Folia Geobotanica 40: 367–384, 2005

GENOME SIZE DISCRIMINATES BETWEEN CLOSELY RELATED TAXA *ELYTRIGIA REPENS* AND *E. INTERMEDIA* (*POACEAE*: *TRITICEAE*) AND THEIR HYBRID

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Abstract: Flow cytometric and karyological investigations were performed on the closely related taxa *Elytrigia repens* and *E. intermedia* (*Poaceae*: *Triticeae*) from the Czech Republic. DNA-hexaploids clearly prevailed among 238 examined plants and amounted to 96.2% of all samples. 2C-values ± s.d. for hexaploid *Elytrigia repens* and *E. intermedia* were estimated at 23.27 ± 0.20 pg and 27.04 ± 0.24 pg respectively. Genome size thus allowed reliable separation of the two species (difference ca. 16%) as well as the identification of hybrid individuals. Natural hybridization in *E. repens* – *E. intermedia* alliance seems to be quite a common phenomenon as indicated from a large proportion (one sixth) of hexaploid samples with intermediate 2C-values. Previously, the crosses were most probably overlooked or misidentified due to their weak morphological differentiation. New nonaploid cytotypes (2n=9x=63) were revealed for both species as well as for the hybrid (determined on the basis of morphological characters only), representing the first records from the field. Fusion of unreduced and reduced gametes of the hexaploids is the most plausible mode of nonaploid origin.

Keywords: C-value, Chromosome number, Cytotype, DNA content, Flow cytometry, Hybridization, Nonaploid, Wheatgrass

Nomenclature: LÖVE 1984

INTRODUCTION

Elytrigia repens (L.) NEVSKI [Syn.: *Agropyron repens* (L.) P. BEAUV., *Elymus repens* (L.) GOULD] and *Elytrigia intermedia* (HOST) NEVSKI [Syn.: *Agropyron intermedium* (HOST) P. BEAUV., *Thinopyrum intermedium* (HOST) BARKWORTH et D.R. DEWEY] are representatives of the family *Poaceae*, tribe *Triticeae* (wheatgrasses). *Triticeae* is a large group comprising approximately 500 taxa divided into 37 genera (LÖVE 1984). The relationships among, and the taxonomy and phylogeny of members of *Triticeae* have triggered a long-term dispute and are still in need of further targeted investigation. Among others, the complexity of the situation is caused by frequent allopolyploid origin of the species that partly share the same genomes (up to dodecaploid plants are known, each combining up to four more-or-less different genomes). Reticulate evolution manifesting itself in the majority of characters is another source of problems. The complexity of the group can easily be distinguished from the taxonomy of the two taxa studied: 137 and 132 synonyms were found for *E. intermedia* and *E. repens*, respectively (CLAYTON & WILLIAMSON 2003). Both

species are rhizomatous perennial grasses that are considered as out-crossing, wind pollinated, and reproduce by seeds and rhizomes (on a local scale).

Elytrigia repens is a native Eurasian species that has become established in most temperate zones of the world. It is one of the most troublesome weeds on cultivated land. In the Czech Republic, the plant is widespread throughout the whole territory from lowlands to the mountain belt, occasionally surviving even above the timberline. It occupies all man-made habitats and arable ground, but occurs also on such natural habitats as steppes, forest margins and tracks. The genome constitution of hexaploid cytotypes (2n=6x=42) was determined as StStH (where St and H designate *Pseudoroegneria* (NEVSKI) Á. LÖVE and *Hordeum* L. genomes respectively) (ASSADI & RUNEMARK 1995). Nevertheless, a more complex genome pattern seems to be plausible. Recent molecular phylogenetic study (MASON-GAMER 2004) revealed at least five distinct lineages, suggesting that allopolyploidy and introgression took place during the evolution of *E. repens*. Chloroplast DNA data identified three potential maternal genome donors (*Pseudoroegneria*, *Dasypyrum* (COSS. et DURIEU) T. DURAND and *Thinopyrum* Á. LÖVE), whilst nuclear DNA data confirmed the previously suggested *Pseudoroegneria* and *Hordeum* as genome contributors of hexaploid plants and, unexpectedly, three additional genome donors were identified: *Taeniatherum* NEVSKI and two donors of unknown identity.

Elytrigia intermedia occurs from France in the west to the Volga river region in the east, with further distribution forming a bend from Turkey and the Caucasus to Iran, Afghanistan, Pakistan, the Pamir Mts. and Altai Mts. in Central Asia (HEGI 1977). The species has also been introduced to North America. In the Czech Republic, its distribution strongly reflects the occurrence of steppe habitats (see Fig. 1). The species colonizes steppes, pine forests on sandy ground, vineyards, orchards and field margins in warm regions. Genome constitution was determined as E^eE^eSt (LIU & WANG 1993) or E^eE^bSt (CHEN et al. 1998), where E^e and E^b designate the closely related *Thinopyrum elongatum* (HOST) D.R. DEWEY and *Th. bessarabicum* (SAVUL. et RAYSS) Á. LÖVE genomes.

The principal morphological characters that distinguish between the cited *Elytrigia* species are as follows: (1) leaf sheath margins – hairy in *E. intermedia* vs. glabrous in *E. repens* (KUBÁT et al. 2002), and (2) glume shape – truncate or very shortly mucronate (never awn-tipped or gradually tapering) in *E. intermedia* vs. awn-tipped or gradually tapering (at least some of each inflorescence) in *E. repens* (BARKWORTH & DEWEY 1985). Nevertheless, many plants from the field combine both features, suggesting that hybridization might have occurred. Hybrid individuals were originally described as *Agropyron* ×*mucronatum* OPIZ (BERCHTOLD & OPIZ 1836) (syn. *Elytrigia mucronata* (OPIZ) PROKUDIN), however, their identification on the basis of morphological characters is uncertain due to large morphological variation of the putative parental species and frequent overlap of character values.

A survey of published karyological data has revealed considerable variation in ploidy levels (based on x=7) for both species (Table 1). Hexaploid cytotype (2n=42) prevails in *E. repens*, however tetraploid (2n=28) and octoploid (2n=56) individuals were also collected in the field (SAKAMOTO & MURAMATSU 1963). PETO (1930) reported 34 and 35 chromosomes for two individuals from Russia and considered these plants hybrids between hexaploid *E. repens* and some species with a lower chromosome set. In addition, one

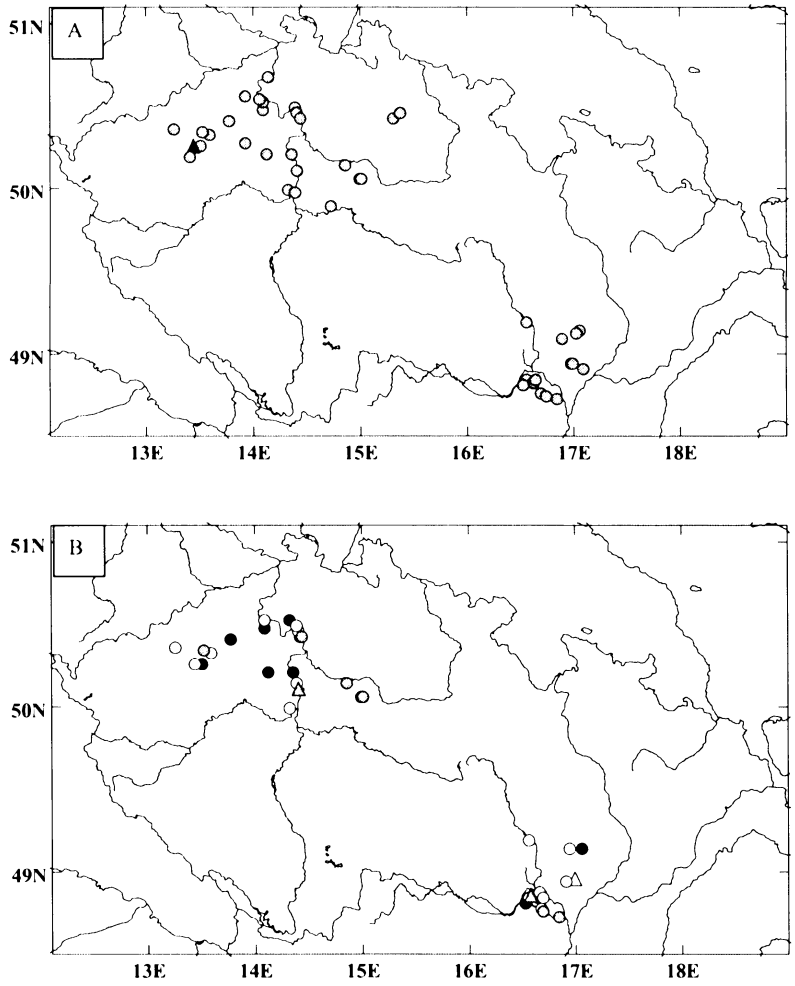

Fig. 1. A Distribution of *Elytrigia repens* cytotypes in the area studied. Shaded dots designate localities of hexaploid plants, the black triangle designates the locality of a nonaploid individual (2n=9x=63). B – Distribution of *E. intermedia* cytotypes and putative hybrids. Open circles designate hexaploid *E. intermedia*, shaded circles designate hexaploid hybrids, black circles designate localities with the co-occurrence of both types. Triangles designate nonaploid plants (open *E. intermedia*, shaded hybrids). The Slovakian locality (no. 18) is omitted.

polyhaploid (2n=21) and one nonaploid (2n=63) plant were detected by DEWEY (1974) in the population of twin seedlings during the experimental germination study. An analogous situation was encountered in *E. intermedia*, where predominant hexaploid and minority tetraploid (BOWDEN 1965) cytotypes occurred. Along with the euploid plants, several aneuploids were detected for both species: 2n=40 for *E. repens* (PETROVA 1975), and 2n=41 and 43 for *E. intermedia* (HARTUNG 1946, BOWDEN 1965, ASSADI 1995). DNA 2C-values of the hexaploid individuals were estimated at 25.96 pg for *E. repens* (BENNETT et al. 1982), and

Table 1. List of published chromosome counts for *Elytrigia repens* and *E. intermedia*. The origin of the analyzed material is provided in brackets (where possible). Superscript numbers refer to the original plant names used in the article.

Elytrigia repens 2n	n	Reference
42		STOLZE 1925[1], MOWERY 1929 (USA-Minnesota)[1], PETO 1929 (Canada)[1], 1930 (Western Canada[1], Denmark[1], Russia-Caucasus Mts.[2]), SCHIEMANN 1929[1], SIMONET 1935[1], VAKAR 1935[1], ROHWEDER 1937[1], SOKOLOVSKAYA & STRELKOVA 1939 (Russia)[1], ÖSTERGREN 1940a[1], b (Sweden)[1], SHARMAN 1943[1], SENN et al. 1947 (Canada)[1], 1949[1], PÓLYA 1948 (Hungary)[1], BEAUDRY 1951 (USA-Wisconsin)[1], HUNZIKER 1954[1], LÖVE & LÖVE 1956 (Iceland)[1], CAUDERON 1958 (France)[1], GILLETT & SENN 1960[1], JONES 1960[1], DEWEY 1961 (USA-Utah)[1], 1970 (USA)[1], 1972[1], 1980 (Iran)[1], BOWDEN 1965 (Canada)[1], GADELLA & KLIPHUIS 1966 (The Netherlands)[1], FERNANDES & QUEIROS 1969 (Portugal)[1], HENEEN 1972[3], KOZUHAROV & PETROVA 1973 (Bulgaria)[1], LÖVE & KJELLQVIST 1973 (Spain)[3], KRUSE 1974[1], ROOS 1975 (Estonia)[5], FREY et al. 1977 (Poland)[1], JOHNSON & JALAL 1977[1], DRULEVA in PROKUDIN et al. 1977 (Ukraine)[5], PETROVA in PROKUDIN et al. 1977 (Ukraine)[5], MURÍN in MÁJOVSKÝ et al. 1978 (Slovakia)[1], POGAN et al. 1980 (Poland)[1], LÖVE 1980a[5],b (Sweden)[6], 1984[8], 1986[9], PROBATOVA & SOKOLOVSKAYA 1980 (Russia-Altai Mts.)[7], 1982 (Russia-Far East)[5], NAPIER & WALTON 1981 (Canada)[1], BELAYEVA & SIPLIVINSKI 1981 (Russia)[1], AROHONKA 1982 (Finland)[3], GUZIK 1984 (Western Russia)[5], LU et al. 1990 (China)[5], SUN et al. 1992 (China)[5], SALOMON & LU 1994 (China-Xinjiang, Tiansan, Balguntai)[3], DEMPSEY et al. 1994 (Great Britain)[7], ASSADI 1995 (Iran)[3], ASSADI & RUNEMARK 1995 (Iran)[5], GOUKASIAN & NAZAROVA 1998[5], LÖVKVIST & HULTGÅRD 1999 (Sweden)[5]
28, 42		AVDULOV 1931[1], ROZANOVA 1940 (Russia-Altai Mts.)[1], HEISER & WHITAKER 1948 (USA-California)[1], SOKOLOVSKAYA & STRELKOVA 1948 (Russia-Altai Mts.)[1], JONES 1957[1], PARFENOV & DMITRIEVA 1988 (Belarus)[5], MIZIANTY et al. 2001 Poland)[3]
28		SINGH 1964 (Great Britain-RBG Kew)[1], GUZIK & LEVKOVSKII 1979 (East Russia)[1]
56		SAKAMOTO & MURAMATSU 1963[1]
21, 42, 63		DEWEY 1974 (USA-Utah)[1]
42, 40		PETROVA 1975 (42 Ukraine, 40 Moldova)[5]
34, 35		PETO 1930 (Russia-Caucasus Mts.)[1]
	21	DEWEY 1967 (USA)[1]
	14, 21	DEVESA et al. 1990 (Spain)[4]
	21+1-2 B	GERVAIS et al. 1999[5]

(original names: *Agropyron repens* (L.) P. BEAUV.[1], *A. repens* var. *glaucescens* ENGL.[2], *Elymus repens* (L.) GOULD[3], *Elymus repens* (L.) GOULD subsp. *repens*[4], *Elytrigia repens* (L.) NEVSKI[5], *Elytrigia repens* subsp. *arenosa* (PETIF) Á. LÖVE[6], *Elytrigia repens* (L.) NEVSKI subsp. *repens*[7], *Elytrigia repens* subsp. *pseudocaesia* (PACZ.) TZVELEV[8], *Elytrigia repens* subsp. *lolioides* (KAR. et KIR.) Á. LÖVE[9]).

Elytrigia intermedia (Table 1. continued)

2n	n	Reference
42		PETO 1930[6], 1936[6], 1938[6], VAKAR 1934[6], 1935[6], 1936[6], PETO & BOYES 1940[6], SIMONET 1935[6], CHIZNYAK 1936[1], ARARATYAN 1938[6], 1939[6], JOHNSON 1938[6], KOSTOFF 1941[6], HARTUNG 1946 (Russia-Caucasus Mts.)[1], LITARDIÈRE 1948 (Corsica)[6], BELL 1950[6], THOMPSON & GRAFIUS 1950[8], PÓLYA 1950 (Hungary)[6], MATSUMURA 1951[6], 1952[6], MATSUMURA et al. 1958a[6], b[6], POPE & LÖVE 1952[2], SACHS 1952[6], BELL & SACHS 1953[6], GAUL 1953a[6], b[6], STEBBINS & PUN 1953 (Turkey)[1], CAUDERON 1954[6], 1958 (France)[6], 1962[1], MURAMATSU 1955[6], TATEOKA 1956[6], LÖVE & LÖVE 1961[6], SAKAMOTO & MURAMATSU 1963[1], SCHULZ-SCHAEFFER & JURA 1967 (Kazakhstan, Uzbekistan)[1], MURÍN in MÁJOVSKÝ et al. 1974 (Slovakia)[5], CHOPANOV & YURTSEV 1976 (Turkmenistan)[11], PETROVA in PROKUDIN et al. 1977 (Ukraine)[5], DRULEVA in PROKUDIN et al. 1977 (Ukraine)[11], PROBATOVA & SOKOLOVSKAYA 1978 (Russia-Caucasus Mts.)[8], LÖVE 1980 (Slovenia)[7], 1984[6,8,9,10], VÁCHOVÁ & FERÁKOVÁ 1980 (Slovakia)[11], NAPIER & WALTON 1981[3, 4], LÖVE & LÖVE 1982 (Italy)[5], PIAO 1982[1], POGAN et al. 1985 (Poland)[1], MIČIETA 1986 (Slovakia)[5], LIMIN & FOWLER 1988[16], LIU & WANG 1993 (Germany)[16], MUJEEB-KAZI et al. 1994[16], ASSADI & RUNEMARK 1995 (Iran)[16], PETROVA & STOYANOVA 1997 (Bulgaria)[12], MIZIANTY et al. 2001 (Poland)[12]
28, 42, 43		BOWDEN 1965 (Canada)[1]
41, 42, 43		ASSADI 1995 (Iran)[13, 14, 15]
43		HARTUNG 1946 (Turkey)[1]

(original names: *Agropyron intermedium* (HOST) P. BEAUV.[1], *A. trichophorum* (LINK) K. RICHT.[2], *A. trichophorum* (LINK) K. RICHT. cv. Greenleaf[3], cv. Chief[4], *Elytrigia intermedia* (HOST) NEVSKI[5], *E. intermedia* (HOST) NEVSKI subsp. *intermedia*[6], *E. intermedia* subsp. *barbulata* (SCHUR) Á. LÖVE[7], *E. intermedia* subsp. *trichophora* (LINK) Á. LÖVE et D. LÖVE[8], *E. intermedia* subsp. *pulcherrima* (GROSSH.) TZVELEV[9], *E. intermedia* subsp. *pouzolzii* (GODRON) Á. LÖVE[10], *E. trichophora* (LINK) NEVSKI[11], *Elymus hispidus* (OPIZ) MELDERIS[12], *E. hispidus* (OPIZ) MELDERIS var. *hispidus*[13], *E. hispidus* var. *podperae* (NÁBĚLEK) ASSADI[14], *E. hispidus var. villosus* (HACK.) ASSADI[15], *Thinopyrum intermedium* (HOST) BARKWORTH et D.R. DEWEY[16]).

