NATURAL HYBRIDIZATION BETWEEN *ELYMUS REPENS* AND *ELYMUS HISPIDUS* ASSESSED BY AFLP ANALYSIS

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ABSTRACT

Hybrid speciation within the genus *Elymus* is analyzed and discussed. The supposed hybrid origin of *Elymus ×mucronatus* between *Elymus repens* and *Elymus hispidus* was tested. In this aim, pollen viability and AFLP variation of putative progenitors and progenies from two natural populations were studied. Pollen fertility of hybrids ranging from 0 to 91.95% (average 59.50%) indicates the occurrence of F1 hybrids and backcross hybrids. Seven *Eco*RI/*Mse*I AFLP primer combinations generated a total of 477 AFLP fragments (300 polymorphic) for 48 parental and hybrid individuals. UPGMA and principal coordinates analysis of the AFLP data set show that hybrids are closer to *E. hispidus* than to *E. repens* and suggest unidirectional introgression towards *E. hispidus*. AFLP fragments of the hybrid taxon were additively inherited from the parental species and no hybrid-specific bands were identified. AFLP analysis validates the hybrid status of *E. ×mucronatus*. Populations in which *E. repens*, *E. hispidus* and their hybrids co-occur still progress from early hybridization stages of F1 hybrids to an advanced hybridization stage of a well-established hybrid swarm. *E. ×mucronatus* is potentially self-sustaining by predominant vegetative reproduction by rhizomes and partial fertility leading to the development of mature seeds. We propose to consider *Elymus ×mucronatus* as a new species of hybrid origin in the Polish flora.

KEY WORDS: AFLP, genetic variation, pollen fertility, unidirectional introgression, *Elymus repens*, *Elymus hispidus*, *Elymus ×mucronatus*.

INTRODUCTION

Hybridization is a well known natural phenomenon and an important mechanism of evolution in plants (Rieseberg 1995; Arnold 1997; Hegarty and Hiscock 2005; Wissemann 2005). However, the frequency of spontaneous hybridization is not universal and differs between plant families and genera, as demonstrated in multiple biosystematic floras (Ellstrand et al. 1996). In natural habitats, many spontaneous hybrids have been found in the tribe Triticeae Dumort., especially between polyploidy species (Nelson and Tyrl 1978; Salomon and Lu 1992; Assadi and Runemark 1995). Hybridization and subsequent introgression of genes of one species into the genomes of others can increase morphological variation of Triticeae species, and in particular of grasses belonging to the genus *Elymus* L. (Brown and Pratt 1960; Nelson and Tyrl 1978; Diaz et al. 1999).

Elymus repens (L.) Gould [Syn.: *Agropyron repens* (L.) P. Beauv., *Elytrigia repens* (L.) Desv. *ex* Nevski] is a cosmopolitan species and occurs almost in the entire world (Hultén and Fries 1986). It is a species characterized by a high tolerance to environmental stress factors and grows in very diverse habitats from wet to strongly dry, and displays great inter- and intrapopulational morphological polymorphism (Prokudin 1982; Szczepaniak 2002a, 2003a; Szczepaniak et al. 2002). Three varieties (var. *repens*, var. *aristatus* and var. *subulatus*) differing mainly in the length of glume awns, lemma awns and the glume shape were distinguished within *E. repens* in Poland (Szczepaniak 2002b, 2003a).

Elymus hispidus (Opiz) Melderis [Syn.: *Agropyron intermedium* (Host) P. Beauv., *Elytrigia intermedia* (Host) Nevski] is included in the group of the Mediterraneo-Irano-Turanian connective element and occurs in the area from Central Asia to Southern and Western Europe, reaching France and Spain (Tsvelev 1976; Melderis 1980). This perennial grass occurs in Poland primarily in xerothermic grasslands of the Festuco-Brometea Br.-Bl. et Tx. 1943

class, which are confined to warm, sunny and dry slopes between fields and to steep escarpments of river valleys, composed of the calcareous or loess substratum (Szczepaniak 2001). Two subspecies (subsp. *hispidus* and subsp. *barbulatus*) or varieties (var. *hispidus* and var. *villosus*, respectively) are distinguished in the Polish flora on the basis of hairless or hairy glumes and lemmas (Szczepaniak 2002b, 2003b; Szczepaniak and Cieślak 2003).

Natural hybridization between *Elymus repens* and *E. hispidus* growing together in the same locality is probable. Two species of hybrid origin were originally and validly described as *Agropyron ×mucronatum* Opiz ex Bercht. (Berchtold and Opiz 1836) [Syn.: *Elymus repens × E. hispidus* var. *hispidus*] and *Agropyron ×tesquicolum* Prokud. (Prokudin 1940) [Syn.: *E. repens × E. hispidus* var. *villosus*]. Prokudin and Druleva (1971, 1972) later described the same species as *Elytrigia ×mucronata* (Opiz ex Bercht.) Prokudin, arisen by interspecific hybridization between *Elytrigia repens* and *Elytrigia intermedia*, and *Elytrigia ×tesquicola* (Czerniak.) Prokudin, a hybrid between *Elytrigia repens* and *Elytrigia trichophora*. The occurrence of hybridization among those species of *Elytrigia* has been suggested on the basis on morphological and anatomical intermediacy of some individuals found in the same habitat as their putative progenitors. Recent flow cytometric and karyological investigations have confirmed common natural hybridization in the *Elytrigia repens – E. intermedia* alliance (Mahelka et al. 2005). The genome size turned out to be a reliable marker for these taxa and hybrid delimitation.

