chromosomes in fragrant orchid has been known for several decades (see e.g. Löve and Löve, 1961) but it was rarely included in the studies.

G. densiflora in its current circumscription seems to be karyologically uniform; exclusively 2n = 4x = 40 (e.g. Marhold et al., 2005; Kubátová et al., 2008). This is in accordance with previouslay reported data from Moravia by Čihalík (Jongepierová and Jongepier, 1989), the data of Averyanov (1979) from Russia (St. Petersburg Region) and those of Agapova (1993) from Russia (St. Petersburg Region) and Estonia. However, these

data contradict to several records published by other authors who reported a tetraploid chromosome number 2n = 80 for *G. densiflora* - either as *G. densiflora* or *G. conopsea* subsp. *densiflora* mainly from Central Europe (Löve and Löve, 1961; Bisse, 1963; Groll, 1965; Mrkvička, 1993; Delforge, 2006). As was already mentioned, *G. densiflora* was described from Sweden and thus the data of Averyanov (1979) and Agapova (1993) concerning diploids are the reports closest to the place from where this taxon was described. The first who mentioned a tetraploid chromosome number for *G. densiflora* were Löve and Löve (1961). They subsequently assigned all previously published 2n = 40 records for *G. conopsea* s.l. to *G. conopsea* s. str. and the ones with 2n = 80 to *G. densiflora*, most likely without consulting any voucher specimens of the chromosome counts or performing any detailed study (and sometimes in contradiction with determination of plants in original papers). Groll (1965) also identified the plants with 2n = 40 as *G. conopsea* subsp. *conopsea* and plants with 2n = 80 as *G. conopsea* subsp. *ensiflora*. He noted, however, that part of the plants with 2n = 40 he studied had a different morphology, with more than 150 flowers per inflorescence, leaves more than 3 cm wide, and a very intensive scent. He classified these plants as *G. conopsea* subsp. *montana*. The morphological characters he provided for these plants perfectly fit *G. densiflora* in current sense. Groll left no voucher specimens (Christoph Dobeš, University of Vienna, pers. comm.), but from his notes on the morphology of the studied taxa we conclude that 2n = 80 reports in his study do not correspond to *G. densiflora*. Similarly, Bisse (1963) and Mrkvička (1993) did not leave or possess any voucher specimens and therefore it is hard to interpret their counts. For proper classification morphological and karyological characters have to be assessed for all individual plants and published data have to be critically reviewed.

Taxon indicated as *G. conopsea* subsp. *montana* Bisse, caused a lot of confusion. There is hardly any agreement on the interpretation of the name of this subspecies. Some authors, e.g. Groll (1965, see above) used this name for what we consider now to be *G. densiflora*. Its holotype has been most probably lost (Procházka, pers. comm.), and other herbarium material mentioned in the protologue (deposited in GFW) is heterogeneous. It remains to be decided if the name should be proposed for rejection because of these ambiguous interpretations or typified anew.

Some authors (e.g. Soó, 1973) recognize subalpine and alpine populations of the Alps and Carpathians as a separate subspecies or variety - *G. conopsea* subsp. *alpina* (Rchb. f.) Soó (= *G. conopsea* var. *alpina* Rchb. f.). This taxon has been sometimes mentioned also in popular literature (e.g. Procházka and Velísek, 1983; Potůček and Čačko, 1996), but not accepted in most recent checklists of vascular plants in Central Europe. Either it is not mentioned at all (Mirek et al., 2002) or treated only as a synonym of *G. conopsea* subsp. *conopsea* (Marhold and Hindák, 1998; Wisskirchen and Haeupler, 1998).

While the situation in ploidy level in *G. densiflora* seems to be less complicated, in current understanding of this species, at least five different cytotypes – 4x, 6x, 8x, 10x, and 12x has been reported for *G. conopsea* (Mrkvička, 1993; Marhold et al., 2005; Kubátová et al., 2008).

The question to be answered is the classification of tetraploid plants with 2n = 80. They were previously confused with *G. densiflora*, but as explained above and as shown in morphometric study by Marhold et al. (2005), they are well different from this taxon. Some authors consider them to belong to *G. conopsea*, but the relationships of diploid *G. conopsea* and these tetraploids remained unclear. Other authors tend to use the name *G. conopsea* subsp. *montana* for tetraploids (Procházka in Kubát, 2002; Marhold et al., 2005). The karyological variation of this group is complicated also by the fact that there are many cases where plants of different ploidy levels co-occur at the same locality. It has been reported from the Czech Republic, Slovakia, and Austria (Marhold et al., 2005; Kubátová et al., 2008). This complicates morphological evaluation of the population samples as it is hard to predict if the population sample is of mixed ploidy levels or not.

Obviously, further comprehensive studies including multi-disciplinary approach (e.g. studies of ploidy levels, population genetics, phylogenetics, ecology) are needed on this plant model.

1.3. *Phragmites australis* (CAV.) TRIN. ex STEUD (*Poaceae*)

1.3.1. Species description, distribution and habitats

Phragmites australis (CAV.) TRIN. ex STEUD (common reed, *Poaceae*) is a clonal grass species with woody hollow culms which can grow up to six meters in height. Leaves are lanceolate, often 20-40 cm long and 1-4 cm wide. Flowers develop by mid summer and are arranged in tawny spikelets with many tufts of silky hair. *P. australis* is wind-pollinated but self-incompatible. Seed set is highly variable and occurs through fall and winter and may be important in colonization of new areas. *P. australis* is a species with a high ability to propagate vegetativelly, while reproduction by seeds occurs only in early stages of settlement, when seeds have suitable conditions for germination and growth (Hürlimann, 1951; Weisner et al., 1993). Germination occurs in spring on exposed moist soils. Vegetative spread by below-ground rhizomes can result in dense clones with up to 200 stems/m^2. Common reed grows in marshes and swamps, along streams, lakes, ponds, ditches, and wet wastelands, often weedy and very difficult to eradicate, as the stoloniferous rhizomes may reach 10 m or more in length. It grows best in firm mineral clays, and tolerates moderate salinity, where water level fluctuates from 15 cm below soil surface to 15 cm above. Common reed tolerates burning if water is above soil surface, but burning is not essential for management. In the tidal environment, it is typically a freshwater species, but its salt tolerance allows it to expand into brackish and even marine environments (Soetaert et al., 2004).

In many parts of the world, *P. australis* is considered to be a highly valuable wetland species (Van der Putten, 1997). Beside its important role in wetlands ecology, *P. australis* has a wide variety of practical uses. It may be grazed or used for i.e. fences, roofs or starch production as well as for medicinal use.

1.3.2. Invasive *Phragmites australis* (CAV.) TRIN. ex STEUD in North America

Common reed is cosmopolitan species native to Eurasia (Den Hartog, 1989; Clevering and Lissner, 1999) but now widespread throughout the world, ranging all over Europe, Asia, Africa, America and Australia, however, the origin of the species is unclear. Until recently the status of the plant as native to North America or introduced has been in dispute. Concerning *P. australis* in North America, the scientists´ opinion is divided: some of them regard *P. australis* as a native North American species which may spread in an uncontrollable way, while others consider it to be a species introduced from Europe (possibly by French settlers) - but rather recent paleobotanical finds of reed remnants in deep and pre-Columbian peat deposits do not support this opinion (Saltonstall, 2002). In view of the aggressive behavior and luxuriant growth of certain reed clones in disturbed habitats in North America (e.g. road ditches) while other clones seem to be non-aggressive, a hypothesis also exists that the non-aggressive reed clones are native American ones while the more aggressive and productive ones are of European origin (Metzler and Rosza, 1987; Tucker, 1990; Marks et al., 1994; Besitka, 1996; Chambers et al., 1999; Saltonstall, 2002). New works has demonstrated the existence of native and introduced genotypes of *P. australis* (e.g. Marks et al., 1994; Saltonstall, 2001, 2002, 2003a, b, c).

P. australis has been an uncommon member of mixed tidal wetland plant communities in North America for at least 3000 years (Niering et al., 1977; Orson et al., 1987). In the 19th but particularly in the late 20th century, *P. australis* began invading fresh and brackish wetlands in North America greatly expanding its range and abundance. Mixed wetland plant communities are replaced by near monocultures of *P. australis* resulting in changed ecosystems, alternation in ecological processes and functions and associated detrimental impacts on native wildlife (Hauber et al., 1991; Havens et al., 1997; Marks et al., 1994; Meyerson et al., 2000). Dense monospecific stands of an invasive non-native genotypes have out-competed other native wetland vegetation types in North America (Chambers, 1997; Havens et al., 1997; Saltonstall, 2002) and common reed is often considered as undesirable pest species (Weinstein and Balletto, 1999). *P. australis* is most abundant along the Atlantic Coast and in freshwater and brackish tidal wetlands of the northeastern United States as far south as North Carolina. It occurs in all eastern

states and populations are expanding, particularly in the Midwest. At present, *P. australis* occurs throughout the entire United States (except Alaska and Hawaii) and southern Canada (Saltonstall, 2002).

Although, hybridization with closely related species is common after an exotic species enters a new area (Anderson and Stebbins, 1954; Lewontin and Birch, 1966), which can quickly change the genetic composition of native populations and cause loss of local adaptations (Anttila et al., 1998; Huxel, 1999) no evidence of hybridization between native and non-native population types of *P. australis* has been detected (Saltonstall, 2003b) despite sympatric growth of both types of reed is known. The population explosion of *P. australis* is often thought of being facilitated by changes in land use patterns and hydrologic regimes, increased disturbances, urbanization and eutrophication (Marks et al., 1994).

Saltonstall (2002) has confirmed the present-day existence of native North American haplotypes (lineages) and introduced European haplotypes as well as their common sympatric growth in North America. A total of 27 haplotypes were identified of which 11 (A-H, S, Z, AA) are native to North America (Saltonstall, 2002). Within the North American populations, a continuum of geographic substructuring exists for the native haplotypes. Types AA, F, Z and S are known historically form the Northeast; types E, G, and H are found throughout the Midwest and types A-D are found in the South and Intermountain West only. Two haplotypes show worldwide distribution (I and M) with M as the most common type in North America, Europe and Asia. The type I is found along the Gulf Coast and also occurs in South America and Asia (for more details see Saltonstall, 2002).

Comparing the genetic structuring of present-day populations with those available in herbarium specimens collected prior to 1910 reveals significant changes in haplotype frequencies in North America. While the herbarium samples show a widespread distribution of native haplotypes across North America, modern populations show a striking range expansion of the M haplotype (for more details see Saltonstall, 2002). Type M has entirely replaced native types in New England and expanded to the southeast where no historic *P. australis* populations were known to occur. Type M (which is most closely related to other European types) had spread to the West and is also becoming prevalent in the Midwest. It is likely that the introduction of type M material has occurred

sometimes in the early part of the 19th century, probably at several Atlantic coast ports. Over the last 150 years, among-population variation in North America has declined significantly and today the genetic structure of North American populations resembles that of European common reed populations.

1.3.3. Native *Phragmites australis* (CAV.) TRIN. ex STEUD in Europe

In Europe *P. australis* is the most common herbaceous dominant of temperate freshwater marshes (Hejný, 1960; Haslam, 1972, 1973; Rodewald-Rudescu, 1974), valued for numerous ecological functions: as a major part of the buffer zone between terrestrial and aquatic ecosystems, for shoreline stabilization, and for providing waterfowl habitat.

Behaviour of common reed is essentially different in European and North American wetlands. While in North America it is considered as an invasive species in Europe we encounter rather decline of its populations. The significant decline in reed belt area has been observed at approximately 60 lakes (Koppitz et al., 2004) and river sites (Ostendorp, 1989; Van der Putten, 1997) in Europe. A lot of ecological factors have been made responsible for the die-back of reed, and many studies have tried to discover ecophysiological mechanisms and tolerance limits of *P. australis* (e.g. Ostendorp, 1989; Van der Putten, 1997; Brix, 1999). As well as changes in land use patterns and hydrologic regimes, increased disturbances, urbanization and eutrophication are often listed as factors causing the population explosion of *P. australis* in North America (Marks et al., 1994) the very same factors are thought to cause declines of *P. australis* in Europe (Van der Putten, 1997). A hypothesis was put forward in the 1990s that in originally diverse populations only such clones would persist that were able to survive under the particular site conditions; this would be reflected by a reduced genetic variability within the population. The resulting populations, consisting of only a few large monoclonal stands, were supposed to be more vulnerable to rapid changes in environmental conditions such as eutrophication, because the low genetic diversity would not provide the stands with a sufficient phenotypic plasticity needed for successful adaptation. This was regarded as a possible explanation for the low genetic variability found in reed populations in some European lakes and for their decline (Eriksson, 1993; Koppitz et al., 1997). The recent die-back of *P. australis* from shoreline habitats of European lakes led to the interest

of biologists. At the same time, comparatively less attention has been paid to reed expansion, which has been observed into other types of habitats, i.e. mainly wet meadows dominated by tall sedges or wetland grasses and into new areas of secondary distribution.

Reed die-back may be caused by a variety of factors, such as eutrophication and its possible impact on carbohydrate storage dynamics (Čížková et al., 1996; Kohl et al., 1998), toxic effects of sapropelic compounds (Armstrong et al., 1996), reduced rhizome ventilation (Armstrong and Armstrong, 1991; Brix et al., 1992; Kohl et al., 1996; Armstrong et al., 1999) or the artificial regulation of the water level (Van der Putten, 1997). Factors as flooding, insect or fungal infestation, mechanical damage and loss of culms can reduce the convective gas flow to the basal parts of the reed plants (Armstrong et al., 1996). This leads to an oxygen deficiency in the roots and the rhizomes, changing the metabolic status of the whole plant. Fermentation processes may become active producing ethanol, lactate and some other compounds (Brändle and Crawford, 1987). Presumably, the shift in central metabolic pools is reflected by changed amino acid patterns in the rhizomes and in the basal culm internodes of *P. australis* (Kohl and Henning, 1987; Haldemann and Brändle, 1988; Kohl et al., 1998).

Recently it has been suggested that low genetic variability might also contribute to reed decline (Kühl and Neuhaus, 1993; Neuhaus et al., 1993; Koppitz et al., 1997). The low genetic variability of natural reed stands observed in some regions is thought to be the consequence of special strategies of colonization (Kühl and Neuhaus, 1993; Koppitz et al., 1997). According to this, reed populations initiated by seeds are initially genetically diverse, but over time become dominated by one or a few clones well-adapted to the prevailing site conditions. If a site is initially colonized by only a few seedlings reflecting different genotypes, which spread vegetativelly thereafter, the resulting stand will necessarily have low genetic diversity. Low diversity can also be expected if initial colonization is effected by a high number of genotypes, but only the well-adapted ones survive and spread (Koppitz and Kühl, 2000).

According to the model of establishment and development of reed stands (Koppitz et al., 1997), the colonization of shores by *P. australis* starts with a variety of new clones. This process is supposed to be the first of three main stages of the establishment of reed clones, the stage of settlement (Kühl and Neuhaus, 1993; Koppitz et al., 1997). In this

stage, the reed colonizes suitable areas by generative propagation. This results in reed stands with high genetic diversity.

In the case of monoclonal or oligoclonal populations, as found in the study Koppitz and Kühl (2000) at many of the water reed sites, the extent of reed die-back of the whole population may be considerable as soon as one of the clones is not adapted to a specific change in conditions. Drastic decline may also be expected at stands where the clones are arranged in clusters or in a row along the shoreline. Moreover, the remarkable differences in performance (in terms of morphology and expansion) of different clones after having been planted at a site with similar site conditions point to a strong influence of the genotype on the development of individual reed clones within a reed stand (Kühl, 1999; Kühl and Zemlin, 2000; Koppitz et al., 2000). Contrary to monoclonal stands, polyclonal (intermixed or mosaic-like) reed stands are less likely to experience a drastic decline after a change in site conditions; instead, 'invisible reed decline' may occur (Kühl and Neuhaus, 1993). If the different clones of a reed stand do not react to stressors in the same way due to different physiological and morphological features, only few clones of the population may be affected by die-back. In that case, some clones would disappear, but the remaining tolerant clones may expand into the gaps. This process would not become apparent as a die-back of the whole population, but it would result in a decrease in the number of clones, and thus a decreased ability to react to stressors and a reduced plasticity of the reed population (Koppitz et al., 1997).

The history of colonization of sites by *P. australis* is important to understand the reactions of reed stands to changes of environmental conditions. Particular clones may exhibit a high degree of plasticity, showing a 'general-purpose genotype' that occupies a wide range of environments, as described for clonal genotypes (Lynch, 1984; Bierzychudek, 1989). However, in general each *Phragmites* clone has probably relatively narrow tolerance limits in relation to its special site conditions. The existence of different ecotypes specifically adapted to local site conditions has already been considered (Björk, 1967; Dykyjová and Hradecká, 1976; Kühl et al., 1997; Čurn et al., 2007). The often described high ecological plasticity of the species *P. australis* (Haslam, 1972; Van der Toorn 1972; Rodewald-Rudescu, 1974; Křiváčková-Suchá et al., 2007) may, consequently, be the result of the high number of reed clones. The considerable differences in genetic diversity as observed e.g. in Koppitz and Kühl (2000) could be one reason for the differing

abilities of reed stands to react to changing site conditions and, eventually, to survive or to die-back. The observable dynamics depends on the reaction of individual clones, e.g. the possibility of tolerant clones to expand into the gaps left by sensitive ones (Čurn et al., 2007). It follows that the extent of reed decline after a deterioration of site conditions may be determined by a variety of factors including genetic diversity, the amplitude of adaptability of each clone, and the type, strength and velocity of the changes.

1.4. *Lythrum salicaria* L. (*Lythraceae*)

1.4.1. Species description, distribution and habitats

Lythrum salicaria L. (purple loosestrife, *Lythraceae*) is an erect perennial herb and it is an example of very important invasive plant species with native/primary area of occurrence in Eurasia and invasive in both North and South America, South Africa, Australia. It persists in a wide variety of environmental conditions; in littoral vegetation of fishponds or streams (Hejný, 1960), rock-pools (Hæggström and Skytén, 1987), mud-flats raising after lowering of water level (Toivonen and Bäck, 1989), fen meadows or woodlands, swamp basins on mineral substratum or open woody margins (Wheeler, 1980).

1.4.2. Causes and conditions of plant invasions

Invasive species are usually described as one of the main causes of biodiversity loss, as they can negatively affect native biota (Diamond, 1989; Stewart, 1991; Strayer et al., 2006). Human-mediated species invasions are increasing worldwide (Mack et al., 2000; Ruiz et al., 2000) and as well as worldwide commerce and travel increase, the threat of invasions by non-native plants, animals, and pathogens also increases. These organisms threaten not only human health and agro-ecosystems but also the natural environment (Schrag and Wiener, 1995). The spread and establishment of invasive organisms are considered to be a major environmental problem. The presence and subsequent domination by invasive species leads to greatly reduced biodiversity and possible changes to ecosystem processes in their new areas of distribution (Vitousek et al., 1997).

Understanding the factors that allow successful establishment of non-native species and subsequent invasions is a key task for conservation biology, and a necessary prerequisite for plans of prevention, screening controls, monitoring, and management (Kolar and Lodge, 2002; Fofonoff et al., 2003; Hulme, 2006; Lodge et al., 2006; Rollins et al., 2006; Strayer et al., 2006). Moreover, knowledge of the origins of non-native species can provide insight into their biology, facilitate the selection of optimal biological control agents, and provide information on the history of invasion and human transport (Collins, et al., 2002; Downie, 2002; Cognato et al., 2005; Goolsby et al., 2006). Determining

the source of non-native species is also essential for ecological and evolutionary studies. For example, data collected from the genuine source provides an evolutionary baseline that allows comparisons of phenotypic traits, allele frequencies, and genetic diversity among introduced and native populations (Wares et al., 2005). Moreover, ecological studies designed to explore determinants of invasion success often require information on the source of introduced individuals to facilitate the selection of appropriate contrast groups (Colautti et al., 2004; Hierro et al., 2005).

After non-native species enters a new area and establishes vital/viable community two possible ways of its development and spreading can occur/arise. Often a time lag is observed in the development of invasive species, which remain close to their point of establishment for decades before range expansion (Cousens and Mortimer, 1995; Kowarik, 1995; Mack et al., 2000). Whether a non-native species becomes invasive depends to a large extent on evolutionary processes (Sakai et al., 2001). In some cases, population growth may be delayed until the species evolves new adaptations through reassortment of existing genetic variability (Brown and Marshall, 1981; Clegg and Brown, 1983). Multiple introductions may be important in providing sufficient genetic variability to allow this differentiation to occur.

1.4.3. Invasion of *Lythrum salicaria* L. in North America

L. salicaria is one of the first plant species which colonize bare ground in recently disturbed wetlands (Hejný, 1960). *L. salicaria* colonizes hydrologically similar habitats in both Europe and North America (Bastlová-Hanzelyová, 2001). However, due to different climatic conditions at similar latitudes, *L. salicaria* grows further south in North America. Therefore, European and North American populations with similar growth characteristics (e.g. shoot height, flower phenology) grow at different latitudes (Edwards et al., 1998). While in native areas *L. salicaria* became a dominant species of plant communities only exceptionally, it was documented as important plant species of invaded wetland habitats in North America (Bastlová – Hanzélyová, 2001). *L. salicaria* has followed the classic pattern of establishment and range expansion of invasive plants (Batra et al., 1986). Although purple loosestrife has an affinity for moist soil zones of wetlands, lakes and rivers in Europe and North America, its dominance in North

America, where it makes up more than 50% of the biomass of emerged vegetation in many areas, is higher than in Europe (Thompson et al., 1987).

Purple loosestrife was firstly, and then probably repeatedly, introduced to the North America in the early 19th century for ornamental and medicinal use. The most frequent occurrence was recorded around the St. Lawrence River. It soon became a popular ornamental plant widely distributed by beekeepers and horticulturists (Stuckey, 1980; Thompson et al., 1987). Although it spontaneously colonized various wetlands mainly in Great Lakes territory, it was relatively sparse until the 1930s when monoculture stands were recorded in Canada in Quebec. Since then, purple loosestrife has spread throughout the NA continent and now it grows in all states of the USA and nine adjacent provinces of Canada (Welk, 2004). *L. salicaria* often forms a monospecific community in North America (Blossey, 1999). Its establishment in North American wetlands is thought to lead to a decrease in the density and diversity of native flora and fauna, may be instrumental in restricting boat traffic in canals and reduce the economic viability of wet meadows for grazing and other economic activities (Thompson et al., 1987).

Typically, *L. salicaria* remains unobtrusive for a long period (at least 20 years) followed by a brief period (of less than 3 years) in which it becomes dominant at multiple locations across a region (Stuckey, 1980). Therefore, a number of management strategies have been implemented (e.g. mechanical disturbance, use of chemical herbicides, and biological agents) to control its invasion (Henne et al., 2005). Besides North America, purple loosestrife has also been introduced into South America (Argentina), New Zealand, and Australia (Wangerin and Schröter, 1937).

Although several hypotheses have been proposed to explain differences in invasion success among species (Kolar and Lodge, 2001; Rejmánek et al., 2005; Wilson, et al., 2007), it has been difficult to find general patterns. Propagule pressure (i.e. the number of individuals introduced and/or the number of introductions performed) is one of the few factors over which a general consensus has been reached: when a high number of individuals are introduced and/or introductions occur on several occasions, the species is more likely to establish and become invasive, for both demographic and genetic reasons (Kolar and Lodge, 2001; Lockwood et al., 2005; Von Holle and Simberloff, 2005; Colautti et al., 2006). High divergence in populations of invasive species indicates multiple introductions from different sources (Sun, 1997). By contrast, species that have entered

and quickly spread into a new area via a small number of introduction events may show lower genetic diversity in the introduced range (Schierenbeck et al., 1995; Ansellem et al., 2000). Often, new populations of invaders are formed by just a few individuals from the native habitat (Elton, 1958), which potentially leads to decreased population level genetic diversity as a result of genetic bottlenecks and founder effects (Nei et al., 1975; Ansellem et al., 2000). However, other studies have shown that the levels of genetic variability among populations in the native and introduced ranges can be similar (Burdon and Brown, 1987). There were probably multiple introductions of *L. salicaria* into North America with the populations coming from different areas in Europe (Stuckey, 1980). Hypothetically, this allowed a greater contact in North America between populations which would normally not interact in Europe. Then a greater possibility of gene transfer between different European populations was possible, which might lead to greater genetic heterogeneity in the invasive North American populations compared to the genetic makeup of the native, Eurasian populations. This hybridization, producing a more heterogeneous genotype in North America, would allow for a more competitive genotype to be produced.

In addition, factors such as adaptive evolution to new environmental conditions, introduction of vigorous genotypes predisposed to successful expansion, introgressive hybridization with native allied species, evolution of increased competitive ability due to a lack of native herbivores, and targeted selection of vigorous genotypes by beekeepers and gardeners have been suggested among potential explanations of successful invasion of purple loosestrife (Thompson et al., 1987; Blossey and Notzöld, 1995; Houghton-Thompson et al., 2005).

There is considerable variation in plant phenology, life history, biomass allocation, and morphology across the primary range of distribution, generally showing a north-south gradient (Olsson and Ågren, 2002; Bastlová et al., 2004). Robust and late-flowering individuals originating from southern geographic areas (e.g. Israel and Spain) were found to be more similar in growth dynamics and phenotype characteristics to invasive North American plants than their counterparts from Central and northern Europe (Bastlová and Květ, 2002).

The greater dominance of purple loosestrife in North American wetlands compared with Eurasia may be due to additional genetic differentiation after it arrived into North America, unveiling previously untapped invasive potential. The alternative hypothesis

is that adaptively significant genes could have introgressed from a close North American relative. Inter- and intra-specific hybridization may be the nucleating event that ends the lag phase (time from introduction to invasion) by providing new sources of adapted genes (Abbott, 1992; Ellstrand and Schierenbeck, 2000). Hybridizations between native and introduced species have often led to the development of new taxa and have even been implicated in the evolution of a number of new invasive species. If hybridization played a role in purple loosestrife becoming invasive, the most likely candidate is *Lythrum alatum* L. (winged loosestrife), a widespread diploid species in North America that has considerable habitat overlap with tetraploid purple loosestrife, but is typically found in drier areas. Winged loosestrife is a shorter, less showy species than purple loosestrife (Blackwell, 1970), and grows in wet meadows as a sub-dominant (Cody, 1978). There is evidence that the genomes of winged and purple loosestrife are compatible even though their ploidies differ, as cultivars of purple loosestrife have been generated by hybridizing the two species (Anderson and Ascher, 1993). Most of the interspecies cultivars are self-sterile, but several studies have shown that they are fully fertile when crossed with the wild species of purple loosestrife (Anderson and Ascher, 1993; Lindgren and Clay, 1993; Ottenbreit and Staniforth, 1994). Although no direct evidence of hybridization in the wild has been observed between winged and purple loosestrife, bees and butterflies are known to move between these species in sympatric populations (Levin, 1970), and morphological characters have been observed in purple loosestrife populations in Minnesota that are not found in Europe (Anderson and Ascher, 1993; Houghton-Thompson et al., 2005).

Other possible mechanism explaining the success of *L. salicaria* is the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Nötzold, 1995), in which the biomass allocation patterns in non-native populations will be shifted, compared to similar patterns in native populations of the same species. While introduced plants should allocate more photosynthates to vegetative growth due the absence of herbivores and other natural control agents in the secondary area native plants have to allocate part of their photosynthates against herbivore defense. Then it is possible that the introduced populations will evolve into a more competitive genotype, compared to native populations of the same species. An alternative to the EICA hypothesis, is that species which successfully invade new geographic areas come from more competitive genotypes of the species in its native area (di Castri et al., 1990). This is more likely

to occur when the involved species has a wide geographic distribution, such as *L. salicaria* (Peacock and McMillan, 1968) and also gene flow/interchange between genotypes and forming of new vigorous can occur after repeated introduction of different genotypes originating in different areas of primary area of purple loosestrife. In this way, the successful invasive populations of *L. salicaria* may come from native, Eurasian genotypes that have certain morphological and life history characteristics, which evolved in response to particular local conditions in the home range, e.g. day length (Ray and Alexander, 1966; Weber and Schmid, 1998).

While many papers studying *L. salicaria* were addressed to its ecology, phenology and morphology there is a lag of studies comprehensive genetic or cytogenetics variability though these methods are recently very popular in plant population biology. Kubátová et al. (2008) published a paper comprising a detailed study of ploidy-level variation in native and invasive populations of *L. salicaria* (see list of papers). It was supposed that the invasiveness of plants from invasive population of purple loosestrife may be induced by a switch in ploidy level. But the invasive spread of North American populations was not triggered by differences in ploidy level since the variability in ploidy level in native population was 2x, 3x, 4x and 6x with highly prevailed tetraploids and plants form invasive North American populations were cytologically uniform and represented only tetraploids.

Purple loosestrife is an interesting biological object and some studies comprising population genetic structure, cytogenetic and morphological variability of this species in both areas of distribution may help to explain its invasive behavior.

2. Application of different methods in plants population biology

2.1. Methods applied and presented in this PhD thesis

Currently, there are many available methods from different branches of biology which can be applied in plant population studies. In this PhD thesis (in the papers and manuscripts included in it) I used three different approaches and/or their combination in the projects where different taxa or species were studied.

In this thesis I utilized morphological, cytogenetic and molecular-genetic approaches to solve different questions from the area of plant biology, taxonomy and population genetics.

In two studies I used morphological markers together with molecular markers to investigate phenotypic and genotypic variation in populations of *Phragmites australis* originating from two human-made lakes of different age and life history. In both cases, clones of *P. australis* and their genotypic variability were identified using RAPD technique. In the first study (Čurn et. al., 2007) morphological and growth characteristics of plants were measured in the field at morphologically distinct homogeneous stands. Then the data gained by using these two above mentioned approaches were compared. In the second study (Křiváčková-Suchá et al., 2007) representative plants were chosen at both investigated localities and transplanted in to the common garden experiment. The clones were propagated vegetatively and cultivated under comparable conditions for one vegetation season. Morphological and growth characteristics of the cultivated plants were recorded and compared with those found in the original reed stands. Preliminary investigation using flow cytometry was carried out for all stands at both lakes involved in this studies (Kubátová et al., unpublished results) to see whether the differences in morphology were not caused by different ploidy levels and what is the cytogenetic structure of studied populations. The third study (Kubátová and Čurn, 2008) was also focused on *P. australis* and methodologically followed the two previously presented studies. However, only DNA markers were utilized here. I aimed this study at direct comparison of different molecular markers as RAPD, microsatellites, AFLP and particularly sequencing of cpDNA. Combination of several DNA techniques allowed

evaluate the data gained from dominant and codominant markers and also allowed to investigate whether the used methods provide similar information about genetic structure and distribution of variation across studied populations.

I utilized flow cytometry to investigate the population cytotype structure in *Gymnadenia conopsea* complex and to solve questions whether the occurrence of ploidy-mixed populations is presented as well as to find out frequency of minor cytotypes and to reveal what is the distribution pattern of different cytotypes within a population (Kubátová et al., 2008).

Flow cytometry was also suitable technique for detailed study of *Lythrum salicaria* populations (Kubátová et al., 2008). In this study was precisely documented variation in ploidy levels in both native and invasive populations of *L. salicaria* in their primary and secondary area of occurrence. The study was addressed to the hypothesis that species invasiveness had been induced by a switch in ploidy level.

2.2. Application of flow cytometry in plant population biology

2.2.1. Potential and exploitation of flow cytometry in study of plant populations

Flow cytometry (FCM) is a unique tool combining a powerful analytical capability to measure simultaneously many optical parameters of cells and subcellular particles, with the potential to isolate purified populations of selected particles (Doležel et al., 2007). The principles of FCM can be simply explain as measurement of proprieties of isolated cells and subcellular particles/organelles flowing in single file within a liquid sheath as they are intercepted by a high-intensity light source focused in a very small region (Robinson and Grégori, 2007). Both analysis and sorting can be carried out at high speed so that statistically relevant samples can be measured and minor subpopulations detected (Doležel et al., 2007). Analysed objects are interrogated in a very short time (a few microseconds) during which multiple signals are collected, mainly light scatter and fluorescence emissions in the visible spectrum (Robinson and Grégori, 2007). The ability to isolate large numbers of particles of the same type then provides enough material for subsequent manipulations, including further growth and culture, as well as analysis with biochemical and genomics techniques (Doležel et al., 2007). The progress in electronics and informatics facilitate flow cytometers readily analyze single particles/cells at rates of up to 100 000 per second. It is thus possible to discriminate particles/cells into clusters based on statistical analyses of the set of parameters collected for each particle (Robinson and Grégori, 2007).