26.25 pg and 25.92 pg for *Thinopyrum intermedium* (HOST) BARKWORTH et D.R. DEWEY subsp. *intermedium* (syn.: *E. intermedia* subsp. *intermedia*) and *Th. intermedium* subsp. *barbulatum* (SCHUR) BARKWORTH et D.R. DEWEY (syn.: *E. intermedia* subsp. *barbulata* (SCHUR) Á. LÖVE) (VOGEL et al. 1999), respectively.

The aim of this study was to examine ploidy variation in two *Elytrigia* species native to the Czech Republic, to find species-specific marker(s) that allow reliable taxa identification, and to state whether the interspecific hybridization occurs as preliminarily assessed on the basis of morphological analysis.

MATERIAL AND METHODS

Field sampling

Two hundred and thirty-eight plants collected at 55 localities were included in the study (see Appendix, Fig. 1). Sampling design was targeted to cover the majority of morphological

variation within each locality. Using the two above-mentioned morphological characters, the material was preliminarily assigned to three groups: *E. repens*, putative hybrids, and *E. intermedia*. All plants were transferred to the experimental garden of the Institute of Botany at Průhonice for further investigation. Vouchers are deposited in the herbarium of the Institute of Botany (PRA).

Chromosome counting

At the first step, chromosomes of the 14 most typical plants representing all three groups (4 *E. repens*, 3 hybrids, 7 *E. intermedia*) from 12 localities were counted (see Appendix). Root tips of the cultivated samples were pre-treated with a saturated solution of p-dichlorbenzene, fixed in a mixture of alcohol : acetic acid (3 : 1) and stained with lacto-propionic orceine. All the plants were found to be hexaploid (2n=6x=42) and served as reference material for cytometric analyses. Karyological investigation was subsequently also applied to the 9 *Elytrigia* samples with relative fluorescence of nuclei deviating from that of hexaploid standards.

Genome size estimation

Genome size (either in relative or absolute units) was determined by flow cytometry using Partec PA II instrument. Sample preparation followed the two-step procedure originally described by OTTO (1990). *Triticum aestivum* var. *lutescens* (ALEF.) MANSF. 'Bezostaya 1' was chosen as an appropriate primary internal standard (with close, but not overlapping genome size); its 2C-value was estimated as 34.4 pg using *Vicia faba* L. (2C=26.9 pg; DOLEŽEL et al. 1992) as an internal standard. Intact leaf tissues of the analyzed plant (ca. 0.5 cm^2) and the internal standard were co-chopped with sharp razor blade in a Petri dish containing 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The suspension was filtered through a 42-µm nylon mesh and centrifuged (150 g/5 min). The supernatant was removed and the pellet was resuspended in 100 µl of fresh ice-cold Otto I buffer. Samples were incubated for 10 min at room temperature, and stained with 1 ml of Otto II buffer (0.4 M Na_2HPO_4 . 12 H_2O) supplemented with ß-mercaptoethanol (2 µl/ml) and fluorochrome. DAPI at a concentration of 4 µl/ml was used for ploidy level estimation (relative genome size), propidium iodide + RNase IIA (both at a concentration of 50 µl/ml) were used to determine absolute genome size. After a few minutes, relative fluorescence intensity (setting *Triticum* as the unit value) of the isolated nuclei was recorded (mostly 5 000 particles). Coefficients of variation of the G0/G1 peak of the plants analyzed varied from 1.23% to 3.11% and from 1.51% to 4.58% for analyses with DAPI and propidium iodide respectively. All samples with relative fluorescence deviating from the range defined for karyologically-confirmed reference material were re-analyzed using corresponding hexaploid *Elytrigia* as internal standard and all of them were also subjected to chromosome counting. For absolute genome size estimation, three hexaploid plants of both *Elytrigia* species were carefully selected according to the following criteria: (1) unambiguous determination on the basis of morphological characters, (2) spatial isolation from its counterpart, (3) cpDNA relevant to particular species (MAHELKA et al., unpubl. results), (4) relative nuclear DNA content matching the range for a given species. Each plant was

re-estimated at least three times on different days to minimize potential random instrumental drift. The genome size terminology follows GREILHUBER et al. (2004). As the ploidy of the samples was mostly inferred from their nuclear DNA content, it should be regarded as the DNA-ploidy level (HIDDEMAN et al. 1984).

RESULTS AND DISCUSSION

DNA-ploidy estimation/analysis

Three distinct groups were obtained when the relative fluorescence of nuclei of the 14 karyologically-proved hexaploids using *Triticum aestivum* var. *lutescens* (2C=34.4 pg) as internal standard was measured. These groups agreed fully with the previous plant determination (*E. repens*, putative hybrids, *E. intermedia*) based on morphological characters. Subsequent cytometric analyses of 224 individuals lacking any chromosome count further confirmed this genome size differentiation. The majority of the material (215 plants) were also DNA-hexaploids. A marked prevalence of the hexaploid cytotype was expected and agreed with records in the literature from other countries (cf. Table 1). Relative fluorescence of nuclei for the whole hexaploid assemblage were as follows (min.–max. (mean)): 0.710–7.728 (0.718), 0.755–0.782 (0.770), and 0.805–0.828 (0.816), corresponding to *E. repens*, putative hybrids, and *E. intermedia* respectively. The average difference between the parental species (using DAPI staining with A-T bases preference) thus amounted to 13.7%, i.e., *E. intermedia* possessed 1.137-fold higher values. The hybrids were located more or less mid-way (Figs. 2, 3). Very narrow intraspecific genome size variation was always found: 2.5% for *E. repens*, 2.9% for *E. intermedia* and 3.6% for the hybrids. Any intermediate fluorescence intensity between putative hybrids and parental species was not observed, indicating the lack of back-crosses due to potential hybrid sterility. The level of fertility in our collection of hexaploid hybrids has not yet been determined. However, ASSADI & RUNEMARK (1995) reported irregular meiosis with only one homologous pair of genomes and 20% pollen fertility in artificial crosses between *E. repens* and *Thinopyrum intermedium*. They supposed that since hybrids are relatively common in the field and reproduce asexually by rhizomes, at least some gene flow via backcrossing to the parents may be expected.

Species-specific DNA values permit the use of genome size as a reliable marker for taxa and hybrid delimitation in any *E. repens* – *E. intermedia* alliance. These data are certainly more robust and unbiased than morphological variation, which forms more a continuum (morphological discrimination between putative hybrids and parental species is vague and affected negatively by subjective judgement). Generally, in some species, ploidy level is therefore the main classification criterion, as demonstrated in *Festuca* (ŠMARDA & KOČÍ 2003). Inconsistencies between morphological and genome size data in our species set were encountered in 10.5% of the hexaploid samples (17 hybrid individuals, 3 individuals of *E. repens*, and 4 individuals of *E. intermedia* were morphologically misidentified). Within all the hexaploid cytotypes, *E. repens* was represented by 101 plants from 45 localities, putative hybrids by 38 plants from 20 localities, and *E. intermedia* by 90 plants from 31 localities (see Appendix, Fig. 1). Both species co-occurred at 21 localities, 8 of them were also inhabited by hybrid individuals. The large proportion of hybrid plants (16.6% of the hexaploids) is quite surprising and points to the weak reproduction barrier between *E. repens* and *E. intermedia*.

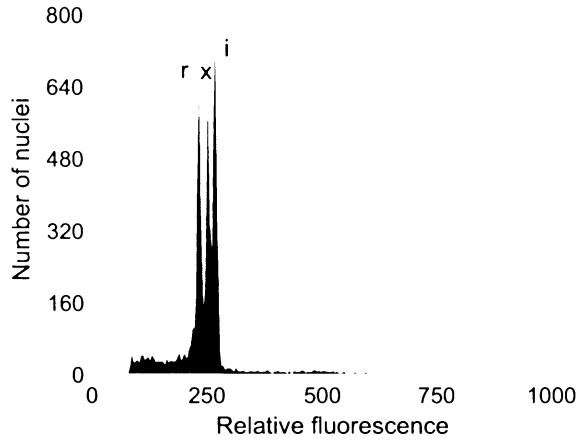

Fig. 2. Histogram of relative DNA content of *Elytrigia repens* (r), putative hybrid (x), and *E. intermedia* (i). Nuclei of the three plants were isolated, stained with DAPI and analyzed simultaneously.

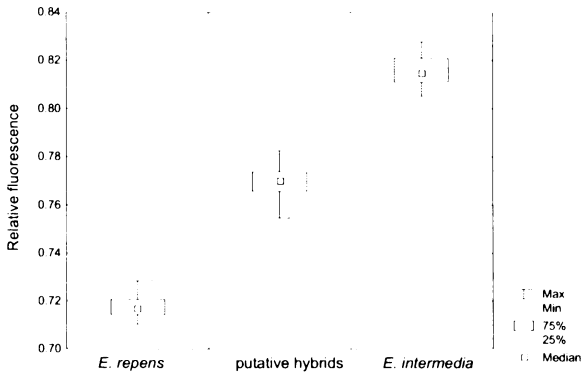

Fig. 3. Box plots illustrating relative fluorescences of nuclei (DAPI-stained) for hexaploid plants of *Elytrigia repens* (101 individuals), putative hybrids (38 individuals), and *E. intermedia* (90 individuals). *Triticum aestivum* was used as internal standard and its genome size was considered as unit value.

However, it should be noted that this is not the frequency of hybrids in the field, as the sampling was not random but targeted to cover the majority of morphological variation within each locality. We are convinced that comprehensive cytometric investigation would reveal hybrid individuals also at other localities within the distribution area of the parental species. Considering the susceptibility of *Elytrigia* to hybridization, the discovery of crosses with other related taxa cannot be ruled out. It is a plausible assumption that genome size data might be very useful in this mission and thus open new prospects in *Triticeae* research.

2C genome size estimation

Average 2C-values ± s.d. estimated using intercalating fluorochrome propidium iodide were 23.27 ± 0.20 pg and 27.04 ± 0.24 pg for *E. repens* and *E. intermedia* respectively, giving a difference of 16.2% (Table 2). A markedly different number of samples measured and/or the unequal proportion of AT/GC base pairs in the two taxa might be responsible for certain discrepancies between the results for DAPI and propidium iodide staining.

Our genome size estimate for *E. intermedia* (2C=27.04 pg) matches quite well the flow-cytometric data published by VOGEL et al. (1999), who reported virtually identical 2C-values (25.92–26.25 pg) for two subspecies of *Thinopyrum intermedium* (syn.: *Elytrigia intermedia*). However, no intraspecific division was possible in our material owing to continuous variation in the morphological characters used for subspecies delimitation; moreover, the formerly distinguished morphotypes lack any separate geographical distribution. On the contrary, the

Table 2. Genome size data for hexaploid *Elytrigia repens* and *E. intermedia* estimated by propidium iodide flow cytometry. *Triticum aestivum* (2C=34.4 pg) calibrated against *Vicia faba* (26.9 pg; DOLEŽEL et al. 1992) was used as internal standard. Three individuals of each species were measured on three different days in order to minimize potential random instrumental drift.

Species	Chromosome number (2n)	2C-value ± s.d. (pg)	C-value (pg)	Cx-value (pg)
Elytrigia repens	42	23.27 ± 0.20	11.64	3.88
Elytrigia intermedia	42	27.04 ± 0.24	13.52	4.51

previous genome size for *E. repens* (2C=25.96 pg; BENNETT et al. 1982) as determined by Feulgen microdensitometry shows 1.12-fold higher values compared to our result, and approaches the estimate for *E. intermedia*. Three theories can be invoked to explain this discrepancy: (1) the existence of genome size variation between *E. repens* from Georgia and the Czech Republic, (2) methodological problems during the preparation of samples for densitometry (related primarily to the temperature and duration of hydrolysis; GREILHUBER 1998), and (3) the use of different internal standard with deviating 2C-value.

Nonaploid plants (2n=9x=63)

Nine individuals (ca. 3.8% of the samples) showed significantly higher fluorescence of nuclei than the reference material of any hexaploid taxon. They also formed three distinct groups, although with smaller inter-group differences than observed among the hexaploids. The mean ratios between the *Triticum* standard and these group members were 1.057 (1 individual from 1 locality, determined morphologically as *E. repens*), 1.107 (range 1.104–1.114, 6 individuals from 2 localities, determined morphologically as putative hybrids), and 1.144 (2 individuals from 1 locality, determined morphologically as *E. intermedia*). Overall variation in relative DNA content (DAPI staining) at nonaploid level thus reached 8.2%. The novel cytotypes were assumed to be DNA-nonaploids. Subsequent chromosome counting confirmed the expected ploidy level and revealed 63 chromosomes in all of them. The mean ratios between nonaploid/hexaploid fluorescence of nuclei for particular groups were 1.473 in *E. repens*, 1.444 in putative hybrids, and 1.406 in *E. intermedia*.

The nonaploid plants were discovered in the field for the first time. The only previous report of this cytotype refers to a single individual arising in artificial conditions. DEWEY (1974) detected one plant with 2n=63 during the cytological investigation of twin seedlings (arising from polyembryonic seeds). The mode of origin of the nonaploid plant was not mentioned by the author. All the plants analyzed in his study represented inbreds from one generation of selfing. The question arises whether the artificial nonaploid would also survive in the natural habitat.