Multivariate statistical analyses of intraspecific morphological variation of *Elymus repens* and *E. hispidus* clearly show intermediate individuals among those species that were defined as specimens of the hybrid origin species *Elymus ×mucronatus* with two varieties: nvar. *mucronatus* [Syn.: *E. repens × E. hispidus* var. *hispidus*] and nvar. *tesquicolus* [Syn.: *E. repens × E. hispidus* var. *villosus*] (Szczepaniak 2003b). These varieties differ only in the degree of glume and lemma hairiness, and occur in the same habitats. Hybrid individuals are often clearly distinguished from their parental species mainly by the presence of ciliate leaf sheath margins, more or less truncate and very shortly mucronate glumes (like in *E. hispidus*), and gradually tapering awned lemmas (like in *E. repens*). Interestingly, there is a great morphological variation among the hybrids examined. This suggests that hybrid individuals may be a hybrid swarm or/and introgressant forms, and that advanced hybridization is taking place.

In the present work, the hypothesis that morphological intermediate individuals have derived from ancient and/or recent hybridization between *Elymus repens* and *E. hispidus* was tested. For this purpose: (1) fertility of presumable hybrids was estimated, (2) AFLP analysis was used to assess the relationship and genetic distinction or its lack between the putative hybrids and their progenitors. The taxonomic status of the hybrids was established.

Molecular techniques have made the detection of hybridization increasingly easier. Allendorf et al. (2001) argue for advantages of genetic markers over morphological data to reveal hybrid individuals. The Amplified Fragment Length Polymorphism (AFLP) method (Vos. et al. 1995) has successfully been used to investigate the gene flow and genomic changes resulting from hybridization and introgression in polyploid taxa of the genus *Achillea* (Guo et al. 2005). Taxonomic distinctions of new species of hybrid origin, e.g., *Cardamine ×enriquei* (Marhold et al. 2002), *Mangifera ×odorata* (Teo et al. 2002), and *Sonneratia*

TABLE 1. Sample size (N) and localities of *Elymus repens*, *E. hispidus* and *E. ×mucronatus* analyzed for AFLP.

	Populations	N	Localities	Position	Habitats
Elymus repens					
R1	var. *aristatus*	5	Poland, Małopolska Upland,	N50°17' E14°20'	Loess slope along road,
R2	var. *repens*	5	Niecka Nidziańska Basin, Smoniowice near Miechów		bordering with cultivation field
R3	var. *repens*	4	Poland, Kielce-Sandomierz Upland, Kielce	N50°53' E20°37'	Excavation of old quarry, on calcareous, stony soil
Elymus hispidus					
H	var. *hispidus*	5	Poland, Małopolska Upland,	N50°17' E14°20'	Loess slope along road,
B	var. *villosus*	3	Niecka Nidziańska Basin,		bordering with cultivation field
H3	var. *hispidus*	4	Smoniowice near Miechów		
B3	var. *villosus*	3	Poland, Kielce-Sandomierz Upland, Kielce	N50°53' E20°37'	Excavation of old quarry, on calcareous, stony soil
Elymus ×mucronatus					
M1	nvar. *mucronatus*	5	Poland, Małopolska Upland,	N50°17' E14°20'	Loess slope along road, bordering
M2	nvar. *mucronatus*	5	Niecka Nidziańska Basin,		with cultivation field
Te	nvar. *tesquicolus*	5	Smoniowice near Miechów		
M3	nvar. *mucronatus*	4	Poland, Kielce-Sandomierz Upland, Kielce	N50°53' E20°37'	Excavation of old quarry, on calcareous, stony soil
Total		48			

×*gulngai* (Zhou et al. 2005) were revealed by AFLP markers. A combination of morphological and molecular approaches, including the AFLP method, helped to determine the degree of genetic distinctiveness, hybridization and clonality for some *Potamogeton* species (Whittall et al. 2004). AFLP markers allowed to identify some hybrid individuals of *Salix* ×*rubens* that were morphologically identical to one of the parental species *Salix fragilis* (Beismann et al. 1997).

MATERIAL AND METHODS

Plant material

Young leaves from specimens morphologically identified a priori as putative hybrids *Elymus* ×*mucronatus* nvar. *mucronatus* (14 individuals), *E.* ×*mucronatus* nvar. *tesquicolus* (5 individuals) and presumable parental species *E. repens* (14 individuals) and *E. hispidus* (15 individuals) were sampled from two localities where they grew together: Smoniowice (Małopolska Upland, S Poland) and Kielce (Kielce-Sandomierz Upland, S Poland) (Table 1). As the plants are strongly rhizomatous, individuals located ca. 6 m distant from each other were collected to avoid sampling from the same clone. Hybrid identification was based on their combination of parental morphological characters that were identified by Prokudin and Druleva (1971, 1972) and Szczepaniak (2003b) as the character set distinguishing two hybrid-origin taxa. Voucher specimens were deposited in the herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences, in Cracow (KRAM).

Pollen fertility

Specimens of *Elymus repens* (5 individuals), *E. hispidus* (7 individuals) and *E.* ×*mucronatus* (15 individuals) were tested for the pollen fertility status using the acetic orcein staining method (Table 2). Spikes of these species were collected from the locality in Smoniowice, and preserved in 70% ethanol solution. Mature anthers from one or two florets from the middle spikelet of the spike were removed onto a microscopic glass slide and macerated in a drop of aceto orcein stain to release pollen grains.

To determine pollen fertility, 300-400 grains were evaluated per individual, and darkly, well-stained pollen grains were recorded as fertile and viable, whereas unstained, very lightly stained or shrunken ones were considered as sterile or non-viable. Pollen fertility was calculated by dividing the number of viable pollen grains by the total number of grains counted in the field of view and averaging them for all the plants in that species. Pollen quality was expressed as the percentage of viable grains detected in each species.

