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Recent Natural Hybridization between Two Allopolyploid Wheatgrasses (*Elytrigia*, Poaceae): Ecological and Evolutionary Implications

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• *Background and Aims* Natural hybridization was investigated between two predominantly allohexaploid wheatgrasses, weedy *Elytrigia repens* and steppic *E. intermedia*, with respect to habitats characterized by different degrees of anthropogenic disturbance.
• *Methods* Using flow cytometry (relative DNA content), 269 plants from three localities were analysed. Hybrids were further analysed using nuclear ribosomal (ITS1-5·8S-ITS2 region) and chloroplast (*trn*T-F region) DNA markers in addition to absolute DNA content and chromosome numbers.
• *Key Results* 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, $>16\,\%$ of randomly collected plants were hybrids. Hexaploid hybrids showed intermediate genome size compared with the parents and additive patterns of parental ITS copies. Some evidence of backcrosses 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.
• *Conclusions* The results show 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. The frequent production of new at least partially fertile cyto- and genotypes provides ample raw material for evolution and adaptation.

Key words: Triticeae, Poaceae, *Elytrigia repens*, *Elytrigia intermedia*, hybridization, polyploidy, chloroplast DNA, internal transcribed spacer, genome size, adaptation.

INTRODUCTION

Hybridization is perceived as an important phenomenon in plant speciation (e.g. Arnold, 1997; Rieseberg *et al.*, 2003; Gross and 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 and 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 the morphology of hybrids is overlapping with that of the parental species. *Elytrigia repens* and *E. intermedia* (Poaceae), on which this study focuses, are examples of such a potential underestimation of hybridization in their native area.

Both species are perennial, outcrossing 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 also within some diploid species, which is a consequence of ancient hybridization events, introgression, lineage sorting of ancestral variation, multiple origins of particular species, or a combination of these (Kellogg *et al.*, 1996; Mason-Gamer, 2004). 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 unparalleled 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 polyploidization generally provides an effective way to escape from sterility (Stebbins, 1940). Within the wheat tribe, about three-quarters of the taxa are of polyploid origin (Löve, 1984).

The predominantly hexaploid *Elytrigia 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;

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Assadi and Runemark, 1995). Moreover, *E. intermedia* is able to cross with wheat. This hybrid (× *Trititrigia cziczinii* Tsvel.) was originally described by Tsitsin (1960) and taxonomically validated by Tsvelev (1973). Since then, many experimental hybridization studies have followed (Sharma and 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 and 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 and 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. This study focuses 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 and Opitz, 1836; Prokudin and Druleva, 1971; Melderis, 1980; Assadi and 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 could lead to the transfer of ecological adaptations between species (Stutz and Thomas, 1964; Arnold and Bennett, 1993; Kim and Rieseberg, 1999; Mahelka, 2006).

The acknowledged ease with which *Elytrigia repens* and *E. intermedia* can hybridize might suggest that they are closely related. Cytogenetic studies revealed the preliminary genome constitution of hexaploid cytotypes ($2n = 6x = 42$) of both species. In *E. repens*, it was determined as StStH, where St and H designate *Pseudoroegneria* (Nevski) Á. Löve and *Hordeum* L. genomes, respectively (Assadi and Runemark, 1995). The genome constitution of *E. intermedia* was determined as E^eE^eSt (Liu and Wang, 1993) or E^eE^bSt (Chen *et al.*, 1998) with E^e and E^b designating the closely related *Thinopyrum elongatum* and *Th. bessarabicum* genomes. More recent studies revealed that both species might have still more complex genomic histories. Mason-Gamer (2004) and Mason-Gamer *et al.* (2005) found at least five distinct lineages in the genome of *E. repens*, revealing the 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. These data show that the two species represent distinct genetic entities that probably share only the St genome from *Pseudoroegneria*. Furthermore, at least some accessions might be further influenced by hybridization and introgression so that both species might actually have multiple origins.