The nonaploids most probably arose by the fusion of reduced and unreduced gametes of the corresponding hexaploids. Relative genome size data for *E. repens* corroborated this hypothesis; the actual 2C-value of the nonaploid was only 1.9% smaller than expected.The relative 2C-value of 9x *E. intermedia*, however, was considerably smaller (by 7.0%) than one

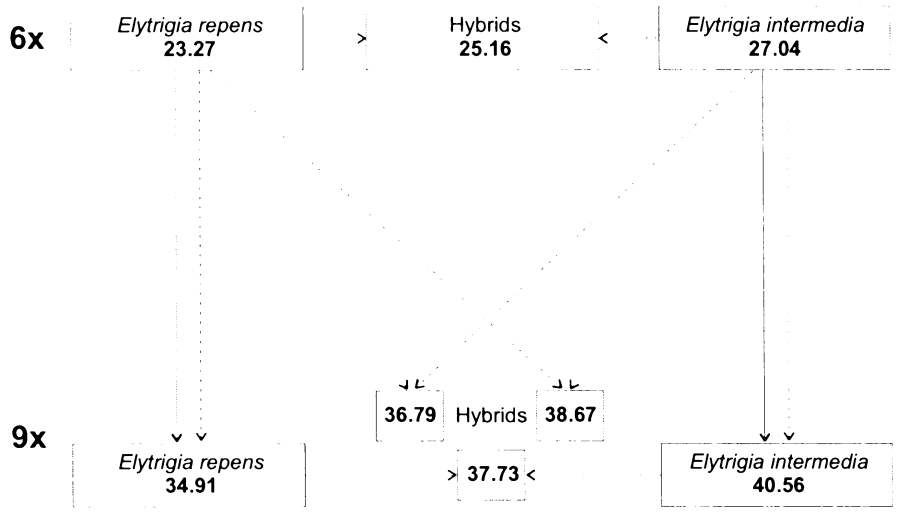

Fig. 4. Potential genesis of nonaploid plants (2n=9x=63). Both *E. repens* and *E. intermedia* are considered as putative parental taxa; involvement of hexaploid hybrids is omitted for lack of evidence of fertility. Solid and dashed lines designate the contribution of unreduced and reduced gametes respectively. Theoretical genome sizes (2C-values; pg) are given.

would predict from simple summation of reduced + non-reduced hexaploid gametes. Regarding the genesis of hybrid individuals, several pathways may theoretically be reconstructed, reflecting the origin of unreduced gametes, the ploidy level of parental plants, and the involvement of hexaploid hybrids (Fig. 4). It seems that the donor of the unreduced gamete in our 9x plants morphologically determined as hybrids was always *E. repens* (under this scenario, the mean 2C-value of the nonaploids was only 1.7% smaller than expected). An alternative hybrid origin after a crossing of 9x *E. repens* with 9x *E. intermedia* is less plausible owing to their sporadic occurrence (though fully consistent with relative genome size data). Similarly, the participation of hexaploid hybrids is questionable because of their apparent sterility, inferred from the lack of 6x back-crosses. One may even on the basis of 2C-values speculate that nonaploid plants morphologically determined as *E. intermedia* might actually have also been hybrid individuals sharing the unreduced genome of hexaploid *E. intermedia* and the reduced genome of *E. repens* (the difference between actual and theoretical values would be 2.7%). Generally, genome size data do not provide the best marker for testing the origin of polyploids. It has been repeatedly demonstrated that both genome size reduction and increase may occur after polyploid formation, resulting in deviations from simple summation of the genome sizes (ECKARDT 2001, LEVY & FELDMAN 2002, BENNETT 2004, LEITCH & BENNETT 2004). Irrespective of the mode of nonaploid origin, some fall in the nuclear DNA content must have taken place during their evolution. Similarly, rapid elimination of some DNA sequences after polyploidization was recorded in related *Triticum* genus (FELDMAN et al. 1997).

Although no dodecaploid plants (2n=12x=84) were found in our study, their occurrence (though very rare) might be expected owing to the production of unreduced gametes by both species of *Elytrigia*.

It is certain that at least some of the nonaploids are partially fertile (MAHELKA, unpubl.). One nonaploid individual yielded several fully developed seeds indicating either backcrossing to the putative hexaploid parents or some degree of self-pollination (considering the rarity of nonaploids in the field). Molecular analysis of all nonaploid plants, their potential progeny as well as hexaploid hybrids is essential in order to elucidate their genesis more certainly. GISH seems to be the most powerful tool to identify actual genome composition.

Acknowledgement: We thank David Short, who kindly improved our English, E. Ibermajerová and E. Morávková for their careful maintenance of the plants in the garden and V. Matějovičová for help with the literature search. Three anonymous reviewers are appreciated for their helpful comments. This study was supported by grant project no. QC 1362 of the Ministry of Agriculture of the Czech Republic, with further support provided by the Czech Science Foundation (grants nos. 206/05/0778 and 206/03/H137), Academy of Sciences of the Czech Republic (grant no. AV0Z60050516) and the Ministry of Education, Youth and Sports (project MŠM0021620828).

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Received 16 December 2004, revision received 24 February 2005, last revision received and accepted 28 March 2005

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APPENDIX

List of populations studied with the number of plants analyzed for each group. The ploidy level of all samples was estimated by flow cytometry. The number of karyologically-confirmed individuals is given in brackets. The location and habitat type of each locality are given. All localities are in the Czech Republic except of locality number 18, which is in Slovakia.

No.	Locality Name (location, habitat)	Coordinates	*E. repens* 6x	*E. repens* 9x	*E. intermedia* 6x	*E. intermedia* 9x	hybrid 6x	hybrid 9x
01.	Rubín 1 (3 km NE of Podbořany town, top of Rubín hill, steppe)	50°15′13.2″ N 13°26′12.4″ E	3		3		0	
02.	Rubín 2 (2.5 km NE of Podbořany town, field margin)	50°15′15.0″ N 13°26′03.3″ E	1	1 (1)	0		0	
03.	Raná (0.5 km SW of Raná village, bottom of Raná hill, steppe)	50°24′34.9″ N 13°46′38.9″ E	3		4		5	
04.	Cikánka (Prague city, near Cikánka bus stop, slope along the road)	49°59′53.8″ N 14°19′32.9″ E	2		2		0	
05.	Brno (Brno city, Kamenný hill, roadside)	49°11′02.5″ N 16°33′05.1″ E	2		1		0	
06.	Kobeřice (Kobeřice village, wasteland near the church)	49°05′34.0″ N 16°53′26.7″ E	1		0		0	
07.	Čejč 1 (1.5 km SE of Čejč village, wasteland)	48°56′16.9″ N 16°59′04.1″ E	1		0		0	
08.	Růžový kopec (2 km NNW of Mikulov, top of Růžový hill, steppe)	48°49′14.3″ N 16°37′31.2″ E	7		14		0	
09.	Jánská hora (2 km W of Dolní Dunajovice village, top of Jánská hora hill, steppe and vineyard)	48°50′54.4″ N 16°33′29.1″ E	1		2		0	
10.	Paví vrch (2 km S of Sedlec village, Paví hill, steppe and field margin)	48°45′50.8″ N 16°41′33.1″ E	1		0		1	
11.	Skalky (1 km W of Sedlec village, Skalky hill, steppe)	48°46′36.2″ N 16°40′23.2″ E	0		1		0	
12.	Malé Žernoseky (Malé Žernoseky village, near the station, grassland)	50°32′24.8″ N 14°03′21.8″ E	1		0		0	
13.	Zlončice (0.5 km S of Dolánky village, steppe)	50°12′40.9″ N 14°21′31.4″ E	2		1		1	
14.	Knovíz (0.5 km NW of the church at Knovíz village, pine forest)	50°12′48.1″ N 14°07′46.4″ E	2		2		1	
15.	Humenský vrch (1 km W of Keblice village, top of Humenský hill, alongside the footpath)	50°28′53.5″ N 14°05′10.1″ E	1		2 (1)		2	
16.	Zebín (2 km NE of Jičín town, top of Zebín hill, steppe)	50°27′12.8″ N 15°22′26.0″ E	4 (1)		0		0	
17.	Vrbčany 1 (1.5 km NE of Vrbčany village, steppe)	50°03′43.5″ N 14°59′56.0″ E	1		1		4 (2)	
18.	Hajnáčka (4 km NE of Hajnáčka village, oak forest)	48°14′50.0″ N 19°59′00.0″ E	0		1		0	
19.	Ješovice 1 (2 km N of Liběchov village, field margin)	50°25′26.0″ N 14°26′17.2″ E	3 (1)		0		1	
20.	Ješovice 2 (1 km WWS of Ješovice village, pine forest)	50°25′45.4″ N 14°25′02.6″ E	0		1		2	
21.	Strači (0.3 km E of Strači village, pine forest)	50°27′04.2″ N 14°24′30.7″ E	1		2		0	
22.	Radouň (0.5 km S of Křešov village, steppic slope along the roadside)	50°29′17.8″ N 14°23′59.3″ E	1		4		0	
23.	Trnovany (0.2 km W of Trnovany village, Robinia grove)	50°19′02.3″ N 13°35′45.7″ E	2		1		0	
24.	Úhošťany (0.5 km NNW of Úhošťany village, Úhošťhill, steppe)	50°21′36.4″ N 13°15′15.8″ E	9 (1)		3		0	
25.	Povrly (1 km W of Povrly village, orchard)	50°40′32.6″ N 14°08′21.8″ E	1		0		0	
26.	Radobýl (1 km NE of Žalhostice	50°31′44.5″ N	2		5 (1)		0	

	village, Radobýl hill, steppe)	14°05′30.7″ E						
27.	Sedlecké skály (Prague city, Sedlecké skály reserve, steppe)	50°08′25.1″ N 14°23′36.9″ E	0		6		0	
28.	Praha, Pecka (Prague city, Pecka reserve, *Robinia* grove)	50°06′26.8″ N 14°24′21.9″ E	1		0		0	2 (2)
29.	Záhoří (2 km NW of Žatec town, steppe)	50°20′24.3″ N 13°31′09.4″ E	5		0		2	
30.	Bezděkov (3 km E of Žatec town, steppe)	50°19′42.1″ N 13°35′22.7″ E	2 (1)		4		0	
31.	Libořice (0.5 km SW of Libořice village, orchard)	50°15′11.0″ N 13°30′26.3″ E	1		1		2	
32.	Přerovský vrch (1.5 km E of Nový Přerov village, Přerovský hill, *Robinia* grove)	48°48′41.6″ N 16°31′00.4″ E	1		2 (1)		1	
33.	Milovická stráň (0.2 km S of Milovice village, Milovická stráň reserve, steppe)	48°50′53.3″ N 16°41′34.8″ E	0		5 (2)		0	
34.	Pavlov – Děvín (0.5 km W of Pavlov village, Děvín hill, steppe)	48°52′27.7″ N 16°39′41.7″ E	0		5		0	
35.	Klentnice (0.5 km SW of Klentnice village, Stolová hora hill, steppe)	48°50′25.2″ N 16°38′24.4″ E	1		5		0	
36.	Valtice (1.5 km SW of Valtice town, vineyard)	48°44′13.1″ N 16°44′13.9″ E	1		0		0	
37.	Poštorná (3 km SSW of Poštorná village station, pine forest)	48°43′34.6″ N 16°50′06.4″ E	2		0		2 (1)	
38.	Hodonín 1 (2 km N of Hodonín town, pine forest margin)	48°54′10.7″ N 17°05′16.1″ E	1		0		0	
39.	Hodonín 2 (1.5 km S of Dubňany village, pine forest)	48°54′08.2″ N 17°05′14.2″ E	2		0		0	
40.	Čejč 2 (0.5 km NE of Čejč village, roadside)	48°56′54.7″ N 16°58′37.4″ E	2		2 (1)		0	
41.	Hovorany (1.5 km NW of Hovorany village, Hovoranské louky reserve, steppe)	48°57′54.2″ N 16°58′25.7″ E	0		2	2 (2)	1	
42.	Kobylí (2 km NE of Kobylí village, Kobylská skála hill, shrubs)	48°56′30.7″ N 16°55′19.1″ E	0		3 (1)		1	
43.	Nesovice (1.5 km W of Nesovice village, Malhotky reserve, steppe)	49°08′53.5″ N 17°03′23.7″ E	2		2		1	
44.	Kloboučky (0.5 km E of Kloboučky village, Baračka reserve, shrubs)	49°07′45.0″ N 17°01′12.5″ E	1		0		0	
45.	Křižanovice (0.5 km E of Křižanovice village, steppe)	49°08′44.6″ N 16°56′54.8″ E	0		2		3	
46.	Milešovka (2 km N of Milešov village, Milešovka hill, along the footpath)	50°33′16.6″ N 13°55′52.4″ E	2		0		0	
47.	Starý Vestec (hill 0.5 km SSE of Starý Vestec village, pine forest)	50°08′17.5″ N 14°51′04.1″ E	2		0		2	
48.	Dolní Dunajovice (2.5 km W of Dolní Dunajovice village, vineyard and field margin)	48°51′22.7″ N 16°34′03.9″ E	5		0		2	4 (4)
49.	Vrbčany 2 (0.5 km N of Vrbčany village station, field margin)	50°03′35.5″ N 15°00′05.6″ E	7		0		2	
50.	Valov (2 km S of Podbořany town, field margin)	50°12′34.5″ N 13°24′54.4″ E	6		0		0	
51.	Zichovec (0.5 km N of Zichovec village, pine forest margin)	50°16′32.2″ N 13°55′27.4″ E	1		0		0	
52.	Drahobuz (0.5 km E of Drahobuz village, roadside)	50°31′32.1″ N 14°19′41.6″ E	0		1		2	
53.	Veliš (0.2 km SE of Podhradí village, Veliš hill, shrubs)	50°25′01.0″ N 15°18′57.9″ E	2		0		0	
54.	Senohraby (Senohraby village, garden)	49°53′56.4″ N 14°43′40.0″ E	1		0		0	
55.	Zbraslav (Prague city, forest margin)	49°58′32.9″ N 14°23′23.4″ E	1		0		0	
Total			101 (4)	1 (1)	90 (7)	2 (2)	38 (3)	6 (6)

Recent natural hybridization between two allopolyploid wheatgrasses (*Elytrigia*, Poaceae): ecological and evolutionary implications

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Abstract

In the tribe Triticeae (family Poaceae), ancient hybridizations have produced a multitude of allopolyploid taxa whose origin is rather well-known. However, the role of recent natural hybridization and introgression under different ecological conditions and the mechanisms generating variability are much less understood.

Using molecular and cytological methods we investigated natural hybridization and gene flow between two predominantly allohexaploid wheatgrasses, weedy *Elytrigia repens* and steppic *E. intermedia*, with respect to habitats characterised by different degrees of anthropogenic disturbance. Weedy *E. repens* was rare in a steppic locality whereas *E. intermedia* was almost absent at two sites of agricultural land-use. Nevertheless, hybrids were common there whereas none were found at the steppic locality, underlining the importance of different ecological conditions for hybrid formation or establishment. At one highly disturbed site, more than 16% of randomly collected plants were hybrids. Hexaploid hybrids showed intermediate genome size compared to the parents and additive patterns of parental ITS copies. Some evidence of backcrosses to either parent was found. The direction of hybridization was highly asymmetric as cpDNA identified *E. intermedia* as the maternal parent in 61 out of 63 cases. Out of nine nonaploid cytotypes (2n=9x=63) which likely originated by fusion of unreduced and reduced gametes of hexaploids, eight were hybrids whereas one was a nonaploid cytotype of *E. repens*. The progeny of one nonaploid hybrid demonstrated gene flow between hexaploid and nonaploid cytotypes. The frequent production of new cyto- and genotypes with at least partial fertility provides ample raw material for evolution and adaptation.

Keywords: Triticeae, polyploidy, gene flow, chloroplast DNA, internal transcribed spacer, genome size

Introduction

Hybridization and introgression are perceived as important phenomena in plant speciation (e.g. Arnold 1997; Rieseberg *et al.* 2003; Gross & Rieseberg 2005). This is mainly evident in hybrids emerging from hybridization involving at least one species non-indigenous to the respective area (Abbott 1992). Such cases are usually well documented and carefully studied, because they represent examples of speciation caught in the act (Ownbey 1950; Rieseberg *et al.* 1990; Gray *et al.* 1991; Ashton & Abbott 1992; Soltis *et al.* 1995; Krahulec *et al.* 2005; Mandák *et al.* 2005). Species co-occurring at the same locality for a longer time may also hybridize; however, their hybrids may be more easily overlooked or misidentified when the parental species are morphologically similar and morphology of hybrids is overlapping with

that of the parental species. *Elytrigia repens* and *E. intermedia* (Poaceae) on which we focus in this study are examples of such a potential underestimation of hybridization in their native area.

Both species are perennial, out-pollinating allopolyploid grasses belonging to the wheat tribe Triticeae (Dewey 1984; Löve 1984). The tribe is especially well-known for the economic importance of its three major crops: wheat, barley, and rye. The tribe's structure is highly reticulate, with distinct genomes/gene lineages occurring within many polyploid , but and also within some diploid species (Kellogg *et al.* 1996; Mason-Gamer 2004). This reticulate structure is likely a consequence of ancient hybridization events, introgression, lineage sorting of ancestral variation, multiple origins of particular species, or a combination of these. These processes resulted in a strong ecological, morphological, and genetic resemblance of many Triticeae taxa (Stebbins 1956; Dewey 1984). Their ability to hybridize with each other is so common that Stebbins noted: 'So many hybrid combinations in one group is unparallelled in the higher plants.' (Stebbins 1956). One consequence of a reticulate structure is that if subsequent hybridization between genetically related species occurs, fertility of the hybrids can be enhanced because their chromosomes may pair more readily, and polyploidisation generally provides an effective way to escape from sterility (Stebbins 1940). Within the wheat tribe, about three fourths of the taxa are of polyploid origin (Löve 1984).