TABLE 2. Comparison of pollen fertility between putative parental species, *Elymus repens*, *E. hispidus* and hybrid *E.* ×*mucronatus* from population from Smoniowice. *N* – number of examined plants. Number of sterile hybrids and with very low fertility ranged from 0 to 5% in bracket.

| Taxa | N | Pollen fertility percentage | |
		Range	Mean ± S.D.
Elymus repens	5	78.72-97.26	88.05±6.71
Elymus hispidus	7	21.33-95.93	80.26±26.34
Elymus ×*mucronatus*	12 (3)	0.00-91.95	59.50±37.73

AFLP analysis

Genetic diversity of *Elymus repens, E. hispidus* and *E.* ×*mucronatus* was assessed using Amplified Fragment Length Polymorphism (AFLP; Vos et al. 1995). Total genomic DNA was extracted from 50 mg of dried leaf tissue following the DNeasy Plant Mini Kit procedure (Qiagen).

AFLP fingerprinting was performed according to the modifications of Bednarek et al. (1999). Genomic DNA was digested with two restriction enzymes (*Eco*RI and *Mse*I). The resulting *Eco*RI and *Mse*I sticky ended fragments were ligated to the complementary ends of synthetic adaptors. The PCR preselective amplification was performed using primers with a single selective nucleotide: *Eco*RI+A and *Mse*I+C. The final selective amplification was performed using seven selective primers pairs: E-AGT/M-CTG, E-AGT/M-CTT, E-AGG/M-CGG, E-AGG/M-CAG, E-ACA/M-CGC, E-ACA/M-CAA and E-ACC/M-CCA, which revealed the greatest number of polymorphic, well-separated, and reproducible bands. The *Eco*RI primer in these reactions was labelled with ^{32}P. Amplified fragments were separated on 7% polyacrylamide gels and exposed to X-ray film (Kodak) at -30°C for 1-2 days.

Data analysis

For the genetic diversity analysis, AFLP fragments were scored as present (1) or absent (0), and used as a raw data matrix. Total number of amplified fragments, the number of polymorphic loci, and the percentage of polymorphic loci were individually scored for parental and hybrid taxa (Table 3). The number of unique, species-specific and hybrid-specific AFLP fragments (e.g. whether they were present with 100% frequency in all the samples of one taxon and absent in all the samples of other taxa) were next detected within *Elymus repens, E. hispidus* and *E.* ×*mucronatus*. The fragments shared between the assumed parental species were identified. Inherited patterns of parental AFLP fragments in the putative hybrids were analyzed in different combinations as: (1) number of shared AFLP fragments between either the supposed parental species and the hybrids (without the second parent) for confirmation of parental contribution in hybridization of *E. repens* and *E. hispidus*, respectively, (2) number of exclusively shared species-specific AFLP fragments between *E. repens* and all the hybrid specimens, and between *E. hispidus* and all the hybrid specimens, for the detection of the potential introgression direction (Table 4).

Genetic similarities among individuals of *Elymus repens*, *E. hispidus* and *E.* ×*mucronatus* were calculated for all AFLP phenotypes data and using the method developed by Nei and Li (1979). Genetic parameters were computed using POPGENE vers. 1.31 (Yeh et al. 1997).

The clustering UPGMA method (unweighted pair group method average) was applied using the matrix of Nei and Li's similarity coefficients (Nei and Li 1979) with the NTSYS-pc software vers. 2.02j and was performed (Rohlf 1993).

Principal coordinates analysis (PCoA, Gower 1966) was based on Euclidean distances matrix and was performed using the MVSP ver. 3.1 (Kovach 1999). UPGMA and PCoA were used to resolve patterns of variation and relationships among and within taxa.

The hierarchical genetic structure (between the carried out and hybrid taxa, among the accessions and within the

TABLE 3. Selected seven primer combinations and polymorphism rates for AFLP analysis of the 3 accessions of *Elymus repens*, 4 accessions of *E. hispidus* and 4 accessions of hybrids *E. ×mucronatus*.

Primers combinations *Eco*RI…/*Mse*I…	All accessions		*Elymus repens*		*Elymus hispidus*		*Elymus ×mucronatus*	
	Total	Polymorphic	Total	Polymorphic	Total	Polymorphic	Total	Polymorphic
	fragments		fragments		fragments		fragments	
AGT/CTG	66	53	46	16	48	17	49	16
AGG/CGG	77	64	53	21	54	14	57	24
ACA/CGC	74	66	66	40	53	24	56	36
ACA/CAA	85	57	67	17	60	4	63	13
AGG/CAG	51	16	45	2	47	7	49	9
AGT/CTT	57	25	48	2	46	5	48	7
ACC/CCA	67	19	59	3	57	3	58	5
Total	477	300 (62.89%)	384	101 (26.30%)	365	74 (20.27%)	380	110 (28.95%)
Mean	68.11	42.86	54.86	14.43	52.14	10.57	54.29	15.71
SD	11.70	21.97	9.26	13.82	5.34	7.93	5.71	10.98

TABLE 4. Number of unique, species-specific AFLP fragments for each *Elymus repens*, *E. hispidus* and *E. ×mucronatus* species and number of shared AFLP fragments between putative parental species and hybrids.