As only two morphological characters, which show large intraspecific variation and frequent overlapping of character values, distinguish between *E. repens* and *E. intermedia* (Melderis, 1980; Barkworth and 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, genome size measurements were recently established as a reliable means of identifying both parents and their hybrids (Mahelka *et al.*, 2005).

In the present paper, using nuclear ribosomal (ITS1-5·8S-ITS2 region) and chloroplast (*trn*T-F region) DNA markers in addition to genome size and chromosome numbers, evidence of natural hybridization between *E. repens* and *E. intermedia* is reported. In particular (*a*) the frequency of hybridization among hexaploid cytotypes with respect to habitat types, i.e. between natural steppic grassland and the agricultural landscape, is compared; (*b*) the origin of nonaploid ($2n = 9x = 63$) cytotypes is proposed; (*c*) evidence of natural hybridization between nonaploid and hexaploid cytotypes is presented; (*d*) the maternal origin of hybrids and nonaploids is identified; and (*e*) the impact of hybridization within habitats of different land-use is discussed.

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 and ranges from lowlands to the mountain belt. It occupies all man-made habitats and arable ground, and also occurs on such natural habitats as steppic grasslands and wet meadows (Chytrý and Tichý, 2003; authors' observations).

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 and Tichý, 2003; authors' observations).

Both species are morphologically variable (Mizianty and Szczepaniak, 1997; Assadi, 1998; Mizianty *et al.*, 2001). Constancy of the morphological characters, their taxonomic significance, correlation with ecological preferences or with genetic variation, remain unexplored. 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 and Opiz, 1836) [syn. *Elytrigia mucronata* (Opiz) Prokudin]. 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 was divided into two sets. (1) To compare between hybridization frequency in a natural habitat and the agricultural landscape, three localities (A–C; described below) were chosen comprising two different habitat types, from which a total of 269 plants was collected (Table 1). At localities A and B, plants were collected predominantly at transect points without bias towards flowering plants. (2) To these were added 33 hexaploid hybrids and five nonaploids from the authors' collection (Mahelka *et al.*, 2005) for more detailed investigation.

Locality A. 'Pouzdřany', a steppic slope in a protected area, characterized by a community of *Festucion valesiaceae* Klika 1931, represents the conserved, natural steppic habitat. The south-facing aspect of the site often causes plants to suffer from droughts. Two transects were sampled (one plant per 5 m): (1) on the top part of the slope, parallel to the boundary between the steppic habitat and an abandoned field (38 plants); (2) from the top to the bottom of the slope along a footpath (74 plants).

Locality B. 'Valtice', a vineyard in an agricultural landscape. (*a*) Three transects were carried out: (1) within the vineyard (12 plants; one per 24 m); (2) along a path adjoining the vineyard (36 plants; one per 5 m); (3) a shrubby vineyard margin ending in a steppic locality adjacent to a cultivated field, transect in orthogonal direction to (1) and (2) (61 plants; one per 8 m). (*b*) An 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, characterized 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.

Test of hexaploid 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: H3, H6, H8, H12, H13, H19, H20, H22–H25, H30, H34, H39, H44, H56, H58, H63; five nonaploids: N3, N5, N6, N8, N9). In the autumn of 2003, spikelets were collected in the experimental garden and flower numbers in spikelets and developed caryopses (one-seeded fruit), 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 to determine the ratio of offspring. In 2002, a total of 195 spikelets was 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 at the 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 DNA content of the whole chromosome complement (holoploid genome size *sensu* Greilhuber *et al.*, 2005; hereafter for brevity the term genome size will be used) of hexaploid hybrids, nonaploids and the nonaploid's offspring, specimens with close but non-overlapping genome size compared with the material analysed were employed as internal standards: *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 genome size of the nonaploid's offspring, both internal standards were used (Table 2). All procedures followed Mahelka *et al.* (2005).