The predominantly hexaploid *E. repens* and *E. intermedia* are no exceptions in this respect, and their ability to hybridize with many other species of the tribe has been observed (Dewey 1984 and references therein; Assadi & Runemark 1995). Moreover, *E. intermedia* is able to cross with wheat. This hybrid (×*Tititrigia cziczinii* Tsvel.) was originally described by Tsitsin (1960) and taxonomically validated by Tsvelev (1973). Since then, many experimental hybridization studies followed (Sharma & Gill 1983; Franke *et al.* 1992; Chen *et al.* 2001; Han *et al.* 2003) in order to transfer desirable traits of the wild grass into the wheat genome (Sharma *et al.* 1995; Friebe *et al.* 1996; Fedak & Han 2005). However, natural hybrids between wheat and *E. intermedia* have not been observed so far. Should those be discovered, hybridization of *E. repens*, one of the most troublesome weeds on cultivated land worldwide (Palmer & Sagar 1963), with the comparably rare *E. intermedia*, combined with abundant production and at least partial fertility of *E. repens* × *E. intermedia* hybrids, might have a considerable impact on risk assessment of genetically modified wheat. Therefore, knowledge about the frequency of hybridization between *E. repens* and *E. intermedia* in nature is not only of interest for science, but also for economy and agriculture. In this context, ecological parameters that could facilitate hybrid formation need to be investigated.

Both *Elytrigia* congeners differ ecologically; however, mainly due to the wide ecological amplitude of *E. repens*, they also co-occur in some types of habitats such as field margins and steppic grasslands in warmer regions. In this study, we focus on several such sites. Although hybridization between them occurs in Central Europe – their hybrid was originally described from the area of the present Czech Republic –, it has attracted little attention. Except for morphology or chromosome pairing in artificial hybrids (Berchtold & Opitz 1836; Melderis 1980; Assadi & Runemark 1995), not much proven evidence of natural hybridization nor of its frequency is currently available. As a consequence of hybridization, the species' introgressive potential can lead to the transfer of ecological adaptations between species (Stutz & Thomas 1964; Arnold & Bennett 1993; Kim & Rieseberg 1999, Mahelka 2006).

Both study species have rather complex genomic histories. Mason-Gamer (2004, 2005) found at least five distinct lineages in the genome of *E. repens*, revealing reticulate and possibly polyphyletic origin of this species. Recently, new insights in the genome composition of *E. intermedia* became available (Kishii *et al.* 2005) but not all potential genome donors have been identified yet. Given the intricate relationships in the presently

worldwide distributed species, processes that occur at a local scale need to be interpreted carefully before generalisations about the respective taxa can be made.

As merely two morphological characters, which show large intraspecific variation and frequent overlapping of character values, distinguish between *E. repens* and *E. intermedia* (Melderis 1980; Barkworth & Dewey 1985; Kubát *et al.* 2002), identification of hybridogenous plants by morphology alone is difficult. As a prerequisite for evaluating the frequency of hybrids in the field, we recently established genome size measurements as a reliable means of identifying both parents and their hybrids (Mahelka *et al.* 2005).

Here, we report evidence of hybridization between *E. repens* and *E. intermedia* under different ecological conditions in the field, using nuclear ribosomal (ITS1-5.8S-ITS2 region) and chloroplast (*trn*T-F region) DNA markers in addition to genome size and chromosome numbers. In particular, we (i) compare the frequency of hybridization among hexaploid cytotypes between natural steppic grassland and the agricultural landscape; (ii) propose the origin of nonaploid cytotypes; (iii) present evidence of natural hybridization between nonaploid and hexaploid cytotypes; (iv) identify the maternal origin of hybrids and nonaploids; and (v) discuss putative evidence of backcrossing in the field.

Materials and Methods

Study species

Elytrigia repens (L.) Nevski [syn.: *Agropyron repens* (L.) P. Beauv., *Elymus repens* (L.) Gould] is widespread throughout the territory of the Czech Republic, occupying a wide range of habitats including man-made sites, dry grasslands, and wet meadows (Chytrý & Tichý 2003).

Elytrigia intermedia (Host) Nevski [syn.: *Agropyron intermedium* (Host) P. Beauv., *Thinopyrum intermedium* (Host) Barkworth et D.R. Dewey] has a more limited distribution, strongly corresponding with the occurrence of steppic habitats. It colonizes dry and warm habitats like steppes and base-rich rocks and also pine forests on sandy ground, vineyards, orchards and field margins in warm regions of the Czech Republic (Chytrý & Tichý 2003).

Both species occur predominantly at hexaploid level in the Czech Republic. Aside from hexaploids, several nonaploids (2n=9x=63) were found earlier (Mahelka *et al.* 2005). In places where natural or semi-natural habitats with *E. intermedia* come into contact with agricultural land-use, both species co-occur and hybridize. The hybrid was originally described as *Agropyron* ×*mucronatum* Opiz (Berchtold & Opiz 1836) [syn. *Elytrigia mucronata* (Opiz) Prokudin]. Melderis (1980) mentioned morphologically intermediate plants locally common in the field and Mahelka *et al.* (2005) provided further evidence for the existence of natural hybrids, based on genome size analysis. The morphology of hybrids is intermediate between the parental species but sometimes overlaps with one or the other parent.

All plants used in this study are cultivated in the experimental garden at the Institute of Botany, Průhonice, Czech Republic.

Sampling strategy

The plant material analysed consists of two sets. 1) For the comparison between hybridization frequency in natural habitat and the agricultural landscape, we chose three localities (A–C; described below) comprising two different habitat types, from which a total of 269 plants were collected (Table 1). At localities A and B, plants were collected predominantly in transect points without bias towards flowering plants. 2) To these we added 33 hexaploid

hybrids and five nonaploids from our collection (Mahelka *et al.* 2005) for more detailed investigation.

Locality A. 'Pouzdřany' – a steppic slope in a protected area, characterised by a community of *Festucion valesiaceae* Klika 1931, represents the conserved, natural steppic habitat. South exposition often causes plants to suffer from droughts. Two transects were sampled (one plant per 5 m): (i) on the top part of the slope, parallel to the boundary between the steppic habitat and an abandoned field (38 plants); (ii) from the top to the bottom of the slope along a footpath (74 plants).

Locality B. 'Valtice' – vineyard, agricultural landscape. 1) Three transects were carried out: (i) within the vineyard (12 plants; one per 24 m); (ii) along a path adjoining the vineyard (36 plants; one per 5 m); (iii) a shrubby vineyard margin ending in a steppic locality adjacent to a cultivated field, transect in orthogonal direction to (i) and (ii) (61 plants; one per 8 m). 2) Additional 20 plants were collected at the adjacent steppic locality in order to cover as much of the morphological variation as possible.

Locality C. 'Dolní Dunajovice' – agricultural landscape, characterised by an alternation of vineyards and cultivated and abandoned fields. Due to discontinuous occurrence of *Elytrigia* species, 28 plants were collected to cover the study area.

Table 1 Localities and distribution of species, hybrids and nonaploids.

Locality	Coordinates	Characterization	No. of plants (transects)		*E. repens*	*E. intermedia*	6x hybrids	9x
A	48°56′23.2′′N 16°38′47.0′′E	steppe, S exposition	(i)	38	4	34	0	0
			(ii)	74	0	74	0	0
B	48°44′13.1′′N 16°44′13.9′′E	1) vineyard	(i)	12	12	0	0	0
			(ii)	36	33	0	3	0
			(iii)	61	46	0	15	0
		2) adjacent steppe		20	9	4	7	0
C	48°51′22.7′′N 16°34′03.9′′E	vineyard, fields		28	19	0	5	4

Test of hybrids' and nonaploids' seed fertility

To assess fertility, all available spike-forming hexaploid hybrids and nonaploids were tested for seed fertility and germinability (18 hexaploids: H-3, 6, 8, 12, 13, 19, 20, 22, 23, 24, 25, 30, 34, 39, 44, 56, 58, 63; five nonaploids: N-3, 5, 6, 8, 9). In the autumn of 2003, spikelets were collected in the experimental garden and flower numbers in spikelets and developed caryopses, if any, were counted. Fertility was calculated as the ratio between caryopses and flowers. Caryopses were tested for germination ability in pots in a greenhouse. Five randomly selected samples of each parental species were tested in the same way and used as a control. Because of their high fertility, only ten randomly selected caryopses were tested for germinability.

Progeny of a nonaploid

The progeny of one hybridogenous nonaploid plant (N7, locality C) was investigated in order to determine the ratio of hybridogenous offspring. In 2002, a total of 195 spikelets were collected from the plant in the field. They produced 20 fully developed caryopses all of which germinated in pots in a greenhouse. Eight of the seedlings died in a 2–6 leaf stage. The other 12 were transferred to the experimental garden and maintained for subsequent analyses.

Genome size analyses

Relative DNA content was measured in all plants for their identification. For determination of absolute genome size of hexaploid hybrids, nonaploids, and the nonaploid's offspring, specimens of two different internal standards with close but non-overlapping genome size compared to the material analysed, were employed: *Triticum aestivum* L. var. *lutescens* (Alef.) Mansf. 'Bezostaja 1' (2C=34.4 pg; Mahelka *et al.* 2005) for hexaploid hybrids and *Vicia faba* (2C=26.9 pg; Doležel *et al.* 1992) for nonaploids. Because of the considerable variation in absolute genome size of the nonaploid's offspring, both internal standards were used (Table 2). All procedures followed Mahelka *et al.* (2005).

Chromosome counting

Chromosome numbers of the four nonaploids and the nonaploid's progeny (12 plants) were counted as described previously (Mahelka *et al.* 2005). Additionally, three hexaploid hybrids with DNA content deviating most from the values typical of hybrids (nos. H-1, H-2, H-63) were counted in order to verify that the plants were not aneuploid.

DNA isolation

DNA was isolated as described in Štorchová *et al.* (2000), but fresh leaves were crushed in liquid nitrogen. Quality and yield of the isolated DNA were checked on agarose gels.

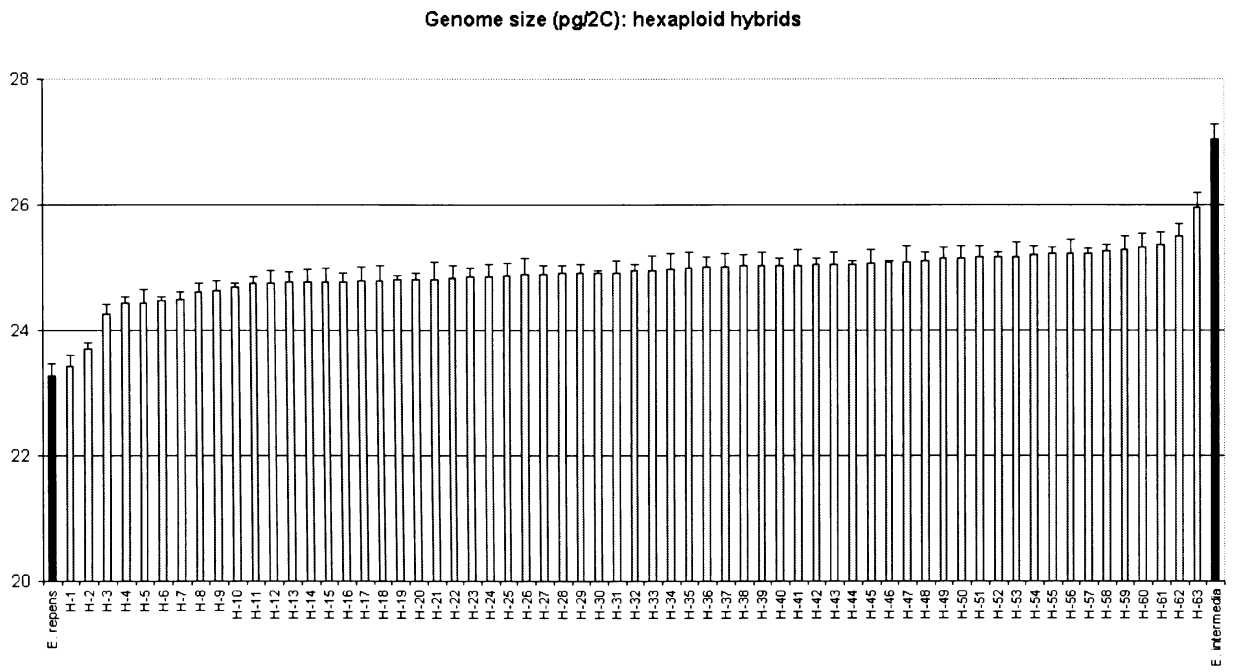

Fig. 1 Absolute genome size of hexaploid *E. repens* × *E. intermedia* hybrids. Values are pg/2C±s.d. Reference values of *E. repens* and *E. intermedia* are shown in black.

Analysis of chloroplast DNA

Based on the knowledge of cpDNA variation in the Triticeae (Mason-Gamer *et al.* 2002 and references therein), the *trn*L intron and the *trn*L-*trn*F intergenic spacer proved to be the most variable regions known so far. A set of *Elytrigia repens* (= *Elymus repens*) data was retrieved from GenBank (accession numbers AY362786–91), but no sequence for *Elytrigia intermedia*

(synonyms included) was available. We therefore assessed intraspecific variation by sequencing these parts for ten samples of each 'pure' parental species, selected according to the following criteria: (i) relative nuclear DNA content matching the range for a given species (genome size); (ii) unambiguous determination on the basis of morphological characters; (iii) representative geographic distribution, plants chosen from distant sites to assess intraspecific variability within the study area.

The *trn*L-*trn*F region was PCR-amplified as follows: reaction volumes of 50 µl contained 5 µl of Mg^{2+}-free reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer (c and f; Taberlet *et al.* 1991), 5–10 ng of genomic DNA, and 1 unit of *Taq* DNA-polymerase (Fermentas, Ontario, Canada). The thermocycling profile was as follows: 94°C/4 min., 40× (94°C/30 s, 53°C/30 s, 72°C/1.5 min), 72°C/10 min. PCR products were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) and sequenced (GATC Biotech, Konstanz, Germany) using the PCR primers. Electropherograms were edited, and alignments adjusted manually in BioEdit (Hall 1999). Sequences representing all the variation found were deposited in GenBank (accession numbers DQ912406–10).

Because of low variability between the parental species, we additionally analysed the *trn*T-*trn*L and *rpl*20-*rps*12 intergenic spacers. PCR amplification of the *trn*T-L was as follows: reaction volumes of 50 µl contained 5 µl of Mg^{2+}-free reaction buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer (a and b; Taberlet *et al.* 1991), 5–10 ng of genomic DNA, and 1 unit of *Taq* DNA-polymerase. The thermocycling profile was: 94°C/3 min., 35× (94°C/1 min., 46.5°C/1 min., 72°C/1 min.), 72°C/10 min. Purification, sequencing and alignment were done like above (GenBank accession numbers DQ914534–36). A single position differed for some *E. intermedia* samples. It created an *Acl*I restriction site. Restriction digests were performed using 12 µl of PCR product, 5 units of *Acl*I enzyme, and 1/10 reaction volume of Tango® buffer (Fermentas), and incubated overnight at 37°C. The products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualised by UV. Initial screening of chloroplast haplotypes was done by PCR-RFLP and all samples not showing the *E. intermedia*-specific mutation in the *trn*T-L were sequenced for *trn*L-F.

The *rpl*20-*rps*12 region was amplified as described by Kaplan & Fehrer (2006) (one sample per species sequenced, GenBank accession numbers DQ914537–38).

Nuclear ribosomal DNA (ITS) analyses

Three samples of each parental species were chosen according to the criteria described above and assessed for intra/interspecific variability (GenBank accession numbers DQ859048–54). PCR amplification of the ITS region was as follows: reaction volumes of 50 µl contained 5 µl of Mg^{2+}-free reaction buffer, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer (ITS 4 and ITS 5, White *et al.* 1990), 5–10 ng of genomic DNA, and 1 unit of *Taq* DNA-polymerase. The thermocycling profile was as follows: 94°C/5 min., 35× (94°C/30 s, 51°C/30 s, 72°C/1 min), 72°C/10 min. As this primer combination yielded some ITS sequences of an unspecified endophytic fungus (GenBank accession number DQ987703), we replaced ITS 5 with a newly-designed Poaceae-specific primer (ITS-Poa-f, 5'-aaggatcattgtcgtgacg-3') spanning the 3' part of 18S rDNA and the 5' end of ITS 1. PCR products were sequenced with the ITS 4 primer. We discriminated between the two species by one *Sma*I restriction site in *E. repens* and one *Hae*III restriction site in *E. intermedia*. For the purpose of RFLP analyses, PCRs were performed in triplicates and equimolar amounts of PCR products were mixed to reduce potential effects of PCR drift and to obtain a more accurate representation of parental copy types. Restriction digests were performed as above, using 10 units of enzyme and incubating overnight at 30°C with *Sma*I and at 37°C with *Hae*III. All 63 hexaploid hybrids, 9 nonaploids, and 12 offspring plants of a nonaploid hybrid were analysed by *Sma*I

RFLP. The nonaploids were additionally analysed by *Hae*III RFLP in order to confirm the contribution of *E. intermedia*. Previously sequenced samples of each parent served as references in the RFLPs.