Flow cytometry was originally a domain of biomedicine research. Its high cost and unavailability of instruments capable for applications in plant biology did not promote the use of FCM in plant sciences. After the pioneering paper of Heller (1973), the first uses of FCM in plant sciences were only reported in 1980s and their numbers did not increase markedly until the early 1990s. Despite a rather slow start, the past two decades resulted in a well-established and flourishing area with many applications in basic and applied research as well as industry (Doležel et al., 2007; Robinson and Grégori, 2007). The use of flow cytometry in plant science has rapidly expanded, particularly in evolutionary biology, ecology, and systematics. The dominant use of FCM in these fields is focused

on the estimation of nuclear DNA content in absolute terms or in relative units, as an indicator of ploidy level Application other than DNA content estimation, e.g. side and forward scatters and particle volume measurement are still relatively scarce. The emphasis on DNA content has been driven primarily by increasing interest in the magnitude and causes of genome size variation and demand for effective and rapid cytotype discrimination. To date, ecologists and evolutionary biologists have exploited only a fraction of the FCM potential, but have already made major advances with far-reaching implications (Suda et al., 2007).

Nowadays, FCM has become an essential tool in the population biologist's kit. In combination with other molecular and phenotypic approaches, it promises qualitative advances in our understanding of genome multiplication and the population biology of vascular plants (Suda et al., 2007). The main applications of FCM in plant population biology and taxonomy and also applications and approaches used in my thesis are: estimation of absolute nuclear genome size, determiantion of ploidy level and cytotype structure of populations.

2.2.2. Estimation of nuclear genome size

The nuclear DNA content varies widely among flowering plants and even between closely related species. However, since most of the variation appears to be a historical legacy it is difficult to determine the sequence of events by which DNA variation occurs (Cullis, 2005). Early attempts to estimate DNA amounts in cell nuclei (Caspersson and Schultz, 1938) preceded the discovery of its central role in heredity. Shortly after, a constancy of DNA amount per organism was established and the term 'C-value' was coined by Swift (1950), referring to the DNA content of an unreplicated haploid chromosome complement (n). Hence, a nucleus in G1 phase of the cell cycle, with two copies of unreplicated genome has a 2C DNA amount (Doležel and Bartoš, 2005). Subsequently, it was found that there was no relationship between DNA C-value and organismic complexity (Mirsky and Ris, 1951). The lack of correlation was later termed 'C-value paradox' by Thomas (1971). The discovery of non-coding DNA and its non-genic, structural function provided a clue to the paradox, but the origin, function and significance of variation in DNA content remain enigmatic. Several theories have been

proposed to explain the 'C-value enigma' (Gregory, 2001). Scientific disciplines, which profit from the knowledge of C-values, are numerous and include molecular biology, systematics and ecology (Bennett et al., 2000). Despite its importance, C-values are known for only a fraction of all plant species, e.g. only 1.4 % of angiosperms (Hanson et al., 2003) with values ranging between 0.065 pg to 125 pg (Bennett and Leitch, 2001; Greilhuber et al., 2005). Regardless this limited number, results show that genome size significantly correlates with many morphological (Vekemans et al., 1996), phenological (Baranyi and Greilhuber, 1999) or ecological (MacGillivray and Grime, 1995) characteristics of taxons. A negative relationship between the genome size and plant weedy status was also shown (Bennett et al., 1998) and one of the theories looking for reasons of plant taxon invasiveness assume importance of relation between the invasiveness and nucleus DNA amount (Rejmánek, 1996). Reasons and consequences of intraspecific variability in genome size (and composition) are often discussed. Although presently used models include stabile amount of DNA within the species (Bennett et al., 2000), molecular mechanisms significantly affecting nuclear DNA amount within the short period of time are also known (Flavell et al., 1974; Kubis et al., 1998). Nuclear DNA amount is undoubtedly a key character, with many uses in various biological fields.

2.2.3. Determination of ploidy level

Although flowering plants (the Angiosperms) vary tremendously in nuclear DNA content, most of this variation is not associated with differences in gene number or gene size. One of important evolutionary force in plants is polyploidy. Polyploidy is a rapid and dramatic mechanism that can double gene content and genome size in a single generation, and most or all flowering plants are either current polyploids or have a polyploidy origin (Wendel, 2000). In fact, it has been suggested that >4% of all speciation events in plants involve polyploidization (Otto and Whitton, 2000). Chromosome counting in dividing nucleus is an unambiguous way to determine the ploidy, or the number of basic chromosome sets in cell nuclei. However, this is time consuming and tissues containing dividing cells may not be readily available. As the nuclear DNA content correlates with ploidy level, estimating relative nuclear DNA content, FCM offers a faster and more convenient method of detecting ploidy level than other methods and enables large-scale

surveys on the landscape, population, individual and tissue scales. Nevertheless, because ploidy level is inferred only indirectly using FCM, karyological and cytometrical results should be distinguished from each other, with the latter designated by the prefix DNA, i.e. DNA ploidy (Suda et al., 2006).

The first use of cytometric assessment of ploidy level was focused on crop plants (de Laat et al., 1987) and this application is still prevailing in plant breeding. Application of FCM in plant breeding helps in several areas as (i) ploidy characterization of gene resources (e.g. in gene banks); (ii) control of ploidy stability at various steps in breeding programmes, including in vitro cultures; (iii) screening for desired cytotypes after ploidy manipulation and hybridization, including the detection of mixoploids (cytochimeras). It is a great advantage of flow cytometric ploidy screening that it may be performed at early ontogenetic stages, then it save both space and time during the breeding process (Väinölä, 2000). Although agriculture and horticulture lead the way in ploidy studies, the last decade has also seen significant progress in wild plant research.

The pioneering work dealing with ploidy heterogeneity in natural populations of *Andropogon gerardii* (*Poaceae*) had already appeared in 1987 (Keeler et al., 1987). In plant biosystematics, ecology, and population biology, FCM is primarily used to address questions of phenotypic manifestation, spatial distribution, and evolutionary significance of genome duplication. Some data indicate that polyploidy may also contribute to invasive behaviour and spread of alien plant species, as documented, for instance, in the genera *Carpobrotus* (Vila and D'Antonio, 1998), *Spartina* (Ainouche et al., 2004) and *Tragopogon* (Soltis et al., 2004). Similarly, ploidy level was found to be the major determinant of hybrid fitness between invasive *Rorippa austriaca* and native *Rorippa sylvestris* (Bleeker and Matthies, 2005). In addition, Bennett et al. (1998) claimed that the proportion of polyploids is higher in weedy species than in other plant groups. Because representative sampling is possible, novel insights into the extent of intra- and interpopulation ploidy variation, niche differentiation, and ecological preferences of individual cytotypes can be gained. Recently, increased attention has attracted surveys of interactions between particular cytotypes and other trophic levels (e.g. pollinators, parasites, symbionts), triggered by the discovery that polyploidy may have important influences on the structure and diversification of terrestrial communities (Thompson et al., 1997). Such conclusions clearly highlight the indispensability of cytotype determination

(most effectively realized by FCM) in any experimental study that may involve heteroploid plant samples. In systematics and evolutionary biology, FCM is an essential tool for quantifying spatial and temporal patterns of ploidy variation and identifying cryptic taxonomic structure. With high resolution and success on gametes as well as somatic tissue, FCM also offers insights into questions of process as well as pattern. Research has focused on mating systems, unreduced gamete production, interactions among intraspecific cytotypes, and dynamics of hybrid zones (Suda et al., 2007).

2.2.4. Assessment of cytotype structure in plant population

Cytotype distribution over vast geographic areas provides useful insight into population biology of multi-ploid taxa. However, large-scale cytotype investigations had been enormously time-consuming until flow cytometry was routinely employed (Lewis et al., 1967). Fortunately, papers based on 1 500 plus individuals are far from the utopia nowadays (e.g. Kubátová et al., 2008). This approach and utilization of FCM in cytotype determination also evoked to raise numerous challenging questions of current biosystematics, such as the overall distribution pattern (sympatry vs. parapatry), investigation of evolutionary forces governing the cytotype co-existence, ecological preferences of individual cytotypes, inter- vs. intra-cytotype competition, or mechanisms of reproductive isolation (Petit et al., 1999).

The unique feature of the polyploid speciation is that a new evolutionary entity can arise abruptly through a seemingly single and rapid genetic event. Because polyploidization potentially confers immediate reproductive isolation, it is widely acknowledged as the major mode of sympatric speciation (Coyne and Orr, 2004). While different ploidy levels may correspond to different taxa (Rosenbaumová et al., 2004; see also Soltis et al., 2007), intraspecific ploidy variation is not a rare phenomenon either (Lihová et al., 2003; Suda et al., 2007).

When more cytotypes occur within the same species, zones of ploidy overlap are often formed. Contact zones are of particular interest to evolutionary biologists because they allow studying mechanisms involved in early stages of polyploid speciation and/or assess selective forces operating in ploidy-mixed populations. Sympatric growth of different ploidy races is mostly explained either by a directional or a balanced selection

(Weiss et al., 2002). The former theory assumes that cytotype intermingling is only a transitional stage and one cytotype will finally be outcompeted (i.e., the minority cytotype exclusion; Levin, 1975). By contrast, the latter type of selection assumes that a long-term co-existence can be maintained due to assortative mating that reinforces isolation between related cytotypes. A number of prezygotic and postzygotic breeding barriers has been identified (Husband and Sabara, 2004), including spatial segregation and microhabitat differentiation of cytotypes (e.g., Felber-Girard et al., 1996), flowering time divergence (Petit et al., 1997), gametic selection (Husband et al., 2002), and selection against triploid hybrids (van Dijk et al., 1992). Evolution of assortative mating in relation to behavioural mechanisms can also be an important component of the successful establishment and/or co-existence of animal-pollinated plant species (Thompson et al., 2004). Two kinds of contact zones are mostly recognized according to their evolutionary history (Petit et al., 1999), i.e., primary (when a new cytotype originates *in situ*) and secondary (when two formerly allopatric cytotypes meet). It has been suggested that reproductive isolation is more readily achieved in the latter one (Petit et al., 1999).

Despite the major role of genome duplication in plant speciation, it is still largely unknown how different factors act in concert to achieve reproductive isolation among ploidy levels in mixed populations. Furthermore, the knowledge of evolutionary and ecological aspects of polyploidization is currently based on simple model systems with two ploidy levels co-occurring in a narrow hybrid zone. While these systems are useful for addressing some patterns and processes involved, they cannot be readily applied to more complex contact zones, which are much more frequent in plants than previously anticipated (Suda et al., 2007). The rapid development of FCM and its application in the assessment of detailed cytotype structure in plant populations caused progress in plant population biology.

2.3. DNA markers in plant population biology

2.3.1. Evolution of DNA marker approaches

Twenty years ago in 1988, based on Southern blot analysis and the restriction fragment length polymorphism technique (RFLP), the so called DNA fingerprinting methodology, was for the first time introduced to plant genome analysis (Weising et al., 2005). In the very first DNA fingerprint report dealing with plants, Ryskov et al. (1988) demonstrated DNA fragment pattern differences between two varieties of barley (*Hordeum vulgare*), acquired after Southern blot hybridization of *Hae*III-digested genomic DNA samples with the M13 probe. The same probe was also used by Rogstad et al. (1988) to generate DNA fingerprints from set of gymnosperms and angiosperms. In a third article appearing in the same year, Dallas (1988) applied the human 33.6 minisatellite probe to distinguish rice cultivars. In 1989, synthetic oligonucleotide probes that recognize simple repetitive DNA sequences (i.e. microsatellites) were introduced as novel method of plant genome analysis. Weising et al. (1989) showed that polymorphic DNA fragment patterns were produced when restriction-digested barley or chickpea DNA was separated by agarose gel electrophoresis, and the dried gels were hybridized with radiolabeled $(GACA)_4$ or $(GATA)_4$ probe. Numerous articles were published subsequently on this so called oligonucleotide fingerprinting approach in plants, showing that the level of detected variation was highly dependent on the chosen probe (Weising et al., 1991; Beyerman et al., 1992; Weising et al., 1992; Depeiges et. al., 1995; Sharma et al., 1995). Plant DNA fingerprinting with mini- and microsatellite-complementary hybridization probe is reviewed e.g. in Nybom (1991; 1993); Weising et al. (1991) or Weising and Kahl (1998).

Two decades ago, since the first introduction of DNA fingerprinting technique to plant genome analysis, molecular biology has also revolutionized ecological research (Freeland, 2005). A major development in plant population genetics has been the significant advance in technologies that allow the direct molecular characterization of genes and gene product. In many respect the development and use of various molecular markers that track changes in individual genes has broadened its applicability across many fields in biology (Coates and Byrne, 2005). Molecular markers allow us, among other

things, to quantify genetic diversity, track the movement of individuals, measure inbreeding, investigate gene flow/population genetic structure/effective population size, identify the remains individuals, characterize new species and retrace historical patterns of dispersal (Freeland, 2005; Coates and Byrne, 2005). The applications are ideal for a broad range of e.g. plant population genetics, conservation genetics, evolutionary studies, plant systematics, invasion biology and are used frequently to address practical ecological questions (Freeland, 2005). In conjunction with these advantages there has been a dramatic increase in studies of intraspecific variation often combining population genetics, phylogenetics and biogeography. Such integrated approaches stimulated the development of new fields of study such phylogeography (e.g. Schaal et al., 1998; Avise, 2000; Kulju et al., 2007), which have made significant contribution to understanding of evolutional and ecological processes in plant population (Schaal and Olsen, 2000).

2.3.2. Molecular markers in plant population genetics

Over the last few decades, the use of molecular markers has dominated studies on population genetic structure and geographic patterns of genetic variation in plants. The range of markers now available allows increased flexibility for investigators to utilize more than one marker varying spatial and temporal scale (Coates and Byrne, 2005). These may range from broad based geographical variation (Thompson, 1999; Schaal and Olsen, 2000) to finer scales metapopulation genetic structure (Manel et al., 2003). Popular molecular marker techniques for population genetic studies include isozymes and an array of DNA based techniques such a restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simples sequence repeats (SSRs) and DNA sequencing. Some of these markers such as, isozymes, RFLPs, microsatellites and DNA sequencing, are codominant and can be analysed as single locus markers, while others, such RAPDs and AFLPs, are dominant multilocus markers (Coates and Byrne, 2005).

Currently there is a list of known DNA molecular techniques but the point of this chapter is not to enumerate all of them so I will shortly describe only those methods I used in the studies presented in this thesis.

2.3.2.1. Dominant DNA (molecular) markers

RAPD - Randomly Amplified Polymorphic DNA)

The randomly amplified polymorphic DNA (RAPD) approach developed by Williams et al. (1990) has become the best known variant of prototype of PCR-based DNA profiling (Weising et al., 2005). RAPDs have been commonly used DNA markers in plant population genetic studies (e.g. Schneller et al., 1998; Kuehn et al., 1999; Selbo and Snow, 2004). The technique uses single arbitrary primers sequences to amplify anonymous regions of genome and can be used to identify and screen numerous polymorphic loci. Since no sequence information is needed, this technique is particularly applicable in cases where little molecular genetic information is available on the target species. Furthermore, the assay is very simple and fast, and many loci can be identified, often with single reaction (Coates and Byrne, 2005). Initially RAPDs were extensively used in plant breeding studies and particularly in genome mapping to identify quantitative trait loci. Subsequently they became increasingly popular in studies of genetic variation in natural populations, often with conjunction with, or as an adjunct to isozyme studies (Peakall et al., 1995). Later, patterns of genetics diversity and population genetic structure using RAPDs had been investigated in a broad range of plant species (Harris, 1999; Nybom and Bartish, 2000; Bussell et al., 2005; Lin et al., 2005; Piluzza et al., 2005; Ren et al., 2005; Čurn et al., 2007).

Although RAPDs provide a fast and cost-effective means of investigating genetic variation, they have a number of limitations (Arnold and Imms, 1998; Harris, 1999). Reproducibility has often been cited as a significant problem since the lack of specificity associated with the use of short arbitrary primers can lead to increased sensitivity to PCR conditions resulting in erratic amplification. However, this problem can be minimized with strictly controlled and standardized reaction condition. Homology is also an important issue, given that comigration of product assumes homology, which may not necessarily be correct (Rieseberg, 1996). This is more likely to be a problem in comparison where higher levels of genetic divergence are involved, such as between taxa, and is less likely to be an issue in population genetic studies. Another limitation is that majority of RAPD

markers are dominant and there is therefore a significant loss of information content compared with codominant markers such isozymes, RFLPs or microsatellites.

AFLP - Amplified **F**ragment **L**ength **P**olymorphism

Amplified fragment length polymorphism (AFLP) (Vos et al., 1995) entered the field of plant population studies several years after RAPD method. As well as RAPD technique AFLPs markers have an advantage over other DNA markers e.g. RFLPs because they are applicable to DNA of any origin without prior sequence information, primer synthesis or library construction. Also, like RAPDs, most AFLPs are dominant markers, but unlike RAPD protocols a single AFLP reaction can survey as many as 100-200 loci (e.g. Mueller and Wolfenbarger, 1999). The polymorphism typically exhibit Mendelian inheritance, enabling their use for typing, identification and mapping of genetic characteristics. The potential to produce hundreds of polymorphic loci per individual and relative ease of applicability to most organisms makes AFLPs one of the most efficient molecular markers for generating polymorphic loci in plant population genetic studies (e.g. Travis et al., 1996; Mueller and Wolfenbarger, 1999; Hedrén et al., 2001; Schönswetter et al., 2004; Houghton-Thompson et al., 2005; Lambertini et al., 2006; Mráz et al., 2007).

Although AFLP analyses do not suffer from the reproducibility problems that potentially exist in the use of RAPDs, they are still largely dominant markers and therefore pose the same difficulties in reliance on the assumption of equilibrium for estimation of population genetic parameters. However, their high resolution and the ease with which one can generate large numbers of genetic markers has seen a steady increase in their application in the analysis of population level variation in plant species (Coates and Byrne, 2005). The high number of loci potentially assayed by the AFLPs could be of great use in paternity and mating systems studies. Despite their dominance, Krauss (1999, 2000) and Kraus et al. (2002) utilized their high polymorphism for successful assigning of paternity and mating patterns in plants.

2.3.2.2. Codominant DNA (molecular) markers

Microsatellites

Microsatellites, also known as simple sequence repeats (SSRs), are now recognized as potentially one of the most useful genetic marker in plant population studies (e.g. Procaccini et al., 2001; Pryor et al., 2001). Microsatellites consist of tandem repeats of a short "motif" sequence, usually one to six bases (Chambers and MacAvoy, 2000). These regions occur frequently and randomly in plant and animal genomes and often have large numbers of moderately frequent alleles (Tautz and Renz, 1984; Powell et al., 1995; Goldstein and Schlötterer, 1999). Thus they show extensive variation between individuals within populations and have been developed for a wide range of purposes in plant breeding, conservation biology and population genetics including forensics, paternity analysis and gene mapping (Jarne and Lagoda, 1996). High variation in these multi-allelic and codominant markers can be used to investigate processes such as migration and colonization, mating and dispersal, genetic drift, bottleneck effect and changes in effective population size (Luikart and England, 1999; Ouborg et al., 1999; Spencer et al., 2000; Sunnucks, 2000). In particular, in plant population genetic studies they have proved to be ideal for assessing gene flow among populations (Chase et al., 1996), and are ideally suited to fine-scale analysis of mating within population (Friar et al., 2000; Konuma et al., 2000).

Unfortunately the significant benefits of such hypervariable codominant markers are offset by the time and effort involved in their development (Squirrel et al., 2003). Sequence information is required to design appropriate primers and such information is generally only available for limited number of commercially important species. For most plant species, microsatellites can only be developed from clones isolated through construction and screening of a genomic library. As pointed out by Squirrel et al. (2003), the development of a working primer set is subject to considerable attrition rate compared with the original number of clones sequenced. This contrasts with, and increase the appeal of, other highly polymorphic markers such AFLPs and RAPDs where generic primers are readily available (Coates and Byrne, 2005).

One approach that has been successfully used in some animal groups involves cross-species amplification of microsatellite loci. However, this approach is much less

successful in plants where the general patterns indicate that cross-species amplification of plant microsatellites will be restricted to closely related species or at least congeners (Peakall et al., 1998; Butcher et al., 2000), although broader cross-transferability has been demonstrated in *Vitaceae* (Arnold et al., 2002).

Microsatellites, similarly as AFLPs have wide range of use and are rapidly becoming the DNA-based markers of choice for plant population genetic structure, mating system, gene flow and phylogeographical or biogeographical studies as well as for project where systematics, taxonomy or hybridization are involved. A number of recent investigations have compared the potential level of resolution and efficiency of these markers in plant population studies (Gerber et al., 2000; Kubátová and Čurn, 2008). As expected, microsatellites are considerably more polymorphic than AFLPs at the locus level, but AFLPs are much more efficient at revealing polymorphic loci. For example, in *Avicennia marina* average expected heterozygosity for AFLPs was only 0.193 compared with 0.780 for microsatellites, but all of the 918 AFLP bands scored were polymorphic (Maguire et al., 2002).

Chloroplast DNA analysis - cpDNA variation

Whereas molecular markers discussed previously are based on various portions of the nuclear genome, another marker that has considerable potential in plant population studies is chloroplast DNA (cpDNA). Compared with the nuclear genome of the higher plants, which consist of a diploid complement of randomly segregating biparentally inherited chromosomes, the chloroplast genome is predominantly uniparentally inherited and consists of a single circular molecule. While in angiosperms the chloroplast genome is generally maternally inherited, in some conifers cpDNA is paternally inherited. A number of different approaches have been utilized to characterize variation in cpDNA. The first and probably most widely used involves RFLP analysis of the entire chloroplast genome. An important factor assisting this approach resides in the relatively high degree of sequence conservation in the chloroplast genome that allows heterologous probes to be utilized across most plant families. With the development of PCR, a number of RFLP studies have been carried out based on PCR-amplified product, thus avoiding the time required for Southern hybridization.

A limitation of cpDNA analysis in many species is the relatively low sequence diversity, which can be attributed to the haploid status of the genome, reduced rate of mutation and lack of recombination (Schaal et al., 1998; Ennos et al., 1999). To address this issue there have been attempts to target sections of the chloroplast genome that have relatively high mutation rates. Useful levels of intraspecific variation have been detected in non-coding regions of the cpDNA genome (reviewed in Shaw et al., 2005). For example, Vaillancourt and Jackson (2000) detected significant levels of polymorphism for eucalypts in sequence studies of the J_{LA} junction between the inverted repeats and the large single-copy regions, with much of the variation due to complex insertion/deletions. Another useful approach with considerable potential involves the analysis of variation using chloroplast microsatellites (Powel et al., 1995; Provan et al., 1999). Despite occasional difficulties in accessing appropriate levels of variation in plant population studies, cpDNA has proved to be extremely useful in providing insights into population and evolutionary processes that could not be delivered by nuclear markers. One of the most fundamental applications of patterns of cpDNA variations has been in the analysis of phylogeographic patterns of population variation (King and Ferris, 1998; Ferris et al., 1999). In addition to uniparental inheritance and lack of recombination, there are a number of reasons why cpDNA may be far more suitable for such studies than nuclear markers. The effective population size of an organelle gene is theoretically half that of nuclear gene, so cpDNA variation is likely to be more sensitive to population differentiation by genetic drift. Also, because the maternally inherited cpDNA will only be dispersed by seed, the genetic differences existing when populations come into contact will probably break down much more slowly than for nuclear markers, leaving a signature of historical relationships for much longer (Ennos et al., 1999).

2.4. Morphological markers versus DNA markers

For centuries, biologists have tried to detect, describe, and explain diversity in the biological world. The formalization of a hierarchical system of nomenclature by Linnaeus (1758) established a framework for describing and categorizing biological diversity. This hierarchical system was initially independent of evolutionary theory, but later workers (e.g. Darwin, 1859; Haeckel, 1866; reviewed by Mayr, 1983) developed the notion that classification should be based on phylogenetic relationships. The twentieth century has seen major conceptual and operational advances in the estimation of phylogeny (e.g. Hennig, 1966; Wiley, 1981; Felsenstein, 1982, 1988), as well as in the analysis of microevolutionary changes (Futuyma, 1986). These two aspects of evolution traditionally have been studied by different kind of biologists: phylogeny by systematists, and microevolutionary changes by population geneticists. However, the two fields have expanded toward a common background, and an understanding of evolution requires a synthesis of the two disciplines.

Investigation of microevolutionary changes and phylogeny require sources of heritable variation. Until the late 1960s, systematics was based largely on analysis of morphological and behavioural variation, approaches that continue to be used in the present with increasing sophistication. However, with the elucidation of the molecular basis of inheritance, biological macromolecules have assumed an increasingly important role in evolutionary studies. Nucleic acids (RNA and DNA) and proteins can provide a broadly applicable set of heritable markers to examine genetic structure of populations or to estimate relationships among taxa. Conversely, such molecular studies have generated massive comparative data bases, providing important insights into evolution of the molecules themselves (reviewed by Macintyre, 1985; Nei, 1987).

Early applications of molecules to systematic problems were largely concerned with proteins. Immunological approaches were pioneered by Nuttall (1904) and achieved prominence with studies of relationships and times of divergence among hominoids (Goodman, 1961; 1963; Sarich and Wilson, 1966; 1967). Early application of electrophoresis and specific histochemical staining revealed a wealth of protein variation within and among species (e.g. Hubby and Throckmorton, 1965; Hubby and Lewontin, 1966; Lewontin and Hubby, 1966). This approach, isozyme electrophoresis,

has generated a massive comparative database. Comparison of amino acid sequences provide the first indication of molecular clock (Zuckerkandl and Pauling, 1962) and have been extensively used to estimate phylogeny (e.g. Goodman et al., 1987). Variation in chromosome structure and number also provides a valuable source of genetic markers within and between species (White, 1973).

Major advances in the manipulation and analysis of nucleic acids have led to the widespread study of DNA and RNA variation. The sequences assayed have come from the nucleus, the mitochondrion, and the chloroplast. Used approaches include hybridization and dissociation of DNA, use of restriction endonucleases to detect base substitutions and rearrangement, and comparison of the primary sequences. Methods for collecting data on nucleic acid differences continue to improve and middle of eighties and discovery of PCR technology mean revolutionary point and introducing of new methods and approaches to this type of studies.

The collection of molecular data and their use have led to several controversies. These controversies include arguments about the relative value of molecular versus morphological data, the constancy of rates of molecular evolution, the neutrality of molecular variants, the types of data that should be collected, the various approaches to analyzing data, and the meaning of "homology" in relation to molecular characters.

There has been considerable debate over whether molecular or morphological features are inherently better sources of information for estimating genetic variation (Patterson, 1978). Some have claimed that molecular characters are relatively weak (e.g. Kluge, 1983), whereas others have claimed that morphological characters are likely to be misleading (e.g. Frelin and Vuilleumier, 1979; Sibley and Ahlquist, 1987). Closer examination shows this to be an empty argument (Hillis, 1987). Comparative studies have shown that morphological changes and molecular divergences are quite independent, responding to different pressures and following different rules (Wilson et al., 1974, 1977). However, the real concerns for the practicing systematists are whether the characters examined exhibit variation appropriate to the question(s) posed, whether the characters have a clear and independent genetic basis, and whether the data are collected and analyzed in such a way that is possible to compare and combine hypotheses derived from them.

The conflicts between molecular and morphological evidence have been overemphasized. The development of molecular markers has not resulted in widespread refutation of hypothesis generated by morphologists. Each approach has distinct advantages and disadvantages. For example, most (but not all) molecular data have a clear genetic basis and the total data set is limited only by genome size; on the other hand, morphological data can be obtained from ancient fossils (e.g. Gauthier et al., 1988) and extensive preserved collections and can be interpreted in the context of ontogeny (Kluge and Strauss, 1985). Although, morphological markers can prove useful in several cases, but they also have obvious disadvantages: (i) morphological variants are not available for most species, (ii) studies utilizing morphological characters are generally limited to only one locus, (iii) recessive alleles can be masked by the dominant ones, or (iv) some of these alleles can display epistatic or pleiotrophic effect, and (v) many characters (e.g., flower colour) have to be scored relatively late in the life cycle. Moreover, because plants are sessile, and hence unable to escape their enemies and changes of the living environment, (vi) they show great phenotypic plasticity. Some of the phenotypically plastic characters can have a low heritability. And proper genetic marker should not depend on the environment. While early studies of genetic variation with morphological characters were relatively few, the establishment of new molecular marker techniques (proteins and DNA markers) has caused the boom in genetics and evolution. Nowadays, workers from many disciplines of plant biology, ranging from ecology through breeding and dispersal (spatial dynamics) to evolution, have integrated the use of DNA markers in their research.

In general, studies that incorporate both molecular and morphological data will provide much better descriptions and interpretations of biological diversity than those that focus on just one approach. Furthermore, it is possible to address some biological problems only with morphological data and other problems only with molecular data (Hillis, 1987; Fernholm et al., 1989).

Recently is still more distinct the multi-disciplinary approach in modern systematics and population biology. In current biology the inquired hypothesis and questions are more often revealed by using various approaches from different fields of biology.

Morphological characters have long been used to identify species, genera and families; to evaluate systematic relationship and to discriminate cultivars, breeding lines

etc. In contrast to molecular markers, morphological characters are often strongly influenced by the environment, and consequently, special breeding programs and experimental design are needed to distinguish genotypic from phenotypic variation. In addition, for some small, non-flowering plant species such mosses and algae, it frequently is difficult to find a sufficient number of morphological characters for a comprehensive study. In contrast, molecular technologies provide almost unlimited numbers of potential markers at the genotypic level.

Correspondence between morphological characters and DNA markers in terms of genetic distance between entities (e.g. populations and cultivars) is sometimes surprisingly high, indicating that both types of data are usually good estimators of genetic relatedness. This is especially true if the morphological descriptors are represented by quantitative rather than qualitative data set (Martinello et al., 2001; Rumpunen and Bartish, 2002). Morphological variability due to qualitative traits, presumably governed by a single or few gene mutations, can actually be much more pronounced than the extent of variation indicated by molecular markers (Levi et al., 2001). Consequently, certain groups of plants (e.g. cultivars, subspecies or species) appear to considerably better defined through the analysis of quantitative morphological traits rather than by DNA markers (Olsson et al., 2000). Sometimes, the evaluated characters have been chosen, on purpose, to reflect plant breeding efforts and may therefore have been subjected to strong selection pressure (Weising et al., 2005).

3. References

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V. Conclusions

I utilize three different methodological approaches in plant population studies to reveal partial questions and to answer introduced hypothesis from different areas of plant population biology. In the paragraphs bellow are my responses to the questions I asked at the beginning of my work.

Manuscript I. - Addressing the flow cytometric puzzle: complex ploidy variation in *Gymnadenia conopsea* complex (*Orchidaceae*).

I.a What is the population cytotype structure based on representative sampling?
Five ploidy levels were detected among 2226 plants analysed. Along with previously known tetra-, octo-, and decaploid individuals, hexaploids and dodecaploids were revealed for the first time.

I.b Do ploidy-mixed populations occur? If so, what is their frequency and which cytotypes are involved?
Ploidy mixing was quite a common phenomenon in *Gymnadenia* populations; more than half of populations (19 out of 36) harboured two or more cytotypes. Two different ploidies were found at 10 localities (=38.5%), three ploidies were found at 6 localities (=16.7%), and four different ploidies grew at two localities (no. 11 – Dolní Čepí, PP Křižník and 28 – Porúbka, Turie). All five cytotypes inhabited locality 20 – Javorník, Jazevčí. While tetra- and octoploid plants formed some cytotype-uniform populations (9 and 8 populations, respectively), rare cytotypes (i.e., 6x, 10x, and 12x) only occurred together with plants of one or both majority ploidies. In addition to sympatric growth of different ploidy levels, plants of *G. * conopsea* and *G. * densiflora* also co-occurred occasionally (6 localities). Their relative abundance varied from rather equal frequency (localities 16, 21, and 27) to marked predominance of one or another taxon (localities 6 and 19). In addition, *G. * conopsea* and *G. * densiflora* sometimes grew in spatially isolated habitats within the same macrolocality (cf. populations 17 + 18, and 31 + 32). Generally, both plants showed slightly different ecological requirements, with the latter taxon preferring more humid sites.

I.c Which minority cytotypes can be found and under which conditions?
Three minority cytotypes were found and their frequencies varied from ~1.3% in hexaploids to ~0.5% in decaploids, and ~0.4% in dodecaploids.

I.d What is the exact distribution pattern of different cytotypes within a population? Do the data suggest genuine sympatry or rather a certain habitat segregation?
Tetraploid and octoploid cytotypes clearly predominated in our sample set and accounted for 60.0% and 37.8% of all plants, respectively. In general, tetraploids corresponding to *G. * conopsea* and *G. * densiflora* occurred at 18 and 15 localities, respectively, hexaploids occurred at 12 localities, octoploids at 20 localities, decaploids at 5 localities, and dodecaploids occurred at 4 localities.