TABLE 1. *Localities and distribution of species, hybrids and nonaploids*

Locality	Co-ordinates	Characterization	No. of plants (transects)	*E. repens*	*E. intermedia*	6*x* hybrids	9*x*
A	48°56′23·2″N 16°38′47·0″E	Steppe, S exposition	38 (1)	4	34	0	0
			74 (2)	0	74	0	0
B	48°44′13·1″N 16°44′13·9″E	(*a*) (1) Vineyard	12 (1)	12	0	0	0
			36 (2)	33	0	3	0
			61 (3)	46	0	15	0
		(b) (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

TABLE 2. *Holoploid 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* (pg/2C) ± s.d.	Chromosome numbers	Morphology	cpDNA	ITS	Potential origin†	
						Scenario 1	Scenario 2
Nonaploids							
N1	34·79 ± 0·19 (Vf)	63	*E. repens*	*E. repens*	*E. repens*	(2*n*)r + (*n*)r	(2*n*)r + (*n*)r
N2	35·64 ± 0·19 (Vf)	63	Hybrid	*E. intermedia*	Both	(2*n*)r + (*n*)i	(2*n*)h + (*n*)r
N3	35·75 ± 0·17 (Vf)	63	*E. repens*	*E. repens*	Both	(2*n*)r + (n)i	(2*n*)h + (*n*)r
N4	35·79 ± 0·26 (Vf)	63	Hybrid	*E. intermedia*	Both	(2*n*)r + (*n*)i	(2*n*)h + (*n*)r
N5	36·05 ± 0·35 (Vf)	63	Hybrid	*E. intermedia*	Both	(2*n*)r + (*n*)i	(2*n*)h + (*n*)r
N6	36·09 ± 0·31 (Vf)	63	*E. repens*	*E. repens*	Both	(2*n*)r + (*n*)i	(2*n*)h + (*n*)r
N7	36·17 ± 0·17 (Vf)	63	Hybrid	*E. intermedia*	Both	(2*n*)r + (*n*)i	(2*n*)h + (*n*)r
N8	37·98 ± 0·31 (Vf)	63	*E. intermedia*	*E. intermedia*	Both	(2*n*)i + (*n*)r	(2*n*)h + (*n*)i
N9	38·03 ± 0·33 (Vf)	63	*E. intermedia*	*E. intermedia*	Both	(2*n*)i + (*n*)r	(2*n*)h + (*n*)i
Progeny of N7							
P1	28·51 ± 0·11 (Ta)	49					
P2	28·04 ± 0·10 (Ta)	50					
P3	28·48 ± 0·08 (Ta)	50		*E. intermedia*			
P4	28·57 ± 0·05 (Ta)	50		*E. intermedia*			
P5	28·64 ± 0·09 (Ta)	51					
P6	28·57 ± 0·26 (Ta)	51					
P7	28·67 ± 0·27 (Ta)	51		*E. intermedia*			
P8	28·77 ± 0·12 (Ta)	51					
P9	28·65 ± 0·24 (Ta)	52					
P10	31·29 ± 0·30 (Vf)	54					
P11	32·80 ± 0·32 (Vf)	54					
P12	35·35 ± 0·37 (Vf)	63					

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

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 H1, H2, H63) were counted 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.

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. Therefore intraspecific variation was assessed by sequencing these parts for ten samples of each 'pure' parental species, selected according to the following criteria: (*a*) relative nuclear DNA content matching the range for a given species (genome size); (*b*) unambiguous determination on the basis of morphological characters; (*c*) 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, the *trn*T-*trn*L and *rpl*20-*rps*12 intergenic spacers were also analysed. 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 as 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 visualized 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 and 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), ITS 5 was replaced 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. The two species were distinguished 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 triplicate 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, nine nonaploids and 12 offspring plants of a nonaploid hybrid were analysed by *Sma*I RFLP. The nonaploids were additionally analysed by *Hae*III RFLP to confirm the contribution of *E. intermedia*. Previously sequenced samples of each parent served as references in the RFLPs.