To estimate the traceability of both parental ITS types in hybrids, we prepared a series of *Sma*I RFLPs, in which PCR products of both parents were mixed in different ratios (98%, 95%, 80%, 65%, 50%, 35%, 20%, 10%, 5%, 2%). To exclude preferential amplification of one or the other parental type in hybrids, we performed several independent amplifications with equal amounts of mixed parental DNAs and examined them by subsequent *Sma*I RFLPs.

Table 2 Genome size, chromosome numbers, morphological identification, chloroplast DNA haplotypes, ITS variants and possible gamete compositions of nonaploid plants and one nonaploid's progeny.

Specimen numbers	**Genome size**[1] **(pg/2C)±s.d.**	**Chromosome numbers**	**Morphology**	**cpDNA**	**ITS**	**Potential origin**[2]	
nonaploids						scenario 1	scenario 2
N-1	34.79±0.19 (Vf)	63	*E. repens*	*E. repens*	*E. repens*	(2n) r + (n) r	(2n) r + (n) r
N-2	35.64±0.19 (Vf)	63	hybrid	*E. intermedia*	both	(2n) r + (n) i	(2n) h + (n) r
N-3	35.75±0.17 (Vf)	63	*E. repens*	*E. repens*	both	(2n) r + (n) i	(2n) h + (n) r
N-4	35.79±0.26 (Vf)	63	hybrid	*E. intermedia*	both	(2n) r + (n) i	(2n) h + (n) r
N-5	36.05±0.35 (Vf)	63	hybrid	*E. intermedia*	both	(2n) r + (n) i	(2n) h + (n) r
N-6	36.09±0.31 (Vf)	63	*E. repens*	*E. repens*	both	(2n) r + (n) i	(2n) h + (n) r
N-7	36.17±0.17 (Vf)	63	hybrid	*E. intermedia*	both	(2n) r + (n) i	(2n) h + (n) r
N-8	37.98±0.31 (Vf)	63	*E. intermedia*	*E. intermedia*	both	(2n) i + (n) r	(2n) h + (n) i
N-9	38.03±0.33 (Vf)	63	*E. intermedia*	*E. intermedia*	both	(2n) i + (n) r	(2n) h + (n) i
progeny of N-7							
P-1	28.51±0.11 (Ta)	49					
P-2	28.04±0.10 (Ta)	50					
P-3	28.48±0.08 (Ta)	50		*E. intermedia*			
P-4	28.57±0.05 (Ta)	50		*E. intermedia*			
P-5	28.64±0.09 (Ta)	51					
P-6	28.57±0.26 (Ta)	51					
P-7	28.67±0.27 (Ta)	51		*E. intermedia*			
P-8	28.77±0.12 (Ta)	51					
P-9	28.65±0.24 (Ta)	52					
P-10	31.29±0.30 (Vf)	54					
P-11	32.80±0.32 (Vf)	54					
P-12	35.35±0.37 (Vf)	63					

[1] (Vf) and (Ta) designate *Vicia faba* or *Triticum aestivum* as internal standards.
[2] *E. repens* gametes are designated 'r', those of *E. intermedia* 'i', and those of F_1 hybrids 'h'.

Results

Flow cytometric analyses and chromosome counts

Initial flow cytometric analysis (relative DNA content) revealed 265 hexaploid plants among 269 plants collected at localities A–C: 123 *E. repens*, 112 *E. intermedia*, and 30 *E. repens* × *E. intermedia* hybrids. Additionally, four nonaploids occurred at locality C (Table 1). Together with material from Mahelka *et al.* (2005), 63 hexaploid hybrids from 20 localities and nine nonaploids from four localities were analysed for absolute DNA content.

Absolute genome sizes of hexaploid hybrids are presented in Fig. 1. All had DNA content intermediate between the parents; plants H-1, H-2 and H-63 could result from backcrosses to one or the other parent (all three are euploid hexaploids according to chromosome counts). Absolute genome sizes and chromosome numbers of nonaploids and of the progeny of one

nonaploid (hybrid N-7) are given in Table 2. Among this progeny, a variety of chromosome numbers was found. Nine plants with chromosome numbers 49–52 were very similar in genome size, two plants with 54 chromosomes had higher genome sizes but different to each other, and one plant with 63 chromosomes had the highest genome size matching the range of other natural nonaploids. These results suggest backcrossing of the mother plant with hexaploids (heptaploid P1, aneuploids P2–P11) and fusion of two reduced gametes of nonaploids (either through self- or out-pollination) (nonaploid P12).

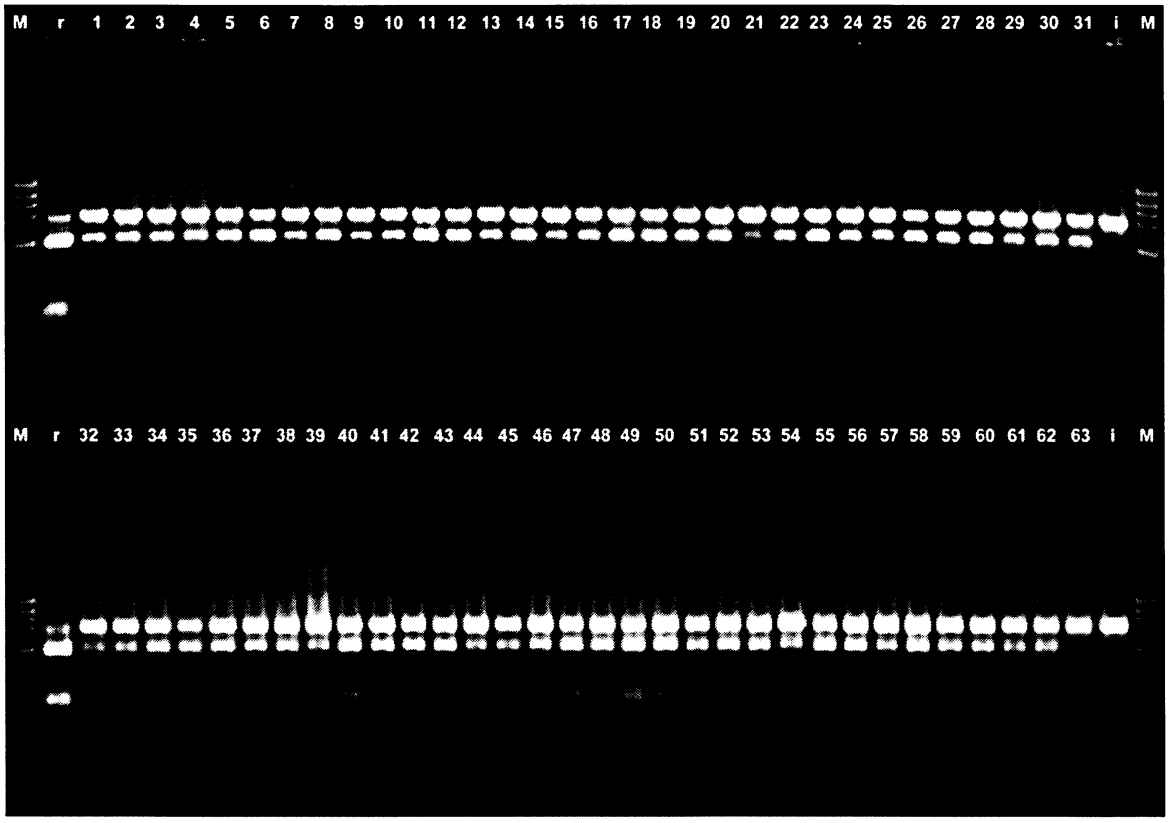

Fig. 2 *Sma*I ITS-RFLP of hexaploid *E. repens* × *E. intermedia* hybrids. Samples are ordered according to their genome size. Letters r and i refer to reference samples of *E. repens* and *E. intermedia*. Approximate lengths of the fragments are 650, 470, and 180 bp.

Chloroplast DNA analyses

Intraspecific variability in 10 accessions of *E. repens* was almost absent (one substitution in *trn*L-F) and our samples fell well into the variation of the GenBank sequences based on North American samples. Intraspecific variability of *E. intermedia* was also very low (two substitutions in *trn*L-F, one in *trn*T-L).

Even interspecific variability of cpDNA was very low for all three markers. While the *rpl*20-*rps*12 intergenic spacer was invariant, only a single mutation occurred in the *trn*T-L region. RFLP screening revealed it in 18 hybrids with the *E. intermedia* chloroplast haplotype. The *trn*L-F sequences differed consistently between *E. repens* and *E. intermedia* only by a 5 bp indel at a tandemly repetitive site which was identified by sequencing.

In 61 cases out of 63 hexaploid hybrids, *E. intermedia* was found to be the maternal parent. In samples H-6 and H-9, the maternal plant was *E. repens*. Out of the nine nonaploid hybrids, *E. intermedia* was identified as the maternal parent in six cases (Table 2). The

nonaploid's progeny expectedly had *E. intermedia*-like cpDNA, confirming maternal transmission of chloroplast DNA.

Nuclear ribosomal DNA (ITS) analyses

ITS copies of all hexaploid parental samples were sufficiently homogenised to provide well-readable electropherograms by direct sequencing. Apart from a few polymorphic sites within each sequence – some reflected interspecific variation, others occurred at otherwise invariant sites –, there was no intraspecific variability within both species. ITS provided us with a taxon-specific marker and was thus usable for inferring recent hybridization events. The parental species consistently differed from each other by 15 substitutions (2.3% sequence divergence). RFLPs revealed a small portion of undigested PCR product in *E. repens* (Fig. 3, arrow) despite manifold overdigestion. Direct sequencing of the undigested fragment (accession number DQ859049) showed one substitution and an adjacent 1-bp indel, both resulting in a loss of the original restriction site, only different from the *E. intermedia*-specific substitution. This mutation occurred in all *E. repens* samples analysed (5 samples) but was too rare to be detectable by direct sequencing of the original PCR product. On the other hand, *E. intermedia* samples contained a small portion (less than ~1%) of ITS copies which were digested with *Sma*I. Direct sequencing of the ~500-bp fragment (Fig. 3, arrow) and BLAST search in GenBank matched an ITS sequence close to the clone AF507808 of *Thinopyrum intermedium* (= *E. intermedia*) (Li *et al.* 2004). This suggests incomplete homogenisation of ITS copies in our *E. intermedia* samples. This minority sequence, undetectable by direct sequencing of the original PCR product, was present in all *E. intermedia* samples analysed (11 samples).

All plants determined as hexaploid hybrids by flow cytometry expectedly displayed an additive pattern of parental ITS copies (Fig. 2). Some samples showed overrepresentation of one or the other parental copy (e.g., H-21, 32, 39, 44, 45, 63 – *E. intermedia*; H-6, 40, 49, 55 – *E. repens*). Generally, *E. repens* copies were more often underrepresented than the *E. intermedia* type.

In nonaploids, restriction digest with *Sma*I showed additive patterns of both parental ITS copies in eight out of nine samples (Tab. 2). No obvious bias between parental ITS copies was detected in any of the samples (not shown). Sample N-1 displayed the RFLP pattern typical of *E. repens*. Restriction digest with *Hae*III excluded the presence of *E. intermedia* ITS in this sample and confirmed the others as true hybrids (not shown). Thus, out of nine nonaploids, eight were hybrids and one (N-1) was a nonaploid cytotype of *E. repens*.

The progeny of one nonaploid hybrid (N-7) was analysed further. *Sma*I RFLPs displayed an additive pattern in all samples, confirming the hybridogenous origin of the offspring plants. Equal or heavily biased copy numbers of both parents were found (Fig. 4). Samples P-5, 9, and 11 displayed an overrepresentation of *E. intermedia* copies, samples P-2, P-6 and P-10 an overrepresentation of *E. repens* copies, the others showed approximately equal amounts of both parental types. Equal or heavily biased copy numbers of *E. repens* or *E. intermedia* ITS suggest F_2 hybrids or backcrosses to the respective parents or, because of irregular meiosis of the nonaploid mother, loss of ITS loci of one or the other parent. The last case is plausible as indicated by samples P-1 – P-9, which have similar genome sizes while displaying a difference of three chromosomes, and thus having different genome composition.

No preferential amplification of either ITS type was detected in PCR-RFLPs with mixed parental DNAs in PCRs (M1 – M3, Fig. 3). Mixing of three PCR products for each sample prior to RFLPs ensured representative amplification, and repeating of a subset of PCR-RFLPs confirmed reproducibility of the patterns with respect to the relative amounts of detected parental ITS copies.

Hybridization between hexaploid parental cytotypes

The frequency of hybridization differed among the three localities (Table 1). At sites of agricultural land-use, hybridization was common whereas no hybrid was found at the steppic locality. One parental species was very rare at either the steppic (*E. repens*) or the agricultural localities (*E. intermedia*). The high proportion of hybrids at locality B-2 likely reflects sampling focused on morphological variation.

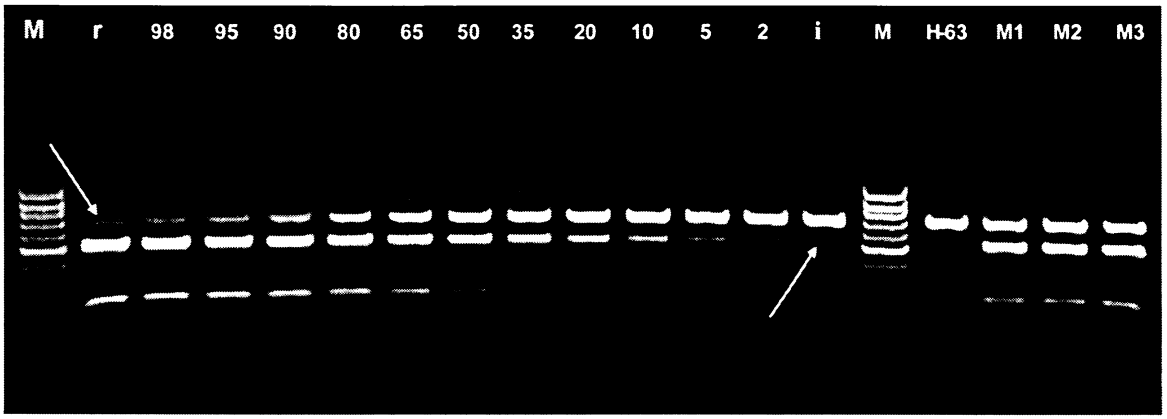

Fig. 3 *Sma*I ITS-RFLP of artificial PCR mixtures and hybrid H-63. From left: PCR products of both parents mixed in different ratios (numbers indicate proportion (percent) of *E. repens* in each sample; letters r and i refer to reference samples of *E. repens* and *E. intermedia*); hexaploid hybrid H-63; M-1 – M-3: *Sma*I ITS-RFLP of PCR amplifications with equal amounts of mixed parental DNAs. For arrows see text (Results). Approximate lengths of the fragments are 650, 470, and 180 bp.

Test of fertility

Five out of 18 investigated hexaploid hybrids and three out of five nonaploids yielded well developed caryopses. Fertility/germinability was: H-6: 1.1%/100%, H-58: 1.3%/50%, H-56: 1.5%/33.3%, H-22: 1.5%/0%, H-63: 28.8%/60%, N-5: 2.0%/100%, N-8: 3.5%/33.3%, N-9: 6.5%/83.3%. Fertility/germinability of five pure *E. repens* and *E. intermedia* samples ranged between 20.6–52.5%/10–100% and 10.3–51.9%/80–90%, respectively. Average values were 41.5%/62% in *E. repens* and 30.9%/88% in *E. intermedia.*

Discussion

Hybrid and putative backcross identification

While genome size is most effective in detecting F_1 hybrids in our study species, we presume that later-generation hybrids in case of backcrossing or introgression might be more problematic to detect because the hybrids' genome size will approach that of one or the other parental species. Additivity of ITS copies matched the results obtained by flow cytometry for all hexaploid hybrids and additionally revealed hybrid origin of most nonaploid plants. This approach enabled us to determine hybrids with a high degree of certainty. We combined RFLP pattern with genome size to find evidence for backcrossing in the field. Flow cytometric data suggested only three candidate plants (H-1, H-2, H-63; Fig. 1) to be potential backcrosses. Out of these, only H-63 showed a congruence of genome size and ITS data. While genome size of this sample was intermediate between the values of other hybrids and *E. intermedia*, ITS displayed a strong bias towards the *E. intermedia* type (about 98%, Fig. 3), almost corresponding with the pure *E. intermedia* sample. The comparably high fertility of

this plant (28.8%) and *E. intermedia*-like morphology suggest that the plant could be either a later generation backcross or *E. intermedia* with a lower genome size. Gene conversion without meiotic cycles (i.e., hybrids persisting vegetatively by rhizomes) is probably unlikely or less efficient to homogenise divergent ITS copies. Therefore, such hybrids with copy numbers biased towards either parental species, but of intermediate genome size, may also be backcrosses or later-generation hybrids. Theoretically, they could have arisen through hybridization of two F_1 hybrids of smaller and larger genome size, resulting in F_2 with intermediate genome size. However, as hybrid fertility is usually low, more complex dynamics of ITS homogenisation or locus loss in F_1 hybrids could as well be responsible for the biased ITS copy numbers.

