Variation in leaf anatomy within the *Elytrigia intermedia* – *E.* ×*mucronata* – *E. repens* (*Poaceae*) hybrid complex

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Abstract. Leaf anatomy provides valuable characters for determining plant species. It is unclear, however, how these characters are inherited in hybrids and whether it is possible to determine the parental species of a hybrid using leaf anatomy. The anatomy of leaf blades within the *Elytrigia intermedia* – *E.* ×*mucronata* – *E. repens* hybrid complex was, therefore, investigated to elucidate its intra- and interspecific variability. A total of twelve characters show considerable differences between the two parental taxa. Their hybrid *E.* ×*mucronata* occasionally combines diagnostic traits of both parental species, but some plants have character variants confined to only one parent. This complicates the use of the suggested characters when attempting to distinguish the hybrid from its parents.

Key words: *Elytrigia* ×*mucronata,* hybrid, leaf blade section, micromorphology, *Triticeae*

Introduction

The tribe *Triticeae* Dumort. comprises approximately 350 taxa (Löve 1984), many of which, such as wheat, barley and rye, are economically valuable. The *Triticeae* are highly heteromorphic, and the appropriate generic concepts for the tribe are subject to a long-term debate (for review, see Barkworth 2000). Since the establishment of the Linnaean system, several treatments have been proposed that more or less reflect the complicated relationships among the members of this group (e.g., Nevski 1933; Hitchcock 1951; Tzvelev 1976; Melderis & al. 1980). Genome analyses of artificial hybrids within the *Triticeae* were performed (e.g., Dewey 1967, 1968), which led to the formulation of the genome-based generic concept (Löve 1984; Dewey 1984 for perennials). Each unique genomic constitution was regarded as a separate genus (Löve 1984). Monogenomic genera comprise only one haplome, while heterogenomic genera combine two or more distinct haplomes. In total, 23 haplomes have been distinguished within the *Triticeae* (Löve 1984). Not all taxonomists, however, accepted this treatment based on one character, and some even voiced their criticism (e.g., Baum & al. 1987; Seberg & Petersen 1998). Subsequently, new classifications reflecting the evolutionary history were proposed (Kellogg 1989; Yen & al. 2005; Barkworth & Bothmer 2009; Yen & Yang 2009).

The genus *Elytrigia* Desv. (*Triticeae*, *Poaceae*) includes perennial, mainly rhizomatous species native to Eurasia. Taxonomists have not reached a consensus on the systematic status of this genus. Its members are often included in the genera *Agropyron*, *Elymus*, *Thinopyrum,* and *Pseudoroegneria* (Melderis 1978; Melderis & al. 1980; Barkworth & al. 2007). In just as many studies, however, *Elytrigia* is regarded as a separate genus (Tzvelev 1976; Edgar & Connor 2000; Wu & al. 2006).

The once-Eurasian species *Elytrigia repens* (L.) Nevski [*Agropyron repens* (L.) P. Beauv., *Elymus repens* (L.) Gould] is now distributed worldwide (Clayton & al. 2006 onwards). It is a highly competitive weed that can colonize a wide range of cultivated or wasteland habitats and even soils polluted with heavy metals (Palmer & Sagar 1963; Brej 1998). On the other hand, the steppe and xerothermic species *Elytrigia intermedia* (Host) Nevski [*Agropyron intermedium* (Host) P. Beauv., *Elymus hispidus* (Opiz) Melderis and *Thinopyrum intermedium* (Host) Barkworth et D. R. Dewey] are distributed across Europe (in the central, southwestern, southeastern and eastern parts), Russia (southwestern parts), Turkey, the Caucasus, Iran, Iraq, Syria, Central Asia (Turkmenistan – Pamir-Alay) and Pakistan (Bor 1968; USDA), and also occur secondarily in North America (Dewey 1983) and New Zealand (Edgar & Connor 2000). Both species are a valuable genetic source for the improvement of wheat (Li & Wang 2009; Zeng & al. 2013).

Hybridization between *Elytrigia intermedia* and *E. repens* is ongoing (Mahelka & al. 2007; Szczepaniak & al. 2007). Their hybrid *Elytrigia ×mucronata* (Opiz) Prokudin [*Agropyron mucronatum* Opiz, *E. apiculata* (Tscherning) Jirásek] has been reported from different parts of the distribution areas of both parental species (Prokudin & Druleva 1971, 1972; Mahelka & al. 2007; Szczepaniak & al. 2007; Angelov & Szczepaniak 2015). All taxa are prevalently hexaploid, nonaploids and, in *Elytrigia ×mucronata,* heptaploids have been also found (Mahelka & al. 2005, 2007). Genome size has been proven as a good marker for identification of the taxa within this hybrid complex: the average 2C values ± s.d. were 23.27 ± 0.20 pg and 27.04 ± 0.24 pg for hexaploid *E. repens* and *E. intermedia*, respectively; the mean values of relative nuclear DNA content were 0.718, 0.770 and 0.816 for *E. repens*, hybrids and *E. intermedia*, respectively, and 1.057, 1.107 and 1.144 for nonaploid cytotypes of *E. repens*, hybrids and *E. intermedia*, respectively (Mahelka & al. 2005).