PCR–RFLPs were used to simultaneously detect the representation of both parental ITS types in hybrids to infer recent hybridization events. As initial digests suggested skewed ratios of parental ITS amplificates despite using replicates to avoid PCR drift (see above), the reproducibility and sensitivity of the method were verified by a semi-quantitative approach as follows: a series of *Sma*I RFLPs, in which separately amplified PCR products of both parents were mixed in different ratios (98 %, 95 %, 80 %, 65 %, 50 %, 35 %, 20 %, 10 %, 5 %, 2 %) was prepared prior to digest (Fig. 1). Additionally to exclude preferential amplification of one or the other parental type in hybrids, several independent amplifications with equal amounts of mixed parental DNAs were performed and they were examined by subsequent *Sma*I RFLPs. No preferential amplification of either ITS type was detected in PCR–RFLPs with mixed parental DNAs in PCRs (Fig. 1, M1–M3). Also, mixing of three PCR products for each sample prior to RFLPs did ensure representative amplification: replication of a subset of PCR–RFLPs confirmed the reproducibility of the patterns with respect to the relative amounts of detected parental ITS copies (not shown). The mixed ratio series (Fig. 1, 98 to 2) allowed an approximate estimation of the relative proportions of parental ITS types present in particular hybrids (e.g. H63, Fig. 1).

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

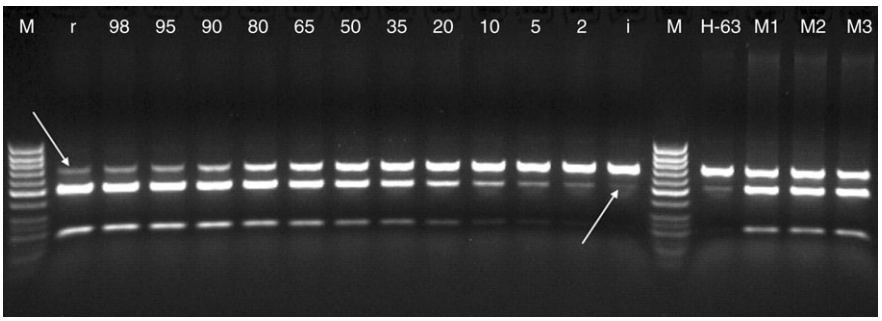

FIG. 1. *Sma*I ITS-RFLP of artificial PCR mixtures and hybrid H63. From left: PCR products of both parents mixed in different ratios [numbers indicate proportion (%) of *E. repens* in each sample; letters 'r' and 'i' refer to reference samples of *E. repens* and *E. intermedia*]; hexaploid hybrid H63; M1–M3: *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.