Paper II. - Phenotypic and genotypic variation of *Phragmites australis*: Comparison of populations in two man-made lakes of different age and history.

II.a The number of clones per unit of population area is lower in the older population than the younger population.
The number of clones was significantly smaller in the Opatovický fishpond (older population) than in the Halámky sand pit (younger population).

II.b The maximum area covered by a single clone is larger in the older population than the younger one.
The largest clonal area was that of clone O-41 at Opatovický fishpond (older population), which was identified with a frequency of 28% among the samples from that site. By contrast, each of the three largest clones of Halámky sand pit had a frequency of 14% among the samples taken from there.

II.c Morphologically homogeneous stands are monoclonal.
Each of the stands sampled for the genetic and morphological analyses was visually homogeneous. The fact that some of the stands were found to be monoclonal while some others were polyclonal contradicts this hypothesis.

II.d Morphological variability is a good indicator of the genetic variability in multiclonal stands and populations.
We did not find a clear relationship between the variation in morphological characteristics, recorded in individual stands, and clone numbers within those stands.

Paper III. - Phenotypic and genotypic variation of *Phragmites australis*: A comparative study of clones originating from two populations of different age.

III.a Are the growth and morphometric characteristics of morphotypes originating from the two populations also preserved in the culture?
Morphological characteristics were preserved to a greater extent in reed plants originating from the Halámky than from the Opatovický population. This is indicated by better separation of clones on the basis of morphological characteristics, by a greater proportion of variation explained by pertinence to clones and by closer correlations in morphological characteristics between cultivated plants and their parent field stands.

III.b Have the growth characteristics of the robust morphotypes a genetic basis?
Preliminary investigation using flow cytometry was carried out for 20 stands of robust reed in Halámky sand pit (Čížková et al., unpublished results) and for all stands in Halámky sand pit and Opatovický fishpond involved in this study (Kubátová et al., unpublished results). It has shown only tetraploid genotypes. This indicates that the robust growth, found in some morphotypes of the Halámky population, is not due to a higher ploidy level.

Manuscript IV. - Phenotypic and genotypic variation of *Phragmites australis*: High level of cryptic variation revealed on the basis of molecular marker analysis non-corresponding to phenotype performance.

IV.a Different molecular markers and morphological characteristics can better reveal genetic structure of studied populations than single marker focused approach.
Combined approach when DNA and morphological markers were analysed lead to clear separation of both studied populations and distinctiveness of both populations was higher using all methods

IV.b The patterns of genotypic variability of two reed populations inhabiting sites differing in both succession stage and substrate conditions are distinct.
The two studied population were clearly distinct using molecular markers.

IV.c The morphological variability of studied populations/stands reflects their genotypic variation and the morphological variability should be an indicator of their genetic variability.
We did not find positive correlation between morphological and genetic variability. Morphologically uniform stands were not monoclonal thus morphological variation may not be used as an indicator of genetically homogenous stands. Number of clones, except those detected by SSRs, is higher in Halámky population (younger population). The average number of clones per reed stand is in this population more than 2.5 in comparison to 1.5 clones per stand in Opatovický fishpond population.

Paper V. - DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale.

V.a What is the level of ploidy variation in both primary and secondary ranges of *L. salicaria* distribution?
Large cytotype variation was found among 1884 progenies of *L. salicaria* raised from 578+ plants sampled at 124 localities across both primary and secondary areas of distribution. Along with previously known diploid and tetraploid plants, DNA hexaploids were revealed for the first time, and DNA triploids were reliably confirmed.

V.b What is the cytotype structure within both native and invasive *L. salicaria* populations?
There was a marked disproportion among frequencies of particular cytotypes, with DNA tetraploids amounting to 86.2% of all progenies. Other ploidies were much rarer (diploids c. 4.5%, triploids c. 0.9% and hexaploids c. 8.4%) and were restricted to the native range. Within the primary area studied, DNA tetraploids were recorded in all European and Middle Eastern countries except Israel. Populations from Israel consisted solely of DNA diploids. DNA hexaploids occurred only in Turkey and rare DNA triploids were encountered in one population from Hungary. Although the majority of populations comprised a single cytotype, progenies of two different ploidy levels within the same population, or even within the same plant, were also detected. DNA

triploids and DNA tetraploids occurred in population H4 near Gödöllo, Hungary, and both DNA tetraploid and DNA hexaploid progenies were raised from seeds collected in populations near Antalya (TR5) and Kutahya (TR6) in Turkey. In contrast, cytotype uniformity was a typical feature of invasive North American populations from 13 states of the USA and provinces of Canada. Only DNA tetraploids were observed among 678 progenies of 221+ plants collected at 60 localities.

V.c Might the switch in ploidy level trigger invasive spread in secondary area of distribution?
Our data show that the invasive spread of North American populations was not triggered by differences in ploidy level.

V.d Is there a difference in genome size between native and invasive populations?
Attempts to reliably determine the genome size in absolute units (DNA picograms) using intercalating propidium iodide have been in vain and gave histograms with high CVs (mostly between 6% and 8%) on a prominent background. Therefore only relative fluorescence values are presented. In all but hexaploid cytotypes, fluorescence intensities increased proportionally with ploidy level. DNA hexaploids showed about 2.4% lower fluorescence intensity than expected, indicating that genome downsizing followed this high-polyploid formation.

VI. List of papers

1. **Kubátová B., Trávníček P., Čurn V., Suda J. (2008):** Adressing the flow cytometric puzzle: complex ploidy variation in *Gymnadenia conopsea* agg. (*Orchidaceae*). – American Journal of Botany (*submitted*).

2. **Čurn V., Kubátová B., Vávřová P., Suchá O., Čížková H. (2007):** Phenotypic and genotypic variation of *Phragmites australis*: Comparison of populations in two man-made lakes of different age and history. – Aquatic Botany 86: 321-330.

3. **Suchá O., Vávřová P., Čížková H., Čurn V., Kubátová B. (2007):** Phenotypic and genotypic variation of *Phragmites australis*: II. A comparative study of clones originating from two populations of different age. – Aquatic Botany 86: 361-368.

4. **Kubátová B. and Čurn V. (2008):** Phenotypic and genotypic variation of *Phragmites australis*: High level of cryptic variation revealed on the basis of molecular marker analysis non-corresponding to phenotype performance. – Molecular Ecology (*submitted*).

5. **Kubátová B., Trávníček P., Bastlová D., Čurn V., Jarolímová V., Suda J. (2008):** DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale. – Journal of Biogeography 35: 167-176.

Adressing the flow cytometric puzzle: complex ploidy variation in *Gymnadenia conopsea* agg. (Orchidaceae) [1]

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Polyploidy, the presence of more than two genomes per cell, has long been recognized to be an important evolutionary force in plants. Since the pioneering works about a century ago (e.g., Lutz, 1907), the number of recognized polyploid plants increased dramatically. Recent estimates suggest that about 70% of angiosperms and up to 95% of pteridophytes underwent one or more rounds of genome duplication in their evolutionary history (Soltis and Soltis, 1999), and genomic data even assume the near ubiquity of polyploidy (Soltis, 2005). The evolutionary success of polyploids may be related to increased heterozygosity, reduced inbreeding depression, broader ecological amplitude, and/or better colonizing ability as compared to their diploid counterparts (Lopez-Pujol 2007; Wendel, 2000; Otto and Whitton, 2000).

The last decade has seen significant progress in our understanding of mechanisms and rates of polyploid formation in both autopolyploids (i.e., polyploids arising within or among populations of the same species) and allopolyploids (i.e., polyploids combining genomes of at least two parental species) (Ramsey and Schemske, 1998). Extensive use of molecular markers changed markedly our perception of dynamics of polyploidy and documented that genome duplication is not a rare, macroevolutionary step but a dynamic and ongoing process (Haufler 2002). In addition, early views on ploidy variation in several plant groups must have been revisited in the light of recent findings that revealed much more prolific cytotype differentiation. While different ploidy levels may diagnose different taxa (Rosenbaumová et al. 2004; see also Soltis et al., 2007), intraspecific ploidy variation is not a rare phenomenon either (Lihová et al., 2003; Suda et al., 2007). When more cytotypes occur within the same species, zones of ploidy overlap are usually formed. Two kinds of contact zones are mostly recognized according to their history (Petit et al., 1999), i.e., primary (when a new cytotype originates *in situ*) and secondary (when two formerly allopatric cytotypes meet). Transitional zones are of particular interest to evolutionary biologists as they allow studying mechanisms involved in early stages of polyploid speciation and/or assessing selective forces operating in ploidy-mixed populations. Sympatric growth of different ploidy races is mostly explained either by a directional or a balanced selection (Weiss et al., 2002). The former theory assumes that cytotype intermingling is only a transitional stage and one cytotype will finally be outcompeted (i.e., the minority cytotype exclusion; Levin, 1975). By contrast, the latter type of selection assumes that a long-term co-existence can be maintained due to assortative mating that reinforces isolation between related cytotypes. Assortative mating can be attained, among

others, by flowering time divergence (Bretagnolle and Thompson, 1996; Petit et al., 1997), microhabitat differentiation (Johnson et al., 2003; Suda et al., 2004), or expression of apomixis, selfing and vegetative propagation (Quarin et al., 2001). In addition, pollinator behaviour is another, only recently acknowledged component that may modulate dynamics of mixed-ploidy populations in animal-pollinated species (Segraves and Thompson, 1999).

An important prerequisite for understanding patterns and dynamics of genome duplication under natural conditions is the knowledge of cytotype distribution at various spatial scales. Distributional data may provide useful insights into the evolutionary history of multiploid taxa, elucidate questions surrounding niche differentiation and intensity of among-cytotype interactions, and assess the role of biogeographic processes (e.g., vicariance, dispersal) for contemporary cytotype pattern (Petit et al., 1999). The field of plant cytogeography has been greatly facilitated by the advent of flow cytometry (Kron et al., 2007; Suda et al., 2007). In contrast to conventional karyological counting, this technique can easily cope with large population samples and thus provides a much more accurate picture of ploidy variation (Lewis et al., 1967; Suda et al., 2007). In addition, the sampling is generally non-destructive, which paves the way for comprehensive studies of rare and endangered plants without the risk of population destruction (Cross and Gravendeel, 2005).

Gymnadenia conopsea (L.) R. Br. s.l. (Fragrant orchid; Orchidaceae) is a Eurasian species that shows considerable variation across its distributional range. Three ploidy levels (2n = 40, 80, and 100) were found to occur in Central Europe, occasionally in cytotype-mixed populations (Marhold et al., 2005). Besides the karyological heterogeneity, differences in plant morphology (Soliva and Widmer, 1999), flowering phenology (Gustafsson and Lönn, 2003), and habitat preference (Möseler, 1987; Gustafsson and Lönn, 2003) have also been reported. The species is self-compatible but pollinator-dependent for fruit setting (Gustafsson, 2000). Attempts to formally classify the variation led to the description of numerous taxa at below-species ranks (Dworschak, 2002) but taxonomic status of most of them remains rather obscure. Recently, two basic variants are recognized, referred to as (at the subspecific level) subsp. *conopsea* and subsp. *densiflora* (Wahlenb.) K. Richt. Generally, the former encompasses early-flowering individuals while the majority of late-flowering plants are identified as subsp. *densiflora*. Both taxa are also distinguishable morphologically, height of the plant, number of flowers in inflorescence, number and width of leaves, and flower dimensions being the most important diagnostic characters (Rose, 1988; Marhold et al., 2005; Gustaffson, 2003; Campbell et al., 2007). However, taxa determination may not always be so straightforward as shown by the incidence of morphotypes corresponding to the nominate subspecies but flowering later in the season (Gustafson and Lönn, 2003; Lönn et al., 2006). Molecular studies justified splitting of *G. conopsea* s.l. into several genetically distinct groups, albeit they often showed only weak correlation with other characters (e.g., Scacchi and Angelis, 1989; Soliva and Widmer, 1999). Critical taxonomic treatment of the group may further be impeded by the fact that several morphotypes/phenotypes/evolutionary units often grow in sympatry (Soliva and Widmer, 1999; Gustafson and Lönn, 2003; Marhold et al., 2005; Lönn et al., 2006).

Co-existence of different phenological variants (e.g., Lönn et al., 2006) and indications of intrapopulation cytotype heterogeneity (Marhold et al., 2005) have stimulated our research on ploidy variation of fragrant orchid in Central Europe. The four

basic questions were addressed: (1) What is the population cytotype structure based on representative sampling? (2) Do ploidy-mixed populations occur? If so, what is their frequency and which cytotypes are involved? (3) Which minority cytotypes can be found and under which conditions? (4) What is the exact distribution pattern of different cytotypes within a population? Do the data suggest genuine sympatry or rather a certain habitat segregation?

Material and Methods

Population sampling: Thirty six *Gymnadenia* populations from the Czech Republic, Slovakia and Austria were sampled during 2004-2007, spanning the geographic range 48° 49' N – 50° 36' N and 13° 39' E – 19° 23' E (Table 1, Map 1). When possible, leaf samples from at least 50 individuals were collected at each locality. The total number of analyzed plants was 2226. The sampling was designed to cover as much morphological and phenological variation as possible, and to include plants from various microhabitats. If spatially separated habitats with different ecological conditions (e.g., dry upper slopes vs. waterlogged valley bottom) occurred at the macrolocality, they were considered as separate localities. Leaf tissue was kept in plastic bags wrapped in moist paper towels and processed within two days.

Sampling on plots: Detailed survey of cytotype distribution was performed at the locality where the most prolific ploidy variation had been found (no. 20 – Jazevčí). Two plots (60 x 8 m and 65 x 5 m, respectively) were defined in parts where *Gymnadenia* plants grew in abundance, every individual was mapped, and a leaf was sampled for ploidy estimation. The total number of analyzed plants was 354.

Flow cytometry: DNA ploidy levels were inferred from relative fluorescence intensities of DAPI-stained nuclei using flow cytometry (FCM). Intact leaf tissue of the analysed plant (ca 0.5 cm^2) was chopped together with an appropriate volume of the internal standard (*Pisum sativum* 'Ctirad', 2C = 9.09 pg; Doležel et al., 1998) using sharp razor blade in a Petri-dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto, 1990). The crude suspension was filtered through a 42 μm nylon mesh and incubated 10 min. at room temperature. Isolated nuclei were stained with 1 ml of Otto II buffer (0.4 M Na_2HPO_4 . 12 H_2O) supplemented with with 4',6-diamidino-2-phenylindole (DAPI) at final concentration 4 μl/ml and ß-mercaptoethanol (2 μl/ml). After a few minutes, relative fluorescence intensity of 5 000 particles was recorded using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp as a source of UV excitation light. Histograms were evaluated using FloMax software, ver. 2.4d (Partec GmbH, Münster, Germany). Up to five *Gymnadenia* plants were analysed together during the large-scale ploidy screening. Our previous experiments showed that such a protocol facilitated unambiguous detection of minority cytotypes. Each plant was re-analyzed separately if mixed-ploidy samples were observed or if the quality of histograms was not considered sufficient (i.e., coefficient of variation, CV, of any peak above 5%).

Chromosome counts: Chromosomes were counted in actively growing root tips of mature plants. Samples were pretreated with a saturated solution of p-dichlorbenzene (three hours, room temperature), fixed in a 3:1 mixture of ethanol and acetic acid (four hours, 4°C), macerated in 1:1 hydrochloric acid:ethanol (30 s, room temperature) and immediately squashed in a drop of lactopropionic orceine. The number of chromosomes was determined in 5-10 complete well-spread mitotic plates using Carl-Zeiss Jena NU microscope equipped with Olympus Camedia C-2000 Z camera. Basic chromosome number n=10 was considered when interpreting chromosome results with respect to ploidy levels.

Statistical analyses: As flow cytometric profiles of *Gymnadenia* samples are very complex (see Interpretation of flow histograms), principal component analysis (PCA) was performed to visualize relationships between measurements and to find groupings, which correspond to different ploidy levels. Ratios between individual peaks of the *Gymnadenia* sample and *Gymnadenia/Pisum* peak ratios were included as variables. The computations were done using the procedure PCA of the SAS statistical package (SAS Institute 2000).

Results and discussion

Interpretation of flow histograms: *Gymnadenia* samples yielded complex flow histograms with several peaks arranged in an endopolyploidy-like fashion. Up to four peaks of *Gymnadenia* nuclei (designated as i, I, II, and III) could have been distinguished on flow histograms (Fig. 1A-C). However, while peaks I and II were always present, peaks i and III often were unrecognizable. For instance, nearly two thirds of tetraploid plants lacked the i-peak, which maked the inference of DNA ploidy level difficult. Not only the absence of some peaks but also unusual peak ratios, which deviated markedly from the genuine endopolyploidy (i.e., 1 : 2 : 4 : 8, etc.), contributed to the challenge of this orchid group (Table 3). Interestingly, two distinct groups of fluorescence intensities were observed when plants tentatively determined as *G. * conopsea* and *G. * densiflora* were analysed. While average peak ratios for the former taxon were 1 : 1.59 : 2.75 : 5.11, the latter taxon showed the following average ratios – 1 : 1.75 : 3.25 : 6.24 (see also Table 3). As any overlap existed between the fluorescence values, these results indicate that FCM profile may be used as a reliable taxonomic marker. Peak ratios in higher ploidy levels matched perfectly those in tetraploid *G. * conopsea*, suggesting that cytotype heterogeneity concerns only this taxon.

To guarantee correct interpretation of resulting histograms with respect to DNA ploidy levels, PCA analysis was performed on flow data (Fig. 2). Well-separated groups of measurements were always obtained whether only histograms with all four *Gymnadenia* peaks distinct (Fig. 2A) or all flow assays (Fig. 2B) were involved.

As was mentioned above taxa of *Gymnadenia* conopsea complex – tetraploid *G. densiflora* and *G. conopsea*, octoploid *G. conopsea (=G.montana)* and minority cytotypes (hexaploids, decaploids and dodecaploids were distinguishable on the basis of FCM profiles. Differentiation of 3 basal taxa and minority cytotypes of *Gymnadenia conopsea* complex was also possible on the basis of their Cx-value and preliminary results

are shown on Fig. 3. and further studies will be focused on precise determination of Cx-values of analysed taxa and evaluation of cytogenetic and morphological data. Some other studies refer that genome size significantly correlates with many morphological (Vekemans et al., 1996), phenological (Baranyi and Greilhuber, 1999) or ecological (MacGillivray and Grime, 1995) characteristics of taxons.

Ploidy variation and population cytotype structure: Five ploidy levels were detected among 2226 plants analysed (Table 2). Along with previously known tetra-, octo-, and decaploid individuals, hexaploids and dodecaploids were revealed for the first time. Tetraploid and octoploid cytotypes clearly predominated in our sample set and accounted for 60.0% and 37.8% of all plants, respectively. Other ploidies were much rarer, and their frequencies varied from ~1.3% in hexaploids to ~0.5% in decaploids, and ~0.4% in dodecaploids. In general, tetraploids corresponding to *G. * conopsea* and *G. * densiflora* occurred at 18 and 15 localities, respectively, hexaploids occurred at 12 localities, octoploids at 20 localities, decaploids at 5 localities, and dodecaploids occurred at 4 localities.

Ploidy mixing was quite a common phenomenon in *Gymnadenia* populations; more than half of populations (19 out of 36) harboured two or more cytotypes (Table 2, Map 1). Two different ploidies were found at 10 localities (=38.5%), three ploidies were found at 6 localities (=16.7%), and four different ploidies grew at two localities (nos. 11 – Dolní Čepí, PP Křižník and 28 – Porúbka, Turie). All five cytotypes inhabited locality 20 – Javorník, Jazevčí. While tetra- and octoploid plants formed some cytotype-uniform populations (9 and 8 populations, respectively), rare cytotypes (i.e., 6x, 10x, and 12x) only occurred together with plants of one or both majority ploidies.

In addition to sympatric growth of different ploidy levels, plants of *G. * conopsea* and *G. * densiflora* also co-occurred occasionally (6 localities). Their relative abundance varied from rather equal frequency (localities 16, 21, and 27) to marked predominance of one or another taxon (localities 6 and 19). In addition, *G. * conopsea* and *G. * densiflora* sometimes grew in spatially isolated habitats within the same macrolocality (cf. populations 17 + 18, and 31 + 32). Generally, both plants showed slightly different ecological requirements, with the latter flowering taxon preferring more humid sites.

To get further insights into the pattern of ploidy distribution at fine spatial scale, detailed cytotype screening was performed on two plots at the locality Javorník, Jazevčí (no. 20) where all five cytotypes had been found during conventional ploidy survey on fifty plants. Grouping of plants of the same ploidy level was observed (see Figs. 4) partly corresponding to geographical configuration and character of localities. Differences in distribution and grouping of cytotypes were found across analysed transects and their distribution in NE part of locality indicate genuine sympatry of different *Gymnadenia* cytotypes.

When more cytotypes occur within the same species, zones of ploidy overlap are often formed. Contact zones are of particular interest to evolutionary biologists because they allow studying mechanisms involved in early stages of polyploid speciation and/or assess selective forces operating in ploidy-mixed populations. Sympatric growth of

different ploidy races is mostly explained either by a directional or a balanced selection (Weiss et al., 2002). The former theory assumes that cytotype intermingling is only a transitional stage and one cytotype will finally be outcompeted (i.e., the minority cytotype exclusion; Levin, 1975). By contrast, the latter type of selection assumes that a long-term co-existence can be maintained due to assortative mating that reinforces isolation between related cytotypes. A number of prezygotic and postzygotic breeding barriers has been identified (Husband and Sabara, 2004), including spatial segregation and microhabitat differentiation of cytotypes (e.g., Felber-Girard et al., 1996), flowering time divergence (Petit et al., 1997), gametic selection (Husband et al., 2002), and selection against triploid hybrids (van Dijk et al., 1992). Evolution of assortative mating in relation to behavioural mechanisms can also be an important component of the successful establishment and/or co-existence of animal-pollinated plant species (Thompson et al., 2004). Two kinds of contact zones are mostly recognized according to their evolutionary history (Petit et al., 1999), i.e., primary (when a new cytotype originates *in situ*) and secondary (when two formerly allopatric cytotypes meet). It has been suggested that reproductive isolation is more readily achieved in the latter one (Petit et al., 1999). Despite the major role of genome duplication in plant speciation, it is still largely unknown how different factors act in concert to achieve reproductive isolation among ploidy levels in mixed populations. Furthermore, the knowledge of evolutionary and ecological aspects of polyploidization is currently based on simple model systems with two ploidy levels co-occurring in a narrow hybrid zone. While these systems are useful for addressing some patterns and processes involved, they cannot be readily applied to more complex contact zones, which are much more frequent in plants than previously anticipated (Suda et al., 2007). The rapid development of FCM and its application in the assessment of detailed cytotype structure in plant populations caused progress in plant population biology.

Chromosome counts: To guarantee reliability of ploidy estimates using flow cytometry, number of chromosomes was determined in 20 plants, representing all cytotypes. This limited number of plants was analysed both in cytotype "uniform" populations (*G. densiflora* and *G. montana* populations from Kuřim and Kurdějov), and from populations with sympatric growth of different cytotypes where plants with minority ploidy levels (determined after FCM analysis) were colected and subjected to karyological analyses. Cytotype distribution over vast geographic areas provides useful insight into population biology of multi-ploid taxa. However, large-scale cytotype investigations had been enormously time-consuming until flow cytometry was routinely employed (Lewis et al., 1967), and only small number of plant not necessary representing real cytotype composition of populations was analysed (Marhold et al., 2005). Modern approach and utilization of FCM in cytotype determination also evoked to raise numerous challenging questions of current biosystematics, such as the overall distribution pattern (sympatry vs. parapatry), investigation of evolutionary forces governing the cytotype co-existence, ecological preferences of individual cytotypes, inter- vs. intra-cytotype competition, or mechanisms of reproductive isolation (Petit et al., 1999).

Conclusions

In this study 36 populations of *Gymnadenia conopsea* complex using flow-cytometry (ploidy level analysis) approach were analysed. Based on analysis of 2226 samples, following patterns of variation were detected:

(1) population of *G. densiflora* are tetraploids, with 2n=40 and are marked by typical profile on FCM histograms differing from other *G. conopsea* populations;

(2) tetra- and octoploid *G. conopsea* plants are also characterised by typical FCM histograms;

(3) on most stands (except *G. densiflora*) sympatric growth of different cytotypes were recorded and this high cytotype diversity would be strongly underestimated if populations were analysed only on the basis of morphological characteristics;

(4) on some stands up to 5 ploidy levels were recorded – tetraploids, hexaploids, oktoploids, decaploids and dodecaploids.

Using of flow cytometry it was possible to analyse cytotypic structure of populations, reveal prevalent occurrence of different cytotypes within analysed populations and their sympatric character. Specific peak ratio acquired on the basis of flow-histograms were calculated and all analysed taxa belonging to *Gymnadenia* conopsea complex s.l. was possible to distinguish on the basis of Cx-value.

Table 1: Characteristic of analyzed populations of *Gymnadenia conopsea* complex from Czech and Slovak Republic and Austria.

No.	Name of locality	Geogr. coordinates		Locality description
		latitude	longitude	
1	Javorník, PR Nad zavírkou (Šumava)	49°7' 44.47"N	13°39' 34.46"E	S Bohemia, Šumava, Nature reserve Nad Zavírkou, mesophytic grasslands on S slopes of the Javornik mountain, 1 km NW of the village Úbislav, close to settlement Směť, S exposure
2	Sudslavice, PR Opolenec	49°5' 13.59"N	13°47' 48.44"E	S Bohemia, Šumavsko-Novohradské podhůří, Nature reserve Opolenec, grasslands and sparse pine forest on limestone ridge above Volyňka river, 1 km E of the village Sudslavice, 4 km N of the town Vimperk
3	Chvalšiny, Svatý Kríž	48°50' 54.6"N	14°12' 0.64"E	S Bohemia, Šumavsko-Novohradské podhůří, mesophytic grasslands on the N slopes of the hill Svatý kříž, 1 km S off the village Chvalšiny
4	Hrádek u Chrašťan	50°29' 18.74"N	13°55' 2.02"E	C Bohemia, Milešovské stredohoří, mesophytic and wet grasslands between Solanská hora hill and ruin of Oltářík castle, 1 km N of the village Chrášťany, S exposure
5	Lísky	50°15' 29.35"N	13°56' 14.57"E	C Bohemia, Džbán, mosaic of wet grasslands, springs and sparse forest on the hill Háje, 1 km NE village Lísky near to Kladno
6	Mělnická Vrutice, NPR Polabská černava	50°20' 32.97"N	14°32' 31.06"E	C Bohemia, Všetatské Polabí, Nature reserve Polabská černava, black bog 1 km SW of the village Mělnická Vrutice
7	Litoměřice, PP Bílé stráně	50°33' 31.05"N	14°7' 59.13"E	N Bohemia, České stredohoří, Nature reserve Bílé stráně, mosaic of xerophytic grasslands on skeletal soils, mesophytic grasslands and shrubs on violent descent in Pokratický creek valley, 1 km N of town of Litoměřice, W exposure
8	Podloučky u Turnova	50°36' 42.07"N	15°12' 46.16"E	N Bohemia, Český ráj, submesophytic grasslands on moderate slopes (in Jizera river valley) 1 km E of the village Podloučky, NE of the town Turnov, S exposure
9	Čížov - Hardegg	48°51' 17.19"N	15°52' 8.79"E	S Moravia, Moravské podhůří Vysočiny, National park Podyjí, skeletal vegetation and mesophytic grasslands in Dyje river valley, 2 km SW of the village Čížov
10	Kuřim, PR Obůrky – Třeštěnec	49°18' 30.79"N	16°30' 13.07"E	S Moravia, Moravské podhůří Vysočiny, wet grasslands in artificial depression in earth cut of unfinished highway, N of the road Kuřim – Čebín, 2 km NW of the town Kuřim
11	Dolní Čepí, PP Křižník	49°28' 57.07"N	16°20' 43.89"E	W Moravia, Českomoravská vrchovina, mesophytic grasslands on violent descent above Svratka river surrounded by mixed forest, 1 km N of the village of Dolní Čepí, W exposure
12	Jobova Lhota u Olešnice, PP V Jezdinách	49°35' 30.47"N	16°26' 19.12"E	W Moravia, Českomoravská vrchovina, Nature reserve V Jezdinách, submesophytic grasslands on the top of hill and mesophytic grasslands on N slopes 1 km NW of the village of Jobova Lhota, N of the town Olešnice
13	Hustopeče, PR Kamenný vrch u Kurdějova	48°57' 49.78"N	16°45' 21.7"E	SE Moravia, Hustopečská pahorkatina, Nature reserve Kamenný vrch, xerophytic grasslands on small hill near the village Kurdějov, E-SE exposure
14	Bořetice, PP Zázmoníky	48°56' 7.56"N	16°51' 17.98"E	SE Moravia, Hustopečská pahorkatina, Nature reserve Zázmoníky, xerophytic grasslands, former vineyards, 2 km N of the village Bořetice, W exposure
15	Kněždub, NPR Čertoryje	48°51' 25.15"N	17°24' 36.9"E	SE Moravia, Bílé Karpaty, Nature reserve Čertoryje, 2 km SE of the village of Radějov – Horní mlýn, submesophytic up to xerophytic grasslands on moderate slope above Járkovec creek, SW exposure, early flowering population of Gymnadenia
16	Velká nad Veličkou, NPR Zahrady pod Hájem	48°53' 3.97"N	17°31' 51.57"E	SE Moravia, Bílé Karpaty, Nature reserve Zahrady pod Hájem, mosaic of xerophytic and mesophytic grasslands, communities on wet and dried-out marl or clay, 1 km NE of the village Velká nad Veličkou, W exposure
17	Javorník, PR Machová	48°49' 45.1"N	17°32' 33.55"E	SE Moravia, Bílé Karpaty, Nature reserve Machová, 4 km S of the village Javorník, mesophytic grasslands on moderate slopes in western part of the reserve, W exposure, early flowering population of Gymnadenia
18	Javorník, PR Machová	48°49' 45.12"N	17°32' 22.66"E	SE Moravia, Bílé Karpaty, Nature reserve Machová, 4 km S of the village Javorník, small area of sloping springs with tuff formation and fens in central part of the reserve, on slopes above Rybický creek, E exposure
19	Horní Němčí, NPR Porážky	48°53' 23.75"N	17°37' 22.86"E	SE Moravia, Bílé Karpaty, Nature reserve Porážky, mesophytic grasslands on N slopes, 3 km E of the village Suchovské mlýny