The studied species have a complicated evolutionary history. An early cytogenetic analysis identified the allopolyploid origins of both *E. repens* (Cauderon 1958; Dewey 1961) and *E. intermedia* (Peto 1936; Vakar 1938), and several genomic constitutions of these species were later proposed (e.g., Dewey 1984; Liu & Wang 1993; Assadi & Runemark 1995). Recently, molecular methods and *in situ* hybridization techniques were used to clarify the genomic constitution of hexaploid *E. repens*, which combines two subgenomes of *Pseudoroegneria* (**St**) and one *Hordeum* (**H**) subgenome. The most likely genomic constitution of hexaploid *E. intermedia* is as follows: one *Pseudoroegneria* (**St**) subgenome, one *Dasypyrum* (**V**) subgenome and one *Aegilops* subgenome (**D**) (Mahelka & al. 2011, 2013). Furthermore, a certain level of heterogeneity has been detected in both species (Mason-Gamer 2008; Mahelka & Kopecký 2010; Mahelka & al. 2011).

Variability of some morphological characters of *E. repens* and *E. intermedia* overlaps. However, recent taxonomical studies have presented two morphological characters as reliable for distinguishing between the species: (1) leaf sheath margins – glabrous in *E. repens*, with macrohairs in *E. intermedia* (Kubát & al. 2002); and (2) glume shape – awn-tipped or gradually thinning out in *E. repens* and truncate or very shortly mucronate in *E. intermedia* (Barkworth & Dewey 1985). Morphological differentiation among the parental species and *E. ×mucronata* is poor, and hybrids and parents often exhibit shared characters.

The examined *Elytrigia* species has typical festucoid leaf structure (Duval-Jouve 1870; Prat 1932; Metcalfe 1960). Runemark & Heneen (1968) distinguished within the *Agropyron-Elymus* complex by three types of anatomical structures. The relationship between genomic constitution and anatomy of leaves and glumes was investigated by Jarvie & Barkworth (1992).

Earlier studies of the *Elytrigia intermedia* and *E. repens* have shown differences between species in leaf anatomy, but intraspecific variations of anatomical characters have seldom been examined. *Elytrigia ×mucronata* was included only in several studies (Prokudin & Druleva 1971, 1972; Szczepaniak 2009) and was determined on the basis of morphological characters. In the present study, the leaf anatomy of *Elytrigia repens* (L.) Nevski, *E. intermedia* (Host) Nevski and their hybrid *E. ×mucronata* (Opiz) Prokudin is compared so as to assess the heritability of these characters in the *Elytrigia ×mucronata* hybrid complex. More specifically, the purpose of this study is:

(1) to investigate the variation in leaf anatomical characters in a broader sampling of *Elytrigia repens* and *E. intermedia*,

(2) to study the displayed leaf anatomical characters in three cytotypes of *Elytrigia ×mucronata*, and

(3) to assess the significance of leaf anatomical characters for determining the parental species and their hybrids.

Material and methods

Leaf anatomy of *Elytrigia repens* (6 plants: $2n = 6x = 42$), *E. intermedia* (22 plants: $2n = 6x = 42$) and *E.* ×*mucronata* (8 plants: $2n = 6x = 42$, 1 plant: $2n = 7x = 49$, 2 plants: $2n = 9x = 63$) from the Czech Republic, Slovakia and Bulgaria was investigated (Appendix 1). Genome size estimation was used for the determination of plants (Appendix 1).

Anatomical studies have been carried out into the transverse sections of leaf and into the leaf blade surface. Leaf blades (both from fresh and herbarium specimens) were fixed in a 1:1 mixture of glycerol and 70 % ethanol. Hand-cut transverse sections were made in the middle of the blade and stained with an aqueous solution of safranin. Slides were examined and imaged with an Olympus BX-50 microscope equipped with an Olympus DP70 digital camera (Olympus, Tokyo).

Terminology of anatomical characters follows that of Ellis (1976, 1979), with the sole exception of crown cells (cellules à pointe courte), a term coined by Prat (1932) for solitary short cells with a conical protrusion of the silicified outer wall of epidermal cells. This distinction means that crown cells are not synonymous for hooks, which include part of the epidermal cell lumen.

Examined characters and distinguished variants

Abaxial surface (1) smooth – Fig. 1C, (2) grooved – Fig. 1E, or (3) ribbed – Fig. 1A. The presence of an abaxial sclerenchymatous projection of the midrib was not evaluated as a ribbed surface. Grooves/ribs were not present below all vascular bundles.

Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle in contact with adaxial epidermal cells (Figs. 1B, D). Fibres of the first and particularly of the second layer of the sclerenchymatous girder under the adaxial epidermis were considered.

Shape of adaxial ribs: (1) extremely flattened – Fig. 2A, (2) rounded – Fig. 2B, (3) flat-topped (sides rounded with flat tops) – Fig. 2D, or (4) square (sides angled with flat tops; a less frequent type) – Fig. 2E. Only ribs above the 1st order vascular bundles were considered. Two types of ribs can occur in one leaf (Fig. 2C).

Depth of adaxial furrows: (1) shallow (<0.25 – less than a quarter of the leaf thickness) – Fig. 2B, (2) medium (0.25–0.5 – a quarter to half of the leaf thickness) – Fig. 2D.

Shape of adaxial furrows (=angle included between two adaxial ribs): (1) narrow (<90°) – Fig. 2D, (2) wide (>90°) – Fig. 2B.