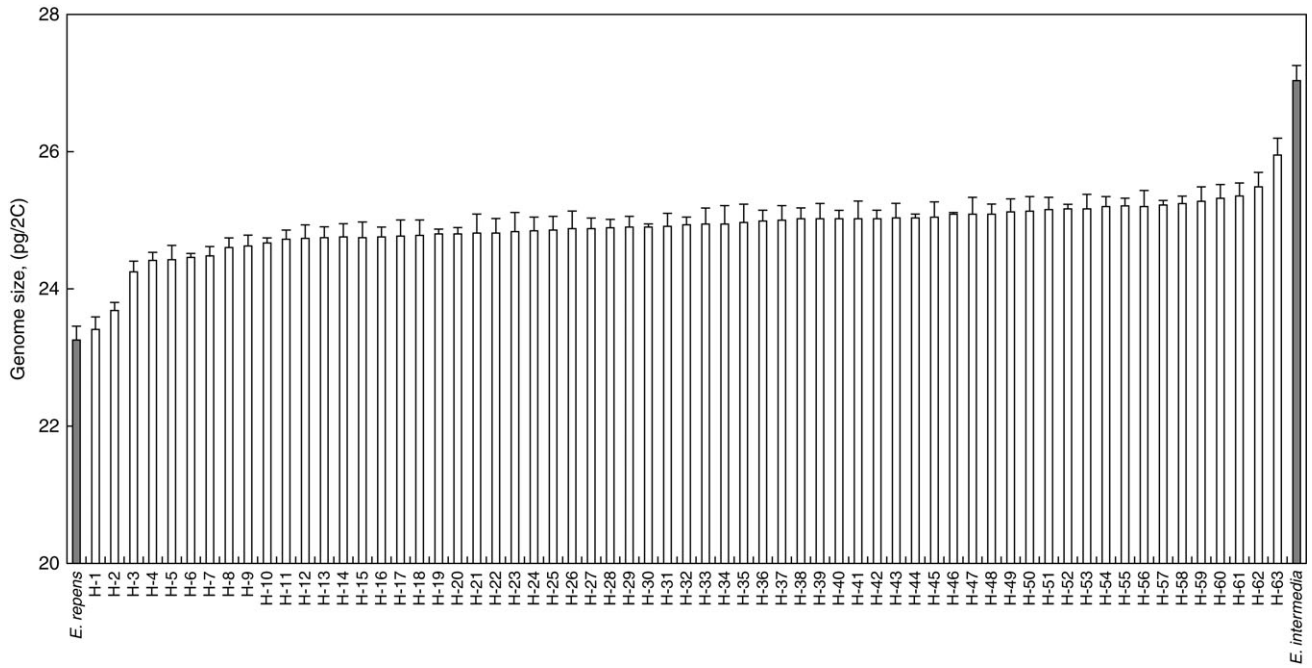

FIG. 2. Absolute genome sizes of hexaploid *E. repens* × *E. intermedia* hybrids. Reference values of *E. repens* and *E. intermedia* are shown in black.

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. 2. All had DNA content intermediate between the parents; plants H1, H2 and H63 deviated from values typical of hybrids, and their genome sizes were approaching either parent (*E. repens* in H1 and H2, *E. intermedia* in H63). All three plants were euploid hexaploids according to chromosome counts. Absolute genome sizes and chromosome numbers of nonaploids and of the progeny of one nonaploid hybrid (N7) 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 were different from 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 outpollination) (nonaploid P12).

Chloroplast DNA analyses

Intraspecific cpDNA variability in ten accessions of *E. repens* was almost absent (one substitution in *trn*L-F), and the present 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 that was identified by sequencing.

In 61 cases out of 63 hexaploid hybrids, *E. intermedia* was found to be the maternal parent. In samples H6 and H9, 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

Despite their allopolyploid origin, ITS copies of all hexaploid parental plants analysed were sufficiently homogenized 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. The parental species consistently differed from each other by 15 substitutions (2·3 % sequence divergence). Thus, ITS provided a taxon-specific marker and was usable for inferring recent hybridization events.

RFLPs revealed a small portion of undigested PCR product in *E. repens* (Fig. 1, left arrow) despite manifold over-digestion. Direct sequencing of this undigested fragment (accession number DQ859049) showed one substitution and an adjacent 1-bp indel, both resulting in a loss of the original restriction site, which was, however, different from the *E. intermedia*-specific substitution. Likewise, *E. intermedia* samples contained a small portion (around