20	Javorník, NPR Jazevčí	48°52' 27.83"N	17°34' 14.36"E	SE Moravia, Bílé Karpaty, Nature reserve Jazevčí, mesophytic grasslands on slopes under Velička river, close to settlement Pod Kozimelkou, 3 km NE the village Javorník, N exposure
21	Březová, PP Kalábová	48°55' 41.66"N	17°43' 51.22"E	SE Moravia, Bílé Karpaty, Nature reserve Kalábová, mosaic of xerophytic, mesophytic and wet grasslands and boggy springs, N of the village Březová, S exposure
22	Vsetín, PPJežůvka	49°20' 59.52"N	18°0' 40.63"E	CE Moravia, Střední Pobečví, Nature reserve Ježůvka, mesophytic grasslands on moderate slopes in Zádilský valley, 2 km NE of the town Vsetín, SW exposure
23	Vsetín - Růžďka, PP Lúčky - Roveňky	49°22' 58.18"N	18°0' 8.22"E	CE Moravia, Střední Pobečví, Vsetínská kotlina, Nature reserve Lúčky-Roveňky, wet grasslands and sloping springs surrounded by spruce forest 1.5 km SE of the village Růžďka, N of the town Vsetín, W exposure
24	Valašské Klobúky, PR Bílé potoky	49°7' 10.67"N	18°1' 19.01"E	CE Moravia, Javorníky, Nature reserve Bílé potoky, submesophytic grasslands on violent descent above Kloboucký creek, 3 km S of the town Valašské Klobouky, W exposure
25	Velké Karlovice, Solanec - Čarták	49°23' 19.97"N	18°14' 30.16"E	CE Moravia, Moravskoslezské Beskydy, mesophytic grasslands in the centre of Beskydy mountains, close to mountain hotel Čarták, 5 km NW of the town Velké Karlovice, W exposure
26	Skalica, SPR Veterník	48°49' 6.8"N	17°13' 54.96"E	W Slovakia, Záhorská nížina, Nature reserve Veterník, xerophytic grasslands on N slopes of the Vintoperk hill, 1 km NW of the village Mokrý Háj, near the town of Skalica
27	Súlov, Vrchteplá	49°8' 0.82"N	18°34' 18.53"E	NW Slovakia, mosaic of xerophytic, mesophytic and wet grasslands and sparse pine forest on S slopes in Vrchteplá valley, 1 km NE of the village Vrchteplá, SW exposure
28	Porúbka, Turie	49°8' 55.37"N	18°44' 3.17"E	NW Slovakia, Lúčanská Malá Fatra, xerophytic grasslands on skeletal soils, mesophytic grasslands and shrubs on violent descent on limestone ridge between the villages of Porúbka and Turie, S of the town Žilina, S exposure
29	Nižné Kamence	49°14' 46.54"N	19°0' 19.74"E	NW Slovakia, Kriváska Malá Fatra, mesophytic grasslands Pod Sokolím, 1 km S of the village of Nižné Kamence, between Belá and Terchová, N exposure
30	Biely potok, Rovná hora	49°15' 52.80"N	19°6' 16.52"E	NW Slovakia, Kysucká vrchovina, mesophytic grasslands on the top and xerophytic grasslands on the slopes of the hill Rovná hora, between the villages of Terchová and Zázrivá, 4 km E of the village Terchová, S exposure
31	Ružomberok, Vlkolínec	49°2' 29.61"N	19°16' 30.92"E	NW Slovakia, Veľká Fatra, submesophytic grasslands on violent descent of Malinné hill above Vlkolínec village, 4 km S of the town Ružomberok, S exposure
32	Ružomberok, wetland in Vlkolínec valley	49°1' 59.48"N	19°16' 12.30"E	NW Slovakia, Veľká Fatra, wetland and sloping springs around Vlkolínec creek, 1 km S of the village Vlkolínec
33	Ružomberok, Vlkolínec valley	49°1' 47.89"N	19°16' 32.06"E	NW Slovakia, Veľká Fatra, mesophytic grassland on slopes in the beginning of Vlkolínec valley, 2 km SE of the village Vlkolínec, S exposure
34	Osádka	49°10' 10.69"N	19°22' 57.67"E	NW Slovakia, Chočské vrchy, mesophytic grassland on slopes above creek, wetlands in alluvial plain, 2 km S of the village Osádka, SE of the town Dolný Kubín, N exposure
35	Lúčky Kúpele	49°8' 1.39"N	19°23' 34.28"E	NW Slovakia, Chočské vrchy, mesophytic grassland and forest in the end of Lúčky valley, N of the village
36	Durrenstein	47-50	15-04	Austria – Ybbstaler Alpen, Lunz am See, mesophytic grasslands and roadsides between Seehof and Mittersee, 3 km S from the Lunzer See; mesophytic grasslands and skeletal slopes above Seebach, 4 km S from the Lunzer See; grasslands on skeletal soils on detrital slopes in the end of Seetal, between Klausse and Ludwigfall, 5 km S from the Lunzer See

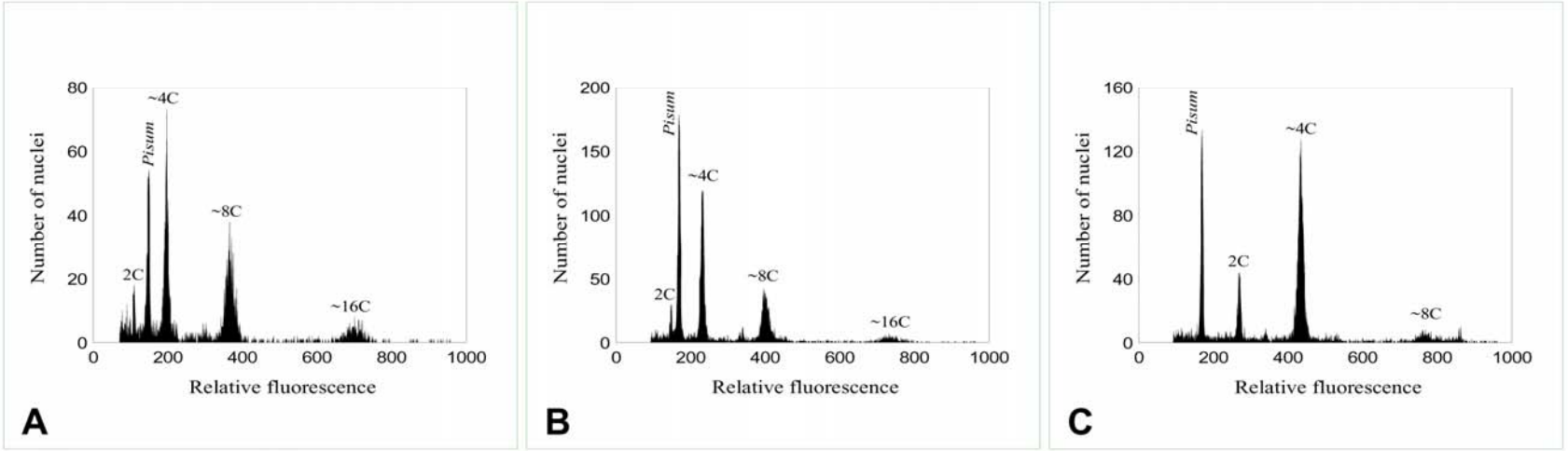
Table 2: Number of analysed plants and frequency of particular ploidy levels.

Site	**Number of analysed plants**	**DNA Ploidy levels**					
		Frequency of ploidy levels					
		4x C	**4x D**	**6x**	**8x**	**10x**	**12x**
1. Javorník, PR Nad zavírkou (Šumava)	60	58		2			
2. Sudslavice, PR Opolenec	70				68	1	1
3. Chvalšiny, Svatý Kríž	14				14		
4. Hrádek u Chrášťan	51		51				
5. Lísky	50		50				
6. Mělnická Vrutice, NPR Polabská černava	50	1	49				
7. Litoměřice, PP Bílé stráně	75	72		3			
8. Podloučky u Turnova	50				50		
9. Čížov, Hardegg	1				1		
10. Kuřim, PR Obůrky – Třeštěnec	50		49	1			
11. Dolní Čepí, PP Křižník	49		5		41	2	1
12. Jobova Lhota u Olešnice, PP V Jezdinách	50		5		44	1	
13. Hustopeče, PR Kamenný vrch u Kurdějova	60				60		
14. Bořetice, PP Zázmoníky	4				4		
15. Kněždub, NPR Čertoryje	50	50					
16. Velká nad Veličkou, NPR Zahrady pod Hájem	26	10	10		6		
17. Javorník, PR Machová	53	50			3		
18. Javorník, PR Machová	60		58	2			
19. Horní Němčí, NPR Porážky	69	10	1	1	57		
20. Javorník, NPR Jazevčí	572	322		10	229	6	5
21. Březová, PP Kalábová	100	49	50	1			
22. Vsetín, PPJežůvka	50	48		2			
23. Vsetín – Růžďka, PP Lúčky – Roveňky	50		50				
24. Valašské Klobouky, PR Bílé potoky	30	30					
25. Velké Karlovice, Solanec – Čarták	50	50					
26. Skalica, SPR Veterník	60				60		
27. Súlov, Vrchteplá	71	34	33	2	2		
28. Porúbka, Turie	60	16		3	40	1	
29. Nižné Kamence	30	29		1			
30. Biely potok, Rovná hora	50	12		2	36		
31. Ružomberok, Vlkolínec	50	2			47		1
32. Ružomberok, Vlkolínec mokřad	50		50				
33. Ružomberok, Vlkolínec stráň kolem cesty	50				50		
34. Osádka	12	8	3		1		
35. Lúčky Kúpele	21		21				
36. Durrenstein	28				28		
∑	**2226**	**851**	**485**	**30**	**841**	**11**	**8**
[%]	**100**	**38,23**	**21,79**	**1,35**	**37,78**	**0,49**	**0,36**

Table3: Results of flow cytometric analyses – expression of peak rations against internal standard and between individual Gymnadenia peaks.

DNA ploidy level	Peak ratios against internal standard (*Pisum sativum*)								Ratios between individual *Gymnadenia* peaks		
	i – *Pisum*		I - *Pisum*		II – *Pisum*		III - *Pisum*		i-I	I-II	II-III
	mean ± SD	*n*	mean ± SD	*n*	mean ± SD	n	mean ± SD	*n*	mean ± SD	mean ± SD	mean ± SD
4x - D	0.744 ± 0.021	53	1.299 ± 0.031	95	2.413 ± 0.063	95	4.630 ± 0.143	90	1.752 ± 0.034	1.857 ± 0.018	1.918 ± 0.022
4x - C	0.841 ± 0.026	78	1.327 ± 0.036	261	2.306 ± 0.062	262	4.275 ± 0.121	242	1.585 ± 0.028	1.738 ± 0.017	1.854 ± 0.024
6x	1.244 ± 0.031	11	1.985 ± 0.050	19	3.447 ± 0.073	19	6.330 ± 0.174	7	1.592 ± 0.029	1.737 ± 0.038	1.846 ± 0.021
8x	1.590 ± 0.040	196	2.547 ± 0.065	198	4.455 ± 0.122	197	8.147 ± 0.205	37	1.602 ± 0.021	1.749 ± 0.021	1.841 ± 0.017
10x	2.001 ± 0.043	5	3.146 ± 0.092	5	5.567 ± 0.168	4	-	-	1.574 ± 0.024	1.742 ± 0.024	-
12x	2.380 ± 0.033	4	3.812 ± 0.047	4	6.699 ± 0.043	2	-	-	1.602 ± 0.004	1.754 ± 0.015	-

Figure 1. Flow cytometric histograms of nuclei isolated from tetraploid (A) *Gymnadenia densiflora*, tetraploid (B) and (C) octoploid *Gymnadenia conopsea* and *Pisum sativum* as an internal standard.



2n=40, *G. densiflora*

2n=40, *G. conopsea*

2n=80, *G. montana*

Map 1: Geographical localization of analyzed populations of *Gymnadenia conopsea* and illustration of cytotype distribution across analysed populations/localities.

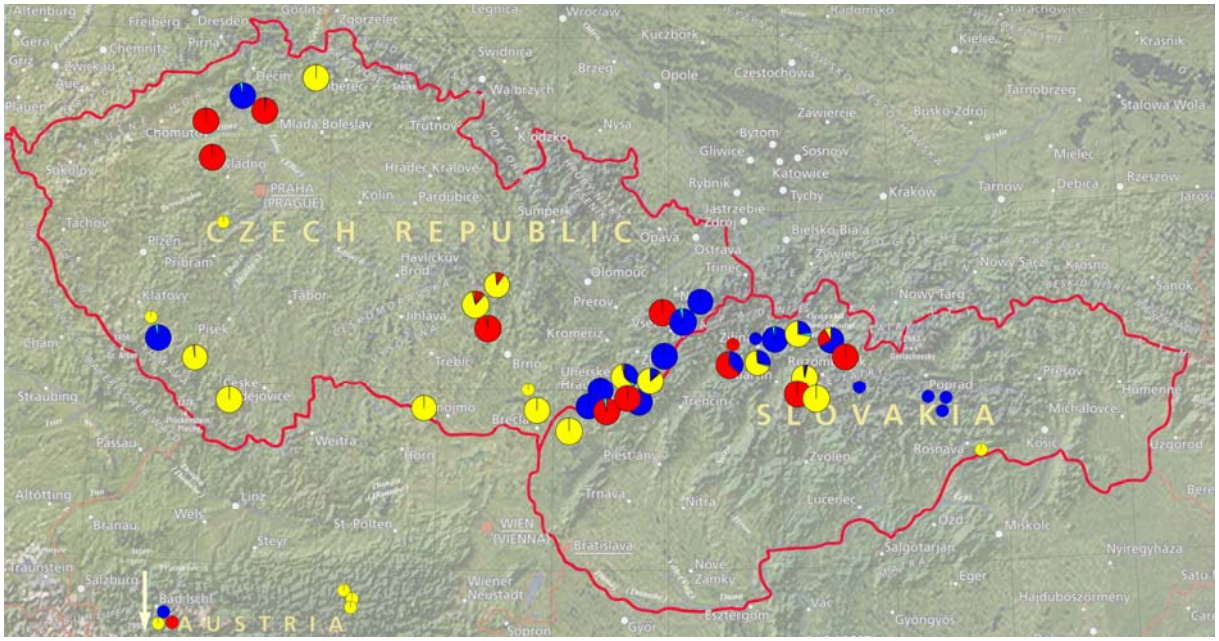
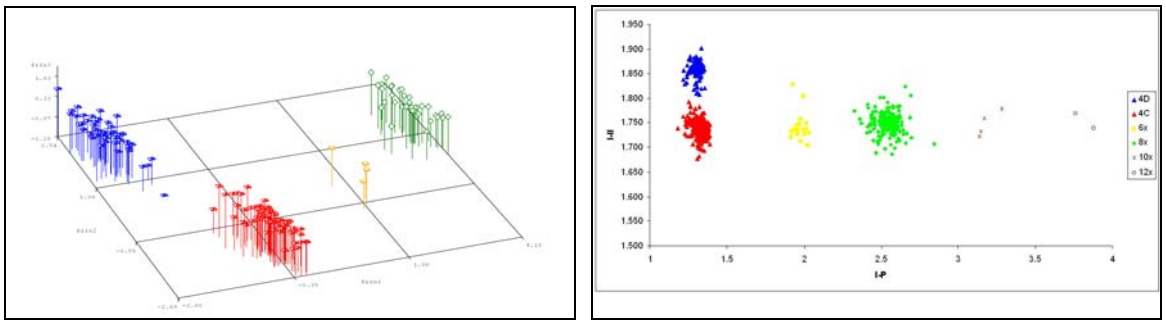


Figure 2: Results of PCA analysis – analysis of only histograms with all four *Gymnadenia* peaks distinct (Fig. 2A) and all flow assays (Fig. 2B).



2A 2B

blue – *G. densiflora*, red – tetraploid *G. conopsea*, green – octoploid *G. conopsea (montana)*, yellow – hexaploid plants, brown – decaploid plants, black – dodecaploid plants.

Figure 3: Differentiation of 3 basal taxa of *Gymnadenia* conopsea complex on the basis of Cx-value (value of „average“ chromosome set).

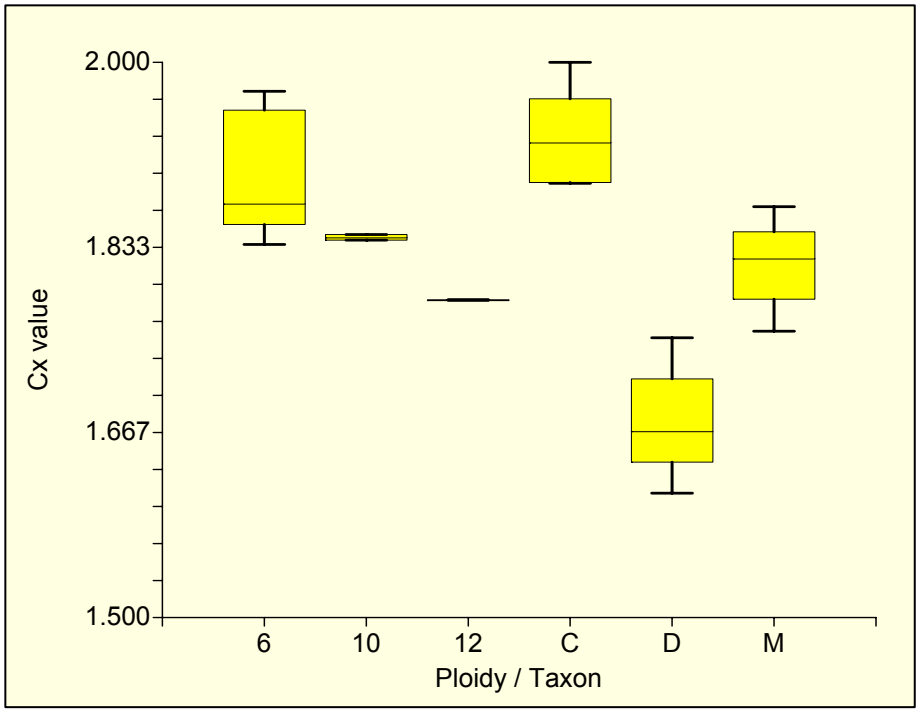
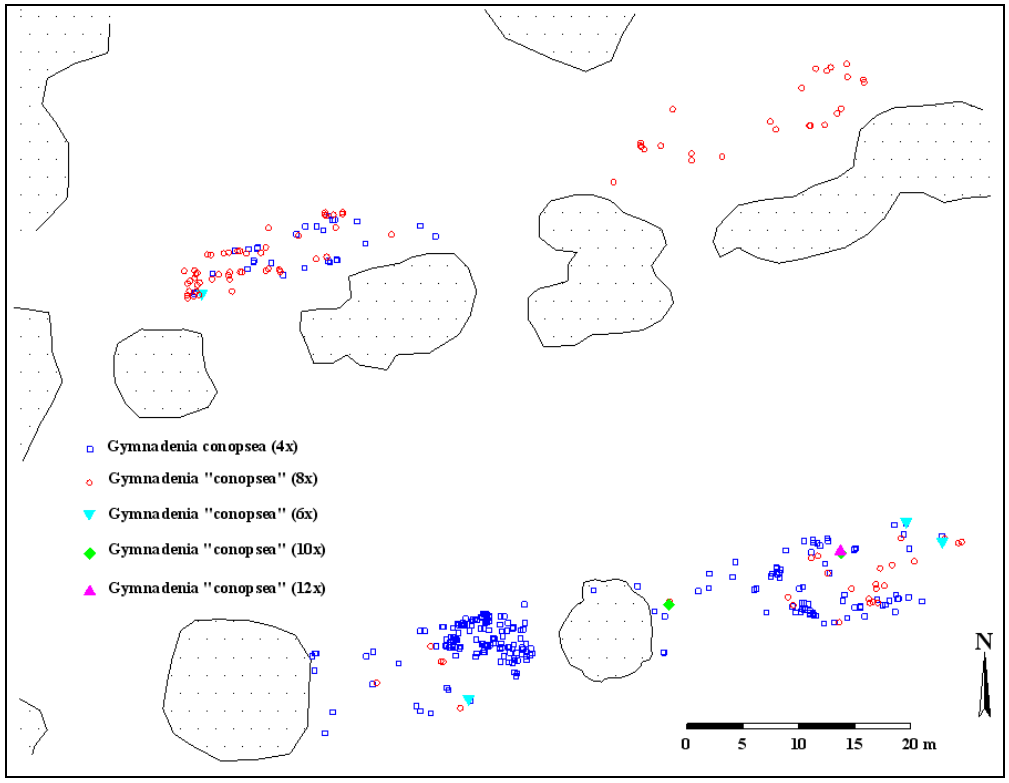


Figure 4: Results of detailed cytotype screening at the locality Javorník, Jazevčí (no. 20).



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ScienceDirect
Aquatic Botany 86 (2007) 321–330
Aquatic botany
www.elsevier.com/locate/aquabot

Phenotypic and genotypic variation of *Phragmites australis*: Comparison of populations in two human-made lakes of different age and history

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Received 3 January 2006; received in revised form 20 November 2006; accepted 27 November 2006

Abstract

Populations of *Phragmites australis* (CAV.) TRIN. ex STEUD. were studied in the littoral zones of two man-made lakes located in the Třeboň Basin (South Bohemia, the Czech Republic): (1) Opatovický fishpond, a shallow artificial lake constructed in 1510–1514 by damming a shallow valley and used since for carp production, and (2) Halámky sand pit, a new lake formed by sand extraction in 1970–1994. Phenotypic variability was assessed on the basis of shoot morphological and growth characteristics, measured at the time of seasonal maximum aboveground biomass. Genotypic variability was detected using RAPDs, which demonstrated a high clonal diversity in both habitats. The clonal diversity would be strongly underestimated if it were based only on morphological differences. Higher genotypic variability was found in the fishpond reed, not corresponding with low variability in its phenotype performance. Based on analysis of 160 samples, four patterns of genotypic variation were detected: (1) Some stands were genetically uniform and were therefore considered to be monoclonal in both populations studied. (2) Some stands consisted of several clones at the Halámky sand pit. However, these clones showed more similarity within the particular stands than with clones of adjacent stands. (3) In the Opatovický fishpond population, multiclonal stands consisted of clones with a low degree of similarity. (4) Identical clones were detected in several neighbouring stands separated by gaps in the Opatovický fishpond population. The findings support a model of colonization postulating that populations initiated by seeds are initially genetically diverse and over time become dominated by a few clones as a result of competition and selection. These processes then decrease both genetic and morphological variability.

Keywords: Phragmites; Phenotypic variation; Genotypic variation; Lake; Clone

1. Introduction

Stands of common reed (*Phragmites australis*) have been extensively studied in various wetland types world-wide. The differences in the structure and production between particular reed stands have been related to the trophic state of their sites (Rodewald-Rudescu, 1974; Dykyjová and Hradecká, 1976; Kühl and Kohl, 1992; Kohl et al., 1998), differences in climatic and meteorological conditions (Haslam, 1971, 1973; Dykyjová and Hradecká, 1976; Zemlin et al., 2000) or the genetic differences between reed clones and populations (Daniels, 1991; Kühl et al., 1997; Koppitz, 1999; Kühl et al., 1999; Lippert et al., 1999; Rolletschek et al., 1999; Guo et al., 2003; White et al., 2004; Bastlová et al., 2006).

Several authors have shown that many aquatic reed stands (i.e. reed stands growing in aquatic habitats, as contrasted with terrestrial ones; see e.g. Güsewell and Klötzli, 2000) have a low genetic diversity (Ziedler et al., 1994; Koppitz et al., 1997). This has been ascribed to the prevailing vegetative propagation of reed since a whole stand can develop from one or a few seeds. According to an alternative hypothesis, the genotypic diversity of some stands may initially be higher and subsequently decrease because of competition and selection (Neuhaus et al.,

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doi:10.1016/j.aquabot.2006.11.010

1993). Nevertheless, few field data exist as yet to test the validity of this hypothesis. This may partly be due to difficulties in finding differently aged populations, subject to the same climatic conditions.

This paper is part of a broader study focused on reed populations of two human-made lakes whose age differs by an order of magnitude. The considerable age difference makes these two populations a good field model to investigate how phenotypic and genotypic diversity change with time, an aspect that has scarcely been investigated. In this study, the following partial hypotheses were tested:

(1) The number of clones per unit of population area is lower in the older population than the younger population.
(2) The maximum area covered by a single clone is larger in the older population than the younger one.
(3) Morphologically homogeneous stands are monoclonal.
(4) Morphological variability is a good indicator of the genetic variability in multiclonal stands and populations.

2. Material and methods

2.1. Site description

Two localities were selected in the Třeboň Basin Biosphere Reserve (Třeboň: 49°05′N, 14°46′E, altitude 430 m), South Bohemia, Czech Republic: Opatovický fishpond and Halámky sand pit.

Opatovický fishpond is a shallow water body constructed in 1510–1514 by damming a shallow valley and used since for carp production. The maximum flooded area is 160 ha. Its maximum length and width are 2.6 and 1.1 km, respectively, and the shoreline length is 9.40 km. The fishpond is fed mainly with mesotrophic to eutrophic water from the Lužnice river catchment. The trophic status of the water has further increased through the fertilization and manuring of the whole fishpond system as well as of the surrounding agricultural land. Well developed reed stands fringe over half of the shore length. Judging from the history of lake management, the reed stands may have been present in the littoral zone of the lake from one to several centuries. Since the 1960s, the reed stands have been subject to a series of scientific studies, which include detailed mapping of their area, production and mineral nutrient economy (Dykyjová and Květ, 1978; Hroudová and Zákravský, 2002). As in most other fishponds of the region, parts of the littoral stands of the Opatovický fishpond were destroyed by cleaning the fishpond bottom of excessive sediments using heavy machinery in the 1970s and 1980s. Only those stands that occurred on a map of the fishpond littoral communities of 1971 (Husák and Hejný, 1978) were included in this study.

Halámky sand pit is a new lake formed by sand extraction, which took place from 1970 to 1994. The area of the lake is 22.89 ha. The maximum length and width are 0.79 and 0.41 km, respectively, and its shoreline length is 2.01 km. The sand-pit is situated in the Lužnice river floodplain and is fed with its water, filtered through a sand barrier. Halámky sand-pit represents a mesotrophic habitat, hosting young and expanding reed stands. All of the reed vegetation there is certainly younger than 35 years, but much of it is probably younger than 20 years. It is likely that it established itself from seed, as the lake does not have direct connection with any watercourse in the catchment.

2.2. Sampling

Well developed, healthy looking and visually homogeneous stands of *P. australis* were chosen in the littoral zones of both lakes. The visual homogeneity was due to the uniformity of shoot heights, angle, width and colour of leaves and panicles, as used previously by Hradecká (1973a, 1973b). The selected stands were mostly bordered by stands of other helophyte species or open water. In one instance, adjacent segments of the reed belt distinctly differed in morphological features (stand height, stem thickness, leaf angle and colour) and were therefore considered to be different stands. Nine and seven reed stands were selected at the Opatovický and Halámky lakes, respectively. Five shoots were sampled from each stand in June 2003 along a transect laid down parallel to the shoreline at a depth of 0.4–0.5 m. These shoots were used for preliminary genetic analyses. The distance between the sampled shoots, within a particular stand, was adjusted according to the length of the smallest stand within each population; these were 10 and 2.5 m at the Opatovický and Halámky lakes, respectively. Total aboveground biomass was harvested from five 0.5 m × 0.5 m quadrates in each stand in August 2003. The quadrates were situated along the same transects and at the same distances as in the previous sampling. Two parallel transects were laid down along the fringes of the reed belts of each lake in September 2003, one being adjacent to the open water edge of the reed belt, while the other followed the terrestrial edge. At Opatovický lake, the water level was 0.2–0.3 m below the soil surface at the terrestrial edge of the reed belt while the water depth was about 0.5 m along the open water edge. At Halámky, the transects were situated at water depths of 0.5 and 2 m, respectively. Five shoots were collected along either transect within a stand (i.e., 10 shoots from each of 16 stands, representing 160 samples in total) and subjected to both morphological measurement and genetic analyses.

2.3. Analysis of molecular markers

Fresh leaf samples were collected in September 2003 as described above. DNA from the leaf samples was isolated by using the Invisorb Spin Plant Mini Kit (INVITEK, Germany) according to standard protocol. DNA samples were stored at −20 °C prior to their use. DNA samples used for primer screening were isolated using the same procedure, from leaf samples collected at the end of June from the same stands as above.

Random primers (OPERON Technologies, CA) were selected for the RAPD analysis. The protocol for the RAPD analysis was adapted from that of Williams et al. (1990). The volume of the final PCR reaction (25 μl) was composed of 1× buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$),

200 μM dNTP, 10 pM of primer, 1 UTaq DNA polymerase (TAKARA) and 25 ng of template DNA. Amplifications were carried out in an MJ Research Thermocycler PTC 100 according to a program that included an initial denaturizing step of 3 min at 94 °C, then 45 cycles of 1 min at 94 °C, 2 min at 35 °C and 3 min at 72 °C followed by final elongation for 10 min at 72 °C. The final step was followed by a holding temperature of 4 °C. PCR products were separated on 1.5% agarose gels in TBE buffer, which included lanes of 100 bp DNA ladder (NEB); DNA bands were visualized after ethidium bromide staining under UV light. RAPD patterns were photographed using an Epson Ultra Cam.

RAPD data files were analysed using the BioProfil 1D++ software (Vilber Lourmat, France) with manual correction. Fingerprint patterns were transformed into a binary character matrix with 1 for presence or 0 for absence of a band at a particular position in a lane. After removing monomorphic bands, genetic distance matrices were generated using Nei and Li similarity metrics and cluster analysis (UPGMA—unweighted pair group method averages) and PCA, PCO and correspondence analysis were performed. These statistical analyses were calculated using MVSP (Kovach Comp.Serv.) and the STATISTICA 6.0 software package (Statsoft).

A total of five random primers (OPA-03, OPA-04, OPA-11, OPB-04 and OPB-08) were surveyed across all samples from the 16 stands. All these primers exhibited a sufficient level of polymorphism. These five primers amplified altogether 88 bands, 75 (85%) of which were polymorphic. Thirteen bands exhibited a low level of polymorphism (polymorphism was detected in only 1–3 plants), so 62 (70%) amplified bands were highly polymorphic. This gave on average 12.4 polymorphic products per one polymorphic primer. The highest polymorphism was detected using primers OPA-04 and OPB-08 and these primers were used for analyses of all 160 reed samples.

Primers OPA-04 and OPB-08 amplified 19 and 16 bands, and only two bands generated by OPB-08 were non-polymorphic. Five additional bands exhibited a low level of polymorphism (3 and 2 using primers OPA-04 and OPB-08, respectively). The 28 highly polymorphic bands represented 80% of all amplified bands. This high level of polymorphism allowed for individual clones to be characterised and distinguished.

The size of the amplified band ranged from 100 to 1500 basepairs. RAPD fingerprints were amplified repeatably (the same results were obtained in three independent PCR experiments). The clear and distinct banding patterns indicate that this method is suitable for genetic studies of *P. australis*.

2.4. Shoot growth and morphology

Characteristics of reed production (density of live, flowering and dead shoots, total aboveground biomass) and morphology (shoot length to the tip of the uppermost leaf, number of internodes per plant, basal diameter, panicle length) were recorded at the time of seasonal maximum aboveground biomass, using recommendations by Dykyjová et al. (1973). In order to facilitate comparisons between the stands, special attention was paid to the tallest shoot within each sample, which had originated from the apical rhizome buds (Dykyjová, 1970; Hradecká, 1973a). Therefore, the shoots were of similar age and, as they all belonged to the first spring cohort of shoots, they were probably little affected by intraspecific competition.

Aboveground biomass samples were collected in late August 2003, as described in Section 2.2. After determination of the morphological characteristics of each sample, its fresh weight was immediately determined on a mechanical balance. Fifty percent of the samples were then selected at random, transported to the laboratory, dried to constant weight in an electrical oven at 80 °C, and weighed. Their fresh and dry weights were used to calculate a linear regression, according to which the dry weights of the remaining samples were estimated.

Nineteen morphological characteristics were recorded for shoots sampled in September 2003, for which the RAPD analysis was performed also (see Section 2.2). The morphological characteristics included the panicle length and dry weight, shoot diameter below the panicle, and lengths and maximum widths of the first to eighth leaf from the shoot apex.

Statistical analyses were carried out using the STATISTICA 6.0 software package (Statsoft). Morphological differences between the stands within each population, based on the data gathered at the time of seasonal maximum aboveground biomass, were first evaluated using one-way analysis of variance. As the condition of homogeneity of variances (tested by the Levene test) was not fulfilled, the data were subjected to the Kruskall–Wallis non-parametric test. The contribution of the measured morphological and growth characteristics to the overall differences between the stands and populations was explored using PCA.

In order to evaluate hypothesis 4, variation coefficients were calculated for all morphological and growth characteristics within each stand. The results were then plotted against the number of clones in those stands. PCA and discrimination analyses were used in order to explore discrimination between clones based on the 19 morphological characteristics of individual shoots.

3. Results

3.1. Genetic diversity of analysed populations

The genetic similarity was estimated for all sample comparisons based on Nei and Li metrics and calculated from the total number of polymorphic bands. This matrix of genetic distances was used to cluster samples, accounting for their degree of genetic similarity. Cluster analysis (UPGMA method) resulted in a dendrogram that separated the two populations in two distinct groups. The level of genetic similarity between individual clones within the Opatovický population was higher than 83% except for two clones located in the most north-eastern part of the lake. The similarity of these two clones with the others was only 70%, but still all the clones formed one group, which was highly distinct from the clones of the Halámky population. In comparison with the Opatovický

population, the level of genetic polymorphism was markedly higher between the clones of the Halámky population. All of the clones again formed one group, but the diversity was higher than in the Opatovický population, with the dissimilarity among clone groups ranging between 15 and 33%. An extreme polymorphism was found in stand B. The group of seven clones found in this stand exhibited a conspicuously high distinctness. The polymorphism within this group was also exceptionally high.

The clones formed two independent and distinguishable groups in the Principal component analysis (PCA) of the RAPD data (Fig. 1A). Like in the cluster analysis, clones from the Opatovický population were aggregated in a relatively compact group. By contrast, the clones from the Halámky population formed an expanded group, whose polymorphism was twice as high as that in the Opatovický clones. Principal coordinates (PCO) and correspondence analysis (CA) gave very similar results (data not shown). Similar results were obtained when both genetic (RAPD) and morphological data were analyzed jointly. Cluster analysis and the PCA, PCO and CA analyses gave results almost coincident with those presented in Fig. 1 (data not shown).