Primers combinations *Eco*RI…/*Mse*I…	No. of species-specific AFLP fragments for *E. repens*	No. of species-specific AFLP fragments for *E. hispidus*	No. of hybrid-specific AFLP fragments	No. of shared AFLP fragments between *E. repens* and *E. hispidus* (without hybrids)	No. of shared AFLP fragments between *E. repens* and hybrids (without *E. hispidus*)	No. of shared AFLP fragments between *E. hispidus* and hybrids (without *E. repens*)	No. of species-specific AFLP fragments exclusively shared between *E. repens* and hybrids (with 100% frequency)	No. of species-specific AFLP fragments exclusively shared between *E. hispidus* and hybrids (with 100% frequency)
AGT/CTG	11	13	0	13	15	31	0	13
AGG/CGG	14	15	0	13	14	32	0	11
ACA/CGC	4	2	0	8	8	18	0	1
ACA/CAA	16	14	0	28	36	49	0	13
AGG/CAG	2	5	0	35	35	40	0	5
AGT/CTT	10	7	0	32	32	41	0	7
ACC/CCA	8	5	0	48	47	53	0	4
Total	65	61	0	177	187	264	0	54
Mean	9.29	8.71	0	25.29	26.71	37.71	0	7.71
SD	5.06	5.19	0	14.51	14.40	11.86	0	4.72

accessions) was assessed using analysis of molecular variance (AMOVA). AMOVA is based on the nonparametric permutation approach (Excoffier et al. 1992) and on pairwise squared Euclidean distances between AFLP phenotypes. AMOVAs were performed using ARLEQUIN version 2.0 (Schneider et al. 2000.

RESULTS

Fertility of the putative parental species and the hybrids

Pollen fertility results obtained using the aceto orcein stain method are given in Table 2. Average pollen viability was comparable for the parental species and ranged from 80.26% (±26.34) for *Elymus hispidus* to 88.05% (±6.71) for *E. repens*. It was interesting to note that *E. hispidus* showed a wide range of pollen viability varying from 21.33 to 95.93%. However, only one specimen, morphologically defined as *E. hispidus* var. *hispidus*, was low fertile, which suggests its hybrid origin.

Pollen fertility among the hybrids was highly variable and ranged from complete sterility 0% to high fertility 91.95% (average 59.50±37.73). One of *E. ×mucronatus* specimens was sterile and two specimens had about 5% fertile pollen grains. Two hybrids showed pollen fertility of less than 50%, while the other seven specimens had about 80% fertile pollen grains. This variation of fertility degree indicates different origins of hybrids that can be F1 individuals (most probably sterile in this case) or backcross hybrids (they exhibit higher fertility), arisen by mating with one or with both parental species or among hybrids.

AFLP polymorphism in the parental species, Elymus repens and E. hispidus, and in hybrids

Seven AFLP primer combinations show a total of 477 clearly identifiable and reproducible bands of all the assumed

progenitors *Elymus repens* and *E. hispidus* and the putative hybrid progenies between them. Of these AFLP bands, 300 (62.89%) were polymorphic and 177 (37.11%) bands were common to all the samples analyzed (Table 3). On the average, 42.86 polymorphic AFLP fragments were detected per primer pair. For 14 individuals of *E. repens* and 15 individuals of *E. hispidus*, 101 (26.30%) and 74 (20.27%) polymorphic bands were detected, respectively. However, putative hybrid-derived samples were more genetically diversified than the putative parents, displaying a total of 110 (28.95%) polymorphic AFLP fragments (Table 3).

The number of legible species-specific fragments produced with each primer pair is given in Table 4. The analysis of the data obtained from scoring the monomorphic species-specific fragments (which were exclusively present in a given taxon) shows 65 fragments unique to *Elymus repens* and a comparable number of 61 fragments unique to *E. hispidus*. The parental species, without the hybrids, shared 177 (37.11%) AFLP fragments. No exclusive hybrid-specific fragments existed in any of the 19 individuals of *E. ×mucronatus*.

Inheritance of the parental banding patterns in the putative hybrids is given in Table 4. A total of 187 (48.70%) *Elymus repens* and 264 (72.33%) of *E. hispidus* AFLP fragments were found in the hybrids. Furthermore, the analysis of species-specific fragments shared exclusively between *E. repens* and hybrid specimens and between *E. hispidus* and the hybrids revealed no common fragments in the former and 54 AFLP fragments in the later case, respectively.

Cluster analysis

Cluster analysis (UPGMA) based on the matrix of genetic similarities among the parents and putative hybrids re-sulted in a dendrogram that clearly distinguished two main groups corresponding to *Elymus repens* and *E. hispidus* with hybrids (Fig. 1). These two clusters were identified at the 67% similarity level. In the dendrogram, individuals of the putative hybrids (*E. ×mucronatus* nvar. *mucronatus* – M1, M2, M3, and *E. ×mucronatus* nvar. *tesquicolus* – Te) were grouped with *E. hispidus* individuals (*E. hispidus* var. *hispidus* – H, H3, and *E. hispidus* var. *villosus* – V, V3) and formed one cluster. Some hybrid derivatives (M1 and M2) grouped within the main cluster formed subclusters between their parents, and were somewhat genetically distinct from *E. hispidus* and other hybrids, which may suggest more advanced hybridization and the beginning of the hybrid stabilization process.