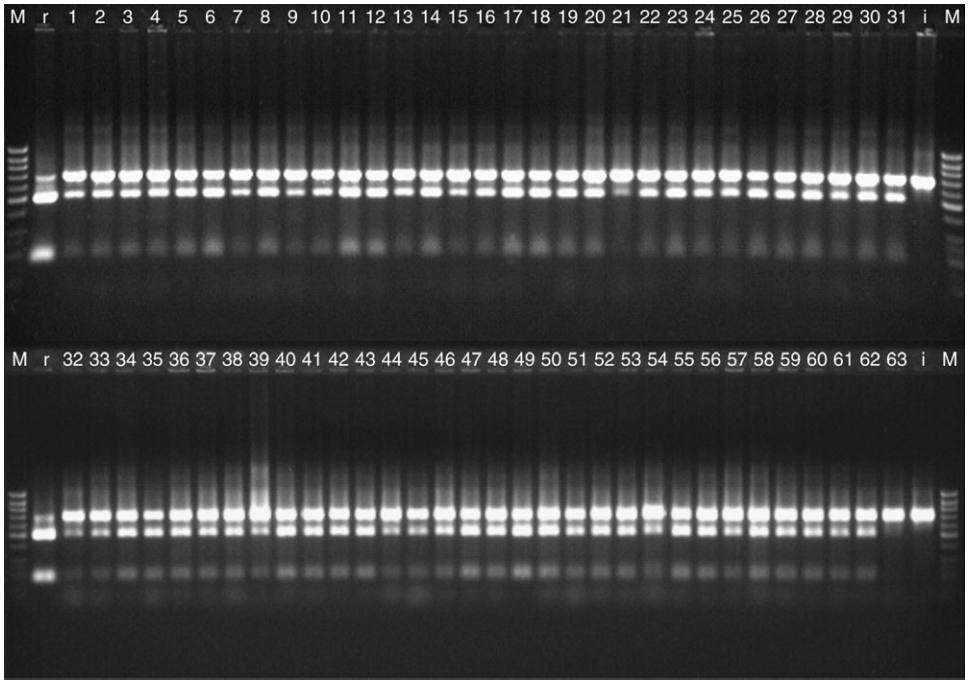

FIG. 3. *Sma*I ITS-RFLP of hexaploid *E. repens* × *E. intermedia* hybrids. Samples are ordered according to their genome size. Letters 'r' and 'i' refer to reference samples of *E. repens* and *E. intermedia*. Approximate lengths of the fragments are 650, 470 and 180 bp.

1%) of ITS copies that were digested with *Sma*I. Direct sequencing of the approx. 500-bp fragment (Fig. 1, right arrow) and BLAST search in GenBank matched an ITS sequence similar to the cloned sequence type AF507808 of *Thinopyrum intermedium* (= *E. intermedia*) (Li *et al.*, 2004) which is very divergent from the major copy type. These minority copies, undetectable by direct sequencing of the original PCR products, were present in several samples of both *Elytrigia* species analysed and suggest a small amount of different ancestral genomes that were not completely homogenized. However, their amount was so low that they did not affect the detection of hybridization between the two species.

All plants determined as hexaploid hybrids by flow cytometry expectedly displayed an additive pattern of parental ITS copies (Fig. 3). Some samples showed overrepresentation of one or the other parental copy.

In nonaploids, restriction digest with *Sma*I showed additive patterns of both parental ITS copies in eight out of nine samples (Table 2). No obvious bias between parental ITS copies was detected in any of these samples (not shown). Sample N1 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 (N1) was a nonaploid cytotype of *E. repens*.

The progeny of one nonaploid hybrid (N7) 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).

Frequency of hybrids and habitat type

The frequency of hybrids differed among the three localities (Table 1). At sites of agricultural land-use, hybrids were common whereas none was found at the steppic locality. One parental species was very rare at both the steppic (*E. repens*) and the agricultural localities (*E. intermedia*). The high proportion of hybrids at locality B-*b* likely reflects sampling focused on morphological variation.

Test of fertility

Five out of 18 hexaploid hybrids and three out of five nonaploids investigated yielded well-developed caryopses. Fertility/germinability was: H6, 1·1 %/100 %; H58, 1·3 %/50 %; H56, 1·5 %/33·3 %; H22, 1·5 %/0 %; H63, 28·8 %/

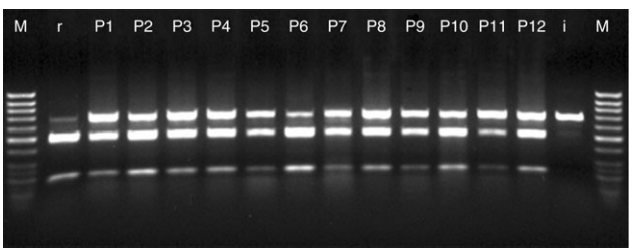

FIG. 4. *Sma*I ITS-RFLP of the progeny of the nonaploid plant N7. Hexaploid *E. repens* (r) and *E. intermedia* (i) were used as reference samples. Samples are ordered according to their chromosome numbers. Approximate lengths of the fragments are 650, 470 and 180 bp.

60 %; N5, 2·0 %/100 %; N8, 3·5 %/33·3 %; N9, 6·5 %/83·3 %. Fertility/germinability of five pure *E. repens* and *E. intermedia* samples ranged between 20·6 and 52·5 %/10 and 100 % and 10·3 and 51·9 %/80 and 90 %, respectively. Average values were 41·5 %/62 % in *E. repens* and 30·9 %/88% in *E. intermedia*.