Relative thickness in two points on the leaf margin – leaf thickness across the middle of the outermost vascular bundle (t_1) was compared with leaf thickness at the most distant place from the end of the leaf, where sclerenchyma extends from the adaxial to the abaxial epidermis (t_2)– Fig. 3A. The following variants were observed: (1) thinning ($t_1 > t_2$) – Figs. 3A–G, (2) identical ($t_1 = t_2$) – Fig. 3H, and (3) thickening ($t_1 < t_2$) – Fig. 3I.

Shape in cross-section margin itself in thinning margins (i.e., $t_1 > t_2$): (1) acuminate – Fig. 3A, (2) caudate – Fig. 3B, (3) narrowly acute – Fig. 3C, (4) acute – Fig. 3D, (5) obtusely pointed – Fig. 3E, (6) rounded – Figs. 3F, G.

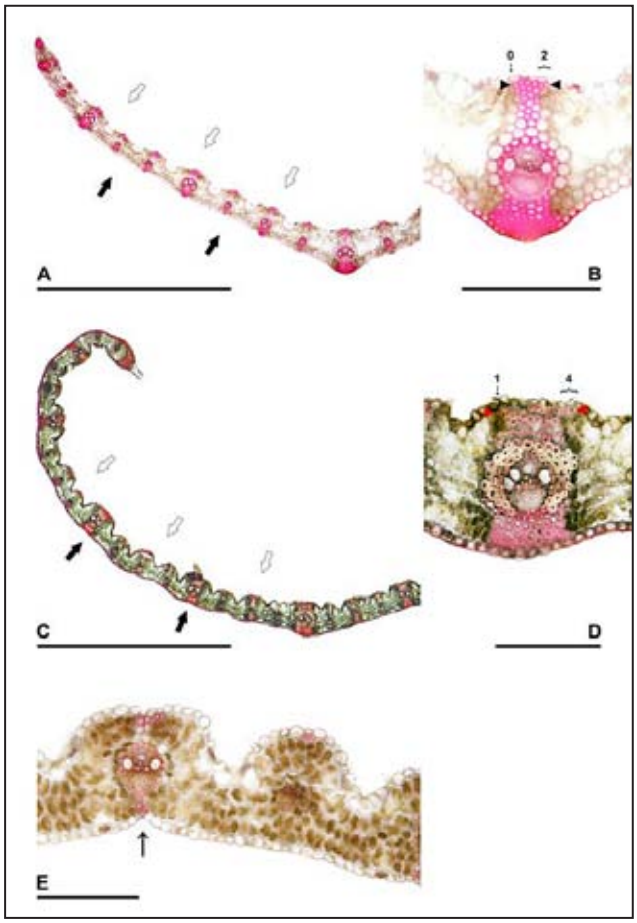

Fig. 1. Abaxial surface (filled arrows), **adaxial surface** (blank arrows) – **A**: *E. repens* (16/1), **C**: *E. intermedia* (BH/2). Scale bar = 1 mm. **Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle in contact to the adaxial epidermal cells** (black or red arrowheads); **median vascular bundle sheath, number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle adjacent to the epidermis and mesophyll** (numbers of fibres are indicated) – **B**: *E. repens* (16/1), **D**: *E. intermedia* (BH/2). **Abaxial surface** – **E**: grooved – *E. intermedia* (C11). Scale bar = 200 μm.

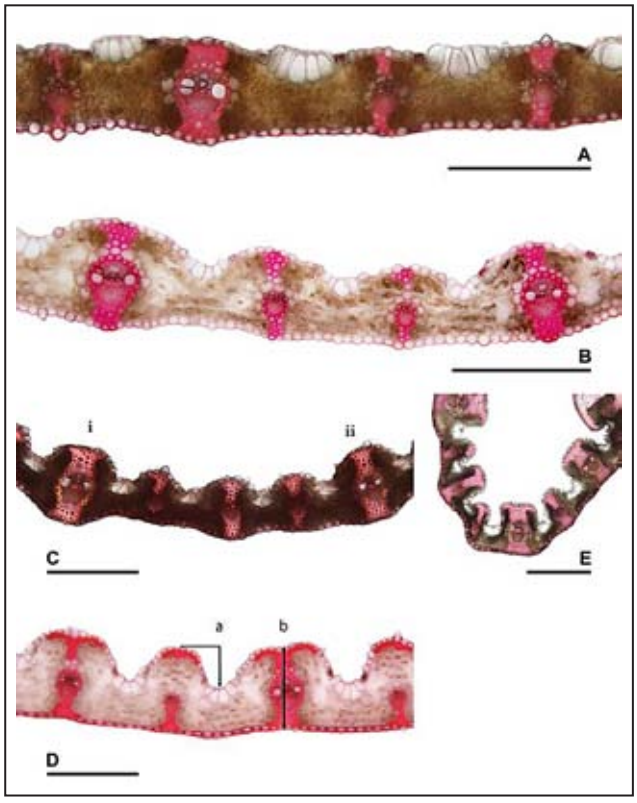

Fig. 2. Adaxial surface (shape of adaxial ribs + depth and shape of adaxial furrows). **A**: *E. repens* (21/3) – extremely flattened, **B**: *E. repens* (16/1) – rounded ribs + shallow and wide furrows, **C**: *E.* ×*mucronata* (3/3) – rounded (**ii**) and flat-topped (**i**) ribs + shallow and narrow furrows, **D**: *E. intermedia* (P/60) – flat-topped ribs + medium and narrow furrows (**a** – depth of furrow, **b** – leaf thickness), **E**: *E. intermedia* (3/2) – square ribs + medium and narrow furrows. Scale bar = 200 μm.