The analysis shows that individuals of *Elymus hispidus* in both varieties and hybrids collected in the same habitat were more similar to each other than to the individuals collected at the other locality. Individuals of *E. hispidus* var. *hispidus* (H3), var. *villosus* (V3) and *E. ×mucronatus* nvar. *mucronatus* (M3) from Kielce are clustered together (Fig. 1). Likewise, the specimens of *E. hispidus* var. *hispidus* (H), var. *villosus* (V) and *E. ×mucronatus* nvar. *tesquicolus* (Te) from Smoniowice are grouped together, which may indicate a gene flow within the species and among the hybrids within one habitat. Furthermore, specimens of *E. repens* var. *repens* (R2) appear to have a closer genetic relationship with *E. repens* var. *aristatus* (R1) from the same habitat in Smoniowice than with *E. repens* var. *repens* (R3) from the locality in Kielce.

Principal coordinates analysis

PCoA results are on the whole similar to those of UPGMA, and clearly show a genetic distinction of *Elymus repens* from *E. hispidus* and the hybrids (Fig. 2). The first th-
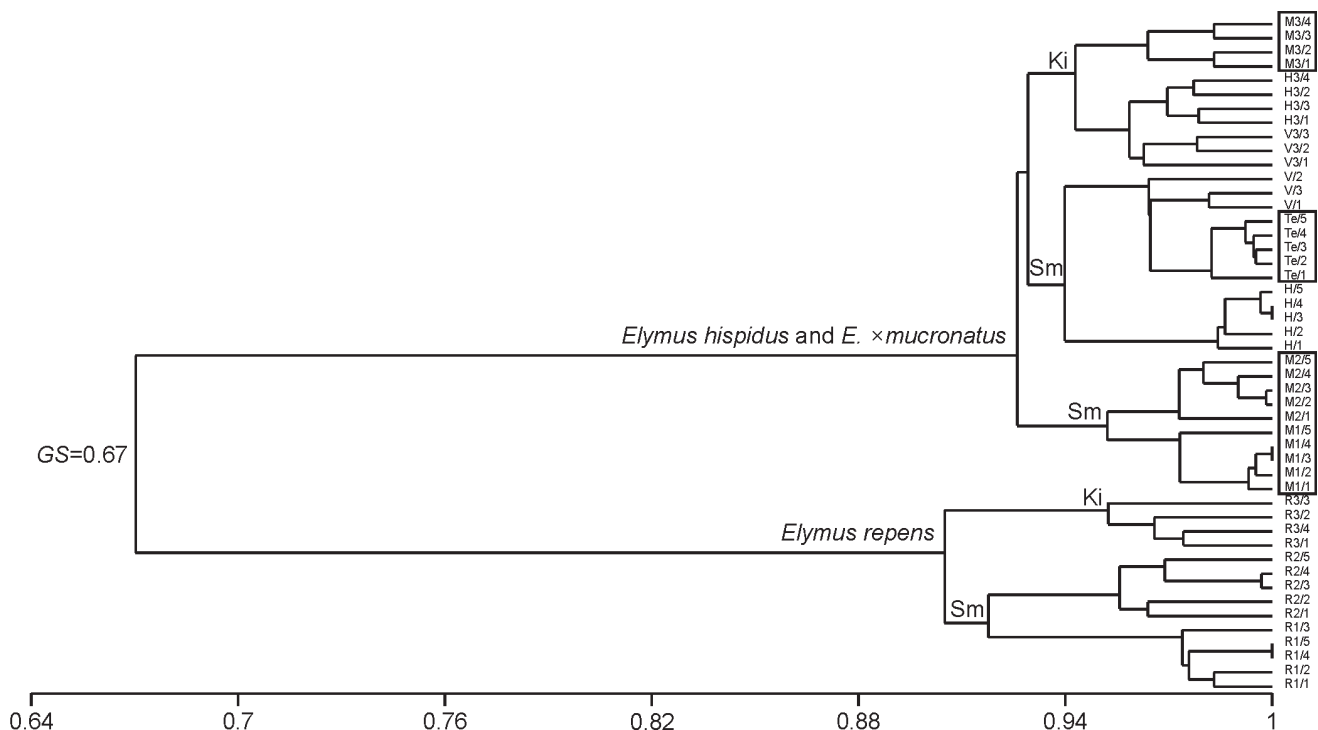

Fig. 1. UPGMA dendrogram of *Elymus repens* (R), *E. hispidus* (H, V) and *E. ×mucronatus* (M, Te) individuals from two localities: Smoniowice (Sm) and Kielce (Ki), based on Nei and Li's similarity coefficients (*GS*) and 477 AFLP fragments. Details of accessions labels correspond to the abbreviations given in Table 1. All the hybrid individuals were marked by frames on the dendrogram.
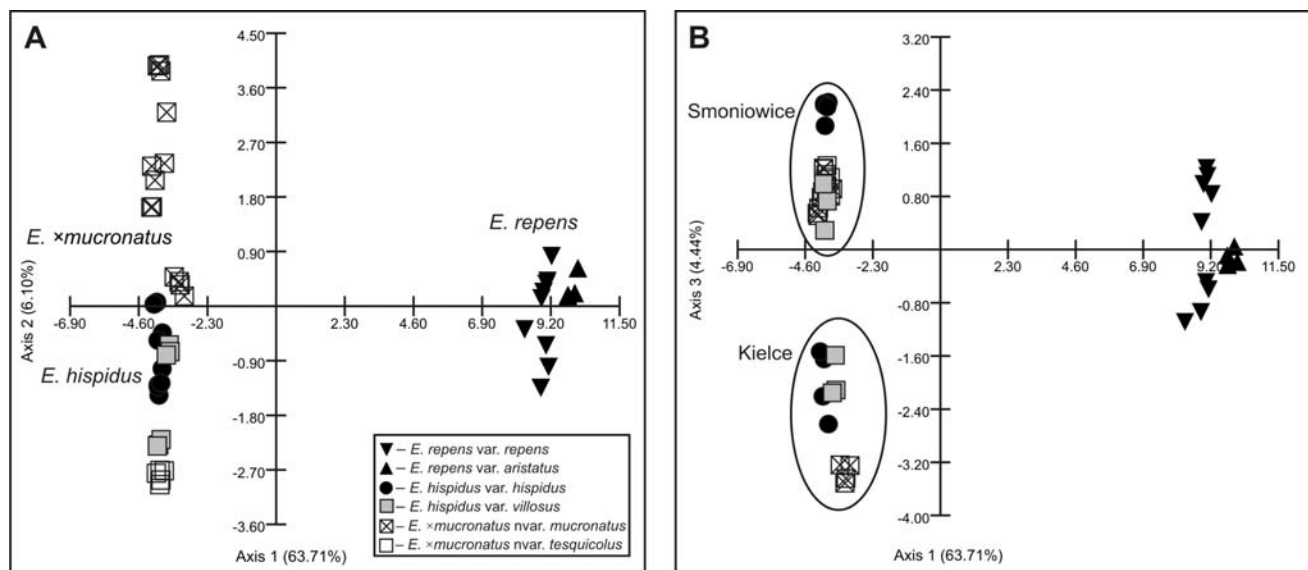
Fig. 2. Principal coordinates analysis (PCoA) of individuals of the parental species *Elymus repens* and *E. hispidus* and their hybrids *E. ×mucronatus* collected in two localities: Smoniowice and Kielce, based on AFLP data. Plot of principal coordinate one against two (A) and three (B).