Origin of nonaploids

Nonaploids can arise by a combination of reduced (n) and unreduced (2n) gametes of parental hexaploid species. Their origin was assessed by a combination of ITS-RFLP, genome size, cpDNA, and morphology (Table 2). Contrary to expectation (2:1 ratio of parental genomes), no obvious bias between parental ITS copies was detected and the results were reproducible (data not shown). The reason is unclear; not much is known about the particular intragenomic processes, but they can often be unpredictable.

One plant (N-1) represented a nonaploid cytotype of *E. repens*. For the origin of the eight nonaploid hybrids, we propose two plausible scenarios (Table 2). Under the first, a stronger maternal influence on the morphology of the plants seems to be apparent: out of six hybrids with lower genome size, two with *E. repens* cpDNA (N-3, N-6) morphologically resembled *E. repens* whereas four with *E. intermedia* cpDNA (N-2, N-4, N-5, N-7) were correctly identified as hybrids. They may all have arisen from 2n (*E. repens*) + n (*E. intermedia*) gametes. Two plants with higher genome size and *E. intermedia* morphology had also *E. intermedia* cpDNA (N-8, N-9). They may represent a composition of 2n (*E. intermedia*) + n (*E. repens*) gametes. Under the second scenario, fusion of unreduced gametes of hexaploid hybrids (with predominantly *E. intermedia*-like chloroplast hapotype) with reduced gametes of parental species is considered because hybrids are partially fertile and might more easily produce unreduced gametes than pure species due to disturbed meiosis (Ramsey & Schemske 1998). Genome sizes in both scenarios roughly match the theoretically expected values, estimated from absolute genome sizes of parental species (Mahelka *et al.* 2005).

The formation of another nonaploid hybrid cytotype which likely arose by fusion of a reduced gamete of *E. repens* and an unreduced gamete of *E. pycnantha* has been described by Refoufi *et al.* (2005). This observation suggests that the formation of unreduced gametes in *Elytrigia* with subsequent hybridization with other species may not be unusual.

Hybridization between nonaploid and hexaploid cytotypes

Data on the progeny of the nonaploid and certain fertility of the nonaploids examined in the experimental garden show that at least partial fertility of nonaploids should be expected. Viability of the nonaploid's offspring in nature is unknown. We did not find such plants growing spontaneously at locality C, from where the nonaploid mother originated. More detailed investigation of localities with nonaploid cytotypes would be desirable in this respect. Recently, we discovered a population of heptaploid (2n=7x=49) cytotypes intermixed with hexaploid *E. intermedia* at another locality (unpubl. observation). Besides heptaploids, several aneuploids (2n=47, 48, 50) were present there, too, similarly to the N-7 offspring recovered from seeds collected in the field. This suggests that such cytotypes can be viable under natural conditions and some of them may persist and take part in further hybridizations.

Hybridization between different cytotypes can apparently generate a large variability of geno- and cytotypes that can serve as raw material for evolution.

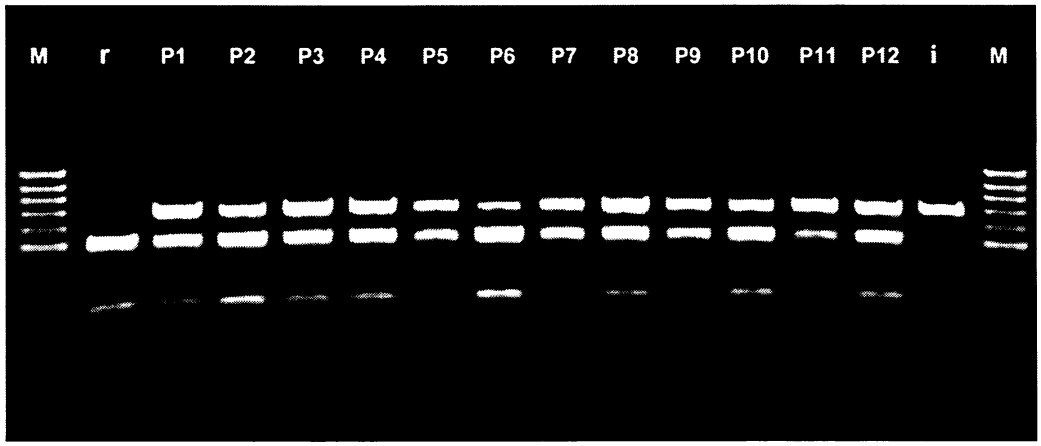

Fig. 4 *Sma*I ITS-RFLP of the progeny of the nonaploid plant N7. Hexaploid *E. repens* (r) and *E. intermedia* (i) were used as reference samples. Samples are ordered according to their chromosome numbers. Approximate lengths of the fragments are 650, 470, and 180 bp.

Ecological and evolutionary implications of hybridization

One of the most interesting aspects concerning hybridization in general is the fate of hybrids after they have arisen. There has been a long debate about hybrid fitness relative to their parents (Barton & Hewitt 1985; Arnold & Hodges 1995). While studies showing decreased hybrid fitness concern mostly animals, there is an increasing number of studies on plants demonstrating that hybrids can be as fit as their parents or even surpass them, at least in some environments (Arnold & Hodges 1995; Krahulcová *et al.* 1996; Wang *et al.* 1997; Campbell & Waser 2001; Rieseberg *et al.* 2003; Campbell *et al.* 2005; Kirk *et al.* 2005a,b). Hybrid fitness in these cases rather displays genotype-by-environment interactions than consistent breakdown. While we did not measure fitness of our natural hybrids, our observations suggest that it may be superior relative to the parents at some intermediate sites, such as transition zones between steppic grasslands and agricultural land. For example, at locality C we found no *E. intermedia* plant out of 28 plants collected; similarly, at locality B-1 we found no plant of this species among 109 plants collected although hybrids were present there. Due to arbitrary sampling at locality C, we may have missed *E. intermedia* plants, but it is plausible that the species is actually rare or even absent at localities B-1 and C. As *E. intermedia* occurs predominantly on adjacent steppes, there is ample opportunity to occasionally form hybrids with *E. repens*. Indeed, influence of the steppic locality adjoining the third transect on the species composition was evident: the closer to the steppe, the higher the proportion of detected hybrids (Table 1). Such hybrids could benefit from acquisition of *E. repens*-specific adaptations to the weedy, disturbed habitats in which *E. intermedia* does not occur. As *E. intermedia* was a mother plant of almost all hybrids, it had to be present at the site at least at the time of hybrid formation. Its rare occurrence at the sites where most hybrids were found probably resulted in a scarcity of conspecific mates and an exposure to an excess of *E. repens* pollen. This can at least partly explain the biased directionality of the cross at localities B-1 and C. Highly asymmetric hybrid formation is not unusual and can be caused by complex genotype-environment interactions (Rieseberg *et al.* 1991; Krahulcová *et al.* 1996; Campbell & Waser 2001; Campbell *et al.* 2005; Kirk *et al.* 2005a,b; Wu & Campbell 2005).

Cytoplasmic incompatibility can be excluded in our case as the reciprocal cross was possible, albeit rare.

The role of hybrids in plant speciation has been an object of discussion (Rieseberg 1997; Gross & Rieseberg 2005). Frequency of hybridization and fertility of hybrids are among the most important aspects in this respect. The frequency of *E. repens* × *E. intermedia* hybrids differed considerably between habitat types, suggesting that different ecological conditions may play an important role in hybrid formation and/or establishment. Our study localities represent two extreme types of habitats: a natural, conserved habitat with nearly no anthropogenic disturbance as well as agricultural habitats with a high degree of anthropogenic disturbance. The latter habitat type with a relaxed competition likely sustained hybrid formation and/or establishment. On the other hand, hybrid formation or establishment in natural steppic populations where *E. intermedia* is common seems to be restricted. As our current study is based on the results from only three localities, no generalisation can be made, and other aspects such as the history of particular localities have to be taken into account. Our data on hybrid seed fertility and germinability under garden conditions have rather informative character as to whether hybrids and nonaploids can produce germinable seeds in principal. However, the data do indicate that at least some F_2 hybrids or backcrosses may be expected in nature as well. While we did not determine male fertility in our hybrids, production of viable pollen of hybrids can be high even in cases of complete seed sterility (Mráz *et al.* 2005). The rather frequent occurrence of hexaploid and nonaploid hybrids in the field and their partial seed fertility suggest that hardly any premating and no strong postmating reproductive barriers exist between the two *Elytrigia* congeners and that hybrids could mediate gene flow in this species complex.

Successful hybridization and potential introgression to one parental species may cause transfer of genetically encoded adaptation whereby genetic diversity of species may be increased (Stutz & Thomas 1964; Arnold & Bennett 1993; Kim & Rieseberg 1999). For example by heterosis and transgressive segregation, hybrid phenotypes may, through new combination of alleles, exceed their parents, at least in some environments (Rieseberg *et al.* 2000, 2003; Campbell *et al.* 2005). The possible number of allele combinations in both *Elytrigia* species is magnified by their allopolyploid origin. Polyploidy per se is often perceived as a process facilitating evolution and adaptation, and the increased number of genetically divergent loci that may enhance environmental adaptability is one of the most often discussed advantages of polyploidy (Wendel 2000 and references therein). According to the preliminary data on genome composition, the two *Elytrigia* species share only one genome, donated by *Pseudoroegneria* (Assadi & Runemark 1995; Chen *et al.* 1998). Theoretically, the hybrid between *E. repens* and *E. intermedia* combines four more or less divergent genomes. F_1 hybrids between the two *Elytrigia* species contain a full genomic complement of both parents and thus their genetic pool may be enriched. Namely *E. intermedia* is known to possess many valuable traits, such as biotic and abiotic resistances, wherefore it is often used in wheat improvement (Fedak 1999; Fedak & Han 2005). Although *E. repens* is rather unexplored in this respect, its ecological amplitude is even wider than that of the former species. Mahelka (2006) showed that the response of the *E. repens* × *E. intermedia* hybrids to flooding tended to be intermediate between that of the parents. This was likely caused by enhanced rhizome production inherited from highly rhizomatous *E. repens*. Such an adaptation may gain high importance after ecological conditions at a locality have changed, e.g. during local floods, which are currently becoming more frequent as a consequence of low-tillage management, especially on heavy soils. In this respect, enhanced rhizome formation in hybrids compared to *E. intermedia* would likely be an adaptive advantage also in habitats frequently disturbed by tillage or ploughing because rhizomes as storage organs maintain damaged plants viable if fragmented and even allow further

propagation. Vegetative propagation may also be important in cases of low fertility, such as in hybrids. Survival of plants at a locality for many years through vegetative propagation increases the chance of hybridization in the future, because 1) multiplication of individuals increases the probabilities simply in a mathematical way; and 2) local ecological conditions change through time whereby the chance to meet a compatible sexual counterpart increases. Moreover, via cultivation of fields, fragmented rhizomes may be easily transported over hundreds of meters from the place where they originated, increasing the chance to find a suitable place for establishment and sexual partner to mate.

In conclusion, we can state that *E. repens* and *E. intermedia* frequently cross at places where they co-occur. Hybrid frequency is likely influenced by habitat type; sites disturbed by human influence sustain hybrid formation and/or establishment. Hexaploid and nonaploid hybrid fertility is not negligible, backcrossing is possible, and the progeny is variable. These processes generate a high diversity of cyto- and genotypes which may adapt to different environmental conditions.

Acknowledgements

We cordially thank Bohumil Mandák for reading the manuscript and for helpful comments and Fred Rooks for language editing. Marie Stará, Ľudmila Tereková and Petr Jurkovský are appreciated for technical assistance in the DNA laboratory. Pavel Trávníček and Jan Suda kindly assisted us in flow cytometric analyses. Eva Ibermajerová, Eva Morávková and Eva Slívová are appreciated for their help in the experimental garden. This study was supported by the Czech Science Foundation (grants 206/05/0778 and 206/03/H137) and the Grant Agency of the Academy of Sciences of the Czech Republic (AV0Z60050516).

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Response to flooding intensity in *Elytrigia repens*, *E. intermedia* (*Poaceae*: *Triticeae*) and their hybrid

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Received 16 May 2005
Revised version accepted 12 October 2005

Summary

Response to flooding intensity in three closely related taxa, *Elytrigia repens*, *E. intermedia* and their hybrid was studied. Plants were exposed to three intensities of flooding for a 30-day period. Response to flooding intensity was estimated by measuring dry mass of the following: total biomass, above-ground living biomass, above-ground dead biomass, below-ground biomass, rhizome and root mass and by the allocation of dry mass into rhizomes and root:shoot ratio. Reduction of nearly all the biomass compartments with increasing flooding intensity was observed in the three taxa. All three taxa can thus be regarded as flood-intolerant. Based on the parameters measured, *E. repens* is regarded as the relatively most flooding-tolerant, *E. intermedia* as the least tolerant, while the hybrid displayed intermediate flooding tolerance. The higher flooding tolerance in *E. repens* was likely related to its ability to accumulate a sufficient mass of rhizomes before flooding, due to higher regeneration ability. *E. repens* also displayed the highest phenotypic plasticity, as deduced from the reaction norms constructed for total biomass and rhizome mass of particular clones of the three taxa studied. This indicates that, on the species level, *E. repens* is better adapted to changing environmental conditions and it can be expected to colonize flooded soils. Both *Elytrigia* species also occur as weeds: *E. intermedia* grows in agricultural environments in warm regions, while *E. repens* infests many different types of habitats. Where they co-occur, hybridization between them may lead to the enrichment of their gene pools with genes responsible for survival of the parental species under extreme conditions; their weedy potential may thus be enhanced.

Keywords: *Agropyron*, allocation, biomass, phenotypic plasticity, reaction norms, stress, waterlogging, weed.

MAHELKA V (2006) Response to flooding intensity in *Elytrigia repens*, *E. intermedia* (*Poaceae*: *Triticeae*) and their hybrid. *Weed Research* **46**, 82–90.

Introduction

Phenotypic plasticity, i.e. variation in morphological and physiological characters induced by different environmental conditions, is one of the major features of plants responsible for their successful survival and propagation in unpredictably changing environments (Schlichting, 1986; Scheiner, 1993). Higher plants are aerobes and without free oxygen they die within several hours or days. Tolerance to anaerobic conditions is rather complex and cannot be explored in its entirety in this paper. However, there are two basic strategies plants use to cope with this stress: firstly, escape from anaerobic conditions and whole plant re-adjustments (such mechanisms are not flooding tolerance *per se*) and secondly, by 'true' flooding tolerance, i.e. by coping with anaerobic conditions through a complex of morphological and physiological adaptations developed to ensure gas exchange between the plant and atmospheric oxygen and thus maintain energy production (reviewed by Armstrong *et al.*, 1994; Blom & Voesenek, 1996; Vartapetian & Jackson, 1997).

Morphological and anatomical mechanisms preventing plants from the consequence of oxygen deficiency include shoot elongation to re-establish aerial contact, stomatal closure, adventitious rooting or aerenchyma formation (Armstrong *et al.*, 1994). Tolerance based on metabolic adaptations, such as the production of alcohol

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dehydrogenase (ADH) (Chan & Burton, 1992; Naidoo *et al.*, 1992; Kato-Noguchi, 1999), is usually thought to be connected with only short-term effects. On the contrary, physiological responses to flooding, such as the compensational ability of plants to acquire additional resources for above-ground growth, as a result of reduced below-ground parts (Rubio & Lavado, 1999) or the ability to control the uptake of toxic high iron and manganese concentrations to the shoots (Ashraf & Yasmin, 1991), provide considerably longer and more significant benefit.