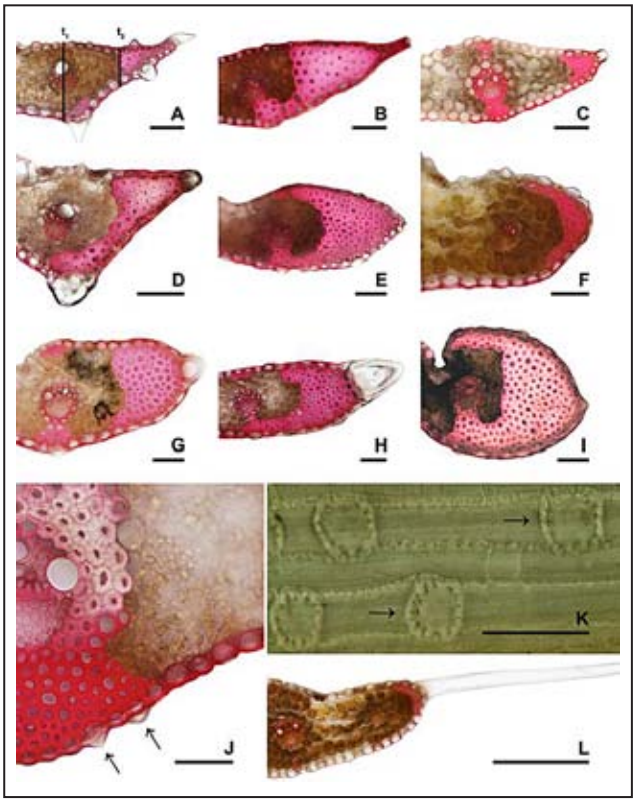

Fig. 3. Relative thickness in two points on the leaf margin, shape in cross-section of the margin (for A–G only) – **A**: thinning, acuminate, *E. repens* (1/4), t_1, t_2 – the compared dimensions, **B**: thinning, caudate, *E. repens* (21/3), **C**: thinning, narrowly acute, *E. repens* (16/1), **D**: thinning, acute, *E. repens* – (3/5), **E**: thinning, obtusely pointed, *E. intermedia* (34/1), **F**: thinning, rounded, *E. intermedia* (C18), **G**: thinning, rounded, *E.* ×*mucronata* (43/2), **H**: identical, *E. intermedia* (8/3), **I**: thickening, *E. intermedia* (3/2). **Crown cells** – **J**: crown cells on cross-section, *E. intermedia* (8/3), **K**: crown cells in surface view, *E. intermedia* (8/3). Scale bar = 50 μm. **Macrohair on margin of leaf blade** (only proximal part is presented) – **L**: *E. intermedia* (C11). Scale bar = 200 μm.

Median vascular bundle sheath – number of layers: (1) two-layered – Fig. 1B or (2) three-layered – Fig. 1D.
Median vascular bundle sheath – cell walls thickness: the inner sheath + the outer sheath – (1) the inner tangential and radial walls thickened + parenchymatous – Fig. 1B, (2) walls distinctly (equally or unequally) thickened + parenchymatous, (3) walls of both sheaths distinctly thickened – Fig. 1D. Occasionally, there is an additional layer of cells with thickened walls – Fig. 1D. The uppermost layer is often interrupted by a girder of sclerenchyma and sometimes is incomplete.
Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle adjacent to the epidermis and mesophyll (seldom adjacent to another layer of sclerenchymatous fibres) – Figs. 1B, D. Fibres of the first layer of the adaxial sclerenchymatous girder were considered.
Crown cells – solitary short cells with a conical protrusion: (1) present – Figs. 3J, K or (2) absent.

Macrohairs on margins of the leaf blade: (1) present – Fig. 3L or (2) absent. Macrohairs are unicellular, at least 3× longer than the base, and visible to the naked eye.

Results

Characters with overlapping variation in *Elytrigia repens*, *E. intermedia*, and *E.* ×*mucronata*

(a) Adaxial and abaxial epidermis – long cells elongated with thickened undulated walls without papillae; short cells solitary (cork cells) or in pairs (cork and silica cells); cork cells tall and narrow, silica cells elliptical; prickle-hairs – both prickles and hooks present; macrohairs unicellular, present or absent; microhairs absent; stomata encompassed by parallel-sided subsidiary cells; bulliform cells in intercostal zones of adaxial epidermis.

(b) Transverse section – flat, V-shaped or convolute at margins in outline; vascular bundles of the first/second/third order rounded, associated with sclerenchymatous girders or strands; single large ribs alternating with 1–3 smaller ribs; bulliform cells in groups (3–6 cells); margins reinforced by sclerenchyma, irregular chlorenchyma.

Distinguishing characters of *Elytrigia intermedia* and *E. repens*

A total of twelve leaf anatomy characters of *Elytrigia intermedia* and *E. repens* were found to distinguish these two species (Table 1). Most characters had unique variants in both parental species. Five characters had unique variants only in *E. intermedia*.