DISCUSSION

Parental species and ITS copy homogenization

ITS sequences of the *Elytrigia repens* and *E. intermedia* specimens analysed were sufficiently homogenized to provide a taxon-specific marker. Preliminary phylogenetic analysis using the major ITS types in a context of related species (not shown) placed the two species in divergent parts of the ITS tree. Because rDNA arrays occur at several chromosomal loci in Triticeae species (Dubcovsky and Dvořák, 1995; Li and Zhang, 2002), interlocus concerted evolution leading to homogenization of the ITS sequences must have occurred in the *Elytrigia* parental species and apparently is nearly complete. The small amounts of unhomogenized ITS sequences detected in both species may still allow some of the ancient hybridization events that have lead to the present genome composition of both allohexaploid grasses to be traced, but this will require a cloning approach and an efficient screening strategy to identify these rare variants.

Hybrid and putative backcross identification

Identification of putative hybrids was based on the combination of two markers – genome size and additivity of parental ITS copies. While this combined approach enabled first or early generation hybrids to be determined with a high degree of certainty (additivity of ITS copies matched the results obtained by flow cytometry for all hexaploid hybrids and additionally revealed hybrid origin of most nonaploid plants), both markers suffer from limitations for inferring introgression, particularly for the following reasons. (*a*) Genome size was most effective in detecting F_1 hybrids in the present study species, revealing a DNA content intermediate between both parents. In contrast, later-generation hybrids or backcrosses might be more problematic to detect because the hybrids' genome size will approach that of one or the other parental species. (*b*) Ribosomal DNA undergoes its own intragenomic evolution (Álvarez and Wendel, 2003), and homogenization of different ITS copies itself may cause an unpredictably biased representation of particular parental copies in hybrid genomes. This process has obviously happened after the initial formation of the allohexaploid parental species (resulting in mainly one ITS variant each) in the present study, but can also occur with remarkable speed in recent hybrids, even within only two generations of backcrossing (Fuertes Aguilar *et al.*, 1999).

Flow cytometric data suggested only three candidate plants (H1, H2, H63; Fig. 2) to be potential backcrosses. Out of these, only H63 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, almost corresponding with the pure *E. intermedia* sample (Fig. 1). Especially the comparably high fertility of this plant (28·8 %) and its *E. intermedia*-like morphology suggest that this plant could be a later generation backcross. This is the only good example among the present data that backcrosses among hexaploids are possible and do exist (but see also the progeny of the nonaploid hybrid N7 below).

However, gene conversion without meiotic cycles (i.e. hybrids persisting vegetatively by rhizomes) is probably unlikely or less efficient to homogenize divergent ITS copies. In this case, the variable proportions of parental ITS variants among the hexaploid hybrids (Fig. 3) may have another explanation. Up to five different genome types (out of at least seven so far discovered in both parental species) whose individual rDNA loci and copy numbers might differ can be arbitrarily recombined during hybridization. On the other hand, it cannot be excluded that 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 homogenization or locus loss in F_1 hybrids could as well be responsible for the biased ITS copy numbers.

Another example of proven backcrosses is the progeny of the nonaploid mother plant N7. Heavily biased copy numbers of *E. repens* or *E. intermedia* ITS suggest loss of ITS loci of one or the other parent, as indicated by the samples P1–P9, which have similar genome sizes while displaying a difference of three chromosomes, and thus having different genome composition.

As long as there is no more information about the genomic processes concerning rDNA loci in both *Elytrigia* species available, these scenarios remain speculative. As a note of caution, several apparent contradictions between genome size and ITS ratio indicate that there is no clear and easy correlation between these approaches and that neither of them seems to be suitable to study introgression: (*a*) the genome size in hybrids H1 and H2 is approaching that of *E. repens* (Fig. 2), but is unaccompanied by ITS sequences skewed to the *E. repens* type (Fig. 3); (*b*) varying ITS ratios exist among hexaploid hybrids of similar genome size (Fig. 3); and (*c*) approximately equal amounts of both parental ITS variants occur in natural nonaploids of markedly different genome size (see below).