Flooding tolerance is of crucial importance in habitats being periodically flooded. In such cases, floods act as a limiting parameter for the presence of certain species (Naidoo *et al.*, 1992; Rubio *et al.*, 1995). Nevertheless, plants from generally non-flooded habitats such as abandoned fields, field margins or roadsides sometimes face oxygen deficiency, because of acute floods after storms, over-irrigation or snow and ice crusts during winter and spring, which all impede gas penetration into the soil. The question arises whether such local, short-term floods of such habitats can limit the distribution of species, which are less flooding-tolerant. Flooding tolerance of species under those conditions would be of adaptive advantage.

This study is part of a project evaluating implications of gene flow within the *Triticeae*, focusing on the *Elytrigia* species. Nowadays, as agricultural practice changes towards reduced-tillage farming, in order to minimize energy input and improve soil conditions (MacIlwain, 2004), agricultural ecosystems change as well. It has been demonstrated that the spectrum of weedy species surviving in fields depends on the management type used (Bilalis *et al.*, 2001; Blackshaw *et al.*, 2001; Moonen & Barberi, 2004; Thomas *et al.*, 2004; Légère *et al.*, 2005). Perennial weedy species grow better under low- or no-tillage management, because their roots or rhizomes are not destroyed by soil tillage. Low-tillage management reduces infiltration of water through the upper layer, especially on heavy soils. As a consequence, an increased frequency of local floods already occurs. Such a trend can be demonstrated by the spread of *Scirpus* (syn. *Bolboschoenus*) *maritimus* species into fields (Hilbig, 1994; Schröder, 1998). The increasing number of localities with this species in agrosystem is likely related with the changing water regime and consequent increasing number of places with standing water (Z. Hroudová, pers. comm.). Knowledge of the ability of species to survive in flooded habitats is thus of increased interest. Flooding tolerance of *Elytrigia repens* is expected, since it is a species growing in a wide variety of habitats, including alluvial flooded meadows and periodically flooded habitats, forming communities of *Agropyro Alopecuretum* Moravec 1965 and *Agropyro–Rumicion crispi* Nordhagen 1940 (Chytrý *et al.*, 2001). On the contrary, response to flooding of *E. intermedia* is unknown, although this species is thought to be flooding-sensitive, as its distribution in the Czech Republic follows mostly steppic habitats (Kubát *et al.*, 2002).

The aim of this study was (1) to assess relative flooding tolerance of plants of three closely related taxa, *Elytrigia repens*, *E. intermedia* and their hybrid in terms of biomass accumulation and (2) to assess the extent of phenotypic plasticity of the three taxa as induced by unpredictably changing environments. The results can help us to predict the ability of the three taxa to spread or survive in habitats where floods act as a parameter limiting the presence of flooding-sensitive species.

Materials and methods

The species

The experimental species, *E. repens* (L.) Nevski [Syn.: *Agropyron repens* (L.) P. Beauv., *Elymus repens* (L.) Gould] and *E. intermedia* (Host) Nevski [Syn.: *A. intermedium* (Host) P. Beauv., *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey] (*Poaceae*), are rhizomatous perennial grasses. The former, which is considered as one of the most troublesome weeds on cultivated land worldwide (Palmer & Sagar, 1963), is widespread throughout the whole territory of the Czech Republic, occupying a wide range of habitats from man-made sites to the natural steppic grasslands. The latter taxon's distribution within the Czech Republic strongly reflects the occurrence of steppic habitats; the species largely colonizes steppes and pine forests on sandy ground, but it also occurs as a weed on vineyards, orchards and field margins in warm regions (Kubát *et al.*, 2002). Where they co-occur, the two species frequently hybridize. The hybrid was originally described as *Agropyron* × *mucronatum* Opiz (Berchtold & Opiz, 1836) (syn. *E. mucronata* (Opiz) Prokudin). However, its name has never been widely accepted. Identification of the hybrid on the basis of morphological characters is uncertain, due to large morphological variation of the putative parental species and frequent overlap of character values. Delimitation on the basis of genome size using flow cytometry turned out to be a more powerful tool (Mahelka *et al.*, 2005). Hereafter, for both *Elytrigia* species and hybrid the term taxa will be used.

To ascertain the ability of these three taxa to survive in flooded soils, a 30-day flooding experiment, which in its strongest regime simulated a real-time flood in the field, was carried out. Involving hybrids in the study extends the knowledge on the ecology of this species

group, and therefore, has an application for the studies of gene flow.

Experimental design

Initially, an experiment with a balanced design with 22 clones of both *Elytrigia* species was planned. Plants had been determined as to species on the basis of only two morphological characters available (Barkworth & Dewey, 1985; Kubát *et al.*, 2002). However, during exhaustive flow-cytometric and karyological investigations (Mahelka *et al.*, 2005), an inconsistency between the morphological and the genome size data was revealed in *c.* 10% of all plants. In effect, some of the putative *E. intermedia* individuals possessed relative genome size matching the values characteristic for *E. repens* × *E. intermedia* hybrids. Since genome size data are certainly more robust than those based on morphology in these species, seven plants previously considered to be *E. intermedia* were re-classified as hybrid individuals. Thus in reality, 22 clones of *E. repens*, 15 clones of *E. intermedia* and 7 clones of hybrids between the two species were included in the experiment.

The plants originated from the author's collection compiled during the summer of 2002. Plants from 27 localities were included in the study. The localities covered the geographic distribution of both *Elytrigia* species within the Czech Republic. Each locality was represented by only one plant of each species (except for two cases), which were randomly selected from the collection and included in the experiment. Each plant can, therefore, be considered a single genotype. All plants were maintained in the experimental garden of the Institute of Botany in Průhonice near Prague in open air until the start of the experiment in the summer of 2003. After this acclimation period, three clone members (ramets) selected for uniformity of size of rhizomes were separated from each plant and transplanted into plastic pots (16 × 16 cm wide, 16 cm high; filled with a homogenized mixture of garden compost and sand (1:1)). Tillers were cut 5 cm above the soil surface to minimize evaporation. All the plants were left outside for 23 days to allow regeneration before flooding was imposed. After 23 days, shoots and leaves of each plant were counted to estimate regeneration ability and for further use as covariate. The experimental design was 3 × 3 factorial and consisted of two blocks, each block comprising the three taxa, randomly assigned within three flooding treatments (each clone was exposed to three intensities of water regime). Factors were taxon and flooding and numbers of clones studied were 22, 15, and 7 in *E. repens*, *E. intermedia* and hybrid respectively. As two individuals of *E. intermedia* died during the regeneration time, 130 experimental units were included. Flooding consisted of three treatments (intensities) arranged as follows: flooded (F) – pots under this treatment were completely submerged with the water level *c.* 2 cm above the soil surface; partly flooded (PF) – pots were submerged so that the water level reached up to half of the pot height; and watered (W) – only the bottom 1 cm (drainage holes) of the pots were submerged in water so that the soil in the pots was saturated by the capillary water. This treatment assured constantly moderate irrigation of all pots and was used as a control. Ideally, the most appropriate control would be to grow the plants under the conditions similar to their ecological optimum. However, *E. repens* grows on a wide variety of habitats and its ecological optimum is, therefore, impossible to define. For brevity, the abbreviations for those flooding treatments will hereafter be used. The experiment continued for 1 month (July). At the end, all the plants were harvested. Above-ground biomass was cut by the soil surface and both fresh and dead biomass were separated. Soil was washed from the below-ground biomass and the biomass was separated into roots and rhizomes. Because it was difficult to distinguish between fresh and dead roots, both were considered together. All the biomass compartments were dried for 48 h in 60°C and weighed. The data was used for comparisons of total biomass, above-ground living biomass, above-ground dead biomass, below-ground biomass, rhizome and root mass, the allocation of dry mass into rhizomes and root:shoot ratio among the three taxa under the three different flooding treatments using an ANCOVA. In order to interpret intraspecific plasticity, expressed by the total biomass and rhizome mass (see *Discussion*), reaction norms, showing the response of each clone to the particular flooding intensities, were constructed for all the three taxa by using non-transformed data.

Statistical analysis

Data were statistically processed using two-way ANCOVA (GLM procedure, type III sum of squares, Statistica™ software, StatSoft, 1998) and Tukey's tests for unequal *n* at a probability level of 0.05 were performed for *a posteriori* comparisons (where significant). In order to include the effect of different regeneration abilities of the plants/taxa on all analyses of biomass production (except the above-ground dead), the number of shoots and leaves emerging from the soil at the start of the flooding experiment was taken as covariate. When above-ground dead biomass was analysed, two questions arose: firstly, whether a plant bore dead biomass and if so, what was responsible for it. To resolve the first question, because this data was not normally distributed after transformation, a log-linear analysis was performed according to Caswell (1989). Data were arranged

into three-way contingency tables with three factors: taxon (T), flooding intensity (F) and numbers of plants of each taxon with dead tissue under each flooding intensity (G). The model included effects of (T), (F) and their interaction (T × F), and was tested by comparing the effects to the saturated model with likelihood ratio test, G^2, which is asymptotically distributed as χ^2 (see Horvitz & Schemske, 1994). The second question was resolved by ANCOVA with dead biomass as dependent variable. Only plants with dead biomass were included into this analysis. Because presence of dead tissue is also the result of natural processes of plants (i.e. senescence), plant size should be considered. Hence, total biomass measured after the flooding experiment was also taken as covariate. The same covariate was used when the allocation of biomass into rhizomes and root:shoot ratio (see *Results*) were analysed. In order to assure data normality, all data was ln-transformed before analyses, except those of allocation into rhizomes, where arcsin $\sqrt{p}$ transformation of percentage (p) data was applied.

Results

At the beginning of the flooding experiment, regeneration and growth capability, based on the number of shoots and leaves emerging from the soil, were found to differ among taxa (16.7 ± 8.7, 8.6 ± 4.1, 8.8 ± 4.9, number of shoots and leaves (mean ± SD) of *E. repens*, hybrid and *E. intermedia* respectively; ANOVA after data ln-transformation: d.f. = 2, MS = 5.67, $F = 18.99$, $P < 10^{-6}$). No differences were found either among plants under different flooding treatments (ANOVA, d.f. = 2, MS = 0.14, $F = 0.46$, $P = 0.63$) or in the taxon × flooding interaction (ANOVA, d.f. = 4, MS = 0.05, $F = 0.15$, $P = 0.962$).

Both taxon and flooding factors had significant effect on all the biomass compartments (Table 1). The taxon × flooding interaction was only significant in above-ground living ($P < 0.05$), above-ground dead ($P < 0.001$) and rhizome mass ($P < 0.001$), suggesting a different response of the taxa to the flooding.

Total biomass, i.e. all the biomass produced by a plant during the 23-day regeneration and 30-day flooding periods, was the highest in *E. repens* and the lowest in *E. intermedia*, whilst the hybrid between both was placed midway (Table 2, Fig. 1A). This applied for all the three flooding treatments: biomass of *E. repens* under each flooding intensity (treatment) was significantly higher compared with both its conspecifics, whilst the differences between hybrid and *E. intermedia* were not significant. When the taxa were considered separately, there was a significant increase in biomass of plants of each taxon under (PF) treatment compared to those under (F) treatment, while biomass of plants under (PF) and control (W) treatments was not significantly different.

A similar pattern was observed in above-ground living biomass, which only included vital leaves and stems. So it can be used as a good indicator of how plants are able to cope with anaerobic conditions. Again, it was the highest in *E. repens* and the lowest in *E. intermedia* at all flooding intensities (Table 2, Fig. 1B). In *E. repens* under the (F) treatment, above-ground living biomass was significantly higher than that of both other taxa under this treatment. When grown under (PF) and (W) treatments, it was significantly higher only when compared with *E. intermedia*.

In order to resolve which factor was responsible for the senescence or decaying of tissue (presence/absence of above-ground dead tissue, Table 2), log-linear analysis was performed (Table 3). It was impossible to distinguish between senescent tissue, which is normally produced during ontogenetic development of a plant, and tissue, which on the contrary is a result of decaying processes induced or enhanced by flooding. Therefore, both were considered together and senescence as a natural process was eliminated by using plant size (total biomass after flooding) as covariate. It was found that the factor responsible for the presence of dead tissue was flooding (Table 3, $P = 0.0016$). Consequently, factors responsible for senescence/decaying intensity (dead tissue mass) were identified by ANCOVA as taxon, flooding

Table 1 Plant parameters of *Elytrigia repens*, *E. intermedia* and their hybrid as affected by three levels of flooding

Source of variation	d.f.	Total biomass	Above-ground living biomass	Below-ground biomass	Rhizomes	Roots	Allocation into rhizomes	Root:shoot ratio	d.f.	Above-ground dead biomass†
Covariate	1	4.79***	2.52***	2.50***	0.43***	1.95***	0.002 NS	0.77***	1	0.09**
Taxon (T)	2	1.71***	1.41***	1.41***	1.69***	0.32***	0.44***	0.28***	2	0.04**
Flooding (F)	2	2.73***	2.05***	2.41***	0.91***	1.41***	0.19***	0.46***	2	0.11***
T × F	4	0.03 NS	0.16**	0.04 NS	0.36***	0.02 NS	0.03 NS	0.06 NS	4	0.06***
Error	120	0.06	0.06	0.03	0.03	0.03	0.02	0.03	66	0.009

Values are mean squares and probability levels (*,**,***, significant at 0.05, 0.01 and 0.001 levels respectively. NS, not significant). See *Materials and methods* for covariates used.
†Only plants with dead tissue were included in the analysis.

Table 2 Plant parameters of *Elytrigia repens*, *E. intermedia* and their hybrid as affected by three levels of flooding

Taxon	Treatment level	Total biomass	Above-ground living biomass	Above-ground dead biomass		Below-ground biomass	Rhizomes	Roots	Allocation into rhizomes	Root:shoot ratio
				Number*	Mass†					
E. repens	F	3.16 ± 0.24c	1.58 ± 0.15c	18 (22)	0.20 ± 0.02a	1.40 ± 0.09c	0.27 ± 0.02a	1.13 ± 0.09bc	0.10 ± 0.02cd	0.92 ± 0.08a
	PF	5.10 ± 0.32de	2.27 ± 0.15d	12 (22)	0.19 ± 0.03a	2.71 ± 0.19d	0.71 ± 0.10b	2.00 ± 0.13d	0.14 ± 0.01d	1.18 ± 0.07ab
	W	5.87 ± 0.36e	2.36 ± 0.18d	11 (22)	0.12 ± 0.02a	3.45 ± 0.22e	1.49 ± 0.16c	1.96 ± 0.15d	0.25 ± 0.02e	1.52 ± 0.09b
Hybrid	F	1.29 ± 0.28ab	0.51 ± 0.12ab	4 (7)	0.16 ± 0.06a	0.68 ± 0.14ab	0.08 ± 0.05a	0.60 ± 0.11ab	0.05 ± 0.03abc	1.30 ± 0.16ab
	PF	2.98 ± 0.54c	1.46 ± 0.31cd	2 (7)	0.10 ± 0.04ab	1.49 ± 0.25c	0.20 ± 0.10a	1.29 ± 0.22cd	0.06 ± 0.03abcd	1.06 ± 0.07ab
	W	3.38 ± 0.52cd	1.43 ± 0.22cd	2 (7)	0.20 ± 0.04ab	1.89 ± 0.30cd	0.41 ± 0.19ab	1.48 ± 0.22cd	0.11 ± 0.03bcde	1.26 ± 0.05ab
E. intermedia	F	1.20 ± 0.16a	0.22 ± 0.12a	12 (13)	0.38 ± 0.04b	0.55 ± 0.06a	0.05 ± 0.02a	0.50 ± 0.07a	0.05 ± 0.03ab	1.10 ± 0.19a
	PF	2.40 ± 0.27bc	1.23 ± 0.17bc	8 (15)	0.13 ± 0.02a	1.10 ± 0.11bc	0.04 ± 0.01a	1.06 ± 0.10bc	0.01 ± 0.004a	1.01 ± 0.13a
	W	2.78 ± 0.34c	1.21 ± 0.16bc	7 (15)	0.09 ± 0.04a	1.52 ± 0.17c	0.16 ± 0.03a	1.36 ± 0.16c	0.06 ± 0.01bc	1.29 ± 0.08ab

Values are mean(g) ± SE. F, pots completely flooded; PF, pots partly flooded; W, pots watered (control), see *Materials and methods*. Values followed by different letters are significantly different at 0.05 level (Tukey HSD test for unequal *n*), a < b < c < d < e.

*Number of plants with dead tissue and total number of plants (in brackets) under particular treatment are shown.

†Only plants with dead tissue were considered when the means were calculated.

and taxon × flooding interaction (Table 1). In conclusion, it was found that dead tissue mass of *E. intermedia* under totally flooded conditions (F) was significantly higher than that of the remaining taxa (based on *post hoc* comparisons).