ree axes accounted for 74.25% of the total genetic variation (63.71% for axis 1, 6.10% for axis 2, and 4.44% for axis 3). The second axis separates some *E. ×mucronatus* nvar. *mucronatus* specimens most clearly (Fig. 2A), similarly to UPGMA, whereas the third axis strongly groups specimens of *E. hispidus* and the hybrids from individual habitats in Smoniowice and Kielce (Fig. 2B).

As shown by the analysis of AFLP polymorphism of studied taxa and UPGMA and PCoA results, the hybrids appear genetically closer to *Elymus hispidus* than to *E. repens*. This is summarized by average genetic similarities between these groups of 92% for the hybrids and *E. hispidus*, and 65% for hybrids and *E. repens*. The parental species are less similar to each other than *E. ×mucronatus* to *E. hispidus* (Table 5). This high genetic similarity of the hybrids to *E. hispidus*, can be formed by unidirectional backcrossing of partially fertile hybrid individuals with one parental species *E. hispidus*.

TABLE 5. Genetic similarity (*GS*) within and between individuals of *Elymus repens*, *E. hispidus* and *E. ×mucronatus* hybrids based on Nei and Li's similarity coefficients (Nei and Li 1979).

Species	Nei and Li's similarity coefficient	
	Range	Mean
E. repens	0.881-0.983	0.936
E. hispidus	0.928-0.997	0.954
E. repens vs. *E. hispidus*	0.630-0.715	0.640
E. repens vs. hybrids	0.631-0.702	0.648
E. hispidus vs. hybrids	0.904-0.957	0.919

Significant genetic diversity (68.21%, *p*<0.006) between parental species *Elymus repens*, *E. hispidus* and hybrids *E. ×mucronatus* was confirmed by AMOVA results based on the grouping of the specimens into particular taxa obtained in UPGMA and PCoA (Table 6). Genetic variation between accessions of individual taxa was also significant (21.68%, *p*<0.001) while the genetic variation within accessions was not statistically significant.

DISCUSSION

The putative parental species *Elymus repens* and *E. hispidus* frequently co-occur in natural and disturbed habitats (present authors' field observations). Some specimens are readily identifiable as "pure" species in such populations while others are morphologically intermediate, clearly having characters of both parental species. Other intermediate plants cannot be identified with certainty because of their great similarity to either *E. hispidus* or *E. repens*. Such two populations were studied in an attempt to determine whether interspecific hybridization occurred between *E. repens* and *E. hispidus* or whether *E. hispidus* exhibited great morphological variation.

Results of previous statistical analyses of some morphological characters such as the lengths of spikes, spikelets, glumes and lemmas did not sufficiently affirm the hybrid origin of *Elytrigia ×mucronata* and *Elytrigia ×tesquicola* (Prokudin and Druleva 1971, 1972). Nonetheless, a detailed biosystematic study of *Elymus repens* and *E. hispidus* confirmed the occurrence of natural hybrids among those spe-

TABLE 6. Summary results of the analysis of molecular variance (AMOVA) based on AFLP data. Plants represented 3 accessions of *Elymus repens*, 4 accessions of *E. hispidus* and 4 accessions of hybrids *E. ×mucronatus*. Levels of significance are based on 1023 iteration steps.

Level of variation	d.f.	Variance components	Percentage of variation	Fixation index	p
Among taxa	2	51.10	68.21	F_{CT}=0.682	<0.006
Among accessions within taxa	8	16.24	21.68	F_{SC}=0.682	<0.001
Within accessions	37	7.57	10.11	F_{ST}=0.899	NS
Total	47	74.91	100		

cies in Poland (Szczepaniak 2002b, 2003b). Hybrid progenies combine both progenitors' morphological, anatomical and genetic characters, and are clearly distinguishable in multivariate statistical analyses (Szczepaniak 2003b).

Pollen quality evaluation and AFLP analysis indicate hybridization between *Elymus repens* and *E. hispidus*. However, the question arises whether we deal with F1 hybrids and/or next generations of hybrids within mixed populations; in other words, how far a population has progressed from early hybridization (F1 only) stages to a well-established hybrid swarm.