Table 1. Characters with partly overlapping or non-overlapping variation in *Elytrigia repens*, *E. intermedia*, and *E. ×mucronata*.

	***E. repens*, $2n = 6x = 42$, n = 6**	***E. ×mucronata*, $2n = 6x = 42$, n = 8**	***E. ×mucronata*, $2n = 7x = 49$, n = 1**	***E. ×mucronata*, $2n = 9x = 63$, n = 2**	***E. intermedia*, $2n = 6x = 42$, n = 22**
Abaxial surface	**ribbed**	ribbed, smooth	smooth	ribbed, smooth	**smooth**, **grooved**
Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle in contact to adaxial epidermal cells	**4**, **5**, 6, 8	4–7, 10, 11, 13–15, 19	12	5, 7, 13	6, 8, **11**–**20**
Shape of adaxial ribs	**extremely flattened**, rounded, flat-topped	rounded, flat-topped, square	rounded, flat-topped	rounded, flat-topped	rounded, flat-topped, **square**
Depth of adaxial furrows	shallow (**0.13**–0.24), medium (0.25–0.33)	shallow (0.18–0.24), medium (0.25–0.45)	medium (0.25–0.33)	shallow (0.17–0.22), medium (0.25–0.33)	shallow (0.18–0.24), medium (0.27–**0.47**)
Shape of adaxial furrows	narrow (75°)*, wide (105°–158°)	narrow (62°–84°), wide (93°–120°)	wide (112°–115°)	wide (122°–145°)	**narrow (65°–83°)**, wide (91°–130°)
Relative thickness in two points on the leaf margin	thinning	thinning, thickening	thickening	thinning, identical, thinning	thinning, **identical**, **thinning**
Shape in cross-section margin itself in thinning margins	**acuminate**, **caudate**, **narrowly acute**, acute, obtusely pointed	narrowly acute, acute, obtusely pointed, rounded	–	acute, obtusely pointed	acute, obtusely pointed, **rounded**
Median vascular bundle sheath – number of layers	2	2	2	2	2, **3**
Median vascular bundle sheath – cell wall thickness	inner tangential and radial walls thickened + parenchymatous; walls distinctly (unequally) thickened + parenchymatous	inner tangential and radial walls thickened + parenchymatous; walls distinctly (unequally) thickened + parenchymatous, walls of both sheaths distinctly thickened	walls distinctly thickened + parenchymatous	walls distinctly thickened + parenchymatous	inner tangential and radial walls thickened + parenchymatous; walls distinctly (un/equally) thickened + parenchymatous, **walls of both sheaths distinctly thickened**
Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle adjacent to the epidermis and mesophyll	**0**, 1, 2	0–4	1	0, 2	1, 2, **3**–**6**
Crown cells	absent	absent, present	absent	absent, present	absent, **present**
Macrohairs on the margins of the leaf blade	absent	absent, present	absent	absent	absent, **present**

Note: Variants present in one species (but not in all individuals) and absent in the second species are indicated in bold.
* The narrow adaxial furrow was observed only once in one specimen of *E. repens*.

Appendix 1. List of localities (country, location, habitat and coordinates) of the examined specimens. The ploidy level of all specimens was assessed by flow cytometry. All specimens (except plants from the localities Babí Hora, Jel'šava and Madara, and heptaploid *E.* ×*mucronata*) were included in earlier studies (Mahelka & al. 2005, 2007).

Country	Locality (location, habitat)	Coordinates	*E. repens*	*E. intermedia*	*E.* ×*mucronata*		
			2*n* = 42	2*n* = 42	2*n* = 42	2*n* = 49	2*n* = 63
CZ	Rubín 1 (3 km NE of Podbořany town, top of Rubín hill, steppe)	50°15′13.2′′ N 13°26′12.4′′ E	1/4	1/1			
	Raná (0.5 km SW of Raná village, bottom of Raná hill, steppe)	50°24′34.9′′ N 13°46′38.9′′ E	3/5	3/2	3/3		
	Brno (Brno city, Kamenný kopec hill, roadside)	49°11′02.5′′ N 16°33′05.1′′ E		5/2			
	Růžový kopec (2 km NNW of Mikulov, top of Růžový kopec hill, steppe)	48°49′14.3′′ N 16°37′31.2′′ E		8/3			
	Paví vrch (2 km S of Sedlec village, Paví vrch hill, steppe and field margin)	48°45′50.8′′ N 16°41′33.1′′ E			10/1		
	Knovíz (0.5 km NW of the church at Knovíz village, pine forest)	50°12′48.1′′ N 14°07′46.4′′ E			14/4		
	Zebín (2 km NE of Jičín town, top of Zebín hill, steppe)	50°27′12.8′′ N 15°22′26.0′′ E	16/1				
	Vrbčany 1 (1.5 km NE of Vrbčany village, steppe)	50°03′43.5′′ N 14°59′56.0′′ E			17/1, 4, 5		
	Strači (0.3 km E of Strači village, pine forest)	50°27′04.2′′ N 14°24′30.7′′ E	21/3				
	Záhoří (2 km NW of Žatec town, steppe)	50°20′24.3′′ N 13°31′09.4′′ E			29/8		
	Pavlov-Děvín (0.5 km W of Pavlov village, Děvín hill, steppe)	48°52′27.7′′ N 16°39′41.7′′ E		34/1			
	Klentnice (0.5 km SW of Klentnice village, Stolová Hora hill, steppe)	48°50′25.2′′ N 16°38′24.4′′ E		35/4			
	Hodonín 2 (1.5 km S of Dubňany village, pine forest)	48°54′08.2′′ N 17°05′14.2′′ E	39/2				
	Hovorany (1.5 km NW of Hovorany village, Hovoranské Louky Reserve, steppe)	48°57′54.2′′ N 16°58′25.7′′ E					41/5
	Nesovice (1.5 km W of Nesovice village, Malhotky natural highlight, steppe)	49°08′53.5′′ N 17°03′23.7′′ E			43/2		
	Čertoryje (4 km SE of Tvarožná Lhota village, Čertoryje National Nature Reserve, meadow)	48°51′34.6′′ N 17°24′32.0′′ E	C17	C11, C18, C24		46/2	
	Valov (2 km S of Podbořany town, field margin)	50°12′34.5′′ N 13°24′54.4′′ E					50/1
	Pouzdřany (1 km NE of Pouzdřany village, Pouzdřanská Step National Nature Reserve – Kolby)	48°56′23.2′′N 16°38′47.0′′E		P/60			
	Babí hora (4.5 km SE of Hluk town, Babí hora National Monument, steppe)	48°57′28.0′′ N 17°33′34.7′′ E		BH/1-4			
SK	Hajnáčka (4 km NE of Hajnáčka village, oak forest)	48°14′50.0′′ N 19°59′00.0′′ E		18/1			
	Jel'šava (3 km NE of Kameňany village, steppe)	48°35′42.5′′ N 20°12′49.2′′ E		JK			
BG	Madara (Madara Plateau, Kapitschan, mountain mesophilic meadow)	43°17′28.4′′N 27°08′00.2′′E		BG-1A/1-3, BG-1B/1-3			