Rhizome mass showed the highest differences among taxa as well as among particular flooding treatments (Table 2, Fig. 1C). The highest mass of rhizomes was in *E. repens* under (W) followed by (PF) treatment, whilst in *E. intermedia* it was negligible under all flooding treatments. The hybrid, midway in rhizome mass, produced significantly less rhizomes under (PF) and (W) treatments compared with *E. repens*, and in general did not significantly differ from *E. intermedia*.

Allocation into rhizomes (Table 2, Fig. 1D) corresponded with the rhizome mass and ranged 10–25%, 5–11% and 1–6% of the total biomass in *E. repens*, hybrid and *E. intermedia* respectively. Under all flooding treatments, *E. repens* allocated significantly more mass into rhizomes than *E. intermedia*. The hybrid displayed intermediate values and did not differ significantly from both.

Root mass in *E. repens* was significantly higher than that in *E. intermedia* under all flooding treatments, root mass of the hybrid was intermediate and did not differ significantly from the other two taxa under particular flooding treatments (Table 2, Fig. 1E). There was significant increase in root mass of plants of all taxa grown both under (PF) and (W) treatments compared to (F) treatment, but there was no increase in root mass of plants under (W) compared to (PF) treatment in *E. repens* and non-significant in hybrid and *E. intermedia* (Fig. 1E).

Root:shoot ratio did not differ significantly among taxa (Table 2, Fig. 1F). In *E. repens*, there was apparent increase as flooding intensity decreased: plants under (W) treatment had significantly higher root:shoot ratio compared with those under (F) treatment, whilst plants under (PF) treatment had root:shoot ratio intermediate. No significant differences between plants under the same flooding treatments were found in hybrid and *E. intermedia*. Since root:shoot ratio as well as allocation patterns change over the course of plant growth and development (Reekie, 1991; Coleman *et al.*, 1994), total biomass of plants measured after the flooding experiment was taken into account and used as covariate. At least the effect of plant size was thus eliminated.

Discussion

The large reduction of the total biomass with increasing flooding intensity observed in the course of the experiment contrasts with the general assumption that total biomass in flood-tolerant species remains unchanged or

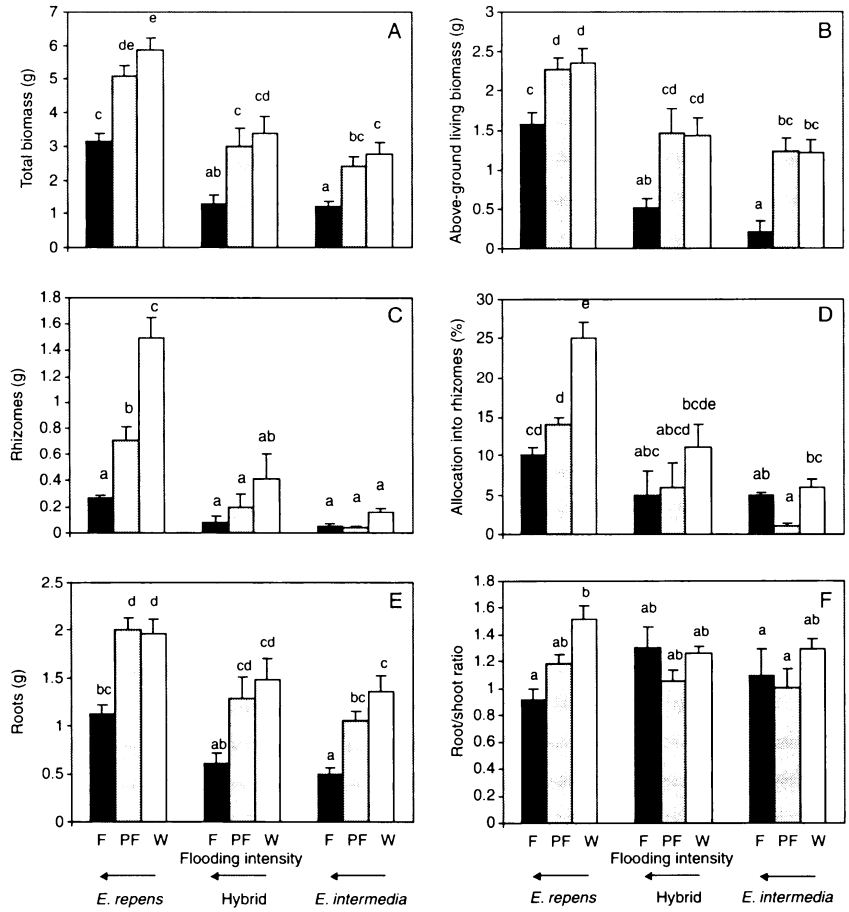

Fig. 1 Means of the measured parameters of *Elytrigia repens*, *E. intermedia* and their hybrid as affected by three levels of flooding (F – pots completely flooded, PF – pots partly flooded, W – pots watered (control), see *Materials and methods*). Bars with different letters are significantly different at 0.05 level (Tukey HSD test for unequal *n*), a < b < c < d < e. Error bars represent SEs. *n* = 22, 15, 7 in *E. repens*, *E. intermedia* and hybrid respectively.

Table 3 Log-linear analysis of the effects of taxon (T) and flooding intensity (F) on the presence of above-ground dead tissue (G)

Model	d.f.	Log-likelihood G^2	*P*
Test: effect of (T)			
TF, G	8	18.254	0.139
TF, TG	6	14.301	
TG	2	3.953	
Test: effect of (F)			
TF, G	8	18.254	0.0016
TF, FG	6	5.415	
FG	2	12.839	
Test: interaction (T × F)			
TF, TG, FG	4	0.866	0.929
TFG	0	0	
TFG	4	0.866	

even increases in flood conditions (Naidoo & Naidoo, 1992; Rubio *et al.*, 1995). On the contrary, in flooding-intolerant species, reduction of biomass in flooded plants was observed (Dias-Filho, 2002). Therefore, all the three taxa studied can be regarded as flooding-intolerant. Absence of flood-tolerating mechanisms is also indicated by the high losses of above-ground living biomass of plants under the strong (F) treatment compared to those under (PF) and (W) treatments. The fact that there was no significant difference between above-ground living biomass under (PF) and (W) treatments in all three taxa (Fig. 1B) indicates that plants (considering root and rhizome mass) under (PF) and (W) treatments had different allocation pattern compared to those under (F) treatment and that these allocated more biomass into the below-ground parts, i.e. roots and rhizomes (Fig. 1B–E). No effective mechanisms to protect the plants from the consequence of oxygen deficiency, such as formation of adventitious roots (Naidoo & Naidoo, 1992; Dias-Filho, 2002) or change in morphology of leaves (Rubio & Lavado, 1999), were observed in the taxa studied. On the contrary, different extents of aerenchyma formation (Rubio *et al.*, 1995; Loreti & Oesterheld, 1996; Grimoldi *et al.*, 1999) might have been responsible for the survival of the plants for such a long time under the flooded conditions. It should be noted that some species, in spite of their tolerance to partial or temporary submergence through the presence of effective mechanisms, can still be susceptible to total submergence imposed both by intense and long-term floods and can subsequently die (Grimoldi *et al.*, 1999).

Plant roots are particularly prone to tissue injury induced by insufficient oxygen supply and initial effect of

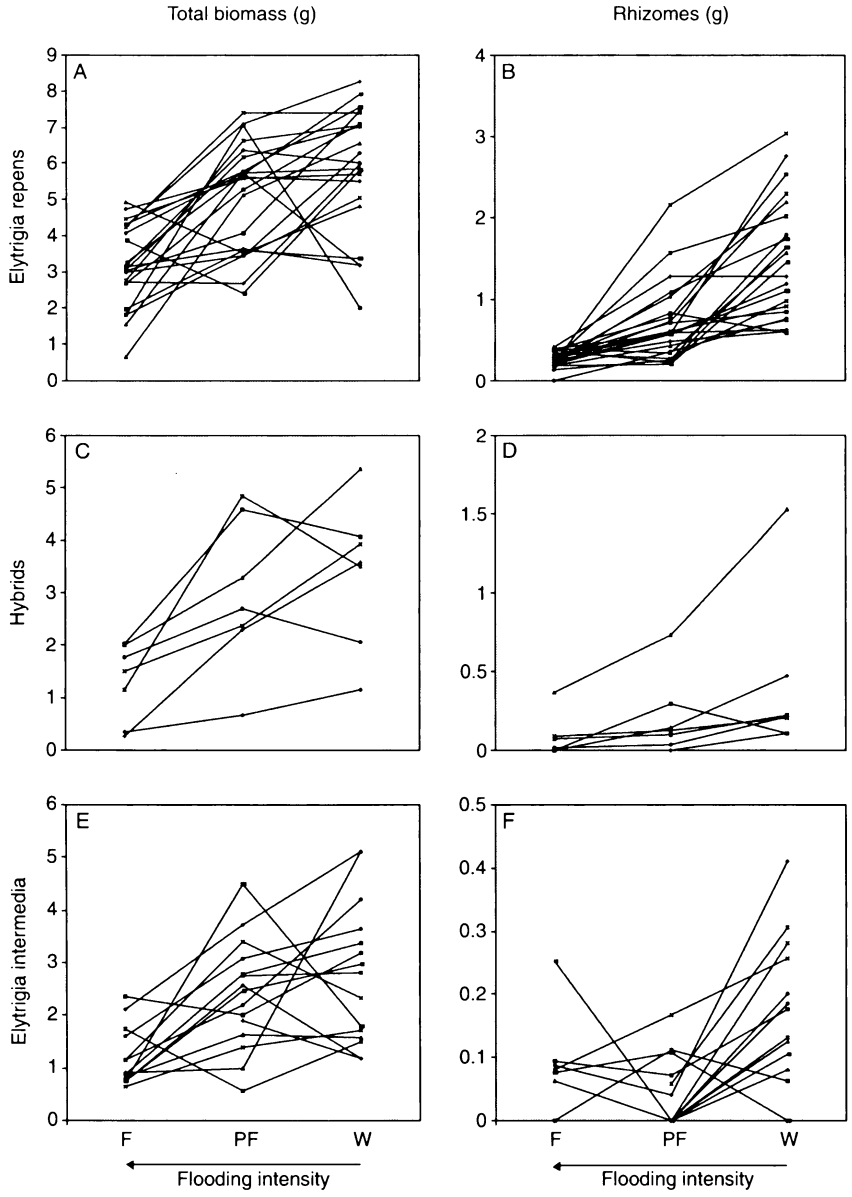

Fig. 2 Reaction norms for total biomass and rhizome mass of *Elytrigia repens*, *E. intermedia* and their hybrid in response to three levels of flooding (F – pots completely flooded, PF – pots partly flooded, W – pots watered (control), see *Materials and methods*). Values plotted are dry weights for each clone of the three taxa under particular intensities of flooding. n = 22, 15, 7 in *E. repens*, *E. intermedia* and hybrid respectively.

flooding is thus apparent in the root system (Blom & Voesenek, 1996). Therefore, the reaction of flooding-tolerant species is to shift at least to some degree allocation of biomass from below- to above-ground parts when flooded and thus to reduce root:shoot ratio (Naidoo & Naidoo, 1992; Neill, 1993; Rubio *et al.*, 1995; Grimoldi *et al.*, 1999). This is related to the effort of plants to increase mass of above-ground aerial shoots, which would provide oxygen to the flooded parts via gas-transport. Within my experimental plants/taxa, such a reaction was observed only in *E. repens* (Fig. 1F).

Enhanced senescence of leaves is a feature accompanying exposure of flood-intolerant plants to flooding (Banga *et al.*, 1997; Dias-Filho & de Carvalho, 2000). Dead tissue mass in the plants studied was a likely consequence of insufficient support from roots, which were poorly developed as a result of the flooding intensity. The significant amount of dead tissue in *E. intermedia* is in accordance with the assumption that this species is the least flood-tolerant.

Rhizomes (i.e. storage organs) with their carbohydrate reserves can engender long-term flooding tolerance in perennial plants (Armstrong *et al.*, 1994). Given the fact that oxygen deficiency is accompanied by increased depletion of carbohydrate reserves in rhizomes, it is likely that plants with higher rhizome production will cope better with oxygen deficiency caused by flooding. A case in point is that of *E. repens*, where regeneration and growth ability were greater than in *E. intermedia*. The significant differentiating factors among the taxa studied were both rhizome mass and allocation into rhizomes (Fig. 1C and D). *E. repens* developing from rhizome (buds) begin to produce rhizomes at the 3 to 4-leaf stage (Fiveland *et al.*, 1972

cited in Werner & Rioux, 1977). No information is, unfortunately, available on *E. intermedia*. Rhizome mass as well as allocation into rhizomes decreased as flooding intensity increased (Fig. 1C and D) in all three taxa with the exception of partly flooded *E. intermedia*. Rhizome mass available at the beginning of a flood thus seems to be the crucial parameter determining the extent (at time and space) of flooding tolerance in *E. repens*–*E. intermedia* group.

No evidence of a direct trade-off between seed and rhizome production was found in *A. repens* (syn. *E. repens*) (Reekie, 1991). Similarly, again in *A. repens*, no significant relationship was observed between total biomass and infructescence allocation (Reekie, 1991). Both rhizome mass and total biomass of plants can thus be adopted as good estimates of fitness in this species. To ascertain how the individual plants coped with the flooding in terms of fitness estimate, reaction norms were constructed (Fig. 2). Some differences were observed. For instance, some partly flooded plants (PF) of *E. repens* produced more total biomass over those watered (W). The reaction of nine plants out of 22 was not consistent, at least under one flooding intensity (Fig. 2A), with the general assumption that total biomass increases as flooding intensity decreases. The response of only three plants varied from the norm when the rhizome mass was taken into account (Fig. 2B). This suggests that maximum fitness in *E. repens* is achieved through rhizome production. A similar pattern appears to arise in the case of hybrids (Fig. 2C and D). It appears that rhizome mass (or even all the biomass compartments) of the plants of all taxa totally flooded (F) did not increase during the flooding experiment and the mass detected after the experiment corresponded with that at the beginning of the flooding. In partly flooded (PF) *E. repens*, there were several clones with an apparent ability to form rhizomes; these plants were considered to be the most flood-tolerant. In *E. intermedia*, rhizome mass of totally (F) and partly flooded (PF) plants was even more limited by the flooding. In totally flooded plants, two plants died during the experiment and seven of 13 did not form rhizomes, while seven of 15 partly flooded plants did not form rhizomes. In watered conditions (W), *E. intermedia* plants formed *c.* 10-fold less rhizome mass than did *E. repens*, although their total biomass was, in fact, comparable.

Since most of the experimental plants originated from common localities with low precipitation, where floods would not be expected (steppe), tolerance to anaerobic conditions can be perceived as phenotypic plasticity. Such plasticity can, within the localities where the plants were collected, be perceived as an adaptive feature, for example, in fields, where plants may be stressed due to heavy rainfall, over-irrigation, or snow and ice crusts during winter and spring which also cause oxygen deficiency in the soil.

The fact that neither the rhizomes nor the aerial shoots of *E. repens* are apparently harmed both by exposure to frost and drought (Palmer & Sagar, 1963), together with the fact that plants with sufficient rhizome mass are able to survive oxygen deficiency (Lenssen *et al.* (2004) reported that *E. repens* survived a 50-day flood), makes it a species with a high-adaptive ability. On the contrary, *E. intermedia* displays rather low phenotypic plasticity, because it is apparently adapted to the narrower range of environmental conditions. It is very likely that rhizome production in *E. repens* is responsible for its distribution among such a wide range of habitats, because it enables this species to survive extreme conditions. In conclusion, the results indicate that *E. repens* is better adapted to changing environmental conditions and especially it may have the potential to colonize flooded soils. It was already pointed out that both *Elytrigia* species also occur as weeds. Whilst *E. intermedia* infests agro-ecosystems in warm regions, *E. repens* is a troublesome weed across many different types of habitats, including wet places. Where they co-occur, hybridization between them may lead to the enrichment of their gene pools with genes responsible for survival of the parental species under extreme conditions. This may enhance their weedy potential.

Acknowledgements

My thanks are due to František Krahulec for reading the manuscript and valuable comments and Conn Bree for English editing. This study was supported by grant project no. QC 1362 of the Ministry of Agriculture of the Czech Republic, with further support provided by the Czech Science Foundation (grants nos. 206/05/0778 and 206/03/H137) and the Grant Agency of the Academy of Sciences of the Czech Republic (grant no. AV0Z60050516).

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