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 (N1) represented a nonaploid cytotype of *E. repens*. For the origin of the eight nonaploid hybrids, two plausible scenarios are proposed (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 (N3, N6) morphologically resembled *E. repens*, whereas four with *E. intermedia* cpDNA (N2, N4, N5, N7) 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 also had *E. intermedia* cpDNA (N8, N9). 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 either parental species is considered because hybrids are partially fertile and might more easily produce unreduced gametes than pure species due to disturbed meiosis (Ramsey and 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 that probably 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 hybrid N7 and a certain fertility of the nonaploids examined in the experimental garden show that at least partial fertility of nonaploids should be expected. The viability of the nonaploid's offspring in nature is unknown. No such plants were found growing spontaneously at locality C, where the nonaploid mother originated. More detailed investigation of localities with nonaploid cytotypes would be desirable in this respect. But recently, a population of heptaploid ($2n = 7x = 49$) cytotypes intermixed with hexaploid *E. intermedia* was discovered at another locality (pers. obs.). Besides heptaploids, several aneuploids ($2n =$ 47, 48, 50) were present there, too, similarly to the N7 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.

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 and Hewitt, 1985; Arnold and 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 and Hodges, 1995; Krahulcová *et al.*, 1996; Wang *et al.*, 1997; Campbell and Waser, 2001; Rieseberg *et al.*, 2003; Campbell *et al.*, 2005; Kirk *et al.*, 2005*a*, *b*). Hybrid fitness in these cases rather displays genotype-by-environment interactions than consistent breakdown. While fitness of the natural hybrids was not measured, the present 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 no *E. intermedia* plants were found out of 28 plants collected; similarly, at locality B-1 no plants of this species were found among 109 plants collected, although hybrids were present there. Due to arbitrary sampling at locality C, it is possible that *E. intermedia* plants were missed, but it is plausible that the species is actually rare or even absent at localities B-*a* 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 hybrids detected (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-*a* 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 and Waser, 2001; Campbell *et al.*, 2005; Kirk *et al.*, 2005*a*, *b*). Complete cytoplasmic incompatibility can be excluded in the present 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 and 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. The present study localities represent two extreme types of habitats: a natural, conserved habitat with nearly no anthropogenic disturbance and agricultural habitats with a high degree of anthropogenic disturbance. The latter habitat type with a

relaxed competition is likely to have 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 the current study is based on the results from only three localities, no generalization can be made, and other aspects such as the history of particular localities have to be taken into account. The present 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 male fertility was not determined in the hybrids in the present study, 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 pre-mating and no strong post-mating 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 and Thomas, 1964; Arnold and Bennett, 1993; Kim and 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 polyploids (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 and Runemark, 1995; Chen *et al.*, 1998). Theoretically, the hybrid between *E. repens* and *E. intermedia* combines genetic material from up to seven different donor species. 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 and 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 with *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 (*a*) multiplication of individuals increases the probabilities simply in a mathematical way; and (*b*) 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 metres from the place where they originated, increasing the chance of finding a suitable place for establishment and sexual partner to mate.

Conclusions and perspectives

In conclusion, it can be stated that *E. repens* and *E. intermedia* frequently cross at places where they co-occur. Hybrid frequency is likely to be 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 that may adapt to different environmental conditions. In the light of weak reproductive barriers and frequent natural hybridization between these species, it cannot be ruled out that introgression may also have contributed to shape the ecological amplitudes of the species.

To further elucidate this, necessary prerequisites are (*a*) to study the (multiple?) origin and genomic composition of both parental species across the study area in more detail, e.g. by the application of single copy nuclear gene markers and/or *in situ* hybridization techniques, and (*b*) to develop a fingerprinting system that allows the identification of vegetatively propagated plants (clones). Based on that knowledge, it will be possible to develop appropriate, sufficiently sensitive markers for studying introgression.

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