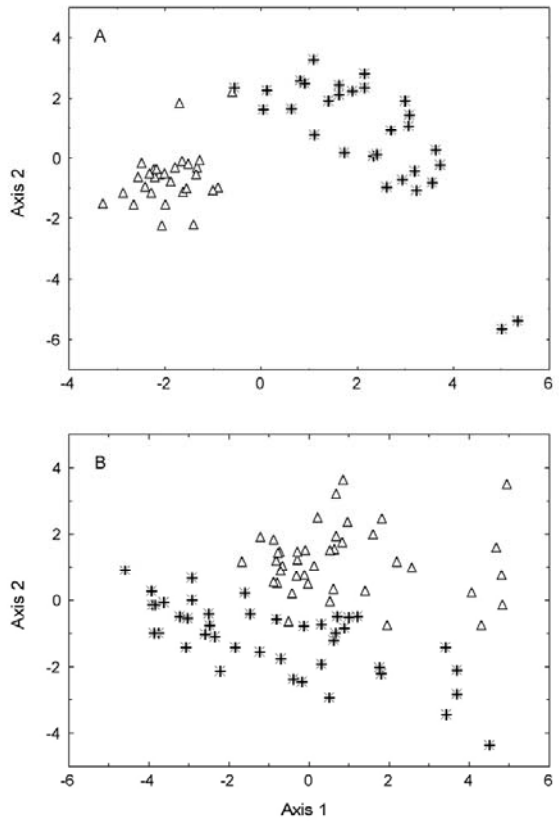

Fig. 1. PCA case scores of *P. australis* stands of the Opatovický (triangles) and Halámky (asterisks) lakes calculated from RAPD (A) and morphological (B) data.

3.2. Clone distribution and genetic variation of stands

All reed samples in some of the investigated stands, exhibited an identical RAPD pattern suggesting that all analysed plants belonged to one clone and, therefore, that this particular stand consists of a single clone. Such monoclonal stands were present both in the Opatovický and Halámky populations (stands E, F*, I and J, N, O, P). A more frequent situation was that stands were composed of higher numbers of clones of different similarity. Considering multiclonal stands only, the number of clones was significantly smaller in the Opatovický than in the Halámky population (t-test, $p = 0.04$). The largest clonal area was that of clone O-41 at Opatovický lake, which was identified with a frequency of 28% among the samples from that site. By contrast, each of the three largest clones of Halámky lake had a frequency of 14% among the samples taken from there.

It was possible to detect four basic patterns of genotypic variation (Fig. 2). (1) In both populations studied, some stands were genetically uniform and, therefore, were assumed to be monoclonal. (2) Some stands consisted of several genotypes at Halámky lake. However, these genotypes were more similar within each particular stand than they were to genotypes in adjacent stands. (3) In the Opatovický population, identical clones were detected in several neighbouring stands separated by gaps. The most conspicuous example of this pattern was represented by clone O-41, which occurred in five stands along the eastern shore. (4) Multiclonal stands in the Opatovický population consisted of genotypes with a low degree of similarity.

3.3. Morphological and growth characteristics of reed stands

Close correlations were found between morphological characteristics describing individual shoots. The highest determination coefficients ($R^2 > 0.80$, $n = 87$, $p < 0.0001$) were found for both mean shoot length versus mean basal culm diameter and maximum shoot length versus maximum basal culm diameter. The first and second PCA axes of all 11 morphological characteristics accounted for 49 and 23% of total variation, respectively. Characteristics associated with the size of individual shoot (mean and maximum shoot length, mean and maximum basal culm diameter, mean and maximum number of internodes) explained 82% of the variability associated with the first axis. Characteristics related to panicle size, shoot densities and the related effect of shoot densities on aboveground biomass explained 87% of the variability associated with the second axis. The plot of case scores separated the two populations with hardly any overlap (Fig. 1B). In comparison with Opatovický, the Halámky population showed more variation along the first axis, which corresponded to a greater variation in shoot size between the stands. Stands located on the extreme left of the first axis were those with robust shoot sizes, which were recorded only in some of the Halámky stands but in none of Opatovický lake. The position of the Halámky population along the second axis

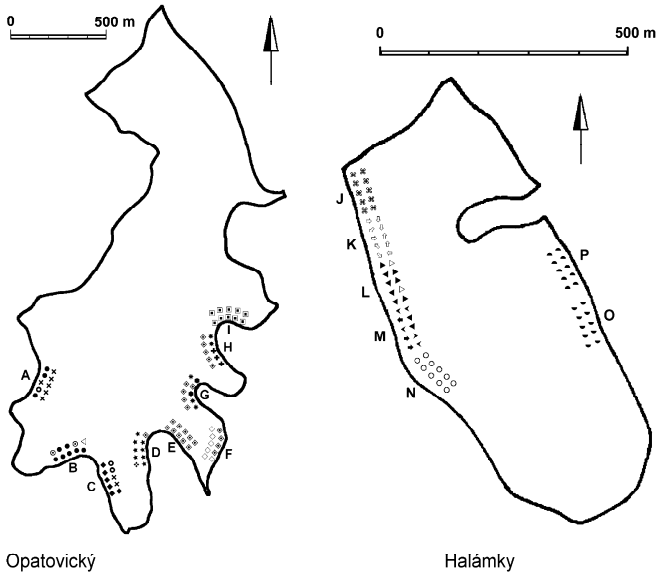

Fig. 2. Spatial distribution of distinct clones (indicated by different symbols) in stands of *P. australis* growing along the shores of Opatovický fish pond (old lake) and Halámky sand pit (new lake), South Bohemia, Czech Republic, as indicated by RAPD analyses performed in 2004.

corresponded to generally smaller panicles and, to some extent, lower shoot densities as compared with the Opatovický population.

Morphological differences were detected between some stands within both populations (Fig. 3). Conspicuous differences were found in shoot length, both between stands within the populations and between ranges covered by whole populations. The maximum shoot heights of individual stands ranged from 2.9 to 4.6 m in the stands studied at Halámky lake, but only from 1.80 to 3.30 m at Opatovický lake. Maximum basal diameters ranged from 6 to 16 mm at Halámky, but only from 6 to 10 mm at Opatovický. The basal diameters were significantly ($p < 0.0001$) correlated with stem lengths. The determination coefficients R^2 were 0.86 and 0.82 for the tallest shoots of Halámky and Opatovický lakes, respectively, and 0.68 and 0.61 for all measured shoots of the two respective lakes. Unlike shoot lengths, panicle lengths were fairly uniform within each population. Differences between stands were found also in shoot densities. Live shoot densities varied from 20 to 120 shoots per m^{-2} at Halámky. At Opatovický lake, values above 100 shoots per m^{-2} were found in all stands and the maximum density of live shoots was over 500 shoots per m^{-2}. On both sites, 55% of all live shoots flowered. The densities of flowering shoots were significantly ($p < 0.0001$) correlated with the densities of all live shoots. The determination coefficients R^2 for these correlations were 0.68 and 0.51 for the Halámky and Opatovický lakes, respectively.

The variation coefficients of morphological characteristics, calculated for separate stands, did not show any clear relation to the numbers of clones within these stands (data not shown). The 19 morphological characteristics of individual shoots, which were further subjected to RAPD analysis, did not discriminate between the clones well, either. In the PCA analysis, performed on the samples from each lake separately, all cases (equivalent to individual shoots) formed one cluster, in which various clones were intermingled (data not shown). The discrimination analysis classified correctly 72 out of 88 cases and 55 out of 63 cases for the Opatovický and Halámky populations, respectively. At Opatovický lake, all samples were classified correctly for 4 clones out of 13. At Halámky lake, all samples were classified correctly for 7 clones out of 12. No conspicuous difference was found between the correctness of classifying monoclonal stands on the one hand and multiclonal ones on the other.

4. Discussion

4.1. Genetic variation

Common reed has not been considered an economically important plant, therefore, knowledge about its molecular biology, especially about its genome, is limited in comparison with crop plant species such as wheat, barley, or lucerne. Much information on the genetic structure and diversity of reed populations has been gained using the RAPD technique. RAPD is an effective method to detect intra- and inter-population variation and is still widely used for these purposes in many plants (Bussell et al., 2005; Lin et al., 2005; Piluzza et al., 2005; Ren et al., 2005). Koppitz et al. (1997), Koppitz (1999), Kühl et al. (1999), Keller (2000), and Xu et al. (2000) also studied genetic relationships both between and within reed populations using the RAPD technique. The genetic structure of reed populations was also studied using other molecular methods, such as microsatellite (SSR) analysis, and chloroplast DNA

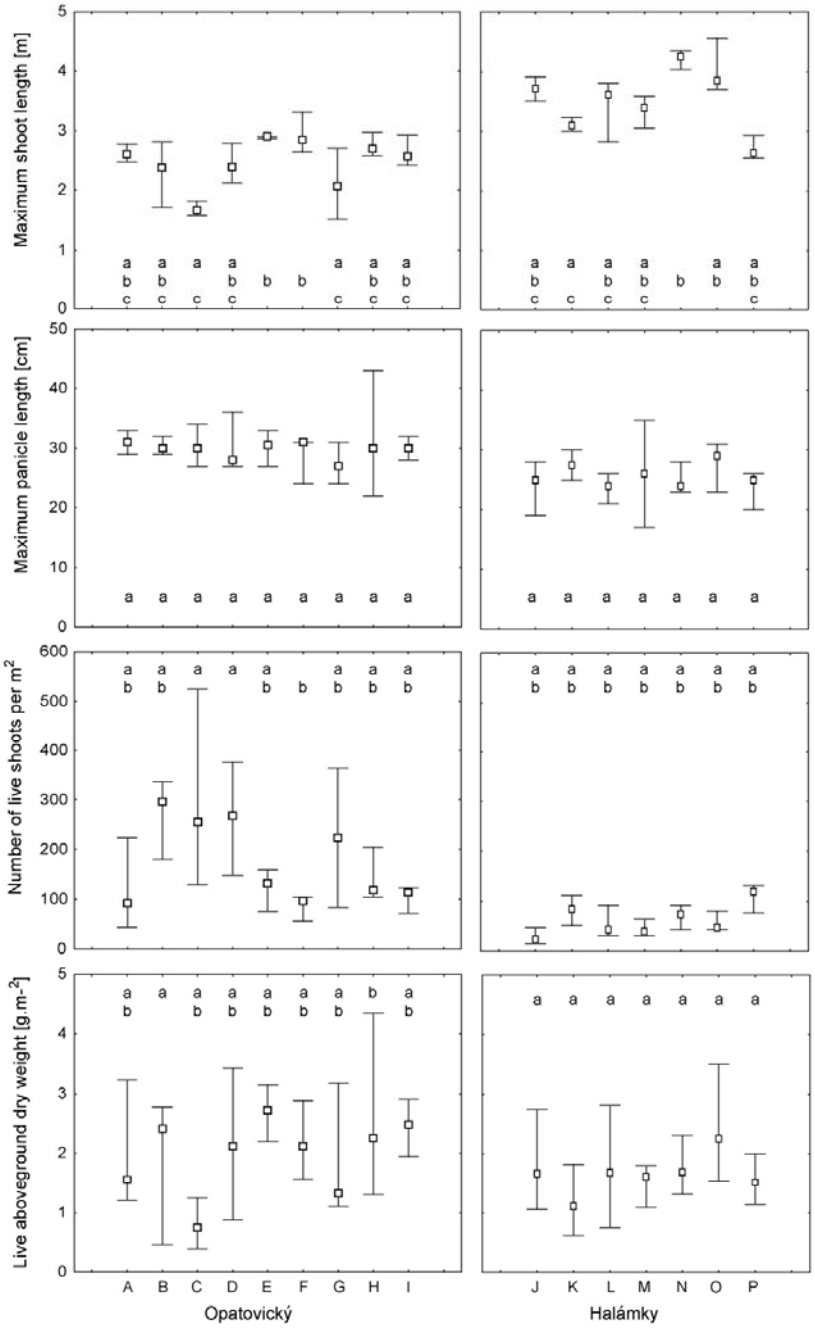

Fig. 3. Morphological and growth characteristics of *P. australis* stands in the Opatovický and Halámky lakes. The squares and bars indicate medians and minimum–maximum values, respectively.

sequencing, because of limitations in the RAPD technique. Saltonstall (2002, 2003) applied these two approaches to evaluate genetic variation in North American populations of reed and detect invasive alien genotypes occurring in North America. In addition to the various methods of DNA analysis, allelic isozyme (allozyme) analysis have proved a useful tool to detect clonal diversity for various plant species including the common reed (Pellegrin and Hauber, 1999; Guo et al., 2003).

Table 1
Numbers of individual distinct clones in stands of *P. australis* along the shores of Opatovický fishpond (old lake) and Halámky sand pit (new lake), South Bohemia, Czech Republic

	Stand								
	A	B	C	D	E	F	G	H	I
Opatovický									
Number of clones along the open water edge	1	3	3	2	1	1	2	1	1
Number of clones along the terrestrial edge	3	1	1	2	1	2	3	2	1
Total number of clones	3	3	3	3	1	2[a]	3	2	1
Clones present only in one stand	–	2	1	2	–	1[a]	1	1	1

	Stand						
	J	K	L	M	N	O	P
Halámky							
Number of clones along the open water edge	1	3	2	3	1	1	1
Number of clones along the terrestrial edge	1	4	2	3	1	1	1
Total number of clones	1	7	3	5	1	1	1
Clones present only in one stand	1	7	2	4	1	1	1

[a] These two clones are very similar, distinctness is less than 0.1%.

4.2. Clone distribution and genetic variation of stands

Genetic analyses indicated that both populations are multiclonal and have high clonal variability. The fact that the clones were grouped geographically may be related to the conspicuous difference in age of the two populations, which in turn may account for different seed sources available during their establishment. Guo et al. (2003) found a positive correlation between genetic and geographical distances for *P. australis* clones of the Yellow River delta, a highly dynamic system, where the time of establishment might also be involved in determining the genetic structure of the populations. By contrast, Lambertini et al. (2006) did not find such a correlation for the common reed within Europe using AFLP analysis. They propose extensive interbreeding and/or introductions by humans as mechanisms that might have obscured the original biogeographical pattern. As for the two populations in our study, a geographical barrier for interbreeding does not seem likely in view of the fairly close geographical distance between the two sites (about 30 km). Rather, a limited establishment success may be related to low seed viability as discussed below in Section 4.4.

Both monoclonal and multiclonal stands were found at either site. A higher number of clones, within a single multiclonal stand, was found at Halámky lake than at Opatovický lake (Table 1, Fig. 2). The numbers of clones found both in individual multiclonal stands (Section 3.3, Table 1) and the whole populations (Table 2) support hypothesis 1, that the number of clones per unit of population area is lower in the older, as compared with the younger, population. The disclosed general pattern of clonal diversity (Section 3.3, Fig. 2) also supported hypothesis 2, that the maximum area covered by a single clone is larger in the older that in the younger population. Some additional support for both hypotheses is provided by the fact that the transect along which the sampling was performed represents a four times longer segment of a reed stand at Opatovický than at Halámky lake. However, it is possible that some clones were not detected at Opatovický lake, where the sampling distance was longer than at Halámky.

As in other European populations studied by Koppitz (1999), different forms of clonal diversity were found: monoclonal or polyclonal stands, and stands with coexisting or intermingling clones. Monoclonal stands (E, I, J, N, O, P) consisted of a single clone, which presumably spread over the area by vegetative propagation. This could be the result of colonization by several clones followed by natural viability selection, as suggested by Koppitz (1999). This model can explain the situation in old, established populations such as that of Opatovický lake. A rather different model should be applied to the development of monoclonal stands at Halámky lake. With respect to the relatively short period of the development of the reed population on this site, it is assumed that these stands were established from one plant and selection probably occurred at the level of suitable conditions for seed germination and/or on the level of juvenile plant establishment.

In polyclonal stands, different clones were found to coexist next to each other without mutual penetration (stands D and F at

Table 2
Clonal diversity of populations of *P. australis* stands growing along the shores of Opatovický fishpond (old lake) and Halámky sand pit (new lake), South Bohemia, Czech Republic

	Opatovický	Halámky
Total number of clones	14	18
Number of monoclonal stands	2 (3[a])	4
Number of clones present only in one stand	9	17
Number of clones occurring in two neighbouring stands	2	1
Number of clones occurring in two non-neighbouring stands	2	0
Number of clones occurring in more than three stands	1 (in five neighbouring stands)	0

[a] See note in table 1.

Opatovický lake). Mixed growth of different clones was also observed; this may have been caused either by spreading of the reed by vegetative propagation and intermingling of rhizomes, or by dispersal of fragments of rhizomes or shoots (stands A, G, H). The presence of the same clone in several stands, like clone O-41 in five stands situated along several hundreds of meters of the shoreline (Fig. 2), might have one more explanation. These currently separate stands may represent remnants of a formerly continuous stand dominated by one clone, which had spread vegetatively in a period of favourable conditions and was fragmented later. The relatively low degree of genetic similarity between this widely spread clone and its geographic neighbours indicate that the diaspores came from less related sources. This could happen if their establishment took place at different times. Although the absolute age of the reed stands investigated is not known precisely, the maximum possible difference in their age can be inferred from the known history of the lakes' management and is likely to be one to several hundred years.

High clonal diversity values were found in three stands at Halámky (K, L, M). This can be ascribed to establishment of several diaspores, which spread vegetatively into the current fairly small patches. This can be explained by the relatively young age of this population. Further development of these stands can bring new and more exact data for modelling reed population development. It seems reasonable to assume that, in such patchy populations, the clones may compete with one another for space, which might result in a successive shift of their borders.

4.3. Morphological variation as related to clonal diversity

The genetic basis of differences between reed morphotypes was first documented for different ploidy levels. Tetraploid is the most common ploidy level, both in the Czech Republic and worldwide (Clevering and Lissner, 1999). A preliminary investigation using flow cytometry was carried out for 20 stands of robust reed in Halámky lake (Čížková et al., unpublished results) and for all stands in Halámky and Opatovický lakes involved in this study (Kubátová et al., unpublished results). It showed only tetraploid genotypes. This indicates that the robust growth, found in some morphotypes of the Halámky population, is not due to a higher ploidy level.

As follows from the sampling scheme, each of the stands sampled for the genetic and morphological analyses was visually homogeneous. The fact that some of the stands were found to be monoclonal while some others were polyclonal (Table 1, Fig. 2) contradicts hypothesis 3, that morphologically homogeneous stands are monoclonal. Neither could we find evidence to support hypothesis 4, that morphological variability is a good indicator of genetic variability in multiclonal stands and populations. We did not find a clear relationship between the variation in morphological characteristics, recorded in individual stands, and clone numbers within those stands. The PCA analysis did not separate the clones on the basis of shoot morphological characteristics. The large overlap of clones in the PCA analysis indicates that the morphological features of some clones are very similar within a population. The discrimination analysis separated the Halámky clones somewhat better than the Opatovický clones (reaching 12 and 18% for these populations, respectively). But the occurrence of errors in the discrimination of many clones, coinciding with the fairly large number of clones present, supports the view that morphometric characteristics may not be a sufficiently precise indication of clonal diversity.

Our findings are in contrast with the results of Kühl et al. (1999), who found that RAPD clearly identified plants belonging to distinctive morphotypes in a shallow lake near Berlin. Also Neuhaus et al. (1993) and Kühl and Neuhaus (1993) reported low levels of genetic variation in particular sites of North German lakes, where one or a limited number of clones formed homogeneous stands, with a single stand covering an area of more than 3000 m^2. This study, based on more detailed sampling of two populations of different age, showed a high level of polymorphism detected by RAPD markers; the genetic similarity between particular clones was found to range from 70 to 100%. This range of genetic variation within polyclonal stands is in correspondence with the results of Koppitz et al. (1997) and Koppitz (1999), who investigated reed stands throughout Europe.

There seems to be little doubt that visually different neighbouring stands are dominated by different clones. However, the results of this study, which related morphological characteristics to particular genotypes using the RAPD technique, found that the genetic variation in reed populations may be higher than has been reported so far and than was previously estimated on the basis of phenotype (Hradecká and Květ, 1973; Hradecká, 1973b; Dykyjová and Hradecká, 1976). Also, more precise sampling and employment of further suitable methods of genetic analysis could disclose possibly higher levels of genetic variation.

4.4. Clonal diversity as related to the long-term stability of reed stands

A hypothesis was put forward in the 1990s (Kühl and Kohl, 1992; Kühl and Neuhaus, 1993; Neuhaus et al., 1993) that in originally diverse populations only such clones would persist that were able to survive under the particular site conditions; this would be reflected by a reduced genetic variability within the population. The resulting populations, consisting of only a few large monoclonal stands, were supposed to be more vulnerable to rapid changes in environmental conditions, such as eutrophication, because the low genetic diversity would not provide the stands with sufficient phenotypic plasticity needed for successful adaptation. This was regarded as a possible explanation for the low genetic variability found in reed populations in some German lakes and their decline (Eriksson, 1993; Koppitz et al., 1997).

Such a reduction of genetic diversity seems to be likely under permanently high water levels and/or in closed stands, because reed seeds need emerged bottoms and light sufficient to establish seedlings (Hejný, 1960; Rea, 1996). Such conditions probably occurred in the German lakes studied. In contrast, the littoral bottom of Opatovický lake became periodically

emerged during the vegetation season until the 1970s, because the management included summer draw-downs every second to 3rd year. These draw-downs may have created opportunities for the generative propagation of reed. However, the establishment success was probably low as reed seedlings were observed only exceptionally in the whole region. This may be explained by a low proportion of viable seeds (Čížková, unpublished results; Ekstam, personal communication). Seed viability is probably reduced by fungal infestation, which is common in this region especially in wet years (Hradecká, 1973a). The cleaning of sediments from fishpond littoral, applied in the 1970s and 1980s, may have contributed to the transport of vegetative pieces of reed to other stands.

The examples of reed populations inhabiting water bodies of contrasting age have shown that both monoclonal and multiclonal stands can exist within both newly formed and rather old populations. In spite of the management impacts considered above, the comparison of the two lakes, in terms of the number of clones and clonal patterns, is in accordance with the hypothesis of Kühl and Neuhaus (1993) and Koppitz and Kühl (2000), suggesting that older populations have a lower clonal diversity. A further study is focused on the relation between the genetic diversity identified by the RAPD method and phenotypic characteristics of cultivated reed clones compared to original field reed stands.

Acknowledgements

This study was supported by Project No. AVOZ60870520 of the Academy of Sciences of the Czech Republic and MSM6007665806 of the Ministry of Education of the Czech Republic. We thank Josef Rajchard for inspiring this research, Jitka Klimešová for advising on the sampling scheme, Jan Květ for helpful comments on the manuscript and Keith Edwards for improving the English.

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Available online at www.sciencedirect.com
ScienceDirect
Aquatic Botany 86 (2007) 361–368
Aquatic botany
www.elsevier.com/locate/aquabot

Phenotypic and genotypic variation of *Phragmites australis*: A comparative study of clones originating from two populations of different age

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Received 24 June 2006; received in revised form 28 December 2006; accepted 4 January 2007

Abstract

Populations of common reed (*Phragmites australis* [Cav.] Trin. Ex Steud.) were studied in the littoral zones of two human-made lakes in the Třeboň Basin (South Bohemia, Czech Republic): (1) Opatovický fishpond, a 500-year-old fishpond, and (2) Halámky sand pit, a new lake formed by sand extraction in the 1970–1990s. Clones were identified using the RAPD method within morphologically different stands. The clones were propagated vegetatively and cultivated under comparable conditions in a common garden experiment for one vegetation season. Morphological and growth characteristics of the cultivated plants were recorded and compared with those found in the original reed stands. The younger (30-year old) population was genotypically more diverse than the older (500-year old) population. In addition, the younger population demonstrated correspondence of genotypic and phenotypic variation, while the older did not. The results indicate that the genetically determined morphological features (especially shoot length) are manifested more fully in the phenotype of the plants of the younger (Halámky) population, whereas environmental factors affect the phenotype to a greater extent in the older (Opatovický) population.

Keywords: Phragmite; Genotypic variation; Phenotypic variation; Lake; Clone

1. Introduction

Distinct morphotypes of *Phragmites australis* are a conspicuous phenomenon found in a recent man-made lake, a flooded sand pit situated near the village of Halámky in the floodplain of the upper Lužnice River (Třeboň Basin Biosphere Reserve, Czech Republic). The reed morphotypes differ from one another by shoot length (2–5 m), basal culm diameter (5–18 mm), leaf width and colour of panicles. Coexistence of reed morphotypes was also observed in the littoral zones of older human-made lakes, constructed one to several hundred years ago, such as the fishponds in the Třeboň Basin (Hradecká, 1973a,b; Obstová, 1989). However, their growth characteristics are more uniform and the extent of morphological variation is comparable in different fishponds. Except for one population (Rod fishpond in the Třeboň Basin Biosphere Reserve; Pechar et al., 2002), the fishpond reed stands do not include robust morphotypes, which have a maximum shoot length over 3.5 m.

The coexistence of morphotypes may indicate that the morphotypes represent different clones, which have spread vegetatively from seedlings that have established themselves under favourable conditions. In addition to research into ploidy levels (reviewed by Clevering and Lissner, 1999), genotypic variation has been studied using the RAPD method (Neuhaus et al., 1993; Koppitz et al., 1997; Koppitz, 1999). These results support the view that the morphological differences have a genetic basis (Čurn et al., 2007). However, scanty information exists as yet about the relationship between genotypic and phenotypic variation.

This paper is part of a broader study focused on reed populations of two human-made lakes whose age differs by an order of magnitude. The considerable age difference makes these two populations a good field model to investigate how

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doi:10.1016/j.aquabot.2007.01.001

phenotypic and genotypic variation change with time, an aspect that has scarcely been investigated. Whereas Čurn et al. (2007) studied the genotypic diversity of the two field populations and the relationship between their morphological and genotypic variation, this study adresses whether the growth and morphometric characteristics of morphotypes originating from the two populations are preserved in culture. Special attention is paid to the robust morphotypes occurring in the young population. Our aim was to test whether growth characteristics of the robust morphotypes have a genetic basis. For this purpose, the relationship in some morphological and growth characteristics was evaluated between plants collected in their natural habitats and plants (originating from the same field stands) cultivated in a common garden experiment under comparable climatic, water level and trophic conditions. The genetic similarity between the plants investigated was assessed on the basis of the RAPD technique.

2. Materials and methods

2.1. Site description

Two localities were selected in the Třeboň Biosphere Reserve (Třeboň: 49°05′N, 14 46′E, altitude 430 m), South Bohemia, Czech Republic:

(1) Opatovický fishpond is a shallow water body constructed in 1510–1514 by damming a shallow valley and used since for carp rearing. The maximum flooded area is 160 hectares. Its maximum length and width are 2.6 and 1.1 km, respectively. Well-developed reed stands occur along over half the shore length.
(2) Halámky sand pit is a new lake formed by sand extraction, proceeding in 1970–1994. The area of the lake is 22.9 ha. The maximum length and width of the lake are 0.79 and 0.41 km, respectively. Its shoreline length is 2.01 km. Reed stands occur in patches along much of the shore. In addition, small islands of reed occur in parts of the lake where the water depth is less than 1.5 m. All the reed vegetation in this lake is certainly younger than 35 years, but much of it is probably younger than 20 years. It most probably established itself from seeds, as the lake is not directly connected with any watercourse.

Further details on the study sites are given by Čurn et al. (2007).

2.2. Sampling

Homogeneous and healthy looking stands of *P. australis* were chosen in the littoral zones of both lakes in the same manner as described by Čurn et al. (2007). Ten and seven stands were selected at Opatovický and Halámky lakes, respectively. The alphabetical labelling of the stands corresponds with that used in the previous paper, where a schematic map with their positions is also given. Three stands (G1, K1 and N1) were included in this study in addition to stands investigated previously. Their labels include the same letters as those of neighbouring stands.

In June 2003, when the reed shoots attained over half of their seasonal maximum length, four shoots were sampled from each stand along a transect laid parallel to the shoreline at a depth of 0.4–0.5 m. At Opatovický lake, the transect copied the water edge of the reed stands. At Halámky, where the shore is steep, the transect was laid near the terrestrial edge of the reed stands. The sampling distances were set at 10 and 2.5 m at Opatovický and Halámky lakes, respectively, in order to account for the different sizes of their reed stands. Ten and seven stands were sampled at Opatovický and Halámku lakes, respectively. The sampled shoots were used for RAPD analysis and were subsequently propagated vegetatively as described below. In August 2003, the total aboveground biomass was estimated for all stands investigated. Shoots were harvested from 0.5 m × 0.5 m quadrats laid along the same transects and at the same distances as in the previous sampling. Five quadrats were sampled in each stand.

2.3. Analysis of molecular markers

Fresh leaf samples were used to characterize the genetic diversity of *P. australis* populations. The leaves were taken from the sampled shoots, 68 individual samples were analysed. DNA from the leaf samples was isolated using Invisorb Spin Plant Mini Kit (INVITEK, Germany) according to standard protocol. DNA samples were stored at −20 °C prior to their use.

Random primers (OPERON Technologies, CA) were selected for the RAPD analysis. The protocol for RAPD analysis has been described in detail by Čurn et al. (2007). PCR products were separated on 1.5% agarose gels in TBE buffer which included lanes of 100 bp DNA ladder (NEB) and DNA bands were visualized using ethidium bromide staining under UV light. RAPD patterns were photographed using Epson PhotoPC 3100Z.

RAPD data files were analysed by the BioProfil 1D+ software (Vilber Lourmat, France) with manual correction. Fingerprint patterns were transformed into a binary character matrix with 1 for presence or 0 for absence of a band at a particular position in a lane. After removing monomorphic bands, genetic distance matrices were generated using Nei and Li and Gower general similarity coefficients. Cluster analysis (UPGMA-unweighted pair group method averages) and principal coordinates analysis (PCoA) were performed subsequently. Values of Nei and Li's coefficient of 0.95 were considered indicative of different clones (Caicedo et al., 1999; Zanotti et al., 2006). Principal component analysis (PCA) was also carried out. These statistical analyses were accomplished using MVSP (Kovach Comp. Serv.) and STATISTICA 6.0 software package (Statsoft).

2.4. Cultivation

The plants were propagated from stem cuttings. After collecting leaf tissue for genetic analyses, all leaves including leaf sheaths (except for the top ones) were removed from the culms. The culms were laid in a shallow layer of water in a

1 m × 2 m plastic tank and shaded. Within 2–3 weeks, new offspring plants were formed from internodes of all shoots. These offspring plants were planted into 1-l plastic pots filled with river sand. For addition of nutrients, a slow releasing granulated fertilizer Osmocote Plus (Type 87000201 CE, Scotts Europe B.V., The Netherlands) was applied at a dose of 6 g per pot. The fertilizer was mixed with the sand at the time of planting. The offspring plants established themselves and had formed well-developed root systems by autumn.

On 15 May 2004, the overwintered plants were sorted into groups according to their parent culms. Two groups were selected from each genotype as determined by the RAPD analyses. Offspring plants were matched in size on the basis of the shoot numbers and heights of the tallest shoots. Four offspring plants of each mother plant were used for comparative cultivation. The aim was to include one clone from each field stand. However, some clones had to be omitted because sufficient numbers of offspring plants were not available. The clones included in the comparative cultivation and their pertinence to original field stands are given in Table 1.

The offspring plants were transplanted into 5-l plastic buckets (1 plant per bucket) filled with river sand. The nutrient dose was adjusted by adding OSMOCOTE Plus, mixed with the sand at the time of transplantation, at a dose of 6 g l^{-1}, i.e., 30 g per bucket. Each bucket was placed in a separate bowl, in which the water was kept at a level of 0.1 m above the bottom throughout the cultivation. The plants were placed in a randomized design in an unshaded outdoor cultivation area. The seasonal course of main meteorological parameters (light, temperature and precipitation) of the 2004 growing season fell within values common for the area. Therefore, it is assumed that the phenotype of the cultivated plants was not affected by exceptional meteorological events.

A solution of $FeSO_4 \cdot 7H_2O$ was used to treat an initiating leaf chlorosis twice during the cultivation (21 May and 13 June). A dose of 28 mg, diluted in 100 ml, was applied per bucket. Twice during the cultivation, the plants were treated with PIRIMOR 25 WG at a concentration of 1 g l^{-1} in order to reduce aphid infestation.

The plants were harvested on 9–12 September 2004. The numbers of green and flowering shoots were counted for each plant. Shoot length to the tip of the uppermost leaf, number of internodes per plant, basal diameter, and panicle length were recorded for the longest shoot and for five randomly chosen shoots. After cutting the aboveground parts, the belowground parts were removed from each bucket and cut into two halves. One half was cleaned from sand with tap water and used for determination of belowground biomass. Samples of both aboveground and belowground biomass were dried in an electrical oven at 80 °C to constant weight and weighed.

Statistical analyses of recorded characteristics were carried out using the software package STATISTICA 6.0 (Statsoft). The effect of mother plants was tested using one-way analysis of variance. As it was not significant, offspring of both mother plants, within a clone, were pooled together and treated as replicates in further calculations. The effects of clone and population on individual growth characteristics were tested using a hierarchical analysis of variance. The overall contribution of differences between the clones to the growth characteristics was explored by PCA analysis.