Unique variants of parental species displayed in three ploidy levels of *Elytrigia* ×*mucronata*

Unique variants of both parental species were combined in four plants of hexaploid *E.* ×*mucronata*: three and one plants displayed unique variants of *E. intermedia* and *E. repens,* respectively. The heptaploid plant of *E.* ×*mucronata* displayed unique variants only of *E. intermedia.* One nonaploid plant of *E.* ×*mucronata* combined unique variants of both parental species and the second plant displayed unique variants of *E. intermedia*.

A comparison of occurrences of the particular variants of studied characters in *Elytrigia repens*, *E.* ×*mucronata* and *E. intermedia* is presented in Appendix 2.

Appendix 2. Comparison of the occurrences of particular variants of studied characters in *Elytrigia repens*, *E.* ×*mucronata* and *E. intermedia*.

Character	Variant	*E. repens*, 2*n* = 6*x* = 42, n = 6	*E.* ×*mucronata*, 2*n* = 6*x* = 42, n = 8	*E.* ×*mucronata*, 2*n* = 7*x* = 49, n = 1	*E.* ×*mucronata*, 2*n* = 9*x* = 63, n = 2	*E. intermedia*, 2*n* = 6*x* = 42, n = 22
Abaxial surface	(1) smooth	–	4	1	1	18
	(2) grooved	–	–	–		5
	(3) ribbed	6	4	–	1	–
Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle in contact to adaxial epidermal cells	4	1	1	–		–
	5	3	1	–	1	–
	6	2	1	–		1
	7	–	1	–	1	–
	8	1	–	–	–	1
	9	–	–	–	–	–
	10	–	1	–	–	–
	11	–	1	–	–	2
	12	–	–	1	–	1
	13	–	1	–	1	2
	14	–	1	–	–	5
	15	–	1	–	–	3
	16	–	–	–	–	2
	17	–	–	–	–	2
	18	–	–	–	–	4
	19	–	1	–	–	1
	20	–	–	–	–	1
Shape of adaxial ribs	(1) extremely flattened	1	–	–	–	–
	(2) rounded	5	6	1	2	7
	(3) flat-topped	2	7	1	1	22
	(4) square	–	1	–	–	2
Depth of adaxial furrows	(1) shallow (<0.25)	3	3	–	2	4
	(2) medium (0.25–0.5)	3	7	1	1	18
Shape of adaxial furrows	(1) narrow (<90°)	1*	5	–	–	16
	(2) wide (>90°)	5	3	1	2	6
Relative thickness at two points on the leaf margin	(1) thinning	6	6	–	1	17
	(2) identical	–	–	–	1	3
	(3) thickening	–	2	1	1	10
Shape in cross-section margin itself in thinning margins	(1) acuminate	1	–	–	–	–
	(2) caudate	1	–	–	–	–
	(3) narrowly acute	1	1	–	–	–
	(4) acute	4	4	–	1	9
	(5) obtusely pointed	1	2	–	1	8
	(6) rounded	–	2	–	–	5
Median vascular bundle sheath – number of layers	2	6	8	1	2	16
	3	–	–	–	–	6

Appendix 2. Cotinuation.