Pollen fertility: implications for hybridization stages

On the whole, average pollen fertility of hybrids *Elymus* ×*mucronatus* is relatively high, and about 50% of pollen grain sets are viable and able to pollinate. At the same time, pollen fertility among the hybrid individuals is extremely varied and ranges from complete sterility of 0% to nearly complete fertility of 90%, sometimes higher than in the parental species (Table 2). Different stages of hybridization can coexist in populations where parental species and hybrids co-occur. The early hybridization stage indicates high sterile of F1 hybrid individuals (25% of analyzed plants). Sterility of F1 hybrids between *E. repens* and *E. hispidus* was also confirmed by Assadi (1995), who studied meiosis in a plant with 2n=42.

Our results show that the populations of *Elymus repens*, *E. hispidus* and hybrids still progress from early hybridization stages (F1) to a well-established hybrid swarm, that is an advanced hybridization stage. A hybrid swarm is a population of individuals that are all hybrids by a varying number of generations of backcrossing with parental species and by mating among hybrids (Allendorf et al. 2001). Low viable F1 hybrids of *E.* ×*mucronatus* are unlikely to mate among themselves. However, backcrossing with either of the high fertility parental species, *E. repens* or *E. hispidus*, is more probable.

Introgression effects blur genetic integrity of parental taxa by the gene flow between their populations. Hybrids, backcrossing with either of the parents, are a good platform for the intertaxa gene flow. Assadi and Runemark (1995) obtained artificial F1 hybrids between *Elymus repens* and *E. hispidus*. The shape of the hybrid's spike and the hairiness of glumes and the lemma were similar to those of *E. hispidus*. The anthers were released and were partly dehiscent, and the pollen fertility was 20%. The authors emphasize high polyploidy of both parents. Therefore, it is not unexpected to find 20% fertile pollen in the hybrids. Since hybrids between *E. repens* and *E. hispidus* are relatively common in the field and propagate vegetatively by rhizomes, at least some gene flow via backcrossing to the parents can be expected. In our study, average pollen fertility of hybrids is higher than that found by Assadi and Runemark (1995), which can be caused by advanced hybridization and a greater frequency of introgressants of following generations than that of F1 hybrids in the populations studied. Fertility is one of the factors stabilizing hybrid-derived individuals and in effect gives rise to new lineages of species or even genera. A single, even partially fertile, hybrid individual may suffice as a progenitor of a new species. However, that tiny fraction, summed over thousands of generations, can make a considerable impact in evolutionary time (Ellstrand et al. 1996).

Genetic evidence for hybridization between Elymus repens and Elymus hispidus

Significant genetic differences between the parental species *Elymus repens* and *E. hispidus* were proved by the AMOVA performed in our study. In comparison with the level of intraspecific genetic variation determined in a previous study (Szczepaniak and Cieślak 2003), a similar level of polymorphic loci within *E. repens* (104 loci vs 101 loci) and a considerably lower number of polymorphic loci within *E. hispidus* (167 loci vs 74 loci) were detected in the present research. Such a decrease in intraspecific genetic variation of *E. hispidus* can be caused by putative interspecific hybridization and the loss of part of the parental genetic pool for hybrid genetic variation.

It is apparent from the AFLP analyses that *Elymus repens* and *E. hispidus* are progenitors of the hybrid taxon *Elymus* ×*mucronatus*. AFLP data show a number of unique fragments of the parental species, but we did not find fragments specific to hybrid taxa (Table 4). Parental AFLP fragments inherited from both parents were present in all of the hybrid individuals. The lack of the accumulation of genetic changes in the hybrids could be explained by relatively recent origin of the hybrids. In this case, micro-evolutionary processes continue in the local habitats. A relatively recent hybrid-derived taxon is unlikely to have unique alleles, and, for biparentally inherited fragments, patterns would be likely to be an additive combination of the two parents (Goldman et al. 2004).

Although cluster analysis based on the AFLP data, can relatively easily distinguish *Elymus* ×*mucronatus* from both parental species, the percentage of similarity with the parental species was very high (65% with *E. repens* and as much as 92% with *E. hispidus*). This provides further evidence for a recent origin of this hybrid species. Does such a high genetic similarity between *E. hispidus* and hybrids suggest that these putative hybrid individuals can be elements of the variability range of *E. hispidus* and locate them at its extreme limit? Such a genetic similarity of individuals of different taxa indicates a weakness of reproductive barriers and the local gene flow between parental species and hybrids. Previous studies of Prokudin and Druleva (1971, 1972) prove that flowering times and rhythms in two putative parental species coincide, which confirms the possibility of their crossing. All of the taxa studied are hexaploids with 2n=42 (Mizianty et al. 2001). Meiosis in *Elytrigia* ×*mucronata* proceeds normally without any disturbances (Prokudin and Druleva 1971). Disturbances found in the reductive division, e.g. in the metaphase and anaphase of the first division, in *Elytrigia* ×*tesquicola* indicate its hybrid derivation (Prokudin and Druleva 1972). Such a relatively regular meiosis in hybrids suggests that their progenitors, *E. repens* and *E. hispidus*, are two phylogenetically similar hexaploid taxa.