2.5. Shoot morphology and growth of field populations

Characteristics of reed productivity (density of live, flowering and dead shoots, total above-ground biomass) and morphology (shoot length to the tip of uppermost leaf, number of internodes per plant, basal culm diameter, panicle length) were recorded in samples of reed shoots collected in late August. Total aboveground biomass included stems, live leaves and panicles. Their dry weight was determined after drying to constant weight at 80 °C. The data were analyzed statistically in the same manner as described above for the cultivated plants. The correlations in individual growth characteristics between plants originating from the field stands and cultivated ones were evaluated using regression analysis.

3. Results

3.1. Genetic diversity of the populations

In our previous studies, DNA samples of *P. australis* from populations from the Czech Republic, Hungary, The

Table 1
Numbers of individual distinct clones identified in *Phragmites australis* stands, from which clones were taken for comparative cultivation

Site	Opatovický									
Stand (water edge)	A	B	C	D	E	F	G	G1	H	I
Number of clones identified in this study	1	1	1	1	1	1	2	1	1	1
Number of clones identified by Čurn et al. (2007)	1	3	3	2	1	1	2	1	1	1
Clones present	**O1**	**O1**	O2	**O3**	**O4**	**O5**	**O6**, O7	**O8**	**O9**	**O10**

Site	Halámky						
Stand (terrestrial edge)	J	K	K1	N	N1	O	P
Number of clones identified in this study	3	1	1	1	3	2	1
Number of clones identified by Čurn et al. (2007)	1	4		1		1	1
Clones present	**H1**, **H2**, H3	**H2**	**H4**	**H5**	**H6**, H8, H9	**H10**	H11

These stands are situated along shores of Opatovický fishpond (old lake) and Halámky sand pit (new lake), Třeboň Basin, Czech Republic. A more detailed analysis of the genetic diversity of these stands is given by Čurn et al. (2007). Cultivated clones are indicated in bold characters.

Table 2
Variation of growth characteristics of cultivated *P. australis* plants originating from Opatovický fishpond (old lake) and Halámky sand pit (new lake)

	Mean	S.D.	Variation coefficient	% variability explained by distribution between clones
Opatovický				
Maximum shoot length (m)	1.77	0.17	9.51	62.10
Maximum base diameter (mm)	5.21	0.68	13.15	26.85
Number of live shoots	26.83	7.43	27.70	49.36
Number of flowering shoots	11.89	5.26	44.20	45.56
Aboveground dry weight (g)	122.56	31.40	25.62	41.33
Belowground dry weight (g)	140.86	44.89	31.87	32.15
R/S	1.17	0.26	22.58	42.50
Halámky				
Maximum shoot length (m)	1.83	0.41	22.63	72.25
Maximum base diameter (mm)	5.51	1.06	19.15	73.30
Number of live shoots	24.89	6.22	25.00	28.89
Number of flowering shoots	2.05	2.71	132.07	72.25
Aboveground dry weight (g)	102.88	40.88	39.73	73.30
Belowground dry weight (g)	91.91	36.61	39.83	18.85
R/S	0.98	0.45	45.42	47.30

Netherlands, Romania, Spain and Sweden were screened with 80 arbitrary decamer primers (OPA, OPB, OPF, OPK kits, Operon Technologies). On the basis of the ability to amplify DNA, the number of polymorphic bands and their intensity and reproducibility of RAPD products, 5 highly polymorphic primers were selected—OPA-03, OPA-04, OPA-11, OPB-04 and OPB-08. These primers were tested across all 68 samples from 17 stands (description of the sampling scheme is given above). These five primers amplified altogether 88 bands ranging from 12 to 20 bands per primer. We found that 74 (84%) of the amplified bands were polymorphic. Thirteen bands (15%) exhibited a low level of polymorphism (i.e., polymorphic bands had a weak staining intensity or polymorphism was detected in only 1–3 plants), so 61 (69%) amplified bands were highly polymorphic. This gave on average 12.2 polymorphic products per one polymorphic primer.

The pattern of RAPD markers was quite variable, out of 88 bands only 14 common bands were found among all 68 tested plants. The population from Halámky lake had three fixed monomorphic bands present in all samples from Halámky which were unique for this population. Only one band with these attributes was found in Opatovický lake.

Within almost all stands from Opatovický (except stand G) and within one half of the stands from Halámky lake, only reed plants with an identical pattern of RAPD markers were found. It is therefore possible that these plants belong to one clone. On the basis of our genetic and phenotypic analyses we suppose these stands to be monoclonal. In Halámky lake, we found more frequently that the stands were formed by a higher number of coexisting and intermingling clones (Tables 1 and 2).

The genetic diversity or similarity was estimated for all comparisons of the reed samples according to Nei and Li metrics and Gower general similarity coefficient; it was calculated from the total number of polymorphic bands. This matrix of genetic distances was used to cluster the samples, based on the degree of their genetic similarity. Cluster analysis (UPGMA method with 95% confidence interval) resulted in a dendrogram (Fig. 1) that grouped the populations according to their geographical origin. The same results of sample clustering were obtained using Nei and Li and Gower GSC matrixes. This dendrogram presents the clustering of all clones identified. Occurrence of particular clones in the field stands is given in Table 1. The clones from all stands from Opatovický lake were clustered in one group while the clones from Halámky lake formed another group. The level of similarity between these two large clusters was about 35%. Likewise, the PCA analysis yielded two independent groups which clearly distinguished the two populations (Fig. 2).

The clones from all stands from the Opatovický population were clustered in one group. The level of genetic similarity among the groups was greater than 80%. In comparison, the level of genetic polymorphism was markedly higher among the clones of the Halámky population. All clones from this site, based on the resulting dendrogram, formed one cluster, but its diversity was greater than in the Opatovický population. An extreme polymorphism was found in the stands J and N1 where three clones were present (Table 1).

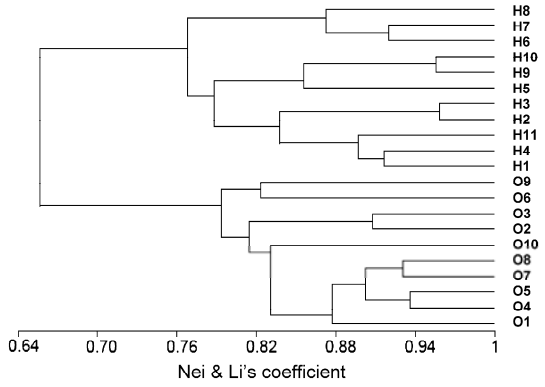

Fig. 1. Dendrogram of individual clones of *Phragmites australis* constructed on the basis of RAPD data, by UPGMA method, resulting from distance matrix calculated with the metric of Nei and Li. The descriptions of clones correspond to those in Table 1.

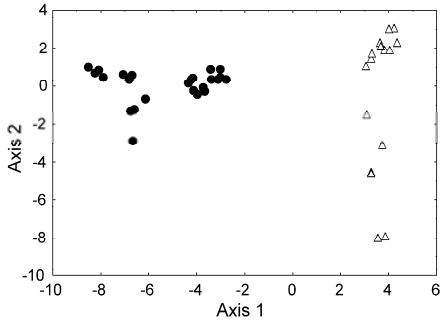

Fig. 2. PCA ordination of *P. australis* clones originating from Opatovický and Halámky lakes calculated from RAPD data. Triangles and circles indicate Opatovický and Halámky population, respectively.

PCoA (principal coordinates analysis) divided the 68 analysed plants into two separate groups, which again differed in their geographical origin. As in the cluster analysis, the clones of the Opatovický population were aggregated in a relatively compact group. In contrast, the clones of the Halámky population formed an expanded group whose polymorphism was twice as high as in the group of Opatovický clones. A more detailed study of the PCoA results has shown that the expanded group containing samples from Halámky is divided into three aggregates whose composition and distances correspond with the results of the UPGMA analysis. The most distinct group of clones (H6, H7 and H8) forms an outlying soft aggregate, like in the UPGMA dendrogram. The clones H5, H9 and H10 also form an independent aggregate.

3.2. Shoot growth and morphology of the cultivated plants

The maximum shoot length was fairly uniform in the cultivated plants of all clones of the Opatovický population while it varied more among the clones of the Halámky population (Table 2). The Halámky population included both the tallest and the shortest shoots of both populations. Panicle length did not differ between the plants either within or between the populations. Shoot numbers were more varied in the Opatovický population than in Halámky. Shoot numbers of plants originating from Halámky were similar to the intermediate shoot numbers of plants originating from Opatovický (data not shown). The numbers of flowering shoots were generally lower for Halámky, than for Opatovický plants (Table 2). Only one plant of 16 belonging to the two tall clones of Halámky flowered. Some clones of the Halámky population had conspicuously lower values of both aboveground and belowground biomass than most of the other clones. The most robust clones of both populations attained a similar aboveground biomass, but the more subtle clones of the Halámky population showed the lowest values. The Halámky clones had generally a lower belowground biomass than the Opatovický clones (Table 2). Root/shoot ratios ranged from 1.0 to 1.5 and from 0.8 to 1.5 for different clones in Opatovický and Halámky, respectively. Distribution of plants between clones accounted for a greater proportion of total variation in growth characteristics (except for the number of live shoots) in Halámky than in the Opatovický population.

In both populations, the first and second PCA axis explained over 45 and 20% of total variation in growth and morphological characteristics, respectively (Fig. 3). In Opatovický population, the differences between the clones coincided most with the variation in aboveground and belowground dry mass, followed by maximum shoot height. These three characteristics together accounted for 75% variation associated with the first axis. The maximum basal culm diameter and the number of live shoots together explained 69% variation associated with the second axis. In the Halámky population, the variation among plants largely coincided with the variation in shoot length, basal culm diameter and aboveground biomass. These three characteristics together accounted for 89% variation associated with the first axis. Belowground biomass, the number of live shoots and the number of flowering shoots together accounted for 92% of variation associated with the second axis. When plants of both populations were analyzed together, they formed one single cluster (data not shown).

Morphological characteristics did not separate the clones of the Opatovický population well (Fig. 3A). Although plants of some clones were situated close to ane another, plants of others were scattered all through the plot. The Halámky clones were separated better (Fig. 3B). The plants formed three groups with

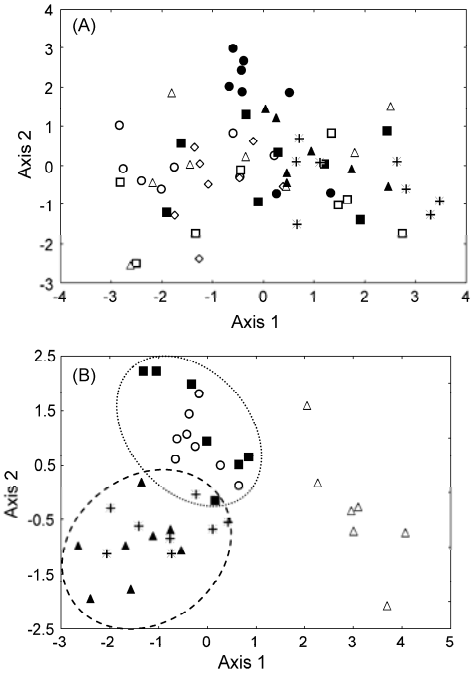

Fig. 3. PCA ordination diagrams of growth characteristics of cultivated *P. australis* plants originating from Opatovický lake (A) and Halámky lake (B). Within a graph, plants indicated by the same symbol belong to the same clone. For the Halámky population, ellipses indicate positions of clones belonging to the same partial cluster in Fig. 1 (dotted ellipse encloses clones H2 (○) and H4 (■); dashed ellipse encloses clones H5 (▲) and H10 (*)).

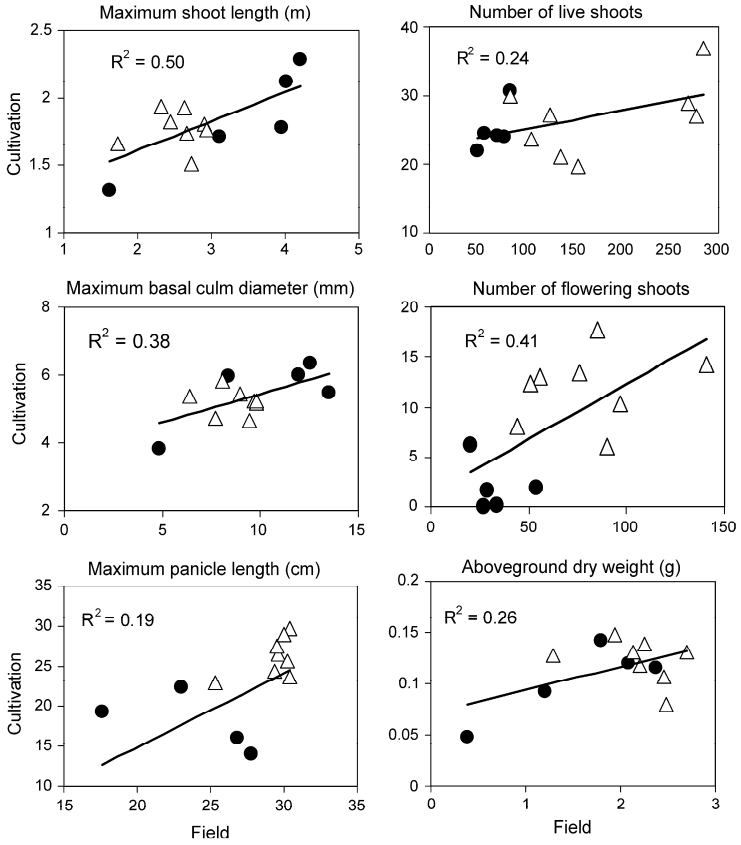

Fig. 4. Correlation in growth characteristics between the field stands and cultivated clones originating from these stands. Values on *x*-axis are means of five independent measurements. Values on *y*-axis are means of eight independent measurements. Triangles and circles indicate Opatovický and Halámky population, respectively. Determination coefficients (R^2) given in bold indicate significant correlations at $P < 0.05$.

little overlap. One group was formed by a single clone (H6), two other groups included two clones each (H2, H4 and H5, H10, respectively). Clones included in the same group in Fig. 3B belonged to the same partial cluster as revealed by UPGMA analysis (Fig. 1).

3.3. Growth of cultivated plants as compared with reed stands in the field

The similarity in the growth and morphological characteristics between the cultivated plants and those of the field stands was evaluated using linear regression analysis. When the plants of both populations were analyzed together, significant correlations between the cultivated and the field plants were found for the maximum shoot length, maximum basal culm diameter, and density of flowering shoots (Fig. 4). Within the Halámky population, the maximum shoot length yielded a significant correlation, explaining 82% of total variation. Correlations in maximum basal culm diameter, maximum panicle length, density of live shoots and aboveground biomass also explained substantial parts of variation within the Halámky population (58, 43, 54 and 73%, respectively). However, these correlations were nonsignificant, which is most probably due to the small number of replicates. Within the Opatovický population, the regressions between cultivated plants and their original field stands explained less than 30% for any of the growth characteristics studied. None of the correlations was significant.

4. Discussion

As indicated by the genetic analyses, both populations were multiclonal and included both monoclonal and multiclonal stands (Table 1). In addition, the populations were separated as distinct clusters, which indicates that they are genetically different (Fig. 1). This is in accordance with our more detailed analysis, carried out for the same populations (Čurn et al., 2007). In comparison with the previous study, this study has shown a smaller number of clones within both populations and also within some stands. This may partly be due to smaller numbers of individual plants analysed (Ellstrand and Elam, 1993; Fischer and Matthies, 1998). In addition, the spring

sampling in this study may have favoured shoots of genotypes that emerge earlier and grow more rapidly than others in spring. This idea is supported by Haslam (1973), who listed the time of emergence and other phenological events among genetically determined features of reed plants. This selection, based on coincident phenological development, might not have taken place if the reed shoots had been sampled in late summer (as in the previous study), when shoots would presumably have reached their final lengths in all clones.

Both genetic and environmental factors affect phenotypic characteristics such as shoot length and basal culm diameter (Haslam, 1973). Yet, few studies have considered both influences on reeds at the same time (Kühl et al., 1999; Koppitz and Kühl, 2000; Guo et al., 2003). The genetic basis of differences between reed morphotypes has previously been documented for different ploidy levels. The most robust morphotypes, which are found in the Danube delta, are octoploid (Pauca-Comanescu et al., 1999). The most common ploidy level, both in the Czech Republic and worldwide, is tetraploid (Clevering and Lissner, 1999). Preliminary investigation using flow cytometry was carried out for 20 stands of robust reed in Halámky lake (Čížková et al., unpublished results) and for all stands in Halámky and Opatovický lakes involved in this study (Kubátová et al., unpublished results). It has shown only tetraploid genotypes. This indicates that the robust growth, found in some morphotypes of the Halámky population, is not due to a higher ploidy level.

Within the tetraploid reeds, phenotypic differences were examined with respect to the geographical origin of reed plants within Europe. Clevering et al. (2001) found significant differences in morphological characteristics of reed seedlings between the northwestern and southeastern European reed populations. Both seedlings and 1-year-old plants originating from southeastern Europe formed fewer but taller shoots as compared with plants originating from northwestern Europe. A similar difference was found between 2-year-old plants originating from different sites across the European latitudinal gradient (Bastlová et al., 2004).

Kühl and Zemlin (2000) found differences in morphological traits in 10 reed clones transplanted from their original habitats to six experimental fields along river banks in Germany. The correlation in morphological traits between plants from the original habitats and from the experimental fields was, however, examined only for two clones and found in only one of them. As indicated by our study, a comparison between the plant performance in culture and in their natural habitats is needed for a better understanding of the interaction between genotype and environmental conditions. Similarly, White et al. (2004) suggested to study the performance of the same genotypes in a range of environmental (i.e., hydrological) conditions.

Phenotypic differences between reed morphotypes were also studied in relation to habitat conditions (Dykyjová and Hradecká, 1973, 1976), but the role of genotype has not been assessed until recently. Clevering (1999) studied the relative effect of genotype and main environmental factors in cultivated young reed plants originating from different populations across Europe. She found that all the plants responded to differences in nutrient supply in a similar manner. Bastlová et al. (2004) came to the same conclusion for 2-year-old reed plants. Kühl and Zemlin (2000) did not find differences in nitrogen content among different reed clones transplanted to the same experimental field, although they found morphological differences. They concluded that the nitrogen content depended primarily on nitrogen availability at each specific site and was not genetically determined. These findings may indicate that mineral nutrition does not represent a key selection factor for the reed populations studied so far. By contrast, White et al. (2004) pointed to considerable morphological differences within reed genotypes as a result of local differences in hydrological regime and/or its consequences.

The main aim of this study was to examine to what degree the differences observed in the field are preserved under controlled conditions, with respect to climate, water supply and nutrition. A weak point of our study may be the fact that each field stand was represented by only one clone in the cultivation. As a result, the effect of some clones on the overall growth was not considered in multiclonal field stands. Nevertheless, as all stands selected were visually homogeneous, it is probable that the clonal heterogeneity of the field stands was not markedly manifested in the plant phenotypes.

According to our results, morphological characteristics were preserved to a greater extent in reed plants originating from the Halámky than from the Opatovický population. This is indicated by better separation of clones on the basis of morphological characteristics (Fig. 3), by a greater proportion of variation explained by pertinence to clones (Table 2) and by closer correlations in morphological characteristics between cultivated plants and their parent field stands (Fig. 4). These findings indicate that genetic variation was more pronounced in the plants of the young expanding population at Halámky. The fact that the relative differences between the clones in shoot length were preserved in the cultivated plants, supports our hypothesis stating that growth characteristics of the robust reed morphotypes have a genetic basis.

The predominant role of genotype in the young population may be ascribed to the relatively short site history accounting for fairly uniform habitat conditions. The substrate is sandy, nutrient poor and the reed plants are provided with nutrients mainly from pore water, slowly but continuously seeping from the Lužnice river bed. The shoot growth can be reduced there by unfavourable environmental conditions, such as the summer flood of 2002 (Křiváčková-Suchá et al., unpublished results), or shading by last years' dead culms, observed in 2005. In contrast to the younger population, environmental factors may have affected the phenotype more in the older population. The longer site history could have brought about more pronounced effects of both intra- and interspecific competition, and a diversification of habitat conditions due to, for example, accumulation of organic matter, which is accelerated by intense eutrophication (Čížková et al., 1996). Products of its anaerobic decomposition can be present in habitats hosting reed-dominated vegetation in toxic concentrations (Čížková et al., 1999) and increased intensity and/or proportion of anaerobic processes in organic matter decomposition coincide with the reed decline on some

sites (Picek et al., 2000, Šantrůčková et al., 2001). As organic matter gradually accumulates on lake bottoms with lake age, the intensity of all the associated effects probably increase during the site history. As a result, stress-tolerant genotypes in the sense of Grime (1979) may have been selected in the older, Opatovický population. This conclusion is supported by our observation that particularly genotypes capable of robust growth were absent from the older population at Opatovický lake.

Acknowledgements

This study was supported by project no. AVOZ60870520 of the Academy of Sciences of the Czech Republic and MSM 60076658-06. We thank Jan Květ for helpful comments on the manuscript.

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Phenotypic and genotypic variation of *Phragmites australis*: High level of cryptic variation revealed on the basis of molecular marker analysis non-corresponding to phenotype performance.

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Abstract

Genetic structure and diversity of two populations of common reed (*Phragmites australis* (CAV.) TRIN. ex STEUD.) of different age and life history located in the Třeboň Basin (South Bohemia, the Czech Republic) were studied using morphological, cytogenetic and molecular approaches. High level of genotypic variability of was detected after analysis of nuclear DNA markers. RAPDs, AFLPs and nuclear microsatellites (SSRs) provided dataset, which exhibited a high clonal diversity on both habitats. In contrast this fact would be strongly underestimated when only morphological approach was used because morphologically homogenous, cytogenetic uniform stands were composed of higher number of genetically different clones. A higher genotypic variability of particular common reed stands has been found in the older fishpond reed population, non-corresponding with a low variability in its phenotype performance. On population level, higher number of clones, except those detected by SSRs, was found in younger sand pit Halámky population. This fact supported the hypothesis of higher variation and divergency in younger population where also the character of internal clonal structure is different. Halámky population has no clear internal structure, detected variability is homogeneously disperged and due to age of this population not subclustered yet. The older population on Opatovický fishpond is internally highly substructured and forms five groups of clones partly corresponding to geographical configuration and management history. The significant role of molecular approach to study of plant population structure is above all in the fact of discovering nature and real genetic structure of populations. Morphologically homogeneous reed stands showed high level of *cryptic* variability non-corresponding to phenotypic character of stands. This variability and also structure of stands and populations can be strongly underestimated using only morphological approach because of only one third of analysed reed stands is really monoclonal.

Introduction

Common reed, *Phragmites australis* (Cav.) Trin. ex Steudel, has a cosmopolitan distribution and is abundant in marsh communities and along the borders of lakes, ponds, and rivers. Common reed is suggested to be native species to Eurasia (Den Hartog 1989; Clevering & Lissner 1999) but now widespread throughout the world, ranging all over Europe, Asia, Africa, America and Australia, however, the origin of the species is unclear. New studies has demonstrated the existence of native and introduced genotypes of *P. australis* in North American wetlands (Marks *et al*. 1994; Saltonstall 2001, 2002, 2003a, b, c). *P. australis* is a species with a high ability to propagate vegetatively, while reproduction by seeds occurs only in early stages of settlement, when seeds have suitable conditions for germination and growth (Hürlimann 1951; Weisner *et al*. 1993). Its

behaviour is essentially different in European and North American wetlands. While in North America it is considered to be an invasive species in Europe we encounter rather decline of its populations. To explain the spread of common reed, or better aggressive European reed genotypes, in North America, it has been suggested that the rapid expansion could be the result of human activities causing habitat disturbances or stresses such as pollution, changes in hydrologic regimes, and increased soil salinity. But the situation will be somewhat more difficult because also its decline is assigned on human activities. A hypothesis was put forward in the 1990s that in originally diverse populations only such clones would persist that were able to survive under the particular site conditions; this would be reflected by a reduced genetic variability within the population. The resulting populations, consisting of only a few large monoclonal stands, were supposed to be more vulnerable to rapid changes in environmental conditions such as eutrophication, because the low genetic diversity would not provide the stands with a sufficient phenotypic plasticity needed for successful adaptation. This was regarded as a possible explanation for the low genetic variability found in reed populations in some European lakes and for their decline (Eriksson 1993; Koppitz *et al*. 1997).

A lot of studies were occupied with ecology, ecophysiology and morphology of common reed, in last years also genetic structure of both native and invasive populations was studied. But it still seems that only complex approach and combining of ecological and molecular data can discover cryptic variation and/or invasion, reveal the "true" structure and variation in reed populations and maybe predict behaviour of reed populations.

In this study, two *Phragmites* populations of known history, age and morphological uniformity were chosen. Both populations were studied from the point of view morphology, ecology and ecophysiology in last decades. To spread and intensify methods used for these studies, different molecular markers were applied and genetic variability of the two reed populations in order to provide further information on the relation between phenotypic and genotypic diversity was analysed. The following hypotheses were surveyed/verified:

(1) different molecular markers and morphological characteristics can better reveal genetic structure of studied populations than single marker focused approach,
(2) the patterns of genotypic variability of two reed populations inhabiting sites differing in both succession stage and substrate conditions are distinct,
(3) the morphological variability of studied populations/stands reflect their genotypic variation and the morphological variability should be an indicator of their genetic variability, so morphologically homogeneous stands are monoclonal,
(4) the number of clones per stand (unit of population) area is lower in the older population than in the younger population and also the maximum area covered by a single clone is larger in the older population than in the younger one.

Material and methods

Site description

Two localities were selected in the Třeboň Biosphere Reserve (Třeboň: 49° 05'N, 14 46'E., altitude 430 m), South Bohemia, Czech Republic:

(A) Opatovický fishpond as a model site occupied by well established stable common reed stand. This locality represents mesotrophic to eutrophic shallow water body constructed in 1510-1514 by damming a shallow valley and used since for carp production. The maximum flooded area is 160 hectares. Its maximum length and width of the shoreline is 2.6 and 1.1 km, respectively. Reed stands occur along over a half of the shore length. In last 50 years the reed stands have been subject to a series of scientific studies, which include detailed mapping of their area, production and mineral nutrient economy (Dykyjová & Květ 1978; Hroudová & Zákravský 2002).
(B) Halámky sand pit is a study model of newly born common reed stand performing extensive morphological variation in early stages of reed stand formation followed by decreasing of variability and "uniforming" of population character. This new oligotrophic lake was formed by sand extraction stopped in 1994. The area of the lake is 22.89 hectares. The maximum length and width of the shoreline is 0.79 and 0.41 km, respectively. Reed stands occur in still isolated patches along much of the shore. In addition, small islands of reed vegetation occur in parts of the lake where the water depth is smaller than 1.5 m. All the reed vegetation there is certainly younger than 35 years, but much of it is probably younger than 20 years. It is likely that it established itself from seeds, as the lake does not direct connection with any watercourse in the catchment. Further details on the study sites are given by Čurn *et al*. (2007).

Sampling

Morphologically (visually estimated) homogenous and healthy looking stands of *P. australis* have been chosen in the littoral zone of both lakes in the same manner as described in our previous paper (Čurn *et al*. 2007). Nine and seven reed stands were selected at Opatovický and Halámky lake, respectively. The alphabetical labelling of stands corresponds to that used in the previous paper, where a schematic map with their positions is also given. Two parallel transects were laid down along the fringes of the reed belts of each lake. One of them was adjacent to the open water edge of the reed belt while the second followed the terrestrial edge. Five shoots were collected along each transect and subjected to both morphological measurement and the genetic analyses.

Analysis of molecular markers

DNA isolation. The leaves were placed in a ziploc bag on ice in cool-box and transferred to laboratory. DNA was isolated from the fresh leaf samples by using Invisorb Spin Plant Mini Kit (INVITEK, Germany) according to the standard protocol. DNA samples were stored at -20°C prior to their use.

RAPD analyses. Random primers (OPERON Technologies, CA) were selected for the RAPD analysis. The protocol for RAPD analysis was adapted from that of Williams *et al*. (1990). The volume of the final PCR reaction (25 µl) was composed of 1× buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM dNTP, 10 pM of primer, 1 U Taq DNA polymerase (TAKARA) and 25 ng of template DNA. Amplifications were carried out in an MJ Research Thermocycler PTC 100 according to the program that included an initial denaturation step of 3 min at 94°C, then 45 cycles of 1 min at 94°C, 2 min at 35°C and 3 min at 72°C followed by final elongation for 10 min at 72°C. The final step was followed by a holding temperature of 4°C. PCR products were separated on 1.5% agarose gels in TBE buffer which included lanes of 100 bp DNA ladder (NEB) and

DNA bands were visualized after ethidium bromide staining under UV light. RAPD patterns were photographed using Epson Ultra Cam 3100Z Imaging System.

SSR analyses. SSR (microsatellite) loci were amplified using 5 primer pairs: *PaGT9*, *PaGT12*, *PaGT14*, *PaGT16* and *PaGT21* (Saltonstall 2003b). The final volume of the PCR reaction was 25 µl. SSR analyses were proceeded under the following conditions: 25 ng DNA template, 1x buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0,01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dNTPs), 25 pM of primer, 1.25 U Taq Purple DNA polymerase (PPP Master Mix, Top-Bio, CZ). Thirty five PCR cycles were performed, with 30 s of denaturation at 94°C, 30 s of annealing at 50-56°C, and 30 s of polymerisation at 72°C followed by final elongation for 5 min at 72°C. PCR products were visualised by ethidium bromide staining after electrophoresis on 3% Synergel and 10% polyacrylamide gels in 1x TBE buffers. SSRs were recorded using Epson Ultra Cam 3100Z Imaging System.

AFLP analyses. Patterns of AFLP variation were evaluated using six primer combinations (*Eco*RI-ACG/*Mse*I-AAA, *Mse*I-ACA, *Mse*I-ACT, *Mse*I-AGA, *Mse*I-AGT, *Mse*I-ATG). AFLP analyses were performed following procedures similar to that of Vos *et al*. (1995) according to modified protocol (Travis & Whester 1995). The volume of 10 µl R/L buffer (100 mM Tris-acetate, pH= 7.5, 100 mM magnesium acetate, 500 mM potassium acetate, 50 mM DTT) containing 5 U *Eco*RI and 5 U *Mse*I restriction endonucleases was added to 40 µl of DNA sample (50 ng DNA). Genomic DNA was digested for 16 hours at 37°C. The volume of 10 µl of ligation master mix (1xR/L buffer, 5 pmol *Eco*RI 3' adaptor, 5 pmol *Eco*RI 5' adaptor, 50 pmol *Mse*I 3' adaptor, 50 pmol *Mse*I 5' adaptor, 1.2 pmol ATP, 1 U T4 DNA ligase) was added to 50 µl of restriction solution and adaptors were ligated for 3 hours in 37°C. After ligation reaction mixture was 10fold diluted with T0.1E buffer. To 5 µl DNA sample (diluted after R/L step) was added 45 µl of master mix (1x PCR buffer, 4 mM $MgCl_2$, 200 µM dNTP's, 75 ng *Eco*RI-A primer, 75 ng *Mse*I-A primer, 1 U Taq DNA polymerase). After initial denaturation at 94°C for 2 min 35 PCR cycles were performed, with 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 60 s of polymerisation at 72°C followed by final elongation for 9 min at 72°C. After PCR reaction mixture was 20fold diluted with TE buffer. The volume of selective amplification reaction (10 µl) was composed of 2.5 DNA sample (diluted after pre-amplification) and 7.5 µl of master mix (1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP's, 5 ng *Eco*RI-ANN-FAM primer, 30 ng *Mse*I-ANN primer, 0.5 U Taq DNA polymerase). After initial denaturation at 94°C for 2 min 10 PCR cycles were performed, with 30 s of denaturation at 94°C, 30 s of annealing at 65°C with touchdown (-1°C/cycle), and 60 s of polymerisation at 72°C followed by 25 PCR cycles with 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 60 s of polymerisation at 72°C followed final elongation for 15 min at 72°C. After selective amplification 1 µl of reaction mixture was mixed with 11 µl of Hi-Di formamide (Applied Biosystems) and 0.4 µl of 400 HD Rox Size Standard (Applied Biosystems), samples were denatured at 94°C for 4 min and subjected to fragmentation analyses on ABI 310 genetic analyser.

cpDNA sequencing. Noncoding cpDNA regions were amplified and sequenced using primer pair (c-d) from Taberlet *et al*. (1991). The intergenic spacer regions separating *trn*L 5' and *trn*L 3' was amplified, total volume of reaction mixture was 25 µl and amplification was performed under the following conditions: 25 ng DNA template, 1x buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0,01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dNTPs), 12.5 pM of primer, 1.25 U Taq Purple DNA polymerase (PPP Master Mix, Top-Bio, CZ). After initial denaturation at 94°C for 2 min 35 PCR cycles were

performed, with 30 s of denaturation at 94°C, 60 s of annealing at 62°C, and 60 s of polymerisation at 72°C followed by final elongation for 10 min at 72°C. The PCR products were separated on 3% agarose gel in TBE buffer. The separated fragments were extracted from gel using JetQuick Gel Extraction kit (Genomed), cloned to sequencing vector using TOPO TA Cloning Kit (INVITROGEN) and sequenced in both directions with BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The samples were run on an ABI Prism 3130 DNA sequencer.