Character	Variant	*E. repens*, 2*n* = 6*x* = 42, n = 6	*E.* ×*mucronata*, 2*n* = 6*x* = 42, n = 8	*E.* ×*mucronata*, 2*n* = 7*x* = 49, n = 1	*E.* ×*mucronata*, 2*n* = 9*x* = 63, n = 2	*E. intermedia*, 2*n* = 6*x* = 42, n = 22
Median vascular bundle sheath –cell walls thickness	(1) inner tangential and radial walls thickened + parenchymatous	5	5	–	–	7
	(2) walls distinctly (unequally) thickened + parenchymatous	1	2	–	–	3
	(3) walls distinctly thickened + parenchymatous	–	–	1	2	4
	(4) walls of both sheaths distinctly thickened	–	1	–	–	8
Number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle adjacent to the epidermis and mesophyll	0	4	2	–	1	–
	1	3	4	1	–	–
	2	1	1	–	1	–
	3	–	1	–	–	–
	4	–	1	–	–	–
Crown cells	(1) absent	6	3	1	1	3
	(2) present	–	5	–	1	19
Macro-hairs on the margins of leaf blade	(1) absent	6	7	1	2	13
	(2) present	–	1	–	–	9

Note: More than one variant of particular characters was present in the examined plants.
* The narrow adaxial furrow was observed only once in one specimen of *E. repens*.

Discussion

Leaf anatomy of *Elytrigia intermedia*, *E. repens* and *E.* ×*mucronata*: present versus earlier studies

Although earlier studies have found the following leaf anatomy characters to be diagnostic among the species, with slight overlaps at most, the present study determined their variation as completely or almost completely overlapping.

Long cells in both *E. repens* and *E. intermedia* have straight or undulating walls without protrusions, although unique observations of papillae on abaxial epidermal cells in *E. repens* have been reported (Runemark & Heneen 1968). The observation of papillae in this case had not been confused with crown cells; the authors had used crown cells properly according to Prat (1932). Meng & Mao (2013) detected papillae in *E. intermedia* but not in *E. repens*. In contrast to the present findings, they did not observe short cells in either species.

Crown cells occurred in the leaf epidermis of *E. intermedia* (except in plants from the Čertoryje locality). No variation concerning the presence of crown cells in *E. intermedia* has been reported (Hejná 1968; Runemark & Heneen 1968). Metcalfe (1960) did not mention explicitly the presence of crown cells, but he did observe conspicuously pitted short cells. The absence of crown cells in the epidermis of *E. repens* has also been noted in the earlier studies (Prat 1933 cit. sec. Metcalfe 1960; Hejná 1968; Runemark & Heneen 1968). In the present study, in agreement with Prat (1933 cit. sec. Metcalfe 1960), crown cells were completely absent from the leaf epidermis of *E. repens,* but were found in parts of the spikelet (palea, lemma and both glumes).

Both prickle hairs and macrohairs occurred on the epidermis of the species, but the presence of macrohairs on the leaf margins of *E. intermedia* was regarded as species specific. Some authors mentioned the presence of macrohairs on the leaf surface only (Krawczyk & al. 2007; Szczepaniak 2009), whereas others have encountered only specimens completely without macrohairs (Hejná 1968; Metcalfe 1960).

Many authors have evaluated the adaxial surface (e.g., Metcalfe 1960; Jarvie & Barkworth 1992; Krawczyk & al. 2007; Szczepaniak 2009), but did not observe all existing variants of shapes of the adaxial ribs, such as the combination of rounded and flat-topped ribs, which occurred in both species, and square ribs (also in combination with flat-topped ribs) in *E. intermedia*. Earlier observations of extremely flattened adaxial ribs in *E. intermedia* (Jarvie & Barkworth 1992) contradict the present results, probably due to misidentification of the analysed accession.

Prokudin & Druleva (1971, 1972) observed a non-overlapping continuum in the amount of leaf sclerenchyma among the members of the hybrid complex:

a low proportion of sclerenchyma in *E. repens*, an intermediate proportion of sclerenchyma in *E.* ×*mucronata* and a high proportion of sclerenchyma in *E. intermedia.* The present results show that variation in the amount of leaf sclerenchyma observed in *E.* ×*mucronata* overlaps with the variations of both parental species.

Distribution of particular character variants – relative thickness in two points on the leaf margin – between species is mostly in agreement with the earlier reports (Duval-Jouve 1870; Prokudin & Druleva 1971, 1972; Krawczyk & al. 2007). The thickening leaf margin observed in *E. repens* (Krawczyk & al. 2007) is confusing, however. All specimens of *E. repens* examined in the present study had thinning leaf margins. Moreover, crown cells, whose absence is characteristic of *E. repens*, were present on the leaf margin of the specimens (Krawczyk & al. 2007). The accession was probably confused with *E. intermedia* or *E.* ×*mucronata*. Some shape variants in cross-sections of the margin itself have not yet been observed.

The leaf margin is reinforced by a girder of sclerenchyma. Krawczyk & al. (2007) found the sclerenchymatous girder to be composed of 30–50 cells in *E. repens* and 50 or more cells in *E. intermedia.* In the material studied here, the number of cells varied from 5 to 90 in *E. repens* and from 10 to 110 in *E. intermedia*.

Prokudin & Druleva (1971, 1972) reported a gradient in the immersion of bulliform cells among the taxa under study: from superficial, to partly immersed, to entirely immersed in *E. repens*, *E.* ×*mucronata* and *E. intermedia*, respectively. The present data, however, show that the degree of immersion can vary within a leaf. Moreover, projecting bulliform cells were also observed in *E. intermedia*, in contrast to the conclusions of Prokudin & Druleva (1971, 1972).