AFLP data show that most hybrids grouped in their own intermediate cluster, which proves their certain genetic distinction and may indicate the beginning of the hybrid stabilization process and speciation (Fig. 1). The genetic results confirm the evidence of biometric analyses of morphological characters set, which clearly distinguishes *Elymus* ×*mucronatus* from the parental species (Szczepaniak 2002b, 2003b). Individuals of *E.* ×*mucronatus* marked as M1 were characterized by more or less truncate glumes,

hairless, sharp lemmas with long awns and ciliate margins of leaves sheaths. Individuals of M2 differ from M1 by the sharply pointed lemmas or lemmas with short awns. Some of these hybrids were fertile; however, vegetative propagation has made a major contribution to the establishment and persistence of *E.* ×*mucronatus.*

Other hybrid individuals are genetically relatively similar to *Elymus hispidus* or *E. repens*, which resulted in a mosaic of morphological parental characters. The results show that the taxonomic status of *Elytrigia* ×*mucronata* and *Elytrigia* ×*tesquicola* may be lowered from hybrid species to intraspecific varieties of *Elymus* ×*mucronatus*. Individuals of *E.* ×*mucronatus* nvar. *mucronatus* and nvar. *tesquicolus* display a continuum of morphological variation (Szczepaniak 2003b), differ only by the degree of hairiness of glumes and lemmas, and are not genetically distinct. A similar level of intraspecific genetic and morphological differences were affirmed within the parental species *E. hispidus* by recognition varieties; var. *hispidus* and var. *villosus* (Assadi 1998; Szczepaniak 2003b) and within *E. repens*; var. *repens*, var. *aristatus* and var. *subulatus* (Szczepaniak 2003a). Morphological varieties of *E. hispidus* and *E. repens* are not reproductively isolated and form fertile hybrids.

AFLP data analyses display that the hybrids are closer to *Elymus hispidus* than to *E. repens* and suggest unidirectional introgressive hybridization between the species. In UPGMA and PCoA, all *E. repens* individuals form their own group, regardless of the occurrence site of particular specimens (Figs 1 and 2). *E. hispidus* and hybrids cluster together, although within subclusters that correspond to individual localities, Smoniowice and Kielce (Fig. 2). Asymmetrical introgression when hybrids contained more nuclear markers from either parental taxa is partially related to the frequency of these parental taxa in individual populations (Burgess et al. 2005). In the populations studied, *E. hispidus* occurred in great abundance throughout the habitat, whereas *E. repens* was found in a much smaller number of individuals only on the habitat edges. It appears that *E. hispidus* occurs primarily in the habitats studied, on the loess in Smoniowice and calcareous soils in Kielce. *E. hispidus* is adapted to a narrow range of environmental conditions and displays rather low phenotypic plasticity (Szczepaniak 2001; Mahelka 2006). The disturbance of agricultural activities in habitats on the field margins (in Smoniowice) and in the quarry neighbouring on a meadow (in Kielce) caused the disappearance of the more susceptible parental species *E. hispidus* and the spread of the better adapted hybrid specimens. Individuals of *E.* ×*mucronatus* are very vigorous and robust, and they have a greater potential to colonize disturbed habitats. This confirms the hybrids' dominant position in the populations studied and explains the difficulty in finding "pure" specimens of *E. hispidus* (present authors' field observations).

Hybridization and introgression can produce shifts in morphology and ecological tolerance, and thus greatly increase the potential of hybrids or introgressants for adaptation in habitats other than optimum for progenitors. As stated, ecological marginal populations of *Carex curvula* are mainly composed of individuals with genotypes resulting from introgressive hybridization. Genotype integrity of this species is maintained in optimal habitats, while introgressed individuals are favoured in marginal habitats (Choler et al. 2004). Studies on flooding tolerance of *Elytrigia repens*,

E. intermedia and their hybrids have shown that hybridization may lead to the enrichment of hybrids' gene pools with genes responsible for the survival of the parental species under extreme conditions (Mahelka 2006).

CONCLUSIONS

AFLP analyses and the level of pollen fertility unambiguously support the hybrid origin of *Elymus* ×*mucronatus*. All of the AFLP fragments of the hybrid taxon were derived from the putative parents; there were no hybrid-specific bands. F1 hybrids not detected morphologically were distinguished from the parental species and backcross hybrids. Unidirectional introgression causes genetic swamping of *E. hispidus* by the hybrid swarm in the populations studied. *E. repens* retains its own genetic integrity to a greater degree than *E. hispidus*, additionally confirmed by AFLP data.

Individuals of the parental species are difficult to find in the habitats studied, and the populations are composed of very vigorous hybrids that spread by extensive long, creeping rhizomes. Some *Elymus* ×*mucronatus* individuals yielded fully developed seeds, observed in natural habitats, also confirming backcrossing to the parental species, or indicating some degree of self-pollination. Therefore, *E.* ×*mucronatus* fulfils molecular criteria for determining hybrid species (Zhou et al. 2005) and could be potentially self-sustaining. It should be considered as a species with the genetic material that can assort among its specimens in their own genome. Furthermore, morphological characters distinguishing hybrids remain stable in homogeneous growth conditions in the Botanical Garden of the Jagiellonian University (present authors' observations). We propose to consider *Elymus* ×*mucronatus* as a new species of hybrid origin in the Polish flora.

The results support the generally expressed viewpoint that generic deliminations within the tribe Triticeae, and especially between *Elymus* species, are problematic and controversial because of the lack of clear-cut morphological differences and weak genetic barriers among taxa (e.g., Baum et al. 1987; Salomon and Lu 1992; Assadi and Runemark 1995; Barkworth 2000). Brown and Pratt (1960) conclude that hybridization and subsequent introgression have occurred many times and in many places, and are still taking place in the populations of *Elymus* species, forming introgressant types within individual species. The present results of AFLP analyses support the view that *Elymus* species, as well as all the Poaceae in a wider context, are predisposed to hybridization, which, among other, affects their enormous evolutionary potential.

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