Data analysis. RAPD and SSR gels were digitized and analyzed by BioProfil 1D+ software (Vilber Lourmat, France) and UltraQuant software (UltraLum, USA) with manual correction. AFLP data were analysed by GenMapper 4.0 software. Fingerprint patterns were transformed into a binary character matrix with 1 for presence or 0 for absence of a band at a particular position in a lane. After removing monomorphic bands, genetic distance matrices were generated using Nei & Li and Gower General Similarity Coefficients and Cluster analysis (UPGMA – unweighted pair group method averages) and PCoA analysis (Principal Coordinates Analysis) were performed. These statistical analyses were calculated using MVSP (Kovach Comp. Serv.) and STATISTICA 6.0 software package (Statsoft). Sequences were analysed by BioEdit software and data were be evaluated using the Fitch parsimony method in combination with heuristic search in PAUP (Swofford 2001).

Flow cytometric analyses

Flow cytometry was employed to estimate DNA ploidy levels. A modified two-step Otto procedure (Otto 1990) with nuclei isolated from young leaves was used for FCM analyses. Small pieces of leaf tissue (pooled samples - 5 plants from each transect) with appropriate volume of leaf tissue of the internal reference standard (*Glycine max* cv. 'Polanka' with 2C DNA content = 2.5 pg, Doležel *et al*. 1994) were chopped together using a new razor blade in a Petri dish containing 0.5 ml ice-cold Otto I buffer (0.1 m citric acid, 0.5% Tween 20). The sample was filtered through a nylon mesh (42 μm) and incubated at room temperature for 15 min. The staining solution consisted of 1 ml Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented with an intercalating fluorochrome (PI - Propidium iodide) and RNase, both in final concentration 50 $\mu g.ml^{-1}$ and an antioxidative compound 2-mercaptoethanol (2 $\mu l.ml^{-1}$). After incubation for 5 min at room temperature, fluorescence intensity in absolute units (pg) of at least 5000 particles was recorded using a Partec CyFLow flow cytometer (Partec GmbH, Münster, Germany) equipped with an green solid-state laser (532 nm). Tetraploid standard reed plants (chromosome number 2n=48) were cultivated in the experimental garden in the Institute of Botany, Prague.

Results

Molecular analyses

RAPD analyses

A total of 120 random primers were surveyed across all analysed samples. On the basis of ability to amplify DNA, the number of polymorphic bands and their intensity and reproducibility of RAPD products, 5 highly polymorphic primers were selected (OPA-03, OPA-04, OPA-11, OPB-04 and OPB-08). These five primers amplified altogether

88 bands and 75 (85%) of these bands were polymorphic. The size of amplified band ranged from 100 to 1500 basepairs. The polymorphism level was different across the group of polymorphic bands, some of them (13) exhibited only low level of polymorphism (polymorphic bands were detected in only 1-3 plants). Among used primers OPB-08 was the most polymorphic primer (primer generating the highest number of polymorphic band with good resolution and also good stability and staining activity) generating 16 bands and only two bands were non-polymorphic.

SSR analyses

Polymorphism of SSR (Simple Sequence Repeats) markers was analyzed using 5 primer pairs. Primer pairs *PaGT14* and *PaGT21* provided non scorable pattern. Primer pair *PaGT14* amplified two zones of microsatellite bands, invariable band at 470 bp and zone of weak stained and unstable bands between 180-210 bp. Bands generated by primer *PaGT21* were weakly stained, formed unstable wide zone 180-350 bp. In this study results of three microsatellite loci are presented, microsatellites amplified using primer pairs *PaGT9*, *PaGT12* and *PaGT16* provided stable, repeatable and well scorable pattern of markers. Number of possible band positions and number of amplified bands for all three microsatellites are given in Table 1.

Amplified bands were scored and altogether 16 possible band positions were statistically evaluated. Nevertheless low polymorphism generated by individual SSR primer after evaluation of the complete dataset it was possible to characterize microsatellite structure of analyzed populations (Fig. 1b). SSR analyses suitably supported results of cryptic diversity detected after RAPD analyse (Fig. 1a). SSR markers manifested high level of stability, were reproducible during all three repeated analyses.

AFLP analyses

AFLPs were analyzed using 6 primer combinations. Summarized results of AFLP analyses are given in Table 2. All six primer combinations generated comparable number of markers: 303 to 356 per primer combination. A little bit larger differences were in abundance of markers in populations – maximal number of markers was detected in *Mse*I- AAA primer (131 markers in plant OP-34), minimal number of 43 markers in primer combinations *Mse*I-ACA (HAL-157) and *Mse*I-ACT (plants OP-19, OP-30, OP-34, HAL-103 and HAL-116). Altogether 2007 possible marker positions and 93,452 of AFLP markers were analysed.

In comparison to RAPDs and especially to SSRs, AFLP markers provided larger dataset and also the level of genetic variability was relatively very high. AFLP technique is able to detect really small distinctness/dissimilarities and this fact should be very important in analyzing of genetic structure of clonal, vegetatively propagated plants. AFLP profiles were stable, well scorable and due to separation of AFLP fragment on genetic analyser it was possible to detect markers/band with high accuracy and carefulness. All six primer combinations gave also similar pattern of distribution across analysed populations and both populations were clearly separated/differentiated (Fig. 2). These results were strongly supported when all six primer combinations were analysed in complex (Fig. 3b). A typical feature was also the character of distribution of variability inside the populations. Plants from Halámky population (newly arised reed stands) formed one more open "homogeneous" cluster without visible inner structure and subgrouping of analysed plants. Range of variability in population from Opatovický lake was similar to previous one, but major differences were found just in the internal structure and variability distribution.

Analysed samples were clustered to several more compact groups, these groups occasionally represented individual reed stands. So if the range of disclosed variability was comparable in both populations, differences were in the internal grouping/structure and optically Opatovický lake population appeared as more compact, less variable and this "phenotypic" impression was in concordance to real phenotypic data.

cpDNA analyses

Analyses of the intergenic spacer regions separating *trn*L 5' and *trn*L 3' chloroplast genes showed absolute uniformity and only one chloroplast haplotype (related to this cpDNA region) was detected.

Cytogenetic analyses

Results from flow cytometry analyses clearly verified uniform presence of only tetraploid genotypes in all analysed stands in both populations. These findings were basal information about cytogenetic and cytotypic structure of investigated populations and also confirmation of uniform ploidy level of analysed plants. Plants from one transect across particular reed stand were pooled and analysed in one FCM analysis. Only clear histograms corresponding to tetraploid plants were recorded so there was no need of analysis of individual non-pooled samples.

Clonal structure and genetic diversity of analysed population

Clonal structure of analysed reed stand in both populations showed unexpected high level of "cryptic" genetic variability. Reed stands mainly on Opatovický lake represent morphologically homogeneous and visually uniform reed population with relatively small differences between individual stands. Stands on Halámky lake were slightly more variable, especially stands N and O where "gigant" plants up to 5 m high were found (for details see Čurn *et al*. 2007) and also differences between individual stands were better visible. In general morphologically reed stands represented homogeneous populations and they were considered to be monoclonal. Table 3 demonstrates results of clonal structure measured by number of distinct clones/genotypes and frequency of monoclonal stand. Total number of clones found out on Opatovický lake population was similar using RAPD, SSR and combined approach (RAPD + SSR + morphology). Microsatellites notwithstanding showed higher diversity and although this marker covers only smaller part of genome it is evident that number of clones present on only one stand is the higher just in microsatellites in this older population with longer life history. Number of clones, except those detected by SSRs, is higher in Halámky population. The average number of clones per reed stand is in this population more than 2.5 in comparison to 1.5 clones per stand in Opatovický lake population. This fact supported the hypothesis of higher variation and divergency in younger population where also the character of internal clonal structure is different – prevalent number of clones is presented only on one stand. And this fact further increase the level of diversity. Smaller number of clones detected by SSRs should be caused by restricted genetic pool and only limited number of different SSR haplotypes founded this new populations. The plants with the same pattern of microsatellite markers were found in both populations, so using this marker both populations are not clearly separated into two independent groups as in RAPDs or AFLPs. This is caused just in consequence of more specific character of this marker but SSRs are still suitable marker for detection of inner structure of

morphologically homogeneous stands and marker disclosing a part of genetic variation in populations.

Combined approach when DNA and morphological markers were analysed lead to clear separation of both studied populations and distinctiveness of both populations was higher using all methods (Fig. 3). In total 19 characteristics of reed production (e.g. density of live, flowering and dead shoots, total aboveground biomass) and morphology (e.g. shoot length to the tip of the uppermost leaf, number of internodes per plant, basal diameter, panicle length) were recorded at the time of seasonal maximum aboveground biomass. Morphological data were analysed and discussed in previous study (Čurn *et al*. 2007) and in this study we used these results as additional information to different molecular approaches. On Fig. 3b is able to see that Halámky population has no clear inner structure, detected variability is homogeneously disperged and due to age of this population not subclustered yet. The older population on Opatovický lake is internally highly substructured forming groups of plants partly corresponding to geographical configuration and management history. Cluster (UPGMA) and principal coordinates (PCoA) analyses of both molecular and morphological markers defined five groups of genotypes in Opatovický population. Group I was formed by only one plant – O-1. Group II was the largest group of 48 plants dispersed across all stands in southern part of Opatovický lake (except stand I). Groups III and IV were almost the same size (16 and 15 plants) and also almost the same range (stands D-G-H; C-D-G-H respectively). According to configuration of lake inlets these four stands are very close each to other and are located just in the mouth of the inlets so this can explain this type of grouping and genotype distribution (possibilities of reed dispersal, lowering and enlarging of variability in established reed stands are given in Čurn *et al*. 2007). Group V is formed by 10 samples from stand I. This uniform monoclonal stand is located in the most North-East par of reed belt in Opatovický lake and this stand was forming after mud removing in the course of lake management. This fact can explain the remoteness of this clone and uniformity of this stand. Also the two other monoclonal stands are located laterally – in one of large lake inlet. These stands are monoclonal, covering extensive area of this part of Opatovický lake and although both these stands are included in Group II they form compact "subgroup" on its edge. Results of these statistical analyses of both molecular and morphological dataset showed high internal structuring and also supported hypothesis that in stable undisturbed conditions reed can form extensive monoclonal stands.

The significant role of molecular markers and their use in study of plant population structure is above all in the fact of discovering nature and real genetic structure of populations and morphologically homogeneous reed stands. All stands in this study showed high level of *cryptic* variability non-corresponding to phenotypic character of stands. This variability and also the structure of stands and populations can be strongly underestimated using only morphological approach because of only one third of analysed reed stands is really monoclonal.

Discussion

Phragmites australis is widely occurred wetland plant often forming extensive stands. Although it is a significant constituent of wetland communities worldwide its behaviour in Old and New World is essentially different. Over the past several decades, populations of the common reed have expanded rapidly in salt marshes of coastal North America and also in coastal wetlands of the Great Lakes creating dramatic changes in

ecological integrity of these wetland communities (Lynch & Saltonstall 2002). Using molecular approach background of this behaviour was disclosed and cryptic invasion of non-native agressive genotypes was revealed (Saltonstall 2002, 2003a, b) and also new hypothesis explaining role of human activities and reaction of compatible genotypes on these new formed conditions were introduced (Saltonstall 2002). On the other side common reed is not only invasive plant species rapidly spreading and injuring native plant communities, it is also plant where significant decline of European populations has been observed at approximately 60 lakes (Koppitz *et al*. 2004) and river sites (Ostendorp 1989; Van der Putten 1997) in Europe. A lot of ecological factors have been made responsible for the die-back of reed, and many studies have tried to discover ecophysiological mechanisms and tolerance limits of *P. australis* (e.g. Ostendorp 1989; Van der Putten, 1997; Brix 1999), also changes in land use patterns and hydrologic regimes, increased disturbances, urbanization and eutrophication are often listed as factors thought to cause declines of *P. australis* (Van der Putten 1997). The recent die-back of *P. australis* from shoreline habitats of European lakes led to the interest of biologists. Reed die-back may be caused by a variety of factors, such as eutrophication and its possible impact on carbohydrate storage dynamics (Čížková *et al*.; 1996; Kohl *et al*. 1998), toxic effects of sapropelic compounds (Armstrong *et al*. 1996), reduced rhizome ventilation (Armstrong & Armstrong 1991; Brix *et al*. 1992; Kohl *et al*. 1996; Armstrong *et al*. 1999) or the artificial regulation of the water levels (Van der Putten 1997). Factors as flooding, insect or fungal infestation, mechanical damage and loss of culms can reduce the convective gas flow to the basal parts of the reed plants (Armstrong *et al*. 1996). This leads to an oxygen deficiency in the roots and the rhizomes, changing the metabolic status of the whole plant. Fermentation processes may become active producing ethanol, lactate and some other compounds (Brändle & Crawford 1987). Presumably, the shift in central metabolic pools is reflected by changed amino acid patterns in the rhizomes and in the basal culm internodes of *P. australis* (Kohl & Henning 1987; Haldemann & Brändle 1988; Kohl *et al*. 1998).

Recently it has been suggested that low genetic variability might also contribute to reed decline (Kühl & Neuhaus 1993; Neuhaus *et al*. 1993; Koppitz *et al*. 1997). The low genetic variability of natural reed stands observed in some regions is thought to be the consequence of special strategies of colonization (Kühl & Neuhaus 1993; Koppitz *et al*. 1997). According to this, reed populations initiated by seeds are initially genetically diverse, but over time become dominated by one or a few clones well-adapted to the prevailing site conditions. If a site is initially colonized by only a few seedlings reflecting different genotypes, which spread vegetativelly thereafter, the resulting stand will necessarily have low genetic diversity. Low diversity can also be expected if initial colonization is effected by a high number of genotypes, but only the well-adapted ones survive and spread (Koppitz & Kühl 2000). According to the model of establishment and development of reed stands (Koppitz *et al*. 1997), the colonization of shores by *P. australis* starts with a variety of new clones. This process is supposed to be the first of three main stages of the establishment of reed clones, the stage of settlement (Kühl & Neuhaus 1993; Koppitz *et al*. 1997). In this stage, the reed colonizes suitable areas by generative propagation. This results in reed stands with high genetic diversity. In the case of monoclonal or oligoclonal populations, as found in the study Koppitz & Kühl (2000) at many of the water reed sites, the extent of reed die-back of the whole population may be considerable as soon as one of the clones is not adapted to a specific change in conditions. Drastic decline may also be expected at stands where the clones are arranged in clusters or in a row along the shoreline. Moreover, the remarkable differences in performance

(in terms of morphology and expansion) of different clones after having been planted at a site with similar site conditions point to a strong influence of the genotype on the development of individual reed clones within a reed stand (Kühl *et al.* 1999; Kühl & Zemlin 2000; Koppitz *et al*. 2000). Contrary to monoclonal stands, polyclonal (intermixed or mosaic-like) reed stands are less likely to experience a drastic decline after a change in site conditions; instead, 'invisible reed decline' may occur (Kühl & Neuhaus 1993). If the different clones of a reed stand do not react to stressors in the same way due to different physiological and morphological features, only few clones of the population may be affected by die-back. In that case, some clones would disappear, but the remaining tolerant clones may expand into the gaps. This process would not become apparent as a die-back of the whole population, but it would result in a decrease in the number of clones, and thus a decreased ability to react to stressors and a reduced plasticity of the reed population (Koppitz *et al*. 1997). The history of colonization of sites by *P. australis* is important to understand the reactions of reed stands to changes of environmental conditions. Particular clones may exhibit a high degree of plasticity, showing a 'general-purpose genotype' that occupies a wide range of environments, as described for clonal genotypes (Lynch 1984; Bierzychudek 1989). However, in general each *Phragmites* clone has probably relatively narrow tolerance limits in relation to its special site conditions. The existence of different ecotypes specifically adapted to local site conditions has already been considered (Björk 1967; Dykyjová and Hradecká 1976; Kühl *et al*. 1997; Čurn *et al*. 2007). The often described high ecological plasticity of the species *P. australis* (Haslam 1972; Van der Toorn 1972; Rodewald-Rudescu 1974; Křiváčková-Suchá *et al*. 2007) may, consequently, be the result of the high number of reed clones. The considerable differences in genetic diversity as observed e.g. in Koppitz & Kühl (2000) could be one reason for the differing abilities of reed stands to react to changing site conditions and, eventually, to survive or to die-back. The observable dynamics depends on the reaction of individual clones, e.g. the possibility of tolerant clones to expand into the gaps left by sensitive ones (Čurn *et al*. 2007). It follows that the extent of reed decline after a deterioration of site conditions may be determined by a variety of factors including genetic diversity, the amplitude of adaptability of each clone, and the type, strength and velocity of the changes.

Acknowledgement

This study was supported by research grant MSM6007665806 of the Ministry of Education of the Czech Republic.

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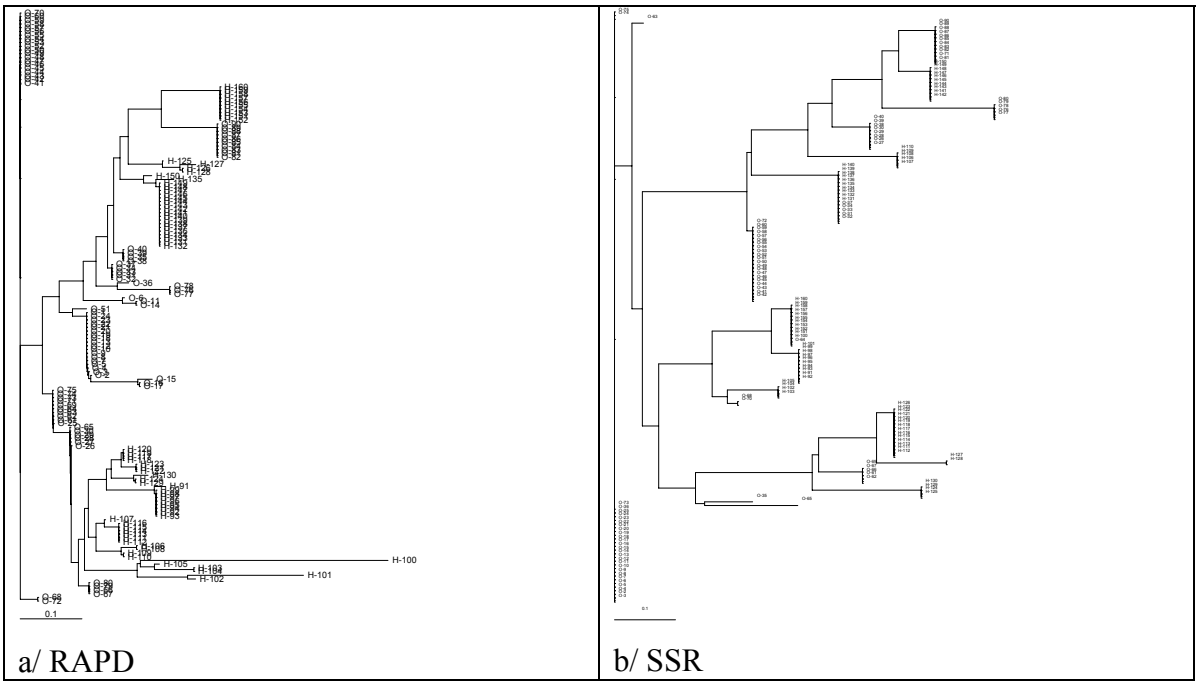
Fig. 1 Cladograms constructed on the basis of RAPD (a) and SSR (b) data, by the neighbour-joining method.



a/ RAPD

b/ SSR

Fig. 2 PCoA case scores of *P. australis* stands of the Opatovický (green circles) and Halámky (blue triangles) lakes calculated from individual AFLP primer combinations.

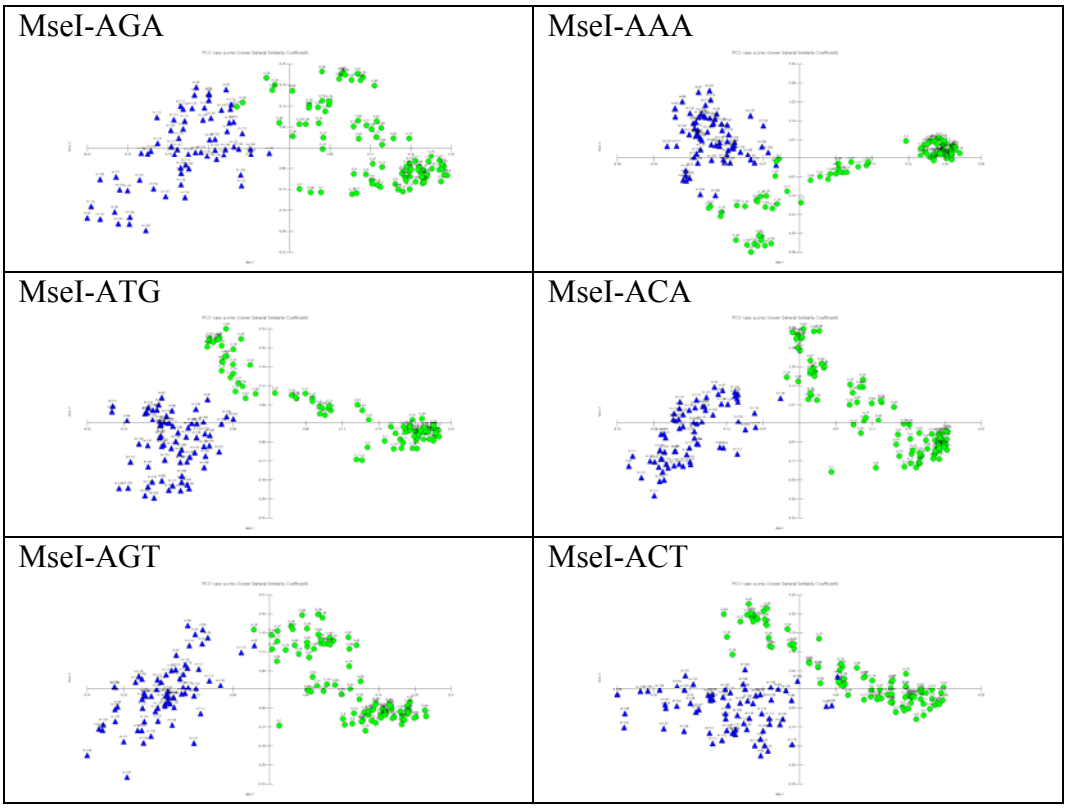


Fig. 3 PCoA case scores of *P. australis* stands of the Opatovický (green circles) and Halámky (blue triangles) lakes calculated from SSR, RAPD and morphological (A) data and from whole dataset including six AFLP primer combinations.

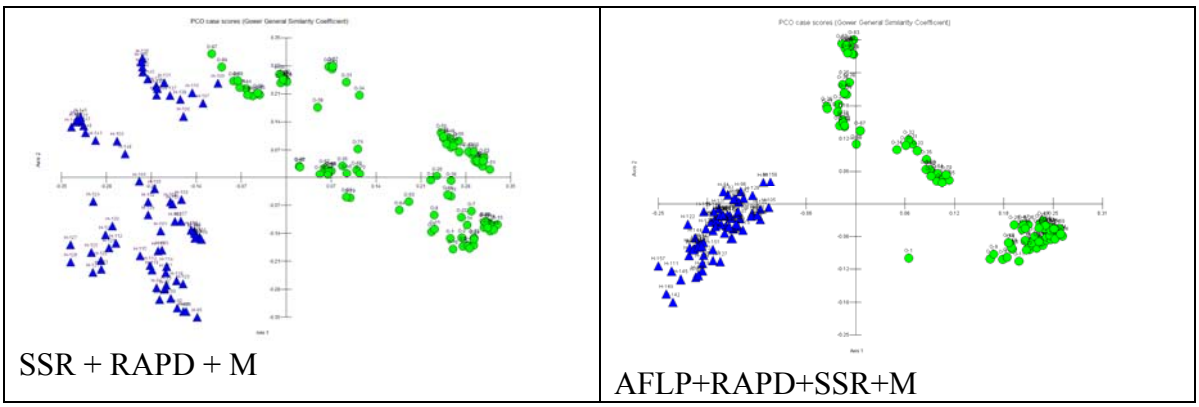


Table 1 Number of possible band positions and number of amplified bands for analysed three microsatellites.

	PAGT09	PAGT12	PAGT16
number of possible band positions	7	3	6
MAXimum number of bands	3	3	4
MINimum number of bands	1	1	1

Table 2 Number of possible band positions and number of amplified bands for analysed AFLP primer combinations.

	*Mse*I primer						
	AAA	ACA	ACT	AGA	AGT	ATG	ALL 6K
total number of alleles	343	356	303	347	327	331	2007
MAXimum number of alleles	131	119	80	124	113	117	638
MINimum number of alleles	62	43	43	51	46	57	305
SUM	18561	16782	9247	17289	15308	16265	93452

MAX, MIN – maximum and minimum number of alleles in one plant, SUM – total number of alleles (markers) in all plants per particular primer combination, ALL6K – results for all six primer combinations

Table 3 Clonal diversity of populations of *P. australis* stands growing along the shores of Opatovický fishpond (old lake) and Halámky sand pit (new lake), South Bohemia, Czech Republic.

	Opatovický			Halámky		
	RAPD	SSR	G+M	RAPD	SSR	G+M
Total number of clones	14	13	16	18	7	18
Number of monoclonal stands	2	5	3	4	4	3
Number of clones present only in 1 stand	9	11	9	17	3	17
Number of clones occurring in 2 neighbouring stands	2	1	1	1	3	1
Number of clones occurring in 2 non-neighbouring stands	2	-	-	-	1	1
Number of clones occurring in more than 3 stands	1	1	3	-	-	-

G+M – genetic (RAPD+SSR) and morphological markers (Čurn *et al*. 2007)

Table 4 Internal structure of clones in Opatovický lake - subgrouping of plants and relevant stands.

	number of plants	Opatovický lake plant No.	stands
Group I	1	1	A
Group II	48	2-25, 36, 41-60, 73-75	A, B, C, D, E, F, H
Group III	16	31-35, 37, 61-65, 68-72	D, G, H
Group IV	15	26-30, 38-40, 66-67, 76-80	C, D, G, H
Group V	10	81-90	I

Journal of Biogeography (J. Biogeogr.) (2008) 35, 167–176

ORIGINAL ARTICLE

DNA ploidy-level variation in native and invasive populations of *Lythrum salicaria* at a large geographical scale

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ABSTRACT

Aim This study aimed to document precisely the patterns of DNA ploidy variation in the native and secondary ranges of *Lythrum salicaria* distribution. The hypothesis that species invasiveness had been induced by a switch in ploidy level was addressed.

Location Europe, Middle East, North America.

Methods DNA ploidy levels of 1884 progenies of 578+ plants collected at 124 localities were determined by DAPI flow cytometry.

Results Large cytotype variation (2*x*, 3*x*, 4*x* and 6*x*) was found across the native area of distribution (64 populations covering 12 European and two Middle Eastern countries). DNA hexaploids were detected for the first time, and rare DNA triploids were reliably confirmed. DNA tetraploids largely prevailed across the native range studied, while DNA diploids and DNA hexaploids were recorded only in Israel and Turkey, respectively. DNA triploid progenies occurred in one population from Hungary (together with DNA tetraploids). Sympatric growth of DNA tetraploids and DNA hexaploids was repeatedly encountered in Turkey. In contrast, cytotype uniformity was a typical feature of the invasive North American plants. Sixty populations, covering 13 states of the USA and provinces of Canada, were characterized by the presence of only DNA tetraploids.

Main conclusions Several *L. salicaria* cytotypes (2*x*, 3*x*, 4*x*, 6*x*) occur in the native range of distribution, with much variation concentrated in the Middle Eastern countries, whereas only DNA tetraploids appeared to occur in North America. Our data show that the invasive spread of North American populations was not triggered by differences in ploidy level. Alternative explanations should be sought.

Keywords

Cytogeography, cytotype, distribution, DNA ploidy level, flow cytometry, invasion biology, *Lythrum salicaria*, purple loosestrife.

INTRODUCTION

Polyploidy, a state of having more than two complete chromosome sets per nucleus, has played a key role in the evolution and diversification of the plant kingdom (Leitch & Bennett, 1997). Polyploid derivatives often differ from diploid progenitors in several cytological, morphological, ecological or physiological characteristics that may alter their fitness and contribute to local adaptation (Ramsey & Schemske, 2002). Generally, polyploids have (1) higher phenotypic variability triggered by genome reorganization, chromosome substitution and new nucleus–cytosol interactions; (2) increased genetic diversity due to possession of more alleles per locus; (3) a higher frequency of heterozygotes, which affords opportunity to mask deleterious alleles and reduces the cost of inbreeding; and (4) enhanced gene expression (gene-dosage effect). Some data indicate that polyploidy may also contribute to invasive behaviour and spread of alien plant species, as documented, for instance, in the genera *Carpobrotus* (Vilà & D'Antonio, 1998), *Spartina* (Ainouche *et al.*, 2004) and *Tragopogon* (Soltis

et al., 2004). Similarly, ploidy level was found to be the major determinant of hybrid fitness between invasive *Rorippa austriaca* and native *Rorippa sylvestris* (Bleeker & Matthies, 2005). In addition, Bennett *et al.* (1998) claimed that the proportion of polyploids is higher in weedy species than in other plant groups.

Lythrum salicaria L. (Lythraceae, purple loosestrife) is a native Eurasian perennial plant with both diploid and polyploid cytotypes, occurring commonly in wetland habitats. The species was introduced into North America in the early 1800s and soon became a popular ornamental plant, distributed widely by beekeepers and horticulturists (Thompson *et al.*, 1987). Although it spontaneously colonized various wetlands, it was relatively sparse until the 1930s, when monoculture stands were recorded in Quebec, Canada. Since then, purple loosestrife has spread throughout the continent and now grows in the conterminous states of the USA and nine adjacent provinces of Canada (Welk, 2004). While the species only rarely becomes a dominant component of wetlands in Eurasia, it often forms a monospecific community in North America (Thompson *et al.*, 1987; Blossey, 1999). As a result, a number of management strategies have been implemented (including mechanical disturbance, use of chemical herbicides and biological agents) to control its invasion (Henne *et al.*, 2005). Nevertheless, the negative impact of purple loosestrife on wetland diversity has been questioned (Hager & McCoy, 1998) or even disproved by some authors (Hager & Vinebrooke, 2004), and more targeted studies are necessary to assess the actual threat for native biota. In addition to North America, the species has been introduced into South America (Argentina), New Zealand, Australia and South Africa (Wangerin & Schröter, 1937; Thompson *et al.*, 1987; D. Richardson, personal communication).

The reasons for the sudden population growth of this species in secondary areas of distribution remain unknown, although factors such as adaptive evolution to new environmental conditions, introduction of vigorous genotypes predisposed to successful expansion, evolution of increased competitive ability due to a lack of native herbivores, targeted selection of vigorous genotypes by beekeepers and gardeners, and even introgressive hybridization with native *Lythrum alatum* have been suggested as potential explanations (Thompson *et al.*, 1987; Blossey & Notzöld, 1995; Houghton-Thompson *et al.*, 2005). Actually, there is considerable variation in plant phenology, life history, biomass allocation and morphology across the primary range of distribution, generally showing a north–south gradient (Olsson & Ågren, 2002; Bastlová *et al.*, 2004). Robust and late-flowering individuals originating from southern geographical areas (e.g. Israel and Spain) were found to be more similar to invasive North American plants in growth dynamics and phenotype characteristics than their counterparts from central and northern Europe (Bastlová & Květ, 2002).