The genus *Elytrigia* Desv. has festucoid leaves (e.g. Runemark & Heneen 1968) characterized, among other things, by a double vascular bundle sheath. The bundle sheath surrounding the median vascular bundle is formed by an inner mestome sheath (the cells, except for the outer tangential wall, have thickened walls) and an outer parenchymatous sheath (Szczepaniak 2009). Some populations of *E. intermedia* has also been observed to have a median vascular bundle sheath composed of one or two layers of cells with distinctly thickened walls (Krawczyk & al. 2007). Median vascular bundle sheaths with distinctly thickened walls of the inner layer or both layers were also observed in *E. repens* and *E.* ×*mucronata*, respectively. Moreover, in *E. intermedia*, the median vascular bundle sheath can be formed by three layers of cells with thickened walls. Quantitative characters, such as the number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle in contact with the adaxial epidermal cells, as well as the number of fibres in the adaxial sclerenchymatous girder of the median vascular bundle adjacent to the epidermis and mesophyll, have not been evaluated yet. The shape of sclerenchymatous girders and the number of layers of sclerenchyma from the epidermis to the vascular bundle were examined in transverse sections in the earlier studies (Metcalfe 1960; Szczepaniak 2009).

Distinguishing characters suggested earlier in the light of the present results

Hejná (1968) concluded that the presence/absence of crown cells is a diagnostic character for distinguishing between *E. intermedia* (crown cells present) and *E. repens* (crown cells absent). In the present study, however, their absence was also observed in *E. intermedia* (in plants from one locality). Because crown cells are not always present in *E.* ×*mucronata*, this character is not useful for identifying *E. intermedia*.

Runemark & Heneen (1968) distinguished three types of anatomical leaf structure within the *Elymus-Agropyron* complex that differ in the shape of epidermal cells, sinuosity of cell walls, presence or absence of crown cells and prickle hairs, and continuity of hypodermal sclerenchyma. They assigned *E. repens* and *E. intermedia* to two different types of leaf anatomy, but no differences, other than crown cells, in the variation of characters used in the mentioned study were found observed in the present study.

Prokudin & Druleva (1971, 1972) studied all representatives of the hybrid complex and found differences in the adaxial leaf surface (and abaxial leaf surface – Prokudin & Druleva 1972 only), leaf margin, degree of immersion of bulliform cells, and amount of sclerenchyma. Their observations partially agree with the present results, but the authors found no overlapping variation between the taxa.

Of the 13 examined characters of leaf anatomy, the type of hairiness, presence of epicuticular wax, volume of the sclerenchymatous tissue, stomata location, and presence of silica crystals in the epidermis were useful characters for distinguishing *E. intermedia* from *E. repens* (Szczepaniak 2009). The variation in these characters was completely overlapping in the material

studied; therefore, these characters are not useful for determining the species under study.

The discrepancies between the present findings and those of the earlier studies can be explained by various reasons. First is the correctness of determination. Morphology alone does not suffice for reliable identification, as it is easy to confuse both species with the hybrid and vice versa. Few species of the *Triticeae* have clear taxonomic identities. Within the tribe, interspecific (intergeneric) hybridization is frequent, and morphologically similar species can have entirely different genomic constitutions. Second, neither any earlier study, nor the present one could have included only primary hybrids. The third reason is the different number of accessions or clones examined. Last, the origin of the material plays a crucial role; for instance, clones found in areas of secondary distribution have limited variation, as do collections in gene banks or botanical gardens.

Are characters stable in different environments?

Variation in the leaf anatomy of *E. repens* was studied under different watering regimes. The experiment yielded no considerable differences in any trait among the watering regimes (unpublished data). The second species, *E. intermedia*, is a xeric grass and does not grow in soils with a high water level (Mahelka 2006).

Is it possible to distinguish hybrids by anatomical characters?

Three ploidy levels of *E.* ×*mucronata* were investigated. The pattern of variation showed a hybrid origin only of several hexaploid and one nonaploid plant of *E.* ×*mucronata.* These plants combined traits that were deemed diagnostic of the parental species; thus, clear identification of these individuals was possible. Other hexaploid individuals, one analysed heptaploid plant and the second nonaploid plant of *E.* ×*mucronata* were indistinguishable from either *E. intermedia,* or *E. repens* by anatomical characters, which makes the suggested characters incapable of differentiating between the taxa.

Prokudin & Druleva (1971, 1972) found *E.* ×*mucronata* to be intermediate between its parents in terms of four (five) anatomical leaf characters. In contrast, Szczepaniak (2009) analysed 13 leaf anatomical characters and found *E.* ×*mucronata* to be more similar to *E. intermedia* than to *E. repens*. As indicated above, neither earlier studies, nor the present one could have used only primary hybrids.

Conclusions

In the present study, considerable differences were found in the leaf blade anatomy between two allohexaploids, *E. intermedia* and *E. repens* (if no hybrids existed, the species could be easily distinguished). The hybrid, *Elytrigia* ×*mucronata*, has either combinations of specific variants of the parental species, or displays variants only of one parental species. Therefore, the use of anatomical characters for determination is limited, as misidentification of the parental species with the hybrid could not be excluded in some cases. To decide which characters are useful for distinguishing between *E. intermedia* and *E. repens*, it is important to study the patterns of their supposed diagnostic traits in *E.* ×*mucronata*.

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