Variation with respect to the number of chromosomes (ploidy level) in *L. salicaria* has also been documented (Buttler, 1984; Mal *et al.*, 1992; Goldblatt & Johnson, 2003). Diploids ($2n = 2x = 30$) appear to occur in the eastern part of the native range (karyological records come from Israel, the Caucasus, the Far East and Japan), while tetraploids ($2n = 4x = 60$) appear to occur in the western part of the native range (karyological records come from Belarus, the Czech Republic, Denmark, Finland, France, Germany, the Netherlands, Norway, Poland, Slovakia and Sweden). In addition to euploid numbers, some hypo-aneuploid plants ($2n = 58$ in Germany and $2n = 59$ in unlocalized European material) and accessory chromosomes ($2n = 60 \pm 1$–$2B$ in the British Isles; Montgomery *et al.*, 1997) have also been observed. Interestingly, Morrison (1963) reported 45 somatic chromosomes (corresponding to triploid cytotype) in European material of unknown provenance. There are also counts $2n = 50$ from northern Germany, north-eastern Poland and Scandinavia, and $2n = 107$ in colchicine-treated plants from Germany (Morrison, 1963); however, these were later queried (Buttler, 1984). Chromosome numbers from the secondary area of distribution are rather scarce, being represented by $2n = 60$ and $2n = 50$–60 (preliminary count) in plants from Ontario (Mal *et al.*, 1992).

Standard karyological treatments used to infer the ploidy level are laborious and time-consuming, which precludes convenient large-scale cytotype screening. To cope with large population samples, cytometric techniques such as flow cytometry (FCM) are much more useful (Doležel, 1997). This approach facilitates unbiased insights into the extent of ploidy variation, accurate assessment of the overall cytotype distribution pattern, and determination of cytotype-specific morphological features and ecological preferences (Burton & Husband, 1999; Baack, 2004; Rotreklová *et al.*, 2005). In addition, juxtaposition of cytotype variation in primary and secondary areas of the species' distribution may be useful for better understanding of potential determinants of invasiveness.

Using FCM, we therefore addressed the following questions: (1) What is the level of ploidy variation in both primary and secondary ranges of *L. salicaria* distribution? (2) What is the cytotype structure within both native and invasive *L. salicaria* populations? (3) Might the switch in ploidy level trigger invasive spread in secondary area of distribution? (4) Is there a difference in genome size between native and invasive populations?

MATERIALS AND METHODS

Plant material

A total of 124 populations of *L. salicaria* from both the primary and secondary range were included in the study (Table S1 in Supplementary Material). The 64 native populations originated from 12 European and two Middle Eastern countries, spanning 32°00′ N–61°52′ N and 06°22′ W–35°07′ E. The 60 invasive populations originated from 13 states of the USA and provinces of Canada, spanning 33°07′ N–53°00′ N and 123°03′ W–69°43′ W (see Fig. 2a,b). Generally, seeds from one to 15 plants per population were collected separately in the

field during 1998–2003 and stored in a refrigerator at 4°C. For 31 populations (seven and 24 in primary and secondary areas, respectively), only pooled seed samples were available. In each sample, seeds of an unknown number of plants per population were mixed. The total number of plants was therefore at least 578 (357+ and 221+ from primary and secondary areas, respectively). The seeds were sown into plastic pots filled with garden soil in early spring 2004 (seeds of each plant in a separate pot, except for the pooled samples) and grown in a glasshouse. During summer, young and healthy progenies were subjected to FCM analyses. Generally, three progenies per plant were analysed to avoid potential ploidy bias due to involvement of non-reduced gametes. More progenies were included in the case of ploidy-heterogeneous samples. The total number of progenies analysed was 1884 (1206 and 678 from primary and secondary areas, respectively).

DNA ploidy-level estimation

Flow cytometry was employed to estimate DNA ploidy levels (the prefix 'DNA' indicates that ploidies were mostly inferred from nuclear DNA content without knowledge of the exact chromosome numbers; Suda *et al.*, 2006). The species studied is, however, rich in secondary metabolites such as polyphenolic compounds (Rauha *et al.*, 2001), which interfere with DNA staining and make FCM analyses difficult. Moreover, the yield of nuclei was very low using the standard protocol due to mucilaginous compounds that trapped the nuclei (the homogenized tissue suspension was extremely viscous). Therefore various plant tissues (leaf laminas of various ages, different parts of stem, inflorescence axis, calyx), buffers (LB01, Otto, Tris·$MgCl_2$), and methodology modifications (addition of chemicals counteracting the negative effects of secondary metabolites, centrifugation, length of staining, etc.) were tested in the first step. A modified two-step Otto procedure (Otto, 1990) with nuclei isolated from young stems yielded the best histograms and was therefore used in all subsequent runs.

Young stem(s) of *L. salicaria* plant(s) analysed, and an appropriate volume of leaf tissue of the internal reference standard (*Zea mays* cv. CE-777, $2C = 5.43$ pg; Lysák & Doležel, 1998) were chopped together using a new razor blade in a Petri dish containing 0.6 mL ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) supplemented with PVP-40 (10 mg mL^{-1}). The sample was filtered through a nylon mesh (42 μm) and incubated at room temperature for 15 min. The staining solution consisted of 1 mL Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) supplemented with an AT-selective fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) at final concentration 4 μg mL^{-1} and an antioxidative compound 2-mercaptoethanol (2 μL mL^{-1}). After incubation for 5 min at room temperature, relative fluorescence intensity of at least 5000 particles was recorded using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with an HBO mercury arc lamp. The instrument was adjusted so that the peak of G0/G1 nuclei of the internal standard was localized on channel 350. The modified methodology yielded *L. salicaria* peaks with coefficients of variation (CV) between 2.5% and 5.0% (CV of the internal standard did not exceed 3.5%). Karyologically checked *L. salicaria* plants with diploid ($2n = 2x = 30$) and tetraploid ($2n = 4x = 60$) numbers of chromosomes from Israel (population IL5) and Hungary (population H1), respectively, were used as reference material to determine fluorescence ratios against the internal standard.

Up to three progenies from three plants per population were analysed together in order to obtain the first insight into ploidy level variation. Our previous experiments on *L. salicaria* showed that the adapted methodology facilitated reliable detection of minority cytotypes present in low proportion (*c.* 10%). Moreover, the lack of endopolyploidy and virtually no mitotic activity of selected stem tissues guaranteed unbiased DNA ploidy level estimates. Nevertheless, each plant was reanalysed separately if mixed samples were suspected, the peaks were asymmetrical, or the CV of the *L. salicaria* peak exceeded the 5% threshold (this threshold was set on the basis of previous single-plant analyses that gave CVs mostly between 3% and 5%).

Chromosome counts

Chromosomes were counted in actively growing root tips of seedlings. Samples were pretreated with a saturated solution of p-dichlorbenzene (3 h, room temperature), fixed in a 3 : 1 mixture of ethanol and acetic acid (4 h, 4°C), macerated in 1 : 1 hydrochloric acid : ethanol (30 s, room temperature) and immediately squashed in a drop of lactopropionic orceine. The number of chromosomes was determined using a Carl-Zeiss Jena NU microscope (Jena, Germany) equipped with Olympus Camedia C-2000 Z camera (Tokyo, Japan).

Statistical analyses

Data were analysed with the SAS 8.1 statistical package using NPAR1WAY and CORR procedures (SAS Institute, Cary, NC, USA). Differences in relative fluorescence intensity between native and invasive populations were analysed by the Kruskal–Wallis test. Spearman' rank correlation coefficient was used to test whether mean fluorescence intensity is related to the position of a population along a geographical gradient.

RESULTS

Large cytotype variation was found among 1884 progenies of *L. salicaria* raised from 578+ plants sampled at 124 localities across both primary and secondary areas of distribution (Table S1). Along with previously known diploid and tetraploid plants, DNA hexaploids were revealed for the first time, and DNA triploids were reliably confirmed (Fig. 1). Nevertheless, there was a marked disproportion among frequencies of particular cytotypes, with DNA tetraploids amounting to 86.2% of all progenies. Other ploidies were much rarer (diploids *c.* 4.5%, triploids *c.* 0.9% and hexaploids *c.* 8.4%) and were restricted to the native range.

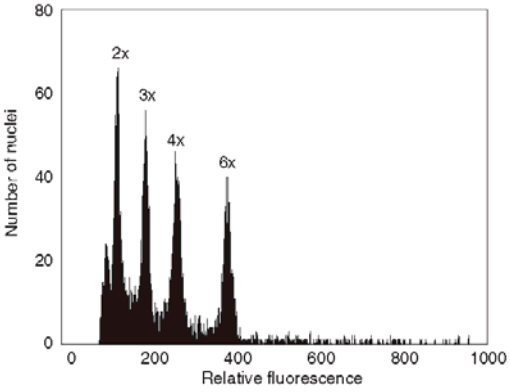

Figure 1 Simultaneous FCM analysis of DAPI-stained nuclei isolated from DNA diploid (population IL3, progeny of plant 1), DNA triploid (H4-3), DNA tetraploid (E1-1) and DNA hexaploid (TR3-1) plants of *Lythrum salicaria*. Actual peak ratios are 1.00 : 1.50 : 2.03 : 2.94.

Within the primary area studied, DNA tetraploids were recorded in all European and Middle Eastern countries except Israel (946 progenies originating from 279+ plants, 50 populations; Table 1; Fig. 2a). Populations from Israel consisted solely of DNA diploids (84 progenies originating from 28 plants, 10 populations). DNA hexaploids occurred only in Turkey (158 progenies originating from 49 plants, six populations) and rare DNA triploids were encountered in one population from Hungary (18 progenies of 1 plant).

Although the majority of populations comprised a single cytotype, progenies of two different ploidy levels within the same population, or even within the same plant, were also detected. DNA triploids and DNA tetraploids occurred in population H4 near Gödöllo, Hungary, and both DNA tetraploid and DNA hexaploid progenies were raised from seeds collected in populations near Antalya (TR5) and Kutahya (TR6) in Turkey.

In contrast, cytotype uniformity was a typical feature of invasive North American populations from 13 states of the USA and provinces of Canada (Table 1; Fig. 2b). Only DNA tetraploids were observed among 678 progenies of 221+ plants collected at 60 localities.

Mean relative fluorescence intensities ± SD for individual cytotypes (setting *Zea mays* as unit value) were as follows: DNA diploids 0.389 ± 0.002 (range 0.383–0.390); DNA triploids 0.581 ± 0.000; DNA tetraploids 0.778 ± 0.013 (range 0.751–0.808); DNA hexaploids 1.139 ± 0.008 (range 1.129–1.156). Fluorescence ratios among the four cytotypes thus averaged 1.00 : 1.49 : 2.00 : 2.93.

Native and invasive tetraploids showed highly comparable mean fluorescent intensities (± SD), amounting to 0.780 ± 0.013 (range 0.754–0.808) and 0.776 ± 0.012 (range 0.751–0.804), respectively. However, the differences were only marginally non-significant using the Kruskal–Wallis test ($P = 0.058$), and slightly lower values were observed in North American plants (Fig. 3).

The relationship between geographical location of tetraploid populations and their mean fluorescence intensity was also tested. The relative fluorescence was not correlated with latitude either in native ($r = -0.142$, $P = 0.327$, $n = 50$) or invasive populations ($r = 0.147$, $P = 0.262$, $n = 60$). On the contrary, nuclei fluorescence intensity in native populations decreased significantly in a west–east direction ($r = -0.769$, $P < 0.0001$, $n = 50$). Invasive North American populations also followed this trend; nevertheless, the west–east fluorescence decrease was less conspicuous and was marginally non-significant ($r = -0.248$, $P = 0.056$, $n = 60$).

DNA ploidy levels inferred from FCM data were confirmed using conventional chromosome counting. Number of chromosomes was successfully determined in one diploid (IL1), one triploid (H4; Fig. 4), three tetraploid (G2, GB1, H4) and two hexaploid (TR2, TR9) populations.

DISCUSSION

DNA ploidy data were obtained for *L. salicaria* plants from substantial parts of both primary and secondary ranges of distribution. Native to Eurasia, the species grows in almost all of Europe except Iceland, the Faeroe Islands, the northernmost parts of Scotland and Scandinavia, the Balearic Islands, Crete, and high mountains (Wangerin & Schröter, 1937). It also extends into some north-west African countries (Morocco, Algeria, Tunis). According to Hultén & Fries (1986), its continuous distribution in Europe spreads between *c.* 36 and 65°N (with isolated occurrence as far as 69°30′ N in Norway). Our European sampling sites were situated between *c.* 37 and nearly 62°N, thus covering a great portion of the south–north gradient. Similarly, the west–east European gradient was also covered representatively, with the westernmost samples originating from Spain (06°22′ W) and the easternmost one from Finland (28°55′ E). The native area of distribution continues through the Middle East, Russia, and China to Japan (*c.* 140°E). However, only limited material from the Asian part of the area of distribution was available (10 localities in both Israel and Turkey). Although these Middle East samples extended our investigated native area to 32°N and *c.* 35°E, more collections from the eastern portion of the distribution range are required for unbiased insight into ploidy variation across the whole of Eurasia. Nevertheless, published chromosome data show that diploids are clearly the dominant, if not the sole, cytotype in the eastern part of the distribution area. Our targeted search for ploidy variation of *L. salicaria* in Asian countries not represented in our material revealed only diploid individuals in Japan (Hokkaido and Honshu: Hara, 1956; Nishikawa, 1989), Primorye Territory of Russia (Sokolovskaya & Probatova, 1985), and the Caucasus (Davlianidze, 1984). Therefore it seems highly unlikely that the invasive North American plants could have originated from eastern Asia.

During the past two centuries, purple loosestrife has been introduced into the North and South Americas, Australia, New

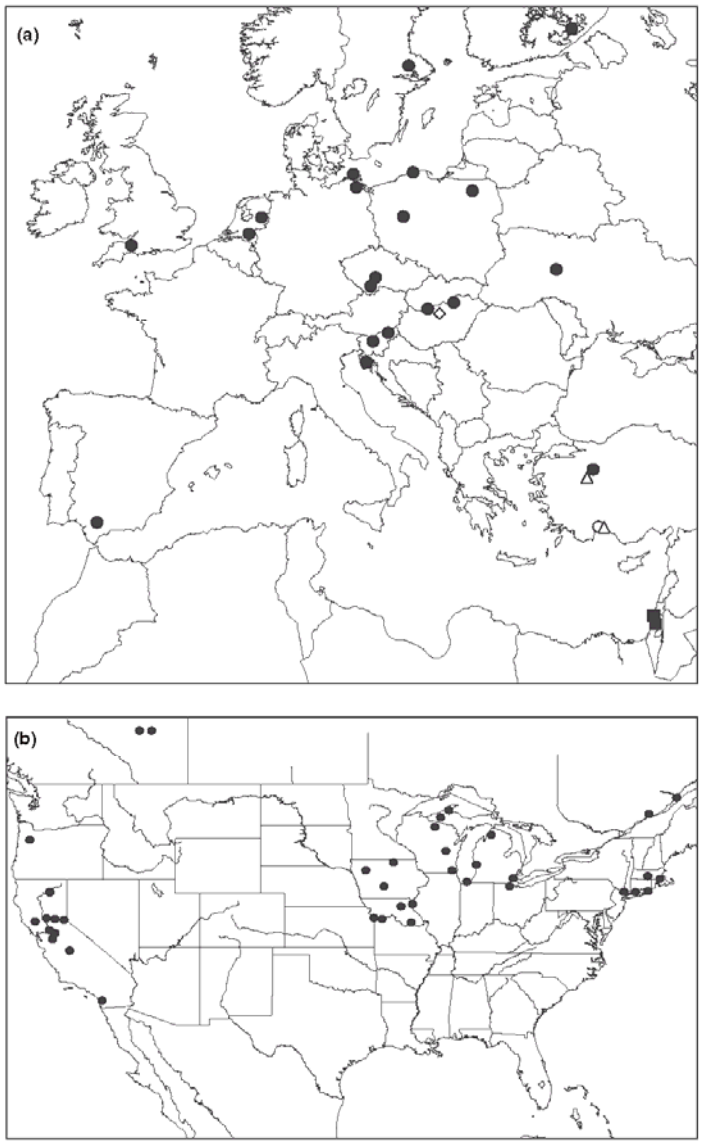

Figure 2 Distribution map of DNA ploidy levels of *Lythrum salicaria* in the area studied. (a) Native range covering 12 European and two Middle Eastern countries (64 populations); (b) secondary range covering 13 states of the USA and provinces of Canada (60 populations). ■, DNA diploids; ●, DNA tetraploids; ○, DNA hexaploids; ◇, mixed 3*x* + 4*x* populations; △, mixed 4*x* + 6*x* populations. The total number of depicted populations is reduced for map clarity (only one symbol is presented for two or more overlapping populations with the same DNA ploidy level).

Zealand, Tasmania and South Africa (Wangerin & Schröter, 1937; Thompson *et al.*, 1987; D. Richardson, personal communication). Its rapid spread is documented most thoroughly in the USA and Canada (Stuckey, 1980), where also our attention was directed. The map of contemporary distribution (Welk, 2004) shows that the species occupies a territory between *c.* 30–53° N and 130–65° W. Our sampling area (33°07′–53°00′ N, 123°03′–69°43′ W) therefore covered nearly the whole of the species' secondary range in North America.

To infer cytotype variation of purple loosestrife, we used primarily FCM instead of laborious karyological treatment. Chromosome counts were obtained for eight populations (including one ploidy-mixed) covering all DNA ploidy levels detected.

There was a marked difference in DNA ploidy-level variation between native and invasive populations. While four cytotypes (2*x*, 3*x*, 4*x*, 6*x*) occurred in Eurasia, we found only DNA tetraploids in North America. This suggests that either the tetraploids were the only ploidy introduced to North America, or they have become the only successful invader in the secondary area (provided more ploidies had been introduced). Nevertheless, even within the primary area studied,

Table 1 Condensed list of analysed *Lythrum salicaria* populations, with DNA ploidy levels and geographical origin (countries with native occurrence of *L. salicaria* are in bold)

Country	Total no. population(s)	Total no. plant(s)	Total no. progenies analysed	DNA ploidy level(s)	Latitudinal range	Longitudinal range
Czech Republic	8	> 42	126	4*x*	48°59′ 49°25′ N	14°21′ 14°50′ E
Finland	1	> 1	3	4*x*	61°52′ N	28°55′ E
Germany	2	2	6	4*x*	53°55′ 54°35′ N	13°09′ 13°20′ E
Hungary	4	19	119	4*x* (3 pop.) 3*x* + 4*x* (1 pop.)	47°36′ 48°10′ N	19°22′ 20°23′ E
Israel	10	28	84	2*x*	32°00′ 32°30′ N	34°46′ 35°07′ E
The Netherlands	2	11	33	4*x*	51°36′ 52°25′ N	05°36′ 06°30′ E
Poland	7	54	162	4*x*	52°28′ 54°40′ N	16°45′ 21°45′ E
Slovakia	2	22	66	4*x*	47°51′ 47°52′ N	18°28′ 18°31′ E
Slovenia	6	> 23	73	4*x*	45°07′ 46°36′ N	14°04′ 15°40′ E
Spain	5	22	66	4*x*	36°57′ 37°05′ N	05°23′ 06°22′ W
Sweden	5	25	81	4*x*	60°02′ 60°12′ N	17°08′ 17°54′ E
Turkey	10	99	316	4*x* (4 pop.) 6*x* (4 pop.) 4*x* + 6*x* (2 pop.)	36°51′ 39°54′ N	29°59′ 31°15′ E
Ukraine	1	5	15	4*x*	49°49′ N	27°48′ E
UK	1	4	56	4*x*	51°02′ N	02°54′ W
Alberta	2	29	87	4*x*	53°00′ N	113°00′ 114°00′ W
California	14	> 45	135	4*x*	33°07′ 41°06′ N	117°05′ 122°37′ W
Connecticut	2	8	24	4*x*	41°08′ 41°75′ N	72°05′ 72°07′ W
Indiana	15	> 15	45	4*x*	41°50′ N	87°00′ W
Iowa	3	15	45	4*x*	41°30′ 43°15′ N	93°05′ 95°20′ W
Massachusetts	1	5	30	4*x*	42°03′ N	71°05′ W
Michigan	5	23	69	4*x*	42°05′ 47°05′ N	83°12′ 89°10′ W
Missouri	7	24	72	4*x*	38°50′ 40°10′ N	91°30′ 94°40′ W
New York	3	12	36	4*x*	41°04′ 41°05′ N	73°07′ 74°05′ W
Ohio	1	5	15	4*x*	41°30′ N	83°30′ W
Oregon	1	> 1	3	4*x*	44°53′ N	123°03′ W
Quebec	3	24	72	4*x*	46°48′ 48°00′ N	69°43′ 72°00′ W
Wisconsin	3	15	45	4*x*	42°40′ 45°50′ N	88°15′ 89°40′ W

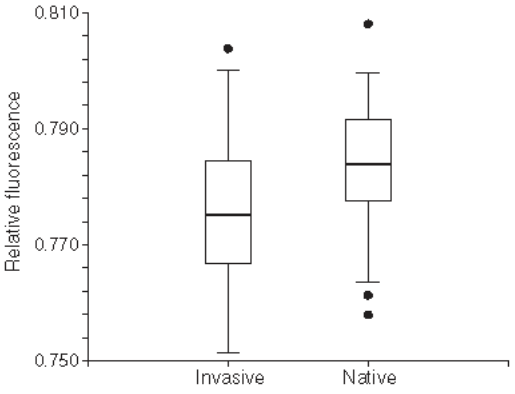

Figure 3 Box-and-whisker plots of relative fluorescence intensities (setting *Zea mays* as unit value) of DNA tetraploid plants originating from both native and invasive populations.

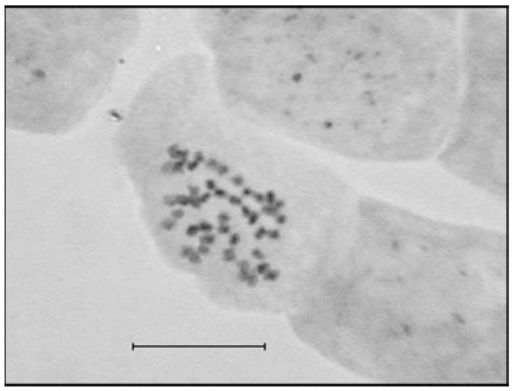

Figure 4 Mitotic metaphase chromosomes of triploid *Lythrum salicaria* from population H4 (Hungary, near Gödöllo) showing 45 somatic chromosomes. Bar = 10 μm.

much of the ploidy diversity was confined to the Middle Eastern countries (Israel: 2*x*, Turkey: 4*x* + 6*x*), whereas their European counterparts were nearly cytotype-uniform. Except for a rare DNA triploid plant encountered in one population from Hungary (near Gödöllo), solely DNA tetraploids were detected in other European populations.

Progenies of one plant from the population near Gödöllo in northern Hungary were consistently estimated as DNA triploids (see also Fig. 4). Due to the uniformity of the progeny's ploidy level, we assume that triploid number of chromosomes already occurred in the parental individual. A corresponding chromosome number ($2n = 45$) was previously reported by Morrison (1963) in European plants of unknown provenance. However, this count has remained largely neglected and even the author considered it as merely aneuploid. Our Hungarian population consisted of DNA triploid and prevailing DNA tetraploid plants (progenies). Both cytotypes were visually indistinguishable from each other, and no differences in seed viability, germination rate, seedling survival and morphological features have been observed (data not shown; confirmed also in experiments of Bastlová *et al.*, 2004). The triploids may have originated either by hybridization between $2x$ and $4x$ plants or via a fusion of unreduced and reduced gametes of a diploid ancestor. However, no diploid *L. salicaria* plant has been detected in Europe, either in our samples or reported previously. The nearest diploid population was confirmed in Israel, *c.* 2100 km away. Nevertheless, we cannot rule out that the minority diploid cytotype still occurs sporadically in Central Europe (or occurred in the past). A detailed cytotype survey, particularly in northern Hungary, is therefore desirable.

Our pattern of *L. salicaria* DNA ploidy distribution corresponds well with published cytogeographical data obtained by a conventional karyological approach. Tetraploids were reported previously from both Europe and North America (Mal *et al.*, 1992), while diploids were reported from Israel (Goldblatt & Johnson, 2003). Although we performed only a few chromosome counts, no support for plants with either 50 or 107 somatic chromosomes (Morrison, 1963; Mal *et al.*, 1992) has been gained from FCM data. Relative fluorescence intensities of all plants assigned among DNA tetraploids corresponded well with those of karyologically confirmed material with $2n = 4x = 60$, and no plant had a fluorescence value exceeding 1.5-fold of that of the tetraploid standard (higher than DNA hexaploids). As other authors have also doubted these old chromosome numbers (Buttler, 1984), they should be discarded until unambiguous confirmation is obtained.

DNA hexaploids were revealed by us for the first time. They occurred, either in pure or mixed populations with DNA tetraploids, in south-western and north-western Turkey. Most plausibly, non-reduced gametes of tetraploid plants were involved in their origin. First, both tetraploid and hexaploid plants were found among progenies of four plants originating from two Turkish populations. Moreover, a related species, *L. alatum*, produced on average 0.35% of unreduced pollen grains (Houghton-Thompson *et al.*, 2005). We can therefore expect that gametes with a somatic number of chromosomes may also occur in *L. salicaria*. Hexaploid cytotypes may possibly hybridize with nearby tetraploids (particularly in mixed populations), giving rise to pentaploid progenies (or other ploidies in the case of unreduced gametes). Although DNA pentaploids have not been confirmed in our study, comprehensive cytotype screening in areas with sympatric growth of $4x$ and $6x$ plants may prove their existence (e.g. Suda & Lysák, 2001). Generally, the Asian part of the *L. salicaria* range deserves further attention to elucidate questions concerning overall ploidy variation, geographical limits of particular cytotypes, extent of cytotype overlap, and width of ploidy-mixed zones.

Previous studies revealed considerable variation in phenology, morphology and growth characteristics in *L. salicaria* populations originating from different latitudes and longitudes in Europe and the Middle East (Olsson & Ågren, 2002; Bastlová & Květ, 2003; Bastlová *et al.*, 2004). We used identical seed sets (collected from the same plants) as in the latter two articles, therefore relationships between phenotypic performance and ploidy level could have been tested reliably. Quite surprisingly, ploidy had little effect on growth characteristics. We can therefore conclude that large phenotypic variation in *L. salicaria* in its native area is not triggered by genome duplication, but may rather be attributed to adaptations to local environmental conditions at the gene level.

Examination of ploidy differences between primary and secondary areas of distribution has recently attracted increased attention from plant scientists, driven by attempts to elucidate potential determinants of invasiveness. One of the earliest papers to explore this issue using flow cytometry was Amsellem *et al.* (2001), although the authors did not find ploidy divergence in *Rubus alceifolius* between its native Asian range and the Indian Ocean islands, where it had been introduced. More similar to our *L. salicaria* results is the ploidy distribution in *Senecio inaequidens* (Lafuma *et al.*, 2003). Indigenous to South Africa, the species encompasses both diploid and tetraploid cytotypes (with two genome size variants) across its primary distribution range, whereas solely tetraploids seem to spread in Europe. Ploidy level might also have played an important role in the expansion of *Phragmites australis* in north-eastern America where, based on the size of guard cells, a recent shift from hexaploids to tetraploids was suggested (Chambers *et al.*, 1999). Yet a different history has been documented in the originally eastern Asian genus *Reynoutria* (Mandák *et al.*, 2003). Introduction to Europe was followed by rapid genesis of novel cytotypes not present in the primary area of distribution.

As the same *L. salicaria* cytotype occurs in both Europe and North America, ploidy level itself has most probably not triggered invasive species behaviour in the secondary area. More likely, crossing among divergent genotypes from multiple introductions caused gene reassortment and induced the adaptive switch in North American populations (Houghton-Thompson *et al.*, 2005). In addition, some *L. salicaria* genotypes may have been pre-adapted to successful colonization of new habitats (Olsson & Ågren, 2002; Bastlová *et al.*, 2004). Houghton-Thompson *et al.* (2005) also tested whether hybridization between *L. salicaria* ($2n = 60$) and *L. alatum* ($2n = 20$) may explain invasive behaviour of North American plants, but found little support for the hypothesis using molecular

markers. Further evidence against hybridization comes from our FCM data, as fluorescence intensities corresponding to potential crosses have not been detected.

Only DAPI-staining (A-T-specific DNA fluorochrome) yielded acceptable *L. salicaria* histograms despite manifold methodology modifications. Attempts to reliably determine the genome size in absolute units (DNA picograms) using intercalating propidium iodide have been in vain and gave histograms with high CVs (mostly between 6% and 8%) on a prominent background. Therefore only relative fluorescence values are presented. In all but hexaploid cytotypes, fluorescence intensities increased proportionally with ploidy level. DNA hexaploids showed about 2.4% lower fluorescence intensity than expected, indicating that genome downsizing followed this high-polyploid formation (Leitch & Bennett, 2004).

Nuclear DNA amount (genome size) has long been known to affect several phenotypic and phenological characters at subcellular, cellular, tissue and organism levels, such as cell volume, mitotic and meiotic cycle duration, minimum generation time in herbaceous plants, and sensitivity to frost (the so-called nucleotypic effect; Bennett *et al.*, 2000). For example, maize races having smaller genomes (and thus more rapid development) were better adapted to growth in northern regions (Laurie & Bennett, 1985). However, no correlation between latitude and relative fluorescence intensity (=DNA amount) has been observed in purple loosestrife, despite its remarkable variability in growth characteristics along the north–south geographical gradient (Bastlová *et al.*, 2004). On the other hand, fluorescence intensity of DNA tetraploids seemed to be negatively correlated with population location along the west–east gradient. The total difference reached about 7.1%. The highest mean fluorescence was observed in population(s) from the UK, followed by Slovenia, the Netherlands and Spain, while populations from Turkey, Finland and Ukraine showed, on average, the smallest fluorescence intensity. It is possible that aneuploid chromosome numbers may elucidate a part of the variation, as one to two accessory chromosomes have been reported in plant material from the British Isles (Montgomery *et al.*, 1997), and hypo-aneuploid plants with 58–59 somatic chromosomes may also exist (Mal *et al.*, 1992). Potentially, relative DNA content may mirror a gradient in climatic conditions from oceanic to more continental zones. A correlation between genome size and environmental variables at the infrageneric level has previously been found, for example, in the genus *Berberis* (Bottini *et al.*, 2000). Nevertheless, we cannot rule out an alternative explanation that differences in relative fluorescence intensity were merely associated with dissimilar levels of secondary metabolites that may have interfered with DNA fluorochromes. Plant material frequently contains endogenous staining inhibitors, often of a phenolic nature, which can strongly affect the measurements (Greilhuber, 2005). As a result, several examples of an apparent variation in nuclear DNA content have turned out to be artefactual. Because purple loosestrife belongs among recalcitrant plant species, we are cautious to draw any decisive conclusion concerning genome size variation until proved independently by other techniques.

Rejmánek (1996) was the first to formulate the theory that invasive spread may be correlated with differences in genome size. Some support for such an effect of nucleotype comes from weedy plants; the probability of being recognized as a weed species was negatively correlated with nuclear DNA amount per genome (Bennett *et al.*, 1998). Interestingly, invasive *L. salicaria* plants seemed to have a slightly lower relative fluorescence intensity (genome size) than their native counterparts (Fig. 3). However, this difference was marginally non-significant and amounted to less than 0.5% of total DNA volume. It is therefore unlikely that such a negligible change in nuclear DNA amount may have led to a dramatic adaptive shift. Moreover, the potentially disturbing effect of secondary metabolites (see above) must also be considered.

In conclusion, our results showed that several *L. salicaria* cytotypes (2*x*, 3*x*, 4*x*, 6*x*) occur in the native area of distribution, with much variation concentrated in the Middle Eastern countries, while only DNA tetraploids seemed to occur in North America. Single-cytotype populations largely prevailed; nevertheless, sympatric growth of 3*x* + 4*x* and 4*x* + 6*x* DNA ploidies was also revealed. There was little correlation between phenotypic variation and DNA ploidy level. Therefore the invasive behaviour of American populations should not be attributed to differences in genome copies, but rather to adaptation to new environment at the gene level. Additionally, we do not consider the apparent difference in genome size an underlying factor of the massive spread of purple loosestrife, despite a certain shift towards lower DNA amounts in North American invasive populations.

ACKNOWLEDGEMENTS

The research was supported by project B6141403 of the Grant Agency of the Academy of Sciences, and partly by the Ministry of Education of the Czech Republic (projects MSM 6007665801, MSM 0021620828 and MSM 60076658-06) and the Academy of Sciences of the Czech Republic (projects AV0Z6005908, AVOZ60050516 and KSK6005114). Markéta Foldynová helped us with sample preparation for FCM analyses. We are also very grateful to all who kindly provided some seeds of *L. salicaria* from both Europe and North America: M. Agami, B. Andersson, N. A. Bingol, R. Burda, K. Edwards, A. Engloner, K. Gilman, J. Květ, J. Królikovska, J. Merilainen, A. Rashid, A. Seliškar, O. Urbanc-Berčič, T. Zoltán and M. Zunzunegui.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

Table S1 Data on localities included in the analyses

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2699.2007.01781.x

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Editor: Peter Linder